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FERMENTATIVE PRODUCTION OF L-GLUTAMIC ACID

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ABSTRACT:

The demand for utilization of L-glutamic acid as a human dietary supplement, flavour enhancer, chemical in biochemical processing, pharmaceutical and cosmetic industries is fastly growing in recent times. It is largely imported amino acid in India and the total worldwide production of L-glutamic acid is more than 1.5 million tons per year through fermentation (Shimuzu and Hirusawa 2006). 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 which the scientific community have pursued with zeal.

Keywords: L-glutamic acid, Microorganisms, substrates, yield, fermentation.

INTRODUCTION

A Survey of literature revealed the various methods of production of L-glutamic acid. In the year 1866, L-glutamic acid was discovered by karl Heinrich Leopold Ritthausen[20]. Later in the year 1907, Kikunae Ikeda identified L-glutamic acid crystals in kombu broth which gives a undeniable flavor in many foods especially in seaweed and he termed this flavor Umami[14].Industrially the L-glutamic acid was produced from vegetable proteins like wheat proteins, soy bean proteins upon acid hydrolysis with Hcl was first reported by Ajinomoto Co., Inc., Tokyo, Japan.

Later a glutamic acid producing bacterium *Micrococcus glutamicus* or *Corynebacterium glutamicum* was discovered by Kinoshita et.al. in 1957 which produces 30 g/L of L-glutamic acid in glucose medium[21]. In the same year Donald A. Kita and Jackson Heights N.Y produced L-glutamic acid by using different strains of *Cephalosporium* through fermentation of substrates like distiller's solubles, cornsteep liquor, wheat gluten etc. and the yield varies from 1 to $3^{1/2}$ g/L[23].

In 1958, Kwei-chao chao and J.W Foster reported that, the *Bacillus* strain 14B22 Utilized 3% glucose medium and liberated 12.5 mg/ml of glutamic acid[25].

Among the tested 250 *Arthrobacter* strains by H.Veldkamp et.al in 1962, they isolated *Arthrobacter globiformis* which produce high yields of glutamic acid ~ 0.45 moles per mole of glucose consumed[45].

Winfred.N.McCutchan et.al. in 1920 found out that *Brevibacterium divaricatum* having a capacity to utilize the enzymatic hydrol obtained after enzymatic hydrolysis of grain sugars and produced 26.2 g/L after 24hrs and 41.5 g/L after 48hrs of incubation time for 181gms of enzymatic hydrol[47].

L-Glutamic acid can be produced by the fermentation of saccharide material using five different strains of *Brevibacterium* consisting of atleast one member of this group desthiobiotin, biotin-d-sulfoxide and biocytin and the yield exceeds 4.0 g/dl.This was first reported by shinichi motozaki et.al. in 1963[39].

A Direct method of production of L-glutamic acid by fermenting a suitable nutrient media with a biotin requiring microorganism *Micrococcus*

glutamicus M-560 was developed by Thomas Philips in 1963. The yield was 40 g/L when grown in 3L molasses medium containing 37.5 parts per billion of biotin and 4000 U/L of penicillin[44].

Isamu Shiio et.al. in 1964 used four different strains of glutamic acid producing microorganisms for L-glutamic acid production from the substrates like sodium acetate, potassium acetate or acetic acid. *Brevibacterium flavum* ATCC No. 13826 produced 15 g/L, *Brevibacterium roseum* ATCC No. 13825 produced 14.8 g/L, *Brevibacteruim lactofermentus* ATCC No. 13869 produced 14.3 g/L and *Corynebacterium acetoacidophilum* ATCC No. 13870 produced 7.3 g/L of glutamic acid[15]

The effect of penicillin on L-glutamic acid by *Corynebacterium hydrocarboclastus* M-104 which assimilates glucose (G-medium) and / or hydrocarbons (H-medium) containing kerosene, n-dodecane, n-Tetradecane and n-hexadecane was first reported by shin-ichiro otsuka et.al. in 1964. The yield of L-glutamic acid was 6.3 g/L in G-medium and were 2.1 g/L, 1.9 g/L, 4.0 g/L and 2.8 g/L in H-medium containing these hydrocarbons[38].

It was reported by Joji Takahashi et.al. in 1964 that L-glutamic acid production by *Corynebacterium* can be induced by the effect of different natural nutrients.The yield was 5 g/L in the medium containing 3% n-paraffins, 0.01% cornsteep liquor mineral salts[18] etc.

A screening test was performed by Wee Chong Tan and Bernard-Malin to isolate a glutamic acid producing microorganism from soil by using a Selective medium containing glucose, urea etc. when it is subjected to U.V and X-ray treatment it liberated 10mgm/ml of broth[46].

John D.Douros, Jr., West Chester et.al. in 1965,described a method for the production of L-glutamic acid using *Nocardia globerula* ATTC15076 byUtilizing hydrocarbons under aerobic conditions at 30^0c for 48-96 hrs. The yield obtained was 1-4 g/L for n-decane with in 36 hrs of incubation time[17].

A screening method had been performed by Ryuichiro Tsugawa et.al. during the year 1965 to isolate a suitable glutamic acid producing microorganism. Among 22 tested strains, *Bacillus brevis* ATCC No.8185 will convert 90% of the DL-hydantoin-5-propionic acid to glutamic acid in a reaction mixture containing 1.0% DL-HPA[36].

L-glutamic acid was produced from unsaturated fatty acids which were reported by Hisoyoshi Okazaki et.al. in the year 1967. The organisms used were *Bacillus thiogenitalis* No. 653 and its Oleic acid requiring mutant D-248. D-248 utilizes oleic acid in presence of 30 µg/L of biotin and liberating 50mg/ml of L-glutamic acid but at the same biotin concentration glutamic acid production by No.563 was reduced to zero. It was also found that No.563 produces glutamic acid 40mg/ml at 5µg/L of biotin concentration present in the medium[12].

In 1970, Y.Nakao et.al. successfully isolated a glycerol auxotroph strain GL-21 from *Corynebacterium alkanolyticum* No.314 by treatment it with N-Methyl_N^1-nitro-N-nitrosoguanidine. When this strain grown in a medium containing n-paraffins, mineral oils and glycerol produced 40 mg/ml of L-glutamic acid[48].

The study of Shigeho Ikeda in 1972 revealed that, an artificially induced mutants like *Brevibacterium ketoglutamicum* S-10 (ATCC 21533), *Corynebacterium hydrocarboclastus* R-17 S-15 (ATCC 21534) and *Arthrobacter paraffineus* S-4 (ATCC 21535) utilized hydrocarbons and their oxidation products present in the nutrient medium containing penicillin and liberated 7g/dl of glutamic acid[37].

The microorganism *Bacillus ammoniagenes* which required both biotin and thiamine along with aminoacids like His or Cys liberated maximum amount of L-glutamic acid i.e more than 50% (w/w) of initial sugar content present in the medium containing wheat bran extract or rice

bran extract. This work was reported by Hong, soon woo, yung chil Hah et.al. in 1974[13].

The mutant strains of genus *Brevibacterium* produced L-glutamic acid by utilizing polyoxy ethylene-sorbitan-mono-palmitate was reported by Takinami et.al. in 1976. The yield was 21 mg/ml for *Brevibacterium lactofermentum* AJ 3611, 19 mg/ml for *Brevibacterium lactofermentum* ATCC 13869, 52 mg/ml and 50 mg/ml for *Brevibacterium flavum* AJ 3612 and *Brevibacterium flavum* ATCC 14067[24].

Haruo Momose and Takashi Takagi in 1978 reported that glutamic acid can be produced by a temperature sensitive mutant strains derived from *Brevibacterium lactofermentum* 2256. Among 159 selected mutant strains produced glutamic acid after a temperature shift from 30˚c to 37˚c.One typical mutant strain, TS-88 produced 2 g/dl of glutamic acid in a biotin rich beet molasses medium with a temperature shift from 30^0c to 40˚c[8].

A novel fermentation process in which L-glutamic acid can be produced with ethanol at increased concentrations from 5 g/L to 25 g/L during fed batch culture and the yield of L-glutamic acid reached to 26 g/L. The organism used was *Brevibacterium divaricatum* NRRL 2311. This method was first explained by Kishimoto michimasa et.al. in 1981 using regression analysis[22].

In 1982, Yoshimura et.al. found out that the mutant strains of *Brevibacterium* or *Corynebacterium* which are resistant to respiratory inhibitors (or)ADP phosphorylation inhibitors produce high yields of glutamic acid from 51 g/L to 52 g/L in glucose medium[30].

The investigated report of Nakazawa et.al. in 1982, revealed that the mutants of genus *Corynebacterium* which were resistant to vitamine-p compounds like esculetin, coumarin, Dicumarol produce high yields of glutamic acid. When these resistant strains grown in 10 g/dl of glucose the yield obtained was in between 5-41% and it was 5-33% when resistant strains grown in log / dl of cane molasses. But at varied temperatures 31.5˚c, 35˚c, 37˚c in presence of 3.6 g/dl of cane molasses the yield of glutamic acid was in the concentration range 26%-37%[10]

A patented work reported by Hiraga et.al. in 1983, described a method for the production of L-glutamic acid using mutant strains of *Brevibacterium* or *Corynebacterium* which were resistant to antibiotics like Decoyinine or Tubercidin produced 15-17.8 g/L of glutamic acid in glucose medium. The same strains produced 47-50 g/L of glutamic acid when grown in sugar cane molasses[11].

In 1985, Yoshimura et.al. patented a workon l-glutamic acid production using mutant strains of *Brevibacterium* or *Corynebacterium* which have an increased superoxide dismutase activity and grows in a medium containing daunomycin or methyl viologen and produced 16-18 g/L of glutamic acid. These strains have liberated 49-51 g/L of glutamic acid in case molasses at 31.5˚c for 36hrs of incubation time[31].

The isolated strain of *Arthrobacter globiformis* from Burdwan soil tend to utilize glucose mineral salt medium and excreted 16.1 g/L of glutamic acid in 120 hrs. This work was reported by D.K. Roy and S.P. Chatterjee in 1988[35].

It was reported by H.J.Henkel et.al. in 1990 that, an immobilized *Corynebacterium glutamicum* grows in a three phase fluidized bed reactor and achieved a maximum productivity of glutamic acid 3 g/L/h in a glucose medium during continuous fermentation[9]. In the same year Yong-Fen Li et.al. developed an immobilized strain of *Corynebacterium glutamicum* T6-13 in Eucheuma gel with cellulose acetate was used for the production of L-Glutamic acid. The conversion of glutamic acid can reach 6.0% in the medium containing 12% of glucose[26].

The maximum yield of glutamic acid 6.86 mg/ml was obtained when 2% glucose medium was fermented for 48 hrs by a *Brevibacterium* Sp. was reported by K.Madhavan Nampoothiri and Dr. Ashok Pandey in 1995[27].

K. Das et.al. in 1995, described the production of L-glutamic acid from palm waste hydrolysate by using a strain *Brevibacterium lactofermentum* ATCC 13869 which utilizes glucose as carbon source and produced 88 g/L of glutamic acid[2].

A novel fermentation process in which L-glutamic acid production can be produced by *Escherichia Coli* strains W3110, GH-1, AJ12628 and AJ12624 which were deficient or low in α-ketoglutaric acid dehydrogenase activity and have low L-glutamic acid decomposing ability, were capable of producing L-glutamic acid 3.0 g/L, 5.0 g/L, 18.5 g/L and 20.0 g/L respectively. This was reported by Tujimoto et.al. in 1995[34].

A novel fermentation process was invented during 1995 by Mototsugu-Shiratsuchi et.al. to establish a simultaneous cultivation method for production of glutamic acid and lysine using *Brevibacterium lactofermentum*AJ12937.The yield of these amino acids was 132 g/L in the medium containing 6000 U/L of penicillin and 146 g/L in the medium containing 4 g/L of PESMP[32].

In 1996, Eiji ono et.al. in 1996, isolated a strain *Escherichia coli* W3110 SUCA :: Km^r / pGK designated as *E.Coli* AJ12949 which was deficient in α-ketoglutarate dehydrogenase activity and amplified PEP carboxylase and glutamate dehydrogenase activities produce high yield of L-glutamic acid 23.3 g/L in glucose medium[6].

The study concluded by Madhavan Nampoothiri,K.,and Ashok pandey in 1996 was that, *Brevibacterium* sp. can be cultivated on sugarcane-baggase enriched with 10%glucose, urea, mineral salts and vitamins for the production of L-glutamic acid using solid state fermentation system.Maximum yield 80 mg glutamic acid/g dry baggase was obtained[28].

The coimmobilized whole cells of *Micrococcus glutamicus* and *Pseudomonas reptilivora* liberated high yields of L-glutamic acid 37.1 Kg/m^3 under optimized medium constituents in glucose medium and optimized physical parameters was first reported by I.Sunitha in the year 1998[41].

A biotin dependent surfactant temperature sensitive mutant strains of *Corynebacterium* or *Brevibacterium* capable of producing L-glutamic acid during shift up temperatures 34˚c, 37˚c, 39˚c and the yield was 0.0, 0.5, 0.9 g/dL for ATCC 13869 and it was 5.8, 8.3, 9.2 g/dL for the strain AJ13029 by utilizing glucose as carbon source. But during time shift up's 8hrs, 12hrs, 16hrs produced 0.5, 0.1, 0.0g/dL of glutamic acid by ATCC 13869 and 8.3, 7.0, 5.4 g/dL by AJ 13029.These strains when subjected to enchancement of gene expression of glutamic acid biosynthesis system with different plasmids expression systems produces L-glutamic acid in the range 33 to 41 g/L. This was the patented work of Kimura et.al. in 1998[5].

The investigated reports of K.Madhavan Nampoothiri and Ashok Pandey in 1999 described that the strain *Brevibacterium* Sp DSM. 20411 utilized cassava starch hydrolysate and accumulated 21g/L of L-glutamic acid. In fed-batch fermentation using 5% w/v sugar concentration L-glutamic acid produced was 25 g/L[33].

Yoshioka et.al. in 1999, invented a method of producing L-glutamic acid with different mutant strains of *Corynebacterium* and *Brevibacterium* using continuous fermentation. *Brevibacterium lactofermentum* ATCC13869 when grown in production medium containing glucose, Biotin, Polyoxyethylene sorbitan monopalmitate etc yielded 56% of L-glutamic acid and the productivity was 5g/L with in 40 hrs. The strain *Brevibacterium lactofermentum* AJ12821 liberated 56% of L-glutamic acid with a productivity of 6.6g/L in 40hrs and the same strain yields 55% with a productivity of 8.3 g/L in 100hrs[43].

In the same year a study on L-glutamic acid production by S.Delaunay, D.Uy et.al. had revealed the importance of PEP carboxylase and pyruvate carboxylase in *Corynebacterium glutamicum* metabolism during a temperature triggered glutamic acid fermentation. In absence of PEP Carboxylase and pyruvate carboxylase activity was sufficient and 70% of glutamic acid was liberated. In

glucose medium containing optimized biotin concentrations.[3].

An aminoacid auxotrophic strain *Bacillus methanolicus* ATCC 55403 by utilizing Methanol & Vit-B_{12} produced L-glutamic acid at a concentration of 5g/L which was reported by Hanson Richard.S. et.al. in the year 2000[7].

Corynebacterium glutamicum 2262, was subjected to several temperature shift up's from 33˚c to 37˚c, 38˚c, 39˚c, 40˚c and 41˚c causes the accumulation of 80g/L of L-glutamic acid in glucose medium under fed batch fermentation. This was explained by S.Delaunay, P.Lapujade et.al. in 2002[4].

In 2004, Sun-uk choi et.al.reported that the strain *Brevibacterium* sp.Tc452 during the shift-up temperature from 30˚c to 38˚c at 25h of cultivation produce maximum yield of L-glutamic acid 41.42 g/L in glucose medium[42].

The investigations carried out by A.N.Jyothi et.al. in the year 2005, revealed that L-glutamic acid can be produced by submerged fermentation of cassava starch using *Brevibacterium divaricatum*. Under optimed parameter conditions the highest glutamate yield of about 3.86% was obtained[19].

Islas-Murguia.L, et.al.in 2006, explained the method of production of L-glutamic acid from the waste of a Mexican lime citrus aurantifolia swingle using *Corynebacterium glutamicum* ATCC 13032 which gave the highest yield of 13.7g/L. The culture medium also contained 2% glucose[16].

A novel fermentation process for the production of glutamic acid from rice hydrolysate was established during the year 2006 by Sun, Z.F., et.al. using temperature sensitive mutant N_1strain derived from parent strain *Corynebacterium crenatum*$D_{6.}$ The average yield of L-glutamic acid was 58.6 g/L[40].

The experimental study carried out by N.M yugandhar et.al. during 2007 revealed that the maximum yield of L-glutamic acid 40.5 mg/ml was obtained with *Brevibacterium roseum* free cells under optimum parameters. The glutamic acid at a concentration of 37.2 mg/ml and 39.6 mg/ml were obtained with immobilized *Brevibacterium roseum* and co-immobilized *Brevibacterium roseum* and *Escherichia intermedia* type 1 strains in a glucose media[49].

G.A Amin et.al. in 2007, described the production of L-glutamic acid from sugarcane baggase using *Corynebacterium glutamicum* ATCC 13022 entrapped into carrageenan gel beads. The best yield was obtained 75.7% at a concentration of 73g when immobilized bioreactor was operated continuously[1].

The study of Mahmoud Tavakkoli et.al. in the year 2009 concluded the fact that date waste juice was the best substrate for the production of glutamic acid. They used *Corynebacterium glutamicum* CECT 690 culture and response surface methodology to predict the effect of fermentation parameters for L-glutamic acid production. The maximum yield was 39.32 mg/ml which was determined by this model and in the second stage the yield was 118.75 mg/ml, 142.25 mg/ml and 95.83 mg/ml at three different air flow rates. [29]

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REFERENCES

1. Amin, G.A., & A. Al- Talhi(2007), *Journal of world Applied sciences*, 2(1), 62-67.

2. Das, K., M.Anis, B.M.N.Mohd. Azemi, N.Ismail(2004),*journal of Biotechnology.&Bio engineering*,Vol.48, issue 5, P:551-555.

3.Delaunay .S, D.uy, M.F. Baucher, J.M Engasser, A.Guyonvarch &J.L. Georgen(1999), *Journal of metabolic engineering*, Vol.1, Issue-4, P: 334-343.

4. Delaunay,S., P. Lapujade, J.M. Engasser. &J.L.Georgen(2002).*J.ind.Microbiol.Biotechnol*.vol:28.

5. Eiichiro kimura; Yoko Asakura; Akinori Uehara; Sumio Inoue; Yoshio kawahara; yasuhiko Yoshihara; Tsu Yoshi Nakamatsu(1998),U.S patent 5846790.

6. Eiji ono; Nobuharu Tsujimoto; Kazuhiko Matsui; Osamu Kurahashi(1996), U.S patent 5573945.

7. Hanson, Richard .S(2000), U.S Patent 6110713.

8. Haruo Momose., and Takashi Takagi(1978),*J. Arg. Biol. Chem*, Vol. 42, No.10, P: 1911-1917.
9. Henkel. H.J; H.J.Johl, W.Trosch, H.Chmiel,(1990) , *Journal of food biotechnology*, Vol.4, Issue-1P:149-154.
10. Hidetsuga Nakazawa, Ichiro Yamane, Eiichi Akutsu(1982), U.S patent 4334020.
11. Hirofumi Hiraga, Minoru yoshimura, Shigeho Ikeda; Hiroe yoshii(1983), U.S patent 4389483.
12. Hisayoshi Okazaki, Toshihiko Kanzaki, Muneharu Doi, Yasuhiro Sumino and Hideo Fukuda(1967), *j.Arg. Biol. Chem.*, Vol 31, No.11, P. 1314-1317.
13. Hong, Soon Woo, Yung Chil HAH and Seung Hee CHA, (1974), *KOR. JOUR MICROBIAL*. Vol. 12, 115-130.
14. Ikeda, K. (2002), *Chem. Senses*, Vol.27, No. 9, 847-849.
15. Isamu Shiio, Koji mitsugi, ShinichiroOtsuka & Toshinao Tsunoda(1964), U.S. Patent 3117915.
16. Islas –Murguia, L.,Perez- Mendoza,J.L., Garcia – Mernandez, F(2000), *Journal of Industrial Microbiology & Biotechnology*, Vol.28, No. 6, P: 333-337.
17. John D. Douros, Jr. West Chester & Andre R. Brillana and Robert W. Eltz (1965), U.S. Patent 3201323.
18. Joji Takahashi, Kaetsu Kobayashi, Yukio imada & Koichi Yamada (1965), *Journal of Applied Microbiology*, Vol. 13, No.1, P:1-4.
19. Jyothi, A.N., K. Sasikiran, Bala Nambisan& C.Balagopalan(2005) , *Process Biochemistry*, Vol.40, Issue 11, 3576- 3579.
20. Karl Heinrich Leopold Ritthausen, *Journal of Toxicology and Environmental health*, 2(2): 471-480.
21. Kinoshita,et al., (1957) Proc. Int. Symp. Enzyme chem., , 464-468.
22. Kishimoto Michimasa, Yoshida Toshiomi, Taguchi Hisaharu(1981), *Journal of fermentation technology*, 59 (1), 43-48.
23. Kita, D.A., (1957),.U.S patent 2789939.
24. Koichi Takinami, Takashi Tanaka, Michiaki Chiba, Hirose(1976), U. S. Patent 3971701.
25. Kwei-Chao Chao and Foster, J.W,(1959) *J. Bacteriol*, 77(6); 715-725.
26. Li, Yong –Fen; Haung Yue; Ye, Lin-Fa; Peng sui & Qing-Qing wen(1990), *Annals of the New York Academy of sciences*.
27. Madhavan Nampoothiri.K., Ashok Pandey(1990), *Rev. Microbiol*,Vol.30, No.3.
28. Madhavan Nampoothiri, K., & Ashok Pandey(1996),*Biotechnology letters*, Vol.18, No.2 P: 199-204.
29. Mahmoud Tavakkoli., zohreh Hamidi – Esfahani & Mohammad Hossein Azizi(2009). *Food & Bioprocess Technology*.
30. Minoru Yoshimura, Yoshihiro Takenaka, Shigeho Ikeda, Hiroe Yoshii (1982), U.S.Patent 4347317.
31. Minoru Yoshimura, Yosuke Koyama, koichi Goto, Sumio Inoue, Shigeho Ikeda, Hiroe Yoshii(1985),U.S patent 4529697.
32. Motatsugu shiratsuchi; Hideo Kuronuma, Yoshio kawaraha, yoshihara , Harufumi Miwa,& Shigeru Nakamori(1995), *J. Biosci. Biotech. Biochem*., 59(1) , 83-86.
33.Nampoothiri,K.M., Pandey, A(1995),.*J.basic. Microbiol*, 35:249254
34. Nobuharu Tujimoto;Yoshimi Kikuchi; Osamu Kurahashi; Yoshiko Kawahara(1995), U.S patent 5378616.
35. Roy, D.K., &S.P Chatterjee(1989), *J.Folia. Microbiologica*, Vol.34, No.1, P: 11-24.
36. Ryuichiro Tsugawa, Shinji Okumura, Tamio ito & Noboru Katsuya(1966) *J. Arg. Biol Chem.*, Vol., 30, No.1, PP: 27-34.
37. Shigeho Ikeda, Ayaaki Ishizaki, yoshio Hirose & Teruo Shiro(1972), U.S. Patent 3674639.
38. Shin – ichiro Otsuka., Ryosuke ishii, Isamu Shiio and Noboru Katsuya (1964), *J. Gen. Appl. Microbial*,Vol. 10, No.2, PP: 179-180.
39. Shinichi Motozaki, Toshinao Tsunoda & Shinji Okumura, Toshinori matsui, Atsuo Kitai, Ryuichiro Tsugawa & Noboru miyachi(1963),U.S.Patent 3096252.
40. Sun,Z.F., Yu,Z.L., Yang Y.F. Article from www.cababstracts plus.org.(2006).
41. Sunitha I., M.V.Subba Rao, C.Ayyanna(1998), *Journal of Bioprocess & Biosystems engineering*. Vol.18, No.5, 353-359.
42. Sun-UK choi., Takuya nihira & Toshiomi Yoshida(2004), *Journal of Bioscience and Bio engineering*, Vol .98, No.3, 211-213.
43. Tatsuya Yoshioka; Toshimasu Ishii; Yoshio kawahara; Yosuke Koyama; Eiko Shimizu(1999), U.S patent 5869300.
44. Thomas Phillips, Edwards Ville & Norman L. Somerson(1963), U.S. Patent 3080297.
45. Veldkamp.H., G. Vandenberg and L.P.T.M Zevenhuizen,(1963) ; *Journal of Antonie- Vanleeuwenhoek*, vol.29, No.1, 35-51.
46. Wee Chong Tan and Bernard Malin(1964), *Butler U.Bot.studies,Berkeley electronic press*, 14(2), 89-103.
47. Winfred, N. Mc Cutchan & Phil H. Hidy. (1962), U.S. Patent, 3061521.
48.Yoshio Nakao, Masakazu Kikuchi, Masaru Suzuki & Muneharu DOI(1972),*J. Arg. Biol Chem.*, Vol. 36, No. 3, PP: 490-496.
49.Yugandhar , N.M.,Ch.A.I.Raju, P.J. Rao, K.Jaya Raju & D.Sri Rami Reddy(2007), *Res. J.Microbiol*., 2,584-589.