ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Methionine Production by Coryneform Bacteria through Fermentation

Venkata Narayana A[1*], Vamsi Priya A[1], Venkata Nadh R[1], Swami AVN[2], Sumalatha B[3], and Vijaya Leela M[4]

[1] Department of Biotechnology, Vignan University, Vadlamudi, Guntur-522 213.
[2] Department of Chemical Engineering, JNTUA, Anantapur-515 002
[3] Department of Chemical Engineering, Vignan University, Vadlamudi, Guntur-522 213.
[4] Department of chemistry, P.N.C & Vijai Institute of Engineering & Technology, Repudi, Guntur- 522 529

ABSTRACT

Production of L-methionine by *Cornybacterium glutamicum* MTCC 2745 was assessed in batch fermentation by varying parameters viz., initial glucose concentration, initial nitrogen concentration, fermentation time, temperature, pH′ initial shaking rate. Nynhydrin test and paper chromatography were used to identify methionine. Methionine estimated by using nitroprusside method. Effect of nitrogen sources on methionine production was examined. The parameters such as concentrations of glucose, ammonium sulphate, K_2HPO_4, $MgSO_47H_2O$, 3,4-dihydroxy benzoic acid and Yeast extract are optimized and maximum yield was 5.6 g of methionine/L.

Keywords: Methionine production, *C.glutamicum*, Fermentation, Optimization

Corresponding author

ISSN: 0975-8585

INTRODUCTION

The history for the production of amino acids by *Corynebacterium* started in the 1950s when Dr Kinoshita was the first to discover that *C.glutamicum* is a superior amino acid producer [1,13], until this time amino acids were produced exclusively by hydrolysis of proteins or by chemical synthesis. These processes are very expensive. Chemical synthesis produces a mixture of D- and L-methionine [14,15], where as hydrolysis of proteins leads to a complex mixture from which methionine must be separated. Chemically produced racemic mixture of methionine isomers can be resolved using continuous flow immobilized enzyme bioreactors containing fungal aminoacylases [16]; nevertheless, the chemical production of the racemic mixture is undesirable as it requires hazardous chemicals such as acrolein, methyl mercaptan, ammonia and cyanide [17]. Although the existing enzymatic processes achieve good yields, they require expensive substrates. Fermentation processes have been able to inexpensively produce many other amino acids, there is a significant interest in developing a microbial process for commercial production of methionine [2,18,19]. In recent times, a lot of research efforts have been increased towards the production of amino acids by fermentation methods. Methionine is an essential amino acid that is required in the diet of humans and livestock. Plant proteins are frequently deficient in methionine and consequently an exclusive vegetable diet may fail to meet nutritional requirements [2]. Such deficiencies can only be overcome by an exogenous supply of the essential amino acid. Methionine deficiency has been linked to development of various diseases and physiological conditions including toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's liver deterioration and impaired growth [3]. Deficiencies can be overcome by supplementing the diet with methionine and, therefore, methionine is of significant interest [4]. The L-form of methionine is used extensively in human medicine for a variety of therapeutic purposes. Methionine extensively used in the poultry and feedstock industry [20-23].

Previously many researchers had been made efforts to commercialize fermentation process for the production of methionine, but nobody could get successful towards increasing methionine concentration (< 4.5 g of methionine/L). This present work was aimed to increase methionine concentration by *C. glutamicum* under optimal fermentation conditions of various parameters.

MATERIALS AND METHODS

Microorganism

C. glutamicum MTCC 2745 powder was obtained from the microbial type collection centre, Chandigarh, India. It was rejuvenated by culturing with nutrient agar slants and stored at refrigeration temperature $4^{O}C$.

Inoculum Preparation

Medium components were sterilized in an autoclave at 121^{o}C for 15 min. A full loop of 24 h slant culture (3% v/v) was transferred aseptically to a 250 mL Erlenmeyer flask containing seed medium (100 mL) with the following composition: Glucose, 40 g/L; Yeast extract, 1 g/L ;$(NH_4)_2SO_4$, 20 g/L; K_2HPO_4 ,2 g/L; $MgSO_4 7H_2O$, 0.25 g/L; 3,4-dihydroxy benzoic acid, 0.3 mg/L and made up volume to 1 litre with distilled water [24]. The pH was adjusted to 7.2 with 1N NaOH.

Fermentation Experiments

The composition of fermentation medium was: Glucose, 40 g/L; Yeast extract, 1 g/L ;$(NH_4)_2SO_4$,20 g/L; K_2HPO_4 ,2 g/L; $MgSO_4 7H_2O$, 0.25 g/L; 3,4-dihydroxy benzoic acid, 0.3 mg/L as diluents added to 1 litre distilled water. The pH was adjusted to 7.2 with 1N NaOH. These medium components were sterilized in an autoclave at 121^{o}C for 15 min. Medium (50 mL) transferred to 250 mL Erlenmeyer flask, then inoculated and incubated on an orbital rotary shaker (130 rev/min) at 30^{o}C for 48 hours.

Analytical Methods

Biomass estimation

After 48 hours, the biomass taken from shake flask experiments was centrifuged at 12000 rev/min for 20 min to bring into pellet form. The biomass was washed twice with sterile distilled water and dried at 65^{o}C and weighed.

Methionine Identification

After cell disruption, Ninhydrin test carried out for 1 mL supernatant fluid, there blue colour was observed. Thereafter, methionine identified by using paper chromatography where R_f value of the ninhydrin-positive spot (bluish-violet) of the supernatant that corresponded with the R_f value of the standard methionine solution was taken to indicate presence of methionine in the broth culture. R_f value obtained in paper chromatography was 0.9 cm which is corroborates with R_f = 0.892 results obtained [25].

Methionine estimation

For the weighed biomass, the same amount of distilled water was added in order to get cell suspension. The cell suspension was disrupted by ultrasonicator (sidulu ultrasonics, sonicator-80, 230 V, 80 KHz), the disruption time was about 15-20 min. The Supernatant fluid containing methioinine was estimated by using nitroprusside method [5].

ISSN: 0975-8585

RESULTS AND DISCUSSION

Effect of Fermentation Time

Methionine concentration increases with fermentation time as shown in Fig 1. Thus, 44 h was chosen as the optimum fermentation time.

Effect of glucose concentration

The effect of varying concentrations (30, 50, 70, 100, 120, 150 g/l) of glucose on methionine production by *C. glutamicum* was examined. In medium keeping all other variables were at constant, varying glucose concentration at desired levels. The preculture was inoculated into shake flask at desired glucose levels. These were incubated for 48 hrs on a orbital shaker. Results showed that methionine production was a function of the initial glucose concentration in fermentation medium as shown in Fig 2. The highest methionine yield (4.6 g/L) was obtained from the fermentation medium containing 100 g/L glucose, beyond which methionine concentration decreased. The decrease was attributed to high substrate concentration exhibited by glucose.

Effect of initial nitrogen concentration

The effect of varying concentrations of ammonium sulphate (20, 40, 60, 80, 100 g/L) on methionine production by *C.glutamicum* was examined. In medium keeping all other variables were at constant, varying nitrogen concentration at desired levels. The preculture was inoculated into shake flask at desired glucose levels. These were incubated for 48 hrs on a rotary shaker, methionine was estimated by using nitroprusside method. Results showed that methionine production was a function of initial nitrogen concentration up to 60 g/L, beyond which methionine production decreased as shown in Fig 3. Nitrogen concentration at 60 g/L, gave the optimum production of methionine. The decrease was attributed due to osmotic pressure exerted by high nitrogen concentration on growth and production of methionine [2].

Effect of varying pH on methionine production

The effect of varying pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) on methionine production by *C.glutamicum* was examined. These p^H values were adjusted with HCl, NaOH by using p^H digital meter. The preculture was inoculated into shake flask at desired p^H levels. These were incubated for 48 hrs on an orbital shaker. Results showed that methionine production was a function of p^H as shown in Fig 4. The optimum p^H value observed as 7 under which methioine production obtained as 4.3 g/L.

Effect of Temperature on methionine production

The effect of varying temperature (26^0C, 28^0C, 30^0C, 32^0C & 34^0C) on methionine production was examined. By varying temperature at desired levels and keeping all other

ISSN: 0975-8585

variables were at constant. The preculture was inoculated into shake flask at desired temperature levels. These were incubated for 48 hrs on an orbital shaker. The optimum temperature value observed as 30^0C, beyond which methionine production decreased as shown in Fig 5.

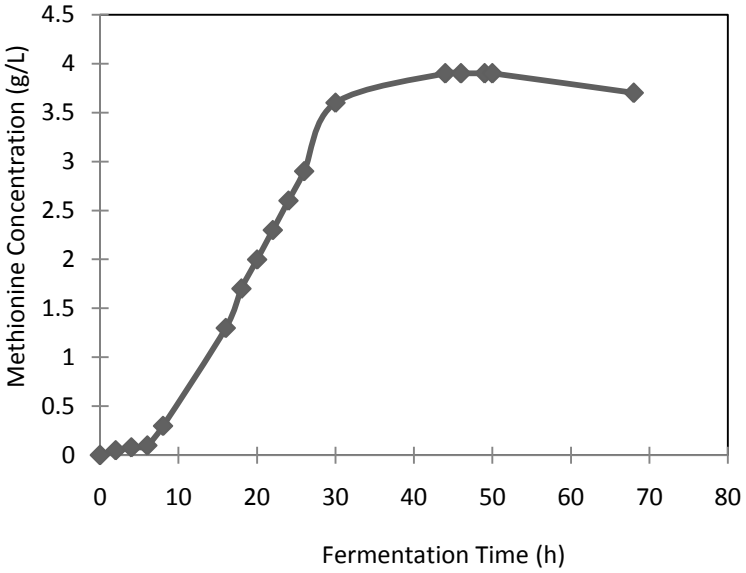

Figure 1: Effect of fermentation time on methionine production with initial glucose concentration 50 g/L.

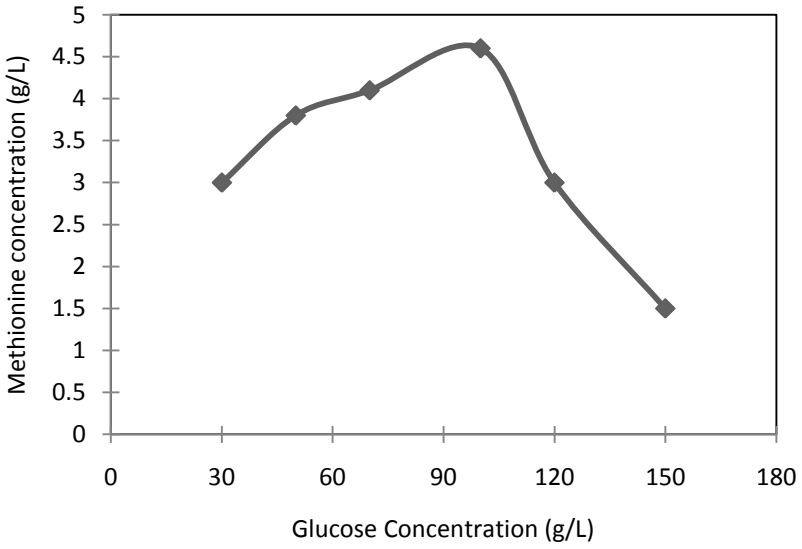

Fig 2: Effect of initial glucose concentration on methionine production

ISSN: 0975-8585

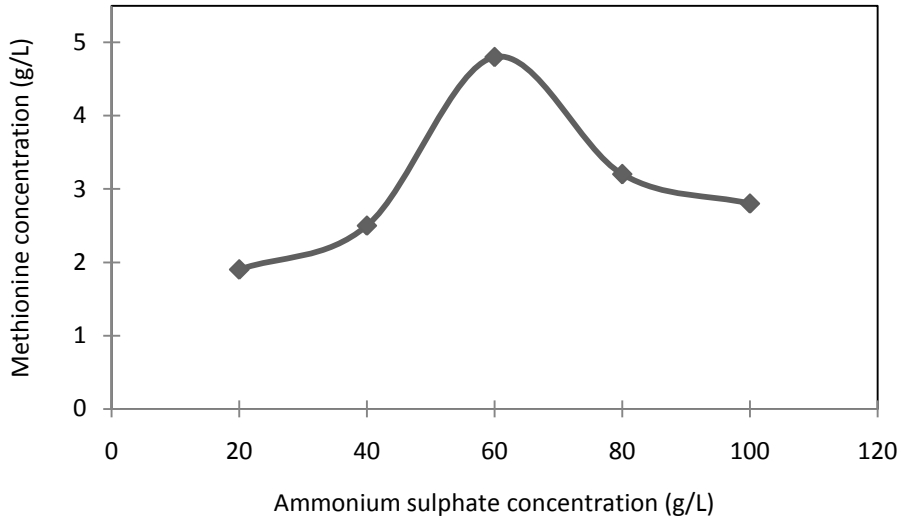

Fig 3: Effect of initial nitrogen concentration on methionine production with initial glucose concentration 50 g/L

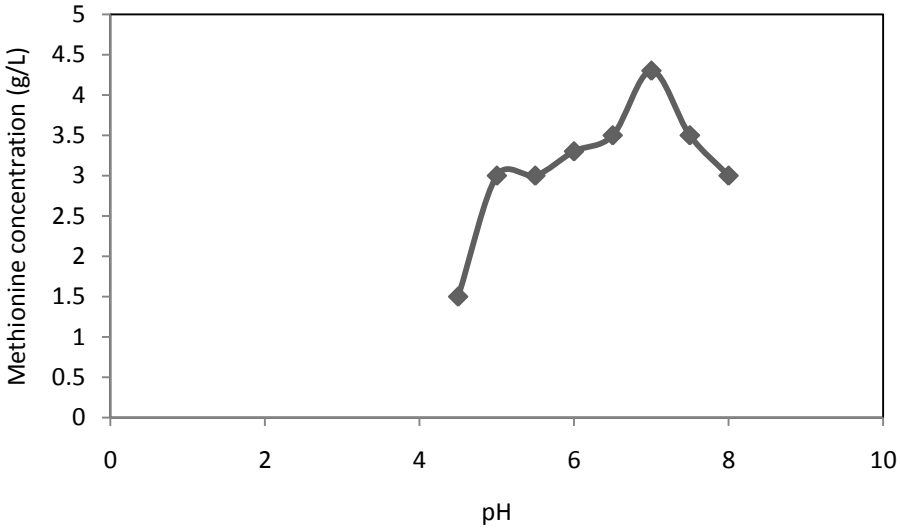

Fig 4: Effect of p^H on methionine production with initial glucose concentration 50 g/L

ISSN: 0975-8585

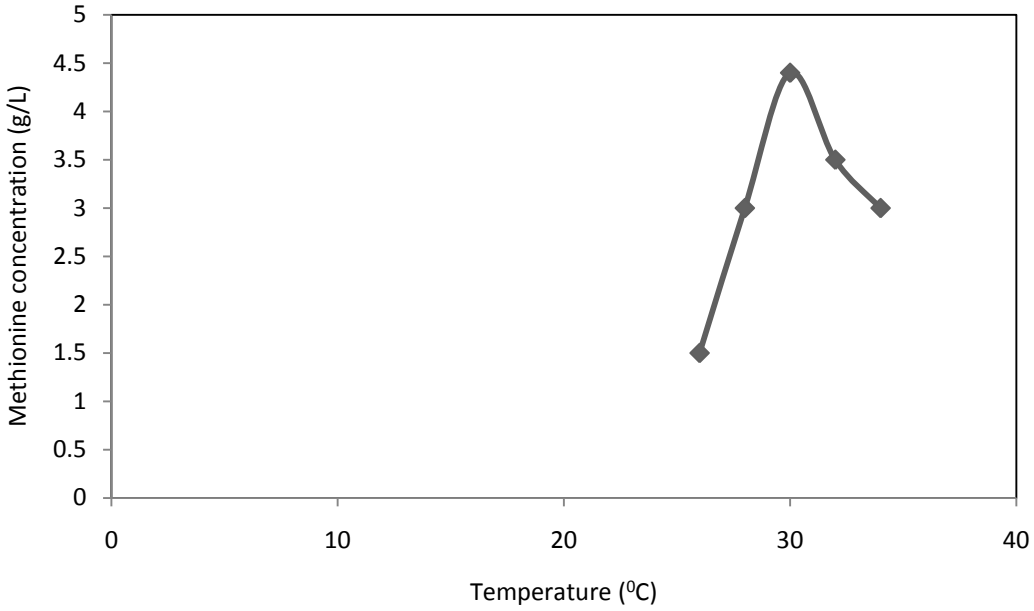

Fig 5: Effect of temperature on methionine production with initial glucose concentration 50 g/L

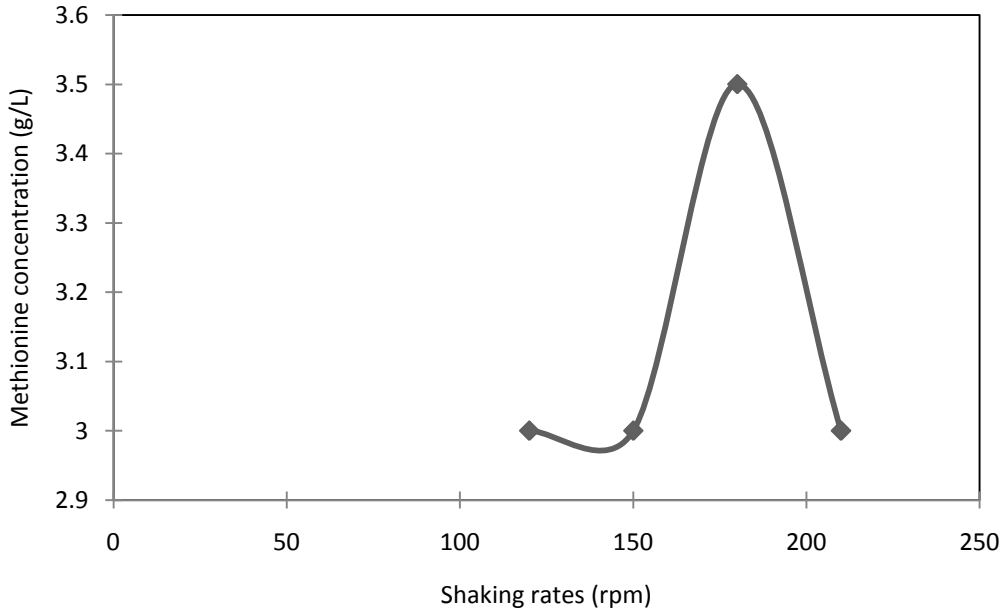

Fig 6: Effect of shaking rates on methionine production with initial glucose concentration 50 g/L

ISSN: 0975-8585

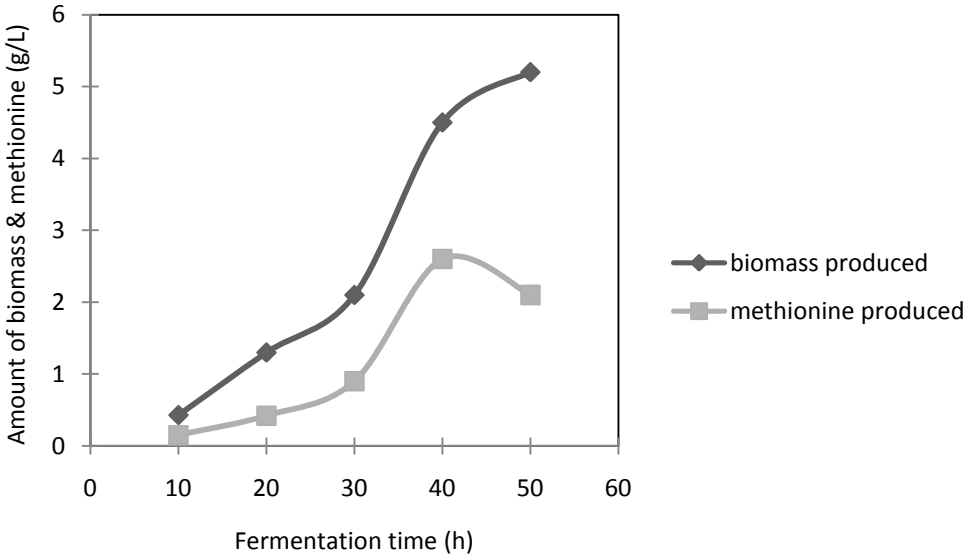

Fig 7: Comparison of amount of biomass and methionine production as a function of fermentation time

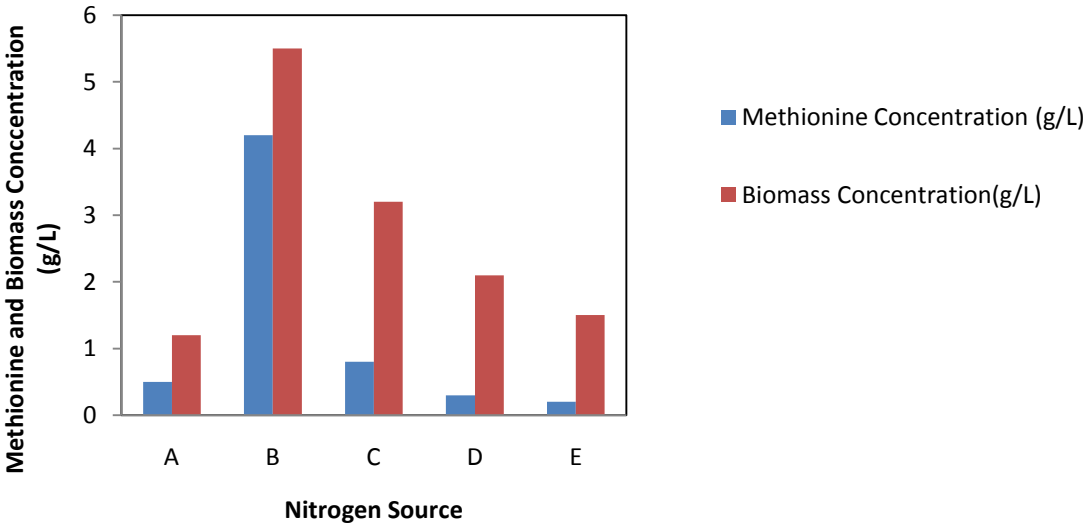

Fig 8: Effect of nitrogen sources on methionine production (initial glucose concentration 50 g/L) A-Urea; B-Ammonium sulphate; C-Ammonium nitrate; D-Ammonium sulphate; E-Sodium nitrate

Effect of shaking rates (rpm) on methionine production

The effect of varying shaking rates on methionine production was observed. By varying shaking rate at desired levels and keeping all other variables at constant. The preculture was inoculated into shake flask at desired shaking rate. These were incubated for 48 hrs on an

orbital shaker. The optimum shaking rate was observed as 180 rpm, under which methionine production obtained as 3.5 g/L. Thereafter methionie production decreased as shown in Fig 6.

Comparison of amount of biomass and methionine production by C.glutamicum as a function of fermentation time

Amount of biomass and methionine production by *C.glutamicum* was estimated as a function of fementation time. Methionine concentration increased directly with rate of biomass concentration (growth associated product formation) up to 40 h, thereafter methionine concentration decreased even though biomass concentration increasing (Non growth associated product formation) where the methionine concentration is function of biomass rather than rate of biomass as shown in Fig 7.

Effect of nitrogen sources on methionine production

The effect of various nitrogen sources (A-Urea, B-Ammonium sulphate, C-Ammonium nitrate, D-Ammonium phosphate, E-Sodium nitrate) on methionine production was examined. Ammonium sulphate was the optimum nitrogen source for methionine production, possibly due to the presence of sulphur which is necessary for synthesis of methionine as shown in Fig 8.

SUMMARY AND CONCLUSIONS

The fermentation conditions for methionine production by *C.glutamicum* MTCC No.2745 were optimized in shake flask on a laboratory scale. The effect of nitrogen sources on methionine production was examined. Ammonium sulphate gave maximum methionine production, possibly due to the presence of sulphur which is necessary for synthesis of methionine. The most favorable conditions for methionine synthesis were fermentation time 44 h, initial glucose concentration 100 g/L, initial nitrogen concentration (ammonium sulphate) 60 g/L, temperature 30^{o} C, pH 7.0 and initial shaking rate 180 rpm.

ACKNOWLEDGEMENTS

We are very grateful to Microbial Type Collection Centre, Chandigarh, india for proving us with *C.glutamicum* (MTCC No 2745) used for this work. We are also thankful to Dr. lavu rathaiah, Chancellor and Prof V. Govardhana Rao, Vice Chancellor of Vignan University for providing laboratory facilities to carry out this work.

REFERENCES

[1] Kinoshita S, Udaka S, Shimino M. J Gen Appl Microbiol 1957; 3: 193-205.
[2] Pham CB, Galvez FCF, Padolina WG. ASEAN Food J 1992; 7: 34-37.
[3] Rose WC, Coon MJ, Lockhart HB, Lambert GF. J Biol Chem 1955; 215: 101-110.
[4] Gomes J, Kumar D. Biotechnol Adv 2005; 23: 41-61.

[5] del Amo GG, Palasi VV and Ruiz. Anales Real Soc Esponi Fishy 1952; 48: 539-544.
[6] Kase, H, Nakayama K. Agr Biol Chem 1975; 39 (1): 153–160.
[7] Kase, H, Nakayama K. Agr Biol Chem 1975; 39 (1): 161-168.
[8] Mondal S, Das YB, Chatterjee SP. Acta Biotechnol 1994; 14 (1): 61-66.
[9] Tosaka O, Takinami K. In: Aida K, Chibata I, Nakayama I, Takinami K, Yamada, H, editors. Biotechnology of amino acid production 1986: pp 152–172.
[10] Sahm H, Eggleling L, Eikmanns B. and Kramer, R. FEMS Microbiol Rev 1995; 16: 243–252.
[11] Hermann T. J Biotechnol 2003; 104: 155–72.
[12] Mondal S, Das YB, Chatterjee SP. Folia Microbiol 1996; 41: 465– 72.
[13] Nakayama K, Kitada S, Kinoshita, S. J Gen Appl Microbiol 1961; 7: 145-154
[14] Mannsfeld SP, Pfeiffer A, Tanner H, Liebertanz. US Patent 04069251; 1978. Leuchtenbeger W. Amino acids-technical production and uses In: Rehm HJ, Reed G, Phuler A, Stadler P. 1996; 6: P. 492
[15] Tosa T, Mori T, Fuse N, Chibata I. Biotechnol Bioeng 1967; 9: 603-8.
[16] Fong CV, Goldgraben GR, Konz J, Walker P, Zank NS. Org Chem Manufact Hazards 1981. p. 115-94.
[17] Umerie SC, Ekwealor IA, Nawabo IO. Bioresour Technol 2000; 75: 249-252
[18] Odunfa SA, Adeniran SA, Teniiola OD, Nordstorm J. Int J Food Microbiol 2001; 63: 159- 63.
[19] Taber H, Rosenthal SM, Tabor CW. J Biol Chem 1958; 233: 907-17
[20] Neuvonen PJ, Tokola O, Toivonen ML, Simell O. Int J Clin Pharmacol Ther Toxicol 1985; 23: 497-500.
[21] Funfstuck R, Straube E, Schildbach O, Tietz U. Med Klin 1997; 92: 574-81. Campbell KCM. US Patent 6187817; 2001
[22] Methionine production by C.glutamicum, Available at: http://d-nb.info/991833252/34 , Accessed on: 26.10.2012.
[23] A simple, rapid and inexpensive procedure to distinguish amino acids and their acids and their esters, Available at: http://www.ias.ac.in/resonance/Mar2002/pdf/Mar2002Classroom2.pdf, Accessed on: 10 Apr. 2013