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ANTICANCER GLUTAMINASE PRODUCTION AND OPTIMIZATION USING HALOTOLERANT ASPERGILLUS FLAVUS CZCU-9, F1H

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ABSTRACT

Isolation of halotolerant glutaminase producing microorganisms was carried out using medium containing different concentrations of NaCl viz. 2.9, 3.5 and 4 %. Only four fungal isolates (F1H, F2H, F3H and F4H) were able to grow at all concentrations has been selected and purified. Production of glutaminase was carried out in broth medium containing 4% NaCl. Quantitaive assay for glutaminase led to 6.43, 5.72, 6.12 and 5.86 U/ml for each isolate extracellularly, respectively. In case of intracellular production assay led to 5.2, 5.46, 5.18 and 5.77 U/ml , respectively. The isolate F1H has showed the highest productivity extracellularly and stability on subculturing so, it was chosen and selected for furthur investigations. The optimized conditions for glutaminase production by isolate F1H was found to be 6 days at 35^{o}C and pH 7 under static and dark incbation conditions with inoculum size of $4.38x10^{6}$ spores/ ml. The optimization led to production of 7.66 U/ml of enzyme with great stability at 4% salt concentration which is a good sign for probability that the enzyme could have unique properties which can be confirmed through more investigatios.The isolate was identified genetically and has shown 100% similarity to Aspergillus flavus CZCU-9 and it was given the name Aspergillus flavus CZCU-9, F1H.

Keywords: Halotolerant, glutaminase, anticancer, extracellular, intracellular. optimization

INTRODUCTION

From the vital enzymes which have potential aim especially near the last past ten years is glutaminase. In addition to its importance as aid in food processing industries, it is very important promising known cure against many types of cancer [1].

Screening of new halotolerant and halophilic isolates producing glutaminase represent new step in getting the enzyme with unique and unexpected properties that can be new hope for more progress in curing process of cancer disease[2].

Howevere, L-glutaminase was reported to be synthesized via bacteria [3], fungi [4], yeast [5], actinomycetes [6].

In addition, the synthesis via *Aspergillus sp* ALAA-2000 was reported [4]. Also, *Vibrio azureus* JK-79 also was good producer of glutaminase [7]. *Aeromonas veronii* has been identified as potential producer of l-glutaminase [8].

Moreover, the production using *Serratia marcescens* and optimization in the light of five variables *viz*., temperature, pH, time and different concentration of galactose and L-glutamine on L-glutaminase production was recorded [9].

Glutamine is important as it is primary tool for donation of its nitrogen which aid in protein, nucleic acid, lipid formation and participate in oxidative metabolism. It was reported that, cancer cells have addiction on glutamine, glutaminase enzyme by its action on hydrolysis of glutamine block the route of cancer cell metabolism as they may die in absence of glutamine in their surrounding medium. Glutaminase is already present in mitochondria but it must be in the level that allows sequential and fast degradation of glutamine [10]. The trend is synthesizing glutaminase enzyme via efficient halotolerant isolate which having a high activity and can be used as agent for increasing the level of glutamine catabolism and stopping cancer

development. This research represent the first step of the overall aim i.e. production and primary optimization of glutaminase using efficient halotolerant isolate.

MATERIALS AND METHODS

1. Isolation

Natural spoiled samples as pickles, detergent processing wastes, infected lemon fruit and soil sample from Sohag Governorate, Egypt were the sources for isolation. Modified medium composed of (g/l) NaCl, 20, 30 &40; $MgSO_4.7H_2O$, 0.1; K_2HPO_4, 3.12; KH_2PO_4,0.28; Glucose, 10; Glutamine, 4.97 and Agar, 20 was prepared and sterilized [11]. The pH of the medium was adjusted at 7. Incubation was at 30ºC for five days in case of fungal isolates and 2 days for getting bacterial isolates on static incubation conditions. The isolates which were able to be sub-cultured with high growth were selected and purified.

2. Production of glutaminase enzyme by the selected isolates

The selected isolates were examined for the ability of glutaminase production. The production was done primarily in 250 ml conical flask containing 50 ml broth medium having the same composition of that used for isolation except agar. Inoculation with 1ml of spore suspension of each isolate was carried out. Incubation was at 30ºC for five days.

3. Separation of the extracellular and intracellular crude enzyme

The extracellular crude enzyme was separated by filtration and the mycelium was washed with distilled H_2O. The intracellular contents of the cells were obtained by ultrasonic disruption of cells by ultrasonic processor (Cole Parmer Ultrasonic Homogenizer CPX 400) over three 15 s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30 min at 4°C to remove cell-debris using Sorvall cooling centrifuge. The supernatants were then used for assay of enzyme [12].

4. Quantitative assay of glutaminase enzyme

The assay was done according to [13]. The reaction mixture was prepared by adding 0.5 m1 of the sample, 0.5 ml of 0.04M L-glutamine solution, 0.5 m1 of distilled water and 0.5 m1 of phosphate buffer 0.1M, pH 8.0. The mixture was incubated at 37°C for 15 min then, 0.5 ml of 1.5 M Trichloroacetic acid was added for stopping the reaction. After that, 0.1 m1 of the mixture was added to 3.7 m1 of distilled water and finally 0.2 m1 of Nessler's reagent was added to complete the volume to 4 ml. the same steps without the sample were done for controls. The absorbance was measured at 450nm using a UV -Visible spectrophotometer. It's known clearly that, one international unit of L-glutaminase was defined as the amount of enzyme that liberates one micromole of ammonia under optimum conditions.

5. Optimization of glutaminase production by isolate F1H

The effect of various process parameters (7) influencing L-glutaminase production by the most potent isolate F1H has been studied at specific range for each. Starting with time from 1-9 days then, temperature from 10-50 oC, different shaking speeds from 0 -200 rpm, dark and light conditions, pH from 1-13, different buffer systems and different inoculum sizes.

RESULTS AND DISCUSSION

I.1. Isolation

The medium used for isolation was for growing intermediate halotolerant micro-organisms on 2.9, 3.5 and 4 % NaCl. The number of isolates on 2.9 % was 7 fungal and 2 bacterial, and on 3.5% was 4 fungal and 2 bacterial, while on 4% was only four fungal isolates. This indicated that, with increasing the concentration of salt the growth of microorganisms decreased. This is due to the presence of gradual increase in salt concentration in the growth environment that lead to influx of cell solutes to the outside medium which lead to change in the morphology and characters of the bacterial cells or even damage and death of the cells as indicated by decreasing the number of isolates

which can still withstand concentration of salt up to 4% [14]. Concerning fungi, the ability to withstand the high salt concentration is increasing total lipids, phospholipids, free fatty acids, free sterols, and the unsaturated fatty acids in fungal mycelia as the NaCl concentration increased in parallel with decreasing neutral lipids and unsaturated fatty acids up to limits that do not lead to distortion of fungal cells [15].

I.2. Quantitative assay of glutaminase

Most researches recorded the synthesis of glutaminase extracellulary only. In this research the finally four selected isolates were able to produce glutaminase extracellularly and intracellularly. Quantitative assay results led to varying enzyme productivity from 5.72 - 6.43 U/ml extracellularly and from 5.18 – 5.77 U/ml intracellularly as shown in table 1. Greater productivity was shown extracellularly with the most potent isolate F1H (6.43 U/ml). This was the chosen isolate for optimization and further researches.

Table 1. Quantitative assay of the extracellular and intracellular samples of selected halotolerant isolates

Isolate code	Production type	Enzyme productivity U/ml
F1H	Extracellular	6.43± 0.15
	Intracellular	5.20± 0.33
F2.h	Extracellular	5.72± 0.40
	Intracellular	5.46± 0.13
F3.h	Extracellular	6.12± 0.18
	Intracellular	5.18± 0.23
F4.h	Extracellular	5.86± 0.15
	Intracellular	5.77± 0.13

I.3. Optimization of glutaminase using halotolerant isolate F1H

The isolate F1H has been shown the highest productivity so, It was selected as the most potent one and was subjected for primary optimization. Seven factors were the basis of optimization *viz*. incubation time, incubation temperature, static and shaking incubation conditions, dark and light conditions, pH, buffer system and inoculum size.

The production of glutaminase by isolate F1H was tested under different shaking speeds incubation viz. 0, 100 and 200 rpm (Fig 1). The maximum productivity was 5.04 U/ml at static conditions. A few researchers gave attention to agitation speed in production of glutaminase microbially as reported by [7], the maximum glutaminase productivity was recorded at 120 rpm via *Vibrio azureus* JK-79 in contrast to the production via the most potent fungal isolate in this research as shaking adversely affect the productivity.

The results in figure 2 indicated that, the glutaminase productivity increased in dark condition than light with productivity 5.7 U/ml. this result may be due to the effect of light on changing microbial activities due to the chemical reactions that affected by light and thus altering the behavior the microorganism [16].

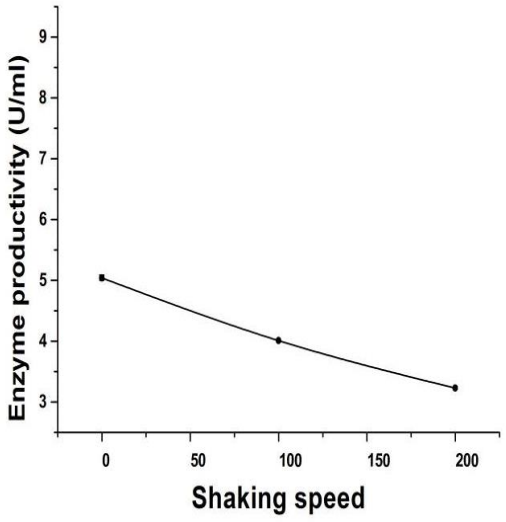

Figure 1. Effect of static and shaking conditions on enzyme productivity for halophilic isolate (F1H).

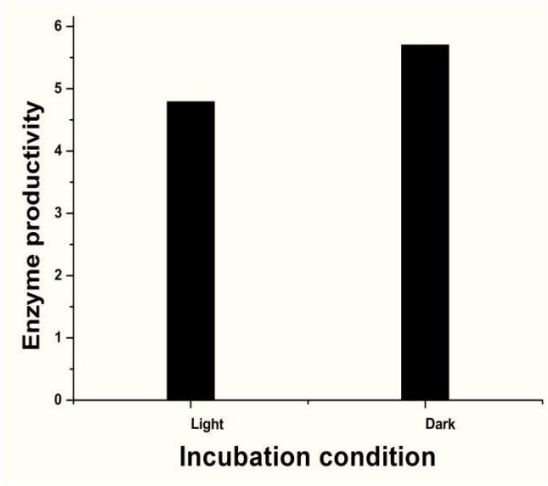

Figure 2. Effect of dark and light conditions on enzyme productivity for halophilic isolate (F1H)

Concerning the effect of pH on glutaminase productivity, the isolate F1H has shown good ability to produce glutaminase over the whole pH range from 1 to 13 with productivity ranging from 0.49 to 5.99 U/ml. The optimum pH for maximum glutaminase productivity was 7 with 5.99 U/ml productivity lesser or greater than this value the enzyme productivity decreased (table 2). It was noticed that, there was another optimum pH value (11) at which the productivity increased again up to 3.83 after decreasing to 3.24 U/ml which may interpreted by the presence of two glutaminase isomers with different optimum pH for their production [17]. This was satisfying with many other researches in which the optimum pH was 7 [18, 19 and 20]**.** The production of glutaminase by other investigators was recorded at other pH values as pH 5 [21 and 22], pH 4.1 [2], pH 6 [23] and pH 8 [24].

Table 2. Effect of different pH values on enzyme productivity for halophilic isolate (F1H)

pH	Final pH	Enzyme productivity U/ml
1	3.23	0.84± 0.26
3	9.38	4.79± 0.89
5	9.55	5.33± 0.95
7	9.66	5.99± 0.95
9	9.85	3.24± 0.36
11	9.59	3.83± 0.05
13	9.62	0.49± 0.09

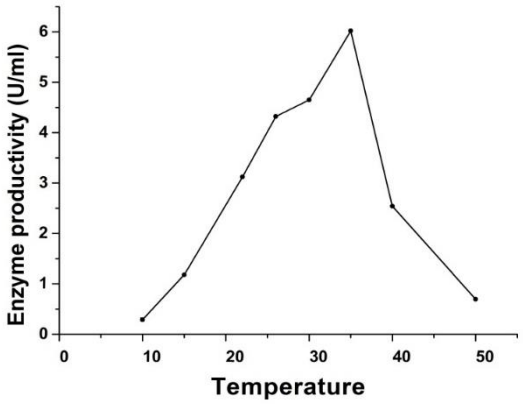

Figure 3. Effect of different incubation temperature on enzyme productivity for halophilic isolate (F1H)

The temperature is an important factor in production of any enzyme as it is controlling all biochemical reactions of the microbial cells [25]. The data represented in figure 3 demonstrated the effect of temperature with range from 10- 50ºC on glutaminase productivity by F1H isolate. The optimum temperature for glutaminase productivity was 35ºC. The productivity increased from 0.29 U/ml at 10ºC up to 6.02 U/ml at 35ºC, further increase in temperature resulted in inhibition of glutaminase productivity. Close to the obtained results many other researches as [2] reported the production of glutaminase via halotolerant isolate at 29.6ºC, 30ºC [26], 27ºC [4] and 37 ºC [7] in contrast to [27] who reported the maximum glutaminase production at higher temperature 40ºC.

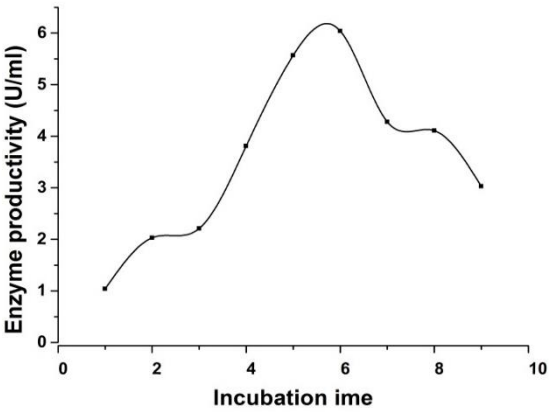

Figure 4. Effect of different incubation time on enzyme productivity for halophilic isolate F1H

Glutaminase productivity was screened over broad time range from 1- 9 days and the highest yield (6.04 U/ml) was obtained on 6thday. The isolate has shown activity starting

from day 1 (1.04 U/ml) followed by gradual increasing up to 6thday, still had the production ability at 9th day reached 3.03 U/ml (figure 4). in another study the optimum glutaminase production time was lower (4.5) days lower and higher incubation time was recorded 4.5 day [28] and 7 days [29] regarding to the isolate used.

In addition, the glutaminase productivity was studied using different buffer systems rather than adjusting pH of the production medium with HCl and NaOH 6N. In this concern, five different buffer systems covering the pH range from 4 to 10 were tested as indicted in table 3. It was noticed that, all used buffer systems had inhibitor effect on glutaminase production. This is may be due to the action of buffer which is to maintain pH constant throughout the overall production time and this was not applicable for the producing isolate [30] so, the most appropriate condition for maximum glutaminas activity was adjusting initial pH at 7 without any buffer.

Moreover, the effect of the inoculum size of isolate F1H used for glutaminase production was studied. In this aspect, different inoculum sizes were used to investigate the optimum for maximum glutaminase production. Data represented in figure 5 has shown that, the highest productivity attained at inoculum $4.38x10^6$ spores/ml. Increasing or decreasing the inoculum above or below this value resulted in lowering the productivity of glutamianse by F1H isolate which may be due to the presence of inadequate level of inoculum in balance with the nutrition, aeration and other factors controlling the production and this condition differs indeed from one isolate to another [31].

It was observed from the results that, all factors affecting glutaminase productivity in noticeable manner. The productivity of the enzyme was increased from 5.04 to 7.66 U/ml after optimization. It is very promising that the production of glutaminase by *Aspergillus flavus* CZCU-9 was carried out in the presence of 4% salt (NaCl) efficiently. In another recent research, screening of halotolerant glutaminase producing isolates was done in Modified M-9 medium containing 20.5 g/L (2.05%) NaCl [2]. Also, investigation of the production of

Table 3. Effect of different buffer systems on enzyme productivity for halophilic isolate (F1H)

Buffer system	pH	Enzyme productivity (U/ml)	Buffer system	pH	Enzyme productivity (U/ml)
I. Citrate	4.6	2.26± 0.22	Citrate-phosphate	4	1.07± 0.15
	4.8	2.17± 0.29		5	1.3± 0.4
	5	2.88± 0.36		6	1.8± 0.28
	5.2	3.44± 0.39		7	4.15± 0.13
	5.4	1.98± 0.32	Phosphate	5.7	2.72± 0.35
				6	4.28± 0.49
				7	3.52± 0.21
				8	3.2± 0.10
II. Phthalate Sodium Hydroxide	4.6	1.11± 0.23	Glycine-NaOH	8.6	3.37± 0.66
	4.8	3.02± 0.47		9.4	2.29± 0.06
	5	1.48± 0.44		10	1.64± 0.18
	5.2	1.10± 0.10		10.6	0.68± 0.10
	5.4	1.27± 0.04			

glutaminase in medium containing 7.5 g/L NaCl 0.75% was reported [9]. Glutaminase from marine bacteria on nutrient broth medium containing 5 g/l (0.5%) NaCl was reported [3] and 2% supplemented medium using *Streptomyces* sp. SBU1 [26].

The most potent halotolerant glutaminase producing isolate (F1H) has been identified genetically based on 18S ribosomal RNA gene partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 complete sequence and 28S ribosomal RNA gene partial sequence and has shown 100% similarity to *Aspergillus flavus* CZCU-9 and it was given the name *Aspergillus flavus* CZCU-9, F1H (table 4).

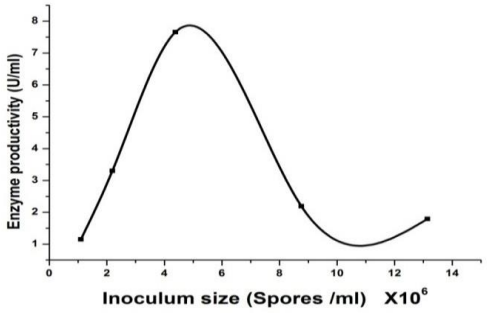

Figure 5. Effect of different inoculum sizes on enzyme productivity for halophilic isolate (F1H)

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Query  1    ATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  526  ATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTC  467

Query  61   GAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGGCCCGTcccccccGGAGAGGGGACGA  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  466  GAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGGCCCGTCCCCCCCGGAGAGGGGACGA  407

Query  121  CGACCCAACACACAAGCCGTGCTTGATGGGCAGCAATGACGCTCGGACAGGCATGCCCCC  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  181  CGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACGGAATTCTGCAAT  240
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Query  361  TCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTGAGAGCCCCCGGCGGCCATGAATGGC  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  421  GGGCCCGCCGAAGCAACTAAGGTACAGTAAACACGGGTGGGAGGTTGGGCTCGCTAGGAA  480
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Query  481  CCCTACACTCGGTAATGATCCTTCCGCAGG  510
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Table 4. Genetic identification of isolate F1H with the percentage of similarities to the resulted strains

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus flavus strain CZCU-9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.	942	942	100%	0.0	100%	MF166768.1
Aspergillus sp. isolate CBA internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inte	942	942	100%	0.0	100%	MF185179.1
Aspergillus flavus strain RF-03 small subunit ribosomal RNA gene, partial sequence; internal transcribed spac	942	942	100%	0.0	100%	MF120213.1
Aspergillus oryzae strain F6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer	942	942	100%	0.0	100%	KX522630.1
Aspergillus flavus isolate RF-02 small subunit ribosomal RNA gene, partial sequence; internal transcribed spa	942	942	100%	0.0	100%	KY933394.1
Aspergillus flavus isolate Sichuan-Rfsb11 18S ribosomal RNA gene, partial sequence; internal transcribed spa	942	942	100%	0.0	100%	KX067887.1
Aspergillus flavus isolate Sichuan-Rfsb10 18S ribosomal RNA gene, partial sequence; internal transcribed spa	942	1003	100%	0.0	100%	KX067886.1
Aspergillus flavus isolate Sichuan-Rfsb05 18S ribosomal RNA gene, partial sequence; internal transcribed spa	942	1075	100%	0.0	100%	KX067885.1
Aspergillus sp. isolate Sichuan-Rfsb01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g	942	942	100%	0.0	100%	KX067884.1
Aspergillus flavus isolate Liaoning-Rfsb135 18S ribosomal RNA gene, partial sequence; internal transcribed s	942	942	100%	0.0	100%	KX067877.1
Aspergillus flavus isolate Anhui-Rfsb31 18S ribosomal RNA gene, partial sequence; internal transcribed space	942	942	100%	0.0	100%	KX067853.1
Aspergillus flavus isolate Anhui-Rfsb27 18S ribosomal RNA gene, partial sequence; internal transcribed space	942	942	100%	0.0	100%	KX067852.1
Aspergillus flavus isolate Anhui-Rfsb15 18S ribosomal RNA gene, partial sequence; internal transcribed space	942	942	100%	0.0	100%	KX067849.1
Aspergillus flavus strain CICC 40866 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	942	942	100%	0.0	100%	KX462776.1
Aspergillus flavus strain CICC 40188 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	942	942	100%	0.0	100%	KX462774.1
Aspergillus flavus strain CICC 40186 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	942	942	100%	0.0	100%	KX462773.1

It is an advantage that the halotolerant glutaminase producer in this research was fungal isolate as most halotolerant glutaminase producing microorganisms proved were belonging to bacterial genera [32]. In this concern, L-glutaminase was produced extracellularly by *Beauveria* sp., isolated from marine sediment using solid state fermentation in the presence of polystyrene as an inert support. Maximal enzyme production (49.89 U/ml) occurred at pH 9.0, 27°C, in a seawater based medium supplemented with L-glutamine (0.25% w/v) as substrate and D-glucose (0.5% w/v) as additional carbon source, after 96 h of incubation was recorded [33]. Solid-state fermentation (SSF) was carried out for the production of extracellular l-glutaminase by the saline tolerant yeast *Zygosaccharomyces rouxii* NRRL-Y 2547 using two agro-industrial substrates, wheat bran and sesame oil cake, which were selected after a screening of four substrates and two moistening media (sea water and 10% NaCl in tap water). Results showed better efficiency of NaCl medium for wheat bran and seawater for sesame oil cake. Maximum L-glutaminase titres (7.5 and 11.61 U/g dry substrate- gds in comparison to 2.2 and 2.17 U/gds as initial values for wheat bran and sesamum oil cake [34].

CONCLUSION

Glutaminase enzyme is an important enzyme on the medical, industrial and economic aspects. It was produced efficiently by selected halotolerant fungal isolate and the primary optimization lead to enhancing the productivity of the enzyme taking into the consideration the possible effects of the mentioned factors as temperature, pH, time, inoculum, incubation conditions as presence or absence of light, agitation speed and using buffer system for adjusting pH. Further optimizations is necessary and studying the salt stability of crude or purified enzyme as main point in many applications which will be under study.

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الملخص العربى :

تم عزل كائنات دقيقة منتجة لإنزيم الجلوتامينيز ومقاومة للملوحة باستخدام وسط غذائى يحتوى على نسب مختلفة من كلوريد الصوديوم (٣,٥,٢,٩و٤٪). تم اختيار أربع عزلات فطرية (ف١هـ, ف٢هـ, ف٣هـ وف ٤ هـ) كأقوى عزلات لها القدرة على النمو بكفاءة على جميع تركيزات كلوريد الصوديوم ومن ثم تنقيتها. تم انتاج إنزيم الجلوتامينيز على وسط غذائى يحتوى على تركيز٤٪ كلوريد صوديوم واختبار وجود الانزيم داخل وخارج الخلايا لكل عزلة. وجد ان انتاجية الانزيم كانت ٦,٤٣, ٥,٧٢, ٦,١٢ و ٥,٨٦ وحدات انزيم/مل خارج الخلايا لكل عزلة بالترتيب و داخل الخلايا ٥,٢, ٥,٤٦, ٥,١٨ و ٥,٧٧ وحدات انزيم/مل بالترتيب. العزلة ف١هـ تم اختيارها كأقوى عزلة منتجة لانزيم الجلوتامينيز خارج الخلايا واخضاعها لمزيد من الدراسات. كانت الظروف الملائمة للحصول على أعلى انتاجية من إنزيم الجلوتامينيز بواسطة العزلة ف١هـ تتضمن التحضين لمدة ٦ ايام فى درجة حرارة ٣٥ درجة سليزية و درجة حموضة ٧ فى ظروف مظلمة وحضان ثابت وبحقن الوسط الغذائى بعدد ٤,٣٨جراثيم/ مل. وجد أن عملية تحسين انتاجية إنزيم الجلوتامينيز فى ظل العوامل السبعة التى تمت دراستها أدت الى زيادة الانتاجية الى ٧,٦٦ وحدات انزيم/مل. تم تعريف العزلة ف١هـ وراثيا ووجد انها بنسبة ١٠٠٪ مشابهة للسلالة اسبرجيللس ڤلافس سى زد سى يو -٩ (*Aspergillus flavus* CZCU-9) وتم اعطائهاالاسم و الرمز الكودى اسبرجيللس ڤلافس سى زد سى يو -٩, ف١هـ (*Aspergillus flavus* CZCU-9, F1H.