IJPSR (2014), Vol. 5, Issue 11 (Review Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 April, 2014; received in revised form, 28 June, 2014; accepted, 10 July, 2014; published 01 November, 2014

A REVIEW ON BIOCHEMICAL AND THERAPEUTIC ASPECTS OF GLUTAMINASE

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Keywords:

L-Glutaminase, Submerged fermentation, solid state fermentation, acute lymphoblastic leukemia, human hepatocellular carcinoma cell line, breast cancer cell line.

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ABSTRACT: One of the prime candidates in the treatment of debilitating human cancers includes a family of enzymes referred to as L- glutaminases. The antitumor activity of these enzymes found effective in countering Acute Lymphoblastic Leukemia (ALL a commonly diagnosed pediatric cancer.). Further it is found to inhibit four human tumour cell lines namely Hep-G2 [Human hepatocellular carcinoma cell line], MCF-7 [Breast cancer cell line], HCT-116 [Colon cell line] and A549 [Human lung Carcinoma].Apart from therapeutic use, it is having a number of other applications like in food industry, analysis and in production of fine chemicals. This review, hence, mainly focuses on the biochemical aspects of L-Glutaminase production, aiming to comprehend the physiochemical characteristics, application of L-Glutaminase and its properties. Processes central to these biochemical aspects, including submerged fermentation and Solid state fermentation of L-glutaminase producing organisms are also discussed.

INTRODUCTION: L-Glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is the enzyme that catalyzes the deamidation of L-glutamine to L-glutamic acid and ammonia [1, 2]. This is an essential enzyme for the synthesis of various nitrogenous metabolic intermediates[3]. L-Glutaminase has received significant attention since it was reported extensively as antileukemic agent [4-6]. Unlike normal cells, leukemic cell do not demonstrate the L-glutamine synthetase, thus it is dependent on the exogenous supply of L-glutamine for their growth and survival [7]. Tumour cells have an absolute requirement for glutamine as a growth substrate. Glutamine is required as a precursor for both DNA synthesis and protein synthesis and may also be used as a respiratory substrate.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(11).4617-34

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(11).4617-34

In experiments where glutamine metabolism in tumour cells has been specifically compared with that in non-transformed cells of the same origin, glutamine metabolism in the tumour cells has been found to be considerably faster. This is true for human hepatocytes and hepatoma cells [8]. L-Glutaminase has received significant attention recently, owing to its potential applications in medicine as an anticancer agent and in food industries [9-10]. Microbial glutaminase have found application in several fields[11]. It has been tried as therapeutic agent in the treatment of cancer [2, 10] and HIV [12]. It is also used as an analytical agent in determination of glutamine and glutamate [14]. However one of the major use of microbial glutaminase in the food industry as a flavour enhancing agent[15]. L- glutaminase is generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauce [16].The gamma glutamyl transfer reactions of L-glutaminase is also useful in the production of the high marketed value specialty chemicals like threonine [14]. To our knowledge, the reports on production of L- glutaminase from *P. expansum* are

scanty. In recent years, L-Glutaminase in combination with or as an alternative to L-asparginase could be used as in enzyme therapy for cancer particularly leukemia[14]. Several attempts were made to produce glutaminase through genetic engineering.

This review focuses on various conditions implemented for the production of L-Glutaminase, in sub-merged fermentation and solid state fermentation, physiochemical characteristics and applications of L-Glutaminase. Biochemical characteristics and purification aspects of the enzyme are dealt with briefly. The aim of the review is to give an overview on microbial production of L-Glutaminase, hitherto.

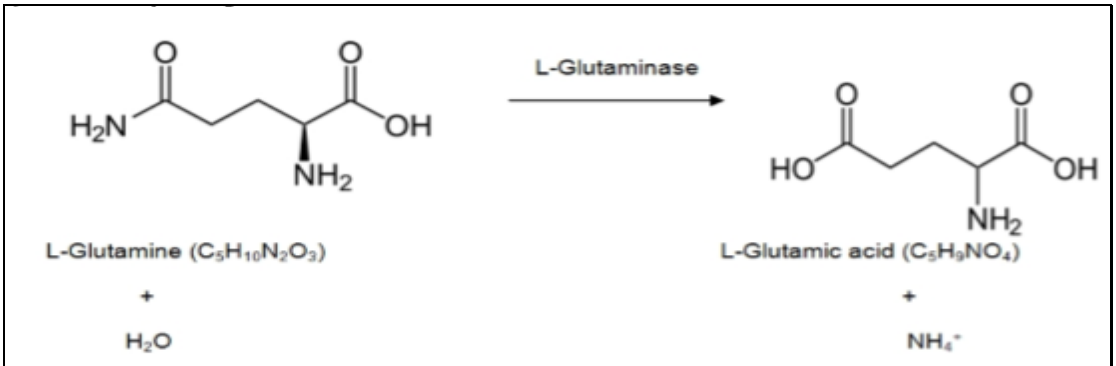

Fig. 1. Schematic representation of mechanism of action of L- glutaminase

OCCURRENCE AND DISTRIBUTION:

L-Glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes[18].It is found to be widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi [19,20,]. L-Glutaminase synthesis have been reported from many bacterial genera, particularly from terrestrial sources like *E. coli* [21], *Pseudomonas sp*[22], *Acinetobacter* [23] *and Bacillus sp* [24]. Although glutaminase have been detected in several bacterial strains, the best characterised were from members of Enterobacteriaceae family.

Among them *E. coli*, glutaminase have been studied in detail[21]. However other members such as *Proteus morganni, P. vulgaris, Xanthmonas juglandis, Erwnia carotovora, E. aroideae, Serratia marcescens, Enterobacter coacae, Klebsiella aerogenes and Aerobacter aerogenes* [25, 19, 26] were also reported to have glutaminase activity. Among other groups of bacteria, species *of Pseudomonas, especially, P. aeruginosa* [27, 28] *P. aureofaciens* [19,] *P. aurantiaca* [22, 29,] *and P. jluorescens* [20] are well recognised for the production of glutaminase. All these strains have been isolated from soil.

Among Yeasts, species of *Hansenula, Cryptococcus, Rhodotorula, Candida scottii* [19] *especially Cryptococcus albidus* [19, 20, 30] *Cryptococcus laurentii, Candida utilis and Torulopsis candida* were observed to produce significant levels of glutaminase under submerged fermentation. *Saccharomyces cerevisiae was also shown to produce glutaminase* [31]. Species of *Tilachlidium humicola, Verticillum malthoasei and fungi imperfecti* were recorded to possess glutaminase activity [19]. Glutaminase activity of soy sauce fermenting *Aspergillus sojae* and *A. oryzae* were also reported [32].

Marine Microorganisms as source of L-Glutaminase: Reports on the synthesis of extracellular L-Glutaminase by marine microorganisms are very limited to marine bacteria including *Pseudomonas jluorescens, Vibrio costicola and Vibrio cholerae* [33, 34] *and Micrococcus luteus* [35] and *marine fungi Beauveria bassiana* [36] only.

Biological role of L-Glutaminase in normal cells and tumor cells

Cancer cells, especially acute lymphoblastic leukemia (ALL) cells cannot synthesize L-Glutamine and hence demand for large amount of L-Glutamine for its growth. The use of amidases deprives the tumor cells from L- glutamine and causes selective death of L-Glutamine dependent tumor cells. L-Glutaminase can bring about

degradation of L-Glutamine and thus can act as possible candidate for enzyme therapy.

Cancer cells require a robust supply of reduced nitrogen to produce nucleotides, non-essential amino acids and a high cellular redox activity. Glutamine provides a major substrate for respiration as well as nitrogen for the production of proteins, hexosamines, and macromolecules. Therefore, glutamine is one of key molecules in cancer metabolism during cell proliferation.

The notion of targeting glutamine metabolism in cancer, originally rationalized by the number of pathways fed by this nutrient, has been reinforced by more recent studies demonstrating that its metabolism is regulated by oncogenes. Glutamine can exert its effects by modulating redox homeostasis, bioenergetics, nitrogen balance or other functions, including by being a precursor of glutathione, the major non-enzymatic cellular antioxidant. Glutaminase (GA) is the first enzyme that converts glutamine to glutamate, which is in turn converted to alpha-ketoglutarate for further metabolism in the tricarboxylic acid cycle. Different GA isoforms in mammals are encoded by two genes, Gls and Gls2.

As each enzymatic form of GA has distinct kinetic and molecular characteristics, it has been speculated that the differential regulation of GA isoforms may reflect distinct functions or requirements in different tissues or cell states. GA encoded by Gls gene (GLS) has been demonstrated to be regulated by oncogenes and to support tumor cell growth. GA encoded by Gls2 gene (GLS2) reduces cellular sensitivity to reactive oxygen species associated apoptosis possibly through glutathione-dependent antioxidant defense, and therefore to behave more like a tumor suppressor. Thus, modulation of GA function may be a new therapeutic target for cancer treatment [37].

MEDIA OPTIMIZATION FOR PRODUCTION OF GLUTAMINASES:

Many studies have been done to optimize cultural conditions for L- Glutaminase production both in batch and continuous fermentation. Production of this enzyme depends on various parameters like concentration of carbon and nitrogen sources, pH of culture medium, temperature, fermentation time and oxygen transfer rate. It has been observed that these parameters vary for different organisms. Properties of various microorganisms are mentioned in table no.1.Glutaminase is mostly obtained by submerged fermentation.

Effect of additional carbon and nitrogen sources on L-Glutaminase production:

Many L-Glutaminase producing microorganisms utilizes L-glutamine as both carbon and nitrogen sources and supplementation of any other carbon and nitrogen sources altered the L-Glutaminase production in most of the L-Glutaminase production fermentations. However, it was reported that addition of glucose enhanced the enzyme production in *C. nodaensis* [38], *Pseudomonas sp.* [39], *Streptomyces rimosus*[40], *Providencia sp.* [41], *T. koningii* [42] *and Beauveria sp.*[17]. Contradicting report on glucose – mediated suppression of the L-Glutaminase production in *Achromobacteraceae*[43], *S. maltophilia NYW-81* [45] was also reported. Sucrose (*Z. rouxii*) and sorbital (*B. bassiana BTMF S10*) also supported L-Glutaminase production in addition to glucose.

This could be confirmed based on the fact that addition of these carbon sources resulted in enhanced L-Glutaminase production; Iyer and Singhal, [36]. Different carbon sources namely glucose, maltose, sucrose, fructose, lactose and soluble starch at 1% (w/v) were added to the basal solid state fermentative medium of *A. oryzae* and they have exerted a considerable effect on the biosynthesis of L-Glutaminase. The maximum enzyme production was promoted by glucose followed by lactose and maltose.

The enhanced production of L-Glutaminase by the incorporation of glucose to the medium may be attributed to the positive influence of glucose as a co-metabolic agent [46] for enhanced enzyme biosynthesis. These results were similar to those reported by production by *Vibrio costicola* by Prabhu and Chandrasekaran[10]. And by *Beauveria sp* by Sabu e tal [17.] *Trichoderma koningii* by Ashraf et al [47].

Most of the L-Glutaminase producing microorganisms utilize the complex organic nitrogen sources rather than inorganic sources for effective enzyme production. It was noticed that addition of yeast extract enhanced the L-Glutaminase secretion by *C. nodaensis* [38], *B. bassiana BTMF S10* [36] and *Z. rouxii* [48,49].While Siva kumar et al [45] observed that S. rimosus prefers

the malt extract for higher L-Glutaminase production. Apart from complex nitrogen sources, inorganic nitrogen compounds such as ammonium sulphate and urea enhanced the enzyme production in *Achromobacteraceae and Providencia sp*. respectively [43]. However, Prabhu and Chandrasekaran [10] and Sabu et al. reported that none of the additional nitrogen source enhances the L-Glutaminase production in solid state fermentation [13,17]. Among the various nitrogen sources, sodium nitrate in the medium promoted enhanced growth of microorganism and consequently the L-Glutaminase production, followed by malt extract and yeast extract .

These results were in similar to those reported by Prashanth Kumar et al [50]. Incorporation of additional nitrogen sources enhance glutaminase yield. Among various nitrogen sources tested ammonium acetate was the best nitrogen source promoted maximum yield 853.84 U/ml for the *Mucor racemosus* strain. The least enzyme yielding nitrogen source was found to be sodium nitrite with an enzyme production of 538.46 U/ml [51].

The supplementation of additional nitrogen sources (either organic or inorganic) such as ammonium nitrate, ammonium sulphate, sodium nitrate, malt extract, yeast extract, urea and peptone had shown a profound impact on the production of L-Glutaminase by *A. oryzae* [52].Among the various nitrogen sources, sodium nitrate in the medium promoted enhanced growth of microorganism and 45.19U/gds of L-Glutaminase production was observed. These results were in similar to those reported by Prashanth Kumar et al., (2009) [50].

Effect of PH: The pH and temperature tolerance of glutaminase from various microorganisms differed greatly. While optimal activities of glutaminase A and B of *P. aeroginosa* were at alkaline pH of 7.5-9.0 and 8.5 respectively [53], glutaminase from Pseudomonas sp was reported to be active over a broad range of pH (5-9) with an optimum near pH 7.0 [54]. Glutaminase of *Pseudomonas acidovorans* showed optimum activity at pH 9.5 and retained 70% activity at pH 7.4 [55].

An intracellular glutaminase from *Cryptococcus albidus* preferred an optimal pH of 5.5- 8.5 [20].Whereas, glutaminase 1 and 11 isolated from marine *Micrococcus luteus* were active at alkaline pH values of 8.0 and 8.5 respectively [35]. Glutaminase from *A. oryzae* and *sojae* recorded pH optima of 9.0 and 8.0 respectively [56]. The intra and extracellular glutaminase from *A. oryzae* were most active and stable at pH 9.0[9].Glutaminase isolated from *Penicillium brevicompactum NRC 829* showed its maximal activity against L-glutamine when incubated at pH 8 [57.]

Effect of temperature: Microbial L-Glutaminase production is generally noticed at mild incubation temperature conditions ranging from 25 to 37^{o}C. The temperature stability of glutaminases also showed wide variation. Glutaminase from *Pseudomonas* showed maximum activity at 37°C and were unstable at high temperatures [58], whereas, the enzyme from *Clostridium welchii* retained activity up to 60°C [59]. Glutaminase from *Cryptococcus alhidus* retained 77% of its activity at 70°C even after 30 minutes of incubation [20].

Glutaminase I&II from Micrococcus luteus had a temperature optima of 50°C and the presence of NaCl (10%) increased the 16 thermo stability [35]. The optimum temperature for activity of both intra and extracellular glutaminases from *A. oryzae* was 45°C while they became inactive at 55°C [9].Glutaminase isolated from *Penicillium brevicompactum* NRC 829 showed its maximal activity at 50^{0}C, further increase in temperature at 70^{0}C retained its activity indicates its thermostable nature [57.] L-Glutaminase obtained from *Aspergillus oryzae* revealed optimum activity in a temperature range of 37 to 45ºC [61].

L-Glutamine was highly deamidated at 60ºC by glutamine amydohydrolase enzyme partially purified from *Penicillium politans NRC 510* [60]. Glutaminase isolated from *P. brevicompactum* NRC 829 indicated that no significant enzyme activity was lost when it was pre incubated at 50ºC to 60ºC for 60 min . L-Glutaminase retained about 92 % of initial activity after incubation (in the absence of substrate) at 70ºC for 30 min. Moreover, L-Glutaminase was still retaining about 66% of the original activity, after incubation at 80ºC for 5 min, which revealed the high thermal stability of L-Glutaminase.

These results indicate the thermophilic nature of the purified amidase enzyme produced by *P. brevicompactum NRC 829* [57]. L-Glutaminase purified from *Aspergillus oryzae* is stable up to 45ºC but lost its activity completely at 55ºC [61].

Prusiner et al.[21] performed the *E. coli* L-Glutaminase stability studies at low temperatures and the authors observed that the exposure of enzyme to cold temperatures resulted in a reversible inactivation of enzymatic activity, while subsequent warming to 24ºC restored the activity and no protein denaturation occurred during this process.

Effect of sodium chloride: Sodium chloride was found to influence the activity of glutaminase from both fungi and bacteria of terrestrial origin. Salt tolerant capacity of various microorganisms is given in table no. 2.Glutaminase from *E.coli, P. fluorescence, Cryptococcus albidus and A. sojae* showed only 65, 75, 65 and 6% respectively of their original activity in presence of 18% NaCl [20]. Similar results were obtained with glutaminase from *Candida utilis, Torulopsis candida and A. oryzae* [31]. Salt tolerant glutaminase have been observed in *Cryptococcus albidus and Bacillus subtilis* [62,63].

Glutaminase I and II with high salt tolerance was reported from *Micrococcus Iuteus* K-3 [35]. High salt-tolerance of L-Glutaminase produced by *Lactobacillus rhamnosus* was reported [64,65] where the presence of 5% (w/v) salt increased L-Glutaminase activity almost two-fold and 90% of the initial activity still remained at 15% (w/v) salt. On the other hand, L- glutaminases from other sources (*Aspergillus oryzae*) are markedly inhibited by high salt concentrations as demonstrated by Yano et al and Sabu A. etal 2000 [9,17].

Effect of various substances and heavy metals: Glutaminase activity was found to be inhibited by various substances and heavy metals. Cetavlon, while accelerating glutaminase of *Clostridium welchii, E.coli and Proteus moranii* in crude extracts and intact cells, inhibited the purified enzyme [66]. Glutaminase of E. coli was found to be sensitive to heavy metals [4] and *Acinetobacter* glutaminase-asparaginase was inactivated by glutamine analogue 6-diazo 5-oxo L-norleucine even at very low concentration while unaffected by EDTA, NH_3, L-glutamate or L-aspartate [43].

Various investigations have shown that glutaminase from *Pseudomonas* was activated by certain divalent anions and cations while inhibited by monovalent anions and by certain competitive inhibitors like NH_3, D and L-glutamic acid and 6-diazo 5-oxo L-norleucine [53]. In the case of fungi both intra and extracellular glutaminase from *Aspergillus oryzae* were inhibited by Hg, Cr and Fe but were not affected by sulphydroxyl reagents. EDTA, Na_2SO_4, and p-c Woromercuribenzoate strongly inhibited the Micrococcus luteus glutaminase I while glutaminase II was inhibited by EDTA, HgCh, Na_2SO_4, CuCh and FeCh[39].

In case of glutaminases isolated from *P. brevicompactum* Among, considerable loss of activity was observed only with Hg2+ and Cu2+ while Na+ or K+ acting somehow as an enhancer . EDTA has no effect on enzyme activity which indicates that L-Glutaminase might not be a metalloenzyme. L-Glutaminase is neither inhibited nor activated by reducing agents compounds including 2-mercaptoethanol (2-ME) and reduced glutathione (GSH) or thiol group blocking (namely iodoacetate) which indicates the absence of evidence for the involvement of SH group(s) in the catalytic site of this enzyme [57].

TABLE: 1 PROPERTIES OF L-GLUTAMINASES FROM VARIOUS MICROORGANISMS

S. NO.	Microorganism	Optimum pH	Optimum Temperature (°C)	Molecular weight Native	subunit	Substrates	References
1	*Escherichia coli*	5	NR	100	28	L-Glutamine,γ-L Glutamylmethylamide,γ -Glutamyl-hydrazide,γ-L-Glutamylhydroxamate,γ LGlutamyl-methoxyamide γ-LMethylLglutamate,γ-L-Ethyl-L-glutamate,γ-L-Thiomethyl-L-glutamateγ-L -Thioethyl-L glutamate	[4,67,68]

2	*Acinetobactr glutaminasificans*	7	NR	132	33	L-Glutamine D-Glutamine L-Asparagine D-Asparagine γ-L-Glutamyl-hydroxamate	[43,67]
3	*Bacillus subtilis*	6	50	55		L-Glutamine D-Glutamine	[63]
4	*Pseudomonas aeruginosa*	7.5-9	NR	137	35	L-Glutamine D-Glutamine L-Asparagine D-Asparagine	[53]
5	*Pseudomonas aurantiaca*	6.8-8	NR	148	47	L-Glutamine L-Asparagine	[19]
6	*Pseudomonas fluorescens*	7	37			glucose, sucrose, maltose, lactose and mannitol, L-tyrosine, L-lysine, L-asparagine and L- glutamic acid	[11]
7	*Escherichia coli*	7.1-7.9	NR	90	35	L-Glutamine γ-L-Glutamyl-hydroxamate	[21]
8	*Pseudomonas aeruginosa*	8.5	NR	67		L-Glutamine, D-Glutamine , L-Theanine , D- Theanine γ-L-Glutamyl-hydrazide	[28]
9	*Bacillus pasteurii*	9	37	100	55	L-Glutamine , D-Glutamine, L-Asparagine	[9]
10	*Rhizobium etli*	NR	NR	106.8	26.9	L-Glutamine	[16]
11	*Micrococcus luteus I*	8	50	86	43	L-Glutamine , γ-L-Methyl-L-glutamate , γ-L-Ethyl-L-glutamate , γ-L-Thio methyl-L-glutamate , γ-L -Thio ethyl-L-glutamate	[35]
12	*Micrococcus luteus II*	8.5	50	86		L-Glutamine	[35]
13	*Pseudomonas nitroreducens*	9	NR	40		L-Glutamine D-Glutamine DL-Theanine Glutathione	[69]
14	*Stenotrophomona s maltophilia NYW-81*	9	60	41		L-Glutamine D-Glutamine L-Asparagine D-Asparagine	[45]
15	Yeast 14 *Debaryomyces sp*	8.5	40	115	50,65	L-glutamine	[64]
16	*Fungi Aspergillus oryzae MA-27-IM*	9	45	113		L-Glutamine D-Glutamine	[70]
17	*Aspergillus oryzae AJ11728*	9	37-45	82		L-Glutamine, D-Glutamine	[71]
18	*Penicillium politans NRC 510*	8	60	133		L-asparagine , D-asparagine , L-glutamine , D-glutamine Nicotinamide , Nicotinamide adenine ,dinucleotide ,Acetamide	[72]
19	*Penicillium brevicompactum*	8.5	50	71		L-Asparagine, L-Glutamine , D-Asparagine , D-Glutamine , NAD Acetamide , Acrylamide	[57]

NR = Not reported

TABLE: 2 SALT TOLERANCE OF L-GLUTAMINASES PRODUCED BY VARIOUS MICROORGANISMS

S.NO.	Microorganism	Residual activity (%)	NaCl concentration (%)	References
1	*Bacillus subtilis*	NR	10	[73]
2	*Aspergillus oryzae*	20	18	[9]
3	*Escherichia coli*	65	18	[63]
4	*Pseudomonas fluorescens*	75	18	[63]
5	*Cryptococcus albidus*	65	18	[63]
6	*Aspergillus sojae*	6	18	[63]
7	*Micrococcus luteus I*	130	16	[35]
8	*Micrococcus luteus II*	100	16	[35]
9	*Lactobacillus rhamnosus*	90	15	[74]
10	*Stenotrophomonasmaltophilia NYW-81*	86	16	[45]
11	*Penicillium politans NRC 510*	75	20	[75]
12	*Penicillium brevicompactum*	95	30	[57]
13	*Vibrio SP. M9*	28.7 U/ml	3.5	[76]
14	*cryptococcus nodaensis*	90	16	[20]

NR = Not reported

PRODUCTION OF L-GLUTAMINASE BY SSF: In recent years, SSF has been emerged as a promising technology for the development of several bioprocesses and products including the production of industrial enzymes on a large scale. The primary advantage of SSF is the fact that many metabolites are produced at higher concentration. Marine microorganisms which are salt tolerant and have the potential to produce novel metabolites are highly suitable for use in SSF by virtue of their rare availability to absorb on to solid support particles.

Some reports suggests that polystyrene, commercially available insulating and packaging material, could be used as an inert solid support for the production of L-Glutaminase by a marine *Vibrio casticola* under SSF while ion exchange resins ,polyurethane foam and computer cards have been used as inert carriers for SSF with fungi. Prema Kashyap et.al reported [77] the production of extracellular L-Glutaminase by the halophilic yeast *Zygosaccharomyces rouxii* under solid- state fermentation, even though there was no report on the production of L-Glutaminase by any yeast under SSF using agricultural by products. In their study they have evaluated several agricultural waste materials as substrates.

They concluded the importance of utilization of natural substrates, viz. wheat bran and sesamum oil cake for the production of glutaminase enzyme. Their study showed that the yield should be increased for industrial use, and this proved the feasibility of SSF and agro-industrial residues for L-Glutaminase production.

Table 3 depicts the various organism and solid substrates used for the production of L-Glutaminase. Renu *et al* [34] compared the L-Glutaminase production in submerged fermentation with solid-state fermentation. The authors observed that solid-state fermentation was preferable to submerged fermentation for L--glutaminase production in terms of yield efficiency, since 25 to 30 fold increase in enzyme production was obtained under solid state fermentation. For the production of L-Glutaminase in solid state fermentation various agro industrial materials were used as solid support.

Many authors reported that wheat bran was found to be a preferable support for enzyme production [10,77].Apart from the wheat bran, rice bran, copra cake powder, ground nut cake powder and sesamum oil cake were used as solid substrates for enzyme production[10]. However Polystyrene beads, impregnated with mineral salts and glutamine were used as solid substrate for glutaminase production. Renu and Chandrasekaran [34] observed that *Pseudomonas fluorescens, Vibrio cholerae, and Vibrio costicola*, from among the strains screened from marine environments of Cochin, produced L-Glutaminase extracellularly in copious amounts. Process conditions for large-scale production of this enzyme were optimized in solid-state fermentation.

The authors studied the impact of operational parameters on L-Glutaminase production by *V. costicola*, and it was observed that maximum enzyme production was achieved in a wheat bran medium containing particles 1.4 to 2.0 mm in size and under optimal conditions which were: a moisture content of 40 to 60 %, pH 6.0, incubation

at 35°C, and with the addition of glutamine at 1.0 % (w w-1).

Later Prabhu and Chandrasekaran [10] observed that during solid state fermentation production of L-Glutaminase by *V. costicola* also simultaneously produced alpha-amylases and cellulases. The authors inferred that wheat bran, which was used as solid substrate, may have been used as substrate and brought about the induction of synthesis of alpha-amylase and cellulase. L-Glutaminase was found to be induced by L-glutamine. The authors later observed that *V. costicola* could grow on an inert carrier such as Polystyrene beads, impregnated with mineral salts and glutamine and produce enzyme under solid-state fermentation [10].

The ability to absorb onto polystyrene appears to be a basic property of marine bacteria. In their natural environment, many species of marine bacteria exist only under adsorbed conditions on detritus or solid substrates. In another investigation by Prabhu and Chandrasekaran [10], the best process parameters influencing L-Glutaminase production by marine *V. costicola* in solid-state process using polystyrene as an inert support were optimized. Maltose and potassium dihydrogen phosphate enhanced enzyme yield by 23% and 18%, respectively, while nitrogen sources had an inhibitory effect. As in the earlier study, leachate with high L-Glutaminase specific activity and low viscosity was recovered.

In the study by Sabu et al. the potential of *Beauveria sp*. for L-Glutaminase production using polystyrene as solid support under solid-state process was evaluated [17]. Future Kashayp *et al.*[77] was produced the L-Glutaminase by the saline tolerant yeast *Z. rouxii NRRL-Y 2547* using two agro-industrial substrates, wheat bran and sesamum oil cake.

The authors observed that NaCl medium for wheat bran and seawater for sesamum oil cake is suitable for moisturizing liquid. Higher L-Glutaminase titres (7.5 and 11.61 U gds-1 for wheat bran and sesamum oil cake, respectively) were produced when SSF was carried out with 64.2% initial moisture content of the substrate, 2 ml of inoculum, and 300C as incubation temperature. External supplementation of fermentation medium with various organic and inorganic nitrogen sources was of no benefit for enzyme production. In recent studies by Sayed found that wheat bran was the best solid substrate for induction of the L-Glutaminase by *Trichoderma koningii*. The maximum yield (45 U gds-1) of L-Glutaminase by *T. Koningii* occurred using wheat bran of 70% initial moisture content, initial pH 7.0, supplemented with D-glucose (1.0%) and L-glutamine (2.0% w/v), inoculated with 3 ml of culture and incubated at 30°C for 7 days.

TABLE 3: VARIOUS FERMENTATION PARAMETERS FOR THE PRODUCTION OF L-GLUTAMINASE IN SOLID STATE FERMENTATION

S.NO.	Organism	Solid Substrate	Medium pH	Incubation temperature (^{0}C)	Activity	Reference
1	*Vibrio costicola ACMR 267*	Polystyrene beads	7	35	232.00 U gds-1	[10]
2	*Beauveria sp. BTMFS 10*	Polystyrene beads	9	27	49,890.00 U L-1	[17]
3	*Zygosaccharomyces rouxii NRRL-Y 2547*	Wheat bran and sesame oil cake	NR	30	7.50 and 11.61 U gds-1	[77]
4	*Actinomucor elegans*	Soya bean curd	NR	25	176.00 U gds-1	[78]
5	*Rhizopus oligosporus*	Soya bean curd	NR	35	187.00 Ugds-1	[78]
6	*Trichoderma koningii*	Wheat Bran	7	30	45 Ugds-1	[42]
7	*Aspergillus oryzae NCIM*	agro residues including wheat bran, rice bran, etc	7	30	27.76 U/gds	[52]
8	*Aspergillus flavus*	agro- industrial by-products like wheat bran, rice	4	30	15.59 U/gds	[79]

		bran or wheat				
9	*Serratia marcescens*	Rice bran	7.17	37.040C,	193.10 IU/ml.	[80]
10	*Bacillus amyloliquifaciens*	agro- industrial by-products	5	60	196.2	[81]
11	*Mucor racemosus*		7	25	969.23 U/ml. T	[51]
12	*Pseudomonas stutzeri P*	agro-industrial waste like green gram husk, Bengal gram husk, cattle feed, wheat bran and groundnut cake	7	37	95.2IU	[83]

NR =Not reported.

Submerged fermentation: Table 4 shows the various bacteria, fungus and yeast used for the production of the L-Glutaminase. Roberts et al [43] isolated the *Achromobacteraceae* from the soil samples and observed that the enzyme from this organism has L-Glutaminase and L-asparaginase activity in a ratio of 1.2:1. The highest yields of enzyme are obtained when cells are grown aerobically in a basal synthetic medium composed of L-glutamic acid, ammonium sulfate, trace minerals, and phosphate buffer.

The authors observed that the temperature between 15 to 25^{o}C is favourable to the organism growth and enzyme production. Extracellular L-Glutaminase producing *Beauveria* sp. BTMF S10 was isolated from marine sediment. The authors observed that this enzyme was inducible and growth associated. The highest yield (46.9 U ml-1) is obtained in a medium supplemented with 1% yeast extract and sorbitol 9% sodium chloride and 0.2% methionine at medium initial pH 9.0 and 27°C. L-Glutaminase production by *Stenotrophomonas maltophilia* NYW-81 was optimized by Wakayama et al[45].

The highest yield was obtained at pH 7.0 and at 30^{o}C temperature. The authors observed that when glutamine is used a sole carbon and nitrogen source the production is high. When glucose is added to the medium it suppresses the L-Glutaminase production in *S. Maltophilia* [45]. Keerthi *et al* [82], observed that the L-Glutaminase produced from *actinomycetes* has a good salt tolerance. They isolated the 20 strains from estuarine fish and observed that *Streptomyces rimosus* was showed highest L-Glutaminase activity. Optimum production of L-Glutaminase was observed at incubation temperature at 27^{o}C, pH 9.0 and glucose and malt extract as carbon and nitrogen sources respectively.

In various investigations of Iyer and Singhal (2008; 2009)[48] observed that the carbon and nitrogen sources for L-Glutaminase production are varied with the organisms. Supplementation of sucrose and yeast extract as carbon and nitrogen source revealed improved L-Glutaminase production frin *Zygosaccharomyces rouxii.* While, higher L-Glutaminase production noticed from *Providencia s*p. with supplementation of glucose and urea as carbon and nitrogen sources. Jambulingam Kiruthika1 *et al* [84] studied L-Glutaminase production pattern under submerged fermentation using novel marine isolate *Vibrio azureus* strain JK-79 (GenBank Acession Number JQ820323) .

It was seen that maximum yield of enzyme production (247 U/ml) was achieved in a seawater based medium at pH 8, 37°C, 1% inoculum concentration and 2% glutamine concentration for 24 h. The medium when supplemented with carbon source, it improved the enzyme production from 247 to 321 U/ml with 1.5% maltose. Addition of 2% soybean meal also improved the L- glutaminase production (289 U/ml).

Jeong-Min Jeon etal[85] isolated glutaminase gene from *L. reuteri KCTC359*, cloned it by PCR, and subsequently introduced into two Korean isolates of *Lactobacillus* species. All the transformants harboring the glutaminase gene from L. reuteri KCTC3594 were able to elevate glutaminase activity.

TABLE 4: VARIOUS FERMENTATION PARAMETERS FOR THE PRODUCTION OF L-GLUTAMINASE IN SUBMERGED FERMENTATION

S No.	Organism	Carbon and nitrogen source	Medium pH	Incubation temperature (^{o}C)	Activity	Reference
1	*Achromobacteraceae*	L Glutamic acid 2.0% and ammonium sulphate50.4%	7.2	25	130.00 U L-1	[43]
2	*Aspergillus oryzae S 2*	Dextrose 0.1%,yeast extract 0.3%,Kcl 0.02%,Nacl 0.01%,Mgcl2 0.02%,starch 0.5%.w/v.	5	35	217.65 U L-1	[44]
3	*Cryptococcus nodaensis*	D-Glucose 3.0% and yeast Extract 0.5%	6.0	28	2060.00 U L-1	[38]
4	*Beauveria bassiana BTMF S10*	L-Glutamine 1.0 %, yeast extract 1.0% and sorbitol 1.0	9	27	46,900.00 U L-1	[36]
5	*Pseudomonas sp BTMS-51*	L-Glutamine 2.0 % and D-glucose- 1.0 %,	6	30	36,050.00 U L-1	[39]
6	*Stenotrophomonas Maltophilia NYW-81*	L-Glutamine 1.0 %	7	30	32,000.00 U L-1	[45]
7	*Streptomyces rimosus*	L-Glutamine 1.0%, Glucose 1.0% and Malt extract 1.0%	9	27	17,510.00 U L-1	[40]
8	*Zygosaccharomyces rouxii NRRL-Y 2547*	Sucrose 1.78%, yeast extract 4.8% and glutamine 0.5 %	7	37	458.68 U L-1	[48]
9	*Providencia sp*	Glucose 1.0 % and urea 0.5%,	8	25	119.23 U L-1	[41]
10	*Zygosaccharomyces rouxii NRRL-Y 2547*	Sucrose 1.78%, yeast extract 4.8% and glutamine 0.5 %	7	37	437.14 U L-1,	[49]
11	*Vibrio SP. M9*	0.5 KCl; 0.5 MgSO4; 1.0 KH2PO4; 0.1 FeSO4; 0.1 ZnSO4; 10 glutamine	7	35	28.7 U/ml	[76]
12	*Streptomyces avermetilis*	Glucose , sodium nitrate, 4% NaCl, $MgSO_4$	8	28	39.3 U/mg	[78]
13	*Penicillium politans NRC 510*	HgCl2, NaF, CaCl2, BaCl2 and CuSO4	8	60	133 U/mg.	[75]
14	*Vibrio azureus JK-79*	2% glutamine concentration, 1.5% maltose, 2% soybean meal	8	37	321 U/ml.	[84,87]
15	*Streptomyces sp-SBU1*	2% NaCl (w/v) and 1% malt extract (w/v) , 1% glucose (w/v) ,	9	30	18.0 U/ml	[88]
16	*Streptomyces labedae*	1.0 KH2PO4; 0.5 MgSO4; 0.1 CaCl2; 0.1 NaNO3; 0.1Na3C6H5O7; 25 NaCl; 10 glucose	7-8	30	12.23U /ml	[89]

Desirable properties of glutaminase enzymes for therapeutic use:

Glutaminase enzymes according to the present invention are therapeutically suitable if they display high enzyme activity at physiologic pH, i.e., between about pH 6.5 and 8.5. Therapeutically suitable glutaminase enzymes must have a low K_M, i.e., between 10^{-6} and 10^{-4} M. Additionally desirable properties of glutaminase enzymes for therapeutic use include:

- High stability at physiologic p H.
- Retains high activity and stability in animal and human sera and blood.
- Cleared slowly from the circulation when injected into animals or humans. A plasma half-life ($t_{½}$) for glutaminase greater than six hours in mice and sixteen hours in humans is desirable.
- Not strongly inhibited by the products of the reaction it catalyzes or by other constituents normally found in body fluids.
- Does not require cofactors or prosthetic groups that can easily dissociate from the enzyme.
- Narrow substrate specificity.
- Effective irreversibility of the enzymatic reaction under physiologic conditions.
- Available from an organism that contains low levels of endotoxin.
- Low immunogenicity.

A number of amino acid-degrading enzymes that do not exhibit antitumor activity also fail to meet at least one of these criteria. For instance, *E. coli* glutaminase has a pH optimum of 5 and essentially no activity at physiologic pH. An ineffective form of E. coli asparaginase has a K_M over 1 mM. Asparaginase enzymes from yeast, Bacillus coagulans, and *Fusanium tricinctum* all have excessively rapid clearance rates in mice. The known mammalian glutaminase enzymes are not suitable for use as therapeutic agents because of their high K_M values (millimolar range), and their requirement for phosphate esters or malate for activation.

The *E. coli* glutaminases (A and B) are also unsuited for therapeutic use because of their high K_M values (millimolar range), low activity at physiological pH (glutaminase A), or requirement for special activating substances (glutaminase B).

One of the most promising therapeutic applications ever proposed for L-Glutaminase is in the treatment of HIV[39]. Roberts et al have patented a therapy for HIV, where L-Glutaminase from Pseudomonas sp. 7A is administered so as to inhibit HIV replication in infected cells. The enzyme brings about inhibition of tumour (melanoma) and DNA biosynthesis in affected cells [12].

Applications of L-Glutaminase: Microbial L-Glutaminases have found several potent applications in various industrial sectors in the recent years. The enzyme, though originally identified as a potent anti-cancer drug with possible applications in enzyme therapy, has been used in food industry for flavour enhancement. Recent applications of the enzyme include its use in biosensors and in the manufacture of specialty chemicals by enzymatic transformations. The important applications are discussed below under the categories- therapeutic applications, applications in food industry, analytical applications, and manufacture of fine chemicals.

GLUTAMINASE AND ITS DEVELOPMENT AS A CHEMOTHERAPEUTIC AGENT:

High rate of glutamine consumption is a characteristic nature of some types of cancerous cells [90]. Based on this character experimental therapies have been developed to deprive L-glutamine to tumour cells; Rosenfold and Roberts Keerthi [91, 48]. Tumour growth regulation can be achieved by inhibition of both protein and nucleic acid biosynthesis in the cancerous cells due to the lack of availability of any component of these macromolecules. Inhibition of the tumour cell uptake of glutamine is one of the possible ways to stop the growth and this is the best accomplished by the use of L-Glutaminase, which breaks down L-glutamine. This in fact, results in a selective starvation of the tumour cells because unlike normal cells they lack properly functioning glutamine biosynthetic machinery [92].

Several studies were made towards using L-Glutaminase in cancer therapy [2, 93, 94, 95, 96, 97]. Warrell et al.[98] used the L-Glutaminase against adult leukemia. One of the major problems encountered in the treatment with microbial L-Glutaminases is the development of immune responses against the enzyme. Also the enzyme introduced intravenously has to act at the tumour site within the short span of time it remains in circulation before being cleared at the kidneys.

In order to avoid the above problems associated with L-Glutaminase mediated treatment of certain types of cancers, several investigations were made. The problems faced in clinical application of A. glutaminase as a drug in the treatment of leukaemia was described by Spires et al [96].

Holchenberg described the human pharmacology and toxicology of A. glutaminase. Jaafar Belgoudi, attempted immobilization of the enzyme in polyethylene glycol and found that the enzyme showed optimal activity over a larger pH range which was due to the matrix effect [99].Giordano et al [100] proposed an extracorporeal administration of the enzyme in acute lymphoblastic leukemia. However, it may be noted that alternative methods for tumour cells are coming up which include the use of phenyl acetate to deplete glutamine [101,102] and the use of gene therapy.

Phenyl acetate was proposed as a drug in the treatment of human cancer even before 30 years [103]. The latter approach was recently tried by the use of an antisense mRNA for phosphate activated L-Glutaminases in Ehrlich ascites tumour (one type of breast carcinoma) [104]. Such improvements in cancer therapy may soon obviate the role of microbial L-Glutaminases as an anti-tumour drug.
Ali Mohamed Elshafeil et al [57] isolated glutaminase from *Penicillium brevicompactum NRC 829.*Using MTT assay, the in vitro bioassay cytotoxic effect of *Penicillium brevicompactum* NRC 829 L-Glutaminase on the growth of four human tumor cell lines namely Hep-G2 [Human hepatocellular carcinoma cell line], MCF-7 [Breast cancer cell line], HCT-116 [Colon cell line] and A549 [Human lung Carcinoma] showed that the crude-enzyme extracts have anti- proliferative activity in different cell lines growth. However, the highest antitumor activity was recorded towards Hep-G2 (65.3%), while the least activity was obtained towards A-549 (33%) when compared with the growth of untreated control cells.

One of the most promising therapeutic applications ever proposed for L-Glutaminase is in the treatment of HIV[39]. Roberts et al have patented a therapy for HIV, where L-Glutaminase from *Pseudomonas sp. 7A* is administered so as to inhibit HIV replication in infected cells. The enzyme brings about inhibition of tumour (melanoma) and DNA biosynthesis in affected cells.

Application of L-Glutaminase in food industry: L-Glutaminase is the most important amino acid in food manufacture for a delicious taste [105,106]. The pleasant and palatable tastes of oriental fermented foods like soy sauce, miso and sufu are considered to be related to the content of L-glutamic acid in them [107,108,109] accumulated due to the hydrolysis of a protein component catalysed by proteolytic enzymes, including L-Glutaminase, protease and peptidases [110]. Hydrolysis of glutamine by L-Glutaminase may also contribute significantly to the high content of L- glutamate in these products [111,112, 113, 114]. Several attempts were made to improve the quality of soy sauce and miso utilizing the action of microbial L-Glutaminases [108,111,114].

Koji mould with highly active L-Glutaminase was used for increasing the L-glutamate content of soy sauce by Yamamoto and Hirooka [112], whereas L-Glutaminase of *C. albidus* was used for the same purpose by Yokotusa et al [20] and Iwasa et al[62]. In another method, the Japan Tokko Koho Company used peptidoglutaminase of B. circulans to improve the flavour of soy sauce [115].Whereas Chou and Hwan [114] used the L-Glutaminase from *Actinomucor taiwanensis* to increase the L-glutamate content of Sufu. Impressive studies were undertaken at the Kikkoman Corporation, Japan for production of glutaminase and its applications. Ushijima and Nakadai [116] used mutation and protoplast fusion techniques for improving L-Glutaminase production by *A. sojae* employed in shoyu fermentation.

Fukushima and Motai [106] achieved continuous production of L-Glutaminase in liquid seasoning using an immobilized *C. albidus*. Sato et al[38] described a fermentation procedure for L-Glutaminase production by *C. nodaensis* in 30L fermenter and an effective method for purification of the enzyme. These processes for L-Glutaminase production and purification have been patented by the company, Sato et al. Several other reports are available on the use of microbial L-Glutaminases in food flavouring.

Processes for continuous conversion of glutamine to glutamate in food preparations, employing either immobilized L-Glutaminase or whole cells of L-Glutaminase producing microbes are also reported by industries and research institutions [117]. Salt tolerant L-Glutaminases are most valuable in the

industrial processes that require high salt environments like the soy sauce fermentation. L-Glutaminases from conventional sources *(A. oryzae*) are markedly inhibited by high salt concentrations as demonstrated by Yano et al. Salt tolerant L-Glutaminases were patented for use in industrial processes [17].

Moriguchi et al[35] have proposed the use of salt tolerant L-Glutaminase from bacteria as a possible alternative, since their enzymes could be halophilic rather than halotolerant allowing for the use of high salt concentrations.

Analytical applications: L-Glutaminase for biosensor application to determine the L-glutamine levels was investigated by Kikkoman Corporation, Japan, Sabu et al, Botre et al. Huang et al.; Mulchandani and Bassi, [17,117,118,119,120] and hybridoma culture media [121] by flow injection analysis.

Analysis of L-glutamine and glutamate levels of the body fluids is important in clinical diagnostics and health monitoring. Enzymatic determination of glutamine and glutamate is more accurate and reliable compared to the older techniques like Nesslerization followed by determination of produced ammonia. Lund [14] described an efficient spectrophotometric method for determination of L-glutamine and L-glutamate using L-Glutaminase and L-glutamate dehydrogenase.

L-Glutaminases are used currently both in free enzyme or immobilized on membranes forms as biosensors for monitoring glutamine and glutamate levels of fluids. The application of L-Glutaminase in clinical analysis hassled to a tremendous boost in the search for L-Glutaminases which are stable over longer periods for use in biosensors, and companies have started to manufacturing highly purified L-Glutaminase enzyme specifically for this purpose. However, the clinically used L-Glutaminases largely come from mammalian sources with a few exceptions.

One of the important producers of analytical grade microbial L-Glutaminase is the Kikkoman Corporation, Japan. This company produces L-Glutaminase using Bacillus sp. and uses in clinical analysis for determination of glutamine in conjunction with L-glutamate oxidase and peroxidase [17].

An amperometric enzymes electrode probe with membrane immobilized L-Glutaminase was described by Villarta et al [117]for determination of serum glutamine levels of humans. Botre et al [118] used L-Glutaminase based biosensor for determination of glutamine and glutamate in pharmaceutical formulations.

The enzyme has also been widely employed in the monitoring of glutamine and glutamate levels in mammalian cell culture media [119,120] and hybridoma culture media [121] by flow injection analysis using biosensors. Free enzyme was used in the determination of glutamine in insect cell culture media by Wang et al[122]. Important applications are also proposed for L-Glutaminase based biosensors in the online monitoring of fermentation [123,124].

Manufacture of fine-chemicals: Theanine (γ-l-glutamyl ethylamide) is one of the major components of amino acids in Japanese green tea and unique as a taste-enhancing amino acid of infused green tea. Recently, increasing attention has been drawn towards the physiological roles of theanine, especially in a clinical point of view because of their ability to suppress stimulation by caffeine, to improve effects of antitumor agents, and their role as antihypertensive agents. In general theanine is synthesized by theanine synthetase (EC 6.3.1.6) in plants.

Tachiki et al. [125] have developed a method of producing theanine from glutamate and ethylamine using a combination reaction of bacterial glutaminases with baker's yeast. Researchers at the Taiyo Kagaku Co., Ltd., Japan, devised a method for continuous production of threonine employing immobilized *Pseudomonas nitroreducens* as source of L-Glutaminase [126,127]. The process reported a threonine yield of 95% on the basis of glutamine consumed. Another US patent entitled "a method for production of threonine using the L-Glutaminase from *P. nitroreducenes*" was granted to Sabu, [17].

Another most important emerging application of L-Glutaminase in industry comes from its use in the manufacture of γ -glutamyl alkamides. L-γ-glutamyl alkamides are prepared by γ-glutamyl transfer from a donor such as glutamine or glutathione to a glutamyl acceptor like ethylamine, methyl amine or glycyl glycine. L-Glutaminases

are found to catalyze these reactions. Several processes based on the microbial L-Glutaminase catalyzed synthesis of γ-glutamyl alkamides have come up in the recent years. Patents have been filled for the threonine production process employing L-Glutaminase from *P. nitreducens, P. adapta and P. denitrificans and Bacillus sp.* [17]. Tachiki et al.[125] used the L-Glutaminase from P. nitroreducens for the production of γ-glutamyl-methylamide in addition to threonine by using methylamine as the of γ-glutamyl acceptor.

Commercial production of microbial L-Glutaminase: The researchers at Kikkoman Corporation, Japan performed some of the remarkable works on production of the enzyme. The research group at the company used mutation and protoplast fusion techniques for improving L-Glutaminase production by the koji mould, *A. sojae*, [116] .A submerged fermentation process for production of thermostable L-Glutaminase by *C. nodaensis*, and its purification was developed by the same company [38] and the process was patented.

The company developed the production of enzyme from the yeasts *Cryptococcus sp, Candida sp, Saccaromyces sp. and Sporromyces* sp. The process claims to yield high titers of L-Glutaminase, which may be exploited commercially. Nevertheless currently the major industrial source of L-Glutaminase remains to be species of Bacilli. The **Table 5** summarizes the L-Glutaminases available in the market.

TABLE 5: COMMERCIAL AVAILABLE MICROBIAL L-GLUTAMINASES

S.NO	Manufacturer	Brand name	Source
1	Amano enzymes inc, Japan	Glutaminase F "Amano 100"	*Bacillus subtilis*
2	Biocatalysts, UK	Flavorpro B73P	*Bacillus sp*
3	Kikkoman Corporation, Japan	GLN	*Bacillus sp*
4	Ajinoto Co Inc, Japan	Glutaminase	*Bacillus sp*
5	Enzyme Development Corporation, Japan	Enzeco	*Bacillus subtilus*

CONCLUSION: Despite the promise of glutaminase as a therapeutic agent, there are currently no therapeutically useful glutaminases available which can be produced cheaply and with little or no contamination by other substances, for example by endotoxins of a host microorganism. Moreover, a suitable enzyme is not available in quantities which are large enough to allow for wide-spread clinical trials.

For a glutaminase to be ideally suited for use in antineoplastic therapy, it should satisfy a variety of criteria. The selected organism should produce the glutaminase in high yield, and it should be capable of being grown in large quantities on a simple and inexpensive medium. The procedures developed for purification of the enzyme should be as rapid and simplified as possible, providing pure enzyme in high yield. The purified enzyme should have long term stability on storage, maximal activity at a physiological pH, and a Km for substrate below the concentration of the substrate in the blood. .

Furthermore, a detailed understanding of the regulation of gene expression based on molecular approaches and other means would contribute immensely towards developing successful strategies for strain improvement which is a prerequisite for any industrially important enzyme. Although some patents had been developed. Further studies and regulatory approvals will enable the introduction of new glutaminase drugs with potential benefits to patients.

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How to cite this article:
Unissa R, Sudhakar M, Reddy ASK and Sravanthi KN: A Review on Biochemical and Therapeutic Aspects of Glutaminase. Int J Pharm Sci Res 2014; 5(11): 4617-34.doi: 10.13040/IJPSR.0975-8232.5 (11).4617-34.