

Research Article

Isolation and identification of killer yeast from fermented vegetables

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Abstract

Nine samples of fermented vegetables (pak-sein, bamboo shoot, sator and cabbage) taken from Pattani and Narathiwat provinces, were examined. The fermented liquid from these samples had pH and NaCl concentration ranges between 3.3-3.9 and 2.4-8.2%, respectively. Eighty two yeast isolates were collected for the killer activity screening against five sensitive reference strains, *Candida tropicalis* TISTR 5045, *Hansenula anomala* TISTR 5113, *Saccharomyces cerevisiae* TISTR 5020, *Saccharomyces cerevisiae* TISTR 5055 and *Torulopsis glabrata* TISTR 5241 by growth inhibition test on YPD agar plate supplemented with 0.003% methylene blue.

Only 13 yeast isolates were killer-sensitive and these were separated into two groups. Isolates W02, W03, W07, W09 and W10 showed killer activity with all five sensitive reference strains, while other isolates W06, C07, C09, C12, B17, B18 and B19 showed killer activity to only four sensitive reference strains, except C10. The isolates showing growth inhibition against food pathogenic bacteria (*Escherichia coli* TISTR 887, *Salmonella typhimurium* TISTR 292, *Staphylococcus aureus* TISTR 118 and *Bacillus cereus* TISTR 868) were W02, W09, W03 and W10. The clear zones of inhibition were about 9.2, 11.0, 6.7 and 9.0 mm, respectively and killer activities of inhibition were about 56.4, 63.5, 38.4 and 48.8 %, respectively. All killer yeasts were identified and revealed as *Candida krusei* using biochemical test.

Keywords: screening, growth inhibition, *Candida krusei*, pathogenic bacteria, Thailand.

Introduction

The presence of yeasts in different kinds of fermented food is common. The production of antimicrobically active toxins or killer yeasts in fermented food is interesting from a biotechnology and food safety perspective [1, 2, 3]. The killer phenomenon in yeast cells was first reported by Bevan and Makower [4]. Killer yeasts can produce toxin proteins or glycoproteins (so-called killer toxins or killer proteins) that can be lethal to susceptible yeast, fungi and bacteria strains [5, 6, 7, 8, 9, 10]. They have been isolated from environmental, clinical, industrial and agricultural sources [2, 11, 12].

Toxin can be interesting as a tool for the development of yeast-based biocontrol in several biotechnological applications such as, use as a model system in fundamental research for studying the mechanisms of regulation of eukaryotic polypeptide processing, secretion and receptor binding [13, 14]. They have also been shown to have inhibitory effects on some wood decay and plant pathogenic fungi [10, 12, 15]. In the medical field, killer yeasts have found application such as, killer yeast from natural environments against some medicinal *Candida* species [16]. In the food industry, killer characteristics can be conferred into yeast strains. The use of killer yeasts as starter culture could protect the product against spoilage yeasts. They have also been considered useful in biological control of undesirable yeasts in the preservation of food [17, 18, 19]. For example, *C. tropicalis* showed inhibitory effects against *S. cerevisiae* in contaminated starter culture of the Turkish baking industry [20] and *Kluyveromyces phaffii* DBVPG 6076 killer toxin against apiculate wine yeast from grape samples [7].

There is little work shown in the literature on the inhibitory effects of killer yeasts against food pathogenic bacteria. Thus, the aim of the present work was to study the killer activity and inhibition of food pathogenic bacteria by yeast strains isolated from fermented vegetables.

Materials and Methods

Fermented vegetables

Nine samples of fermented vegetables, 2 samples each of Pak-Sien (*Cleome ruidosperma* DC.), Pickled stink bean : stor (*Parkia speciosa* Hassk.) and Cabbage (*Brassica oleracea* L.) and 3 samples of Bamboo shoot (*Phyllostachys sp.*) product were purchased from local markets in Pattani and Narathiwat provinces (Table 1). They were brought to the laboratory at room temperature (30±2°C) for determination of pH using pH meter and concentration of NaCl using salinometer.

Culture media

YPD broth (1.0% yeast extract, 2.0% peptone, 2.0% glucose, adjusted with 1N HCl to pH 5.0) was used for killer toxin production. YPD-MB (YPD containing 2.0% agar and 0.003% methylene blue) [20] was used for determination of killer activity.

Isolation of yeasts

The samples were streaked on YPD agar medium with pH 5.0 and incubated at 30°C for 1-2 days. The single colonies were randomly picked and the isolated cultures were purified by representative successive sub-culturing in the same medium. After microscopic examination, purified cultures were grown on slants and stored at 4°C.

Table 1. List of fermented vegetable samples.

Fermented vegetables	Scientific name	Location	pH	Concentration of NaCl (%)	Isolate
Pak-sien	<i>Cleome rutidosperma</i> DC.	Pattani	3.3	2.4	W01-W10
Pak-sien	<i>Cleome rutidosperma</i> DC.	Narathiwat	4.0	2.6	W11-W20
Stink bean	<i>Parkia speciosa</i> Hassk.	Narathiwat	3.9	7.8	S01-S10
Stink bean	<i>Parkia speciosa</i> Hassk.	Narathiwat	3.5	8.2	S11-S18
Cabbage	<i>Brassica oleracea</i> L.	Pattani	3.7	4.2	C01-C12
Cabbage	<i>Brassica oleracea</i> L.	Pattani	3.7	4.0	C13-C20
Bamboo shoot	<i>Phyllostachys</i> sp.	Pattani	3.8	3.8	B01-B08
Bamboo shoot	<i>Phyllostachys</i> sp.	Pattani	3.7	3.8	B09-B16
Bamboo shoot	<i>Phyllostachys</i> sp.	Pattani	3.4	6.0	B17-B24

Screening for killer activity strains

The isolated yeasts from fermented vegetables were screened for killer activity. The killer activity was determined by killer-zone assay in petri dish using YPD-MB [3, 10, 20, 21] buffered to pH 5.0 with 1 N HCl and incubated at 30°C. The single loop of killer-sensitive *Candida tropicalis* TISTR 5045, *Hansenula anomala* TISTR 5113, *Saccharomyces cerevisiae* TISTR 5020, *Saccharomyces cerevisiae* TISTR 5055 and *Torulopsis glabrata* TISTR 5241 were cultivated in YPD broth medium for 24 hr at 30°C. The suspensions were swabbed in YPD-MB plates and incubated for 24-48 hrs until colonies were formed. The strains to be tested were then streak inoculated on the surface of the agar and incubated at 30°C for 24-48 hrs. The killer activity was determined by the occurrence of methylene blue strained dead cells, surrounding the growth of the yeast strains tested for their killer properties. The diameter of the inhibition zone was used as a measure of the yeast killer activity.

Killer activity on food pathogenic bacteria

The killer toxin assay was based on the well-tested method in nutrient agar plate (NA), as described by Polonelli and Morace [5]. The suspension, 10⁵ CFU/ml, of each gram-negative food pathogenic bacteria (*Escherichia coli* TISTR 887, *Salmonella typhimurium* TISTR 292) and each gram-positive food pathogenic bacteria (*Staphylococcus aureus* TISTR 118, *Bacillus cereus* TISTR 868) was swabbed on the NA plate and 40 µl of killer toxin dropped on the surface of Whatman paper discs which were cut into round circles with a diameter of 6 mm for assay killer activity. After the incubation of plates for 24-48 hrs at 35°C, the diameter of the inhibition zone was considered as a measure of inhibition.

Identification of killer yeast

Identification of strains was performed with the API ID 32 C kit (Bio Merieux, France) in accordance with the manufacturer's instructions. The different physiological tests were

assimilation of Sorbitol, D-Xylose, Ribose, Glycerol, Rhamnose, Palatinose, Erythritol, Melibiose, Gulcuronate, Melezitose, Gluconate, Levulinate, Glucose, Sorbose, Glucosamine, Galactose, Actidione, Saccharose, N-Acetyl-glucosamine, DL-Lactate, L-Arabinose, Cellobiose, Raffinose, Maltose, Trehalose, 2-Keto-gluconate, α -Methyl-D-glucoside, Mannitol, Lactose and Inositol.

Results and Discussion

Fermented vegetables

Nine samples of fermented vegetables had pH between 3.3-4.0 and concentration of NaCl between 2.4-8.2 % (Table 1). Eighty two representative yeasts isolates of fermented vegetables. W01-W20, S01-S18, C01-C20 and B01-B24 were collected from Pak-sein, Sator, Cabbage and Bamboo-shoot, respectively.

Screening of killer yeast against sensitive yeasts

A total 82 yeast strains were tested for killer activity in YPD-MB medium and a bluish-coloured zone of dead sensitive yeast strains was apparent surrounding killer yeast colonies after staining with methylene blue (Fig. 1). All strains of yeast isolates behaved as inhibitors, but only 13 of the 82 yeast strains tested in this study i.e. W02, W03, W06, W07, W09, W10, C07, C09, C10, C12, B17, B18 and B19 were separated into two groups. Isolates W02, W03, W07, W09 and W10 showed the highest inhibition of 5 sensitive yeast strains and W06, C07, C09, C12, B17, B18 and B19 showed killer activity to 4 sensitive yeast strains except C10 (Table 2), while, 69 isolates showed killer activity against only 3 sensitive yeast strains or lower. Similar to the results reported by Thaniyavarn and Thammarate [1], the strains of *Hansenula* sp., *Kloeckera* sp. and *Trichosporon* sp. showed killer activity against *Torulopsis glabrata*. The killer yeast strains of *C. tropicalis*, *Debaryomyces hansenii*, *Pichia anomala* and *Kluyveromyces marxianus* are able to kill *Saccharomyces cerevisiae* [3, 20, 21, 22].

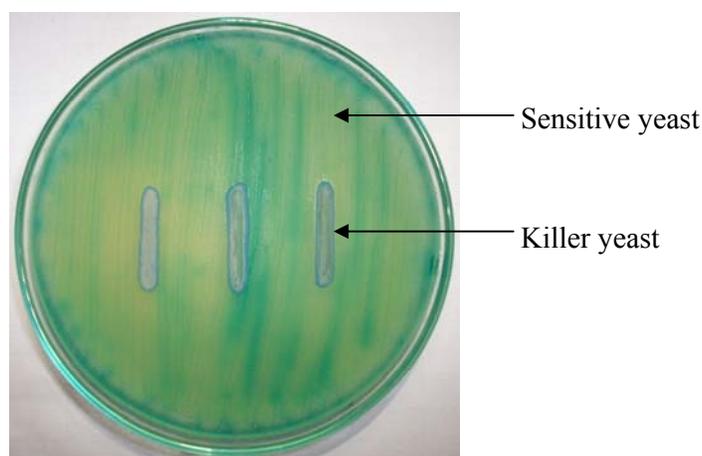


Figure 1. Inhibition zones of sensitive yeast strains surrounding the growth of killer yeast strains in YPD-MB medium.

Table 2. Killer yeast isolate inhibition reference strain.

Yeast isolate	Killer activity				
	<i>S. cerevisiae</i> TISTR 5020	<i>C. tropicalis</i> TISTR 5045	<i>H. anomala</i> TISTR 5113	<i>T. glabrata</i> TISTR 5241	<i>S. cerevisiae</i> TISTR 5055
W02	+ + +	+ + +	+ + +	+ + +	+ + +
W03	+ + +	+ + +	+ + +	+ + +	+ + +
W06	+ + +	+ + +	- - -	+ + +	+ + +
W07	+ + +	+ + +	+ + +	+ + +	+ + +
W09	+ + +	+ + +	+ + +	+ + +	+ + +
W10	+ + +	+ + +	+ + +	+ + +	+ + +
C07	- - -	+ + +	+ + +	+ + +	- - -
C09	+ + +	+ + +	+ + +	- - -	+ + +
C10	+ + +	+ + +	+ + +	- - -	+ + +
C12	+ + +	+ + +	+ + +	- - -	+ + +
B17	+ + +	- - -	+ + +	+ + +	+ + +
B18	+ + +	- - -	+ + +	+ + +	+ + +
B19	+ + +	- - -	+ + +	+ + +	+ + +

Remarks: + + + = Positive activity

- - - = Negative activity

Screening and identification of killer yeast against food pathogenic bacteria

It was also determined that only 13 yeast strains had effective response of killer toxin activity against food pathogenic bacteria. All pathogenic bacteria in food showed sensitivity to killer yeasts. Killer yeast strains W02, W03, W09 and W10 could inhibit *E. coli*, *Salmonella typhimurium*, *S. aureus* and *B. cereus*. The clear zones of inhibition were about 9.2, 11.0, 6.7 and 9.0 mm, respectively (Table 3). The killer activity could inhibit *E. coli* at 56.4%, *Salmonella typhimurium* at 63.5%, *S. aureus* at 38.4% and *B. cereus* at 48.8%, respectively (Table 4). Polonelli and Morace [5] studied yeast strains which showed killer activity against bacteria species such as, *Staphylococcus aureus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella oxytoca*, yeast and mould. The assimilation of carbohydrate test on 6 selected isolates, W02, W03, W06, W07, W09 and W10, were identified as *Candida krusei* by the API ID 32 C system (Table 5) which has been found to dominate in several different types of indigenous fermented food [23, 24, 25].

Table 3. The diameter of clear zones of killer toxin on food pathogenic bacteria.

Killer yeast	Clear zone (mm)			
	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>
W02	9.2 ± 0.79	9.3 ± 0.76	6.3 ± 0.58	6.6 ± 0.52
W03	8.8 ± 0.29	8.3 ± 0.58	6.7 ± 0.58	6.8 ± 0.72
W06	6.5 ± 0.00	9.5 ± 0.87	6.0 ± 0.00	8.7 ± 0.58
W07	6.3 ± 0.29	8.2 ± 1.44	6.0 ± 0.00	7.0 ± 1.00
W09	6.5 ± 0.00	11.0 ± 0.87	6.0 ± 0.00	9.0 ± 1.32
W10	6.5 ± 0.00	10.7 ± 0.29	6.0 ± 0.00	9.0 ± 1.00
C07	6.0 ± 0.00	6.5 ± 0.50	6.0 ± 0.00	0.0 ± 0.00
C09	6.0 ± 0.00	6.3 ± 0.29	6.0 ± 0.00	0.0 ± 0.00
C10	6.0 ± 0.00	6.3 ± 0.29	6.0 ± 0.00	0.0 ± 0.00
C12	6.0 ± 0.00	6.3 ± 0.29	6.0 ± 0.00	0.0 ± 0.00
B17	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	0.0 ± 0.00
B18	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	0.0 ± 0.00
B19	6.0 ± 0.00	6.0 ± 0.00	6.0 ± 0.00	0.0 ± 0.00

Table 4. Killer activity of selected yeasts against food pathogenic bacteria.

Killer yeast isolate	Killer activity (%)			
	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>
W02	56.4 ± 5.82	53.8 ± 4.41	36.4 ± 3.35	35.7 ± 3.82
W03	54.3 ± 0.72	48.1 ± 3.55	38.4 ± 7.33	37.1 ± 3.60
W06	40.0 ± 1.50	54.8 ± 5.11	34.5 ± 5.32	47.1 ± 5.88
W07	39.0 ± 2.37	47.1 ± 6.29	34.5 ± 2.47	38.0 ± 3.32
W09	40.0 ± 0.81	63.5 ± 2.77	34.5 ± 2.47	48.8 ± 7.91
W10	40.0 ± 0.81	61.7 ± 1.42	34.5 ± 2.47	48.8 ± 5.10
C07	36.9 ± 3.25	37.5 ± 1.58	34.5 ± 2.18	0.0 ± 0.00
C09	36.9 ± 3.25	36.5 ± 0.27	34.5 ± 2.18	0.0 ± 0.00
C10	36.9 ± 3.25	36.5 ± 0.27	34.5 ± 2.18	0.0 ± 0.00
C12	36.9 ± 3.25	36.5 ± 0.27	34.5 ± 2.18	0.0 ± 0.00
B17	36.9 ± 1.73	34.6 ± 1.84	34.5 ± 1.04	0.0 ± 0.00
B18	36.9 ± 1.73	34.6 ± 1.84	34.5 ± 1.04	0.0 ± 0.00
B19	36.9 ± 1.73	34.6 ± 1.84	34.5 ± 1.04	0.0 ± 0.00

Control: 70% alcohol.

Table 5. Assimilation of carbohydrate by yeast strains isolated from fermented vegetables.

Assimilation	Reaction					
	W02	W03	W06	W07	W09	W10
Sorbitol	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-
Ribose	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-
Palatinose	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-
Melibiose	-	-	-	-	+	-
Gulcuronate	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-
Levulinate	-	+	+	+	-	-
Glucose	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-
Glucosamine	-	-	-	-	-	-
Galactose	-	-	-	-	-	-
Actidione	-	-	-	-	-	-
Saccharose	-	-	-	+	-	-
N-Acetyl-glucosamine	+	+	+	+	+	+
DL-Lactate	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Maltose	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
2-Keto-gluconate	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	-
Mannitol	+	-	-	-	-	-
Lactose	-	-	-	-	-	-
Inositol	-	-	-	-	-	-

Remarks: + = Positive reaction - = Negative reaction

Conclusions

Eighty two yeast strains in fermented vegetables from Pattani and Narathiwat provinces were isolated. Thirteen killer yeast strains, W02, W03, W06, W07, W09, W10, C07, C09, C10, C12, B17, B18 and B19 could inhibit 4 to 5 sensitive yeast strains. Only six isolates of these, W02, W03, W06, W07, W09 and W10, showed inhibition of food pathogenic bacteria and these were identified as *Candida krusei*.

Future investigation should be focused on the application of killer toxins or killer yeasts for the preservation and control of food pathogenic microorganisms.

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