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Development of a defined starter culture mixture for the fermentation of *sato*, a Thai rice-based alcoholic beverage

Ampa Luangkhlaypho[a], Kobchai Pattaragulwanit[a], Natchanun Leepipatpiboon[b], Chulee Yompakdee[a,*]

[a] Department of Microbiology Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330 Thailand

[b] Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330 Thailand

*Corresponding author, e-mail: Chulee.y@chula.ac.th

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ABSTRACT: *Sato* is a traditional Thai alcohol beverage produced by the fermentation of steamed rice with a microbial starter. The industrial production of *sato* faces problems with inconsistent quality due to the variability in the microbial community of the starter. Previously, an NP1 starter was selected from 114 starter samples for its abilities to liquefy rice, to ferment and produce ethanol, and to give a good flavour and taste to the resultant *sato*. Here, we developed a defined starter culture mixture for *sato* fermentation, according to the composition of the microorganisms identified in the NP1 starter. The oenological parameters during the fermentation with the original NP1 starter and the defined starter culture mixture were compared. The NP1 starter and the alternative starter exhibited similar microbial population dynamics, as determined by conventional cultivation-dependent methods and by cultivation-independent denaturing gradient gel electrophoresis (DGGE) analysis. The profiles of the organic acids, glycerol, and the volatile compounds produced during fermentation were similar. The *sato* fermented with the two different starters gained similarly high scores of sensory evaluation. Based on these data, we concluded that the defined starter culture mixture has a great potential to become an alternative starter to produce high quality *sato* with consistency and may facilitate industrial production of *sato*. The advantages of using the DGGE analysis in combination with the conventional culture method to study the microbial population dynamics during the fermentation process of *sato* are discussed.

KEYWORDS: DGGE, microbial communities, sensory test, lactic acid bacteria

INTRODUCTION

Rice-based traditional alcohol beverages are enjoyed in Asian countries, such as Japan (*sake*), China (*jiu*), Korea (*yakju*), Philippines (*tapuy*), Vietnam (*ruou nep than*), Malaysia (*tapai*), Cambodia (*tapae*), Malaysia and Indonesia (*tapai*), and Thailand (*sato*)[1–3]. The Thai rice-wine, *sato*, is made from glutinous rice mixed with a traditional solid-state starter called *Loogpang*, which is composed of a mixture of various microorganisms grown on rice or rice flour. In the preparation of a traditional starter, rice is ground and thoroughly mixed with certain spices, such as garlic and pepper. Water is added to make a dough-like material and then shaped into a small ball and placed on a bamboo tray[4]. Powder from an old starter is added at the mixing step or sprinkled over the ball. After incubation at room temperature for 2–5 days and drying in the sun, the starter cakes can be kept for many months. Numerous filamentous fungi, yeasts, and bacteria have been isolated from these starter cakes obtained from various geographical sources.

The moulds and the yeasts in the starter play major roles in converting glutinous rice starch into simple reducing sugars and then transforming the sugar into ethanol[5–7] and minor components that are essential for the desirable taste and smell. Moulds isolated from starters have been reported as *Rhizopus* sp., *Mucor* sp., *Amylomyces* sp., and *Aspergillus* sp[6,7].

Yeasts reported were *Pichia* sp., *Saccharomycopsis* sp., *Issatchenkia* sp., *Saccharomyces* sp., and *Candida* sp[5,7]. The bacteria found in *sato* starters are principally lactic acid bacteria (LAB) belonging to the genera *Pediococcus* spp., *Lactobacillus* spp., and *Lactococcus* spp[8]. LAB have been reported to play important roles in food preservation, microbiological stability and the production of aroma-giving compounds in various food products[9]. The LAB and lactic acid found in *Hamei* and *Marcha*, which are starter cultures for rice wine in Manipur and Sikkim in India, showed neither a role in the rice saccharification process nor in the alcohol production (fermentation), but instead were suggested to give flavour to the alcohol beverages[10].

An understanding of the microbial dynamics during the fermentation process will be useful to improve the reproducibility and predictability of the quality of the fermented products. Elucidation of the microbial population dynamics during the fermentation process is therefore crucial. To assess the microbial community, cultivation-dependent techniques have been employed in the past. This type of approach however is limited not only by the long time it takes in obtaining the results, but also in that it may fail to give reliable information on the composition of the microbial communities since some microorganisms are not cultivatable in such environments[11]. Thus a polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) approach can be applied to solve the problem. PCR-DGGE is an electrophoretic method that facilitates separation of double-stranded DNA fragments of the same size but with different sequences[12]. The PCR-DGGE technique shows its power in the analysis of the microbial populations from fermented foods in which some microorganisms are either difficult to cultivate or are uncultivable under these conditions. PCR-DGGE has been used to investigate the microbial communities in several fermented foods, such as *sourdoughs*[13], the Vietnamese alcohol fermentation starter *banh men*[14], fermented milk *zabady*[15], wine[16,17], cheese[18], rice vinegar[19], and *kimchi*[20].

Although *sato* is a traditional Thai alcoholic beverage, the industrial production has not been successful up to the present. This may be due to lack of control on the starter quality and lack of aseptic techniques during the production process of the starter and *sato*. The production process of a starter introduces spatial and temporal variations in the composition of the resultant microbial community that can cause problems for both the *sato* quality and consistency of taste characteristics that stem from these variations. *Sato* makers either produce their own starters or purchase them from starter makers. The traditional way of producing starters is obsolete with lack of quality control on types and numbers of each microorganisms in the starter. The new starters are produced by the mixing of starch, herbs, water and the old starter. Thus it increases the risk of contaminating microorganisms or losing useful microorganisms in the starter every time a new one is made. Hence development of a control formulated defined starter culture mixture is desired for high quality and consistency in the industrial production of *sato*.

Previously, we selected four starters, from an initial survey of 114 starters collected throughout the country, as good sources of starter cultures for *sato* fermentation on the basis of their ability in liquefaction, ethanol production and the flavour production of *sato*[5]. Furthermore, from the four selected starters, the NP1 starter (a sample collected in Nakornpanom province of Thailand) was finally selected as the starter that yielded the best *sato*, gaining the highest evaluation by a set of panellists[5]

In the present study, we composed a defined starter culture mixture according to the microbial species and approximate proportions and densities of the major microorganisms identified in the NP1 starter. We then compared the performance of NP1 starter and the defined starter culture mixture in *sato* fermentation by analysing the population dynamics of microorganisms and the production of various fermentation products in the course of fermentation process, as well as the sensory evaluation of the *sato* produced.

MATERIALS AND METHODS

Source of the starter sample

Previously, four starters were selected based on the liquefaction capacity and ethanol fermentation activities, as well as sensory test of *sato* produced[7]. Among these, a starter collected in Nakornpanom (the NP1 starter) which led to the production of favourable *sato* was selected[7] and is used as the natural starter throughout this study.

Microorganisms used in this study

The mould and yeast strains, *Rhizopus oligosporus*, *Mucor racemosus*, *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera* and *Pichia anomala*, previously isolated from the NP1 starter and identified by Taechavasonyoo et al[7], were used throughout the study. The LAB were isolated in this study from the NP1 starter.

Isolation and identification of LAB in NP1 starter

The LAB were isolated from the NP1 starter by spreading appropriate dilutions onto Man-Rogosa-Sharpe (MRS) selective media (10.0 g/l peptone; 8.0 g/l meat extract; 4 g/l yeast extract; 20.0 g/l glucose; 2.0 g/l K_2HPO_4; 1.0 g/l Tween 80; 2 g/l diammonium hydrogen citrate; 5 g/l sodium acetate; 0.2 g/l $MgSO_4$; 0.04 g/l $MnSO_4$) agar[21]. The plates were incubated in candle jar (under low oxygen condition). The isolates were grouped according to the patterns of chromosomal DNA fragments by RAPD-PCR[22]. A fragment of the 16S rRNA encoding region of a representative isolate of each group was sequenced and the sequences were compared with those available in the NCBI GenBank database. The

rRNA encoding sequence data of the representative isolate has been deposited at GenBank with accession number: JN004055 (*Pediococcus pentosaceus* isolate NP102).

Microbial enumeration of the NP1 starter

Appropriate serial dilutions were made before spreading the sample onto selective media comprised of yeast malt extract (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, and 15 g/l agar, pH 4.5) (YM), rose bengal agar (5 g/l peptone, 10 g/l glucose, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.05 g/l rose bengal, and 20 g/l agar) (RBA) and MRS[21] (under low oxygen condition) plates for enumeration of the yeasts, moulds and LAB, respectively. L-lysine medium (Oxoid) for distinguishing non-*Saccharomyces* yeasts from *Saccharomyces* yeast. Plates were incubated at 30 °C for 2–4 days and then the number of colonies were counted and expressed as colony forming units (CFU) per gram of culture. The experiments were performed in triplicate.

Defined starter culture mixture preparation

To compose the defined starter culture mixture, each strain of the moulds, yeasts and LAB was cultivated beforehand in PDA, YM agar, and MRS agar, respectively, and inoculated at the cell number (colony-forming unit, CFU) found in the original NP1 starter.

Sato fermentation and sample collection

The procedures for the laboratory fermentation of *sato* were performed according to the procedure of Karuwanna with modification[23]. Fifty grams of glutinous rice were soaked in 60 ml of distilled water for 4 h and then steamed for 15 min in an autoclave (121 °C, 15 lb/in^2). The steamed glutinous rice was cooled to 35–40 °C prior to mixing with 2% (w/w) of the test starter and added to a fermentation bottle. Eleven repeated bottles were used for sample taken at each time point of fermentation with defined starter culture mixture or NP1 starter. After incubation at 30 °C for three days, liquid from the saccharification (called *namtoi*) was obtained. Sterile water (200 ml) was then added to allow submerged alcoholic fermentation at 30 °C for ten days to yield the final fermentation product (*sato*). At this point, the remaining rice paste floated covering the surface of the fermentation liquid. This made it difficult to collect homogeneous sample.

To collect homogeneous samples, only liquid part was taken. A 30 ml of fermented liquid sample was collected on day 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, and 13 from each flask, and each sample was subjected to microbiological and chemical analyses.

Sample preparation

For microbiological analysis, each sample was transferred to a Stomacher bag and homogenized with 200 ml of sterile water in a Stomacher Lab-blender 400 (Seward 400, Emergo, England) at high speed without generating extra heat. The appropriate dilutions of samples were made prior to microbial enumeration. For DNA analysis, DNA was extracted from each samples without homogenization. For chemical analysis, clear fermentation broths were used.

Analysis of the oenological parameters

The pH of *sato* during fermentation was measured by a digital pH meter (Seven Easy, Mettler Toledo, USA). Reducing sugar was determined by the dinitrosalicylic acid (DNSA) method[24]. Ethanol concentration was measured using an ebulliometer. Titratable acidity was determined as described by Amerine et al[25]. In all cases the determination and experiments were performed in triplicate.

DNA extraction, amplification and analysis

DNA was isolated from moulds, yeasts and LAB according to the standard method previously described by Burke et al[26], except that the supernatant from the fermented broth samples were subjected to DNA extraction without prior treatment in a Stomacher.

Amplification of the 26S rRNA gene of the fungal community was performed using DNA extracted from the fermented broth as templates with the NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) primers as described by Cocolin et al[16]. A GC clamp (5′-GC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) was linked to the 5′ end of primer NL1 when used for DGGE analysis.

The V3 region of the 16S rRNA gene was selected for bacterial community analysis. The primers used to amplify the V3 regions were 338f (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) as previously described[27,28]. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was linked to the 5′ end of primer 338f when used for the DGGE analysis.

PCR was performed in a final volume of 30 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 mM of each primer, 1.0 U-*Taq* DNA polymerase (New England Biolab), and 2 µl of the extracted DNA (approximately 30 ng).

The reactions were run for 30 cycles: at 95 °C for 60 s, 54 °C for 45 s, and 72 °C for 60 s. An initial 5 min denaturation at 95 °C and a final 7 min extension at 72 °C were used. The PCR amplification of the V3 region used an initial 94 °C for 5 min, followed by a touchdown procedure (in which the annealing temperature was lowered from 65 °C to 55 °C at a rate of 1 °C every cycle) and 10 additional annealing cycles at 55 °C. At each cycle, the elongation step was carried out at 72 °C for 3 min. Prior to performing denaturing gradient gel electrophoresis (DGGE), the PCR-products were analysed for the expected size by electrophoresis in 2% agarose gel.

Denaturing gradient gel electrophoresis

DGGE was performed according to Hu et al[29] using DCode Universal Mutation Detection System (Bio-Rad, CA, USA). About 20 µl of PCR-products were applied to 8% polyacrylamide gel made with linear denaturing ranges from 40% to 60% for moulds and yeasts and from 50% to 60% for LAB. A 100% denaturant contained 7 M urea and 40% (v/v) formamide. The electrophoresis was run in 1 × tris-acetate EDTA (TAE) buffer at 100 V for 14 h under constant temperature 60 °C.

The selected bands on the ethidium bromide-stained DGGE gel were excised and eluted from the gel using 30 µl of sterile distilled water and placed at 4 °C for 18 h. The reclaimed DNA was used as a template for re-amplification of the bands using the same program and same primer pairs without the GC-clamp. The amplified products were then purified by QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer's protocol and sequenced with one of the amplification primers. These sequences were compared with similar sequences in the Genbank database using BLAST analysis (basic local alignment search tool at the website of National Centre for Biotechnology Information (NCBI).)

HPLC analysis of organic acids and glycerol

Organic acids and glycerol were determined using a Bio-Rad Aminex HPX-87H (300 × 7.8 mm i.d.) column equipped with an RI detector. Operating conditions involved a mobile phase of 2 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 55 °C. The standard solutions and *sato* samples were filtered through 0.22 µm membrane filters (Lubitech, China). The standard mixtures contained citric acid, tartaric acid, pyruvic acid, malic acid, succinic acid (Fluka, Switzerland), lactic acid, acetic acid, isobutyric acid, and isovaleic acid (Sigma, German) plus glycerol (Carlo Erba, Italy). All samples were analysed in triplicate.

Analysis of volatile compounds by dynamic headspace and GC-MS

The filtered *sato* samples were transferred into headspace vials (20.0 ml). Volatile compounds were identified by comparison of their mass spectra and retention times with those of the pure standards. The thermal desorber was coupled to a Hewlett Packard Model 5980 gas chromatograph and mass selective detector (MS). The analytical column was an HP-624, with an Agilent 19 091v-402 (25.0 m × 0.2 mm i.d. × 1.12 µm film thickness) capillary column, and using He at 1 ml/min as the carrier gas. The temperature program was 40 °C for 3 min, then risen to 220 °C at 15 °C/min and held for 8 min. The transfer line temperature was 220 °C with a run time of 20 min. The injection temperature was 280 °C, with an injected volume of 1 ml. All samples were analysed in triplicate.

Sensory evaluation

The *sato* was evaluated by a panel of 10 *sato* and wine experts with previous experience in the sensory analysis of *sato* and wines by the method modified from Karuwanna[23]. The subjects were asked to rate the global sensory quality by assigning a total score out of 100 points from the colour (10 points), odour (30 points), flavour (40 points), and impression (20 points). Randomized samples of 25–30 ml were served in clear glasses with three digit random numbers. Evaluations were conducted at 20–22 °C. Each sample was tested in triplicate.

Statistical analysis

An ANOVA was applied and the significant differences were determined by Scheffé tests. All statistical analyses were performed using the software SPSS for Windows, version 16.0.

RESULTS

Isolation, identification of LAB and microbial enumeration of the NP1 starter

In addition to the moulds and yeasts, LAB are also found in many microbial starters, including *sato* starter in general[8] and this specific NP1 isolate, and might contribute to the production of aroma giving compounds. We, therefore, isolated the LAB from the NP1 starter on MRS agar plates under low oxygen condition. DNA extraction was performed from the isolates grown on MRS agar plates. To group the isolates, the DNA of each isolates was subjected to

Table 1 Yeasts, moulds and lactic acid bacteria found in the NP1 starter at an initial stage of fermentation (day 0).[†]

Species	Cell number (CFU/g)
Saccharomyces cerevisiae	$(3.4 \pm 0.1) \times 10^6$
Saccharomycopsis fibuligera	$(8.5 \pm 0.3) \times 10^3$
Pichia anomala	$(8.4 \pm 0.2) \times 10^4$
R. oligosporus	$(3.5 \pm 0.1) \times 10^3$
M. racemosus	$(2.8 \pm 0.2) \times 10^3$
P. pentosaceus	$(6.8 \pm 0.3) \times 10^3$

[†] Microorganisms were enumerated by culturing in yeast malt extract (for yeasts), L-lysine (for distinguishing between *Saccharomyces* and non-*Saccharomyces* yeasts), rose bengal (for moulds) and MRS (for LAB) agar plates.

RAPD-PCR using 10-mer random primer. Three groups were obtained according to RAPD-PCR profiles (data not shown). The representatives of each group were then classified by the amplification and sequencing of a stretch of their 16S rRNA genes[22]. It was found that all the three groups were identified as *P. pentosaceus*.

For composing the defined starter culture mixture for *sato* fermentation, we quantified each species of the microorganisms in the NP1 starter to optimize the inoculation size of each microorganism. The quantification was performed by plating serial dilutions of the sample onto selective media, and enumerated as described in Materials and Methods section. These results are summarized in Table 1.

Based on the microbial population of the NP1 starter, we composed a defined starter culture mixture that consisted of the same isolates of the moulds, yeasts and LAB mixed at the cell number as found in the original NP1 starter (Table 1).

Changes in the oenological parameters during *sato* fermentation

To evaluate the *sato* fermentation produced with the defined starter culture mixture, we quantitatively compared the oenological parameters of *sato* fermented with the NP1 starter and the defined starter culture mixture (Fig. 1a,b). During the first two days of the solid state fermentation, liquefaction was still insufficient to take samples for analysis but from the third day the high sugar syrup (*namtoi*) produced was sufficient for the analysis. The highest concentration of reducing sugar was indeed detected on day 3 for both cultures (100 and 79 g/l for the traditional starter and defined starter culture mixture, respectively), and then, when the submerged fermentation was initiated, the concentration declined abruptly from days 4–7, remaining very low thereafter at $<$ 5 g/l (Fig. 1a,b). This is consistent with the sharp increase in the level of ethanol production during days 3–5 from 5.7–6%(v/v) up to 10–11% (v/v), supporting the ethanol fermentation of the reducing sugar, with the reducing sugar level decreasing with the continued production of ethanol until the reducing sugar became barely detectable towards the end of the fermentation period at days 9–13. Ethanol concentration increased continuously with the initiation of the submerged fermentation to reach approximately 13–15% (v/v) on day 13, and was broadly similar between the two types of ferments.

No significant change in the total acidity was noted over the 13 days of fermentation period (Fig. 1a,b), or between the fermentation broth derived from the NP1 starter and the defined starter culture mixture (Fig. 1a,b), while the pH rose slightly over the 13 day culture period but without any significant difference between the two different starter cultures (Fig. 1a,b).

Microbial population dynamics during *sato* fermentation

To characterize the *sato* fermentation with the NP1 starter and the defined starter culture mixture, we compared the fermentation processes by determining the microbial populations and chemical compositions during the fermentation, and evaluated the final *sato* product by sensory test. The microbial populations were monitored by a combination of conventional cultivation methods and PCR-DGGE analysis.

The profiles of the microbial population dynamics and the kinetics of *sato* fermentation were highly similar between the NP1 starter and the defined starter (Fig. 1c,d). The moulds (*R. oligosporus* and *M. racemosus*) were detectable at the early stages of the fermentation, starting from approximately 10^3 CFU/g on day 0, peaked on day 1 just above and below 10^4 CFU/g and then disappeared from the fermentation fluid after day 3, and were at broadly the same levels between the two fermentation types. The moulds could not be detected after day 3 in the fermentation fluid. This is presumably because, as mentioned in the Materials and Methods section, only the liquid part underneath the incompletely digested rice floating on the surface were taken. Separately from this experiment, the rice paste on the surface of the fermentation fluid were sampled daily and we found that the moulds were still present until the end of fermentation (data not shown). The non-*Saccharomyces* yeasts (*P. anomala* and *Sm. fibuligera*) started at broadly similar levels as the moulds but

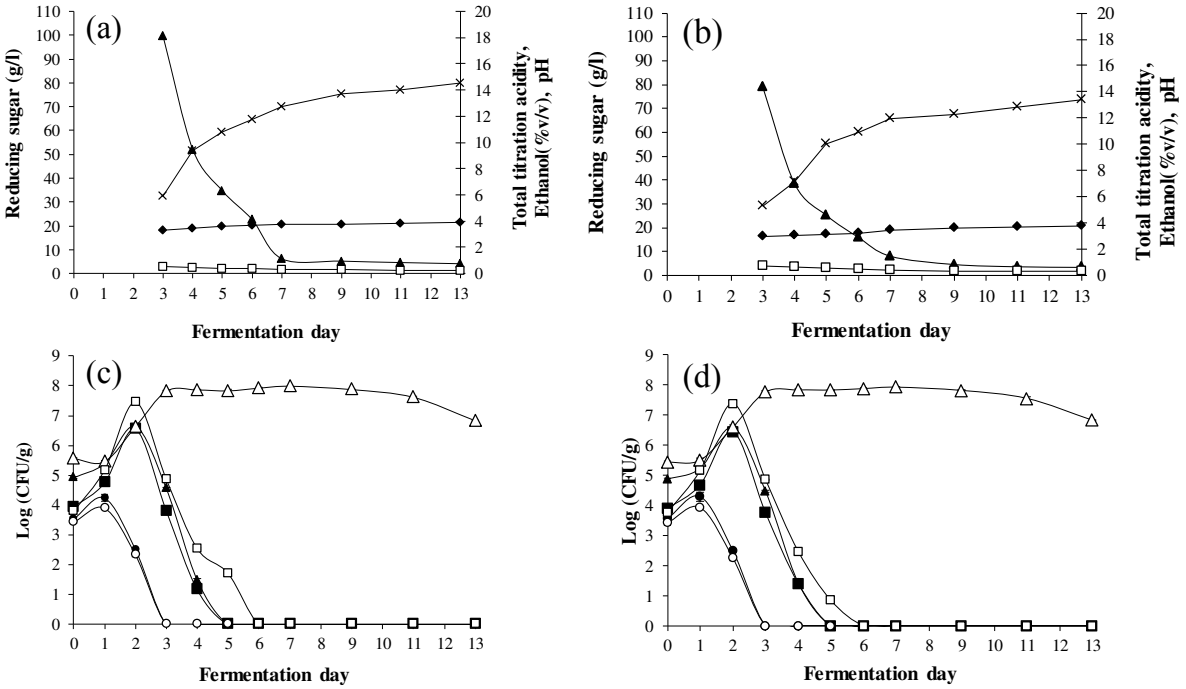

Fig. 1 Analysis of the oenological parameters and microbial population level during the *sato* fermentation by the NP1 starter (a and c) and the pure mixed starter cultures (b and d). Samples of the starter and fermented broth were taken on days 0, 3, 5, 7, and 9. Each sample was prepared for 5 repeated flasks used for sample taken at each time point. The oenological parameters (a and b) analysed were the reducing sugar (closed triangles), total acidity (open squares), ethanol (crosses) and pH (closed diamonds). The liquid samples available on days 0, 1, and 2 were still insufficient for analyses. The mould, yeast and bacteria population levels (c and d) evaluated by the plate culture method for, *S. cerevisiae* (open triangles), *P. anomala* (closed triangles), *Sm. fibuligera* (closed squares), *R. oligosporus* (closed circles), *M. racemosus* (open circles), and *P. pentosaceus* (open squares). Data are shown as the average value determined from three replicates.

increased up to day 2 and so became the dominant species at 5×10^6 CFU/g and then gradually declined to very low levels by day 5. Again, they did not significantly differ between the two fermentation types. The number of *S. cerevisiae* yeast started to increase with the initiation of solid state culture (days 1–2) and increased thereafter until it reached a plateau level on day 3 of just under 10^8 CFU/g, when the non-*Saccharomyces* yeasts started to decline sharply. The population of *S. cerevisiae* remained high from day 3 to day 13 with a gradual decrease after day 7 (Fig. 1c,d). Finally, the LAB (*P. pentosaceus*) was found in the fermentation broth on day 0 at a density of approximately 10^3 CFU/g and increased to reach the maximum level (approximately 10^7 CFU/g) on day 2 and then started to decline sharply to become undetectable by day 6 (Fig. 1c,d).

DGGE profile of the microbial community

The fungal PCR-DGGE profiles of the 26S rRNA gene fragment for the samples collected during the fermentation process are shown in Fig. 2a,b. The microbial population dynamics, as revealed by the DGGE9 profile, were consistent with those determined by the conventional cultivation-dependent methods (Fig. 1c,d).

The DGGE profile during *sato* fermentation with the NP1 starter (Fig. 2a) and the defined starter culture mixture (Fig. 2b) were broadly to largely similar. The DGGE fingerprints of the fungi revealed 5 detectable bands (a–e in Fig. 2a,b). The DNA sequencing results of the bands a–f from the DGGE gels (Fig. 2) matched 100% to the following microorganisms from the GenBank data base: *Sm. Fibuligera* (a); *R. oligosporus* (b); *M. racemosus* (c); *P. anomala* (d); *S. cerevisiae* (e); and *P. pentosaceus* (f) (Table 2). Thus *Sm. fibuligera* and *R. oligosporus* were detectable from day 0 to either day 1 (NP1 starter culture) or day 2 (defined starter culture mixture) and not thereafter. *M. racemosus* was likewise detectable from day 0 to either day 2 (NP1 starter culture) or day 3 (defined starter culture mixture) and not thereafter, while *P. anomala* was detected up to day 2, or weakly at day 3 in the fermentation initiated with the NP1 starter culture. Also,

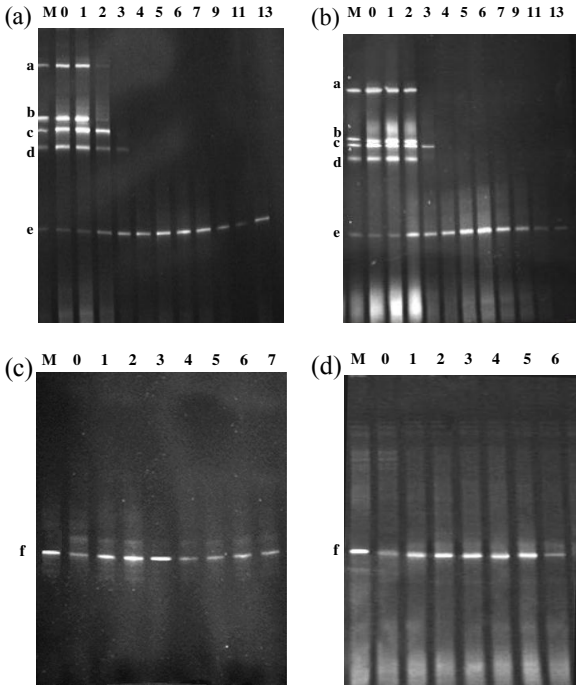

Fig. 2 PCR-DGGE analysis of the microbial community in the fermentation broth collected during the *sato* fermentation process from NP1 starter (a and c) and the defined starter culture mixture (b and d), at the indicated fermentation times from 0–13 days. (a and b): fungal PCR-DGGE analysis of the 26S rRNA gene. The indicated bands a–e corresponded to *Sm. fibuligera*, *R. oligosporus*, *M. racemosus*, *P. anomala*, and *S. cerevisiae*, respectively. (c and d): bacterial PCR-DGGE analysis of the V3 region of the 16S rRNA gene. The indicated bands f correspond to *P. pentosaceus*. M: Markers composed of the PCR-DGGE products from *Sm. fibuligera*, *R. oligosporus*, *M. racemosus*, *P. anomala*, and *S. cerevisiae* (a, b) and *P. pentosaceus* (c, d). Gels shown are representative of three independent amplifications.

as noted with the conventional plating (Fig. 1c,d), *S. cerevisiae* was detectable throughout the fermentation process, but was at a low intensity at days 0 and 1 and gradually increased from day 2 onwards to reach the highest intensity at days 5 and 6, maintaining this intensity until it started to gradually decrease towards the end of the fermentation process, from day 9 to fade by day 13.

The PCR-DGGE profiles derived from the V3 region of the 16S rDNA gene fragment of the LAB collected from the fermentation broth revealed only one detectable band (f in Fig. 2c,d) that was ascribed to an isolate of *P. pentosaceus* (Table 2). This LAB species was detectable from day 0 until days 6–7 in both the defined starter culture mixture (Fig. 2d) and the NP1 starter (Fig. 2c) starters. No PCR products were obtained in the samples collected on days 9–13.

Table 2 DNA sequence analysis of the DGGE bands.

DGGE	Band[†]	Closest relative	% identity[‡]
mould	b	*R. oligosporus*	100%
	c	*M. racemosus*	100%
Yeast	a	*Sm. fibuligera*	100%
	d	*P. anomala*	100%
	e	*S. cerevisiae*	100%
Bacteria	f	*P. pentosaceus*	100%

[†] Bands as indicated on the DGGE gels shown in Fig. 2a,b.

[‡] Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank database.

Table 3 Comparison of organic acid and glycerol produced by defined starter culture mixture and NP1 starter[†].

Organic acid or glycerol	Concentration (g/l)	
	NP1 starter	Defined starter[‡]
citric acid	0.25 ± 0.02	$0.12 \pm 0.01^{*}$
succinic acid	0.46 ± 0.16	0.51 ± 0.04
acetic acid	0.51 ± 0.03	0.68 ± 0.15
lactic acid	5.44 ± 0.10	$0.95 \pm 0.22^{*}$
glycerol	5.98 ± 0.38	$2.92 \pm 0.11^{*}$

[†] Values are mean $\pm$ standard deviation from three determinations.

[‡] Defined starter culture mixture.

[*] Values are significantly different ($p < 0.05$).

Changes in the levels of organic acids and glycerol during *sato* fermentation

A comparison of the levels of the microbial metabolites that may contribute to the flavour and taste of *sato* produced by fermentation with the NP1 starter and the defined starter culture mixture is summarized in Table 3. Citric acid, succinic acid, lactic acid, acetic acid, and glycerol were detected in the *sato* by HPLC. No significant differences were noted in the amount of succinic acid and acetic acid between the *sato* fermentations with the two different starters. However, significantly higher concentrations of glycerol, citric acid and especially lactic acid were noted in the *sato* fermented with the NP1 starter than with the defined starter culture mixture.

The levels of volatile compounds produced during *sato* fermentation

Volatile compounds such as higher alcohols and esters are important for the flavour and aroma of alcoholic

Table 4 Comparison of major volatile compounds produced by defined starter culture mixture and NP1 starter.[†]

Volatile compound	Concentration (mg/l)	
	NP1 starter	Defined starter[‡]
1-propanol	7.1 ± 2.2	$38.9 \pm 6.6^{*}$
isobutyl alcohol	91.0 ± 6.5	$60.2 \pm 5.5^{*}$
1-Butanol	2.88 ± 0.74	3.9 ± 1.3
isoamyl alcohol	96.9 ± 2.9	$131 \pm 13^{*}$
active amyl alcohol	1.06 ± 0.34	1.38 ± 0.27
2-phenylethanol	26.32 ± 0.33	41.8 ± 4.2
ethyl acetate	26.9 ± 2.3	$73.4 \pm 7.8^{*}$
isoamyl acetate	0.14 ± 0.01	$0.32 \pm 0.01^{*}$
propionic acid ethyl ester	0.12 ± 0.00	0.15 ± 0.05

[†] Values are mean $\pm$ standard deviation from three determinations.
[‡] Defined starter culture mixture.
[*] Values are significantly different ($p < 0.05$).

Table 5 Sensory analysis of *sato* produced by defined starter culture mixture and NP1 starter.[†]

Characteristic	Sensory score	
	NP1 starter	Defined starter[‡]
Colour	7.8 ± 0.2	7.7 ± 0.1
Odour	21.9 ± 0.4	21.2 ± 0.1
Flavour	29.3 ± 0.6	29.8 ± 0.2
Impression	14.9 ± 0.3	14.9 ± 0.2
Overall quality	73.8 ± 0.7	73.7 ± 0.3

[†] Values are mean $\pm$ standard deviation from three determinations.
[‡] Defined starter culture mixture.
The values showed no statistical significance ($p > 0.05$).

beverages[25]. The nine volatile compounds detected in the *sato* samples by gas chromatography are listed in Table 4. These are similar to those previously reported for *sato* and *ou*, another kind of Thai rice wine[30,31]. The levels of 1-butanol, 2-phenylethanol, active amyl alcohol and propionic acid ethyl ester in *sato* produced with the two different starters were similar and not significantly different (Table 4). However, significantly higher levels of isoamyl alcohol, ethyl acetate and isoamyl acetate, and significantly lower levels of isobutyl alcohol and 1-propanol were found in the *sato* fermented with the defined starter culture mixture than that with the NP1 starter.

Sensory evaluation

The *sato* products fermented with the defined starter culture mixture and the original NP1 starters were evaluated by a panel of ten *sato* and wine experts. The results of the sensory evaluation were satisfactorily high for all four character scores and the scores for the two *sato* products were comparable in each aspect of their evaluation (Table 5).

DISCUSSION

In the present study, we constructed a defined starter culture mixture according to the composition of the major microorganisms identified in the NP1 starter (species and amount of each microorganism). The driving rationale was that as if the artificially composed starter can serve as a suitable alternative starter for *sato* fermentation in place of the original starter it may then allow for the controlled fermentation with both reduced variation in quality of the *sato* product, and the potential to manipulate it for further improvement in the quality. Indeed, the fermentation with the defined starter culture mixture yielded a high quality *sato* of comparable quality of the *sato* fermented with the original NP1 starter with regard to all aspects of evaluation (i.e., colour, odour, flavour, and impression). The NP1 starter and the defined starter culture mixture starters exhibited broadly similar microbial population dynamics during the fermentation, as determined by conventional cultivation-dependent methods and by the cultivation-independent PCR-DGGE. Moreover, the profiles of oenological parameters, such as the levels of the organic acids and the volatile compounds produced in the course of *sato* fermentation were largely similar, except for the difference in the levels of a few volatile compounds, few organic acids and glycerol, although this did not appear to significantly affect the result of the sensory evaluation (Tables 3 and 4).

In both fermentations, the ethanol levels still continued to increase slightly even after sugar levels decreased to become barely detectable (Fig. 1a,b). The sugar for the fermentation appeared to be supplied from incompletely digested rice paste that floated on the surface of the fermentation fluid. The remaining rice paste could be the substrate for aerobic moulds resulting in the continued generation of sugar. The sugars were then converted to ethanol by yeasts in the fermentation fluids. This type of fermentation is somewhat similar to the so-called parallel combined fermentation or multiple parallel fermentation in *sake* fermentation[32].

The difference on levels of some volatile compounds and organic acids in the *sato* fermented with the NP1 starter and that with the defined starter culture mixture might be caused by the herbs which were added to the starters but not in the defined starter culture mixture. Normally, the recipes for the kind and amount of herbs added to the starter to prevent

contamination from undesired microorganisms are kept secret by the makers. Besides the anti-microbial activity, some herbs may provide aroma as well. We could detect differences in the levels of a few volatile compounds, few organic acids, and glycerol between the NP1 starter and the defined starters while found no significantly difference between the two kinds of *sato* by sensory evaluation, presumably because there is a limit threshold of human sensory perception. In fact, the *sato* produced by NP1 and the define starters showed no significantly difference.

Based on these data, we concluded that the defined starter culture mixture has a great potential as an alternative starter to produce high quality *sato* with consistency, substituting for the traditional highly variable starter, and that this may facilitate industrial production of *sato*. Our result is in agreement with a report of Dung et al[33] that used a defined mixed-culture fungal fermentation starter for the controlled production of Vietnamese rice wine, and obtained a superior flavour and overall acceptability of the rice wine compared to the local commercial rice wines produced from the undefined conventional starter. The use of a defined starter culture mixture will also enable controlled manipulation of the species and amount of the microorganisms mixed in the starter to design *sato* with different bouquet and flavour.

The population dynamics of the microbial community during *sato* fermentation was investigated by cultivation-dependent and -independent molecular methods. The conventional cultivation method of isolating and identifying the microorganisms from the fermentation broth, coupled with PCR-DGGE provides a robust, reliable and precise means to investigate the dynamics of the microbial community during *sato* fermentation. Although the PCR-DGGE electrophoresis by itself provides a broad overview of a microbial community, caution is needed in the interpretation of PCR-DGGE data. An advantage of using DGGE analysis is that it facilitates rapid monitoring and assessment of the dynamics of fairly diverse microbial communities, such as those found in the *sato* fermentation process. In this study, the microbial community revealed by the DGGE technique was consistent with those revealed by the cultivation-dependent technique (Fig. 2), with no evidence of uncultivable microbes present in the NP1 starter community.

The results of this work showed a successful use of a defined starter culture mixture for the production of *sato* at a laboratory scale. Further development of such defined starter culture mixture to be suitable for *sato* production at a commercial scale is needed.

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REFERENCES

1. Aidoo KE, Nout MJR, Sarkar PK (2006) Occurrence and function of yeasts in Asian indigenous fermented foods. *FEMS Yeast Res* **6**, 30–9.
2. Tamang JP (2010) Diversity of fermented beverages and alcoholic drinks. In: Tamang JP, Kailasapathy K (eds) *Fermented Foods and Beverages of the World*, CRC Press, Taylor & Francis Group, New York, pp 85–125.
3. Yamamoto S, Matsumoto T (2011) Rice fermentation starters in Cambodia: cultural importance and traditional methods of production. *Southeast Asian Stud* **49**, 192–213.
4. Lotong N (1997) Koji. In: Wood JB (ed) *Microbiology of Fermented Foods* Vol. 2, 2nd edn, Blackie Academic and Professional, London, pp 658–95.
5. Limtong S, Sintara S, Suwannarit P, Lotong N (2002) Yeast diversity in Thai traditional alcoholic starter. *Kasetsart J (Nat Sci)* **2**, 149–58.
6. Limtong S, Sintara S, Suwanarit P, Lotong N (2005) Species Diversity of molds in Thai traditional fermentation starters (*Loogpang*). *Kasetsart J (Nat Sci)* **39**, 511–8.
7. Taechavasonyoo A, Thaniyavarn J, Yompakdee C (2013) Identification of the molds and yeasts characteristic of a superior *Loogpang*, starter of Thai rice-based alcoholic beverage *Sato*. *Asian J Food Agro Ind* **6**, 24–38.
8. Rittiplang J, Laopaiboon P, Vichitpan K, Danvirutai P (2007) Lactic acid bacteria from indigeneous *Loogpang* samples of Northeastern Thailand and their lactic acid production ability. *Thai J Biotechnol* 44–8.
9. Salminen S, Wright AV (1993) *Lactic Acid Bacteria*. Dekker, New York.
10. Tamang JP, Dewan S, Tamang B, Rai A, Schillingerr U, Holzapfel WH (2007) Lactic acid bacteria in Hamei and Marcha of North East India. *Indian J Microbiol* **47**, 119–25.
11. Duthoit F, Godon JJ, Montel MC (2003) Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl Environ Microbiol* **69**, 3840–8.
12. Ercolini D, Mauriello G, Blaiotta G, Moschetti G, Coppola S (2004) PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. *J Appl Microbiol* **96**, 263–70.

13. Iacumin L, Cecchini F, Manzano M, Osualdini M, Boscolo D, Orlic S, Comi G (2009) Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiol* **26**, 128–35.
14. Thanh VN, Mai LT, Tuan DA (2008) Microbial diversity of traditional Vietnamese alcohol fermentation starters (*banh men*) as determined by PCR-mediated DGGE. *Int J Food Microbiol* **128**, 268–73.
15. Baradei GE, Buchet AD, Ogier JC (2008) Bacterial biodiversity of traditional Zabady fermented milk. *Int J Food Microbiol* **121**, 295–301.
16. Cocolin L, Bisson LF, Mills DA (2000) Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol Lett* **189**, 81–7.
17. Maro ED, Ercolini D, Coppola S (2007) Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *Int J Food Microbiol* **117**, 201–10.
18. Flórez AB, Mayo B (2006) Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE. *Int J Food Microbiol* **110**, 165–71.
19. Haruta S, Shintaro U, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y (2006) Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *Int J Food Microbiol* **109**, 79–87.
20. Lee JS, Heo GY, Lee JW, Oh YJ, Park JA, Park YH, Pyunb YR, Ahn JS (2005) Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *Int J Food Microbiol* **102**, 143–50.
21. de Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–5.
22. Cocconcelli PS, Porro D, Galandini S, Senini L (1995) Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett Appl Microbiol* **21**, 376–9.
23. Karuwanna P (2002) *Wine: Science and Art*. Kasetsart Univ Press, Bangkok. (in Thai).
24. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 420–8.
25. Amerine MA, Ough GS, Singleton VL (1979) *The Technology of Wine Making*, 4th edn, AVI Publishing Company, Westport, Connecticut.
26. Burke D, Dawson D, Stearns T (2000) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. A Cold Spring Harbor Laboratory Press, CA, pp 111–2.
27. Ampe F, ben Omar N, Moizan C, Wacher C, Guyot JP (1999) Polyphasic study of the spatial distribution of microorganisms in pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl Environ Microbiol* **65**, 5464–73.
28. ben Omar N, Ampe F (2000) Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl Environ Microbiol* **66**, 3664–73.
29. Hu Q, Dou M, Qi H, Xie X, Zhuang G, Yang M (2007) Detection, isolation, and identification of cadmium-resistant bacteria based on PCR-DGGE. *J Environ Sci* **19**, 1114–9.
30. Sirisantimethakom L, Laopaiboon L, Danvirutai P, Laopaiboon P (2008) Volatile compounds of a traditional Thai rice wine. *Biotechnology* **3**, 505–13.
31. Chuenchomrat P, Assavanig A, Lertsiri S (2008) Volatile flavour compounds analysis of solid state fermented Thai rice wine (*Ou*). *Sci Asia* **34**, 199–206.
32. Paterson A, Swanston JS, Piggott JR (2003) Production of fermentable extracts from cereals and fruits. In: Lea AGH, Piggott JR (eds) *Fermented Beverage Production*, 2nd edn, Plenum Publishers, NY, pp 1–24.
33. Dung NTP, Rombouts FM, Nout MJR (2005) Development of defined mixed-culture fungal fermentation starter granulate for controlled production of rice wine. *Innovat Food Sci Emerg Tech* **6**, 429–41.