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Note

Determination of a Favorable Medium for Detection of Fungal Extracellular Protease

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To determine a favorable medium for detecting fungal extracellular protease, a plate method was examined. In this study, 12 fungal strains were tested with 4 different types of Czapek Dox agar medium containing skim milk, and the clear zones around the colonies were compared on the different types of medium. Among them, 9 strains produced extracellular proteases. Triton X-100 was required to detect an extracellular protease from *Alternaria alternata,* while in case of *Penicillium frequentans,* saccharose was required. It was found that the media supplemented with both Triton X-100 and saccharose gave acceptable results in terms of extracellular protease detection.

Key words : Extracellular protease/Plate method/Triton X-100/Saccharose.

The extracellular proteolytic enzymes secreted from several fungi play important roles in host infection, disease invasion, and food degeneration (Hankin and Anagnostakis, 1975; Hanzi et al., 1993). Evidence, which shows that the production of extracellular proteases of fungi, especially *Aspergillus fumigatus*, is involved in the known virulence and pathogenicity of the organism, has been presented in recent years (Hanzi et al., 1993). It has been documented that the proteolytic activity of an extracellular alkaline protease from *A. fumigatus* increases proportionally when the fungus invades the brain of a mouse (Barthomeuf et al., 1989; Miyaji and Nishimura, 1977). It has also been suggested that this enzyme plays a possible role in the cleavage of such structural proteins as collagen and elastin (Frosco et al., 1992; Monod et al., 1991; Reichard et al., 1990; Rhodes et al., 1990). On the other hand, some researchers have tried to correlate biochemical events, such as enzyme secretion, with the morphological events of spore germination in order to understand the invasion of host plants by pathogenic fungi (Dodman, 1979; Goodman et al., 1967; Hagerman et al., 1985). There are several methods for the detection and comparison of extracellular enzyme production by the same or different fungal strains (Hagerman et al., 1985; Hankin and Anagnostakis, 1975; Lee et al., 1996). However, the present methods for comparing fungi on the basis of enzyme production are considerably time consuming, especially if the fungi must be obtained in pure culture and the enzymes isolated for direct assay (Hankin and Anagnostakis, 1975; Tsao, 1970). For these reasons, the use of 4 different Czpek Dox agar (CzDA) media containing skim milk has provided a rather simple and rapid method to screen fungi to detect the presence of extracellular protease. The media described here would also be applicable to ecological studies in which enzymatic capabilities of fungi could be compared with those of other microorganisms.

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This study is to determine a favorable medium for the detection of fungal extracellular protease among 4 types of the skim milk-CzDA plate medium.

Twelve fungal strains kept as stock cultures in our laboratory were tested: *Alternaria alternata* YMC (Yonsei Medical Center) 0141, *Aspergillus flavus* YMC 0065, *A. fumigatus* YMC 0064, *Aspergillus niger* YMC 0100, *Aspergillus oryzae* YMC 0063, *Candida albicans* YMC 0078, *Cladosporium herbarum* YMC 0162, *Fusarium solani* YMC 0169, *Penicillium citrinum* YMC 0175, *Penicillium frequentans* YMC 0033, *Rhizopus stolonifer* YMC 0081, and *Rhodotorula rubra* YMC 0080. These fungi were all cultivated on potato-dextrose agar (PDA, Difco) slants with 30 mg/l chloramphenicol (CP, Wako Pure Chemicals), which was added in order to suppress bacterial growth (Tsao, 1970). Cultures were kept at 25℃ for 7 d in the dark prior to testing. For the production of extracellular protease, the strains were inoculated and then grown on 4 different modified CzDA plate media containing skim milk (Difco) at a final concentration of 1.0% (w/v), as nitrogen and carbon source.

TABLE 1. Four different types of skim milk-CzDA medium.[a]

Additional Reagent	CzDA type			
	A	B	C	D
Triton X-100	+[b]	+	−[b]	−
Saccharose	+	−	+	−

[a] Composition of CzDA medium based on the Pitt's formula ($g\ l^{-1}$): $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; Agar, 15 (all reagents of the highest grade available were purchased from Sigma except for the agar from Difco).

[b] +, Added to the medium; −, not added to the medium.

Using the CzDA medium suggested by Pitt's formula (Lee et al., 1996) as a base, 4 different types of CzDA plate medium including 1.0% (w/v) skim milk were prepared with or without the addition of Triton X-100 (Sigma) and/or saccharose (Difco) (Table 1). Triton X-100 and saccharose were added to the media at 0.01% (v/v) and 7.5 g/l of the final concentration, respectively (Lee et al., 1996). When saccharose was added to the media, it was used as another carbon source.

A plate method was examined for detecting extracellular proteases of 12 different fungi. The 4 different types of the skim milk-CzDA medium prepared as described above were used for detection of extracellular protease production, and the ratios of clear zone (or halo) size to colony size were compared on the different types of medium. This ratio was determined by the following equation:

$$R = D_1/D_2$$

where R is the ratio of the clear zone to colony size, D_1 is the diameter of the clear zone and D_2 is the diameter of colony. All tests were made on prepoured plates (90 mm Petri dishes) by point inoculation with

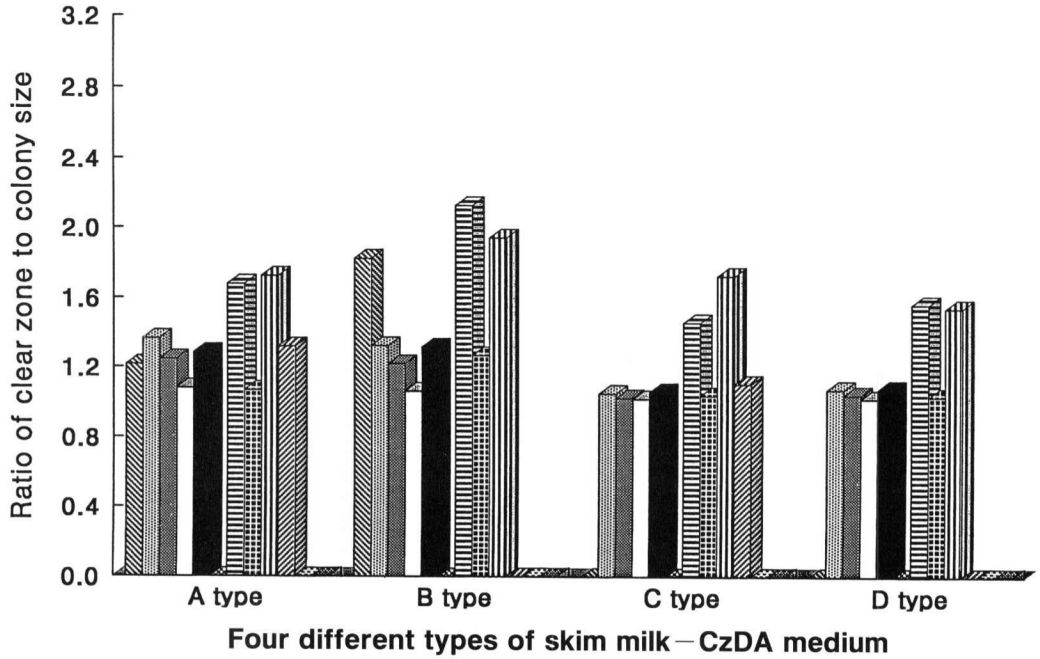

FIG. 1. Comparison of the ratios of the clear zone size to colony size on the 4 different types of the skim milk-CzDA medium for 12 fungal strains which were inoculated and then incubated at 25℃ for 5 d in the dark. Symbols: ▨, *A. alternata*; ▩, *A. flavus*; ▩, *A. fumigatus*; □, *A. niger*; ■, *A. oryzae*; ▩, *C. albicans*; ▤, *C. herbarum*; ▩, *F. solani*; ▥, *P. citrinum*; ▨, *P. frequentans*; ▩, *R. stolonifer*; ▩, *R. rubra*.

all the strains (Hankin and Anagnostakis, 1975). Plates were all incubated at 25℃ in the dark. After 1, 3, 5, and 7 d of incubation, the diameters of the halo and colony were respectively measured for each of the 12 fungal strains at a minimum of 0.5 mm unit. All plating was done in duplicate, and the mean values were treated as follows.

Figure 1 shows the ability of the 12 different fungal strains to produce extracellular protease on the 4 different types of CzDA plate medium containing skim milk. The term extracellular protease production is here intended to mean both synthesis of the enzyme by the fungus and activity of the enzyme in the medium after it is produced (Hankin and Anagnostakis, 1975). Among the 12 fungal strains, *C. herbarum* and *P. citrinum* showed the highest ratio of the clear zone to colony size on all the 4 types of the skim milk-CzDA medium, and the ratio was respectively 2.13 and 1.94, especially on Type B medium. In the case of *A. alternata*, the clear zone was observed on the 2 types of the medium where Triton X-100 had been added, which meant that the extracellular protease was produced only on Types A and B medium by the organism. It has also been reported that in detection of extracellular protease of *Alternaria* strains, addition of Triton X-100 to the media had a favorable effect on the clear zone formation (Lee et al., 1996).

In the case of *P. frequentans*, the halo appeared only on Types A and C media where saccharose had been added, while in the cases of *C. albicans*, *R. stolonifer*, and *R. rubra*, the clear zones were not formed on any type of the medium. On the contrary, in the cases of *Fusarium solani* and *Aspergillus* species such as *A. flavus, A. fumigatus, A. niger,* and *A. oryzae*, the ratios of halo size to colony size were within the range of 1.02-1.37 whether Triton X-100 or saccharose had been added to the media or not. Hanzi et al. (1993) studied the possibility of a relationship among the secreted alkaline proteases from different *Aspergillus* species by using liquid medium containing soluble collagen. In their study, preparation of enzyme concentrates and determination of proteolytic activity were necessary to detect extracellular protease production. It appeared that detection of fungal extracellular protease would be more rapid and effective on a solid, namely a plate medium, than in a liquid medium.

From the results in Fig.1, it is apparent that 9 strains among 12 fungal strains produced extracellular proteases on Type A medium where both Triton X-100 and saccharose had been added, and showed marked variation in their ability to produce extracellular protease according to each type of the medium, while the other 3 strains did not produce extracellular proteases at all. It would seem that it was difficult to use the results of 3 d after incubation to detect extracellular protease production, while the results of 7 d after incubation were nearly similar to

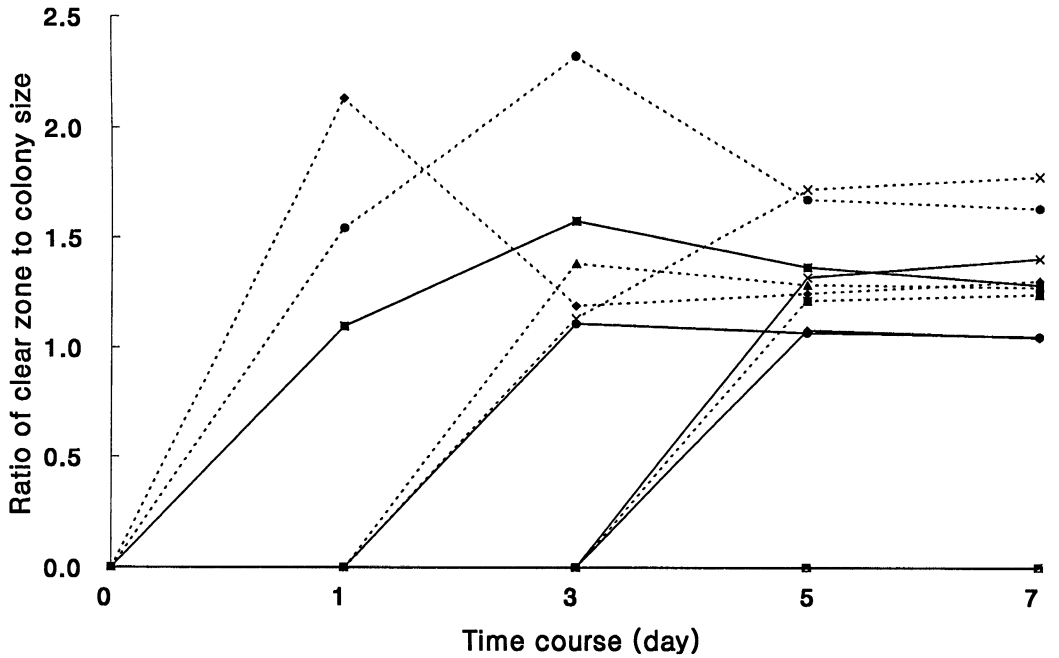

FIG. 2. Change in the ratios of the clear zone size to colony size during time on Type A skim milk-CzDA media for 12 fungal strains which were inoculated and then incubated at 25℃ in the dark. Symbols: ···■··· , *A. alternata*; —■— , *A. flavus*; ···◆··· , *A. fumigatus*; —◆— , *A. niger*; ···▲··· , *A. oryzae*; —△— , *C. albicans*; ···●··· , *C. herbarum*; —●— , *F. solani*; ···×··· , *P. citrinum*; —×— , *P. frequentans*; —○— , *R. stolonifer*; —□— , *R. rubra*.

those of 5 d after incubation. It could be suggested that at least 5 d of incubation is necessary for the detection of fungal extracellular protease by the plate method. As a result, the results of this study demonstrate that extracellular proteases produced by different fungi could clearly be detected on the media used in this study, especially on Type A medium, and show the usefulness and effectiveness of such media in surveys for fungal ability to produce proteolytic enzymes.

Figure 2 demonstrates time course change in the ratio of the clear zone to colony size on Type A skim milk-CzDA medium. *A. flavus, A. fumigatus,* and *C. herbarum* produced extracellular proteases even 1 d after incubation, and the ratios decreased after 3 d of incubation except for *A. fumigatus.* In the cases of *A. oryzae, F. solani,* and *P. citrinum,* the clear zones were formed 3 d after incubation, and the ratios slightly decreased up to 7 d after incubation except for *P. citrinum.* Not until 5 d after incubation did *A. alternata, A. niger,* and *P. frequentans* produce extracellular proteases. Through the results of Fig.2, it was found that the pattern of change in the ratios according to time course was very different among fungal strains, even *Aspergillus* and *Penicillium* species. In relation to these results, it has been documented that the production of enzymes such as protease, cellulase, or pectinase was correlated with the extent of fungal spore germination (Hagerman et al., 1985).

With the exception of *P. frequentans* among 9 fungal strains which produced extracellular protease, the addition of 0.01% (v/v) Triton X-100 to the skim milk-CzDA media (Table 1) made the ratios of the clear zone to colony size significantly high and the clear zones well-defined. Particularly the ratio was the highest on the Type B media where only Triton X-100 had been added (Fig. 1), though these media were considerably unfavorable to the growth of fungal strains (Fig. 3). On the contrary, the saccharose had no effect on the formation of clear zones except on that of *P. frequentans,* but it accelerated the growth of colonies, as another carbon source.

As shown in Fig. 3, the 12 fungal strains revealed different characteristics in growth rates. In particular, the colony size of *C. albicans* and *R. rubra,* known as yeast, was the smallest, which was respectively 5.60-7.50 mm and 3.33-6.33 mm in diameter according to the media used. However, *R. stolonifer* had already grown to 85 mm in width, which was the maximum size, 3 d after incubation (data not shown) on Type C medium supplemented with only saccharose, whereas the colony size of the fungus was less than 50 mm in diameter even 7 d after incubation on Type B medium. On the other hand, *A. alternata, F. solani,* and *P. citrinum* grew as well on Type D medium, without Triton X-100 or saccharose, as on Type C medium. In the cases of *C. herbarum, P. frequentans,* and 4 different species of *Aspergillus,* the growth

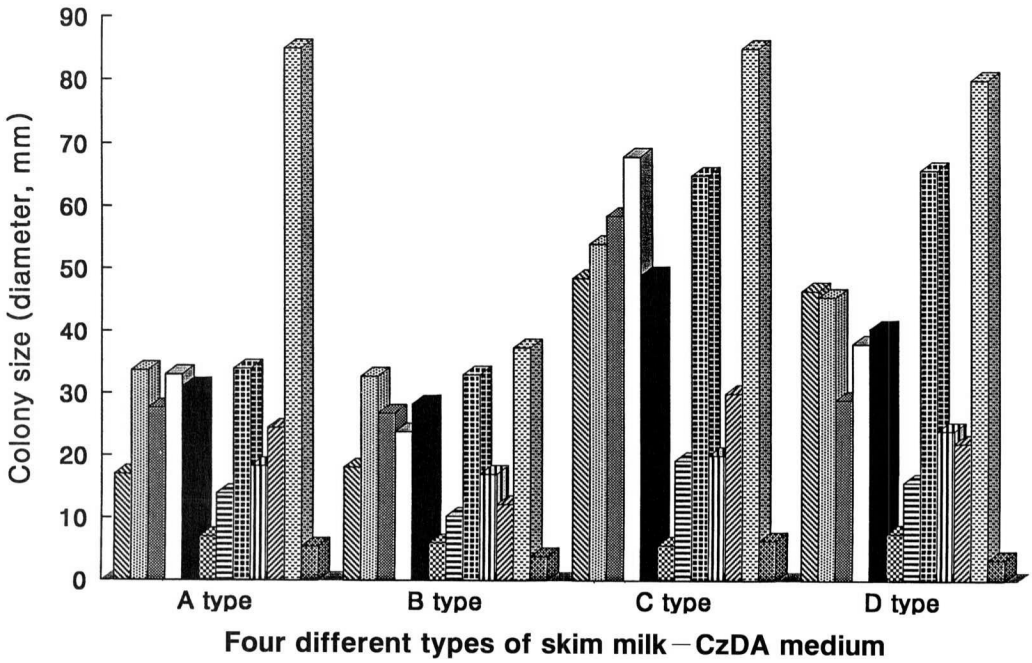

FIG. 3. Comparison of the degree of growth on the 4 different types of the skim milk-CzDA medium for 12 fungal strains which were inoculated and then incubated at 25℃ for 5 d in the dark. Symbols: ▨, *A. alternata*; ▩, *A. flavus*; ▩, *A. fumigatus*; □, *A. niger*; ■, *A. oryzae*; ▩, *C. albicans*; ▤, *C. herbarum*; ▩, *F. solani*; ▯, *P. citrinum*; ▨, *P. frequentans*; ▩, *R. stolonifer*; ▩, *R. rubra*.

rates of colonies were accelerated on the 4 types of medium in the order of C>D or A>B.

Although extracellular proteases were detected on Type B medium with only Triton X-100, and the clear zones around the colonies were well-defined on the media, only 8 strains among 12 fungal strains produced the enzymes on the media, and most of the colonies displayed less growth than on the other media. On Type C medium with only saccharose, all of the strains grew very well, but the extracellular proteases produced by only 8 fungal strains were detected on the medium. In the cases of Type D medium without Triton X-100 or saccharose, the production of extracellular protease was seen only in 7 fungal strains, and it was difficult to observe clear zone formation on the medium though the fungal growth was comparatively noticeable. In conclusion, it was seen that Type A medium with both Triton X-100 and saccharose gave good results in terms of clear zone formation and fungal growth, and the medium was favorable for detecting and screening fungal extracellular protease.

Type A medium used in this study would be able to permit rapid examination of the enzymatic capabilities of fungi, and facilitate recognition of fungi that produce extracellular protease. Type A medium could also be used in surveys of pure cultures for the detection of extracellular enzyme production strains.

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