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Applicability of Yeast Extracellular Proteinases in Brewing: Physiological and Biochemical Aspects

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A general screening survey for expression of extracellular acid proteinase production was performed on over 100 cultures belonging to the genus *Saccharomyces*. Although two strains of *Saccharomyces cerevisiae* showed positive extracellular proteinase phenotypes in plate tests, it was not possible to demonstrate proteolytic activities in cell-free culture supernatants in assays performed at beer pH values. Of several yeasts from other genera examined, *Saccharomycopsis fibuligera* and *Torulopsis magnoliae* produced extracellular proteinases with desirable properties. Proteolytic activities were detected in assays performed at beer pH values and at lower temperature. Brewer's wort served as a highly inducing medium for extracellular proteinase production, with *T. magnoliae* yielding enzyme of highest specific activity. In fact, commencement of enzyme production was detected shortly after the onset of exponential growth in brewer's wort. Inclusion of crude enzyme preparations in brewer's wort inoculated simultaneously with brewer's yeast reduced final ethanol yields slightly and was found to be effective in reducing chill haze formation in bottled beer.

A typical brewing process yields beer with a significant content of unassimilated proteins, and, even though most of these proteins originate from malt, some are derived from hops and from the yeast culture used (11). Certain proteins (cystine-poor proteoses) present have been implicated in foam stability, but others (albumins, barley hordeins, cystine-rich proteoses) contribute to undesirable haze formation upon cool storage (10, 11), thereby altering the physical stability of packaged beer. In efforts to control the chill haze problem, it is common practice in finishing operations to hydrolyze undesired proteinaceous components or to use silica adsorbents or silica hydrogels to adsorb protein and/or polyvinylpolypyrrolidone to remove polyphenols that participate in protein condensation reactions (7, 8). Thus, the construction of a brewer's yeast that secretes into brewer's wort a proteinase that can degrade haze-forming proteins during the course of fermentation would offer a feasible alternative to such methods and hence would be of considerable economic value in chill-proofing beer.

Extracellular proteinase production among various microorganisms is a well-known phenomenon (5, 9, 15, 26). Although much information has accumulated concerning the genetics and biochemistry of intracellular proteolysis in the yeast *Saccharomyces cerevisiae* (for a review, see reference 23), little attention has been given to the possibility that certain strains may be capable of extracellular proteolytic activity. Maddox and Hough (25) noted the presence of proteolytic activity in culture supernatants of a *Saccharomyces uvarum* (*carlsbergensis*) strain, the only documented instance of extracellular proteinase in the genus *Saccharomyces*. Of the few *S. cerevisiae* strains examined, none have been found to secrete proteinases (1, 2, 20, 24). Spencer and Spencer (35), however, observed liquefaction of gelatin in an *S. cerevisiae* brewing strain, but no attempt was made to determine whether a true extracellular proteinase was secreted; in many fungi, acid hydrolysis upon release of organic acid can also result in gelatin liquefaction (1).

A program devised to identify yeasts that produce extracellular proteinases of practical value in brewing should consider that enzymes with the following properties would be desirable: (i) ability to hydrolyze protein in the pH range of 4.0 to 5.5; (ii) activity at brewer's wort fermentation temperatures (10 to 20°C); (iii) resistance to inactivation by indigenous proteinase inhibitors reported to be present in brewer's wort (7) and by ethanol accumulated during brewer's wort fermentation; (iv) specificity towards those proteins reported to constitute principal components of beer haze (6, 22); (v) inability to inactivate other extracellular enzymatic activities of interest, such as glucoamylase used in low-carbohydrate-beer production (36); (vi) reduced or eliminated activity upon pasteurization of packaged beer; and (vii) ability to execute all necessary functions without affecting beer foam stability and organoleptic properties. With these requirements in mind, the present study was undertaken to screen for extracellular acid proteinase production in various *Saccharomyces* spp. In addition, members from other yeast genera (*Saccharomycopsis* and *Torulopsis*) known to produce extracellular proteinases (1, 20, 24, 38) were included, for no evaluation has been reported concerning these organisms as possible sources of the enzyme for use in brewing fermentations and, hence, as candidates for use in subsequent development through spheroplast fusion and/or transformation of brewer's yeast strains that secrete acid proteinases during fermentation.

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MATERIALS AND METHODS

Organisms and maintenance. Strains used in this study were obtained from the Labatt Culture Collection and were as follows (numbers tested): *Saccharomyces bayanus*, 20; *Saccharomyces uvarum (carlsbergensis)*, 6; *Saccharomyces cerevisiae*, 85; *Saccharomyces diastaticus*, 2; *Saccharomyces fragilis*, 3; *Saccharomyces rosei*, 2; *Saccharomyces rouxii*, 2; *Saccharomycopsis fibuligera*, 3; *Torulopsis magnoliae*, 1; and *Aspergillus oryzae*, 1. All cultures were maintained at 4°C on Wickerham's (37) malt extract-yeast

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extract-peptone-glucose (MYPG) agar slant medium, which consisted of the following ingredients (grams per liter of deionized distilled water): malt extract, 3; yeast extract, 3; peptone, 5; glucose, 20; and Bacto-Agar (Difco Laboratories, Detroit, Mich.), 20.

Plate tests for proteinase production. Preliminary assessment of extracellular proteinase production was made by inoculation from overnight MYPG agar slant cultures onto skim milk agar and brewer's wort-gelatin plates. Skim milk agar cultures were examined for clear zones after 2 days of incubation at 27°C, and brewer's wort-gelatin cultures were examined for liquefaction after 3 to 5 days of incubation at 20°C.

Skim milk agar plates were prepared as described previously (33) but with several modifications. To 70 ml of Difco 10% skim milk powder in 0.05 M phosphate buffer, pH 6.5 (autoclaved for 10 min at 100°C), was added 60 ml of a filter-sterilized buffer consisting of 2.4% KH_2PO_4 and 3.5% $Na_2HPO_4 \cdot 7H_2O$. The resultant mixture was combined with 480 ml of an autoclaved solution containing 1% glucose, 0.2% Yeast Nitrogen Base without both ammonium sulfate and amino acids (YNB-N-aa; Difco), and 1% Bacteriological Agar No. 1 (Oxoid Ltd., London, England). Initial culture medium pH was 6.5, and appropriate pH adjustments were made as required. Brewer's wort-gelatin medium (pH 5.5) was prepared by dissolving 120 g of Difco gelatin in 1 liter of steam-streamed 12° Plato (12°P) brewer's wort (°Plato = grams of sucrose per 100 ml, equivalent to carbohydrate content at 20°C) and steam-streaming for 10 min at 100°C.

Liquid culture conditions. Yeasts which showed positive proteinase phenotypes in plate assays were analyzed for extracellular proteinase production in a filter-sterilized synthetic medium containing 1% glucose, 2% bovine serum albumin (Miles Pentex Fraction V Powder, fatty acid poor), and 0.1% YNB-N-aa with inclusion of readily assimilated nitrogen supplied as 5 mM ammonium sulfate, pH 5.5.

Cells were inoculated at a density of 1 g/liter into 30-ml volumes of medium in 300-ml triple-baffled shake flasks (Bellco Glass, Inc., Vineland, N.J.). After 3 days of incubation at 27°C in a New Brunswick Scientific Co. Gyrotory shaker operated at 150 rpm, cells were pelleted by centrifugation, and resultant cell-free supernatants were assayed for proteolytic activity. In small-scale brewer's wort fermentation trials, the same procedure was used, except that 12°P brewer's wort was inoculated with yeast at a density of 3.5 g/liter, and cell-free supernatants were assayed for proteolytic activity after 3 days of incubation at 20°C. Modifications in medium composition and in culture conditions were made as required; these modifications are given in the text.

Enzymatic assay procedure. The assay procedure of McDonald and Chen (27) was used with some modifications in order to analyze cell-free supernatants for acid proteinase production. Volumes (200 μl or less) of cell-free supernatant were added to 2-ml volumes of a hemoglobin substrate solution prepared by dissolving 1.2 g of denatured hemoglobin (standardized for proteinase assays; Nutritional Biochemicals Corp., Cleveland, Ohio) in 100 ml of buffer appropriate for the determination. Assay tubes were attemperated to temperatures appropriate for determinations before enzyme addition. Blanks were prepared in the same manner as assay tubes, except that 4 ml of precipitating agent (8% cold trichloroacetic acid) was added before addition of enzyme. After 1 h of incubation in a circulating water bath at either 20 or 40°C, a 4-ml volume of precipitating agent was combined with the contents of each assay tube. The resulting precipitates of nonhydrolyzed hemoglobin were removed by filtration through Whatman no. 1 filter paper, and 1-ml samples of filtrate were assayed. A 5-ml volume of Lowry reagent (consisting of 100 ml of 2% Na_2CO_3, 10 ml of 10% NaOH, 1 ml of 2.7% sodium potassium tartrate, and 1 ml of 1% $CuSO_4$) was added to each sample. After 10 min at room temperature, 0.5 ml of Folin-Ciocalteau reagent (Fisher Laboratories), diluted 1:1 with deionized glass-distilled water was added. Tubes were immediately vortexed, stored in the dark for 1 h, and then read against blanks at 700 nm in a Pye Unicam SP8-100 UV spectrophotometer. One unit of enzyme activity (PU) is defined as that amount of enzyme which releases the color equivalent of 1 μg of tyrosine in 1 min. All assays were performed in triplicate, and data given represent an average of the three determinations.

Haze determinations. Extracellular proteinase-producing yeasts inoculated into 200-ml volumes of 12°P brewer's wort in 1-liter flasks were incubated at 20°C with shaking for 3 days. Cells were then pelleted by centrifugation, and resultant supernatants were concentrated approximately 100-fold at 2°C under a pressure of 414 kPa with Amicon Diaflo ultrafilters (model 402) fitted with a Diaflo ultrafilter membrane (yM30; 150-mm diameter). After determination of proteolytic activities, volumes of 12°P brewer's wort were supplemented with sufficient crude enzyme concentrate to yield 2 PU/ml and then inoculated with *S. cerevisiae* brewing strain 3001 at a density of 3.5 g/liter. After 5 days of incubation at 20°C with shaking, cells were pelleted by centrifugation, and the resultant beers were filter sterilized and stored under CO_2 in vacuum flasks for 2 days at 4°C. After treatment with silica adsorbent (0.2 g/liter) for 30 min, the beers were vacuum-filtered through diatomaceous earth, filter sterilized, purged with CO_2, bottled, and pasteurized. Bottled beers were forced at 48°C for 1 week in order to accelerate haze formation and stored for 24 h at 0°C before haze analysis. Haze was measured in a standardized radiometer haze meter, type UKIMID, by use of 300-ml samples read at 20°C. The method, "Formazin Turbidity Standards" (4), was used to standardize the haze meter.

RESULTS AND DISCUSSION

Assessment of physiological aspects of extracellular proteinase production in combination with physicochemical studies is essential to determine the suitability of a given yeast strain as a candidate for use in protoplast fusion with brewer's yeast and/or as a source of DNA from which to isolate extracellular proteinase-encoding genes for eventual transformation into brewer's yeast. Of over 100 cultures belonging to the genus *Saccharomyces* screened for extracellular proteinase production by inoculation onto brewer's wort-gelatin and skim milk agar media, two *S. cerevisiae* strains were indicated to produce extracellular acid proteinase (Table 1). Strain 84 produced clear zones on casein agar medium when cultivated at pH 6.5 but not at pH 5.5, and it did not liquefy gelatin in brewer's wort-gelatin medium. Strain 1352, however, hydrolyzed gelatin in brewer's wort-gelatin medium but did not produce clear zones on casein agar plates at either pH value. *A. oryzae*, a mycelial fungal control known to serve as a source of chill-proofing enzyme (8), also expressed activity on brewer's wort-gelatin medium but not on casein agar medium. Differences in the ability to utilize casein versus gelatin may be due to differences in the substrate specificities of the enzymes produced (28, 31) or may be the consequence of parameters known to affect extracellular proteinase production, such as culture medium

pH (16), medium composition (3, 34), and/or incubation temperature (16). As noted in previous screening procedures (1, 24), *S. fibuligera* and *T. magnoliae* liquefied gelatin and produced clear zones on skim milk agar plates.

Positive proteinase phenotypes in plate tests can arise from induction as a consequence of the presence of a protein substrate and/or derepression which occurs upon depletion or omission of one or more nutrilites from culture media (12–15). Given the complex composition of brewer's wort (18, 22, 36), preliminary assessment of extracellular proteinase production in liquid culture was not performed in derepression media lacking readily assimilated sources of carbon, nitrogen, and/or other nutrilites (12–15); instead, the culture medium used contained 5 mM ammonium sulfate and 1% glucose as readily assimilated nitrogen and carbon sources, YNB-N-aa (a source of vitamins and of salts supplying trace elements), and bovine serum albumin as the protein substrate (initial medium pH of 5.5). *S. cerevisiae* 84 and 1352 cells did not produce detectable extracellular proteinases (Table 2), and further experiments using various derepression media are required. *A. oryzae* 1531 cells also did not yield detectable enzyme in synthetic medium, but in this instance the strain did not grow because of complex nutritional requirements (9). Enzymatic activities, however, were evident at all three temperatures in cell-free supernatants from *T. magnoliae* 1536 and from all three strains of *Saccharomycopsis fibuligera*. Upon comparison of specific activities, *T. magnoliae* yielded the highest value (6.31 PU/mg [dry weight] per ml) in assays at 20°C and the lowest value (1.00 PU/mg [dry weight] per ml) in assays at 60°C.

All yeasts which showed detectable extracellular proteolytic activities in synthetic medium at 27°C (Table 2) also did so after cultivation at 20°C in 12°P brewer's wort (Table 3). Again *S. cerevisiae* 84 and 1352 failed to yield proteolytic activities that were detectable under the experimental conditions used. *A. oryzae*, however, did produce extracellular acid proteinase, but yields were low in comparison to the high levels of enzyme production obtained in brewer's wort inoculated with *Saccharomycopsis fibuligera* and *T. magnoliae*. From comparison of data given in Tables 2 and 3, it is evident that specific enzymatic activities were consistently lower after cultivation of *Saccharomycopsis fibuligera* and *T. magnoliae* in brewer's wort than in synthetic medium. Although catabolite repression in brewer's wort would offer a reasonable explanation for the differences observed, the possibility that induction was involved cannot be excluded for it is well known that the extent of extracellular proteinase production can be determined by the initial concentration of protein substrate(s) present in a given medium (14).

In an effort to assess the brewing potential of extracellular acid proteinases of interest, volumes of brewer's wort supplemented with proteinases from *Saccharomycopsis fibuligera* 1570 and from *T. magnoliae* 1536 were inoculated with brewer's yeast in bench-scale fermentations conducted at brewing temperature (20°C); the effects of enzyme addition on haze formation and ethanol production were analyzed. Proteolytic activities remaining upon completion of fermentation were also determined. The proteinase additions rendered bottled beer resistant to haze formation and reduced final ethanol yields slightly (Table 4); however, marked reductions in enzymatic activities from initial values (2 PU/ml) were noted in assays performed upon completion of fermentation. Because crude enzyme preparations were added at the time of inoculation, it is conceivable that the decreases in enzymatic activities involved enzyme instability (19), denaturation by ethanol accumulation (29), and/or inactivation by lytic release of proteinases from brewer's yeast at the conclusion of fermentation (17).

TABLE 1. Comparison of protein substrate utilization ability among various yeasts examined for extracellular proteinase production[a]

Organism/strain	Phenotype[b] on:		
	Brewer's wort-gelatin	Skim milk agar at pH:	
		5.5	6.5
A. oryzae 1531	+	−	−
S. cerevisiae			
84	−	−	+
1352	+	−	−
Saccharomycopsis fibuligera			
1570	+	+	+
1571	+	+	+
1573	+	+	+
T. magnoliae 1536	+	+	+

[a] Brewer's wort-gelatin plates were examined for liquefaction after 3 to 5 days of incubation at 20°C. Casein utilization was determined by examination for the presence of clear zones after 2 days of incubation at 27°C.

[b] Phenotypes: +, utilized substrate; −, did not utilize substrate.

TABLE 2. Comparison of specific activities for extracellular proteinases from various yeasts cultivated in synthetic medium[a]

Organism/strain	Sp act (PU/mg [dry wt] per ml) at:		
	20°C	40°C	60°C
A. oryzae 1531[b]	0	0	0
S. cerevisiae			
84	0	0	0
1352	0	0	0
Saccharomycopsis fibuligera			
1570	1.00	4.98	2.22
1571	1.84	10.54	6.57
1573	1.88	8.38	4.72
T. magnoliae 1536	6.31	11.14	1.00

[a] Yeasts were cultivated for 3 days at 27°C under conditions of aeration in a synthetic medium containing bovine serum albumin as the protein substrate (see Materials and Methods).

[b] Organism did not grow in synthetic medium.

TABLE 3. Comparison of extracellular proteinase production in various yeasts cultivated at 20°C in a 12°P brewer's wort medium[a]

Organism/strain	Biomass (mg [dry wt]/ml)	Proteinase production (PU/ml) at:		Sp act (PU/mg [dry wt] per ml) at:	
		20°C	40°C	20°C	40°C
A. oryzae 1531	7.50	1.11	3.70	0.15	0.50
S. cerevisiae					
84	8.10	0	0	0	0
1352	7.04	0	0	0	0
Saccharomycopsis fibuligera					
1570	18.50	9.72	39.70	0.53	2.20
1571	22.60	3.33	20.20	0.15	0.90
1573	23.70	4.27	27.00	0.18	1.10
T. magnoliae 1536	8.96	14.72	39.40	1.64	4.40

[a] Yeasts were inoculated at 3.5 g/liter into 30-ml volumes of 12°P brewer's wort, and, after 3 days of incubation at 20°C in shake flasks, cell-free culture supernatants were assayed for proteolytic activity at pH 4 and at the indicated temperatures (see Materials and Methods).

TABLE 4. Effect of inclusion of proteinases in 12°P brewer's wort on ethanol production and chill haze

Source of concentrate added to wort[a]	Strain	Residual proteinase activity[b] (PU/ml)	Ethanol production[c] (% vol/vol)	Haze[d] (% of control)
No addition (control)		0	4.01	100
Brewing ale control	3001	0	4.01	98
Saccharomycopsis fibuligera	1570	0.965	3.58	34
T. magnoliae	1536	1.33	3.60	32

[a] Yeasts inoculated into 12°P ale wort were incubated at 20°C with shaking for 3 days, and cell-free supernatants were then assayed for proteinase activity and concentrated approximately 100-fold (see Materials and Methods). Spent-culture supernatants from brewing ale strain 3001 were also concentrated and used as an additional nonproteolytic control.

[b] To 12°P ale wort sufficient concentrate was added to give 2 PU/ml (as determined in assays performed at pH 4 and 40°C). Volumes were then inoculated with brewing ale strain 3001 and, after 5 days of incubation at 20°C, cell-free supernatants were assayed for proteinase activity remaining at pH 4 and 40°C (see Materials and Methods).

[c] Ethanol production was determined by gas chromatographic analysis.

[d] Haze formation was determined by forcing tests conducted at 48°C (see Materials and Methods).

A series of experiments was undertaken to monitor kinetics of extracellular proteinase production in *T. magnoliae* 1536 and *Saccharomycopsis fibuligera* 1570 in relation to growth in brewer's wort (Fig. 1). In the former yeast, enzymatic activities were detected in cell-free supernatants from exponential-phase cultures, and optimal enzyme production was attained late in exponential growth (Fig. 1A). Although extracellular proteinase production in the latter yeast was also evident during logarithmic growth, enzymatic activities continued to increase even after the onset of stationary phase (Fig. 1B). The ability to secrete acid proteinases upon commencement of exponential growth is of importance given the possibility of enzyme inactivation during the course of brewer's wort fermentation (Table 4); moreover, detection of the enzymatic activities at the onset of growth suggests that the particular genes responsible are not subject to complete repression by readily assimilated carbon, nitrogen, and/or other nutrilites at concentrations present in brewer's wort. In fact, polyacrylamide gel electrophoretic analyses have indicated that *T. magnoliae* secretes at least four proteolytic enzymes regulated by induction during the first 24 h of growth (manuscript in preparation). It is at present unclear whether proteolytic activities detected during exponential growth of *Saccharomycopsis fibuligera* 1570 involve secretion of one or several acid proteinases. Nevertheless, from physiological and physicochemical viewpoints both yeasts are indicated to be suitable as candidates for use in protoplast fusion with brewer's yeast and/or as sources of DNA from which to isolate extracellular proteinase-encoding genes for use in transformation. In this regard, it is of particular interest that a gene encoding extracellular proteinase has been cloned recently from *Saccharomycopsis fibuligera* and successfully transformed into *S. cerevisiae* (39).

Large-scale static fermentations will permit further analyses of yeast extracellular proteinases in control of beer stability by facilitating a more detailed evaluation of chill haze and of various other characteristics (foam, color, taste) known to be altered by protein hydrolysis (21). After submission of this manuscript, Nelson and Young (30) reported that certain species belonging to yeast genera (*Candida*, *Debaryomyces*, and *Pichia*) not surveyed in the present study secrete proteolytic enzymes that are also effective in chill-proofing beer.

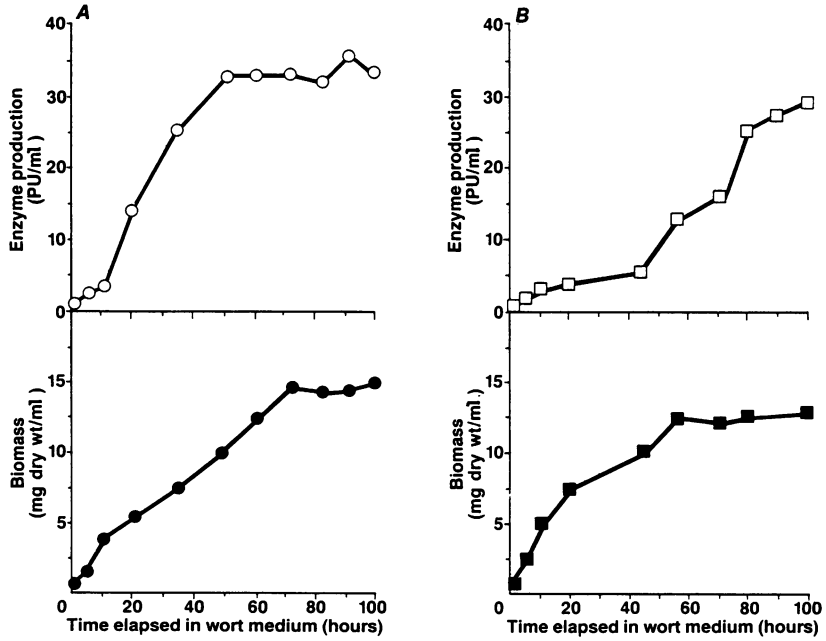

FIG. 1. Kinetics of growth and proteinase production in brewer's wort. (A) Extracellular proteinase production (○) and growth (●) of *T. magnoliae* 1536. (B) Extracellular proteinase production (□) and growth (■) of *Saccharomycopsis fibuligera* 1570. Cells were inoculated at 3.5 g/liter into 200-ml volumes of 12°P brewer's wort in 1-liter shake flasks. Wort cultures were maintained at 20°C, and, at the indicated times, were sampled for biomass and extracellular proteinase production. Assays were performed at pH 4 and 40°C by using denatured hemoglobin as the substrate (see Materials and Methods). Each value plotted represents the average of determinations made in triplicate from each of two experiments.

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