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Isolation, identification and control of osmophilic spoilage yeasts in sweetened condensed milk

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In this paper, six yeast strains were isolated from spoilt condensed milk in the agar culture media with high sugar content. By employing API 20C AUX strip, these six isolates were identified as *Candida pelliculosa* strains. Their growth characteristics were then examined under different culture conditions, including various pH value, temperature, sterilization condition, NaCl and glucose concentrations. Both culture temperature and pH value showed significant influence on the growth of the strains, with the optimum cultural temperature and pH being 33°C and 5.0, respectively. The biomass was evidently depressed by increasing the concentrations of salt and glucose. However, it was also found that the strains tested were able to tolerate high concentrations of NaCl (9-15%) and glucose (60-80%), suggesting that the strains isolated were of osmophilic yeast. To find efficient strategies for controlling the spoilage of condensed milk, a comparative study of the effects of eight antiseptics and heat treatment on these spoilage yeasts were done. Among the tested antiseptics, 0.01% sodium dehydroacetate or 0.03% ethyl p-hydroxybenzoate showed application potential in inhibiting yeasts-caused spoilage of sweetened condensed milk.

Key words: Sweetened condensed milk, spoilage yeast, isolation and identification, control.

INTRODUCTION

Yeasts, one of the most important microorganisms in food industry, have been widely used in the production of bread, beer, wine and other alcoholic drinks for thousands of years. They have also found applications in many products such as ethanol for fuel, yeast extracts, pigments, probiotics and other specific substances for foods and feeds, as well as biochemicals for the pharmaceutical industry. However, except for their well-known fermentation capabilities, they are also responsible for the spoilage of certain foods and beverages (Jakobsen and Narvhus, 1996). The spoilage caused by yeasts may alter both the physical and sensorial properties of foods as a result of their activity (Loureiro and Querol, 1999), which has caused major economic loss in many sectors of food and beverage industries, especially in baking and beverages industry. Current losses to the food industry caused by yeast spoilage are estimated at tens of millions pounds annually in the world (Querol, 2006; Gerez et al., 2010; Serpaggi et al., 2010).

Moreover, food-associated yeasts could be an underestimated source of infections and other unhealthful responses in humans (Fleet, 2007). In 1980s, Fleet firstly reported that yeasts were suspected of being associated with a food-borne gastroenteritis incident (Fleet, 1992).

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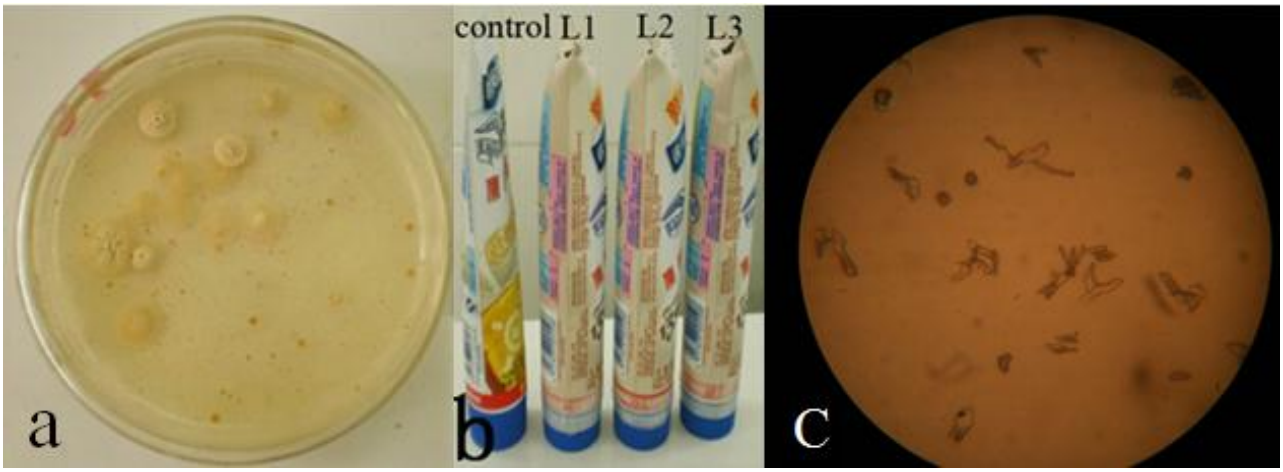

Figure 1. Colony morphologies (a) and light microscrope morphologies (b); spoilt and fresh sweetened condensed milk (c) of the isolates obtained from spoilt condensed milk.

So far, several species, such as *Candida albicans* and *Cryptococcus neoformans*, have been identified as opportunistic pathogens causing a range of mucocutaneous, cutaneous, respiratory, central nervous system and organ infections, as well as general fungemia (Hazen and Howell, 2003). Thus, characterization and control of spoilage yeasts grown in foods have been considered as an important and attractive research area, not only for avoiding economic damages to industries but for reducing public health risks caused by food-borne pathogens (Pitt and Hocking, 2009).

Sweetened condensed milk is one of the most popular dairy products by removing approximately 60% water from milk, in which sugar content is usually about 40-50% (Clark et al., 2009). Since high-sugar content prevents bacterial growth, sweetened condensed milk is usually not sterilized but only pasteurized (Vaclavik and Christian, 2008). Certain osmophilic and osmoduric microorganisms, such as yeasts, osmotolerant micrococci and xerophilic fungi, may grow and cause spoilage in pasteurized foods under certain conditions (Swanson, 2011). Controlling the growth and activity of spoilage yeasts requires a quite good understanding of their characteristics, including physiologic properties, biochemical properties or even genetic responses. Generally, the spoilage caused by yeasts was evidenced with gas production, yeasty flavour and other off-flavours, as well as discolorrations and changes of texture (Brocklehurst and Lund, 1985; Rohm et al., 1992). Up to now, there have been few reports on the characteristics of main microorganisms causing spoilage of condensed milk.

In this research, osmophilic spoilage yeasts were, for the first time, isolated from spoiled condensed milk. The influences of several key cultural factors on the growth of the isolated microorganisms were investigated. In addition, the heat treatment and food preservatives were compared for the control of sweetened condensed milk.

MATERIALS AND METHODS

Fresh and spoilt sweetened condensed milk were provided by a company in Zhejiang Province, China. The spoilt sweeten condensed milk were characterized with swollen packages due to gas production, which was obvious when compared with fresh samples of this product (Figure 1b). Both spoiled and fresh samples were all packaged in plastic tubes like toothpaste packages. They were all stored at 4°C before use. API 20C AUX strip were purchased from BioMérieux, France. All other chemicals were of the highest purity available from commercial sources.

Isolation and identification of osmophilic spoilage microorganisms

The samples of sweetened condensed milk were divided into three groups: Group 1 (spoilt samples with little gas), Group 2 (spoilt samples filled with gas) and Group 3 (normal samples). Ten grams condensed milk were weighed exactly from each sample, dissolved and serially diluted by sterilized physiological saline to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. To protect sugar-tolerant yeasts, 10-20% (w/w) sucrose was added to media and diluents (Beuchat, 1993). Potato dextrose agar (acidified) and nutrient agar were used as typical media for yeast culture (Baroiller and Lapadu-Hargues, 1990). One tenth milliliter of each dilution prepared above was spread on solid nutrient agar media, potato dextrose agar media (PDA media) or high-sugar agar media. Then the PDA media and high-sugar agar media were incubated at 28°C for 48 h and the nutrient agar plates were incubated at 36°C for 24 h.

The purity of the isolated colony was confirmed by microscope observation after being subcultured for three times. Then the strain was inoculated on high-sugar agar slants and stored in a refrigerator at 4°C for further study.

Activated spoilage microorganisms were inoculated in Durham tubes containing high-sugar liquid media, normal condensed milk and physiological saline (control), and then incubated at 28°C. Gas production rates of strains in the tubes were analyzed. After being activated, the isolates were identified primarily according to their morphological, sexual, biochemical and physiological properties. Then API 20C AUX strip (BioMérieux, France) was used for a further identification.

Table 1. Isolation of microorganisms from sweetened condensed milk.

Group	Total plate count (CFU·g^{-1})		Count of yeast by using high-sugar agar medium (CFU·g^{-1})
	Nutrient agar medium	Potato dextrose agar medium	
Group 1	1.7×10^3	1.0×10^3	1.0×10^3
Group 2	3.4×10^3	<1	<1
Group 3	<1	<1	<1

Effect of culture conditions on growth of the isolated strains

Spoilage microorganisms were inoculated on high-sugar liquid mediums and detected with OD_{600} per 4 h to obtain its growth curve. Various concentrations of salt (3-15%, w/v) or glucose (0-80%, v/v) were added into agar broth base. The correct amount of salt or glucose was weighed out for each concentration and added to a volumetric flask. Deionized water was then added to make a total volume of 1 L and this was added to the high-sugar broth agar powder. For test of pH tolerance, the pH of the medium (3.0-7.0) was adjusted using HCl or NaOH and the mediums were incubated at 28°C for 48 h after being inoculated with spoilage yeast. Investigation of the temperature effect on the strains was performed by incubating mediums at different temperatures (20-40°C), followed by analysis of OD_{600} value of strains.

Effect of heat sterilization on spoilage yeasts

Activated cell suspension (10^4 CFU·mL^{-1}) were added to condensed milk, incubated in a water-bath thermostat and heat-treated under the following conditions: (1) 60°C (5-20 min), (2) 70°C (2-10 min), (3) 80°C (2-10 min), (4) 90°C (1-5 min) and (5) 100°C (5-20 s). After heat treatment, the number of microorganisms which may have survived heat treatments were counted using high-sugar agar media incubated at 28°C for 48 h.

Determination of the minimum inhibiting concentrations of candidate antiseptics

Preparation of eight antiseptics solutions: six water-soluble antiseptics including sodium benzoate, potassium sorbate, ethyl p-hydroxybenzoate, calcium propionate, sodium dehydroacetate and R-polysaccharide were dissolved in sterilized water. For nisin (1,000,000 IU·g^{-1}), it was dissolved in 0.02 mol·L^{-1} HCl with pHs at 3.00-4.00. Their final concentrations ranged from 0.01-1.00 g·(100 mL)$^{-1}$. Natamycin was dissolved with 75% ethanol to make corresponding solution at concentrations of 1-100 μg·mL^{-1}.

Screening of the antiseptics

The antiseptics were preliminarily investigated according to their antibacterial activities via modified disc diffusion test (Kim et al., 1995). Two tenth milliliter spoilage microorganisms (10^6 CFU·mL^{-1}) suspension was evenly spread onto high-sugar agar plate mediums using a glass rod spreader. Then Oxford cups that hold antiseptics were placed on planed spots on the surface of the mediums. Plates were incubated at 28°C for 48 h. The diameter of the clear zone around the disc was measured and recorded in milliliters as its antimicrobial activity. Candidate antiseptics without inhibiting zone in this test would not be considered. According to the results obtained from screen test of candidate antiseptics, the antiseptics showing strong inhibiting ability were chosen and tested for their minimum inhibiting concentrations (MIC) test by the same method.

Application of antiseptics in the control of the spoilage yeasts isolated

By MIC test described, two antiseptics were chosen and added into two kinds of condensed milk samples: spoilage sweetened condensed milk and normal sweetened condensed milk inoculated with spoilage yeasts. Then the condensed milk samples were stored at 28°C for 21 days. The spoilage yeast number of condensed milk during the storage was counted with distilled water as control.

Statistical analysis

All reported data are averages of experiments performed at least in 3 replicate. SPSS (Statistical package for Social Sciences, SPSS Inc., Chicago, III., U.S.A) software package was employed for data analysis.

RESULTS AND DISCUSSION

Isolation and identification of osmophilic spoilage microorganisms

To isolate the spoilage microorganisms, the microbes from different samples of sweetened condensed milk, including spoiled samples with little gas (Group 1), spoiled samples filled with gas (Group 2) and normal samples (Group 3), were cultured with three kinds of media (high-sugar agar mediums, potato dextrose agar media and nutrient agar). When compared with normal condensed milk, microorganism count obtained from spoiled condensed milk was obviously higher. However, Table 1 showed that total bacteria counts of spoilage condensed milk samples except for Group 2 were within the upper limit (3×10^3 CFU·g^{-1}) of Chinese national standards for sweetened condensed milk, indicating that bacteria may not be the reason for the spoilage of condensed milk. Then, three strains, named CMY1 to CMY3, were picked out from high-sugar agar mediums and another three strains (CMY4 to CMY6) were screened with potato dextrose agar media. The microorganisms from spoiled condensed milk samples showed similar morphologies as illustrated in Figure1a. On gross observation, the colony looks creamy white in color, exhibited obvious fold and central protuberance. Microscope observation showed the presence of pseudohyphae (Figure 1c). Furthermore, API 20C AUX strip was used to specially detect yeast (Bernal et al., 1998). Identification of API 20C AUX strip further revealed that the six strains isolated (CMY1-6) were *Candida pelliculosa*, an abnormal variant of *Hansenula anomala* which belongs to ascomycetes, endomycetale

Table 2. Gas-producing activities of spoilage microorganism isolated from high-sugar agar culture medium.

Culture time (day)	Gas volume						
	CMY1	CMY2	CMY3	CMY4	CMY5	CMY6	Control
1	+[a]	—[d]	—	—	—	—	—
2	++[b]	+	+	+	+	+	—
3	+++[c]	++	++	++	++	++	—
5	+++	+++	+++	+++	+++	+++	—

[a]+ means less than one-third of Durham tubes were filled with gas; [b]++ means two-thirds of Durham tubes were filled with gas; [c]+++ means all the Durham tubes were filled with gas; [d]- means no gas was produced in Durham tubes.

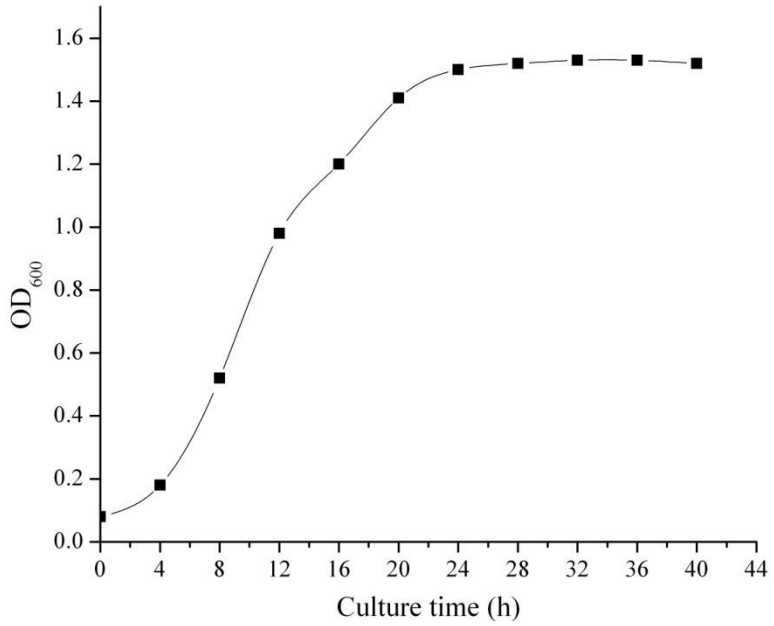

Figure 2. The growth curve of the spoilage yeast CMY1.

and saccharomycetaceae.

One of the most well-known sign of yeast spoilage is the production of gas via fermentation of sugars, resulting in visible swollen containers or even explosion of glass bottled food products (Martorell, 2007). Table 2 shows the gas-producing capabilities of the isolated strains in Durham tubes. Except for CMY1, little gas was produced for all strains tested after a one-day culture. And their gas-formation capabilities became evident when further prolonging the culture time. Among the tested strains, CMY 1 showed the fastest and highest fermenting capability, which was chosen for further researches.

Growth curve of the strain CMY1 of *C. pelliculosa*

Generally speaking, the growth of filamentous fungi could be divided into five phases, namely, the lag phase, the first transition period, the log phase, the second transition period, and the stationary phase. As shown in Figure 2, the growth curve of *C. pelliculosa* CMY1 in high-sugar liquid medium was characterized by the presence of a very short lag phase and high growth rate in the following first transition period, which indicated that the isolated spoilage yeast strain was better able to accommodate itself to the high-sugar environment. Due to a long and smooth transition period observed, the visualization of the start and end points of these periods by using OD changes seemed rather difficult, which is in accordance with the precious reports of the growth curves of other filamentous fungi (Meletiadis et al., 2001). The observed growth curve provided a useful tool to gain insight into the growth characteristics of *C. pelliculosa* strains in high-sugar liquid media.

Effects of culture conditions on the growth of CMY1

To better understand the growth characteristics of *C. pelliculosa* strains in different environmental conditions, the influences of several cultural variables on the biomass of *Candida pelliculosa* CMY1 were investigated.

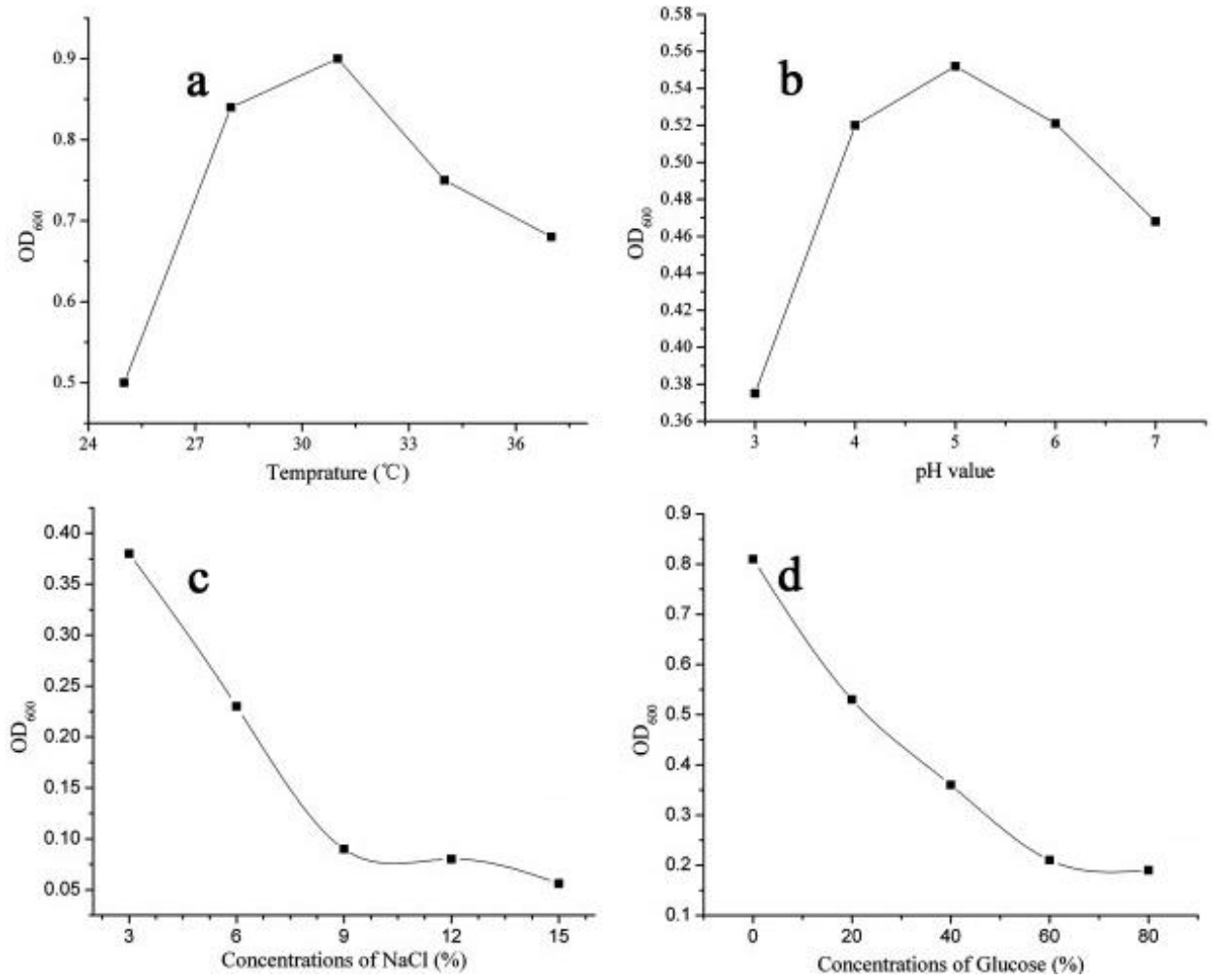

Figure 3. Effects of culture conditions on the growth of CMY1: (a) temperature; (b) pH; (c) concentrations of NaCl; (d) concentrations of glucose.

Figure 3a shows that CMY1 was relatively sensitive to the culture temperature. The highest cell density (OD_{600}) was reached when the culture temperature was about 30°C. Further increase in the culture temperature led to a sharp decrease in the OD_{600} value of the strain. It was also found that CMY1 preferred mild acidic environment, since OD_{600} value reached its highest value when pH value rose to 5.0 (Figure 3b). And the increase of the pH value above 5.0 also resulted in a significant decrease of the biomass of the fungi.

The osmotolerance of the yeast strain was tested by varying the concentrations of NaCl and glucose. Figure 3c to d shows sharp declines when steadily increasing the concentrations of NaCl and glucose to 9 and 60%, respectively. Further increasing the concentrations of NaCl and glucose, however, led to only a small decrease in OD values of the spoilage yeast strain. Within the tested ranges, the isolate could still grow under the environment that contains 9-15% NaCl or 60-80% glucose, showing that it can tolerate high osmotic pressure caused by high concentration of salt or sugar.

Effect of heat treatment on the spoilage yeast strains

Heat treatment is a well-known safe and effective sterilezation method widely used in food industry. Effects of temperature and time of heat treatment on the strain CMY1 mixed in fresh condensed milk were investigated by determining the microbial survival at a specified temperature and time. Table 3 showed that when 60°C was adopted as the temperature of heat treatment, the treatment time of 20 min was required to ensure the population of spoilage yeast survived below t 1 cfu.mL^{-1}. Further increase of the treatment temperature to 70°C led to a significant decrease in the time necessary to accomplish similar pasteurization effect to that at 60°C. When much higher heat treatment temperatures (80-95°C) were employed, the treatment time can be shortened to <1 min. The results clearly depicted the heat tolerance of the yeast strain isolated from spoilt condensed milk, and

Table 3. Effect of temperature and time of heat treatment on the spoilage yeast.

Treatment temperature (°C)	Treatment time (min)	Microbial survival (CFU.mL^{-1})
60	5	39
	10	22
	15	19
	20	<1
70	1	28
	3	<1
	5	<1
	7	<1
	9	<1
80	1	<1
	2	<1
	3	<1
	4	<1
90	0.5	<1
	1	<1
	1.5	<1
95	0.5	<1
	1	<1

Table 4. Inhibitory results of 8 antiseptics for inhibiting CMY1.

Antiseptic	Diameter of inhibition zone around discs (mm)			MIC (%)
	Concentration of antiseptics (%)			
	0.010	0.100	1.000	
Sodium benzoate	—[a]	17	23	0.04
Potassium sorbate	—	10	20	0.05
Ethyl p-hydroxybenzoate	—	12	33	0.03
Calcium propionate	—	—	—	—
R-polysaccharide	—	—	10	—
Sodium dehydroacetate	3	8	21	0.01
Nisin	—	—	—	—

Antiseptic	Concentration of antiseptic(μg·mL^{-1})			MIC (μg·mL^{-1})
	1.0	10.0	100.0	
Natamycin	—	8	10	5.0

The diameter of inhibition zone around the disc was measured in milliliters; [a] means there is no inhibition zone around the disc.

demonstrated that the spoilage yeast can be killed by combination of relatively low temperature and longer holding time, such as 20 min-treatment at 60°C or 5 min-treatment at 70°C.

Effect of antiseptics candidates on the growth of the spoilage yeasts

The use of antiseptics is also considered as a common method for improvement of the shelf-life of foods. In order to develop a non-thermal preservation method for sweetened condensed milk, a serial of antiseptics candidates were chosen and their inhibiting effects on the isolated spoilage yeast were investigated by determination of their minimum inhibiting concentration (MIC). Clear zones formed around Oxford cups on the medium surface were found when five out of eight antiseptics (0.1%) were tested (Table 4). The MIC values of the five antiseptics

Table 5. Effect of ethyl p-hydroxybenzoate and sodium dehydroacetate on the activity of CMY1 in sweetened condensed milk.

Time (day)	0.03% ethyl p-hydroxybenzoate (CFU·mL^{-1})	0.01% sodium dehydroacetate (CFU·mL^{-1})	Control [a] (CFU·mL^{-1})
0	<1	<1	103
3	<1	<1	100
6	<1	<1	1.3×10^{3}
9	<1	<1	8.1×10^{3}
12	<1	<1	5.6×10^{4}
15	<1	<1	1.7×10^{6}
18	<1	<1	4.4×10^{6}
21	<1	<1	8.6×10^{6}

[a] No antiseptics were added.

listed in Table 5 showed that sodium dehydroacetate and ethyl p-hydroxybenzoate had lower effective concentration than others, being of 0.01 and 0.03%, respectively.

Sodium dehydroacetate is a safe food preservative used widely in oriental foods such as wet noodles, pickled vegetables and also in fungal decay control in postharvest fruits and vegetables (Lu, 2009). However, its effectiveness in controlling spoilage of condensed milk has never been reported until now. Therefore, as a promising candidate, sodium dehydroacetate (0.01%) was selected for further testing on the growth of spoilage yeast strains, and compared with ethyl p-hydroxybenzoate (0.03%), by adding them into spoiled condensed milk and normal condensed milk inoculated with CMY1. Table 5 shows that no gas produced or coagulation phenomena emerged after 21-day storage while the control samples were spoilt after been stored for 6 days. Moreover, the growth of CMY1 in condensed milk could also be controlled effectively by the use of either sodium dehydroacetate or ethyl p-hydroxybenzoate. The results indicated that the use of 0.01% sodium dehydroacetate or 0.03% ethyl p-hydroxybenzoate could be a useful method to inhibit the growth of spoilage yeasts and extend the storage time of sweetened condensed milk.

Conclusion

Spoilage yeast strains were separated from samples of spoilt sweetened condensed milk and identified as *C. pelliculosa*. The strains showed particular growth characteristics under osmotic environments, which could tolerant high concentrations of NaCl and glucose. The strains were highly sensitive to the culture temperature and pH values within the tested ranges. The spoilage yeast can be pasteurized by combination of relatively low temperature and longer holding time (60°C, 20 min; 70°C, 5 min). In addition, investigation of the inhibition effects of antiseptics candidates on the growth of the spoilage yeasts showed that, condensed milk could maintain its quality under 28°C for 21 days when 0.01% sodium dehydroacetate or 0.03% ethyl p-hydroxybenzoate was used. Further research is needed to analyze the effect of bacteriostatic agents on the flavor and nutrition of condensed milk.

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