VANILLIN PRODUCTION BY *PHANEROCHAETE CHRYSOSPORIUM* GROWN ON GREEN COCONUT AGRO-INDUSTRIAL HUSK IN SOLID STATE FERMENTATION

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Agro-industrial residues have become an important source for the production of chemical compounds using biological pathways, contributing to preservation of the environment and making the overall process economically supportable. Vanillin is a very important aromatic compound for the food, beverage, and pharmaceutical industries. The aim of the present study was to evaluate the vanillin production by solid-state fermentation on green coconut residue using the basidiomycete *Phanerochaete chrysosporium*. Solid-state fermentation was carried on a support of green coconut husk treated in two different ways: sun-dried and mechanical-pressed. A Plackett-Burman experimental design was used to screen the compounds of liquid medium culture of the vanillin production. Nineteen variables were studied to optimize the culture conditions, and eleven of them were significant. The screening improved the production of vanillin from 44.4 μg/g of support to 52.5 μg/g of support in 24 hours of fermentation. Sun-dried coconut husk was found to be superior to mechanical-pressed coconut husk for production of vanillin. HPLC was used for the quantification of vanillin aroma.

Keywords: Vanillin; Solid-state fermentation; Phanerochaete chrysosporium

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INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of natural *Vanilla planifolia*, which is one of the most widely used flavouring agents in a large range of foods, and also used in some fragrances (Walton et al. 2000; Walton et al. 2003). For a long time, the most important source of vanillin was eugenol, the main component of cloves. Compared with the cost of synthetic vanillin (~US$15Kg^{-1}), natural vanilla extract is relatively expensive, with a current cost of ~US$4000Kg^{-1}, depending on quality. With an increasing interest in natural products, alternative process are being developed that use biotechnological methods involving fungi to produce vanillin (Lesage-Meessen et al. 1996).

Lignin represents one of the main sources of natural aromatics. This insoluble polymer is known to be attacked by microorganisms, particularly by white-rot basidiomycetes, which release aromatic aldehydes such as vanillin when growing on

lignin. Vanillic and ferulic acids are also well-known products of the degradation of lignin related substances by white-rot fungi (Walton et al. 2000; Pandey 2003). Ferulic acid is the most abundant hydroxycinnamic acid in the plant world and occurs mainly in cell walls covalently linked to lignin and other polymers (Harris and Hartley, 1980; Hartley and Harris, 1981; Graf 1992). A number of industrial and food applications have been reported for ferulic acid, especially based on its microbial degradation to vanillin (Gross et al. 1991) and its antioxidant properties (Graf 1992). The high level of ferulic acid hydrolysis from plant cell wall materials rich in this acid such as agro-industrial residues would provide a sufficient natural source of ferulic acid for biotechnological processes (Soccol and Vandenberghe 2003).

Green coconut water is becoming a very attractive product to the Brazilian market, growing 20% in a year. Brazil is the world leader of green coconut production with a planted area of 90,000 ha. The increasing of green coconut consumption generates 6.7 million tons of husk in a year (Embrapa Agroindústria Tropical, 2005).

However, this great production generates a large amount of residues that causes serious environmental problems (Pandev and Soccol 1998; Soccol and Vandenberghe 2003).

The application of agro-industrial residues in bioprocesses provides alternative substrates, and it also helps to solve pollution problems. Biotechnological processes, especially the solid-state fermentation (SSF) technique, have contributed enormously to such utilization (Soares et al. 2000; Soccol and Vandenberghe 2003).

Traditionally, SSF methods have been characterized by the development of microorganisms in a low water environment on a non-soluble material that acts both as physical support and source of nutrients; however it is not necessary to combine the role of support and substrate, but rather to reproduce the conditions of low water activity and high oxygen transference by using a nutritionally-inert material soaked with a nutrient solution (Pandey 2003). Improvement in productivity of metabolic microbial by the organisms is done by manipulating the nutritional parameters, physical parameters, and strain improvement so that it can better be evaluated through experimental design. The Placket-Burman statistical method offers a design where initial screening of the ingredients is done to understand the significance of their effect on the products formation, and then the most suitable ingredients for the increasing of the production of the studied compound are selected for further optimizations (Carvalho et al. 1997; Naveena et al. 2005).

The aim of the present study was to evaluate vanillin production by solid-state fermentation on green coconut agro-industrial husk using the basidiomycete *Phanerochaete chrysosporium*.

EXPERIMENTAL

Materials

Fungal strains and growth media

Phanerochaete chrysosporium CCT-1999 (ATCC 24725) was selected as a suitable organism for bio-processing of SSF due to its ability to degrade lignin effectively

(Kirk and Farrel 1987). The cultures were grown at 28°C ±1°C during five days on Potato Dextrose Agar (PDA) medium, and it was stored at 4°C.

Obtaining the inoculum

The spores were produced during five days on Potato Dextrose Agar (PDA) medium at 28°C ± 1°C, and they were suspended in NaCl 0.9% p/v solution. The spores were quantified in a Neubauer chamber. In solid-state fermentation 1 mL of spore suspension containing 2.7 x 10^6 spores/mL was added (Vane 2003).

Solid support

The solid support consisted of green coconut agro-industrial husk provided by Empresa Brasileira de Pesquisa Agropecuária, Agroindústria Tropical, Brazil. The green coconut husk was treated in two ways: sun-dried and mechanical-pressed. The solid support was submitted to granulometric classification (20 mesh Tyler screen), autoclaved at 121°C for 20 minutes, and stored at room temperature until its use.

Methods

SSF technique

Experiments were conducted in 250 mL Erlenmeyer flasks containing 2g of autoclaved support (sun-dried green coconut husk or mechanical-pressed green coconut husk). It was impregnated to 550% moisture content, with 10 mL of the liquid nutrient medium, and 1 mL of spore suspension, but it didn't develop a submerged fermentation. The liquid nutrient medium contained: 1g glucose; 0.2g KH_2PO_4; 0.05g $MgSO_4.7H_2O$; 0.022g ammonium tartrate; 0.1mg thiamine; 0.02g yeast extract; 0.01g $CaCl_2.H_2O$; 6.7mg veratryl alcohol; and 0.1mL trace solution element in 100 mL of pH 4.5 buffer-solution (Contarini 1992; Vane 2003). The liquid nutrient medium was autoclaved at 121°C for 15 minutes, with the exception of the thiamine and veratryl alcohol. These components had been filtered through cellulose acetate membrane (pore size: 0.25μm; Millipore) and inoculated at the moment of the fermentation.

The Erlenmeyer flasks were incubated at 28°C ±1°C, in the absence of shaking. The samples extractions were done with the mixture of two solvents (60% methanol, 40% water) in each 24 hours until the end of 96 hours of culture. The mixture then was filtered with a cellulose acetate membrane (pore size: 0.25μm, Millipore). All the experiments were done in triplicate.

Screening of liquid medium components using Plackett-Burman design

The Plackett-Burman design was used for screening in order to improve vanillin production. Among the components of the liquid medium culture, sucrose and Polyoxyethylenesorbitan Monooleate (POE-SM) surfactant, were tested for their significance in vanillin production. The effects of 19 variables were considered in 20 experiments with 5 central points and three levels (-1, concentration 0g/L; 0, half of the maximum concentration; +1, maximum concentration used in the liquid medium) (Tables 1 and 2). The Plackett-Burman design was applied in accordance with optimum result of the first stage of the fermentation. The results were analyzed using the Statistica 6.0 (Statsoft) software. Values for vanillin production were expressed in μg/g of the support.

Table 1: Assigned Concentrations of Variables at Different Levels in Plackett-Burman Design for Vanillin Production in SSF

S. no.	Variables with designation (g/L)	-1	0	+1
1	X_1 Glucose	0.0	5.0	10.0
2	X_2 Sucrose	0.0	5.0	10.0
3	X_3 KH_2PO_4	0.0	1.0	2.0
4	X_4 $MgSO_4.7H_2O$	0.0	0.125	0.250
5	X_5 Ammonium tartrate	0.0	0.11	0.22
6	X_6 Thiamine	0.0	0.0005	0.0010
7	X_7 Yeast extract	0.0	0.1	0.2
8	X_8 Veratryl alcohol	0.0	0.0335	0.0670
9	X_9 POE-MS surfactant	0.0	0.05	1.0
10	X_{10} $CaCl_2.H_2O$	0.0	0.5	1.0
11	X_{11} $NaCl.H_2O$	0.0	0.0005	0.0010
12	X_{12} Nitrilotriacetic acid	0.0	0.00075	0.00150
13	X_{13} $MnSO_4.H_2O$	0.0	0.00025	0.00050
14	X_{14} $CoSO_4.7H_2O$	0.0	0.00009	0.00018
15	X_{15} $CuSO_4.5H_2O$	0.0	0.00009	0.00018
16	X_{16} $FeSO_4.7H_2O$	0.0	0.00005	0.00010
17	X_{17} $ZnSO_4.7H_2O$	0.0	0.00009	0.00018
18	X_{18} $NaMoO_4$	0.0	0.000005	0.000010
19	X_{19} H_3BO_3	0.0	0.000005	0.000010

Table 2: Plackett-Burman Design for 19 Variables with Coded Values along with Observed Results for Vanillin Production in SSF

S. no.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	X_{19}	Vanillin production
1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	27,498
2	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	32,936
3	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	31,343
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	25,110
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	27,195
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	30,851
7	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	30,108
8	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	28,821
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	29,317
10	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	27,911
11	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	31,928
12	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	32,694
13	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	30,351
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	26,648
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	23,319
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	25,229
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	27,988
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	26,343
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	27,858
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	24,123
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28,251
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27,956
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28,196
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28,350
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28,500

Analytical procedures

Filtrates were analyzed by HPLC, and the quantification was performed using a vanillin standard. Separation was achieved with a Spherisorb® S5 ODS2 column (150 × 2 mm, Waters Corp., USA) maintained at 40°C using HPLC model Shimadzu, Kyoto, Japan. The mobile phase used a mixture of two solvents with 75% of aqueous formic acid at 0.3% and 25% of methanol, using an isocratic elution with 0.2 mL/min of flow rate. The data were obtained by LCMSsolution (Shimadzu Corp., version 2.00, 2000) software. The vanillin concentration produced (μg/g of support) was determined by:

$$\text{Vanillin} = \frac{\text{(quantification of vanillin for HPLC . volume of the sample obtained)}}{\text{g of support used at the process}}$$

RESULTS AND DISCUSSION

Kinetic Profile of Vanillin Production by *Phanerochaete chrysosporium* on Solid Support

The present study demonstrated the ability of *Phanerochaete chrysosporium* to release compounds from the cell walls of green coconut husk and then to convert it to vanillin, according with the parameters established by solid-state fermentation. The highest production achieved with the mechanical-pressed support happened in 48 hours of fermentation, while just 24 hours was required to obtain the highest production with the sun-dried support, being 28.95 μg/g of the support and 44.4 μg/g of the support, respectively (Fig. 1). It was possible to observe the influence of the type of treatment of the support (sun-dried and mechanical-pressed) on the production, with the highest production rates ($p<0.05$) achieved with the sun-dried support (Fig. 1). Therefore, the solid-state fermentation on sun-dried support was more efficient to produce the compound vanillin in comparison with the mechanical-pressed support.

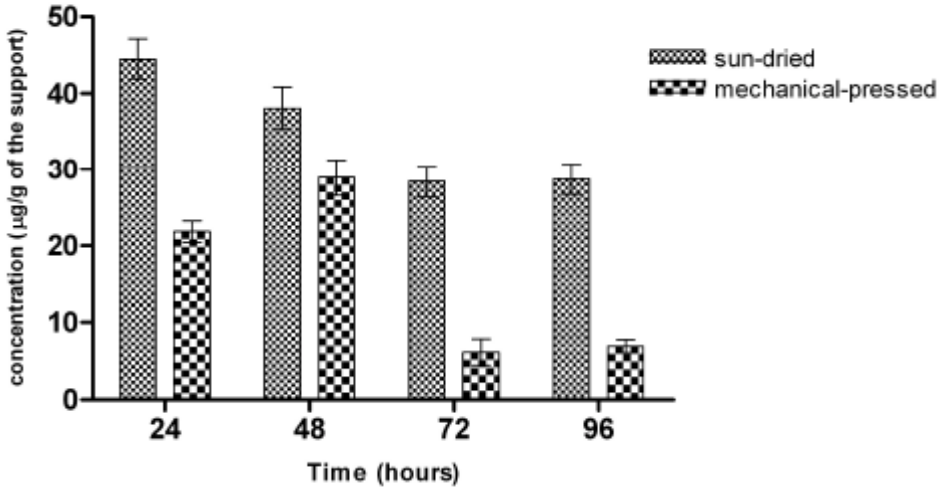

Fig. 1. Kinetic profile of vanillin production by *Phanerochaete chrysosporium* on two solid supports

Several studies have been recently focused on the production of specialty chemicals such as flavor compounds from residues of the agro-industry as a possible way of both disposing them and producing an item of value. Green coconut agro-industrial husk, one of the most widely produced agricultural residues in Brazil (Pandey and Soccol 1998; Carrijo et al. 2002; Soccol and Vandenberghe 2003), can be of interest in vanillin production as it is a potential source of ferulic acid, from which vanillin can be obtained via microbial conversion. Ferulic acid is linked to cell wall polysaccharides through ester bonds, which have to be hydrolyzed to release ferulic acid (Walton et al. 2000; Pandey 2003; Mathew et al. 2006). Hydrolysis can be done through the action of specific enzymes, especially lignolytic enzymes. The white rot fungus *Phanerochaete chrysosporium*, under nitrogen or carbon limitation, produces both extracellular peroxidase lignin (LiP) and manganese peroxidase (MnP) (Podgornik et al. 2001).

Plackett-Burman design

The Plackett-Burman design was implemented just in the case of the sun-dried green coconut husk support to optimize the liquid medium compounds in 24 hours of the solid-state fermentation. In the present study *Phanerochaete chrysosporium* produced a high yield of vanillin in combinations 2 and 12 (Table 2), and the experimental analysis is shown in Table 3. Fourteen variables out of nineteen, namely glucose, sucrose, KH_2PO_4, ammonium tartrate, thiamine, veratryl alcohol, POE-SM surfactant, nitrilotriacetic acid, $MnSO_4.H_2O$, $CoSO_4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $NaMoO_4$, all had values of $p<0.05$ (Table 3), implying that these variables influenced the fermentation process significantly, being part of the experimental model equation. However, three of these variables (Table 3) namely POE-SM surfactant, $ZnSO_4.7H_2O$, and NaMoO4 had negative coefficients over vanillin production, and they must be used on the low level (Table 1) established by the design. The other five variables, $MgSO_4.7H_2O$, yeast extract, $CaCl_2.H_2O$, $NaCl.H_2O$, and H_3BO_3, were found to be insignificant with value $p>0.05$ (Table 3).

The negative coefficients of POE-SM surfactant in this experimental model did not coincide with the data presented for Giese et al. (2004), in which the presence of the surfactant in the liquid medium culture stimulated the activity of lignolytic enzymes in basidiomycetes. However, it can be suggested that in this study, the surfactant did not serve as a stimulant of the activity of lignolytic enzymes in the basidiomycete *Phanerochaete chrysosporium* for release of the ferulic acid of wall cells of solid support for further conversion in vanillin.

Phanerochaete chrysosporium used both carbon sources present in the liquid medium, the glucose and sucrose. The veratryl alcohol also appeared to be an important compound of the liquid medium. This can be explained due to the inductive effect of veratryl alcohol on the microorganism production of lignolytic enzymes, which probably contributed to release the ferulic acid from support cell walls (Leisola et al. 1984; Leisola et al. 1985; Schoemaker and Leisola 1990).

Table 3: Coefficient of each Variable, Confidence interval (CI) at 95% Confidence Level and *p* Value for Production of Vanillin in a 19-Variables Plackett-Burman Design

S. no.	Variables	Coefficient	Coefficient	+ or – (CI)	Value *p*	Significance
1	Glucose	1.71176	1.46206	1.96145	0.000045	significant
2	Sucrose	3.21402	2.96432	3.46371	0.000004	significant
3	KH_2PO_4	0.70418	0.45448	0.95388	0.001437	significant
4	$MgSO_4.7H_2O$	0.12008	-0.12961	0.36978	0.252718	*
5	Ammonium tartrate	2.45332	2.20363	2.70302	0.000011	significant
6	Thiamine	1.56345	1.31375	1.81315	0.000064	significant
7	Yeast extract	0.12833	-0.12136	0.37803	0.226754	*
8	Veratryl alcohol	0.25119	0.00149	0.50089	0.049160	significant
9	POE-SM surfactant	-1.24219	-1.49189	-0.99250	0.000159	*
10	$CaCl_2.H_2O$	-0.20772	-0.45742	0.04197	0.082057	*
11	$NaCl.H_2O$	0.13889	-0.11081	0.38858	0.197392	*
12	Nitrilotriacetic acid	0.39021	0.14051	0.63990	0.012264	significant
13	$MnSO_4.H_2O$	0.25539	0.00569	0.50509	0.046880	significant
14	$CoSO_4.7H_2O$	0.27996	0.03026	0.52966	0.035770	significant
15	$CuSO_4.5H_2O$	0.94921	0.69951	1.19890	0.000456	significant
16	$FeSO_4.7H_2O$	0.59772	0.34803	0.84742	0.002661	significant
17	$ZnSO_4.7H_2O$	-0.69562	-0.94532	-0.44593	0.001505	*
18	$NaMoO_4$	-1.96101	-2.21071	-1.71132	0.000026	*
19	H_3BO_3	-0.14062	-0.39032	0.10908	0.192951	*

*Insignificant.

In accordance with the Plackett-Burman design, confirmatory experiments were done in triplicate in the same conditions imposed in this process of solid-state fermentation on the solid support sun-dried green coconut. The optimization of liquid compounds in the medium increased the vanillin production by 20%, from 44.4 μg/g of support to 52.5 μg/g of support in 24 hours of fermentation. For this study, the Plackett-Burman design not only helped to reduce the number of compounds in the liquid medium, selecting the more significant contributions, to 11 compounds (Table 3) namely glucose, sucrose, KH_2PO_4, ammonium tartrate, thiamine, veratryl alcohol, nitrilotriacetic acid, $MnSO_4.H_2O$, $CoSO_4.7H_2O$, $CuSO_4.5H_2O$ and $FeSO_4.7H_2O_4$ being used on the high level (Table 1) established by the design, but also ensured a better vanillin production, making the process interesting for industrial production.

CONCLUSIONS

Phanerochaete chrysosporium can release compounds from the cell walls of green coconut husk. The sun-dried green coconut husk was a better support solid for the vanillin production by solid-state fermentation. The Plackett-Burman design made it possible to reduce the number of compounds in the liquid medium, removing 8 of them, and it increased the vanillin production by 20%.

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