CHAPTER I

INTRODUCTION

It is well known that the art of winemaking is an ancient one, and wine is still made in a traditional and primitive fashion in many parts of the world. Basically, all one needs are grapes, a vessel in which to process and ferment them, and a storage container; but wine made in this way may vary greatly and unpredictably in quality. The basic purpose of using the advances produced by science and technology is to obtain the highest yield of the desired quality wine. Likewise, the improvement in quality of wine from laboratory test has been selected in order to produce a complete fermentation without the production of off-flavours. One of the significant developments in microbiological control has been in the use of selected yeast cultures.

There are a lot of microorganisms with thermophilic properties and high productivities which still remain in the traditional fermentation products, fruits, flowers and foods of the tropical and sub-tropical zones in Asia. These substrate can be good sources of many yeasts. Screening using highly selective procedures can be used to detect and isolate only the useful yeasts among a large microbial population.

A wine yeast should have the desirable characteristics (a) the efficient conversion of sugar to alcohol; (b) the ability to effect a quick initiation of fermentation e.g. within 48 hours; (c) sulfur dioxide tolerance; (d) the ability to ferment to dryness which

requires that it is alcohol tolerant and can ferment in the presence of relatively high alcohol concentrations; (f) good flocculation ability following fermentation to aid clarification; (g) low foaming; (h) low hydrogen sulfide (H_2S) or mercaptan fermentation; (i) relatively high glycerol production to aid the sensory qualities of the wine; and (j) the production of a desirable fermentation bouquet (Amerine,Kunkee and Singleton, 1979). It is doubtful whether any wine yeast in commercial used has all these characteristics and it is certained that they are not operating in an optimal function. It is proved that wine yeasts vary in their winemaking characteristics, such as yeast for the sparkling wine process must be selected not only for it ability to ferment but also for its ability to settle out rapidly (Kunkee and Amerine, 1970).

In studies of ecology of musts and fermentation, there is also the problem of how much of the flora originated on the grapes and how much they came from the winery equipments. Because grape musts used in the winemaking are usually not pasteurized before fermentation. So, the wineries and winery equipments have a rich flora of their own, which influences the microbial flora of musts and wines processed and stored in them. Wine spoilage can be caused by growth of undesirable yeast in finished wine, by the formation of undesirable alcoholic fermentation due to associative growth of yeasts and acetic acid bacteria leading to the production of large amounts of acetic acid (Kunkee and Amerine, 1970). For many years the winemaker has relied on sulfur dioxide to control microbial growth in wine, and also utilised it as an antioxidant. Sulfur dioxide can effectively inhibit bacterial growth, but its action is strongly pH dependent. However, it is less

effective in preventing yeast growth (Amerine et.al.,1979). It is prime importance for the production of wine, because sulfur dioxide at legal concentration will not prevent further growth of some yeasts. Several yeast strains were resistant to sulfur dioxide and also grew at relatively high concentrations of ethanol (over 18%) (Amerine and Kunkee ,1968)

There are many strains of yeast (desirable and undesirable wine yeasts belonging to Saccharomyces and other genera) other than the yeast strain inoculated which are participating in the fermentation of musts in a winery (Reed and Peppler, 1973). The contamination by wild yeasts is one of the major problems in wine industries because it results in a slow fermentation and inferior quality of the product (Amerine and Kunkee, 1968). Undesirable yeasts in the must can be harmful to the wine (off odor, excessive volatile acid, turbidity, incomplete fermentation, etc.) (Otsuka, Hara and Yoshizawa, 1972). Bottled sweet wine may referment or film can be formed on the surface of table wine during storage if processing is unsuitable. These phenomena are caused by the growth of the pseudo-film forming yeasts (Saccharomyces oviformis, Saccharomyces bayanus, Saccaromyces fermentati, etc.) and film-forming yeasts (Pichia, Hansenula, Candida, etc.) (Reed and Peppler, 1973).

Therefore, it would require special procedure in order to have a fermentation with a single strain. Since Bevan and Makower (1963) observed yeast strains that produce substances by which sensitive yeasts were killed. This phenomenon has received widespread attention. A certain group of the yeast Saccharomyces cerevisiae is known to

secrete a protein toxin to kill sensitive strains of the same species (Wood and Bevan, 1968) and the yeasts, such as Torulopsis glabrata (Bussey and Skipper,1975). Bevan and Makower (1963), who described how strains of these yeast could be classified into one of three phenotypes respected to their killer characteristics: killer, sensitive or neutral. When killer and sensitive cell are grown together in the same culture medium, a high proportion of the latter are killed. Neutral cells are not killed by killer cells, nor do they kill sensitive cells. Cell-contact is not required for the killing reaction to occur as medium in which killer cells have previously been grown and removed by filtration retains its ability to kill sensitive cells. The agent released by killer cells which brings about the death of sensitive cells has been called the killer factor. Somer and Bevan (1969) described a genetic analysis of killer, neutral and sensitive yeast strains that the killer character is under the control of two types of cytoplasmic genetic determinant which give killer and neutral phenotypes. In the absence of cytoplasmic genetic determinant, the phenotype is sensitive. The killer and resistance characters are coded by a double-stranded ribonucleic acid (ds RNA) known 'M ds RNA' of molecular weight 1.2×10^6 MD (Sweeny, Tate and Fink, 1976). A species of ds RNA of molecular weight $2.5 \times 10^6 - 3.0 \times 10^6$, L ds RNA, is also contained in all killer and in most non-killer strains as well (Somers and Bevan,1969). Both M and L ds RNA are seperately encapsulated in intracellular virus-like particles (VLP) (Herring and Bevan, 1974) and are extra-chromosomally transmitted during meiosis (Wickner, 1981). L ds RNA codes for the capsid protein of virus-like particles (Hopper et al., 1977). The M ds RNA species, unlike L, can be eliminated from

killer strains by a variety of treatments, including growth at elevated temperature (Wickner, 1974) or in the presence of sublethal concentrations of cycloheximide (Fink and Styles, 1972). The ability of cycloheximide to cure killer virus suggests a dependence of viral replication on protein synthesis. The Saccharomyces cerevisiae killer factor kills sensitive yeast by a mechanism involving adenosine triphosphate (ATP) leakage, inhibition of macromolecular synthesis, and cell shrinkage (Bussey and Skipper, 1975). No detectable morphological differences exist between the cells or colonies of killer, neutral and sensitive strains nor lysis observed (Wood and Bevan, 1968).

It is possible to change the properties or characteristics of a yeast by altering the genetic material (the detail is shown in Appendix I). Yeast of the genus Saccharomyces (Figure 1), to which most wine yeasts belong are normally diploid,i.e. the nucleus contains two copies of every chromosome. Sporulation can be induced in diploid yeasts (Mortimer and Hawthorne, 1969) and four ascospores are formed in an ascus. Each ascospore receives only one copy of each chromosome by a process called meiosis. During sporulation each chromosome duplicates giving a total of four copies of each chromosome; they are then seperated into four complete sets of chromosomes. On germination the ascospore gives rise to a colony of cells which are all haploid. The haploid state is maintained by the presence of mating factors. A haploid spore is either a or α mating type; mating can take place only between cells of different mating type and this is called a heterothallic system. When cells do mate they fuse forming a zygote in which the nuclei fuse and the diploid state of two complete sets of chromosomes present in the nucleus is restored. The heterothallic life

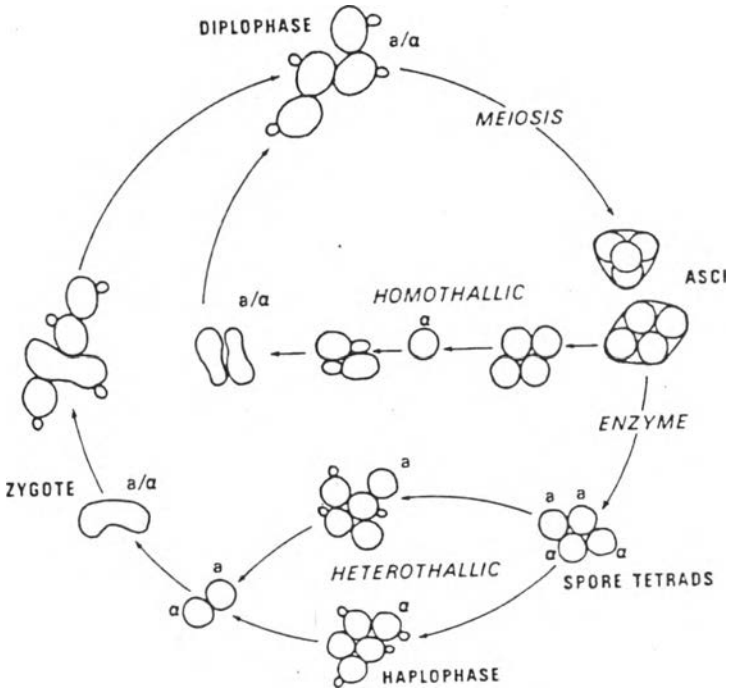

Fig. 1. Life cycle of Saccharomyces species. The diagram illustrates the alternation of haploid phased and the distinctions between heterothallic and homothallic species.

cycle and process of sexual reproduction with the isolation of haploid strains is key importance in the breeding or hybridization of yeast strains with new properties.

Hybridization takes advantage of the heterothallic yeast life cycle by selecting haploid strains of different properties from different parent diploid strains and mating them to form a diploid strain with properties different from that of either parent strain. This permits the selection of desirable characters and the elimination of undesirable characters. Unfortunately many yeasts have a complication to their life cycle in that they are homothallic (Oshima and Takano, 1970). Homothallic yeasts have the ability to switch their mating type from a to α or α to a, at each successive generation following spore germination while they are in the haploid state. Thus, cells of both a and α mating type present in the same culture and can mate to restore the diploid state. This is a problem since haploid cells are necessary for hybridization. The problem is overcome by direct spore-cell mating, mating type switching in homothallic yeast does not occur until the third and fourth generation of growth after spore germination (Takano and Oshima, 1970). Four homothallic spores from the same ascus are micromanipulated into direct contact with heterothallic haploid cells. Mating takes place between compatible spores and cells. The resulting zygote can be isolated by micromanipulation and forms a colony of diploid cells which can be sporulated. Two spores in each ascus are homothallic, but two spores are heterothallic and give stable haploid cultures which can be recognized by lack of ability to sporulate and the formulation of zygotes when mixed with a and α tester strains (Ahmad, 1952).

Yeast strains are used in hybridization as all the genetic information can be induced mutants so that any mutation will be expressed. It can be used to construct strains with very specific nutritional requirements such as amino acid auxotrophs. After hybridization diploid cells can be isolated by micromanipulation or by ensuring that the mated haploids have complementary auxotrophic requirements (Cumming and Fogel, 1978). Hybridization of such yeast strains becomes a matter of introducing suitable marker mutations which can be used in the conjugation with selective media to identify the hybrid clones.

The objectives of this study are :

1. To isolate high temperature tolerant killer yeasts.

2. To breed useful killer wine yeasts which have the ability to kill spontaneous unwanted wild yeasts in wine fermentation.

Sequences of this research

The sequences of this investigation to isolate and hybridize high temperature tolerant killer wine yeasts are as follows:

1. Isolation of high temperature tolerant yeasts.

2. Characterisation of high temperature tolerant killer yeasts.

3. Identification of the selected isolates.

4. Mutagenesis of the selected parental strains.

5. Hybridization of high temperature tolerant killer wine yeasts.

6. Wine fermentation.

7. Wine analysis.

Benefits of this research

The advantages that may be derived from this research work are as follows

1. To obtain characteristics of killer yeasts for wine making.

2. To acquire research experience in hybridization techniques.

3. To improve wine quality by using killer wine yeasts.

4. To be able to apply the killer isolates from this research for other topic of project, i.e. medical science.