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Production of Cellulase Enzyme by *Trichoderma reesei* Cef19 and its Application in the Production of Bio-ethanol

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Abstract: The present study aimed at the production of cellulase enzyme from the cellulolytic fungi *Trichoderma reesei* CEF19 and subsequent application of the cellulase for the fermentation of ethanol. For the same, the cellulolytic fungi, *Trichoderma reesei* CEF19 was isolated and was allowed to produce cellulase enzyme using optimized conditions. The cellulase enzyme was extracted and purified with the help of ammonium sulphate precipitation, dialysis followed by ion exchange chromatography with DEAE-Sephadex column. The purified cellulase enzyme was characterized using SDS-PAGE analysis. The saccharification of the cellulosic substrates was done using the cellulase enzyme. The fermentation of saccharified cellulosic substrates into ethanol was carried out using *Saccharomyces cerevisiae*. From the results obtained, rice straw was found to be the better source for the ethanol production when compared to the other substrates.

Key words: *Trichoderma reesei*, bio-ethanol, rice straw, DEAE sephadex, ITS

INTRODUCTION

The increase in the usage of the fossil fuel results in the global warming and fuel shortages (Zhu and Pan, 2010). Hence, there is an urge for the alternative renewable energy sources like bioethanol. It has been found that the usage of bioethanol from cellulase reduces 90% of greenhouse gases when compared to fossil fuels (Chandel *et al*., 2007). Lignocellulosic materials represent a stable renewable compound which stores the solar energy by recycling and converting atmospheric carbon dioxide through photosynthesis. The usage of such renewable carbon sources will leads to the zero net carbon emission (Foody and Foody, 1991).

The current methods in the production of bioethanol employ food substances and are also found to be requires expensive production strategies (Schubert, 2006). One of the best methods for the bioethanol production is to hydrolyze the cellulosic materials into sugars with the help of microbial enzymes followed by their fermentation into ethanol. This kind of ethanol production is considered as eco-friendly, sustainable and economic method for ethanol production (Chandel *et al*., 2007).

Cellulase is one of the essential enzyme hydrolyses the celluloses into simple sugars. Various microorganisms including bacteria and fungi were revealed to produce cellulase enzyme as they need to utilize the celluloses which can be facilitated by the cellulases. Among the various fungi, *Trichoderma reesei*, *Aspergillus* sp., *Rhizopus* sp. were widely studied for their cellulolytic activity (Chandel *et al*., 2007).

The production cost of bioethanol can be reduced by the saccharification of the substrates with the help of enzymes such as cellulase or enzyme cocktails. The saccharification helps in the breakdown of lignocelluloses into its derivative compounds thus facilitates the ethanol production (Balan *et al*., 2009). In the present study, the application of isolated cellulolytic fungi *Trichoderma reesei* was evaluated for the cellulase production and ethanol fermentation. The comparative evaluation of saccharification was performed with different feed stock using the produced cellulase.

MATERIALS AND METHODS

Isolation and screening for cellulolytic fungi: The fungal strains isolated from wood decaying places near Chennai were screened for cellulase production using CMC agar plates consisting 1% CMC (g L^{-1}: Yeast extract 0.3, $NaNO_3$ 6.5, K_2HPO_4 6.5, KCl 6.5, $MgSO_4.7H_2O$ 3.0, Agar 17.5) (Aneja, 2005). The plates were inoculated with the isolated fungal strains and incubated for two days. After incubation, the plates were allowed to flood with 0.1% Congo red solution and were washed with 1 M NaCl

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for 15 min. The formation of clear zone around the fungi indicates the production of cellulase (Aneja, 2005). From the cellulolytic fungi, the better cellulolytic fungi was selected based on the diameter of the zone of clearance and was proceeded for further studies.

Identification of fungi (Kumari *et al*., 2011): The isolated cellulolytic fungal strain was characterized morphologically and for its molecular identification, the genomic DNA was isolated from the fungi. The ITS region of the DNA was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Liu *et al*., 2007). The amplified DNA was allowed for sequencing using Qiagen Automated sequencer. The sequence was then analyzed for its maximum homology sequence using BLAST tool.

Production of cellulase: The cellulolytic fungal strain *Trichoderma ressei* CEF19 was inoculated into 100 mL of the cellulase production medium (Cellulose 10 g, Peptone 1 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2 g, $CaCl_2$ 0.3 g, $MgSO_4.7H_2O$ 0.3 g, Urea, 0.3 g, Distilled water 1000 mL) (Devi and Kumar, 2012). The production medium was incubated in shaker (120 rpm) for two days. After incubation, the production medium was centrifuged and pellet was removed. The supernatant (culture filtrate) was used as the enzyme source for assay procedures.

Assay of cellulase (Khan and Singh, 2011): The amount of cellulase present in the culture filtrate was assayed using DNS assay. The reaction mixture consists of 0.1 mL of enzyme solution, 1% of CMC in 100 mM Tris buffer was incubated for 15 min at room temperature. The intensity of the color developed was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute under standard reaction conditions.

Extraction and purification of cellulase: The cellulase enzyme produced was then extracted and purified (Li *et al*., 2003). The culture filtrate was extracted with 90% ammonium sulphate precipitation and the precipitate was dissolved in Tris buffer. The precipitate was dialyzed overnight using Tris buffer with three buffer transfers with the same buffer. This partially purified cellulase was further purified using Ion exchange chromatography with DEAE-Sephadex column with Tris buffer as running buffer. About 3 mL fractions were collected and were assayed for the cellulase activity. The fraction with maximum cellulase activity was considered to be having purified cellulase and stored for further usage.

Characterization of cellulase: The molecular weight of the purified cellulase was identified by running the sample on SDS-PAGE along with the marker protein sample (Laemmli, 1970).

Saccharification of cellulosic materials by the cellulase: To facilitate the ethanol production, the cellulose rich materials such as rice straw, corn stover, sugarcane baggase, saw dust. In prior to the production, the saccharification of the substrates were done with the help of the cellulase enzyme (Pasha *et al*., 2012). The substrates were sieved into small meshes and treated with 0.2 M KOH for 24 h and autoclaved. The substrates were then washed to neutral and dried completely. For the enzymatic saccharification, about 12 g of pre-treated substrate was added with 100 mL of cellulase production medium inoculated with cellulolytic *Trichoderma reesei*. The mixture was kept at shaken condition (150 rpm) for 48 h at 55°C. Followed by the incubation, the hydrolysate was collected by filtration and was analyzed for total reducing sugars.

Production of bio-ethanol: The thermotolerant *Saccharomyces cerevisiae* was used for the fermentation of ethanol (Pasha *et al*., 2012). The *Saccharomyces cerevisiae* was allowed to grown on YEPD medium (g L^{-1}: peptone 20, yeast extract 10, dextrose 20) and was used as inoculum for the ethanol production. About 10% inoculum was added to the saccharified hydrolysate and the mixture was incubated at 42°C for 3 days. The samples were isolated at regular intervals and assayed for ethanol production (GC) and leftover sugars (DNS method).

RESULTS AND DISCUSSION

Enzymes from microorganisms are of having high industrial importance and being used as for any commercial applications. Cellulases from fungal sources have been widely used in industrial sector (Jahangeer *et al*., 2005). Apart from the industrial applications, cellulases are also having various applications in environment. They play an important role in the earth's carbon cycle as many plant derived products were cellulosic in nature. The degradation of cellulosic materials is playing an important role in their conversion into various sugars and thereby facilitates the fermentation of Bio-ethanol (Chandel *et al*., 2007).

For the proposed study, the preliminary step was to isolate the cellulolytic fungi. For the same, soil sample was collected from the wood decaying site and was serially diluted and plated on PDA plates. About 17 distinct fungal strains were isolated from the PDA plates and were screened for cellulase production in CMC agar plates. From the 17 strains, 6 strains were found to be cellulolytic through their formation of clear zone upon addition of Congo red solution. Of that, the strain CEF19 showed zone of maximum diameter was selected for the present study.

Through the microscopic observations, the isolate was identified as *Trichoderma* sp. and further identified by sequencing the 5.8 ITS region and compared with the Genbank database with the help of BLAST tool. The ITS sequence of the isolate showed a close relatedness to the *Trichoderma reesei* with 100% similarity. Hence, the isolate CEF19 was confirmed as *Trichoderma reesei* and the sequence was submitted to the Genbank.

The fungus was allowed to produce the cellulase enzyme using the production medium with optimized conditions. During the production of the enzymes, various factors to be considered and are as follows. The incubation time along with the growth rate of the organism, followed by pH, temperature and nutrient sources (carbon and nitrogen) were to be considered during the enzyme production (Kumari *et al*., 2011).

The extracellular cellulase produced was subjected to ammonium sulphate precipitation and dialysis. After the extraction and partial purification, the cellulase was further purified using DEAE-Sephadex chromatography. The purified cellulase enzyme was stored for further analysis and applications. The molecular weight of the purified cellulase was determined using SDS-PAGE analysis and was found to be 38 kda. It was confirmed by the previous reports available (Eriksen and Goksoyr, 1977; Tong *et al*., 1980; Khandke *et al*., 1989; Ganju *et al*., 1990).

The application of the lignocellulosic materials as the substrate for bio-ethanol production was found to be an eco-friendly and economic method (Chandel *et al*., 2007). The bio-ethanol produced in such method was found to be zero emission net carbon and thereby reduces the green house gases. In this present study also various cellulosic materials such as rice straw, corn stover, sugarcane baggase, saw dust were tested for their ability to produce bio-ethanol. The fermentation of bio-ethanol can be fastening up by pre-treating the substrates. The pre-treatment can be done by saccharify such cellulosic substrates into simpler sugars (Balan *et al*., 2009).

In current days, the bio-ethanol production from starch and sugar based biomass has been paid great attention as they are vast available in earth (Roslan *et al*., 2011). In the present investigation, the cellulosic substrates were pre-treated with the help of purified cellulase. The short meshes of substrates treated with cellulase facilitate the hydrolysis of cellulosic materials into reducing sugars and hemicelluloses. The saccharified cellulosic substrates were then allowed to ferment with the help of *Saccharomyces cerevisiae* under prescribed conditions. The bio-ethanol produced was analyzed using GC by retrieving the samples at regular intervals.

The amount of sugars used and bio-ethanol produced in the present study was depends on the nature of the fungi used and the substrate. Of the substrates used, rice straw was found to be the better cellulosic substrate for the bio-ethanol fermentation. The corn stover and sugar cane baggase were also contributed in the bio-ethanol production next to rice straw. The saw dust did not meet the criteria as it fails to produce bio-ethanol as compared to others. Different researchers employed different types of substrates for the Bio-ethanol production. But, lignocellulosic materials were focused recently for bio-ethanol production as they are cheap and abundant (Shimoyama *et al*., 2011).

In the present investigation, the bio-ethanol production was analyzed with the help of the saccharification process by the cellulolytic fungi *Trichoderma reesei* and the production of bio-ethanol by the fermentative yeast *Saccharomyces cerevisiae*. The substrates for the bio-ethanol can be pre-treated either chemically or enzymatically (Balan *et al*., 2009). In the present study, enzymatic sacccharification was employed along with KOH as the substrates are natural biomass and are extremely recalcitrant to enzyme sacccharification. From the previous reports were available which describes the ability of cellulase in the sacccharification of cellulosic substrates for the bio-ethanol production. Since, enzymatic sacccharification is the important step in the bio-ethanol fermentation; we isolated the better cellulase producing fungi (Nipa *et al*., 2006). For the same, we have made an attempt to use the isolated fungal strain *Trichoderma reesei* as the source of cellulase in the sacccharification step. Since the commercial cellulases were expensive, the fungus was used as the cellulase source for the bio-ethanol production.

The results we obtained have demonstrated the significant amount of bio-ethanol was produced by rice

Table 1: Ethanol production efficiency by different substrates

Parameters	Rice straw	Sugarcane baggase	Saw dust	Corn stover
Initial sugars (After saccharification) (g/100 mL)	5.60	4.30	3.20	4.80
Total leftover sugars (After ethanol production) (g/100 mL)	0.80	1.20	1.40	0.90
Total sugars consumed (g)	4.80	3.10	1.80	3.90
Ethanol produced (g/100 mL)	2.27	1.83	1.47	2.00
Fermentation efficiency (yield %)	85.71	72.10	56.25	81.25

straw, followed by corn stover and sugarcane baggase and least was recorded by saw dust (Table 1). The increasing order of bio-ethanol production based on the observation can be represented as follows:

Rice straw>Corn stover>Sugarcane baggase>saw dust

CONCLUSION

From the present investigation, we can conclude that the cellulosic agro wastes such as rice straw, corn stover, sugarcane baggase can be act as the potential substrates for the bio-ethanol production. They are cost effective, abundant and sustainable. The cellulase enzyme produced by the cellulolytic fungi *Trichoderma reesei* was used for the saccharification of the cellulosic substrates in prior to the fermentation of bio-ethanol. From the substrates analyzed, rice straw was found to be an effective substrate for the bio-ethanol production.

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