INCREASE OF PROTEIN CONTENT AND PRODUCTION OF LIGNOCELLULOLYTIC ENZYMES BY SIMULTANEOUS SOLID-STATE FERMENTATIONS OF BREWERY, OLIVE MILL AND WINERY WASTES.

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ABSTRACT

Frequently, the agro-industrial wastes are used as animal feed, however many times these wastes have a poor nutritional quality, mainly they have a low protein content and their digestibility is hard. These disadvantages can be avoided by solid-state fermentation (SSF) of the wastes by filamentous fungi.

Winery, brewery and olive mill wastes were used as solid substrates for the production of lignocellulolytic enzymes and to increase the crude protein content of agro-industrial wastes.

After selection of the fungus in previously study, it was performed an experimental design to evaluate the benefits of using mixtures of wastes in different proportions as solid substrate in SSF by *A. ibericus*. The optimum substrate was a mixture of brewery spent grain and vine-shoot trimmings which achieved and increase the protein content (16.3 %), xylanase (89.33 U/g), cellulose (3.46 U/g) and β -glucosidase (21.91 U/g) activities.

Through this study, it was possible to conclude that the SSF by *A. ibericus* is a suitable biotechnology process to increase the nutritional quality of agro-industrial wastes and to produce value-added products as enzymes in the same low-cost process.

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

INTRODUCTION

Winemaking generates residues characterized by high contents of biodegradable compounds and suspended solids. Grape pomace (GP) or marc is the main residue left after juice extraction by pressing grapes. Disposal of GP has long been a problem for wineries. This by-product is used as animal feed (with low nutritional value), for ethanol production by fermentation and distillation (low level benefit) and/or as organic fertilizers despite causing problems of phytotoxicity due to their high levels on phenolic compounds. In Portugal of about 0.3 million t in 2013 are generated (OIV, 2013).

In olive oil industries, the main system of oil extraction is the continuous 2-phase extraction process that generates as main waste olive pomace (OP) a wet olive cake that cannot be managed easily and most of the solutions proposed for traditional or 3-phase olive waste cannot be applied to OP. In Portugal, it is estimated that 86% of industries produce olive oil by two-phase system (INE, 2015) and generate 0.3 million of t of OP (INE, 2014).

Breweries generate liquid and solid residues that can be revalorized. Beer brewery wastewater (BBWW) is a collective term for the waste-water produced from the cleaning and cooling of fermentation units during the production of beer [1]. Other important solid waste is brewers spent grain (BSG) is a by-product of the brewing process corresponding to around 85% of total by-products generated [2].

The increase of global population causes an increase of food demand. Currently 33 % of croplands are used for livestock feed production, this is a key factor in deforestation. By using agro-industrial wastes as animal feed this problem can be reduced, food supply can increase and the environmental impact of these wastes can be reduced. However, the nutritional quality of these wastes is poor.

SSF is characterized by a fermentation process on a solid support, which has low moisture content. SSF produces a high product concentration but has a relatively low energy requirement [3]. This study seeks novelty simultaneous production of enzymes together animal feed production. After recovery of enzymes from fermented waste, this can be evaluated as animal feed. SSF has been used as pre-treatment to improve the nutritive value of agriculture by-products [4]. The growth of fungi in these by-products causes an increase in protein enriched and feed additives. The agro-industrial-wastes have low protein content (2-6%) and fermented waste can improve it to 8.47-17.08% [5].

MATERIAL AND METHODS

Solid-state fermentation process was carried out in 500 mL Erlenmeyer with 10 g of dry substrate. Moisture level was adjusted to 75 % (wet basis) with urea solution in water. Urea was added to adjust the ratio C/N to 15. When BSG was used as substrate, urea was not added because the C/N ratio was already near 15. Erlenmeyers with solid medium were sterilized at 121 ˚C for 15 minutes.
The inoculum spore concentration was adjusted to 10^6 spores/mL using a Neubauer counting chamber. Each Erlenmeyer was inoculated with 2 mL of spore suspension and incubated at 30 ˚C for 6 days. The extraction of enzymes was performed at the end of each experiment with a solution of 10 g/L NaCl and 5 g/L Triton X-100 at room temperature with agitation (150 r.p.m.) for 1 h. Following, extract was filtered through a net and the liquid fraction was centrifuge at 6000 r.p.m. for 10 minutes. The pellet was added to the filtrated solid and weighted and used for determination of lignocellulosic composition, total nitrogen, ashes and moisture; the liquid was recovered and enzymatic activity, soluble protein, phenolic compounds and reducing sugars were quantified by methods described in Leite et al. [6].

To evaluate the effect of mixture of agro-industrial wastes, a simplex-centroid design it was performed an experimental design (Simplex-centroid design) was carried out. This design consists of mixture runs characterized by all one factor, all combination of two factors at equal levels and all combinations of three factors at equal levels. In addition, a center point with equal amounts of all wastes is studied. Thus, this design allows to test four agro-industrial wastes as substrate and to evaluate the interaction effects among them in SSF. All experiments were performed in duplicate and in randomized run order. In runs with exhausted grape marc (EGM), exhausted olive pomace (EOP) and vineshoot trimmings (VTS). The variables dependents studied were xylanase, cellulose, β-glucosidases activities and the increase of N

RESULTS AND DISCUSSION

Table 1 describes the optimal conditions for each dependent variable and statistical parameters of the models. The Fisher test (F) can show better fit of the model, the higher value of F demonstrated a good fit. The determination coefficients (R^2) were from 0.95-0.98, which demonstrated that a satisfactory adjustment of the model, and indicating that 95-98 % of the variability in the responses could be explained by the model. As it would be expectable, the conditions that maximizes one dependent variable may not be the same that maximizes another dependent variable, for example, the maximum production of xilanases using beechwood as substrate was obtained on assay 13 but in the case of nitrogen, the maximum production was obtained in the assay number 6.
So, in order to select a unique optimal substrate composition that maximize every dependent variable, it was performed an optimization of multiple response using the software Statgraphics plus 5.1. The mixture of BSG and VST was the optimal substrate that maximizes all dependent variables. The theoretical maximum activities for every variable are identified on Table 2. If it compares the maximum enzymes production optimizing each dependent variable by separate (Table 1) and optimizing all at once (Table 2), it can be observed that there were not great differences. Thus, the optimum condition selected can achieved high enzymes activities and a high increase in protein content of fermented waste.

CONCLUSIONS

The mixture of wastes improved the lignocellulolytic enzymes production and protein content in comparison to the use of solids separately. The simplex-centroid design allowed to select the optimum substrate to maximize the enzyme production and the protein content. The multiple

response optimization selected only one combination of wastes that led to a maximum of all dependent variables studied. The optimum waste combination was BSG (0.54 g/g) and VTS (0.46 g/g).

Table 1- Optimum parameters for each dependent variable and statistical parameters.

	Enzyme activities (U/g)					
	Xylanase at 40 ºC	Xylanase at 50 ºC	Cellulase at 40 ºC	Cellulase at 50 ºC	B-glucosidase	Increase of N (%)
BSG (g/g)	0.6	0.53	0	0	0.46	0.5
EGM (g/g)	0	0	0.5	0.38	0	0
VTS (g/g)	0	0.2	0.5	0.38	0.38	0.5
EOP (g/g)	0.4	0.27	0	0.24	0.16	0
OV (U/g)	93.77	98.36	85.71	71.77	22.53	2.61
R^2	0.97689	0.98031	0.95045	0.96016	0.96678	0.95113
R^2 adj	0.95532	0.96194	0.90421	0.92298	0.93579	0.90553
F-ratio	45.3	53.36	20.55	25.83	31.19	20.86
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

BSG- brewery spent grain; EGM- exhausted grape marke; VTS- vine shoot trimings; EOP- exhausted grape pomace; OV- optimum value; R2- coefficient of correlation of a linear regression; R2- adjusted coefficient of determination

Table 2- Optimization of multiple response.

	Enzyme activities (U/g)					
	Xylanase at 50 ºC	Xylanase at 40 ºC	Cellulase at 50 ºC	Cellulase at 40 ºC	β-glucosidase	Increase PROTEIN %
Optimum Value	89.33	86.39	73.46	64.6	21.91	16.13
Experimental	84.92±1.52	87.19±1.72	63.57±3.67	74.43±3.09	21.81 ± 0.91	16.63±0.81
BSG- 0.54; EGM- 0.00; EGM- 0.00; VST- 0.46						

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