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OUR FRIENDS: WINE YEAST AND MALOLACTIC BACTERIA

R. E. Kunkee
Emeritus Professor of Enology
Department of Viticulture and Enology, University of California, Davis CA, USA

ABSTRACT

The awareness, understanding and appreciation of wine microbes by enologists and winemakers can be divided into several familiar eras or time periods. A century and a half ago, microbes were essentially unknown and certainly unappreciated. With Pasteur came the beginning of their study; however, the appreciation and utilization of them for wine production came very slowly. The last four decades have seen an astonishing, and fortunate, expansion in the deliberate utilization of microorganisms for vinifications (both for alcoholic and malolactic fermentations). This utilization was especially stimulated by the commercial availability of "starter cultures". After this time period came a rather retrogressive era: during which a small, but a significant, number of winemakers returned to a more primitive style of vinification. These "naturalists" are electing to use no deliberate inoculations. Each of these time eras will be described with reference to their historical contexts and judgements. Also the definitive microbial research which preceded or accompanied these eras will be discussed. The present era is perhaps the most interesting: the utilization of modern molecular genetics and biotechnology for the manipulation and a better understanding of our "old friends". However, we speculate: The future modifications of wine microbes, or construction of new ones, may not be as remarkable as our fancies might suggest; but the use of biotechnology for identification and typing of microorganisms will certainly bring a welcome stability to the fields of enology and winemaking.

INTRODUCTION

I am so delighted to be here—to speak in front of this chapter of the ASEV, and also to enjoy the charms of your country. I wish to give my highest thanks to Dr. Yokotsuka, and to the other members of the selection committee for inviting me here, both to attend your meeting and to address this organization. Also, it is a welcome opportunity to make acquaintances with fellow scientists, many of whom until now I have known only through their writings.

TOPICS TO BE PRESENTED

It was not easy for me to decide just what would be the most suitable topics about which I should talk. On the one hand, it is necessary, of course, that I talk to you about things I am personally familiar, that is, our own research. On the other hand, I certainly want to cover topics which I hope will be of special interest to you.

Much of my professional career at Davis has been involved with wine spoilage micro-organisms: their identification, their modes of activity and methods of their control. Let me explain. At the beginning of my time in the Department of Viticulture and Enology, fully 80% of the wine produced in California was of the high alcohol Dessert/Appetizer style, that is, containing greater than 14 percent alcohol. These were the wine types mimicking famous ones from Europe: Ports, Sherries and Maderas, for example. That these should be the wines of choice in California at that time is somewhat strange, but seems to be explained by habits developed by the drinking public during the previous period of Prohibition, where the high proof spirits were mostly all that was "available" (except for the legally allowed, but limited, family wine produced at home). Except for a few species of lactobacilli, the high concentrations of alcohol in these "ports" and "sherries" rendered them essentially sterile. That is to say, there were no big problems with microbial spoilage at that time. However, in the late 1960s

began the dramatic change in the California wine picture, the reverse in the choice of style of wines, resulting in the modern picture of almost all of the production being of low alcohol content (14 percent or less). The loss of the ethanol barrier brought about a outburst in microbial spoilage and the production problems associated thereto. My point here is that much of my early career was involved with the solving of these problems. This is, in itself, an interesting story, but I think this is all I will have to say to you about it here today. Those spoilage experiences are probably far removed from the Japanese wine picture, with consideration either with domestically produced wines, or with those imported.

Instead, I wish to say some things more in keeping with the primary role of our society, the ASEV, to show the duel nature of research, fundamental and applied, and the importance of each. Thus, I want to share with you some aspects of two of our research projects on microbial physiology, one having to do with wine yeast and the other with malolactic bacteria.

Before talking about our specific research projects, I feel it is important to discuss some things about the important changes in the California wine scene over the last few decades, with respect to the utilization of "our friends" (that is, the use of microbial starter cultures). These changes actually had their beginnings in the California wine scene, and I take some pride in that. As you know, the practice is now spreading, or has spread, to traditional wine regions. [I must also discuss a reaction to this practice, the "natural" movement, also primarily arising in California.]

In order to lay the foundation for a discussion of employment of starter culture, I need to give a brief history of the early appreciation of wine microbes by winemakers.

BRIEF HISTORY OF STARTER CULTURE UTILIZATION IN WINE-MAKING

As mentioned in the Abstract, it is easy to divide the history of the understanding and utilization of wine microbes into four time periods; and we will examine each of them briefly.

The operative history of the wine microbes (1,2,3,4) begins with Pasteur, and particularly with the publication of his *Études sur le vin* in 1866. Although almost two centuries before, van Leeuwenhoek had observed yeast in wine and beer, and other workers had some notion of biological activities of various microbes, we will start with Pasteur. Not only did he make the discovery of the fermentative, and reproductive, capacities of yeast, but he proved them; and most importantly, he insisted upon them. After making his momentous discovery, it is not surprising that Pasteur should expect yeast to be the most important flavor factor in vinification--even having the idea that grape juice fermented with beer yeast should taste like beer, and vice versa. It should be remembered that Pasteur and his students did not have our advantage of the availability of pure cultures, or a good appreciation of "yeast strains"—these ideas came later with the Robert Koch's employment of solid media. Pasteur's personality was so forceful that his inaccurate assessment of the importance of yeast strain with respect to flavor endured with his students and tended to pervade French enology for at least two decades. We will return again to this notion of yeast strain with regard to wine flavor.

We can now move on to our modern times. It may seem as if we are by-passing a very large body of history. In fact, Pasteur's discoveries were the beginnings of microbiology as a discipline, grounding much splendid research over the next century, even resulting in our knowledge of the glycolytic pathway. Strangely enough, however, scientific progress on wine yeast and its utilization in vinifications was slow. At the turn of the century, Jacquemin, in France, distributed thousands of wine yeast strains (2), but only for a

few years. Shortly before this, Müller-Thurgau in Germany also did some distribution of wine yeasts; and more importantly, he showed that the grape variety, not the yeast strain, was the operative flavor factor (2).

Distribution of wine yeast strains, as slant cultures, became important again in the 1960s, this time in California, by us at Davis. This followed the "endorsement" by Department enologists for the use of "pure yeast" strains—with little idea in those days of the notion of indigenous yeast resident in the wineries. Generally, the distribution was limited to only a few strains of yeast; Montrachet" and "Champagne" (UCD Enology 522 and 595) were the most popular. Recommendations were based mainly on abilities to ferment at a steady rate and to completion—in presence of the rather large amounts of sulfur dioxide, as used in those days, and at the cold temperatures many winemakers were using for white wines. This led to the welcome commercial production, and distribution, outside of the University, of "active dry" yeast, first by one yeast company, and then by several. The use of the active dry cultures, which could be "expanded", but need not be, was an overnight success. And indeed, their use represents today by far the majority on wine produced in California. This "new practice" rather made the old practice of preparing a *pied de cuve* obsolete in California and essentially a lost art. During this same time, much of our microbial research was more directly involved with the malolactic fermentation: its detection and methods of control, based on early work on the isolation, purification and identification of many of the strains of these bacteria (3,5). One of the most famous of these cultures was *Leuconostoc oenos* ML34 (now named *Oenococcus oeni* ML34). This strain has the honor of being the first used as a starter culture to induce a malolactic fermentation in a commercial wine production. This led to distribution of malolactic cultures, as stab cultures, which did require expansion (and personnel with microbiological training to do it). However, the welcome commercial distribution of the bacterial did not come for about another decade. The bacteria could not be preserved by the "active dry" process used for the yeast, but needed to be lyophilized (freeze-dried). This process often resulted in a great loss of viability—especially true for strain ML34. Thus other strains became the strains of choice, particularly PSU1 and MCW.

I want to stress the importance of the current use of starter cultures, both yeast and bacteria, in most of the wines of California. This can be considered an important hallmark of our wine production. The practice spread from California to other "new world" wine regions and eventually to Europe. This is only my second day in Japan, so I do not know the situation here, but I am eagerly looking forward to finding out! I must mention, however, the practice has its detractors, even in California.

I want now to go on to some other topics having to do with our earlier research with yeast and with bacteria.

ETHANOL TOLERANCE IN WINE YEAST

As we have mentioned, the use of yeast starter cultures for wine fermentation is finding worldwide acceptability. These strains were isolated from wines from important wine regions over the last century or more, and they would be expected to be especially suitable for alcoholic fermentation. We need to answer the question whether these "wine yeast strains" are really any different from other yeast strains, say, with regard to fermentation performance in grape must, and thus with regard to ethanol tolerance. Indeed, we found two different kinds of differences between wine yeast and non-wine yeast: 1) their complements of enzyme alcohol dehydrogenase (ADH), and 2) the fatty acid and sterol compositions of their membranes.

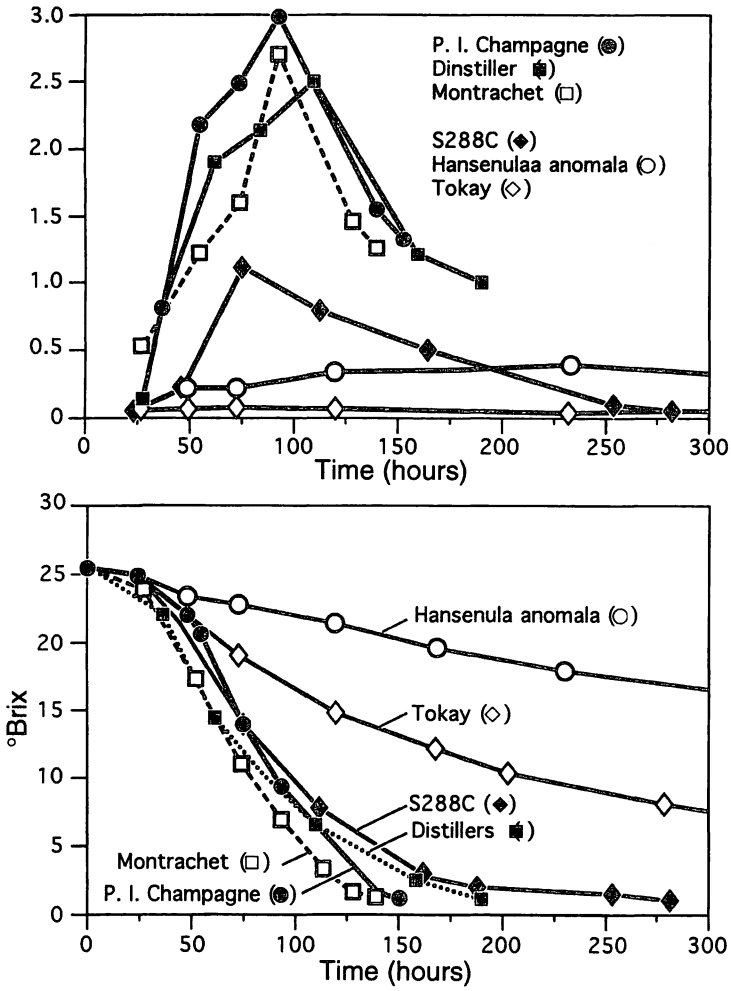

Fig. 1. Top: Specific ADH activities at various times during fermentation, depending upon yeast strain. Bottom: °Brix at various times during fermentations,, depending upon yeast strains.

Alcohol Dehydrogenase Activities

We measured the ADH activities in cell-free extracts of various strains of wine yeast, collected during several stages of an alcoholic fermentation (6). [ADH is the enzyme used by the yeast in the last enzymatic step of ethanol production: the reduction of acetaldehyde to ethanol. Several ADHs are produced by the yeast, depending upon the aerobiosis of the culture and the concentration of glucose, but in the data presented here, we are looking only at ADH-I, the "constitutive" isozyme.] Figure 1 shows the ADH activities of samples of several strains of yeast taken at various times during the fermenta-tions. It can be seen there is a definite division between the non-wine strains (Tokay and S288C) as compared to the others. Not surprisingly, but not shown before, the ADH activities are highest when one would expect them to be, during the fastest fermentation times. [This increased ADH activity would seem to be related to the reoxidation of the coenzyme $NADH^+$, to supply NAD for further glycolysis; or the increased activity may be important in removal of acetaldehyde, thus a detoxifi-cation mechanism.] We then exam-ined various temperatures of fermentation, with a single wine yeast strain, to look further at the relation between fermentation rate and ADH activity. Figure 2 shows the fermen-tation curves at various temperatures, and Figure 3 shows the correspondence between fermentation rates, at the end of fermentations, and the ADH activities (6).

Fatty Acid Composition

Earlier work has shown that ethanol tolerance in yeast is related to formation of so-called "survival factors", long chain saturated and unsaturated fatty acids and sterols, which are formed by the yeast only in the presence of oxygen (4,6,7). We wanted to learn if the wine yeast and

Table 1. Formations of fatty acids, normalized with respect to 16:0, at starts of vinifications and compared to the ends (from 0 to 14 % ethanol), in Montrachet (wine yeast strain) and in S288C (a nnon-wine yeast strain).

	Before	Ethanol	Challenge	After	Ethanol	Challenge
yeast	16:0	18:0	18:1	16:0	18:0	18:1
Montrachet	1	0.15	0.041	1	0.48	0.21
S288C	1	0.095	0.035	1	0.22	0.15

16:0 = palmitic acid　16:1 = stearic acid　18:0 = oleic acid

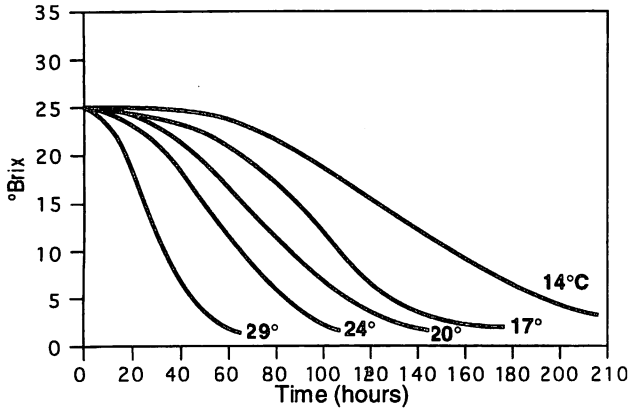

Fig. 2. °Brix at various times during fermentations with Montrachet yeast, depending upon temperatures of the fermentations.

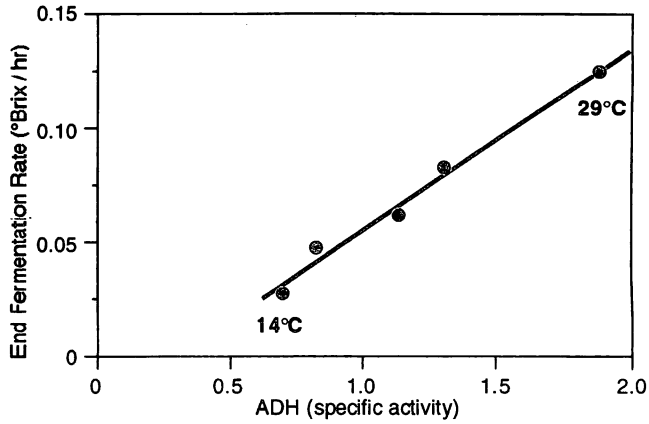

Fig. 3. Specific ADH acctivities at the ends of fermentations with Montrachet yeasts, depending upon temperatures.

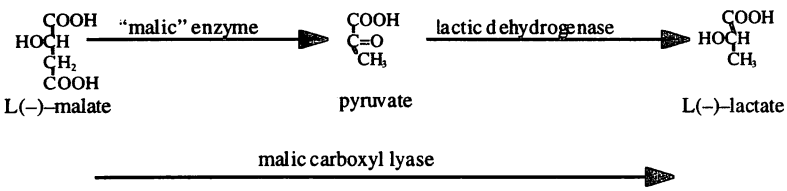

Fig. 4. Enzymatic passways of malolactic conversion:
above: historic 2-enzyme proposal
below: currently accepted 1-enzyme pathway

non-wine yeast strains could also be classified by their complements of these survival factors. In Table 1 are shown amounts of several fatty acids, palmitic, stearic and oleic, at the beginning and at the end of an alcoholic fermentation. The wine yeast strain, Montrachet, shows higher amounts in all cases, and especially higher after the ethanol challenge, as compared to the non-wine Tokay and S288C strains. Thus again we have a clear reason for making a distinction between wine yeast and other yeast.

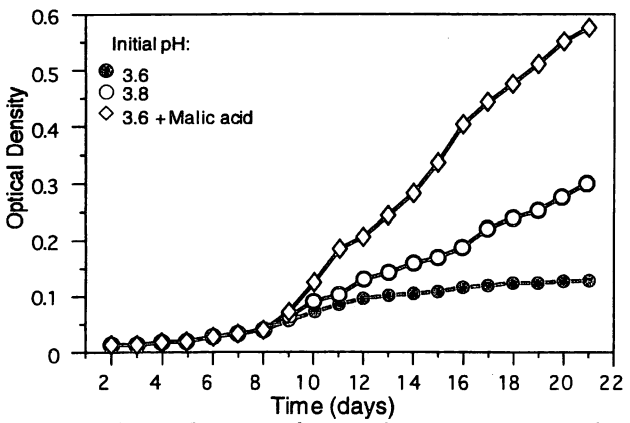

Fig. 5. Stimulation of initial growth rate of PSU-1 bacteria in presence of malic acid, providing stimulation of the initial growth rate in some strains of malolactic bactria.

STIMULATION OF MALO-LACTIC FERMENTATION

We have mentioned early work done at Davis on the selection and identification of strains of the malolactic bacteria (3,5). In ad-dition, we did particular work on the physiology of these bacteria, which are very special in that they are the only members of the global biosphere which decarboxylate L-malic acid directly to L-lactic acid (by the enzyme malate carboxy lyase). [Much earlier work has suggested that this activity arose from the combination of two enzymes (malic oxidase and lactic dehydro-genase) (Figure 4), but our work established it to be one enzyme (8).] As you know, this decar-boxylation has practical use in numerous food and beverage fermentations, as well as in wine making. Although the thermo-dynamics of the decarboxylation show that essentially no energy is produced from malic acid, we found that the presence of L-malic acid brings about a dramatic stimulation of the initial growth of some strains of these bacteria (Figure 5) (in

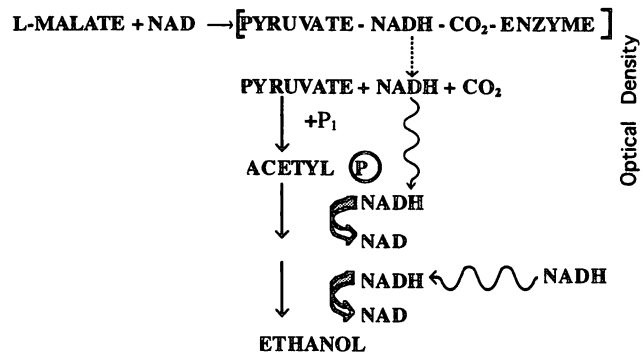

Fig. 6. "Spill off" of extra hydrogen acceptors (acetyl phosphates) in presence of mail acid, providing stimulation of the initial growth rate in some strains of malolactic bacteria.

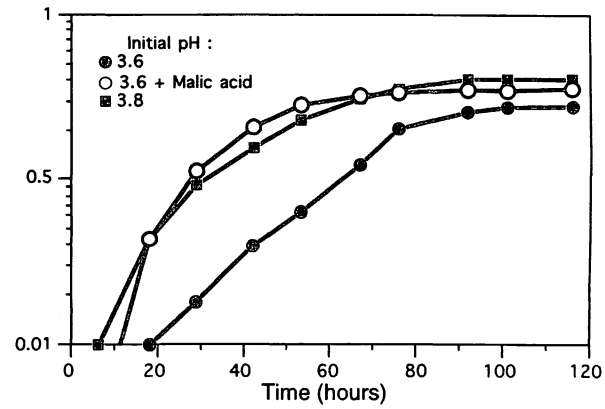

Fig. 7. Lack of stimulation of initial growth rate of Cuc-4 bacteria in presence of malic acid.

addition to the stimulation which might be caused by the increase of pH accompanying the decarboxylation). We also found that the strains which show the stimulation also process the malate oxidase activity. The latter activity, although small, is large enough to provide hydrogen acceptors (in the form of acetyl phosphate coming from a phosphoroclastic splitting of the pyruvate, formed from malate) for reoxidation of NADH (Figure 6). This reoxidation thus serves to bring about a quicker transition of the bacteria out of the resting phase and into the growth phase, *that is*, a stimulation of the initial growth. Other schemes have been suggested to explain the stimulation in the presence malic acid. However the significant part of our research was that the malate oxidase was found in those strains which showed a stimulation by malic acid, and only in those strains (8,9) (Figure 5 versus Figure 7).

USES OF BIOTECHNOLOGY

Much is being said about, and promised with, the application of the new biotechnology, genetic engineering. The potentials loom so large for enology, and indeed for viticulture, it is necessary to include some discussion here.

It is indeed feasible to "manufacture" new wine yeast strains, differing from the other strains only by their enzymatic composition. Any new strains could have increased capacity to produce flavor components, or even to make new flavors. More practically, the new strains might be able to produce increased amounts of glycerol, at the expense of ethanol. More complicated constructions have also been foreseen, the transfer of the genetic material needed for the malolactic reaction from a malolactic bacterium to a wine yeast. In fact, this had been reduced to practice, twice. The first attempt, by us, lacked the necessary mechanism to transport malic into the cell (10). That difficulty seems to have be overcome in the second attempt (11). However, in both cases, non-food grade vectors were used, and thus neither of the new strains can be used commercially. This last point is perhaps the most important. It is probably the biggest determent to the use of these new techniques in any practical way for wine, and other food, production. Another possible, and important, deterrent is the question of public acceptance.

These new technologies have another, and very meaningful, place in the modern enological laboratory—for microbial strain identification. These marvelous methods, involving DNA and RNA homologies and sequencing, are getting easier and cheaper to utilize, and are showing more and more specificity, perhaps to include microbial identification at the "strain" (subspecies) level. (However, we are continuing to investigate the use

of the more conventional technique of fatty acid composition for microbial identifications--which we feel are helpful adjuncts to karyogamic typing).

Equally important as the possible construction of new microorganisms, is the use of the techniques to discover more about the classic microorganisms already in hand. For example, my colleague, Professor Bisson, and other researchers, in studying the uptake of sugars in wine yeast have discovered a family of hexose transporters (HXT). Their conclusions, which would be incomprehensible a few short years ago, show 18 members of this family, and each displaying differences in regulation at both the transcrip-tional and post-translational levels (12).

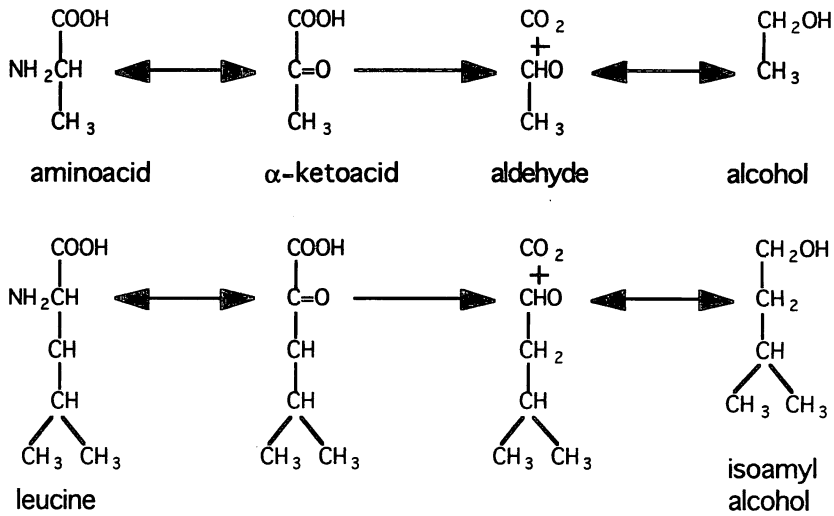

Fig. 8. Above: Ehrlich pathway for general formation of alcohols from amino acids, or from alpha-keto acid intermediates (alanine and pyruvic acid to ethanol, shown).
Below: Pathway for formation of isoamyl alcohol from leucine and alpha-keto isocaproic acid.

As spectacular as results from employment of these new technol-ogies might be, we need to remind ourselves, that much has already been accomplished with classical microbial genetics. For example, we were able to create a mutant of wine yeast which produced very low level of isoamyl alcohol, one of the important components of fusel oil. We did this by isolation of a leucine-less mutant (leucine and isoamyl alcohol are on the same metabolic pathway) (Figure 8). This meant that this mutant organism could not make isoamyl alcohol from the normal anabolic pathways (from glucose), the organisms' main source. We had to use a special trick to produce this mutant, since wine yeast normally have two sets of chromosomes (are homothallic), and both genes of the pair must be deleted at the same nuclear spot. The mutant was originally destined for production of beverage brandy (13), but was found to be more suitable for production of the more delicate eaux de vie, distillates of fruit wines, which demand a lower congener base (Table 2).

NATURAL FERMENTATIONS

In spite of the big success in the employment of

Table 2. Formation of alcohols during vinifications of various varieties of grape musts: by Montrachet yeast strain ("M"); and by the leucine-less mutant of Montrachet ("L") that cannot from iso-amyl alcohol from grape sugars.

		EtOH	iso-amyl	active amyl	iso-butyl	total
Chenin blanc	M	72.8	89.0	22.3	16.3	127.6
	L	72.1	55.8	32.7	40.7	129.2
Sémillon	M	71.4	86.8	14.0	20.8	121.6
	L	70.2	63.3	23.3	37.8	124.4
Thompson Seedless	M	72.3	126.7	34.5	20.4	181.6
	L	68.2	57.8	26.9	33.8	118.5
Cabernet Sauvignon	M	68.9	156.8	38.7	15.9	211.4
	L	70.6	76.2	37.9	41.6	155.7
Carignane	M	71.2	117.6	29.9	31.6	179.1
	L	71.2	56.3	35.1	54.0	145.4

M = Montrachet L = leusine-less mutant

starter cultures for modern wine production, there is a small, but serious, group of sophisticated winemakers who are electing to make no additions of yeast or bacteria, att leas for some of their vinifications. This practice has been named "natural" fermentation, although there are lots of reasons not to use the word natural for this (4). So much has been said, and written, about this "new" methodology, even though the quantity of wine produced thereby is relatively tiny, we feel that we must make some comments about it. Even though these wines are often being touted as not being intentionally inoculated, current research indicates that they, in fact, are being inoculated by indigenous microbes resident in all but the newest and or completely unused wineries (4,14). Natural fermentations can lead to all sorts of problems, the least having to do with weak or incomplete fermentations, giving unfinished or micro-biologically unstable wines; and the most having to do with unwanted odors or flavors (4).

So what is the rationale for this practice? I think it has to do with the disaffection of "so much control" in winemaking procedures. Although these practitioners welcome complete use of "science in the vineyard" (are eager to apply the latest viticultural information), they are uncomfortable with too much "science in the winery". The wines should tend to "make themselves", and the less they are manipulated—the more natural they are—the better. Furthermore, following Pasteur's imagination (see above) that the yeast strain should be the all important flavor factor, the mixture of unknown yeasts, of all sorts, ought to add another dimension to the final flavor of the wine. Are just how good are these new/old styles of wine? The answer is so subjective to be very controversial. Some proponents are eager to insist that they are more flavorful, and have a better mouthfeel (as meaningless as these terms are); however, it has been difficult to subject these wines to rigid objective sensory evaluations.

I believe there is another part of the rationale. With complete control of the vinification procedures, the winemaker can be rather assured that use of the methods which have produced excellent and renowned wines in the past, will predictably allow the continuation of production of wonderful wines in the future. That is, the established control methods will avoid the production of any lesser class of wines. However, the established control methods might also prohibit the production of wines *with* even more outstanding deliciousness, which would be missed if the control methodology were not eased.

CONCLUSIONS

I will conclude on that last controversial note; namely, in such an ancient art such as winemaking, how much control, or science, should be allowed or embraced? I think we all would agree that technology is welcomed, and demanded, in the avoidance of spoilage flavors and odors (assuming we can all agree on what are "spoilage flavors and odors"!). Other than that, I think the answer depends to a great extent on economics: what is the demand for the various types of wines—superb, average and mediocre—?, how much does marketing play a role in this demand? Happily, these are factors which I have been for the most part able to avoid—except for the "economics" of finding research funding!

Thank you for your attention, and again thank you for this wonderful invitation. I am looking forward to the coming events during my stay.

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