ORIGINAL ARTICLE

Induction and inhibition of film yeast from fermented bamboo shoot by seasoning plants

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Abstract

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Three samples of fermented bamboo shoot taken from a village in Amphur Kokpho, Pattani Province, were microbiologically examined. Total viable count was between at 10^4-10^5 cfu/ml while pH range was between 3.4-4.4. Isolation and identification of film yeast on surface of fermented liquid revealed *Saccharomyces cerevisiae* J1, *Candida krusei* J2 and *Candida krusei* J3. When film yeast was cultivated in liquid culture with different NaCl concentrations (0, 2.5, 5 and 7.5% (w/v)), all species tolerated 2.5% NaCl addition. However, growth decreased depending on NaCl concentration. *S. cerevisiae* J1 grew faster than *C. krusei* J2 and *C. krusei* J3. The cultivation of film yeast in medium with different agar concentrations (0.3, 0.5, 1 and 1.5% (w/v)) within 24 h showed that 0.3% was the optimal agar concentration. Seasoning plants (garlic, ginger, galangal, lemon grass, lesser galangal, clove, kaffir lime, garcinia and shallot) were extracted with water (3% (w/v)) and tested for growth inhibition. Results showed the clove extract inhibited all yeast strains within 12 h and after that the efficiency of inhibition was decreased. At low concentration of 0.75% (w/v) clove extract could inhibit film yeast in fermented bamboo shoot.

Key words : bamboo shoot, film yeast, seasoning plant, inhibition

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บทคัดย่อ

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การเกิดและการยับยั้งฟิล์มยีสต์จากหน่อไม้ดองด้วยพืชปรุงแต่งกลิ่นรส

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การวิเคราะห์ปริมาณจุลินทรีย์ในน้ำหมักหน่อไม้ดองจากการหมักของชาวบ้าน จำนวน 3 ตัวอย่าง จากอำเภอโคกโพธิ์ จังหวัดปัตตานี พบว่า มีปริมาณจุลินทรีย์ทั้งหมดและพีเอชอยู่ในช่วง 10^4-10^5 cfu/ml และ 3.4-4.4 ตามลำดับ สามารถคัดแยกและจัดจำแนกฟิล์มยีสต์ที่ผิวน้ำหมักได้เป็น *Saccharomyces cerevisiae* J1, *Candida krusei* J2 และ *Candida krusei* J3 เมื่อศึกษาการเจริญเติบโตในอาหารเหลวที่เติม NaCl ที่ความเข้มข้นต่าง ๆ (0, 2.5, 5 และ 7.5% (w/v)) พบว่า ฟิล์มยีสต์ทุกสายพันธุ์ทนเกลือที่ความเข้มข้น 2.5% ได้ แต่การเจริญเติบโตจะขึ้นอยู่กับความเข้มข้นของเกลือ โดย *S. cerevisiae* J1 เจริญได้เร็วกว่า *C. krusei* J2 และ *C. krusei* J3 ส่วนการเจริญเติบโตในอาหารที่เติมปริมาณวุ้นความเข้มข้นต่าง ๆ (0.3, 0.5, 1.0 และ 1.5% (w/v)) ในเวลา 24 ชั่วโมง พบว่า อาหารที่เติมวุ้น 0.3% มีความเหมาะสมต่อการเจริญเติบโตของฟิล์มยีสต์ ผลของสารสกัดหยาบของพืชปรุงแต่งกลิ่นรสได้แก่ กระเทียม ขิง ข่า ตะไคร้ กระชาย กานพลู มะกรูด ส้มแขก และหอมแดง ด้วยน้ำที่ความเข้มข้น 3% (w/v) พบว่า สารสกัดหยาบจากกานพลูสามารถยับยั้งการเจริญเติบโตของฟิล์มยีสต์ทั้ง 3 สายพันธุ์ได้ ภายใน 12 ชั่วโมง แต่หลังจากนั้นประสิทธิภาพการยับยั้งจะลดลง ซึ่งสารสกัดหยาบจากกานพลูที่ความเข้มข้นต่ำสุด 0.75% (w/v) มีความสามารถในการยับยั้งการเจริญเติบโตของฟิล์มยีสต์จากน้ำหมักหน่อไม้ดองได้

ภาควิชาวิทยาศาสตร์การอาหารและโภชนาการ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตปัตตานี อำเภอเมือง จังหวัดปัตตานี 94000

Fermented bamboo shoot has been consumed by the people in the south of Thailand. The process of fermentation is the traditional preservation method involving the sequential development of various species of lactic acid bacteria. Young edible shoots of bamboo are peeled, chopped to thin piece and added with salt solution. Then, they are kept in a bottle or tank and covered tightly and stored in the ambient temperature (30-35°C) to ferment for 1-2 weeks. Completion of fermentation is indicated by the flavour and taste. Fermented bamboo shoot product can be stored for a year or more. One problem of the product was film yeast on the surface of fermented solution that the consumers dislike after opening the container. Maneewathana, *et al.* (2000) demonstrated that the yeast occurred in 105 pickler brine samples from the retailed stores in the markets and fermented vegetable factories. Many species of yeasts were identified as *Saccharomyces cerevisiae, Pichia etchell/ carsonii, Candida krusei, C. tropicalis, C. lipolytica, C. rugosa, C. holmii, C. utilis, Rhodotorula glutinis, Trichosporon cutaaneum* and *Zygosaccharomyces* sp. The contamination source of yeasts might be the vegetables, humans, soil and insects. Savard *et al.* (2002) isolated the spoilage yeast from fermented vegetables as *S. bayanus* and *S. unisporus*. Film yeast was alterability the odor, colour, taste and texture of products. Villagers or producers or sellers will remove film yeast by skimming, adding salt solution and using chemical such as, benzoic acid and sorbic acid, but there is a need to find safe and effective replacements for chemical preservatives and treatments. The exploration of naturally occurring antimicrobial activity for food preservation is increasing in response to the consumers demand for natural food product and low levels of chemical additives. Particular interest has focused on the potential application of plants. Also, Thai seasoning plants are frequently used as food ingredients and promote antimicrobial activity (Arora and Kaur, 1999; Chomnawang *et al.*, 2005; Siripongvutikorn *et al.*, 2005; Moreira *et al.*, 2005; Mytle *et al.*,

2006). Some research was undertaken to inhibit the growth of food spoilage yeasts. Example, yeasts were totally killed in 1 h by garlic extract but in 5 h with clove (Arora and Kaur, 1999) and essential oil of plant showed anti-yeast activity (Souza *et al.*, 2007). Therefore, seasoning plants may be possible for inhibiting the growth of film yeasts.

The objectives of this study were : (i) to isolate film yeast from fermented bamboo shoot and (ii) to determine the inhibitive efficacy of extracts of seasoning plants.

Materials and Methods

Collection of samples

Three samples of liquid at the surface of fermented bamboo shoot product from villagers in Amphur Kokpho, Pattani Province, were collected aseptically in sterile glass bottles. They were brought to the laboratory at room temperature (32±3°C) for determination of pH using pH meter and total viable counts (FDA, 1998).

Isolation and identification of film yeast

The samples were streaked on YPD agar medium with pH 5.0 (1% yeast extract, 2% peptone, 2% glucose and 2% agar) and incubated at room temperature (32±3°C) for 1-2 days. The single colonies were randomly picked and the isolates were purified by representative successive subculturing in the same medium. After microscopic examination, purified cultures were grown on slants and stored at 4°C. Identification of strains was performed with the API ID 32 C kit (Bio Merieux, Marcy-L'Etoile, France) according to the manufacturer's instructions. The different physiological tests were assimilation of galactose, actidione, saccharose, N-acetyl-D-glucosamine, DL-lactate, L-arabinose, cellobiose, raffinose, maltose, trehalose, 2-keto-D-gluconate, α-methyl-D-glucoside, sorbitol, D-xylose, ribose, glycerol, rhamnose, palatinose, erythritol, melibiose, glucoronate, melezitose, gluconate, levulinate, mannitol, lactose, inositol, glucose, sorbose and glucosamine.

Preparation of inoculum

A loopful of inoculum was taken from a pure culture of film yeast grown on slants and inoculated into 100 ml of YPD broth. Then, it was incubated at room temperature (32±3°C) and on a shaker at 110 rpm for 18 h. The growth so obtained was adjusted to OD_{600} = 1 and used as inoculum.

Liquid and semi-solid cultivation

The experiments (three replications) were carried out with liquid and semi-solid cultivation. In each 250 ml Erlenmeyer flasks, there were 150 ml YPD broth and YPD containing different concentrations of NaCl (2.5, 5.0 and 7.5% (w/v)) and 1% (v/v) of inoculum. They were incubated at room temperature on a shaker at 110 rpm. Cultures were monitored by sampling (5 ml) at the beginning of each experiment and after every 6 h of culture. pH was measured with a pH meter, optical density (OD_{600}) with spectrophotometer and soluble protein determined (Lowry *et al.*, 1951). Semi-solid cultivation was performed using YPD agar medium containing different concentrations of agar (0.3, 0.5 and 1.5% (w/v)). 100 µl of inoculum was dropped and incubated at room temperature for 24 h. The diameter of growth was measured and expressed in milimeters.

Extracts of seasoning plants

Garlic, ginger, galangal, lemon grass, lesser galangal, clove, kaffir lime, garcinia and shallot were bought from the local market in Pattani and various plant parts are shown in Table 1. Crude extracts were prepared according to the method of Shashikanth *et al.* (1981). All the seasoning plants were grounded and extracted with water at 3% (w/v). The extracts were boiled for 10 min and filtered through Whatman no. 1 paper. Nine seasonings were plants parts and the yields of the extracts were recorded as percentage; % extract yield = (final volume/initial volume) x 100. All of the crude extracts were stored in brown bottles at 4°C. The crude extracts were sterilized by filtration (pore size as 0.45 µm cellulose acetate filter) before testing.

Table 1. List of seasoning plants and crude aqueous extract yield used in the antimicrobial assay

Seasoning plants	Plants' parts	aqueous extract yield (%)
Garlic (*Allium sativum* Linn.)	Rhizome	87.5
Ginger (*Zingiber officinale* Rosc.)	Rhizome	81.0
Galanga (*Alpinia galanga* (Linn.) Swartz.)	Rhizome	77.5
Lemon grass (*Cymbopogon citratus* (DC.) Stapf.)	Stem	65.0
Kra-chai (*Boesenbergia rotunda* (L.) Mansf.)	Rhizome	71.0
Clove (*Syzygium aromaticum* (L.) Merr et Perry)	Flower	85.0
Kaffir (*Citrus hystri*)	Leaf	76.5
Garcinia (*Garcinia atroviridis* Griff.)	Fruit	90.0
Shallot (*Allium ascalonicum* Linn.)	Rhizome	88.0

Assay for inhibition of film yeast growth

The antimicrobial test of the crude extracts from seasoning plants was performed by the disc diffusion test (Lennette *et al.*, 1985; Kim *et al.*, 1995). Inocula of film yeasts were obtained from 18 h culture in YPD medium at room temperature (32 ± 3^{o}C) and adjusted to OD_{600} = 1. Sterile filter papers (Whatman No.1, diameter 6 mm) were impregnated with 10 µl of each crude extract and placed in the center of YPD agar plate where the test film yeast with 100 µl was spreaded and incubated at room temperature for 24 h. The diameter of clear zone shown on plates was measured and expressed in millimeters as its antimicrobial activity.

Results and Discussion

Three samples of fermented bamboo shoot had total viable count of 10^{4}-10^{5} cfu/ml and pH 3.4-4.4. The dominant films on the surface of the fermented solutions were isolated. Pure culture colonies developed well on YPD agar medium. All isolates were cream and smooth colonies. The assimilation of carbohydrate test on selected isolates identified *Saccharomyces cerevisiae* J1, *Candida krusei* J2 and *C. krusei* J3 (Table 2). Cell shapes of *S. cerevisiae* J1 and both of *C. krusei* were round and spherical, respectively. This result was according to the other reports. Lactic acid bacteria is the main fermenting organism; after that yeast strains as *S. cerevisiae* and *C. krusei* were found in the food fermentation (Halm *et al.*, 1993; Obiri-Danso, 1994; Olsen *et al.*, 1995; Maneewatthana *et al.*, 2000; Halm *et al.*, 2004). These strains were useful for single cell protein (SCP) production as *C. krusei* SO1 and *Saccharomyces* sp. LK3G. They were a good source of mineral salts, such as iron, manganese, phosphorus and potassium by culturing in sorghum hydrolysate (Konlani *et al.*, 1996). *C. krusei* has been reported as being used for glycerol production (Zhang *et al.*, 2002; Liu *et al.*, 2003; Liu *et al.*, 2005; Liu *et al.*, 2006).

It is known that sodium chloride (NaCl) is normally a preservative and used for product stability. The growth of spoilage yeast are a response. Therefore, the study on growth was carried out in liquid YPD medium. Growth curves of *S. cerevisiae* J1, *C. krusei* J2 and *C. krusei* J3 in the absence and presence of NaCl are shown in Figure 1. In the absence of NaCl, *S. cerevisiae* J1 was able to grow faster than *C. krusei* J1 and *C. krusei* J2. The growth of *S. cerevisiae* J1 was much greater at 24-30 h and the lag phase of both strains of *C. krusei* also slowly increased. In the presence of NaCl, *S. cerevisiae* did not grow well and the growth of all strains decreased according to NaCl concentration increased. All strains tolerated the 2.5% concentration of NaCl and both *C. krusei* J2 and *C. krusei* J3 better tolerated NaCl than did *S. cerevisiae* J1, whereas, the pH was slighly decreased. These results indicate that different yeasts have different relative sensitivity to osmotic

Table 2. Assimilation of carbohydrate of yeast strains

Assimilation	Reaction		
	***S. cerevisiae* J1**	***C. krusei* J2**	***C. krusei* J3**
Galactose	+	-	-
Actidione	-	-	-
Sacchrose	+	-	-
N-acetyl-D-glucosamine	-	+	+
DL-lactate	+	+	+
L-arabinose	-	-	-
Cellobiose	-	-	-
Raffinose	+	-	-
Maltose	+	-	-
Trehalose	+	-	-
2-keto-D-gluconate	-	-	-
α-methyl-D-glucoside	+	-	-
Sorbitol	-	-	-
D-xylose	-	-	-
Ribose	-	-	-
Glycerol	-	+	+
Rhamnose	-	-	-
Palatinose	+	-	-
Erythritol	-	-	-
Melibiose	-	-	-
Glucoronate	-	-	-
Melezitose	+	-	-
Gluconate	-	-	-
Levulinate	-	-	-
Mannitol	-	-	-
Lactose	-	-	-
Inositol	-	-	-
Glucose	+	+	+
Sorbose	-	-	-
Glucosamine	-	-	-

Remark : + = Positive reaction - = Negative reaction

stress (NaCl). Garcia *et al.* (1997) reported that in the presence of 1 M NaCl, *S. cerevisiae* showed a 70% reduction in growth rate while in the case of *C. tropicalis* the reduction of growth rate was 30%. *C. tropicalis* showed a better adaptation to Na than *S. cerevisiae*, because *C. tropicalis* has an adaptative mechanism to increase sodium efflux or restrict sodium uptake during continuous growth in media with NaCl. Glycerol production involves NADH-dependent reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by cytoplasmic glycerol-3-phosphate dehydrogenase (GDP) and subsequent dephosphorylation of glycerol 3-phosphate to glycerol. In the presence of NaCl, GDP activity increased (Andre *et al.*, 1991; Blomberg and Adler, 1989; Albertyn *et al.*, 1994). Yeast can defend against salt stress by the osmolytes such as glycerol. On the basis of our result, we have observed the soluble protein which showed a slight increase in *C. krusei* J2 culture at all NaCl concentrations at 6 h (data not shown). Further research should probably focus on glycerol

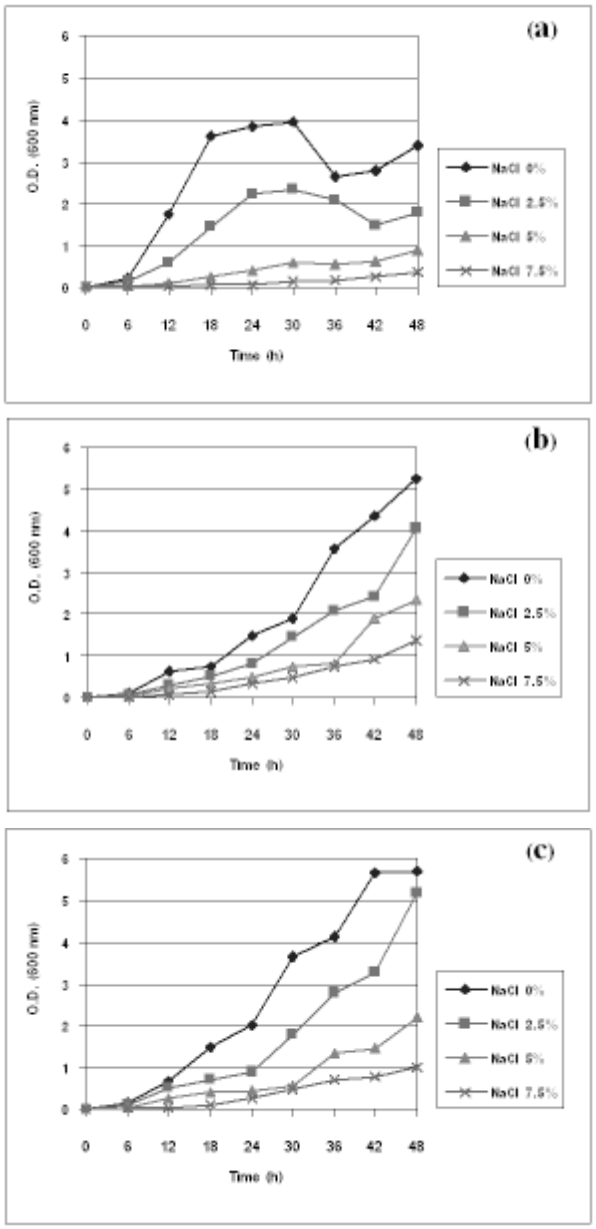

Figure 1. Growth of *S. cerevisiae* J1 (a), *C. krusei* J2 (b) and *C. krusei* J3 (c) in liquid YPD medium and addition of NaCl at differents concentration.

production. On the other hand, Reynold and Fink (2001) reported that bakers' yeast can grow on the semi-solid (0.3%) agar medium. Our study found that both *C. krusei* were well grow on YPD and 0.3% (w/v) agar at 24 h (Figure 2). Whereas,

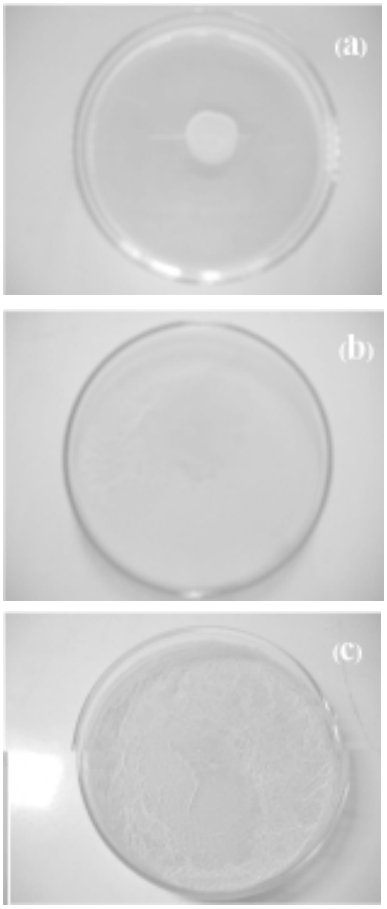

Figure. 2 Growth of *S. cerevisiae* J1 (a) *C. krusei* J2 (b) *C. krusei* J3 (c) on semi-solid medium (YPD and 0.3%(w/v) agar) at 24 h.

S. cerevisiae J1 slowly grow on different agar concentrations.

Inhibitory effects of all crude extracts from seasoning plants against *S. cerevisiae* J1, *C. krusei* J2 and *C. krusei* J3 yeast isolated from fermented bamboo shoot are summarized in Table 3. All film yeasts showed sensitivity to clove extract. *S. cerevisiae* J1 was inhibited as well as *C. krusei* J2 and *C. krusei* J3 within 12 h and the efficiency of inhibition was decreased. The inhibitory activity depended on the time and growth of yeast. Various other workers have reported that the clove activity is an antimicrobial (Arora and Kaur, 1999; Moreira *et al.*, 2005; Mytle *et al.*, 2006; Rahim and Khan,

Table 3. Sensitivity of film yeast to crude extract of 3%(w/v) seasoning plants at 12 h.

Seasoning plants	Film yeast strains		
	***S. cerevisiae* J1**	***C. krusei* J2**	***C. krusei* J3**
Garlic (*Allium sativum* Linn.)	-	-	-
Ginger (*Zingiber officinale* Rosc.)	-	-	-
Galangal (*Alpinia galanga* (Linn.) Swartz.)	-	-	-
Lemon grass (*Cymbopogon citratus* (DC.) Stapf.)	-	-	-
Lesser galangal (*Boesenbergia rotunda* (L.) Mansf.)	-	-	-
Clove (*Syzygium aromaticum* (L.) Merr et Perry)	+	+	+
Kaffir (*Citrus hystri*)	-	-	-
Garcinia (*Garcinia atroviridis* Griff.)	+	-	-
Shallot (*Allium ascalonicum* Linn.)	-	-	-

Note : - = No sensitive, + = Sensitive

Table 4. Inhibition zone of antimicrobial activities of crude extract from clove at differents concentration

Yeast	Inhibition zone (mm)				
	Concentration of clove (%)				
	0	**0.75**	**1.5**	**3.0**	**6.0**
S. cerevisiae J1	-	+(16.7)	+(11.2)	+(30.2)	+(31.5)
C. krusei J2	-	+(26.7)	+(25.7)	+(28.0)	+(27.3)
C. krusei J3	-	+(26.7)	+(27.0)	+(24.0)	+(30.3)

Note : - = No inhibition zone, + = inhibition zone

2006). Clove oil was studied on the yeast *S. cerevisiae*. Yeast cell lysis was shown by the release of substance (Chami *et al.*, 2005). The low concentration was required at 0.75% (w/v) of clove with water extraction (Table 4). Many reports have shown clove extract to be a food preservative. For example, Singh *et al.* (2003) reported the essential oils in clove were highly effective against *Listeria monocytogenes* in hotdogs. Also, Mytle *et al.* (2006) determined that 1% or 2% clove oil in chicken frankfurters inhibited *L. monocytogenes*. Therefore, it is possible to use clove in food fermentation.

Conclusion

The study shows that there are film yeasts (*S. cerevisiae* J1, *C. krusei* J2 and *C. krusei* J3) in fermented bamboo shoot and clove can be considered as a natural preservative because of its antimicrobial activity. To reach an adequate shelf life, fermented bamboo shoot products require a reduced film yeast or native micro-flora. Future investigations should be focused on the use of clove to preserve fermented bamboo shoot along with acceptable colour and flavour for consumers.

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