Increase of content and bioactivity of total phenolic compounds from spent coffee grounds through solid state fermentation by *Bacillus clausii*

Jesús J. Rochín-Medina^{1,2} · Karina Ramírez^{1,2} · Jesús G. Rangel-Peraza² · Yaneth A. Bustos-Terrones²

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Abstract Spent coffee grounds are waste material generated during coffee beverage preparation. This by-product disposal causes a negative environmental impact, in addition to the loss of a rich source of nutrients and bioactive compounds. A rotating central composition design was used to determine the optimal conditions for the bioactivity of phenolic compounds obtained after the solid state fermentation of spent coffee grounds by *Bacillus clausii*. To achieve this, temperature and fermentation time were varied according to the experimental design and the total phenolic and flavonoid content, antioxidant activity and antimicrobial activity were determined. Surface response methodology showed that optimum bioprocessing conditions were a temperature of 37 °C and a fermentation time of 39 h. Under these conditions, total phenolic and flavonoid contents increased by 36 and 13%, respectively, in fermented extracts as compared to non-fermented. In addition, the antioxidant activity was increased by 15% and higher antimicrobial activity was observed against Gram positive and negative bacteria. These data demonstrated that bioprocessing optimization of spent coffee grounds using the surface response methodology was an important

tool to improve phenolic extraction, which could be used as an antioxidant and antimicrobial agents incorporated into different types of food products.

Keywords Spent coffee grounds · Surface response methodology · *Bacillus clausii* · Phenolic content · Antimicrobial activity · Optimization

Introduction

Coffee has become one of the most popular drinks consumed by many people around the world (Mussatto et al. 2011a, b). In recent years, the coffee industry has significantly increased and today, this industry ranks second in the international market after oil. This panorama provides an overview of the impact of this product on export trade for producing countries (Wong Paz et al. 2013). In Mexico, coffee production is distributed in 13 states, where Chiapas, Veracruz, Puebla and Oaxaca are the main producers, with more than 80% of the national production (SIAP-SAGARPA 2016). One of the main problems in this coffee industry is the release of large quantities of by-products (caffeine, tannins, and an important organic load present in the husk and coffee pulp that is discarded and not used for beneficial purposes), which are a source of serious pollution that generates environmental problems and represent a threat to ecosystems, because their hazardous disposal leads to water and soil contamination (Pedraza-Beltrán et al. 2012; Woldesenbet et al. 2015). On the other hand, the spent coffee grounds produced by coffee machines and coffeemakers are wasted with large amounts of nutrients rich in carbohydrates, proteins, pectins, and polyphenols recently considered as a potential renewable resource (López-Barrera et al. 2016).

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✉ Karina Ramírez
kramirez@itculiacan.edu.mx

¹ Departamento de Ingeniería Bioquímica, Instituto Tecnológico de Culiacan, Juan de Dios Batiz 310, 80220 Culiacan, Sinaloa, Mexico

² Division de Estudios de Posgrado e Investigación, Instituto Tecnológico de Culiacan, Juan de Dios Batiz 310, 80220 Culiacan, Sinaloa, Mexico

Solid state fermentation (SSF) is a biotechnology that involves the growth of different microorganisms on solid materials without the presence of free liquid (Hölker and Lenz 2005). This bioprocess has several advantages, such as low cost, low amount of water and energy requirements, less waste generation, does not require strict aseptic conditions, and improves the nutritional value and content of bioactive compounds from residues. The SSF represents a biotechnological alternative that has been used to process a large variety of legumes and cereals, thus improving the nutritional and nutraceutical properties, as well as obtaining food products with acceptable sensorial characteristics (Sánchez-Magaña et al. 2014; Rochín-Medina et al. 2015). This methodology has contributed to generate interest in by-products, such as spent coffee grounds, that could be economically attractive once they are recovered, improved in nutritional and nutraceutical value, and used as a functional ingredient to complement food products.

Bacillus clausii, is a Gram positive microorganism that form spores and has been used as a probiotic that confers health benefits to the host when given in adequate amounts (Urdaci et al. 2004; Cutting 2011). However, there are scarce reports about the optimization of spent coffee grounds using these bacteria to increase the phenolic and flavonoid content and their bioactivity. The aim of this study was to optimize the solid state fermentation conditions of spent coffee grounds using *Bacillus clausii* in order to increase the content of bioactive compounds and to evaluate their antioxidant and antimicrobial activity.

Materials and methods

Materials and bacterial strains

Spent coffee grounds were obtained from Arabica coffee beans medium roasted. *Bacillus clausii* was isolated from a commercial product (Sinuberase®) and cultured in tripticasin soy agar (MCD Lab, Tlalnepantla, Mexico), and stored at $-70\text{ }^{\circ}\text{C}$. *Listeria monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* ATCC 12022, and *Enterococcus faecalis* ATCC 29212 were provided by the National Laboratory for Research in Food Safety (LANIIA) from CIAD Culiacan. Viable bacteria concentrations were estimated by serial dilution method on Trypticase Soy Agar (TSA, Difco, USA).

Optimization: Experimental design and statistical analysis

For the optimization of the parameters to obtain bioactive compounds from fermented spent coffee grounds, the

Response Surface Methodology (RSM) and a central composite rotatable experimental design with two factors in five different levels (fermentation temperature, $X_1 = \text{FT} = 30, 32, 35, 38, 40\text{ }^{\circ}\text{C}$; fermentation time, $X_2 = \text{Ft} = 6, 12, 27, 42, 48\text{ h}$) were used. The statistical design generated 13 treatments. All experiments were performed in four independent experiments and mean values of data are reported. The response variables considered were total phenolic content (TPC), total flavonoid content (TF), and antioxidant activity (AA). Data were experimentally adjusted to second order experimental models for each response variable studied. Subsequently, the non-significant terms ($P > 0.05$) were eliminated from the second-order polynomials and new prediction models were calculated for each response variable. Statistical analysis was performed using Design Expert version 7.0.0 software (Stat-Ease, Minneapolis, MN, USA). The RSM was applied to obtain the maximum values (optimization) of phenolic compounds, total flavonoids and antioxidant activity of fermented spent coffee grounds. Predictive models were used to plot the systems. The overlapping contour graphs of each response variable allowed to determine the optimal temperature and bioprocessing time combination for the production of fermented spent coffee ground flours. The product obtained from the optimum bioprocessing conditions was used in the antimicrobial activity assay.

Spent coffee grounds fermentation

Ten grams of spent coffee grounds were obtained from the coffee filter of a home-made coffee maker; then, they were sterilized and placed in sterile glass petri dishes to start the SSF bioprocess. Samples with a water activity value of 0.96 ± 0.08 and a humidity of $84 \pm 1.02\%$, were inoculated with 1.5×10^8 CFU/mL of *B. clausii*, using the 0.5 McFarland scale and incubated with different temperature ($30\text{--}40\text{ }^{\circ}\text{C}$) and fermentation times ($6\text{--}48\text{ h}$), according to the statistical design. After bioprocess, samples were stored at $-20\text{ }^{\circ}\text{C}$ and protected from light until use.

Phenolic extracts

Extracts from fermented spent coffee grounds were obtained as described previously by Mora-Rochin et al. (2010). Briefly, 1 g (± 0.1 g) of the sample was defatted with 10 mL of hexane and homogenized with 10 mL of a cold ethanol–water solution (80:20 v/v) on an orbital shaker (OVAN noria R, USA) at $30\text{ }^{\circ}\text{C}$ and 50 rpm for 3 h. The mixture was centrifuged ($3000 \times g$ for 10 min) and the supernatant was collected and evaporated to dryness at $35\text{ }^{\circ}\text{C}$ using low pressure. The evaporated residue was reconstituted in 50% methanol, then an aliquot was used to

determine the total phenolic and flavonoid content and the antioxidant activity. Other aliquot was reconstituted in sterile peptone water for antimicrobial evaluation. Data shown are representative of four independent experiments.

Phenolic and flavonoid quantification

Total phenolic content from extracts were determined using the methodology by Singleton et al. (1999). Briefly, 20 μL of the extract was oxidized with 180 μL of the Folin–Ciocalteu reagent. After 3 min, 50 μL of 7% sodium carbonate was added and incubated at room temperature, protected from light. After 90 min, the absorbance was measured at 750 nm using a cell spectrophotometer (Genesys 10 vis, Thermo Scientific). A calibration curve was prepared using gallic acid as standard and the total phenolic content was expressed as mg of gallic acid equivalent (mg GAE)/100 g of sample. For flavonoid quantification, 20 μL of the extract were poured into 80 μL of distilled and subsequently 6 μL of 5% NaNO_2 was added. After 5 min, 12 μL of 10% AlCl_3 was added to the sample followed by 40 μL of 1 M NaOH. Finally, after 30 min the absorbance was measured at 510 nm. A calibration curve was prepared using catechin as standard and the flavonoid content was expressed as mg catechin equivalents (mg CE)/100 g of sample (Xu and Chang 2007). All determinations were evaluated in four independent experiments.

Antioxidant activity

The hydrophilic antioxidant activity of phenols was determined by the ABTS radical cation discoloration test using a cell spectrophotometer (Genesys 10vis, Thermo Scientific). For the ABTS assay the extracts were evaluated against a Trolox standard as a probe, as described by Re et al. (1999). The results of the ABTS assays were expressed as μmol of Trolox equivalents (TE)/100 g of sample. The determinations were evaluated in four independent experiments.

Antimicrobial activity

The disk-diffusion method was used to evaluate the phenolic extracts antimicrobial activity in different strains, such as *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Enterococcus faecalis*. All microorganisms were grown in Brain Heart Infusion broth (BHI; MCD Lab, Tlalnepantla, Mexico) at 37 °C for 24 h. Each microorganism was maintained through cross-striations in trypticase soy agar (MCD Lab, Tlalnepantla, Mexico) adjusted to a density of 1×10^8 CFU/mL using the 0.5 McFarland scale (Schwalbe et al. 2007) in sterile

peptone water. Each tested microorganism was diffused through the medium (Mueller–Hinton agar) by smears. Ten microliters of the fermented or non-fermented phenolic extracts were placed on a sterile disk of 6.5 mm in diameter in contact with the microorganism culture, and incubated at 37 °C for 24 h. Data were obtained by measuring the clear inhibition halos around each strain and reported as millimeters (mm) of inhibition. Three replicates were performed per strain.

Minimum inhibitory concentration (MIC)

The antimicrobial activity was evaluated by the minimum inhibitory concentration methodology according to Wanger (2007), with minor modifications. Briefly, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Enterococcus faecalis* were incubated for 24 h at 37 °C in BHI broth and adjusted to a final concentration of 1×10^8 CFU/mL using the 0.5 McFarland scale. A sample of 100 μL of each bacterial strain was individually poured into a culture tube containing 800 μL of sterile BHI broth. Then, 100 μL of different fermented and non-fermented phenolic extract concentrations (5, 10, 20, 30, 40, 50, 60 mg of phenolic extract/mL) were added to the tube and incubated for 24 h at 37 °C. Inoculated cultures without extract were used as positive controls. Negative controls included culture media with extracts and without bacteria. The MIC was determined as the lowest concentration of phenolic extract which could inhibit visible growth. Three replicates were performed per strain.

Statistical analysis

The antimicrobial evaluation data were analyzed by a single-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons among groups. Mean differences with $P < 0.05$ were considered significant. Statistical analysis was performed using Minitab 16.0 (State College, PA).

Results and discussion

Optimal conditions for solid state fermentation

The total phenol content (TPC), total flavonoids (TF) and antioxidant activity (AA) of 13 experiments with different fermentation temperature and time conditions were evaluated to determine the optimal conditions of spent coffee grounds fermentation by *Bacillus clausii*. As shown in Table 1, values for TPC, TF and AA ranged from 805 to 1121 mg GAE/100 g sample, 365 to 458 mg CE/100 g

Table 1 Experimental design from different solid state fermentation process variable combinations and results from response variables

Assay ^a	Process variables		Response variables		
	Fermentation temperature (°C)	Fermentation time (h)	TPC ^b	TF ^c	AA ^d
1	32	12	880	365	6312
2	39	12	920	409	7789
3	32	42	1010	408	14,765
4	39	42	1121	435	18,321
5	30	27	960	391	11,544
6	40	27	1040	458	15,512
7	35	6	805	365	4021
8	35	48	1090	415	17,490
9	35	27	950	429	14,212
10	35	27	930	420	14,598
11	35	27	925	425	14,256
12	35	27	917	420	14,566
13	35	27	920	421	14,867

^aDoes not correspond to processing order^bTPC Total phenolic content (mg GAE/100 g sample, dw)^cTF Total flavonoid (mg CE/100 g sample, dw)^dAA Antioxidant activity (μmol TE/100 g sample, dw)**Table 2** Simplified statistical analysis of mathematical models

Models of coded response variables	Model	R ²	Lack of fit
$Y_1 = 928.40 + 33.02 X_1 + 91.76 X_2 + 38.05 X_{11} + 11.80 X_{22} + 17.75 X_1 X_2$	< 0.001	0.95	$P = 0.26$
$Y_2 = 423.0 + 20.72 X_1 + 17.46 X_2 - 17.25 X_{22} - 4.25 X_1 X_2$	< 0.001	0.94	$P = 0.36$
$Y_3 = 14499.80 + 1330.57 X_1 + 4754.13 X_2 - 572.15 X_{11} - 1958.40 X_{22} + 519.75 X_1 X_2$	< 0.001	0.95	$P = 0.39$

 Y_1 = Total phenolic content (TPC) Y_2 = Total flavonoid (TF) Y_3 = Antioxidant activity (AA) X_1 = Fermentation temperature (°C) X_2 = Fermentation time (h)

sample (dw) and 4021 to 18 321 μmol TE/100 g (dw), respectively. The analysis of variance of the regression models showed that TPC, TF and AA were significantly depended on the linear and quadratic terms of fermentation temperature (FT) and fermentation time (Ft) as well as their interaction (Table 2). The total variation in TPC (95%), TF (94%) and AA (95%) could be explained by regression models ($P < 0.0001$), where the lack of fit was not significant ($P > 0.05$) and the coefficients of variation of prediction models were < 10%, suggesting that the experimental models were reproducible and well correlated. In general, TPC, TF and AA values were higher along with increasing fermentation temperature and time, with peak values between 37 and 40 °C and 30–48 h for TPC (Fig. 1a, b), 38–40 °C and 25–45 h for TF (Fig. 1c, d); and 35–40 °C and 35–48 h for AA (Fig. 1e, f). The

fermentation time was the factor that exhibited the highest effect on TPC and AA, while the fermentation temperature showed the highest effect on TF.

Optimization process

The best combination of process variables in the production of fermented spent coffee grounds under optimal bioprocessing conditions is shown in an overlaid contour plot for ideal responses (Fig. 2). The center point of the optimization region corresponded to the optimal combination of bioprocess variables (FT = 37 °C and Ft = 39 h) with TPC, TF and AA in the highest values. TPC, TF and AA predicted values, obtained by prediction models and optimal biological processing conditions were 1051 mg GAE/100 g sample (dw), 435 mg CE/100 g

Fig. 1 Response surface and contour plots showing the relationship between temperature and fermentation time on total phenol content (a, b), total flavonoids (c, d) and antioxidant activity (e, f)

sample (dw) and 17,894 $\mu\text{mol TE}/100 \text{ g}$ sample (dw). The statistical model was validated for the optimal fermentation conditions obtained. An experiment under the aforementioned conditions was replicated 10 times on different days (Online Resource 1) and the coefficients of variation of the analyzed variables indicated that the optimal solid state fermentation conditions fitted and were reproducible.

Effect of fermentation on TPC, TF and AA

As shown in Table 3, we demonstrated the fermentation effect on spent coffee grounds compared with samples without bioprocessing. In general, TPC was significantly increased in fermented samples ($P \leq 0.05$) suggesting that *B. clausii* metabolically regulates the release of some phenolic compounds attached to the fiber in the substrate,

Fig. 2 Optimal fermentation zone obtained by an overlaid contour plot for the response variables analyzed

increasing their concentration. Different food-grade bacteria have been evaluated to analyze their potential in the release of phenolic compounds and flavonoids from plant sources, such as cereals and soybean (Chung et al. 2011; Hole et al. 2012). *Bacillus* sp. strains metabolize fiber and release phenolic compounds due to a lignocellulytic activity with a strong correlation between phenolic compounds and production of cellulases and pectinases (Khelil et al. 2016). These enzymes have the ability to break down cell wall components of plant cells, thus achieving ester bond hydrolysis that bind the phenolic compounds to the cell wall after lignin degradation (Hur et al. 2014). As a consequence of this phenomenon, the bound compounds are released and extracted more easily. These results suggest that *B. clausii* during the fermentation of spent coffee grounds could have an enzymatic activity capable of releasing phenolic compounds bound to the fiber that could be extracted and quantified. In addition, the variation of fermentation temperature and time caused changes in the TF content, that was significantly increased ($P \leq 0.05$) 1.14 times compared to samples without bioprocessing. The AA is another parameter that also significantly increased ($P \leq 0.05$) during fermentation. This is mainly due to the increase of phenolic compounds and flavonoids that are positively correlated to this activity. The data obtained by the model of coded response variables showed higher correlations between the response variables. The TPC ($r = 0.91$, $P = 0.001$) and TF ($r = 0.82$, $P = 0.001$) showed a good correlation with the AA, however the TPC and TF presented a low correlation ($r = 0.64$, $P = 0.001$). These results suggest that not all phenolic compounds present in the sample belong to flavonoid compounds, but

also have phenolic acids that may have an antioxidant effect. The phenolic compounds increased after *B. clausii* fermentation of spent coffee grounds have not been reported, however, López-Barrera et al. (2016) reported that spent coffee grounds obtained after beverage preparation with two roasting levels contained flavonoids such as catechin, rutin and quercetin, as well as phenolic acids such as gallic, chlorogenic, caffeic, ellagic, cumaric and ferulic with bioactivity.

Antimicrobial effect by fermented phenolic extracts

Different microorganisms have the ability to cause human health diseases and antibiotic resistance, complicating their control. Thus, it is necessary to evaluate the potential of some natural components present in foods or residues that may have the ability to interact with microorganisms to eliminate them. Here, we evaluated the ability of phenolic extracts, obtained from fermented spent coffee grounds, to inhibit the growth of *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Enterococcus faecalis* using the disk diffusion method that indicates bacteria sensitivity or resistance by the presence or absence of halos of inhibition.

In general, phenolic extracts from fermented to non-fermented spent coffee grounds generated large halos of inhibition in all studied strains, with higher resistance in Gram positive bacteria (Table 3). Fermented spent coffee grounds phenolic extracts showed higher antimicrobial activity compared to non-fermented samples in all strains ($P < 0.05$). *Enterococcus faecalis* was the most resistant strain to non-fermented and fermented spent coffee grounds extracts, while the most sensitive strain was *Shigella flexneri* ($P < 0.05$). However, the antimicrobial effect of fermented extracts was greater in *E. faecalis* with an increase in the inhibition halo diameter of 61%, when comparing with non-fermented extracts, while *Salmonella enterica* serovar Typhimurium showed the lowest increase effect (12%) in the inhibition halo. The improvement in the antimicrobial effect observed could be attributed to the phenolic compounds release during fermentation of spent coffee grounds. These results are in agreement with Kchaou et al. (2013, 2014) studies, who demonstrated that fruit of the date palm (*Phoenix dactylifera* L) extracts, rich in p-coumaric, vanillic and ferulic acids, showed an antimicrobial effect against several pathogens with a broad spectrum of variation related to each microorganism. In addition, a study of the antimicrobial effect of extracts obtained from dates (*Phoenix dactylifera* L.) showed that this effect did not depend on the bacteria morphology and the type of Gram nature (Kchaou et al. 2016). However, in this study, Gram positive bacterial resistance to phenolic

Table 3 Changes in antioxidant property and antimicrobial activity of the fermented spent coffee grounds

	Spent coffee grounds	
	Non-fermented	Fermented
Phenolic content ¹	774 ± 3 ^b	1051 ± 2 ^a
Flavonoid content ²	382 ± 1 ^b	435 ± 1 ^a
Antioxidant activity ³	15,487 ± 129 ^b	17,894 ± 1364 ^a
Antimicrobial activity ⁴		
Gram negative		
<i>Salmonella</i> Typhimurium	7.5 ± 0.1 ^b	8.4 ± 0.8 ^a
<i>E. coli</i>	14.0 ± 0.6 ^b	16.8 ± 1.6 ^a
<i>Shigella flexneri</i>	15.3 ± 0.1 ^b	18.6 ± 0.2 ^a
Gram positive		
<i>Staphylococcus aureus</i>	9.0 ± 0.7 ^b	13.1 ± 0.6 ^a
<i>Listeria monocytogenes</i>	7.8 ± 0.2 ^b	9.8 ± 1.1 ^a
<i>Enterococcus faecalis</i>	4.6 ± 1.0 ^b	7.4 ± 1.0 ^a
Minimal inhibitory concentration ⁵		
Gram negative		
<i>Salmonella</i> Typhimurium	30 ± 1.2 ^b	15 ± 1.3 ^a
<i>E. coli</i>	30 ± 1.0 ^b	15 ± 1.2 ^a
<i>Shigella flexneri</i>	20 ± 1.3 ^b	10 ± 1.4 ^a
Gram positive		
<i>Staphylococcus aureus</i>	40 ± 1.0 ^b	25 ± 1.0 ^a
<i>Listeria monocytogenes</i>	30 ± 1.1 ^b	10 ± 1.0 ^a
<i>Enterococcus faecalis</i>	50 ± 1.0 ^b	30 ± 1.0 ^b

Data are mean ± SD of four independent experiments

Rows with different letters indicate statistical differences ($P < 0.05$)

¹mg GAE/100 g sample, dw

²mg CE/100 g sample, dw

³μmol TE/100 g sample, dw

⁴Halo of inhibition in mm

⁵mg/mL

extracts obtained from fermented spent coffee grounds may be due to the thick layer of peptidoglycan that gives greater resistance to antibiotics than other compounds with biological activity (Mutua et al. 2017).

In contrast to the disc diffusion method, which determines the resistance or sensitivity of a microorganism against a concentration of the phenolic extract, the minimum inhibitory concentration (MIC) indicates the lowest concentration of phenolic extract inhibiting visible growth of microorganisms. The antimicrobial activity of phenolic extracts of spent coffee grounds evaluated by MIC is shown in Table 3. The non-fermented and fermented extracts inhibited the growth of all Gram negative and positive bacteria ($P < 0.05$), with MIC values ranged from 10 to 50 mg of phenolic extract/mL. The lower MIC values for phenolic extracts obtained from fermented spent coffee grounds indicated a better inhibitory activity than extracts

obtained from the non-fermented samples. Among the microorganisms analyzed, *Listeria monocytogenes* showed the highest sensitivity after the exposure to phenolic extracts from fermented samples compared with non-fermented, with a reduction of 67% in the MIC value. In general, these observations showed that all bacteria needed at least 49% less fermented extract concentration to achieve the same inhibitory effect obtained for non-fermented exposed bacteria. Despite this, there are no reports of antimicrobial activity of phenolic extracts obtained from spent coffee grounds. Gullon et al. (2016) studied the antimicrobial effect of pomegranate shell extracts and their derivatives, rich in ellagic acid. These components are also reported in coffee residues, and were exposed to three Gram negative and positive bacteria, and after the evaluation by MIC, values ranging from 20 to 50 mg/mL were obtained, similar to those observed in our study.

Conclusion

In most solid-state fermentation systems, fungi are more suitable than bacterial strains and yeasts; therefore genetically modified bacterial and yeast strains should be developed to improve solid-state fermentation processes. These genetically improved bacterial cultures decrease the time required for fermentation and hence reduce the capital involved. In this study an optimization of operational factors of solid-state fermentation process is provided. A value-addition alternative for spent coffee grounds is proposed by using these residues as substrates in solid-state fermentation.

Temperature and fermentation time were optimized in order to maximize the total phenolic and flavonoid content, and antioxidant and antimicrobial activities. This experimental strategy reduced the time required for fermentation and found the optimal fermentation temperature for this biotechnological process. Optimal conditions for the bioactivity of phenolic compounds obtained after the solid state fermentation of spent coffee grounds by *Bacillus clausii* were: temperature of 37 °C and a fermentation time of 39 h. Under these conditions, total phenolic and flavonoid contents increased by 36 and 13%, respectively, in comparison to non-fermented extracts. Besides, the antioxidant activity was increased by 15% and higher antimicrobial activity was observed against Gram positive and negative bacteria.

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