Studies on the Pretreatment of wheat straw for improve production of Carboxymethyl Cellulase by thermophilic *Trichoderma viride*-FBL1 in Solid State fermentation

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Abstract: Cellulases are important group of enzymes which are used for the conversion of lignocellulosic biomass into a variety of products. *Trichoderma viride*-FBL1 was employed for the production of CMCase enzyme in solid state fermentation using wheat straw as a substrate. The substrate was physico-chemically pretreated with different concentrations of HCl/NaOH to enhance the CMCase yield. 2% HCl for pretreatment of wheat straw was found suitable treatment for maximum enzyme yield. Various cultural conditions were also optimized and optimum parameters found were initial medium pH of 5, incubation temperature of 45^{o}C, substrate level of 15g, initial moisture content of 40%, inoculum size of 5% for seven days of fermentation period. Various extractants such as distilled water, Tab water, Tween-81, 0.2M citrate buffer (pH 4.8) and 0.2M citrate-phosphate buffer (pH 5.0) was used to recover the enzyme from fermented mash and distilled water was found best extractant. The enzyme produced from *Trichoderma viride* show its optimum activity at pH 5 using citrate buffer with incubation time of 15min. [Academia Arena, 2010;2(7):18-30] (ISSN 1553-992X).

Key words: Wheat straw, Pretreatment, CMCase, Trichoderma viride, Solid state fermentation

Introduction

The major polysaccharides (cellulose and hemicellulose) present in the lignocellulosic biomass need to be hydrolyzed with acids or enzymes in order to liberate fermentable sugars (Camassola et al. 2009). In many processes in the enzymatic conversion of lignocellulose biomass to ethanol and other chemical products, a pretreatment stage is required to break the lignin structure and to partially solubilize the polysaccharides (Keller et al. 2003). Cellulose, a major polysaccharide constituent of plant cell walls, is a β-1, 4 linked linear polymer of 8000–12,000 glucose units (Saha 2004) whose natural degradation represents an important part of the carbon cycle within the biosphere. The ability to decompose the cellulosic biomass into glucose, which in turn can be converted into other valuable chemicals and energy (Mukataka et al. 1998) has made cellulases one of the most extensively investigated multicomponent enzyme systems. Cellulases are enzymes that degrade crystalline cellulose to glucose. Three types of cellulases, endoglucanases (EC 3.2.1.4, endo-1,4-β-D-glucanases), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), are considered to be needed to degrade crystalline cellulose to glucose in vivo, and they act synergistically (Henrissat et al. 1985). Endoglucanases are produced by a wide variety of organisms like fungi (Wood 1992) bacteria (Beguin et al. 1992), plants (Ohmiya et al. 1995), and certain insects like termites (Inoue et al. 1997). Fungal endoglucanases are mostly important in the textile and detergent industries, many fungal endoglucanases from members of the subdivision Deuteromycotina, such as *Aspergillus* sp., *Fusarium* sp., *Humicola* sp., *Penicillium* sp., and *Trichoderma* sp., have been purified and characterized (Murashima et al. 2002). Among the cellulolytic fungi, Trichoderma and Aspergillus have been extensively studied particularly due to their ability to secrete cellulose-degrading enzymes (Adsul et al. 2007). Cellulases have been investigated mainly with respect to their industrial use for the bioconversion of agricultural biomass resources into useful products (George et al. 2001) and are commonly used in various industries, including food, brewery and wine, agriculture, textile, detergent, animal feed, starch processing, extraction of fruit and vegetable juices pulp and paper, as well as in research development (Jan and Chen 2003, Gao et al. 2008,Zhou et al. 2008).

Material and Methods

Lignocellulosic Biomass

Wheat straw was purchased from local market of Lahore city, Pakistan which was used as a substrate for the production of CMCase. The substrate was washed and oven dried at 65^{o}C and

then ground to powder form (2mm) by hammer beater mill.

Pretreatment of Substrate

Wheat straw samples (10g) were soaked in different concentration of NaOH and HCl ranging from 1-4% solution at the ratio of 1, 10 (solid, liquid) for 2hr at room temperature (Solomon et al.1999, Gharpuray et al. 1983). After then the samples were autoclaved at 121^{o}C for 15 min. Then samples were filtered and solid residues were washed up to neutrality.

Microorganism

Trichoderma viridi- FBL1 was obtained from Fermentation Biotechnology Laboratory, PCSIR Labs. Complex Ferozpure road Lahore, and was used for the production of CMCase. It was maintained on PDA slants and revived biweekly.

Inoculum Preparation

Inoculum was prepared by adding sterilized distilled water into the 5-day old slant. With the help of inoculating loop the mycelia was mixed and one ml (2 x 10^{8}) of spore suspension was used as inoculum. Inoculum size was measured with heamacytometer as described by Sharma (1989).

Fermentation Methodology

Solid state fermentation was carried out for CMCase production using *Trichoderma viridi*-FBL1. In 250ml conical flask 5g of ground pretreated wheat straw was moistened with diluent (g/l, ammonium sulphate 10. Calcium chloride 0.5, Magnecium sulphate 0.5, Potassium dihydrogen phosphate 4) and then autoclaved at 121^{o}C for 15 min. After sterilization the media was inoculated with 1ml of spore suspension and incubated at 30 $\pm$1 ^{o}C.

Optimization of Initial Cultural conditions

Different cultural conditions like fermentation period (24, 48, 72, 96, 120, 144hr) initial medium pH (4,4.5, 5, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) Temperature (25, 30, 35, 40, 45, 50^{o}C) substrate concentration (5,10,15,20,25and30g) Inoculum size (5, 10,15,20,25,30%) moisture level (20, 40, 60, 80 and 100) were optimized for CMCase production using *Trichoderma viride*-FBL1.

Analytical Methods

Proximate Analysis of Substrate

Total carbohydrates were estimated by the method of Duboise et al. 1956. The moisture, ash contents of the substrate were determined by the methods of AOAC (2005). The lignin content in the biomass was estimated by the method of Milagres (1994). Cellulose was estimated by the methods described by Gopal and Ranjhan (1980).

Enzyme Recovery

CMCase from the fermented mash was extracted by simple contact method as reported by Krishna and Chandrasekaran (1996). In 5g substrate of flask 50ml of distilled water (1,10 solid to liquid ratio) was mixed and placed on shaker at the agitation speed of 150 rpm for 2hrs. After complete mixing it was filtered through muslin cloth and the residues was discarded and the filtrate was used for further analysis.

Estimation of CMCase

500µl of the enzyme sample along with 500µl of 1% (w/v) CMC in 50 mM acetate buffer pH 5 was incubated, in a water bath at 50 °C for 30 min. After incubation 3ml of DNS was added and boiled for 5 minutes and absorbance was taken spectrophotometrically at 540nm. The reducing ends liberated were then measured with DNS (Wood and Bhat 1988)

Effect of pH on enzyme activity

The effect of different pH on CMCase activity was determined by incubating the reaction mixture at different pH values. The pH was adjusted using the following buffers Citrate phosphate (pH 4.8-5.5), Phosphate (pH 6.0-7.0), and Tris-HCl (pH 8.0-9.0). Reaction mixtures were incubated at 50°C for 15 minutes, and the activity of the enzyme was measured spectrophotometrically.

Effect of Temperature on Enzyme activity

The activity of the crude enzyme was measured at different temperature (40, 45, 50, 55, 60, 65, 70, 75 and 80 ^{o}C) for 30 minutes. The reaction mixture was assayed and the CMCase activity was measured with standard assay procedures.

Estimation of Total Proteins

Total proteins in the culture filtrate were estimated by Lowery (1951) method using bovine serum albumin as standard protein.

RESULTS

The proximate analysis of the wheat straw indicated that it contained 39.4% cellulose and 17.3% lignin (table 1).

Table. 1. Proximate analysis of wheat straw

Component	Dry weight (%)
Cellulose	39.4± 0.21
Lignin	17.3± 0.13
Total Carbohydrates	1.54± 0.06
Total Protein	1.57± 0.01
Moisture	5.074± 0.24
Ash	7.201± 0.32

Effect of Pretreatment on CMCase Production

Wheat straw, a lignocellulosic material used in present study contains highly crystalline cellulose and large amounts of arabinoxylan, the major hemicellulosic fraction. Results indicated that (Fig.1A) 2% HCl gave maximum yield (12.8 ± 0.43 U/g) of enzyme while the 4% NaOH gave the enzyme yield of 9.2 ± 0.21 U/g. As the concentration of acid increased decline in enzyme production was observed. Figure 1B describe the general comparison of untreated, acid and base treated wheat straw on CMCase production.

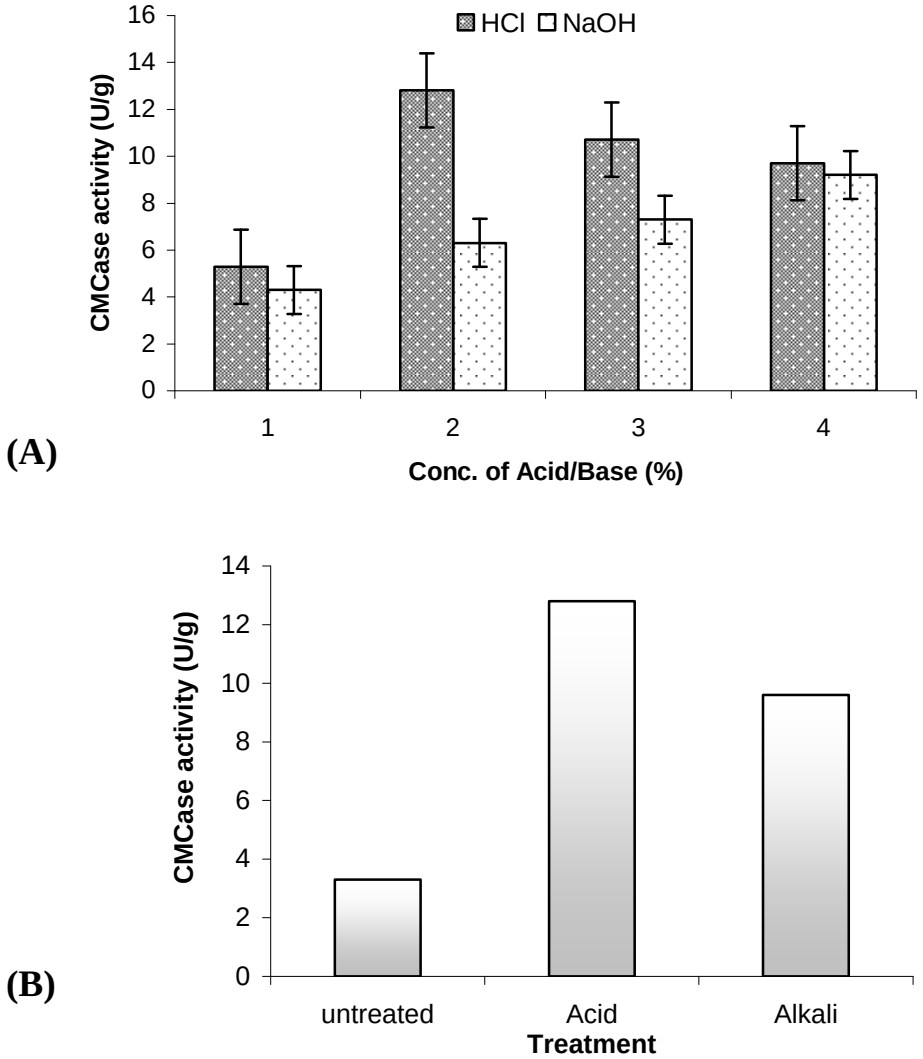


Figure 1. (A) Effect of Different concentration of NaOH/HCl pretreatment on CMCase production from *Trichoderma viride*-FBL1. (B) Comparison of untreated, acid treated and base treated wheat straw on CMCase production.

Effect of different Leaching agent on CMCase activity

The recovery of the enzyme from the fermented mash is very critical hence selection of a suitable extractant is necessary. Different leaching agents were used in this study were distilled water, Tap water, Tween-81, 0.2M citrate buffer (pH 4.8) and 0.2M citrate-phosphate buffer (pH 5.0). It was noted (Fig. 2) that maximum enzyme recovery was obtained with distilled water (14.85 ± 0.12U/g) followed by Tween-81(12.08 ± 0.81 U/g). Tap water and different buffers slightly reduced the enzyme recoveries.

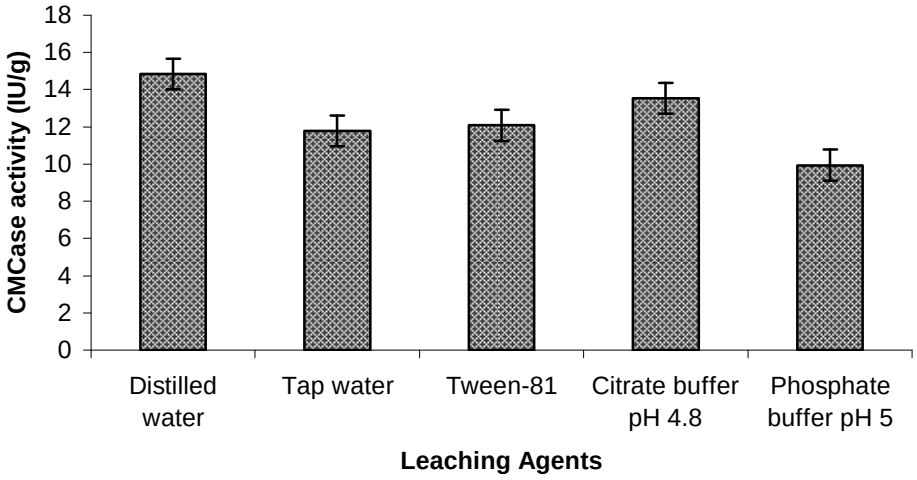


Figure 2. Effect of Different leaching agents on the recovery of CMCase from fermented sugar cane bagasse by *Trichoderma viride*-FBL1 at 30 ^{o}C for seven days.

Time coarse study of CMCase production

Different fermentation experiments were carried out to study the secretion time of CMCase by *Trichoderma viride*-FBL1. Maximum CMCase secretion was obtained during the seventh day of incubation period yielding 8.6 ± 0.32 IU/g. Further increase or decrease in cultivation time reduce the enzyme production (Fig. 3).

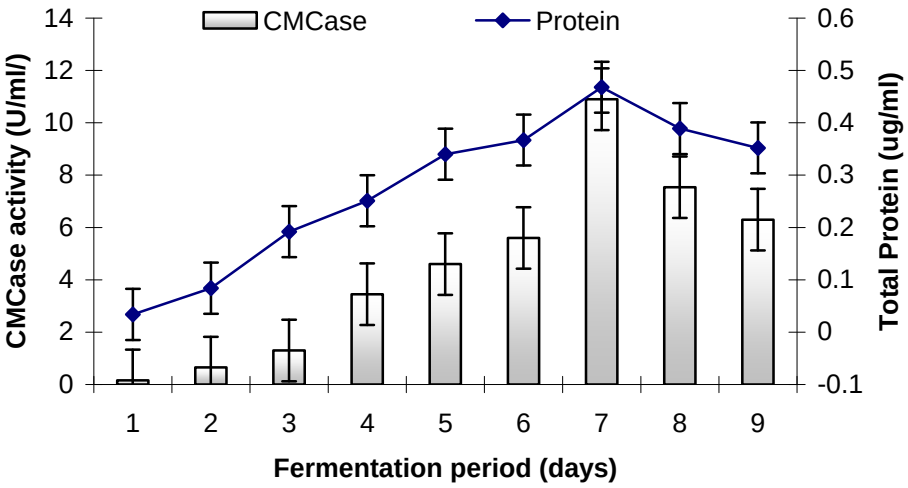


Figure 3. Effect of Different fermentation periods for CMCase production from *Trichoderma viride*-FBL1.

Effect of Initial Medium pH

Different sets of experiments were carried out to test the optimum pH for CMCase production. The initial pH of the medium was varied from 3-8 and adjusted with 1N NaOH/HCl before sterilization and each experiment was carried in triplicates. From these experiments it was observed that maximum CMCase production was found at pH 5 yielding 13.67 ± 0.76 U/g (Fig. 4). Further increase or decrease in initial medium pH affects the enzyme synthesis.

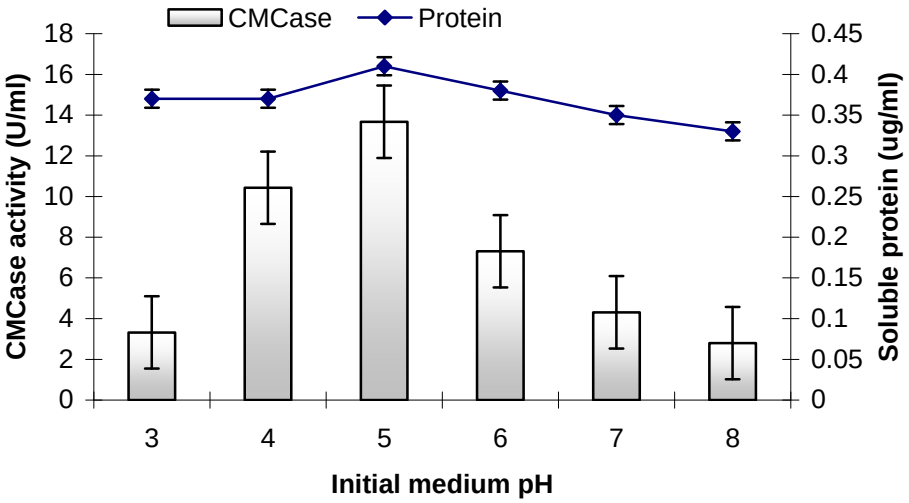


Figure 4. Effect of Different initial medium pH for CMCase production from *Trichoderma viride*-FBL1.

Effect of incubation Temperature

Incubation temperature and pH is very important in enzyme production (Smits et al. 1996). To investigate the optimum incubation temperature for CMCase production a set of experiments were carried out ranging from 25 to 50^{o}C as shown in the figure 5. From the data it was observed that 40^{o}C was found best for CMCase secretion and producing 0.68 mg/ml of protein in the fermented mash.

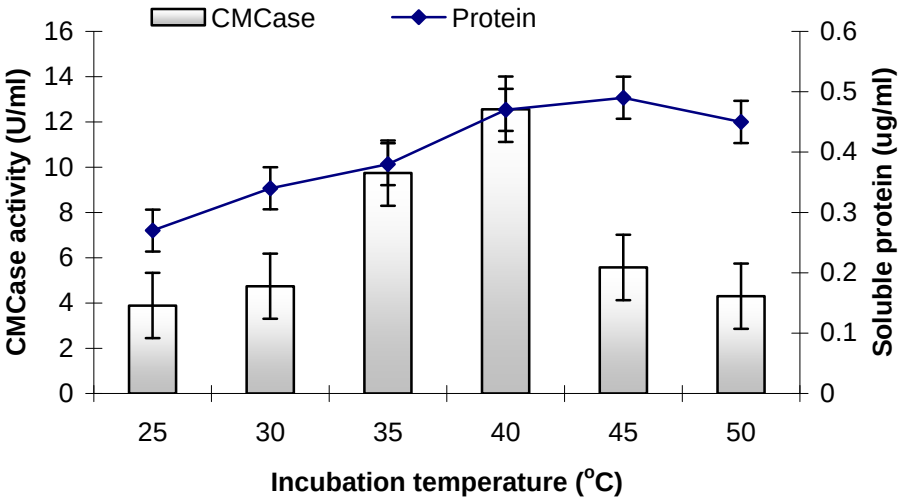


Figure 5. Effect of Different incubation temperatures on CMCase production from *Trichoderma viride*-FBL1.

Effect of Substrate

To investigate the optimum substrate concentration for CMCase production, experiments with different substrate level was conducted and it was observed that 15% substrate concentration was found optimum (Fig. 6).

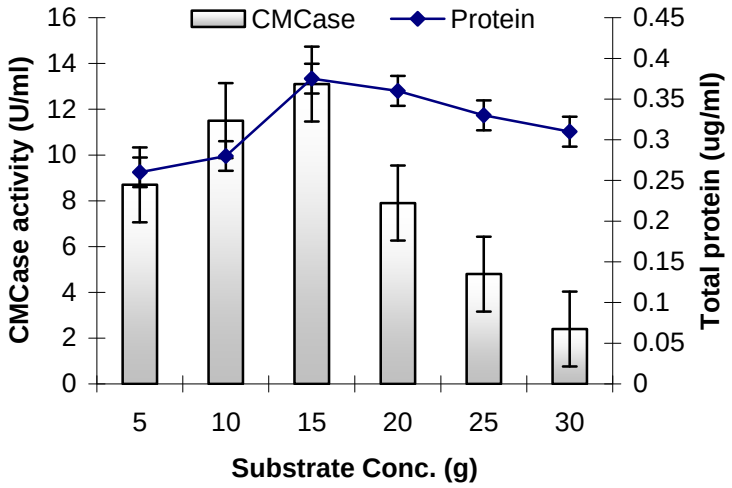


Figure 6. Effect of Different substrate concentrations for CMCase production from *Trichoderma viride*-FBL1.

Effect of Inoculum size

Effect of inoculum size on CMCase production was shown in the figure 7 which indicated that 5% inoculum size gave maximum enzyme yield (12.2 ± 0.21 U/g).

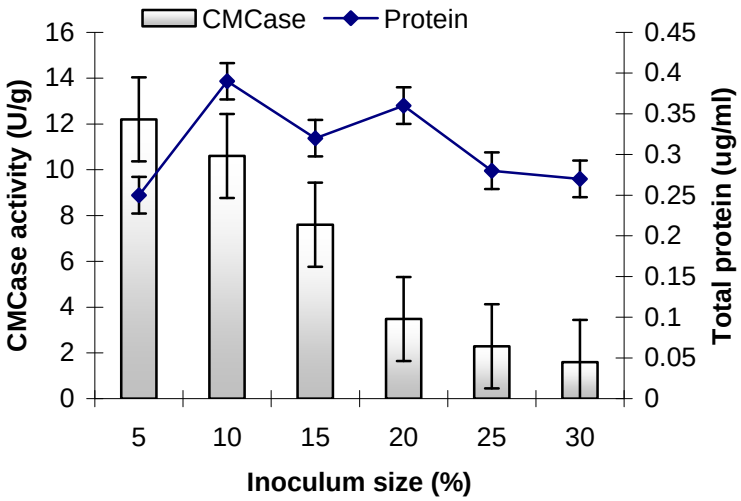


Figure 7. Effect of Different inoculum size on CMCase production from *Trichoderma viride*-FBL1.

Effect of Moisture Level

For CMCase production moisture level was optimized and found that 40% moisture level was best for production as shown in the figure 8. Increased moisture level lowered the enzyme production.

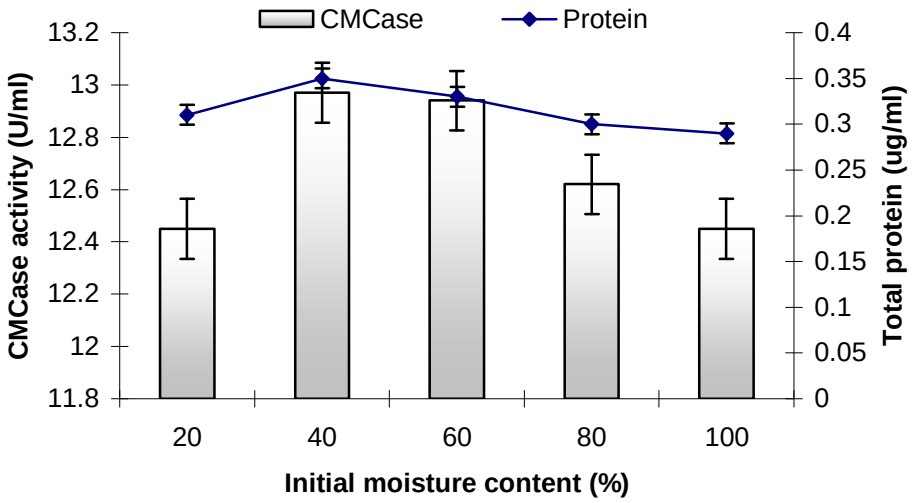


Figure 8. Effect of moisture level on CMCase production from *Trichoderma viride*-FBL1.

Effect of pH on enzyme activity

The CMCase activity was determined at different pH values ranging from 4-9. Maximum activity (16.2 ± 0.32 U/ml) was obtained at pH 5.5 using citrate phosphate buffer, as the pH of the substrate solution was increased the activity was also increased but increased pH toward alkalinity reduces the enzyme activity as shown in the Fig.8. These findings indicated that enzyme produced by *Trichoderma viride* was acidic in nature.

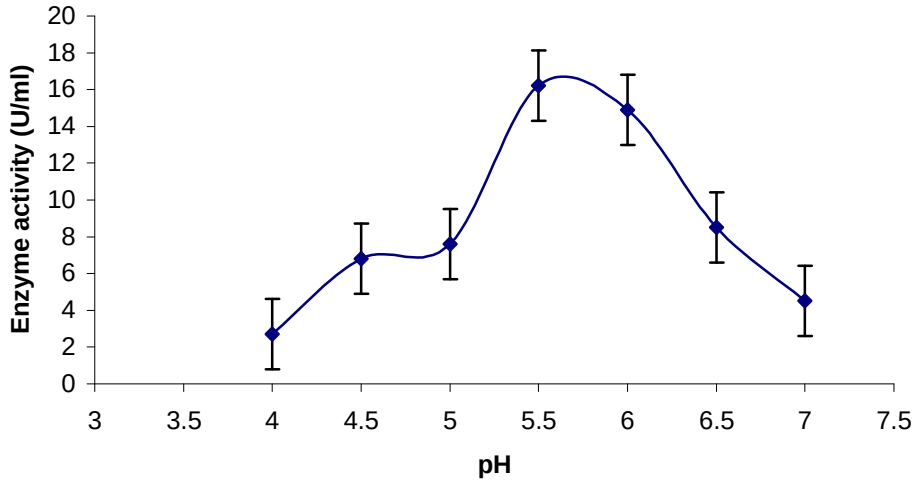
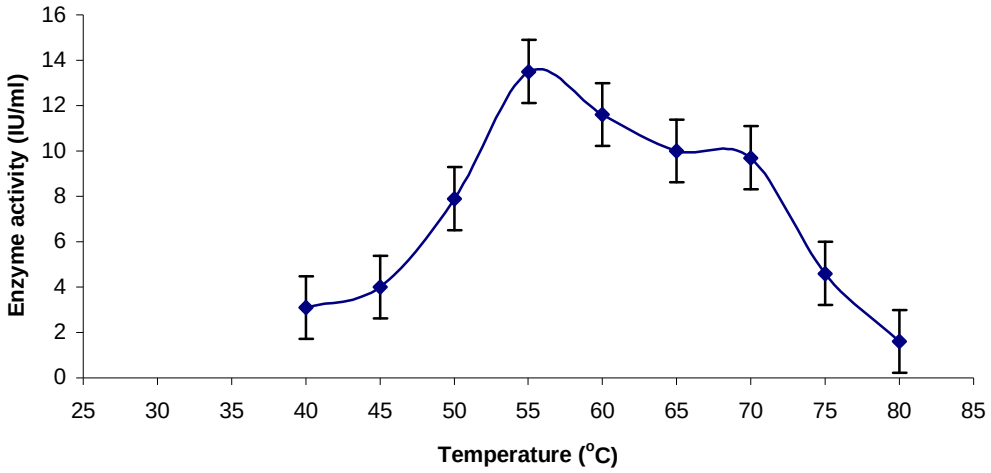


Figure 9. Effect of different pH on CMCase activities isolated from *Trichoderma viride*-FBL1

Effect of Incubation Temperature on CMCase activity

The CMCase enzyme isolated from *Trichoderma viride* was incubated at different incubating temperatures ranging from40- 80° C to check the optimum temperature of enzyme. It was observed that the enzyme activity was gradually increased by increasing temperature and optimum activity (13.5 ± 0.14 U/ml) was obtained at 55°C but as the incubation temperature increases the enzyme activity decreases as shown in the figure 10. At 80°C temperature the enzyme activity was 1.6 ± 0.21 U/ml.



Figure, 10 Effect of incubation temperature on CMCase activities isolated from *Trichoderma viride*-FBL1

DISCUSSION

Already available information of the properties of biomass materials is useful in order to evaluate their suitability as chemical feedstock in different processes. Many studies show that wood morphology and chemical composition vary with location, genetics, and growth conditions. In Pakistan different varieties of wheat straw contained holocellulose 58.5% and lignin 16-17% and ash content in the range of 7.5-8.5 (Ali et al. 1991). Indian wheat straw contained lignin and ash content of 23.0 and 9.99 respectively (Mohan et al. 1988). Norwegians wheat straw consists of 16-20% lignin and 4-9% of ash (Utne and Hegbom 1992). American and Denmark wheat straw contained 16.7 and 20.5% of lignin and 6.6 and 3.7% of ash respectively (Misra1987). Some workers (Reshamwala et al. 1995, Cheung and Anderson 1997, Dewes and Hunsche 1998, Boopathy 1998) reported that wheat straw consist of 30% of cellulose and 50% of hemicellulose and 15% of lignin content.

In the present study wheat straw was pretreated with different concentrations of NaOH and HCl. Main purpose of the treatments was to delignify the substrate. Greater the delignification more cellulose was exposed which was freely available to microbes and hence greater enzyme production. 2% HCl was found optimum for maximum enzyme production. Increase in acid concentration decreased the enzyme production. The enzyme activities decreased which might be due to the formation of such compounds which have inhibitory action on enzyme synthesis by the fungus.

In acid treated samples CMCase production was better which might be due to the rapid hydrolysis of cell wall component into free sugars which are available to the microorganism. In base treated samples enzyme production was comparatively low as compared to acid treated samples. This low production of enzyme might be due to alkalinity in substrate during alkaline treatment which inhibits the growth of the fungus. The major purpose of the pretreatment is to reduce the cellulose crystalinity which might be reduced by various techniques. Steam treatment has been reported to both decrease cellulose crystallinity index and completely solubilize hemicellulose (Puri 1984, Dekker 1985). Moreover, solubilized sugars in steam-treated lignocellulosics are known to occur as a mixture of oligomers (Overend and Chornet 1987). However, the degree of polymerization (DP) of the soluble sugar fraction varied significantly according to the treatment applied. Both the concentration of acid and treatment reaction time was important factors in determining degree of polymerization. Therefore, an enzyme preparation designed to breakdown cell wall

polysaccharides and derived oligomers in steam-treated wheat straw into a mixture of monomeric sugars would require high enzymic activity against crystalline cellulose, arabinoxylan, and xylo-oligomers.

If more concentration of H_2SO_4 (>4.5% H2SO4 on DM basis) was applied, more hemicellulose (>95%) contents should be hydrolyzed (Cunningham et al. 1984, Grohmann et al.1985). Two further aspects that might have influenced the response of the steam treatment are; a) type of substrate and b) amount of water might have influenced the efficiency of heat transfer and acid impregnation. Previous studies (Wong et al. 1988, Grous et al. 1986) have shown that in addition to changes in chemical composition alteration in physical microstructure occurred during treatment. Such physical changes are also partly responsible for improving cell wall hydrolysis by cell-free enzymes. Cell wall swelling can also be affected by impregnating the substrate with sulphuric acid prior the treatment and thereby greater cellulose hydrolysis by cell-free enzymes was achieved (Toussaint et al. 1991).

Pretreatment with dilute hydrochloric acid were also investigated by many workers (Israilides et al. 1978, Mehlberg and Tsao 1979). The major advantage of dilute acid pretreatment is that higher yield of xylose was obtained in this process. Alkaline pretreatments are basically delignification processes and significant amount of hemicelluloses were solublised during the treatment (Millet et al. 1976, Goel and Ramachandram 1983). In comparison with acid – base pretreatment, base is more expansive than acid and concentration of alkali used is generally comparable to or higher than that of the acid (Chemical Marketing reporter, 1994).

Enzyme recovery from the fermented mash is a critical problem. To solve this problem different extractants were used, distilled water followed by Tween-80 and citrate buffer found best extractants. Ikram-ul-Haq et al. 2003 stated that the chemical composition of the buffer might show inhibitory effect on the enzyme activity. Aikat and Bhattacharyya, (2000) also reported highest enzyme yield when potassium phosphate buffer pH 8.0 was used as an extractant, which showed comparatively less activity than distilled water extraction.

Incubation time also affects the enzyme production .As the incubation period increased there might be the depletion of nutrients and production of certain toxic metabolites which inhibit the further microbial growth and physiology resulting in the inactivation of secretary machinery of the enzymes (Nochure et al. 1993). Enzyme production in short incubation period offers the potential for inexpensive production and it varies from enzyme to enzyme from single substrate (Sonjoy et al. 1995). It was found that incubation period needed for enzyme production is shorter in solid state fermentation than in submerged fermentation process (Macris et al. 1989, Illanes et al. 1992, Jiafa et al. 1993). Ahmed at al. 2009 reported the time course of 5days for endoglucanase production from *Trichoderma harzianium.* Gomes et al. 2006 also reported the maximum time of CMCase production of 7days of fermentation period for *T.viride* which was similar to our findings. Zhang et al. 1999 reported the maximum production of cellulase after 6days of fermentation period. Ogel et al. 2001 reported that time course required to reach maximum levels of activity may be affected by several factors, like the presence of different ratios of amorphous to crystalline cellulose.

Optimum pH is very important for growth and metabolic activities of microorganism. Best enzyme production was observed at pH 5. Different workers (Ahmad et al 2009, Margaritis and Merchant 1986, Xia and Cen 1999, Kocher et al. 2008) reported that initial medium pH in the range of 4.5-5.5 was optimum for carboxymethyl cellulose production. Liu and Yang (2007) stated that initial medium pH has strong influence on enzyme production, if the pH is too low or high there was poor growth and the optimum growth and enzyme production was observed at pH 5. Most fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Haltrich et al 1996).

Ahmed at al 2009 reported the optimum fermentation temperature of 28^{o}C for endoglucanase production from *Trichoderma harzianium.* Stutzenberger (1971) studied on cellulytic activities of *Thermonospora curvata* using solid municipal waste as a substrate and reported the optimum cellulose production at 45^{o}C. Margaritis and Merchant (1986) reported the incubation temperature of 44-55^{o}C was optimal for cellulase production which was near to our findings.

Selection of a proper substrate is another key aspect of SSF. In SSF, solid material is non-soluble that acts both as physical support and source of nutrients. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support (Pandey 2003). Low quantity of substrate exhibit low fungal growth and cellulase accumulation while higher substrate level may show extensive growth but cellulase accumulation was reduced. Haq et al. 2006 reported that 10% substrate level was best for CMCase production by using *Trichoderma viride*. Xia and Cen (1999) reported that 30% substrate was best for cellulase accumulation.

Number and density of spores is an important factor in the fermentation experiments. Inoculum size controls and shortens the lag phase, smaller inoculum size increased the lag phase whereas the larger inoculum size increases the moisture content which ultimately decreased the growth and enzyme production (Sharma et al. 1996). The pretreated wheat straw had maximum enzyme production with 10% of inoculum size which was in good agreement with our findings (Fadel 2000). Omojasola and Jilani (2009) worked on cellulose production and reported that maximum glucose production was observed with 8% inoculum size.

Every microorganism requires some moist environment for their growth. In SSF the optimal moisture content depends on the requirement of microorganism, type of the substrate and the types of end products (Kalogeris et al. 2003b). Gao et al. 2008 reported the moisture level of 80% was best for enzyme production. Xia and Cen (1999) reported that cellulase production was maximum when water content of 70% was acquired in koji fermentation. Alam et al. 2005 also reported the moisture level of 50 % was best for CMCase production. High moisture level enhanced fungal growth and cellulase production when lignocellulosic substrates were the carbon sources in the SSF (Kalogeris et al. 2003b, Panagiotou et al. 2003). The demand of moisture level in solid state fermentation differs on enzyme production, substrate, microorganism and particle size of the substrate (Sharma 1989, Fadel 2000, Omojasola and Jilani 2009, Kalogeris et al. 2003b). When the moisture level was too increased the media become clumped and there is poor aeration and poor growth so the enzyme production will decrease (Alam et al. 2005). Muniswaran and Charyulu (1994) observed that high moisture level increases the free excess liquid in the medium which ultimately decrease in growth and enzyme production.

Cellulase enzyme extracted from termite showed the optimum pH of 6.2 (Purwadaria et al. 2003). Peciulyte (2007) isolated cellulolytic fungi from waste paper gradual recycling materials and stated the optimum pH of 4.5, 5.5, 6.5 and 6.0 for *Aspergillus niger* DPK-cl-12, *Gliomastix murorum* var. *murorum*, *Stachybotrys chartarum* DPK-cl-111 and *Penicillium funiculosum* DPK-cl-19 rspectively at 30^{o}C. In an other study (Soni et al. 2008) a wide variation was seen in optimum pH of CMCase produced by different fungus such *Aspergillus sp* showing optimum pH of 6.0, *A. terreus* pH 6.0 and *M.fergusii* T41 showing the optimum pH of 4.0. Lee et al 2008 purify and characterize the cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull and reported the optimum pH of 7.0. Optimum pH of 6.5 for cellulose was also reported by Kim et al. 2009 which were isolated from marine bacterium *Bacillus subtilis* subsp. *subtilis* A-53. The wide variation in pH might be due to the different substrates and different microbial origin.

Cellulase enzyme from different microbes show its highest activities at different temperatures. Cellulase enzymes of termites have optimum temperature in the range of 45-50^{o}C (Purwadaria et al. 2003). In another study (Kim et al. 2009) the CMCase isolated from *Bacillus* sp show the optimum temperature of 50^{o}C. Temperature profile of endoglucanase from *Penicillium chrysogenum* showed that the enzyme was most active at temperature of 48^{o}C (Nwodo et al. 2008). Our findings were in good agreement with Gomes et al. 2006.

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