Assessment of the Effect of Easily-metabolised Carbon Supplements on Xylanase Production by Newly Isolated *Trichoderma asperellum* USM SD4 Cultivated on Oil Palm Empty Fruit Bunches

Kamoldeen A. Ajijolakewu,[a,b] Cheu Peng Leh,[a] Wan Nadiah Wan Abdullah,[a] and Chee Keong Lee [a,*]

Effect of lignocellulosic medium supplemented with selected easily-metabolised carbon sources on microbial xylanase production was assessed. A newly isolated oil-palm-waste-domesticated bio-agent-producing fungus, identified based on rRNA analysis as *T. asperellum* USM SD4 was used as a representative organism. The potential of *T. asperellum* for enhanced xylanase production was evaluated by the statistical optimization of important cultural parameters *via* response surface methodology (RSM). *T. asperellum* showed optimum xylanase activity at pH 7; temperature 27 °C; moisture content 4 mL growth medium (gm): 1 gram dried substrate (gds) and inoculum size 2 x 10^6 spores/mL. Xylanase activity (2,337 IU/gds) attained in this study was far higher than ever reported for *T. asperellum*. Using the set of optimum conditions, the mixture of supplementary sugars to the lignocellulosic medium initiated xylanase repression in a concentration-independent manner. However, the degree of repression depended on the nature and type of respectively added sugar. The repressive effect exerted by monosaccharides (xylose, glucose, and fructose) was greater than exerted by either of dimeric (cellobiose and sucrose) or polymeric (xylan) sugars. Of all added substrates, xylan exerted the least repressive effect. Using xylose as a representative sugar, mechanism of xylanase repression was decisively explained and supported with experimental data.

Keywords: Xylanase; Xylanase repression; Biomass utilization; OPEFB; Trichoderma asperellum USM SD4; Effect of reducing sugars; Response surface methodology

*Contact information: a: School of Industrial Technology, Universiti Sains Malaysia, 1800USM, Penang Malaysia.; b: Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Nigeria, PMB 1515 Ilorin Nigeria; *Corresponding author: cklee@usm.my*

INTRODUCTION

Xylanase is an industrially important enzyme with conspicuous usefulness in several processes ranging from quality food products to paper production (Dobrev *et al*. 2007; Lakshmi *et al*. 2009). It has recently attracted enormous attention due its potential to enhance complete hydrolysis of lignocellulosic biomass (Pathak *et al*. 2014).

Generally, the genus *Trichoderma* and a few other genera such as *Aspergillus, Fusarum*, and *Thermomyces* have been shown as effective candidates for large scale xylanase production (Lakshmi *et al*. 2011; Pathak *et al*. 2014). Of specific importance is the genus *Trichoderma.* Species of *Trichoderma* (*e.g., T. reesei*) have been well investigated for the production of several hydrolytic enzymes including xylanase and

cellulases and are well studied as bio-control agents against plant fungal pathogens (Asad *et al*. 2015). Notable among the species of *Trichoderma is T. asperellum*, which is identified as a prominent mycoparasite non-pathogenic symbiont of plants and is most commonly used to produce biocontrol agents against plant pathogens (Asad *et al*. 2015). Notwithstanding that xylanase is a principal fraction of several cell-walls degrading enzymes by which it exerts its mycoparasitic property, most studies on *T. asperellum* have focused on its bio-control ability at the expense of other industrial uses. Recently, both Bech *et al.* (2015) and Marx *et al.* (2013) separately reported a detailed proteomics of the secretome by *T. asperellum* to show xylanase as an important component enzyme that could be exploited for important industrial processes. However, despite their respective strides, the potential of *T. asperellum* as a suitable fungal isolate for enhanced xylanase has not been well exploited.

Xylanase production is influenced by nutritional, environmental, and other production conditions (Pathak *et al*. 2014). Hence, enhanced xylanase production can be achieved by the optimization of the media composition, especially the carbon sources and the environmental conditions such as temperature, pH, and moisture content. In addition, the genetic diversity of each organism, among other factors, is a serious constraint to the development of the best medium and conditions for metabolite production by any organism. Thus, the optimum cultural and nutritional requirements to produce a specific metabolite cannot be used for the production of another metabolite by the same organism (Lakshmi *et al*. 2009). It is therefore pertinent to study the effects of these parameters, especially the medium composition, through factor optimization, in order to facilitate a cost-effective design for large scale and efficient xylanase production by a newly isolated microbial strain. However, it is not feasible to ascertain optimum production parameters by the classical change-one-factor-at-a-time (OFAT) due obviously to the lengthy time requirements for numerous experiments and the usually inappropriate conclusions drawn as a result of the non consideration of interaction among optimised parameters. In contrast, optimization of parameters by the design of experiment (DOE) addresses most of the constraints that characterize the use of OFAT approach.

Industrially, simple and easily-metabolised carbon compounds such as xylose, xylan, lactose, sophorose, *etc*., are often used as raw materials for xylanase production (Subramaniyan and Prema 2002) usually by submerged fermentation (SMF); and xylose and xylan were particularly identified in previous studies as unique xylanase inducers (Kulkarni *et al*. 1999). However, these simple sugars do not occur freely in nature but are products of the deconstruction of complex substrates such as cellulose and hemicellulose. Hence on the economic scale, utilization of these substrates will unequivocally lead to expensive xylanase enzyme. The cost of the enzyme will indirectly impact processes such as bioethanol production, where it is eventually used, rendering it thereby as a cost- intensive process.

Recently, lignocellulosic substrates are being used as raw materials to develop low-cost media for the production of industrially important chemicals such as ethanol and enzymes (Lakshimi *et al*. 2009). Their suitability as alternative bio-substrates is due to their, inexpensiveness, overwhelming abundance, and the indirect reduction of environmental pollution from their disposal. Among the available agricultural wastes, wheat bran, rice husk, and corn cobs are most often used as major carbon sources in nutrient composition for lignocellulosic enzyme production. However, based on comparatively higher nutritional composition and local abundance, the use of oil palm empty fruit bunches (OPEFB) for general enzyme production is presently gaining

traction. It has in fact been successfully used as raw materials for enhanced xylanase production (Lakshmi *et al*. 2009, 2011).

A review of available literature shows that in order to ensure effective use of these substrates for enzyme production, most reports on the use of lignocellulosic agricultural wastes either have been concentrated on improvement of production methods – solid state fermentation (SSF) and submerged fermentation (SMF) (Pathak *et al.* 2014) – or on the improvement of specific biomass features by assessing the effect of pretreatment, fibre size reduction, and mixture of different lignocellulosic biomass. As a consequence of the remarkable improvement in enzyme yields by mixture of some lignocellulosic substrates, recent studies have focused on exploiting the synergistic effect of mixing several other substrates to achieve higher yields of hydrolytic enzymes. Rice straw and wheat bran (Shenef *et al*. 2010), OPEFB and rice husk (Oke *et al*. 2016), and several other combinations (Pathak *et al*. 2014) have been mixed, leading to a higher enzyme titre. Given the inductive abilities respectively of simple easily-metabolised sugars (monosaccharides, disaccharides and less complex polysaccharides) and lignocellulosic substrates on xylanase production, it is presumed that the use of a mixture of these respective substrates could lead accordingly to a more efficient xylanase production. However, the inductive ability of a mixture of lignocellulosic substrate and easily-metabolised sugars as carbon source for lignocellulosic enzyme production has been poorly investigated, and the mechanism of observed responses is yet unclear. Incidentally, some few earlier researchers (Lakshmi *et al*. 2009; Gaffney *et al*. 2009) have reported the effect of this mixture on xylanase production; all were however plagued with conflicting results. In fact, none of the reports could show the mechanism or at least provide sufficient explanation for their respective observations. Consequently, there is a need for an independent explanation of xylanase induction or repression by reducing sugars in lignocellulosic media.

This study is therefore designed primarily to show the potential of newly isolated *T. asperellum*, a known mycoparasite, as a suitable fungal isolate for enhanced production of xylanase enzyme. More importantly, this study aimed to investigate and critique the effect of the mixture of easily-metabolised carbon sources with lignocellulosic substrate on xylanase production using OPEFB as case study. Optimum conditions for selected process parameters for xylanase production *via* SSF were first determined using a central composite design of the response surface methodology before subsequent determination of respective effect of substrate mixture on xylanase production.

EXPERIMENTAL

Materials

All reagents used were at least AR (analytical reagent) or higher (Guaranteed Reagent) grades from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA), except as stated otherwise.

Microorganism

Isolation and screening

As previously reported (Ajijolakewu *et al*. 2015), a *Trichoderma* species that showed high xylanolytic potential was selected based on a two-level screening of several xylanase-producing fungal species isolated from soil samples around OPEFB dumping sites in Penang, Malaysia. A pure culture of this species was preserved on Potato Dextrose Agar slants at 4 °C with periodic sub-culturing until needed.

Identification and phylogeny

The organism was identified by molecular techniques after a preliminary identification by colonial and morphological approaches. For molecular analysis, genomic DNA was extracted using the CTAB method (Murray and Thompson 1980). The previously described primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*. 1990) were used to amplify the internal transcribed spacer (ITS) region of the rRNA gene. This region included the 5.8S rRNA gene and was located between the small subunit ribosomal RNA and large subunit ribosomal RNA genes of the fungal genome. PCR was done with a total reaction mixture of 25 µL (2 µL genomic DNA, 0.25 µL each of primer, 10 µL of sterile distilled deionised water (ddH_2O), and 12.5 µL master mix (Vivantis Technologies, Selangor, Malaysia). The master mix contained Taq DNA polymerase (0.05 µL), 2x Vibuffer A, 0.4 mM of each dNTP, and 3.0 mM of $MgCl_2$. The PCR was conducted for 35 cycles in an Eppendorf Thermocycler after initial denaturation step at 94 °C for 5 min. Each cycle was characterized by denaturation step at 94 °C for (1 min), an annealing step at 52 °C (1 min), and an extension step at 72 °C for (2 min), with a final extension at 72 °C for 10 min. Sterile deionised distilled water (ddH_2O) was used as a control. To ascertain successful extraction and amplification, both the genomic DNA and amplified PCR products were gel electrophoresed. Sanger sequencing (Genetic Analyzer ABI 3730xl) of the successfully purified DNA products was done at the Centre for Chemical Biology, University Science Malaysia. The combined sequence of ITS-1– 5.8S–ITS-4 gene was compared to those for *Trichoderma* species in GenBank of National Centre for Biotechnology Information (www.ncbi.gov) using BLAST-n search, and the same sequence was subsequently submitted to GenBank to obtain an accession number. Following similarity searches, multiple-sequence alignments of the downloaded similar sequences and the obtained combined sequence were performed using MAFFT alignment software (Katoh and Standley 2013). Aligned sequences were adjusted using Gblock (Castresana 2000; Talavera and Castresana 2007). The phylogenetic analysis was inferred using MEGA 6.0.6 phylogenetic software (Tamura *et al*. 2013). Phylogenetic relationships were determined using the ''Bootstrap M-L'' (maximum likelihood) tree program in MEGA based on Tamura 3-parameter model.

Inoculum development

Spore suspension from a 4- to 5-day-old pure culture on plate was obtained by adding 10 mL sterile distilled water or 10 mL of 1% Tween-80 sterile distilled water and sieving through a 0.5 mm filter to remove mycelia. After appropriate dilutions, inoculum sizes (spores/ mL) were determined by direct microscopic observation using a haemocytometer (Aberkane *et al*. 2002).

Substrate Preparation

Raw OPEFB was used as a sole carbon source for enzyme production. It was selected based on preliminary investigation of the effects of substrate treatment on xylanase production (data not presented). OPEFB fibres were ground to ≤ 2 mm prior to use, and its structural composition was determined as previously described (Ying *et al*. 2014).

Solid State Fermentation

Xylanase was produced in a 250 mL conical flask using SSF in a medium containing 12 mL of modified Mandels and Rees medium (growth medium or gm) and 3 g (oven-dried) raw OPEFB as a carbon source. The growth medium was formulated as follows (g/L); yeast extract (20), K_2HPO_4 (2), $CaCl_2$ (0.3), Tween-80 (2), $MgSO_4$ (0.3), $FeSO_4$ (0.005), $MnSO_4$ (0.002), $ZnSO_4.7H_2O$ (0.0016), and $CoCl_2$ (0.0014). The medium pH was adjusted to 7 before sterilization at 121 °C for 15 min. One millilitre (1 mL) of standardized inoculum (2×10^6 spores/ mL) was aseptically inoculated into the medium, and fermentation proceeded for 48 h at 27 °C. Crude enzyme was extracted with 0.05 M citrate buffer pH 4.8 (45 mL). After the addition of buffer, the flask was shaken at 180 rpm for 15 min in a shaker incubator at 30 °C. Fermented substrates were filtered through Whatman no 1 filter paper to extract the crude enzyme. The filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C, while the supernatant was used as the crude enzyme for the xylanase assay.

Optimization Techniques

Response surface methodology was used to optimize xylanase production by *T. asperellum* USMSD4 using a Central Composite Design (CCD) in Design Expert DX7.0 software (Stat-Ease Inc., Minneapolis, MN, USA). CCD was used to determine the optimum temperature, initial pH, solid-liquid ratio, and inoculum concentration to generate a model for enzyme activity (IU/gds). Three replicates of 30 runs, including 24 factorial points and 6 centre points, were independently analyzed to verify the effects of variables on xylanase synthesis.

The model was analyzed based on ANOVA and regression analyses. The statistical combinations of the real and coded values of the independent variables are shown in Table 1. The enzyme production profile was determined at the completion of each stage of optimization.

Effect of carbon supplements

After the optimization of environmental factors, effects of additional carbon sources on xylanase activity were investigated. Various concentrations (0 to 20% w/w) of six different easily-metabolised carbon sources—glucose, xylose, sucrose, fructose, cellobiose, and xylan—were added to the growth medium as a percentage of the lignocellulosic substrate to assess the corresponding effects on xylanase production.

Analytical Methods

Xylanase activity (IU/gds) was determined quantitatively by the DNSA method using 1% xylan from beech wood as described by Bailey *et al*. (1992). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (xylose) per min under assay conditions. Enzyme activity was expressed as

international unit (IU) per gram of dry fermented substrate (gds). Specific enzyme activity (U/mg protein) and enzyme productivity (U/mgG) were determined based on crude enzyme protein (Ghose 1987) and glucosamine (Swift 1973) content of the fermented crust, respectively.

Table 1. Actual Design for the Optimization of Xylanase Production

Standard Order	Coded Variables				Real Variables				Activity (IU/gds)
	A	B	C	D	Temp. (°C)	Initial Moisture (mL/gds)	Inoculum (x 10^6 spores/mL)	pH	
1	-1.0	-1.0	-1.0	-1.0	25.00	1.00	2	4.00	466.09
2	1.0	-1.0	-1.0	-1.0	35.00	1.00	2	4.00	458.14
3	-1.0	1.0	-1.0	-1.0	25.00	4.00	2	4.00	1923.75
4	1.0	1.0	-1.0	-1.0	35.00	4.00	2	4.00	1698.52
5	-1.0	-1.0	1.0	-1.0	25.00	1.00	3	4.00	461.90
6	1.0	-1.0	1.0	-1.0	35.00	1.00	3	4.00	454.15
7	-1.0	1.0	1.0	-1.0	25.00	4.00	3	4.00	1588.48
8	1.0	1.0	1.0	-1.0	35.00	4.00	3	4.00	1484.23
9	-1.0	-1.0	-1.0	1.0	25.00	1.00	2	7.00	506.50
10	1.0	-1.0	-1.0	1.0	35.00	1.00	2	7.00	454.81
11	-1.0	1.0	-1.0	1.0	25.00	4.00	2	7.00	2330.04
12	1.0	1.0	-1.0	1.0	35.00	4.00	2	7.00	2112.34
13	-1.0	-1.0	1.0	1.0	25.00	1.00	3	7.00	503.17
14	1.0	-1.0	1.0	1.0	35.00	1.00	3	7.00	423.09
15	-1.0	1.0	1.0	1.0	25.00	4.00	3	7.00	2148.82
16	1.0	1.0	1.0	1.0	35.00	4.00	3	7.00	1763.64
17	-2.0	0.0	0.0	0.0	20.00	2.50	2.5	5.50	1029.90
18	2.0	0.0	0.0	0.0	40.00	2.50	2.5	5.50	209.52
19	0.0	-1.3	0.0	0.0	30.00	0.50	2.5	5.50	259.21
20	0.0	2.0	0.0	0.0	30.00	5.50	2.5	5.50	2515.83
21	0.0	0.0	-1.3	0.0	30.00	2.50	0.5	5.50	940.51
22	0.0	0.0	2.0	0.0	30.00	2.50	3.5	5.50	891.85
23	0.0	0.0	0.0	-2.0	30.00	2.50	2.5	2.50	750.95
24	0.0	0.0	0.0	2.0	30.00	2.50	2.5	8.50	1493.42
25	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1377.59
26	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1395.47
27	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1354.88
28	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1053.58
29	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1357.98
30	0.0	0.0	0.0	0.0	30.00	2.50	2.5	5.50	1354.88

Statistical Analysis

Data collected in triplicate were expressed as mean and standard deviations after statistical analysis using SPSS Statistics V22 software (IBM, Armonk, NY, USA). Statistical differences of generated data were determined by one-way ANOVA ($\alpha = 0.05$) followed by a comparison of means using the Duncan's multiple range test.

RESULTS AND DISCUSSION

Identification of Organism

The isolate was confirmed as a novel strain of *T. asperellum* based on ribosomal RNA analyses, similarity searches, and phylogeny. It was subsequently designated *Trichoderma asperellum* USM SD4 with an assigned GenBank accession no. KU878976. The strain has been deposited at the Culture Collection Centre of the School of Industrial Technology, Universiti Sains Malaysia with a strain number same as the GenBank accession number. Figure 1 shows the phylogenetic tree inferred based on comparison of nucleotide sequences of related *Trichoderma* species using the Tamura 3-parameter model.

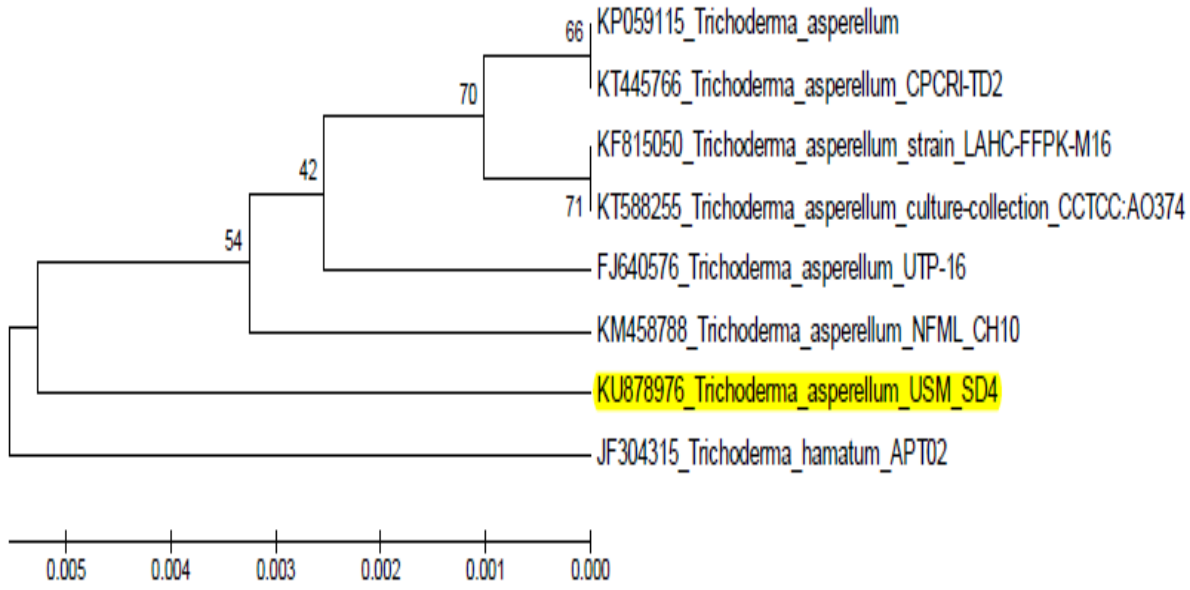

Fig. 1. Maximum likelihood tree inferred from the sequenced internal transcribed spacer gene section of the isolated *T. asperellum* USM SD4. The numbers in the branches of the phylogram indicate bootstrap value in % by 1000-replication multiple; the scale represents the possible substitutions of nucleotides per site.

Optimization of Cultural Condition for Xylanase Production by *T. asperellum* USM SD4

Pre-optimization

Raw OPEFB was used as a substrate for xylanase production based on a preliminary investigation of substrate treatment (data not shown). Composition analysis showed that OPEFB contained 86 ± 1% holocellulose (63% cellulose and 36%

hemicelluloses) and 15% lignin. Prior to optimization, xylanase production peaked at the 2nd day of fermentation with an average activity of 1318 ± 19 IU/gds.

Optimization of xylanase production by design of experiment (DOE)

The effects of incubation temperature, initial pH, initial moisture, and inoculum size on xylanase production by *T. asperellum* were assessed through optimization. Using the parametric variables in Table 2, xylanase production was assayed after 48 h of fermentation based on a previously determined time-course of enzyme production. The responses were determined as xylanase activity (IU/gds) in the various optimization conditions as presented in Table 1.

Table 2. Statistical (ANOVA) Analysis of Reduced Quadratic Model of RSM for the Optimization of Xylanase Production by *T. asperellum* USM SD4

Model Terms	Coefficient	**p-value**	**Significance**
Coefficient of regression equation			
Intercept	1298.91	-	-
A-Temperature	-113.36	*0.0017*	*Significant*
B-Initial Moisture	695.30	*< 0.0001*	*Significant*
C-Inoculum sizes	-75.00	*0.0269*	*Significant*
D-pH	133.0	*0.0004*	*Significant*
AB	-49.06	*0.2186*	*Not Significant*
BD	100.78	*0.0165*	*Significant*
A^2	-129.81	*< 0.0001*	*Significant*
C^2	-39.23	0.0041	*Significant*
Regression analysis of model			
Source	**p-value Prob > F**		**Significance**
Model	< 0.0001		*Significant*
Lack of Fit	*0.3272*		*Not significant*
R^2	0.9608		Good

The individual and interactive effects among the variables resulted in a significant variation in xylanase activity. As indicated in the standard order column, the highest xylanase activity (2515.8 IU/gds) was obtained in run number 20, while the lowest activity (209.52 IU/gds) was shown in run number 18. These values were respectively far higher or lower than the previous activity (1318 ± 19 IU/gds) obtained before parametric optimization. The disparities in these values suggest the importance of optimization of production parameters.

Regression analysis of model

Statistical analyses (ANOVA) of optimization data showed that a reduced quadratic model was reliable to predict xylanase production by *T. asperellum* USM SD4 (Table 2). Using the RSM integrated ANOVA, the following regression equation (Eq. 1) in coded units between xylanase production and the model terms was obtained.

$$\text{Activity (IU/gds)} = 1298.91 - 113.36*A + 695.30*B - 75.00*C + 133.00*D - 49.06*A*B + 100.78*B*D - 141.89*A^2 - 39.23*C^2 \quad (1)$$

Table 2 shows the level of significance of the model terms and equation. The p-

value indicates the level of significance of each model term. Smaller p-values imply that the corresponding co-efficient was significant (Montgomery 2008). Data including the model p-value < 0.0001, the non significant lack of fit (LOF = 0.3272), and the regression coeffecient (R^2 = 0.9608) showed that most of the model terms were significant. With the obtained model, it was easy to predict the individual and interactive effects of respective cultural conditions on xylanase production by the organism.

Analysis of the effects of optimized variables on xylanase production by T. asperellum USM SD4

All linear model terms showed highly significant coefficients, but a significant second order or quadratic effect was only shown by temperature and the inoculum size (Eq. 1). As shown in Table 2, xylanase production was most affected by initial moisture content, followed by the pH and the temperature, but was least affected by the inoculum sizes. All these factors have been earlier reported to directly affects metabolite production *via* SSF. Excess moisture increases aerial mycelial growth, lowers oxygen transfer, and decreases substrate porousity, thereby leading to a lower product yield. However, insufficient moisture reduces nutrients and protein solubility due to reduced surface area of the substrate (Shenef *et al*. 2010). Similarly, higher or extemely low temperature denatures synthesized enzymes as well as necessary enzymes needed for growth and other metabolic activities. Changes in the pH of the culture medium affects permeability of cells, causing enzyme denaturation and affecting enzyme stability. Further, a smaller inoculum size may not be adequate for growth initiation and will definitely prolong the lag phase. In contrast, a larger inoculum could shorten the lag phase, leading to overcrowded growth and competition for limited nutrients without a corresponding increase in xylanase activity (Pathak *et al*. 2014).

The model equation (Eq. 1) showed a strong interactive effect only between the initial moisture and pH. However, despite statistical insignificance, the interaction between incubation temperature and moisture content was nevertheless important for higher xylanase yield. These observations are well respresented in Figures 2a and 2b, respectively. Numerical optimization of the model showed that optimum conditions for xylanase synthesis by *T. asperellum* USM SD4 were: 27 °C; initial moisture ratio (4 mL (gm): 1 gds); pH 7; and total inoculum size of 2×10^6 spores per mL. These conditions were predicted to generate a xylanase activity of 2310.36 IU/gds with 90% desirability.

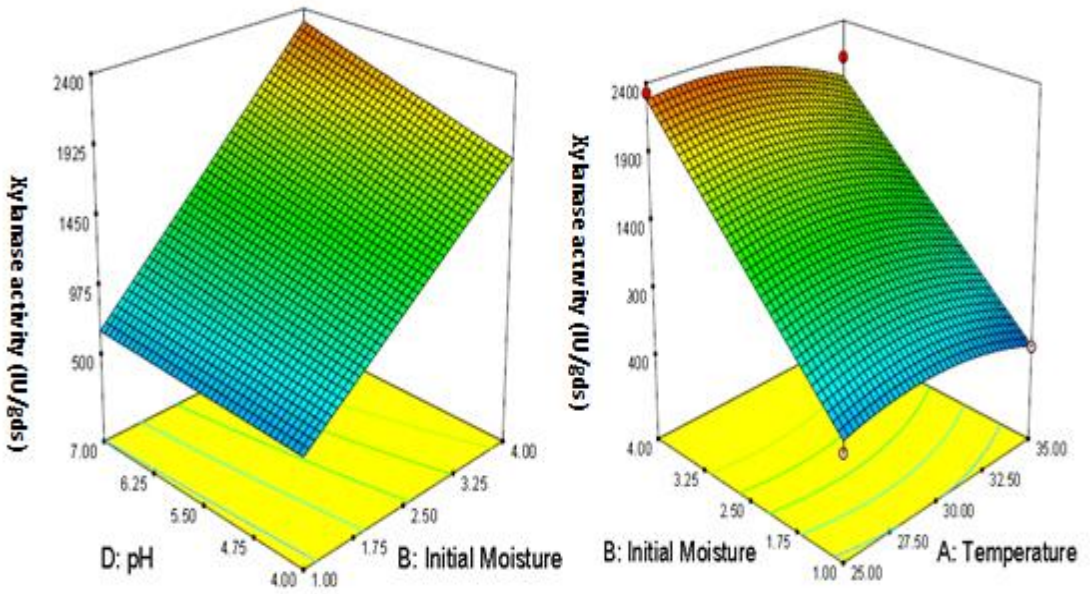

Fig. 2. Effects of (a) pH-Initial moisture interaction and (b) temperature-initial moisture interaction on xylanase activity

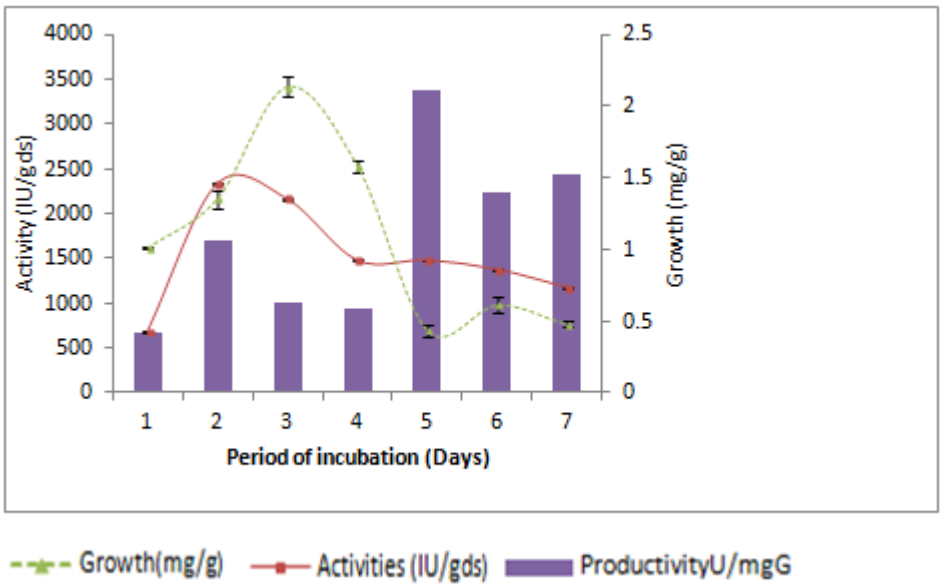

Fig. 3. Time course of enzyme production, growth profile and xylanase productivity using optimized conditions: pH 7, 1 gds: 4 mL (gm), 2 x 10^6 spores/mL and 27 °C

Optimization of xylanase production by T. asperellum USM SD4

The xylanase production profile of *T. asperellum* USM SD4 was evaluated using the selected optimum conditions in order to verify the derived model (Eq. 1). Xylanase biosynthesis was highest on the 2nd day of fermentation (Fig. 3); the xylanase activity value at this point (2337.79 ± 6.6 IU/gds) was 1.2% more than the predicted activity (2310.36 IU/gds). The less than < 5% differences in response values indicate that the model is valid and satisfactory (Montgomery 2008). Based on response surface

optimization, xylanase production by *T. asperellum* USM SD4 was enhanced by 77% relative to the obtained xylanase activity (1318 ± 19 IU/gds) prior to optimization. Comparative analysis of this study and previous reports on the use of *T. asperellum* for xylanase production showed that xylanase activity attained in this study was far higher than ever reported in any other. Xylanase activities reported in earlier studies were very poor in the range 14. U/gds (Marx *et al.* 2013) and 162 U/mL (Asad *et al.* 2015), which is the highest xylanase activity ever reported prior to this study. Finding in this study therefore suggested the potential of *T. asperellum* for enhanced xylanase production

Growth rate and the corresponding xylanase productivity by *T. asperellum* USM SD4 were determined. As shown in Fig. 3, the highest xylanase productivity was achieved on the 5th day of fermentation even though; maximum growth of the organism was attained at 72 h, a day after the peak period for xylanase production. This result suggested that xylanase production by *T. asperellum* USM SD4 is not growth dependent. Earlier report has made similar observation (Sridevi and Charya 2013). The lack of direct correlation between growth rate and xylanase activity was because the growth rate is otherwise dependent on the presence of xylose and other xylan-derived products generated through xylanase-catalyzed hydrolysis of hemicellulose fraction. Hence, the secretion of xylanase is prioritized by the organism to ensure its growth. This reason (dependency on products of xylan hydrolysis) also accounts for the lack of correlation between enzyme productivity and enzyme activity. This is more so that enzyme productivity measures enzyme activity relative to the growth rate of organism

Effect of Additional Carbon Sources on Xylanase Production by *T. asperellum* USM SD4

The effect of six additional carbon sources mixed separately with OPEFB on xylanase production was investigated as earlier described. At all tested concentrations (Fig. 4), all added substrates showed significant repressive effects ($p < 0.05$) on xylanase production.

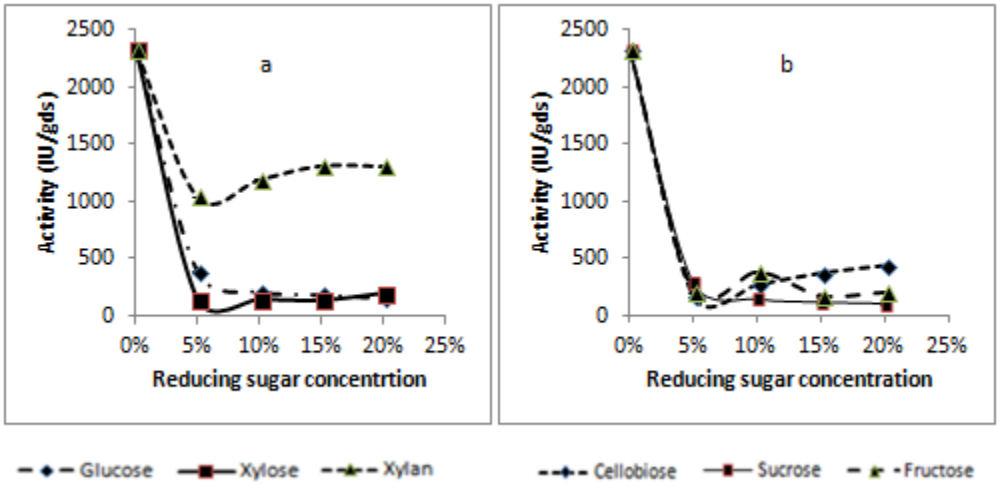

Fig. 4. Effects of addition of (a) glucose, xylose and xylan, (b) cellobiose, sucrose and fructose on xylanase production pH 7, 1 gds: 4 mL (gm), 2 x 10^6 spores/mL and 27 °C. Differences in xylanase activities are significant at $p < 0.05$.

The observed repressive effect was due to catabolic repression of the sugars on xylanase operon. This is because, relative to the complex sugars in OPEFB, all added substrates were either simple sugars or their simple polymeric forms. In addition, the organism might have preferred the newly added pure substrates only for growth enhancement over the complex lignocellulosic polymer. Besides, metabolic shift or expression of diauxic nutrition by all organisms is based on several conditions, including limiting/lack of important nutrients (especially glucose) (Rajaram and Varma 1990; Subramaniyan and Prema 2002), which in this case is available in the form of simple sugar.

The degree of repression was dependent on the nature and type of added substrate. As shown in Figs. 4a and b, repressive effects exerted by monosaccharides (glucose and xylose and fructose) and sucrose were greater than by cellobiose and xylan. Although sucrose is a disaccharide like cellobiose, its higher repressive ability compared with cellobiose could be due to its loose structural units (glucose and fructose), which are readily soluble. In contrast, the disintegration of cellobiose to its glucose units could only be enhanced by the presence of another enzyme (cellobiase). Further still, the least repressive effect was exerted by xylan. Comparatively, this is in contrast to observations in some earlier studies (Subramaniyan and Prema 2002; Dobrev *et al*. 2007) which suggested xylan as an effective xylanase inducer. Hence, xylan could not have been responsible for xylanase repression but definitely a product of xylan hydrolysis. When xylan is hydrolysed by constitutive xylanase, it forms such compounds as xylose and less complex polymeric substrates. These substrates are thought to induce xylanase production (Walsh *et al*. 2007). But xylose accumulation due to further catalysis of these substrates represses further xylanase synthesis, hence the relatively lower xylanase repression.

Of particular interest is xylanase repression by xylan and xylose. This study has shown, in contrast to earlier studies (Subramaniyan and Prema 2002; Dobrev *et al*. 2007), that addition of xylose or xylan to the production medium containing lignocellulosic substrate (in this case OPEFB), no matter the concentration, inhibited xylanase synthesis. Thus, by the provision of these findings, the xylanase inducer could not have been either xylan or xylose but, most probably, the derivatives of xylan hydrolysis and other substrates. Probable xylanase inducers are xylobiose, L-arabinose, and cellulose. Others are lactose, which is often used in the industries for the production of hydrolytic enzymes, and sophorose, which was reported as the most powerful xylanase inducer (Chaudhuri and Sahai 1994; Xu *et al*. 2000; Xiong *et al*. 2004). Possible explanations for these contrasting observations could be differences in the physiological and metabolic properties of the various organisms used, the type of fermentation (SSF or SMF), or more importantly, the composition of the fermentation media including the carbon sources (soluble carbon or lignocellulosic sources) (Kulkarni *et al*. 1999). Reports which suggested inductive ability of xylose and xylan for xylanase production used only the purified forms of these substrates as sole carbon sources *via* submerged fermentation. However in this study, xylanase production was done in fermentation medium composed of lignocellulosic substrate (OPEFB) mixed with respective pure simple sugars as carbon sources. Conversely, other than this study, some other reports on xylanase production using lignocellulosic media (Gaffney *et al*. 2009; Lakshmi *et al*. 2011), have observed that the repression of xylanase by the addition of easily-metabolised sugars-including xylose- only occurred at sugar concentrations higher than 2% and 3% (w/w) of the

lignocellulosic medium. But their reports did not clarify why xylanase synthesis was repressed only at higher reducing sugar concentrations.

Mechanism of xylanase repression by xylose

Two mechanisms of catabolic repression may be responsible for xylanase inhibition by xylose sugar: repression at the transcriptional level or repression by 'inducer' exclusion during glucose transport across the cell membrane (Subramaniyan and Prema 2002). Inducer exclusion is premised upon the natural preference of all organisms for glucose over other carbon sources. Most often, during glucose active transport, xyloses which could have induced xylanase secretion is naturally excluded. However, at the transcriptional level, catabolic repression may be due to auto-inhibition, negative feedback from downstream product accumulation, (Rajaram and Varma 1990; Walsh *et al*. 2007; Lakshmi *et al*. 2009), or the presence of glucose in the fermentation medium (Subramaniyan and Prema 2002). The results in this study suggest that the repression of xylanase biosynthesis is directly related to auto-inhibition as a result of xylose accumulation in the production medium. During auto-inhibition, constitutive xylanases catalyse the hydrolysis of xylan into xylooligomers and xylose sugars. The presence of xylooligomers (xylotriose; xylobiose or others) induces xylanase biosynthesis, while the synthesized xylanase simultaneously hydrolyses both xylan and xylooligomers into xylose. In turn, accumulated xylose sugars repress the genes responsible for xylanase production through negative feedback from the downstream hydrolytic product. A schematic of the proposed mechanism is represented in Fig. 5.

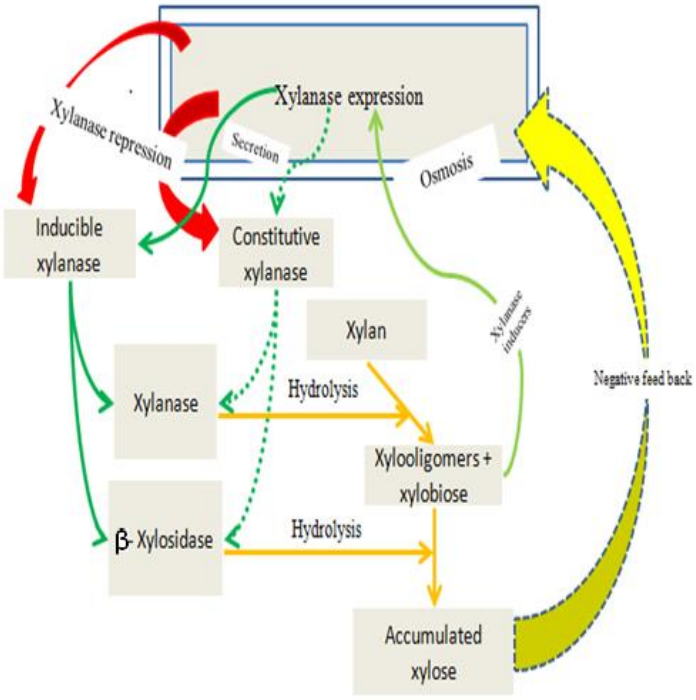

Fig. 5. Mechanism of xylanase repression due to auto-inhibition by xylose sugar. Briefly, xylan is hydrolysed by constitutive xylanases to xylooligosaccharides and xylobiose, which are driven by osmosis or active transport into the cell to induce xylanase genes. Both the induced and constitutive xylanases further hydrolyse xylan to xylooligosaccharides and xylobiose. Xylobiose is catalysed by β-xylosidase to produce xylose sugars. Xylose accumulation leads to auto-inhibition of the xylanase operon due to negative feedback.

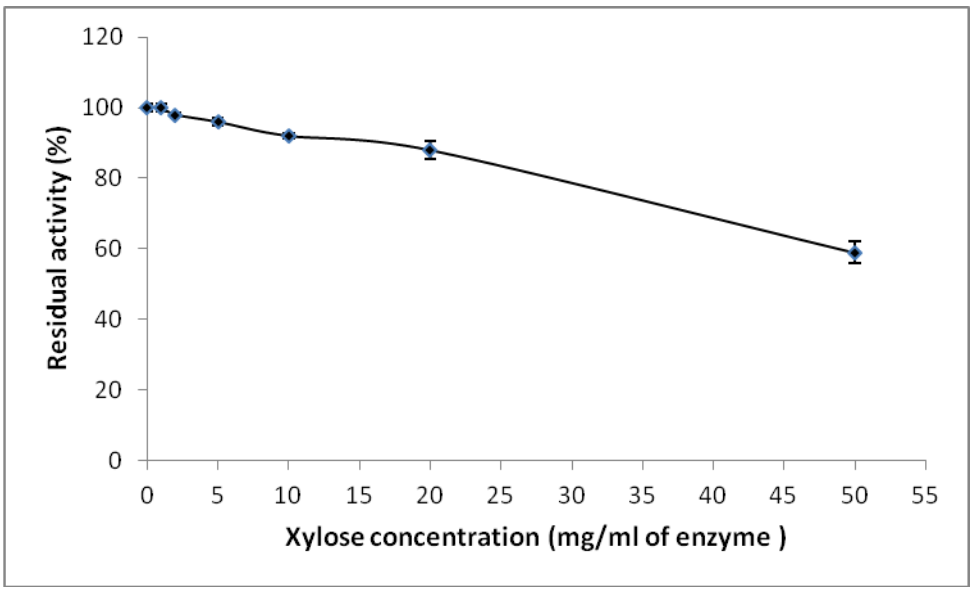

Fig. 6. Effect of different concentrations of xylose on crude xylanase activity (Differences in residual activities are significant at $p < 0.05$)

To demonstrate the auto-inhibition of xylanase due to downstream product accumulation, crude xylanase was charged with different concentrations of xylose sugars (0 to 50 mg/mL of crude enzyme) and assayed (against enzyme blanks) as described. As measured after 10 min of incubation at 30 °C, the residual activities (Fig. 6) progressively declined as xylose concentration increased. Therefore, by this observation, xylanase repression by the addition of xylose or other downstream product to the lignocellulosic production medium is proportional to the extent of concentration of respective sugar in the medium.

CONCLUSIONS

1. A newly isolated oil palm waste domesticated strain of mycoparasitic *Trichoderma*, identified as *T. asperellum* USM SD4 (GenBank accession number KU878976) has been shown as potential organism for large scale xylanase production using cheap lignocellulosic wastes as substrate.
2. Supplementation of xylose or any form of easily-metabolised carbon sources added to lignocellulosic medium as raw materials initiated xylanase repression in a concentration-independent manner.
3. Xylanase repression by the addition of easily-metabolised carbon sources to the lignocellulosic production medium is proportional to the nature and type as well as the extent of concentration of respectively added sugar in the medium.
4. Using OPEFB as an instance, this study has shown that addition of easily-metabolised sugars including xylose and xylan to lignocellulosic medium for xylanase production is not only cost-intensive but would consequently lead to poor enzyme output when considered on industrial scale.

5. Considering its specificity towards OPEFB, lignocellulosic wastes could be used to develop a low cost medium for industrial scale xylanase production by *T. asperellum* USM SD4

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from Universiti Sains Malaysia through Research University (RU) Grant (1001/PTEKIND/811273).

REFERENCES CITED

Aberkane, A., Cuenca-Estrella, M., Gomez-Lopez, A., Petrikkou, E., Mellado, E., Monzon, A., and Rodriguez-Tudela, J. (2002). "Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi," *Journal of Antimicrobial Chemotherapy* 50(5), 719-722. http://dx.doi.org/10.1093/jac/dkf187

Ajijolakewu, K. A., Leh, C. P., Wan Nadiah, W. A., and Lee C. K. (2015). "Screening for xylosyl hydrolase (xylanase) enzymes in soil fungal species; Comparative efficacy of solid state fermentation technique and plate-screening methods," in: *Exploring the Frontiers in Environmental Science and Technology Research (4th International Conference on Environmental Research and Technology)*, Teng T. Yusri Y. and Widad F (eds.), School of Industrial Technology Universiti Sains Malaysia, Penang, Malaysia, pp. 423-428.

Asad, S. A., Tabassum, A., Hameed, A., Afzal, A., Khan, S. A., Ahmed, R., and Shahzad, M. (2015). "Determination of lytic enzyme activities of indigenous *Trichoderma* isolates from Pakistan," *Brazilian Journal of Microbiology* 46(4), 1056-1064. DOI: http://dx.doi.org/10.1590/S1517-838246420140787

Bailey, M. J., Biely, P., and Poutanen, K. (1992). "Interlaboratory testing of methods for assay of xylanase activity," *Journal of Biotechnology* 23(3), 257-270. http://dx.doi.org/10.1016/0168-1656(92)90074-J

Bech, L., Herbst, F.-A., Grell, M.N., Hai, Z., and Lange, L. (2015). "On-site enzyme production by *Trichoderma asperellum* for the degradation of duckweed," *Fungal Genom Biol* 5(2), 126. DOI:10.4172/2165-8056.1000126

Castresana, J. (2000). "Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis," *Molecular Biology and Evolution* 17(4), 540-552. DOI: 10.1093/oxfordjournals.molbev.a026334

Chaudhuri, B., and Sahai, V. (1994). "Comparison of growth and maintenance parameters for cellulase biosynthesis by *Trichoderma reesei*-C5 with some published data," *Enzyme and Microbial Technology* 16(12), 1079-1083. DOI: 10.1016/0141-0229(94)90146-5

Dobrev, G. T., Pishtiyski, I. G., Stanchev, V. S., and Mircheva, R. (2007). "Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design," *Bioresource Technology* 98(14), 2671-2678. DOI: 10.1016/j.biortech.2006.09.022

Gaffney, M., Doyle, S., and Murphy, R. (2009). "Optimization of xylanase production by Thermomyces lanuginosus in solid state fermentation," *Bioscience, Biotechnology, and Biochemistry* 73(12), 2640-2644. DOI: 10.1271/bbb.90493

Ghose, T. (1987). "Measurement of cellulase activities," *Pure and Applied Chemistry* 59(2), 257-268. DOI: 10.1351/pac198759020257

Katoh, K., and Standley, D. M. (2013). "mafft multiple sequence alignment software version 7: Improvements in performance and usability," *Molecular Biology and Evolution* 30(4), 772-780. DOI: 10.1093/molbev/mst010

Kulkarni, N., Shendye, A., and Rao, M. (1999). "Molecular and biotechnological aspects of xylanases," *FEMS Microbiology Reviews* 23(4), 411-456. DOI: 10.1111/j.1574-6976.1999.tb00407.x

Lakshmi, G. S., Bhargavi, P. L., and Prakasham, R. (2011). "Sustainable bioprocess evaluation for xylanase production by isolated *Aspergillus terreus* and *Aspergillus fumigatus* under solid-state fermentation using oil palm empty fruit bunch fiber," *Current Trends in Biotechnology and Pharmacy* 5(4), 1434-1444.

Lakshmi, G. S., Rao, C. S., Rao, R. S., Hobbs, P. J., and Prakasham, R. S. (2009). "Enhanced production of xylanase by a newly isolated *Aspergillus terreus* under solid state fermentation using palm industrial waste: A statistical optimization," *Biochemical Engineering Journal* 48(1), 51-57. DOI: 10.1016/j.bej.2009.08.005

Marx, I.J., Van Wyk, N., Smit, S., Jacobson, D., Viljoen-Bloom, M., and Volschenk, H. (2013). "Comparative secretome analysis of *Trichoderma asperellum* S4F8 and *Trichoderma reesei* Rut C30 during solid-state fermentation on sugarcane bagasse," *Biotechnology for biofuels*, 6(1), 172.

Montgomery, D. C. (2008). *Design and Analysis of Experiments*, John Wiley and Sons, New York, USA.

Murray, M., and Thompson, W. F. (1980). "Rapid isolation of high molecular weight plant DNA," *Nucleic Acids Research* 8(19), 4321-4326. DOI: 10.1093/nar/8.19.4321

Oke, M.A., Annuar, M. S. M., and Simarani, K. (2016). "Enhanced endoglucanase production by *Bacillus aerius* on mixed lignocellulosic substrates," *BioResources* 11(3), 5854-5869. DOI: 10.15376/biores.11.3.5854-5869

Pathak, P., Bhardwaj, N. K., and Singh, A. K. (2014). "Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling," *Applied Biochemistry and Biotechnology* 172(8), 3776-97. DOI: 10.1007/s12010-014-0758-9

Rajaram, S., and Varma, A. (1990). "Production and characterization of xylanase from *Bacillus thermoalkalophilus* grown on agricultural wastes," *Applied Microbiology and Biotechnology* 34(1), 141-144. DOI: 10.1007/BF00170939

Shenef, A., El-Tanash, A., and Atia, N. (2010). "Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran," *Research Journal of Microbiology* 5(3), 199-211. DOI: 10.3923/jm.2010.199.211

Sridevi, B., and Charya, M. S. (2013). "Isolation, identification and screening of potential cellulase-free xylanase producing fungi," *African Journal of Biotechnology* 10(22), 4624-4630.

Subramaniyan, S., and Prema, P. (2002). "Biotechnology of microbial xylanases: enzymology, molecular biology, and application," *Critical Reviews in Biotechnology* 22(1), 33-64. DOI: 10.1080/07388550290789450

Swift, M. (1973). "The estimation of mycelial biomass by determination of the hexosamine content of wood tissue decayed by fungi," *Soil Biology and Biochemistry* 5(3), 321-332. DOI: 10.1016/0038-0717(73)90080-1

Talavera, G., and Castresana, J. (2007). "Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments," *Systematic Biology* 56(4), 564-577. DOI: 10.1080/10635150701472164

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). "MEGA6: Molecular evolutionary genetics analysis version 6.0," *Molecular Biology and Evolution* 30(12), 2725-2729. DOI: 10.1093/molbev/mst197

Walsh, R., Martin, E., and Darvesh, S. (2007). "A versatile equation to describe reversible enzyme inhibition and activation kinetics: Modeling β-galactosidase and butyrylcholinesterase," *Biochimica et Biophysica Acta (BBA)-General Subjects* 1770(5), 733-746. DOI: 10.1016/j.bbagen.2007.01.001

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in: *PCR Protocols: a Guide to Methods and Applications*, Academic Press, Cambridge, MA, USA. pp. 315-322. DOI: 10.1016/b978-0-12-372180-8.50042-1

Xiong, H., Turunen, O., Pastinen, O., Leisola, M., and von Weymarn, N. (2004). "Improved xylanase production by *Trichoderma reesei* grown on L-arabinose and lactose or D-glucose mixtures," *Applied Microbiology and Biotechnology* 64(3), 353-358. DOI: 10.1007/s00253-003-1548-4

Xu, J., Nogawa, M., Okada, H., and Morikawa, Y. (2000). "Regulation of xyn3 gene expression in *Trichoderma reesei* PC-3–7," *Applied Microbiology and Biotechnology* 54(3), 370-375. DOI: 10.1007/s002530000410

Ying, T. Y., Teong, L. K., Abdullah, W. N. W., and Peng, L. C. (2014). "The effect of various pretreatment methods on oil palm empty fruit bunch (EFB) and kenaf core fibers for sugar production," *Procedia Environmental Sciences*, 20, 328-335. DOI: 10.1016/j.proenv.2014.03.041

Article submitted: July 23, 2016; Peer review completed: September 11, 2016; Revised version received and accepted: September 19, 2016; Published: September 26, 2016.
DOI: 10.15376/biores.11.4.9611-9627