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Expression, Purification and Characterization of Recombinant Histidine-tagged L-asparaginase II

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E*SCHERICHIA coli* has two L-asparaginase isozymes that have been designated L-asparaginase I and L-asparaginase II. The amino acid sequences of both are rather dissimilar except for a few regions of significant homology. The sequence corresponding to the mature (*ansB*) was subcloned into pQE-30 expression vector and expressed in *E. coli* M15. The recombinant histidine-tagged (*ansB*) was purified to homogeneity by Ni–NTA affinity chromatography and displayed a single 36.0 kDa band on SDS-PAGE. Results revealed that the recombinant His-tagged L-ASNase II was expressed in an active form and its specific activity was estimated to be 286 U/mg. The optimum temperature was attained at 40°C. The enzyme was maximum at pH 7-8. Also the enzyme was stable at 40-50°C. The purified functional enzyme exhibited a specific activity at 286 U/mg and inhibited the growth of human myeloid leukemia cell line (HL-60) with IC_{50} value of 0.14±0.03 U/ml.

Keywords: Anti-tumor, Cytotoxicity, Expression, His-tag, L-Asparaginase, Leukemia, Purification, Recombinant.

Studying, L-Asparaginase (L-ASNase) has recently gained much attention for its anti-carcinogenic potential due to their high catalytic activity and specificity towards L-asparagine (Duval *et al*., 2002). Beside its therapeutic use, L-ASNase has been used in food industry to reduce acrylamide formation in heat-processed products (Pedreschi *et al*., 2008 and Kukurová *et al*., 2013).

Escherichia coli was found to have two L-asparaginase isozymes that were named L-asparaginase I and L-asparaginase II. Only L-asparaginase II exhibited tumor inhibitory activity, so it has been extensively studied. Trials were conducted to identify microbial sources for L-asparaginase, since its extraction and purification from the guinea pig serum was insufficient. L-asparaginase was isolated and purified from *Escherichia coli*. It was efficient in treatment of the children suffering from acute lymphoblastic leukemia and demonstrated a similar anti-tumor activity to that of guinea pig sera (Jerlstörm *et al.*, 1989).

The anti-neoplastic activity of L-asparaginases is mainly due to the selective depletion of L-asparagine in the blood serum. Unlike normal cells, certain lymphosarcomas and the leukocytes transformed in acute lymphoblastic leukemia (ALL) cannot compensate for the lack of asparagine, because they show a diminished expression of asparagine synthetase. Therefore, these cells

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depend on an extracellular supply of L-asparagine, which they obtain from the circulating pools, for their proliferation. Intravenous treatment with L-asparaginase leads to depletion of the supply of L-asparagine and can thus be cytotoxic for the neoplastic cells causing its selective death. In contrast, normal cells are protected from L-asparagine-starvation due to their ability to synthesize this amino acid in amounts sufficient for their metabolic needs with their own enzyme, L-asparagine synthetase. This behavior of cancer cells was the key of treatment leukemia using L-asparaginase. The cure rate has now improved to 80% in children and 30-40% in adults using L-asparaginase in combination with other existing drugs (Narta *et al.,* 2007 and Sreenivasulu *et al*., 2009).

The purification of recombinant proteins can be improved by using affinity tags. These tags are fused to the protein of interest and used for purification of the tagged proteins selectively *via* affinity chromatography. This method utilizes immobilized metal-affinity chromatography (IMAC) to purify recombinant proteins containing a short affinity tag of polyhistidine residues. IMAC is based on interactions between an immobilized transition metal ion such as Ni^{2+}- and specific amino acid side chains. Histidine exhibits the strongest interaction with immobilized metal ion matrices. (Terpe, 2003).

Polyhistidine tagged proteins can be purified rapidly by IMAC method resulting in about 100 folds enrichment in a single purification step. Affinity-tagged protein purities can be achieved at up to 95% purity by IMAC in high yield. The His-tag combines the advantages of being inert, of low immunogenicity, and of small size (0.84 kDa). Purification using polyhistidine tags was carried out successfully using a number of expression systems, including *Escherichia coli, Saccharomyces cerevisiae*, *Pichia pastoris*, mammalian cells, and baculovirus-infected insect cells (Zakalskiy *et al*, 2012; Sockolosky and Szoka, 2013).

The relatively small size and charge of the His-tag ensure that protein activity is rarely affected. Usually, the affinity tag does not need to be removed following protein purification. But if necessary, the affinity tag can be removed by inserting a protease cleavage site in between the tag and the protein. Some of the commonly used cleavage enzymes include the tobacco etch virus (TEV) protease, thrombin, enterokinase (EK), and factor Xa (fXa), which all belong to the protease class of endopeptidases. Polyhistidine affinity tags are small enough to be incorporated easily into any expression vector by PCR methods. DNA nucleotides coding for the polyhistidine affinity tag can also be created from synthetic oligonucleotides and cloned into an appropriate location in the desired plasmid. Alternatively, there are a wide variety of commercially available expression vectors for expression of polyhistidine-tagged recombinant proteins in different expression systems (Rabbani *et al*., 2010 and Shuguo *et al*., 2012).

Wide variety of immobilized metal matrices is available for use in IMAC. The three-dentate ligand iminodiacetic acid (IDA) was used initially as a matrix for chelating transition metals through three coordination sites. A problem with

the use of IDA matrices is that the metal ion is only weakly bound to this three-coordinate matrix. Metal leaching from the matrix during purification results in lowered yields and impure products. More recently, purification of His-tagged proteins has been facilitated by the development of the commercially available matrices nickel-nitrilotriacetic acid (Ni^{2+}-NTA). It is tetradentate ligand that binds the metal ion with extra carboxylate oxygen compared to IDA. This could give it a superior metal chelating strength (Feng *et al*., 2010).

There have been many attempts for cloning and expression of L-asparaginase (*ansB*) gene from different microorganisms. Wang *et al*. (2001) cloned DNA fragment coding for L-asparaginase from *E. coli* AS1.357 into the expression vector pBV220 and transformed into *E. coli* strains JM105, JM109, TG1, DH5α and AS1.357. The subunit molecular weight of the expressed enzyme was 36,000 Daltons by sodium dodecyl sulfate polyacrylamide gel electrophoresis **(**SDS-PAGE). The recombinant plasmid in *E. coli* AS1.357 remained stable after 72 hr of cultivation and 5 hr of heat induction without selective pressure.

Vidya and Pandey (2012) cloned L-asparaginase II gene that isolated from a moderately thermo-tolerant bacterium belonging to Enterobacteriaceae into pET20b expression vector with a six histidine sequences at the C-terminal end transformed to competent BL21 DE3 cells. The histidine-tagged protein (His-tagged) was purified through nickel affinity chromatography using Ni-NTA spin column. The purified protein showed optimum activities at a temperature of 37°C and in a buffer system of pH 6 to 7. The cloned gene showed 99% similarity with L-asparaginase type II of *E. coli* KO11.

In the case of expression of L-asparaginase from *Erwinia chrysanthemi* 3937 in *E. coli* BL21, the expressed recombinant enzyme was purified to homogeneity in a single-step procedure involving cation exchange chromatography on an S-Sepharose FF column (Kotzia and Labrou, 2007). The sequence of the cloned *ansB* gene from the pathogenic strain *Helicobacter pylori* (993 bp) was subcloned into the pET101 expression vector and transformed to *E. coli* BL21 (DE3) competent cells. The recombinant enzyme purified to homogeneity displayed a single 37 kDa band on a 12% SDS–PAGE (Cappelletti *et al*., 2008).

In this study, the sequence corresponding to the mature L-ASNase II was subcloned into pQE-30 expression vector and expressed in *E. coli* M15. The recombinant histidine-tagged L-ASNase II was purified to homogeneity by Ni–NTA affinity chromatography and displayed on SDS-PAGE. The cytotoxic activity of recombinant L-asparaginase II was assayed and characterization of recombinant L-asparaginase II was conducted.

Materials and Methods

Preparation of plasmid

To prevent self-ligation of plasmid, the terminal 5’-phosphate groups should be removed by using alkaline phosphatase. This dephosphorylation reaction

suppresses self-ligation of vector molecules and decreases the number of empty vectors. The insert DNA with intact 5'-terminal phosphate residues can be ligated efficiently into the dephosphorylated plasmid DNA.

pQE-30 vector was double digested and dephosphorylated using FastDigest *Bam*HI and *Hin*dIII restriction enzymes simultaneously with FastAP Thermosensitive Alkaline Phosphatase according to protocol recommended by the manufacturer. The reaction mixture contained 1 µg of pQE-30 vector, 2 µl of 10X Thermo Scientific FastDigest Buffer, 1 µl of FastDigest *Bam*HI, 1 µl of FastDigest *Hin*dIII restriction enzymes, 1 µl of FastAP Thermosensitive Alkaline Phosphatase and nuclease-free water up to 20 µl. The reaction components were mixed thoroughly and incubated at 37°C for 10 min. The reactions were stopped by heating at 80°C for 20 minutes and electrophoresed into 1% agarose gel in 1X TAE runing buffer for 60 min at 50 V. Then the band of the double digested dephosphorylated plasmid was gel purified as described previously. Gel purified double digested dephosphorylated plasmid DNA was used for subcloning and expression of *ansB* gene.

Subcloning of ansB gene

L-asparaginase II gene was subcloned into pQE-30 expression vector. DNA insert was positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide, a series of six histidine residues that function as a metal binding domain in the translated recombinant protein.

For subcloning of *ansB* gene into pQE-30 expression vector, a ligation reaction was conducted in an 20 µl final volume comprising one µl dephosphorylated plasmid DNA previously digested with *Bam*HI-*Hin*dIII, 2 µl ligase buffer (10x), one µl T4 DNA ligase (Thermo Scientific, USA), and the insert previously released from pGEM-*ansB* construct by double digestion with *Bam*HI and *Hin*dIII. The reaction mixture was brought to the final volume with sterile nuclease-free water and incubated at 22°C for 10 min. For an efficient ligation reaction the amount of plasmid vector was one third of the insert. The resultant plasmid was referred to as pQE-*ansB* and used for transformation of *E. coli* M15 competent cells.

E. coli M15 competent cells were transformed by heat-shock treatment essentially as described by Sambrook and Russel (2001). After transformation into *E. coli* strain M15, clones with correct inserts were checked by colony PCR using vector-specific primers.Positive clones were confirmed by colony PCR according to method described by Güssow and Clackson (1989) with some modifications. PCR product sizes were checked by 1% agarose gel electrophoresis. The positive clones that showed correct PCR product sizes were selected.

Expression of ansB gene

Transformed *E. coli* M15 cells were cultured overnight at 37°C in 5 ml of LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin under vigorous

agitation (200 rpm). This pre-inoculum suspension was then used to inoculate 500 ml fresh LB selection medium, which was agitated until an $O.D._{600}$ reached 0.6. Thereafter, an aliquot of non-induced control cells was collected and reserved for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Expression of His-tagged L-asparaginase II was induced in the left cells by addition of IPTG to a final concentration of 0.4 mM. The cells were cultivated at 37°C for 3 hr in the induction medium and afterwards collected by centrifugation at 7,000 × g at 4°C for 20 min. The resultant pellets were stored at -20°C.

The bacterial pellets were thawed on ice for 15 minutes and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) and incubated on ice for 30 min. Cells were sonicated for 6 cycles (10 s bursts at 200 Watt with a 10 s cooling period between each burst). The cell lysate was centrifuged at 10,000 × g for 20 min. at 4°C and 50 µl cleared lysate supernatant was stored at –20°C for SDS-PAGE analysis.

Purification of recombinant ansB gene

The cell lysate supernatant was purified under native conditions using Ni-NTA resin (Qiagen, Hilden, Germany) following the manufacturer's protocol. Fraction containing recombinant L-asparaginase II was dialyzed by phosphate buffered saline (PBS) and was analyzed by SDS-PAGE and enzyme assay was performed.

Protein expression and purification processes were examined with SDS-PAGE. Protein samples separated on 12% SDS-PAGE gels. Proteins were denatured in the presence of SDS and 2-mercaptoethanol as thiol reducing agent. Gels were stained with Coomassie stain and the protein sizes were determined by comparing the migration of the protein band to a molecular mass standard (Roti®-Mark STANDARD protein marker, Carl Roth GmbH and Co. KG, Karlsruhe, Germany).

SDS-PAGE was conducted according to standard protocols (Sambrook and Russel, 2001). Samples containing the supernatant were mixed with equal amount of 2X sample buffer and boiled for 5 min. followed by centrifugation at maximum speed for 5 min. to settle down insoluble materials. The supernatant was used as sample for SDS-PAGE.

Characterization of recombinant L-asparaginase II

The effect of temperature on recombinant L-asparaginase II activity was assayed at temperatures in the range from 30 to 80°C at pH 8.6. The effect of different pH values on recombinant L-asparaginase II activity was assayed. The enzymatic activity was assessed using different buffers with different pH values ranging from pH 3.0 to10.0. In the pH range from 3 to 5.5, a 50 mM sodium acetate-acetic acid buffer was used, in the pH range from 6 to 8, a 50 mM phosphate buffer was used and in the pH range from 8.5 to 10.0, a 50 mM carbonate buffer was used. Thermal stability was assessed by incubation of

recombinant L-asparaginase II at 40 to 80°C in 0.05 M phosphate buffer (pH 8.6) for 60 min. Subsequently, the samples were assayed for residual activity. The residual activity was calculated by comparing the activity to that of the untreated control enzyme, which is taken as 100 %.

Cytotoxic activity of recombinant L-asparaginase II

Human myeloid leukemia HL-60 cells were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were split every 3–4 days to maintain in an exponential growth.

HL-60 cells were seeded in each well containing 100 µl of RPMI-1640 medium in 96-well plates at 5 x 10^4 cells/well. After 24 hr, different L-asparaginase II levels ranging from 0.001 to 2.0 U/ml were added. Cells were kept in culture for 48 hr before cell viability was assessed. The cell viability was estimated by the 3- [4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann (1983). 10 µl of MTT (5 mg/ml) was added to each well, the plates were incubated at 37°C for 4 hr. Afterwards, 100 µl of acid-isopropanol (0.04 N HCI in isopropanol) was added to all wells and mixed thoroughly. The absorbance was measured within 1 h using an ELISA reader (BioRad, München, Germany) at 570 nm.

All measurements were done in triplicate and cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells. After subtraction of blank values, the leukemic cell survival (LCS) was calculated as previously described (Pieters *et al*., 1990) using the equation:

LCS= (OD treated well/mean OD control wells) X 100

Additionally, the half maximal growth inhibitory concentration IC_{50} value was calculated. IC_{50} represents the inhibitory concentration required to reduce viable cell number by 50%

Statistical analysis

The measured data were subjected to the analysis of variance (ANOVA) appropriate to the design. The significant differences between treatments were compared with the critical difference at 5% level of probability by the Duncan's test using PASW 17.0 statistics software (SPSS Inc).

Results

Construction of expression Vector system

After transformation, several clones (designated *ansB*-1 to *ansB*-7) were picked and checked for successful ligation by colony PCR using primers designed for pQE-30. Clones which produced an amplicon of about 1kb

indicated positive transformants. Six colonies (lanes 1, 2, 3, 4, 6 and 7) were clearly found to be positive for recombinant plasmid carrying *ansB* gene as shown in Fig. 1. Clone *ansB*-4 carrying pQE-*ansB* construct was selected for production of recombinant histidine-tagged L-asparaginase II.

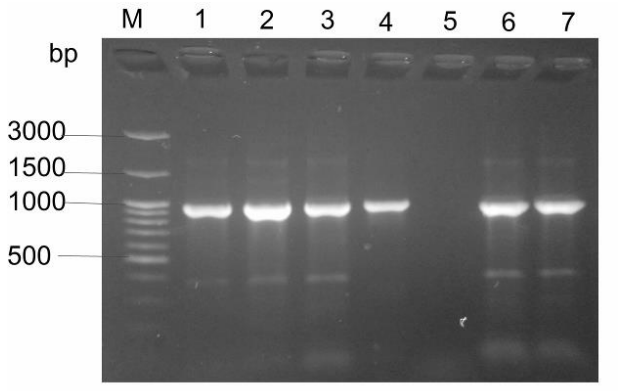

Fig. 1. Screening of transformants containing pQE-*ansB* by colony PCR. Lane M, DNA ladder; lanes 1-7: *ansB*-1 to *ansB*-7 clones. Individual bacterial colonies were picked from LB/ampicillin/ kanamycin plates, resuspended in 100 µl sterile nuclease-free water and boiled. After centrifugation, 1-2 µl of the supernatant was used as a template in the PCR using vector-specific primers. PCR products were analyzed on 1% agarose gel electrophoresis. Lanes 1, 2, 3, 4, 6 and 7 showed PCR product of the expected size (~1kb) considered as successful transformants while lane 5 was unsuccessful transformant.

Overexpression and purification of His-tagged L-asparaginase II in E. coli M15

Analysis of SDS-PAGE gel revealed that a protein had been overexpressed in *E.coli* M15 cells contains pQE-*ansB* vector after induction with IPTG. The molecular weight of the overexpressed protein was 36.0 kDa. The highly purified His-tagged protein was migrated as a single band in SDS-PAGE gel as shown in Fig. 2.

Characterization of recombinant L-asparaginase II

Results revealed that the recombinant His-tagged L-ASNase II was expressed in an active form and its specific activity was estimated to be 286 U/mg. Temperature activity profile showed that the enzyme was active in the temperature range of 30-60°C with maximum activity at 40°C (Fig. 3). The enzyme was found active over a broad pH range (4-10) with maximum activity at pH 7 to 8 (Fig. 4). Results demonstrated that enzyme retained about 100%, 81% and 44% of its initial activity after 60 min incubation at 40, 50 and 60°C, respectively (Fig. 5). Enzyme lost about 85% and 94% of its initial activity when incubated at 70 and 80°C for 60 min, respectively.

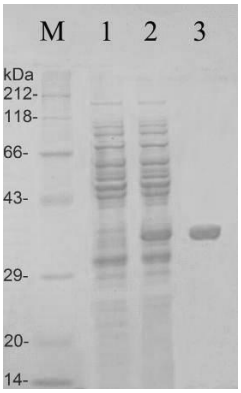

Fig. 2. SDS-PAGE showing expression and purification of His-tagged L-asparaginase II using Ni-NTA affinity chromatography. Lane M, protein marker; lane 1, total soluble proteins of uninduced cells; lane 2, total soluble proteins of induced cells; lane 3, purified His-tagged L-asparaginase II. Transformed *E. coli* M15 cells were cultured in selective LB broth until an $O.D._{600}$ reached 0.6. The expression of His-tagged L-asparaginase II was induced by addition of IPTG. After 3 h, cells were collected and sonicated. The cell lysate supernatant was purified under native conditions using Ni-NTA affinity chromatography. Aliquots from uninduced, induced cells and purified protein were analyzed by SDS-PAGE and Coomassie blue staining.

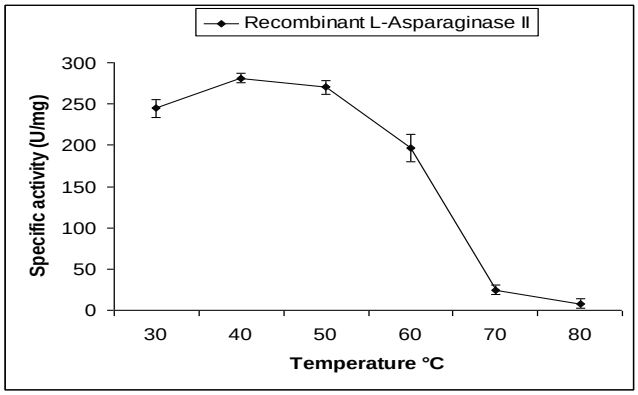

Fig. 3. Temperature profile of recombinant L-asparaginase II. The effect of temperature on the enzymatic activity of recombinant L-ASNase II was assayed at temperatures in the range from 30 to 80°C at pH 8.6.
- Error bars represent standard deviations (SD).

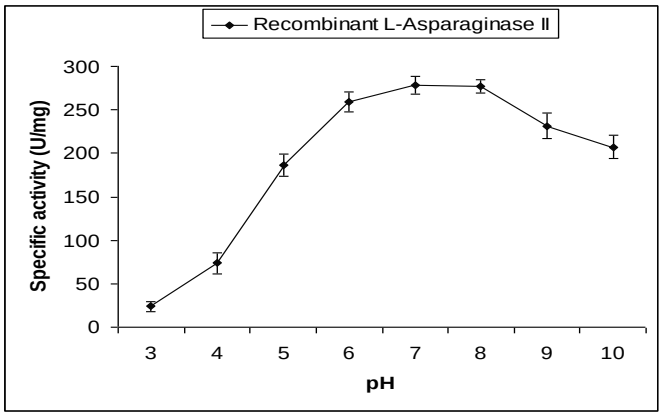

Fig. 4. pH profile of recombinant L-asparaginase II. The effect of pH on the enzymatic activity of recombinant L-ASNase II was assayed at different pH values ranging from pH 3.0 to10.0. Error bars represent standard deviations (SD).

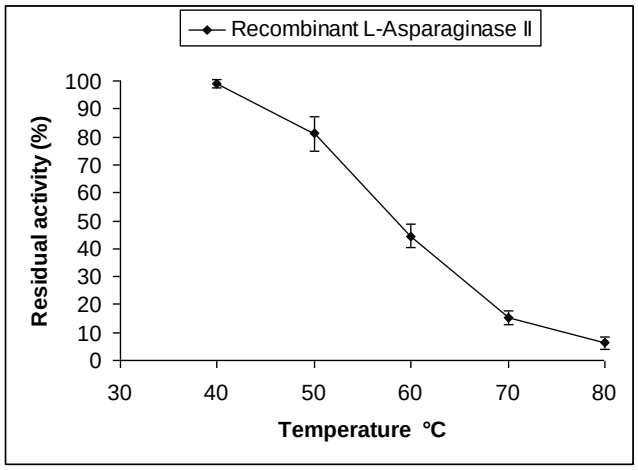

Fig. 5. Thermal stability of recombinant L-asparaginase II. Thermal stability was assessed by incubation of recombinant L-ASNase II at 40 to 80°C in 0.05 M phosphate buffer (pH 8.6) for 60 min, and then enzymatic activity was estimated. Residual activity was calculated by comparing the activity to that of the untreated control enzyme, which is taken as 100 %.

- **Error bars represent standard deviations (SD).**

Cytotoxic activity of recombinant L-asparaginase II

Data obtained from MTT assay indicated a strong dose–response relationship with regard to the cytotoxic activity of L-ASNase (Fig. 6). Results showed that no significant cytotoxic activity was recorded when HL-60 cells were incubated with recombinant L-ASNase in levels ranging from 0.001 to 0.01 U/ml. As the

level of enzyme increased, dramatic decrease in the LCS of HL-60 cells was observed. LCS decreased to about 52%, 14% and 3% when increased levels of the recombinant enzyme (0.1, 1.0 and 2 U/ml, respectively) were applied to HL-60 cell lines. The half maximal growth inhibitory concentration value (IC_{50}) of the recombinant L-ASNase was computed to be 0.14±0.03 U/ml.

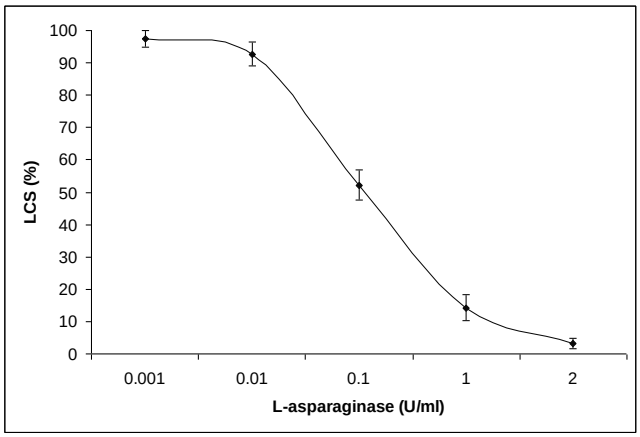

Fig. 6. Cytotoxic effect of recombinant L-asparaginase on cell survival in leukemia cell line. HL-60 cells were treated in triplicate with each concentration for 48 h and the cell viability was determined by the MTT assay.
- Error bars represent standard deviations (SD).

Discussion

The overall objective of this study was the construction of an expression system of L-asparaginase II (*ansB*) gene in frame with N-terminal region coding for six histidine residues to facilitate the protein purification process using immobilized metal-affinity chromatography. Accordingly, the *ansB* gene from a local *E. coli* isolate was cloned into pQE-30 expression vector under the control of T5 inducible promoter and successfully expressed in *E. coli* M15 cells. The expressed functional enzyme bearing N-terminal His-tag was purified in a single step using Ni–NTA chromatography.

With an objective to express His-tagged L-ASNase II enzyme, *ansB* gene was subcloned into pQE-30 expression vector in the right reading frame using *Bam*HI and *Hin*dIII restriction sites incorporated into the PCR fragments through *ansB*-F and *ansB*-R primers. *ansB* gene was subcloned into *Bam*HI/*Hin*dIII digested pQE-30 and subsequently transformed into *E. coli* M15 competent cells. The resulting transformants were validated for the presence of expression construct pQE-*ansB* by colony PCR using vector specific primers. Based on colony PCR screening, clone *ansB*-4 harboring pQE-*ansB* construct was selected

for production of recombinant His-tagged L-ASNase II. L-ASNases from *Escherichia coli, Erwinia carotovora, E. crysanthemi, Helicobacter pylori, Yersinia pseudotuberculosis* and *Bacillus subtilis* was cloned and successfully expressed in various bacterial expression systems (Onishi *et al*., 2011).

In the present work, *ansB* gene was expressed successfully in *E. coli* M15 cells after induction with IPTG. The recombinant His-tagged enzyme was purified to homogeneity by Ni–NTA chromatography and displayed a single ~36.0 kDa band on 12% SDS-PAGE. This value is very close to 34.57 kDa calculated theoretically from the deduced amino acids sequence by ProtParam tool. Slightly larger molecular weight appearing on SDS-PAGE could be due to the presence of His-tag at the N-terminal of the enzyme. These results agree with previously published data indicating that molecular weight of various recombinant L-ASNases on SDS-PAGE ranged from 34 to 37 kDa (Cappelletti *et al*., 2008 and Liu *et al*., 2013). It has been suggested that bacterial L-ASNases are homotetramers with molecular mass in the range 140–150 kDa with a highly conserved overall fold. These four identical subunits were with molecular weight of 35 kDa per subunit and bound mainly by non-covalent forces (Kozak *et al*., 2002 and Wikman *et al*., 2005).

In this study, the specific activity of the purified His-tagged L-ASNase II was found to be 286 U/mg. This value is close to the specific activity of E. coli native L-ASNase (Zhang *et al.*, 2004; Narta *et al.*, 2007). This result suggests that the expressed recombinant enzyme was fully functional and its activity was not affected by presence of the His-tag. Thus there was no need to remove the N-terminal His-tag from the final product because it did not deactivate the enzyme. It has been suggested that His-tag rarely interferes with the structure and function of target proteins, hence no necessity to be removed (Hamdane *et al.*, 2010 and Ralph *et al.*, 2011). In a previous study, recombinant His-tagged L-ASNase II from E. coli was purified in a single step using Ni–NTA affinity chromatography and the specific activity of the purifies enzyme was 190 U/mg (Khushoo *et al.*, 2004).

The influence of temperature on recombinant L- ASNase II activity was assayed in the range from 30 to 80°C. Temperature activity profile showed that the enzyme was active in wide temperature range with maximum activity at 40°C. This value has been reported as the optimum temperature of various L-ASNases (Youssef and Al-Omair, 2008 and Singh *et al.*, 2013). Optimum temperature of recombinant L-ASNase II from E. coli MTCC 739 was at 37°C (Vidya et al., 2011). Regarding pH activity, recombinant L- ASNase II was found active over a broad pH range (4-10) with maximum activity at pH 7 to 8. These results agree with previously published data indicating that optimum pH of L-ASNase is ranging from 6 to 8 (Pokrovskaya *et al*., 2012; Vidya and Pandey, 2012).

In this study, thermal stability was assessed by incubation of recombinant enzyme at 40 to 80°C in phosphate buffer for 60 min. L-ASNase exhibited

thermal stability with about 81% of activity retention after 60 min at 50°C. Likewise, wild-type L-ASNase II from E. coli $DH5_{\alpha}$ retained 71% of its initial activity after 60 min incubation at 50°C (Li *et al.*, 2007). In the present investigation, N-terminal His-tagged L-ASNase II retained about 44% of its initial activity after 60 min at 60°C. This thermal stability was found to be much greater than that of C-terminal His-tagged L-ASNase II from E. coli MTCC 739 which decreased to less than 10% in 1hr of incubation at 60°C (Vidya *et al.*, 2011). These results suggest that N-terminal His-tagged L-ASNase II could be more stable at elevated temperatures compared to C-terminal His-tagged L-ASNase II.

In order to evaluate the cytotoxic activity of L-ASNase against cancer cell line, MTT assay was conducted. This assay is a simple, precise and rapid method to detect living cells in mammalian cell cultures. Evaluation of the cytotoxic activities of different L-ASNases against different cancer cell lines using MTT assay was well documented (Tesfai *et al.*, 2012).

Findings from this study clearly demonstrated that recombinant His-tagged L-ASNase is highly effective against human myeloid leukemia cell line (HL-60). LCS decreased to about 52%, 14% and 3% when increased levels of the recombinant enzyme (0.1, 1.0 and 2 U/ml, respectively) were applied. The enzyme inhibited the growth of HL-60 cell line with IC_{50} value of 0.14±0.03 U/ml. These results concur with those reported by Abakumova *et al.* (2009) who evaluated the cytotoxic activity of recombinant L-ASNase from *Yersinia pseudotuberculosis* using MTT assay and recorded IC_{50} value of 0.2 U/ml.

By comparing effectiveness of recombinant L-ASNases from various sources one can observe that L-ASNase from *E. coli* MG27 exhibits higher activity in suppression of human myeloid leukemia cell line (HL-60) than the enzyme from *Helicobacter pylori* CCUG 17874 reported by Cappelletti *et al.* (2008). IC_{50} value of recombinant L-ASNase from *E. coli* MG27 was 0.14±0.03 U/ml whereas in the case of recombinant L-ASNase from *Helicobacter pylori* CCUG 17874 was 3.4 U/ml. The anti-neoplastic activity of L-ASNase based on the dependence of certain tumor cells on an extracellular supply of L-asparagine. Unlike normal cells, neoblastic cells can not synthesize L-asparagine due to their deficiency in L-asparagine synthetase (Moola *et al*., 1994). Thus, depletion of the circulating pools of L-asparagine by L-ASNase leads to the destruction of the tumor cells, since they are unable to complete protein synthesis.

The findings of the present work revealed that recombinant His-tagged L-asparaginase II was expressed as a functional enzyme, showing feasible thermal stability and was found to be active over broad ranges of temperatures and pH. Furthermore, it exhibited a significant inhibitory effect against leukemic cells suggesting that it could be a promising agent for both therapeutic and industrial applications. Future investigations are aimed for optimization of the expression process to maximize the product at bioreactors level.

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تعبير جينى، تنقية وتوصيف المدمج ل-أسباراجيناز الثاني الموسومة بالهيستادين

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ألإيشيريشيا كولاي تنتج اثنين من نظائر انزيمات ل-أسباراجيناز التي تم تعريفها ل-أسباراجيناز الأول و ل-أسباراجيناز الثاني. تسلسل الأحماض الأمينية للاثنين متماثل على حد سواء باستثناء مناطق قليلة. ل-أسباراجيناز الأول (*ansA*) تعبر جينيا جوهريا في السيتوبلازم ولها قابلية منخفضة لل-أسباراجين. بينما تعبر جينيا ل-أسباراجيناز الثاني (*ansB*) في ظل الظروف اللاهوائية بين الغشائين الخلويين ولديه قابلية عالية لل-أسباراجين. التسلسل المقابل ل (*ansB*) الناضجة تم اسنساخه فرعيا في ناقلات التعبير pQE30 ويظهر في ألإيشيريشيا كولاي M15 . المدمج الموسوم بالهيستادين (*ansB*) تم تنقيته إلى التجانس بواسطة التقارب اللوني–Ni NTA وعرض ٣٦,٠ كيلو دالتون على SDS-PAGE. أظهر الانزيم النقي وظيفيا نشاط معين في ٢٨٦ U /ملغ و منع نمو خلايا سرطان الدم النخاعي البشري (HL-60) بقيمة IC50= 0.03 ± 0.14 U/ مل. وقد تم اختبار النشاط السام للخلايا المؤتلف ل-أسباراجيناز الثاني. وقد أجريت أخيرا توصيف المؤتلف ل-أسباراجيناز الثاني.