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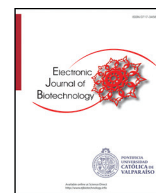
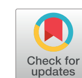


Research article

Ellagic acid production using polyphenols from orange peel waste by submerged fermentation



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ABSTRACT

Background: Biotechnological processes are part of modern industry as well as stricter environmental requirements. The need to reduce production costs and pollution demands for alternatives that involve the integral use of agro-industrial waste to produce bioactive compounds. The citrus industry generates large amounts of wastes due to the destruction of the fruits by microorganisms and insects together with the large amounts of orange waste generated during the production of juice and for sale fresh. The aim of this study was used orange wastes rich in polyphenolic compounds can be used as source carbon of *Aspergillus fumigatus* MUM 1603 to generate high added value compounds, for example, ellagic acid and other molecules of polyphenolic origin through submerged fermentation system.
Results: The orange peel waste had a high concentration of polyphenols, 28% being condensed, 27% ellagitannins, 25% flavonoids and 20% gallotannins. The major polyphenolic compounds were catechin, EA and quercetin. The conditions, using an experimental design of central compounds, that allow the production of the maximum concentration of EA (18.68 mg/g) were found to be: temperature 30°C, inoculum 2×10^7 (spores/g) and orange peel polyphenols 6.2 (g/L).
Conclusion: The submerged fermentation process is an effective methodology for the biotransformation of molecules present in orange waste to obtain high value-added as ellagic acid that can be used as powerful antioxidants, antibacterial and other applications.
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1. Introduction

The production of bioactive compounds using biotechnological methods has gained great interest in recent years. In this context, submerged fermentation processes are important in the food and pharmaceutical industry. The purpose of the process is to take advantage of agro-industrial waste rich in organic compounds that can use as a source of carbon and energy for the growth of microorganisms and the production of bioactive compounds. Bioactive compounds are a broad range of secondary metabolites in plants in which polyphenolic compounds stand out. Polyphenols are classified into condensed, phlorotannins, hydroxystilbenes, hydrolyzable and flavonoids [1]. Hydrolyzable polyphenols are divided into two large groups: gallotannins, molecules formed by a glucose core linked to a galloyl group by an ester bond; and the ellagitannins which are compounds formed by a glucose core linked to the hexahydroxydiphenic acid group by an ester bond [2]. Elagitannins are non-nitrogenated compounds with a molecular weight between 300 and 20,000 Da [3], amorphous, with an astringent taste, weakly acidic, soluble in water and found in the cytoplasm and vacuoles of plant cells as secondary metabolites [4].

There are few works that describe the use of agro-industrial waste to produce polyphenols (ellagitannins) using filamentous fungi in submerged fermentation systems. Some yields may vary because they use other sources of polyphenols, however, there are very similar studies, for example, a continuous bioreactor using elagitanase to hydrolyze ellagitannins from pomegranate husk, achieved a yield up to 175 mg/g of ellagic acid (EA) [5]. Residues from tea production were used as substrate in submerged fermentation to produce EA by *Aspergillus niger* (MTCC 281), the optimal conditions for maximum EA production being at 35°C for 96 h yielding up to 42.35 μg/g of EA [6]. In another study, valonia tannins were used as a carbon source to test the production of EA by *Aspergillus* SHL-6. The maximum yield of EA was reached with 100 mL of culture medium and 5 g of substrate at

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28°C and initial pH between 4.5–5 at 120 rpm for 72 h [7]. Huang et al. [8] tested oak acorns extracts to produce EA by *Aspergillus oryzae* and *Endomyces fibuliger* and a maximum production of EA was found when the two strains were used at 30°C, pH 5.0 and 5.6 g/L of initial substrate concentration, reaching a yield of 32% of EA.

The interest in producing these compounds is due to their biological activities that benefit human health. The anticancer activity of EA with a focus on its stimulating effects on doxorubicin hydrochloride and cisplatin in hepatocellular carcinoma was evaluated. Ellagic acid inhibited tumor growth without causing cardiotoxicity observed in mice [9]. Grirish et al. [10] evaluated the antidepressant effect of EA in mice, using the forced swimming and the tail suspension test. The results showed that EA administered to mice produced a significant reduction in the duration of immobility comparable to fluoxetine, suggesting an effect similar to that produced when an antidepressant is consumed. Because of the increasing incidence of antibiotic-resistant diseases, there is a need to find bioactive compounds that help to control these diseases, for example, raw extracts and fractions of five species of *Terminalia* collected in Tanzania were evaluated for their antimycobacterial effects using *Mycobacterium smegmatis* ATCC 14468 as a model. The results showed that the compounds present in the extracts that inhibit the microorganisms are ellagitannins, gallic acid and EA glycosides [11].

The aim of this work was to evaluate the biotransformation of orange peel polyphenols using a submerged fermentation system with *A. fumigatus* to produce bioactive compounds that are of interest in the food and pharmaceutical industry and other areas.

2. Material and methods

2.1. Orange peel waste

Orange peel waste was obtained from a coffee station at the University of Minho, Portugal. The orange peel waste discarded from the juice extraction process was cut, dehydrated (60°C, 48 h) and crushed until a particle size of less than 1 mm was obtained. This powder of orange peel waste was stored in black bags at room temperature until its use.

2.2. Extraction of polyphenols from orange peel waste

To obtain polyphenolic compounds, we used the methodology described by Buenrostro et al. [12] with some modifications. An aqueous extraction of the orange peel powder was carried out with water at 60°C in a ratio of 1:5 (*w*/*v*) for 30 min. Subsequently, the extracts were filtered through a muslin cloth. The aqueous extract was centrifugated at 3000 rpm for 15 min. To separate the polyphenolic compounds, Amberlite XAD-16 was packed in a glass column and the aqueous extract was fed to the column, using water as eluent to remove all the water-soluble compounds. Subsequently, ethanol was used to recover the polyphenolic compounds. Finally, the solvent was evaporated at room temperature for approximately 12 h and a fine powder was obtained. This powder was identified as total orange polyphenols (TOP) and stored in an airtight container protected from light.

2.3. Acid hydrolysis of orange peel waste

Once the TOP powder was obtained, its chemical characterization was carried out to establish the content of ellagitannins, gallotannins, flavonoids and condensed polyphenols these methodologies are described in points 2.6.2, 2.6.3, 2.6.4 and 2.6.5. Acid hydrolysis of the materials was carried out by methodology described by Ascacio-Valdés et al. [13] with some modifications; mixing 500 mg of TOP in screw-capped test tubes with 1.5 mL of the methanolic sulfuric acid solution (0.190 mL of concentrated sulfuric acid per mL of methanol). The tubes were closed and placed in a heating oven at 80°C for a period of 30 h. After hydrolysis, the tubes were uncovered for solvent removal and 1.5 mL of milli-Q water was added. Samples were placed in 2 mL vials and subjected to sonic vibration for 30 min, then centrifuged (4000 rpm, 10 min). The supernatants were recovered, and the precipitates were re-suspended with 1.5 mL of 96% ethanol. Subsequently, the samples were subjected to sonic vibration for 30 min and filtered through membranes of 0.45 μm for analysis by high performance liquid chromatography (HPLC), all analyzes were performed in triplicate.

2.4. Microorganism

The strain *Aspergillus fumigatus* MUM 1603 was proportionated from MUM (Micoteca of University of Minho, Braga, Portugal). The strain was activated in accordance with Leite et al. [14].

2.5. Experimental design

2.5.1. Kinetic of polyphenols production

The biotransformation process was carried out, at 200 rpm at 30°C, in 125 mL Erlenmeyer flasks reactors packed with a 25 mL volume heterogeneous mixture with the culture medium and orange peel polyphenols. A kinetic evaluation was carried out to determine the maximum tannin production time using a minimum mineral medium Czapeck-Dox previously inoculated with 2×10^7 spores of *A. fumigatus* per gram of material. Forty-two experiments were carried out, in triplicate, from 0–78 h with sampling every 6 h. Once the fermentation time had elapsed, the contents of the Erlenmeyer were passed through a vacuum system with a Whatman No. 41 filter and the obtained liquid was placed in an airtight container and refrigerated until further analysis.

2.5.2. Evaluation of the factors for the EA production

An experimental design of central compounds was used, where the effects of temperature, concentration of spores and polyphenols as a carbon source were evaluated and the amount of EA was taken as the response variable, as shown in Table 1. This design is constituted for a total of 48 treatments and five levels (-1, 0, $+1$) and the α values were -1.6818 and 1.6818. The final fermentation time was 54 h. Once the final fermentation time had elapsed, the contents of the Erlenmeyer were passed through Whatman No. 41 filter paper with the help of a vacuum system. The fermentation extracts were stored in an airtight container under refrigeration conditions until further analysis. All treatments were performed in triplicate. In addition, Table 2 shown the regression coefficients obtained from the experimental design. It is observed that the linear value of the amount of polyphenols is closer to zero, therefore it is the variable that most influences the release of EA.

2.6. Chemical analysis

2.6.1. Characterization of sugars in orange peel waste by HPLC

The samples were analyzed in a HPLC equipment (Jasco AS-2057 plus) and a pump (Jasco 880-PU, Tokyo, Japan) equipped with an infrared detector (Knauer K-2300). A Metacarb 87 H column (300 × 7.8 mm, Varian, USA) was used in a temperature-controlled chamber (Jhones Chromatography model 7971) at 60°C. The separation was carried out under the following conditions: H_2SO_4 mobile phase at 0.005 M and a flow of 0.7 mL/min.

2.6.2. Characterization of polyphenols in orange peel waste by ultrahigh resolution liquid chromatography (UHPLC)

The samples were analyzed by a Shimadzu Nexpera X2 equipped with a diode array detector (Shimadzu, SPD-M20A). The separation was carried out in reverse phase with an Aquity UPLC BEH C18

Table 1
Condensed matrix of central compounds design and response factor.

Treatment	Temperature	Inoculum	Polyphenols	Ellagic acid (mg/g)
A	−1	−1	−1	13.60 (±1.17)
B	−1	−1	1	11.99 (±1.01)
C	−1	1	−1	15.10 (±0.89)
D	−1	1	1	13.59 (±0.08)
E	1	−1	−1	12.64 (±0.62)
F	1	−1	1	3.44 (±1.49)
G	1	1	−1	2.86 (±1.21)
H	1	1	1	0.58 (±0.14)
I	−1.6818	0	0	6.21 (±0.00)
J	1.6818	0	0	0.76 (±0.05)
K	0	−1.6818	0	2.41 (±1.17)
L	0	1.6818	0	2.19 (±0.67)
M	0	0	−1.6818	18.68 (±0.68)
N	0	0	1.6818	14.65 (±1.86)
O	0	0	0	0.90 (±0.26)

Levels					
Factors	(+) α	1	0	−1	(−) α
Temperature (°C)	38	33	30	27	22
Inoculum (spores/g)	2.8×10^7	2.3×10^7	2×10^7	1.7×10^7	1.2×10^7
Polyphenols (g/L)	8.8	8	7.5	7	6.2

column (2.1 mm × 100 mm, 1.7 μm, from Waters) and a precolumn of the same material at 40°C. The injection flow was 0.4 mL/min and the solvents (HPLC grade) were: (A) water/ 0.1% formic acid, and (B) acetonitrile. The elution gradient for solvent B was as follows: 0 to 5.5 min (5%), 5.5 to 17 min (60%), 17 to 18.5 min (100%), 18.5 to 30 min returned to 5%. The phenolic compounds were identified by comparison with the UV spectrum and the retention time corresponding to the standards. The compounds were quantified and identified at 280 nm. For the quantification of ellagitannins, the ellagic acid standard was used as equivalent.

2.6.3. Determination of total hydrolysable polyphenols in submerged fermentation

The determination of total hydrolysable polyphenols was carried out according to the methodology reported [15], with some modifications. Gallic acid was used as standard in a concentration range of 0.2–2 g/L. In a 96-well microplate, 5 μL of the sample (distilled water as a blank) and 60 μL of 15% sodium carbonate (Na_2CO_3) plus 15 μL of the Folin–Ciocalteu reagent were added and mixed. The microplate was incubated at 50°C for 5 min and allowed to cool to room temperature. Absorbance readings were performed on an ELISA microplate analyzer (BioTek) at 700 nm.

2.6.4. Determination of total flavonoids in the submerged fermentation

The determination of total flavonoids was carried out according to the methodology reported [16] with some modifications. Quercetin was used as a standard in a concentration range of 0–1 g/L. In a 96-well microplate, 25 μL of the sample, 200 μL of 60% ethanol and 5 μL of 5% sodium nitrite ($NaNO_2$) were added, allowed to stand for 6 min. Subsequently, 5 μL of aluminum chloride ($AlCl_3$) at 10% was added, allowed to stand for 6 min. Finally, 15 μL of 4% sodium hydroxide (NaOH) was added. Absorbance readings were performed on an ELISA microplate analyzer (BioTek) at 415 nm.

2.6.5. Determination of condensed polyphenols

The determination of condensed polyphenols was carried out according to the methodology reported [17] with some modifications. Catechin was used as a standard in a concentration range of 0–1 g/L. An aliquot of 0.5 mL of the sample was placed with 3 mL HCL/tert-butanol (10% ratio 1:9), and a aliquot of 0.1 mL of $NH_4Fe(SO_4)_2$ in 20% HCl was added. The mixture was sealed hermetically and hat for 1 h at 100°C in a hot water bath. Posteriorly, the samples were cooled and placed in a 96 well microplate, the absorbance readings were performed on an ELISA microplate analyzer (BioTek) at 460 nm.

3. Results and discussion

3.1. Characterization of orange peel waste polyphenols

The percent mass composition of polyphenols present in orange peel waste is shown in Fig. 1. Condensed were 28% of the orange peel waste, followed by 27% of ellagitannins, 25% of flavonoids and 20% of gallotannins. The composition of these percentages come from the hydrolysis of the carbon source since these groups are the main components. Further, there are few works that explain the composition of polyphenols present in agro-industrial waste that can be used in biotechnological processes. However, the potential of the byproducts of the vinification of *Vitis vinifera* L. (orujo) as a promising and renewable source of compounds of nutraceutical interest has been evaluated. They found that the highest proportion of polyphenolic compounds belong to the family of hydrolysable polyphenols, values of 12.3 mg/g were obtained [18]. In another study, it was evaluated the composition of polyphenolic compounds from solid waste from the essential oil industry. Using marjoram residues, the content number of total polyphenols found was 22% [19]. The composition of condensed polyphenols in this study was similar to the reported by Mercado-Mercado et al. [20], where the bio-accessibility of polyphenols from the plant *Hibiscus sabdariffa* was evaluated and it was found that a conventional decoction yields to 26.4% of condensed polyphenols. In another work, it was evaluated the antioxidant activity of polyphenols from different onion varieties using aqueous extractions. The polyphenols obtained were 1.95 mg/g of the total polyphenol content in the Ruby Ring variety, and 0.33 mg/g in the Stanley variety [21].

The polyphenols identified in the orange peel waste in this study were: in greater proportion, catechin, EA and quercetin (2.36, 2.22 and 2.06 mg/g respectively) and in lesser proportion ferulic acid, p-coumaric acid, epicatechin and o-coumaric acid (0.57, 0.45 and

Table 2
Regression coefficients obtained from the design of central compounds.

Factor	Coefficients	Std. Err.	t(38)	p	−95% Cnf. limit	+95% Cnf. limit
Mean/Interc	133.4851	31.4980	4.2379	0.0001	69.7207	197.2495
(1)Temperature (L)	23.4041	12.0892	1.9360	0.0603	−1.0693	47.8774
Temperature (Q)	−11.8332	14.6782	−0.8062	0.4252	−41.5476	17.8811
(2)Inoculum (L)	0.7706	12.0892	0.0638	0.9495	−23.7027	25.2440
Inoculum (Q)	−23.9543	14.6782	−1.6320	0.1109	−53.6686	5.7601
(3)Polyphenols (L)	−0.9838	12.0892	−0.0814	0.9356	−25.4571	23.4896
(3)Polyphenols (Q)	−32.7224	14.6782	−2.2293	0.0318	−62.4368	−3.0081
1L by 2L	−10.9750	15.7953	−0.6948	0.4914	−42.9509	21.0009
1L by 3L	−8.5167	15.7953	−0.5392	0.5929	−40.4926	23.4593
2L by 3L	9.8583	15.7953	0.6241	0.5363	−22.1176	41.8343

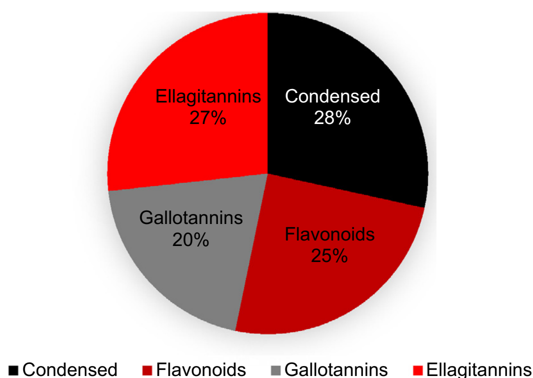

Fig. 1. Composition of tannins presents in the purified polyphenols of orange peel waste.

0.44 mg/g respectively) are shown in Fig. 2. There are some residues of the genus *Citrus* rich in polyphenolic compounds, for example Safdar et al. [22], investigated the content of polyphenols obtained from tangerine peels by means of maceration and extraction assisted by ultrasound and found that for extracts with 100% methanol can be obtained: gallic acid (37.86 μg/g), chlorogenic acid (18.48 μg/g), ferulic acid (50.16 μg/g), caffeic acid (1.28 μg/g) and epicatechin (20.54 μg/g) among others. In another study, polyphenols were extracted by acid hydrolysis from tangerine residues. They were able to obtain yields for extractable and non-extractable hesperidin (259.86 and 182.52 mg/g, respectively) as well as non-extractable gallic acid (36.08 μg/g). In addition, these compounds showed a high antioxidant activity [23].

3.2. Kinetic evaluation of a submerged system to produce polyphenols

The concentration of total polyphenols and flavonoids in relation to the fermentation time is shown in Fig. 3. For the case of flavonoid compounds, there is a slight increase from 0 to 12 h reaching a maximum concentration of 0.76 g/L; later the concentration of flavonoids decreased until reaching 0.42 g/L at 78 h. On the other hand, there are no significant changes in the concentration of total polyphenols until 54 h corresponding to a maximum of 1.68 g/L; later the concentration of polyphenols decreased until it reached 1.26 g/L at 78 h. This behavior may be due to the diauxic phenomenon, where it is related to the growth of the microorganism and the capacity it has to degrade more available sugars and subsequently in other compounds [24]. Based on the results obtained, the time of 54 h was considered to carry out the subsequent analyzes.

Due to the high content of EA in the orange peel samples and the diverse biological properties it presents for the human being, it was decided to study the factors that affect its production in a submerged system of fermentation using orange peel waste.

3.3. Factors affecting the EA production by submerged fermentation

The EA production from the design of central compounds, as shown in Table 1. The results indicate that the highest concentration of EA was obtained in treatments M and N reaching values of 18.68 and 14.65 mg/g respectively. Treatments H, J and O achieved the lowest concentrations of 0.58, 0.76 and 0.90 mg/g respectively. There are few works describing the production of EA from orange peel. In a study conducted by Madeira Jr. et al. [25], the biotransformation phenols from citrus residues by fermentation in solid medium using *Paecilomyces variotii* was evaluated, considering the effect of particle size, water-substrate ratio and temperature using a design of central The highest concentration of EA (0.017 mg/g), a much lower value than the one reported in our study was obtained for a particle size 2.0 mm, mass ratio 2:1 and 32°C.

The Pareto diagram of the estimated effects for the factors evaluated in the design of central compounds is shown in Fig. 4. The dotted line delimits the value of $p < 0.05$, the factors that go beyond this line directly influence the production of EA. In this study, only the concentration of polyphenols in its quadratic effect surpassed this line. The rest of the factors together with their corresponding interactions do not influence the process of the EA production.

Fig. 5a shows the effect of temperature and the inoculum on the EA production. In light gray, it is shown the minimum amount of EA that can be obtained in the process. The deep gray color shows the maximum amount of EA. In the contour diagram a tendency is observed that at the positive axial values of temperature (38°C) and low values of inoculum (1×10^7 spores/g) the maximum production of EA is reached.

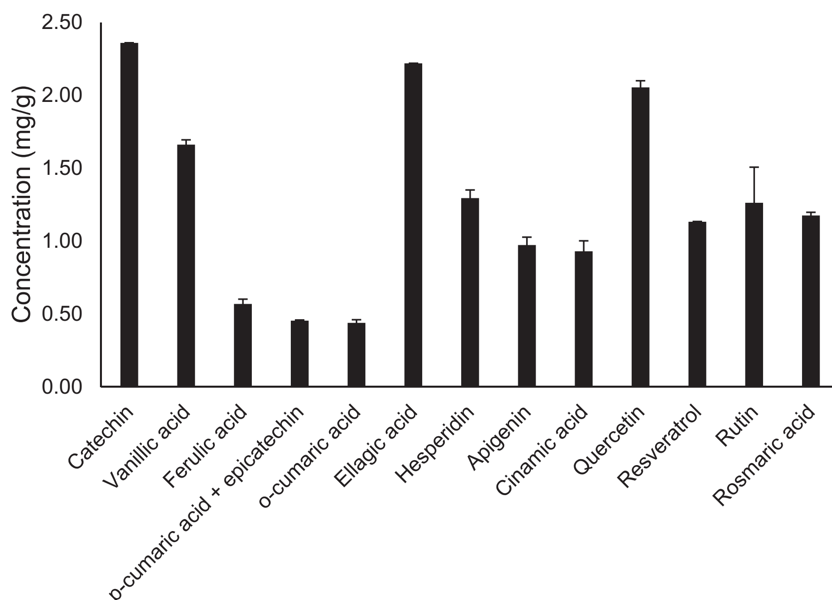

Fig. 2. Concentration of polyphenols in orange peel waste.

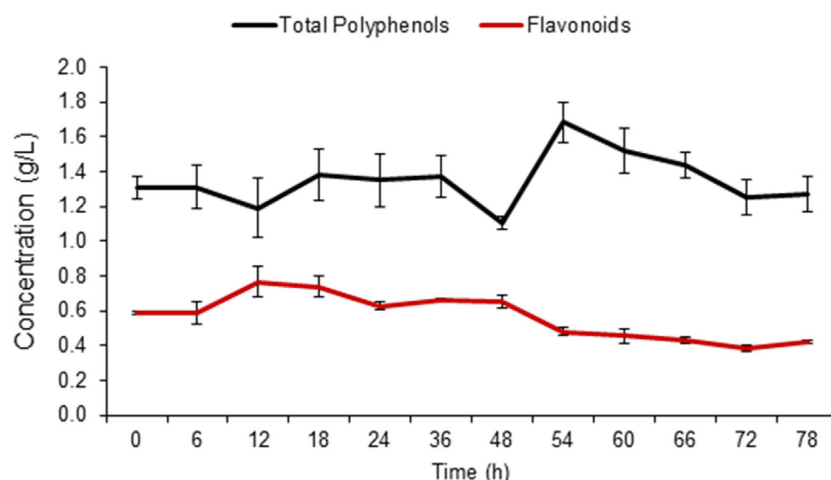

Fig. 3. Concentration of total polyphenols and flavonoids in relation with fermentation time.

In Fig. 5b, the effect of the inoculum values and the concentration of polyphenols on the production of EA is shown. The contour diagram shows that the maximum production of EA is reached at central values of inoculum (2×10^7 spores/g) and central values of polyphenols (7.5 g/L).

In Fig. 5c, the effect of the temperature and the concentration of polyphenols on the production of EA is shown, being shown that the maximum production of EA is reached.at a high temperature (33°C) and central values of polyphenols (7.5 g/L).

Few are the works that explain the effect of the fermentation factors in the submerged fermentation of orange peel polyphenols for the EA production. However, in a similar system, Sepúlveda et al. [26] evaluated the ideal conditions to produce EA using *A. niger* GH1 in a submerged culture of pomegranate peel. They evaluated the influence of substrate, pH and agitation using an experimental design Box–Behnken with five treatments and obtained a maximum production of EA of 20.66 mg/g at a substrate concentration of 7.5 g/L, pH 5.5 and 150 rpm. Also, they studied a submerged fermentation system using *A. niger* GH1 with tannic acid as a source of carbon and energy and found that tannic acid biodegradation leads to other molecules such as gallic acid and pentagaloyl glucose. These biodegradation products are generated due to the physiology of the microorganism and the growing conditions [27]. In another investigation, the submerged fermentation process was studied using low quality green tea waste as a raw material and a strain of *A. niger*. Authors considered factors such as the size of the inoculum and the speed of rotation in the process, using a Box-Bhenken design to maximize their production. They concluded that this bioprocess allows the obtention of black tea with high amounts of phenolic compounds [28]. In a similar study, phenolic compounds from green tea leaves in a solid culture of *Aspergillus fumigatus* were obtained. In this process, a yield of up to 151 g/kg of green tea leaves was achieved being concluded that the microorganism used in the bioprocess has the enzymatic capacity to convert the polyphenols present in the plant material [29]. The use of the submerged fermentation process is of great importance since it is an economic methodology, where low-cost agro-industrial waste can be used to obtain molecules with important biological activities in humans.

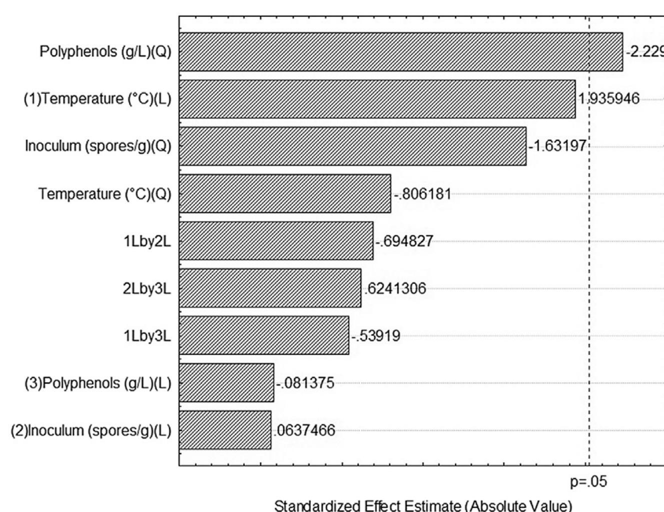

Fig. 4. Standardized effects to factors evaluated in the central compounds design.

4. Conclusion

It was found that orange peel waste had a high concentration of polyphenols, 28% being condensates, 27% ellagitannins, 25% flavonoids and 20% gallotannins. The major polyphenolic compounds were catechin, EA and quercetin. In the submerged fermentation process, it was found that 54 h was the time for obtaining the maximum concentration of total polyphenols. The conditions, using an experimental design of central compounds, that allow the production of the maximum concentration of EA (18.68 mg/g) were found to be - temperature 30°C, inoculum 2×10^7 and orange peel polyphenols 6.2 g/L. The concentration polyphenols of orange peel are the factor that most influences the production of EA. The submerged fermentation process is an effective methodology for the biotransformation of molecules present in orange waste to obtain high value-added polyphenols that can be used as powerful antioxidants, antibacterial and other applications.

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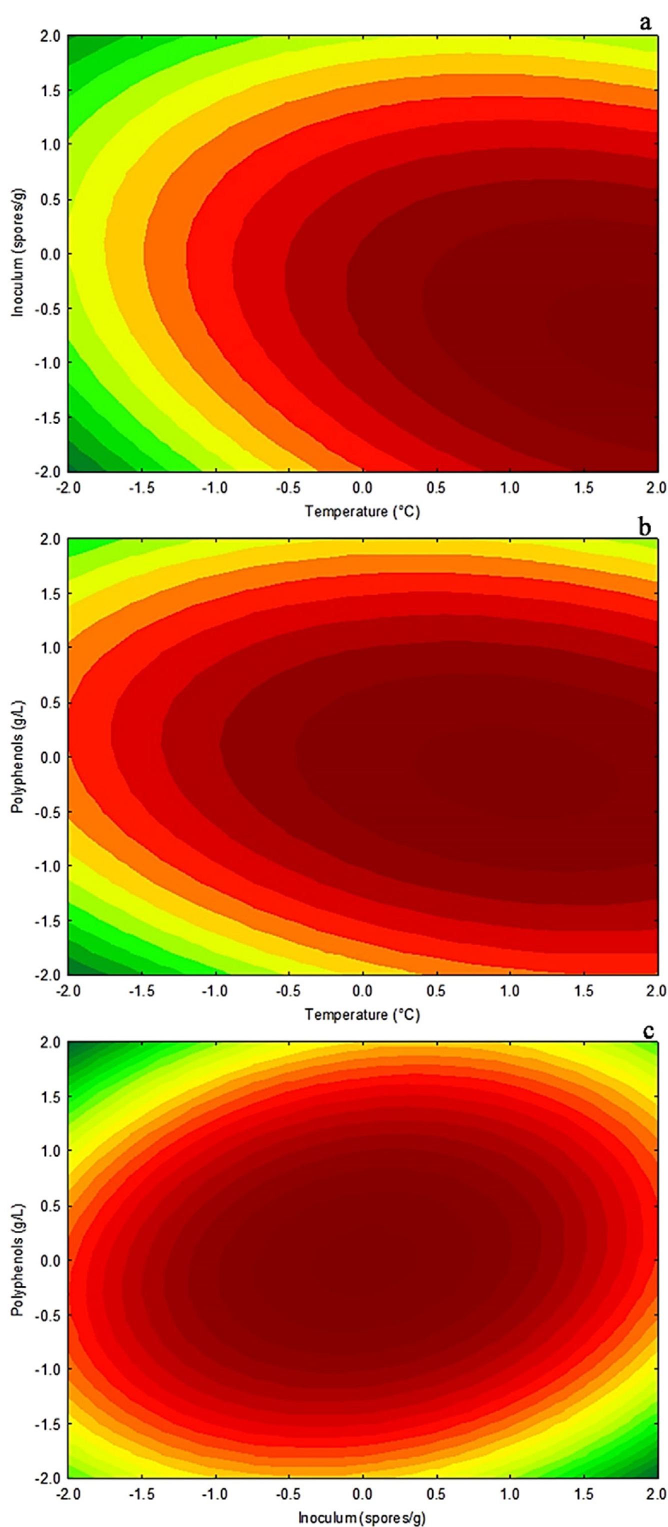

Fig. 5. a shown contour plot of effect the temperature and inoculum on EA production. The fig. 5b shown contour plot of effect the temperature and polyphenols concentration on EA production. Fig. 5c shown contour plot of effect the inoculum and polyphenols concentration on EA production.

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