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Optimization of nutrient medium components for production of a client endo-β-1,4-xylanase from *Aspergillus fumigatus* var. *niveus* using a recombinant *Aspergillus nidulans* strain

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ABSTRACT

The goal of this study was to optimize a nutrient medium to maximize production of endo-β-1,4-xylanase (hereafter referred to as xylanase) using an *Aspergillus nidulans* modified by integration of an AFUMN-GH10 gene from *Aspergillus fumigatus* var. *niveus*. This modification resulted in high-yield secretion and accumulation of recombinant protein. Xylanases are an industrially relevant enzyme class used in food production and bioprocessing. High-titer production of xylanase was achieved by the cultivation of *A. nidulans* in the presence of maltose. A 2-level Plackett-Burman design was used to determine which medium components significantly affected xylanase production. $NaNO_3$, maltose and KCl showed significant effects on xylanase production. These three medium components were further optimized using a 3-level Box-Behnken design, and their optimum levels were maltose, 120 g/L; $NaNO_3$, 12 g/L and KCl, 2 g/L. A xylanase activity of 1,620 U/mL, (12,460 U/g of maltose) was observed when using the optimum medium, which was 280% greater than the maximum level obtained with the basic medium. Pyridoxine concentration in the optimum medium was reduced to limit growth and divert substrate toward enzyme production. Reducing pyridoxine from 1,000 μg/ml to 300 μg/ml increased xylanase activity to 1,790 U/ml. Optimized medium with reduced pyridoxine was then tested in a stirred tank bioreactor, which resulted in 112% more xylanase activity and 43% less dry cell weight compared with the medium containing 1,000 μg/ml of pirydoxine.

1. Introduction

Hemicellulose is a primary component of cell walls, particularly in hardwoods and annual plants (Bhushan et al., 2015; Collins et al., 2005). Xylan is the major component of hemicellulose and made of β-1,4-linked xylose units (Shallom and Shoham, 2003). Endo-β-1,4-xylanases (EC 3.2.1.8) are a class of enzymes that catalyze the hydrolysis of xylan backbone and are mainly grouped in the glycoside hydrolase (GH) families 10 and 11 based on amino acid sequence and catalytic domain analysis; however, these enzymes also have been reported in GH families 5, 7, 8, 16, 26, 30, 43, 52 and 62 (Uday et al., 2016; Verma and Satyanarayana, 2012); (Subramaniyan and Prema, 2002). Xylanases have been reported as enzymes with potential application to improve process in a variety of industries, including production of bread, dough, juice and beer (Bajaj and Sharma, 2011; Butt et al., 2008; Nagar et al., 2012), pharmaceutical production (Topakas et al., 2003), paper and pulp industry (Kumar and Satyanarayana, 2014) and ethanol production (Silva et al., 2012).

Most microorganisms have in their genome genes that encode xylanases (Sutay Kocabas et al., 2015; Wang et al., 2014; Xue et al., 2015); however, xylanases produced by filamentous fungi present more interesting characteristics for industrial application once these microorganisms are able to secrete proteins in higher titers than most other organisms (Okafor et al., 2007; Polizeli et al., 2005). The genus *Aspergillus* has been studied extensively for xylanase production (Cavalieri de Alencar Guimaraes et al., 2013; Kollet and Zlotnik, 2003; Michelin et al., 2010; Peixoto-Nogueira et al., 2009; Segato et al., 2014).

Stirred tank bioreactors are the most widely used reactors for

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growing microorganisms as they enable efficient mass transfer (Kumar and Satyanaana, 2014). Filamentous fungi form biofilms that adhere to bioreactor surfaces (Harding et al., 2009). Rapid growth of filamentous fungi and corresponding biofilm formation can lead to reduced fluid flow and mixing. Reducing growth reduces biofilm formation, which increases mixing and mass transfer through the reactor. Also, reducing growth diverts energy generated from carbon sources from cell mass production into product formation. Growth control can be done by limiting essential nutrients such as vitamins or coenzymes (Müller et al., 2015, 2014; Pardo-Planas et al., 2017b).

In this study we used a recombinant *A. nidulans* strain with a pyridoxine marker as a cell factory to produce an industrially relevant enzyme aiming the optimization in nutrient medium components and concentrations to maximize enzyme production. To reach this goal a Plackett-Burman design was applied to screen the medium components with significant effect on enzyme production, followed by Box-Behnken design with response surface methodology to optimize the concentrations of the significant components. Moreover, multivariate experiments in response surface methodology (RSM) allowed the reduction in experimental units given a precisely result in comparison with those obtained by univariate strategies (Chellapandi and Jani, 2008). This study also aimed to increase xylanase production by controlling growth using dilute pyridoxine concentrations.

2. Materials and methods

2.1. Microbial strains, plasmids and culture conditions

Aspergillus nidulans strain A773 (*pyrG89*; *wA3*; *pyroA4*) was purchased at Fungal Genetic Stock Center (FGSC, USA). The *A. fumigatus* var. *niveus* (AFUMN) strains was kindly provided by Prof. Dr. Rolf A. Prade from Oklahoma State University (Stillwater, OK, USA). *A. nidulans* A773 was cultured in minimal medium as previously described (Segato et al., 2012) and genetically modified by transformation with the pEXPYR plasmid carrying a gene from *A. fumigatus* var *niveus* (AFUMN-GH10) that encodes an endo-β-1,4-xylanase from family GH10 (Couger et al., 2018). The pEXPYR plasmid contain a *pyrG* gene and an induced glucoamylase promoter, which were used to selection, overexpression and secretion of AFUMN-GH10 into the media supplemented by maltose, respectively.

2.2. Inoculum preparation

Spores kept in fungal stock solution (20% glycerol, 10% lactose) at −80 °C were thawed and 20 µl were distributed in a Petri dish containing potato dextrose agar media. Petri dishes were incubated at 37 °C for 2 days. The spores were scraped from the plate and added to 10 mL of distilled water, giving a final concentration of 4×10^8 spores/mL in the spore inoculum (Abdella et al., 2014).

2.3. Culture conditions

The fermentation was performed in minimal medium containing 48 g/L maltose and 10 g/L glucose as carbon sources, 50 mL/L of 20× Clutterbuck salt solution (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4$, 30.4 g/L KH_2PO4), 1 mL/L of 1,000× trace elements solution (22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5.0 g/L $MnCl_2 \cdot 7H_2O$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $Na_2MoO_4 \cdot 4H_2O$, 50 g/L Na_2-EDTA), and 1 mL of a 1 g/l pyridoxine solution (Müller et al., 2015). The medium pH was adjusted to 6.5 using 2 M NaOH and sterilized by autoclaving at 121 °C, 204.7 kPa for 30 min.

For the set of experiments using spores for the inoculum, 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium were inoculated with 50 µL spore inoculum to a final concentration of $4 \text{ X } 10^5$ spores/mL and incubated in an orbital shaker at 37 °C and 225 rpm for 120 h. Samples were withdrawn every 24 h in triplicate, filtered to remove the mycelia and used to assay xylanase activity.

For the set of experiments using cell pellets for the inoculum, cell pellets were prepared by inoculating 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium without maltose with spore suspension to a final concentration of 4×10^5 spores/mL. The flasks were incubated at 37 °C on a rotatory shaker at 225 rpm for 48 h. The resulting pellets were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium and flasks were incubated at 37 °C with agitation at 225 rpm for 120 h. Samples were taken every 24 h and assayed for xylanase activities after removing the mycelia by filtration. All experiments were run in parallel and in triplicate.

2.4. Effect of different nitrogen sources

The effect of the nitrogen source on xylanase production was evaluated by replacing $NaNO_3$ in fermentation medium by yeast extract, peptone, meat extract, corn steep solids, $(NH_4)_2SO_4$ and ý $(NH_4)_2C_4H_4O_6$. The nitrogen mass percent was 0.08% in all fermentation media.

2.5. Multifactorial experiments for optimizing xylanase production

The optimization of medium components concentrations to maximize xylanase production was evaluated by applying a sequential optimization strategy, where the first phase dealt with screening and identifying which components affected xylanase production by recombinant *A. nidulans*. Once the factors affecting xylanase production were determined, the second phase involved ascertaining the component concentration that resulted in the maximum xylanase production. Xylanase production was measured as the xylanase activity measured in the medium.

2.5.1. Screening of factors affecting xylanase production

In the first phase, a Plackett-Burman design (Plackett and Burman, 1946) was employed to screen and determine the most important factors (medium components) that influenced xylanase production. Plackett-Burman cannot be used to measure interactions among factors. This design is especially useful for screening a large number of factors when it is unclear which factors are likely to influence the dependent variable. In the present work, seven assigned factors (maltose, glucose, $NaNO_3$, KCl, $MgSO_4$, KH_2PO_4 and trace elements) were screened in 12 experimental trials. Each factor was examined at two concentrations. Table 1 illustrates the factors examined and the concentration of each factor used in the experimental design. All experiments were carried out for 5 days in duplicate and the mean of the xylanase activity was taken as the response (dependent variable). The main effect of each factor was calculated as the difference between the means of xylanase activity measured in cultivations using +1 and −1 concentrations.

The following first-order model was used for the Plackett-Burman design:

$$Y = \beta_o + \Sigma \beta_i X_i \tag{1}$$

where Y was the response (xylanase activity (U/mL)), β_o was the model

Table 1
Variables and their levels employed in Plackett-Burman design for screening of Variables affecting xylanase production by *Aspergillus nidulans*.

Factor	Low level (−1)	High level (+1)
Maltose	20 g/L	80 g/L
Glucose	5 g/L	15 g/L
$NaNO_3$	2 g/L	8 g/L
KCl	0.1 g/L	1 g/L
$MgSO_4 \cdot 7H_2O$	0.1 g/L	1 g/L
KH_2PO_4	0.5 g/L	3 g/L
Trace elements	0.5 mL/L	2 mL/L

intercept, β_i was the linear coefficient for each factor and X_i is the concentration of each factor.

The magnitude of the coefficient β_i and whether it was positive or negative showed the impact of the corresponding factor on xylanase production. A confidence level of 95% ($p < 0.05$) for each factor was used to determine whether a factor had a significant effect on xylanase production.

2.5.2. *Optimization of significant variables using Box-Behnken design*

The factors identified as having a significant effect on xylanase production from the Plackett–Burman experiments were further optimized with response surface methodology (RSM) using a Box-Behnken design (Box and Behnken, 1960). The design was composed of 15 experiments with three factors, three levels and three center points, and it was used for fitting a second order response surface. The data was then fitted to a second-order polynomial equation and the optimal concentration of each factor for xylanase production was estimated using SAS JMP version 13 (SAS Institute, Cary, NC, USA). Multiple regression through the least squares method was used to fit xylanase production to the following quadratic equation:

$$Y = \beta_o + \Sigma\, \beta_i X_i + \Sigma\, \beta ijXi\, Xj + \Sigma\, \beta_{ii} X_i^2 \tag{2}$$

where Y was the response (xylanase activity), β_o was the model intercept, β_i was the linear coefficient of the model, β_{ii} was the quadratic coefficient, β_{ij} was the interaction coefficient and X_i and X_j are the levels of the factors in coded values.

2.6. *Effect of pyridoxine on enzyme production*

2.6.1. *Effect of pyridoxine on enzyme production in shake flasks*

Cell pellets were prepared as described above. They were then washed to remove residual pyridoxine. Washing was done by centrifuging harvested pellets at 3,750 rpm for 15 min. The supernatant was then discarded and 0.89% NaCl solution was added. This process was repeated twice to remove pyridoxine from pellets. Different concentrations of pyridoxine (0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and1,000 µg/mL) were added to 250 mL shake flasks containing 50 mL production media and they were inoculated with 10% v/v of cell pellets and incubated at 37 °C at 250 rpm for 72 h. Dry cell weight (DCW) (g/L) and xylanase activity (U/mL) were recorded after 72 h.

2.6.2. *Effect of pyridoxine on enzyme production in bioreactor*

Production media (1.5 L) containing 300 µg/mL pyridoxine was prepared in 3 L bioreactor and inoculated with 10% (v/v) cell pellets. Pyridoxine was removed from pellets as described above. A control run was done by inoculation of the washed cell pellets in production media containing 1 mg/mL pyridoxine. Dissolved oxygen (DO) was measured using a dissolved oxygen probe (InPro 6800 Series, Mettler Toledo, Urdorf, CH) and pH was measured by an Ingold gel filled pH sensor (Mettler Toledo).

2.7. *Analytical methods*

Xylanase activity was measured using a 1% beechwood xylan suspension (TCI America, Portland, OR, USA) prepared in 0.95 mL of 0.05M citrate buffer (pH 5). The suspension was mixed with 0.05 mL of crude enzyme extract or enzyme extract diluted with buffer. The reaction mixture was incubated at 50 °C for 15 min and terminated by adding 0.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent. After the addition of DNS, the contents were placed in a boiling water bath for 5 min and then cooled to room temperature. The absorbance was measured against a blank that contained no enzyme at 575 nm using a UV–Vis spectrophotometer (Miller, 1959). The xylanase activity was defined as the amount of enzyme that release 1 µmol of xylose per min. For dry weight measurements, 50 mL of the culture were filtered through a pre-weighed filter (P8 Fisherbrand, 15 cm, Fisher Scientific, Waltham, MA, USA), washed with distilled water and dried to constant weight at 60 °C. The total protein concentration was assayed using a Bradford protein assay kit (BioRad, Hercules, CA, USA) with bovine serum albumin as standard (Bradford, 1976). To determine maltose and glucose a HPLC (Dionex Ultimate 3000, ThermoScientific, Waltham, MA, USA) coupled with an HPX-87P column (300 mm × 7.8 mm) using distilled water as eluent in a flow rate of 0.6 mL/min at 80 °C. Sugars were detected by a refractive index detector (Shodex RI-101, Tokyo, Japan) (Sluiter et al., 2006).

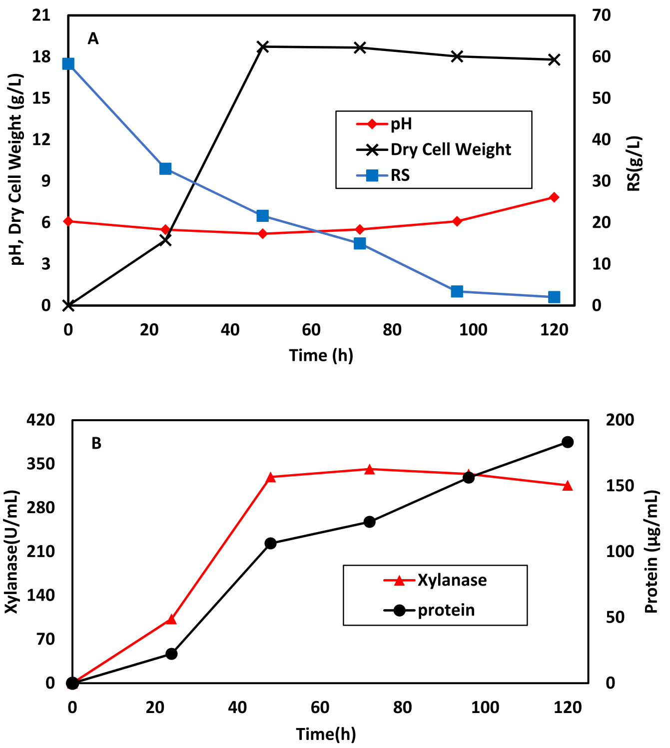

Fig. 1. Dry cell weight concentration, pH and reducing sugar (RS) concentration (A) and xylanase activity and protein conentration (B) during batch fermentation with recombinant *A. nidulans* in shake flasks inoculated with spores.

3. Results and discussion

3.1. *Effect of morphology of inoculum and incubation time on xylanase production by A. nidulans*

Fig. 1 shows batch kinetic data for shake flask experiments using spore inoculation. The pH gradually decreased from 6.17 to 5.06 at 48 h and then increased to 7.85 at 120 h. Cell mass concentration increased to 18.73 g/L at 48 h, then slightly decreased to 17.8 g/L at 120 h. The total reducing sugars concentration decreased from the initial 58 g/L to 2.0 g/L at 120 h. Production of protein and xylanase activity started to increase after 24 h reaching a maximum protein concentration of 183 µg/L after 120 h and a maximum xylanase activity of 342 U/mL after 72 h.

Fig. 2 shows batch kinetic data for shake flask experiments using cell pellet inoculation. The pH gradually decreased from 5.95 to 4.5 at 48 h and then increased to 7.8 at 120 h. Cell mass concentration increased to 23 g/L at 48 h, then slightly decreased to 19.23 g/L. The total reducing sugars decreased from the initial 58 g/L to 1.3 g/L at 120 h. Production of protein and xylanase enzyme started to increase after 24 h with a maximum protein concentration of 294 µg/L observed after 120 h and a maximum xylanase activity of 579 U/mL after 72 h.

Greater enzyme production was obtained with inoculation using cell pellets than with inoculation with spore suspension. This may be due to

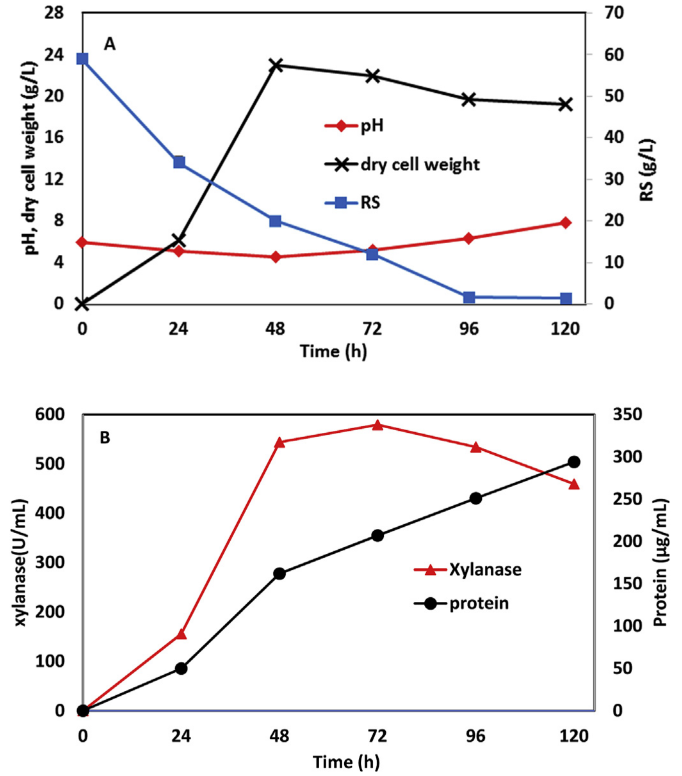

Fig. 2. Dry cell weight concentration, pH and reducing sugar (RS) concentration (A) and xylanase activity and protein conentration (B) during batch fermentation with recombinant *A. nidulans* in shake flasks inoculated with cell pellets.

the cell morphology (mycelia clumps) produced directly from spores, which can result in a low mass transfer rate, poor cell growth, and low enzyme production. A higher mass transfer rate may have been obtained inside the pellets, which would increase both the nutrient mass transfer rate into the cells and the mass transfer rate of xylanase out of the cells and into the medium (Kerns et al., 1986; Talabardon and Yang, 2005; Zhang et al., 2015).

3.2. Effect of nitrogen source on xylanase production by A. nidulans

In the present study, the effect of nitrogen source was tested by replacing $NaNO_3$ in the medium with other nitrogen sources while maintaining a constant nitrogen mass percentage (Goyal et al., 2008). Xylanase production was different among experiments using all inorganic ($(NH_4)_2SO_4$, ý$(NH_4)_2C_4H_4O_6$, and $NaNO_3$) and organic (corn steep liquor (CSL), meat extract, yeast extract and proteose-peptone) nitrogen sources (Fig. 3). It was found that the highest xylanase activity (568 U/mL) was obtained using $NaNO_3$ as nitrogen source, followed by corn steep liquor (380 U/mL).

Kheng and Omar (2005) observed that $NaNO_3$ was the best nitrogen source that they tested for xylanase production using an *A. niger* isolate. Bakri et al. (2008) also found that increasing $NaNO_3$ concentration in media stimulated xylanase enzyme production by *Cochliobolus sativus*. Soundari et al. (2007) stated that $NaNO_3$ was important for xylanase production by various microorganisms. This may be explained by better assimilation of $NaNO_3$ by *A. nidulans*, given that $NaNO_3$ is used as a soil fertilizer and *A. nidulans* is a common soil microorganism (Goyal et al., 2008).

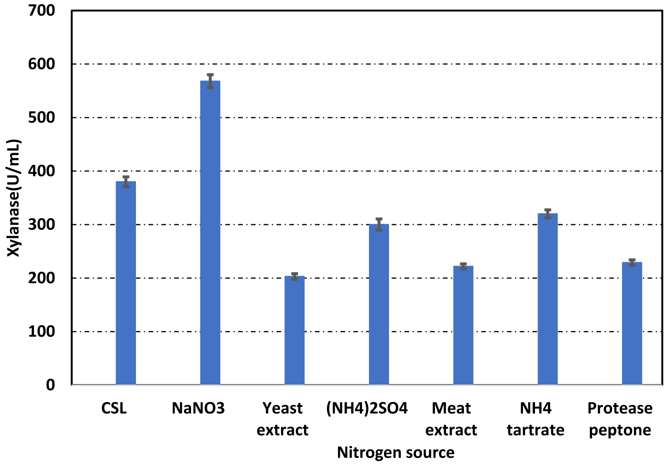

Fig. 3. Production of xylanase enzyme by *A. nidulans* in shake-flasks using various nitrogen sources. Error bars reflect ± 1 standard deviation.

Table 2
Plackett-Burman experimental design matrix for screening of factors affecting xylanase production by *Aspergillus nidulans*.

Maltose (g/L)	Glucose (g/L)	$NaNO_3$ (g/L)	KCl (g/L)	$MgSO_4$ (g/L)	KH_2PO_4 (g/L)	Trace elements (g/L)	Xylanase (U/mL)
80	5	2	1	0.1	3	2	425
80	15	2	0.1	0.1	3	0.5	317
20	15	8	1	0.1	0.5	0.5	576
20	5	8	0.1	0.1	3	0.5	374
20	15	2	0.1	1	0.5	2	201
80	15	8	1	1	3	2	946
80	5	2	0.1	1	0.5	0.5	313
20	5	8	0.1	1	3	2	511
80	15	8	0.1	0.1	0.5	2	800
80	5	8	1	1	0.5	0.5	997
20	15	2	1	1	3	0.5	256
20	5	2	1	0.1	0.5	2	302

3.3. Multifactorial designs for optimizing xylanase production

3.3.1. Screening of significant variables using the Plackett-Burman design

Table 2 illustrates the wide variation of xylanase activity, from (201–997 U/mL) observed in the Plackett-Burman experiment, which emphasized the importance of optimizing medium composition for attaining greater xylanase activity. The main effects of the examined factors on xylanase production are identified and presented in Table 3. The linear correlation model that describes the correlation between the seven factors and the xylanase activity as follows:

Table 3
Estimated effects, corresponding t ratios and p-values for the Plackett-Burman design experiment (* indicates the factor was significant ($p < 0.05$)).

Term	Estimate	Std Error	t Ratio	p-Value
Maltose	124.99793	18.9511	6.6	0.0027*
$NaNO_3$	192.45793	18.95118	10.16	0.0005*
KCl	88.672069	18.95118	4.68	0.0095*
$MgSO_4$	39.532414	20.75998	1.90	0.1296
KH_2PO_4	−23.39126	18.95118	−1.23	0.2847
Trace elements	22.684598	18.95118	1.20	0.2974
Glucose	7.7912644	18.95118	0.41	0.7021

$$\begin{aligned} Xylanase\ activity\ (U/mL) = {} & 494.97 + 124.99\left(\frac{Maltose - 50}{30}\right) \\ & + 7.79\left(\frac{glu\cos e - 10}{5}\right) \\ & + 192.45\left(\frac{NaNO3 - 5}{3}\right) \\ & + 88.67\left(\frac{KCl - 0.55}{0.45}\right) \\ & + 39.53\left(\frac{MgSO4 - 0.55}{0.45}\right) \\ & - 23.39\left(\frac{KH2PO4 - 1.75}{1.25}\right) \\ & + 22.68\left(\frac{Trace - 1.25}{0.75}\right) \end{aligned} \tag{3}$$

The values for the variables in the equation are the concentrations used in each experiment.

From Table 3, factors with p-values < 0.05 were considered to have significant effects on xylanase activity, and thus xylanase production, and these were selected for further optimization using Box-Behnken design. $NaNO_3$, maltose and KCl were determined to be significant factors, with p-values of 0.0005 (t ratio = 10.16), 0.0027 (t-ratio = 6.6) and 0.0095 (t ratio = 4.68), respectively. Each of these factors were observed to have a significant effect on increasing xylanase enzyme production at the higher concentration level tested, which suggested that the optimal concentrations of these compounds for xylanase production would be closer to the higher concentations used in the experiment than the lower concentrations.

3.3.2. Optimization of significant variables using RSM (Box-Behnken design)

To improve the pre-optimization formula for the subsequent optimization step, the variables with a negative-effect on xylanase production obtained from the Plackett-Burman design were fixed at their (−1) coded values, while the variables with a positive-effect on xylanase production were fixed at their (+1) coded values. To identify the optimum response region for xylanase production, the significant factors ($NaNO_3$, maltose, KCl) were tested at three levels to determine the optimum concentration of each component for xylanase production.

Table 4 presents the design matrix for the factors, given in both coded and natural units, and the xylanase activity measured for each experimental run. Table 4 shows the maximum and minimum levels of variables tested in the Box-Behnken design as well as the three center points that were conducted as runs 1, 3 and 4, which contained maltose (120 g/L), $NaNO_3$ (12 g/L) and KCl (2 g/L). The maximum activity was observed at these center points (a mean of 1,566 U/mL). The minimum xylanase activity (1,000 U/mL) was observed in run no. 2 containing maltose (80 g/L), $NaNO_3$ (8 g/L) and KCl (2 g/L).

Table 4
Box-Behnken factorial experimental design and responses of xylanase activity produced.

Maltose(A, g/L)	$NaNO_3$(B, g/L)	KCl(C, g/L)	Xylanase activity(U/mL)	
			Actual Value	Predicted Value
120	12	2	1520	1566
80	8	2	1000	963
120	12	2	1560	1567
120	12	2	1620	1567
120	16	1	1450	1389
160	12	3	1420	1449
120	8	3	1080	1141
80	12	1	1360	1331
160	16	2	1290	1328
160	8	2	1280	1190
120	16	3	1480	1414
80	12	3	1380	1356
80	16	2	1280	1370
160	12	1	1400	1424
120	8	1	1050	1116

Table 5
Estimated effects, corresponding t Ratios and p-values for main effects and interactions among them during the Box-Behnken experiment (* indicates the factor was significant ($p < 0.05$)). A = maltose, B = $NaNO_3$, C = KCl.

Term	Estimate	Std Error	t Ratio	p-Value
Intercept	−3049.167	874.2577	−3.49	0.0175*
A	23.40625	8.413043	2.78	0.0388*
B	444.0625	84.13043	5.28	0.0032*
C	260.83333	278.0207	0.94	0.3912
AB	−0.421875	0.290278	−1.45	0.2059
AC	0	1.16111	0.00	1.0000
BC	0	11.6111	0.00	1.0000
A^2	−0.071615	0.030213	−2.37	0.0639
B^2	−14.97396	3.021305	−4.96	0.0043*
C^2	−62.08333	48.34088	−1.28	0.2553

A second-order polynomial equation was fitted to the experimental xylanase activity data to determine the optimum component concentrations. The model is shown below

(maltose, g/L, (A); $NaNO_3$, g/L (B); KCl, g/L (C)): Xylanase activity (U/mL) $= -3049.166 + 23.4A + 444.06B + 260.83C - 0.421AB + 0.0716A^2 - 14.97B^2 - 62.08\ C^2$ (4)

The main effects of the examined factors on xylanase production were identified and are presented in Table 5, which shows the Student t-distribution, the corresponding p-value, and the parameter estimate. $NaNO_3$ ($p = 0.0032$) and maltose ($p = 0.0388$) were determined to be significant factors. The quadratic effect of the model parameters ($NaNO_{3*}$ $NaNO_3$) was also found to be significant ($p = 0.0043$). From the RSM results, both carbon and nitrogen source acted as limiting substrates and changes in their concentration affected xylanase production (Tanyildizi et al., 2005).

3.3.3. Localization of optimum condition

Fig. 4 depicts the three-dimensional responses for xylanase activity, which were made from selecting two factor and keeping the value of the third factor constant at its central value. Each response surface had a clear peak, which indicated that the model encompassed the optimum region for xylanase production. Fig. 4A is a response surface plot for maltose vs. $NaNO_3$ where concentration of KCl was kept at its zero level (2 g/L). Fig. 4B is a response surface plot for maltose vs. KCl where $NaNO_3$ concentration of was kept at its zero level (12 g/L). Fig. 4C is a response surface plot for $NaNO_3$ vs. KCl where maltose concentration was kept at its zero level (120 g/L). From the plots, it was found that the maximum xylanase activity was obtained at or near the zero level of maltose (120 g/L), $NaNO_3$ (12 g/L) and KCl (2 g/L). This may be explained by catabolite inhibition caused by high concentration of maltose as carbon source (Kremling et al., 2015). Also, high concentrations of nitrogen ($NaNO_3$) might have a toxic effect on enzymes production (Dinarvand et al., 2012).

3.3.4. Model validation

The optimal condition realized from the optimization experiment was validated experimentally and compared with the prediction calculated from the model (Table 4). The experimentally obtained mean xylanase yield of 1,570 U/mL was comparable to the polynomial model predicted value of 1,567 U/mL. The goodness of fit was tested by calculating the determination coefficient (R^2). In this case $R^2 = 0.91$ for the investigated conditions, which indicated that the response model can explain 91% of the total variation in the data. It also showed the relative effects of any two variables when the third is maintained constant. Linear, cross product and quadratic terms were significant at

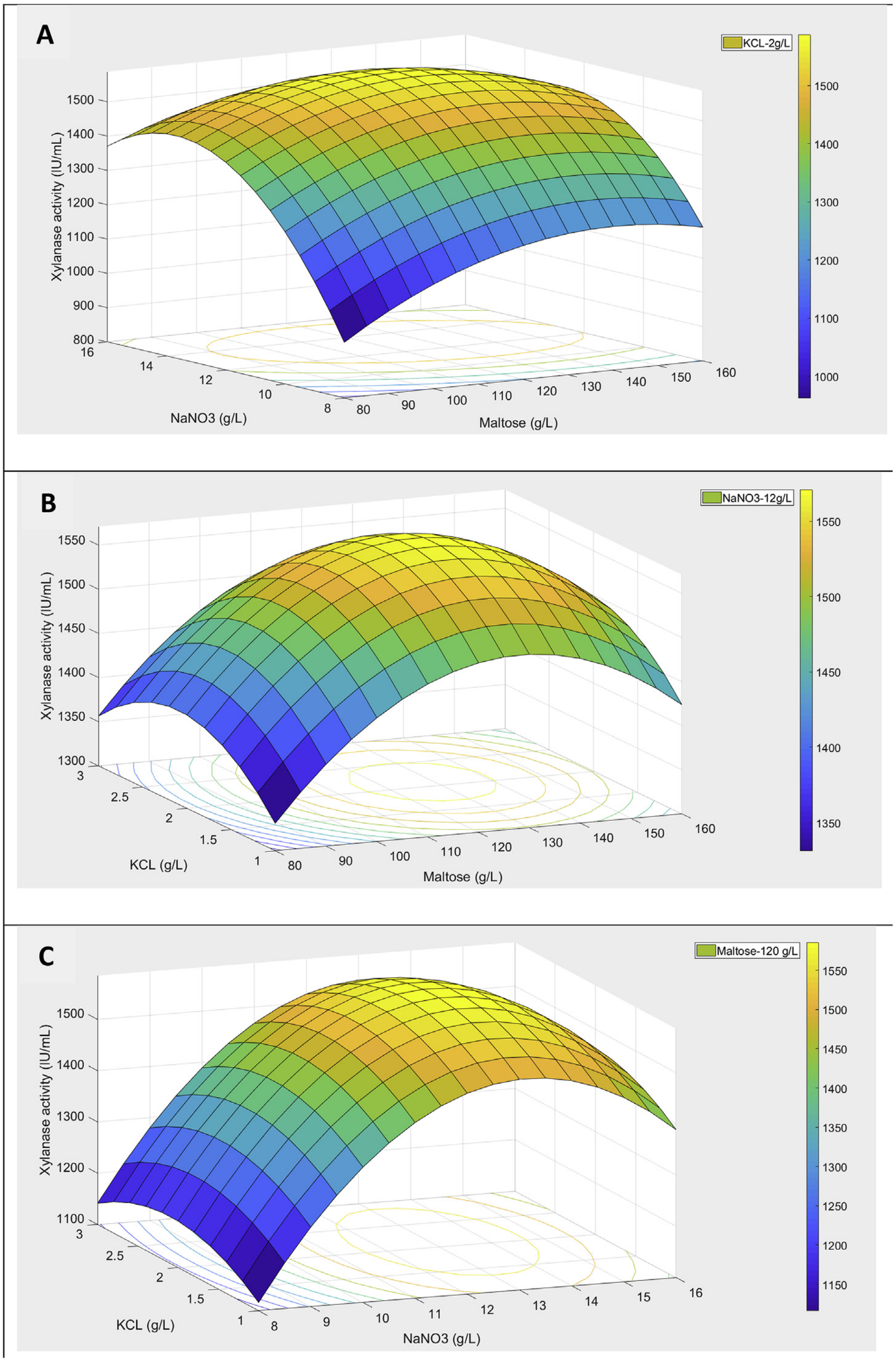

Fig. 4. Response surface curves from Box-Behnken experiments for $NaNO_3$ vs. maltose (A), KCl vs. maltose (B) and KCl vs. $NaNO_3$ (C) for the production of xylanases from recombinant *Aspergillus nidulans*.

the 5% level. Therefore, the quadratic model was selected. A 280% increase in xylanase production compared to the screening media was realized through optimization of the medium components concentrations. Table 6 shows the product concentrations, yields and substrate concentrations for the validation experiment.

3.4. Effect of pyridoxine on enzyme production

3.4.1. Effect of pyridoxine on enzyme production in shake flasks

Previous studies have observed that reducing pyridoxine concentration in enzyme production medium can reduce or eliminate growth of *A. nidulans* A773 while maintaining enzyme production (Müller et al., 2015, 2014; Pardo-Planas et al., 2018, 2017b). Zheng et al. (2015) observed that decreasing riboflavin concentration decreased growth of an auxotrophic *A. nidulans* strain with a riboflavin marker. A strategy of reducing pyridoxine concentration in the optimized medium was examined to determine if it would result in increased xylanase activity.

Fig. 5 shows that absence of pyridoxine resulted in no growth while the addition of pyridoxine in a concentration of 300 μg/mL resulted in a rapid increase in DCW from 0.0 to 20.0 g/L, respectively. Increasing pyridoxine concentration from 300 μg/L to 900 μg/L resulted in smaller

Table 6
Xylanase and substrate concentrations and yields from *Aspergillus nidulans* using the optimum medium as determined using response surface methodology.

Protein concentration (mg/mL)	0.61
Initial glucose concentration (g/L)	10
Initial maltose concentration (g/L)	120
Initial substrate concentration (g/L)	130
Final glucose concentration (g/L)	15
Final maltose concentration(g/L)	9.5
Final substrate concentration (g/L)	24.5
Titer (U/mL)	1,620
Productivity (U/mL/day)	540
Yield (U/g)	12,460
Specific activity (U/mg)	2,564

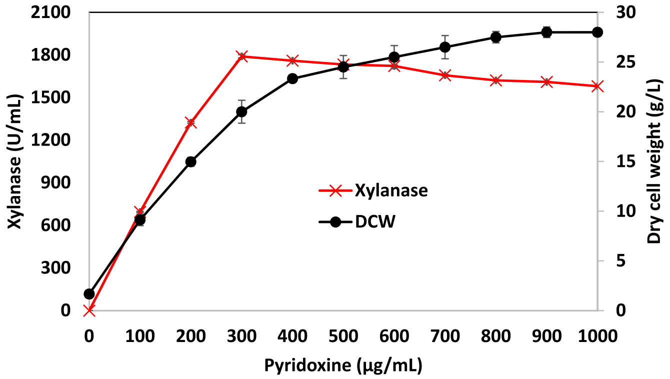

Fig. 5. Effect of pyridoxine concentration (µg/ml) on xylanase enzyme production by *Aspergillus nidulans* in shake flasks.

increases in dry mycelial weight, from 20.0 to 28.0 g/L. DCW was also 28.0 g/L at the maximum pyridoxine concentration of 1,000 µg/L. We observed that color of mycelia was darker in case of pyridoxine concentration of (0, 100 and 200) µg/mL. It started to be light at concentration of 300 µg/mL of pyridoxine. This dark coloration is caused by melanin formation under pyridoxine limitation, which was also observed in previous studies using *A. nidulans* A773 (Müller et al., 2014; Pardo-Planas et al., 2017a). Low cell mass is due to auxotrophic conditions which happened in case of absence of pyridoxine (Mueller, 2012).

Fig. 5 also shows that absence of pyridoxine resulted in no enzyme production since there was no cell growth. Enzyme activity increased rapidly to 1,790 U/mL when pyridoxine concentration was increased to 300 µg/mL. Enzyme activity decreased to 1,580 U/mL as pyridoxine concentration was increased to 1,000 µg/mL, despite an increase in DCW. Therefore, 300 µg/mL pyridoxine was selected as the best pyridoxine concentration for submerged fermentation in bioreactor as the maximum xylanase activity was observed using this pyridoxine concentration.

3.4.2. *Effect of pyridoxine on enzyme production in bioreactor*

Stirred tank bioreactor runs were done using both the original optimized medium with 1,000 µg/mL pyridoxine and optimized medium with 300 µg/mL pyridoxine. Fig. 6 shows the kinetics for the batch fermentation in the bioreactor containing 1,000 µg/mL pyridoxine. The pH was uncontrolled and it increased during the growth phase from the initial value of 5.93–7.16 at 48 h. Then pH decreased in the stationary and death phase from 7.16 to a final value of 6.24 due to release of acidic by-products into the media (Burkert et al., 2005). Other studies also reported the release of organic acids (citric acid, lactic acid and succinic acid) during xylanase production by recombinant *A. nidulans* in shake flasks (Müller et al., 2014; Pardo-Planas et al., 2017b). DCW increased from 3.3 g/L at the beginning of cell cultivation to the maximum 35.0 g/L at 48 h, after which the stationary phase began and DCW decreased to 31.0 g/L at the end of cultivation. DO concentration rapidly decreased in the growth phase from 97% initially to 10% at 24 h and 0.2% at 48 h. This drop is due to cell growth and a rate of oxygen consumption that was greater than the oxygen transfer rate from gas to liquid (Fontana and da Silveira, 2012; Garcia-Ochoa et al., 2010; Ghoshal et al., 2014). DO concentration also decreased because of the rheological changes in the medium caused by the increase in media viscosity due to increase in cell density (Fontana and da Silveira, 2012). DO then slightly increased to a final value of 3%. Protein and xylanase production was observed in the stationary phase and death phase and reached maximum values of 250 µg/mL and 790 U/mL, respectively, at 96 h (Fig. 6B). Schneider et al. (2001) also stated that *Bacillus subtilis* produced xylanase enzyme in the stationary phase. The enzyme activity was lower in the stirred tank bioreactor than that obtained in shake flasks. This is may be due to mycelial mat formation which caused air outlet clogging and a decrease in air supply (Kumar et al., 2009; Garai and Kumar, 2013). The DO concentration was not maintained at 20%, as recommended by Singh et al. (2000) in their study of hemicellulase production by fungi, which likely resulted in reduced enzyme production. Maltose concentration decreased from an initial value of 119.33 g/L to a final value of 19.31 g/L. The glucose concentration increased gradually from an initial value of 9.97 g/L to 19.03 g/l at 48 h, and then decreased again to a final value of 11.93 g/L. This can be explained by hydrolysis of maltose to glucose, which is utilized later by the organism (Müller et al., 2014) (Fig. 6C).

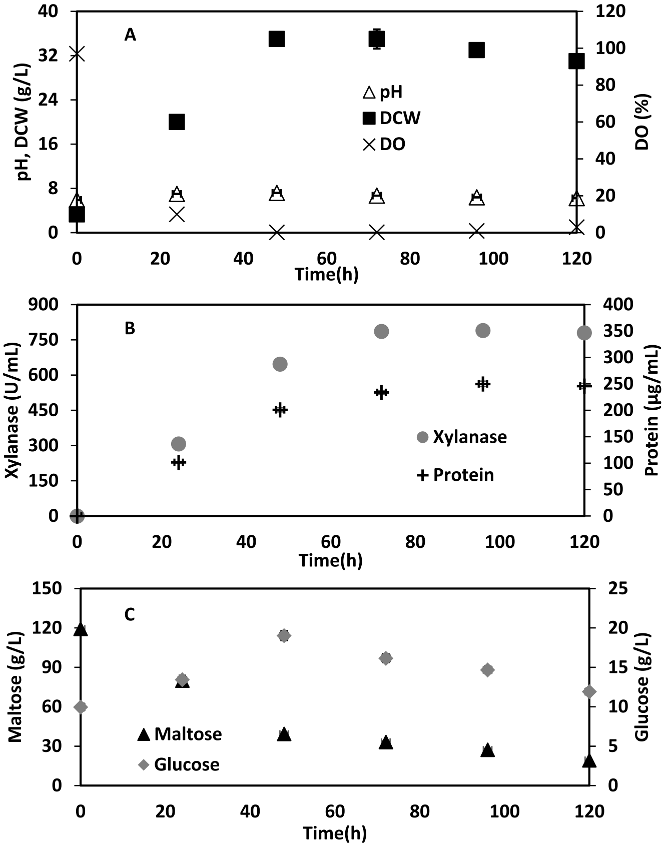

Fig. 6. Batch fermentation kinetics of *Aspergillus nidulans* in a stirred-tank bioreactor with media containing (1000 µg/ml) pyridoxine at 400 rpm and aeration at 1 vvm. Dry cell weight (DCW) concentration, pH and dissolved oxygen (DO) (A); xylanase activity and protein concentration (B); and maltose and glucose concentrations (C) over time are displayed.

Fig. 7 shows the kinetics for the batch cultivation in optimized media containing 300 µg/mL pyridoxine. The pH value increased from the initial value of 5.99–7.00 at 48 h, then decreased to 6.02 at the end

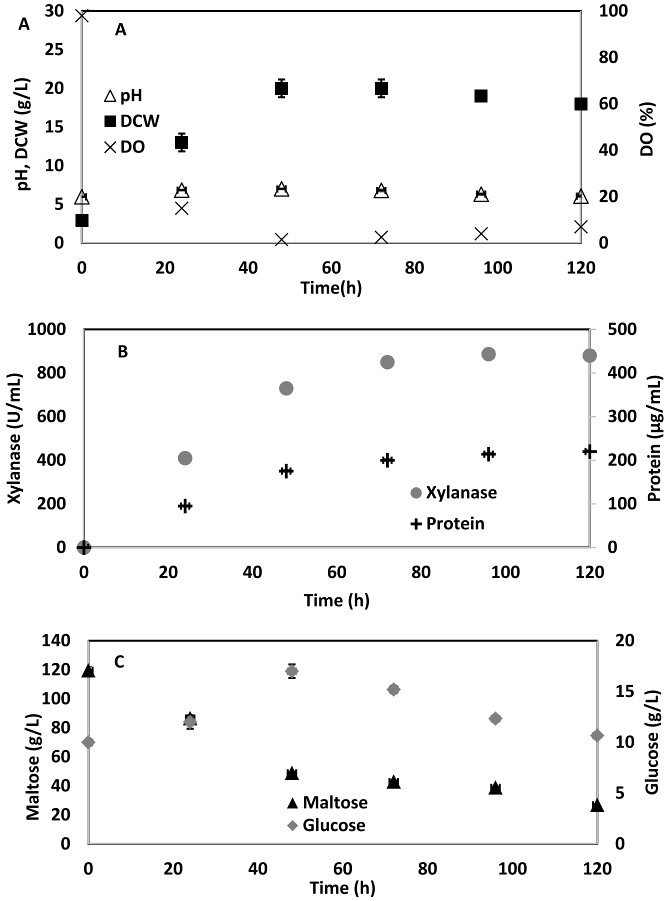

Fig. 7. Batch fermentation kinetics of *Aspergillus nidulans* in a stirred-tank bioreactor with media containing (300 µg/ml) pyridoxine at 400 rpm and aeration at 1 vvm. Dry cell weight (DCW) concentration, pH and dissolved oxygen (DO) (A); xylanase activity and protein concentration (B); and maltose and glucose concentrations (C) over time are displayed.

of cultivation. The pH at the end of cultivation was more acidic than that of the control experiment which contain 1000 µg/mL pyridoxine. This can be explained by an increased release of organic acids (citric acid, lactic acid and succinic acid) due to pyridoxine limitation (Müller et al., 2015; Pardo-Planas et al., 2018). DCW increased from an initial value of 2.9 g/L at beginning of fermentation to reach the maximum value of 20 g/L at 48 h and then decrease slightly to a final value of 18 g/L. The maximum DCW was 43% less than the control experiment (1000 µg/mL) due to lower pyridoxine concentration. DO concentration decreased during the growth phase from an initial value of 98%–15% at 24 h and 1.5% at 48 h. DO then increased to a final value of 7%. Higher DO concentrations were observed during the growth phase than during the control experiment. This is attributed to lower DCW and thus less consumption of dissolved oxygen (Riedel et al., 2013)(Fig. 7A). Xylanase production reached a maximum value of 886 U/mL at 96 h, which was 112% greater than that observed in the control experiment. The specific yield of xylanase activity was 44,300 U/g cells for the reduced pyridoxine medium, which was 207% greater than the yield of 21,300 U/g cells observed in the control experiment. Protein production started at 24 h and increased to reach a maximum of 214 µg/L at 96 h which was lower than that obtained in case of control experiment. The protein produced in the reduced pyridoxine medium had greater activity (4.14 U/µg) than in the control (3.16 U/µg) (Fig. 7B). Maltose concentration decreased from an initial value of 119.67 g/L at a final value of 27.00 g/L. Glucose concentration increased from an initial value of 10.00 g/L to 17.00 g/L at 48 h, then decreased to a final value of 10.67 g/L. Substrate consumption was lower than that in the control due to the decrease in fungal growth by using low pyridoxine concentration (Müller et al., 2014) (Fig. 7C). Xylanase yield on sugar ($Y_{P/S}$) was 9,785 U/g maltose for the reduced pyridoxine medium and 8,642 U/g maltose for the control medium.

3.5. *Comparison of optimization of xylanase production by recombinant Aspergillus nidulans with other fungi*

The recombinant *A. nidulans* strain used in this study produced higher amounts of xylanase activity after medium optimization than most other organisms reported in literature. Another *A. nidulans* strain modified using the pEXPYR vector that expressed xylanase from *Penicillium funiculosum* achieved a maximum xylanase activity of 43.3 U/mL (Müller et al., 2015) with a maximum yield of 781 U/g maltose after 96 h; however, no medium optimization was conducted for this strain. *Streptomyces thermovulgaris* achieved 274 U/mL xylanase activity and with a yield of 10,000 U/g rice straw using central composite design method of optimization (Chaiyaso et al., 2011). Ye et al. (2013) optimized a medium and cultivation for a fungus named “Xw2” using methods similar to this study, which resulted in a maximum activity of 363 U/ml with no yield reported. Naz et al. (2017) used Box-Behnken design to optimize production of xylanase using *Bacillus subtilis* and achieved an activity of 295 U/mL. More recently, Simair et al. (2018) optimized production of xylanase from *Bacillus cereus* TH-050 and achieved an activity of 5,991 U/mL from a xylan-based medium.

4. Conclusions

A linear model was calculated using a Plackett-Burman design to select medium components that had a significant effect ($p < 0.05$) on xylanase production. Maltose (the inducer for xylanase expression), KCl and $NaNO_3$ were the most significant factors. A second-order quadratic model was then used to calculate the optimum medium component concentrations for maximum xylanase production. The optimum concentration for xylanase production were determined to be 120 g/L maltose, 12 g/L $NaNO_3$ and 2 g/L KCl. The xylanase activity obtained using the optimized medium was 1,620 U/mL (12,460 U/g substrate), which was 280% greater than the maximum level obtained with basic medium. This work also made it possible to understand the effect of pyridoxine level on growth and xylanase enzyme production by recombinant *A. nidulans*. Diluted pyridoxine concentrations increased enzyme production by 112% while controlling the growth rate of the strain at 57% of the rate observed with standard 1 mg/L pyridoxine media and thus prevents clogging of bioreactor.

Declarations of interest

None.

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