ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2012; 3(6):1368-1376

ORIGINAL RESEARCH ARTICLE

Fermentative Production of Glutamate by Newly Isolated Soil Bacteria

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Received 14 Jun 2012; Revised 16 Sep 2012; Accepted 28 Sep 2012

ABSTRACT

The demand for L-glutamate production is widely growing due to its greater utilization as flavor enhancer, Human dietary food supplement and in pharmaceuticals. To search out glutamate producing bacteria a total of 238 bacterial strains were isolated from ecologically different soil sources of Province Khyber Pukhtunkhwa. The isolated strains were then screened qualitatively for glutamate production in glucose mineral salt medium. Out of total 238 strains, 59 strains were found as glutamate producers. Among the 59 producer strains, twelve were found as glutamate good producers. All the glutamate good producing bacteria were then further quantitatively examined in production medium using shake flask fermentation.

The percentage of glutamate producing strains were found as 25% in River Indus, 24% in Abbotabad, 23% in Bannu, 22% in Gomal University and 21% in Paharpur soil samples. The percentage of glutamate good producing strains was found as 25%, 23%, 22.2%, 21%, and 13.3% in Bannu, River Indus, Paharpur, Abbotabad and Gomal University soil samples respectively.

Examination of the time courses of all the glutamate good producing strains in the production medium indicated that glutamate yield started after 24hrs of incubation and then continuously increased and found maximum on 4th day and then it decreased gradually.

Strain HBGU-5 produced 15gm/L glutamate while strain HBGU-8 produced 18gm/L, strain HBGU-b2 produced 20gm/L, strain HBGU-P1 and HBGU-P2 produced 22gm/L, strain HBGU-A9 and HBGU-b3 produced 24gm/L, strain HBGU-A7 produced 25gm/L, strain HBGU-A6, HBGU-R2 and HBGU-R9 produced 26gm/l and strain HBGU-R4 produced 30gm/L glutamate on 4th day of incubation. Among these strains, strain HBGU-R4 of River Indus gave highest yield while minimum yield was produced by strain HBGU-5 of Gomal University.

Key words: Soil, Microbes, Glutamate, Production.

INTRODUCTION

L- glutamic acid, an important amino acid having great role in medical sciences, widely used in food industry, pharmaceuticals, animal feedstuffs, cosmetics and other products.

Physiological or anionic form of L-glutamic acid is termed as glutamate and is considered as an exciting molecule of interest by clinicians (Kulkarni et al., 2005). Glutamate acts as an excitatory neurotransmitter in the brain and spinal cord and acts as a precursor for the synthesis of various amino acids and various useful pharmaceutical and healthy products such as proline, ornithine, arginine and glutathione (Meister, 1988) and can be converted into amino acids, that is either glutamine or gamma amino butyric acid (GABA).

Glutamate can be used as fuel by the brain and is used as flavor enhancer in food industry (Garattini, 2000), monosodium glutamate, its salt is present in soy sauces, soups, biscuits, cheese, noodles, vegetable and meat processing (Nampoothiri and Pandey, 1998) and added to variety of foods including Chinese and Japanese foods, also used as food additive, due to its peculiar taste is given then name umami (meaty, delecious taste) (Yamaguchi and Kimizuka, 1979) and is among the five basic tastes of human sense (Kurihara and Kashiwayanagi, 2000).

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Fermentative production of L-amino acids have got great economic and commercial value due to their extensive applications it became a well established branch of industrial (Ajinomoto, 1972) microbiology.

Microbes have been extensively employed and are of great importance due to their beneficial use, and valuable products production during primary and secondary metabolism (Demain, 1990).

Earlier chemical ways or various extraction methods were used for amino acid production. Fermentative production of amino acid started with the isolation of a bacterium, *Corynebacterium glutamicum*, which is Gram positive, rod shaped aerobic and non sporulating bacterium. Firstly, in 1956 it was isolated from soil for production of l- glutamic acid and other amino acids (Herman, 2003) through fermentation.

In Japan, centuries ago sea weeds were extracted and applied as a source of taste improver. Major component found in extracted sea weed was termed kelp later renamed monosodium glutamate (MSG), in 1908 by a chemist Dr. Kikune Ikeda. Afterwards, MSG commercial production started and now annnual commercial production of MSG increased about 1.5 million ton (Kataoka *et al*., 2006).

Microbes can produce L- glutamic acid either by Glutamate dehydrogenase activity as:

α-ketoglutaric acid +NH_4^+ + NADPH → glutamic acid + NADP + H2O

or by glutamate synthase . *Corynebacterium glutamicum, Corynebacterium colluni, Brevibacterium lactofermentum,* and *Brevibacterium flavum,* all industrially important bacteria have these enzymes for glutamate production (Ertan, 1992; Sung *et al*., 1984; Tochikura and T, 1987), these bacterium are also employed for L-Lysine, L-Threonine and L-Ornithine production (Shiio and Nakamori, 1970).

For optically active and biologically required L-Gutamic acid production, this study focuses over microbial utilization, as chemical methods produce recemic mixture of DL-Glutamic acid. L-glutamate is the largest fermentative product as compared to other amino acids production, occupies about 53% of the world market (Eggeling and Sahm, 1999).

Several fermentative techniques have been employed for L- glutamate production (Amin *et al*., 1993; Yoshida et al., 2004). By aerobic fermentation processes *corynebacterium* and *Brevibacterium* produces L-glutamate, requiring oxygen as terminal electron acceptor molecule (Hirose et al., 1985; Takac *et al*., 1998).

During metabolic pathway, these bacteria utilize glucose act as carbon and energy source, and is converted into pyruvic acid through glycolytic pathway, enters into the tri carboxylic acid cycle (TCA cycle) during aerobic glycolysis. An intermediate of the TCA cycle, α-ketoglutaric acid is responsible for L-Glutamate production. Glycolysis can continue during aerobic as well as anaerobic conditions unlike TCA cycle as end product of anaerobic glycolysis is lactic acid or ethanol. So for enhanced glutamic acid production an oxygen transfer strategy, with high selectivity must be applied because production of other metabolic by products depends on supply of oxygen to the cell.

In fermentative production of glutamic acid, there are some triggers such as biotin, required for cell growth, its limited quantity induces L-glutamic acid production (Shiio *et al*., 1962), such as penicillin addition (lactam antibiotics) to growing microbial cells (Nunheimer *et al*., 1970), temperature shift up (Delaunay *et al*., 1999), and (Marquet *et al*., 1986) addition of surfactants.

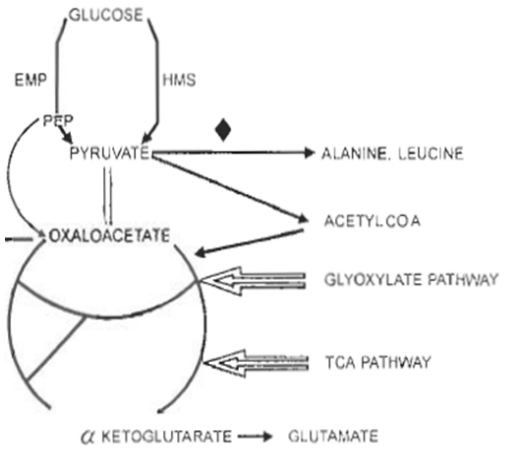
Sano, (2009) reported that today total world production of MSG is approximately 2 million ton (2 billion kg per year) and its demand is increasing at a rate of 10 – 15% per year (Izumi *et al*., 1978).

Besides the existing methods of industrial fermentations, many efforts are being pursued to improve the glutamic acid production especially under the stand point of cheaper available substrates to help go down the production costs. So better and cheaper methods of L-glutamic acid production has always been an issue (Lakshmi and Sarva 2011).

The current study focuses on glutamic acid production by newly isolated soil bacteria from different areas of Khyber Pukhtunkhwa, Province and quantitatively examined its production after optimization of culture conditions.

Biosynthetic pathway for Glutamate production

Fig 1: Biosynthetic pathway for glutamate production by *Corynebacterium glutamicum* (Shah, 1998)



MATERIALS AND METHODS

All the glassware used was sterilized by using autoclave and was of Pyrex Gena Company while the chemicals were of sigma chemical company. All the chemicals used were of analytical grade.

Media compositions:

Nutrient agar medium

Nutrient agar medium composition per 100 ml dH_2O used was as followed.

Peptone 0.5gm, Beef extract 0.3gm, Agar 2.0gm,

Screening medium

Screening medium compositions for L-glutamate production per 100 ml dH_2O was as under.

Glucose 5gm, Calcium carbonate 1gm, Ammonium sulfate 1gm, Potassium di hydrogen phosphate 0.3gm, Di potassium hydrogen phosphate 0.7gm, Magnesium sulfate seven hydrate 0.01gm, Ferrous sulfate seven hydrate 0.2mg, Magnesium chloride tetra hydrate 0.2mg, Thymine hydro chloride 20µg, d-Biotin 10µg,

Basal Seed medium

Glucose 8gm, Calcium carbonate 1gm, Ammonium sulfate 2gm, Potassium di hydrogen phosphate 0.2gm, Magnesium sulfate seven hydrate 0.02mg, Ferrous sulfate seven hydrate 0.3mg, Manganese chloride tetra hydrate 0.3mg, Thymine hydro chloride 10µg,

d- Biotin 10µg,

Production medium

Glucose 10gm, Calcium carbonate 2gm, Ammonium sulfate 2.5gm, Potassium dihydrogen phosphate 0.1gm, Magnesium sulfate seven hydrate 0.05gm, Ferrous sulfate seven hydrate 0.2mg, Magnesium chloride tetra hydrate 0.2mg, Thymine hydrochloride 20µg, d- Biotin 5µg,

All the mediums were autoclaved at 121 ºC, 15p.s.i, for 15 minutes and pH of all the media was set at 7.2. After cooling sterilized glucose and $CaCO_3$ was mixed in salts.

Isolation of Bacterial strain:

Soil samples from different ecological areas Paharpur, Bannu, Abbottabad, River Indus and Gomal University Dera Ismail Khan of province Khyber Pukhtunkhwa were collected. Several soil samples (5gm) were diluted separately into sterile 20 ml normal saline. Nine ml of Normal saline was taken in each test tube and then 1 ml of the soil liquid suspension was serially transferred to each 9ml normal saline containing test tubes. After that 0.1 ml from each test tube was poured into separate sterile nutrient agar medium containing Petri plates and then evenly spreaded over the surface of the plate by means of sterile bent glass spreader. After soil sample application, plates were placed in incubator at 29ºC for 24 hours. Then separate colonies were observed next day and then examined for L-glutamate production (Daoust, 1976).

Culture procedure:

Screening method

Screening media (15 ml) was taken in each 50 ml sterilized flask and inoculated from 24 hours old bacterial streak plate. After inoculation it was incubated in shaking incubator at 30ºC for 72h at 150 rpm. Samples were taken after 72h and centrifuged at 5000rpm for 10 min. Supernatant were then examined for qualitative production of L- glutamate.

Qualitative estimation

Qualitative analysis for L-Glutamate was done by using paper chromatographic technique (Chattopadhyay and Banerjee, 1978).

For paper chromatography Whatman No 1 filter paper having dimensions 19x10 was used, spotted with standard glutamate solution and solution from the supernatant of the sample, 3cm above from the bottom by means of micro capillary tube. At room temperature spots were air dried. Paper was dipped up to 1.5cm in solvent system having 40 ml Butanol, 10 ml acetic acid and 50 ml dH_2O. By using ascending method chromatograms were developed, and solvent was allowed to run for three hours, up to 18cm from bottom. Then after air drying paper was sprayed uniformly by ninhydrin (0.5%) in (95%) acetone. Then paper was placed in oven at 65ºC for 30 min after air-drying. Colored zones of amino acids were produced which indicated the position of amino acids. Through these well defined colored zones

glutamic acid present in the unknown sample was compared with standard glutamic acid colored zone and was identified.

Quantitative estimation of glutamate:

For quantitative estimation of amino acid, 2 ml of amino acid solution was taken in test tube and then 2 ml of buffer ninhydrin reagent was added into it and then it was heated for 15 min in boiling water bath. It was cooled to room temperature. Then 3 ml of 50% ethanol was added. (Plummer, 1972).

Buffer ninhydrin reagent was prepared by taking 0.8gm ninhydrin reagent, 0.12 gm hydrindantin dissolved it in 30 ml of methyl cellosolve (2-methoxy ethylene) and then added 10 ml of 4M acetate buffer of pH 5.5. Then glutamate was quantitatively estimated by taking reading at 570 nm using spectrophotometer.

RESULTS

Soil samples of different ecological areas were taken. Colonies from different soil samples were isolated and screened for glutamate production by using screening media. Each individual colony was qualitatively examined for glutamate production. After qualitative analysis some strains were found as glutamate non producers, some were glutamate producers and some glutamate good producers. Then glutamate producing strains were quantitatively examined by using production medium.

Qualitative examination

Gomal university soil sample:

Net (63) colonies were observed and 15 were glutamate producers. Out of fifteen glutamate producers two were good producers of glutamate. Total number of non producer colonies was forty eight.

Abbotabad soil sample:

Total (58) colonies were observed on the plate and screened out for glutamate production. Three were found glutamate good producers while eleven were found producers and the net outcome was 24%. Total non producers in the sample were forty four.

River Indus soil sample:

Total (52) colonies were observed, 13 were found glutamate producers out of them three were glutamate good producers. Total thirty nine colonies were non producers.

Paharpur soil sample:

Total 41 colonies were observed through spread plate. And checked for glutamate production out of forty-one colonies, nine were found glutamate producers and 32 were found non producers while two were glutamate good producers.

Bannu soil sample:

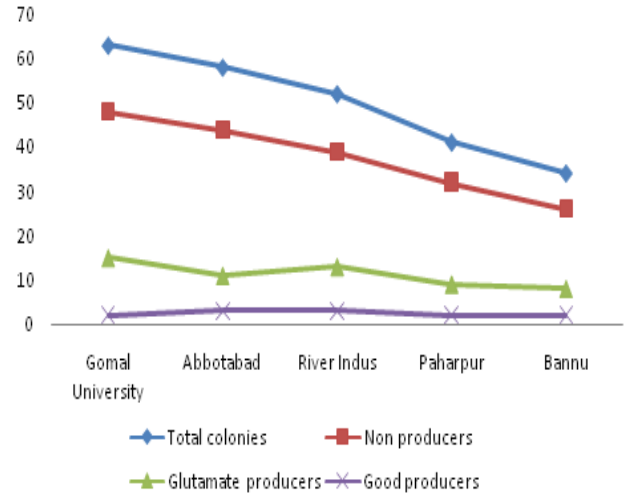
Total 34 colonies were found; out of them eight were glutamate producers. Non producers were twenty six while good producers were two (**Fig 2**).

Table 1: Bacterial strains isolation from various soil samples

Serial number	Gomal Universty sample strains	Abbotabad sample strains	River Indus sample strains	Paharpur sample strains	Bannu sample strains
1	HBGU-1	HBGU-A1	HBGU-R1	HBGU-P1	HBGU-b1
2	HBGU-2	HBGU-A2	HBGU-R2	HBGU-P2	HBGU-b2
3	HBGU-3	HBGU-A3	HBGU-R3	HBGU-P3	HBGU-b3
4	HBGU-4	HBGU-A4	HBGU-R4	HBGU-P4	HBGU-b4
5	HBGU-5	HBGU-A5	HBGU-R5	HBGU-P5	HBGU-b5
6	HBGU-6	HBGU-A6	HBGU-R6	HBGU-P6	HBGU-b6
7	HBGU-7	HBGU-A7	HBGU-R7	HBGU-P7	HBGU-b7
8	HBGU-8	HBGU-A8	HBGU-R8	HBGU-P8	HBGU-b8
9	HBGU-9	HBGU-A9	HBGU-R9	HBGU-P9	HBGU-b9
10	HBGU-10	HBGU-A10	HBGU-R10	HBGU-P10	HBGU-b10
11	HBGU-11	HBGU-A11	HBGU-R11	HBGU-P11	HBGU-b11
12	HBGU-12	HBGU-A12	HBGU-R12	HBGU-P12	HBGU-b12
13	HBGU-13	HBGU-A13	HBGU-R13	HBGU-P13	HBGU-b13
14	HBGU-14	HBGU-A14	HBGU-R14	HBGU-P14	HBGU-b14
15	HBGU-15	HBGU-A15	HBGU-R15	HBGU-P15	HBGU-b15
16	HBGU-16	HBGU-A16	HBGU-R16	HBGU-P16	HBGU-b16
17	HBGU-17	HBGU-A17	HBGU-R17	HBGU-P17	HBGU-b17
18	HBGU-18	HBGU-A18	HBGU-R18	HBGU-P18	HBGU-b18
19	HBGU-19	HBGU-A19	HBGU-R19	HBGU-P19	HBGU-b19
20	HBGU-20	HBGU-A20	HBGU-R20	HBGU-P20	HBGU-b20
21	HBGU-21	HBGU-A21	HBGU-R21	HBGU-P21	HBGU-b21
22	HBGU-22	HBGU-A22	HBGU-R22	HBGU-P22	HBGU-b22
23	HBGU-23	HBGU-A23	HBGU-R23	HBGU-P23	HBGU-b23
24	HBGU-24	HBGU-A24	HBGU-R24	HBGU-P24	HBGU-b24
25	HBGU-25	HBGU-A25	HBGU-R25	HBGU-P25	HBGU-b25
26	HBGU-26	HBGU-A26	HBGU-R26	HBGU-P26	HBGU-b26
27	HBGU-27	HBGU-A27	HBGU-R27	HBGU-P27	HBGU-b27
28	HBGU-28	HBGU-A28	HBGU-R28	HBGU-P28	HBGU-b28
29	HBGU-29	HBGU-A29	HBGU-R29	HBGU-P29	HBGU-b29
30	HBGU-30	HBGU-A30	HBGU-R30	HBGU-P30	HBGU-b30
31	HBGU-31	HBGU-A31	HBGU-R31	HBGU-P31	HBGU-b31
32	HBGU-32	HBGU-A32	HBGU-R32	HBGU-P32	HBGU-b32

33	HBGU-33	HBGU-A33	HBGU-R33	HBGU-P33	HBGU-b33
34	HBGU-34	HBGU-A34	HBGU-R34	HBGU-P34	HBGU-b34
35	HBGU-35	HBGU-A35	HBGU-R35	HBGU-P35	
36	HBGU-36	HBGU-A36	HBGU-R36	HBGU-P36	
37	HBGU-37	HBGU-A37	HBGU-R37	HBGU-P37	
38	HBGU-38	HBGU-A38	HBGU-R38	HBGU-P38	
39	HBGU-39	HBGU-A39	HBGU-R39	HBGU-P39	
40	HBGU-40	HBGU-A40	HBGU-R40	HBGU-P40	
41	HBGU-41	HBGU-A41	HBGU-R41	HBGU-P41	
42	HBGU-42	HBGU-A42	HBGU-R42		
43	HBGU-43	HBGU-A43	HBGU-R43		
44	HBGU-44	HBGU-A44	HBGU-R44		
45	HBGU-45	HBGU-A45	HBGU-R45		
46	HBGU-46	HBGU-A46	HBGU-R46		
47	HBGU-47	HBGU-A47	HBGU-R47		
48	HBGU-48	HBGU-A48	HBGU-R48		
49	HBGU-49	HBGU-A49	HBGU-R49		
50	HBGU-50	HBGU-A50	HBGU-R50		
51	HBGU-51	HBGU-A51	HBGU-R51		
52	HBGU-52	HBGU-A52	HBGU-R52		
53	HBGU-53	HBGU-A53			
54	HBGU-54	HBGU-A54			
55	HBGU-55	HBGU-A55			
56	HBGU-56	HBGU-A56			
57	HBGU-57	HBGU-A57			
58	HBGU-58	HBGU-A58			
59	HBGU-59				
60	HBGU-60				
61	HBGU-61				
62	HBGU-62				
63	HBGU-63				

Fig 2: **Screening of various soil samples for L.Glutamate production**



QUANTITATIVE ESTIMATION AND TIME COURSE OF GLUTAMATE PRODUCTION:

From nutrient agar media selected strains were inoculated into basal seed medium for 24 hrs and then 10ml of the medium was transferred into the 100 ml flask containing 30ml sterile production medium. After every 24hr sample were taken and examined for glutamate production continuously for 7 days.

Strain HBGU-P1 taken from Paharpur soil sample gave maximum glutamate yield of 22gm/l on 4th day. At first day initial concentration of 5gm/l was obtained and then continuous increase up to 4th day and then decreased.

Strain HBGUP2 isolated from Paharpur soil, gave initial yield of 6gm/l and with continuous increase maximum yield of 22 gm/l on 4th day was obtained and after that gradual decrease.

Strain HBGU-b2 collected from Bannu soil sample produced initially 6 gm/l glutamate and with continuous increase up to 4th day, gradual decrease in glutamate upto 7th day. Maximum yield of 20 gm/l was obtained on 4th day.

Strain HBGU-R4 obtained from River Indus soil sample gave maximum yield of 30gm/l on 4th day of incubation. Initial concentration was found as 10 gm/l and after 4th day glutamate concentration decreased and on 7th day it was 18 gm/l.

Strain HBGU-5 taken from Gomal University soil, gave initial yield of 4 gm/l and then increased up to 4th day produced maximum yield of 15 gm /l. Afterwards then continuously decreased and yield was 10gm/l on 7th day.

Initial glutamate yeild of 7 gm/l was given by Strain HBGU-A6, isolated from Abbotabad soil sample and highest glutamate yield of 26 gm/l was observed after 3 days. After 4th day decrease in yeild was observed and on 7th day it was 16gm/l.

Strain HBGU-A7 was isolated from Abbotabad soil sample. Initial concentration was 8gm/l and produced 25gm/l maximally on 4th day. On 7th day it was 18 gm/l.

Strain HBGU-8 isolated from soil sample taken from Gomal University. It gave initial glutamate concentration of 6gm/l, maximum yield of 18gm/l on 4th day and then gradual decrease in glutamate concentration on 7th day was examined as 10gm/l.

Strain HBGU-A9 isolated from Abbotabad soil sample gives initial glutamate concentration of 8gm/l after 24hrs incubation, with continuous increase 24gm/l on 4th day was examined and then gradually glutamate concentration started decreasing and it was 15gm/l on 7th day.

Strain HBGU-b3 isolated from Bannu soil sample, produced 8gm/l glutamate initially, maximum 24gm/l, then after gradual decrease 18gm/l was observed on 7th day.

Strain HBGU-R2 and Strain HBGU-R9, both taken from River Indus soil sample produced maximum yield of 26 gm/l on 4th day of incubation. Initially strain HBGU-R2 produced 8gm/l glutamate and strain HBGU-R9 produced 9 gm/l and after 4th day yield gradually decreased to 18 gm/l on 7th day (**Fig 3**).

7.3 Comparative percentage of glutamate producing strains.

Net percentage of glutamate producing strains in various soil samples were examined and found as River Indus soil sample has the highest capability of yielding glutamate producing strains and the net output was 25%. Total percentage of glutamate producing colonies in Abbotabad soil sample was 24% and in Bannu soil sample 23% while in Paharpur soil source net percentage of glutamate producing strain was 22% (**Fig 4**).

Comparative study of maximum glutamate yield by isolated bacterial strains was done and noticed that different strains have given different maximum yield on 4th day of incubation (**Fig 5**).

Fig 3: Time course for L.Glutamate production by various selected strains

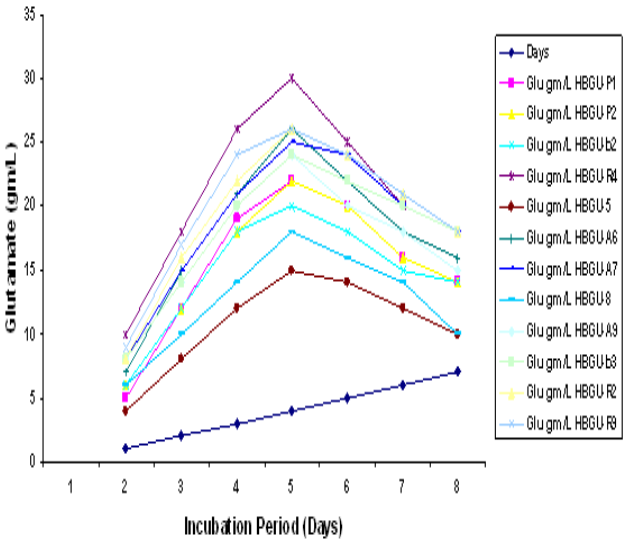


Fig 4: Percentage of glutamate producing strains in soil samples

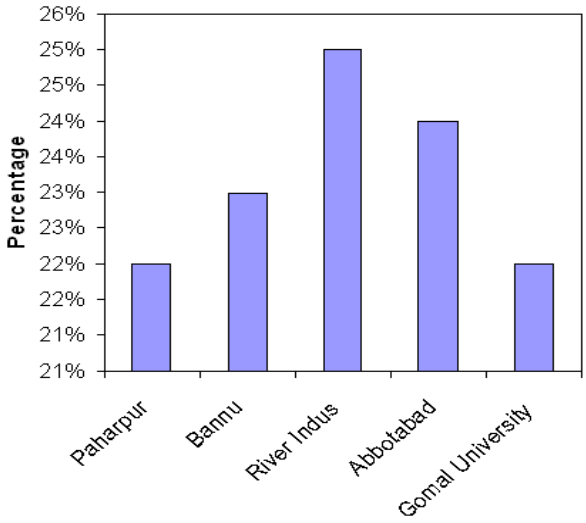
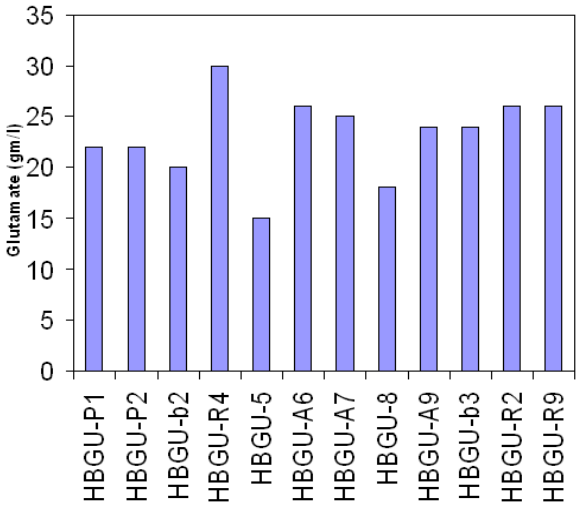


Fig 5: Maximum glutamate yield in gm/L by most potent glutamate producing strain



DISCUSSION

Pharmaceutical products have been produced as primary metabolite by variety of microorganisms and L-Glutamic acid is an important amino acid, worldwide used in various feed stuffs.

Glutamate production by employing living cells is more appreciable for industrial purposes. Glutamate production is not restricted to any specific microbial genera. Although various species of *Streptomyces* genera, *S.aureofaciens*, *S.annulatus*, *S.griseus*, *S.rimosus*, *S.fradie*, *S.olivaceus* have been reported to produce glutamate (Perlman and OBrien, 1958).

In this work fermentative production of glutamate was done due to the fact that through fermentative methods optically active and biologically required L-form of amino acids are produced as compared to chemical methods (Yamada *et al*., 1972) which yield mixture of D and L form amino acids while living cells can only utilize L-form amino acids.

Aim of the work was to isolate bacteria from local soil samples that can fermentatively produce glutamate. For that very purpose soil samples from River Indus, Abbotabad, Bannu, Paharpur and Gomal University Dera Ismail Khan were collected.

Bacteria may have a native ability to produce L-glutamate. Bacteria synthesize glutamate because bacterial cell wall contains D- glutamate as an essential component that is produced from l-glutamate through glutamate racemase (EC 5.1.1.3) or from alpha ketoglutarate by D-amino acid aminotransferase (EC 2.6.1.2) (Yoshimura *et al*., 1993).

Glutamine or glutamic acid is required by the bacteria itself (as a primary metabolite) simply for the construction of cell proteins, particularly in view of the fact that need of these amino acids are of the order of magnitude which would be expected for this reason. (Pollack and Linder, 1942).

Bacterial strains were found capable of producing L-glutamate in the medium having glucose, ammonium salt, minerals and growth factor as by (Otsuka *et al*., 1957).

Bacterial strains having ability to produce glutamate was detected when cells were incubated in screening media and after 24 hours incubation cells were filtered by centrifugation at 5000 rpm for 10 minutes. Number of strains having the ability to release glutamate in Paharpur, Bannu, Abbotabad, River Indus and Gomal University soil samples were found as 9, 8, 14, 13 and 14 out of 41, 34, 58, 52,63 respectively.

As the fermentation proceeds L-glutamate production increased as demonstrated by (Aida et al., 1986).

The highest productivity of L-glutamic acid was obtained by strain HBGU-R4 from river Indus sample on 4th day of incubation when the cell suspension was incubated at 30°C and the pH of the medium was maintained at 7.2. Glutamate production was about 30gm/l with glucose as substrate.

Ability of the microbe to excrete glutamate is also markedly affected by composition of the media. Glucose was used as carbon source. It was found that Glucose acts as good substrate and as carbon source as compared to molasses, starch, sucrose and lactose because of negligible yield of l-glutamate with these substrates (Chao and Foster, 1958).

As it was determined that normal (wild type) bacterial isolates in the molasses based medium containing ammonium sulphate as Nitrogen source under and above optimum conditions upto 96 hours incubation period, produced glutamate 1.63g/l, 1.54g/l, 1.56g/l and 1.53g/l at 24, 48, 72 and 96 hours. (Mali, 2007)

Reason for such low yield of glutamate as compared to yield obtained by this work may be due to use of molasses as substrate. Molasses contains high content of biotin that decreases glutamate production that is why the yield with molasses was low. Even with the incorporation of antibiotics or Tween 80, molasses could not be used as substitute for glucose (Roy and Chatterjee, 1989).

Maximum production in this work was observed at 4th day, after 72hrs. Initial glutamate concentration wasº found very low for each strain (5gm/l) with HBGU-P1 as it was reported by (Sen and Chatterjee,1983) that very low production was observed during initial 24 hrs of fermentation and maximum production of amino acids were observed after 72 h of continuous shaking fermentation.

Potassium di hydrogen phosphate and di potassium hydrogen phosphates were added in the medium to act as buffer and maintain the ph of the media at 7.4.

Different yeild was found with different bacterial strains because genetic potential abilities of each strain vary from the other or it may be due to the fact that optimum growth conditions like pH and temperature varies for each strain. But in this work only single temperature and pH was applied for each and every strain. As it was determined by (Mali, 2007) that *B.cereus* showed best growth and amino acid production at pH 7 and at 30 ºC while maximum growth and amino acid production for *B.subtilis* occur at pH 6 and at 30 ºC.

pH is an important factor affecting microbial growth. As pH changes markedly during fermentation process due to the consumption and conversion of nutrients into the product, if there is no suitable mechanism to control the pH. So for the maintenance of optimal pH, (Yamada, 1972) reported that reagents like calcium carbonate must be added at the beginning of fermentation medium to the culture medium. So for that very purpose 2% calcium carbonate was added to the production medium. (Wang *et al*., 1991) reported that small amount of calcium carbonate must be added as it eliminates the lag phase of cell growth, thus shortening fermentation period.

As Roy and Chatterjee, (1989) observed that under optimal condition in culture flask 16-gm/l glutamate was produced in 120 h and mentioned that that glutamate accumulation in the medium begins during mid exponential growth phase and through adjustment of pH its excretion could be prolonged. So adjustment of pH in the fermentation process at neutral is indispensable and in this work pH was maintained at 7.4.

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