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PRODUCTION AND OPTIMIZATION OF CELLULASE FROM *TRICHODRMA* ISOLATES UNDER LIQUID STATE FERMENTATION (LSF)

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ABSTRACT: Pure cellulose was used as sole carbon source for the production of cellulase by *Trichoderma* isolates under liquid state fermentation (LSF). Carboxymethyl cellulose (CMC) and Congo Red were used and considered only four *Trichoderma* isolates with stronger ability to produce and optimize cellulase. Cellulase production was assayed by measuring the amount of glucose liberated in µmol/ml/min by using the dinitrosalicylic acid reagent (DNS) assay method at 540nm absorbance. To maximize cellulase production, the critical parameters such as carbon source, nitrogen source, cellulose concentration, cultivation temperature and pH on enzyme production were optimized using LSF. The highest cellulase activity was observed after 12 days of incubation on media containing, yeast extract (1%), cellulose concentration (1%) and pH (5.5) from seven *Trichoderma* isolates under LSF. Cellulase synthesis was repressed in the presence of glucose and fructose while it was induced in the presence of maltose and lactose under LSF. It is evident from the present study that the cellulase production extracted to maximum level from *Trichoderma* isolates was active at temperature ranges of 40–60°C and pH values 4.5–6.5. Yeast extract was the preferred nitrogen source to produce cellulase under LSF; and shaking of the culture improved cellulase production by about 2–3 factors higher than a static culture. This indicated that oxygen supply is the critical factor for the growth and enzyme production by *Trichoderma* isolates. Therefore, these cellulase producing *Trichoderma* isolates can be used in food industries, animal feed industries, brewing and wine making, agriculture biomass refining, pulp and paper industries, textile and laundry industries and ethanol production.

Key words/phrases: Cellulase, CMC, Submerged state fermentation, *Trichoderma* isolates

INTRODUCTION

Cellulases are a group of hydrolytic enzymes which are capable of depolymerizing cellulose to smaller molecules (glucose, fructose, *etc*). The complete degradation of cellulose to simpler sugars requires the action of at least three types of enzymes (Gow and Gadd, 1996): endo-β-1,4-glucanase, exo-β-1,4-glucanase (cellobiohydrolase) and β-glucosidase (Aneja, 2005; Zahri *et al*., 2005; Miettinen-Oinonen, 2007). Cellulases chiefly produced by microorganisms such as fungi, bacteria and actinomycetes. *Trichoderma* species is one of the best-known cellulolytic organisms (Chinedu and Okochi, 2003). *Trichoderma* spp are filamentous fungi belonging to a group of largely asexually reproducing soil fungi; includes a wide spectrum of microorganisms that range from high biodegradation potential for instance (*T. reesei*) to facultative plant symbionts (Chet and Baker, 1981 and Kubicek, 2004). Cellulolytic enzymes have been applicable in many industries such as food industries, animal feed industries, brewing and wine making, agriculture biomass refining, pulp and paper industries, textile and laundry industries and ethanol production. However, the cost of cellulase production and optimization profoundly influences the economics of the entire production process (Bhat, 2000). *T. reesei* have developed strains for commercial scale production of cellulase (Murphy and Horgan, 2005). Currently, these enzymes account for approximately 20% of world enzyme market (Bhat, 2003). Enzymes could be produced by liquid state fermentation (LSF) and solid-state fermentation (SSF); the study used LSF. LSF involves the production of enzymes by microorganisms in a liquid nutrient media. Although the use of cellulases in various industries has been

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increasing very rapidly, the cellulases have mainly been crude mixtures causing unacceptable losses of fabric strength and weight. Furthermore, the un-optimized cellulase composition of commercial preparations and non-optimal dosage of the enzymes have led to low reproducibility of the processes. Furthermore, attempts to use these enzymes in the degradation of cellulosic wastes have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition of the enzymes. The aim of this study is to produce and optimize cellulase from *Trichoderma* isolates under LSF. Therefore, this study has initiated to optimize cultivation conditions for maximum cellulase production, to evaluate the effect of different carbon and nitrogen sources and to determine the optimum working conditions to achieve high enzyme production.

MATERIALS AND METHODS

Test fungal isolates

Seven *Trichoderma* isolates were obtained from mycology laboratory, Department of Microbial, Cellular and Molecular Biology, College Natural Sciences, Addis Ababa University. The isolates of *Trichoderma* used in this study were previously isolated, identified and characterized from soil collected from Jimma Zone (western Ethiopia). They were designated as AUT1-7 where AUT stands for Addis Ababa University *Trichoderma* isolate followed by numbers.

Preparation of inoculum

Potato Dextrose Agar (PDA) (Oxoid) was prepared and poured into the Petri dishes. The preserved *Trichoderma* isolates were transferred on to PDA at pH 5.6 and incubated at 30°C. Cultures were aerobically grown for 7 days. After 7 days of incubation, isolates were transferred on to CM-cellulose containing media for screening the potential cellulase producing *Trichoderma* isolates.

Screening of cellulase producing Trichoderma isolates

To screen the potential cellulase producing *Trichoderma* isolates, enrichment procedure was followed in minimal medium comprising (Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2HO$; 0.5g, KCl; 0.5g, CMC; 5g and peptone; 2g with 15g agar pH 5.5 per litter) (Aneja, 2005). After incubation for 3 to 5 days at 30°C on the above mentioned medium, the plates were flooded with 0.1% Congo Red for 15 minutes. Then after the plates were distained with 1M NaCl for 30 minutes. The isolates that showed clearing zones around the colony were considered as cellulase producing *Trichoderma* isolates.

Liquid state fermentation

Cellulase production was carried out using cellulose as a carbon source under LSF. The composition of the medium was in g/L: (Na_2NO_3; 2g, K_2HPO_4; 1g, $MgSO_4$ $7H_2HO$; 0.5g, KCl; 0.5g, Cellulose; 5g and peptone; 2g) in 250ml Erlenmeyer flask (Aneja, 2005). In all conditions, the culture media were autoclaved for 15min, at 121°C. The autoclaved media was inoculated with two plugs (5mm diameter) of *Trichoderma* isolates from 7 days old culture and incubated under continues shaking at 121rpm (Orbital shaker, Gerhardt, Bonn) for 12 days. Then culture broths were filtered off (Whatman No.1 filter paper) and centrifuged (Hermle, Germany), at 10,000 rpm for 15 minutes to remove cell debris. The supernatants were used to assay cellulase by using 1% DNS (Dinitrosalicyclic Acid reagents) method (Ghose, 1987). The optical density (OD) was measured using UV-Spectrophotometer (JENWAY, 6405 UV/Vis. Spectrophotometer, UK) at 540 nm.

Optimization of cellulase production

Effect of temperature

Trichoderma isolates were grown at different temperature in cellulose broth pH 5.5 (15°C, 20°C, 25°C, 30°C, 35°C and 40°C) for 12 days at 121rpm on a shaking water bath (Precision Scientific Company, Chicago, USA); to determine the optimal temperature.

Effect of pH

The effect of pH on cellulase production was conducted by adjusting the cellulose broth at pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 using 1N NaOH and 1N HCl before fungal inoculation.

Cellulose concentration

To study the effect of cellulose concentration on the production of cellulase by *Trichoderma* isolates, different concentration of cellulose were

prepared (0.5–2%) with an interval of 0.5. Then the inoculated flasks were incubated at room temperature for 12 days at pH 5.5 on a shaking condition (Ul-Huque, 1992).

Effect of carbon sources

The effect of carbon sources on the production of cellulase by *Trichoderma* isolates were evaluated by adding 0.2% of different carbon source (glucose, fructose, maltose and lactose) on a growing media containing 0.3% cellulose, 0.2% $NaNO_3$, 1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.05% KCl, and 0.2% peptone.

Effect of nitrogen sources

The effect of nitrogen sources on the production of cellulase by *Trichoderma* isolates were determined by replacing the existing nitrogen with 1% of different nitrogen sources (peptone, ammonium sulphate, sodium nitrate and yeast extract). The control was prepared in the absence of any nitrogen sources.

Time course of enzyme production

To evaluate the effect of cultivation time on cellulase production, *Trichoderma* isolates were grown at room temperature in shaking condition containing cellulose as the main carbon source and the media was adjusted to pH 5.5. Samples were withdrawn from the culture broth at 2-day intervals over a period of 14 days after the culture inoculated.

Cellulase activity assay

Carboxymethyl cellulase (CMCase) was assayed using a modified method described by Mandels *et al*., in 1976 (cited in Dashtban *et al*., 2010). The activity was determined by mixing 0.1ml of enzyme solution with 0.9ml of 0.5% CMC in 50mM of sodium acetate buffer in a 14ml of test tube, pH 5, vortexed for 1min, incubated for 30 minutes at 50°C. The reaction was stopped by adding 2ml of dinitrosalicyclic acid (DNS). To promote full colour development, the mixture was boiled for 15 minutes (95–100°C) in a boiling water bath and cooled in cold water. The formation of reducing sugars was measured using DNS reagents (Ghose, 1987); spectro-photometeric (JENWAY, 6405 UV/Vis. Spectro photometer, UK), absorbance at 540 nm. One unit of cellulase activity was defined as the amount of enzyme producing 1µm of glucose per minute under the specified assay conditions.

Crude enzyme characterization

For the determination of optimum temperature and temperature stability, activity was determined by carrying out the above standard assay at several temperature values. To determine optimum temperature for cellulase activity, the reaction mixture was incubated for 30min in the temperature range of 20°C–80°C (with an interval of 10°C). For temperature stability, each enzyme was incubated at different temperatures from 20°C–80°C (with an interval of 10°C) for 30min before the addition of substrate and the residual activity was measured following the standard assay conditions. A simultaneously prepared enzyme-buffer mix was stored at 4°C for 30min to be used as a control.

Optimum pH for cellulase activity was determined by assaying the cellulase at different pH values (3 to 10). The buffers used were citrate phosphate buffer (pH 3.0 to 7.0), Tris buffer (7.0 to 9.0) and Glycine-NaOH buffer 50mM (pH 9–10) (with an interval of 1 in all pH ranges). For the determination of pH stability, 100µL of enzyme was mixed with 450µL buffers of varying pH and incubated at room temperature for 30min. Residual activity was measured following the standard assay conditions.

The effect of metal ions

To determine the effect of metal ions; salt solutions were used for source of the metal ions NaCl, KCl, $ZnSO_4$, $MgSO_4$ and $CaCl_2$. The concentrations of the ions in the reaction mixture were 5mM. The enzymes were pre-incubated with different metal ions (Na^+, K^+, Zn^{+2} and Ca^{2+}) for 30min at room temperature, and then the enzyme activity was measured under the standard assay conditions.

Statistical analysis

All experiments and enzyme assays were performed in duplicates. Data were statistically evaluated by Excel and SPSS (version 16) programs and results have been presented as mean ±SEM (standard error mean).

RESULTS

Screening of cellulase producing trichoderma isolates

All *Trichoderma* isolates were positive for CMCase and able to grow in CMC agar media (Fig. 1) but the isolates were differing in their ability to produce cellulose degrading enzymes (Figs 1 and 2). The *Trichoderma* isolate (AUT5) was showed the highest hallow zone on the CMC agar media (75 mm) whereas AUT7 showed the least clear zone diameter (9 mm) (Fig. 1). As a result only four isolates AUT1, AUT2, AUT4 and AUT5 were considered for further studies.

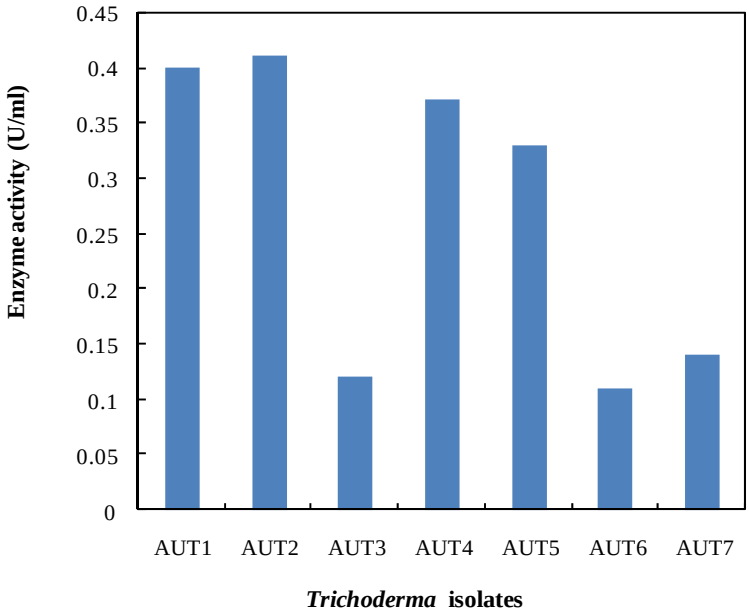

Figure 1. Screening and evaluation of potential cellulases producing *Trichoderma* isolates on CMC agar.

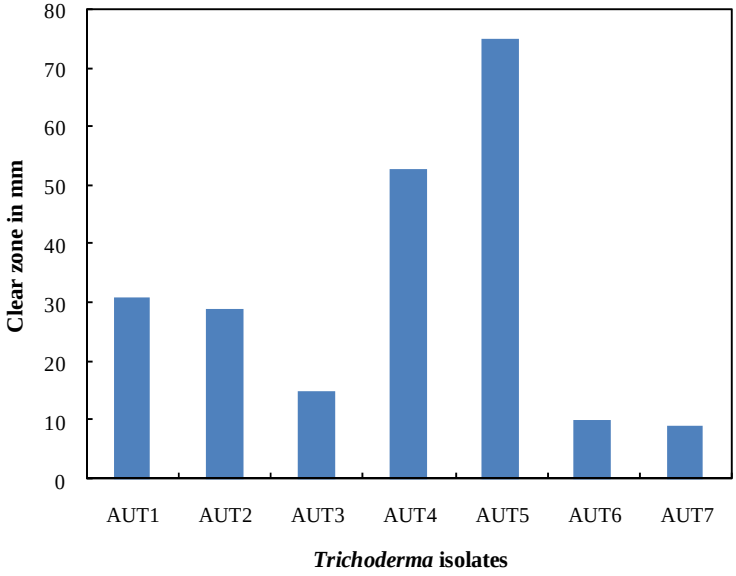

Figure 2. Screening and evaluation of potential cellulases producing *Trichoderma* isolates by DNS assay.

Effect of temperature on cellulase production

The maximum cellulase activities were recorded at 30°C for the selected isolates (Fig. 3). The isolate AUT2 gave the highest cellulase activity (0.544±0.011U/ml) on cellulose broth at 30°C and pH 5.5. Similarly, isolates AUT1, AUT4 and AUT5 produced 0.5025±0.0075 U/ml, 0.4995±0.0045 U/ml and 0.4285±0.0115 U/ml cellulase activity at 30°C and pH values 5.5, respectively. The enzyme activity increased as the temperature increase up to 30°C then after it began to decrease when the temperature rising above 30°C.

Effect of pH on cellulase production

The optimal pH was 5.5 for all isolates (Fig. 4). The cellulase activity of AUT1, AUT2, AUT4 and AUT5, at PH 5.5 were 0.3905±0.0015, 0.3935±0.0045, 0.5805±0.0085 and 0.314±0.0U/ml, respectively. *Trichoderma* isolates AUT4 and AUT5 were showed the highest and the least cellulase activity at pH 5.5, respectively.

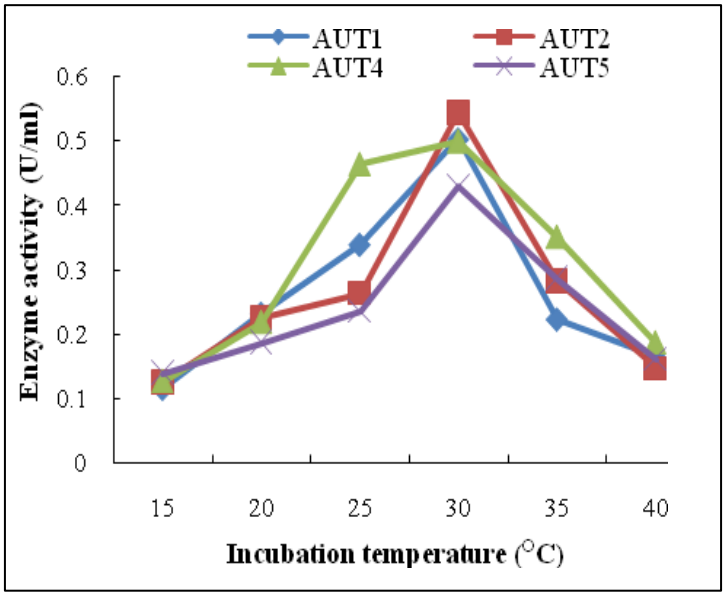

Figure 3. The effect of temperature on the production of cellulase by *Trichoderma* isolates.

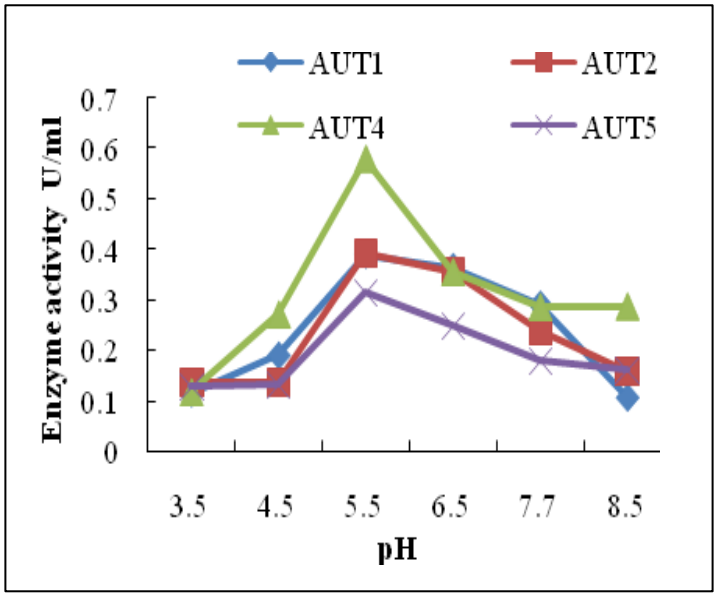

Figure 4. The effect of different pH on the production of cellulase by *Trichoderma* isolates.

Effect of cellulose concentration

It is clearly indicated in Figure 5 that when the concentration of cellulose increased the enzyme activity also increased until the concentration reached at 1%. Maximum cellulase production was obtained at 1% cellulose concentration by all *Trichoderma* isolates. Further, increase in cellulose concentration beyond the level of 1% the production of cellulase begun to decrease. The enzyme activities of the isolates AUT1, AUT2, AUT4 and AUT5 at 1% were 0.625±0.005, 0.609±0.004, 0.785±0.005 and 0.215±0.005U/ml, respectively.

Time course of enzyme production

The cellulase activity increased upto12 days of incubation and decreased, thereafter (Fig. 6). The maximum activity of cellulase was recorded at 12 days by all ***Trichoderma*** isolates but after that a steep decrease was observed by increasing the fermentation time. The enzyme activities of the isolates AUT1, AUT2, AUT4 and AUT5 were 0.3975±0.0055, 0.4033±0.0053, 0.3705±0.0045 and 0.3195±0.0045U/ml, respectively.

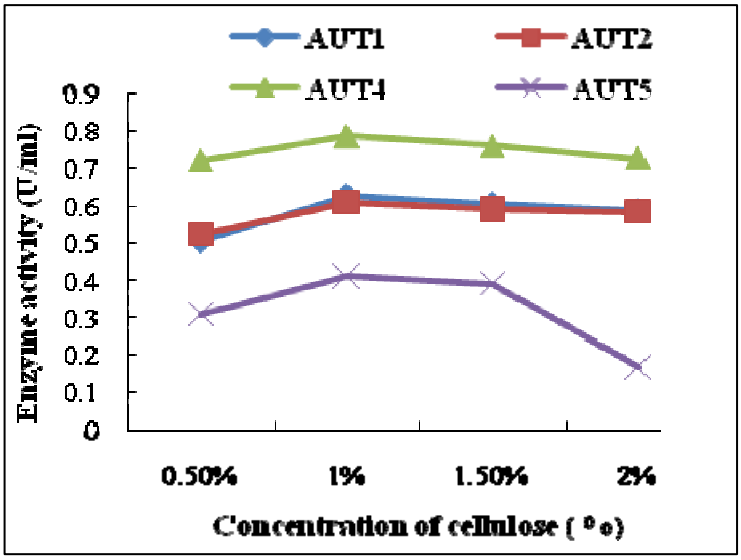

Figure 5. Effect of cellulose concentrations on cellulase production by *Trichoderma* isolates.

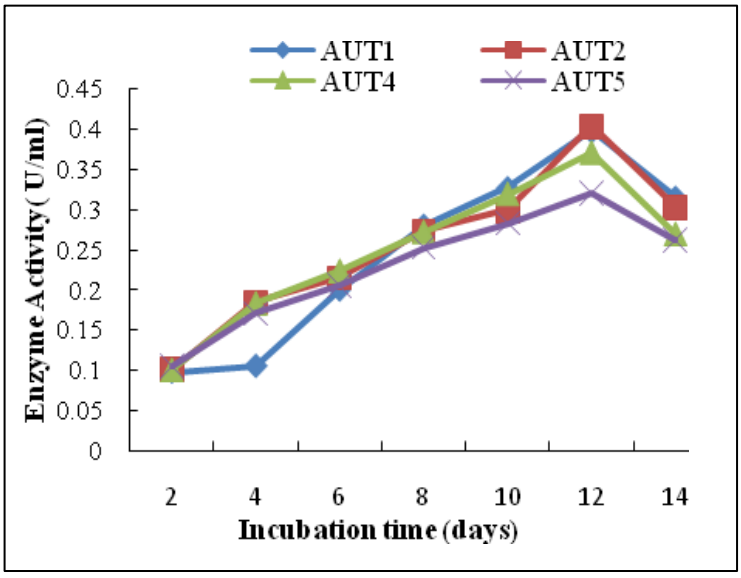

Figure 6. Time course of cellulase production for *Trichoderma* isolates.

Effect of Carbon sources

Carbon source in the medium affects considerably in the synthesis of cellulolytic enzymes by *Trichoderma* isolate in liquid culture (Fig. 7). Lactose and maltose were found to be the good carbon source to induce the production of cellulase by four isolates but glucose and fructose were reduce the production of cellulase. Lactose was a good inducer of cellulase production followed by maltose by all isolates (AUT1, AUT2, AUT4 and AUT5).

Effect of nitrogen s*ources*

The nitrogenous sources have influenced the production of cellulase by *Trichoderma* isolates under LSF. It is evident from Figure 8 that all *Trichoderma* isolates on yeast extract medium was showed the highest cellulase production whereas on sodium nitrate medium showed the least cellulase production. In the control, the cellulase activity was detected.

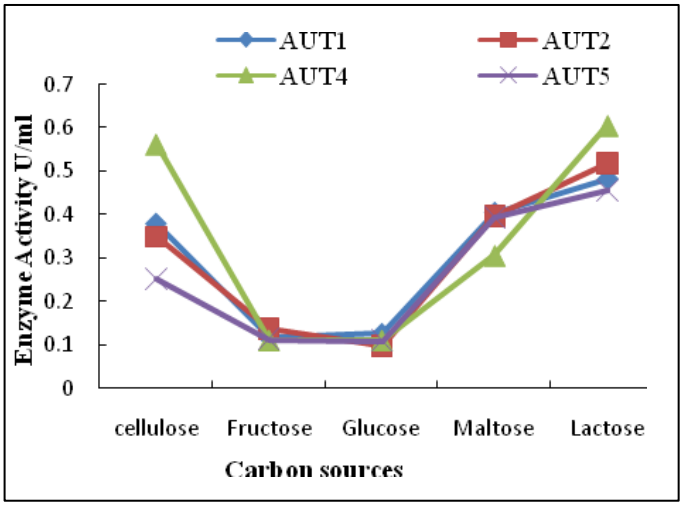

Figure 7. The effect of carbon sources on the production of cellulase by *Trichoderma* isolates.

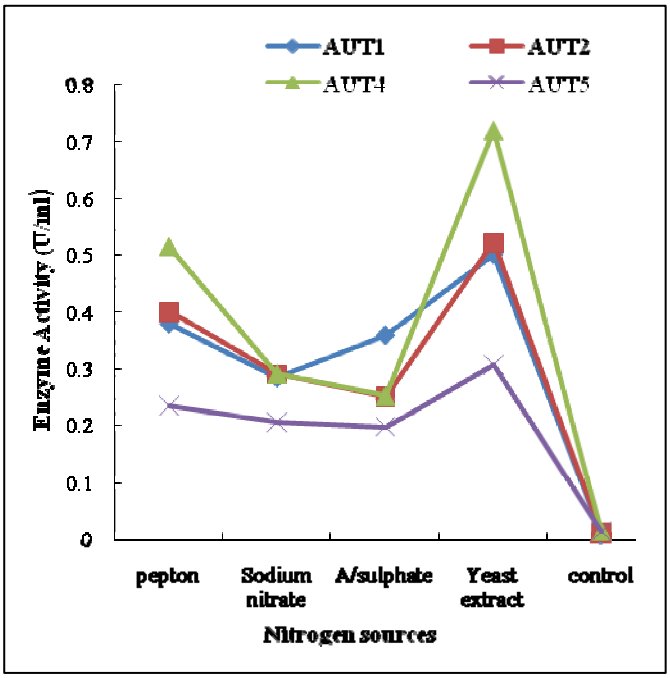

Figure 8. The effect of nitrogen sources on the production of cellulase by *Trichoderma* isolates.

Crude enzyme characterizations

Optimum temperature for activity of cellulase

The optimal temperature for the crude cellulase was found to be 40°C for isolate AUT1, 50°C for isolate AUT2 and 60°C for isolates AUT4 and AUT5 (Fig. 9).

Temperature stability of cellulase

The cellulase was stable at temperature under 40°C for all *Trichoderma* isolates. However, cellulase from AUT1, AUT2, AUT4 and AUT5 maintained 91%, 61%, 52% and 54% activity after 30min of incubation at 40°C, respectively. All isolates at 60°C and above; they retained less than 50% of the enzyme activity as compared to their original activity (Fig. 10).

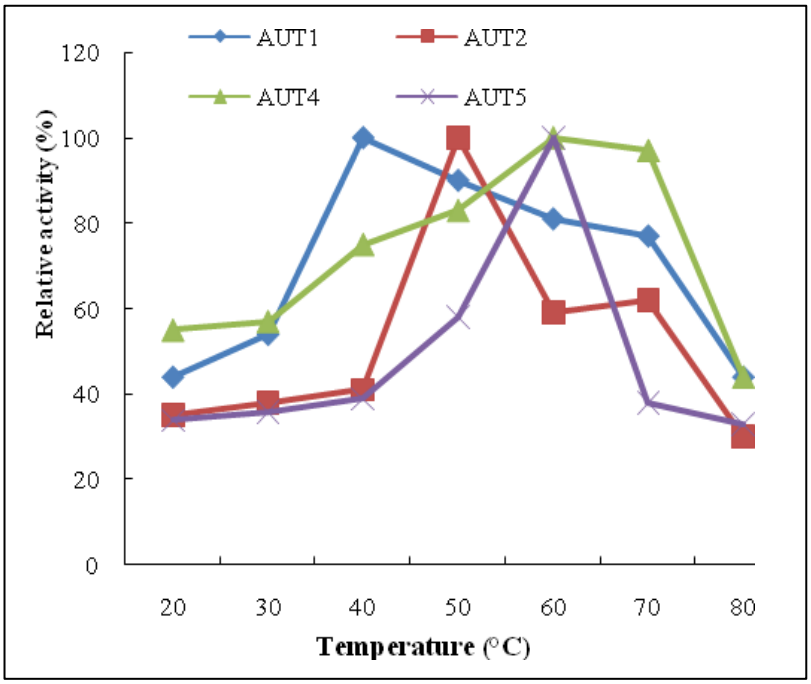

Figure 9. Temperature profile of *Trichoderma* isolates cellulase, assayed at different temperatures.

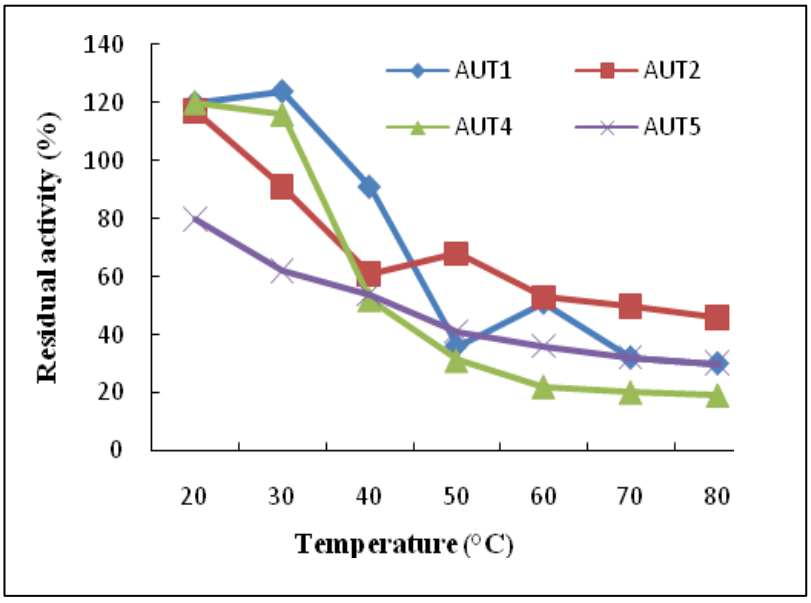

Figure 10. The effect of temperature on the stability of cellulase secreted by *Trichoderma* isolates.

Optimum pH for Activity of Cellulase

The maximum enzymatic activity was obtained at pH range 4.7– 5.4 with optimum pH at 5 for all *Trichoderma* isolates (Fig. 11).

pH stability of cellulase

Trichoderma isolates cellulase was stable in a broad pH range with maximum stability in the pH range of 4.5–6.5 (Fig. 12). However, the enzyme decreased its activity at pH values above 7.5. More than 80% of the residual relative activity retained in the pH ranges of 4.5–6.5. At pH lower than 3 and higher than 11, the enzyme was lost its activity completely.

Effect of metal ions on cellulase activity

The metal ions considerably affect the cellulolytic activity of *Trichoderma* isolates. Of all the metal ions tested Zn^{++} and Mg^{++} brought about a decrease in cellulase activity whereas Ca^{++}, K^{+} and Na^{+} increase the cellulase activity (Table 1). Thus, Ca^{++}, K^{+} and Na^{+} were the recommended cofactors to enhance the cellulase activity of the *Trichoderma* isolates.

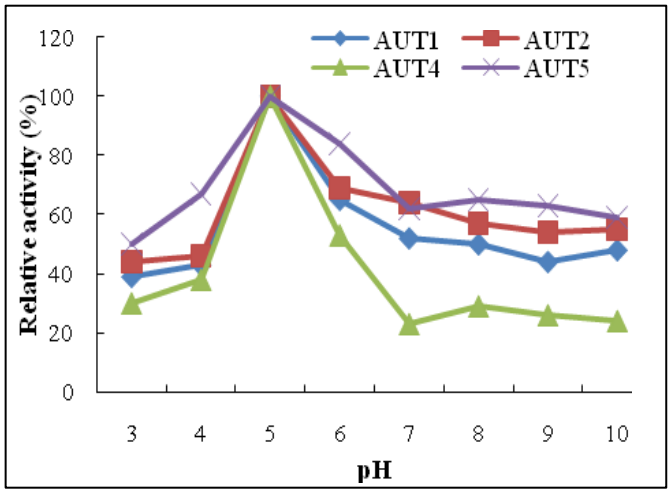

Figure 11. pH profile of *Trichoderma* isolates cellulase at 50°C.

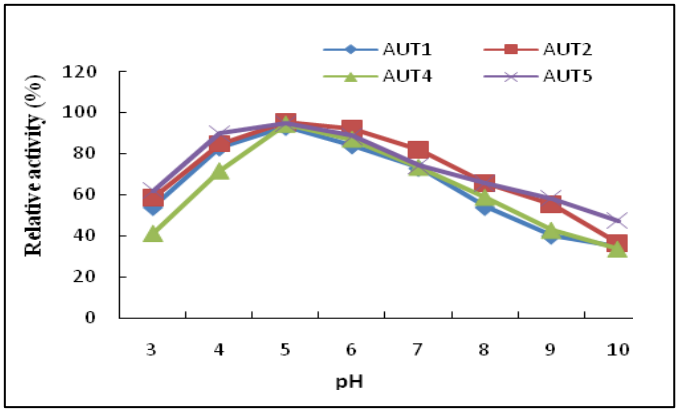

Figure 12. The effect of pH on the stability of *Trichoderma* isolates cellulase extract.

Table 1. The effect of metal ions on the activity of cellulase by *Trichoderma* isolates.

Isolates	Cellulolytic Activity (%of control)					
	Control	Ca^{++}	K^{+}	Mg^{++}	Na^{+}	Zn^{++}
AUT1		112	104.8	87	114	99.4
AUT2	100%	99	108.4	78.6	112.3	78.6
AUT4		101	105	82.8	119.6	78.7
AUT5		125	104	79.9	121	73.2

DISCUSSION

Trichoderma isolates are one of the most important microbial communities responsible for cellulose degradation found abundantly in the plant cell walls. The effect of environmental factors such as temperature, pH and metal ions are found to be important parameters that influence enzyme activities. According to Tholudur *et al*. (1999), cellulase production in cultures is growth associated and is influenced by various factors and their interactions can affect cellulase productivity. Li *et al*. (2009) also stated that optimal temperature for cellulase production depends on the strain variation of the microorganisms as well as the types of nutrient composition. In the present study, the *Trichoderma* isolates were able to grow over a broad range of temperature (15–40°C) and pH (3.5–8.5). However, maximum cellulase production was obtained at 30°C and 5.5 pH. This might be due to better growth of the isolates at these temperature and pH. This result is considerably similar to reported by Shafique and Bajwa (2009) and Li *et al*. (2009) who have indicated that the optimum temperature for maximum cellulase production for *T. reesei* was 30 ± 2°C and the optimum pH for *T. viride* was 5, respectively. However, the result contradicts previous results reported by Gautam *et al*. (2010) who showed that the optimum temperature for cellulase production under LSF is between 40–50°C for *T. viride.* Li *et al*. (2009) has observed that the optimum temperature for cellulase enzyme production from *T. viride* was 50°C. Similarly, Voragena *et al*. (1980) have reported that the optimum pH for maximum cellulase production from *T. viride* was ranges between 4.0 and 5.5. Both high acidic and high basic pH show negative effects, but a medium with low acidic pH, 5.5 was ideal for enzyme production. This might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Haltrich *et al*., 1996).

According to Szakacs *et al*. (2006), cellulases are often inhibited by the presence of high concentrations of their end products. In the present study, lactose and maltose induced the production of cellulase whereas glucose and fructose repress the production of cellulase by *Trichoderma* isolates under LSF. This study, agrees with Szakacs *et al*. (2006) and Baig (2005) who have reported that glucose and fructose represses the production of cellulase activity and lactose, avicel (products of cellulose) and CMC induced the production of cellulase by *Trichoderma* spp. In contrast to the present study, Coban and Biyik (2011) have reported that glucose gave the highest yield, followed by fructose, sucrose and ethanol. Glucose repression of the cellulase system overrides its induction, and de-repression is believed to occur by an induction mechanism mediated by trans-glycosylation of glucose. Yeast extract yielded the highest cellulase production whereas sodium nitrite yielded the least cellulase production. Ahamed and Vermette (2008) have revealed that yeast extract yielded the highest CMCase activity by *Trichoderma reesei* RUT-C30. In contrast to this study, the maximum production of cellulase by *T. viride* was observed in the medium having $NaNO_3$ as the nitrogen source (Khare and Upadhyay, 2011). The organic compounds stimulated higher cellulase yields compared with inorganic compounds. It is believed that simple and organic nitrogen sources like peptone have a stimulatory effect on both the growth rate and cellulase synthesis and thereby shortening the lag phase of the culture (Saxena *et al*., 2007). However, when the organism was grown in liquid media containing inorganic nitrogen sources cellulase productivity was reduced to some extent. This could be attributed to the assumption that ammonium salts may have an inhibitory effect on cellulase production (Saxena *et al*., 2007).

Cellulose with medium viscosity has shown better stimulating effect than cellulose with higher viscosity for cellulase production. High viscosity leads to retard cell division, resulted in low production of metabolites and cellulase secretion (Ul-Haque, 1992). The study found that addition of cellulose at 1% was optimal for cellulase production. It was reported that the optimal cellulose concentration for high cellulase production for *Aspergillus niger* was 1% (Gautam *et al*., 2010), and *T. reesei* was at 0.5–1.5% (Rashid *et al*., 2009). Furthermore increasing the concentration of cellulose beyond the level of 1% resulted in lower cellulase production from *Trichoderma* isolates. This is probably due to the higher viscosity of the medium, which decreases the oxygen supply to the cells. Oxygen is necessary for synthesis of cell membrane components (sterols, non-saturated fatty acids).

Incubation time is one of the most important factors affecting the growth of *Trichoderma* isolates as well as the production of cellulase. This study found that the highest yields of cellulase from the isolates were recorded on the 12thday in SMF using pure cellulose. Khare and Upadhyay (2011) have reported that the maximum production of cellulases by *T. viride* was observed after 6 days of incubation. Sun *et al*. (2010) have observed that the enzyme activity from apple pomace by *Trichoderma* spp. was maximum at 120 hr under LSF. This is probably due to the cease of the growth, the release of simpler sugar and proteases into the medium during the later growth phase. Therefore, it is believed that proper cultivation time allows maximum microorganism growth and product formation to a certain degree in a fermentation system. The time of maximum cellulase production was higher when incubated on pure cellulose compared to agricultural wastes (Ishaque and Kluepfel, 1980).

CONCLUSION

The results from this study have indicated that *Trichoderma* isolates are capable of producing high activities of cellulase (CMCase). The optimum temperature and pH for cellulase enzyme production from *Trichoderma* isolates were 30°C and 5.5, respectively. Yeast extract was the preferred nitrogen source to produce cellulase. The production of cellulase was repressed by simple sugars such as glucose and fructose. Moreover, it may be concluded that the adaptability of the isolates to a wide range of temperatures and pH for its growth and cellulolytic activity, strongly indicates that this *Trichoderma* isolates may be grown in various habitats with different environmental conditions of pH and temperature and can play an important role in cellulose degradation for sustainable development. It will not only help in the production of useful end products including bioethanol from the biodegradation of the low cost enormous stock of cellulose but also help in the disposal of cellulosic wastes which is continuously added to the environment.

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