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Application Prospects for the Innovation of Defined Fungal Starter in Rice Wine Fermentation

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Abstract: The feasibility of pilot-scale manufacture of defined fungal starter and its application in rice wine production from different local starchy materials were investigated. Starter consisting of *Amylomyces rouxii* and *Saccharomyces cerevisiae* gave high performance in winemaking when prepared in conditions of rice flour 80% and cassava flour 20% with 4 incubation days. The starter level at 20% was favourably employed for manufacture with the initial amount of 10 kg mixed-flours. Dry starter granules which were vacuum packed could adapt ambient temperature (approx. 28-32 ℃) during 8 months of storage. The defined starter performed as superior inoculum for winemaking from different agricultural starchy resources. The undesirable bacteria were found at approx. 2 Log CFU/g of dry starter. By morphology, biochemical and physiological growth and the genetic partial 16S analyses, three bacterial isolates were characterized as *Bacillus subtilis/amyloliquefaciens* which may contaminate food but not cause food poisoning and not considered as a human pathogen.

Key words: Defined starter, rice wine, alcoholic fermentation, pilot-scale manufacture, undesirable bacteria.

1. Introduction

The principle of rice wine manufacture consists of the saccharification of steamed rice starch by fungal enzymes and the simultaneous or subsequent alcoholic fermentation by yeasts from traditional starter tablets [1, 2]. The limited knowledge about the microbiological composition of traditional starters in relation to their performance in the fermentation poses an obstacle to industrial development, and thus the development of defined starters containing mixed pure cultures is a priority in food microbiology and technology research. Advantages of defined mixed starter cultures have been described in Refs. [3-5]. Rice wine starters are generally composed of essential and non-essential microorganisms, the presence of moulds and yeasts however is considered essential for this type of fermentation [2, 6].

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In Vietnam, each local producer may have a different way of starter production, depending on available ingredients and local custom and preferences. Following the same long-term research project, the recent studies [7-9] have addressed 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. These studies have successfully identified fungal cultures isolated from various commercial traditional rice wine starters in Vietnam and developed a laboratory-scale process to formulate defined mixed-culture starter granules that produced wine with superior flavours and overall acceptability.

Besides, the raw ingredients for starter manufacture contain a diversity of microorganisms, including spore-forming bacteria that could have survived the heat treatment, some of these genera have been reported as predominant bacteria in fermented cassava

dough [10]. Although a certain number of undesirable bacteria was found in fermented starter dough [7], they gave no clear competitive influences on the growth and the contribution of fungal cultures during the saccharification and the alcoholic fermentation process, and neither expect nor exclusively being interested in mesophillic bacteria, it is obviously useful to characterize these bacterial isolates, particularly for the safety before making a transfer of the new product into practice.

As a logical continuation with the ultimate aim for the application of experimental defined starter processing into practice, this project investigated the manufacture of defined starter granules at pilot-scale trial and their performance in winemaking from different agricultural starchy materials. Factors affecting the storage stability of dry starter and the purification and identification of undesirable bacteria were also included.

2. Materials and Methods

2.1 Culture and Preparation of Strains

The mould of *Amylomyces rouxii* (CBS 111757; LU 2043) and the yeast of *Saccharomyces cerevisiae* (LU 1250) were employed. These strains were isolated [9] from Vietnamese rice wine starters and selected [8] for their superior ability to degrade starch and accumulate alcohol, respectively.

The cultures were grown on slants of Malt Extract Agar (MEA) at 30 ℃ for 5 days (mould) or 2 days (yeast). A suspension of the growing microorganisms was made by adding 5 mL of sterile physiological salt solution (0.85% NaCl) to each slant. The biomass was scraped off the agar by means of inoculating wire.

2.2 Effects of Ingredients and Incubation Time on Starter Processing

A factorial design (2 factors at 3 levels) was used including ingredients (rice flour 100%, rice flour 80% + cassava flour 20%, rice flour 80% + maize flour 20%), and incubation time (3, 4 and 5 days). Each treatment had triplicates. By modifying the appropriate time for the laboratory-scale preparation of defined fungal starter granules [7], the processing was tested as follows: 300 g of ingredients that had been heated at 100 ℃ overnight were prepared in sterile polypropylene bag. The heated ingredients were cooled to 35-40 ℃ and the moisture content at 40% w/w was prepared with the sterile solution containing glucose 1%, lactose 1%, $(NH_4)_2SO_4$ 0.2%, $MgSO_4$ 0.05%, $CaSO_4$ 0.02%, KH_2PO_4 0.1%, acid glutamic 0.05%. A mould suspension 10% v/w of 10^6 spores/mL and a yeast suspension 1% v/w of 10^8 cells/mL were inoculated, mixed well and incubated at 30 ℃. The fermented starter dough was subsequently transformed into granules and dehydrated in a ventilated oven (FED 115, Binder, Germany) on perforated drying trays at 42 ℃ for 17 hours. The dry samples were used as inoculum in the winemaking for required analyses.

2.3 The Feasibility of Pilot-scale Starter Manufacture

As a pilot-scale testing trial the starter preparation was prepared with the initial amount of ingredients at 10 kg per batch. In this procedure, the most favourable conditions of ingredients and incubation time from the previous experiment (2.2) were selected for starter preparation. The final product of starter granules obtained in the starter manufacture was used as stock inoculum. The inoculum levels were tested at 10%, 20% and 30% w/w of ingredients. Each treatment had triplicates.

2.4 Storage Shelf-life of Dry Starter Granules

In the manufacture processing of starter, the final dry starter granules were vacuum packed in sterile polypropylene bags with 0.06 mm thickness using a "Vacupack plus" machine (Krups, type 380, CE, z260590, P.R.C.) and stored at ambient temperature (approx. 28-32 ℃). Analyses of the fungal performance in the winemaking were done after 0, 2, 4, 6 and 8 months of storage.

2.5 Purification and Identification of Bacterial Contaminants

In the production of mixed-culture starter granules, the final product was suspended with sterile physiological salt solution 0.85% and pour-plated into Plate Count Agar (PCA) supplemented with natamycin, incubated at 30 ℃ for 1-4 days, and colonies were isolated and purified on PCA. The colonies that appeared after incubation were counted, calculated as colony forming units (CFU) and expressed as Log CFU/g. Pure isolates of bacteria were stored on MEA. The identification activity was carried out with the support of CBS Institute Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), including following methods: morphological examination, anaerobic growth on TSA, assimilation of carbonate nitrogen compounds by using Biolog GP plates (STAG), and DNA extraction, amplification and sequencing by using DYE-ET terminator cycle sequencing (Amersham Biosciences). Sequence similarity searches were performed with the BLAST program as supported by the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

2.6 Assessment of Defined Starter Granules in the Winemaking Fermented from Different Agricultural Starchy Materials

The ability of defined starter and its validation in comparison with commercial starters were assessed in the winemaking from different agricultural starchy materials. There were two factors including starter inoculation (defined fungal starter, Hai Anh Quang starter and Phuc Hung starter) and starchy materials (purple glutinous rice, Phu Tan glutinous rice, Huong Lai rice and Huyet Rong rice). Each treatment had triplicates.

2.7 Analytical Methods

2.7.1 Assessment of Starter Performance in Winemaking

Fifty grams of agricultural starchy material 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. After soaking they were steamed in an autoclave for 1 hour at 100 ℃. The steamed rice paste was cooled to 30-40 ℃, then inoculated and mixed well with starter 2%. After solid-state fermentation during 3 days at 30 ℃, 70 mL of sterile water was added to the moulded mass to allow for submerged alcoholic fermentation during 4 days at 30 ℃ under anaerobic conditions by replacing the cotton plug with a water lock. Samples were harvested for required analyses.

2.7.2 Chemical Analyses

The pH was measured by a digital pH meter (Sartorius, PB-20, Germany). Total dissolved solids content (mainly sugar) of saccharified liquid was estimated by measuring ^{o}Brix with a manual refractometer (FG 103/113, Euromex Holland). Glucose contents were determined by glucose oxidase test kit (Megazyme, GLC 9/96). Total alcohol content was determined by the distillation method [11]. Biomass was determined by the dry matter method.

2.7.3 Statistical Analysis

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

3. Results and Discussion

3.1 Effects of Ingredients and Incubation Time on Defined Starter Processing

By simplifying the appropriate time for the laboratory-scale preparation of defined fungal starter granules [7], the present process was investigated with the aim to pave the way for transferring the know-how of preparation and application of experimental starter to pilot-scale manufactures into practice. As a cheap and suitable supplement for a good combination with rice flour ingredient that had been mainly employed in the production of alcoholic starters in Vietnam [6], cassava flour and maize flour were tested in turn in the conditions of different incubation time at 3, 4 and 5 days. In all treatments, the fermented starter dough was

obviously adhesive with the mycelium increased gradually during the incubation and fully spread up to 4 days of incubation. However, after 5 days of incubation the mycelium was vanished and the fermented dough became inadhesive. After dehydration the starter granules were harvested with the moisture content of 7.8%. They were employed as inoculums in the winemaking for the assessment of their ability. All starter granules achieved normal and successful wine fermentation from purple glutinous rice with adequate performance during the solid-state fermentation and alcoholic fermentation.

During the incubation, the pH identically rapidly decreased in all treatments and reach in a range of 4.6-5.0 after the alcoholic fermentation, as compared to a pH of 6.02 of the uninoculated control. This pH range was also indicative of the purity and ability of the fungal cultures, since in cultures contaminated with (acidifying) bacteria, the pH usually became considerably lower, in the range of pH 3.0-3.5. Table 1 showed the results of winemaking performance in defined starter granules of different ingredients and incubation time.

During three days of incubation for the saccharification stage, the mycelium appearance and the liquefaction were increasingly obvious in all treatments. The glucose production was also achieved, of which the treatment including rice flour 80% and cassava flour 20% with 4 days of incubation gave the significantly highest content of glucose (20.4% w/v). Actually, the glucose produced during the solid-state fermentation could reach more higher level in all treatments; however, the inoculum comprised mixed fungal pure cultures of the mould *Amylomyces rouxii* and the yeast *Saccharomyces cerevisiae*, therefore as soon as glucose produced by mould, it was consumed and fermented by yeast into ethanol. Concomitantly, in the alcoholic fermentation stage, the rapidly high fermentation rate was clearly observed after 1 day of incubation and the high alcohol contents in the final rice wine product were obtained. In addition, the species *Amylomyces rouxii* belonged to genera of the Zygomycetes and was confined to the order Mucorales, and the mould in this order could produce alcohol under low oxygen conditions [12]. It could support to explain for the rapid and strong fermentation signal at the first beginning time of the alcoholic fermentation.

For the assessment of the fermentative capacity, the treatment including rice flour 80% and cassava flour 20% with 4 days of incubation was also found to give the highest content of ethanol (15% v/v). To combine with the results of the defined starter performance in the saccharification process, this treatment was indicated as favourable conditions for the preparation

Table 1 Winemaking performance in defined starter granules of different ingredients and incubation time.

Ingredient	Incubation time (d)	Results of solid-state fermentation								Results of alcoholic fermentation					
		Mycelium appearance[1] (per d)			Saccharified liquid appearance[2] (per d)			Glucose		Fermentation rate[3] at different time (h)				Alcohol	
		1	2	3	1	2	3	% w/v	SD[4]	24	48	72	96	% v/v	SD
Rice flour 100%	3	+	++	+++	-[5]	+	++	18.18[6] bcd[7]	0.15	10	14	2	0	13 bc	1
	4	+	++	+++	-	++	+++	16.79 ef	0.19	15	16	2	1	13.67 ab	0.57
	5	+	++	+++	-	+	++	17.87 cd	0.71	11	14	1	1	11 d	1
Rice flour 80% + cassava flour 20%	3	+	++	+++	-	+	++	19.01 b	0.73	13	12	1	1	13.33 ab	1.15
	4	+	++	+++	-	++	+++	20.40 a	0.46	19	17	2	1	15 a	1
	5	+	++	+++	-	+	++	17.65 de	0.5	14	11	1	1	12.33 bcd	0.57
Rice flour 80% + maize flour 20%	3	+	++	+++	-	+	++	16.66 ef	0.6	13	15	1	1	12.33 bcd	1.52
	4	+	++	+++	-	++	+++	18.92 bc	1.14	18	15	2	1	14 ab	0
	5	+	++	+++	-	+	++	16.26 f	0.54	12	13	3	1	11.33 cd	1.52

[1] levels of mycelium appearance ranging from + (little) to +++ (very much); [2] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [3] number of gas discharges per 2 minutes; [4] standard deviation; [5] not detected; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level.

of mixed-culture starter granules. In practice, rice and cassava flours are popularly used as ingredients in the production of rice wine starters in Vietnam. Although the practical reasons for using rice and cassava are known [6], no published knowledge is available about the effect of mixing ratio. If only rice flour is used, the starter tablet becomes too compact and hard so that the moulds can grow only on its surface. On the other hand, if only cassava flour is used, the starter tablet becomes too soft and spongy, which is assumed to limit the growth of yeasts.

3.2 The Feasibility of Pilot-scale Starter Processing

In line of preparation for the feasible application of new defined fungal starter processing into practice, in this experiment, the starter production was investigated at a larger amount (10 kg per batch) than those prepared at laboratory-scale processing, by using the selected conditions including rice flour 80% and cassava flour 20% with 4 days of incubation from the previous experiment. The amount of ingredients was divided into smaller parts in which each of 1 kg of ingredients was prepared on a stainless steel tray with a size of 39 × 39 × 9 cm. Three levels of inoculum including 10%, 20% and 30% w/w of ingredients were examined. The starter produced in the previous experiment was employed as the stock inoculation source. The performance of fungal starter granules in the saccharification and the alcoholic fermentation process from purple glutinous rice is presented in Table 2. The incubation temperature was set at 30 ℃; however, the regional ambient temperature was in a range at approx. 28-32 ℃ that was favourable for the growth of moulds and yeasts [8].

Following the same principle of the conversion in the saccharification and the alcoholic fermentation as mentioned in previous experiments, the mixed-culture starter granules produced with a larger amount as pilot-scale trial also gave the similar successful performance in such conversion way.

The treatment with the inoculation level of 10% gave the low performance in saccharification and fermentative abilities in comparison with other two treatments and the difference of alcohol contents was statistically significant at the 95% confidence level. However, the alcohol contents in the treatments with 20% and 30% of inoculation levels gave no significant difference. It was indicated that the level of mixed-culture starter of 20% favourable to be used as stock inoculum for the preparation of starter at larger scale processing.

3.3 Effect of Storage of Dry Defined Starter Granules on Winemaking Performance

Dry starter granules were vacuum packed and stored in polypropylene bags at ambient temperature (approx. 28-32 ℃). The objective was to examine the practical available way of storage and to minimize the influence of the limiting factors for the shelf-life and stability of starter, expectedly to apply easily and effectively into the actual conditions in practice. Dry matter contents and the winemaking performance of defined starter

Table 2 Winemaking performance in defined starter granules of different inoculation levels.

Level of inoculation (% w/w)	Mycelium appearance[1] (per day)			Saccharified liquid appearance[2] (per day)			Fermentation rate[3] at different time (h)				Alcohol	
	1	2	3	1	2	3	24	48	72	96	% v/v	SD[4]
10	+	++	+++	-[5]	+	++	12[6]	6	2	0	11 b^{7}	1.7
20	+	++	+++	+	++	+++	14	8	1	1	14 a	1
30	+	++	+++	+	++	+++	18	9	2	1	14.33 a	1.15

[1] levels of mycelium appearance ranging from + (little) to +++ (very much); [2] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [3] number of gas discharges per 2 minutes; [4] standard deviation; [5] not detected; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level.

Table 3 Effect of storage of dry defined starter granules on their moisture contents and performance in alcoholic fermentation.

Storage period (month)	Moisture content (%)	pH after fermentation	Alcohol	
			% v/v	SD[1]
0	7.8[2]	5	15.33 a[3]	0.57
2	8.5	5.1	13.33 b	1.15
4	9.59	5.2	13 bc	0
6	11.42	4.8	12 bc	2
8	11.38	5.2	11.33 c	0.57

[1] standard deviation; [2] values are means of triplicates; [3] means with different subscripts are statistically significant at the 95% confidence level.

granules before and after the storage are reported in Table 3.

During 8 months of storage at ambient temperature, the moisture levels of defined starter granules had gradually increased. This shows that the packaging may have no good moisture barrier function; however, the difficulty of protection of starter granules against such uncontrolled high ambient humidity and temperature levels is forecasted. It was found that after a certain period of storage, the higher moisture content the less performance of mixed-culture starter in the saccharification and alcoholic fermentation process. This finding is corresponding to the previous research report [7] in which the dry matter content is considerably noted as one of the factors influencing the shelf-life of the starter by effecting on the viability and activity of fungal cultures present in the starter.

Alternatively, the alcohol contents of the final product in winemaking somehow were used as the main factor indicating the assessment of the ability of mixed-culture starter during the storage. The results showed that the alcohol content significantly decreased in the treatment of 2 months of storage, as compared to the alcohol content of 15.3% v/v in the treatment before storage; however, there was no significant difference of alcohol contents in treatments during the storage periods from 2 months until 8 months of storage, in which a certain quite high level of alcohol was still obtained (11.3-13.3% v/v). This was obvious to indicate that, although the stability of mixed-culture starter granules had some challenge during the long storage of 8 months at ambient conditions, they could adapt somehow for their stable performance in winemaking. The pH values after the fermentation were in an identical range of 4.8-5.2 that should be supported for a normal successful alcoholic fermentation. Further trial will be needed into the storage stability of starter granules performed in local rice wine manufactures in order to have a proper recommendation of expiry use of starter granules when they are applied in practice.

3.4 Purification and Identification of Bacterial Contaminants

In the defined fungal starter processing, 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. A certain number of undesirable bacteria was found at approx. 2 Log CFU/g in the final defined fungal starter granules. On the other hand, although some contamination with undesirable bacteria occurred, mould and yeast still grew very well and dominated during the incubation as they performed highly their abilities in the alcoholic fermentation process. It was obvious these undesirable bacteria gave no competitive influences on the growth and the contribution of fungal cultures. However, although neither expect nor exclusively interested in mesophillic bacteria, it is necessary to characterize these bacterial isolates, particularly for the safety before making a transfer of the new product into practice, and this is the aim of this study.

Three bacterial isolates were obtained and named as BN06-31A, BN06-32 and BN06-33. They were characterized on the basis of their morphology, biochemical and physiological growth properties as well as the genetic partial 16S analysis. The results of the genetic analysis of three bacterial isolates were described in Fig. 1. The three bacterial isolates

belonged to the species named *Bacillus subtilis/amyloliquefaciens*. It meant that it was either *B. subtilis* or *B. amyloliquefaciens*. These two species of *B. subtilis* or *B. amyloliquefaciens*. were so much alike (100% identical) that was not possible to distinguish them with the genetic partial 16S results. Also, in the APICHB/20E test and the Biolog GP plate (STAG) test, the results could not differentiate these two species. There were no more tests to discriminate between *B. subtilis* and *B. amyloliquefaciens*. These bacteria might contaminate food but not cause food poisoning and they are not considered as a human pathogen. Particularly, some species of *Bacillus* can be commercially employed for the production of fermented foods, such as *Bacillus natto* in *natto* processing (Japan), *cheonggukjang* (Korea).

3.5 Assessment of Defined Starter Granules in the Winemaking Fermented from Different Agricultural Starchy Materials

In the context of the aim to pave the way for applying the experimental superior fungal starter into practice, in this experiment, the ability of defined starter was tested in the winemaking from different available starchy materials in the region and its validation in comparison with commercial starters were assessed in the winemaking.

Three kinds of starters including defined mixed-culture starter, Hai Anh Quang starter and Phuc Hung starter and four different starchy materials including purple glutinous rice, Phu Tan glutinous rice, Huong Lai rice and Huyet Rong rice were assessed and compared. The results of winemaking performance in defined starter granules and commercial starters of four different starchy materials were presented in Table 4.

During the saccharification stage and the alcoholic fermentation process, the results showed that, in generally, all testing starters similarly performed followed the normal principle in the winemaking from all different kinds of starchy resources. During the incubation in the saccharification process the mycelium appearance and the liquefaction were increasingly obvious, as well as the total high saccharified sugar levels (21-32 °Brix) were obtained. In the alcoholic fermentation stage, the rapidly high fermentation rate was observed after 1 day of incubation, then increased after 2 days and almost no more fermentation signal was observed after 4 days in all treatments.

By assessing the alcoholic contents of the final wine as the main factor indicating the starter ability, the results showed that in the treatments of commercial starter inoculation the alcohol contents differently varied depending on the kinds of starchy materials

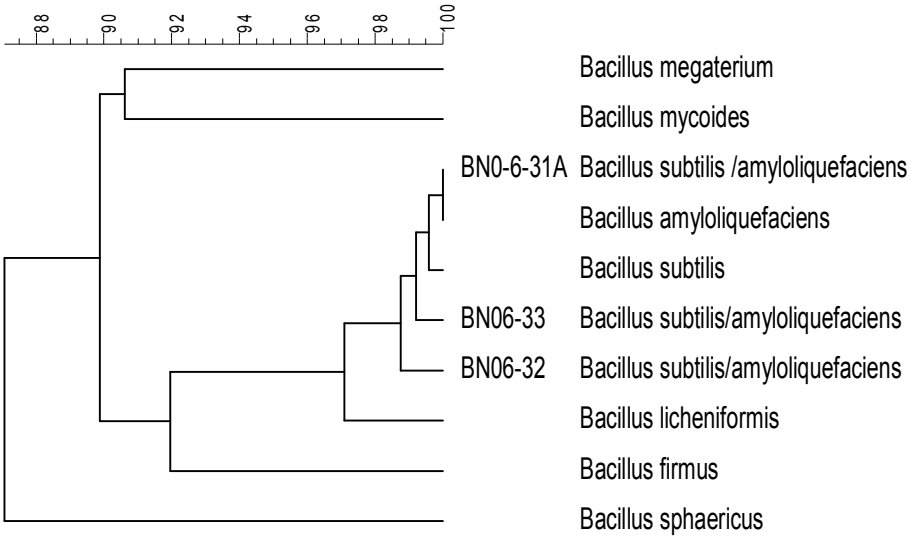

Fig. 1 The genetic analysis of bacterial isolates BN06-31A, BN06-32 and BN06-33.

Table 4 Winemaking performance in defined starter granules and commercial starters of different starchy materials.

Starters	Starchy materials	Saccharified liquid appearance in mould fermentation (per day)[1]			Fermentation rate[2] at different time (h)				Alcohol	
		1	2	3	24	48	72	96	% v/v	SD[3]
D[4]	PGR[5]	+	++	+++	35[6]	14	2	1	14.33 ab[7]	0.57
HAQ[8]	PGR	+	++	+++	28	12	1	0	11 d	0
PH[9]	PGR	+	++	++	24	14	2	1	10.67 d	0.57
D	PT[10]	-[11]	+	++	25	15	4	3	13.33 bc	1.52
HAQ	PT	+	++	++	21	13	2	0	13.67 bc	0.57
PH	PT	+	++	+++	34	10	10	4	14.33 ab	0.57
D	HL[12]	+	++	+++	35	16	2	2	15 a	0
HAQ	HL	+	+	++	26	15	2	2	13.67 bc	0.57
PH	HL	+	+	++	27	14	2	1	13.33 bc	0.57
D	HR[13]	-	+	++	32	13	2	1	14.33 ab	0.57
HAQ	HR	-	+	+	30	12	1	1	12.67 c	0.57
PH	HR	-	+	+	24	13	2	2	10.67 d	1.52

[1] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [2] number of gas discharges per 2 minutes; [3] standard deviation; [4] defined fungal starter; [5] purple glutinous rice; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level; [8] Hai Anh Quang starter; [9] Phuc Hung starter; [10] Phu Tan glutinous rice; [11] not detected; [12] Huong Lai rice; [13] Huyet Rong rice.

employed, whereas the high levels of alcohol contents were stably achieved in all treatments of defined starter performance. This can indicate that the experimental mixed-culture fungal starter granules could be able to be employed effectively as the inoculum for the rice wine fermentation from different kinds of available agricultural starchy materials in the region. The defined starter also received the highest performance for its function during the alcoholic fermentation process, as compared to this of other commercial starters.

4. Conclusion

The mould of *Amylomyces rouxii* (CBS 111757; LU 2043) and the yeast of *Saccharomyces cerevisiae* (LU 1250) were successfully applied as the inoculation source for the defined starter processing. As a logical continuation of previous findings with the ultimate aim for the application of experimental defined starter processing into practice, a pilot-scale testing trial of the starter preparation was prepared following the selected favourable conditions of mixed ingredients, incubation time and inoculum level. During 8 months of storage at ambient temperature (approx. 28-32 ℃) dry starter granules could adapt for their stable performance in winemaking. As the undesirable bacteria, Bacillus subtilis/amyloliquefaciens were found at small amount and gave no competitive effect to the performance of mixed-culture fungal starter granules. The defined starter also performed effectively in winemaking from different agricultural starchy resources. However, in spite of the successful ability for winemaking in testing trials, the defined starter granules should also performed well in the actual conditions of rice wine production at local manufactures. It will therefore be necessary to supply a stock inoculum of defined starter granules to lead local manufactures for further testing in the rice wine fermentation. This aspect is currently examined in another separate research.

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