CHAPTER 7

OPTIMIZATION CONDITION FOR CELLULASE AND XYLANASE PRODUCTION

7.1 Introduction

Cellulase (E.C. 3.2.1.4), is one of the most useful enzymes in industry consist of three major classes: endoglucanases, excoglucanases and β-glucosidases. Cellulose was hydrolyzed by cellulase to hexose sugar, mainly glucose. Cellulase can be produced by fungi, bacteria or actinomycetes, but the most common producer is fungi (Ariffin *et al*., 2006). Most reports for cellulase producer are *Aspergillus* sp. and *Trichoderma* sp. (Milala *et al*., 2005; Wen *et al*., 2005; Zhang *et al*., 2007). Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture (Kuhad *et al*., 2011). Many studies of cellulase were successfully produced on lignocellulosic biomass under solid state fermentation, for example corn straw, rice husk and maize straw were used as substrates for cellulase production under solid state fermentation and found the optimal cellulase secretion by *Aspergillus niger* was shown at 5% concentration of substrates and pH 3.0 (Milala *et al*., 2005).

Xylanase (E.C.3.2.1.8), a group of hemicellulolytic enzymes, is required for the hydrolysis of β-1,4-xylans present in lignocellulosic biomass. These enzymes are important in the bioconversion of hemicelluloase into their consitituent sugars

(Gawande and Kamat, 1999). Their potential applications include pulp bleaching, bread making, clarifying fruit juices and wines, improving the nutritive value of animal feed and ethanol production (Royer and Nakas, 1989; Gilbert and Hazlewood, 1993; Wong *et al*., 1996; Tebka *et al*., 2006; Kongbuntad *et al*., 2006[b]). Many studies on xylanase production have used solid state fermentation by using agricultural residues as solid substrate. For example, Sanghvi *et al*. (2010) produced thermostable xylanase by *T. harzianum* and found that wheat straw produced the highest yields (146 IU/ml) over the various different substrates.

Thermophilic enzymes are better suited for industrial processing of polysaccharides because of their increased activities and stabilities at high temperatures (Kalogeris *et al*., 1998). Recent studies interested in *Thermoascus aurantiacus* because of the thermophilic ascomycete *T. aurantiacus* is able to secrete most of the thermostable hemicellulolytic and cellulolytic enzymes (Brienzo *et al*., 2012). For example, Kalogeris *et al*. (1998) showed type of carbon and nitrogen source, inoculums type, moisture level and particle size enhanced xylanase production by *T. aurantiacus* under solid state culture. Corncob, grasses and corn straw were reported as good substrates for xylanase production (De Silva *et al*., 2005). Moreover, Kawamori *et al*., 1986 found *T. aurantiacus* A-131 could produced cellulase enzyme even without cellulase inducers by using alkali-treated bagasse substrate.

Lignocellulose can be used as a low-priced raw material for enzyme production. Most research on enzyme production has used submerged fermentation, but solid state fermentation has advantages over submerged fermentation, because of less space requirements, lower costs, the abundance of agricultural residue as a substrate for the production of enzymes (Sanghvi *et al*., 2010) and the low technology fermentation

process is particularly suitable for the needs of developing countries (Sathish *et al*., 2008). Thailand is an agricultural country and has abundant agricultural residues from different crop residues. Thus, the use of agricultural residues in cellulase and xylanase production by *Thermoascus aurantiacus* SL16W can be a method for improving residue value and reducing the effect of the residue pollution on the environment.

The purposes of this study were to find out the optimal growth condition and production of cellulase and xylanase that may involve yield measurements of enzyme activity. In addition, the partial characterization of these enzymes was included. Moreover, cellulase and xylanase were used for cellulose and hemicelluloses hydrolysis in bio-ethanol production process in the next experiment.

7.2 Materials and methods

7.2.1 Microorganism

The fungus, *Thermoascus aurantiacus* SL16W (Kongbuntad *et al*., 2006) were used for cellulase and xylanase production in this study.

7.2.2 Lignocellulose substrates preparation

Ten agricultural residues including rice husk, coffee husk, rice straw, corncob, sugarcane bagasse, rice bran, orange peel, banana peel, bamboo pulp, and corn stover were used as substrates for cellulase and xylanase production. Substrates were washed 2-3 times with sterile water and dried in oven at 50-60^{o}C. Dried substrates were ground to a particle size of about 2-5 mm for solid state fermentation.

7.2.3 Solid state cultivation (SSC)

Three mycelia plugs from 10-day-old cultures of *T. aurantiacus* SL16W on PDA were transferred to 250 mL Erlenmeyer flasks containing 3 g solid substrates (agricultural residues) and 15 mL mineral solution (L^{-1}) contained, 3.5 g of $(NH_4)_2.SO_4$, 3 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$ and 0.5 g of $CaCl_2.2H_2O$, pH 6.0 was used as control solvent (Jianmin *et al*., 2008). Cultures were incubated static at 45^oC for 14 days.

7.2.4 Enzymatic assay

Cellulase and xylanase production was determined by analysis of reducing sugar released during hydrolysis of CMC (Wako Pure Chemical Industries Ltd., Japan) and xylan (Sigma-Aldrich, USA), respectively. The assay was carried out in 0.1M phosphate buffer pH 7.0 at 50°C for 30 min. The reducing sugar formed was determined by dinitrosalicylic; DNS method (Miller, 1959). Spectrophotometer was set up at 540 nm. One unit of enzyme activity defined as the amount of enzyme that released 1 µmole of reducing sugar per minute. The activities compared according to the enzyme activity in unit per gram dried substrate (U/gds) and specific activity in unit per milligram protein (U/mg. protein).

7.2.5 Optimization temperature for growth of *T. aurantiacus* SL16W

Pured culture of *T. aurantiacus* SL16W was cultivated on PDA media plates and incubated at different temperatures between 30 to 60^oC under darkness and static condition for 7 days. The diameter of mycelia growth (cm) was measured and spore production on agar media was observed to find out the optimal temperature for growth of *T aurantiacus* SL16W.

7.2.6 Optimization solid substrate for cellulase and xylanase production

To determine the suitable solid substrate for cellulase and xylanase production, ten lignocellulosic biomass included rice husk, coffee husk, rice straw, corncob, sugarcane bagasse, rice bran, orange peel, banana peel, bamboo pulp, and corn stover were used as carbon source in solid state fermentation. Five gram of solid substrates was carried out in 15 ml of distilled water and incubated at 45^{o}C, static condition for 7 day. The relatively cellulase and xylanase activity (%) were calculated.

7.2.7 Experimental design and statistical analysis for solid substrate combination

Three substrates with a high specific activity of cellulase and xylanase were selected and treated by biological pretreatment under solid state fermentation by white-rot fungus (Chapter 6) and substrates were combined using the mixture design experiment, ten different trials following the simplex centroid design for three mixture components. All trials were performed in triplicate, and the mean was determined. The data were analysed for significant difference between trials using analysis of variance (ANOVA). To establish if differences between individual trails were significant ($p < 0.05$) and multiple linear regression analysis was performed using Design-expert 6.0.2 software. The observed values were analyzed and fitted to a mixture quadratic model in Eq. 7.1.

$$Y = \Sigma\beta_{\mathbf{i}}\boldsymbol{X}_{\mathbf{i}} + \Sigma\beta_{\mathbf{ij}}\,\boldsymbol{X}_{\mathbf{i}}\boldsymbol{X}_{\mathbf{j}} \qquad (7.1)$$

Where, Y is the variable response and β is the regression coefficients given by the model and X_i and X_j are the independent factors of the experiment (Akay, 2007).

7.2.8 Optimization nitrogen source for cellulase and xylanase production

Solid state fermentation was carried out in 15 ml of mineral solution (Jianmin *et al*., 2008), 5 g of mixed-substrates (from previous experiment), 5 days-old of *T. aurantiacus* SL16W was inoculated and incubated at 45^{o}C under static condition for 7 days. The effect of various nitrogen sources, ammonium tartrate, $(NH_4)_2C_4H_4O_6$; ammonium sulfate, $(NH_4)_2SO_4$; potassium nitrate, KNO_3; peptone and yeast extract (0.5% w/v) was investigated. The cell-free supernatant, extracted with 50 ml of distilled water was assayed for cellulase and xylanase activity.

7.2.9 Experimental design and statistical analysis for the concentration of mineral solution components for cellulase and xylanase production

Central composite design (CCD) was used to find out the optimal concentration of each component in culture of solid state fermentation for cellulase and xylanase production by *T. aurantiacus* SL16W. Mineral solution (L^{-1}) contained, 3.5 g of $(NH_4)_2.SO_4$, 3 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$ and 0.5 g of $CaCl_2.2H_2O$, pH 6.0 was used as control solution. The different levels of their coded and actual values for CCD following octagon design, the design points (α) for 4 factors as ±2 are shown in Table 7.1. Cellulase and xylanase production using modified mineral solution from CCD experiment were determined compared with control solution.

Table 7.1 Codes and actual levels of the independent variables for design experiment

Variables	Code levels				
	-2	-1	0	1	2
Peptone (%)	0	0.30	0.65	1.00	1.35
$KH_2.PO_4$ (%)	0	0.10	0.30	0.50	0.70
$MgSO_4.7H_2O$ (%)	0	0.01	0.06	0.10	0.15
$CaCl_2.2H_2O$ (%)	0	0.01	0.06	0.10	0.15

All trials were performed in triplicate, and the mean was determined. The data were analyzed for significant difference between treatments using analysis of variance (ANOVA). To establish if differences between individual trails were significant ($p < 0.05$) and multiple linear regression analysis was performed using software Design-expert 6.0.2. The maximum laccase production was taken as the dependent variable of response (Y). The observed values were analyzed and fitted to the second-order model equation is as follows;

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \qquad (7.2)$$

Where, Y is the variable response (enzyme activity) and β is the regression coefficients given by the model and X_i and X_j are the independent factors of the experiment (Akay, 2007). If the curve shape of the response surface plot is elliptical or circular then it is presumed that the correlation between the variables is most significant.

7.2.10 Effect of incubation time on cellulase and xylanase production

Cellulase and xylanase activities were determined after 4 day of incubation until 20 day to find out the optimum incubation time for the maximum production of cellulase and xylanase. The test flask contained 5 g of substrates, 15 ml of mineral solution, 5 days-old of *T. aurantiacus* SL16W was inoculated and incubated at 45^{o}C and enzyme activities were measured by DNS method.

7.2.11 Effect of solid to liquid ratio on cellulase and xylanase production

The effect of solid (mixed-lignocellulosic substrates) to liquid (modified mineral solution) ratio on cellulase and xylanase production was studied. The ratios of 1:1 to 1:10 used to determine the optimal solid to liquid ratio for solid state fermentation by *T. aurantiacus* SL16W and the enzyme activities were measured by DNS method.

7.2.12 Effect of pH and temperature on cellulase and xylanase stability

Crude enzyme produced by *T. aurantiacus* SL16W from 3 agricultural residues were used in this experiment. To determine the effect of pH on enzyme stability, crude enzymes were incubated at room temp 28^{o}C for 24 h in various buffers: 100 mM sodium tartrate buffer (pH 2.0-3.0), 100 mM sodium acetate buffer (pH 3.0-6.0), 100 mM potassium phosphate buffer (pH 6.0-8.0) and 100 mM Tris-SO_4 buffer (pH 8.0-10.0). The thermal stability of crude enzyme cellulase and xylanase was incubated for 60 mins at a temperature range of 30 to 90^{o}C. Then the remaining enzyme activities, cellulase and xylanase were determined using DNS assay by using CMC and xylan as substrates.

7.3 Results and discussion

7.3.1 Optimal temperature for growth of *T. aurantiacus* SL16W

In this study, the fungus *T. aurantiacus* SL16W completely grown on semi-synthetic media, PDA plate in 4th day (colony diameters of 9 cm) at the optimal incubation temperature was 45^{o}C. *Thermoascus aurantiacus* SL16W could produce ascocarp at high temperature, up to 40 ^{o}C at 5th day but no spore reproduction at the temperature up to 50^{o}C or lower temperature at 35^{o}C (Fig 7.1). The brown ascocarps and spores of *T. aurantiacus* SL16W were shown in Fig. 7.2a and b.

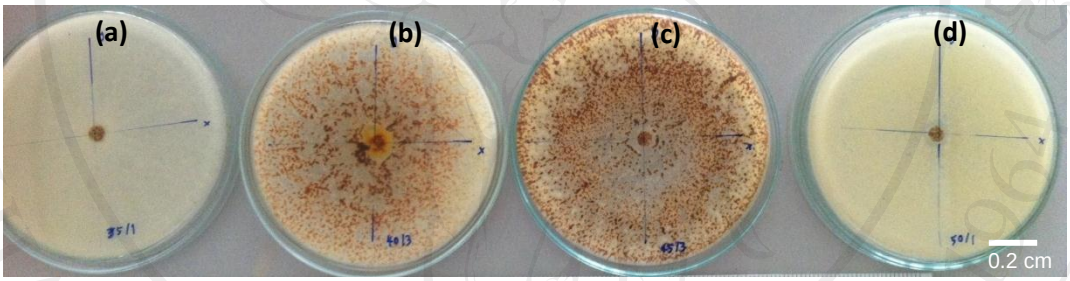

Fig. 7.1 Four-day old of *Thermoascus aurantiacus* SL16W on PDA at different incubation temperature, (a) 35^{o}C, (b) 40^{o}C, (c) 45^{o}C, and (d) 50^{o}C.

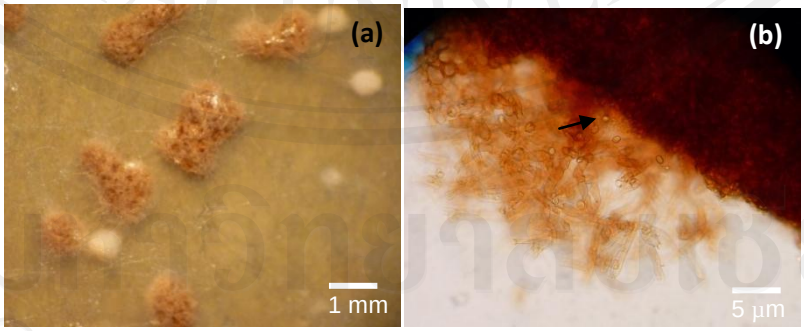

Fig. 7.2 Light micrographs of *Thermoascus aurantiacus* SL16W. (**a**) The ascocarps are orange-brown color grown on PDA under sterio microscope and (**b**) Mycelia and brown ascospores (arrow) under compound microscope.

Thermoascus aurantiacus SL16W is the thermophilic ascomycetes fungus, has been proposed as good microorganism for bioconversion of lignocellulosic biomass to sugars and offer the great potential to be used in industrial scales (Dashtban *et al*., 2009). There are many publications about cellulase and xylanase production by *T. aurantiacus* (Kalogeris *et al*., 1998; Gomes *et al*., 2000; De Silva *et al*., 2005).

7.3.2 Optimal solid substrate for cellulase and xylanase production

In this experiment, 10 lignocellulose substrates (agricultural residues) were used as solid substrate (main carbon source) for cellulase and xylanase production. Among different residues, coffee husk ensured the highest yield of cellulase, 8.72±0.3 U/mg protein. The specific activities of cellulase were 7.16±0.3 and 7.02±1.4 U/mg protein when grown on rice husk and sugarcane bagasse, respectively (Fig. 7.3).

Xylanase activities produced under solid state fermentation by *T. aurantiacus* showed the highest yield by using coffee husk, 86.6±0.3 U/mg protein. The specific activity of xylanase was 54.4±21.0 U/mg protein when grown on sugarcane bagasse and 48.7±5.6 U/mg protein when grown on rice husk (Fig. 7.4).

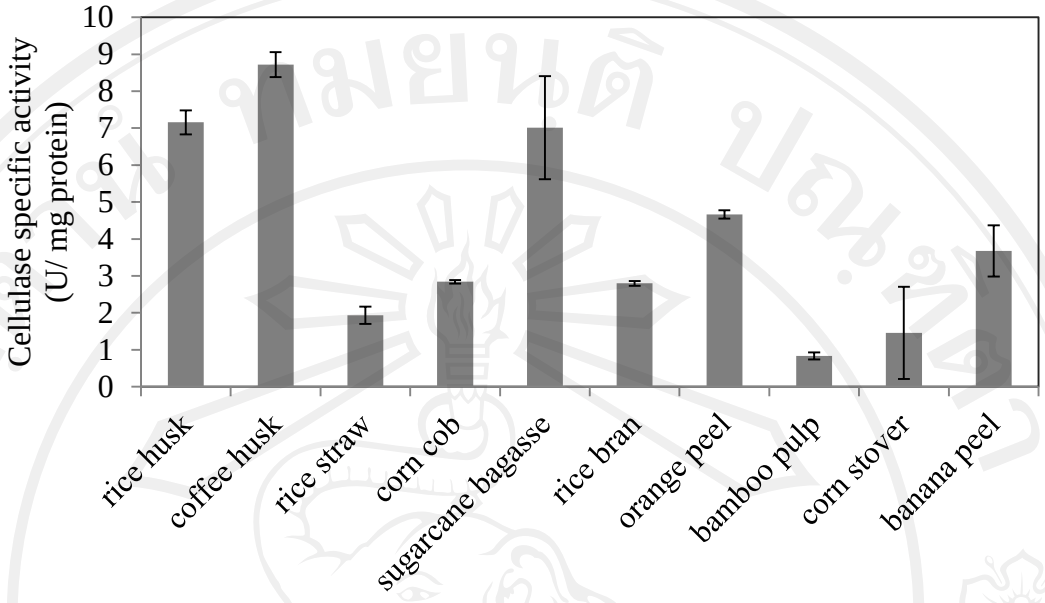

Fig. 7.3 Cellulase production by *Thermoascus aurantiacus* SL16W on various carbon sources, incubated at 45°C for 7 day.

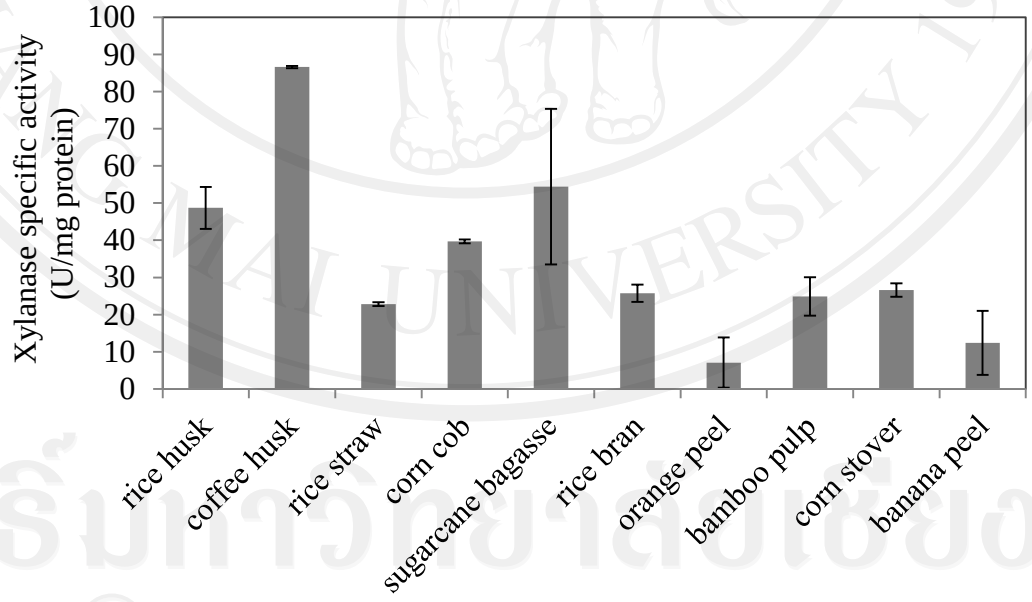

Fig. 7.5 Xylanase production by *Thermoascus aurantiacus* on various carbon sources, incubated at 45°C for 7 day.

The residues from coffee have been reported as substrates for cellulase production (Selvankumar *et al*., 2011) and xylanase (Murthy and Naidu, 2010). Different industrial enzymes such as pectinase, tannase, and caffeinase were also produced by coffee pulps (Pandey *et al*., 2000). Coffee by-products are rich in carbohydrates; 57% w/w of cellulose, 17% w/w of hemicelluloses (Murthy and Manonmani, 2008) and 26.5% w/w of sugars (Brand *et al*., 2000). This substrate is close to the natural habitats of microorganisms and may prove efficient in producing enzymes and metabolites (Jecu, 2000). Sugarcane bagasse has a chemical composition containing 19.2% lignin, 22.9% hemicelluloses, and 43.5% cellulose (Gawande and Kamat, 1999). Thus, bagasse could be used as a substrate for cellulase and xylanase production, there are in agreement with many recent studies (Bigelow and Wyman, 2002: Park *et al*., 2002; Jommuengbout *et al*., 2009). Rice husk could be used as a substrate for cellulase and xylanase production. For example, Lee *et al*. (2008) studied on cellulase production from rice husk by *B. amyloliquefaciens*. Svarachorn (1999), used rice husk as substrate for xylanase production by *Aspergillus niger* strain 4-45-1F. It was shown previously that rice husk is source of polysaccharides (Zemnukhova *et al*., 2004). In addition, Tarley *et al*. (2004) showed from data obtained from elemental analyses that rice husk contains a higher content (% m/m) for carbon (42.6) followed by hydrogen (5.1) and nitrogen (0.8), which are basically composed of cellulose, hemicellulose and lignin (Juliano, 1985).

7.3.3 Experimental design and statistical analysis for solid substrate combination

7.3.3.1 Solid substrates combination ratio for cellulase production

Rice husk, coffee husk and sugarcane bagasse were combined in the mixture design experiment. The observed values of extracellular cellulase activities, protein concentration, and specific activities were shown in Table 7.2. The highest specific activity of cellulase (25.25 U/mg protein) was observed in the fifth trial of the experimental setup. The results of the ANOVA for cellulase production was shown in Table 7.3. The quadratic regression showed the model was significant, the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms. The non significant value 0.49 lack of fit showed that the quadratic model was valid for the present study.

Table 7.2 Cellulase activities, protein concentration, and specific activities from mixture design experiment of three agricultural residues fermented by *Thermoascus aurantiacus* SL16W

Trial	Concentration (%w/w)			cellulase activity* (U/g substrate)	Protein concentration* (mg/ml)	Specific activities* (U/mg protein)
	rice husk	coffee husk	sugar cane bagasse			
1	0.7	0.2	0.2	20.97±1.34 ^{bcd}	0.1±0.02 ^{a}	12.88±2.30 ^{ab}
2	0.0	1.0	0.0	17.65±0.69 ^{cdef}	0.1±0.01 ^{bc}	17.27±4.23 ^{ab}
3	0.2	0.2	0.7	22.16±1.55 ^{bc}	0.1±0.01 ^{de}	22.66±5.59 ^{ab}
4	1.0	0.0	0.0	17.58±0.20 ^{cdef}	0.2±0.03 ^{a}	12.08±2.39 ^{b}
5	0.0	0.0	1.0	26.23±8.68 ^{b}	0.03±0.01 ^{f}	26.54±7.24 ^{a}
6	0.3	0.3	0.3	19.77±1.31 ^{bcde}	0.1±0.0 ^{b}	13.84±1.09 ^{ab}
7	0.2	0.7	0.2	18.92±1.78 ^{bcde}	0.1±0.01 ^{cd}	21.94±1.67 ^{ab}
8	0.5	0.0	0.5	11.94±0.47 ^{ef}	0.04±0.0 ^{ef}	19.59±0.30 ^{ab}
9	0.5	0.5	0.0	17.65±1.48 ^{cdef}	0.1±0.0 ^{a}	13.03±1.49 ^{ab}
10	0.0	0.5	0.5	10.04±1.58 ^{f}	0.02±0.01 ^{f}	24.16±5.59 ^{ab}

Enzyme activities were determined after 7 days of cultivation.

* Results are mean of ±SD of three determinations. Values with the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

Table 7.3 ANOVA for variables substrates for cellulase production fitted to the mixture quadratic model

Source	Mean Square	F value	Prob > F	
model	247.7	5.27	0.025	significant
Linear Mixture (X_1, X_2, and X_3)	230.6	12.26	0.005	
X_1X_2	7.38	0.78	0.405	
X_1X_3	0.021	0.002	0.964	
X_2X_3	9.76	1.04	0.342	
Residual	65.82			
Lack of Fit	39.27	1.11	0.485	not significant
Pure Error	26.55			

R^2 = 0.790; Coefficient of Variance (C.V.%) = 17.00

X_1 = rice husk, X_2= coffee husk and X_3= sugarcane bagasse

On the basis of quadratic polynomial equation of mixture design model in Eq. 7.1, the effect of independent factors; rice husk, coffee husk and sugarcane bagasse on the specific activity of xylanase were analyzed by Design-expert 6.0.2 software was shown in Eq. 7.3;

$$Y = +11.95X_1 +17.99X_2 +24.03X_3 \quad (7.3)$$

Equation 7.3 showed the quadratic model for three components where Y was the specific activity of cellulase (U/mg protein), X_1, X_2, X_3 were rice husk, coffee husk and sugarcane bagasse amount, respectively. The non significant value of the relation between X_1 to X_2 and X_3 were not shown in the equation.

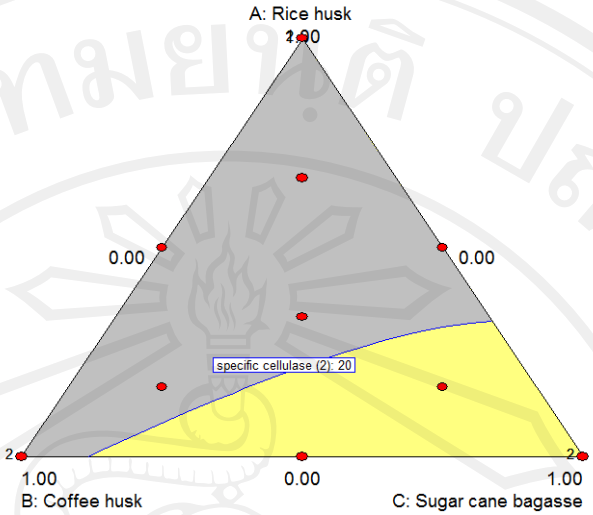

Fig. 7.5 Selected mixture interaction quadratic contour area ternary plots of quadratic model predicted specific activity of cellulase (Units are expressed in U/mg protein) in solid state fermentation.

Figure 7.5 showed information concerning the interaction of solid substrate components on cellulase production in solid state fermentation and the predicted specific activity of cellulase. The increasing amount of sugarcane bagasse and coffee husk led more cellulase production. From the significant quadratic model, indicate that fermentation with 2% (w/w) rice husk; 26% (w/w) coffee husk and 72% (w/w) sugarcane bagasse combination was optimal for cellulase production when the predicted specific activity of cellulase was 24.48 U/mg protein.

7.3.3.2 Solid substrates combination ratio for xylanase production

The observed values of extracellular xylanase activities, protein concentration, and specific activities when combination ratio of rice husk, coffee husk and sugarcane bagasse were tested. The highest specific activity of xylanase (226.4 U/mg protein) was observed in the eighth trial of the experimental setup (Table 7.4). The quadratic

regression showed the model was significant, the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms. The non significant value 0.15 lack of fit showed that the quadratic model was valid for the present study (Table 7.5).

Table 7.4 Xylanase activities, protein concentration, and specific activities from mixture design experiment for three agricultural residues

Trial	Concentration (%w/w)			xylanase activity* (U/g substrate)	Protein concentration* (mg/ml)	Specific activities* (U/mg protein)
	rice husk	coffee husk	sugar cane bagasse			
1	0.7	0.2	0.2	210.3±30.1 ^{a}	0.1±0.02 ^{a}	129.8±33.3 ^{bc}
2	0.0	1.0	0.0	112.6±15.8 ^{d}	0.1±0.01 ^{bc}	106.4±28.2 ^{c}
3	0.2	0.2	0.7	115.5±3.9 ^{d}	0.1±0.01 ^{de}	117.1±17.0 ^{bc}
4	1.0	0.0	0.0	188.1±20.7 ^{ab}	0.2±0.03 ^{a}	119.8±23.6 ^{bc}
5	0.0	0.0	1.0	14.3±0.9 ^{f}	0.03±0.01 ^{f}	12.8±6.2 ^{d}
6	0.3	0.3	0.3	177.1±34.9 ^{abc}	0.1±0.0 ^{b}	124.1±26.1 ^{bc}
7	0.2	0.7	0.2	147.8±17.1 ^{cd}	0.1±0.01 ^{cd}	171.4±16.8 ^{b}
8	0.5	0.0	0.5	138.1±6.7 ^{cd}	0.04±0.0 ^{ef}	226.4±1.5 ^{a}
9	0.5	0.5	0.0	169.3±7.3 ^{bc}	0.1±0.0 ^{a}	125.0±9.2 ^{bc}
10	0.0	0.5	0.5	53.0±15.1 ^{e}	0.02±0.01 ^{f}	128.1±46.3 ^{bc}

Enzyme activities were determined after 7 days of cultivation.

* Results are mean of ±SD of three determinations. Values with the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

Table 7.5 ANOVA for variables substrates for xylanase production fitted to the mixture quadratic model

Source	Mean Square	F value	Prob > F	
model	6398.7	7.1	0.012	significant
Linear Mixture (X_1, X_2, and X_3)	5184.9	5.7	0.034	
X_1X_2	38.0	0.042	0.844	
X_1X_3	17064.9	18.85	0.003	
X_2X_3	3081.3	3.40	0.108	
Residual	905.3			
Lack of Fit	1319.5	3.74	0.154	not significant
Pure Error	353.2			

R^2 = 0.835; Coefficient of Variance (C.V.%) = 26.08

X_1 = rice husk, X_2= coffee husk and X_3= sugarcane bagasse

On the basis of quadratic polynomial equation of mixture design model in Eq. 7.1, the effect of independent factors; rice husk, coffee husk and sugarcane bagasse on the specific activity of xylanase were analyzed by Design-expert 6.0.2 software was shown in Eq. 7.4;

$$Y = +117.065X_1 +114.927X_2 +13.151X_3 +519.633X_1 X_3 \quad (7.4)$$

Equation 7.4 showed the quadratic model for three components where Y was the specific activity of xylanase (U/mg protein), X_1, X_2, X_3 were rice husk, coffee husk

and sugarcane bagasse amount, respectively. The non significant value of the relation between X_1 to X_2 and X_2 to X_3 were not shown in the equation.

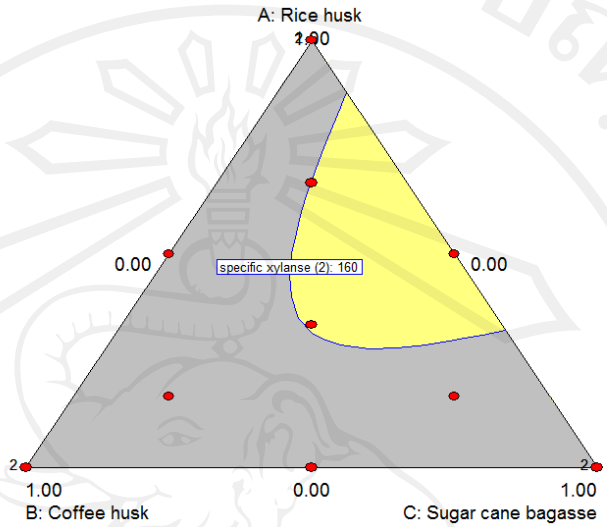

Fig. 7.6 Selected mixture interaction quadratic contour area ternary plots of quadratic model predicted specific activity of xylanase (Units are expressed in U/mg protein) in solid state fermentation

As shown in Fig. 7.6, information concerning the interaction of solid substrate components on xylanase production in SSF and the predicted specific activity of xylanase. The increasing amount of rice husk and sugarcane bagasse led more xylanase production. From the significant quadratic model, indicate that fermentation with 59% (w/w) rice husk; 1% (w/w) coffee husk and 40% (w/w) sugarcane bagasse combination was optimal for xylanase production when the predicted specific activity of xylanase was 199.4 U/mg protein.

Solid substrates combination ratio for both cellulase and xylanse production were determined by using the conjugated area from contour area ternary plots (Fig 7.5 and 7.6). The overlay plot for both enzymes was shown in Fig 7.7, in conjugated area

(arrow point), indicated that fermentation with a combination of 37% (w/w) rice husk; 6% (w/w) coffee husk and 57% (w/w) sugarcane bagasse was an optimal combination ratio for both cellulase and xylanase production when the predicted specific activity of cellulase was 20.0 U/mg protein and the predicted specific activity of xylanase was 157 U/mg protein. The result showed the combined substrates using mixture design experimental model using solid state fermentation increased the maximum activity of cellulase production 1.6 folds, 1.2 folds and 1 folds when compared with each rice husk, coffee husk and sugarcane bagasse control, respectively. Moreover, these combined substates increased of xylanase specific activity as 1.3 folds when compared with the rice husk, 1.5 folds when compared to coffee husk and 12.3 folds when compared to the sugarcane bagasse control.

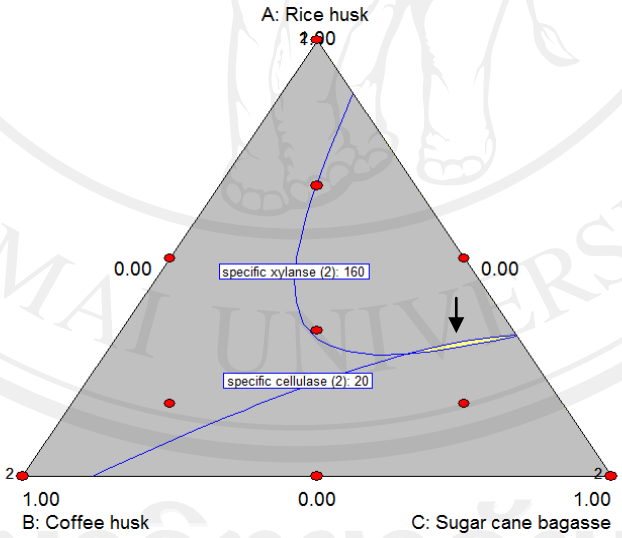

Fig. 7.7 Selected mixture interaction quadratic contour area ternary plots of quadratic model, shared between predicted specific activity of cellulase and xylanase (Units are expressed in U/mg protein) in solid state fermentation.

A mixture experiment involves mixing various proportions of two or more components to make different compositions of an end product (Akay, 2007). This experiment is commonly encountered in industrial product formulation including food processing, chemical formulation, enzyme production, and pharmaceutical drugs (Martinello *et al*., 2006; Moros *et al*., 2005; Zorba *et al*., 2006). Recent studies have found that the mixture design experiment improved the production of cellulase and xylanase. Fang *et al*. (2010) found that coba husk and corn steep liquor in the ratio of 4.5:0.5 % (w/w) had the greatest effect to increased enzyme production (645 U/g dry substrate), with a 2.4% increase of xylanase activity compared with the group with coba husk as the only carbon source. The mixed substrate made more nutrients available for mycelia development and the presence of inducer substrates for enzyme production (Papinutti and Forchiassin, 2007).

7.3.4 Optimal nitrogen source for cellulase and xylanase production

Enzyme production by all microorganisms and *T. aurantiacus* mainly depend on growth conditions and nutrient availability (Sanghvi *et al*., 2010). Cellulase showed high activity within nitrogen sources both organic (peptone and yeast extract) and inorganic (ammonium sulfate, ammonium tartrate and potassium nitrate) around 32-43 U/gds when compared with control (no nitrogen source) 13.08 U/ gds. Xylanase activity, 253.92 and 228.37 U/gds were evident when peptone and yeast extract were added, respectively (Fig. 7.8) These results are in agreement with many those reported where fungi were found to produce higher enzyme activities by adding organic nitrogen sources (Lemos *et al*., 2001; Sanghvi *et al*., 2010).

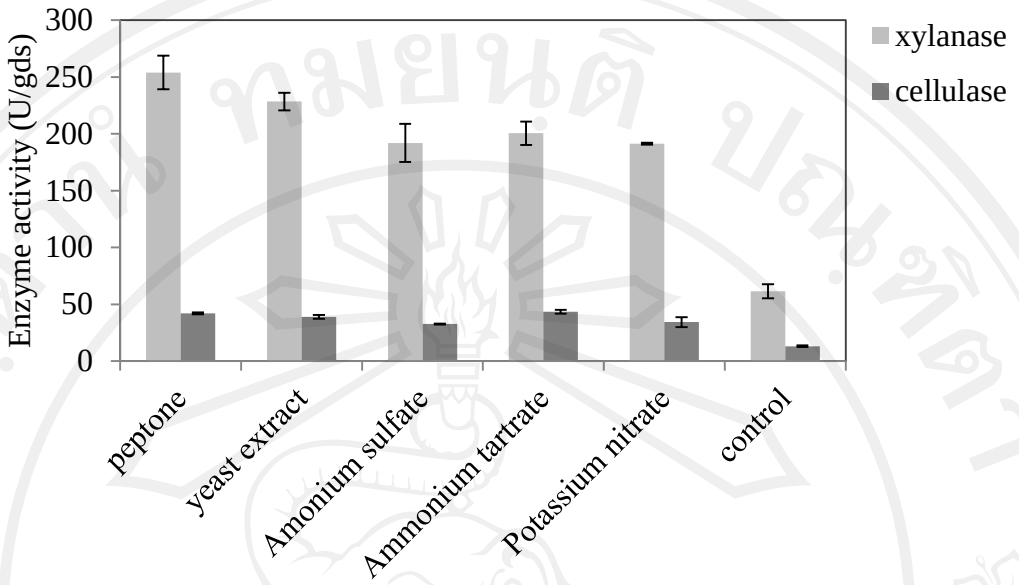

Fig. 7.8 Cellulase and xylanase production in mixed substrates added with various nitrogen sources by *T. aurantiacus* under solid state cultivation. The test flask incubated at 45°C, darkness and static condition for 7 days. Error bars represent the standard deviation from the mean of three replications.

7.3.5 Statistical analysis for the component concentration in mineral solution

7.3.5.1 Component concentration in mineral solution for cellulase production

Statistical testing of the model was performed with the Fisher's statistical test for analysis of variance (the component concentration in mineral solution). The results of the ANOVA for cellulase production are shown in Table 7.6. The quadratic regression showed the model was significant because the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms. The non significant value 0.119 lack of fit showed that the quadratic model was valid for the present study and the value of correlation (R^2) was 0.923.

Table 7.6 ANOVA for response surface quadratic model of media optimization for cellulase production

Source	Mean Square	F value	Prob > F	
model	325.00	5.14	0.027	significant
A	70.38	15.57	0.008	
B	0.21	0.05	0.835	
C	3.02	0.67	0.445	
D	6.30	1.39	0.282	
A^2	50.33	11.13	0.016	
B^2	2.94	0.65	0.450	
C^2	22.86	5.06	0.066	
D^2	7.93	1.75	0.234	
AB	10.49	2.32	0.179	
AC	6.64	1.47	0.271	
AD	51.42	11.38	0.015	
BC	5.65	1.25	0.306	
BD	29.16	6.45	0.044	
CD	0.07	0.015	0.905	
Residual	27.12			
Lack of Fit	17.78	3.80	0.119	not significant
Pure Error	9.35			

R^2 = 0.923; Coefficient of Variance (C.V.%) = 5.09

A = peptone, *B* = KH_2PO_4, *C* = $MgSO_4.7H_2O$ and *D* = $CaCl_2.2H_2O$

Values of Prob > F is less than 0.05 for *A*, A^2, *AD* and *BD* terms indicate model terms are significant in table 7.6. On the basis of quadratic polynomial equation of response surface model (Eq. 7.2), the effect of independent factors; *A* = peptone, *B* = KH_2PO_4, *C* = $MgSO_4.7H_2O$ and *D* = $CaCl_2.2H_2O$ on the cellulase activity were analyzed in Eq. (7.5)

Cellulase activity (U/gds) = + 42.38+ 3.53*A*- 1.84A^2 - 3.94*AD*+2.97*BD* (7.5)

Equation (7.5) shows the quadratic model for four factors effect on specific activity of cellulase (U/gds). The non significant values were not showed in the equation.

8.3.5.2 Component concentration in mineral solution for xylanase production

Statistical testing of the model was performed with the Fisher's statistical test for analysis of variance. The results of the ANOVA for cellulase production are shown in Table 7.7. The quadratic regression showed the model was significant because the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms. The non significant value 0.210 lack of fit showed that the quadratic model was valid for the present study.

Values of Prob > F is less than 0.05 for *A*, A^2, B^2,C^2, *AD*, *BC* and *BD* terms indicate model terms are significant in table 7.7. On the basis of quadratic polynomial equation of response surface model (Eq. 7.2), the effect of independent factors; *A*= peptone, *B* = KH_2PO_4, *C* = $MgSO_4.7H_2O$ and *D* = $CaCl_2.2H_2O$ on the cellulase activity were analyzed in Eq. (7.6)

xylanase activity (U/gds) = +298.12 +25.83*A* -8.48A^2 +6.05B^2 +7.38C^2 - 18.47*AD* +7.17*BC* +24.68*BD* (7.6)

Table 7.7 ANOVA for response surface quadratic model of media optimization for xylanase production

Source	Mean Square	F value	Prob > F	
model	752.88	13.44	0.002	significant
A	3774.68	67.40	0.000	
B	6.96	0.12	0.737	
C	48.17	0.86	0.390	
D	0.29	0.005	0.945	
A^2	1074.87	19.19	0.005	
B^2	547.62	9.78	0.020	
C^2	814.24	14.54	0.009	
D^2	272.53	4.87	0.070	
AB	308.36	5.51	0.057	
AC	19.12	0.34	0.580	
AD	1129.99	20.18	0.004	
BC	411.54	7.35	0.035	
BD	2018.88	36.05	0.001	
CD	248.52	4.44	0.080	
Residual	336.03			
Lack of Fit	81.93	2.36	0.210	not significant
Pure Error	154.09			

R^2 = 0.969; Coefficient of Variance (C.V.%) = 2.51

A = peptone, *B* = KH_2PO_4, *C* = $MgSO_4.7H_2O$ and *D* = $CaCl_2.2H_2O$

The quadratic model of CCD experiment was significant at 0.932 and 0.969 of R^2 of cellulase and xylanase experiment, respectively. At the predict activity of cellulase was 47.25 U/gds and xylanase was 333.06 U/gds, the optimal concentration of mineral solution components were 0.89 % (w/v) of peptone, 0.46 % (w/v) of KH_2PO_4, 0.09 %(w/v) of $MgSO_4.7H_2O$ and 0.07 %(w/v) of $CaCl_2.2H_2O$. The modified mineral solution following CCD experiment presented the increase of cellulase and xylanase activities as 58.03 and 57.86%, respectively when compared with the mineral solution control (Table 7.8).

Table 7.8 Cellulase and xylanase production by *T. aurantiacus* SL16W using modified mineral solution compared with mineral solution control

solvent	Specific activity (U/gds) *	
	Cellulase	Xylanase
Mineral solution control (Jianmin *et al.*, 2008)	19.83±0.13[b]	140.36±8.25[b]
Modified mineral solution (this study)	47.25±0.39[a]	333.06±1.87[a]

Enzyme activities was determined after 14 days of cultivation.

*Results are mean of ±SD of three determination. Values with the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

7.3.6 Optimal ratio of solid to liquid for cellulase and xylanase production

The effect of solid (mixed-lignocellulose substrates) to liquid (modified mineral solution) ratio on cellulase and xylanase production was studied. The results appear in Fig. 7.9, at S/L ratio at 1:4 showed the highest cellulase and xylanase activities. These results are in agreement with those of Kalogeris *et al*. (1998) who have found that solid to liquid ratios 1:4 and 1:5 (initial moisture level above 80%) showed the highest yield of xylanase production. In solid state fermentation must have enough moisture content enhanced fungal growth and enzyme production (Pandey *et al*., 1999). However, too high level of water flood on solid substrate reduced enzyme production because of difficulty in aeration and respiration of microorganisms (Sanghvi *et al*., 2010).

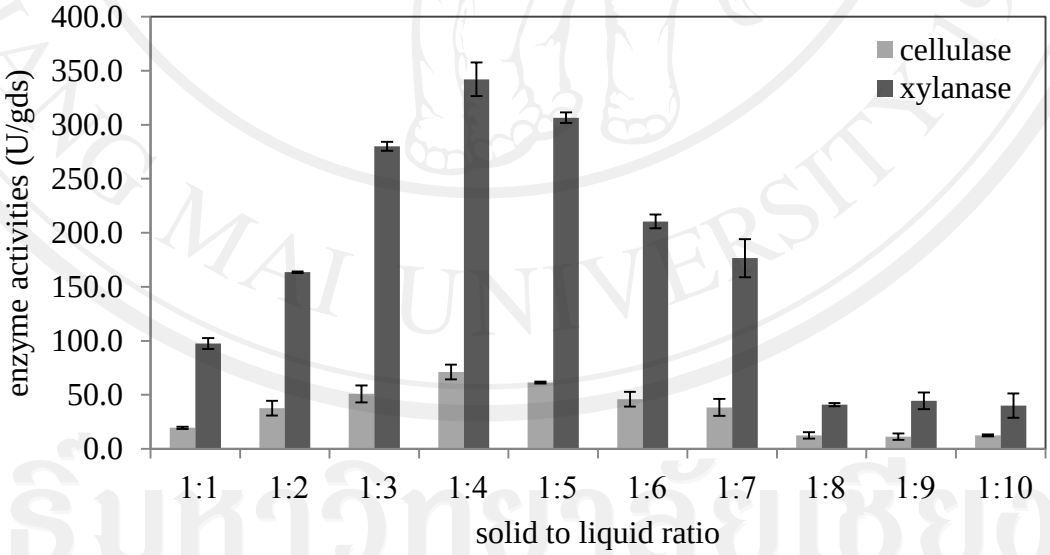

Fig. 7.9 Effect of solid to liquid ratio on cellulase and xylanase production by *Thermoascus aurantiacus* SL16W under solid state fermentation, incubated at 45^{o}C for 7 day.

7.3.7 Optimal incubation time for cellulase and xylanase production

As shown in Fig. 7.10, the cellulase and xylanase production from agricultural residues by *T. aurantiacus* when incubated at 45°C, the maximum activities of cellulase (76 U/gds) and xylanase (714 U/gds) were presented on the 12th and 10th day of incubation, respectively. A further increased in incubation time resulted in a decreased in enzyme production. The results are in agreement with Sanghvi *et al*. (2010) that found the maximum xylanase activity (146 IU/ml) produced on the 12th day of incubation time. Kang *et al*. (2004) found that xylanase production by *Aspergillus niger* has been shown to be closely linked to cellulase production due to the time-course.

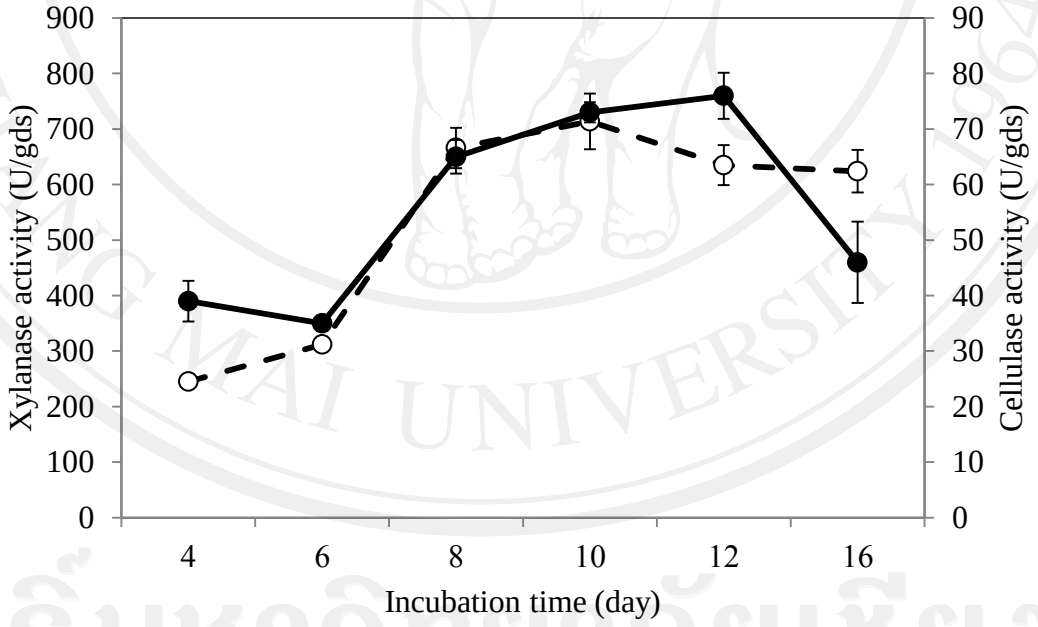

Fig. 7.10 Effect of incubation times on enzyme activity. Cellulase (●) and xylanase (○) production by *T. aurantiacus* SL16W under solid state fermentation incubated at 45°C. Error bars represent the standard deviation from the mean of three replications.

7.3.8 Effect of pH and temperature on enzyme stability

The effect of pH on crude cellulase and xylanase stability was determined. It was found that cellulase had a high stability between pH 6.0 to 8.0, which the enzyme was inactivated, about 40 % of the initial cellulase activity was retained at pH 2.0 and 10.0 (Fig. 7.11). Xylanase activity showed high stability in widely pH range (4.0-8.0) and only 30 % of the initial activity was reduced at pH 2.0, 3.0 and 9.0 (Fig. 7.12).

The thermal stability of cellulase and xylanase activities produced by *T. aurantiacus* were determined. As shown in Fig. 7.13, Cellulase activity was highly stable at temperature 30-50^{o}C, and enzyme activity was slightly decreased when temperature up to 60^{o}C. Xylanase activity showed high stability at high temperature, less than 20% of xylanase activity was deactivated at 65^{o}C, but xylanase activity was retained only 5% when kept at 70^{o}C. Both enzymes presented no activities was retained above 75^{o}C for 1 hr.

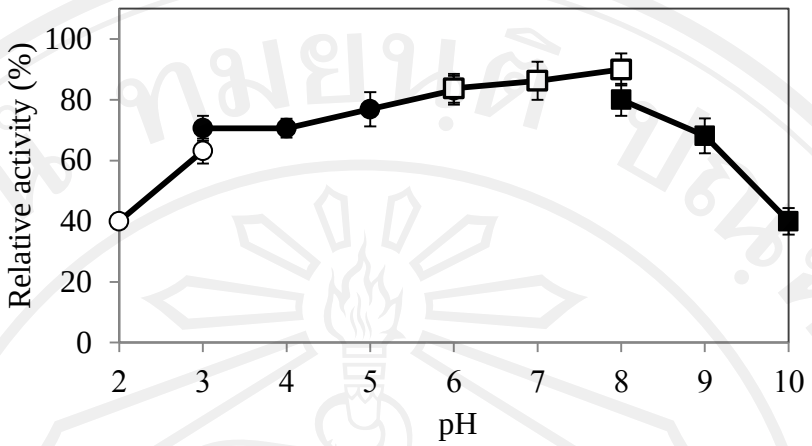

Fig. 7.11 Effect of pH on cellulase stability. Relative activities were determined after incubation of laccase for 24 h in sodium tartrate buffer pH 2.0-3.0 (○), sodium acetate buffer pH 3.0-6.0 (●), potassium phosphate buffer pH 6.0-8.0 (□), and Tris-SO_4 buffer pH 8.0-10.0 (■).

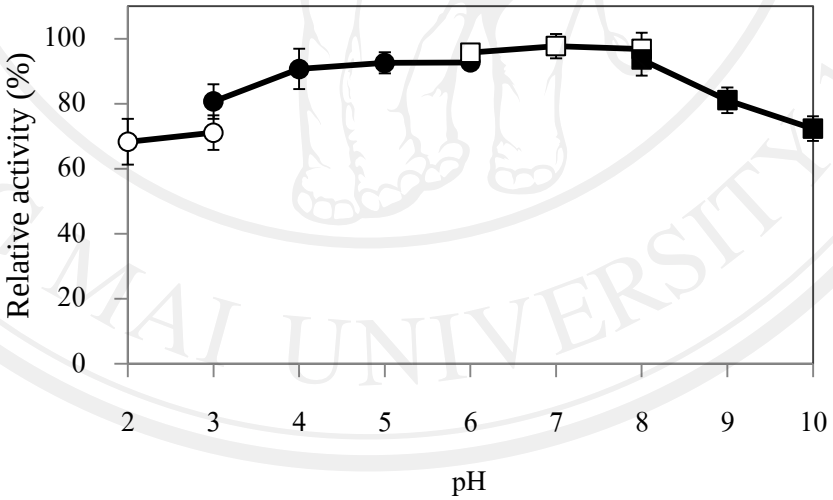

Fig. 7.12 Effect of pH on xylanase stability. Relative activities were determined after incubation of laccase for 24 h in sodium tartrate buffer pH 2.0-3.0 (○), sodium acetate buffer pH 3.0-6.0 (●), potassium phosphate buffer pH 6.0-8.0 (□), and Tris-SO_4 buffer pH 8.0-10.0 (■).

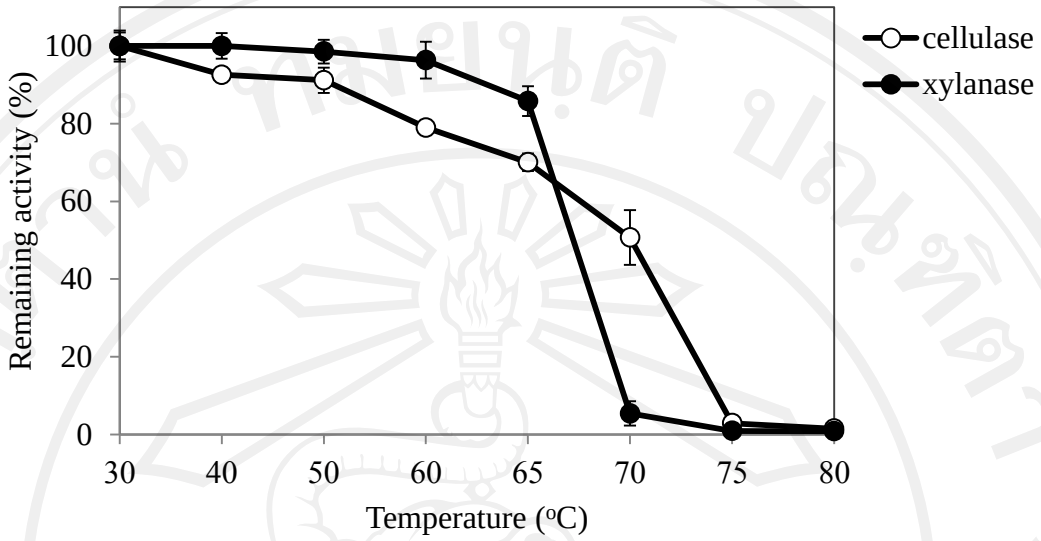

Fig. 7.13 Effect of temperature on the stability of cellulase and xylanase. Remaining activities were determined after incubation of each enzyme for 60 min at various temperatures.

In view of the encouraging results attained described above, the optimal conditions of cellulase and xylanase production were used pretreated substrates (sugarcane bagasse: coffee husk: rice husk, 57:6:37 % w/w) as carbon source, peptone as nitrogen source, solid to liquid (modified mineral solution; 0.89 % of peptone, 0.46 % of KH_2PO_4, 0.09 % of $MgSO_4.7H_2O$ and 0.07 % of $CaCl_2.2H_2O$) ratio as 1:4, pH 6.0-8.0 and incubated at 45^oC for 10-12 days. Cellulase activity was highly stable in pH range 6.0-8.0 and temperature at 30-50^oC for 1 hr. Xylanase activity showed high stability in pH range 4.0-8.0 and temperature at 30-65^oC for 1 hr.

Following the optimum condition for cellulase and xylanase production, the maximum enzyme activities were enhanced as shown in Fig. 7.14. Cellulase activity was 112.3 ± 11.5 U/gds, and xylanase activity was 989.2 ± 61.9 U/gds. Rajesh *et al*.

(2009) produced cellulase from lignocellulosic biomass (rice straw) and found the maximum enzyme activity was 18 U/gds on the 7th day incubation. Gutierrez-Correa and Tengerdy (1998), found *Trichoderma reesei* and *Aspergillus niger* (co-cultured) in solid state fermentation on sugarcane bagasse showed the optimal activites of cellulase (14-15 U/gds) at 30^{o}C incubation. Vimalashanmugam and Viruthagiri (2012) studied on xylanase production from *A. fumigatus* MTCC NO – 343 by using wheat bran as substrate in solid state fermentation and they found the optimum activity as 531 U/gds.

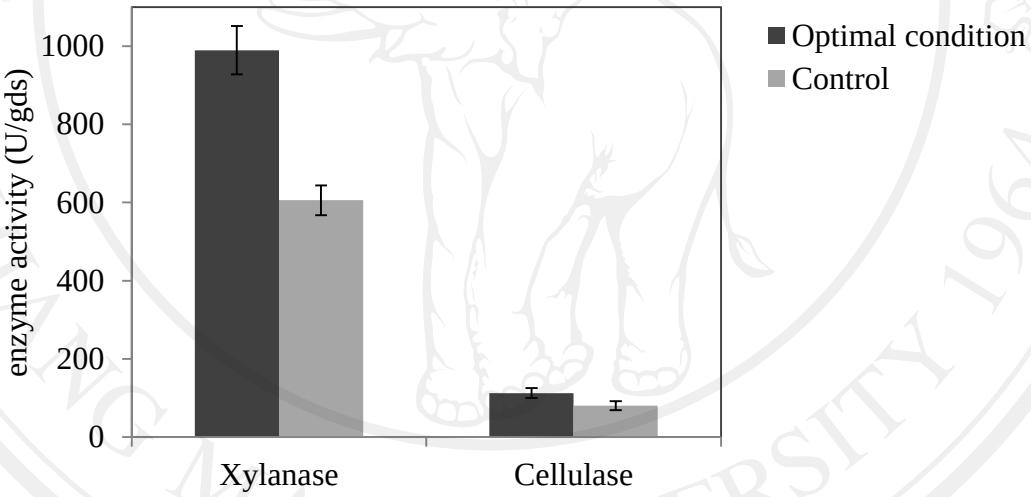

Fig. 7.14 Cellulase and xylanase production by *Thermoascus aurantiacus* SL16W from pretreated mixed-agricultural residues under the optimal condition (in this study) and control (untreated substrates).