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Agaricus blazei as a Substrate for the Production of β-1,3-Glucanase by *Trichoderma harzianum* Rifai

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Summary

Extracellular β-1,3-glucanase was produced by *Trichoderma harzianum* Rifai cultivated in the *Agaricus blazei* (*Agaricus brasiliensis*) extract as a substrate in submerged fermentation. A 2^2-central composite factorial design was developed using the time of culture (x_1/day) and *Agaricus blazei* extract concentration (x_2/(g/L)) as variables, and the results were analyzed using response surface methodology (RSM). The results showed that the *Agaricus blazei* extract concentration was the most important variable in the production of β-1,3-glucanase, and the maximum β-1,3-glucanase activity (0.77 U/mL) was obtained in one day of cultivation. The β-glucan present in the cell wall of *Agaricus blazei* mushroom proved to be a good substrate for inducing the production of specific β-1,3-glucanase by *Trichoderma harzianum* Rifai.

Key words: β-1,3-glucanase, medicinal mushrooms, *Trichoderma harzianum* Rifai, response surface methodology

Introduction

Agaricus blazei is an edible mushroom that belongs to the Basidiomycetes fungal class, and its consumption has increased significantly in Brazil, Japan, China, Korea, Canada, and the USA (*1*). It is made up of essential amino acids, among which arginine is the most abundant, followed by isoleucine, methionine and leucine. Some carbohydrates are also present, including manose, glucose and xylose (*2*), as well as the biopolymer β-glucan, which constitutes part of the cell wall (*3*).

β-Glucans are glucose polymers connected by β-(1→3) glucopyranosyl bonds with β-(1→6) branches, and are considered the main components of the cell wall of filamentous fungi (*4*) and yeasts (*5*). They are also present in cereals, and they are found in higher quantities in barley and oat grains (*6*).

Mushrooms have become attractive as functional foods, and they serve as a source of bioactive compounds (*7*), which have broad beneficial effects, including an increase of immunity, and decrease of blood cholesterol and lipid levels, as well as of blood pressure (*8*). *Agaricus blazei* (*Agaricus brasiliensis*) mushrooms have gained importance in many different countries, mainly because of their nutraceutical and medicinal properties, which come from the β-glucans present in their composition (*9*). Thus, other properties of *A. blazei* have been studied, including their antioxidant (*8*), antitumour (*10*), antigenotoxic (*3*) and anti-diabetic properties (*11*).

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Fungi belonging to the *Trichoderma* genus have been studied to evaluate their production of different enzymes, most commonly cellulases and hemicellulases (*12*), β-glucosidases (*13*), proteases (*14*), lactases (*15*), xylanases (*16*), chitinases (*17*) β-1,3-glucanases (*18*), and other metabolites. Their role in biological control has also been evaluated (*17*). They present rapid growth and have the ability to assimilate various substrates. Also, they are important as wood and herbal material decomposers (*19*).

β-1,3-Glucanase can be produced by several microorganisms such as *Pichia membranifaciens* yeast (*20*), *Bacillus* sp. bacteria using a recombinant *Escherichia coli* (*21*), and *Acremonium* sp. IMI383068 fungi (*22*), which showed distinct levels of β-1,3-glucanase when cultivated in different carbon sources, such as the crude cell wall of *Saccharomyces cerevisiae* (*20*), the cell wall of *Botrytis cinerea* (*21*) and lactose, pustulan and chitin (*22*). Factorial design and analysis by response surface methodology (RSM) have been used by Giese *et al.* (*23*), Donzelli *et al.* (*24*) and Théodore and Panda (*25*) in cultures of *T. harzianum* in order to optimize the production of β-1,3-glucanases in submerged fermentations.

The production of β-1,3-glucanase can be affected by some fermentation parameters, such as agitation, pH, and temperature (*26*). The production of microbial enzymes can be affected by the aeration, incubation time, moisture and fermentative process.

RSM is an empirical model technique used to estimate the relationship between a set of controllable experimental factors and the observed results. It is currently one of the most popular optimization techniques in the field of biotechnology because of its reasonably high efficiency and simplicity. The most common experimental design used in RSM is the central composite design (CCD), which has equal predictability in all directions from the centre (*27*).

The production of β-glucanase is an essential key to evaluate its action in degrading β-(1→3,1→6)-glucans for future production of oligosaccharides that exhibit prebiotic and immunomodulator activity. In this study, an extract of *A. blazei* was used as a substrate to produce specific β-1,3-glucanase by *Trichoderma harzianum* Rifai. The goal of this study is to optimize the production of β-glucanase by *Trichoderma harzianum* Rifai using *A. blazei* as a substrate. RSM was used to optimize the production, with cultivation time (x_1/day) and substrate concentration (x_2/(g/L)) as parameters.

Materials and Methods

Mushroom

The dried *Agaricus blazei* fruiting bodies were purchased from the Commerce of Natural Products Ltd, Uruguaiana, Rio Grande do Sul, Brazil. The composition of 100 g of dry mass was: carbohydrates 44.4, proteins 33.3, dietary fibre 33.3, and (in mg): sodium 6, phosphorus 22, copper 0.21, iron 0.28 and zinc 0.14, according to the manufacturer´s label. Dried material was ground and sieved to obtain homogenous particle size that could pass through the voile cloth.

Microorganism and optimization of β-1,3-glucanase production

The fungus *Trichoderma harzianum* Rifai, isolated from *Aspidosperma* sp. (Peroba) by Barbosa (*28*), was maintained at room temperature in xylose agar slants, incorporating the Vogel's minimal salts medium (VMSM). Before each experiment, the microorganism was transferred to 250-mL Erlenmeyer flasks containing 50 mL of xylose agar with VMSM and incubated at 28 °C for 5 days. Fermentation was carried out in 125-mL Erlenmeyer flasks containing 25 mL of VMSM (*29*) and different concentrations of *A. blazei* extract powder as a substrate. Each flask was inoculated with a suspension containing 10^7 spores, and was kept under constant agitation at 180 rpm and 28 °C, according to the earlier investigation which showed that the best glucanase production was obtained under these conditions. The effects of carbon source and cultivation time on β-1,3-glucanase production by *T. harzianum* Rifai were used as variables in the experimental design presented below.

Factorial design and statistical analysis

The concentration of *A. blazei* and cultivation time for β-glucanase production by *T. harzianum* Rifai were optimized using RSM through a 2^2-factorial central composite experimental design, with five repetitions at the central point and a total of 13 experiments carried out in duplicate (Table 1). The design was chosen according to the growth profile obtained before the optimization. The independent variables studied for β-glucanase production (Y_1/(U/mL)) were as follows: x_1=cultivation time (day) and x_2=*A. blazei* concentration (g/L). Analysis of variance (ANOVA) and multiple regression analysis were also carried out using STATISTICA software v. 6.0, StatSoft Inc, Tulsa, OK, USA.

Table 1. Statistical design of the experiments for two variables for *T. harzianum* Rifai

Experiments	*t*/day (x_1)	*γ*(*A. blazei*)/(g/L) (x_2)
1	–1	–1
2	–1	+1
3	+1	–1
4	+1	+1
5	–1	0
6	+1	0
7	0	–1
8	0	+1
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0

Original independent variables	Levels of variable		
	–1	0	+1
x_1, time of growth/day	1	2	3
x_2, *γ*(*A. blazei* extract)/(g/L)	1.0	2.0	3.0

Analytical methods

β-1,3-Glucanase activity was measured by quantifying the reducing sugars liberated after the hydrolysis of laminarin (β-(1→3,1→6)-glucan produced by the algae *Laminaria digitata*, Sigma-Aldrich, St. Louis, MO, USA). The final assay volume was 0.5 mL, which was made up of 0.35 mL of laminarin (4 mg/mL) in a 25-mM sodium acetate buffer at pH=5.0 and 0.1 mL of crude enzyme. The assay was carried out at 50 °C and stopped after 10 min with 50 μL of 1.0 M NaOH. The method of Somogyi (*30*) and Nelson (*31*) was used to quantify the reducing sugars. One unit of enzymatic activity was defined as the number of μmols of reducing sugars released per minute per mL of enzymatic extract. Glucose was used for the standard curve.

Total sugars were measured using the phenol-sulphuric acid method of Dubois *et al*. (*32*). Fungal biomass was determined gravimetrically by drying to constant mass at 60 °C. pH was measured using a pH meter during fermentation.

Results and Discussion

The variables chosen were based on scientific data from the literature, with the incubation time and substrate concentration as probably the most influential factors in the production of β-1,3-glucanases. These variables were also studied by Giese *et al*. (*23*) and Théodore and Panda (*25*) in the optimization of the production of β-1,3-glucanases using *Trichoderma harzianum*.

Through multiple regression analysis of the experimental data, a second order polynomial equation was obtained for β-1,3-glucanase production by *T. harzianum* Rifai (Eq. 1):

$$\hat{Y}_1 = 0.228276 - 0.123333x_1 - 0.033966x_1^2 + 0.179167x_2 + 0.113534x_2^2 - 0.107500x_1x_2 \quad /1/$$

where $\hat{Y}_1$ is the predicted response, and x_1 and x_2 are independent variables: cultivation time (day) and *A. blazei* concentration (g/L), respectively.

According to the results presented in Table 2, the intercept was significant, indicating that the central point (2 days of cultivation and *A. blazei* extract concentration of 2 g/L) was adequately chosen.

According to the Pareto chart (Fig. 1), the most important variable for β-1,3-glucanase production by *T. harzianum* Rifai was the concentration of *A. blazei*. The analysis of variance for the surface response of Eq. 1 (Table 3) showed that regression was significant ($p<0.05$) and the lack of fit was not significant, indicating that the model can be used for predictive purposes.

Table 2. Analysis of factors and their interaction in the production of β-1,3-glucanase by *T. harzianum* Rifai cultivated in *A. blazei* extract

Factors	Coefficients	S.D.	T	p
Intercept	0.228276	0.008277	27.5795	0.000000
x_1 linear	–0.123333	0.008138	–15.1554	0.000000
x_1 quadratic	–0.033966	0.011995	–2.8317	0.011509
x_2 linear	0.179167	0.008138	22.0163	0.000000
x_2 quadratic	0.113534	0.011995	9.4655	0.000000
$x_1 \cdot x_2$	–0.107500	0.009967	–10.7857	0.000000

S.D.=standard deviation; x_1=time of growth, x_2=*A. blazei* concentration

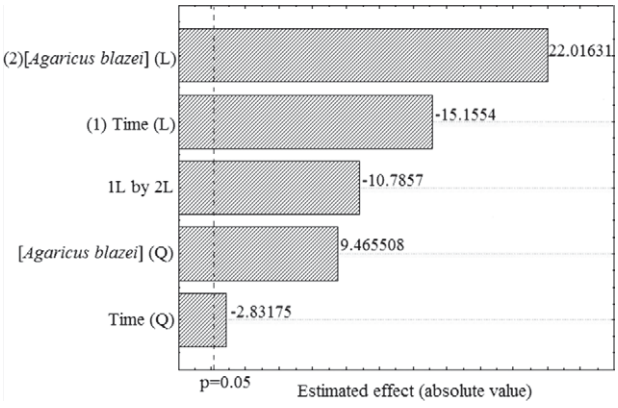

Fig. 1. Pareto chart of standardized effects for β-1,3-glucanase production by *T. harzianum* Rifai cultivated in *A. blazei* extract as substrate. The point at which the effect estimates were statistically significant (at p=0.05) is indicated by the broken vertical line. The linear (L) and squared (Q) effects are also indicated, as well as the linear interaction (1L by 2L) between both effects

Table 3. Analysis of variance (ANOVA) for the production of β-1,3-glucanase by *T. harzianum* Rifai using *A. blazei* extract as substrate

Variation of source	Sum of squares	Degrees of freedom	Mean square	F	p
Regression	0.567742	2	0.283871	33.29297	0.000000
Residues	0.196108	23	0.008526		
Standard error	0.013510	17	0.000795		
Total	0.763850	25			

F=relation (regression/residual), p=relation (deviation of regression/standard error)

The experimental results and predictive activities for β-1,3-glucanase production by *T. harzianum* Rifai using Eq. 1 are presented in Table 4. The highest β-1,3-glucanase activity (0.77 U/mL) was observed on the first day of cultivation using 3 g/L of *A. blazei* extract as substrate. This activity was probably due to the higher concentration of β-glucan present in the extract, as well to the type of glycosidic bond present in the glucans. Concentrations higher than 3 g/L were not tested because of the low solubility of the *A. blazei* extract. In similar studies, Giese *et al*. (*23*) found the highest β-1,3-glucanase activity (1.2 U/mL) produced by the same strain of *T. harzianum* Rifai using 1.5 g/L of botryosphaeran exopolysaccharide (EPS) of a β-(1→3,1→6)-D-glucan type produced by *Botryosphaeria rhodina* as a sole carbon source after 5 days of cultivation. RSM was used, and the results also suggested that β-1,3-glucanase production depended on EPS concentration.

Table 4. Experimental and predictive values for β-1,3-glucanase production by *T. harzianum* Rifai cultivated in *A. blazei* extract as substrate

Experiment	β-1,3-glucanase activity/(U/mL)		
	Experimental	Predicted	
1	0.14	0.15	0.14
2	0.77	0.72	0.71
3	0.09	0.05	0.11
4	0.26	0.22	0.25
5	0.24	0.34	0.32
6	0.12	0.14	0.07
7	0.22	0.19	0.16
8	0.50	0.52	0.52
9	0.20	0.21	0.23
10	0.25	0.24	0.23
11	0.25	0.21	0.23
12	0.22	0.22	0.23
13	0.18	0.24	0.23

Based on the value of R^2, our findings showed that 96 % of the response variability could be explained either by the model, or by the experimental factors and their interactions. The low pure error indicated good reproducibility of the experimental data. RSM was also used by Donzelli *et al.* (*24*) and Theódore and Panda (*25*) to optimize the production of β-1,3-glucanase by some species of *Trichoderma* in submerged fermentation using different β-(1→3,1→6)-D-glucans as the carbon source. Donzelli *et al.* (*24*) found the highest β-1,3-glucanase production by *Trichoderma atroviride* when the initial pH increased from 5.5 to 6.5. The highest β-1,3-glucanase production was obtained by Theódore and Panda (*25*) when *Trichoderma harzianum* was grown in glucose at 30 °C and at initial pH=4.7. However, the level of pH had no significant effect in any of the experimental runs. Carneiro *et al.* (*33*) optimized β-1,3-glucanase production by *Trichoderma reesei* using *Agaricus blazei* extract as a substrate. The best cultivation conditions occurred after 5 days, with maximum activity of 4.35 U/mL and 3 g/L of *A. blazei.* According to the results of this study, it is possible to obtain maximum β-1,3-glucanase production by *T. harzianum* Rifai in 1 day of cultivation and with 3 g/L of *A. blazei* extract (Fig. 2). There are few studies reported in the scientific literature on the optimization of β-1,3-glucanase production by fungi using response surface methodology.

RMS showed to be power technique for optimizing β-1,3-glucanase production by *T. harzianum* Rifai. Traditional methods for optimization are one-factor-at-a-time techniques. Unfortunately, this approach frequently fails to identify the variables that give rise to the optimum response, because the effects of factor interactions are not taken into account in such procedures (*34*).

The highest biomass production by fungi in 3 g/L of *Agaricus blazei* extract was 2.2 g/L, after 24 h of cultivation (Fig. 3). The lowest biomass production observed in 1 and 2 g/L of *Agaricus blazei* extract was 1 and 1.7 g/L after 48 and 24 h, respectively (data not shown). Giese

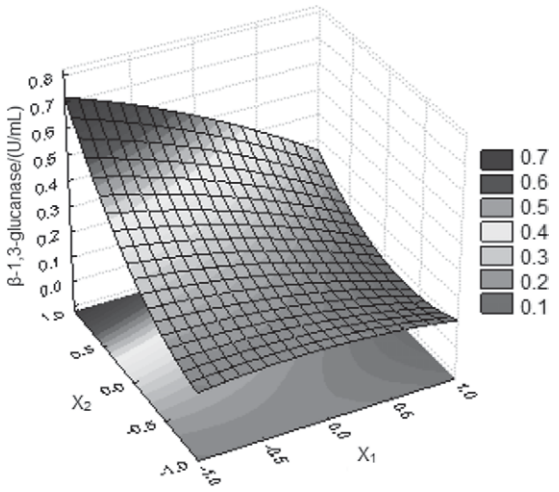

Fig. 2. Response surface for β-1,3-glucanase production by *T. harzianum* Rifai as a function of cultivation time×*A. blazei* extract ($x_1 \cdot x_2$)

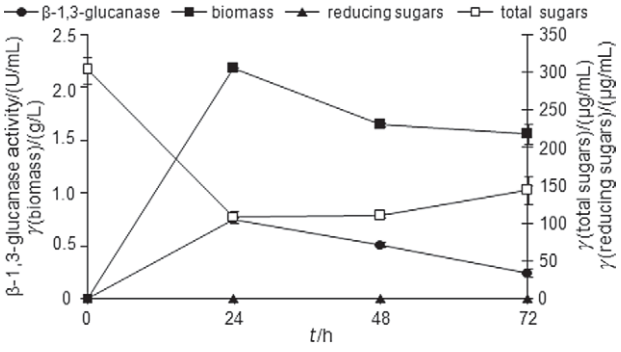

Fig. 3. Growth profile for the production of β-1,3-glucanase by *T. harzianum* Rifai on *A. blazei* extract (3 g/L)

et al. (*35*) also evaluated the production of biomass by *Trichoderma harzianum* Rifai from different carbon sources and observed 7.17 g/L of biomass on the mycelium of *Botryosphaeria rhodina* MAMB-05 as carbon source, after 8 days of cultivation. The level of residual reducing sugars monitored during the growth profile stayed low, which shows an equilibrium between the hydrolysis of β-1,3-glucanase in β-glucan present in the extract of *Agaricus blazei* and glucose consumption by fungi. The high-

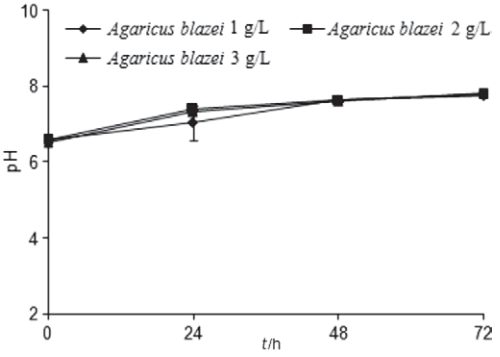

Fig. 4. pH profile during the production of β-1,3-glucanase by *T. harzianum* Rifai on *A. blazei* extract

est enzyme activity was observed by the lowest residual level of glucan in 3 g/L of *A. blazei* extract, measured as total sugar. Slight variations in pH were observed during fermentation (Fig. 4).

Conclusions

The advantage of using RSM analysis is that it allows for a reduction in time and materials known as one-factor--at-a-time, which minimizes possible common errors in traditional techniques. RSM can study many factors simultaneously. *T. harzianum* Rifai grew in *A. blazei* extract as a substrate and produced β-1,3-glucanase. The analysis showed that the highest β-1,3-glucanase production by *T. harzianum* Rifai occurred in 1 day using 3 g/L of the substrate.

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