Vol. 18, No. 2 (2019) 673-680
Revista Mexicana de Ingeniería Química

PRODUCTION OF XYLANASE AND ENDOGLUCANASE BY SOLID-STATE FERMENTATION OF JACKFRUIT RESIDUE

PRODUCCIÓN DE XILANASA Y ENDOGLUCANASA POR FERMENTACIÓN EN ESTADO SÓLIDO DEL RESIDUO DE YACA

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Received: June 17, 2018; Accepted: August 19, 2018

Abstract

To investigate the potential of the jackfruit seed residue in xylanase and endoglucanase production was carried solid-state fermentation by *Penicillium roqueforti* with this residue. Effects of the independent variables water activity, incubation time and temperature in the responses, xylanase and endoglucanase by means of an experimental design 2^3. Kinetic parameters of xylanase were also evaluated. At 90% confidence level it was observed that the water activity had no significant effect on the responses since the time and the incubation temperature had significant effects. The endoglucanase activity was 4.454 U/g at 33 ºC, 100h and water activity of 0.966, the xylanase activity was 3.016 U/g obtained at 33 ºC, 100h and water activity of 0.958. The produced xylanase has K_m = 2.44 mg/mL, V_{max} = 4.59 U/g and K_{cat} = 1.57 1/s. Without the use of any inducer, it was possible to obtain endoglucanase and xylanase by solid-state fermentation of jackfruit residue, demonstrating its biotechnological potential as a substrate.

Keywords: Multienzymatic complex, jackfruit seed, residues.

Resumen

Para investigar el potencial del residuo de la semillas de yaca en la producción de xilanasa y endoglucanasa, fue realizada a fermentación en estado sólido por *Penicillium roqueforti*. Las variables independientes actividad de agua, tiempo de incubación y temperatura se han evaluado mediante un diseño experimental 2^3. También se evaluaron los parámetros cinéticos de la xilanasa. Con un nivel de confianza del 90%, se observó que la actividad de agua no tuvo un efecto significativo sobre las respuestas ya que el tiempo y la temperatura de incubación tuvieron efectos significativos. Las mejores actividades fueron: endoglucanasa 4.454 U/g, a 33 ºC, 100 h y actividad de agua de 0.966, para xilanasa 3.016 U/g a 33 ºC, 100 h y actividad de agua de 0.958. La xilanasa producida tiene K_m = 2.44 mg/mL, V_{max} = 4.59 U/g y K_{cat} = 1.57 1/s. Sin el uso de ningún inductor, fue posible obtener endoglucanasa y xilanasa por fermentación en estado sólido de residuos de jaca, lo que demuestra su potencial biotecnológico.

Palabras clave: Complexo multienzimático, residuo, semilla de yaca.

1 Introduction

Solid State Fermentation (SSF) is the process where the microorganism develops in an environment with little or without free water. This process reveals as a great alternative for the production of enzymes, mainly because of the low cost of production that happens due to the reuse of agro-industrial waste reduction in the downstream stages and reduced investment of maintenance and operation (Behera and Ray, 2016).

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https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n2/Marques
issn-e: 2395-8472

By simulating the environment where naturally occurring, filamentous fungi are the microorganisms best suited to SSF (Yoon *et al*., 2012). Although many studies have been done with fungi such as *Aspergillus niger* and even some species of the genus *Penicillium*, has been little explored the potential *Penicillium roqueforti*, a GRAS (Generally Recognized as Safe) fungi, in the production of enzymes (Andualema and Gessesse, 2012; Musoni *et al*., 2015).

Applications such as the production of biofuels pulp treatment paper treatment effluent manufacturing juices fabric washing and oil extraction has aroused interest for the production of cellulolytic and hemicellulolytic enzymes which are groups of enzymes that degrade cellulose and hemicellulose the most abundant biopolymers in nature. Among the cellulases are endoglucanases (EC. 3.2.1.4) and exoglucanases (E.C. 3.2.1.91), and among the hemicellulases is xylanase (EC.3.2.1.8) (Silva *et al*., 2009; Mansour, 2016). The main difficulty for the application of these enzymes lies in the costs of production due to this fact the research with less costly means of production has intensified as agro-industrial residues that are sources of carbon and of other nutrients necessary for the growth of the microorganism and for the production of the enzyme. Besides reducing the cost in the production of this biocatalyst, reuse such waste means to reduce the disposal of this material of irregular form in the environment, which causes serious environmental risks (Oberoi *et al*., 2008; Bajaj *et al*., 2014; Hansen *et al*., 2015; Behera and Ray, 2016).

These agro-industrial residues, such as jackfruit, are still little used in industrial processes. Although a percentage of this waste is already being used in energy production in industries most of it is still discarded which creates a serious environmental problem. Many studies are already carried out with residues of corn, orange bagasse and other agro-products of global reach, thus motivating the application in the SSF of high local production residues (Musatto *et al*., 2017).

In this context the aim of this work was to investigate the application of the jackfruit residue as substrate of SSF for the cultivation of *Penicillium roqueforti* for both was analyzed by means of experimental factorial planning methodology the influence of water activity temperature and incubation time on endoglucanase, xylanase and FPase activities in the multienzymatic crude extract (MCE) obtained.

2 Materials and methods

2.1 Substrate for fermentation

The samples of jackfruit (*Artocarpus heterophyllus*) was obtained from the local market (Ilhéus, Brazil). After the hygiene and size reduction by cutting the samples of the residues were dried in an oven (TECNAL TE-394/2, Piracicaba, Brazil) at 70 °C for 24 h and milled in a Willey-type knife mill (SOLAB SL31, Piracicaba, Brasil) at an indefinite granulometry. The dried and ground residue were adequately reserved in closed containers of polyethylene from which it was properly withdrawn for the analyzes.

2.2 Cellulose, hemicellulose and lignin content

With dry and ground residue the determination of hemicellulose and lignin cellulose contents (%, g/100g dry matter) in jackfruit residue was carried out according to the methodology described by the Official Association of Analytical Chemistry (AOAC, 1995).

2.3 Microrganism and inoculum

Penicillium roqueforti ATCC 10110 was donated by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Brazil). The preparation of the microorganism and the production of the spore solution followed the methodology used in Marques *et al*. (2017)

2.4 Solid State Fermentation (SSF)

For the SSF, 10 g of the residue were autoclaved in 125 mL Erlenmeyers and inoculated with the spore solution (10^7 spores/g). Incubation was carried out in a bacteriological stove (TECNAL TE-371, Piracicaba, Brazil). An experimental design of factorial type 2^3 (8 different experiments) plus 3 replicates at a central point (Rodrigues and Iemma, 2015) was conducted to evaluate three independent variables: the incubation time at incubation temperature and the water activity of the residue (Table 1).

Table 1. Central Composite Rotatable Design matrix, with real values (coded values are presented in parenthesis), for the factors: temperature (T, °C), time (t, h) and water activity (A_w), and the responses: endoglucanase (END, U/g) and xylanase (XYL, U/g), obtained with the cultivation of *P. roqueforti* ATCC 10110 in residue of jackfruit seed.

TRIAL	T (°C)	t (h)	A_w	END (U/g)	XYL (U/g)
1	27 (-1)	44 (-1)	0.966 (+1)	1,86	1,86
2	27 (-1)	100 (+1)	0.966 (+1)	2.77	2.17
3	27 (-1)	44 (-1)	0.958 (-1)	1,98	1,81
4	27 (-1)	100 (+1)	0.958 (-1)	3,10	2,14
5	33 (+1)	44 (-1)	0.966 (+1)	1.93	1.94
6	33 (+1)	100 (+1)	0.966 (+1)	4,45	3
7	33 (+1)	44 (-1)	0.958 (-1)	1,88	2,32
8	33 (+1)	100 (+1)	0.958 (-1)	3,88	3.07
9	30 (0)	72 (0)	0.962 (0)	3,14	2,09
10	30 (0)	72 (0)	0.962 (0)	3,32	2,17
11	30 (0)	72 (0)	0.962 (0)	3,52	2.01
12	30 (0)	72 (0)	0.953 (-1.68)	1.89	1.67
13	30 (0)	72 (0)	0.971 (+1.68)	3.22	2.05
14	30 (0)	24 (-1.68)	0.962 (0)	3.21	2.34
15	30 (0)	120 (+1.68)	0.962 (0)	2.65	2.97
16	25 (-1.68)	72 (0)	0.962 (0)	2.98	1.42
17	35 (+1.68)	72 (0)	0.962 (0)	3.82	2.13

Response variables were activity of xylanase (XYL, U/g) and endoglucanase activity (END, U/g) produced throughout the fermentations were evaluated and these responses were analyzed with the aid of statistical software STATISTICA^{TM} (version 10.0, from StatSoft, Inc.) in order to evaluate the influence of the independent variables on the response variables.

2.5 *Multienzymatic crude extract (MCE)*

The procedure for obtaining the MCE followed the methodology used in Marques *et al*. (2017), using citrate buffer (pH 4.8, 50 mM) in a ratio of 5:1 volume (mL): weight (g). The MCE containing xylanase activity and endoglucanase activity.

2.6 *Determination of enzymatic activities*

Xylanase activity was determined by the release of reducing sugars result of xylan (from beechwood 1% (m/v)) hydrolysis as described in Santos *et al*. (2012). For endoglucanase activity, the assay has as base the degradation of a 1% (m/v) solution of Carboxymethylcellulose diluted in citrate buffer (pH 4.8, 50 mM), as described in Santos *et al*. (2013).

After the incubation period, all the hydrolysates were analyzed according to the procedure for measurement reducing sugars as describes in the item 2.7.

2.7 *Determination of reducing sugars*

For analysis of reducing sugars, 0.5 mL of the hydrolysate was taken in a test tube in this tube 0.5 mL of DNS (dinitrosalicylic acid) reagent was added. The tubes were immersed in boiling water for 5 min, after which 4.0 mL of distilled water were added for further measurement of absorbance in the spectrophotometer 540 nm (BEL PHOTONICS SF200DM - UV Vis - 1000 nm, Osasco, Brazil) (Adapted from MILLER, 1959).

2.8 *Study of the kinetic parameters of P. roqueforti xylanase*

The kinetic parameters V_{max} (U/g) and K_m (mg/mL) provided by Michaelis-Menten mathematical model were estimated by linearization of the kinetic data obtained by different concentrations of beechwood xylan (2-10 mg/mL). The turnover number was determined as: kcat = V_{max}/U_o, where U_o = 2.906 U/g.

3 Results and discussion

3.1 Cellulose, hemicellulose and lignin contents

Contents of hemicellulose and lignin cellulose were analyzed in the jackfruit residue to determine its composition. Partial bromatological characterization of the jackfruit residue confirms its potential for application as a substrate in solid-state fermentation. Mass percentages obtained for this composition were 29.36% hemicellulose 12.78% cellulose and 5.54% lignin plus 2.70% ash. In comparison, these percentages differ from the values described by Limayem *et al*. (2012) where it is highlighted that agro-industrial residues are composed of 25% - 50% hemicellulose, 37% - 50% cellulose and 5% - 15% lignin. The jackfruit residue used in this work was composed of jackfruit seeds. In percentage terms, the residue was richer in hemicellulose and cellulose than in lignin, which suggests that this residue is a suitable substrate for SSF. As lignin is a compound that has the function of protecting the vegetal structure against chemical and biological attacks (Gonzalo *et al*., 2016), a low lignin content facilitates the action of the microorganisms and their enzymes during the consumption of the vegetal matter of the residue.

3.2 Evaluation of factors influencing the production of endoglucanases and xylanases

The solid-state fermentation (SSF) of the fungus *Penicillium roqueforti* in jackfruit residue was conducted according to the matrix of factorial design, presented in Table 1, for the factors: water activity temperature (T, °C) and incubation time (t, h). Responses analyzed were xylanase activity (XYL, U/g) and endoglucanase activity (END, U/g). The results obtained are shown in Table 2. The effects of the independent variables on the response variables were analyzed at a significance level of 10% ($p < 0.1$) from the responses shown in table 2, to better visualize, pareto graphs were constructed, in order to facilitate the description of the effects of water activity, temperature and incubation time on xylanase (Fig 1.a.) and endoglucanase (Fig 1.b.).

Table 2. Analysis of variance ($p < 0.05$) for the factors: temperature (T, °C), time (t, h) and water activity (A_w), of the response: xylanase (XYL, U/g) and endoglucanase (END, U/g), obtained with the cultivation of *P. roqueforti* ATCC 10110 in residue of jackfruit seed.

XYL

Source	Sum of Squares	Freedom Degrees	Mean Square	*F*	p-value
Regression	2.547	6	0.425	5,450	0.009686**
Residues	0.779	10	0.078		
Lack of Fit	0.766	8	0.096	14,963	0.0641
Pure error	0.013	2	0.006		
Total	3.326	16			
R^2	**0.7658**				
R^2_{adj}	**0.7117**				

END

Source	Sum of Squares	Freedom Degrees	Mean Square	*F*	p-value
Regression	8.879	6	1.48	12,359	0.0004
Residues	1.197	10	0.12		
Lack of Fit	1.125	8	0.141	3,892	0.2204
Pure error	0.072	2	0.036		
Total	10.076	16			
R^2	**0.8812**				
R^2_{adj}	**0.8271**				

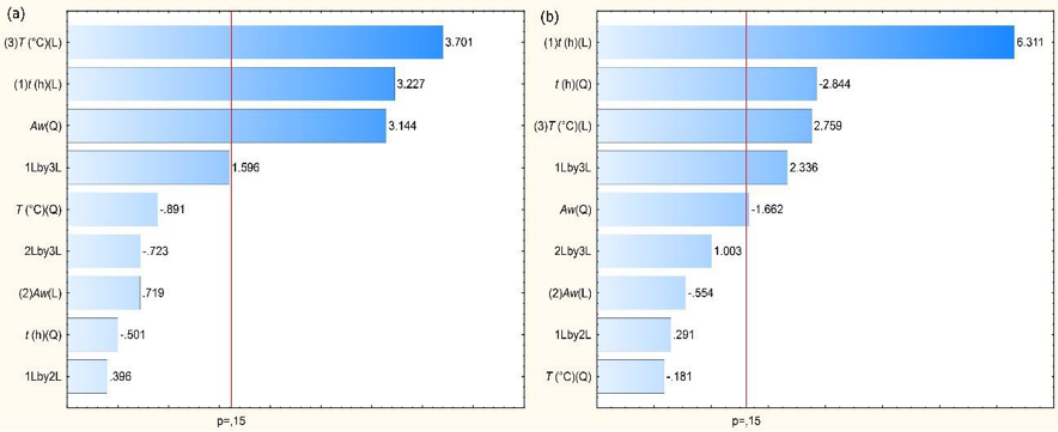

Fig. 1. Pareto graphs constructed from the analysis of the effects on the response variables XYL (a), END (b) during the solid-state fermentation of jackfruit seed residue by *P. roqueforti* ATCC 10110.

The water activity did not present significant effects on the production of the enzymes during SSF. According to Pandey (2003) water activity is the essential parameter to indicate the transport of water mass between the solutes and the microbial cells dealing with the amount of water available for the microorganism to use in its metabolism. Kalai *et al*. (2017) determined in their work that in the range of water activity between 0.83 and 0.99 are the minimum values for germination and optimal growth values of *Penicillium roqueforti* which exalts the non-significance of the water activity factor for the production of the enzymes in a study in the range of water activity evaluated (0.958 - 0.966). Other researchers have also found similar results for the ideal water activity for the development of *P. roqueforti* and other fungi of the genus *Penicillium* (Valik *et al*., 1999, Abellana *et al*., 2001, Plaza *et al*., 2002).

Time and temperature were significant factors in obtaining the enzymes xylanase and endoglucanase as well as the interaction between these two factors. Both effects were positive for the time this evidences the production of these enzymes throughout the growth and development of the fungus. Salles *et al*. (2010) also obtained, in his work with *Aspergillus*, time as a significant factor in the production of cellulases studying in a range of 72h to 168h. Temperature is an important factor for analyzing fungus growth and its metabolism varies according to temperature change (Carlile *et al*., 2001). Since enzymes are products of fungi metabolism, temperature variation is expected to result in higher or lower production of the enzymes. According to Li *et al*. (2009), the highest growth rates of *Penicillium roqueforti* occur at 25 °C. Other works, using the microorganism *P. roqueforti* ATCC 10110, such as Ferraz *et al*. (2017) and Marques *et al*. (2017) determined that the best production of cellulases is between temperatures of 25 °C to 32 °C. Results of this work indicate that with all constant factors, the positive variation in temperature has a growth effect in the production of these enzymes, with the highest production found in temperature of 33 °C.

3.3 Kinetic parameters of the enzyme xylanase

From the linearization (Fig. 2), R^2_{adj} = 0.88, of the kinetic data obtained with the xylanase reaction with the beechwood xylan substrate, the kinetic parameters for *Penicillium roqueforti* xylanase ATCC 10110 produced during cultivation in jackfruit residue were defined. A value of K_m = 2.44 mg/mL, V_{max} = 4.59 U/g and K_{cat} = 1.57 1/s was obtained. The evaluation of the kinetic parameters of xylanase was carried out in a multienzymatic crude extract containing several enzymes. Evangelista *et al*. (2017) found in their work, with genetically modified and purified xylanase, a K_{cat} of 792.8 1/s using xylan as substrate and K_{cat} of 366.4 1/s, using arabinoxylan as substrate. In his work with purified Bacillus subtilis xylanase Irfan *et al*. (2013) obtained a K_{cat} of 850 1/s. Purified enzymes actually have a high catalytic power, however, as described by Saddler and Arantes (2010), the degradation of cellulose and hemicellulose occurs not by the action of an enzyme but through the synergistic action of an enzymatic complex.

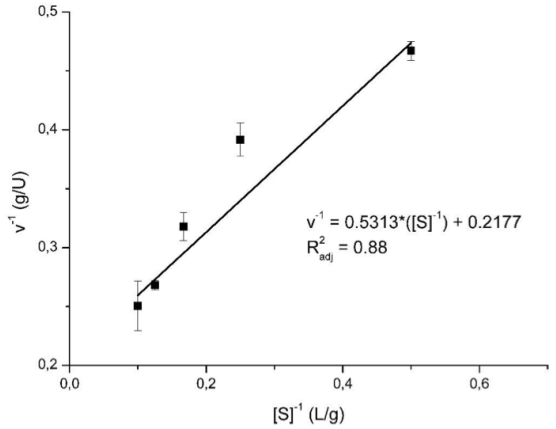

Fig. 2. Linearization of kinetic data for estimation of kinetic parameters (K_m and V_{max}) of xylanase (obtained by cultivation of *Penicillium roqueforti* ATCC 10110 in jackfruit seeds residue) using beechwood xylan as substrate.

Conclusions

Although many studies are being conducted in order to evaluate the use of different agro-industrial residues in the production of compounds of high value added, residues such as jackfruit are still few studied in applications involving solid-state fermentation. *Penicillium roqueforti* (a GRAS fungus) is also poorly exploited in solid-state fermentation still suffering from lack of information on its biotechnological potential in this technique. This work showed that the use of the jackfruit residue is a viable option in the production of enzymes and its use is a contribution to reduce the dumping of this residue in the environment, promoting greater sustainability for the local economy.

Acknowledgements

Os autores gostariam de agradecer à Fundação de Amparo à Pesquisa da Bahia (FAPESB), pelo apoio financeiro, a Universidade Estadual Do Sudoeste da Bahia (UESB) e Universidade Estadual de Santa Cruz (UESC), pelo apoio técnico e administrativo.

Abbreviations

A_w	water activity
CMC	endoglucanase activity, U/g
FPA	atividade papel de filtro, U/g
kcat	constante catalítica, 1/s
K_m	Constante de michaelis, mg/mL
MCE	multienzimatic crude extract
SSF	solid-state fermentation
V_{max}	velocidade máxima de conversão, U/g
XYL	Xylanase activity, U/g

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