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ARTICLE

L-Methioninase from some *Streptomyces* isolates I: Isolation, identification of best producers and some properties of the crude enzyme produced

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Abstract Among 60 isolates of *Streptomyces* tested; only 40 isolates were capable to utilize L-methionine as the only source of nitrogen in medium. In addition, 24 of these isolates could grow in medium amended with L-methionine as a source of nitrogen and carbon. Qualitative rapid plate assay test shows the ability of 18 of these isolates to grow with a pink color surrounding their colonial growth, while 6 of these isolates could grow and utilize L-methionine without any pink color around their colonial growth. Quantitative assay test shows the rate of L-methioninase production by all isolates tested. Permeabilization treatment including chemical and physical methods proved that L-methioninase was found to be extracellularly produced. The results also indicate that L-methioninase production was not correlated with growth rate or L-methionine consumption in medium. On the other hand, quantitative assay test shows that these six isolates were L-methioninase negative and failed to produce any amount of L-methioninase. In addition, results also show that isolates No. 4 and No. 60 were the most suitable for L-methioninase production, these two isolates were characterized and identified as *Streptomyces* sp. DMMMH 4 and *Streptomyces* sp. MDMMH 60 using 16S rRNA with accession No. in gene bank. Furthermore, optimal conditions for enzyme activity produced by the two isolates were established in relation to temperature, pH, reaction time and type of buffer used and its molarities.

1. Introduction

L-Methioninase (methionine γ-lyase) is a pyridoxal phosphate (PLP)-dependent enzyme that catalyzes the direct conversion

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of L-methionine to methanethiol, α-ketobutyrate and ammonia [40]. L-Methioninase has been found in bacteria, some of which are anaerobic, *Porphyromonas gingivalis* [47] and *Treponema denticola* [13] and in eukaryotic pathogens, such as *Entamoeba histolytica* [42]. It is noteworthy that reports describe L-methioninase in the culture filtrates of a few yeasts including *Geotrichem candidum*, *Debaromyces hasenii* and *Saccharomyces cerevisiae* [4]. However, L-methioninase is absent in mammalian cells [4]. Regarding its nature, L-methioninase was characterized from many bacterial species as an intracellular enzyme [39] and from fungal species as an intracellular and extracellular enzyme [39,33]. L-Methioninase has a major role in food industry by imparting a distinctive aroma to many traditional fermented foods including cheese via degradation of L-methionine that releases volatile sulfur compounds (VSCs). The most common (VSCs) found in cheese is methanethiol which derives from the enzymatic degradation of the amino acid L-methionine [19,43] is present in cheese curd. Recently, evidences were reported of the contribution of yeasts to (VSCs) generation in cheese [2]. On the other hand, more attention has been paid to Methionine γ-lyase since it was reported as a potent anticancer against various types of tumor cell lines Breast, Lung, Colon, Kidney and Glioblastoma) [38]. Many researchers [17,1] reported the complete dependence of tumor cells on L-methionine for the proliferation; whereas normal cells are methionine independent [8] under methionine depletion cancer cells were arrested [35]. Consequently, methionine is the main tumor specific target for therapeutic techniques. Thus, therapeutic exploitation of L-methioninase to deplete plasma methionine has been extensively investigated [30]. In addition, the limited distribution of L-methioninase as an intracellular enzyme among all microbial pathogens but not in humans makes this enzyme a promising drug target for antibacterial, antifungal and anti-protozoal therapies [35].

The aim of this study is to screen some *Streptomyces* isolates isolated from Egyptian soils for their ability to produce L-methioninase. In addition, some permeabilization treatments will be carried out on *Streptomyces* cells in order to search for any intracellular enzyme produced inside the cells. Furthermore, some factors affecting L-methioninase activity will be also investigated.

2. Materials and methods

2.1. Isolation and screening of Streptomyces

Streptomyces isolates were isolated from Egyptian soil. A small portion of soil samples were suspended in sterilized water and spread over agar plates containing starch-nitrate agar medium. This medium was amended with 0.1% of yeast extract. 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. In addition, each of these isolates was tested for its ability to decompose and utilize L-methionine as the only source of nitrogen in medium using agar plates containing modified starch-agar medium.

2.2. Media

The following media were used throughout this study. *Starch-nitrate medium [23]: It has the following ingredients (g/L): starch, 20; KNO_3, 2; NaCl, 0.5; K_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.001 and $CaCO_3$, 3. **Modified starch medium: It has the same contents of starch-nitrate medium except adding L-methionine instead of potassium nitrate in medium in such amount that the final concentration of nitrogen (N-base) in the medium remained unchanged. Each of these media was solidified by adding 20 g/L of agar when needed.

2.3. Rapid assay plate method (qualitative assay test)

All grown isolates on modified starch agar medium were picked up and streaked on the modified starch agar plates amended with phenol red at a final 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 as recommended by William and Hariharan [44].

2.4. L-Methioninase assay (quantitative assay test)

L-Methioninase activity was assayed according to the method of Arifi et al. [2], Selim et al. [36] using L-methionine as a substrate. Methanethiol (MTL) produced from substrate reacted with 5,5 dithio-bis-2-nitrobenzoicacid added (DTNB; Sigma–Aldrich) to form thionitrobenzoic acid which was detected spectrophotometrically at 412 nm. The assay mixture contained 20 mM of L-methionine in 0.05 M potassium phosphate buffer pH 7.0, 0.01 mM pyridoxal phosphate (PLP; Sigma–Aldrich), 0.25 mM DTNB and the filtrate or supernatant in a final volume of 1 ml. After 1 h of incubation at 30 °C, the increase in absorbance of the developing yellow color was measured at 412 nm. Controls without filtrate or with heat denaturated filtrate (at 95 °C for 30 min) were prepared separately. Amount of MTL released was calculated according to a standard curve obtained with sodium methane thiolate. One unit (U) of L-methioninase was expressed as the amount of enzyme that releases 1μmole of methanethiol per minute under optimal assay conditions.

2.5. Protein determination

Protein concentration was determined according to the method of Bradford [5] with bovine serum albumin as standard.

2.6. L-Methionine determination

The method of McCarthy and Sullivan [24] was applied.

2.7. Biomass determination

After the fermentation process, the culture was centrifuged at 5500 rpm for 10 min under cooling. The biomass was expressed as gram (wet weight) per flask of fermentation media.

2.8. Identification of the chosen isolates

The most promising isolates were identified by 16S rRNA sequencing data collection. A database containing16S rRNA

gene sequences of all validly published filamentous actinomycetes [18] was compiled from Gene Bank (http://www.ncbi.nlm.nih.gov). All sequences used were longer than 1400 bp. The sequences were grouped by genus according to [10]. Actinomycete strain was grown in 10 ml International *Streptomyces* Project Medium 1 (ISP1) [34] with agitation at 30 °C for 18–24 h and examined by gram stain. Cells (4 ml) were harvested by centrifugation (7500*g* for 2 min), washed once with 500 ml of 10 mM Tris–HCl/1 mM EDTA (TE) buffer (pH 7.7) and resuspended in TE buffer (pH 7.7). The samples were heated in boiling water for 10 min allowed to cool for 5 min and centrifuged (7500*g* for 3 min.). The supernatant was transferred to a clean tube and stored at 4 °C. If melanin or other pigments were produced during growth, cultures were grown in Middle brook 7H9 broth, as these pigments interfered with the PCR.

2.9. Molecular characterization

Chromosomal DNA was extracted with Qiagen kit according to the manufacturer's instruction. 16S rRNA gene was amplified using universal primers F (5′-GTGCCAGCAGCCGCG GTA-3′) and R (5′-TTGTAGCACGTGTGTAGCCC-3′((Ma nfred Kröger, Institute of Microbiology and molecular biology, University of Gießenbei). PCR reaction was achieved in a volume of 50 µl containing 1× green Taq PCR Buffer, 200 mM of each dNTPs, 100 mg BSA, 10 pmole of each primer, 2.5 U of Taq DNA polymerase (Sigma) and 10 ng of DNA.

PCR was achieved by the following conditions: 1 min at 98 °C followed by 35 cycles of 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The PCR product of 16S rRNA was purified (Qiagen kit) and sequenced in Promega company laboratory (Cairo, Egypt). The 16S rRNA sequence was matched with previously published 16S rRNA sequences of bacteria in the NCBI databases using BLAST. Selected sequences of other microorganisms with the greatest similarity to the 16S rRNA sequences of the bacterial isolate were extracted from the nucleotide sequence databases and aligned generating the phylogenetic tree. The 16S rRNA gene sequences of the bacterial isolate which was reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers: LC021308 and LC021309 (Streptomyces sp.). Isolates were called *Streptomyces* sp. DMMMH 4 and *Streptomyces* sp. MDMMH 60.

2.10. Transmission electron microscopy

Electron micrographs of spores were done using transmission electron microscopy, Taking samples using copper grade coated carbon by touching on a JEOL electron microscope (JEM-2100).

2.11. Enzyme release treatments

In order to search for any amount of L-methioninase intracellularly produced premeabilization treatments including chemical or physical methods were applied according to [36,11,9].

2.11.1. Chemical treatments

It was accomplished by treating four day old cells of *Streptomyces* isolates with some solvents. The solvents cell mixture was diluted with 0.1 M potassium phosphate buffer pH 7 and shacked for different times (from 1 to 4 h). The cells were removed by centrifugation and the supernatant was used for L-methioninase determination.

2.11.2. Physical treatments

1- Freezing and thawing method:

Harvested cells were resuspended in 0.1 M potassium phosphate buffer pH 7. The suspension was frozen and rethawed for many cycles. L-methioninase released was determined periodically in the clear supernatant.

2- Grinding with sea sand:

Harvested cells were washed with 0.1 M potassium phosphate buffer pH 7. Cells were resuspended in the same buffer containing PLP as a stabilizing agent as recorded by Ferchichi et al. [12]. This suspension was ground with sterile sea sand in a mortar under cooling. L-methioninase activity was estimated in the clear supernatant.

3. Results and discussion

60 *Streptomyces* isolates were screened for their ability to grow on a modified starch agar medium. The use of L-methionine as a source of nitrogen in medium for the production of L-methioninase by many microorganisms including fungi, yeast or bacteria was reported by many authors, on fungi [19]; on yeast [36] and on bacteria [44,12]. Results show that only 40 isolates could grow and utilize L-methionine as the only source of nitrogen in medium. According to [21] these forty isolates were screened for their ability to grow on agar plate medium containing 0.5% L-methionine as the only source of nitrogen and carbon. Results obtained (data not shown) showed a notable growth and was noticed by 24 of these isolates. Accordingly, these 24 isolates were used for further studies.

3.1. Qualitative and quantitative assay test

These 24 *Streptomyces* isolates were cultivated on modified starch agar plates amended with phenol red (0.07%) (qualitative test). (Table 1) clearly shows only 18 isolates were characterized by the presence of pink color around their colonial growth as evidence for the production of extracellular L-methioninase by these isolates. This phenomenon is not in agreement with that reported by William and Hariharan [44] on *Serratia marcescens* who mentioned that L-methioninase producers were identified as evidenced by the pink color of the colonies (i.e. intracellular).

Furthermore, we can notice from the data illustrated in (Table 1) that the rate of pink color intensity around colonial growth was dependent on the rate of cultivation period (per days) and also on the rate of L-methioninase formation by each isolate tested as shown in (Table 2).

Data illustrated in (Table 2) show the growth rate, protein estimation, enzyme formation, specific activity of the enzyme and L-methionine uptake from medium during different cultivation periods (i.e. 3; 5 and 7 days of growth with shaking 150 rpm at 28 °C). From the data, we can conclude that the

Table 1 Rapid plate assay test (qualitative assay test).

Isolate No.	Color intensity*	Isolate No.	Color intensity*	Isolate No.	Color intensity*	Isolate No.	Color intensity*
1	+ + +	10	–	18	–	33	+
3	+ +	11	–	20	+ +	35	+
4	+ + +	12	+ +	22	+	44	+ +
5	–	13	+	27	+ +	49	–
7	+	16	–	28	+ +	56	+ +
9	+ +	17	+	32	+ + +	60	+ + +

* Pink color.

growth rate was so varied and enzyme formation was highly different and not correlated with each growth or methionine uptake. On the other hand, isolates No. (5, 10, 11, 16, 18 and 49) show the inability to form the pink color around their colonial growth. When assay treatments were carried out on these six isolates, data illustrated in (Table 2) show a notable growth with the inability to produce L-methioninase by all these six isolates. However a notable L-methionine uptake by these isolates was estimated. This phenomenon may be related to that reported by Misono and H. [26], Hagino and Nakayama [14] who mentioned the occurrence of L-methionine decarboxylase (E.C.4.1.1.57) in strains of *Streptomyces* sp. which catalyzes the decarboxylation of L-methionine to form 3-methylthiopropylamine and carbon dioxide which is the oxidative decarboxylation product of L-methionine. Furthermore, data illustrated from (Table 2) clearly show that, the highest enzyme production was obtained after five days of incubation with shaking (150 rpm) at 28 °C thereafter, a slight decrease of enzyme was noticed. In addition, the highest production of L-methioninase was noticed by isolates No. (4 & 60) followed by No. (9, 28, 32, 12 and 3) respectively, although, the growth rate was so varied and not correlated with the rate of enzyme formation. On the other hand, the specific activity was highly different. In addition, results in (Table 2) also show that the productivity of L-methioninase by different *Streptomyces* isolates was not directly proportional to the rate of L-methionine uptake and also with the growth biomass of different isolates. These results are not in agreement with that reported by Khalaf and El-Sayed [19] on the formation of L-methioninase by different fungal strains.

3.2. Permeabilization treatment

Permeabilization treatments including physical and chemical methods clearly indicate the failure of these treatments to prove the presence of intracellular enzyme inside the cells. Accordingly, we can conclude that enzyme formation by all isolates tested was only extracellularly produced.

On the other hand, results also show that isolates (No. 5, 10, 11, 16, 18 and 49) were characterized by its lowest level of L-methioninase formation ranging from zero U/ml isolates (No. 5, 11, 16 and 18) and 2 U/ml by isolates (No. 10 & 49).

According to the data mentioned before, isolates (No. 4 and 60) which were characterized by the highest production of these enzymes (65 & 86 U/ml respectively) and with the highest specific activity (38.9 and 40.9 U/mg respectively) were chosen for further studies.

3.3. Identifications studies

3.3.1. 16S rRNA analysis of Streptomyces sp. LC021308 and LC021309

16S rRNA encoding gene of *Streptomyces* LC021308 and LC021309 was PCR-amplified and sequenced for further characterization. The nucleotide sequence was compared to existing sequences in the databases. A dendrogram showing the result of 16S rRNA analysis is shown in Figs. 1 and 2. The results showed the highest matching of the bacterial isolate to members of the *Streptomyces* group. As presented, the 16S rRNA sequence of the bacterial isolate is the greatest closely associated to *Streptomyces* sp. with a similarity of 99%. This result was identical with the conclusion of the cultural, morphological, biochemical and physiological characterization. This result revealed that the isolate of two *Streptomyces* sp. is a new strain.

The partial 16S rRNA sequence followed by the construction of a phylogenetic tree by the neighbor joining method has revealed that the isolates S4 and S60 were *Streptomyces* sp. The 16S rRNA sequences for *Streptomyces* sp. and the relevant sequences were downloaded and phylogenetic analysis has been carried out with a similarity of 99% with the same species found in gene bank. In laboratories in developed countries, a partial 16S rDNA sequence of a new *Streptomyces* isolate can be obtained quickly and at low cost to give an unambiguous identification of the genus to which the isolate belongs. This is certainly not the case in developing countries, such as South Africa, where high sequencing costs and possible restricted access to sequencing facilities preclude the use of 16S rDNA sequencing as a routine genus-identification tool. PCR-based methods have provided a rapid and accurate way to identify bacteria [28,29,41,3,31,32,7,27]. In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful [6,45]. ARDRA has been shown to be useful in differentiating between bacterial species within a genus, for example, *Clostridium* [28], and in differentiating bacterial strains within a species, for example, *Lactococcus* [29]. It has also been shown to be useful in identifying several medically important species of aerobic actinomycetes belonging to the genera *Actinomadura, Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces* and *Tsukamurella* [45,25]. Small rRNA gene sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. First, the construction of a universal phylogenetic tree classifies organisms into three domains of life: bacteria, Archaea, and Eucarya [20].Second, it revolutionizes the classification of microorganisms, and

Table 2 Quantitative screening of different *Streptomyces* isolates for L-methioninase formation.

Isolate No.	Incubation period/days after																	
	3						5						7					
	Final pH	Growth* wet weight	Protein (mg/ml)	Enzyme formation (unit/ml)	Specific activity (unit/mg)	Methionine** uptake (%)	Final pH	Growth* wet weight	Protein (mg/ml)	Enzyme formation (unit/ml)	Specific activity (unit/mg)	Methionine** uptake (%)	Final pH	Growth* wet weight	Protein (mg/ml)	Enzyme formation (unit/ml)	Specific activity (unit/mg)	Methionine uptake (%)**
1	7.3	1.4	3.1	15	4.8	61	7.8	1.5	3.6	41	11.3	81	8	1.6	4.1	42	10.2	92
3	**7**	**1.1**	**1.530**	**7**	**4.6**	**35**	**6.8**	**1.2**	**1.521**	**19**	**12**	**49**	**6.7**	**1.6**	**2.7**	**37**	**13.7**	**53**
4	**7**	**0.9**	**0.890**	**31**	**34.8**	**42**	**7.2**	**1.1**	**1.670**	**65**	**38.9**	**60**	**7.2**	**1.1**	**1.850**	**63**	**34**	**70**
5	7	0.3	0.112	0	0	14	7.4	0.8	0.557	0	0	29	7.4	1	0.578	0	0	44
7	6.8	0.7	0.400	2	5	20	6.6	1.3	0.908	7	7.7	44	6.5	2	1.880	13	6.9	68
9	**6.9**	**0.7**	**0.322**	**5**	**15.5**	**37**	**6.6**	**1.2**	**2.1**	**42**	**20**	**41**	**6.8**	**2**	**2.4**	**71**	**29.5**	**52**
10	7	0.4	0.080	0	0	8	7	0.7	0.410	2	4.8	24	7	0.8	0.900	2	2.2	26
11	6.8	0.8	2	0	0	25	6.8	1.1	2.8	0	0	41	7	1.1	3	0	0	48
12	7	**1.2**	**0.780**	**8**	**10.2**	**18**	**7.2**	**2**	**1.120**	**15**	**13.3**	**31**	**7.2**	**1.8**	**1**	**14**	**14**	**49**
13	6.5	1.7	2	18	6	24	6.2	2.7	3.4	24	7	50	6.2	3.6	5	33	6.6	88
16	7	0.2	0.057	0	0	27	7	0.6	0.099	0	0	27	7	0.6	0.100	0	0	47
17	7	0.5	0.279	4	14	18	7.7	1.6	1.8	14	7.7	58	8	1.6	1.5	11	7.3	67
18	6.8	0.7	0.250	0	0	42	6.1	1	0.400	0	0	57	5.7	1.5	0.418	0	0	58
20	7	1	0.783	0	0	14	7	1.5	2.4	6	2.5	48	6.7	0.9	2	0	0	53
22	7.2	1	0.9	17	18.8	67	7.4	1.8	1.4	12	8.5	70	7.4	2	2.9	10	3.4	85
27	7	0.4	0.027	0	0	11	7	1	0.804	4	4.9	49	7	1	0.947	4	4.2	55
28	**6.8**	**1.1**	**0.889**	**16**	**17.9**	**40**	**6.8**	**1.7**	**2.7**	**52**	**19.2**	**76**	**6.4**	**2.1**	**3**	**89**	**29.6**	**80**
32	**7**	**0.6**	**1.774**	**2.3**	**1.05**	**44**	**6.7**	**1.3**	**3.9**	**68**	**17.4**	**78**	**6.4**	**1.4**	**3.9**	**67**	**17.1**	**78**
33	7.1	0.6	0.527	10	18.9	60	7.6	1.4	1.4	11	7.8	71	7.5	1.2	1.2	6	5	74
35	6.5	1	2.14	0	0	20	6.3	2.1	2.8	0	0	50	6.3	2	3.4	0	0	64
44	6.9	0.7	0.827	6	7.2	35	6.9	1.8	3	10	3.3	60	6.7	1.8	3.7	9	2.4	67
49	7	0.8	0.114	7	61	62	7	0.9	0.311	2	6.4	74	7	0.8	0.700	2	2.8	78
56	7	1	2.1	0	0	50	7.5	1.8	3.4	0	0	75	7.5	2.6	5	0	0	90
60	**7**	**0.9**	**1.224**	**48**	**39.2**	**32**	**7.2**	**1.2**	**2.1**	**86**	**40.9**	**46**	**7.2**	**1.6**	**2.2**	**86**	**39**	**55**

* Growth (wet weight) was expressed as gram per flask containing 30 ml of fermentation medium.

** Methionine uptake % was expressed as the amount of consumed methionine/initial methionine concentration × 100.

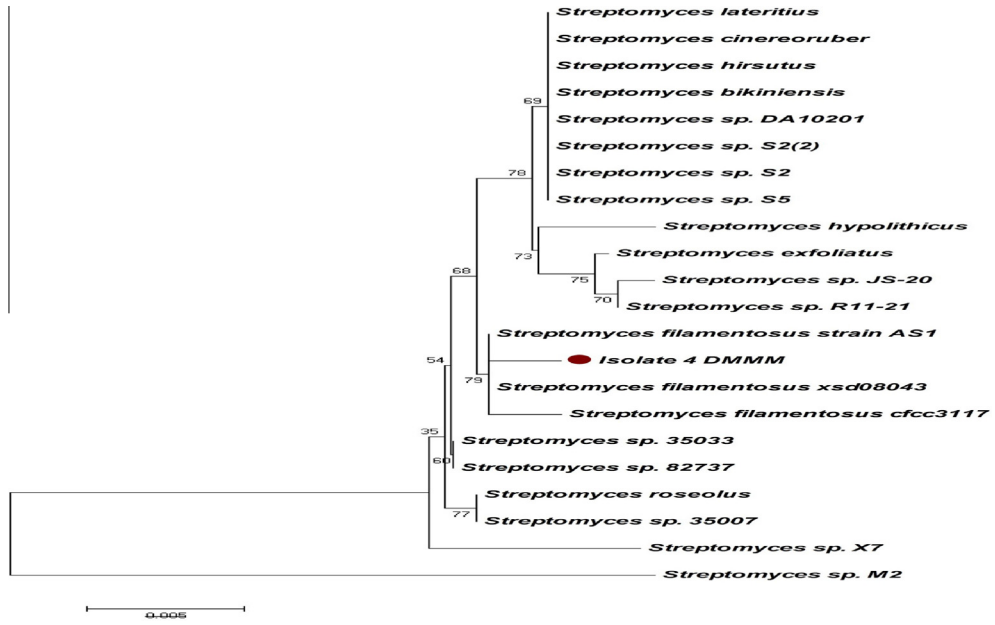

Figure 1 Distance neighbor-joining phylogenetic tree of Egyptian isolate No. 4.

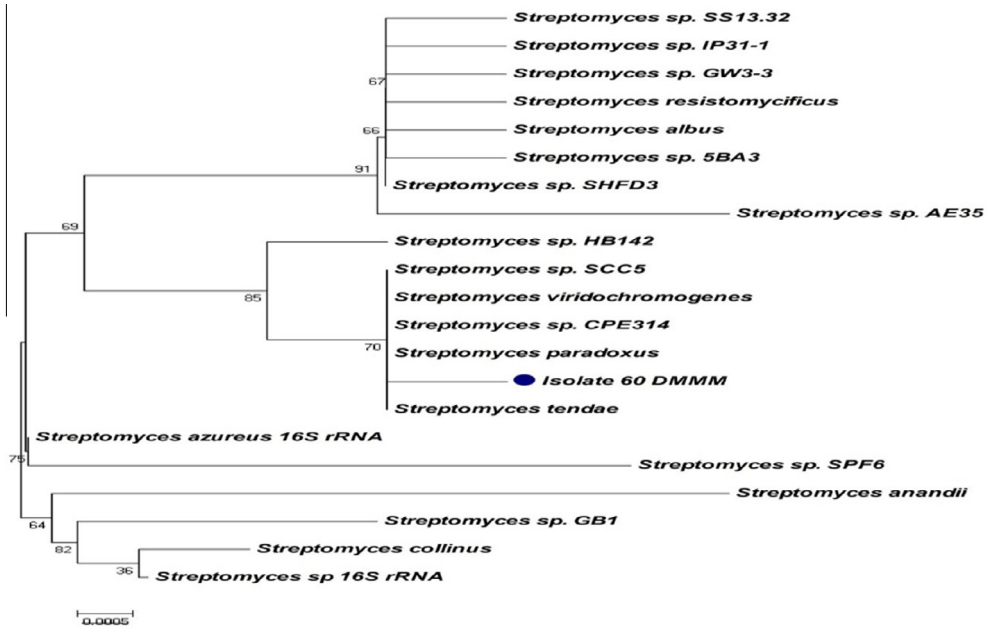

Figure 2 Distance neighbor-joining phylogenetic tree of Egyptian isolate No. 60.

makes the classification of non-cultivable microorganisms possible [16]. Third, it helps to elucidate the relation of unknown bacterial species to known ones. 16S rRNA gene sequencing will continue to be the gold standard for the identification of bacteria, and the automation of the technique could enable it to be used routinely in clinical microbiology laboratories, as a replacement of the traditional phenotypic tests. Modern technologies have made it possible to construct a high density of oligonucleotide arrays on a chip with oligonucleotides representing the 16S rRNA gene sequence of various bacteria. Such a design will facilitate automation of the annealing and detection of the PCR products of 16S rRNA gene amplification, and hence routine identification of most clinical isolates will be possible [46]. The use of 16S rRNA gene sequencing has several advantages. First this study illustrates that the primer design will be useful to identify many bacterial genera.

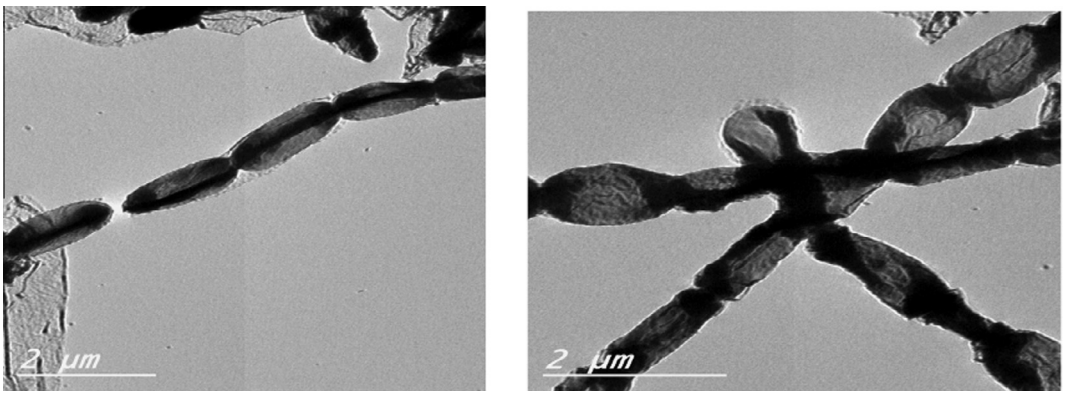

Figure 3 TEM of *Streptomyces* isolate No. 4 (straight or rectus spores).

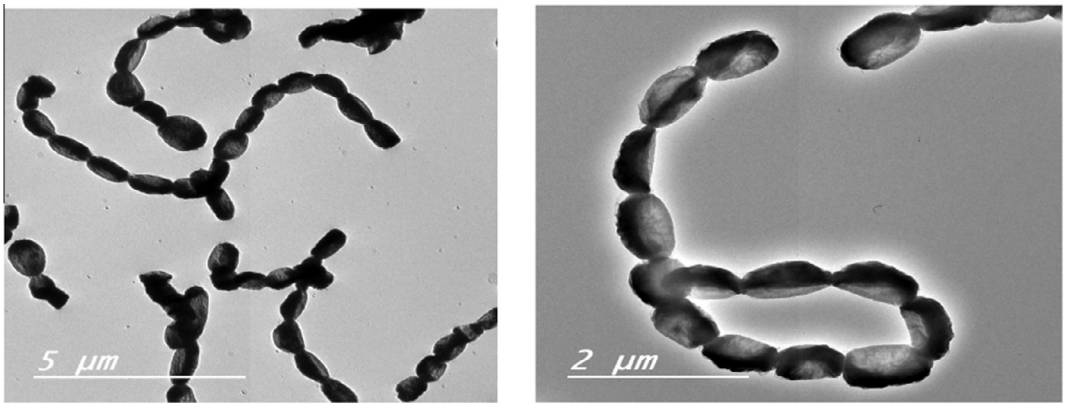

Figure 4 TEM of *Streptomyces* isolate No. 60 (spiral spores).

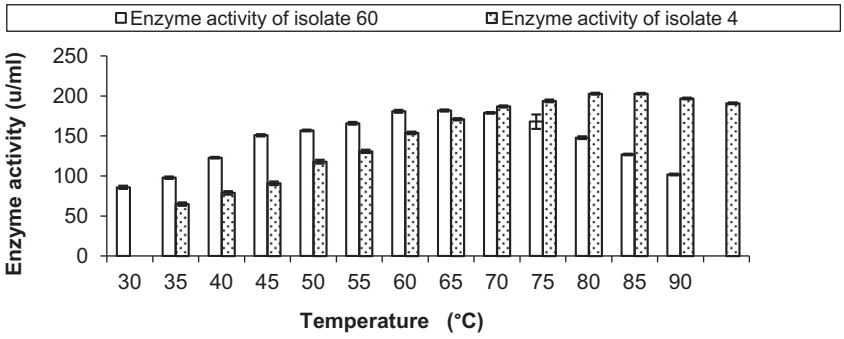

Figure 5 Effect of incubation temperature on the crude L-methioninase.

Transmission by electron microscope (TEM): (see Figs. 3 and 4)

3.4. Some properties of the crude L-methioninase produced by the two identified Streptomyces

Crude enzyme of each isolate was subjected to various experiments in order to, study their activities response which may dependence on their taxonomical criteria and at the same time to optimize their assay conditions needed in L-methioninase production experiments which will be carried out after. Therefore, the following experiments were applied.

3.4.1. Effect of incubation temperature on the crude L-methioninase

Data (Fig. 5) clearly show that (80 °C and 65 °C) were optimal for L-methioninase activity produced by strains No. 4 and No. 60 respectively followed by a slight decrease when higher temperatures were applied. Optimum temperature for L-methioninase produced by several organisms was also reported by many authors. [30] used 25 °C for *Brevibacterium linens*, [22] used 37 °C on *Aspergillus Flavipes* and [36] reported that 45 °C was optimum for L-methioninase activity produced by *Candida tropicalis,* while 37 °C was the optimum temperature for L-methioninase obtained from *Pseudomonas putida* [22].

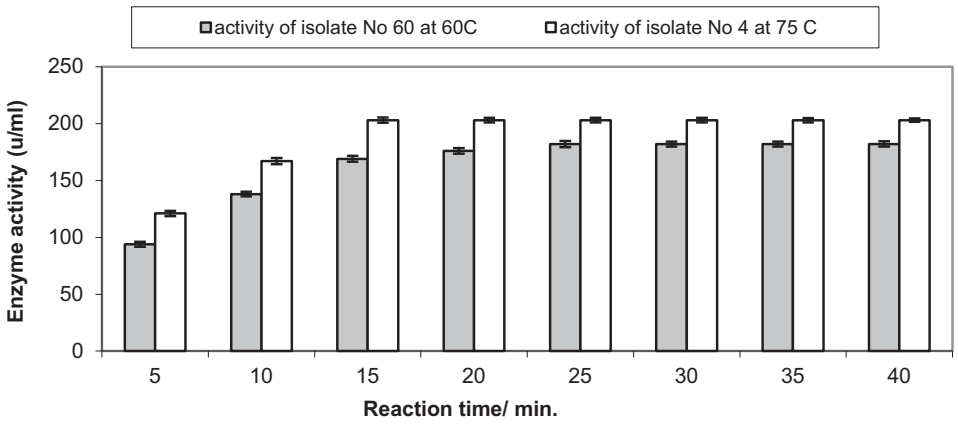

Figure 6 Effect of reaction time on the crude L-methioninase.

Table 3 Effect of pH on the crude L-methioninase.

pH	Buffer	Strain No. 4	Strain No. 60	pH	Buffer	Strain No. 4	Strain No. 60
3.5	A	0	0	6.5	B	182	182
4	A	0	0	7	B	203	182
4.5	A	5	0	7.5	B	194	193
5	A	10	4	8	B	179	184
5.5	A	21	8	8.5	C	108	57
	B	67	78	9	C	8	0
6	B	108	166	9.5	C	0	0

Where: A – citric acid buffer. B – potassium phosphate buffer. C – carbonate buffer.

3.4.2. Effect of reaction time on crude L-methioninase

The progress of enzymatic reaction with time was studied at their optimum conditions. Data illustrated in (Fig. 6) showed the rate of methanethiol released from the hydrolysis of L-methionine. From the results we can conclude that the rate of L-methionine hydrolysis was linear with time up to 15 min for strain No. 4 and 25 min for strain No. 60 thereafter no more hydrolysis was observed. In comparison, L-methioninase obtained from some cheese ripening yeast was reported to be optimally active for 30 min [2] and 10 min for L-methioninase activity for *Candida tropicalis* [36].

3.4.3. Effect of pH on the crude L-methioninase

Crude L-methioninase activity was assayed under various pHs adjusted by using 0.05 M of different buffers (i.e. sodium citrate, potassium phosphate or carbonate buffer) under optimum conditions required for each enzyme produced by the two strains. Data (Table 3) indicates that a notable increase was noticed with the increase of pH reaching its maximum value when pH.7 and pH.7.5 was applied for strain No. 4 and No. 60.respectively. In addition, potassium phosphate buffer proved to be the most suitable for enzyme activity of both strains. Furthermore, the effect of using potassium phosphate buffer with various molarities (0.025–0.4 M) on enzyme activity was also estimated (Fig. 7). Data indicate that 0.15 M and 0.05 M were the best molarities used for enzyme activity produced by strains No. 4 and No. 60 respectively.

As reported by some authors pH 7.5 adjusted by using 0.1 M potassium phosphate buffer was optimum for L-methioninase activity of some cheese lactic acid bacteria [15]. In addition, pH 6.5 adjusted using 0.075 M of sodium citrate buffer was optimal for L-methioninase produced by *Candida tropicalis* [36]. On the other hand, alkaline pH values were reported as the optimum pH for L-methioninase obtained from other yeast isolates [2,37].

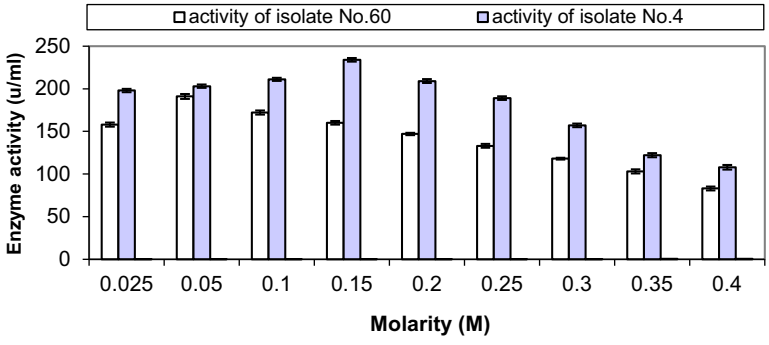

Figure 7 Effect of molarity on the crude L-methioninase.

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