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Isolation, Production Of Novel Enzyme: Amylase From *Penicillium* Sp. And Its Cytotoxic Studies.

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ABSTRACT

Amylases are one of the important enzyme used in various industries. They can be obtained from many sources includes plants, animals and microorganisms. This present study aimed to isolate amylase producing fungi from water samples collected from Kanyakumari District. A total of 14 fungal strains were isolated from three different water samples and 5 isolates exhibited amylase activity. Among five, one best amylase producer was selected for further study. The fungi was identified as *Penicillium sp.* by studying cultural, morphological characters and used for amylase production using jack fruit seed powder as a substrate. The enzyme was purified by column chromatography and then, enzyme activity was determined. The enzyme showed 56517 U/ml of total activity and 63.50 U/mg of specific activity. Also, this enzyme showed cytotoxic effect on MCF-7 breast cancer cell lines *in vitro* conditions.

Keywords: α-amylase, *Penicillium species*, jackfruit seed, solid state fermentation, cytotoxicity.

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INTRODUCTION

Amylases are that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltotriose units [1]. It constitutes a group of industrial enzymes, which alone covers approximately 30% of the enzyme market [2]. They have opened new frontiers of many commercial biotechnological processes, including renewable energy, pharmaceuticals, saccharification or liquefaction of starch, detergent industries, warp sizing of textiles, fibres, paper industries, foodstuffs, baking, clarification of haze formed in beer or fruit juices and for pretreatment of animal feed to improve digestibility [3].

The enzyme can be obtained from several sources, such as plants, animals and microorganisms. Several microorganisms can be obtained for enzyme production. Amylases are known to be produced by a variety of bacteria and fungi and their applications at industrial level have stimulated interest to explore their amylolytic activity in several microbes to be used as Bioresources [4]. However, enzymes from fungal and bacterial sources have dominated in many applications. Filamentous fungi are important organisms for production of useful enzymes and biologically active secondary metabolites [5].

Aspergillus and *Penicillium* are particularly interesting for industrial enzymes due to their easy cultivation, and high production of extracellular enzymes of large industrial potential [6, 7]. They used in a number of industrial processes such as in food, baking, brewing, detergent, textile and paper industries. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medical and analytical chemistry [4]. Therefore, the present investigation deals with screening of fungal strains for amylase production and its cytotoxic effect on cancer cells.

MATERIALS AND METHODS

Sample collection

A water sample was collected from three different places in Kanyakumari District. The samples were collected in sterile plastic sampling containers and transferred to the laboratory for fungal isolation.

Isolation of fungal strains

Serial dilution was made with 1 ml of sample using sterile saline and agitated by vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-5} was spread evenly over the surface of Potato dextrose agar (PDA) medium. The inoculated plates were incubated at room temperature for 3-5 days and the fungal growth were observed.

Screening of 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, pH 5.6. All the isolates were streaked centrally on sterile solidified starch agar plates and incubated at 28°C for 3-5days. After, the plates along with control were flooded with iodine solution and observed for the zone of hydrolysis. The best amylase producers were selected based on the zone of clearance and used for enzyme production.

Morphological identification of fungus

One of the best amylase producing fungal strain was identified by studying cultural, pigmentations, microscopic study by Lacto phenol cotton blue staining technique. Further, it was used for the production of amylase.

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 a control. For this production 10g of powdered jackfruit seed was taken in 250

ml flasks and moistened with nearly 50ml of MSM containing the following in g/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. Then inoculated with 1ml fungal spore suspension and incubated at 28°C for 5 -7 days.

Purification of enzyme

Cell free supernatant was collected by centrifugation, the crude enzyme suspensions were precipitated by ammonium sulphate (75%). The precipitate was collected by centrifuging at 8,000 RPM for 20 min, and resuspended in 100mM Tris buffer pH 6.2. It was dialyzed against the same buffer and freeze-dried. The concentrated sample was passed through a Sephadex G-50 column and eluted with the same buffer at the rate of 15 ml/hours. The collected fractions were subjected to assay of enzyme activity and protein concentration.

Enzyme assay and protein concentration

Enzyme assay of crude and purified enzymes were carried out by DNS method [8] in which 0.5 ml enzyme was reacted with 0.5 ml of substrate (1% starch in 100mM Tris buffer) under standard reaction conditions and the reaction was stopped by adding DNS reagent, amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540 nm with the standard graph plotted by reacting the known concentration of maltose ranging from 0.05mg/ml to 0.5mg/ml. One unit amylase activity was defined as amount of enzyme that releases 1 micromoles of maltose per minute under standard reaction conditions.

Concentrations of protein in crude and purified enzyme were determined by Lowry's method [9] using bovine serum albumin (BSA) as standard. Enzyme activity is expressed as specific activity, which is equivalent to U/mg protein.

Cytotoxic study

MCF-7 breast cancer cell lines were purchased from NCCS Pune were maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5% CO2 in a humidified atmosphere in a CO2 incubator(NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia) for 2 minutes and passed to T flasks in complete aseptic conditions. Extracts were added to grow cells at a final concentration of 1.5µg/ml, 3.1 µg/ml, 6.2 µg/ml, 12.5 µg/ml and 25 µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cells were washed with 1x PBS and then added 30 µl of the MTT solution to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37^0C for 3 hours. MTT was removed by washing with 1x PBS and 200 µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA) and the cell viability was calculated [10].

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RESULTS

Isolation of fungal strains

In this present study, different fungal colonies were isolated from the water samples by the agar plating method. A total of fourteen different fungal colonies (4 from water sample-I; 6 from sample-II and 4 from sample-III) were isolated.

Screening of amylase production

After flooded the plates with Iodine solution, a clear zone around the growth was observed, it indicating the amylolytic activity. Among the 12 isolated fungus only 5 strains were shown amylolytic activity (Table 1).

Table 1. Screening of amylase activity

SL. No.	Strain code	Amylolytic activity
1	S–I (1)	–
2	S–I (2)	–
3	S–I (3)	–
4	S–I (4)	+
5	S–II (1)	+
6	S–II (2)	–
7	S–II (3)	–
8	S–II (4)	+
9	S–II (6)	–
10	S–III (1)	+
11	S–III (2)	+
12	S–III (3)	–

'+' amylase positive; '-' amylase negative

Morphological identification of fungus

Among the five positive strains, best one was selected by based on the maximum size of zone. It was identified by cultural and morphological characterizations. Colony morphology, colour, texture, pigmentation and microscopic observations were studied (Table 2). Based on the results, the fungi strain was probably identified as *Penicillium* species.

Table 2. Cultural and morphological Identification of fungus

SL. No.	Characteristics	Result/inference
1	Source of the sample	Water
2	Growth temperature	28–30°C
3	Growth colour on beginning	Dirty white
4	Growth colour on middle	Dark green
5	Growth colour on later	Green – grey
6	Appearance	Granular/cottony
7	Texture	Velvety
8	Pigments/spots	–
9	Conidiophores	Present
10	Approx. conidiophores	300-400µm
11	Vesicles	Present
12	Shape of vesicles	Club shape
13	Type of vesicles	Uniseriate
14	Phialides	Present
15	Spore type	Conidiospores
16	Arising of Conidia	Central axis / top of the vesicle
17	Conidia arrangements	Chains

Enzyme assay and protein concentration

An enzyme activity, total protein, and specific activities of the amylase enzyme produced by the fungus was calculated. The amylase showed maximum value of total activity, protein concentration and specic activity while jackfruit seed was used as substrate in production medium than compared to starch (control) used (Table 3).

Table 3. Enzyme activity and protein concentration of amylase

The substrate used	Enzyme activity (U/ml)	Total Protein (mg/ml)	Specific activity (U/ mg)
Starch (Control)	24618	410	60.04
Jackfruit seed	56517	890	63.50

Cytotoxic study

Cytotoxicity effects of amylase were studied on MCF-7 breast cancer cell lines. At 1.5, 3.1, 6.2, 12.5 and 25 µl concentrations of amylase, the total viability of cells were 95.4, 74.06, 64.4, 48.7 and 34.8 % respectively (Table 4 & Fig. 1)

Table 4. Cytotoxicity effect of α-amylase

Sample Volume (µl)	Absorbance @ 540nm	% Viability
Control	0.9982	
1.5	0.9529	95.46183
3.1	0.7393	74.06331
6.2	0.6433	64.446
12.5	0.4868	48.76778
25	0.3483	34.89281

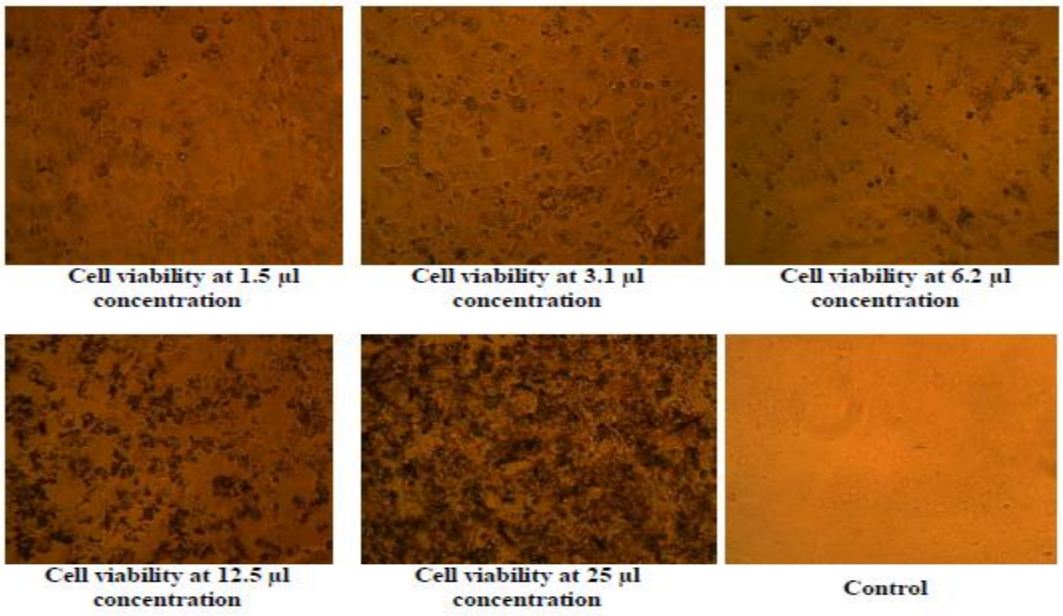

Figure 1. Cytotoxicity effect of α-amylase MCF-7 on breast cancer cell lines

DISCUSSION

Anticancer activity is the effect of natural and synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression. Several synthetic agents are used to cure the disease but they have their toxicity and hence the research is going on to investigate the plant derived chemotherapeutic agents. Anticancer properties of many natural compounds isolated from different Indian plant extracts have

been reported. Research is being carried out throughout the world to find a lead compound which can block the development of cancer in humans [11].

In the present study, three water samples were collected from different areas in Kanyakumari District, a total of fourteen morphologically different fungal colonies were isolated and screened for α-amylase production by starch agar platting. Among the twelve strains tested only five strains exhibited amylase activity. The zone can not in any way be correlated quantitatively with the amount of enzyme produced. The results showed that all isolates possessed a high potential for amylase production. Also, jack fruit seed powder was used as a substrate for amylase production by *Penicillium species*. The total protein, enzyme activity and specific activities of the amylase enzyme produced by various organisms were calculated. The *Penicillium species* produces 63.50 U/mg specific activity of enzyme in jackfruit seed medium. This result was supported by the research of Ibatsam Khokhar *et al*. [12], in their study, four *Penicillium* species viz. *P. janthinellum*, *P. melinii*, *P. velutinum* and *P. waskmanii* showed the amylase production. *Penicillium expansum* produced maximum amylase enzyme when peptone was used as nitrogen source [13]. Finally an attempt has made to study the cytotoxic activity of the α-amylase against MCF-7 breast cancer cell lines. In this study, the α-amylase showed potent cytotoxic effect. At 25µl concentration, the enzyme showed toxic effects in higher level. But in lowest concentration, i.e. at 3.1 µl, it showed less toxic effects. From the present investigation, it is confirmed that amylase of *Penicillium species* have the specificity of cytotoxicity towards MCF-7 breast cancer cells.

CONCLUSION

The fungal strains *Penicillium* species produced more quantity of amylase enzyme with good enzymatic activity and more enzyme production was assessed with jack fruit seed as substrate in production medium. Also, the enzyme has the ability to kill MCF-7 breast cancer cells. Hence this potent fungal strain can also be used in large scale industry for various useful process.

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