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The Mannoprotein of *Saccharomyces cerevisiae* Is an Effective Bioemulsifier

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The mannoprotein which is a major component of the cell wall of *Saccharomyces cerevisiae* is an effective bioemulsifier. Mannoprotein emulsifier was extracted in a high yield from whole cells of fresh bakers' yeast by two methods, by autoclaving in neutral citrate buffer and by digestion with Zymolase (Miles Laboratories; Toronto, Ontario, Canada), a β-1,3-glucanase. Heat-extracted emulsifier was purified by ultrafiltration and contained approximately 44% carbohydrate (mannose) and 17% protein. Treatment of the emulsifier with protease eliminated emulsification. Kerosene-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 5% sodium chloride or up to 50% ethanol in the aqueous phase. In the presence of a low concentration of various solutes, emulsions were stable to three cycles of freezing and thawing. An emulsifying agent was extracted from each species or strain of yeast tested, including 13 species of genera other than *Saccharomyces*. Spent yeast from the manufacture of beer and wine was demonstrated to be a possible source for the large-scale production of this bioemulsifier.

Despite their typically high production costs, emulsifiers derived from natural sources may have certain advantages over the chemically synthesized emulsifying agents. Due to the increasing consumer demand for natural products, bioemulsifiers may eventually become cost-effective for various applications.

All of the known bioemulsifiers from yeasts are produced by yeasts that are capable of growing on water-immiscible substrates such as alkanes or oils. These yeasts include *Candida petrophilum* (12), *Candida tropicalis* (15, 16), *Torulopsis petrophilum* (9), and *Yarrowia lipolytica* (also known as *Candida lipolytica* and *Endomycopsis lipolytica* [6, 7, 20, 22]). The emulsifying agents are produced only in the presence of the water-immiscible substrates and appear to facilitate their metabolism (20).

The bioemulsifiers were extracellular or bound to the cell surface, and all contained carbohydrate and peptide material. Various methods have been used to isolate the bioemulsifiers. These include enzymatic digestion (15, 16), foam fractionation (20), and repeated extractions with chloroform-methanol (6, 7). The low yields obtained, the requirement for hydrocarbon substrates, and the complex procedures for preparation make commercial development of the known yeast-derived bioemulsifiers unlikely.

We undertook this study to increase the yield of yeast-derived bioemulsifiers. We describe a novel bioemulsifier which can be extracted simply and with very good yield from *Saccharomyces cerevisiae*, bakers' yeast. This bioemulsifier was identified as mannoprotein, a major component of the yeast cell wall. The emulsification characteristics of mannoprotein were determined. Emulsifying agents were extracted from a variety of yeasts, including the spent yeast from the production of beer and wine, from yeasts used for single-cell protein, and from 13 yeasts of diverse genera.

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals used were of reagent grade. Zymolase 20T was from Miles Laboratories (Toronto Ontario, Canada); pronase was from Boehringer Mannheim Co. (Montreal, Quebec, Canada). Mineral oil (USP grade 70) was donated by Witco (Montreal, Quebec, Canada). Kerosene and corn oil were purchased in retail stores.

Yeast strains and growth media. Fresh bakers' yeast (*S. cerevisiae*) was obtained from Lallemand Inc. (Montreal, Quebec, Canada). Spent yeast was obtained by allowing the yeast to settle after the primary fermentation from small batches of beer and wine inoculated with commercially available yeast strains. Other yeast species were kindly provided by R. Latta of the Culture Collection of the National Research Council of Canada (Ottawa, Ontario, Canada). *Candida utilis* Y900 was from the collection of Weston Research Centre (Toronto, Ontario, Canada). Pure cultures of all yeast strains were maintained at 4°C on slopes of YPG medium (0.5% [wt/vol] yeast extract, 1% peptone, 1% glucose, and 1.5% agar). Yeasts were grown in YPG liquid medium for 2 days on a rotary shaker (150 rpm) at 28°C. For screening of emulsifier production by intact cells, strains were grown in YCG medium in which 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) replaced peptone.

Extraction and purification of emulsifier. Emulsifier was extracted from yeasts by a modification of the method of Peat et al. (21) for the extraction of mannan. A reducing agent, potassium metabisulfite, was included in the heat-extraction buffer as it increased the emulsifier yield. Fresh bakers' yeast or spent yeast was washed twice in distilled water and suspended at 20% (wet weight; by volume) in 0.1 M potassium citrate–0.02 M potassium metabisulfite. The pH of the mixture was adjusted to 7.0. After the mixture was autoclaved for 3 h (121°C), it was centrifuged at 5,000 × *g* for 10 min at ambient temperature. For crude preparations of emulsifier, 3 volumes of 95% ethanol containing 1% (vol/vol) acetic acid was added to the supernatant. After the solution was cooled at 4°C for 16 h, the precipitate was collected by centrifugation (10,000 × *g*). The crude emulsifier was dried and stored at room temperature. Crude emulsifier from other yeast species was prepared similarly.

For the large-scale preparation of purified emulsifier, the supernatant was concentrated by passage through a hollow-

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fiber ultrafiltration unit (Romicon, Woburn, Mass.) which had a molecular weight exclusion limit of 10,000. The volume of permeate was replaced with distilled water, until 5 volumes had passed through the filter, and then the sample was concentrated to approximately 1/10th the original volume. The purified emulsifier was stored in liquid form at 4°C or was precipitated with ethanol and dried as described above.

Assay of emulsification activity. Emulsification was evaluated as described previously (1, 9). The material to be tested was dissolved in 4 ml of distilled water in a test tube, 6 ml of kerosene (or another water-immiscible liquid) was added, and the tube was vortexed to homogeneity. After 1 h the proportion of kerosene emulsified was compared with the total volume of kerosene added. This parameter was known as the percentage of the kerosene phase that was emulsified. The content of kerosene in the emulsion was calculated by dividing the volume of kerosene in the emulsion phase by the total volume of the emulsion. In the absence of an emulsifying agent, emulsions generated by vigorous mixing separated completely within 1 h.

Screening for the production of emulsifier by intact cells was done by mixing 2 ml of YCG broth culture with 2 ml of water and 6 ml of kerosene and vortexing as described above. Unlike YPG medium, uninoculated YCG medium did not emulsify kerosene.

Enzyme digestions. For the Zymolase 20T treatment, fresh yeast (Lallemand) or yeasts grown freshly on YPG medium were washed twice in distilled water. The cells were suspended at a concentration of 6 mg [wet weight] of yeast per ml in buffer, 67 mM phosphate (pH 7.5), 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride. Zymolase 20T was dissolved in the same buffer and was added to a final concentration of 1 to 2 U/ml (17). The mixture was incubated for 3 h at 25°C with gentle shaking. Insoluble material was then removed by centrifugation for 10 min at 5,000 × *g*. Crude emulsifier was precipitated from the supernatant with ethanol as described above and dried.

For protease treatment, emulsifier (1.4 mg/ml) was dissolved in 50 mM Tris hydrochloride (pH 7.5), and a small volume of toluene was added. Pronase was added to a final concentration of 30 μg/ml, and the mixture was incubated for 24 h at 30°C. This material was tested for emulsification as described above.

Analytical methods. Protein was determined by the dye-binding assay (5), with bovine serum albumin used as the standard. Soluble protein was also measured by determining the A_{280} in a double-beam spectrophotometer (124; Coleman). Carbohydrates were determined by the phenol-sulfuric acid procedure (11), with glucose used as the standard. The sugar components of acid-hydrolyzed emulsifier (0.35 g of emulsifier, 90 ml of 0.1 N H_2SO_4; 121°C, 1 h) were identified by high-pressure liquid chromatography at the Weston Research Centre (Toronto, Ontario, Canada).

Surface tensions of solutions of purified emulsifier in distilled water were measured with an autotensiomat (Fisher Scientific Co., Pittsburgh, Pa.) (1).

Properties of the emulsions. Stabilization of emulsions by emulsifier from bakers' yeast was evaluated over a range of chemical and physical conditions. Purified emulsifier was dissolved in distilled water, and the pH was adjusted to between 2 and 11 with HCl or KOH. After kerosene was added, tubes were vortexed and the emulsions were measured after 1 h. The emulsifier was tested with 0, 0.5, 1.0, 2.5, and 5% (wt/vol) sodium chloride and 0, 10, 25, and 50% (vol/vol) ethanol in the aqueous phase.

For the evaluation of stability, emulsions containing 0.14% (wt/vol) emulsifier in distilled water and kerosene were incubated at 4°C and room temperature for an extended period. Emulsions were subjected to three cycles of heating (40°C, 16 h) and cooling (room temperature, 8 h). The ability of emulsions to withstand three cycles of freezing (−18°C, 16 h) and thawing (23°C, 8 h) was tested in the presence of solutes, including potassium citrate, glucose, potassium chloride, and calcium chloride.

The ability of purified emulsifier from bakers' yeast to emulsify hydrocarbons and organic solvents was tested.

RESULTS

Isolation and identification of emulsifier. Two extraction methods were used to obtain emulsifying agent from cells of *S. cerevisiae*, bakers' yeast. In the first method, washed yeast cells were autoclaved in neutral buffer. An emulsifying agent was released into the aqueous medium and was concentrated by ultrafiltration. The emulsifier was precipitated with 3 volumes of ethanol–1% acetic acid.

The ultrafiltered emulsifier from bakers' yeast contained approximately 44% carbohydrate and 17% protein (Table 1). Analysis of acid-hydrolyzed, purified emulsifier by high-pressure liquid chromatography demonstrated that mannose was the major component of the carbohydrate fraction. No additional sugars were detected. Treatment of the purified emulsifier with the proteolytic enzyme pronase eliminated its ability to stabilize emulsions.

Crude emulsifier was extracted from washed spent beer and wine yeast by the method described above for bakers' yeast. The yield, the protein and carbohydrate content, and the ability to emulsify were comparable to those of the crude emulsifier from bakers' yeast (Table 1).

In the second method, treatment of bakers' yeast with Zymolase enzyme also released an emulsifying agent (Fig.

TABLE 1. Yield and characterization of emulsifer extracted from commercial strains of *S. cerevisiae*

Source	% Yield (g/g [wet wt] of cells) of emulsifier	% Protein content[a]	% Carbohydrate content[b]	Emulsification of a 0.16% (wt/vol) solution	
				% Kerosene phase emulsified	% Kerosene in emulsion
Crude emulsifier from bakers' yeast	17.8	5.4	17	97	75
Purified emulsifier from bakers' yeast	8	17	44	98	78
Crude emulsifier extracted from bakers' yeast by Zymolase	16.7	16.6	38	92	74
Crude emulsifier from spent wine yeast	19.4	4.5	20.3	97	73
Crude emulsifier from spent beer yeast	17.8	2.3	19.2	95	76

[a] Measured by the dye-binding assay (5), with bovine serum albumin used as the standard.
[b] Measured by the phenol test (11), with glucose used as the standard.

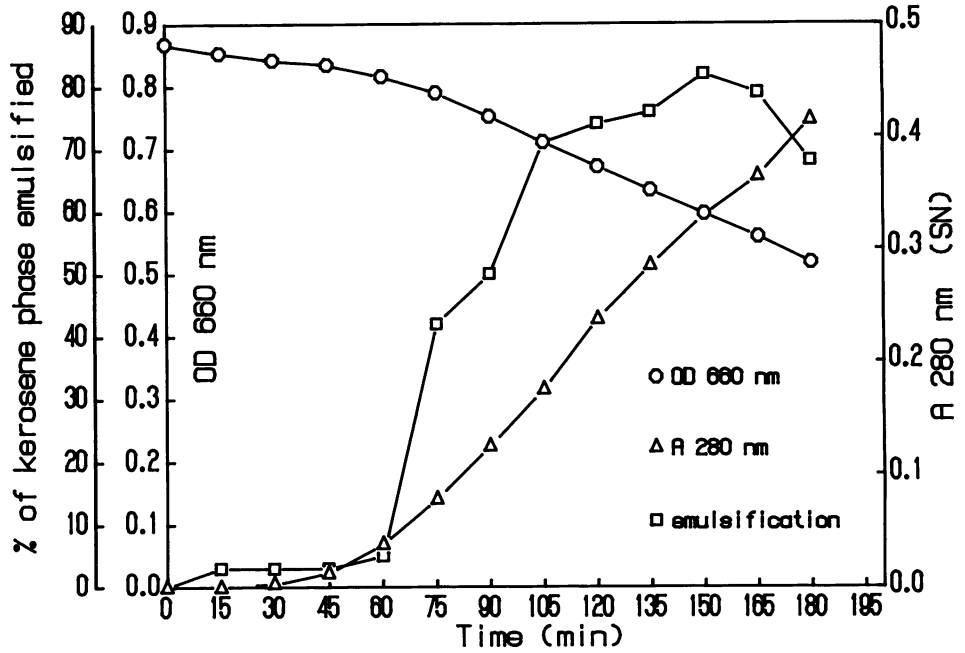

FIG. 1. Release of emulsifier and soluble protein by Zymolase digestion of *S. cerevisiae*, bakers' yeast. Treatment of the digestion supernatant (180 min) with the protease pronase eliminated emulsification. OD_{660}, Optical density at 660 nm.

1). Lysis of the yeast cell walls by Zymolase was monitored by determining the decrease in optical density at 660 nm of the digestion mixture. As lysis proceeded, the concentration of soluble protein (A_{280}) and the emulsifying capacity of the digestion mixture supernatant increased (Fig. 1). Treatment of the digestion supernatant at 180 min with the enzyme pronase completely eliminated its ability to stabilize emulsions. The composition of emulsifier extracted by Zymolase treatment was similar to that of the purified heat-extracted emulsifier (Table 1).

Properties of the emulsions. The purified emulsifier was tested for its stabilization of emulsions under a range of chemical and physical conditions which might be encountered in various applications.

The relation between the fraction of the kerosene phase emulsified and the concentration of purified emulsifier (Fig.

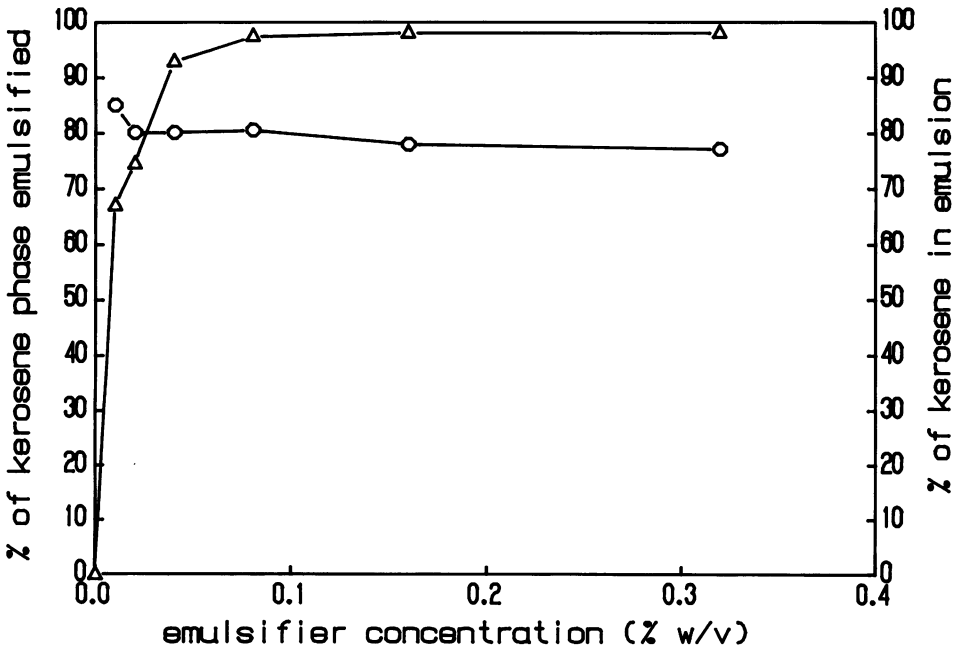

FIG. 2. Relation of the fraction of the kerosene phase emulsified (△) and the percentage of kerosene (by volume) in the emulsion (○) to the concentration of purified emulsifier.

2) provides a basis for comparison of emulsification capacity under different physical and chemical conditions. By using kerosene and distilled water as the the two initial phases, a maximum of 97% of the kerosene phase was emulsified with 0.08% (wt/vol) or more of the purified emulsifier.

To facilitate the detection of the possible detrimental effects of pH, sodium chloride, or ethanol on emulsification, emulsions were made with 0.05% (wt/vol) purified emulsifier (Table 2). The pH of the aqueous phase had little effect on the amount of the kerosene phase emulsified between pH 2 and 11 (Table 2). Below pH 4, the emulsifying agent was insoluble, but it nonetheless stabilized the emulsions. In the presence of 0.5 to 5.0% (wt/vol) sodium chloride in the aqueous phase, stable emulsions were formed. Emulsions made in the presence of salt contained a smaller proportion of the oil phase than did those without salt (Table 2). Stable emulsions were formed in the presence of up to 50% (vol/vol) ethanol in the aqueous phase (Table 2).

Physical treatments known to reduce emulsion stability were tested on emulsions containing more than 0.08% (wt/vol) purified emulsifier. Three cycles of freezing at −18°C for 16 h and thawing at 23°C for 8 h broke the emulsions containing 0.14% (wt/vol) purified emulsifier (Table 3). The addition of 30 mM tripotassium citrate stabilized the emulsions to freezing and thawing (Table 3). In addition, 5 mM calcium chloride or 10 mM glucose, sodium chloride, or potassium chloride prevented the breakage of emulsions containing 0.14% (wt/vol) purified emulsifier during three cycles of freezing and thawing (data not shown).

Emulsions were not disrupted by three cycles of heating to 40°C (16 h) and then cooling to 23°C (8 h). During storage at 4°C, the emulsion height did not change over a 3-month period (Table 3). Emulsion stability during storage at room temperature was improved by the presence of citrate (Table 3).

The emulsifier extracted from bakers' yeast emulsified all of the oils, hydrocarbons, and organic solvents tested (Table 4). Although chloroform appeared to be emulsified, the water content in this emulsion was very low. Emulsions with most other nonaqueous liquids tested contained between 10 and 30% water (Table 5).

TABLE 2. Effect of pH, sodium chloride, and ethanol on emulsions with 0.05% (wt/vol) purified emulsifier

Parameter	% Kerosene phase emulsified	% Kerosene in the emulsion
pH		
2	91	79
3	95	73
4	95	74
5	97	77
6	97	82
7	97	84
8	95	83
9	97	80
10	95	83
11	97	86
% NaCl		
0	95	83
0.5	97	73
1.0	95	76
2.5	97	73
5.0	97	72
% Ethanol		
0	97	80
5	97	84
10	97	86
25	97	77
50	97	82

TABLE 3. Stability of emulsions to three cycles of freezing and thawing and to storage at room temperature and at 4°C in the presence of tripotassium citrate[a]

Citrate concn (mM)	% Kerosene phase emulsified			
	Initial	After 3 freeze-thaw cycles	After 144 h at room temp	After 3 months at 4°C
0	91	15	24	96
2.5	96	8	21	98
5	96	5	38	98
10	96	38	49	98
20	96	89	56	96
30	96	96	64	96
60	96	94	79	96
100	96	94	93	96
250	98	98	100	98

[a] Emulsions were made with 0.14% (wt/vol) purified emulsifier.

The emulsions generated by the emulsifying agents extracted from yeast were of the oil-in-water variety. The surface tension of a 0.04% (wt/vol) solution of purified heat-extracted emulsifier was 61 mNm^{-1}; a 0.4% solution had a surface tension of 55.5 mNm^{-1}. The surface tension of distilled water was 74 mNm^{-1}.

Extraction of emulsifier from diverse yeast species. Broth cultures of 8 yeast strains of 14 were able to emulsify kerosene (Table 6). Yeasts which produced cell-bound or extracellular emulsifiers included *Candida tropicalis*, *Candida utilis*, *Dekkera naardenensis*, *Geotrichum penicillatum*, *Hansenula anomala*, *Kluyveromyces fragilis*, *Metschnikowia lunata*, and *Pachysolen tannophilus*. The heat extraction procedure allowed the isolation of emulsifying agent from all of the yeast strains tested (Table 6). The yields of crude emulsifier varied from 2.9 g/g (wet weight) of cells for *Torulopsis ernobii* to 17.8 g/g (wet weight) of cells for *Saccharomyces cerevisiae*. Protein was detected in all of the crude emulsifiers. The emulsifiers from *D. naardenensis* and *M. lunata* contained approximately 3 times as much protein as that from *S. cerevisiae*; however, they emulsified less kerosene per unit weight (Table 6).

DISCUSSION

Two procedures which solubilize mannoproteins from the cell wall of *S. cerevisiae* (21, 25) have been shown to release

TABLE 4. Oils, alkanes, and organic solvents emulsified with various concentrations of purified emulsifier

Nonaqueous phase	% Nonaqueous phase emulsified with emulsifier concn (% [wt/vol]) of:		
	0.04	0.2	0.4
Kerosene	89	97	97
Vegetable oil	60	82	90
Mineral oil	75	78	100
Pentane	8	80	88
Octane	75	89	100
Hexadecane	83	100	100
Ethyl acetate	0	93	93
Chloroform	100	100	100
Dichloromethane	100	100	100
Toluene	84	98	100
Xylene	90	100	100

TABLE 5. Proportion of the nonaqueous phase in each of the emulsions

Nonaqueous phase	% Nonaqueous phase in the emulsions with emulsifier concn (% [wt/vol]) of:		
	0.04	0.2	0.4
Kerosene	81	80	80
Vegetable oil	45	60	71
Mineral oil	63	51	62
Pentane		91	91
Octane	90	80	82
Hexadecane	86	74	69
Ethyl acetate		83	81
Chloroform	100	100	100
Dichloromethane	100	90	90
Toluene	83	77	74
Xylene	87	74	74

an emulsifying agent simultaneously. After purification, the emulsifying agent had a molecular weight greater than 10,000. It contained mannose and a protein which was necessary for its action as an emulsifier. The emulsifying agent is mannoprotein.

Although mannoprotein has not been used previously as an emulsifier, its structure has been described in the literature. Two general classes of mannoproteins have been identified. Mannoproteins with a structural role contain approximately 90% mannose and 5 to 10% protein (4). Structural mannoproteins are interspersed within a network of glucan to form the outer layer of the cell wall of *S. cerevisiae* (23). The second class of mannoproteins, the mannan enzymes, contain 50 to 70% mannose and the remainder is protein (4). The mannan enzymes include invertase, glucosidase, melibiase, phosphatase, and proteases (2) and appear to be periplasmic since they do not cosediment with cell wall fragments after the mechanical breakage of cells (23).

The structural mannoproteins are among the most abundant macromolecules of the yeast cell; they make up 12 to 14% of the cell dry weight (18, 19, 24). From the high yield of purified emulsifier (8% of the weight of fresh bakers' yeast), it is likely that this consists mostly of structural mannoproteins. The ratio of mannose to protein in the purified emulsifier was 44:17, or 72%, which is slightly higher than that of the mannan enzymes (4). A portion of the carbohydrate may have been lost after fragmentation due to the high shearing forces generated during ultrafiltration.

The heat-extraction procedure is expected to solubilize the structural mannoproteins in the outer layer of the cell wall. Treatment with Zymolase degrades the glucan component of the cell wall, and would therefore be expected to release both the structural mannoproteins as well as the mannan enzymes from the periplasm. This interpretation is supported by the higher proportion of protein in the Zymolase-extracted emulsifier compared with that in the crude heat-extracted emulsifiers (Table 1).

Mannoproteins consist of mannose polymers covalently attached to a protein backbone. The structure of mannoprotein explains its mode of action as an emulsifying agent. The ability of various proteins to stabilize oil-in-water emulsions has been known for a long time (13). Since protease treatment abolished emulsification, the protein component of mannoprotein was essential for its emulsification properties. The presence of hydrophilic mannose polymers covalently attached to the protein backbone provides mannoprotein with the amphiphilic structure common to surface-active agents and many effective emulsifiers (8, 10).

Because of the wide range of conditions under which mannoprotein emulsifier was effective, it is expected that this bioemulsifier could be used in a variety of applications. The ability of emulsions to withstand freezing and thawing is a particularly useful property.

Comparison of mannoprotein with known bioemulsifiers from yeasts. The mannoprotein emulsifier of *S. cerevisiae* has a chemical composition similar to that of the bioemulsifiers produced by alkane-grown yeasts. Purified liposan emulsifier from *C. lipolytica* is a glycoprotein which contains 83% carbohydrate and 17% protein (7). Its major component has a molecular weight of 27,600. The emulsifiers from *C. petrophilum* and *E. lipolytica* also contain carbohydrate and protein (12, 22). Treatment of the emulsifier from *E. lipolytica* with pronase destroys its ability to emulsify (22). These emulsifiers may also be cell wall glycoproteins, and it may be possible to increase their yield by use of the extraction procedures described here.

Many yeast species have a cell wall similar to that of *S. cerevisiae* and contain glycoproteins with a structural role similar to that of the mannoprotein in *S. cerevisiae* (4, 14).

TABLE 6. Heat extraction of emulsifier from various yeast species

Yeast species[a]	Emulsification by whole broth	% Yield (g/g [wet wt] of cells) of emulsifier	% Protein content	Emulsification of a 0.08% (wt/vol) solution	
				% Kerosene phase emulsified	% Kerosene in emulsion
Candida tropicalis NRCC 2774	++	8.5	6.1	64	77
Candida utilis Y900	+	8.7	7.2	68	93
Debaromyces marama NRCC 2621	−	7.6	5.6	57	78
Dekkera naardenensis NRCC 2565	+	7	18.8	78	73
Geotrichum penicillatum NRCC 2623	++	14.5	2.7	64	83
Hansenula anomala NRCC 2505	++	8.3	7.9	65	80
Kluyveromyces fragilis NRCC 2475	+	10	7.6	51	73
Lipomyces starkeyi NRCC 2519	−	11.6	8.2	64	74
Metschnikowia lunata NRCC 2545	++	14.6	13.8	59	79
Pachysolen tannophilus NRCC 2507	+	9	6.5	49	75
Pichia abadieae NRCC 2644	−	18	6.6	24	60
Saccharomyces cerevisiae	−	17.8	5.4	86	77
Torulopsis ernobii NRCC 2663	−	2.9	8.4	27	83
Zygosaccharomyces fermentati NRCC 2743	−	14.8	7	59	76

[a] NRCC numbers indicate the strain reference number at the Culture Collection of the National Research Council of Canada.

The heat-extraction method was used to extract cell wall glycoproteins that are emulsifiers from each of 13 yeast species tested, in addition to *S. cerevisiae*. None of these yeasts were grown on hydrocarbon substrates.

As an emulsifying agent, mannoprotein from *S. cerevisiae* may present certain advantages. The difficulty of removing residual hydrocarbons from bioemulsifiers from alkane-grown yeasts would preclude their use in certain applications. Since *S. cerevisiae* is edible and is used in the manufacture of food and beverage products, it is expected that a mannoprotein bioemulsifier would be nontoxic.

The yield of mannoprotein emulsifier was far greater than those of previously known bioemulsifiers from yeasts. The yield of mannoprotein emulsifier was approximately 8% of the wet weight of yeast biomass. The yield of liposan from a 300-ml broth culture of *C. lipolytica* was 50 mg (7); from an equivalent culture (containing 15 g [wet weight] of *S. cerevisiae* cells), the yield of mannoprotein emulsifier would be approximately 1.2 g. The yields of bioemulsifiers from *C. tropicalis*, *C. petrophilum*, and *E. lipolytica* were also a very small proportion of the yeast biomass (12, 16, 22).

Mannoprotein can be extracted in high yield from yeast cells and processed to a relatively pure dry form by simple procedures. Both the heat extraction and purification by ultrafiltration are amenable to large-scale operations. The spent yeast produced as a by-product in the brewing and wine industries could provide a source of raw material for the mass production of mannoprotein emulsifier. This would eliminate the need to grow the yeast specifically for the production of emulsifier, as is the case with the bioemulsifiers from other yeasts. Currently, spent yeast has low value and is dried for use as a protein supplement in animal feed or is treated as waste with a high biological oxygen demand. In addition, it may be possible to extract glycoprotein bioemulsifier as a high-value product from fodder yeasts that are used subsequently for single-cell protein.

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