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Research Article

Identification of the moulds and yeasts characteristic of a superior *Loogpang*, starter of Thai rice-based alcoholic beverage *Sato*

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Abstract

The traditional Thai alcohol beverage *Sato* uses *Loogpang* (a dry round to flattened ball of glutinous rice) as the microbial starter for fermentation of glutinous rice into rice wine. In the fermentation process of *Sato*, moulds and yeasts play the major roles. The purpose of this study is to select the sources of *Loogpang* that produce *Sato* with a good flavour, to investigate the composition of the moulds and the yeasts and to study their population dynamics during the fermentation process. Four out of 114 sources of *Loogpang* were selected for their high capability of rice liquefaction, ethanol fermentation and formation of good flavour in *Sato*. This study used a morphological method in combination with PCR-RFLP pattern and ITS1-5.8S rRNA-ITS2 sequences for identification of the moulds and the yeasts. The fungal population dynamics studies in *Sato* samples produced from the selected *Loogpang* revealed that the moulds and the non-*Saccharomyces* yeasts were dominant in the early stage and were replaced by *S. cerevisiae* at the later stage (except for *Issatchenkia orientalis*). Based on sensory evaluation of the *Sato* ferment, NP1 *Loogpang* was selected as a superior microbial starter. The results obtained may be useful for concocting a mixed pure culture starter for better controlled fermentation and consistency in *Sato* quality.

Keywords: fermentation, non-*Saccharomyces* yeasts, PCR-RFLP, microbial population dynamics, sensory evaluation, starter culture, rice wine, Thailand.

Introduction

The traditional Thai alcoholic beverage, *Sato*, uses *Loogpang* as the microbial starter to ferment the glutinous rice into rice wine with alcohol content not

exceeding 15%(v/v) (according to the Thai Industrial Standards Institute). *Sato* is normally produced at the household or village level.

Loogpang, a dry round to flattened ball of glutinous rice of 2 to 3.5 cm diameter, is a mixed culture starter of microorganisms, such as moulds, yeasts and bacteria [1], required for the fermentation of rice. The moulds and yeasts play a major role in the production of rice wines. Moulds are mainly responsible for the hydrolysis of starch in the glutinous rice into sugar [1, 2], while the yeasts are reported to be mainly responsible for the subsequent fermentation of the released glucose to ethanol [2, 3] and, along with the lactic acid bacteria (LAB), other fermentation products important for desirable taste and odor qualities [4].

Amongst the microbes of *Loogpang*, the most frequently found filamentous moulds were species of *Rhizopus*, *Mucor* and *Amylomyces* [1], and for the yeasts were species of *Pichia*, *Saccharomycopsis*, *Issatchenkia*, *Saccharomyces* and *Candida* [3, 5].

Each local *Sato* producer has a different recipe for making their starter which, along with seasonal and micro-niche variations, results in a diverse variation in the quality of the products. The quality of *Sato* produced from any given *Loogpang* from local *Sato* producers could not be predicted or guaranteed. Indeed, a similar problem has also been reported in the production of rice wine using *Men*, a Vietnamese rice wine starter [6].

Thus the commercial scale production of *Sato* faces the significant problem of inconsistency in the quality produced from each batch. This inconsistency can largely (if not totally at a commercial scale) be related to the variability of the microorganism communities between different *Loogpangs*. The use of good pure mixed culture of desirable moulds and yeasts should lead to a greater consistency in *Sato* quality between batches, as well as the potential for manipulation to improve the *Sato* characteristics.

Previous studies on *Loogpang* and *Sato* were concerned mostly with the isolation and identification of moulds (into genera level; [1]) and yeasts [3, 5]. However, very little research has focused on upgrading the unidentified starter to a higher quality, well-defined starter towards the production of a superior quality *Sato* at a commercial scale.

This study focuses on identification of the major microbes, moulds and yeasts, in *Loogpang* that were selected on the basis of leading to the production of high quality *Sato*. Further investigation was conducted on the composition and population dynamics of moulds and yeasts during the course of the rice fermentation to *Sato*.

Materials and Methods

Sample collection

A total of 114 different samples of *Loogpang* were collected from small-scale factories and villages in central, northern and northeastern Thailand. Starters were stored at 4°C after collection and prior to screening.

Fermentation process

The traditional process for making *Sato*, somewhat similar to the production process of Balinese rice wine [7] and Vietnamese rice wine [8], was adapted from that described in Karuwanna [9] to the laboratory as follows. Fifty grams of glutinous rice was soaked in 60 ml of distilled water at room temperature for 4 h, followed by steaming in an autoclave. The steamed glutinous rice was cooled to 35 – 40°C prior to mixing with 2 g of *Loogpang* starter. After solid-state aerobic fungal fermentation at 30°C for three days the liquid produced from the *Loogpang* by saccharification (*Namtoi*) was obtained. Then 200 ml of sterile water was added to allow the submerged ethanol fermentation at 30°C in a 500 ml sealed bottle for seven days to obtain the final fermentation product (*Sato*). It should be noted that moulds and remaining rice floated on top of the fermentation broth throughout the submerged fermentation process.

Analytical methods

Glucose levels in the *Namtoi* and *Sato* were analyzed by a glucose oxidase-peroxidase kit (Biotech, Thailand). Reducing sugar was determined by the dinitrosalicylic acid method [10]. The liquefaction was measured by the volume of the total liquid formed on day 3 (data not shown). The ethanol concentration of *Sato* was analyzed by gas chromatography (3800, Varian, USA). The quality of the finished *Sato* was assessed by a panel of three (initial) or ten (final) *Sato* and wine experts.

Microbiological analysis

Five bottles of culture broth were prepared for each *Loogpang* and samples of the fermented broth were taken only once on days 0, 3, 5, 7 and 9 from each bottle. 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. Appropriate serial dilutions were made before spreading onto Rose Bengal agar (RBA) and potato dextrose agar (PDA) plates for moulds, and yeast malt extract agar (YM) (pH 4.5) for yeasts, L-lysine medium agar plates for distinguishing *Saccharomyces* from non-*Saccharomyces* yeasts, respectively. 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.

Identification of moulds and yeasts

Mould isolates were identified based on both morphological and molecular methods. Morphological identification used macroscopic and microscopic morphology, in terms of the size and structure of spores, sporangiophore, rhizoid, chlamydospore and sporangium, as well as culture properties, according to established taxonomic keys and descriptions [11]. Molecular species identification was performed by phylogenetic comparison of the DNA sequences

of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) with those available from known species at the NCBI GenBank database using the sequence similarity criteria of Sujaya *et al*. [7]. Where stated, the sequence similarity approach of Peterson and Kurtzman [12] using the D1 - D2 region of the 26S rRNA gene was additionally performed.

Yeasts were grouped according to the PCR-RFLP patterns of the same ITS region generated by digestion with *Hin*FI, *Hae*III and *Hha*I (see DNA analyses) [13]. A representative isolate of each group was randomly selected for further identification by standard biochemical methods, as outlined by Barnett *et al*. [14] and Kurtzman *et al*. [15], and by phylogenetic analysis of the ITS region [13] as for the moulds.

DNA analyses

DNA was isolated from moulds and yeasts according to the previously described standard method [16]. PCR amplification of the internal transcribed spacers (ITS1 - 5.8S rDNA - ITS2) region was carried out using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, as reported by White *et al*., [17]. The PCR products were purified using a PCR purification kit (Qiagen, Germany) and direct sequenced on both strands using the same primers.

PCR amplification of the D1 - D2 5' region of the large subunit (26S) of the rDNA was performed with the primers NL1 (5'-GCATATCAATAAGCG GAGGAAAAG -3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') as reported by Kurtzman and Robnett, [18].

For PCR-RFLP analysis, the PCR product(s) were digested with 1 U of one of the three different restriction enzymes, *Hha*I, *Hae*III or *Hin*FI (NEB, UK) [13] to completion. After the digested products were resolved and visualized, the patterns were analyzed using the Quantity One Program (Bio-Rad, USA).

To establish the likely identity of each isolate to the species level, the consensus sequences of the representative sample from each of seven groups of 799 yeast isolates plus the 36 mould isolates were searched against the NCBI GenBank database using the BLASTN program [19]. Sequences that showed the highest degree of similarity (assumed closest relatives) were retrieved from GenBank. The ITS sequence data of the representative of each group of moulds and yeasts have been deposited at GenBank with accession numbers: EU798694 (*Saccharomyces cerevisiae* isolate NN691), EU798695 (*Saccharomycopsis fibuligera* isolate NN2501), EU798697 (*Pichia anomala* isolate NN632), EU798698 (*Issatchenkia orientalis* isolate NN2573), EU798699 (*Tolulaspora delbrueckii* isolate NK2324), EU798700 (*Candida glabrata* isolate NK2012), EU798702 (*M. racemosus* isolate NN601), EU798703 (*Mucor hiemalis* isolate NN609), EU798704 (*Rhizopus microsporus* isolate NP101) and EU798705 (*R. oryzae* isolate NK208), while the D1 – D2 region of the 26S rDNA sequence for *P. anomola* isolate NN632 is EU798701.

Sensory evaluation

The flavour of the final *Sato* product was evaluated by sensory testing. In the first screening, *Sato* derived from 114 sources of *Loogpang* were evaluated and *Sato* derived from 19 *Loogpangs* were selected, with the rest being eliminated as they produced an odd or inferior flavour. From these 19 selected *Loogpang* derived *Sato* wines, four were further selected as those that produced *Sato* with favourable flavour by a group of three panelists. The final evaluation of *Sato* from the four selected *Loogpang* was performed by 10 panelists with previous experience in sensory analysis of *Sato* and wines, using the procedure modified from Karuwanna [9]. The subjects were asked to rate the global sensory quality by assigning up to 100 points across four parameters, in aspects of colour (10 points), odor (30 points), flavour (40 points) and impression (20 points). Randomized samples of 25 - 30 ml of each *Sato* were served in clear glasses, each identified to the panelists only as a three digit random number. Evaluations were conducted at 20 – 22°C.

Results

Collection of* Loogpang *samples and screening of starters suitable for* Sato *fermentation

In total, 114 samples of *Loogpang* were collected from 42 provinces located in the north (16), northeast (16) and central (10) regions of Thailand. Each *Loogpang* sample was added as a starter to steamed glutinous rice for *Sato* fermentation, as outlined above. During the first three days of solid-state fermentation, *Namtoi*, a liquid rich in glucose, was produced. From the preliminary examination of the ferments from the 114 *Loogpang*, 95 *Loogpang* were eliminated as they led to one or more poor productions of *Namtoi*, a low ethanol concentration or production of an odd flavour in the *Sato*. The capability of the remaining 19 *Loogpang* in *Sato* fermentation were then assessed by determination of the amount of released glucose in the *Namtoi*, the amount of ethanol produced and the amount of remaining glucose in the *Sato* and by evaluation of the quality of *Sato* by three panelists. From this screening, four *Loogpang* that led to a high liquefaction and glucose release into the *Namtoi*, high ethanol fermentation and production of high quality *Sato* with good flavour were selected. These were samples NN6 and NN25 from Nan province, NP1 from Nakorn Panom province and NK2 from Nongkhai province. The four selected *Loogpang* showed a high productivity of *Namtoi* (37 - 55 ml/ 50 g rice), except for NP1 *Loogpang* which produced only a moderate level of *Namtoi* (28 ml/50 g), and contained glucose at concentrations of 37.9 - 275.8 g/L. These *Loogpangs* led to production of *Sato* with a high ethanol concentration (8.1 - 14.2 % (v/v)) and relatively low remaining glucose levels of 1.55 - 6.30 mg/L.

***Isolation of moulds and yeasts from the selected sources of* Loogpang**

Each of the four selected *Loogpang* was used as a starter for *Sato* fermentation and the broth samples were taken at days 0, 3, 5, 7 and 9 of the fermentation process for the isolation of yeasts and moulds.

Moulds could only be isolated on day 0, at 9×10^3 to 4×10^5 CFU/g, and/or on day 3 (Figure 1, data not shown), with no moulds detected on or after day 3, except in sample NK2, where the moulds could still be detected until the third day of fermentation, possibly reflecting the higher initial amount of mould in this sample compared to the others.

Yeasts were isolated at day 0, at 3×10^5 - 3.2×10^6 CFU/g, and their amounts in all samples increased over the first five days and then either remained constant or slightly increased further from day 5 to day 9, reaching 4.3×10^7 - 5.2×10^9 CFU/g on day 9 (Figure 1, data not shown).

***Identification of the moulds and yeasts in the selected* Loogpang.**

Moulds

Morphological identification of the moulds in the four selected *Loogpang* revealed 36 different mould isolates in total that belonged to two genera, *Mucor* sp. and *Rhizopus* sp. They could be further divided into five probable species groups as follows: *Mucor hiemalis* (12 isolates), *M. racemosus* (17 isolates), *M. indicus* (three isolates; previously known as *Amylomyces rouxii*), *Rhizopus microsporus* (three isolates) and *R. oryzae* (one isolate), according to the keys of Samson *et al*. [11]. One mould from each species group was selected for confirmation of the identification by DNA sequencing and phylogenetic analysis based upon the ITS1 - 5.8S - ITS2 region of rDNA. The representative mould from each group was cultivated on PDA and the mycelia were harvested for DNA extraction. The purified PCR products were directly sequenced. Molecular phylogenetic analysis based upon neighbour joining distance analysis of these consensus sequences and their most similar sequences obtained from BLASTN searches of the NCBI database were performed (data not shown). The molecular identifications of likely mould species, or at least their closest known relatives, were all in agreement with the identifications for the same samples made by the conventional morphological methods (data not shown).

Yeasts

Because of the large number of the yeast isolates, the isolates were first grouped by the RFLP patterns of the PCR amplified ITS1-5.8S-ITS2 region of DNA. The PCR–RFLP patterns attained revealed seven clear different patterns and all isolates were thus grouped accordingly (Table 1). One yeast isolate from each group was selected as a representative of that group and was identified to likely species level by a combination of both conventional biochemical methods [14, 15] (data not shown) and by DNA sequencing of the ITS1-5.8S-ITS2 region [13] (Table 1). From the DNA sequencing results and analysis of the percentage sequence identity to known isolates in the NCBI database, the yeasts in groups 1-3 and 5-7 were identified as *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Pichia anomala*, *Issatchenkia orientalis*, *Tolulaspora delbrueckii* and *Candida glabrata,* respectively (Table 1). No sequence with more than 99% sequence similarity was found for group 4, but it showed 92% similarity to *P. anomala* as the closest sequence in the GenBank database. The D1 - D2 region of the 5' region of the 26S rDNA gene fragment was then PCR amplified and sequenced. The resulting sequence (GenBank accession code EU789701) showed 99% sequence similarity to *Pichia anomala,* which, using the established 99%

criteria for this gene fragment [12], makes the species group 4 also likely to be *P. anomala*, and thus represents a strain polymorphism from those in group 3. A similar strain polymorphism also has been reported in *Hamei*, an Indian rice wine starter [20].

Table 1. Grouping of the yeast isolates from the four selected *Loogpang*.

Gr.	No. of Isolate	Size of PCR product (bp)	DNA fragment (bp)			Species	% Identity*
			*Hin*FI	*Hae*III	*Hha*I		
1	1074	880	360, 350, 180	320, 230, 180, 130	385, 365	*Saccharomyces cerevisiae*	99
2	109	510	220, 140	380	300, 200, 170	*Saccharomycopsis fibuligera*	99
3	83	630	320, 270	630	630	*Pichia anomala*	100
4	9	650	350, 200	650	320, 310, 105	*Pichia anomala*	92**
5	8	500	280, 220	390	190	*Issatchenkia orientalis*	99
6	2	780	395	780	290, 350	*Tolulaspora delbrueckii*	100
7	105	600	285	230	400, 155	*Candida glabrata*	99

*The % identity in DNA sequence of the PCR product of the sample to the indicated species in the GenBank database.
** The D1 - D2 region of the 26S rRNA region showed a 99% DNA sequence identity to that of *P. anomala*.
GenBank accession codes for the isolates of this study are in the text.

Composition of the moulds and yeasts in the selected Loogpang

The composition of the moulds and the yeasts isolated during *Sato* fermentation using the four selected *Loogpang* are summarized in Table 2. Two genera of moulds were found; *Mucor* sp. and *Rhizopus* sp. It was noted that *Rhizopus* sp. was only detected in samples from the northeast region while *Mucor* sp. was found in all selected starters especially *M. racemosus*. Only yeasts in the class Hemiascomycetes, including *Saccharomyces* sp., *Saccharomycopsis* sp., *Candida* sp., *Issatchenkia* sp., *Tolulaspora* sp. and *Pichia* sp., were detected. It was also noted that *S. cerevisiae*, *Sm. fibuligera* and *P. anomala* were found in all samples (Table 2).

Table 2. Composition of moulds and yeasts during *Sato* production from each of the four selected *Loogpang*.

Sample location	Code	*Loogpang* sample	
		Mould	Yeast
North	NN6	*Mucor racemosus* *Mucor hiemalis*	*Saccharomycopsis fibuligera* *Pichia anomala*** *Saccharomyces cerevisiae*
	NN25	*Mucor racemosus* *Mucor indicus*	*Saccharomycopsis fibuligera* *Pichia anomala** *Isatchenkia orientalis* *Saccharomyces cerevisiae*
Northeast	NP1	*Mucor racemosus* *Rhizopus microsporus*	*Saccharomycopsis fibuligera* *Pichia anomala*** *Sacchromyces cerevisiae*
	NK2	*Mucor racemosus* *Mucor hiemalis* *Rhizopus oryzae*	*Saccharomycopsis fibuligera* *Pichia anomala*** *Torulaspora delbrueckii* *Candida glabrata* *Sacchromyces cerevisiae*

*Group 3, **Group 4 from Table 1

***Population dynamics of moulds and yeasts during Sato fermentation with the selected* Loogpang**

To understand the fermentation phenomenon of the *Loogpang*, the population dynamics of the moulds and the yeasts during *Sato* fermentation, together with the changes in the concentrations of glucose (or reducing sugar) and ethanol were determined. The population dynamics data were obtained from the mould and yeast isolates grown on selective media, RBA and YM agar plates, respectively, in combination with the yeast and mould identification data (Tables 1 & 2). The four selected *Loogpang* NN6, NN25, NP1 and NK2 were individually used as the starter for *Sato* fermentation and 200 µl of each sample were taken at day 0, 3, 5, 7 and 9 of the fermentation process and enumerated as described above. The yeasts were further tested in L-lysine medium to distinguish between *Saccharomyces* and non-*Saccharomyces* yeasts [14]. Basically, similar patterns of population dynamics were obtained during the fermentation with the selected *Loogpang* (Fig. 1). The moulds and the non-*Saccharomyces* yeasts were detected only in the early stage of the process (during solid state fermentation and the beginning of submerged fermentation) and *S. cerevisiae* became predominant, replacing the non-*Saccharomyces* yeasts, when the ethanol concentration reached approximately 3 - 6% (v/v) on day 3, except in the case of the NN25 starter where *I. orientalis* was detectable on day 5 and persisted until day 9 (Fig. 1B). The amount of glucose (or reducing sugar) increased in the early aerobic stage (until day 3), and then rapidly decreased during the anaerobic growth phase of the yeast *S. cerevisiae* (Fig. 1). This profile is in agreement with that of the liquefaction of glutinous rice by the amylolytic enzymes produced by moulds [21, 22] and *Sm. fibuligera* [23]. On day 6, *S. cerevisiae* started to utilize glucose and produced ethanol under the anaerobic conditions. Glucose (or reducing sugar) appeared to be generated continuously during the period of ethanol fermentation (a process called parallel combined fermentation by the combined actions of the

moulds in the remaining rice floating on the top of the fermentation broth and the yeasts and bacteria in the fermentation broth), since the ethanol level increased even after the glucose level had dropped to low levels and this was especially obvious with the NN6 and NK2 *Loogpang* (Fig. 1).

***Sensory evaluation of* Sato *fermented with the selected* Loogpang**

The *Sato* fermented with the four selected *Loogpang* was evaluated by sensory test using a panel of ten experts of *Sato* and wines. The results of this evaluation showed that the overall acceptance of *Sato* made with the NP1, NN6, NN25 and NK2 *Loogpang* and the two commercial *Sato* were largely comparable ($p>0.05$) (Table 3). However, the *Sato* produced with the NP1 *Loogpang* gained the highest scores of the sensory test in aspects of flavour, impression and total scores among the 4 selected *Loogpang* and two commercial *Sato*, suggesting that the NP1 *Loogpang* is the superior starter for the production of high quality *Sato*.

Table 3. Average sensory test scores for *Sato* produced from each of the four selected *Loogpang* and compared to those from two commercial *Sato* preparations.

	Average sensory test scores[1]				
	Colour (10)	**Odor** (30)	**Flavour** (40)	**Impression** (20)	**Total score** (100)
NP1	8.2 ± 0.8	22.9 ± 2.5	32.6 ± 3.5	16.1 ± 1.6	79.7 ± 5.7
NN6	7.9 ± 0.7	21.6 ± 3.5	27.5 ± 4.2	14.2 ± 1.9	71.2 ± 7.8
NK2	6.8 ± 1.3	21.8 ± 2.2	28.1 ± 4.1	14.0 ± 2.7	70.6 ± 8.8
NN25	8.1 ± 0.7	23.7 ± 2.2	27.9 ± 4.4	14.6 ± 1.8	74.3 ± 5.6
Com 1[2]	7.9 ± 0.6	22.3 ± 3.8	28.7 ± 3.2	15.4 ± 2.5	74.2 ± 8.2
Com 2[2]	7.5 ± 1.1	20.1 ± 5.7	28.1 ± 5.4	13.8 ± 3.2	69.4 ± 13.5

[1]The sensory taste was performed by a panel of ten *Sato* and wine experts. Data are shown as the mean ± 1 SD and are derived from three repeats. No means within a column are significantly different to the others (P>0.05).
[2]Commercial *Sato*.

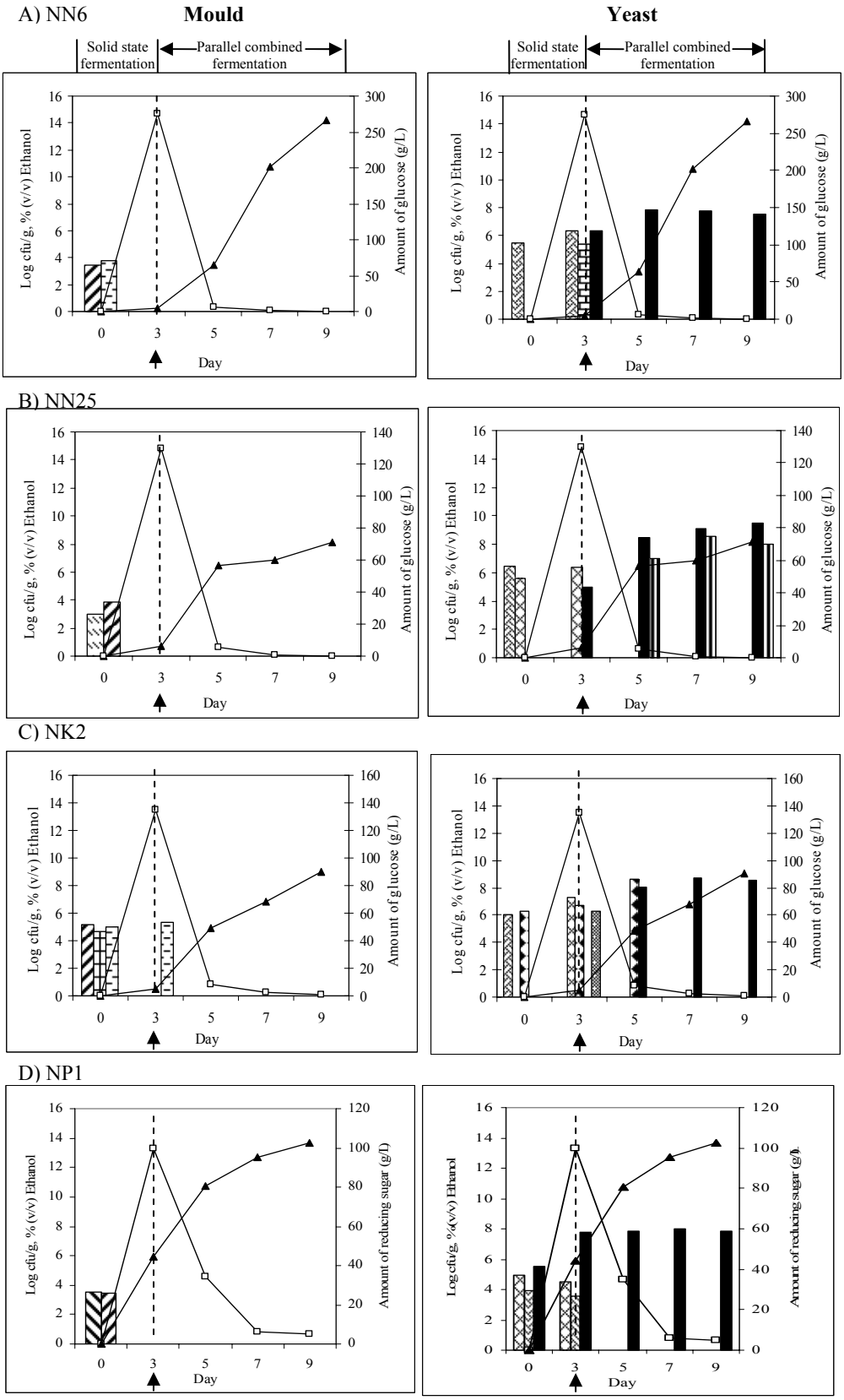

Figure 1. Population dynamics of moulds and yeasts during *Sato* production by four selected *Loogpang* samples.

(A) NN6, (B) NN25, (C) NK2 and (D) NP1. Population dynamics of moulds and yeasts were shown in left and right panels, respectively. *Mucor racemosus* (▨), *M. hiemalis* (▤), *M. indicus* (▧), *Rizopus oryzae* (▦), *R. oligosporus* (▩), *Saccharomyces cerevisiae* (■), *Saccharomycopsis fibuligera* (▩), *Pichia anomala* (group 3) (▩), *P. anomala* (group 4) (▩), *Candida glabrata* (▩), *Issatchenkia orientalis* (▩), *Torulaspora delbrueckii* (▩), % Ethanol (▲) and amount of reducing sugar (□).
Data are shown as the mean ± 1 SD and are derived from three repeats.
The arrow represents the addition of 200 ml of sterile water to begin parallel combined fermentation on day 3.

Discussion

The identification of the microorganisms in the selected sources of *Loogpang* revealed five species of moulds and seven species of yeasts in total. As best can be determined, this is the first report on the identification of moulds in *Loogpang* to the species level using molecular methods in combination with the conventional cultivation-dependent identification. The moulds isolated were all in the order Mucorales, as previously reported for other amylolytic rice fermentation starters [4, 8, 22, 24]. All analyzed yeast isolates were in the class Hemiascomycetes, as has also been found in previous studies of amylolytic starters [4, 8, 24, 25]. Thus, in terms of general mould and yeast compositions at the species level, *Sato* is broadly similar to the starter inoculums of other ethanol fermented Indo-Asian rice-based beverages [5, 22].

All analyzed yeast isolates have also been found in previous studies. For example, the yeasts isolated from *Marcha,* an Indian starter for rice wine, from the Himalayan region (Sikkim and Nepal), were identified as *S. bayanas*, *C. glabrata*, *P. anomala*, *Sm. fibuligera*, *Sm. capsularis* and *P. burtonii* [25]. The yeasts *S. cerevisiae*, *Sm. fibuligera*, *P. anomala* and *C. glabrata*, have been isolated from *Bhaati Jaanr*, a traditional fermented rice beverage of the Eastern Himalayas [4]. *Men*, a Vietnamese rice wine starter, contained *S. cerevisiae, P. anomala* and *C. glabrata* [8], while *S. cerevisiae*, *C. glabrata*. *P. anomala* and *I. orientalis* were the main yeasts found in the fermentation of *brem*, a traditional Balinese rice wine [7]. The most frequent yeast species associated with *Hamei* were *S. cerevisiae*, *P. anomala* and *Trichosporon* sp. [20].

The profiles of population dynamics together with enological parameters during *Sato* fermentation indicate the roles of the microbes on the fermentation. Moulds and the non-*Saccharomyces* yeasts could only be detected at the beginning of fermentation until the late stage of the solid state fermentation (day 3 for moulds and day 5 for the non-*Saccharomyces* yeasts with exception of that for *I. orientalis*). This coincided with the highest amount of glucose (or reducing sugar) being detected on day 3, which may suggest that the amylolytic enzymes secreted by moulds and *Sm. fibuligera* convert the starch in the glutinous rice into simple reducing sugars. The addition of water into the solid state culture on day 3 ended the solid state fermentation and began the parallel combined fermentation in which the moulds from the remaining rice floating on top of the fermentation broth converted starch into sugars simultaneously with the

submerged fermentation by yeasts and the lactic acid bacteria. After water was added on day 3, the amount of sugar sharply decreased until barely detectable by day 5. During the same period, the alcohol concentration increased dramatically to approximately 3.4 - 6.4% (v/v) (Fig. 1), at which the moulds and the non-*Saccharomyces* yeasts (except *I. orientalis*) started to disappear and were replaced by *S. cerevisiae* as the dominant yeast until the end of the fermentation. This was consistent with the continuing increase in ethanol concentration throughout the fermentation. The data is consistent with the role of *S. cerevisiae* in converting sugar into ethanol. Similar observations were also reported in other amylolytic starters [25, 26]. The ethanol level continued to increase even after the glucose level had dropped to a low level, presumably by the parallel combined fermentation. The increase was quite obvious in *Sato* produced from NN6 and NK2 *Loogpang* (Fig.1). The *S. cerevisiae* isolates from the NN6 *Loogpang* may be a higher ethanol producer than those from other *Loogpang* (Fig. 1). The rapid decrease in the number of non-*Saccharomyces* yeasts is most likely due to the toxicity of ethanol, however, it might also be the result of fermentative metabolites or killer-like toxins [27, 28, 29].

The yeast *I. orientalis* was not detected initially in *Sato* fermented with any of the four *Loogpang*, but was detectable at the mid-to-later fermentation stages (days 5 to 9) in the NN25 starter ferment along with *S. cerevisiae,* suggesting that *I. orientalis* is resistant to high ethanol concentrations. In wine fermentation, it has been reported to share the late fermentation stage with *S. cerevisiae* [30], and produces volatile compounds with a desirable aroma and flavour, such as 2-methyl-1-propanol and 3-methyl-1-butanol [30].

The lactic acid bacteria (LAB) found in amylolytic starters have been reported to not play any significant role in either the saccharification process or ethanol production [31]. They may, however, play a role in the taste and aroma of *Sato*. Contribution of the microorganisms identified in this study, as well as LAB, to the taste and aroma of *Sato* remains to be investigated.

Conclusion

The results obtained from this work on identifying a superior *Loogpang* starter, the NP1 *Loogpang*, and the understanding on the roles of major microbes (moulds and yeasts) during *Sato* fermentation are expected to serve as a platform for research into the improvement of the consistency of *Sato* quality produced between batches. The NP1 *Loogpang*, which was shown to be the most suitable source for producing high quality *Sato*, will provide a good source of microorganisms for starter. The composition of mixed pure culture starter based on the microbial composition of the NP1 *Loogpang* will be reported elsewhere.

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