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Role of Alcohol and Metal Ions as a Trace Elements on Biosynthesis of L-asparaginase from *Aspergillus terreus*

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The manufacture of enzymes, like L-asparaginase with less adverse effects in the treatment of cancer is an important facet of pharmaceutical industry. Yeast and filamentous fungi are commonly reported in scientific literature to produce L-Asparaginase with less adverse effects than prokaryotic microorganisms. L-Asparaginase production was investigated in the filamentous fungi *Aspergillus terreus* KLS2 a soil isolate from different regions of Gulbarga and produces 6.05IU. The process economization were employed to achieve higher yield of L-Asparginase through solid state fermentation (SSF) and here we made an attempt to incorporate metal ions as a trace elements sources such as manganous sulphate, zinc sulphate, copper sulphate and ferrous sulphate and glycerol as alcohol source to the production medium. The maximum enzyme production i.e 6.66IU and 6.42IU were observed by using 0.01% manganous sulphate and zinc sulphate and 7.11 IU were observed by using 0.50% of glycerol.

Key words: L-Asparaginase, Carob pod, Solid state fermentation,Metal ions and Glycerol.

Enzymes as drugs have two important features that distinguish them from all other types of drugs being used in the clinic. First, the enzymes often bind and act on their targets with great affinity and specificity and secondly, these are the catalytic moieties, which convert multiple target molecules to the desired products simultaneously. These two features make enzymes specific and potent drugs that can accomplish therapeutic effects in the body that other small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for treatment of a wide range of disorders [1].

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In the 21st century having the knowledge on human genome, enzyme particularly of microbial origin, are envisaged to play a crucial role in the diagnosis, curing, biochemical investigation and monitoring of many dreaded diseases.

The enzyme L-asparaginase has been studied extensively over the past few years, because of its ability to inhibit several biological functions. A pre- requisite for making an effective medication for the treatment of cancer is the fundamental difference between normal cells and cancer cells must be defined. The chemotherapeutic agent must exploit this cellular difference in such a way that normal cells are spread and only cancer cells are injured. L-asparaginase exploits the unusually high requirement tumour cells have for the amino acid asparagines [2].

L-asparaginase production using microbial systems has attracted considerable attention, owing to the cost-effective and eco-friendly nature. A wide range of microorganisms

such as filamentous fungi, yeasts, and bacteria have proved to be beneficial sources of this enzyme [3-7]. L-Asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate[8].

The present study will highlights the effect of metal ions on L-asparaginase production by using carob pod as a substrate through solid state fermentation from *Aspergillus terreus* KLS2. There were no reports on solid state fermentation by using carob pod. Hence we made an attempt to produce with different metal ions with different concentration for their maximum yield.

MATERIALS AND METHODS

Microorganism

Aspergillus terreus KLS2 were isolated from different soil samples from various places of Gulbarga were used for isolation of *Aspergillus terreus* strains as per the method of Seifert[9]. The isolated strains were tentatively identified in the laboratory as described by Rapper and Fennell [10] and were maintained on potato dextrose agar (PDA). Further confirmation sent for Agarkar Research Institute, Pune.

Production of L-asparaginase

The isolated strains were screened and used potential strain for the production of L-asparaginase through solid state fermentation by using carob pod as a substrate with influence of additional supplement of nutrients such as metal ions, alcohol and phosphate sources.

Preparation of carob pod medium

The deseeded carob pods were dried at 52°C for 6 hrs in an oven. Then the kibble was chopped into small particle size of 2 mm and pulverized in a waring blender at high speed. Thus obtained carob substrate was analyzed for total fermentable sugars and pH was adjusted to 4.5 units. Then the required amount of substrate was taken in a 250 ml Erlenmeyer flask and rehydrated using distilled water to get desired moisture level [11]. The cotton-plugged flasks were autoclaved at 121°C for 15 min and allowed to cool.

Fermentation studies

The production of L-asparaginase was carried out by using 20 g of carob pod as a substrate under solid state fermentation. The moisture content of the flask is 65% were maintained and inoculated 1 ml of inoculum ($1x10^7$spores/ml). The content of the flask were mixed thoroughly by beating the flasks on the palm of hand and incubated in slanting position at 35 ^{0}C for 7 days. The pH 4.5 was maintained throughout the fermentation process.

Influence of trace metal ions on L-asparaginase

The different trace metals such as Cu^{+2}, Zn^{+2}, Mn^{+2} and Fe^{+2} were supplemented with the percentage of 0.1%, - 0.3% with increment of 0.1%. All these metal ions were prepared by using double distilled water and added individually and kept for fermentation as described earlier.

Influence of alcohol source on L-asparaginase production

A set of conical flasks containing 20 g of carob substrate was prepared for solid state fermentation as described earlier and supplemented with glycerol at concentration of 0.1%, 0.25%, 0.50% and 1.0%.

Influence of phosphate source on L-asparaginase production

A set of conical flasks with 20 g of carob substrate were prepared for SSF and supplemented with different phosphate sources like, diammonium hydrogen phosphate, dipotossium hydrogen phosphate and potassium dihydrogen phosphate at concentration ranging from 0.05 to 0.20 M with increments of 0.05 M.

Extraction of L-asparaginase

The samples were withdrawn periodically at 24 hrs in aseptic condition 1 gm of mouldy substrate was taken into a beaker and distilled water (1:10) was added to it. The contents of flasks were allowed to have contact with water for 1 hr with occasional stirring with a glass rod. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay.

Assay of L-asparaginase

Assay of enzyme was carried out as per Imad *et al.* [12]. 0.5 ml of 0.04 M asparagine was taken in a test tube, to 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M TCA

(Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA.
The enzyme activity was expressed in International unit.

International Unit (IU)

One IU of L-asparaginase is the amount of enzyme which liberates 1 micro mol of ammonia per ml per minute (µmole/ml/min).

RESULTS AND DISCUSSION

Production of L-Asparaginase

In the present study, *A. terreus* were isolated and named serially from KLS1 to KLS35. The potential strains were selected on the basis of pink zone around the colony by plate assay method. Among these *Aspergillus terreus* KLS2 were used as potential strain for the production of L-asparaginase through solid state fermentation. The fermentation studies were indicated that the L-asparaginase production was maximum 6.05 IU at 72 hr fermentation period. Similar reports were reported by Sutthinan Khamna *et al* [13] reported that the maximum L-asparaginase production was observed at pH 7.0 and temperature 30^0 C at 178 hr of fermentation period.

Influence of metal ions

The metal ions such as manganous sulphate, copper sulphate, zinc sulphate and ferrous sulphate were employed for the production of L-asparaginase by *A. terreus* KLS2 strain. The results of the studies pertaining to the influence of metal ions on the production of L-asparaginase are represented in Fig. 1-4.

The addition of metal ions were done in the percentages of 0.01, 0.02 and 0.03%. Manganous sulphate and zinc sulphate acted as best source of metal ions for the production of L-asparaginase at 0.01% at 72 hrs of fermentation period, the enzyme production observed was 6.66IU and 6.42IU respectively. Copper sulphate and ferrous sulphate were less inducers of L-asparaginase production, the enzyme production observed was 4.367IU and 4.37IU respectively at 0.01% at 72 hrs of fermentation period.

Trace elements have profound effect on the growth and physiological activities of the organisms. In general trace elements play a key role in the metabolism of organisms. Few metal ions need to be supplemented to a fermenting medium, as they are essential for cell mass formation and also acts as a co-factor for several biosynthetic enzymes. Robinson and Berk [14] have reported the supplementation of metal ions like copper, zinc, and manganese to the H82 medium in the concentration ranging 0.001-0.002% and obtained maximum L-asparaginase production of 1.73, 1.74 and 1.71 IU/ml by supplementation of 0.001 concentration of copper, zinc, and manganese respectively. As such our result coincides to the results of Robinson and Berk [14].

Influence of alcohol source

Under the present study the alcohol, i.e., glycerol was supplemented at the concentration

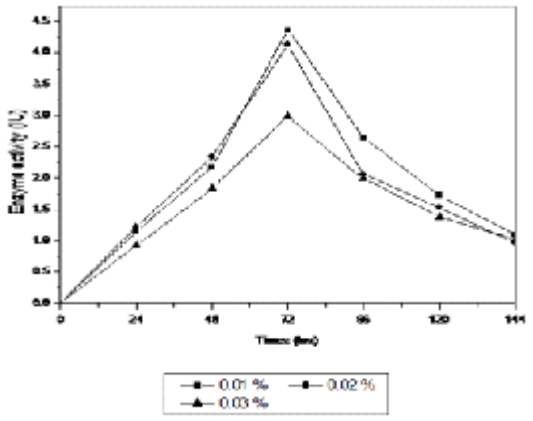

Fig. 1. Influence of copper sulphate on production of L-asparaginase from *Aspergillus terreus* KLS2

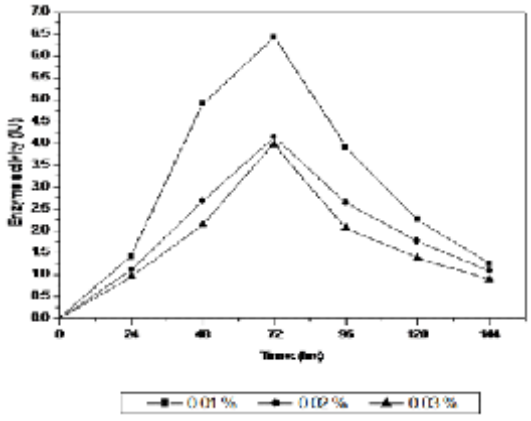

Fig. 2. Influence of Zinc sulphate on production of L-asparaginase from *Aspergillus terreus* KLS2

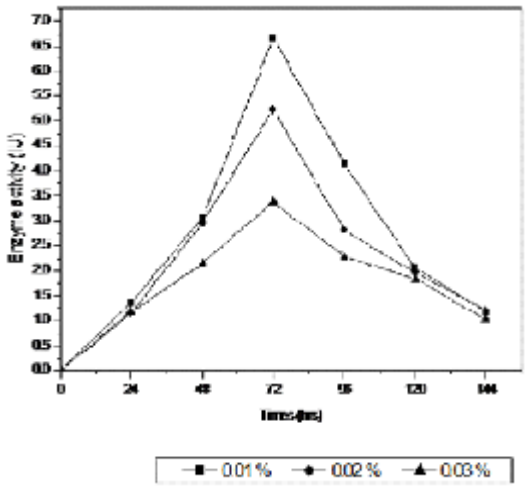

Fig 3. Influence of Manganous sulphate on production of L-asparaginase from *Aspergillus terreus* KLS2

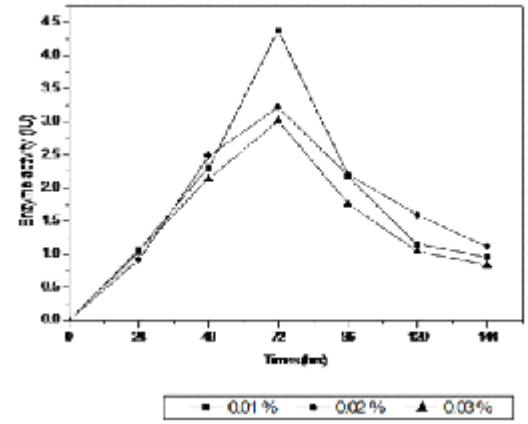

Fig. 4. Influence of Ferrous sulphate on production of L-asparaginase from *Aspergillus terreus* KLS2

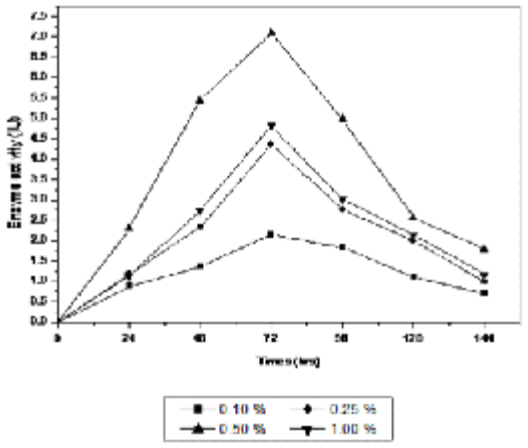

Fig. 5. Influence of Alcohol (Glycerol) on production of L-asparaginase from *Aspergillus terreus* KLS2

0.10, 0.25, 0.50 and 1.0% on deseeded carob pod in order to evaluate their effect on L-asparaginase production. The results on the effect of glycerol on L-asparaginase production with *A. terreus* KLS2 strain on carob pods are presented in Fig 4. The results revealed that maximum L-asparaginase production of 7.11 IU was obtained on supplementation of 0.50% of glycerol at 72 hrs of fermentation period.

Robinson and Berk [14] supplemented alcohols like glycerol in the concentration between 0.05 - 1.00% in (submerged fermentation) H82 medium. In all the cases the enhancement of L-asparaginase production was observed but the maximum production was observed at 0.50%. Singh and Sukumaran [15] have supplemented 0.4% of glycerol into the medium for the production of L-asparaginase by using wild and mutant *E. coli* strains and obtained 2 IU/ml and 9.00 IU/ml of L-asparaginase respectively.

Addition of lower alcohols to the solid substrate medium enhances the production of microbial metabolite [16]. The optimum amount of alcohols depend upon the composition of the fermentation medium. Generally, alcohols have been shown to act on membrane permeability in microorganisms by affecting their growth and sporulation [17].

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