Wastes: Solutions, Treatments and Opportunities III – Vilarinho et al. (Eds)
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Improvement of antioxidant compounds extraction by SSF from agro-food wastes

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ABSTRACT: Mediterranean agro-industrial wastes are generated in huge amounts, mainly from olive mills, wineries and breweries. These wastes management poses serious environmental problems to the regions where they are generated. The objective of this work is to use the agro-industrial wastes as sources of phenolic compounds, improving its extraction from mixtures of olive pomace, brewer's spent grain and vine-shoot trimmings by solid-state fermentation (SSF) with *A. niger* and to obtain enzymes such as xylanases, cellulases and β-glucosidase. The results allowed to obtain the combination of wastes that maximized enzymes production and increased total phenolic compounds and antioxidant activity by SSF. Therefore, SSF showed to be an interesting valorization strategy to exploit agro-industrial wastes following the concept of circular economy.

1 INTRODUCTION

The industries of olive oil (2.8 Mt), wine (14.9 Mt), and beer (9.5 Mt) generate the majority of agro-industrial wastes in Mediterranean area. Mediterranean wastes represents respectively 92.8%, 51.2% and 5.3% of the total wastes generated annually by these industries in the world (FAOSTAT, 2014).

These wastes have generally no value for new applications, thus actions are being taken to change the society to a more environmentally friendly and resource-conserving. Circular economy is a concept that is gaining popularity because searches to reuse the wastes in a closed-loop (Ingrao et al., 2018; Korhonen et al., 2018).

Vegetables, fruits and beverages are the major sources of phenolic compounds in the human diet. The agricultural and food industries generate substantial quantities of phenolic-rich by-products, which could be a valuable natural sources of antioxidants (Balasundram et al., 2006). The main Mediterranean wastes are brewery spent grain (BSG) from brewery industry, olive pomace (OP) from olive oil industry, and vine-shoot trimmings (VST) from winery. Recently, it has been observed the increasing of the interest of scientific researchers for the study of biological properties of plants and active principles responsible for their therapeutic effects (Junio et al., 2011; Silva & Fernades Júnior, 2010).

Phenolic compounds can be extracted by conventional solvent extraction, such as microwave-assisted, Soxhlet, maceration, ultrasounds, high hydrostatic pressure and supercritical fluid extractions, among others (Ignat et al., 2011). However, the total recovery of them can be difficult because those compounds are present as insoluble bound to form conjugates with sugars, fatty acids or amino acids (Dey & Kuhad, 2014). In addition, the public awareness of health and environment along with safety hazards associated with the use of organic solvents in food processing and the possible solvent contamination of the final products together with the high cost of organic solvents, led to the development of a new and clean technology (Lafka et al., 2011).

Currently, enzymatic treatment for extraction of natural phenolics is a technique quite useful. Several microorganisms have the ability to produce a variety of enzymes under solid-state fermentation (SSF) (Dey & Kuhad, 2014). Besides that, SSF can be used for the production of some industrially important phenolic compounds, for the improvement of antioxidant potentials of solid substrates by increasing total phenolic compounds (TPC), and also for the bioavailability enhancement of them (Dey et al., 2016).

Filamentous fungi have the highest adaptability for SSF and are able to produce high quantities of enzymes with high commercial values (Dulf et al., 2017). *Aspergillus niger* has been used in many SSF studies. This fungi synthesize several food and industrial enzymes (cellulases, xylanase, *β*-glucosidase, protease, pectinase, *…) and has a significant role in the hydrolysis of phenolic* conjugates (Dulf et al., 2017). The enzymes break the lignocellulosic cell walls and transform insoluble phenolics into soluble-free phenolics (Bhanja et al., 2009; Đorđević et al., 2010; Dulf et al., 2016; Wang et al., 2014; Zheng et al., 2009).

This study evaluated the use of SSF as a clean strategy to extract antioxidant phenolic compounds from mixtures of agro-industrial wastes, producing lignocellulolytic enzymes linked to the release of phenolic compounds from lignocellulosic material, such as xylanase, cellulase and *β*-glucosidase.

2 MATERIAL AND METHODS

2.1 *Raw material*

Two olive pomaces were used, the organic crude olive pomace (COP^{org}) and the exhausted olive pomace (EOP) that were collected from olive oil industry (Trofa, Portugal). Brewer's spent grain (BSG) was obtained from the beer industry (Vila Verde, Portugal) and vine-shoot trimming (VST) from the winery industry (Ourense, Spain) during the 2016/2017 season. These residues were dried at 65°C during 24 hours and stored at room temperature.

2.2 *Microorganisms*

Aspergillus niger CECT 2088 from CECT (Valencia, Spain) culture collection was used. It was revived on malt extract agar (MEA) plates. To obtain inoculum for SSF, the selected fungi were cultured on MEA slants, and incubated at 25°C for 6 days.

2.3 *Solid-state fermentation*

SSF process was carried out in 500 mL Erlenmeyer with 10 g of dry substrate (wastes mixtures) sterilized at 121°C for 15 minutes. Compositions of media were defined in Table 1. Moisture level was adjusted to 75% (w/w) in wet basis with distilled water and urea was added to adjust de ratio C:N to 15. The inoculation were performed following the methods described by Salgado et al. (2014).

SSF were incubated at 25°C for 7 days. The extraction of enzymes and phenolic compounds was performed with distilled water at room temperature in an L:S ratio of 5 and with agitation for 1 h. Following, extracts were centrifuged (4000 g, 15 min), filtered through Whatman N°1 filter paper and stored at 4°C. Controls of each run were performed without inoculation of fungi.

2.4 *Simplex centroid mixture design*

To evaluate the effect of mixture of agro-industrial wastes, it was implemented an experimental design (Simplex centroid mixture design). This design consists in a mixture run 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 was studied. Thus, this design allowed to test four agro-industrial wastes as substrate and to evaluate the interaction effects among them in SSF (Table 1).

All experiments were performed in duplicate and in randomized order. In runs with COP^{org}, BSG, VST and EOP. The dependent variables studied were xylanase, cellulose, *β*-glucosidases

Table 1. Residues mixtures, obtained from Simplex centroid mixture design.

Run	A	B	C	D	COP^{org} (g)	BSG (g)	VST (g)	EOP (g)
1	1	0	0	0	10	0	0	0
2	0	1	0	0	0	10	0	0
3	0	0	1	0	0	0	10	0
4	0	0	0	1	0	0	0	10
5	0.5	0.5	0	0	5	5	0	0
6	0.5	0	0.5	0	5	0	5	0
7	0.5	0	0	0.5	5	0	0	5
8	0	0.5	0.5	0	0	5	5	0
9	0	0.5	0	0.5	0	5	0	5
10	0	0	0,5	0,5	0	0	5	5
11	0.33	0.33	0.33	0	3.33	3.33	3.33	0
12	0.33	0.33	0	0.33	3.33	3.33	0	3.33
13	0.33	0	0.33	0.33	3.33	0	3,33	3.33
14	0	0.33	0.33	0.33	0	3.33	3,33	3.33
15	0.25	0.25	0.25	0.25	2.50	2.50	2.50	2.50

activities and the increase of TPC and antioxidant capacity. A control of each experiment was performed without inoculation of fungus.

2.5 *Analysis of total phenolic compounds, antioxidant capacity and enzymes activity*

TPC, antioxidant activity by DPPH method, cellulases, xylanases and β-glucosidase activity was measured using the methods described by Leite et al., 2019.

3 RESULTS AND DISCUSSION

One of the main characteristics of SSF is the solid substrate. It acts as a physical support and source of nutrient during enzyme production. Thus the mixture of substrates can improve the enzymes growth and production, because it is difficult to acquire all the essential nutrients from the single substrate (Doriya & Kumar, 2018).

Simplex-centroid design allowed to optimize the combination of agro-industrial wastes as substrate for SSF in order to maximize the production of enzymes and the increase of TPC and antioxidant activity (Table 2).

Xylanase activity ranged from 55 to 710 U/g and the maximum value was found in 12th run consisting of 33% (w/w) of COP^{org}, 33% (w/w) of BSG and 33% (w/w) of EOP. Cellulase and β-glucosidase activity varied from 17 to 57 U/g and 43 to 262 U/g (Table 2) respectively. Maximum values were found in 5th run consisting of 50% (w/w) of COP^{org} and 50% (w/w) of BSG. On the other hand, the maximum values for the variation of antioxidant activity and TPC were found for 1st run (Table 2) in COP^{org}. Current experimental findings suggest that olive pomace (COP^{org} and EOP) and BSG have positive effects on enzymes production. COP^{org} is the best waste to obtain phenolic compounds with antioxidant activity.

Table 3 describes the optimal conditions for each dependent variable. It was concluded that the developed model can calculate the response accurately, with R^2 coefficients of 0.977 (xylanase activity), 0.960 (cellulase activity), 0.989 (β-glucosidase activity), 0.970 (variation of antioxidant activity) and 0.981 (variation of TPC).

In order to select a unique optimal substrate composition that maximize every dependent variable, it was performed an optimization of multiple response. The mixture of COP^{org} (42%, w/w), BSG (46, w/w) and EOP (12 w/w) was the optimal substrate that maximizes all dependent variables, with

Table 2. Enzymes activities, antioxidant capacity and TPC.

Run	Xylanase activity (U/g)	Cellulase activity (U/g)	β-glucosidase (U/g)	Variation of Antioxidant Activity	Variation of TPC
1	55 ± 3	25.1 ± 0.6	95 ± 5	26.6 ± 0.5	2.69 ± 0.45
2	395 ± 22	49 ± 4	214 ± 3	−3.2 ± 0.0	1,66 ± 0.06
3	83 ± 1	22 ± 2	43 ± 1	0.6 ±0.4	−0,34 ± 0.08
4	192 ± 2	20 ± 1	76 ± 4	−6.8 ± 2.2	0,73 ± 0.29
5	461 ± 6	56.2 ± 0.5	237 ± 9	4.6 ± 1.3	1,89 ± 0.07
6	82 ± 6	22 ± 1	84 ± 3	−1.7 ± 0.4	0,08 ± 0.11
7	69 ± 1	27 ± 6	84 ± 0	−9.5 ± 2.8	−0,05 ± 0.39
8	319 ± 5	57 ± 5	221 ± 11	−6.3 ± 1.2	0,91 ± 0.14
9	529 ± 19	56 ± 5	142 ± 1	1.7 ± 3.1	0,65 ± 0.32
10	63 ± 1	49.5 ± 0.0	98 ± 5	−3.6 ± 0.8	−0,02 ± 0.05
11	353 ± 38	17 ± 2	157 ± 3	−0.7 ± 0.5	−0,53 ± 0.18
12	710 ± 29	49.8 ± 0.5	262 ± 5	2.1 ± 1.4	−0,13 ± 0.52
13	104 ± 13	24 ± 0	95 ± 2	4.2 ± 3.6	0,01 ± 0.17
14	425 ± 16	54 ± 6	158 ± 8	−3.3 ± 0.8	−1,14 ± 0.23
15	652 ± 72	40 ± 3	158 ± 7	9.1 ± 4.7	−0,81 ± 0.17
16	547 ± 9	38 ± 2	148 ± 2	10.4 ± 1.2	−0,59 ± 0.06
17	649 ± 72	44.7 ± 0.3	167.4 ± 4.8	9.2 ± 0.4	−0,38 ± 0.06

Table 3. Optimum parameters for each dependent variable and statistical parameter.

	Xylanase activity (U/g)	Cellulase activity (U/g)	β-glucosidase (U/g)	Variation of Antioxidant Activity	Variation of TPC
	790.47	60.33	273.16	26.66	2.70
COP^{org} (g)	0.26	0.00	0.33	1.00	0.00
BSG (g)	0.44	0.55	0.49	0.00	0.00
VST (g)	0.00	0.24	0.00	0.00	0.00
EOP (g)	0.30	0.21	0.17	0.00	1.00
R^2	0.977	0.960	0.989	0.970	0.981

Table 4. Optimization of multiple response.

	Xylanase activity (U/g)	Cellulase activity (U/g)	β-glucosidase (U/g)	Variation of Antioxidant Activity	Variation of TPC
Optimum value	667.691	57	267.12	5.925	0.968

the theoretical maximum activities on Table 4. Significant differences can be observed when are compared the maximum enzymes production of each dependent variable (Table 3) and optimizing all at once (Table 4).

This work shows that mixtures of wastes are an effective solution for the improvement of SSF by filamentous fungi, leading to the enhancement of enzymes production and to the liberation of phenolic compounds, which is in accordance with several works in the literature (Kumar et al., 2018; Ohara et al., 2017; Oliveira et al., 2017; Sousa et al., 2018).

Zimbardi et al. (2013) optimized the production of β-glucosidase, β-xylosidase and xylanase. The maximal production occurred in the wheat bran, but Sugarcane trash, peanut hulls and corncob enhanced β-glucosidase, β-xylosidase and xylanase production, respectively. Maximal levels after optimization reached 159.3 ± 12.7 U g^{-1}, 128.1 ± 6.4 U g^{-1} and 378.1 ± 23.3 U g^{-1}, respectively. Salgado et al. (2015) also observed an increase of production of the lignocellulolytic enzymes with the mixture of crude olive pomace, VST and Exhausted Grape Marc (EGM).

Cai et al. (2011), Razak et al. (2015) and Singh et al. (2010) reported that the use of filamentous fungi in SSF enhances the phenolic compound release in cereals due to the produced enzymes action.

Therefore, SSF has been proven to be an excellent process for the improvement of antioxidant properties and nutritional quality, of a great variety of vegetables and cereals, including agro-industrial wastes (Dey et al., 2016).

4 CONCLUSIONS

The mixture of wastes and its use as substrate in SSF improved the extraction of antioxidant phenolic compounds and the production of lignocellulolytic enzymes in comparison to the use of each waste alone. The simplex centroid mixture design allowed to optimize the wastes combination to maximize the extraction of antioxidant phenolic compounds or the production of lignocellulolytic enzymes. The optimization of multiple response selected only one combination of wastes that led to a maximum of all dependent variables studied. The best wastes combination was composed of COP^{org}, BSG and EOP. SSF showed to be a suitable and clean technology to extract antioxidant compounds from agro-industrial wastes.

ACKNOWLEDGEMENTS

Paulina Leite is recipient of a PhD fellowship (SFRH/BD/114777/2016) supported by the Portuguese Foundation for Science and Technology (FCT). José Manuel Salgado was supported by grant CEB/N2020 – INV/01/2016 from Project "BIOTECNORTE – Underpinning Biotechnology to foster the north of Portugal bioeconomy" (NORTE-01-0145-FEDER-000004). This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2019 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte.

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