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Production of Hydrolytic Cellulase Enzyme by Isolate *Aspergillus flavus* OR and *Trichoderma reseei* Using Rice Straw as the Feedstock Material

REEMA NINGTHOUJAM, PANKAJ JANGID AND HARISH KUMAR DHINGRA*

Mody University of Science & Technology, Lakshmangarh, Sikar-332 311 (Rajasthan), India
**(e-mail : harishdhingra2000@gmail.com; Mobile : 79884 76239)*

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ABSTRACT

The potential of *Trichoderma reseei* and isolate *Aspergillus flavus* OR for production of cellulase enzyme was checked by using rice straw as the lignocellulosic substrate. A maximum cellulase yield of 0.054 IU/ml at 120 h incubation was achieved using the *T. reesei.* Further incubation decreased the cellulase activities which proved that incubation period played a major role in the enzyme production process and should be monitored very carefully. *A. flavus* OR gave the highest cellulase activity of 0.042 IU/ml at 192 h. Based on the results of the hydrolytic enzymatic activity, both the microorganisms were seen as an efficient cellulose degrader and could replace the commercial enzymes which are not cost-effective. Rice straw also acted as a promising feed stock in the production of energy. The bioconversion of lignocellulosic biomass received global attention in the past few decades and is now a subject of intensive research in the field of developing liquid fuels such as bioethanol.

Key words : *Trichoderma reseei*, *Aspergillus flavus*, cellulase, rice straw

INTRODUCTION

One of the most abundant and significant components found in the plant cell wall is the cellulose. Cellulose is the linear polymer of glucose which is linked together by β-1,4-glycosidic bonds. Conversion of lignocellulosic biomass into valuable products requires the use of cellulase enzymes which makes the production steps more costly. Cellulase is the third largest enzyme used in industry due to its wide applications and mainly used in the enzymatic hydrolysis of conversion of lignocellulosic biomass to bioethanol. Cellulases are association of free enzymes which include endoglucanases, exoglucanases and cellobiases. Cellulases enzymes can saccharify cellulose present in lignocellulosic materials which release glucose and further upon fermentation can be converted into cellulosic ethanol (Marques *et al.*, 2018). The process in the production of cellulase enzyme should be made economic feasible. Cellulases are secreted by varieties of microorganisms like bacteria and fungi (Hmad and Gargouri, 2017). The enzymes produced by these microorganisms differ in their mode of action among which the fungi are the preferred ones as they yield more enzymes as compared to bacteria and have the potential to produce complete cellulase complex also. The *Aspergillus* genus is reported as one of the major sources in the cellulolytic production which is a ubiquitous fungal species and is found mostly in soil (Fakruddin *et al.*, 2015). It plays an important role in the decaying and decomposition and is well characterized (Kumar *et al.*, 2016; Sohail *et al.*, 2016). Another well-known fungus, which is one of the most extraordinary producers of cellulases and hemicellulases, is the *Trichoderma reesei* (Chen *et al.*, 2021). *T. reesei* among the *Trichoderma* species is best studied (Schmoll *et al.*, 2016), mainly due to its wide application in biotechnology (Gudynaite-Savitch and White, 2016; Arnau *et al.*, 2020). *T. reesei* harbours complex regulatory mechanisms enabling to fine-tune the expression and secretion of enzymes towards the substrate characteristics, an energy-conserving strategy for feedstock degradation (Novy *et al.*, 2019). Cultivation of industrial fungi has been a goal for high efficiency value-added products since time immemorial. Fungal strain improvement by genetic modification would result in large scale cellulase production. Using fungal enzyme consortia in solid waste management and handling has been shown effective as an alternative opportunity that is well-organized, environmentally sustainable with low cost (Sarsaiya *et al.*, 2017).

In the present investigation, novel fungal

isolate *A. flavus* OR and *T. reseei* were checked for their cellulolytic activity. A cheap feedstock i. e. the rice straw was selected where it was found abundantly and which when burnt caused air pollution and is a serious case of concern. For most developing countries as well as for some developed countries, the sustainability of agricultural and rural waste management is very much important. However, bioethanol produced from biomass is viewed as an attractive, renewable source of energy to fuel industry (Ningthoujam and Dhingra, 2021).

The objective of this study was the isolation of novel fungus that has the ability to produce cellulase on an industrial level scale where technical constraints, durations and economy should be considered at the same time.

MATERIALS AND METHODS

The substrate used in this study was rice straw, a lignocellulosic substrate collected from a nearby field from Imphal East, Manipur. Rice straw is abundantly available, relatively cheaper and is a rich source of carbon. The rice straw was chopped into pieces, grinded and milled and washed extensively with tap water to remove all the soluble contents. It was dried for two days at 65°C to remove the available moisture content (Selvaraj and Vasan, 2017). *T. reesei* (MTCC 4876) was procured from MTCC Chandigarh for comparing its cellulolytic activity with the novel fungal isolate which was obtained from soil containing dead and decaying plant materials. These two microorganisms were maintained on Potato Dextrose Agar (PDA) supplemented with streptomycin and was incubated at 30°C for five days.

DNA isolation from microbial samples was done using the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee Stores Pvt. Ltd. Amplification of the internal transcribed spacer (ITS) region was performed using PCR with the universal primers ITS1(5' TCCGTAGGTGAAC CTGCGG 3') and ITS4 (5' TCCTCCGCTTAT TGATATGC 3'). 5 µL of isolated DNA was added in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). The PCR was performed using the following thermal cycling conditions.

The DNA template was heated to 95°C. This broke the weak hydrogen bonds that held DNA strands together in a helix, allowing the strands to separate creating single stranded DNA. The mixture was cooled to 55°C. This allowed the primers to bind (anneal) to their complementary sequence in the template DNA. The reaction was then heated to 72°C, the optimal temperature for DNA polymerase to act. DNA polymerase extended the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Single-pass sequencing was performed on each template using below 18s rRNA universal primers. BLAST (Basic Local Alignment Search Tool) was performed using NCBI (National Centre for Biotechnology Information) blast similarity search tool (*http://www.ncbi.Nlm.nih.gov/BLAST*). The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminated poorly aligned positions and divergent regions (removed alignment noise). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. The program Tree Dyn 198.3 was used for tree rendering.

The enzymatic saccharification of the rice straw was carried out by adding each fungus separately in 100 ml reagent screw cap bottle by incubating 10 g of the rice straw at 30°C and kept for 192 h in shaking incubator at 150 rpm. The reducing sugar (which was equivalent to glucose) thus produced in the hydrolysate was determined by the DNS method (3, 5- Dinitrosalicyclic acid).

RESULTS AND DISCUSSION

The fungus isolate was identified at the molecular level as *A. flavus* OR and the gene sequence of the isolate is shown in Fig. 1. Also, the phylogenetic tree analysis is shown in Fig. 2. The accession number obtained was OK330386 and was deposited in the GenBank database.

Fig. 3 shows the enzyme activities of *T. reseei* and *A. flavus OR*. The figure shows the effect of different time intervals on the production of cellulase enzyme. Yield of cellulase

>Contig OR
CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGAC
ACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAAT
GGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAAT
TCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCG
AGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAA
AGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGC
CCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGAT
ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCAGGTAACGGCG

Fig. 1. Gene sequence of isolate *Aspergillus flavus* OR.

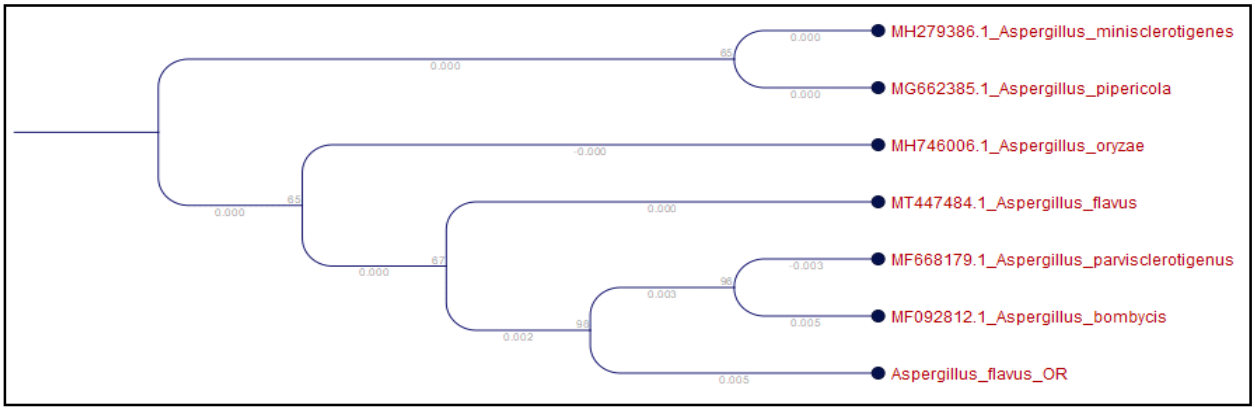

Fig. 2. Phylogenetic analysis of *Aspergillus flavus* (OR).

enzyme was measured after every 24 h and was measured up to the extent of 192 h. *A. flavus* OR grown on rice straw gave the highest cellulase activity of 0.042 IU/ml at 192 h. It was seen that *T. reseei* gave the highest yield of 0.054 IU/ml at 120 h incubation. Further incubation decreased its yield. From this investigation, it was concluded that both these microorganisms were able to produce cellulase enzyme and could reduce the cost of the enzyme hydrolysis process.

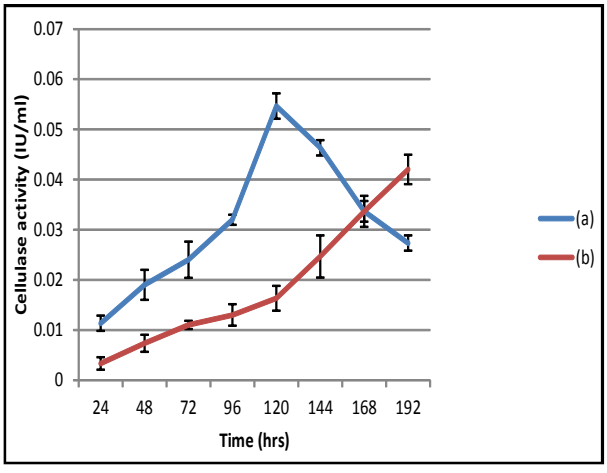

Fig. 3. Cellulase activity in *Trichoderma reseei* (a) and *Aspergillus flavus* (b).

CONCLUSION

In this study, *A. flavus* OR isolated from soil sample was found to be a potential cellulase producer and was comparable with the enzymatic yield produced by *T. reseei* even though it took a little longer period of time. Rice straw was thus found to be a promising carbon source. *A. flavus* OR has the capability of degrading the lignocellulosic biomass and releasing efficient sugars.

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