# Defined fungal starter granules for purple glutinous rice wine

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# Contents

# Abstract

The Mekong Delta region of South Vietnam is particularly known as a production area of purple glutinous rice wine (*Ruou Nep Than*). The latter differs from regular rice wine for its sherry-like taste and flavour and its attractive brown-red colour. Wine is made from purple glutinous rice at home or by small cottage industries, by washing, soaking, steam-cooking, cooling and inoculation of the cooled rice with powdered rice-wine starter (*Men*). The fermentation takes place in two stages, i.e. an aerobic phase which is dominated by mycelial fungi from *Men* that convert rice starch into glucose, and an anaerobic phase during which an active alcoholic fermentation is caused by the yeasts originating from *Men*. Of pure isolates from Vietnamese rice wine starters *Amylomyces rouxii* (CBS 111757) and *Saccharomyces cerevisiae* (LU 1250) were selected as a powerful glucose producer and a superior fermentative strain, respectively. These were shown to be compatible in mixed cultures, which is of importance for the production of starters with good quality.

The development of a laboratory-scale process to formulate defined mixed-culture starter granules was established. The process was based on traditional starter manufacturing methods and modified where appropriate, as determined by optimization experiments. The herbs "Tieu Hoi" (Fennel: *Foeniculum vulgare* Miller) and "Dinh Huong" (Clove: *Syzygium aromaticum* L.) as supplementary ingredients were observed to stimulate the yeast and mould growth. Starter mould viability and its enzymatic activity were found to be quite stable during a 3 months storage test. Yeast activity is most likely the limiting factor for shelf-life and stability of starter. The dry matter content of starter granules is one of the factors influencing the shelf-life of the starter.

Based on Vietnamese standard 3215-79 the wine produced with experimental dehydrated defined mixed-culture starter was assessed as superior compared with commercially available rice wines, particularly because of its flavour and overall acceptability.

# Foreword

In order to complete my PhD research not only my hard work, but also the kind contribution in one way or another from many people in The Netherlands and in Vietnam were involved. For me no words can be enough to show my deep gratefulness since without their help I could not have reached my goal successfully. This is a good opportunity for me to express my respectful gratitude for their support.

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*Ngo Thi Phuong Dung*

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# Chapter 1

# General introduction

Fermented foods have been very popular since ancient times all over the world. In modern times fermented foods are important subjects for scientific research and many aspects of fermented foods have been published (Ko, 1982; Hesseltine and Wang, 1986; Steinkraus, 1989).

Alcoholic beverages, including beers, wines and spirits, are an essential type of indigenous fermented products. They play an important role in human spiritual and cultural life. Many kinds of indigenous alcoholic beverages are produced and consumed world-wide. Rice wine, a highly popular traditional fermented beverage, will be the major topic in the present thesis. Depending on regional ingredients and manufacturing procedures, rice wines are known under a variety of local names. The manufacture of rice wine can be characterized as a biological process whereby rice (*Oryza sativa* L.) is converted into wine by physical, microbiological and biochemical operations including steaming, inoculation with starter, mashing and fermentation. Depending on the fermentation performance, the alcohol content differs and can reach up to 15% v/v (about 12% w/v). By distillation, products with approximately 50% v/v (about 40% w/v) alcohol can be obtained.

In Vietnam, the production of rice wine is a source of income for farmer families in rural areas. Presently, it is manufactured at home-scale using solid-state starters in tablet form, and the wine is prepared under non-sterile and marginally controlled conditions. Depending on the kind of rice used as ingredient in the winemaking procedure, and different regional processing methods, several alcoholic beverages are known commercially. In Vietnamese, *Ruou* means wine. In the north and south we can find *Ruou De* or *Ruou Nep*: these are fermented from rice or glutinous rice followed by distillation. In particular in the Mekong Delta of the south there is *Ruou Nep Than* (purple glutinous rice wine) which is fermented from *Nep Than* (purple glutinous rice) without distillation. In mountainous districts, such as Da Lat, Buon Me Thuot, and Dac Lac, an ethnic minority (*Thuong* people) produce *Ruou Can* that is fermented from rice or maize or cassava with or without distillation.

The government of Vietnam wishes to develop biotechnology to improve its people's living conditions. The development of small-scale technology to process agricultural products by fermentation is one of the major parts of the programme to improve the socio-economic situation in Vietnam. The Ministry of Science, Technology and Environment is responsible for reorganizing biotechnology-related institutions into a National Network. The Biotechnology Research and Development Institute (BiRDI) was selected among a total of 12 research units to be a member of this network. BiRDI is part of the Can Tho University (CTU), and its mission is to generate knowledge of

biotechnological processes that are of importance to the Mekong Delta region. The ultimate aim is to contribute to the development of local industry and improve livelihood of the population of the region. Activities of BiRDI are in the area of plant biotechnology and food (bio)technology. The present research falls within the scope of these on-going activities.

**Traditional rice-based alcoholic beverages**

Although numerous rice-based traditional alcoholic beverages have different compositions according to formulation and processes used, the principle of their manufacture can be characterized as a biochemical modification of cereal starches brought about by micro-organisms in which fungi (yeasts and moulds) play essential roles. Moulds produce the amylases that degrade the starch into dextrins and sugars, and yeasts convert these sugars to alcohol (Lim, 1991; Motarjemi and Nout, 1996; Nout and Aidoo, 2002). Table 1 summarises some traditional fermented alcoholic beverages from various countries. This table mentions raw materials and major functional fungi involved in the fermentation process. In this general introduction in line with the scope of this thesis, emphasis will be laid on the alcoholic fermentation process for the preparation of wine from purple glutinous rice (PGR), its small-scale production process and the manufacturing method of traditional starter for rice wine in Vietnam.

In Vietnam, regular rice wine is made from white rice or white glutinous rice and is distilled after fermentation, yielding a colourless liquor with a bland taste. PGRW (Purple Glutinous Rice Wine) is also a well-known and popular traditional product in Vietnam; however, it differs strongly from regular rice wine. PGRW is particularly interesting for its brown-red colour and sherry-like flavour which make it a very attractive and characteristic product compared with the colourless and neutrally flavoured regular wine from white rice. PGRW is twice as expensive because of the more expensive ingredient PGR.

PGRW is made at a home- or cottage-level as a source of income for farmer families and it is commercialized in two qualities: crude cloudy wine containing sediment, and clear filtered wine. The traditional production processes are shown in Figure 1. Starch-based starters are powdered, mixed with gelatinized PGR and incubated under ambient conditions. After an initial period of uncontrolled aerobic solid-state fermentation, the now moulded mass is mixed with water, and is allowed to undergo submerged alcoholic fermentation. After filtration, the final product is a clear purple liquid containing a mixture of glucose, ethanol and other soluble matter. Normally, the alcohol content in the final product is approximately 7-10% v/v (about 6-8% w/v), which would be insufficient to preserve the wine. Therefore a volume of concentrated (distilled) alcohol is often added to the wine based on producers' and consumers' requirements for the level of alcohol and the required storage life. The yield, flavour and overall success obtained with these uncontrolled fermentation operations vary considerably and cannot be predicted or guaranteed. This poses a problem for local producers who expressed their opinions during our visits to collect samples of starters and wines.

**Table 1. Traditional fermented rice-based beverages in various countries***

| Product | Country | Raw material | Functional yeasts and moulds |
|---|---|---|---|
| Brem Bali, Arak, Tuak, Ciu | Indonesia | Rice, glutinous rice, sap of palm trees, cane-sugar | *Amylomyces* spp., *Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp. |
| Bubod, Roselle, Lambanog, Tuba, Tapoi, Tapuy | Philippines | Rice, roselle fruit, palmyra juice | *Aspergillus* spp., *Endomycopsis* spp., *Hansenula* spp., *Endomycopsis fibuliger, Rhodotorula glutinis, Debaromyces hansenii, Candida parapsilosis, Trichosporon fennicum, Saccharomyces ellipsoideus* |
| Bupju, Takju, Yakju | Korea | Rice, glutinous rice, barley, wheat, millet | *Aspergillus oryzae, Aspergillus sojae, Mucor* spp., *Rhizopus* spp., *Saccharomyces cerevisiae, Hansenula anomala, H. subpelliculosa, Torulopsis sake, T. inconspicua, Pichia polymorpha* |
| Fenni, Sonti, Ruhi, Madhu, Jnard | India | Rice, Cashew apple | *Mucor, Rhizopus* |
| Mie-chiu, Shaohing | China | Rice, wheat, barley | *Aspergillus oryzae, Rhizopus* spp., *Saccharomyces cerevisiae* |
| Mirin, Sake, Shochu, Umeshu | Japan | Rice, maize, barley, plum | *Aspergillus oryzae, Aspergillus awamorii, Saccharomyces sake, Hansenula anomala* |
| Ruou De, Ruou Nep, Ruou Nep Than, Ruou Can, Ruou Vang | Vietnam | Rice, (purple) glutinous rice, maize, cassava | *Mucor* spp., *Rhizopus* spp., *Aspergillus* spp., *Saccharomyces ellipsoideus, Saccharomyces cerevisiae, Endomycopsis fibuliger, Hansenula anomala, Torulopsis candida* |
| Sato, Ou, Nam-Khao | Thailand | Rice, glutinous rice | *Mucor* spp., *Rhizopus* spp., *Candida* spp., *Saccharomyces* spp. |
| Tapai, Samsu | Malaysia | Rice, glutinous rice | *Amylomyces rouxii, Rhizopus* spp., *Endomycopsis* spp. |

* based on data of (Hesseltine, 1983; Sakai and Caldo, 1985b; Wang and Fang, 1986; Luong, 1998; Haard et al., 1999; Nout and Aidoo, 2002)

Purple glutinous rice
↓
wash
↓
soak 6-8 hours
↓
steam/cook until gelatinized
(time depends on quantity of rice)
↓
cool
↓
inoculate ← grind into powder ← starter (*men*)
↓
aerobic solid-state fermentation 1-2 days
(the moulded mass normally fills 2/3 of the height of its container)
↓
add water to just submerge the moulded mass
↓
alcoholic fermentation 3-5 days
↓
grind and mix ← add alcohol
↓
mature at least 15 days (for stabilization of quality and flavour)

filtration

crude turbid wine          clear filtered wine

**Figure 1. General outline of the traditional process for purple glutinous rice wine**

**Fermentation starters**

The traditional home-scale technologies to produce rice-based fermented beverages can vary according to the different regions; however, their process usually involves the use of solid-state starters. These starters, which normally combine three groups of micro-organisms namely yeasts, moulds and bacteria, convert starchy materials to fermentable sugars and subsequently to alcohol and organic acids (Sakai and Caldo, 1985a; Hesseltine et al., 1988; Nwosu and Ojimelukwe, 1993). There are several starter cultures available in the markets in most Asian countries as summarized in Table 2. They are prepared under non-sterile conditions by producers lacking microbiological training. In the preparation of starter cakes, rice or wheat is ground and thoroughly mixed with spices which are believed to play a major role in preventing growth of undesirable micro-organisms. A mixture of

garlic, pepper, rhizomes, onion and root is used in the preparation of the starter and producers regard their recipes as secret passed from generation to generation. The ratio of ground rice to mixed spices is about 14:1. Water is added to make a dough-like material which is shaped into small balls or cakes of about 4 cm in diameter and 1cm thick. Dry powdered starter from previous batches is sprinkled over the cakes. The latter are then placed on a bamboo tray, covered with a cloth and incubated at ambient temperature for 2-5 days during which the dough rises slightly and will be covered with fungal mycelia. The cakes are air- or sun-dried and have a shelf life of several months. To control microbial activities of the mixed cultures contained in starter cakes, pure cultures of the selected strains of *Rhizopus* spp. (mainly *R. oryzae*) have been used for industrial production of enzyme starters, in particular, for the distillery industry, and mycelial fungi and yeasts with amylolytic activities have been studied (Nout and Aidoo, 2002).

The preparation and the use of fermentation starters as a source of inoculum are important in the manufacture of PGRW. It is recognized by winemakers that the choice of starter tablets influences the yield and quality of wine. Each locality in Vietnam has its own way of starter production depending on available ingredients and fancy interest; however, the products in principle must contain a combination of micro-organisms able to perform the microbiological and biochemical reactions that convert rice into wine (Phuoc, 1979; Luong, 1998; Phuc, 1998). There are three main kinds of Vietnamese traditional starters: starters without and with added supplements containing oriental herbs, and starters with added supplement containing leaves with strong essential oils. The latter supplements are used to produce fragrance and keep down the growth of undesired micro-organisms in the final products. Particularly, the role of the oriental herbs in the traditional starter preparation has been studied with respect to their effect on the microflora of starter during the manufacture (Hieu, 1990). It was observed that 33 of 35 investigated oriental herbs stimulate the growth of yeasts and moulds. There appeared to be a synergistic effect. A complex mixture of 10 medicaments is applied in practice and is effective and economic. The raw ingredients can be either rice flour or cassava flour or combinations of rice flour and cassava flour; however, the mixed flours are preferred by local producers. The traditional process of starter production is schematized in Figure 2.

The limited knowledge about traditional starters poses an obstacle to industrial development, and thus, these starters attract attention of researchers in food microbiology and technology. This has resulted in studies concerning the selection of safe and storable superior starters for small-scale fermentation processes. Single, mixed or multiple-strain cultures have been investigated to select compatible strains and good combinations of complementary functionality. These were examined for viability when processed and stored in simple starch-based starters (Ko, 1972; Cronk et al., 1977; Hesseltine, 1983). Advantages of defined mixed starter cultures have been mentioned (Holzapfel, 1997; Ndip et al., 2001; Siebenhandl et al., 2001), and include: enhanced yield, improved hygiene, predictability of fermentation processes, and control of safety and quality. In addition, genetically modified starter and protective cultures have been developed. By recombinant DNA technology, "tailor-made" starter cultures which would meet technical and metabolic requirements necessary for a specific fermentation can be developed, and by the gene

disruption technique, undesirable properties such as mycotoxin or antibiotic production may be eliminated (Geisen and Holzapfel, 1996; Holzapfel, 2002; Ulgen et al., 2002).

**Table 2. Starters for alcoholic beverages used in various parts of Asian countries\***

| Product | Country | Functional yeasts and moulds |
|---|---|---|
| Bakhar | India | *Mucor* spp., *Rhizopus* spp., *Amylomyces* spp. |
| Bubud | Philippines | *Mucor* spp., *Rhizopus* spp., *Amylomyces* spp., *Candida* spp., *Saccharomyces* spp., *Endomycopsis* spp., *Torulopsis* spp. |
| Chu | China | *Aspergillus oryzae, Rhizopus* spp. |
| Look-pang | Thailand | *Mucor* spp., *Chlamydomucor oryzae, Candida* spp, *Saccharomycopsis.* |
| Men ruou, Men com ruou | Vietnam | *Mucor* spp., *Rhizopus* spp., *Aspergillus* spp., *Penicillium* spp., *Saccharomyces* spp., *Endomycopsis* spp., *Hansenula* spp., *Torulopsis* spp., *Rhodotorula* spp. |
| Murcha | Nepal | *Rhizopus* spp., *Mucor* spp., *Saccharomyces cerevisiae, Endomycopsis fibuliger.* |
| Nurook | Korea | *Aspergillus oryzae, A. niger, Rhizopus* spp., *Penicillium* spp., *Mucor* spp., *Hansenula anomala, Pichia anomala.* |
| Ragi | Indonesia | *Mucor* spp., *Rhizopus* spp., *Amylomyces* spp., *Aspergillus* spp., *Penicillium* spp., *Candida* spp., *Saccharomyces* spp., *Endomycopsis* spp., *Hansenula* spp., *Torulopsis* spp., *Rhodotorula* spp. |
| Tapai | Malaysia | *Amylomyces rouxii, Rhizopus* spp., *Endomycopsis* spp. |

\* based on data of (Steinkraus, 1989; Nout, 1992; Tamang and Sarkar, 1995; Basuki et al., 1996; Steinkraus, 1997; Lee and Fujio, 1999)

**The role of mycelial fungi and yeasts**

Three groups of micro-organisms, namely mycelial fungi, yeasts and bacteria are associated in the performance of traditional starter tablets. In the present thesis, the role of the mycelial fungi and yeasts will receive major attention as they are considered crucial for starch degradation and alcoholic fermentation. In the solid-state fermentation the moulds bring about saccharification of the rice starch, and the sugars thus formed are fermented into alcohol by the yeasts, so the quality of the final products depends mainly on the activities of these micro-organisms. One of the approaches for improving the ethanol production would be to establish the optimum conditions for simultaneous saccharification and fermentation with a mixed culture of amylolytic and ethanol-producing micro-organisms. As listed in Table 2, the major moulds in traditional starters are *Amylomyces rouxii, Rhizopus* spp. and *Mucor* spp. , and the common yeasts are *Saccharomyces cerevisiae, Hansenula* spp., *Endomycopsis fibuligera* and *Candida* spp. The moulds produce α-amylase and amyloglucosidase (also called gluco-amylase) that hydrolyse starch to dextrins and maltose but mainly to glucose (Cook et al., 1991; Crabb, 1999; Nout and Aidoo, 2002). The raw starch is gelatinized by cooking, liquified by α-amylase and saccharified to glucose by amyloglucosidase. Therefore, both enzymes are of concern in

the starch degradation but in different reaction pathways. α-amylase cleaves starch randomly at the 1,4-α-glycosidic bonds, giving maltooligo-saccharides as final products.
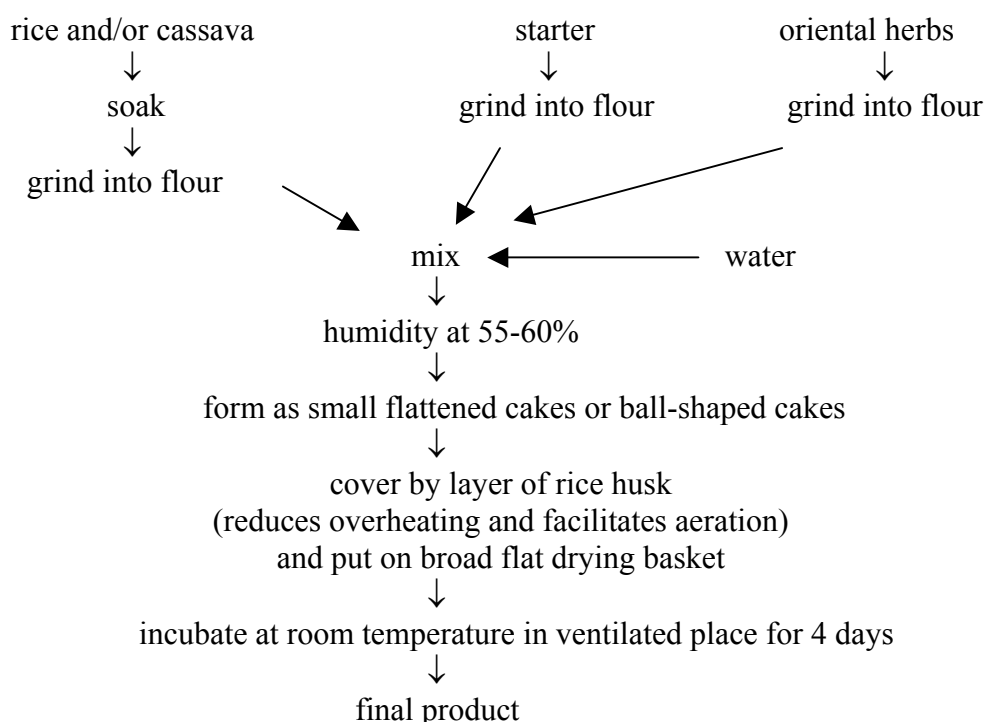
```
   rice and/or cassava              starter              oriental herbs
           ↓                           ↓                      ↓
         soak                   grind into flour        grind into flour
           ↓
   grind into flour

                              mix  ⟵——————  water
                               ↓
                       humidity at 55-60%
                               ↓
             form as small flattened cakes or ball-shaped cakes
                               ↓
                     cover by layer of rice husk
               (reduces overheating and facilitates aeration)
                   and put on broad flat drying basket
                               ↓
           incubate at room temperature in ventilated place for 4 days
                               ↓
                         final product
```

**Figure 2. Traditional process for preparing alcoholic starter**

Amyloglucosidase liberates single D-glucose monomers in the β-form from the non-reducing end of starch and preferentially hydrolyses 1,4-α-glucosidic bonds and with a considerably lower reaction rate 1,6-α-glucosidic linkages (Schindler et al., 1998). For the measurement of α-amylase activity, two commonly used methods include (a) the visualization of the degradation zone in rice-starch agar by flooding with iodine solution and (b) using chromogenic substrates releasing coloured products upon enzyme reactions. Amyloglucosidase activity can be determined, for instance, using the subtrate p-nitrophenyl β-maltoside, plus β-glucosidase by the release of free p-nitrophenol which can be measured spectrophotometrically. A recent study (Shigechi et al., 2002) examined the necessity of mixtures of both α-amylase and amyloglucosidase enzymes for the complete starch degradation. It was reported that in comparison with strains producing only glucoamylase, (recombinant) *Saccharomyces cerevisiae* strains, expressing both α-amylase and glucoamylase, grew faster under aerobic conditions, degraded starch better, and produced more ethanol.

Yeasts are significant in winemaking because they conduct the alcoholic fermentation, but some could also spoil wines during their storage or negatively affect wine quality through autolysis (Fleet, 1993). Ethanol is the main product resulting from yeast sugar fermentation; it is important to note that at certain concentrations ethanol can

inhibit cell growth and viability of yeasts. Ethanol has been reported to have different inhibitive effects on yeast cells (Casey and Ingledew, 1986; D'Amore et al., 1990; Sharma, 1997). One of the major target sites of ethanol is the plasma membrane of yeast and other micro-organisms. At the plasma membrane level, the influx of nutrients as well as the excretion of products (including ethanol) is controlled. At certain high concentrations, ethanol causes altered membrane organization and permeability. This results in leakage of essential co-factors and coenzymes which are essential components for the activity of enzymes involved in glycolysis and alcohol production, resulting in the inhibitory effect of ethanol on fermentation. Besides, a number of other factors also affect yeast fermentation performance, such as osmotic pressure, substrate feeding, nutrient supplementation, temperature, and intracellular ethanol accumulation (Sharma et al., 1996; Peres and Laluce, 1998; Wang and Sheu, 2000). The nature of ethanol tolerance is rather complex and no universal method of measurement of ethanol tolerance exists. Methods that could be used to assay and define ethanol tolerance are for example, to monitor the effects of ethanol on batch culture growth, fermentative ability, and cell viability; tolerance could also be defined as the maximum level of ethanol produced. Each method has its own advantages and disadvantages and shows that alcohol affects the above activities in different ways. For example, cell growth and viability are inhibited by ethanol, whereas only at relatively high concentrations, ethanol also affects the fermentative capacity, which makes fermentation the most ethanol tolerant metabolic activity. Thus, when measuring ethanol tolerance it is essential to mention the experimental conditions (D'Amore et al., 1990). These authors also mentioned that cultivation conditions, e.g. osmotic pressure, mode of substrate feeding, temperature and medium composition, influence cell viability and ethanol production at high sugar concentrations. Maximum viability and ethanol concentration could be achieved using fed-batch or infusion-feeding schemes. When the feeding scheme is modified so that glucose is added at consecutive moments during the fermentation (fed-batch), an increased fermentation rate was observed. However, high substrate concentrations in the range of 15 to 25% (w/v) sugar can significantly inhibit yeast growth and fermentative activity. This effect can be related directly to increased osmotic pressure and reduced water activity, as well as indirectly as a result of the high levels of ethanol produced during the fermentation (Casey and Ingledew, 1986). Therefore, optimized fermentation conditions are required to improve yeast performance and ethanol tolerance.

**Aim and outline of this thesis**

The present thesis addresses the problem of poor and variable quality of traditional starter tablets, by understanding and quantifying the impact of microflora in these starters, concentrating on mycelial fungi and yeasts and by assessing the option to prepare stable mixed cultures of selected compatible strains.

During preliminary field work, processes for the manufacture of starter tablets and rice wine were observed, and representative samples of starter tablets were collected from leading producers in the Mekong Delta area. Based on results of screening of starters for their viability and biochemical activity, the best starters are analysed for their microflora

and pure cultures of moulds and yeasts are obtained (Chapter 2). The role and identity of the predominant mycelial fungi and yeasts in the saccharification of cooked rice starch and the alcoholic fermentation of saccharified rice are studied in Chapters 3 and 4. Compatibility and viability of promising combinations of moulds and yeasts to be grown in rice dough as affected by oriental medicinal herbs are examined in Chapter 5. Subsequently, the technical feasibility of preparing defined mixed starter cultures for PGRW and their stability in a dehydrated tablet environment are reported in Chapter 6.

Chapter 7 presents a preliminary assessment of winemaking performance of experimental mixed pure-culture starters compared with commercial starters according to Vietnamese standard.

Chapter 8 is a general discussion of the fungal performance of starters for rice wine fermentation, and innovation of starter manufacturing method as well as future prospects for the application of granulated starters in the Mekong Delta.

# Chapter 2

# Functional properties of some traditional Vietnamese starch-based rice wine fermentation starters (*Men*)

**Abstract**

In this study 29 samples of Vietnamese commercial starch-based rice wine starters were collected from leading starter producers in the Mekong Delta region which is particularly known for its manufacture of purple glutinous rice wine (*Ruou Nep Than*). The latter differs from regular rice wine by its sherry-like taste and flavour and its attractive brown-red colour. The starters were screened for their ability to liquefy cooked purple glutinous rice (*Nep Than*), and to produce high alcohol levels. We selected 6 rice wine starters for their significantly higher ethanol accumulation, superior ability to liquefy cooked rice, and attractive flavour and colour of the resulting wine. Ethanol contents reached 12% w/v, a sweet alcoholic fragrance was noticed and the colour varied from red to lightly brown. Total mould, yeast and bacteria counts were 3.4 - 6.0, 5.8 - 7.2 and 2.6 - 6.2 log CFU/g of starter dry weight sample, respectively.

## INTRODUCTION

*Men ruou* (*Men*) is the local name of Vietnamese starter that is used in the manufacture of popular traditional fermented beverages, such as rice wine and purple glutinous rice wine (PGRW). PGRW differs from regular rice wine which is made from white rice or white glutinous rice and distilled after fermentation, yielding a colourless liquor with a neutral taste. In contrast, PGRW is popular for its sherry-like taste and flavour and its attractive brown-red colour. In the Orient, similar starters for rice wine were described as starch-based products that are shaped into small balls, cakes or powder, containing three major groups of micro-organisms namely yeasts, moulds and bacteria (Hadisepoetro et al., 1979; Steinkraus et al., 1983; Ardhana and Fleet, 1989; Steinkraus, 1989). Three main kinds of Vietnamese traditional starters (*Men*) are known: starters (a) without and (b) supplemented with oriental medicinal herbs and (c) starters supplemented with leaves with aromatic essential oils. In the Mekong Delta region, starters of type (b) predominate. The mentioned supplements produce fragrance and reportedly keep down the growth of undesired micro-organisms in the final products. Particularly, the oriental medicaments in the traditional starter preparation have been studied (Hieu, 1990; Phuc, 1998) for their effect on the starter microflora. The starter ingredients can be either rice flour or cassava flour or

combinations thereof; mixed flours are preferred by local producers. The yield and quality of wine are known to be influenced by the solid-state starters used; however, hardly any knowledge exists about the relation between their specific formulation, their microbiological composition and their performance as starters. In principle, moulds produce amylases that break down the starch into fermentable sugars, and yeasts convert the sugars to alcohol. Bacteria, mainly lactic acid bacteria, most likely occur as opportunistic contaminants. Therefore, in principle, our interest focuses on the role of individual moulds and yeasts in the liquefaction and alcoholic fermentation, geared towards the development of superior defined mixed starters in solid-state tablets. Single, mixed or multiple strain mixed cultures have been described for application in various food fermentations (Hesseltine, 1983; Holzapfel, 1997, 2002).

Each locality in Vietnam has its own way of production of starch-based rice wine starter, depending on available materials and preferences. With the aim to pave the way for upgrading the traditional starter tablet technology for winemaking, screening of starters for their functionality in production of high alcohol yields was carried out with rice saccharification and alcoholic fermentation stages as crucial assessment criteria.

## MATERIALS AND METHODS

### Collection of samples

During field visits, processes for the manufacture of starter tablets and rice wine were recorded and twenty-nine samples of dry commercial starters were obtained from small-scale factories and markets in different provinces. Sampling was focused particularly on the leading producers in the Mekong Delta region. The dry starters were subjected to microbiological analysis immediately after collection. The places of manufacture that are used as the commercial names of starters as well as the places of collection and the period of storage prior to analysis are shown in Table 1; however, the date of manufacture could not always be determined. Samples were stored at 4ºC during the experimental work.

### Fermentation test

We modified the traditional process for making PGRW for use at laboratory scale, as follows: (1) fifty grams of purple glutinous rice and 60ml of distilled water in a 500-ml conical flask covered by a cotton plug were soaked (2) for 4 hours at room temperature (25ºC). After soaking they were steamed (3) in an autoclave for 1 hour at 100ºC. The gelatinized rice paste was cooled to 35-40ºC (4), then inoculated and mixed well with 2 g of powdered starter (5). After solid-state fermentation during 3 days at 30ºC (6) for fungal growth under aerobic conditions, 70ml of sterile water was added (7) to the moulded mass to allow for submerged alcoholic fermentation for 7 days at 30ºC under anaerobic condition in which the cotton plug was replaced by a water lock (8). All of the fermented rice mass was homogenized (9) using a Stomacher Lab-blender (Seward 400, Emergo,

England) and clear liquid was harvested after centrifugation (10) at 3660 rpm for 20 minutes by using a centrifuge Mistral 3000i (rotor windshield 43124-708 BS 4402, Leicester, United Kingdom); clear liquid (supernatant) was decanted (11). Each sample was tested in triplicate.

## Analytical methods

Numbers in brackets refer to the manufacturing stages mentioned in "Fermentation test" above. The pH after soaking (2), after solid-state incubation (6) and after submerged incubation (8) was measured by a digital pH meter WTW pH 525. Flavour after (6) and after (8) was assessed subjectively by a small expert panel. Colour of clear final liquid was measured by Colorimeter Tricolor LFM3, (Dr. Lange) in which the L*, a*, b* system (DIN 6174, CIE-LAB standardized in 1976) was used. Glucose and ethanol after incubation (6) and after incubation (8) were analysed by HPLC Organic Acid Analysis Column-Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm (BIO-RAD).

## Microbiological analysis

For microbiological analysis, a sample of 1 g of each selected starter was transferred to a stomacher bag and homogenized with 99 ml sterile saline (NaCl 0.85% w/v) in a Stomacher Lab-blender 400 for 1 minute at high speed and appropriate serial dilutions were performed using the same diluant. 1 ml-portions of the appropriately diluted suspension were mixed with molten (45ºC) medium and made into pour-plates.

Moulds were enumerated on Czapek-Dox Agar rendered selective for fungi by addition of oxytetracycline. The medium contained (g/L) sodium nitrate 2, potassium chloride 0.5, magnesium glycerophosphate 0.5, ferrous sulphate 0.01, potassium sulphate 0.35, sucrose 30, agar 20. To 900ml of basal medium 100ml containing 100 mg sterile filtered solution of oxytetracycline was added. Oxytetracycline Glucose Yeast Extract Agar was used for yeast count. The ingredients (g/L) were yeast extract 5, glucose 20, agar 12. Oxytetracycline was added to this medium as above.

MRS medium was used to determine the lactic acid bacteria (LAB) count. It contains (g/L) peptone from casein 10, meat extract 8, yeast extract 4, D (+) glucose 20, di-potassium hydrogen phosphate 2, tween 80 1, di-ammonium hydrogen citrate 2, sodium acetate 5, magnesium sulfate 0.2, manganese sulfate 0.04, agar technical 12. To 1 L medium 2 g natamycin dissolved in 40ml of sterile water was added to prevent fungal growth.

All plates were incubated at 30ºC for 2 to 4 days. The colonies that appeared after incubation were counted and calculated as colony forming units (CFU) per gram of dry weight sample.

*Isolation and purification of moulds:*

After completing step (6) of the fermentation test (mentioned above) a piece of moulded rice covered with mycelium was transferred onto a plate containing sterile Czapek-Dox

Agar supplemented with oxytetracycline, incubated at 30ºC for 3 days and resulting colonies were purified on Czapek-Dox agar.

**Table 1. List of commercial starters**

| No. | Name | Place collected | Period of storage prior to analysis (weeks) |
|---|---|---|---|
| 1 | Long xuyen 1 | Long xuyen | 3 |
| 2 | Chau doc 1 | O mon | 3 |
| 3 | Long xuyen 2 | Thot not | 3 |
| 4 | Nga bay | Can tho | 3 |
| 5 | Dong nai | Soc trang | 4 |
| 6 | Bac lieu 1 | Bac lieu | 3 |
| 7 | Bac lieu 2 | Bac lieu | 3 |
| 8 | Ho Chi Minh | Ben tre | 3 |
| 9 | Chau doc 2 | Ben tre | 3 |
| 10 | Go den 1 | Ben tre | 3 |
| 11 | Chau doc 3 | Ben tre | 3 |
| 12 | Van loi | Cai lay | 5 |
| 13 | Go den 2 | Go den | 5 |
| 14 | Long xuyen 3 | Ho Chi Minh | 5 |
| 15 | Tay ninh | Ho Chi Minh | 5 |
| 16 | Phu nguyen | Lai vung | 4 |
| 17 | Tra vinh 1 | Tra vinh | 4 |
| 18 | Tra vinh 2 | Tra vinh | 4 |
| 19 | Sa dec 1 | Vinh long | 4 |
| 20 | Vung liem | Vung liem | 4 |
| 21 | Cao lanh | Cao lanh | 3 |
| 22 | Sa dec 2 | Cao lanh | 3 |
| 23 | Xuan thanh | Tra vinh | 4 |
| 24 | Tan hiep | Kien giang | 4 |
| 25 | Ngo, Trung quoc | Can tho | 4 |
| 26 | Huong thuy | Cai rang | 3 |
| 27 | Thot not | Cai rang | 3 |
| 28 | Soc trang | Cai rang | 3 |
| 29 | Lang Van-Ha bac | Cai rang | 3 |

All starters were classified as type (b) (see Introduction).

*Isolation and purification of yeasts:*

After completing step (8) of the fermentation test (mentioned above) appropriate dilutions were made into pouring plates with Oxytetracyclin Glucose Yeast Extract Agar which was incubated at 30ºC for 1 or 2 days. Colonies were purified on Glucose Yeast Extract agar.

*Isolation and purification of LAB:*

During step (6) of the fermentation test, 1ml of liquid released by the moulded mass was diluted and pour-plated into MRS supplemented with natamycin, incubated at 30ºC for 4 days, and colonies were isolated and purified on MRS agar.

## Statistical analysis

Experimental data were statistically analysed using Statgraphics Plus Version 5, Manugistics, Inc., Rockville, USA.

## RESULTS AND DISCUSSION

## Manufacturing procedures of rice wine and starter tablets

During the field work of starter collection, information about the small scale processes for the production of rice wine and the preparation of starters was obtained. Though each producer has his own way for winemaking depending on his individual experience and regional available raw materials, in principle they follow the same process in which powder of starch-based starters is mixed with gelatinized rice and incubated under ambient conditions. After an initial period of aerobic solid-state fermentation during which the liquefaction and the saccharification takes place, the moulded mass that normally fills 2/3 of the height of its container, is mixed with water and allowed to undergo submerged alcoholic fermentation. The final product is either a colourless liquor if distillation is applied after fermentation, or crude turbid wine or clear filtered wine. The starchy raw materials can be whole or broken rice (*Oryza sativa* L.), glutinous rice or purple glutinous rice. Both steaming and cooking are applied for starch gelatinisation. Most producers prefer cooking because it takes less time until the starch has completely gelatinized, although they have to be well experienced in adding an appropriate volume of water for cooking. Processors who apply a combination of two or three different starters claim that their final product will be of better quality, for instance, the sweet alcoholic taste is stronger and the flavour is more attractive. They have experienced that each different starter has its own advantage. All of them agree that the incubation period required for the fermentation depends on the weather of the area which is characterized by two seasons: the dry and the rainy periods. The temperature is normally around 30-33ºC, and ranging between 28ºC and 40ºC. The hotter the weather, the shorter the incubation time. Recently, producers tend to prefer polyethylene vessels instead of the old-fashioned large glazed terracotta jars as fermentation containers because the former are cheap, and more convenient to use. Without distillation, the alcohol content of the final product is approximately 6-8% w/v, which is quite low and not adequate for preservation. To overcome this problem, producers apply different ways to increase the alcohol content, either by adding refined or crude cane sugar after an initial period of aerobic solid-state fermentation, or by adding distilled concentrated alcohol to the final product based on

consumers' requirements. Some producers mix the moulded rice-mass with distilled alcohol instead of water, followed by submerged alcoholic fermentation for a few months, and filtration. To this clear filtered liquid, cane sugar can be added and the solution is cooked, and filtered again; this kind of wine can be preserved for a year and keeps its taste.

Similar as for the manufacture of regular rice wine, the alcoholic fermentation starters are produced in different ways based on the availability of raw materials and preferences. In principle, the traditional process of starter production is as follows: rice flour and/or cassava flour at a moisture content of 55-60% is mixed with starter culture with or without added supplements such as oriental medicinal herbs or leaves containing strong essential oils; next, the resulting dough is shaped into small, flat or globular cakes of about 2 cm diameter, allowed to incubate at room temperature in a ventilated place for 4-10 days to obtain the final product. In the Mekong Delta area, a combination of rice flour and cassava flour is a popular base and a supplement of Chinese medicinal herbs is preferred in the preparation. No information about leaves containing essential oils has been reported so far, since this information is protected as a hereditary right of mountain people who manufacture these starters. Predominant herbs applied in the Mekong Delta include *Cinnamomum cassia* Blume, *Illicium verum* Hook, *Foeniculum vulgare* Will, *Amomum* Tsao-Ko Grev. Et L., *Glycyrrhiza uralensis* Fisch, *Myristica fragrans* Hout, *Mentha arvensis* L., and *Eugenia aromstioca* Baill (Hieu, 1990; Phuc, 1998).

**Initial testing of starters**

In general, all tested starters could to a certain extent convert purple glutinous rice into wine. After the first three days of incubation under aerobic conditions, liquefaction and saccharification were clearly observed: liquid of high glucose content was released by the moulded mass which showed abundant mycelial growth in certain cases. During the next stage of 7 days incubation under anaerobic conditions, the alcoholic fermentation took place as shown by gas bubbling through the water lock.

Table 2 shows the mean values of three replications of the screening experiment in which liquefaction, pH, glucose, ethanol and colour measurements are given. The ethanol content obtained after alcoholic fermentation is an important screening criterium. Statistical analysis revealed significantly ($p < 0.05$) different alcohol levels.

Differences of liquefaction patterns could also be observed. There was a positive correlation between the extent of liquefaction and the production of ethanol. In cases of starters 2, 6, 15, 20, 23 and 29 the results showed that with more liquid formed during the aerobic solid-state fermentation, higher ethanol levels were obtained in the final products. This can be explained by strong activities of both moulds and yeasts in the microflora of the corresponding starters. However, high levels of reducing sugars were not necessarily completely converted into ethanol; for instance, in starters 2, 10, 11, 14 and 18 we observed significant residual levels of sugars suggesting that the yeasts in these starters have limited abilities to accumulate ethanol.

The initial pH of soaked purple glutinous rice decreased from 5.6 to the range of 3.8-4.6 after the first three days of incubation and then remained quite stable around 3.5-4.3 until the end of the fermentation.

By visual observation, the colour of the final products was described as red to lightly brown. The colour was also measured by Colorimeter using the L*a*b*-system for quantification, in which L = luminosity, a = red-green balance, and b= yellow-blue balance. The results show that the choice of starters did not strongly influence the colour of the wine.

The flavour of the fermenting rice was recorded after 3 days and at the end of the fermentation. After 3 days, a sweet fragrance was dominant in all treatments except with starters 9 and 28 which caused a slightly sour flavour, whereas slight alcoholic flavour was noticed with starters 2, 12, 20, 23 and 29. The final products had a strong alcohol flavour in all cases, and sweet odours still remained particularly in starters 1, 2, 3, 20 and 29.

During the fermentation process, glucose contents were high after the first three days but glucose was exhausted in most cases at the end of the fermentation, whereas ethanol increased, confirming that the starch is first converted to sugars and subsequently to alcohol. With starters 2, 12, 20, 23 and 29, glucose contents were rather low after mould fermentation but they were accompanied by significant levels of ethanol. We conclude that in these cases, the yeasts had started already to convert glucose as soon as it was produced and continued exhausting it towards the end of the fermentation. In contrast, the yeasts of starters 9, 10, 11, 14 and 18 only weakly fermented glucose that had been formed at high levels, resulting in low ethanol content and a significant level of residual sugars left in the final product. In the case of starters 2, 6, 15, 20, 23 and 29 we observed a complete fermentation, i.e. exhaustion of glucose. In all treatments, ethanol was detectable already after the first few days of the process, indicating that the production of rice wine using a starch-based starter is indeed the result of mixed and simultaneous fermentation. We also observed that all yeasts in the microflora of starters were able to grow under aerobic conditions; however, the significant increase of ethanol levels later during the process shows that fermentation by the yeasts is favoured by anaerobic conditions.

In conclusion, a limited selection of interesting starters was made for further studies. Starters 2, 6, 15, 20, 23 and 29 were selected because of their capacity for rice liquefaction, their significantly higher ethanol accumulation, as well as the flavour and the colour of their wines.

## Microflora composition and isolation of micro-organisms

The microbiological composition of the six selected starters reported as Log CFU/g of dry weight sample is shown in Table 3. Although it is known that mould counts represent their germination capacity rather than their biomass, in this case the use of CFU counts is appropriate as the germination activity is essential for starter performance. Yeasts had the highest count ($10^7$ CFU/g sample).

**Table 2. Performance of Vietnamese traditional rice wine starters**

| Starter | Liquefaction* after mould fermentation | Liquefaction* after alcoholic fermentation | pH after mould fermentation | pH after alcoholic fermentation | Glucose (% w/v) after mould fermentation | Glucose (% w/v) after alcoholic fermentation | Ethanol (% w/v) after mould fermentation | Ethanol (% w/v) after alcoholic fermentation** | Colour L | Colour a | Colour b |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ++ | | 3.8 | 3.6 | 19.3 | 0.6 | 5.3 | 10.2 cdef | 50.7 | 27.5 | 17.1 |
| 2 | +++ | | 4.1 | 3.9 | 12.9 | 0.1 | 8.2 | 11.0 b | 43.8 | 30.6 | 20.4 |
| 3 | + | | 4.6 | 3.6 | 20.2 | 0.3 | 5.0 | 9.6 hik | 45.8 | 29.7 | 20.8 |
| 4 | + | | 4.4 | 3.6 | 17.9 | 0.5 | 4.8 | 9.7 ghik | 45.6 | 29.8 | 22.0 |
| 5 | + | | 4.6 | 3.5 | 18.6 | 0.4 | 4.9 | 9.8 fghi | 42.8 | 31.8 | 22.4 |
| 6 | +++ | | 4.6 | 4.2 | 21.7 | 0.1 | 5.6 | 11.2 b | 42.5 | 33.1 | 22.2 |
| 7 | + | | 4.4 | 3.7 | 17.5 | 1.2 | 4.8 | 10.5 c | 51.5 | 21.2 | 21.7 |
| 8 | ++ | | 3.9 | 3.7 | 14.4 | 1.1 | 6.6 | 9.9 efghi | 49.1 | 26.4 | 20.7 |
| 9 | + | | 4.4 | 3.5 | 24.5 | 0.9 | 4.2 | 9.5 ik | 48.4 | 29.6 | 19.1 |
| 10 | + | | 4.4 | 3.5 | 24.1 | 0.7 | 4.5 | 10.0 defg | 46.4 | 30.6 | 19.7 |
| 11 | ++ | | 4.0 | 3.5 | 24.2 | 0.6 | 4.6 | 9.6 hik | 49.4 | 26.8 | 18.8 |
| 12 | ++ | | 4.1 | 3.9 | 4.4 | 0.7 | 9.6 | 9.8 fghi | 54.5 | 20.8 | 17.7 |
| 13 | ++ | | 4.1 | 3.9 | 22.3 | 0.6 | 5.4 | 10.3 cd | 52.1 | 24.2 | 17.3 |
| 14 | ++ | | 4.6 | 3.7 | 24.3 | 1.1 | 5.3 | 9.9 efgh | 46.0 | 29.6 | 21.2 |
| 15 | +++ | | 4.5 | 4.3 | 21.8 | 0 | 6.6 | 11.1 b | 45.0 | 29.7 | 21.3 |
| 16 | ++ | | 4.5 | 4.1 | 18.4 | 0.2 | 7.6 | 10.3 cd | 47.7 | 26.1 | 21.6 |
| 17 | + | | 4.1 | 3.4 | 20.1 | 1.1 | 4.5 | 9.6 hik | 47.2 | 30.4 | 18.9 |
| 18 | + | | 4.2 | 3.7 | 24.3 | 2.3 | 4.9 | 8.8 l | 49.1 | 27.1 | 20.5 |
| 19 | ++ | | 4.6 | 4.1 | 18.1 | 0.4 | 5.8 | 10.3 cd | 48.7 | 24.9 | 22.9 |
| 20 | +++ | | 4.4 | 4.3 | 12.9 | 0 | 9.3 | 11.7 a | 46.4 | 29.8 | 22.2 |
| 21 | +++ | | 4.6 | 4.2 | 16.2 | 0.4 | 6.9 | 10.1 cdefg | 50.8 | 22.2 | 22.1 |
| 22 | +++ | | 4.6 | 4.1 | 12.9 | 0.6 | 7.2 | 10.2 cde | 48.6 | 26.2 | 20.9 |
| 23 | +++ | | 4.4 | 4.3 | 9.5 | 0.1 | 10.1 | 12.0 a | 44.6 | 29.5 | 19.7 |
| 24 | + | | 4.6 | 4.1 | 22.4 | 0.4 | 5.8 | 10.5 c | 49.1 | 25.1 | 22.1 |
| 25 | ++ | | 4.6 | 4.4 | 20.9 | 0.5 | 6.0 | 10.3 cd | 54.9 | 18.8 | 21.0 |
| 26 | ++ | | 4.5 | 4.3 | 13.5 | 0.6 | 7.5 | 10.2 cdef | 54.8 | 20.0 | 19.9 |
| 27 | + | | 4.6 | 3.6 | 20.6 | 1.1 | 5.8 | 9.4 k | 46.8 | 27.7 | 21.6 |
| 28 | ++ | | 4.6 | 3.6 | 20.2 | 0.9 | 5.4 | 9.5 hik | 43.1 | 31.6 | 23.2 |
| 29 | +++ | | 4.4 | 4.3 | 11.7 | 0.1 | 10.0 | 12.0 a | 45.4 | 28.7 | 19.4 |

*levels of liquefaction ranging from + (little) to +++ (very much); starters shaded grey are those selected for further study.

**means with difference subscripts are statistically significant at the 95% confidence level.

In general, lactic acid bacteria (LAB) were found to be present at relatively low levels compared to yeasts and moulds. A total of 119 strains, comprising 53 moulds, 51 yeasts and 15 bacteria, was isolated for identification and further study.

**Table 3. Total mould, yeast and lactic acid bacteria (LAB) counts (log CFU/g dry weight) of selected fermentation starters**

| Starter | Moulds | | | Yeasts | | | LAB | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | Mean values | A | B | Mean values | A | B | Mean values |
| 2 | 5.8 | 6.2 | 6.0 | 7.4 | 6.8 | 7.1 | 5.8 | 6.6 | 6.2 |
| 6 | 5.0 | 3.6 | 4.3 | 7.0 | 6.6 | 6.8 | 4.2 | 5.2 | 4.7 |
| 15 | 3.7 | 3.1 | 3.4 | 6.1 | 5.5 | 5.8 | 2.9 | 2.3 | 2.6 |
| 20 | 5.5 | 4.7 | 5.1 | 7.1 | 7.3 | 7.2 | 4.8 | 4.0 | 4.4 |
| 23 | 5.1 | 4.1 | 4.6 | 6.5 | 7.3 | 6.9 | 4.1 | 3.9 | 4.0 |
| 29 | 4.8 | 4.2 | 4.5 | 7.0 | 6.4 | 6.7 | 3.6 | 3.0 | 3.3 |

A and B are duplicates

# Chapter 3

# The contribution of mycelial fungi

# during the saccharification of rice

**Abstract**

In the liquefaction and saccharification process, which is one of the two essential stages in the alcoholic fermentation of Vietnamese purple glutinous rice wine, the starchy materials are degraded into sugars, mainly glucose. In this study, the role of the enzymatically active mycelial fungi in the starch conversion, and optimum conditions for their function were examined. Eight of 53 moulds isolated from Vietnamese rice wine fermentation starters were selected for their ability to degrade starch, and were studied further for their glucose production and amyloglucosidase activities during the saccharification of purple glutinous rice (*Oryza sativa* var. *glutinosa*). Isolates were identified as *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (an atypical form of *A. rouxii*), *Rhizopus oligosporus* and *Rhizopus oryzae*. *Amylomyces rouxii* was able to produce up to 25% (w/w of fermented moulded mass) glucose during saccharification with an amyloglucosidase activity up to 0.6 U/g of fermented moulded mass. Based on statistical analysis, optimum conditions for saccharification were defined as follows: incubation temperature 34ºC, incubation time 2 days and inoculation level 5 Log spores/g of steamed rice.

## INTRODUCTION

The liquefaction and saccharification of rice by aerobic solid-state fermentation is one of the two important and essential stages of the preparation of rice wine. In this process, starchy materials are degraded and converted to sugars of which glucose is the main component. The starch, composed of amylose and amylopectin molecules associated by hydrogen bonding (Leach, 1965; Nout, 1992; Wasserman and Yu, 2003), is first soaked to soften and hydrate the starch granule for subsequent gelatinization by steaming or cooking to make it more readily available for enzymic hydrolysis (Snow and O'Dea, 1981). Enzymes which hydrolyse starch are available from animal, plant, and microbial sources; microbes are increasingly and widely used as important enzyme sources for production of starch-degrading enzymes. These enzymes are mainly used in the food and beverage industry. Many micro-organisms including fungi and bacteria can produce amylolytic enzymes, particularly α-amylase and glucoamylase (Underkofler, 1976; Ray, 2001; Shigechi et al., 2002). In the present chapter, fungi receive major attention as they are

considered crucial for starch degradation in rice wine manufacture. In the solid-state fermentation of gelatinized rice, the moulds bring about liquefaction by α-amylase, and saccharification by amyloglucosidase of rice starch to glucose. Therefore, both enzymes are of concern in the starch degradation but in a different mode. α-Amylase cleaves starch randomly at 1,4-α-glycosidic bonds, giving maltooligo-saccharides as final products (Wong and Robertson, 2003). Amyloglucosidase liberates single D-glucose monomers in the β-form from the non-reducing end of starch and preferentially hydrolyses 1,4-α-glucosidic bonds and with a considerably lower reaction rate 1,6-α-glucosidic linkages (Hoa, 1998; Schindler et al., 1998; Ha, 2000; Reilly, 2003).

This chapter presents a comparative study of starch degrading activities of pure strains of moulds isolated from Vietnamese commercial rice wine starters. Powerful glucose producers are identified and culture conditions are optimized for maximum starch degrading activity.

## MATERIALS AND METHODS

### Cultures

A total of 53 pure isolates of moulds previously isolated from six selected Vietnamese commercial fermentation starters were examined.

### Screening test of starch degradation

With the aim to obtain a limited selection of moulds with high enzyme activities, all 53 pure cultures of moulds were tested for their ability to degrade soluble starch, in rice starch agar medium. The medium contained 0.5% of glutinous rice flour, 0.1% of peptone (Oxoid, L34) and 1.5% of agar technical (Oxoid, L13). A piece of mould pure culture was transferred to the centre of a plate of starch agar by means of an inoculating wire and incubated at 30ºC for 48 hours. Visualization of starch degradation was done by flooding with a 0.25% iodine solution. Clearing of the typically blue coloration of the starch with iodine indicated starch degradation.

### The saccharification process with selected moulds

*Procedure of saccharification test*

Fifty grams of purple glutinous rice and 60ml of distilled water in a 250 ml conical flask covered by a cotton plug, were soaked for 4 hours at room temperature (22ºC). After soaking they were steamed in an autoclave for 1 hour at 100ºC. The steamed rice paste was cooled to 35-40ºC, then inoculated and mixed well with pure cultures, which had been grown for 4 days at 30ºC on a slant of malt extract agar (Oxoid, CM59) and suspended in a certain volume of sterile physiological salt solution 0.85% to get the final level of $10^6$

spores/g of rice paste. The inoculated rice paste was incubated for 4 days at 30ºC and then harvested for analyses.

*Degradation products*

Glucose and ethanol were determined by HPLC, using the organic acid analysis column-Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm (BIO-RAD, Thermo Separation Products Inc. USA).

*Enzyme activity*

Amyloglucosidase was assayed by using a test kit (Megazyme, RAMGR3) of p-Nitrophenyl-β-maltoside according to (McCleary et al., 1991).

## Identification of isolated moulds

A limited selection of enzymatically active moulds was identified based on morphological examination and cultural properties. The species of moulds were classified according to taxonomic keys and descriptions (Ellis et al., 1976; Hesseltine, 1991; Samson and Hoekstra, 2002).

## Factors affecting mould growth and starch degrading activity

The experiment was set up in a factorial design (three factors) at three levels: spore inoculum (using a spore suspension of $10^8$ spores/ml by microscopic count, three levels of spore inoculation were made at 4, 5, and 6 Log spores/g of steamed rice paste), incubation time (for 2, 3 and 4 days) and incubation temperature (20ºC, 30ºC and 40ºC). Each treatment had duplicates.

## Statistical analysis

Experimental data were analysed statistically using Statgraphics Plus Version 5, Manugistics, Inc., Rockville, USA and the software of the General Algebraic Modeling System (GAMS), Washington, DC, USA.

## RESULTS AND DISCUSSION

## Gelatinization of purple glutinous rice starch

Gelatinization was monitored by observing the loss of birefringence under a polarization microscope at time intervals of 15 minutes until the birefringence of the native starch granules was lost completely. The soaked starch - purple glutinous rice in this case - was completely gelatinized after steaming at 100ºC for 1 hour. Indeed, this time-temperature

combination is sufficient for rice starch gelatinization. Although individual granules of each starch species have a specific gelatinization temperature range even up to 121ºC for 1 hour (in case of potato starch), the maximum temperature reported for rice starch gelatinization is 80ºC (Leach, 1965).

**Liquefaction of gelatinized purple glutinous rice starch**

Table 1 shows the results of the mould growth (indicated as mycelium appearance) and the diameters of the starch degradation zones in duplicate. Most strains grew very well on glutinous rice starch agar, but this does not automatically imply that they are good at starch degradation. However, it can be concluded that strains that actively degraded starch, always grew well.

Statistical analysis revealed significant ($p < 0.05$) differences in degrading ability. From the screening test of the starch degradation, 8 strains of moulds that were isolated from 4 different starters significantly had the highest ability to convert starch, and therefore these 8 strains were studied in more detail for their saccharification, and other factors affecting their performance.

**Saccharification of purple glutinous rice starch**

The eight mould strains selected for strong starch degradation as shown in Table 1 were studied further for their capacity to saccharify starch. This was done by inoculating these moulds into a previously gelatinized glutinous rice mass followed by incubation at 30ºC. With all strains except 6.5, liquefaction was obvious after one day of incubation, as a liquid phase appeared, which increased with time of incubation. Samples of liquid and solid mass were taken daily and analysed for pH, glucose and amyloglucosidase activity.

During the incubation, the pH rapidly decreased to a range of 4.0 - 4.7, as compared to a pH of 6.2 of an uninoculated control. This pH range is also indicative of the purity of the mould cultures, since in cultures contaminated with (acidifying) bacteria, the pH usually becomes considerably lower: about pH 3.0 - 3.5.

Production of glucose and amyloglucosidase activity are presented in Figures 1 and 2, respectively. All strains gave the highest glucose contents after 3 days of incubation. Three groups of moulds, producing different levels of glucose could be distinguished; in the order from high to low levels as follows: group 1 including strains 6.9, 20.2, 20.3 and 20.7; group 2 including 15.2, 15.4 and 15.5; group 3 including 6.5. The differences between these three levels were statistically significant at the 95% confidence level. The highest glucose level reached was 25% (w/w). There is also a good correlation between glucose levels and amyloglucosidase activities. Strains producing high glucose levels also had significantly higher enzyme activities. In other words, similar to the order of glucose production, the same three groups with different amyloglucosidase activity were distinguished. The highest amyloglucosidase activity found was 0.6 U/g of fermented moulded mass.

**Table 1. Performance of moulds in starch liquefaction**

| strain no. | mycelium appearance [1] | diameter of clear zone in iodine test (cm) [2] | | |
|---|---|---|---|---|
| | | A [3] | B | Mean values |
| 2.1 | ++ | 4.1 | 4.5 | 4.3 mno |
| 2.2 | + | 4.2 | 4.5 | 4.4 lmn |
| 2.3 | + | 3.1 | 2.7 | 2.9 rs |
| 2.4 | ++ | 4.5 | 4.5 | 4.5 klmn |
| 2.5 | + | 3 | 4 | 3.5 pq |
| 6.1 | ++ | 5 | 5.4 | 5.2 fghi |
| 6.2 | ++ | 4.5 | 4.8 | 4.7 iklm |
| 6.3 | ++ | 5.5 | 5.2 | 5.4 efgh |
| 6.4 | ++ | 5.1 | 5.6 | 5.4 efgh |
| 6.5 | +++ | 7.2 | 7 | 7.1 c |
| 6.6 | ++ | 5.3 | 5.2 | 5.3 efgh |
| 6.7 | ++ | 5.3 | 5.5 | 5.4 efgh |
| 6.8 | + | 2.4 | 2.6 | 2.5 st |
| 6.9 | +++ | 7.1 | 7 | 7.1 c |
| 6.10 | ++ | 4.8 | 5 | 4.9 hikl |
| 6.11 | +++ | 5.6 | 4.9 | 5.3 efgh |
| 15.1 | ++ | 3.6 | 3.7 | 3.7 pq |
| 15.2 | +++ | 9 | 8.9 | 9.0 a |
| 15.3 | ++ | 2 | 2 | 2.0 t |
| 15.4 | +++ | 8.7 | 8.8 | 8.8 ab |
| 15.5 | +++ | 9.2 | 9.3 | 9.3 a |
| 15.6 | ++ | 2 | 2.1 | 2.1 t |
| 15.7 | +++ | 2 | 2 | 2.0 t |
| 20.1 | ++ | 5.5 | 5 | 5.3 efgh |
| 20.2 | ++ | 7.2 | 7.2 | 7.2 c |
| 20.3 | +++ | 8.1 | 8.5 | 8.3 b |
| 20.4 | +++ | 2.5 | 2 | 2.3 t |
| 20.5 | +++ | 3.5 | 4 | 3.8 opq |
| 20.6 | +++ | 3 | 3.5 | 3.3 qr |
| 20.7 | ++ | 7 | 7.2 | 7.1 c |
| 20.8 | +++ | 2.8 | 2.2 | 2.5 st |
| 20.9 | +++ | 2 | 2 | 2.0 t |
| 23.1 | ++ | 5.4 | 5.4 | 5.4 efgh |
| 23.2 | ++ | 5 | 5.2 | 5.1 ghi |
| 23.3 | ++ | 5.3 | 5.5 | 5.4 efgh |
| 23.4 | +++ | 2.5 | 2.1 | 2.3 t |
| 23.5 | +++ | 2.3 | 2 | 2.2 t |
| 23.6 | +++ | 6 | 6.5 | 6.3 d |
| 23.7 | ++ | 5.2 | 6 | 5.6 efg |
| 23.8 | +++ | 2.2 | 2.5 | 2.4 st |
| 23.9 | +++ | 2 | 2.1 | 2.1 t |
| 29.1 | ++ | 5.5 | 5.8 | 5.7 efg |
| 29.2 | ++ | 5.6 | 6 | 5.8 de |
| 29.3 | + | 5.1 | 5.1 | 5.1 ghi |
| 29.4 | + | 4.1 | 4 | 4.1 nop |
| 29.5 | ++ | 5 | 5.6 | 5.3 efgh |
| 29.6 | ++ | 5 | 5.5 | 5.3 efgh |
| 29.7 | + | 5 | 5 | 5.0 hik |
| 29.8 | ++ | 5.7 | 5.2 | 5.5 efgh |
| 29.9 | + | 3 | 4 | 3.5 pq |
| 29.10 | ++ | 6.1 | 5.3 | 5.7 def |
| 29.11 | ++ | 6 | 6.5 | 6.3 d |
| 29.12 | +++ | 6.5 | 6 | 6.3 d |

Moulds were inoculated on glutinous rice starch plates and incubated 30ºC for 48 hours.
[1] levels of mycelium appearance ranging from + (little) to +++ (very much); [2] there is a statistically significant difference amongst the medians at the 95% confidence level. 19 homogenous groups were identified using the order of alphabet. The standard error is 0.204. Strains shaded grey are those selected for further study; [3] A and B are duplicates.

However, after 4 days of incubation, glucose decreased whereas amyloglucosidase still increased slightly. This can be explained by consumption of glucose during longer incubation, caused by depletion of other nutrient sources, whereas amyloglucosidase activity remained intact or was even increased.

In this experiment only pure moulds were used as inocula, so ethanol production was not expected because the conversion from sugars to ethanol would be primarily performed by yeasts. However, the analytical results showed that small quantities of ethanol (approx. 1.5% w/w) were found in all samples. This seems little in comparison with the amount of glucose produced by moulds; the phenomenon can be explained by the ability of some moulds to ferment sugar into ethanol under anaerobic conditions (Hawker, 1966).
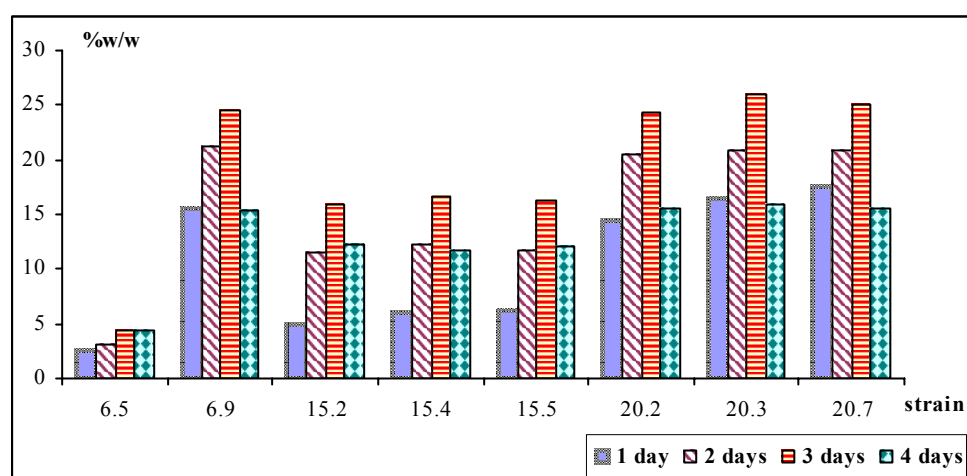


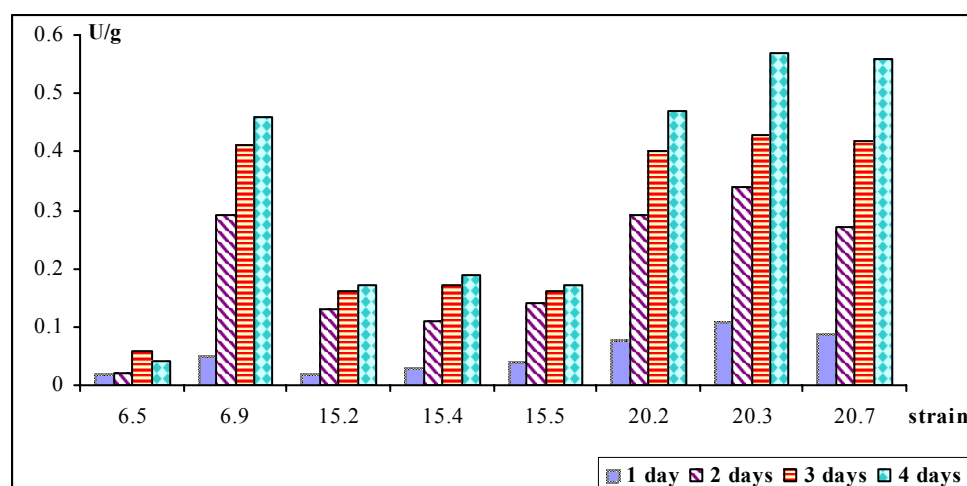**Figure 1. Glucose production by moulds in the saccharification**



**Figure 2. Amyloglucosidase activity of moulds in the saccharification**

**Identification of selected mould isolates**

As shown in Figures 1 and 2 and as mentioned in the previous section, three main groups of moulds were distinguished according to their abilities to produce glucose and amyloglucosidase. All eight selected mould isolates were characterized. Table 2 summarizes the morphological and cultural properties of the selected moulds that formed the basis of their identification. Four species were distinguished, namely *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (an atypical form of *A. rouxii*), *Rhizopus oligosporus* and *Rhizopus oryzae*. These species were also found earlier in amylolytic fermentation starters used in the production of alcoholic beverages in various Asian countries (Hesseltine and Wang, 1986; Phuc, 1998; Nout and Aidoo, 2002). The results show that strains that gave the same performance in the starch degradation and saccharification belonged to the same species. *Amylomyces rouxii* appears to be the favourite mould for starch conversion. All species belong to genera of the Zygomycetes, which are commonly found in foods; these are confined to the order Mucorales. The moulds in this order can produce alcohol under low oxygen conditions (Hawker, 1966; Cronk et al., 1977). These findings explain the presence of low but significant levels of ethanol in saccharified rice.

**Table 2. Distinctive morphological and cultural properties of identified moulds**

| Properties | *Amylomyces rouxii* | *Amylomyces* aff. *rouxii* | *Rhizopus oligosporus* | *Rhizopus oryzae* |
|---|---|---|---|---|
| Isolates | 6.9, 20.3, 20.7 | 20.2 | 6.5 | 15.2, 15.4, 15.5 |
| Colony | white, 3-6 mm high | most characteristics look very alike *A. rouxii*, but is an atypical form, such as sporangia maturing with small apophysis on the top | pale brownish-grey, up to 1 mm high | whitish, brownish-grey with age, about 10 mm high |
| Rhizoids | none | | brownish, very short | brownish, opposite the sporangiophores |
| Sporangiophores | formed mostly in restricted marginal areas, rarely branched | | solitary, or in groups of up to 4, non-striate | solitary, or in groups of up to 5, striate |
| Sporangia | abortive, globose, usually not maturing | | globose, brownish-black at maturity | globose, dark-brown |
| Chlamydospores | very abundant, single and in series, predominantly intercalary | | abundant, single or in short chains | abundant |
| Columella | subglobose, smooth | | globose, with funnel-shaped apophysis | ovoid or globose, slightly rough-walled |
| Growth | optimum at 35-40ºC | | optimum at 30-35ºC | optimum at 30-35ºC |

**Optimization of glucose production by *Amylomyces rouxii***

Because of its powerful glucose production and enzymatic activity, *Amylomyces rouxii* (strain no. 20.3, CBS 111757) was selected for the study of factors affecting saccharification performance. According to the statistical design, in total there were 27

treatments which are presented in Table 3 with the corresponding results of pH, glucose levels and amyloglucosidase activity. Again, pH values in the range of 4.3-4.7 indicated a successful solid-state fermentation, free from contamination with acidifying bacteria. The results showed that the incubation temperature had a strong effect, whereas the incubation time and the inoculation levels did not show any influence on the pH values. pH levels were almost the same in the case of incubation at 30ºC or 40ºC, in which a pH-range of 4.3-4.8 was found. However, this range was significantly different from that obtained in case of incubation at 20ºC. There is a correspondence between glucose levels and final pH values. Combinations producing high glucose levels had final pH of 4.3-4.8 and poor glucose producers had final pH values of 5.5-6.2. *Amylomyces rouxii* species are favoured by temperatures around 30ºC but still show good growth and enzyme activity at 37ºC and 40ºC (Ellis et al., 1976). The low pH of approx. 4.0 is also preferred for the production of microbial amylolytic enzymes (Underkofler, 1976).

The liquefaction was temperature dependent; for example, at 20ºC, no liquid was formed due to the poor growth during solid-state fermentation, whereas at 30ºC and 40ºC much liquid was produced in all cases after 2 days of incubation. Therefore, the sampling for determination of glucose and amyloglucosidase was made on the totality of the moulded mass, instead of only the liquid, in order to keep all samples in the test analysed under the same conditions. However, samples of liquid were also taken for analysis of glucose and amyloglucosidase to examine any difference compared with samples of solid moulded mass. It was found that both glucose and amyloglucosidase levels in liquid were much higher (even twice in some cases) than those in solid samples. This phenomenon can be understood based on the principle of starch conversion. After the complete gelatinization by steaming soaked purple glutinous rice at 100ºC, resulting in the dissolution of starch granules to form a viscous suspension, the viscosity of the paste was reduced by starch hydrolysis with α-amylase, and finally saccharified to glucose (Chaplin, 2003). Therefore the glucose concentration is higher in liquefied extract fraction, and lower in the residue fraction of the saccharified moulded mass. A correlation between glucose levels and amyloglucosidase activities was again observed. Combinations producing high glucose levels also had high enzyme activity. However, at the same favourable incubation temperature, glucose accumulation was significantly influenced by both the incubation time and inoculation level, whereas amyloglucosidase activity was influenced by incubation time only. Combinations inoculated with different spore concentrations gave almost identical amyloglucosidase activity.

Based on the results of statistical analysis of Statgraphics Plus Version 5, GAMS optimizing software was applied to determine the optimum conditions for the moulds to produce glucose. These are a combination of incubation temperature of 34ºC, incubation time of 2 days and inoculation level of 5 Log spores/g of steamed rice paste.

Table 4 indicates the statistical significance of each of the factors and the statistically significant differences at the 95% confidence level between pairs of means. The relationship between glucose and independent variables is described in Table 5 resulting in the equation of the fitted model from which the surface plot of the optimum conditions for glucose production is made and shown in Figure 3.

**Table 3. Variables affecting saccharification in solid-state fermentation**

| | Testing factors | | | | Result of Solid-State Fermentation | | | |
|---|---|---|---|---|---|---|---|---|
| | Incubation temperature | Incubation time | Inoculation levels (Log | | Glucose | | Amyloglucosidase | |
| | | | | | Solid | Liquid | Solid | Liquid |
| No. | (ºC) | (days) | spores/g) | pH | (% w/w) | (% w/v) | (U/g) | (U/ml) |
| 1 | 20 | 2 | 4 | 6.2 | 0.07 | | 0 | |
| 2 | 20 | 2 | 5 | 6.1 | 0.09 | | 0 | |
| 3 | 20 | 2 | 6 | 5.9 | 0.16 | | 0 | |
| 4 | 20 | 3 | 4 | 6.0 | 0.23 | No | 0.05 | No |
| 5 | 20 | 3 | 5 | 5.8 | 0.24 | liquid | 0.05 | liquid |
| 6 | 20 | 3 | 6 | 5.7 | 0.7 | formed | 0.06 | formed |
| 7 | 20 | 4 | 4 | 5.8 | 0.8 | | 0.06 | |
| 8 | 20 | 4 | 5 | 5.6 | 2.5 | | 0.06 | |
| 9 | 20 | 4 | 6 | 5.5 | 2.8 | | 0.06 | |
| 10 | 30 | 2 | 4 | 4.6 | 15.1 | 28.3 | 0.34 | 0.55 |
| 11 | 30 | 2 | 5 | 4.5 | 21.9 | 34.8 | 0.36 | 0.55 |
| 12 | 30 | 2 | 6 | 4.5 | 14.4 | 28 | 0.32 | 0.54 |
| 13 | 30 | 3 | 4 | 4.6 | 15.9 | 27.5 | 0.35 | 0.54 |
| 14 | 30 | 3 | 5 | 4.3 | 20 | 33 | 0.37 | 0.59 |
| 15 | 30 | 3 | 6 | 4.4 | 14.6 | 26.1 | 0.32 | 0.54 |
| 16 | 30 | 4 | 4 | 4.4 | 13.9 | 24.2 | 0.34 | 0.68 |
| 17 | 30 | 4 | 5 | 4.6 | 14.5 | 30.6 | 0.36 | 0.67 |
| 18 | 30 | 4 | 6 | 4.7 | 12.1 | 25.7 | 0.34 | 0.65 |
| 19 | 40 | 2 | 4 | 4.7 | 13.4 | 23.4 | 0.17 | 0.37 |
| 20 | 40 | 2 | 5 | 4.6 | 16.2 | 30.9 | 0.18 | 0.38 |
| 21 | 40 | 2 | 6 | 4.6 | 12.1 | 26.6 | 0.18 | 0.35 |
| 22 | 40 | 3 | 4 | 4.6 | 13 | 28 | 0.28 | 0.58 |
| 23 | 40 | 3 | 5 | 4.6 | 14.1 | 30.3 | 0.27 | 0.57 |
| 24 | 40 | 3 | 6 | 4.7 | 13.9 | 27.2 | 0.27 | 0.56 |
| 25 | 40 | 4 | 4 | 4.8 | 13.2 | 25 | 0.24 | 0.49 |
| 26 | 40 | 4 | 5 | 4.8 | 15.5 | 27.4 | 0.23 | 0.44 |
| 27 | 40 | 4 | 6 | 4.8 | 14.2 | 25.6 | 0.24 | 0.5 |

Moulds were inoculated at the indicated levels to gelatinized purple glutinous rice starch in conical flasks with a cotton plug, and incubated at the indicated time-temperature combinations, after which samples of saccharified rice paste (solid moulded mass including liquid and only liquid when available) were analysed for pH, glucose contents and amyloglucosidase activity. In each treatment values are means of duplicates.

**Table 4. Statistical analysis of variance and multiple range tests for glucose by temperature, time and inoculum level (Log spores/g of steamed rice)**

| Source | Analysis of variance [1] | | | | | Multiple range tests [2] | |
|---|---|---|---|---|---|---|---|
| | Sum of squares | Df | Mean square | F-Ratio | p-Value | LS Mean | Homogeneous groups |
| Main effects | | | | | | | |
| Temperature (ºC) | 2394.95 | 2 | 1197.47 | 367.09 | 0.0000 | | |
| 20 | | | | | | 0.848889 | a |
| 40 | | | | | | 13.95 | b |
| 30 | | | | | | 15.8167 | c |
| Time (days) | 1.89006 | 2 | 0.94503 | 0.29 | 0.7498 | | |
| 4 | | | | | | 9.94444 | d |
| 3 | | | | | | 10.2967 | d |
| 2 | | | | | | 10.3744 | d |
| Inoculum level (Log spores/g) | 58.4192 | 2 | 29.2096 | 8.95 | 0.0005 | | |
| 6 | | | | | | 9.43444 | e |
| 4 | | | | | | 9.50556 | e |
| 5 | | | | | | 11.6756 | f |
| Residual | 153.319 | 47 | 3.2621 | | | | |
| Total (corrected) | 2608.58 | 53 | | | | | |

[1] The analysis decomposes the variability of glucose production into contributions due to various factors. The p-values indicate the statistical significance of each of the factors. Because of p-values less than 0.05, temperature and inoculum level have a statistically significant effect on glucose production (95% confidence level); [2] The test applies a multiple comparison procedure to determine which means are significantly different. The homogenous groups are identified by identical symbols.

**Table 5. Statistical analysis of multiple regression for (A) relationship between glucose production and independent variables and for (B) relationship between variables**

**(A)**

| Parameter | Estimate | Standard error | T Statistic | p-Value |
|---|---|---|---|---|
| CONSTANT | -137.752 | 26.8875 | -5.12327 | 0.0000 |
| Temperature | 5.96989 | 0.796645 | 7.49379 | 0.0000 |
| Time | 1.16167 | 7.96645 | 0.14582 | 0.8847 |
| Log spores | 22.1525 | 6.97168 | 3.1775 | 0.0028 |
| Temperature*Temperature | -0.0841722 | 0.00534573 | -15.7457 | 0.0000 |
| Time*Time | -0.137222 | 0.534573 | -0.256695 | 0.7986 |
| Log spores*Log spores | -2.20556 | 0.534573 | -4.12583 | 0.0002 |
| Temperature*Time | -0.06025 | 0.234543 | -0.256883 | 0.7985 |
| Temperature*Log spores | -0.0295 | 0.143938 | -0.204949 | 0.8386 |
| Time*Log spores | 0.123333 | 1.43938 | 0.085685 | 0.9321 |
| Temperature*Time*Log spores | 0.00425 | 0.0462953 | 0.0918019 | 0.9273 |

**(B)**

| Source | Sum of squares | Df | Mean square | F-Ratio | p-Value |
|---|---|---|---|---|---|
| Model | 2461.12 | 10 | 246.112 | 71.77 | 0.0000 |
| Residual | 147.456 | 43 | 3.42921 | | |
| Total (corrected) | 2608.58 | 53 | | | |

R-squared= 94.3473%; Standard error of Est.= 1.85181; Mean absolute error= 1.18625; p-Value is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.
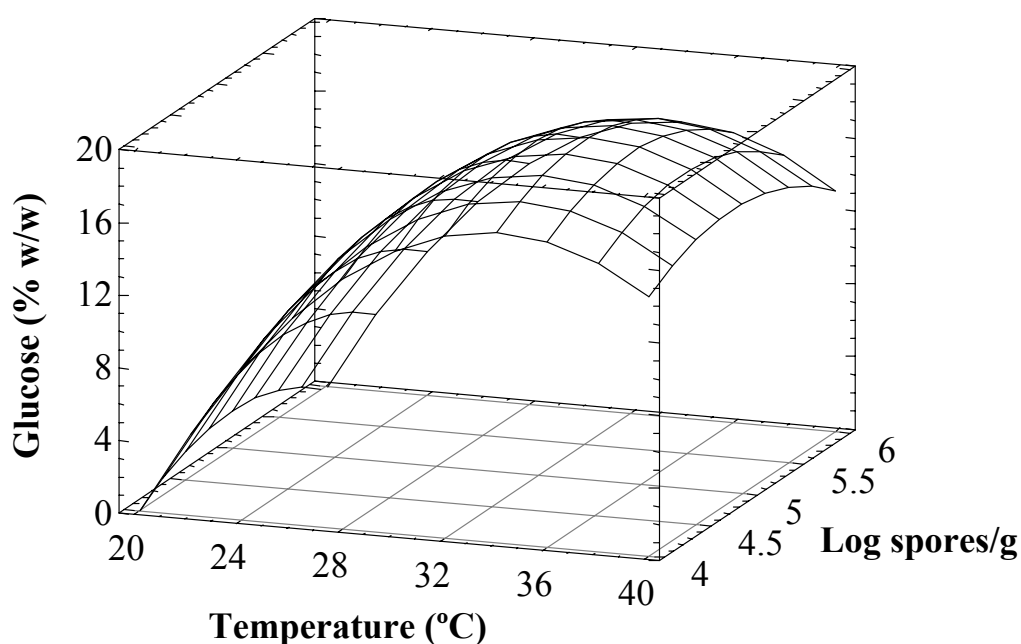
**Figure 3. Effect of incubation temperature (range 20-40ºC) and inoculation level (range 4-6 Log spores/g of steamed rice) on concentration of glucose produced (optimum incubation period was 2 days)**

This chapter addresses the conversion of starch involving three main stages, namely gelatinization, liquefaction and saccharification. The first requirement is to solubilise the starch by heating in order to break hydrogen bonds in the starch granules. Gelatinization is achieved typically by heating in water at 90-100ºC (Hall, 2001). In the present study, the purple glutinous rice starch was gelatinized by steaming at 100ºC for 1 hour which resulted in complete gelatinization. The starch hydrolysis during the liquefaction was evaluated by measuring α-amylase activity using rice-starch agar. Since α-amylase only cleaves starch randomly at 1,4-α-glycosidic bonds resulting in liquefaction and yielding maltooligo-saccharides as final products, it belongs to the liquefying category that is the primary stage in the starch degradation. The maltodextrins formed by α-amylase during the liquefaction are then further saccharified by amyloglucosidase, liberating single D-glucose. Measurement of the glucose contents is the final step for evaluation of starch conversion. In this study both enzymes were examined as produced by *Amylomyces rouxii*, *Amylomyces* aff. *rouxii*, *Rhizopus oligosporus* and *Rhizopus oryzae*. These mould isolates were selected for their high starch degradation in rice starch agar medium. However, in further starch saccharification four isolates belonging to *Amylomyces* species gave significantly higher results of glucose and amyloglucosidase compared with isolates belonging to *Rhizopus* strains. This difference can be explained by (a) lower amyloglucosidase activity of *Rhizopus* spp. (Underkofler, 1976), or (b) higher consumption by *Rhizopus* of glucose for mycelium production. Therefore, successful starch degradation

in starch agar is not necessarily a guarantee for good saccharification (Schindler et al., 1998; Araujo et al., 2004), as the efficiency of saccharification is correlated closely with the glucogenic enzyme system rather than with the activity of α-amylase (Underkofler, 1976).

In order to accurately analyse the yield of the starch conversion, some criteria need to be considered, such as complete gelatinization of starch, specificity and purity of enzymes, complete hydrolysis of starch to glucose, measurement of glucose produced from hydrolyzed starch and minimization of interference (Hall, 2001). In the present study, the analysis of starch degradation was carried out following the above-mentioned critical factors. Besides, the findings in this chapter also provide optimum conditions including spore inoculum, incubation temperature and incubation time for growth and function of moulds for the maximum hydrolysis of starch to glucose. This study did not address in detail the limiting factors to glucose production or the improvement of the yield of starch conversion. However, it can be seen clearly that following the enzymatic process by the enzymatically active mould *Amylomyces rouxii* under optimum conditions, the maximum content of glucose will be obtained. In a further study, the selected *Amylomyces rouxii* strain will be examined for its compatibility with fermentative yeasts in order to formulate a defined mixed-culture starter. The choice of starter is very important in the alcoholic fermentation of rice wine since it strongly influences the yield and quality of wine.

# Chapter 4

# Fermentative capacity and ethanol tolerance of yeasts

# in the alcoholic fermentation of rice wine

**Abstract**

One of the most important and best-known industrial fermentations is that in which alcohol is produced from sugars by yeasts. It is usually conducted with yeasts belonging to the species *Saccharomyces cerevisiae* which is predominant in the production of traditional fermented beverages. In this study, the characteristic pattern of an alcoholic fermentation performed by yeasts, and their potential of alcohol production, their ethanol tolerance as well as the optimum conditions for their activity were investigated. Of 51 yeasts isolated from Vietnamese rice wine starters, five strains selected as superior alcohol producers were characterized as *Saccharomyces cerevisiae* and two representative isolates of poor ethanol producers belonged to *Candida glabrata* and *Pichia anomala*. The *S. cerevisiae* strains were studied further for their fermentation rate and ethanol tolerance. They performed successfully in the alcohol fermentation with complete depletion of a relatively high initial percentage of glucose in which 8.8% (w/v) ethanol was produced from the initial reducing sugars at 20% (w/v). Fermentative capacity of *S. cerevisiae* was limited at ethanol concentration of 14% (w/v) in a fed-batch test that was carried out under conditions of excessive glucose supply. The yeast growth was completely inhibited by ethanol concentrations of 9 to 10% (w/v) in challenge tests with added ethanol. Based on statistical analysis, optimum conditions for the fermentation were as follows: incubation temperature 28.3ºC, incubation time 4 days and inoculation level 5.5 Log cells/ml of saccharified rice liquid.

## INTRODUCTION

In nearly all regions of the world fermented alcoholic beverages - strongly linked to people's culture - are produced in diverse forms and tastes and are consumed since ancient times (Lim, 1991; Phuc, 1998). Starting from starchy raw materials such as rice starch, saccharification and alcoholic fermentation are the two major steps involved in the alcohol production. In this conventional two-stage process, starch is enzymatically hydrolyzed by moulds to glucose which can subsequently be fermented to ethanol mainly by yeasts (Nout and Aidoo, 2002). In fact, some yeasts can degrade starch but this trait is not widespread (De Mot and Verachtert, 1987; Laluce et al., 1988; Ulgen et al., 2002). Years of experience and study have shown that yeasts are predominant and essential in winemaking because of their alcoholic fermentation and their effects on wine quality through secondary

metabolites and autolysis products (Fleet, 1998; Querol et al., 2003). Various yeasts are found to be involved in wine production (Tam, 2000; Ray, 2001; Nout and Aidoo, 2002). However, the most beneficial and important yeasts in terms of desirable food fermentation belong to the genus *Saccharomyces*, especially *S. cerevisiae* (Battcock and Ali, 1993). Ethanol is the major desired product resulting from the fermentation, but it also inhibits the yeast cells by various mechanisms (Casey and Ingledew, 1986; Jones, 1989; D'Amore et al., 1990). It inhibits cell growth, viability and fermentation activity, although to different extents. Therefore, the higher the ethanol tolerance of the yeast, the more alcohol it can accumulate. One of the major target sites of ethanol in yeasts and other micro-organisms is the plasma membrane (D'Amore, 1992). At certain high concentrations, ethanol causes altered membrane organization and permeability. This results in leakage of cofactors and coenzymes which are essential components for the activity of enzymes involved in glycolysis and alcohol production, resulting in the inhibitory effect of ethanol on fermentation. A number of other factors also affect yeast fermentation performance, such as osmotic pressure, substrate feeding, nutrient supplementation, temperature, and intracellular ethanol accumulation (Jones et al., 1981; D'Amore, 1992). The nature of ethanol tolerance is rather complex and no universal method of measurement of ethanol tolerance exists. Methods that could be used to assay and define ethanol tolerance are for example, to monitor the effects of ethanol on batch culture growth, fermentative capacity, and cell viability. Tolerance could also be defined as the maximum level of ethanol produced. The experimental conditions should be necessarily mentioned when measuring ethanol tolerance, since each method has its own advantages and disadvantages which shows that alcohol affects the above activities in different ways (D'Amore et al., 1990). Optimized fermentation conditions are required to improve yeast performance and ethanol tolerance.

The objectives of this study were to examine the fermentative capacity and ethanol tolerance of yeasts isolated from Vietnamese rice wine starters and to identify them and to determine the optimum conditions for maximum fermentation activity.

## MATERIALS AND METHODS

### Cultures

A total of 51 pure isolates of yeasts previously isolated from six selected Vietnamese commercial fermentation starters were examined.

### Preparation of inoculum

The culture which had been grown for 2 days at 30ºC on a slant of malt extract agar (Oxoid, CM59) was suspended in a certain volume of sterile physiological salt solution 0.85% to obtain a level of $10^8$ cells/ml (microscopic count) to be used as inoculum.

**Preparation of saccharified purple glutinous rice liquid**

Saccharified purple glutinous rice liquid was prepared as follows: fifty grams of purple glutinous rice and 60 ml of distilled water in a 250 ml conical flask covered by a cotton plug, were soaked for 4 hours at room temperature (22ºC). After soaking, rice and water were steamed in an autoclave for 1 hour at 100ºC to obtain a paste. The steamed rice paste was cooled to 35-40ºC, then inoculated (5 Log CFU/g paste) and mixed well with *Amylomyces rouxii* (strain no. 20.3, CBS 111757), which had been grown for 4 days at 30ºC on a slant of malt extract agar (Oxoid, CM59), and was suspended in sterile physiological salt solution 0.85%. After incubation for 3 days at 30ºC, the saccharified mass was centrifuged to collect the clear saccharified liquid. The dissolved solids content was adjusted to 20ºBrix by adding sterile water.

**Screening for alcoholic fermentation**

To select yeast strains for further studies of their fermentation rate and ethanol tolerance, 51 pure cultures of yeasts that had been isolated previously were screened for their ability to produce ethanol from saccharified purple glutinous rice liquid. The procedure was as follows: fifty ml of saccharified purple glutinous rice liquid at 20ºBrix in a 250 ml conical flask covered by a cotton plug were sterilized at 115ºC for 10 minutes. The sterile liquid was inoculated ($10^6$ yeast cells/ml rice liquid) and mixed well. The inoculated liquid was incubated at 30ºC for 5 days under anaerobic conditions by replacing the cotton plug with a water lock. At the end of the fermentation, alcohol content, residual glucose and pH were measured.

**Fermentation rate**

The fermentation rate of a yeast isolate selected for its high ethanol production was monitored by measuring gas production, glucose consumption and ethanol production in samples taken every 6 hours during the incubation.

**Ethanol tolerance**

High ethanol producing yeasts were examined for ethanol tolerance in fed-batch fermentation and challenge experiments, as follows:

*Fed-batch fermentation*

As soon as the gas production rate decreased, sterile glucose solution was supplemented by adding 5 ml of stock glucose solution 50% (w/v), which had been prepared and sterilized by sterile filter 0.2 μm (S&S), to 50 ml of saccharified liquid. The supplementation with additional glucose was continued until the yeasts failed to respond by gas production. The levels of glucose and ethanol were determined at certain time points: immediately after inoculation, just before adding glucose, and later at time intervals of 5 days up till 1 month of fermentation. The gas production rate was recorded daily.

*Challenge with added ethanol*

Pure ethanol was added to saccharified liquid to levels of 0, 5, 10, 15 and 20% (w/v). Numbers of viable yeast cells at time zero and after 3 days of fermentation at 30ºC were measured by plate counting on malt extract agar medium (Oxoid, CM59). Inoculation was done to a level of $10^4$ cells/ml (microscopic count) in this experiment.

**Factors affecting fermentation capacity**

The experiment was set up in a factorial design (three factors) at three levels: yeast inoculum (using a yeast suspension of $10^8$ cells/ml by microscopic count, three levels of yeast inoculation were made at 4, 5, and 6 Log cells/ml of saccharified liquid, incubation time (2, 3 and 4 days) and incubation temperature (20ºC, 30ºC and 40ºC). Each treatment had duplicates.

**Identification of yeasts**

Five representative high-glucose fermenting yeasts and two representative poor-glucose fermenting yeasts were identified on the basis of their cell morphology, biochemical and physiological growth properties (Rohm et al., 1990; Yarrow, 1998; Middelhoven, 2000).

*Macro- and micro-morphological examination*

The texture, appearance, color and margin of colony as well as the cell shape, asexual reproduction and filament formation were noted by microscopic examination.

*The fermentation of glucose*

The ability of yeasts to ferment glucose was detected by examining the cultures for production of gas over a period of one week. Tubes were prepared by placing a small inverted Durham tube in a 16 x 120mm test tube and adding about 6ml of a 2% solution of D-glucose dissolved in a 1% solution of yeast extract. After autoclaving at 115ºC for 10 minutes the insert should be completely filled with liquid. Yeast was then inoculated with a loopful and the tubes were incubated at 25ºC until gas was visible in the insert or up to 1 week if no gas was produced.

*Growth temperature*

The growth of yeasts was tested by streaking the culture on a slant of glucose yeast extract agar (Oxoid, CM545) and incubated for one week at different temperatures: 25ºC, 30ºC, 37ºC, 40ºC, 42ºC and 45ºC.

*Assimilation of carbon compounds*

The assimilation of carbon compounds was evaluated using API ID 32 C strips (Bio Mérieux S.A., France) in which 32 cupules containing 32 different dehydrated

carbohydrate substrates were inoculated with a suspension of yeast to be tested. After 24-48 hours of incubation at 30ºC, growth (turbidity) in each cupule is evaluated by visual comparison with a negative control.

*Assimilation of nitrogen compounds*

Assimilation of $KNO_3$, L-lysine, cadaverine, creatine and D-glucosamine was tested in glass petri dishes. The procedure is as follows: dissolve 10g of bacteriological agar (Oxoid, L11) in 900 ml of distilled water, then sterilize at 121ºC for 15 minutes and cool to 50ºC in water bath. To this agar solution 100ml of 10-fold concentrated filter sterile yeast carbon base (Difco 239110) is added and mixed, before pouring into a sterile glass petri dish that already contains 1ml of a suspension of the testing yeast. The dish is then gently swirled round to mix thoroughly. After the agar has solidified, a few crystals of ammonium sulphate (positive control) are deposited in the centre of the dish whereas a few crystals of the five above-mentioned nitrogen compounds are deposited at five different sites around the perimeter of the dish. All dishes are incubated at 25ºC for 4 days and checked daily for the growth of yeasts.

*DNA extraction, amplification and sequencing*

Of all yeast strains, genomic DNA was isolated, and the internal transcribed spacer (ITS) and large subunit (LSU) 26S RNA regions were amplified by PCR using the primers V9G and RLR3r. After purification by GFX colums, sequencing PCR's were done with ITS1 forward, ITS4 reverse, NL1 forward and RLR3r reverse primers, followed by purification with sephadex, finally followed by a sequencing run on the ABI prism 3700, according to the procedures described elsewhere (Kurtzman et al., 2003; Han et al., 2004).

*Analysis of identification results*

The species of yeasts were classified by following the taxonomic keys, references and the on-line BioloMICS software available on the CBS website (http://www.cbs.knaw.nl) (Kurtzman and Fell, 1998; Barnett et al., 2000; Smith et al., 2002).

**Analytical methods**

The pH was measured with digital pH meter WTW pH 525. Glucose and ethanol were determined by HPLC, using an Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm (BIO-RAD, Thermo Separation Products Inc. USA). Total dissolved solids content (mainly sugars) of saccharified liquid was estimated by measuring ºBrix with a manual refractometer (FG102/112, Euromex-Holland).

**Statistical analysis**

Experimental data were statistically analysed using Statgraphics Plus Version 5, Manugistics, Inc., Rockville, USA and the software of the General Algebraic Modeling System (GAMS), Washington, DC, USA.

**RESULTS AND DISCUSSION**

**Screening for alcoholic fermentation**

The fermentative capacity of the 51 yeast isolates is presented in Table 1. Three groups of yeasts, producing different levels of ethanol could be distinguished. The differences between these three levels were statistically significant at the 95% confidence level. Six isolates, belonging to group 1 and 2 originated from the same rice wine starter whereas the remainder of forty-five isolates in group 3 came from the other five starters.

Besides the fermentative nature of strains, some other observations could be made. After one day of fermentation, gas production was observed clearly in group 3 and there was a frothy layer of suds on the surface of saccharified liquid. After 4 days of fermentation no more bubbling was observed and therefore sampling for analysis was done after 5 days of fermentation. In groups 1 and 2, a certain amount of glucose remained in the final products: approx. 6-8% (w/v) whereas glucose was consumed completely in all flasks of group 3. The ethanol contents were 4-5.9% (w/v) in groups 1 and 2 and 8.2-8.8% (w/v) in group 3. This indicates a good correlation between the amount of glucose consumed and the amount of ethanol produced. The final products of group 3 had a strong alcoholic and weak fruity flavour whereas the flavours of samples in groups 1 and 2 were weakly alcoholic and slightly sour. The sour flavour of groups 1 and 2 is in agreement with the somewhat lower final pH in these groups. These data suggest that isolates of groups 1 and 2 are more sensitive to alcohol, or might have oxidized some sugar into e.g. gluconic or acetic acid resulting in low pH whereas isolates of group 3 would be strong fermentative yeasts. To select high ethanol producing isolates for further studies, a limited number of five isolates was chosen randomly from group 3, since all of them gave almost the same ethanol contents. The only point to be considered was that each of these five isolates had been isolated originally from a different rice wine starter.

**Table 1. Fermentative nature of 51 yeast isolates from Vietnamese rice wine starters [1]**

| Group of strains (number of isolates) | Alcohol content (% w/v) | Residual glucose content (% w/v) | pH |
|---|---|---|---|
| Group 1 (1 isolate) | 4.3 | 8 | 3.0 |
| Group 2 (5 isolates) | 5.2-5.9 | 6-8 | 3.0-3.2 |
| Group 3 (45 isolates) | 8.2-8.8 | 0 | 3.7-3.9 |

[1] Fermentation tests were carried out on saccharified purple glutinous rice liquid at 20°Brix in conical flasks with a water lock. Analysis was done after five days of incubation at 30°C.

**Fermentation rate**

One representative yeast isolate (*Saccharomyces cerevisiae*, strain no. 2.1, LU1250) producing the highest ethanol level in the screening test, was monitored for its fermentation rate. This was estimated by the gas production rate as measured in a calibrated water lock (the average volume of a gas bubble was 0.8 ml).

Gas production rate, glucose consumption and ethanol production during the fermentation are shown in Figure 1. A sudden increase in gas production rate was observed after 24 hours of fermentation and a significant decrease after 60 hours. The figure shows the characteristic pattern of an alcoholic fermentation with complete depletion of a relatively high initial concentration of glucose. However, this data does not yet provide information on the potential limit of alcohol production and alcohol tolerance.
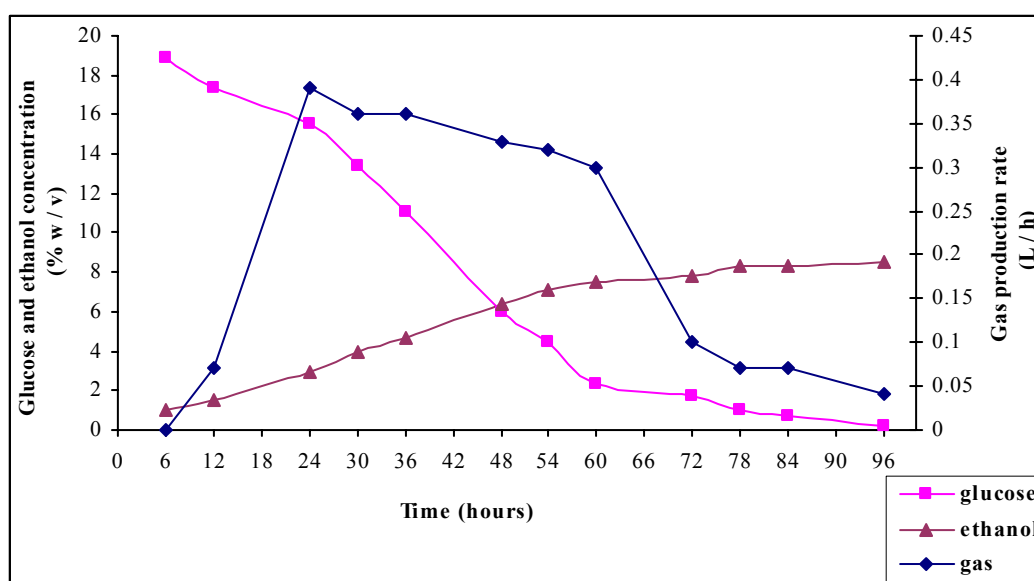


**Figure 1. Gas and alcohol production by one of the *Saccharomyces cerevisiae* strains (strain no. 2.1, LU1250)**

The fermentation test was carried out as described in Table 1 but gas production was at time intervals of 6 or 12 hours, when also samples were taken for analysis of glucose and ethanol.

**Alcohol tolerance**

*Fed-batch fermentation*

One of the methods to estimate ethanol tolerance, is to measure the capacity of yeasts to produce ethanol under conditions of unlimited but not excessive glucose supply in a fed-batch fermentation. The ethanol concentration and substrate (sugar) concentration interact to determine the inhibitory effect of ethanol (Jones, 1989). This effect has been explained in terms of osmolality. With an increase in the osmotic pressure of the medium, yeast viability and fermentative ability decrease due to the accumulation of high levels of intracellular ethanol (D'Amore and Stewart, 1987) which is toxic to the yeast cells

(Nagodawithana and Steinkraus, 1976; D'Amore et al., 1988). In order to maintain cell viability and continued ethanol production from maximum amounts of sugar, and simultaneously keep the osmotic effect of the substrate as low as possible, fed-batch or infusion-feeding conditions during fermentation can be used.

In the present experiment, the ethanol tolerance of yeasts was evaluated under conditions that glucose was not limiting. Five *Saccharomyces cerevisiae* strains, i.e. strains no. 2.1 (LU1250), 15.7 (LU1252), 20.4, 23.9 and 29.2 were tested. At the beginning of fermentation, saccharified liquid at 20ºBrix (approx. at 21% (w/v) of glucose analysed by HPLC) was prepared and followed by five successive additions of 5 ml glucose 50% w/v to saccharified liquid during the fermentation, as soon as the gas production was observed to start decreasing. The moments at which glucose was added were after 2, 4, 6, 9 and 11 days of fermentation as indicated in Figure 2. The last supplement of glucose was at 11 days of fermentation, after which no increase of gas production rate was observed. This indicated that beyond 11 days of fermentation, newly added glucose did not result in renewed activity, and that the fermentation rate was now limited by other factors, hypothetically by the alcohol concentration.

The changes of gas production that were observed up to 15 days of fermentation are shown in Figure 2. The results indicate that the increasing gas production was statistically significant at 95% confidence level after adding glucose until 11 days of fermentation, and from this time it decreased gradually to zero at 15 days until the end of fermentation, respectively.
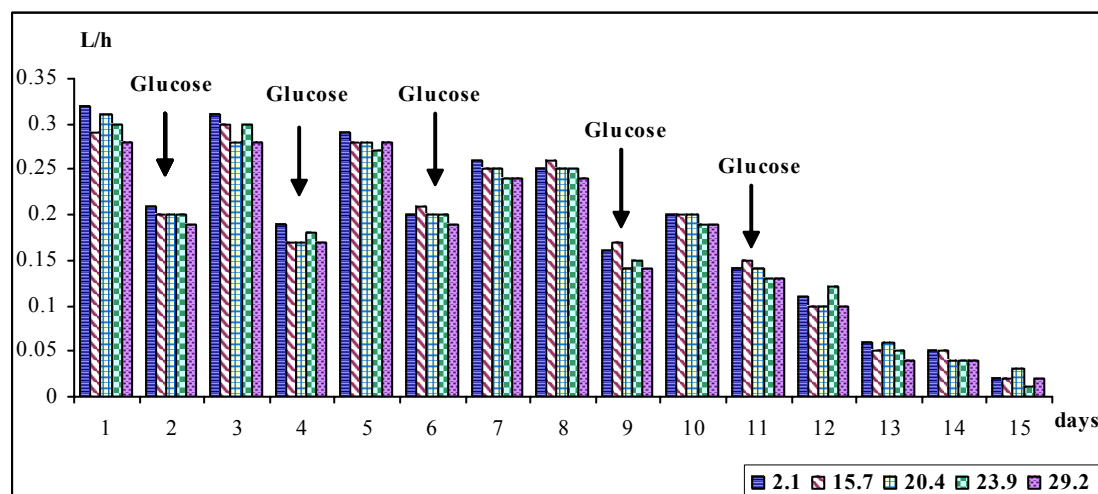


**Figure 2. Gas production rate of five strains (2.1, 15.7, 20.4, 23.9, 29.2) of *Saccharomyces cerevisiae***
Glucose approx. 5%w/v was added to fermented saccharified liquid at day 2, 4, 6, 9 and 11 of the fermentation when the decrease of gas production was observed. From 15 days until the end (29 days) of fermentation no more gas production was observed.

Although the supplementation with glucose lasted until 11 days of fermentation, the sampling was still continued at time intervals of 5 days up till one month of fermentation

for analyses of glucose and ethanol concentrations. Similar as for gas production, glucose and ethanol analyses showed that all five yeast isolates again performed in the same way. Yeasts could consume the supplemented glucose for ethanol production very well up to 11 days of fermentation time, resulting in gradually increasing ethanol contents (significantly at 95% confidence level). However, after this period some variations of glucose and ethanol contents were found. In comparison with glucose and ethanol levels after 11 days of fermentation, after 14 days of fermentation the glucose consumed was significantly higher at 95% confidence level whereas there was no significant difference in the ethanol produced. But then later, at 19 days of fermentation it was found that ethanol content had increased again significantly at 95% confidence level and was stable up to the end of sampling (29 days). The ethanol tolerance estimated by the fed-batch fermentation was at the level of approx. 14% (w/v) ethanol, since a quantity of residual glucose content was still found in the fermented liquid (approx. 3% w/v) but no more ethanol was produced. However, a certain high glucose concentration can inhibit yeast growth and fermentative activity directly as a result of high osmotic pressure and low water activity, and indirectly as a result of the high ethanol levels produced during such fermentation in which direct substrate inhibition of fermentative ability generally becomes significant somewhere in the range of 15 - 25% w/v sugar (Casey and Ingledew, 1986).

The fermentation yield was used as an indicator to express the capacity to consume glucose and produce ethanol. A yield of 100% is used as a theoretical reference, based on two molecules of ethanol being produced from one molecule of glucose. Therefore, the fermentation yield was calculated by actual output divided by theoretical output, multiplied with 100%. Figure 3 shows the almost identical performance of the five *Saccharomyces cerevisiae* strains during the fed-batch fermentation. The figure shows that in 50 ml of saccharified liquid at 20ºBrix the equivalent glucose level provided at the beginning of fermentation was 10.5 g. The samplings for analysis were done at 0, 4, 6, 9, 11, 14, 19, 24 and 29 days of fermentation. The supplementation of glucose was successful up to the fourth addition. The yield of fermentation of the five yeast isolates ranged from 84 -98%. The last (fifth) supplement of glucose did not immediately result in increasing fermentation activity, until after one week later, presumably due to some delay needed to restore their fermentation capacity. The fermentation yield then decreased until the last sampling. By adding five times 5 ml of stock glucose solution 50% w/v, the original 50 ml of saccharified liquid was increased to a final volume of 75 ml. The highest ethanol content was found at 10.1 g whereas a certain amount of glucose supply was still remaining (2.3 g).

In practice, the actual observed ethanol yield in alcoholic fermentation can vary from 75% to 93% depending on raw materials and starters as well as the technological process (Thuong and Hang, 2000). Due to the optimization of starters and processing conditions, this yield increased gradually asymptotic to the theoretical level but does not reach 100%. In this study, the alcoholic fermentation was conducted by pure superior fermentation yeasts under controlled sterile conditions; the ranges of fermentation yield were consequently quite high.
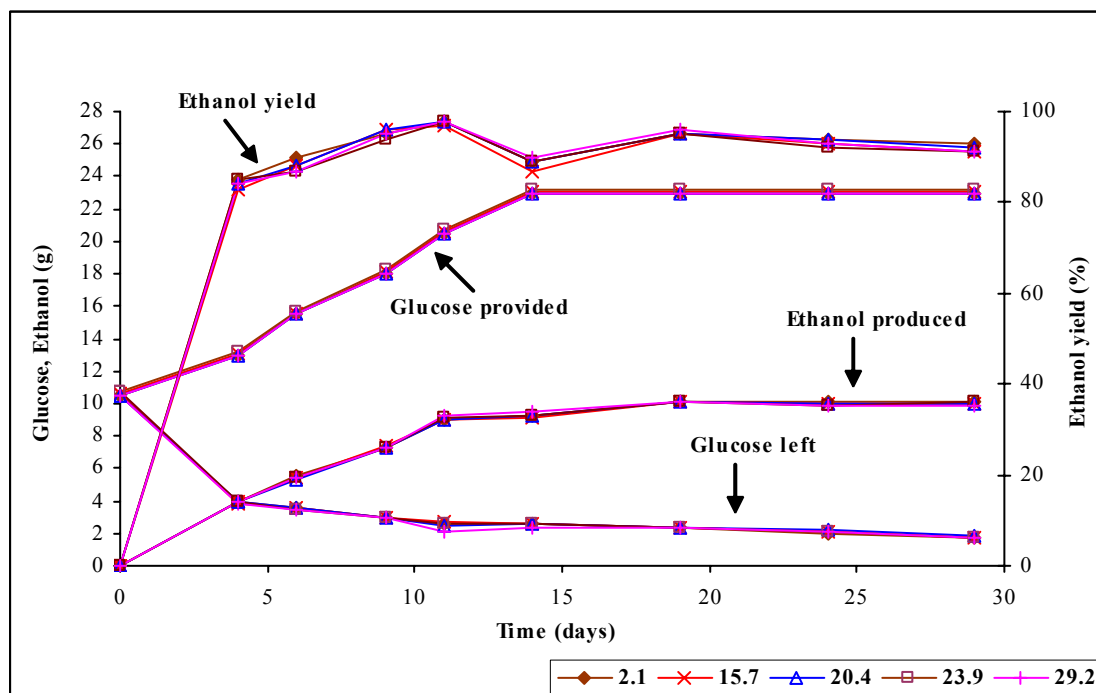
**Figure 3. The performance of five *Saccharomyces cerevisiae* strains in fed-batch fermentation**

Each of five isolates (2.1, 15.7, 20.4, 23.9 and 29.2) was tested in 50 ml of saccharified liquid at 20ºBrix followed by five additions of glucose 5% w/v in fermented saccharified liquid at 2, 4, 6, 9 and 11 days of the fermentation. The final volume of fermented saccharified liquid was 75 ml.

*Challenge with added ethanol*

Another method utilized to define ethanol tolerance of yeasts is to examine their growth and viability in growth media containing different levels of ethanol as inhibitory factor (Casey and Ingledew, 1986). A control without ethanol was included. In this test, yeast inoculation was at the level of $10^4$ cells/ml (microscopic count) in saccharified purple glutinous rice liquid. This level of inoculation was applied to enable the monitoring of growth as well as death. The numbers of viable yeast cells at the start and after 3 days of fermentation at 30ºC were determined by plate counting and were reported as log CFU/ml (Figure 4). In the control without ethanol added yeasts grew very well resulting in a range of 8.1-8.6 log CFU/ml. Yeasts still grew well in a medium supplemented with 5% (w/v) ethanol, resulting in a higher level of viable yeast cells compared with the inoculation level. However, the decline of yeasts was found starting from the level of 10% (w/v) up to 20% (w/v) ethanol. Based on the data in Figure 4, the ethanol tolerance as defined as the ethanol concentration where no growth could be detected anymore, was determined as 9-10% (w/v) of ethanol. So, this was quite a lower level compared with that found in the fed-batch experiment in which ethanol was produced up to 14% (w/v). This difference can be explained by the way ethanol was added: at the beginning of the fermentation causing a shock for the yeast resulting in cell (membrane) damage whereas in the fed-batch fermentation, yeasts had time to adapt to increasing ethanol levels.
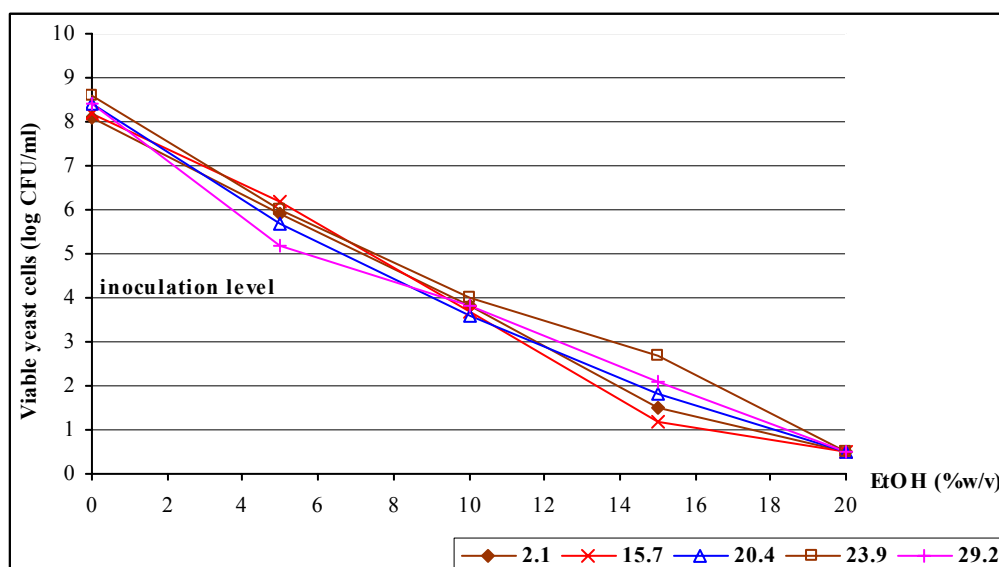
**Figure 4. Growth and decline of five strains of *Saccharomyces cerevisiae* exposed to various concentrations of ethanol**

Yeasts were inoculated at a level of $10^4$ cells/ml in saccharified purple glutinous rice liquid to which various levels of ethanol were added. After three days of incubation at 30ºC, viable yeast cells were determined by plate-counting.

**Optimization of ethanol production by *Saccharomyces cerevisiae***

Because of its superior ethanol production and ethanol tolerance, *Saccharomyces cerevisiae* (strain no. 2.1, LU1250) was selected for the study of factors influencing its fermentative capacity. According to the statistical design, in total there were 27 treatments which were described together with the results of pH and ethanol levels in Table 2.

It was found that a final pH in the range of 3.7-3.9 could be associated with a successful alcoholic fermentation. A pH of approximately 4.0 is considered typical for fermented rice wine (Cronk et al., 1977). The variables "incubation time" and "inoculum levels" gave no clear effect on pH values at incubation temperatures of 20ºC and 30ºC. The relation between pH values and the levels of yeast inoculum was observed particularly at 40ºC, when higher levels of yeast inoculum resulted in higher pH values. Also in the case of incubation at 40ºC, a relation between ethanol contents and the levels of yeast inoculum was clearly observed. The higher the inoculum, the lower ethanol content was produced. A possible explanation for this phenomenon could be that *Saccharomyces cerevisiae* can grow at 40ºC but at this temperature considerably more energy is required for maintenance. The optimum temperature for growth and performance in most yeast species is in the range of 20-30ºC (Yarrow, 1998). Under conditions of alcohol fermentation at a favourable temperature there is no clear effect of the different levels of yeast inoculum on the ethanol production, and indeed the statistical analysis also indicated this. The results also showed that at an incubation temperature of 20ºC, higher inoculation levels correlated with significantly higher ethanol production. Therefore, the level of yeast inoculum can have a clear effect on the ethanol production at sub-optimum incubation temperatures.

Although *S. cerevisiae* grew and fermented very well at either 20ºC, 30ºC or 40ºC, the incubation temperature around 30ºC was preferable. Besides, the incubation time was also found to have significant influence on the ethanol production.

**Table 2. Factors affecting fermentation capacity: experimental treatments and fermentation results**

| No. | Testing factors | | | Result of Alcoholic Fermentation | |
|-----|-----------------|---|---|----------------------------------|---|
| | Incubation temperature (ºC) | Incubation time (days) | Inoculation levels (Log cells/ml) | pH | Ethanol (% w/v) |
| 1 | 20 | 2 | 4 | 4.5 | 0.6 |
| 2 | 20 | 2 | 5 | 4.3 | 2.1 |
| 3 | 20 | 2 | 6 | 4.2 | 2.7 |
| 4 | 20 | 3 | 4 | 4.2 | 2.7 |
| 5 | 20 | 3 | 5 | 4.1 | 5.3 |
| 6 | 20 | 3 | 6 | 4.1 | 4.7 |
| 7 | 20 | 4 | 4 | 4.2 | 4.0 |
| 8 | 20 | 4 | 5 | 4.1 | 6.6 |
| 9 | 20 | 4 | 6 | 4.1 | 7.2 |
| 10 | 30 | 2 | 4 | 3.8 | 4.7 |
| 11 | 30 | 2 | 5 | 3.7 | 5.9 |
| 12 | 30 | 2 | 6 | 3.8 | 5.3 |
| 13 | 30 | 3 | 4 | 3.7 | 7.8 |
| 14 | 30 | 3 | 5 | 3.7 | 8.5 |
| 15 | 30 | 3 | 6 | 3.8 | 8.5 |
| 16 | 30 | 4 | 4 | 3.8 | 9.7 |
| 17 | 30 | 4 | 5 | 3.9 | 9.7 |
| 18 | 30 | 4 | 6 | 3.8 | 9.9 |
| 19 | 40 | 2 | 4 | 3.7 | 2.8 |
| 20 | 40 | 2 | 5 | 4.2 | 2.2 |
| 21 | 40 | 2 | 6 | 4.4 | 1.9 |
| 22 | 40 | 3 | 4 | 3.6 | 5.3 |
| 23 | 40 | 3 | 5 | 4.0 | 3.4 |
| 24 | 40 | 3 | 6 | 4.2 | 2.7 |
| 25 | 40 | 4 | 4 | 3.6 | 5.9 |
| 26 | 40 | 4 | 5 | 3.8 | 4.6 |
| 27 | 40 | 4 | 6 | 4.3 | 4.0 |

Yeast cells were inoculated at the indicated levels in saccharified purple glutinous rice liquid (20ºBrix) in conical flasks with a water lock and incubated at the indicated time-temperature combination, after which samples were analysed for pH and ethanol contents. In each treatment, values are means of duplicates.

**Table 3. Analysis of variance and multiple range tests for ethanol by temperature, time and inoculum level**

| Source | Analysis of variance [1] | | | | | Multiple range tests [2] | |
|---|---|---|---|---|---|---|---|
| | Sum of squares | Df | Mean square | F-Ratio | p-Value | LS Mean | Homogeneous groups |
| Main effects | | | | | | | |
| Temperature (ºC) | 189.583 | 2 | 94.7913 | 103.76 | 0.0000 | | |
| 40 | | | | | | 3.64444 | a |
| 20 | | | | | | 3.98333 | a |
| 30 | | | | | | 7.77778 | b |
| Time (days) | 125.98 | 2 | 62.9902 | 68.95 | 0.0000 | | |
| 2 | | | | | | 3.13333 | c |
| 3 | | | | | | 5.43333 | d |
| 4 | | | | | | 6.83889 | e |
| Inoculum level (Log cells/ml) | 2.66259 | 2 | 1.3313 | 1.46 | 0.2432 | | |
| 4 | | | | | | 4.83333 | f |
| 6 | | | | | | 5.21111 | f |
| 5 | | | | | | 5.36111 | f |
| Residual | 42.9376 | 47 | 0.913566 | | | | |
| Total (corrected) | 361.163 | 53 | | | | | |

[1] The analysis decomposes the variability of ethanol production into contributions due to various factors. The p-values indicate the statistical significance of each of the factors. Because of p-values less than 0.05, temperature and time have a statistically significant effect on ethanol production (95% confidence level); [2] The test applies a multiple comparison procedure to determine which means are significantly different from which others. The homogenous groups are identified by identical symbols.

**Table 4. Analysis of multiple regression for (A) relationship between ethanol production and independent variables and for (B) relationship between variables**

**(A)**

| Parameter | Estimate | Standard error | T Statistic | p-Value |
|---|---|---|---|---|
| CONSTANT | -53.8648 | 7.40288 | -7.27619 | 0.0000 |
| Temperature | 2.61556 | 0.219339 | 11.9247 | 0.0000 |
| Time | 1.91111 | 2.19339 | 0.871306 | 0.3884 |
| Log cells | 4.46528 | 1.9195 | 2.32627 | 0.0248 |
| Temperature*Temperature | -0.0396389 | 0.00147183 | -26.9317 | 0.0000 |
| Time*Time | -0.447222 | 0.147183 | -3.03855 | 0.0040 |
| Log cells*Log cells | -0.338889 | 0.147183 | -2.3025 | 0.0262 |
| Temperature*Time | 0.0916667 | 0.0645762 | 1.41951 | 0.1630 |
| Temperature*Log cells | -0.0270833 | 0.0396302 | -0.683401 | 0.4980 |
| Time*Log cells | 0.7625 | 0.396302 | 1.92404 | 0.0610 |
| Temperature*Time*Log cells | -0.02625 | 0.0127464 | -2.0594 | 0.0455 |

**(B)**

| Source | Sum of squares | Df | Mean square | F-Ratio | p-Value |
|---|---|---|---|---|---|
| Model | 349.985 | 10 | 34.9985 | 134.63 | 0.0000 |
| Residual | 11.178 | 43 | 0.259954 | | |
| Total (corrected) | 361.163 | 53 | | | |

R-squared= 96.905%; Standard error of Est.= 0.509857; Mean absolute error= 0.379527; p-Value is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level.

GAMS optimizing software was applied to determine the optimum conditions for the fermentation. These are defined as follows: incubation temperature of 28.3ºC, incubation time of 4 days and inoculation level of 5.5 log cells/ml of saccharified liquid. Table 3 presents the statistical significance of each of the factors and the statistically significant differences at the 95% confidence level between pairs of means. The relationship between ethanol and independent variables is summarized in Table 4, resulting in the equation of the fitted model from which the surface plot of the optimum conditions for ethanol production is made as shown in Figure 5.
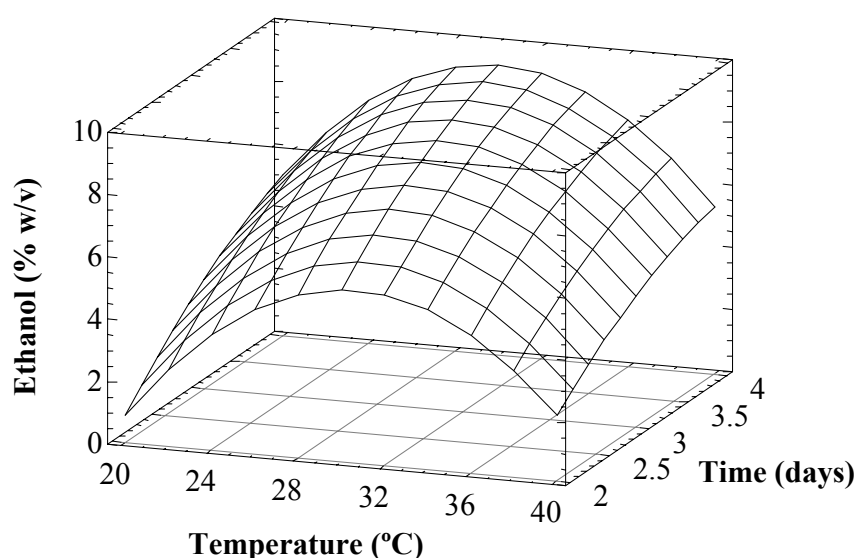


**Figure 5. Effect of incubation temperature (range 20-40ºC) and incubation period (range 2-4 days) on concentration of ethanol produced (optimum inoculation level was 5.5 Log cells/ml of saccharified liquid)**

**Characterization of yeast isolates**

As shown in Table 1, three main groups of yeasts were distinguished in the screening test based on their ethanol production. Five representative high-ethanol producing yeasts were selected for further studies as reported above and for identification. Besides, 2 representative isolates belonging to the other two groups that had the lower ethanol producing capacity were also included in the classification. The results of the identification are summarized in Table 5.

The 7 yeast isolates belonged to three species namely *Saccharomyces cerevisiae*, *Candida glabrata* and *Pichia anomala*. The poor ethanol producers were identified as *C. glabrata* and *P. anomala*. The five randomly selected isolates from a group that showed superior fermentation ability all belonged to the species *S. cerevisiae*. Although other yeasts such as *Saccharomycopsis fibuligera* (Sandhu et al., 1987; Knox et al., 2004) have been identified in rice starters as well, it is not surprising to encounter *Saccharomyces*

*cerevisiae,* as numerous studies document that the species *S. cerevisiae* is predominant in alcoholic fermented beverages (Battcock and Ali, 1993; Barnett et al., 2000). Also, the behaviour of strains 2.1, 15.7, 20.4, 23.9 and 29.2 in the fed-batch and ethanol challenge experiments are typical for *S. cerevisiae* (Walker, 1998).

This chapter examines the alcoholic fermentation conducted by yeasts that are isolated from rice wine starters, and their fermentation potential under the stressful conditions of accumulating ethanol. Indeed, defining the ethanol tolerance of yeast is difficult, and therefore it is usually adapted to meet the individual's needs and interests (Jones et al., 1981; D'Amore et al., 1990). Tolerance can be defined differently, such as the amount of ethanol that is produced, the rate of cell growth and viability, the ratio of fermentative activity in a medium without and with a certain level of ethanol, or the highest ethanol concentration produced under defined conditions.

In this present study, ethanol tolerance was estimated as the amount of ethanol produced as the result of glucose fermentation and as the rate of viability loss of yeasts in the presence of different levels of ethanol as inhibition factor. Five isolates selected from a group of forty-five superior fermentation producers were characterized as *Saccharomyces cerevisiae*. These five isolates originated from five different original rice wine starters selected from the earlier study in this thesis. Besides, two representative isolates of poor ethanol producers were also identified as *Candida glabrata* and *Pichia anomala,* and they originally came from one and the same rice wine starter (No.6), different from the other five starters mentioned above. In fact, all these six rice wine starters had been selected for their superior ability to liquefy cooked rice, significantly higher ethanol accumulation, and attractive flavour and colour of the resulting wine. However, the pure cultures of moulds as well as of yeasts that were studied in previous and present chapters, performed very differently in starch saccharification and alcoholic fermentation. This is not surprising since original rice wine starters are not defined pure culture starters, but contain a mixed microflora. In addition, the interactions among these micro-organisms in starched-based starters, especially the compatibility and interactions between yeasts and moulds, are of importance for the production of starters with good quality. Although the individual pure moulds and yeasts can have powerful activities, they should also combine well in mixed culture starter production. It is therefore necessary to examine the mutual compatibility of pure and mixed cultures of moulds and yeasts. This aspect will be addressed in the next chapter.

Under practical conditions of alcoholic fermentation, the ethanol yield cannot reach the theoretical level of 100%. The actual lower yield of ethanol is due to factors such as synthesis of cell material and some loss of ethanol due to evaporation. However, one important factor regarding the aim of producing a high level of alcohol is that fermentative yeasts, not oxidative yeasts, are required since fermentative yeasts consume higher sugar levels and convert sugar into alcohol rather than biomass.

**Table 5. Summary of characteristics of identified yeasts**

| Properties | *Saccharomyces cerevisiae* | *Candida glabrata* | *Pichia anomala* |
|---|---|---|---|
| Isolates | 2.1, 15.7, 20.4, 23.9, 29.2 | 6.3 | 6.6 |
| Group in the screening test | 3 | 2 | 1 |
| Macro- and micro-morphology | | | |
| Reddish diffusing pigment | - | - | - |
| Colony texture | butyrous | butyrous | butyrous |
| Colony appearance | smooth | smooth | smooth |
| Colony colour | cream or white | cream or white | white |
| Colony margin | entire | entire | entire |
| Cell shape | oval or round | oval or round | oval or round |
| Asexual reproduction | budding, multilateral | budding | budding |
| Filaments | no hyphae | no hyphae | no hyphae |
| Fermentation of glucose | + | + | + |
| Acetic acid production | - | - | - |
| Carbon assimilation | | | |
| Glucose | + | + | + |
| Galactose | + | - | + |
| Sucrose | + | - | + |
| DL-lactate | + | - | + |
| Raffinose | + | - | + |
| Maltose | + | - | + |
| Trehalose | + | + | + |
| Methyl α-D-glucoside | + | - | + |
| 2-Keto-gluconate | - | + | - |
| D-Xylose | - | - | + |
| Glycerol | - | - | + |
| Palatinose | - | - | + |
| Erythritol | - | - | + |
| Melezitose | - | - | + |
| Cellobiose | - | - | + |
| Mannitol | - | - | + |
| Sorbitol | - | - | + |
| Esculin | - | - | + |
| N-Acetyl-glucosamine | - | - | + |
| Nitrogen assimilation | | | |
| $KNO_3$ | - | - | + |
| L-lysine | - | - | + |
| Cadaverine | - | - | + |
| Creatine | - | - | - |
| D-glucosamine | - | - | - |
| Growth | | | |
| 25ºC | + | + | + |
| 30ºC | + | + | + |
| 37ºC | + | + | + |
| 40ºC | + | + | - |
| 42ºC | - | + | - |
| 45ºC | - | + | - |
| Growth in presence of cycloheximide 0.01% | - | - | - |
| Sequences matching with those of documented strains | 572/572 bases with U44806 NRRL Y-12632 *Saccharomyces cerevisiae* 26S ribosomal RNA gene partial sequence | 678/678 bases with AY198398 CBS 138 *Candida glabrata* 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence | 539/539 bases with U74592 NRRL Y-366 *Pichia anomala* 26S ribosomal RNA gene partial sequence |

+ positive, - negative, none of the yeast isolates assimilated the following carbon sources: Ribose, Rhamnose, Melibiose, Glucuronate, Gluconate, Levulinate, Sorbose, Glucosamine, L-Arabinose, Lactose, Inositol.

Considering the results of six starters selected as described in Chapter 2, it is noteworthy that the best yeasts were isolated from starters 2, 15, 20, 23 and 29 and the best moulds from starters 6 and 20. Thus starter 20 contains both best moulds and yeasts, and indeed this starter was one of the three starters which significantly gave the highest ethanol production and best performance in the screening test.

# Chapter 5

# Interaction of mixed fungal pure cultures in rice dough in the presence of oriental herbs

**Abstract**

Fungi are essential for the performance of starters for rice wine fermentation. In the Mekong Delta region in Vietnam, traditional rice wine starters contain herbs as ingredient. However, little is known about the importance of herbs for the activity of starters. This study examined the interaction of moulds and yeasts isolated from Vietnamese commercial rice wine starters in mixed pure culture fermentations. The effect of some representative oriental herbs on the growth of moulds and yeasts was investigated in view of the future production of a defined experimental rice wine starter. Three moulds including *Rhizopus oryzae*, *Amylomyces* aff. *rouxii* and *Amylomyces rouxii,* and one yeast *Saccharomyces cerevisae* were examined individually and in a combination of mixed cultures to study their interaction and compatibility. There were no combinations showing mutual inhibition. Ten representative oriental herbs used traditionally in starter production were studied. The herbs "Tieu Hoi" (Fennel; *Foeniculum vulgare* Miller) and "Dinh Huong" (Clove; *Syzygium aromaticum* L.) were observed to stimulate the yeast and mould growth.

## INTRODUCTION

A variety of traditional fermented rice-based beverages as well as their corresponding starters in various parts of Asian countries have summarily been described (Hesseltine and Wang, 1986; Lim, 1991; Phuc, 1998). Whereas the importance of traditional rice wine fermentation starters for the yield and quality of the final products is recognized, little knowledge is available about their microbiological composition in relation to their performance in the fermentation. This limitation poses an obstacle to industrial development, and thus the development of starters attracts the attention of researchers in food microbiology and technology (Hesseltine, 1983; Nout, 1992; Holzapfel, 1997; Ray, 2001; Zorba et al., 2003). Rice wine starters are generally composed of essential and non-essential micro-organisms; the presence of moulds and yeasts however is considered essential for this type of fermentation (Lotong, 1998; Luong, 1998; Trang et al., 1999; Nout and Aidoo, 2002).

In Vietnam, each locality has its own way of starter production for glutinous rice wine manufacture, depending on available ingredients and local habits. Presently, the rice

wine starters are manufactured at home scale, by solid-state fermentation methods resulting in dehydrated shelf-stable starters in tablet form. Generally, there are three main kinds of Vietnamese traditional starters: (a) plain starters, (b) starters supplemented with oriental herbs, and (c) starters supplemented with leaves containing aromatic essential oils. In the Mekong Delta region, starters of type (b) predominate for they are preferred because of their fragrance and it is believed that they limit the growth of undesirable (spoilage) micro-organisms in the wine.

In this study, the compatibility of moulds and yeasts isolated from Vietnamese commercial rice wine starters, and selected for their saccharification and alcohol production respectively, is examined in defined mixed culture fermentations. The effect of representative oriental herbs on the growth of moulds and yeasts is also investigated with the purpose to include them as ingredients in defined mixed-culture experimental rice wine starters.

## MATERIALS AND METHODS

### Cultures

Three mould strains, i.e. *Rhizopus oryzae* strain no. 15.2 (M1), *Amylomyces* aff. *rouxii* strain no. 20.2, CBS 111760 (M2) and *Amylomyces rouxii* strain no. 20.3, CBS 111757 (M3) and one yeast, *Saccharomyces cerevisae* strain no. 2.1, LU1250 (Y) were used. All four strains had previously been isolated from Vietnamese rice wine starters.

### Preparation of cultures and inoculation

The cultures were grown on slants of Malt Extract Agar (Oxoid, CM59) at 30°C for 2 days in case of the yeast, and for 5 days in case of the moulds. A suspension of the growing micro-organism or its spores was made by adding 5ml of sterile physiological salt solution (0.85% w/v NaCl) onto each slant. The fungal spores and the yeast cells were scraped off the agar by means of an inoculation wire. For mould inoculation, a suspension prepared from one slant was used, and for yeast inoculation 1ml of a suspension was applied.

### Oriental herbs

Ten representative oriental herbs used in this study are listed in Table 1. The data are based on a textbook of medicinal plant and herb medicine in Vietnam (Loi, 2001) and information from the local seller.

### Preparation of herb extract

5 g of each tested herb was added to 100 ml of distilled water and extraction was done in a waterbath at 50°C for 1 hour. The extract solution was then filtered over a filter paper and

over a 0.2μm (S&S) sterile membrane filter. Mixtures of extracts were made by pooling equal aliquots of each herb extract.

**Table 1. Oriental herbs used in this study**

| Nr. | Vietnamese name | English /Common name | Scientific name | Part used for extraction |
|---|---|---|---|---|
| 1 | Nhuc dau khau | Mace | *Myristica fragrans* Houtt | Seed |
| 2 | Bach truat | Bai zhu | *Atractylodes macrocephala* Koidz | Tuber |
| 3 | Nhuc que | Cinnamon twig | *Cinnamomum cassia* Blume | Outer bark |
| 4 | Thao qua | Cardamom | *Amomum tsao-ko* Crev. et Lem. | Seed |
| 5 | Cam thao | Licorice root | *Glycyrrhiza uralensis* Fish | Root |
| 6 | Bac ha | Mint | *Mentha arvensis* L. | Leaf |
| 7 | Te tan | Ginger | *Asarum sieboldii* Miq. | Root and Leaf |
| 8 | Uat kim | Tumaric | *Curcuma longa* L. | Tuber |
| 9 | Tieu hoi | Fennel | *Foeniculum vulgare* Miller | Flower |
| 10 | Dinh huong | Clove | *Syzygium aromaticum* L. | Flower |

**Procedure of compatibility testing of moulds and yeasts**

*Effects of yeast on mould*

Fifty grams of purple glutinous rice and 60ml of distilled water in a 250 ml conical flask covered by a cotton plug, were soaked for 4 hours at room temperature (22ºC). After soaking, the mixture was steamed in an autoclave for 1 hour at 100ºC. The steamed rice paste was cooled to 35-40ºC, then inoculated with pure cultures, which had been prepared as described above, and mixed well. There were six treatments including M1, M2, M3, M1Y, M2Y, M3Y and a control without inoculum. The uninoculated and inoculated rice pastes were incubated for 3 days at 30ºC and then harvested for analyses.

*Effects of mould on yeast*

Fifty ml of medium containing 20% glucose, 1% yeast extract and 0.2% $(NH_4)_2SO_4$ in a 250 ml conical flask covered by a cotton plug were sterilized at 115ºC for 10 minutes. Mould and yeast cultures which had been prepared as described above, were inoculated into cooled (35-40ºC) sterile medium. There were four treatments including Y, YM1, YM2, YM3 and a control without inoculum. After incubation at 30ºC for 3 days under anaerobic conditions for which the cotton plug had been replaced by a water lock, the samples were harvested for analyses.

**Effect of oriental herbs on the growth of micro-organisms**

Fifty ml of medium containing 20% glucose, 1% yeast extract and 0.2% $(NH_4)_2SO_4$ in a 250 ml conical flask covered by a cotton plug, were sterilized at 115ºC for 10 minutes. To

this medium, 1ml of sterile extract of herb extract was added. There were 8 treatments with different solutions including A(1, 2, 3), B(4, 5, 6), C(7, 8, 9, 10), D(1-10), E(7), F(8), G(9), H(10) and a control without herb extract; numbers in brackets refer to the description of oriental herbs given in Table 1 . For each treatment the inoculation was made with mould and yeast cultures which had been prepared as described above. There were 3 treatments of inoculum including *S. cerevisae* (Y), *A. rouxii* (M3), *S. cerevisae* and *A. rouxii* (YM3) and a control without inoculum. After incubation at 30ºC for 48 hours, the samples were harvested for plate counts and measurement of biomass.

**Analytical methods**

The pH was measured by a digital pH meter WTW pH 525. Glucose contents were determined by glucose oxidase test kit (Megazyme, GLC 9/96). Ethanol contents were determined by a UV-method kit (Boehringer, Mannheim, cat. no. 176290). Amyloglucosidase was assayed by using a test kit (Megazyme, RAMGR3) of p-nitrophenyl-β-maltoside (McCleary et al., 1991). The plate counts were made on Oxytetracycline Glucose Yeast Extract Agar (Oxoid, CM545) for yeasts. Biomass was determined by the dry matter method.

**Statistical analysis**

In all experiments each treatment had a duplicate and experimental data were statistically analysed using Statgraphics Plus Version 5 (Manugistics, Inc., Rockville, USA.)

**RESULTS AND DISCUSSION**

**Compatibility of moulds and yeasts**

*The effects of yeast on mould*

In order to detect effects of yeast on mould, the gelatinized paste of purple glutinous rice was inoculated with three individual moulds, and with combinations of these three single moulds with the yeast. Because of extensive liquefaction during the incubation, samples of liquid could be taken after 3 days of incubation at 30ºC for determination of pH, volume of liquid produced, glucose, ethanol and amyloglucosidase activity. The uninoculated control did not show any changes during the incubation.

The pH values in all treatments showed a sharp decrease from 6 to 4.2 - 4.6 after 3 days of incubation, which can be considered typical in solid-state fermentations by moulds and yeasts (Underkofler, 1976; Hesseltine, 1983; Battcock and Ali, 1993). The glucose and ethanol contents, amyloglucosidase activity and volumes of liquid produced by single moulds and by combinations of mould plus yeast, are presented in Figure 1. The results

show that glucose levels in "mould-only" flasks, were significantly higher than in "mould plus yeast". Concomitantly, ethanol produced by mould only was significantly less than in mixed cultures of mould and yeast. In mixed cultures of mould and yeast, glucose produced by mould is consumed and fermented by yeast into ethanol.

While all three moulds produced amyloglucosidase, *Amylomyces rouxii* strain 20.3 (M3) was the best producer of amyloglucosidase and glucose. This conclusion agrees with the data presented in Chapter 3 about the saccharification of rice. All three moulds also produced a certain amount of ethanol under the low oxygen conditions of the experiment. This is a common feature of moulds belonging to the order of Mucorales (Hawker, 1966; Ellis et al., 1976; Luong, 1998). The combination of this mould with *Saccharomyces cerevisae* strain 2.1 (M3Y) also gave the highest alcohol concentration and the largest volume of free liquid.

Somewhat less amyloglucosidase activity was produced by all three moulds, when (part of) the glucose was simultaneously fermented to ethanol by the yeast.

All three mould strains tested are compatible with the yeast strain, *i.e.* the moulds show the same saccharification and liquefaction ability in the presence of the yeast.
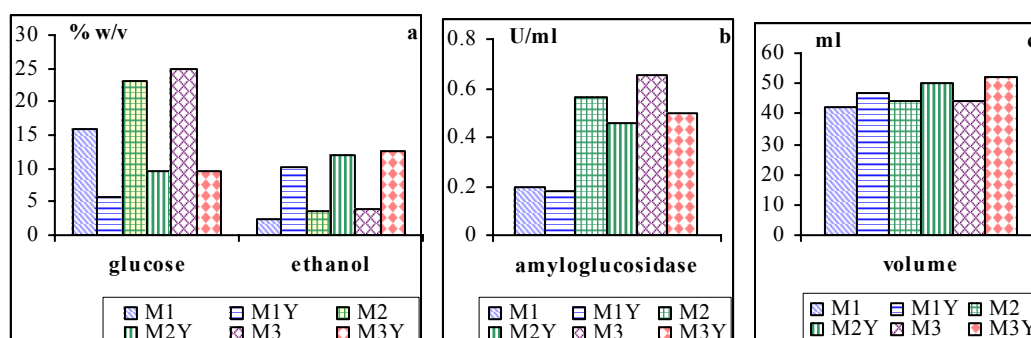


**Figure 1. The effects of yeast on mould in liquefaction and saccharification of gelatinized purple glutinous rice starch. (a) glucose and ethanol contents; (b) amyloglucosidase activity; (c) volume of liquid produced**

Each of three moulds namely *Rhizopus oryzae* (M1), *Amylomyces* aff. *rouxii* (M2) and *Amylomyces rouxii* (M3) were grown individually and in a mixed culture with yeast *Saccharomyces cerevisae* (Y). The samples were analysed after 3 days of incubation at 30ºC.

*The effects of mould on yeast*

Following the same principle of inoculation, medium was inoculated with yeast only and with a combination of yeast and each of the three moulds. After 3 days of incubation at 30ºC samples were taken for determination of pH, glucose, ethanol and biomass. The uninoculated control showed pH of 6 and a glucose content of 19.2% (w/v), which was the same as the initial medium. In the other flasks the pH value rapidly dropped to a constant value of 3.8 – 4.0, which may be expected in successful alcoholic fermentations (Hesseltine, 1983).

The ethanol contents and biomass produced are presented in Figure 2. The glucose was consumed completely after 3 days of incubation. Ethanol produced by yeast only, was significantly higher than by yeast plus mould. On the contrary, biomass formed by yeast only, was significantly lower than biomass obtained with mixed cultures, especially with M1. These data indicate that both yeast and mould grew very well in the medium. However, when mould was present, it competed with yeast for glucose, resulting in slightly lower ethanol in mixed cultures. Due to its very fast growth with thick mycelium, *Rhizopus oryzae* (M1) consumed high amounts of glucose for mycelium production, yielding most biomass when present in a mixed culture. In contrast, the highest ethanol level [9.6% (w/v)] was obtained in the flask with yeast only, whereas ethanol levels produced by mixed cultures were in the range of 8.3-8.6% (w/v). This can be explained by the yeast converting glucose mainly to ethanol and carbon dioxide, with production of moderate amounts of biomass, in contrast to the moulds that oxidise glucose for the greater part to carbon dioxide and water, yielding much more energy to produce biomass.

In conclusion, although some competition for glucose was obvious, there were no combinations that really inhibited each other. Combining these results with the results of the effect of yeast on moulds, it can be concluded that all strains tested are compatible with each other and can be used for the design of starters.
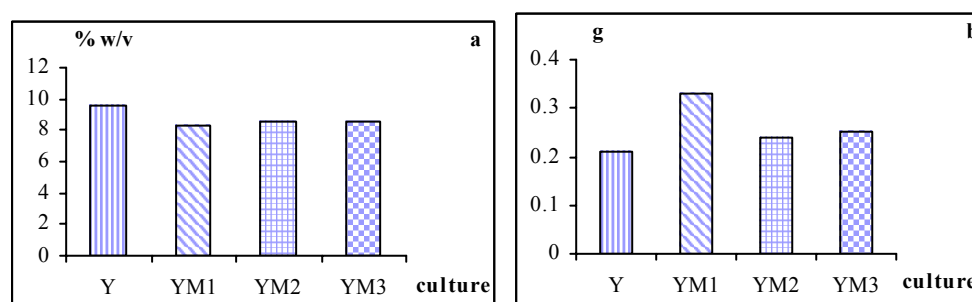


**Figure 2. The effects of mould on yeast in mixed-culture fermentation. (a) ethanol; (b) biomass**

The yeast *Saccharomyces cerevisae* (Y) was grown in glucose medium singly and in mixed cultures with each of three moulds namely *Rhizopus oryzae* (YM1), *Amylomyces* aff. *rouxii* (YM2) and *Amylomyces rouxii* (YM3). Samples were taken and analysed after 3 days of incubation at 30ºC.

**The effects of oriental herbs on the growth of micro-organisms**

In the Mekong Delta region in Vietnam, traditional rice wine starters are usually supplemented with oriental herbs. Based on the experience of leading local producers and the few available literature references (Hieu, 1990; Loi, 2001), ten representative oriental herbs were selected for this study. The aim was to find out the effect of any specific herb on the growth of mould and yeast in the production of rice wine fermentation starters.

While these herbs are primarily added to starters for the fragrant flavour which they convey to the rice wine, they may have antibacterial properties as well, thereby protecting the rice wine fermentation against failure. The possible antibacterial activity of these herbs was not part of this study.

Various combinations of herbal extracts and single extracts were studied for their effect on growth of the mould and yeast (Figure 3). Fermentation medium without any herbal extract served as a control. It can be concluded that herbs 9 and 10 (cf. Table 1) have a stimulatory effect on biomass and also on yeast count. Particularly, herb 9 (*Foeniculum vulgare*) proves to be stimulatory in biomass production of mould and yeast. It is not known which chemical compound(s) in this watery extract of the flower (the part used for extraction) of this herb, commonly known as Fennel, causes this growth stimulation. It could be its mineral content, particularly its Mg, Mn, salt contents (Walker, 1998) since the experimental medium is probably low in salt.

Both "Tieu Hoi" (*Foeniculum vulgare*) and "Dinh Huong" (*Syzygium aromaticum*) are commonly applied in the traditional process of rice wine making in Vietnam because of their assumed antibacterial properties and their fragrant flavour. Both herbs will be applied in our further studies of fermentation starter development and preparation.
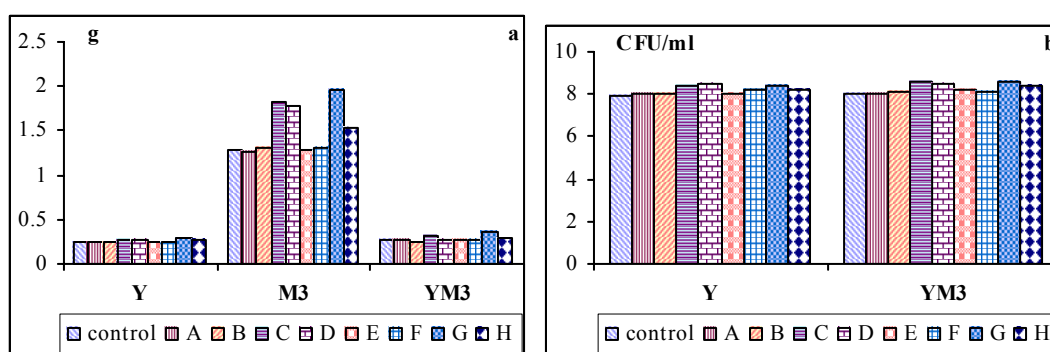


**Figure 3. The effect of oriental herbs on (a) the biomass of mould and yeast and (b) the yeast count**

One control and 8 treatments without and with herbs were employed including A(1 + 2 + 3), B(4 + 5 + 6), C(7 + 8 + 9 + 10), D(1-10), E(7), F(8), G(9), H(10). Numbers in brackets refer to the description of oriental herbs mentioned in Table 1. After incubation at 30ºC for 48 hours the growth of mould was determined by biomass and the growth of yeast was determined by both biomass and plate count.

In conclusion, we found that the tested pure cultures of moulds and yeast were compatible in mixed-culture fermentations. In mixtures with traditionally used herbs, we found that some herbs stimulate fungal biomass formation. It is unknown whether this is of relevance to the fermentation. Probably of more importance is their contribution to the flavour of rice wine.

# Chapter 6

# A laboratory-scale process for the preparation of defined mixed-culture starter for purple glutinous rice wine

**Abstract**

For the successful manufacture of purple glutinous rice wine it is essential to dispose of a defined starter culture. Fungi – moulds and yeasts - are the essential micro-organisms active in such starters. This study describes the development of a laboratory-scale process to formulate a shelf-stable mixed-culture starter. The process was based on traditional starter manufacturing methods and modified where appropriate as determined by optimization experiments. The mixing ratio of flours, i.e. 80% rice flour and 20% cassava flour to be used as raw ingredients, the decontamination of the flour mixture by dry heating at 100ºC, the moisture content of dough of 30% and the incubation period of 24 hours were optimum conditions obtained through experimentation and were adopted as part of the process. Granulation of fermented starter dough, followed by drying at 45ºC achieved rapid dehydration and maximum survival of starter micro-organisms. The effects of storage of starter granules on viability of mould and yeast and their performance in winemaking were also examined. Mould viability and its enzymatic activity were found to be quite stable during 3 months storage. Yeast activity is most likely the limiting factor for shelf-life and stability of starter. The dry matter content is one of the factors influencing the shelf-life of the starter.

## INTRODUCTION

As in various other East-Asian countries, rice wine is popular in Vietnam and the traditional starter used for its preparation is called *Men ruou* (*Men*). This kind of starter cakes has been reported to contain variable mixtures of fungi, mostly Mucorales and yeasts (Tamang and Sarkar, 1995; Lotong, 1998; Nout and Aidoo, 2002). Depending on available ingredients and habits, each locality in Vietnam has its own way of producing starter (Phuc, 1998; Tam, 2000); however, the traditional home-scale preparation of starch-based starters is carried out under non-sterile conditions by producers lacking microbiological training. During the preparation of starter cakes, the ingredients - rice flour, cassava flour or their mixtures - are ground and thoroughly mixed with spices and herbs which are believed to prevent the growth of undesirable micro-organisms. The producers keep their recipes secret and pass them on from one generation to the next. Particularly in the Mekong Delta, a mixture of oriental herbs is preferred as supplement in the preparation of starters, whereas some ethnic minorities in the mountainous districts

prefer to add leaves with aromatic essential oils. Water is then added to form a dough-like mass with a moisture content of 55-60%, and the whole mass is inoculated by dusting dry powdered starter from previous batches over it, followed by thorough mixing. The inoculated dough is shaped into small flattened or ball-shaped cakes about 4 cm in diameter and 1cm thick. These are then covered with a thin layer of rice husks; according to producers, this would reduce overheating and facilitate aeration. The incubation takes place on a bamboo tray in a ventilated place at ambient temperature (about 30ºC) for 4 days. The cakes are now available to be used as inocula in winemaking (Luong, 1998; Phuc, 1998). These commercial undefined starters can have a shelf-life of several months; their quality varies considerably and therefore no prediction or guarantee can be given about the yield and quality of wine.

The present study investigates the technical feasibility of preparing stable mixed-culture starters containing selected compatible strains of mould and yeast, by appropriate modifications of the traditional starter manufacturing method.

## MATERIALS AND METHODS

### Preparation of rice and cassava flours

Rice flour was prepared by dry grinding of polished white rice with a hammer-mill type grinder (SM 100 confort, Retsch, Germany). Cassava flour was prepared as follows: fresh cassava tuber was peeled, chopped into small pieces, washed, sun-dried and ground into flour using the grinder mentioned above.

### Preparation of inoculum

The mould *Amylomyces rouxii* strain no. 20.3 (CBS 111757; LU2043) and the yeast *Saccharomyces cerevisae* strain no. 2.1 (LU1250) were grown on slants of malt extract agar (Oxoid, CM59) at 30ºC for 5 days (mould) or 2 days (yeast). A suspension of the growing micro-organisms was made by adding 5ml of sterile physiological salt solution (0.85% NaCl) to each slant. The biomass was scraped off the agar by means of an inoculating wire.

### Preparation of sterile herb extract

Sterile extract of two oriental herbs, namely "Tieu Hoi" (Fennel; *Foeniculum vulgare* Miller) and "Dinh Huong" (Clove; *Syzygium aromaticum* L.) was prepared as follows: 5 g of herb was added to 100 ml of distilled water, and individual extracts were made in a waterbath at 50ºC for 1 hour. The extract solutions were then filtered over a filter paper and over a 0.2μm (S&S) sterile membrane filter. Mixtures of extracts were made by pooling equal aliquots of each herb extract.

**Adjustment of required moisture content of dough**

A volume of sterile water was added to mixed flours to reach the required moisture content according to the following equation:

$$\text{moisture content (g/g)} = \frac{am + bn + x}{m + n + x}$$

in which a = moisture content (g/g) of rice flour, m = weight (g) of rice flour, b = moisture content (g/g) of cassava flour, n = weight (g) of cassava flour, x = volume (ml) of sterile water needed to obtain the moisture content in the dough.

**Preparation of wine using fermented dough as a starter**

Fifty grams of purple glutinous rice and 60 ml of distilled water in a 250 ml conical flask covered by a cotton plug were soaked for 4 hours at room temperature (25ºC). After soaking they were steamed in an autoclave for 1 hour at 100ºC. The gelatinized rice paste was cooled to 35-40ºC, then inoculated with 2 g of fermented dough and mixed well. After solid-state fermentation during 2 days at 30ºC, 70ml of sterile water was added to the moulded mass to allow for submerged alcoholic fermentation during 3 days at 30ºC under anaerobic conditions for which the cotton plug had been replaced by a water lock. Samples were harvested for required analyses.

**Mixing ratios and dry heat treatments of rice and cassava flours**

Fifty grams of mixed rice and cassava flours in a 250 ml conical flask covered by a cotton plug were heated in an electric oven overnight (17 hours). In total there were 18 treatments of different mixing ratios and different dry heating temperatures. These treatments are presented together with the microbiological counts in Table 1. The heated flour mixtures were cooled to 35-40ºC, and based on the above-mentioned equation the desired volume of sterile water was added to reach the required moisture content of 50% (w/w). To the resulting dough, 1ml of sterile herb extract was added. Next, the dough was inoculated with a suspension obtained from 2 slants of mould culture and 2 ml of yeast suspension. The inoculated dough was mixed well and incubated at 30ºC for 24 hours. Samples for plate counts were taken at incubation time 0 and 24 hours. Each treatment had two duplicates.

**Effect of dough moisture content and incubation time**

Forty grams of rice flour and ten grams of cassava flour in a 250 ml conical flask covered by a cotton plug were heated at 100ºC in an electric oven overnight (17 hours). The heated flour mixture was cooled to 35-40ºC and doughs of 40, 50, 60 and 70% (w/w) moisture content were prepared using the equation above. To the doughs 1 ml of sterile herb extract was added, and they were inoculated as described above. The inoculated dough was mixed

well and incubated at 30ºC for different periods of 24, 48 and 72 hours. Samples were taken after 24, 48 and 72 hours of incubation, for plate counts and evaluation of winemaking performance in which the respective fermented doughs were used as starter (see above). Each treatment had triplicates.

**Granulation of wet starter dough, and drying conditions for starter granules**

One hundred and twenty grams of rice flour and thirty grams of cassava flour in a 500 ml conical flask covered by a cotton plug were heated at 100ºC in an electric oven overnight (17 hours). The heated flour mixture was cooled to 35-40ºC and doughs of 30 and 40% (w/w) moisture content were prepared. To each dough 3ml of sterile herb extract was added, and it was inoculated as described above. The inoculated dough was mixed well and incubated at 30ºC for 24 hours. The fermented starter dough was now transformed into granules of 3-4 mm diameter, by mild rotation on steel sieves (pore size 4 mm) that had been previously disinfected with 70% ethanol. The granules were distributed on a perforated drying tray and dehydrated in the oven at 40ºC and 45ºC up to 5 hours. Samples were taken during drying at 1 hour intervals up to 5 hours for determination of dry matter and of winemaking performance. For each treatment the remainder of dry starter granules was vacuum packed using a "Vacupack plus" machine (Krups, type 380, CE, z260590, P.R.C.) and used for storage experiments.

**Storage stability (shelf-life) of dry starter granules**

Dried starter granules obtained in the previous experiment were stored under two different conditions: in a refrigerator at 4ºC and in a dark cabinet at room temperature (approx. 30ºC). Mould and yeast counts were performed after 0, 1, 2 and 3 months of storage. The winemaking process mentioned above was used to evaluate the performance after 3 months of storage.

**Microbiological analysis**

A sample of 1g of each treatment was transferred to a stomacher bag and homogenized with 99ml sterile saline (NaCl 0.85% w/v) in a Stomacher Lab-blender (Seward 400, Emergo, England) for 1 minute at high speed, and appropriate serial dilutions were performed using the same diluent. 1-ml portions of the appropriately diluted suspension were mixed with molten (45ºC) medium and made into pour-plates. Czapek-Dox Agar (Oxoid, CM95), Oxytetracycline Glucose Yeast Extract Agar (Oxoid, CM545) and Plate Count Agar (Oxoid, CM325) were used for mould, yeast, and total mesophilic counts, respectively. All plates were incubated at 30ºC for 2 - 4 days. The colonies that appeared after incubation were counted, calculated as colony forming units (CFU) per gram of sample and expressed as Log CFU/g.

**Chemical analysis**

pH was measured with a digital pH meter WTW pH 525. Alcohol content was determined by the distillation method (So and Nhuan, 1991). Total dissolved solid content (mainly sugars) of saccharified liquid was estimated by measuring ºBrix with a manual refractometer (FG102/112, Euromex-Holland).

**Statistical analysis**

Experimental data were analysed statistically using Statgraphics Plus Version 5, Manugistics, Inc., Rockville, USA.

**RESULTS AND DISCUSSION**

**Effects of mixing ratio and dry heat treatment of rice and cassava flours on the growth of micro-organisms**

Both rice and cassava flours can be used as mixed ingredients in the process of alcoholic starter production in Vietnam. However, except for the explanation of the reasons of use (Luong, 1998) of rice and cassava, no relevant scientific knowledge is available about the effect of mixing ratio. If only rice flour is used, the starter cake becomes too heavy, hard and solid so that moulds can grow only on the surface. On the other hand, if only cassava flour is used then the starter cake becomes too soft and spongy, which would limit the growth of yeasts. In commercial practice local starter producers prefer to use cassava as a supplement because it is cheap and combines well with rice flour. Cassava flour is also used industrially as a raw material for starch extraction, for alcohol production, for bread making or as sun-dried chips for animal feeding (Kimaryo et al., 2000). The dry heating of mixed flours at the start of the process was carried out with the aim to reduce the number of undesirable spoilage micro-organisms during the process of starter production. The effect of the mixing ratio and the dry heat treatment on the growth of micro-organisms is shown in Table 1.

Although only pure cultures of mould and yeast were used to inoculate the starter dough, the count of mesophilic bacteria was also determined to detect the presence of undesirable contaminants. Due to the standardized preparation of inoculum, the mould and yeast counts at the onset of incubation were always approx. 4.6 Log CFU/g for mould and 6.5 Log CFU/g for yeast. A certain number of undesirable bacteria (ranging 1.7-3.9 Log CFU/g) was also found at the start of incubations, and these developed to 3.6-5 Log CFU/g after 24 hours of incubation. The significantly higher level of bacterial contamination was found in cases of lower temperature of dry heat treatment as well as in cases of higher levels of cassava flour. This can be explained by the presence of heat-resistant bacterial spores which could have survived the heat treatment; these genera have been reported as predominant bacteria in fermented cassava dough (Miambi et al., 2003).

On the other hand, although some contamination with undesirable bacteria occurred, mould and yeast still grew well and dominated during incubation. After 24 hours of incubation, the levels developed up to 6.1 Log CFU/g for mould, and 8.2 Log CFU/g for yeast. Neither the mixing ratio of flours, nor the temperatures of dry heating had a significant effect on yeast count, whereas only the dry heating temperature had a significant impact on mould growth. Mixed flours heated at 100ºC allowed for significantly higher levels of mould CFU than in flour heated at the lower temperatures. Although for moulds the number of CFU does not fully reflect the biomass formed, the effect of heating temperatures may be explained by a stronger reduction of competitive bacteria that otherwise would inhibit the growth of mould. The growth of yeast was not much affected by competitive undesirable bacteria during incubation, possibly because of its faster multiplication than that of the mould.

In conclusion, the dry heating temperature at 100ºC is successful in reducing the number of undesirable bacteria without overheating the rice and cassava flours – as judged by the absence of browning – therefore, it can be applied as a decontamination treatment in the process of starter production. The mixing ratio of 80% rice flour to 20% cassava flour was selected as most appropriate as both mould and yeast can grow very well with minimum competition from bacteria and the binding properties of cassava are best in this mixture. The dried starter granules should not be too soft or crumbly because this would lead to losses and dustiness when used in practice.

**Table 1. Effect of mixing ratio and dry heating temperature of mixed flours: experimental treatments and microbiological composition**

| No. | Mixing Ratio (%) | | Dry heating temperature (ºC) | Microbiological Composition (log CFU/g fermented dough) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rice | Cassava | | Incubation time 0 h | | | Incubation time 24 h | | |
| | | | | mould | yeast | bacteria | mould | Yeast | bacteria |
| 1 | 100 | 0 | 60 | 4.7 [1] | 6.5 | 2.1 kl [2] | 5.5 fgh | 7.9 bc | 4 fg |
| 2 | 100 | 0 | 80 | 4.6 | 6.6 | 2 l | 5.3 h | 8 abc | 4 fg |
| 3 | 100 | 0 | 100 | 4.6 | 6.5 | 1.7 m | 5.9 abc | 8.1 ab | 3.7 hi |
| 4 | 80 | 20 | 60 | 4.7 | 6.5 | 2.8 ghi | 5.3 h | 7.9 bc | 4.5 cd |
| 5 | 80 | 20 | 80 | 4.7 | 6.6 | 2.7 hi | 5.7 def | 8.1 ab | 4.3 de |
| 6 | 80 | 20 | 100 | 4.6 | 6.5 | 2.3 k | 6 ab | 8.2 a | 4 fg |
| 7 | 60 | 40 | 60 | 4.5 | 6.5 | 3.1 ef | 5.6 efg | 7.9 bc | 4 fg |
| 8 | 60 | 40 | 80 | 4.7 | 6.6 | 2.9 fgh | 5.7 def | 7.9 bc | 3.9 gh |
| 9 | 60 | 40 | 100 | 4.6 | 6.5 | 2.6 i | 6 ab | 8 abc | 3.6 i |
| 10 | 40 | 60 | 60 | 4.6 | 6.5 | 3.4 cd | 5.3 h | 7.6 d | 4.6 bc |
| 11 | 40 | 60 | 80 | 4.5 | 6.6 | 3.3 de | 5.5 fgh | 7.9 bc | 4.2 ef |
| 12 | 40 | 60 | 100 | 4.6 | 6.6 | 3 fg | 5.8 cde | 8 abc | 4 fg |
| 13 | 20 | 80 | 60 | 4.7 | 6.5 | 3.9 a | 5.5 fgh | 7.9 bc | 5 a |
| 14 | 20 | 80 | 80 | 4.6 | 6.6 | 3.6 bc | 5.8 cde | 8 abc | 4.8 ab |
| 15 | 20 | 80 | 100 | 4.5 | 6.5 | 3.5 cd | 6.1 a | 8 abc | 4.2 ef |
| 16 | 0 | 100 | 60 | 4.6 | 6.6 | 3.9 a | 5.4 gh | 7.8 cd | 5 a |
| 17 | 0 | 100 | 80 | 4.6 | 6.6 | 3.8 ab | 6 ab | 8 abc | 5 a |
| 18 | 0 | 100 | 100 | 4.6 | 6.5 | 3.4 cd | 6 ab | 8.1 ab | 4.5 cd |

[1] values are mean of duplicates; [2] means with different subscripts within a column are statistically different at the 95% confidence level.

**Effect of dough moisture content and incubation time on the growth of micro-organisms and on winemaking performance**

The dough moisture content has been reported as an important parameter during the preparation of starch-based starters for alcoholic fermentation (Steinkraus et al., 1983; Lotong, 1998). It should be favourable for the growth of mould and yeast: too low or too high moisture contents may result in poor viability of cultures and to failure of fermentation processes. We examined the effect of 4 moisture levels, i.e. 40, 50, 60 and 70%. In commercial practice, a level of 50-55% is used in the traditional production of Vietnamese alcoholic fermentation starters. In order to determine a favourable incubation period, the fermenting dough was sampled for analysis after 24, 48 and 72 hours of incubation. The microbiological composition (mould, yeast and bacteria) of fermented dough of various moisture contents at incubation intervals is shown in Figure 1. It is shown that incubation periods longer than 24 h are not favourable for the composition of fermented dough. At 24 hours of incubation, mould and yeast had reached a maximum of 5 and 7-8 Log CFU/g, respectively. However, prolonged incubation for 48 and 72 h. resulted in a slight decline of viable mould and yeast with concomitant increase of undesirable bacteria. The levels of bacteria increased gradually during the incubation from 3 to 7 Log CFU/g, which may be expected to compete with the growth of desirable mould and yeast.
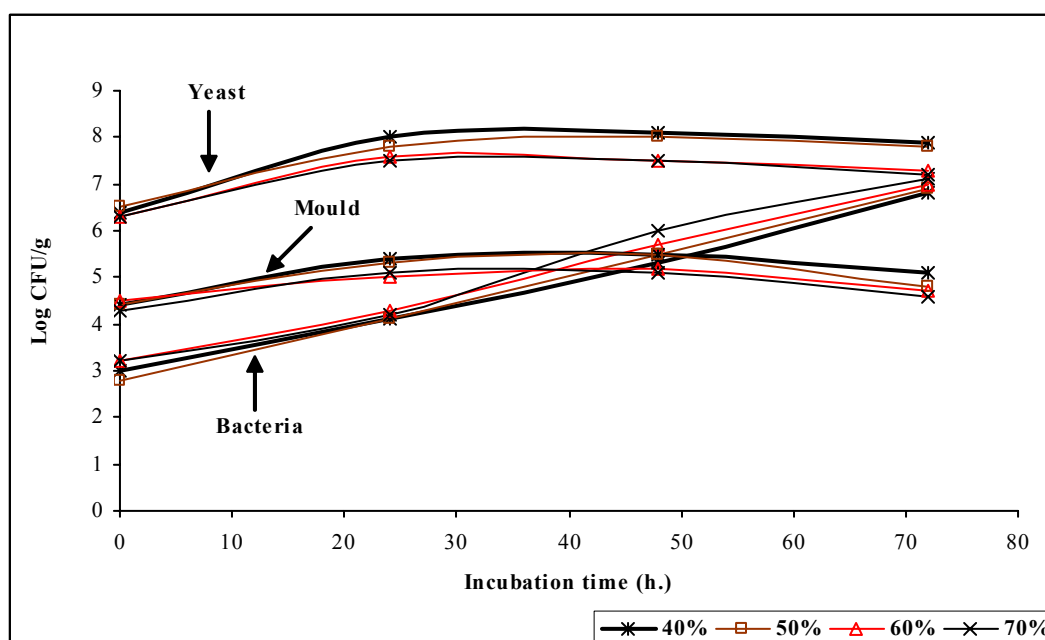


**Figure 1. Microbiological changes during fermentation of starter dough with varying moisture content**

Figure 1 also shows that the fungi tend to multiply best in dough of 40% moisture content; on the other hand, bacterial growth is more successful at the higher moisture levels.

The doughs fermented during 24, 48 and 72 hours were tested as starter in winemaking, mainly judging by flavour and alcohol content. The bacteria count in the wine was also determined to examine the fate of bacteria which were present as starter contaminants. Table 2 shows that most of these doughs gave a pleasant flavour that was quite strong alcoholic, aromatic and sweet, except in one case (70% moisture content, incubated for 72 h) which gave a sour but only very slight alcoholic flavour. For all starter doughs, the wine alcohol contents were significantly lower with longer dough incubation times; starter dough of 40% moisture content showed significantly higher wine alcohol content especially after incubation of 24 or 48 h. The table shows that a certain number of bacteria still remained in the final product, and that there was a positive correlation between the bacteria counts in fermented dough and in final wine in which the viability of bacteria significantly increased gradually during the dough incubation.

The concomitant growth of bacteria resulted in competition and limitation of the growth and the winemaking performance of desirable mould and yeast; this became more important when doughs underwent prolonged incubation. It appeared that the combination of 40% moisture content and short (24h) incubation provided optimum conditions for the maximum development of functional fungi with minimized bacterial interference. An added advantage of the lower moisture content, is that less water needs to be removed in the later stage of dehydration.

**Table 2. Winemaking performance and bacterial levels in starter doughs of different moisture contents and incubation periods**

| Moisture Content (%) | Incubation period (h) | Flavour assessment[1] | Alcohol content (% v/v) | Mesophilic bacteria (Log CFU/g fermented dough) |
|---|---|---|---|---|
| 40 | 24 | +++ | 19.8 [2] a[3] | 4.6 f |
| 40 | 48 | +++ | 19.8 a | 6.2 e |
| 40 | 72 | +++ | 18.8 b | 7.2 b |
| 50 | 24 | +++ | 19.8 a | 4.7 f |
| 50 | 48 | +++ | 18.8 b | 6.3 de |
| 50 | 72 | ++ | 13.8 d | 7.5 b |
| 60 | 24 | +++ | 18.8 b | 4.7 f |
| 60 | 48 | +++ | 18.8 b | 6.6 cd |
| 60 | 72 | ++ | 12.3 e | 7.9 a |
| 70 | 24 | ++ | 18.8 b | 4.7 f |
| 70 | 48 | ++ | 17.2 c | 6.7 c |
| 70 | 72 | + | 11.5 f | 7.9 a |

[1] flavour ranging from + (do not like much because of slightly alcoholic flavour and unpleasant flavour) to +++ (like very much for its strong alcoholic flavour and aromatic sweet smell); [2] values are means of triplicates; [3] means with different subscripts are statistically different at the 95% confidence level.

**Effect of granulation and dehydration on viability of functional fungi in a laboratory-scale starter production process**

In the previous section it was indicated that 40% moisture content (the lowest level tested) gave promising results with respect to microbiological composition and wine quality. In view of required drying, it would be of interest to apply even lower moisture levels.

Therefore we compared doughs of 30% and 40% moisture content for their behaviour during granulation, drying and the effect on fungal viability. Wet fermented dough granules were prepared and spread on a perforated drying tray to be dehydrated at 40 and 45ºC in an electric oven during periods up to 5 hours. The starter granules were sampled for analysis of moisture level and fungal viability at time intervals of 1 h. The changes of dry matter levels and viable counts of mould and yeast are presented in Figure 2.
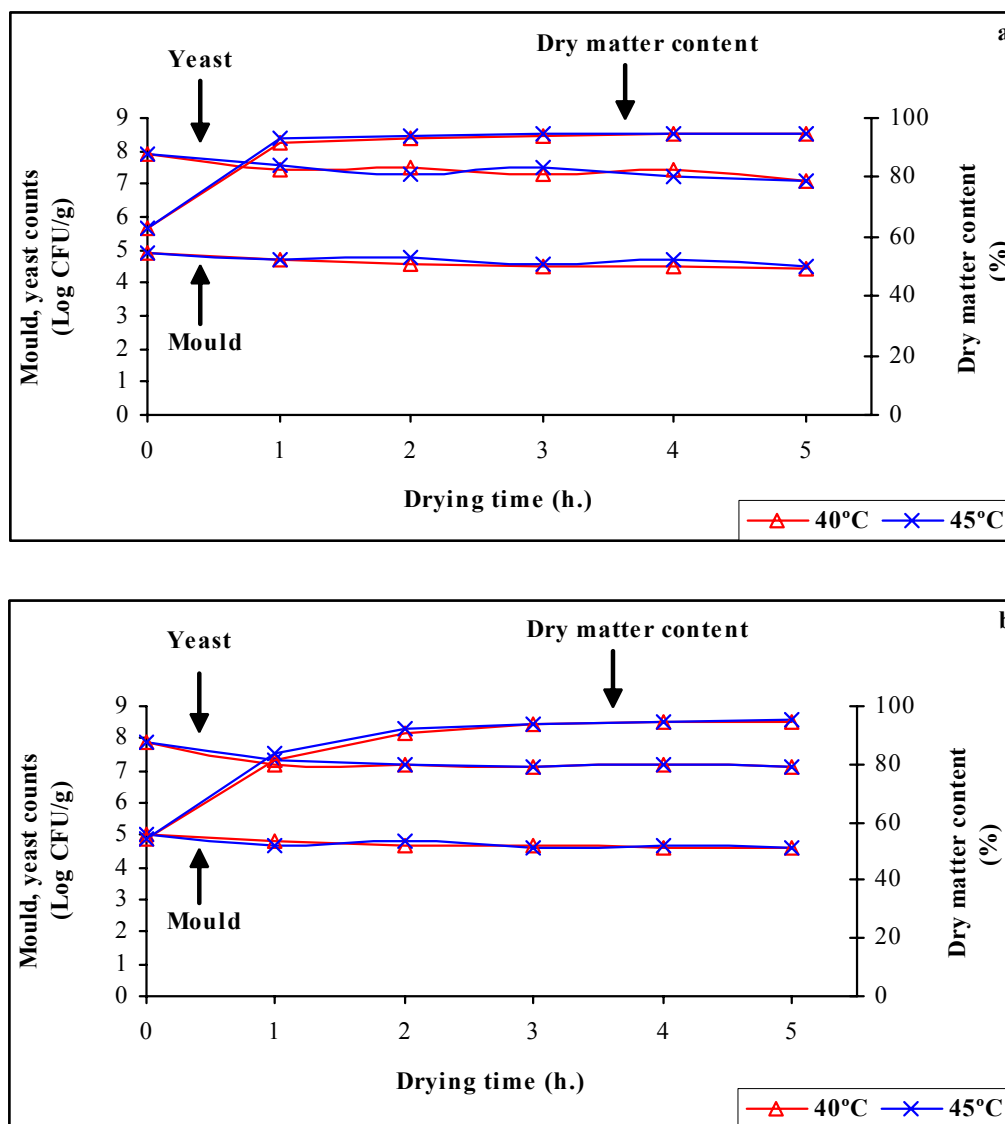


**Figure 2. Dry matter contents and fungal viability in starter granules during dehydration (a) 30% moisture content and (b) 40% moisture content of dough**

Wet fermented dough granules were spread on perforated drying tray for drying at 40 and 45ºC in oven for up to 5 hours.

The dry matter contents of fermented dough granules at the beginning before drying were a little lower in both cases of doughs at 30% and 40% moisture content. They were 63% (instead of 70%) and 54% (instead of 60%), respectively. These results were

caused by a certain amount that was added to the dough including herb extract solution and inoculum of mould and yeast suspension. Figure 2 shows that with the combination of 30% moisture content and 45ºC drying temperature, the maximum dry matter content of 95% is reached in the shortest time (3 h of drying). Moisture of 30 or 40% and drying conditions did not significantly influence the number of fungal colony forming units. This indicates that the granulation results in a rapid drying; this is also favourable for the survival of the starter micro-organisms.

In order to evaluate the viability of surviving fungi in the dried starter granules, they were tested for their winemaking performance. Table 3 presents the criteria used to assess winemaking performance: liquefaction, ºBrix, pH, alcohol content, and flavour. No significant optimum combination of processing factors was observed in relation with wine quality. All starter granules achieved normal and successful wine fermentation with adequate liquefaction, high levels of reducing sugars, normal pH levels, typical flavour and high alcohol contents of the final products.

Whereas no differences were observed in the performance of doughs of 30% and 40% moisture content, the advantage of the lower moisture content is that drying can be achieved more rapidly and cheaper, and that this (30% m.c.) dough is less sticky and easier to granulate.

By consequence, for the preparation of dried granulated starter, the moisture level of 30% and drying temperature of 45ºC were selected for further process developments.

## Table 3. Winemaking performance of dried starter granules

The starter granules made from dough of 30 and 40% moisture content and dried at 40 and 45ºC during up to 5 hours of drying were tested in winemaking. Samples for analysis were taken after 2 days of solid-state fermentation, and after 3 days of alcoholic fermentation.

| Combinations of factors | | | Winemaking performance | | | | |
|---|---|---|---|---|---|---|---|
| | | | Solid-state fermentation | | | Alcoholic fermentation | |
| Moisture Content (%) | Drying Temperature (ºC) | Drying Time (h) | Liquefaction | ºBrix | pH | Flavour | Alcohol Content (% v/v) |
| 30 | 40 | 0 | All samples | 17 [1] | 4.5 | All samples | 18.8 |
| 30 | 40 | 1 | gave the | 16 | 4.4 | gave the same | 19.7 |
| 30 | 40 | 2 | same | 16 | 4.5 | preferable | 19.7 |
| 30 | 40 | 3 | positive | 17 | 4.5 | flavour which | 19.7 |
| 30 | 40 | 4 | liquefaction | 17 | 4.5 | was strong | 18.8 |
| 30 | 40 | 5 | in which a | 16 | 4.5 | alcohol and | 19.7 |
| 30 | 45 | 1 | certain | 17 | 4.5 | sweet | 19.7 |
| 30 | 45 | 2 | amount of | 16 | 4.5 | smelling | 19.7 |
| 30 | 45 | 3 | liquid came | 16 | 4.4 | | 19.7 |
| 30 | 45 | 4 | out from the | 17 | 4.5 | | 18.8 |
| 30 | 45 | 5 | moulded | 17 | 4.4 | | 19.7 |
| 40 | 40 | 0 | mass after 2 | 17 | 4.5 | | 19.7 |
| 40 | 40 | 1 | days of solid- | 17 | 4.5 | | 19.7 |
| 40 | 40 | 2 | state | 17 | 4.5 | | 19.7 |
| 40 | 40 | 3 | fermentation | 17 | 4.5 | | 18.8 |
| 40 | 40 | 4 | | 17 | 4.5 | | 19.7 |
| 40 | 40 | 5 | | 16 | 4.5 | | 19.7 |
| 40 | 45 | 1 | | 17 | 4.5 | | 18.8 |
| 40 | 45 | 2 | | 17 | 4.4 | | 19.7 |
| 40 | 45 | 3 | | 17 | 4.4 | | 19.7 |
| 40 | 45 | 4 | | 16 | 4.4 | | 18.8 |
| 40 | 45 | 5 | | 17 | 4.5 | | 19.7 |

[1] values are mean of duplicates

**Effect of storage of dry starter granules on fungal viability**

Dry starter granules were vacuum packed and stored at 4ºC (refrigerator) and at approx. 30ºC (room temperature). The viability of mould and yeast at the start of the storage period and after 1, 2 and 3 months of storage was determined by culturing as shown in Table 4. At both storage temperatures, mould and yeast counts remained almost the same. In granules made of 40% moisture content dough, yeast counts after storage were slightly decreased. Overall, there was no clear effect of storage on the viability of mould and yeast.

**Table 4. Fungal viability in dry starter granules after 0, 1, 2 and 3 months of storage**

| MC[1] (%) | T[2] (ºC) | t[3] (h) | Mould count (Log CFU/g starter) | | | | | | | Yeast count (Log CFU/g starter) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | t0[4] | t1 | | t2 | | t3 | | t0 | t1 | | t2 | | t3 | |
| | | | | 4ºC[5] | 30ºC | 4ºC | 30ºC | 4ºC | 30ºC | | 4ºC | 30ºC | 4ºC | 30ºC | 4ºC | 30ºC |
| 30 | 40 | 0 | 4.9[6] | 4.7 | -[7] | 4.8 | - | 4.6 | - | 7.9 | 8 | - | 8 | - | 8.1 | - |
| 30 | 40 | 1 | 4.7 | 4.5 | 4.6 | 4.4 | 4.6 | 4.3 | 4.4 | 7.4 | 7.4 | 7.3 | 7.4 | 7.2 | 7 | 7.1 |
| 30 | 40 | 2 | 4.6 | 4.6 | 4.6 | 4.5 | 4.5 | 4.4 | 4.4 | 7.5 | 7.3 | 7.4 | 7.4 | 7.2 | 7.1 | 7.2 |
| 30 | 40 | 3 | 4.5 | 4.4 | 4.5 | 4.6 | 4.4 | 4.5 | 4.4 | 7.3 | 7.2 | 7.2 | 7.2 | 7.1 | 7.1 | 7.2 |
| 30 | 40 | 4 | 4.5 | 4.5 | 4.3 | 4.4 | 4.4 | 4.3 | 4.3 | 7.4 | 7.3 | 7.2 | 7.3 | 7.1 | 7.2 | 7.2 |
| 30 | 40 | 5 | 4.4 | 4.3 | 4.2 | 4.3 | 4.2 | 4.3 | 4.2 | 7.1 | 7.2 | 7.1 | 7 | 7 | 7.1 | 7.1 |
| 30 | 45 | 1 | 4.7 | 4.5 | 4.5 | 4.6 | 4.3 | 4.4 | 4.2 | 7.6 | 7.4 | 7.5 | 7.2 | 7.2 | 7.2 | 7 |
| 30 | 45 | 2 | 4.8 | 4.5 | 4.7 | 4.6 | 4.5 | 4.4 | 4.4 | 7.3 | 7.3 | 7.4 | 7.3 | 7.1 | 7.2 | 7 |
| 30 | 45 | 3 | 4.6 | 4.3 | 4.4 | 4.5 | 4.4 | 4.4 | 4.5 | 7.5 | 7.3 | 7.3 | 7.2 | 7.1 | 7.1 | 7 |
| 30 | 45 | 4 | 4.7 | 4.5 | 4.5 | 4.3 | 4.3 | 4.3 | 4.4 | 7.2 | 7.1 | 7.2 | 7.1 | 7 | 7.1 | 7 |
| 30 | 45 | 5 | 4.5 | 4.4 | 4.5 | 4.4 | 4.4 | 4.4 | 4.4 | 7.1 | 7.1 | 7.1 | 7 | 6.8 | 7 | 7 |
| 40 | 40 | 0 | 5 | 4.8 | - | 4.7 | - | 4.7 | - | 7.9 | 8 | - | 8 | - | 8 | - |
| 40 | 40 | 1 | 4.8 | 4.8 | 4.6 | 4.5 | 4.5 | 4.4 | 4.5 | 7.2 | 7.1 | 6.9 | 7 | 7 | 7 | 6.9 |
| 40 | 40 | 2 | 4.7 | 4.6 | 4.6 | 4.5 | 4.2 | 4.4 | 4.2 | 7.2 | 6.9 | 6.8 | 6.7 | 6.9 | 6.9 | 7 |
| 40 | 40 | 3 | 4.7 | 4.7 | 4.5 | 4.4 | 4.3 | 4.2 | 4.3 | 7.1 | 7.1 | 7 | 6.9 | 7 | 7.1 | 7 |
| 40 | 40 | 4 | 4.6 | 4.6 | 4.5 | 4.5 | 4.3 | 4.4 | 4.1 | 7.2 | 7.1 | 7 | 7.1 | 6.8 | 6.9 | 6.8 |
| 40 | 40 | 5 | 4.6 | 4.7 | 4.4 | 4.3 | 4.1 | 4.2 | 4.1 | 7.1 | 6.9 | 7 | 7 | 7 | 7 | 6.9 |
| 40 | 45 | 1 | 4.7 | 4.6 | 4.6 | 4.3 | 4.2 | 4.2 | 4.2 | 7.3 | 7.1 | 6.8 | 6.9 | 6.9 | 6.9 | 6.7 |
| 40 | 45 | 2 | 4.8 | 4.7 | 4.7 | 4.5 | 4.3 | 4.3 | 4.4 | 7.2 | 7 | 7 | 7.1 | 7 | 7 | 6.8 |
| 40 | 45 | 3 | 4.6 | 4.6 | 4.5 | 4.2 | 4.1 | 4.2 | 4.2 | 7.1 | 7.1 | 7 | 6.8 | 6.8 | 6.9 | 7 |
| 40 | 45 | 4 | 4.7 | 4.7 | 4.6 | 4.4 | 4.3 | 4.3 | 4.1 | 7.2 | 7 | 6.9 | 6.9 | 6.8 | 7 | 7 |
| 40 | 45 | 5 | 4.6 | 4.5 | 4.5 | 4.2 | 4.3 | 4.1 | 4.1 | 7.1 | 6.8 | 6.9 | 7 | 7.1 | 7 | 6.9 |

[1] moisture content of dough; [2] drying temperature; [3] drying time; [4] storage period in months; [5] storage temperature; [6] values are means of duplicates; [7] wet starter granules stored only at 4ºC

The starter granules stored for 3 months were also tested for their winemaking performance. In Table 5, the winemaking performance and dry matter content of starter granules before and after 3 months of storage are compared. During 3 months of storage at 4ºC, moisture levels had decreased slightly whereas they had increased significantly after 3 months at 30ºC. This shows that the packaging needs some further improvement in order to better protect the granules against high ambient humidity levels.

The alcohol contents in wine made with starter granules after 3 months of storage were significantly lower compared with those produced with fresh granules. Alcohol contents in wine from granules stored at 4ºC were significantly higher than the corresponding ones stored at 30ºC. However, in general the alcohol contents produced after 3 months of storage were still quite considerable: 15-18% (v/v) with granules stored at 4ºC and 10-14% (v/v) with granules stored at 30ºC. In addition, other criteria for wine quality showed the usual performance of 3 months stored granules: good liquefaction after

the first solid-state fermentation and strong alcohol and sweet flavour of final product were observed. Considering the mould counts and high levels of °Brix, it can be concluded that the viability and enzyme activity of the mould in dry starter granules are stable during storage.

From this experiment it can be concluded that the yeast activity is most likely the limiting factor for shelf-life and stability of the starter granules. It is not known what is the reason for the relative storage sensitivity of the yeast; possibly, the moisture content of the granules during storage plays a role. Higher moisture contents of starter stored at room temperature related significantly to lower wine alcohol contents, compared with dry starter stored at 4°C.

**Table 5. Dry matter and winemaking performance of dry starter granules before (t0) and after 3 months (t3) of storage**

Conditions of factors included 30% and 40% moisture content, drying time at intervals of 1 hour up till 5 hours, drying temperature at 40 and 45°C, storage temperature at 4 and 30°C and storage period at 0 (t0) and 3 (t3) months.

| MC[1] (%) | T[2] (°C) | t[3] (h) | Moisture content (%) | | | °Brix | | | Alcohol content (% v/v) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | t0[4] | t3 4°C[5] | 30°C | t0 | t3 4°C | 30°C | t0 | t3 4°C | 30°C |
| 30 | 40 | 0 | 37.3[6] | 28.5 | -[7] | 17 | 22 | - | 18.8 | 13.6 | - |
| 30 | 40 | 1 | 8.3 | 7.1 | 9.2 | 16 | 22 | 22 | 19.7 | 16.2 | 13.6 |
| 30 | 40 | 2 | 6.8 | 6 | 9 | 16 | 23 | 22 | 19.7 | 16.2 | 12 |
| 30 | 40 | 3 | 6 | 6 | 9 | 17 | 22 | 21 | 19.7 | 17.9 | 13.6 |
| 30 | 40 | 4 | 5.4 | 5.4 | 8.5 | 17 | 22 | 22 | 18.8 | 16.2 | 11.1 |
| 30 | 40 | 5 | 5.2 | 5.5 | 8.3 | 16 | 21 | 21 | 19.7 | 16.2 | 12.8 |
| 30 | 45 | 1 | 7 | 6.6 | 8.3 | 17 | 22 | 23 | 19.7 | 16.2 | 13.6 |
| 30 | 45 | 2 | 5.8 | 5.7 | 8 | 16 | 23 | 22 | 19.7 | 17.1 | 11.1 |
| 30 | 45 | 3 | 5.2 | 4.9 | 8.5 | 16 | 22 | 21 | 19.7 | 17.1 | 13.6 |
| 30 | 45 | 4 | 5.1 | 5 | 8.7 | 17 | 21 | 21 | 18.8 | 16.2 | 12.8 |
| 30 | 45 | 5 | 5 | 5 | 8.8 | 17 | 21 | 22 | 19.7 | 17.9 | 14.5 |
| 40 | 40 | 0 | 45.6 | 34.7 | - | 17 | 22 | - | 19.7 | 15.4 | - |
| 40 | 40 | 1 | 18.7 | 11.7 | 19.8 | 17 | 21 | 21 | 19.7 | 17.1 | 12 |
| 40 | 40 | 2 | 9.5 | 8.5 | 10.5 | 17 | 21 | 21 | 19.7 | 17.1 | 12.8 |
| 40 | 40 | 3 | 6.3 | 5.9 | 9.4 | 17 | 22 | 22 | 18.8 | 16.2 | 11.1 |
| 40 | 40 | 4 | 5.6 | 5.7 | 9.3 | 17 | 21 | 22 | 19.7 | 17.9 | 12.8 |
| 40 | 40 | 5 | 5.5 | 5.5 | 9.3 | 16 | 21 | 21 | 19.7 | 17.1 | 12.8 |
| 40 | 45 | 1 | 16.2 | 9.2 | 18.8 | 17 | 22 | 22 | 18.8 | 16.2 | 12 |
| 40 | 45 | 2 | 7.9 | 6.4 | 9.7 | 17 | 23 | 22 | 19.7 | 15.4 | 10.2 |
| 40 | 45 | 3 | 6.1 | 6 | 9.4 | 17 | 22 | 23 | 19.7 | 16.2 | 11.1 |
| 40 | 45 | 4 | 5.3 | 5.4 | 9.1 | 16 | 22 | 21 | 18.8 | 17.9 | 13.6 |
| 40 | 45 | 5 | 5 | 5.1 | 8.9 | 17 | 22 | 22 | 19.7 | 17.1 | 12.8 |

[1] moisture content of dough; [2] drying temperature; [3] drying time; [4] storage period in months; [5] storage temperature; [6] values are means of duplicates; [7] wet starter granules stored only at 4°C

In conclusion, a defined mixed-culture shelf-stable granulated starter for alcoholic fermentation was successfully developed at a laboratory-scale that could be used for the preparation of Vietnamese rice wine. There are few reports that are related to the present study (Hieu, 1990; Luong, 1998; Phuc, 1998), which suggest favourable process conditions that are very different from our findings. These include, a dough moisture content of 50-60%, dough fermentation for 2-4 days, followed by drying small flattened cakes of

fermented dough in a ventilated place for a few days. Unfortunately, no scientific data or relevant experimental results from these studies are available for comparison. Our defined starter should now be tested in a consumer trial before a transfer of the new product is to be made into practice.

# Chapter 7

# Sensory evaluation of purple glutinous rice wine produced with defined experimental starter

**Abstract**

Purple glutinous rice wine is a typical fermented beverage in the Mekong Delta area in Vietnam, appreciated for its colour, flavour and taste. We compared commercially available rice wines ("Bien Hoa", "Can Tho" and "Go Den" brands) with wine produced with our experimental (referred to as "Experimental") dehydrated defined mixed-culture starter of the mould *Amylomyces rouxii* and the yeast *Saccharomyces cerevisae*. The wines were judged according to Vietnam standard method 3215-79 by a panel consisting of 20 experts. The "Experimental", "Bien Hoa" and "Can Tho" wines received scores for colour, clearness, flavour and taste that were significantly higher than those for "Go Den" wine, mainly because of their attractive natural colour, pleasant alcoholic flavour as well as the specific sherry-like taste of purple glutinous rice wine. The "Go Den" wine was considered unacceptable because of its colour that was perceived as "artificial" and its excessive alcohol content; however, it could still be safely offered for sale. The "Experimental" wine scored very well on flavour. A preference ranking supported the evaluation results, and indicated that some practices, such as the use of artificial colour and increasing the alcohol content by addition of distilled rice alcohol, were not appreciated by panellists.

## INTRODUCTION

Traditional fermented rice-based beverages are highly popular in various countries and as such they represent an important category of indigenous fermented products (Phuc, 1998; Haard et al., 1999; Nout and Aidoo, 2002). They play a role in human spiritual and cultural life. They are produced predominantly at artisanal small-scale level. Nowadays, the development of small-scale technology to process agricultural products by fermentation is one of the major parts of the programme to improve the socio-economic situation in Vietnam. The ultimate aim is to contribute to the development of local industry and improve livelihood of the population of the region. Therefore, the quality of such products including rice wine should be examined professionally using officially acknowledged and standardized techniques. Among the variety of methods to examine and evaluate wine, such as sensory observations, microscopic examination, fining test, and chemical analyses (Amerine and Cruess, 1960), sensory evaluation and acceptability tests are important (rapid and low-cost) methods to provide relevant and reliable information about the quality and acceptability of beverages or food (Guinard and Mazzucchelli, 1996; Legin et al.,

2003). In general, three methods are mostly applied in sensory analysis: the difference methods, the priority methods and the predilection exploration method. The difference methods include triangle test, paired comparison test, duo-trio test, score test, and A-Not A test. The priority methods apply paired comparison test, score test, and order test. The predilection exploration method is used to find out the attitude and the preference of a large number of consumers in different geographic areas (Meilgaard et al., 1991; Parolari, 1996).

In this chapter, the sensory assessment of purple glutinous rice wine produced with defined experimental dry starter is evaluated following official Vietnamese sensory evaluation techniques which belong to the category of the score test. Some representative kinds of commercial rice wines manufactured with traditional alcoholic starters are included for comparison.

## MATERIALS AND METHODS

### Preparation of inoculum

The mould *Amylomyces rouxii* strain no. 20.3 (CBS 111757) and the yeast *Saccharomyces cerevisae* strain no. 2.1 (LU 1250) were grown on slants of malt extract agar (Oxoid, CM59) at 30ºC for 5 (mould) and 2 days (yeast). A suspension of the growing micro-organisms was made by adding 5ml of sterile physiological salt solution (0.85% NaCl) to each slant. The biomass was scraped off the agar by means of an inoculating wire.

### Preparation of defined experimental starter

The study of compatibility in mixed cultures and of the effect of oriental herbs and the process for preparing defined mixed starter cultures were described in Chapters 5 and 6, respectively.

The defined experimental starter was prepared as follows. A mixture of 120 g of rice flour and 30 g of cassava flour in a 500 ml conical flask covered by a cotton plug was heated at 100ºC in an electric oven overnight (17 hours). The heated flour mixture was cooled to 35-40ºC and 41.5ml of sterile water was added to reach the required moisture content of 30%. Three ml of sterile extract of oriental herbs containing "Tieu Hoi" (*Foeniculum vulgare* Miller) and "Dinh Huong" (*Syzygium aromaticum* L.) was added. The extract was prepared by adding 5 g of each herb to 100 ml of water and heating in a water bath at 50ºC for 1 h, followed by filtration over a filter paper and over a 0.2µm (S&S) sterile membrane filter. Mixtures of extracts were made by pooling equal aliquots of each herb extract. Next, inoculation was performed with a suspension obtained from 6 slants of mould culture and with 6ml of yeast suspension. The inoculated dough was mixed well and incubated at 30ºC for 24 hours. The fermented starter dough was granulated (3-4 mm diam size) by rotation on previously disinfected steel sieves. The granules were distributed on a perforated drying tray and dehydrated at 45ºC during 3 h. The dry starter

granules were vacuum packed using a Vacupack-Plus machine (Krups, type 380, CE, z260590, P.R.C.) and stored at 4ºC.

**Preparation of purple glutinous rice wine with defined experimental starter**

One hundred grams of purple glutinous rice and 120 ml of distilled water in a 500-ml conical flask covered by a cotton plug were soaked for 4 hours at room temperature (25ºC). After soaking they were steamed in an autoclave for 1 hour at 100ºC. The gelatinized rice paste was cooled to 35-40ºC, then inoculated and mixed well with 4 g of defined experimental starter dry granules, prepared as described above. After solid-state fermentation during 3 days at 30ºC, 140 ml of sterile water was added to the moulded mass to allow for submerged alcoholic fermentation for 3 days at 30ºC under anaerobic condition in which the cotton plug was replaced by a water lock. After 2 weeks of maturation, all of the fermented rice mass was homogenized using a Stomacher Lab-blender (Seward 400, Emergo, England) and clear liquid was harvested after centrifugation at 7500 rpm for 20 minutes (Rotanta 46R, type 4810, Hettich Zentrifugen, D78532 Tuttingen, Germany). The clear wine was used for sensory evaluation tests.

**Sensory evaluation and analysis**

The Vietnamese standard method 3215-79 formulated by the National Committee for Sensory Evaluation for the quality of food products (Thu, 1989) was used. Depending on the product, quality attributes are allocated a weight factor that illustrates its perceived importance for the acceptability of the product. At first, panellists will score individual quality attributes using a scale of $0 - 5$. Scores for a certain attribute given by all panellists are averaged, and the average is corrected using the corresponding weight factor. Subsequently, the weighted average scores are totalled. Table 1 describes quality criteria categories, weight factors, and scoring ranges applicable to sensory evaluation of rice wine according to Vietnamese standard. The total of weighted scores corresponds with acceptability categories such as excellent, good, fair etc. as shown in Table 2.

The evaluation process was carried out in a 20ºC conditioned room, using identical clean glassware, coded and numbered with using tri-figures. For each kind of wine a volume of 4 litres used for testing was prepared in one big bottle, mixed very well and divided into 200 ml portions in smaller bottles which had been rinsed with the corresponding wine before dividing. These small bottles were coded and kept stable at 20ºC for at least 2 h before testing. Twenty examiners who are well-experienced in sensory evaluation of wine were invited. The examiners were requested to have only a moderate meal at least 2 h before the sensory evaluation session, and from that time point to refrain from eating, smoking and using flavoured products such as perfumes, cosmetics and soap. Before tasting a sample, the examiners had to drink some mineral water. Colour, clearness, flavour and taste of the wine were evaluated by each examiner.

**Table 1. Quality criteria categories, weight factors, and scoring ranges applicable to sensory evaluation of rice wine according to Vietnamese standard 3215-79 (Thu, 1989)**

| Criterion | Weight factor | Scoring range (unweighted) | Description of scores |
|---|---|---|---|
| Colour and clearness | 0.8 | 5 | Liquid is very clear, no turbidity, no unusual small objects, colour is perfectly specific for the product |
| | | 4 | Liquid is very clear, no turbidity, a little bit of unusual small objects, colour is specific for the product |
| | | 3 | Liquid is clear, more unusual small objects, colour is a bit different from the specific colour of the product |
| | | 2 | Liquid is rather turbid, contains many unusual small objects, colour is very different from the specific colour of the product |
| | | 1 | Liquid is turbid, containing much sediment, many unusual crude objects, the colour is not specific for the product |
| | | 0 | Liquid is very turbid, the colour is dirty, the product is spoiled |
| Flavour | 1.2 | 5 | Fragrant, harmonious, perfectly specific for the product |
| | | 4 | Not perfectly harmonious, fragrant and perfectly specific for the product but it takes time to realize |
| | | 3 | Slightly to strong undesirable flavour, not very specific for the product |
| | | 2 | Strong undesirable flavour, very little specific for the product |
| | | 1 | Very strong and obviously undesirable flavour, not specific for the product |
| | | 0 | Unpleasant and undesirable flavour of spoiled product |
| Taste | 2.0 | 5 | Harmonious, gentle, excellent taste, perfectly specific for the product |
| | | 4 | Not perfectly harmonious, good taste, specific for the product |
| | | 3 | Not harmonious, fair taste, little specific for the product |
| | | 2 | Bitter, pungent, weak taste, very little specific for the product |
| | | 1 | Unpleasant and undesirable taste of spoiled product |
| | | 0 | Product completely spoiled and corrupt, impossible to taste |

**Table 2. Quality categories based on sensory evaluation of wine**

| Quality category | Total of weighted scores |
|---|---|
| Excellent | 19.6-20.0 |
| Good | 18.2-19.5 |
| Fair | 15.2-18.1 |
| Acceptable | 11.2-15.1 |
| Poor (not acceptable but can still be offered for sale) | 7.2-11.1 |
| Very poor (not acceptable for sale but can possibly be used after appropriate reprocessing | 4.0-7.1 |
| Spoiled (impossible to use or to reprocess) | 0-3.9 |

**RESULTS AND DISCUSSION**

Four different kinds of purple glutinous rice wine were included in the sensory evaluation; their respective characteristics are summarized in Table 3. Three flasks of "Experimental" rice wine were taken at random for pH measurement and ethanol determination by the distillation method (Duong, 1971; So and Nhuan, 1991). The pH value and ethanol content of these three samples were identical, *i.e.* pH 3.9 and ethanol level at 20% (v/v). This indicates that the "Experimental" wine was representative for the wine obtained previously in small-scale defined mixed-culture fermentations.

**Table 3. Purple glutinous rice wines evaluated in sensory test**

| No. | Name | Shelf-life | Period of storage prior to analysis | Alcohol content (% v/v) | Characteristics |
|-----|------|-----------|-------------------------------------|-------------------------|-----------------|
| 1 | "Experimental", produced at BiRDI | (1) | 2 weeks | 20 | Experimental wine produced as described in methods section. |
| 2 | "Go Den" | 6 months | 2 months | 34 | Commercial wine produced artisanally by local producer, with artificial red colour and distilled alcohol added. The final wine is not centrifuged, both liquid and wine dregs are mixed in the fermentation containers. At the time of purchase, the supernatant wine is decanted and sold. This wine was bought directly from its local producer. |
| 3 | "Bien Hoa" | 3 years | 1 year | 13 | Commercial wine produced in a factory. The clear wine was bottled and labeled. This wine was bought from a retail outlet. |
| 4 | "Can Tho" | 2 weeks | 1 week | 32 | Commercial wine produced artisanally by local producer, with distilled alcohol added. The final wine is not centrifuged, both liquid and wine dregs are mixed in the fermentation containers. At the time of purchase, the supernatant wine is decanted and sold. This wine must be used fresh. This wine was bought directly from its local producer. |

(1) not determined

Table 4 summarizes the results of sensory evaluation and the totals of weighted scores for the 4 wines. For each criterion the unweighted scores given by 20 panellists were averaged, multiplied with the respective weight factor and totalled.

The scores given by the twenty panellists indicated that the panel was adequately sensitive and able to distinguish the different products; the same evaluation results or very slight differences were obtained with blind duplicates. The "Experimental" wine, "Bien Hoa" wine and "Can Tho" wine obtained significantly higher scores on all attributes, compared with "Go Den" wine mainly because of colour and clearness, but they scored

also higher on flavour and taste. The "Experimental" wine obtained the best scores for flavour.

**Table 4. Sensory evaluation of experimental and commercial purple glutinous rice wines**

| Attributes | WF[1] | "Experimental" | | "Go Den" | | "Bien Hoa" | | "Can Tho" | |
|---|---|---|---|---|---|---|---|---|---|
| | | UAS[2] | WAS[3] | UAS | WAS | UAS | WAS | UAS | WAS |
| Colour and clearness | 0.8 | 3.95[4] | 3.16 | 2.38 | 1.90 | 4.3 | 3.44 | 3.15 | 2.52 |
| Flavour | 1.2 | 3.90 | 4.68 | 2.45 | 2.94 | 3.0 | 3.60 | 3.35 | 4.02 |
| Taste | 2.0 | 3.15 | 6.30 | 2.95 | 5.90 | 3.2 | 6.40 | 3.25 | 6.50 |
| Total of weighted average scores | | 14.14 a[5] | | 10.74 d | | 13.44 b | | 13.04 c | |
| Quality category | | acceptable | | not acceptable, but can still be sold | | acceptable | | acceptable | |
| Acceptability score | WF | n[6] | Scores | n | Score | n | Score | n | Scores |
| Don't like at all | 1 | 1 | 1 | 3 | s | 0 | s | 2 | 2 |
| Don't like | 2 | 2 | 4 | 8 | 3 | 5 | 0 | 3 | 6 |
| So-so, just fair | 3 | 4 | 12 | 6 | 16 | 9 | 10 | 4 | 12 |
| Like quite a bit | 4 | 7 | 28 | 2 | 18 | 3 | 27 | 5 | 20 |
| Like very much | 5 | 6 | 30 | 1 | 8 | 3 | 12 | 6 | 30 |
| | | | | | 5 | | 15 | | |
| Total | | 20 | 75 | 20 | 50 | 20 | 64 | 20 | 70 |

[1] WF: weight factor

[2] UAS: unweighted average score (n=20)

[3] WAS: weighted average scores

[4] values are means of 20 evaluation results by 20 examiners with duplicates

[5] values with different subscripts are statistically significant at the 95% confidence level

[6] number of panellists

Although it is not a required item of the sensory evaluation of wine by Vietnamese standards, panel members were also asked to evaluate the samples according to their acceptability. As shown in Table 4, acceptability scores indicate that the panel members did not significantly prefer any of the wines, except that they did not appreciate the "Go Den" wine. As shown in Table 3, it is clear that the commercial rice wines produced at home-scale have excessively high alcohol contents because of the addition of distilled rice alcohol. Panellists commented that they did not appreciate the unnatural (artificial) colour and the excessively high alcohol content (achieved by addition of distilled rice alcohol) of this wine. This is in good correspondence with the scores given for quality attributes. It must be noted here that several small-scale wine producers manipulate the quality of their wine: to cut the cost of the required purple glutinous rice, it is blended with the - considerably cheaper - white glutinous rice and artificial red colour; poor saccharification and/or fermentation yields are masked by adding – cheap – distilled alcohol obtained from white glutinous rice. Apparently, experts able to detect the differences, prefer the unadulterated wine.

Purple glutinous rice wine is a typical product of the Mekong Delta area; in this region the development and upgrading of small-scale industry to add value to agricultural products - for instance by fermentation - is part of the Government policy aimed at improving the livelihood of Vietnamese people. Up till now, yield, flavour and acceptability of local rice wine prepared by traditional - uncontrolled - methods are variable and unpredictable. With the aim of a future transfer of know-how of biotechnological processes to wine starter manufacturers, we developed our "Experimental" wine at the laboratory scale. In the previous chapters, technical aspects such as saccharification and alcohol production were discussed. However, we considered the acceptance by consumers to be an essential requirement for our new product. Because of the typical character of the product it was considered appropriate to use the Vietnamese standard method. Although a panel of limited size was used, its members were very well acquainted with the quality requirements of the product, and preliminary trials showed that the panel gave consistent and reproducible judgements. The results of our experiment indicate that the "Experimental" wine received adequate – acceptable – scores and it received the highest scores for its flavour.

# Chapter 8

# General Discussion

This study addresses the problem of poor and variable quality of traditional starter tablets by understanding and quantifying the impact of microflora in these starters, concentrating on mycelial fungi and yeasts, and by assessing the option of preparing stable mixed cultures of selected compatible strains. It was found that a limited selection of pure moulds and yeasts offering powerful glucose production and active alcoholic fermentation, respectively, are compatible in mixed-culture fermentations. This study also describes the improved processing as well as increased insight into the stability of single pure and mixed starter components, when processed into starter granules, dried and stored.

**Fungal performance of starters for rice wine fermentation**

The manufacture of rice wine can be understood scientifically as a biological process whereby agricultural products, such as rice or (purple) glutinous rice, are converted into wine by microbiological and biochemical reactions during steaming, inoculating with starter, mashing and fermentation. The choice of alcoholic fermentation starters is one of the most important factors influencing the yield and quality of the final products in winemaking. There are several amylolytic starter cultures available in the markets in most Asian countries (Hesseltine and Wang, 1986; Steinkraus, 1989; Nout and Aidoo, 2002). *Men ruou* (*Men*) is the local name of Vietnamese starters of which three main kinds are commonly known (Luong, 1998; Phuc, 1998). In the Mekong Delta region, starters which are supplemented with oriental herbs are the most common. Not surprisingly, all 29 samples of commercial starch-based rice wine starters studied in Chapter 2 are classified as the above-mentioned type. However, in principle any kind of rice wine starters must contain a combination of micro-organisms able to perform the microbiological and biochemical reactions that convert rice into wine.

Fungi are essential for the performance of starters for rice wine fermentation. In the solid-state fermentation the moulds bring about saccharification of the rice starch, and the sugars thus formed are fermented into alcohol by the yeasts, so the quality of final products depends mainly on the activities of these micro-organisms. These two essential stages were examined in Chapters 3 and 4 in which the performance of pure moulds and yeasts that had been isolated from six selected Vietnamese commercial fermentation starters (in Chapter 2) was described.

In Chapter 3 significantly different levels of glucose production and amyloglucosidase activity among pure isolates of moulds were observed. Although all *Amylomyces* strains and *Rhizopus* strains showed active starch degradation in rice starch

agar medium, *Amylomyces rouxii* was found as the most powerful glucose producer with highest enzyme activity whereas *Rhizopus oligosporus* showed the lowest levels. This difference can be caused by either lower amyloglucosidase activity of *Rhizopus* spp. (Underkofler, 1976) or higher consumption by *Rhizopus* of glucose for mycelium production. The efficiency of saccharification is correlated closely with the glucogenic enzyme system rather than with the activity of α-amylase (Underkofler, 1976).

Two clearly different groups of yeasts in ethanol fermentation were also distinguished (Chapter 4). Five strains of *Saccharomyces cerevisiae* were found as superior fermenters, and other strains including *Candida glabrata* and *Pichia anomala* performed less efficiently. Combining the results of Chapters 2, 3 and 4, it can be clearly seen that the pure cultures of moulds and yeasts isolated from selected superior starters, even originally from the same starter (mould strains 6.5 and 6.9) performed very differently in starch saccharification and in alcoholic fermentation. This phenomenon obviously indicates that original rice wine starters are not defined pure culture starters, but contain a mixed microflora.

This study also defined optimum conditions including incubation temperature, incubation time and inoculation level for the maximum activity of a powerful glucose producer (*Amylomyces rouxii* strain) and a superior fermentative and ethanol tolerant yeast (*Saccharomyces cerevisiae* strain). Optimum conditions for saccharification were defined as follows: incubation temperature 34ºC, incubation time 2 days and inoculation level 5 Log spores/g of steamed rice. For the fermentation, optimum conditions were as follows: incubation temperature 28.3ºC, incubation time 4 days and inoculation level 5.5 Log cells/ml of saccharified rice liquid. For starch saccharification at a favourable temperature, there is no clear effect of the different incubation times on the glucose production. This can be explained by the predominant effect of inoculation levels of spores. At the same favourable incubation temperature higher spore levels lead to more mycelium growth that enters deeply inside the moulded mass of inoculated steamed rice to change the conditions in the medium, for instance anaerobic condition, resulting in limited enzyme production and activity. On the contrary, for the alcoholic fermentation at a favourable temperature, the incubation time and not the inoculation level, gives significant effects on the ethanol production. It can be understood that yeasts can grow rapidly at a favourable temperature, so after 2 days under anaerobic condition the lowest level of yeast inoculum has increased sufficiently. But from there on, the time becomes important because in anaerobic conditions the longer the incubation time the more ethanol is produced. This explanation is supported by measurements of fermentation rate (Chapter 4) in which ethanol content increased gradually during the fermentation. Of course, at a certain time and at a certain ethanol level, yeast cannot ferment glucose into ethanol anymore since viability loss of yeast in the presence of high levels of ethanol becomes an inhibition factor. This point is determined by the ethanol tolerance of yeast (Casey and Ingledew, 1986; Jones, 1989; D'Amore et al., 1990).

Although the individual pure moulds and yeasts can have powerful activities, the interactions and the compatibility of these cultures needed to be examined for they are important for the production of starters with good quality. This aspect was addressed in

Chapter 5 indicating that although some competition for glucose occurs, there were no combinations that really inhibited each other. All strains tested are compatible with each other and can be used for the design of starters.

Other fungal properties, especially those that relate to product quality, are: release of pink-brown colour into the wine, overproduction of glucose resulting in sweetness, and production of esters resulting in fruity fragrances. In fact, the colour release resulting in the attractive dark red colour of the final products was also examined in this study to find out any correlation with the ability of starch degradation. Samples of liquid formed in the solid- state incubation of the wine fermentation were subjected to colour measurements. Different UV/visible spectrophotometric approaches were taken using for instance, a Novaspec II spectrophotometer (Amersham pharmacia biotech AB SE-751 84, Biochrom Ltd. Cambrige), a scanning spectrophotometer Cecil CE 2020 (Cecil instruments Ltd., Cambridge, UK), and a UV/VIS spectrophotometer Lamda 12 (Perkin Elmer, Netherlands). However, a suitable wavelength for colour of purple glutinous rice wine could not be detected. Probably in this kind of wine there is no single compound that can absorb any range of colour wavelength. In addition, there was a problem in preparation of samples since samples of purple glutinous rice wine are too concentrated and must be diluted for colour measurement, but wine cannot be diluted for measuring its colour probably because of the collodial nature of the colouring matter (Gayon, 1974). It is recognized that the colour of wine depends on different factors such as pH, the age of wine, alcohol content, enzyme activity of starters, etc… in which effects of pH and the age of wine have been reported to be the most significant (Sudraud, 1958). The low pH increases colour extraction of wine; different ages of wine have different wavelengths of absorbance. Therefore, the detailed study of colour release requires a separate study in order to obtain effective results. In the scope of the present study the colour was evaluated by visual judgment by panel members as reported in Chapter 7.

The flavour of wine is also a sensory perception that varies with the chemical composition of the product and is judged subjectively by members of taste panels (Fleet, 2003). The flavour of purple glutinous rice wine is described in Chapter 7. Besides, because of the fact that yeasts have a dominating influence on the production of wine flavour (Fleet, 1993) four representative yeast isolates obtained in Chapter 2 were tested for flavour development. This experiment was a part of an MSc. thesis (Caprioli, 2003) in which headspace gas chromatography (HS-GC 8000 TOP, ThermoFinigan, Milan, Italy) was applied. The compounds present in the flavour of purple glutinous rice wine were identified, including ethanol, pentanol, isobutanol, methanol, propanol and acetone. These compounds were found in all wines produced with various yeasts (two *Saccharomyces cerevisiae* strains, *Candida glabrata* and *Pichia anomala*).

**Innovation of the starter manufacturing method**

Advantages of defined mixed starter cultures have been described in literature (Ray, 2001; Siebenhandl et al., 2001; Holzapfel, 2002). Most important are improvements of yield and achieving constant quality of the final products.
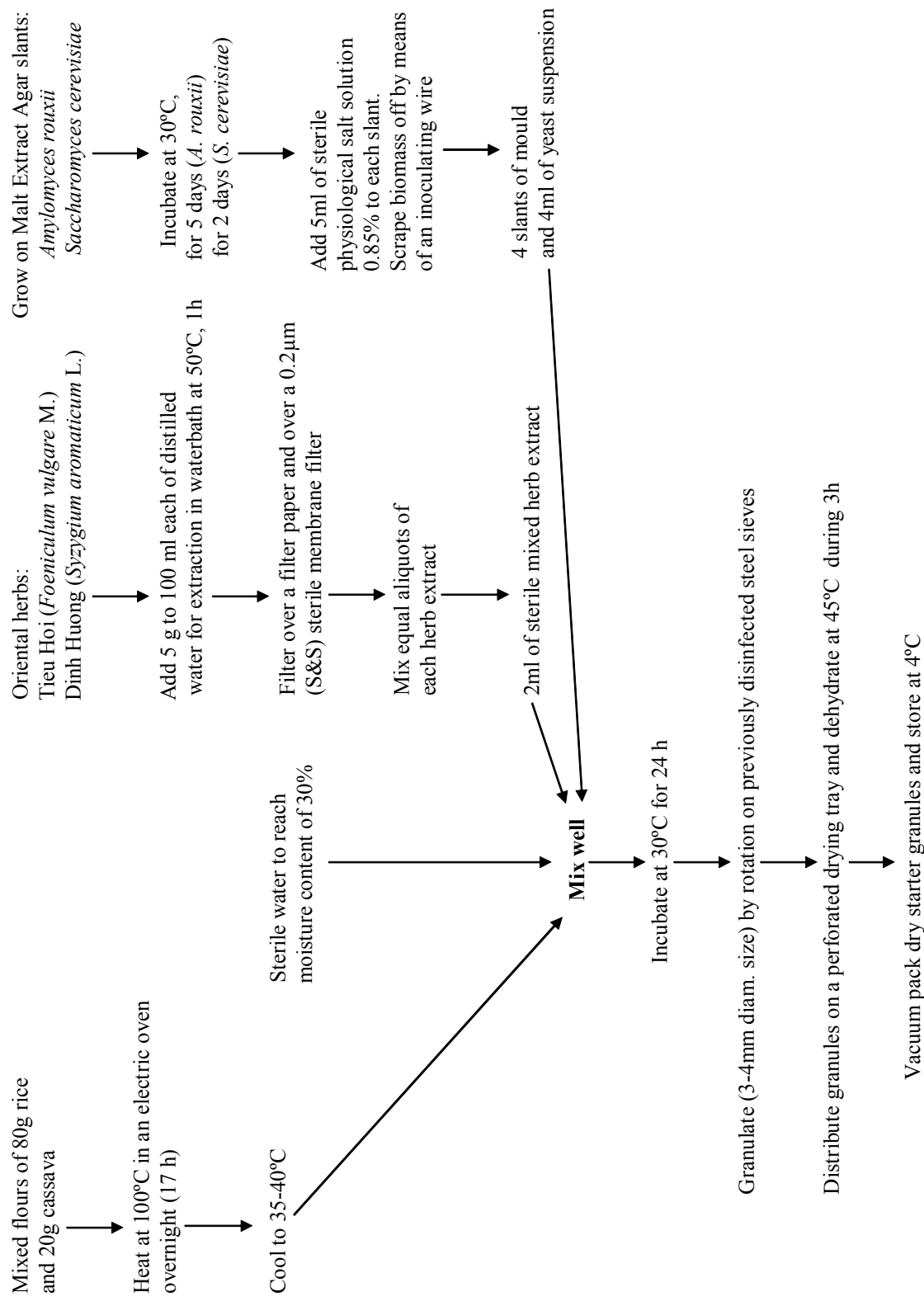
Grow on Malt Extract Agar slants:
*Amylomyces rouxii*
*Saccharomyces cerevisiae*

Incubate at 30°C,
for 5 days (*A. rouxii*)
for 2 days (*S. cerevisiae*)

Add 5ml of sterile
physiological salt solution
0.85% to each slant.
Scrape biomass off by means
of an inoculating wire

4 slants of mould
and 4ml of yeast suspension

Oriental herbs:
Tieu Hoi (*Foeniculum vulgare* M.)
Dinh Huong (*Syzygium aromaticum* L.)

Add 5 g to 100 ml each of distilled
water for extraction in waterbath at 50°C, 1h

Filter over a filter paper and over a 0.2μm
(S&S) sterile membrane filter

Mix equal aliquots of
each herb extract

2ml of sterile mixed herb extract

Mixed flours of 80g rice
and 20g cassava

Heat at 100°C in an electric oven
overnight (17 h)

Cool to 35-40°C

Sterile water to reach
moisture content of 30%

**Mix well**

Incubate at 30°C for 24 h

Granulate (3-4mm diam. size) by rotation on previously disinfected steel sieves

Distribute granules on a perforated drying tray and dehydrate at 45°C during 3h

Vacuum pack dry starter granules and store at 4°C

**Figure 1. Flow diagram for the preparation of defined mixed-culture starter granules**

For the manufacture of purple glutinous rice wine it is essential to dispose of a defined starter culture for the same reason. Fungi - moulds and yeasts - are the essential micro-organisms in such starters. Chapter 6 describes the development of a laboratory-scale process to formulate a shelf-stable mixed-culture starter. All possible factors affecting the preparation and storage of starter were examined as well as the interactions among these factors. Figure 1 describes a flow diagram for the production of a defined mixed-culture shelf-stable granulated starter preparation. It is recognized that yeast activity is most likely the limiting factor for shelf-life and stability of starter. The dry matter content is one of the factors influencing the shelf-life of the dried granulated starter. Unfortunately, no scientific data or relevant experimental results are available for comparison. Our defined starter should now be tested in a consumer trial before a transfer of the new product is to be made into practice.

In this study, three kinds of commercially available rice wine were compared with wine produced with our experimental dehydrated defined mixed-culture starter of the mould *Amylomyces rouxii* and the yeast *Saccharomyces cerevisae* (Chapter 7). The wines were judged according to Vietnam standard 3215-79 by a panel consisting of 20 experts. The "Experimental" wine was one of three wines obtaining significantly higher scores on all attributes, and also obtained the best scores for flavour. Although this was a preliminary trial with a panel of limited size, it provided very positive evidence of the advantages of applying the pure mixed culture starter in the rice wine making.

**Future prospects for the application of granulated starters in rice wine fermentation**

Upgrading of traditional starter tablet technology for winemaking from purple glutinous rice could solve quality problems for local producers. This kind of rice wine has been strongly linked to people's culture since ancient times, especially in the Mekong Delta area. However, presently it is still manufactured at home-scale using solid-state starters in tablet form; the wine is prepared under non-sterile conditions. This practice produces low yields of wine of variable quality that can not be controlled.

The Biotechnology Research and Development Institute (BiRDI) of Can Tho University (CTU) is one of research units in the National Network organized by the Vietnamese Ministry of Science, Technology and Environment. In line with one of the essential general objectives of the Government policy for improving the socio-economic conditions in Vietnam, the present research was part of the BiRDI research programme, related to food.

The formulated starter granule preparation that is established based on results obtained in this study will be applied and improved further. Findings of the present thesis can be employed in teaching and research in BiRDI and CTU, especially in courses within the Food Technology specialization. Informative data of the study can be effectively employed for some current research topics in BiRDI, such as a study of mould and yeast biodiversity and functionality in starters for alcoholic fermentation, viability studies on mycelial fungi and their spores used in solid-state fermentations, enzymology and physiology of enzyme-producing micro-organisms. The ideas for further studies mentioned

in this thesis can be worked out by MSc. or BSc. students as part of the requirements for obtaining their degrees.

To obtain a first confirmation about whether our starter technology would be adequate for transfer to starter manufactures, we produced our "Experimental" wine at laboratory scale, as described in Chapter 7 which obtained a very positive sensory evaluation. BiRDI has regular contacts with leading local producers of starters and rice wine. This makes it a convenient means to exchange ideas and transfer scientific findings to the entrepreneurs. The home-scale production units are accessible for further trials to test new products. Future marketing of new (starter) products will be of benefit to rice wine producers and the BiRDI alike.

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# Summary

In the Mekong Delta region in Vietnam, purple glutinous rice wine (*Ruou Nep Than*) is well-known and particularly interesting because of its sherry-like taste and flavour, and its attractive brown-red colour. It is presently manufactured at home-scale using solid-state starters in tablet form; the wine is prepared under non-sterile conditions. This practice produces low yields of wine of variable quality. Whereas it is recognized that the choice of starter influences the yield and quality of wine, there is no knowledge of the relationship between their microbiological composition and their performance as starters in which fungi (mycelial fungi and yeasts) are the essential functional microflora. Therefore, the present thesis addresses the problem of poor and variable quality of traditional starter tablets by understanding and quantifying the impact of microflora in these starters, concentrating on mycelial fungi and yeasts and by assessing the option of preparing stable mixed cultures of selected compatible strains.

During preliminary field work, processes for the manufacture of starter tablets and rice wine were observed, and 29 representative samples of Vietnamese commercial starch-based rice wine starters were collected from leading producers in the Mekong Delta area. In Chapter 2 these starters were screened for their ability to liquefy cooked purple glutinous rice (*Oryza sativa* var. *glutinosa*), and to produce high alcohol levels. A limited number of six rice wine starters were selected for their significantly higher ethanol accumulation, superior ability to liquefy cooked rice, and attractive flavour and colour of the resulting wine. Ethanol contents reached 12% w/v, a sweet alcoholic fragrance was noticed and the colour varied from red to lightly brown. Total mould, yeast and bacteria counts were 3.4 - 6.0, 5.8 - 7.2 and 2.6 - 6.2 log CFU/g of dry weight sample, respectively. A total of 119 strains, comprising 53 moulds, 51 yeasts and 15 bacteria, was isolated.

In Chapter 3 pure isolates of moulds are examined for the role of the enzymatically active mycelial fungi in the starch conversion, and optimum conditions for their function. During the liquefaction and saccharification process - one of the two essential stages in the alcoholic fermentation of Vietnamese purple glutinous rice wine - the starchy materials are degraded into sugars, mainly glucose. Eight of 53 moulds isolated from Vietnamese rice wine fermentation starters were selected for their ability to degrade starch, and were studied further for their glucose production and amyloglucosidase activities during the saccharification of purple glutinous rice. Isolates were identified as *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (an atypical form of *A. rouxii*), *Rhizopus oligosporus* and *Rhizopus oryzae*. *Amylomyces rouxii* was able to produce up to 25% (w/w) glucose during saccharification with an amyloglucosidase activity up to 0.6 U/g of fermented moulded mass. Based on statistical analysis, optimum conditions for saccharification were defined as follows: incubation temperature 34ºC, incubation time 2 days and inoculation level 5 Log spores/g of steamed rice.

The other stage is that in which alcohol is produced from sugars by yeasts. Yeasts belonging to the species *Saccharomyces cerevisiae* are predominant in numerous traditional fermented beverages. In Chapter 4, the characteristic pattern of an alcoholic fermentation performed by yeasts, and their potential of alcohol production, their ethanol tolerance as well as the optimum conditions for their activity were investigated. Of 51 yeasts isolated from Vietnamese rice wine starters, five selected strains from a group of forty-five superior alcohol producers were characterized as *Saccharomyces cerevisiae* whereas two representative isolates of poor ethanol producers were identified as *Candida glabrata* and *Pichia anomala*. The *S. cerevisiae* strains were studied further for their fermentation rate and ethanol tolerance. They performed successfully in the alcohol fermentation with complete depletion of a relative high initial percentage of glucose in which 8.8% (w/v) ethanol was produced from the initial reducing sugars at 20% (w/v). Fermentative capacity of *S. cerevisiae* was limited at ethanol concentration of 14% (w/v) in a fed-batch test that was carried out under conditions of unlimited glucose supply. The yeast growth was completely inhibited by ethanol concentrations of 9 to 10% (w/v) in challenge tests with added ethanol. Based on statistical analysis, optimum conditions for the fermentation were as follows: incubation temperature 28.3ºC, incubation time 4 days and inoculation level 5.5 Log cells/ml of saccharified rice liquid.

Chapter 5 addresses the interactions and the compatibility of selected moulds and yeasts as a prerequisite for the formulation of mixed starters. Three moulds including *Rhizopus oryzae*, *Amylomyces* aff. *rouxii* and *Amylomyces rouxii,* and one yeast *Saccharomyces cerevisae* were examined individually and in a combination of mixed cultures. There were no combinations showing mutual inhibition; all strains tested are mutually compatible and can be used for the design of starters. In this chapter oriental herbs that are commonly applied as supplementary ingredients in starter production were also studied. Ten representative oriental herbs were studied for their effects on the growth of moulds and yeasts in view of the future production of a defined experimental rice wine starter. The herbs "Tieu Hoi" (Fennel; *Foeniculum vulgare* Miller) and "Dinh Huong" (Clove; *Syzygium aromaticum* L.) were observed to stimulate the yeast and mould growth. These herbs are also appreciated as herbal medicines.

For the successful manufacture of purple glutinous rice wine it is essential to avail of a defined starter culture. Chapter 6 describes the development of a laboratory-scale process to formulate a shelf-stable mixed - pure culture starter. The process was based on traditional starter manufacturing methods and modified where appropriate as determined by optimization experiments. The mixing ratio of flours, i.e. 80% rice flour and 20% cassava flour to be used as raw ingredients, the decontamination of the flour mixture by dry heating at 100ºC, the moisture content of dough of 30% and the incubation period of 24 hours were conditions obtained though experimentation that were adopted as part of the process. Granulation of fermented starter dough, followed by drying at 45ºC achieved rapid dehydration and maximum survival of starter micoorganisms. The effects of storage of starter granules on the viability of mould and yeast, and their performance in winemaking were also examined. Mould viability and its enzymatic activity were found to be quite stable during a 3 months storage period. Yeast activity is most likely the limiting

factor for shelf-life and stability of starter. The dry matter content of the granules is one of the factors influencing the shelf-life of the starter.

The wine produced with experimental dehydrated defined mixed-culture starter of the mould *Amylomyces rouxii* and the yeast *Saccharomyces cerevisae* was compared with commercially available rice wines (Chapter 7). The wines were judged according to Vietnamese standard 3215-79 by a panel consisting of 20 experts. The "Experimental", "Bien Hoa" and "Can Tho" wines received scores for colour, clearness, flavour and taste that were significantly higher than those for "Go Den" wine, mainly because of their attractive natural colour, pleasant alcoholic flavour as well as the specific sherry-like taste of purple glutinous rice wine. The "Go Den" wine was considered unacceptable because of its artificial colour and excessive alcohol content; however, it could still be safely offered for sale. The "Experimental" wine scored very well on flavour. An acceptability assessment supported the evaluation results, and indicated that some practices, such as the use of artificial colour and increasing the alcohol content by addition of distilled rice alcohol, were not appreciated by panellists.

The know-how of preparing and application of defined mixed-culture starter granules should be transferred to starter manufacturers. Regular contacts with leading local producers of starters and rice wine will not only benefit the industrial development but will also give the much-needed feed-back to improve and update academic teaching and research activities.

# Samenvatting

*Ruou Nep Than* (RNT) is een rijstwijn bereid uit paarse kleefrijst, en is populair in het Mekong deltagebied van Vietnam vanwege zijn sherry-achtige smaak en geur, en aantrekkelijke bruinrode kleur. RNT wordt kleinschalig gemaakt door middel van een vastestoffermentatie waarvoor starterpillen (*Men*) worden gebruikt als enststof; de wijnproductie vindt onder niet-steriele omstandigheden plaats. De traditionele methode levert lage wijnopbrengsten van wisselende kwaliteit. Hoewel het uit ervaring bekend is dat de keuze van de starterpillen van invloed is op de opbrengst en kwaliteit van de wijn, is er weinig bekend over het verband tussen de wijnkwaliteit en de microbiologische samenstelling van de pillen, waarin fungi (schimmels en gisten) de belangrijkste microflora zijn. Daarom richt dit proefschrift zich op het probleem van slechte en onvoorspelbare kwaliteit van traditionele starterpillen, teneinde de invloed van de microflora van deze starters te begrijpen en kwantificeren. Speciale aandacht gaat uit naar de schimmels en gisten, en de mogelijkheid om stabiele gedefinieerde mengcultures te maken van geselecteerde verenigbare stammen.

Gedurende eerder uitgevoerd praktijkonderzoek werden RNT bereidingsprocessen beschreven en 29 representatieve monsters van commerciële Vietnamese zetmeelbevattende rijstwijnstarterpillen verkregen van vooraanstaande producenten in de Mekong delta. In hoofdstuk 2 worden deze starters vergeleken m.b.t. hun vermogen om gekookte paarse kleefrijst (*Oryza sativa* var. *glutinosa*) te vervloeien, en hoge alcoholgehaltes te vormen. Een zestal starterpillen werd uiteindelijk geselecteerd op grond van hun alcoholproductie, vervloeiing van gekookte rijst en aantrekkelijke geur en kleur van de wijn. Ethanolgehalten bereikten 12% m/v, een zoet alcoholische geur en een kleur tussen rood en lichtbruin werden verkregen. Het totaalgehalte aan schimmelsporen, gisten en bacteriën bevond zich respectievelijk tussen 3,4 – 6,0, 5,8 – 7,2 en 2,6 – 6,2 log kve/g starter drogestof. Uit deze starters werden 53 schimmel-, 51 gist- en 15 bacteriestammen geïsoleerd.

Hoofdstuk 3 behandelt de eigenschappen van de schimmelreincultures, vooral hun enzymatische omzetting van zetmeel en de hiervoor optimale omstandigheden. Tijdens de vervloeiing en versuikering – één van de twee essentiële stadia in de bereiding van Vietnamese RNT rijstwijn – wordt het rijstzetmeel afgebroken, voornamelijk tot glucose. Acht van de 53 schimmels uit de starters werden geselecteerd op grond van hun zetmeelafbrekend vermogen en ze werden verder onderzocht m.b.t. hun glucoseproductie en amyloglucosidaseactiviteit tijdens de versuikering van paarse kleefrijst. De reincultures werden geïdentificeerd als *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (een atypische vorm van *A. rouxii*), *Rhizopus oligosporus* en *Rhizopus oryzae*. *Amylomyces rouxii* produceerde tot 25% m/m glucose tijdens versuikering, met een amyloglucosidaseactiviteit tot 0,6 U/g gefermenteerde schimmelrijst. Statistische analyse leverde de volgende optimumomstandigheden voor versuikering: bebroedingstemperatuur 34ºC, fermentatietijd 2 dagen en beëntingsniveau van 5 log kve/g gestoomde rijst.

Het tweede essentiële stadium is de omzetting door gisten van suikers in alcohol. Gisten van het soort *Saccharomyces cerevisiae* komen veelvuldig voor in traditionele gefermenteerde dranken. Hoofdstuk 4 beschrijft het onderzoek m.b.t. de alcoholische fermentatie door de gisten, hun alcoholproducerend vermogen, hun ethanoltolerantie, en de optimum omstandigheden voor hun activiteit. Van de 51 giststammen die eerder werden geïsoleerd uit starterpillen bleken 45 zeer goede alcoholvormers te zijn. Vijf representatieve stammen uit deze groep werden allen geïdentificeerd als *Saccharomyces cerevisae*. Twee representanten van de overige slecht-tot-matig alcoholvormende gisten werden geïdentificeerd als *Candida glabrata* en *Pichia anomala*. De *S. cerevisiae* stammen werden onderzocht m.b.t. hun fermentatiesnelheid en ethanoltolerantie. Deze waren in staat tot een volledige omzetting van een beginconcentratie van 20% m/v glucose onder vorming van 8,8% m/v ethanol. Het fermenterend vermogen van *S. cerevisiae* werd geremd nadat 14% m/v ethanol was gevormd in een "fed-batch" fermentatie experiment, waarbij glucose niet limiterend was. In challengeproeven met toegevoegde ethanol werd de groei van *S. cerevisiae* volledig geremd bij 9 tot 10% m/v ethanolconcentratie. Statistische analyse leverde als optimumomstandigheden voor de fermentatie de volgende combinatie: bebroedings-temperatuur 28,3 ℃, fermentatietijd 4 dagen en beëntingsniveau 5,5 log gistcellen per ml versuikerde rijstvloeistof.

Hoofdstuk 5 betreft de interacties en compatibiliteit van geselecteerde schimmels en gisten ter voorbereiding van het maken van mengcultuurstarters. Drie schimmelstammen, *Rhizopus oryzae*, *Amylomyces* aff. *rouxii* en *Amylomyces rouxii*, en één giststam *Saccharomyces cerevisae* werden afzonderlijk en in mengsels getest. In geen van de combinaties trad onderlinge remming op, d.w.z. de stammen waren onderling compatibel en konden worden gebruikt voor de samenstelling van mengcultures. Tevens werden in dit hoofdstuk een aantal oosterse kruiden onderzocht die vaak als ingrediënt worden toegevoegd in de fabricage van starterpillen. Tien representatieve oosterse kruiden werden getest m.b.t. hun invloed op de groei van de genoemde schimmels en gist met het oog op de productie van experimentele rijstwijnstarter op basis van gedefinieerde mengcultures. De kruiden "Tieu Hoi" (Venkel; *Foeniculum vulgare* Miller) en "Dinh Huong" (Kruidnagel; *Syzygium aromaticum* L.) bleken de groei van de gist en schimmels te bevorderen. Deze kruiden worden overigens ook gebruikt in de Vietnamese kruidenapotheek.

Voor een goede en betrouwbare bereiding van RNT wijn is een gedefinieerde starter onontbeerlijk. Hoofdstuk 6 beschrijft de ontwikkeling van een houdbare meng-reincultuur starter, gefabriceerd op laboratoriumschaal. Het proces was geïnspireerd door traditionele starterbereidingsprocessen, met aanpassingen op grond van de eerder vermelde optimumomstandigheden. De mengverhouding van 80% rijstmeel en 20% cassavemeel als grondstof, de decontaminatie van dit mengsel door droge verhitting bij 100 ℃, het deegvochtgehalte van 30% en de bebroedingstijd van 24 uur vloeien voort uit eigen experimenten en zijn onderdeel van het experimentele proces. Granulering van het gefermenteerde starterdeeg gevolgd door drogen bij 45 ℃ leverde snelle droging op met maximale overleving van starter micro-organismen. Een bewaarproef werd uitgevoerd m.b.t. de overleving van schimmel en gist, gevolgd door een wijnbereiding om de kwaliteit

van de starter te beoordelen. De schimmel en bijbehorende enzymactiviteit konden een bewaartijd van 3 maanden goed doorstaan. Gistactiviteit was verminderd; dit is waarschijnlijk een factor die de houdbaarheid van de starter beperkt. Het vochtgehalte van de gedroogde starterkorrels is één van de factoren die de houdbaarheid bepalen.

De wijn geproduceerd met de experimentele gedroogde gedefinieerde starter met reincultures van de schimmel *Amylomyces rouxii* en de gist *Saccharomyces cerevisiae*, werd vergeleken met commercieel verkrijgbare rijstwijnen (Hoofdstuk 7). De wijnen werden beoordeeld volgens de Vietnamese standaardmethode 3215-79 door een panel van 20 personen die bekend waren met het product. De "experimentele", "Bien Hoa" en "Can Tho" wijnen werden significant beter beoordeeld op kleur, geur en smaak, dan "Go Den" wijn, voornamelijk dankzij hun aantrekkelijke natuurlijke kleur, aangename alcoholgeur en specifieke sherry-achtige smaak, eigen aan RNT wijn. De "Go Den" wijn werd als onacceptabel beoordeeld op grond van de kunstmatige kleur en het te hoge alcoholgehalte; deze wijn levert echter geen speciaal gevaar op, en mag dus wel verkocht worden. De geur van de "experimentele" wijn werd als zeer goed beoordeeld. Een acceptatieproef bevestigde de bovengenoemde kwaliteitsbeoordelingen, en gaf aan dat sommige locale praktijken zoals het gebruik van kunstmatige kleurstoffen en het verhogen van het alcoholgehalte door toevoeging van gedestilleerde rijstalcohol niet door het panel werden gewaardeerd.

De kennis m.b.t. de vervaardiging en toepassing van gedefinieerde mengcultuur starterkorrels dient te worden overgedragen naar producenten van rijstwijnstarters. Regelmatige contacten met vooraanstaande plaatselijke producenten van starters en rijstwijn zijn gunstig voor de industriële ontwikkeling en zullen ook de hoognodige terugkoppeling opleveren die nodig is om het academisch onderwijs en onderzoek actueel en relevant te houden.

# Tóm tắt

Ở Việt nam tại vùng Đồng bằng sông Cửu long, *Rượu Nếp Than* (RNT) là một thức uống có cồn rất phổ biến và đặc biệt được ưa thích do hương vị thơm ngon và màu đỏ đẹp hấp dẫn. RNT được sản xuất hoàn toàn thủ công sử dụng nguồn giống chủng là dạng bánh men làm rượu, và quá trình chuẩn bị thực hiện ở điều kiện không được vô trùng. Sự sản xuất này đã dẫn đến kết quả năng suất rượu thấp và chất lượng rượu không ổn định. Trong khi việc sử dụng nguồn giống chủng cho thấy ảnh hưởng chủ yếu đến năng suất và chất lượng rượu, thì những kiến thức về mối liên hệ giữa thành phần hệ vi sinh vật hiện diện trong bánh men làm rượu và sự hoạt động của chúng trong đó nấm mốc và nấm men có vai trò chủ yếu vẫn chưa được hiểu biết nhiều. Vì vậy, luận án này nhấn mạnh giải quyết vấn đề còn hạn chế về chất lượng kém và không ổn định của viên men làm rượu cổ truyền, bằng cách tìm hiểu và định lượng ảnh hưởng của hệ vi sinh vật hiện diện trong viên men rượu, đặc biệt tập trung nghiên cứu nấm mốc và nấm men, và bằng cách đánh giá qui trình chuẩn bị sản xuất tổ hợp giống ổn định gồm những dòng nấm mốc và nấm men thuần được chọn lọc do chúng có hoạt tính cao và có khả năng tương hợp với nhau.

Trong thời gian đi tìm hiểu thực tế, những qui trình sản xuất viên men làm rượu và sản xuất rượu lên men truyền thống từ gạo/nếp đã được quan sát tìm hiểu, và 29 loại viên men làm rượu của những địa điểm sản xuất tiêu biểu của vùng Đồng bằng sông Cửu long đã được thu thập. Trong Chương 2, những viên men này được sơ tuyển chọn lọc dựa vào khả năng làm đường hoá nếp than (*Oryza sativa* var. *glutinosa*) đã được nấu chín và khả năng sản xuất độ cồn cao. Kết quả có sáu loại viên men rượu được tuyển chọn bởi khả năng sản xuất cồn cao (khác biệt có ý nghĩa so với các loại viên men khác), khả năng đường hoá tốt và sản phẩm rượu có mùi và màu hấp dẫn. Rượu có nồng độ cồn đạt 12% w/v, có mùi thơm ngọt và màu nâu đỏ. Mật số tế bào nấm mốc, nấm men và vi khuẩn theo thứ tự là: 3.4 - 6.0, 5.8 - 7.2 và 2.6 - 6.2 log CFU/g trọng lượng khô của viên men. Tổng cộng có 119 dòng phân lập được thuần chủng bao gồm 53 dòng nấm mốc, 51 dòng nấm men và 15 dòng vi khuẩn.

Trong Chương 3, những dòng phân lập thuần chủng của nấm mốc được sử dụng để nghiên cứu về vai trò và hoạt tính enzim của nấm mốc trong quá trình biến đổi tinh bột, và điều kiện tối ưu hóa cho hoạt động của chúng. Trong suốt quá trình đường hóa – là một trong hai giai đoạn chủ yếu của quá trình lên men rượu – nguồn nguyên liệu tinh bột được phân giải tạo ra đường, chủ yếu là đường glucoz. Tám trong số 53 dòng nấm mốc được phân lập từ viên men rượu Việt nam đã được tuyển chọn bởi có hoạt tính phân giải tinh bột cao, và chúng được nghiên cứu chi tiết về khả năng sản xuất đường glucoz và hoạt tính amyloglucosidaz trong quá trình đường hóa từ nếp than. Những dòng nấm mốc tuyển chọn được định danh thuộc các loài sau: *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (một dạng biến đổi của *A. rouxii*), *Rhizopus oligosporus* và *Rhizopus oryzae*. Trong quá trình đường hóa *Amylomyces rouxii* có khả năng sản xuất gluco đạt đến nồng độ 25% (w/w) và hoạt

tính amyloglucosidaz đạt đến 0.6 U/g khối mốc ủ. Kết quả phân tích thống kê xác định điều kiện tối ưu hóa cho sự đường hóa của nấm mốc là: nhiệt độ ủ 34ºC, thời gian ủ 2 ngày và nồng độ giống chủng 5 Log bào tử /g nếp hấp.

Một giai đoạn chủ yếu khác trong quá trình lên men rượu là sự chuyển hoá từ đường thành cồn bởi nấm men. Sự hiện diện của nấm men thuộc loài *Saccharomyces cerevisiae* chiếm đa số trong phần lớn các loại thức uống lên men truyền thống. Trong Chương 4, tính chất đặc trưng của hoạt tính nấm men trong quá trình lên men rượu, khả năng sản xuất rượu và khả năng chịu đựng độ cồn cao cũng như điều kiện tối ưu hóa cho hoạt tính của nấm men đã được nghiên cứu. Từ 51 dòng nấm men được phân lập từ viên men rượu Việt nam, năm dòng nấm men đã được tuyển chọn từ nhóm gồm 45 dòng nấm men có khả năng sản xuất rượu cao và năm dòng nấm men này được định danh thuộc loài *Saccharomyces cerevisiae*. Hai dòng nấm men tiêu biểu khác thuộc nhóm gồm các dòng nấm men có khả năng sản xuất rượu thấp được định danh thuộc loài *Candida glabrata* và *Pichia anomala*. Năm dòng nấm men *S. cerevisiae* được nghiên cứu chi tiết về khả năng lên men rượu và tính chịu đựng độ cồn cao. Kết quả cho thấy quá trình lên men rượu thành công bởi hoạt động của các dòng nấm men này với sự tiêu thụ và biến đổi hoàn toàn lượng đường khử ban đầu ở nồng độ 20% (w/v) và sản xuất ra ethanol với nồng độ là 8.8% (w/v). Trong thí nghiệm thử quá trình lên men với điều kiện cung cấp bổ sung glucoz vào dung dịch đường trong suốt quá trình lên men, kết quả cho thấy khả năng lên men của *S. cerevisiae* bị giới hạn khi nồng độ ethanol đạt 14% (w/v). Trong thí nghiệm thử khả năng tính chịu đựng ethanol của nấm men, kết quả cho thấy sự sinh trưởng của nấm men hoàn toàn bị hạn chế khi ethanol đạt nồng độ 9-10% (w/v). Kết quả phân tích thống kê xác định điều kiện tối ưu hóa cho sự lên men của nấm men là: nhiệt độ ủ 28.3ºC, thời gian ủ 4 ngày và nồng độ giống chủng 5.5 Log tế bào /ml dung dịch đường khử.

Chương 5 nghiên cứu những tác dụng lẫn nhau và sự tương hợp với nhau của những dòng nấm mốc và nấm men được tuyển chọn vì đây là điều kiện cơ sở cần thiết phải có cho thí nghiệm sau về sự sản xuất ra nguồn giống chủng gồm tổ hợp nấm mốc và nấm men. Ba dòng nấm mốc gồm *Rhizopus oryzae*, *Amylomyces* aff. *rouxii* và *Amylomyces rouxii,* và một dòng nấm men *Saccharomyces cerevisae* được nghiên cứu từng dòng riêng lẽ và trong tổ hợp gồm nấm mốc và nấm men. Không có tổ hợp nào cho thấy sự hạn chế lẫn nhau, tất cả các dòng nấm mốc và nấm men thử nghiệm đều có thể hỗ tương lẫn nhau và được áp dụng để tạo thành nguồn giống. Trong chương này một số dược thảo được áp dụng phổ biến như là yếu tố bổ sung trong quá trình sản xuất viên men rượu cũng được nghiên cứu. Mười loại dược thảo tiêu biểu đã được nghiên cứu về sự ảnh hưởng của chúng đối với sự sinh trưởng của nấm mốc và nấm men với mục đích ứng dụng sau này cho sự sản xuất thử men làm rượu với nguồn giống đã được xác định. Kết quả cho thấy " Tiểu Hồi" (Fennel; *Foeniculum vulgare* Miller) và "Đinh Hương" (Clove; *Syzygium aromaticum* L.) có tác dụng kích thích sự sinh trưởng của nấm mốc và nấm men. Trong thực tế sản xuất những dược thảo này cũng được sử dụng phổ biến như là thành phần thuốc thảo mộc có tác dụng ức chế sự phát triển của các vi sinh vật không có ích.

Sử dụng được nguồn giống chủng gồm nấm mốc và nấm men xác định có hoạt tính cao là điều chủ yếu trong sự sản xuất thành công RNT. Chương 6 mô tả sự phát triển qui trình sản xuất ở mức độ phòng thí nghiệm tạo ra men làm rượu gồm tổ hợp giống thuần

chủng ổn định. Qui trình này dựa trên phương pháp cổ truyền sản xuất viên men rượu và được cải tiến ở một số bước thích hợp dựa vào những kết quả tối ưu đạt được trong các thử nghiệm. Tỷ lệ pha trộn bột gồm 80% bột gạo và 20% bột khoai mì được sử dụng như là nguồn nguyên liệu ban đầu, sự khử nhiễm hỗn hợp bột bằng cách sấy khô ở 100ºC, độ ẩm bột nhào 30% và thời gian ủ 24 giờ là những điều kiện đạt được từ sự thử nghiệm và được ứng dụng trong qui trình. Sự tạo thành hạt nhỏ từ khối bột nhào lên men và được sử lý bằng cách sấy khô ở 45ºC đạt được sự khử nước nhanh chóng và vẫn giữ được sự tồn tại tối đa của những vi sinh vật trong nguồn giống chủng. Ảnh hưởng của sự tồn trữ hạt men giống đối với mật số tồn tại của nấm mốc và nấm men, và hoạt tính của chúng trong sản xuất rượu đã được nghiên cứu. Kết quả cho thấy sự tồn tại và hoạt tính enzim của nấm mốc khá ổn định trong suốt thời gian 3 tháng tồn trữ. Hoạt tính của nấm men có thể là yếu tố hạn chế hơn hết đến tính bền vững và ổn định của men giống. Hàm lượng chất khô của hạt men giống là một trong những yếu tố ảnh hưởng đến tính bền vững của men giống.

RNT được sản xuất từ tổ hợp giống thuần thử nghiệm gồm nấm mốc *Amylomyces rouxii* và nấm men *Saccharomyces cerevisae* được đánh giá so sánh với RNT được bán trên thị trường (Chương 7). Một nhóm gồm 20 người tham gia qui trình đánh giá chất lượng rượu dựa theo tiêu chuẩn Việt nam 3215-79 với phương pháp đánh giá cho điểm. Các loại RNT "thử nghiệm", "Biên Hòa" và "Cần Thơ" đạt được số điểm về màu sắc, độ trong, mùi vị cao hơn có ý nghĩa so với số điểm của RNT "Gò Đen", chủ yếu là do màu sắc rượu đẹp tự nhiên, mùi hương cồn dễ chịu cũng như vị thơm ngọt đặc trưng của RNT. Rượu "Gò Đen" không đạt ở mức độ được chấp nhận bởi vì màu sắc không tự nhiên đặc trưng của sản phẩm và độ cồn dư thừa cao; tuy nhiên, rượu này vẫn được đánh giá thuộc ở mức độ an toàn để bán ra cho người tiêu thụ. Rượu "thử nghiệm" đạt được điểm cao về mùi hương. Kết quả đánh giá cảm quan về sự chấp nhận sản phẩm đã củng cố cho những kết quả ghi nhận vừa nêu trên, và cho thấy rằng một số tập quán như sử dụng màu hóa học và gia tăng nồng độ cồn trong rượu bằng cách bổ sung lượng cồn chưng cất cao độ thì không được ưa thích và đánh giá cao.

Những hiểu biết về qui trình chuẩn bị và ứng dụng men làm rượu với dạng hạt gồm tổ hợp nấm mốc và nấm men được xác định có hoạt tính cao cần nên được chuyển giao ra thực tiễn cho những người sản xuất men làm rượu. Sự hợp tác thường xuyên với các cơ sở địa phương sản xuất bánh men rượu và rượu gạo/rượu nếp không những rất có lợi trong vấn đề phát triển công nghiệp mà còn có được những ý kiến đóng góp và kinh nghiệm thực tiễn cần thiết nhằm mục đích cải tiến và hiện đại hóa thông tin trong lĩnh vực giảng dạy và nghiên cứu.

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# Curriculum Vitae

Ngo Thi Phuong DUNG was born on 27 October 1959 in Can Tho City, Vietnam. After primary, secondary and high school education in Can Tho, she attended Can Tho University (CTU), Department of Biology in 1977, and received her Bachelor degree in November 1981. After graduating from CTU she started working as a researcher and instructor in the Biotechnology Research and Development Institute (BiRDI), CTU. In 1993, supported by the VH-24 collaboration programme between CTU and WAU (Wageningen Agricultural University, The Netherlands), she had the first training abroad in microbiology research for five months in the Department of Microbiology, WAU. From 1996 to 1998, financially supported by the Ministry of Foreign Affairs (NUFFIC), The Hague, The Netherlands (MHO-7 project, phase 1), she completed her Master study in Biotechnology in WAU. In 2000 she attended a practical course on Food Fermentation at the Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University. In the framework of MHO-7 project, phase 2 she had a chance for further study and in 2001 she started the PhD sandwich project, which is described in this thesis. This research was carried out at the Laboratory of Food Microbiology, Wageningen University as well as at BiRDI in Vietnam. The field and laboratory work in Vietnam was also partly supported by a grant of the International Foundation for Science, Stockholm, Sweden (grant Number E/3322-1).

# Training and Supervision completed

| Activities | Place | Year | Period | Credit points |
|---|---|---|---|---|
| Attend the VLAG course FF | WU | 2000 | 3 weeks | 3 |
| Take the field work for PhD thesis | MD, VN | 2000 | 1 week | 1 |
| Prepare the research proposal | WU | 2001 | | 4 |
| Attend the didactical course | CTU | 2001 | 3 weeks | 3 |
| Take part in teaching the course FF as part of the MHO-7 program | CTU | 2001 | 2 weeks | 2 |
| Attend the workshop on Curriculum MSc-Biotechnology | BiRDI, CTU | 2002 | 1 day | 0.2 |
| Take part in teaching the course FF as part of the MHO-7 program | CTU | 2002 | 2 weeks | 2 |
| Attend the course "Introduction to food- and airborne fungi" | CBS, Utrecht | 2002 | 1 week | 1 |
| Take the international excursion "The Introduction Day" | WU | 2002 | 1 day | 0.2 |
| Attend VLAG course "Management of food safety and microbiological risks" | WU | 2002 | 1 week | 1 |
| Attend the course FE as part of the MHO-7 program | CTU | 2003 | 2 weeks | 2 |
| Take part in teaching the course FF as part of the MHO-7 program | CTU | 2003 | 2 weeks | 2 |
| Attend literature study program | BIBIOT, WU | 2004 | 1 day | 0.2 |
| Attend the course of detecting pathogens in food by molecular techniques | RIKILT | 2004 | 2 weeks | 2 |
| Attend two workshops of NUFFIC – MHO project | BiRDI & CTU | 2004 | 1 week | 1 |
| Attend the course of detecting pathogens in food by microbiological techniques | PI, HCMC | 2004 | 1 week | 1 |
| Total of credit points | | | | 25.6 |
| Other activities: Co-supervising 3 MSc-Biotechnology theses (2001 & 2002) and working as the 1st secretary of the MHO-7 program (2000-2004) | | | | |

Notes:

BIBIOT: Biotechnion Library, WU; BiRDI: Biotechnology Research and Development Institute; CBS: Centraalbureau voor Schimmelcultures; CTU: Can Tho University; FE: Food Enzymology; FF: Food Fermentation; MD: Mekong Delta; PI, HCMC: Pasteur Institute, Ho Chi Minh City; RIKILT: Institute of Food Safety, WUR; VN: Vietnam; WU: Wageningen University.

# Acknowledgements