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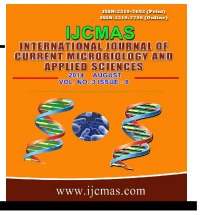

Original Research Article

Cellulase production and purification of mutant strain *Trichoderma viride*

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A B S T R A C T

Trichoderma viride under solid state fermentation technique using cheap and an easily available agricultural waste material, wheat bran, was used to produce cellulase enzyme. The improved mutants of *Trichoderma viride* were obtained by physical and chemical mutation using UV and microwave radiation followed by EMS (Ethyl methane sulfonate). The enzyme recoveries were observed as 13.38 mg/g and 12.46 mg/g and their activities 1391 U/g and 1525.34 U/g for ammonium sulphate and acetone respectively, and their corresponding specific activities were 144 U/mg and 111.63 U/mg.

Keywords

Trichoderma viride, Mutation, Cellulase activity

Introduction

Life on earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component**.** Today cellulase is great attention due to the large industrial application. Cellulase enzyme is used in many industries for the preparation of medicines, waste treatment, food production, paper industries, perfumes, backing etc,. (Beauchemin et al., 2003). Many filamentous fungi are known to produce the enzyme cellulase, Commercial cellulase preparations are from most often used species *Trichoderma viride* and *Trichoderma ressei* as they are popular as it contains high activities of both exo-glucanase and endo-glucanase but low levels of *β*-glucosidase, (Leite et al.,2008). The enzymatic saccharification of cellulose so far has not been reached to the level of conversion of cellulose to glucose by the microbial enzymes. Therefore, the strains have been mutagenized and genetically modified to obtain an organism capable of producing high levels of cellulase (Mandels and Andreotti, 1978; Durand et al., 1988; Szengyel et al., 2000). The several approaches to increase enzyme activity, like physical and chemical mutation to obtain enhanced cellulase producing strains have been given a high priority (Kotchoni and Shonukan, 2002). Previously used physical mutagens include Ultraviolet light, microwaves and high energy ionizing radiations (Li XH et al., 2010; Xu F et al., 2011). Chemical mutagenesis had been carried out by treatment with Nitrosoguanidine, Ethyl Methane Sulphonate, Diethyl sulphonate and colchicines (Bhargavi and Singara, 2010).

Improved strains were success for industrial application of cellulase production. Therefore, aim of the present study is to investigate high level production of extra cellular carboxymethyl cellulase (CMC) through mutating *Trichoderma viride* by combining both the physical and chemical methods, and purification of cellulase by Ammonium sulphate and cold Acetone methods.

Materials and Methods

Fungal isolate

Trichoderma viride strain was isolated from paddy rhizosphere soil samples, the colonies were screened for (CMC) carboxymethyl cellulose agar plates. The 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.

Mutagenesis with microwave treatment

The spores from the screened fungal culture were mutated with microwave oven (maximum power: 700W; microwave frequency: 2450MHz) used to radiate the 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

Microwave treated culture was suspended with 10 ml of distilled water (5×10^5 conidia/ml), conidial suspension was transferred to the sterilized Petri plates and exposed to ultraviolet irradiation for 40 min with 5 min time interval under UV lamp having a wavelength of ג = 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 done with appropriate dilutions of 50, 100, 150, 200, 250 and 300 µg ml -1 of ethyl methyl sulfonate (EMS) stock followed by UV-irradiation according to the method described by Morikawa et al., (1985) and Asdul et al., (2007) with modifications. After the mutagenesis, it was kept at room temperature for 24 h. About 200 µl of the treated spore suspension was subsequently spread on the screening plates. The mutagenesis strain was calculated the enzyme production up to 12 generation.

Production media preparation

The production media contained 20 g wheat bran; 0.5% glucose; 0.2% NH_4NO_3; pH adjusted to 4.5 and maintained at 40% moisture content in 250 ml conical flask so as to obtain a 1-2 cm layer of mixture without free liquid. The flask was sterilized by autoclaving, cooled, mixed thoroughly then added 1ml spore suspension and incubated the flask for 7days at 25ºC. After the incubation, the enzyme was extracted by following the standard procedures.

Cellulase production

Enzyme assay using DNS (Dinitrosolicyclic acid) was performed as below. About 0.8 ml of enzyme substrate (CMC);(0.25 g CMC was add 24.75ml of 0.015M sodium acetate buffer pH 4.8) was taken in a test tube then added 0.8 ml of culture filtrate. Test tubes were incubated in water bath at 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 absorbance of the samples was read at 540 nm in spectrophotometer. The amount of reducing sugar was determined using a standard graph.

Purification by Ammonium sulphate method

The modified method of De-Moraes et al., (1999) was followed for the enzyme purification. All procedures of the cellulase purification were carried out at 4ºC. The culture supernatant was separated by centrifugation process, by using 10,000 rpm for 15 minutes. After obtaining clarity to maximum level, solid crystals of ammonium sulphate was added to the crude enzyme extract until it was 80 % saturated and kept for 4 - 6 hours. The resulting precipitate was collected by centrifugation at 10,000 rpm for 15 min. After centrifugation the supernatant was kept separate and sediments were dissolved in small amount of 0.015 M Sodium acetate buffer (pH 4. 8). The solution was kept in a dialysis bag and after sealing securely, dialyzed against distilled water with 4 regular changes of the water after every 6 h. Total proteins and activity of partially purified cellulase were determined before and after dialysis of ammonium sulfate precipitation as mentioned before.

Acetone precipitation

Cold acetone was added to the crude enzyme at different ratio (1:3, 1:4, 1:5, 1:6 and 1:7). The tubes were incubated at -20ºC for overnight. After the incubation the content was centrifuged at 10,000 rpm for 15 minutes at 4°C. The precipitate was re-suspended with minimal amount of 0.015M sodium acetate buffer pH-4.8. (Ekundayo Opeyemi Adeleke et al., 2012).

Estimation of proteins

(Lowry et al., 1951)

The Lowry's method was followed for the estimation of protein. One gram of the fungal biomass along with substrate was taken and 10 ml of buffer was added and centrifuged. The supernatant was used for protein estimation. Five ml of stranded reagent was added to the 1 ml of test sample and mixed with the contents and incubated for 10 minutes at room temperature. After the incubation, 0.5 ml of 1N Folin-phenol reagent was added to the test solution and incubated at dark place for 30 min and read calorimetrically at 660nm. Solution of bovine serum albumin was used for obtaining the standard calibration curve

Results and Discussion

Mutagenesis

The cellulase enzymes excreted by *T.viride*, which was grown in solid-state wheat bran fermentation medium, exhibited significantly maximum carboxymethyl cellulase activity.

The main effect of mutagenic agents (X– rays, UV– rays, nitrous acid, dimethyl sulfonate, ethyl methane sulfonate (EMS) and acridine mustards) is to induce a lesion in or modification of the base sequence of DNA molecule; a mutation appears if this lesion remains un repaired (Devehand and Gwynne, 1991). The mutagenesis was achieved through micro wave followed by

UV rays and EMS in *T.viride* for higher cellulase production. 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 wild strain *T.viride* spores were exposed to microwave (maximum power: 700W; microwave frequency: 2450MHz) for different times (15–150 Sec), using the ice bath to eliminate heating effect of the oven on the spores as the *T.viride* spores were very sensitive to microwave. However, the rate of positive mutation as to the survival colonies increased as the time increased. On ultraviolet irradiation for various time intervals, 65 mutant derivatives were isolated out of 292 survivals, for their ability to hydrolyze the cellulose on agar plates more efficiently and significantly in comparison to parental strain. The mutant strains were inoculated to the production media. The cellulase activity was increased by the mutant strain obtained after 25 minutes exposure to UV rays compared with untreated strain. The enzyme activity recorded for wild type was 1350.72 U/g and for mutant strain was 1461.24 U/g. Further increase in UV exposure time caused a decline in activity, and also in surviving population. The spores exposed to 35 and 40 min were totally killed.

The compound mutagenesis was carried out to isolate hyper producer mutant derivatives of *T. viride* using different doses of EMS (50-300 μg/ml) and UV-irradiation at 254 nm for 20 min. The results were presented in Table 2. After the chemical mutagenesis, 85 mutant derivatives were isolated out of 232 survivals. The mutant spores were inoculated with production medium. The EMS concentration of 200 μg/ ml evidenced the most promising concentration as it illustrated significantly higher cellulase activity 1543.37 U/ g than the wild type 1350.72 U/ml. After the mutagenesis, the stability of these mutants for cellulase production was determined by successive sub-culturing for 12 generations. After each subculture, mutants were tested for their ability to stably produce cellulase by solid state fermentation.

In the present study, *T. viride* was mutagenized and genetically modified to develop a mutant strain capable of exhibiting high levels of cellulase activity. This is because of 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.

The improved enzyme yield to date was obtained through continuous efforts in strain improvement (Yamanobe et al., 1987), and medium optimization. *Aspergillus cellulolyticus* has been exploited as an industrial microorganism for cellulolytic enzyme production in Japan.

The successful story of *A. cellulolyticus* indicates that there is a great possibility to further improve mutant strain of *T. viride* and to enable it as an ideal cellulolytic enzyme producer for industrial purposes (Bailey and Tahtiharju, 2003; Villena and Gutierrez- Correa, 2006). EMS is strong mutagenic agent and induces permanent changes in DNA structure (frame shift mutation). Similar research was conducted by Hamad et al., (2001), where they reported that chemical treatment is more efficient in inducing high level mutations as compared to UV irradiation. Likewise, Mohsin, (2006) worked on strain improvement of thermophilic fungi (*Humicola insolens*) by using both physical and chemical mutagens.

Table.1 Effect of UV mutation on different time interval of cellulase activity

Sl .no	Exposure (min)	Number of survivor	Number of improved mutant	Cellulase activity Ug^{-1}
1	0	All	0	1350.72±0.86
2	5	86	21	1349.43.±0.62
3	10	68	18	1343.43±0.40
4	15	54	13	1340.42±0.33
5	20	43	8	1395.37±0.23
6	25	21	4	1461.24±0.27
7	30	17	1	1400.91±0.69
8	35	3	Nil	942.29±0.59
9	40	Nil	Nil	346.67±0.86

Each value is an average of three replicates; ± indicated the standard error from mean value

Table.2 effect of chemical mutation on cellulase production

S. no	EMS. con (µg/ml)	Number of survivor	Number of improved mutant	Cellulase activity (U/g)
1	0	Nil	0	1350.72±0.86
2	50	89	35	1350.96±0.86
3	100	71	18	1351.53±0.38
4	150	38	20	1406.40±0.26
5	200	20	9	1543.37±0.15
6	250	12	3	1234.45±0.40
7	300	2	Nil	716.53±0.12

Each value is an average of three replicates; ± indicated the standard error from mean value

Table.3 Cellulase activities of mutant strains for 12 generations

Generation	Cellulase activity U/ g
1^{st}	1543.37±0.15
2^{nd}	1543.16±0.26
3^{rd}	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

Figure.3 Purification of enzyme from Ammonium sulphate 30% saturation; 40% saturation; 50% saturation; 60% saturation; 70%saturation

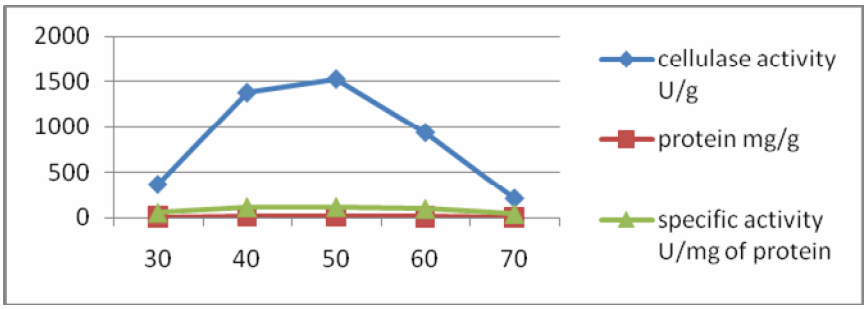
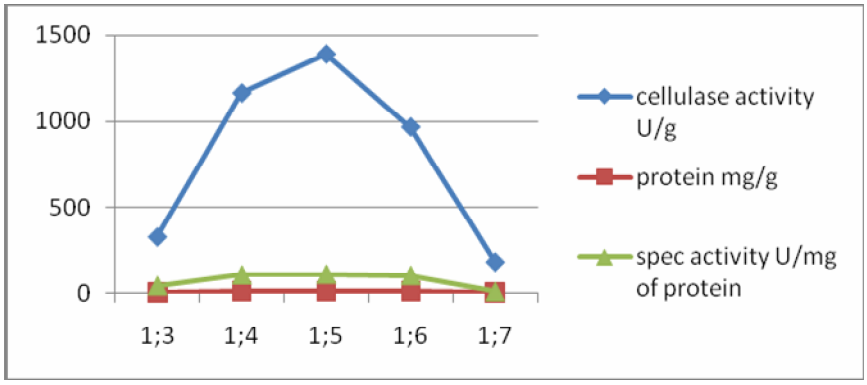


Figure.4 Purification of cellulase enzyme from Cold Acetone



In this method to isolate some mutant strains for better enzyme excreting efficiency. It was reported that when fungi were grown with mutagens at sub lethal concentrations, secretary enzyme production increased, but in this study, the killing rate was nearly 100% after UV treatment for than 35 min.

Purification

Two types of purification methods were done, such as cold acetone and Ammonium sulphate precipitation methods. The cold acetone was added to the crude enzyme at 1:3 to1:7 ratios of supernatant and incubated for overnight at-20ºC and calculated the enzyme activity. The maximum recovery was found for 1:5 ratio. The cellulase activity, protein and specific activities were 1391 U/gm, 12.38 mg/g and 111.63 U / mg respectively in the acetone precipitation.

The dialyzed ammonium sulphate precipitation step was done, after salting out of crude enzyme solution with different concentrations of ammonium sulphate saturation at 30 % to 70 %. The recovery of enzyme was higher at a saturation point of 50 % ammonium sulfate. The recovery of enzyme yield was 1525.34 U/gm while the specific activity was 144 U/mg respectively. The result was presented in figure 2

In the present study, crude enzyme was purified with cold acetone and ammonium sulphate dialysis method. The ammonium sulphate dialysis was better than acetone

purification in recovery of enzyme. The gene responsible for the production of cellulase may be over expressed due to mutation, as a result increase in enzyme activity (Gaedner et al., 1991; Chand et al., 2005).

Guowei Shu et al., (2013) worked on *Trichoderma viride* through solid state fermentation and crude enzyme was purified by ammonium sulphate precipitation method, whose cellulase activity was 73.6%. Hafiz Muhammad Nasir Iqbal et al., (2011) reported that the crude enzyme was partially purified by ammonium sulfate precipitation in two fractions of 0% - 50% and 50% - 80%. At 80% saturation, higher recovery of specific activity of 48 U/mg and 1.07 fold purification was achieved. Shanmugapriya (2012) reported that specific activity of the crude sample was found to be 6.57 Umg^{-1} of dialysed sample and ammonium sulphate precipitated sample was found to be 4.21U/mg.

In this study both physical and chemical agents such as microwave, UV and EMS was employed on *Trichoderma viride* isolate to obtain mutant strains. These mutant strains were enhanced the production of acid cellulase on SSF. Saving the economy of the country and meeting industrial sector demand. *Trichoderma viride* is capable of producing cellulase in the heap of wheat bran. The purified cellulase from this organism may be adapted for large scale industrial applications in the bioconversion of agricultural wastes.

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