Use of Immunological Methods in the Interspecies Relationships of Genus *Saccharomyces* and its Possible Application in Detection of Contaminating Strains[1]

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ABSTRACT

Immunological methods have been used in order to detect contaminating yeast and metabolic variants during the brewing process. This paper describes some of our results using this methodology and comparing established brewing yeast strains with several contaminants isolated during the brewing process. Six yeast strains of the genus *Saccharomyces* were used, two of them well characterized: *S. uvarum* CC15i30 and *S. cerevisiae* var. *ellipsoideus*. The other four strains were isolated during the brewing process; two of them (3/24 and 5/24) produce diacetyl; the other two strains (A51 and A66) produce variable amounts of phenol. Agglutination technique was rather coarse for our purposes and we decided to rely mostly on the more powerful analytic immunodiffusion technique in gel, described by Ouchterlony. Two series of tests were run: in one, antigens were placed in the center well and the antisera in the surrounding wells, and in the other, the reverse was done. The first test differentiated *S. uvarum* from *S. cerevisiae* var. *ellipsoideus*, since *S. uvarum* has antigens which are not shared with *S. cerevisiae* var. *ellipsoideus*. Antigens of contaminants 3/24, 5/24, A51, and A66 show a considerable cross reactivity with their antisera, but seldom react with *S. uvarum* and *S. cerevisiae* var. *ellipsoideus* antisera. By means of the second test, with *S. uvarum* antiserum placed in the center well, *S. uvarum* was differentiated from *S. cerevisiae* var. *ellipsoideus*, but when antiserum from *S. cerevisiae* var. *ellipsoideus* was placed in the center well, it was not possible to differentiate them.

Key words: *Antigen-antibody reactions, Antigenic relations, Immunodiffusion, Immunological pattern, Yeast strains.*

Contamination of the brewing process by wild yeast or by metabolic variants is an annoying problem yet to be solved. However, the spoilage of large volumes of beer can be avoided if one is able to determine contamination early enough in the brewing process. In other situations, it is compulsory to determine the taxonomic situation of the contaminant yeast using morphological colony, metabolic, and other criteria (15). This last procedure, although very informative, is sometimes variable, cumbersome, and time-consuming. These are important reasons to justify the search for other methods that could provide tools to rapidly and reliably differentiate a contamination with wild yeast.

One of the approaches that has been explored during the last few years is the extraordinary specificity of antigen-antibody reactions as applied to identification of bacteria and pathogenic fungi in diagnostic medical bacteriology and mycology (8,10,11,14). This methodology has greatly speeded up diagnosis in clinical medicine and it appears to be an important advance (2,7). Several techniques have been used to demonstrate antigen-antibody reaction, namely, agglutination, complement fixation, precipitation in different systems, immunofluorescence, etc. (1,5,9).

A review of the current methods of detecting contamination and spoilage in the brewing process finds only a very few papers dealing with immuno-serological procedures applied to this problem. One reason might be that the taxonomic status of sporogenous and nonsporogenous (10,12) yeasts is confusing, and another could be that immunology is such a young science that it has not been used in all its potential. Whatever the reason for the limited utilization of this valuable methodology, several workers have used these procedures for identification of yeasts, as outlined by Campbell (3,4), Campbell and Brudzynski (6), and Sandula *et al.* (16), with the purpose of studying the antigenic relations of brewing yeasts. Immunofluorescence has also been applied by Richards and Cowland (13) for the rapid detection of contaminants in the brewing process.

The immunoserological approach has been used in this work in order to detect contaminant yeasts and metabolic variants during the process. In some cases, the interest was in determining the relation of a particular isolate to the original fermentation strain. At the beginning, an agglutination technique was used, but it was found to be rather coarse for our purposes and it was decided to rely mostly on the more powerful analytic immunodiffusion in gel, described by Ouchterlony (7). In this work, results obtained using this methodology and by comparing established brewing yeast strains with several contaminants isolated during the brewing process are reported.

METHODS

Yeast Strains

Six yeast strains of the genus *Saccharomyces* were used, two of them taxonomically well characterized: *Saccharomyces uvarum* CC15i30, and *S. cerevisiae* var. *ellipsoideus* from our own collection. The other four strains used in the experimental work were isolated during the brewing process; two of them, strains 3/24 and 5/24, at the peak of fermentation in our pilot plant, and the other two, A51 and A66, from yeast left at storage. Strains 3/24 and 5/24 produce high concentrations of diacetyl; A51 and A66, isolated during a quality control sampling, were recognized by colony morphology and proved by fermentation to produce variable amounts of phenol.

The strains were cultured on wort agar and propagated in flasks of 500 ml with 150 ml of sterile MYGP (malt extract, 3 g; yeast extract, 3 g; micological peptone, 5 g; glucose, 10 g; and distilled water, 1000 ml) -aerated broth, incubated at room temperature for 48 hr. The yield of cells was centrifuged and washed three times with sterile MYGP broth and resuspended in 100 ml of 0.15*M* NaCl.

Antigen Preparation

For preparation of the antigens, the cellular suspensions were diluted with sterile 0.15*M* NaCl solution to obtain a count of 1×10^8 cells/ml; these suspensions were cooled at 0° C and sonicated with Biosonic III (Bronwill Scientific, Rochester, NY 14601) equipment at a frequency of 20 kilocycles/sec for 8 min, maintaining the temperature at approximately 0° C by adding Dry Ice. The efficiency of sonication was controlled by observing microscopically the ratio of disrupted yeast cells during and at the end of the process. (About 90% of the cells were disrupted.) The cell walls were washed three times with sterile 0.15*M* NaCl by centrifugation and resuspended to the original volume.

Sedimentation was allowed for 30 min to separate the coarse materials, and the supernatant was recovered for the experiments. This supernatant was used as antigen for immunodiffusion tests as an immunogen for rabbits to obtain the antisera.

Antisera Preparation

Two New Zealand rabbits were selected for each yeast antigen. Each antigen was mixed previously with complete Freund adjuvant and immunization was achieved by subcutaneously injecting 1.0 ml of the antigen mixture. To accomplish immunization for each antigen, the remaining five injections were applied weekly. The first injection was applied using 1 ml, the next two injections using 2 ml, and the last two injections using 3 ml. This series of injections was repeated for each antigen. Ten days after the last injection, the

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rabbits were bled from the marginal vein. The serum was separated, merthiolate was added to prevent contamination, and storage was at 1°–2°C.

The sera were tested with their specific antigens by an agglutination technique to determine their antibody titer. Petri dishes (50-mm i.d.) were used for the immunodiffusion test. The gel was prepared dissolving 1.0 g of Oxoid-Ion No. 25 Agar (from Oxoid Limited, London, or Wilson Diagnostic, Inc., Glenwood, IL 60425) in 90 ml of 0.15*M* NaCl buffered with 0.05*M* phosphate (NaH_2PO_4, Na_2HPO_4) at pH 7.2. This mixture was heated in a boiling water bath until a clear solution was obtained, which was then autoclaved for 15 min at 15 lb/sq. in. After cooling to 45°C, 1 ml of 1% merthiolate solution was added and the volume made up to 100 ml with double distilled water. Finally, 5 ml of this agar gel was poured into each petri dish and, after hardening, wells were made with a cork borer 5 mm in diameter at a distance of 8 mm from center to center. The bottoms of the wells were sealed with hot agar.

Two series of tests were run: one in which the antigens were placed in the center well and the antisera in the surrounding wells; the other in which antiserum was placed in the center well and the antigens were placed around it.

RESULTS

Figure 1 shows the pattern used in most of the experiments, in relation to the distance among wells.

Since *S. uvarum* strain CC15i30 is our main working species, it is interesting to consider the behavior of its antigen complex against antisera of the other strains which were used in this work.

A line of precipitation between the antigen and its homologous antiserum, and also between the antigen and the antisera for A51 and *S. cerevisiae* var. *ellipsoideus*, is shown in Fig. 2. No other reactions were obtained with this distribution. The lines among these strains are continuous but cannot be considered as identity lines.

Another test modifying the distances between wells of the experiment just mentioned shows some continuity in line given by A51 and *S. cerevisiae* var. *ellipsoideus*. Furthermore, this line is not continuous with 15i30. This must be assigned to a specific antigen-antibody line, which is not shown by the others. Since there are different antigens in the yeast 15i30, the experiment could be used to differentiate among these strains (see Fig. 3). The reactions of var. *ellipsoideus* antigen complex with its own antisera and with antisera of the other strains are shown in Fig. 4. We have found a

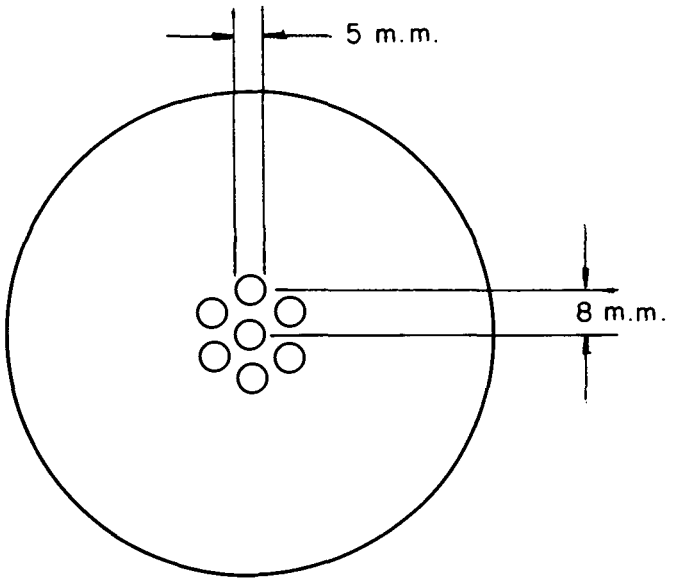

Fig. 1. Pattern used in the experiments.

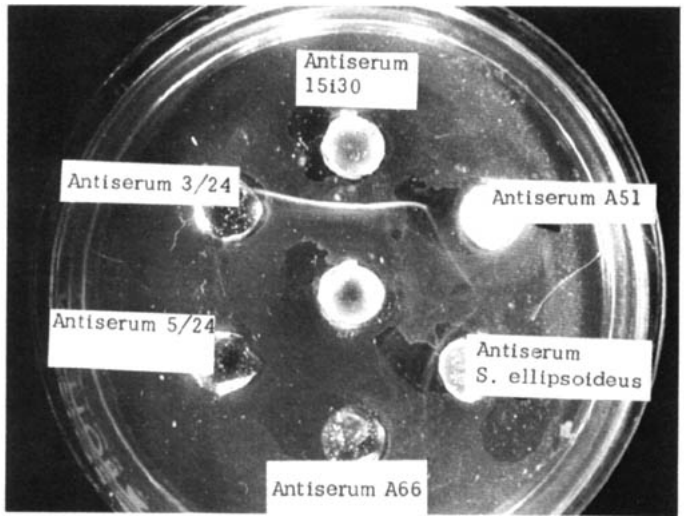

Fig. 2. Center well. Antigen 15i30.

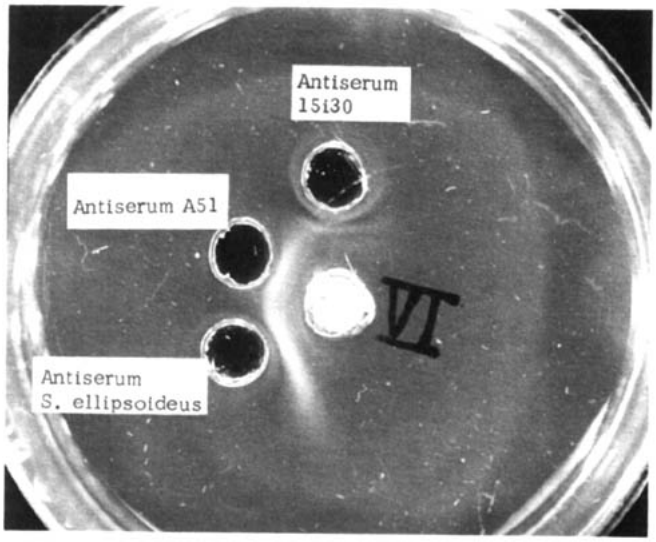

Fig. 3. Center well. Antigen 15i30.

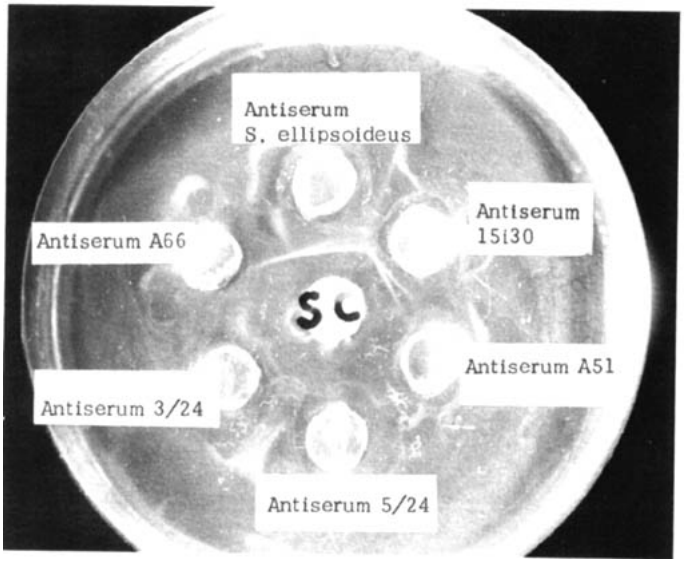

Fig. 4. Center well. Antigen *S. ellipsoideus*.

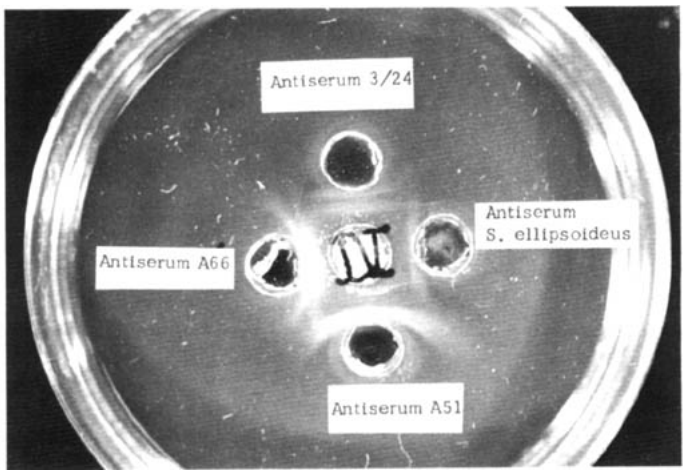

Fig. 5. Center well. Antigen 3/24.

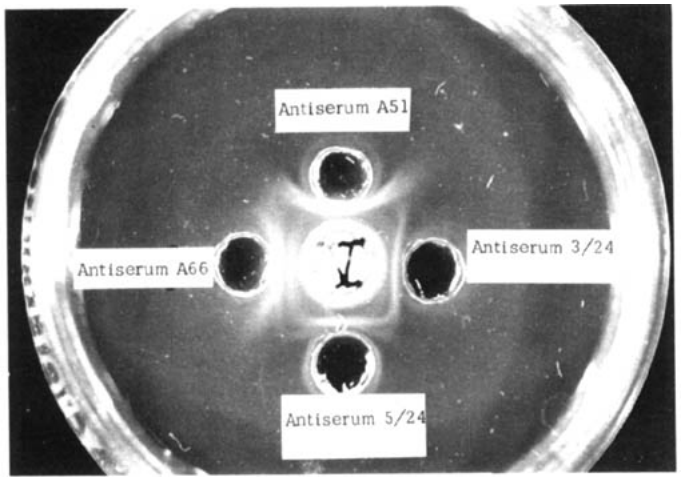

Fig. 6. Center well. Antigen A51.

reaction of partial identity between antisera to *ellipsoideus* and *uvarum* strains (meaning that we have a very similar antigen but with two different determinants). The reaction of the *ellipsoideus* antigen and antiserum A51 is also apparent.

Antigens from the contaminant strain 3/24 are not shared with antigens from other contaminant strains, as their antisera have proved. This is depicted in Fig. 5.

The antigen of another contaminant strain (A51) shows a complex antigen-antibody pattern against its homologous antiserum and contaminant strains A66, 3/24, and 5/24 (Fig. 6). This means that strain A51 has antigens that also exist in the other strains, but they are of different specificity. There may be some sort of relation, since all these strains belong to the genus *Saccharomyces*, although they behave as different antigen-antibody systems.

Table I presents a simplification of the basic relations among other strains tested, placing the antigen in the center well against all antisera. Antigen 15i30 reacts with its homologous antiserum and also against *ellipsoideus* and A51 antisera, but not with the others. The antigen from *ellipsoideus* reacts with 15i30, with its homologue, and also with A51.

Antigens of contaminants 3/24, 5/24, A51, and A66 show a considerable cross reactivity with their antisera. In some cases, they present identity reactions while, in others, independent antigen-antibody lines reveal the presence of several unrelated antigens. The antigens of these contaminants seldom react with *S. uvarum* 15i30

TABLE I
Basic Relationships among Strains
(Antigens in Center Well)

	Antiserum					
Antigen	*S. uvarum* **(15i30)**	*S. cervisiae* **var.** *ellipsoideus*	**3/24**	**5/24**	**A51**	**A66**
S. uvarum (15i30)	SHR[a]	SR[b]	...	...	SR	...
S. cerevisiae var. *ellipsoideus*	PIR[c]	SHR	...	...	IR[d]	...
3/24	...	...	SHR	SR×2	SR	SR IR
5/24	...	IR	...	SHR	IR	IR
A51	...	...	SR	IR	SHR	SR×2
A66	SR	...	SR	...	...	SHR IR

[a]SHR = Specific homologous reaction.
[b]SR = Specific reaction.
[c]PIR = Partial identity reaction.
[d]IR = Identity reaction.

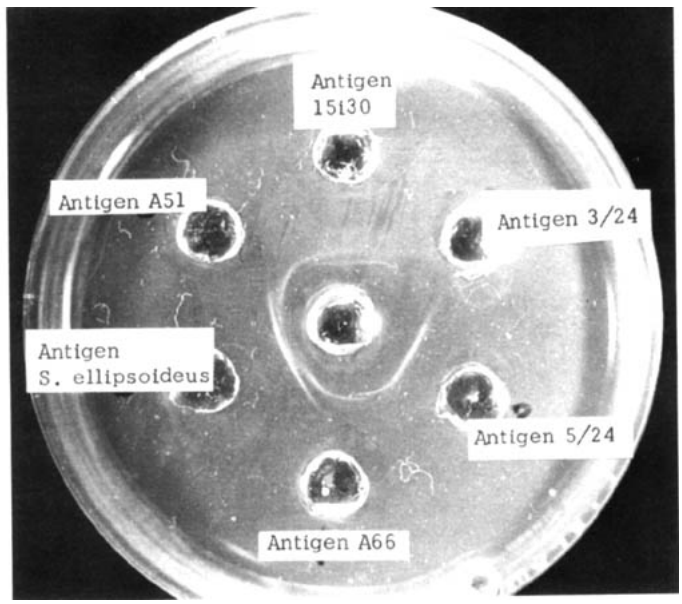

Fig. 7. Center well. Antiserum 15i30.

and *S. cerevisiae* var. *ellipsoideus* antisera.

Only antigens derived from A51 and A66 show reactivity with *S. cerevisiae* var. *ellipsoideus* and *S. uvarum*, respectively.

If antiserum of *S. uvarum* 15i30 is placed in the center well and it is tested against antigens of the other contaminant strains, some interesting results are obtained. Figure 7 shows that serum 15i30 reacts with its homologous antigen and with the antigen derived from strain A51, forming a line of identity. Furthermore, antigen from *S. cerevisiae* var. *ellipsoideus* shows a line of partial identity plus an antigen-antibody line. It also shows reactivity with antigens from strains A66 and antigen 5/24.

The activity of antiserum for *S. cerevisiae* var. *ellipsoideus* reacts with its homologous antigen, and also with antigen of 15i30 but not with antigen of other strains, as shown in Fig. 8. Finally, when the distribution of the antigens is changed to place the 15i30 antigen next to *ellipsoideus* antigen, the two lines formerly seen appear to form a line of identity, indicating that this antiserum cannot distinguish between the two strains (Fig. 9).

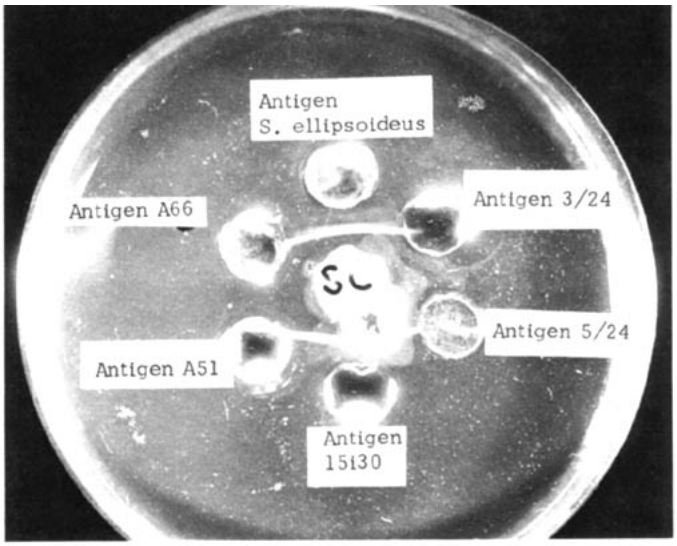

Fig. 8. Center well. Antiserum *S. ellipsoideus*.

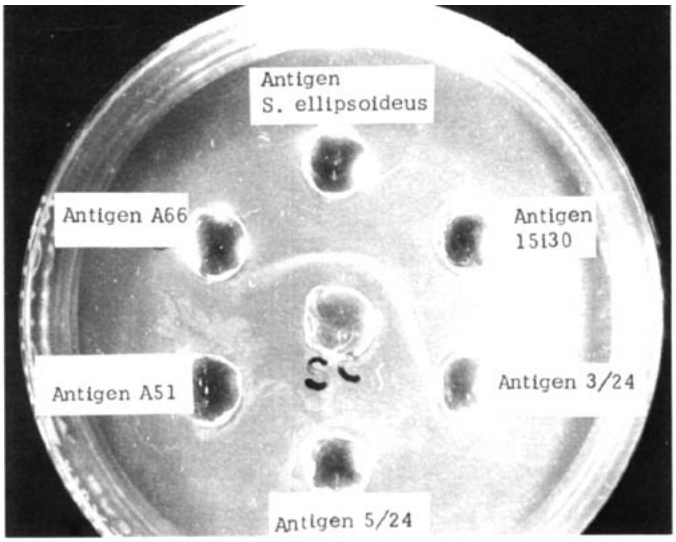

Fig. 9. Center well. Antiserum *S. ellipsoideus*.

DISCUSSION

The present observations with crude antigens from established yeast strains, and also with supposedly contaminant strains belonging to the genus *Saccharomyces,* confirm the work of Campbell and Brudzynski (6) and Sandula and coworkers (16) in that there is a considerable overlapping of antigens between *S. uvarum* and *S. cerevisiae* var. *ellipsoideus,* at least with the strains studied. However, in our plates, mixed with an overlapping pattern, it was possible to detect antigenic differences which permitted separation of the two *Saccharomyces* strains. The A51 contaminating strain appears to be more closely related to *S. cerevisiae* var. *ellipsoideus,* although it also shows some reactivity against our *S. uvarum* 15i30. This difference can hardly be significant, since *S. uvarum* and *S. cerevisiae* var. *ellipsoideus* present cross reactivity.

Unabsorbed *S. uvarum* antiserum can be used to distinguish at least partially between the *S. cerevisiae* var. *ellipsoideus* and *S. uvarum* strains investigated, but the *ellipsoideus* antiserum cannot be used for this purpose. It may be possible to absorb *uvarum* antiserum with *S. cerevisiae* var. *ellipsoideus* cells, with the hope that some specific reactivity would remain, but it still remains to be seen if it cross-reacts with A51, A66, and 5/24. This approach should be important, since A51 and A66 yeast strains produce phenol while *S. uvarum* does not, and differentiation among them could be accomplished.

One interesting feature seen in the plates is that antiserum against one of the contaminating strains had antibodies for antigens present in the others, but they were not shared among them. This immunological pattern points to the complexity of the antigens of the cell wall of these wild yeasts.

It is remarkable that antigens from contaminating wild strains reacted rarely and poorly with the *S. uvarum* and *S. cerevisiae* var. *ellipsoideus* strains used, permitting a differentiation between these strains and contaminating yeasts.

It is important to point out that there are no predictable relations between physiological and immunological properties. Therefore, the demonstration of close relations between culture yeast strains and other contaminating strains is no proof of similar metabolic behavior; antigens are only structural markers in the cell wall, while metabolic end products are the interaction of many biochemical pathways controlled by entirely different genetic mechanisms.

The present work supports the conclusion of Sandula *et al.* (16) in that the use of immunological methods should not be overestimated and perhaps should not be extrapolated for other properties of the cell. Immunological analysis is a very powerful technique which can be used for phylogenetic and taxonomic purposes, but it should be used with great caution if it is to be used as a reagent for differentiating wild and culture yeast strains.

This work has confirmed previous results reported by Sandula *et al.* (16) and, in addition, has proved that it is possible to differentiate *S. uvarum* from *S. cerevisiae* var. *ellipsoideus*.

Acknowledgment

The collaboration of José L. Zanic is acknowledged.

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[Received June 1, 1976.]