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L-methionine γ-lyase From Thermo-tolerant Fungi: Isolation, Identificatio Of The Potent Producers, And Statistical Optimization Of Production Via Response Surface Methodology

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Abstract

In the current study, thermo-tolerant fungal isolates were screened for their ability to metabolize L-methionine by L-methionine γ-lyase (MGL). Among 63 fungal isolates, fifteen isolates exhibited ability to MGL induction as evidenced by qualitative assay method using phenol red. Then, quantitative assay method indicates that fungal isolates coded as 26 & 37 were the most potent for MGL production. Fungal mycelium treatment under the sonication process proved that isolate No. 37 could be release MGL with maximum activity, meanwhile isolate No. 26 has been produced MGL extracellularly under the same culture conditions. Thereafter, characterization and identification of these two isolates was carried out by morphological, microscopic examination and molecular techniques. In consequence of this characterization, isolate No. 26 & 37 were identified as *Aspergillus fumigates* and *Rhizomucor miehei* respectively. Different culture conditions were screened by Plakket-Burmn design to define the significant parameters that affect the induction of MGL of the two fungal strains. Using the response surface method model, independent culture parameters were optimized through a central composite design (CCD) to maximize the induction of extracellular and intracellular MGL by *A. fumigatus* and *R. miehei*. Statistical optimization using Response surface methodology (RSM) revealed that yeast extract, incubation period and temperature were significant factors for MGL production by *R. miehei* where MGL was 8.27 U/mg. Additionally, glycerol, PH, period and inoculum size were the most efficient factors affect MGL by *A. fumigatus* where MGL was 12.37 U/mg.

Keywords: Thermo-tolerant fungi; L-methionine γ-lyase; Identification; Optimization; Response Surface Methodology; Central Composite Design.

Introduction

Temperature is considered one of the most important factors which affect the growth of fungi [1]. Thermophilic fungi require 45°C as optimum temperature to grow. The nature of thermophilic and thermotolerant fungi based on their minimum and maximum growth temperature [2]. Thermophilic and thermotolerent fungi are wildly used to produce novel and thermostable enzymes as proteases, cellulases, xylanases and methionase [3-5]. MGL (EC4.4.1.11) is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the direct conversion of L-methionine to methanethiol, ammonia and α-ketobutyrate (Selim et al., 2015; Abdelraof et al., 2019). Microbial MGL has been studied extensively for bacterial strains,

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comparing to little studies concerning the eukaryotic source (fungal strains). Microbial MGL has been discovered in different of microorganisms including bacteria, fungi and yeast. The bacterial MGL has imparted much attention by a lot of researchers, comparing to the eukaryotic MGL sources. As it appeared from the previous studies the importance MGL producing bacteria are *Pseudomonas putida* and *Pseudomonas ovalis* [6], *Aeromonas sp*. some strains of filamentous fungi are reported to be extracellular MGL producer belonged to Aspergillus genera [7]&[8]. MGL plays an important role in food industry by showing a distinctive aroma to many traditional fermented foods including cheese through a degradation of L-methionine that releases volatile sulfur compounds. The most common found in cheese is methanethiol which derives from the enzymatic degradation of the amino acid L-methionine is present in cheese curd [7]&[9]. MGL is one of microbial enzymes it has high therapeutic value where it was reported as a potent anticancer agent against various types of tumor cell lines: breast, colon, lung, kidney, and glioblastoma [10]&[11]&[12]. Many human cancer cell lines and primary tumors have an absolute requirement for L-methionine, an essential amino acid, to survive and proliferate [11]&[13]&[12]. On the other side, normal cells have the ability to grow on amino acid homocysteine, instead of methionine, pointing to their active methionine synthase [12]. Unfortunately, the therapeutic efficiency of bacterial MGL has scarcely occurred without some evidence of toxicity and immunogenic reactions, especially with regard to multiple doses, which may restrict their clinical utility [14]&[7].different attempts have been made to overcome the high immunogenicity and rapid clearance of L-methioninase from plasma including immobilization with biocompatible polymers such as polyethylene glycol (pegylation) that one of the most applicable techniques for providing MGL with less immunogenic properties [15]&[7]. On the other side, the production of MGL by eukaryotes including fungi is the most promising biotechnological process. This results in therapeutic enzymes with fewer immunogenic and allergic reactions, which may be contributes to the higher specificity to their substrates compared with the substrate analogues, displaying a less troublesome during the course of tumor therapy [6]&[16]. In addition, the limited distribution of MGL as an intracellular enzyme among all microbial pathogens but not in humans makes it a promising drug target for antibacterial, antifungal and anti-protozoal therapies [17]&[9]&[6]. Statistical optimization using RSM is considered very important for enhancing the production where RSM incorporates the interaction effects of variables and aids us in simultaneously optimizing several process parameters within a minimal number of experimental runs [19]. The aim of this study is mainly focused on screening for L-methioninase-producing fungi and then optimizing the culture media for enhanced production of the enzyme using a statistical design.

2.Materials and Methods:

2.1. Materials

The source of chemicals and reagents used in this study were as follows: L-methionine from (Merk, Germany). Methanethiol used as sodium methanethiolate; Pyridoxal-5- phosphate (PLP); 5,5-Dithiobis-2-nitrobenzoicacid (DTNB); Commassi Brilliant Blue G-250 and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Sigma, St. Louis, USA). Sephadex G-100 and DEAE-cellulose were purchased from Pharmacia, Biotechnology (Sweden). Potato dextrose agar (PDA) (Condlab, Spain). All other chemicals were of the highest analytical grade.

2.2. Isolation of thermo-tolerant fungi

Soil and water samples were collected from Al Buwaidah - Bahariya Oasis Wells, Egypt. The collected soil and water samples were characterized by high temperature (38^oC-50^oC) used for the isolation of the fungal species on potato dextrose agar (PDA) medium. Soil and water samples were inoculated onto the PDA plates and incubated at 50◦C for 5–7 days. The fungal growth was observed and purified, and then the purified isolates were preserved at 4 ◦C for further study.

3.2. MGL assay procedure

MGL activity measurement was assessed based on the demethiolation reaction method Selim, Elshikh [9] Methanethiol (MTL) release from L-methionine was immediately reacted with 5,5 dithio-bis-2-nitrobenzoicacid added (DTNB) resulting in a yellow colored product(i.e. thionitrobenzoic acid)absorbance at 412 nm. The assay reaction was contained 20 mM of L-methionine in 0.05 M potassium phosphate buffer pH 7.0, 0.01 mM pyridoxal phosphate (PLP), 0.25 mM DTNB and the filtrate or supernatant in a final volume of 1 ml. After 1 h of incubation at 30^o C.

Controls without filtrate or with heat denatured filtrate (at 60^{o} C for 10 min) were prepared separately. Sodium methanethiolate was used as a standard reference to determine the amount of MTL released. One unit (U) of MGL was expressed as the amount of enzyme that releases 1µmole of methanethiol per minute under optimal assay conditions.

2.3. Protein determination

Protein concentration was determined according to the method of Bradford [18] with bovine serum albumin as standard.

2.4. Screening of fungal isolates for MGL production

All fungal isolates were screened for L-methioninase production according to qualitative followed by quantitative screening. Firstly, qualitative screening was carried out using rapid assay plate method [9], where all grown isolates on PDA agar medium were picked up and streaked on the modified Czapek-Dox agar plates. It has the same contents of Czapek-Dox agar medium amended with L-methionine instead of sodium nitrate in medium, in which the final amount of nitrogen (N-base) content in the medium remained unchanged. The medium was supplemented with 0.007% phenol red and the pH of the medium was adjusted to 6.5 using 1 N sodium hydroxide solution. The screening plates were incubated for 7 days at 28°C, MGL producing isolates were identified as evidenced by the pink color of the colonies or around the growth [9]. Finally, the selected fungal isolates were screened quantitatively under shaking incubation for 7 days using L-methioninase assay method [9]. The isolates were incubated in modified Czapek-Dox broth amended with L-methionine at 28^{o}C under shaking condition for 7 days. After incubation, the fungal biomass was harvested, washed with 0.1 M of potassium phosphate buffer pH 7 and sonicated at Sonicator (Vibra-Cell 72405) for 20 min with a 10 s on/10 s off pulse cycle at 60 W, and the resultant extract was used as an intracellular source of MGL, while the culture filtrate was used as the source of extracellular one.

Identification of the most potent isolates

The most potent fungal isolates were identified morphologically and genetically. Morphological identification of the fungus was carried out with observing the morphological characteristics (color, texture, and appearance) and microscopic characteristics using light microscope [19-22]. DNA was extracted from agar cultures using Quick-DNA Fungal Microprep Kit (Zymo research; D6007) following the manufacturer's protocol and supported by Sigma Scientific Services Company (Egypt). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo; K1051). The primers used were Forward ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and Reverse ITS4-R (5′- TCCTCCGCTTATTGATATGC-3′) according to method used by [23].

2.5. Statistical modeling and Optimization of MGL production

The preferred culture medium for the selected fungal strains was then used for furtherstatistically optimization of MGL.Experimental design was conducted via response surface methodology (RSM) by Minitab 2017 software; Plackett-Burman factorial design (PBD)experimental design, doing the experiment, data analysis and validation of the results [24].

2.5.1. Plackett–Burman design (PBD)

The Plackett-Burman experimental design was used to determine the major factors influencing methionase production[25]. Table 1 shows factors and their levels which are usedin Placket burmanfor MGLproduction, where nine numerical factorsof basal medium were used; factors are peptone, yeast extract, L-methionine, $MgSO_4$, incubation temperature, pH, inoculum size, glycerol and incubation period. The experimental design comprised a total of 24 experimental trials; among these, one run was carried out at the center point values and the remaining runs were conducted by combinations of high (+) and low (−) levels of all variables. In the Plackett-Burman experimental design, two levels were used to determine whether the maximum production was obtained at lower or higher concentration of the variables by comparing them with the experimental results obtained from center point values. A 24-run experiment was generated based on the rule R=n+1 where R is the run numbers and n is the number of variables (Table 2). The PB experimental design based on the following first-order model:

$Y= \beta 0 + \Sigma Bi\ Xi$

Where: Y represents the response (L-methioninase activity U/ml), β0 is the model intercept, Bi is the linear coefficient, xi is the level of independent variable, and k is the number of involved variables.

Table 1: Factors and their levels which are used in PBD for MGL production.

Factor Name	Units	Low level	High level
Peptone	g/L	1	3
Yeast extract	g/L	0.5	2
L-methionine	g/L	2	5
$MgSO_4$	g/L	0.5	1
Temperature	ºC	25	35
pH		5	7
Inoculum size	%	1	3
Glycerol	mL/L	0.5	3
Incubation period	Days	5	10

2.5.2. Central composite design (CCD)

After the identification of factors which affect MGLproduction by PB design, three variables (incubation time, yeast extract, and temperature) in *Rhizomucor miehei*were chosen for response surface methodology of central composite design (CCD). The CCD resulted in total of 20 experimental trials (Table 4). Other media components and fermentation conditions were chosen at the significant level concentrations from the PB design and four variables (incubation time, inoculation size, glycerol, and pH) in *Aspergillus fumigatus*were chosen for response surface methodology of central composite design (CCD). The CCD resulted in total of 31 experimental trials (Table 5). The results of the CCD were expressed by the following second order polynomial using a multiple regression technique according to the following equation: $Y= \beta 0 +\Sigma \beta i Xi + \Sigma \beta ii X2i + \Sigma \beta ij Xi Xj$ Where: Y is the predicted response, β0 is the intercept term, βi is the linear coefficients, βii is the quadratic coefficients, βij is the interactive coefficients, and xi and xj are the coded independent variables.

2.5.3. Validation of the results

After the theoretical optimization of the nine factors for maximum production of MGL by the selected fungi, the optimum conditions were experimentally applied in the lab level and compared with the theoretical results. The application of the conditions was performed in three replicas and recorded as (mean ± standard deviation).

3. Results and discussion

3.1. Isolation and screening of MGL-producing thermotolerant fungi

Totally, 63 thermo-tolerant fungal isolates were isolated from soil and water samples. All fungal isolates were screened qualitatively according to rapid assay plate method [9]. Since, L-methionine was used as a sole nitrogen source in order to select the methionine-degrading fungal isolates. Results illustrated in (**Table 1**), showed 15 fungal isolates have the capability to methionine decomposition, which identified as evidenced by the pink color around the colonial growth (i.e. extracellular) or in colonies (i.e. intracellular). Selim et al. [9] reveled the rate of pink color intensity was based on the rate of cultivation period and also on the rate of L-methioninase formation by each isolate. Supplementation of culture medium with L-methionine as a sole nitrogen source instead of a main nitrogen source was studied by many authors to determine the methionine-degrading microorganisms such as fungi [19]; on yeast [36] and on bacteria [44,12]. After that, the fifteen fungal isolates were screened quantitatively using modified PDB and DOX broth media. As shown in Table (2), two fungal isolates coded as 37, and 26 proved to be the most potent producer of MGL. Since, 37 isolate was induction of MGL intercellularly, while 26 was providing MGL extracellularly.

Table 2: Quantitative Screening of MGL by the selected fifteen isolates.

Isolate No.	PDB broth media			DOX broth media		
	Protein mg/ml	**Activity Unit/ml**	**Specific activity Unit/mg**	**Protein mg/ml**	**Activity Unit/ml**	**Specific activity Unit/mg**
6	0.50	2.46	4.92	0.58	3.80	6.55
12	0.35	2.59	7.40	0.52	3.34	6.40

13	0.52	2.32	4.40	0.66	3.86	5.84
21	0.28	2.32	8.28	0.39	3.67	9.41
26	0.48	7.06	14.70	0.49	7.38	15.06
30	0.32	2.46	7.68	0.46	3.78	8.21
36	0.40	3.40	8.50	0.40	3.90	9.75
37	0.38	3.82	10.05	0.37	4.02	10.86
42	0.29	1.91	6.58	0.43	3.20	7.44
43	0.41	3.28	8.00	0.39	3.40	8.71
44	0.65	1.50	2.30	0.72	3.06	4.25
46	0.36	2.32	6.44	0.48	3.62	7.54
50	0.30	2.32	7.73	0.41	3.23	7.87
51	0.42	3.00	7.14	0.42	3.50	8.33
53	0.44	3.28	7.45	0.39	3.34	8.56

3.2. Morphological and molecular identification of the most potent thermotolerant fungi

The two fungal isolates 26 and 37 were identified using morphological and genetical methods as shown in Figure 1. According to macromorphological identification and microscopic characteristics, the fungal isolate 37 was identified as *Rhizomucor miehei*, where colonies showed a rapid growth rate with whitish gray color, sporangiophores were brownish and unbranched, ranged (674–934 µm) in length and (11–14 µm) in diameter, sporangia were spherical in shape ranged (26 - 32 µm) in diameter. Sporangiospores were ellipsoidal (5.2–6.6 × 5.2–5.2µm), slightly brown and rough-walled. On the other hand, the fungal isolate 26 was identified morphologically as *Aspergillus fumigatus*, where colonies were powdery gray blue with heavy sporulation, conidiophores were unbranched with 286 to 390 µm in length and 7.8 to 10.4 µm width, vesicle were uniseriate dome shape, with (18.2–20.8 × 23.4–31.2 µm) in size, conidial head size (26.0–31.2 × 39–46.8 µm), conidiospores were spherical with 2.5-3.1 µm. Molecular identification was carried out to confirm the morphological identification. the molecular identification results revealed that the two fungal isolates 26 & 37 were identified as *A.fumigatus* and *R. miehei*, respectively. Identified strains were subsequently recorded in gene bank with accession numbers MZ407596 & MW648933 respectively as shown in Figure 1E. Previous studies reported that *Aspergillus* spp. could produce L-methionase as *Aspergillus flavipes* [32,33], *Aspergillus ustus* [11], *Aspergillus flavus*, *Aspergillus tamari*, *Aspergillus oryzae*, and *Aspergillus parasiticus* [34].

3.3. Effect of different media on MGL production by *R. miehei* and *A. fumigatus*

From results in (Table 3), we can notice that the highest level of enzyme production (i.e. related to specific activity) (11.21 U/mg) and (15.34 U/mg) with good growth were obtained when *R. miehei* and *A. fumigatus* mycellium were grown in Basal medium [29] followed by methioninase glucose media medium [7] (10.93 U/mg) and (15.22 U/mg). On the other hand, the use of TGY medium [30] or basic medium were correlated with the lowest yield of both growth and enzyme formation. Accordingly, the basal medium proved to be the most suitable for both growth and enzyme production by the two selected fungal strains and therefore it was chosen and used for further optimization studies.

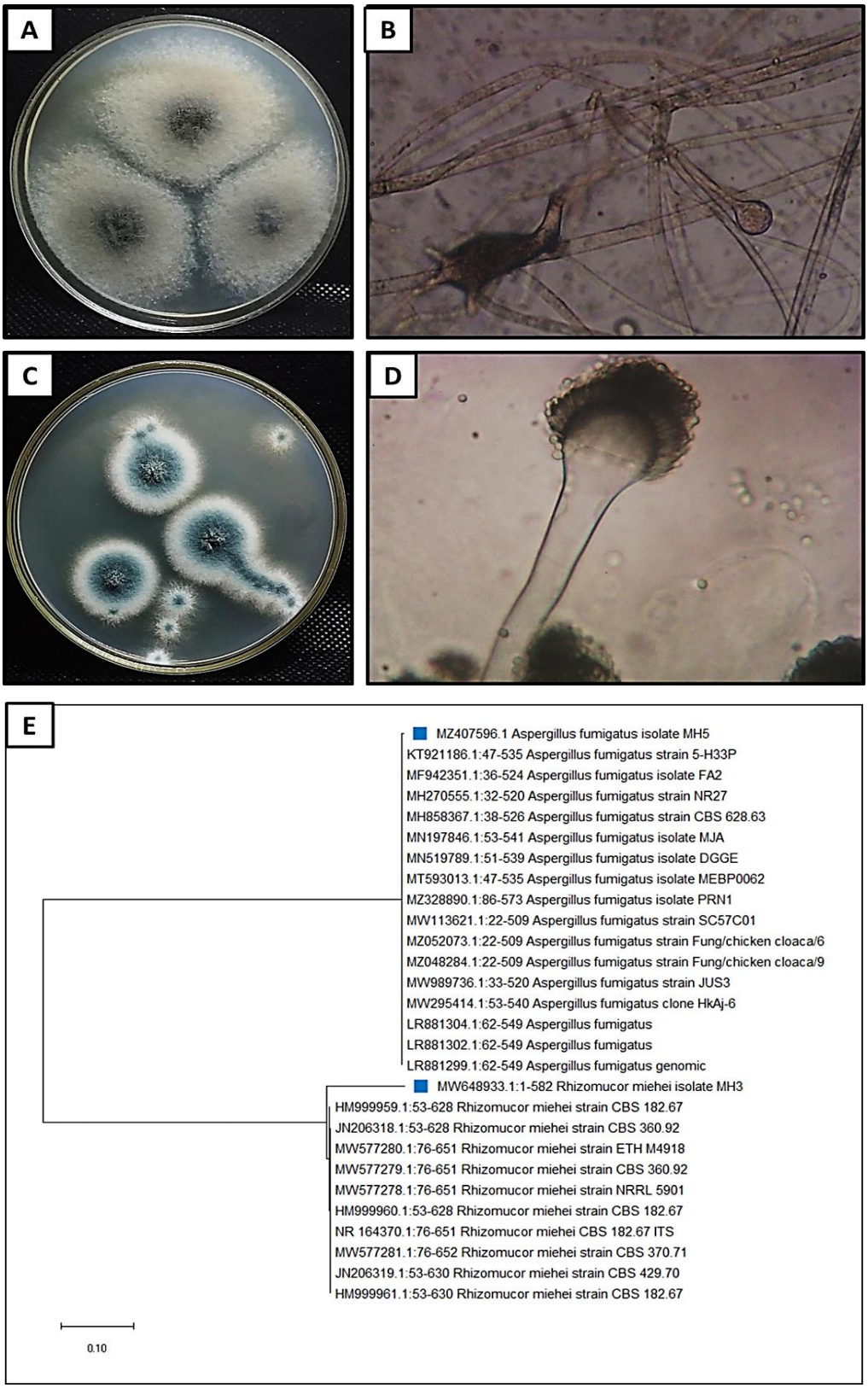

Figure1: Identification of fungal isolates 37 (A&B) and 26 (C&D)

Table (3): Effect of different media on MGL production

Fungal strain	Specific activity Unit/mg		
	TGY media	Basal media	Methioninase glucose media
A. fumigatus	13.10	15.34	15.22
R. miehei	9.78	11.21	10.93

3.4. Statistical optimization of MGL production

3.4.1. Plackett-Burman design

The PBD design is an efficient way to select of significant physicochemical parameters, required to improve MGL production, among a large number of process variables. In the current study, PBD design with 24 runs includes nine variables, examined at two levels and coded as (−1) for low level and (+1) for high level. MGL average activity for the different trials was calculated as (U/ml). The MGL activity in *R. miehei* and *A. fumigatus* recorded a wide variation from 0.10 to 2.40 U/ml and 0.31 to 5.10 U/ml respectively as shown in Table 4. The maximum MGL production in *R. miehei* was achieved in run 16 while in *A. fumigatus* was achieved in run 13 as shown in Table 2. Results of PBD of *R. miehei* revealed that three factors affected significantly on MGL production, these factors were incubation period, temperature and yeast extract as shown in Figure 2A, thus these factors are selected for CCD. On the other hand, four factors affected significantly methionase production by *A. fumigatus* as shown in Figure 2B, consequently these four factors were selected for CCD optimization.

Table 4. PBD for screening of the medium components and conditions for MGL production by ***R. miehei***

Trial no.	Meth.	Gly.	Pept.	YE	PH	Period	Temp.	Inoculum Size	Mgso4	Enzyme activity	Protien	Specifiec activity
1	2	0.5	1	0.5	5	5	25	1	0.5	0.68	0.46	1.47
2	5	3	1	2	7	5	35	1	0.5	0.79	0.45	1.75
3	2	3	3	2	5	10	35	1	1	1.02	0.36	3.30
4	5	3	3	0.5	7	10	25	3	0.5	0.75	0.43	1.74
5	5	0.5	3	0.5	5	5	35	3	1	0.10	0.37	0.27
6	5	3	1	2	5	5	25	3	1	0.52	0.45	1.15
7	2	3	3	0.5	7	5	25	1	1	0.69	0.36	1.91
8	2	3	3	0.5	7	5	25	1	1	0.59	0.47	1.25
9	2	0.5	3	2	7	5	35	3	0.5	0.93	0.57	1.63
10	5	0.5	3	2	5	10	25	1	0.5	2.40	0.33	7.20
11	2	3	1	0.5	5	10	35	3	0.5	1.86	0.38	4.89
12	5	0.5	1	0.5	7	10	35	1	1	0.26	0.42	0.62
13	2	0.5	1	2	7	10	25	3	1	1.15	0.70	1.64
14	5	0.5	3	2	5	10	25	1	0.5	0.81	0.39	2.07
15	2	3	3	0.5	5	10	35	1	1	0.99	0.24	4.12

16	2	3	1	0.5	5	10	35	3	0.5	0.45	0.36	1.27
17	5	0.5	3	2	5	5	35	3	1	0.81	0.60	1.35
18	2	0.5	3	0.5	7	5	35	3	0.5	0.91	0.58	1.56
19	5	0.5	1	2	7	10	35	1	1	0.58	0.35	1.65
20	5	3	1	2	7	5	35	1	0.5	1.92	0.60	3.2
21	2	0.5	1	0.5	5	5	25	1	0.5	1.08	0.29	3.72
22	5	3	3	0.5	7	10	25	3	0.5	0.78	0.26	3.00
23	2	0.5	1	2	7	10	25	3	1	0.46	0.28	1.64
24	5	3	1	2	5	5	25	3	1	0.82	0.61	1.34

Table 5. PBD for screening of the medium components and conditions for MGL production by ***Aspergillus fumigatus***

Trial no.	**Meth.**	**Gly.**	**Pept.**	**YE**	**PH**	**Period**	**Temp.**	**Inoculum Size**	**Mgso4**	**Enzyme activity**	**Protein**	**Specific Activity**
1	2	0.5	1	0.5	5	5	25	1	0.5	1.13	0.34	3.32
2	5	3	1	2	7	5	35	1	0.5	1.58	0.41	3.85
3	2	3	3	2	5	10	35	1	1	0.67	0.32	2.09
4	5	3	3	0.5	7	10	25	3	0.5	3.12	0.49	6.36
5	5	0.5	3	0.5	5	5	35	3	1	5.10	0.61	8.36
6	5	3	1	2	5	5	25	3	1	1.42	0.46	3.08
7	2	3	3	0.5	7	5	25	1	1	0.64	0.47	1.36
8	2	3	3	0.5	7	5	25	1	1	1.17	0.44	2.65
9	2	0.5	3	2	7	5	35	3	0.5	3.99	0.55	7.25
10	5	0.5	3	2	5	10	25	1	0.5	2.71	0.41	6.60
11	2	3	1	0.5	5	10	35	3	0.5	1.40	0.33	4.24
12	5	0.5	1	0.5	7	10	35	1	1	1.28	0.30	4.26
13	2	0.5	1	2	7	10	25	3	1	2.29	0.41	5.58
14	5	0.5	3	2	5	10	25	1	0.5	3.56	0.41	8.68
15	2	3	3	0.5	5	10	35	1	1	1.16	0.15	7.73
16	2	3	1	0.5	5	10	35	3	0.5	0.31	0.18	1.72
17	5	0.5	3	2	5	5	35	3	1	2.41	0.52	4.63
18	2	0.5	3	0.5	7	5	35	3	0.5	1.77	0.55	3.21
19	5	0.5	1	2	7	10	35	1	1	1.38	0.46	3.00
20	5	3	1	2	7	5	35	1	0.5	1.34	0.55	2.43
21	2	0.5	1	0.5	5	5	25	1	0.5	2.07	0.54	3.83
22	5	3	3	0.5	7	10	25	3	0.5	2.57	0.36	7.13
23	2	0.5	1	2	7	10	25	3	1	1.67	0.33	5.06
24	5	3	1	2	5	5	25	3	1	1.27	0.49	2.59

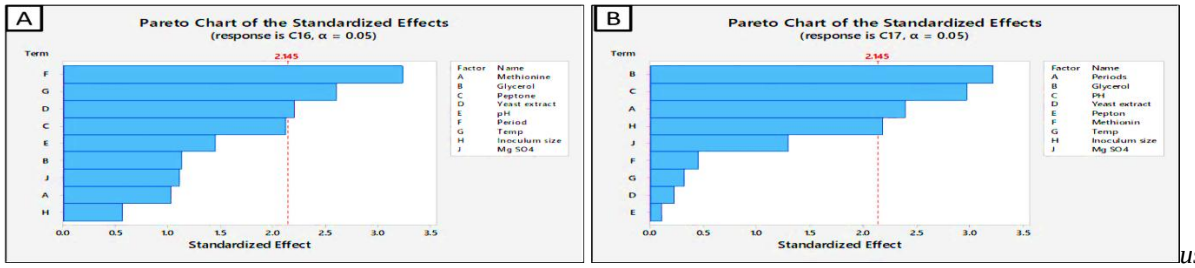

Figure 2: Pareto charts of *Rhizomuco rmiehei*(A), *Aspergill fumigatus* (B) show significant factors affecting methionase production

3.4.2. Central Composite Design

The next step in the optimization of the medium was to determine optimum levels of significant variables to maximize MGL production. Based on the results of PBD design, three factors showed maximum effect on MGL production by *Rhizomucor miehei* (incubation time, yeast extract, and temperature). CCD with 20 runs was designed for optimization of MGL production by *Rhizomucor miehei*. Table 5 illustrated that run no. 19 is the best for MGL production (4.89 U/mL), where this run contains yeast extract 0.3 g/L, incubation period 8 d and temperature 37^{o}C. The analysis of variance (ANOVA) was used to estimate the significance of the models[31]. For each terms in the models, a large F-value and a small P-value would show a more significant effect on the respective response variable[32]. Result of ANOVA revealed that, incubation period as a single factor among other linear terms has a significant effect on the production ($p<0.05$) and F-value is 61.39. Only the square term of incubation period has a significant effect ($P<0.05$) and F-value is 13.36. Furthermore, two way interactions (Yeast extract*Period) and (Period*Temperature) have a significant effect where $p<0.005$ and F-value are 5.11 & 8.16 respectively (Table 6). Regression equation was as follow:

MGL activity = -3.5 - 35.2 Yeast extract + 1.11 Period + 0.50 Temperature + 0.26 (Yeast extract*Yeast extract)
+ 0.1400 (Period*Period)
+ 0.0114 (Temperature*Temperature)
+ 1.231 (Yeast extract*Period) + 0.613 (Yeast extract *Temperature) - 0.1469 (Period*Temperature).

On other hands, in the case of *Aspergillus fumigatus*, four factors (period, inoculum size, glycerol, and pH) were selected as significant factors affect MGL production, where CCD with 31 runs was designed. Table 7 showed that run no. 5 is the best for MGL production (8.55U/mL), where this run includes glycerol 0.5 mL/L, pH 7, incubation period 10 d and inoculum size 5 %. ANOVA results illustrated that, the incubation period has a significant effect on MGL production, but all other single factors, square and two way interaction have not significant effects (Table 8).

Regression Equation in Uncoded Units

MGL activity = 32.4 + 0.1 Glycerol - 1.13 PH - 4.25 Period - 0.22 Inoculum size + 5.03 Glycerol*Glycerol - 0.033 PH*PH
+ 0.1184 Period*Period
+ 0.117 Inoculum size*Inoculum size - 1.83 Glycerol*PH + 0.49 Glycerol*Period + 0.73 Glycerol*Inoculum size + 0.199 PH*Period + 0.186 PH*Inoculum size - 0.206 Period*Inoculum size.

Table 6. Central composite design matrix for the MGL production by *Rhizomucor miehei*

Trial no.	YE	Period	Temp.	Specific activity U/ mg	Protein Mg/ml	Enzyme activity U/ ml
1	0.7	12	33	0.56	0.09	6.22
2	0.163641	10	35	0.60	0.24	2.5
3	0.3	8	33	3.52	0.43	8.10
4	0.5	10	31.6364	0.68	0.28	2.42
5	0.5	10	35	0.99	0.31	3.19

6	0.7	12	37	0.56	0.26	2.15
7	0.5	10	35	1.32	0.26	5.07
8	0.5	10	35	1.34	0.25	5.60
9	0.5	10	35	0.87	0.32	2.71
10	0.5	10	35	0.69	0.32	2.15
11	0.83635	10	35	0.75	0.27	2.77
12	0.5	10	35	1.00	0.22	4.54
13	0.7	8	37	3.67	0.44	8.34
14	0.5	10	38.3636	0.87	0.24	3.62
15	0.5	6.636	35	3.96	0.44	9.00
16	0.3	12	33	0.79	0.29	2.70
17	0.3	12	37	0.14	0.23	0.60
18	0.7	8	33	0.99	0.24	4.12
19	0.3	8	37	4.89	0.53	9.22
20	0.5	13.36	35	0.50	0.21	2.38

Table 7. ANOVA of the full quadratic model for the MGL of *Rhizomucor miehei*

Source	DF	Sum of squares	Mean square	F-value	P-value
Model	9	32.30	3.58	10.61	0.000
Linear	3	22.57	7.52	22.25	0.000
Yeast extract	1	0.8011	0.8011	2.37	0.155
Period	1	20.76	20.76	61.39	0.000
Temperature	1	1.01	1.01	3.00	0.114
Square	3	4.55	1.51	4.48	0.031
Yeast extract*Yeast extract	1	0.0015	0.0015	0.00	0.948
Period*Period	1	4.519	4.519	13.36	0.004
Temperature*Temperature	1	0.03	0.03	0.09	0.772
2-Way Interaction	3	5.18	1.72	5.11	0.021
Yeast extract*Period	1	1.94	1.94	5.74	0.038
Yeast extract*Temperature	1	0.48	0.48	1.42	0.261
Period*Temperature	1	2.76	2.76	8.16	0.017
Error	10	3.38	0.338		
Lack-of-Fit	5	3.058	0.6117		
Pure Error	5	0.323	0.0648		
Total	19	35.6911			

Table 8. Central composite design matrix for the MGL production by *Aspergillus fumigatus*

Trial no.	**Glycerol level**	**PH level**	**Period level**	**Inoculum Size level**	Specific activity U/ mg	Protein Mg/ml	Enzyme activity U/ ml
1	0.5	9	10	3	1.15	0.27	4.25
2	0.1	7	10	3	6.08	0.48	12.66
3	0.5	7	6	3	7.60	0.52	14.61
4	0.5	7	10	3	1.17	0.29	4.030

5	0.5	7	10	5	8.55	0.54	15.83
6	0.3	6	12	4	2.21	0.23	9.60
7	0.3	6	8	4	4.14	0.44	9.40
8	0.5	7	10	3	2.96	0.51	5.80
9	0.3	8	12	4	1.60	0.44	3.63
10	0.5	7	10	3	3.09	0.35	8.82
11	0.7	8	12	4	1.5	0.33	4.54
12	0.7	8	8	4	6.65	0.48	13.85
13	0.5	7	10	3	1.94	0.43	4.51
14	0.7	8	8	2	7.28	0.55	14.21
15	0.5	7	10	3	5.83	0.50	11.66
16	0.3	6	8	2	1.66	0.46	3.60
17	0.3	8	8	2	1.80	0.33	3.27
18	0.7	6	8	2	5.22	0.49	10.65
19	0.5	5	10	3	1.04	0.27	3.85
20	0.3	6	12	2	1.52	0.40	3.80
21	0.9	7	10	3	5.72	0.55	10.40
22	0.5	7	14	3	5.2	0.44	11.81
23	0.7	6	12	4	1.59	0.52	3.05
24	0.7	6	12	2	0.95	0.37	2.56
25	0.5	7	10	3	3.03	0.42	7.21
26	0.7	8	12	2	2.37	0.30	7.90
27	0.3	8	12	2	1.71	0.38	4.50
28	0.5	7	10	1	1.83	0.42	4.35
29	0.7	6	8	4	3.20	0.44	7.27
30	0.5	7	10	3	2.03	0.51	3.98
31	0.3	8	8	4	3.13	0.44	7.11

Table 9. ANOVA of the full quadratic model for the MGL of *Rhizomucor miehei*

Source	DF	Sum of squares	Mean square	F-value	P-value
Model	14	88.469	6.3192	1.69	0.158
Linear	4	72.137	18.0342	4.81	0.010
Glycerol	1	0.282	0.2817	0.08	0.788
PH	1	0.062	0.0620	0.02	0.899
Period	1	71.553	71.5531	19.08	0.000
Inoculum size	1	0.240	0.2400	0.06	0.803
Square	4	7.461	1.8653	0.50	0.738
Glycerol*Glycerol	1	1.157	1.1575	0.31	0.586
PH*PH	1	0.030	0.0303	0.01	0.929
Period*Period	1	6.416	6.4164	1.71	0.209
Inoculum size*Inoculum size	1	0.394	0.3944	0.11	0.750
2-Way Interaction	6	8.871	1.4786	0.39	0.872
Glycerol*PH	1	2.132	2.1316	0.57	0.462
Glycerol*Period	1	0.608	0.6084	0.16	0.692
Glycerol*Inoculum size	1	0.342	0.3422	0.09	0.766
PH*Period	1	2.528	2.5281	0.67	0.424

PH*Inoculum size	1	0.555	0.5550	0.15	0.705
Period*Inoculum size	1	2.706	2.7060	0.72	0.408
Error	16	59.994	3.7496		
Lack-of-Fit	10	30.810	3.0810	0.63	0.751
Pure Error	6	29.183	4.8639		
Total	30	148.463			

Validation and production of MGL

The MGL productivity has been practically optimized and the optimum parameters have been carried out to validate the CCD model. The results had shown the validation of the model at different conditions. The optimum conditions of *Rhizomucor miehei* were methionine 2, glycin 3.0, pepton 1.0 yeast extract 0.163 g, PH 5.0 period 6.63 d, temperature 38.36 ^{o}C, inoculum size 3 and $MgSO_4$ 0.5 with maximum MGL close to that predicted (8.27 U/mg) (Figure 3), while conditions of *A. fumigatus* were glycerol 0.9, PH 5.0, period 6 d and inoculum 5% with experimental MGL productivity 12.37 U/mg .

The influence of inoculum size on L-methioninase production by *Aspergillus ustus* was carried out by [8] they reported that, the using of 2 ml spore suspension was the most useful for enzyme yield. Also, El- Sayed [33] investigated the effect of inoculum size and inoculum age on MGL production and growth of *Aspergillus flavipes* under solid-state fermentation. He found that, the increase of inoculum size was correlated with a gradual increase of MGL production and growth yield was observed reaching optimum values when 2.5 ml inoculum of 6 days old culture was used. With further increasing of inoculum size (4 ml), and inoculum age (after 6 days) a detectable reduction of both enzyme productivity and growth rate of *A. flavipes* was observed. This phenomenon may be attributed to nutrient limitation or accumulation of some non-volatile, self-inhibiting substrates as explained by [34]. On the other hand, neutral pH 7.0 was found to be optima for of MGL production by *Brevibacterium linens* [35] while pH 8.5 was favored for MGL production by *Clostridium sporogenes* [36]. In agreement with our results, Kagkli, Bonnarme [37] studied the effect of initial pH on MGL production by *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. They found that, optimum MGL production was observed at pH 5.5. Generally, the acidic initial pH was reported for MGL production by yeast [38]. In addition, Arfi, Landaud [39] used initial pH 5.0 for MGL production by *Geotrichum candidum*. Furthermore, the effect of initial pH on MGL production by Aspergillus ustus was investigated by [8]. They revealed that the productivity of MGL increased with increasing the alkalinity of the medium to reaching its maximum yield at alkalinity pH 8.5.

The influence of incubation time on MGL production studied by many authors, Abu-Tahon and Isaac [8] shows that the increase of alkaline MGL accumulation by *Aspergillus ustus* was observed after eight days of incubation. While Hamed, Elsoud [40] reported that, the highest induction of MGL from *Collectrichum globosum* was found after incubation for seven days. On the other hand, Spinnler, Berger [38] reported that, MGL production by several cheese ripening yeasts (i. e. *Geotrichum candidum*; *Saccharomyces crevice* and *Kluyveromyces lactis*) was obtained after 48 h of incubation. In addition, they mentioned that this incubation period corresponds to the maximum growth of their yeasts. Furthermore, Arfi, Landaud [39] studied the effect of incubation period on MGL production by *Geotrichum candidum*. They found that, *G. candidum* showed maximum growth and MGL formation after 48h of incubation. In addition, Selim, Karm Eldin [41] reported that 48 h incubation period was optima for both growth and enzyme production by *Candida trobicalis*.

The effect of using various carbon sources for MGL production by other microorganisms was studied by many authors. Khalaf and El-Sayed [7] found that, glucose was the best carbon source for MGL production by *Aspergillus flavipes*. While the use of citric acid; cellulose or potassium oxalate were correlated with a significant decrease for enzyme production. The use of glucose as the most favored carbon source for MGL production by cheese ripening yeast *Geotrichum candidum* was also

reported by Arfi, Landaud [39]. On the other hand, sucrose exerted the highest increase in the enzyme production as compared with other carbon sources by many microorganisms including *Aspergillus ustus* Abu-Tahon and Isaac [8]; *Pseudomonas ovalis* [6].

The constitutive nature of this enzyme has been reported for *Pseudomonas putida* [14]; for *Geotrichum candidum* [42] and for *Candida trobicalis* [41]. In addition, Nakayama, Esaki [29] reported MGL production by *Pseudomonas putida* was obtained when grown in a medium containing peptone. In accordance of our results, the inducible synthesis of this enzyme by other microorganisms was reported by many authors Abu-Tahon and Isaac [8] showed that none of the incorporated nitrogenous compounds had a detectable effect on alkaline MGL production by induction of MGL by *Aspergillus flavipes* was evaluated by Khalaf and El-Sayed [7]. They found that, L-methionine was observed to be the optimum inducer of MGL biosynthesis by using 0.2% w/v of L-methionine in basal medium.

In this respect, Arfi, Landaud [39] used peptone and yeast extract for production of MGL by Geotrichum candidum. Moreover, Ferchichi, Hemme [35] mentioned that addition of yeast extract and peptone to growth medium was necessary to maintain acceptable growth rate of Brevibacterium linens MGL production. The effect of using some organic nitrogen sources in combinations like peptone and yeast extract on the production of other enzymes by some microorganisms was reported by many authors [43, 44]. They reported that, peptone and yeast extract are complex ingredients containing several essential amino acids as well as many other growth promoting factors which are necessary for cell growth.

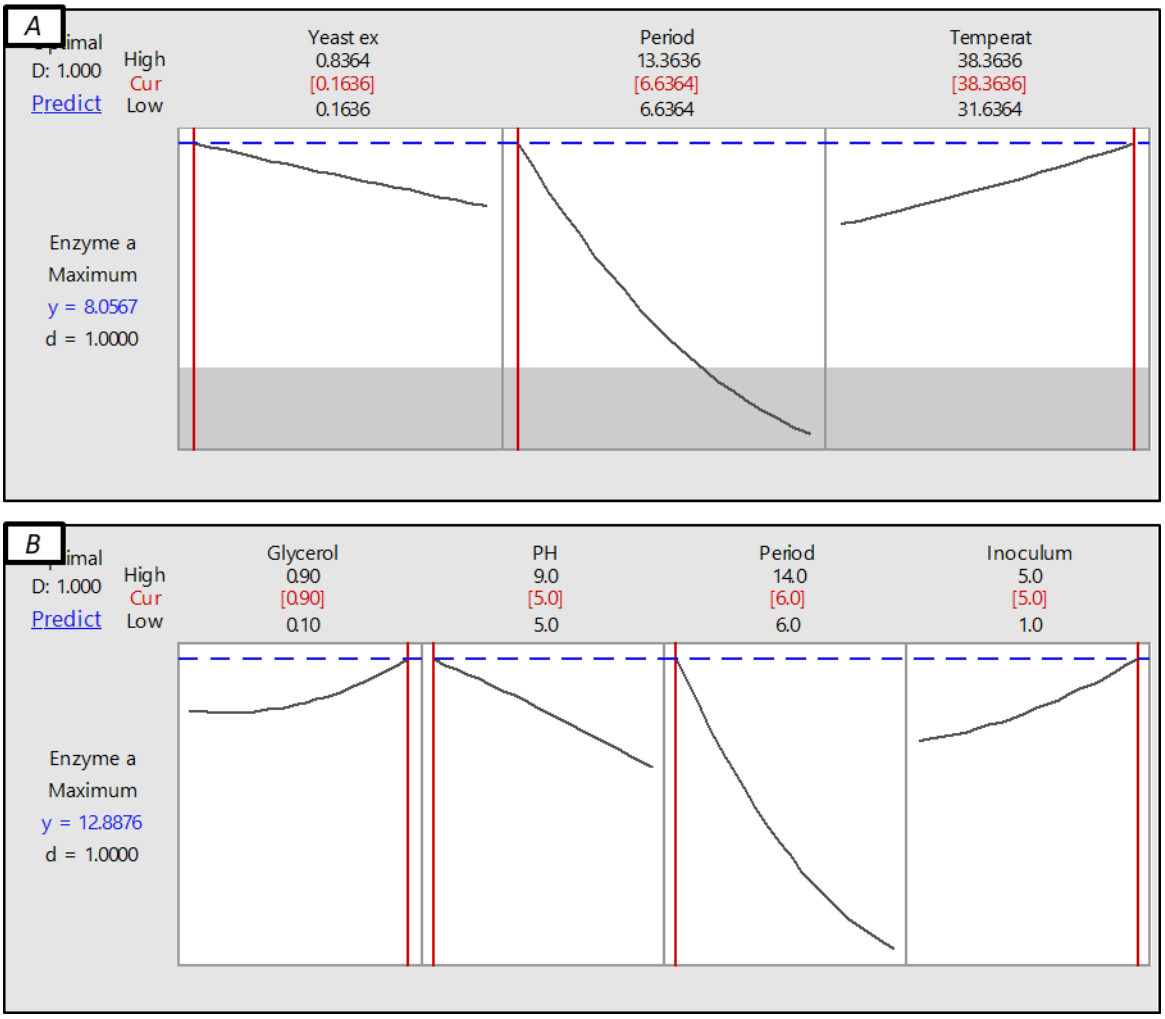

Figure 3: Response optimizer for *R. miehei and A. fumigatus* for MGL production

4. Conclusion

In the current study, MGL producing thermotolerent fungi *R. miehei and A. fumigatus* were isolated and identified morphologically and genetically. Moreover, statistical optimization using RSM was carried out. Result illustrated that yeast extract, incubation period and temperature are significant factors affect MGL production by *R. miehei* where MGL was 8.27 U/mg. Additionally, glycerol, PH, period and incoculum size are the most efficient factors affect MGL by *A. fumigatus* where MGL was 12.37 U/mg. Eventually, the isolated thermotolerent fungi *R. miehei and A. fumigatus* in this study are promising for MGL production which can be used in medical and pharmaceutical applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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