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Yeast Diversity in Thai Traditional Fermentation Starter (Loog-pang)

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ABSTRACT

From 38 samples of loog-pang-kao-mag, 31 of them contained yeasts in the range of 3.9×10^4 - 2.9×10^7 cfu/g while 7 samples did not. In 19 samples of loog-pang-lao yeast numbers were 2.9×10^4 – 5.0×10^7 cfu/g. Forty-three isolates of yeast were obtained from all samples of loog-pang-kao-mag and 49 were obtained from loog-pang-lao. Among them, 31 isolates from loog-pang-kao-mag and 20 isolates from loog-pang-lao were identified as *Saccharomycopsis fibuligera.* The other yeast species presented in both types of loog-pang were *Pichia anomala* (8), *Issatchenkia orientalis* (6), *P. burtonii* (6), *P. fabianii* (4), *Candida rhagii* (4), *C. glabrata* (3), *Torulaspora globosa* (3), *P. mexicana* (2) and 1 isolate each of *P. heimii*, *Rhodotorula philyla*, *Saccharomyces cerevisiae*, *T. delbrueckii* and *Trichosporon asahii. S. fibuligera* was the single yeast species found in 22 samples (70.97%) of loog-pang-kao-mag and 7 samples (36.84%) of loog-pang-lao. In addition, 7 samples (22.58%) of loog-pang-kao-mag consisted of *S. fibuligera* together with 1-2 species of other yeast, while 9 samples (47.37%) of loog-pang-lao consisted of *S. fibuligera* and 1-4 other yeast species. Forty-six from 47 isolates of *S. fibuligera* revealed strong amylolytic activity, express as ratio of clear zone diameter and colony diameter on soluble starch agar, in the range of 1.93-3.25. However, most of them produced low ethyl alcohol, *i.e.*, less than 2 %v/v from 18 % glucose at 48 hours. On the contrary, other yeast species showed low amylolytic activity but high or moderately high in alcohol fermenting ability. Among the isolates that fermented high alcohol contents were *T. globosa* YKM032 (6.03%v/v), *I. orientalis* YMK036 (6.01%v/v), *P. burtonii* YKM034 (6.00%v/v) and *P. burtonii* YL048 (5.99%v/v). The only one isolate of *S. cerevisiae* produced relatively low ethyl alcohol at 4.68%v/v.

Key words: yeast, diversity, identification, traditional fermentation, starter, yeast cake, loog-pang

INTRODUCTION

"Loog-pang", commonly is known as "Chinese yeast cake" to the Western people, is a Thai term for dry form of "fermentation starter" for production of traditional fermented products from starchy raw materials, *i.e.*, *kao-mag* (alcoholic sweetened rice), *lao* (rice wine) and *num som sai chu* (vinegar) (Lotong, 1992). The use of this type of fermentation starter is believed to be originated in China and have been transferred to many countries in Asia. Various local names are used in Asian countries, such as *banh men* in Vietnam, *chu* in Chinese, *koji* in Japanese, *nuruk* in Korean, *murcha* in Indian, *ragi* in Indonesia, *ragi tapai* in Malaysia and *bubod* in Philippines. These starters are apparently mixed cultures of molds, yeasts and bacteria grown on rice or other cereals. In certain localities native herbs were added (Batra and Millner, 1974; Saono, 1982; Lotong, 1998 and Thanh *et al.*, 1999). *Saccharomycopsis fibuligera* is a common yeast species found in many starter cakes including

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loog-pang. While *Pichia anomala* is presented in *bubod*, *loog-pang* and *murcha*, *Candida* spp. are the common yeast in *ragi*. *Saccharomyces* spp. has been found only in some samples of *banh men*, *bubod*, *loog-pang* and *ragi* (Chatisantien, 1977; Chaowsungket, 1978; Dijen, 1972; Saono, 1982; Lotong, 1998 and Thanh *et al.*, 1999). Among microorganisms presented in these starter cakes only some species play important roles in product formation (Lotong, 1992; Haard et al. 1999). Though there are some reports concerning with microorganisms in loog-pang and their possible roles in product formation but only few isolates of microorganism were collected and maintained properly. From past to present, loog-pang is difficult to acquire because its preparation process is known only to certain household. Due to the limited knowledge some key microorganisms tend to resulted in decrease in the quality of loog-pang. Therefore, isolation, selection and preservation of the key microorganisms in loog-pang which could be used as pure cultures for production of fermented products are prime importance. This work reports the isolation and identification of yeasts from loog-pang, including their amylolytic activity and ethyl alcohol fermenting abilities.

MATERIALS AND METHODS

Sources of loog-pang

Thirty-eight samples of loog-pang-kao-mag and 19 samples of loog-pang-lao were collected from different provinces in Thailand. They were stored in refrigerator until use.

Isolation of yeast

To obtain as many yeast species as possible, isolation was carried out by 3 protocols namely, enrichment in acidified (pH 3.7-3.8) YM broth (0.3% yeast extract, 0.3% malt extract and 0.5% glucose), enrichment in YM broth containing 300 g/l glucose and preparation of fermented products from each type of loog-pang followed by isolation from fermented mash. Pure cultures obtained were preserved on YM agar and stored in a refrigerator.

Identification of yeast

Morphological, physiological and biochemical characteristics of yeast isolates were examined according to the standard methods as described by Yarrow (1998). Assimilation of carbon compounds were tested by API 20 C AuxTM (Bimerieux, France).

Standard plate count of yeast in loog-pang

Number of yeast in loog-pang was determined by standard plate count technique using YM agar and spread plate method. Colonies were counted after incubation for 2 days at room temperature.

Amylolytic activity

Active yeast culture grown on YM agar was point-inoculated on the surface of soluble starch agar (4 % soluble starch, 5 % yeast extract and 1.5 % agar) at 4 positions. Two replications were performed on 1 isolate. After incubation for 72 h at room temperature the culture plate was flooded with Lugol's iodine solution (2 g iodine, 2 g ammonium sulfate and 300 ml deionized water) for 1 min and the diameters of clear zone and colony were measured. The amylolytic activity was expressed as ratio of clear zone diameter to colony diameter.

Ethyl alcohol fermentation

Active yeast cultures grown on yeast extract peptone dextrose (YPD) agar (1 % yeast extract, 2% peptone, 2% glucose and 1.5% agar) for 24 h were transferred to 50 ml of YPD broth in 250 ml Erlenmeyer flasks and incubated on rotary shaker (170 rpm) at room temperature for 16-20 h. They were then used as inoculum by transferring appropriate amounts to 100 ml of YPD broth

containing 18 % glucose to obtain assigned initial cell concentration. The fermentation was carried out on a rotary shaker at 70-80 rpm at room temperature for 48 h. The growth was measured as optical density at 660 nm by spectrophotometer (Shimadzu UV-190, Japan). Ethyl alcohol concentration was measured by gas chromatography (Shimadzu GC-9A, Japan) using propanol as an internal standard.

RESULTS AND DISCUSSION

Standard plate count of yeast in loog-pang

A total of 38 samples of loog-pang-kao-mag and 19 samples of loog-pang-lao were collected from different provinces of Thailand. Most of the samples were collected from central and northeastern regions. All loog-pang-kao-mag, except in 7 samples without yeast, contained yeasts in the range of 3.9×10^4-2.9×10^7 cfu/g while in loog-pang lao they were 2.9×10^4 - 5.0×10^7 cfu/g. However, most samples contained 10^5-10^6 cfu/g (Table 1 and 2) which were similar to the earlier reports in *nurak* (Kim, 1968), *bubod* (Tanimura *et al*., 1978) and *ragi* (Hadisepoetro *et al*., 1977).

Isolation and identification of yeasts from loog-pang

The isolation of yeasts were carried out using 3 protocols, to obtain as many kinds of yeast as possible, resulted in 43 and 49 isolates from loog-pang-kao-mag and loog-pang-lao, respectively. Identification following taxonomic keys from "The Yeasts: a Taxonomic Study, 4th edition" (Kurtzman and Feel, 1998) revealed that most isolates obtained from loog-pang-kao-mag were *Saccharomyces fibuligera* (31 isolates) (Table 1). The remaining isolates were *Torulaspora globosa* (2), *Issatchenkia orientalis* (3), *P. burtonii* (2) and one isolate each was *C. rhagii*, *P. fabianii*, *P. anomala*, *Rhodotorula philyla* and *T. delbrueckii*. Many samples of loog-pang-kao-mag (22) contained only *S. fibuligera* while the other 7 samples consisted of *S. fibuligera* together with other 1-2 species of yeast. Only 2 samples did not have *S. fibuligera* but *T. delbrueckii* or *T. globosa* was presented (Table 3). In loog-pang-lao 20 from 49 isolates of yeast were *S. fibuligera* (Table 2). The other isolates were *P. anomala* (7), *P. burtonii* (4), *C. glabrata* (3), *C. rhagii* (3), *I. orientalis* (3), *P. fabianii* (3), *P. mexicana* (2), *P. heimii* (1), *T. globosa* (1) and *Trichosporon asahii* (1). It should be noted that only *S. fibuligera* was found in 7 samples while in other 9 samples contained *S. fibuligera* and other 1-4 yeast species. There were 2 samples that were devoid of *S. fibuligera* whereas *P. anomala* was found in the other 6 samples (Table 4). Surprisingly, *S. cerevisiae* was found only in 1 sample of loog-pang-lao, which may result from long term storage of loog-pang. However, this finding agrees with Chatisantien (1977) and Chaowsungket (1978). Many of the species reported in this work were also commonly found in other fermentation starters, for example, *S. fibuligera* and *I. orientalis* in starter cakes, *P. anomala* in *bubod*, *murcha* and *ragi*, *P. fabianii* in rice *koji* and *S. cerevisiae* in *banh men* and *ragi* (Dijen, 1972; Saono, 1982; Lotong, 1998 and Thanh *et al.*, 1999).

Amylolytic activity of yeast isolates obtained from loog-pang

Investigation on all isolates of *S. fibuligera* revealed strong amylolytic activity on starch agar, 1.93-3.25, expressed as ratio of clear zone diameter and colony diameter, except YKM025 that the ratio was 1.18. while the other yeast species showed relatively low activities (ratio as 1) and 2 isolates of *T. globosa* did not have activity at all (Table 1 and 2). The strong amylolytic activity of *S. fibuligera* agrees with the roles of this yeast in loog-pang on the hydrolysis of starch to sugar that has been indicated by many authors (Sukhumavasi *et al.*, 1975; Chatisantien, 1977 and Chaowsungket, 1978).

Table 1 Yeast in loog-pang-kao-mag.

Sample no.	No. of yeast (cfu/g)	Isolation				Identification	Amylase activity (diameter of clear zone/diameter of colony)	Ethanol conc. (%w/v)
		No. of isolate	Method	Incubation (day)	Yeast isolate code			
1	1.8×10^6	1	YM_{30}	2	YKM001	*Saccharomycopsis fibuligera*	2.84	2.89
2	3.3×10^6	1	KM	1	YKM002	*Torulaspora delbrueckii*	1.00	1.77
5	2.2×10^6	2	YM (pH 3.5)	2	YKM003	*Pichia fabianii*	1.00	1.79
			YM (pH 3.5)	2	YKM004	*S. fibuligera*	2.44	1.58
6	3.3×10^6	2	YM (pH 3.5)	2	YKM005	*S. fibuligera*	2.61	1.55
			YM (pH 3.5)	2	YKM006	*P. anomala*	1.00	3.95
13	3.0×10^7	3	YM (pH 3.5)	2	YKM007	*S. fibuligera*	2.58	1.14
			YM_{30}	2	YKM008	*S. fibuligera*	2.38	1.32
			YM_{30}	2	YKM009	*T. globosa*	0	3.69
15	7.2×10^6	1	YM (pH 3.5)	2	YKM010	*S. fibuligera*	2.33	1.80
18	0	0	-		-	-	-	-
19	2.5×10^5	1	KM	2	YKM011	*S. fibuligera*	2.20	1.48
20	0	0	-		-	-	-	-
21	1.7×10^6	1	YM_{30}	2	YKM012	*S. fibuligera*	2.42	2.18
23	0	0	-		-	-	-	-
24	3.9×10^4	1	KM	2	YKM014	*S. fibuligera*	2.39	1.67
25	2.2×10^6	2	YM (pH 3.5)	2	YKM015	*S. fibuligera*	2.30	1.27
			YM_{30}	2	YKM016	*Rhodotolura philyla*	1.00	0.04
27	5.1×10^6	1	YM_{30}	2	YKM017	*S. fibuligera*	2.64	1.32
31	3.3×10^7	2	YM_{30}	2	YKM018	*S. fibuligera*	2.50	1.04
			KM	2	YKM019	*S. fibuligera*	2.38	2.71
34	8.5×10^6	1	YM (pH 3.5)	2	YKM020	*S. fibuligera*	2.22	2.34
37	5.0×10^4	1	YM_{30}	2	YKM021	*S. fibuligera*	2.77	1.67
59	2.9×10^7	1	YM_{30}	2	YKM022	*S. fibuligera*	2.82	0.56
60	0	0	-		-	-	-	-
64	3.4×10^6	1	YM (pH 3.5)	2	YKM023	*Issatchenkia orientalis*	1.00	2.75
65	1.3×10^6	2	YM (pH 3.5)	2	YKM024	*Candida rhagii*	1.00	2.16
			YM (pH 3.5)	2	YKM025	*S. fibuligera*	1.18	1.01

Table 1 (continued).

Sample no.	No. of yeast (cfu/g)	Isolation				Identification	Amylase activity (diameter of clear zone/diameter of colony)	Ethanol conc. (%w/v)
		No. of isolate	Method	Incubation (day)	Yeast isolate code			
66	7.0×10^6	1	YM (pH3.5)	2	YKM026	*S. fibuligera*	2.53	2.40
67	0	0	-		-	-	-	-
68	4.5×10^5	1	YM_{30}	2	YKM027	*S. fibuligera*	2.54	1.83
69	0	0	-		-	-	-	-
70	0	0	-		-	-	-	-
71	1.7×10^5	1	KM	2	YKM028	*S. fibuligera*	2.19	1.36
74	7.2×10^6	1	YM (pH 3.5)	2	YKM029	*S. fibuligera*	2.76	0.99
77	4.9×10^6	1	YM_{30}	2	YKM030	*S. fibuligera*	2.59	0.96
78	4.2×10^6	2	YM (pH 3.5)	2	YKM031	*S. fibuligera*	2.45	0.93
			YM_{30}	2	YKM032	*T. globosa*	0	4.76
79	6.7×10^5	1	KM	3	YKM033	*S. fibuligera*	2.03	1.94
84	7.0×10^5	5	YM_{30}	2	YKM034	*P. burtonii*	1.00	4.74
			YM (pH 3.5)	2	YKM035	*S. fibuligera*	2.25	1.81
			KM	7	YKM036	*I. orientalis*	1.00	4.75
			KM	7	YKM037	*P. burtonii*	1.00	2.46
			KM	7	YKM038	*I. orientalis*	1.00	2.71
22	7.4×10^6	1	YM_{30}	2	YKM039	*S. fibuligera*	1.93	0.93
39	9.0×10^4	1	YM_{30}	2	YKM040	*S. fibuligera*	2.33	0.84
40	1.0×10^4	1	YM_{30}	2	YKM041	*S. fibuligera*	1.98	1.39
63	5.4×10^6	1	YM_{30}	2	YKM042	*S. fibuligera*	3.06	0.90
72	6.8×10^5	1	YM_{30}	2	YKM043	*S. fibuligera*	3.25	0.67
73	5.9×10^5	1	YM_{30}	2	YKM044	*S. fibuligera*	2.79	0.79

Remark: YM (pH 3.5) = Enrichment in acidified YM broth (pH 3.5).
YM_{30} = Enrichment in acidified YM broth containing 30% glucose.
KM = Preparing alcoholic sweeten rice fermentation (kao-mag) using loog-pang-kao-mag and isolated from kao-mag.

Table 2 Yeast in loog-pang-lao.

Sample	No. of yeast (cfu/g)	Isolation				Identification	Amylase activity (diameter of clear zone/diameter of colony)	Ethanol conc. (%w/v)
		No. of isolate	Method	Incubation (day)	Yeast isolate code			
3	9.7×10^5	4	YM (pH 3.5)	2	YL001	*Pichia anomala*	1.00	3.12
			YM (pH 3.5)	2	YL002	*P. anomala*	1.00	3.51
			YM (pH 3.5)	2	YL003	*Saccharomycopsis fibuligera*	2.71	0.39
			L	3	YL004	*Saccharomyces cerevisiae*	1.00	3.70
4	1.3×10^6	2	YM_{30}	2	YL005	*S. fibuligera*	2.33	0.56
			YM (pH 3.5)	2	YL006	*S. fibuligera*	2.11	0.62
7	2.7×10^5	1	YM (pH 3.5)	2	YL007	*S. fibuligera*	2.08	0.91
12	4.3×10^6	1	YM (pH 3.5)	2	YL008	*S. fibuligera*	2.47	0.73
14	8.8×10^6	2	L	3	YL009	*S. fibuligera*	2.64	0.82
			L	7	YL010	*P. anomala*	1.00	2.50
17	5.8×10^6	1	L	3	YL011	*S. fibuligera*	2.30	0.52
26	3.8×10^6	3	L	3	YL012	*Candida glabrata*	1.00	1.91
			YM (pH 3.5)	2	YL013	*S. fibuligera*	2.78	1.47
			L	3	YL014	*S. fibuligera*	2.24	1.31
28	1.8×10^6	2	YM (pH 3.5)	2	YL015	*S. fibuligera*	3.13	1.53
			YM (pH 3.5)	2	YL016	*C. glabrata*	1.00	2.85
30	1.8×10^7	3	YM_{30}	2	YL017	*Torulaspora globosa*	1.00	3.06
			YM (pH 3.5)	2	YL018	*Tichosporon asahii*	1.00	0
			L	1	YL019	*S. fibuligera*	2.47	0.49
33	5.00×10^7	2	YM_{30}	2	YL020	*P. anomala*	1.00	2.25
			L	3	YL021	*P. heimii*	1.00	2.59
48	3.00×10^5	1	L	7	YL022	*S. fibuligera*	2.35	1.85
57	1.20×10^4	1	YM (pH 3.5)	2	YL023	*S. fibuligera*	2.56	2.05
58	3.00×10^6	2	YM_{30}	2	YL024	*P. anomala*	1.00	4.17
			YM (pH 3.5)	2	YL025	*S. fibuligera*	2.32	1.96
81	4.60×10^6	5	YM (pH 3.5)	2	YL026	*S. fibuligera*	2.11	1.72

Table 2 (continued).

Sample	No. of yeast (cfu/g)	Isolation				Identification	Amylase activity (diameter of clear zone/diameter of colony)	Ethanol conc. (%w/v)
		No. of isolate	Method	Incubation (day)	Yeast isolate code			
			YM (pH 3.5)	2	YL027	*C. rhagii*	1.00	2.34
			L	1	YL028	*S. fibuligera*	2.75	1.22
			L	10	YL029	*C. rhagii*	1.00	1.69
			L	10	YL030	*P. burtonii*	1.00	2.53
83	1.46×10^6	3	YM (pH 3.5)	2	YL031	*S. fibuligera*	2.24	1.49
			YM (pH 3.5)	2	YL032	*P. fabianii*	1.00	1.83
			L	3	YL033	*P. anomala*	1.00	2.30
85	4.02×10^6	4	YM (pH 3.5)	2	YL034	*S. fibuligera*	2.57	1.54
			YM (pH 3.5)	2	YL035	*C. rhagii*	1.00	2.64
			L	3	YL036	*P. mexicana*	1.00	2.85
			L	3	YL037	*Issatchenkia orientalis*	1.00	2.97
86	6.60×10^6	5	YM_{30}	2	YL038	*S. fibuligera*	2.36	1.01
			YM_{30}	2	YL039	*P. fabianii*	1.00	3.00
			L	3	YL040	*I. orientalis*	1.00	3.20
			L	3	YL041	*P. fabianii*	1.00	2.91
			L	3	YL042	*C. glabrata*	1.00	2.38
87	5.42×10^6	6	YM_{30}	2	YL043	*P. burtonii*	1.00	2.56
			YM (pH 3.5)	2	YL044	*P. mexicana*	1.00	2.84
			L	1	YL045	*P. burtonii*	1.00	2.56
			L	3	YL046	*P. anomala*	1.00	3.57
			L	3	YL047	*I. orientalis*	1.00	4.04
			L	3	YL048	*P. burtonii*	1.00	4.73
49	1.33×10^6	1	YM_{30}	2	YL049	*S. fibuligera*	3.07	1.91

Remark: YM (pH 3.5) = Enrichment in acidified YM broth (pH 3.5).
YM_{30} = Enrichment in acidified YM broth containing 30% glucose.
L = Preparing rice wine (lao) using loog-pang-lao and isolation from fermenting mash

Table 3 Summary of yeast species in loog-pang-kao-mag.

Yeast	No. of samples
S. fibuligera	22
S. fibuligera + *T. globosa*	2
S. fibuligera + *C. rhagii*	1
S. fibuligera + *P. anomala*	1
S. fibuligera + *P. fabianii*	1
S. fibuligera + *R. philyla*	1
S. fibuligera + *P. burtonii* + *I. orientalis*	1
I. orientalis	1
T. delbrueckii	1

Table 4 Summary of yeast species in loog-pang-lao.

Yeast	No. of samples
S. fibuligera	7
S. fibuligera + *C. glabrata*	2
S. fibuligera + *P. anomala*	2
S. fibuligera + *P. anomala* + *S. cerevisiae*	1
S. fibuligera + *T. globosa* + *T. asahii*	1
S. fibuligera + *C. rhagii* + *P. burtonii*	1
S. fibuligera + *P. anomala* + *P. fabianii*	1
S. fibuligera + *C. rhagii* + *P. mexicana* + *I. orientalis*	1
S. fibuligera + *C. glabrata* + *P. fabianii* + *I. orientalis*	1
P. anomala + *P. heimii*	1
P. anomala + *P. burtonii* + *P. mexicana* + *I. orientalis*	1

Ethyl alcohol fermentation of yeast isolates obtained from loog-pang

The study on ethyl alcohol fermentation in 18% glucose medium using shaking cultivation at room temperature after 48 h of fermentation revealed that most isolates of *S. fibuligera* produced relatively low ethyl alcohol (less than 2%v/v). Eleven isolates of this species produced 2.01-3.00 %v/v whereas 3 isolates produced more than 3.00%v/v, *i.e.*, YMK001 (3.66%v/v), YMK019 (3.43%v/v) and YMK026 (3.04%v/v). Most of the remaining yeast species produced ethyl alcohol less than 4%v/v and 2 isolates, *i.e.*, *R . philyla* and *T. asahii* could not ferment. High ethyl alcohol fermenting yeast were found in seven isolates i.e. *P. anomala* YL024, , *I. orientalis* YL047, *P. anomala* YKM006, *T. globosa* YKM009, *P. anomala* YL046, *P. anomala* YL002 and *I. orientalis* YL040 at 5.28, 5.11, 5.00, 4.67, 4.52, 4.44 and 4.05%v/v, respectively. The isolates that produced exceptionally high ethyl alcohol were *T. globosa* YKM032 (6.03%v/v), *I. orientalis* YMK036 (6.01%v/v), *P. burtonii* YKM034

(6.00%v/v) and *P. burtonii* YL048 (5.99%v/v). The only one isolate of *S. cerevisiae* produced ethyl alcohol at 4.68%v/v. Yeast species that produce low concentration of ethyl alcohol might have another role in contribution of desirable flavor of the products.

In order to maintain diversity of these yeasts, all isolates were preserved by lyophilization and cryopreservation for further studies.

CONCLUSIONS

The results of this work showed that most samples of loog-pang-kao-mag and loog-pang-lao comprised of *S. fibuligera,* which showed strong amylolytic activity. Its character related to the role of this yeast in starch hydrolysis during product formation. About half of the samples contained only *S. fibuligera.* This may due to low quality of these loog-pangs and/or long term storage in undesirable conditions. Though most of *S. fibuligera* isolates could produce only low concentration of ethyl alcohol they might contribute to a certain extent on ethyl alcohol production. Moreover, the results of this work indicated that *S. cerevisiae* might not be the main ethyl alcohol producer in loog-pang but other yeast species could be the major players, for example *T. globosa, I. orientalis, P. burtonii* and *P. anomala*.

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