academicJournals

Vol. 9(9), pp. 588-597, 4 March, 2015
DOI: 10.5897/AJMR2014.7317
Article Number: ED2819151033
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimization of L-glutaminase synthesis by *Aspergillus oryzae* NRRL 32657 in submerged culture

Ahmed M. Alian, Wael A. Bazaraa*, Nagwa M. El- Shimi and Reda M. Mohamed

Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

Received 2 December, 2014; Accepted 9 February, 2015

Various fungal strains have been screened for L- glutaminase synthesis. *Aspergillus oryzae* NRRL 32657 proved to be the highest producer (24.19 U.ml^{-1}) with the largest pink zone diameter (8 mm). Production of L- glutaminase (E.C. 3.5.1.2. L-glutamine amidohydrolase) by *A. oryzae* in batch submerged system was optimized. Several factors: incubation period, source and concentration of carbon, source and concentration of nitrogen, initial pH level, incubation temperature, amino acids source and concentrations and sodium chloride concentration were tested for their effect on enzyme synthesis. The highest L- glutaminase synthesis (46.53 U. ml^{-1}) was achieved when *A. oryzae* was allowed to grow aerobically (30ºC for 3 days) in a buffered medium (pH 7.0) containing 2.5% lactose, 2% yeast extract, 0.5% L- glutamine and 0.5% sodium chloride.

Key words: L-glutaminase, *Aspergillus oryzae*, submerged fermentation, optimization.

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Iyer and Singhal, 2009). The action of glutaminase plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes (Nandakumar et al., 2003). Several species of bacteria: *Pseudomonas* sp, *Vibrio costicola* and *V. cholerae* (Renu and Chandrasekaran, 1992), *Vibrio* sp. M9 (Durai et al., 2014), *Bacillus subtilis* and *B. licheniformis* (Cook et al., 1981), *Beauveria* sp. (Sabu et al., 2000), and *Micrcoccus luteus* (Moriguchi et al., 1994); actinomycetes (Teja et al., 2014) and *Streptomyces rimosus* (Sivakumar et al., 2006); molds: *A. oryzae* (Chou et al., 1993), *A. wentii* (Siddalingeshwara et al., 2011), *A. flavus* (Nathiya et al., 2011b) and *Trichoderma koningii* (Pallem et al., 2010) and yeasts: *Debaryomyces* sp. (Durá et al., 2002), *Zygosaccharomyces rouxii* (Kashyap et al., 2002; Iyer and Singhal, 2008 and 2010) were reported to produce L- glutaminase. Glutaminases are classified into two types: intracellular and extracellular and the majority is extracellular (Kashyap et al., 2002; Iyer and Singhal, 2008 and 2010). Recently, microbial L-glutaminases have found several potent applications in various industrial sectors. The enzyme, though originally identified as a potent anti-cancer drug with possible applications in enzyme therapy (Sabu et al., 2000), has been used in food industry to enhance flavor and as a replacement of monosodium glutamate specially in Chinese foods (Wakayama et al., 2005).

Monosodium glutamate (MSG) gives the taste “umami”

*Corresponding author. E-mail: waelbazaraa@hotmail.com.

which has been widely recognized as the fifth basic taste besides sweet, acid, salty and bitter. It has been widely used as a flavor enhancer in the food industry. However, there are some questions about its safety, since it may cause some side effects for some people such as wheezing, changes in heart rate and difficulty in breathing (Farombi and Onyema, 2006). Therefore, the need to develop a safer natural flavor enhancer as an alternative to MSG has been increased. In the present study, a stepwise optimization process for fermentation parameters to maximize L-glutaminase production by *A. oryzae* NRRL 32657 has been conducted.

MATERIALS AND METHODS

Fungal strains

The investigated strains: *A. oryzae* NRRL 5590, *A. oryzae* NRRL 32614, *A. oryzae* NRRL 32657, *A. parasiticus* NRRL 1988, *Zygosaccharomyces rouxii* NRRL Y-12622 and *Z. rouxii* NRRL Y-2547 (Northern Regional Research Laboratories, Peoria, IL, USA) and *Saccharomyces cervicea* (Department of Microbiology, Faculty of Agriculture, Cairo University, Giza.) were allowed to grow on malt extract agar (Merck, Darmstadt, Germany) at 30ºC for 3 days. Cultures were then stored in a refrigerator at 4ºC till use. Cultures were activated monthly on malt extract agar.

Spores suspension

For the preparation of *A. oryzae* NRRL 32657 spores, the mold was inoculated on malt extract agar slants and incubated for 5 days at 30°C. After incubation, spores were scrapped and inoculated into 50 ml of saline solution containing of 0.1% tween 80. The collected spores were microscopically counted (1.5×10^7 spores.ml^{-1}), stored at 4°C and utilized as stock inoculum.

Media

Screening medium

The glutamine agar medium (GAM) (Siddalingeshwara et al*.,* 2010) was utilized for the production of the enzyme and it contained (gL^{-1}): glutamine, 10; KH_2PO_4, 0.1; K_2HPO_4, 1.0; NaCl, 0.5; Mg $SO_4.7H_2O$, 1.0; phenol red indicator, 0.125 and agar 15.

L-glutaminase production medium

The medium used for growth and production of glutaminase was referred to Wakayama et al*.* (2005). The medium contained (gL^{-1}): yeast extract, 17.5; glucose, 20; glutamine, 5; KH_2PO_4, 1.5; K_2HPO_4, 3; NaCl, 5 and Mg $SO_4.7H_2O$, 5 at pH 7.0. Fifty milliliters of the medium were placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. The sterilized flasks were inoculated with 1 ml of the previously prepared spores suspension, placed in a rotary shaker (100 rpm) and the growth was aerobically carried out at 30ºC for 3 days. At the end of the incubation period, the mycelia were recovered from each flask by filtration on Whatman no.1 (Whatman Ltd., Maidstone, England) and used for determination of biomass (mg.ml^{-1}). Glutaminase activity was then determined in the culture filtrate.

Methods

Dry weight of the mold mycelia

After filtration, mycelia were washed twice with distilled water and dried at 70°C to constant weight.

Assay of L-glutaminase

L-Glutaminase activity was determined using L-glutamine as substrate and the released ammonia was measured using Nesseler's reagent. 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 phosphate buffer (0.1 M, pH 7.0). Then the mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 0.5 ml of 1.5 M trichloro acetic acid. 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 430 nm using a spectrophotometer, Model 6300 (Jan way LTD., Essex, U.K). A standard graph was plotted using ammonium chloride (Imada et al*.,* 1973). One international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmol of ammonia under optimum conditions.

Factors regulating glutaminase production

Incubation period

To determine the optimum incubation period for glutaminase production, fermentation flasks were incubated for different time durations (1 to 7 days) and then enzyme activity was daily analyzed.

Carbon source

Glucose, fructose, sucrose, lactose, maltose, mannitol and dextrin (1%, each) were separately added to the fermentation medium.

Carbon source concentration

Different levels of the best carbon source "lactose" (0.5 to 4.0%) were tested.

Nitrogen source

Organic source (peptone, tryptone, yeast extract, beef extract and urea containing 15, 13.2, 9.5, 12.5 and 46.6 % nitrogen, respectively) and inorganic source (ammonium nitrate, sodium nitrate, ammonium sulfate and ammonium chloride) were evaluated for their effect on growth of the tested strain and glutaminase synthesis. The amounts of nitrogen sources were adjusted to give final nitrogen concentration of 0.2%.

Nitrogen source concentration

To determine the optimum concentration of the best nitrogen source, different levels (0.5 to 3.5%) of yeast extract were added to the growth medium.

Effect of temperature

The effect of temperature on glutaminase production was studied

Table 1. Screening of different strains for their glutaminase activity

Strains	Enzyme activity (U.ml^{-1})	Pink zone (mm)
Aspergillus parasiticus NRRL 198	19.36^{b*}±0.03	5.00^{b}±0.06
Aspergillus oryzae NRRL 32614	4.96^{e}±0.03	2.00^{f}±0.03
Aspergillus oryzae NRRL 32657	24.19^{a}±0.10	8.00^{a}±0.01
Aspergillus oryzae NRRL 5590	10.73^{c}±0.00	4.00^{c}±0.00
Zygosaccharomyces rouxii NRRL Y-12622	11.50^{c}±0.02	4.00^{c}±0.05
Zygosaccharomyces rouxii NRRL Y-2547	8.23^{d}±0.00	3.00^{d}±0.02
Saccharomyces cerevisiae	5.19^{e}±0.01	2.50^{e}±0.01

* Means followed by different superscripts within columns are significantly different at the 5% level.

by carrying out the growth at different temperatures (20, 30 and 40°C).

Effect of pH

To study the effect of the initial pH values of the medium on strain growth and glutaminase activity, different pH levels were tested (3, 4, 5, 6, 7, 8, 9 and 10).

Effect of amino acids

Different amino acids (0.5%, w/v) that is glutamine, asparagine, arginine, lysine, glycine, proline, methionine, tryptophan and glutamic acid were separately added to the growth medium with and without yeast extract, tested for their effect on the enzyme synthesis and different levels (0.02 to 0.19% nitrogen) of the best amino acid (glutamine and glutamic acid) without yeast extract were tested.

Effect of sodium chloride concentration

Impact of sodium chloride on enzyme production was determined by adding different concentrations of NaCl (0.25-20 %, w/v) to the fermentation medium.

RESULTS AND DISSCUSSION

Screening of different fungal strains for glutaminase activity

The seven tested strains were screened for their abilities to produce glutaminase. Such screening of strains was based on the semi qualitative method (Katikala et al*.,* 2009; Siddalingeshwara et al*.,* 2010). Cultures were grown on the glutamine media supplemented with a dye indicator (phenol red). The indicator is pH sensitive. Normally it gives yellow color to media in acidic and neutral conditions and gives the pink color to the media when the pH changes from acidic to alkaline condition. The pink zone around colony indicates the pH alteration which originated from ammonia accumulation in the medium. The results from plate assay and the determination of enzyme activity in the culture filtrate are presented in Table 1.

The obtained results show that different strains varied in their ability to produce glutaminase after three days of cultivation. The highest glutaminase activity was recorded for *A. oryzae* NRRL 32657 (24.19 U.ml^{-1}) followed by *A. parasiticus* NRRL 1988 (19.36 U.ml^{-1}), *Z. rouxii* NRRL Y-12622 (11.50 U.ml^{-1}) and *A. oryzae* NRRL 5590 (10.73 U.ml^{-1}), while the lowest was for *A. oryzae* NRRL 32614 (4.96 U.ml^{-1}). Therefore, *A. oryzae* NRRL 32657 was selected for further experiments. With respect to the relation between the qualitative and quantitative methods, the obtained results indicate a high correlation (r = 0.97) between both techniques. Therefore, the pink zone qualitative detection method could give an indication about the approximate glutaminase activity. These results are in agreement with those obtained by Katikala et al. (2009) and Siddalingeshwara et al. (2010) who used both methods to test the ability of different strains for production of glutaminase.

Factors regulating glutaminase production

Effect of incubation period

Enzyme activity was measured every 24 h time intervals (Figure 1). With regard to the different incubation periods, the maximum enzyme production (24.5 U.ml^{-1}) was noted at the 3rd day of fermentation and the activity gradually decreased after that till the end of the incubation period reaching 44.9% of the maximum.

Such reduction in activity was probably due to presence of proteolytic activity which was able to degrade the L- glutaminase present in medium (Kashyap et al*.,* 2002). Also, organism might have reached a stage from which it could no longer balance its steady growth with the availability of nutrient resources (Siddalingeshwara et al*.,* 2011). Similarly, 3 day of fermentation was the time of choice to maximize glutaminase production by *Aspergillus* sp. (Prasanth et al*.,* 2009) and *Aspergillus wentii* (Siddalingeshwara et al*.,* 2011).

Effect of carbon source

Varying the carbon source in culture medium affected

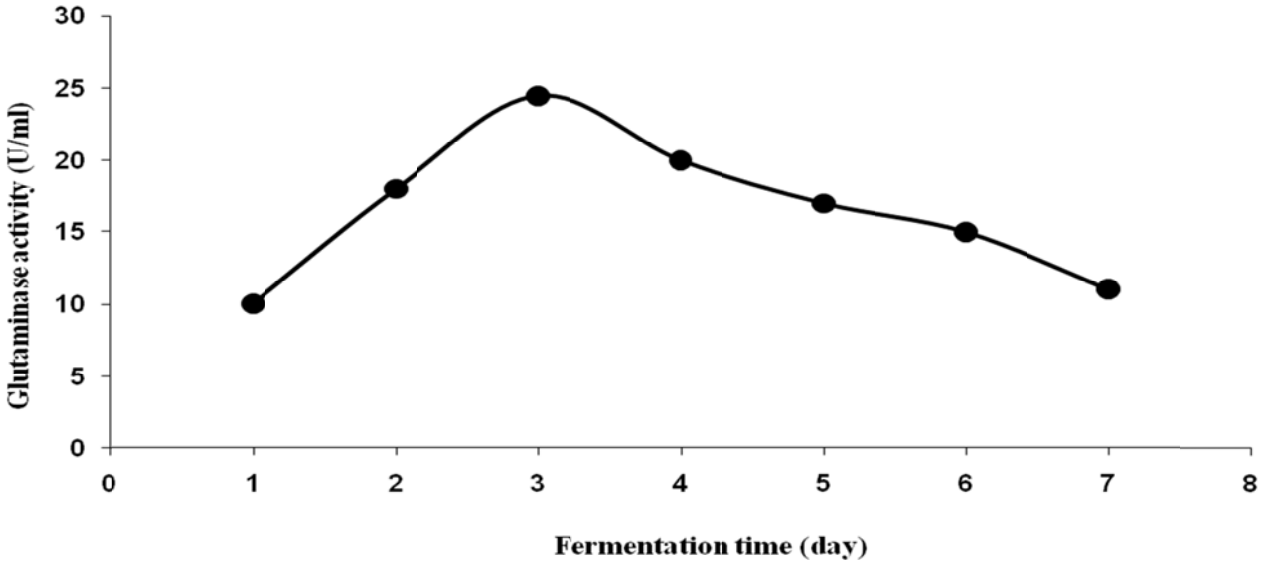

Figure 1. Effect of incubation period on glutaminase production by *Aspergillus oryzae* NRRL 32657.

Table 2. Effect of different carbon sources on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Carbon source	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) * ±SD
Glucose	8.0	9.16	15.24^{d}±0.1
Fructose	8.2	11.62	15.50^{d}±0.3
Sucrose	8.2	11.24	11.97^{f}±0.3
Maltose	8.0	13.78	14.86^{e}±0.4
Lactose	9.0	5.38	38.97^{a}±0.1
Mannitol	8.6	6.70	32.24^{b}±0.5
Dextrin	8.6	7.66	21.40^{c}±0.1

*Means followed by different superscripts within columns are significantly different at the 5% level.

glutaminase production by *A. oryzae*. Results (Table 2) show no relationship between biomass and glutaminase synthesis but the highest pH value (9) was obtained at the highest enzyme activity, while the lowest pH values (8.0 and 8.2) were obtained at the lowest recorded enzyme activity. Maximum glutaminase activity (38.97 U.ml^{-1}) was obtained with lactose followed by that obtained with mannitol (32.24 U.ml^{-1}).

It is of interest to report that both activities were achieved with slowly assimilated sugars (lactose and mannitol). On contrary, the presence of fast metabolized sugars such as glucose, fructose, sucrose and maltose resulted in catabolite repression and therefore, the glutaminase synthesis was reduced to 39.1, 39.8, 30.7 and 38.1% of the maximum obtained activity (38.97 U.ml^{-1}), respectively. The utilization of dextrin as carbon source resulted in an intermediate activity (54.9% of the maximum) and probably due to the enzymatic hydrolysis of dextrin to units of glucose which resulted in catabolite repression. Similarly, Yuasa et al. (2003) reported that the use of relatively slowly assimilated carbon sources such as lactose, mannitol and sorbose eliminated the catabolite repression occurred with the use of fast assimilated carbon sources such as glucose. Also, Prasanth et al. (2009) reported that the replacement of glucose with lactose improved the glutaminase synthesis by *Aspergillus* sp. from 22.97 to 27.64 U.ml^{-1}. On the other hand, carbon sources such as glucose and maltose enhanced glutaminase production by *Trichoderma koningii* in solid-state fermentation using sesamum oil cake (Pallem et al., 2010).

Effect of lactose concentration

The obtained results (Figure 2) indicate that lactose concentrations affected biomass as well as L- glutaminase activity.

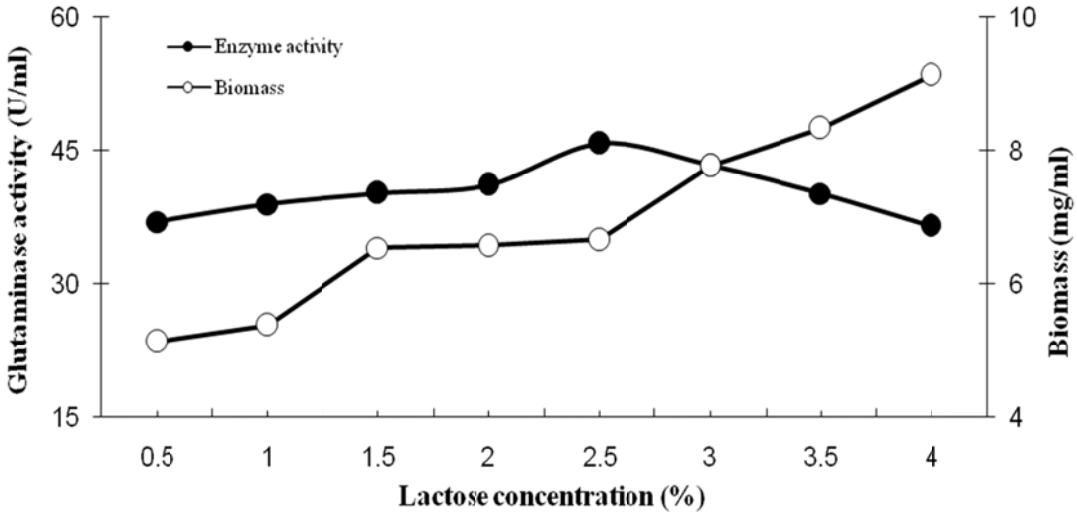

Figure 2. Effect of lactose concentrations on biomass and glutaminase produced by *Aspergillus oryzae* NRRL 32657.

Table 3. Effect of different nitrogen sources on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Nitrogen source	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) * ±SD
Peptone	8.4	5.84	27.32^{c}±0.04
Tryptone	8.8	7.57	35.50^{b}±0.01
Yeast extract	9.0	6.66	45.78^{a}±0.01
Beef extract	9.0	5.21	20.12^{d}±0.03
Urea	8.6	3.85	17.50^{e}±0.02
$(NH_4)_2SO_4$	7.6	3.16	5.35^{h}±0.01
NH_4NO_3	8.6	4.16	10.42^{g}±0.00
$NaNO_3$	9.0	4.23	13.12^{f}±0.00
NH_4Cl	7.0	2.18	3.37^{i}±0.03

*Means followed by different superscripts within columns are significantly different at the 5% level.

Gradual increase in biomass from 5.13 to 9.13 mg.ml^{-1} was noted when the level of lactose was elevated from 0.5 to 4.0%. Also, a gradual increase in glutaminase activity was noted reaching its maximum value (45.78 U.ml^{-1}) at lactose concentration of 2.5% after which a gradual decrease occurred reaching 36.5 U.ml^{-1} at lactose level of 4.0%. Since 2.5% lactose resulted in the highest enzyme synthesis, such concentration was utilized for further experiments.

Effect of nitrogen source

The changes in biomass, medium pH, and enzyme synthesis are shown in Table 3. The results indicated that, for all tested nitrogen sources, the pH values of the fermentation medium increased at the end of incubation period (3 days) comparing to the initial pH (7.0), except the use of NH_4Cl where the pH level remained constant (7.0). The highest pH value (9) was detected when yeast extract, beef extract and sodium nitrate, were used as to nitrogen source.

Data in the same table clearly show that the presence of organic nitrogen (except urea) in the fermentation medium was more favorable for the growth of *A. oryzae* NRRL 32657. The maximum biomass (7.57 mg. ml^{-1}) was obtained in the presence of tryptone as an organic nitrogen source. The obtained biomass in the presence of yeast extract and peptone was 6.66 and 5.84 mg.ml^{-1}, respectively. However, the obtained biomass in case of using urea was the lowest among the tested organic sources and this was probably due to that urea didn't contain growth factors such as vitamins and minerals as present in peptone, tryptone, beef extract and yeast

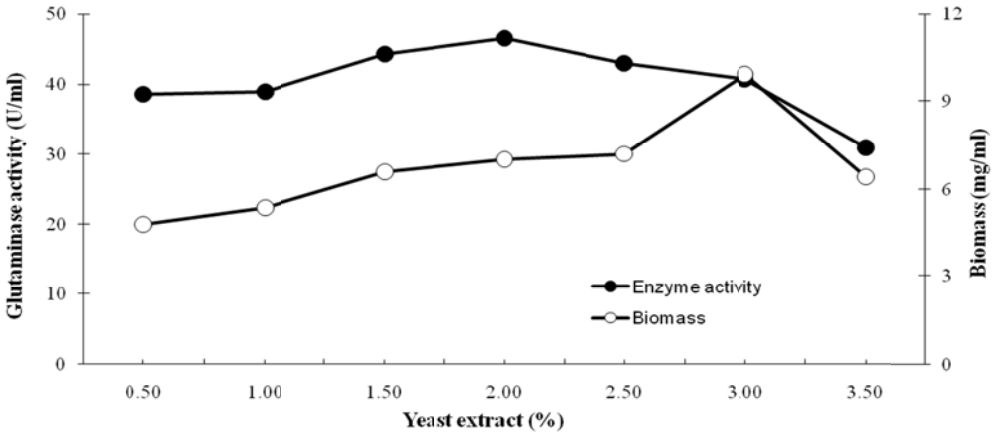

Figure 3. Effect of yeast extract concentrations on biomass and glutaminase produced by *Aspergillus oryzae* NRRL 32657.

extract (Bazaraa and Al-Dagal, 1999). On the other hand, inorganic nitrogen sources did not support good mold growth and the obtained biomass ranged from 2.18 to 4.23 mg. ml^{-1}. Concerning the effect of nitrogen source on glutaminase synthesis, data (Table 3) reveal that, organic nitrogen sources significantly enhanced more enzyme synthesis than the inorganic sources. Among the organic sources, yeast extract was found to be the best nitrogen source for glutaminase production (45.78 U. ml^{-1}) followed in descending order by tryptone (35.50 U. ml^{-1}), peptone (27.32 U. ml^{-1}), beef extract (20.12 U. ml^{-1}) and urea (17.5 U. ml^{-1}) with significant differences between them. These results are in agreement with those reported by Iyer and Singhal (2008), who reported that, the tested organic nitrogen sources were preferable than the inorganic sources and yeast extract was found to be the best nitrogen source for glutaminase synthesis by *Z. rouxii*. . In addition, these results correlate well with those reported by Nathiya et al. (2011a) who indicted that, maximum glutaminase synthesis by *Penicillium brevicompactum* was obtained when yeast extract was used as an organic nitrogen source in fermentation medium. Moreover, Nathiya et al. (2011b) recorded that the maximum L-glutaminase production from *Aspergillus flavus* was achieved when yeast extract was utilized as nitrogen source. Also, Suresh Kumar et al. (2013) reported that the maximum glutaminase yield (25 IU.ml^{-1}) was obtained in the presence of yeast extract. On the other hand, the use of sodium nitrate as the nitrogen source led to the highest glutaminase production by *A.oryzae* (Prasanna and Raju, 2013) and *Streptomyces arvermitilis* (Abdallah et al*.,* 2013). While, Kashyap et al. (2002) and El-Sayed (2009) reported that, the supplementation of agro-wastes with organic sources such as (peptone, yeast extract, malt extract and beef extract) and inorganic sources such as ($(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$) did not improve glutaminase synthesis.

Effect of yeast extract concentration

A positive correlation between biomass and yeast extract concentration was noticed (Figure 3). The obtained biomass increased as yeast extract concentration increased and recorded the highest amount of biomass (9.94 mg. ml^{-1}) in the presence of 3.0% yeast extract and decreased thereafter. Results (Figure 3) also reveal that the synthesis of glutaminase by test organism was influenced by the concentration of yeast extract. The maximum production (46.53 U.ml^{-1}) was achieved at 2.0% yeast extract concentration. Utilizing higher yeast extract concentration above 2% resulted in lower enzyme synthesis, where 42.89, 40.68 and 30.86U.ml^{-1} were obtained at yeast extract concentration of 2.5, 3.0 and 3.5%, respectively. Similar trend for glutaminase production as affected by nitrogen source concentration was obtained by Nathiya et al. (2011a) using *Penicillium brevicompactum*.

Effect of different amino-acids

Amino acids were reported as common growth factors required for the production of enzymes as major nitrogen source (Suresh Kumar et al*.,* 2013). Hence, to study the effect of additional amino acids on glutaminase production, different amino acids were separately added to the medium (Table 4). It was observed that the medium supplementation with different amino acids variously affected the growth, pH and enzyme synthesis. Among the tested amino acids, L-glutamine supplementation followed by glutamic acid resulted in the highest synthesis of L- glutaminase (46.53 and 42.80 U. ml^{-1}, respectively). Results (Table 4) confirm that the addition of the tested amino acids in absence of yeast extract resulted in lower values for final pH, biomass and enzyme activity comparing to in the presence of yeast

Table 4. Effect of different amino acids on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

Amino acids	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1})±SD*	
			With**	without
Glutamine	9.0 (8.6) ***	7.02 (2.07)	46.53^{a}±0.01	24.50 ^{a} ±0.00
Asparagine	8.8(7.8)	5.76 (0.83)	32.04^{c}±0.03	18.40 ^{c} ±0.01
Arginine	8.6(8.0)	6.04 (1.08)	26.20^{d}±0.00	15.20 ^{d} ±0.02
Lysine	8.4(7.5)	6.36 (0.82)	20.80^{e}±0.01	8.90 ^{e} ±0.00
Glycine	9.0(7.5)	6.33(1.64)	13.64^{f}±0.02	6.70 ^{f} ±0.01
Proline	8.6(8.0)	8.14(5.56)	11.59^{g}±0.03	5.80 ^{g} ±0.03
Methonine	8.4(7.5)	5.71(1.20)	20.50^{e}±0.02	8.60 ^{e} ±0.01
Tryptophan	8.4(7.5)	6.36(1.41)	20.31^{e}±0.01	8.30 ^{e} ±0.00
Glutamic acid	8.4(8.0)	7.61(2.08)	42.80^{b}±0.00	20.03 ^{b} ±0.01

* Means followed by different superscripts within columns are significantly different at the 5% level. ** With yeast extract or without yeast extract. *** Data between parentheses were for enzyme production in media without the addition of yeast extract.

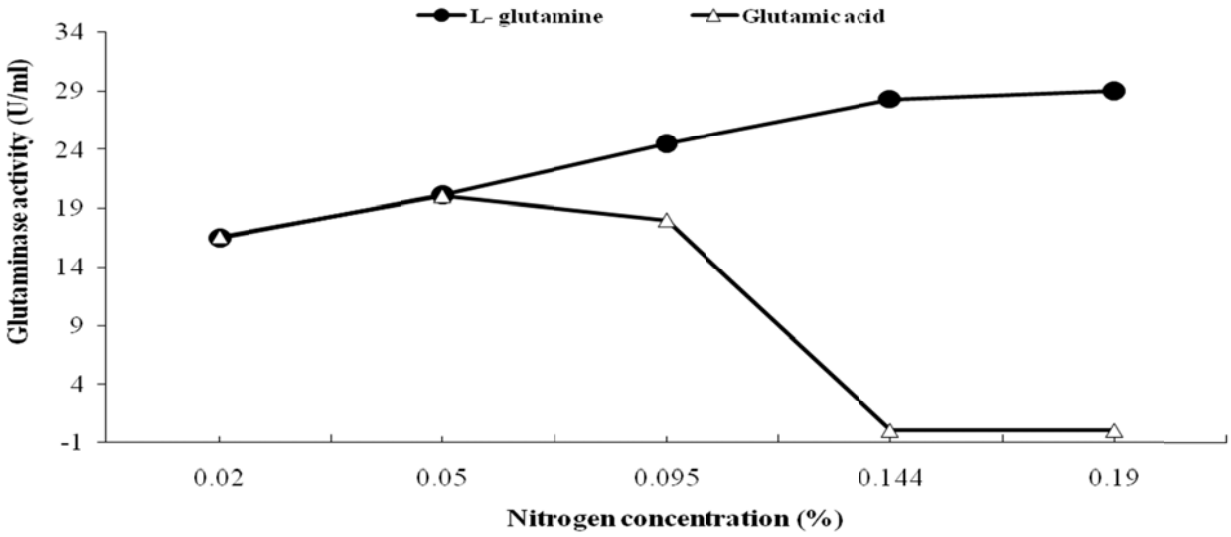

Figure 4. The effect of different glutamine and glutamic acid concentrations (as nitrogen percentage) on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

extract. Data reveal that the biomass ranged from 0.082 to 5.56 mg. ml^{-1} (in absence of yeast extract) and the highest value (5.56 mg. ml^{-1}) was observed in the case of proline. Therefore, the presence of yeast extract in medium was essential for maximum biomass (8.14 mg. ml^{-1}) and glutaminase synthesis (46.53 U.ml^{-1}) since it contained the required growth regulators (Nathiya et al., 2011a). Concerning the effect of amino acids on glutaminase production by *A. oryzae* without the addition of yeast extract, it was found that the highest glutaminase synthesis (24.50 U. ml^{-1}and 20.03 U. ml^{-1}) was obtained with the addition of L- glutamine and glutamic acid, respectively.

To determine the optimal L-glutamine and glutamic acid concentrations for glutaminase activity, various concentrations of nitrogen (ranged from 0.02 to 0.19%) were separately added to the fermentation medium. Results presented in Figure 4 indicate the relation between glutaminase activity and the nitrogen concentration of L-glutamine and glutamic acid. The increase in nitrogen concentration in glutamine from 0.02 to 0.144% led to an increase in glutaminase activity from 16.5 to 28 .3 U. ml^{-1} and persisted after that. On the other hand, in case of the use of glutamic acid the maximum activity (20.03 U. ml^{-1}) was achieved at 0.05% nitrogen, decreased to reach 18.01 U.ml^{-1} at 0.095% nitrogen and a complete loss of activity was noted at the highest tested concentrations (0.144% and 0.190%). Prabhu and Chandrasekaran (1997)

Table 5. Effect of initial pH on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657.

pH	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1}) ±SD*
3	3.0	0.00	0.00^{f}±0.00
4	4.0	0.00	0.00^{f}±0.00
5	8.6	4.51	18.30^{d}±0.02
6	8.6	7.00	29.50^{b}±0.01
7	9.0	7.02	46.53^{a}±0.01
8	9.2	10.01	19.92^{c}±0.00
9	9.0	0.00	0.00^{f}±0.00
10	10.0	0.00	0.00^{f}±0.00

*Means followed by different superscripts within columns are significantly different at the 5% level.

studied the effect of the additional amino acids on glutaminase production by marine *V. costicola* in solid state fermentation. They reported that all tested amino acids (except glutamine), had a negative impact on glutaminase synthesis. El-Sayed (2009) reported that L-glutamine was the best tested amino acid in enhancing glutaminase synthesis by *Trichoderma koningii*. He explained that this result may be due to the action of glutamine as an inducer.

Effect of incubation temperature

Data indicate that, glutaminase synthesis was apparently variant at different temperatures, and at 30°C the maximum glutaminase synthesis (46.53 U. ml^{-1}) was achieved. A sharp reduction in such activity (63.0 and 41.3% considering 46.53 U. ml^{-1} as 100%) was noted when *A. oryzae* was allowed to grow at 20 and 40°C, respectively. On the other hand, maximum growth (7.61 mg ml^{-1}) was achieved at 20°C. Similarly, Kashyap et al. (2002) indicated that maximum glutaminase production (11.61 U per g dry solids) by *Z. rouxii* was obtained when the fermentation was carried out at 30°C. Obtained results agreed well with those reported for glutaminase synthesis by *T. koningii* (EL-Sayed, 2009), *Vibrio* sp. (Prakash et al*.,* 2010) and *A. oryzae* NCIM 1212 (Prasanna and Raju, 2013). On the other hand, incubation temperatures 33 and 40°C were selected as optimal temperature for the production of glutaminase by *T. koningii* and *P. fluorescens* (Pallem et al*.,* 2010; Chitanand and Shete, 2012).

Effect of pH value

Results presented in Table 5 confirm that *A. oryzae* NRRL 32657 grew well in buffered medium that ranged from 5 to 8. Data reveal that the maximal biomass values (10.01 and 7.02 mg. ml^{-1}) were observed at initial pH values 8 and 7, respectively. Meanwhile, pH values 3, 4, 9 and 10 were not suitable for the growth.

The maximum glutaminase production of 46.53 U. ml^{-1} was obtained at the initial pH value of 7.0. This may be attributed to the balance of ionic strength of plasma membrane (El-Sayed, 2009). These results are in coincidence with that reported by Prasanna and Raju (2013). Also, Balagurunathan et al. (2010) stated that the optimum pH value for glutaminase production by *Streptomyces olivochromogenes* was 7.0. Abdallah et al. (2012) reported that, maximum enzyme productivity (13.47 U.ml^{-1}) was recorded at pH 8.0 and no synthesis was detected at pH 3, 4 and 11. On the other hand, Nathiya et al. (2011b) reported that, the maximum glutaminase production by *Aspergillus flavus* (38.69 U/g) was observed at initial pH 4.0 and a loss of more than 50% of enzyme production was observed at initial medium pH of 7.0.

Effect of NaCl concentration

The relation between salt tolerance of the test organism and glutaminase production was studied and results were recorded in Table 6. Increasing NaCl concentrations from 0.25 to 2.0% dramatically affected mold growth (Table 6). A complete inhibition was noted at 17.5% NaCl, while a good resistance was obvious up to 2.5%. On the other hand, glutaminase activity was highest (46.53 U. ml^{-1}) at 0.5% NaCl and decreased thereafter with a complete loss of activity at 17.5% NaCl. Since growth of mold was very limited or non at pH above 10.0. Therefore, no change in pH level was detected. Suresh Kumar et al. (2013) reported that the production of L-glutaminase from *Serratia marcescens* was maximized at 0.75% NaCl. On the other hand, Krishnakumar et al. (2011) and Abdallah et al. (2013) found that the maximal L-glutaminase activities produced by marine alkalophilic *Streptomyces* sp.-SBU1 and *Streptomyces avermitilis* were observed in a medium supplemented with 2 and 3% NaCl (w/v),

Table 6. Effect of NaCl concentrations on glutaminase synthesis by *Aspergillus oryzae* NRRL 32657

NaCl (%)	Final pH	Biomass (mg.ml^{-1})	Enzyme activity (U.ml^{-1})±SD*
0.25	8.9	7.13	41.63^{b} ±0.02
0.50	9.0	7.02	46.53^{a}± 0.01
1.00	9.0	7.00	39.42^{c}±0.00
1.50	8.8	6.92	31.00^{d}±0.02
2.00	8.4	6.85	25.40^{e}±0.01
2.50	7.6	6.70	17.36^{f}±0.01
5.00	7.4	4.32	16.23^{g}±0.03
7.50	7.2	2.05	15.50^{h}±0.02
10.0	7.2	1.65	13.38^{i}±0.01
12.5	7.0	0.44	4.70^{j}±0.02
15.0	7.0	0.31	1.21^{k}±0.00
17.5	7.0	0.00	0.00^{l}±0.00
20.0	7.0	0.00	0.00^{l}±0.00

*Means followed by different superscripts within columns are significantly different at the 5% level.

respectively. Therefore, it could be concluded that 0.5 % of NaCl concentration was the optimum for the production of glutaminase from *Aspergillus oryzae* NRRL 32657.

The study indicated the relation between glutaminase activity and the different optimization steps for maximizing glutaminase synthesis by *A. oryzae* as following: fermentation time was tested as the first factor and the activity was 10 U.ml^{-1} after one day of fermentation and increased to reach 24.5 U.ml^{-1} at the 3rd day of fermentation. Second, lactose was chosen as the best carbon source and activity increased from 24.5 to 38.97 U.ml^{-1}. In the third step of optimization, increasing lactose concentration to 2.5% resulted in an additional 27.9% increase in activity. Optimization of nitrogen source, nitrogen source concentration, yeast extract concentration, amino acids, incubation temperature, initial pH value of media and NaCl concentration did not significantly affect the activity under the specified tested conditions. Maximum glutaminase synthesis by the test organism after the stepwise optimization (46.53 U. ml^{-1}) was almost 4.7 folds that of 10 U.ml^{-1} obtained at the beginning of optimization after one day of fermentation. Results were compared with others in literature and among results of synthesis of glutaminase by mold in literature, only *Penicillium brevicompactum* showed higher glutaminase synthesis (66.7 U.ml^{-1}) (El-Shafei et al*.,* 2014). Otherwise, the tested *A. oryzae* NRRL 32657 showed higher or equal results. On the other hand, the bacterial strains *Vibrio costicola* and *Vibrio sp.* significantly showed higher activity as reported by Prabhu and Chandrasearan (1997) and Prakash et al. (2010), respectively.

Conflict of interests

The authors did not declare any conflict of interest.

Conclusion

It could be concluded that *A. oryzae* NRRL 32657 could be considered as a promising mold strain for the production of glutaminase. More research in the area of mutation, protoplast fusion and genetic engineering are needed to enhance the activity of glutaminase from this source.

REFERENCES

Abdallah NA, Amer SK, Habeeb MK (2012). Screening of L-glutaminase produced by actinomycetes isolated from different soils in Egypt. Int. J. ChemTech Res. 4:1451-1460.

Abdallah NA, Amer SK, Habeeb MK (2013). Production, purification and characterization of L-glutaminase enzyme from *Streptomyces avermitilis*. Afr. J. Microbiol. Res.14:1184-1190.

Balagurunathan R, Radhakrishnan M, Somasundaram S (2010). L-glutaminase producing *Actinomycetes* from marine sediments – selective isolation, semi quantitative assay and characterization of potential strain. Aust. J. Basic Appl. Sci. 4: 698-705.

Bazaraa WA, Al-Dagal MM (1999). Optimization of extracelluar inulinase production of *Kluyveromyces marxianus* strain NRRL-2415. Egypt. J. Microbiol. 2: 269-282.

Chitanand MP, Shete HG (2012). Condition optimization and production of extracellular L- glutaminase from *Pseudomonas fluorescens*. Int. J. Pharm. Bio Sci. 3:155-162.

Chou CC, Yu RC, Tsai CT (1993). Production of glutaminase by *Actinomucor elagans, Actinomucor taiwanensis* and *Aspergillus oryzae*. J. Chin. Agric. Chem. Soc*.* 31:78-86.

Cook WR, Hoffman JH, Bernlohr RW (1981). Occurrence of an inducible glutaminase in *Bacillus licheniformis.* J. Bacteriol*.* 148: 365-367.

Durá MA, Flores M, Toldra F (2002). Purification and characterisation of a glutaminase from *Debaryomyces* spp. J. Food Microbiol. 76:117-126.

Durai S, Selvaraj B, Manikkam R, Ramasamy B (2014). Production and optimization of L- glutaminase from *Vibrio* sp. M9 isolated from mahabalipuram marine sediments. World J. Pharm. Res. 3:2117-2126.

El-Sayed ASA (2009). L-glutaminase production by *Trichoderma koningii* under solid state fermentation. Ind. J. Microbiol. 49:243-250.

El-shafei AM, Hassan MM, Ali NH, Abou zeid MA, Mahmoud AD, El-ghonemy DH (2014). Purification, Kinetic Properties and Antitumor Activity of L-Glutaminase from *Penicillium brevicompactum* NRC 829. Br. Microbiol. Res. J. 1:97-115.

Farombi EO, Onyema OO (2006). Monosodium glutamate-induced oxidative damage and genotoxicity in the rat: modulatory role of vitamin C, vitamin E and uercetin. Hum. Exp. Toxicol. 25: 251- 9.

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* NRRL-Y 2547: Effect of aeration, agitation regimes and feeding strategies. Chem. Eng. Technol. 33:52-62.

Kashyap P, Sabu A, Pandey A, Szakacs G, Soccol CR (2002). Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochem. 38:307-312.

Katikala PK, Bobbarala V, Tadimalla P, Guntuku GS (2009). Screening of L-glutaminase producing marine bacterial cultures for extracellular production of L-glutaminase. Int. J. ChemTech Res. 1:1232-1235.

Krishnakumar S, Rajan AR, Ravikumar S (2011). Extracellular production of L-Glutaminase by marine alkalophilic *Streptomyces sp*.-SBU1 isolated from Cape Comorin Coast. Ind. J. Geo-Marine Sci. 5: 717-721.

Moriguchi M, Sakai K, Tateyama R, Furuta Y, Wakayama M (1994). Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. J. Ferment. Bioeng. 77: 621-625.

Nandakumar R, Yoshimune K, Wakayama M, Moriguchi M (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. J. Mol. Catal. B Enzym. 23:87-100.

Nathiya K, Sooraj SN, Angayarkanni J, Palaniswamy M (2011b). Optimised production of L-glutaminase: A tumour inhibitor from *Aspergillus flavus* cultured on agroindustrial residues. Afr. J. Biotechnol. 63:13887-13894.

Nathiya K, Sooraj SN, Palaniswamy M (2011a). Statistical optimization of key process variables for enhanced L-glutaminase production by *Penicillium brevicompactum.* Res. Art. Biotechnol. Bioinf. Bioeng. 4: 423-430.

Pallem C, Manipati S, Somalanka SR (2010). Process optimization of L-glutaminase production by *Trichoderma koningii* under solid state fermentation (SSF). Int. J. Appl. Biol. Pharm. Technol. 1:1168-1174.

Prabhu GN, Chandrasekaran M (1997). Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* in solid state fermentation using polystyrene as an inert support. Process Biochem. 32:285-289.

Prakash, PJ, Poorani E, Anantharaman P, Balasubramaniam T (2010). L-glutaminase production and the growth of marine bacteria. Res. J. Microbiol. 4:168-172.

Prasanna KL, Raju KJ (2013). Production of L-glutaminase by *Aspergillus oryzae* NCIM 1212 under solid state fermentation using agro – residues. J. Chem. Biol. Phys. Sci. 2:261-269

Prasanth KK, Prabhakar T, Sathish T, Girija SG, Moges F, Lakshmi SG (2009). Studies on extracellular L-glutaminase production by isolated halophilic *Aspergillus sp*. J. Pharm. Chem. 3:4-7.

Renu S, Chandrasekaran M (1992). Extracellular L-glutaminase production by marine bacteria. Biotechnol. Lett. 14:471-474.

Sabu A, Keerthi TR, Kumar SR, Chandrasekaran M (2000). L-Glutaminase production by marine *Beauveria* sp. under solid state fermentation. Process Biochem. 35:705-710.

Siddalingeshwara KG, Devi DN, Pramoda T, Vishwanatha T, Sudipta KM, Mohsin SK (2010). Rapid screening and confirmation of L-Glutaminase producing novel *Aspergillus wentii*. Int. J. ChemTech Res. 2: 830-833.

Siddalingeshwara KG, Sunil DP, Pramoda T, Vishwanatha T, Ramaesha I, Jayaramu M, Dwarakanath V, Naveen M, Sumanth BK (2011). Studies on fermentation kinetics for the synthesis of L-Glutaminase – A tumour inhibitor from microbial origin. J. Pharm. Biomed. Sci. 22:1-4.

Sivakumar K, Sabu MK, Manivel PR, Kannan I (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.

Suresh Kumar S, Muthuvelayudham R, Viruthagiri T (2013). Medium optimization for production of L-glutaminase (EC.3.5.1.2) by *Streptomyces griseus* under submerged fermentation. Int. J. Sci. Eng. 1:1-5.

Teja DD, Devi FS, Harsha N, Vishala SS, Lakshmi PKS (2014). Production of L- glutaminase from marine ecosystems and optimal conditions for maximal production by Actinomycetes. Int. J. Adv. Res. 2:485-491.

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

Yuasa A, Okamura H, Kataoka J (2003). Microbial culture with enhanced glutaminase activity and utilization thereof. U.S. Patent, No. 3, 0124646 A1.