S04-25

EVALUATION OF SUBSTRATE COMPOSITION FOR LIGNOCELLULOLYTIC ENZYMES PRODUCTION BY SOLID STATE FERMENTATION OF WASTES FROM OLIVE OIL AND WINE INDUSTRIES

Salgado, José Manuel[1,2*]; Moreira, Claudia[1], Abrunhosa, Luis[1]; Venâncio, Armando[1]; Domínguez, José Manuel[2]; Belo, Isabel[1]

[1]IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

[2]Department of Chemical Engineering, Sciences Faculty, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, Spain

* Corresponding author. *E-mail*: jmsalgado@ceb.uminho.pt.

Abstract

Wastes from olive oil and wine industries (as exhausted grape mark, vineshoot trimmings, two-phase olive mill waste, vinasses and olive mill wastewater were evaluated for lignocellulolytic enzymes production (as cellulases, xylanases and feruloyl esterases) by solid state fermentation with *Aspergillus niger, Aspergillus ibericus* and *Aspergillus japonicus*.

To study the effect of different substrates in enzymes production a Plackett-Burman experimental design was presented. The variables that had a higher positive effect in lignocellulolytic enzymes were urea, time and exhausted grape mark. The mixture of two-phase olive mill waste with exhausted grape mark and vineshoot trimmings had maxima activity of cellulases, xylanases and feruloyl esterases.

Keywords: solid state fermentation, lignocellulolytic enzymes, agro-industrial wastes.

INTRODUCTION

Lignocellulolytic enzymes (LCE) are use in saccharification processes, for the subsequent production of ethanol by fermentation. Principal LCE are cellulases, xylanases, ligninases and pectinases. Since last two decades, cellulases and xylanases are gaining enormous attention for their potential in biotechnology processes (Deswal et al., 2012). On an

industrial scale, xylanases are produced by *Aspergillus* sp.(Couri et al., 2000). The use of enzymes increases the costs of biotechnology processes for ethanol production. Therefore, the exploitation of agro-industrial wastes as low-cost substrate for the production of enzymes can contribute to make those processes more profitable.

Fungi produce a complete set of cellulases: cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) andβ-glucosidases (EC 3.2.1.21) that are necessary to efficiently hydrolyse cellulose (Tabka et al., 2006). A synergistic action of these enzymes is required for the complete hydrolysis of cellulose (Pothiraj et al., 2006).

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan (Collins et al., 2005).Feruloyl esterases (FAEs, E.C. 3.1.1.73), also known as ferulic acid esterases, are a subclass of carboxylic acid esterases (E.C. 3.1.1.1) (Kumar et al., 2011).

In the present work, we evaluate different wastes from olive oil and wine industries: two-phase olive mill waste (TPOMW), exhausted grape mark (EGM), vineshoot trimmings (VTS), vinasses, olive mill wastewater (OMW) for cellulases, xylanases and feruloyl esterases production by solid state fermentation with three strains of *Aspergillus*.

METHODOLOGY

Characterization of raw material

The residues were collected from industries in the area in season 2011/2012 and stored at -20 ºC.

Agro-industrial wastes were characterized by quantitative acid hydrolysis in a two-stage acid treatment (the first stage with 72 wt % sulfuric acid at 30 ºC for 1 h, the second stage after dilution of the media to 4 wt % sulfuric acid at 121 ºC for 1 h) (Bustos et al., 2004). For chemical oxygen demand (COD) determination the test kits from Hach Lange LCK114 (150 – 1000 mg L^{-1}) were used according the manufacturer method. Reducing sugars were determinate by dinitrosalicylic acid (DNS) method (Miller, 1959). Lipid (total fat contents) were extracted with diethyl ether, in a Soxtec System HT2 1045-extraction unit, after samples lyophilisation (Official Methods of Analysis, 2007). Total phenols were assessed by the Folin–Ciocalteau Method (Commission Regulation (EEC) N◦2676/90) using caffeic acid as standard.

Solid state fermentations

Fermentations were carried out in Erlenmeyer flask of 500 mL with 30 g of dry solid substrate. Table 1 shows composition of each medium. Independent fermentations were performed with *Aspergillus niger, Aspergillus ibericus* and *Aspegillus japonicus*. For the inoculation, spores of fungus growth in growth medium slant tubes (20 g/L of malt extract, 1 g/L of peptone, 20 g/L glucose and 20 g/L agar) were suspended in a sterilized solution composed by 0.1% of peptone and 0.01% of Tween 80 Each flask was inoculated with 2 mL of the spore suspension and incubated at 25 ºC for 7 or 14 h.

The extraction of enzymes was performed at final time (7 or 14 h) of each experiment with asolution composed of 1% of NaCl and 0.5% of Tritom-X100 at 4 ºC, with agitation for 2 h. After, extract was centrifuged (4000 rpm, 10 min) and filtered.

Plackett-Burman experimental design.

To evaluate the substrate composition, seven independent variables were screened in eight combinations (Table 1) organized according to the Plackett–Burman design (Plackett and Burman, 1946).

Table 1. Experiments of Plackett-Burman design.

Runs	EGM/TPOMW (g/g*)	VTS/ TPOMW (g/g*)	OMW (mL/g*)	Vinasses (mL/g*)	Nutrients** (mL/g*)	Urea (g/g*)	Time (h)
1	1	1	0.5	1	0.5	0.01	14
2	1	1	0	1	0	0	7
3	1	0	0.5	-1	0.5	0	7
4	1	0	0	-1	0	0.01	14
5	0	1	0.5	-1	0	0.01	7
6	0	1	0	-1	0.5	0	14
7	0	0	0.5	1	0	0	14
8	0	0	0	1	0.5	0.01	7

*g of dry solid substrate

**Basal medium:3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L $CaCl_2.2H_2O$, 1mL/L of trace metal solution, 0.1 g/l peptone, 0.1 g/L yeast extract.

Enzymatic activities

Endo-1,4-ß-Glucanase (Cellulase) activity was determined with the enzymatic kit Azo-CM-Cellulose S-ACMC 04/07 (Megazyme International, Ireland). Endo-1,4-ß-Xylanase activity was determined with the enzymatic kit Azo wheat arabinoxylan AWX 10/2002 (Megazyme International Ireland). Feruloyl esterase activity was determined according to Mastihuba et al. (2002).

RESULTS

Characterization of raw material

Before carrying out the solid state fermentations, raw materials were characterized (Table 2). The results of characterization of EGM, VTS, TPOMW were expressed as grams of cellulose, hemicelluloses (xylan, arabinans, acetyl groups), lignin (Klason) per g of dry solid. VTS presented a higher content of celluloses (0.3 g/g of dry solid) and hemicelluloses (0.1 g/g). VTS are likely to be valued through fractionation steps with diluted acids to obtain hemicellulosic hydrolyzates, which can be fermented to lactic acid and xylitol (Bustos et al., 2005; Salgado et al., 2012).

Table 2. Characterization of wastes

Solid waste	Olive Bagasse	Vineshoot trimmings	Grape bagasse
Celluloses (g/g)	0.07 ± 0.24	0.30 ± 0.03	0.14 ± 0.01
Hemicelluloses (g/g)	0.04 ± 0.21	0.10 ± 0.01	0.06 ± 0.01
Lignina (g/g)	0.58 ± 0.41	0.37 ± 0.02	0.58 ± 0.01
Reducingsugars (mg/g)	24.30 ± 1.42	55.35 ± 0.05	3.00 ± 0.01
Protein (mg/g)	0.30 ± 0.03	1.27 ± 0.03	1.30 ± 0.00
Total phenols (mg/g)	2.57 ± 0.04	1.25 ± 0.04	0.19 ± 0.01
Lipids (mg/g)	102.46 ± 0.04	29.6 ± 0.00	21.3 ± 0.00

Lignocellulolytic enzymes production

The influence of substrate composition in enzyme production was studied using a Plackett-Burman experimental design. TPOMW and mixture with EGM and VTS were added as solid substrate, another wastes OMW and vinasses were used as nutritional supplement and they were compared with other nutrients (basal medium and urea), time effect was also evaluated. Table 3 shows enzymatic activities of each microorganism in all experiments.

Table 3. Experiments of Plackett-Burman design.

	Aspergillusniger			*Aspergillusibericus*			*Aspergillusjaponicus*		
Runs	CA (U/g*)	XA (U/g*)	FEA (U/g*)	CA (U/g*)	XA (U/g*)	FEA (U/g*)	CA (U/g*)	XA (U/g*)	FEA (U/g*)
1	5.20	2.30	89.53	5.50	1.52	12.09	6.77	0.49	0.00
2	0.00	0.05	0.00	0.00	0.15	0.00	0.02	0.04	0.00
3	0.08	0.02	0.00	0.00	0.11	0.00	0.06	0.03	0.00
4	3.36	2.52	58.05	3.76	1.74	7.49	4.50	0.39	3.03
5	1.80	3.06	8.51	1.33	1.34	5.90	2.36	0.11	2.18
6	0.10	0.03	0.00	0.03	0.05	0.00	0.02	0.09	0.00
7	0.11	0.06	0.00	0.10	0.13	0.00	0.01	0.00	0.00
8	1.79	1.65	32.10	1.90	0.95	0.51	1.54	0.00	0.00

*g of dry solid substrate

CA: Cellulose activity

XA: Xylanase activity

FEA: Feruloyl esterase activity

Mixture of three solid residues presented the maxima of lignocellulolytic activity. The variables that had a higher positive effect in three fungi were urea, time and EGM addition.

CONCLUSIONS

Different enzymes were produced according to the substrate used. Mixture of TPOMW with other wastes of lignocellulosic nature favors the production of cellulases, xylanases and feruloyl esterases. *A. niger* was more effective in producing the three enzymes. In future studies, we will optimize the variables with a higher effect detected in Plackett-Burman design.

ACKNOWLEDGEMENTS

José Manuel Salgado is grateful for Postdoctoral fellowship (EX-2010-0402) of Education Ministry of Spanish Government.

Luís Abrunhosa acknowledges the Portuguese Fundação para a Ciência e Tecnologia (FCT) for the financial support through the grant SFRH/BPD/43922/2008.

REFERENCES

Bustos, G., Moldes, A. B., Cruz, J. M., & Domínguez, J. M. (2004). Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by lactobacillus pentosus. *Journal of the Science of Food and Agriculture, 84*(15), 2105-2112.

Bustos, G., Moldes, A. B., Cruz, J. M., &Domínguez, J. M. (2005). Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using lactobacillus pentosus.*Biotechnology Progress, 21*(3), 793-798.

Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilicxylanases.*FEMS Microbiology Reviews, 29*(1), 3-23.

Couri, S., Da Costa Terzi, S., Saavedra Pinto, G. A., Pereira Freitas, S., & Augusto Da Costa, A. C. (2000). Hydrolytic enzyme production in solid-state fermentation by aspergillusniger 3T5B8. *Process Biochemistry, 36*(3), 255-261.

Deswal, D., Sharma, A., Gupta, R., &Kuhad, R. C. (2012). Application of lignocellulolytic enzymes produced under solid state cultivation conditions.*Bioresource Technology, 115*, 249-254.

Kumar, C. G., Kamle, A., Mongolla, P., & Joseph, J. (2011). Parametric optimization of feruloyl esterase production from aspergillusterreus strain GA2 isolated from tropical agro-ecosystems cultivating sweet sorghum. *Journal of Microbiology and Biotechnology, 21*(9), 947-953.

Mastihuba, V., Kremnický, L., Mastihubová, M., Willett, J. L., &Côté, G. L. (2002). A spectrophotometric assay for feruloyl esterases.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426-428.

Plackett, R. L., Burman, J. P. (1946). The design of optimum multifactorial experiments. *Biometrika*, *33*, 305–325.

Pothiraj, C., Balaji, P., &Eyini, M. (2006). Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. *African Journal of Biotechnology, 5*(20), 1882-1885.

Salgado, J. M., Rodríguez, N., Cortés, S., & Domínguez, J. M. (2012). Coupling two sizes of CSTR-type bioreactors for sequential lactic acid and xylitol production from hemicellulosichydrolysates of vineshoot trimmings.*New Biotechnology, 29*(3), 421-427.

Tabka, M. G., Herpoël-Gimbert, I., Monod, F., Asther, M., &Sigoillot, J. C. (2006). Enzymatic saccharification of wheat straw for bioethanol production by a combined

cellulasexylanase and feruloyl esterase treatment. *Enzyme and Microbial Technology, 39*(4), 897-902.