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Isolation and Molecular Identification of Fungi Producing L-methioninase Enzymes Isolated from Makkah Region Soil

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Abstract

Out of 24 fungal isolates recovered from plant rhizosphere in north and south of Jeddah city in Saudia Arabia, eight isolates were able to produce a large amount of L-methioninase enzyme. The higher producers fungal isolates were identified by sequencing of 18S rRNA using DNA sequencing of ITS-1 and ITS-4 primers and deposited in the GenBank nucleotide sequence database under accession numbers as: *Nothophomagossy piicola* LT592943.1, *Aspergillus oryzae* XR002735719.1, *Penicillium oxalicum* LC369124.1 *Penicillium oxalicum* LT558935.1, *Aspergillus niger* DQ915806.1, *Penicillium sp*. KP872507.1, *Aspergillus awamori* MH062890.1, *Penicillium sp*.KP256500.1, *Rhizopus oryzae* GU126375.1 *Penicillium oxalicum* MH171483.1(AAD2) produced the highest amounts of L-methioninase (2.5825 U/min/ml), followed by isolates *Nothophomagossy piicola* LT592943.1AAA1(2.1780U/min/ml) after five days of incubation.

Keywords: Fungi, L-glutaminase, 18S rRNA

Introduction

L-methioninase (METs) catalyses the decomposition of methionine, an indispensable amino acid into ammonia, α-Keto glutarate and methanethiol [1]. Bacterial L-metioninase has gained much consideration as it displays anti-propagative action to different kinds of cancerous cells [2]. Treatment through

permeability ascertained that L-methioninase can be produced in vitro in microbes [3; 4]. There are virtually no wide-ranging investigations about the prospective of filamentous fungi used to produce L-methioninase [5].

L-Methioninase has been filtered and considered from several bacterial species such as *Pseudomonas putida,* [6; 7], *Brevibacterium linens* [8], *Citrobacter freundii* [9, 10], and *Clostridium sporogenes* [11]. Furthermore, L-Methioninase enzyme was refined from *Arabidopsis thaliana* [12] and *Streptomyces variabilis*3MA [13].

The in vitro L-methioninase enzyme idealization and production applying quite a lot of agronomical-industrial remains from *Aspergillus flavipes* MTCC 6337 applying the technique of SSF is investigated [4], *Penicillium notatum* [5]. *Geotrichum candidum* [14] was used as an in vivo and in vitro enzyme [15]. *A. flavipes, Aspergillus sp., Penicillium sp.,* and *Humicola fuscoatra* [4]. A lot of extracted yeast from different places IN Egyptian soils *Candida tropicalis* was known as the major active separate. Findings indicated that the enzyme was produced in vivo [3].

The L-methioninase is expectant as an antitumor factor as L-methionine compound is necessary for the development of pernicious cells [2]. Tan et al. [16] reported that increased reliance on methionine of cancer cells is a cancer-specific metabolic disorder. Fu and Tian [17] reviewed that increased methionine dependence in tumour cells is a specific metabolic disorder. This metabolic disorder is also the purpose of selective cancer therapy.

Actually, L-methionine could possibly be reused through homocysteine re-methylation in normal cells [18]. . Many kinds of cancer cell not able to live in culture empty of L-methionine although homocysteine is existing [19]. L-methionine enzyme was primary studied as a tumor-choosy treating purpose in extracellular trails [20]. As a result, in the battle against cancer, it possibly will increase the medicine effectiveness so other wises traight forward directed to cancer therapy and might weaken chemo-therapy poisonousness [21; 22].

Methods

Collection of Samples

Different soil samples were collected from some plants rhizosphere from Makkah region, Jeddah province (Jeddah city, Khulais and Bahra). The soil samples were collected from the surface and depth of 10cm from different plants rhizosphere.

All samples were transported labeled in clean individual to the laboratory, 10g of each sample was suspended in 90ml of sterile distilled water in a 250ml Erlenmeyer flask. The soil sample was agitated in rotary shaker [23] at 150rpm for 20mins.The suspension was allowed to stand for 30sec before it was serially diluted. Aliquots of 0.1ml of suspension was evenly spreaded on Modified Czapek Dox Agar. The plates were incubated at 28C for 72h.

Purification of isolated fungal strains

Colonies of fungal were selected randomly and were purified by transferring part of the edges shoots fungal foreign colony to Petri dishes containing Czapek yeast extract agar medium) Glucose/:2.00, Amino acid: 10.00, Kh2po4:1.52, KCl: 0.52, $MgSO4.7H_2O$:0.52, $FeSO4.7H_2O$: 0.01(and incubated at (28 ± 2°C) for 3-7days, the purified isolates were maintained by periodical transfer on Czapek yeast extract agar at 4°C for other studies [24].

Detection of L-enzymes activity of fungal isolates (Qualitative assay)

Appropriately fungal isolates were screened for the ability of L-methioninase production by a qualitative rapid plate assay procedure using (Czapek yeast extract agar medium) and Glutamine, asparagine, methionine and arginine were used as carbon sources with each enzyme respectively. Czapek yeast extract agar medium was autoclaved and plates prepared. The pH of the medium was adjusted at 6±0.2 **(**using 1N NaOH and 1N HCl) and phenol red (2g in 100ml ethanol) with final concentration of 0.009% just before pouring the plates, phenol red as an indicator was added to the media, as yellow color zone around the colonies were reflected L- methioninase [25].

The plates were then incubated in an inverted position at 28±2ºC for 48 to 96 hours. All fungal were tested by the plate assay. Producing colonies were selected on the basis of formation of yellow zone around the colonies for L-methioninase [26]. Well isolated colonies and purified and subcultures from each plate were selected for further studies.

Quantitative assay of the L-methioninase by rapid plate method:

The potential quantitative estimation of the enzymes in culture filtrate was done by agar well diffusion method. The fungal Cell-Free Filtrate was harvested by centrifugation at 6000 rpm min-1 for 30 min then filtrate by filter paper. Agar plates were prepared by pouring 20 ml of MCD media in to sterile Petri dish. After plates solidify 7mm wells were punching, using sterilized cork borer, 100µl of sample was loaded in the wells, the plates kept in upright position at 28±2C˚ for 24 to 48 hrs [27].

Quantitative assay of the L-methioninase by Nesslerization:

The L-methioninase production was detected by determining the amount of ammonia released from L-methionine spectrophotometrically after Nesslerization as described by Imada et al. [28] with some adaptations. The optimal reaction system includes 1 ml of 1% L-methionine in 0.5 M phosphate buffer (pH 7.0), 0.1 ml of pyridoxal phosphate, and 1 ml of raw enzyme. The reaction system was incubated at 30°C for 1 h. The enzymatic activity was blocked by adding 0.5 ml of 1.5 mol/L trichloroacetic acid or by boiling for 5 min. The system was centrifuged at 5,000 rpm for 5 min to eliminate the precipitated protein. 0.1 ml of prior mixture was added to 3.7 ml of distilled water and the liberated ammonia was detected utilizing 0.2 ml of Nessler reagent, and the deve-

loped colored compound was measured at 480 nm using spectrophotometer. Enzyme and substrate blanks were utilized as controls.

One unit of L-methioninase was measured as the amount of enzyme that releases ammonia at 1 μmol/h under standard examination conditions. The specific activity of L-methioninase was expressed as the activity of enzyme in terms of units per mg of protein [29].

Molecular identification

Genomic DNA extraction:

DNA was isolated from fungal isolates using **DNeasy® fungi Mini Kit**

Polymerase chain reaction (PCR)

ITS-u1 and ITS-u4 universal oligonucleotide primers were utilized as described [30]. Forward ITS1 (5`TCCGTAGGTGAACCTGCGG3`) and reverse ITS4 (5`TCCTCCGCTTATTGATATGC3`) primers were ordered and synthesized to recover full-length gene.

Database research

The obtained sequencing results exposed to a BLAST search program (http://www.ddbj.nig.ac.jp/search/blast-j.html) for identification of the similarities with the genes in the gene bank.

Formula for the calculation of enzyme activity:

$$\text{Enzyme activity (U/ml)} = \frac{\text{μmoles of urea released}}{\text{time of enzyme action} * \text{volume of enzyme (ml)}}.$$

Results

Isolation of fungal strains:

Based on serial dilution 20 fungal isolates from different soil samples, fungal isolates AAA was obtained from north Jeddah, while fungal isolates AAB obtained from the samples from east Jeddah, also the fungal isolates AAD isolated from south Jeddah. Three fungal isolates AFA was obtained from the samples collected from Khulais and four fungal isolates AGL was isolated from Alkhumra samples.

Various fungal strains which were isolated from soil samples were preserved on the modified Czapek dox agar (medium) for further studies.

Qualitative assay of L-methioninase activity of fungal isolates:

Fungal isolates ability for L- methioninase activity was tested on modified MCD supplemented with L-Met, as the sole nitrogen sources. The primary screening was carried out using phenol red as the pH indicator; Phenol red dye is turn to yellow in the acidic medium. Existence of pink color region around the colonies on MCD plates with L- methionin as a nitrogen source was due to the liberation of corresponding enzyme.

The isolates in Figures (1) showed L-methioninase activity were AAA1, AAB1, AAB4, AAD2, AAD3, AAD5, AFL2, AGL1 and AGL4.

4.3. Quantitative assay of L-methioninase activity in Cell-Free Filtrate of the selected fungal isolates by diffusion method:

The cell free filtrate of four selected fungi was evaluated L-methioninase. production by agar well diffusion assay on solid MCD plates with red phenol, after 24h and 48h of incubation, the measurement of yellow zone in millimetres were recorded based on the diffused of L-methioninase in the agar, of fungal isolates during 24and 48 h.

Results in Table(1) and Fig.(1) show the diffusion of L- methioninase that produced by all isolates show, all the tested fungal isolates produced L-methioninase after 24h of incubation, It noticed the increase of the incubation period to 48h revealed to increase the production of the enzyme. The maximum amount of L-methioninase was produced by isolates, AAD2, AAA1, AAD3 and isolateAGL4, the diameter of the diffusion enzyme 58mm, followed by isolates AGL2 and AFL2 the diameter of the zones 37mm.

Table (1): Quantitative assay of L-methioninase activity in Cell-Free Filtrate of the selected fungal isolate by diffusion method

Fungal isolates	Zone diameter in (mm) after	
	24 h	48 h
AAA1	20	58
AAB1	20	34
AAB4	12	34
AAD2	18	58
AAD3	17	58
AAD5	11	35
AFL2	12	37
AGL1	14	37
AGL4	16	58

Quantitative assay of L-methioninase activity produced by the selected fungal isolates by Nessler's reaction.

L-methioninase production by the selected fungal isolates was assayed quantitatively for 7 days of incubation. Results in Figure (2) show, the fungal isolates produced different amounts of L-methioninase increased by increasing incubation periods, maximum amounts of the enzyme was accumulated after isolate AAD2 which produced the highest amount 2.5825U/min/ml, followed by isolates AAA1 (2.1780U/min/ml) and AAD3(1.9755U/min/ml) and AGL4 (1.64 U/min/ml) after five days of incubation, the amounts decreased at incubation time under or above these periods.

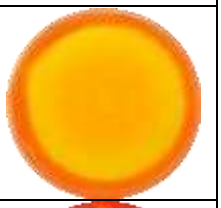
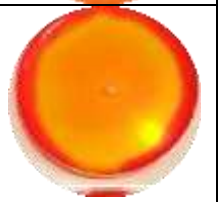
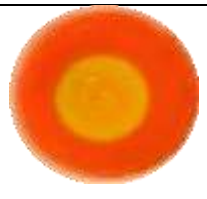
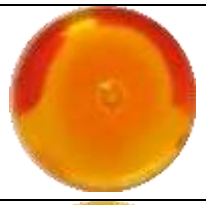
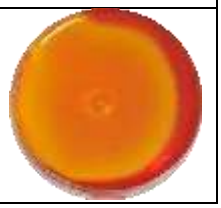
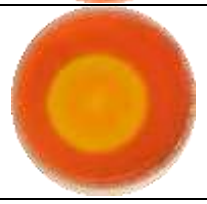
Fungal Isolates	**12H** Control plate	**24H**	**Fungal Isolates**	**12H**	**24H**
AAB1			AAD5		
AAD2			AGL1		
AAB4			AAD3		
AAA1			AFL2		
AGL4					

Figure (1): Quantitative assay of L-methioninase activity in Cell-Free Filtrate of the selected fungal isolate by diffusion method

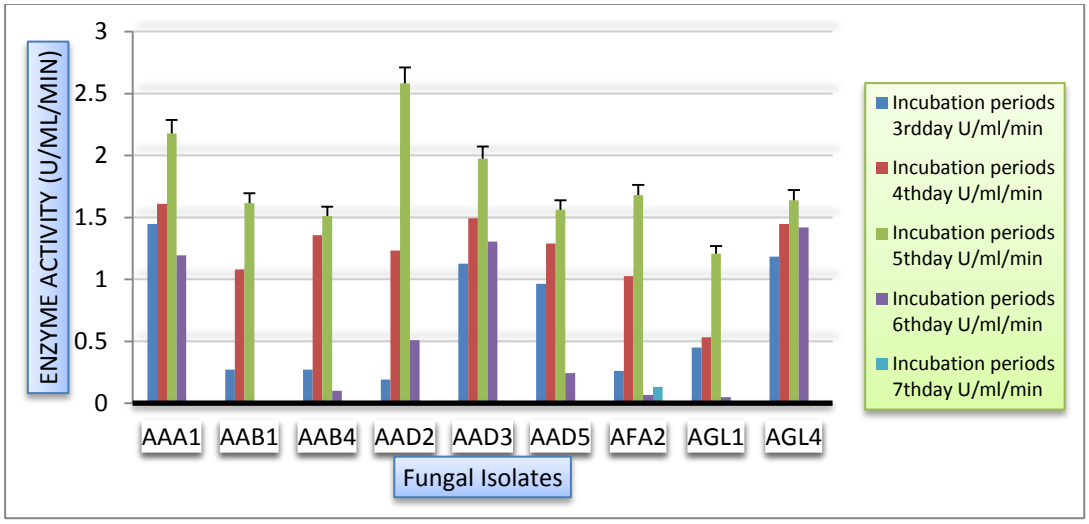


Figure (2): Quantitative assay of L-methioninase activity produced by the selected fungal isolates by Nessler's reaction.

Molecular identification of fungal isolates:

The fungal isolates were subjected to molecular analysis and identification using DNA sequencing of ITS-1 and ITS-4 primers. First, the 750 bp amplicons were separated on 1% agarose gels to prove their accuracy and specificity. Then, the fragments with the molecular sizes representing the ITS-1 and ITS-4 primers purified for sequencing Table (2).

Table (2): DNA sequencing of 18s rRNA using universal primer for fungal isolates

Fungal isolate	Name and Accession of No. of the most related strain in NCBI GenBank		Identity (%)	Coverage (%)
AAA1	LT592943.1	*Nothophomagossy piicola*	100%	100%
AAB1	XR_002735719.1	*Aspergillus oryzae*	91%	100%
AAB4	LC369124.1	*Penicillium oxalicum*	100%	100%
AAD2	LT558935.1	*Penicillium oxalicum*	100%	100%
AAD3	DQ915806.1	*Aspergillus niger*	100%	100%
AAD5	KP872507.1	*Penicillium* sp.	90%	100%
AFA2	MH062890.1	*Aspergillus awamori*	100%	100%
AGl1	KP256500.1	*Penicillium* sp.	100%	100%
AGL4	GU126375.1	*Rhizopus oryzae*	90%	100%

Discussion

In most of the microbial assay programs for enzyme production, the microorganisms are the first isolated from certain conditions by routine isolation methods and then examined for enzymatic activity by procedures as zone of color change or zone of clearance on agar medium supplemented with suitable substrate [31].

The examination and identification of filamentous fungi able to secrete extracellular enzymes with biotechnological ability are activities of large importance [32].

The primary screening was carried out using phenol red as the pH indicator. Phenol red stain is pink at alkaline pH and change to yellow at acidic pH. Existence of color region around the colonies on MCD plates with L-methionin nitrogen sources was caused by the releasing of corresponding enzyme. Screening for enzymatic activity by techniques as zone of color change or zone of clearance on agar medium supplemented with an appropriate substrate [33; 34].

The maximum amount of L-methioninase was produced by isolates, AAD2, AAA1, AAD3 and isolateAGL4, followed by isolates AGL2 and AFL2. These results were in agreement with various results that found by Gulati et al. [35] and Swapna et al. [36].

The present data are in agreement with the studies that found the ammonia formation rises alkalinity of the L-methionine-agar medium the colour changed to pink-red, this result was agree with that found by El-Sayed et al., [22]; Selim et al. [3] and Abu-Tahon and Isaac [37].

L-methioninase production by fungi under submerged fermentation (SmF) by *Aspergillus* sp. [38], *Debaryomyces hansenii* [5;14] has been found that SSF used for the production by *A. flavus*.

The nine isolates were molecular analyzed and identified using DNA sequencing of IT1and ITS4 primers. According to Nocker et al. [39] amplification, IT1, and ITS4 sequences of fungi can produce 750bp amplicon. The DNA sequences analyzed using the Blast alignment tools of GenBank. They showed isolates were identified with similarity percentages 99%-100% as: *Nothophomagossy piicola* LT592943.1, *Aspergillus oryzae* XR002735719.1, *Penicillium oxalicum* LC369124.1 *Penicillium oxalicum* LT558935.1, *Aspergillus niger* DQ915806.1, *Penicillium* sp. KP872507.1, *Aspergillus awamori* MH062890.1, *Penicillium sp.KP256500.1, Rhizopus oryzae* GU126375.1

The 18S rRNA gene-based polymerase chain reaction (PCR) for the identification of airborne fungi has recounted; however, this technique needs sprecise hybridization and analyses of the oligonucleotide. Investigations appraised a PCR technique dependent on a different cluster of primers intended to 18S rRNA gene for fast identification of an aetiology of pan-fungal in bacterial keratitis [40; 41].

From (45%) of isolates that produced L-methioninase. *Penicillium oxalicum* MH171483.1 (AAD2) produced the highest amounts of L-methioninase (2.5825 U/min/ml), after five days of incubation, the amounts decreased at incubation time under or above this period. Khalaf and Alsayed [5] reported specific activity in case of *Aspergillus flavipes* was 10.78 U/min/ml. Raimbault et al. [42] and Khalaf and Alsayed [5] reported the production of L-methioninase under submerged conditions.

The optimum alkaline L-methioninase productivity (10.8 U/min/ml) was produced by *A. ustus*, followed by *A. ochraceus*, *Fusarium proliferatum*, *Penicillium crustosun*and *Trichoderma longibrachiatum* [37]. L-methioninase production by *Serratia proteamaculans* isolated from seashell sample was 0.0282U/min/ml [43].

The fermentation is a metabolic process in many microorganisms and involves oxide-reduction reactions resulting in the breakdown of complex organic compounds into simpler by-products and energy the microbes carry out this metabolic action by the release of 27 extracellular enzymes [44]. Culture broths produced on L-methionine glucose media have shown good L-methioninase production under submerged conditions [15]. This media gives the necessary minimal nutrition required and provides L-methionine as carbon source which is used by bacteria, as growth substrate will allow its growth.

This media allows the growth of even those bacteria which can use glucose and grow, therefore to further screen the obligate L-methionine degraders, 100% L-methionine basal agar where L-methionine is the only source of carbon, nitrogen [38]. The enzyme activity was estimated in culture broth utilizing Nessler's reagent [45].

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