

# Statistically optimized production of extracellular L-methionine $\gamma$ -lyase by *Streptomyces* Sp. DMMM60 and evaluation of purified enzyme in sub-culturing cell lines

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## ARTICLE INFO

### Keywords:

L-methionine  $\gamma$ -lyase  
Beet molasses  
Plackett–burman design  
Purification  
Anticancer

## ABSTRACT

The optimal conditions for the production of L-methionine  $\gamma$ -lyase (MGL) by a soil bacterium, *Streptomyces* DMMM60, were established and their effects were compared using Plackett–Burman design (PBD). Accordingly, the optimal conditions of beet molasses, yeast extract, L-methionine,  $MgCl_2$ , Tween 80, initial pH, aeration, temperature, inoculum size and incubation period for the maximum MGL production (60.7 U/mg) were 23.5 g/L, 0.79 g/L, 0.87 g/L, 0.85 g/L, 0.41%, 8.3, 50 ml, 33 °C, 5.2 ml, and 4 day, respectively. Initial pH was found to be the most significant factor for the production of MGL based on the calculated percentage of participation P (%) from an analysis of the variance (ANOVA). Additionally, MGL was purified from the supernatant of *S.* DMMM60 using heat treatment, DEAE-cellulose and Sephadex G<sub>100</sub>, and the purified enzyme displaying 3.15-fold in comparison with the crude enzyme. Purified enzyme exhibiting a single band of 55 kDa subunits on the SDS-PAGE and the enzyme had maximum activity in pH 8 at 45 °C with ionic stability within pH range 6.5–8.5 and thermal stability below 60 °C. *In-vitro*, MGL showed a significant cytotoxic effect against cancer cell lines whereas those for normal cells was demonstrated a negligible toxicity.

## 1. Introduction

L-methionine  $\gamma$ -lyase (MGL) (EC 4.4.1.11) is a multifunctional catalytic enzyme that requires pyridoxal-5'-phosphate (PLP) as a coenzyme to catalyze the direct demethylation and demethylation of L-methionine to methanethiol,  $\alpha$ -ketobutyrate and ammonia (Tanaka et al., 1985; El-Sayed, 2010). Microbial L-methioninase has been discovered in a variety of microorganisms including bacteria, yeast and fungi. The bacterial L-methioninase has brought much attention by a lot of researchers, comparing to the eukaryotic L-methioninase sources. As it appeared from the previous studies the importance L-methionine  $\gamma$ -lyase producing bacteria are *Pseudomonas putida* and *Pseudomonas ovalis* (Tanaka et al., 1976), *Aeromonas* sp. (Tanaka et al., 1985), *Citrobacter freundii* (Manukhov et al., 2005), *Lactococcus lactis* (Martinez-Cuesta et al., 2006), and *Clostridium sporogenes* (Kreis and Hession, 1973) as an intracellular producers. Moreover, a few strains of filamentous fungi are reported to be extracellular L-methionine  $\gamma$ -lyase producer belonged to *Aspergillus* genera (Ruiz-Herrera and Starkey, 1969; Khalaf and El-Sayed, 2009; Abu-Tahon and Isaac, 2016).

Significant current effort in cancer studies are being focused on designing new forms of chemotherapy which use the properties of cancer cells are unique when compared with normal cells. A distinctive property of various types of cancer cells is methionine dependence (Tan et al., 2010). There are many reports which confirmed that normal cells be capable of grow on homocysteine instead of methionine, attributed to their active methionine synthase. However, cancer cells devoid of the active methionine synthase, thus depend upon external methionine supplementation from the diet (Hoffman, 1997). Consequently methionine could be the main tumor specific target for therapeutic techniques. Therefore, therapeutic utilization of L-methionine  $\gamma$ -lyase to deplete plasma methionine is apparently a promising and an alternative strategy in cancer treatment (Yoshioka et al., 1998).

Utilize of new microorganisms with high L-methionine  $\gamma$ -lyase production efficiency, development of novel strategies to improve the yield using cheap substrates may lower the production cost which can be highly appreciable for large scale production. Therefore, to find the new group of L-methionine  $\gamma$ -lyase with preferable efficiency, specificity and activity for several applications, investigation of L-methionine  $\gamma$ -lyase

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using other groups of microbes is desirable. Survival possibility in wide range of pH, temperature and growth at different agro-industrial by-products makes *Streptomyces* species an excellent candidate for study of inexpensive L-methionine  $\gamma$ -lyase production. In our previous study, *Streptomyces* DMMMH4 and *Streptomyces* DMMMH60 have been explored for extracellular L-methionine  $\gamma$ -lyase production under submerged fermentation (Selim et al., 2015).

The majority of the L-methionine  $\gamma$ -lyase produced by microorganisms is intracellular in nature, except for a few which secrete L-methionine  $\gamma$ -lyase outside the cell wall (El-Sayed, 2010). For industrial and medical purposes, extracellular L-methionine  $\gamma$ -lyase is more suitable compared to the intracellular type for many of reasons, including higher accumulation in culture broth under normal conditions, easy extraction and downstream processing. In addition extracellular L-methionine  $\gamma$ -lyase produced is mostly soluble, biologically active and contains an authentic N-terminus and it is relatively free from endotoxins, all properties which result in the minimization of adverse effects (Suganya et al., 2017). The consideration of employing this enzyme from *Streptomyces* strains as being a main specific cancer target for therapeutic techniques has been scarcely studied until now. Recently, the utilization of response surface methodology for maximum L-methionine  $\gamma$ -lyase production by *Trichoderma harzianum* was investigated via Central composite design and artificial neural network (Nisha et al., 2019).

Nevertheless, the optimization of fermentation condition parameters using Plackett-Burman design for contribute to the highest L-methionine  $\gamma$ -lyase production by *Streptomyces* species under submerged conditions hasn't been reported to date. Accordingly, the aim of the present study was to optimization of fermentation conditions for the extracellular production of L-methionine  $\gamma$ -lyase by *Streptomyces* DMMMH60 under submerged fermentation using a Plackett–Burman design. The study was also intended to enzyme purification, characterization and anticancer evaluation.

## 2. Materials and methods

### 2.1. Chemicals

L-methionine and starch were obtained from Merck (Germany), trichloroacetic acid, bovine serum albumin, Coomassie brilliant blue, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), Pyridoxal-5'-phosphate (PLP), were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Nessler's reagent was purchased from LOBA Chemie (Mumbai, India). All other chemicals used were of the highest grade available.

### 2.2. Microorganism and inoculum preparation

*Streptomyces* DMMMH60 strain was used throughout this study was isolated from agricultural Egyptian soil and identified as described by (Selim et al., 2015) and deposited in the DDBJ/EMBL/Gene Bank nucleotide sequence databases (Gen bank accession no. LC021309). The *S.* DMMMH60 culture was preserved on Starch-nitrate Agar slants medium comprised (Waksman, 1959) (g/L): Starch, 20; KNO<sub>3</sub>, 2; NaCl, 0.5; K<sub>2</sub>HPO<sub>4</sub>, 1; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.001; CaCO<sub>3</sub>, 3 and agar, 20. The initial pH of the above media was adjusted to 7.0 before sterilization. The inoculated slants were incubated at 30 °C for 7 days to obtain a heavy sporulated growth. After that time, *S.* DMMMH60 suspension was harvested by scraping the surface of sporulating slants in 10 ml of sterile distilled water, concentration of the spores was approximately about 10<sup>6</sup>–10<sup>7</sup> CFU/ml.

### 2.3. Preparation of culture medium using beet molasses hydrolysate

The molasses used in this study was kindly supplied by the Sugar and Integrated Industries Corporation, Al-Howamdia, Egypt. It consisted of about 25% total solid and 55% of this solid represented total

sugar. The beet molasses hydrolysate used as carbon and energy sources to synthesis of MGL by *Streptomyces* Sp. was prepared according to the method that reported by (Cakar et al., 2014) and the total carbohydrate concentration was determined by phenol-sulfuric acid method (Dubois et al., 1956). The crude molasses was diluted 2-fold (w/v) with distilled water and adjusted to pH 3.0 with 6 M H<sub>2</sub>SO<sub>4</sub>. Then, molasses was heated at 60 °C for 1 h. The pH was then adjusted pH 1.0 and continuously heated at 60 °C for 2 h. The molasses solution was centrifuged at 6000 ×g for 20 min to separate solid materials. Before sterilization of molasses, the pH of the hydrolysate was adjusted to 7.0 with 10 M NaOH. This treatment was designated the H<sub>2</sub>SO<sub>4</sub>-heat treatment and the supernatant was termed as H<sub>2</sub>SO<sub>4</sub>-heat-treated molasses and stored at 4 °C for further use. In addition, molasses hydrolysate medium was amended with L-methionine and yeast extract as a co-inductive agent in such amount that the final concentration of nitrogen (N-base) in the medium remained unchanged. To analyze reduced sugar at the end of the cultivation period, DNS assay (Miller, 1959) was used.

Flasks containing the modified starch medium (Selim et al., 2015) were also inoculated at the same time as a reference medium.

### 2.4. Biomass determination

At the end of cultivation, the culture was centrifuged at 10,000 ×g for 10 min and then the precipitate cell pellets were washed with distilled water. The washed mycelia was filtered with a pre-weighed filter paper and dried at 105 °C until a constant weight prior to measuring the biomass yield of culture. The dry cell weight (DCW) was expressed as a gram per liter of culture medium. The obtained supernatant was then used as a source of enzyme for further studies.

### 2.5. Extracellular MGL activity assay and protein estimation

L-methionine  $\gamma$ -lyase (MGL) activity in the clear supernatant was assessed from the amount of methanethiol (MTL) formed using L-methionine as a substrate as described by (Laakso and Nurmikko, 1976) and (Selim et al., 2015). MTL amount was calculated according to a standard curve obtained with sodium methanethiolate. One unit (U) of MGL was expressed as the amount of enzyme that generated 1  $\mu$ M of MTL per minute under optimal assay conditions. Another MGL activity assay involved the determination of ammonia formed with Nessler's reagent as described previously (deamination method) (Khalaf and El-Sayed, 2009). One unit (U) of MGL activity was taken as the amount of the enzyme that liberates 1  $\mu$ m of ammonia per minute. The extracellular protein content was estimated by the method of (Bradford, 1976) using bovine serum albumin as standard. Specific activity of MGL was defined by the mean of enzyme activity (U) per milligram of protein (mg) of each sample.

### 2.6. Statistical modeling and Optimization of MGL Production by *Streptomyces* DMMMH60 through Plackett-Burman design

The production and statistical optimization of MGL by *Streptomyces* DMMMH60 Sp. has been performed in four sequential steps; Plackett-Burman experimental design, doing the experiment, data analysis and validation of the results (Venkata Mohan et al., 2007). Before statistical modeling, the different factors were tested for the optimum maximum and minimum levels of study based on One-factor-at-a-time method (data not shown). The medium components before optimization and conditions were checked for MGL production, and then were considered for further optimization studies. Modeling of MGL by *S.* DMMMH60 has been carried out using Plackett-Burman factorial design (PBD). Table (1) shows the PBD with ten numeric factors; Pretreated beet Molasses, yeast extract, L-methionine, MgCl<sub>2</sub>, Tween 80, Incubation temperature, pH, Inoculum size, Aeration and Incubation period. For Plackett-Burman design and analysis, Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, version 7.0.0) have been used.

**Table 1**  
Plackett-Burman experimental design of ten variables for evaluating factors influencing MGL production.

Factor code	Factor Name	Units	Low level	High level
A	Pretreated beet Molasses	g/L	20	35
B	Yeast extract	g/L	0.5	2
C	L-methionine	g/L	0.25	1
D	MgCl <sub>2</sub>	g/L	0.5	1
E	Tween 80	%	0.1	0.5
F	Incubation Temperature	°C	30	40
G	pH		6	8.5
H	Inoculum size	%	4	10
J	Aeration	ml	30	120
K	Incubation period	days	3	5

The Plackett-Burman experimental design was used to determine the major factors influencing MGL production. The experimental design comprised a total of 15 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.

## 2.7. Statistical data analysis

After doing the experiments, Analysis of variance (ANOVA) has been used for estimation of the statistical parameters and numerical optimization of the culture conditions. A probability value criterion of less than (0.05) was used as parameter for statistical significance.

## 2.8. Validation of the results

After the theoretical optimization of the ten factors for maximum production of MGL by *S. DMMM60*, 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 replicates and recorded as (mean ± standard deviation).

## 2.9. MGL purification

The aqueous extract of *S. DMMM60* culture broth was collected by cooling centrifuge (10,000 × *g* for 20 min at 4 °C). Crude enzyme was partially purified by heating at 60 °C with stirring for 15 min. After cooling for 5 h at 0 °C, the denatured proteins were removed and the enzyme was fractionated using DEAE-cellulose column (ion-exchange chromatography), after the column was washed with 1 L of the potassium phosphate buffer (pH 7.4, 50 mM) containing 50 mM NaCl, the enzyme was eluted with a linear gradient of (0, 50, 100, 150 and 200 mM) NaCl dissolved in the same buffer containing 10 μM PLP. The most active homogenous fractions were collected and applied into a Sephadex G<sub>100</sub> column (gel filtration chromatography) equilibrated with 50 mM potassium phosphate buffer (pH 7.5). The enzyme was eluted with the same buffer containing 10 μM PLP, and the active fractions were gathered and lyophilized. The homogeneity and molecular weight of the lyophilized enzyme were conducted using SDS-PAGE as reported by (Laemmli, 1970). In addition, the absorption spectrum of purified L-methionine γ-lyase was determined by using Agilent UV/Vis scanning spectrophotometer to ensure the purity of L-methionine γ-lyase. A diluted sample of purified enzyme in 20 mM potassium phosphate, pH 7.5, at a concentration of 75 mg/ml was made. The absorption spectrum between 200 and 600 nm was measured using 20 mM potassium phosphate pH 7.6, buffer containing pyridoxal 5- phosphate, as the blank (El-Sayed, 2011).

## 2.10. Biochemical properties of *S. DMMM60* MGL

The effect of pH value on MGL activity was studied by incubating the reaction mixture at pH values ranging from 4 to 10.7 using 50 mM acetate buffer (pH 4–6), 50 mM potassium phosphate buffer (pH 6–8.0), and 50 mM carbonate buffer (pH 8.5–10.7). To determine its pH stability, the purified enzyme was pre-incubated in buffers of different pH values at 28 °C for 24 h prior to measuring the residual activities. The optimal temperature for MGL activity was measured by incubating the reaction mixtures in 50 mM Potassium phosphate buffer (pH 7.8) at different temperatures from 30 to 80 °C for 20 min. The residual enzyme activity was calculated as the percentage of its maximal activity. Thermal stability assays were performed by pre-incubating the purified enzyme at several temperatures ranging from 30 to 80 °C. Aliquots were drawn at desired time intervals (10–100 min), and the remaining activity was assayed by the standard method. In addition, the effects of metal ions and activators/inhibitors on the enzyme activity were evaluated after the enzyme was pre-incubated with (10 mL<sup>−1</sup>) of each of one them separately for 1 h at 28 °C, and the residual activities were determined by the optimum assay method.

## 2.11. In vitro effect of MGL on different human cell lines

The cytotoxicity of purified MGL was evaluated by the thiazolyl blue tetrazolium bromide (MTT) assay (Mosmann, 1983) on human HepG2 (liver), MCF-7 (breast), A549 (lung), PC3 (prostate), CACO (intestinal), HEP-2 (larynx), HELA (cervical) and HCT116 (colon) cancer cell lines and on human HFB4 (melanocyte) normal cell line as control. Anticancer activity was expressed by median growth inhibitory concentration (IC<sub>50</sub>). The cells were seeded in 96-well plate at a cell concentration of 1 × 10<sup>4</sup> cells per well in 100 μl of growth medium. The cells were grown and maintained in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100U/ml) and streptomycin (100 μg/ml). The cells were treated with different concentrations of MGL (0.25–10 μg) and incubated at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. After 24 h of incubation, MTT was added to each well, and the plates were incubated for 4 h. Then, the optical density was measured at 590 nm using a plate reader to determine the number of viable cells and the percentage of viability was calculated as:  $[1 - (OD_t / OD_c)] \times 100\%$ , where OD<sub>t</sub> is the mean optical density of wells treated with the tested sample and OD<sub>c</sub> is the mean optical density of untreated cells. For microscopic observation, Olympus inverted microscope was used to evaluate the treated and non-treated cells.

## 3. Results

Growth of *Streptomyces* DMMM60 on beet molasses hydrolysate medium supplemented with L-methionine and yeast extract was noted as profuse and uniform and the bacterial hyphae completely covered the entire medium within 48 h of incubation. In the reference medium (modified starch medium), *S. DMMM60* produced (24.9 U/mg) of MGL activity at 120 h, while produced (32.7 U/mg) of MGL activity at 96 h in beet molasses hydrolysate medium before optimization of process variables.

In the Plackett-Burman design, the independent variables (medium components and cultural conditions of molasses hydrolysate medium) were studied with their respective high and low levels (Table 2). The results of contribution of the different factors show that pH has the maximum contribution percent (47.05%) followed by Tween 80 (27.05%), aeration (7.65%), AB (6.56%), inoculum size (2.05%) and MgCl<sub>2</sub> (1.86%) and the significant medium components showing *P* values < 0.05 significance level obtained by regression analysis. The rest of the terms have contribution values of less than 1%.

On the other hand, In Table 3 the ANOVA of MGL production showed that the model and the model factors; pretreated beet molasses

**Table 2**Actual, predicted, and residual values for L-methionine  $\gamma$ -lyase according to Plackett-Burman design.

Order Run	MGL Specific activity (U/mg)			Dry Cell Weight (g/L)	Reducing Sugar Concentration (g/L)		pH	
	Actual	Predicted	Residual		Initial	Final	Initial	Final
1	44.7	43.52667	1.173333	3.26	27.5	12.7	7.25	8.4
2	18.6	18.6	0	3.88	20	7.2	6	7.5
3	55.7	55.85	-0.15	3.61	20	5.1	8.5	8.8
4	31.7	31.55	0.15	2.42	20	13.8	6	7.3
5	43.08	43.52667	-0.44667	3.11	27.5	16.8	7.25	8.3
6	38.3	38.3	0	4.01	35	21.7	8.5	8.4
7	44.8	44.8	0	4.81	35	16.3	8.5	8.3
8	41.3	41.15	0.15	3.43	35	26.4	8.5	8.2
9	29.7	29.7	0	3.92	35	20.1	6	7.4
10	17.4	17.55	-0.15	2.27	35	14.7	6	7.7
11	44.7	44.85	-0.15	4.62	20	8.7	8.5	8.7
12	45.7	45.55	0.15	6.11	20	4.2	8.5	8.5
13	42.8	43.52667	-0.72667	3.22	27.5	12.9	7.25	8.4
14	34.2	34.2	0	3.18	20	10.3	6	7.6
15	46.1	46.1	0	4.38	35	27.3	6	7.2

**Table 3**

ANOVA for MGL production according to Plackett-Burman design.

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F <sup>a</sup>
Model	1458.835	10	145.8835	195.4437	0.0005
A-Pretreated beet molasses	14.08333	1	14.08333	18.86778	0.0225
B- Yeast extract	0.163333	1	0.163333	0.218822	0.6718
C-L-methionine	9.245	1	9.245	12.38575	0.0389
D-MgCl <sub>2</sub>	30.68056	1	30.68056	41.10349	0.0077
E-Tween 80	447.005	1	447.005	598.8635	0.0001
G-pH	777.4939	1	777.4939	1041.627	< 0.0001
H-Inoculum size	33.89389	1	33.89389	45.40847	0.0067
J-Aeration	126.405	1	126.405	169.3479	0.0010
K-Incubation period	11.36056	1	11.36056	15.22001	0.0299
AB	108.375	1	108.375	145.1926	0.0012
Curvature	91.56291	1	91.56291	122.6691	0.0016
Residual	2.239267	3	0.746422		
Lack of Fit	0.135	1	0.135	0.128311	0.7545
Pure Error	2.104267	2	1.052133		
Cor Total	1552.637	14			

<sup>a</sup> Values of "Prob > F" less than 0.0500 indicate model terms are significant.

(A), yeast extract (B), L-methionine (C), MgCl<sub>2</sub> (D), Tween 80 (E), pH (G), inoculum size (H), aeration (J), incubation period (K) and AB are all significant terms. The curvature "F-value" of 122.67 indicates that there is a significant curvature which is a measure between difference of the average of the center points and the average of the factorial points. The lack of fit is not significant, which is a good criterion for the model. The R<sup>2</sup> (0.998) of the model is in a good agreement with the adjusted-R<sup>2</sup> (0.993). In addition, the model adequate precision ratio is 49.56 which indicate an adequate signal. These parameters reveal that the model can be used to navigate the space of the model with a good accuracy and precision (see Fig. 1).

The three dimensional (3D) response surface plots-generated by Design-Expert software shown in Fig. 2 represent the relationships and effects of different experimental variables (factors) on MGL productivity. Best experimental variables levels for maximizing MGL production were predicted through analysis of these plots in combination with numerical optimization for each variable and desirability analysis.

### 3.1. Final equation of MGL by *Streptomyces sp.* in terms of actual factors

MGL Specific activity (U/mg) = 20.22305556 - 1.561111111 × Pretreated beet Molasses - 31.01111111 × yeast extract + 2.866666667 × L-methionine + 7.833333333 × MgCl<sub>2</sub>

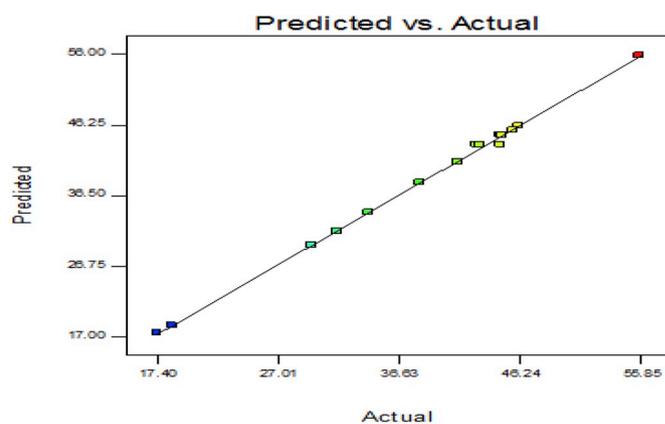


Fig. 1. The relation between predicted and actual results MGL.

+ 37.375 × Tween 80 + 7.886666667 × pH - 0.686111111 × Inoculum size - 0.088333333 × Aeration - 1.191666667 × Incubation period + 1.133333333 × Pretreated beet Molasses × yeast extract.

### 3.2. Validation of the results

The production of L-methionine  $\gamma$ -lyase has been numerically optimized and the optimum conditions have been applied practically to validate the Plackett-Burman model. The results shown in Table (4) reveal the validation of the model at different conditions. The conditions of the test number 5 showed the maximum MGL productivity with experimental specific activity of 60.7 U/mg.

### 3.3. Purification of *S. DMMM60* MGL

After cultivation of the submerged culture of *S. DMMM60*, the cell-free extract was centrifuged at 10,000 × g for 20 min and used as the source of extracellular crude enzyme. The starting volume for the purification was 50 ml of the concentrated crude enzyme solution containing 38.6 mg of protein with a specific L-methionine  $\gamma$ -lyase activity of (60.7 U/mg). This crude enzyme solution was partially purified by heat treatment at 60 °C for 10 min; the denatured proteins were precipitated by cooling centrifuge and discarded. Active supernatant was applied to a DEAE-Cellulose column and the enzyme eluted using a gradient of NaCl (50–200 mM L<sup>-1</sup>), the maximum enzyme activity was assessed at 100 mM NaCl. Fractions 9 to 12, which had maximum enzyme activity were pooled, concentrated, and subjected to Sephadex

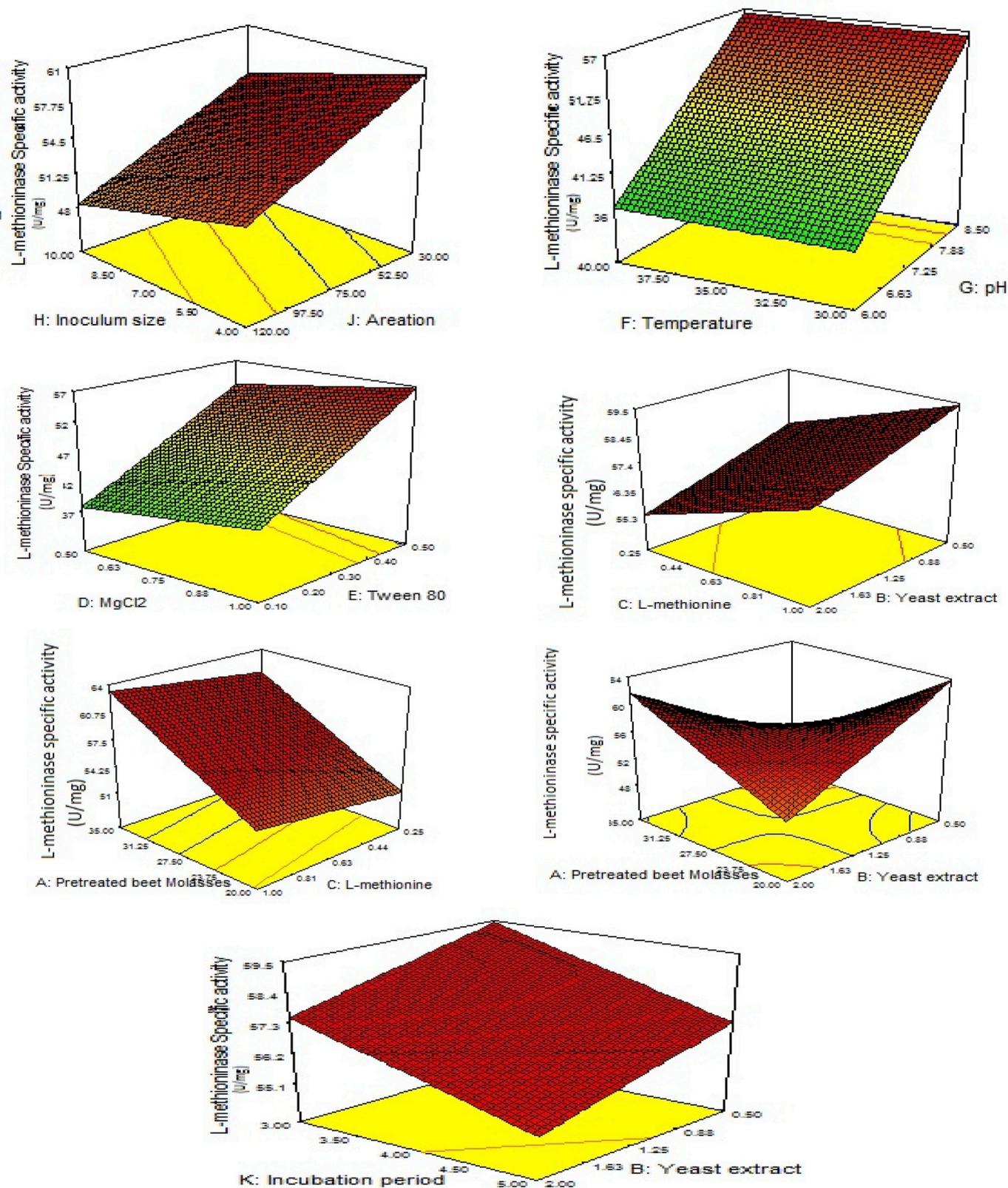


Fig. 2. Interactions and effect of different factors on MGL production.

G<sub>100</sub>column. As summarized in (Table 5), purification of MGL resulted in a 3.15-fold increase in specific activity with a good recovery of about 44.1%. As can be seen in (Fig. 3a), the purified MGL was migrated as a single band on SDS-PAGE with an approximate molecular mass of

55 kDa. On the other hand, the absorption spectrum of the purified enzyme showed a maximum peak at 280 and 420 nm as shown in (Fig. 3b).

**Table 4**  
Validation of the Plackett-Burman using different points.

Test Number	Conditions										L-methionine $\gamma$ -lyase Specific Activity (U/mg)	
	Pretreated beet Molasses (g/L)	Yeast extract (g/L)	L-methionine (g/L)	MgCl <sub>2</sub> (g/L)	Tween 80 (%)	Temperature (°C)	pH	Inoculum size (%)	Aeration (ml)	Incubation period (day)	Theoretical	Experimental
1	20.59	0.91	0.92	1	0.43	31.53	8.41	6.92	69.72	4.76	57.33	55.4
2	21.7	0.66	0.8	0.6	0.44	35.54	8.48	4.51	77.58	4.77	56.86	57.9
3	22.84	1.58	0.59	0.89	0.36	30.12	8.47	5.12	30.16	3	55.75	58.7
4	24.89	1.13	0.43	0.8	0.49	38.84	8.39	7.31	35.15	4.51	56.54	57.2
5	23.52	0.79	0.87	0.85	0.41	33.03	8.38	5.27	50.5	4.22	57.54	60.7

**Table 5**  
Purification profile of *Streptomyces* DMMM60 MGL.

Purification Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	52.6	3189	60.6	100	1
Heat treatment	29.8	2833	96.6	88.8	1.59
DEAE-Cellulose	11.9	2128	178.8	66.7	2.16
Sphadex-G <sub>100</sub>	5.4	1407	260.5	44.1	3.15

### 3.4. Biochemical characterization of the purified MGL

The effect of pH on the activity of the purified MGL was tested in the range of pH 4–10.5 using different buffer systems. As shown in (Fig. 4a) MGL has been found to be active at broad pH intervals (7.0–9.0) and the pH optimum was found at 8. Enzyme stability studies at a series of pH solutions (Fig. 4a) showed that MGL was displayed its significant catalytic stability in the range of pH 6.5 to 8.5, a slight decrease in the enzyme activity was observed at pH 9.0 while at pH 5.0 the enzyme retained about 53.8% of its initial activity. The effect of temperature on the purified MGL activity was performed at 30–80 °C for 20 min at pH 8. The thermo dependence curve of MGL activity indicated that the enzyme displayed activities between 35 and 55 °C, with its highest activity at 45 °C (Fig. 4b). The enzyme activity lost about 43.5% and 71.8% of its initial activity at a reaction temperature of 70 °C and 80 °C, respectively. Moreover, heat stability curve of MGL (Fig. 4c) illustrated high thermo stability with more than 80% of its initial activity retaining after 1 h incubation at 60 °C. Furthermore, after heating the purified enzyme at 70 °C for 1 h, the enzyme still retained about 57.9% of its original activity.

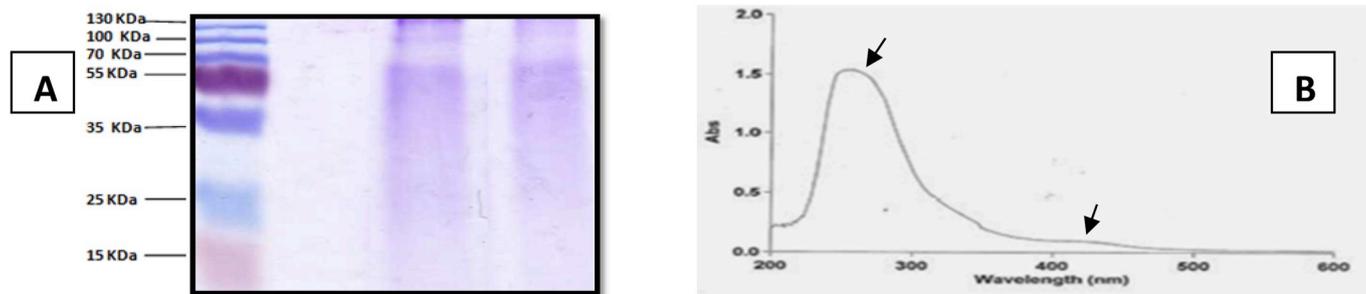
The influences of inhibitors/activators and metal ions on an enzyme activity were usually considered as significant properties for its classification (Table 6). Among 13 metal ions tested, did not significantly activated effects on purified enzyme, whereas a considerable loss of MGL activity more than 50% was noted with Cr<sup>+2</sup>, Cd<sup>+2</sup>, Cu<sup>+2</sup> and Ni<sup>+2</sup>, Hg<sup>+2</sup> respectively. The enzyme activity was completely

denatured in the presence of benzoic acid and Iodoacetate (thiol-reducing agent), as reflected by the enzyme activities 8.2%, 12.4% respectively. Besides, purified MGL was strongly inhibited by PMSF, SDS, and DTT, retaining only 52.3%, 32.4%, and 27.9% of its original activity, respectively. Also, hydroxylamine, Mn<sup>+2</sup>, Fe<sup>+3</sup> had slightly inhibitory effect of enzyme activity with by about 14.5, 11.6 and 9.2% respectively. Otherwise, the enzyme activity was not affected in the presence of each of Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>+2</sup>.

As shown in (Table 7), *S. DMMM60* MGL was found to be potent anticancer agent had a high efficiency, since the tumor growth of all lines was inhibited by adding 1 unit/ml of purified enzyme. A colon carcinoma cells was the most sensitive, 0.05 U/ml followed by lung carcinoma, 0.17 U/ml, prostate carcinoma 0.17 U/ml, breast carcinoma, 0.35 U/ml, hepatocellular carcinoma 0.43 U/ml, cervical carcinoma 0.61 U/ml, larynx carcinoma, 0.76 U/ml, and intestinal carcinoma 0.90 U/ml. The safety pattern of purified MGL was examined on human melanocytes cells which had a much higher IC<sub>50</sub> 9.7 U/ml when compared to the cancer cell lines. Inverted microscope was used to demonstrate the morphological changes of the cancer cells (Fig. 5). In the control samples, the surface of cells was safety, integrity, smooth, and undamaged. After incubation with purified MGL for 24 h, we observed some holes and multiple dents formed on the cell surface, cellular rounding up, shrinkage, and loss of cell adhesion. These results could be supported the theory that methionine starvation causes the death of cancer cells more than normal cells.

## 4. Discussion

The preference of sugar beet molasses as co-metabolic agent for MGL biosynthesis could be related to their direct usability in different metabolic pathways as carbon and energy sources and it was clear with the sugar consumption by the tested organism. The implication that sugar beet molasses is a best stimulator of extracellular MGL by *Streptomyces* DMMM60 coincide with that reports for alkaline L-methioninase production by *Aspergillus ustus* (Abu-Tahon and Isaac, 2016), emphasizing their feasibility as co-metabolic agent for the initiation of bacterial growth. Therefore, the enzyme production and biomass yield



**Fig. 3.** (A) SDS-PAGE showing purified MGL from *S. DMMM60*. Lane 1. DEAE-Cellulose column, Lane 2. Sephadex G<sub>100</sub> column (B) Absorption spectrum of the purified enzyme.

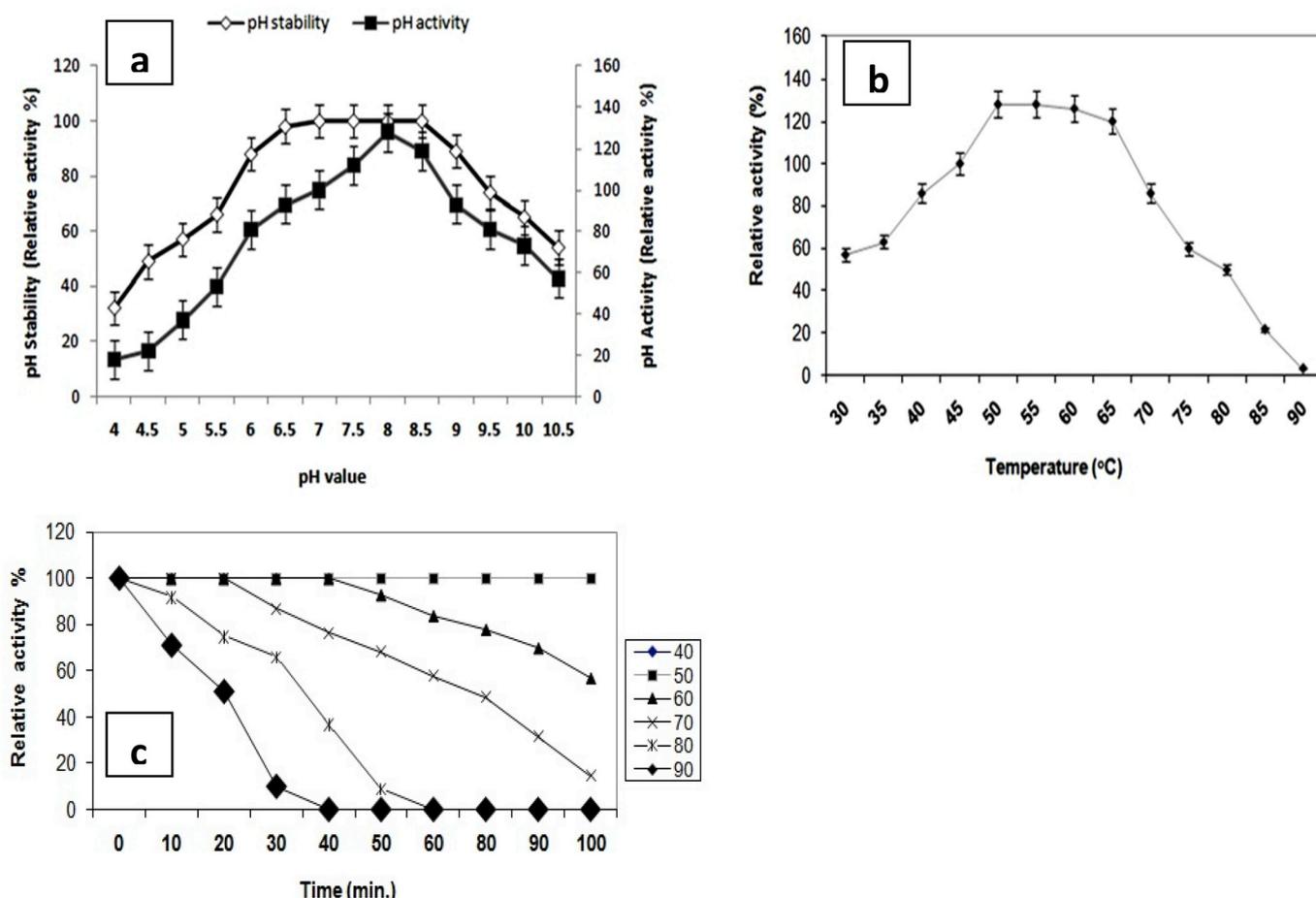


Fig. 4. Effect of pH and temperature on the activity and stability of purified MGL (a) pH activity and stability, (b) temperature activity, (c) temperature stability.

Table 6  
Effect of metal ions and inhibitors on purified MGL activity.

Metal ions (Chloride salt) (10 mM)	Relative Activity (%)	Inhibitors (10 mM)	Relative Activity (%)
Control	100	EDTA	100
Na <sup>+</sup>	100	SDS	32.4
K <sup>+</sup>	97.2	DTT	27.9
Mg <sup>+2</sup>	100	PMSF	52.3
Mn <sup>+2</sup>	88.4	β-mercaptoethanol	61.3
Zn <sup>+2</sup>	89.7	Tween 80	100
Ca <sup>+2</sup>	96.8	Tween 20	97.9
Ba <sup>+2</sup>	58.7	Benzoic acid	8.2
Li <sup>+2</sup>	44.9	Iodoacetate	19.4
Cu <sup>+2</sup>	27.3	Hydroxylamine	55.5
Co <sup>+2</sup>	68.9		
Cd <sup>+2</sup>	30.8		
Hg <sup>+2</sup>	12.7		
Fe <sup>+3</sup>	98.8		

Table 7  
IC<sub>50</sub> of purified MGL of each of human cell line.

Compounds (µg/ml)	Human cell lines (IC <sub>50</sub> ) (µg/ml)								
	HELA	HEP-2	A549	HCT-116	PC3	MCF-7	HepG2	CACO	HFB4
MGL (U/ml)	7.7 (0.61)	9.5 (0.76)	2.22 (0.17)	0.69 (0.05)	2.2 (0.17)	4.48 (0.35)	5.46 (0.43)	11.3 (0.904)	113.7 (9.7)

significantly increased with the increase in the initial concentration of pretreated beet molasses from 20 to 35 g/L. However, the enzyme was slightly decreased when the initial concentration was more than 35 g/L and this may be closely related to their increased of self-inhibiting substances. The current study indicates that *S. DMMMH60* MGL should be an inducible enzyme. In addition, the supplementation of the culture medium with yeast extract had considerably high MGL activity beside the main nitrogen source (L-methionine), this may have been caused by the fact that, yeast extract rich with methionine which could serve as an induction agent. Consequently (El-Sayed, 2009) reported that chicken feathers and soya bean meal are the most suitable substrates with L-methionine for L-methionine γ-lyase biosynthesis by *Aspergillus flavipes* and who suggested that may be correlated with their high sulfur-amino acid content of these components. Enzyme production in different incubation period showed that a gradual increase on MGL production and biomass yield was observed reaching optimum values at 4 day of incubation period suggesting that Tween 80 can improve and accelerate the enzyme accumulation during incubation period. This accelerated was likely because of the highly shortened of growth and increasing productivity, for further increasing of incubation time, a detectable

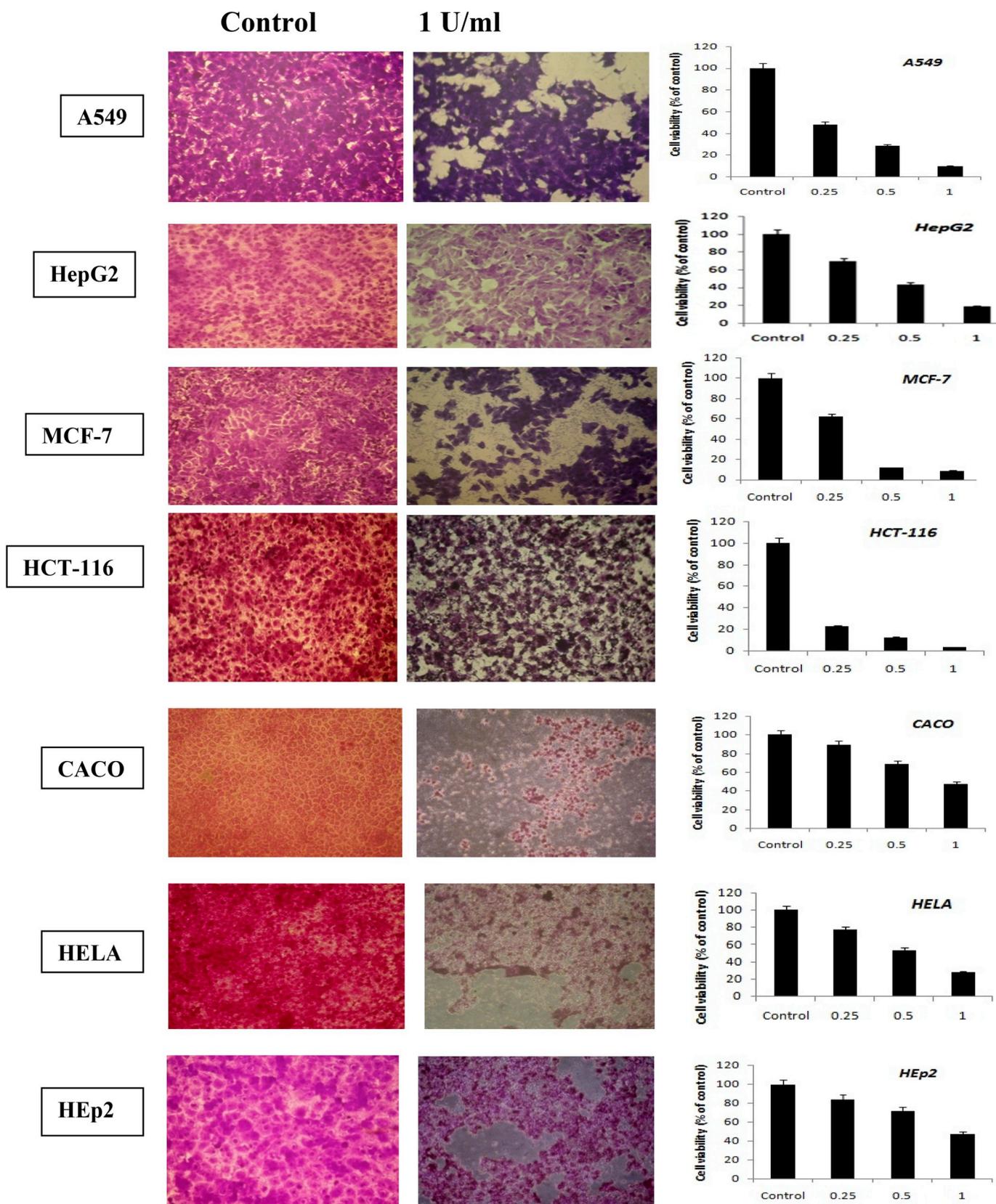


Fig. 5. Cytotoxicity effect of the purified L-methionine  $\gamma$ -lyase on different cancer cells after 24 h of treatment under inverted microscope.

reduction of both enzyme productivity and growth rate of *S. DMMMH60* could be observed and this might be attributed to nutrient limitation or accumulation of some acids resulted by molasses fermentation. These phenomena suggested that methionine is classifying

as a non-polar protein, adding hydrophilic nonionic surfactant seems to be usefulness to improve methionine solubility and to overcome mass transfer problem. Also, the positively acting of Tween 80 may be related to an improved the permeability of the cell membrane thus permitting

more enzymes being secreted outside the cells, as described for other microorganisms (Zeng et al., 2006). Hence, it was possible to produce MGL effectively by Tween 80 without the time-consuming inoculum preparation. On the other hand, the highest MGL productivity was achieved at pH 8.3, as the pH decreased or increased, enzyme yield was found to be affected. The pH has the maximum contribution percent (47.05%) in the Plackett-Burman design and this could be due to the electric charge of the cells and the oxidation-reduction potential that can affect nutrient absorption and enzymatic reaction (Elkhwaga, 2018). In addition, the maximum MGL yield was achieved using potassium phosphate buffer (0.05 M) might be related to monovalent cation  $K^+$  that may regulate cellular transport and membrane depolarization of cells (Khalaf and El-Sayed, 2009). In aerobic condition processes, oxygen transfer plays a very important role in the cell proliferation and/or product formation and this due to the low solubility of oxygen in medium. The oxygen concentration required for the maximum enzyme production by *S. DMMM60* was observed at the highest aeration rate (50 ml/250-ml flask). In this respect (Musengi et al., 2013), suggested that the lower growth in the larger culture volume was due to oxygen transfer limitations, resulting in decreased peroxidase production by *Streptomyces* strain. Furthermore, inoculation size was found to have strong effects on both enzyme yield and biomass synthesis. In this study, an increase in inoculum density would ensure fast propagation and biomass synthesis. However, high inoculum size (more than 6%) led to a considerable reduction of enzyme yield and growth rate due to nutrient limitations or increase in initial moisture contents resulted in lower  $O_2$  transfer, ultimately leading to poor product biosynthesis (El-Sayed, 2009). On contrast, low inoculum size leading to decrease in enzyme productivity and this could be attributed to the minimize number of cells that may need to longer time to grow for using the substrate. The development of MGL was observed when the culture medium was supplemented with  $MgCl_2$ , and the positive effect of magnesium ion might be attributed to their contribution as osmotic modulator and cofactor for enzyme activity as reported by (Khalaf and El-Sayed, 2009).

In this study, extracellular MGL was purified from an aqueous extract of *Streptomyces DMMM60* to homogeneity and subsequently characterized. Purification scheme was successfully completed in three steps using heat treatment, DEAE-cellulose and Sephadex  $G_{100}$ , with a 44.1% yield. Related to our results, similar protocols have been used for MGL purification from bacterial and fungal species (Manukhov et al., 2005). purified MGL with 21% yield from *Citrobacter freundii* by heat treatment at 60 °C followed by separation on DEAE-cellulose column and Sephacryl S-200HR column. The sequential purification steps appeared from the gel-electrophoretic profile showed that the purified MGL was migrated as a single band on SDS-PAGE with an approximate molecular mass of 55 kDa. The homogeneity of the purified enzyme was demonstrated by the appearance of a single band and ensured the efficiency of the purification scheme. The purified *S. DMMM60* MGL exhibited two absorption maxima at 280 nm, indicating a protein containing aromatic amino acids, and at 420 nm due to the aldimine linkage of the pyridoxal phosphate aldehyde group and the lysine amino group on the PLP-enzyme binding domain. The absorption spectrum of purified MGL was typical with that reported by (Dias and Weimer, 1998; El-Sayed, 2011). In the present study, the maximum *S. DMMM60* MGL activity was displayed at pH. 8 indicating that enzyme belong to the alkaline L-methionine  $\gamma$ -lyase group. In general, pH optima of this enzyme from other sources are in the range of pH 6.5 to 7.5 (Tanaka et al., 1976; El-Sayed, 2011), only L-methionine  $\gamma$ -lyase from *Aspergillus ustus* (Abu-Tahon and Isaac, 2016) and *Clostridium sporogenes* (Kreis and Hession, 1973) have been reported to have a higher activity optimum at pH 8.5. At pH values below or above the optimum, the affinity between the enzyme and its substrate may also be affected by the reaction pH value where the enzyme may not be saturated with substrate thereby disrupting the enzyme-substrate intermediate (El-Sayed, 2011). The pH stability of the purified enzyme was showed

maximally at pHs ranging from 6.5 to 8.5, reflecting its remarkable stability under alkaline condition. A slight decrease in the enzyme activity was observed at pH 9.0 while at pH 5.0 the enzyme retained about 53.8% of its initial activity; the significant inhibitory effect on L-methionine  $\gamma$ -lyase activity at acidic conditions (3.5–5.5) suggests denaturation of the enzyme subunits or dissociation of the PLP coenzyme (El-Sayed, 2011). Similar pH stability curve was obtained for MGL purified from *Pseudomonas putida* and *Brevibacterium linens* from 7 to 8 (Tanaka et al., 1976; Dias and Weimer, 1998) indicating a similar conformational tertiary structure of the *S. DMMM60* MGL. Furthermore, highest activity of the purified enzyme was recorded at 45 °C. In this finding, the optimum temperature for *S. DMMM60* MGL was closeness to that reported for purified enzyme from cheese lactic acid bacteria at 37 °C (Hanniffy et al., 2009) and *Pseudomonas putida* at 37 °C (Lishko et al., 1993). Similarly, *Clostridium sporogenes* MGL activity increased slightly when heated for 15 min at 50 °C or for 10 min at 60 °C with a rapid loss of activity after further heating at 60 °C (Kreis and Hession, 1973). From the heat stability profile, it could be noticed that, the purified enzyme was more stable up to 50 °C for 100 min and not loss of activity followed by a notable decrease of the enzyme stability after 1 h at 60 °C was carried out. Furthermore, after heating at 90 °C for 30 min, the enzyme still retained about 18% of its original activity. The effect of metal ions and inhibitors showed that, a considerable loss of enzyme activity more than 50% was noticed with  $Cr^{+2}$ ,  $Cd^{+2}$ ,  $Cu^{+2}$  and  $Ni^{+2}$ ,  $Hg^{+2}$  respectively, suggesting interaction with surface thiols of the enzyme and/or an internal aldimine of the co-enzyme (PLP) (El-Sayed, 2011). No inhibition was occurred in the presence of EDTA, which indicated that no metallic cofactors were needed. The enzyme activity was completely denatured in the presence of benzoic acid and this was correlated with the change in the enzyme structure by formation of benzoic acid-enzyme complex as suggested by (Singh et al., 2013). It was also noticed that, the extent of enzyme inhibition by thiol-reducing agent (Iodoacetate) indicating the presence of –SH group in the active site of the enzyme. Besides, MGL was strongly inhibited by PMSF, SDS, and DTT, retaining only 52.3%, 32.4%, and 27.9% of its original activity, respectively. In accordance with the data reported by (El-Sayed, 2011), the dramatic inhibition of MGL by these compounds could be correlated with the presence of a cysteine/disulfide bond which preserves the molecular catalytic folding state of the enzyme. Moreover, the negative effect of enzyme activity with hydroxylamine may be due to the dissociation of pyridoxal phosphate from holo-methioninase, which is similar to other PLP enzymes (El-Sayed, 2011). Anticancer activity of the purified enzyme was evaluated in-vitro using different cancer and normal cell line. The observed results generally indicated that, the activity of MGL against cancer cell lines was highly effectiveness while the cytotoxic effect toward normal cells was negligible. Our results corroborate the earlier findings of (Tan et al., 2010), they treated twenty-one different human cancer cell lines and seven human normal cell lines in-vitro with recombinant MGL. They found that high cytotoxic activity of MGL towards cancer cell lines compared to the normal cell lines and suggested the cells from different kinds of cancer are methionine dependent which can also affect their DNA methylation. The exact mode of MGL anticancer is fully understood by (Hoffman, 1997; Tan et al., 2010) who suggested that, the amount of methionine needed by the cancer cells is much greater than that required by the normal cells, this may be related to the increased protein synthesis and enhanced *trans*-methylation reactions. This ensures that multiple biochemical reactions necessary for the fast proliferation of cancer cells and have notably action on DNA lead to sharp damage of cell membrane which undergoing apoptosis resulted in the death of the cells. Inverted microscope for the treated and untreated samples was also supported this phenomenon, whereas some holes and multiple dents formed on the treated cell surface, cellular rounding up, shrinkage, and loss of cell adhesion.

## 5. Conclusion

Although many bacterial and fungal species have been reported to produce MGL in great detail, there are no reports focused on this enzyme activity of *Streptomyces* Sp. have yet been given. Therefore, the Plackett–Burman experimental design was used to optimize parameters affecting the production of MGL by *Streptomyces* DMMH60 under submerged conditions. The significant realization of this research is the selection of readily available components and a reduced cost of the medium. According to this study, the proposed model as designed by PBD proved to be significant in statistical terms and showed enhanced production of MGL in the presence of optimized medium. Purified MGL with a relative molecular weight of 55 kDa has ability to remain stable over the wide range of pHs (6.5–8.5) and temperatures (30–50 °C). MGL was found to be active against all cancer models in a dose-dependent manner suggested that, this enzyme could be a potential candidate for achieve complete tumor regression. Future studies are required to investigate the stabilization of enzyme via immobilization to configure it for use in-vivo.

## Conflicts of interest

The authors declare no conflict of interest.

## Acknowledgment

The author expresses their sincere thanks to National Research Centre of Egypt for providing the necessary research facilities.

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