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Optimizing Different Vitamins for L-Glutamic Acid Production by *Brevibacterium* Strain NIAB SS59

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Abstract.- The effect of different vitamins on glutamate (Glu) fermentation by *Brevibacterium* strain was explored. Riboflavin and thiamine appeared to be the most potent vitamins, whereas the others, too, played a significantly positive role in improving the volumetric and specific rates of Glu production. An overall 2.7-4.5 fold increase in Glu production was recorded. Regarding time scale, maximum production was observed at around 72 h and 30±1°C. It was inferred that vitamins have a vital role in amino acid fermentation and their importance in this regard cannot be denied.

Key words: Vitamins, *Brevibacterium*, glutamic acid, fermentation

INTRODUCTION

Vitamins as essential component are found in almost all the naturally occurring foodstuffs. Normally, these are either not manufactured or do exist in minute quantities in the body and hence are supposed to be supplied with the diet as vital ingredients (Lieberman and Bruning, 1990; Anthea *et al.,* 1993). Vitamins are used in a variety of ways expanding their functionality (Kutsky, 1973) and are so vital for normal body functions that sometimes even slightest of scarcity may cause an everlasting dent (Rohde *et al.,* 2007). Most often vitamins, such as A, B-complex, C, D, E and K are obtained from food (vegetables, meat, eggs, liver, fruit). However, some of them (vitamin D, K, biotin) may also be obtained from other sources like sunlight and gut flora (Dietary Reference Intakes: Vitamins, The National Academies, 2001). Not only that vitamins do keep intact in the body even after the completion of growth and development, they also facilitate the individual to proficiently and actively utilize the chemical energy provided by food, and consume fat, carbohydrate and protein needed for respiration (Bender, 2003). In fact, vitamins are not the energy suppliers, but perform an active role as a catalyst during the metabolic regulation and energy transformation (Bolander, 2006).

Amino acids cover the foremost proportion among the primary metabolites yielded by microorganisms (Ekwealor and Obeta, 2007). Glutamic acid (Glu) is one of the non-essential amino acids that can be synthesized by the human body through appropriate nutrition. It is supposed to be nature's 'brain food' because of its ultimate role not only as excitatory neurotransmitter but also in a number of other processes, such as boosting mental competence, speeding up the curative action of different sorts of ulcers, overcoming weariness as well as helping cure schizophrenia, alcoholism and thirst for sugar. It plays a vital role in the sugar and fat metabolism and assists in the transportation of potassium across the blood-brain barrier. Although, high dosage of Glu may induce headache as well as other neurological symptoms, those afflicted with personality disorder may benefit from it. Generally, protein-rich plant food, dairy products, meat, poultry, fish and eggs are considered to be the rich source of Glu (Russel, 1994, 1999). It is claimed to be the biggest source of intestinal energy as around

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95% of the Glu is metabolized by the intestinal cells during the first pass *(*Reeds, 2000). Some of the very obvious uses of Glu are as food additive, feed supplement, flavour enhancer as well as therapeutic agent (Yugandhar *et al.,* 2006). The production of its salt, mono sodium glutamate, being the largest amino acid product is increasing enormously at a rate of around 1.5 million tons as its demand is increasing at a rate of almost 6% per annum (Ganguly and Banik, 2011; Yugandhar *et al.,* 2007).

Use of microbes through biotechnology has made it really convenient and economical to upgrade the waste or byproducts into value added products, such as amino acids. Today, microbes have turned out to be so powerful that none of the compounds can be manufactured by their help or at least by involving them **(**Ritter, 2004). As much as microbes are getting involved in the production of amino acids, such as Glu, lysine, valine, a number of media are being devised and optimized in order to get maximum yield. Vitamins have played an imperative role in this context for Glu production. Ghosh and Sen (1996) opted for this approach through microbes and displayed the application of non-conventional feedstock for its conversion to glutamate. This study instigated us to examine a strain of *Brevibacterium* for Glu production, and to optimize different vitamins for its yield enhancement. The results of this study helped us conclude that it was a potential strain for industrial use with a little genetic manipulation.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Brevibacterium strain NIAB SS-59, originally isolated from potato field, was grown in glucose-trypticase fermentation medium (L-6) that contained (g/100 ml): glucose, 5.0; trypticase, 0.075; KH_2PO_4, 0.07; K_2HPO_4, 0.04; $CaCO_3$, 0.2; $(NH_4)_2SO_4$, 0.3; $MgSO_4.7H_2O$, 0.03; thiamine HCl, 5 mg/l; biotin, 60 µg/l. The inoculum was prepared in glucose-yeast extract seeding medium (GYE) that consisted of glucose, 0.5% and yeast extract, 0.3%, used at 10% v/v.

Fermentation conditions

A 24hr-old culture of *Brevibacterium* strain was inoculated in the freshly prepared production medium (L-6) for Glu fermentation in Erlenmeyer flasks (250 ml), each containing 50ml of the medium. A set of 3-5ml fractions was intermittently removed from the fermented broth and nephalometrically assessed for the bacterial growth. The fermentation lasted for a maximum of 96 h in a gyratory shaker at 150rpm and 30±1°C. Thereafter, the growth was harvested, the broth made cell-free through centrifugation at 3,000rpm x g, and filtered through Millipore filter (0.45µm) in order to avoid any chance of residues or cell particle.

Amino acid analysis

Initially, paper chromatography was applied for the qualitative analysis of the fermented amino acids. The paper Whatman 1 with n-butanol: acetic acid: water (4:1:5) was used. These results were further confirmed through paper electrophoresis and by polychromatography followed by spectrophotometry (Nadeem *et al.,* 2011). The soluble sugars were assessed by DNS method (Miller, 1959).

Optimising Glu production

Growth pattern of the subject strain was studied in L-6 medium, and the growth curves were plotted on the basis of dry cell weight against the OD (Nadeem *et al.,* 2002).

In order to find out the most suitable vitamin and optimise its concentration for maximum Glu production, L-6 (pH 7) was used as the fermentation medium. It was enriched with three different concentrations (0.001, 0.01, 0.1µg/ml) of thiamine, pantothenic acid, pyridoxine HCl, riboflavin and amino-benzoic acid, adding separately and aseptically. The pure culture (NIAB SS-59) was inoculated and fermented under the same set of fermentation conditions as described above. On the basis of vitamin influence upon the subject strain, the trend of amino acid production was monitored after every 24 h till the end.

Kinetic study

Organism was grown as described earlier in time course study. Samples were collected periodically for determination of dry cell mass, glucose and Glu. All kinetic parameters were determined after Aiba (Aiba *et al.*, 1973). The

differential equations which captured the kinetics of cell mass formation (X), glucose consumption (S), and Glu synthesis are given below:

$$dX/dt = \mu X \quad (1)$$
$$d\,\text{Glu}/dt = Q_{Lys} \times X \quad (2)$$
$$-dS/dt = -\mu X/Y_{X/S} \quad (3)$$
$$dP/dS = Y\,\text{Glu}_{/S} \quad (4)$$
$$dP/dX = Y\,\text{Glu}_{/X} \quad (5)$$

where Q Glu, q_P, $Y_{X/S}$, $Y_{Glu/S}$, and $Y_{Glu/X}$ are the volumetric rate of glutamate formation, specific rate of glutamate production, substrate consumption yield coefficient with respect to growth, glutamate synthesis based on substrate uptake and cell mass formation, respectively.

Statistical analysis

The data obtained were subjected to analysis of variance technique and in case of significant difference, a one way ANOVA test was applied using GrapgPad Instat3.0 software.

RESULTS AND DISCUSSION

Response of the strain towards the fermentation medium was good. As compared to the lag phase, the log phase was long enough and it lasted upto nearly 48 h, where the maximum dry mass (20.16g/l) was obtained. Thereafter, the decline phase began, which gradually lasted till the end (Fig. 1).

Vitamins are the members of enzymes or coenzymes prosthetic group. Some of the commonly known vitamins are *p*-aminobenzoic acid, thiamine (B_1), riboflavin (B_2), nicotinic acid (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin, cyanocobalamine (B_{12}), folic acid, lipoic acid, inositol, vitamin K, mevalonic acid and haemin. Normally, these are required in a very minute quantity (10^{-12} mol l^{-1} to 10^{-6} mol l^{-1}) that may vary in certain conditions, such as stress (Dunn, 1985). Under the condition where only a single vitamin is required, it is advisable to add a pure vitamin rather than to enrich the medium with a combination of cheaper vitamins, as for example in case of vinegar production, where calcium pantothenate is included in media formulation (Beaman, 1967). Biotin is supposed to be the pre-requisite in case of Glu production, whereas some other strains do need thiamine (Kinoshita and Tanaka, 1972). In fact, like all other supplements, vitamins do have their own importance regarding amino acids fermentation. Vitamins normally perform a catalytic role inside the bacterial cells. They may behave as a constituent of co-enzymes or they may act as the prosthetic group of enzymes, but whatever the case may be, their actual role in amino acid production is still to be defined (Ekwealor and Obeta, 2007).

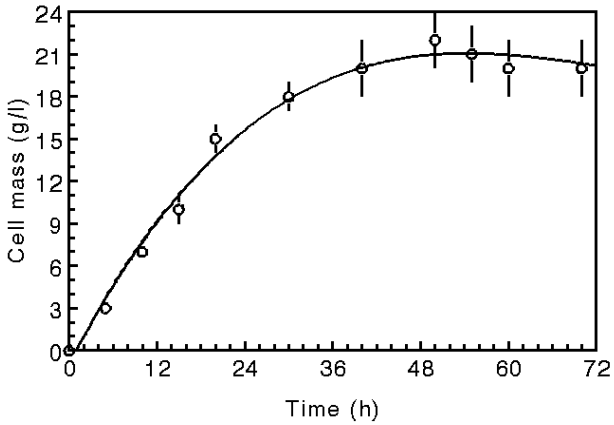

Fig. 1. Growth curve of NIAB SS-59 on the basis of dry cell mass in medium, L-6. Error bars show standard deviation among *n*=3 experiments.

Naturally existing carbon and nitrogen sources hold as a whole or a fraction of the requisite vitamin(s). However, in case of deficiency, the scarcity is met by providing the required constituents (Rhodes and Fletcher, 1966). Similar was the case in present study, where the fermentation medium was blended with various vitamins, and their performance/ impact upon Glu production was keenly observed. It was found that almost all the vitamins had positive influence upon Glu production. However, intensity of yield varied. Riboflavin and thiamine showed the best results reflecting an almost 4.0-4.5 fold increase in Glu production. Thiamine had slight edge on riboflavin in that the organism produced almost an equal amount of Glu under the same set of conditions, utilizing one-tenth of thiamine concentration as compared to that of riboflavin. The peak production

time also was almost the same (72 hr) in both the cases (Table I).

The behaviour of pantothenic acid was unique as the organism produced almost the same amount of Glu (20.1-21.7g/l) with all the three concentrations. It reflected that the organism was insensitive, or at least less sensitive, to change in the vitamin concentration. It was also noticed that at lower concentration of pantothenic acid the strain attained the peak production level in lesser time (48 hr). With amino benzoic acid and pyridoxine HCl (0.01µg/ml each) the strain produced 19.7 and 16.4g/l Glu by the 72 h of fermentation, and it was almost 2.7-3.2 fold increase in that produced by the parent (Table I).

A similar study to explore the influence of different vitamins upon Glu production was also done by Ghosh and Sen (1996). They claimed that sub-lethal doses of vitamins were more effective for enhanced Glu production. They also reported thiamine to be the most potent vitamin for Glu enhancement, whereas aminobenzoic acid and nicotinic acid as the least effective for the same. They also stressed upon the importance of pantothenic acid during the logarithmic phase as its absence badly affected the rate of Glu production. Our study also coincided with their findings as here also thiamine was the most effective vitamin; however, riboflavin also favoured Glu production equally. Moreover, a 3.5-fold increase in Glu production was also observed by the addition of pantothenic acid in the fermentation broth (Table I).

Analysis of variance (Table I) showed that the different concentrations of vitamins had statistically significant influence on Glu and cell mass production as well as substrate consumption. Both, riboflavin (Rf, 0.01µg/l) and thiamine (Th, 0.001µg/l), supported the same amount of Glu/l followed by pantothenic acid (PhCl, 0.001µg/l), aminobenzoic acid (ABA, 0.01µg/l) and pyridoxine-HCl (0.01µg/l); the latter two were non-significantly different with respect to enhancing Glu formation.

Since riboflavin, aminobenzoic acid and pyridoxine-HCl were used under the same set of working conditions, their effect on different kinetic parameters was studied as described in materials and methods. Representative time course production of Glu, cell mass (g/l) and substrate present in fermentation medium is shown in Figure 2.

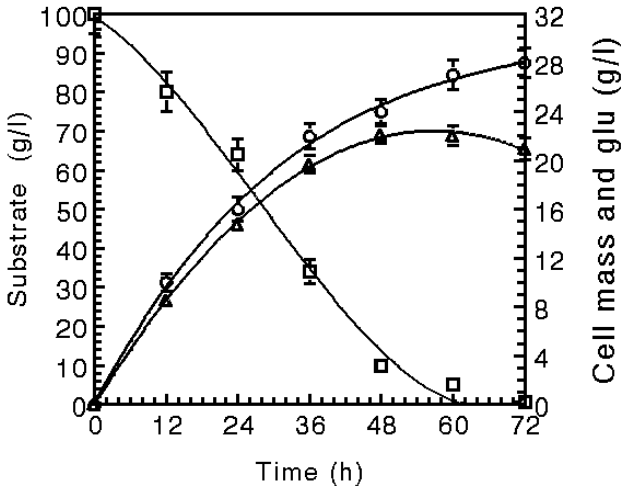

Fig. 2. Representative time course production of Glu, cell mass and glucose present in the L-6 medium following growth of NIAB SS-59.Medium contained glucose (100 g/l) supplemented with 0.01 µg riboflavin/l. Error bars show standard deviation among *n*=3 experiments

Maximum Glu and cell mass were recovered after 60h growth of *Brevibacterium* strain. Total glucose in the medium was completely consumed by the organism and it produced cell mass (22.5 g/L) and 30g Glu from 100g glucose. The shorter fermentation time for maximum Glu production is highly profitable for its cost effective production at industrial scale and will be improved by further studies.

Effect of the same concentration of vitamins on product formation kinetic parameters was highly significant, *e.g.,* on Q_{Glu} (*P*- values > 0.001), $Y_{Glu/X}$ (*P*>0.001), q_{Glu} (*P*>0.001) as was its effect (*P*>0.001) on Glu yield ($Y_{Glu/S}$). Riboflavin was the significantly better product formation supplement, while both ABA and pyridoxine-HCl supported almost the same values of all product formation parameters, though both of them supported significantly higher values of all product formation parameters than those supported by the control medium. *i.e.*, medium without the addition of vitamin supplementation (Table II). When the effect of the above vitamin was compared on substrate

Table I.- Response of NIAB SS-59 to different vitamins regarding glutamic acid production.

Vitamin	Conc. (µg/ml)	Sampling time (h)	Ave. Glu production (g/l)	Other amino acids produced
Riboflavin	0.01	72	27.0±2[a]	Lys, Ala,Val, Ile
Thiamine	0.001	72	26.9±2 [a]	Lys, Arg, Ala, Val, Ile
Pantothenic Acid	0.001	48	21.7±2 [b]	Lys, Ala, Val, Ile
Amino-benzoic acid	0.01	72	19.7±1.2 [bc]	Lys, Ala Val, Ile
Pyridoxine HCl	0.01	64	16.4±1.3 [bc]	Lys, Ala, Val, Ile
Control	0.00	72	6.0±0.1 [c]	Lys, Ala,Val, Ile

$P > 0.001$
Means in column 4 followed by different superscripts differ significantly according to Tukey-Kramer test using GraphPad Instat3.0 software.

Table II.- Effect of different vitamins in glucose (100 g/l medium) based medium on kinetic parameters of glutamate production by *Brevibacterium* sp.

Kinetic parameters	Vitamins (0.01 µg/l)				
	Rf	ABA	PHCl	Control	P-value
Q_{Glu} (g/l h)	0.96±0.04[a]	0.69±0.015[b]	0.56±0.03[c]	0.26±0.01[d]	>0.001
$Y_{Glu/S}$ (g/g S)	0.30±0.03[a]	0.22±0.03[b]	0.18±0.03[b]	0.067±0.02[c]	> 0.001
$Y_{Glu/X}$ (g/ g cells)	1.6±0.31[a]	0.94±0.14[b]	1.1±0.06[b]	0.60±0.04[c]	>0.0008
q_{Glu} (g/ g h)	0.23±0.02 [a]	0.22±0.03[a]	0.13±0.02 [b]	0.049±0.002 [c]	>0.001
Q_S (g/l.h)	0.99.±0.04 [a]	0.72±0.02 [b]	0.6±0.02 [c]	0.24±0.02[d]	>0.001
Q_X (g/l.h)	0.96±0.03 [a]	0.63±0.03 [b]	0.52±0.02 [c]	0.20±0.01[d]	>0.001
$Y_{X/S}$ (g/g)	0.27±0.01[a]	0.19±0.01 [b]	0.15±0.01 [c]	0.14±0.01 [c]	>0.001

± Stands for standard deviation among n=3 experiments. Rf, ABA and phcl stand for riboflavin, aminobenzoic acid and pyridoxine HCl, respectively. Mean followed by different letters in a row differ significantly according to Tukey-Kramer test.

consumption parameters, almost the same effect was observed. Values of Q_S and Q_X, both, were significantly higher in the presence of riboflavin as vitamin supplement followed by both ABA and pyridoxine-HCl. Effect on $Y_{X/S}$ was significantly higher (P<0.001), followed by that of ABA, while the effect of pyridoxine-HCl was comparable with the control medium (without any supplement). The Glu formation kinetic parameters are much higher than the values previously reported in literature (Leuchtenberger *et al.,* 2005; Trotsche *et al.,* 2003).

Studying the effects of nutritional factors is not new. Even the old studies depict the interest of scientists in this context. Takahashi and his group (Takahashi *et al.,* 1965) worked on the stimulatory role of thiamine for Glu fermentation. They reported 3-5 µg/l of thiamine for production and 50 g/l for growth as the optimal concentration. They also reported that the excessive amount of the vitamin in the fermentation medium was a hindrance in Glu production; however, population density of the bacteria was increased. In the present study, although the strain, NIAB SS-59, needed far lesser concentration (1.0µg/l) of thiamine for enhanced production of Glu, its response to the higher concentrations of vitamin was exactly alike to that reported earlier by Takahashi and coworkers. A similar finding was also reported by Sen and Chatterjee (1989).

The interest of workers regarding the effect of different vitamins on bacterial performance leading to amino acid production is still alive, and workers around the world are still zealously working on this aspect. Ekwealor and Obeta (2007) also investigated the influence of vitamins upon amino acid fermentation. According to them riboflavin and folic acid instigated the organism for amino acid production, whereas biotin was responsible for increase in the yield. In the recent study, riboflavin and thiamine were found to be the two most effective vitamins for the enhanced yield of Glu.

Concluding, the importance of vitamins for amino acid production cannot be denied and the same was strongly supported by this study where all

of the vitamins tested played their role somehow or the other in the enhancement of Glu production. Even pyridoxine HCl, which appeared to be least effective, caused more than 2.5-fold increase in the amino acid production. The similar study was also done with some other strains and a more or less similar trend was observed in all the cases with slight variation of vitamin preference to the subject strain (data not presented).

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