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Full Length Research Paper

Characterization of cellulase producing from *Aspergillus melleus* by solid state fermentation using maize crop residues

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The production of cellulase by the yellowish orange sclerotia producing species, *Aspergillus melleus* UPAR01 on lignocellulosic material by solid state fermentation (SSF) was investigated. The first experiment was conducted to find out the colony radial growth rate (*Kr*) of fungus on solid medium using potato dextrose agar (PDA) and incubated at 30°C in the dark. The result shows that the average *Kr* value of *A. mellues* strain approximately was 0.77±0.03 cm/day. When the fungus was used to produce cellulase using maize crop residues as the sole carbon source by SSF at 30°C for seven day, the values of FPase, endoglucanase, β-glucosidase, and xylanase were achieved at 0.284±0.04, 9.45±0.33, 1.20±0.12, 12.58±0.08 U/mg protein, respectively. The optimal pH and temperature (°C) for the enzymatic activities was expressed by response surface methodology (RSM). The data shows that the optimum pH range was between 5.5 and 5.8 and the optimum temperature ranged from 53 to 59°C. In addition, none of the metal ions and ethylene-diaminetetra-acetic acid (EDTA) induced cellulase and xylanase activities.

Key words: *Aspergillus melleus*, cellulase, solid state fermentation (SSF), maize crop residues.

INTRODUCTION

Cellulase is a group of enzymes that synergistically work to hydrolyze cellulose to glucose. It is composed of endoglucanase (endo-1, 4-β-D-glucanase, EC 3.2.1.4), exoglucanase (1,4-β-D-glucancellobiohydrolase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase or cellobiase, EC 3.2.1.21) (Joo et al., 2009; Gao et al., 2008). Although xylanase (EC 3.2.1.8) is not part of the cellulase system but this enzyme is needed in the hydrolysis of lignocellulosic biomass which provides an appreciable amount of hemicellulose or xylan (Kamble and Jadhav, 2011).

Solid state fermentation (SSF) of lignocellulosic material for production of cellulase and xylanase is an attractive means to produce enzymes because of its

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lower capital investment and lower operating cost. Several studies have indicated that agricultural residues including maize crop residues can be used as lignocellulosic carbon source for the production of cellulase (Liming and Xueliang, 2004; Zhu et al., 2009; McMillan et al., 2011) including solid state fermentation of castor bean (*Ricinus communis* L.) for cellulase production by *Aspergillus japonicas* URM5620 (Herculano et al., 2011). Both bacteria and fungi are known to produce cellulase, however, fungal enzymes are generally complete comprising all the cellulosic activities (Stockton et al., 1991). *Aspergillus* species are well known to be good for producing cellulases and xylanase and many species have been studied, including *A. terreus* (Gao et al., 2008), *A. niger* (Ncube et al., 2012), *A. fischeri* (Senthikumar et al., 2005), *A. niveus* and *A. ochraceus* (Betini et al., 2009).

The aims of this experiment were to investigate the enzymes kinetics including optimal pH, temperature and kinetics based on the optimization by Response Surface Methodology (RSM).

MATERIALS AND METHODS

Fungal cultures

The fungus *Aspergillus melleus* UPAR01 used in this investigation was originally isolated from maize crop area, Phayao province, Thailand (2010) using modified *Aspergillus flavus* and Parasiticus Agar (AFPA) (Cotty, 1994). The culture was transferred and preserved in Potato Dextrose Agar (PDA) slants at 4°C in the culture collection of the Biotechnology program, School of Agriculture and Natural Resources, University of Phayao, Thailand. To prepare spore suspension, *A. melleus* fully grown spores (approximately seven days incubation on PDA at 30°C) were suspended in 0.85% (w/v) sodium chloride to prepare the homogenous spore suspension (10^6 spore/ml).

Media preparation

Potato Dextrose Agar (PDA) and Malt Extracted Agar (MEA) were purchased from Difco, USA. V8 medium (Cotty and Misaghi, 1984) consisted of 5.0% (v/v) V8 juice and 2.0% (w/v) agar. Modified *A. flavus* and Parasiticus Agar (AFPA) (Cotty, 1994) consists of 1.0 %(w/v) peptic digest of animal tissue, 2.0 %(w/v) Yeast extract, 0.05 % (w/v) Ferric ammonium citrate, 1.0 ml of 0.2% (w/v) dichloran in ethanol and 1.5 % (w/v) agar. Semi-synthetic medium (SS) consists of 5.0 g of lignocellulose substrate followed with 40 ml of the SS medium in a 250 ml Erlenmeyer flask. The SS medium contains 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, 0.2% (v/v) Tween 80 and 0.5 ml of all each trace elements stock solution including 0.0005 % (w/v) $ZnSO_4.7H_2O$, 0.0005% (w/v) $FeSO_4.7H_2O$, 0.002% (w/v) and 0.005% (w/v) $MnSO_4.H_2O$. The pH of all media was adjusted to 5.5, and sterilized by autoclaving at 120°C for 15 min.

Substrate preparation

Maize crop residues (stalk, stover, and hub) were collected from Dok Kham Tai district, Phayao province, Thailand. Sample was dried in a hot air oven at 60°C for 72 h and then ground and passed through 2 mm sieve. The sample was analyzed for lignocellulosic compositions by the detergent method (Goering and Van Soest, 1970) and was used as substrate for enzyme production.

Characterization of *A. melleus*

A. melleus was identified according to taxonomic schemes proposed by Klich (2002). Macroscopic characteristics, including colony diameter, colony colors, colony texture, conidial color, sclerotia, reverse color, and soluble pigment, were observed by naked eye. Fine structural characteristics, including seriation, vesicle shape, stipe length, and the shape, size and texture of conidia, were observed under a dissecting microscope.

The molecular investigation was examined in the laboratory test. *A. melleus* was inoculated into Malt Extracted Agar (MEA) plate at 25°C for 7 days in the dark, and then its DNA was extracted from the culture using MoBio-UltracleanTM Microbial DNA Isolation Kit. Fragment containing part of the β-tubulin gene was amplified using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995). The PCR fragments were sequenced with the ABI Prism® Big DyeTM Terminator v. 3.0 Ready Reaction Cycle sequenced Kit. Samples were analyzed on an ABI PHISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package. For storage, isolates were grown on V8 medium (5% V8 juice and 2%agar) (Cotty and Misaghi, 1984), from which 3-mm plugs of sporulating culture were taken and placed in vials containing 5 ml of 0.01% Triton X-100 (TX).

Measurement of colony radial growth rate (*Kr*), mycelia agar plug (0.2 cm) of *A. melleus* was inoculated onto the center of 20 ml of PDA in Petri dish and then incubated at 30°C for 7 days in the dark. Fungus was measured for the radial growth of colonies (cm) by a ruler with repeated 3 times for 3 days. Typically, the fungal mycelia had grown and reached into the end of the plate approximately in 6 to 7 days. After the last measurements, the *Kr* was determined according to the method described by Morrison and Righelato (1974). The linear regression was $Kr = dr/dt$ in which dr/dt was colony radial growth rate (cm) obtained during $time_o$ (observed time) and $time_i$ (initial time), respectively.

Production of enzymes

Solid State Fermentation (SSF) process was used in this study. The maize crop substrate (5.0 g) was weighed and put into a 250 ml Erlenmeyer flask with 40 ml of the Semi-synthetic medium (approximately 63% moisture content using Extech SDL550 Moisture Content Meter, Datalogger, USA). Culture medium was inoculated with 2.0 ml of *A. melleus* UPAG01 spore suspension (10^6 spore/ml) and incubated at 30°C for 7 days. After incubation, crude enzymes were extracted by adding 50 ml of 0.01% (w/v) Tween 80 in 50 mM sodium citrate buffer pH 5.0 and mixed for 1 h on a rotary shaker at 150 rpm. The suspension was then centrifuged and the same buffer was added to the clarified supernatant to make a total volume 100 ml. The suspension contained the crude enzymes used for further studies.

Determination of enzyme activities

Cellulase (FPase, endoglucanase and β-glucosidase) and xylanase activities were assayed according to the method described by Ghose (1987) and Ghose and Bisaria (1987). The reaction mixtures were determined by incubating 0.5 ml of enzyme solution with 50

Table 1. Variables and their level for 2^2 factorial central composite designs for cellulase and xylanase activities (U/ml).

Sample number	Natural variables		Coded variables	
	pH*	Temp (°C)	χ_1	χ_1
1	4.0	45	-1	-1
2	4.0	75	-1	1
3	8.0	45	1	-1
4	8.0	75	1	1
5	3.172	60	-1.414	0
6	8.828	60	+1.414	0
7	6.0	38.74	0	-1.414
8	6.0	81.21	0	+1.414
9	6.0	60	0	0
10	6.0	60	0	0
11	6.0	60	0	0
12	6.0	60	0	0
13	6.0	60	0	0

mM citrate buffer pH 5.0 containing one piece of 1X6 cm Whatman No.1 filter paper for FPase, 0.5 ml of 2.0 %(w/v) carboxymethylcellulose (CMC) for endoglucanase and 0.5 ml of 1.0% (w/v) xylan for xylanase, respectively. The reaction mixtures were incubated at 50°C for 60 min (FPase) and 30 min (endoglucanase and xylanase). β-glucosidase activity was assayed according to the method described by Sternberg et al. (1977). The reaction mixture was determined by incubating 0.5 ml of enzyme solution and 0.5 ml of 4 mg/ml D-salicin, followed by incubation at 50°C for 30 min.

After incubation, all reactions were assayed by estimating the reducing sugar liberated using DNS reagent (Miller, 1959), and were calculated in international units of enzyme activity. One unit of cellulase (FPase, endoglucanase, and β-glucosidase) is defined as the amount of enzyme that liberates 1.0 µmol of glucose per 1.0 min under the assay conditions. One unit of xylanase is defined as the amount of enzyme that liberate 1.0 µmol of xylose per 1.0 min under the assay conditions.

Lowry method (Lowry et al., 1951) was used in this study for protein determination. The amount of total proteins (mg/ml) was performed by means of standard curve obtained from solutions containing (0.0-2.0 mg/ml) bovine serum albumin (BSA).

Kinetic characterization (pH and temperature) by response surface methodology effect of pH and temperature

Sodium citrate (50 mM) buffer pH 3.172, 4.0 and 6.0 and 50 mM sodium phosphate buffer pH 8 and 8.8 were examined in this study. The statistical approach with Response Surface Methodology (RSM) was used to determine the optimal conditions to obtain the highest activity of enzymes. The experiments were based on 2^2 full factorial Central Composite Design (CCD) with augmented points in two independent variables including χ_1: pH, and χ_2: temperature values, while enzyme activity (γ; U/ml) was the dependent variable as described in Table 1.

All measurements were performed in three replicates, and data were expressed as average values, and analyzed using Minitab 5.1 software. The lower *p*-value and insignificant lack of fit was obtained with a quadratic model that suggested good fit. Higher coefficients of regression (95%) suggested that there was good agreement between predicted and estimated enzymatic activities. The results of RSM were used to fit a second-order polynomial equation that represents the behavior of the system; $\gamma = \beta_0+\beta_1\chi_1+\beta_2\chi_2+\beta_{11}\chi_1^2+ \beta_{22}\chi_2^2+\beta_{12}\chi^1\chi^2$

Effect of metal ions

The effects of metal ions which included Mg ($MgSO_4$), Ca ($CaCl_2$), Mn ($MnSO_4$), Fe($FeSO_4$), Co($CoCl_2$), Cu($CuSO_4$), Zn($ZnSO_4$), and ethylene-diaminetetra-acetic acid (Na-EDTA) were investigated by incorporating them to 0.1 M final concentration mixture prior to determination of enzyme residual activities.

Km and *Vmax*

Four types of substrates, Avicel®, CMC, salicin and xylan were also chosen to test the substrate specificity of FPase, endoglucanase, β-glucosidase and xylanase, respectively. After the selection of the optimum pH for enzyme activities, the Michalis-Menten for *Km* and *Vma*x values were determined by the investigation using varying substrate concentrations ranging from 0 to 0.25 %(w/v), and the assays were conducted in the same manner of cellulases and xylanase determination.

RESULTS AND DISCUSSION

The AFPA medium was modified by Pitt et al. (1983). This medium was used for *Aspergillus* spp. isolation by several authors including Reddy et al. (2009) and Foley et al. (2014). The medium was used to select *Aspergillus* sp. including *A. flavus* or *A. parasiticus* because both fungi produced yellow orange color when viewed from the reversed side of the plate. Yellow orange color of colony was observed because medium help improved color production on the reverse of the plate due to the optimal concentration of a more soluble iron salt and the addition of yeast extract (Pitt et al., 1983). In this study, *A. melleus* UPAG01 color change of the reverse phase on AFPA was similar as *A. flavus* or *A. parasiticus* by appearance of yellow orange color reverse colony. Conidia and mycelium germination of isolates *Aspergillus* could be observed in these AFPA medium except groups of *A. nomius.* Damann et al. (2004) showed AFPA medium did not support sporulation by *A. flavus*. *A. melleus* showed dark yellow reverse colony similarity as *A. flavus* and Foley et al. (2014) reported that *Aspergillus* spp. produced yellow orange color when viewed from the reversed side of the plate. However, *A. melleus* UPAG01 produced yellow radiate conidia heads (50-150 µm) which enable clear differentiation from *A. flavus* or *A. parasiticus* (green conidia). The microscopic morphology showed that the hyphae (2-3 µm diameter) were septate, hyaline, and biserate with smooth wall globose conidia (2-3 µm diameters) which was reported by Christensen (1982) and Klich (2002). In addition, this strain produced abundantly yellow to brown sclerotia on 5% V8 medium and PDA (Klich, 2002) (Figure 1). When *A. melleus* UPAG01 DNA was extracted, to identify the species of

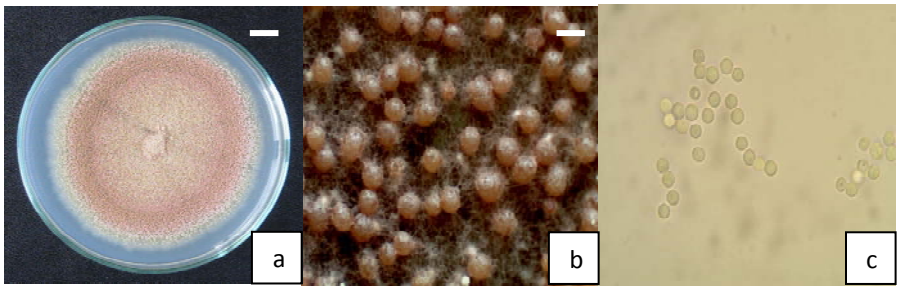

Figure 1. Morphological characteristics of *A. melleus* UPAG01 (a) conidia (pale yellow) and sclerotia (dark brown) on PDA (bar=1 cm)(b) scerotia on PDA (bar=0.1 mm) (c) Conidia under microscope.

Table 2. Equations analysis for optimal pH and temperature (coded values).

Enzymatic acticity (U/ml); γ	Fitted Equation	pH optimum (χ_1)	Temperature (°C) optimum (χ_2)
FPase; γ_1	$0.1872-0.013\chi_1-0.010\chi_2-0.061\chi_1^2-0.066\chi_2^2$	-0.059	-0.130
Endoglucanase; γ_2	$1.538-0.262\chi_1-0.401\chi_2-0.250\chi_1^2-0.400\chi_2^2$	-0.832	-0.220
β–Glucosidase; γ_3	$0.145-0.016\chi_1-0.017\chi_2-0.030\chi_1^2-0.035\chi_2^2$	-0.246	-0.70

Aspergillus was followed according to the method described by Glass and Donaldson (1995). The sequences were compared on GenBank using BLAST and in a large fungal database of CBS-KNAW Fungal Biodiversity Centre with sequences of most of the type strains. The results had a 100% match in all databases with the type strain of *A. melleus* sample that is a fungus in the Subgenus *Circumdati*, Section *Circumdati*.

For the measurement of colony radial growth rate (*Kr*), several reports showed that *Aspergillus* sp. had wide range of *Kr* values that depended on species, strains and culture conditions. The *Kr* values of *Aspergillus* strains isolated from honey bee and *A. flavus* ATTC 16872 were 0.228 and 0.24 cm/day, respectively (Yoder et al., 2008; Lopez-Malo et al., 1998). High or low values of pH and chemical substances which included bicarbonate, vanillin and avermectin (more than 0.01% w/v) affected colonies formation by *Aspergillus*, and lead to the reduction of the values of *Kr* (Lopez-Malo et al., 1995; Rodr´ıguez-Urra et al., 2009; Danmek et al., 2011). In this study, PDA with pH at 6.0 was used because several reports discussed PDA as suitable for the culturing of *Aspergillus* and had no effect in the range between pH at 5.5 to 7.5 (Brancato and Golding, 1953; Cotty, 1988; Thompson, 1990; Yoder et al., 2008). In addition, Gupta et al. (2012) showed that PDA is the most commonly used as laboratory medium for fungi due to its good and balanced nutrient content. *A. melleus* UPAG01 grew well and produced white mycelia and brown conidia with dark-brown sclerotia until the end of the PDA plate within 6 days at 30°C in the dark same as the other *Aspergillus* spp. including *A. flavus* and *A. niger* (Reddy et al., 2009; Foley et al., 2014). Data showed the beginning of exponential phase at the second day of incubation. Therefore, linear regression of *A. melleus* UPAG01 was calculated using the relationship between colonies radial growth (cm) versus time (day) that at this time. Average *Kr* value of all *A. melleus* UPAG01 for 6 days was 0.77±0.03 cm/day.

Among the selected maize crop residue, it contained the high cellulose (33.15%dw) hemicellulose (44.67%dw) and low lignin content (4.91 %dw). The residue was selected as a carbon source for enzyme suitable for production of cellulase and xylanase by *A. melleus* UPAG01. Under acidic conditions with pH 5.5 and 7 days of incubation, *A. melleus* UPAG01 produced 0.28±0.04, 9.45±0.33, 1.20±0.12 and 12.58±0.08 U/mg of FPase, endoglucanase, β-glucosidase, and xylanase, respectively. The high yield might result from its lignocellulosic materials present in inducers and nutrients. Several reports indicated that cellulase and xylanase production were favored in the acidic range of pH 4 to 6 (Brijwani et al., 2010; Romero et al., 1999; Tishkov et al., 2013). Similarly, Pothiraj et al. (2006) and Mrudula and Murugammal (2011) reported that *A. terreus* and *A. niger* produced high value of cellulase activities in the sixth to eight day of incubation by Solid State Fermentation (SSF). For optimal pH and temperature, the overall second order polynomial equation for the activities as measured in term of Unit (U/ml) is shown in Table 2. The three dimensional plots of the combined parameters between χ_1 and χ_2 on the enzyme activities are shown in Figure 2. The negative significant coefficient of χ_1, and χ_2 values are expressed as a linear effect; this is meant for decreasing enzyme activities (γ).

For the other terms, two quadratic terms of χ_1^2, and χ_2^2

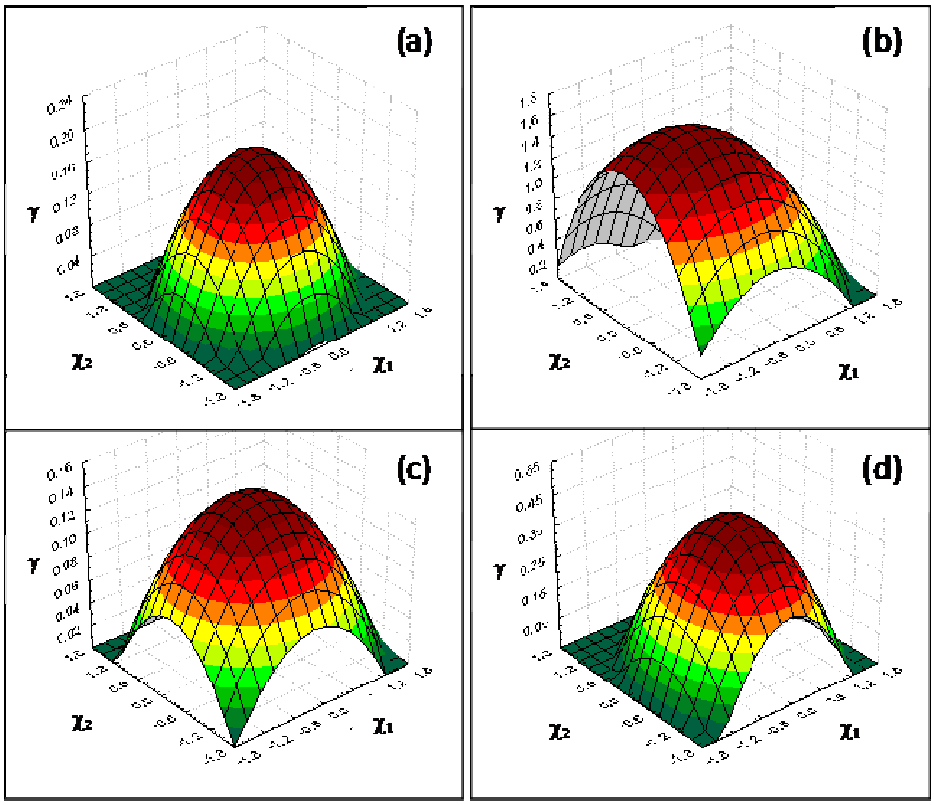

Figure 2. Three dimensional plots of the combined effects between pH (χ_1) and temperature (χ_2) on enzyme activities (γ; U/ml) from *A. melleus* UPAG01: FPase (a), endoglucanase (b), β-glucosidase (c), and xylanase (d)

were found to be expressed similarly as linear effect whereas interaction term ($\chi_1\chi_2$) was not significant. The optimal pH and temperature range for cellulase and xylanase activities production by *A. melleus* UPAG01 were found to be at pH 5.5 to 5.8 and at temperature of 53 to 58°C which was similar as the other *Aspergillus* species including *A. terreus* (Gao et al., 2008), *A. fumigatus* (Das et al., 2013) and *A. oryzae* (Riou et al., 1998; Hoa and Hung, 2013) when using the Equation in Table 2, respectively. The results of interaction between the temperature and pH revealed that increasing both temperature (>60°C) and pH (>6.0), leads to a decrease of enzyme activity. Under strong acidic (pH 3.17) and alkali conditions (pH 8.82) at high temperatures (>75°C), the enzyme activities were much lower. Other reports mentioned that the optimal pH and temperature for cellulase and xylanase activities from the other fungi, *A. terreus* (Gao et al., 2008), *A. fumigatus* (Das et al., 2013) and *A. oryzae* (Hoa and Hung, 2013), were in the pH range between 4.5 to 6.0 and temperature range between 50 to 60°C.

The effect of metal ions and substances on cellulase and xylanase of *A. melleus* UPAG01 are summarized in Figure 3. None of the metal ions and substances induced both cellulase and xylanase activities. The high values inhibitions of all enzymes were found in the presence of Cu and EDTA similarly as the results obtained by Tejirian and Xu (2010) and Quay et al. (2011). Approximately 50% of relative activities were found in the present of Cu ion (52±2.96% in FPase, 57±5.23% in endoglucanase, 48±0.71% in β-glucosidase and 46±0.75% in xylanase, respectively). The inhibition of enzymes by Cu ion might be affected by the high Cu concentration (0.1 M final concentration). However Tavares et al. (2013) showed that cellulase from *Aspergillus* spp. including *A. nidulan* could be induced when using low and optimal Cu concentration (0.018 M). In addition, Cu and EDTA ion (0.015 mM), activated cellulase activity (endoglucanase) from *Aspergillus awamori* VTCC-F099 by up to 55% (Nguyen and Quyen, 2010).

Crude *A. melleus* UPAG01 enzymes appears to be a good candidate, which is very useful for their applications to degrade lignocellulosic material. The relationship between enzyme activities and substrate concentrations produced typical Michaelis-Menten curves. *Km* values were found to be 0.09 (FPase), 0.668 (endoglucanase), 0.069 (β-glucosidase), and 2.176 g/L (xylanase) whereas *V*max values were found to be 0.003 (FPase), 0.044 (endoglucanase), 0.083 (β-glucosidase), and 1.071 $\mu mol.min^{-1}.ml^{-1}$ (xylanase) when each Avicel®, CMC,

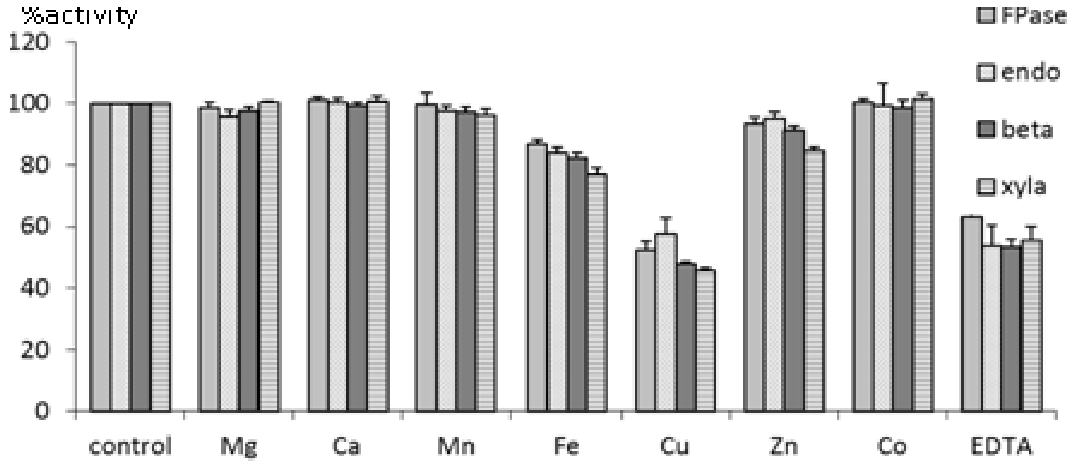

Figure 3. Effects of metal ions and Ethylene Diamino Tetraacetic acid (EDTA) on the activity of the enzymes from *A. melleus* UPAG01.

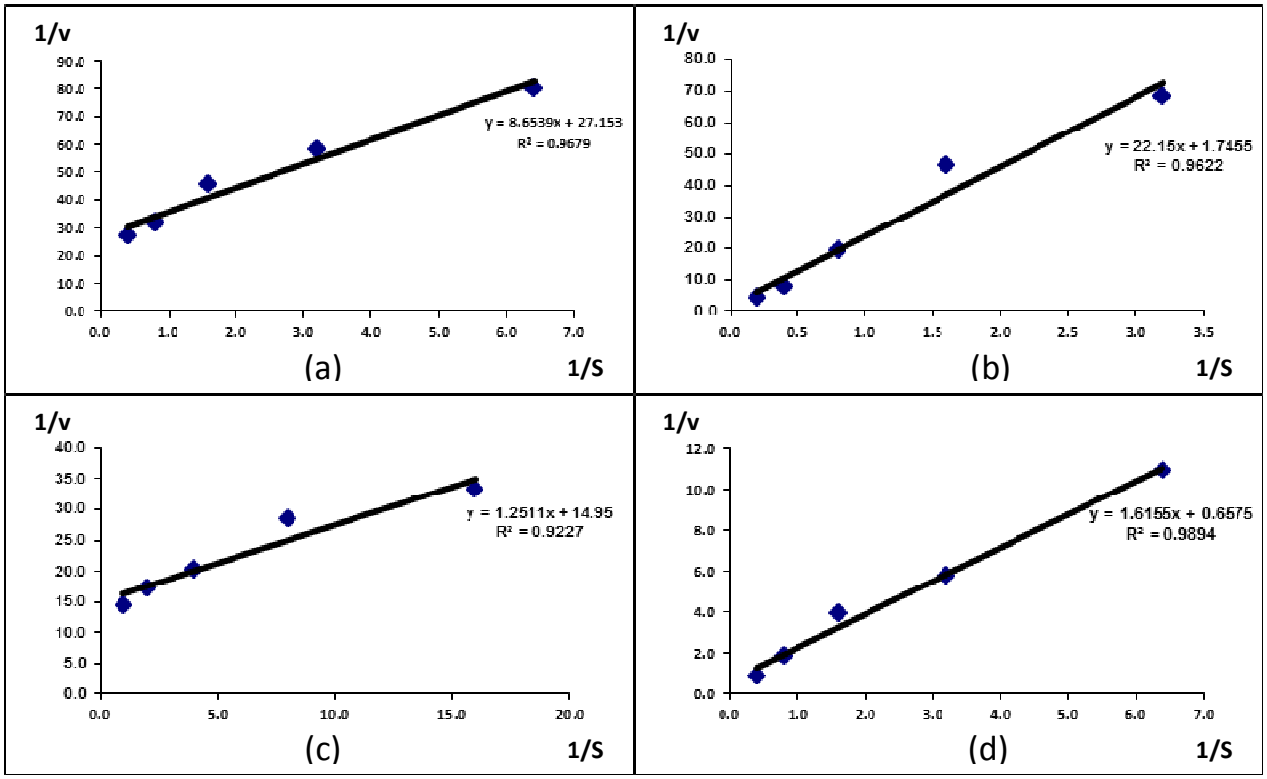

Figure 4. Michaelis-Menten curves of *A. melleus* enzymes determined by the investigation using varying substrates including Avicel® (a), CMC (b), salicin (c) and xylan (d).

salicin, and xylan was used as the substrate (Figure 4). For this observation, selection of the appropriate storage solvent (sodium citrate) and specificity with substrates seems to be crucial for cellulase and xylanase in order to maintain high levels of enzymes for either storage and in biodegradation reactions of lignocellulosic materials.

Conclusion

According to the investigation, the use of the lignocellulosic material, maize crop residue, as the sole carbon source was a candidate for the production of cellulase and xylanase. The unique enzymes from *A. melleus* could be used for hydrolysis of local agricultural biomass.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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