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Extracellular enzyme production by *Rhizopus* and *Mucor* species on solid media

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Solid media were employed to determine the presence and absence of extracellular enzyme production by two genera of fruit-rot fungi, *Rhizopus* and *Mucor*. The results of this investigation revealed that phosphatase was released into the cultural medium by all the fungi examined; however, only *R. oryzae*, *R. tritici*, *M. mucedo*, and *M. piriformis* showed the possibility of being high producers of the enzyme. Protease, urease, ribonuclease, pectate lyase, and polygalacturonase, at varying levels of activity, were detected, in the majority of the fungi, in the cultural medium.

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Des milieux solides ont été utilisés pour déterminer la production ou non d'enzymes extracellulaires chez deux genres de moisissures, *Rhizopus* et *Mucor*. Les résultats obtenus démontrent que la phosphatase est libérée dans le milieu de culture par toutes les moisissures étudiées, cependant, seuls *R. oryzae*, *R. tritici*, *M. mucedo* et *M. piriformis* démontrent qu'ils pourraient produire l'enzyme en quantités élevées. La protéase, l'uréase, la ribonucléase, la pectate lyase et la polygalacturonase ont été détectées, à différents taux d'activité, chez la majorité des moisissures dans le milieu de culture.

[Traduit par le journal]

Introduction

Numerous fungi are known to produce degradative enzymes which are important in the breakdown of organic matter, in host infection, and in food deterioration (Hankin and Anagnostakis 1975). The use of solid media for the detection of enzyme production by fungi has made it possible to rapidly screen large populations of fungi for the absence or presence of specific enzymes (Berkenhamp 1973; Hankin and Anagnostakis 1975; McIntyre and Hankin 1978; Federici 1982). We report here on the ability of two genera of fruit-rot fungi, *Rhizopus* and *Mucor*, to release extracellular enzymes into the cultural medium.

Materials and methods

Species of *Rhizopus* and *Mucor* used in this work were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and the Department of National Health and Welfare (Ottawa, Canada). Stock cultures were maintained at 5°C on slants of potato dextrose agar (PDA).

The semiquantitative tests for enzymatic activities, as described by Federici (1982), were performed on prepoured plates. The plates were inoculated with mycelia plugs from 5-day-old cultures on PDA medium. The mycelia plugs, which were cut with a No. 2 cork borer, were placed in the center of the test media. When not specified, plates were incubated at 27°C for 3–5 days in the dark and tests were done when colonies reached 4–6 mm in diameter.

Deoxyribonuclease activity was detected on DNase test agar (Difco) supplemented with 1% yeast extract (Difco), while RNase production was tested by the method reported by Hankin and Anagnostakis (1975). After incubation the plates were flooded with 1 *N* HCl; DNA and RNA depolymerization were shown by clear zones surrounding the colonies. Amylase production was tested on Bacto nutrient agar (Difco) containing 0.2% soluble starch (Merck) (Hankin and Anagnostakis 1975). After incubation, the plates were flooded with an iodine solution and a yellow halo around the colonies in an otherwise blue medium indicated enzyme production. Urease production was tested on Christensen's agar (Seeliger 1956) and urea hydrolysis was indicated by the appearance of a deep pink color during incubation. Lipolysis was assessed on Sierra's (1957) medium with the use of Tween 60 and 80 (Fisher Scienctific Co.) as substrates. Activity was indicated by the appearance of deposits of calcium salts formed by liberated fatty acids. Pectolytic activity was determined as described by Hankin and Anagnostakis (1975). The medium at pH 7 was used to detect pectate lyase production and at pH 5 to detect polygalacturonase activity. For all tests, plates were flooded with a 1% aqueous solution of hexadecyltrimethylammonium bromide (Fisher Chemical Co.). This reagent precipitates intact pectin in the medium, and thus, clear zones around a colony in anotherwise opaque medium indicated degradation of the pectin. Proteolytic activity was determined as described by Hankin and Anagnostakis (1975). Complete degradation of the gelatin was seen as a clearing in the somewhat opaque agar around colonies. When the plates were flooded with an aqueous saturated solution of ammonium sulfate, a precipitate formed which made the agar more opaque and enhance the clear zones around colonies. Phosphatasic activity was tested on plate count agar (Difco) supplemented with 2% 0.01 *M* phenolphtalein diphosphate, sodium salt (Boehringer Mannheim GmbH), as described by Hankin and Anagnostakis (1975). After incubation the plates were opened and inverted over a container of ammonium hydroxide. Colonies of phosphatase-producing strains turned pink to red.

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TABLE 1. Extracellular enzymes produced by *Rhizopus* and *Mucor* species on solid media

Fungi examined	ATCC No.	Enzymes detected									
		RNase	DNase	Protease	Amylase	Lipase	Phosphatase	Urease	Polygalac-turonase	Pectate lyase	Total no.
Rhizopus arrhizus	6204	+++	++	−	−	+	+	−	++	+	6
R. chinensis	12276	+	−	+	−	−	+	+	+	−	5
R. circinans	1225	−	−	+	+	−	++	−	+	+	5
R. japonicus	24863	−	−	+	+	+	+	+	−	−	5
R. kazanensis	8998	−	−	+	−	−	++	+	+	−	4
R. oryzae	22957	−	−	+	−	−	+++	+	+	−	4
R. pymacus	11559	++	−	+	−	−	+	+	+	−	5
R. stolonifer	12939	+++	−	+	+	−	+	+	+	+	7
R. tritici	1230	−	−	+	−	−	+++	+	−	+	5
R. sp. 66-81-2		+	−	+	−	−	++	+	−	+	5
Mucor hiemalis	8690	+	+	+	+	−	++	++	++	++	8
M. mucedo	9836	+++	+	−	+	−	+++	++	−	−	6
M. piriformis	16296	−	+	−	+	−	+++	+	+	+	7
M. racemosus f. *racemosus*	7935	+	+	+	+	−	+	+	++	++	8

NOTE: All experiments were carried out in duplicate. −, No detection; +, 1−2.5 mm (low); ++, 2.5−9.5 mm (moderate); +++, 9.5 and above (high).

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Intensity of enzymatic activity was measured as the diameter of the halo around the colonies (Federici 1982). The symbols +, ++, and +++ indicate, respectively, a diameter of 2 mm, between 2 and 8 mm, and more than 8 mm. The lack of enzyme production is indicated by the symbol −. A completely subjective estimate of the enzyme production based on color intensity is represented by the symbols in the case of urease.

Results and discussion

The ability of the members of Zygomyctes to produce extracellular enzymes on solid media is shown in Table 1. As seen in the data, the minimum number of enzymes detected on solid media was four in *R. kazanensis* and *R. oryzae* and the maximum was eight in *M. hiemalis* and *M. racemosus* f. *racemosus*.

Ribonuclease production was detected in *R. arrhizus*, *R. chinensis*, *R. pymacus*, *R. stolonifer*, *R. tritici*, *R.* sp. 66-81-2, *M. hiemalis*, *M. mucedo*, and *M. racemosus* f. *racemosus*, while DNase was detected in *R. arrhizus*, *M. hiemalis*, *M. mucedo*, *M. piriformis*, and *M. racemosus* f. *racemosus*. *Rhizopus arrhizus*, *R. stolonifer*, and *M. mucedo* showed a possibility of being high producers of RNase, while *R. arrhizus* was the only fungus which showed the possibility of being a moderate producer of DNase.

Proteolytic activity was detected in all of the fungi except *R. arrhizus*; however, none of the fungi showed the possibility of being high producers of the enzyme protease.

Three species of *Rhizopus* (*R. circinans*, *R. japonicus*, and *R. stolonifer*) and four species of *Mucor* (*M. hiemalis*, *M. mucedo*, *M. piriformis*, and *M. racemosus* f. *racemosus*) secreted amylase into the cultural medium, but did not show a high level of amylolytic activity.

Lipolytic activity was detected in only two of the fungi, *R. arrhizus* and *R. japonicus*.

All *Rhizopus* and *Mucor* species were shown to have phosphatase activity; however, the highest level of activity was detected in *R. oryzae*, *R. tritici*, *M. mucedo*, and *M. piriformis*.

Urease activity was detected in all fungi except *R. arrhizus* and *R. circinans*. Two *Mucor* species (*M. hiemalis* and *M. mucedo*) were the only fungi that showed a moderate level of enzyme activity.

Pectinolytic activity was here detected as polygalacturonase and pectate lyase. Polygalacturonase was detected in all fungi except *R. japonicus*, *R. tritici*, *R.* 66-81-2, and *M. mucedo*. *Rhizopus arrhizus*, *M. hiemalis*, and *M. racemosus* f. *racemosus* showed the possibility of being moderate prioducers of polygalacturonase. Pectate lyase was detected in *R. arrhizus*, *R. circinans*, *R. stolonifer*, *R. tritici*, *R.* 66-81-2, *M. hiemalis*, *M. piriformis* and *M. racemosus* f. *racemosus* being considered as moderate producers of the enzyme.

The results of this study show the wide array of extracellular enzyme activity and the level of their production in two groups of Zygomycetes, *Rhizopus* and *Mucor*, which have been associated with the disintegration of soft fruits during postharvest infection (White and Fabian 1953; Beneke *et al.* 1974; Archer 1979; Dennis and Harris 1979).

The extracellular enzymes produced by these fungi play an important role in the infection process of fruits in storage. It is conceivable that knowledge of the patterns of enzymes secreted by these two genera of postharvest spoilage fungi, *Rhizopus* and *Mucor*, might be of interest to the fruit storage industry in an effort to continue to provide proper control measures against this group of spoilage fungi in storage.

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