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STUDIES ON THE MICROBIAL PRODUCTION OF L-GLUTAMIC ACID

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INTRODUCTION

L-amino acids are building blocks of the important food components proteins, which the body needs more than any other factor. From a biological point of view life is uniquely characterized by its association with proteins. The main biological function of L-amino acids is the protein synthesis. They are also used by cells to synthesis compounds having adverse other roles in the living systems. The indispensable L-amino acids in the diet mixture are important source of nitrogen in animal body. However, when the indispensible L-amino acids are fed in sufficient quantity, the nitrogen of L-amino acids may be replaced in the diet by ammonium source. Considering the nutritive Values and to meet high demand of L-amino acids, many advanced technological methods of L-amino acids synthesis are being developed. Most significant developments, particularly in Japan, where miracles were made using microbes and all kinds of raw materials for the fermentation process in order to produce L-amino acids have been very favorable for L-amino acid fortifications of foods [1].

Konbu (Kelp like sea weed) has been widely used as an important traditional seasoning source in Japan. In 1908, the taste of Konbu was identified as being due to L-glutamic acid. Based on this discovery, the industrial production of Mono Sodium L-Glutamate (MSG) was identified by Ajinomoto Co. in 1909 [2].Initially, L-glutamic acid was produced by the acid hydrolysis of wheat gluten or Soybean protein [3]. A half century after this discovery, it was reported that considerable quantities of L-glutamic acid accumulated in bacterial culture[4]. The research and development carried out mainly in Japan, resulted in the successful and economical production of L-glutamic acid by the fermentation process. The significance of the establishment of microbial production of L-glutamic acid cannot be overestimated. Such essential metabolites as L-amino acids or nucleotides were considered not to be accumulated in microbial culture due to regulatory mechanisms in the cell[5]. The discovery of L-glutamic acid fermentation stimulated a wide variety of research aimed at isolation of wild strains and the genetic derivation of

mutants which could accumulate large amounts of primary metabolites [6].Fortunately, fundamental knowledge about biosynthesis of L-amino acids and their regulatory mechanisms had already been focused by different scientists [7-21]. L-amino acids are constituents of proteins and play vital roles in the living organisms. There have been increased demands for L-amino acids for use in the areas of food and feed additive and drug manufacturing [22-26]. L-amino acids are used in foods as flavor enhancers, seasoning and nutritional additives [27]. MSG, the most important commercial L-amino acid product, is widely used as a flavor enhancer [25,28]. L-alanine and L-glycine are used as flavoring agents [22]. The essential L-amino acids which are often present only in insufficient amount in cereals are L-lysine, L-threonine and L-methionine [24]. L-lysine and DL methionine are now used practically to increase the efficiency of protein utilization of animal feeds [22]. The reducing power of L-cysteine is utilized to improve bread quality and backing process [25]. In the field of medicine L-amino acids are used for infusions and as the therapeutic agents [23]. In the past, based on their nutritive values, essential L-amino acids were the principal component of the infusion mixtures, only L-glycine among non-essential L-amino acids being used to increase the nitrogen content [25]. However, it has already been proved that non-essential L-amino acids like L-glutamic acid also have nutritional significance and development of L-amino acid infusions has been stimulated [23].Based on the physiological properties, some L-amino acids are also being used therapeutically. For example, L-glutamate is used for gastric ulcer treatment,L-asparagine is effective in the development of hyperammonemia and liver disorder by increasing the arginase activity in liver [23,26]. L-amino acid derivatives are widely used in chemical factories. Among them MSG is most common [23]. L-glutamic acid derivatives are also widely used in cosmetics, synthetic leathers, surface active agents, fungicides and pesticides [26]. One typical example is use of poly- γ -methyl glutamate in the manufacture of synthetic leather [22].L-γ glutamate derivatives are widely used in different industries. For example, poly γ-methyl glutamate is used in synthetic lather

production, MSG in cosmetic production, methyl, ethyl and tert-tutyl esters as surface active agent, maize gluten as herbicides etc [22, 26].

Interest in L-glutamate production on large-scale was stimulated by the increasing demand for MSG as a flavor enhancing agent According to the report of Ajinomoto Co. (Japan) in 2010, the global demand for MSG was accounted about 2.16 million tons per annum and its market demand was rising about 6.0% per year[21]. Currently China is the World's largest MSG producing and consuming country. Chinese production and consumption accounted for approximately 73% and 67% of world production and consumption in 2009, respectively. Chinese exports accounted for approximately 37% of the World's MSG exports in 2009. Asian production accounted for approximately 91% of World MSG production in 2009. In the world, large MSG producing countries include China, Japan, Taiwan, Korea, France, Brazil and Indonesia. China and Indonesia are relatively recent entrants in the market.In India, the annual demand level was 5,000 tons per annum and its growth rate was 9.0 to 10% per annum. But the demand was met up mainly by its export. However, in view of the abundant availability of indigenous raw materials, India is in a position to set up globally competitive project for L-glutamic acid / MSG Considering the above data, we tried to develop a method for the industrial production of L-glutamic acid from sugar using a mutant strain of *Micrococcus glutamicus* .Hope it will be helpful to meet the future demand of L-glutamic acid in our country and to some extent of the world.

To meet the diversified demands mentioned above-L-amino acids including L-glutamic acid are now commercially produced. The production methods developed so far may summarize as follows:

(A) Chemical synthesis method

(B) Bio-synthesis of L-amino acids

(C) Protein hydrolysis and

(D) Microbial methods including

a. ***Direct production of L-amino acids from carbon sources such as glucose,***
b. ***Precursor addition methods and***
c. ***Enzymatic methods.***

Therefore, biological production of L-amino acids including L-glutamic acid includes:

(i) Biosynthesis and excretion of L-amino acids from carbohydrates, as in the case of L-glutamic acid from glucose,

(ii) Salvage synthesis of L-amino acids from intermediate metabolites for example, L-tryptophan accumulation from anthranillic acid,

(iii) and

Enzymatic synthesis of L-amino acids from their substrates, for example L-aspartic acid from fumaric acid and ammonia by aspartase of *E. coli*[29].

With selection of a suitable strain, careful determination of culture medium and optimization of cultural condition, L-amino acid production can precisely be controlled and enhanced [30-33].

Even though L-glutamic acid can be produced either by a chemical or biological method, later is more economic, even though relatively low yields are obtained during the extraction of L-glutamic acid, requiring specific installations and the use of expensive products [29, 34]. The stereo specificity of L-amino acids and the steady increase of their market demands. Thus, L-glutamic acid producing strains of Gram +ve bacteria like *Micrococcus glutamicus, Brevibacterium flavum, Brevibacterium lactofermentum* etc have been used for the last more than fifty years for the industrial production of L-glutamic acid [35-47]. Interest in microbial production of L-amino acids has increased greatly since development of large scale microbial processes to make L-glutamic acid. Though the large scale manufacture of inexpensive L-amino acids could stimulate market development, much of recent interests are due to the potential production of natural isomers at a cost permitting their use as dietary supplements and in chemical industries [48-69]. The possibilities and supplementing diets with L-amino acids have been presented since the finding that some of these compounds, essential for life could not be synthesized by the body [27]. However, we owe much of our knowledge of microbial production of a non-essential L-amino acid, L-glutamic acid.

DISCOVERY AND ISOLATION OF L-GLUTAMIC ACID FROM NATURAL SOURCES

While engaged on the study of various vegetable proteins Ritthausen (1866) hydrolyzed with sulfuric acid what then was called wheat gluten or gluten fibrin, and now would be called gliadin. At the end of the reaction, calcium hydroxide was added in excess to precipitate sulfate, oxalate in excess to precipitate calcium, lead carbonate in excess to precipitate oxalate and hydrogen sulfide in excess to precipitate the lead. The final clear filtrate was concentrated to a low bulk when crystals of tyrosin mixed with another and more soluble substance separately. On careful treatment with warm water, the latter was separated and was deposited from the chilled aqueous extract in crystalline form. This turned out to be a new L-amino acid, which, in view of its source from gluten, Ritthausen named "L-glutamic acid" [70].

CHEMICAL PROPERTIES OF L-GLUTAMIC ACID

Chemical names: 2-Aminopentanedioic acid, 1– Aminopropane 1, 3 dicarboxylic acid

α – Amino L-glutamic acid

Trivial name: L-glutamic acid

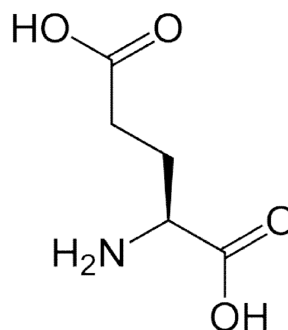
Abbreviation:

Glu or E; Glx or Z represents either L-glutamic acid or glutamine

Empirical formula:

$C_5H_9O_4N$; C, 40.82%; H, 6.17%; O, 43.50% and N, 9.52%. 147.14

Structure of L-glutamic acid:



L-glutamic acid

$[M]_D$ of L-antipode: + 177° in H_2O and +46.8^o in 5(N) HCl; C=2, T=25°C

The carboxylate anion of L-glutamic acid is known as L-glutamate, and this is one of the 20 proteinogenic L-amino acids. It is not among the human essential L-amino acids. Its codons are GAA and GAG. As its name indicates, L-glutamic acid has a carboxylic acid component to its side chain. At pH 7.0, the amino group is protonated and one or both of the carboxylic groups will be ionized. Hence, the species has a charge of – 1 and is referred to as L-glutamic acid [43].

The bulk of L-glutamic acid produced throughout the world now depends on

direct fermentative processes using both free and immobilized microbial cells [41]. Although other methods namely, chemical synthesis, protein hydrolysis etc also have been employed to some extent [46]. However, chemical method generally yields D L-glutamic acid mixture [51]. Thus fermentative methods were proved to be most suitable.

MICROBIAL PRODUCTION

Interest in microbial production of L-amino acids has increased greatly since development of large scale microbial processes to make L-glutamic acid. The economical production of L-glutamic acid is of considerable commercial importance since the monosodium salts thereof is highly useful as a flavoring agent in many food products [24]. Several methods of obtaining or producing L-glutamic acid are reported in the scientific and patent literature [96]. Most of these are either chemical methods which lead to the racemic form of L-glutamic acid and require a resolution step to obtain the natural form, or isolation methods where in L-glutamic acid is reconverted from various natural sources [43]. There have also been reports on the production of L-glutamic acid from α-Ketoglutatic acid [6]. All of these methods however leave much to be desired in that they are expensive, low yielding or dependent upon difficulty available starting materials [43]. Though the large scale manufacture of L-glutamic acid could stimulate market development, much of the recent interest is due to the potential production of natural isomers at a cost permitting their use as flavoring agent or other economic products.

Microbial strains employed in L-glutamic acid production

A wide variety of microorganisms can produce L-glutamic acid by fermentation from various carbon sources : *Micrococcus glutamicus* [102], *Pseudomonas flurorescensus* [103], *Bacterium ketoglutaricum* [104], certain coliform bacteria [105]; *Kluyvery citrophila* [106], [107]; *Escherichia coli* [108]; *Bacterium ketoglutaricum* [109]; *Agrobacterium* sp., *Erwinia* sp, *Serratia* sp., *Deboromyces* sp. and other yeast and molds [110]; *Proteus vulgaris, Pseudomonas ovalis* and *Serratia marcescens* [111]; *Aspergillus niger, Aspergillus wentii, Apioporthe corui, Viridostroma* sp., *Endoconidiophora adipose, Stachybotrys atra, Syncephalastrum*

racemosum, Thielaviopsis sp., *Phoma* sp. [112]; *Aeromonas* sp. [113]; *Xanthomonas* sp. [114]; *Bacillus pumilus, Bacillus subtilis, Aerobacter aerogenes, Serratia marcescens, Micrococcus pysogenes, Xanthomonas pruni, Pseudomouas aeruginosa* [115]; *Micrococcus varians* [116]; *Microbacterium Salicinovorum nov* [117]; *Corynebacterium lilium* [118]; *Corynebacterium callunae* [119]; *Corynebacterium herculis* [120]; *Brevibacterium diverticum* [121]; *Brevibacterium aminogenes* [122]; *Brevibacterium flavum* [46]; *Breviacterium lactofermentum* [51]; *Brevibacterium saccharolyticum* [46]; *Brevibacterium roseum* [123]; *Brevibacterium lactofermentum* [51]; *Brevibacterium saccharolyticum* [46]; *Brevibacterium roseum* [123]; *Brevibacterium immariophilum* [124]; *Brevibacterium ammoniagenes* [125]; *Brevibacterium alanicum* [126]; *Brevibacterium thiogenitalis* [127]; *Microbacterium flavum var. glutamicum* [26]; *Microbacterium ammoniaphilum* [128] etc.

However, production of L-glutamic acid from carbohydrates and different sources of nitrogen, *Micrococcus glutamicus* was proved to be the best microorganism. Kinoshita isolated it in early 1956 by a new screenining method which is described below:

The principle of the screening method is derived from the bio-autographic technique which was applied directly to the microbial colony on an agar plate. A series of various, defined test media, and one containing nutrient medium, were all inoculated with bacterial isolates of various origins, each at the same relative position on the plate. The organisms were fully grown, and those present on the taste media then killed by subjecting the plates to a strong dose of ultraviolet irradiation. The plates were layered with basal agar medium for L-glutamic acid bio-assay containing the assay organism *Leuconostoc mesenteroides* strain P-60, diapered in the medium. During incubation at 37°C, a halo of growth developed around each colony which had produced L-glutamic acid during the initial incubation. Thus, the bacteria were selected by picking the area of growth on the nutrient agar plate corresponding to the site where colonies were surrounded by halos on the test plates. About 500 bacterial isolates were

tested in this way. With the test medium (glucose, 5.0 gm; KH_2PO_4, 0.05 gm; $MgSO_4.7H_2O$, 0.01 gm; Urea, 0.8 gm; $FeCl_3.6H_2O$, 0.004 gm; water, 100 ml; pH 7.2) approximately 1/10 of the colonies were surrounded by very faint halos and 4.0% by clearly visible halos. Only a few colonies formed deuse halos. All halos–forming and some non-halo-forming organisms were grown in the same medium in liquid culture, with shaking, for several days. Analysis of the culture filtrates revealed that about 2/3 of the forming bacterial which had grown well produced L-glutamic acid (mostly 0.4 to 0.5 mg / ml). None of the non-halo-forming bacteria produced any L-glutamic acid, only one organism accumulated a large amount. The pure culture of this organism yielded about 10 mg of L-glutamic acid per ml of medium after 2 days of incubation. This organism was later identified as a new species, which was named *Micrococcus glutamicus*. It is a Gram-positive, spherical cell, ranging from about 0.5 to 3.0 µm in diameter and typically appears in tetrads. It has a substantial cell wall, which may comprise as much as 50% of the cell mass [123,129,130]. The genome of *Micrococcus glutamicus* is rich in guanine and cytosine (GC), typically exhibiting 65 to 75% GC content. *Micrococcus glutamicus* often carry plasmids (ranging from 1.0 to 10 MDa in size) that provide the organism useful traits [102]. The taxonomy of *Micrococcus glutamicus* is as follows:

- Domain: *Bacteria*
- Kingdom: *Bacteria*
- Phylum: *Actinobacteria*
- Class: *Actinobacteria*
- Subclass : *Actinobacteridae*
- Order: *Actinomycetales*
- Suborder: *Micrococcineae*
- Family: *Micrococcus*
- Species: *glutamicus*

The life and activities of microbial cells have developed as a compromise, dictated by conditions of environmental and nutritional constrains and competition to which they have been routinely exposed in their natural ecosystem [131]. Although when transferred to laboratory culture, the conditions are usually for removed from those prevailing in natural environment, regulatory mechanisms still operate to prevent, “wastful” expenditure of energy

[132]. Thus, it is not surprising that microbial metabolites of possible industrial interest are normally produced initially at very low levels [133]. If the product turns out to be of commercial value, programmes of media development and strain improvement are initiated. The over production of desired metabolite can be achieved by the genetic removal of feed-back control, which is possible through mutation [134]. Mutations resulted from events which are beyond the cell's ability to genetic damage [135]. The molecular nature of the mutation at the levels of DNA is not really important [136]. The common encountered base pair substitution arising from transitions or trans-versions and frame-shift mutations are actually transmitted for generation to generation [137]. What is important is the rate at which mutation itself: Is it "tight or leaky"? Is it conditional that is, the activity, osmotic or temperature remedial? And it is reversible? In most cases tight mutations are required because activity is totally abolished. Non-revertibility is also desirable because it decreases the rate of "Strain degeneration". Mutation can influenced by many factors, e.g., the type of mutagen, dose, physiological state of the culture, plating conditions and repair inhibitors [138-145]. Mutagen act by producing alterations in DNA, because DNA is found in autonomous plasmids, as well as integrated into the intact genophore [146]. Treatment of an organism with a mutagen may cause alteration of episomes and plasmids as well as chromosomal genes [141]. Different mutagens have different mechanism of action. Therefore, they produce diverse results and must be used under particular conditions [137]. Chemical mutagens can induce genetic changes: (i) the direct induction of base inspiring by means of misincorporation of mutagenic base analogue into the DNA or (ii) by alteration of existing base in situ or indirectly via induced misrepairing mechanisms [139]. Ethyleneimine (azaridine) reacts with DNA in vitro, mainly at the N7 position of guanine and N3 of adenine, then imidazole ring opening of the modified genuine results in formation of formamidopysimidine (FaPy) residues [146]. Ultra violet irradiation has been recommended as a mutagen of first choice [142]. It can induce

both base pair substitution and frame shifts mutation [142-147,148]. The ratio of mutation to lethality is usually high and UV irradiation (light) is relatively safe mutagen for the experimenter [149]. The mutagenic effects of UV ray can be enhanced by insuring that the irradiated culture is not exposed to photo reactivating light and by plating immediately after mutagenesis to prevent pre-replication repair [150-154]. Both lethality and mutation have been shown to decrease when culture is incubated in dark under non grown condition [155]. The intra-strand cyclobutane pyrimidine dimer is the predominant DNA lesion produced by UV irradiation (250 nm) [147]. Many other lesions have been reported such as hydration across (5, 6) double bond of pyrimidines and dimmers with the L-amino acid cysteine [144-146].

In cellular system, the pyrimidine dimer is the major cause of lethal and mutagenic damage[133]. Cells have evolved several systems to deal with these lesions. The first is photo-reactivation or photo-repairs and secondly pyrimidine dimer can be excised from DNA by excision-repair mechanism [136]. The third mechanism is post replication repair occurs after DNA synthesis [146].

The UV irradiation is an extremely convenient mutagen, provided that the cells to be treated are appreciably transparent to it (some strongly pigmented fungal spores) [141]. Mutagenic wave lengths lie between 200 and 300 nm, the peak of absorption by nucleic acids [147]. It is thus an extremely fortunate coincidence that a convenient source of UV, a low pressure mercury vapor (“germicidal”) lamp, emits a very high proportion of its energy at 254 nm, close to the most effective wave length [141]. High pressure mercury vapor lamps spread their energy more uniformly over a wide range of wave length [142]. Killing by UV irradiation is in mainly (haploid) organisms, exponential, often with a marked initial lag, probably at least in part due to the operation of repair mechanisms. The proportions of particular mutant closes amongst the survivors in general increase approximately linearly with dose until, at dose giving very low level of survival, the proportions may fall [147]. A guide to determine the dose of UV ray that will

leave the highest proportion of mutant survivors is to plot a survival curve and to use the highest dose that results in exponential killing; that is before any slowing down in the rate of killing with increasing dose is apparent [155]. For reproducibility of experiments, and to prevent increase in viable count of strong suspensions, it may be necessary to avoid exposure to long wave length UV irradiation or the shorter wave lengths of the visible spectrum (sun light and light from fluorescent tubes are effective sources) during the period immediately following the irradiation in order to avoid photo reactivation [156]. The enzyme reversal of a proportion (often upto a maximum of about 60%) of the mutagenic and killing effect of a given UV dose. The action sprectrum for photo-reactivation is not the same in all organisms (peak at 375 nm and 435 nm for *Escherichia coli* and *streptomyces griseus* respectively), but it can probably be assumed that wave lengths longer than about 525 nm will have negligible effect [157]. Thus, post irradiation manipulations may be carried out in yellow light [138]. An ordinary yellow coated light bulb may be a good enough source for this purpose, or alternatively a dark room safe light with Wratten Number OB filter can also be used [141]. If a higher intensity of illumination needed, or a large room has to be illuminated, a convenient and inexpensive source is a sodium vapour lamp (emitting 589 nm) of the killing use in street light [147].

Photoreactivation occurs unpredictably amongst micro-organisms; for e.g. a certain wild type isolates K673 of *Streptomyces coelicolar* was strongly photoreactivable, while another wild type of some "species", A3(2) was not [157]. It is therefore worth testing an organism that is to be irradiated extensively to seed whether the extra complications designed to avoid photo-reactivation are required.

Mechanism of L-glutamic acid fermentation

The extensive studies by Kinoshita and associates on the mechanism of L-glutamic acid formation by the organism they named *Micrococcus glutamicus,* have been summarized by Kinoshita (1959), Asai *et al*.(1959) and Takanaka *et al.* (1960) [158-160]. They believed the following reactions occur : (1) Sugar metabolism proceeds via the Embden

Meyerhof Parnas (EMP) pathway and the (2)Hexose Monophosphate Shunt (HMS); at low aeration rates, the EMP pathway is dominant, and lactic acid accumulates instead of L-glutamic acid; with sufficient air, the HMS syatem is predominant and L-glutamic acid accumulates. Once, L-glutamic acid if formed, the organism showed little ability to degrade it.

According to them, when the HMP system predominates, glucose is oxidized to gluconate. From 6-phosphogluconate and ribose 5-phosphate, pyruvate and acetate are formed. This organism cannot synthesize L-glutamic acid from malate or citrate. Malate apparently results from the enzyme reaction first described by required for citric acid formation. Citric acid, formed acetate and oxalate, is oxidized to L-glutamic acid via two NADP-specific dehydrogenases, i.e., isocitrate dehydrogenase and L-glutamate dehydrogenase, which are closely coupled in the presence of ammonium ion. The reaction rates are such that apparently much of the citric acid and formed is trapped and taken to L-glutamic acid.

Biotin also may operate by altering metabolic pathways, such as determining whether the EMP pathway or the HMS predominates. With low concentration of biotin, the HMS is activated. This supplies the NADPH required for the NADPH-linked coupling reactions involved in L-glutamate formation [161,162].

In addition, the acetic acid and dicarboxylic acid oxidizing activities of cells grown in biotin-rich and biotin deficient medium were reported to be quite different. Oxidation of these acids is much more active by biotin rich cells [163]. For example, succinate oxidation was found to be low in biotin deficient cells [164]. Thus, the flow of carbon to fumarate and aspartate, can be partially blocked, and part of the carbon usually drained off in this pathway can be shunted back to L-glutamic acid production [165].

There is a disagreement as to the exact function(s) of biotin in the L-glutamic acid fermentation. Oishi and Aida (1965) have reported that with *Brevibacterium ammoniagenes* 317-1, biotin deficiency led to an insufficient shift of sugar metabolism from the EMP path way to

the HMS to account for the increased L-glutamic acid accumulation [166]. Moreover, detailed comparisons by Otsuka, Miyajima and Shiio (1965) of glucose pathway to L-glutamic acid in *Micrococcus glutamicus* and *Brevibacterium flavum* grown in a medium low in biotin showed that glucose was metabolized aerobically, by both organisms, by EMP and HMS routes in an approximate ratio of 85 : 15 [167]. Furthermore, activities of enzymes involved in L-glutamate formation were found not be changed significantly by biotin content of the medium in which the organism were growth [168]. An exception was isocitrate lyase activity which was high in cell free extracts from *Micrococcus glutamicus* grown in biotin rich medium but almost absent from cells grown in low biotin containing medium [169]. A marked increase in permeability to L-glutamate in cells of both organisms when grown in low biotin containing medium over cells grown in biotin rich medium was also found [170]. Shiio *et al*. (1959) had previously reported a permeability change in *Brevibacterium flavum* grown in biotin deficient medium. One of the functions of biotin appears definitely to be at the cell permeability level [171]$6H_{12}O_6 + 2.3O_2$ --> $0.82C_5H_9O_4N + 1.94CO_2$ ……… (1)

Oxygen is consumed in large amounts. Therefore, quantitative discussion on oxygen transfer during the fermentation is indispensible for both the optimization of operation and the scale up.

The following equations are the simplest expression of the basis of discussing oxygen transfer during the fermentation:

${}^{r}a_b = k_d(P_B – P_L) - k_rM\ (P_L \geq P_{Lcrit}) < kM\ (P_L < P_{Lcrit})$ ………. (2)

Where ${}^{r}a_b$ is the rate of cell respiration (moles of O_2 / ml / min); k_d is the Oxygen absorption coefficient (mole/ml/min/atm); P_B is the gas phase Oxygen absorption coefficient (mole/ml/min/atm); P_B is the gas phase Oxygen tension (atm); k_r is the cell's Oxygen demand (mol / min / gm of cell); M is the cell density (gm / ml); and P_{Lcrit} is the critical level of liquid phase oxygen for cell respiration (atm). Rate of cell respiration (${}^{r}a_b$) equals k_rM at P_L levels above P_{Lcrit}; in other words, cell respiration is satisfied when oxygen supply is sufficient to maintain P_L above P_{Lcrit} [172].

Values of P_{Lcrit} are usually less than 0.01 atm, too low to be determined with conventional membrane coated oxygen electrodes. The determination of extremely low P_{Lcrit} values was indispensible; therefore a new method was developed in which both P_L and the redox potentials of culture medium were measured simultaneously [174]. This value was 0.002 atm for *Brevibacterium flavum* .

Lack of quantitative analysis on oxygen transfer sometimes leads to inaccurate conclusions. For example, L-glutamic acid producing bacteria are known to excrete large quantities of lactic acid in biotin rich medium [175]. However, the analysis of oxygen transfer clarified the true factor: it was due not to excess biotin but to oxygen deficiency that lactic acid became the dominant product in a biotin rich medium [176]. In biotin rich medium cell density become high which resulted in a high oxygen demand of culture broth consequently, the amount of oxygen was insufficient [177]. In fact cell did not accumulate lactic acid even in biotin rich media when sufficient oxygen was supplied. In L-glutamic acid fermentation, the P_L level is closely related to product fermentation as shown in Fig.9. The production yield markedly decreased when the P_L level was zero as measured by conventional membrane coated oxygen electrodes. This implies that aeration and agitation must be controlled so as to keep P_L slightly above zero for the effective production of L-glutamic acid. In this culture of high productivity, cell respiration was satisfied. Limited oxygen supply resulted in lactic acid or succinic acid accumulation at the expense of L-glutamic acid formation [178]. Oxygen tension in the growth phase also seriously influenced the product formation [179]. Oxygen deficiency in the growth phase did not bring about any marked change in L-glutamic acid formation, where as a high aeration condition seriously reduced the L-glutamic acid producing capacity of the cell [180]. Although the productivity of the other L-amino acids were also influenced by oxygen supply, the extent or the mode of the influence was diverse according characteristics of biosynthetic pathways of each L-amino acid [181]. Fig10 shows the relation between the degree of oxygen satisfaction (${}^{r}a_b$ / k_rM)

and relative productivity of each L-amino acid.For example, oxygen shortage caused inhibition of both L-glutamic acid and L-lysine production, but the extent of the inhibition was more serious in L-glutamic acid fermentation than in L-lysine fermentation [182]. Moreover, the maximum production of L-leucine was observed when the cell respiration was slightly repressed [183]. Aerobic microorganisms required oxygen to reoxidize NADH to form NAD^+ and to generate ATP for growth and metabolism [184]. L-glutamic acid is biosynthesized by the way of pyruvate, citrate & α-ketoglutarate. Formation of 5.33 moles of NADH and generation of 2.0 moles of ATP are accompanied with assimilation of 1.0 mole of glucose [185]. The amount of NADH formed in the L-glutamic acid fermentation is more than in L-lysine formation due to 2.66 moles [186]. This suggests that the inhibition of product formation due to oxygen shortage was more serious in L-glutamic acid fermentation. However, extremely deficient condition of oxygen shortage was more serious in L-glutamic acid fermentation. However, extremely deficient condition of oxygen might inhibit the cell to reoxidize 2.0 moles of NADH and lead to accumulation of lactate in place of L-amino acids [187].

Immobilized microbial cells

Immobilization of whole cells is now a well-established method in the field of enzyme technology, both scientifically and industrially. The scientific status is documented by an ever increasing member of publication; the industrial breakthrough is demonstrated by the successful performance of several industrial microorganisms [188].

The development mainly occurred during the past decade, while the first land mark papers, which bridged the gap between some old but rather accidentally design of an immobilized cell system, appeared around 50 years age [189].

The really important point was the observation that enzymes are active and stable for long period of time, if kept within the cellular domain together with all other cell constituents, whether the cells are dead or viable, but in a resting state [190]. The development of immobilized cell system happened on the following lines, first of all, single enzyme catalyzed reactions were

considered where the preservation of cell viability was not a prerequisite or where cell viability was destroyed deliberately by heat treatment or chemical treatment to improve the catalyst stability [191]. It should be mentioned however that cell viability could also be advantageous in these groups of applications, because reactivation or enhancement of an activity by cell growth becomes possible. The next step in heterogeneous bio-catalysis was the controlled immobilization of viable cells, which were however kept in the resting state and were able to catalyze multi-enzyme and co-factor dependent reactions [192]. It is obvious that this group of reactions really in the domain of immobilized whole cells, where no practical alternative exists on the immobilized enzyme level [193]. The final step was the immobilization of living and growing immobilized cells, where (limited) growth is a necessary perquisite for the bioconversion [194]. This development was not only important for the expansion of the field of microbial cell immobilization, but ever more was the key point to include plant cells and mammalian cells as well [195]. Comparing the task of cell immobilization on the one hand and that of enzyme immobilization on the other, the size of the cells makes it much easier to develop for simple immobilization techniques such as entrapment [196]. With increasingly high demands for the cell physiology in connection with viable and growing cells, the task is more difficult, but in most cases solutions could be presented. In addition to the principal strategies of (a) Biocatalyst aggregation (cross linking flocculation), (b) Attachment of the biocatalyst to a pretreated carrier (absorption, covalent bonding) and (c) Entrapment (porous network, microencapsulation), the concentration and the spatial distribution of the biocatalyst species can be influenced by the cell growth within a carrier (eg. Cell growth from immobilized spores)[197]. Further, by gradual change in reaction conditions, the physiology and activity of the immobilized species can be adapted to new levels in continuous processes [198].

The overall composition of an immobilized cell system in chemically less well defined than an immobilized

enzyme preparation [199]. Careful alteration has to be given to the loss of cell matter or reaction by products into the reaction medium and their action as toxins [200].

There are various methods available in the field of immobilization of whole microbial cells, namely adsorption, covalent bonding, cell to cell cross linking, microencapsulation, entrapment in polymeric network, gelatin of polymers, precipitation polymers, poly-condensation, polymerization and iontropic gelation of polymers[195]. However, most wide used methods namely gelation of polymer using agar end iontropic gelation using calcium alginate are extensively reviewed in this section.

Agar method

Gelation is a temperature controlled phase transition in polymer solvent systems, where a homogenous polymer solution is transformed to a homogenous gel without change in composition [201]. Usually, gelation occurs by lowering the temperature, but systems are known where the reverse effect is observed [202]. Systems of the latter type can be of interest in connection with the application of thermophilic organisms [203]. The cells are mixed with the polymer solution at an appropriate concentration and are completely immobilized by entrapment in the solidified gel [204].

Agar is mainly used in this method [205]. Small particles of regular size have been prepared using specific forms in which the solution is solidified by cooling [206]. A much more practical procedure however is the use of a water immiscible oil phase (paraffin oil, soy bean oil, butyl phythalate) for the dispersion of the solution and the conversion of this emulsion into a solid / liquid dispersion by cooling the whole system. In this way, small spherical particles in a controlled diameter range can be obtained, depending on the degree of mixing in the liquid / liquid system [207].

The very simple method of gelation suffers from the fact that rather soft, mechanically unstable gels are obtained [208]. Therefore, a technical application of this method is very unlikely. The situation may be different, however, in the application of microbial cells, were the very mild immobilization conditions

(if the temperature of gelation is acceptable) are of higher importance than the mechanical property [209]. The reversibility of gelation may be of interest if the cells or intracellular products have to be recovered [208].

Calcium alginate method

If a water soluble polyelectrolyte is mixed with the appropriate, usually multivalent, counter ions, solidification by poly-salt formation occurs [210]. In connection with the formation of highly water swollen structures of controlled morphology, the term “ionotropic gelation” was introduced. The most well-known example is the Ca-alginate gel, which is obtained by gelatin of Na-alginate solution in a $CaCl_2$ bath [211].

One has to distinguish between poly-anions and poly-cations since they determine the stability regions of the respective poly-salts: poly-anion salts become unstable at higher pH values, while poly-cation salts are redissolved at lower pH. Another important aspect for biochemical transformation is the stability against phosphate buffer: Alginate gels are completely redissoved, while chitosan gels require the presence of phosphate to stabilize the network [210].A large variety of polymers with different structure and functional groups exists with regard to poly-anions. Alginate is the most important and most widely used products [212].

Since the entrapment of a controlled amount of cells is very simple and generally various cells could be immobilized with a complete preservation of viability [213].

Iontropic gelation is a reversible procedure [214]. This can be advantageous in connection with recovery of the entrapped species or in connection with multi step immobilization procedures, e.g. in microcapsule formation, or epoxy bead preparation [215, 216]. Attempt have been made, on the other hand, to stabilize the ionic gel e.g. by covalent cross linking [217]

The application of alginate for the purpose of whole cell immobilization was first reported in 1975 [218]. In these experiments, All counter ions were used, while Kierstan and Bucke (1977) in the subsequent paper introduced the now widely used Ca-alginate combination [219]. As with k-carrageenan, the alginates are produced from sea weed

and they are readily available as food additives. Alginates are hetero-polymer carboxylic acids, coupled by 1→4 glycosidic bonds of β-D mamuronic (M) and α-L-gulcoronic acid (G) units. The uronic acids are arranged in a block wise fashion along the chain. The same macromolecule contains the duty homo-polymeric blocks, (M)n and (G)n, together with the blocks of alternative sequence (MG)n [220]. Alkali and magnesium alginates are soluble in water whereas alginic acids and the salts of polyvalent metal cations are insoluble [221]. By simple dropping of a sodium alginate solution into a $CaCl_2$ solution, rigid near spherical gals are formed by ionotropic gelation, in which the interaction of alginates and the strength and texture of the resulting gels are dominated by association of (G) n sequences, while the (M)n and (MG)n blocks play only a subordinate part in the gel network [222]. But independent of the type of uronic acid, both of them held Ca ions strongly enough so that Ca-alginate gel themselves have no tendency to redissolve [223]. However, in the presence of any mono-valention like Na^+, a minimum amount of Ca^{+2} has to be present to prevent swelling of the gel [224-227]. This minimum Na^+ / Ca^{+2} ratio may vary from 5 : 1 (M–type) to 20 : 1 (G–type) [223]. Further more, different molecular weight samples are available, which determine the solution viscosity at a given polymer concentration [228,229]. Typical value may be found between 1.0% and 8.0% of Na-alginate [223].

Ca-alginate beads can be prepared in a broad range of particle size. The typical biocatalyst particles will be in the size range of 0.5 to 3.5 mm [230]. The particle size is primarily determined by the surface tension of the alginate solution and not by the inner diameter of the extrusion dies [231]. The most effective way to prepare smaller particles is to use a controlled blow off stream of compressed air concentric to the extrusion die [232]. If special care is given to the construction of the coaxial droplet forming device, very small particles in the size ranging between 10 and 120 µm can be obtained [225].

A special property of the Ca-alginate system is the fact that the particle size can be reduced by partial or complete drying, since the resulting shrunken

particles reuse well to only a limited extent if re-equilibrated with aqueous media [223].

The preparation bath used for the solidification of Na-alginate solution droplets usually contains a 2.0% $CaCl_2$ solution, although this may be reduced or increased if required [225]. The application of counter ions other than Ca^{+2} has been demonstrated recently by using Al^{+3} in a pilot scale study [227]. The temperature of gelation can be chosen between 0 and 80°C, depending on the nature of the cells. Biomass loading in the direct entrapment step can be varied up to 30% wet weight [233]. By applying the drying procedure and subsequent reduction of the particle size, the cell loading can be increased to values of 100% wet weight and even higher [224-226]. Furthermore, the problem of scale-up of the process of biocatalyst preparation should be mentioned. If the single sampler pipette for droplet formation is substituted by a plate with 42 outlets, each with an inner diameter of 0.4 mm, combined with compressed air tubing's, about 5.0 kg alginate solution can be processed [234].

Fermentation processes

The fermentation method is a production process in which a specific L-amino acid is synthesized in large amounts by a specially selected microorganism in culture. The selected microorganism is cultured with carbohydrates and ammonia and releases the L-form of the L-amino acid into the culture medium. The cell produces glutamate from 2-oxo-glutamate (2-oxo-pentanediolic acid) by reductive ammonia fixation that uses the enzyme glutamate dehydrogenase, a normal cellular constituent [161].

The L-glutamic acid is one of the most important commercial L-amino acid. Its sodium salt, MSG, is used as flavor enhancer, since it heightens and intensifies the organoleptic properties of the food stuff without adding significant flavor of its own. For more than a few decades, *Corynebacterium glutamicum* (initially *Micrococcus glutamicus*) has been used for L-amino acid production including the commercially important products of glutamate, glutamine and lysine. Among the L-amino acids mentioned earlier, L-glutamic acid is the largest fermentative product, which occupies about 53% of the world's L-

amino acids market [235]. It is also particularly important in food industries and widely used as an important starting substance for the synthesis of various and useful pharmaceutical and health products.

Micrococcus glutamicus was discovered in 1957 by Kinoshita *et al.,* and provided a novel method for producing the natural L-amino acids including L-glutamic acid [236]. It is a Gram +ve, non sporulating bacterium that may be isolated from the soil and widely used in the industrial production of L-amino acids such as L-glutamic acid and L-lysine [102]. There are several bacterial strains mentioned earlier that also over produce L-glutamic acid, however, *Micrococcus glutamicus* is typically used in the commercial production of this L-amino acid, thus, represents one of the major industrial fermentation microorganisms [236].

In order to achieve high L-glutamic acid titres and productivities, the fermentation is operated with a rapid initial growth period followed by an intense L-glutamic acid production phase. Khan *et al*., (2005) determined that the growth of *Micrococcus glutamicus (Corynebacterium glutamicum)* did not follow the simple Monod's Kinetics [237]. The growth inhibition by the product (L-glutamic acid) occurs during the fermentation. Also, the substrate (glucose) limitation at the lower concentration and inhibition at the higher concentrations are exhibited with respect to the cell growth. A modified form of Monod's equation along with a product inhibition term is able to define the growth Kinetics at the low substrate concentrations. However, this model appears to fail at high substrate concentration, when the substrate inhibition also occurs. At very high concentrations like 350 gm/L of glucose, the growth is almost zero, which may be due to severe substrate inhibition.

One of the key factors that limit the L-glutamic acid production is its excretion from the microorganism to the culture medium. At increase in the permeability of the bacterial membrane would be necessary to allow the excretion of the metabolite several strategies can be used cultivation of the bacteria in a biotin limited medium or the addition of detergents or antibiotics in presence of excess biotin [238-240]. More recently,

the L-glutamic acid over production was also obtained with temperature sensitive strains cultivated at higher temperature [241]. Biotin is a Co-factor of acetyl Co-A carboxylase, the first enzyme in the synthesis of oleic acid, which is involved in the culture medium, since this influences the occurrence of the deficient phospholipids membrane. Biotin limitation was the first process used for the production of L-glutamic acid, and final concentration between 10 – 30 gm/L has been attained in batch cultures of different strains [242,243]. The limiting concentration for biotin depends on the strain, the nature of the carbon source and its concentration. About 20 µg / L of biotin are sufficient to allow *Corynebacterium glutamicum* growth, L-glutamate excretion occurs only when biotin concentration fall down to 3.0 µg / L [244].

The carbon source has an important influence on kinetics and stoichiometry of the cell growth and L-amino acid production by *Corynebacterium glutamicum.* The culture media based on the glucose, fructose or sucrose are typically used for this production. Similar results have been detected when the glucose, fructose or glucose plus fructose and sucrose were employed as the carbon sources [245]. Also, the selection of an in expensive carbon source is essential for economic L-amino acid manufacture, since it represents a major part of the variable operation costs. Thus, the use of complex sugar substrates such as cane molasses, beet molasses, or corn, wheat or cassava hydrolyzates has become common practice, the type of hydrocarbon often being depend on the geographical location of the production plan while the molasses is more usual in the Europe, South Africa and China, starch hydrolysate from the corn (corn syrup) is the most important carbon source in the North America [246-248]. Momose and Takagi (1978) and Sun *et al*., (1989) used complex media (Soybean hydrolysates or rice hydrolyste coupled with corn syrup and rape oil) [249,250]. Final L-amino acid concentration of 70 gm/L has been reported on the glucose of 88 gm/L on the palm waste hydrolysates [251]. Vijayalakshmi and Sarvamangala (2011) investigated the effect of fruit substrate of *Muntingia Calabura Linn* on L-glutamic acid

production using different bacterial strain like *Corynebacterium glutamicum* DSM 20300^T and *Arthrobacter globiformis* MTCC 4299 and obtained maximum 15.1 mg/ml L-glutamic acid with *Corynebacterium glutamicum* DSM 20300^T at pH 7.0, temperature 30°C was maintained for 48h incubation time with urea, biotin and penicillin optimum concentration were being 2.0 gm/L, 1.0 μg/L and 1.0 IU/ml respectively [252].

In order to facilitate the exploitation of the low cost substrates (i.e. molasses), whose natural biotin content is much higher than that required for the operation at biotin-limitation conditions, the addition of surfactants have much proposed. High L-glutamic acid production (upto 100 gm/L on the beet molasses) has been achieved by this strategy [253,254]. However, the consumption of large amounts of the surfactants, the introduction of potential contaminants during the fermentation may be resulted. (Surfactants are difficult to sterilize) the extreme sensibility of the surfactant / biomass ratio are major drawbacks to this process. Also, ratio is the major drawbacks to this process. Also, the amount of added surfactant or more precisely the ratio of the surfactant concentration to the biomass concentration is a key parameter. It has to be high enough to increase the membrane permeability, but not so large that it would lead to be cellular death and thus, to a decrease in L-glutamic acid production [241].

Nampoothiri and Pandey (1996) reported maximum L-glutamic acid production by *Brevibacterium roseum*, using glucose as carbon source and urea as nitrogen source. About 90% available glucose was consumed after 48h of fermentation time, and the main accumulation of the glutamate occurred after the maximum growth of the microorganism. They found that 0.5% urea was an optimal concentration for the L-glutamic acid synthesis, while high concentrations of urea inhibited the cell growth, may be due to excess of ammonium ion released by the hydrolysis of urea [255]. Similar results were obtained by Jyothi *et al*., (2005) with a hydrolysate from the cassava residues containing a un-expectable starch content of about 45-55%. The estimation of the reducing sugars in the culture broth at periodic

intervals showed that the consumption of glucose increased with the fermentation time and about 80% to 90% were consumed after 48h [256].

L-amino acids can be produced in the bioprocess applying batch, feed batch, repeated fed batch or continuous production techniques. Nowadays most glutamate production is carried out by batch or fed batch fermentations utilizing free suspended cells of *Corynebacterium glutamicum* in stainless steel stirred tank bioreactors of up to 500 m^3. In fact, the fed batch mode is still the preferred technology because, it allows easy control of the nutrients concentration, reducing the influence of this variable on the process productivity of the yield, due to the occurrence of unwanted effort such as substrate inhibition or overflow metabolism. As mentioned above, the typical overflow metabolites are lactate or acetate [257-268]. The L-glutamic acid content in the medium increased with time of incubation. The L-glutamate was detectable in the medium after 12h and attained stationary level after 48h. Joseph and Rao (1973) in their study with *Micrococcus glutamicus* obtained maximum glutamate yield in a potato hydrolysate medium, when 0.5% urea was used as nitrogen source. The selectivity of the nitrogen source by different microbial strains and the difference in the carbon source could be the reason for different yields observed [258]. Most reports mention 25–35°C as optimum temperature range for cultivating coly form bacteria, although some strains with increase thermo tolerance were also described [262,263]. Increased temperature has been used by some authors to induce the L-glutamic acid production in certain strains [262]. Delaney *et al*. (2002) demonstrated that increasing the culture temperature from 33 to 36, 38, 39 or 40°C resulted in final L-glutamic acid titres superior to 80 gm / L, the best channeling of the carbon flow towards the L-glutamic acid synthesis having been found L-glutamic acid synthesis having been found at 39 & 40° C. Moreover, this study concluded that the higher temperature, the slower the growth ratio and the higher the lactate accumulation [267].

Another important factor determining the L-glutamic acid productivity could be the oxygen concentration. A

sufficient oxygen supply might help to reduce the accumulation of by products, such as lactic acid and succinic acid both metabolites indicating an energetic imbalance. However, in the above mentioned temperature up shocks strategy, oxygen transfer could not be held to be responsible for the metabolic shift. Indeed, though pO_2 levels felt as low as 10% of air saturation during the period of intense metabolic activity immediately after the temperature shift, the diminished metabolic activity during the period in which organic acids were produced was such that the pO_2 increased up to 60% air saturation at the end of the fermentation [263].

Recently, Xiao *et al.*(2006) have reported that higher fumarate concentration could be achieved in *Corynebacterium glutamicum* cultures, by maintaining a constant low level of the dissolved oxygen concentration. However, by product lactate has also severely accumulated in these conditions. The analysis of the activity changes of the two key enzymes (glutamal and lactate delydrogenases) and the metabolic network flux showed that the lactate over production was brought above by metabolic flux in the tricarboxylic acid cycle (TCA cycle) too low to balance the glucose glycolysis rate. In order to avoid this problem, a balanced metabolic control by the respiratory quotient was proposed. By operating at these optimize conditions, the TCA metabolic flux rate was regulated at an appropriate level to achieve the metabolic balance among the glycolysis, glutamate synthesis, and TCA metabolic flux, thus, increasing maximal glutamate concentration by about 15% and completed repressing the lactate accumulation [268].

A continuous fermentation process could lead to more efficient L-amino acid production at industrial scale, but the development of an adequate microbial immobilization protocol would be necessary. The immobilized cells provided some advantage over freely suspended cells, such as simpler reuse of the biomass, easier liquid solid separation and minimal clogging to continuous flow systems [255,256]. However, relatively few reports are found on utilization of immobilized cells systems for the production of L-glutamic acid. The living cells of bacteria were

entrapped in the polyurethane foams in a vertical rotating immobilized cell reactor, entrapped in k-carrageenan gel beads, Calcium alginate or absorbed on to porous glass beads [269-279].

In the most cases, the main drawback was associated to the oxygen requirements for these associated to the oxygen requirements for these microbial systems, due to the internal mass transfer limitations in the immobilization supports [280]. Moreover, during the long term operation using such bioreactors, high cell density could lead to the starvation of the cells nearest support surfaces, and biotin and penicillin might accumulate inside the cells [281]. Due to this reason, it seems that the immobilized cell strategy is not economically competitive with the currently available technology.

Finally, the need of a cost efficient downstream process has to be stressed, since it is crucial to reduce the investment and production cost in L-glutamic acid production. The usual downstream sequence included a first step involving cell separation from the culture medium, followed by the purification of the L-glutamic acid and crystallization. Crystallization technique such as cooling/vacuum crystallization are economically favorable but could not be applied for all the L-amino acids. The solubility of the L-glutamic acid is barely affected by the temperature as shown by a flat solubility curve making the cooling crystallization unsuitable for this product [282]. Due to the amphiteric character of L-amino acids, efficient crystallization depends also on the pH during the operation. Thus, L-glutamic acid is crystallized from the spend medium by lowering the pH to its isoelectric point of pH 3.2 using hydrochloric acid.

ISOLATION AND PURIFICATION

The crystals of L-glutamic acid hydrochloride were separated from the liquid by filtration and re-dissolved water. This solution was again filtered to eliminate humus. The pH was then adjusted to the isoelectric point of L-glutamic acid (pH 3.2) with sodium or potassium hydroxide, and this solution was stored for one weak to allow L-glutamic acid to crystallize out. This step notably increased the purity of the crystals for the following reason. There are two polymorphs in L-glutamic acid

crystals: a metastable granular α-form (7) and a stable, thin, plate like β-form [283]. The α-form grows better than β-form in solutions containing other L-amino acids and, growing by its specific hydrogen bonding network, the dominant (001) face of the α-form selectively incorporates L-glutamic acid molecules at both the L-α-L-amino acid and the γ-carboxyl residues [284]. Because the solution of the crude in the early production method still contained other L-amino acids, the α-form of L-glutamic acid was the dominant crystal formed at pH 3.2. Purity was improved because the grown α-form crystals did not contain other L-amino acids.

The separation of L-glutamic acid, α-form crystals were re-dissolved in water and placed into an enamel-jacketed iron wire vessel-sodium bicarbonate was added to adjust the solution to a neutral pH (litimus paper was used). The monosodium glutamate was then decolorized by adding activated carbon and filtering. The filtered, clear solution was then concentrated by heating and cooled in the enameled vessel, causing MSG crystals to form & precipitate. When separated from the solution, the lump of MSG crystals was cracked by hammer into powder and separated from any adhered mother liquor by centrifugation. This final MSG powder was dried, sieved, and packed as the final product [285].

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