

# Allergens of *Curvularia lunata* during cultivation in different media

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**Background:** Despite enormous efforts toward the standardization of fungal extracts, only a few extracts have been characterized that are relevant for the diagnosis and immunotherapy of allergy-associated disorders.

**Objective:** The goal of this study was to determine the optimum growth condition of *Curvularia lunata*, an important fungal allergen for quality raw material, and to analyze the *C. lunata* extract for IgE- and IgG-binding proteins.

**Methods:** *C. lunata* was grown in synthetic (Czapeck Dox medium), semisynthetic (Sabouraud's broth [SB]), and natural media (potato dextrose [PD]) for different periods of time. The extracts were probed for allergenic and antigenic activity, with pooled patient sera and polyclonal antibodies raised against *C. lunata* by means of ELISAs, immunoblots (in vitro), and intradermal tests (in vivo).

**Results:** The growth of *C. lunata* was better in semisynthetic media (ie, SB) compared with other types of media. Dry weight and protein content was maximum in the 7-day culture of SB. ELISA with pooled sera from *C. lunata*-sensitive patients exhibited that cultures grown in SB for 11 to 13 days and PD plus 1.0% agar for 5 days were the most potent. Intradermal tests with 11- to 13-day SB culture extract showed maximum skin reactivity in allergy patients. Immunoblots with patient sera showed 10 to 14 IgE-binding proteins in 5- to 15-day SB cultures. Analysis of 5- to 15-day SB extract with rabbit sera showed 10 to 16 IgG-binding proteins.

**Conclusions:** The extract from 11- to 13-day SB cultures were most biologically potent (intradermal tests) and showed high antigenic and allergenic reactivity (ELISA and immunoblot). The addition of yeast extract did not affect the dry weight and protein content of the *C. lunata* extract. Furthermore, addition of agar in PD medium increases the dry weight and protein content of the fungal mat. Synthetic media was not suitable for mass cultivation of *C. lunata*. (J Allergy Clin Immunol 1999;104:857-62.)

**Key words:** Allergens, *Curvularia lunata*, fungus, standardization, characterization, extract, source material

Airborne fungal spores have been recognized as important triggers of respiratory allergies.<sup>1,2</sup> Aerobiologic surveys show that fungal spores are present in the

## Abbreviations used

|      |                                |
|------|--------------------------------|
| CDM: | Czapeck Dox medium             |
| MW:  | Molecular weight               |
| PD:  | Potato dextrose                |
| PDA: | Potato dextrose plus 1.0% agar |
| SB:  | Sabouraud's broth              |
| YE:  | Yeast extract                  |

atmosphere worldwide. Fungi, such as *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, and *Fusarium* species, belonging to fungi imperfecti have been found to be largely responsible for IgE-mediated allergic reactions in predisposed individuals.<sup>3-6</sup> The genus *Curvularia* has been reported to be an important inhalant allergen and also causes respiratory mycoses, such as allergic bronchopulmonary aspergillosis.<sup>1,7-9</sup> Studies from North America<sup>10,11</sup> and the Asian countries<sup>12-15</sup> reported the high prevalence of *Curvularia* species in the atmosphere and sensitivity toward its allergens in atopic subjects. Clinical studies carried out with *Curvularia lunata* extracts in India and abroad exhibited markedly positive skin reactions (2+ to 4+) in 7% to 16% of patients with nasobronchial allergy.<sup>1,10,11</sup> A recent report from Thailand showed sensitivity to *C. lunata* in 26% of patients with allergic rhinitis.<sup>14</sup>

Aqueous allergen extracts prepared from the fungal raw materials are heterogenous in nature and vary in their potency and composition.<sup>16,17</sup> The composition of allergen extracts depends mainly on the source materials, extraction procedures, and storage conditions.<sup>18-20</sup> However, the extracts made from undefined raw materials have been used for many years for the diagnosis and immunotherapy of type I allergic disorders. In recent years considerable emphasis has been placed on standardization of allergenic extracts to maintain batch-to-batch consistency in allergen content and biologic potency. For this reason, intensive studies of growth characteristics during cultivation of allergenically important fungi are required to obtain adequate source material followed by allergen isolation and characterization.<sup>21-24</sup> Fungal growth is affected by incubation temperature, nutrient media, time period of cultivation, and the type of culture conditions (ie, stationary or shake culture). Conditions for optimal growth and sporulation were reported to differ within and among fungal species.<sup>24-26</sup> Potency of the allergen extracts may change by altering the above parameters. This study was therefore undertaken to study the growth pattern of *C. lunata* in 7 different nutrient

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media. The extracts generated from media cultures on different days were characterized for their biochemical properties and immunologic activity.

## METHODS

### Cultivation of *C lunata*

Pure culture of *C lunata* (No. 4624) was obtained from the Indian Agricultural Research Institute (Delhi, India). It was subcultured in 2% agar slants in potato dextrose (PD) media and incubated at 28°C until sufficient sporulation occurred. *C lunata* was cultivated in Czapeck Dox medium (CDM), Sabouraud's broth (SB), SB plus 50 mg of yeast extract (YE), SB plus 100 mg of YE, PD, PD plus 0.5% agar, and PD plus 1.0% agar (PDA). Erlenmeyer flasks (500 mL) containing 100 mL of medium were inoculated, each with 2 mL of spore-mycelial suspension (approximately 2500 spores) and incubated under stationary conditions at 28°C. The spore-mycelial mass (fungal mat) was collected every alternate day from the fifth day onward (5, 7, 9, 11, and 23 days) for measuring dry weight, protein content, and carbohydrate content and for immunobiochemical studies. The cultures grown in SB were designated as SB5 (fifth-day extract), SB7 (seventh-day extract), SB9 (ninth-day extract), SB11 (11th-day extract), SB13 (13th-day extract), and SB15 (15th-day extract), whereas PDA cultures were named as P5 (fifth-day extract), P7 (seventh-day extract), P9 (ninth-day extract), and P11 (11th-day extract). The pH of the medium was measured after removing the spore-mycelial mass.

### Harvesting of fungal mat and preparation of extracts

The mat was washed with distilled water to remove the medium and freeze-dried, and the dry weight was determined. The allergens were extracted at a 1:20 dilution (wt/vol) in 50 mmol/L  $\text{NH}_4\text{HCO}_3$  containing 1 mmol/L phenyl methyl sulfonyl fluoride and 2 mmol/L EDTA at 4°C for 4 hours. The extracted material was lyophilized, stored at -20°C, and used for in vivo and in vitro experiments.

### Protein and carbohydrate estimation

Protein content of the extracts was estimated by the method of Lowry et al<sup>27</sup> after phosphotungstic acid precipitation.<sup>28</sup> Carbohydrate content was determined in extracts by the phenol-sulphuric acid method.<sup>29</sup>

### Skin tests and collection of patient sera

To determine the biologic potency of extracts prepared from different days' cultures, intradermal tests were performed on patients with nasobronchial allergy at the V. P. Chest Institute (Delhi, India). For skin tests, the lyophilized extracts were reconstituted at 1:500 dilution (wt/vol) in PBS and filtered through 0.22- $\mu\text{m}$  filters. About 0.02 mL of each extract was injected intradermally into the forearm of patients, raising a bleb of 2 mm at the injection site. PBS was injected as a negative control, and histamine diphosphate (100  $\mu\text{g}/\text{mL}$ ) was injected as a positive control. The skin test responses were graded after 20 minutes on the basis of the wheal size produced in comparison with that produced by the negative control. The sera of patients with positive intradermal test responses (wheal size more than  $10 \times 10 \text{ mm}^2$ ) to *C lunata* extract were collected with their consent. Sera from some non-*C lunata*-sensitized persons were also collected to be used as negative controls.

### Rabbit anti-*C lunata* sera

Antisera to *C lunata* (rabbit antibodies) were produced by immunizing New Zealand White rabbits. About 100  $\mu\text{g}$  in 0.25 mL of extract was mixed with an equal amount of CFA. The emulsion thus

obtained was injected intramuscularly at 5 to 6 different sites. Four weeks after immunization, the animals were boosted with the same concentration of protein in incomplete Freund's adjuvant. After 4 secondary boosters, the animal was bled from the ear vein, and the blood was collected. The serum was separated and used for ELISA and immunoblot. The immunization protocol and handling of animals was approved by the institute's animal care and use committee.

### ELISA

To evaluate the potency of culture extract obtained on different days, ELISA was performed with pooled patient sera positive to *C lunata* according to the method of Voller et al.<sup>30</sup> Briefly, each well of the microtiter plate was coated with 1  $\mu\text{g}$  of *C lunata* protein in 100  $\mu\text{L}$  of carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS-0.1% Tween 20, the free sites were blocked with 3% BSA for 1 hour at room temperature. This was followed by incubation with 100  $\mu\text{L}$  of patient sera (1:10 vol/vol dilution) at 4°C overnight. The plate was washed again and incubated for 4 hours at room temperature with a 1:1000 dilution (vol/vol) of anti-human-IgE peroxidase (Sigma). The color was developed with *o*-phenylenediaminobenzidine. The reaction was stopped after 40 minutes and read at 490 nm in an ELISA reader (Dynatech).

### Protein profile of *C lunata* extracts by SDS-PAGE

An amount of 100  $\mu\text{g}$  of protein of extract each day from SB culture of *C lunata* was resolved on 10% SDS-PAGE.<sup>31</sup> The separated proteins were stained with Coomassie brilliant blue and destained.

### Immunoblot with rabbit-human antisera

SDS-PAGE-separated proteins were electrophoretically transferred to nitrocellulose membrane in Tris-glycine buffer (pH 8.0; 25 mmol/L Tris and 192 mmol/L glycine) containing 25% methanol for 4 hours at 150 mA. The strips were cut out and blocked with 3% BSA for 1 hour at room temperature. The strips were washed with PBS-Tween 20 three times and incubated overnight with 1 mL of patient sera (dilution 1:5, vol/vol) or anti-*C lunata* rabbit antibodies (dilution 1:2000, vol/vol). After washing with PBS-Tween 20, allergenic-antigenic proteins were detected by using anti-human IgE-peroxidase (1:1000 vol/vol, Sigma) or goat anti-rabbit IgG peroxidase (1:2000 vol/vol, Sigma). The bands were visualized by using diaminobenzidine (1 mg/mL) and hydrogen peroxide (0.3%) in 0.05 mol/L acetate buffer (pH 5.0).

## RESULTS

*C lunata* was cultivated in CDM (synthetic media), SB, SB plus 50 mg of YE, SB plus 100 mg of YE (semi-synthetic media), PD, PD plus 0.5% agar, and PDA (natural media) to discover a suitable media and conditions for optimum yield of raw material with high allergenic-antigenic content. The dry weight of the fungal mat in SB, SB plus 50 mg of YE, PDA, PD, and PD plus 0.5% agar media was maximal on day 7, whereas in SB plus 100 mg of YE, it was maximal on day 11 (Fig 1, A). The yield of mycelial-spore mass in PDA culture was relatively less than that found in SB, SB plus 50 mg of YE, and SB plus 100 mg of YE (Fig 1, A). The dry weight of fungal mat in CDM was greater on day 13, but the growth of *C lunata* was extremely poor in CDM compared with other media. Hence further studies with CDM were not performed.

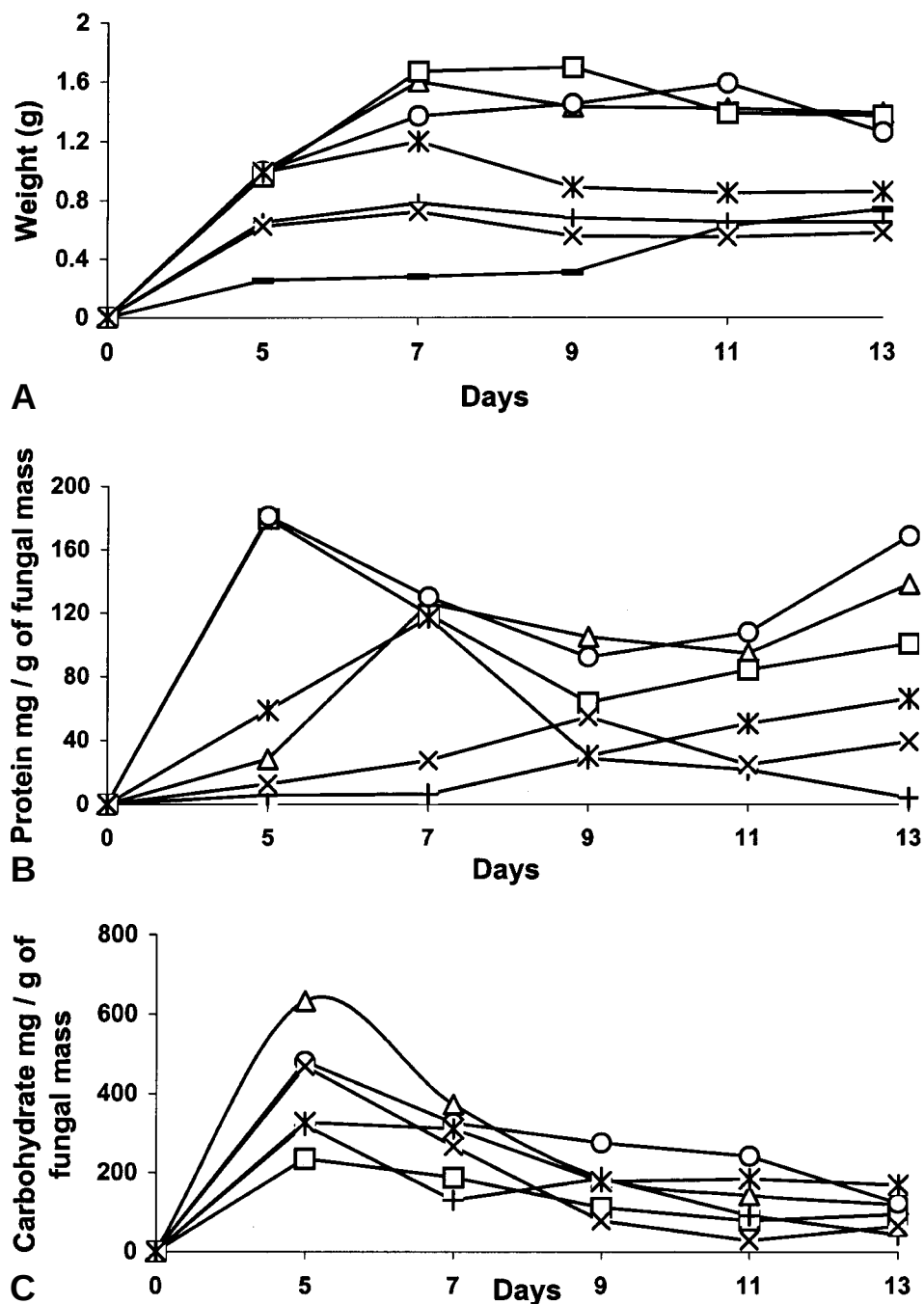


**FIG 1.** Characteristics of *C. lunata* grown for different time periods (5-13 days) in synthetic (CDM), semisynthetic (SB), and natural media (PD). Measurements shown are for dry weight (**A**), protein content (**B**), and carbohydrate content (**C**). Bars, CDM; triangles, SB; boxes, SB plus 50 mg of YE; circles, SB plus 100 mg of YE; plus signs, PD; x, PD plus 0.5% agar; and asterisks, PDA.

The maximum protein content was observed on day 5 in SB plus 50 mg of YE and 100 mg of YE cultures, whereas in SB and PDA, it was obtained on day 7. The protein content was maximal on day-9 culture of PD and PD plus 0.5% agar media; however, it was less compared than that found with SB and PDA (Fig 1, B). The total carbohydrate content in the fungal mat was greater dur-

ing the initial growth phase (ie, until day 8) and then it decreased gradually (Fig 1, C). There was decrease in pH from 6.5 to 4.3 from day 5 to day 11 in the culture filtrate of SB, SB plus 50 mg of YE, and SB plus 100 mg of YE (data not shown).

Cultures SB5 through SB15 and P5 through P11 were selected for further studies on the basis of their higher

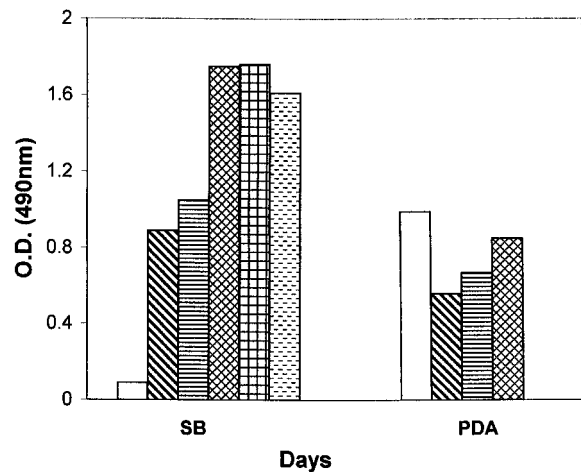


**FIG 2.** IgE-specific ELISA with pooled patient's sera (1:10 dilution) with *C. lunata* in SB (5-15 days) and PDA (5-11 days) extracts. Open boxes, Fifth day; hatched boxes, seventh day; boxes with horizontal lines, ninth day; boxes with crossed lines, 11th day; checkered boxes, 13th day; and boxes with dashes, 15th day.

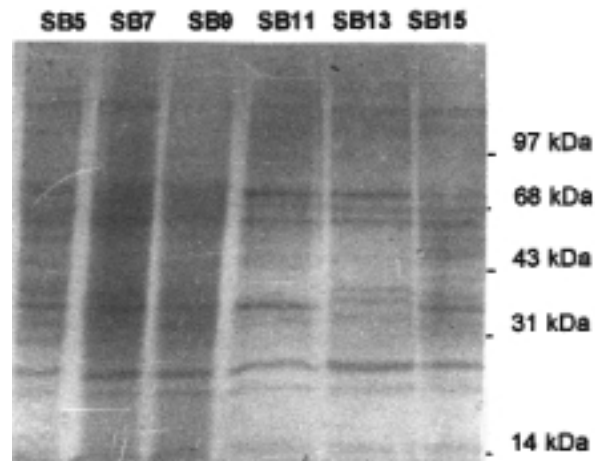


**FIG 4.** Immunoblot analysis of different days' extracts of *C. lunata* grown in SB exhibiting IgE-binding components determined by using pooled sera from patients allergic to *C. lunata* (1:5 dilution) in SB5 (fifth day extract in SB) through SB15 (15th day extract in SB) extracts.

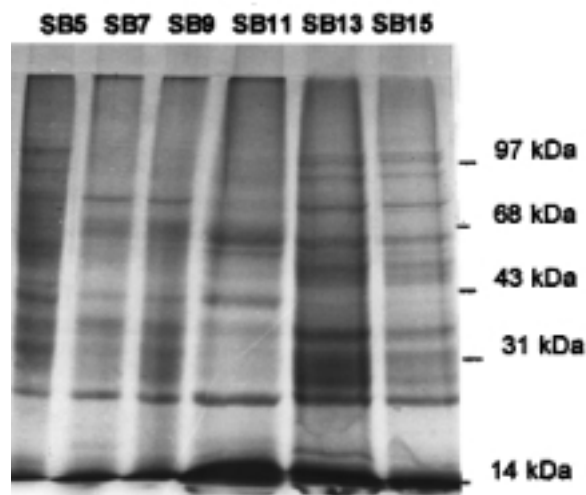


**FIG 3.** SDS-PAGE pattern of different days' extracts of *C. lunata* grown in SB in SB5 (fifth day extract in SB) through SB15 (15th day extract in SB) extracts.

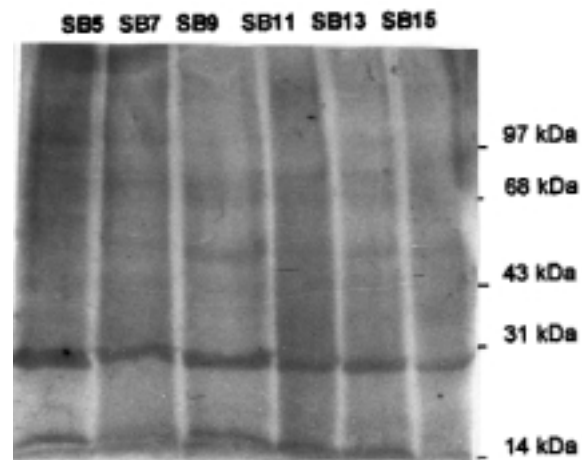


**FIG 5.** Immunoblot analysis of different days' extracts of *C. lunata* grown in SB exhibiting IgG-binding components determined by using anti-*C. lunata* rabbit sera (1:2000 dilution) in SB5 (fifth day extract in SB) through SB15 (15th day extract in SB) extracts.

protein content and dry weight. To investigate the allergenicity of spore-mycelial extract, an IgE-specific ELISA was performed by using pooled patient sera (ie, positive intradermal test response to *C. lunata*; Fig 2). SB11 and SB13 extracts from SB cultures showed maximum IgE binding by ELISA (OD, 1.75). In the case of PDA cultures, P5 and P11 extracts showed high reactivity (OD, 0.99 and 0.85, respectively). For in vivo screening of these extracts, intradermal tests were performed on 41 patients with nasobronchial allergy. Mild positive skin reactions were frequent with all the extracts tested. However, SB11 and SB13 extracts showed severe skin reactivity (2+ to 4+) in a greater number of patients (4 [9.7%]) than other extracts of SB and PDA cultures.

Extracts such as P5, P7, and P9 caused 2+ skin reactivity in one patient each. SB cultures exhibited greater potency than PDA cultures, and therefore the allergenic-antigenic activity of SB extracts were studied.

Fig 3 shows the SDS-PAGE profile of *C. lunata* extracts prepared from SB cultured on different days. *C. lunata* extracts from SB cultures (SB5-SB15) resolved into 17 to 21 stained protein bands, whereas cultures grown in PDA showed 12 to 16 protein bands (data not shown). The bands with molecular weights (MWs) of 15, 23, 26, 30, 33, 41, 45, 53, 61, 65, and 70 kd were prominent in extracts of 5- to 15-day SB cultures. To determine IgE reactivity of SDS-PAGE-separated *C. lunata* proteins, immunoblot analysis with pooled patient sera was

performed. Western blotting revealed 10 to 14 allergenic proteins in SB5 through SB15 extracts (Fig 4). A number of allergenic components with MWs of 15, 26, 30, 33, 38, 45, 58, and 65 kd were found to be common in these extracts. However, a 26-kd protein was observed as the most prominent band in SB extracts.

The antigenic profile of the *C lunata* extracts in SB and PDA was investigated by specific IgG immunoblot with sera raised in rabbits. A total of 10 to 16 IgG-binding proteins were visible in SB5 through SB15 extracts (Fig 5), whereas PDA extracts showed 5 to 13 antigenic bands (data not shown). The protein component with MWs of 15, 23, 26, 30, 33, 38, 41, 45, 48, 50, 61, and 70 kd showed IgG reactivity in all the extracts.

## DISCUSSION

In the last 2 decades considerable progress has been made to standardize some allergenic extracts and to prepare in-house reference standards.<sup>16,17,23,32</sup> However, most of the fungal extracts available for diagnosis and immunotherapy need standardization of source material for their allergen content and biologic potency by adequate in vivo and in vitro methods. This study was undertaken to standardize the growth conditions of *C lunata*, an important fungal allergen, to obtain quality raw material for preparing extracts with high IgE-binding activity, which is useful in diagnosis of allergy to this fungus.

To discover the optimum growth conditions, *C lunata* was cultivated in 3 types of media: synthetic, semisynthetic, and natural media. Analysis of the growth characteristics of *C lunata* revealed that the composition of culture media and duration (time period) affects considerably the quality and quantity of raw materials. The growth of *C lunata* in pure synthetic medium was poor, as measured by dry weight and protein content (Fig 1, A and B). Vander Heide et al<sup>33</sup> and Dixit et al<sup>34</sup> have demonstrated that the growth of fungi, such as *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, and *Epicoccum* species, was relatively impaired in synthetic media, resulting in lower yields compared with semisynthetic medium or natural medium. The addition of YE has been reported to improve the growth and increase the metabolism and yield of other fungi, such as *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria* species.<sup>33</sup> Also, *Aspergillus fumigatus* cultures grown without YE remained in the early growth phase and were not able to complete the growth cycle.<sup>33</sup> In our study the addition of YE in SB showed no significant effect on the growth of *C lunata*. During the initial growth period (days 5-7), the culture filtrate turned acidic, and the total protein content was high in the fungal mat. This could be due to the active secretion of several enzymes and proteins during the active-growth phase. The carbohydrate content was also high in 5- to 7-day cultures. The later growth period (days 9-11) showed a slight increase in pH of the culture filtrate, an increase in protein content, and a decrease in carbohydrate content. Between the 2 phases, there was little change in pH and a decrease in protein/carbohydrate content. Early cultures of

*Aspergillus* and *Penicillium* species have also been reported to exhibit higher protein/carbohydrate content than the older cultures.<sup>33</sup>

Among the natural media tested, PDA cultures showed maximum yield and protein content. The cultures grown in PD plus 0.5% agar and PD alone exhibited less growth and were therefore not selected for further studies. The data exhibited an increase in protein content with increase in agar concentration. Dixit et al<sup>34</sup> have also demonstrated PDA as an optimum growth medium that facilitates spore formation in *Epicoccum nigrum*.

Studies on *Aspergillus*, *Alternaria*, *Cladosporium*, and *Fusarium* species have shown the presence of inhalant allergens both in spores and mycelia.<sup>33-35</sup> The present study was carried out with the spore-mycelial mass (fungal mat) antigens or allergens because it was difficult to separate spores from mycelium. Moreover, the patients are exposed to spore and mycelial antigens alike, and thus both can act as inhalant allergens. The potency of extract from different days was evaluated by ELISA, and SB9 through SB15 cultures grown in SB showed higher IgE binding than other extracts. The PDA culture showed maximum IgE-specific binding with the P5 extract. Intradermal tests on allergy patients revealed more skin reactivity (2+ to 4+) with SB11 or SB13 extracts than with extracts prepared from other culture. SB5 through SB11 extracts showed high potency with ELISA and intradermal tests and was used for further studies. Other workers have also assessed the potency of different fungal extracts by means of ELISAs and skin tests.<sup>36-37</sup>

SB5 through SB15 extracts on SDS-PAGE showed proteins with MWs of 15, 26, 33, 45, 65, and 70 kd as the prominent bands. A band of 33-kd protein was intense after the 11th day. The cultures in SB from days 11 to 15 showed better resolutions on SDS-PAGE. A report on *E nigrum* showed 21 and 22 bands from mycelium and spore extracts, respectively, in the range of 16 to 110 kd. A *fumigatus* culture filtrate extracts exhibited proteins in the molecular range of 12 to 100 kd, with prominent bands at 70, 50, 34, 28, and 14 kd.<sup>38</sup>

Antigenic-allergenic activity of various *C lunata* extracts was investigated with anti-*C lunata* rabbit sera and *C lunata*-sensitive patient sera, respectively. SB5 through SB15 extracts from SB cultures showed greater potency, as determined by both in vivo (intradermal test) and in vitro (ELISA) tests, and therefore allergenic profile of the same was checked with pooled patient sera. Immunoblot analysis revealed 10 to 14 allergenic proteins in SB5 through SB15 extracts with MWs of 15, 23, 26, 30, 33, 38, 41, 45, 53, 61, 65, and 70 kd. A protein of 26 kd was found to be highly immunoreactive and present in all the patient sera tested (data not shown). Verma and Gangal<sup>35</sup> have reported 12 to 18 IgE-binding proteins from spore, mycelial, and culture filtrate extracts of *Fusarium solani*. *Alternaria* extract was reported to contain 30 IgE-binding components, and 4 protein components (85, 56, 42, and 31 kd) bind IgE in sera of more than 50% of patients. A 31-kd protein exhibited IgE antibodies in 95% of the tested patients.<sup>39</sup> Aukrust and

Borch<sup>40</sup> reported a 13-kd major allergen and a 25-kd intermediate allergen from *Cladosporium herbarum*.

SB5 through SB15 extracts showed 10 to 16 IgG-binding proteins (MWs of 15, 23, 26, 33, 38, 41, 45, 53, 61, 65, and 75 kd). A 26-kd protein component was visualized as a strong band on immunoblot with anti-*C lunata* antibodies in all the extracts. Second- and third-phase (day 9 onwards) cultures in SB media exhibited more IgG-binding proteins. Vander Heide et al<sup>33</sup> have demonstrated more antigenic material in cultures from day 20 than from early culture (day 5) for *A fumigatus*, *C herbarum*, *P notatum*, and *A alternata*.<sup>33</sup> A study on *F solani* (11-day culture) exhibited 9 to 37 antigenic bands in spore, mycelium, and culture filtrate extract,<sup>35</sup> whereas Longbottom<sup>5</sup> showed 15 IgG-binding proteins in *A fumigatus* from day-5 culture. In *E nigrum* (7-10 day culture), of 44 SDS-PAGE bands, 38 to 40 bands were reactive to patient sera.<sup>34</sup>

In conclusion, *C lunata* grown for at least 11 days in SB yields better source material with high biologic potency. The extract (SB11-SB13) contains most of the allergenic-antigenic proteins and hence can be selected for diagnostic and therapeutic uses in *C lunata* allergies.

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## REFERENCES

- Agarwal MK, Shivpuri DN. Fungal spores—their role in respiratory allergy. *Adv Pollen Spore Res* 1974;1:78-128.
- Salvaggio J, Aukrust L. Mold induced asthma. *J Allergy Clin Immunol* 1981;68:327-46.
- Aukrust L, Aas K. The diagnosis of immediate type allergy to *Cladosporium herbarum*. *Allergy* 1978;33:24-9.
- Kleine-Tebbe J, Worm M, Jeep S, Matthies F, Lowenstein H, Kunkel G. Predominance of the major allergen (Alt a 1) in *Alternaria* sensitized patients. *Clin Exp Allergy* 1993;23:211-8.
- Longbottom J. Identification of antigenic components reacting with both IgG and IgE Ab of patients with allergic bronchopulmonary Aspergilliosis. *Clin Exp Allergy* 1983;53:354-62.
- Horner WE, Helbling A, Salvaggio JE, Lehrer SB. Fungal allergens. *Clin Microbiol Rev* 1995;8:161-79.
- Elliot MW, Taylor AJ. Allergic bronchopulmonary aspergillosis. *Clin Exp Allergy* 1997;27:55-9.
- Lake FR, Froudast JH, McAleer R, Gillon RL, Tribe AE, Thompson PJ. Allergic bronchopulmonary fungal disease caused by *Bipolaris* and *Curvularia*. *Aust N J Med* 1991;21:871-4.
- Travis WD, Kwon-Chung KJ, Kleiner DE, Geber A, Lawson W, Pass HI. Unusual aspects of allergic bronchopulmonary fungal disease: report of two cases due to *Curvularia* organisms associated with allergic fungal sinusitis. *Hum Pathol* 1991;22:240-8.
- Galant S, Berger W, Gillman S, Goldsobel A, Incaudo G, Kanter L. Prevalence of sensitization to aeroallergens in California patients with respiratory allergy. *Ann Allergy Asthma Immunol* 1998;8:203-10.
- Corey JP, Kalseruddin S, Gungor A. Prevalence of a mould-specific immunoglobulins in a Midwestern allergy practice. *Otolaryngol Head Neck Surg* 1997;117:516-20.
- Singh BP, Mukerjee PK, Nath P. Allergic significance of air borne fungal spores of allergy patient's residences. *Proc Indian Natl Sci Acad* 1981;B-47:78-82.
- Singh BP, Singh AB, Nair PKK, Gangal SV. Survey of airborne pollen and fungal spores at Dehra Dun, India. *Ann Allergy* 1987;59:229-34.
- Pumhirum P, Towiwat P, Mahakit P. Aeroallergen sensitivity of Thai patients with allergic rhinitis. *Asian Pac J Allergy Immunol* 1997;15:183-5.
- Nagendra PKV, Pramila R. Studies on aero-allergens trapped by Boem's personal sampler and their application in sensitivity tests on allergy patients. *Indian J Allergy Appl Immunol* 1998;12:5-11.
- Bush RK, Yunginger JW. Standardization of fungal allergens. *Clin Rev Allergy* 1987;5:3-21.
- Dhillon M. Current status of mould immunotherapy. *Ann Allergy* 1991;66:385-92.
- Martinez J, Torres JM. Characterization of exoantigens of *Aspergillus fumigatus*. Study of factors influencing their quality. *Allergol Immunopathol (Madr)* 1985;13:501-8.
- Esch RE. Allergen source materials and quality control of allergenic extracts. *Methods* 1997;13:2-13.
- Lehrer SB, Salvaggio JE. Allergen: standardization and impact of biotechnology—a review. *Allergy Proc* 1990;11:197-208.
- Everardo AM, Walderez G, Mahasti SM, Dulcinea SPA, Claudete RP, Julio C. Biochemical, antigenic and allergenic characterization of crude extracts of *Drechslera (Helminthosporium) monoceras*. *Mycopathologia* 1995;131:75-81.
- Kauffman HF, Heide SV, Laan SV, Hovenga H, Beaumont F, Vries KD. Standardization of allergenic extracts of *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 1985;76:168-73.
- Savolainen J. A standardized densitometric immunoblotting analysis of *Candida albicans* protein allergens. *Clin Exp Allergy* 1995;25:357-63.
- Vijay HM, Young NM, Jackson GE, White GP, Bernstein IL. Studies on *Alternaria alternata* allergens. V. Comparative biochemical and immunological studies of three isolates of *Alternaria tenuis* cultured on synthetic media. *Int Arch Allergy Appl Immunol* 1985;78:37-42.
- Shafiee A. New synthetic medium for the production of *Alternaria* allergens. *Ann Allergy* 1978;40:220-2.
- Savolainen J, Viander M, Einaasson R, Koivikko A. Allergic variability of different strains of *Candida albicans*. *Int Arch Allergy Appl Immunol* 1989;90:61-6.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
- Singh BP, Sridhara S, Arora N, Gangal SV. Evaluation of protein assay methods for pollen and fungal spore extracts. *Biochem Int* 1992;27:477-84.
- Dubois M, Gillis KA, Hamilton JK, Robers RA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350-6.
- Voller A, Bidwell D, Barlette A. Microplate enzyme immunoassay for the immunodiagnosis of virus infection. In: Rose N, Feldman H, editors. *Manual of clinical immunology*. American Society of Microbiology; 1976. p. 506.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 1970;227:680-5.
- Savolainen J, Kalimo K, Einarsson R, Viander M, Terho EO. In-house reference (IHR) preparations of *Candida albicans* allergen extract. A standardized extraction procedure. *Allergy* 1998;53:359-66.
- vander Heide SV, Kauffman HF, Vries KD. Cultivation of fungi in synthetic and semi-synthetic liquid medium. *Allergy* 1985;40:586-91.
- Dixit AN, Lewis WH, James W. The allergens of *Epicoecum nigrum*. *J Allergy Clin Immunol* 1992;90:11-9.
- Verma J, Gangal SV. *Fusarium solani*: immunochemical characterization of allergens. *Int Arch Allergy Immunol* 1994;104:175-81.
- Corsico R, Cinti B, Feliziani V, Gallesio MT, Liccardi G, Loreti A, et al. Prevalence of sensitization to *Alternaria* in allergic patients in Italy. *Ann Allergy Asthma Immunol* 1998;80:71-6.
- Carol E, O'Neil, Marjorie L, McCants BA, John E, Salvaggio MD, et al. *Fusarium solani*: prevalence of skin reactivity and antigenic/allergenic analysis. *J Allergy Clin Immunol* 1986;77:842-9.
- Banerjee B, Chetty A, Joshi AP, Sarma PU. Identification and partial characterization of diagnostically relevant antigens of *Aspergillus fumigatus*. *Asian Pac J Allergy Immunol* 1990;8:13-8.
- Paris S, Fitting C, Latge JP, Herman D, Guinnee MT, David B. Comparison of conidial and mycelial allergens of *Alternaria alternata*. *Int Arch Allergy Appl Immunol* 1990;92:1-8.
- Aukrust L, Borch SM. Partial purification and characterization of two *Cladosporium herbarum* allergens. *Int Arch Allergy Appl Immunol* 1979;60:68-79.