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Research Article

Production of Anticancer Agent L-glutaminase from *Aspergillus flavus* Mutated by Physical Mutagens under Solid State Fermentation Conditions, Immobilization and Characterization of the Free and Immobilized Enzyme.

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ABSTRACT:
L-glutaminase has gained a considerable attention in recent years due to its potential applications in medicine as an anticancer agent, as an efficient anti-retroviral agent and as a biosensor. Potentialities of twenty three fungal isolates were investigated for their ability to produce L-glutaminase using modified modified Czapek Dox's agar plate. *Aspergillus flavus* AUMC 8653 was picked out as the most potent fungal strain for the enzyme production then exposed successively to gamma and UV-radiation (physical mutagens) to enhance L-glutaminase production. Mutant *A. flavus* M-19 showed the maximum production of L-glutaminase with a 2.77- fold increase in production compared to the wild-type strain. Wheat bran was selected as a promising natural substrate for production enhancing of L-glutaminase on solid state fermentation by *A. flavus* M-19. The optimized process parameters of effect of temperature 35 ^{o}C, initial pH 7, moisture content 70%, supplementation of the salt basal medium with glucose (1%) and beet molasses (20% v/v) achieved maximum productivity of L-glutaminase 45.0±0.97 U/mg protein. highest enzyme preparation precipitated with ammonium sulphate (70% saturation). The enzyme was efficiently immobilized by covalent binding with activated charcoal giving an immobilization yield of 82.66%. Optimum pH values were 7.5 and 7 for free and immobilized enzyme, respectively. While, Optimum reaction temperatures were 30°C and 35°C for free and immobilized enzyme, respectively. After incubation for one hour pH 8 at 30°C, the relative activity of free enzyme decreases to 60% whereas for the immobilized enzyme decreased to 44% at 35°C. The free enzyme retained about 57% of its original activity for 30 min at 40 °C. While, the immobilized enzyme retained about 50 % at 50^{o}C for 30 min.

Key words: L -glutaminase, *Aspergillus flavus*, Mutation, Wheat bran, Inert support, Characterization.

INTRODUCTION

The enzyme L-glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which produces L-glutamic acid and ammonia from L-glutamine. The human genome carries the codes for glutaminase isoforms, the kidney isoform

(KGA/GLS1) and the liver isoform (LGA/GLS2) [1].

L-glutaminase is an innervate anti-leukemic agent. Unlike normal cells, leukemic cell do not demonstrate the L-glutamine synthetase, thus, it depends on the exogenous supply of L-glutamine to produce a-ketoglutarate, to provide their Krebs cycle fuels, and to also produce intermediates for formation of lipids, nucleosides and other biomolecules needed for their proliferation and survival [2, 3, 4]. Therefore, blood L-glutamine serve as metabolic precursors for nucleotide and protein synthesis of tumor cells [5]. Consequently, L-glutaminase causes selective death to L-glutamine dependent tumor cells by blocking the energy route for their proliferation [6]. In experiments the glutamine catabolic rate in tumor cells was faster than that of healthy cells [7].

Several microorganisms, including bacteria, yeast, moulds and filamentous fungi have been recorded to produce L-glutaminase [8, 9, 10], while the most potent producers are fungi [11]. On commercial scale, glutaminases are synthesized mainly by *Aspergillus* and *Trichoderma* sp. [12, 13, 14, 15]. Glutaminase plays a critical role in the cellular nitrogen metabolism of both, prokaryotes prokaryotic and eukaryotic cells [8, 16].

Recently, L-glutaminase recorded a great attention as a therapeutic agent against cancer and HIV [17, 18, 19], as a bio-sensing agent in testing glutamine levels in mammalian and hybridoma cell lines [8, 20] and production of some chemicals such as theanine and as a flavor enhancer in industry of food[21].

L-glutaminase improves the flavor of fermented foods by raising their glutamic acid percentage and thereby imparting a palatable taste [22, 23]. The use of L- glutaminase as a flavor enhancer in Chinese foods has canceled the use of monosodium glutamate which acts as an allergen for individuals [2, 13, 24, 25].

Mutational studies by physical and chemical means play a role in strain improvement because they are qualified to increase productivity and economically feasible technology allow us to draw the results effectively Sadhu et al. [26]. Mutational improvement for fungal strains allows the organism to thrive in stressful conditions, such as starvation in respect to a particular nutrient, slowly making the organism more resilient and allowing it to be modulated into a more desired form [27, 28]

With respect to solid state fermentation (SSF), it is important to know that, this technology has a great advantages such as being less economically, needs small vessels, low moisture solid substrates were used, providing a selective environment for growth and extracellular enzymes production by various fungi [29]. Thus, a searching for other fungal species for overproduction of L-glutaminase using agro industrial solid substrates is pursued. Recently, solid state fermentation has been utilized as a promising bioprocess for enzymes production and other economical metabolites.

The engagement of enzymes to inert materials is a kind of immobilized method; it is practical and suitable method for much industrial application [30]. There are many advantages for the immobilization of cell and enzyme, such are isolation and purification of enzyme is avoided, retention of enzyme and reduces the cost because the immobilized enzyme can be used many cycles, increasing the stability of enzyme toward the environment conditions such as PH, temperature and ionic strength, product is not contaminated with the enzyme and Reduces effluent disposal problems [31].

The objective of this study was to select fungus with good ability to produce L- glutaminase and optimize physio-chemical parameters to get the maximum yield of L-glutaminase in SSF and study some of catalytic characters of the free and immobilized enzyme.

MATERIALS AND METHODS

2.1. Isolation of L-glutaminase producing fungi

Fungi used in this work were isolated from different soil samples obtained from Kingdom of Saudi Arabia and Sharm El-Sheikh, Egypt. The

dilution plate technique was used for fungal isolation [32]. The purified isolates were identified by Assiut University Mycological Centre (AUMC), Egypt. All cultures were placed with their accession numbers. These fungi were cultivated on Czapek's Dox medium every 4 weeks and incubated at 30°C for seven days.

The obtained fungi were subjected for rapid assay of L-glutaminase production by plate assay method described by [33]. Modified Czapek's Dox (MCD) medium [34] with the following composition 0.2% glucose, 1.0% L-glutamine, 0.15% K_2PO_4, 0.05% KCl, 0.05 % $MgSO_4.7H_2O$, 0.003% $CuNO_3$. $3H_2O$, 0.005% $ZnSO_4.7H_2O$, 0.003% $FeSO_4.7H_2O$, 1.8% agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. Control plates were MCD medium containing $NaNO_3$ as nitrogen source. The plates were incubated for five days at 30°C. The development of L- glutaminase producing fungi was indicated by the linear growth measurement and formation of a pink area around the fungal colonies.

2.2. Strain improvement by mutagenesis

Spores of *A. flavus* (10^5-10^6 spores/mL) were harvested from a 7 day-old spores grown on Czapek's Dox medium following exposure to different doses (0.25~2.5 kGy) of Co^{60} γ-rays emitted by an Indian gamma cell located at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. Survivors were grown by spreading 1.0 mL of the treated spores on MCD medium and incubating at 30 °C for 7 days. Mutants with the largest clear zones around the colonies were selected and re-irradiated with Co^{60} γ-rays. The best surviving mutant was then exposed to ultraviolet -radiation (power: 30W, wavelength: 260 nm at a distance of 20 cm for 2~30 min). A mutant displaying high glutaminase activity was selected, and stability of the enzyme production was studied for nine generations by successive inoculations of the strain on MCD medium.

2.3. Solid-State Fermentation (SSF)

Seven agro-industrial residues namely corn cobs, ground nut shell, rice bran, soya bean meal, sugar cane bagasse, wheat bran and wheat straw were examined for their power as substrate in SSF for production of enzyme. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 5 g substrates moistened with 10 ml of MCD containing 0.15% K2HPO4, 0.05% KCl, 0.05% MgSO4.7H2O, 0.003% CuNO3.3H2O, 0.005% ZnSO4.7H2O, and 0.003% FeSO4.7H2O. All flasks were sterilized at 121°C for 20 min. Each flask was inoculated with one ml spore suspension of *Aspergillus flavus* M-19 and then incubated at 30°C for 5 days.

2.4. Enzyme extraction

After finishing incubation process the crude L-glutaminase was extracted by adding 50 ml of sterilized water, shaking for 30 min followed by centrifugation for 30 min.

2.5. Chemicals and Buffers

L-glutamine, L-methionine, bovine serum albumin, Nessler's reagent, Folin reagent, soya bean meal and corn steep liquor were purchased from Sigma Chemicals Company (St. Louis, MO, U.S.A.). Buffers were prepared according to the method recorded by [35]. Cane and beet molasses were obtained from Hawamdiah sugar factory, Egypt. Crude whey was collected from the rural area of Kalyoubia governorate, Egypt. Other chemicals were also of the best analytical grade.

2.6. L- glutaminase Assay

Enzyme productivity was calculated following the method of [36]. This method measures the liberated ammonia from L- glutamine in the enzyme reaction by the Nessler's reaction. The absorbance measured at 450 nm. The amount of liberated ammonia was calculated by standard curve of ammonium sulfate. One unit of L-glutaminase is the amount of enzyme which induces the synthesizing of 1µmol of ammonia per min.

2.7. Protein determination

The protein amount of the enzyme preparations was evaluated by the method of [37]. All

assessments were achieved in triplicates and the averages were recorded.

2.8. Optimization parameters for highest L-glutaminase productivity

The *A. flavus* was studied for further investigations. Various parameters were optimized and once the optimization has been done for a factor, it was incorporated into the experiment for the optimization of the next parameter. Incubation temperature (25~50°C), initial pH of the medium (5~9; pH was adjusted using 1N NaOH or 1N HCl) and initial moisture content of the substrate (40~90%). The effect of several carbon sources by supplementation of salt basal medium with 1% (w/v) galactose, glucose, fructose, maltose, lactose, sucrose and dextrose was studied. The impact of supplementation of salt basal medium with various nitrogen sources 1% (w/v) beef extract, peptone, urea, yeast extract ammonium sulphate and ammonium chloride. The effect of addition variant by-products (corn steep liquor, whey, beet molasses and cane molasses) as additives (20%, v/v) was also tested.

2.9. Enzyme purification

Enzyme purification was started with precipitation of 400 ml of crude enzyme by gradual addition of $(NH_4)_2SO_4$ using the range of saturation from 50 to 90%.

2.10. **Immobilization of L-glutaminase**

Different supports namely activated charcoal, carboxymethyl cellulose, chitin DEAE-cellulose and sephadex G-25 and were employed for L-glutaminase immobilization according to modified method of [38]. Experimentally, 200 mg of each support was shacked in 5 ml Citrate-phosphate buffer (0.1 M, pH 7) containing 2.5% glutaraldehyde at room temperature for 2h. The carriers were filtered off and washed with distilled water to eliminate the excess of glutaraldehyde then each treated carrier was incubated with 5 ml of Citrate-phosphate buffer including 1 ml of enzyme preparation. After being shaken for 2 h at 30°C, the unbounded enzyme was abstracted by washing with distilled water until no protein or activity was detected in the wash. The immobilization yield was calculated by the following equation:

% yield = immobilized enzyme (I) / Added enzyme (A) – Unbounded enzyme (B)

2.11. Catalytic properties of the free and immobilized L-glutaminase

The optimum pH of the free enzyme and immobilized enzyme activity were investigated by measurements at 30 ^{o}C in different buffers covering the pH range of 6-11. These buffer solutions were 0.1 M Citrate-phosphate, pH 6.0-8.0 and 0.2 M Glycine-NaOH, pH 8.5-11.0. The enzyme stability was recorded by monitoring its remaining activity after incubation for different periods in various pH values. Optimum temperature of the enzyme activity was determined by incubating enzyme at various temperatures extending between 20 to 60^{o}C in optimum recorded pH for free and immobilized enzyme. Thermostability of the free and bond enzyme was estimated after incubating them at variant temperatures for different durations in the same buffers.

2.12. Statistical Analysis:

The recorded data were analyzed with SPSS version 20, in which the equations of the hypothesis tests, including the mean, standard deviation, and T-statistics value were used.

RESULTS

3.1. Screening of fungal isolates for L-glutaminase production

Twenty three fungal isolates were investigated for their capability to produce L- glutaminase belonged to nine genera, namely, *Aspergillus, Cunninghamella, Emericella, Fusarium, Humicola, Paecilomyces, Penicillium, Rhizopus and Trichoderma* by using plate assay method (Table 1). L- glutamine in CDM also served as an enzyme inducer. The highest productivity of L-glutaminase was recorded for *Aspergillus flavus, Aspergillus fumigates, Paecilomyces lilacinus, Aspergillus ochraceus, Penicillium chrysogenum.* The current study was conducted to select the most potent fungus to produce L-glutaminase and

optimize the condition of production. It was obvious that *A. flavus* exhibited highest growth (30.0 ±0.12 mm), maximum pink area diameter of (13.0±0.19 mm) and (6.3±0.23 U/ml).

3.2. Screening of mutants for L-glutaminase production

Wild-type strain *A flavus* was subjected to mutagenesis by using Co^{60} γ-rays and UV-irradiation (data not shown). Seven mutants were selected (M-9, M-12, M-16, M-19, M-20, M-21 and M-21) which showed over production of L-glutaminase. Mutant M-19 was selected as the potent mutant for production of L-glutaminase (17.5±0.66 U/ml) compared to the wild-type strain (Table 2).

3.3. Genetic stability of the selected mutant strain

The stability of L-glutaminase production by selected mutant M-19 was studied by successive subculturing of the mutant for nine generations. The mutant maintained the same production yields after being subcultured nine times; indicating hereditary stability of the mutation has (Table 3).

Table 1. Screening for L-glutaminase production by investigated fungal species

Region of isolation	Fungal species	Colony diameter (mm)	pink area diameter (mm)	AUMC No.
Kingdom Saudi Arabia	*Aspergillus flavus* Link	30.0±0.22$^{\bullet}$	13.0±0.19$^{\bullet}$	8653
	Aspergillus fumigatus Fresenius	25±0.22***	11.0±0.13***	8594
	Aspergillus niger Van Tieghem	13±0.22***	0.5±0.04***	8593
	Aspergillus ochraceus Wilhelm	19±0.27***	8.0±0.09***	8670
	Aspergillus sydowii (Bainier &Sartory)	10.0±0.5***	-	8660
	Aspergillus terreus Thom	11.0±0.4***	4.0±0.07***	8605
	Cunninghamella phaeospora Boedijn	12±0.16***	4.0±0.08**	8662
	Emericella nidulans (Eidam) Vuillemin	14.0±0.12***	0.3±0.12**	8640
	Emericella quadrilineata (Thom &Raper)	10.0±0.1***	-	8636
	Fusarium proliferatum Matsush	8.0±0.3***	0.2±0.08***	8617
	Fusarium solani (Martius) Saccardo	13.0±0.08	-	8615
	Humicola grisea Traaen	11.0±0.22***	-	8598
	Humicola insolens Cooney & Emerson	13±0.15***	4.0±0.03***	8607
	Penicillium chrysogenum Thom	16.0±0.09***	0.7±0.07***	8656
	Penicillium corylophilum Diercks	13.0±0.21***	-	8601
	Penicillium duclauxii Delacroix	9.0±0.09***	2.0±0.18**	8667
	Rhizopus stolonifer (Ehrenberg) Vuillemin	5.0 ±0.9**	-	8671
Ras mohammed (Egypt)	*Aspergillus niger* Van Tieghem	9.0±0.08***	2.0±0.14**	10146
	Aspergillus ustus (Brinier) Thom & Church	12.0±0.31***	4.0±0.05***	10151
	Penicillium citrinum Thom	8.0±0.17**	3.0±0.18**	10147
	Penicillium crustosun Thom	7.0±0.14***	-	10148
	Paecilomyces lilacinus Thom	21.0±0.14***	9.0±0.06***	10149
	Trichoderma longibrachiatum Rifai	3.0±0.12***	-	10155

Data represent the mean of 3 different readings ± standard deviation. *** Highly significant, $p \leq 0.01$ and ** Significant, $p \leq 0.05$. Data was statistically compared using t-test (N = 3). The obtained data were statistically compared to the highest value obtained which is marked as ●.

3.4. Screening of different agro-industrial residues for L- glutaminase production under SSF

Among seven natural substrates employed for the production of L- *glutaminase* (data not shown), wheat bran was recorded as the most potential substrate for the L- *glutaminase* production using *A. flavus* M-19 giving maximum enzyme activity (22.1 ± 0.38 U/mg protein) followed by Sugar cane bagasse (19.84 ± 0.10 U/mg protein), Rice bran (12.4 ± 0.11 U/mg protein) and Wheat straw (7.4 ± 0.09 U/mg protein). While the lowest enzyme productivity was recorded for Corn cobs followed by Ground nut shell and Soya bean meal.

Table 2: L-glutaminase production under liquid state cultivation by *A. flavus* and its most potent mutants.

strain		pink area diameter (mm)	L-glutaminase activity (U/ml)
wild	-	13.0±0.19	6.3±0.23
Mutants			
	M 9	19.0±0.19	12.6±0.31***
	M 12	24.0±0.22	14.1±0.21***
	M 16	22.0±0.31	13.0±0.17***
	M 19	28.0±0.11	17.5±0.66•
	M 20	24.0±0.27	15.1±0.67***
	M21	18.0±0.12	12.7±0.79***
	M22	16.0±0.32	10.66±0.23**

Table 3: L-glutaminase activities of most potent mutant *A. flavus* M-19 for nine generations.

	1st	2nd	3rd	4th	5 th	6 th	7 th	8 th	9 th
L- glutaminase activity (U/ml)	17.5 ±0.37	17.78 ±0.19	18.1 ±0.44	17.34 ±1.12	16.7 ±1.07	17.23 ±0.49	17.0 ±0.15	17.6 ±0.42	17.3 ±0.56

Optimization of fermentation parameters for maximum L-glutaminase production

Various experiments were carried out to find out the effect of various physio-chemical parameters for improvement the production of *L-glutaminase by A. flavus* M-19 under SSF culture.

3.5. Effect of incubation temperature

The impact of incubation temperatureon enzyme production was investigated, a maximum enzyme activity of 25.3±0.25 U/mg protein was obtained by *A. flavus* M-19 at 35°C (Fig. 1).

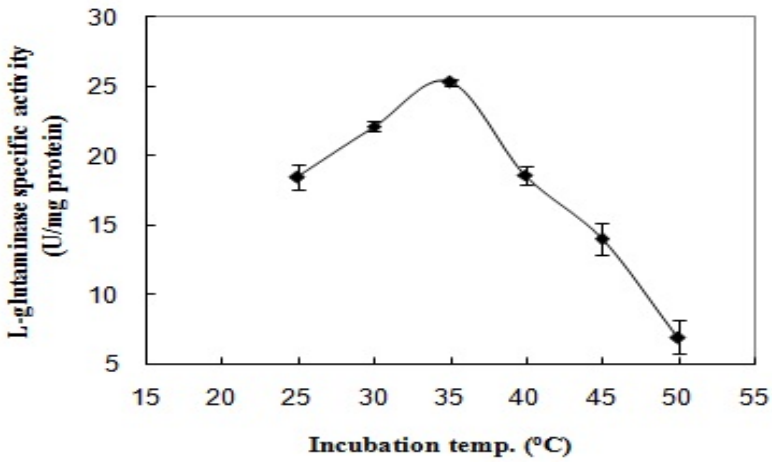

Fig. 1. Effect of incubation temperature on production of L-glutaminase of *A. flavus* M-19

3.6. Influence of initial pH

The maximum L- glutaminase e production was obtained at an initial pH 7 giving 33±0.12 U/mg protein (Fig. 2).

3.7. Effect of moisture level

In the current study, the highest L- glutaminase production 33±0.12 U/mg protein was obtained at 70% initial moisture content (Fig. 3).

3.8. Influence of different carbon sources

Results on the influence of supplementation of SSF medium with various carbon sources such as galactose, glucose, fructose, maltose, lactose, sucrose and dextrose on enzyme production are shown in the (Fig. 4).

Among the tested carbon sources, glucose was found to be the most suitable carbon source to maximize the production of L- glutaminase giving 40.5±0.77 U/mg protein.

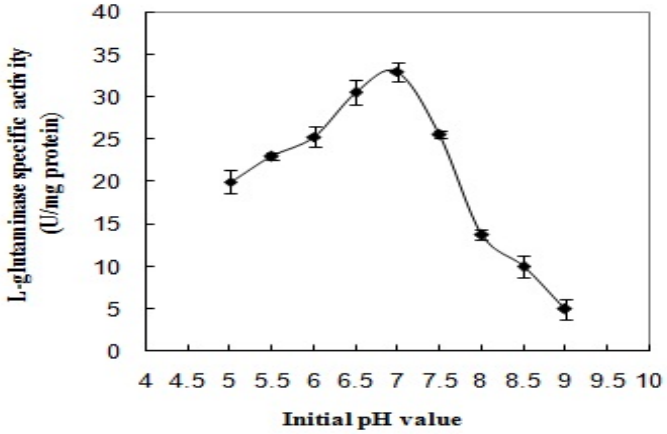

Fig. 2. Influence of initial pH on production of L-glutaminase of *A. flavus* M-19

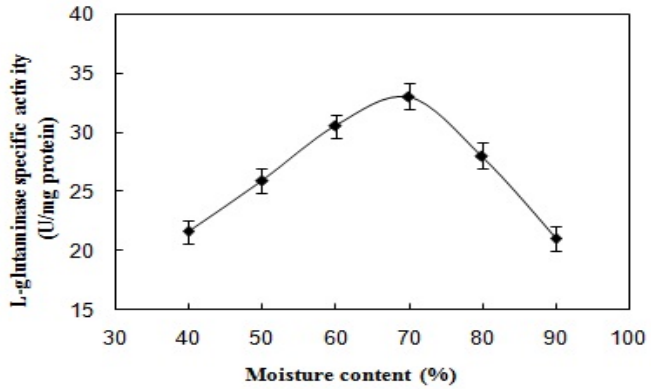

Fig. 3. Effect of Moisture content on production of L-glutaminase of *A. flavus* M-19

3.9. Influence of different nitrogen sources

When the influence of organic and inorganic nitrogen sources on L- glutaminase production was investigated, it was observed that all the additional nitrogen sources had a negative effect on the synthesizing of L-glutaminase (Fig. 5).

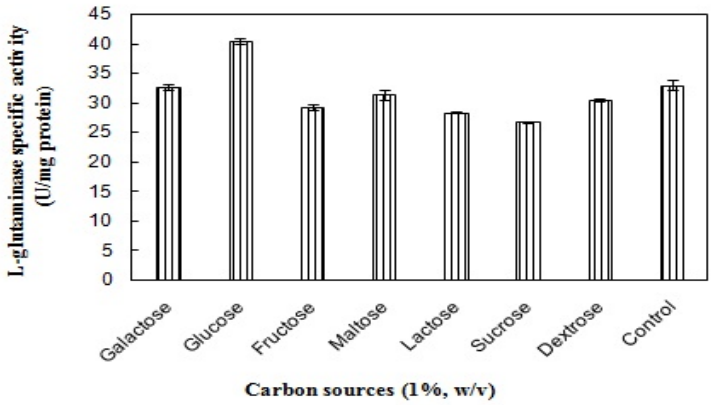

Fig. 4. Influence of carbon sources on production of L-glutaminase of *A. flavus* M-19

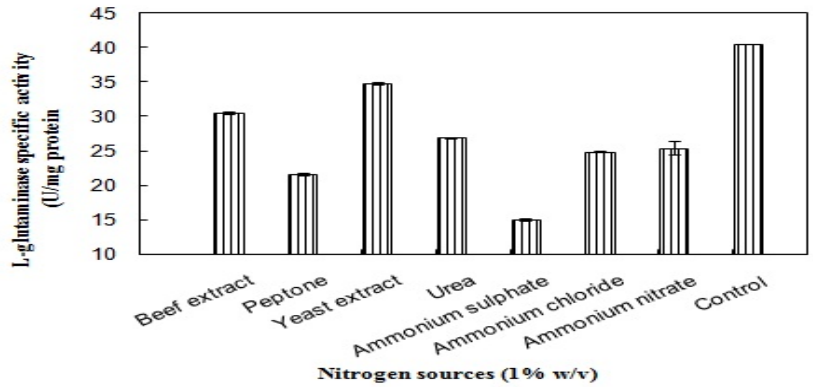

Fig. 5. Influence of nitrogen sources on production of L-glutaminase of *A. flavus* M-19

3.10. Effect of different by-products

The influence of different natural additives such as beet and cane molasses, corn steep liquor and whey on L- glutaminase production. The present study found that beet molasses caused a considerable stimulatory effect on L- glutaminase production 45.0±0.97 U/mg protein by *A. flavus* M-19 (Fig. 6).

3.11. Enzyme purification

The results of L -glutaminase purification showed that Precipitation of proteins from the crude enzyme preparation with 70% saturation of ammonium sulfate provided a fraction containing the highest enzyme preparation (83.26 U/mg proteins).

3.12. Immobilization of L-glutaminase

Five supports, pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein. Immobilization of *A. flavus* M-19 L- glutaminase by covalent binding was achieved by cross linking between the enzyme and tested carriers throughout glutaraldehyde. The results (Fig. 7) indicated that the highest immobilization yield (82.66%) was detected with activated charcoal as a carrier followed by carboxymethyl cellulose (70 %), DEAE-cellulose (64.8 %), chitin (50.55 %) and sephadex G-25 (44%).

3.13. Effect of pH on enzyme activity and stability of free and immobilized L-glutaminase

The results (Fig. 8) reveal that the free enzyme showed a great activity at pH 6-8 with the maximum at pH 7.5, while immobilized enzyme showed its greatest activity at pH 7. The free L-glutaminase retained full activity after one hour of incubation at pH 7.5 and about 60% of the original activity after one hour was restored at pH 8 (Fig. 9). While the immobilized enzyme retained 44 % of the original activity after one hour at pH 8 (Fig. 10).

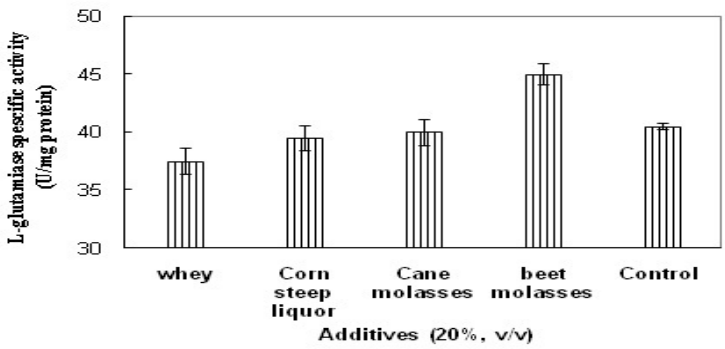

Fig. 6. Influence of different additives on production of L-glutaminase of *A. flavus* M-19

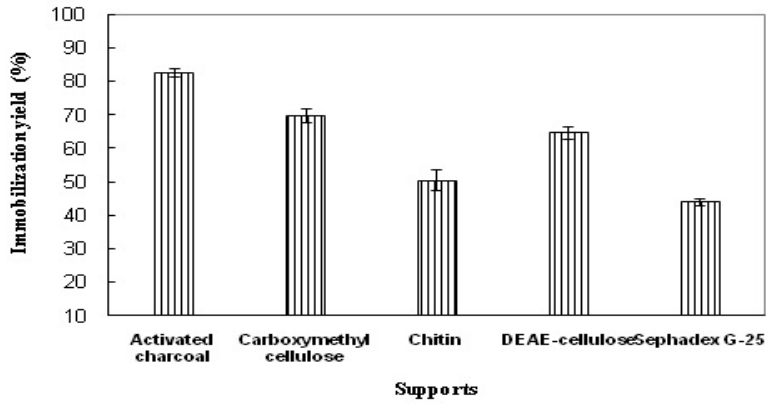

Fig. 7. Effect of different supports on immobilization yield of L-glutaminase of *A. flavus* M-19

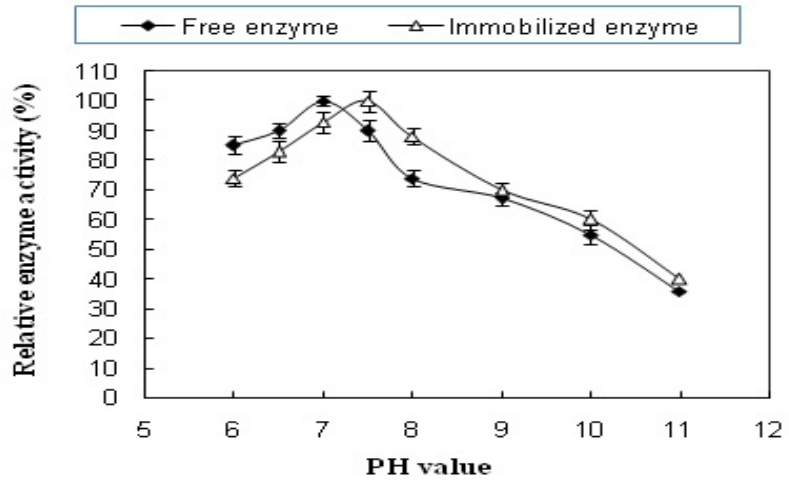

Fig. 8. Effect of pH on enzyme activity of free and immobilized L-glutaminase of *A. flavus* M-19

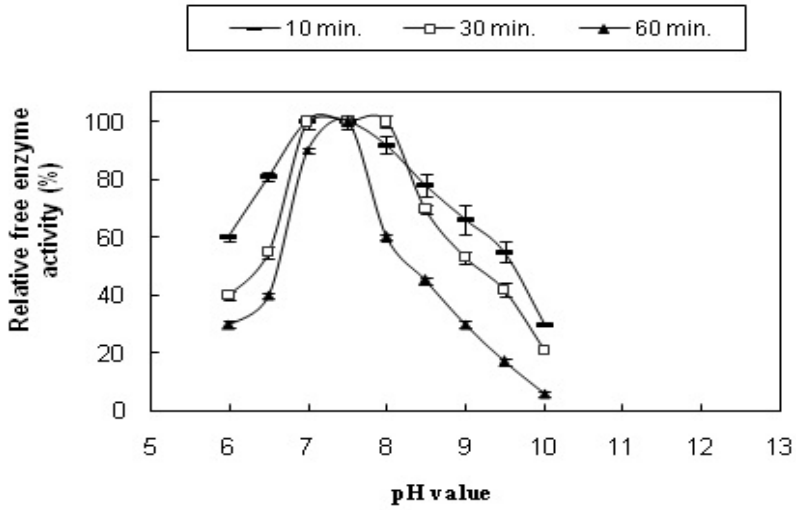

Fig. 9. Effect of pH on enzyme stability of free L-glutaminase of *A. flavus* M-19

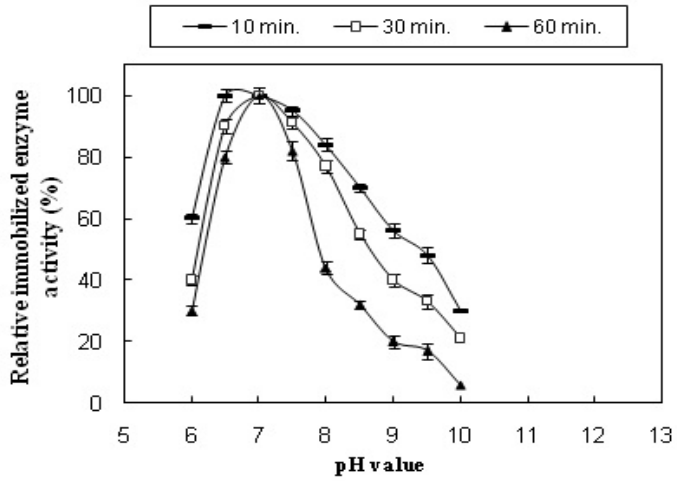

Fig. 10. Effect of pH on enzyme stability of immobilized L-glutaminase of *A. flavus* M-19

3.14. Effect of temperature and thermal stability of free and immobilized L-glutaminase

The optimum temperature for the immobilized enzyme increased to 35°C compared to 30°C for the free enzyme (Fig. 11). The results of stability showed that the free enzyme retained about 57% of its original activity for 30 min at 40 °C (Fig. 12). While the immobilized enzyme could tolerate up to 40°C for an 30 min without loss of more than 27% of its initial activity and also retained about 50% of this activity at 50°C (Fig. 13).

DISCUSSION

L-glutaminase is a potent anti-leukemic agent. Unlike normal cells, leukemic cell do not demonstrate the L-glutamine synthetase, thus, it depends on the exogenous supply of L-glutamine to produce α -ketoglutarate, to provide their Krebs cycle fuels, and to also produce intermediates for formation of lipids, nucleosides and other biomolecules needed for their proliferation and survival [2-4]. Therefore, searching for a new enzyme with unique therapeutic properties is a challenge for many biotechnology laboratories. *A. flavus* showed maximum L-

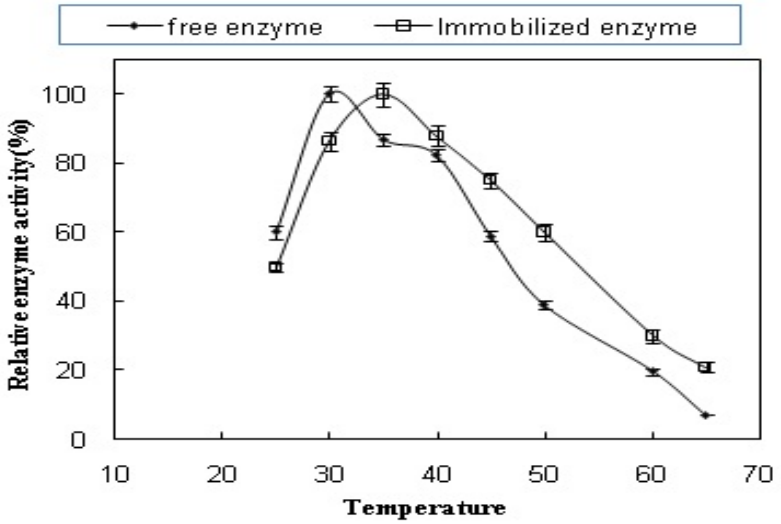

Fig. 11. Effect of temperature on enzyme activity of free and immobilized L-glutaminase of *A. flavus* M-19

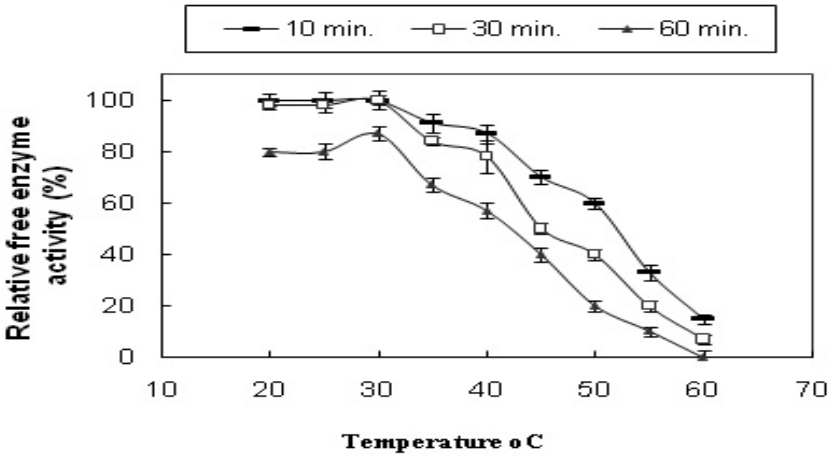

Fig. 12. Effect of temperature on enzyme stability of free L-glutaminase of *A. flavus* M-19

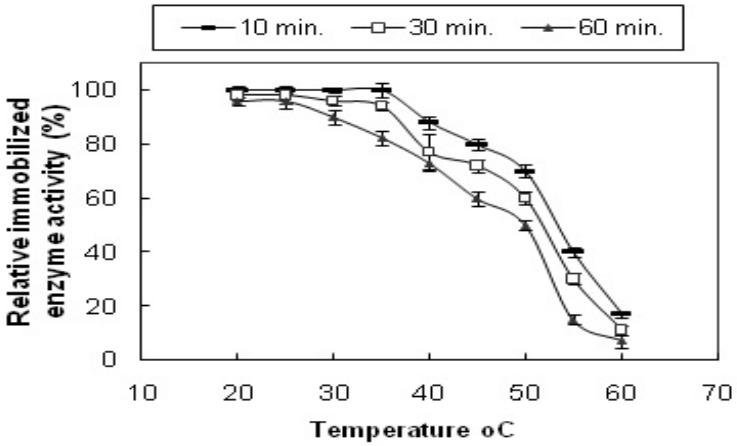

Fig. 13. Effect of temperature on enzyme stability of immobilized L-glutaminase of *A. flavus* M-19

glutaminase production as compared with other fungal strains. Similar results were obtained [14, 39] who recorded that A. *flavus* was the potent fungus for L- glutaminase production.

. The result of mutagenesis showed that the mutant M-19 showed the maximum production of L-glutaminase with a 2.77- fold increase in production compared to the wild-type strain. Our results were in accordance with that reported by Jesura et al. [40] who reported mutation techniques for glutaminase inhancement from *Aeromonas veronii.* Hence this mutant was picked out for the further investigation under SSF.

Selection of natural substrate is one critical role for the L- *glutaminase* production because they mimic the conditions under which the microbe grows natured and it reduced energy requirement for agitation with high extracelluar product concentration [8]. Wheat bran was considered as the best natural substrate because it is consider as a complete nutritious meal for microorganisms having all the ingredients and remains unfastened even under moist conditions providing a large surface area [41]. Moreover the chemical structure of wheat bran shows that it includes various soluble sugars like glucose, xylose, arabinose, galactose, etc. which help in starting the growth and accelerate microorganisms replication [42]. This finding was in accord with observations with those observed for *Aspergillus oryzae* [43,44], *Zygosaccharomyces rouxii* 8 and *Trichoderma Koningii* [14].

Microbial enzyme biothsynthesis is known to be a function of the prevailing culture and environmental condition. The incubation temperature is a significant environmental factor for L- glutaminase production by microorganisms because it organizes microbial growth and consequently enzyme secretion. Comparable results were obtained by Han et al. [45] who recorded that the most favorable temperature for L- glutaminase production from *Rhizopus oligosporus* was 35°C.

The initial pH of the production medium is an important parameter affecting the enzyme production since it can indirectly act on the fungal growth by affecting the availability of medium nutrients. This finding was in coincidence with that reported by El-Sayed [14] and Iyer and Singhal [10] who recorded that the optimum pH of L- glutaminase production by *Trichoderma koningii* and *Zygosaccharomyces rouxii* NRRL-Y 2547, respectively was 7.0 under SSF conditions. While, the maximum L- glutaminase productivity by *Streptomyces rimosus* obtained at pH 9.0 reported by [46].

Generally the moisture level of substrates in SSF processes varies between 30-85% has a marked effect on growth kinetics of fermenting [46]. Similar results were obtained by Kashyap et al. [8] and El-Sayed [14] who found that 70% and 65% initial moisture content was the optimum for L-glutaminase production from *A. flavus* and *Zygosaccharomyces rouxii*., respectively. While the optimum initial moisture content recorded for *A. fumigates* by [39] was 80%.

Among the different carbon sources that added to the basal medium glucose causes the maximum induction in L-glutaminase production. Stimulatory effect of glucose may be attributed to the positive effect of glucose as a cometabolic agent [47]. Our results were in agree with observations made by [8,14,48].

Concerning the effect of different nitrogen sources, all the additional sources caused inhibition of L-glutaminase production. Ther epressor effect of all nitrogenous sources on enzyme production by the experimental fungal isolate could be attributed to to the preferential utilization of these nitrogen nutrients comparing to the wheat bran. Thus, the enzyme production being not justifi ed by the solid fungal culture in the presence of these organic and inorganic nitrogenous compounds [14]. Convenient results were also recorded by [8,14,49].

The stimulatory effect of beet molasses may be attributed to the presence of several vitamins such as thiamine, riboflavin, pyridoxine, folic acid and biotin in beet molasses [50]. Similar result was obtained by Abu-Tahon and Isaac [51] who recorded that addition of beet molasses to *Aspergillus ustus* caused maximum production of L-methioninase.

The results of precipitation procedure showed highest enzyme preparation precipitated with ammonium sulphate (70% saturation). This finding was in coincidence with that reported by Desai et al. [52] who recorded the same concentration of ammominum sulphate for L-glutaminase purification from *Streptomyces* sp.

There are many advantages for the immobilization of cell and enzyme, such are isolation and purification of enzyme is avoided, retention of enzyme and reduces the cost because the immobilized enzyme can be used many cycles, increasing the stability of enzyme toward the environment conditions such as PH, temperature and ionic strength, product is not contaminated with the enzyme and reduces effluent disposal problems [31]. Activated carbon is extremely porous so it is characterized by a large surface area available for chemical reactions [53]. Charcoal was recorded as the best carrier of enzyme immobilization by [53,54].

The free and immobilized L-glutaminase of *A. flavus* M-19 was found to have good activity at a neutral value. This optimum pH for free enzyme is equal to that reported from *Pseudomonas aeruginosa* [55] .While that recorded to immobilized enzyme is in agreement with the previous results on other L-glutaminase from

Saccharomyces cerevisiae [56], *Streptomyces* sp. [52] and *Aspergillus sojae* [57].

With respect to the effect of temperature on the activity and stability of L-glutaminase, the obtained results confirmed that immobilized L-glutaminase achieved its optimal activity at 35°C while the free enzyme recorded 30°C. This temperature shift could be due to the formation of a molecular cage around the enzyme, which protected the enzyme molecules from the temperature [58].The optimum temperature 35°C was reported for L-glutaminase of various fungal species [55,59,60].

CONCLUSION

As glutamine amidohydrolase broadens its wide applications used in the food and pharmaceutical industry and can work as anti –leukemic agent, there is always a scope for novel glutaminase with better characteristics. Therefore the present study was focused on the exploring of the potentiality of *Aspergillus flavus* M-19 for production o L-glutaminase using wheat bran as a substrate which is an agro-industrial residue, easily available and economical. Supplementation of the salt basal medium with glucose (1%) and beet molasses (20% v/v) achieved maximum productivity of L-glutaminase 45.0±0.97 U/mg protein

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