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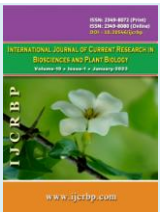

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Novel and Efficient Plate Assay Method for the Screening of L-Asparaginase Producing Microorganisms

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Screening, microorganisms, L-asparaginase, nutrient asparagine agar, potato dextrose asparagine agar, Gram's iodine

Abstract

Microbes producing L-asparaginase are usually screened on plates containing L-asparagine and phenol red. The contrast of the colored zone formed by microorganisms varies from yellow and pink in this method and we cannot differentiate between L-asparaginase producers and non-producers. Later an improved method was developed for screening of extracellular L-asparaginase producing microbes with bromothymol blue as pH indicator instead of phenol red. The reported results were not very sharp for the isolates producing extracellular L-asparaginase as they produce dark blue colored zones at alkaline pH. The present method is novel, more sensitive, simple and rapid than the existing methods for screening of both bacteria and fungi producing L-asparaginase extracellularly. Nutrient asparagine agar and potato dextrose asparagine agar media stained with Gram's iodine solution were used for detection of extracellular L-asparaginase producing bacteria and fungi respectively. Plate detection assay with Gram's iodine exhibited clear zones around the colonies producing L-asparaginase. The present method is more accurate for screening of potent isolates based on the diameter of hydrolyzed zones formed. The reported method can be utilized to identify the prominent isolates producing L-asparaginase.

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Introduction

L-asparaginase is a hydrolase that catalyzes the conversion of L-asparagine and is a potential chemotherapeutic agent used in the treatment of acute lymphoblastic leukemia (Piatkowska-Jakubas *et al.*, 2008). This enzyme is also used in food industry for degradation of acrylamide formed during food processing at high temperatures (Mohan Kumar *et al.*, 2013). The formation of acrylamide in fried foods especially in potato chips is neurotoxic (Medeiros Vinci *et al.*, 2012). The reaction between free asparagine and reducing sugars results in the formation of acrylamide in heated foods. L-asparaginase prevents the acrylamide formation by inhibiting the reaction of L-asparagine with reducing sugars (Ciesarová *et al.*, 2006).

L-asparaginase is present in animals, birds and plants (Wriston and Yellin, 1973). The enzyme is extensively distributed in fungi (Dange and Peshwe, 2015) and yeasts (Dunlop and Roon, 1975). The commercial industrial production of enzyme is carried out using

Escherichia coli and *Erwinia chrysanthemi* (Keating *et al.,* 1993). Properties of L-asparaginase have made the enzyme an important chemotherapeutic agent for treating lymphoproliferative and lymphoma diseases. In recent years this enzyme has gained importance in pharmaceutical industry for the treatment of lymphoblastic leukemia, malignant diseases of the lymphoid system and Hodgkin's lymphomas (Appel *et al.,* 2007).

The enzymes produced by microbes are mostly intracellular with low yield and difficult to extract and purify. In an earlier study, phenol red plate assay method was reported for screening of extracellular L-asparaginase producing microbes (Gulati *et al.,* 1997). Later an improved plate assay method was developed for screening of L-asparaginase producing microorganisms using bromothymol blue (BTB) (Mahajan *et al.,* 2013).

The enzyme production is often insufficient for therapeutic and other beneficial applications. Thus, there is a high demand for rapid screening of microbes producing extracellular L-asparaginase in high yields. With this objective, we intend to utilize this novel technique to screen soil samples for the isolation of potent isolates producing high yields of extracellular L-asparaginase. In the present investigation a novel, sensitive, accurate and rapid plate assay method for screening of extracellular L-asparaginase producing microorganisms with Gram's iodine solution is reported.

Materials and Methods

Collection of soil samples

Soil samples were collected from paddy field crops located in Chittoor district, Andhra Pradesh, India. The samples were collected at random from each plot to make a composite sample. The soil samples were collected into sterile test tubes and brought to the laboratory for microbial analysis. Each sample was serially diluted to tenfold dilution series with 0.85% sodium chloride. One ml aliquot of 10^{-6} dilution was used for agar plating method.

Isolation of strains

The serially diluted 10^{-6} samples were plated on newly developed nutrient asparagine agar (NAA) medium. NAA medium was used for isolation of bacteria producing extracellular asparaginases. The composition of the medium is (gm/L): peptone, 5.0; beef extract, 3.0; sodium chloride, 5.0; L-asparagine, 10.0 and agar, 20.0. pH was adjusted to 7.0.

Fungi producing asparaginase were screened on potato dextrose asparagine agar (PDAA) medium containing the following ingredients (gm/L): potato (starch), 4.0; dextrose, 20.0; L-asparagine, 10.0 and agar, 20.0. pH was adjusted to 5.6 with 1N hydrochloric acid.

After sterilization, streptomycin sulphate antibiotic at 30 mg/1000 ml was added to the PDAA medium at 40 °C-50 °C.

Gram's iodine solution was prepared by dissolving the following ingredients (gm/300 ml of distilled water): potassium iodide, 2.0 and iodine, 1.0.

Bacterial isolates were inoculated onto NAA medium and incubated at 37 °C for 24 hr. The fungal isolates were inoculated onto PDAA plates and incubated at 25 °C for 72 hr. Uninoculated plates served as controls.

Identification of isolates

From 10^{-6} dilutions, one of the plates having unselected isolates was used as a check for the qualitative estimation of hydrolyzed zone with Gram's iodine. A total of 110 bacterial and 80 fungal strains were isolated randomly from the remaining plates of the designed medium.

The selected promising isolates were identified as *Bacillus licheniformis* and *Bacillus subtilis* while the fungal isolate was identified as *Aspergillus niger* on the basis of 16S rRNA gene and gene sequence analysis with Midilabs. The identified isolates were labeled as IICT-PN-7 (*Bacillus licheniformis*), IICT-PN-8(*Bacillus subtilis*) and IICT-PN-9 (*Aspergillus niger*). Bacterial isolates were maintained on nutrient agar where as fungal isolates on potato dextrose agar media. These were used for further studies.

The selected isolates were subcultured on NAA and PDAA media and preserved in refrigerator for testing. The identified isolates were subjected to streak-plate method with Gram's iodine for the detection of the hydrolyzed zones. The two best isolates among them were selected.

Determination of L-asparaginase activity

Bacterial L-asparaginases were quantitatively estimated with nutrient asparagine broth as production medium of pH 7.0. Potato dextrose asparagine broth of pH 5.6 was used for determination of fungal L-asparaginases. The production of L-asparaginase was carried out in Erlenmeyer flasks incubated on rotary shaker at 220 rpm and 30 °C. Five ml quantities of the samples were withdrawn at intervals of 12 hrs. The enzyme activity was evaluated by Nesslerization procedure (Shifrin *et al.,* 1974) with 189 mM L-asparagine as substrate and recording the absorbance in an Agilent Cary spectrophotometer at 436 nm.

Results and Discussion

Applications of L-asparaginase are increasing constantly. Anticancer activity (Darwesh *et al.,* 2022; Benchamin *et al.,* 2019) and use of L-asparaginase in food processing (Baskar *et al.,* 2019) is extensively studied. The present investigation was carried out to develop a simple and accurate plate assay method for screening of microorganisms producing L-asparaginase. The selected isolates produced high yield of the enzyme.

The collected samples were serially diluted for plating to determine L-asparaginase activity exhibiting isolates. Pour-plate method for bacterial colonies was performed with 1 ml aliquot of 10^{-6} dilution in NAA medium (Fig. 1 c) and fungal colonies in PDAA medium (Fig. 2 c). Uninoculated plates of NAA medium was set as a control for bacteria (Fig. 1 a) and uninoculated PDAA plate as control for fungi (Fig. 2 a).

The plates were flooded with prepared Gram's iodine solution after 24 hr of incubation for bacterial isolates. Clear hydrolyzed zones were developed around the colonies in pour-plate (Fig. 1 d) and streak-plate of *Bacillus licheniformis* (Fig. 1 f) and *Bacillus subtilis* (Fig. 1 h). Uninoculated plate stained with Gram's iodine solution did not exhibit clear hydrolyzed zone (Fig. 1 b).

PDAA plate inoculated with *Aspergillus niger* (Fig. 2 e) and pour-plate method of 10^{-6} dilution in PDAA medium (Fig. 2 c) exhibited growth after incubation of 72 hr. Clear zones were observed around the fungal colonies in pour-plate flooded with Gram's iodine solution (Fig. 2 d) and plate inoculated with *Aspergillus niger* (Fig. 2 f). Uninoculated PDAA plate flooded with Gram's iodine solution was completely stained (Fig. 2 b).

Maximum hydrolyzed zones of 5.5 mm and 16.1 mm were observed for bacterial and fungal colonies respectively. A sharp, clear, distinct and prominent zone of hydrolysis was observed in the plates inoculated with *Bacillus licheniformis, Bacillus subtilis* and *Aspergillus niger*.

Both pour-plate and streak-plate methods exhibited hydrolyzed zones on NAA and PDAA media indicating the enzyme production capabilities of the isolates.

In the evaluation of identified isolates to produce extracellular L-asparaginases, the initial enzyme production began at 12 hr in bacteria and at 48 hr in *Aspergillus niger*. The isolate *Bacillus licheniformis* produced higher amount of L-asparaginase of 4.7 IU/ml and *Bacillus subtilis* exhibited the enzyme activity of 3.2 IU/ml at 24 hr. With *Aspergillus niger* an enzyme yield of 2.8 IU/ml was obtained at 72 hr.

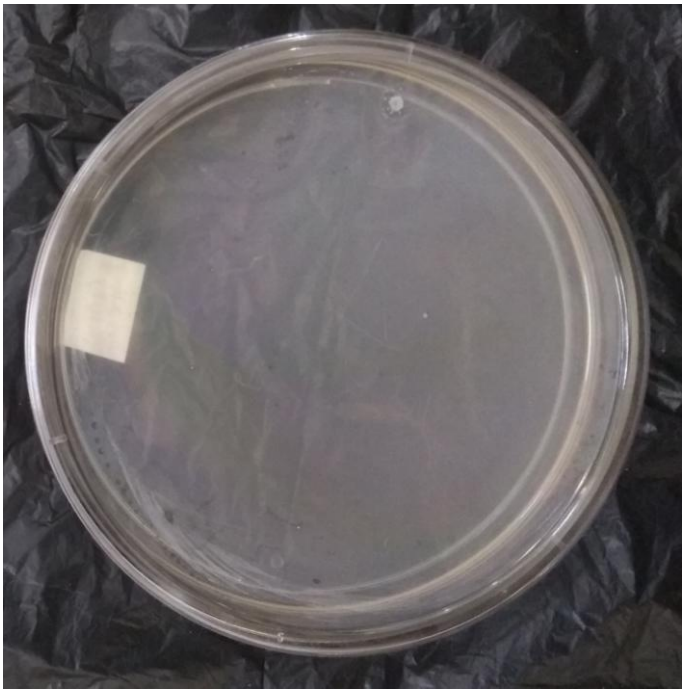
The earlier reports for screening of extracellular L-asparaginase producing microorganisms with phenol red (Gulati *et al.,* 1997) and BTB (Mahajan *et al.,* 2013) were not effective. In the present method with Gram's iodine solution prominent isolates can be identified based on hydrolyzed zone diameter formed. This is one of the main advantage of the present method over phenol red and BTB assays.

A direct correlation existed between the zone diameter and enzyme activity. The present plate assay is advantageous as the production of L-asparaginase can be directly visualized from the plates. The isolates were also evaluated for their efficiency to produce extracellular L-asparaginase in fermentation broth by shake flask method. *Bacillus licheniformis* (IICT-PN-7) and *Bacillus subtilis* (IICT-PN-8) showed 100% relative activity at 24 hr where as *Aspergillus niger* (IICT-PN-9) at 72 hr.

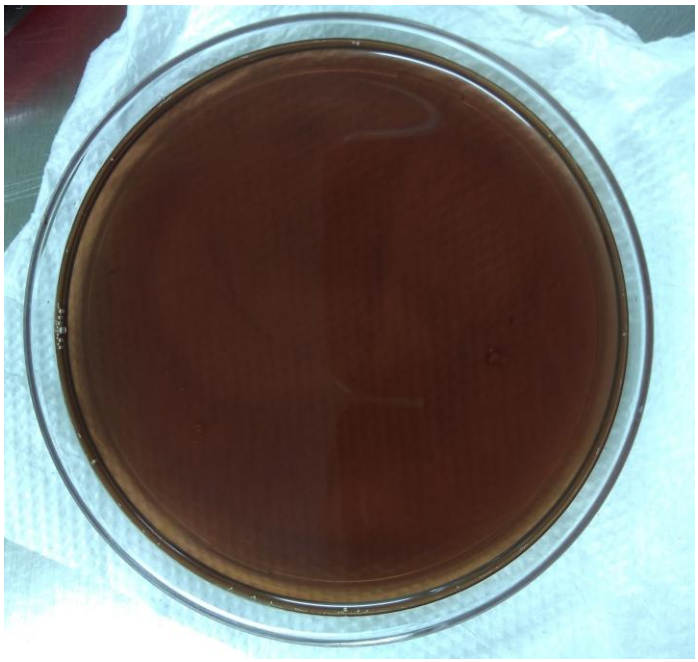
The results indicated that the newly developed screening method for extracellular bacterial L-asparaginase in NAA medium and fungal L-asparaginase in PDAA medium with Gram's iodine solution is efficient, rapid and accurate. The screening method developed can be employed for the isolation of potent isolates producing L-asparaginase.

Fig. 1. Screening of L-asparaginase producing bacteria on NAA plates flooded with Gram's iodine

a. Control

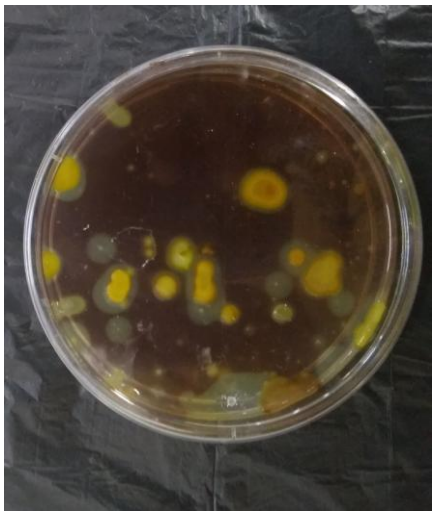


b. Control plate of L-asparaginase stained with Gram's iodine

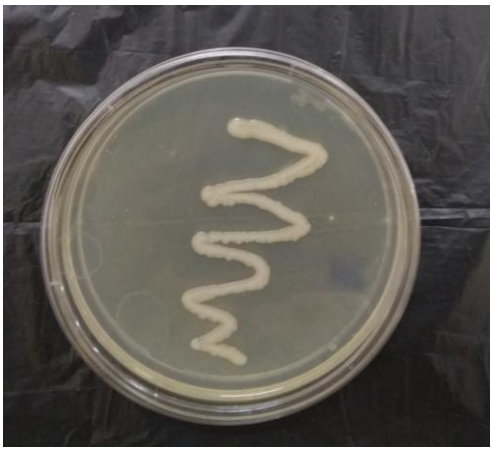


c. Pour-plate method of bacteria in NAA medium

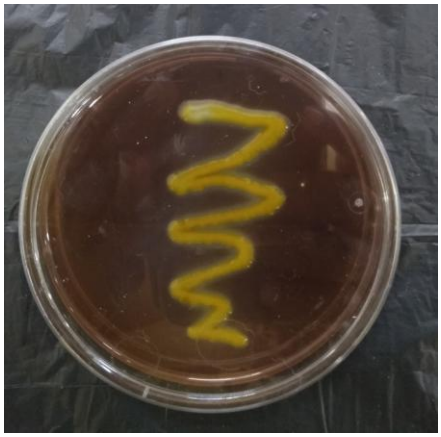
d. Formation of clear hydrolyzed zones with unselected bacterial strains in NAA medium by pour-plate method stained with Gram's iodine solution



e. Streak-plate method in NAA medium with *Bacillus licheniformis* (IICT-PN-7)



f. L-asparaginase activity exhibited by *Bacillus licheniformis* (IICT-PN-7) in NAA medium stained with Gram's iodine solution



g. Streak-plate method in NAA medium with *Bacillus subtilis* (IICT-PN-8)

h. L-asparaginase activity exhibited by *Bacillus subtilis* (IICT-PN-8) in NAA medium stained with Gram's iodine solution

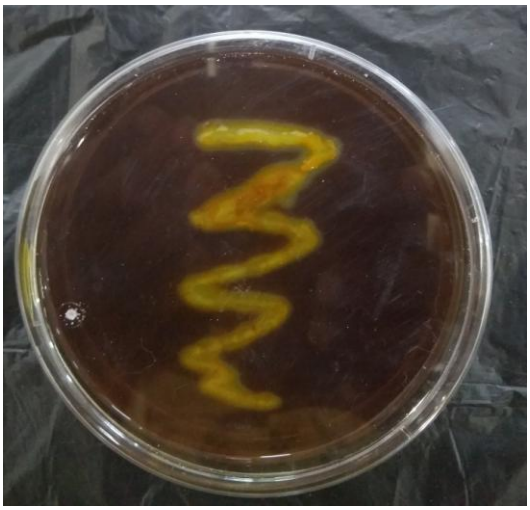
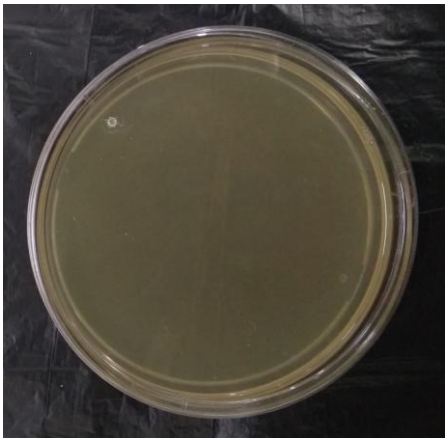
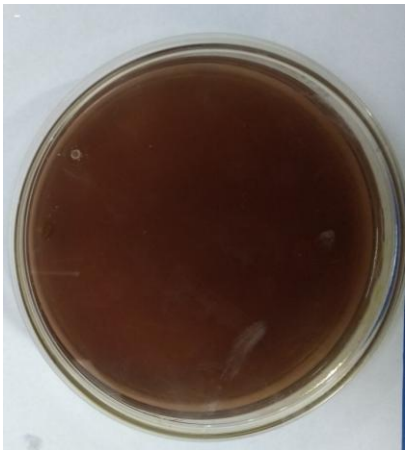


Fig. 2. Screening of fungi on PDAA plates flooded with Gram's iodine

a. Control

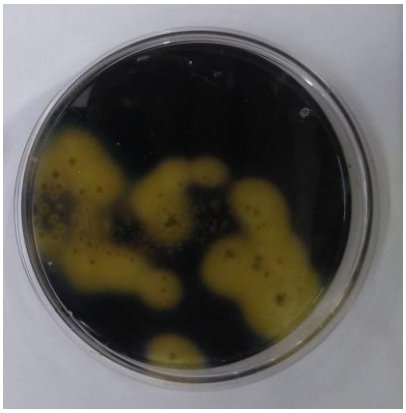


b. Control plate stained with Gram's iodine

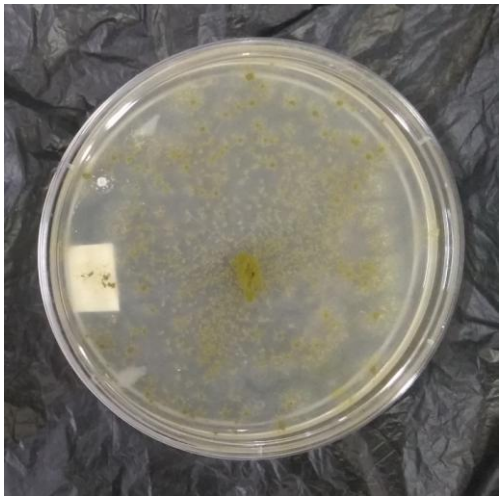


c. Pour-plate method of fungi in PDAA medium

d. Formation of clear hydrolyzed zones with unselected fungal strains in PDAA medium by pour-plate method stained with Gram's iodine solution



e. PDAA medium inoculated with *Aspergillus niger* (IICT-PN-9)



f. L-asparaginase activity exhibited by *Aspergillus niger* (IICT-PN-9) in PDAA medium stained with Gram's iodine solution

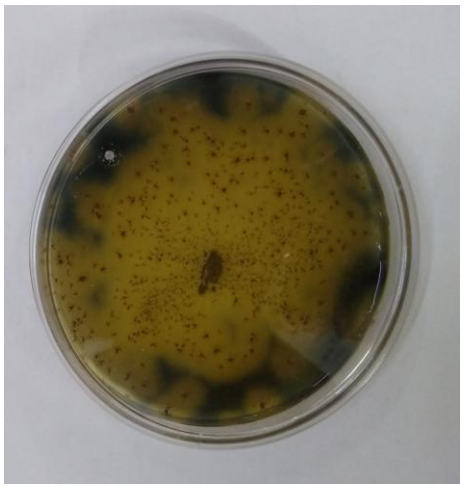
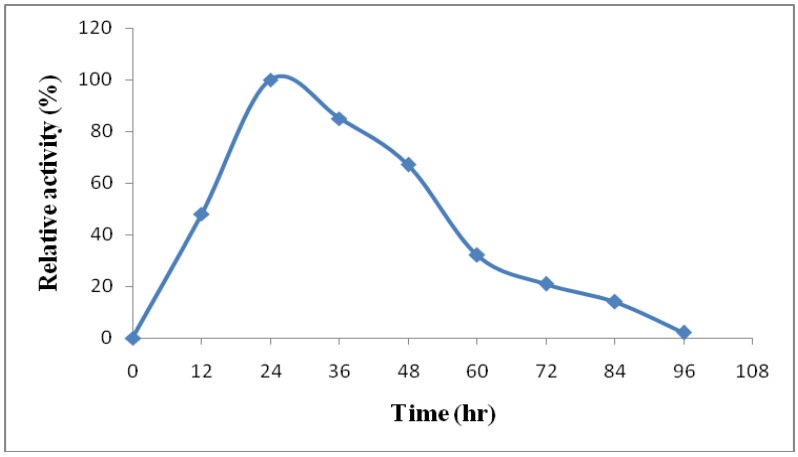
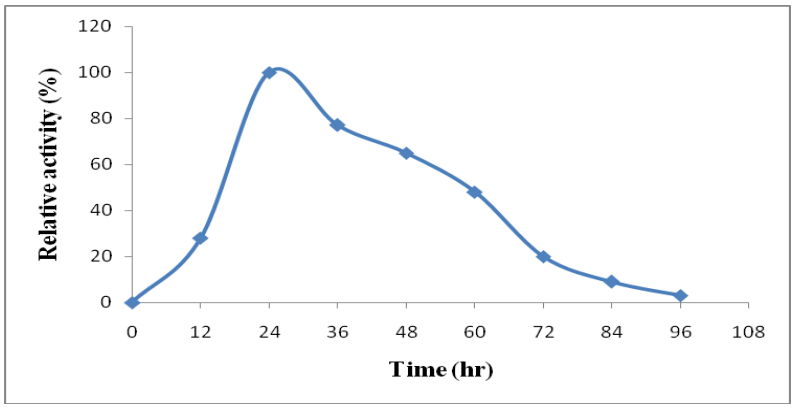


Fig. 3. Production of L-asparaginase by the identified isolates

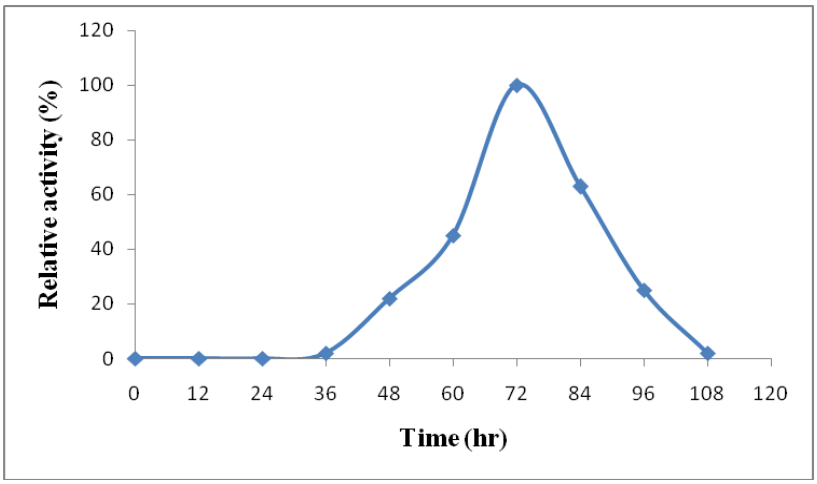
a. *Bacillus licheniformis* (IICT-PN-7)



b. *Bacillus subtilis* (IICT-PN-8)



c. *Aspergillus niger* (IICT-PN-9)



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