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Comparative analysis of purified anti-leukemic L-asparaginase enzyme from *Trichoderma* spp.

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ABSTRACT

L-asparaginase has a therapeutic property and is widely used in chemotherapy or treatment of acute lymphoblastic leukemia, besides its use in the food processing industry. A comparative study of fungal L-asparaginase production and characterization has been performed. Among the several isolates, two cultures *Trichoderma viride HK01* and *Trichoderma koningii HK02* were further selected based on their capacities of producing L-asparaginase. These cultures were identified using morphological, staining, and ITS sequencing approach. The molecular weight of purified L-asparaginase as estimated by SDS gel electrophoresis was ~69 kDa and ~72 kDa from *T. viride HK01*and for *T. koningii HK02*, respectively. The apparent K_m of purified L-asparaginase from *T. koningii HK02* and *T. viride HK01* was 0.0528 mM and 0.0565 mM and V_{max} were 55.24 and 71.13 U/mL, respectively. The optimum temperature for purified L-asparaginase was 37°C for *T. viride HK01* and 40°C for *T. koningii HK02*. Likewise, the optimum pH was 7.0 and 8.0 for *T. koningii HK02* and *T. viride HK01*, respectively. Mercury and lead have completely inhibited the enzyme activity from both the fungi. Manganese has increased the enzyme activity in *T. koningii* HK02. The IC_{50} was 2.0 and 2.5 Units/mL for L-asparaginase from *T. koningii* HK02 and *T. viride* HK01, respectively. Thus, observed results suggested that purified L-asparaginase have required properties for possible treatment against the cancerous-cells.

1. INTRODUCTION

L-asparaginase (EC 3.5.1.1) is an enzyme, consist of tetrameric peptides, which catalyzes the hydrolysis of free L-asparagine amino acid into an L-aspartate (aspartic acid) and ammonia [1,2]. Under normal physiological conditions, this hydrolytic reaction is mostly non-reversible [3]. This enzyme has a huge medicinal and clinical application, along within food processing industries. According to an estimate, it is among one of the largest groups of therapeutic enzymes accounted for ~40% of the total sale worldwide as anti-leukemic and anti-lymphoma agents and its demands are increasing several folds every year [4,5].

Normal human (eukaryotic) cells are capable of synthesizing L-asparagine (which is an essential amino acid) using L-asparagine synthase and hence cells are protected from L-asparagine starvation. However, in leukemic cells, L-asparagine is not synthesized as they lack L-asparagine synthetase [6]. The lymphatic tumor cells require large amount of L-asparagine for their uncontrolled growth and they rely on their exogenous source for survival. During therapeutic treatment of lymphatic tumor, intravenous injections of L-asparaginase degrade the exogenous L-asparagine in the blood. This selective starvation of tumor cells results in impairment of protein synthesis leading to the cell death [7,8]. The acute lymphoblastic leukemia (ALL) which is commonly observed in children, are effectively treated with L-asparaginase [9,10]. L-asparaginase is also used in for therapeutic treatment of acute lymphoblastic leukemia, lymphosarcoma, acute myelomonocytic and myelocytic leukemia, chronic lymphocytic leukemia, reticulosarcoma, melanosarcoma, and Hodgkin disease [11,12].

All these years, L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* were widely used for its chemotherapeutic properties. However, soon it was found that, its long-term use leads to various unwanted side effects which results in exerting normal cell toxicity along with patient's hypersensitivity and rapid clearance of the enzyme from the blood stream [12,13]. It was also observed that the toxicity of L-asparaginase was mainly because of bacterial endotoxins and glutaminase activity in enzyme preparations [14]. L-asparaginase was found to cause many adverse effects mainly hepatotoxicity, along with fever, skin rashes, pancreatitis, diabetes, hepatic dysfunction, neurological seizures, leukopenia, etc. [15,16]. To avoid such side effects, its purification is considered as an essential step for its biological and physical characterization [11]. For effective therapeutic

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use of L-asparaginase having fewer side effects, the enzyme preparation should be free from any impurities and contamination requiring efficient purification step [12].

Numerous studies have been carried out to minimize the allergenic reactions caused by various impurities in L-asparaginase during the application [17]. Among the many studies, Manivasagan *et al.* [18] suggest that actinomycetes could be a potential source for L-asparaginase enzyme and especially *Streptomyces* spp. have been studied well for many bioactive compounds over these years. However, as mentioned above new source of L-asparaginase was always there and therefore, this study was performed to isolate new fungal strains capable of producing high quality and quantity of L-asparaginase enzyme for it potential therapeutic use. There were few studies on production of L-asparaginase from *Trichoderma* species, but rare studies have been reported on its complete purification and physicochemical characterization. The enzyme L-asparaginase was purified from selected *Trichoderma* spp. in this study and characterized for its optimum activity under selected abiotic parameters. Moreover, the possible cytotoxicity of purified L-asparaginase was also assessed using leukemia cell lines.

2. MATERIALS AND METHODS

2.1. Sample Collection

The samples (soil and agriculture waste) for isolation of fungi capable of producing L-asparaginase enzyme was collected from the agricultural farm near Patan-Chanasma highway, Gujarat, India (23°47'36.8"N; 72°07;20.9"E). Soil samples below the surface (~ 2 – 6 cm) were collected from different locations across the farm and transported to lab immediately and stored at 4°C until its use.

2.2. Screening of L-asparaginase Producing Fungi

The L-asparaginase producing fungi were screened using serial dilution followed by plating method. The collected samples serially diluted in normal saline and spread on modified Czapek-Dox agar medium (g/L; glucose, 2.0; potassium dichloride, 0.52; potassium dihydrogen phosphate, 1.52; magnesium sulfate, 0.52; and trace nutrient: Copper nitrate, iron sulfate, zinc sulfate; and agar 20.0; pH = 6.2) containing L-asparagine as sole source of nitrogen. The medium was also supplemented with phenol red (0.009 %, v/v) as a pH indictor and 0.02 % (w/v) tetracycline. The plates were incubated at room temperature for 72 h. The L-asparaginase producing fungi were screened on the basis of zone of clearance (formation of pink zone) around the fungal growth.

2.3. Isolation and Identification of L-asparaginase Producing Fungi

The L-asparaginase producing fungi were preliminary screened and isolated on the basis of zone of clearance (formation of pink zone) around the fungal growth. The fungal growth with bigger zone of clearance were selected, further screened and purified by repeated streaking on fresh media. The selected fungi were identified using polyphasic approach and ITS gene sequencing. The ITS gene sequence from both the fungi were submitted to NCBI database, with GenBank Accession No. of OL824937 and OL824938, respectively, for *Trichoderma koningii HK02* and *Trichoderma viride HK01*.

2.4. Production and Purification of L-asparaginase

Fungal culture was grown in production medium under optimized conditions for bulk production of L-asparaginase enzyme. The mycelial growth was separated from the culture supernatant by centrifugation.

2.4.1. Ammonium sulfate precipitation

To initiate the L-asparaginase purification from culture supernatant (production medium), it was initially centrifuged at 5000 × g for 25 min and supernatant was mixed with finely powdered ammonium sulfate under cold conditions until its completely dissolves to give 45% saturation and incubated overnight at 4°C. The precipitates were harvested at 11000 × g for 25 min and supernatant was again saturated with ammonium sulfate at 55–85% saturation. The resultant precipitates were collected by next round of centrifugation and dissolved in Tris-HCl buffer (50 mM, pH = 8.0). The dissolved precipitate was dialyzed in a pre-treated dialysis tube and obtained precipitates were discarded by centrifugation. Post-dialysis the proteins from the samples were estimated using Lowery method [19].

2.4.2. Dialysis

The precipitates obtained after ammonium sulfate precipitation (i.e., partially purified enzyme) were dialyzed in a pre-treated dialysis tube (21 mm × 5 m)overnight at 4°C, against the same buffer. The precipitate produced after dialysis was separated and discarded by centrifugation.

2.4.3. Size exclusion and ion exchange chromatography

The dialyzed fractions were further purified through size exclusion chromatography using Bio-Gel P100 (Bio-Rad, USA) and collecting nearly 150–200 aliquots using 50 mM HEPES (pH = 5.5–6.0) as elution buffer. The eluents were further purified using pre-equilibrated (0.4 M NaCl) ion exchange chromatography by column of CM-Sephadex C50 (Sigma-Aldrich). The fractions were eluted using same buffer with discontinuous gradient of NaCl ranging from 0.1 M to 0.9 M at a flow rate of 0.5 mL/min, with each fraction of 2 mL. Fractions eluted for each gradient of NaCl were pooled separately and assayed for protein content and L-asparaginase enzyme activity. The active fractions with high enzyme activity were used for purity testing using SDS-PAGE and cytotoxicity assays.

2.4.4. L-asparaginase enzyme assay

L-asparaginase is an extracellular enzyme. The enzyme activity was performed according to the modified protocol of Imada *et al.* [20]. The assay system consists of 0.5 mL of 0.5 M phosphate buffer (pH = 7), 0.5 mL of substrate (0.04 M L-asparagine), 0.5 mL of purified enzyme, and final volume make up to 2 mL with distilled water. The reaction system was incubated at 37°C for 30 min and assay was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). The ammonia produced was measured by adding 0.2 mL Nessler's reagent into a reaction system of 0.1 mL of filtrate and 3.75 mL distilled water, incubated for 10 min under room temperature and recording the absorbance at 436 nm (Shimadzu UV-1800 UV-Vis spectrophotometer) against the blank. The blank consists of all reagents except that the purified enzyme and was added after the TCA addition. The amount the ammonia produced was calculated using the standard curve of ammonium sulfate. The unit activity of L-asparaginase was defined as the amount of enzyme required to produce 1 µmol of ammonia per mL per min under standard enzyme conditions. The protein content was measured using BSA as a standard.

The enzyme activity was calculated using following equation:

$$\text{Units / mL} = \frac{(\mu\text{mole of Ammonia released})(2.20)}{(0.2)\ (30)\ (0.1)}$$

Where, 2.20 = Final volume of reaction system, 0.2 = Volume taken for OD, 0.1 = Enzyme aliquote, 30 = Incubation time (in min)

2.4.5. Protein estimation and enzyme yield

The total protein content of the cell free extracts and eluted fraction was estimated by Lowry method [19], with BSA as a standard and reading the absorbance at 660 nm.

The purification factor was calculated by using following equation:

$$\text{Purification factor} = \frac{\text{Specific activity of enzyme at given purification step}}{\text{Initial specific activity of crude enzyme}}$$

Also, the enzyme yield was calculated using following equation:

$$\text{Enzyme yield \%} = \left(\frac{\text{Initial unit activity of enzyme}}{\text{Unit activity of enzyme at given purification step}}\right) \times 100$$

2.5. Characterization of L-asparaginase

2.5.1. Molecular weight determination using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Post-purification of L-asparaginase enzyme, the homogeneity and purity along with the molecular weight of the purified L-asparaginase enzyme was determined by SDS-PAGE using method developed by Laemmli [21], under non-reducing conditions. The gel system consists of separating gel of 6% stacking gel and 12.5% of separating gel. The separated protein bands after electrophoresis were visualized by silver staining.

2.5.2. Effect of abiotic factors on L-asparaginase activity

The abiotic factors influencing the enzyme production were assessed by optimizing one parameter at a time by keeping remaining factors unchanged. The effect of incubation temperature on L-asparaginase production was evaluated at different temperatures, from −20°C to 60°C, (with pH of the medium 7.0) by inoculating loop-full of spores. The effect of initial pH of the medium on L-asparaginase production was studied in the entire pH range 0–14 at 37°C for 3 days. The effect of different metal ions, namely, Magnesium (Mg^{+2}), Manganese (Mn^{+2}), Zinc (Zn^{+2}), Copper (Cu^{+2}), Iron (Fe^{+2}), Cobalt (Co^{+2}), Lead (Pb^{+2}), and Mercury (Hg^{+2}) at a concentration of 1 mM each was also assessed on L-asparaginase production under optimized conditions.

2.6. *In vitro* Cytotoxicity Assay

To evaluate the cytotoxicity (anti-proliferative property) of purified L-asparaginase enzyme from both the fungi, WTS-8 assay was performed on THP1-Human monocytic leukemia cell lines, procured from NCCS, Pune, India. Cells were grown on Roswell Park Memorial Institute (RPMI-1640) media supplemented with 10 % Fetal Bovine Serum (FBS) in standard T-25 flask (Thermo Fisher Scientific, USA). The matured cells were harvested at 300 × g by aspirating in a 5 mL centrifuge tube. RPMI media containing 10% FBS was used to adjust cell count, to have ~ 10,000 cells in 50 µL suspension. This 50 µL suspension was suspended in 96 well microtiter plate (Thermo Fisher Scientific, USA) and incubated at 37°C for 24 h by maintaining CO_2 concentration at 5%. After growth period, 50 µL of purified L-asparaginase enzyme was added to respective wells in different concentration (×2) and further incubated for 24 h maintaining same conditions. To estimate the viable cells, 10 µL cell counting Kit-8 reagent was added to each cell after 24 h and plate was further incubated for 3 h at 37°C by maintaining CO_2concentration at 5%. The content from microtiter plate was read at 450 nm in plate reader. The cell growth inhibition (%) was calculated by considering the amount of L-asparaginase enzyme required to inhibit the cell growth by 50%, that is, IC_{50}, (the cells were observed using compound microscope, XDFL series, Sunny Instruments, China and ImageJ (Fiji) V1.53j as imaging software) which was generated through dose-response curve for the cell line and following equation.

$$\text{\% Inhibition} = 100 - \left(\frac{\text{Absorbance of test}}{\text{Absorbance of control (i.e. non-treated)}}\right) \times 100$$

2.7. Statistical Analysis

The obtained results were analyzed with ANOVA. The statistical analysis was performed using PAST 3.0.

3. RESULTS AND DISCUSSION

3.1. Screening and Isolation of L-asparaginase Producing Fungi

The screening of L-asparaginase producing fungus was performed by plate assay method. The assay method consists of L-asparagine as a sole source of nitrogen and phenol red as a pH indicator. During the growth of potent fungal culture, the change in the color of medium surrounding the cell mass into pink (from yellow) indicates the production of L-asparaginase, [Figure 1] which was due to release of ammonia resulting into the increasing in the pH of the medium. The L-asparaginase catalyzes the conversion of L-asparagine into L-aspartate and ammonia by the deamidation reaction [22].

In this study, ~20 such fungal isolates were screened capable of producing L-asparaginase. Among them two fungal cultures HK01 and HK02 were further selected based on their ability of higher production of L-asparaginase. A broad diameter of pink color zone surrounding the fungal cell mass indicates the organism's capability producing L-asparaginase enzyme. It was also observed that, in liquid culture, the mycelial growth can form relatively large spherical pellets and may result in better yield of L-asparaginase as compared to the mycelial growth as free filaments [Figure S1] Moreover, the extracellular enzyme suspension has been tested for glutaminase activity and it

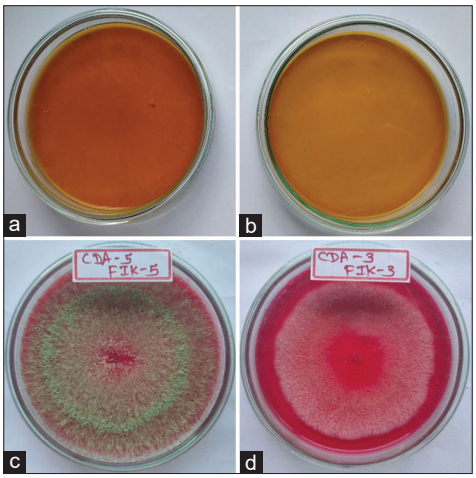

Figure 1: The plates showing the L-asparaginase enzyme activity of (a) *Trichoderma koningii* HK02 and (b) *Trichoderma viride* HK01.

was found that, culture suspensions of both the fungi were free of glutaminase activity [Figure 2].

The isolates were identified using polyphasic approach as noted above. According to the ITS, sequencing results culture HK01 was identified as *T. viride HK01*and HK02 as *T. koningii HK02*. This identification was also supported by the results of morphological staining method.

3.2. Purification of L-asparaginase

Enzyme purification of L-asparaginase was performed using crude culture filtrates/culture suspension of *T. koningii HK02* and *T. viride HK01*. The ammonium sulfate precipitation and dialysis were used for partial purification of L-asparaginase. Enzyme was further purified using size-exclusion chromatography. The purification was started using protein concentration of 176.7 µg having 56.64 µM/mL/min of enzyme activity and 320.5 Unit/mg of protein of specific activity of L-asparaginase from *T. koningii HK02*. Similarly, purification of L-asparaginase from *T. viride HK01* was started with 148.4 µg of protein concentration with 70.51 µM/mL/min of enzyme activity and 475.1 Unit/mg of protein of specific activity.

The ammonium sulfate precipitated and dialyzed concentrated enzyme had a specific activity of 344.7 Unit/mg of protein with protein concentration of 163.08 µg and enzyme yield was 99.25% and purification factor was 1.08 for L-asparaginase from *T. viride HK01* [Table 1]. Similarly, for *T. koningii HK02*, the ammonium sulfate precipitated and dialyzed concentrated L-asparaginase has a specific activity of 508.1 Unit/mg of protein with protein concentration of 137.8 µg and enzyme yield was 99.3% and purification factor was 1.07.

The enzyme preparation obtained after dialysis and ammonium sulfate precipitated was further purified using gel-filtration chromatography yielding several fractions, containing L-asparaginase in few of them with enzyme peak in latter fractions. For *T. viride HK01* after gel-filtration chromatography, the specific activity of purified L-asparaginase increased several folds to 1172.1 Unit/mg of protein with protein concentration of 59.6 µg. Similarly, for *T. koningii HK02* the specific activity of purified L-asparaginase also increased several folds to 1171.2 Unit/mg of protein with protein concentration of 48.6 µg. In the last step of purification, CM-Sephadex C50 (ion exchange chromatography) column showed total L-asparaginase activity of 3009 Unit/mg of protein with 23.3 µg of protein content for *T. viride HK01*and for *T. koningii HK02* its was 1919.4 Unit/mg of protein with protein concentration of 29.43 µg [Table 1].

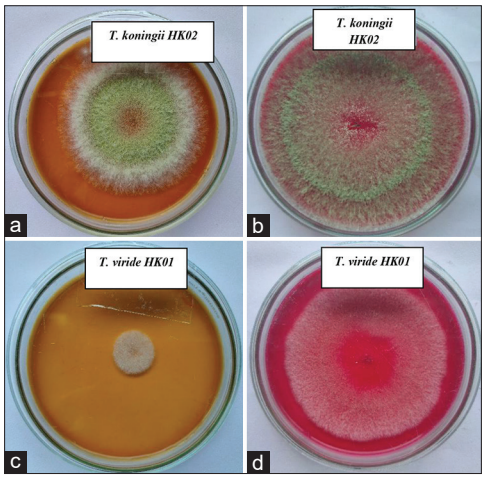

Figure 2: The L-glutaminase activity of purified L-asparaginase. (a) *Trichoderma koningii* HK02 Glutaminase negative (b) *Trichoderma koningii* HK02 L-asparaginase positive; (c) *Trichoderma viride* HK01 glutaminase negative (d) *Trichoderma viride* HK01 L-asparaginase positive.

In their study, Sahu *et al.* [22] have purified the L-asparaginase enzyme from an actinomycete (strainLA-29) which was isolated from the fish gut. They achieved 18-fold purification of the enzyme, which showed the specific activity of ~ 13.57 IU/mg of protein. Also in another study 99.3 and 106 fold purification of L-asparaginase enzyme was obtained by Narayana *et al.* [23] and El-Bessoumy *et al.* [24], respectively, from *Streptomyces albidoflavus* and *Pseudomonas aeruginosa*.

3.3. Characterization of L-asparaginase

3.3.1. Molecular weight determination using SDS-PAGE

As shown in Figure 3, the single band on SDS-PAGE of L-asparaginase after purification steps shows that enzyme was purified successfully. The single bands on SDS-PAGE also confirmed that there was no detectable contamination in the purified enzyme and electrophoresis process has resolved only one distinctive band. A defined molecular weight marker ranging between molecular mass of ~14 and 97 kDa was also separated along with purified L-asparaginase protein on SDS-PAGE.

L-asparaginase enzyme is a tetrameric protein [1] and on SDS-PAGE only single band was visualized, which suggest that purified L-asparaginase might be a homotetramer. Furthermore, on comparing the purified band with standard molecular weight markers, the apparent molecular weight of L-asparaginase enzyme from *T. viride HK01* was ~69 kDa and for *T. koningii HK02* it was ~72 kDa [Figure 3].

Similarly, L-asparaginase obtained from *Rhizopus* spp. [25] and *Aspergillus niger* [8] was 48 and 66 kDa, respectively. The molecular weight of purified L-asparaginase from *Aspergillus terreus* was about 94 kDa [26,27] which was comparatively higher than L-asparaginase of *Trichoderma* spp. obtained in this study. Furthermore, L-asparaginase with higher molecular weight as high as 216 kDa was also observed from *Cylindrocarpon obtusisporum*, a mesophilic fungus [28]. Different studies reported variation in the molecular weight of L-asparaginase enzyme, primarily due to environmental adaptation and genetic variations.

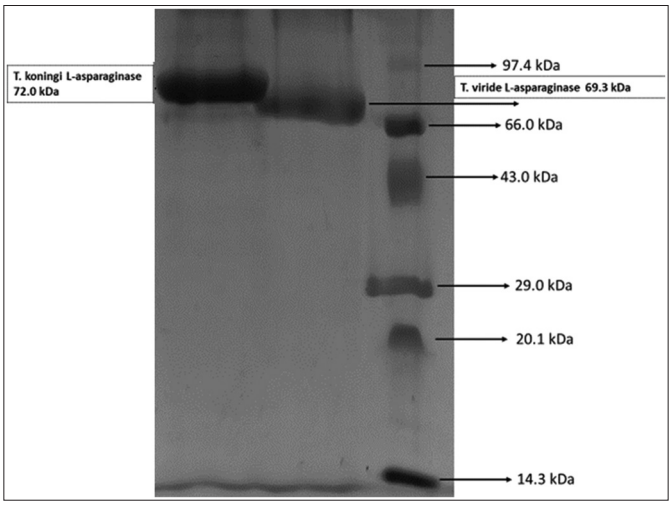

Figure 3: Sodium dedecyl sulfate-polyacryalamide gel electrophoresis of purified L-asparaginase enzyme.

Table 1: Summary of the purification procedure of the enzyme L-asparaginase.

No.	Sample	Protein concentration (µg)		Enzyme activity (µm/mL/min)		Specific activity (unit/mg of protein)		Enzyme yield (%)		Purification factor	
		T. koningii HK02	*T. viride HK01*	*T. koningii HK02*	*T. viride HK01*	*T. koningii HK02*	*T. viride HK01*	*T. koningii HK02*	*T. viride HK01*	*T. koningii HK02*	*T. viride HK01*
1	Crude sample	176.7	148.4	56.64	70.51	320.5	475.1	100	100	1	1
2	Pellet after $(NH_4)_2SO_4$ Precipitation	164.31	141.3	54.33	67.91	330.6	480.6	95.92	96.31	1.03	1.01
3	Sample after dialysis	163.08	137.8	56.23	70.02	344.7	508.1	99.25	99.3	1.08	1.07
4	Size exclusion chromatography (Bio-Gel P)	48.6	59.6	56.08	69.86	1153.9	1172.1	99	99.07	3.65	2.47
5	CM-Sephadex C50 (Elution 0.4 M NaCl)	29.43	23.3	56.49	70.11	1919.4	3009	99.73	99.43	5.98	6.33

T. koningii: Trichoderma koningii, T. viride: Trichoderma viride

3.3.2. Kinetics of the purified L-asparaginase enzyme

The estimated K_m and V_{max} of purified L-asparaginase enzyme are observed from Table 2. Thakur *et al.* [29] studied the L-asparaginase from *Mucorhiemalis*, whose K_m and V_{max} calculated were 4.3 mM and 625 U/mL, respectively. A higher K_m of 12.5 mM and V_{max} of 104.16 U/mL of L-asparaginase of *Aspergillus aculeatus* [30]. However, lower K_m of 1 mM was also obtained in a study by Raha *et al.* [28] for L-asparaginase enzyme obtained from *Cylindrocarpon obtusisporum*. The lower K_m observed in this study suggest the higher affinity of purified L-asparaginase enzyme toward its substrate, which should be highly effective towards tumor cells.

3.4. Effect of Abiotic Parameters on L-asparaginase

3.4.1. Temperature profile

The purified L-asparaginase enzyme from *T. koningii HK02* and *T. viride HK01* exhibits the optimum activity at 40°C and 37°C with unit activity of 58.66 µM/mL/min and 71.08 µM/mL/min, respectively [Figure 4]. In case of L-asparaginase enzyme from *T. koningii HK02*, the specific activity of the purified enzyme decreased at both the sides of optimum temperature. The enzyme activity gradually increased with increasing temperature from 10°C to 37°C, with increase in unit activity from 7.31 µM/mL/min to 55.01 µM/mL/min. On increasing the temperature from 40°C to 50°C, the activity of L-asparaginase for *T. koningii HK02* decreases sharply from 58.66 µM/mL/min to 14.02 µM/mL/min. L-asparaginase purified from *T. viride HK01*, also exhibited similar pattern with increase in temperature from 10°C to 30°C, activity of the enzyme also increased from 6.88 µM/mL/min to 64.41 µM/mL/min. The specific activity initially declined from 71.08 µM/mL/min to 66.87 µM/mL/min at 40°C, but it declined sharply at 50°C to 19.93 µM/mL/min. The specific activity of purified L-asparaginase enzyme from both the fungi was <1 µM/mL/min at higher temperature of 60°C. For the results, it can be observed that the unit activity of L-asparaginase enzyme was higher for *T. koningii HK02* as compared to *T. viride HK01*.

The optimum temperature around 37°C for L-asparaginase enzyme makes it suitable for the treatment of tumor patient for the removal of asparagine. These results are in line with the studies of Dange and Peshwe [30], Sarquis *et al.* [31] who observed that the optimum temperature for the L-asparaginase enzyme purified from *A. terreus* KLS2 and *Streptomyces gulbargensis* was 37°C and 40°C, respectively. Furthermore, in recent studies purified L-asparaginase from *Lasidiplodia theobromae* optimum temperature of 37°C [32].

Table 2: Kinetic parameters of purified L-asparaginase enzyme.

Value	*Trichoderma koningii* HK02	*Trichoderma viride* HK01
K_m	0.0528 mM	0.0565 mM
V_{max}	55.24 U/mL	71.13 U/mL

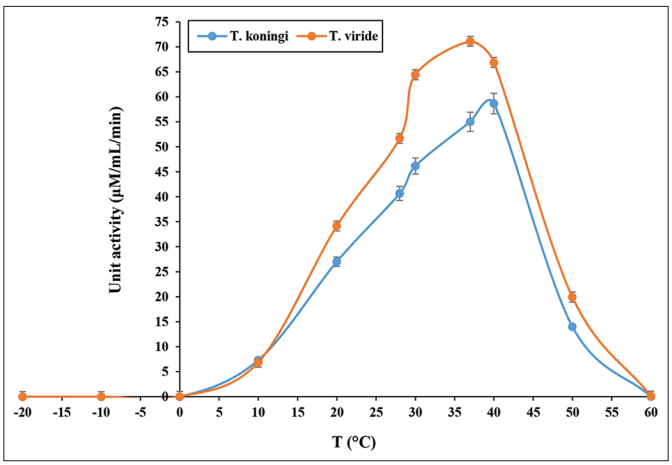

Figure 4: Effect of various temperatures on activity of purified L-asparaginase enzyme.

3.4.2. Effect of pH

The optimum pH or the maximum activity of purified L-asparaginase enzyme for *T. koningii HK02* and *T. viride HK01* was pH 7.0 and 8.0, respectively [Figure 5]. The estimated maximum unit activity of purified L-asparaginase enzyme from *T. koningii HK02* was 56.9 µM/mL/min and 71.61 µM/mL/min from *T. viride HK01*. As observed in temperature, the activity of the purified enzyme also decreased on both the side of the optimum pH for both the fungi. The enzyme activity decreases gradually from 56.9 µM/mL/min to 36.56 µM/mL/min on reducing pH from 7.0 to 5.0 for purified L-asparaginase from *T. koningii HK02*. It decreases sharply by further reducing pH to more acidic scale (i.e. pH = 3.0) where enzyme activity was as low as 2.8 µM/mL/min. On increasing pH toward basic scale, the enzyme activity also gradually decreases to 21.13 µM/mL/min at pH = 9.0 and the enzyme became inactive at pH = 10.0, where activity was near to zero.

For L-asparaginase enzyme purified from *T. viride HK01*, the activity also decreases on acidic range, decreases from 71.61 µM/mL/min to

26.18 µM/mL/min by reducing pH from 8.0 to 6.0. It decreases sharply to 2.21 µM/mL/min at pH 4.0. While on basic range, the decrease in activity was gradual till pH 10.0, but decrease sharply thereafter and activity was near to zero at pH 12.0. Thus, the results suggest that purified L-asparaginase enzyme from *T. koningii HK02* showed considerable activity toward acidic pH while purified L-asparaginase enzyme from *T. viride HK01* more active toward basic pH.

It was observed that, at neutral and alkaline pH amidases enzymes like L-asparaginase was more stable exhibiting optimum activity [33]. One of the studies also suggests that optimum activity for such amidase was in pH range of 5.0–9.0 [33]. L-asparaginase enzyme purified from *Streptomyces* sp. PDK7 [34] had optimum pH of 8.5, whereas the optimum pH of *S. albidoflavus* was observed at pH = 7.5 [23].

3.4.3. Effect of metal ions

Various metal ions are co-factors for many microbial enzymes, by acting as electron donor or electron acceptor and affecting overall enzyme stability and activity [35]. In the present study effect of eight metals ion as mentioned above on purified L-asparaginase enzyme were accessed. As anticipated, lead and mercury had completely inhibited the L-asparaginase activity from both the fungi [Figure 4]. In the presence of metals, like cobalt the activity of L-asparaginase was sharply decreased, to 5.82 µM/mL/min from to 57.34 µM/mL/min (control) for the enzyme purified from *T. koningii HK02*. Furthermore, the enzyme activity decreased to 9.7 µM/mL/min from 71.63 µM/mL/min (control) for the enzyme purified from *T. viride HK01*. Whereas, in the presence

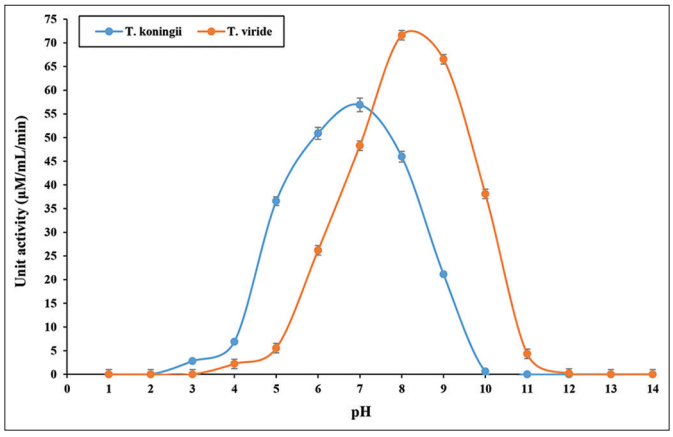

Figure 5: Effect of various pH on activity of purified L-asparaginase enzyme.

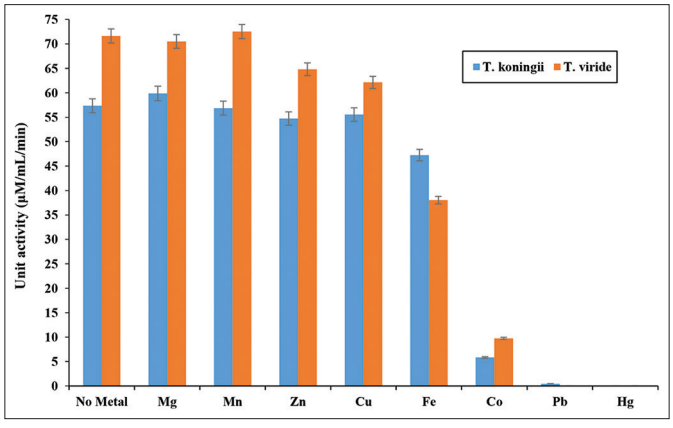

Figure 6: Effect of various metal ions on activity of purified L-asparaginase enzyme.

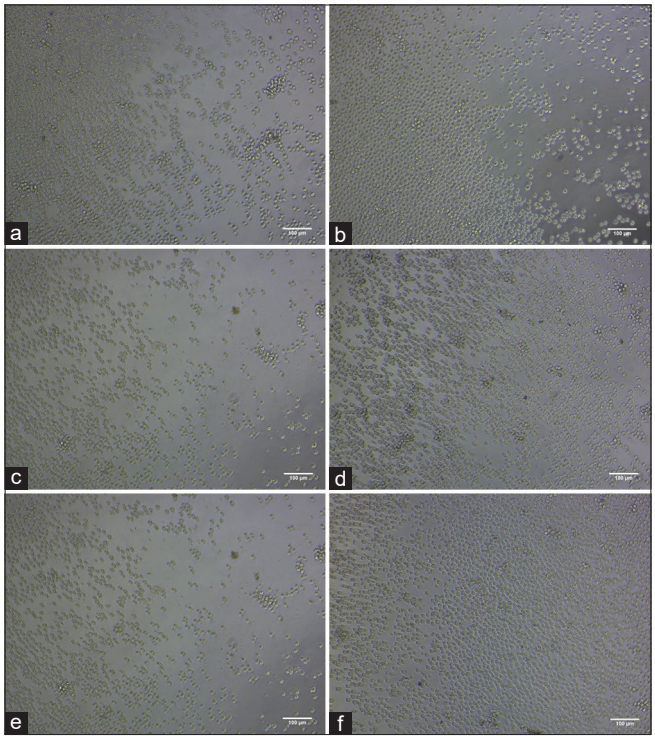

Figure 7: Cytotoxicity assay on THP1-Human monocytic leukemia cell line using purified L-asparaginase enzyme (a) 0.25 Units/mL, (b) 0.50 Units/mL, (c) 1.0 Units/mL, (d) 2.0 Units/mL, (e) 2.5 Units/mL, (f) control (without enzyme) of *Trichoderma koningii* HK02.

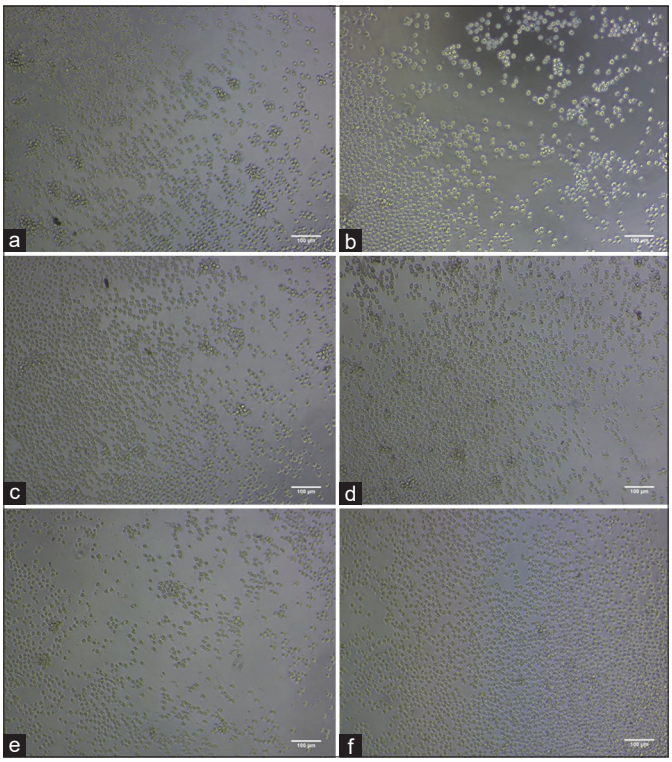

Figure 8: Cytotoxicity assay on THP1-Human monocytic leukemia cell line using purified L-asparaginase enzyme (a) 0.25 Units/mL, (b) 0.50 Units/mL, (c) 1.0 Units/mL, (d) 2.0 Units/mL, (e) 2.5 Units/mL, (f) control (without enzyme) of *Trichoderma viride* HK01.

of manganese ions the activity of purified L-asparaginase from *T. koningii HK02* was increased to 59.87 µM/ml/min in presence of manganese from 57.34 µM/mL/min (control). In the presence of other metal ions such as zinc, copper, and iron, the L-asparaginase enzyme from activity *T. koningii HK02* decreased by many folds as observed from Figure 6. Similarly, the activity of purified L-asparaginase from *T. viride HK01* in the presence of zinc, copper, and iron also decreased by several folds. However, the observed results also suggest that the effect of manganese and magnesium on L-asparaginase activity from *T. viride HK01*was negligible.

3.5. *In vitro* Cytotoxicity Assay

Since purified L-asparaginase enzyme has a potential application for anti-cancer properties, its toxicity to human cells must be accessed. The IC_{50} for L-asparaginase purified from *T. koningii* was found to 2.0 Units/mL, whereas that from *T. viride HK01* was 2.5 Units/mL. The results suggest that purified L-asparaginase enzyme from both the fungi were equally effective against the cancerous cell lines [Figures 7 and 8].

4. CONCLUSION

The purified L-asparaginase enzyme was ~69 kDa and ~72 kDa in molecular size respectively from *T. viride HK01* and *T. koningii HK02*. It exhibited the temperature of 37°C and 40°C and pH of 8.0 and 7.0 for its optimum activity from *T. viride HK01*and *T. koningii HK02*, respectively. The purified L-asparaginase from both the sources did not showed glutaminase activity. Thus, the results from the above study suggested that the isolated and purified L-asparaginase enzymes could be the potential agents for treatment of acute lymphoblastic leukemia.

5. ACKNOWLEDGMENT

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6. AUTHORS' CONTRIBUTIONS

KL designed the study, performed the experiments and wrote the manuscript. HB developed the concept of the study and edited the manuscript. Both the authors read and approved the final manuscript.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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SUPPLEMENTARY DATA

Comparative analysis of purified anti-leukemic L-asparaginase enzyme from *Trichoderma* spp.

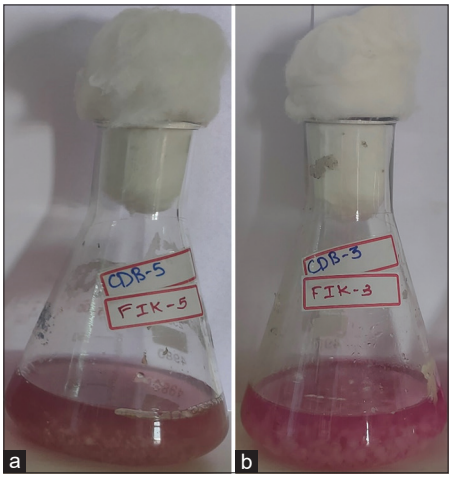

Figure S1: The flasks showing the L-asparaginase enzyme activity of (a) *Trichoderma koningii* HK02 and (b) *Trichoderma viride HK01*.