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### RESEARCH ARTICLE

## PARTIAL PURIFICATION AND CHARACTERISATION OF L-ASPARAGINASE FROM *GORDONIA* SP KM067296 ISOLATED FROM LONAR LAKE (MAHARASHTRA, INDIA).

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L-asparagine amino hydrolase, halo-tolerant, *Gordonia terrae*, Purification, anti-leukemic.

### Abstract

A unique extracellular L-Asparaginase or L-asparagine amino hydrolase (E.C.3.5.1.1) was studied as a potent anti-leukemic candidate. Lonar lake provides an extraordinary environment for halo-tolerant and alkali tolerant microbes. Many researches have been carried out on its microbial flora but the study on L-asparaginase producing species have not been reported very extensively and an attempt for the same is made in this study. In the present study new potential bacterial L-asparaginase producer *Gordonia terrae* KM067296 has been studied for enzyme production and partial purification and characterization of the enzyme was also carried out. The crude enzyme extract was partially purified using ammonium sulfate precipitation, dialysis, and gel filtration. The partially purified L-asparaginase showed specific activity of 38.61 IU/mg and was purified up to 14 fold post gel filtration and showed maximal activity against L-asparagine when incubated at pH 7.4 at 37°C for 30 minutes with substrate concentration of 0.04 M. The activity of L-asparaginase was enhanced by MgCl<sub>2</sub> and was inhibited by EDTA. The molecular weight of the enzyme was found to be approximately 12 kDa. A Line weaver Burk analysis showed a Km value of 0.061 mM and Vmax of 0.57 IU. This study revealed *Gordonia terrae* KM067296 as a prospective source for high yielding and highly substrate specific production of L-asparaginase.

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### Introduction:-

L-Asparaginase (L-asparagine amino hydrolase, EC 3.5.1.1) represents one of the most biotechnologically and biomedically important class of therapeutic enzymes contributing for about 40% of the total worldwide enzyme sales (Eishafei et al., 2012). L-asparaginase, an amidase hydrolyses the amide bond in L-asparagine to give aspartic acid & ammonia (Aljewari et al., 2010) and play vital role in the metabolism of all living organisms (Anese M, 2011). It keeps nitrogen balance and the level of amino acids within cells. L-asparaginase has been broadly studied in plants, animals, rodents and microorganisms of which the most commonly utilized microorganisms for L-asparaginase production are *Erwinia caratovora*, *Bacillus spp.*, *Corynebacterium glutamicum*, *Aspergillus terreus*, *Withania somnifera* and *E.coli* (Kumar and Verma, 2012) however tumour inhibitory activity of L-asparaginase has been reported with *E.coli*, *Erwinia aroideae* and *Serratia marcescens* that have reached up to clinical trials. Chronic

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administration of such an enzyme protein, in general, produces the corresponding antibody in the tissues, resulting in the anaphylactic shock or neutralization of drug effect, thus exploring novel serologically different L-asparaginase is a need of the hour (Jois et al., 2013).

L-asparaginase is an enzyme drug choice for acute lymphoblastic leukemia in children used in combination chemotherapy. Normal cells possess an enzyme L-asparagine synthase which satisfies for the requirement of the amino acid unlike in leukemic cells which have trivial or no L asparagine synthase. This deficit leads to the inhibition of DNA and RNA synthesis, blocking protein synthesis and destruction of cell function and cell death (Chande and Bhat, 2014). It is widely used in acute lymphoblastic leukemia, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, and reticulosarcoma and melanosarcoma regimen. Apart from its therapeutic value, L-asparaginase also finds application in food industry. It has been found that L-asparaginase reduces the formation of carcinogenic acryl amides in deep fried potato recipes. Addition of asparaginase before baking or frying the food, results in conversion of asparagine into common amino acid, aspartic acid and ammonium. As a result, asparagine is unavailable to complete Maillard reaction, and thereby the formation of acryl amide is greatly lowered (Friedman, 2003). The present study aims to isolate, purify and characterize L-asparaginase from selected bacterial species.

Microbes residing in supreme habitat like alkaline or saline areas tend to express proteins with different characteristics than those inhabiting in the usual environment. Lonar Lake located in Buldhana district of Maharashtra appears to be one of the marvels of state and is considered to be the world's third largest crater formed due to a meteor impact. It is a saline soda lake with pH in the range of 10-12, serves as a distinctive habitat for isolation of halophiles and alkaliphilic organisms (Chande and Bhat, 2014). Studies on L-asparaginase producing isolate from Lonar Lake have not been reported very extensively and an attempt for the same is made in this study. The current study was aimed at exploring *Gordonia terrae* strain DSM43249 as a source of L-asparaginase.

### Materials and Methods:-

Soil samples were collected in sterile containers from four side of Lonar lake (19°58'36"N 76°30'30"E, Buldhana, Dist. Maharashtra, India) in the month of December. The pH of the lake was noted immediately at the site. Luhana's method (Luhana et al., 2013) was modified for carrying out production assay. It was grown at 37°C in shaker incubator and then transferred to Erlenmeyer flask for 72h at 37°C in M-9 media containing (per 1L distilled water): Na<sub>2</sub>HPO<sub>4</sub>, 6.0g; KH<sub>2</sub>PO<sub>4</sub>, 3.0g; NaCl, 0.5g; L-asparagine, 1%; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.12g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.01g; peptone 0.6%; yeast extract, 1%; pH-7.0. At the end of incubation time, the samples were centrifuged at 10,000rpm for 15min, at 4°C, and then cell free supernatant was used in further purification steps. All the assays were performed in triplicates.

### Enzyme Assay:-

The enzyme was assayed by direct nesslerization method. One unit of L-asparaginase activity (IU) is defined as the amount of enzyme which liberates 1 µmol of ammonia per min at 30°C and pH 7.4 (Luhana et al., 2013).

### Estimation of Protein:-

The protein contents were estimated by the method of Lowry using bovine serum albumin as the standard (Lowry, 1951).

### Purification of L-Asparaginase:-

The activity of extracellular and intracellular crude enzyme was assayed. The extracellular concentration of the enzyme was significantly higher and hence it was used for further purification and characterization. The supernatant from the production media (crude enzyme) was subjected to serial ammonium sulfate precipitation 0%-40%, 40%-60% and 60%-80% and 80%-100% and enzyme assay for each fraction was carried out. Maximum activity was found in 60% saturation and hence this fraction was dialyzed against Tris buffer (50mM, pH 8.4) with three buffer changes at 4°C overnight. Gel filtration chromatography was done for dialyzed sample using Sephadex G-75 column (1×25m) equilibrated and eluted with 150ml Tris-HCl (50mM, pH 8.4). The separated proteins were visualized by PAGE (Laemmli, 1970) using Coomassie brilliant blue R-250 staining solution and the molecular weight of L-asparaginase was determined using standard molecular weight marker (medium range 97-14KDa; Sigma-Aldrich).

### Characterization of L-Asparaginase:-

The optimum conditions for the enzyme activity were determined by assaying at different temperatures (10-45°C) pH (4-10) (Sabbagh et al., 2013) and incubation time (10-60 minutes). The enzyme kinetics as measured by the Michaelis constant ( $K_m$ ) is defined as the substrate concentration at half the maximum velocity, the rate of enzymatic reactions, by relating reaction rate to the concentration of a substrate. The Michaelis constant ( $K_m$ ) value of the purified enzyme was estimated in a range of L-asparagine concentrations of 0.02M- 0.08 M. Line Weaver-Burk plot analysis was carried out to calculate the apparent  $K_m$  and  $V_{max}$  value of purified L-asparaginase. The activity was determined in different concentrations of metal ion  $MgCl_2$  and metal ion chelator ethylenediamine tetra acetic acid (EDTA) in the concentration of 0.2-0.8mg/ml in the reaction mixture (Mohapatra et al., 1995).

### Results and Discussion:-

#### Identification of Isolate:-

The potential isolate was identified by 16S rRNA sequencing method (Chromus Biotech Pvt.Ltd., Bangalore). The sequence was aligned with representative sequence. The isolate was found to be *Gordonia sp* KM067296.

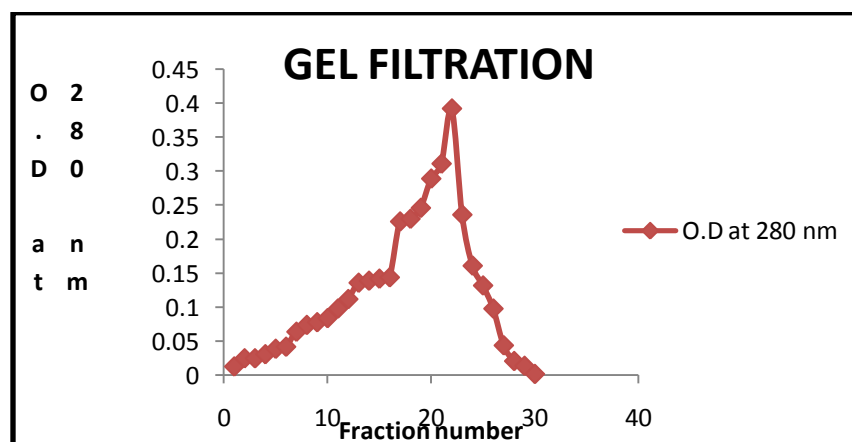
#### Purification of L-asparaginase:-

The sequential multi steps purification procedures has been summarized in Table 1.

**Table 1:-** Enzyme activity after different stages of purification.

Sample	Volume (ml)	Enzyme Activity (IU/ml)	Protein (mg/ml)	Total Activity(IU)	Specific Activity(IU/mg)	Fold Purification	% yield
Crude Extract	200	54.61	19.5	10922	2.80	1	100
80% Dialysis	8	32.77	5.5	262.16	5.95	2.12	2.4
Gel Filtration	2	21.16	0.548	42.32	38.61	13.79	0.4

Figure 1 shows the elution profile of the partially purified L-asparaginase from Sephadex G-75 (Sigma-Aldrich) column. A sharp distinctive peak of L-asparaginase activity, which fits with only one protein peak, was noticed. The most active fractions with specific activity 38.61IU/ mg and about 14-fold purification were pooled together, concentrated and stored at -20°C.

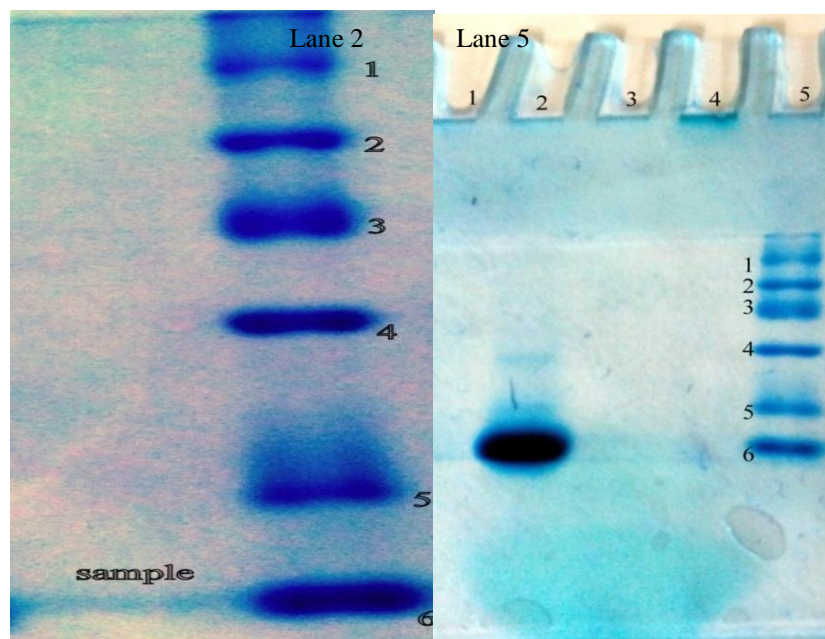


**Figure 1:-** Elution profile of Gel filtration chromatography of L-asparaginase on Sephadex G-75.

Total protein was analyzed at 280 nm. The fractions were assayed for enzyme activity. Fraction No.21 had highest specific activity of 38.61IU/ mg of protein

SDS PAGE revealed only a single distinctive protein band for the preparation of L-asparaginase with an apparent molecular weight of  $12 \pm 2$  kDa. In this respect, L-asparaginase purified from *Pseudomonas* MB-405, *Thermus thermophilus* and *Escherichia coli* were with smaller MW values ranging from 33-34 kDa (Manna S, 1995) (Soares A.L, 2002). In figure 2b, Lane 5 shows bands of various protein molecular weight markers, Lane 2 showing one band

of purified L-asparaginase from *Gordonia* sp **KM067296** obtained through sephadex G-75 chromatography separation with an apparent molecular weight of 12 KDa.



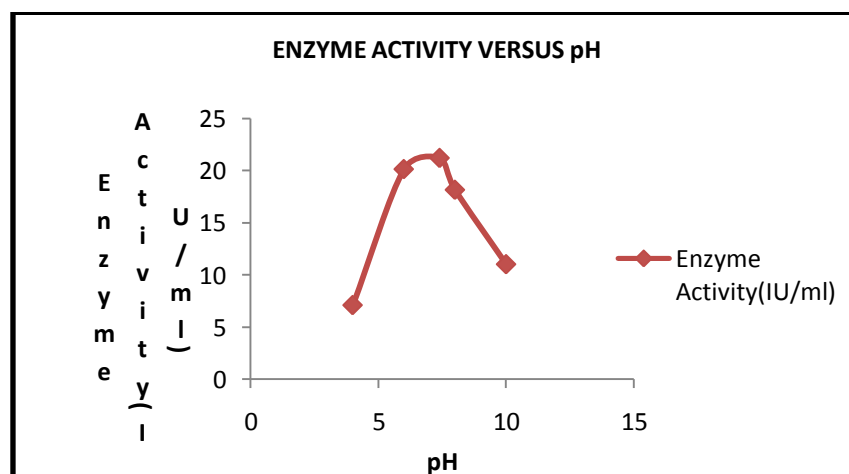
**Figure 2a:-**Separation of protein bands through SDS PAGE

**Figure 2b:-**Separation of protein bands through Native PAGE

Characterization of Partially Purified L-asparaginase Enzyme

#### Effect of pH on enzyme activity:-

Results revealed that the pH 7.4 was optimal for L-asparaginase from *Gordonia terrae*. These results coincide with that of Dhevagi and Poorani (2006) who reported the maximal L-asparaginase activity in *Streptomyces spp.* which was between pH 7.0-8.0, whereas pH 5.0-9.0 were reported earlier to be optimum for amidase activity (Ohshima et al., 1976).

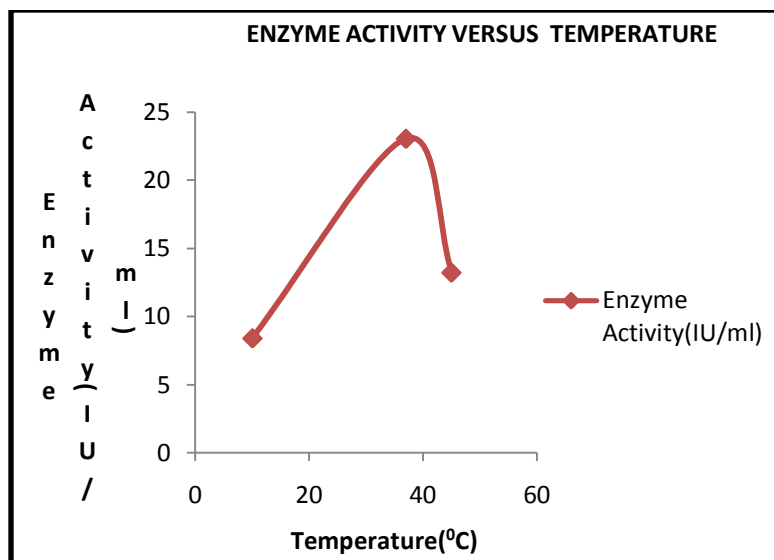


**Figure 3:-** L-asparaginase activity from *Gordonia terrae* in the range value of pH 4-10 in various buffers.

#### Effect of temperature on enzyme activity:-

The purified L-asparaginase was active at a range of temperature from 10°C to 40°C with an optimum at 37°C, but its activity started decreasing as it approached 45°C. These results were in agreement with a previous study where

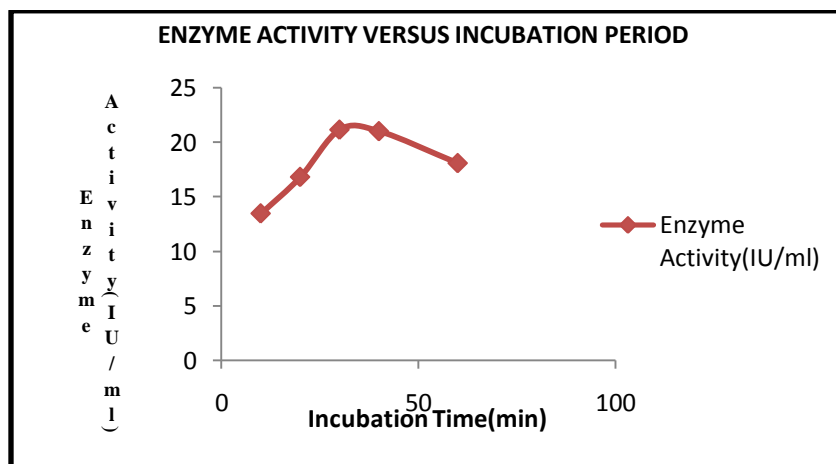
the temperature for optimum L-asparaginase activity obtained from *Pseudomonas stutzeri* MB-405 was 37°C. L-asparaginase from *Erwinia spp.* showed maximum activity at 35°C (Borkotaky and Bezbaruah, 2002).



**Figure 4:-** Graph showing activity of L-asparaginase from *Gordonia terrae* at temperatures range of 10-60°C in Tris-HCl (pH-7.4)

#### Effect of Incubation time on enzyme activity:-

The L-asparaginase activity increased as the incubation time increased up to 45 min, after which decrease in enzyme activity was seen wherein highest enzyme activity was observed at 30 mins. Similar results were recorded for L-asparaginase activity from chicken liver (Sayed E.M, 2011). The incubation of L-asparaginase purified from *Ps.aeruginosa* at 37°C for different time intervals showed that the activity reached its maximum at 30 min (Bessoumy et al., 2004).

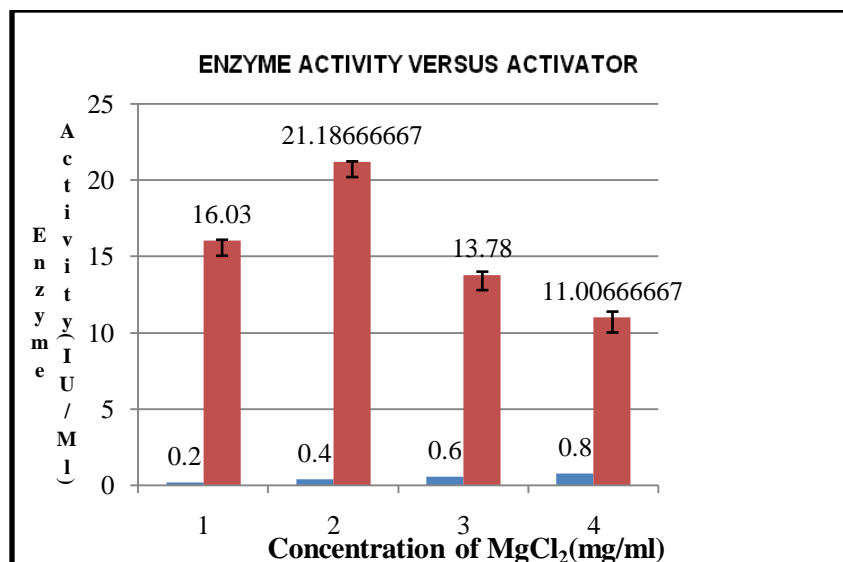


**Figure 5:-**Effect of incubation time on L-asparaginase activity from *Gordonia terrae* at different incubation period range of 10-60 minutes in Tris-HCl buffer (pH-8.4).

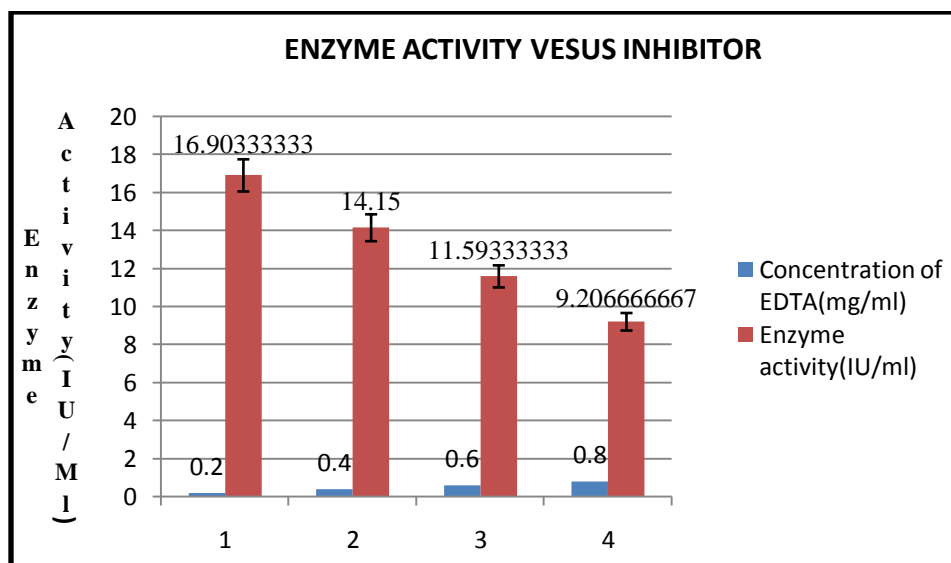
#### Effect of metal ions on enzyme activity:-

Among the salts tested, considerable loss of activity was observed only with EDTA. The highest inhibition value was recorded with a concentration of 0.8 mg/ml. There are reports that L-asparaginase extracted from *Bacillus spp.* was strongly inhibited by EDTA (Mohapatra et al., 1995). Addition of  $MgCl_2$  enhanced the enzyme activity to

certain extent after which decrease in enzyme activity was seen. These results are in agreement with results reported for L-asparaginase from *Erwinia caratovora* (Warangkar and Khobragade, 2010).



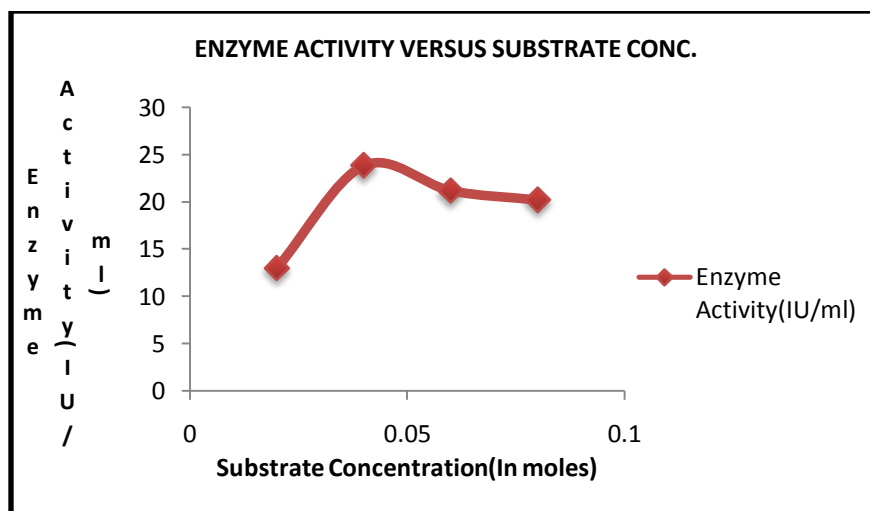
**Figure 6:-** Effect of activator L-asparaginase activity from *Gordonia terrae* at different concentration of activator ( $MgCl_2$ ).



**Figure 7:-** Effect of Inhibitor on L-asparaginase activity from *Gordonia terrae* at different concentrations of inhibitor (EDTA).

#### Effect of substrate concentration on enzyme activity:-

A Line weaver Burk analysis showed a  $K_m$  value of 0.061mM and  $V_{max}$  of 0.57 IU. Higher  $K_m$  values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported (Chang and Farnden, 1981). On The other hand, a lower  $K_m$  value (0.058 mM) was obtained for L-asparaginase from *Erwinia chrysanthemi* 3937 (Kotzia and Labrau, 2007).



**Figure 8:-** Enzyme activity of L-asparaginase isolated from *Gordonia terrae* different substrate concentrations

### Conclusion:-

The present study focused on production, partial purification and characterization of L-asparaginase from *Gordonia terrae* strain KM067296. The isolated *Gordonia terrae* has the ability to produce a significant amount of extracellular L-asparaginase. The present study indicates the isolated *Gordonia terrae* strain KM067296 used in this study will be a potential source for extracellular L-asparaginase enzyme. The kinetic parameters for optimal production of biologically active L-asparaginase have been ascertained. The high catalytic activity of the enzyme at physiological pH and temperature and its considerable stability over a wide range of pH and temperature makes it highly favorable to be exploited as a potent anticancer agent. Studies on the enzyme relating to purification and characterization would open new avenues in the application of the enzyme in the healthcare industry and for pharmaceutical, therapeutic, commercial purposes.

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