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Der Pharmacia Lettre, 2016, 8 (20):68-74
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

In vitro cardio-protective activity of amylase from *Aspergillus niger* on H9C2 cardiomyoblast cells

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ABSTRACT

This research is aimed to investigate the cardio protective effect of amylase. Doxorubicn was used to induce toxicity in H9C2 (cardiomyoblast) Cells and were exposed to 6.25 to 100 µg/ml of amylase from Aspergillus niger. After treatment the percentage of cell viability was studied by cytotoxicity assay by direct microscopic observation and by MTT method. The obtained results showed that amylase significantly increased the percentage of cell viability in a concentration dependent manner. Concentration of 50 µg/ml of amylase shows moderate activity whereas 100µg/ml was found to have potent activity. Cell viability at 6.25, 12.5, 25, 50 and 100 µg/ml was recorded as 42.2, 48.5, 50.7, 60.8 and 62.4% respectively by MTT assay. The data revealed that the treatment with amylase induced cell viability.

Keywords: *Aspergillus niger*, α-amylase, SDS – PAGE, cardio-protective effect

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INTRODUCTION

Alpha amylases are extracellular enzymes that randomly cleave the ∞ 1, 4 - linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units[1]. Studies on fungal amylases especially *Aspergillus niger* has been concentrated because of their ubiquitous nature and non-fastidious nutritional requirement [2]. Enzymes such as amylases and proteases are widely used in industry for the manufacture of pharmaceuticals, foods, beverages and confectioneries as well as in textile and leather processing, and waste water treatment [3-5]. Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituent [6-8]. Nowadays, the new potential of using microorganism as biotechnological source of industrially relevant enzymes has stimulated interest in exploration of extracellular enzymatic activities in several microorganisms [9]. *Aspergillus niger* was produced more amylase yield on agricultural waste. Since agricultural waste is very cheap, easily available source from mill factories and more suited for solid state cultivation of *Aspergillus niger* for amylase production [6]. Usability of waste loquat kernels as substrate in microbial culture media for the production of α-amylase was investigated for the first time in the present study [10]. Amylases are hydrolytic enzymes that stand out as a class of enzymes which are of useful applications in the brewing, textile, detergent and pharmaceutical industries [11, 12]. Therefore, the present study focus to screen fungal strains for amylase production and its cardio protective effect on H9C2 (cardiomyoblast) Cells.

MATERIALS AND METHODS

Sample collection
Three water samples were collected from different places in Kanyakumari District. Samples were collected in sterile plastic sampling containers and transferred to the laboratory for fungal isolation.

Isolation of fungal strains
The fungi were isolated by serial dilution method in which isolates of fungi were obtained by serial dilution [13]. 1ml of collected water sample was taken and serial dilution was made up to 10^{-5} using sterile saline water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-5} was taken and spread evenly over the surface of Potato dextrose agar (PDA) medium. The inoculated plates were incubated at room temperature for 3-5 days and observed for fungal growth.

Screening of Fungal Isolates for Amylase Production
All the four fungal isolates were screened for amylase production efficiency in starch agar media comprising the following in g/ L. yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15, and pH 5.6. All the isolates were streaked centrally on sterile solidified starch agar plates and incubated at 28°C for 3-5days, after that all the plates along with control were flooded with iodine and observed for zone of hydrolysis. The best amylase producers were selected based on the zone of clearance and used for enzyme production.

Production of amylase
Production of amylase was carried out in Erlenmeyer flasks using jack fruit seed powder as substrates and starch powder was used as control. For this production 10gm of powdered jackfruit seed was taken in 250 ml flasks and moistened with nearly 50 ml of MSM containing the following in gm/L (0.8 g NaCl , 0.8 g KCl , 0.1 g $CaCl_2$, 2.0g Na_2HPo_4, 0.2g $MgSo_4$, 0.1 g $FeSo_4$, 8.0g Glucose, 2.0 g NH_4Cl, pH 6.2). Flasks were autoclaved, cooled to room temperature, inoculated with 1ml fungal spore suspension and incubated at 28°C for 5 -7 days.

Extraction of Crude Enzyme
Crude enzyme was extracted from fermented media by adding 100 ml of 100 mM Tris buffer pH 6.2, agitating the flask in shaker at 180 rpm for 1hour, the mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4^0C for 5 min. The supernatant was collected and treated as crude enzyme [14].

Estimation of protein
The protein concentration of the enzyme was estimated by Lowry et al., [15].

Purification of amylase
The crude enzyme suspensions were precipitated by ammonium sulphate (75%). Purification using ammonium sulphate (% w/v) at 60, 80 and 100 on amylase enzyme from *Aspergillus niger* strain was reported [16]. The precipitate was collected by centrifuging at 8000 RPM for 20 min and resuspended in 100mM Tris buffer pH 6.2. It was dialysed against the same buffer and freeze dried. The concentrated sample was passed through Sephadex G-50 column and eluted with the same buffer with the flow rate of 15 ml/ hours [17].

Molecular weight determination
The molecular mass of amylase was analysed by 10% acrylamide gel electrophoresis. The proteins were visualized using coommassie blue and the molecular weight was determined by comparing with molecular weight markers.

In vitro cardio-protective determination by MTT assay
H9C2 (cardiomyoblast cell line) was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:
Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of enzyme or compound stock:
1 mg of each enzyme was added to1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Cytotoxicity Assay by Direct Microscopic observation:
Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:
Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

RESULTS

Isolation of fungal strains
In this present investigation different fungal colonies were isolated from the collected water samples by agar platting method. A total of fourteen morphologically different fungal colonies were isolated (Figure 1).

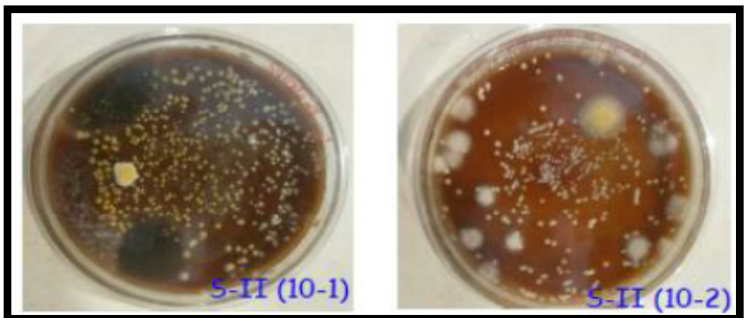


Figure 1: Isolation of fungus

Screening of Fungal Isolates for Amylase Production
The ability of fungus that can produce amylase was determined by starch agar platting. The fungal growth on the plate was flooded with Iodine solution; a clear zone around the growth was indicating the amylolytic activity (Table 1).

Table 1: Screening of amylolytic activity

SL. No.	Strain code	Amylolytic activity
1	S-II(1)	++
2	S-II(2)	-
3	S-II(3)	-
4	S-II(4)	+
5	S-II(6)	-
6	S-III(3)	-

'+' good activity; '-'no activity

Extraction and purification of ∞-amylase

The enzyme from the culture filtrate was precipitated by ammonium sulphate; the precipitate was resuspended in tris buffer (100 mM, pH 6.2). Then it was dialyzed against the same buffer and passed through a Sephadex G-75 column and eluted with the same buffer at the rate of 15 ml/hour. Later, the crude enzyme is subjected to chromatography and gel filtration [18].

Molecular weight determination

The molecular mass of amylase was analysed by 10% acrylamide gel electrophoresis and the stained proteins with coomassie brilliant blue R-250 showed single band which was found to have approximate molecular weight of about 44-46 KDa (Figure 2). Presence of amylase was verified by SDS-PAGE analysis, showing a single band of approximately 43 kDa [1].

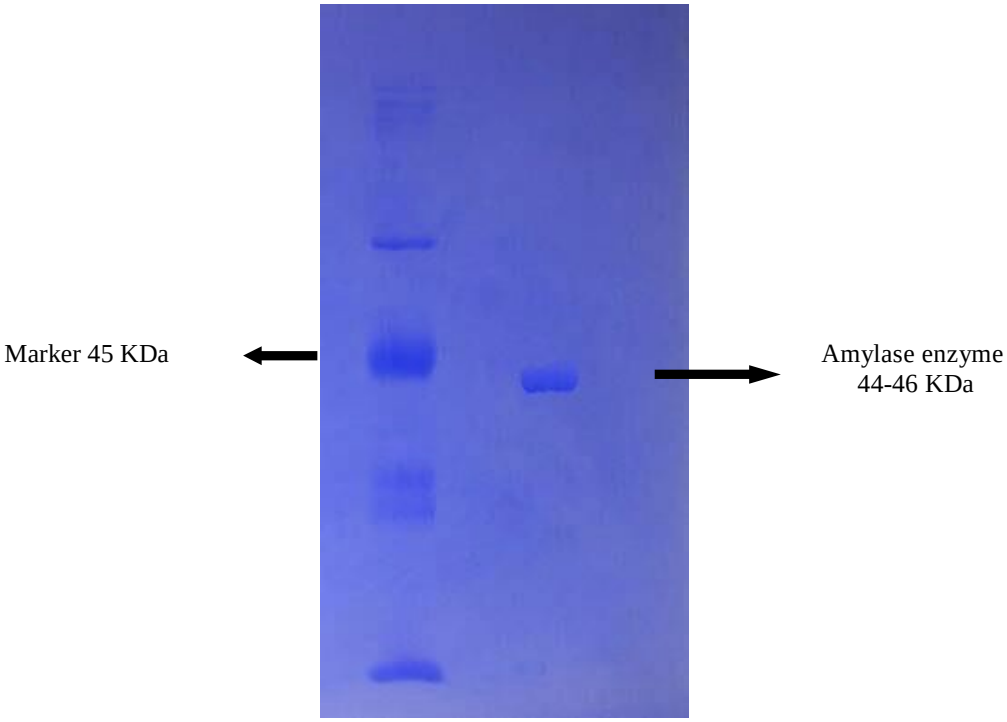


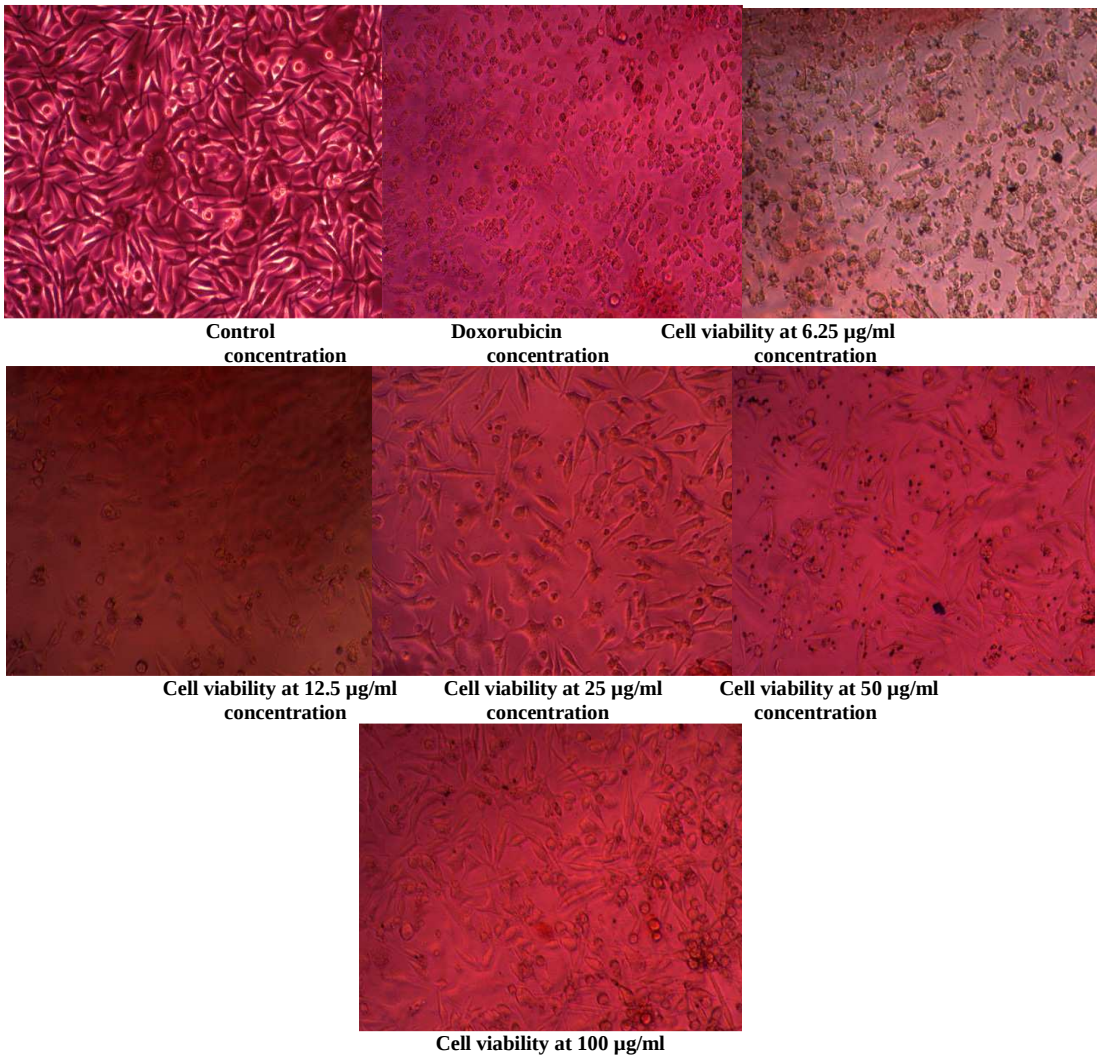
Figure 2: SDS-PAGE analysis of ∞-amylase

MTT assay

Cytotoxicity of amylase was assessed using MTT assay, after exposing the H9C2 cardiomyoblast cells at 6.25 to 100 µg/ml concentrations, Amylase induced significant increase in cell viability in a concentration dependent manner (Table 2). Concentration of 50 µg/ml of amylase shows moderate activity whereas 100 µg/ml was found to have potent activity. Cell viability at 6.25, 12.5, 25, 50 and 100 µg/ml was recorded as 42.2, 48.5, 50.7, 60.8 and 62.4% respectively by MTT assay.

Table 2: Cardio-protective activity of amylase

Sample Concentration (µg/ml)	Average OD at 540nm	Percentage of cell Viability
Control	**0.6425**	
Doxorubicin	0.2366	36.8249
6.25	0.2711	42.19455
12.5	0.3115	48.48249
25	0.3258	50.70817
50	0.3908	60.8249
100	0.4011	62.42802



Control concentration **Doxorubicin concentration** **Cell viability at 6.25 µg/ml concentration**

Cell viability at 12.5 µg/ml concentration **Cell viability at 25 µg/ml concentration** **Cell viability at 50 µg/ml concentration**

Cell viability at 100 µg/ml concentration

Figure 3: Morphological changes in H9C2 cardiomyoblast cells exposed to various concentration of amylase

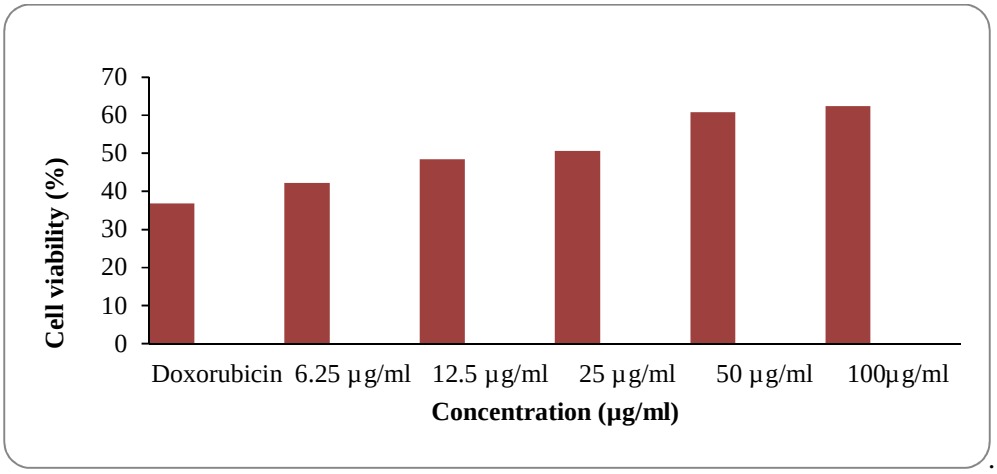


Figure 4: In vitro cardio-protective determination of amylase by MTT assay

CONCLUSION

Doxorubicin-induced cardiomyopathy is strongly linked to an increase in cardiac oxidative stress, as evidenced by reactive oxygen species (ROS) induced damage such as lipid peroxidation, along with reduced levels of antioxidants and sulfhydryl groups. Myofibrillar deterioration and intracellular calcium dysregulation are also important mechanisms commonly associated with doxorubicin-induced cardiac toxicity. Doxorubicin is used to induce toxicity [19]. Cell viability at 6.25, 12.5, 25, 50 and 100 µg/ml concentration of amylase was recorded as 42.2, 48.5, 50.7, 60.8 and 62.4% respectively by MTT assay. Amylase induced significant increase in cell viability in a concentration dependent manner. Concentration of 50 µg/ml of amylase shows moderate activity whereas 100µg/ml was found to have potent activity. The spectrum of amylase applications has expanded into many fields, such as clinical, medical and analytical chemistry [20]. In the light of modern biotechnology, Amylases are now gaining importance in biopharmaceutical applications [21]. From the present investigation it is confirmed that amylase from *Aspergillus niger* is having potent cardio-protective effect and can be used in clinical field.

Acknowledgement
We would like to show our gratitude to Murugan. S, Department of Biochemistry, Lekshmipuram college of arts and science, Neyyoor for comments that greatly improved the manuscript.

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