ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 05, pp.50212-50221, May, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

OPTIMIZATION OF B-GLUCOSIDASE PRODUCTION BY *ASPERGILLUS NIGER* IN SOLID-STATE FERMENTATION USING RESPONSE SURFACE METHODOLOGY

***Ilyes Dammak, Sonia Khoufi and Sami Sayadi**

Environmental Bioprocess Laboratory, Center of Biotechnology of Sfax, BP"1177", Sfax 3018, Tunisia

ARTICLE INFO

Article History:
Received 27th February, 2017
Received in revised form
15th March, 2017
Accepted 24th April, 2017
Published online 23rd May, 2017

Key words:
Wheat bran,
Fungal enzyme,
Fractional factorial,
Box-Behnken,
Substrate,
Enzyme.

ABSTRACT

Sequential optimization strategy, based on statistical experimental design, was employed to improve the production of β-glucosidase by *Aspergillus niger* ATCC 16404 using solid-state fermentation (SSF) technique. Different nitrogen sources were tested for *A. niger* culture using wheat bran as solid medium. The highest enzyme production per kg of dry substrate (DS) (1.65×10^5 U/kg DS) was obtained with soybean peptone. By using fractional factorial design, urea, calcium chloride, soybean peptone concentration and inoculum spore number were selected as parameter with high significant effect on β-glucosidase production. Optimum process conditions were evaluated by response surface methodology (RSM). In this respect, the Box-Behnken design was employed. The optimum conditions were found to be 0.095 kg/kg DS for urea, 0.025 kg/kg DS of calcium chloride, 0.119 kg/kg DS of soybean peptone and inoculum spore number of 8.5×10^{11} spore/kg DS to achieve a β-glucosidase production yield of about 2.87×10^5 U/kg DS after 7 days of SSF at temperature 30°C.

Citation: Ilyes Dammak, Sonia Khoufi, Sami Sayadi, 2017. "Optimization of β-glucosidase production by *Aspergillus niger* in solid-state fermentation using response surface methodology", *International Journal of Current Research*, 9, (05), 50212-50221.

INTRODUCTION

β-glucosidases (EC 3.2.1.21; β-D-glucopyranosideglucohydrolases) are enzymes that hydrolyze the β-glucosidic linkages between glucose and aglycon groups, such as those comprising the norisoprenoids, volatile phenols and other benzyl derivatives, aliphatic alcohols, sesquiterpenes, resveratrol and monoterpenes as found in grape musts and other fruit juices, as well as short chain oligosaccharides and disaccharides (Bhatia, 2002). These enzymes, ubiquitous among microorganisms (bacteria, yeasts and fungi) and also present in different fruits including grapes (Tate, 2006), find potential applications in (1) flavor enhancement of fruit juices and wine through the release of aroma rich compounds from natural non-odorous and non-volatile glycoside precursors (Daenen, 2008), (2) synthesis of glucosides and fucosides with various potential applications in pharmaceutical, cosmetic and detergent industries (Zhang*et al*., 2007), (3) hydrolytic removal of aglycone moiety from flavanoid and isoflavanoid glycosides (Ribeiro, 2007), and (4) biosynthesis of oligosaccharides (Bruins, 2003). The application of β-glucosidases at industrial scale requires an effective process for the production of high amounts of enzyme with low cost.

****Corresponding author:*** **Ilyes Dammak,**
Environmental Bioprocess Laboratory, Center of Biotechnology of Sfax, BP"1177", Sfax 3018, Tunisia.

This could be obtained from either submerged or solid-state fermentation (SSF) techniques. SSF appeared as an interesting alternative method for enzymes production (Couto, 2005). This kind of bioprocess offers advantages over submerged fermentation, especially for the fungal cultures, as there is higher productivity per unit volume, reduced energy requirements, lower capital investment, low wastewater output, and low downstream processing cost (Hölker, 2004). The selection of an adequate support for performing SSF is essential step, since the success of the process depends on it. Agricultural residues such as grasses, tree wastes and many other green plants whose disposal is considered as an environmental problem can represent large renewable resources for enzyme production by filamentous fungi (Pandey, 2002). The simplicity of filamentous fungi cultivation and desired physicochemical properties of produced enzymes, gives them advantages over the other living organisms. Certain fungi, such as *Aspergillus* and *Trichoderma*, are almost used at the industrial scale for food processing, regarding their recognized safety for human health (Hu *et al*., 2008). The optimization of enzyme production has the most common goals of minimizing cost, maximizing production, and process efficiency. This is one of the major quantitative tools in industrial decision-making. A factorial design of experiments by response surface methodology (RSM), that employed variables that had the most influence in enzyme production and analysis, stands as a better approach for improving enzyme production because it determines the interactions between

theselected fermentation parameters (Tavares *et al*., 2006). In determining the optimum conditions of many processes, the RSM is one of the most useful statistical approaches. It makes the optimization process more efficient and effective by exploring the response surfaces covered in the experimental design (Montgomery and Mastrangelo 1991). RSM has been confirmed to be a useful statistical technique for the investigation and optimization of complex reaction conditions. In the current study, extracellular β-glucosidase production by *Aspergillus niger* ATCC 16404 in wheat bran-based solid medium has been optimized using experimental designs. A screening of the most essential factors is achieved with a fractional factorial design, as the first step. In a second step, Box-Behnken design is employed to analyze response surfaces as a function of some of the important parameters and then to select the optimal solid-state conditions for the production of β-glucosidase.

MATERIALS AND METHODS

Substrate and chemicals

This study employed wheat bran purchased from a local market as a support substrate for SSF. The wheat bran sample had an average particle size of 1-5 mm. The wheat bran had a chemical composition that (% w/w) was as follows: water content 16; total carbohydrates44.6 (composed of hemicelluloses (notably arabinoxylans (30%), cellulose (10-15%), and starch (10-20%)), proteins 6.12; minerals 3.46, fat content 0.39 and other components 29.4 (such as lignin and cutin) (Beaugrand*et al*., 2004; Maes, 2001). In this study, all the chemicals used were of analytical grade; sodium acetate, sodium citrate, calcium chloride, urea and magnesium sulfate were purchased from Merck (Darmstadt, Germany); p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenol (pNP) and soybean peptone from Sigma-Aldrich (ChemieGmbh, Germany).

Microorganism

Aspergillus niger ATCC 16404 was acquired from the collection of microorganisms in the Center of Biotechnology of Sfax (Tunisia). It was propagated on potato dextrose agar (PDA) slants at room temperature with periodic transfer (after 7 days). Inoculum was prepared on 250 mL flask with 40 mL PDA medium. 50 mL Tween 80 (0.05% v/v) was added after 3 days of incubation at 30°C. The spores were suspended under agitation using amagnetic stirrer and then counted in a Malassez chamber (Maes, 2001). Stock cultures were retained in spore suspension frozen in 25% (v/v) glycerol for short-term storage before inoculation of medium.

Solid medium preparation

The experiments were performed in 250 mL flasks with 100 g of wheat bran moistened with the required volume of buffer (sodium acetate or citrate phosphate buffer 20 mM, pH 4.0) containing various nutritional factors according to the experimental designs shown in Table 1. The medium was sterilized at 121°C for 20 min. After cooling, the substrate was inoculated with inoculum sizes according to Table 2 obtained from spore suspensions prepared as described above. The contents were then mixed thoroughly, and then the mediums were incubated statically in complete darkness at 30°C for 1 week.

Fungal enzyme preparation

The enzyme was extracted with sodium acetate or citrate-phosphate buffer 50 mM, pH 4.0 (20 mL buffer/g substrate) by shaking the mixture for 1 h at 160 rpm at room temperature. The suspension was filtered and centrifuged at 4°C, centrifugation force 8,000 *g* for 20 min. The suspension was filtered and centrifuged and the supernatant was used in enzyme assays

β-glucosidaseactivity assay

β-glucosidase activity was determined according to Norkrans(1950), by measuring the hydrolysis of p-nitrophenyl β-D-glucoside (pNPG). The reaction was initiated by adding 0.1 136 mL of pNPG solution (5 mM) and kept for 10 min at 50°C, then the reaction was stopped by the addition of 2 mL of sodium carbonate solution (1 M, pH 10.8). The absorbance was measured at 405 nm using spectrophotometer UV/Vis Spectronic (Thermofisher, France), and translated to µmol of p-nitrophenol (pNP) using a standard graph prepared under the same conditions. The β-glucosidase activity was expressed as enzyme unit (U) per kg of dry substrate. One unit of β-glucosidase activity is defined as the amount of enzyme required to release 1µmol of pNP per min under the above assay conditions.

Protein assay

The Bradford method was used to quantify the proteins content (Bradford, 1976) and bovine serum albumin BSA (Sigma Aldrich) was used as a standard.

Medium optimization

The optimization of β-glucosidase production's yield in solid-state medium has been carried-out in two steps as mentioned subsequently.

Screening of essential cultural factors using fractional factorial design

For systems with a great number of variables, different approaches of experimental factorial designs can be applied to achieve a screening of critical variables and to estimate their main effects on the responses (Cela *et al*., 2009; Lewis, 1998). For this study, the fractional factorial experimental (28//32) design was used to find out the most significant factors of medium components affecting β-glucosidase production by *A. niger* under SSF. It allows the investigation of eight factors tested at two different levels in 32 experimental runs (Table 1). The following parameters were tested: initial moisture content, inoculum size, urea, soybean peptone, calcium chloride, magnesium sulfate concentrations, and wheat bran particle size. The medium composition varied according to Table 2. The choice of the given levels to each factor was based on previous literature works and preliminary experiments (Daroit *et al*., 2007). From the 32 experimental runs, we could estimate the significance of each factor, using the method of least square (Job *et al*., 2010). After this screening step, the process variables that did not have a significant effect on β-glucosidase production (tested by applying *t*-test) were screened out. The remaining factors affecting β-glucosidase production were further optimized using RSM.

Table 1. Experimental ranges for the fractional factorial screening design

Factors	Levels	
	Low level (-)	High level (+)
Initial moisture content (%) (A)	60	80
Urea (kg/kg DS) (B)	0.05	0.09
Inoculum size(spore/kg DS) (C)	5×10^{7}	200×10^{7}
Moisturizing agent* (-) (D)	SCB[1]	SAB[2]
$CaCl_2$ (kg/kg DS) (E)	4.5×10^{-3}	13.4×10^{-3}
$MgSO_4$ (kg/kg DS) (F)	4.5×10^{-3}	13.4×10^{-3}
Soybean peptone (kg/kg DS) (G)	0.05	0.09
Wheat bran particle size (m) (H)	$<10^{-3}$	$> 2\times10^{-3}$

*50 mM, pH 4.0
[1] SCB: sodium acetate buffer
[2] SAB: sodium citrate buffer

Table 2. Experimental conditions of the fractional factorial screening design and the corresponding experimental β-glucosidase production responses

Run no.	Moisturizing agent	Initial moisture content (%)	Urea (kg/kgDS)	Calcium chloride (kg/kgDS)	Magnesium sulfate (kg/kgDS)	Soybean peptone (kg/kgDS)	Wheat bran particle size (m)	Inoculum size (spore/kgDS)	Experimental β-glucosidase productions (U/kgDS)
1	SAB	0.6	0.05	13.4×10^{-3}	4.5×10^{-3}	0.09	$> 2\times10^{-3}$	5×10^{7}	6.481×10^{4}
2	SCB	0.8	0.05	4.5×10^{-3}	13.4×10^{-3}	0.09	$<10^{-3}$	5×10^{7}	4.879×10^{4}
3	SCB	0.6	0.05	4.5×10^{-3}	4.5×10^{-3}	0.05	$<10^{-3}$	5×10^{7}	8.307×10^{4}
4	SAB	0.6	0.05	4.5×10^{-3}	13.4×10^{-3}	0.09	$<10^{-3}$	200×10^{7}	1.071×10^{5}
5	SAB	0.6	0.09	4.5×10^{-3}	13.4×10^{-3}	0.05	$> 2\times10^{-3}$	5×10^{7}	5.845×10^{4}
6	SCB	0.8	0.09	4.5×10^{-3}	13.4×10^{-3}	0.05	$<10^{-3}$	200×10^{7}	6.3×10^{4}
7	SAB	0.6	0.09	13.4×10^{-3}	4.5×10^{-3}	0.05	$<10^{-3}$	200×10^{7}	2.464×10^{5}
8	SCB	0.6	0.05	13.4×10^{-3}	13.4×10^{-3}	0.05	$> 2\times10^{-3}$	200×10^{7}	2.089×10^{5}
9	SAB	0.8	0.09	13.4×10^{-3}	13.4×10^{-3}	0.09	$> 2\times10^{-3}$	200×10^{7}	2.034×10^{5}
10	SCB	0.8	0.05	13.4×10^{-3}	4.5×10^{-3}	0.09	$<10^{-3}$	200×10^{7}	9.921×10^{4}
11	SCB	0.6	0.09	13.4×10^{-3}	13.4×10^{-3}	0.09	$<10^{-3}$	5×10^{7}	8.336×10^{4}
12	SCB	0.6	0.09	4.5×10^{-3}	4.5×10^{-3}	0.09	$> 2\times10^{-3}$	200×10^{7}	2.044×10^{5}
13	SCB	0.8	0.09	13.4×10^{-3}	4.5×10^{-3}	0.05	$> 2\times10^{-3}$	5×10^{7}	5.779×10^{4}
14	SAB	0.8	0.05	13.4×10^{-3}	13.4×10^{-3}	0.05	$<10^{-3}$	5×10^{7}	7.464×10^{4}
15	SAB	0.8	0.09	4.5×10^{-3}	4.5×10^{-3}	0.09	$<10^{-3}$	5×10^{7}	3.864×10^{4}
16	SAB	0.8	0.05	4.5×10^{-3}	4.5×10^{-3}	0.05	$> 2\times10^{-3}$	200×10^{7}	6.8×10^{4}
17	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	9.013×10^{4}
18	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	9.786×10^{4}
19	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	9.81×10^{4}
20	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	7.407×10^{4}
21	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	6.628×10^{4}
22	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	9.285×10^{4}
23	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	9.792×10^{4}
24	SCB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	5.386×10^{4}
25	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	5.886×10^{4}
26	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	8.664×10^{4}
27	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	4.878×10^{4}
28	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$<10^{-3}$	100×10^{7}	4059×10^{4}
29	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	7529×10^{4}
30	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	7.057×10^{4}
31	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	5.336×10^{4}
32	SAB	0.7	0.07	8.95×10^{-3}	8.95×10^{-3}	0.07	$> 2\times10^{-3}$	100×10^{7}	8.964×10^{4}

Optimization of selected factors using response surface methodology

The screening data revealed four factors (inoculum size, calcium chloride, soybean peptone and urea concentrations) affecting the β-glucosidase production. We achieved the optimization of β-glucosidase production by employing the response surface methodology (RSM).The response surfaces covered in the experimental design is explored by this such as Hoke design designs, *D*-optimal designs, Box-Behnken designs and others, approach, thereby making the optimization process more effective and efficient (Box, 1978). The central composite are the most frequent designs in optimization problems involving three or more factors (Sarabia, 2009). Box-Behnken design is suitable to discover curvatures in a multi-dimensional space but involve a large number of experiments that exceed three factors. To obtain the best settings for the four selected factors, we applied the Box-Behnken design in a hypercube experimental region (Rosales, 2007). The design comprised of 29 experiments where each parameter was tested at three levels and in multiple combinations with the other parameters (Table 3).The whole set of experiments was carried out in triplicate

and mean response was employed for more analyses. A second-order polynomial model was then fitted into the experiment data using the experimental design software Design-Expert® 8.0.6 (Stat-Ease Inc., Minneapolis, MN).

The following second order polynomial model adequately describes β-glucosidase production for easy prediction of β-glucosidase production in the experimental surface,

$$\hat{y}= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + +\beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_1X_4 + \beta_{34}X_3X_4+\beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 \quad (1)$$

Where
$\hat{y}$:predictedβ-glucosidase production response (U/kg DS)
X_j: coded variables (-)
β_0:predictedβ-glucosidase production in the center of experimental surface (U/kg)
β_j:model coefficient of main factor effect (-)
β_{jk}: model coefficient of interaction effect between 2 factors(-)
β_{jj}: model coefficients for cross-interactioneffect(-)
X_j are the coded variables relating the natural variables U_j by the following equation,

$$X_j = (U_j - \text{center [j]}) / (\text{step of variation [j]}) \quad (2)$$

where

$$\text{center [j]} = (U_{j,high} - U_{j,low})/ 2 \quad (3)$$

$$\text{step of variation [j]} =(U_{j,high} + U_{j,low}) / 2 \quad (4)$$

$U_{j,high}$ and $U_{j,low}$ are the two extreme levels (highest and lowest) for each given natural variable U_j. The coded variables$X_j$are equal to (-1) and (+1) when the levels of natural variable U_j are $U_{j,low}$ and $U_{j,high}$, respectively (Table 3).The experimental results of β-glucosidase production (y_i) for an experiment (i) could be assumed as the average value of β-glucosidase production and the systematic experimental error

$$y_i = \eta_i + e_i \quad (5)$$

where
η_i : experimental β-glucosidase production (U/kgDS)
e_i : systematic experimental error (U/kgDS)

The model coefficients β_0, β_j, β_{jk} and β_{jj} are estimate dusing the least squares fitting of the estimated results to the experimental results obtained in the 24 design points of the four factors of Box-Behnken experimental design (Table 4).Four additional experiments (runs 25 to 29) were also added to subsequently check the validity of the fitted model before a predictive use of it in the studied context (Baati, 2001). The fitted model was used to evaluate the relative sensitivity of the response to the variables in the entire study context and to find the best experimental conditions. The relationships between the experimental β-glucosidase production responses and the variables (X_1, X_2, X_3, X_4) are illustrated graphically by plotting the response surfaces curves and the overlaid contour plots.

RESULTS AND DISCUSSION

Many local agro-industries byproducts have been examined based on their capacity to produce extracellular β-glucosidase for potential industrial application (Tan, 2012; Vervoort *et al*., 2016). Based on our first investigation, wheat bran was chosen as solid substrate to conduct experiments. Due to the important role of culture condition factors such as the initial moisture content and nitrogen source, we have decided to pick the best condition of these two factors.

Effect of initial moisture content

Initial moisture content has a major function in fungus growth in SSF. Indeed, it directly affects the germination of spores, mycelia growth and the making of enzymes (Gervais, 2003). Therefore, *A. niger* was cultivated on a solid medium containing only wheat bran with initial moisture contents ranging from 40 to 90% which includes the water content of wheat bran (0.12 kg/kg DS) and the water from the suspension of spores inoculum. The initial moisture content of the culture medium was adjusted by using sodium acetate buffer (pH 4.0). Fig. 1 shows the making of β-glucosidase as a function of initial moisture contents. Indeed, the initial moisture content of 60% provides a better cultural condition for β-glucosidase production, with 1.57×10^5 U/kg of dry substrate (DS) successfully obtained after7 days of static culture. However, we noted a swift decline of β-glucosidase production for initial moisture content values higher than 60%. Elyas *et al*. (2010) also showed this fact with the extracellular β-glucosidase of *Aspergillus*-AS 58 cultivated in wheat bran as solid substrate. Similarly, Ellaiah *et al*. (Ellaiah *et al*., 2002) indicated that an initial moisture content of 60% is optimal for the making of glucoamylase by using newly isolated *Aspergillus* species.

Effect of nitrogen source

The effect of nitrogen source on β-glucosidase production by *A. niger* after 7 days of static culture in wheat bran is shown in Fig. 2a. The maximum β-glucosidase production was obtained when soybean peptone was used as the nitrogen source added to the prepared solid medium, which yielded a β-glucosidas production of 2.25×10^5 U/kg DS. These could be explained to be the result of the C/N ratio of wheat bran sample (18/1), which is insufficient for fungus growth. According to López *et al*. (2003), the recommended C/N ratio for fungus growth is about (20/1). Besides, Lahoz *et al*. (1983) recommend nearly (29/1) for a better *A.niger* growth and primary metabolites production.

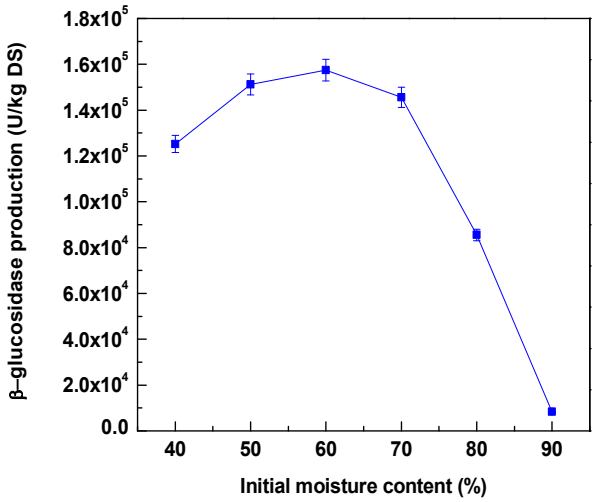

Fig.1. Effect of initial moisture content on β-glucosidase production by *A.niger* ATTC-16404 in solid substrate with temperature, 30°C and pH, 4.0

Table 3. Experimental ranges for the Box-Behnken design

Variables	Factors	Coded level		
		-1	**0**	**+1**
X_1	Inoculum size (spore/kg DS)	10^9	5×10^{11}	10^{12}
X_2	Calcium chloride (kg/kg DS)	7×10^{-3}	16×10^{-3}	25×10^{-3}
X_3	Urea (kg/kg DS)	0.07	0.105	0.140
X_4	Soybean peptone (kg/kg DS)	0.07	0.105	0.140

Table 4. Experimental conditions of the Box-Behnken design and the corresponding experimental β-glucosidase production response

Exp. run	Inoculum size (spore/kg DS)	Calcium chloride (kg/kg DS)	Urea (kg/kg DS)	Soybean peptone (kg/kg DS)	Experimental β-glucosidase productions (U/kg DS)
1	10^{12}	7×10^{-3}	0.105	0.105	1.124×10^5
2	10^9	25×10^{-3}	0.105	0.105	0.943×10^5
3	5×10^{11}	7×10^{-3}	0.105	0.140	1.305×10^5
4	5×10^{11}	16×10^{-3}	0.07	0.140	1.738×10^5
5	5×10^{11}	25×10^{-3}	0.07	0.105	1.61×10^5
6	5×10^{11}	25×10^{-3}	0.140	0.105	0.776×10^5
7	10^{12}	16×10^{-3}	0.105	0.07	0.852×10^5
8	5×10^{11}	16×10^{-3}	0.140	0.07	0.629×10^5
9	5×10^{11}	16×10^{-3}	0.07	0.07	0.714×10^5
10	10^{12}	16×10^{-3}	0.07	0.105	0.938×10^5
11	10^{12}	25×10^{-3}	0.105	0.105	1.924×10^5
12	5×10^{11}	25×10^{-3}	0.105	0.140	1.429×10^5
13	10^9	16×10^{-3}	0.105	0.07	1.129×10^5
14	10^9	16×10^{-3}	0.140	0.105	0.929×10^5
15	5×10^{11}	16×10^{-3}	0.105	0.105	1.686×10^5
16	5×10^{11}	7×10^{-3}	0.140	0.105	0.986×10^5
17	10^9	16×10^{-3}	0.07	0.105	1.186×10^5
18	5×10^{11}	7×10^{-3}	0.07	0.105	1.838×10^5
19	10^9	16×10^{-3}	0.105	0.140	1.405×10^5
20	5×10^{11}	25×10^{-3}	0.105	0.07	1.614×10^5
21	10^{12}	16×10^{-3}	0.140	0.105	0.933×10^5
22	10^9	7×10^{-3}	0.105	0.105	1.361×10^5
23	5×10^{11}	7×10^{-3}	0.105	0.07	1.2×10^5
24	5×10^{11}	16×10^{-3}	0.140	0.140	0.7×10^5
25	5×10^{11}	16×10^{-3}	0.105	0.105	1.767×10^5
26	5×10^{11}	16×10^{-3}	0.105	0.105	1.705×10^5
27	5×10^{11}	16×10^{-3}	0.105	0.105	1.905×10^5
28	5×10^{11}	16×10^{-3}	0.105	0.140	1.638×10^5
29	5×10^{11}	16×10^{-3}	0.105	0.105	1.952×10^5

Time progression of β-glucosidase production by *A. niger*

Fig. 2b demonstrated the time progression of β-glucosidase production by *A. niger* under static culture in wheat bran. The production of β-glucosidase was not significant until the third day of culture, which coincided with the rapid establishment of vegetative growth throughout the solid substrate. Afterwards, the spores of *A. niger* started to form mycelium and the activity of β-glucosidase increased rapidly. The β-glucosidase production in wheat bran supplemented with soybean peptone, showed a maximum level of about 2.25×10^5 U/kg DS after 7 days of static culture (Fig. 2b), followed by a slight decrease of β-glucosidase production, which could be attributed to the proteolysis of β-glucosidase by the act of self-produced proteases released by *A. niger* during its growth on wheat bran. In addition, a slight increase of β-glucosidase production was observed after 10 days of static culture, this could result from the cell lysis of the mycelium at the end of the cell cycle and release of endocellular β-glucosidase in the medium (Woodward, 1982). According to these results, the β-glucosidase production by *A. niger* needed to be optimized by varying the other culture factors. Therefore, the experimental statistical approach has been adopted. Firstly, a screening (fractional factorial) design was applied to choose the most important culture factors affecting β-glucosidase production. Secondly, the selected culture factors have been optimized using RSM for optimal β-glucosidase production by *A. niger*.

Determination of significant factors

Table 5 reports the coefficient values (the weight associated to each factor level) calculated, as described above, and significance analyzed using *t*-test. These outcomes are illustrated by the histograms shown in Fig. 3, which represent the differential effects of each factor when putting into consideration the two different levels taken two by two. Asdisplayed in Table 5 and Fig. 3, the inoculum size, the initial moisture content, and calciumchloride concentration exhibits

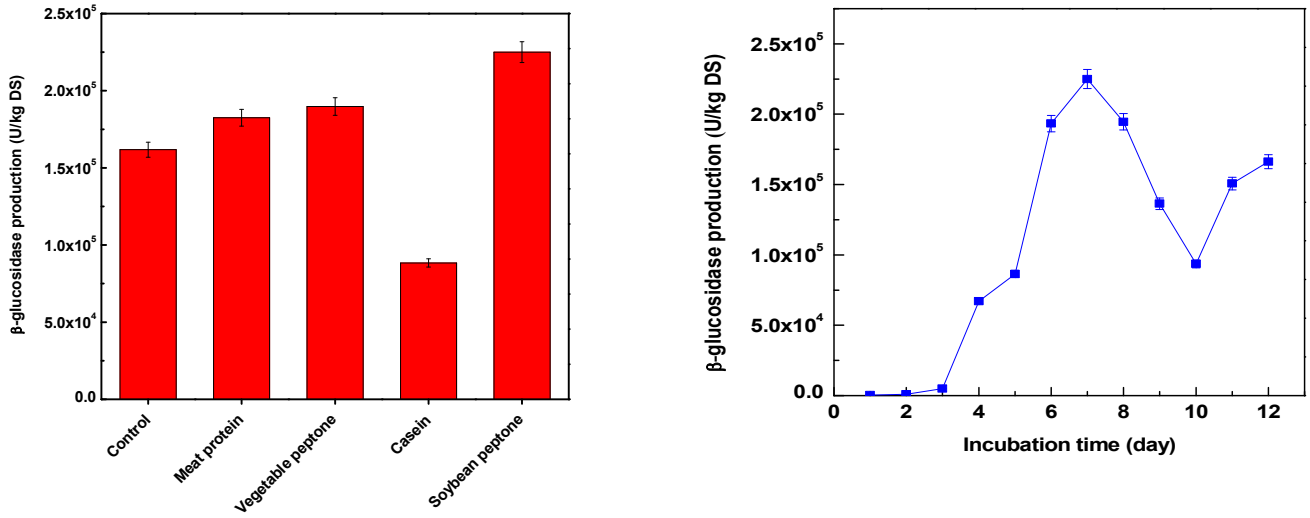

Fig. 2. (a) Comparison of β-glucosidase production by *Aspergillus niger*ATCC-16404 grown on wheat bran, added with different organic nitrogen sources. Initial moisture content was fixed at 60% (w/w). (b) Time course of β-glucosidase production by *Aspergillus niger*ATCC-16404 grown on wheat bran added with soybean peptone (initial moisture content, 60%; temperature, 30°C; pH 4.0)

considerable effects on the β-glucosidase production compared to other factors. A main parameter needed in controlling the growth of microorganism and production of metabolite in SSF is the initial moisture content(Farinas, 2015).In this study, the utmost β-glucosidase productions were achieved when the initial moisture content was 60%. Consequently, the initial moisture content was fixed at 60% in the optimization step of this study. Urea concentration (B), magnesium sulfate concentration (E), buffer type (F), soybean peptone concentration (G), and substrate particle size (H) seem to have no significant effect on the response. However, it was chosen toinclude the two factors: urea and soybean peptone concentrations. First, urea and soybean peptone have a relatively high positive effects on the response (Fig. 2a). Second, the role of both nitrogen sources in the conservation of the protein structures and the stabilization of the activities of several enzymes has been well-documented (Zhang *et al*., 2007; Sharma *et al*., 2015; Yang *et al*., 2016). In the same way, many reports have been given about the effect of inoculum size in fungal growth and productivity (Jain *et al*., 2015; Ravichandra*et al*., 2016; Tasar, 2016). Low growth and productivity results when the inoculum concentration is too high or low. Alonger cultivation time is needed when the inoculum size is small. Crowded and nutritional deficiency rapidly forms when there is a large size of inoculum in the culture. Not long, a mycelium mat covers the culture medium and causes poor substrate aeration.The significant and interaction effects are those that exceed the limit of the Student test (equal to 2.16 with a confidence level of 95% (Fig. 3)).Table 5 shows a significant ($p > 0.05$)positive effect of the inoculum size and calcium chloride concentration. Elyas *et al*. (Elyas *et al*., 2010b) described similar results on the production of β-glucosidase by *Aspergillus* SA-58. Conversely, a significant ($p > 0.05$) negative effect was observed with initial moisture content ranging from 60 to 80% (w/w). Rodríguez *et al*. (1986) also obtained similar results. The authors recommended optimum initial moisture content of 56% for *A. niger*growth in a packed bed bioreactor.From the obtained results, it was revealed that three static culture factors are the most important in β-glucosidase production: the inoculum spore size, the initial moisture content and the calcium chloride concentration. Based on previous observations, we have set the initial moisture content at 60% for optimizing the factors urea, calcium chloride,soybean peptone concentrations and inoculum size.

Optimization design

According to the results of the fractional factorial design, non-significant factors are set at their best levels. In order to optimize β-glucosidase production, we have employed RSM. The experimental design chosen was the Box-Behnken plan, for four factors. Factor levels were selected according to theoutcomes of previous results and prior experiments. The operating conditions of the 29 experimental ran in Box-Behnken design are shown in Table 4.The experience in the center points was repeated 5 times (runs 25, 26, 27, 28 and 29) to estimate the systematic experimental error. β-glucosidase production measured after 7 days of static culture are displayed in the last column of Table 4. A second order polynomial model was setup to determine the relationship between the four factors (calcium chloride, urea, soybean peptone concentrations, and inoculum spore number) and the response.

The resulting estimated model, expressed in coded variables is

$$\hat{y} = -72051{,}8 - 9{,}072\times10^{-6}X_1 + 294{,}5\,X_2 + 939{,}45\,X_3 + 788{,}01\,X_4 + 6{,}78\times10^{-7}X_1X_2 + 3{,}61\times10^{-8}X_1X_3 + 7{,}3\times10^{-8}\,X_1X_4 + 0{,}15\,X_2X_3 - 2{,}3\,X_2X_4 - 1{,}94X_3X_4 - 1{,}24\times10^{-14}X_1^2 - 11{,}35\,X_2^2 - 3{,}95\,X_3^2 - 2{,}54\,X_4^2 \quad (6)$$

Statistical analysis and validation of the model

The importance of the model equation was estimated by the analysis of variance (ANOVA) using Fisher test (Table 6). The significance describes the factors' effects on β-glucosidase production when changing factors' levels based on a reference state. This analysis showed that the regression is statistically significant at a confidence level of 95%. The sum of squares that resulted from lack of fit was then evaluated with 10 degrees of freedom. The validity of the obtained model was verified by the Fisher test:

$$F_{exp} = \frac{8{,}32\times10^{7}/10}{5{,}74\times10^{6}/4} = 5{,}8 < F_{0,05}(10,4) = 5{,}96 \quad (7)$$

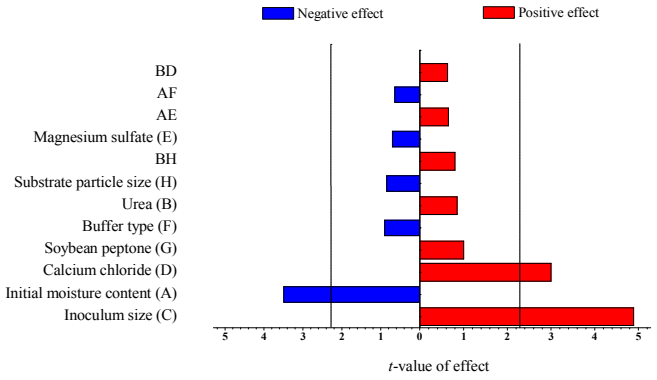

Fig. 3. Graphical study of the effects of different tested variables on β-glucosidase production in wheat bran after 7 days of solid-state fermentation

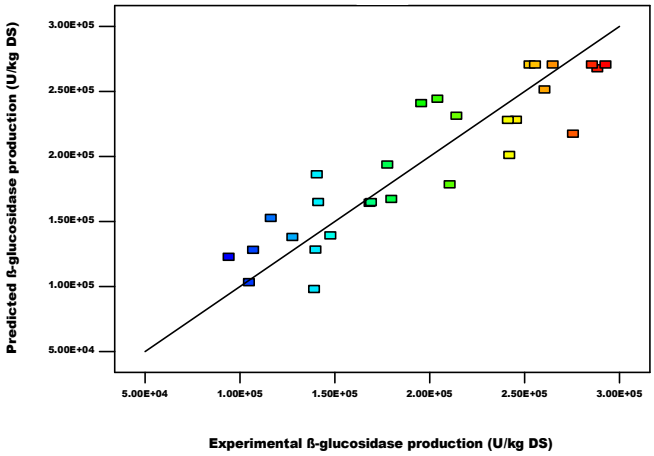

Fig. 4. Correlation between experimental and predicted β-glucosidase production computed from the obtained predictive model

In addition, Fig. 4 shows the good correlations between predicted and experimental results, confirming the validity of the model. The experimental values are very close to those calculated using the model equations. Indeed, the differences between calculated and measured responses are not statistically significant when using the *t*-test. We can then draw a conclusion that the second order models are suitable to describe the response surfaces and can be employed as a prediction equation in the studied domain.

Interpretation of the response surface model

To find the optimum, we have adopted a numerical optimization to discover a point that optimizes the desirability function. Desirability is a mathematical function used to find the optimum of a response; it should range from zero (outside of the limits) to one (at the goal).In our case, the goal is to discover the best set of factors' levels that will provide the maximum production of β-glucosidase. The solution of desirability function gave the optimum production of β-glucosidase, with the value of desirability index 0.76. It corresponds to a maximum production of β-glucosidase at 2.87×10^5 U/kg DS. This point is situated inside the experimental domain. Fig. 5 shows the features of the response surface in each direction of the experimental domain. From these curves, it can be inferred that the maximization of the β-glucosidase yield requires relatively a high level of X_1 (X_1 = 0.75), a high level of X_2 (X_2 = 1), a low level of X_3 (X_3 = 0.35), and the a high level of X_4 (X_4 = 0.7). This corresponds to the following settings of the natural variables: inoculum size $=8.5\times10^{11}$ spore/kg DS, calcium chloride = 0.025 kg/kg DS, urea = 0.095 kg/kg DS and soybean peptone = 0.119 kg/kg DS. Fig. 6 illustrates graphically the evolution of the β-glucosidase yield versus two variables, while the other two variables were held constant. The maximum production of β-glucosidase at 2.87×10^5 U/kg DS, is situated inside the experimental domain (contour plots). Fig. 6a shows that with the inoculum size 5×10^{11}spore/kg DS and soybeanpeptone concentration of 0.105 kg/kg DS, β-glucosidase production can be enhanced from 2.6×10^5 to 2.87×10^5 U/kg DS by the increase of the concentration of the inoculum size and calcium chloride until a concentration of inoculum size of 8.5×10^{11}spore/kg DS. A negative effect of inoculum size on response is seen after this concentration. As reported by many researchers (Kashyap*et al*., 2002; Sun*et al*., 2010), an increase of the inoculum size guarantees a speedy proliferation of biomass and enzyme synthesis.

Table 5. Estimates and statistical analysis of coefficients, (A)initial moisture content, (B) urea, (C) inoculum size, (D) calcium chloride, (E) magnesium sulfate, (F) moisturizing agent, (G) soybean peptone, and (H) wheat bran particles size

Factor	Coefficient estimate	Standard error	*F*-value	*p*-value*(Prob > F)
Initial moisture content (A)	-1858.22	4.36	0.043	0.043*
Urea (B)	914.14	4.63×10^{-3}	0.947	0.947
Inoculum size (C)	2757.42	10.02	0.006	0.006*
Calcium chloride (D)	1559.90	2.99	0.103	0.029*
Magnesium sulfate (E)	-577.16	2.82×10^{-5}	0.996	0.996
Moisturizing agent (F)	-967.62	0.24	0.628	0.628
Soybean peptone (G)	1053.49	1.21×10^{-3}	0.973	0.973
Wheat bran particles size (H)	-849.83	0.05	0.827	0.827
AB	-587.95	0.50	0.491	0.491
AC	-811.79	0.95	0.345	0.345
AD	433.51	0.27	0.610	0.610
AE	842.51	1.02	0.327	0.327
AF	-206.57	0.06	0.808	0.808
AG	816.35	0.96	0.342	0.342
AH	716.13	0.74	0.403	0.403

* Values of "Prob > F" less than 0.05 indicate model terms are significantat the level 95%.

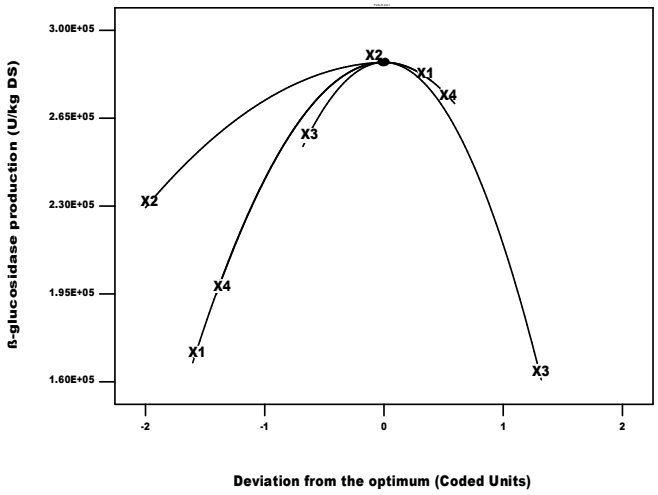

Fig.5. Variation of β-glucosidase production from the estimated optimum as function of the tested factors (X_1: inoculum size, X_2: calcium chloride, X_3: urea, and X_4: soybean peptone)

Nonetheless, after a certain point, the enzyme production could decline because of the exhaustion of nutrients, which results in decrease in metabolic activity. From Fig. 6b, it was observed that the enzyme yield enhances essentially by increasing urea concentration. However, a concentration more than 0.095 kg/kg DS cause inhibition of enzyme synthesis. This fact was also reported by Daroit *et al*. (2007) who found that the presence of urea has the crucial role on the production of β-glucosidase at high level. It is also clear from Fig. 6b that there is a gradual increase in the enzyme yields upon increasing the concentration of calcium chloride. Thus, it was implied that a high concentration of calcium chloride (0.025 kg/kg DS) was favorable for the production of β-glucosidase by *A. niger*. The role of calcium in the maintenance of the protein structures and the stabilization of the activities of several enzymes has been well-documented (Ertan *et al*., 2015; Sutherland, 1996).

Table 6. Analysis of variance (ANOVA) of the Box-Behnken design response.

Source of variation		Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value* (Prob >*F*)
Regression		3.98×10^{8}	14	2.84×10^{7}	4.48	0.0041*
Residual		8.89×10^{7}	14	6.35×10^{6}		
	Lack of fit	8.32×10^{7}	10	8.32×10^{6}	5.80	0.0525
	Error	5.74×10^{6}	4	1.43×10^{6}		
Total		4.87×10^{8}	28			

* Values of "Prob >*F*" less than 0.05 indicate model terms are significant at the level 95%.

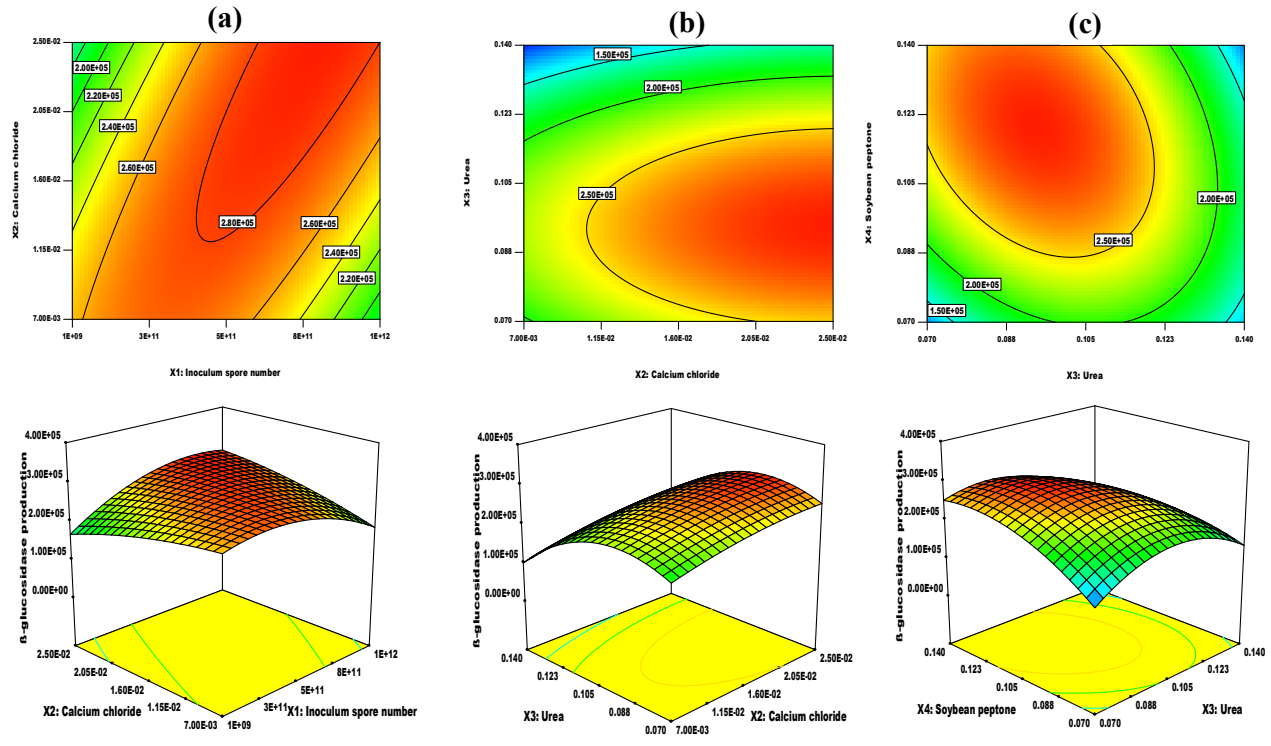

Fig. 6. Contour plots (top) and (bottom) response surface curves of interaction effect between (a) inoculum size and calcium chloride, (b) calcium chloride and urea, (c) soybean peptone and urea on the β-glucosidaseproduction response

These results were in agreement with others, for example, Colin *et al*. (2010) who reported that adding 0.5 g/L of calcium chloride to the medium increases the production of extracellular lipase by *A. niger* MYA 135. The authors explained the increase of enzyme production resulting from the secretion occurring through pathways regulated by the concentration of Ca^{2+}, for eukaryotes. These authors noted that the increase in Ca^{2+}concentration activates intracellular protein CaM kinase, leading to the release of extracellular lipase. Fig. 6c shows that with an inoculum size of 5×10^{11} spore/kgDS and a calcium chloride concentration of 16×10^{-3} kg/kg DS, the β-glucosidase production can be enhanced from 1.5×10^{5} to 2.87×10^{5} U/kg DS by the increase of the concentration of urea and soybean peptone until a concentration of 0.095 kg/kg DS is reached for urea, and 0.119 kg/kg DS for soybean peptone. After these concentrations, a negative effect of both nitrogen sources on response is observed. This fact was also reported by Hatzinikolaou and Macris (1995), who noted that urea and peptone concentrations remarkably affected the production of glucose oxidase.

Optimization

As the results of the numerical analysis, with desirability function, agree with those of the contour plot study, it can be inferred that there is no a masked optimum: the one predictedby few sections of contour plot analysis represents a real optimum for the entire experimental domain.The Design-Expert® software predicted the maximum β-glucosidase yield to be 2.87×10^{5}U/kg DS in the optimized conditions (calcium chloride, 0.025 kg/kg DS; urea, 0.095 kg/kgDS; inoculum spore number, 8.5×10^{11}spore/kg DS and soybean peptone, 0.119 kg/kg DS). Supplementary experimental runs were conducted under the selected optimal conditionsfor validation. It led to an experimental β-glucosidase production of about 2.81×10^{5}U/kg DS which is acceptable to the predicted value (2.87×10^{5} U/kg DS). The optimized β-glucosidase production obtained in this work was higher than those obtained by other recognized high β-glucosidase producer fungi, i.e. β-glucosidase production by *Penicilliumcitrinum* YS40-5 in SSF condition is about 1.6×10^{5}U/kg DS (Ng, 2010), *Paecilomycesvariotii* MG3, 153 U/g DS(Job, 2010), and *Aspergillus niger*KK2 (10^{5}U/kg DS)(Kang*et al*., 2004).

Conclusion

Statistical optimization of solid-state fermentation conditions to obtain a high β-glucosidase yield by *Aspergillus niger* ATCC16404 has been effectively achieved using fractional factorial and Box-Behnken designs. The optimal conditions for the production of β-glucosidase were determined as follows:calcium chloride, 0.025 kg/kg DS; soybean peptone, 0.119 kg/kg DS; urea, 0.095 kg/kg DS and inoculum spore number, 8.5×10^{11} spore/kg DS within 7 days of static culture at 30°C. Under these conditions, the experimental β-glucosidase production was found to be about 2.87×10^{5} U/kg DS. The strategy implemented in this research proved to be an effective and powerful tool for screening, optimization, and modelling solid-state fermentation. Enhanced production of *A.niger* β-glucosidase using the statistical methodology tool stated in this paper will aid in several uses at industrial levels.

Acknowledgements

This research was supported by the Tunisian Ministry of Higher Education, Scientific Research and Technology. The authors thank Prof.HafedhDhuibfor providing the microbial strain used in this study.

REFERENCES

Baati, R., Kamoun, A., Chaabouni, M., Sergent, M., Phan-Tan-Luu, R. 2006. Screening and optimization of the factors of a detergent admixture preparation, *Chemometr.Intell. Lab. Syst.*80 198-208.

Beaugrand, J., Reis, D., Guillon, F., Debeire, P., Chabbert, B. 2004. Xylanase-mediated hydrolysis of wheat bran: evidence for subcellular heterogeneity of cell walls, *Int. J. Plant Sci.* 165 553-563.

Bhatia, Y., Mishra, S., Bisaria, V. 2002. Microbial β-glucosidases: cloning, properties, and applications, *Crit. Rev. Biotechnol,* .22 375-407.

Box, G.E., Hunter, W.G., Hunter, J.S. 1978. Statistics for experimenters: an introduction to design, data analysis, and model building, *JSTOR*.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*72 248-254.

Bruins, M., Strubel,M., Van Lieshout, J., Janssen,A., Boom, R. 2003. Oligosaccharide synthesis by the hyperthermostable β-glucosidase from *Pyrococcus furiosus*: kinetics and modelling, *Enzyme Microb. Tech.* 33 3-11.

Cela, R., Claeys-Bruno, M., Phan-Tan-Luu, R. 2009. Screening strategies in comprehensive chemometrics, Brown TR, Walczak S. Oxford: Elsevier.

Colin, V.L., Baigori, M.D., Pera, L.M. 2010. Effect of environmental conditions on extracellular lipases production and fungal morphology from *Aspergillus niger* MYA 135, *J. Basic Microbiol.*50 52-58.

Couto, S.R., Sanromán, M.A.2005. Application of solid-state fermentation to ligninolytic enzyme production, *Biochem. Eng. J.* 22 211-219.

Daenen, L., Saison, D., Sterckx, F., Delvaux, F., Verachtert, H. and Derdelinckx, G. 2008. Screening and evaluation of the glucoside hydrolase activity in Saccharomyces and Brettanomyces brewing yeasts, *J. Appl. Microbiol.,* 104 478-488.

Daroit, D.J., Silveira, S.T., Hertz, P.F., Brandelli, A. 2007. Production of extracellular β-glucosidase by Monascus purpureus on different growth substrates, *Process Biochem.,* 42 904-908.

Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., Srinivasulu, B. 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species, *Process Biochem.,* 38 615-620.

Elyas, K., Mathew, A., Sukumaran, R.K., Ali, P.M., Sapna,K. Kumar, S.R., Mol, K.R. 2010. Production optimization and properties of beta glucosidases from a marine fungus *Aspergillus*-SA 58, *New Biotechnol*., 27 347-351.

Ertan, H., Cassel, C., Verma, A., Poljak,A., Charlton, T., Aldrich-Wright, J., Omar, S.M., Siddiqui, K.S., Cavicchioli, R. 2015. A new broad specificity alkaline metalloprotease from a *Pseudomonas* sp. Isolated from refrigerated milk: role of calcium in improving enzyme productivity, *J. Mol. Catal. B-Enzym.*113 1-8.

Farinas, C.S. 2015. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector, *Renew. Sust. Energ. Rev.,* 52 179-188.

Gervais, P., Molin, P. 2003. The role of water in solid-state fermentation, *Biochem. Eng. J.,* 13 85-101.

Hatzinikolaou, D., Macris, B. 1995. Factors regulating production of glucose oxidase by *Aspergillus niger*, *Enzyme Microb. Tech.,* 17 530-534.

Hölker, U., Höfer, M., Lenz, J. 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, *Appl. Microb.d Biotechnol.*64 175-186.

Hu, G., Heitmann, J.A., Rojas, O.J. 2008. Feedstock pretreatment strategies for producing ethanol from wood, bark, and forest residues. *Bioresources.,* 3 270-294.

Jain, K.K., Dey,T.B., Kumar,S., Kuhad, R.C. 2015. Production of thermostable hydrolases (cellulases and xylanase) from *Thermoascus aurantiacus* RCKK: a potential fungus, *Bioprocess Biosyst. Eng.,* 38 787-796.

Job, J., Sukumaran, R.K., Jayachandran, K. 2010. Production of a highly glucose tolerant β-glucosidase by *Paecilomyces variotii* MG3: optimization of fermentation conditions using Plackett–Burman and Box–Behnken experimental designs, *World J. Microb. Biot.,* 26 1385-1391.

Kang, S., Park, Y., Lee, J., Hong, S., Kim, S. 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass, *Bioresource Technol.,* 91 153-156.

Kashyap, P., Sabu, A., Pandey, A., Szakacs,G., Soccol, C.R. 2002. Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation, *Process Biochem.,* 38 307-312.

Lahoz, R., Reyes, F., Gomez, P., Martinez, M. 1983. Lytic enzyme activity in autolysing mycelium of *Aspergillus niger*, *Zeitschrift für allgemeine Mikrobiologie*23 17-25.

Lewis, G.A., Mathieu, D., Phan-Tan-Luu, R. 1998. Pharmaceutical experimental design, CRC Press.

Lopez, J.C., Pérez, J.S., Sevilla, J.F., Fernández, F.A., Grima, E.M. Chisti, Y. 2003. Production of lovastatin by *Aspergillus terreus*: effects of the C: N ratio and the principal nutrients on growth and metabolite production, *Enzyme Microb. Tech.*33 270-277.

Maes,C., Delcour, J. 2001. Alkaline hydrogen peroxide extraction of wheat bran non-starch polysaccharides, *J. Cereal Sci*. 34 29-35.

Montgomery, D.C. and Mastrangelo, C.M. 1991. Some statistical process control methods for autocorrelated data. *J. Qual. Technol.*, 23 179-193.

Ng, I.S., Li, C.W., Chan,S.P., Chir, J.L., Chen, P.T., Tong, C.G., Yu,S.M., Ho, T.H.D. 2010. High-level production of a thermoacidophilic β-glucosidase from *Penicillium citrinum* YS40-5 by solid-state fermentation with rice bran, *Bioresource Technol.,* 101 1310-1317.

Norkrans, B. 1950. Influence of cellulolytic enzymes from Hymenomycetes on cellulose preparations of different crystallinity, *Physiologia Plantarum* 3 75-87.

Pandey, G., Kapur, M. 2002. Design and development of a common synthetic strategy for a variety of 1-N-iminosugars, *Org. Lett.*4 3883-3886.

Ravichandra, K., Yaswanth, V., Nikhila, B., Ahmad, J., Rao, P.S., Uma, A., Ravindrababu, V., Prakasham, R. 2016. Xylanase Production by Isolated Fungal Strain, *Aspergillus fumigatus* RSP-8 (MTCC 12039): Impact of Agro-industrial Material as Substrate, *Sugar Tech.*18 29-38.

Ribeiro, M., Mandarino, J., Carrao-Panizzi, M., De Oliveira, M., Campo, C., Nepomuceno, A., Ida, E. 2007. Isoflavone content and β-glucosidase activity in soybean cultivars of different maturity groups, *J. Food Compos. Anal.,* 20 19-24.

Rosales, E., Couto, S.R., Sanromán, M.A. 2007. Increased laccase production by *Trametes hirsuta* grown on ground orange peelings, *Enzyme Microb. Techn.*40 1286-1290.

Sarabia, L., Ortiz, M. 2009. Response surface methodology, *Compr. Chemometr.*1 345-390.

Sharma, P., Chaturvedi, A., Sharma, L. 2015. Parametric optimization for extracellular tannase production in submerged fermentation by isolated Aspergillus species, *Int. J. Curr. Microb. App. Sci.* 4 232-239.

Sun,H., Ge,X., Hao,Z., Peng, M. 2010. Cellulase production by Trichoderma sp. on apple pomace under solid state fermentation, *Afr. J. Biotechnol.*9.

Sutherland, G.R., Aust, S.D. 1996. The effects of calcium on the thermal stability and activity of manganese peroxidase, *Arch. Biochem. Biophys.*332 128-134.

Tan, I.S., Lee, K.T. 2015. Immobilization of β-glucosidase from *Aspergillus niger* on κ-carrageenan hybrid matrix and its application on the production of reducing sugar from macroalgae cellulosic residue, *Bioresource Technol.*184 386-394.

Tasar, O.C., Erdal, S., Taskin, M. 2016. Chitosan production by psychrotolerant *Rhizopus oryza*e in non-sterile open fermentation conditions, *Int. J. Biol. Macromol.*89 428-433.

Tate, D. Reynolds, A.G. 2006. Validation of a rapid method for measuring β-glucosidase activity in fermenting muscat grape musts, *Am.J. Enol. Viticult.*57 60-68.

Tavares, A.P., Coelho, M.A., Agapito, M.S., Coutinho, J.A., Xavier, A.M. 2006. Optimization and modeling of laccase production by *Trametes versicolor* in a bioreactor using statistical experimental design. *Appl. Biochem. Biotech.,*134 233-248.

Vervoort, Y., Herrera-Malaver, B., Mertens, S., Guadalupe, V. Medina, J. Duitama, L. Michiels, G. Derdelinckx, K. Voordeckers, K.J. Verstrepen. 2016. Characterization of the recombinant *Brettanomyces anomalus* β-glucosidase and its potential for bioflavouring, *J. Appl. Microbiol.,* 121 721-733.

Woodward, J., Wiseman, A. 1982. Fungal and other β-d-glucosidases—their properties and applications, *Enzyme Microb. Tech.,* 4 73-79.

Yang, J., Jiao, R.H., Yao, L.Y., Han, W.B., Lu, Y.H. R.X. 2016. Tan, Control of fungal morphology for improved production of a novel antimicrobial alkaloid by marine-derived fungus Curvularia sp. IFB-Z10 under submerged fermentation, *Process Biochem.,* 51 185-194.

Zhang, C., Li, D., Yu, H., Zhang, B., Jin, F. 2007. Purification and characterization of piceid-β-D-glucosidase from *Aspergillus oryzae*, *Process Biochem*., 42 83-88.

Zimbardi, A.L., Sehn, C., Meleiro, L.P., Souza, F.H., Masui, D.C., Nozawa, M.S., Guimarães, L.H., Jorge, J.A., Furriel, R.P. 2013. Optimization of β-glucosidase, β-xylosidase and xylanase production by *Colletotrichum graminicola* under solid-state fermentation and application in raw sugarcane trash saccharification, *Int.J.Mol. Sci.,* 14 2875-2902.

****