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Comparative studies on the enzyme activities of wild and mutant fungal strains isolated from sugarcane field

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Abstract: Three fungal species namely, *Aspergillus fumigatus, Penicillium chrysogenum* and *Verticillium terrestre*, isolated from sugarcane field soil, revealed the extracellular production of amylase, cellulase and lipase. Improvement in elucidation of these enzymes was achieved by developing mutants after exposure to UV light.

Keywords: Amylase, cellulase, lipase, extra cellular enzyme, fungi, *A. fumigatus, P. chrysogenum, V. terrestre*.

Introduction

Fungi are the chief source for various industrially important enzymes as they are able to synthesize and secrete large amounts of extracellular enzymes. The exponential increase in the application of enzymes in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement through strain improvement and medium optimization for higher enzymatic yield. Improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the process and may also possess some specialized desirable characteristics.

A variety of industries employ microbial amylase, cellulose and lipase. Various forms of amylases are used to convert starch into different sugars. Amylase, cellulase and lipase from microbial sources are used in textile, paper and detergent industries (Peij *et al.,* 1998; Tsukagoshi *et al.,* 2001). α- Amylases β amylase and glucoamylase are commonly produced by various species of *Aspergillus* and used as sources of industrial amylases (Boyee, 1986). The most widely used thermostable enzymes are the amylases in the starch industry (Emmanuel *et al.,* 2000; Sarikaya *et al.,* 2000).

Cellulase has enormous potential as a renewable source of energy (Coral *et al.,* 2002). Viniegra *et al.* (2003) reported the advantages of fungal enzyme production in solid state over liquid fermentation systems. Pandey *et al.,* (2000) reported that solid state fermentation holds tremendous potential for enzymes production.

Lipases from fungi (Essamri *et al.,* 1998) have been widely used for biotechnological applications in dairy industry, oil processing, production of surfactants and preparation of pure pharmaceuticals (Viniegra *et al.,* 2003). Novel enzymes were developed by the combined use of microbial screening and rational protein engineering (Sarikaya *et al.,* 2000).

UV light has been shown to be lethal and mutagenic in a variety of organisms, including fungi. The correlation between the quantity of energy absorbed by DNA and the observed biological effects (survival and mutation frequency) are illustrated in the wavelength region between 254 and 320 nm. UV irradiation was found to be best for the improvement of strains like *Aspergillus niger* for maximum production of various enzymes (Kang *et al*., 1999). In recent years, attempts have been made for the overproduction of microbial enzymes by induced mutagenesis. Suntornsuk and Hang (2008) have reported that the strain improvement in *Rhizopus oryzae* by UV, resulted in the production of more glucoamylase by a mutant than the parent strain. Mutational experiments were performed to produce morphological mutants from *Aspergillus wentii* Wehmer (IMI 17295) by UV and X-ray irradiation. Among the morphological mutants, five representative types were recognized. Marked variation existed in amylase activity between the morphological mutants and the wild type (Basu & Chakrabarty, 1974).

In tests for rhizosphere competence, two of the cellulase minus strains were found to have significantly enhanced rhizosphere competence after UV mutagensis (Melo *et al*., 1997). Mutant strains from *Aspergillus niger* UAM-GS1 were produced by UV radiation to increase their hemicellulolytic and cellulolytic activity production. The mutant strains showing more enzymatic activity were thus labelled as GS1-S059 and GS1-S067. These strains also showed the largest relationship between diameter of hydrolysis zone and colony diameter (Soledad De Nicolás-Santiago *et al*., 2006).

Sandana Mala *et al*. (2001) have reported 2.53 times more of lipase production from *Aspergillus niger* by UV mutants than the parental strain. In *Rhizopus* sp., the lipase yield of the best UV mutant $BTUV_3$ was 164% higher than the parent strain ($BTNS_{12}$) and 180% higher than the wild strain (BTS-24) (Bapiraju *et al*., 2004). Ellaiah *et al*. (2002) reported 156% increase in lipase yield of *Aspergillus niger* by UV mutagenic treatment.

The purpose of the present investigation is to enhance the production of industrially important enzymes like amylase, cellulase and lipase by subjecting the fungal strains to random mutagenesis by UV. The enzyme activities of selected fungal strains were compared between wild and UV mutants after exposing them to UV light.

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Materials and methods

The soil samples used in the present study were collected from sugarcane fileds of Eraiyur, Perambalur Dt., Tamil Nadu, India. The samples were kept in sealed polythene bags and stored at 4°C till the experimental period. The physico- chemical- parameters of soil samples such as pH, temperature, electrical conductivity and turbidity were determined by standard procedures (Eaton *et al.,*1995). The soil samples were serially diluted (Aneja, 1994) pour plated on rose bengal chloramphenicol agar (pH 6.8 ±0.2; Himedia, Mumbai). The plates were incubated at 30°C for 72 hrs. The fungal colonies appeared on the plates were isolated, purified and subjected to microscopic studies for identification, (Gillman, 1957).

Detection of enzyme activity

Screening for α-amylase activity: The isolates were grown on starch agar plates (pH 6.8 ±0.2; Himedia, Mumbai) and incubated at 30°C for 72 hrs. After 72 hrs, the fungal mat was removed from the plates and the plates were flooded with iodine solution.

Screening for cellulase activity: The isolates were grown on Czapek mineral salt agar medium (pH 6.8 ± 0.2; Himedia, Mumbai) with 0.5% carboxymethyl cellulose (CMC) as a substrate and incubated at 30°C for 72 hrs. After 72 hrs, the fungal mat was removed from the plates and the plates were flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide.

Screening for lipase activity: The microbial utilization of lipid compounds was detected in the lipase screening medium by the formation of clear zones.

α - Amylase assay (Peter Bernfield, 1985): The fungal isolates were inoculated into sterilized starch broth and incubated at 28°C in a rotary shaker for 6 days. After 6 days the culture filtrate (1 ml) was added to 5ml of 10mM calcium chloride ($CaCl_2$) solution and kept for 3 hrs at room temperature. The extract was centrifuged at 5400 rpm at 4°C for 20 minutes in a refrigerated centrifuge. The supernatant was used as an enzyme sources. Starch solution (1 ml) was pipetted out and added to 1 ml of diluted enzyme source, incubated at 27°C for 15 minutes. The reaction was stopped by the addition of 2 ml of dinitro salicylic acid reagent and was kept in boiling waterbath for 5 minutes. Potassium sodium tartarate (1 ml) was added then cooled in running tap water, Solution was made upto 10 ml and OD was measured at 560nm. Standard was prepared with 10-100 µg of maltose.

Cellulase assay (Mukherjee & Majimdar, 1993): The isolates were inoculated into modified Czapek's cellulose medium and incubated at 28°C in a rotary shaker for 6 days. Carboxymethyl cellulose (0.45 ml; 0.5%) was added to 0.5ml of enzyme extract, the tubes were incubated at 55°C for 15 minutes. DNS reagent (0.5ml) was added and kept in boiling water bath for 5 minutes. Potassium sodium tartarate (1ml) was added and cooled it. The mixture was made up to 5 ml by addition of distilled water. The OD was measured at 540 nm. The readings were compared with standard graph (glucose 50 - 100 µg).

Lipase assay (Safiar, 1999): The isolates were inoculated into the lipase production medium and incubated at 28°C in a rotary shaker for 6 days. Olive oil (250 µl) was transferred to a test tube containing 2 ml of phosphate buffer and sample (1 ml) was added to it. The mixture was vortexed for 15 seconds and incubated at 37°C in water bath under static conditions for 30 minutes. The reaction was stopped by adding 1 ml of conc. HCl and vortexed for 10 seconds, benzene (3 ml) was added and vortexed for 90 seconds, the aqueous and organic phases were allowed to separate. Benzene layer (2 ml) was withdrawn and transferred to a test tube containing 1 ml of cupric acetate and the mixture was vortexed for 90 seconds centrifuged at 5000 rpm for 10 minutes in refrigerated centrifuge. The organic layer was again withdrawn adjusted to estimate the liberated free fatty acids by measuring the OD at 715 nm.

Total protein estimation

The protein content of the samples was estimated by Lowry method (Lowry *et al.,* 1951). To the test sample 5 ml of alkaline solution (alkaline sodium carbonate; copper sulphate and sodium potassium tartarate) was added mixed thoroughly and allowed to stand at room temperature for 10 minutes. Folin - phenol reagent (0.5 ml) was added and kept at room temperature for 30 min. After 30 minutes the OD was measured. Bovine serum albumin was used as standard.

Strain improvement by UV mutation

The fungal strains were subjected to UV radiation (254 nm) to see its effect on enzyme activity. The isolates were cultured in potato dextrose agar plates and subjected to UV (254 nm) irradiation for 3, 5,10 and 15 minutes. The exposure was carried out at distance of 20.0 cm away from the center of the Germicidal lamp (UV light source). Immediately after exposure, mycelial discs (7 mm) were cut and inoculated into the specific enzyme production medium and incubated for 6 days. Mycelial discs from unexposed plates were inoculated into production medium and served as control. After incubation, various enzyme assays were performed by using the similar procedure.

Results and discussion

The fungal isolates from soil samples were identified and confirmed as *Aspergillus fumigatus, Penicillium chrysogenum* and *Verticillium terrestre* by the Mycology Division of the Centre for Advanced Studies in Botany, University of Madras. The physical parameter of the soil sample was analysed and

Table 1. Physical parameters of the soil sample

Parameters	Values
Temperature (°C)	30°C ± 1°C
pH	8.2 ± 0.2
Total dissolved solid (ppt)	0.11 ± 0.03
Turbidity (NTU)	1037 ± 11.0
Conductivity (ms)	0.23 ± 0.001

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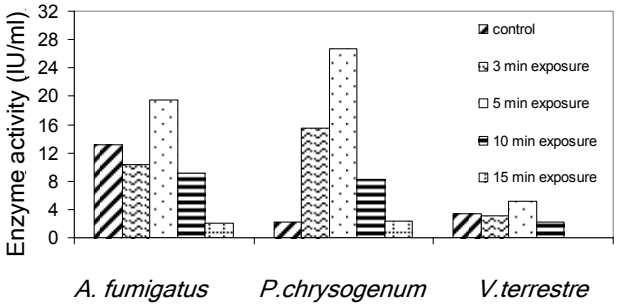
presented in Table 1. Screening for the production of amylase, cellulase and lipase by plate assays revealed the production of these enzymes by the formation of discolouration zones in agar plates.

The enzyme activity of the wild and UV-mutated fungi was assayed and presented (Fig. 1, 2 & 3). Strain improvement experiments are usually achieved through mutation, selection, or genetic recombination. UV rays are important inducers of strain mutations. The pyrimidines (thymine and cytosine) are especially sensitive to modifications by UV rays absorption. This may result in the production of thymine dimers that distort the DNA helix and block future replications (Sambrook *et al*. 2000). In many cases, mutations by UV are harmful, but occasionally it may lead to a better adapted organism to its environment with improved biocatalytic performance. The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool. The challenge is to isolate those strains which are true mutants that carry beneficial mutations (Parekh *et al*., 2000).

Fig.1. Effect of UV irradiation on amylase activity



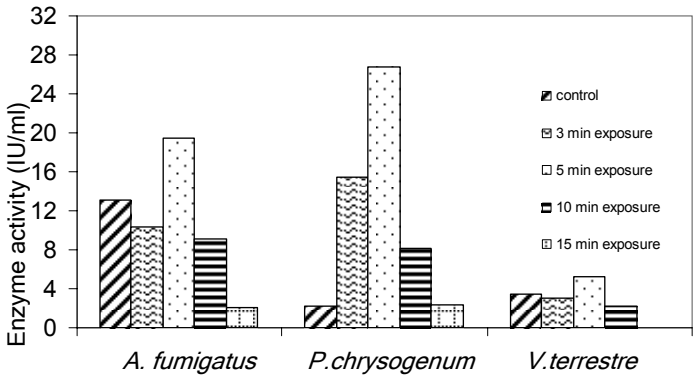
Each fungal strain responded differently in their production of enzymes after exposure to UV light. The mutant strain of *P. chrysogenum* showed highest amylase and lipase activity where as mutant *A. fumigatus* showed high cellulase activity compared to other fungi. Mutated strains of *A. fumigatus* and *V. terrestre* did not show any significant improvement in the production of lipase.

Of all the three fungi tested, *V. terrestre was* found to be poor producer of all the enzymes both in wild and mutated condition. This may be attributed to their genetic make up and subsequent physiological conditions (Parekh *et al*., 2000).

The effect of UV exposure on fungal strains in our studies showed that exposure of these fungi under UV for 5 minutes significantly enhanced the activities of all the enzymes tested. It is in conformation with the earlier work reported by Sandana Mala *et al*. (2001) where exposure of *A. niger* to 4 min improved its lipase activity several fold than 12 min exposure. Bapiraju, *et al.* (2004) reported that UV induced mutant of *Rhizopus* sp. showed higher lipase activity than the parent strain. Strains of *Trichoderma harzianum* with reduced production of cellulase-complex enzymes [beta]-glucosidase and endoglucanase were used for UV-light mutagenesis. In tests for rhizosphere competence, two of the cellulase minus strains were found to have significant enhancement after UV mutagensis (Melo *et al*., 1997),

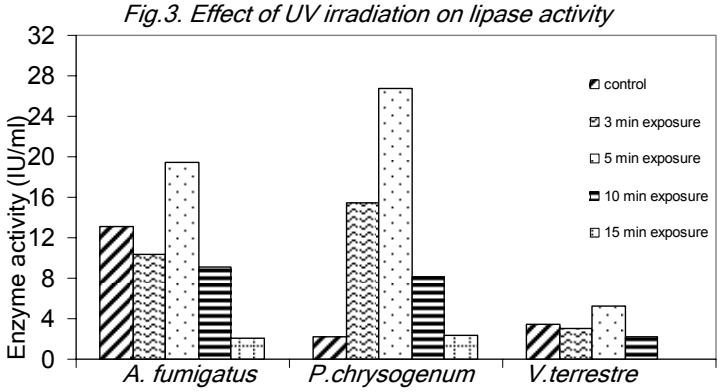
Our studies confirmed that long period of exposure to UV light (>5 minutes) proved to be detrimental to the enzyme production. With increase in exposure time, the production of all the enzymes was reduced. These results are in conformation with an earlier study with *A. niger* JGI 24 in which increased time of UV exposure resulted in decreased alpha amylase production. On 20 min exposure to UV, amylase activity was found to be zero (Varalakshmi *et al*., 2009). Gocke and Kiefer (1978) have reported that the synthesis of two inducible enzymes in UV irradiated cells was determined during 8 h post-irradiation incubation. In contrast to the reduction of synthesis shortly after irradiation, the effect after a longer period of incubation depends on the radiation sensitivity of the strain.

Fig.2. Effect of UV irradiation on cellulase activity



Enhancement of amylase, cellulase and lipase activity was observed in all the strains after exposure to UV radiation. However *V. terrestre* responded very poorly. This effect is attributed to possible changes in the promoter zones of the genes coding for these enzymes due to the ultraviolet exposure. The radiation might have deregulated the transcription of the mRNA corresponding to these enzymes, leading to an increased production. Since ultraviolet radiation affects mainly the hydrogen bonds of

Fig.3. Effect of UV irradiation on lipase activity



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pyrimidic bases (cytosine + thymine) the most vulnerable regulatory sequences must have been those containing the highest concentration of C + T (Soledad De Nicolás-Santiago *et al*., 2006). It can be assumed that amylase, cellulase and lipase production might be under the control of such regulon. Further work is required to check the stability of enhanced production of these enzymes.

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