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Solid-State Fermentation in Brewer's Spent Grains by *Fusarium fujikuroi* for Gibberellic Acid Production

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Abstract: The search for new production methodologies of gibberellic acid (GA_3), such as solid-state fermentation (SSF), and the use of agro-industrial waste are important to lower production costs. Therefore, the aim of this study was GA_3 production by *Fusarium fujikuroi* on SSF mode using brewer's spent grains (BSG). BSG presents in its composition components that are known to be excellent inducers of metabolite production, showing, this way, its potential to be used as the substrate in biotechnological processes. Optimization of GA_3 production was carried out using a 2^2 central composite design, considering the effects of moisture content, temperature, and fermentation time. The highest mycelial growth and GA_3 production (0.82 $g.Kg^{-1}$) was obtained in the condition of 80% moisture content, 28 °C in 96 hours of fermentation. These results suggest that the SSF using BSG as the medium for the growth of *F. fujikuroi* is a viable way to GA_3 produce.

Keywords: gibberellic acid; brewer's spent grains; solid-state fermentation; *Fusarium fujikuroi*.

1. Introduction

Gibberellins are phytohormones resulting from secondary metabolites. They are also important biotechnological products, with great economic value, used in agriculture as natural plant growth hormones [1]. They play a role in breaking flowering dormancy, increasing flowering initiation, increasing stunted plants, spurring germination processes [2-5]. Gibberellins are chemically composed of diterpenoids consisting of tetracyclic ent-gibberellane carbon skeletal structure arranged in four or five rings [6,7].

Gibberellic acid (GA_3; $C_{19}H_{22}O_6$; MM = 346.37) is chemically characterized as a tetracyclic dihydroxy-γ-lactone acid-containing C1–C2 double bond, C10 γ-lactone ring and an OH group in C13; it is one of the most important compounds of gibberellins and plays an important role in plant growth as a natural hormone. Plants and some microorganisms, such as fungi and bacteria, can produce this molecule. For the agro-industrial sector, it has promising applications because it is related to plant growth. Since its discovery, studies have focused on increasing yield, productivity, and, mainly, reducing production costs so that its use is not restricted. It is applied in crops, orchards, and ornamental plants, playing a role in seed germination, response to abiotic stress, fruit growth enhancement, stem elongation, flowering,

barley malting, and interactions with other phytohormones that lead to other physiological effects [8,9].

GA_3 is presently produced on a commercial scale through submerged fermentation by *Fusarium fujikuroi*. However, low yields and costly downstream processes greatly increase the expenses of GA_3 production by submerged fermentation [10,11]. This technological route's disadvantages lead to the prospecting of new processes aimed mainly at increasing the amount of product obtained per reactor volume, with solid-state fermentation (SSF) for the GA_3 production being an interesting alternative. With the SSF, it is usually possible to obtain greater amounts of the desired metabolite (GA_3), as it represents the conditions closest to these microorganisms' natural habitat. These conditions hinder unwanted contamination by bacteria. The literature shows that SSF has economic advantages in the face of submerged fermentation. As examples, the authors cite a wide range of agro-industrial wastes used in the process and that greater production of biomass and metabolites is achieved [12-15].

An agro-industrial country, such as Brazil, generates large amounts of wastes. These come from the industrial processing of sugarcane, citrus, corn, rice, soy, coffee, castor, sunflower, barley, malt, cassava, among others, and they have great potential for use in bioprocesses to produce high value-added bioproducts.

Several authors have been conducting studies to produce GA_3 by SSF using agro-industrial waste as substrates. Among the agro-industrial waste used, we can cite wheat bran [16-18], cassava [19], coffee husk [20], soybean hulls [21], citric pulp [10,21,22], pigeon pea pods, sorghum straw, corncobs [23], corn stalks [24], jatropha seed cake [25], and rice bran and malt [26].

Worldwide the quantity of craft beer industries is increasing. Brazil in 2019 reached the mark of 1,2091 registered breweries. The growth in the number of establishments has been constant over the past 20 years, with an average growth rate of 19.6% per year. This growth rate has recently grown, being 36.4% if the last 5 years' period is analyzed [27-31]. Malt bagasse comes from the process of obtaining the must by boiling ground malt and adjuncts. Research shows quantities of 14-20 Kg of residue per 100 liters of beer produced [32,33], and the chemical composition of this residue varies as per the variety and harvesting time of the barley, malt grinding conditions, and type of adjuncts (corn, rice, wheat, and sorghum) incorporated in the brewing process [34]. Thus, the brewery wastes from the craft beer industry represent a serious environmental hazard. The brewing process generates a wide variety of wastes such as brewer's spent grain, hot trub, and residual yeast, whose amount generated cannot be reduced due to their generation stages, which are indispensable to the production process [35]. These residues have several components with significant nutritional value, such as carbohydrates, proteins, amino acids, phenolic compounds, vitamins, fibers, and minerals, as they have a high organic matter content [36-39]. Since they are abundant in nutrients such as carbon, nitrogen, and mineral salts, they are an excellent medium for the growth of fungi used in SSF processes. Otherwise, if disposed of improperly, it can cause significant environmental damage. Therefore, an interesting alternative is to use the brewery wastes as culture media in biotechnological processes, thus being within the green chemistry concept. Given the foregoing, this work aimed to optimize the phytohormone GA_3 production by solid-state fermentation of brewer's spent grain by *Fusarium fujikuroi*.

2. Materials and Methods

2.1. Substrates.

The brewer's spent grain (BSG) was kindly provided by On Tap brewery (São José - SC/Brazil). The BSG was transported to the laboratory at room temperature. It was dried at 50 °C until constant mass. Then, BSG was stored in hermetically sealed bags at room temperature. The dry BSG was characterized: moisture (135 °C for 2 h), protein (Kjeldahl method), fat (Soxhlet extraction with ethyl ether), starch (absorbance at 510 nm), and total sugars (Lane Enyon) [40].

2.2. Inoculum preparation.

Fusarium fujikuroi IOC 4380 was kindly donated by Coleção de Culturas de Fungos Filamentosos – CCFF (Filamentous Fungus Culture Collection), FIOCRUZ - IOC (Rio de Janeiro, Brazil) and maintained in commercial culture medium potato dextrose agar - PDA (Kasvi, Bazil).

F. fujikuroi was activated on PDA Petri dish at 28 °C for 10 days. Then to define the time and quantity of discs to be used for the inoculum preparation, tests were performed on 100 mL Erlenmeyer with 30 mL of potato dextrose broth (Himedia, Brazil) enriched with 2 $g.L^{-1}$ $(NH_4)_2SO_4$, 1 $g.L^{-1}$ $FeSO_4.7H_2O$, 1 $g.L^{-1}$ $MnSO_4.H_2O$, 0.5 $g.L^{-1}$ $MgSO_4$ and pH 5-5.5 [1]. Erlenmeyer flasks were sterilized in an autoclave at 121 °C for 15 min. After, 2 or 3 discs (Ø 12 mm each) taken from *F. fujikuroi* culture in PDA were added. The tests were conducted in an orbital shaker (TECNAL, TE-240, Brazil) at 120 rpm and a temperature of 28 °C for up to 96 hours [41]. The biomass was determined by the dry matter methodology, in which the culture medium was filtered on quantitative filter paper, and the oven (Solab, Brazil) dried at 80 °C until constant weight. All tests were performed in triplicate.

For the GA_3 production assays, the inoculum was prepared in 250 mL Erlenmeyer, where each one contained 100 mL of culture medium and 3 discs under the same conditions described above. The biomass was separated from the culture medium by filtration using a quantitative filter paper, and then the biomass was added to the BSG to start fermentation.

2.3. Solid state fermentation (SSF) of brewer's spent grain (BSG).

SSFs in 500 mL flasks were carried out, which 50 g of BSG were supplemented with 40 $g.Kg^{-1}$ glucose, 1 $g.Kg^{-1}$ $FeSO_4.7H_2O$, 0.5 $g.Kg^{-1}$ $MgSO_4$, 1 $g.Kg^{-1}$ $MnSO^4.H_2O$, and 0.2 $g.Kg^{-1}$ $ZnSO_4.7H_2O$ and pH 5-5.5, all supplementation components were added relative to BSG mass [1]. Flasks were sterilized in an autoclave at 121 °C for 15 min. Then, in each flask was added the inoculum, prepared as described in item inoculum preparation (item 2.2), at a ratio of 15% to the mass of BSG and homogenized [10,20].

For the optimization step, a 2^2 central composite design (CCD) was adopted [42], with a duplicate of the factorial assays and triplicate experiments at a central point, totalizing 11 experiments, in which the independent variables studied were: moisture content (60 to 80%) and temperature (28 to 35 °C), keeping constant the inoculum ratio of 15% to the mass of BSG, fermentation time of 96 hours, and aeration by diffusion. Statistical analyses were performed using online software Protimiza Experimental Design (http://experimental-design.protimiza.com.br/) and a level of significance of 95% ($p<0.05$).

After fermentation conditions optimization for the GA_3 production, a kinetic experiment was carried out under the following conditions: moisture content of 80% and temperature of 28 °C. At the end of each determined time (24 to 120 hours), the GA_3 production was evaluated.

2.4. Analytical procedure.

2.4.1. Fungal growth.

Microbial growth in the BSG was determined according to the adapted Standard Methods G21-15 [43]: 0 - absence of growth; 1 - little growth, small fragments of mycelium thrown into the culture medium; 2 - moderate growth, the appearance of fine pellet on the surface of the culture medium; 3 - great growth, the appearance of mycelium in more than half of the medium.

2.4.2. Extraction of GA_3.

For the extraction of GA_3, 15 g of the fermented were used, with phosphate buffer (pH = 8.0) ratio 1:3 (w:v) and then placed under orbital shaking at 120 rpm and 25 °C for 20 min. Afterward, liquid-liquid extraction was performed using 1:1 (v:v) ethyl acetate. The organic phase was recovered by using separating funnels and then route-evaporated at 50 °C and 10 rpm for reducing the volume of solvent (5% - initial volume) [10,44]. Then, the crystals of GA_3 were dissolved in 10 mL of ethanol (Merck, 99% purity) [45].

2.4.3. GA_3 content.

The GA_3 content was measured by the spectrophotometric method described by Holbrook *et al*. [46] and Berríos *et al*. [44]. After extraction, 1 mL of the sample was transferred to a volumetric flask (10 mL), and the volume was adjusted with HCl at 3.75 M. Then, the absorbance at 254 nm was measured and analyzed by a GA_3 (Sigma-Aldrich, 90% purity) calibration curve for determining the GA_3 production ($g.Kg^{-1}$ of BSG).

2.4.4. Water activity (a_w).

The a_w of the BSG was determined on the Aqualab equipment (Decagon Devices). After calibrating the equipment, 3 ± 0.2 g of the sample were placed in the instrument, and the reading was performed automatically.

3. Results and Discussion

3.1. Brewer's spent grain (BSG) characterization.

The dry BSG (dry basis) is composed of 8% moisture and 19.4% protein, 14.3% fat, 15.9% starch, and 12.8% total sugars. The results agree with the literature where, for Santos *et al*. [22], the moisture was between 8.7 and 10.8%, protein content 24.2%, fat content 3.9%, and ash content 3.4%. For Cordeiro *et al*. [32], the humidity was 75.4%, ashes 1.2%, carbohydrates 15.4%, total proteins 5.37%, total fats 2.43%, and crude fiber 3.98%. Mathias *et al*. [37] reported the brewer's spent grain composition showed high moisture (86%), ash of 3.8%, total organic carbon of 52%, total nitrogen of 4.3%, total protein of 26.9 and, carbon to nitrogen ratio of 12.1. For Mussatto *et al*. [47], the protein and extractive components were

22.4% and 4.6% ash in brewer's spent grain. Thus, BSG is well-balanced in terms of amino acids, fatty acids, and carbon sources [36,37]; that is, BSG can be used as the alternative substrate in the biotechnological process, as in the fungal GA_3 production.

3.2. Inoculum growth.

The amount of mycelial mass present in the inoculum is one of the main factors observed in fermentation processes. Therefore, *F. fujikuroi* growth kinetics was evaluated to determine the maximum mycelial mass in the shortest time. For this, the microorganism grown in commercial PDA, 2 or 3 discs, was transferred to potato dextrose broth with mineral supplementation at 28 °C and 120 rpm for up to 96 hours for growth estimation. Figure 1 shows that the largest amount of dry mass was found in 48 hours using 3 discs (6.3 $g.L^{-1}$). For both assays, it was noted that in 48 hours, the microorganism reaches the maximum growth in mycelial mass, and, after this time, a small increase in mycelial mass was observed (Figure 1). Thus, 3 discs containing *F. fujikuroi* and inoculum grew for 48 hours were adopted in the work sequence (mycelial mass <6 $g.L^{-1}$).

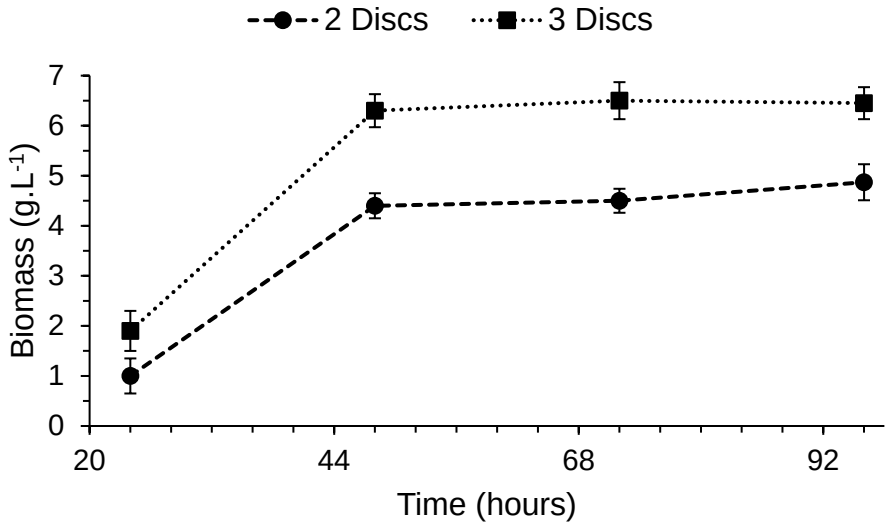

Figure 1. Kinetic evaluation of *Fusarium fujikuroi* mycelial growth for inoculum preparation. Experimental condition: potato dextrose broth with mineral supplementation at 28 °C and 120 rpm.

3.3. Optimization of GA_3 production by SSF.

For Singhania *et al*. [48] and Rios-Iribe *et al*. [49], the physical factors that affect the growth of microorganisms and GA_3 production are temperature, moisture, pH, agitation, aeration, and/or water activity. Thus, it is essential for the biotechnological processes to search for the best process conditions. For this, the experimental design methodology was used for the GA_3 production optimization by SSF. The matrix of the 2^2 CCD with coded and real values of the independent variables and the responses in GA3 production and mycelial growth are shown in Table 1.

Table 1. The matrix of the 2^2 CCD experimental design (coded and real values) for optimization of GA_3 production and mycelial growth by SSF of BSG in 96 hours.

Run	Moisture content – X_1 (%)	Temperature – X_2 (°C)	GA_3 production ($g.Kg^{-1}$)	Predicted GA_3 production ($g.Kg^{-1}$)[1]	Relative error (%)[2]	Mycelial growth[3]
1	-1 (60)	-1 (28.0)	0.54	0.51	5.56	2
1†	-1 (60)	-1 (28.0)	0.53	0.51	3.77	2
2	1 (80)	-1 (28.0)	0.80	0.79	1.25	3

Run	Moisture content – X_1 (%)	Temperature – X_2 (°C)	GA_3 production ($g.Kg^{-1}$)	Predicted GA_3 production ($g.Kg^{-1}$)[1]	Relative error (%)[2]	Mycelial growth[3]
2†	1 (80)	-1 (28.0)	0.82	0.79	3.66	3
3	-1 (60)	1 (35.0)	0.45	0.42	4.44	1
3†	-1 (60)	1 (35.0)	0.44	0.42	2.27	1
4	1 (80)	1 (35.0)	0.37	0.35	5.41	1
4†	1 (80)	1 (35.0)	0.38	0.35	7.89	1
5	0 (70)	0 (31.5)	0.46	0.52	-13.04	2
6	0 (70)	0 (31.5)	0.47	0.52	-10.64	2
7	0 (70)	0 (31.5)	0.47	0.52	-10.64	2

† genuine replicates.

[1] Calculated according to Equation 1.

[2] $Relative\ error\ (\%) = \left(\frac{Experimental\ production - Predicted\ production}{Experimental\ production}\right) * 100$

[3] 0 - absence of growth; 1 - little growth, small fragments of mycelium thrown into the culture medium; 2 - moderate growth, the appearance of fine pellet on the surface of the culture medium; 3 - great growth, the appearance of mycelium in more than half of the medium.

The main variables for fermentation, for both SSF and submerged, are the moisture content and a_w, limiting microbial growth and, consequently, metabolite production and the product's efficiency. Also, the effect of aw may be different on the growth and metabolite production [50]. With this, the a_w of BSG in different moisture contents was evaluated. BSG aw with the moisture content of 60, 70, and 80% was greater than 0.98. These a_w levels make feasible the growth of bacteria, yeasts, and mold, including *F. fujikuroi*. Corona *et al*. [50] confirmed this fact when carrying out studies on the influence of a_w to produce GA_3 by *F. fujikuroi*, showing that the optimal range of a_w is between 0.98 and 0.99. With these results, the a_w of this work provides conditions for good mycelial growth.

The visual characteristic of microbial growth under the experimental conditions of Table 1 can be visualized in Figure 2. Growth was classified as moderate (2) in runs 1, 5, 6, and 7 (Figure 2a,e), as a result of the appearance of a fine pellet on the surface of the BSG and the mycelial mass was present in less than half of the biomass used. For run 2, it was noted an excellent (3) mycelial growth throughout the BSG (Figure 2b), where it was also observed the greater GA_3 production. In runs 3 and 4 (Figure 2c,d), little (1) colonization of the substrate of fermentation was observed due the low mycelial growth, indicating that the conditions that used higher temperature were not propitious for the growth of *F. fujikuroi*. These results are following the study of Machado *et al*. [51], who evaluated the growth kinetics of *F. fujikuroi* and GA_3 production using solid substrates and observed that a great mycelial growth occurred at 28 °C and high moisture content.

Independent of the moisture content and temperatures employed, by visual analysis, it is possible to state that the *F. fujikuroi* could grow in the BSG. However, higher GA_3 production was achieved using high humidity (80%) and lower temperatures (28 °C). According to Machado *et al*. [20], GA_3 is a secondary metabolite. Thus, there is no direct relation between fungal biomass and GA_3 production, which is aligned with experimental design data, where production occurred in all conditions evaluated. Nevertheless, analysis of CCD data indicates that fungal growth is a relevant factor to be analyzed to achieve high production of GA_3 (Table 1). According to Cuali-Álvarez *et al*. [52] and Díaz *et al*. [53], when producing GA_3 by *F. fujikuroi*, it could be inferred that fungal growth was an important factor to obtain higher concentrations of the metabolite when using sewage sludge and welsh onion waste.

Among the conditions studied, the variation in GA_3 production ranged from 0.37 to 0.82 $g.Kg^{-1}$ (Table 1). This Table shows the good experimental reproducibility, which showed similar values in the central points (runs 5, 6, and 7), with a GA_3 production of 0.46, 0.47, and 0.47 $g.Kg^{-1}$, respectively.

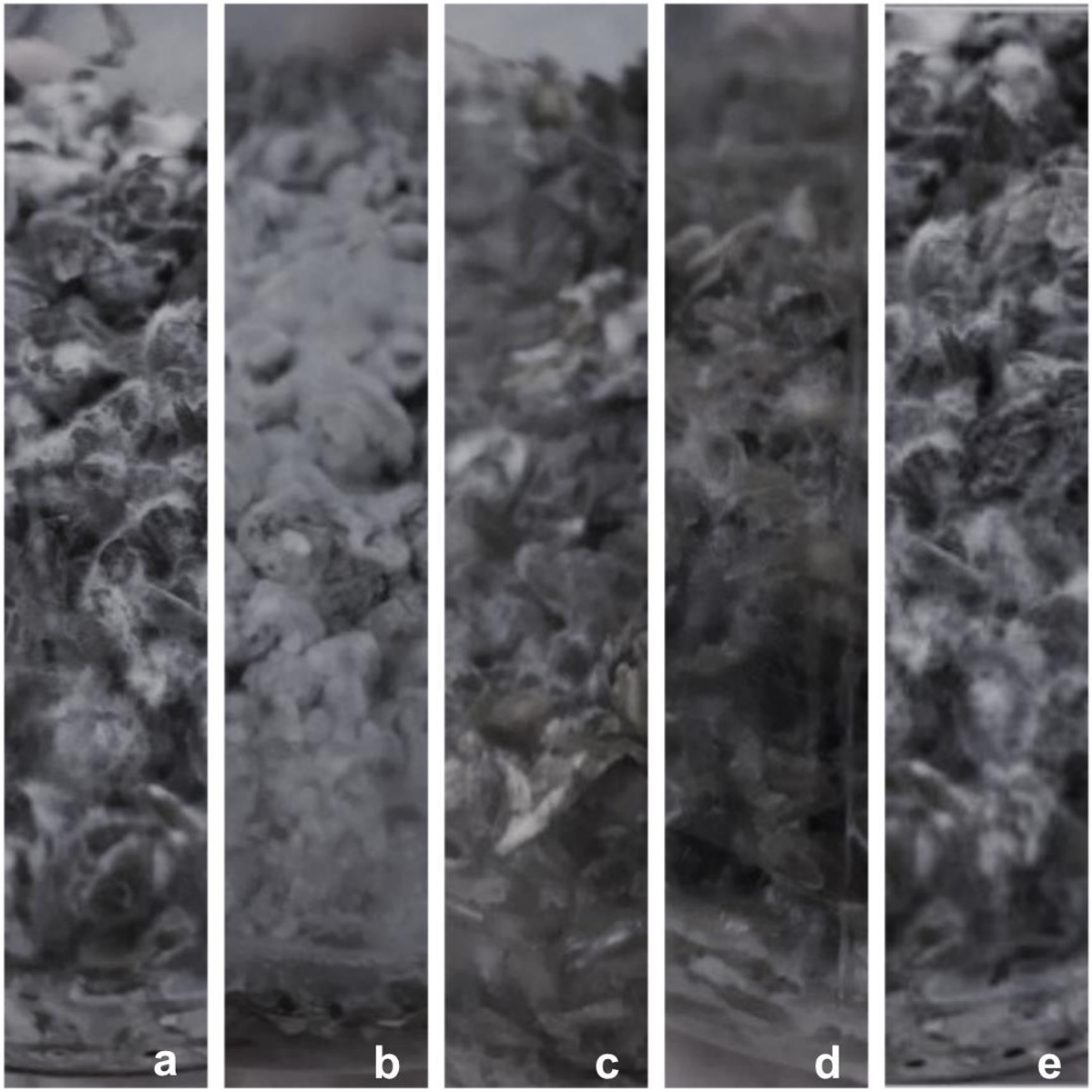

Figure 2. *Fusarium fujikuroi* mycelial growth by SSF of BSG under the experimental conditions of Table 1.

It can be observed the temperature showed a significant negative effect, where with the decrease of the temperature, the GA_3 production was increased. On the other hand, a positive effect on GA_3 production was observed for the studied range's moisture content (see runs 1 and 2). Werle *et al*. [26] and Machado *et al*. [20] also observed a significant positive effect for moisture on the GA_3 production by SSF. With the decrease in temperature and increase in moisture content, the production of GA_3 also increases.

These results (Table 1) were similar to those found by Werle *et al*. [26] using a mixture of crude rice bran and malt waste (0.93 g GA_3.Kg of the substrate), and better than those obtained by Machado *et al*. [20], using cassava bagasse and coffee husk (0.49 g $GA_3.Kg^{-1}$ of the substrate), and inferior to those found by Corona *et al*. [50] with wheat bran and starch (4.5 - 5.0 g $GA_3.Kg^{-1}$ of the substrate), Rodrigues *et al*. [10] from sugarcane bagasse, soy bran, soy and coffee husk, citric pulp and cassava bagasse (0.1 - 5.8 g $GA_3.Kg^{-1}$ of the substrate), and De Oliveira *et al*. [22] using citric pulp (7.60 g $GA_3.Kg^{-1}$ of the substrate).

With the CCD results, analysis of variance (ANOVA) was performed to evaluate the effect of variables on GA_3 production. This analysis carried out an F-test, indicating whether there are significant differences between the averages. The values found in this test were tabled

$F_{0.95;3;7}$ = 4.34 and calculated F = 40.58 and, as the calculated F is 9.35 times higher than tabled F, it means that there are statistically significant differences between the averages ($p \leq 0.05$). The probability of significance (*p-value*) was 0.00008. It was also possible to verify the percentage of total variance model, evaluated through the R^2, which showed a value of 94.5%, resulting in an empirical mathematical model (Equation 1) expressing the GA_3 production ($g.Kg^{-1}$) as a function of moisture content (%) and temperature (°C).

$$GA_3 Production\ (g * Kg^{-1}) = 0.52 + 0.05 * X_1 - 0.13 * X_2 - 0.09 * X_1 * X_2 \quad (1)$$

Where X_1 denotes moisture content and X_2 temperature.

Figure 3 shows the response surfaces generated from the statistical analysis. It can be observed that the results were consistent, as previously discussed. Thus, the optimized conditions to produce GA_3 (0.82 $g.Kg^{-1}$) indicated by this study are those of the run 2 of Table 1, namely moisture content of 80%, the temperature of 28 °C, and fermentation time of 96 h.

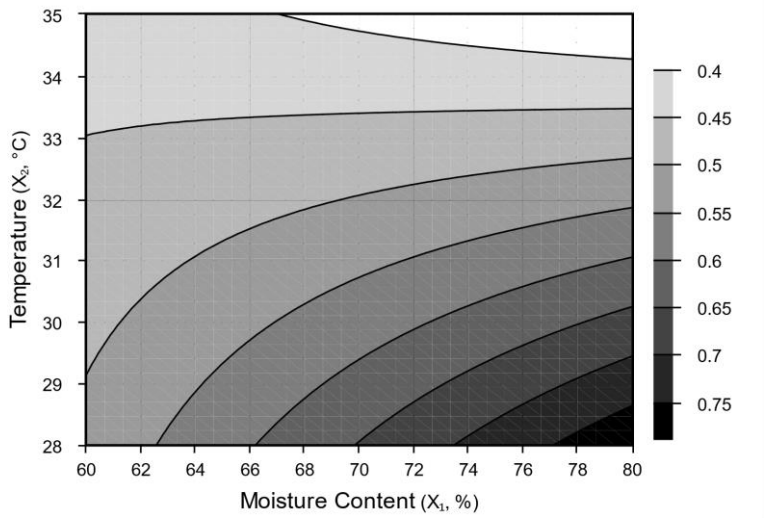

Figure 3. Contour plot of GA_3 production by SSF of BSG as a function of moisture content and temperature. Experimental data and conditions are shown in Table 1.

A single solid substrate was used, and a higher GA_3 production was achieved in this work. However, Camara *et al*. [8], addressing the current advances in the GA_3 production, reported that higher metabolite production values are achieved when more than one solid substrate is used. Thus, showing that the BSG has great potential to produce GA_3 by SSF.

3.4. GA_3 production kinetics.

A kinetic experiment was carried out at the optimized experimental conditions (moisture content of 80% and temperature of 28 °C) for BSG bioconversion in GA_3 by SSF, since it provided satisfactory production results and great mycelial growth. The kinetics showed that for the first days of fermentation of BSG for GA_3 production was slow, reaching approximately 0.43 $g.Kg^{-1}$ in 72 hours. After these 72 hours there was a considerable increase (~2-fold) in the production of GA_3, reaching a maximum production of 0.82 $g.Kg^{-1}$ in the total 96 hours of fermentation. After 96 hours, there was a decrease in GA_3 production (0.45 $g.Kg^{-1}$) of approximately 55%, due to its decomposition into other compounds, according to Machado *et al*. [20], is a characteristic behavior of kinetics of secondary metabolites such as GA_3. It can be inferred that this decomposition of the product in other components is also due to its instability to certain environmental conditions encountered [54].

GA_3 production kinetics of this study is like those reported by Gelmi *et al*. [54], Hollmann *et al*. [55], and the patent deposited by Vidhya and Balu [56].

4. Conclusions

The brewer's spent grain showed great potential for use as a substrate in biotechnological processes, mostly due to excellent inducers of metabolites production (protein content and carbon sources). *F. fujikuroi* was able to grow in the BSG spontaneously and had maximum production of GA_3 (0.82 $g.Kg^{-1}$). These results demonstrate that the SSF using BSG as the medium for the growth of *F. fujikuroi* is a viable way for the GA_3 production. It is necessary to carry out investigations of increase of scale-up, given the use of SSF for industrial GA_3 production.

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Conflicts of Interest

The authors declare no conflict of interest.

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