

Optimization of production conditions for xylanase production by newly isolated strain *Aspergillus niger* through solid state fermentation of oil palm empty fruit bunches

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ABSTRACT

Response surface (RSM) optimization of selected cultural parameters and the effect of various nitrogen sources on xylanase production by novel strain of *Aspergillus niger* via solid state fermentation of oil-palm-empty-bunches (OPEFB) were assessed. The newly isolated oil-palm-field-domiciled strain of *Aspergillus niger* was identified based on rRNA analysis as *Aspergillus niger* USM SD2 (GenBank accession no. KU882054). RSM was used to generate regression model for xylanase activity by this organism; while the effect of non-inclusion of incubation period among selected cultural conditions was assessed based on the xylanase production profile of the organism after optimization. Results showed that optimization model terms were significant with regression coefficient (R^2) value of 0.95. Xylanase activity was optimum at 25 °C, initial moisture-substrate ratio 4:1; pH 6.3 and total inoculum size, 2×10^6 spores/ml. At optimum production period, xylanase production was enhanced by more than 100% of its value prior optimization. Moreover, the use of yeast extract (Y/E) enhanced xylanase production better than any other nitrogen sources evaluated. Consequently, at its optimum concentration (15 g/l), the addition of Y/E enhanced xylanase production such that final activity (3246 IU/gds) was 160% and 29% higher than activities (1250 IU/gds) prior to and after (2512 IU/gds) parametric optimization respectively. Further analyses also showed that xylanase production by *A. niger* USM SD2 was independent of period of incubation and, hence, its growth rate.

1. Introduction

Xylanase is an industrially important hydrolytic enzymes which catalyses the hydrolysis of xylan to xylo-oligosaccharide and pentose sugars and has wide industrial applications. In the food industry, it is used to increase the nutritive and energy values of food and juices, improves the digestibility of poultry feed and as food additives in bakery (Prasad Uday et al., 2016; Uday et al., 2016). It is also used for the extraction of plant oils and extra polymeric substances, constituent of laundry detergent and as a bleaching agents in the pulp and paper industry (Ang et al., 2013; Dobrev et al., 2007; Fang et al., 2010; Lakshmi et al., 2009). It is particularly important in the bioethanol industry where it is used in synergy with cellulase enzymes for the complete hydrolysis of usually alkaline-treated lignocellulosic biomass, to release glucose, xylose and other pentose sugars for eventual fermentation to bioethanol (Uday et al., 2016; Ajijolakewu et al., 2017).

Xylanase is an inductive enzyme, hence, it is affected by the genetic diversity and metabolic conditions of producing organisms. Published

data (Ajijolakewu et al., 2016; Ang et al., 2013; Pal and Khanum, 2010; Pathak et al., 2014; Saleem, 2014) have shown that xylanase production is dependent on types of organism; as well as the nutritional, physiological and biochemical properties of the respective organisms. Generally, due to their abilities to produce high extracellular enzymes with outstanding industrial qualities such as wide temperature and pH of activity, the ease of enzyme production and availability of the fungal isolates (Deshpande et al., 2008; Umsza-Guez et al., 2011); fungal species are often employed for the production of lignocellulosic enzymes. Among several non-pathogenic organisms, filamentous fungi such as *Aspergillus* spp (Ang et al., 2013; Shah and Madamwar, 2005), *Trichoderma* spp (Pathak et al., 2014; Rezende et al., 2002) were the most investigated for xylanase production; but strains of *Aspergillus niger* are specifically important for the production of xylanase and other accessory enzymes (Deshpande et al., 2008; Dobrev et al., 2007; Shin et al., 2011). Apart from the properties of organism, xylanase production is affected by environmental conditions of pH; temperature; initial moisture; inoculum sizes; period of incubation and the nutritive

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components of the growth medium (Prasad Uday et al., 2017). Moreover, as observed by Uday et al. (2017) who reported the optimization of physical and morphological regime for improved cellulase free xylanase production by fed batch fermentation using *Aspergillus niger* (KP874102.1) in order to address the catabolic repression occasioned by high concentration of xylan in the growth medium, optimization of media composition is vital to improve both the activity and the volumetric productivity of general metabolites including enzymes such as xylanases. It is hence very clear that the best cultural conditions and nutritional requirements to produce a specific metabolite cannot be used for the production of the same metabolite by different organisms or for the production of another metabolite by the same organism as earlier reported (Ajijolakewu et al., 2016). Therefore, it is necessary to study the effect of these parameters on respective organisms through factor optimization for efficient xylanase production.

Methods such as the classical change-one-factor-at-a-time (OFAT) and optimization study by design of experiment (DOE) have been used (Ajijolakewu et al., 2016). Although, easy to conduct, conventional OFAT does not consider interactive effects among factors, and as such causes unreliable and inaccurate conclusions in addition to the fact that it is time consuming and cost-implicative when large variables, such as those mentioned above, are considered. All these constraints are addressed by the use of statistical optimization of parameters by DOE as reported and observed by (Bhunia and Dey, 2012; Montgomery, 2008). DOE ensures enhanced production of target products and saves time and cost through a properly planned optimization process which identifies significant factors from a lump via preliminary screening exercise and which influences the activities of the concerned organism via a combinatorial interactive effect among significant factors for optimal performance towards enzyme production. However, this is notwithstanding that the uniqueness of each organism requires a repeat of all stages in optimization for the synthesis of specific metabolite as earlier observed.

More often than not, when DOE is used for optimization of enzyme production, most earlier works (Oberoi et al., 2014; Pal and Khanum, 2010; Pathak et al., 2014) would consider the incubation period as an important parametric factor. However, all organisms are thought to show characteristic responses to certain changes in cultural conditions (in this case the interactive effect among factors) which may not be properly accommodated within the time range often used during parametric optimization. The above concept of 'stress response' (Hilker et al., 2015) in organisms, could probably have accounted for why some other earlier reports (Latifian et al., 2007; Yang et al., 2006) on xylanase modelling did not consider period of incubation. Moreover, since OPEFB like many other non wood lignocellulosic biomass is a poor source of nitrogen. There is therefore a need to consider additional nitrogen sources due to its importance on growth and metabolic activities of organisms.

Our earlier study (data not shown) has revealed that auto-hydrolysed or water-treated OPEFB was more appropriate for xylanase production by this organism when compared with raw and OPEFB biomass treated by other pretreatment techniques. Therefore, this study was primarily designed to optimize selected process parameters for xylanase production by a new strain of *Aspergillus niger* cultivated on oil palm empty fruit bunches using the central composite design (CCD) of response surface statistical optimization technique. It was further aimed to verify the applicability of non consideration of incubation period during statistical optimization of process parameters and the effects of various selected alternative nitrogen sources on xylanase production by this organism.

2. Materials and methods

2.1. Materials

All reagents used in this study are at least Analytical Reagent or

Guaranteed Reagent Grades, and are from Sigma (St. Louis, MO USA) or Merck (Darmstadt, Germany) except otherwise stated.

2.2. Microorganism

2.2.1. Isolation and screening

Aspergillus niger USM SD2 was selected based on its xylanolytic potential from among consortium of other fungal species isolated from soil samples around OPEFB dumping sites as earlier described (Ajijolakewu et al., 2015). Pure culture was preserved on Potato Dextrose Agar slants at 4 °C with periodic sub-culturing.

2.2.2. Identification and phylogeny

In this study, both the traditional and molecular identification through genomic analyses were used to identify the new isolates. Traditional method employs the morphological and colonial properties of the organisms which include structure, shape, pattern, colour and some other specific features of organisms to classify and identify phylogenetic relationship among organism. Even-though it is more error-prone compared to molecular method (Hamsher et al., 2013) and is such a subject of continuous academic review; it however provides a strong background for a successful identification by molecular approach. On the other hand, and molecular identification methods used gene nucleotide sequence data of respective organism for studying phylogeny and to provide genealogically correct classifications. Sequenced data are used to infer evolutionary relationship by using evolutionary trees which are interpolated through phylogenetic statistical hypotheses to compare a wide consortium of organisms within and without the conventional boundaries of evolutionary biology (Nadler, 1995). However due to the problem of overlapping morphological characteristics of several species within the same genus or inter-genus species complex, an acceptable recent identification requires the combination of the traditional and molecular methods of identification. Molecular identification method employs nucleotide sequence data of respective organism for studying ecological relationship and to identify phylogeny for proper classifications of organisms using some statistical hypotheses.

Sequel to colonial and morphological approaches isolate was identified by molecular technique. Genomic DNA of the fungus was extracted using the CTAB method (Murray and Thompson, 1980). A pair of primer designed by White et al. (1990), the ITS-1 (5'-TCCGTAGG-TGAACCTGCGG-3') and ITS-4 (5'-TCCTCGCTTATTGATATGC-3') was used to amplify the internal transcribed spacer (ITS) region of the rRNA gene which include the 5.8S rRNA gene and which fall between small subunit ribosomal RNA and large subunit ribosomal RNA genes of fungal genome. PCR was done in a total reaction mixture containing 25 µL (2 µL genomic DNA, 0.25 µL each of respective primer, 10 µL of sterile distilled deionised water (ddH₂O) and 12.5 µL master mix (Vivantis Technologies, Malaysia). Master mix's contents in respective proportion were Taq DNA polymerase (0.05 µL) 2 × Viburbuffer A, 0.4 mM each dNTPs and 3.0 mM MgCl₂. The PCR was conducted for thirty five 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), an extension step at 72 °C for (2 min) with a final extension at 72 °C for 10 min immediately following the last cycle. Sterile de-ionized distilled water (ddH₂O) was used as control. To ascertain successful extraction and amplification, both the Genomic DNA and amplified PCR products were gel electrophoresed. Thereafter, Sanger sequencing (Genetic Analyzer ABI 3730xl) of the successfully purified DNA products was done at the Centre for Chemical Biology, University Science Malaysia and the sequencing result was subsequently submitted to GenBank to obtain accession number. The combined sequence of ITS-1-5.8S-ITS-4 gene was compared to those of other *Aspergillus* species from standard laboratories available in the GenBank (NCBI) using BLAST-n search. Following similarity search, multiple-sequence alignments of similar

sequences 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 (Tamura et al., 2013) phylogenetic software. Phylogenetic relationships were determined using the “Bootstrap M-L” (Maximum Likelihood) tree program in MEGA based on Tamura 3-parameter model.

2.2.3. Inoculum development

A 10 ml of 1% (v/v) Tween-80-sterile distilled water was added to a 4- to 6- day old pure culture on agar plate to obtain spore suspension. Suspension was sieved through 0.5 mm filter to remove mycelia. After appropriate dilutions, inoculum sizes (spores/ml) was determined by direct microscopic observation using haemocytometer as earlier described (Aberkane et al., 2002; Rodriguez-Tudela et al., 2003).

2.3. Substrate preparation

Auto-hydrolysis treated OPEFB was used as sole carbon source for enzyme production. It was selected based on preliminary investigation of most suitable type of chemical pre-treatment of substrate (data not presented) for xylanase production. The treatment conditions used were steam under pressure (autohydrolysis) at temperature 170 °C for 1hr. After pre-treatment, substrates were grinded to ≤ 2 mm prior to use as carbon source and respective structural composition was determined as previously described (Ying et al., 2014).

2.4. Solid state fermentation

Xylanase biosynthesis was performed in a 250 ml conical flask using SSF in a production medium containing 12 ml modified Mandels and Rees medium (growth medium or gm) and 3 g (Oven dried bases –odb-) pretreated OPEFB as carbon source. Composition of the growth medium includes (g/l) (except stated otherwise): 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), $ZnSO_4 \cdot 7H_2O$ (0.0016), and $CoCl_2$ (0.0014). The medium pH was adjusted to 6.3 before sterilization at 121 °C for 15 mins. One millilitre (1 ml) of standardized inoculum (2×10^6 spores/ml) was aseptically inoculated into the medium and fermentation was allowed to proceed for 4 days at 25 °C. Crude enzyme was extracted with 0.05 M citrate buffer pH 4.8 (45 ml). After the addition of buffer, the whole flask was kept in a rotary incubator at 180 rpm (30 °C) for 15 min. Fermented substrates were filtered through Whatman no 1 filter paper to extract the crude enzyme. Filtrate obtained was centrifuged at 10,000 rpm for 15 min at 4 °C, while the supernatant was collected as the crude enzyme.

2.5. Optimization techniques

Response surface methodology was used for the optimization of conditions for xylanase production by *A. niger* USM SD2 using Central Composite Design (CCD) of the Design expert DX7.0 (Stat-Ease, 2007). Optimization of xylanase production was done for five days based on preliminary data prior optimization. Twenty four factorial points and 6 centre points (30 runs) in triplicates were independently done to verify the effects of variables on xylanase production. The statistical combination of the real and coded values of the independent variables are shown in Section 3.2.2.

Optimum temperature, initial pH, solid-liquid ratio and the most efficient inoculum concentration derived from CCD optimization were used as functions to generate model for enzyme activity (IU/gds). The model was analysed based on ANOVA and regression analyses.

2.5.1. Nitrogen supplementations

After the optimization of environmental factors, effects of alternative and increased nitrogen sources on xylanase activity were

verified. Various concentrations of yeast extract (Y/E), mycological peptone, ammonium sulphate and urea were investigated at different concentrations (0–30 g/l or 0–0.05 M depending on respective source) as possible alternative nitrogen sources for improved xylanase production by *A. niger* USM SD2.

2.6. Analytical methods

Xylanase activity (IU/gds) was determined quantitatively by the DNSA method as described by Bailey et al. (1992) using 1% xylan (w/v) from beech wood (Sigma) as substrate. Enzyme activity, expressed as international unit (IU) per gram of dry fermented substrate (gds), was defined as the amount of enzyme which liberate 1 μ mol of reducing sugar as xylose per min under the assay conditions. Specific enzyme activity (U/mg protein) and enzyme productivity (U/mgG) were determined based on crude enzyme's protein (Ghose, 1987; Lowry et al., 1951) and glucosamine (Swift, 1973) content of the fermented crust respectively. Enzyme productivity is a measure of enzyme production relative to growth of organism during fermentation. It was derived by the division of xylanase activity by the corresponding growth on a specific assay period. Time course for enzyme production was determined at the completion of each stage of optimization

2.7. Statistical analysis

Data collected in triplicates were reported as the mean and standard deviations based on statistical analysis using IBM SPSS Statistics V22. 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

3. Results and discussion

Despite the various industrial importance of xylanases, its production is yet, compared to cellulase, at developmental stage. This is because, xylanases like other hydrolases is affected by the types of organism; as well as the nutritional, physiological and biochemical properties of the producing organism. Hence, recent researches (Ajijolakewu et al., 2016; Bhunia and Dey, 2012; Montgomery, 2008; Prasad Uday et al., 2017) are not only engrossed in the search for novel organisms which could be employed on a large scale production of xylanases but also on the identification of suitable cultural conditions through factorial optimization of significant factors for enhanced production of xylanases by the isolated organisms. In this work, selected process parameters were optimized for optimal xylanase production by a newly isolated and characterized strain of soil fungi cultivated on oil palm empty fruit bunches using the central composite design (CCD) of response surface statistical optimization technique. Results of these analysis and various other investigated parameters are as presented in the following section.

3.1. Identification of organism

Based on the results of ribosomal RNA analyses, similarity search and phylogeny test, the isolated strain was confirmed as a novel strain of *Aspergillus niger*. It was subsequently designated *Aspergillus niger* USM SD2 with an assigned GenBank accession number KU882054. This strain was deposited at the Culture Collection Centre of the school of Industrial Technology, Universiti Sains Malaysia under the strain number *A. niger* USM SD2 KU882054. Fig. 1 shows the phylogenetic tree derived from MEGA 6.0.6 phylogenetic software using Tamura 3-parameter model. Phylogenetic relationship was based on the sequences of eleven other *Aspergillus* species obtained from GenBank as shown

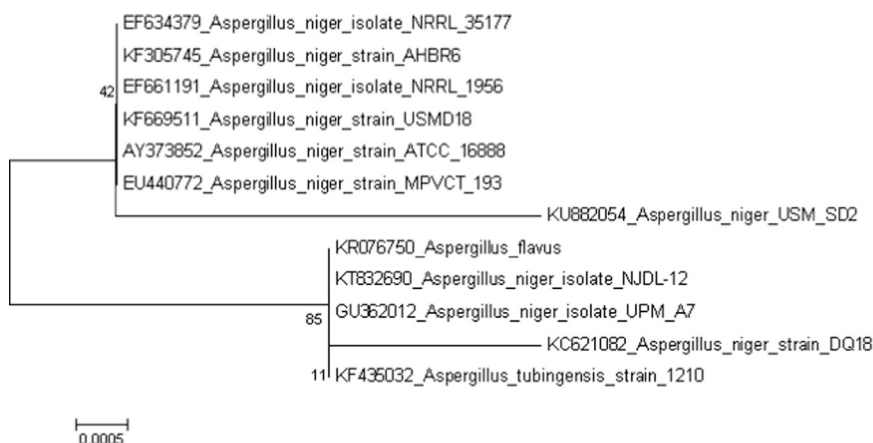


Fig. 1. Maximum likelihood tree inferred from the sequenced internal transcribed spacer gene section of the isolated *A. niger* USM SD2. Number in the branch of phylogram indicates bootstrap value in % by 1000-replication multiple; while scale shows possible substitutions of nucleotides sequence per site.

3.2. Optimization cultural conditions for xylanase production by *A. niger* USM SD2

3.2.1. Pre-optimization

Based on results of the preliminary investigation of suitable chemical pretreatment of OPEFB (data not shown), autohydrolysis-treated OPEFB was used as the substrate for xylanase production by *A. niger* USM SD2. Structural analysis of treated fibre showed that it is composed of 80.31% holocellulose (cellulose-95.6%; and 4.4% hemicelluloses) and 19.2% lignin. Fig. 2 shows the time course of xylanase production prior to optimization.

As shown in Fig. 2, xylanase production peaked at the 4th and 5th day of fermentation, which correspond to the stationary phase, with average activity (1250 ± 10 IU/gds) (Fig. 2). This peak period is similar with the ranges reported in literatures for other *Aspergillus* species (Fang et al., 2010; Umsza-Guez et al., 2011).

3.2.2. Optimization of production conditions by design of experiment (DOE)

Central composite design (CCD) (Design Expert 7.0) was used to optimize xylanase production by *A. niger* USM SD2. In this study, the individual and interactive effects of temperature, pH, moisture content and inoculum sizes on xylanase production were investigated. Even though, period of incubation is one of the most important environmental conditions that are often optimized according to previous works (Lakshmi et al., 2011; Pathak et al., 2014) for xylanase production; this study was designed without consideration of period of incubation in order to verify the validity of optimization without considering the incubation period. Table 1 shows statistical combination of the real and coded values of the independent variables. Three replicates of 30 runs,

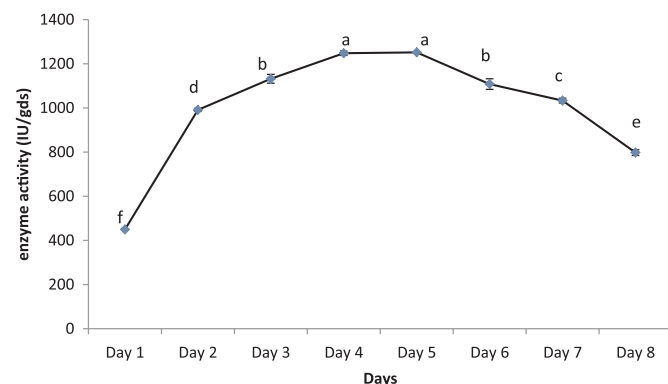


Fig. 2. Time course using water-treated substrate and un-optimized conditions: pH6, 1gds:3 ml (gm), 2×10^6 spores/ml and 30 °C. (Lower case letters indicate level of significance at $p \leq 0.05$; Error bars indicate standard errors of the means of three replicates).

which include 24 factorial points and 6 centre points, were independently carried out to verify the effects of respective variables on xylanase production by *A. niger* USM SD2.

Using the parametric variables as shown in Table 1, assay of xylanase production was done on the 5th day of fermentation based on previously determined time-course of enzyme production (Fig. 2). Responses determined as xylanase activity (IU/gds), using the respective optimization conditions, were also presented in Table 1. As shown, the individual and interactive effects among the variables resulted in a significant variation in xylanase activity.

As indicated in the standard order column, highest xylanase activity (2167 IU/gds) was obtained in the third run, while lowest activity (167.0 IU/gds) was shown in run number 19. The most obvious reasons for this difference in xylanase production is the changes in temperature and moisture content. The third run used the best combination of temperature (25 °C) and moisture (4 ml per gram of substrate), while the 19th run used a higher temperature (30 °C) and the moisture content was near absence (0.5 ml). Variations in factorial levels and interactive effects among these factors are reflected in the corresponding response values obtained (Table 1) which were respectively far higher or lower compared with the previous activity (1250 IU/gds) (Fig. 2) obtained without parametric optimization. The disparities in these values suggest the importance of optimization of production parameters.

3.2.3. Regression analysis of model

A second order polynomial model was shown as the best model to predict xylanase production. Statistical analyses (ANOVA) of the data (Table 2) revealed that a reduced quadratic model was most reliable to predict xylanase production by *A. niger* USM SD2. Based on the RSM integrated ANOVA, the following regression equation (in coded units), between xylanase activity and the model terms was derived:

$$\text{Activity (IU/gds)} = +1042.27 - 333.76*A + 458.56*B - 77.99*C - 61.06*D - 148.08*A*B - 81.87*D^2 \quad (1)$$

The level of significance of the model terms and equation are as shown in Table 2. The item p-value shows the level of significance of each term, while correlation coefficient (R^2) shows how observed variations could be explained by the generated model equation. According to Montgomery (2008), smaller p-value and a higher R^2 respectively implied that the corresponding co-efficient is significant. In other words, if model p-value as shown in Table 2 is less than 0.0001, then the probability of having a higher F-value due to noise is 0.0001. Moreso, an $R^2 = 99$ indicates that 99% of the observed variations results could be explained by the generated model equation. All data shown including, model p-value (Prob > F) less than 0.0001; the non significant lack of fit (LOF = 0.1367) and the regression coefficient ($R^2 = 0.9542$) indicate that model terms are significant. With the model obtained, it was easy to predict the individual and interactive effects of

Table 1
Actual design for the optimization of xylanase production.

Standard order	Coded variables				Real variables				Xylanase activity (IU/gds)
	A	B	C	D	Temp (°C)	Moisture-solid ratio (ml: g)	Inoculum ($\times 10^6$ spores/ml)	pH	
1	−1.0	−1.0	−1.0	−1.0	25.0	1.0	2.0	5.0	884.6
2	1.0	−1.0	−1.0	−1.0	35.0	1.0	2.0	5.0	396.6
3	−1.0	1.0	−1.0	−1.0	25.0	4.0	2.0	5.0	2167.0
4	1.0	1.0	−1.0	−1.0	35.0	4.0	2.0	5.0	1096.6
5	−1.0	−1.0	1.0	−1.0	25.0	1.0	6.0	5.0	737.2
6	1.0	−1.0	1.0	−1.0	35.0	1.0	6.0	5.0	370.7
7	−1.0	1.0	1.0	−1.0	25.0	4.0	6.0	5.0	1827.5
8	1.0	1.0	1.0	−1.0	35.0	4.0	6.0	5.0	1018.1
9	−1.0	−1.0	−1.0	1.0	25.0	1.0	2.0	9.0	611.2
10	1.0	−1.0	−1.0	1.0	35.0	1.0	2.0	9.0	276.0
11	−1.0	1.0	−1.0	1.0	25.0	4.0	2.0	9.0	1771.8
12	1.0	1.0	−1.0	1.0	35.0	4.0	2.0	9.0	793.5
13	−1.0	−1.0	1.0	1.0	25.0	1.0	6.0	9.0	553.5
14	1.0	−1.0	1.0	1.0	35.0	1.0	6.0	9.0	249.2
15	−1.0	1.0	1.0	1.0	25.0	4.0	6.0	9.0	1721.4
16	1.0	1.0	1.0	1.0	35.0	4.0	6.0	9.0	716.1
17	−2.0	0.0	0.0	0.0	20.0	2.5	4.0	7.0	1674.9
18	2.0	0.0	0.0	0.0	40.0	2.5	4.0	7.0	348.5
19	0.0	−1.3	0.0	0.0	30.0	0.5	4.0	7.0	162.0
20	0.0	2.0	0.0	0.0	30.0	5.5	4.0	7.0	1932.0
21	0.0	0.0	−1.5	0.0	30.0	2.5	1.0	7.0	1291.5
22	0.0	0.0	2.0	0.0	30.0	2.5	8.0	7.0	763.3
23	0.0	0.0	0.0	−2.0	30.0	2.5	4.0	3.0	651.7
24	0.0	0.0	0.0	2.0	30.0	2.5	4.0	11.0	821.8
25	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1072.1
26	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1048.6
27	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1124.5
28	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1285.0
29	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1120.5
30	0.0	0.0	0.0	0.0	30.0	2.5	4.0	7.0	1082.8

Table 2
Statistical (ANOVA) analysis of reduced quadratic model of RSM for the optimization of xylanase production by *A. niger* USM SD2.

Model terms	Coefficient	p-value Prob > F	Significance
<i>Coefficient of regression equation</i>			
Intercept	1042.27	–	–
A-Temperature	−333.76	< 0.0001	Significant
B-Initial moisture	458.56	< 0.0001	Significant
C-Inoculum sizes	−77.99	0.0094	Significant
D-pH	−61.06	0.0305	Significant
AB	−148.08	0.0001	Significant
D ²	−81.87	0.0025	Significant
<i>Regression analysis of model</i>			
Source	p-value Prob > F		Significance
Model	< 0.0001		Significant
Lack of fit	0.1367		Not significant
R ²	0.9542		Good

respective cultural conditions on xylanase production by the organism.

3.2.4. Analysis of the effects of cultural conditions on xylanase production by *A. niger* USM SD2

The selected cultural conditions investigated in this study were incubation temperature; initial pH of the medium; initial moisture content and the inoculum sizes. As shown in Table 2, linear coefficient of all these cultural terms showed high significance. However, only the pH showed a significant second order or quadratic effects. The implication of this is that any change in the values of these terms will significantly affect xylanase production by this organism. Among all the factors considered, both temperature and initial moisture showed the greatest effect ($p < 0.0001$) on xylanase production; while production was least affected by the initial pH of growth medium as shown in Table 2. However, all these factors have generally been identified as highly significant parameters affecting the rate of microbial synthesis of

enzyme and other metabolites (Beg et al., 2001). It is a common knowledge that a very high or extremely low temperature denatures an already synthesized enzymes and other necessary enzymes needed for microbial metabolic activities. Similarly, the moisture content determines the growth rate and other physiological activities of organisms. Gervais and Molin (2003) had observed that an increased moisture increases aerial mycelial growth, lowers oxygen transfer and decreases substrate porosity. On the other hand, low moisture reduces nutrients and protein solubility due to reduced surface area of the substrate (Shenef et al., 2010). Moreover, changes in medium pH affects the permeability of microbial cells, affect enzyme stability and also causes denaturation of secreted enzymes (Ajijolakewu et al., 2016). Lastly, a smaller inoculum may not be adequate for growth initiation, delays the lag phase as well as enzyme synthesis (Fang et al., 2010); while a high inoculum sizes could only shorten the lag phase but will lead to a possible competition for limiting nutrient due to overcrowded growth of the organism per unit substrate (Pathak et al., 2014).

According to the model equation (Eq. (1)), interactive effect exist only between the temperature and initial moisture, while other factors showed individual effects on xylanase activity. The implication is that, for optimal xylanase yield by this organism, there must be an interaction between the temperature and initial moisture content at their respective best values. The RSM plots below represented as Fig. 3a–d show the interactive effect. At optimum interaction between temperature and initial moisture content (Fig. 3a), the RSM plots show that the predicted optimum interactive conditions for xylanase production by *A. niger* USM SD2 are 25 °C and initial moisture ratio (4 ml (gm): 1 gds). Based on this figure, it could be inferred that a lower initial moisture content or higher temperature is not suitable for xylanase production by *A. niger* USM SD2. Meanwhile, at this optimum interaction, xylanase activity was 2116 IU/gds. However, changes in the values of other cultural factors at the optimum interaction between temperature and moisture content lead to a corresponding changes in

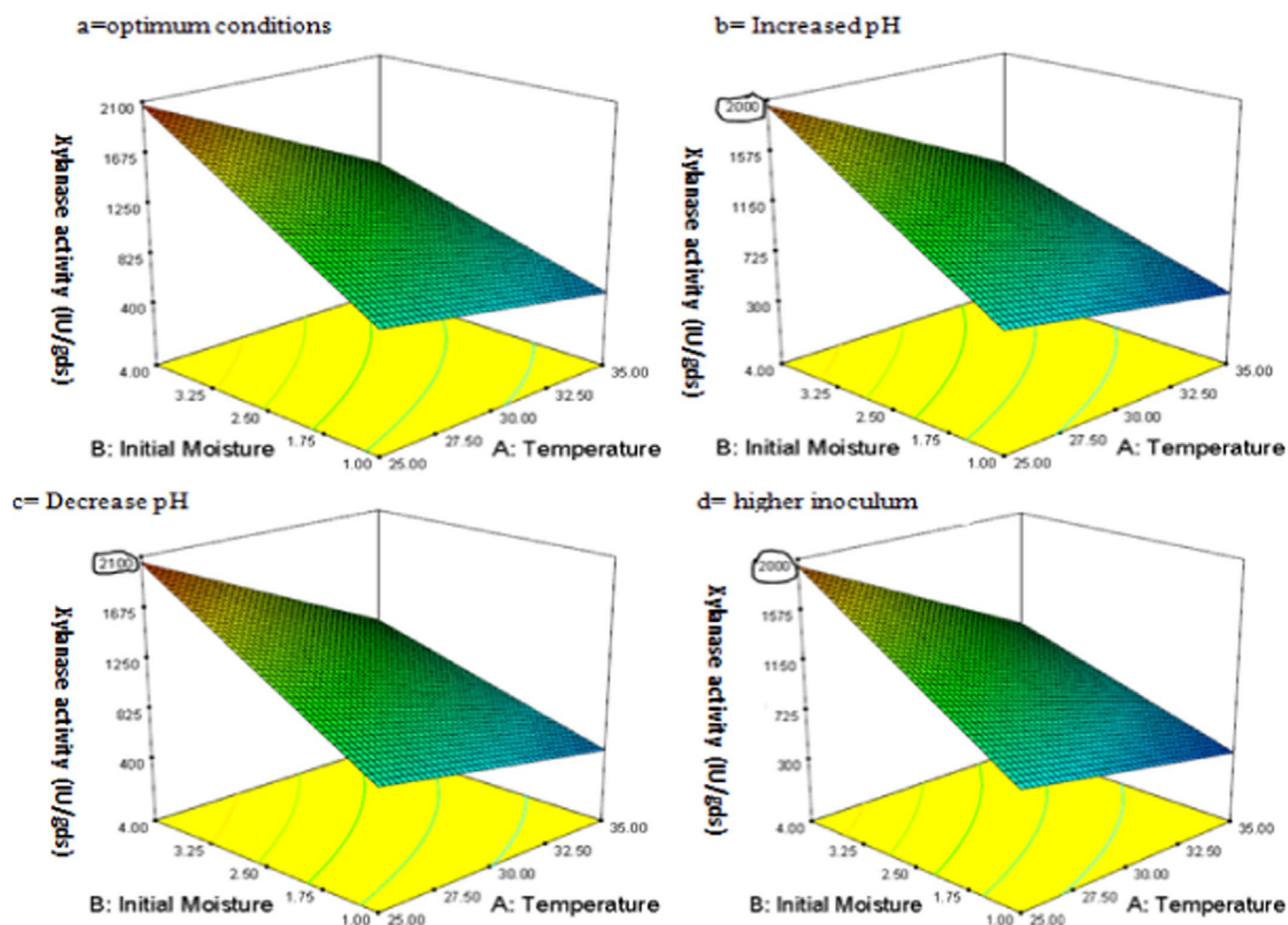


Fig. 3. a–d: Effects of selected cultural conditions on xylanase activity by *A. niger* USM SD2.

Table 3
Suggested optimum conditions by design expert.

No.	Temperature (°C)	Moisture ratio (ml: g)	Spores per ml inoculum ($\times 10^6$ spores/ml)	pH	Activity (IU/g)	Desirability	
1	25	4: 1	2×10^6	6.3	2071.88	1	Selected
2	25	4: 1	2×10^6	6.4	2071.82	1	
3	25	4: 1	2×10^6	6.5	2071.83	1	

xylanase yield. These are well demonstrated in the 3-D surface response graph as shown in Fig. 3b–d. As shown, an increase in pH beyond pH7 (Fig. 3b) or inoculum sizes beyond 2×10^6 spores/ml (Fig. 3d) caused slight reduction in the level of xylanase activity from 2116 IU/gds to 2000 IU/gds.

On the other hand, a decrease in pH value towards pH 5 (Fig. 3c) did not show significant effect on xylanase yield. This confirms the lower significance of the medium's initial pH as indicated by its co-efficient (0.0305) compared to factors such as incubation temperature and initial moisture content of the medium with significant coefficients below 0.0001 (Table 2). It also suggests that a fairly acidic pH range is suitable for xylanase production by *A. niger* USM SD2. Some other strains of *Aspergillus niger* and other *Aspergillus* species (Bakri et al., 2008; Lakshmi et al., 2009; Pal and Khanum, 2010; Shenef et al., 2010) have been reported to produce enzyme within the purview of fairly acidic pH range (5–7).

3.2.5. Verification of model equation and optimization of xylanase production conditions by *A. niger* USM SD2

The validity of the derived model (Eq. (1)) was verified by numerical optimization of the selected production conditions. Suggested optimum production criteria (Temperature (°C) – 25; inoculum size (2×10^6 spores/ml); pH 6.3; Initial moisture-substrate ratio of 4 ml (gm): 1 gds) with 100% desirability as shown in Table 3 were selected.

These conditions were predicted to generate a xylanase activity of 2072 IU/gds. Using these criteria, three independent replicates of xylanase production was done and xylanase activity value (2116.90 ± 7.37 IU/gds) with about 2.1% variation from the predicted value was obtained. This suggests that the model equation was satisfactory, because any differences in response values less than 5% is considered statistically insignificant (Montgomery, 2008). Therefore, relative to the obtained xylanase activity (1250 IU/gds) prior to optimization, xylanase production by *A. niger* USM SD2 increased by about 70% (i.e. from 1250 IU/gds to 2117 IU/gds) due to parametric optimization.

To verify the derived model (Eq. (1)) and to assess the effect of non

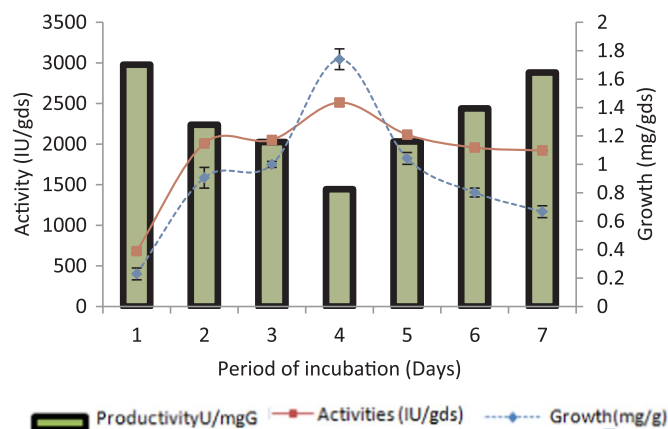


Fig. 4. Time course of enzyme production, growth profile and xylanase productivity using optimized parametric conditions: pH6.3, 1gds:4 ml (gm), 2×10^6 spores/ml and 25 °C. Key:

Productivity (IU/mgG) — Activities (IU/gds) — Growth (mg/g).

inclusion of incubation period in the optimization design, time-course of enzyme production was done using the set of optimum conditions as stated above. Results are as shown in Fig. 4.

Time course of xylanase production (Fig. 4) showed that 4th day was the peak period for enzyme production, even though, optimization was done for five days during parametric optimization by CCD. Nevertheless, this new peak was within the pre-optimization peak range -i.e. 4th and 5th day- according to earlier data (Fig. 2, Section 3.2). Average xylanase activity on the 4th day increased to 2511.6 IU/gds (Fig. 4), which is about 19% higher than xylanase activity (2116.9 IU/gds) obtained on the 5th day and more than 100% higher than the activity obtained (1250 IU/gds) prior to optimization. However, despite the change in peak period, enzyme activity on the 5th day (2116.9 IU/gds) remained constant (Fig. 4). This observation confirms the validity of the derived optimum conditions. It also suggest that optimization without consideration of incubation period is valid, provided time course of enzyme production would be done after optimization, using the optimized conditions.

As shown in Fig. 4, xylanase productivity by *A. niger* USM SD2 was highest on the first day of incubation; while the lowest productivity corresponded to the peak fermentation period (4th day) of enzyme activity. Productivity is a measure of enzyme production relative to the growth rate of organism. Further, the organism attained maximum growth on the 4th day which corresponds to the peak period for xylanase activity. Based on this result, it could be argued that xylanase production by *A. niger* USM SD2 is directly proportional to its growth profile. This initial finding contradicts previous works (Quiroz-Castaneda et al., 2011; Sridevi and Charya, 2013) which suggested that xylanase production is not a factor of growth of organism. However, in line with another report (Tlecuil-Beristain et al., 2008), this initial observation suggests that xylanase production is growth dependent. The concept of growth dependency is discussed further evaluated under Section 3.3.2.

3.3. Effect of different nitrogen (N_2) sources on xylanase production by *A. niger* USM SD2

3.3.1. Effect of different nitrogen (N_2) sources and concentrations

Nitrogen is the second most important nutrient to all organisms after carbon. Its importance in the growth of organisms is well established (Brijwani et al., 2010). Source of nitrogen is however as crucial as nitrogen itself. This is because, organisms cannot utilize the gaseous free nitrogen except in its combined forms. Different concentrations of three major organic and one inorganic nitrogen sources were investigated as a possible alternative nitrogen source to enhance xylanase production by *A. niger* USM SD2. Nitrogen sources were used

individually and in different concentrations. Results are as shown in Fig. 5a–c.

As shown in Fig. 5, all the nitrogen selected sources supported xylanase production. Increase in concentration lead to a corresponding increase in xylanase activity up to an optimum beyond which xylanase production progressively dropped. However, different concentrations of each nitrogen source was required to achieve highest xylanase activity. For yeast extract, concentrations between 15 and 20 g/l showed highest activity (3214 IU/gds); while 20 g/l of peptone (3208 IU/gds), 0.03 M of Urea (3208 IU/gds) and 0.02 M of Ammonium sulphate (2673 IU/gds) were required to achieve their respective highest xylanase activity. Any concentration above these ranges lead to a fall in the xylanase activities (Fig. 5a and b). A previous studies have also reported enzyme inhibition due to high concentration of nitrogen (Jatinder et al., 2006). Excess nitrogen concentration affects medium pH which consequently affects microbial metabolism (Lakshmi et al., 2009).

Comparatively (Fig. 5c), all the organic nitrogen sources were better than ammonia which is the only inorganic source used. This is in agreement with observation by previous reports (Fadel, 2000; Pathak et al., 2014) who have made similar observation. However, no significant difference ($p < 0.005$) was observed on xylanase production at the highest concentrations of the respective organic nitrogen sources. Meanwhile, at all concentrations, ammonium sulphate least supported xylanase activity as shown Fig. 5b and c. Based on these analyses, yeast extract (15 g/l) was selected as the sole nitrogen source for subsequent enzyme production.

3.3.2. Xylanase production profile of *A. niger* USM SD2 based on optimized conditions

Xylanase production profile of *A. niger* USM SD2 was determined based on Using the set of optimum cultural conditions (parametric optimization and nitrogen sources) as earlier stated. Fig. 6. shows the time course of enzyme production.

As shown in Fig. 6, there was a significant increase in xylanase activity (3246 ± 18) and the growth rate of organism compared to prior respective values. Also, xylanase production was peaked at the 4th day of fermentation as previously observed after parametric optimization. However, contrary to earlier observation (Section 3.2.5; Fig. 4) where both xylanase activity and growth peaked at the fourth day, maximum growth was obtained on the 5th day. This change in the growth peak period thus thought to be due to an increased nitrogen concentration in the production medium. Moreover, unlike growth which is mostly dependent on the presence of both the carbon and the nitrogen sources; xylanase production is governed by other physiologic factors such as catabolite repression. Therefore, it could be said that, increased nitrogen concentration would not only affect the growth of

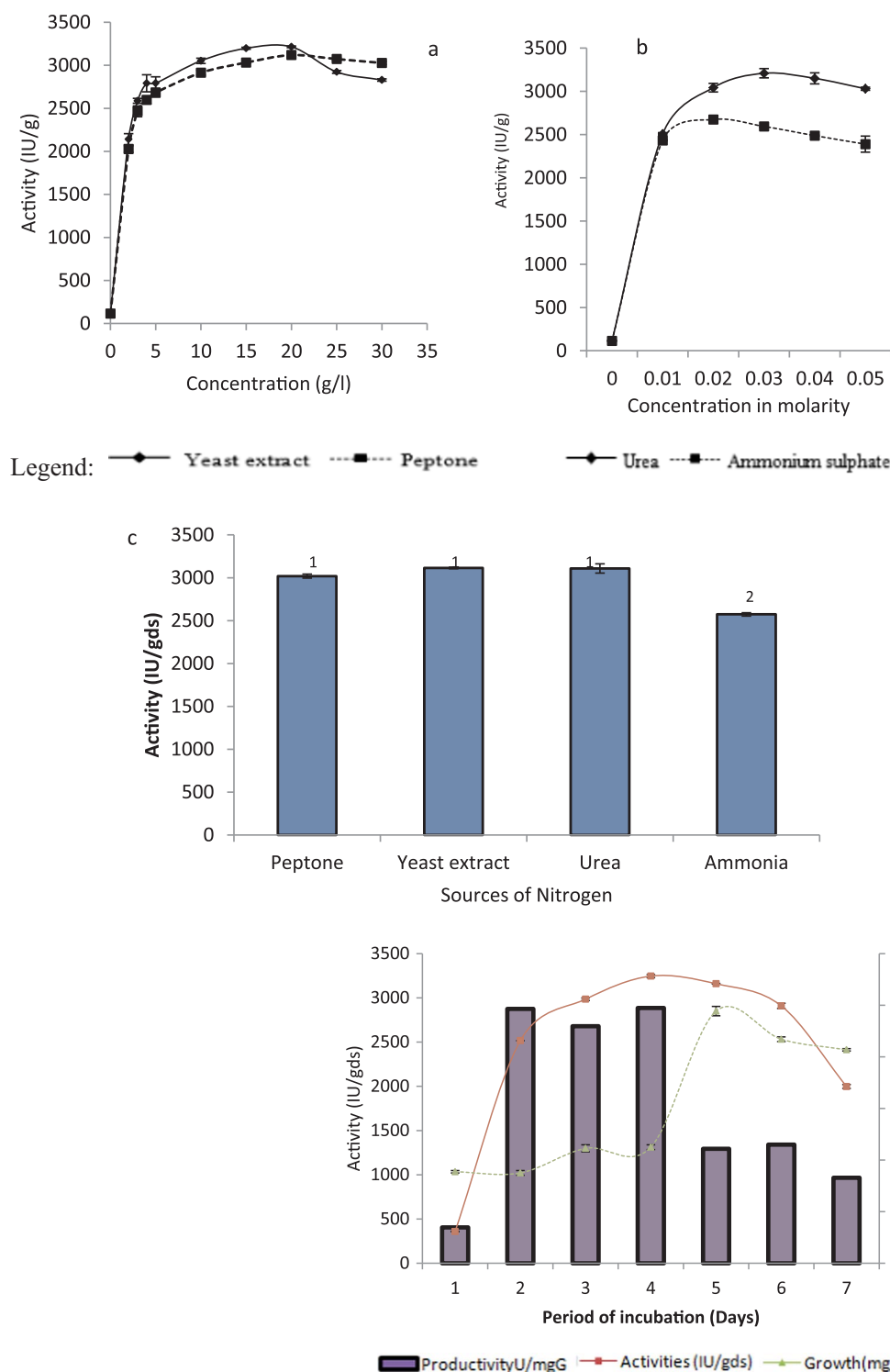


Fig. 5. a & b = Effects of different nitrogen sources on xylanase production by *A. niger* USM SD2. c = comparative effect of different nitrogen sources on xylanase production at the highest respective concentration of nitrogen source (pH6.3, 1gds:4 ml (gm), 2×10^6 spores/ml and 25 °C). Superscripts (1–3) show that differences in xylanase activities are statistically significant at $p < 0.005$.

Fig. 6. Time course of enzyme production, growth profile and xylanase productivity using the scaled-up optimized parametric conditions: pH6.3, 1gds:4 ml (gm), 2×10^6 spores/ml and 25 °C Key ProductivityU/mgG Activities (IU/gds) Growth(mg/g).

organism and the corresponding xylanase production, but could makes xylanase biosynthesis less dependent on growth rate of organisms.

Analysis of the crude enzyme showed xylanase activity = 3246 ± 18 IU/gds; total protein = 3.11 mg/g and specific activity = 1043.73 U/mg protein/gds. Also, based on changes in growth pattern, enzyme productivity was thus highest on the fourth day and lowest on the first day (Fig. 6) of enzyme production.

Evaluation of these results show that, xylanase activity by *A. niger*

USM SD2 was improved by about 160% and 29% compared to the previous activities prior and after parametric optimization respectively.

4. Conclusion

New strain of *Aspergillus niger* was isolated and identified as *A. niger* USM SD2 with GenBank accession number KU882054.

Response surface optimization of cultural conditions and improved

nitrogen concentration has been shown to enhance xylanase production by *A. niger* USM SD4 by about 160% compared to the activities prior to parametric optimization.

It has been demonstrated that optimization of cultural parameters without consideration of incubation period is valid, provided time course of enzyme production would be done after optimization using the set of derived optimized conditions.

This study has shown that higher nitrogen concentration not only affects the growth rate of organism and xylanase activity but could make both conditions independent of each other.

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References

- Aberkane, A., Cuenca-Estrella, M., Gomez-Lopez, A., Petrikou, E., Mellado, E., Monzon, A., Rodriguez-Tudela, J., 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. *J. Antimicrob. Chemother.* 50 (5), 719–722.
- Ajijolakewu, K.A., Leh, C.P., AbdulLah, W.N.W., Lee, C.K., 2016. Assessment of the effect of easily-metabolized carbon supplements on xylanase production by newly isolated *Trichoderma asperellum* USM SD4 cultivated on oil palm empty fruit bunches. *BioResources* 11 (4), 9611–9627.
- Ajijolakewu, K.A., Leh, C.P., Wan Nadiyah, W., 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: International Conference on Environmental Research and Technology (ICERT 2015), Penang, Malaysia. School of Industrial Technology Universiti Sains Malaysia, 27–29 May 2015, pp. 423–428.
- Ang, S.K., E.M, S., Y, A., A.A, S., M.S, M., 2013. Production of cellulases and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. *Process Biochem.* 48 (9), 1293–1302.
- Bakri, Y., Al-Jazairi, M., Al-Kayat, G., 2008. Xylanase production by a newly isolated *Aspergillus niger* SS7 in submerged culture. *Pol. J. Microbiol.* 57 (3), 249–251.
- Beg, Q., Kapoor, M., Mahajan, L., Hoondal, G., 2001. Microbial xylanases and their industrial applications: a review. *Appl. Microbiol. Biotechnol.* 56 (3–4), 326–338.
- Bhunia, B., Dey, A., 2012. Statistical approach for optimization of physiochemical requirements on alkaline protease production from *Bacillus licheniformis* NCIM 2042. *Enzym. Res.* 2012.
- Brijwani, K., Oberoi, H.S., Vadlani, P.V., 2010. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem.* 1 (45), 120–128.
- Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17 (4), 540–552.
- Deshpande, S., Bhotmange, M., Chakrabarti, T., Shastri, P., 2008. Production of cellulase and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid state fermentation (SSF) of water hyacinth (*Eichhornia crassipes*). *Indian J. Chem. Technol.* 15 (5), 449.
- Dobrev, G.T., Pishtytski, I.G., Stanchev, V.S., Mircheva, R., 2007. Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design. *Bioresour. Technol.* 98 (14), 2671–2678.
- Fadel, M., 2000. Production physiology of cellulases and β -glucosidase enzymes of *Aspergillus niger* grown under solid state fermentation conditions. *Online J. Biol. Sci.* 1 (5), 401–411.
- Fang, T.J., Liao, B.C., Lee, S.C., 2010. Enhanced production of xylanase by *Aspergillus carneus* M34 in solid-state fermentation with agricultural waste using statistical approach. *New Biotechnol.* 27 (1), 25–32.
- Gervais, P., Molin, P., 2003. The role of water in solid-state fermentation. *Biochem. Eng. J.* 13 (2), 85–101.
- Ghose, T., 1987. Measurement of cellulase activities. *Pure Appl. Chem.* 59 (2), 257–268.
- Hamsher, S.E., LeGresley, M.M., Martin, J.L., Saunders, G.W., 2013. A comparison of morphological and molecular-based surveys to estimate the species richness of chaetoceros and *Thalassiosira* (Bacillariophyta), in the Bay of Fundy. *PLoS One* 8 (10), e73521.
- Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., Hincha, D.K., Kunze, R., Mueller-Roeber, B., Rillig, M.C., 2015. Priming and memory of stress responses in organisms lacking a nervous system. *Biol. Rev.*
- Jatinder, K., Chadha, B.S., Saini, H.S., 2006. Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using Response Surface Methodology. *World J. Microbiol. Biotechnol.* 22 (2), 169–176.
- Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30 (4), 772–780.
- Lakshmi, G.S., Bhargavi, P.L., 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. *Curr. Trends Biotechnol. Pharm.* 5 (4), 1434–1444.
- Lakshmi, G.S., Rao, C.S., Rao, R.S., Hobbs, P.J., 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. *Biochem. Eng. J.* 48 (1), 51–57.
- Latifian, M., Hamidi-Esfahani, Z., Barzegar, M., 2007. Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. *Bioresour. Technol.* 98 (18), 3634–3637.
- Lowry, O., Rosenbrough, N., Farr, A., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Montgomery, D.C., 2008. Design and Analysis of Experiments. John Wiley & Sons.
- Murray, M., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8 (19), 4321–4326.
- Nadler, S., 1995. Advantages and disadvantages of molecular phylogenetics: a case study of ascaridoid nematodes. *J. Nematol.* 27 (4), 423.
- Oberoi, H.S., Rawat, R., Chadha, B.S., 2014. Response surface optimization for enhanced production of cellulases with improved functional characteristics by newly isolated *Aspergillus niger* HN-2. *Antonie Van Leeuwenhoek* 105 (1), 119–134.
- Pal, A., Khanum, F., 2010. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour. Technol.* 101 (19), 7563–7569.
- Pathak, P., Bhardwaj, N.K., Singh, A.K., 2014. Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopy waste paper recycling. *Appl. Biochem. Biotechnol.* 172 (8), 3776–3797.
- Prasad Uday, U.S., Bandyopadhyay, T.K., Bhunia, B., 2016. Rapid development of xylanase assay conditions using Taguchi methodology. *Bioengineered* 7 (6), 424–431.
- Prasad Uday, U.S., Bandyopadhyay, T.K., Goswami, S., Bhunia, B., 2017. Optimization of physical and morphological regime for improved cellulase free xylanase production by fed batch fermentation using *Aspergillus niger* (KP874102.1) and its application in bio-bleaching. *Bioengineered* 8 (2), 137–146.
- Quiroz-Castaneda, R.E., Perez-Mejia, N., Martinez-Anaya, C., Acosta-Urdapilleta, L., Folch-Mallol, J., 2011. Evaluation of different lignocellulosic substrates for the production of cellulases and xylanases by the basidiomycete fungi *Bjerkandera adusta* and *Pycnoporus sanguineus*. *Biodegradation* 22 (3), 565–572.
- Rezende, M.L., Barbosa, A.D.M., Vasconcelos, A.F.D., Endo, A.S., 2002. Xylanase production by *Trichoderma harzianum* rj41 by solid state fermentation on sugarcane bagasse. *Braz. J. Microbiol.* 33 (1), 67–72.
- Rodriguez-Tudela, J.L., Chrysanthou, E., Petrikou, E., Mosquera, J., Denning, D.W., Cuenca-Estrella, M., 2003. Interlaboratory evaluation of hematocytometer method of inoculum preparation for testing antifungal susceptibilities of filamentous fungi. *J. Clin. Microbiol.* 41 (11), 5236–5237.
- Saleem, R., 2014. Xylanase, laccase and manganese peroxidase production from white rot fungi. *Iran. J. Energy Environ.*
- Shah, A.R., Madamwar, D., 2005. Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. *Process Biochem.* 40 (5), 1763–1771.
- Shenef, A., El-Tanash, A., Atia, N., 2010. Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. *Res. J. Microbiol.* 5 (3), 199–211.
- Shin, H.D., Vo, T., Chen, R., 2011. Novel *Aspergillus* hemicellulases enhance performance of commercial cellulases in lignocellulose hydrolysis. *Biotechnol. Prog.* 27 (2), 581–586.
- Sridevi, B., Charya, M.S., 2013. Isolation, identification and screening of potential cellulase-free xylanase producing fungi. *Afr. J. Biotechnol.* 10 (22), 4624–4630.
- Swift, M., 1973. The estimation of mycelial biomass by determination of the hexosamine content of wood tissue decayed by fungi. *Soil Biol. Biochem.* 5 (3), 321–332.
- Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* 56 (4), 564–577.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30 (12), 2725–2729.
- Tlecuitl-Beristain, S., Sánchez, C., Loera, O., Robson, G.D., Díaz-Godínez, G., 2008. Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: production of a novel laccase isoform. *Mycol. Res.* 112 (9), 1080–1084.
- Uday, U.S.P., Choudhury, P., Bandyopadhyay, T.K., Bhunia, B., 2016. Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. *Int. J. Biol. Macromol.* 82, 1041–1054.
- Umsza-Guez, M.A., Díaz, A.B., Ory, Id, Blandino, A., Gomes, E., Caro, I., 2011. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. *Braz. J. Microbiol.* 42 (4), 1585–1597.
- White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 18 (1), 315–322.
- Yang, S.Q., Yan, Q.J., Jiang, Z.Q., Li, L.T., Tian, H.M., Wang, Y.Z., 2006. High-level of xylanase production by the thermophilic *Paecilomyces thermophila* J18 on wheat straw in solid-state fermentation. *Bioresour. Technol.* 97 (15), 1794–1800.
- Ying, T.Y., Teong, L.K., Abdullah, W.N.W., Peng, L.C., 2014. The effect of various pre-treatment methods on oil palm empty fruit bunch (EFB) and kenaf core fibers for sugar production. *Procedia Environ. Sci.* 20, 328–335.