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Highly Efficient Conversion Biomass of Saccharum munja for Cellulases and Xylanase Production to Ethanol Repression by Newly Isolated Trichoderma atroviride AD-130

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Abstract

In this study the filamentous fungi Trichoderma atroviride AD-130 was evaluated for production of cellulases as well as xylanase. An attempt has been made to optimize the cultural and nutritional conditions for cellulases and xylanase production by *Trichoderma atroviride* AD-130 under submerged fermentation. The lignocellulosic biomass of *Saccharum munja* was used as carbon source for cellulases and xylanase production. Whole fermentation process was carried out in 250 mL Erlenmeyer flasks with agitation speed of 170rpm. The maximum titers of cellulases (FPase 1.01 U mL^{-1}, CMCase 2.69 U mL^{-1}, β-glucosidase 0.82 U mL^{-1}) and xylanase (82.99 U mL^{-1}) were obtained on 5th and 4th day respectively when *Trichoderma atroviride* AD-130 was grown at initial medium having pH 6.0 at 30°C using 2.0% (w/v) *Saccharum munja* biomass as substrate.

Key words : *Trichoderma atroviride* AD-130, *Saccharum munja*, Cellulase, Xylanase, Ethanol.

Substantial research efforts have been made for biochemical conversion of agricultural lignocellulosic biomass (LB) into ethanol. The general process for converting LB into bioethanol take in steps like feedstock pretreatment, enzymatic hydrolysis, sugar fermentation, separation of lignin residue, and the recovery and purification of ethanol to meetfuel specifications (Meng *et al*., 2021). However, the cost ofethanol production from LB is relatively high based on current technologies, and the major challenges are the low yield and high cost of the enzymatic hydrolysis process (Vaez, S., *et al*., 2021) which is achieved by a sequence of reactions with the main components of cellulase complex enzymes [endoglucanases (EG), cellobiohydrolases (CBH), β-glucosidases (BGL)] responsible for converting cellulosic part into fermentable sugars. But, in

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annual plants and hardwoods ,xylan, the most abundant non-cellulosic polysaccharide accounting for 20-35% of the total dry weight in biomass must also be hydrolysed in the presence of decomposing enzyme, viz., xylanase (Wang, Z. *et al*.,2020, Seema Devi *et al*., 2012).

Thus, the complex enzymes 'cellulases and xylanase' play crucial role in the degradation of lignocellulosic materials. Currently the cost of enzymes is also too high and research is continuing to bring down the cost of enzymes as in many bioconversion strategies, the cellulases required for biomass conversion may account for as much as 40% ofthe total process cost (Ghazanfar M *et al*., 2022). Therefore, large-scale low cost production of cellulases is very important through microorganism selection and improved fermentation process conditions (Yusuf, A.. A and Inamboo, F.L. 2021). Moreover, the ability offilamentous fungi to secrete large amounts ofcellulolytic proteins has motivated their extensive use for the production of industrial enzymes (Sivamani, S *et al*., 2018). Among the cellulolytic fungi, *Trichoderma* spp. and *Aspergillus* spp. Have been widely studied for their ability to secrete high levels of cellulose degrading enzymes. Hence, the filamentous fungus *Aspergillus* was envisaged to be one of the major agents of decomposition and decay for the present study owing to its potential to produce a broad range of enzymes including cellulases as well as xylanase (Aggarwal *et al*., 2022).

The production of cellulases and xylanase is greatly influenced by media components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature and moisture (Pabon *et al*., 2020). In order to obtain maximum enzyme production, development of a suitable medium and culture conditions is necessary. Although the use of expensive substrate is one of the major problems in cellulases production by fermentation but the choice of substrate for enzyme production ultimately governs the cost of production.

Reduction in the production cost and improvement in cellulases yield could also be achieved using appropriate and low cost carbon and nitrogen sources in the formulation of fermentation medium (Shanmugam R *et al*., 2022). Therefore, the use of abundantly available and cost-effective agricultural by products must allow reduction of the overall production cost of the decomposing enzymes which could be resolved by isolating cellulolytic strains with high levels of cellulases as well as xylanase productivity, optimizing fermentation conditions while making useof cheaper agricultural and industrial substrates as carbon or nitrogen source in the fermentation medium (Anil K *et al*., 2014, Seema Devi *et al*.,2015, 2016). Hence, the optimization of all the process parameters was being considered as pre-requisite to make the process of enzyme production cost-effective at large scale and submerged fermentation process was preferably selected because of more nutrients availability, sufficient oxygen supply and less time required for the fermentation than other fermentation techniques (Seema Devi *et al*., 2012).

This study was aimed at evaluating growth conditions that affect the production of cellulases and xylanase by using *Trichoderma* atrovirideAD-130 and optimizing their cultivation conditionsunder submerged fermentation (SmF) using lignocellulosic biomass (LB) of *Saccharum munjaas* substrate.

Materials and Methods

Collection of substrate : Lignocellulosic biomass (LB) of *Saccharum munja* was collected from Tilyar Lake on National Highway (NH) 10, Rohtak which is located at a latitude of 30°1`N and longitude of 75°17`E. The collected LB was washed with distilled water and then

dried at 70°C till constant weight. The feedstock was oven-dried and size-reduced to pass through a 2 mm sieve and stored in sealed plastic bags at room temperature for carrying out further experiments.

Microorganism : *Trichoderma atroviride* AD-130 was isolated from soil sample was cultivated and maintained on potato dextrose agar (PDA) at 4°C.

Preparation of Spore Suspension : The slants of seven days old cultures were wet by adding 10 mL of sterilized distilled water. The spores were scratched by sterile wire loop to break clumps and obtained homogenous spore suspension. One milli liter of spore suspension containing 1x107 spores was used as inoculum.

Medium Preparation for Enzyme Production : The selected fungus, *viz*., Trichoderma atrovirideAD-130 was grown in Mandels and Sternburg (1976) media in Erlenmeyer flasks. The media contained (per liter of distilled water): proteose-peptone 1.0 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, Urea 0.3 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.3 g, $FeSO_4.7H_2O$ 5.0 mg, $MnSO_4$. $7H_2O$ mg, $ZnSO_4.7H_2O$ 1.4mg and $CoCl_2$ 0.002 g. Then, 50mL of the liquid media containing 1.0 g of LB was placed in 250 mL Erlenmeyer flasks and sterilised at 121°C for 15 minutes. After sterilization, the media was allowed to cool and inoculated with 1.0mL spore suspension of the fungus containing approximately 1 x 10^7 spores per milliliter counted with the help of haemocytometer and flasks were incubated in shaker at 180rpm. The effect of different variables, viz., pH, incubation period and temperature were studied on cellulases and xylanase productionby'one variable at one time' approach.

Enzyme Extraction : One millilitre of the culture filtrate was withdrawn under aseptic conditions at desired intervals and centrifuged at 10,000rpm for 10minutes at 4°C to remove unwanted particles and spores. The supernatants obtained after centrifugation were used as enzyme source and assayed for filter paperase (FPase), carboxymethyl cellulase (CMCase), β-glucosidase and xylanase.

Enzyme assay : The FPase and Carboxymethyl cellulose (CMCase) activities were measured according to IUPAC method of Ghose (1987). Xylanase and -glucosidase assays were performed according to the method of Ghose and Bisaria (1987). The reducing sugar concentration was estimated by DNSA method (Miller, 1959).

Results and Discussion

Effect of Incubation Period on Enzyme Production of Trichoderma atrovirideAD-130 : In Figure 1, the effect of incubation period at regular intervals upto seven days on various cellulases and xylanase production by *Trichoderma atroviride*AD-130 on ligno-cellulosic biomass of *Saccharum munja* under submerged cultivation has been shown. FPase, CMCase, β-glucosidase and xylanase activity (U mL^{-1}) ranged from 0.4 to 0.99, 0.71 to 2.09, 0.25 to 0.67 and 41.23 to 65.86 respectively. Trichoderma atrovirideAD-130 gave maximum production of FPase (0.99 U mL^{-1}), CMCase (2.09 U mL^{-1}) and β-glucosidase (0.67 U mL^{-1}) on fifth day while the maximum production of

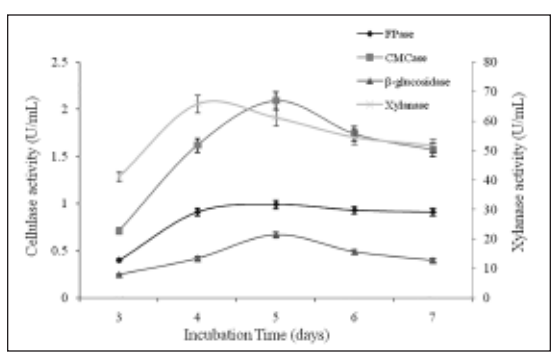

Fig. 1. Effect of incubation period on cellulase and xylanase (U mL^{-1}) *Trichoderma atroviride* AD-130

xylanase (65.86 U mL^{-1}) was achieved on 4th day.

Thus, it could be predicted from the effect of incubation period on the production of various enzymes, *viz*., FPase, CMCase, β-glucosidase and xylanase released by *Trichoderma atroviride*AD-130 under submerged cultivation on lignocellulosic biomass of *Saccharum munja* that the fungus expressed its maximum cellulase activity on 5th day and xylanase activity on 4th day. Corroborative results were obtained by many researchers like Ahmed *et al*. (2008) could achieve maximum production of FPase, CMCase and β-glucosidase from T. Harzianum at 120 hours while Gupta R. et al., (2009) and Sun F. and Chen H. (2007) obtained maximum cellulases production from *A. niger* and *A. phoenix* respectively at 120 hours incubation period and Sukumaran *et al*., (2005) reported maximum xylanase production from *Aspergillus foetidus* on the fourth day while maximum xylanase Production by *Penicillium sclerotiorum* during 5 days was reported by (Zhu, J.Y., and Pan, X.J.,2010). Substantiating the results, other researchers found different incubation times for maximum cellulases production as the time-course required to reach maximum level of cellulases and xylanase activity might be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Gundupalli *et al*., 2022).

Effect of pH on Enzyme Production of *Trichoderma atroviride*AD-130 : Figure 2 also represents the effect of pH on the production of cellulase and xylanase by the fungus *Trichoderma atroviride*AD-130 grown under submerged cultivation using lignocellulosic biomass of *Saccharum munja*. The production of FPase, CMCase, β-glucosidase and xylanase (U mL^{-1}) by *Trichoderma atroviride*AD-130 has been found to be varying from 0.81 to 1.01, 0.78 to 2.69, 0.39 to 0.82 and 39.75 to 82.99. FPase, CMCase, β-glucosidase and xylanase activity of Trichoderma sp. R-4 was found to increase along with the increase in pH upto 6.0. Decrease in production of FPase, CMCase, β-glucosidase and xylanase was observed beyond pH 6.0.

The maximum production of FPase, CMCase, β-glucosidase and xylanase by the fungus *Trichoderma atroviride*AD-130 grown under submerged cultivation at pH 6.0 has been found to be 1.01, 2.69, 0.82 and 82.99 U mL^{-1} respectively.Corroborative results were also obtained by many researchers, for example, Sarkar *et al*., (2012) found that *Aspergillus fumigates* exhibited maximum cellulases and xylanase activity at 4.0 and 5.0 pH respectively, Knob and Carmona (2008) found maximum xylanase production by *Penicillium sclerotiorum* at pH 6.5 while Badal C.S. and Michael A.C. (2008). found highest xylanase production by *Aspergillus niger* at initial pH 7.0. This could have been owing to the change in morphology of microorganism at a particular pH which in turn might have affected the extracellular secretion of enzymes (Arti Devi *et al*., 2022) as the cellulases and xylanase are inducible enzymes and induction efficiency of substrates depends on their chemical composition and morphological structure.

Effect of Temperature on Enzymes Production : Incubation temperature is a very

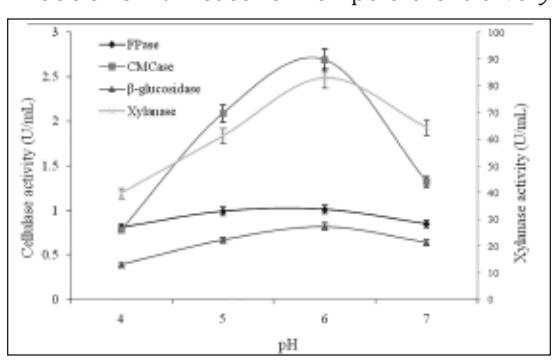

Fig. 2. Effect of pH on cellulase and xylanase production by *Trichoderma atroviride* AD-130

important physical factor which influences the metabolic activities of microorganisms. The effect of temperature on cellulase production was determined by incubating the culture flasks of *Trichoderma atroviride*AD-130 at different temperatures of 24, 27, 30, 33 and 37°C. The initial pH of fermentation medium was adjusted to 6.0 and culture flasks were incubated at 180 rpm. Figure 3 shows the effect of different incubation temperatures (24-37°C) on cellulase and xylanase production by *Trichoderma atroviride*AD-130.

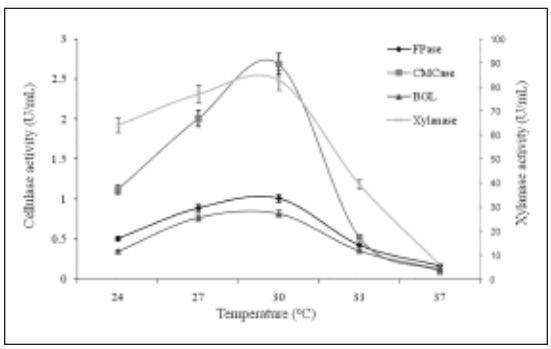

Fig. 3. Effect of temperature on cellulase and xylanase production by *Trichoderma atroviride* AD-130

Cellulase and xylanase production by *Trichoderma atroviride*AD-130 was found to increase gradually when incubation temperature increased from 24 to 30°C. The maximum production of FPase 1.01 U mL^{-1}, CMCase (2.69 U mL^{-1}), β-glucosidase (0.82 U mL^{-1}) and xylanase (82.99 U mL^{-1}) by *Trichoderma atroviride*AD-130 was observed at 30°C. Further increase in incubation temperature from 30 to 33°C or above resulted in decreased cellulase and xylanase production.Contrary to the above results, different cellulolytic fungi were found to produce maximum cellulases at 28°C, for example, *T. Viride* (Zhou *et al*., 2008), *T. Harzianum* (Ahmad Idi and Mohamad S. Eva (2011), T. Asperullum (Chen Y *et al*., 2010) and *A. niger* (Harun M.Y *et al*.,2011) yielded maximum cellulases at 28°C.On the other hand, Baig *et al*. (2004) reported maximum cellulases production by *T. Reesei* at 30°C while (Ahamed, A., and Vermette, P 2008) found maximum cellulases and xylanase activity by Aspergillus fumigates at 30°C. However, as many researchers had reported on variety of optimum temperatures, it could be suggested that the optimal condition for cellulases production depended not only upon the variation of the microorganism (Sukhang *et al*., 2020) but also the temperature variation of the culture conditions.

Conclusions

There is a growing demand for cellulases and xylanases in the market for a variety of applications, among which the bioconversion of lignocellulosic biomass for ethanol production is the fore most one.The results of present study revealed that maximum production of FPase, CMCase, β-glucosidase and xylanase by the fungus *Trichoderma atroviride*AD-130 grown under submerged cultivation at pH 6.0 has been found to be 1.01, 2.69, 0.82 respectively on5thday and xylanase 82.99 U mL^{-1} on 4^{th} day at 2%(w/v) substrate concentration, 30°C, 170 rpm and initial pH 6.0 of the basal medium under SmF. Use of low costlignocellulosic biomass of *Saccharum munja* as substrate make the process of enzyme production more cost effective. Further investigations are required to enhance the cellulolytic enzyme activity by means of carbon and nitrogen sources from submerged fermentation.

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