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Gibberellic Acid Production by Solid-State Fermentation in Coffee Husk

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Abstract

Five strains of *Gibberella fujikuroi* and one of *Fusarium moniliforme* were screened for the production of gibberellic acid (GA_3) in coffee husk, and based on the results, one strain, *G. fujikuroi* LPB-06, was selected. The comparative production of GA_3 by solid-state fermentation and submerged fermentation indicated better productivity with the former technique, mainly with pretreated substrate. The GA_3 accumulation was 6.1 times higher in the case of solid-state fermentation. Considering the C:N relation, higher yields of GA_3 were achieved using a mixed substrate comprising coffee husk and cassava bagasse (7:3, dry wt), increasing the results twice. Supplementation of an optimized saline solution containing 0.03% $FeSO_4$ and 0.01% $(NH_4)_2SO_4$ enhanced the accumulation of GA_3 1.7 times in the fermented substrate. Under the finally optimized condition, the culture gave a maximum of 492.5 mg of GA_3/kg of dry substrate, with a pH of 5.3, moisture of 75%, and incubation temperature of 29°C. GA_3 yield was almost 13 times more than the initial results.

Index Entries: Gibberellic acid; *Gibberella fujikuroi*; biosynthesis; solid-state fermentation; coffee husk; alkali hydrolysis.

Introduction

Gibberellins (GAs) are a group of diterpenoid acids that function as plant growth regulators influencing a range of developmental processes in higher plants. Presently, more than 100 compounds of this family are known. One of them, gibberellic acid (GA_3), has received greatest attention

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because of its significant application as a plant growth promoter. It affects stem elongation, germination, elimination of dormancy, flowering, sex expression, enzyme induction, and leaf and fruit senescence *(1)*. GA_3 is a high-valued plant growth regulator with various applications in agriculture. Its high price, however, has limited its use to high-premium crops.

The industrial process currently used for the production of GA_3 is based on the submerged fermentation technique. In spite of the use of the best process technology, the yield of GA_3 is considered low. As early as 1979, it was stressed that the submerged fermentation process used for its production was approaching a saturation point beyond which cost reduction would be impossible. The presence of product in dilute form in submerged fermentation was recognized as a major obstacle in the economic manufacture of the product, mainly owing to the consequent higher costs of downstream processing and disposal of wastewater. Moreover, the cost of separation of the microbial cells from fermentation broth using centrifugation or microfiltration has been reported to involve between 48 and 76% of the total production cost of the microbial metabolite by submerged fermentation *(2,3)*. Thus, it was considered logical only to look beyond the conventional submerged technique and explore other alternatives to achieve a more economic process. Lower production costs could lead to a more extensive application of GA_3 in agriculture and consequent benefits.

Recently, different studies have been carried out to decrease production costs, using several approaches such as screening of the fungal strains to isolate hyperproducer strains; optimization of the nutrients and culture conditions; development of alternative production processes such as immobilized cells and fed-batch culture; and minimization of the cost of the extraction procedure *(4)*. Another technique, solid-state fermentation, has also been investigated to increase the yields of GA_3 and thus to decrease production and extraction costs *(5)*. Indeed, the solid-state fermentation technique has shown several economic advantages over the submerged fermentation process in the production of microbial biomass and metabolites and in the value addition of agroindustrial byproducts *(6–11)*.

Brazil is the largest producer of coffee in the world. Its production in 1999 was 24.8 million bags. During the industrial processing of coffee cherries, several residues are generated, representing about 50% of total mass, which practically do not find any useful application. Instead, their disposal is a major environmental concern. These products are rich in organic matter, which could make them suitable for bioconversion processes. However, these residues also contain toxic substances, such as phenolic compounds (tannins, chlorogenic acid, and caffeic acid) and caffeine, which inhibit their application. If the toxicity could be overcome, useful products could be produced from these residues by cultivating microbes in submerged fermentation or solid-state fermentation *(9,10,12,13)*.

The present work was undertaken to explore the possibility of using coffee husk as solid substrate for the production of GA_3 using the solid-state fermentation technique. Studies were carried out to select the best strain of

Gibberella fujikuroi, which was further used in solid-state fermentation to optimize the fermentation parameters such as supplementation of minerals and nitrogen to the substrate, initial pH and moisture of the substrate, and incubation temperature, for improved yields of GA_3. Studies were also performed on the pretreatment of the substrate to determine whether it improves fermentation efficiency.

Materials and Methods

Microorganisms and Inoculum

Five strains of *Gibberella fujikuroi* and one of *Fusarium moniliform*, from LPB Collection, Curitiba, were used to select the best GA_3 producer. These were maintained on potato-dextrose agar slants at 4°C and were grown twice in succession in Czapek Dox medium on a rotary shaker (200 rpm) at 30°C for 48 h to develop the inoculum. The inoculum was stored at 4°C.

Solid Substrate

Coffee husk was ground and sieved to obtain particles between 0.8 and 2.0 mm. It was autoclaved at 121°C for 20 min.

Submerged Fermentation

The substrate for submerged fermentation was prepared by adding 1 L of distilled water to 200 g of coffee husk and autoclaving at 100°C for 60 min. The extract was filtered through a muslin cloth. The solid residue was dried for 24 h at 65°C for 20 h and used as substrate for solid-state fermentation to determine whether this could still be used as substrate in solid-state fermentation. The liquid residue was used for submerged fermentation studies.

Pretreatment of Coffee Husk

Two hundred grams of dry coffee husk was mixed with 1 L of KOH solution with different concentrations of alkali and autoclaved for different times. The husk after autoclaving was filtered and washed with distilled water until all adhering alkali were removed.

To test the influence of the pretreatment factors on the production of GA_3, two statistical experimental designs were used. The parameters studied with the $3^{(2-0)}$ experimental design were the concentrations of KOH (5, 20, and 35 g/L) and different extraction times (15, 30, and 45 min).

The second design was defined to optimize the conditions of the pretreatment established by the first experimental design. The results of GA_3 and the statistical analysis that related the most significant factor were observed. Lower KOH concentrations (0.5, 2.5, and 5 g/L) were used with the same extraction times (15, 30, and 45 min).

Solid-State Fermentation

Solid-state fermentation was carried out by placing 10 g of coffee husk in 250-mL Erlenmeyer flasks and inoculating it with the microbial suspension prepared as already described. Except for optimization of the physical parameters, all the solid-state fermentation experiments were carried out with a substrate pH of 4.5, moisture at 65%, 15% inoculum, and incubation at 29°C for a period of 7 d. Substrate (coffee husk) pretreatment was done with 2.5 g of KOH/L and autoclaving as already described for 45 min.

In another set of experiments, coffee husk was mixed with cassava bagasse (7:3, dry wt basis), and the mixed substrate was supplemented with a mineral salts–acid solution (3.5% HCl) containing 0.03% $FeSO_4$ and 0.01% $(NH_4)_2SO_4$ and autoclaved at 121°C for 30 min.

Estimation of GA_3

Thin-Layer Chromatography

After fermentation, GA_3 was extracted with ethyl acetate at pH 2.5. The extract was concentrated under reduced pressure to 10–15 mL and was subjected to thin-layer chromatography (TLC) for confirmation of the presence of GA_3. The mobile phase was chloroform, ethyl acetate, acetic acid (40:60:5). After evaporation of the solvent, the residue was dissolved in 80% methanol in water (about 8 mL), and the mixture was applied to a preconditioned silica C-18 column (300 mg). The volume of the eluate was then completed to 10 mL.

High-Performance Liquid Chromatography

The fermented substrate was extracted with phosphate buffer, pH 8.0. The extract was purified with C-18 silica packed in a Pasteur pipet and then analyzed. The high-performance liquid chromatography (HPLC) system used was a Varian composed of a pump (9012Q), a diode array detector (9065), and an autosampler (AI200). The data were processed using Varian Workstation 5.1. The column was a C18 (5 µm, 4.5 × 250 mm) and the mobile phase was methanol and water (40:60).

Optimization of Nutritional Factors

C:N Relation

To change the original coffee husk C:N (which was about 5:1), cassava bagasse was used. Cassava bagasse contains amide, resulting in a very high C:N relation. Cassava bagasse was added at five different concentrations (15, 30, 45, 60, 75, and 90%). In the experiments conducted after this study, the substrate used was composed of 30% cassava bagasse and 70% pretreated coffee husk.

Optimization of Saline Solution

To test the influence of the presence of certain salts, two statistical experimental designs were used. In the first, the parameters studied with

Table 1
Addition of Saline Solutions
for First Experiment

Salt added	Salt concentration (g/100 mL H_2O)
KH_2PO_4	10
K_2SO_4	20
$MgSO_4$	10
$ZnSO_4$	10
$CuSO_4$	10
$FeSO_4$	10
$(NH_4)_2SO_4$	2

Table 2
Optimization of Physical Parameters for First Experiment

Level	Temperature (°C)	Moisture (%)	pH
–1	26	60	4.0
0	28	65	4.5
+1	30	70	5.0

Table 3
Optimization of Physical Parameters
for Second Experiment

Level	Moisture (%)	pH
–1	70	5.0
0	75	5.3
+1	78	5.5

a $2^{(7-4)}$ experimental design were on the presence or absence of seven salts reported to influence the production of gibberellins in the concentrations shown in Table 1. The second optimization experiment included two salts that were found useful in the first experiment at three different concentrations with a $3^{(2-0)}$ experimental design: 5, 30, and 55 g of $FeSO_4$/100 mL and 0.5, 10, and 20 g of $(NH_4)_2SO_4$/100 mL.

Optimization of Physical Parameters

The parameters studied were initial moisture and pH of the substrate and incubation temperature. To test the influence of these parameters, two experimental designs were used. The first was a $2^{(3-1)}$ (levels at Table 2), and since it showed that temperature had no significant influence, the second experiment (Table 3) was a $3^{(2-0)}$ design, varying only pH and moisture.

Table 4
Presence of GA_3 Produced by Fermentation, Analyzed with TLC[a]

Strain	Submerged fermentation of husk extract	Solid-state fermentation	
		Coffee husk	Treated residue
LPB-1	+++	++++	++++
LPB-2	++++	++	+++
LPB-3	++	+++	+++
LPB-4	+	+	++
LPB-5	+	+	++
LPB-6	++++	++++	+++++

[a]The number of plus signs indicates the degree of fluorescence; that is, the more the plus signs, the better the fluorescence.

Kinetics of GA_3 Production

To study the metabolism of GA_3 production in solid-state fermentation, and to confirm the results obtained in the optimizations, the kinetics of production were monitored for 12 d using the optimized conditions. In addition to the concentration of GA_3 in the fermented substrate, final moisture, pH, and loss of weight of the substrate were analyzed. Twenty-four Erlenmeyer flasks were prepared, and every 24 h, two of them were taken for analysis.

Results and Discussion

Screening of Fungi

Among the six strains screened, *G. fujikuroi* LPB-6 produced the highest quantity of GA_3 at the end of a 7-d incubation period in the three systems of fermentation (submerged fermentation using coffee husk extract and solid-state fermentation with untreated coffee husk and the solid residue obtained after the filtration of extract for submerged fermentation). The comparative production determined with TLC is shown in Table 4, which also shows that the solid residue obtained after the extraction of coffee husk was the best substrate for GA_3 production (considering that a better fluorescence indicated higher production of GA_3). This could probably be explained by the fact that during extraction of coffee husk, water-soluble components of coffee husk were removed and this facilitated better growth and activity of the fungal culture. It is known that benzoic and cinnamic acids and derivatives are plant growth inhibitors. These compounds may also inhibit the biosynthesis of gibberellins by *G. fujikuroi*. Coffee beans are known to contain large amounts of caffeic and chlorogenic (cinnamic acid derivatives) as well as tannic acids (a benzoic acid derivative). Therefore, the removal of these compounds by alkali could improve yields of gibberellic acid in coffee husk fermentation.

The results obtained with HPLC analysis (30 mg of GA_3/L in submerged fermentation, 18.3 mg of GA_3/kg in solid-state fermentation in

Table 5
Influence in GA_3 Production by Variation in C:N Relation by Addition of Cassava Bagasse

Cassava bagasse (%)	C:N relation	mg GA_3/kg substrate
15	5.9	160.7
30	7.3	230.5
45	9.3	206.5
60	12.9	62.5
75	20.8	62.4
90	52.5	60.2

coffee husk, and 38.4 mg of GA_3/kg in solid-state fermentation in the extracted residue, data not shown) further confirmed the results obtained with TLC. These results prompted us to attempt an optimization of the pretreatment conditions.

Pretreatment Optimization

The pretreatment was optimized with a factorial plan, utilizing the Statistica program. The first experiment showed that the concentration of alkali had the most significant effect, and production increased with a decrease in KOH concentration. The best production—99.7 mg of GA_3/kg of substrate—was achieved for a 45-min treatment with a solution of 5 g/L of KOH. Hence, in the second optimization lower concentrations of KOH were used, which showed 2.5 g/L of KOH for 45 min as the most suitable, resulting in 112.6 mg of GA_3/kg of substrate (data not shown). A smaller alkali concentration (0.5 g/L) was probably insufficient for removal of the inhibitors present in coffee husk, leading to lower GA_3 production. The increase in the extraction time increased the response variable; however, longer treatments were not done because time was not a significant variable at the 5% level.

Nutritional Parameters

C:N Relation

Cassava bagasse was mixed in different quantities with the coffee husk to obtain varying C:N ratios. As shown in Table 5, higher titers of GA_3 were obtained with the substrate enriched with 30% cassava bagasse, resulting in a C:N relation of 7.3, reaching a production of 230.6 mg of GA_3/kg of substrate. In subsequent experiments, therefore, pretreated coffee husk admixed with 30% cassava bagasse was used as the substrate.

Enrichment with Salts: First Optimization

The results, as recorded in Fig. 1, showed that supplementation of salts had a negative effect on the production of GA_3. This could probably be explained by the fact that many such salts occur naturally in coffee husk,

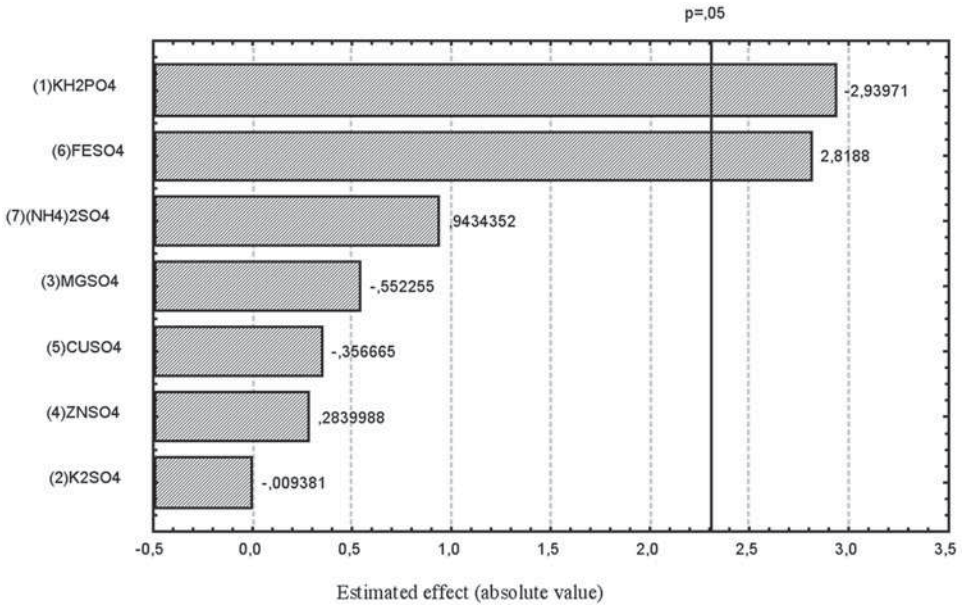

Fig. 1. Pareto chart of effects of first optimization of nutritional parameters with seven variables in two levels. The response variable is given as mg of GA_3/kg of substrate.

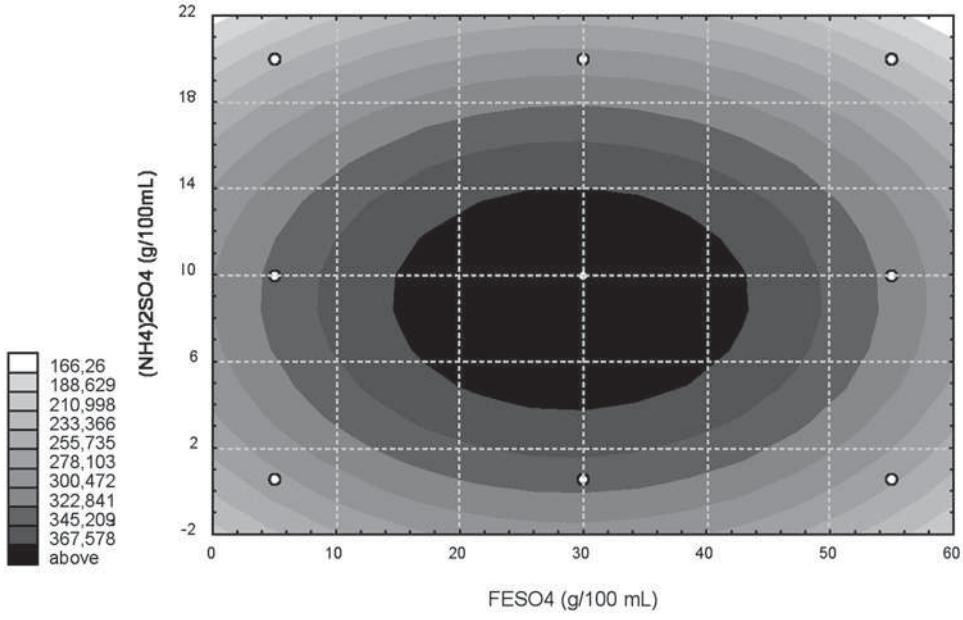

Fig. 2. Contour response to GA_3 production as function of $(NH_4)_2SO_4$ and $FeSO_4$ concentrations (levels given in mg of GA_3/kg of substrate). $R^2 = 0.9532$.

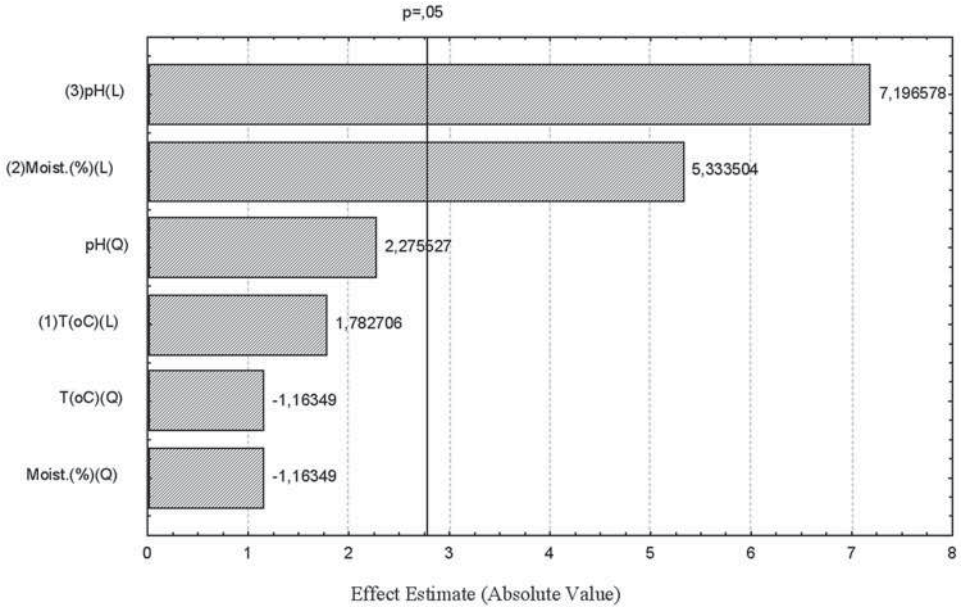

Fig. 3. Pareto chart of effects for first optimization of physical parameters. The response variable is given as mg of GA_3/kg of substrate.

and external addition of these could become inhibitory for the growth and metabolism of the microorganism.

Enrichment with Salts: Second Optimization

The concentration of the salts ($FeSO_4$ and $[NH_4]_2SO_4$) was varied with a view to determine the most positive influence on the production of GA_3. Maximum production was obtained (389 mg of GA_3/kg of substrate) with the saline solution containing 30 mg of $FeSO_4$ and 10 mg of $(NH_4)_2SO_4$/100 mL, as shown in the contour response in Fig. 2.

Physical Parameters

First Experiment

The Pareto chart of effects in Fig. 3 shows that the variable of larger influence on the production of GA_3 was the pH, followed by the initial moisture of the substrate. The pH levels used were 4.0, 4.5, and 5.0. As demonstrated by the Pareto chart of effects, the production of GA_3 increased with the increase in pH. According to Kumar and Lonsane *(3)*, the pH was one of the most important factors for gibberellin production, mainly for definition of produced gibberellin. They suggested the pH range of 3.5–5.8 as suitable for an increase in GA_3 production.

Another parameter in this study was the moisture of the substrate. The levels used were of 60, 65, and 70%. Here, also, the Pareto chart demonstrated that there was a gradual increase in the production of GA_3 with an increase in the initial moisture contents of the substrate. Generally, in solid-

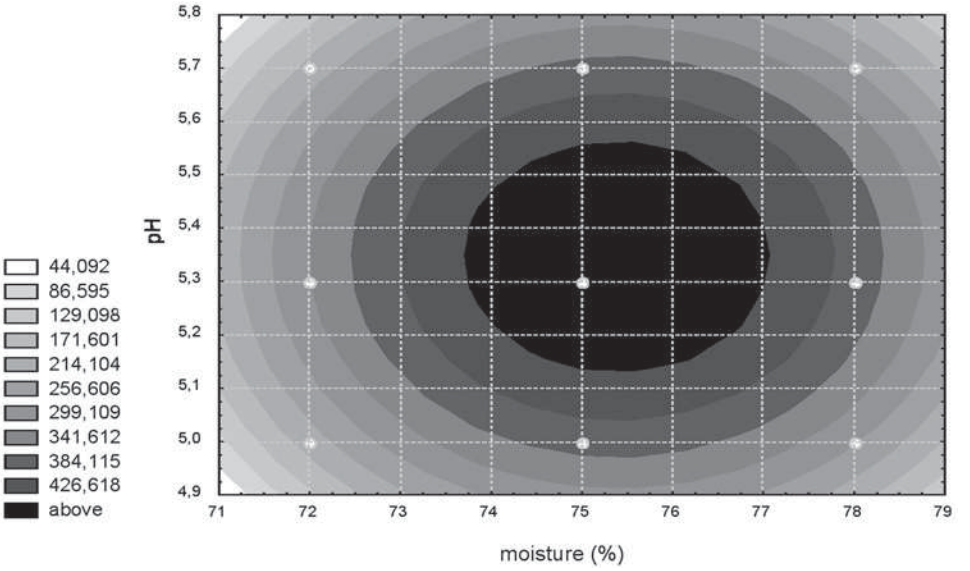

Fig. 4. Contour response to GA_3 production as function of pH and initial moisture of substrate (levels given in mg of GA_3/kg of substrate). $R^2 = 0.83125$.

state fermentation moisture of the substrate is one of the most important factors, because it is a function of the capacity of retention of the substrate, and does not correspond, necessarily, to the needs of the microorganism growing in it *(14,15)*. According to Raimbault et al. *(16)*, values between 35 and 80% should be used, depending on the microorganism and substrate.

The third factor of the study was the temperature of incubation. None of the temperatures used significantly affected GA_3 production. Thus, in subsequent experiments, the temperature was maintained at 29°C.

Second Experiment

With this optimization, under optimized conditions of substrate pH and moisture of 5.0 and 70%, respectively, and incubation at 29°C, a production of 492.5 mg of GA_3/kg of substrate was achieved (Fig. 4).

Kinetics of GA_3 Production

During the course of fermentation, the pH of the substrate changed from an initial value to 5.5 during the first 24 h and then remained between 5.4 and 5.6 during the rest of the fermentation. The substrate moisture was maintained constant at 75%. Tomasini et al. *(17)* had observed a similar behavior during solid-state fermentation with cassava bran; they had an initial pH of 6.0, which in the first 10 h decreased to 5.3, but remained constant until the end of fermentation. In submerged fermentation, generally no change, or at least no major change in pH has been observed (irrespective of the nature and concentration of nitrogen source such as urea pH of 5.77–6.18, glyine pH of 4.20–4.62, ammonium nitrate pH of 4.68–5.22, and ammonium acetate pH of 5.44–5.79 *[18]*).

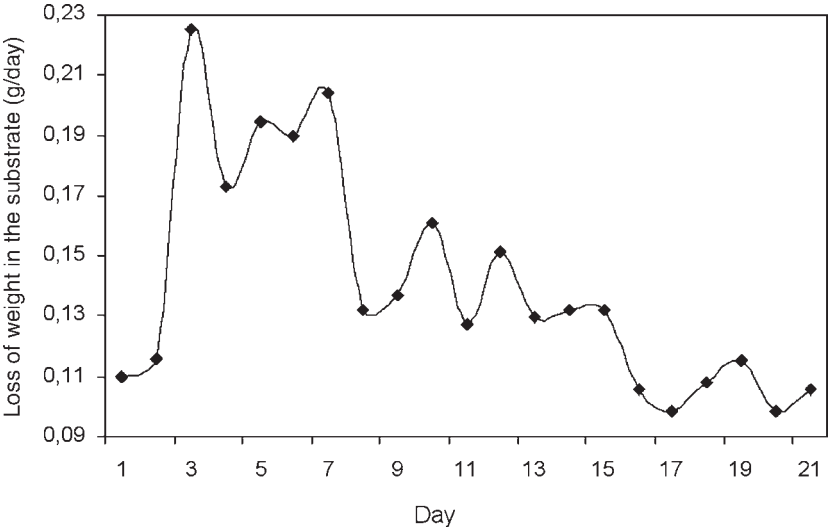

Fig. 5. Loss of weight in substrate during fermentation.

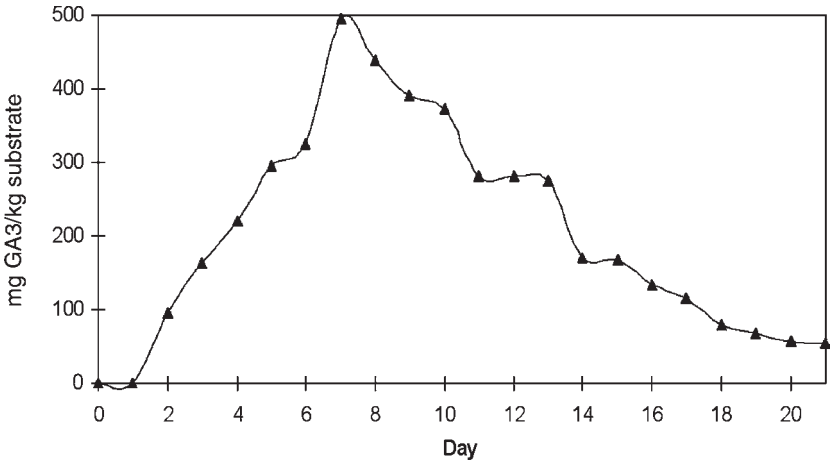

Fig. 6. Gibberellic acid production during fermentation.

Figure 5 shows the loss of weight in the substrate during the course of fermentation, which apparently could be divided into four zones, 0–3, 4–7, 8–14, and 15–21 d, with values of 0.11, 0.20, 0.14, and 0.11 g/d, respectively. Considering that the loss of weight in substrate could be related to growth of microorganism, there was an adaptation zone until the third day, followed by a phase with better growth of microorganism until the seventh day. After that there started a gradual decrease in growth in the second and third week of fermentation.

Figure 6 indicates that the best production of GA_3 was after the seventh days of fermentation, reaching 494 mg of GA_3/kg of substrate. Since this value was very close to the one achieved in the optimization, it proved

the reproducibility of the method. An interesting aspect shown was the delay of the metabolite concentration after the seventh day (about 1.8 mg/[kg of substrate·h]). This phenomenon was observed by other investigators who worked on gibberellic acid production in solid-state fermentation. Tomasini et al. *(17)* using cassava bran achieved maximum production after 35 h, after which there was a delay of about 11 mg/kg of substrate/h. Bandelier et al. *(5)* working with wheat bran had observed a delay of 18 mg/(kg of substrate·h), after the tenth day of fermentation. Pastrana et al. *(19)* presented a hypothesis for this phenomenon. They attribute this behavior to the mass transfer restrictions (or oxygen diffusion) determined by the relative proportions between biomass and free water in between the particles. However, this hypothesis could only explain the stagnation in the productive process of the metabolite and not its delaying. Perhaps this could be explained by the continuation of the biosynthesis, changing gibberellic acid in another gibberellin, or other organic compounds.

Conclusion

From the results, it can be concluded that a mixture of coffee husk and cassava bagasse could be a good substrate for the production of gibberellic acid by solid-state fermentation. With judicious selection of nutritional and physical factors, the yield of GA_3 was improved and we obtained 492.4 mg of GA_3/kg of substrate, which could be considered reasonably high when compared with the other findings as reported in the literature on solid-state fermentation.

Acknowledgments

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