Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(4):1290-1307

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Partial characterization of L-asparaginase-producing by *Streptomyces* sp. SAH1_CWMSG isolated from Rizosphere soil in Egypt

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ABSTRACT

Fifty Streptomyces isolates were collected and screened for their capability for L-asparaginase (L-ASNase) activity. The SAH1_CWMSG isolate was found to be a promising L-ASNase producer. The SAH1_CWMSG strain possesses phenotypic characteristics typical of Streptomyces. Further phylogenetic analysis of the 16S rRNA gene sequence confirmed (99%) similarity with Streptomyces ghanensis. The strain was named Streptomyces ghanensis SAH1_CWMSG (Gen Bank ID: KT953341).The crude enzyme showed an antifungal activity against common fungal strains. In vitro, anticancer evaluation revealed that the pure enzyme exerted its action in HepG2 and MCF-7 cancer cell lines and showed promising anticancer activity compared to doxorubicin. SAH1_CWMSG strain produced L-ASNase activity of 13 IU under submerged fermentation by shake flask. The maximal activity of 106.90 IU was obtained using ammonium sulfate as nitrogen source at 28°C and 120 h on 200 rpm. The purified enzyme has K_m and V_{max} of 27 mMol and 69 IU/mL, respectively, with molecular weight of 38 kDa by SDS-PAGE. This is the first report on the production and characterization of L-ASNase by the SAH1_CWMSG strain. These results provided additional information on L-ASNase production and its application as an anticancer agent.

Keywords: *Streptomyces ghanensis* SAH1_CWMSG, Antimicrobial and anticancer activity, Phenotypic and phylogenic identification, Asparaginase activity, characterization, production.

INTRODUCTION

The previous studies have been affirmed that extensive variety commercial agricultural foods arranged by warming medications contain high levels of acrylamide, a potential human carcinogen [1]. Thusly, it is imperative to lessen the acrylamide substance to moderate levels, which could relate to an unimportant malignancy hazard in these items [2]. A pretreatment of potato cuts and bread batter with ASNase before fricasseeing or heating counteracts acrylamide development [3]. L-ASNase (L-asparagine amido hydrolase E.C.3.5.1.1) can specifically lessen the level of free L-asparagine by hydrolyzing it to L-aspartic and ammonia, there by particularly expelling one of the vital acrylamide precursors.

Asparagine is a nutritional requirement of both normal and cancer cells. Normal cells produce enzyme asparagine synthetase, which is able to synthesize asparagine from aspartic acid, whereas, in cancer and tumor cells this enzyme is present in low levels [4]. L-ASNase has likewise been utilized for making an indicative biosensor, as the measure of ammonia released by the activity of the enzyme specifically associates to the level of L-asparagine in a patient's blood [5].

The powerful utilization of L-ASNase for acrylamide moderation has been shown utilizing L-ASNase II from Escherichia (E.) coli, which was initially created as a pharmaceutical and chemotherapeutic operators in the treatment of intense lymphoblastic leukemia for more than 30 years [6].

Several groups of microorganisms, for example, bacteria, fungus, yeast, *Streptomyces* and green algae have been ended up being capable wellsprings of L-ASNase for restorative, convenient clinical use and industrialized applications. In spite of the fact that they were confined from plant and some animal sources, microorganisms are a superior wellspring of L-ASNase in light of the accompanying favorable circumstances: bulkiness production aptitude and economic factors. Microorganisms are effortless to influence to achieve enzymes with preferred character; it is simple to extract and purify the enzyme [7].

Streptomyces strain remains a focus of systematic research because it is still a rich source of commercially significant compounds such as antibiotics, enzymes, enzyme inhibitors, antitumor, antibacterial, antifungal, antioxidant, antiviral and pharmacologically active agents [8]. They are recognized as a comparatively less-explored source for L-ASNase and therefore act as candidates for the production of L-ASNase [9]. Several Streptomyces species such as *S. karnatakensis, S. venezualae, S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-ASNase production [10-11]. The ASNase yield from these *Streptomyces* strains varied depending on the producer strains, culture and cultivation conditions [12-13].

The aim of the present study was to isolate and fully identify the most active L-ASNase producing *Streptomyces* strain. Further analysis deals with the optimization of different parameters for L-ASNase production. The molecular weight of the purified enzyme was measured and the activity of the purified enzyme as antitumor agent in vitro and antimicrobial activity was determined.

EXPERIMENTAL SECTION

Isolation and Screening for L-ASNase

A total of five soil samples were collected from different sites of wheat plants at Mashtool El Sook, El Sharkia Governorate, Egypt. 100µL from each serial dilution up to 10^{-7} were taken and spread on starch nitrate agar (SNA) plates and incubated at 28°C for 5-7 days [14]. After incubation, the individual *Streptomyces* colonies were picked out and sub-cultured into SNA plates. Then the pure colonies were maintained in ISP2 agar slant and kept at 4°C until further use.

Microorganisms isolated from soil samples were screened for L-ASNase activities on Asparagine Dextrose Agar (ADA) medium containing 0.009%w/v of phenol red. The color change of the medium from yellow to pink is an indication of the extracellular L-ASNase activity [15].

Biological activities of culture broth

Antimicrobial spectrum

The strains and media used

Bacillus subtilis (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 7839) and *Pseudomonas aeruginosa* (ATCC 9027) were used as bacterial test strains. The bacteria were slanted on nutrient agar (Merck, Darmstadt, Germany). On the other hand, *Candida albicans* (ATCC 10231), *Saccharomyces cervecia* (ATCC 9763), *Aspergillus niger* (ATCC 16404), *Fusarium oxyspoium* (ATCC 62506), *Fusarium solani* (ATCC 36031), and *Alternaria* sp. (ATCC 20084) were used as antifungal test strains. They were slanted and mentioned on the potato Dextrose Agar medium (Lab M Limited, Bury, Lancashire, UK). Mueller-Hinton agar (Lab M., Bury, Lancashire, UK), following the manufacturer's instructions, was used for the bioassay.

Antimicrobial Bioassay– well diffusion method

The antibacterial screening was essentially by the agar well diffusion method described by Jorgensen and Turnidge [16], with some modification. The test bacterial and fungal cultures were inoculated into freshly prepared Mueller-Hinton agar plates (bacterial strains) and SDA plates (fungal and yeast strains). Then the wells of 9 mm were made and loaded with 100 µL of cell free culture supernatant. The plates containing bacterial and fungal strains were incubated at 37ºC for 24 hrs and 25ºC for 48 hrs, respectively. After incubation, the zone of inhibition was measured and expressed as millimeter in diameter.

Disk diffusion susceptibility test

The disk diffusion susceptibility method was carried out according to Jorgensen and Turnidge [16]. The test was performed by applying SAH1-CWMSG-strain inoculum to a starch nitrate agar plate. Six standard antibiotic disks e.g. Tetracycline, Neomycin, Oxytetracycline, Vancomycin, Rifamycin and Streptomycin were placed on the inoculated agar surface. Plates were incubated for 24-48 hrs at 28°C prior to measurement of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter.

Anticancer activity
Cell lines and culturing

Breast MCF-7, liver HepG2 and lung A549 cancer cell lines as well as the normal cell line (human normal melanocyte, HFB4) were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 $^{\circ}$C in a humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 mL of complete culture medium.

In Vitro anticancer assay

The anticancer activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [17]. Cells were inoculated in 96-well micro-titer plate (10^4 cells/ well) for 24 h before treatment with the tested compound to allow attachment of the cell to the wall of the plate. The tested pure enzyme was dissolved in DMSO at 1 mg/mL immediately before use and diluted to the appropriate volume just before addition to the cell culture. Different concentrations of tested pure enzyme and doxorubicin were added to the cells. Triplicate wells were prepared for each individual dose. The cells were incubated with the pure enzyme for 48 h at 37°C and in an atmosphere of 5% CO_2. After 48 h the cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and the attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated. The results were compared to the ant-proliferative effects of the reference control doxorubicin.

Strain identification
Phenotypic identification

The characterization of the SAH1_CWMSG strain followed the guidelines adopted by the International *Streptomyces* Project (ISP) [18]. The melanin pigment production, nitrate reduction, utilization of C and N sources, and the culture characteristics were studied. Colors were assessed and diaminopimelic acid isomers in the cell-wall were analyzed. The electron microscope study was carried out using a scanning electron microscope (JEOL JSM 5300, JEOL Techniques Ltd., Japan). The growth of the SAH1_CWMSG strain at different temperatures (26, 28, 32, 37, 40, 45 and 50°C) and salinity (0, 2, 4, 7, 10 and 13 % NaCl) was studied by using the ISP 2 medium. All the plates were incubated for 7-14 days.

Genotypic Identification
DNA isolation and PCR amplification

An overnight culture of the SAH1_CWMSG strain grown at 28 ºC was used for the preparation of genomic DNA. DNA extraction was done by using the protocol of the Gene JET genomic DNA purification Kit (Thermo K0721) as following the manufacturer of the kit. The PCR amplification of the 16S rDNA region was carried out following the manufacture of the Maxima Hot Start PCR Master Mix (Thermo K1051). The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16S rDNA region. The domain bacteria-specific primer 27F (forward primer) was 5'AGAGTTTGATCMTGGCTCAG3' and the universal bacterial primer 1492R (reverse primer) was 5'TACGGYTACCTTGTTACGACTT3' [19].

The PCR reaction was performed with 5µl of genomic DNA as the template, 1µl of 16S rRNA Forward primer, 1µl of 16S rRNA reverse primer, 18 µl Water, nuclease-free and 25 µl Maxima® Hot Start PCR Master Mix (2X) in a 50µl reaction mixture as follows: activation of 2 Taq polymerase at 95 °C for 2minutes, 35 cycles of 95 °C for 1minute, and 65°C and 72 °C for 1minute each were performed, finishing with a 10-minute step at 72 °C. After completion, the PCR products were electrophoresed on 1 % agarose gels, containing ethidium bromide (10mg m1/l), to ensure that a fragment of the correct size had been amplified.

DNA sequencing, phylogenic analysis and tree construction

The amplification products were purified with the K0701 GeneJET™ PCR Purification Kit (Thermo**).** Afterward, the samples become ready for sequencing in an ABI Prism 3730XL DNA sequencer and analysis on GATC Company. Sequencing reaction was performed with the primers 518F 5' (CCA GCA GCC GCG GTA ATA CG) 3' and 800R 5' (TAC CAG GGT ATC TAA TCC) 3' using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Diformamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence alignment was prepared with DNASTAR software programs (DNASTAR. INC., Madison, Wis.).

Phylogenetic data were obtained by aligning the nucleotides of different 16S RNA retrieved from the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. The classifier was trained on the new phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) proposed by Wang *et al.* [20], (http://rdp.cme.msu.edu/classifier/classifier.jsp). Phylogenetic and molecular evolutionary analyses were conducted using MEGA6. A rooted phylogram was derived from the distance matrices using the neighbor-joining method through the Molecular Evolution Genetics Analysis (MEGA6) software version [21]. All analyses were performed on a bootstrapped data set containing 1000 replicates (generated by the program).

Optimization of cultural and environmental conditions

L-ASNase production media and cultivation conditions

Six different types of broth media were used in this study for primary evaluation of their L- ASNase activity. The compositions of these media were as follows, with some modification: Medium1 composed of (g/L): starch, 20; KNO_3, 2; asparagine,10 ; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4$, 0.7; NaCl, 0.5; $FeSO_4$, 0.1; CaCO3. Medium 2 composed of (g/L): soluble starch 50; polypeptone, 10; beef, 0.5; NaCl, 0.5. Medium 3 composed of (g/L): Sucrose, 30; polypeptone, 5; beef, 5; yeast extract, 2; malt extract, 2; K_2HPO_4, 1.5; KH_2PO_4, 5; $MgSO_4$, 0.5. Medium 4 composed of (g/L): Glucose, 3; NaH_2PO_4, 6; KH_2PO_4, 3; NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 0.015; asparagine, 3. Medium 5 composed of (g/L): Dextrose, 2; starch, 20; Asparagine, 14; KNO_3, 2; yeast extract, 2; KH_2PO_4,2; $MgSO_4$, 0.1; NaCl, 0.1; $FeSO_4$, 0.01. Medium 6 composed of (%): 1glycerol, 1 Asparagine, 0.1 K_2HPO_4, 0.1 trace salt solution (0.01% $FeSO_4.7H_2O$, 0.01% $MnCl_2.4H_2O$, and 0.01% $ZnSO_4.7H_2O$). All the nitrogen sources in these media were omitted and replaced by 1 % L-Asparagine.

For all media used, the pH was adjusted to 7.0 before sterilization. The carbon source and amino acid were sterilized separately and added to the fermentation medium before inoculation. Fifty mL of these liquid media were dispensed into each 250 mL Erlenmeyer flask and autoclaved at 121 °C for 20 min. The flasks were inoculated in duplicates

with 5% of the vegetative cells from a seven-day-old culture. The inoculated flasks were kept at 28 °C on a rotary shaker (New Brunswick Scientific Co., NJ, USA) at 200 rpm for 120 h. The un-inoculated fermentation medium was used as a negative control during the experiment. The contents of each flask were harvested by centrifugation at 8000 rpm for 10 min and the supernatant was analyzed for enzyme activity and cell growth.

Effect of different nitrogen sources on L-ASNase production

Correspondingly, various organic and inorganic nitrogen sources were investigated for their effect on growth and L-ASNase production. The organic nitrogen sources were yeast extract, peptone extract, beef extract, malt extract, soy bean and soluble casein. While inorganic nitrogen sources included KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2H_2PO_4$, $(NH_4)_2HPO_4$ and $(NH_4)_3PO_4$, all having constant nitrogen content equivalent to Asparagine 1% (w/v). The respective nitrogen sources were inoculated as sole nitrogen sources in the medium. The culture media after inculcation were incubated at 200 rpm in a rotary shaker on 28°C for 120h. The respective cell growth and L-ASNase activity were determined.

Effect of different carbon sources on ASNase production

Various carbon sources (xylose, glucose, fructose, arabinose, mannose, raffinose, galactose, sucrose, maltose, lactose, cellobiose, starch, inulin, cellulose and glycerol) were also investigated for their effect on the growth and L-ASNase activity. All carbon sources were sterilized separately and added to the basal medium after autoclaving. Medium without carbon source is serving as negative control. The culture media after inculcation were incubated under the shake flask conditions as mentioned before. The cell growth and L-ASNase activity were determined.

Biomass determination

The biomass was determined as mycelia dry weight after centrifugation (8 000 g for 10 min) of culture broth in duplicate, and dried at 105 ºC overnight until constant weight.

L-ASNase enzyme assay

L-ASNase activity was measured by the following method [22].

Step 1: The reaction mixture containing 0.5 mL of 0.04 M L-asparagine, 0.5 mL of 50 mM Tris buffer, 0.5 mL of an enzyme preparation and distilled water to a volume of 2.0 mL, was incubated at 37ºC for 30 min. The reaction was then stopped by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). Blank tubes were run by adding the enzyme preparation after the addition of TCA.

Step 2: To 3.7 mL of distilled water, 0.1 mL of the above mixture (Step 1) and 0.2 mL of Nessler's reagent were added at room temperature and the absorbance was measured immediately at 425 nm using a spectrophotometer, and the amount of liberated ammonia was determined. One international unit of L-ASNase is defined as the amount of enzyme that liberates 1 µ mole of ammonia per minute under the assay conditions [23].

The protein content measurement

The protein content was estimated by using Lowry *et al*. [24] with bovine serum albumin as standard.

Enzyme extraction and purification

The purification of fermented broth was carried out at 4°C [25] by the following steps.

Ammonium sulfate precipitation

The crude enzyme was saturated with 45% ammonium sulphate. The saturated enzyme solution was kept overnight at 4°C and precipitate enzyme was centrifuged at 8000 rpm for 10 min at 4°C.The precipitate was discarded and the supernatant was brought to 85% ammonium sulphate saturation. The precipitate of this step was centrifuged at 8 000 rpm for 10 min at 4 °C and stored in dialysis bags at 4°C [26].

Dialysis

The obtained fractions was dissolved in 0.05M Tris buffer pH 8.0 and dialysed against the same buffer, until the dialysate was free form ammonia. After dialysis, the samples were used for protein estimation and enzyme assay.

Gel filtration on Sephadex column

The dialyzed fraction (value) was applied to a Sephadex G-50 column (25 x1.5 cm) pre-equilibrated with a 0.01M Tris buffer pH 8.5 and eluted by the same buffer at a flow rate of 60 mL/h. Fractions showing L-ASNase activity were pooled and concentrated by lyophilization.

SDS-polyacrylamide gel electrophoresis of purified L-ASNase

The active fractions obtained from the purification process were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the determination of the molecular weight of L-ASNase by using standard molecular markers [27] using silver nitrate stain.

Effect of substrate concentration on L-ASNase activity and calculation of K_m and V_{max} values

In order to characterize the L-ASNase produced by the strain of SAH1_CWMSG, the pure enzyme (0.1mg/mL) was incubated at 30 min with different concentrations of asparagine. The asparagine concentration was varied from 0.02 M to 0.1 M. The K_m and V_{max} values of L-ASNase were calculated from the reciprocal plots of substrate concentration versus reaction velocity.

RESULTS AND DISCUSSION

Strain Isolation

About fifty isolates of *Streptomyces* were isolated from different sources including rhizosphere soil on starch nitrate (ST) medium. All Streptomyces isolates were screened on a medium containing asparagine and phenol red for L-ASNase recognition. Growth (colony diameter) and L-ASNase activity (pink zone diameter) were taken note.

Ten *Streptomyces* isolates grew well and formed a pink zone around their growth. Thus this qualitative method was used as a screening test for L-ASNase production. The diameter of the pink areas ranged from 20-30 mm and maximum value was recorded for the isolate SAH1_CWMSG as shown in Fig. 1. The isolation of rare and uncommon *Streptomyces* has become an increasingly important part of natural product discovery.

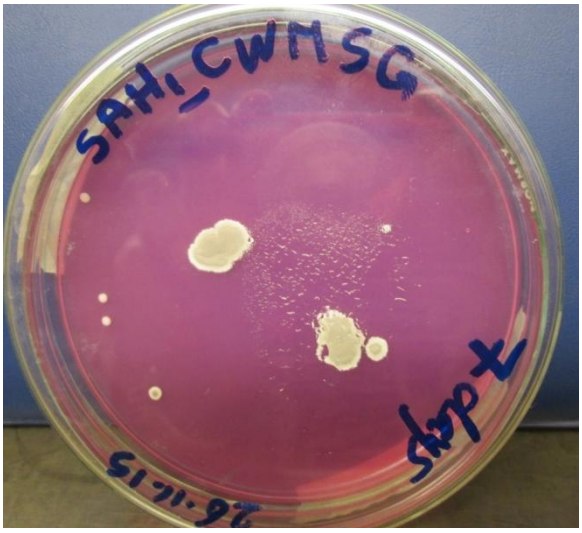


Figure 1. Qualitative screening for L-ASNase production for *Streptomyces* sp. SAH1_CWMSG

Out of ten *Streptomyces*, two were high producers of L-ASNase, which was detected in a solid medium using the plate assay method with phenol red as indicator. There was a direct correlation between L-ASNase production and the diameter of the red zone produced [28] The plate assay method has many advantages as the method is quick and L-ASNase production can be visualized directly from the plates without performing time consuming assays [29]. The best active isolate was SAH1_CWMSG, which belongs to the gray series and was isolated from the rhizosphere soil of wheat plants, grown in Al Sharkia, Egypt. *Streptomyces* had been known to possess the ability to produce bioactivities and enzymes including L-ASNase [30].

Cultures giving a positive test with the plate assay method were then checked for enzyme activity quantitatively. Among several potential L-ASNase enzyme producing isolates, an isolate designed SAH1_CWMSG showed the highest enzyme activity and was utilized for further work.

Biological activity

Emphasized screening of isolate for antimicrobial activity

SAH1_CWMSG strain showed different activities against bacterial strains as *Staphylococcus aureus* (13 mm) in an agar medium. SAH1_CWMSG strain showed also antifungal activity against all tested fungi including *Candida albicans*, *A. niger, Fusarium oxysporium, Fusarium solani* and *Alternaria sp.* with inhibition zone ranging from 13-20 mm and as shown in Table 1.

In vitro anticancer activity of L-ASNase

As shown in Table 2, the anticancer of the pure enzyme was tested using SRB assay in MCF7, HepG2, and A549 cell lines as well as a normal cell line (human normal melanocyte, HFB4). For comparison, doxorubicin was also tested, while DMSO treatments were used as control cancer cells. The results revealed that the pure enzyme did not exert any activity against lung cancer A549 cell line. Moreover, the tumor cells showed normal growth in the culture system and DMSO did not seem to have any noticeable effect on cellular growth. Additionally, the pure enzyme exhibited lower toxicity to the normal HFB4 cell line.

Studying the anticancer activity of the pure enzyme against MCF-7 and HepG2 cell lines, revealed that the compound was found to be more potent than doxorubicin as an anticancer agent, with IC_{50} values of 2.81±0.32 and 3.10±0.35 µg/mL in MCF-7 and HepG2 respectively, versus 2.85±0.30 and 3.50±0.41 µg/mL for doxorubicin in MCF-7 and HepG2 respectively. These results were in accordance with those results obtained from many *in vitro* anticancer studies that proved that the MTT assay was the most reliable method to analyze the anticancer property. The cytotoxicity of L-ASNase on MCF-7 cells, Jurkat and Molt-4 (human T-lymphoblastic leukemia), MCF-7 (human breast adenocarcinoma), LnCap (human prostate carcinoma), NGUK1 (Rat Gasser node neurinoma) were determined by the MTT assay [31]. L-ASNase is an important anticancer agent used worldwide in first and second line treatment of acute lymphoblastic leukemia, lymph proliferative and malignancies [32].

In conclusion, the pure enzyme exerts anti-carcinogenic activity in hepatic HepG2 and breast MCF-7 cancer cell lines by reducing cell proliferation and resulted in significant growth inhibition, which revealed promising activity compared to the activity of the commonly used anticancer drug, doxorubicin. The present study reveals that among the human cancer cell lines tested, HepG2 cells are slightly more sensitive to the tested compound than MCF-7 cells.

Table 1. Antimicrobial activity (IZ mm) of L-ASNase activity produced by *Streptomyces* sp. SAH1_CWMSG

Test organism		IZ (mm)
Bacteria		
Bacillus subtilis	ATCC 6633	00
Staphylococcus aureus	ATCC 6538	13
Escherichia coli	ATCC 7839	00
Pseudomonas aeruginosa	ATCC 9027	00
Yeasts		
Candida albicans	ATCC 10231	13
Saccharomyces cervecia	ATCC 9763	00
Fungi		
Aspergillus niger	ATCC 16404	14
Fusarium oxyspoium	ATCC 62506	13
Fusarium solani	ATCC 36031	20
Alternaria sp.	ATCC 20084	20

Table 2. *In vitro* anticancer activity of the purified L-ASNase produced by *Streptomyces* sp. SAH1_CWMSG on different cell lines

Compounds	IC_{50} (µg/mL)			
	MCF-7	HepG2	A549	HFB4
Doxorubicin	2.85±0.30	3.50±0.41	4.43±0.50	85.60±9.10
DMSO	N.A.	N.A.	N.A.	89.90±9.00
Pure (L-ASNase)	2.81±0.32	3.10±0.35	N.A.	82.90±9.18

Data were expressed as Mean ± Standard error (S.E.) of three independent experiments. N.A. is no activity

Strain identification

Conventional identification of strain SAH1-CWMSG

Cultural and Morphological characteristics

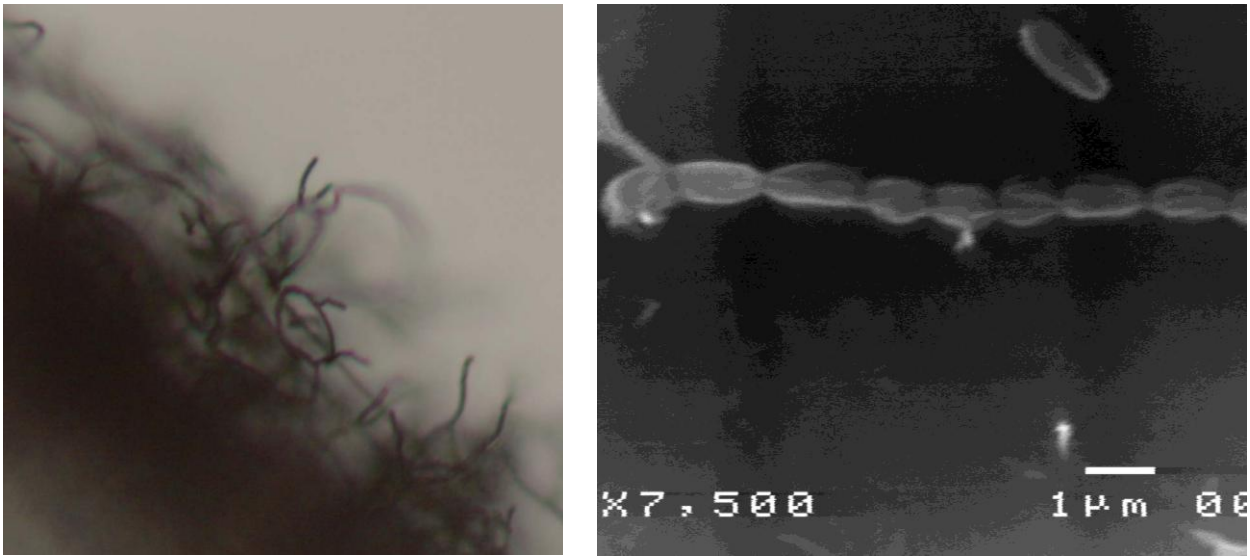
The chemotaxonomic and phenotypic analyses demonstrated that the strain exhibited characteristics similar to those of other members of the genus *Streptomyces.*

Table 3 shows that the growth of the SAH1_CWMSG strain varies from abundant to fair depending on medium composition. The growth was abundant on media ISP-2, ISP-3, ISP-4, ISP-7 and weak on the other media. The color of aerial mycelium ranged from off-white to gray. Consequently, the aerial mycelium is assigned to the gray series. The substrate mycelium differed from one medium to another depending on the medium composition. The color of substrate mycelium was beige with ISP medium no. 1, 4, 6, 8, 9 and it was brown when using ISP medium no. 2, 5 and 7. Spore masses were matched against the seven color wheels of Tresner and Backus [33], as used in the ISP [18].

Table 3. Cultural characteristics of the *Streptomyces* sp. SAH1_CWMSG at 14 and 21 days

	Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1	Tryptone yeast extract broth (ISP 1)	Weak	Off white	Beige	None
2	Yeast -malt extract agar (ISP 2)	Abundant	Gray	Brown	None
3	Oat meal agar (ISP 3)	Abundant	Gray	Grayish	None
4	Inorganic-trace salt- starch agar (ISP 4)	Abundant	Gray	Beige	None
5	Glycerol asparagine agar (ISP 5)	Weak	Gray	Light brown	None
6	Peptone yeast extract iron agar (ISP 6)	Weak	Gray	Beige	None
7	Tyrosine agar (ISP 7)	Abundant	Off White	Brown	None
8	Nutrient agar	weak	Gray	Beige	None
9	Czapek's agar	weak	Gray	Beige	None

Microscopically, it was observed that the morphology of the spore chains of aerial mycelium is of the rectus-flexible (RF) type (Fig. 2a). According to the shape of the spore chains observed under light microscopy, the isolates were grouped as RF, spiral (S) and retinaculum apertum (RA), [34]. The micrograph in Fig. 2b shows that the individual spores are cylindrical with a smooth surface, which was determined according to the categories of Tresner and Davies [35], who found that spore surface is one of the descriptive characterizations for each type of culture.



(a) **(b)**

Figure 2. Light microscopy images of the aerial mycelium showing a straight spore chain (G x 400) for 14 days at 28 °C (a), Scanning electron micrographs showing smooth spore-surface ornamentation (X7, 500) of *Streptomyces* sp. SAH1_CWMSG grown on starch nitrate medium for 21 days at 28 °C (b).

Physiological and biochemical characteristics

Table 4 shows that SAH1_CWMSG isolate does not produce melanin pigment, while degraded protein and starch on the media used. As well as the results were positive for nitrate reduction and gelatin liquefaction. Other categories were tested and considered to ascertain the species classification of a new isolate strain as recommended by Holt and Shape [36]. The cell wall of the isolate was confirmed to contain the LL-diaminopimelic acid (LL-DAP) type [37], a typical constituent of *Streptomyces.* Furthermore, the isolate can utilize all C-sources of the tested sugar using ISP-9 by different degrees in the utilization. Strain SAH1_CWMSG was growing well in a medium containing most of the amino acids used. However, a weak growth was recorded on a medium containing L-Cysteine as an N-source. Furthermore, the results in Table 4 showed that a good growth of SAH1_CWMSG was noticed in a temperature range of 26-40°C and the presence of 0-4 % NaCl, while there was no growth at 45-50°C and 10-13% NaCl.

The characteristics of SAH1_CWMSG strain were compared to published descriptions of various *Streptomyces* species in the morphological, physiological and biochemical characters [18]. In conclusion, SAH1_CWMSG belongs to the gray series group, with negative melanin pigment, spiral hyphae and a smooth spore surface.

Table 4. **Morphological, Physiological and biochemical characteristics of *Streptomyces* sp. SAH1_CWMSG**

Character	Results	Character	Utilization
Morphological characteristic		**N-Source**	**Utilization**
Spore chains	RF (Straight)	DL-Methionine	++
Spore surface	Smooth	DL-Iso-leucine	++
Color of aerial mycelium	Gray	L-arginine	+++
Physiological characteristics		L-Lysine	+++
Melanin production	Negative	L-Glutamic	++
Action of milk	No coagulation on 14 days	L-histidine	++
Nitrate reduction	Positive	Ph- alanine	++
Gelatin liquefaction	Positive	L-aspargine	++
Starch hydrolysis	Positive	L-valine	++
Cell wall hydrolysis		L-cystaine	+
LL-Diaminopimelic acid	Positive	Glycine	++
Sugar pattern	ND	Proline	++
Utilisation of C-source		Ornithine	++
No carbon	-	Tyrosine	+++
D-glucose	+	DL-serine	+++
D-xylose	+	**Antibiotics susceptibility**	mm
L-arabinose	++	Tetracycline	9*
L-rhamnose	++	Neomycin	17**
D-fructose	++	Oxytetracycline	7*
D-galactose	++	Vancomycin	21**
raffinose	+	Rifamycin	8*
D-Mainitol	++	Streptomycin	25**
Myo-inositol	+	**NaCl Sensitivity**	
Salicin	++	0-4%	Abundant
Sucrose	+++	7%	Weak
Temperature tolerance (ºC)		10-13%	No growth
26-40 °C	Abundant		
45-50 °C	No growth		

*+++ = good growth, ++ = Moderate growth, + = weak growth, - = negative, ND= not detected, *= Resistant, **=Highly Resistance*

In particular, SAH1_CWMSG does not resemble any known *Streptomyces* strains completely. Classification and identification of *Streptomyces* based on morphological and biochemical characterization are not enough in most cases. Therefore, molecular data, predominantly rRNA gene sequences have been introduced [38].

Molecular identification of strain SAH1_CWMSG

The 16S rRNA is a powerful tool for phylogenetic analysis and species differentiation of the genus *Streptomyces.* It can be used as a genetic method in parallel to conventional taxonomic methods, including numerical, phonetic and other genetic analyses [39]. Therefore, in this work, phylogenetic analyses based on the 16S rRNA gene sequence of the isolate were partially sequenced in parallel to conventional methods.

PCR amplification of the 16S rRNA gene

The 16S rRNA gene of SAH1_CWMSG was amplified using the *Streptomyces*-specific PCR primers previously mentioned in Materials and methods. The PCR product of the isolate showed a band at 1.500 kb (data not shown).The specificity of the PCR was affected by factors such as the primers, the properties of the gene regions flanking the target site, the annealing temperature in the PCR reaction and the reaction conditions [38]. The primer pair used was F27/R1492-amplified DNA according to Edwards *et al.* [19].

Phylogenetic analysis

The alignment of the 16S rRNA nucleotide sequence of SAH1_CWMSG consisted of 760bp. The 16S rRNA reported gene sequence was matched in the gene bank database through the NCBI BLAST (www.ncbi.nlm.nih.gov). A comparison between the 16S rRNA sequence of this strain with those members in the genomic database bank was performed. This comparison indicated a high level of sequence similarity (99%) with many *Streptomyces* species.

The multiple sequence alignment and phylogenetic tree are represented and illustrated graphically in Fig. 3. The phylogenetic tree was derived from the distance matrices using the neighbor-joining method. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [21]. In conclusion, the phylogenetic analysis coupled with a conventional method of SAH1_CWMSG indicated that the closest strain is *Streptomyces ghanaensis* strain CSSP718. Therefore, *Streptomyces ghanaensis* SAH1_CWMSG was proposed as its name.

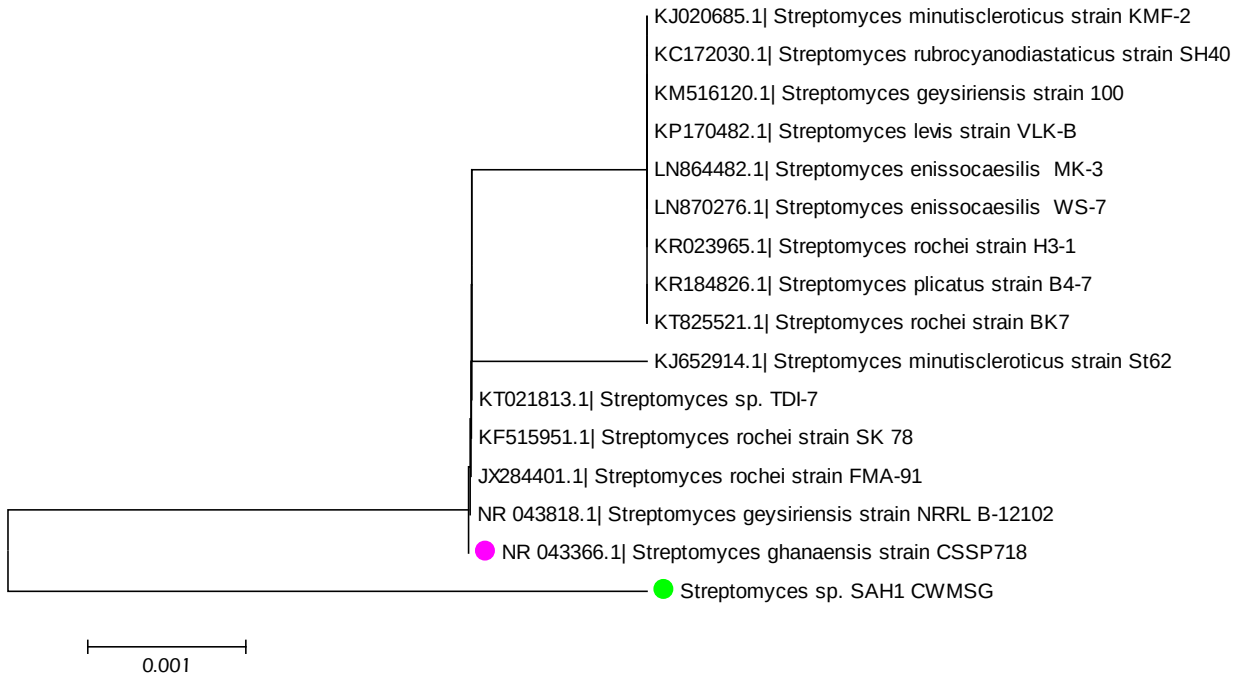


Figure 3. Neighbor joining tree based on 16S rRNA gene sequences showing relationship between *Streptomyces* sp. SAH1_CWMSG and 16 isolates and closely related type strains of the genus Streptomyces. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. Bar 0.01 nucleotide substitutions per site

Nucleotide sequence Gen Bank ID

The nucleotide sequence of the 16S rRNA gene of *Streptomyces* sp. SAH1_CWMSG has been deposited in Gen Bank under the accession number ID: KT953341.

Media screening for L- ASNase production by SAH1_CWMSG strain

Streptomyces sp. SAH1_CWMSG, a soil isolate, was assessed for creation of L-ASNase activity, with numerous functions, chiefly anticancer action. The *Streptomyces* was subjected to submerged fermentation by "shake flask "strategy.

Sex media with different compositions were screened to find out the appropriate one for ASNase production. The results were displayed in Fig. (4), indicating the level of ASNase activity ranged from 8.1 to 13 IU. Media No. 3, 4, 5 support high enzyme production and the maximum was achieved on medium No. 6 correlated with lose in biomass. On the other hand, a high yield of cell growth accompanied with minimum ASNase production was observed in medium 1. Also, the most suitable pH range for maximum enzyme production was 6 -7.5. Our results are in agreement with Kumar *et al.* [14], who obtained maximum ASNase production of 14.56 IU at 4 and 2g/L of L-asparagine and glucose, respectively. Also these results are greater than that obtained by DeJong [40], who achieved 1 IU of L-ASNase/100 mL of a 4% peptone medium using *Streptomyces griseous* ATCC 10137.

Effect of different nitrogen sources on ASP production by *Streptomyces* sp. SAH1_CWMSG

Nitrogen source is a limiting nutrient and plays a key role in L-ASNase production. The supplementation of additional nitrogen sources, either organic or inorganic, to the production medium showed a profound impact on the production of L-ASNase by the SAH1_CWMSG strain.

The results illustrated in Fig. (5) showed the L-ASNase activity by SAH1_CWMSG strain varied with different nitrogen sources and ranged between 38.65 and 72.99 IU. Among them, culture medium amended with $(NH_4)_2SO_4$ favored maximal L-ASNase activity (72.99 IU) [41] who reported 1.0% ammonium sulfate recorded maximum production of L-ASNase by the isolated fungusVS-26. In addition, these results were greater than those obtained by Basha *et al.* [12], who obtained L-ASNase activity of 49.2 IU. On the other hand, the organic nitrogen sources enhanced biomass production of the SAH1_CWMSG strain but were less effective than the inorganic nitrogen source on the L-ASNase activity.

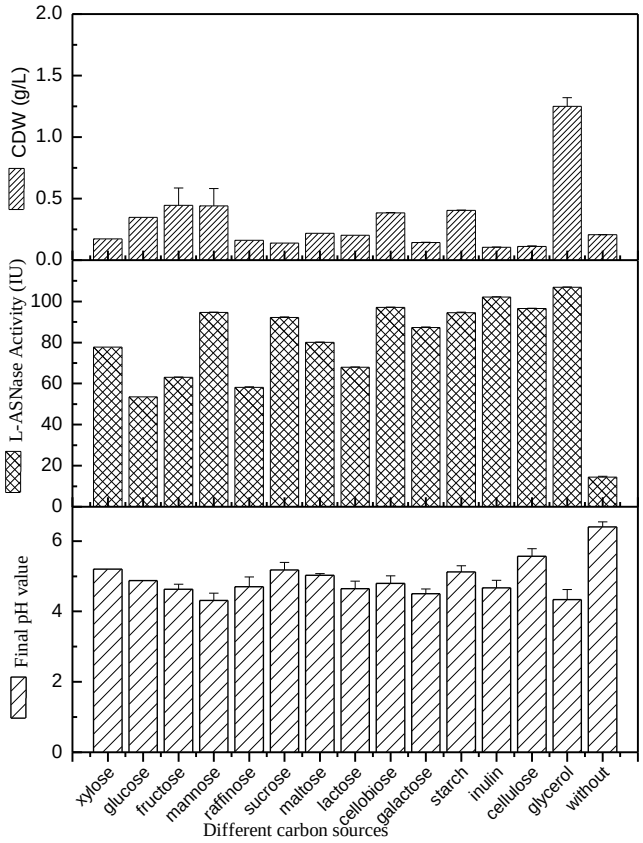


Figure 4. Effect of different media on L-ASNase production by *Streptomyces* sp. SAH1_CWMSG

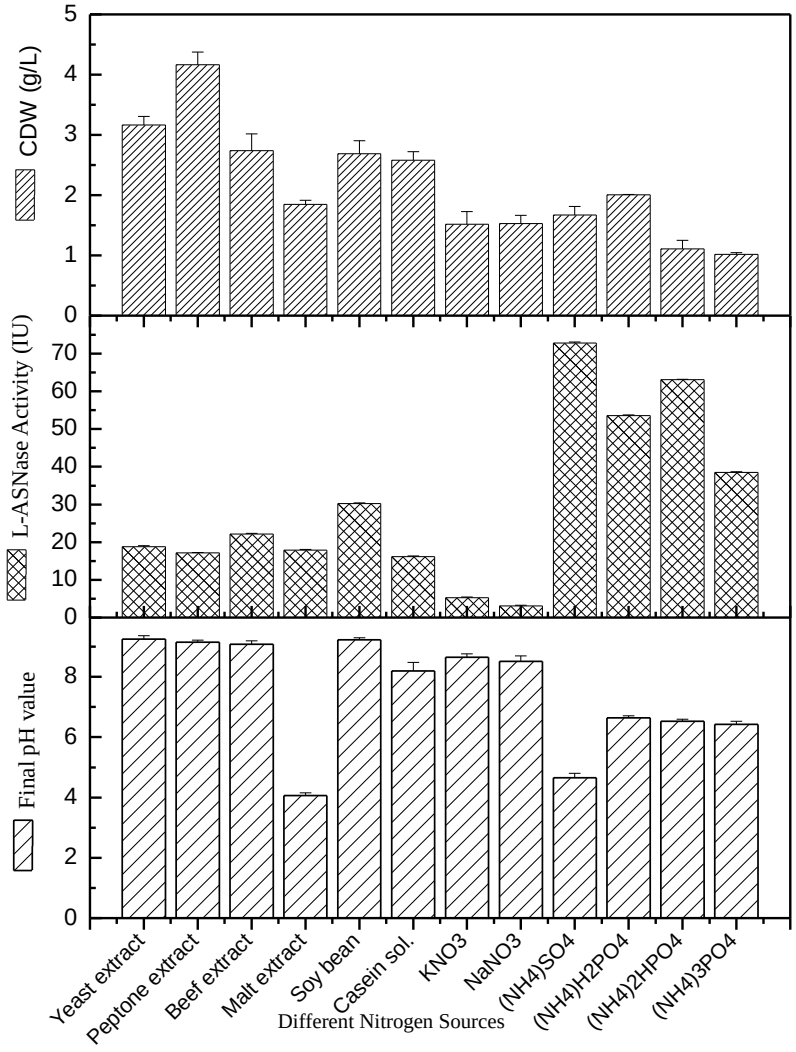


Figure 5. Effect of different nitrogen sources on ASNase production by *Streptomyces* sp. SAH1_CWMSG

Effect of different carbon sources on L-ASNase production by SAH1_CWMSG strain

Different carbon sources like xylose, glucose, fructose, arabinose, mannose, raffinose, galactose, sucrose, maltose, lactose, cellobiose, starch, inulin, cellulose and glycerol were amended in the production medium to determine their impact on L-ASNase activity by *Streptomyces* sp. SAH1_CWMSG.

As compared to various carbon sources tested, L-ASNase production was high (106.92 IU) in the production medium containing glycerol (1.0%) as the carbon source (Fig. 6). On the other hand, glucose and raffinose recorded the lowest enzyme activity of 53.46 and 58.32 IU respectively. These results were in accordance with Abdel-Fatah [42], who indicated that L-ASNase production by *Streptomyces longsporusflavus* was marginally favorable with glycerol and there was no significant effect with glucose and lactose. After medium optimization, the enzyme activity (106.92 IU) was enhanced by more than 7 fold that of the basal medium.

Enzyme extraction and purification

Ammonium sulfate precipitation and gel filtration on sephadex G-50 column chromatography

Data in Table (5) and Fig. (7) showed that the ammonium sulfate 45 and 85 % concentrated enzyme preparation had a protein content of 1.73 and 2.20 mg with specific activities of 126.30 and 364.86 IU/mg proteins, showing a purification fold of 2.94 and 8.50. The enzyme recovery at this step was 27.5 %. When the ammonium sulfate concentrate was passed through sephadex G-50 column, L-ASNase activity and protein were determined in each fraction. The total enzyme activity of the active fractions (6, 7 and 8) from column G-50 was 1297.65 IU with specific Activity 581.62 IU/mg.

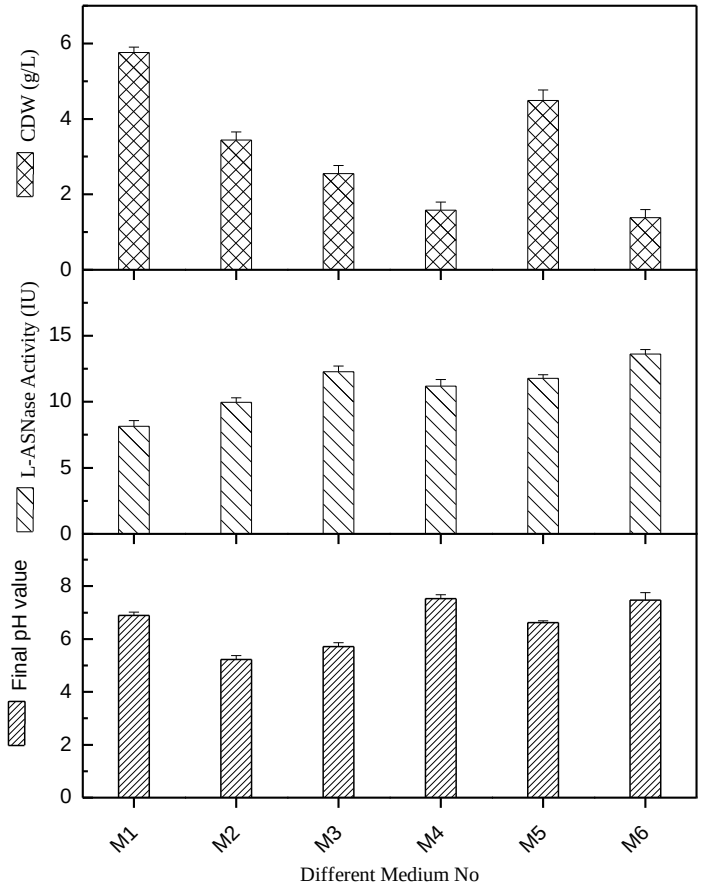


Figure 6. Effect of different carbon sources on ASNase production by *Streptomyces* sp. SAH1_CWMSG

Table 5. Purification profile of L-ASNase activity by *Streptomyces* sp. SAH1_CWMSG

Purification step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Purification folds	Yield %
Control (Crude Extract)	67.93	2916.00	42.93	1.00	100.0
Amm.Sulfate 45%	1.73	218.90	126.30	2.94	7.50
Amm.Sulfate 85%	2.20	801.90	364.86	8.50	27.50
Amm.Sulfate 100%	0.93	07.29	7.82	0.18	0.25
Sephadex G-50	2.23	1297.65	581.62	44.5	13.55

The results in Table 5 clearly indicate that the purification fold increased from 8.5 to 13.55 fold using 85% ammonium sulfate and sephadex G-50 column, respectively. The specific activity of the enzyme increased with each step of purification with a minimum loss in quantity, giving a final recovery of 44.5%. In this study, the purification process of the L-ASNase enzyme was carried out by subsequent steps such as ammonium sulfate precipitation, dialysis and gel filtration on sephadex G-50 column, according to the previous work of Dharmaraj [13].

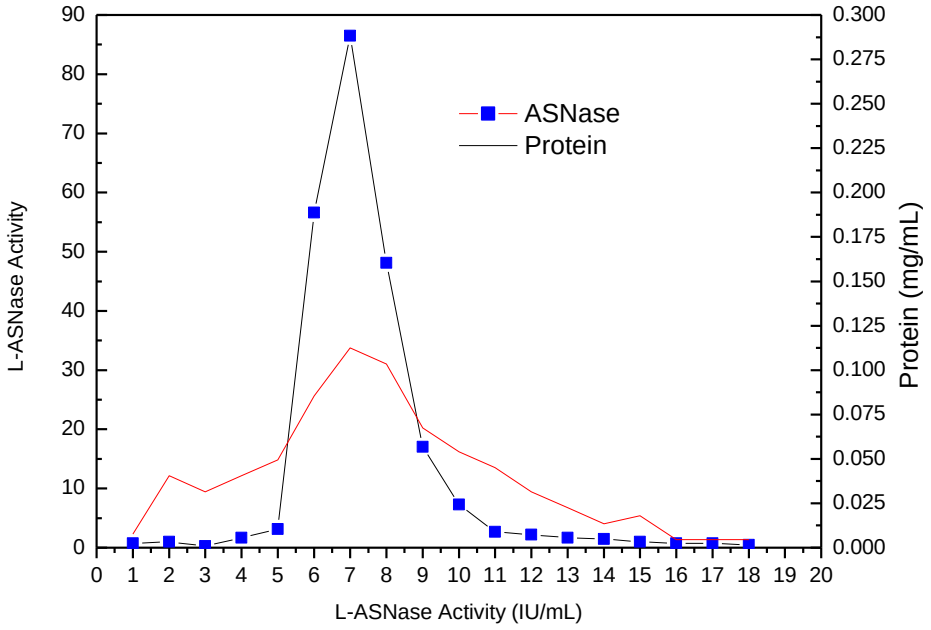


Figure 7. Elution profile of preparations obtained from *Streptomyces* sp. SAH1_CWMSG by ammonium sulphate (80%saturation) using Sephadex G-50 gel filtration chromatography

The K_m and V_{max} calculation

L-ASNase activities were measured under optimum assay conditions. The results obtained in Fig. 8 were plotted on a graph of the enzyme activity (IU/mL) against different concentrations of substrate. The best yield was recorded at 0.06 M substrate concentration as shown in Fig. 8. The K_m and V_{max} were calculated to be 27 mMol and 69 IU/mL respectively. This indicates that the high affinity of the enzyme to the substrate. L-ASNase from different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. The calculated values of K_m and V_{max} values were in agreement with Kumari *et al.*[4] who found that L-ASNase produced by *Streptomyces griseoluteus* sp. WS3/1 has K_m and V_{max} value of 0.0377M and 9.69 IU/mL$_*$min respectively.

SDS-BAGE polyacrylamide gel electrophoresis

After every purification procedure, the peak fractions with the highest enzyme activity were analyzed using SDS-PAGE. Two protein bands were visible in this study corresponding to two subunits with molecular weight about 29 and 38 kDa as shown in Figure 9. The molecular weight of the L-ASNase obtained from the SAH1_CWMSG strain is in agreement with those results by Bansal, *et al.* [43] who mentioned that the enzyme from *Pyrococcus furiosus* was found in dimer form as determined by SDS-PAGE. Also these results were in agreement with those results by Narayana, *et al.* [44], who mentioned the enzyme structure has the molecular weight of 37kDa and 34 kDa, as revealed by SDS-PAGE from *S. albidoflavus* and gel filtration of L-ASNases exist in dimer, hexamer or in monomer form when isolated from various sources.

In addition, the molecular weight of L-ASNase from *Streptomyces* has been reported to be between 33kD to 200kD [45]. The variability in the molecular weight of L-ASNase in different organisms may be inferable to its genetic diversities [46].

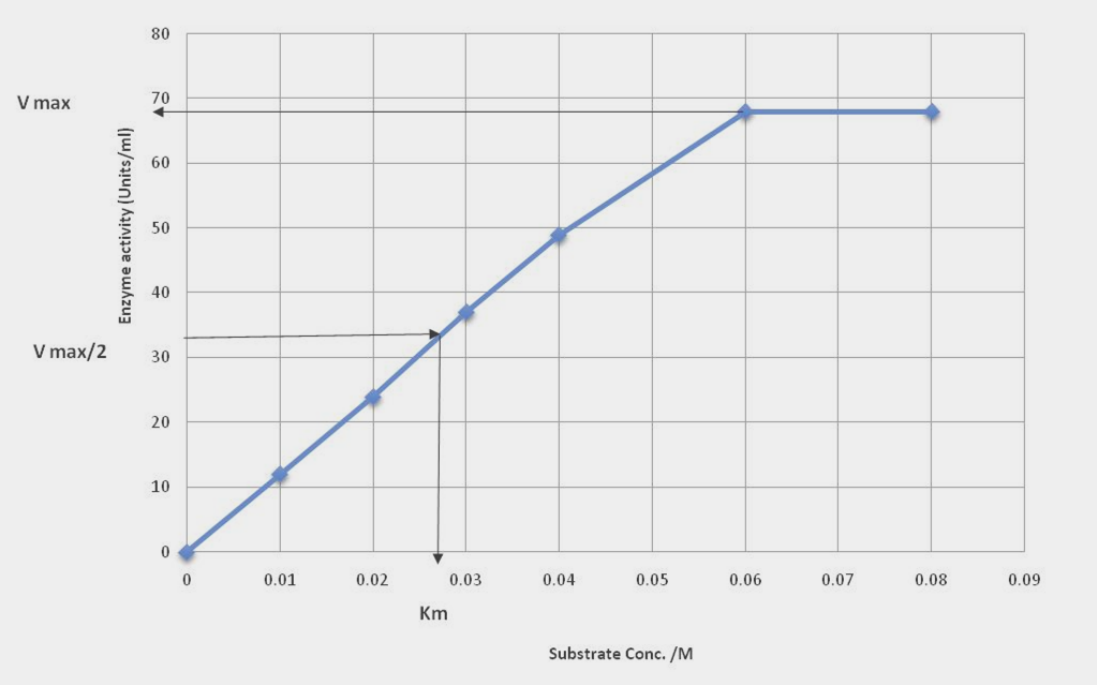


Figure 8. The K_m and V_{max} values of L-ASNase from *Streptomyces* sp. SAH1-CWMSG

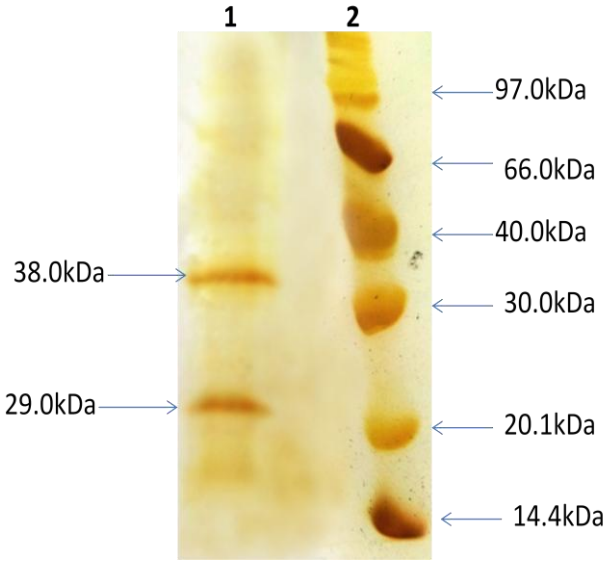


Figure 9. SDS-PAGE analysis of L-asparaginase produced by *Streptomyces* sp. SAH1_CWMSG. Lane 1, Sephadex G50 purified enzyme concentrate of L-ASNase; lane 2, standard protein marker

CONCLUSION

The present study indicates the local soil isolate SAH1_CWMSG can be used as a potential source of L-ASNase production. The isolate was identified as *Streptomyces ghanensis* SAH1_CWMSG (ID: KT953341) on the basis of polyphasic data. Furthermore, the purified L-ASNase showed antifungal activity and dormant anticancer activity against the MCF-7 cell line and should be considered for further pharmaceutical use as an anticancer agent. The maximal enzyme activity of 106.92 IU was obtained using ammonium sulfate at 28°C and 120 h on 200 rpm. The L-ASNase from its culture filtrate was purified using Sephadex G-100 and its molecular weight was determined by SDS-PAGE. The enzyme kinetic parameters were also determined. Although there have been many reports on the subject of L-ASNase producing microorganisms, this is the first report on L-ASNase production by *Streptomyces ghanensis* SAH1_CWMSG.

Acknowledgment
The authors are thankful to the National Research Center at Dokki, Giza, Egypt, for providing all facilities to support and conduct the present research successfully.

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