Utilization of Lignocellulose-based Orange Peel Waste for Induced Sporulation of *Trichoderma asperellum via* Box-Behnken Matrix Design

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The feasibility of using orange peel residues as a substrate for induced sporulation of *Trichoderma asperellum* was evaluated. The Box-Behnken matrix (BBM) design was used to screen the effects of several parameters, including the effect of pH, inoculum, and moisture under solid-state fermentation culture conditions. The study was performed in two experimental steps (screening and optimization). Moisture content and pH were determined to be the most influential parameters on spore production during the screening stage. A Box-Behnken design was used to optimize and to define the interaction of the selected parameters. The moisture content was determined as the most significant parameter affecting spore production. An inoculum of 1×10^6 spores g^{-1}, pH 6.07, and moisture content of 69.0% was the combination of conditions observed to achieve the maximum production of 2.04×10^9 spores g^{-1}. The experimental value of 2.16×10^9 spores g^{-1} (from the experimental model) showed a good fit to the regressed model, with a standard error of 5%. Based on this work, a high yield of spores was obtained at 144 h of cultivation time, indicating that it is a feasible approach to use orange peel as a substrate for biomass and spore production.

Keywords: Lignocellulose; Orange peel waste; Fungi; Sporulation; Optimization

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INTRODUCTION

During the past several years, substantial advancements in green chemistry principles have developed for the cleaner production and revalorization of naturally occurring lignocellulose biomass. Lignocellulosic biomass refers to crops, crop residues, *etc.*, and are composed of carbohydrates, cellulose, and hemicellulose.

Because approximately 70% of lignocellulosic biomass is comprised of sugar monomer units, it makes an excellent candidate for many purposes, including being used as the substrate for fermentation processes to obtain value-added products (Arevalo-Gallegos *et al.* 2017; Asgher *et al.* 2017; Bilal *et al.* 2017). *Trichoderma* is a filamentous fungus present in soil everywhere around the world. It is a very popular microorganism in agriculture mainly due to its capabilities to act as a highly efficient biofertilizer and biopesticide.

Several *Trichoderma* species have been reported as biocontrol agents (BCA's), and they can show different action mechanisms, including competition for space due to its fast mycelial growth, antibiosis through the production of secondary metabolites, and mycoparasitism mediated by the production of cell wall-degrading enzymes (Joshi *et al.* 2016; Sain and Pandey 2016; Saravanakumar *et al.* 2016).

The market of biopesticides is growing quickly in comparison with conventional chemical pesticides. Biopesticides are offering a more ecological way for the management of pests, but they also face several challenges, such as increased barriers for the use of biological products, a lack of awareness toward agricultural biological products, and most importantly a lack of global availability (Olson 2015). To enhance the feasibility of using biopesticides, it is necessary to increase the spore production of species of interest. Several authors have reported different alternatives to achieve higher spore production, in which the cell structure is more adapted to grow in a field and resist environmental conditions *in situ* (Shah and Pell 2003).

Solid-state fermentation (SSF) consists of a fermentation process involving solid material in the absence (or near absence) of free water. However, enough water should be adsorbed by the substrate to support the growth and metabolism of the microorganism (Pandey 2003). This technology is an alternative that offers high spore production for different species of filamentous fungi with a minimal cost of separation; species that have been studied for use in SSF include *Beauveria bassiana, Metarhizium anisopliae,* and *Trichoderma asperellum* (de la Cruz-Quiroz *et al.* 2017; Muñiz-Paredes *et al.* 2017). The SSF method allows for the use of different agroindustrial residues, such as sugarcane bagasse, corn cob, and wheat bran, as a carbon source for fungal development, for the induced production of value-added products (Mishra *et al.* 2016). Several parameters are considered as the key factors for fungal development and spore production under SSF culture conditions (Lopez-Perez *et al.* 2015). These factors include temperature, substrate, pH, inoculum, and moisture, among others (Cavalcante *et al.* 2008; Nuñez-Gaona *et al.* 2010; Zhang and Yang 2015).

The approach of using *Trichoderma* on orange peel as a substrate for SSF has been focused mainly on the production of enzymes such as pectinase and cellulase, among others (Ng *et al*. 2014; Irshad *et al*. 2014), but there is a lack of information concerning the production of fungal biocontrol agents, which have high importance as biocontrol products. Therefore, in order to valorize the local wastes and promote an alternative to reduce the pollution caused by the organic residues, the present study was focused on the evaluation of the potential to use orange peel as substrate in SSF and determining the culture conditions that maximize the spore production of *T. asperellum*. The effects of pH, inoculum, and moisture were evaluated under SSF culture conditions.

EXPERIMENTAL

Materials

Microorganism and culture conditions

The *T. asperellum* was provided by the Food Research Department of the Universidad Autónoma de Coahuila (Saltillo, México). The fungus was cultivated and preserved in a milk-glycerol 8.5% solution.

Sterilized potato dextrose agar (PDA) was used to reactivate the fungi (samples were incubated at 121 °C for 15 min). The tube slants were inoculated with the fungal strains and incubated at 29 °C for 6 days. For the stock culture and preservation of the strain, the slants were preserved at 4 °C.

Raw material – Orange peel

The orange peel (OP) substrate was obtained from juice producers from Montemorelos, Nuevo Léon, Mexico. The material was oven-dried at 50 °C for 24 h to 48 h, ground, fractioned (300 μm to 1680 μm), and stored in the desiccator under low moisture conditions for further evaluation. This resulting material was used as a substrate for SSF without any pretreatment.

Solid state fermentation

The orange peel was sterilized at 121 °C for 30 min. After cooling down the substrate in sterile conditions, it was inoculated with a concentrated spore suspension of 2×10^7 spores g^{-1} dry matter.

A set of 500-mL Erlenmeyer flasks was used as a reactor, and each one was loaded with 7 g of substrate to adjust the initial moisture content to 60%. The kinetic culture runs were incubated at 29 °C for 168 h. All of the treatments were performed in triplicate and monitored on a daily basis. This kinetic study was followed to determine the time of maximum spore production by *T. asperellum*.

Methods

Screening factors for spore production via Box-Behnken Matrix

The aim of using a Box-Behnken matrix (BBM) was to determine the most influential factors for the spore production from *T. asperellum* under SSF conditions. Each reactor was packed with 7 g of the substrate and the moisture, pH, and inoculum were adjusted according to the BBM at a minimum (-1), basal (0), and maximum (1) level (Table 1). The cultures were incubated at 28 °C for 144 h.

Optimization of spore production via Box-Behnken matrix

The most important factors in spore production were identified and selected from the previous section. The second step towards optimization was performed with a Box–Behnken design of experiment (DOW) employed to maximize the culture conditions that increase the spore production.

Each reactor was loaded with 7 g of substrate, and the moisture and pH were adjusted according to the BBM at a minimum (-1), basal (0), and maximum (1) level (Table 2). The cultures were incubated at 28 °C for 144 h.

Table 1. Box–Behnken Matrix Used to Determine the Influence of Moisture, pH, and Inoculum for the Spore Production of *T. asperellum* Under SSF Conditions

Run	A	B	C	Spores/g (Dry wt.)	Standard Deviation
1	1	0	1	1.13×10^9	2.16×10^8
2	0	0	0	1.84×10^8	1.53×10^6
3	0	-1	1	$< 1 \times 10^5$	$< 1 \times 10^5$
4	-1	1	0	1.38×10^9	1.23×10^8
5	0	0	0	3.40×10^8	1.01×10^8
6	0	-1	-1	$< 1 \times 10^5$	$< 1 \times 10^5$
7	-1	0	1	1.21×10^9	9.65×10^7
8	-1	0	-1	$< 1 \times 10^5$	$< 1 \times 10^5$
9	1	1	0	2.18×10^9	1.66×10^8
10	0	0	0	1.75×10^8	1.27×10^7
11	-1	-1	0	$< 1 \times 10^5$	$< 1 \times 10^5$
12	0	1	1	1.81×10^8	2.29×10^7
13	1	-1	0	$< 1 \times 10^5$	$< 1 \times 10^5$
14	0	1	-1	1.43×10^9	6.48×10^7
15	1	0	-1	1.29×10^8	1.79×10^7

Code	Factors	High Level	Basal Level	Low Level
A	pH	7	6	5
B	Moisture (% wt/v)	60	50	40
C	Inoculum (spores/g substrate)	1×10^7	5×10^6	1×10^6

Table 2. Box–Behnken Matrix Used to Determine the Influence of pH and Moisture for the Spore Production of *T. asperellum* Under SSF Conditions

Run	A	B	Spores/g (Dry wt.)
1	7	70	2.11×10^9
2	6	90	$< 1 \times 10^5$
3	8	70	1.28×10^9
4	6	50	3.42×10^8
5	6	70	2.37×10^9
6	8	50	5.47×10^8
7	7	90	$< 1 \times 10^5$
8	7	50	3.93×10^8
9	8	90	$< 1 \times 10^5$

Code	Factors	High Level	Basal Level	Low Level
A	pH	8	7	6
B	Moisture (% wt/v)	90	70	50

Spore determination

The fermented matter (1 g) was added to 100 mL of diluted Tween 80 (0.01%) in an Erlenmeyer flask. A magnetic stirrer was used to release the spores from the solid matter and to homogenize the suspension. The spores were counted using a hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

Experimental design and statistical analysis

The statistical analysis was performed using Centurion XV software (Statpoint Technologies, Inc., Warrenton, VA, USA). An analysis of variance (ANOVA) was used to evaluate the spore production response at 95% of confidence level. A statistical Box-Behnken design (Box and Behnken 1960) and combined response surface methodology was employed to optimize and evaluate the effects of pH (at 5, 6, and 7), moisture level (at 40%, 50%, and 60%), and inoculum (1×10^6, 5×10^6, and 1×10^7 spores g^{-1}) on spore production per gram of orange peel (dry wt.).

RESULTS AND DISCUSSION

The strain of *T. asperellum* evaluated showed a long phase without spore formation at 72 h, and it was only after 96 h that the fungal strain began to sporulate. However, the mycelium was observed from the 48-h culture (data not shown). The spore production increased exponentially from 96 h to 144 h of culture, reaching a maximum value of 9.99×10^8 spores g^{-1} (dry wt.). At the end of the culture 168 h, it is possible to observe a slight stationary phase, keeping the spore concentration at 8.99×10^8 spores g^{-1} (Fig. 1). The mycelium and spores generated during the fermentation process were homogeneous over the entire surface of the substrate. The culture time was fixed at 144 h for the next experiments.

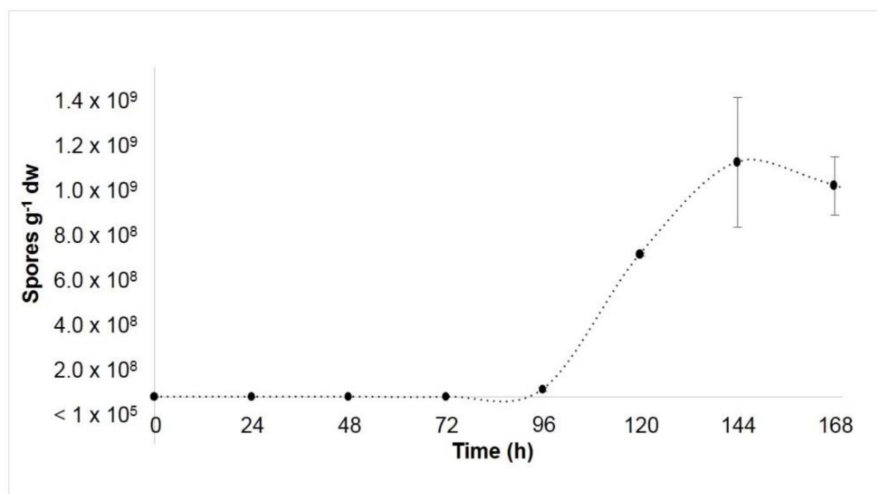

Fig. 1. Kinetic chart of the spore production of *T. asperellum* using orange peel as a substrate

The OP is composed of high levels of organic carbon mainly integrated into cellulose (13.61%), hemicellulose (6.10%), and lignin (2.10%) (Ververis *et al.* 2007). The low percentage of lignin in OP makes the substrate more available for fungal hydrolysis. Therefore, the biodegradation of cellulose and hemicellulose into glucose is a key role in the SSF process of fungal biomass development. Several parameters are commonly evaluated and optimized for SSF such as temperature, inoculum size, moisture, water activity, aeration, the nature of the solid substrate pH, and the particle size of the substrate, among others (Lopez-Perez *et al.* 2015). The selection of microorganisms and substrate is

also very relevant for this purpose. For each combination of microorganisms and substrate, the culture conditions must be optimized mainly based on factorial design experiments and response surface methodology to identify the critical factors and their interactions (Thomas *et al.* 2013).

Screening Factors for Spore Production *via* Box-Behnken Matrix

The present study was performed in 15 runs to identify factors that significantly affect the spore production on SSF using OP by *T. asperellum*. Table 1 presents the effects of the observed independent variables on the spore production determined at 144 h of culture. The SSF by *T. asperellum* resulted in a production amount that ranged from 1.75×10^8 to 2.18×10^9 spores g^{-1} in the first experimental section. The low level of moisture content (40%) was too low for fungal development; therefore the spores were not produced. High spore production was obtained with 50% and 60% moisture content levels. The levels of inoculum and pH evaluated showed a varied production of spores.

Analysis of variance is shown in Table 3. The inoculum showed a positive effect on spore production; however, statistically the values were not significant (Fig. 2).

Table 3. Analysis of Variance of the Screening Factors for Spore Production

Source	GL	SC Sec.	Contribution	SC Adjust.	MC Adjust.	F-value	p-value
Model	9	9.7E+18	72.06%	9.7E+18	1.08E+18	5.73	0.001
Lineal	3	7.0E+18	51.58%	7.0E+18	2.33E+18	12.31	0.000
pH	1	1.1E+17	0.82%	1.1E+17	1.11E+17	0.59	0.452
Moisture	1	6.7E+18	49.67%	6.7E+18	6.75E+18	35.56	0.000
Inoculum	1	1.4E+17	1.08%	1.4E+17	1.46E+17	0.77	0.390
Square	3	1.7E+18	12.66%	1.7E+18	5.73E+17	3.02	0.054
pH*pH	1	1.2E+18	9.07%	1.3E+18	1.30E+18	6.87	0.016
Moisture*Moisture	1	4.6E+17	3.44%	4.5E+17	4.50E+17	2.37	0.139
Inoculum*Inoculum	1	1.9E+16	0.14%	1.9E+16	1.96E+16	0.10	0.751
Interaction of 2 factors	3	1.0E+18	7.83%	1.0E+18	3.54E+17	1.87	0.168
pH*Moisture	1	2.1E+17	1.57%	2.1E+17	2.13E+17	1.13	0.301
pH*Inoculum	1	4.4E+16	0.33%	4.4E+16	4.45E+16	0.23	0.634
Moisture*Inoculum	1	8.0E+17	5.93%	8.0E+17	8.05E+17	4.24	0.053
Error	20	3.7E+18	27.94%	3.7E+18	1.89E+17	-	-
Lack of fit	3	3.7E+18	27.40%	3.7E+18	1.24E+18	285.54	0.000
Pure error	17	7.3E+16	0.54%	7.3E+16	4.34E+15	-	-
Total	29	1.3E+19	100.00%				

Model summary	S	R^2.	R^2 (adjusted)	R^2 (predicted)
	435877198	72.06%	59.49%	28.64%

Statistical analysis revealed that the main effects on spore production response were caused by the moisture (*p* = 0.0000) and the quadratic pH (*p* = 0.0002) terms (Fig. 2).

The Pareto plot showed the moisture as a factor with a positive effect on spore production, which meant that the spores produced by *T. asperellum* increased if the moisture levels increased above 60%. The quadratic effect of pH was positive and statistically significant. This behavior suggested that an increase in the levels of pH increased the spore production. The regression results expressed by Eq. 1 includes only the significant variables on spore production in SSF by *T. asperellum* according to the Pareto chart of Fig. 2.

$$\text{Spore g}^{-1} = 1.10 \text{ x } 10^{10} + (4.72 \text{ x } 10^{9} * pH) + (6.47 \text{ x } 10^{7} * Moisture) + (4.00 \text{ x } 10^{8} * pH^{2}) \quad (1)$$

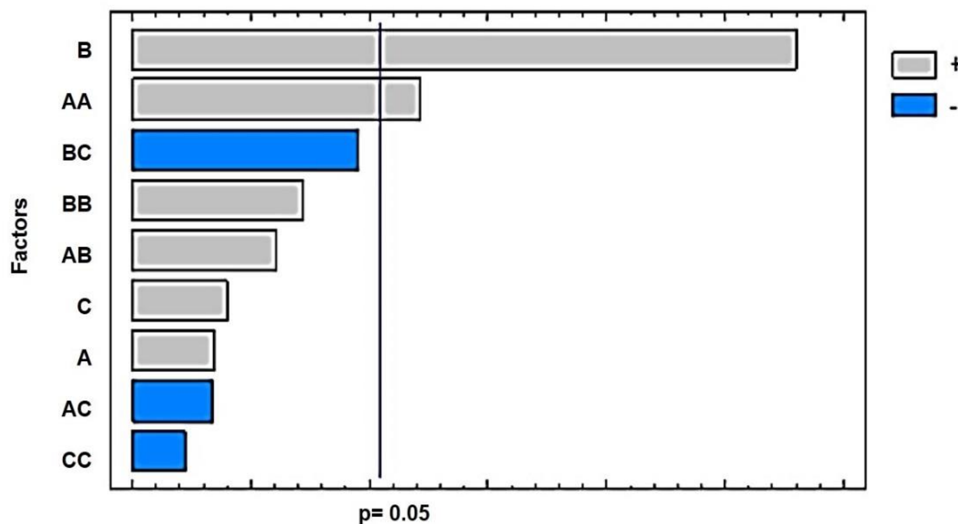

Fig. 2. Standardized effects of pH, moisture, and inoculum on spore production in SSF by *T. asperellum* according to Box-Behnken statistical design; A- pH; B- moisture; C- inoculum; AA- quadratic effect of pH; AB- interaction effect of pH and moisture; AC- interaction effect of pH and inoculum; BB- quadratic effect of moisture; BC- interaction effect of moisture and inoculum; and CC- quadratic effect of inoculum

The rest of the experimental factors were not statistically significant ($p > 0.05$), and they were excluded from the model. According to the response surface obtained in the current experimental region, the maximum value of the response variable was 2.043×10^{9} spores g^{-1}, which was obtained at the high levels of pH and moisture content (Fig. 3). The factor level combination of pH 7, 60% moisture, and 1×10^{6} spores g^{-1} of inoculum resulted in the maximum spore production. It was observed that as the pH and moisture increased, the spore production also increased. A maximum spore production was observed at the highest pH and moisture conditions tested in this experiment. To see the spore production behavior beyond the recorded parameters, *i.e.,* 60% moisture and pH 7, an additional design of experiment was arranged, aiming to find a higher spore production in the next experimental region, *i.e.,* moisture level from 50% to 90% and pH from 6 to 8.

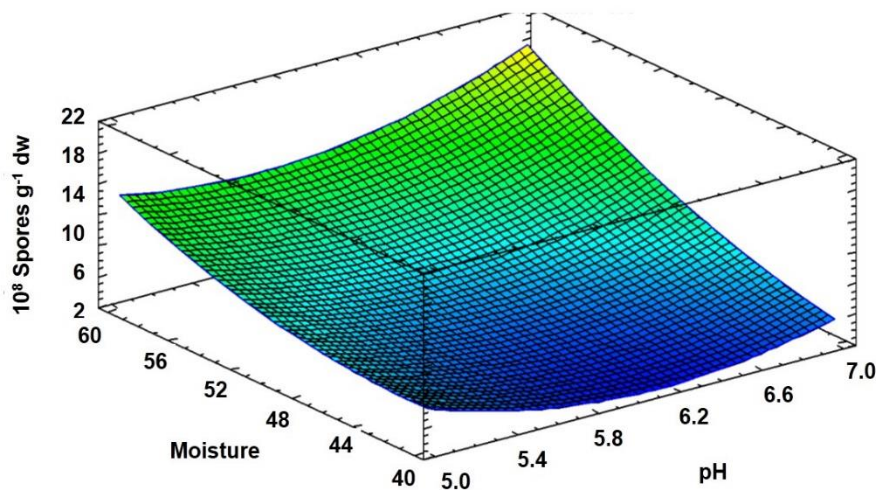

Fig. 3. Surface plot of the interaction effect between moisture and pH on the spore production of *T. asperellum*

Optimization for Spore Production *via* BBM

Based on the results obtained in the previous section, higher levels of moisture and pH were evaluated as two independent variables for the optimization of spore production (Table 2). The analysis of variance is shown in Table 4. The concentration of inoculum was fixed at the low level (1×10^6 spores g^{-1}) because this factor did not show any significant effect on the variable response. The maximum production of 2.37×10^9 spores g^{-1} was obtained when the pH and moisture were adjusted to 6 and 70%, respectively.

Table 4. Analysis of Variance of the Optimization Conditions for Spore Production

Source	GL	SC Adjust.	MC Adjust.	F- value	p- value
Regression	3	8.10E+18	2.70E+18	12.77	0.000
Moisture	1	6.75E+18	6.75E+18	31.94	0.000
Moisture * Moisture	1	1.23E+18	1.23E+18	5.83	0.023
Error	26	5.49E+18	2.11E+17		
Lack of fit	5	1.69E+18	3.39E+17	1.87	0.142
Pure Error	21	3.80E+18	1.81E+17		
Total	29	1.35E+19			

The pH and moisture showed a negative linear effect on spore production; however, statistically the values were not significant (Fig. 4). The quadratic effect of moisture was negative and statistically significant. The range of pH evaluated did not show a significant influence on the spore production. However, the analysis suggested that levels of moisture higher than 70% resulted in a devastating reduction in the development of mycelial growth, and therefore on the production of spores. No interaction between the factors evaluated was observed. According to the response surface obtained in the current experimental region, the maximum value of the response variable was 2.04×10^9 spores g^{-1}, which was

obtained at the factor level combination of a 6.07 pH, 69.02% moisture level, and 1×10^6 spores g^{-1} of inoculum (Fig. 5). The spore production response was analyzed by response surface regression using the quadratic model shown in Eq. 2 as a function of the process variables:

$$\text{Spore g}^{-1} = -1.82 \text{ x } 10^{10} + (5.86 \text{ x } 10^{8} * Moisture) - (4.26 \text{ x } 10^{6} * Moisture^{2}) \quad (2)$$

No significant variables were excluded from the quadratic model. The high R^2 value (92.3% for spore production response) and error values for the spore production (averaging only 5%) indicate a good fit of the data to the regression models Eq. 3:

$$Error\ \% = \frac{[Mesured - Model]}{Mesured} * 100 = \frac{[2.16 \text{ x } 10^{9} - 2.04 \text{ x } 10^{9}]}{2.04 \text{ x } 10^{9}} * 100 = 5\ \% \quad (3)$$

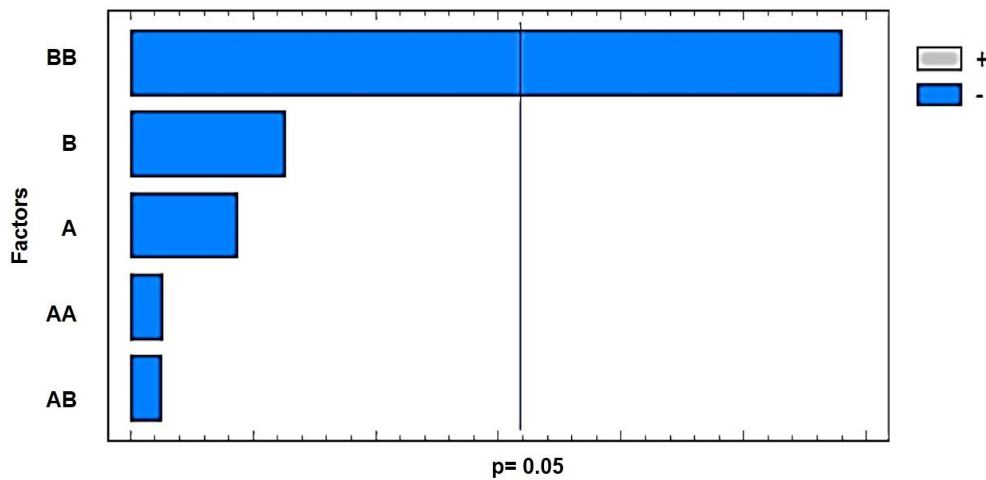

Fig. 4. Standardized effect of the independent variables (pH and moisture) on spore production in SSF by *T. asperellum* according to Box-Behnken statistical design; where, A- pH; B- moisture; AA- quadratic effect of pH; BB- quadratic effect of moisture; AB- interaction effect of pH and moisture

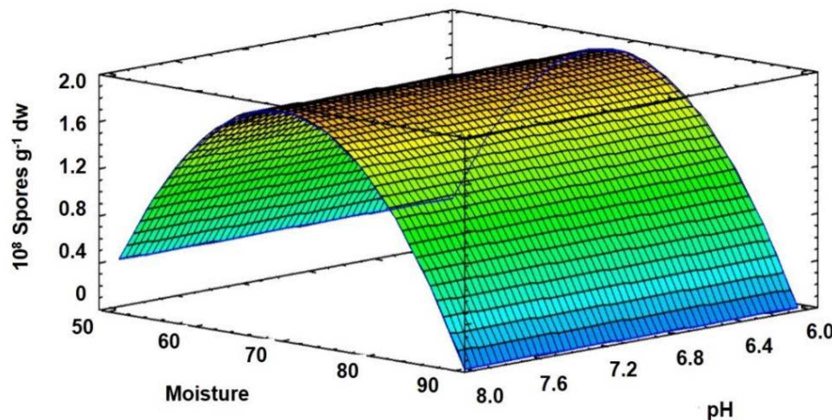

Fig. 5. The interaction effect between moisture and pH on the spore production of *T. asperellum*

It can be noted that a close agreement between the experimental results and the theoretical values predicted by the equations was present, so the spore production can be adequately described from the results obtained using the Box-Behnken design.

To produce filamentous fungi, the inoculum level appeared to be an important parameter on SSF because it was closely related to the high spore production and the reduction of the culture time. Commonly an inoculum concentration of 1×10^7 spores g^{-1} is recommended (Arzumanov *et al.* 2005; Nuñez-Gaona *et al.* 2010). In the present study, the statistical analysis suggested that it was possible to choose an inoculum level between 10^6 and 10^7 spores g^{-1} that resulted in the same spore production. At this stage, using a concentration of 1×10^6 spores g^{-1} reduced the inoculum starter by 10 times for production of spores in an SSF by *T. asperellum*. Inoculum levels from 1×10^3 to 1×10^9 spores g^{-1} have been evaluated for the spore production of *Trichoderma harzianum* through SSF of corn stover and wheat bran by Zhang and Yang (2015). Zhang and Yang indicated that the production of higher values of spores (7 to 8×10^9 spores g^{-1}) when the inoculum levels of 1×10^6, 1×10^7, and 1×10^8 spores g^{-1} were used. A marked decrease in spore production was pointed out when the inoculum level was 1×10^9 spores g^{-1}. Inoculum levels have also been evaluated for other fungal biocontrol agents such as *Beauveria bassiana* by Nuñez-Gaona *et al.* (2010), who tested concentrations of 1×10^6, 7×10^6, and 5×10^7 spores g^{-1} through SSF of wheat bran. The three levels of inoculum evaluated resulted in high spore production (1.08×10^{10} to 1.53×10^{10} spores g^{-1}). The levels of production were particularly homogeneous; however, high levels of inoculum generated the spore production in less time (285 h, 232 h, and 148 h. Xie *et al.* (2016) evaluated inoculum levels of 1×10^5, 1×10^6, 1×10^7, and 1×10^8 spores per gram of wet substrate (gws^{-1}) to produce *Isaria javanica* through SSF of brown rice. An index of 2×10^9 spores g^{-1} was achieved when the SSF was inoculated with 1×10^8 gws^{-1}. Nevertheless, the rest of the inoculum levels resulted in concentrations between 6.7×10^8 to 1×10^9 spores g^{-1}.

Fungal strains showed better development in a range of pH that was slightly acidic. In SSF lower pH values are important for avoiding the growth of undesirable microorganisms (Sargin *et al.* 2013). Wide ranges of pH were suitable for the spore production of *T. asperellum* under the tested conditions. Nevertheless, spore production was enhanced with a pH of 6. This effect of pH was like that reported by Zhang and Yang (2015), reaching 8.64×10^9 spores g^{-1} by the strain *Trichoderma harzianum* TS1 using wheat straw and wheat bran as substrates. Wheat-bran malt sprout mixture was the substrate for the SSF by *T. harzianum* EGE-K38, generating 3.7×10^9 and 4.6×10^9 spores g^{-1} at pH 4 and 7, respectively (Sargin *et al.* 2013). However, Zhang and Yang (2015) reported that the pH of the substrate without adjustment (5.8) resulted in the best spore production (1.3×10^{10} spores g^{-1}). The pH values of 4.5, 5.5, and 6.5 were evaluated for the growth of *Trichoderma koningii*. The microbial development was determined by fungal radial growth in chitosan detector agar, obtaining the best value of 73 mm at pH 5.5 (da Silva *et al.* 2012). According to Zhang and Yang (2015), pH was suitable to create the environmental conditions for fungal development in solid culture. In this way, better and more stable enzyme activity was achieved, thereby degrading the solid substrates, improving the uptake of nitrogen, and ultimately producing higher numbers of spores. It is important to note that the adjustment of pH is only feasible at the beginning of the SSF process. The heterogeneous system and lack of adequate equipment to determine the pH in solid materials make it hard to adjust and monitor this parameter during the fermentation

process (Krishna 2005). To overcome the variability of pH buffer solutions with no influence on biological activity are also recommended (Pastrana 1996).

In the present culture system, water content was observed as the fundamental parameter to induce the metabolic activity of the fungi, and therefore the production of its spores. It is known that water is necessary for the diffusion of nutrients as well as for maintenance of the stability and function of biological structures such as proteins, carbohydrates, and nucleotides (Singhania *et al.* 2009; Farinas 2015). In this way, the growth of fungi in SSF is closely related to the water adsorbed on the solid substrate. The suitable values of moisture for SSF depend on the intrinsic characteristics of the microorganism used and the physical structure of the solid substrate, such as the porosity or the specific surface area (Farinas 2015). That is why each substrate should be evaluated to identify the optimal values of moisture on a SSF system for a specific value-added product. Water absorption capacity is a very important parameter that indicates the feasibility of the material to absorb water. High levels of water absorption are preferred because it is easy to adjust the water content in the SSF and stimulate fungal growth (Buenrostro-Figueroa *et al.* 2014). Several studies have described the effect of the initial substrate moisture content on the fungal development and production of value-added products by different fungal strains cultivated under SSF. Most researchers have reported that a moisture content lower than 60% and higher than 80% are not favorable for fungal growth on SSF (Zhang *et al.* 2015).

In the present study, moisture was the most significant factor at the screening stage. A range of moisture from 40% to 60% was evaluated. No fungal growth was observed at the lower moisture level (40%), which may have been due to the lack of nutrient diffusion. Low nutrient diffusion is a limiting factor for fungal development and desired products, such as biomass, enzymes, and secondary metabolites, among others (Yoon *et al.* 2014; Farinas 2015). Low water content also affects the degree of swelling and produces higher water tension (Veerabhadrappa *et al.* 2014). The analysis of the optimization in the present study indicate that if water content increase by more than 60%, more spores could be produced in the system. Therefore, higher ranges of moisture were evaluated, resulting in 69.0% as the optimum level of the experimental analysis, generating a production of 2.04 $\times 10^9$ spores g^{-1}. A water content greater than 70% resulted in stacking of the solid substrate and no fungal growth was observed. The effect mentioned above implies a decrease of porosity of the solid material, creates problems with the oxygen diffusion, and therefore inhibits fungal development (Zhang *et al.* 2013; Veerabhadrappa *et al.* 2014). Additionally, an excessively humid environment could be a factor for creating the culture conditions for unwanted microorganisms, increasing the risk of contamination (Yoon *et al.* 2014; Farinas 2015).

Moisture content is commonly evaluated on this kind of culture system for several reasons. However, *Trichoderma* strains are tolerant to a wide range of water requirements. Many authors use 70% to 75% of moisture content as a constant value on SSF. Important results have been reported following this percentage of moisture such as Witkowska *et al.* (2016), who reported 6.07 $\times 10^9$ using a mix of sugar beet pulp, wheat bran, and apple pomace by *T. atroviride* TRS40 on SSF after adjusting moisture to 75% and pH to 7.45. Cavalcante *et al.* (2008) evaluated the effect of different water contents on SSF to produce spores from four *Trichoderma* strains testing rice, corn bran, and wheat bran as substrates. They reported high spore production by *T. harzianum* (2.8 $\times 10^9$ g^{-1}) and *T. viride* (2.4 $\times$

10^9 g^{-1}) using wheat bran with a moisture content of 68.41%. An increase in the value of moisture over 73.13% resulted in a strong decrease in the spore production by all species studied. Regardless of the type of value-added product desired, it is necessary to evaluate at least the main factors involved in the culture conditions in SSF. As seen in this experiment there were initial culture condition. However, these varied mainly by the type of solid substrate and fungal microorganism being evaluated. A quick example of this would be the production of *Beauveria bassiana* (1.8×10^9 spore g^{-1}) using a moisture content of 51% to 54%, lower values than those used with the *Trichoderma* strains mentioned above (Mishra *et al.* 2016).

CONCLUSIONS

1. Orange peel as a substrate allows the adjustment of the moisture content of the system up to 70%, where beyond this value, the conditions were not feasible for SSF.
2. Moisture was a key factor for the spore production by *T. asperellum*. An inoculum of 1×10^6 spores g^{-1}, pH of 6.07, and a moisture content 69.0% was the best combination to achieve the maximum production of 2.04×10^9 spores g^{-1} in the experimental model.
3. The experimental value of 2.16×10^9 spores g^{-1} showed a good fit with the data; the regression model had an error value of 5%.
4. The OP resulted a feasible solid substrate for the spore production by *T. asperellum* under SSF culture conditions.

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