Biochemical studies and biological activities on L-glutaminase from rhizosphere soil *Streptomyces rochei* SAH2_CWMSG

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Background and objective
L-glutaminase (L-GLUNase) is a potential anticancer enzyme that hydrolyzes amide bond of L-glutamine to give glutamate and ammonium ion. It is used as an antioxidant, a flavor enhancing agent and a biosensor for glutamine level measurement. The aim was to produce L-GLUNase in high yield from a promising local *Streptomyces* isolate for many pharmaceutical applications.
Materials and methods
A total of 20 *Streptomyces* isolates for their capacity of L-GLUNase production were screened. A potent L-GLUNase producer, SAH2_CWMSG isolate, was identified by phenotypic and phylogenetic analysis. L-GLUNase was purified using ammonium sulfate followed by gel filtration on Sephadex G-100. The purified L-GLUNase was characterized, and its application as an antimicrobial, anticancer, and antioxidant agent was investigated.
Results and conclusion
The phylogenetic analysis of SAH2_CWMSG strain confirmed that the SAH2_CWMSG strain was most similar to *Streptomyces rochei* (99%). It produced L-GLUNase activity of 58 U/ml under shake flask submerged fermentation. The purified L-GLUNase has the molecular weight of 55 kDa and K_m and V_{max} value of 1.314 mmol/l and 95.24 μ Me/min, respectively. Of the various physiochemical parameters tested, pH 7.5 and temperature 40°C were optimal for the enzyme activity. On the contrary, 10 mmol/l of Mn^{+2} showed a slight increase in L-GLUNase activity. A promising *Streptomyces* sp. fully identified as *S. rochei* SAH2_CWMSG (Gen Bank ID: KU720627) is an efficient source of L-GLUNase production. Therefore, it can be potentially used as enzyme supplement, which has many industrial and pharmaceutical applications.

Keywords:
biological activities, L-glutaminse characterization, phenotypic and phylogenetic identification, *Streptomyces* rochei SAH2_CWMSG

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Introduction

L-glutaminase (L-glutamine amidohydolase E.C 3.5.1.2) is a potential anticancer enzyme that hydrolyzes amide bond of L-glutamine to give glutamate and ammonium ion. L-glutaminase assumes a noteworthy part in the nitrogen metabolism of both prokaryotes and eukaryotes. L-GLUNase has pulled in much attention as of late for its wide application in pharmaceuticals as well as being a hostile agent toward leukemia [1]. L-GLUNase exhibits its anticancer effect by depleting L-glutamine from the tumor cells, prompting their death as they are dependent on this amino acid [2]. For example, L-GLUNase purified from Purified L-GLUNase obtained from *Aspergillus flavus* could stop a breast carcinoma [3].

L-GLUNase is actually a vital ordinary antioxidant that helps in avoiding human infections. Moreover, it is not related to lethal and cancer-causing effects like those of artificial antioxidants [4,5], not very many reports are accessible for the utilization of bacterial and fungal L-GLUNases as antioxidant mediators [4–6].

L-GLUNase is also used as an efficient antiretroviral agent [7], along with its use in food industry as a flavor and aroma-enhancing agent [8]. Another important application of glutaminase is that it also plays an important role in biosensor as a monitoring agent for glutamine level measurement [9].

It can be obtained from, animal, plant and microbial cells. Nevertheless, the greatest sources of it are the microorganisms, demonstrating the simple generation of a coveted compound in significant sum with

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soundness and hereditary material control [10]. Among the microorganisms producing bioactive compounds, 73% are *Streptomyces* and 27% being rare actinomycetes. *Streptomyces* are well recognized to produce valuable medicines, particularly antibiotics and anticancer mediator, and industrial products like enzymes for revenue-generating discovery platform [11,12]. A lot of information has been presented for microbial L-GLUNase producers like *Streptomyces rimosus* [13], *Streptomyces avermitilis* and *Streptomyces labedae* [14], *Streptomyces gresius* [15], *Streptomyces enissocaesilis* DMQ-24 [4], and *Streptomyces canaries* [16].

In the present work, an endeavor was made to isolate and fully identify an efficient local *Streptomyces* isolate for L-GLUNase production in high yield, representing a promising isolate. The purified enzyme was characterized and its biological activities were investigated.

Materials and methods

Isolation, screening and quantitative assay of L-glutaminase

Five soil samples were collected in sterile bags from the rhizosphere area of wheat plants at Mashtool El Sook, El Sharkia Governorate, Egypt. Overall, 100 μl of each serial dilution up to 10-7 was taken and spread on starch nitrate agar (SNA) plates and incubated at 28°C for 5–7 days [17]. After incubation, the pure individual *Streptomyces* colonies were picked out, maintained in ISP-2 agar slant and kept at 4°C.

Screening of isolates for L-GLUNase activities in minimal glutamine agar medium containing of 0.009% w/v of phenol red was done. Isolates showing maximum zone of color change (from yellow to pink) were selected as a possible strain for further studies [18].

Potential *Streptomyces* isolate identification

Conventional taxonomy

Morphological, biochemical, cultural, and physiological characterization of the potential strain SAH2_CWMSG was performed and recommended by the International *Streptomyces* Project (ISP) [19]. Microscopic depiction was performed with cover slip culture. Formation of aerial, substrate mycelium and spore arrangements on mycelium was monitored under a phase-contrast microscope (Nikon Eclipse E600, New York, USA) at 200 magnification and scanning electron microscopy (JEOL JSM 5300; JEOL Techniques Ltd, Tokyo, Japan). The cultural characteristics such as growth, coloration of aerial and substrate mycelia and formation of soluble pigment were investigated in seven different media, including ISP-2–ISP-7 using the procedures as recommended by the ISP. Biochemical and physiological characterization analyses such as melanin pigment production, nitrate reduction, gelatin liquefaction, and starch hydrolysis were also performed as suggested by the ISP. The growth rate in NaCl (2–13%) and survival at 50°C were also evaluated. Furthermore, the capability of the isolates to utilize various carbon and nitrogen sources was estimated using ISP-9 agar medium. Based on the aforementioned characteristics, genus level identification of the potential strain was made by Bergey's Manual of Systematic Bacteriology [20].

Disk diffusion susceptibility test

The disk diffusion susceptibility of the selected isolate was carried out according to Jorgensen and Turnidge [21]. The test was performed by applying SAH2-CWMSG strain inoculum to a SNA plate. Six standard antibiotic disks, for example, tetracycline, neomycin, oxytetracycline, vancomycin, rifamycin, and streptomycin (Bioanalyse, Ankara, Turkey), were placed on the inoculated agar surface. Plates were incubated for 24–48 h at 28°C before measurement of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter.

Phylogenetic identification

DNA isolation and PCR amplification

An overnight culture of the SAH2-CWMSG strain grown at 28°C 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; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) following the manufacturer's instructions on the kit. The PCR amplification of the 16S rDNA region was carried out following the manufacturer's instruction regarding the Maxima Hot Start PCR Master Mix (Thermo K1051). The 16 s rDNA was amplified by PCR (PCR system 9700; Applied Biosystemes, Perkin–Elmer, Foster City, California, USA) using primers designed to amplify a 1500 bp fragment of the 16S rDNA region. The domain bacteria-specific primer 27 F (forward primer) was 5′-AGAGTTTGATCMTGGCTCAG-3′ and the universal bacterial primer 1492 R (reverse primer) was 5′-TACGGYTACCTTGTTACGACTT-3′ [22].

The PCR reaction was performed with 5 μl of genomic DNA as the template, 1 μl of 16 s rRNA forward primer, 1 μl of 16 s rRNA reverse primer, 18 μl

water, nuclease-free and 25 μl Maxima Hot Start PCR Master Mix (2×) in a 50-μl reaction mixture as follows: activation of 2 Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, and 65°C and 72°C for 1 min each, and finally, a 10-min steep at 72°C. After completion, the PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 mg ml/l), to ensure that a fragment of the correct size had been amplified.

DNA sequencing, phylogenetic analysis and tree construction

The 16S rRNA sequence analysis and phylogenetic tree construction by neighbor-joining method was performed for species-level confirmation of *Streptomyces* spp. The classifier was trained on the new phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) proposed by Wang *et al.* [23], (http://rdp.cme.msu.edu/classifier/classifier.jsp).

The amplification products were purified with the K0701 GeneJET PCR Purification Kit Thermo (Thermo Fisher Scientific Inc.). Afterwards, the samples become ready for sequencing in an ABI Prism 3730XL DNA sequencer and analyzer of GATC Company (GATC Biotech Ltd., London, UK). Sequencing reaction was performed with the primers 518 F 5′-CCA GCA GCC GCG GTA ATA CG-3′ and 800 R 5′-TAC CAG GGT ATC TAA TCC-3′ using a PRISM Big Dye Terminator v3. 1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Diformamide. 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). The sequence alignment was prepared with DNASTAR software programs (DNASTAR Inc., Madison, Wisconsin, USA).

Phylogenetic data were obtained by aligning the nucleotides of different 16S RNA retrieved from the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. The sequences with 98–100% homology were considered for molecular taxonomy analysis. Multiple sequence alignment was performed in the 16S rRNA sequence generated in this study and sequences from GenBank database with the CLUSTAL W program. Phylogenetic tree was constructed using the neighbor-joining and maximum-parsimony tree making methods in Mecular Evolutionary Genetic Analysis (MEGA version 6.0) software [24], based on bootstrap values of 500 replications.

Production medium and cultivation conditions for L-glutaminase

The broth medium was used in this study for primary evaluation of L-GLUNase activity (g/l): glucose, 3; NaH_2PO_4, 6; K_2HPO_4, 3; NaCl, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 0.015; and glutamine, 3. The pH of the medium was adjusted to 7.0 before sterilization. The carbon source and amino acid were sterilized separately and added to the fermentation medium before inoculation. Overall, 50 ml of the liquid medium was dispensed into 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 7-day-old culture. The inoculated flasks were incubated at 28°C on a rotary shaker (New Brunswick Scientific Co., Edison, New Jersey, USA) at 200 rpm for 120 h. The uninoculated fermentation medium was used as a negative control during the experiment. The contents of each flask were harvested by centrifugation at 10.000 rpm for 10 min, and the supernatant was analyzed for enzyme activity and cell growth.

Assay of L-glutaminase

L-GLUNase activity was determined using the method of Imada *et al.* [25], utilizing L-glutamine as substrate, and the released ammonia was measured using Nessler reagent. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml Tris-HCl buffer (0.05 mol/l; pH 8). Then the mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.5 ml of 1.5 mol/l Tri-chloroacetic acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added and incubated for 10 min at room temperature. A standard graph was plotted using ammonium sulphate. One international unit of L-GLUNase was defined as the amount of enzyme that liberates 1 μmol of ammonia per milliliter per minute.

The protein content measurement

The protein content was estimated by using the Lowry *et al.* [26] with BSA (1 mg/ml) as standard.

Extraction and purification of L-glutaminase

The bulk-produced culture broth under optimal conditions was chilled and centrifuged at 10 000 rpm for 10 min to remove the cell debris and the supernatant, referred as cell-free extract. The enzyme was precipitated by slow addition of ammonium sulphate (25–100%) by continuous gentle stirring on a magnetic stirrer overnight at

4°C with occasional changes of Tris buffer pH 8. The chilled mixture was then subjected to centrifugation at 15 000 rpm for 15 min. The supernatant was discarded carefully, and the precipitate was dissolved in a minimum amount of 0.05 mol Tris-HCl buffer pH 8.0. The precipitated protein obtained after treatment of the crude enzyme extract with ammonium sulfate (75% saturation) was dissolved in 0.05 mol Tris-HCl buffer pH 8 and dialyzed (with gentle stirring) in cold same buffer for 24 h with changing the buffer four times. After dialysis, the enzyme solution (1 ml containing 13.3 mg protein and with a total activity of 405.6 units) was applied to Sephadex G-100 column, and the protein was eluted by the identical buffer at a flow rate of 60 ml/h [27].

Physiochemical characterization of purified L-glutaminase

Effect of temperature on enzyme activity and stability

The effect of reaction temperature on L-GLUNase activity was tested by incubating the reaction mixture at different temperatures ranging from 30 to 55°C in 0.05 mol Tris-HCl buffer pH 8. The thermal stability of the purified enzyme was determined by incubating the enzyme solution at different times (15–60 min) at various temperatures (40, 50, 60, 70, and 80°C) in the absence of substrate. The enzyme was removed and cooled to the room temperature, and the residual activity was measured by the standard assay method as previously mentioned.

Effect of pH on L-glutaminase activity and stability

The optimum pH for L-GLUNase activity was determined using 0.05 mol sodium citrate (pH 4.0–7.0), 0.05 mol potassium phosphate (pH 6.5–8.0) buffers and 0.05 mol Tris-HCL buffer (pH 7–9). After incubating each reaction at 45°C for 30 min, enzymatic activity was detected. The pH stability of the enzyme was determined by preincubating the enzyme solution at different pH values ranging from 4.0 to 9.0 pH with 0.05 mol sodium citrate buffer for 2 h at 4°C. At the end of the preincubation time, the pH value of enzyme solution was readjusted to pH 7.5 and then residual enzyme activity was assayed by the standard method.

Reaction time for enzyme activity

L-GLUNase was incubated with its substrate; the greater amount of product will be formed. The reaction of the enzyme was preceded from 0 to 30 min. The enzyme activity was assayed by the standard method to select an appropriate incubation time.

Determination of purified L-GLUNase molecular weight by SDS-page

The purity of L-glutamine, protein was tested by SDS-PAGE using Coomassie brilliant blue dye using standard molecular markers according to the procedure of Laemmli [28] and Ali *et al.* [29].

Calculation of K_m and V_{max} values for L-GLUNase

To characterize the L-GLUNase produced by SAH2_CWMSG strain, the pure enzyme (10–100 mg/ml) was incubated for 30 min with different concentrations of glutamine (1–10 mmol/l). The K_m and V_{max} values of L-GLUNase were calculated from the graph of substrate concentration versus reaction velocity.

Effect of metal ions and other reagents on L-GLUNase activity

The effects of some metal ions, for example, magnesium chloride ($MgCl_2$), potassium chloride (KCl_2), magnesium chloride ($MnCl_2$), ferrous chloride ($FeCl_2$), cobalt chloride ($CoCl_2$), sodium chloride (NaCl), nickel chloride ($NiCl_2$); iodoacetate, SDS; glutathione; and EDTA on L-GLUNase activity were tested. The effect of these compounds on enzyme activity was assessed by incubating enzyme solution with 10 mmol/l concentrations of each compound for 20 min before addition of substrate. After a preincubation time, enzymatic activity was determined under optimal assay conditions.

Biological activities of L-GLUNase

Antimicrobial bioassay – well diffusion method

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). The antibacterial screening bioassay was made by the agar well diffusion method described by Jorgensen and Turnidge [21] using Mueller-Hinton agar (Lab M Limited, Bury, Lancashire, UK). On the contrary, *Candida albicans* (ATCC 10231), *Saccharomyces cervecia* (ATCC 9763), *Aspergillus niger* (ATCC 16404), *Fusarium oxyspoium* (ATCC 62506), *Fusarium solani* (ATCC 36031), and *Alternaria* spp. (ATCC 20084) were used as antifungal test strains. The fungal test strains were slanted and maintained on the potato Dextrose Agar medium (Lab M Limited).

Anticancer activity

Cell lines and culturing

Three human cancer cell lines are used throughout this study, namely, Caucasian breast adenocarcinoma (MCF-7), hepatocellular carcinoma (Hep-G2), and colon carcinoma (HCT-116) beside one normal cell

line, namely, human epithelial retina cells, which were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM for cancer cell lines) and DMEM-F12 for normal cell line, supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded at a concentration of 2×10^6 in a 25 ml tissue culture flask and incubated at 37°C till 80–90 confluent sheet.

In vitro ant proliferative assay

The anticancer activity was measured *in vitro* using the MTT assay according to the previously reported standard procedure [30,31]. 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 an appropriate volume just before addition to the cell culture. Cells were incubated alone or with enzyme at different concentration (1000, 500, 250, 125, and 62.25 μg/ml). After 48 h of incubation cell with enzyme, 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 results were compared with the antiproliferative effects of the reference control doxorubicin.

Antioxidant activity of the purified L-glutaminase by DPPH assay

The purified enzyme was tested for the scavenging effect on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Sajitha *et al.* [6] with slight modifications. One milliliter of serial dilution (100–500 mg) of L-GLUNase was added to 8 ml of 0.004% (w/v) DPPH in ethanol (95%). The mixture was then incubated at 37°C for 60 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm, using Agilent Technologies, Cary Series UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA). Ascorbic acid was used as standard. The DPPH radical scavenging activity (% inhibition) was calculated by the following formula:

$$(\%\text{Inhibition}) = \left(\frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right) \times 100$$

Results and discussion

Isolation and detection of *Streptomyces* producing L-GLUNase

Approximately 20 isolates of *Streptomyces* were isolated from rhizosphere soils of wheat plant on SNA medium and tested on a medium containing glutamine and phenol red for L-GLUNase detection. Isolates growth and L-GLUNase production (pink zone) were observed.

Seven *Streptomyces* segregates developed well-shaped pink color around their growth. Of these seven isolates, SAH2_CWMSG was selected showing a dark pink color as presented in Fig. 1. SAH2_CWMSG was isolated from the rhizosphere of the wheat plant, grown in Al Sharkia Governorate, Egypt.

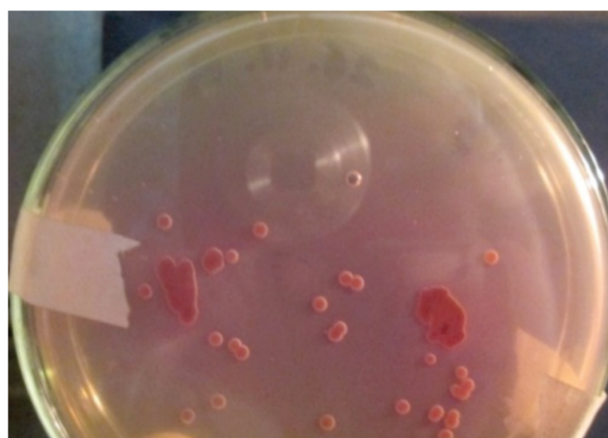
Identification of *Streptomyces* isolate

Conventional taxonomy of SAH2-CWMSG isolate

Morphological properties: Table 1 demonstrates that the growth of the SAH2_CWMSG strain differs from weak to abundant based on the medium contents. The growth was feeble on media ISP-1, ISP-6, and nutrient agar and strong on the other media. The color of elevated mycelium extended from bright gray to dim gray. Thus, the aerial mycelium is appointed to the gray series. The substrate mycelium varied upon the medium constituent. The color of substrate mycelium was beige with ISP medium no. 1 and 6 and nutrient agar, and it was dark when utilizing ISP medium no. 2, 5, and 7. Spore masses were coordinated against the seven color wheels of Tresner and Backus [32], as utilized as a part of the ISP.

Microscopically, it was noted that the morphology of the spore chains of aerial mycelium is of the spiral type

Figure 1



Qualitative screening for L-GLUNase production for *Streptomyces rochei* SAH2_CWMSG.

(Fig. 2a). As indicated by the state of the shape of the spore chains detected under light microscopy, the isolates were assembled as RF, spiral (S) and retinaculum a pertum (RA) [20]. The micrograph in Fig. 2b demonstrates that the individual spores are cylindrical with a smooth surface, which was resolved by the classes of Tresner and Davies [33], who found that spore surface is one of the expressive portrayals for each kind of culture.

Physiological and biochemical characteristics

Table 2 demonstrates that SAH2_CWMSG does not deliver melanin color, while it degraded protein and starch on the media utilized. Additionally, the results were positive for nitrate reduction, milk coagulation and gelatin liquefaction. Different classifications were tried and considered to build up the species classification of a new isolate as recognized by Holt *et al.* [34].

The cell wall of the isolate was affirmed to contain the ll-diaminopimelic acid (ll-DAP) type [35], a classic constituent of *Streptomyces*. Besides, the isolate can use all carbon sources of the examined, sugar utilizing ISP-9 by various degrees of the usage as represented in Table 2. Strain SAH2_CWMSG was growing well in a medium containing most of the amino acids used (Table 2). Moreover, a frail growth was noticed on medium including L-cysteine as a nitrogen source.

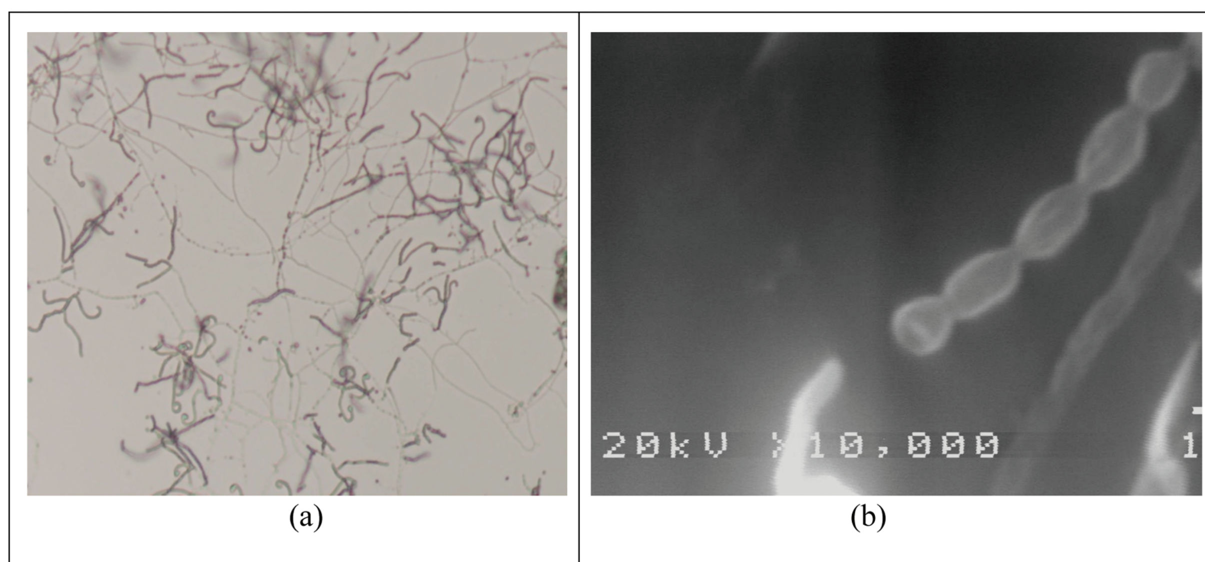
The outcomes in Table 2 demonstrated that the abundant growth of SAH2_CWMSG was seen in a temperature scope of 26–45°C and the presence of 0–7% NaCl, whereas there was no growth at 50°C and 10–13% NaCl.

The characteristics of SAH2_CWMSG strain were matching with *Streptomyces* spp. published in Bergey's

Table 1 Cultural characteristics of the *Streptomyces rochei* SAH2_CWMSG at 14 and 21 days

	Medium no.	Growth	Color of		
			Aerial mycelium	Substrate mycelium	Diffusible pigments
1	Tryptone yeast extract broth (ISP-1)	Weak	Light gray	Beige	None
2	Yeast-malt extract agar (ISP-2)	Abundant	Dark gray	Brown	Brown
3	Oat meal agar (ISP-3)	Abundant	Dark gray	Grayish	None
4	Inorganic-trace salt-starch agar (ISP-4)	Abundant	Gray	Grayish	None
5	Glycerol asparagine agar (ISP-5)	Abundant	Light gray	Brownish	None
6	Peptone yeast extract iron agar (ISP-6)	Weak	Beige	Beige	None
7	Tyrosine agar (ISP-7)	Abundant	Gray	Brown	Brown
8	Nutrient agar	Weak	Gray	Beige	None
9	Czapek's agar	Abundant	Gray	Garish	None

Figure 2



Light microscopy images of the aerial mycelium showing a rectus-flexible (straight spore chains) type (G×400) for 14 days at 28°C (a). Scanning electron micrographs showing smooth spore surface ornamentation (×7, 500) of *Streptomyces rochei* SAH2_CWMSG grown on starch nitrate agar medium for 21 days at 28°C (b).

Table 2 Physiological, morphological, biochemical properties and amino acids utilization of *Streptomyces rochei* SAH2_CWMSG

Characteristics	Results
Morphological characteristics	
Spore chains	Spiral
Spore surface	Smooth
Color of aerial mycelium	Gray
Physiological characteristics	
Melanin production	Positive
Action of milk	No coagulation in 14 days
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Starch hydrolysis	Positive 14 days
Cell wall	ll-Diaminopimelic acid
Carbon utilization	
No carbon	–
D-glucose	+
D-xylose	++
l-arabinose	++
D-fructose	++++
Raffinose	+
D-Mainitol	+++
Meso-inositol	+++
Salicin	+++
Sucrose	+
Temperature tolerance (°C)	
26–40°C	Abundant
45	Very weak
50°C	No growth
Amino acids	
DL-Methionine	++
DL-Iso-leucin	++
L-arginine	+++
L-Lysine	+++
L-glutamic	+++
L-histidine	++
Ph-alanine	++
L-aspargine	++
L-valine	++
L-cystaine	+
Glycine	++++ ++ +++ ++
Prolene	++
Ornithine	++
Tyrosine	+++
DL-Serine	+++
NaCl % resistance	
0	Gray, abundant
2–4	White, grayish, very good
7	Yellow, weak
10–13	No growth
Antibiotic susceptibility (mm)	
Rifamyacin (RD 5 μg)	14
Vancomycin (VA 30 μg)	40
Streptomycin (S 10 μg)	00
Neomycin (N 30) μg	30
Tetracycline (TE 5 μg)	20
Nalidixic acid (NA 30 μg)	15
Novobiocin (NV 30 μg)	40
Cefodizime (CDZ 30 μg)	00

ND, not detected. +++Good growth. ++Moderate growth. +Weak growth. –Negative. *Resistant. **Highly resistance.

manual with respect to morphological, physiological, and biochemical characters [19]. In brief, SAH2_CWMSG fit into the gray series group, with negative melanin pigment, spiral hyphae and a smooth spore surface. Classification and identification of *Streptomyces* established on morphological and biochemical characterization are insufficient in most cases; consequently, molecular information, mainly rRNA gene sequences, has been announced [34].

Genotypic identification of SAH2_CWMSG isolate

The 16S rRNA is a strong tool for phylogenetic analysis and species diversity of the genus *Streptomyces*. It can be used as a genomic technique in parallel to traditional taxonomic methods, including numerical, phonetic, and other genomic analyses [36]. Consequently, in this study, molecular biology methods depending on the 16S rRNA gene sequence of the isolate were partly sequenced in equivalent to traditional methods.

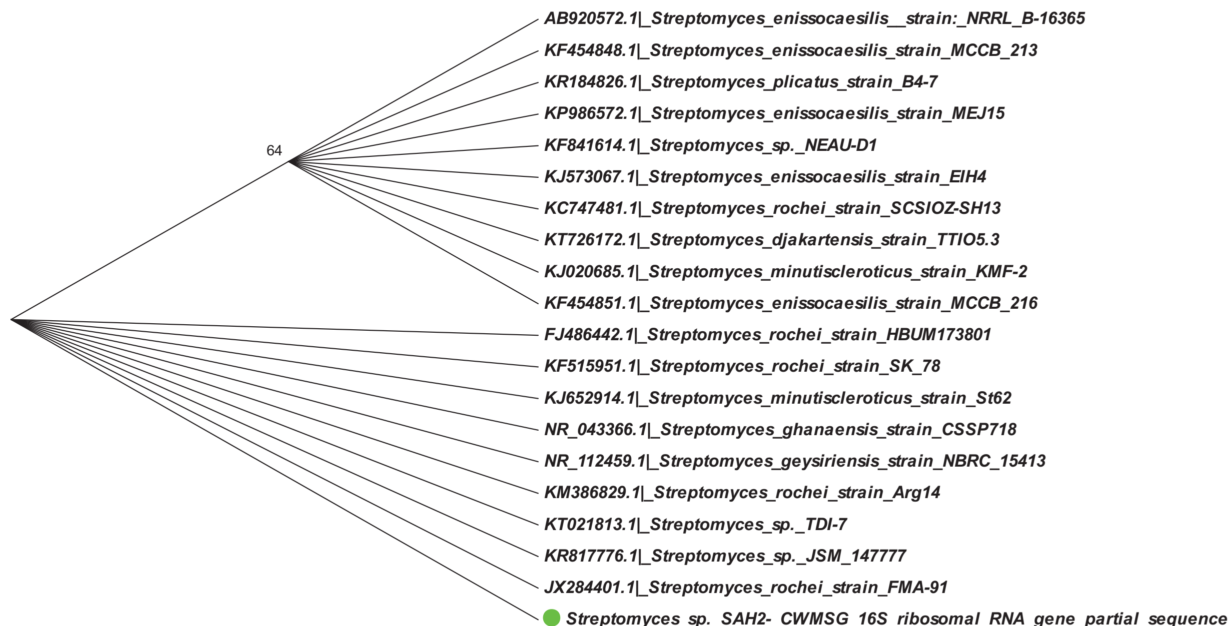
PCR amplification and phylogenetic analysis

The 16S rRNA gene of SAH2_CWMSG was amplified by the *Streptomyces*-specific PCR primers stated previously in materials and methods. The specificity of the PCR was influenced by components as the primers, the characters of the gene regions flanking the objective site, the annealing temperature in the PCR reaction and the reaction environments [37]. The primer pair used to be F27/R1492-amplified DNA, consistent with Edwards *et al.* [22].

The alignment of the 16S rRNA nucleotide sequence of SAH2_CWMSG comprised 1268 bp. The 16S rRNA stated gene sequence was coordinated in the gene bank database through the NCBI BLAST (http://www.ncbi.nlm.nih.gov). A correlation between the 16S rRNA sequence of this strain and those individuals in the genomic database bank was accomplished. This correlation demonstrated an extraordinary level of sequence similarity (99%) with *Streptomyces* spp.

The phylogenetic tree (Fig. 3) resulted from the distance matrices by the neighbor-joining approach and directed by MEGA6 [24]. The analysis included 16 nucleotide sequences. All sites in the missing gaps and misplaced information were removed. In summary, the phylogenetic investigation combined with an ordinary scheme of SAH2_CWMSG demonstrated that the nearest strain is *S. rochei* strain FMA-91. Thus, *S. rochei* SAH2_CWMSG was suggested as its name.

Figure 3



Neighbor-joining tree based on 16S rRNA gene sequences showing relationship between *Streptomyces rochei* SAH2_CWMSG and 19 isolates and closely related type strains of the genus *Streptomyces.* Only bootstrap values above 50% (percentages of 500 replications) are indicated. Bar, 0.01 nucleotide substitutions per site.

Table 3 Effect of different concentrations of ammonium sulfate on L-GLUNase activity, protein content, and specific activity

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification folds	Yield %
Crude (100 ml)	116 550	3915.000	0.03	1.00	100.00
00–50 (10 ml)	3330	0338.330	0.10	3.02	8.64
51–75 (10 ml)	5328	0314.166	0.06	0.58	92.86
76–100 (10 ml)	1998	0058.000	0.03	0.49	18.46

Nucleotide sequence Gen Bank ID

The nucleotide sequence of the 16S rRNA gene of SAH2_CWMSG strain has been submitted in Gen Bank underneath the accession number ID: KU720627.

Production of L-GLUNase by submerged fermentation

Submerged fermentation is the regularly used process for L-GLUNase production by numerous microbial strains [38,39]. In this current work, SAH2_CWMSG strain produced L-GLUNase activity of 58 U/ml/min in a specific glutamine medium under the shake flask submerged fermentation on 28°C at 120 h and 200 rpm. These results were better than those stated by Sivakumar *et al.* [15] who showed that the maximum L-GLUNase production of 17.5 IU was obtained from *S. rimosis* culture. As well as, they are greater than the results acquired by Mousumi and Dayanand [4] who exposed that the maximum L-GLUNase production (31.55±0.020 IU) was achieved in a batch bioprocess with all optimized conditions under submerged fermentation from *Streptomyces enissocaesilis* culture.

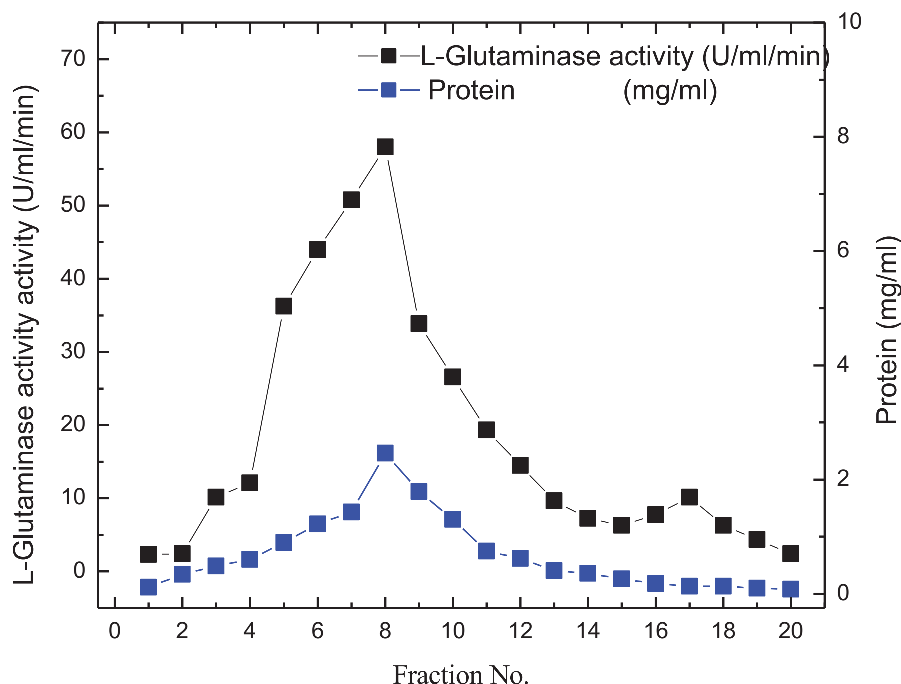
Extraction and purification of L-glutaminase

Ammonium sulfate fractionation

L-GLUNase produced by SAH2_CWMSG strain was purified using ammonium sulphate. The best fractions (6–12) yield 75% with respect to the crude enzyme (Table 3). At ammonium sulphate 75% saturation, the maximum total activity of L-GLUNase (314.166 U), specific activity (0.6 U/mg), yield (92.86%), and the purification folds (0.58) of the purified enzyme were recorded.

The former conclusions matched with that revealed by Balagurunathan *et al.* [40] who established that at 80% ammonium sulfate saturation, maximum yield of L-GLUNase was produced by *Streptomyces olivochromogenes*. Moreover, Abdallah *et al.* [41] reported that at the same ammonium sulphate saturation, the maximum total activity of the L-GLUNase (122.3 U/ml), specific activity (9.7 U/mg),

Figure 4



Elution profile of preparations obtained from *Streptomyces rochei* SAH2_CWMSG by ammonium sulphate (80% saturation) using Sephadex G-100 gel filtration chromatography.

and yield (6.6%) from *Streptomyces avermitilis* were obtained with purification fold of 2.0.

Absorbance at 280 nm indicated two peaks as shown in Fig. 4 at fractions 1–5 and 6–12. Enzyme activity estimation showed high L-GLUNase activity at peak no. 2 (fractions 6–12) only.

Physiochemical properties of L-glutaminase

The properties analyzed involved effect of temperature (30–55°C), thermal stability (40–80°C), pH (5–9), pH stability, effect of substrate concentration (L-glutamine: 1–10 mmol/l), reaction time, kinetic determination, metal ions, and other reagents on enzyme activity.

Optimum reaction temperature and thermal stability

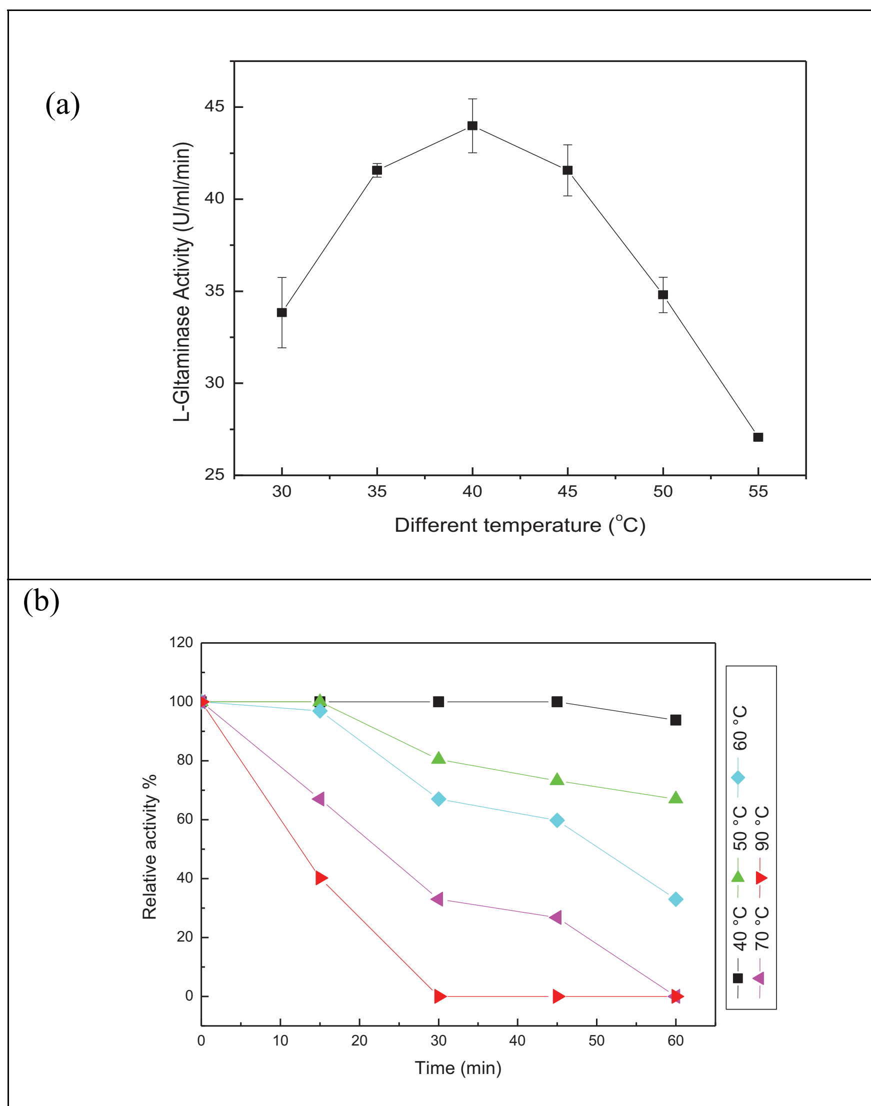
The relation between purified L-GLUNase activity and reaction temperatures (30–55°C) was carried out. Maximum enzyme activity produced by SAH2_CWMSG strain was achieved at 40°C (Fig. 5a), after which a gradual decrease in enzyme activity was noticed by increasing the reaction temperature, and almost 61% of the activity was lost at 55°C. The results obtained were in accordance with Abdallah *et al.* [41] and Desai *et al.* [42] who mentioned that L-GLUNases from *S. avermitilis* and *Streptomyces* spp. showed optimal activities at temperatures ranged from 30 to 40°C. On the contrary, Iwasa *et al.* [43] reported that L-GLUNase from *Cryptococcus albidus* ATCC 20293 exhibited maximal yield at 70°C.

The residual activity of L-GLUNase enzyme was determined at different temperatures (40–80°C) for different periods of time (15–60 min), and the relative activity was calculated. At 40°C, enzyme activity (99%) was very stable after 60 min, whereas at 50°C, the enzyme loses 20 and 27% of its activity when incubated for 30 and 45 min, respectively. At 80°C, it retains only 40% of its activity after incubating for 15 min and completely loses its activity after 30 min (Fig. 5b). Koibuchi *et al.* [44] mentioned that L-GLUNase from *Aspergillus oryzae* was stable up to 45°C and loses its activity completely at 55°C. Singh and Banik [45] found that the purified L-GLUNase from *Bacillus cereus* MTCC 1305 retained 50% and 20% of its stability at 50 and 55°C, respectively, after 30 min of incubation time. In all cases, we noticed that the enzyme activity decreased by increasing the incubation temperature and incubation period.

Optimum reaction pH and stability

The effect of the pH level of the reaction mixture on L-GLUNase was investigated via different buffers. The results revealed that the maximum activity was obtained at pH 7.5 using Na-citrate buffer (Fig. 6a). This is in accordance with Moriguchi *et al.* [46] and Rashmi *et al.* [47] who stated that the pH 7.0 was optimal for the L-GLUNase by the *Micrococcus luteus*,

Figure 5



Effect of different temperature (a) and thermal stability (b) on L-GLUNase activity produced by *Streptomyces rochei* SAH2_CWMSG. The results are presented in enzyme activity.

whereas Iwasa *et al.* [43] described that pH 6.0 was the optimum in case of *Cryptococcus albidus*.

Results in Fig. 6b clearly reveal that L-GLUNase stability increased by increasing the pH, reaching its highest stability at pH 7.5 followed by pH 7.8, after incubation overnight at 4°C. At pH 8, the enzyme retained 69% of its activity when incubated overnight. These results coincide with Roberts *et al.* [48] who achieved the highest stability of L-GLUNase from *Pseudomonas aeruginosa* at pH 7.5. Alternatively, Weingand-Ziadé *et al.* [49] denoted that L-GLUNase showed highest stability at pH 7.0 from *Lactobacillus rhamnosus*.

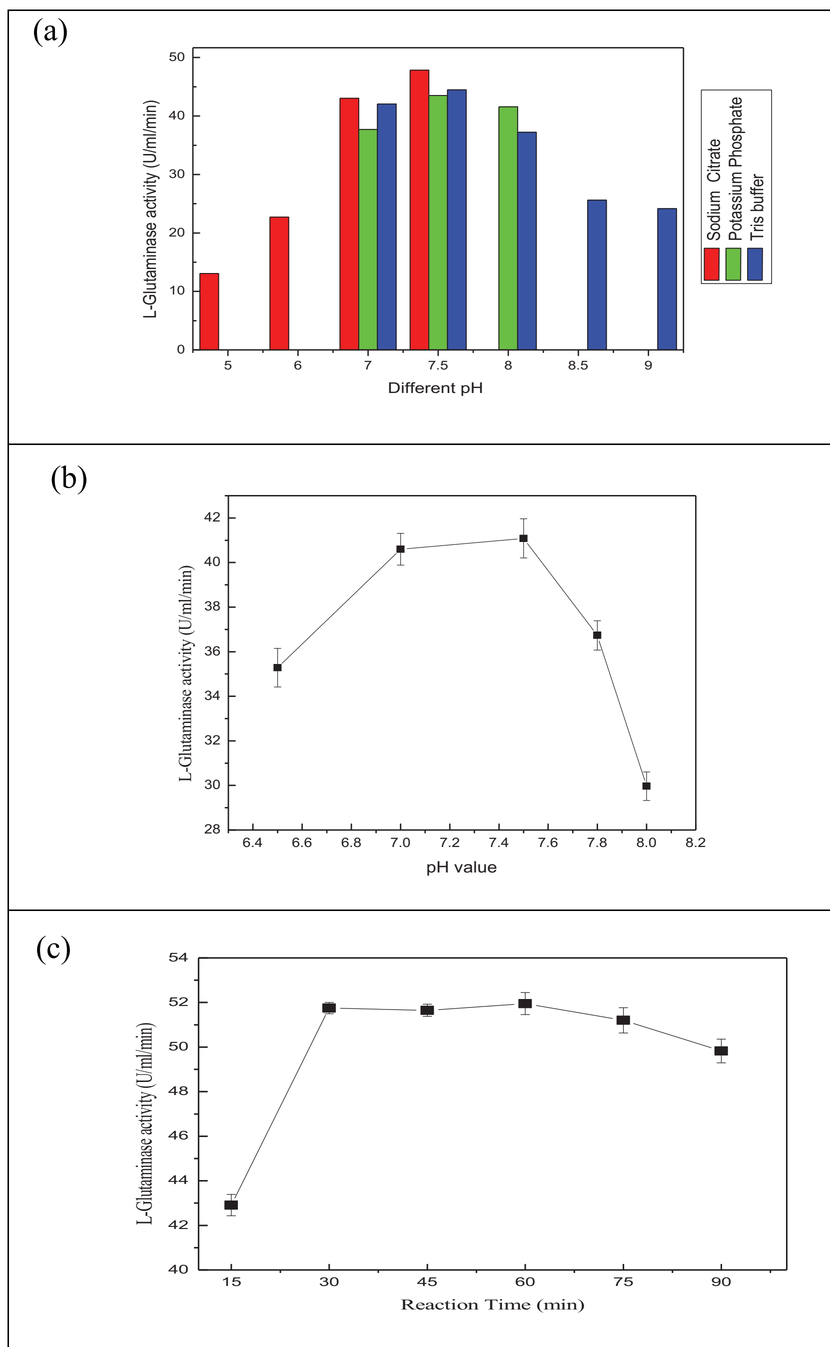
Reaction time for enzyme activity

The results in Fig. 6c showed that the maximum glutaminase activity of 52 U/ml was achieved by incubating the enzyme with glutamine substrate for 30 min at 40°C. The increasing of the incubation period was correlated with decreasing enzyme activity in a gradual manner. These results were in agreement with Fifi [16] who indicated that the pure L-GLUNase showed a maximal activity, in contrast to L-glutamine when incubated at pH 8.0 on 40°C for 30 min.

Calculation of K_m and V_{max} values for L-GLUNase

L-GLUNase activity increased with increasing of l-glutamine concentration, reaching its maximum velocity. The K_m of 1.314 mmol/l and V_{max} of 95.24 μMol/min values were anticipated from Lineweaver–Burk plots by using equation derived from non-linear regression analysis of the curve as shown in Fig. 7. These results showed lower K_m and higher V_{max}, which indicates the high affinity of L-GLUNase towards its specific L-glutamine substrate.

Figure 6



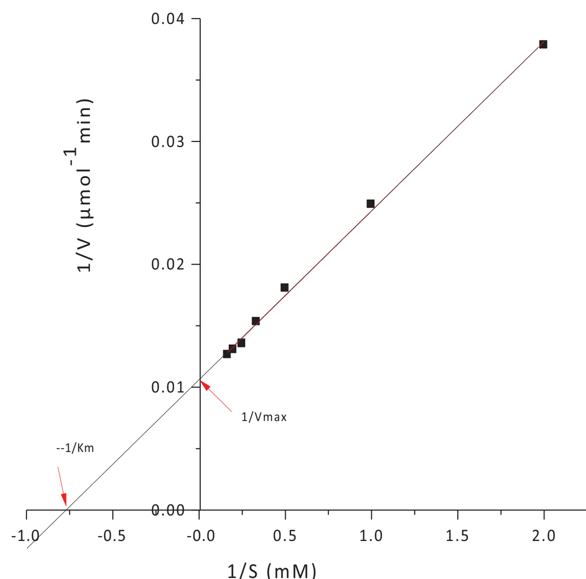
Effect of different initial pH (a), different buffers (b) and reaction time (c) on L-GLUNase activity produced by *Streptomyces rochei* SAH2_CWMSG. The results are presented in enzyme activity.

Our results were in agreement with Singh and Banik [45] who found that the V_{max} of pure L-GLUNase by *Bacillus cereus* MTCC 1305 to be 100 μmol/min/ml. On the contrary, our results contradicted with that of B?lb?l and Karakuş [50] who mentioned that the K_m and V_{max} values were 0.49 mm and 13.86 U/l, respectively, for L-GLUNase from *Hypocrea jecorina*. Our results indicated that 70 mmol/l was the optimal glutamine concentration for maximum L-GLUNase activity of 95.24 μMol/min. In agreement with these results, Huertá-Saquero *et al.* [51] reported 80 mmol/l was the optimal glutamine concentration for maximum glutaminase activity from *Rhizobium etli*.

Molecular weight determination of L-GLUNase by SDS-PAGE electrophoresis

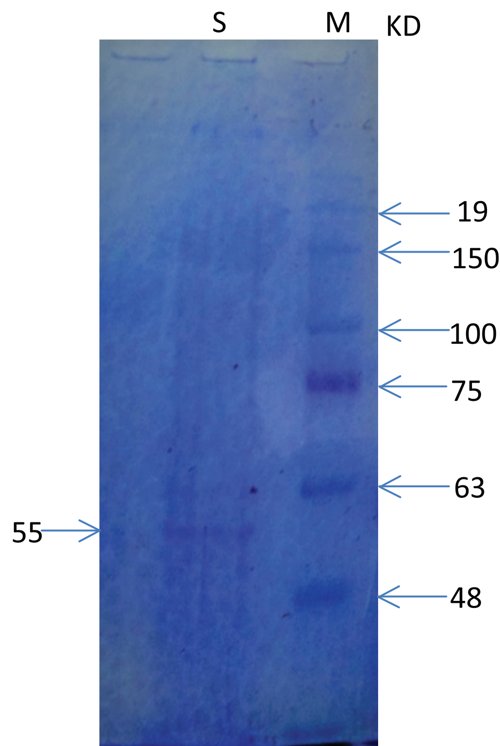
The purified enzyme indicated a single band on SDS-PAGE as shown in Fig. 8, and the molecular weight of L-GLUNase was determined to be 55 kDa. These results were in accordance with several authors such as Abdallah *et al.* [41] and Bazaraa *et al.* [52], who denoted that the purified

Figure 7



Lineweaver–Burk (double-reciprocal) plot of 1/V against 1/S giving intercepts at $1/V_{max}$ and $-1/K_m$ of L-GLUNase activity produced by *Streptomyces rochei* SAH2_CWMSG.

Figure 8



SDS-PAGE analysis of L-GLUNase produced by *Streptomyces rochei* SAH2_CWMSG. Lane 1, dialyzed enzyme concentrate of ammonium sulfate.

L-GLUNase from *S. avermitilis* and *Aspergillus oryzae* NRRL 32567 exhibited a single band on SDS-PAGE, and the molecular weight was estimated to be 50 and 68 kDa, respectively. In contrast, Wakayama *et al.* [53] and Jeon *et al.* [54] denoted that the molecular weight of purified L-GLUNase from *Micrococcus luteus* K-3 and *Lactobacillus reuteri* were 48 and 70 KDa, respectively. The variation of L-GLUNases Mecular weight from diverse sources proposed that it is microorganism specific.

Effects of metal ions and some compounds on L-GLUNase

The effect of metal ions and some compounds on L-GLUNase were tested, and the results were illustrated in Fig. 9. The enzyme activity was partially inhibited by all metal ions used, except Mn^{+2}, which activated it. These outcomes were in accordance with those of Yulianti *et al.* [55] and Dubey *et al.* [56] who mentioned the positive effect of Mn^{+2} on L-GLUNase, whereas the addition of other metal ions, for example, Zn^{+2}, Fe^{+3} and Ca^{+2}, decreased the enzyme activity. On the contrary, Jeon *et al.* [54] noted that iodoacetate inhibited L-GLUNase activity produced by *Lactobacillus reuteri* KCTC3594.

Biological activities

Antimicrobial activity screening of SAH2_CWMSG isolate

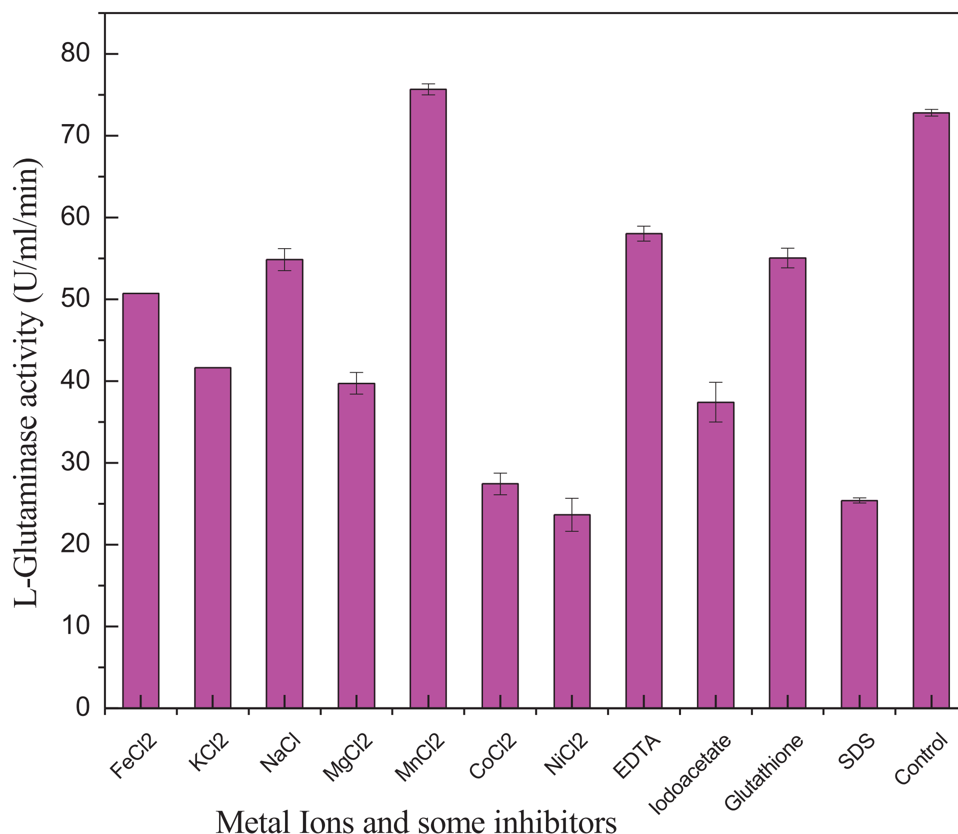
SAH2_CWMSG isolate did not show any activities against bacterial strains used, but showed antifungal activity against all tested fungi as *Fusarium solani*, *A. niger*, *Candida albicans*, *Saccharomyces cervecia*, *Fusarium oxysporium*, and *Alternaria* spp., with inhibitory diameter zone going from 13 to 20 mm, as displayed in Table 4.

In vitro antiproliferative activity of L-GLUNase

The anticancer activity of L-GLUNase was verified in contradiction of four kinds of human cancer cell lines by MTT assay *in vitro*. The results in Table 5 showed that the pure enzyme has significant efficiency against Hep-G2 cell (100%), MCF-7 cells (97.5%), and HCT-116 cell (100%) at 1000 µg/ml with IC_{50} of 279.7, 405.1, and 354.2 µg/ml, respectively. Unfortunately, the pure enzyme exhibited the same toxicity to the normal epithelium retina cell line (RPE-1), but still the IC_{50} was greater than IC_{50} of HEPG-2 cell line.

These results were in accordance with Nathiya *et al.* [3] who mentioned that L-GLUNase purified from a bacterium was able to stop a breast carcinoma with IC_{50} of 256 µg/ml. Similarly, Fifi [16] indicated that L-GLUNase has a noteworthy efficiency contrary to Hep-G2 cell (IC_{50}, 6.8 µg/ml) and a reasonable cytotoxic result against HCT-116 cell (IC_{50}, 64.7 µg/ml). The obtained outcome demonstrated the cytotoxicity of L-GLUNase counter to two cell

Figure 9



The effect of various metal ions and other reagents on L-GLUNase activity produced by *Streptomyces rochei* SAH2_CWMSG. The results are presented in enzyme activity.

Table 4 Antimicrobial activity (IZ mm) of L-GLUNase activity produced by *Streptomyces rochei* SAH2_CWMSG

Test organism	IZ (mm)
Bacteria	
Bacillus subtilis ATCC 6633	00
Staphylococcus aureus ATCC 6538	00
Escherichia coli ATCC 7839	00
Pseudomonas aeruginosa ATCC 9027	00
Yeasts	
Candida albicans ATCC 10231	00
Saccharomyces cervecia ATCC 9763	20
Fungi	
Aspergillus niger ATCC 16404	13
Fusarium oxyspoium ATCC 62506	15
Fusarium solani ATCC 36031	18
Alternaria sp. ATCC 20084	20

Table 5 The cytotoxic effect of SAH2_CWMSG strain L-glutaminase on 4 human tumor cell lines using MTT assay exposed to different concentrations of the drug for 48 h

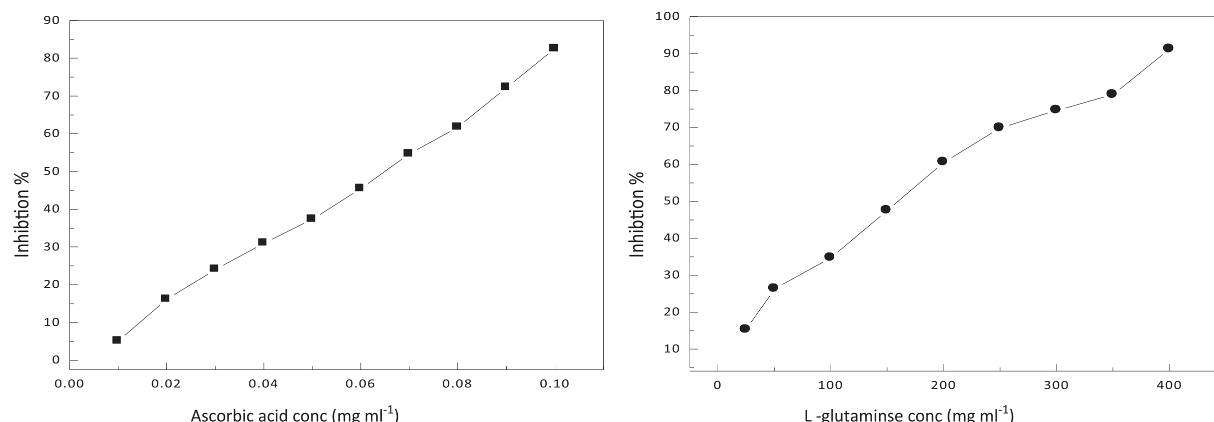
Cell line	HEPG-2	MCF-7	HCT-116	RPE-1
Percent mortality at 1000 (μg/ml)	100	97.5	100	100
IC_{50} (μg/ml)	279.7	405.1	354.2	429.2

lines and also showed that the toxic effect was dose response. The IC_{50} results stated have been assumed in more experimentation.

Antioxidant activity

The antioxidant properties are very vital owing to the deleterious role of free radicals in biological systems. DPPH assay is the most commonly used spectrophotometric procedures for determination of the antioxidant ability of plant extracts and pure compounds owing to its simple, fast, sensitive, and reproducible approach [57]. The IC50 value of L-GLUNase is 165 mg/ml, whereas the IC50 of L-ascorbic acid is 0.65 mg/ml (Fig. 10a and b). The enzyme was found to scavenge free radicals produced *in vitro*, and these findings showed L-GLUNase as an antioxidant agent. These outcomes were in agreement with Liyana-Pathirana and Shahidi [58], who revealed that L-GLUNase can scavenge radicals produced *in vitro* by DPPH assay demonstrating L-GLUNase as antioxidant. Our studies revealed that the scavenging activity increases with the increasing of L-GLUNase concentration.

Figure 10



Antioxidant activity for L-glutaminase from *Streptomyces rochei* SAH2_CWMSG using DPPH assay.

Conclusion

L-GLUNase produced from the potent *S. rochei* SAH2_CWMSG (Gen Bank ID: KU720627) holds proper features in comparison with others formerly described in literature. It is active and steady over a widespread range of pH and temperatures. L-glutamine represented the best substrate for enzyme activity. SAH2_CWMSG strain proved to be an appropriate source of L-GLUNase, which has antifungal, anticancer and antioxidant activities and could, therefore, be potentially used as enzyme supplement which has many applications in industrial and pharmaceutical fields. Further studies will be conducted in our laboratory for maximal optimization and scaling up its production.

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Conflicts of interest

There are no conflicts of interest.

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