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Screening for temperature tolerance of some *Aspergillus* spp.

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Abstract

Three species of *Aspergillus* viz., *A. fumigatus*, *A. nidulans* and *A. sparsus* were isolated from different substrates at high temperature (45 ^{o}C) and were subjected for thermotolerance screening. Fresh and dry weight, absorbance by extract/spore suspension, spore count and enzyme activity were the parameters used to evaluate fungal species under different temperatures regimes i.e. 25±2 ^{o}C, 40±2 ^{o}C, 50±2 ^{o}C and 60±2 ^{o}C. Maximum thermotolerance was observed in *A. fumigatus* then *A. sparsus* and *A. nidulans*.

Key words: Absorbance, *Aspergillus*, enzymes and temperature.

Introduction

The *Aspergillus* genus is vast group of organism of cosmopolitan occurrence. Species of *Aspergillus* are important medically and commercially. Some species can cause infection in humans and other animals. Members of the genus are also sources of natural products that can be used in the development of medications to treat human disease (Denning, 1998). Some are also employed in a variety of fermentation processes; Alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients rather than from grapes or malted barley using species of *Aspergillus* (Du *et al.*, 2008).

Some species of genus are an important component of thermotolaremt mycoflora isolated from a variety of substrates from Lahore (Nazir *et al.*, 2007). The present study is an extension of the pervious work and is focus on evaluation of thermotolerant ability of isolated *Aspergillus* species.

Materials and Methods

Isolations

Species of *Aspergillus* were isolated from various substrates like compost, leaf manure, farmyard manure and dung and culture on malt extract agar medium.

Thermotolerance Screening

Thermotolerance of species was tested on liquid broth. Simple 2% malt extract broth was prepared for this purpose and twelve flasks for each fungus were taken which were autoclaved with medium at 121 ^{o}C for 20min then inoculated with disc of 0.5 mm of pure culture of isolates under aseptic conditions. Flasks were incubated on four temperatures viz., 25±2 ^{o}C, 40±2 ^{o}C, 50±2 ^{o}C and 60±2 ^{o}C. Three replicates for each temperature were prepared. After seven days of incubation fungal biomass was separated by filtration. Fungal extract was preserved for further biochemical studies. Fresh weight of fungal biomass was taken and then fresh biomass was dried at 60 ^{o}C for 24 hours and afterward dry weight was noted. Absorbance of fungal extract was determined at 500 nm, 550 nm and 600 nm on spectrophotometer.

Quantification of spore number

The number of the spore per ml was determined by hemacytometer. Isolate of *Aspergillus* inoculated on Malt extract agar culture medium and incubated at 25±2 ^{o}C, 40±2 ^{o}C, 50±2 ^{o}C, and 60±2 ^{o}C. After 04 days the spores were harvested by adding 10 ml sterilized distilled water in the petriplate. 50 µl of spore suspension was added on the hemacytometer covered by glass cover slip. Spores were counted under the microscope and data was recorded.

Enzyme activity test

Microbial strains

Fungal cultures were maintained on the Malt extract agar (MEA). For enzyme activity test 7 days old culture was used.

Growth conditions

To maintain the fungal growth media used had: Yeast extracts = 14.3 g $(NH_4)_2SO_4$ = 2.1 g, $MgSO_4 \cdot 7H_2O$ = 0.3 g, $CaCl_2 \cdot 2H_2O$ = 0.3 g, $FeSO_4 \cdot 7H_2O$ = 0.5 g, KH_2PO_4 = 10 g and Main carbon source = 20 g. Pectin and cellulose were used as main carbon source.

Broth assay

Broth medium was prepared for enzyme activity test. Pectin and cellulose were used as carbon sources. Conidial suspension of isolated fungi was adjusted by hemacytometer @10^6 spore ml^{-1}. This conidial suspension was poured into each flask @ 50 µl which were incubated at 45±2 °C. After 3 days of incubation period, 05 ml broth was taken, for pectin stained with 50 µl, 0.05% ruthenium red solution for ½ hour and change in color was checked. Same the procedure was done with Congo red dye for cellulolytic enzymes.

Results and Discussion

Isolation

Three species of *Aspergillus* viz. *A. fumigatus, A. nidulans* and *A. sparsus* were isolated from different substrates at high temperature (45 ^oC).

Fresh and dry weight comparison

The growth of *Aspergillus* spp varied greatly at different temperatures and their incubation period. The fungal biomass decreased at elevated temperature. Thermotolerance decreases with increase of temperature: maximum growth was determined at 40 ^oC for *A. fumigatus* and *A. sparsus* while for *A. nidulans* it was recorded at 25 ^oC. Above 50 ^oC fungal biomass decreased and at 60 ^oC negligible growth was recorded. Maximum value was considered as optimum value for other temperature regimes for every *Aspergillus* sp. In *A. fumigatus* 41% reduction occurred than the optimum value (40 ^oC) whereas 79.60% reduction in fresh biomass of fungal isolated at 60 ^oC noted when compared with optimum that kept at 40 ^oC while at 25 ^oC the fresh biomass exhibited an increase of 31.78% and 76.43% when compared with 50 ^oC and 60 ^oC fresh biomasses respectively. A reduction of 13.50% form the optimum observed at this (25 ^oC) temperature. Growth of *A. sparsus* when compared with its optimum temperatures mycelial growth increased with increase in temperature up to 40 ºC followed by decline. The optimum temperature for fungal mycelial growth considered as 40 ºC (*A. sparsus*) since the mold showed optimum to normal growth at 25 ºC where the percentage reduction from optimum was 5.53% while at 50 ºC the fungus showed 77.61% growth which is 22.38% less from the optimum (40 ºC) moreover at 60 ºC.

A regular decrease in weight of biomass recorded when temperature increased in *A. nidulans*. Maximum growth was recorded at 25 ºC and at 40 ºC 16.34% from optimum (40 ^oC). Mean biomass weight of mold noted at 50 ºC and 60 ºC with reduction 73.97% and 89.22% from optimum was observed these temperatures (Fig. 1).

Absorbance comparison

Thermotolerance of fungi was checked by absorbance at 500, 550 and 600 nm wavelengths. The absorbance of fungal extract increased when the temperature was increased and 50 ºC extract considered as optimum for *A. fumigatus* and *A. sparsus*. The rate of absorbance of *A. fumigatus* at 550 and 600nm increased with an increase in temperature. An absorbance of *A.* sparsus, 1.158 noted for 50 ^oC extract which reckoned as optimum followed by 60 ºC extract (0.937), 25 ºC extract (0.747) and 40 ºC extract (0.704) similarly *A.* sparsus at 550nm also exhibit same trend of absorbance as in 500nm while 600nm *A.* sparsus extracts exhibit a decreasing trend of absorbance values with the highest absorbance (0.515) recorded for 25 ºC extract, followed by 50 ºC extract (0.487), 40 ºC extract (0.479) and 60 ºC extract, whose absorbance value was 0.267. A trend of increase followed by decline in absorbance was visualized in *A. nidulans*. Maximum absorbance was noted for 60 ºC extract at all wave lengths (500, 550 and 600 nm). The rate of absorbance at this wavelength initially decreased followed by increase (Fig. 2).

Quantification of spore number

Spore production depended on temperature, period of incubation and culture medium, when provided all given factors to *Aspergillus* spp. it came to knowledge that number of spores is dependent on temperature; with the increase in temperature spore abundance decreased. The most suitable temperature for maximum spore production reckoned 25 ^oC (3.5×10^8) for *A. fumigatus* while at this temperature *A. sparsus* and *A. nidulans* generated 1.5×10^6 and 9.0×10^6 number of spores respectively. At 40 ^oC *A. sparsus* generated 2.5×10^7 numbers of spores whereas 2×10^8 calculated for *A. fumigatus*, which is, less then other temperatures exhibit a trend of reduction in number of spores. At 50 ^oC *A. fumigatus* generated 2×10^7spore while at 60 ^oC no growth and sporulation occurred of all isolates of *Aspergillus* spp. (Fig. 3).

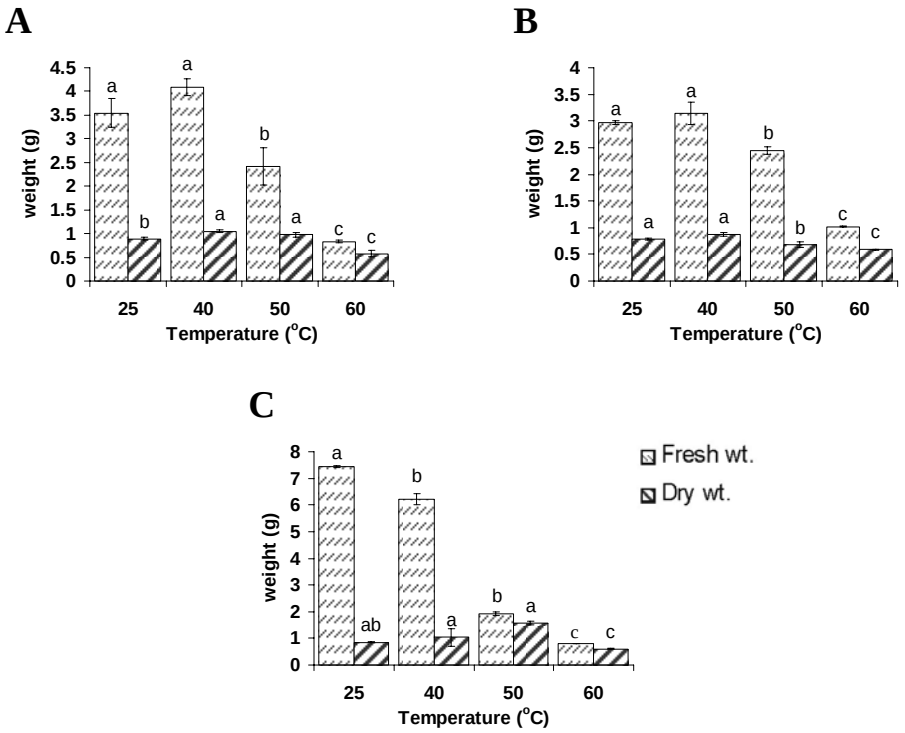

Fig.1: Fresh weight and dry weight comparison of **A:** *A. fumigatus*; **B:** *A. sparsus*; **C:** *A. nidulans*

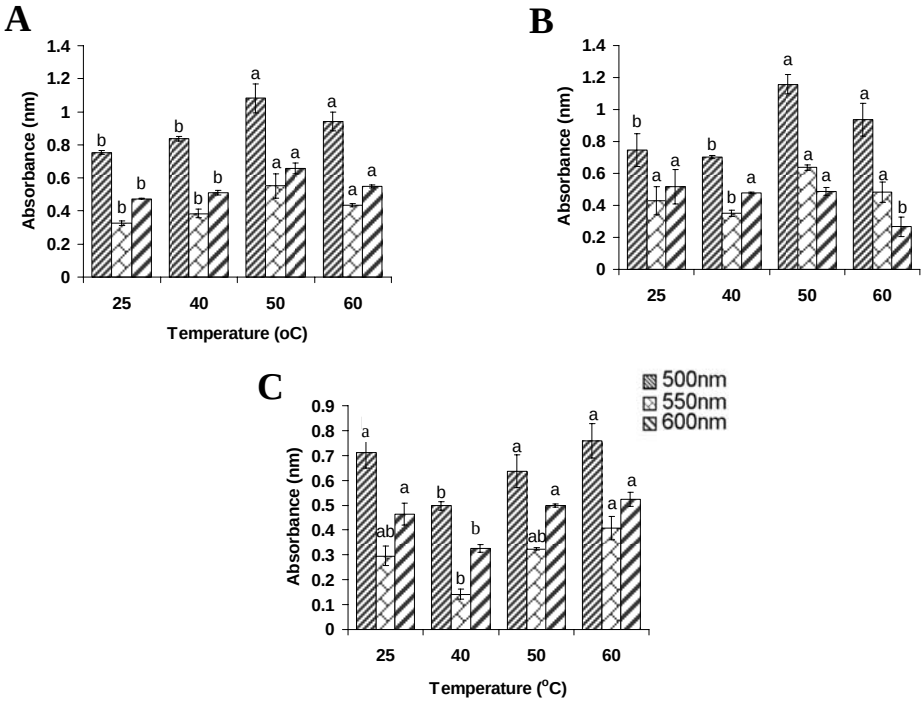

Fig. 2: Absorbance comparison of **A:** *A. fumigatus*; **B:** *A. sparsus*; **C:** *A. nidulans*

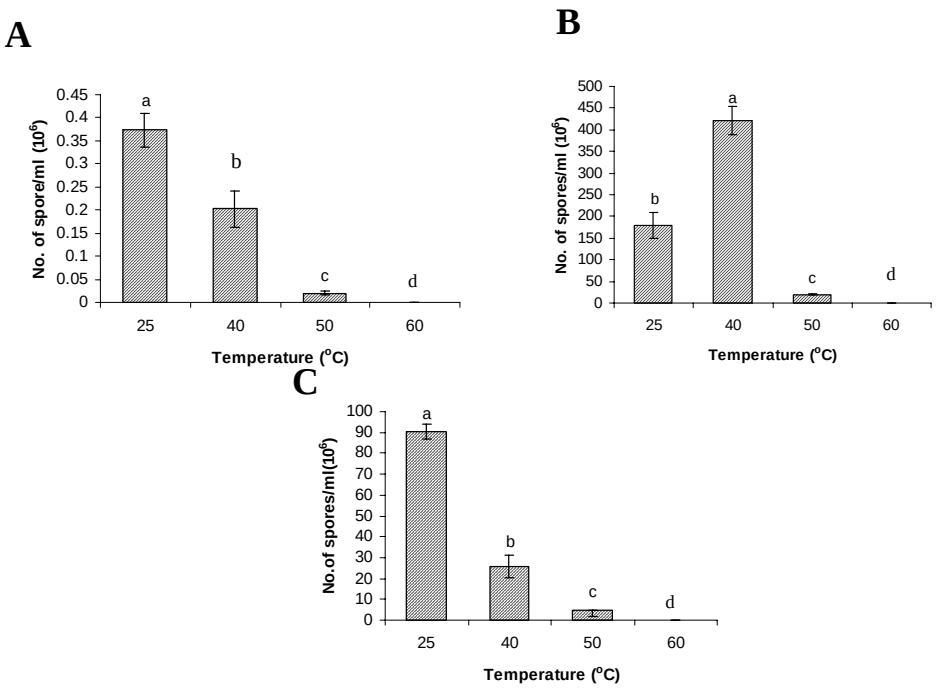

Fig.3: Spore production comparison of **A:** *A. fumigatus*; **B:** *A. sparsus*; **C:** *A. nidulans*

Enzyme activity test

Enzyme activity tested for two enzymes, pectinolytic and cellulolytic enzyme. Pectin was degraded by pectinolytic enzyme at 40 ^{o}C and on staining solution with ruthenium red; discoloration of dye occurred which showed the presence of pectinolytic enzyme in the solution. When the reaction of dye was done for 60 ^{o}C extract discoloration was not occurred for all tested molds (*A. fumigatus*, *A. nidulans* and *A. sparsus)*. Discoloration intensity was low at 25 ^{o}C and 50 ^{o}C that demonstrate that at this temperature pectinolytic enzyme was produce but not at that level which can discolor the stain but discoloration of 50 ^{o}C and 40 ^{o}C extract of *A. nidulans* was clearly visualized.

For the detection of cellulolytic enzyme solutions of different temperatures stained with Congo red dye, maximum discoloration was visualized at 40 ^{o}C and 25 ^{o}C for *A. fumigatus*, *A. nidulans* and *A. sparsus* which show slight change in dye color at 25 ^{o}C extract while no visible change in dye color observed in 50 ^{o}C and 60 ^{o}C extracts of both fungi that demonstrated cellulolytic enzyme production but not in detectable quantity, which can discolor the dye. The degrading enzymes might present at every temperature but working depends on temperature and other parameters.

Three different species of *Aspergillus* (*A. fumigatus A. sparsus* and *A. nidulans*) were isolated from different substrates viz. compost, leaf manure, farmyard manure and dung at 45 ^{o}C. Thermotolerant range of these species checked at different temperatures viz., 25±2 ^{o}C, 40±2 ^{o}C, 50±2 ^{o}C and 60±2 ^{o}C. Maximum growth was recorded at 40 ^{o}C in case of *A. fumigatus* and *A. sparsus* while *A. nidulans* generated maximum growth at 25 ^{o}C and very minute growth was observed at 60 ^{o}C while satisfactory growth was noted at 50 ^{o}C. Which means *Aspergillus* species can tolerate temperature up to 50 ^{o}C.

Absorbance is another parameter which was considered for the evaluation of thermotolerance of these selected fungi (Brian, *et al*., 2006). This was adopted as indirect measure to compare sporulation ability at variable regimes. Absorbance was checked at three wavelengths i.e., 500 nm, 550 nm and 600 nm. 500 nm, which comes under the range of visible light, and result, have shown maximum absorbance by all selected fungi, at 550 nm. The rate of absorbance was much less than 600 nm. Absorbance rate reveals interesting variation in the growth rate (Hsiao and Björn, 1982). Number of spores per ml was an additional parameter to demonstrate the thermotolerance of selected fungi. Number of spores was inversely related to the growth of the mold (Wolk 2000). As the temperature increased the rate of spore formation declined. The carbon sources used in experiment were cellulose and pectin for detection of cellulolytic and pectinolytic enzymes. Metabolic activity has always been considered as vital features to elevate the performance of an individual. The production of extracellular enzyme is effected by environmental conditions; temperature affects the production of enzyme which was much faster. When the degradation of the substrate i.e. pectin was studied its disintegration shown at every temperature except 60 ^{o}C Hansen (2001). Maximum discoloration of the dye (ruthenium red) was done at 40 ^{o}C. Similarly in the case of cellulolytic enzyme the degradation was done at every temperature except 60 ^{o}C.

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