STUDIES ON L-GLUTAMIC ACID PRODUCTION USING *BREVIBACTERIUM* SP.

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in Biotechnology

BY

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UNDER THE SUPERVISION OF

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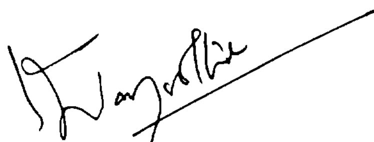
July 1997

DECLARATION

I, Madhavan Nampoothiri do hereby declare that the thesis entitled "**Studies on L-glutamic acid Production using *Brevibacterium* sp.**" is an authentic work accomplished by me under the supervision of Dr. Ashok Pandey, Head, Biotechnology Division, Regional Research Laboratory (Council of Scientific and Industrial Research), Trivandrum, and also declare that the contents in the thesis have not previously formed in any form for the award of any degree, diploma, associateship, fellowship or other similar title or recognition.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.



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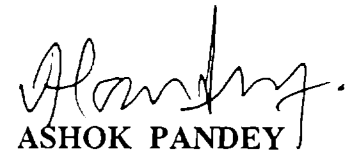
CERTIFICATE

This is to certify that to the best of my knowledge and belief, the thesis entitled "**Studies on L-glutamic acid Production using *Brevibacterium* sp.**", is a record of bonafide research carried out by **Mr. Madhavan Nampoothiri**, under my supervision and also that the contents in this thesis have not previously formed in any form for the award of any degree, diploma, associateship or other similar title or recognition.



ASHOK PANDEY

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I convey my indebtedness to my beloved parents for their care and overwhelming encouragement which greatly helped me for the successful completion of this work. I am also thankful to my sister and relatives. And above all, I submit myself before the Almighty who has given me the might to lead a restless life for the pursuit of knowledge.

***MADHAVAN NAMPOOTHIRI*, K.**

the world of microbes is not only strange but stranger than what we imagine

PREFACE

The thesis entitled "**Studies on L-glutamic acid Production using *Brevibacterium* sp.**" is carired out by the author at Regional Research Laboratory (CSIR), Trivandrum during the period from July1992 to June 1997.

Amino acids are now widely used for their flavour enhancing, nutritional, physiological and chemical properties involving the food, feed, agrochemical and pharmaceutical industries. Among the various methods for the production of amino acids, eg. protein hydrolysis, chemical synthesis and microbiological methods, the latter has the advantage of providing the optically active and biologically required L-form of amino acids from cheap carbon and nitrogen sources and hence the fermentation technology represents and will remain the key position in the amino acid industry.

Glutamic acid is the most important commercial amino acid obtained exclusively by fermentation. It is a typical type of aerobic fermentation and still the batch/fed batch process is the most popular fermentation method. Owing to the importance of the particular industrial fermentation much efforts are still going on in the line and thus, an attempt has been made to develop an indigenous technology for the production of L-glutamic acid using a strain of *Brevibacterium* sp. (DSM 20411).

The non-pathogenic coryneform bacteria belonging to the genera Corynebacterium and Brevibacterium are the chief bioconverters used for the large scale production of amino acids and nucleotides. Apart from that these Coryneform

bacteria are widely used in many biotransformations such as the transformation of steroids, terpenoids, degradation of hydrocarbons etc, they are known for the production of a number of useful enzymes including amidases, nitrilases, proteases and esterases and all of them can play vital roles in biotransformation experiments.

The thesis comprises a set of experiments mainly focused on the improvement of L-glutamic acid fermentation. Much attention has been given to use of locally available raw materials, culturing the organism on inert solid substrates and also immobilization of the bacterial cells from the view point of long term utilization of biocatalyst and continuous operation of the stabilized system. Studies were also carried out for the down stream processing for the extraction and purification of L-glutamic acid. An attempt was made to study the morphological features of the microorganism including the cell premeability. In relation with the accumulation of glutamic acid within the cells an approach was made to study the behaviour of the *Brevibacterium* cells when they are exposed to hyper osmotic environment. Attempts were also made to study the requirement of iron and production of siderophores by this microbial strain. The search for a suitable nitrogen source for glutamate fermentation ended with a promising result that they got a potent urease activity and it can be utilized for many biotransformation studies.

The entire thesis is presented in three sections, viz. introductory section, experimental section and the concluding section.

The introductory section (A) contains a general intrduction, an extensive survey of the recent literature available on the subject, followed by the significance and specific objectives of the present investigation.

The experimental section (B) deals with various experiments conducted and each one is described under separate sub-sections. In addition to a general materials and method section (B.1), each sub-section has its own brief introduction, materials and methods, and results and discussion.

The third and final section (C) contains the summary and prospects of the present work, bibliography and a synoptic bio-data of the author, including the list of research publications.

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SECTION A

A.1 INTRODUCTION

In recent years, microorganisms have found their application in the production of a variety of metabolites which are playing a very important role in employment, productivity, trade, economics and the quality of human life throughout the world. The use of microorganisms for large scale industrial processes is not new, although it has assumed renewed emphasis in recent years. Today, industrial biotechnology involves not only improved fermentation techniques and processes but also a host of other important fields such as food production, fine chemical production, therapeutic production etc. and thus it has offered new horizons to revolutionize world economy.

The various products of microbial fermentation and metabolism include primary and secondary metabolites. The primary metabolites are low molecular weight compounds (~ 1500 daltons) necessary for microbial growth[1]. Some of them are the building blocks of macromolecules, while others participate in the synthesis of coenzymes. Among the most important in industry are amino acids, organic acids, purine and pyrimidine nucleotides and vitamins. Generally, microbial cells do not overproduce primary metabolites, as this would be a wasteful process and decrease the survival ability of the cell. There are, however, microbial strains that show aberrations in their regulation of production of these metabolites and they are the starting strains for industrial processes.

Amino acids are rather simple organic compounds that contain at least one amino group and one carboxylic function in their molecular structure. Among the naturally occurring amino acids the protein forming α-amino acids are most widely

distributed and of considerable economical interest. In addition, the α-amino acids found in proteins are optically active and occur as L-enantiomers.

Amino acids can be produced by a number of ways[2]: (1) protein hydrolysis method, (2) chemical synthesis (3) microbiological methods including (a) direct production of amino acids from a carbon source where the organism synthesizes amino acids and accumulates them in the medium (eg. L. glutamic acid, L-lysine), (b) precursor addition methods where the organism synthesizes amino acids from intermediate metabolites (eg. L-serine from glycine), (c) enzymatic production of amino acids from their immediate substrates as in the case of L-aspartic acid synthesis from fumaric acid and ammonia.

The biotechnological manufacture of amino acids plays an important role, since these structural units of proteins are important as additives to foodstuffs and fodders. Fields of application in medicine, as substances imparting aroma, intermediates in chemical industry and in cosmetics are also important. However, the use of amino acids is based on their nutritional value, taste, physiological activities and chemical characteristics.

In recent years, a considerable amount of interest has been displayed in various parts of the world for the production of amino acids by fermentation processes. The impetus for these advances originated chiefly from the interest in the nutritional applications of glutamic acid and lysine[3]. Among the various methods for the production of amino acids, the microbiological method has the upperhand in terms of yielding optically active and biologically required L-form of amino acid from cheap carbon and nitrogen sources[4] and hence the fermentation

technology represents and will remain in the key position in the amino acid industry.

L-glutamic acid is one of the naturally occurring non-essential amino acid with two carboxylic groups either in bound form as a constituent of protein together with other amino acids or in free form. It has got a wide spectrum of commercial use as food additive, feed supplement, infusion compound, therapeutic agent and precursor for the synthesis of peptides or agrochemicals and will be discussed in details in the next sub-section.

Till mid-fifties, L-glutamic acid was mainly produced by the acid hydrolysis of wheat gluten or soybean protein, the rich sources of glutamic acid[5]. It was more costly, using expensive wheat gluten or soybean protein as raw materials, and moreover, resulted in large amount of by-products such as starch or amino acid mixtures.

In 1957, Kinoshita *et al*[6] isolated a L-glutamic acid producing microorganism, *Micrococcus glutamicus* (synonym of *Corynebacterium glutamicum*) and subsequent research brought about the economic production of L-glutamate by the fermentation process. Production of L-glutamic acid is now a large branch of fermentation industry and provides a significant impetus to the development of microbiological production of primary metabolites. It is a typical type of aerobic fermentation and still the batch/fed batch process is the most popular fermentation method[7]. Morevoer, it is the most important commercial amino acid which can be obtained exclusively by fermentation. The objective is mainly achieved by using some bacterial strains belonging to the genera Corynebacterium, Brevibacterium, Microbacterium and Arthrobacter[8]. All amino

acid accumulating microorganisms which are currently known are aerobes (predominantly facultative rather than obligate aerobes), suggesting that the accumulation of amino acid depends on a metabolic balance which may be affected by environmental factors.

Accumulation of L-glutamic acid is mainly governed not by its biosynthesis but by its excretion[9]. Different explanations have been put forward for the changes responsible for triggering glutamate efflux and will be discussed in detail. An another interesting feature is that no other cases are known of the fermentation where a wide range of possible alternative products can be formed[10] and thus it provides an excellent example of the effects which can be obtained by artificial regulation of the environmental conditions.

The organization and regulation of amino acid biosynthetic pathways attracted the largest interest of biochemical and genetic research in the past few years and still the microbial production of amino acid is a complicated bioprocess. Fermentative production of amino acid is typically a recovery cost intensive process, in which the product concentration is a critical factor for the development of the process[11,12]. In the fermentation process of amino acid production, enhancement of productivity (g of product/liter/hour), as well as yield improvement, is important to achieve the commercial production of amino acid on an industrial scale.

A.II REVIEW OF LITERATURE

Prelude

Amino acids are almost tasteless. However, they exhibit synergistic flavour enhancing properties and are precursors of natural aromas. The flavour enhancing property of *Konbu*, a Kelp like seaweed traditionally used as a seasoning source in Japan was recognized as being due to L-glutamic acid[13]. Until 1950's no appropriate commercial processes for the natural amino acids were existed except by the hydrolysis of wheat gluten, soybean cake or from other natural protein rich materials.

In 1957, two groups independently isolated a soil microorganism belonging to the genus, Micrococcus (synonym of Corynebacterium) and was found potent enough to excrete considerable amount of L-glutamic acid when grown on a medium containing glucose and an inorganic ammonium salt[6,14]. This remarkable discovery led to an extensive screening and breeding programmes for developing strains and also efficient fermentation processes for the production of commercially important amino acids including L-glutamic acid.

(i) Microbial strains

Fermentative production of amino acids got a very reputed status by the discovery of an efficient glutamic acid producer, *Corynebacterium glutamicum* by Kinoshita et al in 1957[6]. Even before that several screenings have been made of amino acid excretion by bacteria, yeast, fungi and actinomycetes[15-19]. Asai *et al* (1959)[20] reported that those which could detectably accumulate amino acids

include *Escherichia coli, Bacillus subtilis, Aspergillus oryzae, Penicillium chrysogenum, Rhodotorula glutinis, Serratia marcescens*. In the eukaryotic group, yeasts of the genera Hansenula, Candida and the baker's yeast, *Saccharomyces cerevisiae* are all potent, for amino acid excretion[21-23]. Microbial production of some amino acids (eg. L-glutamic acid) involves either one stage, using a single organism or by two stages, involving the production of an intermediate compound and its conversion to the particular amino acid (eg. α-ketoglutaric acid to L-glutamic acid)[24,25]

However, only a limited number of bacterial species were used to obtain production strains for the commercial manufacture of amino acids and they belonged to the corynebacterium-Brevibacterium group of coryneform bacteria[26-37]. The Bergy's Manual of systematic Bacteriology (1986)[38] included the corynebacteria in Section 15 as irregular, non-sporing, gram positive rods. The section, however, contained a diverse collection of 22 taxa.

A number of wild strains that have been isolated as L-glutamic acid producing bacteria are shown in Table A1. The Guanine-Cytosine (GC) content of strains belonging to Corynebacterium, Brevibacterium, Arthrobacter and Microbacterium varies from 46-72% which is not enough to characterise one genus[39] DNA-DNA hybridization studies shows that there are only very minor differences between *Brevibacterium flavum, Brevibacterium lactofermentum and Corynebacterium glutamicum* species and hence it has been proposed to consider them as one corynebacterium glutamicum species[40,41]. Taxanomical identification

and differentiation are mainly based on several criteria where the coryneform morphology, the facultative anaerobic growth and the mycolic acid, meso-diamino pimelic acid (mDAP) and arabino galactan content in the cell walls are dominant factors for classification[38,42-45].

Table A1. Microbial strains producing L-glutamic acid[6]

Genus	Species
Corynebacterium	*C. glutamicum*
	C.lilium
	C. callunae
	C. herculis
Brevibacterium	*B. divaricatum*
	B. aminogenes
	B. flavum
	B. lactofermentum
	B. saccharomyticum
	B. roseum
	B. immariophilum
	B. alanicum
	B. ammoniagenes
	B. thiogenitalis
Microbacterium	*M. salicinovolum*
	M. ammoniaphilum
	M. flavum var. glutamicum
Arthrobacter	*A. globiformis*
	A. aminofaciens

A wide spread or even ubiquitous occurrence of Corynebacterium and related genera has to be assumed from the available data[44]. Corynebacteria have been found in soil or aquatic habitats, on plants, in feces, on animal or human skin or in dairy products such as cheese surfaces. The non-specific growth requirements and physiology makes it difficult or impossible to design selective media for

primary isolation, cultivation and storage of representative members of this group. Rich to very rich media, in some cases containing growth factors such as D-biotin, thiamine or p-aminobenzoic acid have been suggested. Preservation at 70°C in the presence of glycerol (20% or dimethyl sulfoxide (7%) is suitable for long term storage.

(ii) Coryneform Bacteria - A Potent Group for Biotechnology

The non-pathogenic Coryneform bacteria belongs to the members of the genera corynebacterium, Brevibacterium and of the related genera Arthrobacter and microbacterium are widely used for industrial production of amino acids, and nucleic acids[46,47]. Other applicable potentials for industrial uses of coryneform bacteria were depicted in Table A2.

Table A2. Biotechnological applications of coryneform bacteria

Application	References
Cheese industry	48
Biotransformation of steroids	49,50
Biotransformation of terpenoids	51
Degradation of hydrocarbons	52,53
Degradation of Fluorine	54
Production of emulsifying agents	55
Production of antibiotics (Corynecins)	56
Production of bacteriocin like compounds	57,58
Production of amidases and nitrilases	59,60
Production of proteases	61
Production of esterases	62
Fumarase activity	63
Urease activity	64,65

(iii) The Genus Brevibacterium

This genus was proposed by Breed, with *Brevibacterium linens* as type species, for a number of gram positive rods formerly classified as Bacterium spp.[66] *Brevibacterium linens* has a coryneform morphology and chemotaxonomic. Numerous phenetic evidences indicate that it is a good species. *Brevibacterium linens* could thus form the nucleus of a redefined genus Brevibacterium as suggested by Yamada and Komagata[67].

Table A3. General characteristics of the genus Brevibacterium

Characteristics	Specifications
Cell morphology	Rod-Coccus cycle
Gram stain	+
O_2 requirement	Strictly aerobic
Motility	
Catalase	+
Peptidoglycan:	
Group	A
Diamino acid	Meso-DAP
N-Glycolyl residue	
Wall arabino galactan polymer	
Mycolic acids	
Major fatty acid types[b]	S, A,I
Major Menaquinones[c]	MK-8$(H)_2$, MK 7-(H2)
Habitat and pathogenicity	Cheese, skin
Mol% G + C	60-70%

+ 90% or more strains are positive
- 90% or more strains are negative
a - designated as Schleifer and Kandler (1972)[69]
b - S, Straight chain saturated; U, monosaturated; A, anteiso-methyl-branched; I, Iso-methyl branched
c - Symbolism of Collins and Jones (1984)[70]

This particular genus was included in the 9th edition (latest) of Bergey's Manual of determinative bacteriology (1994) in group 20 (p.571) as irregular non-sporing gram positive rods[68]. Four different species are mentioned and they are *B. casei, B. epidermis, B. iodinum and B. linens*. Additional species that have been poorly studied or are of uncertain generic position were also treated, and some are now included under the genera Arthrobacter, Corynebacterium and Microbacterium. Table A3 shows the salient features of the genus Brevibcaterium[68].

(iv) Regulatory Mechanisms of L-glutamic acid Biosynthesis

Most of the enzymes of the Embden-Meyerhof pathway and glucose 6-phosphate dehydrogenase,one of the enzymes of the hexose monophosphate oxidative cycle have been demonstrated in the cell free extract of glutamate producing *Brevibacterium flavum*[71]. The result suggests that the operation of both EMP and HMP pathways in the organism for the aerobic oxidation of glucose Fig. A1 shows the regulation of L-glutamic acid biosynthesis.

Two enzymes have been shown to play key roles in the biosynthesis of L-glutamic acid[72]. (i) Phosphoenolpyruvate carboxylase (PEPC): It catalyzes carboxylation of phosphoenolpyruvate to yield oxaloacetate. It is repressed by both L-aspartic acid and L-glutamic acid.(ii) α-Ketoglutarate dehydrogenase (KDH) in L-glutamate overproducing strains the low KDH content limits further oxidation of α-Ketoglutarate to carbon dioxide and succinate thus forming the formation of L-glutamic acid.

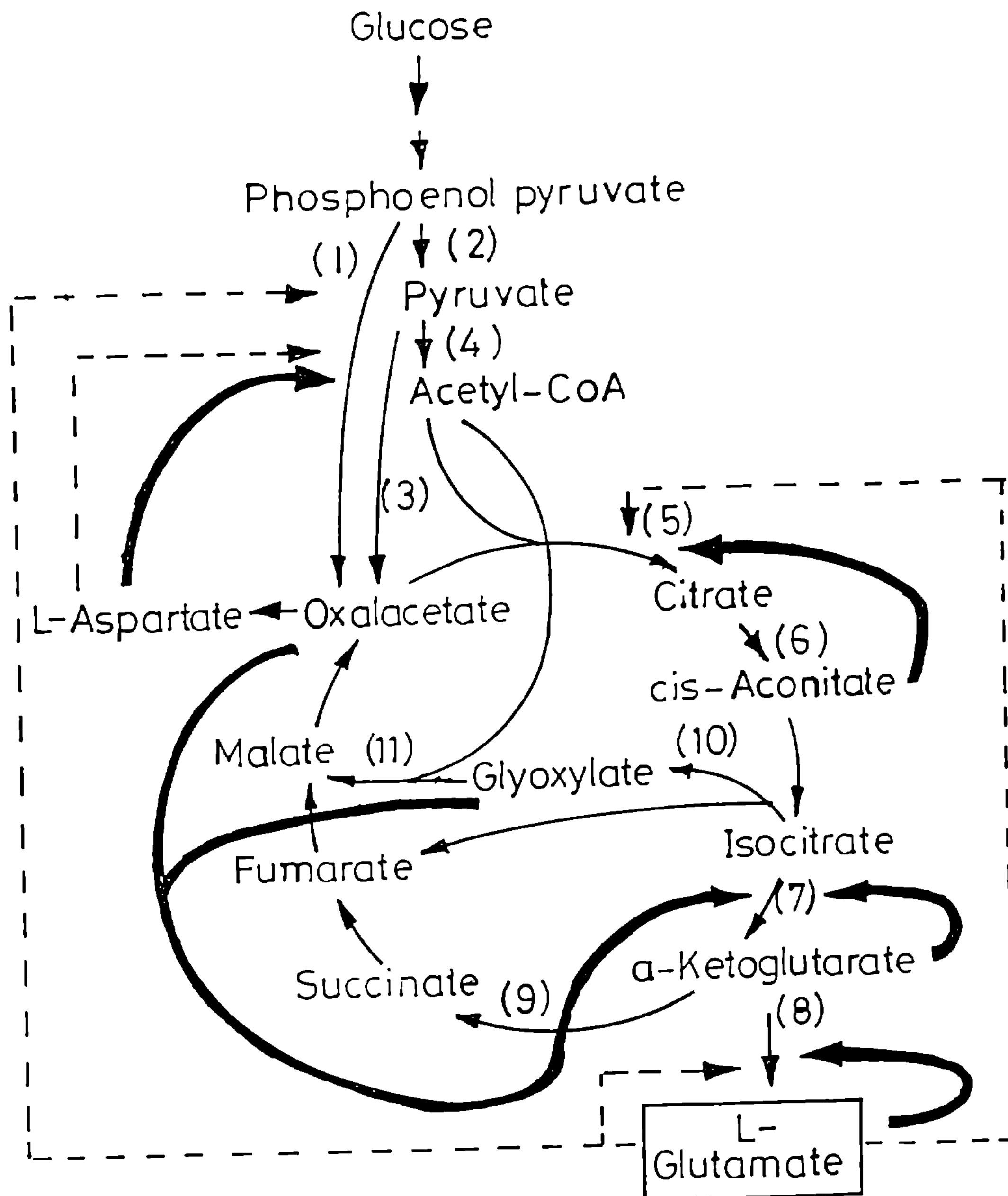


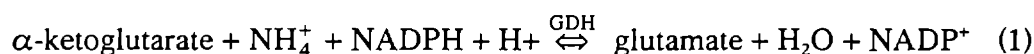
Fig.A1. Regulation of L-glutamic acid biosynthesis[71]

⟶ feedback inhibition, -----> repression, enzyme (coding gene: 1. phosphoenolpyruvate carboxylase (ppc), 2. pyruvate kinase (pyk), 3. pyruvate carboxylase (pyc), 4. pyruvate dehydrogenase (pdh), 5. citrate synthase (glt A), 6. aconitase (cit B), 7. isocitrate dehydrogenase (icd), 8. L-glutamate dehydrogenase (gdh), 9. α-ketoglutarate dehydrogenase (ace E), 10. Isocitrate lyase (ace A), 11. malate synthetase (ace B).

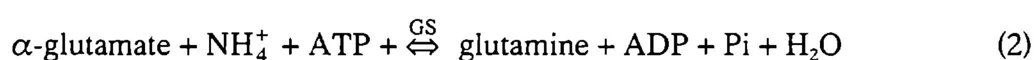
In acetate containing media, the formation of enzymes in the glyoxylate cycle is stimulated (isocitrate lyase, Malate synthase) and the metabolic switch of the TCA cycle to the glyoxylate cycle may be enhanced[73]. A strain of *Microbacterium ammoniaphilum* produced 50% of L-glutamic acid formed from glucose via phosphenol pyruvate, Citrate and α-ketoglutarate and the other 42% via the TCA of the glyoxylate cycle[74].

It has been further postulated that the low activity and high instability of the α-ketoglutarate dehydrogenase favours the conversion of α-ketoglutarate by glutamate dehydrogenase (GDH), especially in the presence of high NH_4^+ concentrations[72]

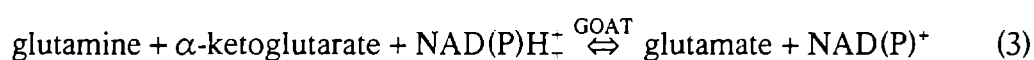
In microorganisms, L-glutamate can be formed either by the glutamate dehydrogenase (GDH) or by the coupled reactions of glutamate synthetase (GS) and glutamate synthase (GOGAT)[75].



$$\alpha\text{-ketoglutarate} + NH_4^+ + NADPH + H+ \overset{GDH}{\Leftrightarrow} \text{glutamate} + H_2O + NADP^+ \quad (1)$$



$$\alpha\text{-glutamate} + NH_4^+ + ATP + \overset{GS}{\Leftrightarrow} \text{glutamine} + ADP + Pi + H_2O \quad (2)$$



$$\text{glutamine} + \alpha\text{-ketoglutarate} + NAD(P)H_+^+ \overset{GOAT}{\Leftrightarrow} \text{glutamate} + NAD(P)^+ \quad (3)$$

All the three enzymes have been detected in the industrially important glutamic acid bacteria, *C. glutamicum, C. callunae* and *B. flavum*[44,76-84].

Recently, the gene encoding GDH has been cloned from both *C. glutamicum* and *C. melassecola*[76,84]. Sequence analysis of the *C. glutamicum* GDH reading frame showed that the *gdh* gene consists of 1344 bp encoding 448 - amino acid residue polypeptide. Glutamate synthesis in these bacteria is dependent on the GDH activity[44]. Since (i) GDH defective mutants of B. flavum were described as glutamate auxotrophic and as showing negligible glutamate secretion[71,80], (ii) GS/ GOGAT system of B. flavum is repressed under the ammonia concentrations used for cultivation of these organisms[80], (iii) GDH activity in *C. callunae* and *B. flavum* is orders of magnitude higher than GC/GOGAT system[77,79]. To verify this hypothesis, Bormann *et al.*[83] constructed two C. glutamicum strain, one being glutamate dehydrogenase (GDH) negative and the other possessing 11-fold higher specific GDH activity than the parental wild type and used to analyse the role of GDH. The results indicate (i) GDH is dispensable for glutamate synthesis required for growth and (ii) that although a high level of GDH increases the intracellular glutamate pool, the level of GDH has no influence on glutamate secretion.

Now-a-days, genetic engineering and amplification of relevant structural genes have become fascinating methods for the construction of strain with desired genotype. Thus, by cloning and expressing the various genes of the L-glutamate pathway, we can produce strains with increased productivity.

(v) Glutamate Excretion

Accumulation of L-glutamic acid is mainly governed not only by its biosynthesis but also by its excretion. Generally, the intracellular accumulation of

glutamate does not reach levels sufficient for feedback control in glutamate overproducers due to rapid excretion of glutamate. The mechanism of glutamate transport into the medium has been subject of a long controversy in the literature. So far, three different mechanisms have been proposed in L-glutamate efflux.

In the past, the mechanism of glutamate excretion was simply explained as a 'leakage' or 'overflow' phenomenon[85]. The 'leak' model is based on the observation that glutamate efflux can be induced by various treatments such as biotin limitation, addition of surfactants or addition of sublethal amounts of β-lactam antibiotics such as penicillins all of which correlate with alterations in the cell membrane[86-88]. This suggest that alternations of membrane permeability due to changes in fatty acid composition of the cell membrane particularly in the content of phospholipids and oleate, together with low activity of the α-ketoglutarate dehydrogenase complex, were the factors responsible for high level of L-glutamate excretion[34]. Fig. A2 is a hypothetical diagram which illustrates the mechanism of L-glutamate excretion by alterting the cell permeability.

The second hypothesis is based on general model for many efflux processes in bacteria, namely, inversion of the uptake process induced by changes in chemical potential or regulation, or by uncoupling due to changes in the membrane structure[89] According to Clement *et al.* (1984)[89], in the case of phospholipid deficiency there is an 'uncoupling' in the glutamate transport system, resulting in 'reversal' of its action. Instead of the uptake of glutamate into the cells, it begin to pass out into the medium through the same channel as shown in Fig.A3.

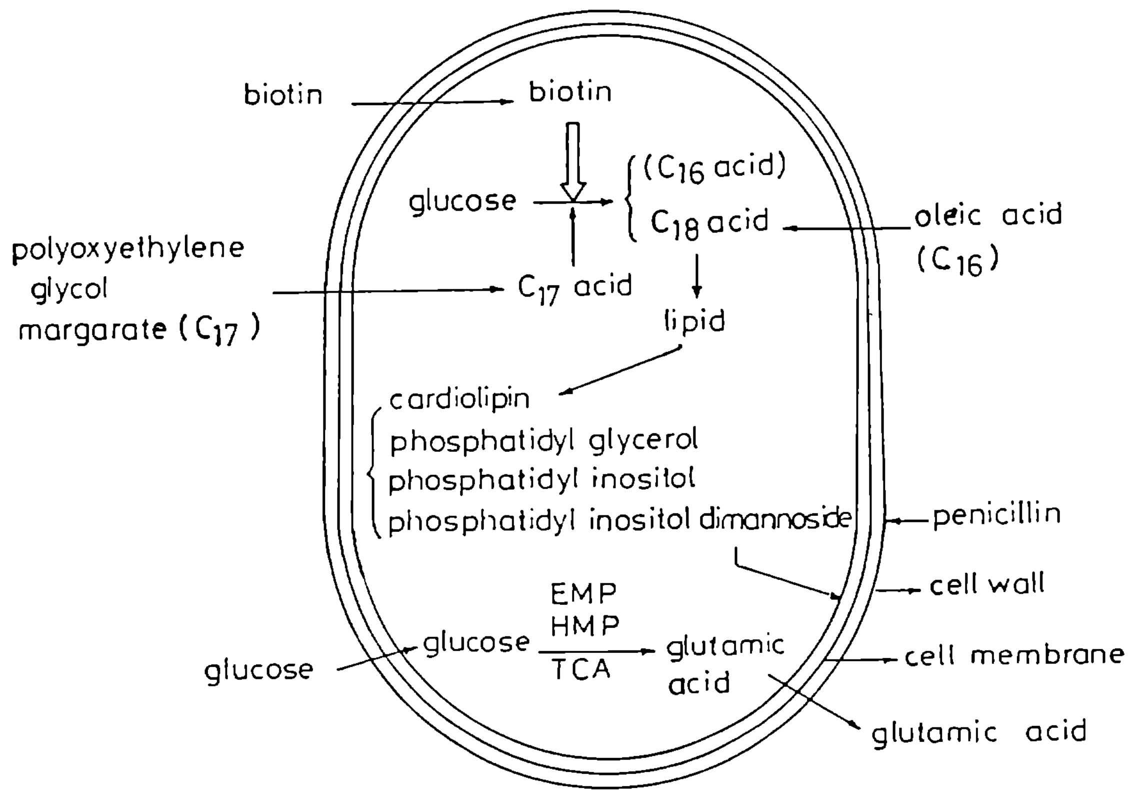


Fig.A2 Mechanism of L-glutamic acid excretion by altering cell permeability[193]

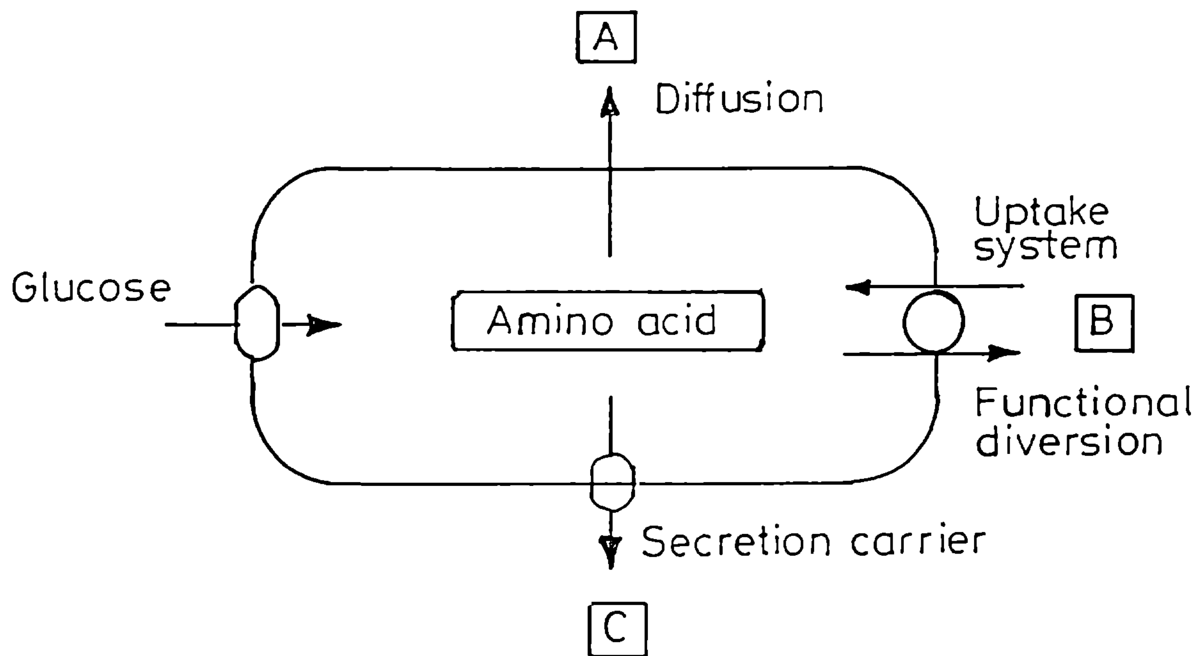


Fig.A3 Three methods (A-C) explaining the mechanisms of amino acid secretion[170]

Recently, rather convincing experimental evidences were presented showing the existence of a specific glutamate carrier for excretion because several of their observations did not agree with either 'leakage' or the 'inversion' model[90-94]. Some of the important findings are,

(1) *C. glutamicum* cells were able to export glutamate against an existing gradient, which makes it unlikely that the internal/external glutamate ratio can be the driving force for glutamate efflux[90].

(2) Glutamate export proceeded even in the absence of a membrane potential[90].

(3) The transport of glutamate was not coupled with the movement of H^+, K^+ or Cl^- ions, as would be expected in the leak model[90].

(4) No transport of amino acids other than glutamate was observed. It shows the carrier specificity[91].

(5) No changes were observed in the fatty acid of phospholipid contents when the cells were switched between non-producing and producing states[92].

(6) No specific membrane component that regulated glutamate efflux[92].

(7) The observed differences between the excretion and uptake rates ruled out inversion of the uptake system[96,97].

(8) No change in anisotropy of membranes was observed during any period of fermentation. Hence Neuback *et. al* (1993)[95] concluded that glutamate secretion was not coupled to differences in membrane facility.

(9) Plakanov *et al.*(1989)[94] referred to a specific secretion system common to glutamic acid and tetracycline.

(10) Gutmann *et al.* (1992)[90] determined that there was a positive correlation between the secretion rate and the intracellular ATP pool, indicating that ATP or a high energy compound would be involved in the activity of the secretion system.

(11) Presence of ABCD gene cluster encoding the Glutamate uptake system of *C. glutamicum*, was reported by Kronemeyer *et al.* (1995)[98]. But no component of the ABCD is involved in export.

Any way, isolation and characterization of the specific glutamate carrier, cloning and characterization of the gene(s) encoding the glutamate transport system are necessary to provide further evidence for the active transport model.

(vi) Glutamic acid Production from Non-carbohydrate Materials

After the realisation of the production of L-glutamic acid based on glucose as substrate by Kinoshita et al., increasing efforts were going on to replace the starting materials with cheaper, more constant supplies. Table A4 shows some of the non-carbohydrate materials tried as the carbon source for L-glutamic acid

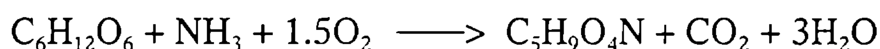
production. But none of them were not good as that of carbon sugars and moreover, in most cases the wild strains were not able to utilize these substrates effectively. So the corresponding auxotrophic mutants were required and obviously it was a tedious process with comparatively little profits.

Table A4. L-glutamic acid production from non-carbohydrate materials

Substrate	Microogranisms	References
Acetic acid	*B. flavum* *B. thiogenitalis*	99-102
Hydrocarbons	*C. hydrocarboclastus* *A. paratineus* *C. alkandyticum* *C. petrophilum*	103,164
Methanol	*M. methylovora*	105
Ethanol	*Brevibacterum* sp. B-136	106
Ethanol	*C. alkanolyticum*	107
Oleic acid	*B. thiogenitalis*	108
α-Ketoglutaric acid	*PS. ovalis* *Erwinia, Bacillus,* *Micrococcus, Aerobacter,* *Aspergillus, Aeropus*	110
l-Amino butyric acid fumaric acid	*B.pumilus*	111
DL-hydration-5-propionic acid	*B. brevis*	112
2-Furonic acid	*Pseudomonas* sp. 5839	113
DL-pyrrolidane carboxylic acid	*P. alealigenes*	114

(vii) By-products of Glutamic acid Fermentation

Theoretically, the yield of glutamic acid from glucose is 81.74% (w/w)[9]



$$C_6H_{12}O_6 + NH_3 + 1.5O_2 \longrightarrow C_5H_9O_4N + CO_2 + 3H_2O$$

This represents a 100% molar conversion or 81.7% weight conversion of sugar to glutamic acid. As many byproducts and as high cell mass are formed, the yield is generally lower.

There are numerous factors which can change the course of glutamic acid fermentation, including biotin or ammonium ion concentration. Various organic compounds, dissolved oxygen concentration, pH and temperature as shown in Table A5.

Table A5. Fermentation conversion of gutamate-producing bacteria by various environmental factors

Factor	Fermentation conversion	References
Oxygen	Lactate or succinate <——> glutamate (insufficient aeration) (adequate aeration)	15
NH_4^+	a-Ketoglutarate <——> glutamate (lack) (neutral or weakly alkaline)	116
pH	N-acetyl glutamate <——> glutamate (neutral or (acidic) weakly alkaline)	117
Biotin	Lactate or succinate <——> glutamate (saturated) (lack)	116
Temperature	Lactate <——> glutamate (37°C) (30°C)	10

(viii) Recent attempts in L-glutamic acid Fermentation

The natural excretion of L-glutamic acid is an exceptional phenomenon. All other amino acids can only be produced in mutants with distinct alterations in particular biosynthetic pathways or in their regulation[118]. Earlier, mutant selection was mainly based on selection of blocks in unwanted pathways and feedback deregulated alterations in the allosteric forward enzymes via suitable amino acid analogues[34]. Recent developments in recombinant DNA technology has made possible genetic recombination between different species, and the increasing of the biosynthesizing activity of a microorganism by increasing the gene copy member or improving the micro-organism in relation to the substrate and environmental conditions, such as temperature. Some applications of the technique to amino acid production has been reported already[119-121].

Breeding programmes today largely involve genetic engineering for the detection and manipulation of the key-step biosynthetic enzymes, a large number of genes which have already been cloned and analysed[122-126]. Several kinds of plasmids of Brevibacterium and a plasmid of corynebacterium relating to spectinomycin resistance were found to be suitable as a possible vector system[127-130]. Construction of a chimera plasmid involving a gene associated with L-glutamic acid biosynthesis was performed with *B. lactofermentum*[130]. The plasmid PAM 330 is a cryptic plasmid in *B. lactofermentum* which is being used industrially for the fermentative production of various amino acids including

L-glutamic acid. This plasmid has some desirable features as a cloning vector such as multicopies and the presence of several unique restriction sites.

To increase glutamic acid yield, a recombinant DNA molecule was constructed in a *C. glutamicum* consisting of a plasmid PCG4 (29 Kb), encoding streptomycin resistance (Smr), and spectinomycin resistance (SPCr) a high copyno plasmid PCG_1 (3.0 Kb) or PCG_2 (6.6 Kb) which were isolated from *C. glutamicum*[131]. A technique for protoplast fusion was established in *B. flavum* to obtain a recombinant using nutrient requirements, rifampicum resistance and streptomycin resistance as a selection marker[132]. Thus, success is likely in the near future with respect to development of host-vector systems in glutamic acid bacteria and cloning of genes for amplifying particular enzyme function that are limiting steps in the biosynthetic pathways of desired amino acids. A few recent reports are there regarding the breeding of glutamate producing strains using the protoplast fusion technique[133-135]. Electroporation is now widely used as a more convenient and much more efficient method of transformation. It is more reliable, rapid and not strain specific. A number of factors including pulse voltage, amount of DNA and cell concentration can affect the efficiency or transformation[136,137].

As useful mutants, an oleic acid auxotroph[108], a penicillin resistant mutant, a glycerol auxotroph[139] have been successfully employed for L-glutamic acid production. Another approach was focused on the developments of thermophilic bacteria. A temperature sensitive mutant, was derived from *B. lactofermentum* No.2356. This mutant strain produced glutamic acid when temperature was shifted

from 30°C to 37°C during cultivation[140]. It was concluded that by controlling only temperature during fermentation, 'physical control method', glutamic acid production could be realized. A strain of *C. thermo ammonogens* is reported to accumulate L-glutamic acid at a temperature above 43°C[141]. Further strain development resulted in mutants either having sensitivity in cell permeability or having the capability of increased carbon dioxide fixation or having a too low ion activity level in pyruvate dehydrogenase[141-143].

A new type of fermentation process was reported which uses a strain to overproduce L-glutamic acid and L-lysine simultaneously[144]. The cultivation of an auxotropic regulatory mutant of *B. lactofermentum* in a medium supplemented with polyoxyethylene sorbitan amonopalmitate as surface active agent resulted in the accumulation of both lysine and glutamic acid.

A multi-step inoculation procedure followed by the fed-batch mode in the main fermentation is still the preferred technology for the production of L-glutamic acid. Alternative processes such as production in air lift fermenters[145-148], fluidized fermenters[149,150], or by cell recycling techniques[151] are not competitive so far. Amin *et al.* (1994)[152] designed a vertical rotating immobilized cell reactor of the bacterium *C. glutamicum* for the continuous production of glutamic acid in a three phase fluidized bed reactor. The limited oxygen transfer rate was the major disadvantage of the fluidized bed process.

An aerobic cross-flow filtration system for cell recycling was developed with the aim of accomplishing glutamate fermentation with built-in electrodialysis. Cell

recycling glutamic acid fermentation using two different types of cross-flow filtration units with air supply was associated with a remarkable recovery in the rate of substrate consumption and glutamate fermentation as compared to cell recycled fermentation without the supply of air which gave very poor results[151].

Moreover, there might be a potential for improvements in L-glutamate fermentation by employing techniques such as computer supported control of the process[153-156], Fuzzy expert systems[153,155,156] and simulation of relevant effects in fermentation such as the simulation of the effect of mixing, scale up and pH value regulation during glutamic acid fermentation[157,158].

Methods for extraction and purification of amino acids from fermentation broths were described by Samajima[159] The application of biosensors are very promising in glutamic acid fermentation[160,161]. Specific enzyme electrodes were already desired and widely used for detecting and quantifying the amount of glutamic acid present in the fermented broth.

(iv) Applications of L-glutamic acid

(a) As a Flavour enhancer

Dr. Ikeda in 1908 discovered the flavour-intensifying property of Monosodium glutamate ($C_5H_8NO_4Na.H_2O$)[13]. It is crystalline white, transparent and very soluble in water (73% w/v). Now it is used commercially as a flavour enhancer, usually in combination with nucleotides inosinate, to provide an expansion and extension of taste in processed food such as soups, biscuits, noodles, chinese foods, meat and vegetable processing etc. Eventhough there are three types of monosodium

glutamate, the L, D, and LD, type but only the L-type has the flavour intensifying property. The concentration of monosodium glutamate used in salted food is generally between 0.2-0.5%. MSG is also used as an intermediate in the manufacture of Folic acid, an important vitamin. Glutamic acid mother liquor in MSG production is being used in the manufacture of sauce and as soil conditioner, fertilizer etc.

In the minds of many, the safety/toxicity of monosodium glutamate (MSG) is considered a controversial subject. MSG is a natural component in many foods such as mushrooms, tomatoes and peas. The free glutamate consumed daily as MSG typically equals about 1/1000 of the total glutamate present in the body. The main use of MSG is as a food ingredient and so its safety when used in the diet is the most important aspect of its safety for use.

The turnover of MSG in the body is approximately 5-10 g per hour since it is readily transaminated to α-ketoglutarate, which is used in the Kreb's cycle for conversion to energy in several organs[162]. Unrealistically large doses can elicit mild transient sensations such as light-headedness, stiffness/tightness/weakness of the limbs and warmth and burning of the skin, face or scalp in few cases[163]. The demonstration that injected or force-fed neonatal rodents given extremely high doses of MSG showed evidence of brain lesions had led to much additional research to determine any possible link between neurotoxicity and human use of MSG. However, no evidence from animal tests indicate that MSG in the diet causes brain damage in humans. Both short term and chronic toxicity studies on MSG in the diet of several species of doses upto 4% (approx 6-8 g/kg body wt per

day) showed no special toxic effects and no evidence of carcinogenicity and mutagenicity[164].

The joint expert committee on Food Additives (JECFA) of the United Nations and Agricultural organisation (FAO) and the world health organization (WHO) have placed MSG in the most favourable category for a food ingredient[165]. The institute of Food Technologies (IFT) of USA also sees no health hazard from MSG. IFT acknowledges that some persons indeed report adverse reactions to MSG (such as chinese Restaurant Syndrome) that these are the exceptions and not the rule.

Ajinomoto Co. Inc. in Japan, one of the leading manufacturers of MSG in the world have clarified that MSG has not been banned in any country in the world nor is any ban contemplated. Following the recommendations of JECFA, the Codex Alimentaries Commission of the FAO/WHO of the United Nations have classified MSG and the ammonium, calcium and potassium salts of L-glutamic acid as being toxicologically cleared for use in food with the exception for infants below 12 weeks of age[165]. In Western countries, it is used as a flavour enhancer and in Japan, plain MSG is marketed only to the food processing industries as an additive. Table salt is coated with 10% powdered MSG to improve the caking tendency of the salt and to enhance the flavour.

(b) In Pharmaceutical industry

In the Pharmaceutical industry amino acids are required in high quality at a rate of more than 5,000 t a^{-1}. Many therapeutic agents are derived from natural or non-natural amino acids. L-arginine and its salt with L-glutamic acid is effective in

the treatment of hyperammonemia and hepatic disorders. Parenteral nutrition with L-amino acid infusion solution is a well-known component of clinical pre- and post-operative nutrition therapy. Besides the essential amino acids, a standard infusion solution contains L-glutamic acid[166].

(c) As a Neurotransmitter

All organs in the human body contain MSG as it is found in the blood stream at about 5-9 μmol/100 ml[167] By far, the highest levels are found in parts of the brain at about 100 μmol/100 ml[168]. The brain synthesises its own glutamate from glucose. This is an excitatory neurotransmitter, namely We need glutamate (or MSG if sodium is considered to be the counter ion) to think a fact which is worthwhile pondering.

L-glutamate produces excitation of practically all cells tested in the mammalin Central Nervous System (CNS). Its depolarising action on neuronal membrane is associated with an increase in both Na^+ and K^+ conductance, which may result in turn from displacement of Ca^{2+} from critical sites at the neuronal membrane where certain permeability is controlled. Its concentration is particularly high at certain sites, including the forebrain, cerebellum and dorsal roots and dorsal gray matter of the spinal cod.

(d) Miscellaneous Applications

N-acetyl glutamate is commercially available as a biodegradable surfactant with low skin irritation properties which is valued as an additive in cosmetics, soaps and shampoos[169] Oxopyrolidine carboxylic acid, another derivative of L-glutamic acid, is used as a natural moistening factor in cosmetics. Amides of acyl glutamate are utilized as gelatinizing agent and have application in oil dispersion for marine antipollution purposes.

A.III SIGNIFICANCE AND OBJECTIVES OF THE PRESENT INVESTIGATION

The commercial applications of L-amino acids and especially that of L-glutamic acid were already mentioned in detail. Recent literature survey shows that amino acids that enter new markets with a growing demand will benefit from the existing experiences and the potential that fermentation technology can offer today. The whole market is estimated to amount to about 3 billion US$ in 1995, covering 38% for food, 54% for feed and 8% for other applications such as medicine and cosmetics[170].

Table A6. Sample importers of Glutamate - 1995[172]

Name of the importer	Quantity in Kg	CIF value in Rs.	Country
The Anglo French Drug Co.	1,000	1,63,471	Japan
Energy Pharma Ltd.	20,000	8,12,571	China
Kunal Impex	20,000	7,64,772	Taiwan
Kumal Impex	20,000	6,62,802	France
Themis Chemicals Ltd.	20,000	7,18,803	Taiwan
General Traders	5,443	95,125	Indonesia
Sha Shantilal indermall	2,177	78,160	Indonesia
Union Trading Co.	1,814	65,127	Idonesia
Dujodwala Resins & Terpene Ltd.	17,500	5,63,222	South Korea
R.K. Chemicals	36,000	11,64,365	Taiwan
Madchl Chemical Pharmaceutical P Ltd.	20,000	7,62,223	China
Arandi Ltd.	17,840	9,58,020	Indonesia
Addlife Pharma Ltd.	17,000	8,39,656	South Korea
Kiran Kotak & Co.	16,000	7,73,250	South Korea
Themis Chemicals Ltd.	40,000	20,87,675	Taiwan

With the major markets in animal nutrition and food additives, the world wide demand for amino acid is increasing by 5-10%[171]. Glutamic acid and MSG are not produced in India and the growth rate in demand is 9-10% per annum[172]. Currently, the annual world-wide demand for MSG is more than 800,00 tonnes per annum and in India it is 500 tonnes per annum, which is met by imports only[172]. In early 1990's the total expenditure for importing amino acids, the country has spent about $41,656[173]. Tables A6 and A7 show some of the statistical data regarding the import of L-glutamic acid (or MSG) during 1995-96 at Bombay and Madras ports.

Table A7 Sample importers of Glutamate - April 1996[174]

Name of the importer	Quantity in Kg	CIF value in Rs	Country
Deluxe Sale Corporation	8,500	4,43,800	Republic Peoples of Korea (RPK)
Deluxe Sales Corporation	8,500	4,43,800	RPK
Neo Sack Ltd.	17,000	9,99,300	RPK
Global Impex	35,412	18,04,500	Indonesia
Madchl Chem. & Pharm. Ltd.	17,000	9,00,600	RPK

In order to produce L-glutamic acid indigenously and to reduce the dependence on the imported supply of this amino acid, much attention is required on the R&D, especially on microbiology (isolation and maintenance of potent strains), regulation (characterization and manipulation of biosynthetic pathways) and bioprocessing engineering (optimization of process conditions, suitable down stream steps etc) for this.

The present work was undertaken by us to study the process of L-glutamic acid production using a strain of *Brevibacterium* sp. (DSM 20411).

The specific objectives were:

(1) To optimize the growth conditions for *Brevibacterium* sp. (DSM 20411)

(2) To evaluate and optimize the physical, chemical and biological requirements for the production of L-glutamic acid

(3) To optimize the immobilization parameters for *Brevibacterium* sp. from the view point of long term utilization of biocatalysts and continuous operation of stabilized system for L-glutamic acid production.

(4) To cultivate *Brevibacterium* in solid cultures and to evaluate its efficiency for glutamate production.

(5) To utilize newer raw materials for L-glutamic acid production from the stand point of savings in production cost.

(6) Recovery and purification of glutamic acid from the fermented broth using chromatographic techniques.

The details of these experimental studies and their results and comments are discussed in Section B (Experimental) as successive chapters.

SECTION B

B.I MATERIALS AND METHODS

This chapter deals with the materials and methods (Analytical and Experimental) which were most common and repeatedly used throughout this work. In addition, each of the experimental chapter has its own materials and method, where the specified materials, experimental and analytical details have been mentioned.

MATERIALS

Glasswares and Chemicals

All glasswares used were of corning glass. All the chemicals used in the analytical methods and the complex nutrient components used for the preparation of microbial media were of analytical grade with maximum available purity supplied by Hi-media (Bombay), BDH, E-Merck (Bombay), CDH (New Delhi), Sd. Fine Chemicals (Bombay) SISCO (Madras), Ranbaxy (Ahmedabad), Nice (Cochin); Corn steep liquor and Tweens were procured from Sigma, USA.

METHODS

Microorganism: A strain of *Brevibacterium* sp. (DSM 20411) was used throughout in the studies. It was procured from DSM-Deutsche Sammlung von Mikroorgnismen und Zellkulturen GmbH, Germany.

Growth medium: Agar slants or plates were prepared to maintain the pure culture for a short period of 2-3 weeks. It was grown on a medium containing (g/l) casein

peptone 10, yeast extract 5, glucose 5, sodium chloride 5, agar powder 5 and distilled water 1 L (pH 7.2-7.4).

Culture preservation: For short term preservation, the agar slants were stored at 4°C in a refrigerator.

Long term preservation was carried out in glycerol. For that, 20 ml of a 20 h old culture was centrifuged (8000 g for 10 meter) and the cell mass was resuspended in a minimum quantity of sterile water and it was mixed (1:1) with pre autoclaved glycerol (50 %) contained in a screw caged polythene tube. Once again it was thoroughly mixed and the mixture was properly sealed and preserved at -20°C.

Inoculum and Incubation: A loopful of cells from a freshly grown culture of *Brevibacterium* sp. were transferred to 250 ml conical flask containing 50 ml growth medium as above. The flask was incubated at 30±1°C for 20 h with 180 rpm. This was used as inoculum. This was used to inoculate agar slants for preservation also.

Fermentation

Unless and otherwise mentioned, fermentation was carried out by taking 50 ml of the liquid medium, in 250 ml Erlenmeyer flask. After inoculating (20 h old culture, 5% v/v inoculum size), the flasks were incubated at 30±1°C for a stipulated period on a rotary shaker at 180 rpm. Samples were withdrawn as whole flask at

desired time intervals for analysis. The results reported are the average of four sets of experiments.

Gram staining[175]

Using bacterial suspensions of different ages, few smears were prepared on a clean dry glass slides. After drying the smears were stained with crystal violet for a minute. After draining out the excess stain and rinsing the slide in tap water, the slides were flooded with gram's iodine solution and allowed to remain for half a minute. The slides were then rinsed in tap water and decolourised in alcohol. In the next step, the slides were counterstained with safranin for 30 seconds. Then once again rinsed in tap water and blot dried in air. Properly dried slides were then examined under the oil-immersion objective of a compound microscope.

Cell count: Viable cells in the growth medium was determined by plate-count (colony count) technique using a colony counter. In this method, 1 ml of an appropriately diluted (10^1-10^8) cell suspension was poured into pre-sterilized petri plate. Autoclaved growth medium containing agar (40°C) was poured into the petri dish containing the cell suspension and was thoroughly mixed with the inoculum by rotating the plate. After solidification of the medium, the plate was inverted and properly sealed and incubated for 24 h at 30±1°C. The number of colonies developed on each plate were counted using a colony counter.

Determination of Bacterial Growth

(a) By turbidimetric method: A known volume of bacterial suspension (fermented broth) at required time intervals was collected and after adequate dilution the absorbance at 610 nm (optical density, OD) was noted in a UV spectrophotometer.

(b) By dry weight method: For this purpose, 1 ml of the bacterial suspension was transferred into a clean dry Eppendouf tube which was previously weighed. The sample was centrifuged using a microcentrifuge. The supernatant was decanted completely and the tube with the pellets was dried in the hot air oven at 80°C for over night. The weight of the tube was noted again and the weight of pellet after drying was calculated.

Assay Methods

DNS (Dinitro salicylic acid) method for the determination of reducing sugars[176]

Materials

(a) Dinitorsalicylic acid reagent: DNS reagent is prepared by dissolving (by stirring) 100 g potassium sodium tartarate, 0.25 g sodium sulphate, 1.0 g phenol and 5 g 3,5-dinitrosalicylic acid in 250 ml 2N NaOH solution and diluted to 500 ml with distilled water.

(b) Standard glucose solutions

Procedure

A known quantity (50 μL, diluted if necessary) was taken in a 25 ml test tube and made up to 2.5 ml with distilled water, to this 3 ml DNS reagent was added and thoroughly mixed. The contents were heated on a boiling water bath for 12 minute. After cooling, 16 ml distilled water was added and the contents were mixed well and the colour developed was read at 540 nm against the blank using a spectrophotometer.

The amount of reducing sugars present in the sample was calculated using a standard graph obtained by using various known concentrations of glucose.

Phenol-sulphuric acid method for total carbohydrates[177]

Materials

(a) 5% phenol

(b) Conc. sulphuric acid

(c) Standard glucose solutions

Stock - 100 mg in 100 ml distilled water

Working standard - 10 ml of stock diluted to 100 ml with distilled water

Procedure

Pipetted out 500 μL of adequately diluted sample into a clean dry test tube and made up to 1 ml with water. Added 1 ml of phenol solution (5%) to each tube followed by 5 ml sulphuric acid. While adding sulphuric acid, the tubes were kept

bath at 25-30°C for 20 minutes. The blank solution was prepared by taking 1 ml distilled water. The colour developed was read at 490 nm using a spectrophotometer.

Protein estimation by Lowry's method[178]

The method developed by Lowry *et al.* is sensitive to give a moderately constant value and hence largely followed.

Materials

(a) 1% copper sulphate solution (A)

(b) 2% sodium potassium tartarate (B)

(c) 2% sodium carbonate in 0.1 N sodium hydroxide (C)

An alkaline mixture solution was prepared by mixing A, B and C in the ratio 1:1:98.

(d) Folin-Ciocalteau reagent: The commercially available reagent solution was diluted with equal volumes of distilled water before use.

(e) Protein solution: weighed accurately 50 mg of bovine serum album and dissolved in distilled water and made upto 50 ml in a standard flask.

Procedure

Pipetted out 100 μl of adequately diluted sample in to a clean dry test tube and made up to 1 ml. A tube with 1 ml of water served as the blank. 4.5 ml of alkaline mixture solution was added in to each tube and mixed well. The solution was allowed to stand for 10 minutes. 0.5 ml Folin's reagent was added and thoroughly

mixed and incubated at room temperature in the dark for 30 minutes. The blue colour developed by the reaction mixture was measured at 660 nm using a spectrophotometer.

A standard graph was prepared by using a series of known concentration of standard BSA and the concentration in the sample was calculated by comparing with it.

Qualitative Analysis of L-glutamic acid

Thin layer chromatography (TLC)[179]

For preparing silica gel plate, 25 g silica gel G (Sigma, USA) and 50 ml chloroform/water (1:1) in a stoppered 250 ml Erlenmeyer flask were mixed with vigorous shaking for 30 minutes. The suspension was poured on to the plates which were air-dried overnight. The dry layer has a thickness of about 0.15 mm. Fermented sample (1 ml) was centrifuged in a Eppendouf centrifuge and the supernatant (10μl) was spotted on the silica plates prepared as above. L-glutamic acid (1 mg/ml with 10% n-propanol) was used as standard for comparison.

A solvent mixture of n-butanol:glacial acetic acid: water (4:1:1 v/v) was used as the mobile phase. Spots were detected by sprayiing the plates with ninhydrin/acetic acid/butanol mixture (300 mg ninhydrin, 3 ml glacial acetic acid and 100 ml n-butanol). The sprayed plates were kept for 30 minutes at 60°C or 10

minutes at 110°C. The developed spots were compared with that of standard sample (by calculating the R_f values).

In addition to TLC, paper chromatography[180] was also conducted to confirm the presence of L-Glutamic acid in the supernatant. The whole procedure was same as that of TLC. After the run, the paper was removed and allowed to dry. Ninhydrin reagent was sprayed evenly to the paper and then it was dried using a hair dryer. The developed spots were compared with that of the authentic standard sample. The R_f value was calculated.

Quantitative determination of L-glutamic acid[181]

When the culture supernatant was analysed qualitatively by TLC and paper chromatography, it showed only one spot which was identical to the authentic sample of L-glutamic acid. Hence, the ninhydrin colour reaction method was used for the quantitative estimation of L-glutamic acid.

Reagents

(a) Ninhydrin: commercially available ninhydrin powder was used.

(b) Ninhydrin solution: 0.80 g of reagent $SnCl_2.2H_2O$ (stannous chloride) was dissolved in 500 ml of the citrate buffer. This solution was added to 20 g of ninhydrin dissolved in 500 ml of methyl cellosolve. A small quantity of ninhydrin solution was prepared freshly according to the need as per the above mentioned concentration.

(c) 0.2 M citrate buffer (pH 5.0): 21.008 g of reagent grade citric acid monohydrate is dissolved in 200 ml of N NaOH and diluted to 500 ml.

(d) Methyl cellosolve: Commercially available methyl cellosolve was used. 1:1 diluted solution was prepared with distilled water before use so as to get a clear solution.

(e) Diluent solvent: Equal volumes of water and reagent grade n-propanol were mixed.

Procedure

100 μl of the sufficiently diluted sample was pipetted out to a test tube containing 1.0 ml of ninhydrin solution. The tube was covered with an aluminium cap and the contents were mixed. The tubes were heated for 20 minutes in a boiling water bath. 5 ml of diluent was then added to each tube and properly mixed and kept for 15 minutes. The intensity of the blue colour developed was measured at 570 nm using a UV spectrophotometer. Appropriate correction for blank was made either by reading blank solutions against diluent and zeroing the instrument on the blank reading so obtained or by means of an appropriate blank solution in conventional manner.

The standard curve was prepared with 0.1 ml samples of L-glutamic acid at 6 concentrations from 0.5 to 2.0 mM.

Determination of the free amino acids in bacterial culture by the method of Yemm and Cocking[182]

Reagents

(a) 0.2 M citrate buffer (pH 5.0) - 21.0 g of citric acid monohydrate is dissolved in 400 ml 0.5 N NaOH and the solution diluted to 500 ml.

(b) Commercially available methyl cellulose

(c) 0.01 M potassium cyanide - 0.1628 g of potassium cyanide was dissolved in 250 ml distilled water

(d) 0.25 N perchloric acid

(e) Ninhydrin methyl cellosolve 5 g Ninhydrin is dissolved in 100 ml methyl cellosolve

(f) Aqueous ethanol 60% (v/v)

Procedure

A known volume (10 ml) of a washed bacterial suspension was centrifuged and the pellets were resuspended in 0.25 N perchloric acid (1.5 ml) at 4°C for 3 minutes. The suspension was centrifuged (8000 g, 10 minutes) at 10°C and the supernatant fluid decanted into 5 ml graduated tubes. The residue was re-extracted with 0.25 N perchloric acid (1.0 ml), the suspensions centrifuged and the supernatant fluid added to the first extract. The combined extract was neutralised with 1N NaOH to pH 5.0 and diluted with water to 5 ml. Neutralised extract (0.5 - 1.0 ml) was mixed with citrate buffer (0.5 ml), ninhydrin reagent (0.2 ml) and

KCN-methyl cellosolve (1 ml) in a test tube. The tube was stoppered, heated vigorously in a boiling water bath for 15 minutes and cooled in cold water for 5 minutes. The solution was diluted with aqueous ethanol (3 ml) and well mixed. Absorbances were measured at 570 nm against a blank of distilled water. A standard curve was made by using various concentrations of alanine (2-20 μg). The test values obtained in terms of alanine were converted into α-amino nitrogen concentration by multiplying the values by 14/89. Free amino acid concentration was expressed as micromoles/g dry weight of bacterial cells.

Colorimetric determination of lactic acid[183-185]

Reagents

(a) 20% solution of $CuSO_4.5H_2O$

(b) 4% solution of $CuSO_4.5H_2O$

(c) Solid calcium hydroxide

(d) Concentrated sulfuric acid

(e) 1.5% solution of p-hydroxydipiphenyl in 0.5% NaOH

Procedure

To 1.0 ml of aliquot, added 0.1 ml of 4% $CuSO_4.5H_2O$solution and 6 ml of conc.H_2SO_4 through the walls of the tube and mixed well. All the tubes were kept in boiling water bath for 5 minutes and were cooled to below 20°C. To each tube parahydroxy diphenyl was added (0.1 ml) and mixed thoroughly so as to get uniform solution of precipitated reagent. The tubes were kept at 30°C for 30

minutes. During the incubations, tubes were shaked well for 2-3 times. After incubation excess reagent was dissolved by heating the tubes in boiling water bath for 90 minutes. The tubes were cooled and noted the optical density at 560 nm. A standard graph was prepared using different concentrations of lactic acid (1-10 mg). The test values were compared with this standard graph and calculated the concentration of lactic acid in each sample.

INSTRUMENTAL METHODS OF ANALYSIS

(i) Optical microscopy

Polarised optical microscope (Nikon, HFX, Japan) was used to observe the bacterial cells after staining. Stained bacterial cells of different ages were observed and the morphology of the cells were studied. The bacterial cells were photographed using a camera attached to the microscope.

(ii) Scanning Electron Microscopy (SEM)

The SEM studies were carried out using a Jeol Model 35 C scanning electron microscope of an accelerated voltage of 15 KV or 12 KV. The specimen used for this studies include cross sections of calcium alginate beads with entrapped bacterial cells, bacterial cells growing on the surface of an inert solid substrate and glutamic acid crystals in the broth. The dehydrated specimen (after a series of alcoholic treatment with different percentages of ethyl alcohol) were coated with gold (100 A°) and scanned.

Instruments used

A number of instruments were oftenly used for conducting the work successfully. They are listed below:

Scanning Electron Microscope	Jeol, JSM-35C Japan
Polarised Optical Microscope	Nikon, HFX, Japan
IR spectrometer	Perkin Elmer model 882
UV-spectrophotometer	Shimadzu, UV-16A, Japan
Fermenter	BIOFLO III, New Brunswick Scientific, Edison, NJ, USA
Laminar Flow Chamber	Thermodyne, HCL-104, India
Cooling Centrifuge	Remi Rm-12C, India
Incubator shaker	MB-orbit-Environ Shaker,SK-1009R, India
Incubator shaker	Certomat, MO, Germany
Incubator	MB, Laboratory incubator, DTC-1, India
Weighing Balance	Mettler, PM-200, Switzerland
pH meter	Systronics, pH system 361, India
Colony Counter	Lapiz, India
CO_2 indicator	Riken Keiki Co Ltd. RI-411 A, Japan
Hot air oven	Kemi, India
Peristaltic pump	Eyela Microtable pump, MP-S, Japan
Waterbath	Superfit, India
Magnetic stirrer	Remi, ZMLH, India
Vortex Mixer	Superfit, VM 301, India
Ultrafitration unit	Millipore, USA

B.2 OPTIMIZATION OF GROWTH AND PRODUCTION CONDITIONS OF *BREVIBACTERIUM* SP. IN LIQUID MEDIA

Introduction

The constituents of a growth medium for microorganisms must satisfy the elemental requirements for cell biomass and metabolate production and there must be an adequate supply of energy for biosynthesis and cell maintenance. Microbial cells continuously strive by modifying their environment to achieve and maintain the optimal conditions for their growth. Temperature, pH, dissolved oxygen and sufficient supply of substrate, nutrients, salts, vitamins are among the important factors for achieving optimum cell growth. The rate at which the carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites[186].

Attempts were made to study the growth of *Brevibacterium* sp. in media with different constituents and also the influence of physical, chemical and biochemical parameters on the growth and activity of the microbial culture.

Materials and Methods

Microorganism and growth medium: A strain of *Brevibacterium* sp. (DSM 20411)[187] was used in this study. Culture was grown as mentioned in Section B1.

To study the general morphology and growth pattern, the organism was cultured by taking 20 ml of the above medium, but without agar in 100 ml Erlenmeyer flask. After sterilization (121°C, 15 minutes) the flasks were inoculated

with 20 h old culture (5% v/v inoculum size) and the fermentation was carried out for a stipulated period of time.

To view the morphology of the bacterial cell, cultures of different ages [from log (20 h) and stationary phases (48 h)] were used. The cells were properly smeared on a clean dry glass slides and after gram staining (see Section B1), they were observed under the oil immersion objective of a polarised optical microscope.

Bacterial growth was noted intermittently by turbidimetric method (see Section B1).

Formulation of Production Medium: In most of the amino acid fermentation studies, the components of the seed and production medium were the same except slight changes in the concentration[188]. Five different media (M1-M5) compositions were tried as shown in Table B1.

Table B1. Different media compositions used for L-glutamic acid production

Medium-M_1		Medium-M_2		Medium-M_3	
Glucose	2.0 g	Glucose	2.0 g	Glucose	2.0 g
$NaNO_3$	0.5 g	Peptone	0.5 g	Urea	0.5 g
KH_2PO_4	0.18 g	KH_2PO_4	0.18 g	KH_2PO_4	0.18 g
TH	10 μg	Yeast extract	0.5 g	TH	10 μg
MS	1 ml	TH	10 mg	MS	1 ml
		MS	1 ml		

Medium-M_4		Medium-M_5	
Conc. molasses	2% (v/v)	Glucose	2 g
$NaNO_3$	0.5 g	$NaNO_3$	0.5 g
KH_2PO_4	0.18 g	KH_2PO_4	0.18 g
TH	10 μg	TH	10 μg
		CSL	0.1% (v/v)
		Tween-80	0.01% (v/v)

Dissolved in 100 ml distilled water; pH 7.2)

TH — Thiamine hydrochloride
CSL — Corn steep liquor
MS — Mineral solution which contains $FeSO_4.8H_2O$, $MnSO_4.4H_2O$, $MgSO_4.4H_2O$, $ZnSO_4.6H_2O$ and NaCl each 1 mg

Optimization of Physical, Chemical and Biochemical Parameters

Effect of pH: pH of M5 was set at different initial values (pH 4, 5, 6, 7, 8, 9, 10 and 11). 20 ml of the medium was used for the experiment. It was inoculated with 20 h old culture and the fermentation was carried out at 30±1°C on a rotary shaker (180 rpm) as mentioned earlier for a desired time interval.

Effect of temperature: For studies on effect of temperature, after the inoculation, the flasks (with 20 ml M5 medium) were incubated at different temperatures (20, 25, 30 and 35°C) for a stipulated period on a rotatory shaker agitated at 180 rpm.

Effect of ratio of volume of the medium and flask: For the study, two different types of sets were prepared. In one set, the volume of the medium (M5) was kept constant at 20 ml with variation in the volume of flasks from 100 ml to 500 ml. In another set, the volumes of the medium (M5) were 50 and 100 ml in 250 and 500 ml flasks, respectively. After inoculation, the flasks were incubated at 30±1°C on a rotary shaker with an rpm of 180 for a desired period of time.

Effect of agitation: For studies on the effect of agitation, fermentation was carried out at four different agitation speeds (60, 180, 350 and 500 rpm). Flasks were prepared by taking 50 ml medium in 250 ml flasks, inoculated and incubated at 30±1°C (180 rpm) for a stipulated period of time.

Effect of inoculum size: 50 ml M5 medium in 250 ml conical flask was inoculated with different volumes (0.5, 2, 5 and 10% v/v) of 20 h old culture. Fermentation was carried out for a stipulated period of time at 30±1°C at 180 rpm.

Effect of different carbon sources: To study the effect of different carbon sources, in M5 medium, glucose was replaced individually by fructose, sucrose, lactose, maltose, xylose and starch at 2% w/v. Mixed substrate fermentation was also tried where glucose was partly replaced with fructose or sucrose (1% glucose + 1% fructose or 1% sucrose). Apart from these carbon sugars, two non-carbohydrate carbon sources, sodium acetate (1%) and glycerol (1%), were also tried to see the efficacy of the organism to utilize these substrates.

Effect of corn steep liquor and yeast extract: Different concentrations of corn steep liquor (0.05, 0.1, 0.25, 0.5 and 1%) was used in M5 to see its effect on microbial growth and glutamic acid production. To see the effect of yeast extract the corn steep liquor in M5 was replaced with various concentrations (0.5, 1 and 2%) of yeast extract.

In all experiments, samples were withdrawn at desired time intervals as whole flasks and the results reported are the average of four sets of experiments.

Analytical Methods

The bacterial growth was determined by measuring optical density of culture broth at 610 nm. Soluble sugars were analysed by DNS method. Phenol sulphuric acid method was applied for the detection of total carbohydrates in the samples. Lactic acid was determined by colorimetric method. Thin layer chromatography and paper chromatography were used for the qualitative determination of glutamic acid. Quantitatively, it was estimated with Ninhydrin colour reaction by measuring the absorbance at 570 nm. Detailed description of all these procedures are given in Section B1.

Resutls and Discussion

Morphology and Growth pattern

Bacterial cells were observed through the microscope as shown in Figures B1 (a) and (b). Cells in young cultures (exponential phase) were irregular rods of variable length but in general ~ 0.6-1.0 μm in diameter. Cells were arranged singly

A (18 h)

Fig.B1. Photomicrographs of *Brevibacterium* cells stained with grams stain.

B (48 h)

or in pairs and often at an angle to give 'V' formations. Cells from older cultures (48 h or more) were composed largely of coccoid cells. The presence of rod and coccus forms was a characteristic feature of the genes *Brevibacterium*[68]. When they were in the productive state, the cells became swollen rods. The cells tested are gram positive, non-sporulating, non-motile and non-flagellatted.

Fig. B2 shows the growth pattern of *Brevibacterium* sp. in defined media. Apparently, within 24 h of cultivation, the culture attained maximum cell density.

Effect of Medium Composition: Among the different media tried (Table B1), M2 and M5 showed comparatively better growth of the organism (Fig.B3). One possible explanation might be that these two media contained nutrient rich substances like yeast extract in M2 and corn steep liquor in M5. These nutrients might have provided the vital growth promoting substances which would have resulted in better growth.

Fig. B4 shows the level of L-glutamic acid accumulated in all the five media tried. A maximum of 1.13 mg/ml was obtained in M5 after 48 h fermentation. Both growth and production were seems to be the best in M5 and thus, in subsequent studies, only this medium was used.

In qualitative analysis using TLC, the culture filtrate (M5) developed only one spot which was identical with the authentic sample of L-glutamic acid (Fig.B5). Hence, for quantitative assay, we used Ninhydrin colour reaction method.

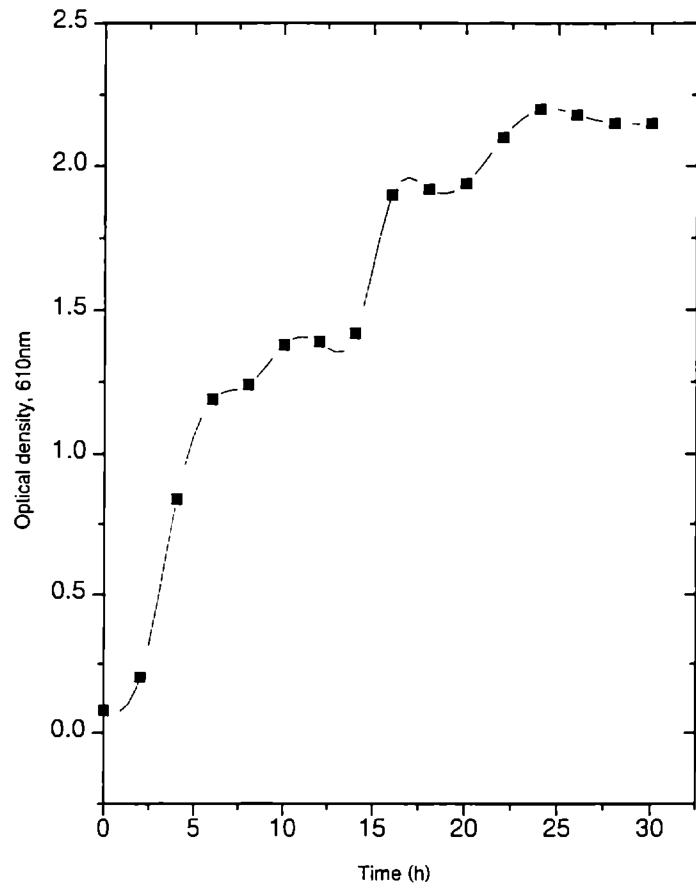


Fig.B2. Growth profile of *Brevibacterium* sp.

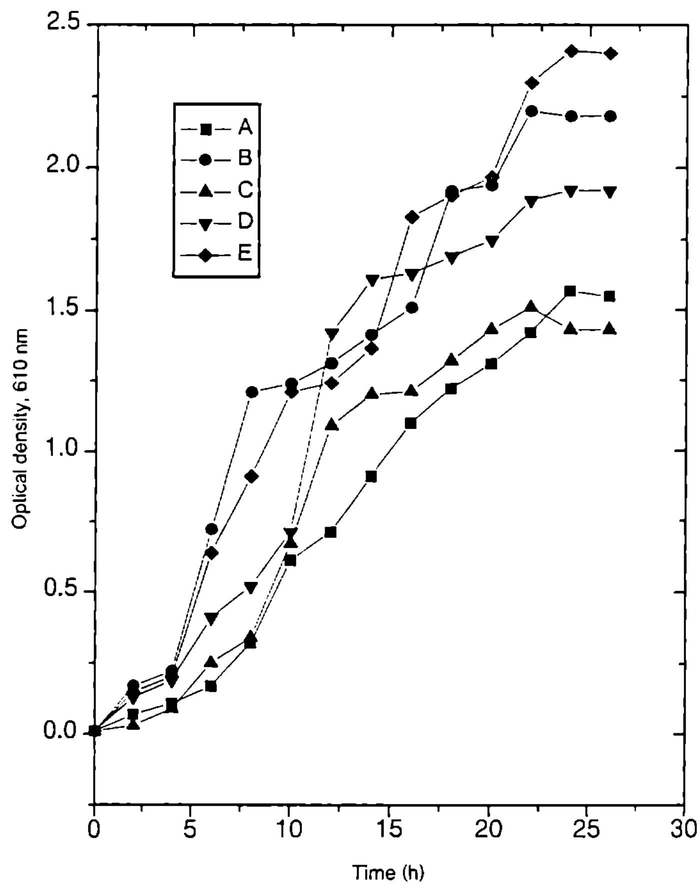


Fig.B3. Growth profile of *Brevibacterium* sp. in different production media: A: M1, B: M2, C: M3, D: M4 and E: M5

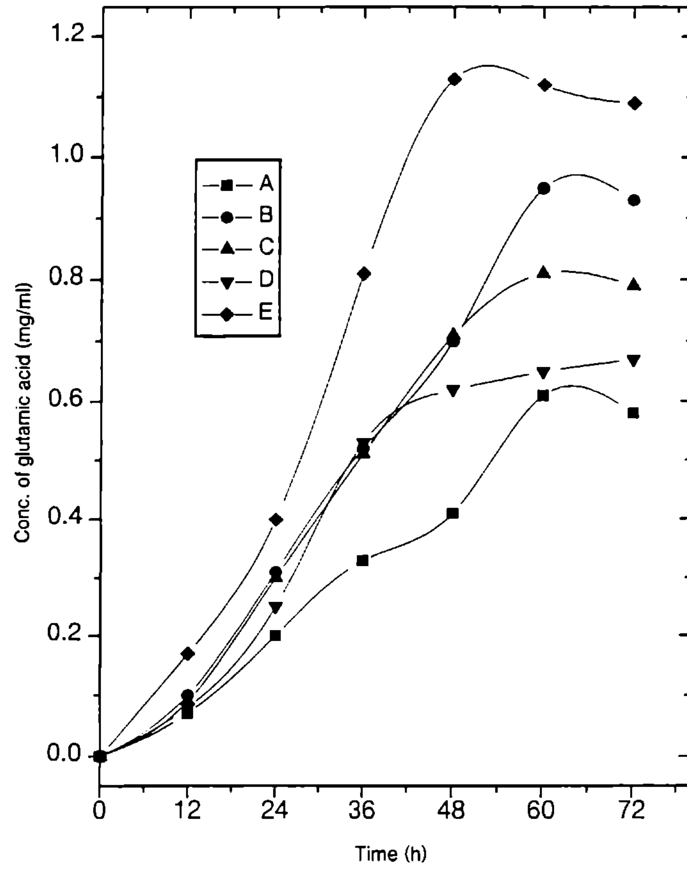


Fig.B4. Glutamic acid production by *Brevibacterium* sp. in different production media. A: M1, B: M2, C: M3, D: M4 and E: M5

Fig.B5. **Qualitative detection of L-glutamic acid in the culture broth by paper chromatography**

A - Standard **B - Sample**

Effect of pH and temperature: The conversion of sugar to amino acids results a change in the pH of the production medium. Failure to control the pH finally causes definite injury to the culture and reduces its productivity. Fig. B6 reveals that optimum pH for glutamic acid fermentation ranged between 7 and 8. Data recorded in Table B2 shows the effect of temperature on glutamic acid production by *Brevibacterium* sp. Maximum yields were obtained at 30°C.

Table B2. Effect of temperature on L-glutamic acid production by *Brevibacterium* sp.

Temperature °C	Glutamic acid mg/ml
20	0.51
25	0.86
30	1.13
35	1.02

Effect of dissolved oxygen: It is well known that dissolved oxygen levels affect the metabolic state of microorganisms. These effects are usually seen as changes in respiration and growth rate of the organism[189,190]. Other intracellular effects includes changes in cytochrome levels, hydrogenase levels, TCA cycle intermediates level and changes in gross cellular composition[191,192].

The degree of aeration in shaker flasks is a function of the shaking speed, the stroke of displacement of the shaker, the degree or baffling of the flasks, the

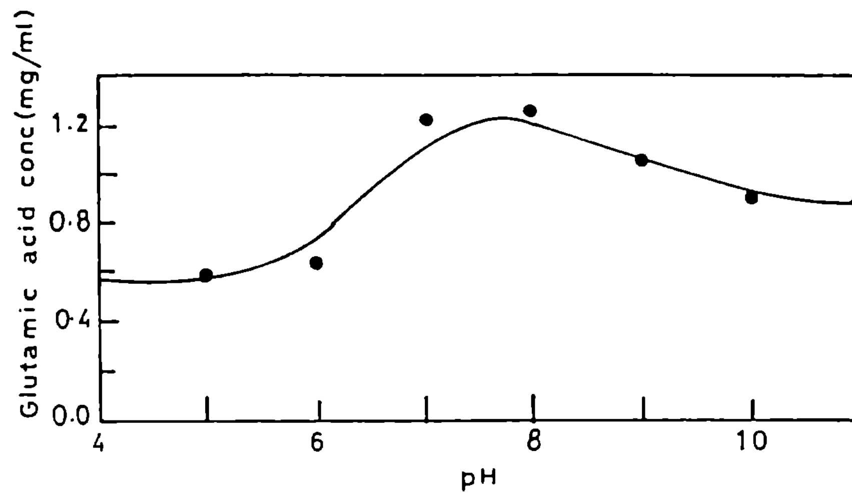


Fig.B6. Effect of pH on glutamic acid production by *Brevibacterium* sp. (48 h)

type of closure used etc. Conventional method for shaking cultures involves the control of oxygen supply by altering the liquid volume (working volume) i.e. by changing the ratio between the volume of the flask and the volume of medium used[3]. Variation of the liquid volume in the flasks leads to changes in agitation intensity as well as on oxygen supply.

It was interesting to note that glutamic acid production has increased due to changes in ratio of volume of the medium to volume of the flask (Table B3). It was also evident that it was not the ratio of the volume of the medium to flask volume which was critical for the glutamic acid production, as different yields have been obtained with 1:5 ratio of volume of medium to flask volume. The data thus indicated that it was the working volume of the medium at specific medium volume to flask volume ratio, which was important. Thus, the best yield was obtained when 100 ml of the medium (M5) was taken in 500 ml flask, which resulted in the production of 6.86 mg/ml glutamic acid.

Table B3. Effect of ratio of volume of the medium and flask in Glutamic acid production (48 h)

Volume of the flask, ml	Ratio of volume of medium : flask volume	Glutamic acid production, mg/ml
500	1:25	1.17
250	1:12.5	1.32
150	1:7.5	3.04
500	1:5	6.86
250	1:5	5.78

It was found that both over abundant and meagre aeration were undesirable for L-glutamic acid production. In comparison with the conditions having 500 rpm and 350 rpm, the dry cell density and specific growth rate during the exponential phase was lower in 60 rpm condition (Table B4) showing that the organism were under oxygen limitation. The dissolved oxygen level with 180 rpm should be less than that of 500 rpm and 350 rpm, even then the maximum dry cell density and specific growth rate were almost the same. These results showed that, the oxygen supply was sufficient for fermentation when the rpm was 180.

Table B4. Effect of Agitation on cell growth of *Brevibacterium* sp.

Agitation speed (rpm)	Maximum cell dry density (mg/50 ml)	Specific growth rate (h^{-1}) at log phase
500	4.90	0.12
350	5.70	0.11
180	5.50	0.13
60	330	0.09

The lactate and amino acid concentration profiles are shown in Table B5. For oxygen rich conditions (350 and 500 rpm), the maximum concentrations of L-glutamic acid obtained were 1.73 mg/ml (350 rpm) and 1.48 mg/ml (500 rpm). The maximum lactate concentration were 0.2 mg/ml and 0.22 mg/ml, respectively.

Table B5. Performance of glutamic acid fermentation with different agitation speeds

Agitation speed (rpm)	Conc. of glutamic acid, mg/ml			Conc. of lactic acid, mg/ml		
	24 h	48 h	72 h	24 h	48 h	72 h
500	0.90	1.48	1.40	0.33	0.22	0.10
350	1.12	1.73	1.71	0.36	0.24	0.11
180	2.71	6.73	6.28	3.10	1.20	0.75
60	0.28	0.55	0.57	6.20	4.13	1.23

In oxygen limited growth (60 rpm), lactate was accumulated up to a maximum of 6.2 mg/ml and then depleted. The concentration of glutamic acid was comparatively poor, maximum of 0.57 mg/ml after 72 h (Table B5). Accumulation of lactic acid was an indication of oxygen limited conditions. Utilization of lactate by the organism indicated that they underwent a preferred substrate (glucose) starvation. Since very little amino acid was produced, it can be assumed that most of the lactate was utilized for cell maintenance.

When we examined the lactate concentration and amino acid production of 180 rpm fermentation, the maximum concentration of lactic acid was 3.1 mg/ml after 24 h followed by an uptake of lactate. The maximum glutamic acid formation (6.73 mg/ml) was noted at 180 rpm after 48 h fermentation.

The striking differences in yields suggested that the oxygen supply rate was an important regulating factor on the metabolism of *Brevibacterium* sp. The main path way of glutamate synthesis involves combination of oxidative degradation of glucose and anaerobic citrate decomposition[193]. So the balance of aerobic and

anaerobic combination is one of the important factors which control the yields of glutamate.

When considering the rate of 180 to 500 rpm, amino acid production decreased as oxygen supply rate increased. This suggested that a lower oxygen supply rate during the growth phase was better in order to get higher productivity in the production phase. At sub-optimum oxygen level (partially oxygen limited state, 180 rpm), the culture produced more lactate than in the oxygen sufficient conditions. This suggested that the organism utilised glucose more efficiently at low oxygen supply rate. The lactate thus produced by the partial oxygen limited cultures was then consumed by the organism during the production phase for cell maintenance and also for amino acid production. The result indicated that the final amino acid concentration on the partially oxygen limited condition was nearly three times higher than the oxygen sufficient conditions.

Inoculum size: The data recorded in Table B6 reveals that of the different inoculum sizes attempted, 5% v/v gave the best results, in terms of readily growth and maximum glutamate secretion. Higher inoculum size (10% v/v) did not show any further utility. To reach the production phase at the earliest , the inoculum must be available in sufficiently large volume and the length of the lag phase is affected by the size of the inoculum and its physiological condition.

Table B6. Effect of inoculum size on growth and glutamic acid production by *Brevibacterium* sp.

Inoculum size (% v/v)	Optical density, 610 nm		Glutamic acid, mg/ml	
	24 h	48 h	24 h	48 h
0.5	1.21	1.67	1.73	3.32
2.0	1.72	1.74	2.22	4.90
5.0	1.93	1.91	2.96	6.20
10.0	1.83	1.87	2.84	5.56

Effect of different carbon sources: Table B7 shows that the different carbon sources used and the corresponding cell growth of the culture during 72 h of cultivation. Except starch, all other carbon sources supported the cell growth of the culture. The culture attained maximum cell density with glucose, fructose and sucrose within 24 h of cultivation while with lactose and xylose it took 48 h.

Table B7. Growth of *Brevibacterium* sp. on various carbon sources (set B)

Carbon source	Optical density		
	24 h	48 h	72 h
Glucose	2.23	1.97	1.82
Sucrose	2.09	1.89	1.81
Fructose	2.04	1.87	1.80
Maltose	1.96	1.79	1.74
Lactose	0.99	1.13	1.11
Xylose	0.82	0.91	0.87
Starch	no growth	no growth	no growth

Fig.B7 shows that the effect of various carbon sources on glutamic acid production by *Brevibacterium* sp. In this case, two sets of flasks were prepared, set A, with 20 ml medium in 250 ml flask (1:12.5) and set B, with 50 ml medium in 250 ml flask (1:5). The yields, in general, were high in set B when the ratio of the

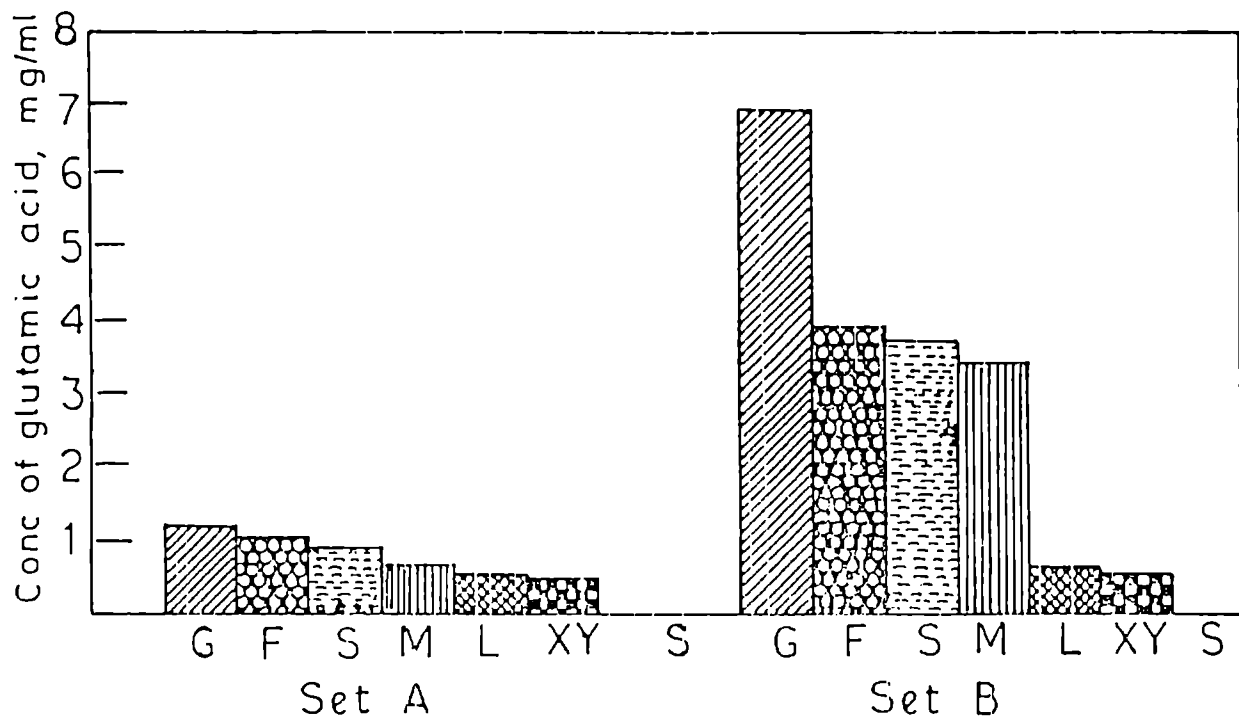
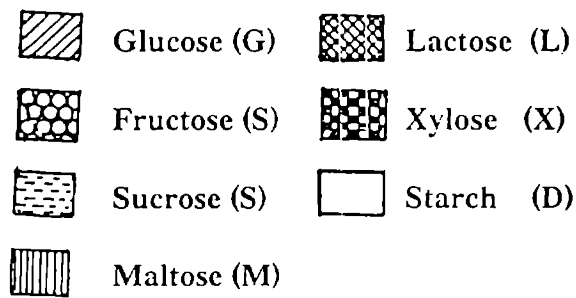


Fig.B7. Effect of various carbon sources on glutamic acid production by *Brevibacterium* sp. (48 h)



volume of the medium used and that of the flask was 1:5. Maximum glutamic acid was produced with glucose in both cases (1.13 and 6.86 mg/ml in set A and B, respectively). When fructose, sucrose and maltose were used as the substrate (Set B), the yields of amino acid varied between 49-56% compared to that with glucose. Although the cell growth with lactose and xylose was not very poor (Table B7), glutamic acid production was significantly poor (0.60 and 0.55 mg/ml, respectively) with these two and with starch no sign of amino acid production was noticed.

It was interesting to note that during the first 48 h of fermentation, the rate of glucose consumption by microorganism in set A was slower than in Set B (70.1 and 91.6%, respectively). After 96 h, however, the culture has consumed more than 90% glucose in both cases (90.5 and 94.7%, respectively) (Fig.B8). Regarding utilization of various carbon sources for production of amino acids, Shiio *et al*[194] reported that the yields strongly affected by the metabolic pathways specific to respective carbon source and the regulating metabolisms of these pathways.

To determine the suitable concentration of carbon source in the medium, four different concentrations of glucose (1, 2, 5 and 10%) were tested and the results obtained are plotted in Fig.B9. evidently, 2% concentration of glucose was most effective. A maximum titre of 6.86 mg/ml glutamic acid was obtained in 48 h which corresponded to about 42.0% conversion (assuming 81.74% as the theoretical conversion and by taking initial glucose concentration into account). Considering the glucose used up by the bacterium in 48 h (which was 91.6% of

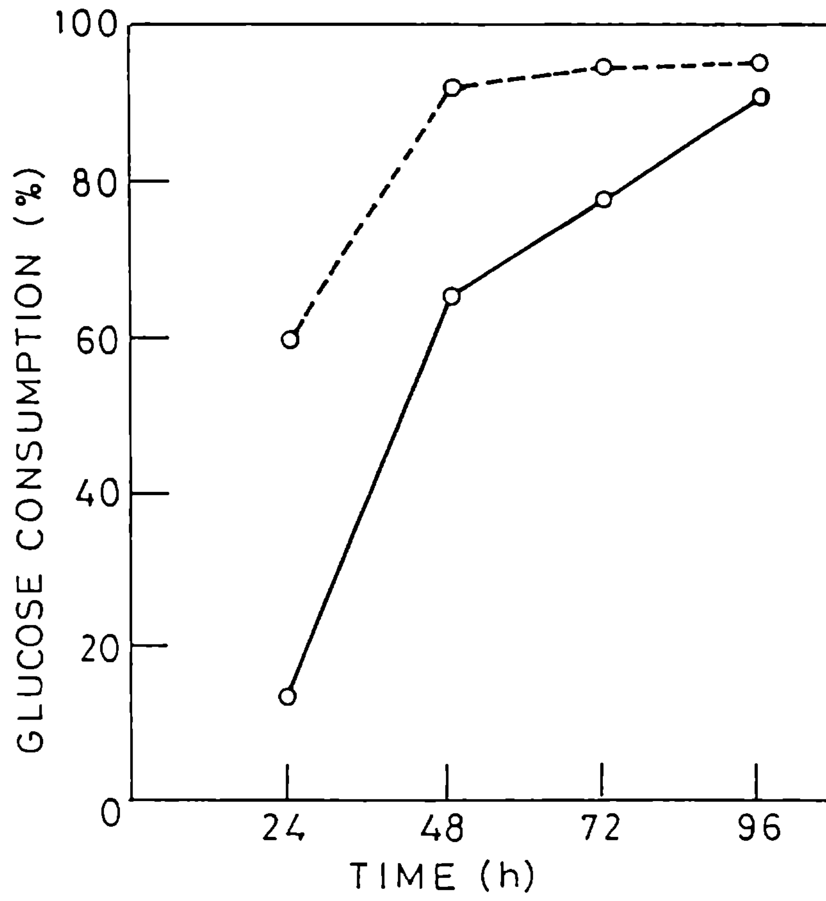


Fig.B8. Glucose consumption by *Brevibacterium* sp.

o——o Set A o- - - -o Set B

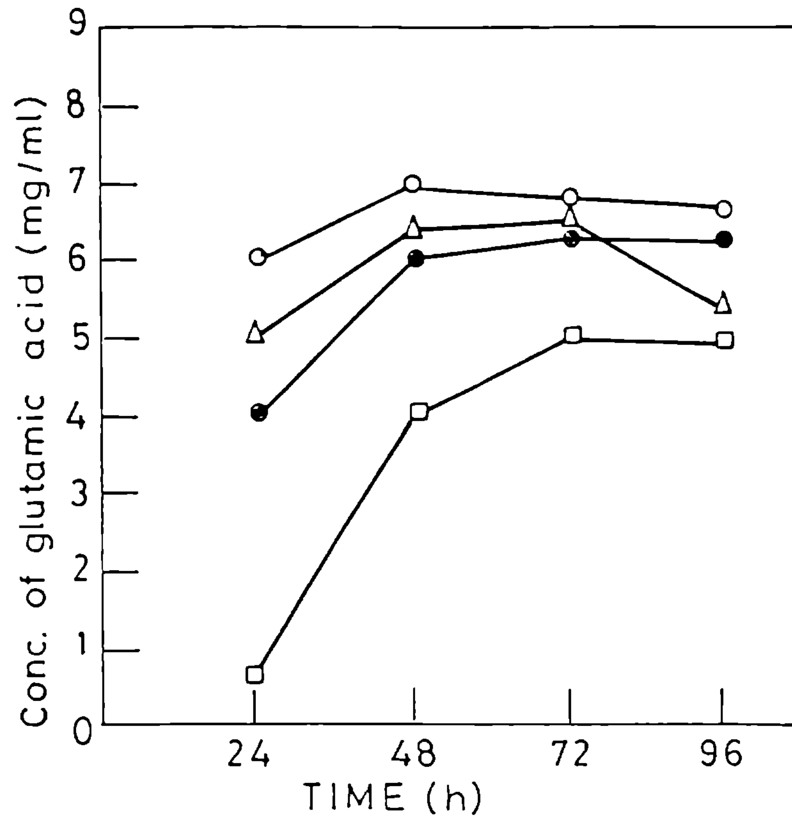


Fig.B9. Effect of concentration of glucose on glutamic acid proudction by *Brevibacterium* sp.

□ 1% glucose • 5% glucose

o 2% glucose Δ 10% glucose

initial concentration), the conversion efficiency was 45.8%. In the case of higher concentrations (5 and 10% glucose), maximum yields were obtained after 72 h and were 6.26 and 6.54 mg/ml, respectively. These corresponded to 15.32 and 8.0% conversion efficiencies (on the basis of initial glucose concentration). While on the basis of consumed substrates the conversion efficiencies were 59.83 and 39.67% for 5 and 10% glucose concentrations, respectively. Evidently, higher glucose concentrations interfered with the culture's activity and it could not utilize the substrate effectively (Fig.B10). As far as cell growth of the culture was concerned, after 48 h, the ODs with 2, 5 and 10% initial glucose were 1.93, 1.86 and 1.53, respectively, which also confirmed that 2% initial glucose concentration is the most suitable for the microorganism.

It is known that an ideal substrate concentration in a fermentation process results in higher conversion efficiencies and optimum substrate utilization[195]. To this effect, we achieved good success with more than 90% substrate consumption by the microorganism.

Mixed substrate fermentation: Yet in another study, we monitored the glutamic acid production by mixed substrates. In this, glucose was partly replaced with fructose or sucrose and the results are recorded in Table B8. None of these could exert any desirable impact on the microbial culture for glutamate synthesis.

Table B8. Mixed substrate fermentation for glutamic acid production, mg/ml

Substrate	Time (h)	
	48	72
Glucose (2%)	6.86	6.78
Sucrose (2%)	3.74	4.07
Fructose (2%)	3.85	4.46
Glucose (1%) + Sucrose (1%)	4.93	4.56
Glucose (1%) + Fructose (1%)	4.70	4.21

Non-carbohydrate Sources: From Fig. B11, it was evident that glucose served as the best carbon source when compared with the non-carbohydrate carbon sources, glycerol and acetate. Maximum production (49 µmol/ml) of glutamic acid was obtained after 48 h. Using glycerol, the maximum production were 10.4 µmol/ml after 72 h while only 8.23 µmol/ml was produced with the acetate medium. So the efficiency of the strain to utilize these substrates was not promising.

Effect of Yeast Extract and Corn Steep Liquor: Enrichment of the medium with supplementation of both yeast extract (Table B9) and corn steep liquor (CSL) (Table B10) upto a certain concentration enhanced glutamate accumulation.

Table B9. Effect of yeast extract on growth and glutamic acid production by *Brevibacterium* sp.(48 h)

Nutrient	Concentration (%w/v)	Optical density, 610 nm	Glutamic acid, mg/ml
Control		0.94	2.87
Yeast extract	0.5	1.62	4.10
	1.0	1.96	5.75
	2.0	2.12	4.27

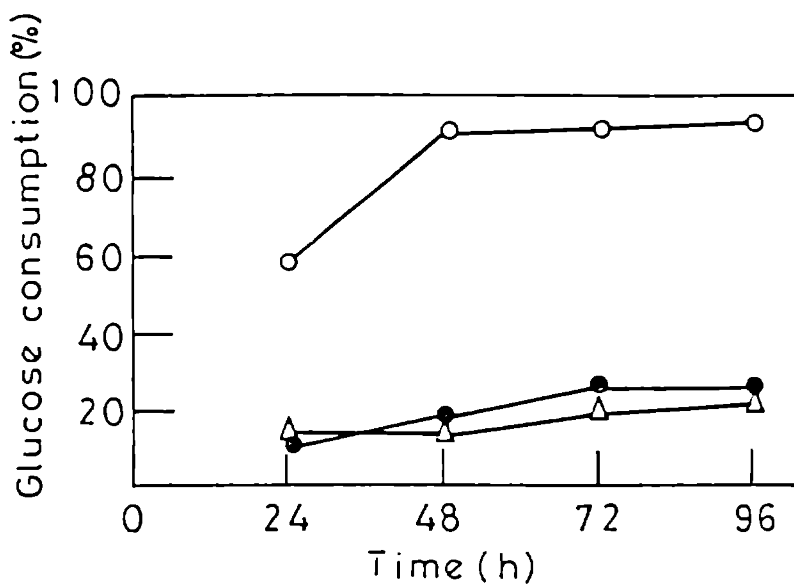


Fig.B10. Effect of concentration of glucose on its consumption by *Brevibacterium* sp.

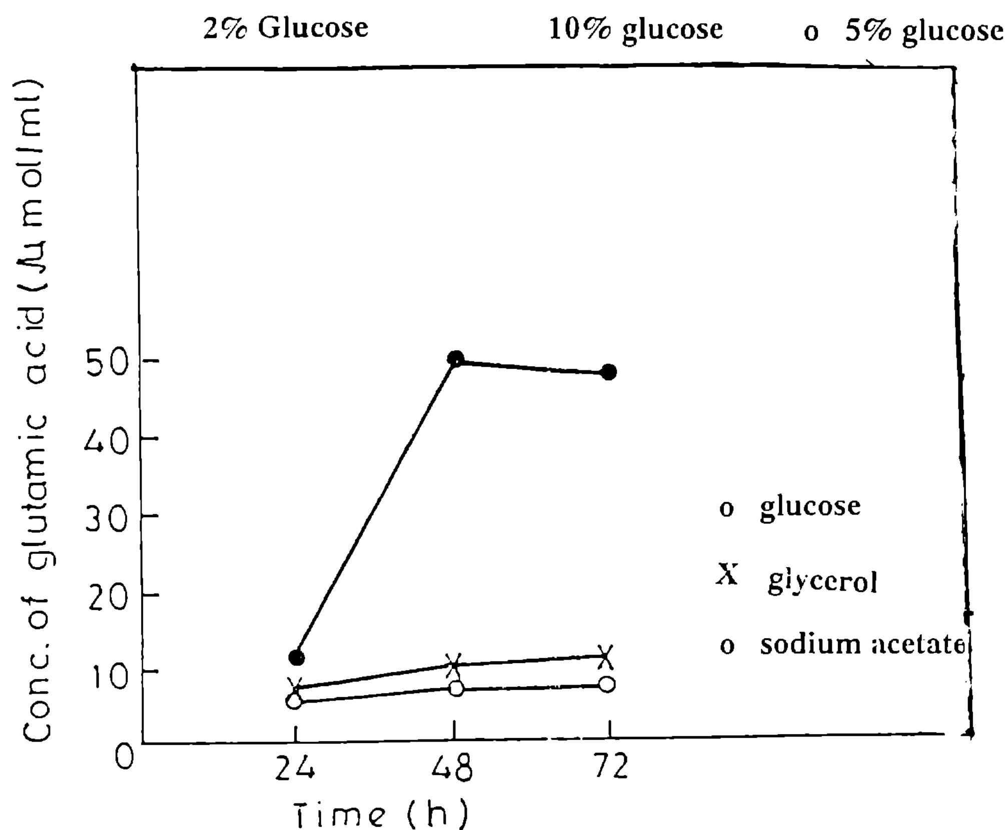


Fig.B11. Effect of different carbon sources on glutamic acid production by *Brevibacterium* sp.

The bacterial growth was considerably increased by the addition of these nutrient sources. Maximum of 5.75 mg/ml glutamate was accumulated when the yeast extract concentration was 1% and for corn steep liquor the maximum (7.10 mg/ml) titre was obtained with 0.25%. It is evident from the data (Table B10) that as the concentration of CSL increased the bacterial growth as well as the concentration of lactic acid were also correspondingly increased, but the secretion of glutamate was affected adversely beyond 0.25%.

Table B10. Effect of corn steep liquor (CSL) on L-glutamic acid fermentation by *Brevibacterium* sp. (48 h)

Concentration of CSL (% v/v)	Optical density, 610 nm	Glutamic acid, mg/ml	Residual sugar concentration, mg/ml	Conc. of lactic acid, mg/ml
Control	0.92	2.82	8.20	0.78
0.05	0.970	4.37	6.28	0.56
0.1	1.345	5.84	5.71	0.91
0.25	1.750	7.10	2.65	1.12
0.5	2.150	6.63	1.86	2.42
1.0	2.212	4.21	1.18	3.72

Both yeast extract and corn steep liquor contains a mixture of vitamins, bases, minerals, salts and amino acids. All these nutrients enhanced the growth of the organisms. But the concentration of some of the vitamins such as biotin was very critical since it affects the growth and the cells permeability which triggers the glutamate efflux. The optimum cell permeability might have been attained at 1% yeast extract or 0.25% corn steep liquor and hence the maximum glutamate secretion also.

B.3 MEMBRANE PERMEABILITY AND GLUTAMATE EXCRETION

Introduction

The integrity of cell surfaces is important in primary metabolite production mainly for two reasons, first in terms of the ability of the cell to excrete the over synthesized product and second in determining the tolerance and hence survival of an organism making the toxic product such as ethanol or butanol.

Accumulation of L-glutamic acid is mainly governed by its excretion not by its biosynthesis and hence glutamate fermentation provides an example of a case where cell permeability is a vital factor in regulating the out come196,197. During growth on glucose, the overproducers accumulate glutamate intracellularly until saturation was reached. Accumulation then ceases, due to feedback regulation unless the permeability barrier is altered to facilitate exit of the amino acid.

The present work is intended to study the effect of d-biotin, various Tweens (20, 40, 60 and 80), penicillin G and oleic acid on growth and extracellular formation of L-glutamic acid by *Brevibacterium* sp.

Materials and Methods

Microorganism: A strain of *Brevibacterium* sp. (DSM 20411) was used for the study.

Fermentation: Fermentation was carried out by taking 50 ml of M5 medium without corn steep liquor. To study the effect of d-biotin the above medium was supplemented with various concentrations of biotin (00-50 μg/L). To see the effect of oleic acid, the biotin was replaced with various concentration of oleic acid. The effect of penicillin G and Tweens were studied in a biotin rich medium (25 μg/L biotin). After inoculation, the flasks were incubated at 30±1°C (180 rpm) for a stipulated period. Samples were withdrawn as whole flask and the results obtained are the average of three sets of experiments.

Analytical methods

Bacterial growth, reducing sugar, lactate, glutamic acid were determined as mentioned earlier (see Section B1).

Results and Discussion

As the biotin concentration in the culture medium increased, it promoted the growth of the bacterial cells also. Maximum cell growth was noted when the biotin concentration was 10 μg/L (Fig.B12). In the absence of biotin the growth was poor.

From Table B11 it is very clear that the maximum extracellular accumulation of L-glutamate (6.90 mg/ml) occurred when the biotin concentration was 2.5 μg/L. The production was considerably reduced as the concentration of biotin increased. On the other hand, lactic acid, one of the byproduct of glutamate was started to accumulate as the concentration of biotin increased.

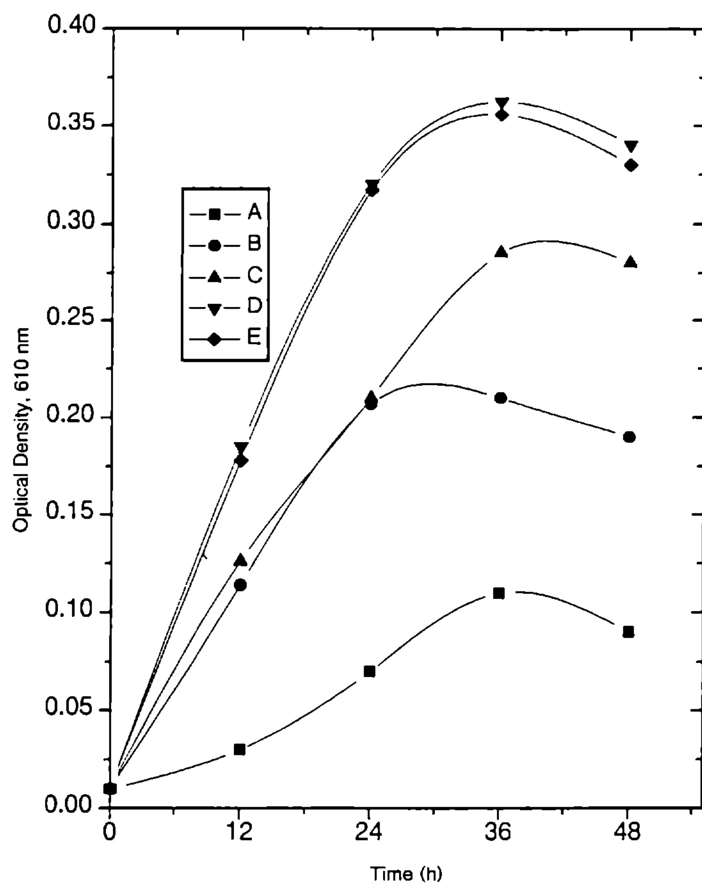


Fig.B12. Growth of *Brevibacterium* sp. as a function of the biotin concentration. A: 0 μg/L, B: 0.5 μg/L, C: 2.5 μg/L, D: 10.0 μg/L and E: 25.0 μg/L

Table B11. Role of Biotin on glutamic acid fermentation using *Brevibacterium* sp. (48 h)

Conc. of biotin (μg/L)	Growth, OD at 610 nm (X10)	Residual glucose (%)	L-glutamic acid, mg/ml	Lactic acid, mg/ml	pH
00	0.09	10.3	0.75	0.31	8.70
0.5	0.10	5.20	4.75	0.42	8.63
1.0	0.216	4.40	5.32	0.80	8.50
2.5	0.280	3.70	6.90	1.10	8.15
5.0	0.297	2.80	4.10	1.80	8.28
10.0	0.338	2.10	3.31	2.50	7.81
25.0	0.317	1.87	1.21	2.71	7.20
50.0	0.315	1.41	1.14	3.74	6.86

The addition of oleic acid to the medium supported the growth and glutamate production. The maximum production (4.45 mg/ml) was noted when the medium was supplemented with 50 mg/L of oleic acid (Table B12) and thereafter as the concentration of oleate increase the production decreased. Addition of oleic acid was accompanied with long lag period in the growth while the biotin caused more rapid growth hence the cells were harvested after 60 h of incubation while it was 48 h for the biotin supplemented medium.

Table B12. Glutamate formation in glucose-oleate medium by *Brevibacterium* sp. (60h)

Oleic acid mg/L	L-glutamic acid mg/L
0	0.81
10	1.95
50	4.45
100	3.14
200	1.10

Table B13 shows that the effect of addition of penicillin G depended on the concentration as well as on the addition of time. Addition of 2 units/ml after 9 h of incubation resulted in the maximum glutamate production of 6.50 mg/ml. Addition of penicillin at the time of inoculation or after 15 h of incubation were not much promising. It showed that the addition of β-lactum antibiotics such as penicillin G should be immediately after the lag phase to make it effective.

Table B13. Effect of penicillin G on glutamic acid production in a biotin rich medium by *Brevibacterium* sp.

Penicillin added unit/ml	Glutamic acid, mg/ml			
	Time of Penicillin addition after inoculation (h)			
	00	7	9	15
0	0.70			
1.0	0.80	4.70	3.70	1.30
2.0	2.40	5.10	6.50	2.21
5.0	2.0	4.50	6.33	2.10
10.0	1.80	2.85	3.13	1.10

Similarly to a biotin rich medium (25 µg/L) various concentrations of Tweens (20, 40, 60 and 80) were added at two different time intervals. One was at the time of inoculation and the other was after 9 h. Addition of 0.5% Tween 60 (polyoxyethylene sorbitan monostearate) after 9 h of incubation resulted in the maximum glutamate titre, 4.50 mg/ml (Table B14). Tween 40 (palmitic acid ester) and Tween 80 (oleic acid ester) also induced the secretion of glutamate. On the other hand. Tween 20 (lauric acid ester) was not that much effective.

Table B14. Effect of Tweens on glutamic acid production in a biotin rich medium by *Brevibacterium* sp.

Tweens added (%)	Glutamic acid, mg/ml							
	Time of addition after incubation							
	0 h				9 h			
	T.20	T.40	T.60	T.80	T.20	T.40	T.60	T.80
0.05	1.41	1.93	1.41	1.95	1.20	2.56	3.11	2.51
0.25	1.10	1.30	1.80	1.20	1.80	3.63	3.80	3.40
0.5	1.40	1.95	2.10	1.80	2.10	3.95	4.10	3.65
1.0	0.82	0.92	1.01	0.73	0.90	1.72	1.93	1.58

Most of the coryneform bacteria are naturally auxotrophic for biotin[38]. In these organisms, glucose is converted to α-ketoglutarte via the EMP pathways and the TCA cycle, then to glutamate by reductive amination. Biotin is a co-factor of acetyl CoA carboxylase, the first enzyme in the synthesis of oleic acid by repressing acetyl CoA carboxylase[198,199] Limited amounts of biotin or C16-C18 saturated fatty acids cause incomplete biosynthesis of oleic acid resulting in a decrease in phospholipid concentration and thus causes marked changes in the lipid composition of cell envelopes[200,201] As a result, the physical properties of the cell membrane are modified so as to become permeable to glutamic acid. Hence, the inability for glutamate excretion in the cells grown in the biotin rich medium might be mainly due to a diminution in the cellular permeability of glutamate. It was found that biotin is an essential factor for oleate biosynthesis and thus the requirement of biotin was in fact replaceable by oleate. Our findings on this particular strain were also in the same line.

Sommerson and Phillips[202] reported that the addition of Penicillin G to the growing culture containing excess biotin caused the production of glutamate in *Micrococcus glutamicus*. The cells which grew after the addition of penicillin did not synthesise cell wall completely and frequently exhibit elongation and swelling. The role of penicillin is thus to inhibit the synthesis of the cell wall, leaving the cell membrane unprotected and thus breaking the permeability barrier by mechanical damage to the cell membrane.

Smith *et al. (1961)*[203] observed that *Ashbya gossypii* can produce appreciable amounts of riboflavin in a synthetic medium only when non-ionic surface active agents were added. The explanation was that the lowering of the interfacial tension between the cell membranes and the aqueous medium may alter the permeability of the cell in such a way as to increase the rate of excretion of the riboflavin. However, Shiio *et al* (1963)[204] on their study of the effect of Tweens on the glutamate formation was shown to be greatly affected by the nature of fatty acid part of the Tween. The addition of Tween 60 to the growing culture affected the cellular permeability and thus the formation of glutamate. On the other hand, the addition of Tween 60 to the reaction mixture with washed cells has no effect. These facts led to the inference that surfactants interfere with the formation of cell membranes.

In Summary, biotin, oleic acid, penicillin G and Tweens affect the synthesis of normal cell membrane at different steps, respectively and make the cell membrane permeable to glutamic acid. The mechanism of glutamate transport into the medium has been subject of a long controversy. The 'leak'

model is based on the observation that glutamate efflux can be induced by various treatments which correlate with alteration in the cell membrane.

Our findings were also in accordance with this hypothesis. However, more studies were required to say authentically about any of the various hypothesis regarding the glutamate efflux. But, of the many factors, cell permeability is also one which trigger the secretion of glutamate.

B.4 ACCUMULATION OF GLUTAMIC ACID IN RESPONSE TO OSMOTIC STRESS

Introduction

The response of bacteria to hyperosmotic environments have received increasing attention in recent years. In many fermentation processes, the cells are exposed to osmotic stress because of both medium composition and product accumulation in the broth. Microorganisms have evolved many ways to cope up with environmental stresses. Low molecular weight substances are synthesized or transported in response to extracellular osmolality[205] Brown has termed these inorganic ions or smaller organic molecules as 'compatible solutes'[206].

The osmotic responses of gram negative bacteria, notably *Escherichia coli* were well studied. The primary response of *E. coli* to osmotic upshocks by addition of NaCl was the accumulation of K^+ ions with a concomitant increase of the glutamate intracellular content[207]. In a subsequent phase, these compounds are replaced by one or several neutral compatible solutes such as trehalose, proline and betaine[207-211].

The physilogical responses of gram positive bacteria to a high osmolality environment has been comparatively less studied[217,218]. Gram positive bacteria have been reported to accumulate the neutral amino acid proline, gamma amino butyrate, glutamine and ecotines[206,212-216].

Osmotic pressure of the medium has been reported to be an important operational parameter in glutamic acid fermentation[217,218]. In this study, inorder to gain a better understanding of the process of glutamate overproduction by *Brevibacterium* sp., we investigated the influence of a high medium osmolality on the growth and physiology of the bacteria. The main objective was to determine the internal content (total free amino acid and glutamic acid) of the bacteria during the growth phase under osmotic stress conditions.

Methods

Fermentation: 24 h old culture of *Brevibacterium* sp. (5% v/v) was used to inoculate 50 ml of M5 medium having various concentrations of NaCl (0-2 M) taken in 250 ml Erlenmeyer flasks. After inoculation the flasks were incubated at 30°C for a stipulated period of time (48 h) on a rotary shaker.

After incubation, the cells were harvested by using a cooling centrifuge (8000 g, 10°C). The pellets obtained from 50 ml of the culture was washed twice with distilled water and then resuspended in a minimum amount of distilled water.

Intracellular free amino acids were extracted with trichloroacetic acid (TCA) method of Sutherland and Wilkinsen (1969)[219] Extraction was performed at 4°C for 30 minutes with the cold trichloroacetic acid (TCA) at a final concentration of 10%. After centrifugation at 4°C, supernatant was extracted with cold ether (10 ml/100 ml TCA extract) for 5 minutes. Ether layer was removed by a separating funnel and the aqueous layer re-extracted with a further aliquot of ether. The process was repeated until the pH of the aqueous layer was

approximately 5.0. Ether solution was discarded in each case. Finally neutralization was achieved by the careful addition of 0.01 N KOH. The cell extracts thus obtained were analysed to find out the total amino acids content and glutamic acid.

Analytical methods for the determination of bacterial growth, total amino acids, and reducing sugar are mentioned earlier (Section B1) Glutamic acid in a known volume of the extract was separated by paper chromatography and the spot was extracted with 0.4% Cu $(NO_3)_2$ in methanol and it was quantified.

Results and Discussion

When *Brevibacterium* sp. was cultivated at various concentrations of NaCl (0.5 M, 1.0 M, 1.5 M and 2.0 M), the observed fermentation kinetics are shown in Fig.B13 (biomass) and in Fig.B14 (glucose consumption). The addition of NaCl first resulted in an arrest of cell growth and glucose consumption. However, the cell

Table B15. Free amino acid concentrations (mM/g dry wt) of *Brevibcaterium* sp. grown in various concentrations of NaCl

Amino acid	Conc. of NaCl					
	Without NaCl	0 M NaCl	0.5 M NaCl	1.0 M NaCl	1.5 M NaCl	2.0 M NaCl
Total free amino acids	282.8	371.5	464.1	423.9	443.9	347.9
Glutamic acid	142.3	244.6	335.9	273.1	199.8	144.5
Cell yield dry weight (mg/L)	690.0	798.0	840.0	910.0	720.0	615.0

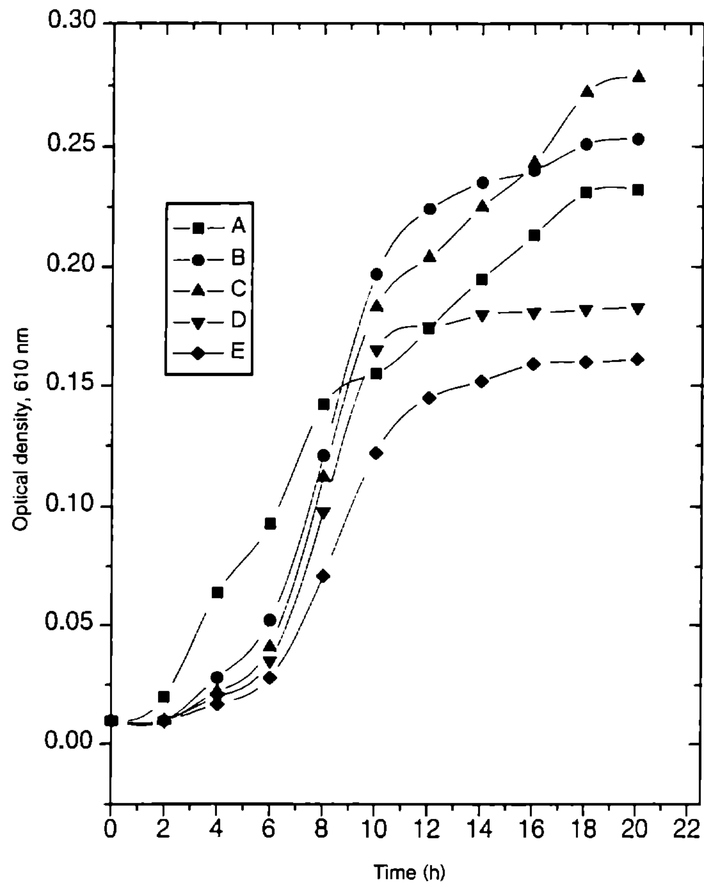


Fig.B13. Effect of NaCl concentration on the growth of *Brevibacterium* sp.
A: without NaCl, B: 0.5 M, C: 1.0 M, D: 1.5 M and E: 2.0 M

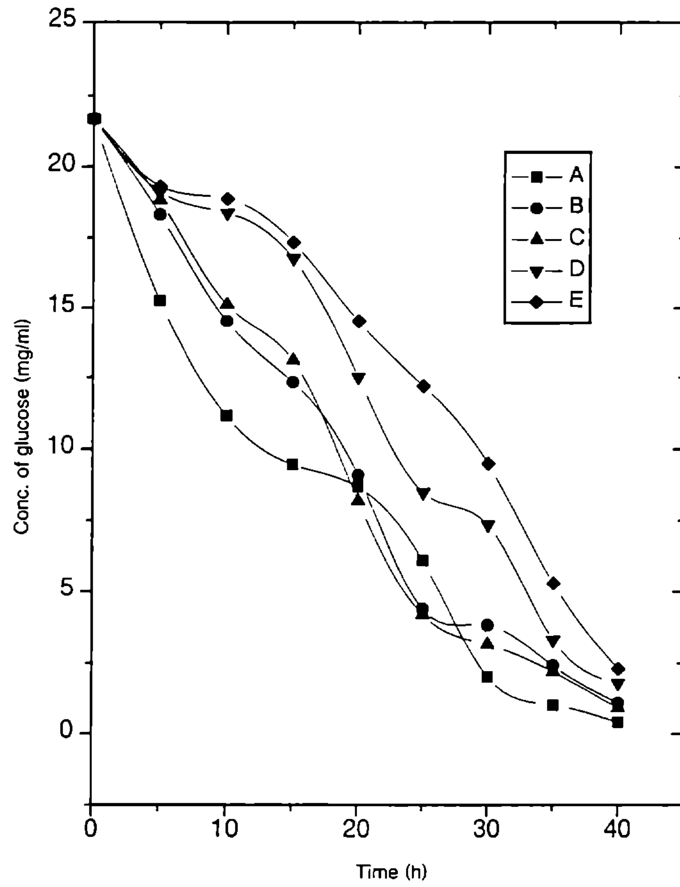


Fig.B14. Effect of a saline upshock on the glucose consumption of *Brevibacterium* sp. A: without NaCl, B: 0.5 M, C: 1.0 M, D: 1.5 M, and E; 2.0 M

growth was found to resume within few hours and to last until the total consumption of glucose. Cell growth was maximum when the medium contained 1.0 M NaCl and any further increase in salt concentration resulted in a linear decrease of cell growth (Table B15).

After a momentary interruption of cell growth, lasting for few hours, *Brevibacterium* sp. was found to grow even in a high salt concentration such as 1.5 M -2.0 M. Increasing the medium salinity, however, resulted in comparatively poor growth, i.e. reduced glucose to biomass conversion yields. The decrease of growth yields implied that bacteria were devoting an increased proportion of their carbon and energy source to osmotically induced processes such as uptake of ions and the synthesis of compatible solute. Similar results of salt addition have been reported for other microbial strains[220,221].

The most accumulated free amino acid was glutamic acid upon the growth conditions examined (Table B15). It was maintained at high concentrations even when cells were grown in the absence of NaCl (nearly 50% of the total amino acid accumulated) and with the increase of the external NaCl concentration it increased and showed the maximum (335.9 mM/g dry weight) at 0.5 M NaCl. It means 72.6% of the total amino acids accumulated was glutamic acid. However, it linearly decreased to 144.5 mM of dry weight in the presence of 2 M NaCl, which is almost equal for that in the absence of NaCl.

Growing *Corynebacterium glutamicum* under salt stress conditions was found to result in a higher activity of the glutamate and proline synthesis pathway, with as a consequence, an intracellular accumulation of proline, the end product of the pathway[222]. Glutamate accumulates to act as an osmolyte to increase the intracellular concentration of solutes to reduce loss of water by osmosis[223]. Glutamate also acts as a counter iron for the K^+ that accumulate under salt stress conditions[224]. It has been shown that K^+-glutamate stimulates protein- DNA interactions, suggesting that the accumulation of glutamate could be involved in regulation of expression of functions made in response to osmotic stress[225,226].

Further studies are required to investigate the effect of elevated salt concentrations on the metabolism of *Brevibacterium* sp. when as performed in glutamate production processes with added surfactants. Upon the addition of surfactants, the medium osmolality is further increased by the excretion of glutamate. This is expected to elucidate the respective role of osmotic pressure and of membrane permeability modifications on the selectivity of the amino acid secretion and thus, to provide a more rational basis for the optimization of the glutamate production.

B.5 IRON REQUIREMENT AND SEARCH FOR SIDEROPHORES IN *BREVIBACTERIUM* sp.

Introduction

Iron is required as a co-factor for a variety of basic biochemical mechanisms and is generally accepted that all living organisms, except lactic acid bacteria have an absolute requirement for the metal[227] Iron, an essential element for microbial growth exists in nature predominantly in the insoluble ferric(III) oxidation state which is not readily available for assimilation. To solubilize and sequester ferric iron, the iron requiring microorganisms have evolved efficient high affinity iron acquisition systems[228]. Components of the system include extracellular production of siderophores, which are specific iron(III) chelating compounds and specific membrane receptor proteins for uptake of the iron siderophore complex.

Siderophores are defined as low molecular weight, ferric specific ligands designed for solubilization, transport and storage of iron in microorganism[229] More than one hundred type of siderophores have already been reported which are produced by different bacteria and fungi[230].

Knowledge of the nutritional requirements of a bacterial strain is of crucial importance to devise growth media for specific purposes. This is the case for industrial fermentation as well as for basic laboratory research with many aspects of carbohydrate and amino acid metabolism. The effects of some chelating agents and autoclaved sugar on the growth of *Micrococus glutamicus* in synthetic

medium have been described by Nakayama *et al.*(1964)[231]. Ferrichrome was effective in promoting growth in synthetic medium and could be replaced by hydroxy aspergillic acid or large amounts of ascorbic acid or iron salts. Apart from that no such studies were reported in Coryneform bacteria.

Thus we decided to define the role of iron, a key nutritional factor in bacterial physiology and production of siderophores by this strain.

Materials and Methods

Media: Liquid YPG medium containing yeast extract, peptone and glucose was used for the growth studies. For the studies requiring a chemically defined medium, a number of media were tried and finally, the casamino acid (CAA) medium was chosen for further studies. It contained (g/l) casamino acid 5, K_2HPO_4 1.18 and $MgSO_4.7H_2O$ 0.25.

These media (YPG and CAA) were depleted of iron (or contaminating iron) by 8-hydroxyquinoline (5% in chloroform) treatment[232]. It was carried out by mixing the medium with the hydroxyquinoline-chloroform solution, followed by vigorous shaking in a suited borosile container. The treated media (YPGQ or CAAQ) were tested (after autoclaving) for the absence of 8-hydroxyquinoline by the CAS assay.

Effect of synthetic chelating agent: For the studies involving synthetic chelating agent, to obtain the iron decreased medium, various concentrations of ethylene diamine dihydroxyphenylacetic acid (EDDHA) was supplemented in liquid or

solid media. For iron sufficient conditions of growth, the media, after autoclaving were supplemented with sterile iron chloride at 100 μM final concentrations. Freshly grown cultures of *Brevibacterium* sp. (20 h old) in YPGQ or CAAQ medium were used as inoculum. Static and agitated (180 rpm) cultures were performed at 30°C, in petri-dishes with 25 ml solid medium or in 100 ml Erlenmeyer flask containing 20 ml liquid medium.

Preparation of CAS agar plates[233]: To prepare 1L of blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron(III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA (hexadecyltrimethylammonium bromide) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved and mixed with 960 ml of sterilized CAA medium and is poured into the petriplates. Each plate received 25 ml of blue agar.

Preparation of CAS reagent

6 ml volume of 10 mM HDTMA solution was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron(III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl) and 7.5 ml 2 mM aqueous CAS solution was slowly added under stirring. 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M. HCl was carefully added. This buffer solution was rinsed into the volumetric flask which was then filled with water to afford 100 ml CAS assay solution. The solution was stored in dark, preferably in polyethylene bottle.

Detection of siderophores[234]: Siderophore production was detected by growing the bacteria as single colonies on the chrome-Azurol-S (CAS)-agar medium or directly from the spent culture supernatants using CAS reagent or by adding an excess of iron (1 ml of 2M $FeCl_3$/500 ml culture supernatant) to visualize the formation of coloured siderophore-iron complex. To detect siderphores in culture supernatant, 0.5 ml of this (after centrifugation) was transferred to a 8 ml tube. To this, 0.5 ml of CAS reagent was added and change in colour of the mixture was noted. For comparison, a tube with Desferol as standard was also used.

Effect of iron (Fe^{3+}) on the growth of *Brevibacterium* sp.: To monitor the effect of Fe^{3+} on the growth of *Brevibacterium* sp., CAAQ medium (after autoclaving) was supplemented with different concentration of (0-100 mM) of iron using 20 mM sterile $FeCl_3$ solution. Freshly grown cultures of *Brevibacterium* sp. (20 h old) in YPGQ or CAAQ medium was used as inoculum. Agitated cultures were performed at 30°C in 100 ml Erlenmeyer flasks with 20 ml medium.

Effect of iron concentration on glutamic acid: To study the effect of iron on glutamic acid production, the usual production medium, M5 was treated with 8-hydroxyquinoline to remove all the irons and then it was supplemented with various known concentrations of irons. Growth and glutamate production was analyzed as mentioned earlier.

Results and Discussion

Effect of synthetic chelating agents

Brevibactrium sp. grew well in YPG medium and reached to stationary growth phase after 20 h (Fig. B15). Addition of EDDHA at 10-1000 μg/ml resulted in decreased cellular growth, as shown in Fig.B16. From the plate studies, it was evident that as the concentration of EDDHA increased, the number of colonies as well as the size of the colonies decreased. Even in the heavy streaked area the colonies were widely scattered showing the poor growth (Fig. B17).

The reduction in the cellular growth was apparently due to the formation of Fe-EDDHA complex on EDDHA concentration (Fig.B16). When YPG medium was completely depleted with iron(8-hydroxyquinoline treatment), the cell growth was adversely affected as shown in Fig.B15 (O.D. at 610 nm, 0.08 in comparison to 0.129 in control, i.e. in YPG medium after 24 h of incubation). Addition of iron (100 mM), however, reverted this effect to a large extent (Fig. B17).

Detection of siderophore production: An assay of culture supernatant (from both CAA and YPG medium) showed positive results (i.e. change of colour from blue to purple-orange in case of CAS assay, and a brownish-red coloration with iron chloride), indicating the production of siderophores by the bacteria.

The secretion of siderophores by *Brevibacterium* sp. was more clearly revealed by the CAS-Agar plate studies. As shown in Fig.B18, a clear purple

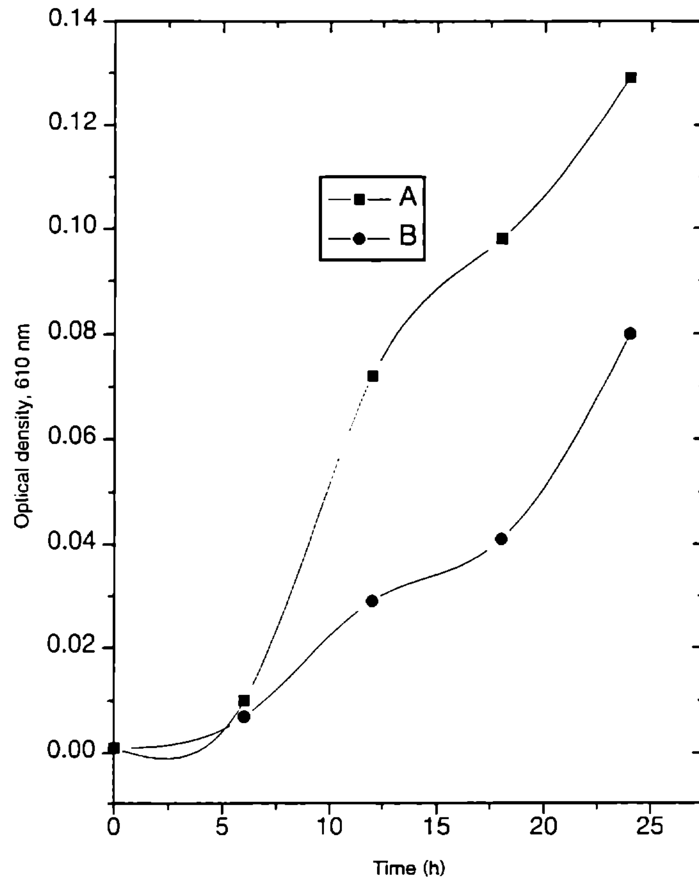


Fig.B15. Growth pattern of *Brevibacterium* sp. in YPG and YPGQ media. A: YPG and B: YPGQ

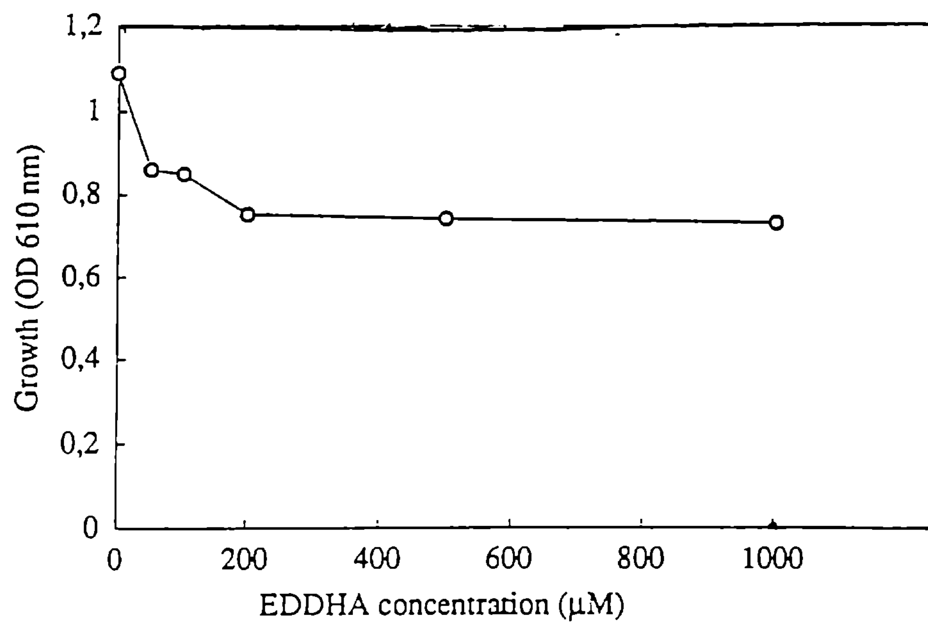


Fig.B16. Effect of EDDHA on the growth of *Brevibacterium* sp.

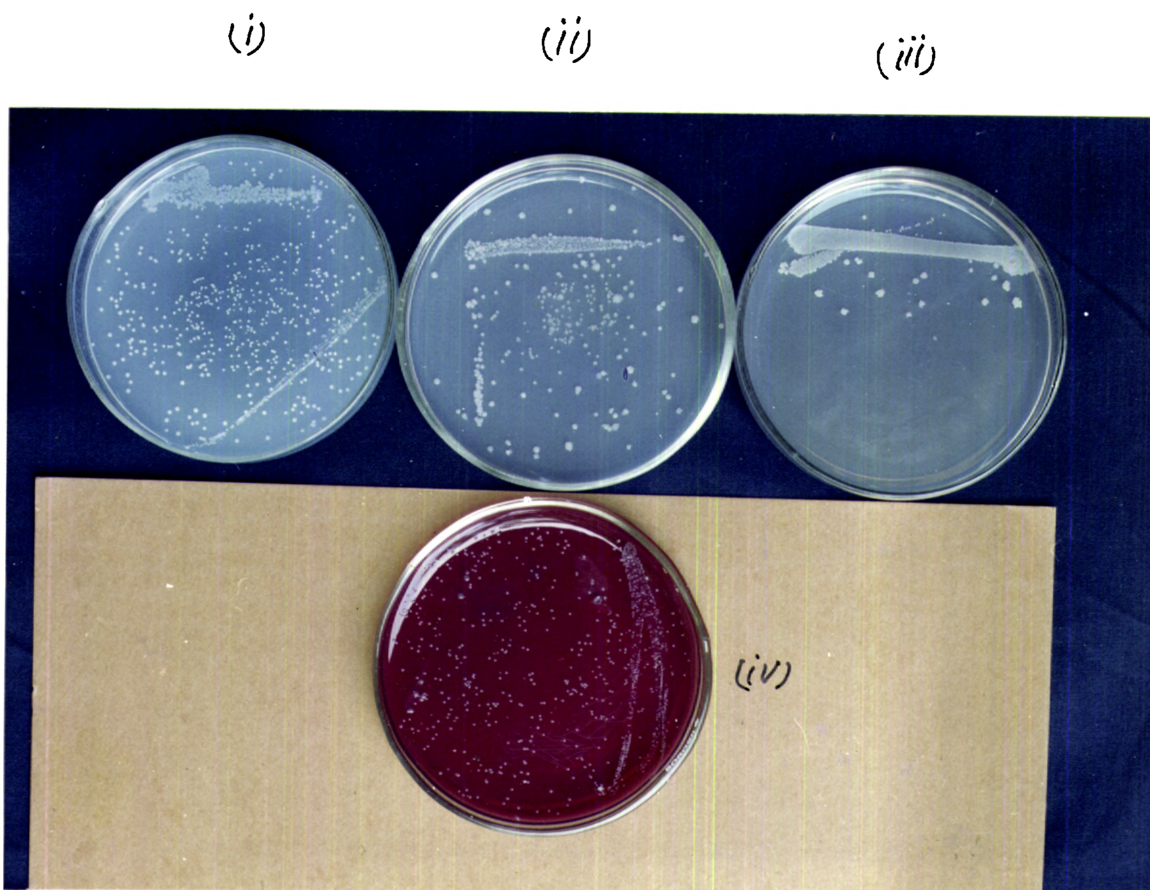


Fig.B17. Effect of EDDHA on the growth of *Brevibacterium* sp.

(i) 100 μM (ii) 250 μM (iii) 500 μM

(iv) with surplus iron

Fig.B18. Formation of orange haloes around the bacterial colonies in CAS - agar plates

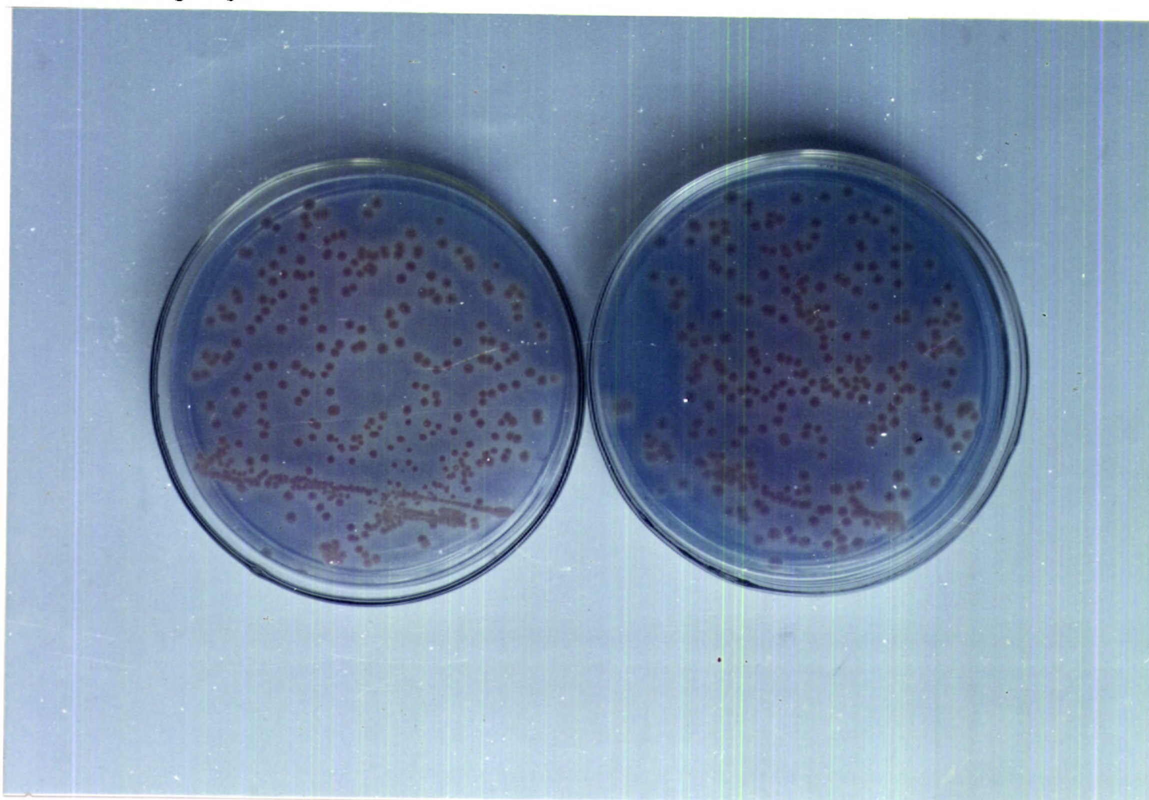
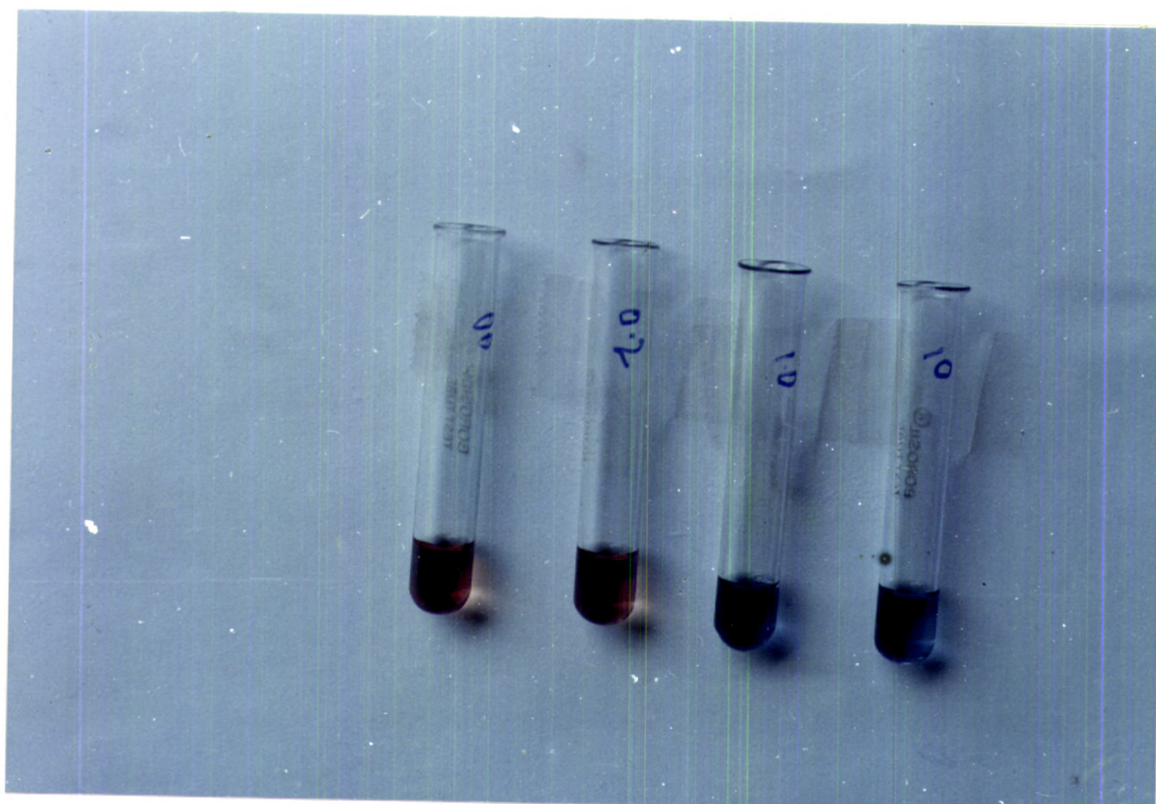
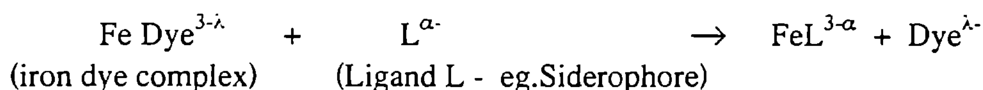


Fig.B20. CAS assay for the detection of siderophores



orange halo was developed around the colonies. This was an indication of siderophore production by the colonies,



$$\underset{\text{(iron dye complex)}}{Fe\,Dye^{3-\lambda}} + \underset{\text{(Ligand L - eg.Siderophore)}}{L^{\alpha-}} \rightarrow FeL^{3-\alpha} + Dye^{\lambda-}$$

In the presence of siderophore, free dye was released and was accompanied by the colour change, colony turns from blue to orange.

Effect of iron (Fe^{3+}) on the growth and Glutamic acid production by of *Brevibacterium* sp. : Fig.B19 describes the growth pattern of the bacterial strain in CAAQ medium as a function of iron supplementation and it is evident that addition of iron helped to growing of cells.

When the supernatant (24 h) obtained from the samples [control (without iron) and 0.2 mM Fe^{3+}] were subjected to CAS assay, it turned positive (Fig.B20) indicating the production of siderophores by the cells and that the cells were iron starved. Supernatant obtained from the sample with 10 mM concentration of Fe^{3+}, however, showed negative result with CAS assay (remained as clear blue solution) which indicated sufficient iron availability as required by the bacterial culture.

The culture growth was nearly arrested affected by the hydroxyquinoline treatment of M5 medium. One possible explanation was that in the treated medium, in addition to iron some other vital growth factors might also been removed[235]. Hence the growth was affected considerably.

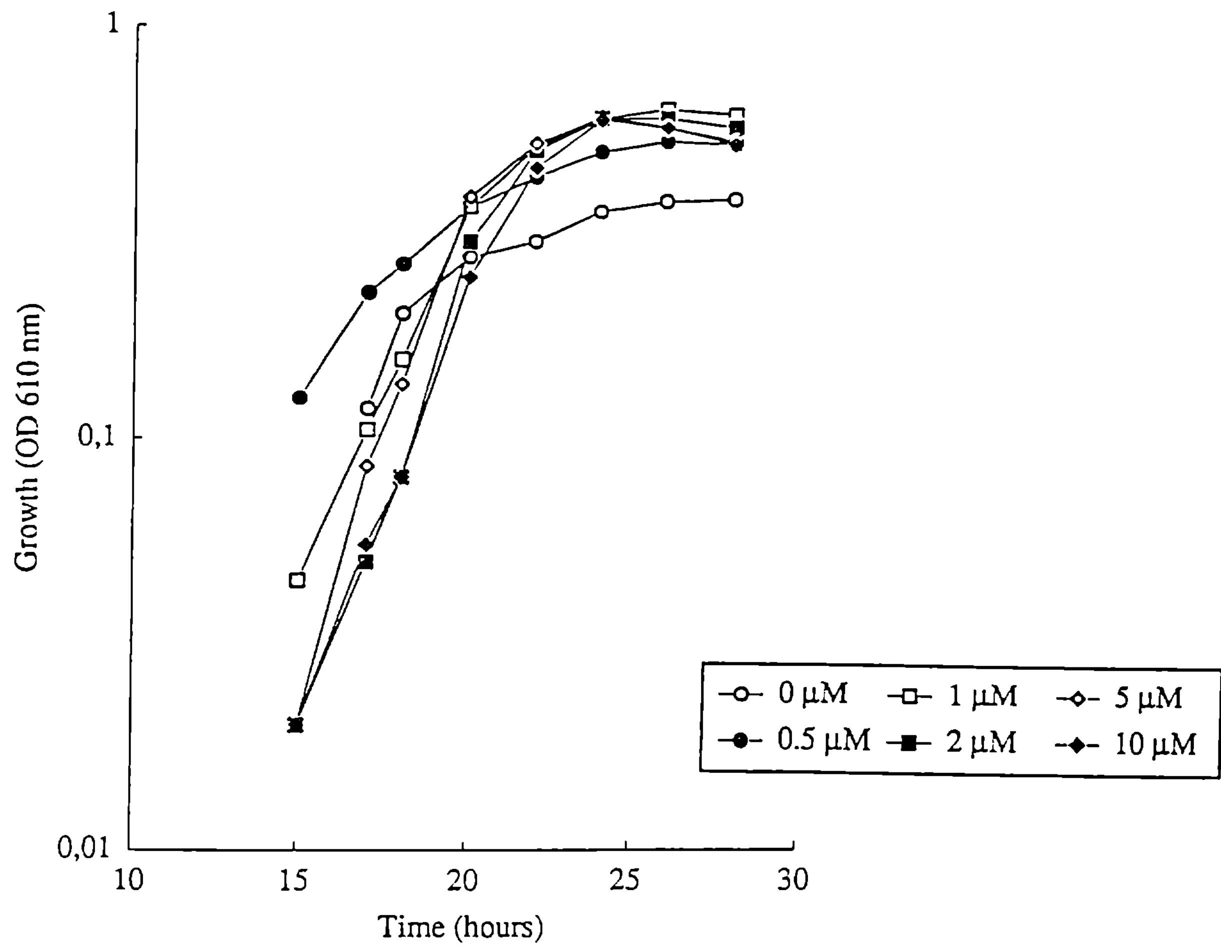


Fig.B19. Growth of *Brevibacterium* sp. as a function of iron concentration

As a second attempt after autoclaving, the treated media was supplemented with 0.5% w/v of sterilized yeast extract solution plus various known concentration of iron and was inoculated (5% v/v) with 20 h old culture from CAAQ medium and was allowed to ferment in the typical conditions as mentioned earlier (Section B1). Yeast extract was selected because it contains most of the essential growth factors and it normally contains less contaminating iron[235]. In comparison with normal growth medium (M5) a less cell growth was noted in all the flasks with varying amount of iron concentration (Table B16). After 72 h fermentation the flasks were removed and samples were collected as whole flasks and the amount of glutamic acid produced in corresponding to various concentration was determined and is shown in Table B16. It was obvious that addition of iron upto 10 mM enhanced the accumulation of glutamic acid and

Table B16. Effect of iron concentration on glutamic acid production by *Brevibacterium* sp.

Concentration of iron (μM)	Growth OD, 610 nm x 10 (20h)	Glutamic acid mg/ml (72 h)
Control (00)	0.087	1.13
0.5	0.091	1.57
1.0	0.097	2.10
2.0	0.104	2.63
5.0	0.109	3.15
10.0	0.112	3.38
20.0	0.107	3.25
50.0	0.109	3.30
M5 Medium	0.197	6.71

thereafter no such effect was noticed. A maximum of 3.38 mg/ml of glutamate was obtained with 10 mM initial iron concentration. Compared to the usual M5 medium the production was less.

Recently, the effects of various iron complexing substances on the growth of *C. glutamicum* in synthetic medium were investigated by Liebl *et al.* (1989)[236] *C. glutamicum* has an absolute requirement for the presence of an iron complexing compound as a growth factor for rapid and abundant growth in synthetic medium. The requirement can be met by adding low concentration (10^{-5} M) of dihydroxyphenols (catechol, protocatechuate) or relatively high concentrations (0.1%) of citrate to the medium or by autoclaving a small amount of glucose together with other media components.

According to Liebel *et al.*[236], *C. glutamicum* is not able to produce strong iron-binding compounds like many other microorganisms. Instead, *C. glutamicum* seems to be siderophore auxotrophic, a phenotypic trait which is also found with several other bacterial species.

Based on these findings, it can be concluded that iron is essential for growth of *Brevibacterium* sp. which produced siderophores in iron starved conditions. As a growth factor, it will be always good to have a sufficient amount of iron as a medium component in glutamic acid fermentation. More studies are required for the isolation, purification, characterization of these siderophores produced by the *Brevibacterium* sp.

B.6 UREASE ACTIVITY OF *BREVIBACTERIUM* SP.

Introduction

Urease (urea amidohydrolase, EC. 3.5.1.5) catalyses the hydrolysis of urea and is a very interesting enzyme because of the high efficiency of its action. It deserves attention mainly because of its wide applications in urea sensors for the routine determination of urea in blood, in urine, and in waste water; in the treatment of uraemia; in the treatment of waste water containing urea from fertilizer plants and in the study of enzymatic kinetics as a model enzyme, soluble or immobilized[237,238]. Moreover, urease from Jack bean was the first enzyme crystallized[239] in addition to being the first enzyme shown to contain nickel[240].

Many microorganisms, including the bacteria *Helicobacter pylori, Staphylococcus saprophyticum, Lactobacillus fermentum* and the mould *Aspergillus niger*, contain urease activity[241-244]. In addition, cultures which produce glutamic acid and lysine, eg. *Arthrobacter* sp., *Microbacterium* sp. and *Corynebacterium* sp. may also possess a potent urease activity. The production of amino acids by these coryneform bacteria is a process of great commercial significance and an adequate supply of a suitable nitrogen source is essential for glutamic. acid fermentation. Coryneform bacteria are also used in other biotechnological applications including biotransformations.

This Chapter deals with the urease activity of glutamic acid producing *Brevibacterium* sp.

Materials and Methods

Media composition: The medium used for the production of glutamic acid and urease contained (g/litre) glucose 20, urea (sterilized by microfiltration) 5, KH_2PO_4 1.8, thiamine hydrochloride 10 μg, corn steep liquor 0.25%,Tween 80 1 ml, mineral solution 10 ml ($FeSO_4.7H_2O$, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCl, each 10 mg) and distilled water. Different concentrations of urea (0.15, 0.25, 0.5, 1.0 and 2.0%) were used to determine its effect on urease activity and glutamic acid production. Other carbon sources including sucrose (2%), fructose (2%), glycerol (1%) and sodium acetate (1%) were also used in order to study their influence on growth and urease activity.

Fermentation: 50 ml of medium was used in 250 ml capacity Erlenmeyer flasks. After inoculating with the cell suspension of *Brevibacterium* sp. (20 h old culture, 5% v/v inoculum size), flasks were incubated at 30°C for the stipulated period on a rotary shaker at 180 rpm. Samples were withdrawn intermittently as whole flasks and the results reported are the averages of four sets of the experiments.

Urease assay: Both crude and partially purified enzyme samples were assayed. After the stipulated period of fermentation, cultures were harvested by centrifugation at 5000 rpm for 20 min. The supernatants obtained were partially purified by $(NH_4)_2SO_4$ fractionation (40-60% saturation), followed by extensive dialysis against phosphate buffer (0.2 M) at 4°C for 24 h.

Urease activity was routinely assayed by measuring ammonia liberated from urea using Nesslers' reagent[245] The reaction mixture contained 4 ml 0.05 M

phosphate buffer (pH 7), 5 ml 0.25 M urea and 1 ml of enzyme solution in a total volume of 10 ml. The reaction was carried out for 40 min at 35°C. After incubation, 1 ml of reaction mixture was withdrawn into a 50 ml standard flask containing 0.2 ml trichloroacetic acid (TCA) and diluted to 40 ml with distilled water. To this 1 ml Nesslers' reagent was added. The contents of the flask were mixed well and made up to 50 ml with distilled water. The absorbance of the solution was measured at 405 nm (UV A spectrophotometer, Shimadzu, Japan).

For the studies on the effect of pH on urease activity, the pH of the reaction mixture was set at different values with phosphate buffer and then incubated as described earlier. For the studies on the effect of temperature on urease activity, the reaction mixture was incubated at different temperatures.

One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol ammonia per minute per ml of the enzyme solution. Specific activity was determined and expressed as μmol of ammonia liberated per minute per milligram of protein.

Analytical methods: Bacterial growth was determined by measuring the absorbance of appropriately diluted culture broths at 610 nm. Soluble sugars and soluble proteins were estimated by methods of Miller and Lowry *et al.* respectively. Glutamic acid was estimated quantitatively by a ninhydrin colour reaction. Urease activity was confirmed qualitatively using Christensen's urea-agar medium[246]. A colorimetric indo-phenol method was used for measuring urea[247]

Christensen's Urea Agar Medium: The medium contained (g/L) of peptone 1.0 g, glucose 1.0 g, NaCl 5.0 g, KH_2PO_4 2.0 g, phenol red 0.012 g, agar 20 g and distilled water.

The ingredients were combined and the pH was adjusted to 6.8-6.9. The agar was dissolved by boiling and sterilised by autoclaving. After cooling to 50°C aseptically added sufficient, 20% solution of urea (sterilized by filtration) to give a final concentration of 2% urea. The contents were mixed well and aseptically dispensed 2-3 ml volumes into sterile small tubes or 25-30 ml into a petriplate and allowed to solidify.

Estimation of urea by indo-phenol method: Urease catalyzes the rapid hydrolysis of urea. The ammonia formed reacts with hypochlorite and phenol to give the blue dye, indophenol which gives an absorbance signal between 530 and 650 nm proportional to urea concentration.

Reagents

1. Urease (10 KU/L) - dissolved 10 U lyophilized urease from jack bean in 0.5 m sodium phosphate buffer, 20 mmol/l, pH 7.0 and added 0.5 ml glycerol.

2. Urea standard solution (0.5 mmol/l), dissolved 30.0 mg urea in 1 L distilled water.

3. Phenol/nitroprusside (Phenol, 106 mmol/L, nitrogrusside, 0.17 mmol/l): dissolved 25 mg sodium nitroprusside and 5 ml liquid phenol in 500 ml water.

4. Hypochlorite (NaOCl, 11 mmol/l; NaOH, 125 mmol/L); dissolved 2.5 g sodium hydroxide in water, added 2.5 ml of a sodium hypochlorite solution containing about 13% active chlorine and made up to 500 ml with water.

Measurement

Pipetted 0.1 ml urease suspension into two test tubes marked as standard and sample. 0.2 ml standard urea solution was added into the standard test tube while 0.2 ml sample was added into the test tube marked as the sample tube. The contents were mixed, tubes were stoppered and incubated (40°C) for 15 minutes. After incubation, 5 ml phenol solution and 5 ml hypochlorite solution were added to each test tube. Contents were mixed and incubated for 30 minutes. The contents of each tube were poured into cuvettes and read the absorbance at a wave length between 530 and 650 nm using a spectrophotometer.

Results and Discussion

Figure B21 shows the growth pattern and urea consumption by *Brevibacterium* sp. over a period of 48 h. Acetate and glycerol supported growth, but growth and urea consumption were higher with glucose as the sole carbon source. In media containing glucose, maximum growth was attained at 24 h while it took 36 h to reach a maximum with glycerol and acetate. Similarly, 98% of urea was consumed within 36 h when the bacteria was cultivated in glucose medium. With glycerol and acetate (even after 48 h fermentation), the consumption of urea was only 64 and 42% respectively.

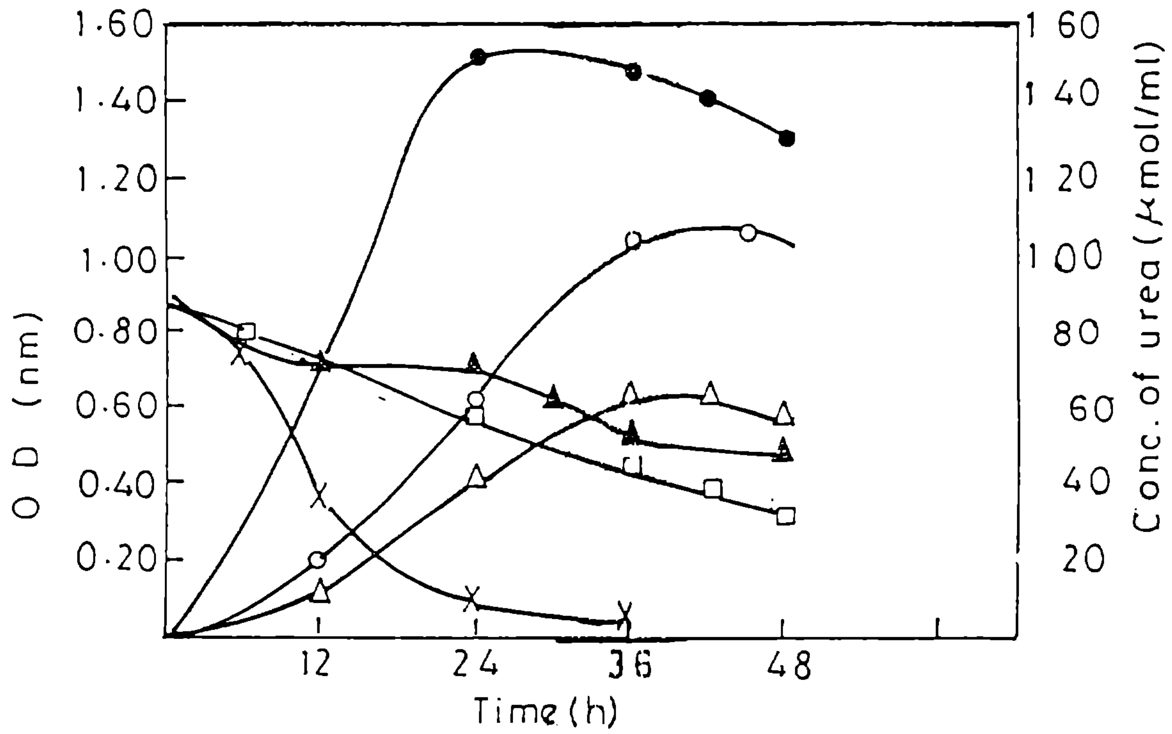


Fig.B21. Effect of different carbon surces on cell growth and urea consumption by *Brevibacterium* sp.

- ● Cell growth in glucose medium
- o Cell growth in glycerol medium
- Δ Cell growth in acetate medium
- X Urea consumption in glucose medium
- ▲ Urea consumption in acetate medium
- □ Urea consumption in glycerol medium

Effect of urea consumption on glutamic acid production

Glutamic acid fermentation can be characterized as the fermentation of nitrogen assimilation, uptake of ammonia being the key process. Addition of urea of 0.5% initial concentration looked suitable for glutamic acid production (Fig.B22). Table B17 contains data on the growth of *Brevibacterium* sp. at different concentrations of urea. High concentrations (1% or more) of urea inhibited growth. This might be due to excess ammonium ions released by the hydrolysis of urea, although it has been reported that a larger amount of ammonium ions were necessary for glutamic acid production; its high concentrations were inhibitory to growth and glutamate production. It is apparent from Fig.B23 that glutamic acid accumulated in the medium with a decrease in glucose and urea concentrations. The main accumulation of glutamate occurred after maximum growth by which time urea was almost totally decomposed to ammonia.

Table B17. Growth pattern of *Brevibacterium* sp. on different concentrations of urea

Percentage of urea	Cell growth (610 nm)		
	24 h	48 h	72 h
0.25	1.029	0.790	0.570
0.5	1.210	0.892	0.720
1.0	1.05	0.810	0.698
2.0	0.970	0.720	0.498

Urease activity

Urease activity was confirmed qualitatively using Christensen's urea-agar medium (Fig.B24) and change in the colour of the medium from yellow to pink

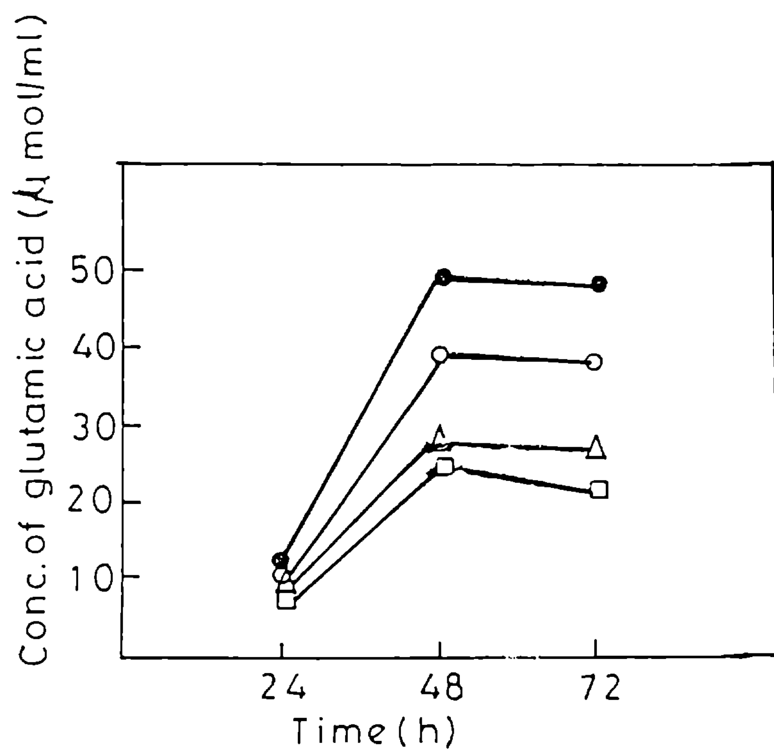


Fig.B22. Effect of urea concentration on glutamic acid production by *Brevibacterium* sp.

● 0.5% urea   o 1.0% urea

Δ 0.25% urea   ◻ 2.0% urea

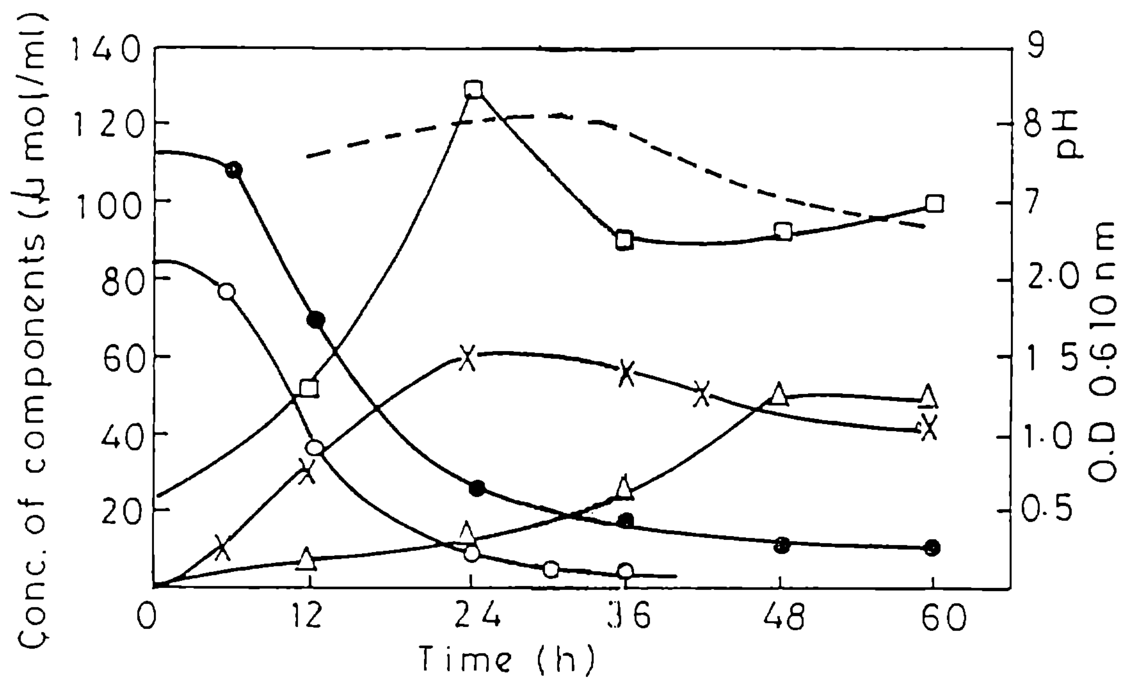
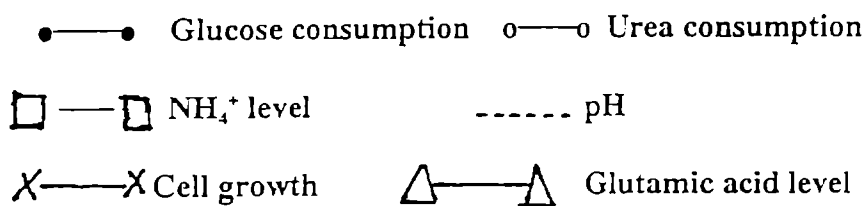


Fig.B23. Accumulation of glutamic acid in culture medium of *Brevibacterium* sp.



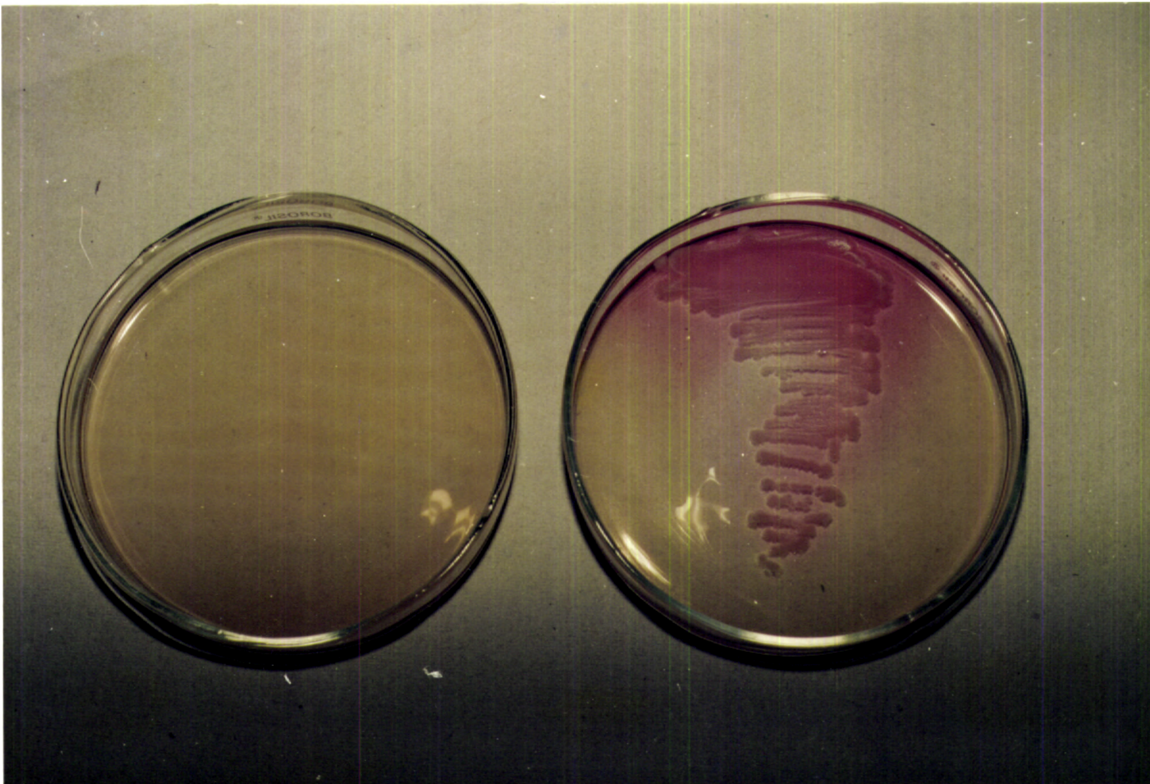


Fig.B24. Qualitative test for urease production by *Brevibacterium* sp.

was due to the pH change caused by the liberation of ammonia by the urease activity of the organism.

Table B18 shows the specific activity of crude urease enzyme when cells were cultured using different carbon sources. With an initial concentration of 0.5%, urea a maximum specific activity (0.228 mmol ammonia/mg protein) was obtained after 24 h when glucose was used as the carbon source. Results obtained with sucrose and fructose were more or less same but enzyme activity was considerably decreased when cells were cultured on glycerol or acetate.

Table B18 . Specific activity of the crude urease enzyme when the *Brevibacterium* cells were cultured under different carbon sources

Fermentation period (h)	Glucose	Sucrose	Fructose	Glycerol	Acetate
18	0.214	0.207	0.200	0.114	0.126
24	0.228	0.215	0.207	0.131	0.129
30	0.185	0.193	0.168	0.132	0.131
36	0.157	0.153	0.152	0.127	0.130

The initial concentration of urea in the culture medium influenced urease activity. Table B19 shows urease activity at different time intervals with different initial concentrations of urea. With 0.15 and 0.25% urea, enzyme activity reached a maximum in the early log phase (12 h), while with 0.5 and 1.0%, maximum activities were attained during the late log phase.

Table B19. Urease activity of *Brevibacterum* sp. with different initial concentrations of urea in the medium

Fermentation period (h)	0.15% Urea			0.25% Urea			0.5% Urea			1.0% Urea		
	Total activity	Protein (mg/ml)	Specific activity (U/mg protein)	Total activity	Protein (mg/ml)	Specific activity (U/mg protein)	Total activity	Protein (mg/ml)	Specific activity (U/mg protein)	Total activity	Protein (mg/ml)	Specific activity (U/mg protein)
6	0.346	1.35	0.256	0.332	1.30	0.255	0.166	1.20	0.138	0.152	1.10	0.126
12	0.568	1.58	0.359	0.603	1.55	0.389	0.318	1.60	0.198	0.291	1.50	0.194
18	0.638	2.30	0.277	0.680	2.70	0.254	0.622	2.90	0.214	0.525	0.26	0.201
24	0.610	3.31	0.184	0.610	3.35	0.184	0.776	3.40	0.228	0.548	3.20	0.171
30	0.513	3.48	0.147	0.525	3.50	0.150	0.664	3.58	0.185	0.568	3.45	0.164
36	0.403	3.51	0.114	0.430	3.55	0.121	0.568	3.60	0.157	0.541	3.50	0.154
42	0.291	3.72	0.078	0.332	3.80	0.087	0.525	3.90	0.134	0.283	3.60	0.078
48	0.180	3.18	0.048	0.221	3.78	0.058	0.305	3.88	0.080	0.249	3.59	0.069

Table B20. Specific activity of urease after partial purification

Fermentation time (h)	Specific activity (U/mg protein)	
	I	II
6	0.351	0.173
12	0.427	0.252
18	0.307	0.261
24	0.288	0.270
30	0.262	0.239

I - partially purified enzyme from the culture containing 0.25% initial urea concentration.
II - partially purified enzyme from the culture containing 0.5% initial urea concentration

Samples from cultures having 0.25 and 0.5% initial concentrations of urea were chosen to study urease activity with partially purified enzyme (Table B20). Up to 30 h of fermentation, the specific activity of the enzyme was most promising with 0.25% urea concentration (maximum activity 0.427 mmol ammonia/mg protein after 18 h). The maximum enzyme activity with 0.5% urea concentration was 0.27 mmol ammonia liberated per mg protein after 24 h. A possible reason for this is that the amount of proteins present (Table B19) in the fermented broth with 0.25% of initial concentration of urea were comparatively less.

Effect of temperature and pH on urease activity

Table B21 shows the effect of temperature and pH on urease activity. Five different temperatures from 25 to 50°C were studied to determine the influence of temperature on enzyme activity. The optimum temperature for urease activity was 35°C and 50% activity was lost when the reaction was carried out at 50°C. To monitor the effect of pH on urease activity, the specific activity of the partially purified enzyme was studied at pH from 6.4 to 7.4. Maximum enzyme activity

was obtained at pH 7.0. It was, therefore, concluded that the optimum urea concentration for enzyme activity was 0.25%, while 0.5% was optimal for glutamic acid accumulation.

Table B21. Effect of temperature and pH on urease activity

Effect of temperature		Effect of pH	
Temperature (°C)	Specific activity (U/mg protein)	pH	Specific activity (U/mg protein)
25	0.367	6.4	0.351
30	0.386	6.6	0.372
35	0.427	6.8	0.394
		7.0	0.427
40	0.382	7.2	0.410
50	0.213	7.4	0.396

In one mode of regulation by environmental conditions, urease expression is regulated by the global nitrogen control system. In *Klebsiella* in the presence of poor nitrogen sources such as proline, arginine or histidine, synthesis of urease is activated (i.e. under conditions of nitrogen starvation)[248]. In a second mode, urease expression in organisms such as *P. mirabilis* is induced by the presence of the substrate urea[249] In the present case, maximum activity was noticed with a low concentration of initial urea (0.25% in comparison with 0.5%), while glutamate production was maximum with 0.5%. So a nitrogen starved condition (a low initial concentration of urea, 0.25%) may be the reason for higher enzyme activity.

Cousineau and Chang reported[250] the fermentation of amino acids from urea and ammonia by a sequential enzyme reaction using a micro encapsulated

multi enzyme system. A multi enzyme system consisting of urease, glutamate dehydrogenase and glucose 6-phosphate dehydrogenase acts by converting urea into ammonia which, in the presence of NADPH, is then incorporated into alpha ketoglutarate to form glutamate with glucose 6-phosphate dehydrogenase to recycle the co-factor. We assume that this sort of enzyme combination may be present in this *Brevibacterium* sp. which also utilizes urea as a good nitrogen source and excretes glutamic acid into the culture medium.

B.7 SOLID STATE FERMENTATION FOR L-GLUTAMIC ACID PRODUCTION USING *BREVIBACTERIUM* SP.

Introduction

Solid-State Fermentation (SSF) has gained importance in recent past due to its many advantages over submerged fermentation (SmF)[251]. Solid-state (substrate) fermentation is generally defined as the growth of microorganisms on solid materials in the absence of or near-absence of free water. The substrate, however, must contain enough moisture, which exists in the absorbed form within the solid matrix[252] or in other words, solid-state cultivation deals with the controlled growth and metabolism of microorganisms on and/or inside water insoluble materials, in the presence of varying free water amounts[253] SSF processes are used on a commercial scale for the production of fermented foods, fungal metabolites and for bioconversion of organic wastes into useful products[254].

SSF carried out on inert support materials, which differs from the process of microbial growth on or in solid particles floating in a liquid medium, has been regarded as one of the future development of the SSF systems[255]. The use of solid inert material impregnated with suitable liquid media would provide homogenous aerobic conditions throughout the fermenter and the purity of the product would also be comparatively high. Literature survey revealed that a few attempts have been made on SSF processes employing inert supports. Meyrath (1966)[256] reported high yields of dextrinogenic amylases with *Aspergillus oryzae* on vermiculite impregnated with starch solution. Successful production of citric acid on solid

inert material by *A. niger* was reported by Lakshminarayana *et al.* (1975)[257].
Oriol *et al.* (1988)[258] otpimized the various parameters for culturing filamentous fungi on sugar cane bagasse impregnated with a liquid glucose medium. Auria *et al.* (1990)[259] developed a process using ion exchange resin, Amberlite IRA 900, as inert carrier for *A. niger*. Recently, Kobayashi *et al.* (1991)[260] and Zhu *et al.* (1994)[261] used polyurethene foam (PUF) as inert support for culturing *Aspergillus oryzae* and *Penicillium citrinum* respectively. Since the discovery of *Micrococcus glutamicus* by Kinoshita *et al.* (1957)[6], many bacteria have been reported to produce L-glutamic acid directly from sugar in high yields in liquid fermentation. But to the best of our knowledge, no attempts have so far been made to use amino acid producing bacteria in SSF systems.

In this chapter, we described our findings on cultivation of *Brevibacterium* sp. grown on cane bagasse as inert support for production of L-glutamic acid which is such first report on the subject.

Materials and Methods

Microoganism and culture media: Culture preservation conditions and growth medium were similar as described earlier.

Inert carrier: Sugar cane bagasse was washed, sieved, autoclaved and dried by the processing methodology of Saucedo-Castaneda *et al.* (1992)[262]. Milled sugar cane bagasse, obtained from a local sugar mill, was sieved to obtain the particles of 0.45-3.00 mm size. After washing it twice with deionized water (using ten times water volume each time) to remove soluble sugars and other soluble

constituents adhering to the particles, it was dried in a hot air oven at 60°C for 30 h and stored at room temperature in sealed polythene bags. For each set of the experiment, an adequate amount of the bagasse was autoclaved at 121°C for 30 min.

Production medium: The production medium used for impregnating the bagasse contained: glucose 2 g, urea 1 g, KH_2PO_4 0.18, mineral solution 1 ml (prepared by dissolving 1 mg each of $FeSO_4.7H_2O$, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCl in 100 ml distilled water), thiamine hydrochloride 100 μg, corn steep liquor 0.25% (w/v) and two drops of Tween-80 in 100 ml distilled water (pH 7.2). It was sterilized at 121°C for 15 minutes. In the studies on the effect of glucose concentration in the medium, however, instead of 2%, varying concentrations of glucose, as shown in the relevant text, were used.

Fermentation: Fermentation was carried out by taking 2 g of dried bagasse in 250 ml conical flask. This was impregnated with 18 ml of pre-inoculated (20 h old culture of *Brevibacterium* sp. at 5% v/v level.) production medium and mixed well. The flasks were incubated at 30°C for 120 h. Samples, as whole flask in duplicate, were withdrawn after each 24 h. The results shown are the average of four sets of the experiments.

Extraction of the fermented matter

Fermented matter (5 g, wet wt) was mixed thoroughly on a magnetic stirrer with 25 ml of distilled water for 30 min and the contents were filtered through ordinary filter paper. The residue was again treated similarly and both the filtrates

were combined. The filtrate, so obtained, after centrifugation at 5000 rpm for 10 min was used for various assays as below.

Analytical methods: Soluble sugars and proteins were analysed in the filtrate by the methods of Miller and Lowry *et al.* (1951), respectively. TLC was used for the qualitative detection of L-glutamic acid. The filtrate tested only one spot which was identical with authentic sample of L-glutamic acid (similar to our findings in liquid fermentation), hence ninhydrin colour reaction method was used for quantitative estimation. The yields of glutamic acid are reported as mg obtained from one g dry fermented substrate (mg/gds). Solid pH measurements were made by the method of Moore and Johnson (1967)[263].

Results and Discussion

When compared (after 72 h) with the control flask (with uninoculated cane bagasse), a number of striking changes such as change in colour, from white to yellow. occurrence of a pleasant fruity smell, adherence of the substrate to the wall of the flask etc were observed, when the bagasse was incoculated with Brevibacterium cells (Fig.B25). It clearly indicated the growth of the bacterium on the surface of the bagasse.

Fig.B26 shows the glucose consumption pattern by *Brevibacterium* sp. in SSF system. With the course of time, there was gradual decrease in the concentration of sugar which was used by the bacterial strain and this indirectly showed the growth of the culture. The amount of soluble proteins well correlated with this. We studied some of the important parameters as under.

Fig.B25. Growth of *Brevibacterium* sp. on the surface of inert cane bagasse

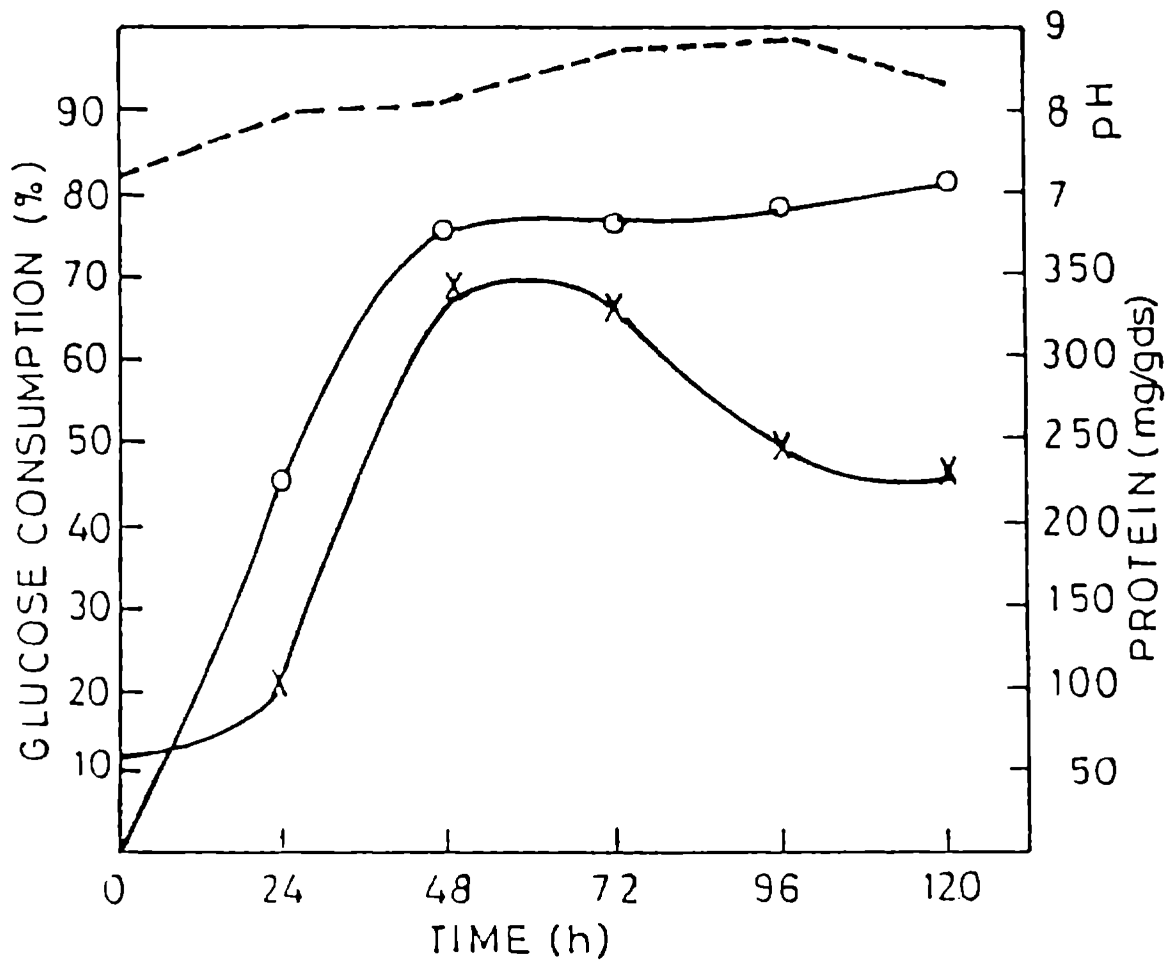


Fig.B26. Glucose consumption, protein and pH during Glutamic acid production in SSF by *Brevibacterium* sp. (120 h)

------- pH o——o Glucose consumption, % X——X Protein

Influence of initial moisture level: Five different initial moisture levels, as shown in Table B22, were set in the substrate. The total substrate in each flask was always 20 g (wet wt). Although, bacterial activity, as observed by glutamic acid production, occurred even at lowest experimental moisture content, i.e. 65-70%, maximum production was observed with 85-90% moisture substrate in 96 h. By this time, 78% of the glucose was consumed by the bacterial culture and the conversion rate was 29.88% (on the basis of glucose consumed and that 81.74% is the theoretical conversion of glucose to L-glutamic acid). There are reports which describe SSF with inert support impregnated with relatively a large

Table B22. Influence of initial moisture on glutamic acid production in Solid State Fermentation by *Brevibacterium* sp.

Initial moisture level (%)	Ratio between moistening agent (ml) & cane bagasse (gm)	Glucose consumption (%)	Glutamic acid (mg/gds)	Protein mg/gds	pH
65-70	14:6	65.7	33.3	133.2	7.9
70-75	15:5	66.7	42.2	200.9	8.1
75-80	16:4	69.7	48.3	242.7	8.2
80-85	17:3	77.2	62.7	306.8	8.2
85-90	18:2	78.3	75.4	354.3	8.6

quantities of liquid medium of low sugar concentration[261]. For the homogenous distribution of the nutrients, the moistening agent (production medium) was required in high amounts and so the initial moisture level too. The amount of moistening agent was shown to influence the physico-chemical properties of the solids[264]. Very low moisture levels lead to poor microbial growth and poor accessibility to nutrients. At the same time, high substrate moisture results in

decreased substrate porosity which in turn prevents oxygen penetration. This may facilitate bacterial contamination. At higher moisture contents (90% or more) the support may form clumps which also affect bacterial activity adversely.

Effect of particle size of the substrate: The physical morphology, especially porosity and particle size of the substrate, govern the accessible surface area to the organism and it is of great importance in SSF. As shown in Table B23, particles of four different individual sizes and three of mixed sizes were used in the present study. Maximum glutamic acid production (82.22 mg/gds) was obtained with the substrate containing particles of four different sizes in equal amounts. Among the individual particle size substrate, the best yields (43.67 mg/gds) were resulted by 1 mm particles substrate. Thus, evidently, substrates with mixed particle size were better choice. With smaller particles, the available surface area for microbial growth was larger but the inter-particle space and hence porosity became less. With bigger particles substrate, the situation was reverse. These two opposing factors probably interact together and thus determine the growth and activity of microorganisms[265]. These results are also in similar lines. The role of oxygen transfer into void space affects the microbial growth and a compromise has always to be made on composition of substrates with mixed particle sizes for optimal activity and mass transfer effects[266,267].

Table B23. Effect of particle size of sugar cane bagasse on glutamic acid production in Solid State Fermentation by *Brevibacterium* sp. (72 h)

Particle size (mm)	Distribution (%)	Glucose consumption (%)	Glutamic acid (mg/gds)	Proteins (mg/gds)	pH
0.450	100	69.20	29.49	72.00	7.68
1.000	100	73.50	43.67	114.12	8.24
2.000	100	70.58	30.08	72.82	8.19
3.000	100	65.32	27.36	81.40	8.12
1 and 0.450	50 each	72.00	46.42	241.13	8.18
1 and 2.00	50 each	68.73	50.10	310.58	8.20
0.45, 1.00, 2.00 and 3.00	25 each	70.10	82.22	337.38	8.22

Effect of inoculum size: Three different inoculum size, viz. 0.5, 2.0 and 4.0 ml (containing $7x10^7$ cells/ml), were used to inoculate 20 g wet substrate. The results are shown in Fig.B27. An increase in inoculum size from 2.5% (v/wet wt of substrate) to 10% did not have much impact on the production level, although a slight increase was noted. Increasing the inoculum size to 20%, resulted faster glutamic acid formation during the early phases of fermentation (upto 48 h), but after 96 h the yields were less when compared with the two others. Marginal decrease seen with larger inoculum doses could be probably due to shortage of nutrients available for the large number of cells.

Effect of initial pH: The pH of the production medium was adjusted to different values to achieve the medium pH as 5.5, 6.5, 7.5 and 8.5 before mixing with the bagasse. The results obtained are shown in Fig.B28. The maximum production was noted with the substrate having 7.5 initial pH.

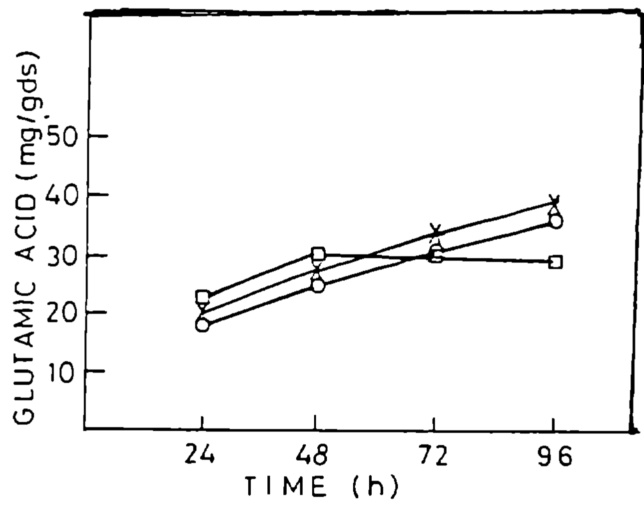


Fig.B27. Efffect of inoculum size on glutamic acid production by *Brevibacterium* sp.

o——o 2.5% v/w X——X 10.0% v/w

20.0% v/w

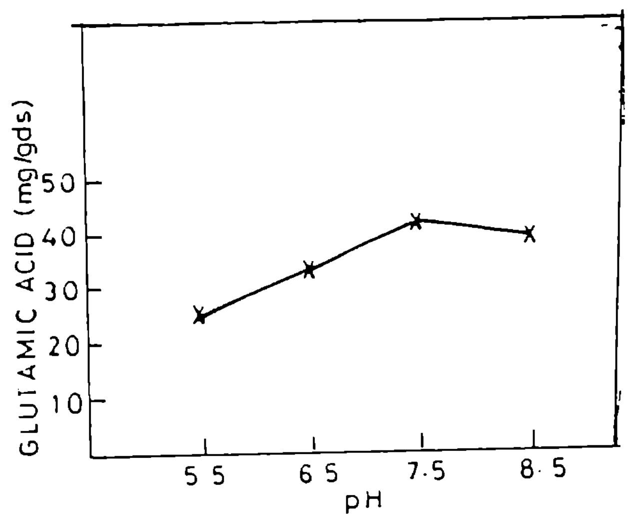


Fig.B28. Effect of initial pH on glutamic acid production in SSF by Brevibacterium sp. (120 h)

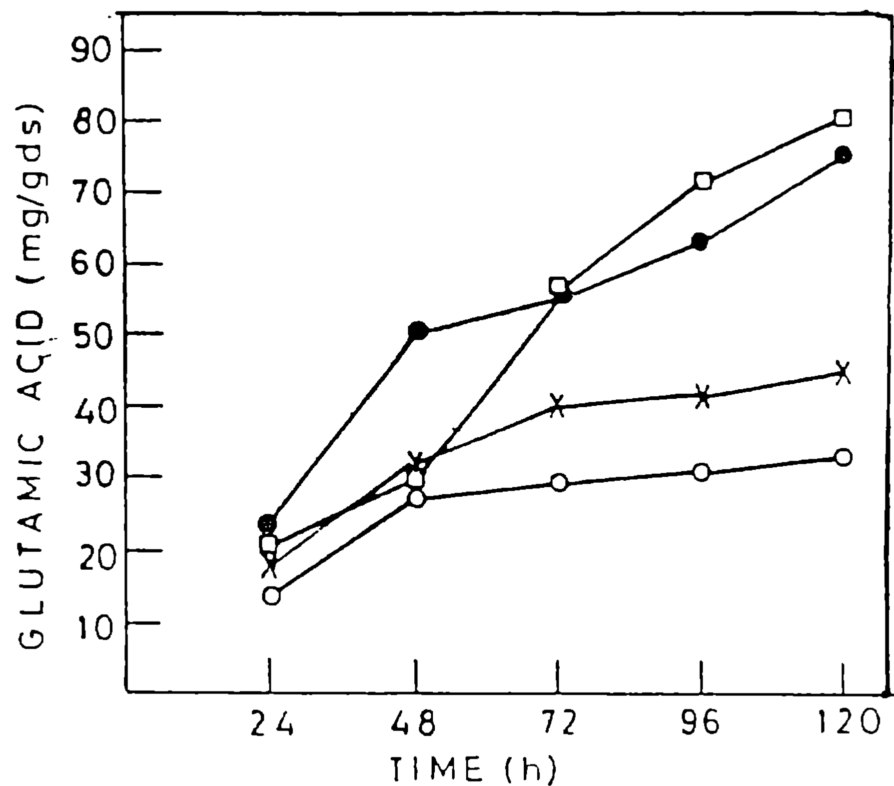


Fig.B29. Effect of concentratio of glucose on glutamic acid production in SSF by Brevibacterium sp. (120 h)

o______o 1% Glucose ● ______● 5% Glucose

X______X 2% Glucose □——□ 10% Glucose

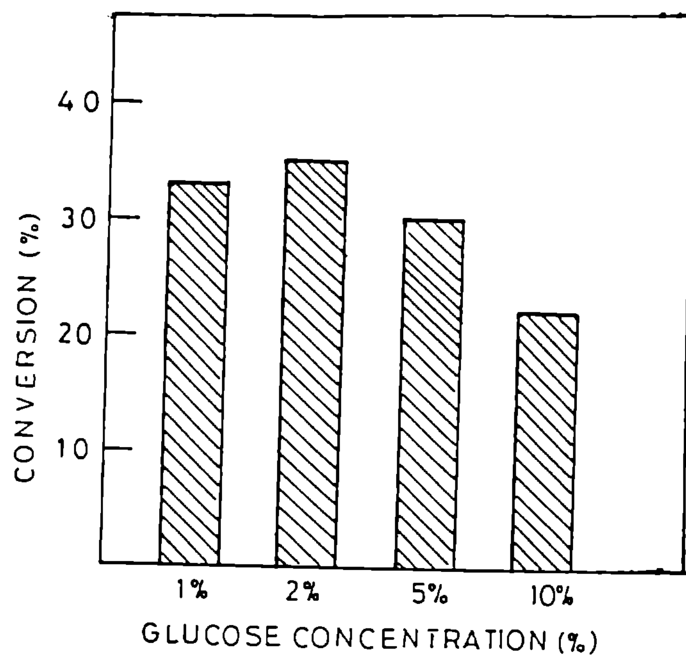
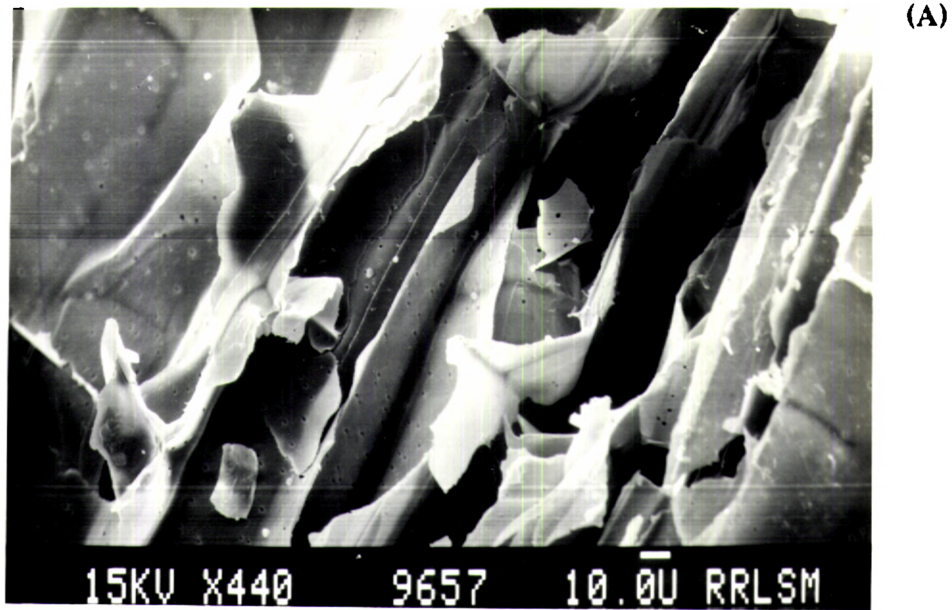


Fig.B30. Effect of initial glucose concentration on its conversion to glutamic acid in SSF by *Brevibacterium* sp. (120 h)

(A)



(B)

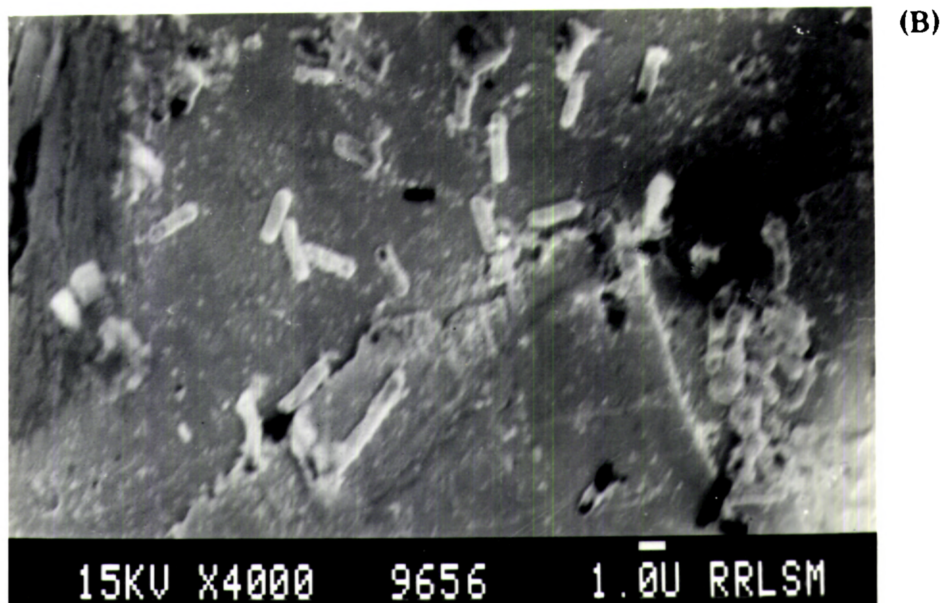


Fig.B31. Scanning electron micrograph showing the growth of *Brevibacterium* cells on the surface of inert cane bagasse

(A) 00 h (B) 72 h

Effect of initial glucose concentration: It is known that an ideal substrate concentration in any fermentation process results higher conversion efficiencies and optimum substrate utilization. Results of our study on the effect of using 1, 2, 5 and 10% glucose in production medium showed maximum glutamic acid with 10% glucose (Fig.B29). In this case, glucose consumption was 80% but the conversion efficiency was 22.03%, as evident from Fig.B.30. Maximum conversion (34.75%) was obtained with 2% glucose but this was marked with poor substrate utilization (68.5%). Considering both the factors as crucial, 5% glucose concentration appeared to be the best in which case the bacterial culture consumed 81% substrate with 30% conversion efficiency. In SSF systems utilizing inert supports, adequate availability of energy source (carbon) and nutrients is very crucial. If the carbon source is too less, it affects nutrients uptake due to improper availability resulting from the inadequate distribution and if it is too high, it affects the growth and conversion efficiencies probably by a feedback mechanism.

SEM studies: Growing cells on the surface of the inert substrate were clearly viewed as shown in Fig. B31A&B.

In conclusion, a culture method where substrate and support are separate and an absorbed liquid is used, offers a number of advantages such as the use of monomeric carbohydrates immediately available for the organism, degradation of the solid matrix during growth can be avoided thus ensure constant geometric conditions and it make easier comparison with submerged cultures, thus this technique can be effectively used for the production of primary metabolites such as amino acids on a large scale.

B.8 IMMOBILIZATION OF *BREVIBACTERIUM* CELLS FOR THE PRODUCTION OF L-GLUTAMIC ACID

Introduction

In the last few years, apart from the genetic manipulation of organisms, several other attempts have been made to design different strategies to optimize the glutamate fermentation. These include cell recycling[151], fuzzy supervisory-control systems[153], automation of the process[155], culturing of the microorganism in solid substrates[268], nutrient formulation[269,270] and the use of different raw materials[271,272].

Immobilization has been considered as a useful and attractive technique for the production of microbial products. Immobilization of cells offers certain advantages such as high productivities due to cell contractions obtained in the gel bead, easy and less costly down stream processing and lessening of the chances of contamination etc. But its application has been highlighted from the view point of long term utilization of biocatalysts and continuous operation of stabilized systems[273]. A number of amino acids have been produced using this methodology. These include L-aspartic acid[274], L-isoleucine[275], L-serine[276], L-Lysine[277] For the first time, immobilized *Corynebacterium glutamicum* cells were employed to produce L-glutamic acid from glucose by Slowinski and Charm[278] (1973). Amin *et al.*[279] (1993) made an attempt to study the formation of by-products during glucose conversion to glutamic acid using *Corynebacterium glutamicum* immobilized in polyurethane foam. Entrapment of protoplast of *Brevibacterium flavum* in matrices of agar-acetyl cellulose filtering is another attempt to produce L-glutamic acid[280].

The present study was intended to standardize the various optimal immobilization parameters for a strain of *Brevibacterium* sp. for L-glutamic acid production in batch, repeated batch and continuous mode operation. As a prelude, a study was conducted to evaluate the diffusional properties of a few gels such as calcium alginate, agar and polyacrylamide.

Materials and Methods

Diffusional studies for the selection of gels: As a prelude to investigate the efficiency of the gel entrapped *Brevibacterium* sp. for L-glutamic acid production, a study was planned to evaluate the diffusional properties of a few gels viz. calcium alginate, agar and polyacrylamide.

Glucose (mol. wt. 180), a low molecular weight substrate as glutamic acid (mol. wt. 147) was employed for the comparison of the diffusional properties of alginate (2% w/v), agar (2% w/v) and polyacrylamide beads (2% w/v).

Polyacrylamide beads were prepared according to the method of Woodward (1985)[281] 1.98 g acrylamide and 0.1 g methylene bis acrylamide in 11 ml distilled water was supplemented with ammonium persulphate solution (15 mg in 3 ml distilled water) and 0.1 ml of tetramethyl ethylene diamine (TEMED). The cubes were cut from the gel after plating in petri-plates and were washed in distilled water before use. The details for the preparation of alginate and agar beads are mentioned on subsequent pages. The only difference was that the beads did not contain any

microbial cells. Glucose at 200 mg level was added as molecular weight marker to all type of gels before making the beads.

The beads were then transferred to conical flasks containing 50 ml of distilled water and kept on a rotary shaker (180 rpm) at 30±1°C. Samples were withdrawn at different time intervals and the amount of glucose diffused out from each type of bead were determined.

Medium for L-glutamic acid production: Production medium contained (g/L): glucose, 5.0; urea, 5; KH_2PO_4 1.8; and (ml/L): mineral solution, 10 ml ($FeSO_4.7H_2O$, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCl each 10 mg), corn steep liquor, 0.25% v/v ml and one drop of Tween 80, in distilled water (pH 7.2). The medium was autoclaved at 121°C for 15 minutes. Glucose and urea were sterilized separately by microfiltration.

Cultivation of microbial cells: Inoculum was prepared from the freshly grown culture as described earlier (Section B1). 2.5 ml of culture broth were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of the growth medium and incubated at 30°C (180 rpm). Fresh cells were harvested from the broth by centrifugation at 10,000 g (10 minutes, 10°C) and washed twice with 0.2% (w/v) KCl solution.

Cell immobilization in alginate: Entrapment of cells using sodium alginate was performed as follows. Centrifuged *Brevibacterium* cells (0.25-2.0 g wet weight) were mixed thoroughly with 20 ml of sterile sodium alginate solution (2-5% w/v). The mixture was extruded as drops into a solution of $CaCl_2$ (0.5 M). Bead size (2-5 mm)

was controlled by the gauge number of the hypodermic needle used during extrusion. The beads were cured in the same solution at room temperature for an hour and stored in a freshly prepared 0.1 M $CaCl_2$ solution at 4°C. Biocatalysts having different storage periods were tried during the experiments.

Immobilization in agar: Immobilization in agar was done by following the method of Gogoi *et al.* (1991)[282]. A known amount of cells was mixed into aqueous agar solution (3-5% w/v) at 41°C. The mixture was quickly placed dropwise onto a hydrophobic phase (sunflower oil) whereby spherical beads were instantaneously formed.

Enumeration of viable cells: The alginate gel beads of a known weight were washed twice in sterile physiological saline and suspended in 10 ml of 0.1 M phosphate buffer (pH 7.3). The suspension was stirred using a magnetic stirrer at room temperature for 15 minutes, after which the alginate gels had completely dissolved. The cell suspension obtained was diluted and spread on nutrient agar plates. The colonies were counted after the incubation of plates for 24 h at 30°C. Similarly agar beads after proper rinsing with sterile saline, were mixed thoroughly in 10 ml distilled water at 41°C. Appropriate dilutions of the resulting cell agar mixture were plated out on nutrient agar[283]. All counts were performed in triplicate.

Batch fermentation: For batch fermentation, immobilized *Brevibacterium* cells were cultivated in 500 ml Erlenmeyer flasks containing 100 ml production medium. Fermentation was carried out over 120 h at 30°C (150 rpm).

Production medium was supplemented with 0.01-0.05 M $CaCl_2$ solution to monitor the effect of calcium ions on the performance of immobilized (in alginate) cells.

Repeated Batch Fermentation: Fermentation was carried out as above and after each 120 h the biocatalysts were aseptically removed from the fermentation medium and then washed 3-4 times with sterile distilled water. They were used again, together with fresh medium, in the same experimental conditions.

Continuous production of L-glutamic acid.: For continuous production of L-glutamic acid using immobilized biocatalyst a packed column reactor was used as shown in Fig.B32. It consisted of a glass column having 40 cm length and 3 cm diameter which was loosely packed with the biocatalysts. Top and bottom meshes were used to prevent the wash out of biocatalysts. Production medium was injected from the bottom of the column using a peristaltic pump at a flow rate of 0.25-1.0 ml/minute. The entire fermented medium coming out of the bioreactor was mixed with the reservoir containing medium. The fermentation was continued for 24 h, after which the fermented medium was replaced with fresh medium.

In all cases, the results are represented as the average of three sets of experiments.

SEM studies: SEM studies were conducted as mentioned in Section B.1 to view the growth of the cells within beads at different periods of incubation.

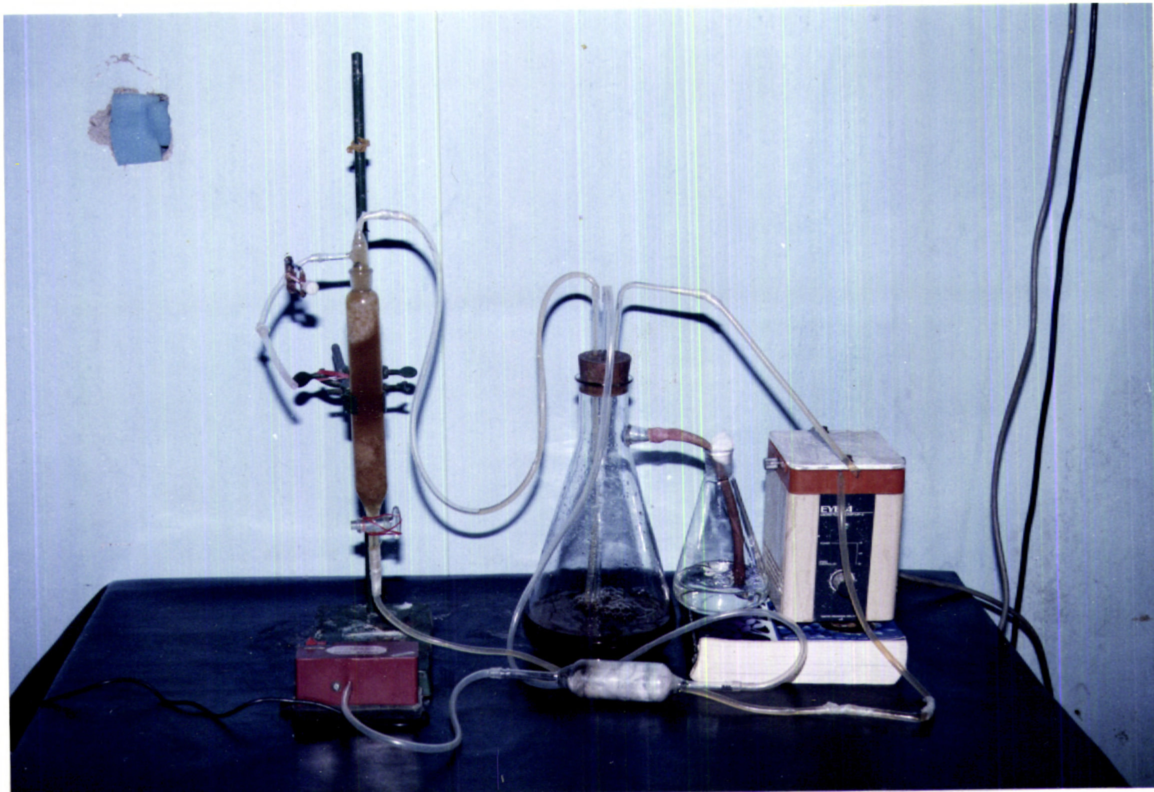


Fig.B32. Set up of the packed column bioreactor for the production of L-glutamic acid using immobilized *Brevibacterium* cells

Analytical methods: Estimation of reducing sugars and L-glutamic acid were done as mentioned earlier in Section B.1. To determine the cell leakage from the beads, the bacterial growth was determined by measuring the absorbance of approximately diluted culture broths at 610 nm.

Results and Discussion

Diffusional Studies

Table B24 quantitatively describes the efficiency in terms of diffusional property among alginate, agar and polyacrylamide beads. For low molecular weight substrate like glucose, the diffusion was relatively faster in alginate and agar beads with polyacrylamide showing less glucose diffused out in the given period of time. Diffusional limitations encountered in the polyacrylamide beads might be due to smaller pore size in the gels. The present study thus convincingly established the superiority of alginate and agar beads over polyacrylamide beads. In subsequent studies, thus alginate and agar beads were selected for the immobilization of *Brevibacterium* cells.

Table B24. Diffusion pattern of glucose

Time of incubation (minute)	Glucose mg/50 ml		
	Alginate	Agar	Polyacrylamide
00			
30	186.00	185.7	151.0
60	193.5	191.0	153.6
90	196.8	191.8	153.7
120	198.0	193.0	154.1

Optimization of immobilization in Ca-alginate (Batch cultures)

Calcium alginate gel beads are easy to produce on a large scale without any sophisticated equipment. It is non-toxic and available at low prices. On the other hand, there are a number of immobilization parameters that need to be optimized.

Alginate concentration

Three concentrations (2%, 3% and 5% w/v) of sodium alginate were used to prepare the beads. Fig.B33 shows the effect of alginate concentration on cell leakage and L-glutamate production. The maximum concentration of L-glutamate (7.41 mg/ml) was obtained with 3% calcium alginate beads after 96 h fermentation. Even though the cell leakage was comparatively less with much harder (5% w/v alginate) beads the production was decreased. Shimmyo *et al.* (1982)[284] and Nasrin *et al.* (1989)[277] have also reported similar findings, in other fermentations.

Effect of calcium ions

Table B25 shows the effect of concentration of calcium ions in the medium on L-glutamic acid production by the immobilized cells. Addition of calcium salts up to 0.03 M slightly increased the production of glutamate without much affecting the immobilized cells. At 0.05 M concentration an inhibition in the number of viable cells, and hence a decrease in the production, were noted. It has been reported that calcium alginate gel is unstable in the presence of phosphates and certain cations such as Mg^{++} or K^{+}, which are major nutrients of living microbial cells[285] However, the solubilizing effect of these agents can be overcome by supplementing the growth medium with $CaCl_2$.

Table B25. Effect of calcium chloride concentrations on biomass and glutamic acid production (96 h) with alginate beads

$CaCl_2$ concentration (M)	Viable cells/10 beads	L-glutamate (mg/ml)
Control	$57x10^7$	6.4
0.01	$53x10^7$	6.7
0.02	$50x10^7$	7.0
0.03	$49x10^7$	7.4
0.05	$44x10^7$	6.2

Influence of storage period of beads

Beads were stored for 4 and 24 h. As is evident from Fig.B34, the prolonged storage of beads led to significant increase in and a steady, L-glutamic acid production. This was probably due to improvement of the gel solidity attained with the consequent decrease of cell release from the beads. According to Lu & Chen (1988)[285] the cells encapsulated in properly solidified beads had a better storage stability than the free cells.

Effect of bead size

Beads of three diameters (2, 3 and 5 mm) were prepared. A maximum of 7.40 mg/ml L-glutamic acid (batch mode) was obtained using beads of 3 mm diameter (Fig.B35) and a considerable reduction (28.3%) in the production was noted with 5 mm bead size.

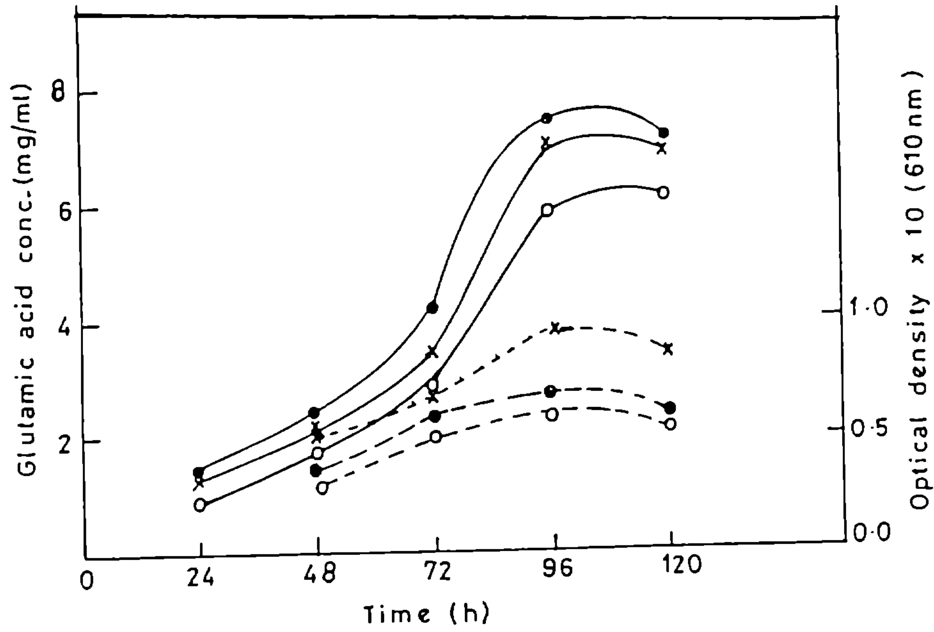


Fig.B33. Effect of alginate concentration on cell leakage and L-glutamic acid production

—— glutamic acid production

X——X 2%

●——● 3%

o——o 5%

cell leakage

X——X 2%

●-----● 3%

o-----o 5%

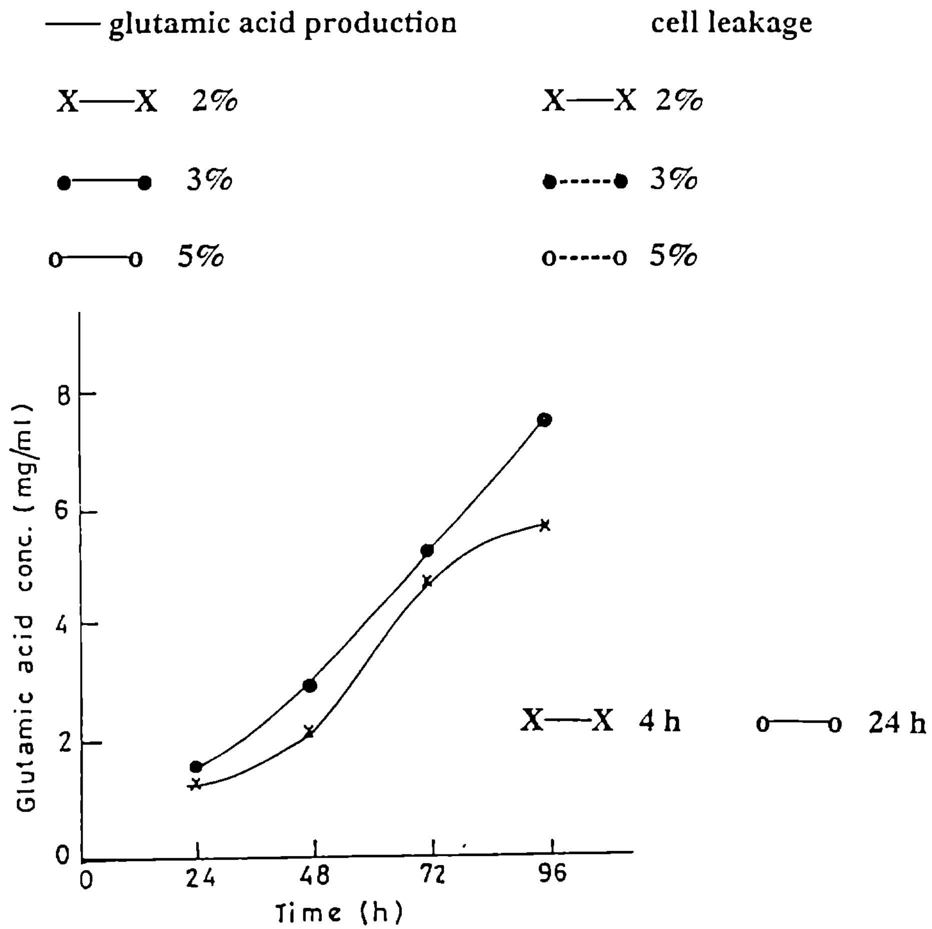


Fig.B34. Effect of storage period of alginate beads on L-glutamic acid production

Effect of initial cell loading

Table B26 shows the effect of initial biomass concentration on glutamate production. Concentrations varying from 0.25-2.0 g per 20 ml gel were tried. It was evident that both the glucose consumption and glutamate production were affected by the initial cell loading. Around 90% of the available glucose was consumed after 96 h when the biomass concentration was 0.75 g and this resulted in maximum L-glutamic acid (7.40 mg/ml). A drastic reduction in the glucose consumption (67%) and hence in production was recorded when the initial biomass was raised to 2 g wet cells. It has been reported by Gosmann & Rehm (1986)[286] that with increasing cell concentration in gels, oxygen was consumed faster than it could diffuse into the beads. At this point, diffusion became the limiting factor.

Table B26. Effect of initial biomass concentration on glutamate production using Brevibacterium cells entrapped in alginate

Wet weight of cells/20 ml gel	Glucose (C) mg/ml	Glutamic acid mg/ml	Glucose (C) mg/ml	Glutamic acid mg/ml	Glucose (C)	Glutamic acid mg/ml
	48		72 h		96 h	
0.25	23.3	2.2	33.4	3.4	37.3	4.1
0.5	26.5	2.7	36.0	4.0	40.8	6.0
0.75	29.1	2.30	41.4	5.2	45.4	7.4
1.0	28.8	3.1	42.6	5.0	44.6	7.0
2.0	21.6	2.3	30.6	3.4	33.8	5.2

Glucose (C) - glucose consumed

Optimization of Immobilization in agar (batch cultures)

Effect of concentration of agar for preparation of beads: Three concentrations (3, 4 and 5% w/v) were used. Compared to alginate beads, an increase of 37% in the production (11.78 mg/ml) was obtained after 96 h with 4% agar beads (Fig.B36). As the concentration increased, the cell leakage was reduced. The cell leakage with agar beads was comparatively less than with alginate beads.

Effect of bead size: Fig.B37 shows the concentration of glutamate obtained with each size bead. In contrast to results obtained with alginate, immobilization in agar beads of 4 mm diameter was most effective.

Effect of initial biomass concentration: The optimum biomass was 0.75 g wet cells/20 ml gel as in alginate, although with higher product yields. Around 95% of the available glucose was consumed after 96 h with this cell loading. Higher initial loading (2 g wet cells) resulted in a reduction in the production (Table B27) as well as in the glucose consumption.

The main metabolic limitation with the immobilized system is diffusion. With both alginate and agar there appeared to be good mass transport (consumption of glucose, excretion of glutamate). However, the cell leakage was minimum with agar beads and so the beads were more stable.

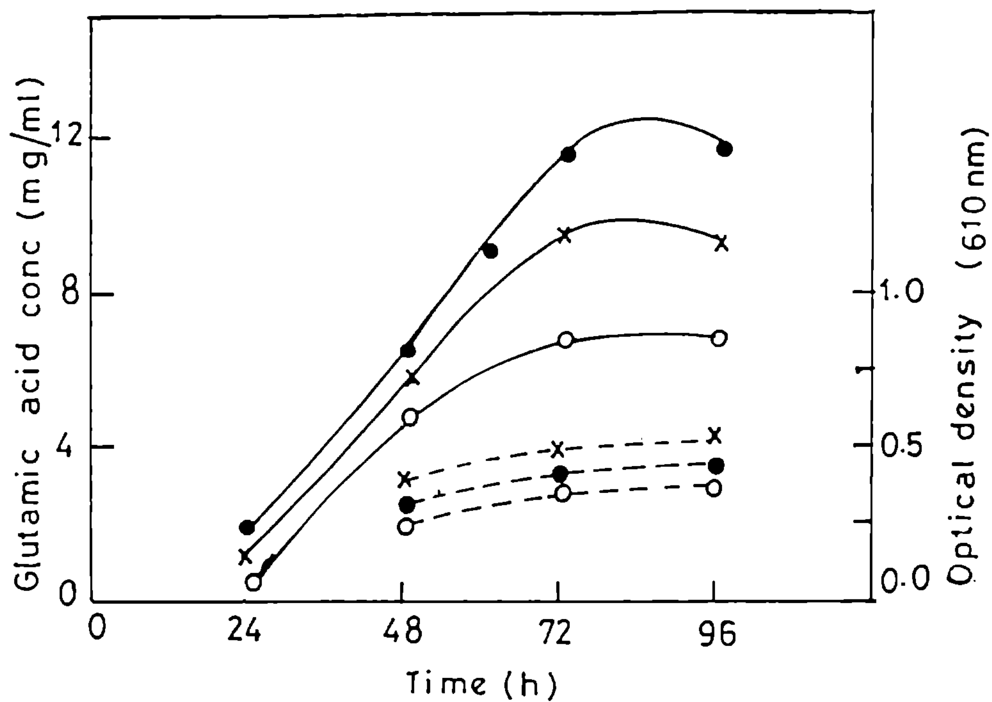
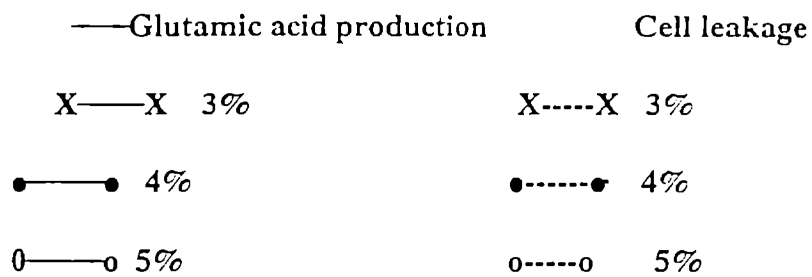


Fig.B36. Effect of agar concentration on cell leakage and L-glutamic acid production



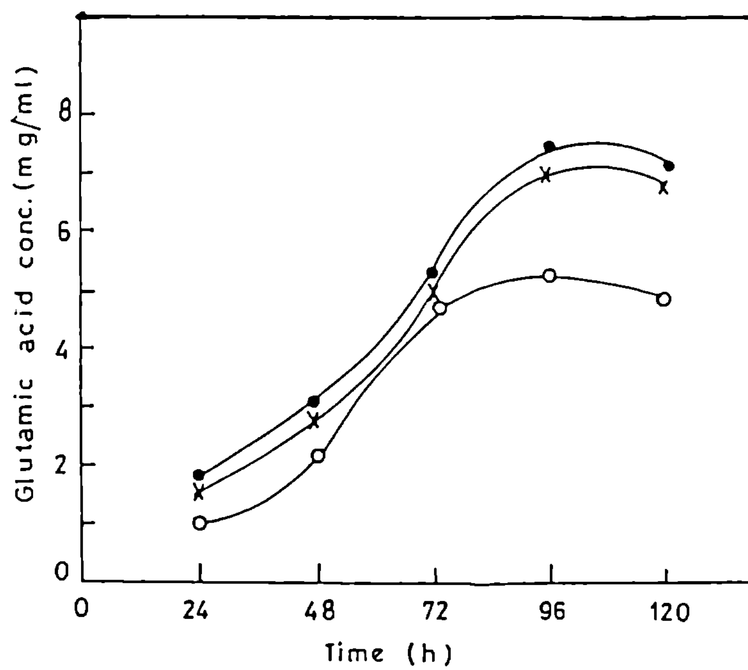


Fig.B35. Infuence of bead size (alginate) on L-glutamic acid production

X——X 2 mm o——o 5 mm •——• 3 mm

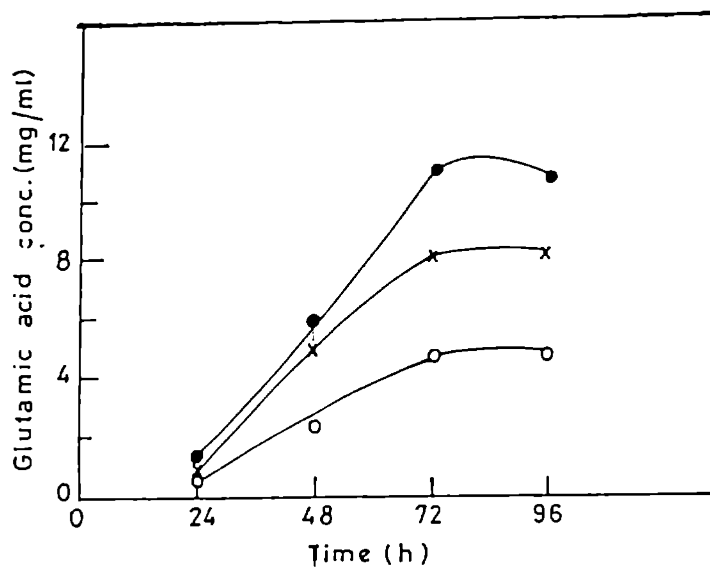


Fig.B37. Influence of bead size (agar) on L-glutamic acid production

X——X 3 mm •——• 4 mm o——o 5 mm

Table B27. Effect of initial biomass concentration on glutamate production using *Brevibacterium* cells entrapped in agar

Wet weight of cells (g)/ 20 ml gel	Glucose (C) mg/ml	Glutamic acid mg/ml	Glucose (C) mg/ml	Glutamic acid mg/ml	Glucose (C) mg/ml	Glutamic acid mg/ml
	48 h		72 h		96 h	
0.25	24.7	5.8	34.4	7.9	40.7	10.6
0.5	27.9	6.1	37.5	8.7	43.1	11.2
0.75	30.8	6.2	44.0	8.9	48.2	11.8
1.0	29.8	6.0	40.3	8.9	46.1	9.1
2.0	22.2	4.0	31.2	5.3	35.8	6.5

Glucose (C) - Glucose consumed

Repeated use of entrapped *Brevibacterium* cells

The entrapped cells were cultivated for 25 days in five batches and each batch lasted for five days. Results are shown in Fig.B38. From the 3rd to 5th batch, L-glutamate was produced at the maximum level (8.7 mg/ml using alginate beads and 13.3 mg/ml with agar beads). As shown in the Fig.B38, (in both cases) leakage of cells scarcely increased during repeated cultivation.

Continuous Production of L-glutamic acid

For constructing continuous production systems with immobilized growing cells, one should consider the cell holding ability of gels on account of its importance and in comparison with alginate the cell retaining capacity of agar beads are much better. Moreover, the agar gel is characterized by a favourable permeability matrix

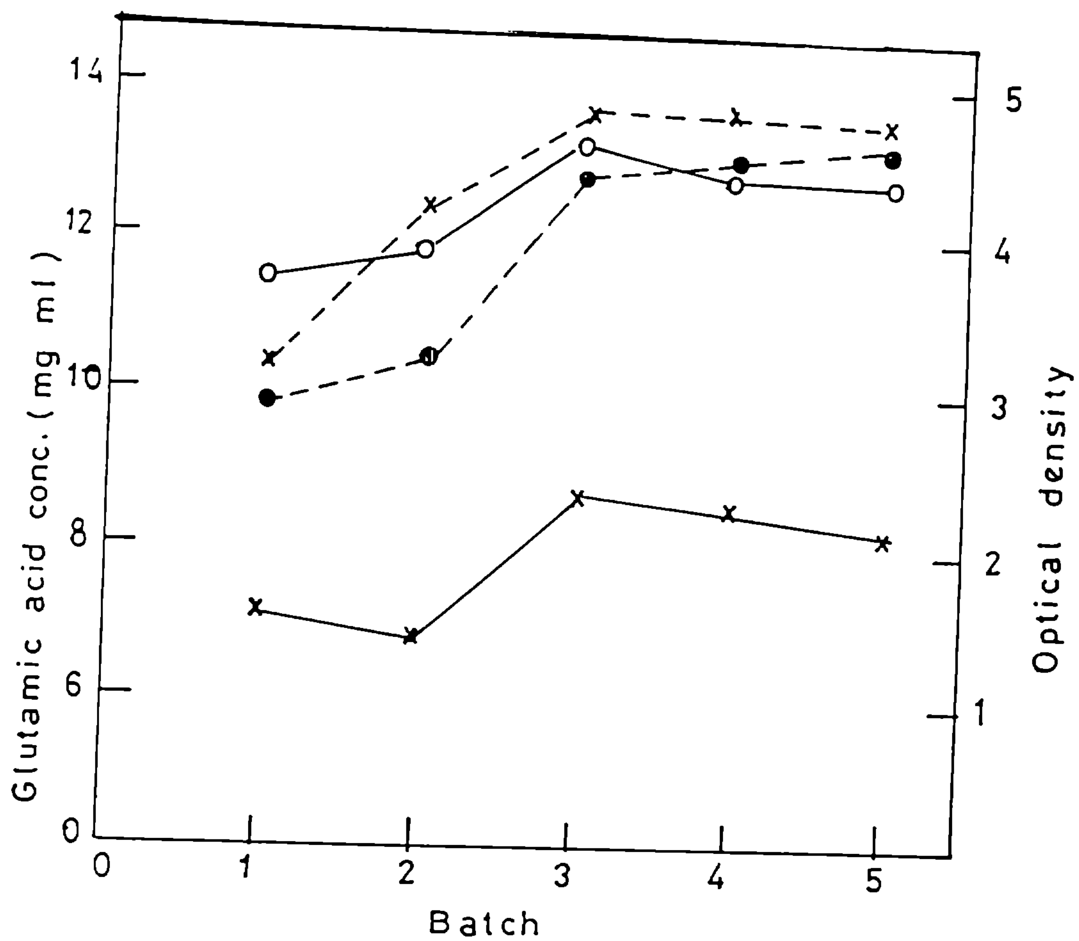


Fig.B38. Repeated batch fermentations with immobilized cells

X——X Glutamate production with cell entrapped alginate beads

X-----X Cell leakage with alginate beads

o——o Glutamate production with cell entrapped agar beads

•-----• Cell leakage with agar beads

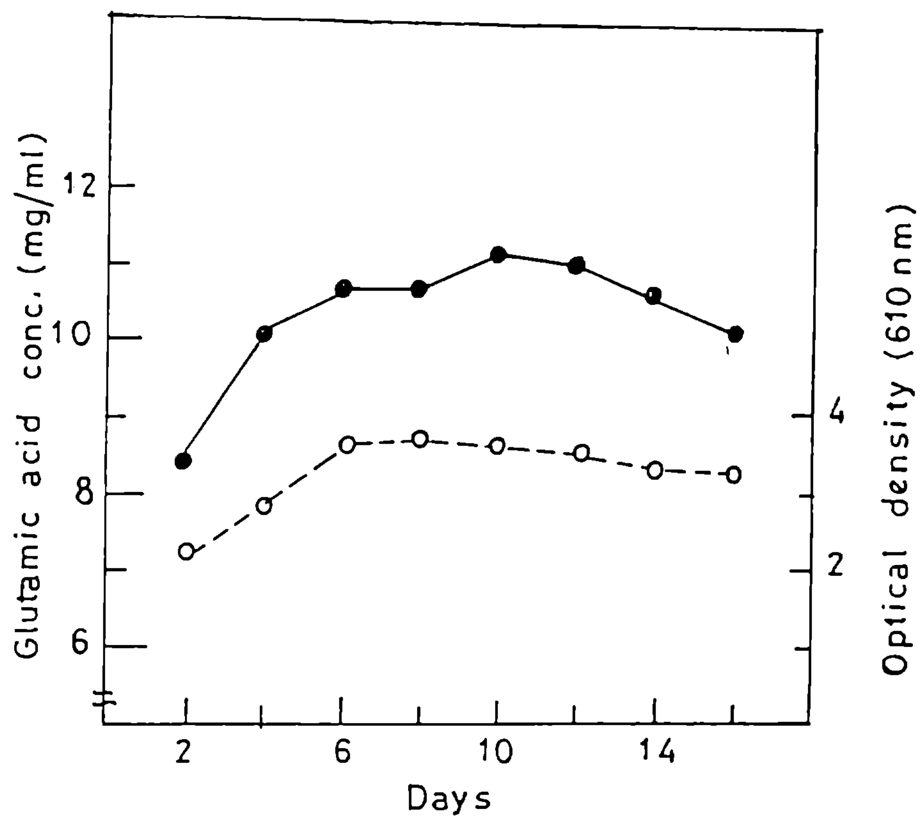


Fig.B39. Continuous production of L-glutamic acid by immobilized growing *Brevibacterium* cells

●——● L-glutamic acid production

o-----o Cell leakage

density relationship for the production of gel entrapped cell system. Thus, continuous production of L-glutamic acid was performed using the agar-entrapped cells in a packed column bioreactor. Fig.B39 shows that the L-glutamate productivity was maintained within a range of 10-11 mg/ml from day 2 to day 16. During continuous fermentation, leakage of cells was more or less at the same level from the 6th day onwards.

Four different flow rates were tried. As is evident from Table B28, production was maximum (10.5 mg/ml) when the flow rate was 0.5 ml/ml.

Table B28. Influence of flow rate on glutamate production in continuous mode

Flow rate ml/minute	Glutamate production mg/ml
0.25	9.2
0.50	10.7
0.75	10.5
1.00	8.6

When the column was packed to different heights using different quantities of the agar beads a maximum yield of 10.70 mg/ml glutamic acid was obtained with column with 20 g beads, occupying 14 cm of the column (Table B29). When the column was filled with 30 g agar beads occupying 21 cm of the column, the production was reduced by 30%.

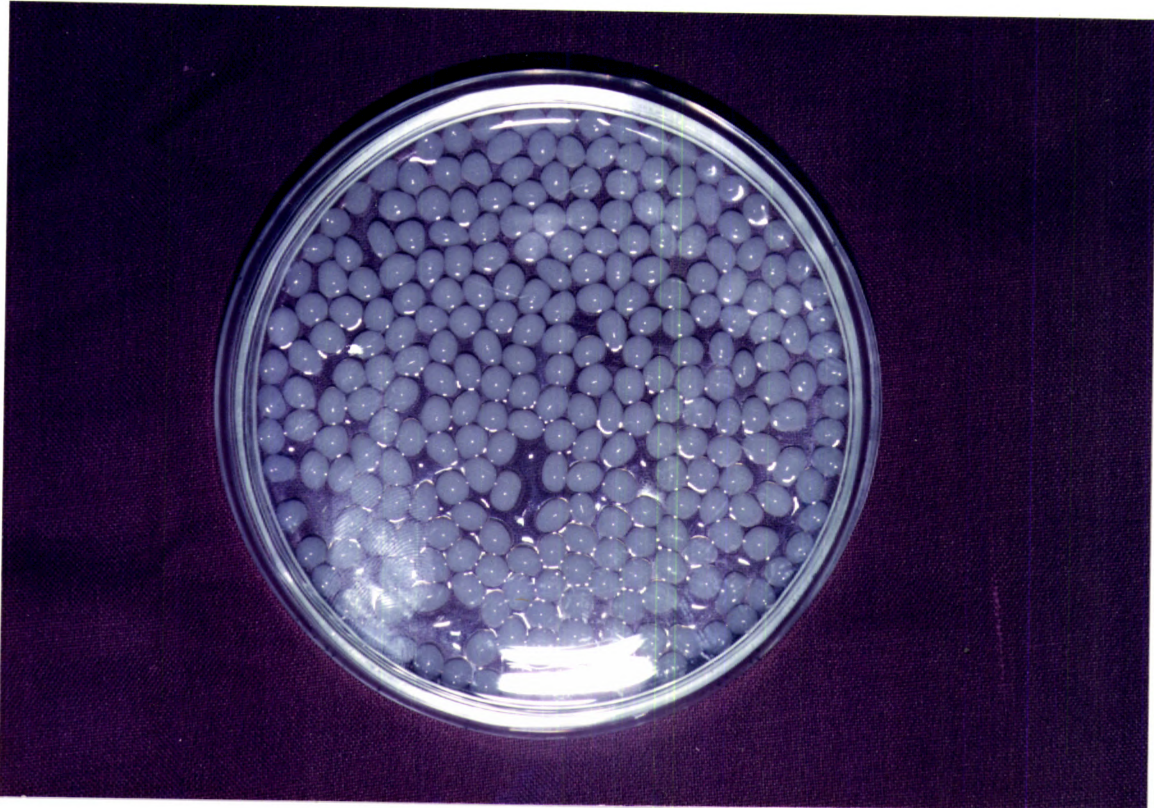
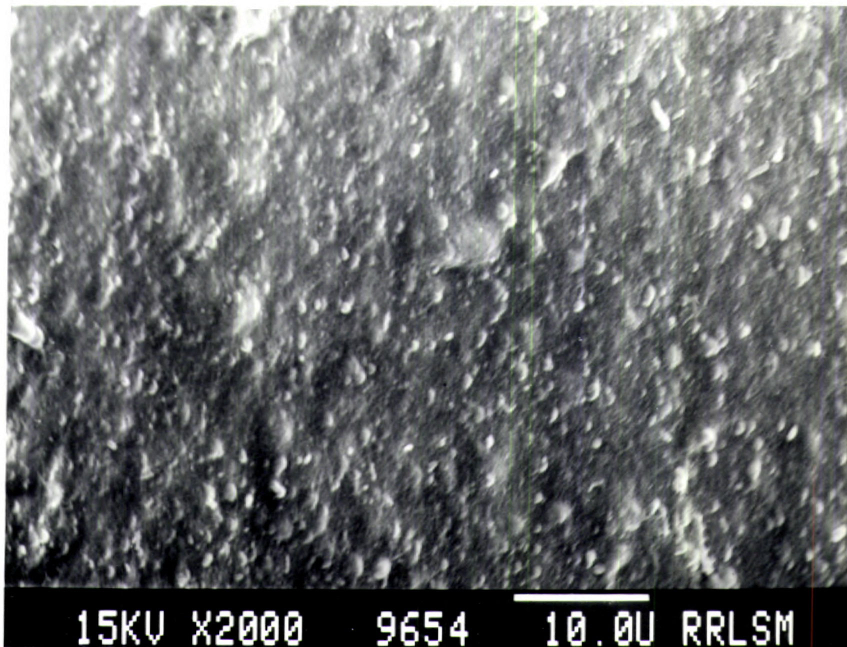


Fig.B40. Scanning electron micrograph showing

(A) Cross section of alginate beads (00 h)



(B) Cross section of alginate beads (72 h)

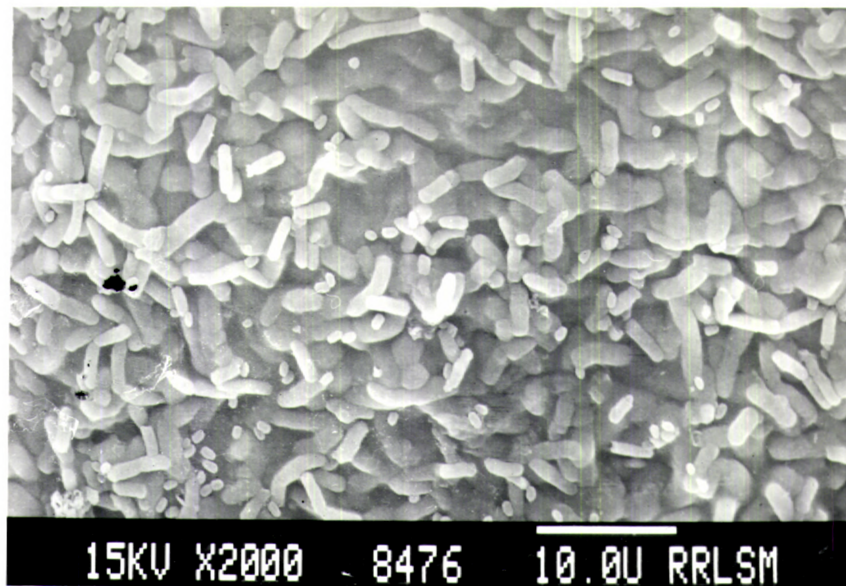
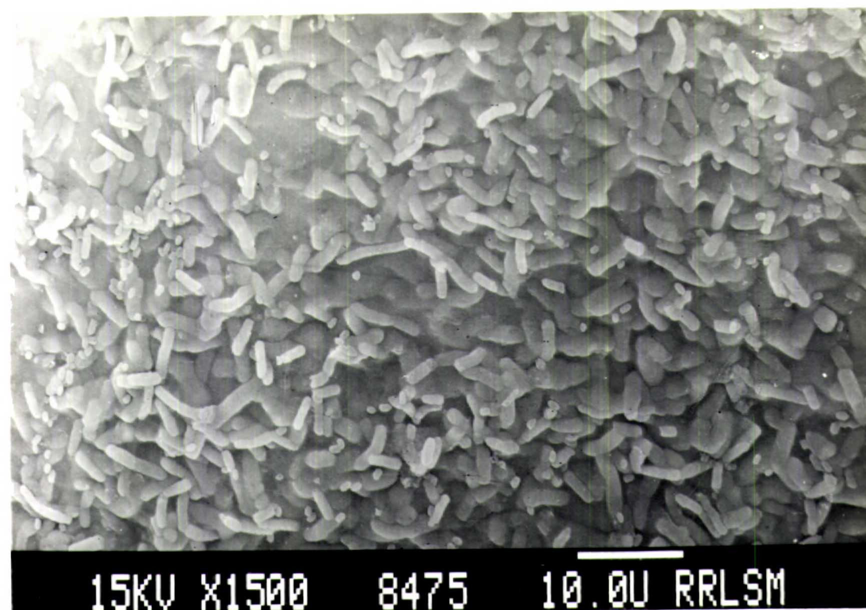


Fig.B40. Scanning electron micrograph showing the growth of *Brevibacterium* cells within the beads



(C) Cross section of agar beads (72 h)

Table B29. Effect of initial biomass and packed column height on glutamate production in continuous mode

Amount of agar beads (gram wet weight)	Packed column height (cm)	Initial biomass gram (wet weight)	Glutamate produced (mg/ml)
10	6.5	0.38	8.0
15	9.5	0.56	8.7
20	14.0	0.75	10.7
25	18.0	0.94	10.6
30	21.0	1.13	7.5

The inference from these data was that the operational stability of the biocatalyst indicated the possibility of the application of immobilized *Brevibacterium* cells for long term use in repeated batch mode and continuous process for the production of L-glutamic acid. Gel concentration has a significant effect, and the optimum concentration for alginate beads was 3% and for agar 4%. Storage of alginate beads (24 h) increased the gel consistency and durability. The immobilization procedure was most effective when the bead size was smaller (3 mm) for alginate beads and for agar bead it was 4 mm. In both cases, the optimum initial biomass was 0.75 g wet cells/20 ml gel.

SEM Studies

In comparison with the control, the cross section of the aged beads shows a highly densed growth of the bacterial cells (Figs.B40A,B&C) A clear morphology of the cells, rod-coccus cycle were visible in the SEM photographs.

B.9.1 BIOCONVERSION OF CASSAVA STARCH TO L-GLUTAMIC ACID USING *BREVIBACTERIUM* SP.

Introduction

Several strains of *Corynebacterium* and *Brevibacterium* are used as cost effective bioconverters which have been exploited by the fermentation industry to produce various amino acids, including L-glutamic acid. After the realisation of the overproduction of L-glutamic acid by *Brevibacterium* sp. based on glucose and other related sugars like maltose, sucrose, fructose etc, an attempt to develop a microbial process for glutamate production employing locally available starchy tubers as the raw material. Cassava (*Manihot esculenta* Crantz) popularly known as tapioca, is one of the major tuber crops of the world, being cultivated extensively in tropical countries and obviously provides a major source of calories to millions of people in the world. On dry weight basis, tapioca contains about 80-82% starch (fresh roots have 22-30% starch) of which 55-60% is recoverable as starch[287] In preparations of sweeteners, cassava starch had definite advantages like easy gelatinization on heating (ease of liquefaction), less interference from other ingredients like fat, protein etc. Cassava starch although has potential commercial importance[288], the presence of cyanide in small quantities is a drawback in its utilisation. Attempts have been made for detoxification of tapioca during its processing by using microbial strains of *Corynebacterium* and *Brevibacterium*[289] The present study reveals the possibilities of utilizing starch for the fermentative production of L-glutamic acid using a strain of *Brevibacterium* sp.

Materials and Methods

Preparation of cassava starch: Fresh cassava tubers obtained locally were washed and peeled. The process of starch extraction from these tuber consisted of wet milling the washed cassava roots followed by washing the starch from the fibrous mass. Starch was sedimented in settling containers and air dried.

Hydrolysis of cassava starch: Liquefaction of the starch slurries (5% w/v) was carried out with a thermostable α-amylase (Termamyl liquid 120, Novo Industries, Bagsvared, Denmark, produced from a strain of *Bacillus licheniformis*). The enzyme was having 120 KNU/g activity [one Novo α-amylase unit (NU) is defined as the amount of enzyme that hydrolyse 5.26 g starch/h under Novo's standard conditions]. pH of the slurry was adjusted to 6.0 and reaction was carried out in a stirred reactor with enzyme at 85°C for 2 h. In order to obtain higher D.E. (dextrose equivalent) values, the resulting dextrose solution was cooled to 60°C. The pH was adjusted to 4.8-5.0 and saccharified with a fungal glucoamylase [AMG 300 liquid, Novo Industries, which has an activity of 300 AGU/ml (one Novo amylo-glucosidase is defined as the amount of enzyme that splits one micromole of maltose per minute at 25°C)]. Reaction was carried out for 18-24 h at 60°C and was then stopped by heating to 90°C for 10 minutes. The hydrolysate was double filtered using a nylon cloth and later by using whatman No.1 filter paper so as to get the clear hydrolysate. Hydrolysate of different DEs were prepared by the intermittent analysis of reducing sugars. The cassava starch was also hydrolyzed using HCl as described elsewhere.

Fermentation: Until and unless mentioned, all the studies were performed with hydrolysate prepared by enzymatic hydrolysis. Prior to fermentation, the hydrolysate was diluted to a suitable reducing sugar concentration. For preparing fermentation medium, two different media compositions were tried. In one, cassava starch hydrolysate (85-90 DE) was diluted to 5% initial sugar concentration and was supplemented with 0.7% $NaNO_3$. The pH of the medium was set at 7.2 with 1N NaOH solution. The other medium contained $NaNO_3$, 7.0 g (or urea 0.5 g), KH_2PO_4 0.128 g, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO_4.4H_2O$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCl, each 1 mg), 1% v/v corn steep liquor and one drop of Tween 80 in 100 ml starch hydrolysate (pH 7.2). This second medium was termed as complete medium. Fermentation was carried out by taking 50 ml of above media in 250 ml Erlenmeyer flasks. After autoclaving all the flasks were inoculated with 2.5 ml (5% v/v) ml cell suspension (10^8 cells for ml) of *Brevibacterium* sp. (20 h old culture) and incubated at 30°C for stipulated period on a rotary shaker (180 rpm). Sample, as whole flask in duplicate, were withdrawn at desired time intervals for analysis. The results reported are the average of four sets of experiments.

Studies in Fermenter: As an attempt to evaluate more about the economic feasibility of the process and also to know more about the behaviour of *Brevibacterium* sp., studies were carried out in a 5 L fermenter (BIOFLO III, New Brunswick Scientific, Edison,N.J., USA) provided with all the accessories for the control of fermentation conditions. As a preliminary step, the optimum parameters obtained in shake flask studies were maintained throughout the period of fermentation such as pH 7-8, temperature 30°C, agitation speed 180 rpm. Dissolved

oxygen level was maintained at 60% of air saturated medium Fermentation was carried out in batch mode with a working volume of 2.5 L. Samples were withdrawn intermittently for analysis.

In addition to the batch mode, fed batch process was also tried where the initial concentration of reducing sugars in the medium was 50 g/l, in the stage when the concentrations fell to 20 g/l, starch hydrolyzate solution (conc. of reducing sugars, 100 g/L) was supplied to keep the sugar concentration constant in the medium (50 g/l).

Analytical methods

Bacterial growth was determined by measuring the optical density (OD) of the culture broth at 610 nm. Soluble sugars were analysed by DNS method. Total carbohydrates in the sample was detected by phenol sulphuric acid method. Starch content was determined by hydrolyzing the substrate with 10% HCl and estimating the glucose content by DNS reagent. TLC was used for the qualitative detection of L-glutamic acid. The TLC plate with ninhydrin showed only one spot which was identical with authentic sample of L-glutamic acid hence, ninhydrin colour reaction method was used for quantitative estimation of L-glutamic acid in these studies. All these methods are already described earlier.

Results and Discussion

Liquefaction and saccharification of cassava starch have been carried out either with HCl or enzymes and the results showed that the hydrolysate obtained with the enzymatic treatment supported better growth of the culture (OD at 610 nm

was 1.85 after 24 h instead of 1.03 in acid treated hydrolyzate). Enzymatic hydrolysis of starch has been reported better in comparison with the acidic hydrolysis by other authors also[288]. The hydrolysis of starch by the use of enzymes improved the efficiency of the process and the properties of the product. Hence, further studies were carried out with the hydrolysate prepared by enzymatic method.

Figure B41 shows the L-glutamic acid production with two different media using *Brevibacterium* sp. There was much difference in the yields of L-glutamic acid in two media and after 60 h, the yields of L-glutamic acid was almost two times more in complete medium. There are many reports which stated the importance of several growth factors essentially required by *Corynebacterium* for glutamic acid production[34,231]. The results obtained in cassava starch hydrolysate supplemented with inorganic nitrogen only resulted in poor growth and glutamic acid yields, which showed the requirements of other growth factors to be added in the medium. In further studies, hence, we used the second, i.e. complete medium.

Growth and glutamic acid production based on the hydrolysate having different DE values

Figure B42 shows the growth pattern of *Brevibacterium* sp. on cassava starch hydrolysate. In general, higher DE hydrolysate supported better growth of the culture which was maximum with the 85-90 DE hydrolysate (O.D. 1.92 at 18h). Apparently, rate of cell growth was directly related to the DE values of the hydrolysate as higher the DE value, lesser was the time to achieve optimum cell growth. In the case of 15-20 DE hydrolysate, the growth was marked with delayed lag phase and continued upto 36 h.

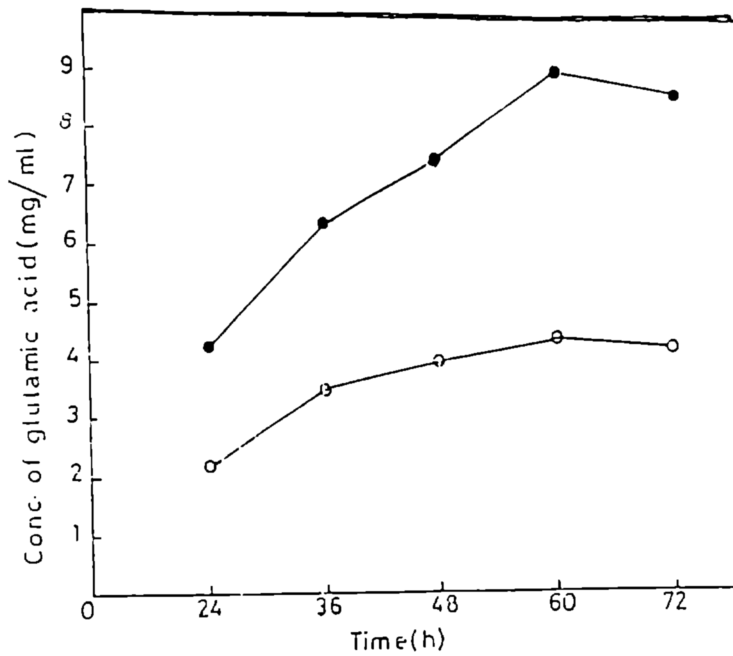


Fig.B41. Effect of media composition on L-glutamic acid fermentation

•——• complete medium o——o medium with nitrogen only

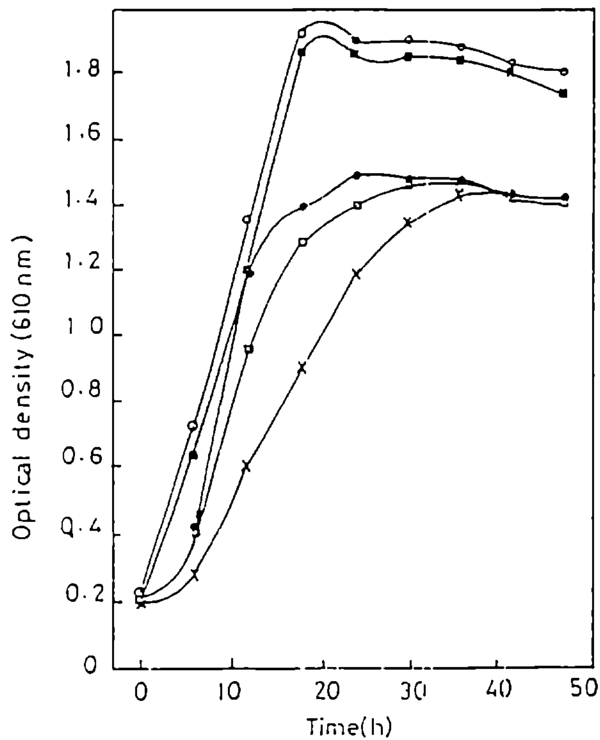


Fig.B42. Growth pattern of *Brevibacterium* sp. in cassava starch hydrolysate with different DE values

X——X 15-20 DE □——□ 30-35 DE •——• 45-50 DE o——o 85-90 DE

■——■ 60-65 DE

Figure B43 shows the consumption of reducing sugars by *Brevibacterium* sp. at different DE values. The pattern was similar in all cases with a consumption of more than 85% sugars (94% with DE 85-90). Figure B44 shows the glutamate production at different time intervals. A maximum of 8.80 mg/ml L-glutamic acid was obtained after 60 h fermentation with the medium having 85-90 DE. While considering the percentage conversion of sugars to L-glutamic acid (based on glucose consumed and 81.74% as the theoretical conversion rate), it was maximum (33.95%) with the hydrolysate having DE value 45-50 as shown in Fig.B45. Apparently, with 85-90 DE hydrolysate, the conversion was lowest (~ 30%). Thus, if conversion factor has to be considered as a major criterion, a low DE value hydrolysate, i.e. 45-50 DE would be sufficient for L-glutamic acid production. There are reports in the literature where a wide variety of applications have been mentioned for low DE starch hydrolysate[290]. As soluble, easily digestible carbohydrates, low dextrose equivalent starch hydrolysate appear to fit the need of formulated foods such as, food bars, cheeses, cheese powders, milk substitutes, dairy spreads, spray dry diet powders, and intermediate moisture foods. In this context, our findings are significant. Yet, another advantage of partially hydrolyzed starch was that the glucose could be made available in a kind of controlled release process, which avoided the kind of repression which normally one faces with fermentation feedstocks[291].

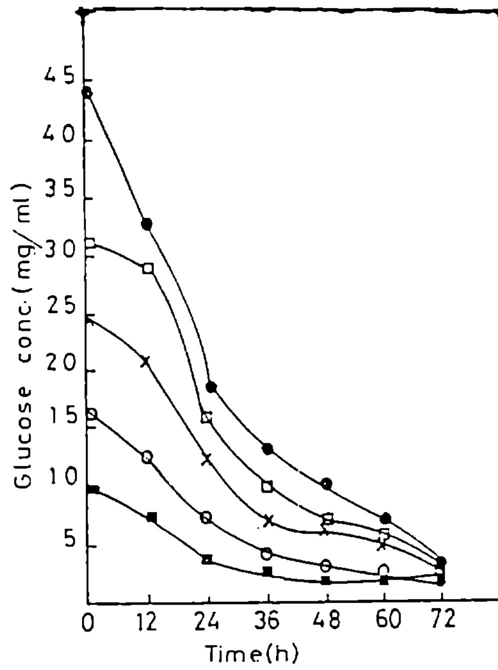


Fig.B43. Consumption of sugars by *Brevibacterium* sp. in different DE value starch hydrolysate

■—■ 15-20 DE o—o 30-35 DE X—X 45-50 DE

□—□ 60-65 DE ●—● 85-90 DE

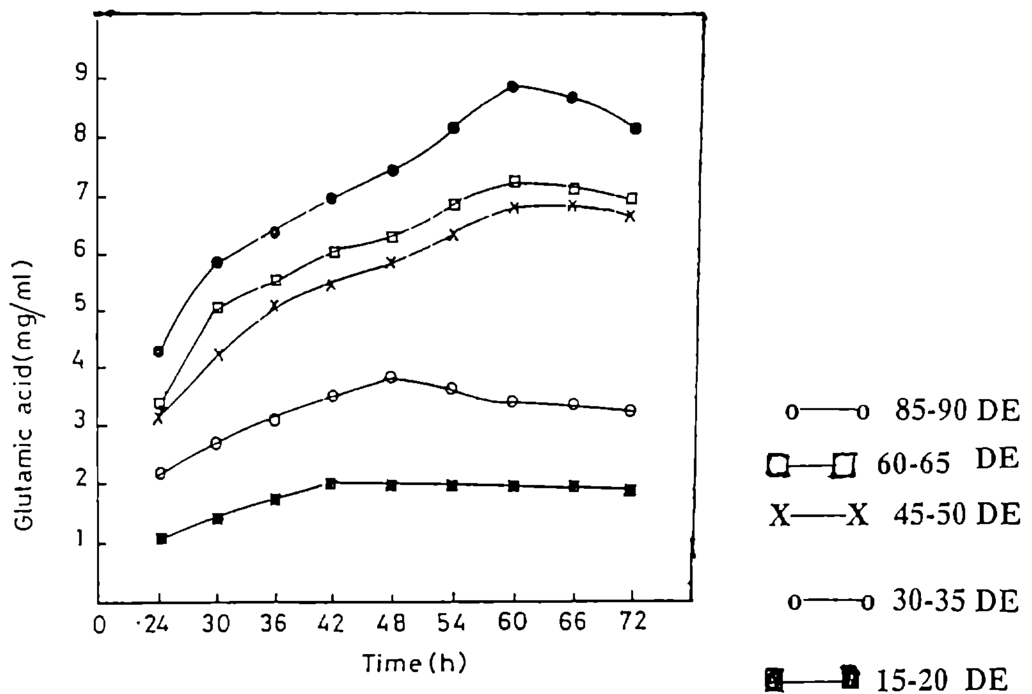


Fig.B44. Yields of L-glutamic acid in different DE value starch hydrolysate

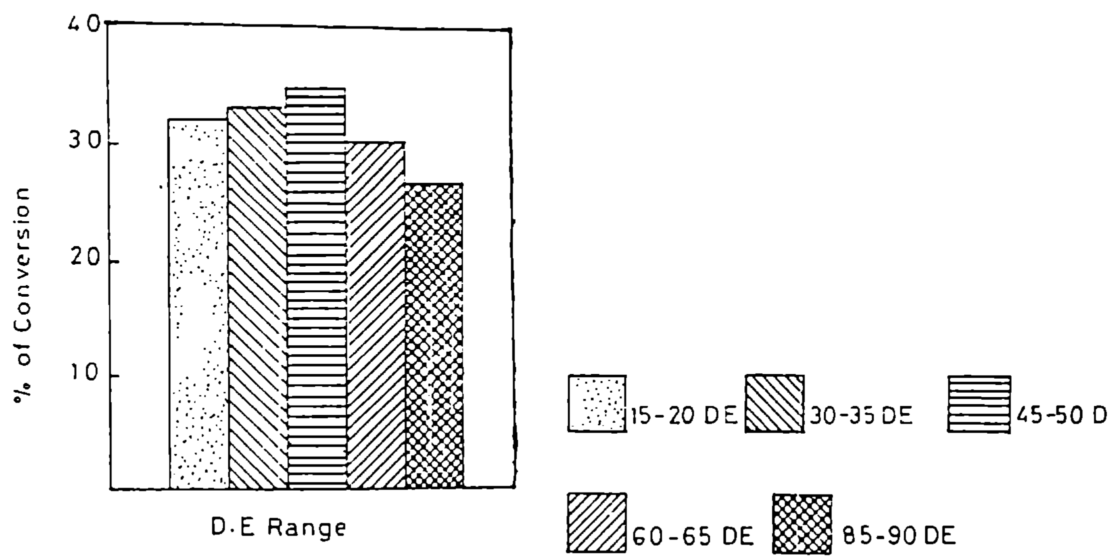


Fig.B45. Percent conversion of cassava starch hydrolysate to L-glutamic acid

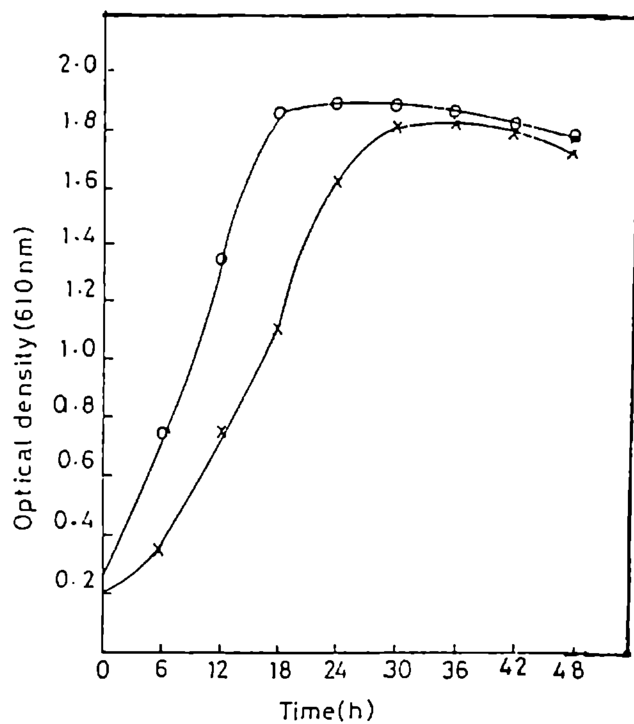


Fig.B46. Effect of supplementation of different nitrogenous compounds on growth of *Brevibacterium* sp.

o——o sodium nitrate X——X urea

Influence of nitrogen source on glutamate yield

Two nitrogen sources, namely sodium nitrate (inorganic) and urea (organic) were tested for their suitability to promote glutamate production in cassava starch hydrolyzate medium. The initial sugar concentration was made 5% by suitably diluting the hydrolysate (85-90 DE). The nitrogenous compounds were added in such a way to keep the C/N ratio as constant. Fig.B46 shows the cellular growth of *Brevibacterium* sp. measured as OD in media with $NaNO_3$ and urea. Highest growth was attained by the culture in 20 and 30 h with $NaNO_3$ and urea, respectively. It is worth to note here that this strain possessed good urease activity also. Table B30 shows the glutamate production with these nitrogen sources at different concentrations. Evidently, $NaNO_3$ appeared slightly better choice over urea giving an yield of 8.80 mg/ml glutamic acid after 60 h fermentation. With urea, maximum yield was 8.20 mg/ml after 66 h fermentation.

Table B30. Effect of different nitrogen sources on glutamate yields (60 h)

Amount of N-source added (% w/v)		Yields of L-glutamic acid (mg/ml)	
$NaNO_3$	Urea	$NaNO_3$	Urea
0.70	0.25	8.80	4.31
1.40	0.50	6.73	8.20*
2.80	1.00	4.15	5.20

*Yields after 66 h fermentation (8.12 after 60 h)

Studies in fermenter

Table B31 shows the summary of the fermentation process (batch mode) such as cell growth, substrate consumption and the corresponding product formation. From the data it was evident that more than 95% of the reducing sugars were consumed and within 40h fermentation and the accumulation of glutamic acid

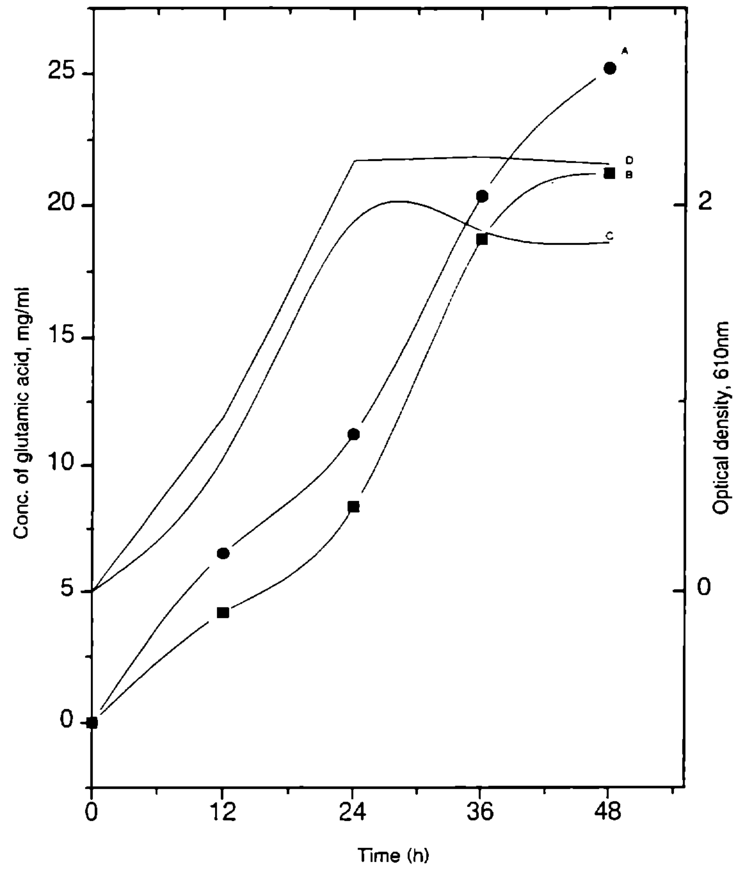


Fig.B47. Comparison of the growth profile and glutamic acid production in batch and fed batch processes.
A: conc.of glutamic acid in fed batch
B: conc. of glutamic acid in batch
C: OD in batch, D: OD in fed batch

was nearly 21 g/L which was approximately two and half fold more than what was obtained in shake flask studies. Based on the glucose consumed (also by assuming 81.74% as the theoretical conversion) we got conversion of about 66.4%. Generally, the classical methods used to optimize cultures in shake flasks does not take into account interaction between factors and can thus lead to a erroneous optimum. In a fermenter that problem was avoided and it may be one of the reasons for higher conversion and moreover the higher working volume also.

Table B31. The overall changes during L-glutamic acid fermentation using *Brevibacterium* sp.

Time (h)	Optical density, 610 nm	Conc. of glutamic acid, mg/ml	Glucose consumption, %)
0	0.01		
12	1.65	4.15	21.85
24	2.12	8.35	41.64
36	2.07	24.70	89.78
48	1.98	25.2	92.73
60	1.95	25.14	93.10

Figure B47 shows a comparison of bacterial growth and L-glutamic acid production in batch and fed batch process. A maximum of nearly 25 g/L of glutamate was obtained in fed batch process which was 16% more than the batch mode. So the maintenance of an active biomass constantly for a long period could enhance the accumulation of glutamate to a certain extent. It is worth to refer the conclusion of Wang *et al.* (1994)[292]. According to them, by adopting a substrate feeding strategy, using an oxystat the final phenyl alanine productivity was improved by 62.5% than in a batch culture. An oxystat is defined as a simple and inexpensive control method where a limited quantity of substrate was provided for feeding under low dissolved oxygen tension. It will avoid the substrate inhibition during the course of fed-batch operation.

B.9.2 RECOVERY OF L-GLUTAMIC ACID FROM CASSAVA STARCH HYDROLYSATE BY ION-EXCHANGE RESIN COLUMN

Introduction

In the extraction and purification of amino acids from fermentation broths, properties such as ionic dissociation constants (as amphoteric compounds), solubilties, molecular sizes, adsorption-desorption characteristics etc can be utilized partial chemical modification or chemical alteration can often give bigger differences in physicochemical properties and lead to more effective extraction and purification.

Amino acid fermentation broths have characteristics which are different from those of protein hydrolysates and it makes the down stream processing comparatively easier. Fermented broths are aqueous solutions of amino acids and in most cases, a significant amount of a single amino acid can be accumulated in the fermentation broth in its free and salt forms and other contaminant amino acids are usually small in number and quantity. Moreover, amino acids produced in fermentation broths are usually the optically active L-form and therefore, an optical resolution step is not required.

The glutamic acid is usually present in the fermentation broth either in free form or as a salt. Suhua reported the use of liquid ammonia (to adjust the pH) in extraction of glutamic acid as zinc glutamate. Xianzhong *et al.* isolated glutamic acid from fermentation broth by crystallization using HCl. Glutamic acid was also

extracted from the fermentation broth by flocculation with the addition of polymers containing COOH or SO_4 group. Recently, some reports have appeared, where glutamic acid was extracted by aqueous two phase partition and by membrane filtration.

An attempt has been made to separate glutamic acid from the fermentation broth by employing ion-exchange chromatography and also by making use of its low solubility at the isoelectric point.

Materials and Methods

Filtration and Centrifugation of Broth

Two batches each consisting of 2.5 L fermentation broth obtained from batch fermentation in fermenter was filtered using a micro-filtration unit fitted with 50 mm membrane diameter with a pore size of 45 μm under vacuum using a pump. Both the filtrates were combined and then centrifuged at 10,000 rpm for 10 minutes to get the clear supernatant, which was then used for the recovery of the product.

Separation process by Ion-Exchange column

Preparation of Resin: Spherical particles of cation exchange resin, Amberlite IR 120 plus (Hi-media) was used. Prior to use the resins was pre-conditioned according to the method of Morre and Stein. The resin (100 g) was washed thoroughly for two times with 4N HCl. After two washes with distilled water (colloidal materials may appear in the filtrate), the resin is washed with 2N NaOH until the filtrate was alkaline. The resulting material (sodium salt of the resin) was

suspended in 3 times its volume of 1N NaOH and heated over a steam bath for 2 h with occasional shaking. The supernatant fluid was decanted after about 30 minutes of settling and replaced with fresh hot 1N NaOH. This procedure is repeated 2 times. The resin was filtered and washed with 2 L of distilled water to make it free of alkali. The resin was filtered and stored as the moist sodium salt.

Packing the column: Resin (as above) was placed in a column (3x50 cm, width x length) containing distilled water and filled upto 25 cm^3. The excess water was removed using a siphon.

Separation process by ion-exchange column: The chromatographic conditions were selected to minimize the inhibitory effect of co-existing inorganic ions on the adsorption of amino acids by ion-exchange resins, following the method of Samejima[159]

Removal of impurities from the broth was done by filtering and centrifuging the broth. The pH of the broth dropped from 7.5 to 4 (with 1N HCl) which was the most important factor affecting the adsorption of glutamic acid on the resin because the ionic forms vary with the pH.

The main processes increased in the column were adsorption and elution. In the adsorption process, the broth used was adjusted to a suitable pH of 1.8-2.0 using hydrochloric acid to change the glutamic acid so that the ion exchange between the glutamic acid and the resin could occur. The broth was continuously recycled at a flow rate of 20 ml/min (retention time 50 minutes) until glutamic

acid fully adsorbed in on the column and leaving other ions. In the elution process, the pH was increased to 3.8-4.0 by treating the fermented broth with urea and sodium hydroxide. This was done to release the glutamic acid bound on the resin by changing the glutamic acid charge.

Crystallization: After adsorption and elution, the eluent containing a high amount of glutamic acid was acidified to pH 3.2 (isoelectric point of glutamic acid) with hydrochloric acid. Storage at 20°C for 48 h resulted in the formation of crystals of glutamic acid. After evaporation of the eluent the dry solid crystals were obtained.

Analytical methods: Estimation of glutamic acid and reducing sugars were done by ninhydrin colour reaction and by DNS method, respectively. The product purity was reconfirmed by IR spectrum using pure glutamic acid as a standard.

Results and Discussion

Recovery of Glutamic acid

The fermented broth contained various impurities such as bacterial cells, macromolecules, pigments, inorganic substances, organic substances etc. which were removed by filtration and centrifugation. Summary of the purification steps using filtration and the ion exchange process is given in Table B32.

Glutamic acid was recovered in pure form by cation exchange resin. The elution profiles of the glutamic acid concentration from the column are shown in Fig.B48. Symmetrical peaks of glutamic acid concentration and absorbance were

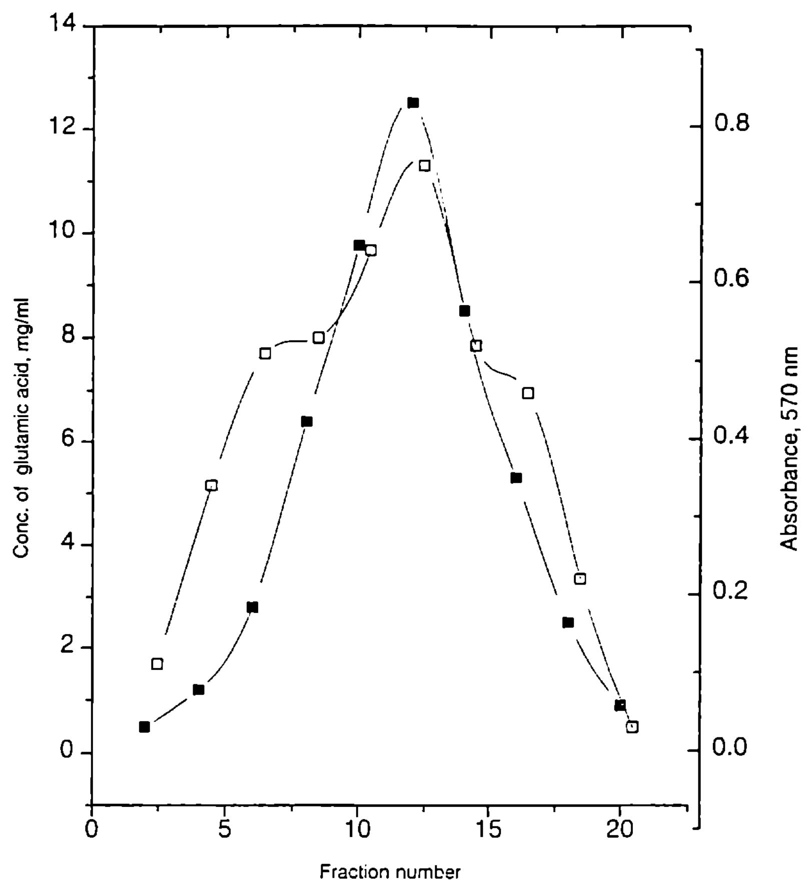


Fig.B48. Glutamic acid recovered at different elution volumes thrugh ion-exchange resin column

◇—◇ Conc. of glutamic acid, mg/ml

■—■ Absorbance, 570 nm

Fig.B50. IR-spectra of standard (I) and purified (II) samaples of L-glutamic acid

obtained. Glutamic acid was recovered for the highest yield taking fractions of 8 to 17 (total elution volume of 200 ml) through the ion-exchange column.

Table B32. Down stream steps for the recovery and purification of glutamic acid from the fermentation broth

Process	Cation-exchange process
Purification steps	* Culture solution
	* Filtration, pH 7.5-8.0
	* Adsorption to cation exchanger (Amberlite, IR 120 plus)
	* Elution with base (NaOH and urea)
	* Crystallisation

Filtration: membrane pore size 45 μm; Centrifugation: 10,000 g for 10 minutes;
Ion-exchange column: 3 cm x 50 cm

By charging the pH to the isoelectric point (3.2) and by the subsequent cooling of the eluent the glutamic acid was crystallized out as shown in the fig.B49. The dry solid crystals were obtained by centrifugation and evaporation of the product.

The purity of the final product which observed and reconfirmed using 12 spectrum showed a similar peaks at the same frequencies. All functional groups of the product (NH_2, COOH and CH_2) showed a frequency similar to standard glutamic acid as shown in Fig.B50.

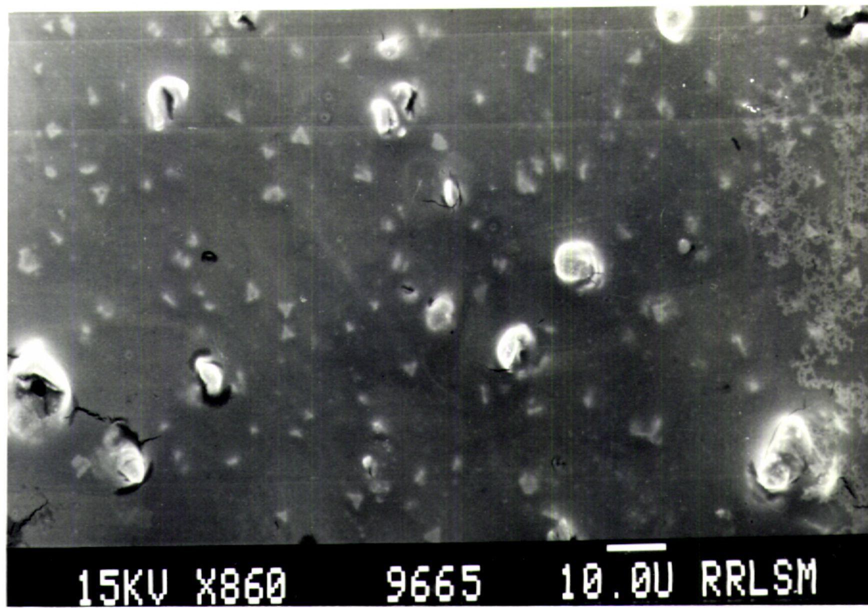


Fig.B49. Scanning Electron micrograph of L-glutamic acid crystals in concentrated fermentation broth

Ion-exchange chromatography is known to be a very effective tool for the analytical purposes and small scale laboratory work. Resins of various types have been used to separate amino acids into groups acidic, basic and neutral. The adsorption and elution characteristics of amino acids on ion-exchange resins are more or less affected by environmental factors, such as pH, ionic dissociation of amino acids, contaminating ions and ionic strength. By making use of various combinations of such factors, ion-exchange resin can be effectively utilized for concentration and separation of glutamic acid.

SECTION C

SUMMARY

The review of the literature showed that in recent years, a considerable amount of interest has been displayed in various parts of the world for the production of amino acids by fermentation process. L-amino acids have a wide spectrum of commercial use as food additives, feed supplements and infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agro-chemicals. During the last decade, dynamic developments in both the areas, in the market (applications) and in industry (methods of production) have occurred. The whole market is estimated to have about 3 billion US dollars in 1995, covering 38% for food, 54% for feed and 8% for other applications such as medicine and cosmetics. The objective is mainly achieved by using some bacterial strains belonging to the genus *Corynebacterium, Brevibacterium, Microbacterium* and *Arthrobacters*.

Glutamate fermentation is a typical aerobic type and the aerobic oxidation of glucose involves both EMP and HMP pathways followed by TCA cycle. Two enzymes have been shown to play a key roles in the biosynthesis of L-glutamic acid.

(i) Phosphoenolpyruvate carboxylase and

(ii) α-Ketoglutarate dehydrogenase

The low activity and high instability of α-ketoglutarate dehydrogenase favours the conversion of α-ketoglutarate by glutamate dehydrogenase (GDH), especially in the presence of high NH_4^+ concentration. In microorganisms,

L-glutamate can be formed either by the glutamate dehydrogenase or by the coupled reactions of glutamine synthetase (GS) and glutamate synthase (GOGAT).

The mechanism of glutamate transport into the medium has been a subject of long controversy in the literature. So far, three different mechanisms have been proposed in L-glutamate efflux. They are 'leakage' or 'overflow phenomenon', inversion of the uptake process induced by changes in chemical potential or regulation, or by uncoupling due to changes in the membrane structure. Recently, rather convincing experimental evidences were presented showing the existence of a specific glutamate carrier.

Increasing efforts are still going on to replace the starting materials of cheaper cost and constant supplies. There are numerous factors which can change the course of glutamic acid fermentation and is a most remarkable conversion and no other cases are known of the fermentation where such a wide range of possible alternative products.

A multi-step inoculation procedure followed by the fed-batch mode is the main fermentation and is still the preferred technology for the production of L-glutamic acid. Alternative processes such as production in air lift fermenters, fluidized fermenters or by cell recycling techniques are not competitive so far. However, there might be potential for improvements in L-glutamate fermentations by employing techniques such as computer supported control of the process, Fuzzy expert systems and simulation of relevant effects in fermentation such as the

simulation of the effect of mixing, scale up and pH value regulation during glutamic acid fermentations.

As useful mutants, an oleic acid auxotroph, a penicillium resistant mutant, a glycerol auxotroph have successfully been employed for L-glutamic acid production. Yet another approach was focused on the developments of thermophilic bacteria. Other useful techniques employed were protoplast fusion and electroporation. Recent developments in recombinant-DNA technology has made possible genetic recombination between different species and the increasing of the biosynthetic activity of microorganism by increasing the gene copy number or improving the microorganism in relation to the substrate and environmental conditions. Some applications of the technique to amino acid production has been reported. Recent efforts have made it possible to isolate some plasmids of glutamic acid bacteria.

In India, the current annual demand for MSG was around 500 tonnes per annum. which is presently met by imports. The demand is increasing about 10% per year. Apart from glutamate, we are importing L-lysine, glycine, L-aspartic acid,L-methionine and L-dopa etc. Thus much attention is required on R&D line for the production of amino acids.

The present work was undertaken with a view to study the fermentative behaviour of *Brevibacterium* sp. for the production of L-glutamic acid.

The first subsection of the experimental section (B.1) deals with the materials and methods (Analytical, Experimental and Instrumental) which are most common and repeatedly used throughout this work. The following subsections deal with various experiments conducted.

A strain of *Brevibacterium* sp.(DSM 2041) was used throughout the studies. It was grampositive, non-sporulating, non-motile and non-flagallated. Cells in young cultures (experimental phase) were irregular rods of variable length. Cells were arranged singly or in pairs and often at an angle to give 'V' formation. Cells from older cultures (72 h more) were composed largely of coccoid cells.

Growth profile studies revealed that apparently within 24 h of cultivation the culture attained maximum cell density.

A number of growth media were evaluated to find out a suitable production medium for L-glutamic acid. Finally, medium M5 which contained 2 g glucose, 0.5 g, $NaNO_3$, 0.18 g KH_2PO_4, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO.4H_2O$, $MgSO_4.4H_2O$, $ZnSO_4.2H_2O$ and NaCl each 1 ml), 10 μg thiamine hydrochloride, corn steep liquor (0.1% v/v) and Tween 80 (0.01% v/v) in 100 ml distilled water (pH 7.2) was used.

In quantitative analysis, the culture filtrate gave only one spot which was identical with the authentic sample of L-glutamic acid. Under optimized conditions with glucose as carbon sources at pH 7.5 and 30°C incubation, the culture produced 1.13 mg/ml glutamic acid after 48 h fermentation in shake flasks.

Change in ratio of the volume of fermentation medium and flasks volume greatly affected the glutamic acid production. The data indicated that it was the working volume of the medium at specific medium volume to flask volume ratio, which was important. Thus the best yield was obtained when 100 ml of the medium (M5) was taken in 500 ml flask, which resulted in the production of 6.86 mg/ml glutamic acid.

It was found that both abundant and meagre aeration were undesirable for L-glutamic acid production. In comparison with the conditions having 500 and 350 rpm agitation, the dry cell density and specific growth rate during the exponential phase was lower in 60 rpm conditions showing that the organism were under oxygen limitation. The dissolved oxygen level with 180 rpm would have been less than that of 500 and 350 rpm, even then the maximum dry cell density and specific growth rate were almost the same. Moreover, the maximum glutamic acid formation (6.73 mg/ml) was noted at 180 rpm after 48 h fermentation. In oxygen limited growth (60 rpm), lactate was accumulated up to a maximum of 6.2 mg/ml and then deplated.

Among the different inoculum sizes attempted, 5% v/v (20 h old culture) gave the best results in terms of readily growth and maximum glutamate secretion. A very high inoculum size (10% v/v) did not show much impact in the shake flasks experiments.

Experiments were carried out to find the efficiency of *Brevibacterium* sp. to utilize different carbon sources (glucose, fructose, sucrose, maltose, lactose, xylose and starch) for its growth and for the production of glutamic acid. Except starch, all the carbon sources supported the growth of the culture, which showed a preferential choice of growth and activity when grown on glucose as sole carbon source. Mixed substrate fermentation did not improve the yield of amino acid. Maximum glutamic acid production (6.86 mg/ml) were obtained when 2% glucose medium was fermented under optimized conditions for 48 h. It corresponded to about 42.0% conversion (assuming 81.74% theoretical conversion), taking initial glucose concentration in to account. Considering the glucose used up by the bacterium in 48h (which was 91.6% on initial concentration) the conversion efficiency was 45.8%. The efficiency of the strain to utilize non-carbohydrate carbon sources such as glycerol and sodium acetate was not promising.

Enrichment of the medium with supplementation of both yeast extract and corn steep liquor to a certain concentration enhanced glutamate accumulation. Maximum of 5.75 mg/ml glutamate was accumulated when the yeast extract concentration was 1% and for corn steep liquor the maximum titre (7.10 mg/ml)was obtained with 0.25%. As the concentration of CSL increased the bacterial growth as well as the concentration of lactic acid also correspondingly increased, but the secretion of glutamate was affected adversely beyond 0.25%.

Glutamate fermentation provided an example of a case where cell permeability was a vital factor in regulating the outcome. Biotin oleic acid,

penicillin G and Tweens (20, 40, 60 and 80), affected the synthesis of normal cell membrane at different steps and made the cell membrane permeable to glutamic acid.

As the biotin concentration in the culture medium increased, it promoted the growth of the bacterial cells also. Maximum cell growth was noted when the biotin concentration was 10 µg/L. On the other hand, glutamic acid production decreased with increase in the biotin concentration. Maximum extracellular accumulation of L-glutamic acid (6.90 mg/ml) occurred when the biotin level was 2.5 µg/L. Lactic acid started to accumulate as the concentration of biotin increased. The addition of oleic acid to the medium supported both growth and production. Maximum production (4.45 mg/ml) was noted when the medium was supplemented with 50mg/L of oleic acid.

The addition of penicillin G or Tweens (20, 40, 60 and 80) induced the accumulation of L-glutamic acid in a biotin rich medium. Addition of 2 units/ml penicillin after 9 h of incubation resulted in the maximum glutamate production of 6.50 mg/ml. Addition of penicillin at the time of inoculation or after 15 h of incubation were not much promising. Similarly addition of 0.5% Tween 60 after 9h of incubation resulted in the maximum glutamate titre, 4.50 mg/ml.

Osmatic pressure of the culture medium represented one of the major operational parameters for an efficient production of glutamate. After a momentary interruption of cell growth and substrate consumption, lasting for few hours, *Brevibacterium* sp. was found to grow even in a high salt concentration such as 1.5

-2.0M. Growth was maximum when the medium contained 1M NaCl and any further increase in salt concentration resulted in a linear decrease of cell growth. The most accumulated free amino acid was glutamic acid upon the growth conditions examined. It was maintained at high concentrations even in control samples and with the increase of the external NaCl concentration it increased and showed the maximum (335.9 mM/g dry weight) at 0.5 M NaCl. It means 72.5% of total amino acids accumulated (464.1 mM/g dry weight) was glutamic acid. However, it linearly decreased to 144.5 mM of dry weight in the presence of 2 M NaCl which is almost equal for that in the absence of NaCl.

Studies on the iron requirement of *Brevibcaterium* sp. revealed that iron was essential for growth of *Brevibacterium* sp. and it produced siderophore. A reduction in the cellular growth was noted in the presence of synthetic chelating agents such as EDDHA which was apparently due to the formation of Fe-EDDHA complex and thus devoiding the bacteria from iron uptake. An assay of culture supernatant (from iron deficient medium) with CAS showed positive results indicating the production of siderophores by bacteria which was further confirmed in CAS-Agar plate studies. Addition of iron up to 10 mM to the modified M5 medium (hydroxyquinoline treated to remove contaminated iron and then supplemented with 0.5% v/v or sterilized yeast extract solution) enhanced the accumulation of glutamic acid (3.38 mg/ml of glutamate with to 10 mM iron concentration) and thereafter no such effect was noticed. Compared to the usual production medium (M5) the yield was very poor.

Urease (urea amidohydrolase, EC. 3.5.1.5) activity was found in the fermented broth of *Brevibacterium* sp. Maximum glutamic acid production (49 μmol/ml) was obtained after 48 h with 2% glucose and 0.5% urea. 98% of the available urea was utilized within 36 h of fermentation. The initial concentration of urea in the medium influenced urease activity, maximum specific activity (0.427 mmol of ammonia liberated per mg protein) of the partially purified enzyme being noted after 12 h of fermentation with 0.25% initial concentration of urea. Urease was most active at pH 7.0 and the optimum temperature was 35°C in the presence of 0.05 M phosphate buffer.

A resurgence of interest in solid state fermentation (SSF) system has been witnessed in the last decade due to numerous economic advantages over conventional submerged fermentation (SmF). However, solid state fermentation carried out on inert support materials has been regarded as one of the future development of the SSF system. Solid state fermentation system was used to cultivate *Brevibacterium* sp. on sugar cane bagasse impregnated with a medium containing glucose, urea, mineral salts and vitamins for producing L-glutamic acid. Maximum yields (82.22 mg glutamic acid per g dry bagasse with biomass and substrate mg/gds) were obtained when bagasse of mixed particle size (0.45 to 3.00 mm) was moistened at 85-90% moisture level with the medium containing 10% glucose. Since the discovery of *Micrococcus glutamicus* by Kinoshita *et al.* (1957)[7], many bacteria have been reported to produce L-glutamic acid directly from sugar in liquid fermentation. But to the best of our knowledge, no attempts have so far been made to use amino acid producing bacteria in SSF systems.

Application of immobilized growing microbial cells has been currently highlighted from the view points of long-term utilization of biocatalysts and continuous operation of stabilized systems. Studies were performed to elucidate the optimal conditions (gel cncentration, bead size, initial biomass, storage period) for immobilization of growing cells of *Brevibacterium* sp. in alginate and agar gels and to evaluate the immobilized biocatalysts in batch, repeated batch and continuous mode in a packed-column bioreactor for L-glutamic acid production. Gel concentration has a significant effect and the optimum concentration for alginate beads was 3% and for agar 4%. Storage of alginate beads (24 h) increased the gel consistency and durability. The immobilization procedure was most effective when the bead size was smaller (3 mm) for alginate beads and for agar beads it was 4 mm. In both cases, the optimum initial biomass was 0.75 g wet cells/20 ml gel. The entrapped cells were cultivated for twenty five days in five repeated batches (each batch lasted for five days). Maximum L-glutamic acid production in batch and repeated-batch modes was 7.4 and 8.7 mg/ml, respectively with alginate beads, and 11.8 and 13.3 mg/ml respectively with agar beads. Under optimized conditions (flow rate, initial cell loading), L-glutamate productivity was maintained within a range 10-11 mg/ml for two weeks, in continuous mode in a packed column bioreactor with agar immobilized cells. Stability of biocatalyst indicated the possibility of the application of immobilized *Brevibacterium* cells for a long term use.

After the realization of the overproduction of L-glutamic acid by *Brevibacterium* sp. based on glucose and other related sugars like maltose, sucrose,

fructose etc., an attempt was made to develop a microbial process for glutamate production employing locally available starchy tubers as the raw material. Cassava (*Manihot esculenta* Crantz), popularly known as tapioca, on dry weight basis, it contained about 80% starch of which 60% can be recovered. Starch hydrolyzates of different dextrose equivalents (D.E.) were peppered by the enzymatic hydrolysis of dried cassava starch. The rate of cell growth was directly related to the D.E. values of the hydrolysate as higher the DE value, lesser was the time to achieve optimum growth. Maximum glutamic acid production (8.8 mg/ml) was obtained when a suitably diluted hydrolysate having the dextrose equivalent (DE) 85-90, was supplemented with $NaNO_3$ (0.7%), KH_2PO_4 (0.18 w/v%) and a mineral solution containing Mg, Mn, Fe, Zn, NaCl and 0.1% v/v corn steep liquor and was fermented for 60 h under agitation at 30°C. Highest conversion rates (33.9%) was obtained with the hydrolysate having 45-50 DE value. Supplementation of the hydrolysate with inorganic nitrogen alone was not effective.

As an attempt to evaluate more about the economic feasibility of the above process, studies were carried out in a 5 L fermenter. Fermentation was carried out in batch mode with a working volume of 2.5 L. More than 90% of the reducing sugars were consumed within 48 h of fermentation and the accumulation of glutamic acid was nearly 21 g/L which was approximately two and half fold more than what was obtained in shake flask studies. By maintaining an active biomass constantly for a long period through fed-batch process resulted in a maximum titre of 25 g/L of glutamate which was 65% more than batch mode.

The last sub-section (B.9.2) of the experimental section deals with the extraction and purification of glutamic acids from the fermented broth. An attempt was made to separate glutamic acid from the broth by employing ion-exchange chromatography and also by making use of its low solubility at the isoelectric point. After adsorption to a cation exchange resin (Amberlite IR 120 plus) and elution (with NaOH and urea), the eluent containing a high amount of glutamic acid was acidified to pH 3.2, isoelectric point of glutamic acid with HCl. A cooling process at 20°C for 48h enhanced the formation of crystals of glutamic acid. After evaporation of the eluent, the dry solid crystals were obtained. The purity of the final product was reconfirmed using IR spectrum. All functional groups of the product (NH_2, COOH and CH_2) showed a frequency similar to standard glutamic acid.

PROSPECTS OF PRESENT INVESTIGATIONS

Prospects of the present investigation are very promising since this particular strain acts as a cost effective bioconverter for the production of L-glutamic acid and, at the same time, the organism has got potent hydrolytic enzymes like urease and most probably a wide spectrum of other amidases like glutaminase etc. which can be used for many biotransformation studies.

Strain improvement studies can be conducted to isolate the hyper producers of glutamic acid. By protoplast fusion techniques, a variety of new hybrids can be produced and they may utilize newer raw materials such as starchy substances (directly), cellulosoic or lignocellulosoic substances. The efficiency of solid state fermentation can be checked with these improved strains or by culturing the organism in various types of inert substrates such as ion-exchange resin etc for L-glutamic acid production. By using this strain, a search for auxotrophic and regulatory mutants were always possible and thus to channel the metabolic pathway in the biosynthesis of desired amino acids other than glutamic acid such as L-lysine, L-threonine, L-tryptophon, L-isoleucine, etc. By making use of the advantages offered by the recombinant DNA technology it is possible to clone one or more structural genes of the pathway in multicopy plasmid, thus increasing the dose of the corresponding enzyme and it would lead to the overproduction of corresponding amino acids. By using innovative bioreactors and by further automation of the process. the amino acid production can be enhanced. Simultaneous to this, it will be worth to screen the amidases/nitrilases activities of *Brevibacterium* sp. or mutant of strains for biotransformation studies. To summarise, it is an industrial microorganism with tremendous potential for many biotechnological applications.

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LIST OF PUBLICATIONS

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B. Original Papers

2. Effect of different carbon sources on growth and glutamic acid fermentation by *Brevibacterium* sp. **Nampoothiri, K.M.** and Pandey, A., *J. Basic Microbiol.*, 35(4), 249-254 (1995).

3. Glutamic acid fermentation using *Brevibacterium* DSM 20411. **Nampoothiri, K.M.** and Pandey, A., *J. Food Sci. Technol.*, **32**(5), 406-408 (1995).

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7. Bioconversion of tapioca starch to L-glutamic acid using **Brevibacterium** sp. **Nampoothiri, K.M.** and Pandey, A. (communicated).

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