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Trichoderma Species Isolation, Strain Improvement and Cellulase Production

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Abstract
Problem: The cellulase enzyme production by Trichoderma species was a major problem in bioethanol production through lignocellulosic biomass, as Trichoderma reesie produces low enzyme yield. **Approach:** The fungi were isolated from the soil samples from the sugarcane juice centre and dead and decaying plant wood. Screened for cellulase production and the most efficient strain was identified as Trichoderma reesei. To improve enzyme yield, strain improvement was carried out through protoplast fusion followed by sequential mutagenesis using UV radiation and EtBr treatment. **Findings:** Three stable strains (CMR1, CMR2, CMR3) were obtained. Among these, CMR3 strain shows the highest cellulase activity under SSF and SMF. On the 7th day of SSF, CMR3 recorded maximum activity 135±5.4 IU/g endoglucanase, 25±1.25 IU/g exoglucanase, and 45±2.3 IU/g β-glucosidase. On the 7th day of SMF, CMR3 strain recorded maximum activity 75±3.7IU/g endoglucanase, 19±0.95 IU/g exoglucanase, and 34±1.7 IU/g β-glucosidase. Overall enzyme yield under SSF was 1.8-fold higher than SMF. **Conclusion:** The strain improvement trough protoplast fusion and sequential mutagenesis of Trichoderma reesie enhanced cellulase production the CMR3 strain produces high enzyme yield.
Keywords: Protoplast fusion; endoglucanase; exoglucanase; β-glucosidase; sequential mutagenesis, Strain improvement, cellulase, UV radiation

Introduction

The rising utilisation of fossil fuels over the decades has increased environmental pollution, led to several health issues. Bioethanol is considered a better alternative to fossil fuels due to their environmental sustainability, energy security, and economic viability. Sustainability of bioethanol is possible if it relies on cellulosic biomass (Broda et al. 2022). The bioethanol production from the cellulosic biomass involves different steps of pretreatment, hydrolysis, and ethanol production. Hydrolysis of biomass is crucial for producing fermentable sugar, which is converted into ethanol through fermentation (Das et at. 2023). Two methods, acid hydrolysis and enzyme hydrolysis used to break down cellulose biomass. Acid hydrolysis is commonly used but has some problems like releasing toxic waste and hard to recover useful sugars. Enzymatic hydrolysis is more effective and safer for the environment (Vasić et al. 2021). Cellulose must be enzymatically broken down into its building block, the Glucose unit, which can be fermented to ethanol. Cellulase enzyme hydrolyses the cellulose chain to glucose monomer, A process mediated by a group of cellulolytic enzymes such as endoglucanase, exoglucanase, and β-glucosidase. Cellulase acts in combination to degrade the β-1,4-glucosidic linkage in cellulose (Ilić et al. 2023).

Cellulases are produced by a variety of organism, including bacteria, fungi (Iram et at. 2021). Among these, filamentous fungi are the most widely used due to their high secretion capacity and ability to produce a complete cellulase. The organisms, such as Trichoderma ressiand Aspergillus niger, are mostly studied, but each strain has limitations. T. ressi produces high levels of endoglucanase and exoglucanase but lacks β-glucosidase activity (Keshavarz et al.2016).A.niger is high in β-glucosidase production but shows lower activity of other cellulase component (Yoon et al. 2014). In order to stabilize the production of cellulase, protoplast fusion is one of the key strategies in the fungal strain improvement technique.

The present study is to isolate and screen cellulase producing fungi, and to enhance enzyme production through strain improvement, particularly protoplast fusion and sequential mutagenesis. Also assess cellulase production efficiency under solid state fermentation (SSF) and submerged fermentation (SMF) using sugarcane bagasse as substrate.

Materials and methods

Sample collection:

The soil samples from the sugarcane juice centre and dead and decaying plant wood were collected in aseptic bags and suspended in sterile normal saline.

Isolation of cellulase producing fungi:

The samples were diluted, 10^{-6} fold diluted 100 μl sample was spread on Mandel's solid medium and incubated at room temperature. The fungi showing a clear zone around the colonies were selected. The selected fungal isolates were grown on Mandel's solid medium. The 8 isolates were selected from the screening. These 8 isolates were inoculated in Mandel's broth and checked for cellulase production. The fungi showing good enzyme production on Mandel's broth were selected for further studies.

Colony characteristic:

The Isolated fungal strains were observed for colony morphology on Mandel's solid medium.

Microscopy:

The fungal isolates grown on Mandel's solid medium were identified by staining with lactophenol cotton blue. The hyphae were selected with sterile forceps and placed on a clean slide, lactophenol cotton blue was added, placed cover slip on the slide and observed under the microscope.

Inoculum preparation:

Wild-type and mutant strains were inoculated on Potato Dextrose agar (PDA) plates and incubated at room temperature. After 72hr of incubation, spores were collected using sterile water containing 0.1% of Tween 80 to get 1×10^8 ml/l spores. Spore suspension was used as an inoculum for cellulase production.

Substrate preparation:

Sugarcane bagasse was collected from sugarcane juice centre, dry for 3 days under the sun light and ground using grinder, filtered to the size of 2-5nm.

Cellulase production by solid state fermentation:

SSF was performed in a 2000 mL flask using ground 70g of sugarcane bagasse. Mandel's medium was prepared by adding ($MgSO_4$ 0.3g/l, $(NH_4)_2SO_4$ 1.4g/l, KH_2PO_4 0.3g/l, $CaCl_2$ 0.3g/l, peptone 0.75g/l, yeast extract 0.25g/l, $FeSO_4 \cdot 7H_2O$ 0.005 g/l, $MnSO_4H_2O$ 0.0016g/l, $ZnSO_4 \cdot 7H_2O$ 0.0014g/l, $CoCl_2$ 0.002g/l, carboxy methyl cellulose 10g/l, tween 80 2ml) and making the volume to 1L with distilled water. To sugarcane bagasse. 387ml of Mandel’s medium was added to make up the moisture level up to 70%. The prepared media was autoclaved at 15lbs for 15 min. Added 20ml of inoculum to the sterile media (Moisture level 75%) and incubated for 1 week at 25 °C.

Cellulase production by submerged fermentation

SMF was carried out in a 500 mL flask, in 100ml of Mandel's solution, took 10g of ground sugarcane bagasse. The medium was autoclaved at 15lbs for 15 min. Added 3ml of inoculum to the sterile medium and incubated for 1 week at 25 °C.

Extraction of enzyme

Crude cellulase was extracted using cirtate (pH 6.8) from SSF at a 1:100 dilution, directly from SMF and centrifuged the extracts at 10,000 rpm for 10 min, the supernatant was taken. The cell free supernatant was filter by a watchman filter paper, and the filtrate was crude enzyme.

Enzyme assay for cellulase

Filter paper assay

The cellulase activity was assayed by the method of Hankin et al. (1975). To 1ml of 0.05M citrate buffer, 1×6cm filter paper and 0.5 ml of enzyme was added, in a test tube and incubated at 50 °C for 60 min The reaction was stopped by adding 3ml of

DNS reagent, boiled for 5 min, and measured OD value was measured at 540nm in a colorimeter.

CMC assay

CMC assay was used for the estimation of endoglucanase of cellulase enzyme, this assay was carried out by the method of wood et al. (1977). 0.5ml of enzyme was added in a test tube, and 0.5ml of CMC solution was incubated at 50 °C for 60 min the reaction was stopped by adding 3ml DNS reagent, boiled for 5 min, and measured OD value was measured at 540nm in a colorimeter.

p-nitrophenyl β-D-glucopyranoside (pNPG) assay

The β-glucosidase activity was estimated by p-nitrophenyl β-D-glucopyranoside (Breuilet al. 1986). The substrate solution was prepared by dissolving pNPG to make 50mM.The cellulase enzyme sample was mixed with 0.5ml of the pNPG solution, the mixture was incubated at 50 °C for 30 min. After incubation, the reaction was stopped by adding 2.0 ml of 1M sodium carbonate (Na_2CO_3), the colour changed to yellow, and measured OD value was measured at 405nm in a colorimeter.

Protoplast fusion

Preparation of protoplast

Protoplast preparation was performed according to the modified method of Peberdy et al. (1980). Fungal culture was grown for 48hrs and diluted to approximately 1×103. The spores were centrifuge and washed three time with distilled water and suspended in protoplast solution (0.6M KCl, 10mM $CaCL_2$, 50mM phosphate buffer, chitinase 5mg/ml), incubated at 30 °C for 1hr. Samples were drawn at an interval of 10 min, upto 1 hr to know the time at which maximum protoplast formation has occurred. The protoplast formation was confirmed by methylene blue staining and hypertonic lysis of the suspension, and by observing the change in the cells shape to spherical and shining white. The suspension was filter through nylon mesh (30 µm).

Protoplast purification

The purification of protoplast was achieved by centrifugation of filtrate at 1000 g for 10 min, removed supernatant and osmatic medium (500Mm KCl) with trapping buffer (0.6M sorbitol, 50mM tris-HCl (pH 7.0), 10mM $CaCl_2$) was added in equal amount, centrifuged at 3000 g for 10 min.

Protoplast fusion

The fusion of protoplast between P2 and P5 strains was carried out by the modified method of Lynch et al. (1989). The protoplast was suspended at a 1:1 ratio within 1ml of fusion buffer (0.6 M KCl, 10mM $CaCl_2$, 50mM phosphate buffer). 200 µl of protoplast suspension was loaded in electrophoretic cuvette, and the formation of a pearl chain under the uniform AC electric field at 250 V/cm, and 2MHz was examined under a microscope. Electrical breakdown pulses (4kV, 1cmDC) applied every 10s to initiate the cells fusion. Subsequent to fusion, the cuvette was supplemented with regeneration minimal medium (glucose 20g/l, K_2PO_4 1g/l, KH_2PO_4 0.46g/l, thiamine-HCl 0.12g/l, asparagine 2g/l, $MgSO_4{\cdot}7H_2O$ 0.5g/l). After incubation for 8hrs at 30 °C. Cells were spread on Mandel's solid medium and incubation at 30 °C for 72 hours. The fusant cells were picked as per their growth on Mandel's solid medium.

Sequential mutagenesis

UV mutagenesis

UV mutagenesis was performed following to the method described by Abdul Hameed et al. (2012) method. Fusant CMR1 spores were washed and resuspended in 1ml of 100mM phosphate buffer at pH 7.0, the cells suspension prepared at 1×10^6/ml and exposed to the UV radiation for 10 min with 30 W lamp. After irradiation, the cells were kept in the dark for 1hr and 100µl suspension was spread on Mandel's solid medium and incubated at 30 °C.

EtBr mutagenesis

EtBr incorporation was carried out by Pasha et al. (2005) method. EtBr was added in the Mandel's medium in a sublethal concentration (1µg/ml). After sterilisation, and ethidium bromide was added and plates were prepared. UV-treated CMR2 strain spores were suspended in sterile normal saline. 100µl suspension was spread on the plates and incubated at 30 °C.

Stability studies of mutants

Mutant strains obtained from protoplast fusion followed by sequential mutagenesis (UV and EtBr) were studied for their stability of cellulase production for a period of 2 months (30 generations).

Mutants after every fermentation were inoculated on PDA slant and used for the next fermentation.

Results

Many isolates were grown on Mandel's solid medium. Eight isolates (P1-P8) were selected by the size of clear zone around the colonies for enzyme studies. Among them P1, P2, P4, P5, and P7 were identified as Trichoderma species, and P3, P6, and P8 as Aspergillus species. From 8 isolate the enzyme production by the P2 strain showed higher production of endoglucanase and exoglucanase and the P5 strain showed higher production of β-glucosidase compared with other strains in Table 1. These two strains (P2, P5) were used for further studies.

Fungal characteristics

The colony morphology of the P2 strain was appeared as dark green colonies. Microscopic observation revealed septate hyphae with highly branched conidiophores. The morphological and microscopic observation revealed that the species was identified as Trichoderma reesei.

The colony morphology of P5 was characterised as bright green spores and dense woolly colonies. Microscopic observation demonstrated hyphae with highly branched conidiospores forming a tree like structure and a cluster of elongated conidia. The morphological and microscopic observation revealed that the species was identified as Trichoderma viride.

Protoplast fusion and sequential mutagenesis

The selected strains (P2, P5) were isolated on Mandel's solid medium with cellulose as a carbon source and used to successive protoplast fusion and mutagenic treatment with UV and EtBr. After protoplast fusion CMR1 strain, native T. reesei was selected, which was further UV mutated to get CMR2 strain and this UV mutant was further mutated by EtBr incorporation method, got CMR3 strain. Hence three improved mutants were isolated (CMR1, CMR2, CMR3). Mutant strains were selected according to the size of the clearing zone surrounding the colonies on Mandel's solid medium.

Mutant stability for cellulase production

The selected mutant strains (CMR1, CMR2, CMR3) remain stable in cellulase production for the duration of 30 generations.

Enzyme production

The enzyme production by wild type strains on the 7th day they were shown in table 1. The P2 strain (Trichoderma reesei) produces 46±2.3IU/ml of endoglucanase, 8±0.4 IU/ml of exoglucanase, and 16±0.8 IU/ml β-glucosidase and P5 strain (Trichoderma viride) produces 38±1.9 IU/ml of endoglucanase, 4 ± 0.2IU/ml of exoglucanase and 21±0.9IU/ml of β-glucosidase, P2 strain showed higher production of endoglucanase and exoglucanase, P5 strain showed higher production of β-glucosidase compared to other strains. The result presented in table 2 shows the production of endoglucanase, exoglucanase and β-glucosidase activity by mutant strains obtained through protoplast fusion and sequential mutagenesis. The mutant strain CMR3 yielded higher enzyme production than the other mutants and wild type strains, the mutant strain CMR3 shows maximum activity 135± 5.2IU/g endoglucanase, 251.25 IU/g exoglucanase and 45±2.3 IU/g β-glucosidase was observed under SSF, In SMF maximum activity was 74±3.7 IU/ml endoglucanase, 19 ± 0.95IU/ml exoglucanase and 34±1.7 IU/ml β-glucosidase was observed.

Table 1.cellulase production by wild type strains

Strain	Endoglucanase	Exoglucanase	β-glucosidase
(SMF IU/ml)			
P1	28±1.4	5±0.25	12±0.6
P2	46±2.3	8±0.4	16±0.8
P3	31±1.55	3±0.15	9±0.44
P4	25±1.25	6±0.3	13±0.6
P5	38±1.9	4±0.2	21±0.9
P6	35±1.75	7±0. 35	17±0.8
P7	42±2.1	5±0.25	14±0.7
P8	29±1.45	2±0.1	8±0.4

Table 2.cellulase production by fusant and mutant strains

Strain	endoglucanase	Exoglucanase	Β-glucosidase
SSF IU/g			
CMR1	120±4.8	20±1.0	39±1.9
CMR2	132±5.2	24±1.2	44±2.2
CMR3	135±5.4	25±1.25	45±2.3
SMF IU/ml			
CMR1	61±3.0	12±0.6	25±1.25
CMR2	69±3.4	15±0.75	29±1.45
CMR3	74±±3.7	19±0.95	34±1.7

Discussion

Cellulase enzyme plays a crucial role in conversion of cellulosic biomass in fermentable sugar used for the production of bioethanol. Fungal cellulase is an alternative for the degradation of waste cellulose. The fungal enzymes are sustainable solution to chemical hydrolysis. The enzymatic hydrolysis increases the interest with the growing demand for bioethanol production to shift from fossil fuel (Raheja et al. 2025).

A diverse range of microorganisms are capable of cellulose degradation, among the cellulolytic microorganisms, filamentous fungi are the most prolific producer of cellulases. Among fungi, Trichoderma and Aspergillus species are the most efficient cellulase producers (cherry et al. 2003). complete hydrolysis of cellulose requires the combined effort of endoglucanase, exoglucanase, and β-glucosidase (Mafa et al. 2021).

Although Trichoderma reesei are the most studied cellulolytic fungi, T, reesei shows high yield capacity for endoglucanase and exoglucanase but low in β-glucosidase, however, strain improvement by protoplast fusion and mutations are employed for strain improvement. Protoplast fusion and EtBr and UV mutagenesis proved to be effective in producing stable, high-yielding mutants. Sequential mutagenesis of Aspergillus species improved the enzymatic activities of endoglucanase, exoglucanase, and β-glucosidase compared to wild-type strains (Xian et al. 2025). In strain improvement through protoplast fusion and sequential mutagenesis, three mutant strains (CMR1, CMR2, CM3) were obtained. The CMR3 strain shows superior cellulase activity under SSF and SMF.

The enhanced enzyme activity of the mutant strain compared to wild-type strain highlights the significance of strain improvement for the fungal strain. In this study T,reesei CMR3 strain produced 135±5.4 IU/g endoglucanase, 25±1.25 IU/g exoglucanase, and 45±2.3 IU/g β-glucosidase under SSF, and under SMF CMR3 strain produced 74±3.7 IU/ml endoglucanase, 19±0.95 IU/ml exoglucanase, and 341.7 IU/ml β-glucosidase, which was greater than the wild type strain. (Ligodiet al. 2023) reported that a mutant strain of Trichoderma species produced 120 IU/g endoglucanase, 24 IU/g exoglucanase, and 38 IU/g β-glucosidase under SSF. Similarly,(Peng et al. 2021) reported that the mutant strain of Trichoderma afroharzianum MEA12 produced 48.6 IU/ml endoglucanase, 16.22 IU/ml exoglucanase, and 28.4 IU/ml β-glucosidase under SMF.

In the present study, the stability of T,reesei mutant CMR3 shows enhanced cellulase production across multiple generations, confirms that protoplast fusion followed by sequential mutagenesis as a successful strain improvement approach. Similarly, the stability has been observed in previous studies, (Papzanet al. 2021) reports that mutant Trichoderma species obtained through mutagenesis showed enhanced and stable cellulase production for multiple generations, confirmed the long-term stability of the improved strain.

Furthermore, SSF yielded higher cellulase activity compared to SMF using sugarcane bagasse as substrate. The CMR3 strain under SSF enzyme production was 1.8-fold higher than SMF. The SSF is more effective for cellulase production is due to close contact between fungal hyphae and solid substrate, leading to better enzyme secretion. (Raghuvanshi et al. 2014) used wheat straw and rice straw as substrate with mutated Trichoderma and yielded 1.4-.1.5-fold higher than wild type.

This study reveals that strain improvement through protoplast fusion and sequential mutagenesis in Trichoderma sp ssignificantly enhanced cellulase production. The SSF proved more effective than the SMF, with sugarcane bagasse as a cost-effective and sustainable, and economical cellulase production.

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