

Optimising Carbon and Nitrogen Sources for L-Glutamic acid Production by *Brevibacterium* strain NIAB SS-67

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Abstract.- Glutamic acid has attracted attention for its pharmaceutical and food applications. There is a 13.3% annual market growth and needs extensive research work on its over-production using new organisms or innovative technology. To increase glutamate yield and productivity, we used *Brevibacterium* strain NIAB SS-67 and got 38 g glutamic acid/L of glucose-trypticase (L-6) medium (pH 7.0) at 30 °C. Further enhancement in glutamate production was sought by optimising C and N sources using research techniques. Out of nine C sources (glucose, galactose, dextrin, lactose, maltose, sorbose, xylose, fructose, sucrose) and six N sources (urea, ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, potassium nitrate and sodium nitrate) tested, 12% dextrin and 2% ammonium sulphate revealed the best results, yielding up to 60.8 g glutamic acid /L (50% enhancement) after 48 h fermentation. Product yield (0.23 g/g on glucose medium) increased to 0.61 g/g with glutamic acid volumetric productivity of 2.1 g/L h, which is 1.75-fold higher than that reported on other *Brevibacterium* strains, grown under identical growth conditions

Key words: Glutamate, fermentation, *Corynebacterium glutamicum*, carbon, nitrogen.

INTRODUCTION

L-Glutamic acid (Glu) is a non-essential, acidic and genetically coded amino acid. It is considered to be nature's "brain food" for its ability in improving mental capacities; helps speed the healing of ulcers; gives a "lift" from fatigue; and helps control alcoholism, schizophrenia and the craving for sugar. Also it plays a key role in the stabilization of the active conformation of intracellular androgen receptor (AR) and in androgen and anti-androgen activities (Georget *et al.*, 2006).

Glu has developed as an industrially important amino acid for its ever-increasing demand as taste enhancer and is produced by microbial fermentation (Ghosh and Sen, 1996; Sano, 2009). Today, total world production of glutamate by fermentation is estimated to be 2 million tons/y (Sano, 2009). However, future production growth will likely require further innovation, namely, selection of new organisms, their improvement and/or innovation in culturing the organisms and enhancing excretion of glutamate in the fermentation medium (Choi *et al.*,

2004; Shirai *et al.*, 2005; Amin and Al-Talhi, 2007).

Corynebacterium glutamicum, was originally used as L-glutamate producing bacterium (Hirasawa *et al.*, 2001; Hwang *et al.*, 2008). For enhanced extracellular amino acid production, changes in cellular metabolism and/or regulatory control are required. L-Glutamate excretion by *C. glutamicum* is induced by biotin limitation, treatment with penicillin, or by addition of fatty acid ester surfactants (Hirasawa *et al.*, 2001; Choi *et al.*, 2004; Amin and Al-Talhi, 2007). Glu is excreted by many bacteria in response to different stress conditions, and is particularly influenced by the concentration of C and N in the medium. Recently Jyothi *et al.* (2005) used *Brevibacterium divaricatum* for production of glutamic acid from cassava starch factory residues and showed application of non-conventional feedstock for its conversion to glutamate. These studies prompted us to test a *Brevibacterium* strain in our culture collection for Glu production, and the optimization of C and N sources for enhanced Glu fermentation using various carbon and nitrogen sources in a classical amino acid production medium (L-6) using search technique. These studies led us to conclude that this strain is a useful strain for furthering its potential for industrial application after its genetic modification.

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MATERIALS AND METHODS

Microorganism and fermentation condition

Brevibacterium strain 1 NIAB SS-67, previously isolated from a potato field was purchased from NIAB Bacterial Culture Collection and was grown in glucose-trypticase fermentation medium L-6 as per instructions of suppliers. This medium contained (g/100 ml): glucose, 1.0; trypticase, 0.075; CaCO₃, 0.20; KH₂PO₄, 0.07; K₂HPO₄, 0.04; MgSO₄.7H₂O, 0.03; (NH₄)₂SO₄, 0.30; biotin, 60 µg/l; thiamine HCl, 5 mg/l. Inoculum of the organism was prepared in glucose yeast extract (GYE) (0.5% glucose and 0.3% yeast extract liquid medium) broth and used at 10% (v/v). The fermentation was carried out in 250 ml Erlenmeyer flasks at 30±1°C in a rotary shaker (150 rpm), and lasted for a maximum of 72 h. Aliquots of fermented broth were removed at regular time intervals to analyze the bacterial growth and amino acid production. The bacterial growth was measured by nephelometer, whereas Glu was detected by paper chromatography, paper electrophoresis and by polychromatography, followed by spectrophotometry (Nadeem *et al.*, 2001).

Optimising Glu production

Growth pattern of *Brevibacterium* NIAB SS-67 was studied glucose-trypticase (L-6) medium. The growth curves were plotted on the basis of dry cell weight vs optical density of cells in saline (Nadeem *et al.*, 2002).

For optimising the C source, we used L-6 medium (pH 7) containing Trypticase (0.75%), CaCO₃ (2%), KH₂PO₄ (0.07%), K₂HPO₄ (0.4%), MgSO₄.7H₂O (0.03%), (NH₄)₂SO₄ (0.03%), Biotin (60 µg/l), Thiamine HCl (5 mg/l) and the C source (10 %) either glucose, galactose, lactose, maltose, sorbose, xylose, fructose, sucrose or dextrin. The C sources were sterilized separately and added to the autoclaved fermentation medium. A similar medium was used for optimisation of N source, except that the C source was glucose (10%) and the N source (3%) either urea, ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium phosphate, potassium nitrate and sodium nitrate. The culture (NIAB SS-67) was inoculated and fermentation

carried out under conditions described earlier. The Glu production was monitored every 24 h interval till 72 h.

Calculation of kinetic parameters

Kinetic parameters, namely maximum volumetric rate of glutamate formation (Q_p), product yield (Y_{P/S}), specific product yield (Y_{P/X}) and specific rate of glutamate formation were determined by applying equations 1-4.

$$dP/dt = Y_{P/X} \cdot dX/dt \quad (1)$$

$$Y_{P/X} = dP/dX \quad (2)$$

$$Y_{P/S} = -dP/dS \quad (3)$$

$$q_p = 1/X \cdot dP/dt \quad (4)$$

Statistical analysis

All experiments were replicated in three flasks and the data are presented as the mean of three independent experiments. Tukey-Kramer one way ANNOVA (Instat 3 software) was used to determine the significant differences among mean values at the 5 % level of confidence

RESULTS

Growth pattern on L-6 media

The lag phase was very short (1 h), followed by the log phase that extended up to 40 h. The maximum dry cell mass (10.5 gL⁻¹) was observed at 32-40 h, followed by a decline phase that started at 56 h and continued till 72 h (fig not shown).

Optimizing C and N source for Glu production

Among the tested C sources, only dextrin and maltose favoured Glu production; dextrin being much more effective than maltose. With dextrin, the amino acid production started within initial 8 h and increased gradually up to 48 h when 40 g/L of Glu was observed as compared to only 32 g/L with maltose. Regarding the growth of the organism, the dry cell mass produced with dextrin, fructose and galactose was higher (10.0-11.3 g/L) than xylose, lactose, maltose, sorbose and sucrose (8.2-9.5 g/L), whereas glucose produced the least (8.8 g/L) after 48 h incubation (Fig. 1).

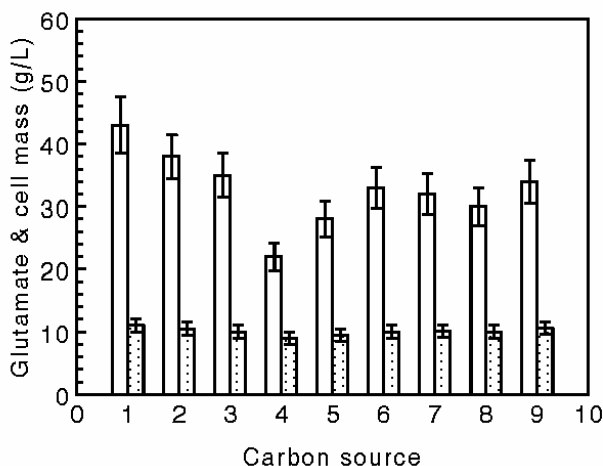


Fig. 1. Effect of dextrin (1), maltose (2), lactose (3), glucose (4), sorbose (5), fructose (6), sucrose (7), galactose (8) and xylose (9) as carbon sources on the growth and production of Glu by NIAB SS-67

Optimisation of dextrin concentration for Glu production was carried out using 20-120 g dextrin /L against a glucose-control. With all concentrations of dextrin tested, the peak production of Glu was observed at 48 h, whereas with glucose-control the peak was delayed to 72 h. A 120 g dextrin /L was found optimum for the Glu production showing peak production (40.9 g/L) at 48 h (Table I). Although, at 60 and 80 g dextrin/L, the isolate also produced 30.1 and 38.0 g/L of Glu, respectively, the values are higher than that observed for glucose-control, 21.9 g/L, at 72 h. Though the dry cell mass with 8 as well as 12% dextrin was statistically non-significant, but both were significantly different from control, 2, 4 and 6 % dextrin respectively with $P= 0.005$ (Table I). Glu yield was significantly higher at 40 g/L but at 120 g dextrin/L, cell mass was significantly higher than control (Table I). Since 120 g dextrin gave significantly higher glutamate content in the fermentation broth was opted for further optimization studies.

Regarding N source, Glu production was not supported by sodium nitrate, whereas KNO_3 and NH_4NO_3 were lesser effective than $(NH_4)_2SO_4$, NH_4Cl , and urea, which produced higher Glu during 48 h fermentation (Fig 2). Ammonium sulfate proved to be the best N-source and its different concentrations were tested for Glu production in L-6

Table I- Growth and Glutamic acid (Glu) production by *Brevibacterium* sp. NIAB SS-67 at different dextrin concentrations

Dextrin concentration	Max. cell dry mass	Max. Glu produced	Y _{P/S}
(g/L)	(g/L)	(g/L)	(g/g)
20	8.0c (24) ^a	10d (24) ^b	0.50b
40	9.40c (24)	24.4c (48)	0.61a
60	10.5b (48)	30.1b (48)	0.51b
80	11.0a (48)	38.0a (48)	0.48c
120	11.3a(48)	40.9a (48)	0.46c
Control*	8.8c (72)	21.9c (72)	0.27d

Each value is a mean of n=3 experiments. Means followed by different letters differ significantly at $P \leq 0.05$ using Kramer's one way ANNOVA.

^aAt harvest time of maximum Glu production

^bFigures in parentheses represent the harvest time

*L-6 medium containing 100 g/L glucose as C source, and 3 g/L $(NH_4)_2SO_4$ as N source

P values for column, 2, and 3 were 0.005, 0.0001 and 0.0001 respectively using Kramer one way ANNOVA in InStat 3 software.

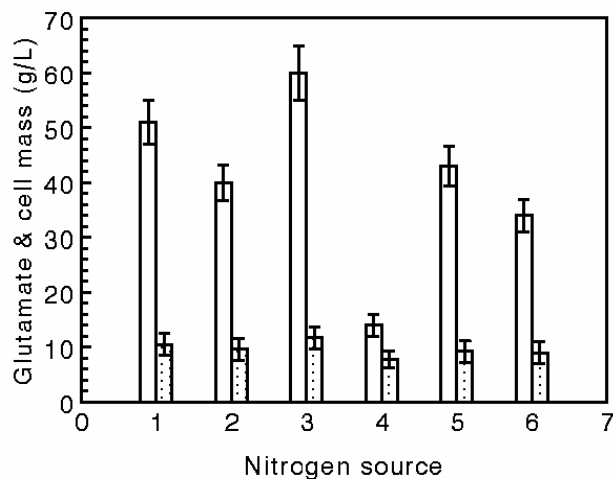


Fig. 2. Effect of different nitrogen sources (1 urea; 2 ammonium chloride; 3 ammonium sulphate; 4 sodium nitrate; 5 ammonium phosphate and 6 potassium nitrate) upon glutamate production by *Brevibacterium* sp.NIAB SS-67

medium with 120 g dextrin/L as the C source. This medium containing 0.03% $(NH_4)_2SO_4$ served as control. Ammonium sulphate concentrations from 1.0-4.0% differed significantly with P values of 0.0005, 0.0029 and 0.0001 in enhancing glutamate

product yield (Y_{P/S}), specific glutamate yield Y_{P/X}) and productivity respectively. Only 5.0% ammonium sulphate repressed formation of glutamate significantly (Table II). (NH₄)₂SO₄ at 2% supported the highest glutamate yield and productivity, which though were not significantly different from 1, 3, and 4% nitrogen source was opted for further studies. Finally, in L-6 medium containing 12% dextrin and 2% (NH₄)₂SO₄, NIAB SS-67 was regrown in triplicate to study the kinetics of cell mass and glutamate formation, the test organism yield glutamate up to 60.9 g glutamate/L after 48 h fermentation with Q_p of 2.1 g/L.h (Table III) and was comparatively better yield and productivity.

Table II.- Ammonium sulphate (AS) concentration-dependent kinetic parameters of glutamate production by *Brevibacterium* sp. following growth on 12.0 % dextrin in L-6 medium (pH 7.0) at 30°C in shake flask cultivation.

AS concentration (%)	Y _{P/S} (g/g)	Y _{P/X} (g/ g cells)	Q _P (g/L.h)
1	0.48a	4.82a	1.81a
2	0.55a	5.12a	2.00a
3	0.51a	4.94a	1.84a
4	0.49a	4.51a	1.64a
5	0.41b	4.12b	1.43b
P	0.0005	0.029	0.0001

Each value is a mean of three readings. Means followed by same letters do not differ significantly at $P \leq 0.05$.

Table III.- Kinetic parameters for glutamate production by *Brevibacterium* sp. following growth on 12.0 % dextrin and 2.0 % ammonium sulphate in L-6 medium (pH 7.0) at 30° C in shake flask cultivation.

Kinetic parameter	Parameter value
Y _{P/S} (g/ g)	0.55
Y _{P/X} (g/ g cells)	5.2
Q _P (g /L.h)	2.1
q _P (g/ g cells. h)	0.181

Each value is a mean of n=3 readings. Standard deviation among replicates was less than 5% and has not been presented.

DISCUSSION

For almost 50 years now, biotechnological production processes have been used for industrial

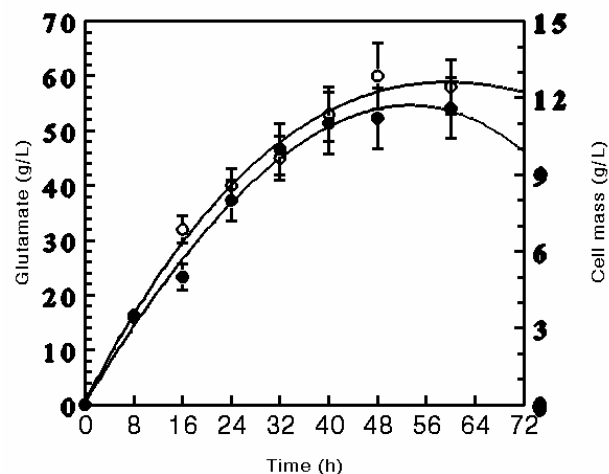


Fig. 3. Time course production of glutamate and cell mass (g/L) in dextrin (12%) and ammonium sulphate (2.0%) in L-6 medium at 30°C

production of amino acids. Market development has been particularly dynamic for the glutamate as flavour-enhancer, and the animal feed amino acids L-lysine, L-threonine, and L-tryptophan, which are produced by fermentation processes using high-performance strains of *Corynebacterium glutamicum* and *Escherichia coli* from sugar sources, such as molasses, sucrose or glucose (Leuchtenberger *et al.*, 2005). All the amino acids are important for nutrition, seasoning, flavouring and as precursors for pharmaceuticals, cosmetics and other chemicals. They may be produced either by isolation from natural materials (originally from the hydrolysis of plant proteins) or by chemical, microbial or enzymatic synthesis, the latter two give rise to optically pure amino acids (Ghosh and Sen, 1996). It is well established that rate of carbon metabolism can influence the formation of biomass or production of primary or secondary metabolites. Though bacteria can utilize a vast range of carbon compounds including molasses, corn steep liquor, bagasse, distillery sludge, starch waste, whey, palm waste, rice hydrolysate and carbohydrates, including starch, sucrose, glucose, fructose (Nadeem and Ahmad, 1999; Ruklisha and Ionina, 1998; Das *et al.*, 1995; Zhifeng *et al.*, 1989) but carbohydrates are preferred in microbial fermentation processes. Owing to the high starch content and organic nature of agro-

industrial wastes, also an attempt was made by Jyothi *et al.* (2005) to utilize cassava for the production of glutamic acid employing submerged fermentation using *Brevibacterium divaricatum* (Jyothi *et al.*, 2005).

Glucose has been found as the most suitable carbon source for amino acids, particularly glutamic acid, production. Many workers (Sen *et al.*, 1992) have reported glucose, specifically, as the best carbon source. However, fructose and sucrose have also been reported in this context (Kiefer *et al.*, 2002; Sen *et al.*, 1992). But on the basis of particular nutritional versatility of the organisms under study suitable carbon sources may vary, as happened in the study under discussion, where dextrin appeared to be the most supportive C source. Easily metabolizable carbon sources normally cause catabolite repression and thus support lower yields (Kiefer *et al.*, 2002). Our work corroborated previous studies (Kiefer *et al.*, 2002; Choi *et al.*, 2004).

Regarding nitrogen source, majority of the industrially used microorganisms can utilize inorganic or organic (amino acids, protein or urea) sources of nitrogen. Ammonia is, frequently, the preferred nitrogen source in many laboratory and industrial scale fermentations, where it is added as ammonium chloride or ammonium (Das *et al.*, 1995). Commonly utilized nitrogen sources are Soya bean meal, yeast extract, corn steep liquor, distilleries solubles etc. As reported by many workers, urea supported maximum growth. The glutamic acid synthesis reduced in decreasing order by the addition of $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NH_4OAC and NH_4NO_3 , respectively (Sen *et al.*, 1992). In the present study, the best results were observed in case of ammonium sulphate, as reported by Li *et al.* 1. Maximum volumetric productivity (2.0 g/L h) was observed with 2% $(\text{NH}_4)_2\text{SO}_4$ and is significantly higher than that observed by Choi *et al.* (2004). Maximum glutamate content (60 g/L) was obtained with 2% nitrogen source in the medium and is 0.62 mol/mol substrate consumed and is 3-fold higher than that reported earlier (Kinoshita *et al.*, 2004). Previously other authors used an optimization strategy based on desirability function approach (DFA) together with response surface methodology (RSM) to optimize production medium (Li *et al.*,

2007), where they tried different combinations of glucose and $(\text{NH}_4)_2\text{SO}_4$ to attain the optimal medium as well as studied the effect of temperature, pH, inoculum size, speed of agitation and nitrogen sources.

Different workers overtime adopted different approaches and strategies to enhance the production of desirable amino acid(s). Fermentation problems often force to reach a compromise between different experimental variables in order to achieve the most suitable strategy applied in industrial production. The importance of the use of multi-objective optimization methods lies in the ability to cope with this kind of problems (Li *et al.*, 2007).

Choi *et al.* (2004) enhanced glutamate production by temperature- shift cultivation. Amin and Al-Talhi (2007) used immobilized cell reactor for complete consumption of substrate to result in hyper-production of glutamate. The difference in glutamate production between two *Corynebacterium* spp was caused by the difference in the degree of decrease in oxoglutarate dehydrogenase complex (ODHC) specific activity, along with difference in K_m values of isocitrate dehydrogenase, glutamate dehydrogenase, and ODHC explained the mechanism of flux redistribution at the branch point of 2-oxoglutarate (Shirai *et al.*, 2005) during hyper-production. Recently Hasegawa *et al.* (2008) demonstrated that a decrease in pyruvate dehydrogenase (PDH) activity catalyzes the conversion of pyruvate to acetyl-CoA. Furthermore, an increase in pyruvate carboxylase (PC) activity is evident under glutamate-overproducing conditions, which may lead to an increase in carbon flux from pyruvate to oxaloacetate. Their data suggest that a novel metabolic change occurs at the pyruvate node, leading to a high yield of glutamate through adequate partitioning of carbon flux. Thus we may increase yield and productivity of glutamate by increasing secretion of glutamate from the cells by increasing flux of carbon to both pyruvate and oxoglutarate branch points.

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