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Optimization of some parameters influencing *Thermoascus aurantiacus* growth: effects of lignin-related compounds

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Statistical designs were used to optimize some parameters affecting the growth rate of a Brazilian strain of *Thermoascus aurantiacus*. The mycelial growth rate was measured using the horizontal tube method. Temperature of incubation and initial pH were the major factors affecting the growth rate. They were optimal at 6.0 and 48°C, respectively. The maximum growth rate was obtained in solid Czapek modified medium containing 1.5% glucose and 38.4 mEq L^{-1} $NaNO_3$. Under these conditions, the growth rate of *T. aurantiacus* was 5.16 ± 0.10 mm h^{-1}. Lignin-related compounds such as tannins and extractive substances added at 0.1% (w/v) to the minimal Czapek medium increased growth rate 14% and 29%, respectively.

Keywords: *Thermoascus aurantiacus*; thermophilic fungus; growth rate optimization; statistical-design optimization; lignin-related compounds

Introduction

A strain of *T. aurantiacus* isolated from chip piles of *Eucalyptus* wood [1] exhibits properties different from those found for other strains reported in the literature. It degrades extractives efficiently and partially removes lignin from *E. grandis* wood. Moreover, it exhibits inductive phenoloxidase activity which was not reported for other *T. aurantiacus* strains [11].

Microbial growth and metabolite yield (protein, antibiotic, pigment) can be improved through modifications of the environment in which a microorganism is grown. An ideal growth medium for industrial microorganisms should be simple and economically attractive [9]. Process optimization by classical methods involves modification of one independent variable at a time, while all others are fixed at a certain level. These methods do not guarantee true optimal conditions and do not allow for study of interactive effects of variables on the yield. An alternative approach is to apply statistical mathematical methods that consider all combinations of independent variables simultaneously and at multiple levels in a short time [4,7,9].

The aim of this work was to develop a minimal and simple growth medium for this strain of *T. aurantiacus*, through selection and optimization of the concentrations of carbon and nitrogen sources, the initial pH and incubation temperature. Fungal growth was evaluated by the longitudinal growth rate of the mycelial front on a solid medium using the horizontal tube method [8,16]. Optimization of the different parameters influencing the growth rate of *T. aurantiacus* was obtained by a combination of two factorial designs (2^4) and a sequential simplex method. Finally, *T. aurantiacus* growth was evaluated on solid medium containing selected lignin-related compounds, under the previously optimized conditions.

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Materials and methods

Microorganism

Thermoascus aurantiacus, a Brazilian strain, was isolated from *Eucalyptus* wood as previously described [1]. Stock cultures were grown on potato dextrose agar (PDA) and Czapek agar (Difco, Detroit, MI, USA) for 3–4 days and stored at 4°C. Inocula used were 5-mm diameter agar-mycelium discs of fungal growth grown on Czapek agar plates at 50°C for 3–4 days.

Media and culture conditions

T. aurantiacus was grown at 50°C in Petri plates containing YpSs [6], PDA and Czapek agar media and evaluated for appearance and rate of growth for 3 days. Measurements of colony radial growth rates were made by measuring the diameter of colonies in three directions and the average of three plates was considered.

Czapek modified medium (pH 7.3) was chosen to study the parameters affecting growth rate of fungus containing (g L^{-1}): agar, 15.0; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5, and KCl, 0.5. This medium was supplemented with 0.1 ml L^{-1} of a trace element solution [8] containing (mg L^{-1}): $CuSO_4 \cdot 5H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.2; $MnSO_4$, 0.02; and $ZnCl_2$, 0.15. The Czapek basal medium contained variable concentrations of carbon and nitrogen sources. The initial pH of the medium was adjusted with HCl or NaOH after autoclaving it at 121°C for 15 min.

Measurement of fungal growth rate

Fungal growth was determined by measuring the longitudinal growth rate of mycelium on solid medium or as dry weight from growth in liquid medium. The choice of the best growth conditions was evaluated by the horizontal tube method [8,16]. Growth rate was monitored by measuring

Table 1 Definition of variables and levels for the first 2^4 FD

Variable	Level (+)	Level (−)	Centre point[a]
Temperature (°C)	55.0	45.0	50.0
pH	7.8	5.8	6.8
Glucose concentration (%)	1.5	0.5	1.0
$NaNO_3$ concentration (mEq L^{-1})	44.0	4.0	24.0

[a]The experimentally determined growth rate at this point was 3.93 ± 0.2 mm h^{-1}.

the position of the advancing mycelial front for at least 4 days. Growth rates were calculated from the slope of the curve of distance against time, and expressed as mm h^{-1}. In liquid medium, the growth rate was expressed as mg ml^{-1} of dry weight. All experiments were done in triplicate and average values are reported.

Statistical treatment

The carbon and nitrogen concentrations, initial pH and incubation temperature were optimized using a sequence of two factorial designs and simplex methods. The factorial design (FD) [2,3] adopted was 2^4 (four independent variables and two levels). For the first 2^4 FD, the central value and interval between the levels were chosen according to literature [15] and experimental results (Table 1). For the second 2^4 FD, these values were chosen according to first FD results (Table 2). The four independent variables considered were: carbon (C) and nitrogen (N) concentrations, incubation temperature (T) and initial pH of medium (pH). The complete design (two FD) consisted of 32 experimental points. The interaction effects between the variables were determined according to Strange [19]. The simplex technique (ST) [12,17] consists of a geometrical figure defined by experimental points equal to one more than the number of independent variables ($n + 1$). In our study $n = 3$, and the simplex took the form of a tetrahedron. Optimization was achieved by moving this geometrical figure in the direction of improved response values (rate of growth) using a computational routine denominated 'Amoeba'. Simplex vertices were obtained from the second 2^4 FD.

Growth of T. aurantiacus on lignin-related compounds

T. aurantiacus was grown on solid Czapek modified medium designed after the statistical treatment. The Czapek modified medium containing 1.5% glucose, 38.4 mEq L^{-1} $NaNO_3$ (pH 6.0) was supplemented with a final concentration of 0.1% (w/v) of lignin-related compounds, added as a *N,N*-dimethylformamide suspension (0.1% v/v). The lignin-related compounds tested were: acetovanillone, vanillin and guaiacol (Aldrich, Milwaukee, USA), tannic acid (Merck, Hohenbruns, Germany), extractives (ethanol-soluble fraction) of *E. grandis* (Brazil) obtained following methods outlined by Machuca and Durán [11] and tannins of *Pinus radiata* D Don (Chile), obtained from J Baeza, Chemical Renewable Resources Laboratory, Universidad de Concepción, Chile. The horizontal tubes were incubated at 48°C for 4–5 days. Controls containing 0.1% (v/v) *N,N*-dimethylformamide in the absence of a lignin-related compound were prepared and incubated under the same conditions as the test cultures.

Table 2 Definition of variables and levels for the second 2^4 FD

Variable	Level (+)	Level (−)
Temperature (°C)	53.0	48.0
pH	7.3	6.3
Glucose concentration (%)	1.2	0.7
$NaNO_3$ concentration (mEq L^{-1})	34.0	14.0

Results

T. aurantiacus growth

The best sporulation of *T. aurantiacus* was attained on PDA and YpSs. However, the best growth rate was attained on Czapek medium. The growth rates on PDA, YpSs and Czapek media were: 3.75, 3.52 and 4.10 mm h^{-1} (colony diameter), respectively. Thus, Czapek medium was chosen for selection and optimization of some parameters which affect *T. aurantiacus* growth. During the initial culturing in a Czapek medium, the hypha formed a white mycelium which turned dark after 2 days. After 3 days, numerous dark brown asci and a large amount of orange exudate covered the agar surface.

By using the horizontal tube technique to measure growth rate, it was possible to find a linear correlation between the advancement of mycelial front and incubation time, up to 120 h of growth (Figure 1). This linearity was obtained in all experiments carried out using the tube method. After inocula standardization good reproducibility was obtained in terms of size and age.

Selection and optimization of some parameters affecting the growth rate

The selection and optimization of the best carbon sources was obtained by addition of 1.5% (w/v) of different, simple

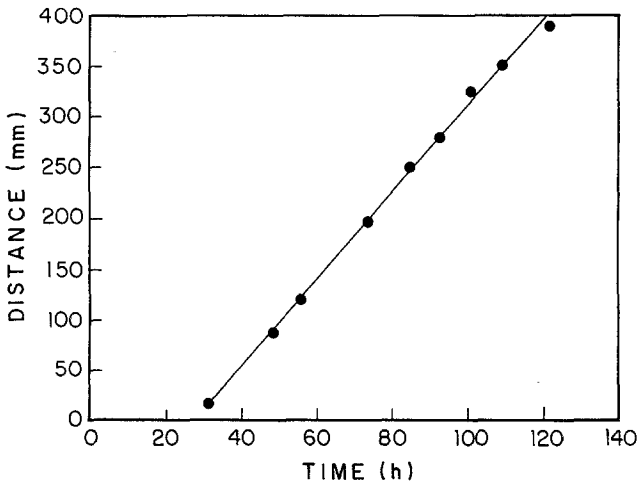

Figure 1 Curve of advancement of the *T. aurantiacus* mycelial frontier (mm) against incubation times (h) obtained through the horizontal tube method. The fungus was cultivated on Czapek modified medium (pH 6.8), containing $NaNO_3$ (24 mEq L^{-1}) and glucose (1.0%) at 50°C.

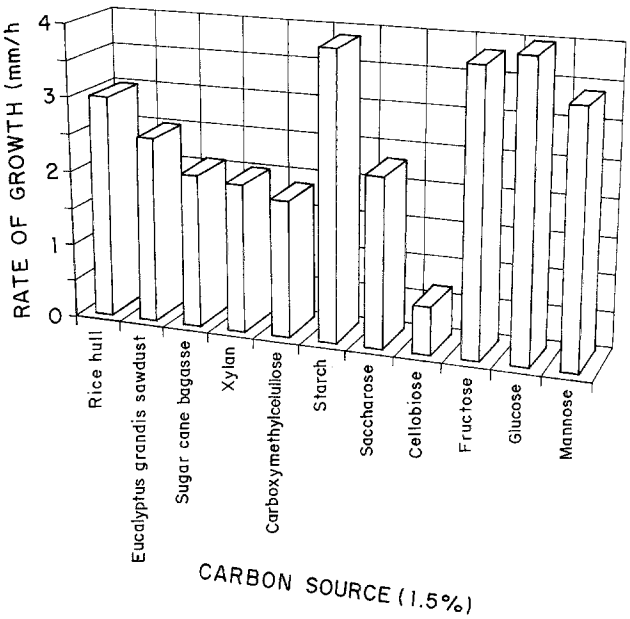

Figure 2 Effect of different carbon sources on the growth rate of *T. aurantiacus*. The growth rate was determined using the horizontal tube method and Czapek modified medium (pH 7.2), containing 1.5% (w/v) of different carbon sources and $NaNO_3$ (24 mEq L^{-1}) as the nitrogen source. The incubation was at 50°C.

and complex substrates in a basal Czapek medium containing $NaNO_3$ as the nitrogen source. In the same manner, 24 mEq L^{-1} of different nitrogen sources (inorganic and organic) were added to the Czapek medium, containing glucose as the carbon source. The pH of the media was not adjusted (pH ~7.2 after autoclaving) and the temperature was 50°C. The Brazilian strain of *T. aurantiacus* grew in media containing different carbon and nitrogen sources (Figures 2 and 3). The fact that *T. aurantiacus* grew well in simple sugars, polysaccharides and lignocellulosic materials suggests that it produces a wide spectrum of enzymes. When inorganic nitrogen sources were added, the growth rates were similar but were improved relative to urea. The best carbon and nitrogen sources were glucose and $NaNO_3$.

In a Czapek medium containing 1.5% glucose and 24 mEq L^{-1} $NaNO_3$ (pH 7.2, not adjusted), *T. aurantiacus* did not grow below 30°C and over 55°C a decreased growth rate with dehydration of the culture after 3 days was observed (Figure 4). The best growth rate was between 45 and 50°C.

The basal Czapek medium was optimized to obtain the maximal growth rate by adjusting glucose and $NaNO_3$ concentrations, initial pH, and incubation temperature. The first FD (2^4) was applied with the aim of finding a region of optimal growth and determining if there was interaction between the variables. The second FD (2^4) was applied with the aim of reducing the region of optimal growth, defined by the first FD, to get a better approximation of the maximum point of growth. Finally, ST was applied in order to find the maximum point of growth within the defined region by the two FDs. The fungal growth rate in the centre point (Table 1) was experimentally determined to be 3.93 ± 0.2 (mm h^{-1}). The results of the first FD show a region (experiments 13 and 16) where values of rates near the centre point were obtained (Table 3). From data listed in Table 3, the principal and interaction effects of the second and third order were calculated (Table 4). The fungal growth rate was more affected by temperature and pH than by glucose and $NaNO_3$ concentration. The variable interactions T × pH, pH × (glucose) and (glucose) × ($NaNO_3$) have a significant effect over the growth rate. The interaction effects of third order between the variables was also calculated; however, the results did not differ significantly and are not included in Table 4.

The variable values for the second FD (2^4) (Table 2) were chosen between the variable values of the first FD (2^4) and the centre point (Table 1). The fungal growth rate was improved after the second FD and attained similar or

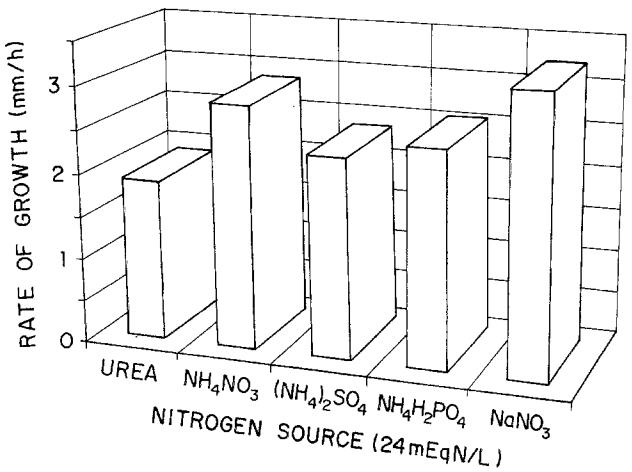

Figure 3 Effect of different nitrogen sources on the growth rate of *T. aurantiacus*. The growth rate was determined using the horizontal tube method and Czapek modified medium (pH 7.2), containing 24 mEq L^{-1} of different nitrogen sources and glucose (1.5%) as the carbon source. The incubation was at 50°C.

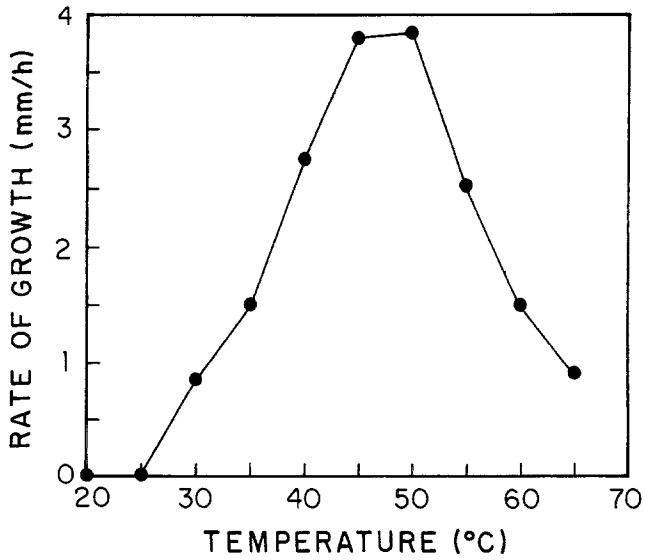

Figure 4 Effect of incubation temperature on the growth rate of *T. aurantiacus*. The growth rate was determined using the horizontal tube method and Czapek modified medium (pH 7.2), containing $NaNO_3$ (24 mEq L^{-1}) and glucose (1.5%) as nitrogen and carbon sources, respectively.

Table 3 Experimental results for the first 2^4 FD

Experiment	T[a]	pH[b]	C[c]	N[d]	Rate of growth (mm h^{-1})
1	+[e]	+	+	+	2.51 ± 0.18
2	+	+	+	−	0.66 ± 0.08
3	+	+	−	−	1.72 ± 0.16
4	+	+	−	+	0.82 ± 0.15
5	+	−	+	+	1.65 ± 0.03
6	+	−	+	−	0.76 ± 0.03
7	+	−	−	−	1.45 ± 0.07
8	+	−	−	+	2.07 ± 0.08
9	−	+	+	+	2.76 ± 0.15
10	−	+	+	−	2.63 ± 0.02
11	−	+	−	−	1.73 ± 0.07
12	−	+	−	+	1.07 ± 0.17
13	−	−	+	+	3.46 ± 0.04
14	−	−	+	−	2.91 ± 0.09
15	−	−	−	−	2.95 ± 0.01
16	−	−	−	+	3.49 ± 0.01

[a]Incubation temperature (°C).
[b]Culture medium pH.
[c]Glucose concentration (%).
[d]$NaNO_3$ concentration (mEq L^{-1}).
[e](+): high level; (−): low level. See Table 1.

Table 4 Principal and interaction effect values for the first 2^4 FD

Effect	Estimated value
Principal effects	
Temperature (°C)	−1.17
pH	−0.61
Glucose concentration	0.26
$NaNO_3$ concentration	0.31
2nd order interaction effects	
T × pH	0.55
T × (Glucose)	−0.38
T × ($NaNO_3$)	0.24
pH × (Glucose)	0.55
pH × ($NaNO_3$)	−0.27
(Glucose) × ($NaNO_3$)	0.48

higher yields than that of the centre point (3.93 mm h^{-1}). Again, experiments carried out at (−) level of temperature (48°C), gave the best results (Table 5). Varying the $NaNO_3$ concentration from 14 to 34 mEq L^{-1} did not affect the fungal growth rate; however, the incubation temperature and the medium pH continued to exert a significant effect. The interaction effect of second order decreased when compared with those of the first FD and third order interactions between the variables were not observed. The diminished interaction effect of second order may be due to the fact that these values are near the maximum growth point after the second FD (Table 6).

Within the experimental region of optimal growth, defined by the second FD, the maximum growth point was examined using ST. The geometric figure was defined by four better results in Table 6 (experiments 9, 13, 14 and 16), and took a tetrahedral shape. The temperature variable was fixed at 48°C since, at this temperature, the best results of the second FD were observed.

The points generated by the ST (Table 7) were carried

Table 5 Experimental results for the second 2^4 FD

Experiment	T[a]	pH[b]	C[c]	N[d]	Rate of growth (mm h^{-1})
1	+[e]	+	+	+	1.67 ± 0.06
2	+	+	+	−	1.81 ± 0.11
3	+	+	−	−	1.52 ± 0.13
4	+	+	−	+	1.06 ± 0.01
5	+	−	+	+	2.08 ± 0.12
6	+	−	+	−	2.65 ± 0.03
7	+	−	−	−	2.32 ± 0.19
8	+	−	−	+	2.83 ± 0.21
9	−	+	+	+	3.07 ± 0.04
10	−	+	+	−	2.85 ± 0.07
11	−	+	−	−	2.74 ± 0.05
12	−	+	−	+	2.56 ± 0.07
13	−	−	+	+	3.91 ± 0.05
14	−	−	+	−	3.87 ± 0.04
15	−	−	−	−	2.91 ± 0.02
16	−	−	−	+	4.05 ± 0.06

[a]Incubation temperature (°C).
[b]Culture medium pH.
[c]Glucose concentration (%).
[d]$NaNO_3$ concentration (mEq L^{-1}).
[e](+): high level; (−): low level. See Table 1.

Table 6 Principal and interaction effect values for the second 2^4 FD

Effect	Estimated value
Principal effects	
Temperature (°C)	−1.25
pH	−0.92
(Glucose)	0.24
($NaNO_3$)	0.07
2nd order interaction effects	
T × pH	−0.04
T × (Glucose)	−0.12
T × ($NaNO_3$)	−0.23
pH × (Glucose)	0.14
pH × ($NaNO_3$)	−0.21
(Glucose) × ($NaNO_3$)	−0.18

Table 7 Simplex optimization in solid and liquid Czapek medium for *T. aurantiacus*

Vertex	Variables			Rate growth (mm h^{-1})	Dry weight (mg)
	pH[a]	C[b]	N[c]		
1	6.80	1.45	34.0	4.20 ± 0.30	15.8 ± 1.10
2	5.55	1.08	24.0	4.55 ± 0.05	81.9 ± 0.90
3	4.68	1.01	19.0	4.11 ± 0.10	70.6 ± 0.85
4	6.18	1.01	49.0	4.34 ± 0.10	15.9 ± 0.92
5	6.11	0.92	36.5	4.67 ± 0.10	16.9 ± 1.20
6	6.00	0.78	37.8	4.74 ± 0.02	47.7 ± 0.76
7	5.97	1.46	38.4	5.16 ± 0.03	62.5 ± 0.60
8	5.81	1.84	40.6	4.60 ± 0.15	35.4 ± 0.45

[a]Culture medium pH.
[b]Glucose concentration (%).
[c]$NaNO_3$ concentration (mEq L^{-1}).

out experimentally in a solid medium as well as in a liquid one in order to compare growth in both media. After ST application, the growth rate values were higher than those obtained after the second FD (Table 7). No statistical correlation was found between the growth rate in solid medium and mycelial mass production. After point 5, the new values generated by ST with their respective yields begin to be very close which indicates the proximity of the maximum growth point within the experimental region defined by FD. The movement of ST was stopped at experimental point number 8, and point 7 was considered as the maximum growth for *T. aurantiacus*.

Effect of lignin-related compounds on the growth of T. aurantiacus

After defining a minimal Czapek medium which supported the maximum growth rate, different lignin-related compounds were tested in order to study their effect on growth of *T. aurantiacus*. *T. aurantiacus* exhibited a greater tolerance to lignin-related compounds as phenols, tannin and their derivatives, and to a fraction of ethanolic extractives, at a concentration of 0.1% (w/v). Of all the lignin-related compounds tested, only acetovanillone produced a 24% fungal growth rate inhibition. Tannin from *P. radiata* D Don (Chile) and ethanolic extractives from *E. grandis* (Brazil) stimulated the *T. aurantiacus* growth rate by 14% and 29%, respectively, as compared with the controls. Guaiacol and tannic acid increased the growth rate slightly (Table 8). However, 0.1–0.15% (w/v) tannic acid resulted in a total inhibition of *Lentinus edodes* and *Volvariella volvacea* growth [5]. With guaiacol and tannic acid, *T. aurantiacus* formed a brown zone in the medium after 2–3 days, which is indicative of production of extracellular phenoloxidases. The appearance of coloured zones around mycelium on solid medium containing phenolic compounds such as gallic or tannic acid has been employed for a long time as a simple plate-test for screening of lignin-degrading or ligninolytic enzymes [10,13,14].

Discussion

The ease of measuring growth on a solid media surface, together with good reproducibility and linear response growth rate, make the horizontal tube method a convenient technique for the study of different parameters related to *T. aurantiacus* growth. Through application of two statistical methods, a minimal Czapek medium was defined, where the glucose and $NaNO_3$ concentrations, initial medium pH and the incubation temperature were optimized to get the maximal growth rate of the Brazilian strain of *T. aurantiacus* (Table 9).

With 32 experiments generated from two FD, it was possible to determine a region of maximum growth for *T. aurantiacus*. The effect of the four variables was studied simultaneously, in four different values for each variable in a short period of time. Besides the rapidity of FD, the effect of each variable and the effect of the interaction of different variables on the growth rate could be studied. The application of ST, with eight experiments carried out in sequential form, led to the point of maximum fungal growth within the defined region by FD. The growth rate at this point was 5.16 ± 0.10 mm h^{-1}, which is 1.3-fold higher than that of the centre or reference point.

The Brazilian strain of *T. aurantiacus* exhibited pH and optimal temperature of growth lower than those reported by Rosenberg [15] for the Miehei strain (pH 6.8; temperature 52.5°C); however, this pH value is higher than that of the *levisporus* variety of the same fungus (pH 4.0; temperature 50°C) [20].

T. aurantiacus exhibited great tolerance to lignin-related compounds which, depending on their concentrations, can act as growth inductors or inhibitors of many fungi [5,18]. Higher growth rates of *T. aurantiacus* were observed when tannins of *P. radiata* D Don and ethanolic extractives of *E. grandis* were added to the growth medium. Previous results showed that when *E. grandis* sawdust was treated with *T. aurantiacus*, 64.4% of the ethanol-soluble substances were degraded in 21 days [11]. The fact that the Brazilian strain of *T. aurantiacus* grows well with different lignocellulosic substrates (rice hull, sawdust of *E. grandis* and sugar cane bagasse) and with lignin-related compounds may be linked to the ability of this fungus to produce extracellular ligninolytic enzymes (phenoloxidases, laccases, peroxidases, etc). These results are in agreement with previous results on the enzymatic profile of the Brazilian strain of *T. aurantiacus* in liquid culture [11].

The optimization of some parameters for *T. aurantiacus* growth was developed with the purpose of getting the maximum fungal growth rate on a solid medium. Study of the effect of these parameters on fungal growth in liquid medium and on production of phenol oxidases by *T. aurantiacus* is in progress.

Table 8 Effect of lignin-related compounds on growth rate of *T. aurantiacus*

Compost (0.1%)	Growth rate (mm h^{-1})[a]
None	5.00 ± 0.01
Guaiacol	5.25 ± 0.30[b]
Vanillin	4.97 ± 0.04
Acetovanillone	3.78 ± 0.25
Tannic acid	5.15 ± 0.05[b]
Tannins *P. radiata*	5.70 ± 0.10
EtOH-extractives *E. grandis*	6.43 ± 0.18

[a]Values represent the mean of three replicates.
[b]Development of brown colour in the culture medium after 2–3 days incubation.

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Table 9 Optimized parameters for *T. aurantiacus* growth rate

Variable	Optimum
Temperature (°C)	48.0
pH	6.0
Glucose concentration (%)	1.5
$NaNO_3$ concentration (mEq L^{-1})	38.4

computational program Amoeba. This work was financially supported by CAPES and FAPESP.

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