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Extracellular Proteinases of Yeasts and Yeastlike Fungi[1]

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Approximately 800 yeasts and other fungi, representing over 70 species, were tested for extracellular caseinolysis. Isolates of a variety of genera, including *Aureobasidium*, *Cephalosporium*, *Endomycopsis*, *Kluyveromyces*, and numerous sporobolomycetes, demonstrated significant proteolytic activity. Caseinolysis was not necessarily correlated with gelatin liquefaction or with albuminolysis. Numerous fungi showed significant proteolysis at 5 C. The most active organisms were isolates of *Candida lipolytica*, *Aureobasidium pullulans*, *Candida punicea*, and species of *Cephalosporium*. Taxonomic and ecological implications of proteolytic activity are discussed.

Although yeasts and yeastlike fungi frequently are isolated from proteinaceous substrates, it is commonly believed that they lack significant extracellular proteolytic activity. Gelatin liquefaction has been reported frequently, but this property often is attributed to acid hydrolysis following production of organic acids. In only a few studies have yeasts been tested for extracellular proteolysis with substrates other than gelatin. Lodder and Kreger van Rij (7) mentioned several yeasts, including *Candida lipolytica* and *Candida pseudotropicalis*, that apparently peptonize milk. Peptonization of milk has been noted also for *Candida punicea* and *Candida curiosa* (6). More recently, Staib (11) reported data on strains of *Candida albicans* that utilized human serum albumin, presumably by extracellular proteinases.

Studies in our laboratories on yeasts of aquatic origin (9) indicated that certain taxa characteristically elaborate extracellular proteinases. To ascertain the extent of this activity, over 790 isolates of yeasts and yeastlike fungi, representing 20 genera and approximately 73 species, were examined for their ability to attack casein; representative caseinolytic strains were tested also for gelatin liquefaction and albuminolysis.

MATERIALS AND METHODS

Fungi were obtained from the collections of the Department of Microbiology, University of Miami, Coral Gables, Fla., and the Department of Biology, Georgia State College, Atlanta, Ga. The organisms included cultures from various terrigenous, freshwater, and marine habitats (1, 8, 9), and from clinical sources. Cultures were maintained on an agar medium of 2.0% glucose, 4.0% peptone, and 0.5% yeast extract. For test purposes, cells from 7-day slants of the stock medium were streaked onto an agar medium containing 2.0% casein, 0.3% beef extract, 0.5% tryptone, and 0.1% glucose (Fig. 1). The casein was dissolved in distilled water, sterilized at 110 C for 10 min, cooled, and added to the remaining medium at 47 C with constant agitation. The final *p*H was between 6.3 and 6.7. Based on prior experiments, incubation temperatures of 18 or 24 C were employed. After 3 weeks, proteolysis was recorded as zones of clearing at the periphery of the colonies.

Representative organisms were tested for casein hydrolysis at a *p*H range between 4.7 and 8.0, and at temperatures of 5, 18, 23, 27, 30, and 37 C. The *p*H of the casein medium was adjusted with 0.01 N HCl or 0.01 N NaOH; the medium stabilized with 0.25% $CaCO_3$ and dispensed in 20-ml portions per plate. Inocula consisted of 0.01-ml cell suspensions from a 48-hr shake culture grown in yeast nitrogen base (YNB) broth (Difco) with 0.5% glucose added. After incubation for 8 days, hydrolysis was recorded as the diameter (mm) of cleared zone from the colony edge; zones greater than 3 mm in diameter were arbitrarily considered representative of significant levels of extracellular proteinase(s). Strains with significant activity were tested subsequently for hydrolysis of gelatin and albumin.

The gelatin medium (5 ml/tube) contained 10% gelatin, 0.5% glucose, 0.5% $(NH_4)_2SO_4$, and vitamins and trace elements according to the method of Wickerham (13). Gelatin hydrolysis was determined by depth (mm) of liquefaction after the culture was cooled to 5 C. For preparation of the albumin agar, 5.0 g of reagent-grade egg albumin was carefully dissolved, with minimal stirring, in 100 ml of distilled water. This solution was then sterilized with ethylene

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oxide, heated to 47 C, and added (final albumin concentration, 1.0%) to a basal medium similar to that employed with casein. Inoculum preparation and incubation were as described for the casein tests.

To obtain cell-free filtrates (YNB broth), the culture liquors of selected yeasts were filtered through sterile 0.45-μ cellulose ester membranes (Millipore Corp., Bedford, Mass.). The filtrates from 24-hr cultures were tested for ability to liquefy gelatin and to clot 1.0% casein solution that had been buffered at *p*H 6.0 with McIlvaine's citric acid phosphate buffer.

RESULTS AND DISCUSSION

Caseinolytic activity of the various organisms is given in Table 1. All isolates of *Kluyveromyces*, *Endomycopsis*, *Cephalosporium*, and *Aureobasidium*, and most sporobolomycetes and trichosporons attacked casein, whereas caseinolysis was not observed for representatives of *Saccharomyces*, *Hansenula*, *Hanseniaspora*, *Torulopsis*, and *Rhodotorula*. Considerable strain variation in degree of caseinolysis was observed, particularly for isolates of the genera *Kluyveromyces*, *Sporobolomyces*, and *Trichosporon*. It is especially interesting that all five species (33 isolates) of *Kluyveromyces* were caseinolytic, because these species represent a conglomerate of discrete physiological (vitamin requirements) and morphological types.

Of a total of 19 species of *Candida*, comprising 200 isolates, only *C. pseudotropicalis*, *C. lipolytica*, *C. punicea*, *C. aquatica*, and *C. curiosa* showed caseinolysis. *C. pseudotropicalis* is the imperfect stage of *Kluyveromyces fragilis*; all members of this genus showed caseinolytic activity. The taxonomic status of *C. lipolytica* is in question. The significant proteolytic activity of this species, in addition to its commonly demonstrated capacity to degrade various hydrocarbons, further indicates its unique position among the common species of *Candida*. The failure of Dion (2) to obtain activity for *C. lipolytica* may be attributed to his use of an incubation temperature 3 C above the critical growth temperature of the organism. *C. punicea* is, in fact, a sporobolomycete (1) in that ballistospore production can be demonstrated readily on corn meal agar at 15 C and 90% relative humidity. *C. curiosa*, *C. aquatica*, and fungi with similar physiological and morphological properties (e.g., *Rhodotorula infirmo-miniata*, *Candida nivalis*, and *Rhodosporidium toruloides*) appear more closely related to various families of the *Heterobasidiomycetes* than to the majority of the species of *Candida*.

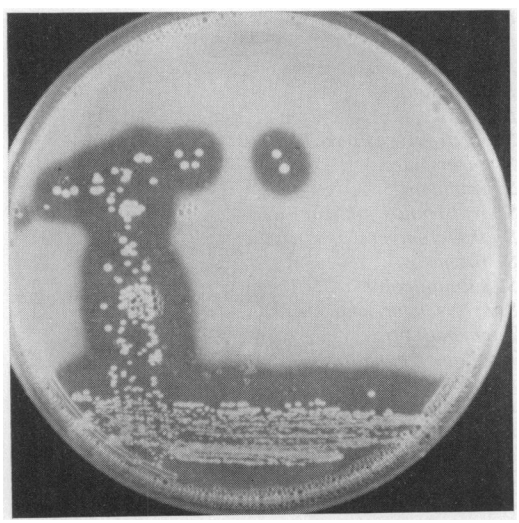

FIG. 1. *Caseinolytic activity of Candida punicea after 48 hr at 18 C on initial streak transfer to casein agar (clearing ca. 6 mm).*

Only 9 of the 107 isolates of the various sporobolomycetes lacked caseinolytic activity. The majority of these fungi were isolated initially from aquatic environs. The proteolytic pink yeasts mentioned recently by Kazanas (5) were most likely sporobolomycetes. Our earlier work on several hundred sporobolomycetes, not identified to species, demonstrated the striking capacity of this group to attack casein. The nine isolates which did not attack it had been maintained in culture for periods up to 5 years before testing. Previously, we noted that variability for caseinolytic activity occurred among certain isolates of *Sporobolomyces* (9). Such strain variability has been noted for other taxa (e.g., *Sporotrichum schenckii*).

Sixty-eight representatives of *Debaryomyces hansenii* were tested because this organism is often isolated from meat products and is one of the most common species found in the oceans. Of the 20 isolates obtained from meat products, only 1 showed caseinolysis. Two positive strains were recovered from our North Sea investigations, and the only other proteolytic isolate was of human origin.

Most caseinolytic isolates produced sharply defined regions of lysis; however, certain isolates of *Cryptococcus*, *Dioszegia*, *Taphrina*, and *Trichosporon* produced diffuse halos only 2 to 3 mm wide. Among the isolates of *Trichosporon cutaneum*, the majority of which were of human origin, proteolysis varied from the more common diffuse halo type to a type that shows, within 5 days, sharply defined zones greater than 15 mm. Casein hydrolysis by intracellular enzymes has been reported for several isolates of *Trichosporon* (3, 12). Groninger and Eklund (4) attributed the caseinolysis of their isolates of *Trichosporon* to an intracellular or "surface-bound" enzyme. It is

possible that proteinases associated with cell membranes produced the diffuse halos observed with certain of our yeasts.

Conditions for optimal enzymatic expression varied with the different organisms. Although optimal activity for the various fungi was between *p*H 5 and 7, casein hydrolysis for selected species occurred over the *p*H range 4.7 to 8.5 with most optima near *p*H 6.5. Incubation temperature generally was more critical than *p*H. Usually, optimal activity was correlated with the temperature that gave maximal growth. In a few strains (e.g., *Protomyces indunatus*), casein hydrolysis occurred only at temperatures above those for optimal growth. The vast majority of the fungi that were caseinolytic at 18 C were also proteolytic at 5 C; a few strains were caseinolytic at 0 C.

Selected caseinolytic strains were tested for their ability to hydrolyze gelatin and albumin (Table 2). The most active hydrolysis of casein was exhibited by isolates of *A. pullulans*, *C. lipolytica*, *C. punicea*, and species of *Cephalosporium*. Isolates of these fungi completely liquefied gelatin, and were the only taxa that exhibited albumin hydrolysis. Comparatively few strains were tested for albuminase and, except for the

TABLE 1. *Hydrolysis of casein by yeasts and yeastlike fungi*

Species	No. of isolates tested	No. of caseinolytic isolates	Species	No. of isolates tested	No. of caseinolytic isolates
Aureobasidium pullulans	26	26	*Kluyveromyces aestuarii*	23	23
Bullera alba[a]	2	2	*K. africanus*	1	1
Candida albicans	35	0	*K. drosophilarum*	2	2
C. aquatica[a]	1	1	*K. fragilis*	6	6
C. curiosa[a]	1	1	*K. polysporus*	1	1
C. diddensii	20	0	*Phialophora dermatitidis*	1	0
C. guilliermondii	15	0	*Pichia fermentans*	3	0
C. intermedia	4	0	*P. polymorpha*	3	0
C. krusei	18	0	*Protomyces inouyei*[a]	1	1
C. lipolytica	3	3	*P. inundatus*[a]	1	1
C. mycoderma	10	0	*P. pachydermus*[a]	1	1
C. natalensis	6	0	*Rhodosporidium toruloides*	3	0
C. parapsilosis	37	0	*Rhodotorula aurantiaca*	3	0
C. polymorpha	5	0	*R. glutinis*	16	0
C. pseudotropicalis	1	1	*R. graminis*	11	0
C. pulcherrima	2	0	*R. infirmo-miniata*[a]	28	0
C. punicea[a]	1	1	*R. lactosa*	2	0
C. reukaufii	1	0	*R. minuta*	12	0
C. sorbosa	1	0	*R. pallida*	5	0
C. tenuis	3	0	*R. pilimanae*	6	0
C. tropicalis	34	0	*R. rubra*	20	0
Cephalosporium spp.	9	9	*Saccharomyces carlsbergensis*	4	0
Cladosporium werneckii	1	0	*S. cerevisiae*	6	0
Cryptococcus albidus	14	0	*S. fructuum*	3	0
C. lactativorus	2	0	*Sporidiobolus johnsonii*[a]	7	5
C. laurentii	8	0	*Sporobolomyces pararoseus*[a]	37	37
C. neoformans	8	3	*S. roseus*[a]	58	51
Debaryomyces hansenii	68	4	*S. salmonicolor*[a]	2	2
Dioszegia spp.[a]	3	2	*Sporotrichum schenckii*	24	2
Endomycopsis capsularis	2	2	*Taphrina* spp.[a]	11	8
E. fibuliger	2	2	*Torulopsis candida*	12	0
E. selenospora	2	2	*T. ernobii*	5	0
Geotrichum candidum	5	4	*T. glabrata*	35	0
Hanseniaspora uvarum	15	0	*Trichosporon capitatum*	7	2
Hansenula anomala	8	0	*T. cutaneum*	46	43
H. californica	2	0	*T. fermentans*	2	0
H. ciferrii	3	0	*T. penicillatum*	1	0
H. saturnus	22	0			
H. suaveolens	3	0			

[a] Incubation at 18 C; others at 24 C.

hydrolysis shown for the four species, the results were inconclusive.

Correlations were not apparent in ability of the fungi to hydrolyze the three substrates, for yeasts attacking casein did not necessarily liquefy gelatin. *Kluyveromyces aestuarii* and numerous strains of *Sporobolomyces* showed significant caseinolytic activity, but negligible hydrolysis of gelatin. Conversely, strains of *R. infirmo-miniata* produced active gelatinases but showed little or no caseinolytic activity.

C. punicea did not grow on the albumin agar; cleared zones were produced in this medium beneath 0.2-μ porosity membranes that contained colonies previously grown on casein agar. *C. punicea* actively hydrolyzed casein at temperatures from 5 to 20 C. Blocks of agar (ca. 6 mm square) were removed aseptically from the casein-cleared zones under membranes bearing colonies of *C. punicea* and transferred to fresh casein agar at various *p*H values. Hydrolysis occurred at 5 and 18 C, from a *p*H range of 4.5 to 7.0, with optimal activity at *p*H 6.5.

A few yeasts produced extracellular proteinase(s) when grown on Wickerham's defined yeast nitrogen base medium (YNB). Cell-free filtrates of *C. lipolytica* (UM 127A), *A. pullulans* (C-348), and *Cephalosporium* sp. (IMS 634) completely liquefied the gelatin medium within 48 hr at 25 C. In casein, the filtrates of *C. lipolytica* and *A. pullulans* (1 ml added to 5 ml of casein) produced clotting at 10 and 30 min, respectively, at 25 C. The filtrate of the *Cephalosporium* sp. was inactive in casein. Filtrates of *Kluyveromyces* spp. and *Sporobolomyces* spp. were negative for gelatin and casein.

Although this report is concerned mainly with yeasts and yeastlike fungi, a few taxa of "molds" are included. The isolates of *Cephalosporium* which hydrolyzed albumin were of marine origin and all produced a unicellular budding stage, particularly in liquid media. Two *Cephalosporium* isolates that were inactive on albumin were of terrestrial origin and were not observed to produce a yeast phase. Species of *Cephalosporium* have been reported to give extracellular hydrolysis

TABLE 2. *Proteolytic activity of selected yeasts and yeastlike fungi on casein, gelatin, and albumin*[a]

Species	Casein			Gelatin		Albumin	
	No. of isolates	Cleared zone	Optimal temp	No. of isolates	Depth	No. of isolates	Cleared zone
		mm	C		mm[b]		mm
Aureobasidium pullulans	26	18–53	24	8	5–35[b]	4	3–20
Bullera alba	2	8–14	18	2	—	1	—
Candida lipolytica	3	25–50	18	3	35	3	10–15
C. punicea	1	38	18	1	35	1	+
Cephalosporium sp.[c]	7	12–43	24	5	35	7	1–2
Cephalosoporium sp.	2	48	30	2	35	2	—
Dioszegia spp.	2	6–15	18	1	8	1	—
Endomycopsis capsularis	2	7	24	2	35	1	—
E. fibuliger	1	14	30	1	35	1	—
E. selenospora	1	6	24	1	35	1	—
Kluyveromyces aestuarii	18	8–26	27	8	0–2	8	—
K. drosophilarum	2	8–14	30	2	—	2	—
K. fragilis	2	10–21	27	6	2–10	3	—
Protomyces inouyei	1	8	18	1	—	1	—
P. inundatus	2	7–9	18	2	—	1	—
P. pachydermus	1	6	18	1	—	1	—
Sporidiobolus johnsonii	5	12–25	18	3	0–3	1	—
Sporobolomyces pararoseus	37	5–29	18	16	0–9	2	—
S. roseus[c]	54	10–33	18	8	0–12	2	—
S. roseus	2	5–13	30	2	—	1	—
S. salmonicolor	1	8	24	1	—	1	—
Sporotrichum schenckii	1	6	24	1	35	1	—
Taphrina spp.	2	6–7	18	2	2	2	—
Trichosporon capitatum	1	14	30	1	35	1	—
T. cutaneum	3	14–20	30	3	35	3	—

[a] The *p*H of the casein medium was 6.5. The incubation temperatures for the gelatin and albumin tests were as given in Table 1.

[b] Complete liquefaction at 35 mm.

[c] *Cephalosporium* spp. and *S. roseus* are each listed in two groups on the basis of temperature optima.

of casein and gelatin (10). *Geotrichum candidum*, although lacking a yeast stage, was studied in view of its superficial similarity to *Trichosporon* spp. *Sporotrichum schenckii* is a dimorphic pathogen of man and lower animals, and its yeast stage generally is formed on enriched media at 37 C. However, several of our isolates produced a limited yeast phase at room temperatures, particularly in liquid media. The strain of *S. schenckii* that showed significant extracellular proteolysis was isolated from a cold-stored meat product. Both *Cladosporium werneckii* and *Phialophora dermatitidis* existed primarily in unicellular budding stages and are superficially similar to *Auerobasidium pullulans*.

Correlation of extracellular proteinase production with other physiological properties of the various organisms was not attempted, but certain systematic relationships were suggested. Proteolysis was shown by yeasts and other fungi of both basidiomycetous and ascomycetous relationship, although no broad phylogenetic distinctions were evident. Nevertheless, contrary to reports in literature extant, it is apparent that extracellular proteolytic activity is not uncommon among various taxa of yeasts and yeastlike fungi.

A number of the fungi examined in this survey are psychrophilic or have been associated with cold-stored proteinaceous products (3, 5, 6). Many of these fungi are proteolytic and occur in the natural environment of the animal prior to its processing for food. In view of their extracellular proteolysis, it is probable that certain yeasts contribute to active spoilage of meat and fish products. These proteolytic species commonly are found on food products in association with cold-tolerant mesophiles. The latter, which are frequently nonproteolytic fungi, grow more rapidly on most media that are used for growing yeasts. The use of the casein medium and low incubation temperatures for isolation of yeasts associated with cold-stored proteinaceous products may provide a more valid procedure for ascertaining the role of yeasts in spoilage of various frozen food products.

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