Can Tho University Journal of Science
website: sj.ctu.edu.vn

PREPARATION OF FUNGAL ALCOHOLIC STARTER USING RICE MALT AS SUBSTRATE

Ngo Thi Phuong Dung, Nguyen Minh Doi and Huynh Xuan Phong
Biotechnology Research and Development Institute, Can Tho University, Vietnam

ARTICLE INFO

Received date: 09/08/2015
Accepted date: 26/11/2015

KEYWORDS

Amylomyces rouxii, fungal alcoholic starter, fermentation, rice malt, Saccharomyces cerevisiae

ABSTRACT

In this study, rice malt was used as the substrate to culture Saccharomyces cerevisiae for the preparation of defined fungal alcoholic starter. Defined mould starter using Amylomyces rouxii was prepared on the substrate of maize powder and rice husk. The different ratios of rice powder and rice malt were examined: A1 (rice malt only), A2 (1:0.5), A3 (1:1), A4 (1:2), and A5 (1:3). The results showed that the treatment of only rice malt gave the highest ethanol concentration (11.3% v/v) and the yeast population at 8.13 log CFU/g. The mould starter (8.74 log CFU/g) was added into the yeast starter at different ratios (0.5%, 1.0%, 1.5% and 2.0% w/w) for testing the fermentation capacity. Maximum ethanol concentration was 13.7% v/v when the defined fungal starter supplemented with 1% of mould starter and the incubation time for solid-state fermentation was 2 days, and 4 days for alcoholic fermentation stage. The yield of pilot-scale production (8 kg of rice) was found at 1.23 liters of rice white distilled spirit (ethanol concentration was 30% v/v) per one kilogram of rice. Aldehyde concentration of final distilled product was 33.8 mg/L and the quality score was obtained at 14.2/20 by sensory evaluation. This research was indicated the promising application of defined fungal alcoholic starter in local rice white distilled spirit manufactures.

Cited as: Dung, N.T.P., Doi, N.M. and Phong, H.X., 2015. Preparation of fungal alcoholic starter using rice malt as substrate. Can Tho University Journal of Science. 1: 47-52.

1 INTRODUCTION

In Vietnamese, rice white distilled spirit processing, alcoholic fermentation starter, called "*men*", plays a very important role because it regulates the yield of fermentation and the quality of the products. Some common mould strains in alcoholic starters are *Amylomyces rouxii*, *Rhizopus* spp., *Mucor* spp., *Aspergillus* spp.; and the popular yeast strains include *Saccharomyces cerevisiae, Hansenula* spp., *Endomycopis* spp. (Steinkraus, 1997; Nout and Aidoo, 2002). Using undefined alcoholic starters may lead to negative effects on fermentation yield and quality of rice white distilled products. In the recent research (Dung and Phong, 2011), the defined starter consisting of *A. rouxii* and *S. cerevisiae* has been found to give high yield and stable performance in winemaking from different agricultural starchy resources. *S. cerevisiae* has high ethanol production and stable fermentation capacity under many stresses, and food safety properties. The three main groups of microorganisms, namely mycelial fungi, yeasts and bacteria, are associated in the performance of traditional starters (Hesseltine *et al.*, 1988; Lim, 1991). The role of the mycelial fungi and yeasts receive major attention as they are considered crucial to starch degradation and alcoholic fermentation. Recently, the study of alcoholic starters with amylase enzyme supplement is also locally interested due to

the promising finding of their easy to use, stable fermentation and high yield of final product (Lan, 2011). The aim of this study is to produce the defined fungal alcoholic starter from *A. rouxii* and *S. cerevisiae* using rice malt as substrate.

2 MATERIALS AND METHODS

2.1 Materials and cultures

– Microbiological media: Potato Glucose Agar (PGA, potato (20%), glucose (2%), agar (2%), $(NH_4)_2SO_4$ (1%)), Malt Extract Agar (MEA, malt extract 3%, peptone 0.5%, agar 1.5%; Oxoid, UK), Sabouraund (glucose 4%, peptone 10%, agar 2%; Merck, Germany), Czapek-Dox (sucrose 3%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05%, $FeSO_4$ 0.001%; Merck, Germany).

– Cultures: *Saccharomyces cerevisiae* (2.1, LU1250), *Amylomyces rouxii* (20.3, LU2043) (Food Biotechnology Laboratory, BiRDI, Can Tho University).

– Materials: rice IR 50404 and maize VN 25-99 were purchased from Binh Minh district, Vinh Long province.

2.2 Methods

2.2.1 Production of defined yeast starter using rice malt as substrate

Rice grains were washed, soaked, and incubated in wet sack for germination in ambient condition. After incubating 4 days, rice malts were dried at 42°C for 1 day and grinding because amylase enzyme had the highest activity after incubating 4 days (Dung *et al.*, 2012). *S. cerevisiae* was cultured in PGA medium for 24 hours at 30°C. Added 3 mL of physiological salt solution (0.85% w/v of NaCl, sterilized at 121°C for 15 minutes) into petri dish and collected 1 mL of yeast suspension into tube containing 9 mL of the same solution. Serial dilution of yeast suspension was prepared and yeast density determined by haemacytometer to get the yeast inoculum at 10^7 cells/mL.

Different ratios of rice powder and rice malt including A1 (rice malt only), A2 (1:0.5), A3 (1:1), A4 (1:2), and A5 (1:3) were used as substrates for culturing *S. cerevisiae*. Adding 80 mL of yeast suspension into 100 g of substrates, mineral solution was added for 40-50% (w/v) of moisture content. The dough-like mass was incubated at 30°C for 2 days. Then it was dried and grinded into granules. Yeast density in different treatments of defined yeast starter was determined by using plate counting method on Sabouraud dextrose agar.

Screening for the fermentation capacity of yeast starters: Rice was prepared as follows: 50 g of rice and 60 mL of distilled water in a 250-mL flask covered by cotton plug were soaked for 4 hours. After soaking, soft rice was steamed in autoclave for 1 hour at 100°C. Steamed rice was cooled to 35-40°C and inoculated with 0.5 g of defined yeast starters. The inoculum was mixed well and incubated at 30°C for 7 days with air-lock flasks. Ethanol concentration, Brix and pH were determined.

2.2.2 Production of defined mould starter using Amylomyces rouxii

A. rouxii was grown for 5 days at 30°C on Malt extract agar (MEA, Oxoid, UK). Spore suspension was prepared by adding 5 mL of physiological salt solution (0.85% w/v of NaCl, sterilized at 121°C for 15 minutes) into petri dish. The spores were scraped off the agar by an inoculation loop.

Substrate of defined mould starter production was prepared as follows: One hundred grams of maize powder and rice husk (10% w/w) was dried overnight at 100°C in 500 mL flask and then added 50 mL of mineral solution ($(NH_4)_2SO_4$ 0.2%; KH_2PO_4 0.1%; $MgSO_4$ 0.05%; $CaSO_4$ 0.02%). The mixture was mixed well, soaked for 1 hour, sterilized at 100°C for 1 hour, and cooled to 35-40°C. Spore suspension was added into the substrate and mixed well. After incubation in 4 days at 30°C, inoculated mass was dried at 42°C for 17 hours, then grinded into granules for further experiments (Tai, 2006). Mould density of defined mould starter was determined using plate counting method on Czapek-Dox agar (Dung, 2004).

Determination of saccharification capacity of mould starter: Fifty grams of rice were soaked in 60 mL of distilled water in a 250 mL flask (covered by cotton plug) for 4 hours and steamed in autoclave for 1 hour at 100°C. Steamed rice was cooled to 35-40°C and inoculated 1 mL of mould inoculum (10^6 spores/mL). The inoculated mixture was incubated at 30°C for 3 days. The total dissolved solids (ºBrix) of fermenting liquid was determined by manual refractometer (FG102/112, Euroes-Holland) and the volume was measured after centrifuged the whole saccharified rice at 6,000 rpm in 10 minutes.

2.2.3 Evaluate the ratios between mould starter and yeast starter

Defined fungal starter was produced by mixing defined yeast starter and defined mould starter. There were 4 levels of defined mould starter sup-

plemented into defined yeast starter: 0.5%, 1%, 1.5% and 2% (w/w). Steamed rice was cooled to 35-40°C, then inoculated with different treatments of defined fungal starter at the same inoculated ratio (1% w/w) and mixed well. Ethanol concentration, Brix and pH were measured after 7 days of incubation at 30°C.

2.2.4 Effect of the incubation time for solid-state fermentation and alcoholic fermentation

A factorial design (2 factors at 4 levels) was used: time for solid-state fermentation (1, 2, 3 and 4 days), time for alcoholic fermentation (3, 4, 5 and 6 days). Each treatment had triplicates. The favorable ratio of mould supplement from the previous experiment was applied for the fermentation testing.

2.2.5 Production of rice wine at pilot scale

The experiments were carried out with eight kilograms of rice at Biotechnology Research and Development Institute (BiRDI) and at the local manufacture in Binh Minh district, Vinh Long province. Defined fungal alcoholic starter was applied with the favorable conditions established from the previous experiments.

2.2.6 Analytical methods and statistical analysis

The pH was measured with a digital pH meter (PB-20, Sartorius, Germany). Total dissolved solids content of saccharified liquid (°Brix) was estimated by manual refractometer (FG102/112, Euro,es-Holland). Alcoholic content was determined using the distillation methods (So and Thuan, 1991). Final product of rice wine was chemically analyzed to determine ester, acid, aldehyde, methanol and furfural by using the method of Vietnam National Standard 7043:2002. Sensory of rice wine was evaluated by using the Vietnamese standard 3217:79. Experimental data were statistically analyzed using Statgraphics Centurion XV, Manugistics Inc., USA.

3 RESULTS AND DISCUSSION

3.1 Fermentation capacity of defined yeast starters

Yeast densities of all defined yeast starters were in a range of 8.13-9.26 log CFU/g that was found to be higher in comparison with the standard of yeast density in alcoholic starter is 8 log CFU/g (Hien *et al.*, 2006). The result showed that the ethanol concentrations gradually increased with the increasing of rice malt as well as the yeast density in the starter combinations after 7 days of fermentation (Fig. 1).

Although the treatment A1 (0:1) had the lowest yeast density, ethanol content was the highest one because it had the highest content of rice malt. *S. cerevisiae* could not use starch molecule for fermentation, but it was able to use shorter molecules such as glucose, saccharose, fructose, maltose, raffinose, and galactose (Hien *et al.*, 2006). Amylase enzyme from rice malt broke the glycoside bond of starch molecule to form smaller molecules that yeast used for fermentation. Particularly, treatment A1 starter gave the highest ethanol concentration (11.3% v/v) with significant difference at 95% confidence level in comparison with other treatments.

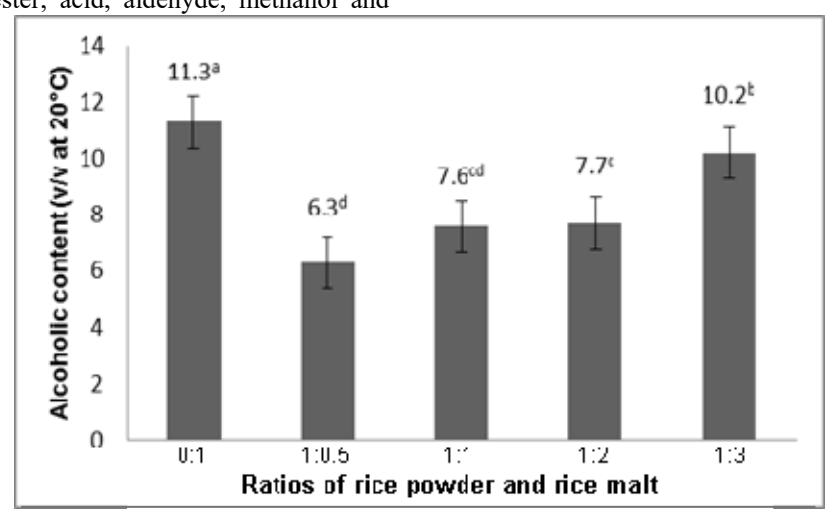

Fig. 1: Alcoholic contents from defined yeast starter

Note: Mean values of triplication with different subscripts are statistically different at the 95% confidence level

In the study of rice malt supplement into defined fungal alcoholic starter, the suitable ratio between defined fungal alcoholic starter and rice malt was 1:3 and the ethanol concentration was 6.5% (v/v) (Lan, 2011). Therefore, using rice malt as substrate was more advantageous because of higher ethanol concentration.

3.2 Production of *Amylomyces rouxii* starter

A. rouxii mould starter was produced using maize powder and rice husk (10% w/w) as substrate. Maize powder was the starch source for mould growth and husk was used for making aerobic condition. Mould density reached 8.74 log CFU/g which was higher than those found in commercial alcoholic starters (3.4-6.0 log CFU/g) (Dung *et al*., 2007). Therefore, defined mould starter could be added into defined yeast starter for rice wine fermentation. The results of saccharification capacity indicated that the mycelium of mould developed and covered rice mass rapidly after incubating for 1 day. Volume and Brix value of liquid released from rice mass were 42.5 mL and 13°Brix, respectively. Therefore, mould starter could be supplemented into yeast starter for rice wine fermentation.

3.3 The ratios of mould starter supplement into yeast starter

Because defined mould starter had high mould density (8.74 log CFU/g), it was added into yeast starter with small ratios at 0.5%, 1.0%, 1.5% and 2.0% (w/w). The result was illustrated in Table 1. When the ratio of mould supplement was 1.0% (w/w), ethanol concentration was the highest one (12.7%, v/v) and differed from others significantly at 95% confidence level. It showed that mould starter supplement with 1.0% (w/w) ratio gave the highest function of fermentation.

Defined fungal alcoholic starter with rice malt supplement could give ethanol concentration at 12% v/v (Lan, 2011), so using rice malt as substrate was little better than supplementing rice malt into defined fungal alcoholic starter. The defined fungal alcoholic starter with 1.0% (w/w) mould supplement was continuously screened for determination of fermenting duration.

Table 1: The result of mould starter supplement testing

Ratio	Final Brix	Final pH	Ethanol concentration (%, v/v at 20°C)
0.5%	6.5	3.96	11.7^{b}
1.0%	6.5	3.91	12.7^{a}
1.5%	7.0	3.93	11.7^{b}
2.0%	7.5	3.94	11.5^{b}

Note: Mean values of triplications with different subscripts within a column are statistically different at the 95% confidence level

3.4 Duration for solid-state fermentation and alcoholic fermentation

To utilize the benefits of amylase enzyme in reducing fermenting duration, time for solid-state fermentation was 1, 2, 3, and 4 days; time for alcoholic fermenting was 3, 4, 5, and 6 days. As the results in Table 2, when solid-state fermentation time increased from 1 day to 3 days, alcoholic content also increased slightly. However, ethanol concentration was low if solid-state fermentation extended to 4 days (treatments 13 to 16). Besides, ethanol concentration was also decreased when alcoholic fermentation took place for 5 and 6 days. There were two main reasons of alcoholic content decreasing. Firstly, long solid-state fermentation made aerobic condition for contamination of aerobic microorganisms. Secondly, when fermentation finished, ethanol could be converted into acetic acid with the presence of acetic acid bacteria and oxygen (Hien *et al.*, 2006). Treatment number 6 was the most optimal one because it not only gave the highest concentration of ethanol (13.7% v/v) but also took less time (6 days). Therefore, incubation time for solid-state fermentation was 2 days and incubation time for alcoholic fermentation was 4 days.

In a study of Nhan (2010) on rice malt starter, the ethanol concentration from rice distilled products was obtained only 5.3-6.3% v/v. In another research by Lan (2011) on producing the alcoholic starter using rice malt as a source of enzyme amylase and the distilled final product was found at 14.7% (v/v) of ethanol, but the fermentation time was longer for 10 days.

Table 2: Correlation of solid-state fermentation and alcoholic fermentation to ethanol concentration

Treatment	Fermentation time (day)		Brix	pH	Ethanol concentration (%, v/v at 20°C)
	Solid-state fermentation	Alcoholic fermentation			
1	1	3	7.3	3.43	10.2^{g}
2	1	4	6.3	3.46	12.2^{de}
3	1	5	6.5	3.42	11.8^{ef}
4	1	6	6.5	4.41	11.5^{f}
5	2	3	7.0	3.48	11.7^{ef}
6	2	4	5.5	3.62	13.7^{a}
7	2	5	5.5	3.54	13.3^{ab}
8	2	6	6.0	3.58	12.7^{cd}
9	3	3	7.0	3.64	11.8^{ef}
10	3	4	5.5	3.69	13.2^{abc}
11	3	5	5.5	3.54	12.8^{bc}
12	3	6	6.0	3.68	12.7^{cd}
13	4	3	6.0	3.68	10.3^{g}
14	4	4	5.5	4.94	10.7^{g}
15	4	5	6.0	3.42	11.3^{f}
16	4	6	6.0	3.95	10.7^{g}

Note: Mean values of triplication with different subscripts within a column are statistically different at the 95% confidence level

3.5 Production of rice wine at laboratory scale

3.5.1 Chemical analysis of rice wine product

Rice white distilled spirit was analyzed for determining some substances as required in the Vietnam National Standard 7043:2002 including ester, acids, aldehyde, methanol, and furfural. Final products produced in BiRDI consisted of less concentration of undesirable substances (aldehyde, methanol, and furfural) than these produced in local manufacture (Table 3).

Defined fungal alcoholic starter gave less concentration of undesirable substances than commercial starter, especially; methanol concentration was accepted according to the Vietnam National Standard 7043:2009. Defined fungal alcoholic starter using rice malt as substrate gave rice wine with less aldehyde concentration than rice wine produced from defined fungal alcoholic starter with rice malt supplement (Lan, 2011). Aldehyde concentration is very important because it influences product safety, commercial alcoholic starter cannot overcome this problem. Therefore, defined fungal alcoholic starter was effective to reduce aldehyde concentration. However, when fermenting in local manufacture, defined fungal alcoholic starter give higher concentration of aldehyde. Although two distillation systems were batch mode, the distillation process at local manufacture was carried out quickly and temperature was not controlled during the distillation.

Table 3: Chemical analysis of rice wine products from BiRDI and local manufacture

Location	Substances concentration (mg/L)				
	Ester (ethyl acetate)	Acids	Aldehyde (acetaldehyde)	Methanol	Furfural
BiRDI	67.7	0.12	33.8	0.05	1.31
Local manufacture	135	0.78	135	0.08	1.99
Vietnam National Standard 7043:2002	≤200	-	≤50	≤0.1	-

Note: "-" Not determined

3.5.2 Sensory evaluation of rice wine product

Rice white distilled products from BiRDI and local manufacture were used for sensory evaluation by ten examiners. Final scores were calculated and analyzed following Vietnam National Standard 3217:79, the result was shown in Table 4. All rice wine products were acceptable by sensory evaluation. The products from defined fungal alcoholic

starter and commercial starter used by local manufacture had fair sensory when producing in local manufacture.

Comparing with defined fungal alcoholic starter with rice malt supplement, defined fungal alcoholic starter using rice malt as substrate gave less sensory when applying in BiRDI but higher sensory when applying in local manufacture. It proposed that we need to control the level of undesirable substances lower than standard (safety aspect) but maintain the special taste (sensory aspect) of traditional rice wine.

Table 4: Sensory evaluation of final product

Location	Clearness and colour		Smell		Taste		Final score	Quality ranking[3]
	(1)	(2)	(1)	(2)	(1)	(2)		
BiRDI	3.8	3.04	3.6	4.32	3.4	6.8	14.2	Medium
Manufacture	4.8	3.84	3.8	4.56	3.8	7.6	16.0	Fair

Note: [1]Average score without multiplying with significant value; [2]Average score with multiplying with weight factor (clearness and colour 0.8, smell 1.2, taste 2.0); [3]Vietnam National Standard 3217:79

4 CONCLUSIONS

Defined yeast starter *S. cerevisiae* using rice malt as substrate with 1% (w/w) of defined mould supplement gave high fermentation capacity after 2 days for solid-state fermentation and 4 days for alcoholic fermentation. The results of chemical analysis and sensory evaluation of final products indicated the promising application of defined fungal alcoholic starter in local rice white distilled spirit manufactures.

ACKNOWLEDGMENTS

This research was jointly sponsored by the Ministry of Science and Technology of Vietnam (contract nr. 09/2014/HĐ-NĐT); the Advanced Program in Biotechnology, Can Tho University; and the New Core to Core Program.

REFERENCES

Directorate for Standards, Metrology and Quality, 1979. Vietnam National Standard 3217:79. Liquors, Sensory evaluation. Ministry of Science and Technology. Ha Noi (in Vietnamese).

Directorate for Standards, Metrology and Quality, 2009. Vietnam National Standard 7043:2009. White spirit, Specification. Ministry of Science and Technology. Ha Noi (in Vietnamese).

Dung, N.T.P., 2004. Defined fungal starter granules for purple glutinous rice wine. Dissertation. Wageningen University. Wageningen.

Dung, N.T.P., Nhan, B.D., Phong, H.X., 2012. Production of alcoholic starter from *Saccharomyces cerevisiae* and amylase enzyme of rice malt. Can Tho University Journal of Science. 21a: 11-18 (in Vietnamese).

Dung, N.T.P., Phong, H.X., 2011. Application prospects for the innovation of defined fungal starter in rice wine fermentation. Journal of Life Sciences. 5 (4): 255-263.

Dung, N.T.P., Rombouts, F.M., Nout, M.J.R., 2007. Characteristics of some traditional starch - based rice wine fermentation starter (men). LWT/Food Science and Technology. 40: 130-135.

Hesseltine, C.W., Rogers, R., Winarno, F.G., 1988. Microbiological studies on amylolytic oriental fermentation starters. Mycopathologia. 101: 141-155.

Hien, N.T., Luong, N.D., Binh, G.T., 2006. The technology of production of monosodium glutamate and traditional fermented products. The University of Science and Technology. Ha Noi (in Vietnamese).

Lan, N.T.B., 2011. Study of defined alcoholic starter supplemented with enzyme of rice malt and its application in local rice wine manufacture. Master thesis. Can Tho University. Can Tho (in Vietnamese).

Lim, G., 1991. Indigenous fermented foods in South East Asia, ASEAN Food Journal. 6: 83-101.

Luong, N.D., 1998. Microbiological Technology: Production of fermented drinks.. Microbiological Technology. HCM University of Technology. HCM City (in Vietnamese).

Nhan, B.D., 2010. Production of alcoholic rice white spirit starter from indigenous yeasts and enzyme amaylase from rice malt. Bachelor thesis. Can Tho University. Can Tho (in Vietnamese).

Nout, M.J.R. and Aidoo, K.E., 2002. Asian fungal fermented food. In: Osiewacz, H.D. (Editor). The Mycota, Vol. X: Industrial Applications. Springer-Verlag, New York.

So, P.V., Thuan, B.N.T., 1991. Analysis of cereals and food. The Polytechnic University. Ha Noi, Vietnam (in Vietnamese).

Steinkraus, K.H., 1997. Classification of fermented foods: worldwide review of household fermentation techniques. Food Control. 8: 311-317.

Tai, H.V., 2006. Production of yeast and mould starters for rice wine fermentation. Master thesis. Can Tho University. Can Tho (in Vietnamese).