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Fermentative Production of Lysine by *Corynebacterium glutamicum* from Different Carbon Sources

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ABSTRACT- Production of lysine by *Corynebacterium glutamicum* (PTCC 1532) from different agricultural by-products (molasses and pulpy waste date) was compared to glucose as raw materials. For this purpose, ammonium sulphate was selected as a constant nitrogen source. The effect of different nitrogen sources was also investigated with glucose as a constant carbon source. The production of L-lysine was examined qualitatively and quantitatively using thin layer chromatography (TLC). Results of fermentation experiments showed that the maximum yield corresponded to molasses (48 g L^{-1}) for the fermentation period of 96 hours. For other substrates the yield was lower and the period of fermentation exceeded that for molasses.

Keywords: Lysine, Molasses, Pulpy Wastes Date, Fermentation, *Corynebacterium glutamicum*

INTRODUCTION

Agricultural by-products may be used as low-cost carbohydrate sources for microbial production of high value added products such as amino acids (2). Amino acids are the basic bioelements of proteins, which are the most important macromolecules for human and animal functions. Out of the 20 L-amino acids ecumenically found in most of living organisms, L-lysine is one of the 9 amino acids which are essential for human and animal nutrition. Its demand has been steadily increasing in recent years (12). It is the second produced amino acid in a large industrial scale with several hundred thousand tones of production (about 800,000 tones) per year (1 and 6).

Lysine is generally recognized as the most deficient amino acid in the food supply of both man and domestic meat producing animals. Since animal feed, such as grain and defatted oil seeds, contain only a small quantity of lysine, poultry, cattle and other live stock are unable to synthesize this amino acid. As a result, it must be added to this feed stuff to provide an adequate diet (16). Moreover, it is used to enrich human foods which lack this essential amino acid. It is the first limiting amino acid in virtually every cereal grain known to man, thus it has large potential for improving the protein quality of cereal-based human diets especially in third world countries owing to their high dependence on cereal foods. Supplementation of wheat based foods with L-lysine improves their protein quality, resulting in improved growth and tissue synthesis. In addition, it has pharmaceutical applications both in the formulation of diets with a balanced amino acid composition and in the infusion of amino acids. It has been

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reported that addition of 0.4% L-lysine (HCl salt) and 0.15% L-threonine to wheat flour yields material with the same nutritional value as milk, while a mixture of 0.1% lysine and DL-methionine can be effectively used to strengthen animal fodder (15).

Lysine can be produced in different ways including chemical synthesis, extracting from protein hydrolyzate, enzymatic method, fermentation method, protoplast fusion technique and recombinant DNA technology (1 and 12). Among these methods, fermentation is the most economical and practical means of producing lysine, as in this method low temperature, low pressure and low-cost carbon sources are used and a biological form of lysine (L-lysine) is produced (4). *Corynebacterium glutamicum* is widely used for the industrial production of amino acids especially L-glutamate and L-lysine (8). This organism is able to use a variety of carbohydrates, alcohols and organic acids as single sources of carbon and energy for growth and also for the amino acid production (9 and 14). However, for industrial fermentations, the use of complex sugar substrates such as cane molasses, beet molasses, or hydrolysates from corn, wheat or cassava became standard. The type of the sugar used depends on the geographical location of the production plant (7 and 8). As nitrogen sources, various inorganic and organic salts and compounds such as ammonium salts and other similar compounds, urea, natural proteolytic organic substances such as peptone, casein hydrolysate, yeast extract, corn steep liquor, soybean protein hydrolysate, and various other extracts of vegetal and animal tissues may be employed. In addition to carbon and nitrogen sources, the culture media employed for production of this amino acid normally contains usual inorganic nutrients and essential elements for growth of micro-organisms (3).

Utilization of agricultural by-products as substrates for fermentation might offer an inexpensive alternative for microbial products such as amino acids. Although there are several reports on lysine production from this bacterium, to the authors' knowledge, there are no reports on the utilization of pulpy waste date substrate despite of its availability and its low cost in our country, Iran. Molasses, as a valuable product and not as waste of sugar factories can also be considered as fermentation media. The main producing countries of molasses are USA, Japan, Netherlands and UK, utilizing it largely for animal feed. It has been reported that about 480000 tones of molasses are produced annually in Iran which can be a good source of carbon and mineral compounds.

In the present study, the use of these agricultural by-products (molasses and pulpy waste date) as alternative substrates for production of L-lysine by *C. glutamicum* has been investigated.

MATERIALS AND METHODS

Microorganism

C. glutamicum is a gram-positive, non-sporulating and non-motile bacterium, with polymorphic short rods producing yellowish colonies. It requires biotin in order to grow, and a cultivation temperature of approximately 30°C. Most strains are able to utilize acetic acid, ethanol, glucose or sucrose to produce amino acid (17). *C. glutamicum* (PTCC 1532) was purchased from Persian Type Culture Collection.

Seed culture preparation

Essential materials for seed culture contained glucose (20 g/L), peptone water (10 g/L), yeast extract (10 g/L), NaCl (2.5 g/L), $MgSO_4.7H_2O$ (0.25 g/L), $MnSO_4.H_2O$ (0.1 g/L), K_2HPO_4 and KH_2PO_4 (1.0 g/L), and biotin (0.01 g/L) (18).

Basic culture preparation

Basic culture contained a substrate (molasses, pulpy waste date or glucose) 100 g/L, $MgSO_4.7H_2O$ (2.85 g/L), KH_2PO_4 and K_2HPO_4 (0.5 g/L), $MnSO_4.H_2O$ (0.016 g/L), $CaCO_3$ (20 g/L), $(NH_4)_2SO_4$ (46 g/L), urea (5 g/L), biotin (0.01 g/L) and L-leucine (0.4 g/L); the culture was prepared in 100 ml volumetric flasks. The pH was adjusted to 7.0 with 1 N NaOH and the medium was sterilized at 115°C for 10 min (18).

Activation and screening

The bacteria were activated by incubating at 30°C for 2 days in Nutrient Broth media. Then tri-sector cultivation was performed in Plate Count Agar media in order to isolate the bacteria.

Cultivation and lysine production

The inoculum was introduced in seed culture and was incubated in a rotary shaker at 120 rpm and 30 °C. After 18 hours, the seed culture was pipetted in a basic medium with the same condition at 180 rpm. Lysine accumulation was determined by sampling from the broth culture within 5 days and performing some experiments (18).

Selection of the best nitrogen source

In order to find the best nitrogen source for the microorganism growth and lysine accumulation; ammonium sulphate of basal medium was replaced with equimolar concentration of the various nitrogen sources such as ammonium chloride, ammonium dihydrogen phosphate, ammonium acetate and potassium nitrate whilst the carbon source did not vary and was considered constant as glucose.

Effect of different carbon sources

For this purpose, three carbon sources (molasses, pulpy waste date and glucose) were used as substrates in the basic culture whilst the nitrogen source (ammonium sulphate) was constant. Other processes were carried out as follows.

Qualitative assay

Ascending Thin Layer Chromatography was employed for the detection of L-lysine in the culture broth. The solvent systems used included n-butanol: acetic acid: water (1:2:4, v/v). The spots were visualized by spraying with a solution of 0.5% ninhydrin in butanol (4).

Quantitative estimation

Quantitative estimation of L-lysine in the supernatant fluid was determined by the same method mentioned above. Ascending thin layer chromatography was performed in plates with a diameter higher than the qualitative method in order to accumulate lysine

preparatively. After isolation of lysine from the other components of media on plates, silica gel was scraped from plates and diluted with water. In order to separate the micro-organisms from the silica gel, centrifugation was done at 2000 rpm for 15 min. The supernatant containing soluble L-lysine was lyophilized using SPEDI-VAC-GAUGE freeze drier. The lysine produced was obtained in brown crystalline powder after one day (4).

Statistical analysis

All experiments were carried out in triplicates. The data were analyzed using the ANOVA procedure of SPSS, Version 13. Means were compared by Duncan's multiple range tests (DMRT, $p< 0.05$).

RESULTS AND DISCUSSION

Medium composition is a very important factor strongly influencing fermentation processes, often being the object of extensive process development and optimization studies. The culture medium must satisfy the requirements of microbial growth and production in a suitable manner.

Lysine producing bacteria need ample supply of a suitable nitrogen source, as this amino acid contains about 19.16% nitrogen (15). For this purpose the same concentrations of different nitrogen sources were studied. As shown in Fig.1, maximum L-lysine (22 g/L) was accumulated when ammonium sulphate was used. This result was similar to the finding of Ekwealor and Obeta (4) who reported that ammonium sulphate was the best nitrogen source for microorganism growth and lysine accumulation. They stated that lysine production is a function of nitrogen concentration up to 4.0%, beyond which the accumulation of lysine decreased. This decrease may be attributed to osmotic pressure exerted by high nitrogen concentration which may have adversely affected the organism's growth and lysine accumulation (4). Thus, ammonium sulphate with a concentration of 4.0% was selected for the latter stages.

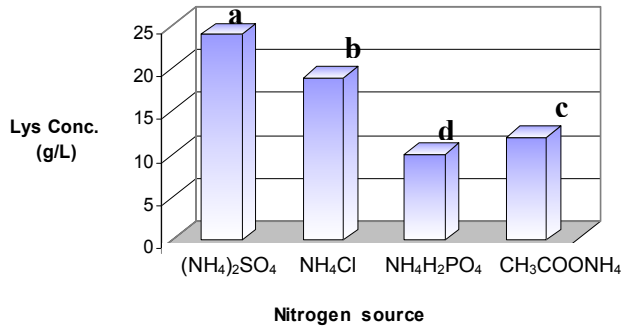

Figure 1. Comparison of lysine production yield from different nitrogen sources while glucose was used as a constant carbon source

Corynebacterium and related microorganisms can be used for inexpensive production of amino acids from cheap renewable carbon sources by direct fermentation (1 and 12). Therefore, in addition to glucose, molasses and pulpy waste date were used

as the carbon sources in this study, and the production yield was compared in different time intervals (0-120 h). Shah (15) and Ferreira and Duarte (5), stated that the initial sugar concentration of 10% gave the highest production yield; while over 10%, the cell mass and L-lysine yield decreased (15 and 5). This was related to the sensitivity of bacteria to osmotic pressure of the medium and for this reason the fermentation product accumulated in the medium was low (above 10% sugar). After clarification, waste molasses and pulpy waste date have been used for the production of L-lysine to make the fermentation process economically feasible. Molasses, pulpy waste date and glucose with a concentration of 10% were used. This was in accordance with the work of Nakamaya et al., (10) who also used molasses at 10% sugar for *C. glutamicum* (10).

Fig 2. shows the results of paper chromatography techniques of molasses and pulpy waste in comparison with each other in different time intervals (0-120 h). Spots in plates demonstrated that the production of lysine increased with increasing time in both substrates. It is also shown that the medium before fermentation (the first column in figure 2) contains little amounts of aspartic acid which decreased as the fermentation time increased. It seems likely that this decrease may be attributed to the consumption of aspartic acid by microorganisms for growth or its conversion to lysine (3 and 6). This figure also shows that the maximum production yield corresponded to the medium containing molasses within 96 hours, followed by glucose and pulpy waste date.

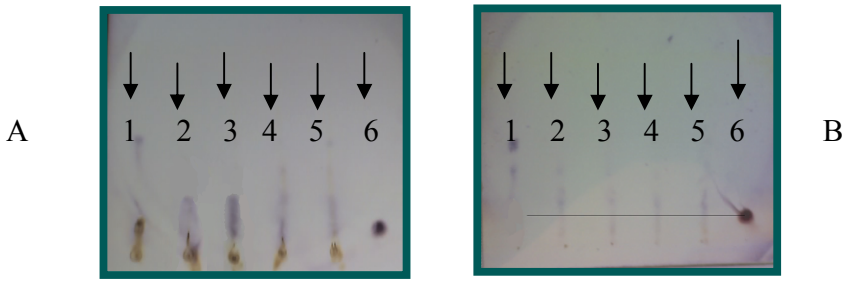

Figure 2. Comparison of lysine production from different carbon sources (A: molasses and B: pulpy waste date) in different time intervals (0-120 h); 1) blank medium without microorganisms growth; 2) Lysine production after 120 hours in medium containing each substrate; 3) Lysine production after 96 hours in medium containing each substrate; 4) Lysine production after 72 hours in medium containing each substrate; 5) Lysine production after 48 hours in medium containing each substrate; 6) 0.5% lysine standard solution

The amount of lysine produced from molasses and pulpy waste in comparison with glucose is shown in Fig. 3 and Table 1. The maximum production yield for molasses obtained in 96 h, after which lysine reduction occurred, might be related to its consumption by bacteria. Nakamaya et al., (10) also used molasses (as 10% glucose) in a mineral salt medium, recording a yield of 39.5 g/L (10). From the two other substrates including pulpy waste date and glucose, maximum lysine production was obtained within 120 hours. Pulpy waste date has a relatively low content of monosaccharide, therefore, the production of L-lysine was found to be 15.3 to 45.7 g/L in the fermentation broth within 5 days. For glucose, a maximum L-lysine production was reached after 5 days which was similar to the study by Nasri et al., (11). However, Plachy and Ulbert (1985) showed the maximum production of L-lysine (45 g/L) to be after the 4^{th} day in a 20 L fermentor by the mutant strain of *C. glutamicum* (13).

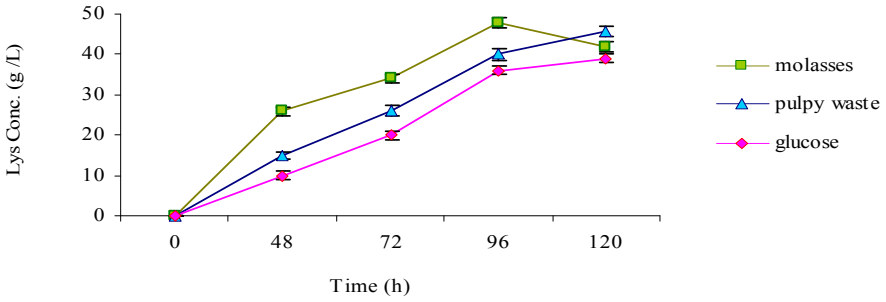

Figure 3. Lysine concentration values versus time using different carbon sources

The maximum weight of lysine produced from molasses, pulpy waste date and glucose within two days were 48 g/L, 45.7 g/L and 38.8 g/L, acquired within 96, 120 and 120 hours, respectively.

Table 1. Lysine production using different carbon sources at various incubation times*

Substrate	Lysine concentration (g/L) at different incubation times (h)[a-e]				
	0	48	72	96	120
Molasses	0.0 [e]	26.0 [d]	34.0 [c]	48.0 [a]	42.0 [b]
Pulpy waste date	0.0 [e]	15.3 [d]	26.0 [c]	40.4 [b]	45.7 [a]
Glucose	0.0 [e]	10.0 [d]	22.2 [c]	36.0 [a]	38.8 [b]

[a-e] **In each row different superscript letters indicate significant differences (p<0.05)**
[*] **Each point is the average of three replicates**

CONCLUSIONS

The fermentative method has the important advantage of yielding the optically active L-form of lysine directly. The demand for L-lysine in food, animal feeds and pharmaceutical industry is still increasing. To meet this demand, the amino acid industry is attempting to improve the production technology by reducing costs and utilizing unusual resources. Taken together, the results of this study indicated that ammonium sulphate with a 4.0% concentration was the best nitrogen source for microorganism growth and lysine accumulation. Moreover, molasses was the best substrate for lysine production, and in comparison with other carbon sources had the highest yield in the minimum fermentation time. Pulpy waste date can also be introduced as a good substrate since it produces a high amount of lysine within 120 h which is nearly equal to that of molasses.

REFERENCES

1. Anastassiadis, S. 2007. L-lysine fermentation. Resent Patents Biotechnol. 1**:** 11-24.

2. Buzzini, P. and A. Martini. 1999. Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. Bioresource Technol. 71**:** 41-44.

3. Coello, N., L. Brito and M. Nonus. 2000. Biosynthesis of L-lysine by *Corynebacterium glutamicum* grown on fish silage. Bioresource Technol. 73**:** 221-225.

4. Ekwealor, A. and A. N. Obeta. 2005. Studies on lysine production by *Bacillus megaterium.* African J. Biotechnol. 4**:** 633-638.

5. Ferreira, M. C. and J. Duarte. 1991. Glucose utilization by lysine-producing fluoroacetate-sensitive mutants of *Corynebacterium glutamicum.* Appl. Biochem. Biotechnol. 27**:** 251-257.

6. Haleem shah, A., A. Hameed, and G. Majidkhan. 2002. Fermentative production of L-lysine: bacterial fermentation-I. J. Med. Sci. 2**:** 52-157.

7. Hermann, T. 2003. Industrial production of amino acids by coryneform bacteria. J. Biotechnol. 104 **:** 155–172.

8. Ikeda, M. 2003. Amino acid production process. *Adv.* Biochem. Eng. Biotechnol. 79**:** 1–35.

9. Kircher, M., and W. Pfeerle. 2001. The fermentative production of L-lysine as an animal feed additive. Chemosphere. 43: 27-31.

10. Nakayama, K. J. A. and K. J. A. Araki. 1973. Process for producing L-lysine: US3708395.

11. Nasri, M. et al. 1989. Production of lysine by immobilized living Corynebacterium sp cells. Biotechnol. Lett. 11**:** 865-870.

12. Nelofer, R., Q. Syed, and M. Nadeem. 2008. Statistical Optimization of Process Variables for L-lysine Production by *Corynebacterium glutamicum.* Turk. J. Biochem. 33 (2)**:** 50–57.

13. Plachy, J., S. Ulbert, J. Pelechova and V. Krumphanzl. 1985. Fermentation production of L-homoserine by *Corynebacterium* sp. and its possible use in the preparation of threonine and lysine. Fol. Microbiol. 30:485 - 492.

14. Seibold, G., M., Auchter, S. Berens, J. Kalinowski and B. J. Eikmanns. 2006. Utilization of soluble starch by a recombinant *Corynebacterium glutamicum* strain: growth and lysine production. J. Biotechnol. 124: 381–391.

15. Shah, A. H. 1998. Studies on the Microbial Production of Lysine. Ph.D thesis. Quaid-i-Azam University, Islamabad.

16. Tosaka, O. 1983. The Production of L-Lysine by Fermentation. Trends in Biotechnol. 1: 70-74.

17. Tryfona, T., and M. T. Busturd. 2005. Fermentative production of lysine by *Corynebacterium glutamicum*: transmembrane transport and metabolic flux analysis. Process biochem. 40: 499 -508.

18. Weuster-Botz, D., R. Kelle, M. Frantzen and C. Wandrey. 1997. Substrate controlled fed-batch production of L-lysine with *Corynebacterium glutamicum*. Biotechnol. Prog. 13: 387-393.

تولید تخمیری لیزین به وسیله کورینه باکتریوم گلوتامیکوم از منابع کربنی مختلف

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چکیده– در این پژوهش تولید لیزین توسط کورینه باکتریوم گلوتامیکوم، بر روی منابع کربنی مختلف از ضایعات کشاورزی (شامل ملاس و پالپ خرما) در مقایسه با گلوکز بررسی شد. برای این منظور در ابتدا پس از بررسی اثر منابع نیتروژنی مختلف، سولفات آمونیوم به عنوان بهترین منبع نیتروژنی انتخاب گردید. ارزیابی کمی و کیفی لیزین تولیدی توسط روش کروماتوگرافی لایه نازک بالا رونده انجام شد. نتایج آزمایش های تخمیر نشان داد که بالاترین بازده تولید مربوط به ملاس به میزان ٤٨ گرم بر لیتر در طی ٩٦ ساعت تخمیر بود. برای دیگر سوبستراها بازده تولید کمتر و زمان تخمیر طولانی تر از ملاس بود.

واژه های کلیدی: لایسین، ملاس، پالپ ضایعات خرما، تخمیر، کورینه باکتریوم گلوتامیکوم

* به ترتیب استادیار و دانشجویان پیشین کارشناسی ارشد

** مکاتبه کننده