EFFECT OF UV RAYS AND CHEMICALS ON CELLULASE PRODUCTION OF *ASPERGILLUS NIGER* IN SUBMERGED FERMENTATION USING RICE HUSK AS SUBSTRATE

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Abstract

Cellulases are employed in variety of sectors such as paper, textile, pharmaceuticals, food, leather and detergent. Microorganisms secrete cellulases while growing on cellulosic materials that have been proved effective in various industries. The efficiency of microbial cellulase production can be improved by treating microbes with different mutagens. In the present study *Aspergillus niger* spores were exposed to UV radiation, ethidium bromide, and nitrous acid and cellulase activity was assessed after each treatment. Wild strain *Aspergillus niger* was capable toproduce 0.18 IU/ml/min CMC-ase and 0.27 IU/ml/min FP-ase activities. Short-wave UV light (254 nm) exposure for 30 minutes resulted in a 39% increase in CMC-ase and 33% increase in FP-ase activities, while long-wave UV light (366 nm) exposure resulted in a 33% increase in both CMC-ase and FP-ase activities, compared to the wild strain. CMC-ase and FP-ase activities after 0.25 mg ml^{-1} ethidium bromide treatment increased about 22% and 16% respectively than the wild strain whereas 0.25 M nitrous acid treatment gave 100% increase in CMC-ase activity and a 48% increase in FP-ase activities. In recent research, nitrous acid has been found more effective in raising cellulase activity to a greater extent as compared to UV exposure and ethidium bromide treatment. This study suggests that mutagenesis of fungal strain could provide a new choice for the processing of bioresources in the future to produce enhanced cellulase production.

Key words: *Aspergillus niger*, cellulases, submerged fermentation, mutagens, UV radiations.

Introduction

Lignocellulosic biomasses are inexpensive natural resources on earth and its valorization is key component for sustainable development (Gusakov, 2013). These are basically composed of lignin, cellulose and hemicellulose. The cellulose and hemicellulosic contents of the lignocellulosic biomass can be transformed into value added products such as fermentable sugars, bio ethanol and organic acid by using cellulolytic enzymes (Ahorsu et al., 2018, Jiménez-Quero et al., 2020). High cost of cellulases are commonly considered as one of the main obstacles in the economic valorization of lignocellulosics substrates into fermentable sugars (Wu et al., 2011), but it can be made more cost-effective by employing microbial enzymes. Many species of fungi secrete cellulase enzymes while degrading plant biomass (Suto & Tomita,

2001). On lignocellulosic substrates in submerged fermentation, filamentous fungi particularly *Aspergillus* and *Trichoderma* spp. have been reported to produce cellulases (Baig & Saleem, 2012, Kuhad et al., 2016).

All four classes of cellulase enzymes have been identified in *Aspergillus species* (Vries & Visser, 2001). Over-production of cellulases can be achieved by mutagenizing high cellulase secreting strains. (Yu et al., 2020, Hidayati et al., 2021). Several approaches including physical mutagens (ultraviolet, X and other radiations) and/or chemical mutagens (nitroglycerin, ethyl bromide, nitrous acid etc.) are often employed to improve strains (Chand et al., 2005, Raghuwanshi et al., 2014, Peng et al., 2021). Vegetative hyphae and spores are commonly treated with various mutagens in order to obtain better mutants among the progeny that survive (Chand et al., 2005).

Higher cellulase activities have been reported previously from the mutants obtained after exposure of fungal conidia to ultraviolet (UV) radiations (Parekh et al., 2000). Previous studies have shown increase in cellulase production by using mutants obtained after UV exposure as compared to the wild strain *Aspergillus niger* (Kang et al., 1999;Jafari et al., 2017). Chemical mutagens may induce mutations within a sequence. Chemicals such as nitrous acid have been commonly used to induce mutations (Sidorkina et al., 1997, John & Madhavan, 2008, Abdullah et al., 2015).

Previous studies have shown increase in the rate of enzyme production by growing fungi with mutagens at sub-lethal concentrations (Rudravaram et al., 2003). Mutation based strain improvement is a successful strategy for increasing the cellulase production efficiency of various microorganisms, but it is mostly a trial and error procedure that takes a long time and effort (Iftikhar et al., 2010). The objective of this study was to observe how chemicals and UV treatments affected *Aspergillus niger's* cellulase production efficiency. The cellulase activities of the treated ones were compared to the wild strain in order to get hyper cellulase producing mutants.

Materials and Methods

Fungal strain: Pure culture of *Aspergillus niger* (FCBP 0002) was acquired from the fungal culture bank, Institute of Agricultural Sciences at the University of the Punjab, Lahore and used throughout the study.

Revival of Fungal strain: The wild strain of *Aspergillus niger* was refreshed from stock culture by streaking on PDA plates and slants under aseptic conditions and incubated at 30°C for 4-7 days.

Spore suspension: Spore suspension was prepared from 7 days old culture slant by slightly scratching the spores on the agar surface with a sterile needle and by adding 5 ml sterilized saline (0.5% NaCl) solution.

Spore counting: The Neubauer haemocytometer was used to calculate the spore

concentration. For production of cellulases, a concentration of $2.25x10^6$ spores per ml was used.

Screening: To study the cellulolytic activity of wild and treated strains of *Aspergilus niger,* 100 ul of spore suspension was injected into the well created in the center of CMC agar plate and were kept at 28℃ for two days. Plates were then stained for 30 min with 1% Congo red dye and destained for 15 minutes with 1 M NaCl solution. (Onsori et al., 2005).

Fermentation medium: Vogel's medium (pH 5) was employed as the fermentation medium, which contained (g/100ml): KH_2HPO_4, 0.50; $(NH_4)_2SO_4$, 0.40, Trisodium citrate, 0.25; NH_4NO_3, 0.20; yeast extract, 0.20; $MgSO_4$. $7H_2O$, 0.02; Peptone, 0.10; and 2 g rice husk.

Ultraviolet treatment: Forty-two PDA plates were prepared by adding 0.1 ml spore suspension on each. By using a germicidal UV lamp in a black box, half prepared plates were exposed to long wavelength U.V light (366 nm) and half plates were subjected to short wavelength U.V light (254 nm) from a distance of 11 cm (Adsul et al., 2007) for 10-60 minutes. Plates used as control received no UV exposure. After treatment plates were incubated at 30℃ for 4-5 days while being wrapped in black paper to ensure mutation. The experiment was conducted in triplicate.

Ethidium bromide treatment: To begin the slight germination of spores, 1ml of spore suspension (2.25×10^6 spores ml^{-1}) was added to 9 ml of fermentation medium and incubated for 4 hours. Fifteen samples were prepared (triplicate for each treatment) in such manner. After centrifuging the tubes, the supernatant was discarded in sterile environment then samples were treated with 1 ml of 0.1, 0.25, 0.5 and 1 mg of EtBr solution respectively, control samples did not receive the EtBr solution. Samples were shaken for 30 min at 30ºC in an orbital shaker (Chand, 2005). After treatments, these underwent a 15 min, 10,000 rpm centrifugation. Supernatant was removed, discarded and washed with sterile distilled water and 0.1 ml of each treated suspension were plated on PDA plates and incubated at 30ºC for 4 days. Control suspensions were prepared in the same manner except the addition of EtBr solution.

Nitrous acid treatment: Different concentrations of $NaNO_2$ (0.07, 0.1, 0.25, 0.5, 1 M) in acetate buffer (0.2 M, pH 4.5) were prepared. 1ml of spore suspension ($2.25x10^6$ spores ml^{-1}) was added to 9 ml of fermentation medium in a centrifuge tube and incubated for 4 hours to start the slight germination of spores, eighteen samples were prepared in this way (triplicate for each treatment). Following centrifugation, each tube received a freshly made 0.07, 0.1, 0.25, 0.5, and 1 M solutions of $NaNO_2$ in acetate buffer, except the control tube, which received only the acetate buffer (Carlton & Brown, 1981). The solution was thoroughly shaken for 10 minutes then the reaction was terminated by adding 4ml of phosphate buffer (0.2 M, pH 7.1), centrifuged at 10,000 rpm, and the supernatant was discarded (Azin & Noroozi, 2001). The remaining residues of nitrous acid

were then removed from treated spores by centrifuging at 10,000 rpm, while being rinsed with distilled water. After suspending in saline, 0.1 ml of spores were plated on PDA plates and incubated at 30ºC for 5-7 days. Control was treated identically except for the omission of $NaNO_2$ treatment.

Fermentation: When growth appeared on PDA plates of treated and untreated samples, mycelial discs (8 mm) were cut and introduced into fermentation medium. Flasks were kept on an orbital shaker at 30ºC for seven days.

Sample collection: During fermentation period, small amount of sample was drawn aseptically from each flask after every 24 hour for seven days and cellulolytic activities were estimated in terms of FP-ase and CMC-ase units.

Enzyme assays: To determine the Filter paper activity, 0.5 ml of each culture supernatant was taken in test tube, incubated with a rolled '1 by 6 cm' filter paper strip (Whatman No. 1) and 1 ml of 0.05 M citrate buffer (pH 5.5), for 30 min at 50ºC Vijayaraghavan and Vincent (2012). After that 3.0 ml of DNS reagent was added in test tube, throughly mixed and boiled for exactly 5 minutes in a water bath. The generated colour is measured and compared to the spectro zero at 540 nm. International units were used to calculate and express cellulase activity. One unit of FP-ase activity corresponded to the amount necessary to form 1 milligram of glucose per minute from filter paper at 50 degrees Celsius.

CMC-ase activity was measured as described by Ariffin et al., (2006), 0.5 ml of each sample along with 0.5 ml of 1% carboxymethyl cellulose substrate solution was taken in a test tube, covered with aluminium foil and incubated for 30 minutes at 50ºC. After incubation 3.0 ml DNS reagent was added, mixed thoroughly, boiled for exactly 5.0 min in a water bath and absorbance was measured at 540 nm.

Statistical analysis

All analyses were carried out in triplicate and the results were presented as mean. The acquired data was subjected to a one-way analysis of variance, and the Duncan multiple range analysis was used to separate the means that were significantly different. The test p-value is 0.05.

Results

Cellulolytic activity of wild strain *A. niger*

The CMC-ase and FP-ase activities of wild strain *A. niger* were 0.18 IU/ml/min and 0.27 IU/ml/min respectively at third day of incubation.

Cellulolytic activities after UV treatment

CMC-ase, FP-ase activities on third day of incubation and diameter of clearing zones of strain *A. niger* after 10-60 min UV (366nm and 254 nm) treatment shown in the table 1 and 2. Maximum CMC-ase and FP-ase activities observed after 30 minutes of UV exposure.

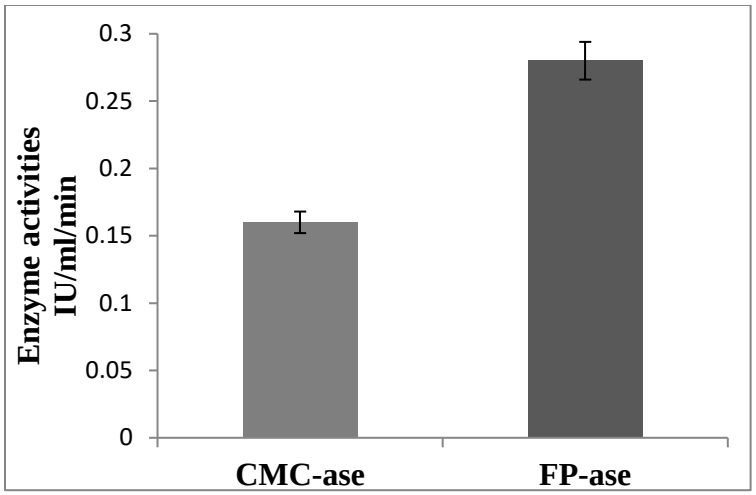

Figure 1. Cellulolytic activity of Parental strain *A. niger*

Table 1: CMC-ase, FP-ase activities and clearing zones diameter on CMC agar plates *Aspergilus niger* after 10-60 min UV (366 nm) exposure

Strains	Enzyme activities (IU/ml/min)		Diameter of clearing zone (mm)
	CMC-ase	FP-ase	
Wild type/ control	0.18^{bc}±0.02	0.27^{cd}±0.02	29^{b}±2.0
UV: 10 min	0.17^{bc}±0.02	0.26^{d}±0.02	28^{bc}±1.6
UV: 20min	0.20^{b}±0.01	0.32^{b}±0.01	30^{b}±1.8
UV: 30 min	0.25^{a}±0.03	0.37^{a}±0.02	40^{a}±1.6
UV: 40 min	0.19^{b}±0.03	0.29^{c}±0.02	38^{ab}±1.2
UV: 50 min	0.15^{c}±0.04	0.19^{e}±0.01	15^{d}±2.4
UV: 60 min	0.09^{d}±0.02	0.17^{ef}±0.01	18^{c}±1.7
$LSD_{0.05}$	0.04	0.03	2.42
CV	1.71	0.81	0.71

LSD: Least significance deviation
CV: Coefficient of variance
[The values having different letters are statistically significant (P=0.05) according to Duncan's multiple range test]

Table 2: CMC-ase, FP-ase activities and clearing zones diameter on CMC agar plates of *Aspergilus niger* after 10-60 min of UV (254 nm) irradiation

Strain	Enzyme Activities (IU/ml/min)		Diameter of Clearing zone (mm)
	CMC-ase	FP-ase	
Wild type/ control	0.18^{c}±0.02	0.27^{cd}±0.02	29^{d}±2.0
UV: 10 min	0.19^{bc}±0.02	0.28^{c}±0.02	35^{c}±2.4
UV: 20min	0.21^{b}±0.01	0.29^{c}±0.03	34^{cd}±1.6
UV: 30 min	0.24^{a}±0.02	0.36^{a}±0.03	44^{a}±1.6
UV: 40 min	0.24^{a}±0.01	0.32^{b}±0.01	38^{b}±1.6
UV: 50 min	0.21^{b}±0.03	0.24^{d}±0.03	37^{b}±2.0
UV: 60 min	0.18^{c}±0.02	0.20^{e}±0.02	36^{bc}±2.8
$LSD_{0.05}$	0.03	0.03	2.8
CV	1.06	0.92	0.64

LSD: Least significance deviation
CV: Coefficient of variance
[The values having different letters are statistically significant (P=0.05) according to Duncan's multiple range test]

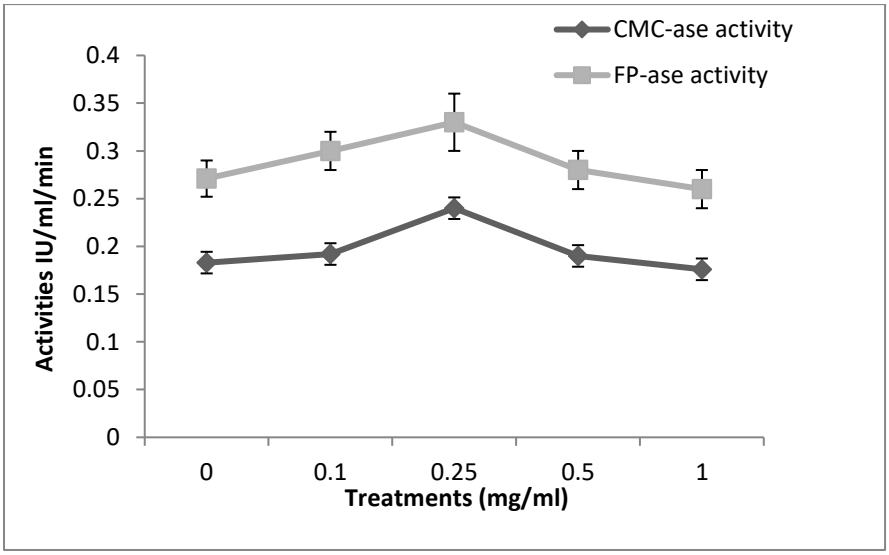

Figure 2: Comparison of cellulase activities between parental strain *A. niger* and EtBr treated cultures

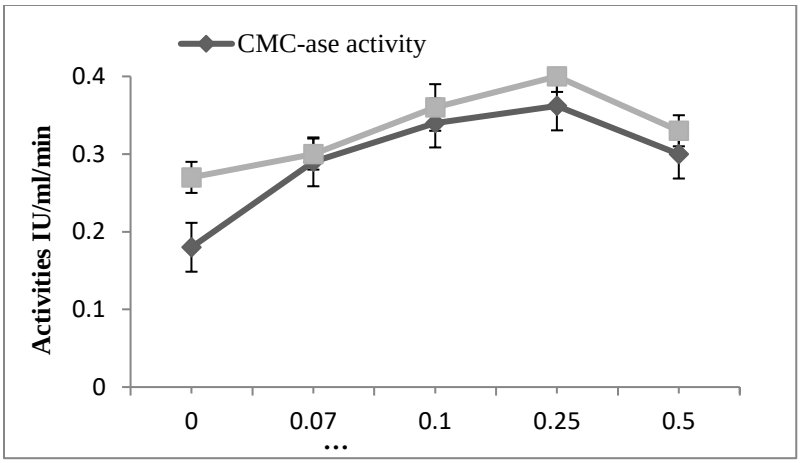

Figure 3: Percentage comparison between parental strains of *Aspergilus niger* and its nitrous acid treated culture for CMC-ase and FP-ase activities

Cellulolytic activities after treatment with Ethidium bromide

Comparison of CMC-ase and FP-ase activities after treatment with different concentrations of ethidium bromide shown in figure 2. Maximum cellulase activities observed at 0.25 mg ml^{-1} concentration.

Cellulolytic activities after treatment with nitrous acid

Percentage comparison of CMC-ase and FP-ase activities after different concentrations of nitrous acid shown in figure 3. Highest cellulase activities observed at 0.25 mg ml^{-1} nitrous acid concentration which has shown 47 mm of enzyme clearance zone on petri plate.

Discussion

The application of mutagens and hyper producing culture selection has been found effective in increasing cellulase production by using microorganisms (Vu et al., 2011, Raghuwanshi et al., 2014). Vu et al., (2011) found 8.5-fold increase in cellulase yield by using mutant *Aspergillus* sp. as compared to wild strain grown on medium containing wheat bran. In the present study spores of wild strain *Aspergilus niger* was physically exposed to UV light at 254 nm and at 366 nm for 10-60 min and treated with chemicals like ethidium bromide and nitrous acid. The efficient mutant strains was selected on the basis of plate screening followed by enzyme activities in submerged fermentation and results were compared with wild type strains. Exposure to UV light (366nm) for 30 min. has resulted increase in activity of CMC-ase and FP-ase by 33% while the remaining mutants obtained after UV treatment exhibited either similar or reduced activities (Table 1). Hidayati et al., 2021 obtained mutants by treating *Trichoderma sp.* (PK1J2) to gamma rays at 300 Gy, which show 90% increase in endoglucanase, 50% in FPase and 30% increase in βglucosidase activity as compared to parental strains.

During UV exposure (254 nm), maximum increase in cellulase activity was observed in the mutants obtained after 30 min and 40 min exposure times, however prolonging the exposure time beyond this resulted in decrease in cellulase activity. Mutant get after 30 min exposure time showed 39% increase in CMC-ase activity and 33% increase in FP-ase activity and mutants obtained after 40 min of exposure showed 33% increase in CMC-ase activity and a 20% increase in FP-ase activity as compared to the parental strain, while the remaining mutant exhibited similar or reduction in activity (Table 2).

UV radiations stimulates the electrons, which causes additional bonds to form between nearby pyrimidine in DNA (Sambrook and Russell, 2001). Zones of diffusion of around 40 mm and 44 mm was recorded as compared to the 29 mm zone of diffusion of the parental strain (Supplementary Figure 1). Liu et al., (2011) reported 3.96 times higher cellulase activity by treating the *Aspergillus nidulans* with UV light. Kumar, (2015) reported 57.4% increase in endoglucanase and 12.8% increase in β-glucosidase activities after developing mutant by using UV rays. These result have shown that UV exposure (30 and 40 minutes) has helped in enhacing the cellulase activities.

In the current study, treatment of spores with 0.25 mg ml^{-1} ethidium bromide (Figure 2) led to an increase in the activity of CMC-ase and FP-ase activities that was around 33% and 22% higher than the wild strain. According to Gomez-Diaz et al., (1997), treatment of cells with ethidium bromide cause physiological stress and modification of DNA that can lead to hyper production of several enzymes. The data obtained from chemical treatment revealed that nitrous acid has been proved more effective than the parental strain, as it increased cellulase activity by 100% (Figure 3).

This might be due to the fact that nitrous acid is strong mutagenic agent that can cause oxidative deamination of nitrogenous bases of DNA (Sidorkina et al., 1997) that might result in enhanced cellulase production. . John and Madhavan, (2008) used nitrous acid to induce mutation in Lactobacillus strains, they found that it was more beneficial than insertion mutations. When compared to wild strains, the mutant strains exhibit greater growth and higher lactic acid production in a shorter fermentation duration. Abdullah et al., (2015) found 1.7 fold high enzymatic activity in mutant produced by treating with nitrous acid as compared to parental strain. Likewise Hamad et al., (2001), described that chemical treatments are more effective than UV radiation in inducing high-level mutations. Chand et al., (2005) produced mutants, that exhibit 5-fold rise in β-glucosidase and 1 fold increase in both CMCase and FP-ase, after mutagens treatments.

Peng et al., (2021) reported that mutagenesis followed with improved laboratory evolution is a viable way to boost cellulase

production from fungi. Thus cost-effective cellulases can be produced by inducing mutation and employing lignocellulosic substrates.

Conclusion

Aspergillus niger was mutated by using UV light, ethidium bromide and nitrous acid. Mutagenesis and selection of cellulase over producing mutants has been found effective for production of significant yield of cellulases. Both physical and chemical mutagens have been found effective in increasing cellulase yield but nitrous acid has significantly contributed to the excessive production of cellulases. By employing lignocellulosic wastes and mutagens, cost-effective production of cellulases can be achieved which could be used in industries and bioethanol applications. This strategy could be an alternative of conversion of lignocellulosic waste into useful products.

Conflict of interest

Authors declare no conflict of interest.

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