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L- ASPARAGINASE FROM *STREPTOMYCES* sp. ISOLATED FROM THE RHIZOSPHERE OF A PALM TREE, ITS SEPARATION, PURIFICATION AND ANTITUMOR ACTIVITY

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ABSTRACT

Screening procedure was initiated in an effort to find organisms that would produce higher yields of L-asparaginase (L-Asp). In this screening, 100 bacterial isolates were examined for L-Asp production. Eleven isolates produced L- Asp on modified M9 medium containing asparagine as nitrogen source and phenol red as an indicator. The diameter of the produced pink zone was ranged from 8.9 to 23.3 mm. In liquid medium, the isolate AFP47 was the most active isolate in intracellular L-asparaginase production with 4.73 U/mg dry cells. The isolate AFP47 was identified using morphological, physiological and chemical characters as *Streptomyces* sp AFP47. Identification was confirmed with 16S rDNA and similarity level of 99% was found to genus *Streptomyces*. L-asparaginase was extracted from the cells and purified using column chromatography. The purified enzyme has 140 kDa and exhibited antitumor activity against Leukemic target cell HL60 with LD_{50} of 150 U/ml.

KEY WORDS:**L-Asparaginase; *Streptomyces*; actinomycetes; antitumor; enzyme**

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INTRODUCTION

Asparaginase was "discovered" about 35 years ago when scientists found that guinea pig serum (Kidd) suppressed the growth of lymphosarcomas[1]. Asparaginase catalyzes the hydrolysis of asparagine to aspartic acid[2]. Tumor cells, especially acute lymphoblastic leukemia cells, have low levels of asparagines synthetase that catalyzes the synthesis of asparagine. Therefore, tumor cells require an external source of asparagine. Asparaginase destroy all the asparagine that does manage to get synthesized in a tumor cell or that comes in from other sources, therefore, the cells die because they do not get asparagines which is needed for protein building. Although L-asparaginase inhibits tumor cell growth[3], studies of this phenomenon have been limited, usually because sufficient quantities of the enzyme were not available. For clinical trials, this enzyme has been obtained from microorganisms, *Escherichia coli*[4], *Erwinia cartovora*[5], *Enterobacter aerogenes*[6], *Corynebacterium glutamicum*[7], *Thermus ther mophilus*[8] and *Serratia marcescens* [9]. It is also produced from, guinea pig serum[1], chicken livers and Pisum sativum[10]. About 71% of bacterial isolates produce L- asparaginase [11] and the reported yields of this enzyme from bacterial was about 80 to 950 international units per g (dry weight) of cells[12]. The enzyme with the greater affinity to tumor cells, asparaginase II, appears to be located in the periplasmic space between the bacterial plasma membrane and the cell envelope[13]. The aim of the present studies was to characterize L-asparaginase producing bacterial isolate using biochemical and phylogenetic analysis, study factors affecting L-asparaginase activity, and determine the molecular weight of the purified enzyme. The activity of the purified enzyme as antitumor agent in vitro was determined.

MATERIALS AND METHODS

(i) Isolation, purification and identification of actinomycetes

Rhizosphere soils, marine shrimps and fresh and marine water samples were collected from Jeddah. Isolation of actinomycetes was carried out using starch nitrate medium (ST) [14], ST with 5mg/l Streptomycin, ST with 5mg/l Amphotericin B or ST with 5mg/l of both Streptomycin and Amphotericin B and incubation at 28°C for 14 days.Visible colonies were picked and subcultures on the same medium until pure cultures were obtained. The most active isolate in L-Asp production was grown on yeast extract-malt extract agar (ISP2) for microscopic observations using light and scanning electron microscopes. Cultural characteristics were studied on solid ISP media [14] and Glucose-asparagine agar[15] after incubation for 7-14 days at 28°C. For chemotaxonomic studies, mycelia and cells were harvested by centrifugation, washed and lypholized. The amino acid in the cell wall and sugar analyses of whole cell hydrolysates were performed as described by Hasegawa *et al.* [16] and Staneck and Roberts [17], respectively. Polar lipids and fatty acid were extracted and detected by previously described methods[18, 19]. Melanoid pigment production, utilization of different carbon and nitrogen sources and other physiological and biochemical tested were determined[14, 20, 21, 22, 23].

(ii) Screening for L-Asparaginase production

A rapid plate assay for screening L-asparaginase producing actinomycetes was used. A pH dye-based procedure was used and the results are obtained within 24 to 48 hr [24].

(iii) L-asparaginase production in liquid medium:

Each actinomycete isolate was grown 250 ml Erlenmeyer flasks containing 48 ml of Dextrose- Asparaginase medium[19] with dextrose 0.5% and L-asparagine 1.5% as carbon and nitrogen sources respectively. Each flask was inoculated with $4x10^5$ CFU/ml, previously grown in Starch nitrate broth at 30°C

in 24 hr. At the end of growth period, the cells were washed once with 0.05 M Tris-HCL buffer (pH 7.4), resuspended in the same buffer and disrupted using ultrasonication (Dakshin probe sonicator) for 10 min. The supernatant was collected after centrifugation at 10,000 rpm for 15 min at 4ºC and the cell free extract was subjected to intracellular L-asparaginase assay.The pellet obtained by centrifugation was dried at 60°C for 24 hr and dry weight was determined.

(iv) L-asparaginase assay

Sample *(*0.2 ml) the cell free extract was made up to 2.0 ml with 0.05 M Tris-HCl buffer (pH 7.4), containing 20 µM of L-asparagine. The reaction mixture was incubated for 15 min at 37°C in a water bath and the reaction was stopped by the addition of 0.1 ml of 1.5 M trichloroacetic acid[24]. The liberated ammonia was determined spectrophotometrically at 500 nm by nesslerization. One IU of asparaginase is the amount of enzyme, which liberates 1 µM of ammonia in 1 min at 37°C.

(v) Phylogenetic analysis of 16S rDNA sequence

Genomic DNA from the selected bacterium AFS47 was extracted using QIAamp DNA Mini Kit[25]. The primers were designed based on the highly conserved region of 16S rDNA from various bacteria[26]. The 16S rDNA gene was amplified using PCR and the PCR products were purified using QIAquick PCR purification kit (QIAGEN). The DNA sequence was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using the BLAST program.

(vi) Purification of L-asparaginase

Purification of L-asparaginase was carried out after enzyme precipitated using 80% Ammonium sulfate at 4ºC[27]. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4°C. Purification was carried out using Sephadex G-100 column and elution was performed using 0.01 M phosphate buffer pH 8.5 followed by CM-Sephadex C50 ion-exchange chromatography. The active fractions were collected, dialyzed, concentrated, lyophilized for subsequent step[28] and enzyme was assayed. The protein concentration was determined by the modified Lowry's method[29]

(vii) Molecular weight determination

PAGE a slab gel electrophoresis was carried out using a 15% polyacrylamide gel (pH 7.8) with a 5% top gel (pH 6.2). After electrophoresis in a Tris-glycine buffer (pH 8.3) at 200 V for 7 hr at 70°C, molecular weight of the purified enzyme was determined from a standard of proteins [30].

(viii) Anti-tumor Activity of the L-asparaginase

Leukemic target cell HL60 (acute promyelocytic leukemia) was cultured at 37°C in 5% CO2, in RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma Chemical Co.), L-glutamine, and antibiotics. Antitumor activity of different concentration of the purified L- asparaginase was determined[31] and % of inhibition was calculated [32]

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RESULTS

About 100 isolate of actinomycetes were isolated from different sources including fresh and marine water, rhizosphere soil and marine shrimps on starch nitrate (ST) medium with or without antibiotic addition. All the obtained actinomycete isolates were screened on medium containing phenol red for L-asparaginase detection. Growth (colony diameter) and L-asparaginase production (pink zone diameter) were measured (mm). Eleven actinomycete isolates grow well and formed a pink zone around their growth (Table 1). The pink zone resulted from the degradation of asparagine in the growth medium by asparaginase to aspartate and ammonia that changed the color of phenol red to pink. The diameter of the pink areas were ranged from 13.8-15.6 mm and maximum values were recorded for the isolate AFP47 (Fig.1A). In the liquid medium (pH 6.6), the growth of the

eleven selected actinomycete isolates was recorded. The pH was increased by the growth of all the tested isolates and was ranged from 6.94-8.73. The growth (mg/ml) and L-asparaginase production (U/mg dry wt.) were differing significantly from isolate to isolate (table 2). The productivity of L-asparaginase was slightly enhanced when the cells were disrupting using sonication.

The best L-asparaginase producer (4.73 U/mg dry weights) was the isolate AFP47 that recorded antitumor activity against LD60 cell line. It was selected for more detailed studies. The isolate AFP47 (Fig 1 B) was grown in different growth media and the aerial, substrate mycelia were described in addition to soluble pigment production. The growth was ranged from heavy, moderate to poor (table 3). Starch nitrate agar and glucose asparagine agar were good media for growth and typical morphological development. Moderate growth was found in Inorganic salts-starch iron agar, Oatmeal agar (ISP3) and Yeast extract-malt extract agar. Scanty (poor) growth was detected on glycerol asparagine agar and Tyrosine agar. Gray or pale gray pigment was detected in all tested media. Microscopic observation of the isolate AFP47 showed substrate and aerial mycelium bearing a straight chain of conidia that had rough surface (Fig 1B). The diameter of the spore was ranged from 4-5 and 6-9 µm. No zoospore, sporangium, sclerichia or fragment hyphae were present. Some physiological characters were recorded in Table 4. The bacterium grew aerobically, strongly catalase and oxidase positive. Melanin pigment was detected on ISP6, ISP7. Temperature growth range was approximately 10-45ºC and pH range was 6-9. The isolate AFP47 was resistant to a wide variety of antibiotics, including, penicillins and cephalosporins (Table 4). It was sensitive to tetracyclines, gentamycin, rifampin and Kanamycin. Galactose, mannitol, glucose, lactose, L- arabinose and sucrose are good carbon sources while suitable nitrogen sources were ammonium chloride, sodium nitrate, amino acids, yeast extract and peptone (Table 5). The isolate AFS47 showed excellent antifungal activities against all tested fungi including *Aspergillus favus, A. niger, Fusarium oxysporium, Penicillium italicum* and *Rhizopus nigricans* with inhibition zone ranged from 15-30 mm (Data not shown). Analyses of whole cell hydrolysates, revealed the presence of only L-isomer of diaminopimelic acid in addition to glucose (table 6), indicating a wall chemotype IV. Whole cell sugar pattern was glucose, indicating the whole cell sugar pattern as type A. Since the analysis of phospholipids has been using two dimension thin layer chromatography indicated the presence of phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine (phospholipids pattern type PII). Saturated fatty acids (branched and unbranched) were detected in cell hydrolysate using gas chromatography with no mycolic acids. The results 16SrDNA showed 95 % similarity to genus *Streptomyces* sp. The identified bacterium was grown in liquid medium and L-asparaginase was extracted from the cells and purified using column chromatography. The purified enzyme had 140 kDa (Fig 2).The anti-tumor activity was also determined and the results were shown in table (6). Different dilutions of the purified material were used ranging from 25 to 250 µg/ml. It was found that as the concentration of the tested material increased the % of inhibition of carcinoma cells increase, until it reached to 100 % at 250 µg/ml.

Table 1
Growth (colony diameter, mm) and L- asparaginase production (pink zone diameter, mm) of 11 actinomycete isolates.

Actinomycete isolates	Source of isolation	Isolation media	Growth (Colony diameter, mm)	L- asparaginase detection (pink zone diameter, mm)
AFW5	Water	M1	15.2 ± 0.63	18.3 ± 0.12
AFW12	Water	M1A	14.6 ± 0.44	8.9 ± 0.92
AMW42	Marine water	M1A	13.8 ± 0.42	9.2 ± 0.33
AFP47	Soil 1	M1	15.6 ± 0.62	26.5 ± 4.22
APM56	Soil 1	M1B	14.8 ± 0.42	13.3 ± 2.20
AFP62	Soil 2	M1	14.9 ± 0.12	11.1 ± 1.22
AFP 72	Soil 2	M1C	14.6 ± 0.22	10.4 ± 1.00
AFP 76	Soil 3	M1B	13.8 ± 0.92	10 ± 0.67
AFS 87	Soil 4	M1C	14.2 ± 1.20	8.05 ± 0.34
AFS95	Soil 5	M1C	14.2 ± 2.22	12.55 ± 0.45
AFM98	Shrimp	M1C	14.8 ± 1.0 2	23.3 ± 0.45
F value			19.8*	11.9*

Soil 1: Rhizosphere of palm tree, Soil 2: Rhizosphere of tomato plant, Soil 3: Rhizosphere of Jojoba, Soil 4: Rhizosphere of wheat, Soil 5: non rhizosphere soil, M1: Starch nitrate medium (ST), M1A: ST with 5mg/l Streptomycin, M1B: ST with 5mg/l Amphotericin B, M1C: ST with 5 mg/l of both Streptomycin and Amphotericin B

Table 2
Growth, L-asparaginase production and antitumor activity of the selected actinomycete isolates

Bacterial isolate	Final pH	Growth (dry weigh, mg /ml)	L-asparaginase production (U/mg dry weight)	Anti-tumor activity against LD60
AFW5	7.09	1.322 ± 0.11	4.02 ± 0.01	-
AFW12	7.43	4.648 ± 0.11	3.09 ± 0.15	-
AMW42	6.94	3.965 ± 0.11	2.84 ± 0.19	-
AFP47	8.73	4.637 ± 0.11	4.73 ± 0.41	+
APM56	8.51	5.953 ± 0.11	3.9 ± 0.31	-
AFP62	8.4	4.863 ± 0.11	2.98 ± 0.20	-
AFP 72	7.92	4.215 ± 0.11	3.74 ± 0.41	-
AFP 76	8.23	4.812 ± 0.11	3.88 ± 0.33	-
AFS 87	7.12	3.664 ± 0.11	3.03 ± 0.21	-
AFS95	7.61	5.901 ± 0.11	4.21 ± 0.31	-
AFM 98	7.71	4.924 ± 0.11	4.05 ± 0.37	+
LSD	0.001	0.339	0.348	

Each value is the mean of three readings ± SD.

Table 3
Cultural characteristics of the actinomycete isolate

Agar media	Growth	Color of aerial mycelium	Color of substrate mycelium	Color of soluble pigment
Starch-nitrate	Heavy	Pale gra	Pale yellow	Pale gray
Yeast extract-malt extract (ISP-2)	Moderate	Dark gray	Yellowish gray	Pale gray
Oatmeal medium (ISP-3)	Moderate	Light grey	Pale gray	Gray
Inorganic salts-starch iron (ISP-4)	Moderate	Grey	Pale gray	Gray
Glycerol-asparagine (ISP-5)	Scanty	White	White	Pale gray
Tyrosine (ISP-7)	Scanty	Pale grey	Gray	Gray
Glucose- asparagine	Heavy	Grey	Dark gray	Pale gray

Table 4
Physiological properties of the actinomycete isolate AFP47

Characteristic	Result	Characteristic	Result
Melanin production		Tolerance to NaCl	5-12%
On ISP medium 6	+	pH range	6-9
On ISP medium 7	+	Growth temperature	10 - 45°C
Decomposition of		Nitrate reduction	+
Xanthine	+	H_2S production	+
Hypothanthine	+	Resistance to antibiotic	
Urea	+	Penicillin	+ve
Casien	+	Cephalosporine	+ve
Hydrolysis activities:		Kanamycin	-ve
Chitin	+	Rifampin	-ve
Gelatin	+	Tetracyclines	-ve
Pectin	+	Gentamycin	-ve

Table 5
Utilization of carbon and nitrogen sources by the selected actinomycete isolate AFP47

Carbon source	Utilization	Nitrogen source	Utilization
Galactose	+	Na NO_3	++
L- Arabinose	+	NH_4Cl	++
Mannitol	+	$NaNO_2$	-
D-Sorbitol	-	Valine	+
Glucose	+	Phenyl alanine	+
Sucrose	+	Peptone	+
Lactose	+	Yeast extract	+

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

Table 6
The biochemical tests (sugar, amino acid, phospholipids, and fatty acid composition of the cell wall or cell hydrolysate) of the selected isolate AFP47

Type of the reaction	Results
Sugar in the cell hydrolysate	
Glucose	+
Amino acids in the cell wall	
Diaminopimelic acid (DAP)	L-Form
Glutamic acid	+
Lysine	-
Phospholipids	
Phosphatidylethanolamine	+
Phosphatidylcholine	+
Phosphatidylinositol	+
Fatty Acids	Iso and antiso fatty acids

+: Present, - : Absence

Table 7
Effect of different concentrations of the purified L-asparaginase on the viability of tumor cell line In vitro.

Dose (µg/ml)	% of inhibition of cell viability
25	10
50	25
100	40
150	60
200	85
250	100

Figure 1

The selected actinomycete isolate AFP47 on screening medium containing phenol red as indicator (A), on starch nitrate medium (B) and under scanning electron microscope (C)

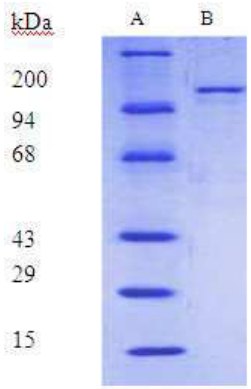

Figure 2.

Polyacrylamide gel electrophoresis of the purified L-asparaginase, Lane A: protein standard; Lane B: purified intracellular enzyme

DISCUSSION

Actinomycetes are abundant in nature and have been isolated from water, fodder, wastes and various types of soils including rice paddy, lake mud and water, deciduous forest, tropical forest, wasteland and cave soils [19,33,34]. Out of 100 actinomycetes, 11 % were highly producer of L-asparaginase that was detected in solid medium using the plate assay method with phenol red as indicator. Similarly, out of 40 bacterial isolates, only six strains (15%) showed significant L-asparagianse production[35] and a direct correlation between L-asparaginase production and diameter of the red zone produced[35]. The plate assay method have many advantages as the method is quick and L-asparaginase production can be visualized directly from the plates without performing time consuming assays[24] . The best active isolate was AFS47 which belonging to gray serious and was isolated from rhizosphere soil of palm tree, grown in Al Madinah, Saudi Arabia. Malibari [37] and Ara et al. [38] reported that the highest densities of actinomycetes was found in soil samples, collected from Al Mademah city which may due to the organic materials and exudates derived from the plant roots. Moreover, Tewtrakul and Subhadhirasakul[39] further support this statement, where they obtained a correlation relationship between the diversity of actinobacteria with the type of plants and soil organic matter content. Actinomycetes had been known to possess the ability to produce bioactivities such as pesticides, herbicides, antibiotics[40,41] and enzymes including Asparaginase. The most active L-asparaginase producer isolate has wall chemotype IV, whole cell sugar type A, phospholipids PII pattern and saturated fatty acids. According to morphological, physiological, biochemical and 16S rDNA analysis, it was identified as species belonging to genus *Streptomyces*[20].

Genus Streptomyces is providing a good source of L-asparaginase production when compared to bacterial and fungal sources[35] and about 38% of the actinomycete isolates produced L-asparaginase[42]. Using gel electrophoresis, the molecular weight of the enzyme was recorded as 140 kDa whatever the molecular weight was 97.4 kDa[43], 160 kDa[44] and 80 kDa[7] for L- asparaginase from *Streptomyces tendae* TK-VL 333, *Pseudomonas aeruginosa* 50071 and *Corynebateriun glutamicum*.Effective depletion of L- asparagine results in cytotoxicity for leukaemic cells and tumour inhibitory activity has been demonstrated only with asparaginases from *E. coli, Erwinia aroideae* and *Serratia marcescens*[45]. The administration of such enzyme that is a protein for a long duration, in general, produces the corresponding antibody in the tissues, resulting in anaphylactic shock or neutralization of drug effect. Therefore, the use of new serologically different L-asparaginase with a similar therapeutic effect is highly desirable and actinomycetes may be a potential source of high yield, high substrate specificity L-asparaginase, which act as an anti-leukaemia agent[3].

CONCLUSION

In conclusion, actinomycetes are a good source of L- asparaginase enzymes that exhibit antitumor activities.

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