Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EVALUATION OF ANTITUMOR ACTIVITY OF GLUTAMINASE FREE L-ASPARAGINASE FROM INDIGENOUS BACTERIAL STRAINS FOR POTENTIAL CHEMOTHERAPEUTIC APPLICATION

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ABSTRACT

A total of 154, bacterial strains were isolated from rhizospheric soil, river water and were screened for L-asparaginase activity, out of which 54 bacterial strains were found to be L-asparaginase positive strains. These L-asparaginase positive strains (54) were further screened for their glutaminase activity. Among them six bacterial strains exhibiting glutaminase free L-asparaginase activity were selected and identified as *Pseudomonas otitidis*, *Ochrobactrum anthropi, Paenibacillus lactis, Enterobacter cloacae, Bacillus subtilis* and *Escherichia fergusonii* on the basis of morphological, cultural, biochemical characteristics and 16S rRNA gene sequencing. L-asparaginases from these bacterial strains were evaluated for their antitumor activity against breast cancer (T47D, MDA-MB-231) and T-lymphoblast acute lymphoblastic leukemia (MOLT-4) cell lines and also for their *in vitro* serum and trypsine digestion half-life. Extracellular L-asparaginase from *P. otitidis* showed maximum 82.5±0.02% and 28.84±0.02% growth inhibition towards T47D and MDA-MB-231 cells, respectively and 37 h and 30 min *in vitro* serum and trypsin half life respectively. These results demonstrated that *P. otitidis* L-asparaginase possess effective antitumor activity, prolonged *in vitro* serum and trypsin digestion half-life as compared to previous reports. Thus, glutaminase free L-asparaginase from *P. otitidis* can be further explored for improving L-asparaginase therapy and other biotechnological applications.

KEYWORDS:Acute Lymphoblastic Leukemia, Glutaminase, L-Asparaginase, Rhizosphere, MTT assay, Serum half life

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INTRODUCTION

L-Asparaginase (L-Asparaginase amidohydrolase E.C. 3.5.1.1) is an enzyme of high anti-neoplastic value; due to which it is used in the treatment of children with ALL and other lymphoproliferative malignancies[1]. This enzyme is also used in food processing industries, for the production of acrylamide free foods[2]. The anti-neoplastic action of L-asparaginase is explained on the fact that certain tumor cells, more specifically ALL tumor cells lack or are unable to synthesize the non-essential amino acid L-asparagine *de-novo* due to absence of L-asparagine synthetase but they require huge amount of L-asparagine to keep up their rapid malignant growth. To fulfill their nutritional requirement they use serum L-asparagine. L-Asparaginase, as a chemotherapeutic drug rapidly hydrolyses amide group of the serum L-asparagine into L-aspartate and ammonia, under physiological conditions[3]. As a result, ALL tumor cells selectively die due to rapid and complete depletion of the serum L-asparagine while, most of the normal cells remain unaffected due to the presence of L-asparagine synthetase[4]. For this reason, L-asparaginases have been used since 1970, and have become an important, integral part of modern combinational chemotherapy protocols of pediatric ALL[5]. L-Asparaginases are widely distributed in nature including microbes, animal tissues and plants species but, microorganisms are the more efficient, inexpensive and leading sources of this enzyme. However, the biochemical and chemical kinetic properties of the enzyme vary with genetic nature of the organisms. To date, L-asparaginases isolated from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) has been used in clinical purposes[6], but L-asparaginase from both these sources have intrinsic glutaminase activity[4]. They hydrolyzes glutamine of circulating system to monosodium glutamate, which is responsible for a wide range of serious toxicity including leucopenia, immunosuppression, acute pancreatitis, thromboembolysis, hyperglycemia and neurological seizures etc[7]. Most of the microbial L-asparaginases reported, are intracellular, thus their extraction is labour intensive with low yield and excess loss of enzyme activity. These complications are responsible for high cost of this potent enzymatic drug which restricted their clinical applications. Therefore, for the successful treatment of ALL and other lymphoid malignancies, low cost glutaminase free L-asparaginase is highly desirable which have similar or more effective chemotherapeutic property than the previously reported ones. The existing problem related to therapy of this noble drug can be solved with the isolation of glutaminase free L-asparaginase producing bacterial strains from natural habitats. Keeping this in view, the rhizospheric soil and river water samples were screened for glutaminase free L-asparaginase producing bacterial strains and their antitumor potential was evaluated.

MATERIALS AND METHODS

Anhydrous L-asparagine, glutamine, trichloroacetic acid (TCA), Folin-Ciocalteu's phenol reagent were purchased from HiMedia, Mumbai, India. Primers, *Taq* DNA polymerase and dNTP, purification kit were obtained from Bangalore Genei (India). *E. coli* L-asparaginase, RPMI-1640 medium, Fetal bovine serum (FBS), 3-(4, 5,-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, were purchased from Sigma chemical Co., USA. All the chemical used were of analytical grade and purchased from standard sources.

Isolation and screening of L-asparaginase producing bacterial strains

Total 16 rhizospheric soil and 6 water samples were collected from various sites of State Forest Research Institute, agriculture fields and different sites of river Narmada, Jabalpur, Madhya Pradesh, India. Samples were enriched into growth medium containing (g/l) 15g L-asparagine and 10g yeast extract[8]. L-Asparaginase producing bacterial strains were isolated by plating serially diluted enriched sample on modified M-9 medium containing (g/l) 6g $Na_2HPO_4.2H_2O$, 3g KH_2PO_4, 0.5g NaCl, 5g L-asparagine, 2ml 1M $MgSO_4.7H_2O$, 1ml 0.1M $CaCl_2.2H_2O$, 2g glucose, 20g agar,

0.009% phenol red, at 37ºC for 16 h of incubation[9]. Pink zone forming bacterial colonies were isolated, purified on LB agar medium. Further, purified colonies were point inoculated on modified M-9 medium and the zone index was calculated as:

$$\text{Zone index} = \frac{\text{Diameter of zone produced by L-asparaginase (mm)}}{\text{Diameter of bacterial colony (mm)}}$$

The cultures were maintained in LB agar slants at 4°C for further use.

Cultivation medium and crude enzyme preparation

For quantitative estimation of the enzyme activity, modified basal semi-synthetic broth medium containing (g/l): 5.20g glucose, 2.07g L-asparagine, 6g $Na_2HPO_4.2H_2O$, 1.77g KH_2PO_4, 0.37g $MgSO_4.7H_2O$, 0.015g $CaCl_2.2H_2O$, 1.0g yeast extract 1.0g peptone (pH 7), was used[10]. The 24 h old bacterial culture was aseptically transferred in 20 ml pre-autoclaved modified basal semi-synthetic broth medium in 250 ml Erlenmeyer flask and incubated at 37ºC in orbital shaking incubator at 180 rpm for 24 h. After incubation cells, were removed by centrifugation at 10,000 rpm for 5 min at 4°C and cell free supernatant was used to analyze extracellular L-asparaginase and glutaminase activity.

L-Asparaginase and glutaminase assay

The L-asparaginase activity was measured by the standard method of Wriston[11] using Nesslerization reaction. The reaction was started by adding 0.1 ml crude enzyme preparation in 0.9 ml of pre-warmed 0.01M L-asparagine prepared in 0.05M Tris-HCl buffer (pH 8.6), mixed by vortexing and incubated for 30 min at 37°C. The reaction was terminated by the addition of 0.1ml, 1.5 M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 10,000 rpm for 5 min at room temperature and the precipitate was separated. 0.25 ml Nessler's reagent was added into tubes containing 0.5 ml supernatant and 1.75 ml distilled water. The content of the tubes was vortexed and incubated at room temperature for 10 min. The optical density was determined at 480 nm against the blanks that contains TCA before enzyme addition. The ammonia produced was calculated on the basis of a standard curve obtained with ammonium sulfate as the standard. One L-asparaginase unit (IU) is defined as the amount of enzyme that librates 1µmol of ammonia under standard assay conditions. Glutaminase activity was determined by Nesslerization method[12] using glutamine as the substrate instead of L-asparagine.

Identification of bacterial strains

Glutaminase free L-asparaginase producing six bacterial strains were characterized on the basis of morphological, cultural and biochemical characteristics following Bergey's Manual of Systematic Bacteriology[13] and results were analyzed by Probabilistic Identification of Bacteria (PIB) computer kit[14]. Further, 16S rRNA gene amplification was done using two universal *eubacterial* oligonucleotide primers, 8F, 5'-AGAGTTTGATCCTGGCTCAG-3' 1492R 5-GGTTACCTTGTTACGACTT-3', according to the conditions previously described by Weisburg et al (1991)[15]. The amplified PCR products were purified using the purification kit (Bangalore Genei) according to the supplier's instructions and sequenced by using an automated sequencer. The DNA similarity was determined by BLAST search tool and sequences are submitted in the National Centre of Biotechnology Information (NCBI), GeneBank database.

Cell cultures and treatment

Human T-lymphoblast acute lymphoblastic leukemia cell line MOLT-4 and Breast cancer cell lines; MDA-MB-231 and T47D were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown in RPMI-1640 medium containing 10% FCS, 100U penicillin/100 µg streptomycin/ml, in CO_2 incubator (Thermocon Electron Corporation, USA) at 37°C with 98% humidity and 5% CO_2 gas environment. Cells were treated with filter sterilized 0.1 IU/ml crude L-asparaginase solution during the logarithmic growth phase.

In vitro cytotoxicity against human cancer cell lines

The cytotoxic effect of L-asparaginase was measured against human cancer cell lines including MDA-MB-231, T47D and MOLT-4 by using the MTT assay, according to the method of Mosmann[16]. Briefly, $1.2x10^4$ to $1.5x10^4$ exponentially growing cells were seeded in 96-well microtiter plates, containing 0.1 ml RPMI-1640 medium, with10% fetal bovine serum. Plates were incubated at 37°C in CO_2 incubator with 5% CO_2. Further, 0.1ml/well crude L-asparaginase (0.2 IU/ml stock) was added and after 48 h of incubation cell viability was measured by adding 10µl/well of MTT dye (5mg/ml stock). Plates were again incubated at 37°C in CO_2 incubator for 4 h. The formazan blue crystal, formed by viable cells, was dissolved with 150 µl DMSO. The rate of color production was measured at 570 nm with ELISA reader. The results are expressed as the percent of cell growth inhibition relative to that of untreated control cells.

Cell survival % = (O.D Treated well/Mean O.D control well) x 100%

In vitro serum half life

In vitro half life of L-asparaginase in human blood serum was measured by adding 0.4 ml enzyme (2 IU) into 3.6 ml human blood serum. After mild homogenization, the reaction mixture was incubated at 37±1°C and the enzyme activity was determined in 0.1 ml reactive plasma containing L-asparaginase enzyme at regular intervals of 3 h till the enzyme was found to be active[17].

In vitro trypsin digestion half life

3 IU (0.6 ml) of L-asparaginase was added in 1.6 ml of 0.05M phosphate buffer (pH 7.4) and then 80 µl trypsin solution (5 mg/ml stock) was added. Reaction mixture was homogenized vigorously, incubated at 37±1°C and the enzyme activity was determined in 0.1 ml reactive trypsin containing L-asparaginase enzyme at regular intervals of 5 min till the enzyme was found to be active [17].

Statistical analysis

Statistical analysis was done by using student t-test and p* value<0.001 was considered to be significant.

RESULTS

Isolation and screening of L-asparaginase producing bacterial strains

A total of morphologically different 154, bacterial strains were isolated from rhizospheric soil, river water and were screened for L-asparaginase activity. Among them 54 bacterial strains were found to be L-asparaginase positive strains on the basis of pink zone formation on modified M-9 agar medium (Fig 1) and their colony size and zone diameter were measured. All positive bacterial strains incubated at 37°C produced extracellular L-asparaginase ranging from 0.17 to 2.22 IU/ml (data not shown) in the medium containing L-asparagine as a sole carbon source. Further, all 54 bacterial strains were screened for their intrinsic glutaminase activity and 6 bacterial strains were found to be glutaminase free L-asparaginase producers. All the six glutaminase free L-asparaginase producing bacterial strains i.e. BGCC#2385 (GG1), BGCC#2391 (LG2), BGCC#2388 (BO1), BGCC#2389 (CO1), BGCC#2390 (CO3) and BGCC#2386 (CA3) showed 0.24, 0.88, 0.59, 0.79, 0.89, and 0.84 IU/ml enzyme activity, respectively and were used for further studies (Table 1).

Figure 1
L-***Asparaginase screening plates (a) control without phenol red (b) control $NaNO_3$ instead of L-asparagine (c) control without inoculation (d) L-asparaginase producing colonies (e) primary screening of L-asparaginase producers.***

Table 1
L-asparaginase activity of glutaminase free L-asparaginase producing bacterial strains isolated from rhizospheric soil and river water.

Bacteral isolates code	Source	Qualitative L-asparaginase activity			Quantitative L-asparaginase activity			Glutaminase activity (IU/ml)
		Zone size (mm)	Colony size (mm)	Zone index (mm)	L-Asparaginase activity (IU/ml)	Protein concentration (mg/ml)	Specific activity (IU/mg)	
GG1	river water	5	3	1.66	0.24	2.69	0.09	-
LG2	river water	7	5	1.4	0.88	1.75	0.50	-
BO1	rhizosphere	8	3	2.66	0.59	1.86	0.31	-
CO1	rhizosphere	13	9	1.44	0.79	2.13	0.37	-
CO3	rhizosphere	6	3	2	0.89	1.71	0.52	-
CA3	rhizosphere	10	6	1.66	0.84	2.72	0.30	-

Identification of bacterial strains
Glutaminase free L-asparaginase producing six bacterial strains were identified on the basis of morphological, cultural, biochemical tests and 16S rRNA gene sequence analysis. The partial sequence of each strain aligned and clustered with sequences from NCBI database. Results of the morphological, cultural, biochemical and 16S rRNA gene sequencing analysis confirmed the identity of these bacterial strains as *E. fergusonii* (GG1), *P. lactis* (LG2), *P. otitidis* (BO1), *E. cloacae* (CO1), *O. anthropi* (CO3) and *B. subtilis* (CA3) the 16S rRNA gene sequences were submitted to GeneBank database accession number KF607093, KF607091, KF607097, KF607094, KF607096 and KF607095, respectively. All six bacterial strains are maintained in Bacterial Germplasm Collection Centre (BGCC), Bacteriology Laboratory, Department of P. G. Studies and Research in Biological Science, R. D. University, Jabalpur (M.P.) and assigned their BGCC number as BGCC#2385 (*E. fergusonii*), BGCC#2391 (*P. lactis*), BGCC#2388 (*P. otitidis*), BGCC#2389 (*E. cloacae*), BGCC#2390 (*O. anthropi*) and BGCC#2386 (*B. subtilis*) (Table 2).

Table 2
Identification of glutaminase free L-asparaginase producing bacterial strains isolated from rhizospheric soil and river water.

Characteristics	Bacterial strains					
	GG1	LG2	BO1	CO1	CO3	CA3
Shape	rod	Rod	rod	rod	rod	rod
Gram staining	-	+	-	-	-	+
Motility	+	-	+	+	+	+
Catalase	+	-	+	+	-	+
Oxidase	-	-	+	-	+	+
Indole	+	-	-	-	-	+
Methyl red	-	+	-	-	+	-
Voges Proskauer	-	-	-	+	+	+
Citrate	-	-	+	+	-	+
H_2S (TSI)	+	-	-	-	-	+
Urease	+	-	-	-	+	+
Glucose	+	+	+	+	+	+
Galactose	+	-	-	+	+	+
Mannose	+	+	-	+	+	+
Fructose	-	+	-	+	+	+
Sucrose	+	+	-	+	+	-
Maltose	+	+	-	+	+	+
Lactose	-	+	-	+	-	-
Arabinose	+	+	-	+	-	+
Trehalose	+	+	-	+	+	+
Cellulose	+	+	-	+	+	-
Millibiose	-	+	-	+	-	-
Dulcitol	+	-	-	-	-	+
Mannitol	+	+	-	+	-	+
Adonitol	+	-	-	-	+	-
Rhamnose	+	-	-	+	+	-
Inuline	-	-	-	-	-	-
Raffinose	+	+	-	+	-	-
Xylose	+	+	-	+	-	+
Salicin	+	-	-	+	-	+
Lysine	+	-	-	-	-	-
Ornithine	+	-	+	+	-	-
Arginine	-	-	+	+	+	-
Nitrate reductase	+	+	-	+	-	+
Esculine	-	+	-	+	-	-
Starch hydrolysis	-	+	+	+	-	+
Potassium cyanide	-	-	-	+	-	+
Gelatin hydrolysis	+	-	+	-	-	+
Gluconate	-	-	-	-	-	+
Malonate	-	-	-	+	-	+
16S rRNA gene sequence length (bp)	1203	1484	748	1473	780	1501
NCBI Accession no	KF607093	KF607091	KF607097	KF607094	KF607096	KF607095
BGCC no	BGCC#2385	BGCC#2391	BGCC#2388	BGCC#2389	BGCC#2390	BGCC#2386
Identified species	*E. fergusonii*	*P. lactis*	*P. otitidis*	*E. cloacae*	*O. anthropi*	*B. subtilis*

Cytotoxic activity of glutaminase free L-asparaginase

The antineoplastic activity of crude glutaminase free L-asparaginase produced by six selected bacterial strains were studied using breast cancer cell line (T47D, MDA-MB-231) and T-lymphoblast acute lymphoblastic leukemia (MOLT-4) cell line which resulted in appreciable inhibition of the cell growth. The T47D cells were found to be more sensitive towards L-asparaginase produced by *P. otitidis*, *P. lactis* and *E. fergusonii* in which 82.5±0.02%, 63.72±0.01% and 84.54±0.04% growth inhibition was recorded, while only 8.02±0.09% growth inhibition was observed by *E. coli* L-asparaginase (procured from Sigma chemical Co., USA.) which was used as reference preparation. Percent growth inhibition of MDA-MB-231 cells was found to be 78.7±0.09%, and 68.83±0.07%, by *E. cloacae* and *O. anthropi* L-asparaginase, respectively. However, MOLT-4 cells were found to be less sensitive for all selected bacterial L-asparaginases except *E. fergusonii* (Fig 2).

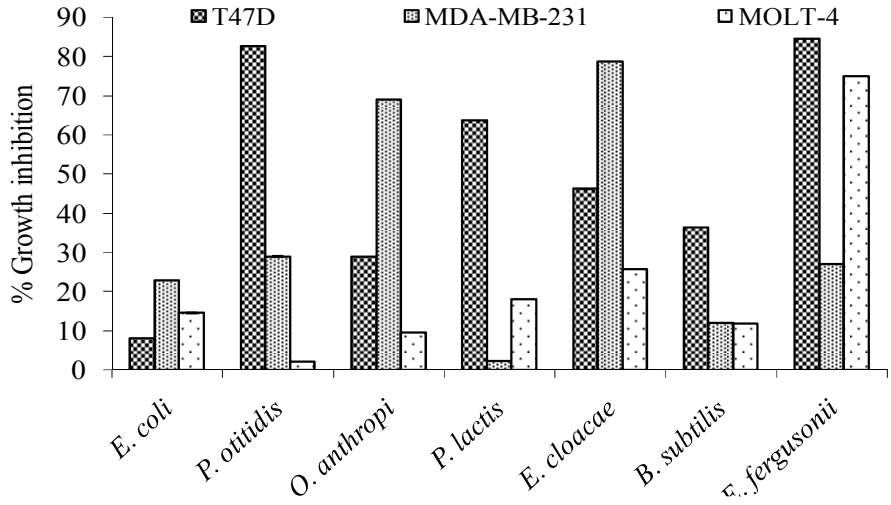

Figure 2
Antitumor activities of glutaminase free L-asparaginases towards T47D, MDA-MB-231 and MOLT-4 cancer cell lines. E. coli L-asparaginase (procured from Sigma chemical Co., USA.) was used as reference preparation.

In vitro serum half life

Pharmacokinetics of L-asparaginase considerably depends upon *in vitro* serum half-life. Maximum *in vitro* serum half life of 37 h and 21 h was exhibited by *P. otitidis* and *E. cloacae* L-asparaginase, respectively. However, it was found to be 18 h for *O. anthropi*, *P. lactis,* and *E. fergusonii* while, it was only 15 h for *B. subtilis* L-asparaginase (Table 3).

In vitro trypsin digestion half life

L-asparaginases obtained from all six bacterial strains were analyzed for their *in vitro* trypsin digestion half life. Trypsin digestion half life of 30 min was recorded for *P. otitidis* L-asparaginase and 21 min for *E. cloacae* L-asparaginase. It was found to be 20 min for *E. fergusonii* and *P. lactis* L-asparaginase while, only 17 min and 15 min were observed for *O. anthropi* and *B. subtilis* L-asparaginases (Table 3).

Table 3
In vitro serum and trypsin digestion half life of glutaminase free L-asparaginases

Bacterial Strains	*In-vitro* serum half-life (h)	*In vitro* trypsin digestion half-life (min)
P. otitidis	37	30
E. cloacae	21	21
E. fergusonii	18	20
P. lactis	18	20
O. anthropi	18	17
B. subtilis	15	15

DISCUSSION

Microorganisms are the main sources of therapeutic protein; used in treatment of patients with heart attacks, strokes, cystic fibrosis, Gaucher disease, diabetes, anemia, hemophilia, inflammation and cancer including ALL and other lymphoproliferative malignancies[18]. The most important therapeutic features of enzymatic drugs that distinguish them from other types of therapeutic agent is that it often bind and act on their targets with great affinity, specificity and enormously high catalytic efficiency to convert target molecules into the desired product(s)[19]. Isolation and screening of bacteria from natural habitats had lead to the discovery of many novel and useful secondary metabolites, particularly enzymes, antibiotics, vitamins and enzyme inhibitors. The rhizosphere is the narrow zone of soil and act as a reservoir for microbial diversity. Among

the screened bacterial strains (154) only 54 (35.06%) were able to form pink zone in plates containing modified M-9 medium, indicative of L-asparaginase producing strains. The bacterial L-asparaginase hydrolyzes L-asparagine into L-aspartate and ammonia. Due to which acid base indicator dye phenol red converts into pink color at basic pH. Among the 54 L-asparaginase producing bacterial strains, 43 (79.62%) were isolated from rhizospheric soil and rest of the 11 (20.37%) were isolated from river water. The maximum zone index 2.6 mm was recorded in bacterial strain BGCC#2388, isolated from rhizosphere of *Brassica oleracea*. These results indicate that rhizosphere can be a suitable source for isolation of L-asparaginase producers and our findings are in accordance with previous reports[20,21,22].

The intrinsic glutaminase activity of L-asparaginase is responsible for serious toxicity including hepatotoxicity, hemostatic disorders and hyperglycemia[23]. During the present study out of 54 L-asparaginase producing bacterial strains only 6 bacterial isolates i.e. *E. fergusonii, P. lactis, P. otitidis*, *E. cloacae, O. anthropi* and *B. subtilis* did not exhibited intrinsic glutaminase activity. The L-asparaginases from *E. coli* (Elspar® marketed by Merck & Co.) and *Erwinia chrysanthemi* (*Erwinia*, marketed by Ogden BioServices Pharmaceutical Repository in United States) have been used clinically. Unfortunately, both sources exhibited 2-10% glutaminase activity[4]. Hence, glutaminase free L-asparaginase is highly desirable and can significantly improve treatment outcomes.The occurrence of L-asparaginase have been reported in most of microbial species but their biochemical and enzyme kinetic properties such as enzymatic activity, substrate specificity, molecular weight, glutaminase and antitumor activities vary with genetic nature and culture condition[24]. L-asparaginases obtained from *Bipolaris* sp. BR438, chicken liver, yeast and *Bacillus coagulans* [25,26,27,28] does not possess antitumor activity, while, on other hand *Bacillus subtilis; Saccharomyces cerevisiae*, *Mycobacterium tuberculosis* and *Escherichia coli* possess two type of L-asparaginases and only one exhibited antitumor activity [29,30,31,32]. The MTT assay is a rapid and selective method used as indicator of mitochondrial dehydrogenases activity and widely used in cell proliferation analysis. This assay is based on the conversion of MTT to formazan by reduction of NADH or NADPH dehydrogenases. In T47D cells 82.5±0.02%, 46.15±0.97%, 28.84±0.06%, 63.72±0.01%, 84.54±0.04% and 36.23±0.21% reduction in cells proliferation was recorded by *P. otitidis*, *E. cloacae, O. anthropi, P. lactis, E. fergusonii* and *B. subtilis* L-asparaginase, respectively. The MDA-MB-231 cells showed significant reduction in cell growth of 28.84±0.02%, 78.7±0.09%, 68.83±0.07% and 26.81±0.01% by *P. otitidis*, *E. cloacae, O. anthropi* and *E. fergusonii* L-asparaginases, respectively while only 2.11±0.16% and 11.92±0.33% growth inhibition was observed by *P. lactis* and *B. subtilis* L-asparaginase, which indicated that L-asparaginase of both the organism are less toxic to MDA-MB-231 cells. The MOLT-4 cells are found to be less sensitive for all selected bacterial L-asparaginases except 74.97±0.11% reduction in cells growth was exhibited by *E. fergusonii* L-asparaginase.

Our results indicated that glutaminase free crude L-asparaginase obtained from six bacterial strains possess effective antitumor activity towards the T47D and MDA-MB-231 cell lines as compared to MOLT-4 cell line. 70% and 93% reduction in cell viability of HeLa and BT20 (breast cancer) cell lines, respectively, against 0.5 IU/ml concentration of purified *Thermus thermophilus* L-asparaginase was reported by Prista et al (2001)[33]. However, at same concentration K562 (chronic myelogenous leukemia) cells are less sensitive to *Erwinia chrysanthemi* L-asparaginase (Erwinase)[33]. The crude L-asparaginase obtained from chicken liver doesn't possess antitumor activity at 0.7 to 1.0 IU/ml concentration[26]. In the present investigation the cells growth inhibition data clearly indicates that even very low concentration of crude L-asparaginases (0.1IU/ml) from these bacterial strains posses strong cytotoxic activity in tested cell lines (T47D and MDA-MB-231), which can be enhanced after purification. The MOLT-4 cells showed highest cells viability; which might be due to an increased rate of asparagine synthesis in response to asparagine depletion by these cells[34]. These results are also supported by the study of Huston et al (1997)[35] according to which, for human leukemia cell line MOLT-4, NALL-1 and BALL-

1 amino acid deprivation leads to change in transcriptional regulation and increase in asparagine synthetase mRNA protein and enzymatic activity. Serum contains several pancreatic enzymes among them trypsine and elastase-I are major one. The level of trypsine and elastase-I enzymes present in serum increases many folds during L-asparaginase therapy[36] which may hydrolyse the clinically administrated L-asparaginase. Therefore, results in reduction of L-asparaginase bioavailability for hydrolysis of serum L-asparagine. During, trypsine digestion assay the *P. otitidis* L-asparaginase lost 50% of its original activity after 30 min of incubation; which is significantly high as compared to commercial *E. coli* L-asparaginase[17]. In human, native L-asparaginase preparations (*E. coli* and *Erwinia chrysanthemi* L-asparaginase) are degraded by the plasma with a half life ($t_{1/2}$) of 18 to 24 h, making it necessary to administer the enzyme every 2-5 days to replenish continuous asparagine depletion[37]. Thus, for suitable and cost effective chemotherapy prolonged serum half life is required. Hence, we analyzed *in vitro* serum half-life and found that the blood clearance of the *P. otitidis* L-asparaginase was 37 h, which is lower than that of purified *Cladosporium* sp. L-asparaginase[1] but is slightly higher than commercialized native *E. coli* L-asparaginase[17]. These properties of glutaminase free L-asparaginase from *P. otitidis* makes it a potential source for the treatment of cancer in future after further investigations.

CONCLUSION

L-Asparaginase is an integral part of modern combination chemotherapy protocols of ALL but intrinsic glutaminase activity of this enzymatic drug is responsible for numerous side effects. Hence, now days much emphasis is given on glutaminase free L-asparaginase production from new microbial sources. The enzyme from *P. otitidis* was found to be more effective for *in vitro* cytotoxic activity against T47D and MDA-MB-231 cell lines. Reasonably high antitumor activity of L-asparaginase tested in solid tumor cell lines suggests applicability of this enzyme for treatment of solid tumors in humans. The prolonged *in vitro* serum and trypsine digestion half-life of glutaminase free L-asparaginase of *P. otitidis* is significantly high as compared to previous reported ones. Hence, on the basis of glutaminase free activity, effective antitumor property, prolonged *in vitro* serum and trypsine digestion half life of L-asparaginase obtained from *P. otitidis* may act as a leading compound for the development of potent anticancer drug with more cure rates and lesser side effects. Further, purification, characterization and antitumor activity of glutaminase free L-asparaginase from *P. otitidis* needs to be explored.

ACKNOWLEDGEMENT

Authors are thankful to Head, Dept. of P.G. Studies and Research in Biological Science, R.D. University, Jabalpur for providing Lab facilities and Dr. Fayaz A. Malik, Scientist, Department of Pharmacology and Cancer Biology, Indian Institute of Integrative Medicine (IIIM-CSIR), Jammu, India, for providing Laboratory facilities for screening of antitumor activity of enzymes. Authors are also thankful to M.P. Biotechnology Council, Bhopal for providing financial support.

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