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Fungi Allergens Produced by Solid-State Fermentation Process

Optimization and Allergen Characterization

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Abstract

Allergenic extracts were produced from *Drechslera (Helminthosporium) monoceras* biomass cultured by solid-state fermentation using wheat bran as the substrate. The main fermentation variables were selected by statistical design, and the optimized biomass yield (1.43 mg/[g of dry substrate · d]) was obtained at pH 9.5 and 45.8% moisture. The allergenic extracts were produced from crude extract by protein precipitation and polyphenol removal. Proteins in the range of 16–160 kDa were identified in the extracts. Their reactions in patients were characterized by in vivo cutaneous tests (positive in 40% of the atopic patients) and by dot-blotting assays.

Index Entries: Allergenic extract; *Drechslera (Helminthosporium) monoceras*; solid-state fermentation; statistical experimental design; wheat bran; proteins; biomass.

Introduction

Allergy-related diseases may be considered a worldwide public health problem. Asthma and rhinitis are the most frequent clinical manifestations. Allergenic extracts are of fundamental importance to the diagnosis and therapy of allergies. The spores and mycelium fragments of molds in the atmosphere are considered to be one of the most potent allergenic agents.

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Their allergenic compounds are usually proteins that induce in humans the formation of IgE isotype antibodies when inhaled, swallowed, or injected *(1)*. About 340 genera of molds associated with respiratory allergies are listed in the literature. *Alternaria*, *Cladosporium*, *Aspergillus*, and *Penicillium* are the most extensively studied genera, and their extracts have been characterized and standardized *(2)*.

Previous studies showed intense allergic reactions to *Drechslera (Helminthosporium) monoceras* extracts in asthmatic patients, as measured by cutaneous tests *(2,3)*. These extracts were obtained from the fungi biomass cultured by liquid fermentation. The main antigens were identified as 14.4, 36, and 60 kDa proteins, and these extracts were successfully used in allergy diagnosis *(2,3)*.

D. monoceras is a saprophyte fungi that is found in the soil and is associated with pathogenicity in plants such as maize, oats, wheat, sugarcane, and grasses. This characteristic suggests that solid-state fermentation using agricultural residues as substrates could be a suitable process for saccharification and fermentation, owing to the similarity to its natural habitat. In addition, it could be a less expensive process to produce allergenic extracts on a large scale. Solid-state fermentation can be a powerful process for microorganism growth using agroindustrial residues, and it is more advantageous in many ways than liquid fermentation, especially when yeasts or filamentous fungi are used *(4)*. In recent years, several processes that utilize agroindustrial residues as raw materials for the production of bulk chemicals and value-added products, such as ethanol, single-cell protein, edible mushrooms, enzymes, organic acids, amino acids, and biologically active secondary metabolites, have been reported *(5)*.

This article describes a statistical process optimization for production of *D. monoceras* biomass cultured by solid-state fermentation and presents the characterization of the allergenic extracts obtained.

Materials and Methods

Microorganism and Inoculum

The strain ICBUSP K-1-16, CBS 15426 of *D. monoceras* used was obtained from the culture collection of the Laboratory of Microbiology, Biomedical Science Institute, University of São Paulo, Brazil, and maintained at 25°C on potato dextrose agar submerged in mineral oil. The subculture was made in a 1000-mL Erlenmeyer flask containing slant agar with 4% wheat bran and nutrients in accordance with modification of the Czapeck broth (CB) made by Yunginger et al. *(6)* (2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.01 g of $FeSO_4$, 15 g of maltose, 15 g of dextrose, 10 g of tryptone, 1000 mL of distilled water, pH of solution: 6.8–7.0). The inoculum was obtained from 14-d-old colonies through suspension with sterile water of the biomass from the agar surface. Biomass concentration in the inoculum was estimated by absorbance measurements at 550 nm using a standard curve relating absorbance and dry biomass weight.

Kinetic Characterization

Solid-state fermentation was carried out in Raimbault-type fixed-bed columns *(7)*, and the experimental setup was adapted from Moraes *(8)*. Wheat bran was used as the substrate and enriched with nutrients in accordance with the CB composition. The mixture was sterilized for 15 min at 121°C and adjusted to a final pH and moisture content. The medium was inoculated with a predetermined cell mass concentration and distributed among 12 columns (35 mm diameter × 200 mm height). Each column was individually aerated, and the set of columns was submerged in a thermostatic bath at 25°C for temperature control. Fermentation took 12 d, with the removal of a column as a sample every 24 h.

The kinetic behavior of fermentation was characterized by biomass production, sugar depletion, water activity, and moisture contents. Biomass concentration was determined by protein dosage using the Bradford method *(9)*, and the total reducing sugars by dinitrosalicylic acid reagent *(10)*. Both analyses were conducted with the crude extract obtained from the fermented solid. Yield was calculated at 7 d of fermentation and expressed as a ratio of milligrams of protein produced per gram of dry substrate per day (mg/[g of dry substrate · d]). The efficiency of glucose conversion in the biomass was estimated by the yield coefficient, $Y_{X/S}$ (mg of protein/mg of glucose).

Crude and Allergenic Extracts

Crude extracts were obtained after drying the fermented samples at 37°C for 72 h. Extraction was achieved by the addition of deionized water to solids at a 1:15 (w/v) ratio, adjustment of pH to 9.0, and incubation in a shaker at 30°C for 16 h, according to Saraiva *(11)*. The suspension was filtered and centrifuged at 10,000*g* for 10 min.

The allergenic extracts were prepared from the supernatant crude extract by protein precipitation and removal of salts, polyphenols, and other interfering compounds. Proteins were precipitated using ethanol (70% final concentration) or saturated ammonium sulfate and shaken for 1 h at 0°C. After removal of the supernatant the precipitate was dissolved in 20 m*M* phosphate buffer (pH 7.0) and centrifuged for more efficient separation of insoluble solids. The protein solution was desalted using Sephadex G-25 M columns. The eluted proteins were then dialyzed (3500 mol wt cutoff membrane) in 20 m*M* phosphate buffer (pH 7.0) for 48 h with three changes of the buffer to remove polyphenols and other interfering compounds. Polyphenols were determined using a spectrophotometric method and a catechin standard curve, as described by Price and Butler *(12)*.

Optimization Strategies

Optimal operating conditions for biomass production by solid-state fermentation were determined using statistical experimental design and the surface response analysis methodology *(13)*. Initial moisture (*M*) and

pH of medium, inoculum concentration (*Co*), substrate particle size (*dp*), and airflow rate (*F*) were the variables studied. The experimental response was the biomass yield obtained after 7 d of fermentation and expressed in terms of total protein per gram of dry substrate (mg/[g of substrate · d]).

First, the influence of the variables on biomass production was evaluated using a two-level fractional factorial design, 2^{5-2}. The variables were tested at the levels –1, 0, and +1, which correspond to pH 7.0, 9.0 and 11.0, *M* = 40, 50, and 60%; *F* = 1, 2, and 3 L/h; *dp* = 0.35, 0.59, and 0.84 mm; and *Co* = 0.2, 0.4, and 0.6 g/L, respectively. Eight runs were performed according to the 2^{5-2} design (levels –1 and +1), and three runs were added to the central point (level 0).

The strategy for optimization of the most significant variables included an initial central composite design, an evaluation of the maximum slope trajectory, and a second central composite design of the experiments. The software STATISTICA (version 5.5) was used for calculating the effects of variables, statistical models, and analysis of variance (ANOVA).

Characterization of Allergenic Extracts

Proteins of high and low molecular mass were identified in the allergenic extracts by soldium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) *(14–16)*, using 10 and 15% polyacrylamide gels, respectively. Protein concentration was determined using the Bradford method *(9)*. Antigenicity of extracts was evaluated by cutaneous prick tests, and the most reactive human sera were collected for dot-blotting analysis.

Results and Discussion

Effects of Process Variables

The statistical effects estimated for the five variables studied are shown in a Pareto chart (Fig. 1). It was observed that the most significant effects on biomass yield were obtained for the pH and *M* variables with a confidence level of 90%.

Optimization of Variables

Initial Central Composite Design

An initial central composite design of experiments was carried out for the main variables pH and *M*. The variables were tested at the levels $-2^{1/2}$, –1, 0, +1, and $+2^{1/2}$, which correspond to pH 5.0, 5.6, 7.0, 8.4, and 9.0, and 30, 33, 40, 47, and 50% for M, respectively. Four runs were performed according to the 2^2 design (levels –1 and +1), three runs to the central point (level 0), and four runs to the star design (levels $-2^{1/2}$ and $+2^{1/2}$) *(13)*. The real values of pH and *M* were calculated from the previously defined coded values (X_1 and X_2), according to Eq. 1 and 2.

$$X_1 = (\text{pH} - 7.0)/1.4 \quad (1)$$

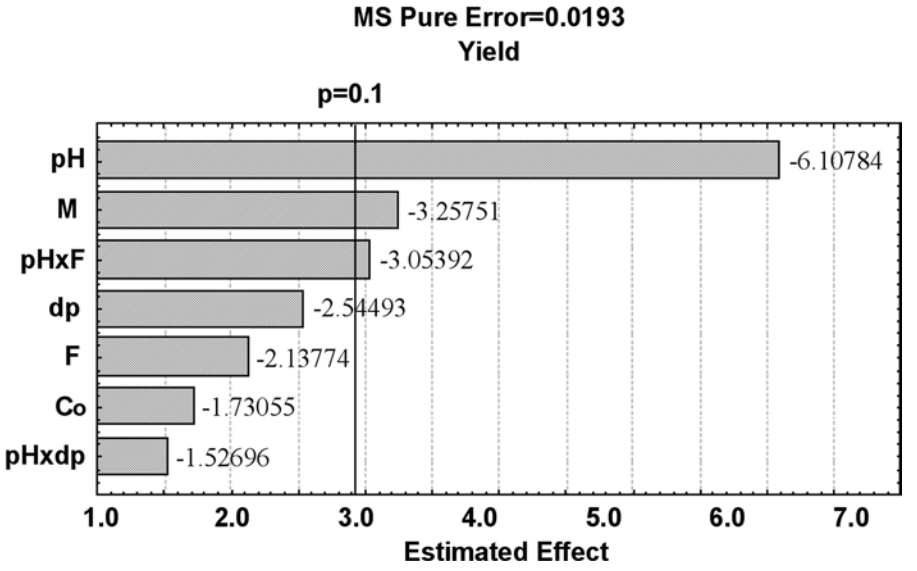

Fig. 1. Pareto chart of effects (at 90% of confidence level) for two-level fractional factorial design (2^{5-2}).

$$X_2 = (M - 40)/7 \quad (2)$$

The biomass yield results obtained for the initial central composite design varied from 0.7 (corresponding to the experiment at $X_1 = -2^{1/2}$, $X_2 = 0$) to 1.56 mg/(g of dry substrate · d) (corresponding to the experiment at $X_1 = +1$, $X_2 = +1$). The values used in the experiments for *F*, *dp*, and *Co* were 2 L/h, 0.59 mm, and 0.4 g/L, respectively. The experimental data for yield as a function of the coded variables pH and *M* (X_1 and X_2) could be better fitted by a linear model (Eq. 3), indicating that yield tends to increase with pH and *M*. The linear model was validated by ANOVA with a confidence level of 95%, and the correlation coefficient value obtained ($R^2 = 0.977$) was considered satisfactory for this kind of experiment:

$$\text{Yield} = 1.187 + 0.21 \cdot X_1 + 0.08 \cdot X_2 + 0.04 \cdot X_1 \cdot X_2 \quad (3)$$

Maximum Slope Trajectory

Making constant values for yield, expressions for X_1 against X_2 were obtained from Eq. 3, which represent straight lines (with the same slope), for each value of yield. From the central point ($X_1 = 0$, $X_2 = 0$) and going perpendicularly to straight lines, the maximum slope trajectory was obtained *(13)*. In algebraic terms, this trajectory can be determined from the ratio of X_2 to X_1 in Eq. 3, which corresponds to 0.38 in this case. Attributing different values to X_2 and X_1, according to this ratio, five experiments were carried out with pHs ranging from 7.8 to 11.2 and *M* from 41.6 to 48%. The highest yield (1.47 mg/[g of dry substrate · d]) was achieved for the experiment corresponding to pH 9.5 and *M* = 44.8%.

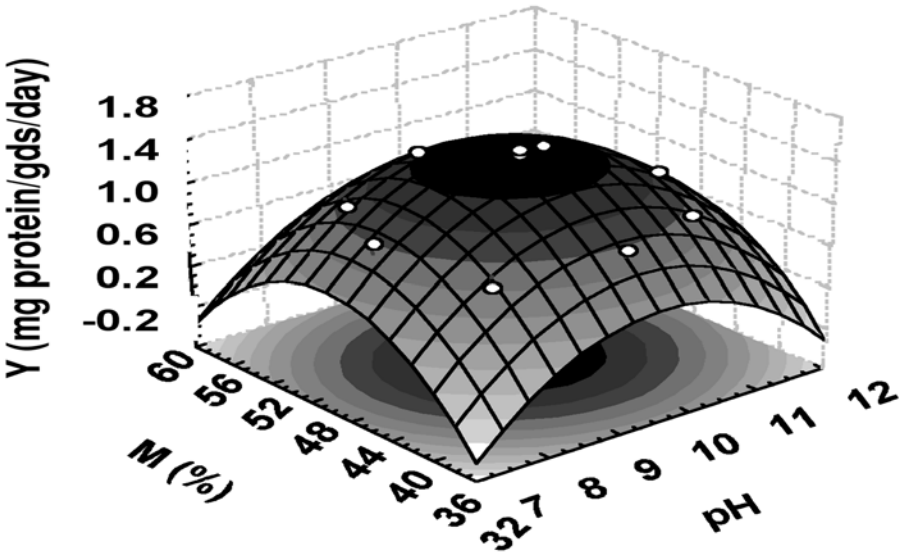

Fig. 2. Response surface for yield obtained from Eq. 6 for real values of pH and *M*.

Second Central Composite Design

Based on the results obtained from evaluation of the maximum slope trajectory, a second central composite design was developed for determination of the optimal region. The variables were tested at the levels $-2^{1/2}$, –1, 0, +1, and $+2^{1/2}$, which correspond to pH 7.5, 8.1, 9.5, 10.9, and 11.5, and 35.1, 38.0, 45.0, 52.0, and 54.9 % for *M*, respectively. A total of 11 runs were performed, similarly to the initial central composite design. The coded values were calculated from Eqs. 4 and 5. The experimental results obtained for yield varied from 0.74 (corresponding to the experiment with $X_1 = -1$, $X_2 = -1$) to 1.46 mg/(g of dry substrate · d) (corresponding to the experiment with $X_1 = 0$, $X_2 = 0$). The experimental data could be fitted with a nonlinear model (Eq. 6) with the coded values of pH and *M*:

$$X_1 = (\text{pH} - 9.5)/1.4 \tag{4}$$

$$X_2 = (\text{M} - 45)/7 \tag{5}$$

$$\text{Yield} = 1.43 - 0.274 \cdot X_{12} + 0.057 \cdot X_2 - 0.241 \cdot X_{22} \tag{6}$$

The model was validated by ANOVA with a confidence level of 95% and experimentally. The correlation coefficient value obtained ($R^2 = 0.954$) was considered satisfactory for this kind of experiment. The results were reproducible under controlled conditions within experimental error for solid state fermentation.

Figure 2 shows the surface response for yield in terms of real values of pH and *M* (calculated from Eq. 4 and 5). Thus, an optimum region was determined with a maximum point of yield (1.43 mg/[g of dry substrate ·d])

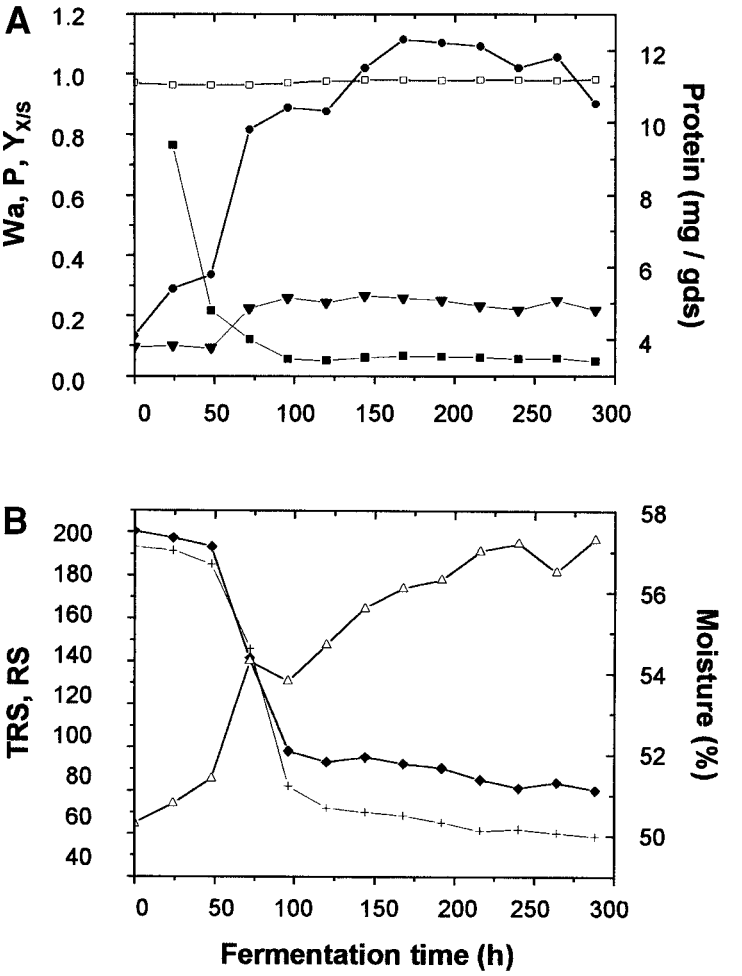

Fig. 3. Time course of solid-state fermentation. (**A**) Water activity (—□—), polyphenols (m*M*, —▼—), $Y_{X/S}$ (mg protein/mg glucose, —■—, and protein (mg/g of dry substrate [gds], —●—); (**B**) total reducing sugars (TRS) (mg glucose/gds, —◆—), reducing sugars (RS) (mg glucose/gds, —+—), and moisture (%, —△—).

obtained at pH 9.5 and 45.8% *M*, which corresponds to coded values of $X_1 = 0$ and $X_2 = 0.116$.

Fermentation Kinetics

Figure 3A, B shows the time course profiles for protein, polyphenols, water activity, conversion efficiency ($Y_{X/S}$), moisture, and sugars for the fermentation carried out under the optimized conditions. The protein profile shows the fungal growth. It may be considered that the log phase starts after 48 h and the conidial phase appears after 192 h. Sugars were consumed during fermentation but were not totally depleted. No significant changes were obtained for water activity and moisture during fer-

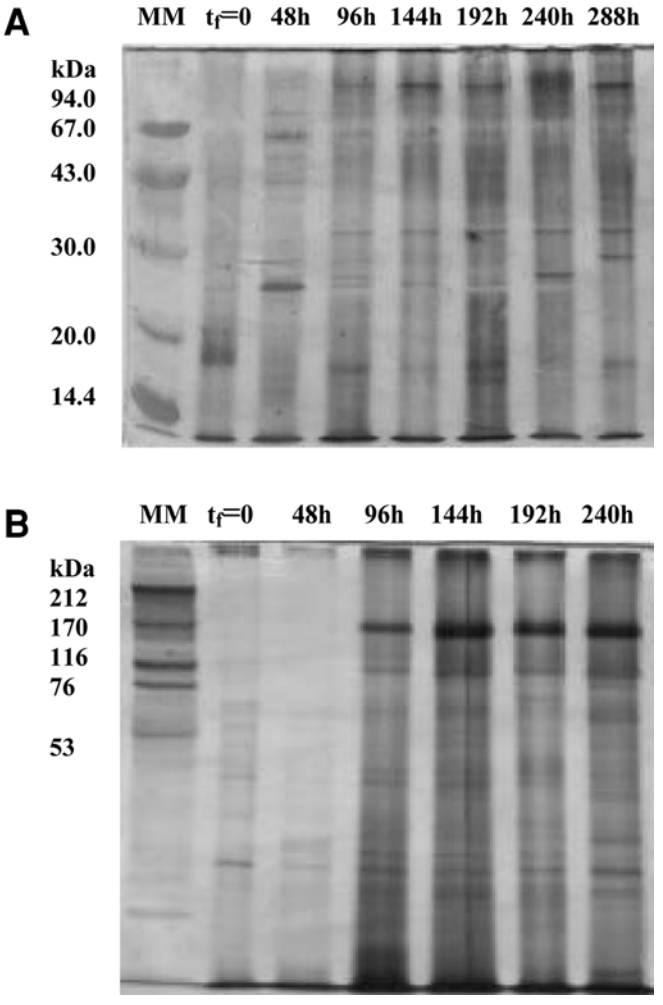

Fig. 4. SDS-PAGE of allergenic extracts, revealed by silver strain. (**A**) Fifteen percent polyacrylamide gel; (**B**) 10% polyacrylamide gel. MM, molecular marker; t_f, fermentation time (h).

mentation, as expected. Polyphenol concentration increased with time in a behavior similar to that of protein concentration. It was also observed that $Y_{X/S}$ decreased during fermentation. These results indicate an oxygen limitation in the system, and, therefore, if maintenance requirements were negligible, the main reduction in the yield parameter is owing to anaerobic conversion. In addition, the increase in polyphenols during fermentation indicates that polyphenols are probably products of anaerobic conversion or maintenance metabolism.

Characterization of Allergenic Extract

Figure 4 shows the SDS-PAGE of extracts obtained with 0, 48, 96, 144, 192, 240, and 288 h fermentation times. The lower molecular mass proteins

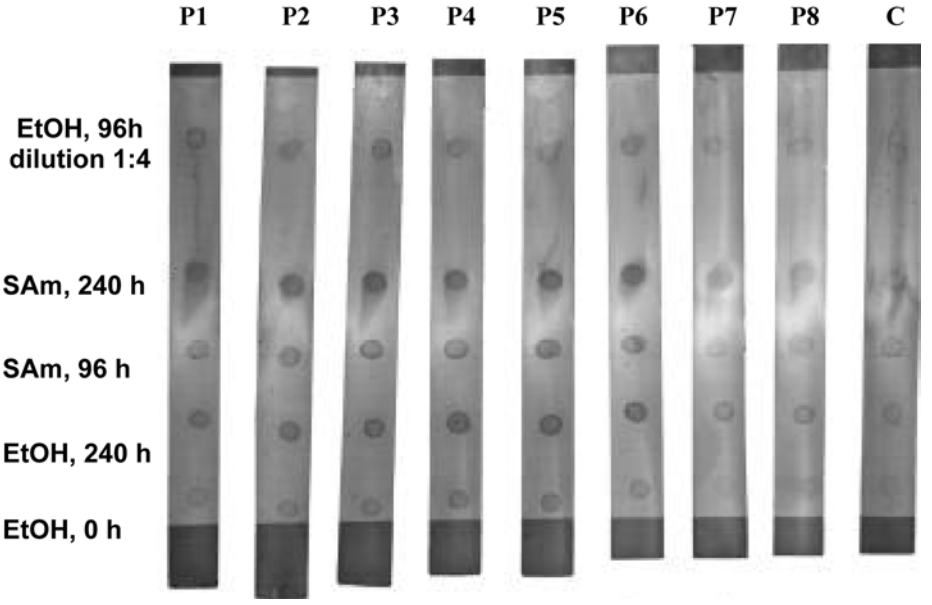

Fig. 5. Dog-blotting analysis of allergenic extracts of *D. onoceras* (extracts obtained by precipitation with EtOH or ammonium sulfate [SAm] at 0, 96, and 240 h of fermentation) for sera of patients (P1–P8) who had positive reactions in skin tests. C, control.

(Fig. 4A) were identified in an approximate range of 16.1–76.4 kDa. The highest molecular mass proteins (Fig. 4B) were in the range of 103.3–157.3 kDa. In both cases, the complete protein spectrum appears after 96 h of fermentation. The pool of proteins obtained in this work includes the molecular masses 14.4, 36.0, and 60 kDa, identified by Menezes et al. *(2)* as being the most allergenic fractions produced by liquid fermentation.

Cutaneous reactivity tests (prick tests) using the allergenic extracts were carried out in 33 atopic patients with asthma symptoms. Medium reactions were observed in 40% of the cases. High reactivity was observed in eight patients, and their sera were collected for dot-blotting analysis. Figure 5 shows the dot-blotting analysis of allergenic extracts of *D. monoceras* for sera of the patients with higher reactivities (P1–P8). Water was used as the control in the experiments. These results showed good agreement with prick tests, and it can be observed that in comparison to the control, patients P1–P6 showed higher reactivity to allergenic extracts.

Conclusion

The production of *D. monoceras* biomass by solid-state fermentation is possible and the allergenic extracts obtained were reactive in cutaneous tests and dot blotting. The optimized process conditions are useful for further scale-up studies. The kinetic behavior of biomass growth suggests

oxygen limitations, and the oxygen supply to the system and mass transfer are important aspects to be studied in further work. The production of allergenic extracts under controlled conditions is reproducible, and the standardized extracts are valuable for the diagnosis and therapy of allergies.

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