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Short communication

Comparison of β-1,3-glucanase production by *Botryosphaeria rhodina* MAMB-05 and *Trichoderma harzianum* Rifai and its optimization using a statistical mixture-design

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ABSTRACT

Botryosphaeria rhodina MAMB-05 produced β-1,3-glucanases and botryosphaeran when grown on glucose, while *Trichoderma harzianum* Rifai only produced the enzyme. A comparison of long-term cultivation (300 h) by *B. rhodina* demonstrated a correlation between the formation of botryosphaeran (48 h) and its consumption (after 108 h), and de-repression of β-1,3-glucanase synthesis when glucose was depleted from the nutrient medium, whereas for *T. harzianum* enzyme production commenced during exponential growth. Growth profiles and levels of β-1,3-glucanases produced by both fungi on botryosphaeran also differed, as well as the production of β-1,3-glucanases and β-1,6-glucanases on glucose, lactose, laminarin, botryosphaeran, lasiodiplodan, curdlan, Brewer's yeast powder and lyophilized fungal mycelium, which were dependent upon the carbon source used. A statistical mixture-design used to optimize β-1,3-glucanase production by both fungi evaluated botryosphaeran, glucose and lactose concentrations as variables. For *B. rhodina*, glucose and lactose promoted enzyme production at the same levels (2.30 U mL^{-1}), whereas botryosphaeran added to these substrates exerted a synergic effect favorable for β-glucanase production by *T. harzianum* (4.25 U mL^{-1}).

1. Introduction

Non-cellulose degrading β-glucanases are ubiquitously distributed among yeasts and fungi and play important physiological roles in cell wall differentiation [1]. They constitute primarily the β-1,3-glucanases, but also β-1,6-glucanases and accessory β-glucosidases. Their production in fungi is controlled through regulatory mechanisms involving substrate induction and catabolite repression depending upon the substrate [2].

Botryosphaeria rhodina produces a β-glucanolytic enzyme complex [3], and an exopolysaccharide botryosphaeran [4] that has been used as carbon source for the production of β-glucanases [3], and also as a substrate for β-glucanases [5]. Botryosphaeran is a 1,3;1,6-β-D-glucan with approximately 22% side branching comprising β-glucosyl and gentiobiosyl residues linked by β-1,6-bonds [4].

It is unclear whether a correlation exists between botryosphaeran production and β-glucanase synthesis in *B. rhodina*, and whether botryosphaeran can de-repress β-glucanase synthesis in glucose-grown cultures. We sought to address this problem, and in this work report on the effects of glucose-limitation on β-1,3-glucanase production by glucose-grown cultures of *B. rhodina* under long-term cultivation. *Trichoderma harzianum*, by contrast, does not produce any exopolysaccharide, but is considered a good producer of β-glucanases [6]. It was therefore of interest to perform a comparative study between *T. harzianum* and *B. rhodina*, which produces an exopolysaccharide, to clarify some physiological aspects. Similarly, the effects of long-term growth by both fungi on botryosphaeran as sole carbon source on production of β-1,3-glucanases are also reported. Statistical analysis by the response surface method can be associated with mixture-design as an approach to evaluate the effects of a combination of two or more medium components in order to find the component proportions giving an optimal response for a selected variable [7]. As high enzyme titres resulted from growth on glucose and lactose, we developed a mixture-design experiment using mixtures of these carbohydrates together with botryosphaeran as inducer to formulate a growth

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Table 1
Statistical mixture-design matrix defining conditions for carbohydrate formulation on optimizing β-1,3-glucanase production by *Botryosphaeria rhodina* and *Trichoderma harzianum* grown for 8 days.

Exp.	Carbon source (g L^{-1})			β-1,3-Glucanase activity (U mL^{-1})	
	Glucose (x_1)	Lactose (x_2)	Botryosphaeran (x_3)	*Botryosphaeria rhodina*	*Trichoderma harzianum*
1	0	0	2	0.079 ± 0.01	2.979 ± 0.09
2	10	0	0	2.318 ± 0.07	3.853 ± 0.19
3	0	10	0	2.238 ± 0.08	4.243 ± 0.07
4	5	0	1	0.816 ± 0.02	3.289 ± 0.20
5	5	5	0	2.218 ± 0.01	4.247 ± 0.21
6	0	5	1	0.837 ± 0.02	4.006 ± 0.07
7	3.3	3.3	0.7	1.259 ± 0.01	3.621 ± 0.08
8	1.7	1.7	0.13	0.744 ± 0.01	3.932 ± 0.01
9	6.7	1.7	0.03	1.664 ± 0.06	4.095 ± 0.11
10	1.7	6.7	0.03	1.624 ± 0.05	4.139 ± 0.05

medium to optimize the production of β-1,3-glucanases by both fungi.

2. Materials and methods

2.1. Materials

β-1,3-Glucans included: botryosphaeran (*B. rhodina* [4]), bacterial curdlan (Megazyme) and algal laminarin (Sigma). β-1,6-Glucans were lasiodiplodan (*Lasiodiplodia theobromae* [8]), and pustulan (lichen [9]). Lyophilized fungal mycelium (LFM) was prepared from *B. rhodina* [3].

2.2. Microorganism and cultivation

B. rhodina MAMB-05 was grown on Vogel minimal salts medium (VMSM)-glucose-agar [3] for 5 days at 28 °C, and three 7-mm plugs taken for inoculation purposes. *T. harzianum* conidia were harvested from cultures grown on VMSM-xylose-agar [3] for 7 days at 28 °C, and 1×10^7 spores/flask used for inoculation.

Erlenmeyer flasks containing VMSM and a carbon source were inoculated and the fungal cultures shaken (180 rpm) at 28 °C. In growth profile experiments, both fungi were grown on glucose (10 g L^{-1}) or botryosphaeran (1.5 g L^{-1}) for 300 h, and flasks removed at various time intervals. In experiments evaluating enzyme production on various carbohydrates, the fungal isolates were grown on glucose, lactose, Brewer's yeast powder (BYP) or LFM at 10 g L^{-1}, and botryosphaeran, laminarin, lasiodiplodan or curdlan at 1.5 g L^{-1} for 8 days. All experiments were carried out in duplicate, and values reported represent average values ±SD.

2.3. Analytical techniques

Assay of β-1,3-glucanase and β-1,6-glucanase activities were measured against laminarin and pustulan, respectively [5]. Fungal biomass and botryosphaeran was determined gravimetrically [10]. Reducing sugars were determined by the cuproarsenate method [11].

2.4. Statistical experimental design

Conditions to optimize β-1,3-glucanase production by *B. rhodina* and *T. harzianum* were performed using a statistical mixture-design matrix [7] with three components as carbon sources in the formulation with 10 experimental runs (Table 1). In a mixture experiment the sum of the component fractions must be equal to unity and their proportions must be non-negative. The restrictions on the levels of each factor are expressed as follows:

$$\sum_{i=1}^{q} x_i = 1 \quad \text{(i.e., 100\%)} \tag{1}$$

where x_i represents the proportion of the ith component in the mixture, and q is the number of components. The most typical canonical models to represent the mean of the response variable (Y) as a function of the factors x_i ($q = 3$) using regression coefficients b_i, b_{ij} and b_{ijk} are shown in Eqs. (2)–(4),

Linear model:

$$\hat{y} = \sum_{i=1}^{q} b_i x_i \tag{2}$$

Quadratic model:

$$\hat{y} = \sum_{i=1}^{q} b_i x_i + \sum_{i}^{q} \sum_{<1} b_{ij} x_i x_j \tag{3}$$

Special cubic model:

$$\hat{y} = \sum_{i=1}^{q} b_i x_i + \sum_{i}^{q} \sum_{<1} b_{ij} x_i x_j + \sum_{i} \sum_{<1}^{q} \sum_{<k} b_{ijk} x_i x_j x_k \tag{4}$$

In these equations, b_i, the linear value of component i, represents the estimated response to the ith pure component, whereas b_{ij} is the coefficient of the additive blending of components i and j. The b_{ijk} coefficient describes non-linear blending owing to the simultaneous presence of three components. The linear model is used when the effects of the components in the mixture are additive, and the response variable can be defined as linear combination of their components. The quadratic model considers antagonistic ($b_{ij} < 0$) or synergic ($b_{ij} > 0$) interactions between pairs of components of the mixture. The cubic special model is able to consider interactions between three components. The independent variables for optimization of β-1,3-glucanase production (Y_1, U mL^{-1}) were: x_1 (glucose, g L^{-1}); x_2 (lactose, g L^{-1}), and x_3 (botryosphaeran, g L^{-1}) as described in Table 1. Analysis of variance (ANOVA) and multiple regression analyses were performed using STATISTICA Version-6 (StatSoft, Inc. 2001).

3. Results and discussion

3.1. Comparison of β-1,3-glucanases production under starvation conditions

B. rhodina produced β-1,3-glucanases on glucose as sole carbon source [3], and also produced botryosphaeran, which reached maximum levels during the stationary phase (96 h) of growth [10]. In extending our studies on β-1,3-glucanases, it was of interest to

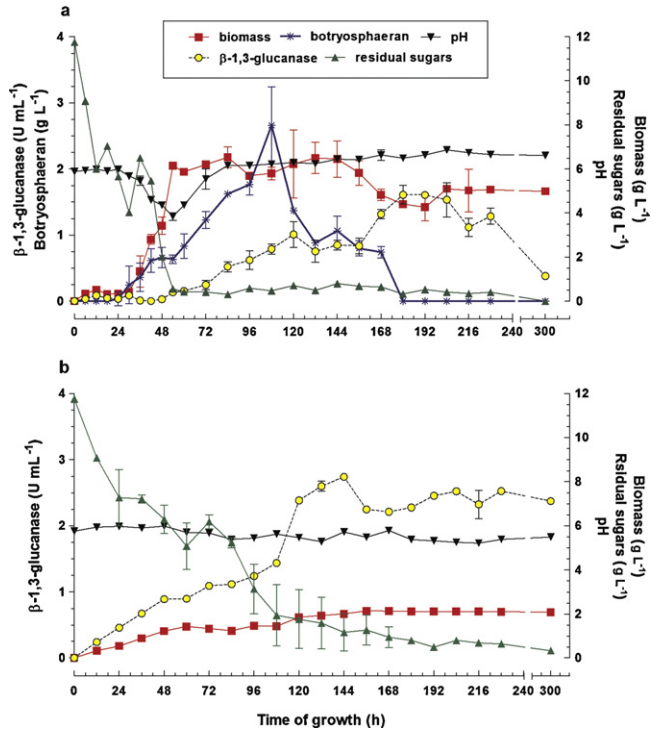

Fig. 1. Profile of growth and production of β-1,3-glucanases by (a) *Botryosphaeria rhodina*, and (b) *Trichoderma harzianum* when cultivated on glucose as sole carbon source.

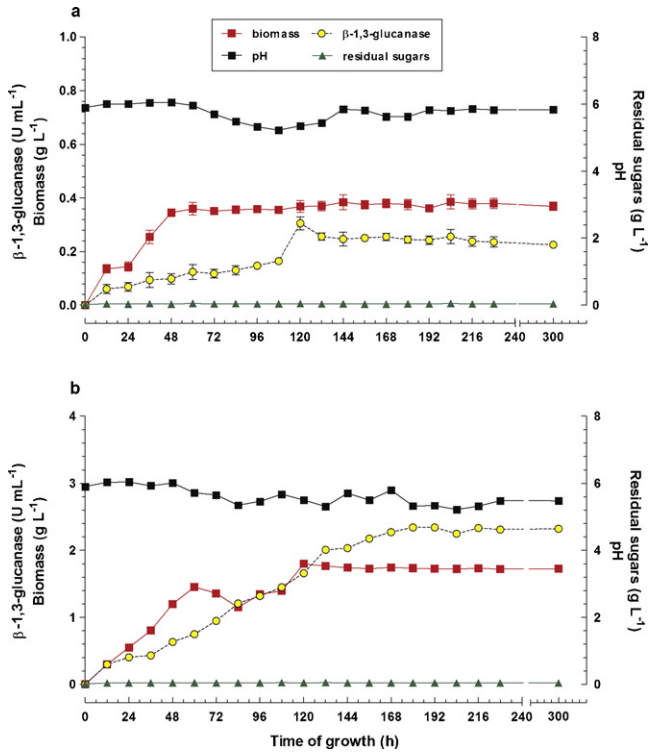

Fig. 2. Profile of growth and production of β-1,3-glucanases by (a) *Botryosphaeria rhodina*, and (b) *Trichoderma harzianum* when cultivated on botryosphaeran as sole carbon source.

examine the course of β-1,3-glucanase and botryosphaeran production during long-term fermentation by *B. rhodina* grown on glucose. This would determine whether de-repression of β-1,3-glucanase synthesis could occur by botryosphaeran (as inducer) once glucose was depleted from the nutrient medium as occurs under starvation conditions. The growth time was extended to 300 h, at which time glucose would be completely exhausted from the growth medium. *B. rhodina* was grown on glucose and the production of β-glucanase monitored over this period (Fig. 1a). Stationary phase was reached by 48 h as indicated by the biomass profile. Glucose had almost totally disappeared from the ECF by 60 h, and at this stage botryosphaeran production commenced and continued to increase up to 108 h of growth (0.24 g g^{-1} glucose), and thereafter its content in the ECF gradually declined, being totally depleted by 180 h of growth, presumably utilized as a carbon source to support fungal growth.

β-1,3-Glucanase production commenced during the stationary phase when the residual glucose concentration was near-depleted from the medium (after 50 h), and gradually increased reaching highest titres (1.3 U mL^{-1}) at 192 h of cultivation (0.8 U h^{-1} g^{-1} glucose); this occurred when the fungus was presumably in the death phase. Up until this stage, the concentration of botryosphaeran had steadily declined in the ECF, and this was related to its hydrolysis by the β-1,3-glucanases produced; the botryosphaeran-degradation products being consumed by the fungus. Botryosphaeran can serve as a substrate for the β-glucanolytic enzyme complex produced by *B. rhodina* [3]. Between 144 h and 192 h, there was a decline in the mycelial biomass level and this correlated with a rise in β-1,3-glucanase activity; presumably the mycelium, which contains 1,3:1,6- and 1,6-linked β-D-glucans as constituents of the fungal cell wall [12], was degraded by the β-glucanases produced. The results indicated that de-repression of β-1,3-glucanase synthesis occurred and appeared to be related to the consumption of botryosphaeran produced during long-term fungal growth, as well as utilization of the polysaccharide components in the mycelium. A similar observation was reported for *Sclerotium glucanicum*, which produced β-glucanases, as well as an exopolysaccharide (scleroglucan, a 1,3;1,6-β-D-glucan) [13].

β-1,3-Glucanase synthesis in fungal species is regulated through induction and/or catabolite repression [3,6]. With *B. rhodina*, the production of β-1,3-glucanases occurred only in the presence of low levels of glucose, being regulated by catabolite repression. Similar observations have been reported for *Neurospora crassa* [14] and *Acremonium persicinium* [15]. By contrast, *Trichoderma viride* and *Saccharomyces cerevisiae* produced β-glucanases not repressed by glucose [16].

The profile of growth and production of β-1,3-glucanase by *T. harzianum* on glucose showed a distinct difference by comparison to *B. rhodina* (Fig. 1b). β-1,3-Glucanase production commenced during the exponential growth phase being secreted into the ECF, and was not repressed by glucose. This finding was in agreement with observations reported for other *T. harzianum* strains [17]. While *B. rhodina* β-1,3-glucanase appeared to be repressed by glucose, β-1,3-glucanase production by *T. harzianum*, in contrast, occurred much earlier (commencing after 12 h). Maximum production of β-1,3-glucanase (2.7 U mL^{-1}) by *T. harzianum* occurred at 144 h (1.8 U h^{-1} g^{-1} glucose) and enzyme titres were 1.7-fold higher than those of *B. rhodina*. Maximum specific growth rate was 0.02 h^{-1} for *T. harzianum*, and 0.05 h^{-1}, *B. rhodina*. In *Trichoderma* spp., β-1,3-glucanase synthesis appears be to regulated at different glucose concentrations [17].

3.2. Comparison of β-1,3-glucanase production on botryosphaeran

β-1,3-Glucanase production by *B. rhodina* on botryosphaeran commenced during the exponential growth phase and enzyme activity increased up to 120 h (0.3 U mL^{-1}) and then leveled off (Fig. 2a). Enzyme activity was detectable at 300 h and

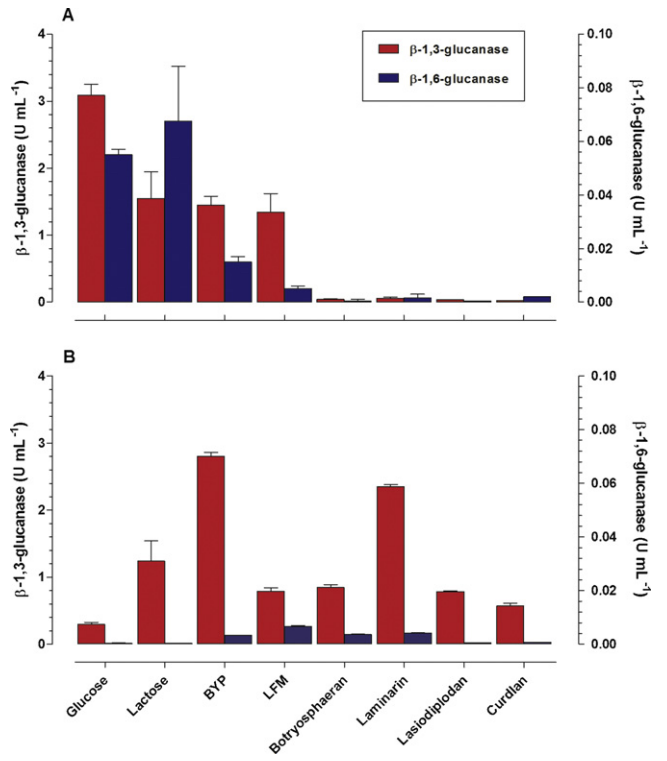

Fig. 3. Effect of different carbon sources on the production of β-1,3-glucanases and β-1,6-glucanases by (a) *Botryosphaeria rhodina*, and (b) *Trichoderma harzianum* grown for 8 days.

appeared stable, not declining with time. For *T. harzianum*, β-1,3-glucanases were secreted principally during the exponential growth phase (Fig. 2b) and reached maximum titres (2.3 U mL^{-1}) at 168 h. The level of residual sugars during the growth period demonstrated an equilibrium between enzymatic hydrolysis of botryosphaeran by the β-1,3-glucanases induced, and consumption of the hydrolysis products. Under the conditions of growth, fungal mycelium and β-1,3-glucanase remained stable.

3.3. Effect of different carbon sources on β-glucanase production

Different carbohydrates can serve as substrates for the production of botryosphaeran by *B. rhodina* [10]. Seven different substrates as carbon sources were compared with glucose for their ability to produce β-glucanases by *B. rhodina* and *T. harzianum* when grown under similar conditions over 8 days (Fig. 3). Both fungal isolates grew on all of the substrates. *B. rhodina* produced higher biomass on lactose (4.7 g L^{-1}) and glucose (3.9 g L^{-1}), whereas the highest amount of biomass produced by *T. harzianum* occurred on glucose (7.88 g L^{-1}) and LFM (7.17 g L^{-1}). Lowest biomass production by both fungi occurred on all of the β-glucans examined. Highest β-1,3- and β-1,6-glucanase activities, respectively, occurred on glucose (3.1 U mL^{-1}, 0.06 U mL^{-1}) and lactose (1.5 U mL^{-1}, 0.07 U mL^{-1}) for *B. rhodina*. At 8 days growth, glucose in the medium was near-limiting (Fig. 1a), and enzyme synthesis was catabolically de-repressed under these conditions, with botryosphaeran produced serving to induce β-glucanase formation. *B. rhodina* is known to produce botryosphaeran when cultivated on lactose [10].

Fungal cell wall material (LFM and BYP) was capable of producing high β-glucanase activities (Fig. 3). The cell wall constituents of yeasts (and fungi) comprise 1,3;1,6- and 1,6-linked β-D-glucans [1], and these are also present in the mycelium of *B. rhodina* [12]. Botryosphaeran, laminarin, lasiodiplodan and curdlan, by comparison, served as relatively poor inducers of β-1,3-glucanases in *B. rhodina*.

In *T. harzianum*, highest β-1,3-glucanase activities occurred on LFM (2.8 U mL^{-1}) and laminarin (2.4 U mL^{-1}). Enzyme titres on botryosphaeran (0.8 U mL^{-1}) and BYP (0.7 U mL^{-1}) were somewhat lower, but higher than those for *B. rhodina*. Enzyme induction patterns by the different substrates were significantly different for the two fungal species. Induction of β-1,3-glucanases by substrates such as 1,3-β-D-glucans and microbial cell walls has been extensively described [2]. Low levels of β-1,6-glucanase were produced by *B. rhodina* and *T. harzianum*.

3.4. Evaluation of β-1,3-glucanase production using statistical mixture-design

Both fungi produced β-1,3-glucanases on glucose as well as lactose as carbon source, and a statistical mixture-design was developed to evaluate the best concentration of these sugars, and the effects of adding botryosphaeran (inducer) to nutrient medium containing glucose and lactose on β-1,3-glucanase production by *B. rhodina* and *T. harzianum*. The response was obtained as a function of proportions of each components in the mixture (Table 1). For *B. rhodina*, the quadratic model is given by Eq. (2):

$$Y_1 = \underset{(\pm 0.046)}{\mathbf{2.32}}x_1 + \underset{(\pm 0.046)}{\mathbf{2.24}}x_2 + \underset{(\pm 0.046)}{0.08}x_3 - \underset{(\pm 0.53)}{\mathbf{2.43}}x_1x_2 - \underset{(\pm 0.53)}{0.026}x_1x_3 - \underset{(\pm 0.53)}{0.03}x_2x_3 \quad (5)$$

Standard error estimates are presented in parenthesis directly below their corresponding model coefficients. Bold face coefficients indicate those that are significant at the 95% confidence level. The equation shows that addition of botryosphaeran to the culture medium was not significant to enhance β-1,3-glucanase production by *B. rhodina*. The equation also showed that $x_1 \approx x_2$ indicating that glucose and lactose promoted enzyme production at similar levels. There was an antagonist effect between both of these substrates, which decreased β-1,3-glucanase production. The mean square lack of fit/pure error ratio was 0.0778; much smaller than the $\nu_1 = 1$, $\nu_2 = 7$ and 95% confidence *F*-distribution value (5.59) indicating no significant lack of fit. Furthermore, the mean square regression/residual ratio was 354.31; much larger than the $\nu_1 = 5$, $\nu_2 = 8$ and 95% confidence *F*-distribution value (3.69) showing that the regression was highly significant. The response surface plot is depicted as a contour (Fig. 4a). The maximum enzyme production value is predicted to occur for a pure glucose or pure lactose nutrient medium. An additional run was made to test the predictive ability of the model. The predicted result was 2.3 U mL^{-1} as maximum activity of β-1,3-glucanase using glucose as sole carbon source, and the experimental value obtained for *B. rhodina* was 2.1 U mL^{-1}.

A special cubic model was obtained for *T. harzianum*, which showed that $x_2 > x_1 > x_3$, where lactose promoted higher enzyme production (Eq. (3)).

$$Y_1 = \underset{(\pm 0.102)}{\mathbf{3.85}}x_1 + \underset{(\pm 0.102)}{\mathbf{4.24}}x_2 + \underset{(\pm 0.102)}{\mathbf{2.97}}x_3 - \underset{(\pm 1.49)}{1.99}x_1x_2 + \underset{(\pm 1.49)}{\mathbf{7.09}}x_1x_3 + \underset{(\pm 1.49)}{\mathbf{5.28}}x_2x_3 - \underset{(\pm 10.52)}{\mathbf{45.03}}x_1x_2x_3 \quad (6)$$

A synergic effect existed between botryosphaeran with each of the other substrates, and favorable for β-1,3-glucanase production. Combined effect of all substrates evaluated resulted in a large decrease in β-1,3-glucanase activity. The effect of adding botryosphaeran on lactose or glucose substrates promoted enzyme production under the conditions tested (Fig. 4b). The r^2 value implied 93% of the variability in the observed response

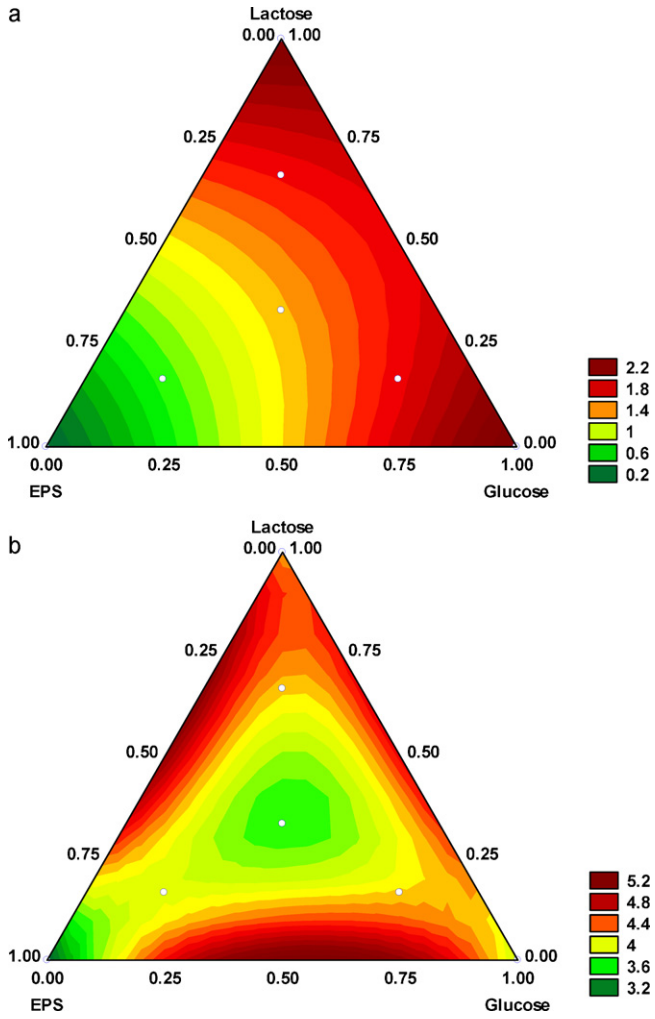

Fig. 4. Response surface contours of β-1,3-glucanase production. (a) Special quadratic model for *Botryosphaeria rhodina* and (b) special cubic model for *Trichoderma harzianum*. EPS is botryosphaeran.

values can be explained by the model. The pure error was low indicating good reproducibility of the experimental data. In conclusion, β-1,3-glucanase production by *T. harzianum* was not repressed by glucose and lactose. The synthesis of β-1,3-glucanases by *B. rhodina* appeared to be regulated by catabolite repression and increased significantly after the growth phase as a function of the presence of botryosphaeran in the nutrient medium.

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