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Breeding an Amylolytic Yeast Strain for Alcoholic Beverage Production

Ming-Chung Cheng · Rei-Chu Chang · Der-Feng Dent · Pao-Chuan Hsieh

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Abstract A starch-utilizing, yeast-like fusant was successfully created from fused protoplasts of S*chizosaccharomyces pombe* and *Monascus anka*, and the feasibility of using this fusant as a new strain for alcoholic beverage development was reported. The new fusant utilized various carbon sources more efficiently than its parent cells did. Rice koji prepared separately by cultivating the fusant and its parental strains on rice was compared to explore the effect of yeast strain on the production of α-amylase, glucoamylase, and acid protease that are crucial in wine making using cereal grains. It was found that the fusant produced greater levels of the above-mentioned enzymes than its parental strain does. Consequently, the usage of this fusant in the alcoholic fermentation of polished rice was found to reduce approximately 50% consumption of added glucoamylase than when its parental strain was used. Besides, at the end of fermentation, the fusant yeast resulted in a mash with distribution of flavor components very different from that produced by its parental strains. Thus, the fusant can be used as a new yeast strain for creating novel alcoholic beverages.

Keywords Protoplast fusion · S*chizosaccharomyces pombe* · *Monascus anka* · Glucoamylase consumption reduction · Flavor components · Novel alcoholic beverages

Introduction

Chinese rice spirits are conventionally produced from rice grain by the Amylo process, in which the starchy raw materials have to go through the four steps of liquification, saccharification, fermentation, and distillation that are laborious and time-consuming. A new yeast strain with the capability of simultaneously performing saccharification and fermentation would simplify the brewing process and reduce production costs.

M.-C. Cheng (✉) · D.-F. Dent · P.-C. Hsieh
Department of Food Science, National Pingtung University of Science and Technology, No.1, Shuefu Road, Neipu, Pingtung, 912, Taiwan, Republic of China
e-mail: pea007@ms15.hinet.net

R.-C. Chang
Department of Food Science and Technology, Tajen University, No. 20, WeiShin Road, Yanpu, Pingtung, 907, Taiwan, Republic of China

Flavor components play important roles in alcoholic beverages, and are mostly generated from yeast metabolism [1, 2]. Therefore, a challenge for manufacturers of alcoholic beverages is to breed new strains to develop novel styles of alcoholic beverages in order to meet the demands of consumers [3]. Traditional techniques, such as screening, mutation, and hybridization for strain improvement, are not yet regulated under current laws governing genetically modified food. Many researchers found that they were able to neglect differences between the parental strain's species [4], mating types [5], and ploidy [6], to obtain interspecific [7] and intergeneric [8] protoplast fusions for strain improvement, and positive results were reported [9].

Monascus anka has been used for thousands of years in China as a strain for producing red koji, red rice wine, and other fermented food products. Steam-cooked non-glutinous rice is inoculated with *M. anka* to produce amylolytic enzymes. They are able to break down starch into simple fermentable sugars that can undergo fermentation to produce alcohol [10]. *Schizosaccharomyces pombe* was originally used in manufacturing millet beer; it can directly use simple sugars to produce alcohol, and functions to lower acidity during wine production [11]. In this study, we used the method of protoplast fusion to fuse *M. anka*, which has the capability of directly using starch for fermentation to produce alcohol, with *S. pombe*, which is unable to directly use starch for fermentation, to breed a yeast-like fusant that was able to directly use starch for alcohol production. When this fusant was stabilized, various properties were analyzed, including its appearance, its carbon and nitrogen source utilization, and its capabilities for fermentation and metabolism in producing enzymes. In addition, the feasibility of its application towards creating new styles of rice spirits was explored.

Materials and Methods

Strains and media *S. pombe* CCRC 21461, *Trichoderma viride* CCRC 32054, and *M. anka* CCRC 31540 were obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu City, Taiwan. Yeast malt (YM) broth contained 0.3% yeast extract, 2% peptone, 1.0% dextrose, and 0.3% malt. Starch broth consisted of 2% potato starch. The regeneration media consisted of 2% soluble starch, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 4.47% KCl, and 1.5% or 0.4% agar. The minimum essential broth [12] contained a 1% carbon source and 0.67% of yeast extracts, but no amino acids were added. The fermentation broth contained 10% glucose, 0.5% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$, and 0.1% $MgSO_4 \cdot 7H_2O$, at pH 6.0. The PEG-Ca^{2+} solution consisted of 40% polyethylene glycol (PEG) 4000, 0.6 M KCl, and 40 mM $CaCl_2$ in 10 mM Tri-HCl buffer, at pH 6.0. The above reagents were all purchased from Sigma Chemical (St. Louis, MO, USA). The malt extract wort was made by steeping malt in 60 °C water overnight and filtering it for further use. The reducing sugar content was 8.6%.

Preparation of inocula One loopful of 2-day-old slant culture of *S. pombe* was inoculated into 250 ml flasks containing 100 ml YM broth and shaken at 30 °C for 48 h at 100 rpm. The *S. pombe* culture was then reinoculated into YM broth at a rate of 5% (*v*/*v*) and shaken at 30 °C for 12 h at 100 rpm. One loopful of 5-day-old slant culture of *M. anka* was inoculated into 250 ml flasks containing 100 ml starch broth and shaken at 30 °C for 5 days at 100 rpm. The culture of *M. anka* was then reinoculated into starch broth at a rate of 5% (*v*/*v*) and shaken at 30 °C for 48 h at 100 rpm. Both cultures were separately centrifuged at

15,000×*g* for 20 min at 30 °C; and the pellets thus obtained were washed with sterile water and centrifuged again. The harvested *S. pombe* cells and *M. anka* mycelia were respectively resuspended in sterilized water for protoplast preparation.

Preparation of protoplast and protoplast fusion Protoplasts were prepared and fused from *S. pombe* and *M. anka* according to the methods of Lin et al. [13]. A cell wall-degrading enzyme prepared from *T. viride* cultured in 1:1 mixture of sawdust and barn, and extracted with 0.4 M KCl. *M. anka* and *S. pombe* cells, were incubated separately with the above-mentioned cell wall degrading enzyme at 40 °C for 40~60 min at 50 rpm. The isotonic stabilizer used was 0.5 M KCl. The rate of protoplast formation (%) was calculated by the protoplast formation divided by the total cells counted. The regeneration rate (%) of the protoplast was calculated by regrown cells divided by protoplasts selected under various controlled conditions [12].

The two types of protoplasts (*S. pombe* and *M. anka*) were mixed in a 0.5 M KCl solution at a ratio of 1: 1, and the mixed solution was centrifuged at 15,000×*g* for 10 min. The mixed protoplast was then harvested and diluted to 10^7~10^8 protoplasts/ml by PEG-Ca^{2+} solution, and it was then incubated at 30 °C for 10 min. The resulted mixture was centrifuged at 15,000×*g* for 10 min again, and the resulted pellets were harvested and resuspended in isotonic stabilizer to adjust the concentration of protoplasts at about 10^7~10^8/ml for the fused protoplast preparation[13].

The protoplast fusants were cultured using the sandwich-embedded method. Two milliliter of protoplast fusion preparation solution was added to a test tube containing 2 ml of 1.5% solidified regeneration medium with holes as the base of the sandwich, and then covered with 2 ml of melted regeneration liquid medium as the top of the sandwich, and the sandwich was incubated at 30 °C for 2~3 days. Both fused and regenerated cells grew and broke out of the top agar under anaerobic conditions. The fused cells were selected microscopically, transferred to a starch regeneration broth, and incubated at 30 °C with shaking at 100 rpm for 2~3 days. The cells were monitored daily by microscope, and then the cells were loop-transferred to a new regeneration agar and incubated at 30 °C for 1~2 days. The stable fusion cells of *S. pombe* and *M. anka*, called the fusant, was used to explore its ability to utilize starchy materials, and its ability in production of alcohol.

Utilization of carbon and nitrogen sources The fusants obtained were transferred to YM slants and stored at 4 °C. After 4, 8, 12, and 24 weeks of storage, the stability of the fusants was microscopically examined, and the selected fusants were transferred to minimum essential broth containing 2% starch to examine substrate utilization [13]. The properties of assimilation of the new fusants were determined by incubating the culture at 30 °C by shaking at 100 rpm in minimum essential media (MEM) containing various carbon and nitrogen sources. The progress of cell growth was monitored by measuring its optical density at 660 nm after 72 h of incubation.

The inoculation rates were 2% and 5% carbon sources selected from D, L-arabinose, cellobiose, cellulose, galactose, α-methyl D-glucoside, fructose, glucose, inositol, insulin, lactose, maltose, D-mannitol, melezitose, melibiose, potato starch, raffinose, L-rhamnose, ribose, L-sorbose, succinic acid, tartaric acid, salicin, trehalose, and xylose that replaced starch as the carbon source in the regeneration media. Various nitrogen sources, at 0.5% loading, were selected from beef extract, KNO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, and peptone that replaced the yeast extract in the regeneration broth.

The properties of fermentation were determined by incubation at 30 °C without shaking in MEM containing various carbon and nitrogen sources. A test with a Durham tube was

used as CO_2 production indicator of the properties of fermentation. After incubation under fermentation for 24, 48, and 72 h, if the quantity of CO_2 produced in the Durham tube was >2/3, then it was labeled "+++", "++", and "+", respectively, if the CO_2 produced was <2/3 after 72 h of fermentation, then it was labeled "−".

Determination of the fermentation properties using glucose, malt extract, and corn starch S. pombe and the fusant were separately inoculated at a rate of 6% into a 50 ml fermentation broth containing 10% glucose, malt extract, or corn starch in 250 ml Erlenmeyer flasks. Fermentation profiles were monitored by measuring the weight reduction of the mash [14]. A fermentation bung plug with concentrated sulfuric acid was inserted into the flask and incubated at 30 °C. The weight was measured every 24 h, and the flask was shaken periodically during incubation. The number of days required for the weight to stop decreasing was the number of days of fermentation. Using the weight reduction of the broth to estimate its fermentation ability, the rate of fermentation was calculated by dividing the reduction in weight of the broth by the total sugars in the fermentation broth for a theoretical value of the weight of CO_2.

Preparation of rice koji and crude enzyme The fusant and parental strains were grown on steamed rice at 30 °C for 5~6 days. Three grams of rice koji were sampled daily, and the enzymes in it were extracted with 30 ml of 10 mM acetate buffer (pH 5.0) containing 0.5% NaCl, at room temperature, for 3 h. After filtration (Toyo filter paper no. 2; Advantec Toyo, Tokyo, Japan), the filtrate was analyzed.

Determination of glucoamylase (EC3.2.1.3) Activity Glucoamylase activity of rice koji filtrate was determined according to the method of Goto et al. [15] with some modifications. The glucoamylase activity was measured in 0.1 M sodium acetate (pH 5.0) as the release amount of reducing sugars from 1% soluble starch (Sigma). One unit of enzymatic activity of glucoamylase was defined as the amount of enzyme required to produce 1 μM of reducing sugar per min. After incubation, the amount of reducing sugar was estimated by the dinitro-salicylic acid (DNSA) method [16].

Determination of acid protease activity The acid protease activity of rice koji filtrate was determined according to the method of Bhumiratana et al. [17] with some modifications. The generated amino acids were reacted with Folic–Ciocalteu's phenol reagent for a colorimetric reaction, and a standard curve was established using 20, 50, and 100 μg tyrosin/ml, then the OD_{660nm} was determined to represent the enzyme activity. 1 μg of tyrosine generated at 38 °C for 60 min was defined as one activity unit.

Determination of α-amylase (EC 3.2.1.1) activity The α-amylase (EC 3.2.1.1) activity of rice koji filtrate was assay by the modified method of Yamaguchi et al. [18]. Two milliliter of 0.5% soluble starch in 0.04 M phosphate buffer (pH 6.0) was mixed with 0.1 ml of the filtrate. After appropriate incubation at 40 °C, 0.2 ml aliquot of the reaction mixture was added to 5 ml of 2×10^{-4} M I_2-KI solution. The absorbance of the mixture was measured with a spectrophotometer at wavelength of 700 nm. Hydrolysis of 0.1 mg of soluble starch in 1 min was defined as one unit of the α-amylase activity.

Ethanol tolerance of yeast According to the methods of Asano et al. [14], 50 ml of fermentation broth containing 10% glucose was added to a 250-ml Erlenmeyer flask, and 95% alcohol was added to adjust the alcohol concentrations to 0%, 5%, 6%, 7%, 8%, 9%, 10%,

15%, and 20%. The broth was inoculated with 6% of the fusant and *S. pombe* that had been pre-incubated for 5 and 2 days, respectively, and a fermentation bung with concentrated sulfuric acid was inserted into the Erlenmeyer flask and incubated at 30 °C. The phenomenon of CO_2 loss resulting in weight reduction of the fermentation broth in the Erlenmeyer flask was used to calculate the rate of fermentation in order to assess the alcohol tolerance of the strains.

Fermentation test in Erlenmeyer flasks Polished rice grains (300 g) were individually added to a 4-l Erlenmeyer flask at a concentration of 15% (*v*/*v*), and 1% HCl (12 N) was added. After liquification at 121 °C for 20 min, the mixture was cooled to 60 °C. Glucoamylase at 0%, 0.02%, 0.05%, and 0.1% was added to the mash, and held at 60 °C for 60 min, then cooled to 35 °C for later use [19–21]. The glucoamylase used was Amano GNL-4200 (Amano Enzyme, Nagoyo, Japan) with an enzyme activity of 9,800 units/ml. The saccharifying mash was inoculated with 6% of the parental strains, or the fusant, and the broth was fermented at room temperature (28~32 °C) at 80 rpm, and samples were collected for analysis of alcohol content and residual sugar in the mash. The number of days of incubation required for the alcohol concentration of the mash to stabilize was recorded.

Analysis of mash and rice spirits The alcohol concentration of the mash was analyzed with a gas chromatograph-flame ionization detector (GC-FID) using a Hewlett Packard 5890 SeriesII system (Hewlett–Packard, Wilmington, DE, USA). The amount of reducing sugars was estimated by the DNSA method [16]. After the mash was distilled, the alcohol concentration of the distillate was adjusted to 30%. A 10-ml sample was collected, and 0.1 ml of 1% cyclohexanol was added as the internal standard, then the flavor components were analyzed by GC-FID (Agilent 6890). The chromatographic conditions were as follows: a Chrompack CP-Wax 57 C.B column (50 m×0.25 mm I.D., film thickness, 0.2 μm), split injection mode (1:5), an injection temperature of 220 °C, a column temperature of 70 °C for 5 min that was increased by 3.5 °C min^{-1} to 200 °C for 20 min, a carrier gas rate of 6.3 mL min^{-1} with an FID temperature of 250 °C, and an injection volume of 1 μL.

Results and Discussion

Protoplast Fusion and its Properties

Our observation indicated that our yeasts *S. pombe* and *M. anka* were young enough, and they formed protoplast easily in 12 h under the assistance of cell wall-degrading enzyme produced by *T. viride*. The two types of protoplasts underwent cell wall regeneration in YM media containing a 0.5 M KCl isotonic solution at 30 °C for 48 h, after which they were tested for regeneration using the protoplast with a 100% rate of protoplast formation. The protoplast regeneration rate ranged from 0.01~0.15%, averaging approximately 0.095%.

The two types of protoplasts were treated with PEG to induce fusion, making use of the characteristics of *S. pombe*, which cannot utilize starch, by using starch as the sole carbon source in the selective regeneration medium, in order to culture and screen the fusant for starch utilization and fermentation abilities. With the sandwich method, the mixed solution of the two protoplasts was poured onto the agar, placed in the solidified medium with 2% agar on the top and base, and incubated under anaerobic conditions at 30 °C for 72 h to obtain the fusant. After screening and subculturing, the fusants were grown on potato starch solidified media, the different profiles of the fusants were monitored, and fusants with starch utilization ability were selected.

Under microscopic observation, it was obvious that the morphology of the protoplasts was significantly differed from the original strains. The appearance of *S. pombe* had changed from long rod into a spherical shape (Fig. 1a, b), while *M. anka* had changed from mycelium-like (filamentous) into a spherical appearance (Fig. 1c, d). Both two protoplasts had a ball-like appearance and were smaller than their parental cells size (Fig. 1b, d), whereas all of the fusants had an ovoid-shaped appearance, and their sizes were smaller than the parental strains (Fig. 1e).

To examine the utilization of nutrients by the fusant, 25 different carbon sources were studied, and it was found that the fusants exhibited marked changes in their utilization and fermentation of carbon sources, of which the fusants could use 24 carbon sources, which was much more extensive than their parental strains that only use seven of the 25 carbon sources. For the fermentation of carbon sources, the fusants were also found to utilize 11 of the 25 carbon sources, while the parental strains could only use five (Table 1). In terms of nitrogen utilization, the fusant was found to grow far better in organic nitrogen source than

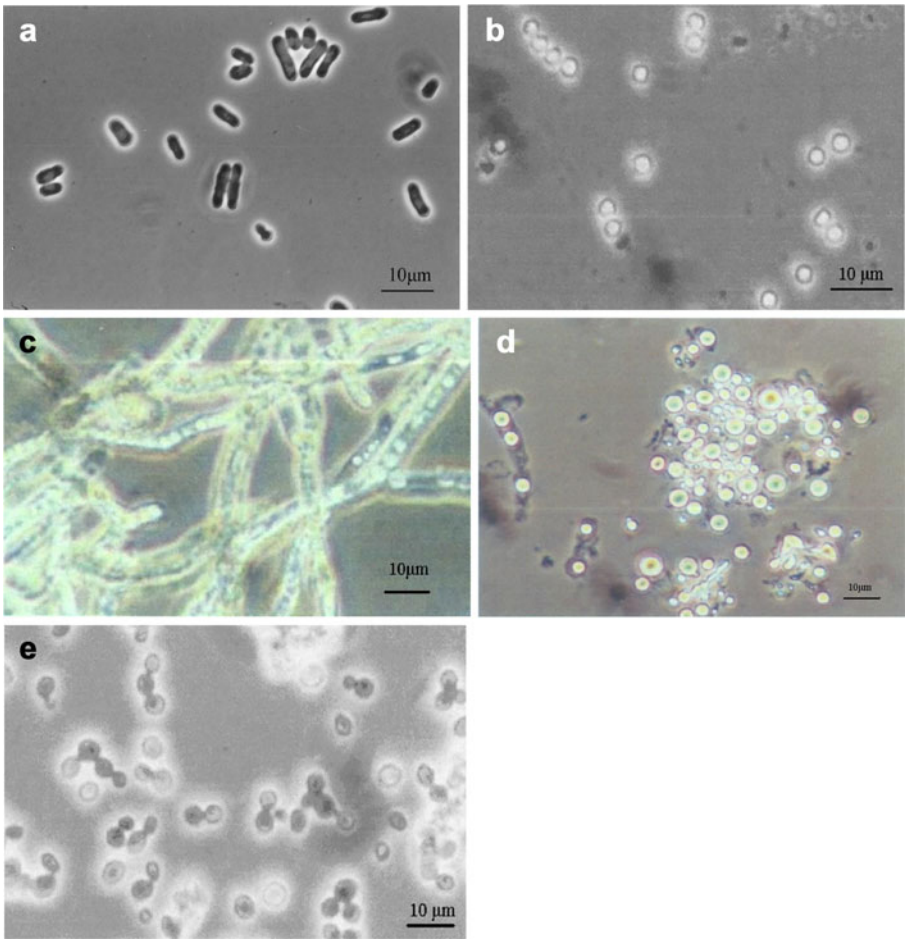

Fig. 1 **a** *S. pombe* CCRC21461, **b** protoplasts form from *S. pombe*, **c** *M. anka* CCRC31540, **d** protoplasts form from *M. anka*, and **e** fusant I formed from *S. pombe* and *M. anka*

inorganic nitrogen sources. In comparison, its parental strain *S. pombe* seem to grow almost equally well in both organic and inorganic nitrogen sources (Table 2).

Determination of the Fermentation Efficiency

Assessment of the fermentation capacities of the fusant and parental strains were separately performed on synthesized media with glucose and potato starch as the carbon

Table 1 Properties of assimilation and fermentation of the fusant and *S. pombe* in defined media containing a sole carbon source

Carbon source [a]	Assimilation		Fermentation	
	Fusant	*S. pombe*[b]	Fusant	*S. pombe*[c]
D-arabinose	+	−	−	−
L-arabinose	+	−	−	−
Cellobiose	+	−	−	−
Cellulose[d]	+++	−	+	−
Fructose	+++	++	+++	++
Galactose	+++	−	+++	−
Glucose	+++	+++	+++	+++
Inositol	+	−	+	−
Lactose	++	−	++	−
Maltose	+++	+++	+++	+++
D-Mannitol	+	−	−	−
Melezitose	+	−	++	−
Meliobiose	+	−	−	−
α-methyl D-glucoside	+++	+++	−	−
Potato starch	+++	−	++	−
Raffinose	+++	+	+++	+
L-Rhamnose	+++	++	−	−
Ribose	+	−	−	−
Salicin	−	−	−	−
L-sorbose	+	−	−	−
Succinic acid	++	−	+++	+++
Sucrose	+++	+++	−	−
Tartaric acid	+++	−	−	−
Trehalose	+	−	−	−
D-xylose	+	−		

[a] Medium composition (1 L): 20 g carbon source, 5.0 g yeast extract, 1.0 g KH_2PO4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 7H_2O$, and 0.1 g NaCl

[b] Determined after 72 h in shaking culture at 30 °C: $O.D._{660\,nm} \geq 1.0$, +++; $O.D._{660\,nm} \geqq 0.6$, ++; $O.D._{660\,nm} \geqq 0.2$, +; and $O.D._{660\,nm} < 0.2$, −

[c] Determined after 24 h in stationary culture at 30 °C, gas produced in a Durham tube over 2/3, +++; after 48 h, gas produced in Durham tube over 2/3, ++; after 72 h, gas produced in Durham tube over 2/3, +; after 72 h less than 2/3, −

[d] Carbon source was cellulose, it was not dissolved in medium so count cell, After 72 h in shaking culture at 30 °C, 5×10^5 cell/ml:+, 5×10^6 cell/ml:++, 5×10^7 ell/ml:+++, less than 5×10^5 cell/ml:−

Table 2 Various nitrogen source for the growth of the fusant and *Schizosaccharomyces. pombe*

Nitrogen source[a]	Fusant	*S. pombe*
$(NH_4)_2HPO_4$	–[b]	–
$(NH_4)_2SO_4$	+	++
KNO_3	–	++
NH_4NO_3	–	++
NH_4Cl	–	++
Peptone	–	+++
Yeast extract	+++	+++
Beef extract	+++	+++

[a] Medium composition (1 L): 20 g potato starch, 5.0 g nitrogen source, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 7H_2O$, and 0.1 g NaCl

[b] Determined after 72 h in shaking culture at 30 °C, $O.D._{660\,nm} \geq 1.0$, +++; $O.D._{660\,nm} \geqq 0.6$, ++; $O.D._{660\,nm} \geqq 0.2$, +; $O.D._{660\,nm} < 0.2$, −

sources, and on natural media with malt extract as the base. As shown in Fig. 2a, the fusant and parental strains had very rapid rates of fermentation on the glucose media, and fermentation was completed after only about 4 days. The calculated fermentation efficiency of the fusant and parental strains showed minor differences, at 93.68% and 94.09%, respectively. From Fig. 2b, the fusant and parental strains had very slow rates of fermentation on the fermentation media using potato starch as the carbon source; after 25 days of observation, until the weight of media had stopped decreasing, the calculated fermentation efficiencies were 56.03% and 0%. From Fig. 2c, for the media with extracted malt liquid, the strains was fermented for around 11 days and as the weight reduction of the media slowed, the calculated fermentation efficiencies were 74.86% and 82.64%, with the fusant performing better than the parental strains. However, in these three types of media, the growth lag phases of the fusant were all shorter than those of the parental strains.

Enzyme Activity Assays

Brewing of alcoholic beverages in oriental countries is performed by parallel complex fermentation using starchy materials. In this process, various kinds of enzymes in rice koji and wheat koji are used to dissolve and saccharify steamed rice, and then the yeast cells can be used to ferment the simple sugars into alcohol [22]. Among these enzymes, α-amylase, glucoamylase, and acid protease, which are able to participate in breaking down starchy materials into simple sugars and other components that yeast can use to ferment, are of great importance [23, 24]. The fusant and its parental strains were separately inoculated on steamed rice to make rice koji in order to investigate their performances in enzyme production. The activity of glucoamylase was not observed until day 2, and its activity peaked on day 6 at 165.0 units/g. The parental strain *S. pombe* had only trace activity, which was still close to 0 units/g on day 6, while the other parental strain *M. anka* had up to 907.6 units/g of activity on day 6 (Fig. 3a). As for the activity of α-amylase, although the activity at 2.66 units/g of the fusant, being markedly less than 22.69 units/g of *M. anka* (Fig. 3b), was still superior to that of the other parental strain *S. pombe* that had almost no enzyme activity. In terms of acid protease production, when

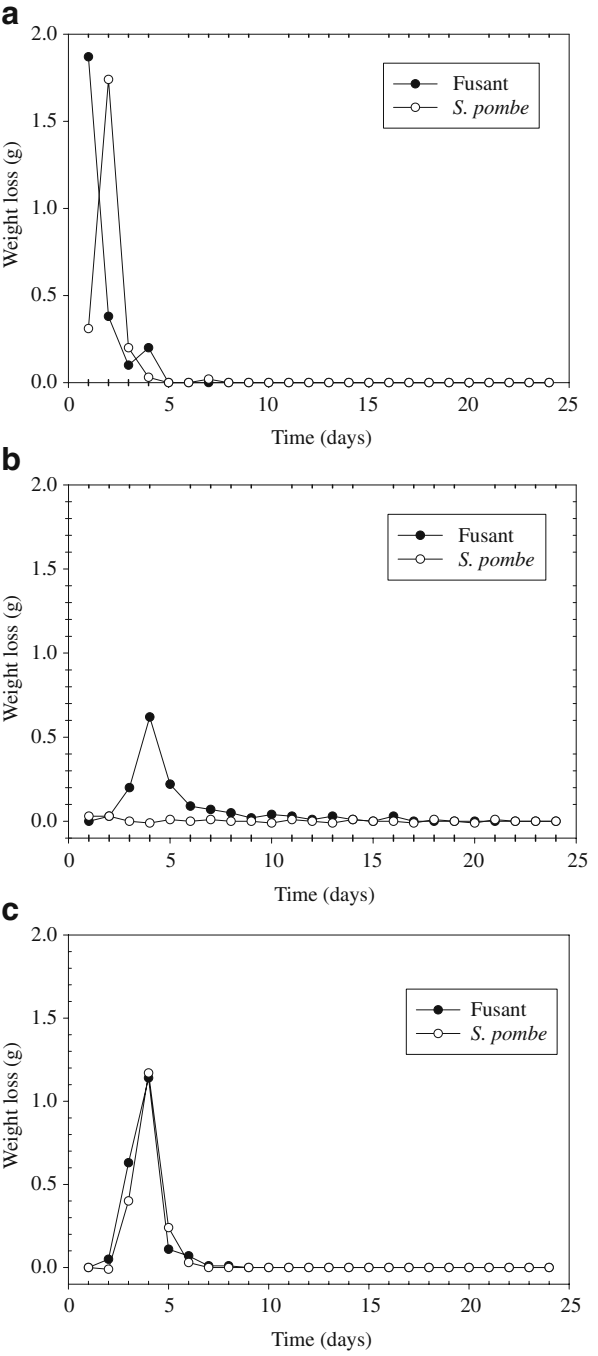

Fig. 2 Weight loss of *S. pombe* and the fusant in different media: glucose fermentation media (**a**), starch fermentation media (**b**), and malt extract wort (**c**)

incubated until day 6, the fusant produced acid protease at 682.75 units/g levels which was much higher than 38.23 units/g of its the parental strain *S. pombe*, but was slightly lower than 1,208.25 units/g of the other parental strain *M. anka* (Fig. 3c). Therefore, the three kinds of enzyme produced from the fusant are associated with the constituents of the

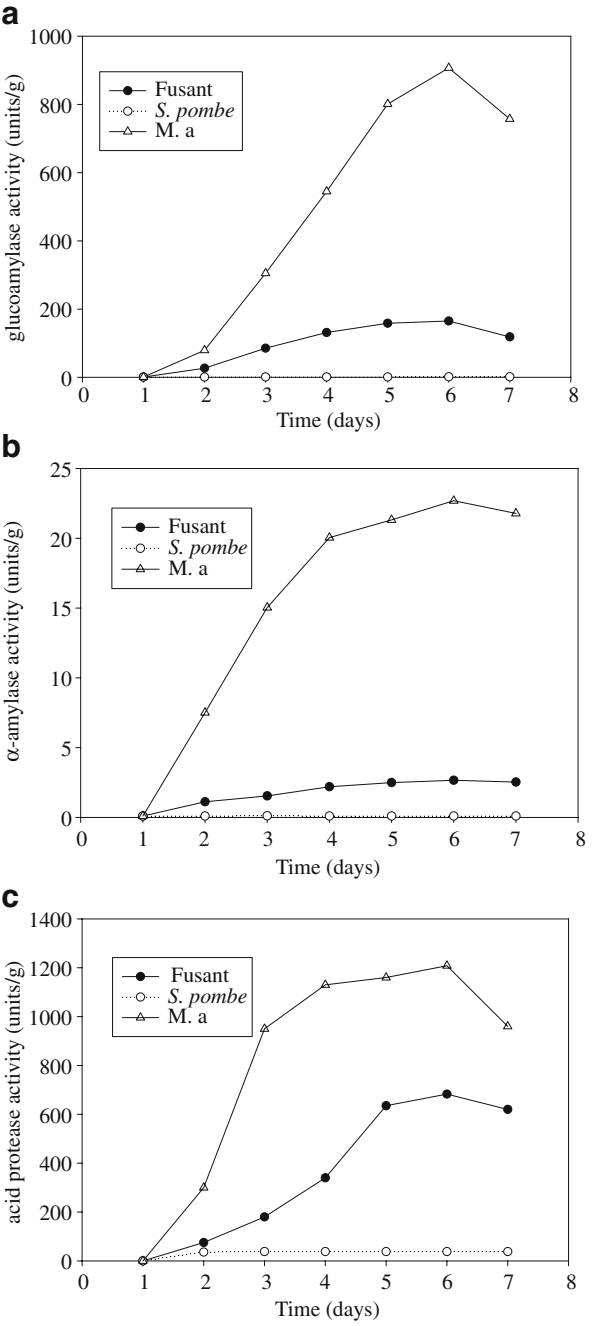

Fig. 3 Enzyme production profile during *S. pombe, M. a* and the fusant incubated in brown rice medium at 30 °C. **a** Glucoamylase, **b** α-amylase, and **c** acid protease

rice grain breakdown. As was reported by Imada et al. [7], the characteristics of interspecific fusant come from the distribution of the parental strains' traits, and frequently result in activity levels falling between those of the two parental strains, i.e., *M. anka* and *S. pombe*, in our case.

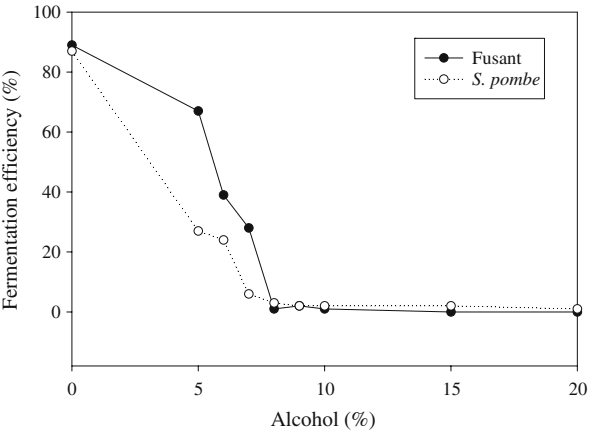

Fig. 4 Fermentation efficiency of *S. pombe* and the fusant under different alcohol concentration

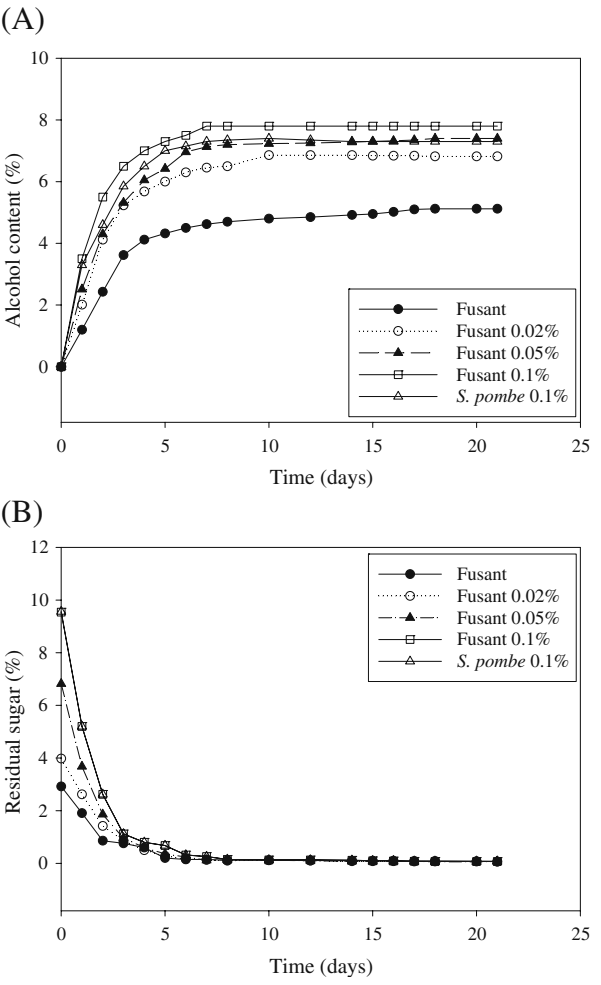

Fig. 5 Fermentation profiles of *S. pombe* and the fusant under different ratios of enzyme addition in rice mash. **a** Alcohol concentration and **b** residual sugar. Note that the profile of fusant with 0.1% glucoamylase superimposed with that of *S. pombe* with 0.1% glucoamylase

Ethanol Tolerance of the Yeast

To produce high concentrations of alcohol, the yeast strains must be able to survive in the presence of ethanol [25, 26]. To evaluate the ethanol tolerance of the fusant strain, we took advantage of the fact that yeast usually uses sugar for fermentation, and carbon dioxide constitutes the main carbon flux from sugars during alcoholic fermentation. By using the method of medium-weight reduction, the fermentation rate can thus be detected by the rate of carbon dioxide emissions [14]. Figure 4 presents the fermentation capacities of *S. pombe* and the fusant in the presence of different levels of alcohol in the media. In 7% alcohol, the fermentation capacity of the fusant was 27.6% which was better than the 2% fermentation capacity of its parental strain *S. pombe*.

Brewing Test of the Fusant and the Parental Strains

Steamed rice inoculated with the strains was used to conduct fermentation experiments, due to the direct utilization of rice for fermentation of the fusant. A clear trend was observed in the decrease of the saccharification time at high enzyme [27]. In order to assess the fusant's efficiency in rice fermentation, after liquifaction of rice with dilute acid, different proportions (0%, 0.02%, 0.05%, and 0.1%) of glucoamylase were added separately, and their fermentation profiles were compared to that of its parental strain *S. pombe* containing normal loading of 0.1% glucoamylase. As shown in Fig. 5, with an increase in the amount of the glucoamylase added, more sugar was released from the mash. In the batch with the addition of 0.1% glucoamylase, the sugar content in the mash reached >9.5% on day 1, and the process of alcohol fermentation occurred very fast. The fermentation was quite vigorous on days 2–4, and reached a peak on day 7. The alcohol generated by the fusant after the addition of 0.1% glucoamylase reached 7.8%, followed by 7.4% alcohol in the fusant with 0.05% glucoamylase, and 7.3% alcohol in the parental strain *S. pombe* with 0.1% glucoamylase. The next highest alcohol content 6.82% was fermented by the fusant with addition of 0.02% glucoamylase, whereas the worst alcohol production of 5.12% was seen in samples with no glucoamylase added. It was thus estimated that approximately 50% of the glucoamylase usage could be reduced if the parental strain *S. pombe* was replaced by

Table 3 Flavor contents (ppm) of rice spirits made from fermentation by *S. pombe* and the fusant

Flavor compound	*S. pombe*	Fusant
Acetalaldehyde	488	213
Ethyl acetate	333	33
Isoamylacetate	494	104
Isoamylalcohol	475	123
n-Propanol	15	15
1-Butanol	370	1,167
2-Methyl butanol	87	439
3-Methyl butanol	497	1,609
Ethyl lactate	69	161
Propanoic acid	182	149
Caproic acid	122	255
2-Phenyl ethanol	14	103

the fusant for polished rice fermentation. Judged from the observation that glucoamylase degraded gelatinized starch to simple sugar for yeasts fermentation, and our fusant cell displayed glucoamylase, α-amylase, and acid protease which may enhance the distribution of the other constituents in the starchy material-like rice grain. Similar situation have been found where the arming yeast cells displaying glucoamylase and α-amylase increased the surface contact between starch granules and yeast cells, and thus increased the alcohol production rate as reported by Khaw et al. [23].

The flavor components of the rice spirits that were fermented by the fusant were analyzed. From the results shown in Table 3, the contents of acetaldehyde and isoamyl alcohol, typically unpleasant components, were lower than those fermented by the parental strains. In terms of the primary aroma that was conventionally present in distilled rice spirits, the spirit fermented by the fusant yeast demonstrated a significant increase in 2-phenylethanol content representing rose fragrance, and ethyl lactate content indicating a frankincense fragrance. Besides, there were differences in the distributions of all flavor components between spirit fermented by the fusant and its parental strain. Therefore, we find it very feasible to utilize the fusant in new spirit development.

Conclusions

In this study, the difference between the parental strain and fusant was compared in terms of the shape of cell, their ability to utilize a variety of nutrients source for assimilation and fermentation, their fermentation efficiency, and efficiency of producing three kinds of enzyme-glucoamylase, α-amylase and acid protease for brewing. It was found that, through the method of protoplast fusion, a starch utilization yeast was successfully bred from *S. pombe* and *M. anka.* For the production of rice spirits, the fusant was found to reduce approximately 50% consumption of glucoamylase compared to its parent strain. After fermentation was completed, the liquor was analyzed for trace elements; it still retained the primary aroma of rice spirits, whereas there were differences in the distribution of the flavor components compared to the parental strains; therefore, the new strain bred from protoplast fusion can be applied to develop novel alcoholic products.

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