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Isolation and production of clinical and food grade L-Asparaginase enzyme from fungi

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Abstract
The present study deals with the isolation and optimization of an extracellular L-asparaginase from a fungal microorganism screened from marine soil. Optimization and production of *Trichoderma viride* sp. was performed by submerged fermentation and maximum enzyme production occurred on the third day at pH 6.5 and temperature of 37 °C which showed to be an ideal condition for the marine enzyme. Optimization of the various other carbon and nitrogen source supplements as nutritive additives further enhanced the production when 0.5% substrate was supplemented with 0.5% peptone and 0.6% maltose. Good scavenging property and the ability to lower the acrylamide levels in food stuffs makes it a valuable enzyme in pharmaceutical and food industries.

Keywords: L-asparaginase, optimization, production, marine, antioxidant, acrylamide-mitigation agent.

1. Introduction
A tetrameric L-asparaginase (E.C.3.5.1.1) is a hydrolytic enzyme that catalyzes the breakdown of L-asparagine to form L-aspartic acid and releases ammonia [1]. The substrate, L-asparagine is as an essential amino acid which is required for the development and growth of cancer cells. L-asparagine is synthesized by the normal cells with the help of L-asparagine synthetase. The abnormal cancer cells distinguish themselves from normal cells in low level of L-asparagine [2]. For survival, the tumor cells require greater amounts of L-asparagine, as they deplete all the L-asparagine from the circulatory blood leading to death of leukemic cells [3].
L-asparaginase is an enzyme drug which is used to reduce the circulatory L-asparagine level, thereby stopping tumor cell growth which is widely used in the treatment of acute lymphoblastic leukemia (ALL) [4]. The enzyme is also commercially available and is marketed under the brand names like Preventase and it is extensively used as the potent antileukemic agent. L-asparaginase also serves as a very good food-processing agent as it reduces the level of acrylamide formation during the manufacture of starchy food products a d is marketed as Acrylaway [5, 6].
Extracellular L-asparaginase production under submerged fermentation at the commercial scale requires optimization of several process parameters so that an appreciable yield can be achieved with a reduction of enzyme production cost. There are very few reports on the production of L-asparaginase by fungal species. L-asparaginase is also in great demand in clinical applications and in food processing industries. We report the isolation and optimization of fermentation conditions for L-asparaginase by *Trichoderma viride* sp. production from marine soil. The paper also highlights the applications of the enzymes demonstrating the antioxidant activity and the property to lower the acrylamide content.

2. Materials and Methods
2.1 Isolation and screening of L-asparaginase-producing fungi
Soil was used as the source of microorganism which was collected from five different locations within Karnataka. Marine soil was obtained from the coastal belt from the estuarine region of Mangalore beach. While the potato soil, dump yard soil, garden soil was collected from the surroundings in Bangalore and fruit-vegetable soil was from the fields of Kengeri. For screening the fungi producing L-asparaginase a serial dilution and spread plating method on the modified Czapek-Dox agar plate containing (w/v) glucose (0.2%), L-asparagine (1%), agar (2%), $MgSO_4 \cdot 7H_2O$ (0.052%), KCl (0.052%), KH_2PO_4 (0.152%), $CuNO_3 \cdot 3H_2O$ (trace),

$FeSO_4$ (trace) and $ZnSO_4 \cdot 7H_2O$ (trace). Phenol red (0.009%, v/v) was added to the medium (pH 6.2) before being poured into petri plates. The plates were incubated for three days at room temperature (28 ± 2 °C). The development of pink colouration after the incubation period revealed positive L-asparaginase producing fungi [7].

2.2 Identification of fungal isolate

The positive isolates were selectively chosen, regrown to obtain a pure culture on the medium as per Gulati *et al*., (1997) [7]. A tentative identification based on morphological and microscopic features was performed in the laboratory by Lactophenol Cotton Blue test [8]. The fungus was then identified [9] and the fungal isolate was sent to the National Fungal Culture Collection of India (Agharkar Research Institute, Pune) for identification.

2.3 Inoculum preparation and crude enzyme production

The inoculum was prepared by growing the isolate on the modified Czapek-Dox agar plates for 72 h at 28 °C. 2 inoculum discs taken from the culture plate edge with a sterile 5 mm diameter cork-borer [3] was suspended in 100 ml of the modified Czapek-Dox medium. The incubation was carried out at 28 ± 2 °C and on the third day, the culture broth was filtered with Whatman no.1 filter paper and the filtrate served as the crude extracellular enzyme solution.

2.4 L-asparaginase enzyme assay and protein estimation

Determination of L-asparaginase activity was performed as per Imada *et al*., (1973) [10] and the protein was estimated by Lowry *et al*., (1951) [11] method.

2.5 Optimization of production parameters for L-asparaginase

Various factors and fermentation conditions such as pH, temperature, substrate concentration, carbon and nitrogen additives were optimized one factor at a time and the optimized factor was incorporated in the subsequent experiments.

2.5.1 Time course of enzyme production

The fungal culture was inoculated in the modified Czapek-Dox broth under submerged fermentation conditions and the enzyme activity of L-asparaginase was monitored for six days.

2.5.2 Effect of initial pH

Effect of initial pH of production medium was adjusted in the range pH 5.5-7.5 using a LI 120 digital pH meter (Model LI-120, ELICO, India) and the required pH was prepared by the addition of acid or alkali solution to the medium before autoclaving. The culture flasks were autoclaved at 15 Ib/inch and 121 °C for 15 min and inoculated with inoculum disc (5 mm × 2) under sterile conditions. After three days of incubation, the enzyme activity was determined according to Imada *et al*., (1973) [10].

2.5.3 Effect of initial temperature

The production medium of pH 6.5 was inoculated with the culture under sterile conditions. Three different temperature conditions at 30 °C, 37 °C and 45 °C were chosen and incubated for 3 days and the enzyme activity was estimated by the method of Imada *et al*., (1973) [10].

2.5.4 Effect of L-asparagine concentration on enzyme production

The fermentation medium was prepared with different concentrations of L-asparagine 0.25, 0.50, 0.75 and 1% (w/v). The flasks were incubated at 37 °C for 3 days. The enzyme activity was checked as described earlier.

2.5.5 Effect of carbon source and optimization of maltose concentration

The effect of different carbon sources on enzyme production was conducted by substituting glucose in the basal medium with the carbon source under study. The monosaccharides (D-galactose, fructose), disaccharides (maltose, lactose, sucrose) and polysaccharide (starch) were inspected. Glucose was used as control. The prepared culture flasks were inoculated with the inoculum under sterile conditions and incubated at 37 °C 3 days. The enzyme activity was estimated as per Imada *et al*., (1973) [10].

Maltose disclosed to be the best carbon source which was further scrutinized at different concentrations ranging from 0.2 - 1%. The production medium was prepared with different concentrations of maltose weighed separately and sterilized at 121 °C, 15 Ib/inch for 15 min. The medium was further subjected to incubation at 37 °C for a period of 3 days and the L-asparaginase assay was carried out as Imada *et al.*, (1973) [10].

2.5.6 Effect of nitrogen source supplement on enzyme production

The enzyme production was investigated in the presence of various nitrogen sources (0.5%, w/v). The nitrogen sources used were peptone, tryptone, beef extract, yeast extract, urea and sodium nitrate. The medium without any nitrogen source served as control. The flasks were incubated at 37 °C for 3 days and the enzyme activity was determined as mentioned previously.

2.5.7 Production with optimized parameters for L-asparaginase by submerged fermentation

The fermentation medium of pH 6.5 containing 0.5% L-asparagine, 0.5% peptone, 0.6% maltose was inoculated with two 5 mm inoculum discs using a sterile cork-borer and incubated at 37 °C for 3 days. The broth was centrifuged at 12,000 rpm for 10 min and the supernatant was collected and served as the cell free extract.

2.6 Determination of antioxidant activity of L-asparaginase

L-asparaginase was tested for its antioxidant potential by the ABTS assay. The antioxidant activity was carried out as per Re *et al*., (1999) and Monica *et al*., (2013) [12, 13] with slight modifications. L-ascorbic acid was used as a standard.

2.7 Acrylamide reduction potential of L-asparaginase

Fresh potatoes procured form the market were peeled and finely sliced bearing a thickness of 6mm. These potato slices were pre-treated with L-asparaginase [14] for 45 min at room temperature and deep fried in oil at 150 °C for 6 min. After which, 0.5 g of fried potato was thoroughly crushed with 1 ml of 0.05 M phosphate buffer (pH 7.0) and incubated at room temperature and at 37 °C for 1 h. The enzyme reaction was arrested by the addition of TCA and ammonia liberation was estimated as per Imada *et al*., (1973) [10]. The control samples were fried and were processed without enzyme pre-treatment.

The acrylamide reduction potential of L-asparaginase was carried out using a combination method employed by Kumar and Manonmani (2013) and Pedreschi *et al*., (2008) [5, 14] with modifications.

2.8 Statistical analysis
Biological triplicates and technical replicates were evaluated for L-asparaginase activity. ANOVA (analysis of variance) and DMRT (Duncan's multiple range test) were used to find out the differences among each treatments by launching the SPSS at a significance level of 5%.

3. Results and Discussion
3.1 Isolation, screening and identification of L-asparaginase-producing fungi
The development of pink colour is positive for production of L-asparaginase by a rapid plate assay. The fungal organism which produced pink colour around the colonies was selected as a potential strain (Figure 1). The Lactophenol cotton blue stained organism was microscopically observed and identified as a filamentous fungi based on its morphological features and the isolate was confirmed as *Trichoderma viride* sp. at National Fungal Culture Collection of India, Pune.

Fig 1: L-asparaginase positive colony grown on modified Czapek Dox agar pH 6.2 at room temperature (28 ± 2 °C).

3.2 Optimization of culture conditions for the production of L-asparaginase by submerged fermentation
3.2.1 Time course for enzyme production
The enzyme activity was checked for six days to determine the maximum enzyme production and optimum activity was observed on the 3rd day at room temperature (28 ± 2 °C). The extended incubation time resulted in the fall of enzyme activity (Figure 2).

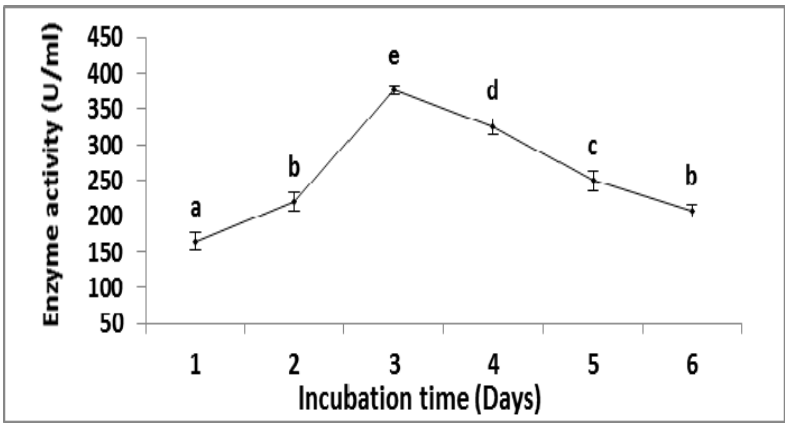

Fig 2: The culture age of *Trichoderma viride* sp. grown on fermentation medium of pH 6.2 containing 1% (w/v) L-asparagine and inoculated with the inoculum discs (5 mm). The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.2 Effect of initial pH for enzyme production
The effect of initial pH on the enzyme production was determined to identify the most relevant pH to enhance the enzyme production. The enzyme activity was highest for pH 6.5 and a decline was observed at pH 7.0 and pH 7.5 (Figure 3).

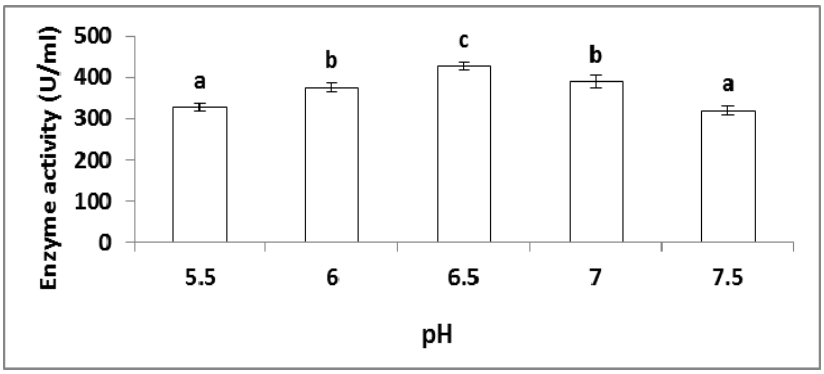

Fig 3: Influence of initial pH for L-asparaginase production tested in a fermentation medium containing 1% (w/v) L-asparagine and incubated at room temperature (28 ± 2 °C) for 3 days. The medium with pH 6.2 was taken as control. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.3 Effect of initial temperature on enzyme production
The influence of initial temperature was investigated to determine the maximum activity, which was observed at 37 °C temperature (Figure 4).

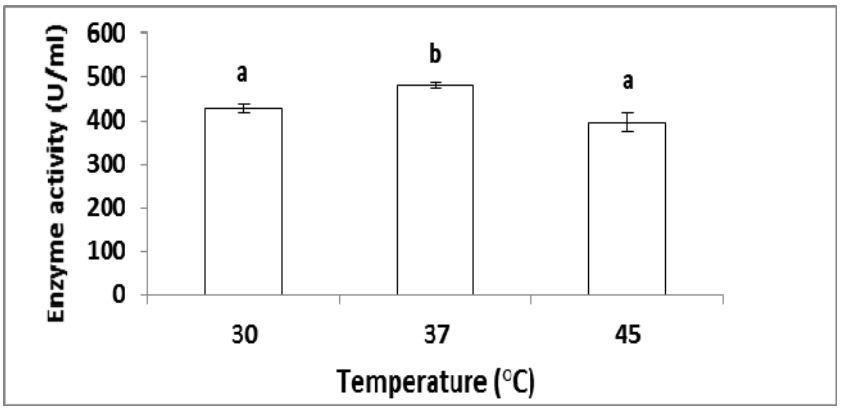

Fig 4: Influence of initial temperature for L-asparaginase production. The fermentation medium (pH 6.5) consisted of L-asparagine (1%, w/v) incubated at various temperatures for 3 days. The culture flask at 30 °C was taken as control. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.4 Effect of L-asparagine concentration on enzyme production
The impact of varying concentrations of the substrate L-asparagine was analysed. The maximum enzyme production was seen at 0.5% (w/v) at par with 0.75%. Higher concentration tends to lower the enzyme activity (Figure 5).

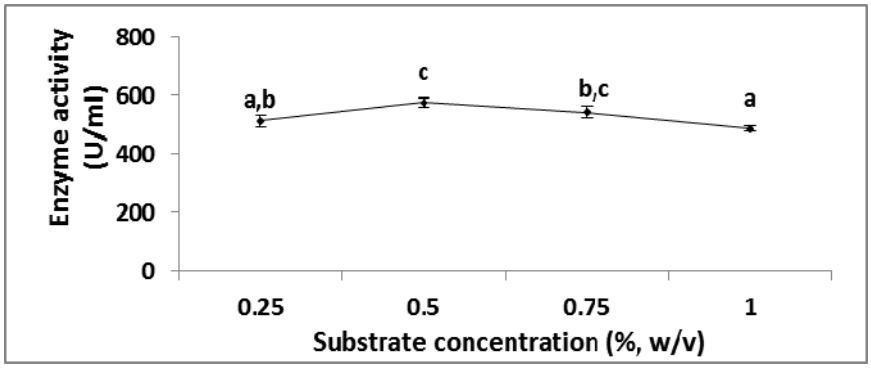

Fig 5: Influence of L-asparagine concentration. The production medium of pH 6.5 was incubated at 37 °C for 3 days. 1% (w/v) L-asparagine served as control. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.5 Effect of carbon source on enzyme production
The effect of various carbon sources showed maltose to have the highest enzyme activity, followed by fructose. But, D-galactose showed very low enzyme activity (Figure 6).

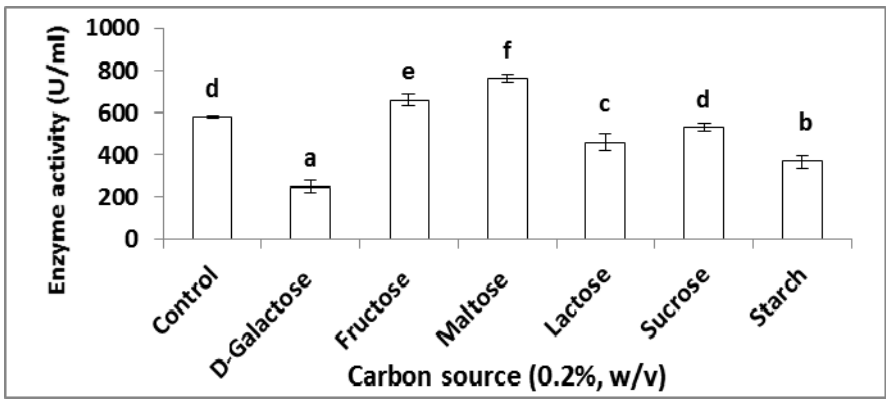

Fig 6: Influence of carbon source on L-asparaginase production. The production media (pH 6.5) comprising of L-asparagine (0.5%, w/v), peptone (0.5%, w/v) was incubated at 37 °C for 3 days. The medium with glucose (0.2%, w/v) acted as control. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.6 Effect of maltose concentration on enzyme production

Maltose was tested at concentrations in the range 0.2 - 1% for enzyme production. 0.6% disclosed to have optimum enzyme activity and slight loss of activity was observed at higher concentrations of maltose (Figure 7).

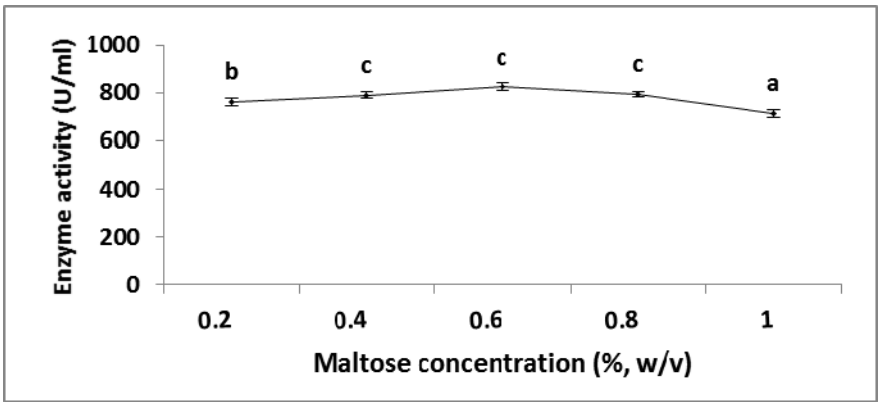

Fig 7: Influence of maltose concentration on L-asparaginase production. The fermentation media of pH 6.5 constituting 0.5% (w/v) L-asparagine, 0.5% (w/v) peptone, and 0.6% (w/v) maltose and incubated at 37 °C for 3 days. The culture medium with 0.2% (w/v) maltose was considered as control. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.7 Effect of nitrogen source supplements on enzyme production

The effect of nitrogen source supplement was tested in the medium containing 0.5% L-asparagine. The medium without the tested nitrogen source served as control. Peptone showed the highest enzyme activity than the other nitrogen sources (Figure 8). Lower activity was seen with yeast extract.

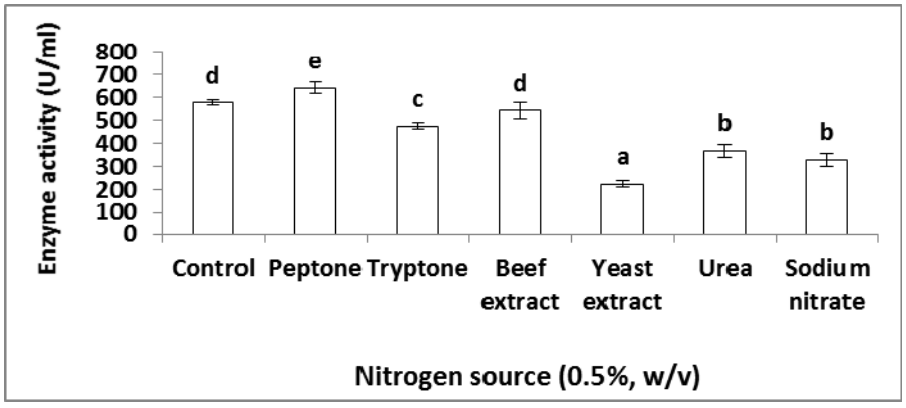

Fig 8: Influence of nitrogen source supplements for L-asparaginase production. The fermentation medium of pH 6.5 encompassing L-asparagine (0.5%, w/v) was incubated at 37 °C for 3 days. The values with different letters on error bars are significantly different at $P_{0.05}$.

3.2.8 Production of L-asparaginase by submerged fermentation

The various parameters which were investigated and optimized with regard to the physical and nutritional conditions were utilized for the bulk production of L-asparaginase. The production medium was supplemented with 0.05% of surfactant (tween 20) and the organism was cultivated under the optimized conditions (Table 1).

Table 1: Summary profile of optimization for L-asparaginase production

Optimization conditions	Optimized parameters
Time course	3rd day
pH	6.5
Temperature	37 °C
Substrate concentration	0.50 %
Nitrogen source supplement	Peptone (0.5%)
Carbon source	Maltose
Maltose concentration	0.6%

3.3 Antioxidant activity of L-asparaginase

L-asparaginase was tested for its anti-oxidant potential by performing the ABTS assay. ABTS assay for estimating the anti-oxidant potential demonstrated an effective removal of free radicals. The enzyme is an anti-neoplastic agent and hence should depict anti-oxidant potential [15]. The enzymatic scavenging activity showed linear correlation with the ascorbic acid scavenging activity standard. There was linear relationship between the scavenging activity% and increasing enzyme concentration. As the concentration of enzyme was increased, the % inhibition or % scavenging activity was found to increase linearly. It was observed that at lower concentrations of the enzyme and standard ascorbic acid, the scavenging activity of the enzyme was 88% whereas standard was 60% (Figure 9). The purified enzyme therefore displayed a very good antioxidant/scavenging activity Thus L-asparaginase for *Trichoderma viride* sp. can be demonstrated as an antileukemic agent. Typical correlation was observed for

L-asparaginase of *Aspergillus flavus* sp. [16] and that of *Mucor hiemalis* sp. [13].

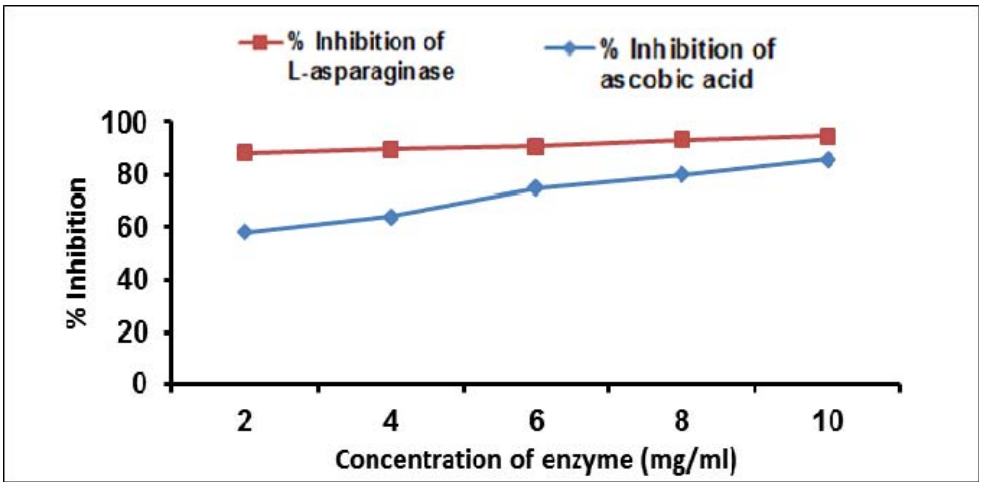

Fig 9: L-asparaginase and L-ascorbic acid scavenging activities by ABTS assay

3.4 L-asparaginase as an acrylamide-mitigation agent

It is evident that L-asparagine is converted into L-aspartic acid and ammonia upon the action of L-asparaginase enzyme. In a similar manner, the L-asparagine present in the starchy potatoes also liberates ammonia upon enzyme action. Interestingly, a very potent carcinogen content is formed when potatoes are deep-fat fried in oil as the L-asparagine gets converted into acrylamide [6]. As the formation of acrylamide is certain to happen in fried potatoes when it is exposed to high temperatures, the L-asparaginase enzyme can be used to lower the levels of this deadly carcinogen. In our study at 37 °C, the enzyme treated fried potato exhibits an activity 94.26 U/ml which is lower when compared to the non-treated fried potato (403.27 U/ml) and this reduction could be due to the L-asparaginase mitigation power (Figure 10). Acrylamide reduction was also seen in cases of Kumar and Manonmani, 2013 and Granda *et al*., 2004 [14, 6].

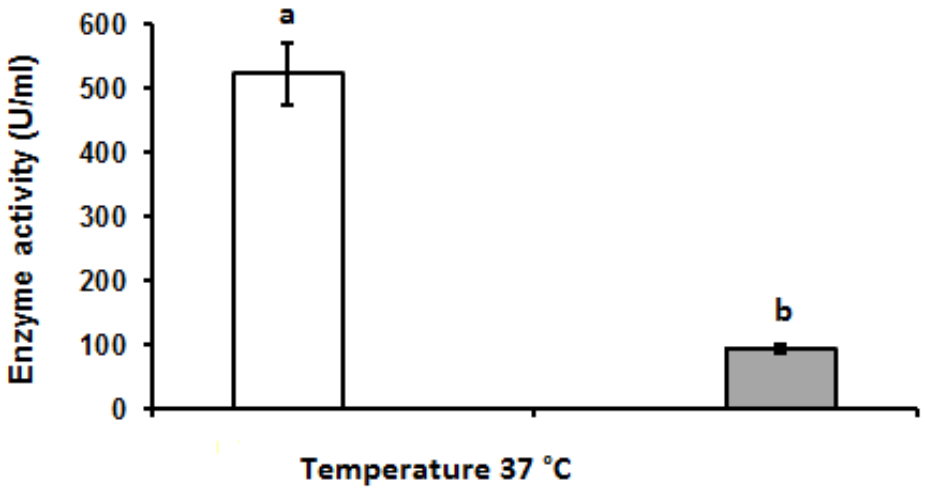

Fig 10: Acrylamide reduction potential of L-asparaginase. The values with different letters or numbers on error bars are significantly different at $P_{0.05}$.

4. Conclusion

Marine soil is a rich source of L-asparaginase producing microorganisms. *Trichoderma viride* sp. isolated from marine soil and optimized for production parameters showed increased in the yield. However this is a preliminary study and further requires enzyme purification of the enzyme. The enzyme properties such as high radical scavenging activity and acrylamide mitigation make fungal L-asparaginase a potential candidature in industries as an antitumour and acrylamide-mitigation agent.

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