academicJournals

Vol. 12(50), pp. 6944-6953, 11 December, 2013
DOI: 10.5897/AJB2013.13107
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production of L-glutaminase and its optimization from a novel marine isolate *Vibrio azureus* JK-79

Jambulingam Kiruthika[1]* and Nachimuthu Saraswathy[2]

[1]Government College of Technology, Coimbatore, Tamil Nadu, INDIA
[2]Kumaraguru college of Technology,Coimbatore, Tamil Nadu, INDIA.

Accepted 20 September, 2013

L-Glutaminase is an amidohydrolase which is produced by a variety of micro-organisms including bacteria, yeast and fungi. It is currently used in the treatment of acute lymphoblastic leukemia (ALL) and human immunodeficiency virus (HIV). Another potential application of L-glutaminase is as flavor enhancing agent in soy sauce fermentation. Even though L-glutaminase activity was reported in numerous micro-organisms, production of L-glutaminase from marine bacteria is very scanty. With this view, in the present research work, L-glutaminase production pattern was studied under submerged fermentation using novel marine isolate *Vibrio azureus* strain JK-79 (GenBank Acession Number JQ820323) based on one-factor-at-a-time approach. The 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). The above results indicate the scope for production of salt tolerant L-glutaminase using this novel marine bacterial strain.

Key words: L-Glutaminase, *Vibrio azureus* JK-79, submerged fermentation, optimization of fermentation, one-factor-at-a-time approach.

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolases E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine to glutamic acid and ammonia (Nandha et al., 2003). In recent years, it has gained much attention due to their potential application as anti-cancer agent (Roberts et al., 1970) and flavor enhancing agent (Yokotsuka, 1985).

Another most promising application of L-glutaminase is its usage in the treatment of human immunodeficiency virus (HIV) (Roberts et al., 1972). L-Glutaminase is also used in biosensors to monitor L-glutamine level in mammalian and hybridoma cell lines (Huang et al., 1995; Mulchandi et al., 1996). The tremendous application of L-glutaminase in various fields always prompted a search for a better source of the enzyme. Although L-glutaminase can be derived from both plant and animal sources, microbial source is generally preferred for industrial production due to their economic production, consistency, ease of process modification and optimization (Sabu, 2003).

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

*Corresponding author. E-mail: mailkiruthika@yahoo.com; 919944935597.

Abbreviations: HIV, Human immunodeficiency virus; **ALL,** acute lymphoblastic leukemia; **SWG,** sea water glutamine; **BSA,** bovine serum albumin.

Table 1. Composition of various fermentation media.

Medium No.	Medium	Composition of medium
1	Mineral Salts Glutamine medium (MSG) Renu and Chandrasekaran (1992)	L-glutamine-1%, D-Glucose-0.5%,Nacl-3%, KH_2PO_4 -0.1%, $MgSo_4.7H_2O$-0.05%, $CaCl_2$-0.01%, $NaNo_3$-0.01%, trisodium citrate- 0.01% and Distilled water-100ml , pH 6
2	Wakayama et al. (2005)	L-Glutamine - 2.0%, K_2HPO_4- 0.1%, KH_2PO_4- 0.1%, $MgSO_4$ $7H_2O$-0.01%, NaCl- 0.1% and Yeast extract -0.05%, Distilled water-100ml and pH 6.0
3	Sato et al. (1999)	D-Glucose -3.0%, yeast Extract- 0.5%, $MgSO_4$ -0.1% and KH_2PO_4 -0.1%, Distilled water-100ml and pH 6.0
4	Kumar and Chandrasekaran (2003)	L-Glutamine -1 %, D-glucose-0.05%, Distilled water-100 ml and pH 6.0
5	Sea water Glutamine (SWG) Kumar and Chandrasekaran (2003)	L-Glutamine -1 %, D-glucose-0.05%, aged sea water-100 ml and pH 6.0

candidate for enzyme therapy (Tanaka et al., 1988).

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 (Sabu et al., 2003). The marine biosphere is the richest habitat of microorganisms especially bacteria and also is the one which is less characterized. Hydrolytic enzymes from halotolerant micro-organisms provide an interesting alternative for therapeutic purposes as they are capable of functioning under conditions that leads to precipitation or denaturation of most proteins from terrestrial bacteria. Further, sea water, which is saline in nature and chemically closer to human blood plasma, could provide biomolecules especially enzymes that could have no or less side effects when used in therapeutic applications (Iyer and singhal, 2009).

L-glutaminase activity was reported in various terrestrial micro-organisms such as *Escherichia coli, Pseudomonas sp, Acinetobacter sp, Bacillus sp, Proteus morganni, Cryptococcus, Candida* and *Aspergillus oryzea* (Sabu, 2003). Apart from terrestrial sources, few marine micro-organisms were also known to synthesize L-glutaminase and include *Pseudomonas flurosence, Micrococcus luteus, Vibrio costicola* and *Beuveria bassiana* (Chandrasekaran, 1997).

In spite of its demonstrated potential as antileukemic agent, L-glutaminase is generally regarded as a key enzyme that controls the taste of fermented food such as soya sauce by increasing the glutamic acid content, there by imparting a unique flavor to the food (Nandakumar et al., 2003). Thus, salt tolerant and heat stable L-glutaminase demands not only search for potential strain, but also economically viable bioprocess for its large scale production (Nagendraprabhu G et al., 1995). From the literature, it is evident that only few reports are available on the extracellular production of L-glutaminase from marine bacteria and since there is an excessive requirement for salt and thermo tolerant L-glutaminases, a search for a potential marine strain that hyper produce this enzyme with novel properties and an economically viable bioprocess is pursued. Thus the present study, focuses on the L-glutaminase production from a potential and novel isolate *Vibrio azureus* JK-79 (JQ820323) under submerged fermentation and optimization of the process parameters and nutritional factors of fermentation for enhanced enzyme production.

MATERIALS AND METHODS

Micro-organism and culture maintenance conditions

The *V. azureus* JK-79 (JQ820323) used in this study was isolated from marine sediment collected from Parangipettai costal area (Lat. 11°.29' N; Long. 79°.46'E) (Kiruthika and Saraswathy, 2013). The culture was maintained in Zobell's marine agar slant (Himedia, India) at 4°C and was periodically sub-cultured.

Inoculum preparation

A loopful of culture from 24 h old Zobell's marine agar slant was inoculated on 10 ml of inoculum medium containing (g/l of aged sea water (30 ppt of salinity)): Peptone, 5; yeast extract, 1; NaCl, 2.45; and L-glutamine, 1. The inoculated medium was incubated at 37°C on rotary shaker at 120 rpm for 24 h.

Production media

Enzyme production was carried out in 500 ml Erlenmayor flasks. Five different types of production media (Table 1) were evaluated for the production of L-glutaminase enzyme. All the five different media were inoculated with the prepared inoculums at 10% level and incubated in an incubator at 37°C, 120 rpm for 24 h. After incubation 10 ml of the culture was collected and harvested by centrifugation at 10,000 rpm for 20 min and the supernatant was used as sample for enzyme assay.

Enzyme assay

Glutaminase was assayed according to the method described in the study of Imada et al. (1973). An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1 M, pH 8). The mixture was incubated at 37°C for 30 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloro acetic acid.

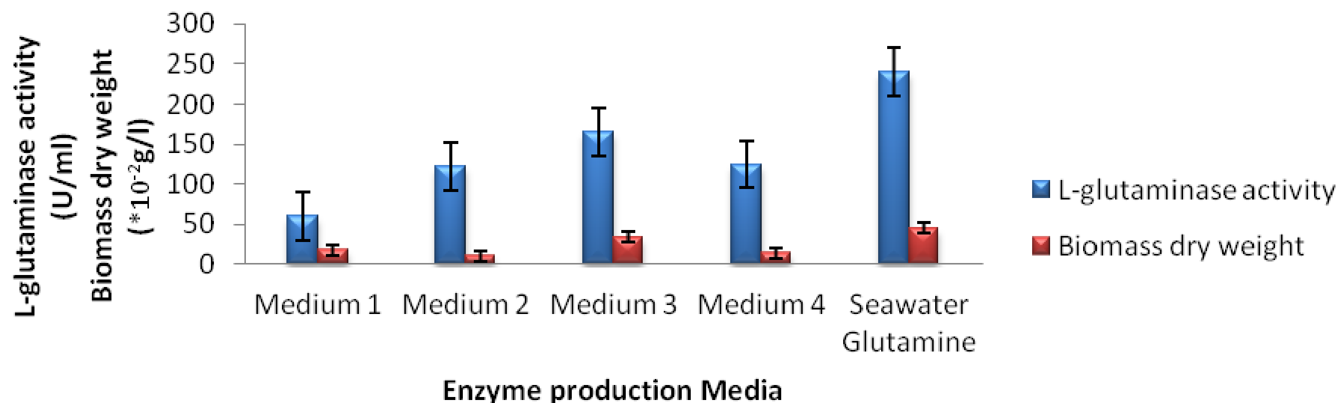

Figure 1. L-Glutaminase production from *Vibrio azureus* JK-79 and biomass concentration on different fermentation medium.

To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 450 nm using a UV-visible spectrophotometer (Spectronic-Genesys5).

The liberated ammonia content was measured and one international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmole of ammonia under optimal conditions. Assays were done in triplicates and the mean enzyme activity was expressed as units/ml of culture supernatant (U/ml).

Optimization of process parameters for L-glutaminase production

The sea water glutamine (SWG) medium was used as the basal medium and the process parameters were optimized one after another. After optimization of each parameter, it was included in the next study at its optimal level. The pH of the medium (5-9), incubation temperature (25-45°C), incubation time (24-120 h), inoculums concentration (1-5%) ,additional carbon source (D-fructose, sucrose, lactose, D-glucose, maltose and starch at 1% w/v), additional nitrogen sources (peptone, yeast extract, soybean meal, beef extract, meat extract, tryptone, ammonium sulphate, ammonium chloride, sodium nitrate and potassium nitrate at 1% w/v), and different amino acids (methionine, phenylalanine, histidine, cystein, L-aspargine, L-glutamic acid and L-glutamine at 1% w/v) were optimized for L-glutaminase yield. All experiments were conducted in triplicates and the mean values were alone taken into consideration.

Biomass estimation

Biomass was estimated in terms of dry weight and total cell protein. To determine the dry weight, the fermentation media was withdrawn at regular intervals of time and the turbidity was evaluated at 600 nm. From the absorbance, the dry weight was determined from the standard curve of absorbance vs. dry weight. Total cell protein was measured using the method of Herbert et al. (1971) which is essentially a modified Lowry's method and expressed as total cell protein (µg/ml). 0.5 ml of the sample was added to a test tube containing 0.5 ml of 1N NaOH. The tubes were closed with aluminium foil and the contents were boiled for 5 min in a water bath at 100°C. The mixture was cooled rapidly under tap water and 2.5 ml of reagent (0.5% sodium carbonate + 0.5% cupric sulphate solution in 1% sodium potassium tartarate). The tubes were kept for 10 min which was followed by addition of 0.5 ml of Folin's reagent. A blank containing 0.5 ml of distilled water instead of sample and a set of standard protein (bovine serum albumin) solutions were treated in the same way including the heating stage. After keeping for 30 min, allowing full colour development, the absorbance was measured at 750 nm in UV-visible spectrophotometer against the blank.

Enzyme protein

Protein content in the sample was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard and the values were expressed in (µg/ml).

RESULTS AND DISCUSSION

The microbial production of the enzyme depends on the genetic nature of the organism, the physio-chemical parameters, the fermentation medium components and their concentration. Hence, optimization of the above conditions is important to get maximal yields and to develop effective bioprocess system for industrial application. Many authors reported increased enzymes yield upon optimization of bioprocess conditions (Iyer and Singhal, 2008; 2009; 2010).

In the preliminary stage, a suitable fermentation medium or production medium was selected for the novel isolate *V. azureus* JK-79. Five different medium as given above were evaluated for the L-glutaminase production. Among the five media, maximal L-glutaminase production (240 U/ml), biomass (0.4561 g/l), enzyme protein (130 µg/ml) and total cell protein (170 µg/ml) were obtained from sea water glutamine (Figures 1 and 2) compared to other medium. Hence, it was preferred as optimum medium for the production of L-glutaminase.

Effect of pH on the medium

The pH of the fermentation medium is reported to influence the growth of any microbial strain and subse-

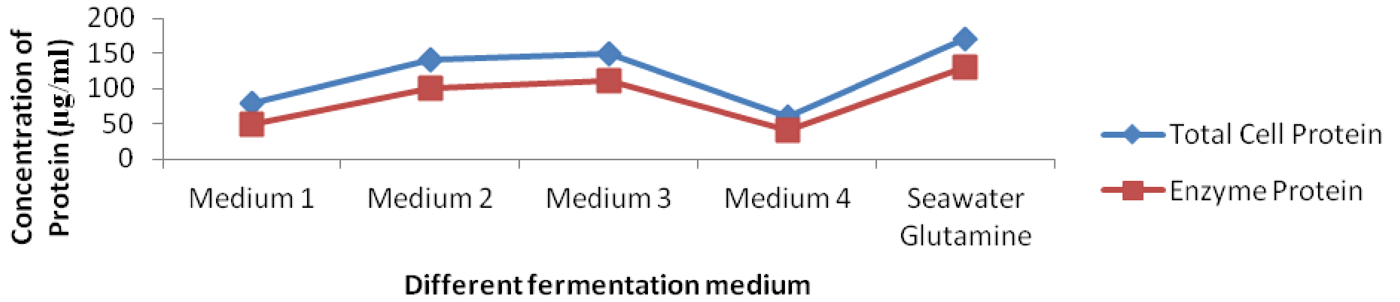

Figure 2. Total cell protein (TCP) and enzyme protein (EP) concentration on various fermentation media during the production of L-glutaminase from *Vibrio azureus* JK-79.

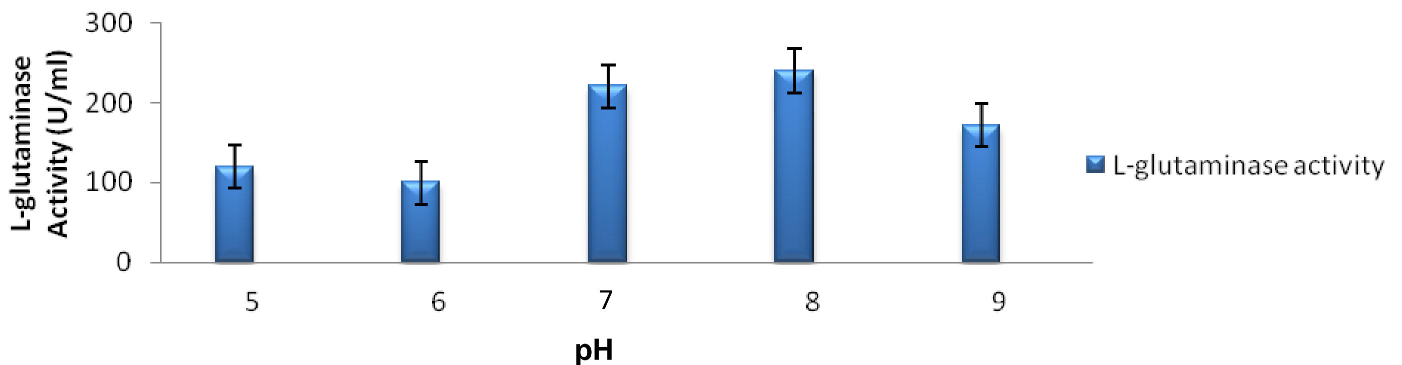

Figure 3. Effect of pH on enzyme production at 37°C and 120 rpm.

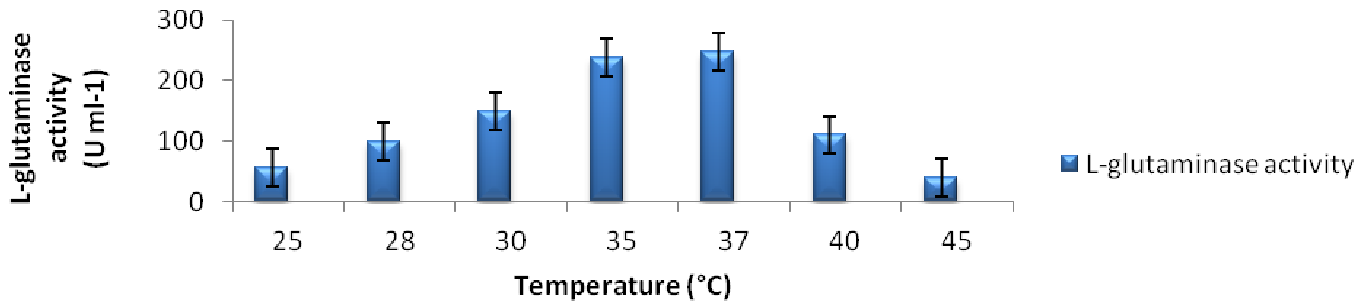

Figure 4. Effect of temperature on L-glutaminase production from *Vibrio azureus* JK-79 at pH 8 and 120 rpm.

quent metabolic product formation. In general, L-glutaminase production by most of the microbial organisms under submerged fermentation conditions is observed to be optimum in the pH range 5.0 to 9.0. The results presented in Figure 3 indicate that pH of the fermentation medium influence the enzyme production. Thus maximal enzyme production was observed at pH 8.0 (241.56 U/ml). Either increase or decrease in the pH of the medium resulted in decreased enzyme production. Results also suggest this marine bacterium is alkalophilic in nature.

Effect of temperature

Incubation temperature influenced the rate of L-glutaminase production by marine *V. azureus* JK-79. Thus the maximal enzyme production (242 U/ml) was observed at 37°C. Variation in temperature in either way resulted in decrease of L-glutaminase production (Figure 4). The loss of activity is more at higher temperature when compared to the lower temperatures. Based on the literature, the optimum temperature for L-glutaminase production is varied with micro-organism used. It was

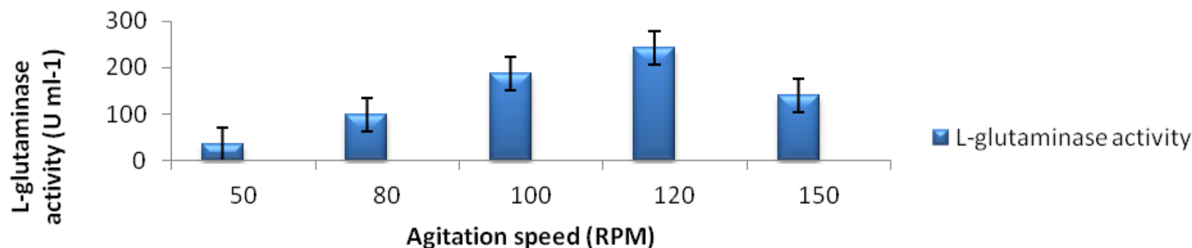

Figure 5. Effect of agitation speed on enzyme production at 37°C and pH 8.

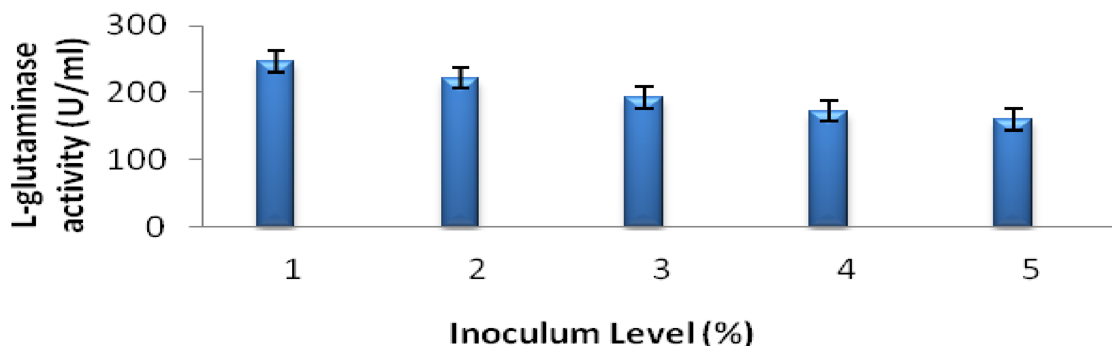

Figure 6. Effect of different inoculums levels on enzyme production at optimal conditions of pH 8, 120 rpm and 37°C.

observed that 27°C was optimum for the enzyme production by *Beauveria* sp. BTMP S10 (Keerthi et al., 1999) and *Streptomyces rimosus* (Siva Kumar et al., 2006).

Effect of agitation

The initial inoculums size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation (Subba et al., 2009). To evaluate same, experiments were planned with increasing inoculums concentration from 1 to 5%. The results (Figure 5) indicate that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (246 U/ml) was observed at 1% initial inoculums supplementation.

Effect of inoculum size

The initial inoculum size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation (Subba et al., 2009). To evaluate same, experiments were planned with increasing inoculums concentration from 1 to 5%. The results (Figure 5) indicated that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (246 U/ml) was observed at 1% initial inoculums supplementation.

Effect of incubation period

After optimization of various process parameters, a time course experiment was carried out to monitor the rate of L-glutaminase production by this novel isolate. Results presented in Figure 6 evidence that L-glutaminase production increase progressively until 24 h, when maximal enzyme production (246.86 U/ml) was recorded. The observed is in accordance with literature reports, where it is noticed that the optimum incubation period for marine *V. costicola* (Nagendra Prabhu and Chandrasekaran, 2003) were also within 24 h.

Effect of additional carbon sources

Incorporation of additional carbon sources into the enzyme production medium at 1% level, resulted in a significant increase in the enzyme production compared to the control (158 U/ml), which contained only glutamine (Figures 7 and 8). Among the various carbon sources tested, maltose supported maximal enzyme yield (301 U/ml), biomass (0.576 g/l), total cell protein (1020 µg/ml) and enzyme protein (240 µg/ml).

All the other carbon sources also showed considerable

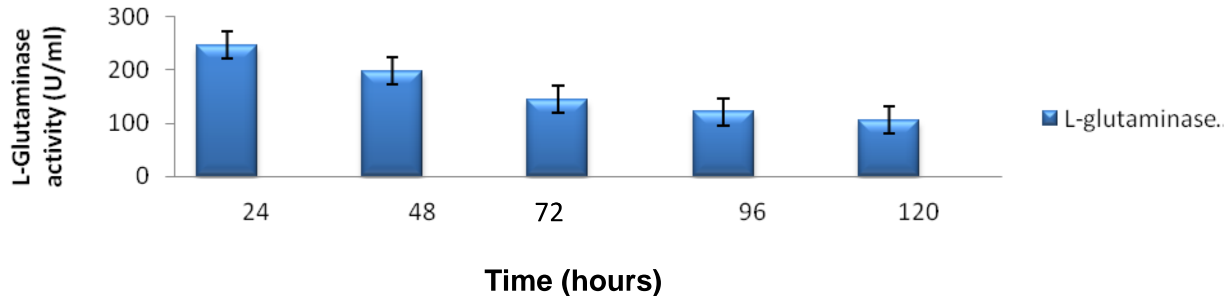

Figure 7. Effect of incubation period on enzyme production.

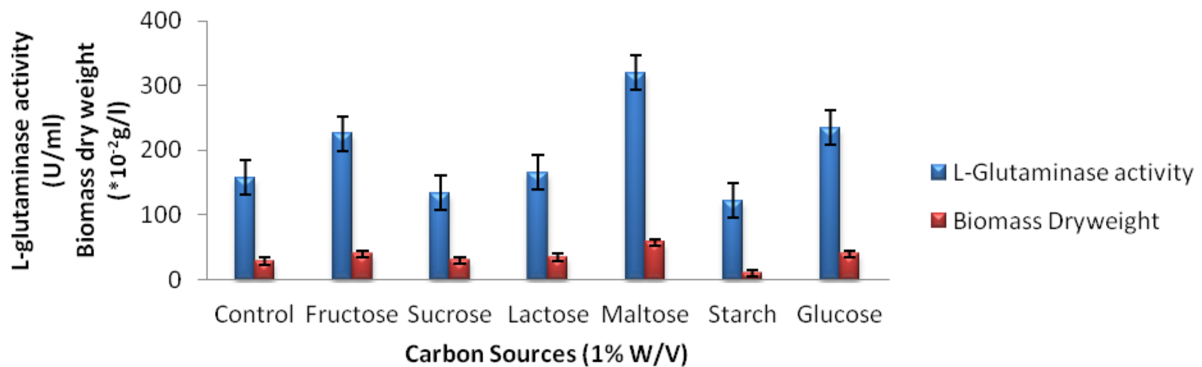

Figure 8. Effect of additional carbon sources on the enzyme production

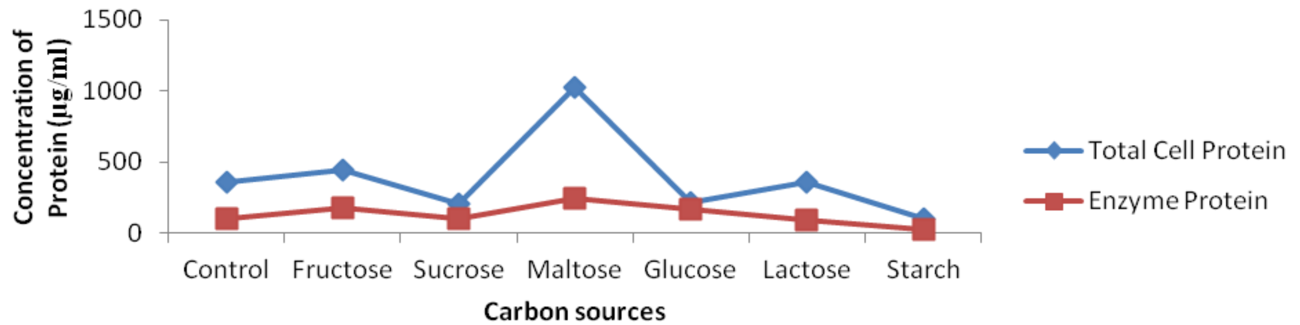

Figure 9. Concentration of TCP and EP in different carbon sources.

amount of enzyme production. Starch was the least effective as a carbon source (122 U/ml). From the result, it was observed that all the other carbon sources except starch (0.102 g/l) supported biomass production, indicating that starch could act as a growth repressor for this marine bacterial strain. The optimum concentration of maltose for maximal enzyme synthesis (321.34 U/ml) was determined to be 1.5% (Figure 9).

Effect of additional nitrogen sources

Incorporation of additional nitrogen sources, along with glutamine in the enzyme production medium influenced the rate of L-glutamine production by marine *V. azureus* JK-79. From the results presented in Figures 10 and 11, it was inferred that among the organic nitrogen sources tested, soybean meal supported maximum enzyme production (226 U/ml), total cell protein (710 µg/ml) and enzyme protein (360 µg/ml). Further studies on optimization of soybean meal indicated that 2% (w/v) was optimal for maximal enzyme production (289 U/ml) and further increase in the concentration of soybean meal resulted in decrease in enzyme production (Figure 12). From the result (Figure 13), it was observed that none of the inorganic nitrogen sources promoted the enzyme

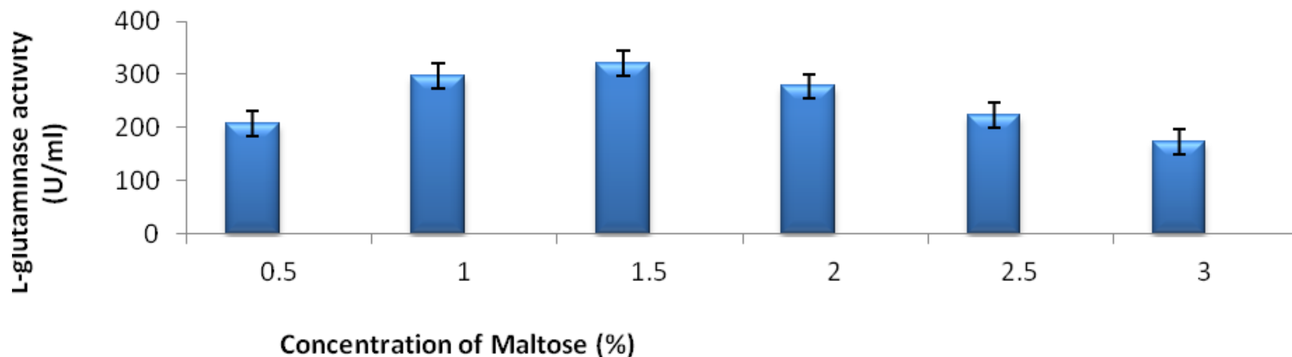

Figure 10. Concentration of maltose on enzyme production.

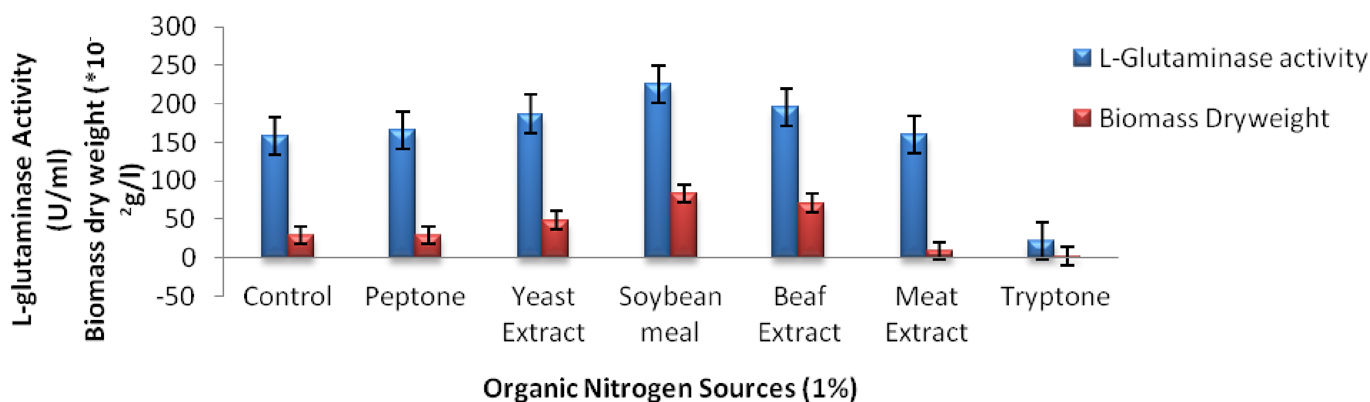

Figure 11. Effect of additional nitrogen sources on enzyme production.

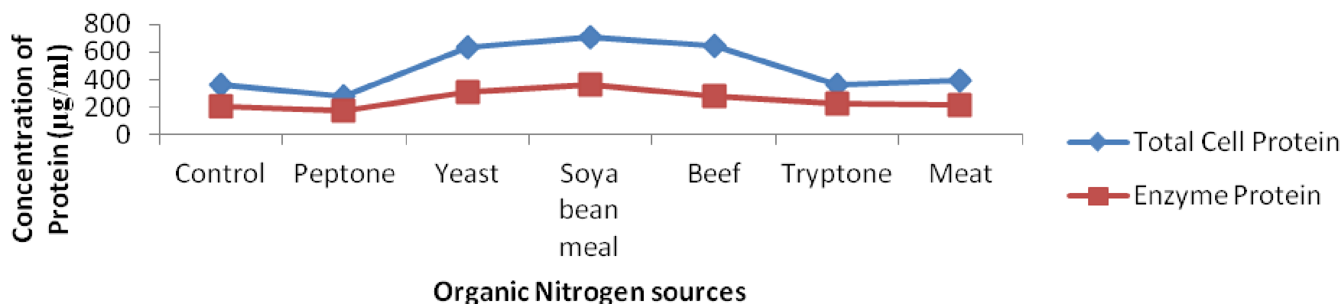

Figure 12. Concentration of TCP and EP on additional nitrogen sources.

synthesis as compared to organic nitrogen source.

Effect of amino acids

Need for amino acids as inducer compound for enhanced enzyme production was evaluated by incorporating different amino acids along with carbon source (D-glucose) in the enzyme production medium. The results presented in Figures 15 and 16 show that L-glutamine supported enhanced level of enzyme production (248 U/ml), total cell protein (195 µg/ml) and enzyme protein (164 µg/ml). None of the amino acids supported either biomass growth or the enzyme production. Since L-glutamine promoted maximal enzyme production compared to other amino acids, optimal concentration of L-glutamine was evaluated. From the results (Figure 17), it was evident that 2% concentration promoted maximal enzyme

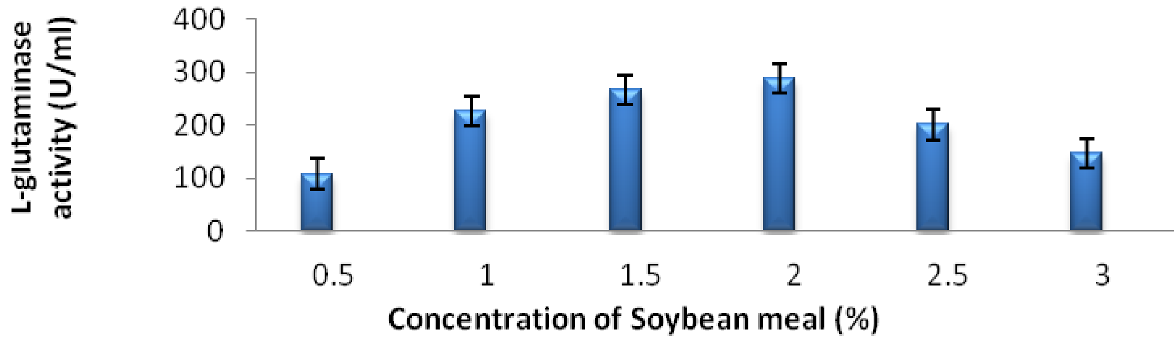

Figure 13. Effect of different concentration of soybean meal on enzyme production.

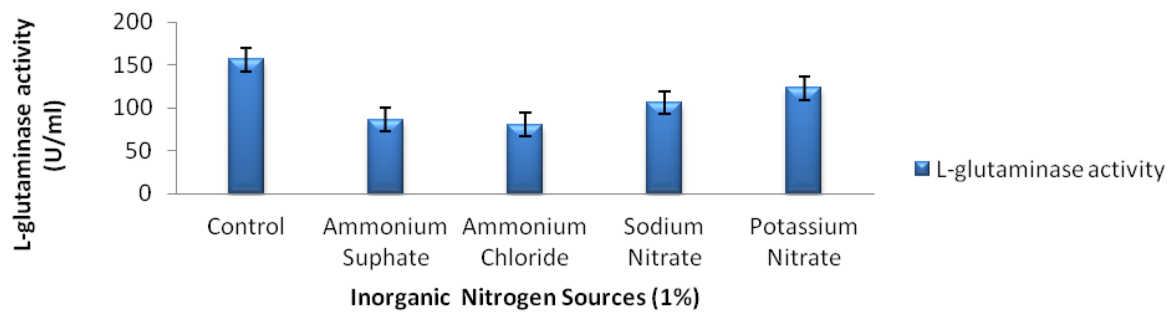

Figure 14. Effect of different inorganic nitrogen sources on enzyme production.

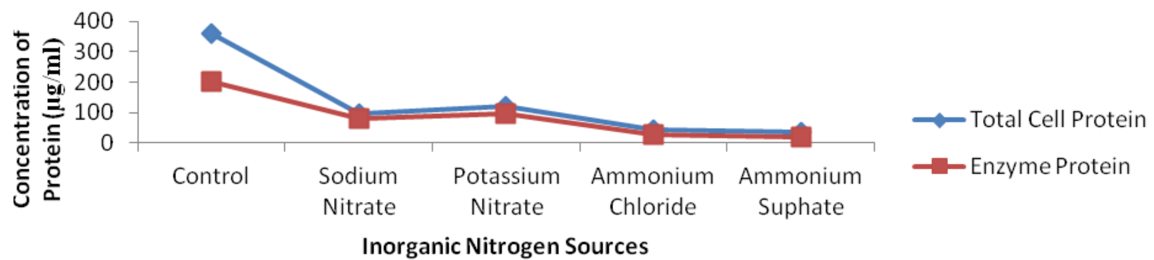

Figure 15. Concentration of TCP and EP on different inorganic nitrogen sources.

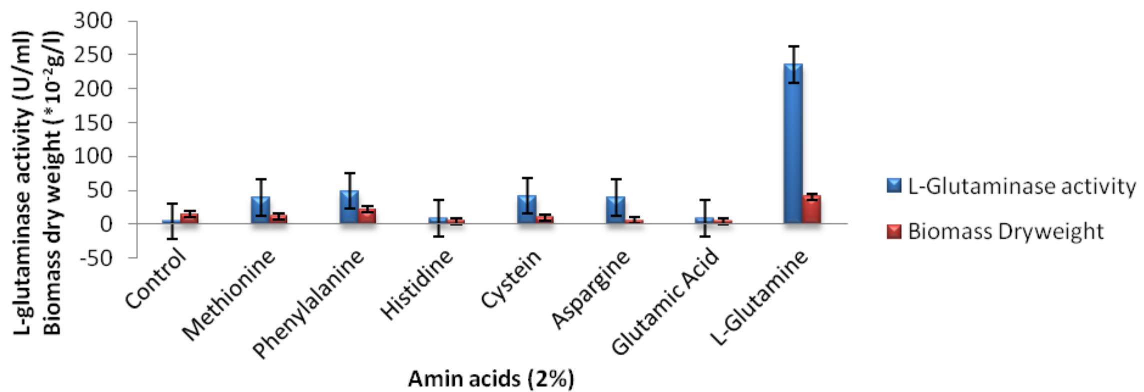

Figure 16. Effect of amino acids on enzyme production.

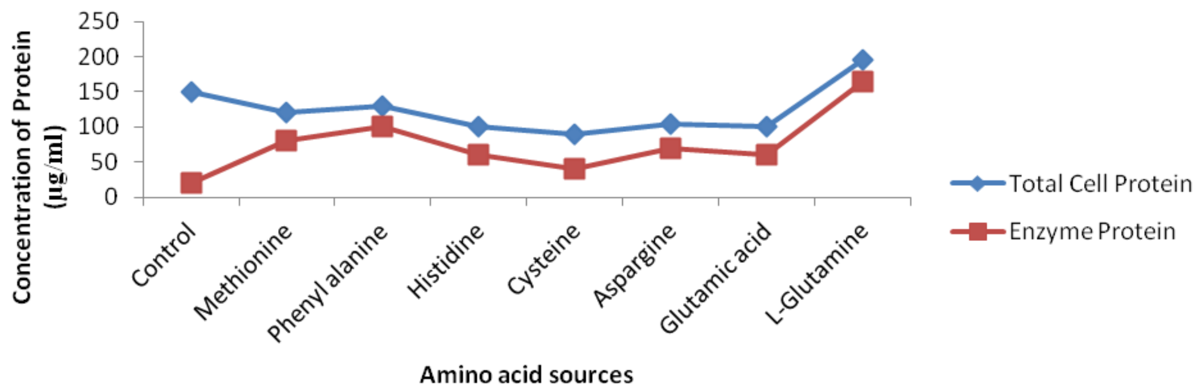

Figure 17. Concentration of TCP and EP on various amino acids.

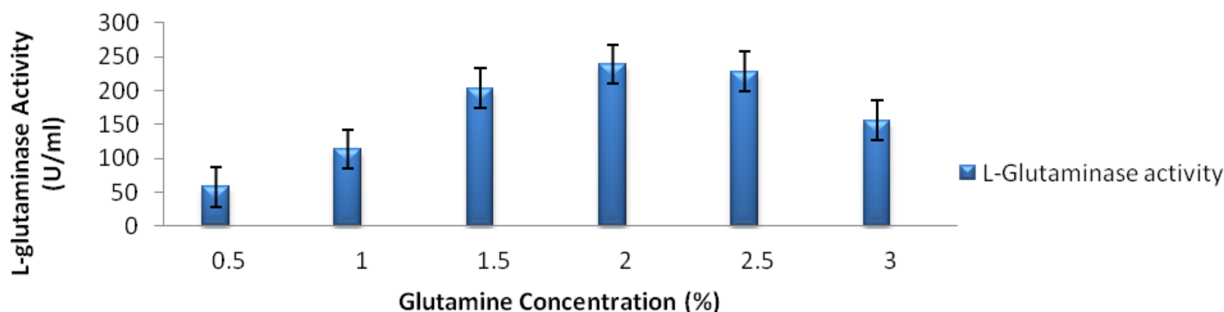

Figure 18. Effect of different glutamine concentration on enzyme production.

production (249 U/ml). Further, in general concentration upto 2% were observed to support the enhanced enzyme production.

Conclusion

The potential of isolated novel strain *V. azureus* JK-79 for L-glutaminase production was analyzed under sub-merged fermentation with different process parameters and medium constituents. Maximum production was noticed at pH 8, 37°C, 120 rpm, with 1% inoculums size, 2% glutamine concentration, 1.5% maltose, 2% soybean meal and 24 h of incubation period. Under optimal conditions, the glutaminase production improved to 321 U/ml. The results of the present study indicate this novel strain has immense potential as an industrial organism for the production of L-glutaminase as extracellular enzyme employing submerged fermentation. Further, it was observed that from the course of the present study, sea water could be used as an ideal fermentation medium for L-glutaminase production.

REFERENCES

Chandrasekaran M (1997). Industrial enzymes from marine microorganisms- Indian scenario. J Mar Biotechnol. 5:86-89.

Herbert D, Phipps PJ, Strange PE (1971). Chemical analysis of microbial cells. Methods Microbiol. VB:249-344.

Huang YL, Khoo SB, Yap MGS (1995). determination of glutamine in mammalian-cell culrures with a flow-injection analysis wall-jet electrode system. Anal. Lett. 28 (4):593-603.

Imada A, Igarasi S, Nakahama K, Isono M (1973). Asparaginase and glutaminase activities of microorganisms. J. Gen. Microbiol. 76:85-99.

Iyer P, Singhal RS (2008). Production of Glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology, Bioresour. Technol. 99:4300-4307.

Iyer P, Singhal RS (2009). Screening and selection of Marine isolate for L-glutaminase Production and Media Optimization Using response surface Methodology'. Appl biochem biotechnol, 159:233-250.

Iyer P, Singhal RS (2010). Glutaminase production using *Zygosaccharomyces rouxii* NRRRL-Y 2547: effect of aeration, agitation regimes and feeding strategies. Chem. Eng. Technol. 33:52-62.

Keerthi TR, Suresh PV, Sabu A, Rajeevkumar S, Chandrasekaran M, (1999). Extracellular production of L-glutaminase by alkalophilic *Beauveria bassiana* BTMF S10 isolated from marine sediments. W. J. Microbiol. Biotechnol. 15:751-752.

Kiruthika J, Saraswathy N (2013). Selective isolation and Molecular identification of L-glutaminase producing marine bacteria. Res. J. Biotechnol. 8(8):64-69.

Kumar SR, Chandrasekaran M (2003). Continuous production of L-glutaminase by an immobilized marine *Pseudomonas sp* BTMS-51 in a packed bed reactor. Proc. Biochem. 38:1431-1436.

Lowry OH, Rosebrough NN, Farr AL, Randall RY (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193:265-275.

Mulchandi A, Bassi AS (1996). Determination of Glutamine and glutamic acid in mammalian-cell cultures using tetrahiafulvalene modified enzyme electrodes. Biosensor. Bioelectron. 11(3) :271-280.

Nagendraprabhu G and Chandrasekaran M, (1995). Polystrene an inert carrier for L-glutaminase production by marine *Vibrio costicola* under solid state fermentation. World. J. Microbial. Biotechnol. 11:683-684.

Nandakumar R, Yoshimune K, Wakayama M, Moriguchi M (2003). Microbial Glutaminase: biochemistry, molecular approaches and applications in the food industry. J. Mol Cat b: enzymatic. 23:87-100.

Prabhu GN, Chandrasekaran M (1995). Polystrene- an inert carrier for glutaminase production by marine *Vibrio costicola* under solid state fermentation. World J. Microbiol Biotechnol. 11:683-684.

Prabhu GN, Chandrasekaran M (1996). L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using different substrate. J. Marine Biotechnol. 4:176-179**.**

Renu S, Chandrasekaran M (1992). Extracellular L-glutaminase production by marine bacteria. Biotechnol Lett. 14:471-74.

Roberts J, Holcenberg JS, Dolowy WC (1970). Antineoplastic activity of highly purified bacterial glutaminase. Nature 227:1136-1137.

Roberts J, Holcenberg JS, Dolowy WC (1972). Isolation, Crystallization and properties of *Achromobacteraceace* glutaminase-asparginase with antitumor activity. J. Biol. Chem. 247:84-90.

Sabu.A, (2003). Sources, properties and applications of microbial therapeutic enzymes. Ind .J. Biotechnol. 2:334-341.

Sato I, Kobayashi H, Hanya Y, Abe K, Murakami S, Scorzetti G, Fell JW, (1999). *Cryptococcus nodaensis* sp nov, a yeast isolated from soil in Japan that produce a salt-tolerant and thermostable glutaminase. J. Ind. Microbiol. Biotechnol. 22:127-132.

Siva kumar K, Sahul MK, Manivel PR, Kannan L, (2006). Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, Chanos chanos (Forskal, 1775). Ind. J. Exp. Biol. 44:256-258.

Subba Rao CH, Sathish T, Ravichandra P, Kumar TP, Prakasham RS, (2009). Characterization of thermo and detergent stable serine protease from isolated *Bacillus circulans* and elevation of eco-friendly applications. Proc. Biochem. 44:262-268.

Tanaka S, Robinson Ea, Appellla E, Miller M, Ammon HL, Roberts J, weber IT, Wolodawer A (1988). Structures of amidohydrolases aminoacid sequence of a glutaminase-asparginase from *Acientobacter glutaminasificans* and preliminary crystallographic data for an asparginase from *Erwinia crysanthemi*. J. Biol. Chem. 263(18):8583-8591.

Wakayama M, Yamagata T, Kamemura A, Bootim N, Yano S, Tachiki T, Yoshimune K, Moriguchi M (2005). Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. J. Ind. Microbiol. Biotechnol. 32:383-390.

Yokotsuka T (1985). Fermented protein foods in the Orient, with emphasis oh shoy and miso in Japan. Microbiology and Fermented Foods. Wood B.J.B (ed) Elseiver Applied Science, London. 197-247.