

Antitumor L-Glutaminase Production by Rare Actinomycetes Obtained from Marine Water Using Submerged Fermentation

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ABSTRACT

Rare actinobacteria have received significant attention which are commonly categorized as strains other than *Streptomyces* or isolates with less frequency of isolation under normal parameters. These rare actinomycetes may produce important enzymes and some antibiotics. L-glutaminase is an amidohydrolase, produced by a variety of microorganisms including bacteria, yeast and fungi and catalyzed the deamination of L-glutamine to glutamic acid and ammonia. It has extensive applications as an antitumor agent. About 11 actinomycetes isolates were obtained from saline water from the Red Sea Coast on starch nitrate agar containing 10 % NaCl and some antibiotics (25 µg /ml nystatin, 25 µg/ml novobiocin and 25 µg/ml cycloheximide. at 45°C. All the isolates were screened for L- glutaminase production using phenol red as indicator. Out of 11 isolates, 5 showed excellent production and the isolate MM11 was the most active one. According to morphological and physiological characters, it was identified as identified as species of genus *Streptomyces*. Identification was confirmed using 16SrDNA and the isolate was identified as *Streptomyces* sp. MM11 and was similar to *Streptomyces barkulensis* strain RC1831 with 95% similarity level. Thus, it was identified as *Streptomyces barkulensis* MM11. Maximal enzyme production was detected in medium containing L-glutamine as carbon and nitrogen sources, respectively at pH 9.0, 40°C and after 7 days. It was clear that addition of yeast extract decreased the enzyme production. The enzyme was collected; partially purified using column chromatography. The molecular weight was determined to be 44 kD. Brine shrimp lethality test was used to predict the cytotoxic effect of the L-glutaminase. The obtained enzyme showed no toxicity and excellent antitumor activities against two cancer cell lines. In conclusion, using submerged fermentation, L-glutaminase was produced by *Streptomyces barkulensis* MM 11 using maltose and glutamine as carbon and nitrogen sources and optimizing the growth conditions enhanced the enzyme production which can be used as antitumor agent with no toxicity.

KEY WORDS: L-GLUTAMINASE, ANTITUMOR, TOXICITY, STREPTOMYCES, 16SRRNA, SCREEN, ISOLATION, ANTIBIOTIC.

ARTICLE INFORMATION

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Received 12th July 2020 Accepted after revision 28th Sep 2020

Print ISSN: 0974-6455 Online ISSN: 2321-4007 CODEN: BBRCBA

Thomson Reuters ISI Web of Science Clarivate Analytics USA and Crossref Indexed Journal



NAAS Journal Score 2020 (4.31) SJIF: 2020 (7.728)

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Online Contents Available at: <http://www.bbrc.in/>

DOI: <http://dx.doi.org/10.21786/bbrc/13.3/37>

INTRODUCTION

Actinomycetes are a heterogeneous group of Gram-positive bacteria with high G+C content in their DNA >55%. They are one of the most diverse groups of filamentous or non-filamentous bacteria, among which some genera produce spores and exhibit powdery growth (Laycock et al., 2013 Takahashi and Nakashima, 2018). The phylum actinobacteria represents one of the largest among the 30 major phyla within domain Bacteria. There are 6 classes, 18 orders, 14 suborders, 63 families and 374 genera were recorded (Subramani and Aalbersberg, 2012). The genus *Streptomyces* belongs to actinomycetes and contains the largest number, about 600 species (Han et al., 2015). It has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups (Solanki et al., 2008).

Rare actinobacteria are commonly categorized as strains other than *Streptomyces* or actinobacterial strains with less frequency of isolation under normal parameters ((Berdy, 2005, Baltz, 2006). Isolation of actinomycetes for novel compounds from conventional habitats had led to rediscovery of known compounds. However, there is still an urgent need for discovering novel secondary metabolites to combat the problem of arising number of resistant pathogenic bacteria and increasing need of more efficient enzymes in pharmaceutical industries (Rangseekaew and Pathomaree, 2019).

Consequently, the search for novel products has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual or unexplored habitats. Almost every group of organisms isolated from marine environment has unique structures. New and rare actinomycetes have been isolated from the marine environment. Rare actinomycetes may be a source of L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) which is a hydrolytic enzyme that catalyzes the deamination of L-glutamine to glutamic acid and ammonia. L-glutaminase has received significant attention with respect to its extensive applications in pharmaceuticals as an anti-leukemic agent and in food industry as a flavor enhancer (Nakadai and Nasuno, 1989, Nandakumar et al., 2003, Dhevagi et al., 2017 Subramani and Sipkema, 2019).

Another great application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian (Balagurunathan et al., 2010). L-glutaminases are widely distributed in bacteria, yeast and fungi (Nandakumar et al., 2003). L-glutaminases production have been reported from *E. coli*, *Bacillus subtilis* (Dubey et al., 2015), *Proteus morganni*, *Proteus vulgaris*, *Xanthomonas juglandis*, *Erwinia carotovora*, *Erwinia aroideae*, *Serratia marcescens*, *Enterobacter coacae*, *Klebsiella aerogenes* and *Aerobacter aerogenes* (Wade et al., 1971). Also, L-glutaminase synthesis has been reported from *Streptomyces rimosus* (Keerthi et al., 1999), *Streptomyces sp.-SBU1* (Krishnakumar et al., 2011) and *Streptomyces avermitilis* (Abdallah et al., 2013). L-glutaminase was

isolated from *Penicillium crustosum*, *Emericella nidulans* and *Mucor circinelloides*, grown on the selective medium and the effects of pH, L-glutamine concentrations, temperatures, and incubation periods on glutaminase production was studied. They recorded maximum production at pH 8, temperature 30 °C, with 0.6% of the L- glutamine. The enzyme was extracted and purified with gel filtration and ion exchange (DEAE Sephadex A50). The molecular weight has 70 kDa and showed cytotoxic activities against two cell lines (LD50: 0.067 - 0.079 mg/ml), (Khalil et al., 2020).

Recently, Masisi et al. (2020) studied the role of glutaminase in cancer, primarily focusing on breast cancer and they address the role played by oncogenes and tumour suppressor genes in regulating glutaminase. They also discussed the current therapeutic approaches to targeting glutaminase. L-glutaminase productions from microbial sources are become urgent need to over produce the enzyme with new and novel character (Prabhu and Chandrasekaran, 1997). The present study reported the production, purification and characterization of extracellular glutaminase enzyme for biotechnological applications from one of the rare actinomycetes.

MATERIAL AND METHODS

Samples collections for rare actinomycete isolation:

Marine water samples were collected from Red Sea Cost in Jeddah city, western region, Saudi Arabia. The collected samples were taken to laboratory in sterile plastic bags and stored at 4°C until used. From each sample, 0.1 ml was spread on each Petri dish plate containing Starch nitrate medium prepared with 10 % NaCl and containing 25 µg /ml nystatin, 25 µg/ml novobiocin and 25 µg/ml cycloheximide. All plates were incubated at 45°C for 5 days. The colonies which showed powdery growth were selected and transferred to slants of the same medium and preserved at 4°C. For long preservation (more than six months), strains were kept in 20% glycerol and stored at -80°C for further study (Aly et al., 2015).

Screening and selection of L-glutaminase producing isolates:

The strains were preliminary tested for L-glutaminase production by streaking on minimal glutamine agar medium (MGA) plates, containing (g/l): KCl, 0.5; MgSO₄.7H₂O, 0.5; KH₂PO₄, 1.0; FeSO₄.7H₂O, 0.1; ZnSO₄.7H₂O, 1.0; glutamine 5 as the sole carbon and nitrogen source and phenol red 0.012 as a pH indicator. All plates incubated at 45°C for 5 days. Formation of pink zones around the microbial growth indicated the positive reaction (Balagurunathan et al., 2010, Balagurunathan and Subramanian, 1993). Secondary screening for L-glutaminase production in liquid medium was carried out by inoculating the strains that showed positive result in rapid screening, in medium containing (g/l): L-glutamine, 20; yeast extract, 0.5; K₂HPO₄, 1.0; KH₂PO₄, 1.0; MgSO₄.7H₂O, 0.1; NaCl, 1.0 (Wakayama et al., 2005). After growth, the clear supernatant was used as crude enzyme (Dura et al., 2002). L-glutaminase production was measured according L-glutaminase assay method described by (Imada et al., 1973). One international unit

of L-glutaminase was defined as the amount of enzyme that liberates one μMol of ammonia under optimum conditions. The enzyme yield was expressed as Units/ml (U/ml).

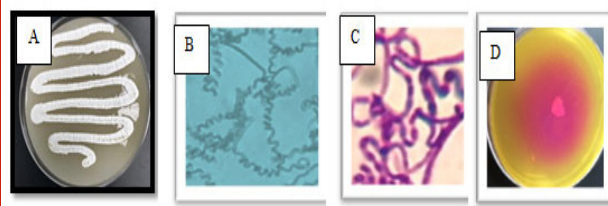
Identification of the bacterial isolate: The bacterial isolate that showing the highest L-glutaminase production was identified using morphological, physiological, biochemical and molecular studies. Molecular characterization was determined after extraction of DNA (Kumar et al., 2010). PCR amplification of the 16S rDNA of the *Streptomyces* sp. was performed using two primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAACC-3') as recommended by Hall et al. (1999).

L-glutaminase assay: Imada et al. (1973) was used for L-glutaminase assay and the mixture was incubated at 37°C for 30 min. The addition of 0.5 ml of 1.5 M trichloroacetic acid was used to stop the reaction. Enzyme activity was determined (U/ml). One unit of L-glutaminase is the amount of the enzyme that produced a μMol of ammonia.

Effect of growth factors on L-glutaminase production: Impact of different parameters, temperature, pH value, and yeast extract and incubation period on L-glutaminase production by *Streptomyces barkulensis* was investigated (Aly et al., 2017).

Purification of the L-glutaminase: After precipitation with 80% NH_4SO_4 , the enzyme was purified using Sephadex G100 column chromatography and DEAE-Cellulose column chromatography. The active peak was collected, lyophilized and enzyme molecular weight was detected (Aly et al., 2017).

Figure 1: The selected isolate MM 11 on starch nitrate agar containing three antibiotic (A), on slide agar (B), stained with Gram stain (C) and screened for L-glutaminase using phenol red as indicator (D)



Toxicity and antitumor activity: The brine shrimp lethality test was used to predict the cytotoxic effect of the natural products (Meyer et al., 1982). Varying concentrations of L-glutaminase was added to sea water, containing a counted number of live brine shrimp larvae. Control brine shrimp larvae were incubated in a mixture of sea water. After 24hr., the average number of larvae that survived in each vial was determined. The mean mortality level was plotted against the logarithm of concentrations, the concentration killing fifty percent of the larvae (LC_{50}) was determined (Meyer et al., 1982). Similarly, the antitumor activity against Ehrlich carcinoma and Lymphoma cell

line were determined. The cells were grown in RPMI 1640 medium (Sigma, USA) with 10% fetal calf serum (Gibco, USA) at 37°C under a humidified atmosphere consisting of 95% air and 5% CO_2 for 48 hr. Cells were treated with different doses of the L-glutaminase for 24 hours, centrifuged for 2 min at 1500 g and counted using hemacytometer after staining with trypan blue and removing the supernatant. The percentage of cell viability was assessed to determine the 50 % lethal dose by which 50% of cells are killed (LD_{50}) (Al-Footy et al., 2016).

RESULTS AND DISCUSSION

About 11 actinomycetes isolates were obtained from saline water of the Red Sea Coast in Jeddah city. The isolation medium was starch nitrate agar supplemented with 10% NaCl and 25 $\mu\text{g/ml}$ nystatin, 25 $\mu\text{g/ml}$ novobiocin and 25 $\mu\text{g/ml}$ cycloheximide. The incubation temperature was 45°C. All the isolates were screened for L-glutaminase production using.

Figure 2: The phylogenetic tree of the isolate MM11 and the most related genera

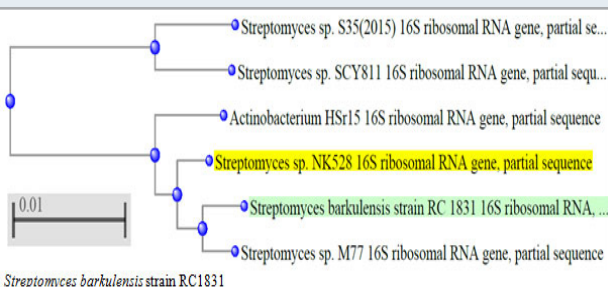
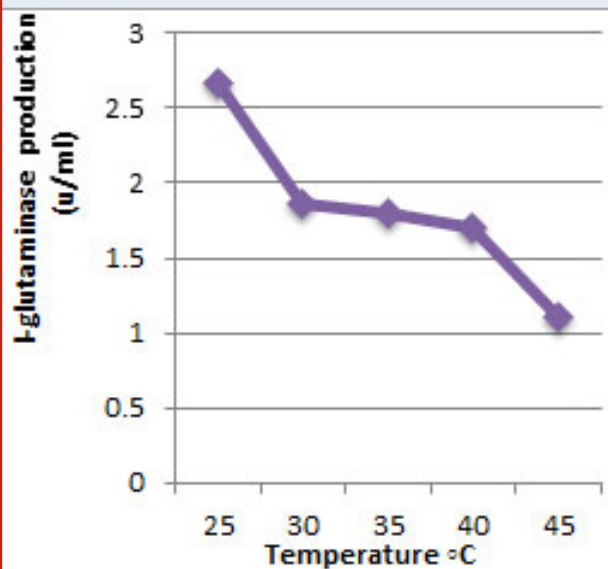


Figure 3: Effect of the temperature on production of L-glutaminase in broth medium by the isolate MM11



L-glutamine as carbon and nitrogen sources. Phenol red was added to the medium as indicator. Out of 11 isolates, 5 bacterial isolate showed the maximum growth and the diameter of the pink zone was ranged from 22 to 37 mm

(Table 1). The most active isolates were grown in liquid medium containing L-glutamine for 5 day. The isolate MM11 was the most active isolate for L-glutaminase production (Figure 1). The selected isolate was grown on different agar media, growth, color and pigment production were recorded (Table 2). Moreover, some physiological characters and resistant to some antibiotics were determined (Table 3). The growth on different carbon and nitrogen sources were reported in Table 4.

Figure 4: Effect of the pH value on production of L-glutaminase in broth medium by the isolate MM11

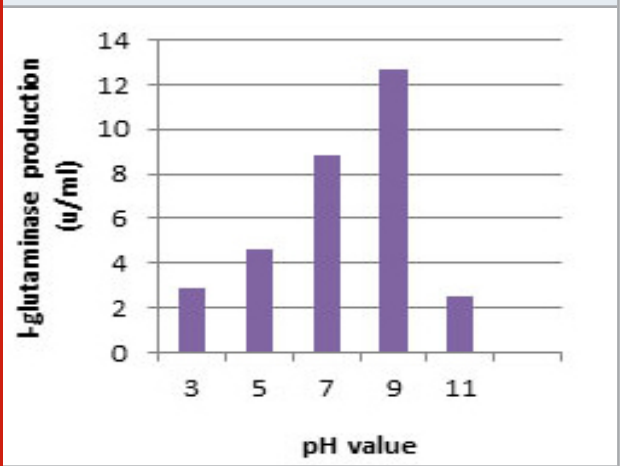


Figure 5: Effect of the yeast extract addition on production of L-glutaminase in broth medium by the isolate MM11

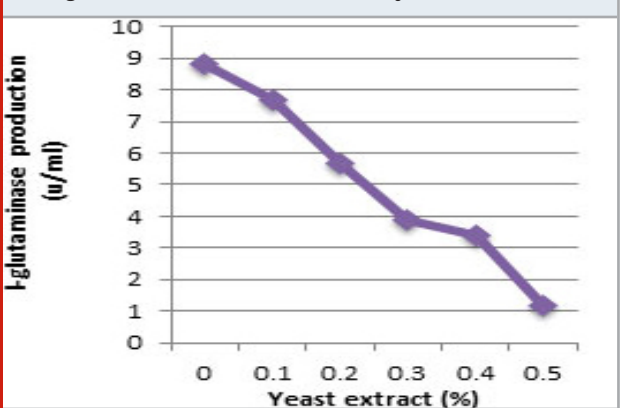


Figure 6: Effect of the incubation period on the production of L-glutaminase in broth medium by the isolate MM11

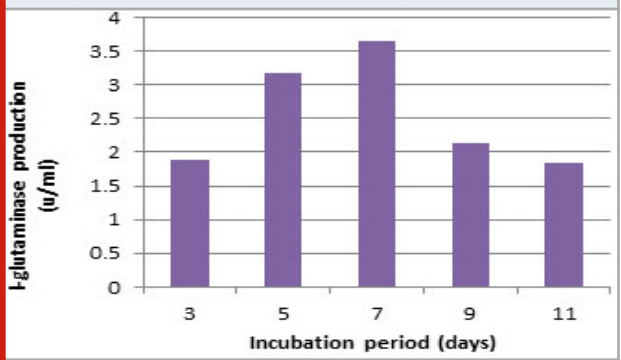


Figure 7: Elution profile of L-glutaminase of the isolate MM11 after sephadex G-100 column chromatography (A) and DEAE-Cellulose column chromatography (B).

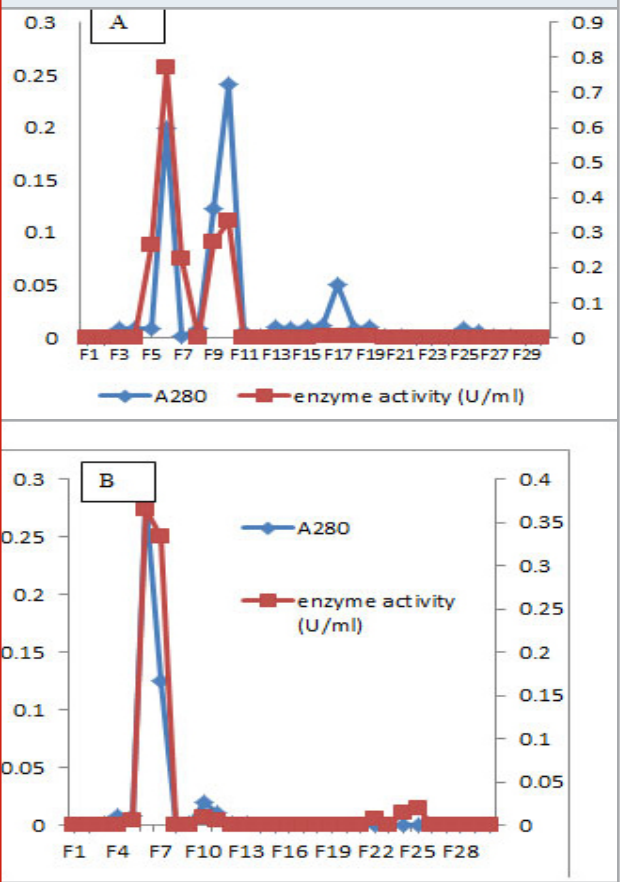
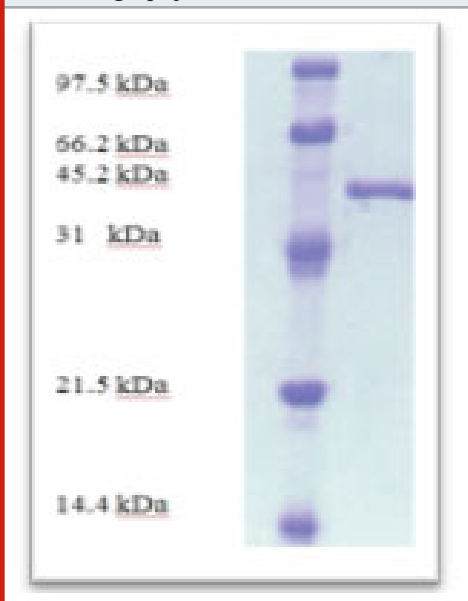


Figure 8: SDS-PAGE analysis of the purified enzymes after DEAE-cellulose column chromatography



According to morphological and physiological characters, the most active isolate was identified as species belong to genus *Streptomyces*. Identification was confirmed using 16SrRNA and the isolate was identified as *Streptomyces barkulensis* (Figure 2). Maximum L-glutaminase production was detected in broth medium contained L- glutamine as carbon and nitrogen sources at 45°C, pH 9.0, no yeast extract addition and after 7 days (Figures 3, 4, 5 and 6). Addition of yeast extracts decreased L-glutaminase production. In broth medium, the selected bacterium was grown in Lab. scale production, the

enzyme was collected and purified. After precipitation with 80% NH₄SO₄ the crude enzyme was partially purified using column chromatography. Molecular weight of the purified L-glutaminase was determined using gel electrophoresis. The molecular weight was of the pyre enzyme was determined to be 44 kDa (Figure 8). Brine shrimp lethality test was used to predict the cytotoxic effect of the L-glutaminase. The obtained enzyme showed no toxicity and excellent antitumor activities against two cancer cell line (Table 5).

Table 1. Source of some bacterial isolates from marine water and their L-Glutaminase production on minimal agar medium (Pink zone diameter/ mm) and in liquid broth medium (u/ml).

BACTERIAL ISOLATE	SOURCE	COLOR OF THE ISOLATE	GROWTH ON AGAR MEDIUM	L- GLUTAMINASE DETECTION	
				ON SOLID AGAR (DIAMETER OF PINK ZONE, MM)	IN LIQUID BROTH MEDIUM (U/ML)
MM1	MARINE WATER	BLUE	+++	33±3.31	12.10 ±2.19
MM5	MARINE WATER	DARK GRAY	+++	25±0.94	6.68 ±1.39
MM9	MARINE WATER	BLUE	+++	24±5.31	10.01±1.22
MM11	MARINE WATER	YELLOWISH WHITE	+++	37±3.39	18.14 ±2.04
MM13	MARINE WATER	DARK GRAY	+++	22±0.39	6.13 ±1.34
LSD 6.66					

Table 2. The selected actinomycetes MM11 on different media after growth for 5 days at 30°C.

MEDIA USED	GROWTH	COLOR OF AERIAL MYCELIUM	COLOR OF SUBSTRATE MYCELIUM	PRESENCE OF SOLUBLE PIGMENT
STARCH- NITRATE AGAR	HEAVY	PALE YELLOW	YELLOW	NO PIGMENT
YEAST EXTRACT- MALT EXTRACT AGAR (ISP-2)	HEAVY	WHITE	YELLOWISH WHITE	NO PIGMENT
IN-ORGANIC SALTS- STARCH IRON AGAR (ISP-4)	POOR	WHITE	WHITE	NO PIGMENT
GLYCEROL ASPARAGINE AGAR (ISP-5)	HEAVY	WHITE	WHITE	NO PIGMENT
TYROSINE AGAR (ISP-6)	MODERATE	WHITE	YELLOWISH WHITE	NO PIGMENT
E-MEDIUM (ISP-9)	POOR	WHITE	WHITE	NO PIGMENT

Table 3. Antibiotic susceptibility and physiological and biochemical tests of the selected isolate MM11

TESTS	RESULT	TESTS	RESULT
GELATINE PRODUCTION	—	AMIKACIN	SENSITIVE
MELANIN PRODUCTION	—	CEFTAZIDIME	RESISTANT
STARCH HYDROLYSIS	+	AZTREONAM	RESISTANT
CATALASE	+	PIPERACILLIN	RESISTANT
OXIDES	+	IMIPENEM	SENSITIVE
INDOLE TEST	+	CIPROFLOXACIN	SENSITIVE
METHYL RED TEST	+	AMPICILLIN	SENSITIVE

The chance of isolating new actinomycete strains with novel character is difficult and need special methods to be isolated. Addition of NaCl to the isolation medium enhanced salt tolerant actinomycetes. Similarly, addition of antibiotics to the growth medium enhanced rare actinomycete isolation. Selective isolation of actinomycetes for L-glutaminase production is of great interest for preparing new antitumor agents. Out of 11 actinomycetes isolates, 5 isolates (45.5%) were highly producer of L-glutaminase and the most active isolate was *Streptomyces barkulensis*. Screening was performed based on the activity of glutaminase (33 u/ml). Similarly, Out of 102 actinomycete isolates, only 6 *Streptomyces* isolates recorded L-glutaminase activities (Abdallah et al., 2012).

Detection of L-glutaminase in this study was recorded by diameter of the pink zone (mm) and the same method was used (Abdallah, et al., 2012). Similarly, L-glutaminase was obtained by *Streptomyces avermitilis* (Omura et al., 2001) and *Streptomyces labedae* (Han et al., 2012). Effect of various physicochemical factors on L- glutaminase production by *Streptomyces species* was detected. It was found that under the best growth conditions, rapid L-glutaminase production was found (Tobin et al., 2001). On the other hand, there is a dire need for the discovery of new drugs to effectively target the life-threatening diseases like cancers. The application of enzymes in diverse biotechnological industries tends to the discovery of novel enzymes. Members of the class actinobacteria especially *Streptomyces* spp. have long been recognized as prolific sources of useful bioactive enzyme like

Table 4. Effect of different carbon and nitrogen sources in ISP-9 medium on growth of the selected isolate MM11.

CARBON SOURCE	UTILIZATION	NITROGEN SOURCE	UTILIZATION
NEGATIVE CONTROL	++	AMMONIUM SULFATE	+
POSITIVE CONTROL (GLUCOSE)	+++	AMMONIUM	
CHLORIDE	+		
SUCROSE	++	SODIUM NITRATE	++
STARCH	+++	POTASSIUM NITRATE	+
LACTOSE	+++	GLYCINE	++
DEXTROSE	-	PEPTONE	+++
MALTOSE	+	YEAST EXTRACT	+++
GLYCEROL	+	VANILLIN	+++
XYLOSE	-	ASPARAGINE	+++

+++ : Good utilization, ++: Moderate utilization, +: Poor utilization, -: No utilization

Table 5. Toxicity (mg/ml) against *Artimia salina* and the antitumor activities (LD₅₀, mg/ml) of l-glutaminase.

Tested Product	Toxicity against <i>Artimia salina</i> (LC ₅₀)	Antitumor activity (LD ₅₀)	
		Lymphoma cell line	Erlich cell line
Filtrate	≥77.0*	≥77.0*	≥77.0*
L-glutamimase	≥55.3*	5	7.5
Control (cis platin)	≥ 3.0	3.6	≥ 3.6

* : significant results compared to contro

L-glutaminase (Nathiya et al., 2011). Therefore, current actinomycetes isolation programs are reoriented toward largely unexplored and extreme environments. The

present study has been focused on the isolation and identification of different actinomycetes from extreme habitats in Saudi Arabia. Marine environments were studied in order to unravel the diversity of actinomycetes and determine their potential as a resource for biotechnological applications. The findings obtained from this study revealed that the most active isolate was of genus *Streptomyces* (Nonomura, 1974).

L-Glutaminase, an amidohydrolase enzyme has been a choice of interest in the treatment of lymphoblastic leukaemia. The isolate was identified as *Streptomyces* sp. Effect of physicochemical factors namely temperature, pH, yeast extract concentration, and incubation period on the production of L-glutaminase from the *Streptomyces* was carried out. The enzyme production was found to be optimum in medium containing L-glutamine as carbon and nitrogen source at pH 9, temperature 45°C The L-glutaminase produced from *Streptomyces* sp. was purified by ammonium sulphate precipitation, dialysis method and ion exchange chromatography.

After the purification of the enzyme by ion exchange chromatography, it has 44 kDa. Similar results were obtained by (Okami (1986), Omura et al., 2001).

CONCLUSION

Rare actinomycetes were found in extreme environments and need special method to be isolated. *Streptomyces barkulensis* isolated from marine water in medium containing three different antibiotics. It was characterized and identified. The previous isolate showed excellent activity of L-glutaminase production. Optimization of growth conditions enhanced the production process. Using Lab. scale production the enzyme was purified and characterized. It showed no toxicity and moderate antitumor activity. Thus, it can be developed for medical uses.

ACKNOWLEDGEMENTS

This Project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (G-415-247-37). The authors therefore acknowledge with thanks DSR for technical and financial support.

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