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*A N I N T E R N A T I O N A L J O U R N A L*

# A New Alternative to Produce Gibberellic Acid by Solid State Fermentation

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## ABSTRACT

*Gibberellic acid ($GA_3$) is an important hormone, which controls plant's growth and development. Solid State Fermentation (SSF) allows the use of agro-industrial residues reducing the production costs. The screening of strains (four of Gibberella fujikuoroi and one of Fusarium moniliforme) and substrates (citric pulp, soy bran, sugarcane bagasse, soy husk, cassava bagasse and coffee husk) and inoculum preparation study were conducted in order to evaluate the best conditions to produce $GA_3$ by SSF. Fermentation assays were carried out in erlenmeyers flasks at 29°C, with initial moisture of 75-80%. Different medium for inoculum production were tested in relation to cells viability and $GA_3$ production by SSF. F. moniliforme LPB 03 and citric pulp were chosen for $GA_3$ production. The best medium for inoculum production was citric pulp extract supplemented with sucrose. $GA_3$ production by SSF reached 5.9 g /kg of dry CP after 3 days of fermentation.*

**Key words:** gibberellic acid, *Fusarium moniliforme,* solid state fermentation, inoculum, agro-industrial residues

## INTRODUCTION

Gibberellic acid ($GA_3$) is an important member of the gibberellins family and acts as a natural plant growth hormone, controlling many development processes, which is gaining great attention all over the world due to its effective use in agriculture, nurseries, tissue culture, tea gardens, etc. (Davies, 2004; Shukla et al., 2005). The cost of $GA_3$ has restricted its use to preclude application for plant growth promotion, except for certain high value plants. Reduction in its production costs could lead to wider applications for a variety of crops (Kumar and Lonsane, 1989; Tudzynski, 1999; Linnemannstons et al., 2002).

Industrially $GA_3$ is produced by submerged fermentation (SmF) using the ascomycetous fungus *Gibberella fujikuroi*, renamed *Fusarium fujikuroi* (O'Donnell et al., 1998). The cost of $GA_3$ production using SmF is very high, mainly due to extremely low yield and expensive downstream processing.

Therefore in recent years the possibility of using solid state fermentation (SSF) has attracted a great deal of attention (Machado et al., 2004; Corona et al., 2005). In fact, the SSF technique has shown a number of economic advantages over SmF process in the production of microbial biomass and metabolites and the valorization of agro-industrial by-products (Pandey et al., 2001; Soccol and Vandenberghe, 2003; Soccol et al., 2006). Various

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processes of $GA_3$ production using SSF have been studied (see Table 1).
The main objective of the present investigation was select strains and substrates and study the inoculum preparation for $GA_3$ production by SSF using agro-industrial residues.

**Table 1** - Different strategies $GA_3$ production in SSF using waste and/or by products

| Substrate/ Support | Estrategy | Bioreactor | Production | References |
|---|---|---|---|---|
| Glucose and Glicina | - | Stirred fermentor (6L) | 520 mg/L | Hollmann et al., 1995 |
| Wheat meal | Fed-batch operation | Pilot-scale reactor (50L) | 3 g/kg | Bandelier et al., 1997 |
| Glucose and rice meal | | erlenmeyer flasks (500mL) | 2,862 g/L | Escamilla et al., 2000 |
| Coffee husk and cassava bagasse | Optimization of physical and chemical conditions | erlenmeyer flasks (250mL) | 492,5 mg/kg | Machado et al., 2002 |
| Glucose and rice meal | | erlenmeyer flasks (250mL) | 1 g/L | Shukla et al., 2005 |
| Wheat meal and soluble starch | Optimization of physical chemical conditions | Glass columns | 4.5-5 g/kg dry basis | Corona et al., 2005 |

## MATERIALS AND METHODS

### Microorganism

Strains of *Gibberella fujikuroi* LPB 02, LPB 05, LPB 06, LPB Bca and *Fusarium moniliforme* LPB 03, were conserved in PDA (Potato Dextrose Agar) slants, where the microorganism was cultured. These strains were incubated at 28ºC for six days and kept at 4ºC for up to two months.

### Substrates

Six agro-industrial residues were used as substrate in this study: citric pulp (CP), soy bran (SB), sugarcane bagasse (SCB), soy husk (SH), cassava bagasse (CB) and coffee husk (CH). These substrates were previously dried, triturated in a records mill and bolted to get a particle size between 0.8 and 2 mm.
CH was pre-treated with an alkaline solution (KOH 0,25%, 100°C for 45 min) in order to eliminate toxic substances naturally present in the CH (Machado et al., 2002).

### Solid State Fermentation (SSF)

Substrates were impregnated with the nutritive solution containing $FeSO_4.7H_2O$ and $(NH_4)_2SO_4$, in order to have an initial moisture content of 75-80%. The pH was adjusted to 5.0 – 5.5 and the support was then inoculated (10% - 20% v/w). After homogenization, the inoculated support was transferred to 250 mL erlenmeyer flasks. Flasks were incubated for 7 days at 29°C.
The screening of strains and substrates was conducted using an experimental design (six factor simplex-lattice design). The mixture of six agro-industrial residues (previously described) in a 1:1 ratio was studied for the five strains (Table 2). The inoculum used in this step (15% v/w) was obtained by growth in Czapek broth medium for seven days.

**Table 2 -** Six factor simplex-lattice design for individual tests using different residues as substrates

| Assay | CP | CB | SB | SH | CH | SCB | LPB 02 strain* | LPB 03 strain* | LPB 05 strain* | LPB 06 strain* | LPB BCA strain* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1 | 0 | 0 | 0 | 0 | 0 | 3.9 | 5.7 | 3.8 | 3.1 | 3.5 |
| **2** | 0 | 1 | 0 | 0 | 0 | 0 | 0.5 | 0.5 | 0.3 | 1.1 | 0.8 |
| **3** | 0 | 0 | 1 | 0 | 0 | 0 | 0.0 | 3.8 | 0.0 | 0.0 | 3.4 |
| **4** | 0 | 0 | 0 | 1 | 0 | 0 | 3.7 | 2.8 | 1.8 | 1.8 | 2.8 |
| **5** | 0 | 0 | 0 | 0 | 1 | 0 | 3.1 | 0.8 | 2.9 | 2.8 | 3.3 |
| **6** | 0 | 0 | 0 | 0 | 0 | 1 | 0.7 | 1.0 | 2.4 | 1.5 | 0.9 |
| **7** | 1/2 | 1/2 | 0 | 0 | 0 | 0 | 1.5 | 2.9 | 0.3 | 1.3 | 1.9 |
| **8** | 1/2 | 0 | 1/2 | 0 | 0 | 0 | 4.0 | 1.0 | 1.9 | 0.0 | 0.0 |
| **9** | 1/2 | 0 | 0 | 1/2 | 0 | 0 | 3.9 | 5.0 | 3.7 | 3.0 | 3.0 |
| **10** | 1/2 | 0 | 0 | 0 | 1/2 | 0 | 2.4 | 4.3 | 1.1 | 3.1 | 4.0 |
| **11** | 1/2 | 0 | 0 | 0 | 0 | 1/2 | 2.0 | 1.5 | 0.0 | 1.1 | 0.7 |
| **12** | 0 | 1/2 | 1/2 | 0 | 0 | 0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| **13** | 0 | 1/2 | 0 | 1/2 | 0 | 0 | 3.5 | 3.1 | 0.5 | 0.0 | 1.5 |
| **14** | 0 | 1/2 | 0 | 0 | 1/2 | 0 | 1.4 | 2.2 | 2.2 | 2.2 | 3.0 |
| **15** | 0 | 1/2 | 0 | 0 | 0 | 1/2 | 0.7 | 1.3 | 2.2 | 0.9 | 1.1 |
| **16** | 0 | 0 | 1/2 | 1/2 | 0 | 0 | 1.2 | 1.1 | 2.6 | 4.7 | 3.2 |
| **17** | 0 | 0 | 1/2 | 0 | 1/2 | 0 | 1.2 | 0.9 | 3.6 | 4.3 | 3.5 |
| **18** | 0 | 0 | 1/2 | 0 | 0 | 1/2 | 1.1 | 0.8 | 1.5 | 0.0 | 0.8 |
| **19** | 0 | 0 | 0 | 1/2 | 1/2 | 0 | 3.4 | 3.6 | 3.1 | 1.3 | 2.6 |
| **20** | 0 | 0 | 0 | 1/2 | 0 | 1/2 | 0.0 | 0.0 | 2.2 | 2.3 | 3.0 |
| **21** | 0 | 0 | 0 | 0 | 1/2 | 1/2 | 2.3 | 2.2 | 1.1 | 0.4 | 1.7 |

citric pulp (CP), soy bran (SB), sugarcane bagasse (SCB), soy husk (SH), cassava bagasse (CB) and coffee husk (CH) . * Production of GA3: g of GA3/kg of dry waste.

**Development of inoculum production**

Different medium for inoculum production (pellets with posterior growth in Czapek broth medium, PDA medium, Czapek broth medium, citric pulp extract) were tested in relation to cells viability of the selected strain and $GA_3$ production by SSF.

**PDA Medium**

The mycelia of the selected fungus was produced in 125 mL erlenmeyer flasks containing 30 mL PDA medium from the stock culture and incubated at 28ºC for seven days. The mycelia suspension was recovered by scraping using 30 mL of a 0.01% Tween 80 solution. The obtained suspension was then stored at 4ºC for up to seven days.

**Pellets growth with posterior growth in czapek broth medium**

The mycelia of the selected fungus was produced in a petri dish containing 20 mL PDA medium and incubated at 28ºC for seven days. Five discs of 6 mm of the solid culture was inoculated in 125 mL erlenmeyer flasks containing 30 mL of Czapek broth medium and incubated at 30ºC and 120 rpm for seven days.

**Czapek broth medium**

The mycelia of the selected fungus was produced in PDA slants that were incubated at 28ºC for seven days. The mycelia suspension was produced by scraping using a platinum strep for inoculation in 125 mL erlenmeyer flasks containing 30 mL of Czapek broth medium, which were incubated at 30ºC and 120 rpm for seven days.

**CP extract**

CP extract was produced using 5 g of CP (previously dried and triturated in a record mill and bolted to get a particle size smaller than 0.8 mm), with the addition of 50 mL of water. The mixture was boiled during 30 min and then filtrated.

The media, which was in 125 mL erlenmeyer flasks containing CP extract (1:10 in water), was inoculated by scraping the platinum strep over the medium from the stock culture. Flasks were incubated at 30ºC and 120rpm for 7 days.

**Cells Viability**

Each strain was inoculated in a 125 ml erlenmeyer flask containing 20 ml of the studied medium. Every 24 hours one erlenmeyer flask was withdrawn. 100µL of the mycelia suspension were

transferred to petri dishes containing PDA medium. Successive dilutions were made in order to have between 30 to 300 colonies/Petri dish. Colonies were then counted and the viability was calculated (Banwart, 1982).

**Analytical Methods**

15g of the fermented and homogenized material was added of 45 mL phosphate buffer (pH 8.0) and mixed for 20 minutes. 30 mL of the filtered, which was previously purified according to the Holbrook method (Holbrook et al., 1961), was acidified with HCl 30% in a 100 mL volumetric flasks containing 10 mL absolute ethanol. Samples were analyzed in a spectrophotometer, after 75 minutes at 20ºC, where $GA_3$ was measured at 254 nm.

## RESULTS AND DISCUSSION

**Screening of Strains and Substrates for $GA_3$ Production**

In this study the selection of a $GA_3$ productive strain and a suitable substrate or a mixture of substrates was carried out. The production of $GA_3$ by solid-state fermentation was then determined.

Table 2 presents the results of $GA_3$ production with the five studied strains using the combinations of the six substrates. Concerning the substrates, CP presented very good levels of $GA_3$ production with all strains (Table 2). Regarding the strains, the best $GA_3$ accumulation was obtained by *F. moniliforme* LPB 03 (5.7g of $GA_3$/kg of CP) followed by *G. fujikuroi* LPB 06 (4.7g of $GA_3$/kg of SB: CH) which were selected for the next steps of the study.

Analyzing the results obtained for the substrates and their mixtures, the best combination was CP and SH with has shown productions over 3.0 g $GA_3$/kg of dry substrate for all strains. Contrarily, the combination of CB: SB did not present any production except when the strain LPB02, that produced only 1.1g of $GA_3$/kg of dry CB:SB. If used separately, the substrates CB and SCB have not showed significant results of production under the conditions studied.

The production of gibberellins starts when nitrogen is depleted and continues when a sufficient carbon concentration is available in the substrate (Escamilla et al., 2000). In this way, substrates must provide an initial mycelia growth in a nitrogen-limited balanced medium, the beginning of $GA_3$ production after nitrogen exhaustion and an extended metabolite production in the presence of sufficiently available carbon substrate. In fact, the best production was reached using CP. This fact can be due to the presence of 5% and 19% of proteins and carbohydrates, respectively (Rodrigues, 2006) and, consequently, a high C/N ratio.

SB, SH and CH have a large amount of N in its formulation which gives them a very low relationship C/N (Kagawa, 1995; Machado et al., 2001) compared to CB and CP (Table 3). Changes in the C: N ratio were determinant to find the best strain and substrate for $GA_3$ production. So this study aimed to guide the following steps to optimize this process.

**Table 3 -** Protein and carbohydrate composition of the substrates.

| | CP* | CB* | SB* | SH* | CH* | SCB* |
|---|---|---|---|---|---|---|
| Proteins | 5% | 0.24% | 48% | 13 – 14 (%) | 10.9% | 0 – 0.3% |
| Carbohydrates | 19% | 67% | - | - | 17.4% | 39.7–49% |
| Reference | Rodrigues, 2006 | Vandenberghe et al., 2000 | IMCOPA, 2007 | POLINUTRI, 2007 | Souza et al., 2001 | Castro et al., 2008 |

* citric pulp (CP), soy bran (SB), sugarcane bagasse (SCB), soy husk (SH), cassava bagasse (CB) and coffee husk (CH).

**Development of inoculum production**

The development of a biotechnological process passes through different important steps. One of them is the inoculum production, which generally involves the study of cells viability, the optimization of medium composition and the genetic amelioration of the strain. Some details of the medium used for cell reproduction (inoculum production) are sometimes not really verified. However, a very significant gain in productivity can be reached with these procedures.

Tests were performed so as to choose the best medium for inoculum production using the selected strain *F. moniliforme* LPB 03. Fig 1

shows the kinetics of viability according to the different methods of inoculum production. Czapek broth proportioned the highest viable cells count reaching up to $10^9$ colonies / mL of medium in the $7^{th}$ day of growth.

The viability of each method of inoculum production was tested with respect to $GA_3$ production by SSF using CP as substrate. The production of $GA_3$ was determined in the $4^{th}$ and $7^{th}$ days using with 10, 15 and 20 % (v/w) of inoculum. Good results of $GA_3$ were attained with all different methodologies of inoculum production. The best results were obtained using 15% (v/w) of the inoculum grown in Czapek medium (5.3 g / kg of CP) in the $4^{th}$ day of fermentation. The methodologies of pellets formation, with subsequent growth in Czapek medium, and the PDA medium, have shown good results using both 10% of inoculum rate (3.10 g/kg of CP and 2.40 g/kg of CP, respectively), Although these two methodologies can not be economically justified at industrial scale. PDA and Czapeck are expensive mediums. The inoculum which was grown in CP extract, though less rich in nutrients, also showed good results in the $4^{th}$ day of fermentation (3.01 g / kg of CP) (Table 3). However, 20% of inoculum rate was used in this case, that could be seen as a limiting factor for its industrial application.

**Table 3** - $GA_3$ production in SSF using different media for inoculum growth.

| Inoculum (inoculum rate) | $4^{th}$ day of fermentation* | | | $7^{th}$ day of fermentation* | | |
|---|---|---|---|---|---|---|
| | 10% | 15% | 20% | 10% | 15% | 20% |
| Czapek medium | 2.42 | 5.30 | 1.50 | 3.10 | 0.60 | 1.67 |
| PDA medium | 2.40 | 1.86 | 1.13 | 3.97 | 3.00 | 3.84 |
| Pellets growth with posterior growth in Czapek medium | 3.10 | 2.92 | 3.19 | 3.37 | 1.13 | 0.7 |
| CP extract | 0.83 | 1.77 | 3.01 | 2.14 | 1.46 | 1.66 |

* Production defined in g of $GA_3$/kg of CP.

Fig 1 shows the number of viable cells of the strain *F. moniliforme* LPB 03 cultivated in different media using different methodologies of inoculum production. Cells count reached $10^8$cells/mL in 48 h using Czapeck medium. The same concentration was attained with the medium composed by CP extract only in 96 h. The other medium and methodologies, Pellets and PDA, showed higher concentrations of viable cells after 144 and 120 h, respectively.

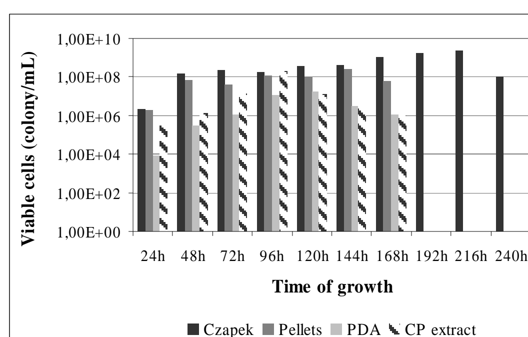


**Figure 1** - Viables cells counting of inoculum produced using different methodologies of cultivation and media.

The development inoculum production of the strain *F. moniliforme* LPB 03 was successfully conducted using different media and methodologies. However, only Czapeck medium and CP extract showed better characteristics for the production of high concentrations of viable cells.

Since one of the objectives of this work was the use of agro-industrial wastes and/or byproducts, some complementary tests were performed with

CP extract. In this case, some components of the Czapek medium (30 g/L sucrose, 3 g/L $NANO_3$, 1 g/L $K_2HPO_4$, 0.5 g/L $MgSO_4$, 0.5 KCl and 0.01 g/L $FeSO_4$) were added to CP extract so as to analyze the significant effect of each on inoculum production, according to a $2^{6-3}$ experimental design. The presence and the absence of each component were then examined.

According to Pareto diagram (Fig 2), sucrose was the most significant component at p level < 0.05 ($R^2$ = 0,7049). Further studies were carried out for the production of inoculum using CP extract added of three concentrations of sucrose (30, 35 and 40 g/L), with subsequent use in SSF for $GA_3$ production. Higher cell concentrations were attained at 4th day with all studied CP extracts (Fig 3).

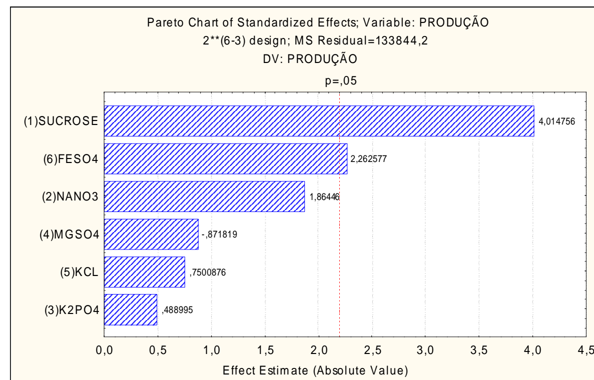


**Figure 2 -** Pareto chart of effects of the components adde to CP extrat medium

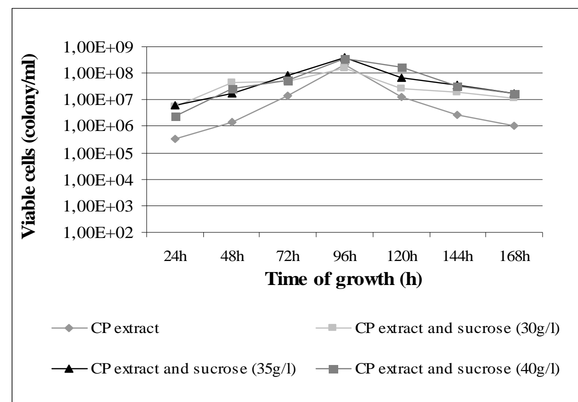


**Figure 3** - Cells viability for inoculum produced in CP extracts.

Then, three inoculum rates (10, 15 and 20% v/w) were used. Higher concentrations of viable cells were obtained with CP extract added of 35g/L of sucrose using 15% of inoculum rate (data not shown).

The study of inoculum stability was carried out when the inoculum suspension was maintained under refrigeration (4° C). It was observed a decrease in viability only after 5 days of storage (Fig 4). This fact allows the use of inoculum till 4 days.

Optimized conditions for inocolum production (15% v/w, CP extract added of 35 g/L sucrose) were used in a kinetic study of $GA_3$ production with CP as substrate in solid-state fermentation by *F. moniliforme* LPB 03. The best production was reached in the 3rd day of fermentation (5.8 g of $GA_3$/kg of CP) (Fig 5). This production is comparable to the reported production of $GA_3$ as it can be seen in Table 1. As an example, the production of $GA_3$ obtained by Corona et al. (2005) reached 4.5-5 g/kg dry basis using wheat meal and soluble starch.

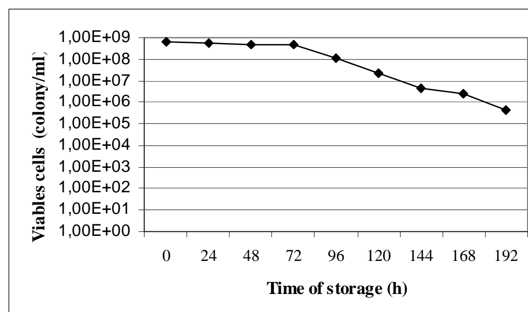


**Figure 4** - Viability of inoculum of *F. moniliforme* LPB 03 produced using CP extract after storage stored under refrigeration.

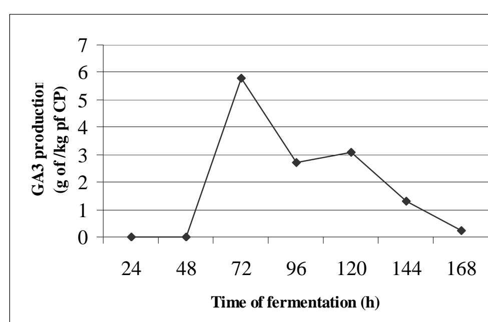


**Figure 5 -** Kinetic of $GA_3$ production by SSF using CP as substrate by *F. moniliforme* LPB 03.

After this study, the same production of $GA_3$ which was obtained using Czapek broth as a medium for inoculum growth was achieved. Besides, inoculum production time was decreased in 48h (from 144h with Czapek broth medium to 96h with CP extract). $GA_3$ production in SSF *F. moniliforme* LPB 03 was also reduced in 24h, which represents an economically significant gain for the process.

## CONCLUSIONS

The development of a new process passes through several steps of study and optimization. In this article, the screening of a productive strain and solid substrate/support were concluded. Citric pulp and *F. moniliforme* LPB 03 were chosen as substrate/support and strain, respectively, for $GA_3$ production in SSF. Thus, the optimization of inoculum production was performed where CP extract added of sucrose was chosen for cells' cultivation.
Some patents that present the difficulty to produce 1-5 g of $GA_3$/L are reported (Escamilla et al., 2000). In this work, a production 5.8 g of $GA_3$/kg of CP was reached in 3 days even before any phisico-chemical optimization of the fermentation process. Moreover, CP has been proved to be a rather promising susbtrate to be used in the production of $GA_3$ by SSF. Besides it is a sub-product of the juice industry which is produced in abundance. So the costs of this process of $GA_3$ could be reduced, making it economically viable.

## RESUMO

O ácido giberélico ($GA_3$) é um importante hormônio vegetal. A fermentação no estado sólido (FES) utiliza resíduos agro-industriais reduzindo os custos de produção. Neste trabalho a seleção de cepas (quatro de *Gibberella fujikuoroi* e uma de *Fusarium moniliforme*) e substratos (polpa cítrica, casca de soja, bagaço de cana, farelo de soja, bagaço de mandioca e casca de café) e o estudo da preparação do inóculo foram conduzidos para otimizar as condições de produção de $GA_3$ por FES. Os ensaios foram realizados em frascos de erlenmeyer a 29°C, com umidade inicial de 75-80%. Diferentes meios para a produção do inóculo foram testados em relação à viabilidade das células

e produção de $GA_3$ por FES. *F. moniliforme* LPB03 e polpa cítrica foram escolhidos. O melhor meio para a produção de inóculo foi o extrato de polpa cítrica. A produção por FES alcançou 5.8 g de $GA_3$/kg de polpa cítrica após 3 dias de fermentação.

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