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Research Article

Physical and chemical mutation of cellulase producing fungi *Trichoderma viride*

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Abstract

Cellulase producing fungi *Trichoderma viride* was cultured and fermented on the solid state wheat bran fermentation medium. The cellulase activity of *T. viride* fermented in this solid state were 72 hrs, 5.0 and 25ºC, respectively, screening of the strain was Carboxy methyl cellulose sodium (CMC-Na) and Congo red were used to stronger ability to produce enzymes. The wild strain was produce enzyme activity was (1350 U/g), the wild compound mutagenesis by physical and chemical method like microwave, ultraviolet and EMS. The mutation strain showed higher level of cellulase activity (1461.24 U/g) and (1543.45 U/g) respectively.
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Keywords: *Trichoderma viride*, Microwave treatment, UV irradiation, EMS treatment and Cellulase activity.

Introduction

The industrial revolution generated an increasing need for energy that was fueled mainly by fossil fuels. With the progress of industrialization, petroleum was in great demand. As a consequence, serious environmental problems have arisen (Aristidou and Penttila, 2000; Jeffries and Jin, 2000; Zaldivar *et al.*, 2001). The recent development of biotechnology, there has been vast interest to use cellulose - digestive microorganisms to convert cellulosic biomass to glucose that can be used in different applications. Many microorganisms that produce various cellulolytic enzymes have been studied for several decades. Bacteria compare with fungi capable of producing extracellular enzymes responsible for degradation of cellulose are known, some of them being highly cellulolytic, which include species of *Aspergillus* sp., *Trichoderma* sp., *Sclerotium* sp. and are also being considered for commercially exploitation (Yeoh *et al.*, 1984 and Pointing, 1999). The genus of *Trichoderma* has been especially famous for producing cellulolytic enzymes with relatively high enzymatic activity (Miettinen - Oinonen and Suominen 2002).

However, main problem in cellulase production by fermentation is the utilization of expensive substrates. These technologies have hardly been realized in practice because of their high running cost and low yields of this enzyme. Therefore, investigation on the ability of microbial strains to utilize inexpensive substrate and improvement of enzyme productivity is thus an important object for research. (Beg *et al.*, 2000; Senthilkumar *et al.*, 2005). Wheat bran, the agricultural by-product is a cheaply available resource in India, and has potential as an industrial fermentation substrate. In the current work, wheat bran was used as substrate in order to reduce the cost of cellulase production. The aim of this present study was to obtain high levels of extracellular cellulases by mutating the *Trichoderma viride* using physical and chemical methods.

Materials and Methods

Fungal isolate

Trichoderma viride strain was isolated from paddy rhizosphere soil samples, the colonies were screened for carboxymethyl cellulase (CMC) agar plates were flooded with 1% Congo red solution for 1 hour then de-stained with 1M NaCl solution for 15 minutes. The screened culture was maintained on potato dextrose agar plates at 25ºC for 7 days, spores were developed and stored at 4ºC.

Production media preparation

The production media contained 20 g of wheat bran; 0.5% glucose; 0.2% NH_4NO_3; pH 4.5; and maintained 40% moisture content in 250 ml conical flask at obtained a 1 – 2 cm layer of mixture without free liquid. The flask was sterilized by autoclaving, cooled at room temperature, mixed thoroughly then added 1 ml spore suspension and incubate the flask for 7 days at 25ºC after the incubation calculate the enzyme yield.

Cellulase production

Cellulase enzyme production was carried out by using DNS (Dinitrosolicyclic acid) method of Miller (1959). One ml of substrate filtrate was collected from the production medium by centrifugation. 0.8 ml of enzyme substrate (CMC); (0.25 g CMC was add 24.75 ml of 0.015M sodium acetate buffer pH 4.8) was taken in a test tube than add 0.8 ml of culture filtrate. Test tubes were incubated in water bath 50°C for 10 min. After incubation, 2.4 ml of DNS was added in the test tube and boiled for 10 min. After boiling, immediately the contents were allowed to cool at room temperature. After cooling, the samples absorbance was read at 540 nm in spectrophotometer. The amount of reducing sugar was determined using a standard graph.

Mutagenesis with microwave treatment

The screened fungal spores were suspended with 10 ml of distilled water (5×10^5 spores/ ml), spore suspension was transferred to the sterilized petriplates and exposed to microwave oven (maximum power: 700 W; microwave frequency: 2450 MHz) was used to radiate the single spore suspensions. The spores were heated with 15, 30, 60, 90, 120 and 150 sec. After that heating immediately the contents was allowed to cool at room temperature (Xing-hua Li *et al.*, 2009)

UV mutagenesis

Spores were exposed to ultraviolet irradiation for 40 min with 5 min time interval under UV lamp having a wavelength of ƛ = 254 nm and 220 V at 50 Hz. The distance between lamp and suspension was adjusted to 20 cm for each trial (Hamad *et al.*, 2001). After the time intervals, 200 µl of the conidial suspension was transferred to PDA agar plate with the addition of 0.1% Triton X-100 and L-sorbose as colony restrictors. Plates were then incubated at 25 ± 2°C for 7 days, and mutant colonies were replicated on the screening medium.

Mutation by EMS (Ethyl methane sulfonate)

Spores were washed with 0.05% Tween 80 solution and they were counted under the microscope. Spore suspension was adjusted to 10^8 spores/ml. Random mutagenesis was appropriate dilutions of 50, 100, 150, 200 and 250 were prepared from 300 µg ml^{-1} of ethyl methyl sulfonate (EMS) treatment followed by UV-irradiation according to the method described by Morikawa *et al.* (1985) and Asdul *et al.* (2007) with modifications it was kept at room temperature for 24 hrs followed by UV-irradiation at 254 nm for 20 min corresponding to a lethal rate of 84%. 200 µl of the treated spore suspension was subsequently spread on the screening plates.

Result and Discussion

Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity (Lynd *et al.*, 2005; Penttila *et al.*, 1986; Lowe *et al.*, 1987; Tomme *et al.*, 1988). Enzymes produced microorganisms are commercially available for agricultural use. However, *Trichoderma* is thought to be cellulase producers, and crude attempts to use these enzymes in the degradation of cellulosic wastes have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition of the enzymes. The selection of these cellulase producing strains was based on the larger diameter of clear zone surrounding the colonies on plate screening medium as compared to wild strain. The range for UV and chemical mutagenesis was selected based on the previous studies published (Li XH *et al.*, 2010; Shafique *et. al.*, 2011; *Zaldívar*, M *et al.*, 2001). Improvement of strain by mutations is an old age culture used for successful method (Kumakura *et al.*, 1984; Chadha and Garcha, 1992; Hayward *et al.*, 2000; Bailey and Tahtiharju, 2003; Villena and Gutierrez-Correa, 2006).

The fungal strain *T. viride* was mutagenized and genetically modified to develop a mutant strain capable of exhibiting high levels of cellulase activity because fungal strains have a unique character to pass over the environmental stress including chemical and irradiative mutagenesis and are highly susceptible to various physical as well as chemical mutagenic agents. This practice has become a routine in the field of biotechnology to develop a mutant through random mutagenesis (Azin and Noroozi, 2001; Mohsin, 2006). In the present study, wild strain was isolated by its ability to grow on CMC-Na agar plate and hydrolytic zone was subjected to successive mutagenic treatments with microwave and ultraviolet. The wild *T. viride* strain spores were exposed to microwave (maximum power: 700 W; microwave frequency: 2450 MHz) for different times (15 – 150 sec), using the ice bath to eliminate heating effect of the oven on the spores. The lethality rate of *T. viride* spores exposed to microwave responding under varying times is illustrated. With 90 sec of exposure to microwave, the lethality rate of *T. viride* spores crossed 80% and after 150 sec it was approximately 100%. It suggested that the *T. viride* spores were very sensitive to microwave. However, the rate of positive mutation as to the survival colonies increased as the time increases. After the microwave treatment, spores were exposed to ultraviolet irradiation for 40 min with 5 min time interval under UV lamp having a wavelength of ƛ = 254 nm and 220 V at 50 Hz. The distance adjusted to 20 cm. the spores were transferred to screening plate. The efficient mutants were further assessed in fermentation medium. The strain designated as exhibited maximum cellulase activity 1461.24 U/g at an exposure time of 25 min of UV irradiation (Figure - 1).

Furthermore, improvement in enzyme production by EMS mutagenesis was carried out to isolate hyper producer mutant derivatives of *T. viride* using different doses of EMS (50 - 300 µg/ ml) for 20 min of UV irradiation. After the chemical mutation, spores were ability to hydrolyze the cellulose on agar plates more efficiently and significantly in comparison to parental. These selected mutants were subjected to quantitative analysis by DNS method. The 200 µg/ ml of EMS concentration (Figure - 2) evidenced the most promising concentration as it illustrated significantly higher cellulase activity (1543.37U/ g) than the wild type (1350.72) by mutant strain.

The perusal of data obtained from chemical treatment revealed that EMS proved more effective as it enhanced cellulase activity to the greater extent as compared to UV. This might be due to the fact that EMS is strong mutagenic agent and induces permanent changes in DNA structure. Similar research was conducted by Hamad *et al.* (2001), Mohsin (2006), Shazia Shafique *et al.* (2011) where they reported that chemical treatment is more efficient in inducing high level mutations as compared to UV irradiation. The mutation of the strain was stable.

Figure - 1: cellulase activity of mutant strains mutated by the compound effect of microwave and ultraviolet

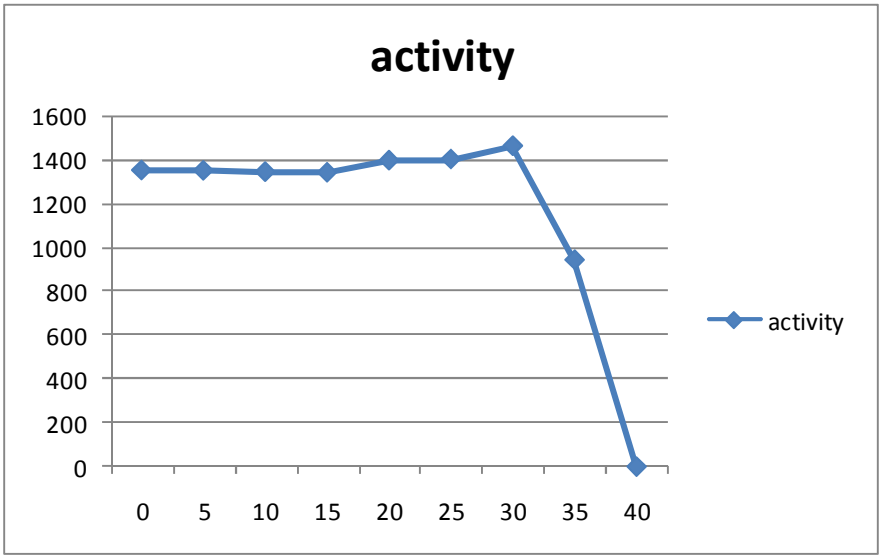

Figure - 2: Cellulase activity of mutant strains mutated by the compound effect of EMS and ultraviolet

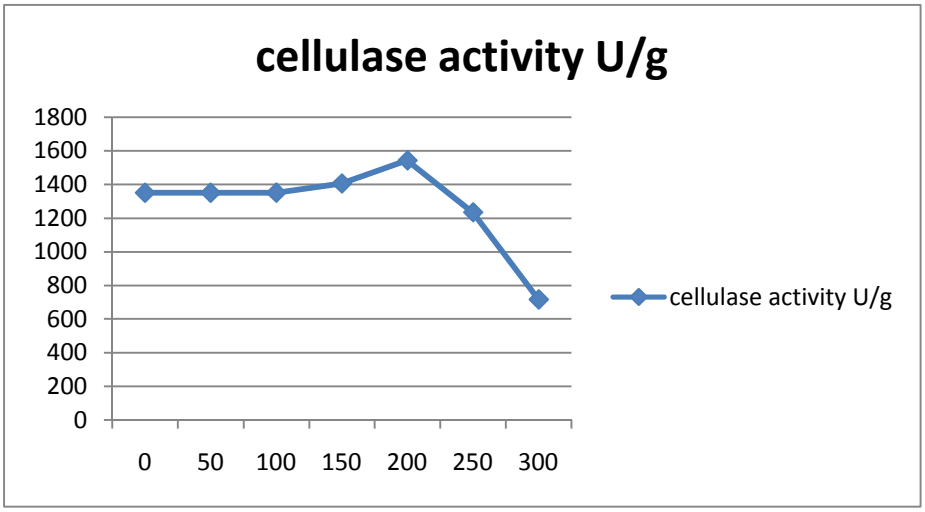

Table - 1: Comparison of cellulase activity on wild and mutant strains

Sample	Activity U/g	Protein mg/g	Spec. activity U/mg of protein
Wild strain	1350.72±0.86	38.57±0.29	35.01
Physical mutation	1461.24±0.27	30.70±0.13	47.59
Chemical mutation	1543.45±0.15	31. 63±0.23	48.79

Table – 2: Cellulase activities of mutant strains for 12 generations

Generation	Cellulase activity U/ g
1st	1543.37±0.15
2nd	1543.16±0.26
3ed	1542.73±0.50
5^{th}	1543.52±0.43
7^{th}	1542.22±0.27
9^{th}	1542.29±0.77
12^{th}	1543.08±0.83

In this study, both physical such as UV irradiation, and chemical, EMS was employed on an isolate *Trichoderma viride* to obtain mutant strains. Both of these treatments affected the morphological and genetically of fungal mutant strains. These mutant strain were enhanced the production of acid cellulase on SSF. Saving the economy of the country and meeting industrial sector demand.

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