Middle East Journal of Agriculture Research, 3(4): 1205-1213, 2014
ISSN 2077-4605

Lignocellulose Degrading Enzymes of *Pleurotus sapidus*

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ABSTRACT

The present study was performed for the production of some lignocellulose degrading enzymes from *Pleurotus sapidus* during the submerged cultivation. Moreover, characterization of lignocellulose degrading enzymes were evaluated. Basal medium containing 1% rice straw was the best for maximum lignocellulose degrading enzymes secretion by *P. sapidus.* Corn stalks were the most suitable carbon source for the production of cellulase, xylanase and laccase. While, saw dust gave low enzymes productivity. Maximum activity of cellulase, xylanase, veratryl alcohol oxidase (VAO) and laccase was obtained at concentration of 20 g/L of corn stalks. The most suitable concentration of yeast extract for maximum production of cellulase was 2 g/L. Moreover, maximum activity of xylanase, veratryl alcohol oxidase (VAO) and laccase was obtained at 2.5 g/ L of yeast extract. Maximum activity of cellulase and xylanase was obtained after 6 days of cultivation. On the other hand, veratryl alcohol oxidase (VAO) and laccase enzymes reached the maximum activity after 9 and 3 days of cultivation, respectively. The optimum temperature for cellulase and laccase activity was 50°C. While, xylanase and VAO enzymes reached the maximum activity at 60°C and 55°C, respectively. Concerning the optimum pH, cellulase and xylanase enzymes showed maximum activity at pH 3.5 and pH 4.0, respectively. However, pH 5.5 and pH 3.0 were optimum for the activity of VAO and laccase, respectively.

Key words: Lignocellulose biodegradation, *P. sapidus*, cellulase, xylanase, veratryl alcohol oxidase (VAO) and laccase.

Introduction

Cellulose, a principle component of all plant materials, is considered one of the most abundant renewable resources in the world (Cen and Xia, 1999). Moreover, xylan, the second most abundant polysaccharide and consists of β-1,4 linked xylopyranosyl residues.

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated. Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%), a group of carbohydrates in which xylan forms the major class, and lignin (20-30%). Lignin is a branched polymer of substituted phenylpropane units joined by carbon and ether linkages (Subramaniyan and Prema, 2002).

Cellulases are the enzymes responsible for the cleavage of the β-1,4-glycosidic linkages in cellulose (Schwarz, 2001). The complex structure of xylan needs different enzymes for its complete hydrolysis. The depolymerisation action of endo-xylanase results in the conversion of the polymeric substance into xylo-oligosaccharides and xylose. Endo- β-1,4- xylanases that depolymerise xylan by the random hydrolysis of xylan backbone (Subramaniyan and Prema, 2002).

Lignin content in lignocellulosic substrates negatively influences the hydrolysis reaction. Cellulases are irreversibly adsorbed on lignin and that prevents their action on cellulose (Lai *et al.,* 1990). Two families of lignolytic enzymes are widely considered to play a key role in the enzymatic degradation: 1- oxidation enzymes (laccase (phenol oxidase) & veratryl alcohol oxidase (VAO)), 2- peroxidase enzymes (lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase) (Krause *et al*., 2003; Malherbe and Cloete, 2003).

Veratryl alcohol oxidase (VAO) enzyme is able to oxidize a number of aromatic alcohols to aldehydes and reduced O_2 to H_2O_2. The role of the oxidase enzyme in biodegradation might be to produce H_2O_2 during oxidation of lignin fragments (Bourbonnais *et al.*, 1997). Laccases are multicopper oxidases that catalyze oxidation of various substituted phenolic compounds, aromatic amines and even certain inorganic compounds by using molecular oxygen as the electron acceptor.

Their substrate versatility makes laccase highly interesting for various applications, including textile dye bleaching, pulp bleaching and bioremediation, where enzymatic catalysis could serve as a more environmentally being alternative than the currently used chemical processes (Kiiskinen, 2005).

The unique capability of white-rot fungi to attack lignified biopolymers has attracted intense research efforts for decades. *P. sapidus* is a member of the oyster mushroom family and secretes a broad set of extracellular enzymes when cultivated on lignified bio-polymers Matis *et al,.* (2005).

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The aim of this work is to study the production conditions of lignocellulose degrading enzymes by *P. sapidus* during the submerged cultivation on medium containing corn stalk. Moreover, characterization of lignocellulose degrading enzymes were evaluated.

Materials and Methods

Microorganism and lignocellulosic materials

The fungus *P. sapidus* was obtained from Plant pathology Dept., Fac. of Agric. at Moshtohor, Benha Univ., Egypt. The original culture was maintained on potato dextrose agar (PDA) slant. Stock cultures were kept at 5˚C. Lignocellulosic materials such as rice straw, wheat straw, bagas, saw dust and corn stalks were obtained from the farm of the Fac. of Agric. at Moshtohor.

Media

Four media were used for screening the highest production of lignocellulose degrading enzymes by *P. sapidus*, the used media were: M1:Czapek-Dox liquid medium containing 1% rice straw (Coral *et al*., 2002); M:Simplified minimal medium containing 1% rice straw (El-Shafai and Rezkallah, 1998); M3:Potato dextrose agar (PDA) containing 1% rice straw and M4:Basal medium containing trace elements and 1% rice straw, (Lelliott and Stead, 1987), each medium was adjusted to pH 6.

Fermentation and optimization studies

About 95 mL of the production medium were dispended into 250 mL Erlenmeyer flasks, sterilized and inoculated with 5 mL of a 3-days-old fungal inoculum of *P. sapidus*. The inoculated flasks were incubated at 25˚C under shaking at 150 rpm for 7 days. The cultures were centrifuged at 4000 rpm for 30 min at 4˚C. The supernatant was used for measurement of the lignocellulose degrading enzymes activity. Optimization of lignocellulose degrading enzymes production by *P. sapidus* was carried out in the presence of some factors such as production media, different lignocellulosic materials as a carbon source, nitrogen source and its concentration and time course.

Concentration of lignocellulose degrading enzymes

Culture of *P. sapidus* was centrifuged at 4000 rpm for 30 min at 4°C and the clear supernatant was used as source of crude enzymes. Lignocellulose degrading enzymes were concentrated by ultrafiltration technique (JumbosepTM Centrifugal Devices, exclusion limit 10 kDa) at 4000 rpm and 4 °C. Following the ultrafiltration, protein concentration and lignocellulose degrading enzymes activity in the retentate and filtrate were measured.

Enzymes assay and protein determination

Endoglucanase activity was determined according to the method described by Bailey (1981). Xylanase activity was determined by measuring the amount of the reducing sugars liberated from xylan as described by Ahmed *et al*., 1997. Reducing sugars was determined using dinitrosalicylic acid method (Miller *et al*., 1960).

For determination of veratryl alcohol oxidase (VAO) activity, the reaction mixture contained 1mL crude enzymes and 5 mL substrate (1mM veratryl alcohol) in 50 mM Na-acetate buffer, pH 3.5, 40°C. Oxidation of the substrate was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde. One unit of the enzyme is defined as the amount producing 1 µ mol of veratraldehyde per minute under the assay conditions (Tien and Kirk, 1988).

Laccase activity was determined by oxidation of 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Majcherczyk *et al.*, 1999). The reaction mixture contained 1mL enzyme solution and 5 mL substrate (5mM ABTS) in 50mM Na-acetate buffer pH 3.5 and 40°C. Oxidation of ABTS was followed by absorbance increase at 420 nm. Protein concentration was determined according to Lowry *et al.*, (1951).

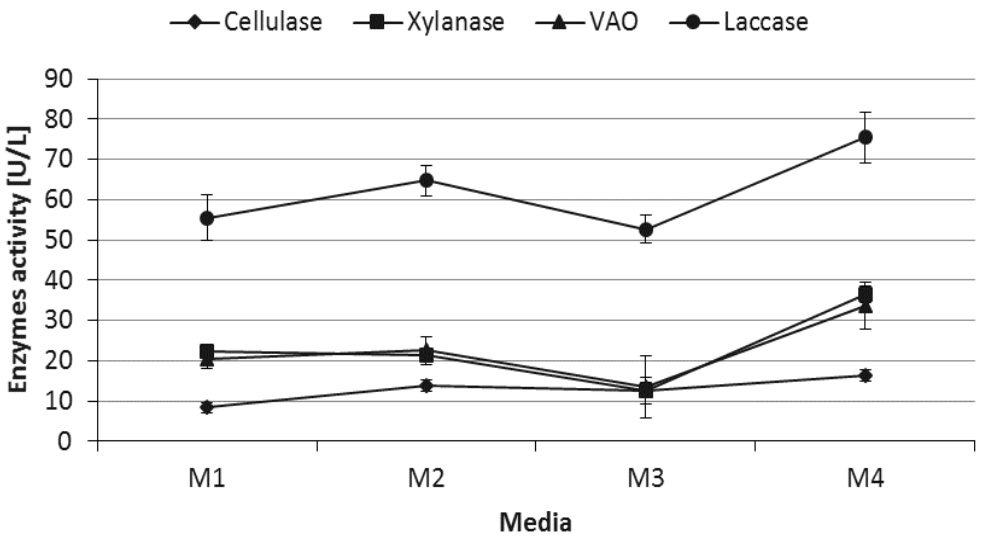
Results and Discussion

Optimization of lignocellulose degrading enzymes production by P. sapidus

-Fermentation media

The influence of different media on lignocellulose degrading enzymes production by *P. sapidus* was investigated. Four different media were tested for their ability to support growth and enzymes production (Fig. 1). Data indicated that Basal medium containing 1% rice straw was the best for maximum lignocellulose degrading enzymes secretion by *P. sapidus*.

This may be attributed to the presence of Tween 80 within the constituents of the medium, which supports the release of the enzymes complex into the medium by causing an increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes.



M1: Czapek-Dox containing 1% rice straw *M2: Simplified minimal medium containing 1% rice straw*
M3: Potato dextrose agar (PDA) containing 1% rice straw *M4: Basal medium containing 1% rice straw*

Fig.1. Diagram showing the effect of different media on the lignocellulose degrading enzymes production.

The obtained results are in accordance with those reported by Vyas (2004) who demonstrated that the addition of Tween 80 as surfactant led to higher cellulase activities. In addition, the presence of trace elements solution in the basal medium may encouraged the growth of the used fungus resulting in an increase of lignocellulose degrading enzymes.

-*Carbon source*

This experiment was focused on the effect of different lignocellulosic materials on the secretion of lignocellulose degrading enzymes by *P. sapidus.* Five different materials were tested, rice straw, wheat straw, bagas, saw dust and corn stalks (Fig.2). Cellulase, xylanase and laccase enzymes showed the highest activity with using corn stalks as carbon source. On the other hand, veratryl alcohol oxidase showed the best secretion when rice straw was used followed by corn stalks.

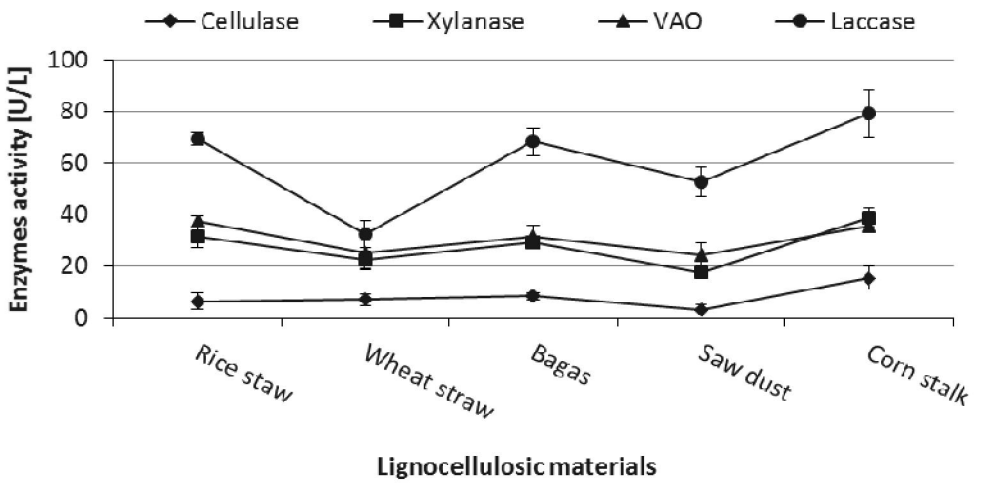


Fig.2: Diagram illustrating the effect of different lignocellulosic materials on the lignocellulose degrading enzymes production.

-*Corn stalks concentrations*

The production of hydrolytic enzymes is directly related to the available substrate (Nybroe *et al*., 1992). Lignocellulose degrading enzymes are inducible enzyme system. Therefore, an increase in the concentration of a particular substrate may stimulate the specific enzyme production by microorganisms.

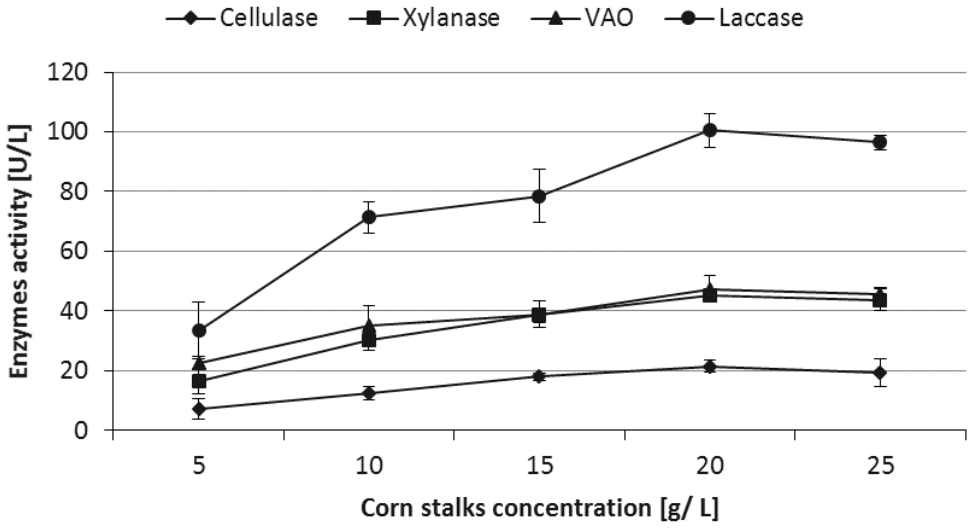


Fig.3: Effect of corn stalk concentration on the lignocellulose degrading enzymes production.

In the present study, the effect of different concentrations of corn stalks on lignocellulose degrading enzymes production by *P. sapidus* was investigated. Corn stalks concentrations (5, 10, 15, 20 and 25 g/L) were added to the basal medium. The investigated enzymes were determined and the results were graphically illustrated in Fig. (3).

As expected, lignocellulose degrading enzymes secretion increased with an increase of corn stalks concentration. The optimum concentration of corn stalks was found to be 20 g/ L for all investigated enzymes activity.

A further increase in corn stalks concentration beyond 20 g/ L did not result in a further increase in enzymes activity. The decrease in enzymes productivity at higher corn stalks concentration may be attributed to substrate inhibition as described by Guedon *et al.,* (2002). They reported complex media with high substrate concentrations to be unfavorable for *Clostridium cellulolyticum*, as the bacterium was unable to utilize excessive substrate.

-*Yeast extract concentration*

The used basal medium was supplemented with 1 – 3 g/L yeast extract. Lignocellulose degrading enzymes activities were determined. Data illustrated in Fig. (4) obviously indicate that nitrogen concentration greatly affected enzymes secretion. Maximum rate of cellulase enzyme production by *P. sapidus* was found to be 2 g/ L of yeast extract. With regard to xylanase, veratryl alcohol oxidase and laccase enzymes, the maximum activities were obtained when 2.5 g/ L of yeast extract was used.

Previous experiments on the effect of various nitrogen sources on cellulase production demonstrated a substantial increase in the enzyme activity when the media were supplemented with yeast extract (Vyas, 2004). In contrast, the growth of *Trichoderma reesei* on production medium without nitrogen source increased cellulase production (Turker and Mavituna, 1987).

-*Time course*

The time course of lignocellulose degrading enzymes was studied to determine the point of time with maximum activity. Data illustrated in Fig (5) showed the effect of different incubation periods on enzymes productivity by *P. sapidus*. From the recorded results, it was found that cellulase and xylanase activities progressively increased with the incubation period from 1-15 days and reached the maximum activities after 6 days of cultivation.

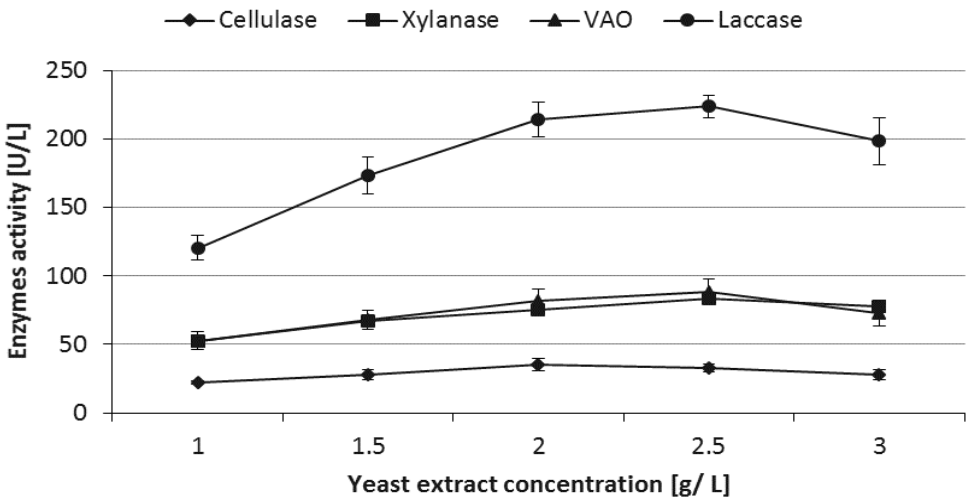


Fig.4: Diagram showing the effect of yeast extract concentration on the lignocellulose degrading enzymes production.

After that, the enzymes activity began to decrease. These results are in good agreement with those reported by Garcia-Kirchner *et al.*, (2002) they grew *Penicillium* sp. and *Aspergillus terreus* for 6 days for the maximum yield of cellulolytic and xylanolytic activities.

The highest veratryl alcohol oxidase and laccase activities were obtained after 9 and 3 days, respectively. The aforementioned results are in accordance with those obtained by Akpinara and Urekb (2014), they investigated the ligninolytic ability of *Pleurotus eryngii* to produce laccase and veratryl alcohol oxidase enzymes through solid-state fermentation using apricot and pomegranate agroindustrial wastes. They recorded that, the highest veratryl alcohol oxidase activity was after 10 days of cultivation in apricot cultures.

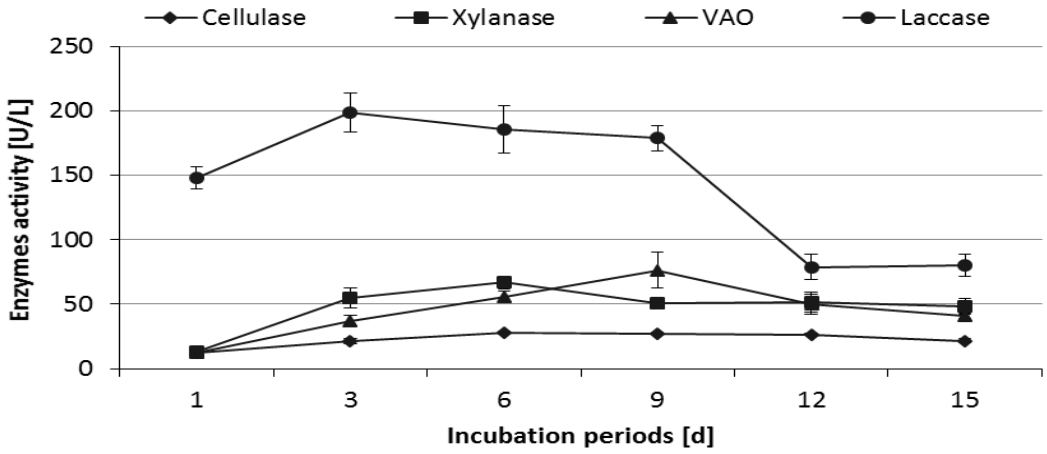


Fig.5: Effect of incubation period on the lignocellulose degrading enzymes production.

Comparison between basal medium Vs. optimized medium composition for production of lignocellulose degrading enzymes by P. sapidus

The optimum conditions for lignocellulose degrading enzymes production by *P. sapidus* were concluded in Table (1).

P. sapidus exhibited the highest enzymes activity which increased to be about two fold higher as compared with enzymes yields obtained in basal medium. Therefore, optimized basal medium was chosen to be used for lignocellulose degrading enzymes production for the succeeding work.

Table 1: Comparison of basal medium Vs. optimized medium composition

Basal medium	Constituents (g/L)	Optimized medium	Constituents (g/L)
Rice straw	10	Corn stalk	20
Yeast extract	1.0	Yeast extract	2.5
KH_2PO_4	0.2	KH_2PO_4	0.2
KCl	0.2	KCl	0.2
$MgSO_4.7H_2O$	0.2	$MgSO_4.7H_2O$	0.2
Tween 80	1 ml	Tween 80	1 ml
Trace element solution	1 ml	Trace element solution	1 ml
Enzymes	Activity (U/L)	Enzymes	Activity (U/L)
Cellulase	16.38	Cellulase	28.3
Xylanase	36.48	Xylanase	66.9
Veratryl alcohol oxidase	33.63	Veratryl alcohol oxidase	76.5
Laccase	75.43	Laccase	189.5

Concentration of lignocellulose degrading enzymes produced by P. sapidus.

The culture supernatant of *P. sapidus* was concentrated by ultrafiltration and the obtained results were recorded in Table (2).

Table 2: Concentration of lignocellulose degrading enzymes produced by *P. sapidus.*

	Initial supernatant	Retentate
Volume (mL)	1000	43
Activity (U/mL)		
Cellulase	0.036	0.46
Xylanase	0.075	1.17
Veratryl alcohol oxidase	0.073	1.12
Laccase	0.210	2.89
Protein (mg/mL)	0.570	2.85
Specific activity (U/mg)		
Cellulase	0.063	0.16
Xylanase	0.132	0.41
Veratryl alcohol oxidase	0.128	0.39
Laccase	0.368	1.014

Characterization of lignocellulose degrading enzymes

Protein after concentration was 2.85 mg/mL. The obtained specific activities were 0.16, 0.41, 0.39 and 1.014 U/mg for cellulase, xylanase, veratryl alcohol oxidase and laccase activities, respectively.

The concentrated enzymes produced by *P. sapidus* were biochemically characterized. Optimum temperature, optimum pH and temperature stability were investigated.

-Optimum temperature

Optimum temperature was studied for lignocellulose degrading enzymes produced by *P. sapidus*. For estimation of the temperature optima, the activity was determined by carrying out standard assays at several temperatures degrees (30-65°C). Under the assay conditions used, crude preparations of lignocellulose degrading enzymes showed high activity over a wide range of temperature between 30 and 65°C. Data illustrated in Fig. (6). showed that, the optimum temperature for cellulase and laccase was 50°C. On the other hand, the optimal temperature for the activities of veratryl alcohol oxidase and xylanase enzymes were found to be 55°C and 60°C, respectively. These results are in good agreement with those reported by Rao *et al*., (2003) and Wang *et al*., (2008).

-Thermal stability

Thermal stability was studied for lignocellulose degrading enzymes produced by *P. sapidus*. The loss of enzyme activity depends both on time and temperature, it is therefore important to investigate the stability of the enzyme with respect to these factors.

To estimate the temperature stability, the residual activity was determined after incubation for 12 h. at the enzymes temperature optima. The obtained data were illustrated and recorded in Figs.7 and Table 3.

Data show that laccase retained 88.54 and 71.43% of the initial activity after 2 and 12 h incubation at its optimal temperature, respectively. This indicates high thermostability of laccase enzyme. These results are in good agreement with those reported by Mukhopadhyay and Banerjee (2014), they studied the purification and biochemical characterization of a newly produced laccase from *Lentinus squarrosulus* MR13 and reported that the enzyme was stable at temperatures between 25°C and 55°C and decreased rapidly when the temperature was above 65°C.On the other hand, veratryl alcohol oxidase was rapidly inactivated and retained about 44.74 and 5.83% of the initial activity after 2 and 12 h, respectively.

Cellulase retained about 86.54 and 15.45% of the initial activity after 2 and 12h., respectively. About 17.44% residual activity was obtained for xylanase after 12h. The loss of enzyme activity can be attributed to protein denaturation. The aforementioned results are in accordance with those obtained by Reis *et al*., (2003), Whiteley *et al*., (2003) and Wang *et al*., (2008) who stated that the loss of enzyme activity at elevated temperatures is a consequence of thermal denaturation of protein.

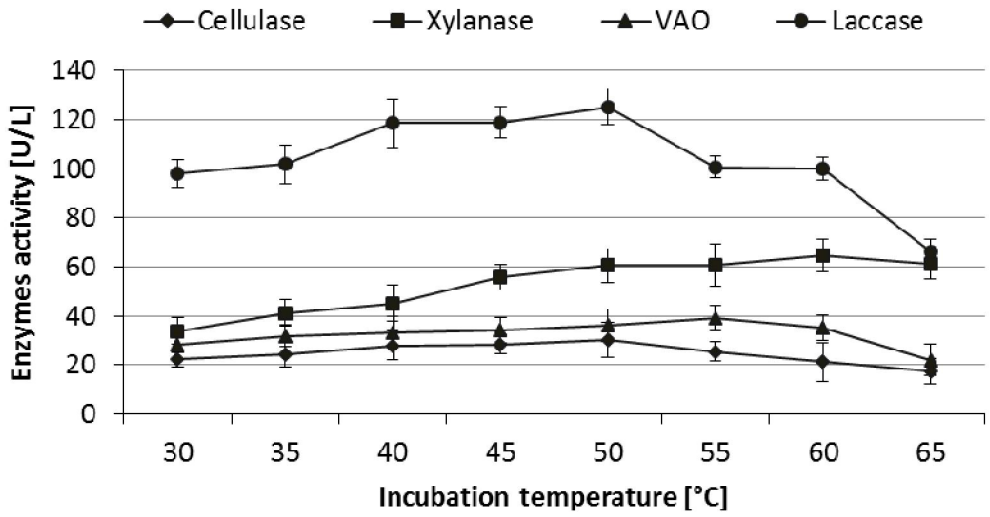


Fig.6: Diagram illustrating the effect of the temperature on lignocellulose degrading enzymes activity.

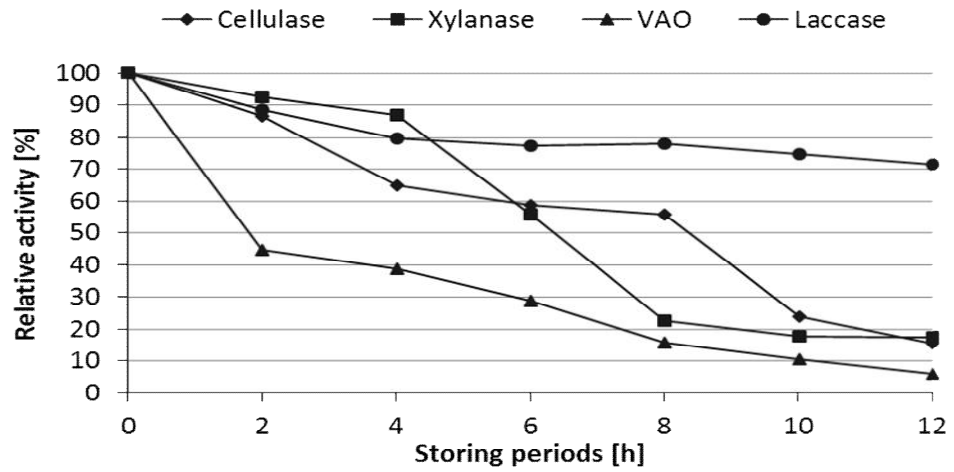


Fig.7. Thermal stability of lignocellulose degrading enzymes activity.

-Optimum pH

The ionization state of amino acid residues of an enzyme depends on the pH value. Since catalytic activity is dependent on the state of ionization of these residues, enzyme activity is consequently pH dependent. Enzymes are often active over a narrow pH range with a specific pH optimum at which their catalytic activity is maximal (Wilson, 2000).

Table 3: Thermal stability of lignocellulose degrading enzymes

Enzyme	Residual activity [%]	
	2[h]	12 [h]
Cellulase	86.54	15.45
Xylanase	92.45	17.44
Veratryl alcohol oxidase	44.74	5.83
Laccase	88.54	71.43

Therefore, it was of interest to determine the pH optima of the lignocellulose degrading enzymes under investigation (Fig.8). pH 4.0 was favorable for the activity of the xylanase enzyme. Cellulase and laccase exhibited the maximum activity at pH 3.5 and pH3, respectively. These results are in good agreement with those obtained by Fadel (2001), Coral *et al*., (2002) and Mukhopadhyay & Banerjee (2014), they studied the biochemical characterization of a newly produced laccase from *Lentinus squarrosulus* MR13 and reported that the enzyme was stable in a broad range of pH (pH 4–9). Veratryl alcohol oxidase exhibited high activity in the pH range from 3.0 - 6.0 with an optimum at pH 5.5. The aforementioned results are in accordance with those obtained by Wolfenden and Wilson, 1982.

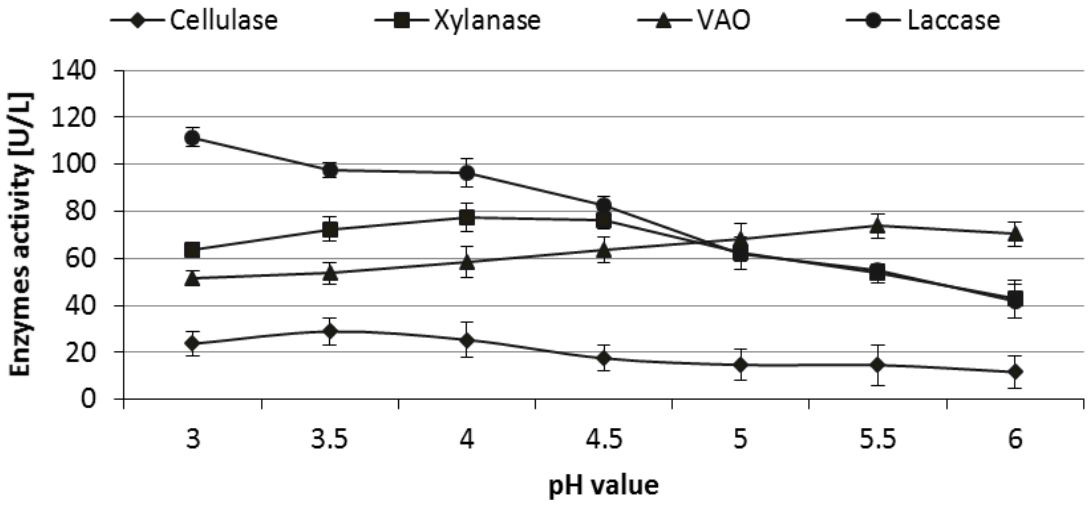


Fig.8: Effect of pH on lignocellulose degrading enzymes activity.

Acknowledgment

The authors would like to express sincere appreciation to all staff members of Agric. Botany Dept., Fac. of Agric. at Moshtohor, Benha Univ., for their kind cooperation.

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