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Chapter 4

Fungal Biodegradation of Agro-Industrial Waste

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Additional information is available at the end of the chapter

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1. Introduction

Fungi possess an efficient hydrolytic system capable to convert lignocellulosic material to essential metabolites for growth. Usually, these fungi secrete enzymes, including cellulases (cellobiohydrolases, endoglucanases), hemicellulases (xylanases) and β-glycosidases. In terms of enzyme novelty, interest is focused on not only finding enzymes which could break down lignocellulose much more rapidly, but also enzymes which could withstand pH, temperature and inhibitory agents. Mutant strains of *Trichoderma reesei* have been selected that produce extracellular cellulases up to 35 g/l [1,2]. It has been suggested that increasing the specific enzyme activity is the most likely approach to improving the commercial prospects of lignocellulose hydrolysis [3].

Lignocellulose consists of lignin, hemicellulose and cellulose [4,5]. The major components of lignocellulosic biomass are cellulose (C6 sugars), hemicellulose (C6 and C5 sugars) and lignins (polyphenols) [6]. Large amounts of lignocellulosic "waste" are generated through agricultural practices, paper-pulp industries, and can pose an environmental pollution problem. Lignocellulosic waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon [7]. However, plant biomass considered as "waste" can potentially be converted into various different value added products as illustrated in (Fig 1)including biofuels, chemicals, animal feed, textile and laundry, pulp and paper [8,9,10,11]. Production of ethanol and other alternative fuels from lignocellulosic biomass can reduce urban air pollution, decrease the release of carbon dioxide in the atmosphere, and provide new markets for agricultural wastes [12].

One effective approach to reduce the cost of enzyme production is to replace pure cellulose with relatively cheaper substrates, such as lignocellulosic materials. There are reports of successful attempts to produce cellulases on lignocellulosic materials [13]. Environmental pollution is a worldwide threat to public health. Biological degradation, for both economic and ecological reasons, has become an increasingly popular treatment of agricultural and

industrial waste [14]. Continuous accumulation of industrial wastes poses a serious environmental problem [15,14,16].

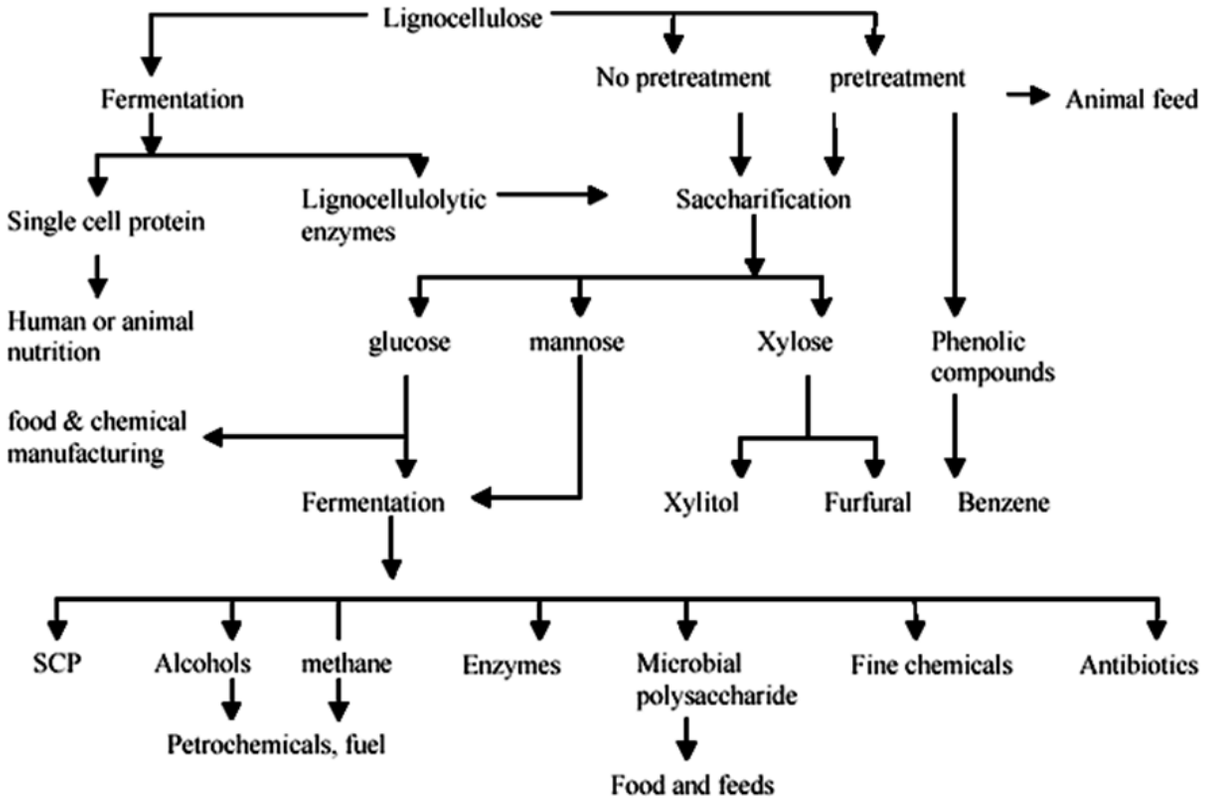


Figure 1. Lignocellulose bioconversion into value-added by-products Howard *et al*., (2003)

1.1. Cellulose

Cellulose is composed of insoluble, linear chains of glucose units linked by β-1, 4-glucosidic bonds (Fig 2). It is composed of highly crystalline regions and (non-crystalline) regions forming a structure generally resistant to enzymatic hydrolysis, especially the crystalline regions [17]. The cellulose fibers are usually embedded in an amorphous matrix of hemicellulose and lignin [18]. The presence of lignin in the biomass lowers the biodegradability both of the cellulose and hemicellulose [19]. Numerous pretreatment methods including biological methods have been developed for separation of lignocellulosic to cellulose, hemicellulose, and lignin [6]. Biological methods based on the enzymology have been suggested [20,21]. According to [22] the main problem in cellulose hydrolysis is the secondary and tertiary structures, not the primary linkage structure.

Chemically, cellulose $(C_6H_{10}O_5)_n$ molecules are linear glucans [23,24,18]. Partial hydrolysis of cellulose produces a range of oligosaccharides including cellobiose, cellotriose, and cellotetrose [25,26]. Intra-molecular hydrogen bonds between hydroxyl groups on the same cellulose chains produce the high viscosity, and rigidity associated withcellulose polymers. The hydroxyl groups at the ends of each cellulose chain have different chemical properties. At one end of the cellulose chain the number one carbon contains an aldehyde hydrate

group with reducing activity, while at the other end the number four carbon is an alcoholic hydroxyl with non-reducing activity. In native cellulose the cellulose chains are oriented in a parallel super molecular structure (Fig 2) in which inter-molecular hydrogen bonding between contiguous cellulose molecules, results in a sheet-like structure in the cellulose fibers [6].

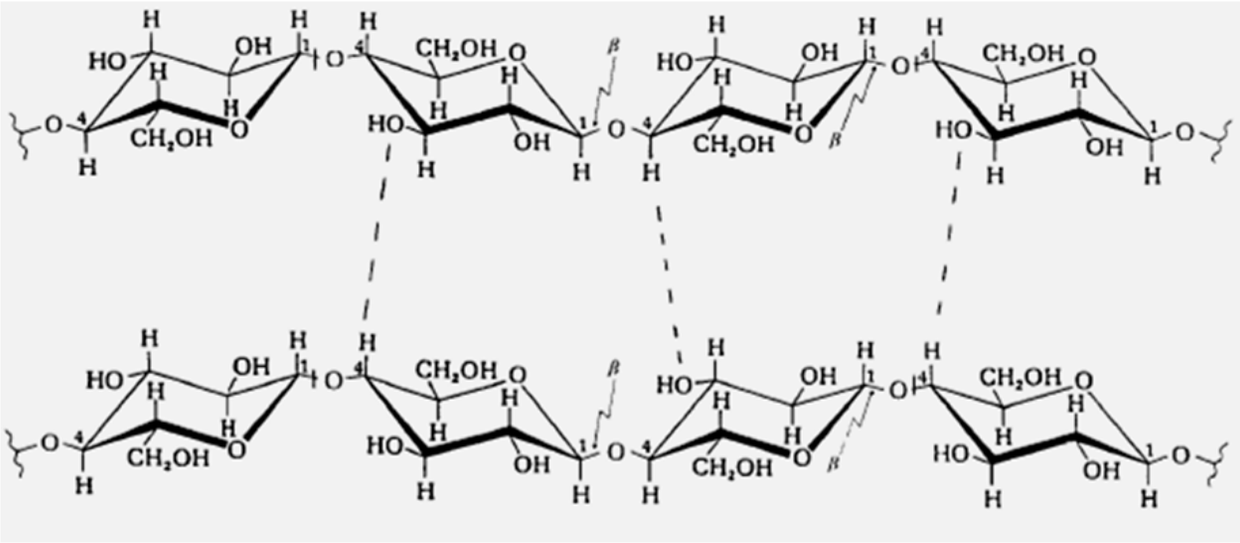


Figure 2. Structure of cellulose

The hydrogen bond strength is 25 KJ/mol, which is almost one hundred times stronger than Van der Waals forces (about 0.15KJ/mol), but less than one-tenth the strength of the O-H covalent bond (460 KJ/mol) [27]. Both inter and intra chain hydrogen bonds, which result in a sufficiently packed structure that prevents penetration, not only by enzymes, but also by small molecules, such as water. However, some regions, e.g. non-crystalline regions, permit penetration by larger molecules, including cellulases [28]. Obviously, reduction of crystallinity of cellulose and removal of lignin and hemicellulose are important goals for any pretreatment process [29].

1.2. Cellulolytic substrates under study (Wood dust).

Cellulose and hemicellulose are macromolecules from different sugars. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions [30,5]. Cellulose makes up about 45% of the dry weight of wood.

Hemicellulose is a complex carbohydrate polymer and makes up 25–30% of total wood dry weight. It consists of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by ß-1,4- and occasionally ß-1,3-glycosidic bonds [31]. Another major difference is the degree of polymerization, that hemicellulose has branches with short lateral chains consisting of different sugars [12].

Wood is an essential material for man. It is a material source for pulp and paper production, manure in the agricultural sector and fuel in the energy sector. Wood debris and by-product of wood processing pollutes the environment [32]. Wood wastes and their disposal have environmental concern worldwide especially when these wastes are biodegradable to useful goods [33]. DuPont, a company with environmental waste management, stated that 'waste manufacturing may be a product looking for a market [34]. The use of biological degradation have greater advantages over the use of chemical degradation because biotechnological synthesized products are less toxic and environmentally friend [35]. Sawdust as a lignocellulosic material can undergo enzymatic degradation to produce protein, glucose, and subsequently ethanol [33].

1.3. Cellulases

Cellulases are a group of hydrolytic enzymes capable of hydrolysing cellulose to smaller sugar components like glucose [36]. In industry, these celluloytic enzymes have found novel applications in the production of fermentable sugars, ethanol, organic acids, detergents, pulp and paper industry, textile industry and animal feed [37,38,39, 40, 41,16]. Today, these enzymes account for approximately 20% of the world enzyme market, mostly from Filamentous fungi, in particular *Trichoderma* and *Aspergillus* [42]. Cellulases can be divided into three major enzyme activity classes [43,44,45]; endoglucanses or endo1,4β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (D-glucoside glucohydrolase) (EC 3.2.1.21) [46,47,48].

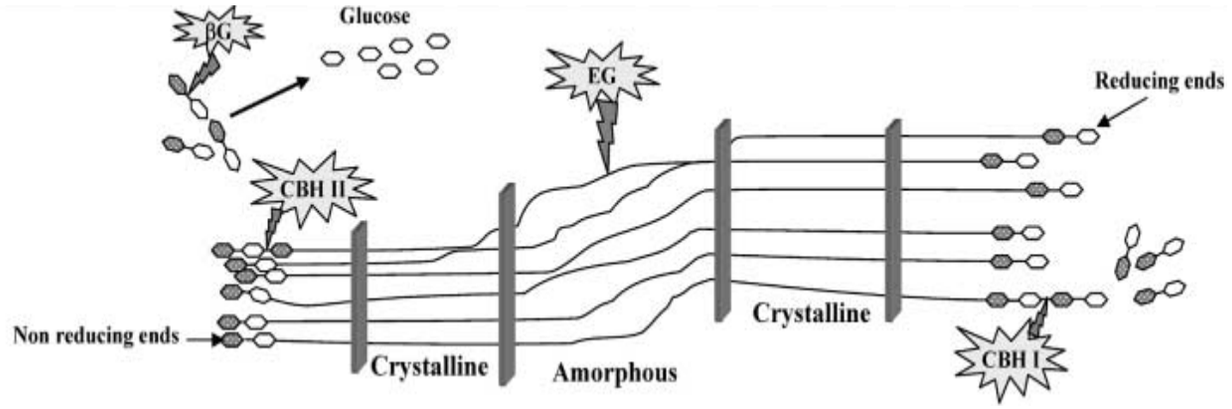
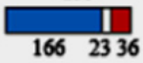


Figure 3. Different type of cellulase and their mode of action on cellulose

Extensive research efforts have been focused on lowering the cost of enzyme production, such as: (1) Screening for organisms with novel enzymes; (2) Improvement of existing industrial organisms and enzyme engineering; (3) factors such as substrate, culturing conditions and recycling of enzymes [49]. Strain improvement, choice of substrate and culture conditions in relation to improvement of fungal cellulolytic enzyme production were also studied [50]. Other investigators have looked for cheaper substrates [51,52,48].

1.4. Cellulase structure

Most fungal cellulases consist of two domains, i.e. a larger catalytic domain and a smaller cellulose binding domain (CBD) Table (1). These domains are joined by a glycosylated linker peptide [53,54]. The catalytic domain contains an active site [55]. The presence of CBD is essential to the degradation of solid crystalline cellulose [56,57]. Many studies have shown that removal of the CBD typically results in a decrease of about 50-80 % of the activity of fungal cellulases [58,59]. The biochemical role of a CBD is to keep the enzyme catalytic unit close to the substrate surface [60].

Enzyme	Family	Amino acid residues	Molecular mass kDa	Isoelectric point (pI)	Structural organization[b]
EGI	7	437	50–55	4.6	
EGII	5	397	48	5.5	
EGIII	12	218	25	7.4	
EGIV	61	326	(37)[a]	–	
EGV	45	225	(23)[a]	2.8–3	
EGVI	unknown*	unknown*	95–105	5.6–6.8	unknown*
CBHI	7	497	59–68	3.5–4.2	
CBHII	6	447	50–58	5.1–6.3	

a The molecular mass calculated from amino acid sequence
b ■ , the catalytic domain; □ , linker region; ■ , CBD
* gene not described

Table 1. Properties of cellulases from Trichoderma reesei (Saloheimo et al., 1997).

1.5. Cellulolytic organisms

A wide variety of microbes including bacteria, fungi and actinomycetes are involved in the decomposition of cellulose, of which fungi have generally been considered to be the main cellulolytic organisms [61,62]. By 1976 more than 14000 fungi active against cellulose and other insoluble fibres had been collected [63,1]. Many fungi are capable of growing on cellulose as the sole carbon source, including those normally found on wood [64]. Fifty two fungal isolates with cellulolytic activities were isolated on Czapex' dox with a filterpaper and CMC agar media [65]. The cellulase systems of the mesophilic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* are the most thoroughly studied [66]. The production of cellulase by the members of genus *Fusarium* were investigated by several workers [67,68,69]. Filamentous fungi, particularly *Aspergillus* and *Trichoderma* species, are well known as efficient producers of these cellulases [70,71]. *Trichoderma* species are common soil inhabiting fungi with a strong ability to degrade cellulose [72,6]. Trichoderma reesei

produce two exo-glucanases (Table1) and at least six endoglucanases and two β-glucosidases [57,54,73]. The filamentous fungus *Trichoderma reesei* has a long history in the production of hydrolytic enzymes, which was widely used in the textile, pulp and paper industries [3,38].

1.6. Enzyme applications

1.6.1. In pulp and paper

Novel enzyme technologies can reduce environmental problems, eliminating caustic chemicals for cleaning paper machines, reduce manufacturing costs and create novel high-value products. Today recombinant DNA has allowed the cloning of enzymes modified for temperature and pH stability [74]. Cellulases have been used in many processes in the paper industry [75,76,77]. Endoglucanase treatment of pulps was shown to decrease the viscosity and chain length and increase the reactivity of a pulp made from eucalyptus and acacia [78]. Despite the progress achieved, more effort is needed for lignocellulosic enzymes and/or microorganisms to have significant industrial impact [74].

1.6.2. Enzymatic deinking

One of the greatest challenges in paper recycling is removal of contaminants; some of the most problematic contaminants are polymeric inks and coating. Toners such as those used in laser and xerographic copy machines are thermally fused to the surface of the printed page [79]. Cellulases are particularly effective in facilitating the removal of toners from office waste papers [30]. Kim et al.,(1991) showed that crude cellulases applied to pulps facilitate deinking [80].

1.6.3. Textile industry

Cellulases are important tools in the textile industry. They provide an economical and ecological way to treat cotton and cotton-containing fabrics. Pretreatment with cellulases reduces the pill-formation and increases the durability and softness of the fabric. Currently textile enzymes have a market value equivalent to 12% of the industrial enzyme market [81]. Today a commercial endoglucanase cellulase product, Cellulsoft Ultra L (expressed in genetically modified *Aspergillus*) is available from Novozymes [82].

Endoglucanases have been used to release the microfibrils from the surface of dyed cellulosic fabrics and thus to restore the original colors [83,84]. Trichoderma viride cellulase that removal of surface fibrils from linen fabrics can be accomplished without high weight losses or reduction in tensile strength [85].

1.7. Bio-fuel

In the 20th century, the world economy has been dominated by technologies that depend on fossil energy, such as petroleum, coal, or natural gas to produce fuels, chemicals and power

[86]. However, fossil energy sources are not infinite. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 [87]. A search for other energy sources is advisable. Biomass is a potential renewable energy source that could replace fossil energy for transportation [86]. Ethanol is used as a chemical feedstock. Brazil produces ethanol from the fermentation of cane juice whereas in the USA corn is used. In the USA, gasoline fuels contain up to 10% ethanol by volume [9] Ethanol blended with gasoline (10:90) reduces carbon monoxide emissions [12]. By December 2011 there were nearly 10 million E85-capable vehicles (85% ethanol) on U.S roads [88]. Production of fuel ethanol from fermenting sugar or crops such as corn has dropped to around 800 thousand barrels per day (kb/d) in the US, according to the International Energy Agency's latest Oil Market Report (OMR) 2012.

1.8. Enhancing the feeding value of Agro-industrial by-products

Agro-industrial by-products are wastes, often causing environmental pollution and hazard when left unutilized. As grain production remains insufficient to meet human and animal needs; the alternative is to employ feed ingredients which do not have direct human food value [89]. Changes in the protein and cellulose of agro-industrial by-products after fermentation with *Aspergillus niger*, *Aspergillus flavus* and P*enicillium sp*. in solid state [89] indicate the possibility of enhancing the feeding value of these by-products. Biological treatment of agricultural residues is a new method for improvement of digestibility [90]. Attempts are being made to improve the digestibility of lignocellulosic feeds by the use of microbial additives [91].

2. Materials and methods

2.1. Organism, cultivation and growth conditions

Trichoderma sp FJ937359 previously isolated from Sharkia, Egypt, was cultivated on Czapek medium containing (1%) wood dust as a sole carbon source. The culture was incubated for 14 days at 30°C on orbital shaker at 150 rpm. At the end of the incubationtime, residues were removed and the filtrate was centrifuge at 5000 rpm. The resulting clear supernatant was used as the source of crude enzymes [92].

2.2. Determination of enzyme activity

Endoglucanase activity was routinely measured according to [93]. One ml appropriate filtrate dilution, was added to 1 ml of 1% carboxymethyl cellulose (CMC) dissolved in 50 mM sodium acetate buffer, pH 5.0. After incubation at 50 °C for 60 min, the reaction was stopped by addition of 3 ml dinitrosalicylic acid. After 10 min in a boiling water bath, the enzymatic hydrolysis of CMC was determined at 540 nm. One unit of CMCase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose equivalents min^{-1}.

2.3. Determination of protein concentration

The protein content of the crude enzyme was assayed by Folin-phenol reagent, using bovine serum albumin as a standard [94].

2.4. Purification of endoglucanases (CMCases)

Trichoderma sp. Shmosa Tri FJ937359 culture filtrate was used as source of crude enzyme. Proteinwasprecipitated byslow addition of ammonium sulphate until the desirable saturation 80%. The obtained precipitated protein was resuspended in known volumeof 0.1 M citrate phosphate buffer (pH 5.0) and dialyzed via cellophane bags (MWCO 10 KDa) against citrate-phosphate buffer (pH 5.0). A pharmacia sephadex G 100 column (2.5 x 90 cm) was used for purification of the dialyzed enzyme. The solid phase consists of sephadex G_{100} (10 gm) swollen in 0.1 M citrate phosphate buffer (pH 5.0) for 24 hour at 4.0°C, sodiumazide (0.02%) was added to prevent any microbial growth. Fractions were collected and assayed for CMCase activity and protein content.

2.5. Estimation of enzymes molecular weights

For molecular weight determination, the enzyme preparations and known molecular weight markers were subjected to electrophoresis (SDS-PAGE) with 10% acrylamide gel; 0.2% CMC was incorporated into the separating gel prior to the addition of ammonium persulphate [95]. After electrophoresis, the gel was stained with Coomassie Blue R dye. For CMCase activity, the gel washed at room temperature in solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%), solution B (sodium phosphate buffer, pH 7.2) for 1 h, respectively then solution C (sodium posphate buffer, pH 7.2, containing 5 mM b-mercaptoethanol and 1 mM EDTA) at 4 °C overnight. The gel was then incubated at 37 °C for 4 h, stained with 1% Congo red for 30 min, destained in 1 M NaCl for 15 min [96], clear bands indicated the CMCase activitiy.

2.6. Enzyme characteristics

2.6.1. Effect of substrate concentration on CMCase I and II activities

The (CMCase) activity was carried out for the two enzymes at different concentrations of the substrate (CMC): 0.12, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 % at pH 5.0 using citrate-phosphate buffer. Activity was determined using DNS reagent (3, 5-dinitrosalicylic acid) [93].

2.6.2. Effect of reaction time on CMCase I and II activities

(CMCase) activity was determined for the two enzymes CMCase I and II at different incubation time of reaction mixture; 15, 30, 60, 90, 120and 150min. Activity was determined as previously described.

2.6.3. Effect of reaction temperature on CMCase I and II activities

The enzymatic activity of (CMCase) was estimated for CMCase I and II at the following reaction temperatures 20, 30, 40, 50, 60, 70 and 80°C.

2.6.4. Thermal stability of CMCase I and II

The thermal stability of (CMCase)activity was tested by preheating of enzymes at 40, 50, 60 and 70 °C. Activity was estimated every 30 min.

2.6.5. Effect of pH-value of reaction mixture on CMCase I and II activities

The pH optima of the (CMCase) enzymes were determined at a pH range from 2 to 9, using citrate–phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 3.0 to 7.0), and Tris buffer (0.08 M Tris, 0.1 M HCl, pH for pH 8 and 9.0. The (CMCase)activity at each pH-value was estimated as previously described.

2.6.6. pH-stability of CMCase I and II

The pH stability of purified (CMCase)enzymes was determined by preincubation of enzymes at a pH range from 2 to 9for 24 hours [97] then activities were assayed as previously described at each pH value.

2.6.7. Effect of some metallic ions on CMCase I and II activities

The effect of KCl, NaCl, $CaCl_2$, $CoCl_2$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MgSO_4.7H_2O$, as well as EDTA and SDS at a concentration of 20 ug/ml of reaction mixture on CMCase I and II. Activities were investigated at optimum conditions as described before.

2.6.8. Enzyme substrate specificity

The specificity of the enzymes for their substrate was investigated using different substrates: CMC (control), Chitin, Starch, β. glucan, Xylan and Cellobiose.

3. Experimental results

3.1. Cellulase production

There is a growing demand for specific, efficient and cheap cellulases. Therefore it is important to gain more information about the production, purification and activity of these enzymes produced by microorganisms. Cellulases yield appear to depend on factors like carbon source, pH value, temperature, aeration, growth time [98]. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favorable for the microorganism for over-production of the desired metabolite [99]. Sawdust is reported to be more suitable for cellulase production as it gave the highest yield of

enzyme compared to bagasse and corncob [100]. The highest cellulase productivity with sawdust may be due to its very high percentage of cellulose which is the major component of cell walls of wood.

The use of inexpensive biomass resources as substrates can help to reduce cellulase production cost [52]. Research efforts have been undertaken to replace the expensive carbon and nitrogen sources with cheap raw materials in the media to bring down the production cost of cellulases [48]. The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling. The negative attitude in which wastes are viewed as valueless, has been replaced by a positive view in which wastes are recognized as raw materials of potential value [49,101]. Production of low-cost cellulolytic enzymes using inexpensive growth media has been investigated by different research workers [102,103]. Lignocellulosic materials from crop residues, wood, and wood residues are considered as the least expensive sources of cellulosic substrates [104,105]. In our study wood dust was selected as substrate for CMCase productionby *Trichoderma sp.* (Tri) Shmosa Tri FJ937359. This selection was due to wood dust being widely available, relatively inexpensive and a highly cellulolytic substrate.

3.2. Cellulases purification and activity

The crude enzymes obtained from culture filtrate of Trichoderma sp. Shmosa Tri FJ937359 grown on wood dust was subjected to a purification protocol. Results showed in table (2) indicated that specific activity increased to 62.86 U/mg (1.26 fold) by salting out compared with the crude enzyme. Many workers used ammonium sulphate for precipitation of Cellulase [106,107]. The results showed also that there are two peaks I and II for CMCase with specific activities 170.53 and 166.51 U/mg, respectively. The peaks were used for subsequent characterization and properties of enzymes. These purification techniques are comparable to those reported by [108,109,110,111] using sephadex G-100; sephadex G-150; sephadex G -50 and G -200 and superdex-200 HR respectively.

Purification steps		Protein (mg/ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/ mg)	Recovery (%)	Fold
Crude enzyme		0.340	595	29505	49.58	100	1
Precipitate with $(NH4)_2SO_4$		0.237	47.4	2980	62.86	10.09	1.26
Gel filtration	Peak I	0.132	3.3	562.75	170.53	1.90	3.43
Sephadex G_{100}	Peak II	0.152	3.8	632.75	166.51	2.14	3.35

Table 2. Purification profile (CMCases) produced by Trichoderma sp. grown on wood dust

3.3. Enzymes molecular weights

The molecular weights of CMCases were estimated using the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that two clear bands appeared (I, II) when stained with 1% Congo red and destained in 1 M NaCl

(zymogram method), indicating the presence of CMCase activity (Fig 4). The results showed also that the molecular weights of the 2 enzymes I, II were, 58 KDa and 34 KDa, respectively Fig (4). These findings are in agreement with other investigators that reported the presence of different isoenzymes with different molecular weights of CMCase produced by various microorganisms [112,113,114]. Our results are also in comparable to these obtained by Holt and Hartman (1994), they use a zymogram method to detect endoglucanases from Trichoderma reesei [115]. The molecular weight of EG endoglucanase purified from the culture filtrate of Trichoderma sp. C-4, was 51 kDa [106]. The EGs of the mesophilic fungi *Trichoderma reesei* and *Phanerochaete chrysosporium* have molecular weight ranges from 25 to 50 kDa [12]. However carboxymethyl cellulase purified from Trichoderma viride, was examined by (SDS-PAGE) and the molecular weight was 66kDa [116]. Two endoglucanases were purified to homogeneity (EG-III and EG-IV), from the culture filtrate of a mutant strain Trichoderma sp. M7, the molecular weights determined to be 49.7 and 47.5 kDa [114]. The difference in the molecular weights of CMCase enzymes may due to the biological aspect of the fungal strains, these aspects include intrinsic genomic traits, identity of the gene encoding enzyme and the proteomic level.

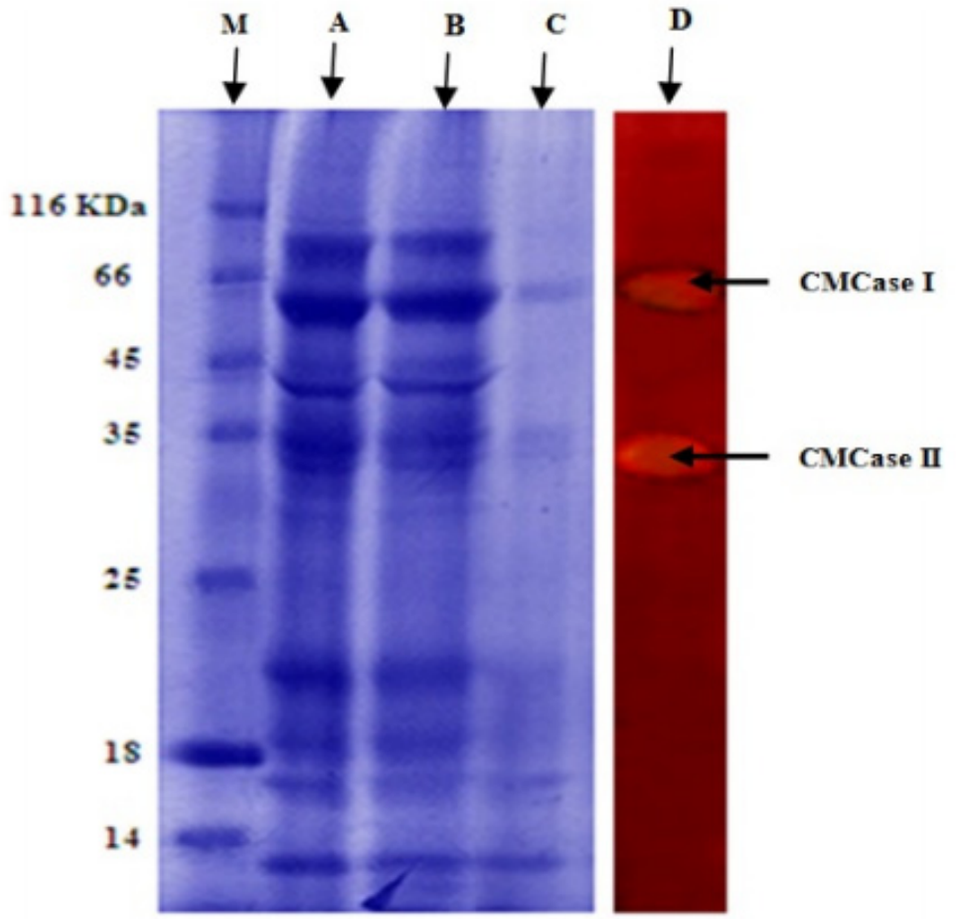


Figure 4. SDS-PAGE profile of CMCase (I,II) from Trichoderma sp. (M) Standard protein markers, (A) Crude enzyme preparation, (B) dialyzed enzyme preparation, (C) active fractions of column chromatograph and (D) zymogram stain with Congo red

3.4. Effect of substrate concentration on CMCase I and II activities

The results in Fig. (5) illustrate that the optimum specific activities of CMCase I and II were obtained at a substrate concentration of 1% (w/v). Increasing substrate concentration beyond 1 % (w/v) caused a decrease in the specific activities of both enzymes. This is probably because at high substrate concentration, many substrate molecules are around the enzyme molecules, crowding the active site or may be bound to regions which are not the active site. These results are supported by Bakare, et al., (2005) who reported that the activities of cellulases are greatly influenced by the concentration of the substrate [107]. At a fixed enzyme concentration, an increase in the concentration of substrate results in an increase in enzyme activity until a saturation point is reached beyond which enzyme activity decreases. Our results showed also that the Km value of CMCase I and II were 4.0 and 3.1 mg per ml, respectively. Km values denote the amount of substrate needed to achieve half the maximal initial reaction rate [107]. Petrova et al., (2009) purified two endoglucanases EG-III and IV from Trichoderma sp. M7, which exhibited Km's of 2.9 and 3.8 mg/ml, respectively [114].

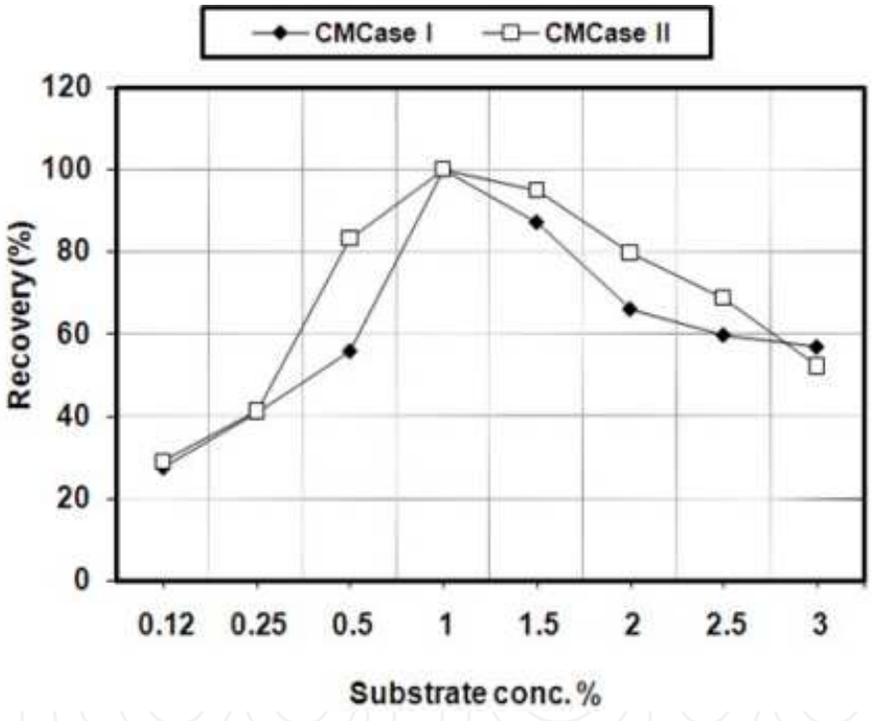


Figure 5. Effect of substrate concentrations on the activities of the purified (CMCase I) and (CMCase II

3.5. Effect of reaction time on CMcase I and II activities

The results presented in Fig (6) showed that the highest specific activities of CMCase I and CMCase II were recorded after one hour. Our results also indicatethat CMCase I and II retained 25.2 and 42.3 % of their activities respectively, after 3 hours of reaction. Our data is in agreementwith previous studies that recorded the amount of reducing sugar produced under the action of cellulases; for Trichoderma koningii and Aspergillus niger reducing sugars gradually increased with the increase in incubation time and reach the maximum at 60 min [67,117].

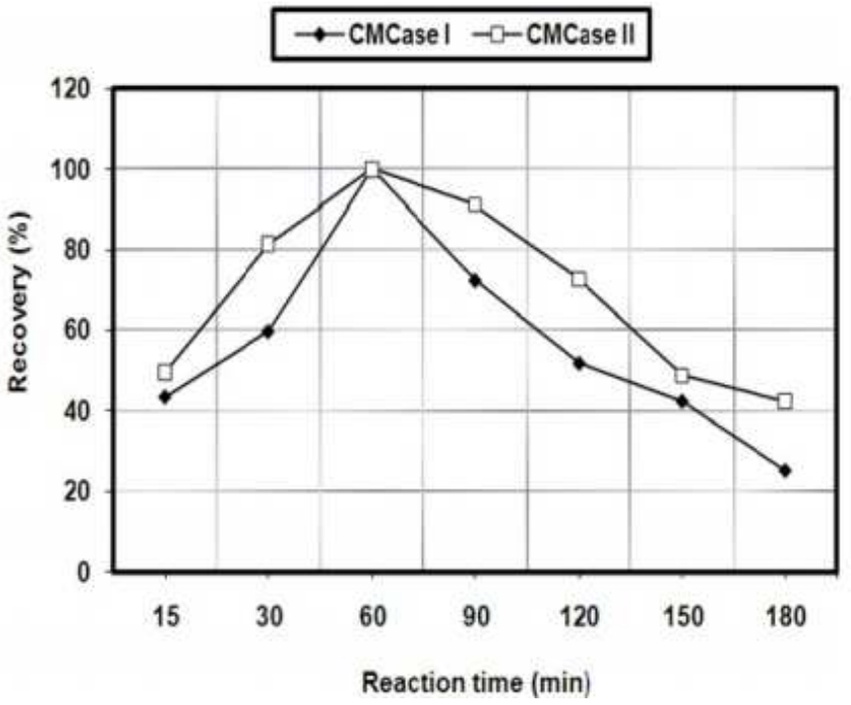


Figure 6. Effect of reaction time on the activities of the purified (CMCase I) and (CMCase II).

3.6. Effect of reaction temperature and thermal stability on CMcase I and II

Data in Fig (7) illustrates that the specific activities of CMCase enzymes increased with increasing reaction temperature, reaching optimum values at 50°C for both CMCase I and II.

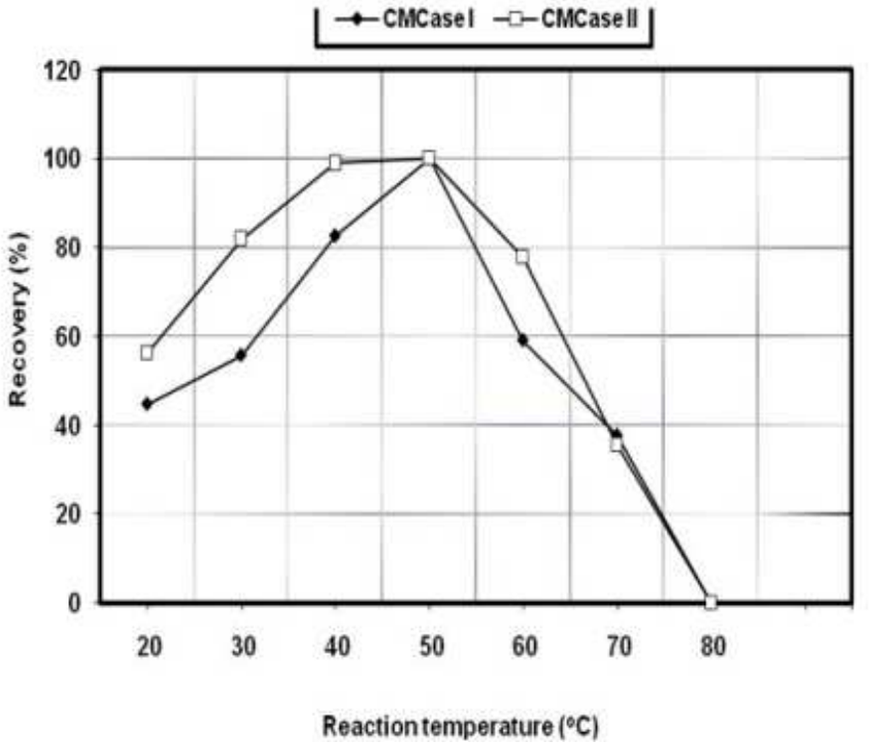


Figure 7. Effect of reaction temperature on the activities of the purified (CMCase I) and (CMCase II)

In general, cellulases have high temperature optima when compared with other enzyme systems [108]. Cellulolytic enzyme activities increased with increasing temperatures up to 60° to 65°C for the three enzyme components from *Trichoderma longibrachiatum* [118]. Ülker and Sprey, (1990) purified the low molecular weight endoglucanase from Trichoderma reesei and found the optimal temperature to be 52°C [119]. Petrova et al., (2009) stated that the optimal temperature values for two purified endoglucanases EG-III and EG-IV from Trichoderma sp. M7, were found to be 60°C and 50°C, respectively [114]. Our results show that the thermal inactivation of the purified CMCase I and II enzymes increased with increasing preheating temperature as well as exposure time (Fig 8). The enzymes can withstand 60 min at 50ºC without loss of enzymatic activity. The results also illustrate that CMCase I and II retained 14.0 and 26.5 % of their original activities after 90 min at 70°C. Our findings are in agreement with that reported for CMCase and FPase from Trichoderma sp. A-001 that lost 20–33% of their activities when kept at 60°C for 4 hours before assaying, and a beta-glucosidase that lost 37% of its activity when maintained at 70°C for 4 h [120]. Endoglucanase purified from the culture filtrate of Trichoderma sp. C-4 was assayed at various temperatures ranging between 30°C and 70°C and the optimum temperature was found to be 50°C. The enzyme showed stability at 50°C for 60 min but lost 50% of its maximal activity after 10 min at 60°C [106]

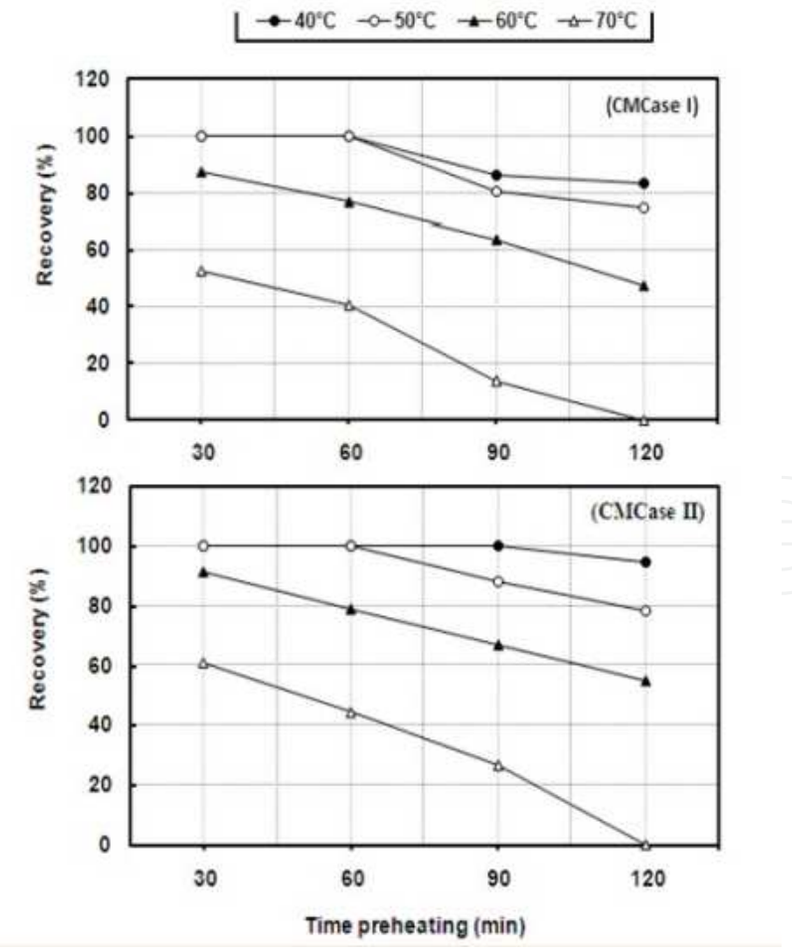


Figure 8. Thermal stability of CMCase I and II

The optimum temperature for C1cellulase of *Trichoderma viride* was found to be 40 °C [121]. El-Zawahry and Mostafa (1983) recorded that the optimum temperature for cellulases activities produced by Trichoderma viride ranged from 50 to 60 °C [67].

3.7. Effect of PH values of reaction mixture and PH stability on CMCase I and II

Results in Fig. (9) show that the specific activities of CMCase I and II increase gradually with increasing pH of the reaction mixtures,reaching a maximal value at pH 5.0 for both CMCase I and II, consistent with other researchers [106,114]. Kalra et al., (1986) reported that purified enzyme preparations from Trichoderma longibrachiatum showed an optimal pH of 5.0 for CM cellulose [118]. However, Carboxymethyl cellulase purified from Trichoderma viride, showed an optimum pH 4.0 at 50 °C [116]. We also found that both CMCase I and II retained 23.2 and 22.9% of their activity at pH values as high as 9.0, respectively. The results in Fig (10) show that CMCase I was active at room temperature after 24 hrs over a broad pH range (3.0-9.0). On the other hand CMCase II was relatively stable in pH range (4.0-6.0). In general, the pH-stability curves of the enzymes are much broader than the pH-activity curves [108].

Fungal cellulases, in general, are stable at over the pH range 3 – 8 and usually active over the pH range 3.5 to 7, in citrate, phosphate or acetate buffers [112]. The optimum activities of C1 and Cx cellulases produced by *Trichoderma viride* was obtained at pH 5.0 [67]. Catriona et al. (1994) reported that the pH range over which the cellulases were highly active is fairly broad (pH 5.0 – 7.0) [123]. The instability of these enzymes at very low or very high pH values is due to the fact that they are proteins which are generally denatured at extreme pHvalues [124].

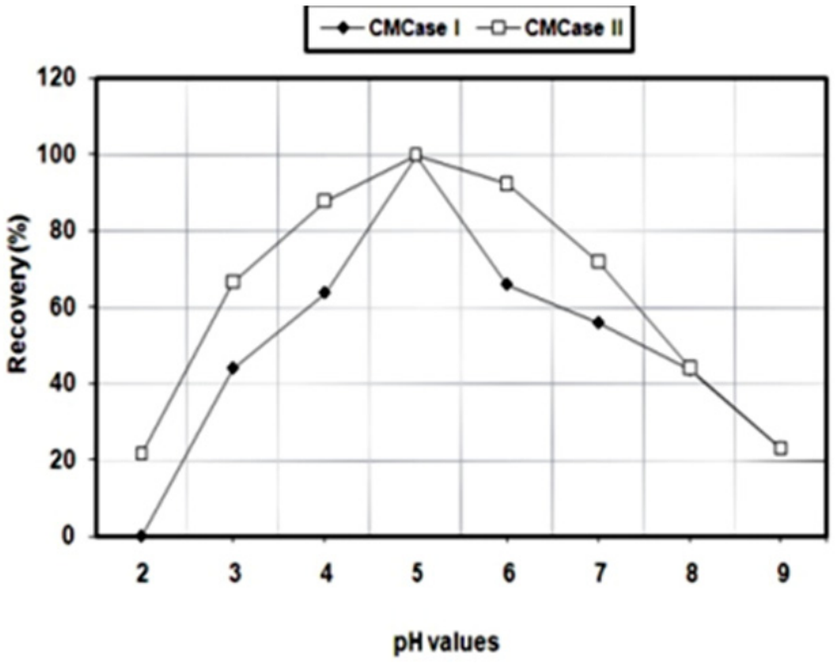


Figure 9. Effect of pH values of reaction mixture on the activities of the purified (CMCase I) and (CMCaseII)

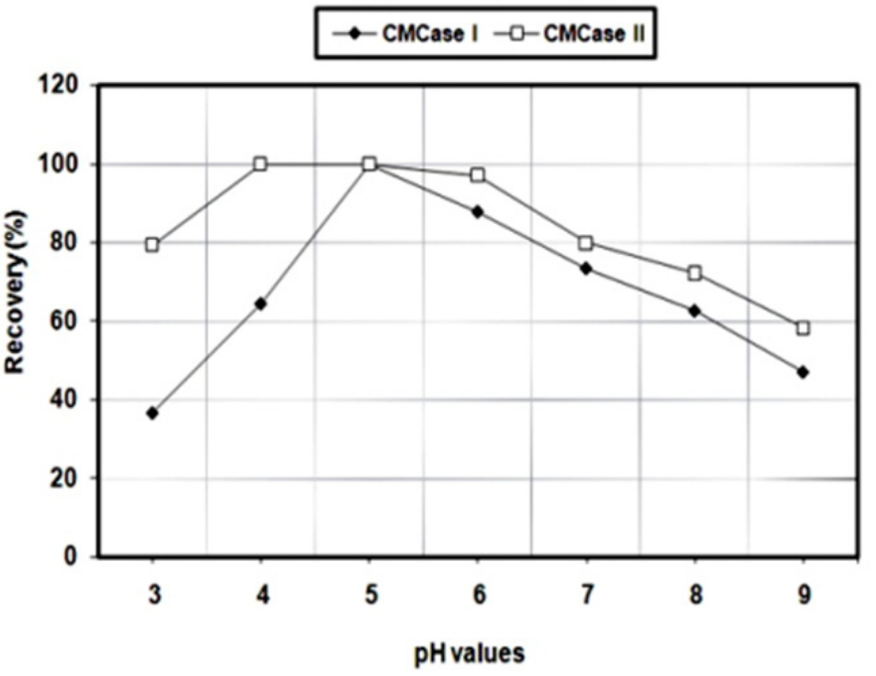


Figure 10. pH stability of CMCase I and II

3.8. Effect of some metallic ions and chemicals substances on CMCase I and II

The obtained results in Fig. (11) show the inhibitory effect of metallic ions on the activities of CMCase I and II. CMCase I was found to be more sensitive than CMCase II. Co^{+2} was the most inhibitory ion for CMCase I, while Hg^{+2} was the most inhibitory ion for CMCase II. Heavy metal ions such as Hg+2 and Ag+ have significant or complete inhibitory effect on

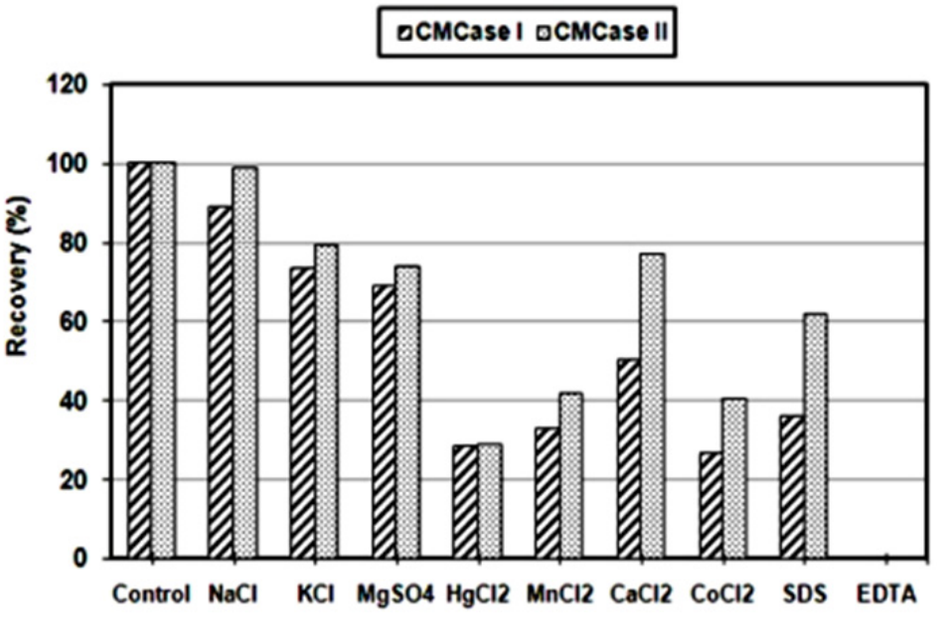


Figure 11. Effect of some metallic ions and chemicals on CMCase I and II produced by Trichoderma sp. grown

CMCase purified from Trichoderma viride [116]. Petrova et al., (2009) proved that Mn+2, Cu+2 and Pd+2 strongly inhibited EG-III and EG IVendoglucanases purified from Trichoderma sp [114]. These inhibitory effects may be due to the toxic effect of these ions. Our results also show complete inactivation of the two enzymes in presence of EDTA. EDTA is known as an ionic chelator [125] and its inhibition ability indicates that specific ions might be actively involved in the catalytic reaction of the enzyme [126]. EDTA inhibited enzyme activity at all concentrations while by low concentrations of Na+ and Mg++ stimulated enzyme activity [123].

3.9. Substrate specificity of CMCase I and II enzymes

Both enzymes clearly have high hydrolytic activity towards carboxymethyl cellulose CMC,neither showed any hydrolytic activity against chitin, starch and cellobiose (Fig 12). On the other hand both CMCase I and II had relatively low hydrolytic activity towards β glucan and xylan. Ülker and Sprey, (1990) purified endoglucanase from Trichoderma reesei, gave a strong increase in CMC-fluidity but the enzyme had no specificity toward crystalline cellulose (Avicel) or xylan [119]. Purified endoglucanases from Trichoderma sp. EG-IV catalyzed the hydrolysis of Na-CMC whereas EG-III displayed high activity towards xylan [114]. Although *Km* values may serve only denote the amount of substrate needed to achieve half the maximal initial reaction rate [108], *Km* is a measure of the apparent affinity of an enzyme for its substrate [107]. An endo-1,4-β-D-glucanase I was purified from *Bacillus circulans* F-2. It showed a high-level of activity towards carboxymethyl cellulose (CMC) as well as *p*-nitrophenyl-b-D-cellobioside, xylan, Avicel and filter paper [127].

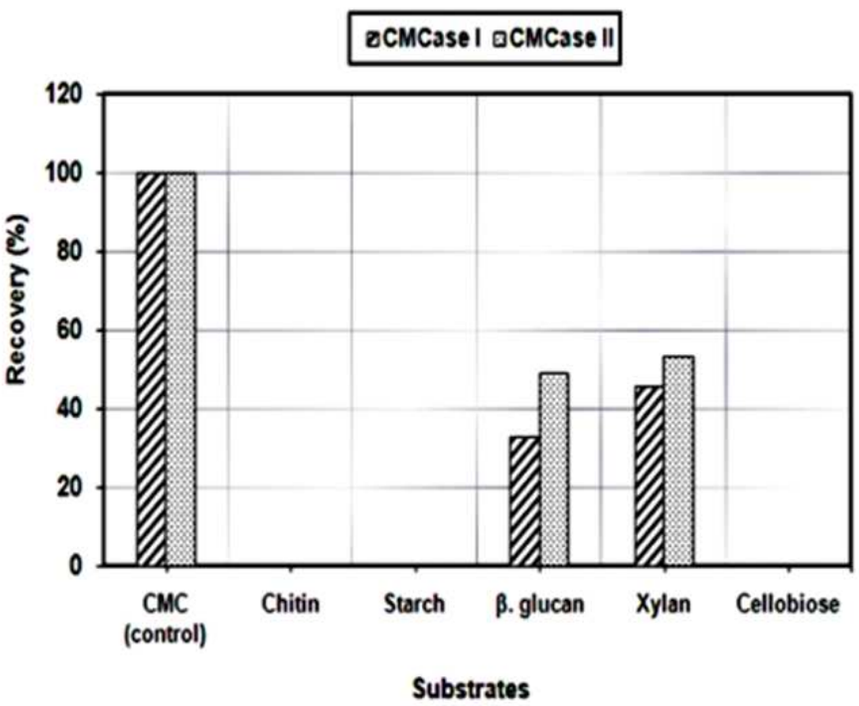


Figure 12. Substrate specificity of CMCase I and II

Finally, it could be concluded that fungi in generalconsidered to be potential candidates for the production of cellulases which seemed to be very important for different biotechnological aspects. The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness of energy conservation and recycling. In our study wood dust was selected as substrate for CMCase productionby *Trichoderma sp.* (Tri). Wood dust is a waste by-product from wood processing, as a result it is widely available, relatively inexpensive, and a highly cellulolytic substrate. Moroever, as compared to thermal degradation of agricultural, industrial wastes, biological degradation has great potential for both economic and ecological reasons.

Our result showed that using wood dust as substrate *Trichoderma sp.* (Tri) produce high levels of endoglucanases (CMCase I and II). In terms of enzyme novelty from an applications perspective, interest is focused on not only finding enzymes which could break down lignocellulose much more rapidly, but also enzymes which could withstand pH, temperature and inhibitory agents. Our result showed that both purified CMCase I and II withstand high temperature 70°C even after 90 min, making them good candidates for novel industrial applications.

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