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EVALUATION OF METHODS FOR SCREENING OF EXTRACELLULAR CELLULASE IN FILAMENTOUS FUNGI

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تقييم طرق مسح نشاط أنزيم السيليوليز خارج الخلوي في بعض الفطريات الخيطية

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الخلاصة

من الواضح أن العديد من الكائنات الحية الدقيقة قادرة على إنتاج الأنزيمات السليلوزية خارج الخلوية. ومن أهمها الفطريات الخيطية المحللة للمخلفات السليلوزية بفعالية أكبر وذلك من خلال معدلاتها الأيضية النشطة. وتعتبر الأنواع التابعة لجنس ترايكوديرما *Trichoderma* من أفضل هذه الكائنات الخيطية. وتناولت الكثير من الأبحاث الأنظمة الأنزيمية السليلوزية حيث ركزت دراستها على الأنواع التابعة لهذا الجنس.

تهدف هذه الدراسة إلى استخدام الثلاث طرق المختلفة الخاصة للمسح السريع للكشف عن الأنشطة الأنزيمية السليلوزية للفطريات وهذه الطرق هي :

فحص انتشار الآجار *Agar difffusion test* ، تقانة المنطقة الشفافة *Cleared - zone Technique* وطريقة آزور السليلوز *Cellulose - azure Method* وأجريت الدراسة على ثمان وعشرين نوعا تابعا لخمسة عشر جنسا من أجناس الفطريات الخيطية من ترب مختلفة في المملكة العربية السعودية.

Key words: Saudi Arabia, Mycoflora Extracellular Cellulase, Screening.

ABSTRACT

Twenty-eight fungal species belonging to fifteen genera were isolated from Saudi Arabian soil and were screened for the production of extracellular cellulase enzymes. Cleared-zone technique, cellulose azure method and agar diffusion test for rapid screening were used. The cleared-zone technique was found to be much less reliable as compared to other methods. *Trichoderma viride, T. pseudokoningii* and *Aspergillus fumigatus* were the best producers of cellulase enzyme. The data obtained also suggest that caution is needed using these methods. The disadvantages of using one method for screening, which has been a common practise up to now, are discussed.

INTRODUCTION

Many microorganisms are capable of producing extracellular cellulases. Important among these are the filamentous fungi which digest cellulosic wastes rapidly through their high metabolic rates. While *Trichoderma* species and their mutants are regarded as the best producers of cellulases to-date (1.7) and much studies have been centred on them, other fungi like *Fusarium* and *Penicillium* species were also reported (8-11). In another direction, manipulation of culture conditions to maximize cellulase production has received much interest (12, 13, 14, 4, 5). Most of these works have focussed on the *Trichoderma* cellulase system, with comparatively fewer reports on other fungal species. Different methods are available for rapid screening of cellulolytic activities of fungi like insoluble cellulose incorporated into the agar medium and the clearing of the semi-opaque cellulose are used as an indication of cellulolytic activity (15). Mineral agar medium overlaid with cellulose-azure (0.05 g) as substrate was also used to screen extracellular cellulase activities and the blue dye is released from the cellulose azure and diffuses into the medium (16). Tan *et al.* (6) reported that fourty strains of filamentous fungi including species of *Trichoderma, Aspergillus, Penicillium* and *Fusarium* secreted extracellular cellulases on cellulose-azure substrate. Twenty-nine fungal species isolated from dung samples of five herbivorous animals collected from arid and semi-arid areas of Iraq were capable of producing cleared zones on cellulose agar medium (17). More recently, Leinhos and Buchenauer (18) used the agar diffusion test to examine cellulolytic activity of *Verticillium chlamydosporium, V. gonioides, V. lecanii, V. psalliotae, V. tenuipes* and *Alternaria alternata.* In the present study cleared-zone technique, cellulose-azure method and agar diffusion test were used to screen extracellular cellulolytic activities of fungi isolated from Saudi Arabian soils (19).

MATERIALS AND METHODS

Chemicals

Carboxymethylcellulose-Na salt (CMC, low viscosity, Fluka, Switzerland), crystalline cellulose (Avicel) and cellulose-azure (Winlad, U.K.) were used for screening cellulolytic activities. All the other chemicals used were of Analar grade.

Screening for Cellulolytic Ability of Fungi Organisms

Thirty isolates of the genera *Aspergillus, Penicillium, Fusarium, Alternaria, Drechslera, Ulocladium, Chaetomium, Curvularia, Trichoderma, Cochliobolus, Rhizopus, Mucor, Phoma, Emericella,* and *Stachybotrys* were screened for extracellular cellulase activities. The fungal species were isolated from Saudi Arabian soil during the period of May-August 1993 (19). Modified Czapek Dox agar medium as described by Abdel-Hafez et al. (20) was selected to isolate fungi.

Cleared-Zone Technique

The medium used for cleared-zone technique contained (g/L), 2 g KH_2PO_4; 1.4 g $(NH_4)_2\ SO_4$; 0.3 g $Mg\ SO_4.\ 7H_2O$; 0.3 g $CaCl_2$; 4 mg $FeSO_4.\ 7H_2O$; 1.6 mg $Mn\ SO_4.\ H_2O$; 1.4 mg $ZnSO_4.\ 7H_2O$; 2 mg $CoCl_2$; 0.8 g peptone; 2 ml Tween 80; 10 g cellulose; and 15 g agar. Urea (0.3 g) was added after autoclaving (21). Mycelial discs of 4 mm diameter cut from the edge of the active colonies were used as inocula. Quadruplicate sets of plates were innoculated for each fungus. The plates were incubated at 28°C for 7 days. At the end of the incubation period the plates were flooded with a mixture of HCl/iodine solution which was prepared by mixing 1 ml 0.1 M HC1 with 5 ml 1% (w/v) I_2 in 2% (w/v) KI (15). A cleared yellow zone around the colony against a dark brown background was considered as a positive test for cellulase.

Agar Diffusion Test

Fungi were grown in 250 ml flasks, each flask receiving 50 ml of the above cellulolytic medium (21) with 1% Avicel as a carbon source. Each flask was inoculated with three 4 mm mycelial discs from 7 day-old cultures and triplicate sets of cultures were prepared for each fungus. Cultures were incubated in an orbital incubator operating at 120 strokes/min at 28°C. After 7 days, the solid fractions (mycelia and residual cellulose) were separated from the culture broth by centrifuging the samples at 4,000 rpm for 10 min. The culture supernatant represented the enzyme source.

For the agar diffusion test, the following medium was used: 1% carboxymethyl cellulose and 2% agar in 0.1 mol/L phosphate buffer (pH 6.0). Enzyme preparations (0.2 ml) were added into agar holes (9 mm diam) and the plates incubated for 30 hr at 28±2°C. Triplicate sets of plates were prepared for each fungus. After incubation, the plates were overlayered with Cu-acetate solution 10% and the radius of the degradation zones was measured (18).

Cellulose-Azure Method

The mineral agar medium plates contained per 100 ml, $(NH_4)_2\ SO_4$ 0.1 g; KH_2PO_4 0.1 g; $MgSO_4\ 7H_2O$ 0.02 g; Difco agar 1.5 g and overlaid with cellulose-azure (0.05 g) as a substrate (16). Fungal cellulolytic activities were assessed on the basis of their ability to utilize cellulose-azure and subsequently releasing the blue azure-dye into the basal mineral agar. The relative cellulolytic activity of each species was scored by comparing the intensity of the blue colour of the mineral medium with a standard blue colour scale of 1 to 10 (maximum) over an incubation period of 10 days. All tests were set up on the same day and periodically inspected for the release of the dye. Three replicates were set up for each isolate and uninoculated medium held under the same conditions served as control.

RESULTS AND DISCUSSION

The objective of these experiments was to determine the cellulolytic ability in isolated fungi. Throughout this study different methods were used for comparative purposes and the tested isolates were screened for extracellular cellulase activities. All the isolated fungi had different abilities to produce extracellular cellulases.

From the cleared-zone technique it was difficult to determine the percent cleared-zone with respect to colony size as an indication of the enzyme activity level. The disadvantage of this method could be observed in the difficulty of observing the clearing zone. Most of the isolates did not show clearance in the visual scoring for strong cellulolytic activity (Table 1). In the cellulose-azure method, the results of the rapid screening for extracellular cellulase activity showed that overall tested fungal isolates secreted the enzyme. Some isolates caused the release of some of the blue dye from the cellulose within 3-4 days but others took considerably longer time. The final results were recorded after 10 days of incubation (Table 2).

Table 1
Cellulolytic activity using cleared-zone technique

Fungal species	IMI Number	Cellulolytic Activity*
Aspergillus fumigatus Fres.	356162	++
A. niger van Tiegh.	356160	++
A. flavus Link	356149	++
A. terreus Thom		++
Alternaria alternata (Fr. :Fr.) Keissler		
Alternaria sp.		
Curvularia sp.		–
Cochliobolus spicifer R.R. Nelson		+
C. lunatus R.R. Nelson & F.A. Haasis	356138	+
Chaetomium sp.	356145	++
Drechslera sp.		++
Emericella nidulans (Eidam) Vuill.		++
Fusarium moniliforme Sheldon	356153	++
F. solani (Martius) Sacc.	356146	+
Mucor sp.	356159	++
Penicillium chrysogenum Thom.	356165	++
P. griseofulvum Dierckx	356166	++
Penicillium sp.		
Phoma sp.		+
Rhizopus sp.		–
Stachybotrys sp.		++
Trichoderma viride Pers. :Fr.		++
T. harzianum Rifai		++
T. koningii Oudem.	356151	++
T. pseudokoningii Rifai	356158	++
Ulocladium atrum Preuss	356164	+
U. septosporum (Preuss) E.G. Simmons	350894	+
Ulocladium sp.	356143	+
	356142	

IMI number = International Mycological Institute Number.
*++ : strong cellulolytic activity; + weak cellulolytic activity;
–; no cellulolytic activity.

Table 2
Intensity of blue azure dye released by fungal isolates on mineral agar medium

Fungal species	Days									
	1	2	3	4	5	6	7	8	9	10
Aspergillus fumigatus Fres.	0	1	4	6	7	8	8	9	10	10
A. niger van Tiegh.	0	0	1	1	2	3	3	4	5	5
A. flavus Link	0	0	1	2	3	4	5	5	6	7
A. terreus Thom	0	0	0	0	1	2	2	3	3	4
Alternaria alternata (Fr. :Fr.) Keissler	0	0	0	0	0	1	1	2	3	3
Alternaria sp.	0	0	0	0	0	1	2	3	4	4
Curvularia sp.	0	0	0	0	0	0	1	2	2	2
Cochliobolus spicifer R.R. Nelson	0	0	0	0	1	1	2	3	4	4
C. lunatus R.R. Nelson & F.A. Haasis	0	0	1	2	3	4	5	5	6	7
Chaetomium sp.	0	0	0	1	2	2	3	4	5	5
Drechslera sp.	0	0	0	1	2	3	3	4	4	4
Emericella nidulans (Eidam) Vuill.	0	0	0	0	1	2	2	3	4	4
Fusarium moniliforme Sheldon	0	0	0	1	2	3	4	4	5	6
F. solani (Martius) Sacc.	0	0	0	0	0	1	2	3	4	4
Mucor sp.	0	0	0	0	0	0	1	1	1	1
Penicillium chrysogenum Thom.	0	0	0	1	1	2	3	3	4	4
P. griseofulvum Dierckx	0	0	0	0	1	2	3	4	4	4
Penicillium sp.	0	0	0	0	1	1	2	3	4	4
Phoma sp.	0	0	0	0	0	0	1	1	2	3
Rhizopus sp.	0	0	0	0	0	0	1	2	1	1
Stachybotrys sp.	0	0	0	0	0	1	1	2	4	6
Trichoderma viride Pers. :Fr.	0	2	3	6	8	10	10	10	10	10
T. harzianum Rifai	0	0	0	4	5	6	7	7	8	8
T. koningii Oudem.	0	0	1	3	4	5	6	7	7	8
T. pseudokoningii Rifai	0	0	4	7	7	8	9	10	10	10
Ulocladium atrum Preuss	0	0	0	0	1	2	3	3	4	4
U. septosporum (Preuss) E.G. Simmons	0	0	0	1	2	3	4	5	6	6
Ulocladium sp.	0	0	0	0	0	1	2	2	3	4

Number 1-10 (maximum) intensity scale of the blue azure dye released from cellulose-azure into basal mineral azure medium through cellulolytic activity of fungal isolates.

Twenty isolates gave moderate results, seven showed moderate to strong activity, with three isolates of *Aspergillus fumigatus, Trichoderma viride* and *T. pseudokoningii* giving the most positive results. None, however, caused as fast a release of the dye as *T. viride* (Table 2). Some typical examples are shown in Fig. 1.

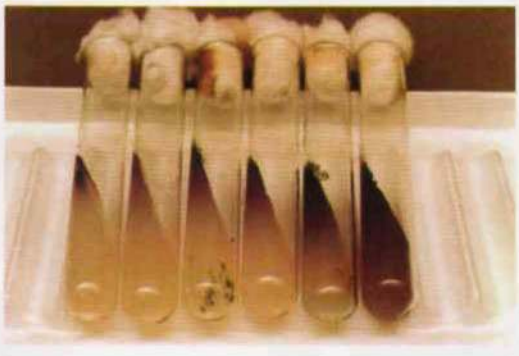

Fig. 1: Intensity of blue azure dye released from cellulose-azure into mineral agar medium through cellulolytic activity of *T. viride*.

All isolates were able to degrade carboxymethyl cellulose (CMC) in agar diffusion tests by production of cellulolytic exoenzyme (Table 3). *Trichoderma viride, T. pseudokoningii, Aspergillus fumigatus* and *A. flavus* produced large lytic zones (7-8 mm in radius) around the fungal colony on CMC agar, and the maximum (10 mm) was seen in T. viride. The other isolates produced smaller lytic zones (2-5 mm) beyond the fungal colonies. Heat-treated enzyme preparations used as controls proved to be inactive in the agar diffusion test.

Table 3

Cellulolytic activity of 7-day-old culture filtrates of fungal isolates
(Agar diffusion test).

Fungal species	IMI Number	Cellulolytic Activity*
Aspergillus fumigatus Fres.	356162	8.0 ± 0.3
A. niger van Tiegh.	356160	4.0 ± 0.2
A. flavus Link	356149	7.0 ± 0.1
A. terreus Thom	–	3.0 ± 0.4
Alternaria alternata (Fr. :Fr.) Keissler	–	2.0 ± 0.3
Alternaria sp.	–	2.0 ± 0.3
Curvularia sp.	–	2.0 ± 0.4
Cochliobolus spicifer R.R. Nelson	356138	4.0 ± 0.8
C. lunatus R.R. Nelson & F.A. Haasis	356145	5.0 ± 0.7
Chaetomium sp.	–	4.0 ± 0.2
Drechslera sp.	–	3.0 ± 0.1
Emericella nidulans (Eidam) Vuill.	356153	4.0 ± 0.5
Fusarium moniliforme Sheldon	356146	5.0 ± 0.1
F. solani (Martius) Sacc.	356159	4.0 ± 0.1
Mucor sp.	–	0.0
Penicillium chrysogenum Thom.	356165	4.0 ± 0.3
P. griseofulvum Dierckx	356166	4.0 ± 0.11
Penicillium sp.	–	2.0 ± 0.3
Phoma sp.	–	0.0
Rhizopus sp.	–	0.0
Stachybotrys sp.	–	4.0 ± 0.7
Trichoderma viride Pers. :Fr.	356151	10.0 ± 1.1
T. harzianum Rifai	356158	5.0 ± 0.3
T. koningii Oudem.	356164	5.0 ± 0.1
T. pseudokoningii Rifai	350894	8.0 ± 0.7
Ulocladium atrum Preuss	356143	4.0 ± 0.3
U. septosporum (Preuss) E.G. Simmons	356142	5.0 ± 1.0
Ulocladium sp.	–	2.0 ± 0.6

*Radius of the lysis halos (mm) with carboxymethylcellulose as enzyme substrate in the agar diffusion assay (n=3); (mean ± SD); IMI number = International Mycological Institute Number.

In the agar diffusion test, only *T. viride, T. pseudokoningii* and *A. fumigatus* exhibited high cellulolytic activity. Also, in cellulose-azure method, *Trichoderma* species were generally the earliest cellulase producers (Table 2) showing observable dye release between 3-4 days after inoculation. Similar observations have also been made (6). The levels of enzyme activity were also high, and the blue intensity at the end of 10 days ranging from 7 to the maximum 10 was seen in *T. viride* and *T. pseudokoningii,* but in *T. koningii* and *T. harzianum* were less than 10. Dye release was slow between 4-5 days after inoculation with *Aspergillus* spp. comparable with *Trichoderma* spp., and only one species of *Aspergillus* (*A. fumigatus*) exhibited maximum cellulase activity of 10 at the end of 10 days of incubation. Although species of *Penicillium* have been widely isolated from nature by the use of cellulose media (22-24), few have been assessed for their cellulolytic ability. Highly cellulolytic species reported including *P. verruculosum* (11) and *P. funiculosum* were a source of commercial production of cellulase (10). However, throughout this study, *Penicillium* spp. were considered as moderately cellulolytic. *Aspergillus flavus, F. moniliforme, U. septosprum, C. lunatus, Chaetomium* sp., *T. harzianum* and *T. koningii* show moderate to strong activities of dye release which were detected slightly between 4-5 days. Other species have also been demonstrated to degrade cellulose-azure, but at a less extent comparable with *Trichoderma viride, T. pseudokoningii* and *A. fumigatus.*

CONCLUSIONS

Cellulolytic enzymes were examined in the agar diffusion test. Although a survey of the extracellular enzyme production by filamentous fungi using the qualitative techniques serves as a convenient way of demonstrating some types of extracellular enzymes in a relatively short time, most enzymes can be detected visually only beyond the periphery of the colony. Enzymes may be produced directly under the fungal colony and their detection is especially difficult. Some others may be bound to the fungal cell wall. Zone clearing in the cellulose Czapek agar plate was indistinct and extremely difficult to identify. So comparison of data suggests that some caution should be made to the use of cellulose agar test medium, otherwise an inaccurate view of enzyme production by certain fungi may result. However, a preliminary study with the isolates of a fungal species revealed that the production of cellulolytic enzymes was induced by cellulose. The isolates may vary in the production of enzymes, while the isolates of *T. viride, T. pseudokoningii* and *A. fumigatus* exhibited consistent high cellulolytic activities.

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