# OPTIMIZATION OF 6-PENTYL-α-PYRONE PRODUCTION BY SOLID STATE FERMENTATION USING SUGARCANE BAGASSE AS RESIDUE

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Solid state fermentation (SSF) has been used as a model for the study of metabolism and physiology of microorganisms. The aim of the present study was to enhance 6-PP production by *Trichoderma harzianum* 4040 in solid state fermentation using sugarcane bagasse as a residue. A fractional factorial design was used to select the components of the nutrient solution. The fermentation was carried out during 9 days, and the aroma extraction was done on the third, fifth, seventh, and ninth days using organic solvent. On the seventh day the major concentration of 6-PP was found. The variables glucose, sucrose, and $MgSO_4$ were found to be significant statistically ($p > 0.05$) as components of the nutrient solution used in the production of 6-PP by filamentous fungi in SSF using sugarcane bagasse as a residue. GC-MS was used for quantification of 6-PP aroma.

*Keywords: Solid state fermentation; 6-pentyl-α-pyrone; Sugarcane bagasse; Trichoderma harzianum.*

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## INTRODUCTION

As a major constituent of all plant materials, cellulose forms about half to one-third of plant tissues and is constantly replenished by photosynthesis. One of the largest cellulosic agro-industrial by-products is sugarcane bagasse, a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugar cane. It is a lignocellulosic residue (by-product) of the sugar industry and is almost completely used by the sugar factories themselves as fuel for the boilers. Several processes and products have been reported that utilize sugarcane bagasse as a raw material. Bagasse consists of approximately 50% cellulose and 25% each of hemicellulose and lignin. Chemically, bagasse contains about 50% a-cellulose, 30% pentosans, and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0%, respectively. Such biomass resources have been used in bioconversion processes using microbial cultures (Pandey et al. 2000; Pandey 2003).

Agro-industrial residues are usually considered as the best substrate for solid-state fermentation process, mainly due to low cost. Worldwide concern with environmental issues has heightened the importance of using agro-industrial residues (Hölker and Lenz

2005). Several authors have cited economical and practical advantages of SSF. These include high productivity, low capital investment, reduced energy requirement, low wastewater output, improved product recovery, and elimination of foam products (Lonsane et al. 1985; Mudgett 1986).

A large number of microorganisms including bacteria, yeasts, and fungi have been used for cultivation on bagasse. However, filamentous fungi, especially basidiomycetes are the preferred choice for enzyme production and protein enrichment and have been employed most widely (Pandey *et al.* 2000). The *Trichoderma* fungi are largely found in soil, being used in agriculture as a biological control of plants, especially against phytopathogenics fungi. Many *Trichoderma* species produce important secondary metabolites, as lactone 6-pentyl-α-pyrone (Wiest et al. 2002). Besides its coconut-like aroma, lactone has antibiotic properties, and its toxicity is related to its capacity of being adsorbed by hydrophobic cellular membrane (Bonnarme 1997). This metabolite has attracted considerable attention as a biocontrol agent due to its powerful antifungal activity (Cooney 1997).

This work aimed to optimize 6-PP production by *Trichoderma harzianum* 4040 by SSF using sugarcane bagasse as a residue. The optimization was carried out by fractional factorial design to select the medium components of nutrient solution for this purpose.

## EXPERIMENTAL

### Microorganism and Media

*Trichoderma harzianum* 4040 obtained from the collection of Mycology Department of Oswaldo Cruz Foundation (FIOCRUZ) was grown and periodically transferred onto Potato Dextrose Agar (PDA - Himedia Labs, Mumbai, India) medium, and stored at 4°C.

A spore suspension was prepared after 7 days of culture at 28ºC, resuspended in saline solution (0.9% NaCl), and quantified in a Neubauer´s chamber.

Nutrient solution for kinetic profile contained ($L^{-1}$): glucose, 30 g; $(NH_4)_2SO_4$, 0.94g; $MgSO_4.7H_2O$, 1.5 g; $KH_2PO_4$, 1.0 g; KCl, 0.5 g; $CaCl_2.2H_2O$, 0.008 g; $FeSO_4.7H_2O$, 0.01 g; and $ZnSO_4.7H_2O$, 0.001 g.

### Solid State Fermentation

SSF was conducted without stirring in 250 mL Erlenmeyer flasks containing 4.5 g of sugarcane bagasse supplemented with 10 mL of nutrient solution and 1 mL of spore suspension containing 4 x $10^6$ spores/mL. The incubation temperature was 28ºC ± 1ºC. Sugarcane bagasse and nutrient solutions were individually autoclaved at 121ºC during 20 min before inoculation.

The best day for 6-PP production was determined by kinetic profile during third, fifth, seventh, and ninth days of SSF. After this, the same conditions of SSF were used to optimize the components of the nutrient solution in concentrations defined by experimental design.

## Moisture Determination

0.5g of fermented sample was maintained at 60°C for 24h to determine the moisture level using dry weight. The following equation was used to calculate the moisture level:

$$\text{Moisture (\%)} = \frac{(\text{wet weight} - \text{dry weight}) * 100}{\text{wet weight}} \quad (1)$$

## Analytical Procedures

*Extraction of 6-PP*

The extraction of aroma produced by fungi in SSF was carried out using dichloromethane as organic solvent (1g of sample added of 10 mL of solvent) (Sarhy-Bagnon et al. 2000). Samples were then stirred for 20 minutes, and supernatants were filtered over a 0.25 ⌈m Millipore filter. Samples were stored at -18ºC until the moment of analysis.

*GC-MS analysis*

An Agilent 6890N model gas chromatograph fitted with a quadripolar mass spectrometer with ionization by electronic impact (70 eV) was used for quantitative analysis of 6-PP. The 6-PP was separated on a DB-5 column (internal diameter: 0.32 mm, length: 30 m, film thickness: 0.25μm). 1 μL of sample in split mode was applied with a split ratio of 1:100 to the end of the run time. The column was maintained at 40ºC for 2 min, followed by a slope-wise increment of 20ºC/min until 120ºC which was maintained for 2 min, and then to 210ºC at 10ºC/min. Injector and detector temperature was 250ºC. Helium was used as a carrier gas with a flow rate of 1.2 mL/min.

A standard-curve was constructed for 6-pp quantification. The compound interest concentration produced (μg/g of residue) was determined by:

$$[\text{6-PP}] = \frac{(\text{quantification of compound for CG-EM}) * (\text{volume of the sample obtained})}{\text{g of the residue}} \quad (2)$$

## Experimental Design

A fractional factorial design $2^{9-5}$ was used to study the effects of 9 variables in 16 experiments with 3 central points and three levels (-1, concentration 0 g/L; 0, half of the medium concentration; +1, maximum concentration used in the liquid medium) (Table 1) (Lundstedt et al. 1998). The response variable was 6-PP concentration on the seventh day of SSF. Statistical analyses were performed using *Statistica 7.0* (Statsoft Inc., Tulsa, OK, USA).

**Table 1.** Experimental Domain for the Fractional Factorial Design

| Factors | Mininum level (-1) | Central (0) | Maximum level (+1) |
|---|---|---|---|
| G: Glucose (g/L) | 0.0 | 15.0 | 30.0 |
| S: Sucrose (g/L) | 0.0 | 15.0 | 30.0 |
| M:$MgSO_4.7H_2O$ (g/L) | 0.0 | 0.75 | 1.5 |
| Z: $ZnSO_4.7H_2O$ (g/L) | 0.0 | 0.001 | 0.0005 |
| N: $(NH_4)_2SO_4$ (g/L) | 0.0 | 0.47 | 0.94 |
| F: $FeSO_4.7H_2O$ (g/L) | 0.0 | 0.005 | 0.01 |
| C:$CaCl_2.2H_2O$ (g/L) | 0.0 | 0.004 | 0.008 |
| K: KCl (g/L) | 0.0 | 0.5 | 0.25 |
| P: $KH_2PO_4$ (g/L) | 0.0 | 0.5 | 1.0 |

## RESULTS AND DISCUSSION

### Kinetic Profile of 6-PP Production

The present study demonstrated the ability of *Trichoderma harzianum* 4040 to produce the coconut-like aroma from sugarcane bagasse, according the parameters established by SSF.

The kinetic profile shows that the highest aroma concentration (254 µg/g of residue) was reached on the seventh day of fermentation (Fig. 1).

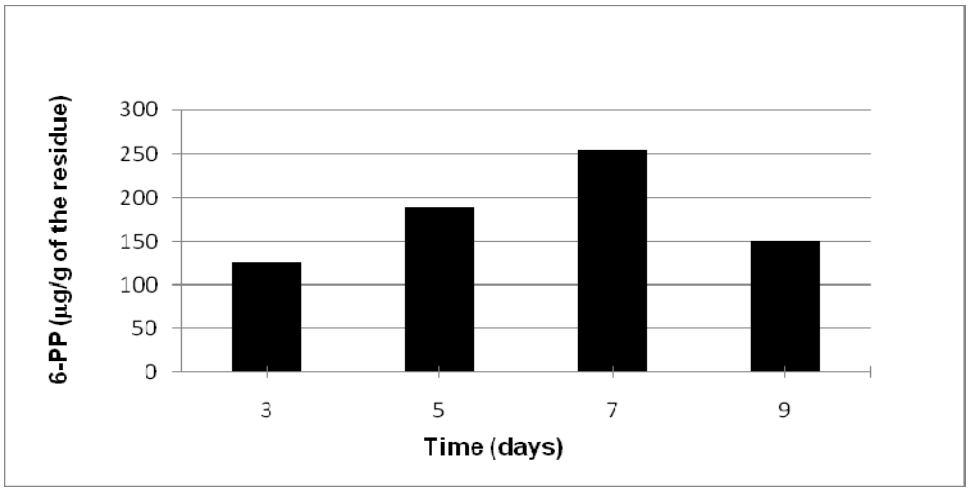


**Fig. 1.** Kinetic profile of 6-PP production by SSF

A lot has been said about the toxic effect of the 6-PP towards microorganisms and the consequent decrease of the compound production. Martins (2003) verified that there was no 6-PP inihibition over the *Trichoderma harzianum* 4040 at concentration levels up to 100 ppm. Prapulla et al. (1992) and Serrano-Carreon et al. (1992) reported that the

toxic effect occurs at aroma concentrations above 100 ppm, and it resulted in the reduction of the production of coconut-like aroma after the 7th day of the fermentation process presented on this study.

The aroma production decreased on the ninth day of culture with a constant decrease of moisture (Fig. 2). The initial moisture level of SSF process was 56%, and the moisture level decreased almost 2% for every two days of fermentation. On the seventh day, it decreased almost 5%.

Martins (2003) also verified that the microorganism growth was slower in semi-solid media for *T. Harzianum* 4040, due to reduced water activity, which may influence the production of metabolites. According the same author, the aroma production also decreased when the moisture decreased. However, further investigations should be performed to establish the relationship between the moisture decreasing during the process and the aroma production by the microorganism during the SSF.

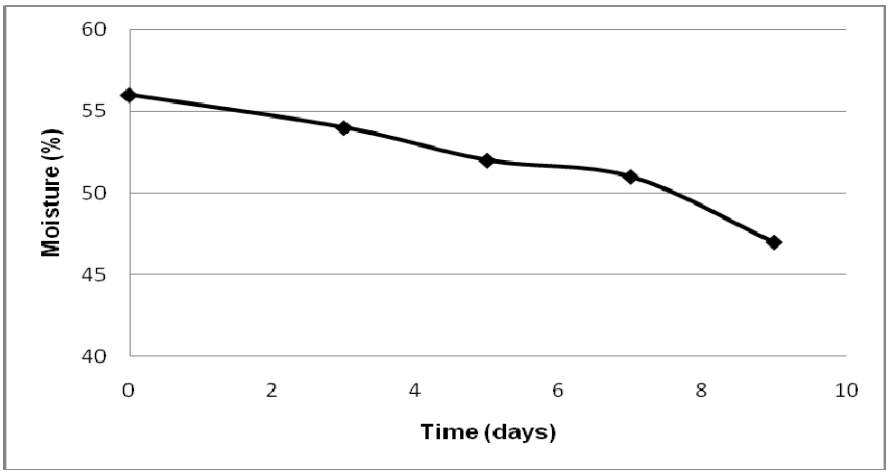


**Fig. 2.** Moisture content during the SSF process

## Experimental Design

A fractional factorial design was used to evaluate the effects of the compounds from liquid culture medium used as nutrient solution. The experimental designs were chosen intending to include the components concentrations used by Martins (2003), Ramos (2006), and Ramos et al. (2008) to produce 6-PP by solid-state fermentation in green coconut residue. The level -1 was designated as a 0 g/L concentration in a liquid medium to evaluate whether a substance could be out of a nutrient solution.

Sucrose variable was evaluated as a possible substitute for glucose to reduce the process cost. The results reached by Sarhy-Bagnon et al. (2000) showed that the microorganism was able to use sucrose from sugarcane bagasse during solid-state fermentation.

Table 2 shows the results obtained with 16 experiments, in which the 6-PP highest production rates were reached on combinations 15 and 16 of the experimental design. Besides, production rates were higher in the presence of $MgSO_4.7H_2O$.

The model suggests that glucose is the most significant carbon source for 6-PP production. One of the possible reasons is that glucose is a disposable monosaccharide

easier to obtain than sucrose, a disaccharide formed by glucose and fructose molecules which needs a period of time for carbohydrate hydrolysis.

During the experiments using glucose as the only carbon source (runs from 9 to 12), the 6-PP final concentration was larger than those obtained using only sucrose (runs from 5 to 8). However, the highest 6-PP final concentrations were reached when both carbon sources were used together (runs 15 and 16).

**Table 2.** Experimental Runs and Response Values of the Fractional Factorial Design. Fermentation Period: Seventh Day

| Run | G | S | M | Z | N | F | C | K | P | 6-PP (*) |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1 | 27.9 |
| **2** | -1 | -1 | -1 | 1 | -1 | 1 | 1 | 1 | -1 | 35.4 |
| **3** | -1 | -1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | 40.9 |
| **4** | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 46.7 |
| **5** | -1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | -1 | 134.5 |
| **6** | -1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 1 | 152.4 |
| **7** | -1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 188.5 |
| **8** | -1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 177.9 |
| **9** | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 212.3 |
| **10** | 1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 | 223.4 |
| **11** | 1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | 1 | 233.6 |
| **12** | 1 | -1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 245.7 |
| **13** | 1 | 1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 | 207.6 |
| **14** | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | -1 | 217.8 |
| **15** | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | 289.6 |
| **16** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 323.5 |
| **17 (C)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 189.7 |
| **18 (C)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 199.7 |
| **19 (C)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 207.3 |
| *⌈g/g of residue | | | | | | | | | | |

Based on effect evaluations of compounds from nutrient solution according to a Pareto chart (Fig. 3), it could be observed that $(NH4)_2SO_4$, $ZnSO_4.7H_2O$, $CaCl_2.2H_2O$, $KH_2PO_4$, KCl, and $FeSO_4.7H_2O$ were not at a significant level ($p > 0.05$). However, glucose, sucrose, and $MgSO_4.7H_2O$ were the significant variables ($p < 0.05$). Glucose x sucrose and glucose x $(NH4)_2SO_4$ were the only significant ($p < 0.05$) interactions.

The optimized data for 6-PP was validated by analysis of variance (ANOVA). The ANOVA showed a high $R^2$ (0.9988), an Adjusted $R^2$ (0.9892), and also *p*-values (significance $p < 0.05$) (Table 3).

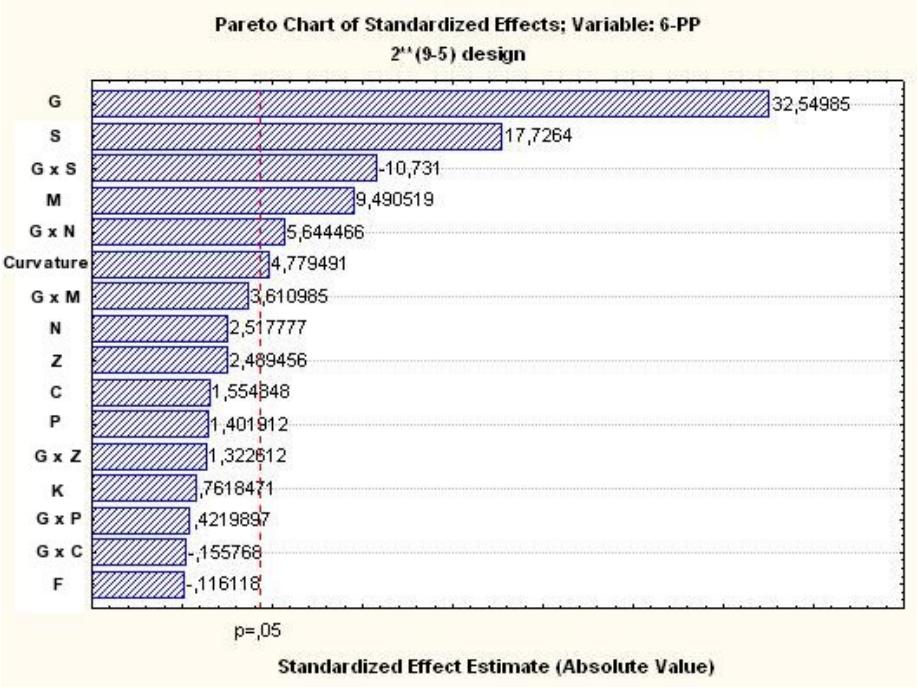


**Fig. 3.** Pareto chart for the response variable 6-PP

**Table 3.** ANOVA for 6-PP Produced Response from the Fractional Factorial Design ($R^2$ = 0.9988 and Adjusted $R^2$ = 0.9892)

| Factor | Sum of squares | df | Mean squares | F-ratio | *p*-value |
|---|---|---|---|---|---|
| *G*: Glucose (g/L) | 1780.0 | 1 | 1779.97 | 22.844 | 0.041096 |
| *S*: Sucrose (g/L) | 82555.7 | 1 | 82555.66 | 1059.493 | 0.000943 |
| *M:*$MgSO_4.7H_2O$ (g/L) | 24484.4 | 1 | 24484.43 | 314.225 | 0.003167 |
| *Z:* $ZnSO_4.7H_2O$ (g/L) | 7018.3 | 1 | 7018.25 | 90.070 | 0.010921 |
| *N*: $(NH4)_2SO_4$ (g/L) | 482.9 | 1 | 482.90 | 6.197 | 0.130506 |
| *F*: $FeSO_4.7H_2O$ (g/L) | 494.0 | 1 | 493.95 | 6.339 | 0.128123 |
| *C*:$CaCl_2.2H_2O$ (g/L) | 1.1 | 1 | 1.05 | 0.013 | 0.918168 |
| *K:* KCl (g/L) | 188.4 | 1 | 188.38 | 2.418 | 0.260229 |
| *P:* $KH_2PO_4$ (g/L) | 45.2 | 1 | 45.23 | 0.580 | 0.525732 |
| G by S | 153.1 | 1 | 153.14 | 1.965 | 0.295989 |
| G by M | 8972.8 | 1 | 8972.83 | 115.154 | 0.008572 |
| G by Z | 1016.0 | 1 | 1016.02 | 13.039 | 0.068864 |
| G by N | 136.3 | 1 | 136.31 | 1.749 | 0.316943 |
| G by C | 2482.5 | 1 | 2482.53 | 31.860 | 0.029983 |
| G by K | 1.9 | 1 | 1.89 | 0.024 | 0.890518 |
| Error | 13.9 | 1 | 13.88 | 0.178 | 0.714066 |
| Total SS | 155.8 | 2 | 77.92 | | |

The interaction between the carbohydrates glucose and sucrose had a negative effect, because when one was highly concentrated, the other one needed to be in a lower concentration in the nutritive medium. Glucose was the most significant sugar and it must be used in higher concentration than sucrose.

Glucose and ammonium sulfate had a positive interaction, and both must always be associated. Central point replicas were used to calculate the experimental error. They showed that the curvature was significant ($p$ = 0.041). Equation (3) represents the studied design (normalized variables):

$$[6\text{-PP}] = 176.5474 + 71.8313\ G + 39.1188\ S + 20.9437\ M - 23.6813\ G\ X\ S + 12.4562\ G\ x\ N. \quad (3)$$

Predicted $x$ observed values, which can be seen in Fig. 4, confirmed that the proposed model well represented the experimental data, with the points almost disposed in line.

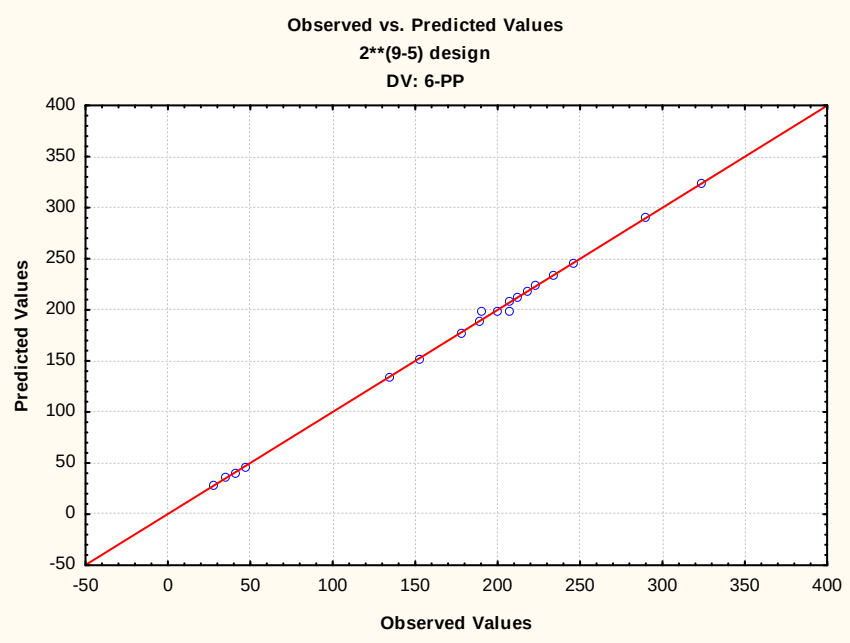


**Fig. 4.** Correlation among predicted and observed values

Glucose, sucrose, and $MgSO_4.7\ H_2O$ were shown to be statistically significant (p> 0.05) as components of the nutrient solution used in the production of 6-PP by *T. harzianum* 4040 in SSF using sugarcane bagasse as a residue. An increase in the aroma production was observed in comparison with Martins (2003), who obtained 191.23 µg/g on the 7$^{th}$ day of fermentation. The same strain was used, but with a nutrient solution that was qualitatively and quantitatively richer. The strain used in both studies was a wild strain isolated from beach sand. Oda *et al* (2009) obtained higher yields in aroma production (7.1g/L) using another species of the genus *Trichoderma*, and the highest

production was obtained using a genetically modified strain. However, it is not possible to do an effective comparison between the results obtained by those authors with results of the present study, because the *T. harzianum* 4040 is a wild strain isolated from beach sand. Besides that, Oda *et al.* (2009) used a liquid medium with a different nutrient compound in a submerged system, presenting results in units not comparable to culture in SSF.

## CONCLUSIONS

1. *Trichoderma harzianum* 4040 is able to produce 6-PP using sugarcane bagasse as residue in SSF.
2. The maximum production of 6-PP was obtained on the seventh day of the SSF process.
3. The sucrose substitution by glucose was not so effective, because the 6-PP production was increased when glucose was used as the carbohydrate of the nutrient solution.
4. The experimental design showed that 6-PP production is more efficient when glucose concentration in the nutrient solution is higher than the sucrose level, because there is a negative interaction between these carbohydrates.
5. The variable $MgSO_4.7H_2O$ is significant for the nutrient solution.
6. The variable $(NH4)_2SO_4$ is significant for the nutrient solution when associated with glucose.

## ACKNOWLEDGMENTS

The authors are grateful for the support of the CNPq – Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FUJB (Fundação Universitária José Bonifácio) and FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro).

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Article submitted: July 17, 2010; Peer review completed: August 24, 2010; Revised version received and accepted: September 7, 2010; Published: Sept. 8, 2010.