Pakistan J. Zool., vol. 41(4), pp. 261-267, 2009.

Optimization of Fermentation Conditions for Enhanced Glutamic Acid Production by a Strain of *Corynebacterium glutamicum* NIAB BNS-14

Bushra Niaz, Shahid Nadeem, H. Muhammad Muzammil, Junaid A. Khan and Tahir Zahoor
BCD, NIAB, P.O. Box 128, Faisalabad (BN, SN, HMZ, JAK), Institute of Food Science & Technology, University of Agriculture, Faisalabad (TZ)

Abstract.- *Corynebacterium glutamicum* NIAB BNS-14 was used for enhancing glutamic acid production for which eight different carbon (glucose, fructose, sucrose, maltose, lactose, dextrin, sorbose, and galactose) and seven nitrogen sources (ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, urea, potassium nitrate and sodium nitrate) alongwith various concentrations of biotin (0.5, 1.0, 2.5, 5.0, 10, 15µg/ml) were used. The strain gave maximum production as 8.5g/l of glutamic acid at 10% fructose and 3% ammonium nitrate with 10µg/ml biotin at 30°C and 150rpm after 72hrs of fermentation. The cell mass obtained was 21.1g/l.

Key words: *Corynebacterium glutamicum,* glutamic acid, optimization of fermentation conditions.

INTRODUCTION

Glutamic acid is used by the body to build proteins. In addition, it is the most common excitatory (stimulating) neurotransmitter in the central nervous system as well as may impose protective effects on the heart muscles in heart patients. Intravenous injections of glutamic acid (as monosodium glutamate) have been observed to boost up exercise tolerance and heart function in people suffering from stable angina pectoris (Thomassen *et al.,* 1991; Zello *et al.,* 1995). However, the fundamental role of glutamic acid in the stabilization of the active conformation of the human androgen receptor and in androgen and antiandrogen activities has very well been demonstrated by Georget *et al.* (2006).

Several strains of *Corynebacterium glutamicum, C. lilium, C. callunae, C. herculis, Brevibacterium flavum, B. lactofermentum, B. divaricatum, B. ammoniagenes, B. thiogenetalis* and *Microbacterium ammoniaphilum* are considered among the potent glutamic acid-producing strains (Abe and Takayama, 1972; Kikuchi and Nakao, 1986). However, *Corynebacterium* and *Brevibacterium* are being used and exploited as the cost effective bioconverters by the fermentation industry in this context (Aida, 1986). Presently, almost the entire bulk of glutamic acid marketed is being produced through fermentation (Sunitha *et al.,* 1998). A research programme was initiated to determined and enhance the potentiality of *C. glutamicum*, NIAB BNS-14, for glutamic acid production through fermentation. The present study mainly emphasizes upon the selection and optimization of suitable carbon and nitrogen sources as well as the effect of biotin upon growth and production of glutamic acid by *C. glutamicum* NIAB BNS-14.

0030-9923/2009/0004-0261 $ 8.00/0

MATERIALS AND METHODS

Isolation and characterization of bacteria

Soil and water samples were collected from the vicinity of NIAB, Faisalabad, and processed for screening of bacteria capable of producing glutamic acid. Nutrient agar was used for isolation and propagation of the isolates (Ahmad and Nadeem, 1993).

In order to isolate coryneforms from the bulk, the colonies which were smooth, entire, circular, dull to slightly glistening and pale yellow to yellow in colour on nutrient agar were selected and biochemically tested for different sugars (glucose, fructose, maltose, sucrose, dextrin, sorbose) and methyl red (Cruickshank *et al.,* 1975). Fresh peptone-water, 5ml, mixed with 0.5ml of 10% sugar solution and methyl red as indicator, was taken in 50x7mm Durham tubes (inverted position), in 150x12.5mm test tubes plugged with cotton wool. A loopful of the desired culture was inoculated in this medium and incubated at 37°C for 24-48hrs

depending upon the mode of growth of the organism. Gas production or change in the colour of the medium was observed and on the basis of these results, the strains of *C. glutamicum* were selected and processed further.

Experimental

Experiments were conducted to find out the potential of the strain to utilize different carbon (glucose, fructose, sucrose, maltose, lactose, dextrin, sorbose, and galactose) and nitrogen (ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, urea, potassium nitrate and sodium nitrate) sources for its growth and for the production of glutamic acid. A glucose-based fermentation medium (Costa-Ferreia and Duarte, 1992) with slight modification (designated as L-6) was taken as 50ml per 250ml Erlenmeyer flasks**.** In addition, sterile solution of biotin with different concentrations (0.5, 1.0, 2.5, 5.0, 10, 15µg/ml) was also aseptically added; a control with no biotin was, too, operated under the same set of fermentation conditions for comparison. The freshly grown culture was inoculated in the medium and incubated in rotary shaker at 30°C and 150rpm for a maximum of 96hrs. The fermentation broth was monitored at regular intervals whilst the broth was centrifuged to make it cell-free and the supernatant was filtered through Millipore filter (0.45µm) for complete removal of cells and other residues. The qualitative analysis of glutamic acid produced in the fermentation broth was done by paper chromatography followed by paper electrophoresis (Nadeem *et al.,* 2004). The quantitative estimation was done after Nadeem *et al.* (2001) and Yaqoob *et al.* (1999).

Growth curve

In order to work out the optimum growth stage for the strain under study and co-relate it with the product formation, growth curve of the cells was drawn on the basis of dry weight percentage of the fresh cells. The culture was crumbed from a freshly prepared slant (40hr) of *C. glutamicum* and suspended it into 10ml of sterile 0.01M citrate buffer of pH 7.0. It was well shaken to segregate the clumps and for proper mixing. Serial dilutions were made. The population density was determined after plating out 1ml of the final dilution; the count was adjusted to 10^7- 10^8cells/ml (Sunitha *et al.,* 1998). A 5% (v/v) of the cell suspension was inoculated in 50ml glucose-yeast extract medium (glucose 0.02g, yeast extract 0.3g in 100ml distilled water, pH adjusted to 7.0 and autoclaved), in 250ml Erlenmeyer flask and incubated at 30^{o}C and 150rpm. The samples were monitored on hourly basis, harvest pH was noted, and the broth was centrifuged at 10,000 x g to get the pellet. Well sterilized petri plates, alongwith their lids, were weighed (Weight A) and the pellets were placed in them. The Petri plates, including the lids as well as the fresh pallets, were weighed again (Weight B) and put into an oven at 70^{o}C for at least two days. Thereafter, the Petri dishes, including the dried cells and the lids, were weighed for the 3rd time (Weight C). From the three weights (A, B, C), the dry weight percentage was calculated as: (C-A) / (B-A) x 100.

RESULTS AND DISCUSSION

Bacterial isolation

In the present study, 55 bacterial isolates were obtained from local natural habitats. Among them, 37 were isolated from soil whereas 18 were obtained from water. All of the isolates were tested for the production of amino acids. However, the main emphasis was on glutamic acid. An isolate, NIAB BNS-14 producing 2.5g/l of glutamic acid, was selected for further study. As a whole, 19 isolates produced glutamic acid varying in quantity with a production percentage of 34.55. Alanine was found to be the most frequently produced amino acid in the fermentation broth.

Bacterial identification

Since *C. glutamicum* is widely used for the large-scale fermentative production of many kinds of amino acids, particularly glutamic acid and lysine (Nishio *et al.,* 2004), more attention was paid to isolate this very bacteria in the present study. The isolates, for the identification of *C. glutamicum,* were tested against a number of sugars and it was observed that: 13 isolates were glucose positive (+), 4 lactose positive (+), 2 xylose positive (+), 3 sorbose positive (+), 7 maltose positive (+), 13 sucrose positive (+), 9 dextrin positive (+), 7 starch

positive (+), 6 galactose (+) and 13 isolates were found to be fructose positive (+). On the basis of these results it was concluded that 4 of the tested isolates were *C. glutamicum* (data not shown).

Selection of carbon source

Among various carbon sources studied, the best results were shown by fructose producing upto 3.7g/l glutamic acid in the fermentation broth. It was followed by maltose and glucose, which produced 2.7 and 2.5 g/l of glutamic acid, respectively (Fig.1). The best glutamic acid production (3.7g/l) appeared at 10% fructose, which gradually decreased with the increase in concentration as 2.4g/l at 12% and 2.2g/l at 16% (Fig. 2).

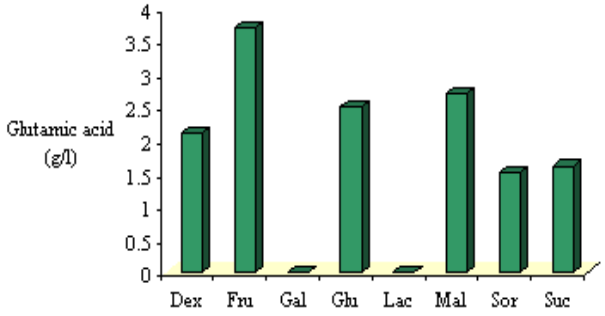

Fig. 1. Effect of different carbon sources on the production of glutamic acid by *Corynebacterium glutamicum* NIAB BNS-14; Dex, dextrin; Fru, fructose; Gal, galactose; Glu, glucose; Lac, lactose; Mal, maltose; Sor, sorbose, Suc, sucrose.

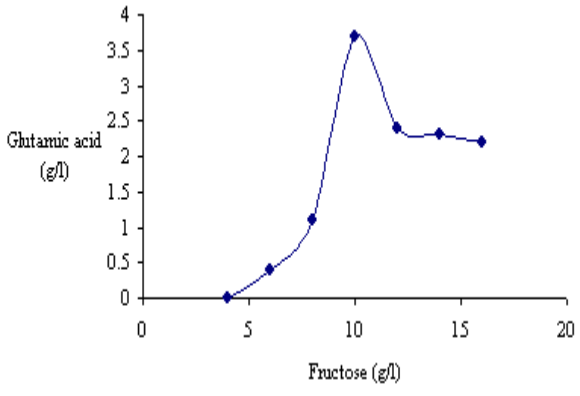

Fig. 2. Effect of different concentrations of fructose upon glutamic acid production by *Corynebacterium glutamicum* NIAB BNS-14.

According to Stanier *et al*. (1987) and Veldkamp (1970) the nature of carbon and energy source plays a vital role for the fermentative organisms. According to them sugars are often preferred but other organic compounds of similar oxidation level are also suitable. In this study different carbon sources were tested for their influence upon the performance of bacterial strains regarding their growth and glutamic acid production. It was clearly observed that performance of the organism changed with the change in carbon source as well as its concentration. It was also found that there was a direct correlation between population density of the cells and the glutamic acid produced by them. *i.e*. the amino acid production increased with the increase in cell number.

Selection of nitrogen source

Nitrogen sources have been observed to influence the mode of fermentation process. For this purpose, difference N sources with different concentrations were investigated. The graph of glutamic acid production by *C. glutamicum* NIAB BNS-14 with nitrogen sources tested (mentioned above) varied. Though, this variation remained within a narrow range, 4.5-5.5g/l, the peak production time fairly assorted. Moreover, concentration of the source also affected the production potential of the strain. The maximum production of glutamic acid, 5.5g/l, was obtained with 3% ammonium nitrate followed by 4.9g/l with 3% ammonium phosphate in the fermentation broth (Fig. 3); in both the cases the peak production appeared after 96hrs of incubation. As a whole a nearly two-fold increase was observed in glutamic production by changing nitrogen source and adjusting its concentration in the medium (Fig. 4).

The capability of breaking down organic nitrogen sources, such as amino acids and ammonium ions NH_4^+, into smaller units is an important factor regarding the media formulation. Bacteria have the natural ability to utilize nitrogen from NH_4^+. The NH_4^+ is an ideal source of nitrogen in many fermentations, where it is utilized as $(NH_4)_2SO_4$ or NH_4Cl (Dunn, 1985). Ammonium salts often introduce an acidic environment as NH_4^+ is consumed liberating the free acid. Several

bacteria can exclusively utilize ammonia as the nitrogen source, whereas some others draw it out from nitrates through a mechanism, illustrated by Brown *et al*. (1974**),** where an enzyme nitrate reductase, converting nitrates to NH_4^+, is repressed in the presence of ammonia. Because of this reason ammonia or NH_4^+ is supposed to be an ideal nitrogen source. This preference has also been confirmed in the present study, where NH_4^+ produced better results than the Na and K sources. Similar studies were also conducted by Ghosh and Sen (1996), who stressed upon the ample amount of suitable nitrogen source for glutamic acid fermentation. Roy and Chatterjee (1989) also worked on the same lines and reported ammonium chloride as the best suitable N source. Conclusively, ammonium salts have been reported suitable for glutamate production and the results of present study are quite in-line with these findings.

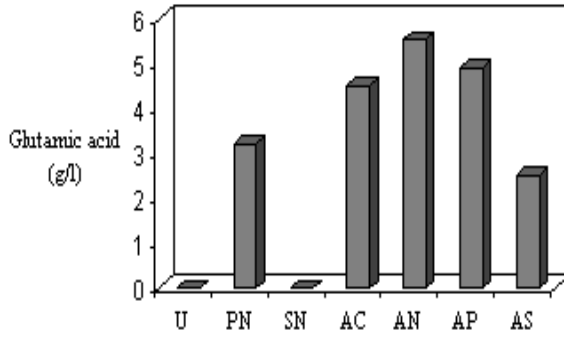

Fig. 3. Effect of different nitrogen sources on glutamic acid production by *Corynebacterium glutamicum* NIAB BNS 14; U, urea; PN, potassium nitrate; SN, sodium nitrate; AC, ammonium chloride, AN, ammonium nitrate; AP, ammonium phosphate; AS, ammonium sulphate.

Influence of biotin

In the present study, the maximum excretion of glutamic acid by *C. glutamicum* NIAB BNS-14 was observed at 10µg/ml biotin concentration. Glutamic acid in the broth started appearing after almost 24hr of incubation and gradually increased upto 72 hr, where an amount of 4.2g/l was observed (Table I).

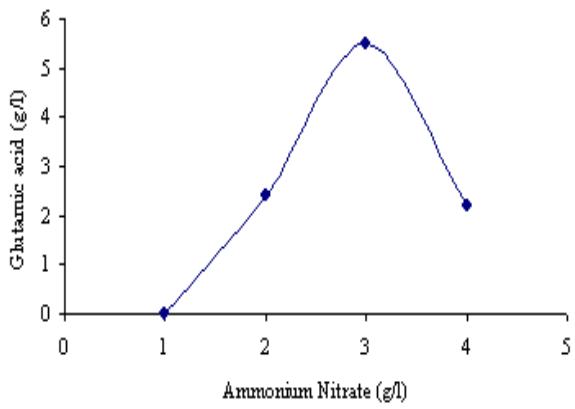

Fig. 4. Effect of different concentrations of ammonium nitrate upon glutamic acid production by *Corynebacterium glutamicum* NIAB BNS-14

Table I.- Effect of different concentrations of biotin on glutamic acid (Glu) production by *Corynebacterium glutamicum* NIAB BNS-14

Concentration (µg/ml)	Sampling time (Hrs.)	Harvest pH	Glu (g/l)
0.5	24	7.1	-
	48	6.5	-
	72	6.8	-
	96	7.0	-
1.0	24	7.6	-
	48	6.4	-
	72	6.9	-
	96	7.1	-
2.5	24	7.2	-
	48	6.2	-
	72	6.8	-
	96	6.9	-
5.0	24	7.3	-
	48	6.4	-
	72	6.7	0.7
	96	7.0	0.5
10.0	24	7.0	0.5
	48	6.3	1.3
	72	6.5	4.2
	96	7.0	2.2
15.0	24	7.1	-
	48	6.2	0.3
	72	6.6	1.3
	96	7.2	0.9
Control	24	7.2	-

Table II.- Glutamic acid produced by *Corynebacterium glutamicum* NIAB BNS-14 under optimized conditions.

Strain	Fermentation time (Hrs.)	Harvest pH	Average glutamic acid production (g/l)	Other amino acids produced	Dry cell mass (g/l)
NIAB BNS-14	24	5.6	0.5	Lysine, Alanine	10.7
	48	6.6	4.8	Lysine, Alanine	15.3
	72	6.6	8.5	Lysine, Alanine	21.1
	96	6.8	3.2	Lysine, Alanine	17.3
Control	24	7.4	1.6	Lysine, Alanine	11.5
	48	7.5	2.1	Lysine, Alanine	12.9
	72	7.4	4.1	Lysine, Alanine	17.8
	96	7.4	2.0	Lysine, Alanine	13.6

It is broadly accepted that low levels of biotin are required for extracellular accumulation of glutamic acid by *C. glutamicum*. Though, the real mechanism behind it is still unknown, it is considered that change in membrane permeability might be the main cause (Kinoshita, 1999). A lot of effort has been made in this context (Shiio *et al.*, 1963; Philips and Somerson, 1963). One of the basic reasons that permit bacteria to excrete out glutamic acid is the nutritional requirement for biotin (Nakayama, 1987). Tanaka *et al.* (1960) claimed that reasonable amounts of glutamic acid are accumulated in the fermentation broth with limited supply of biotin. Demain and Birnbaum (1968) were also of the same view. Kikuchi *et al.*, (1973) related this excretion to the decrease in phospholipid content of cell membrane. According to them, the decrease in phospholipid content most likely introduces changes in membrane structure relaxing enough the permeability barrier accommodating an increase in the excretion of glutamate from the cells. Simultaneously, excessive biotin in the medium is also not helpful as in such a case the fermented glutamic acid does not permeate out of the cell membrane and accumulates intracellularly (Kinoshita, 1985).

The most appropriate biotin concentration in the fermentation medium has always been a point of profound interest and quite a bulk of efforts in this regard has been attributed to many workers, who have stressed upon the "sub-optimal" concentration of biotin for maximum excretion of glutamic acid from the cell (Kinoshita, 1985, 1999; Nakayama, 1987; Hirose *et al.*, 1985). The logic behind is that excessive biotin supply instigates the optimal cell growth resulting into high lactate production, whereas glutamic acid is best excreted under sub-optimal growth level.

Some additional factors, such as the strain, kind and concentration of the carbon source, should also be kept in mind while working out the most suitable biotin concentration. So, it can be inferred that biotin requirement may vary from strain to strain but the basic principle of using "sub-optimal" concentration, however, remains the same. The findings of Kinoshita (1999), Ghosh and Sen (1996), Nakayama (1987) and Hirose *et al.* (1985) confirm this statement as the suitable biotin concentrations reported by them (2.5-5.0 μg/ml, 0.001μg/ml, 5μg/l, 0.5μg g^{-1} of dry cells, respectively) also vary. In the present study, where several different concentrations were employed the maximum excretion of glutamic acid was observed at 10μg/ml, neither below nor above to this level favoured glutamic acid production. A decrease in the excretion level of glutamic acid from the cells was noticed with further increase in biotin concentration in the fermentation broth (Table I).

Finally, when grown under the optimised conditions the overall glutamic acid production by the strain increased upto 8.5g/l with 21.1g/l dry cell weight after 72hr of fermentation at 30°C and 150rpm (Table II).

CONCLUSION

The study shows that comparatively better yields of glutamic acid can be obtained from *C. glutamicum*. It was also confirmed that optimization

and adjustment of different nutritional ingredients play a vital, and often positive, role in glutamic acid fermentation resulting an increase in production as well as cell mass. Maximum production of glutamic acid was obtained when different carbon and nitrogen sources with a limited supply of biotin were used.

REFERENCES

ABE, S. AND TAKAYMA, K., 1972. Amino acids producing microorganisms, variety and classification. In: *The microbial production of amino acids* (eds. K. Yamada, S. Kinoshita, T. Tsunoda and K. Aida), vol. 24, pp: 3-38. Kodanash Ltd., Tokyo.

AHMAD, M.S. AND NADEEM, S., 1993. Screening of bacterial isolates for amino acid fermentation. *The Nucleus*, **30**: 45-49.

AIDA, K., 1986. An overview of the microbial production of amino acids. In: *Biotechnology of amino acid production* (eds. K. Aida, I. Chibata, K. Nakayama, K. Takinami and Yamada, H.), vol. 24, Kodansha Ltd., Tokyo.

BROWN, C.M., MACDONALD, D.S. AND MEERS, J.F., 1974. Physiological aspects of microbial inorganic metabolism. *Advan. Microb. Physiol*., **11**: 1-52.

COSTA-FERREIRA, M. AND DUARTE, J.C., 1992. Amino acid accumulation by an analogue-sensitive mutant of *Corynebacterium glutamicum. Biotechnol. Lett.,* **14**: 1025-1028.

CRUICKSHANK, R., DUGUID, J.P., MARMION, B.P. AND SWAIN, R.H.A**.,** 1975. *Medical microbiology*, 12th edn. (revised print), vol. II. Churchill Livingstone, Edinburgh.

DEMAIN, A.L. AND BIRNBAUM, J., 1968. Alteration of permeability for the release of metabolites from the microbial cell. *Curr. Top. Microbiol. Immunol*., **46**: 1-25.

DUNN, G.M., 1985. Nutritional requirements of microorganisms. In: *Comprehensive biotechnology*, (eds. M. Moo-Young, A.T. Bull and H. Dalton), Vol. 1, pp. 113-126. Pergamon Press, New York, Sydney.

GEORGET, V., BOURGUET, W., LUMBROSO, S., MAKNI, S., SULTAN, C. AND NICOLAS, J.C., 2006. Glutamic acid 709 substitutions highlight the importance of the interaction between androgen receptor helices H3 and H12 for androgen and antiandrogen actions. *Mol. Endocrin*., **20**: 724-734.

GHOSH, R. AND SEN, S.K., 1996. Extracellular accumulation of glutamic acid by *Bacillus cereus* P11. *Adv. Fd. Sci*. (CMTL), **18**: 87-91.

HIROSE, Y., ENEI, H. AND SHIBAI, H., 1985. L-glutamic acid fermentation. In: *Comprehensive biotechnology* (ed. M. Moo-young), pp. 593-600. Pergamon Press, Oxford.

KIKUCHI, M. AND NAKAO, Y., 1986. Glutamic acid. In*: Biotechnology of amino acid production.* (eds. K. Aida, I. Chibata, K. Nakayama, K. Takinani, and H. Yamada), Kodansha, Tokyo, pp. 110-116.

KIKUCHI, M., KANAMARU, T. AND NAKAO, Y., 1973. Relation between the extracellular accumulation of L-glutamic acid and the excretion of phospholipids by penicillin-treated *Corynebacterium alkanolyticum*. *Agric. Biol. Chem*., **37**: 2405-2408.

KINOSHITA, S., 1985. Glutamic acid bacteria. In.: *Biology of industrial microorganisms.* (eds. A.L. Demain and N.A. Solomon), pp:115-142. Benjamin Cummings, London.

KINOSHITA, S., 1999. Glutamic acid producing microorganisms. In: *Encyclopedia of bioprocess technology: Fermentation, biocatalysis, and bioseparation.* (eds. M.C. Flickinger and S.W. Drew), John Wiley and Sons, Inc., pp. 1330-1336.

NADEEM, S., KHAN, S.H., NIAZ, B., MISHRA, A. AND KHAN, J.A., 2004. Production of L-lysine by a mutant of *Corynebacterium glutamicum*, NIAB SK-179, in cane molasses medium. *Biosci. Res*., **1**: 34-37.

NADEEM, S., IKRAM, A., RANA, S.M., YAQOOB, N., QURESHI, M.J. AND SHAKOORI, A.R., 2001. Enhanced L-lysine production by an *Escherichia coli* mutant WARN 30255 after MNNG treatment. *Int. J. Agric. Biol*., **3**: 448-450.

NAKAYAMA, K., 1987. Amino acids. In: *Prescott & Dunn's Industrial microbiology* (ed. G. Reed), 4th edn., pp. 748-801, CBS Publishers and Distributors, India.

NISHIO, Y., NAKAMURA, Y., USUDA, Y., SUGIMOTO, Y., MATSUI, K., KAWARABAYASI, Y., KIKUCHI, H., GOJOBORI, T. AND IKEO, K., 2004. Evolutionary process of amino acid biosynthesis in *Corynebacterium* at the whole genome level. *Mol. Biol. Evol*., **21**: 1683-1691.

PHILIPS, T. AND SOMERSON, N.L., 1963. *Microbial technology*, p. 313, Reinhold Pub. Corp., New York.

ROY, D.K. AND CHATTERJEE, S.P., 1989. Production of glutamate by *A. globoformis. Folia Microbiol.,* **34**: 11p.

SHIIO, I., OTSUKA, S. AND TAKINAMI, M., 1963. Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. *J. Biochem*., **51**: 56-62.

STANIER, R.Y., ADELBERG, E.A. AND INGRAHAM, J.L., 1987. Microbial growth. In: *General microbiology*, 4th edn. pp: 275-292; Macmillan, London.

SUNITHA, I., SUBBA RAO, M.V. AND AYYANNA, C., 1998. Optimization of medium constituents and fermentation conditions for the production of L-glutamic acid by the coimmobilized whole cells of *Micrococcus glutamicus* and *Pseudomonas reptilivora. Bioproc. Eng.*, **18**: 353-359.

TANAKA, K., IWASAKI, A. AND KINOSHITA, S., 1960. Studies on L-glutamic acid fermentation. Part V. Biotin and L-glutamic acid accumulation by bacteria. *J. Agric.*

Chem. Soc. Japan, **34:** 593-600.

THOMASSEN, A., NIELSEN, T.T. AND BAGGER, J.P., 1991. Antiischemic and metabolic effects of glutamate during pacing in patients with stable angina pectoris secondary to either coronary artery disease or syndrome X. *Am. J. Cardiol*., **68**: 291-295.

VELDKAMP, H., 1970. Enrichment cultures of prokaryotic organisms. In: *Methods in microbiology* (eds. J.R. Norris and D.W. Ribbons), vol. 6, pp: 303-361. Academic Press, Inc., New York.

YAQOOB, N., NADEEM, S. AND AHMAD, M.S., 1999. Improvement of *Escherichia coli* for lysine overproduction through mutagenesis. *The Nucleus*, **36**: 105-108.

ZELLO, G.A., WYKES, L.F. AND BALL, R.O., 1995. Recent advances in methods of assessing dietary amino acid requirement for adult humans. *J. Nutr*. **125**: 2907-2915.

(Received 16 January 2007, revised 19 April 2009)