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Production, Purification and Characterization of L-Methioninase from *Streptomyces Variabilis* 3MA2016

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ABSTRACT

Fourty five Streptomycetes isolates were screened for production of L-methioninase. Best nine needed a pink color around their settlement as proof to the processing of extracellular L-methioninase. These isolates were quantitatively checked of L-methioninase production and the promising isolate was subjected to identification showed that the strain named *Streptomyces variabilis* 3MA2016. Ultimate L-methioninase production by *S. variabilis* 3MA2016 was after 7 days of incubation at temperature 30°C at pH 7.0 at L-methionine concentration 0.4 % after usage of glucose as a carbon source and peptone as organic nitrogen source. Purification of L-methioninase was done by different methods. The molecular weight of the purified enzyme was ~ 45 KDa. The perfect temperature of the purified L-methioninase was 30 ºC and the enzyme was stable up to 40 ºC for 1 h. But, at extreme temperature up to 80 °C leads to denaturation of the enzyme. The optimum pH of L-methioninase was about 7.0 and stable in pH range of 7 to 8. The enzyme was inhibited by PMFS, EDTA, Tris and glycine, respectively from 78% to 3%. The (K_m) and (V_{max}) was 0.43 and 34.60, individually.

Keywords: Production - purification - characterization - L-methioninase - *streptomyces* sp.

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INTRODUCTION

The requisition about enzyme advances with pharmaceutical research, advancement and manufacturing is a developing field. The idea of curative enzymes need been around for no less than 50 years. For example, in 1960 restorative enzyme play as a part of replacement therapies for hereditary deficiencies [1] Enzymes likewise medications need two significant characteristics that recognize them from the greater part different sorts of pills. First, enzymes usually attach and focus on their objects with huge affinity and specificity. Second, enzymes are reactant furthermore modify different focus particles of the needed results. These two features make enzymes settle on particular powerful medications that might finish restorative biochemistry in the body. These features have resulted in the improvement of numerous enzyme drugs for an extensive range of disorders.

The best surety for L-methioninase, however, will be practically potentially in blending therapy, where it has the potential to selectively sensitize tumor cells to large portions classes of presently utilized chemotherapy. In this way, L-methioninase might enactment not main likewise a widespread disease Drug, as well as similarly as a widespread modulator from claiming other chemotherapy medications. A substitute usage of L-methioninase to heart disease, the place L-methioninase might have been utilized to those medications for hyperhomocysteinemia [2, 3, 4]. Also the individuals profound established diminishment of L-methionine from 0.86 with 0.17% of the diet, acquires something like a 30% more lifespan in male Fischer 344 rats. So, it might a chance to be used for maturing since those data uncover that the unremitting use from L-methioninase could titrate serum L-methionine levels moreover conceivably raise the life span [5].

L-methioninase which was first reported in 1973 [6] is apyridoxal phosphate-dependent enzyme that catalyzes the direct α- and β-elimination of L-methionine to α-ketobutyrate, methanethiol, and.ammonia [7]. Large number tumor units have a supreme prerequisite to plasma L-methionine, since normal cells.are resistant to the restriction of exogenous L-methionine [8]. L-Methionine exhaustion needs an expansive range of antitumor activities [9]. Under L-methionine exhaustion, cancer cells were captured in the late S-G2 period because of those pleiotropic impacts furthermore underwent apoptosis. So, restorative misuse for L-methioninase should drain plasma L-methionine need been extensively investigated [10]. Growth of different tumors for example Lewis lung carcinoma [11], human colon cancer lines [12], glioblastoma [13], and neuroblastoma [14] was captured by L-methionine. L-Methionine on mix with anticancer pills for example, such that cisplatin, 5 fluorouracil, nitrosourea, also vincristine shown synergistic antitumor impacts once rat and mankind's tumors done mouse models [15, 16, 17, 18].

L-methioninase might have been purified also described from a number bacterial species including *Pseudomonas.putida*, *Aeromonas* sp., *Citrobacter freundii*, *Brevibacterium linens*, *Lactococcus lactis* and *Clostridium sporogenes* [19]. The restorative reaction for bacterial L-methioninase will be connected with secondary immunogenicity, low substrate specificity, also perilous impacts of the Kidney furthermore liver [17]. A couple investigations were accounted for the characterization for L-methioninase from streptomycetes. Streptomycetes are spoken to on nature those biggest number of species and varieties, which contrast significantly over their morphology, physiology, and biochemical activities. *Streptomyces* picked up exceptional imperativeness because of their limit to process bioactive metabolites and enzymes [20]. From the above, it may be unequivocally apparent that L-methioninase needs enormous possibility. Those right now accessible brands in advertise are exact unreason ability What's more subsequently there will be a dire requirement for new sources for those processing of the enzyme. So, it was the trend towards isolating some *streptomyces* sp. having ability producing L-methioninase, production, purification and characterization of L-methioninase.

MATERIAL AND METHODS

Isolation of streptomycetes

Streptomycetes isolated using the serial dilution method [21]. *Streptomyces* isolates were isolated from soil and marine at Starch nitrate agar media [22] which has the following ingredients (g/L): starch, 20; KNO_3, 2; NaCl, 0.5; K_2HPO_4, 1; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, 0.001 and $CaCO_3$, 2.0. Agar, 20, distilled water, 1000 (sea water (50%) in case of marine samples) pH 7.0-7.4. The plates were incubated at 28°C for seven days, pure colonies of *Streptomyces* that appeared were picked up and maintained on starch-nitrate agar slants and stored at 4 °C.

Rapid assay plate method

Fourty five *streptomyces* samples were tested for its ability to decompose and utilize L-methionine as the only source of nitrogen in a modified starch agar medium amended with phenol red with concentration of 0.07% (W/V) just before pouring the plates. The pH of this medium was adjusted to 7.0. L-methioninase producing isolates were identified as evidenced by the pink color of the colonies or around the growth, resulting from the production of ammonia by the action of L-methioninase on L-methionine [23].

Screening for L-methioninase producer isolates

Nine streptomycetes isolates which have the ability to maximum production of L-methioninase were quantitatively screened for their L-methioninase productivities using different three media (A, B and C):

Medium (A) contains (g/L): starch, 20; NaCl, 0.5; K_2HPO_4, 1; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, 0.001; $CaCO_3$, 3 and L-methionine, 10; pH 7.0 -7.4 [24].

Medium (B) contains (g/L): KCl, 0.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1; $FeSO_4.7H_2O$, 0.1; $ZnSO_4$, 0.1; NaCl, 25 and L-methionine, 10; pH 7.0 -7.4 [25].

Medium (C) contains (g/L): glucose, 5; peptone, 5; beef, 3; NaCl, 5 and L-methionine, 10; pH 7.0 -7.4 [26].

L-Methioninase assay

Activity of L-methioninase was determined by Nesslerization method [27] with some modifications. Standard contains 1 ml of 1% L-methionine in phosphate buffer (0.2 M) (pH 7.0), 0.1 ml of pyridoxal phosphate and 1 ml of crude enzyme and incubated at 30°C for 1 h, then stopped the activity by adding 0.5 ml of 20 % trichloroacetic acid. Then, centrifuged at 5,000 rpm for 5 min. to remove the precipitated protein, 0.1 ml of above mixture was added to 3.7 ml of distilled water and 0.2 ml of Nessler reagent. The developed colored was measured at 480 nm using UV/VIS-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). One unit (U) of L-methioninase was defined as the amount of enzyme that liberates ammonia at 1 µmol/min under optimal assay conditions. The specific activity of L-methioninase was expressed as the activity of enzyme in terms of units per milligram of protein.

Determination of extracellular protein

The protein concentration of the prepared crude was estimated by Bradford reagent [28] using bovine serum albumin as a standard protein. Absorbance at 280 nm was used for determining protein in column elutes [29].

Identification of the promising isolate

Morphological, physiological and biochemical identification

The promising isolated streptomycete which showed high production of L-methioninase activity was subjected to identification by morphological, physiological and biochemical tests as spore chain morphology by light microscopy, Spore surface ornamentation by examining spores using transmission electron microscope (TEM) using copper grade coated carbon by touching on electron microscope (JEM-HR-2100- made in Japan), colour of spore mass, Pigmentation of substrate mycelium, diffusible pigments , melanin pigment production on peptone-yeast extract-iron agar and tyrosine agar and utilization of different carbon sources using D-glucose (positive control), D-fructose, sucrose, rhamnose, D-mannitol, D-xylose, raffinose, L-inositol, galactose and L-arabinose for determined the ability of strains to use different carbon sources. Carbon sources were added to the basal medium at 1.0% (w/v) [30, 31].

Molecular identification

The most promising streptomycete isolate has high L-methioninase activity was identified by 16S rRNA sequencing. Chromosomal DNA was extracted and 16S rRNA gene was amplified using universal primers F (5´-GTGCCGCAGCCGCGGTA-3´) and R (5´-TTGTAGCACGTGTGTAGCCC-3´) (Manfred Kroger, Institute of Microbiology and molecular biology, University of Gießenbei). The PCR product of 16S rRNA was purified to remove contaminants and sequenced then the 16S rRNA sequence was compared with published 16S rRNA sequences of *Streptomyces* sp. in the NCBI databases using BLAST program. (http://www.ncbi.nlm.nih.gov). Selected sequences of the greatest similarity to the 16S rRNA sequences of the *Streptomyces* isolate were aligned and generating the phylogenetic tree. The 16S rRNA gene sequences of the *Streptomyces* sp. was reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases.

Medium optimization for production of L-methioninase

Optimization of the components of medium required for maximum L-methioninase production by *Streptomyces* strain was evaluated at broth medium (C). Subsequently the medium component studied included the effect of different incubation time (4, 5, 6, 7, 8 and 9 days), different pH (3, 4, 5, 6, 7, 8 and 9 adjusted with 1 N HCl or 1 N NaOH), different temperatures (20, 25, 30, 35 and 40 °C), different L-methionine concentration (0, 0.4, 0.8, 1.2 and 1.6 %), different additional carbon sources (glucose, xylose, arabinose, manitole, galactose, fructose, sucrose, lactose, maltose and starch at 1% w/v) and different nitrogen sources (yeast extract, malt extract, peptone, beef extract, sodium nitrate, ammonium oxalate, ammonium chloride, ammonium molybidate and ammonium sulfate).

Purification of L-methioninase

The proteins were precipitated from the supernatant with ammonium sulfate (0–80%) saturation. L-Methioninase active fraction was pooled, centrifuged (5,000 rpm, 30 min) and the precipitate was dissolved in minimal amount of 0.2 M phosphate buffer, pH 7.0 and dialyzed overnight at 4 °C against the same buffer. The dialyzate was loaded on a (1.5 × 60 cm) Sephadex G-100 equilibrated with 0.2 M phosphate buffer, pH 7 and eluted with one liter of the same buffer at flow rate 0.5 ml/min. Elute fractions (5 ml) was collected for measurement of absorbance at 280 nm and the enzyme activity was assayed [27]. The active fractions were pooled and dialyzed against the same buffer. The protein content was measured and the L-methioninase activity was assayed as described before.

Characterization and properties of the purified L-methioninase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of the purified L-methioninase was determined according to the method [32].

Effect of temperature on the activity and stability of L-methioninase

The effect of reaction temperature on L-methioninase activity was determined by incubating the reaction mixture at different temperatures ranging from 20 to 80 °C in 0.2 M phosphate buffer. The thermal stability of the purified enzyme was determined by preincubating the enzyme solution for up to1 h at various temperatures (from 30°C to 80°C) in the absence of substrate. At different times (10–60 min), aliquots were removed and cooled and the residual activity was measured by the standard assay method as previously mentioned.

Effect of pH on the activity and stability of L-methioninase

The optimum pH for the purified L-methioninase activity was determined using 0.2M citrate phosphate buffer pH (3.0-5.0), phosphate buffer pH (6.0-8.0) and Tris-HCl pH (9.0-11.0). After incubating each reaction at 30 ºC for 1hour, enzymatic activity was detected. The pH stability of the enzyme was determined by preincubating the enzyme solution at different pH values ranging from 5.0 to 9.0 for 2 h. at 4 ºC. At the end of

preincubation time, the pH value of enzyme solution was readjusted to pH 7.0 and then residual enzyme activity was assayed by the standard method.

Effect of inhibitors on L-methioninase activity

Compounds tested for their inhibitory effects included glycine, phenylmethylsulfonyl fluoride (PMSF), Tris, and EDTA. The inhibitory effect of these compounds on enzyme activity was assessed by incubating enzyme solution with 5mM concentration of each compound for 20 min before addition of substrate. After preincubation time, enzymatic activity was determined under optimal assay conditions [33].

Substrate concentrations

Different concentrations of L-methionine (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 % w/v) have been used and the apparent K_m value of the purified enzyme was determined through studies relating substrate concentrations to the velocities of the reaction. The apparent K_m and V_{max} values were determined from Lineweaver–Burke plots [34].

RESULTS AND DISCUSSION

Rapid assay for production of L-methioninase

Rapid plate assay method will be utilized to screening the capacity about 45 streptomyces isolates for processing of L-methioninase. It was revealed that just 9 streptomyces isolates were portrayed toward that appearance for pink shade around their colonial growth as proof and has greatest ability for the production of extracellular L-methioninase Table (1) and Figure (1). Different authors expressed those microorganisms including bacteria [35], yeast [36] and fungi [37] having the capacity to utilize L-methionine as a nitrogen source also produce L-methioninase enzyme. These result were in agreement with that mentioned by author [24] used that method for detection of L-methioninase production showed that there are 18 isolates had pink colour around their colonial growth as indicate that for the production of extracellular L-methioninase.by these isolates.

Table 1. Screening for production of L-methioninase by rapid plate assay test (qualitative assay test)

Sample	Results	Sample	Results	Sample	Results
1	-	16	++	31	++
2	+	17	-	32	-
3	-	18	-	33	++
4	-	19	-	34	-
5	-	20	-	35	++
6	+	21	++	36	+
7	-	22	-	37	-
8	+	23	++	38	-
9	-	24	-	39	-
10	-	25	+	40	++
11	-	26	++	41	+
12	-	27	-	42	+
13	-	28	-	43	++
14	-	29	-	44	-
15	+	30	-	45	+

Figure 1. Screening of isolates by rapid plate assay test on production of L-methioninase

Quantitative screening for production of L-methioninase

Nine *Streptomyces* isolates were characterized by the presence of strong pink colour around their colonial growth as were screened quantitatively for production of L-methioninase at three different media A, B and C (Table 2, 3 and 4). Protein estimation, enzyme formation and specific activity were measured for every isolate and it was illustrated that isolate (no. 40) showed that the highest specific activity at three different media A, B and C was 32.92, 19.71 and 37.48 (U/mg), respectively. It was shown that there are two streptomycetes isolates were the most suitable for L-methioninase production identified as *Streptomyces* sp. DMMMH 4.and *Streptomyces* sp. MDMMH60 [24].

Table 2. Quantitative screening of *Streptomyces* isolates for L-methioninase formation at medium (A)

Samples	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
16	95.5	4.4	21.08
21	41.4	4.1	10.09
23	57.9	3.9	14.8
26	27.3	2.5	10.92
31	65.6	2.8	23.42
33	16.4	1.4	11.71
35	75.5	4.2	17.97
40	98.8	3.0	32.92
43	71.7	4.5	15.93

Table 3. Quantitative screening of *Streptomyces* isolates for L-methioninase formation at medium (B)

Samples	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
16	83.7	5.2	16.09
21	54.8	3.7	14.81
23	69.9	4.0	17.45
26	29.1	2.9	10.03
31	53.9	3.1	17.38
33	25.3	1.9	13.3
35	61.2	5.6	10.92
40	74.9	3.8	19.71
43	69.1	5.3	13.03

Identification of the promising isolate

Several endeavors have been made to overcome the diversification of criteria and techniques applied in the description, classification and identification of streptomycetes. Huge variations of genotypic and phenotypic methods are currently used for the identification and classification of microorganisms [38]. But the most useful method for establishing the relatedness of higher taxa is the comparative analysis of the ribonuclease-resistant oligonucleotides of the 16S ribosomal RNA (rRNA) [39]. Morphological, physiological and biochemical tests were used for identified the promising isolate no. 40 showed it has a spiral spore chain

morphology under light microscopy (Figure 2), smooth spore.surface ornamentation by using TEM (Figure 3). While, colour of spore mass was brownish gray with pale yellow coloured for substrate mycelium and diffusible pigments was pale brown. Therefore, melanin pigment was not produced on peptone-yeast extract-iron agar and tyrosine agar. Utilization of carbon sources showed that this isolate was grown at all carbon sources used expects raffinose only. The nucleotide sequence obtained by molecular identification 16s rRNA was compared with existing sequences in the databases. The 16S rRNA sequence of the isolate is the greatest closely associated to *Streptomyces* sp. with a similarity of 99%. This result was identical with the conclusion of the morphological, biochemical and physiological characterization. So, this result revealed that this isolate was a new strain called *Streptomyces variabilis* 3MA2016 and have accession number KY290458. A phylogenetic tree shows the result of 16S rRNA analysis (Figure 4).

Table 4. Quantitative screening of *Streptomyces* isolates for L-methioninase formation at medium (C)

Samples	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
16	117.3	6.7	17.51
21	67.1	5.2	12.91
23	91.4	5.8	15.76
26	38.5	3.9	9.87
31	68.9	3.5	19.18
33	44.7	5.6	7.98
35	103.6	4.0	25.90
40	138.7	3.7	37.48
43	106.8	5.9	18.1

Figure 2. Photomicrograph showing spiral sporephores hyphae
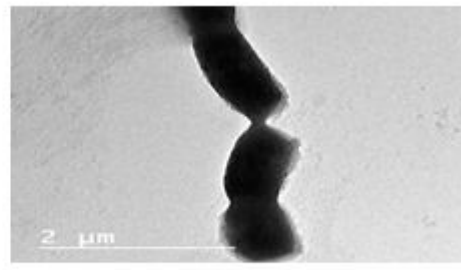
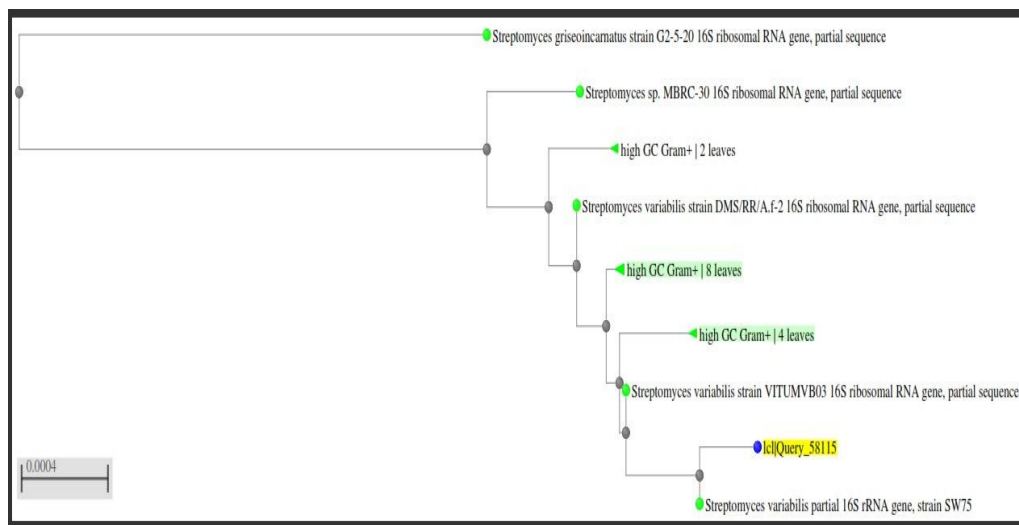Figure 3. TEM photomicrograph showing smooth spore surface

Figure 4. Phylogenetic tree of *Streptomyces variabilis* 3MA2016

Effect of some factors on the production of L-methoninase

Effect of time

Incubation time test was influenced on L-methoninase production by *Streptomyces variabilis* 3MA2016. Results showed that L-methoninase production increase constantly until 7 days, where the maximal enzyme production was (4.698 U/ml) then enzyme activity shortened after 7 days (Figure 5). These results proved that the connection between enzyme productivity and incubation period is fickle depending on the organism. It was noticed that the greatest production of L-methoninase by *Aspergillus flavipes* MTCC 6337 at day 8 of fermentation [40].
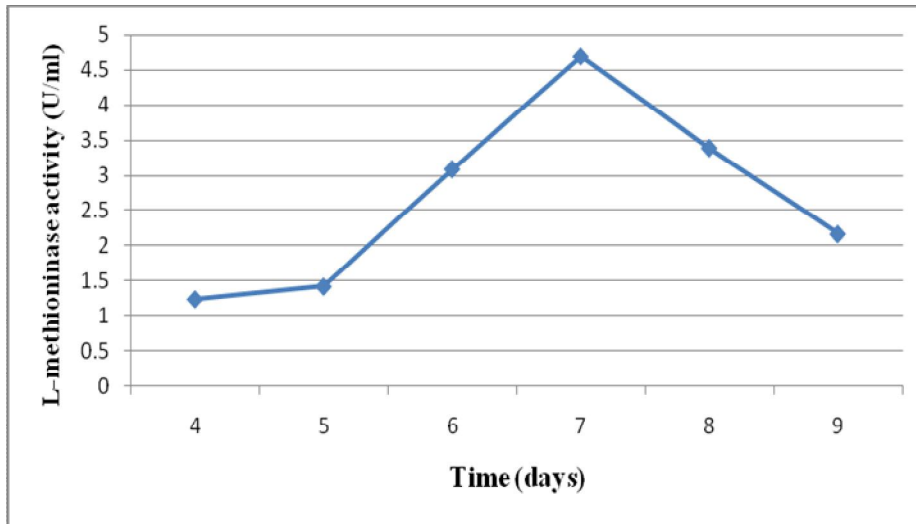
Figure 5. Effect of incubation time on L-methoninase production

Effect of pH

Figure (6) showed that the maximal enzyme production was noticed at pH 7.0 was (5.1 U/ml), either excess or lowering in the pH of the medium resulted in decline in production of the enzyme. It was stated that maximum L-methoninase productivity was obtained at pH 8.0 by *Aspergillus flavipes* MTCC 6337 [40].
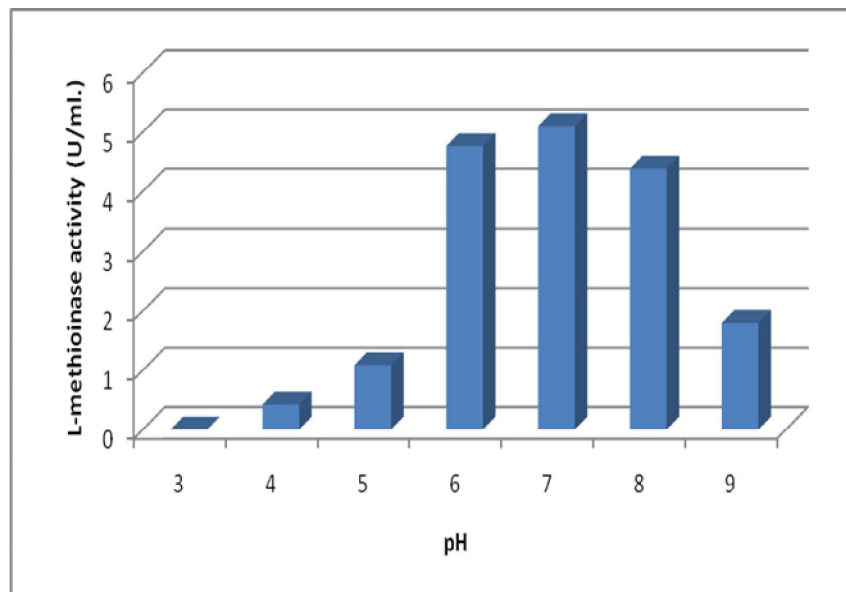
Figure 6. Effect of pH on L-methoninase production

Effect of Temperature

Incubation temperature of the fermentation medium is informed to effect the growth of any microbial strain, thus the maximal enzyme production (4.983 U/ml) was noticed at 30°C. Diversity in temperature in any way leads to reduce of L-methioninase production which may be due to the denaturation of microbial strain at higher temperatures (Figure 7). These results showed that there are relationships between temperature and enzyme productivity as mentioned by [40] mentioned that maximum values of L-methioninase was obtained at temperature 30 °C by *Aspergillus flavipes* MTCC 6337.
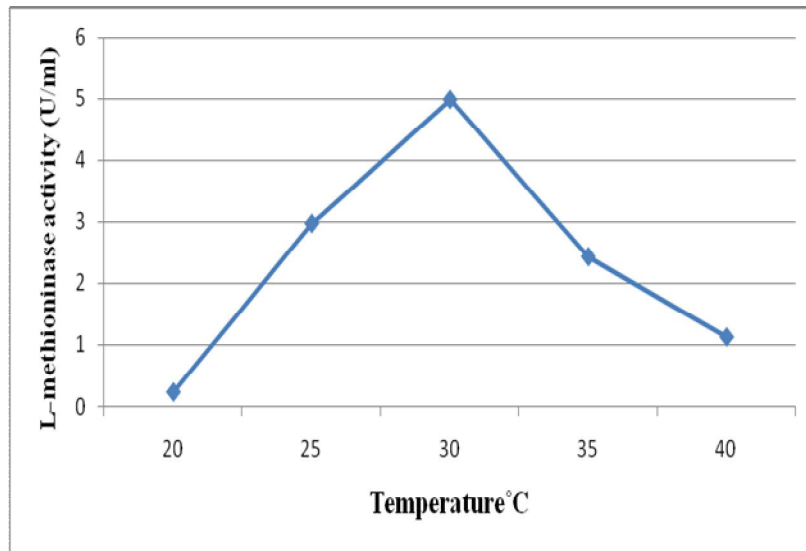
Figure 7. Effect of Temperature on L-methioninase production

Effect of L-methionine concentration

L-Methionine is an inducer substance for L-methioninase production. So, integrating various concentrations of L-methionine was used in the enzyme production medium. The results (Figure 8) showed that 0.4% (w/v) concentration gave maximal enzyme production (4.88 U/ml). But, by increase in L-methionine concentration, there was a decline in the enzyme production. This is may be due to the repressor effect of L-methionine at higher concentrations. The maximum L-methioninase yield was obtained with L-methionine concentration of 0.8% (w/v) by *Aspergillus flavipes* MTCC 6337 [40].
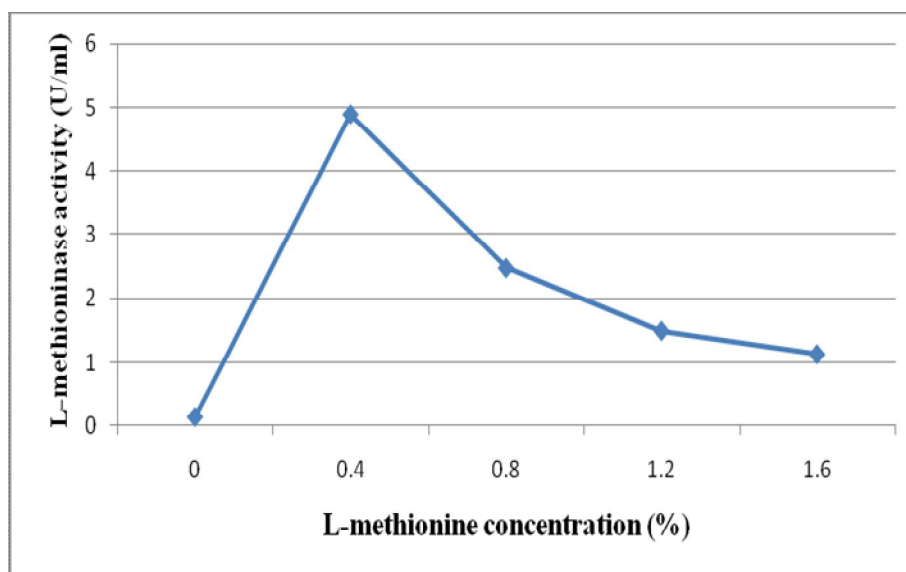

Figure 8. Effect of L-methionine concentration on L-methioninase production

Effect of different carbon sources

Different carbon sources were added into medium at 1% level resulted in an increase in the enzyme production. Glucose was supported maximal enzyme yield (6.622U/ml) and all the other carbon sources also showed considerable amount of enzyme production. Arabinose was the least effective as a carbon source (0.25 U/ml) (Figure 9). These result similar to [40] reported that among the different carbon sources supplemented glucose 1% (w/v) promoted maximal L-methioninase production by *Aspergillus flavipes* MTCC 6337.
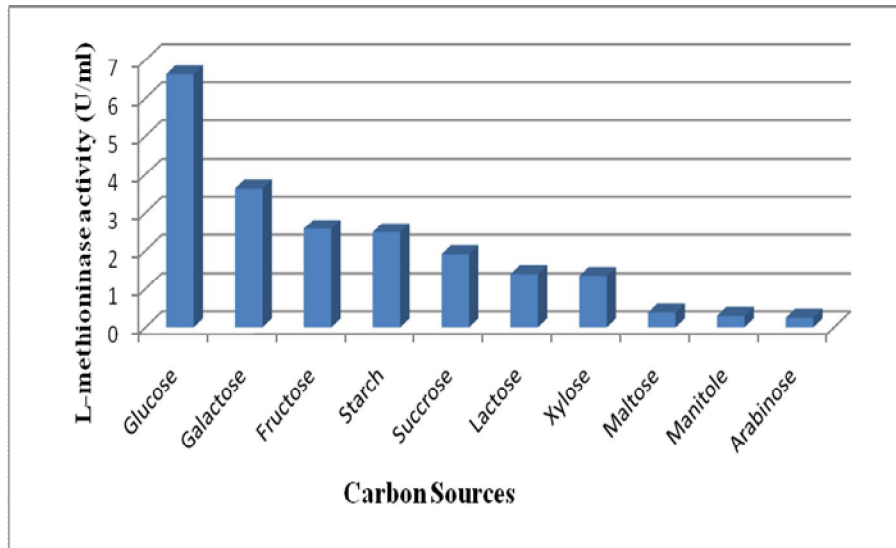
Figure 9. Effect of different carbon sources on L-methioninase production

Effect of different nitrogen sources

Effect of additional organic and inorganic nitrogen sources into medium resulted in a respectable increase in the enzyme production. From all the diverse nitrogen sources tested, peptone was supported maximal enzyme output (7.247 U/ml). All the other nitrogen sources also showed various amount of enzyme production. Ammonium oxalate was the least effective as a carbon source (0.661 U/ml) Figure (10). It was revealed that L-methionine might have been the most proper nitrogen sources for L-methioninase production by *Aspergillus flavipes* MTCC 6337 [40].
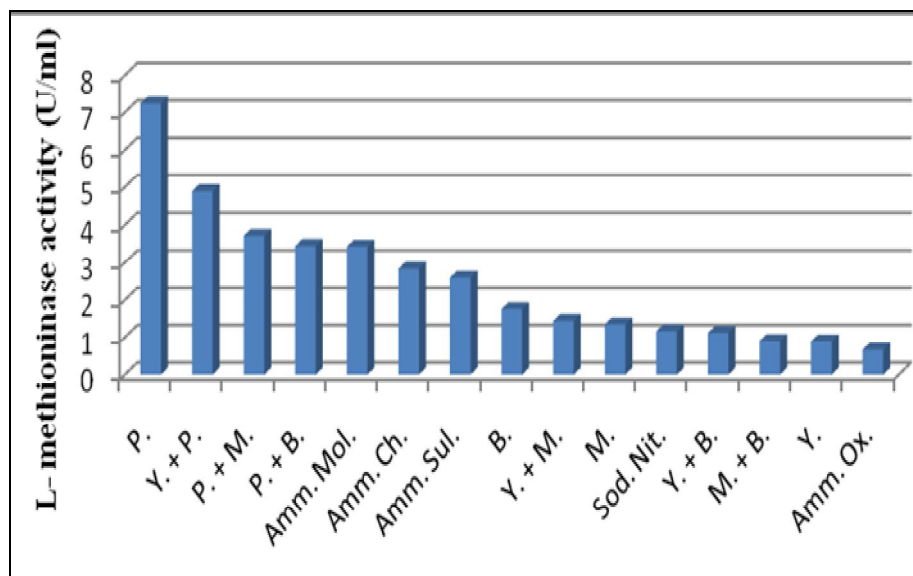

Figure 10. Effect of different nitrogen sources on L-methioninase production

Purification of L-Methioninase

The purification of L-methioninase is important from the viewpoint of developing a better understanding of the functioning of the enzyme. Precipitation is regularly utilized technique for the isolation and recovery of proteins from unrefined biological mixtures. Fractions from 80% ammonium sulfate saturation demonstrated high enzyme activity and specific activities in comparison with crude L- methioninase as noticed in Table (5). The obtained L- methioninase produced after partially purified using 80% ammonium sulfate was subjected on column of gel filtration using Sephadex G-100. The elution diagram of the enzyme is illustrated graphically in Figure (11). The obtained result showed a single peak co-eluting with L-methioninase activity. The purification results are summarized in (Table 5) with a purification fold 27.811 and recovery of 43.432 %. L-methioninase precipitated by ammonium sulfate from *Brevibacterium linens* [41]. While, L- methioninase from *Aspergillus flavipes* is purified using ammonium sulfate precipitation followed gel-filtration chromatography [42].

Table 5. Purification profile of L-methioninase from *Streptomyces variabilis* 3MA2016

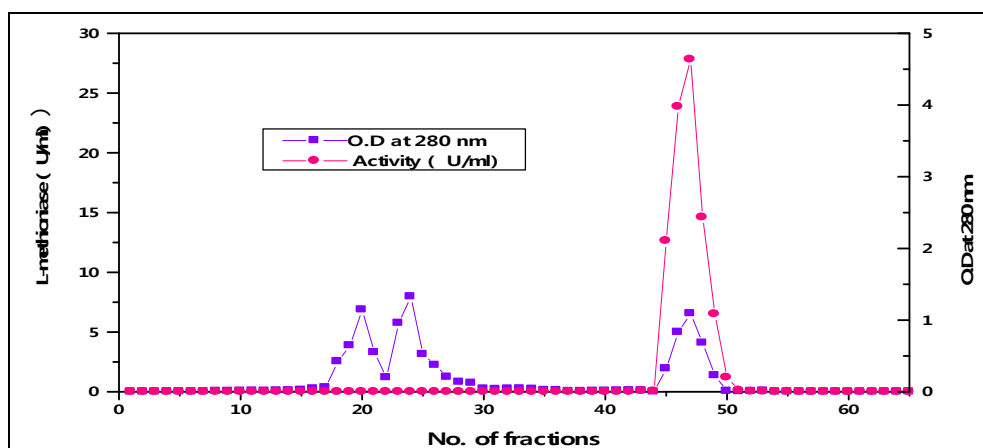| Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification Fold
Crude	1215.72	270.19	4.433	100	1
$(NH_4)_2SO_4$ (40%)	85.17	110.54	0.770	7.005	0.171
$(NH_4)_2SO_4$ (60%)	122.05	112.83	1.0810	10.030	0.240
$(NH_4)_2SO_4$ (80%)	759.65	8.36	90.867	62.480	20.197
Sephadex G-100	528.02	4.22	125.123	43.432	27.811

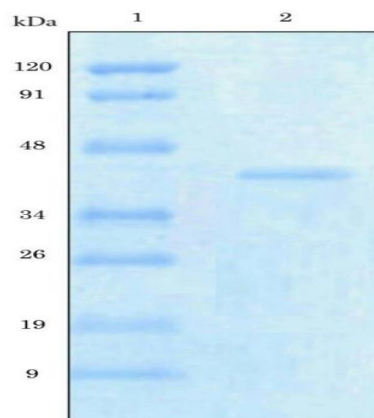Figure 11. Elution profile for the purification of L-methioninase

Figure 12. SDS-PAGE of L-methioninase produced by *Streptomyces variabilis* 3MA2016
From left to right: Lane1, standard molecular weight marker
Lane 2, final purified L-methioninase obtained by Sephadex G-100

The molecular weight of the purified L-methioninase was determined under denaturing conditions using 10% SDSPAGE. Figure (12) showed the electrophoretogram of the purified L-methioninase as determined using SDSPAGE, the molecular weight of the purified L-methioninase was found to be about 45 KDa. Many authors stated the same results, where they found that the molecular weight of L-methioninase purified from bacterial and fungal sources could range between 43 and 48 kDa [43, 44]. Also, it was stated that the molecular weight of L-methioninase purified from *Citrobacter freundii* might have been in range from 43.0 to 45.0 kDa per subunit [43].

Effect of some parameters on the activity and Stability of L-methioninase

Effect of temperature on the activity and stability of L-methioninase

The ideal temperature of the purified enzyme was 30°C (26.355 U/mL). At lower and higher temperatures, a gradual decrease in enzyme activity was observed until reach at 80 °C a complete inhibition was noticed as seen in Figure (13). This result may be explained by the fact that the temperature raises the reaction velocity and also influence the rate of enzyme destruction, producing a continuous fall in the concentration of active enzyme. *Aspergillus flavipes* exhibited maximum activity at 35 °C followed by a gradual decrease until reach 60 °C. However, L-methioninase of *Brevibacterium linens* showed optimum activity at 25 °C [45].
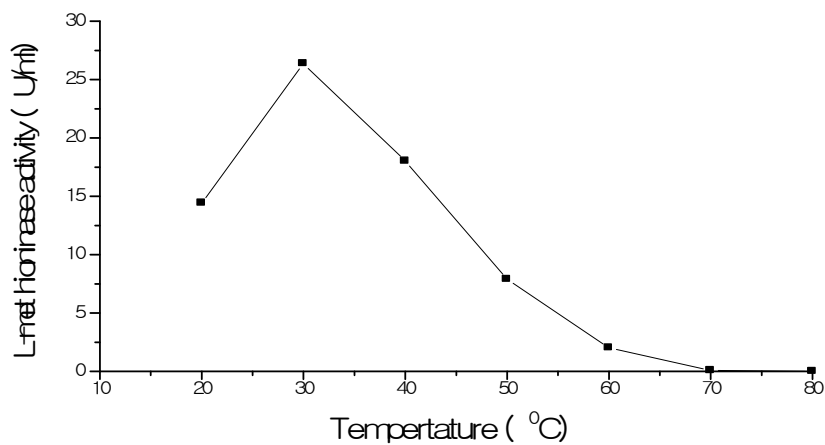

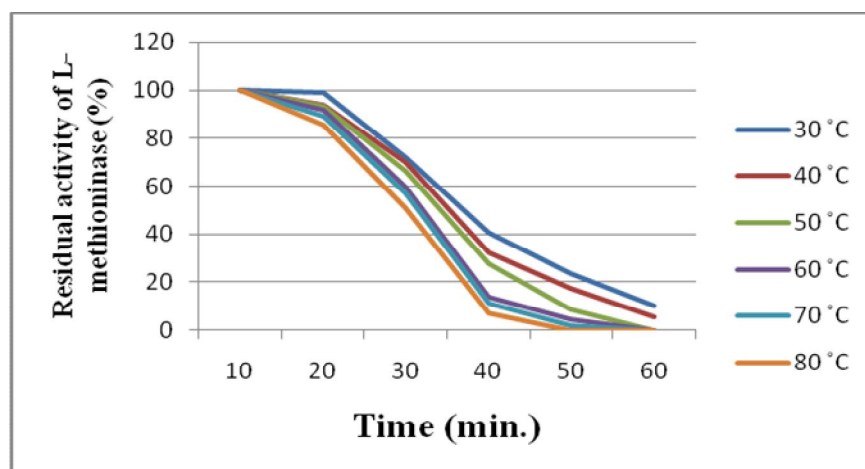Figure 13. Effect of Temperature on the purified L-methioninase

Figure 14. Effect of temperature on the stability of purified L-methioninase

Thermostability profile of the enzyme revealed that the L-methioninase enzyme was steady at temperature up to 40 °C for 1 hour of pre-incubation period as noticed in Figure (14). But higher temperature up to 80 °C causes denaturation of the enzyme. The same results observed in *Brevibacterium linens* BL2 and they observed that the enzyme was labile at temperatures higher than 30 °C [41], while on the contrary L-methioninase purified from *Aeromonas* sp., *C. tropicalis* and *Streptomyces* sp. DMMMH4 was thermostable up to 80 °C [24, 46, 47].

Effect of pH on the activity and Stability of L-methioninase

The result (Figure 15) indicated that the optimum pH of L-methioninase was about 7 using phosphate buffer with activity (26.319 U/mL), further increase or decrease of pH beyond the resulted decline in enzyme activity. It was stated the same result where the optimum pH of *Pseudomonas ovalis* was 7.2. While, others stated that the pH of L-methioninase from *Streptomyces* sp. DMMMH4 and *C. tropicalis* was 6.0 and 6.5, receptively [48]. On the other hand, the alkaline range of pH correlated with maximum enzyme activity for bacteria, *Pseudomonas putida* [3] and *Citrobacter freundii* [49].
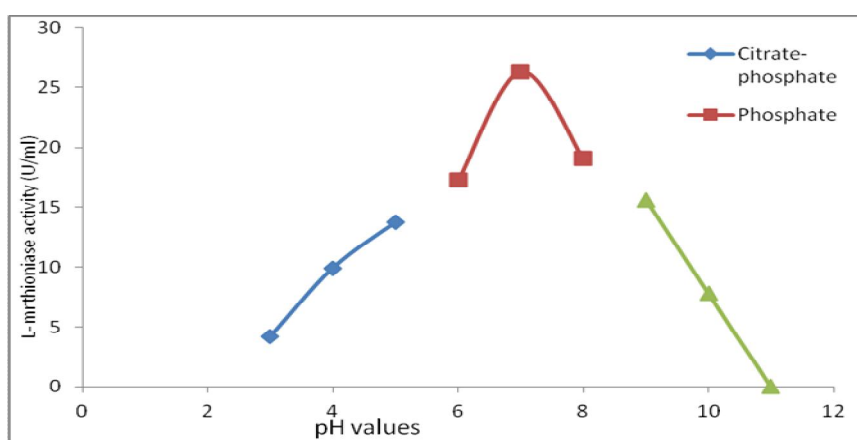

Figure 15. Effect of pH on the purified L-methioninase

To investigate the pH stability of the enzyme, the enzyme solution was previously incubated at different pHs for 2h at 4 ºC. The enzyme was stable in pH range of 7 to 8. It was cleared that the stability of the enzyme was decreased at higher and lower pHs (Table 6). The same result was observed by many authors [7,41]. It was cleared that the stability of the enzyme was decreased at higher and lower pHs and this was explained by author who said that the lower stability of the enzyme at a higher and lower pHs may be attributed to the dissociation of pyridoxal-5-phosphate or unfolding of the enzyme active site [42].

Table 6. Effect of pH on the Stability of purified L-methioninase

pH	Residual activity (%)
5	62.47
6	88.52
7	100
8	93.24
9	89.60

Effect of inhibitors on L-methioninase Activity

The enzyme was inhibited by PMFS, EDTA, Tris and glycine respectively from 78 to 3% as seen in figure (16). On the contrary EDTA has no effect on L-methioninase produced by *Streptomyces* sp. DMMMH4 and *Brevibacterium linens* [33, 41]. The inhibition effect of PMFS, Tris and glycine was observed by others, where

they explained the inhibition occurred by Ttris due to the amino group of Tris molecule which affect on the enzyme activity [48, 50].
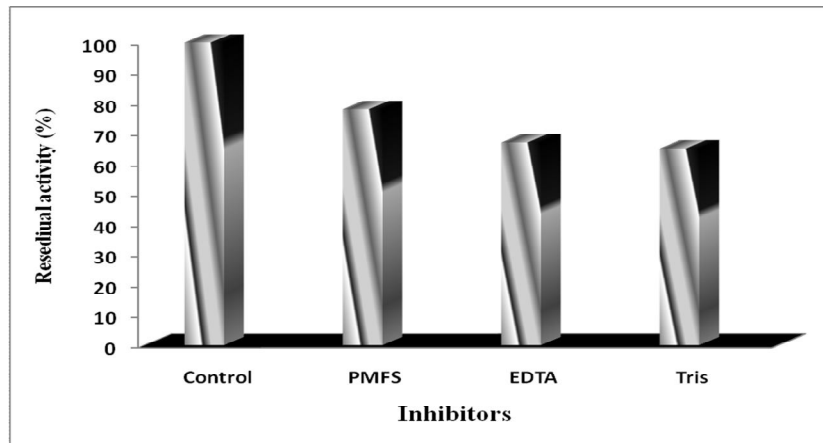

Figure 16. Effect of inhibitors on L-methioninase activity

Effect of L-methionine and kiniects properties

As noticed in Figure (17) the activity increased by increasing the concentration of L-methionine until reached 1% (26.402 U/mL), while the increase of the activity was barely noticed by increasing the substrate concentration this indicates that the active center of the enzyme.became saturated.with its substrate at concentrations above 1% w/v. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) was 0.439 and 34.602, respectively. It was reported that the specificity of fungal L-methioninase was higher than that observed for bacterial enzymes [47].
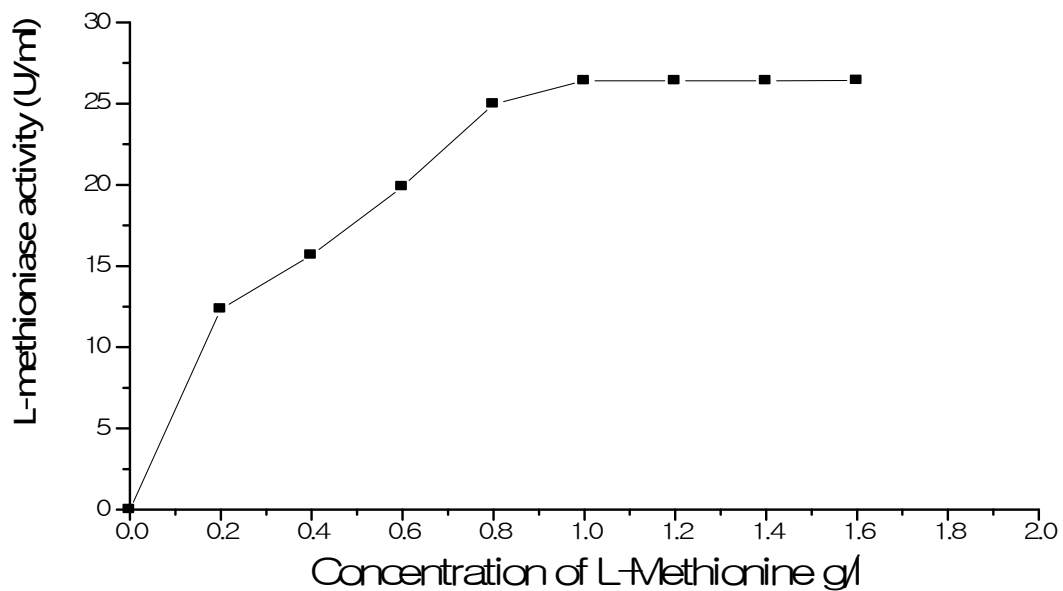

Figure 17. Lineweaver-Burk plot of L-metioninase activity with L-methionine

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