

## Control of Spoilage Microorganisms in Soybean Milk by Nipagin Complex Esters, Nisin, Sodium Dehydroacetate and Heat Treatment

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**Abstract.** Soybean milk is a kind of highly perishable Asian traditional soy product, and this study was envisaged to extend its shelf-life through the application of hurdle technology. Twelve strains of bacteria were isolated from samples of spoilage soybean milk and were identified as *Klebsiella oxytoca* (S 5, S 7, S 8), *Serratia marcescens* (S 9, S 10, S 14, S 15, S 17), *Klebsiella ozena* (S 16, S 18), and *Serratia plymuthica* (S 21, S 25, S 28). To control these spoilage microorganisms, eight commonly used food preservatives were tested by Oxford Cup inhibition method. Results showed that sodium dehydroacetate, nipagin complex esters and nisin significantly inhibited the growth of the isolated strains with their MIC (minimum inhibition concentration) values ranging from 0.004% to 0.05%. Levels of heat treatment duration, together with concentrations of sodium dehydroacetate, nipagin complex esters, and nisin were employed as hurdle factors. The optimum conditions for improvement of the shelf-life were gained from orthogonal tests as follows: heat treatment at 100 °C for 10 minutes, and nisin 0.004%, nipagin complex esters 0.008%, dehydroacetate 0.002%. The fresh soybean milk applied with the optimum formula could be maintained under upper limit of the microbial count (100 CFU/mL, DB44/425-2007 China) for more than 6 days at 25 °C, and 11 days at 4 °C. Here in our study, hurdle technology approach significantly improved the shelf-life of soybean milk.

**Keywords:** soybean milk; spoilage microorganisms; hurdle technology; shelf-life; isolation and identification

### 1. Introduction

Soybean milk, a drink popular in the hawker culture of China and many other Southeast Asian countries, is recognized as the most inexpensive protein source of high nutritional quality. Soybean milk is extracted from integrate soybean seeds, containing all the nutrients in the seeds such as soy proteins and soy flavonoides [1]. Soybean milk has about the same amount of protein as cow's milk, though the amino acid profile differs [2]. As this drink is cholesterol free and low in energy, it could enhance health benefits in terms of reducing body weight and blood lipids [3]. The American Academy of Paediatrics also considers soybean milk a suitable alternative for children who cannot tolerate human or cow's milk, or whose parents opt for a vegan diet.

However, soybean milk is of high susceptibility to spoilage microorganisms and is easy to transmute if without interventions of food control. To study the spoilage microorganisms in the soybean milk and develop an effective method for inhibiting them is crucial for the industrialization process of soybean milk. Sources of spoilage microorganisms are believed to be raw ingredients or environmental contaminants [4]. Generally, high concentration of single food preservative is needed for a lethal effect on the spoilage microbiota, but it often exceeds the threshold acceptable to consumers. An attractive alternative to high dose of single food preservative is using hurdle factors by several food preservatives or preservation conditions, such as

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temperature, pH, aw, Eh, Pres, competitive flora [5]. Combined hurdle factors can generate synergic effects on the growth and propagation of spoilage microorganisms, and this process for food stabilization allows the same microbiological security in food by using a much lower amount of each preservative compound or a lower intensity of the physical treatment involved [6]. Thermal treatment is routinely employed to sterilize soy products and maintain their quality. While considering minimizing the damage of food flavour, taste and texture in the sterilization process, reduction in the heat duration and processing temperature are particularly important [7].

In this research, the main spoilage microorganisms in soybean milk were, for the first time, isolated and identified. In additions, the influence of heat treatment and food preservatives on the growth of the isolated microorganisms was investigated to develop new control strategies for the spoilage of soybean milk.

## **2. Methods and Materials**

### **2.1. Isolation and Identification of Spoilage Bacteria**

Four samples of spoilage soybean milk (package inflated and spoilage was sensorial detectable, provide by a company in Shenzhen, China) were used in this study. These samples were of three different brands and were all stored at 25 °C before use. For the isolation process, half millilitre solution of each sample was diluted stepwise up to  $10^{-5}$  with sterile water and 0.2 mL of each dilution was spread on nutrient agar (Difco) plates. After incubation at 37 °C for 24~48 hours, strains on plates were isolated by shape, colour of colonies. Each strain was inoculated on slant nutrient agar, and then was stored at 4 °C for further studies. These isolates were identified primarily according to morphology, gram and spore stain, catalase test, and API system (bioMérieux, France), and further identified following the API system index and Bergey's Manual of Systematic Bacteriology.

### **2.2. Preparation of Fresh Soybean Milk, Inoculum and Antiseptics Experiments**

All the fresh soybean milk used in this study was provided by the same company as referred above. The obtained fresh soybean milk was stored at 4 °C for use in the following experiments. Bacteria used in antiseptic inhibitive tests were enriched in nutrient broth media for 24 h at 37 °C. Cell suspensions of bacteria were diluted with peptone water to provide initial cell counts of about  $10^5\sim 10^6$  CFU/mL. Based on different inhibitive spectrum of antiseptics and Chinese food regulation "Food additives hygiene standards (GB2760-2007)", eight antiseptics were primarily selected as candidates. Water soluble antiseptics including sodium dehydroacetate, nipagin complex esters, calcium propionate,  $\xi$ -polylysine, potassium sorbate, sodium diacetate were dissolved into sterilized water. Chitosan 470 was dissolved with HAc, and Nisin ( $10^6$  IU/g) was dissolved efficiently using 0.02 mol/L HCl.

### **2.3. Screening Test of Candidate Antiseptics**

Screening test of candidate antiseptics was carried out by modified disc diffusion method. Oxford cups were used to hold antiseptics. An aliquot of 1.00 mL spoilage microorganisms ( $10^5\sim 10^6$  CFU/mL) suspension was evenly spread onto nutrient agar (Difco) plates medium using a glass rod spreader. The Petri dishes were left at room temperature for 40 min to dry the agar surface. Then Oxford cups were placed on planed spot on the surface of the medium. Plates were incubated at 37 °C for 48 hours. The diameter of the clear zone around the disc was measured and recorded in millimetres as its antimicrobial activity. Six oxford cups per plate and three plates were used.

### **2.4. Minimum Inhibition Concentration (mic) Test**

For determining the minimum inhibition concentration of the antiseptics, respective solution of gradient concentrations was made up for further tests. Since concentrations of 0.01 g/100 mL, 0.10 g/100 mL, 1.00 g/10 mL had already been tested in the screen test of candidate antiseptics, more exact and minor gap concentrations were used in MIC test. Cell suspension of half millilitre isolates was inoculated into Muceller Hinton broth (Merk, Darmstadt, Germany) containing 0.000% to 0.010% antiseptics and incubated at 37 °C for 48 hours with shaking at 140 rpm. Viable cell counts (log CFU/mL) were made on nutrient agar by a pour plate method after incubation at 37 °C for 24 hours. The MIC was taken as the lowest concentration of antimicrobial agents that inhibit bacterial growth.

## 2.5. Application of Hurdle Technology to Control Spoilage Microorganisms

Effective antiseptics and thermal sterilization at 100 °C were selected as hurdles. Orthogonal-test design was carried out and the tests were arranged scientifically to solve multiple-factor optimization requirements with reduced numbers of tests conducted. Microbial count changes of each single test was checked at 48h. Verification experiment was performed to test the efficiency of the optimum formula in controlling the spoilage of fresh soymilk. Finally, soybean milk was cultured at 25 °C and 4 °C respectively and viable bacterial count on nutrient agar plates were recorded to discover the efficiency of the formula for preserving the soybean milk in the real conditions.

## 3. Results and Discussions

### 3.1. Isolation and Identification of Spoilage Bacteria

Twelve strains were isolated from 4 samples of spoilage soybean milk according to characteristics of colonies. Based on test results from API 20E (for gram-negative) and API 50 CHB (for gram positive) and additional test of shape, gram stain, catalase, spore stain, these 12 stains were identified as *K.oxytoca* (S 5, S 7, S 8), *S.marcescens* (S 9, S 10, S 14, S 15, S17), *K.ozena* (S 16, S 18), and *S.plymuthica* (S 21, S 25), respectively. The corresponding proportion of each bacteria isolate was *K.oxytoca* 9.38%, *S.marcescens* 40.62%, *K.ozena* 21.88%, and *S.plymuthica* 28.12%. The spoilage microorganisms isolated from the spoilage soybean milk in this experiment differed somewhat from one relating report previously [8]. They found that *B.licheniformis*, *B.pumilus* and *B.borstelensis* were the microorganisms that caused the spoilage of soybean milk. These dissimilar results probably caused by different samples used. *K.oxytoca* also contributed to the spoilage of tofu [9], and it is the toughest bacterial to control in food products. *S.marcescens*, *K.ozena*, *S.plymuthica* were probably brought by using the soybeans and water that were contaminated by these microorganisms because these *Enterobacteriaceae* bacteria are widely existed in the water, air and vegetal foods [4].

### 3.2. Screening and Mic Tests for Control of Spoilage Microorganisms

Inhibition effects of selected antiseptics were considered if clear zone were formed around oxford cups on the medium surface. Results of screen tests (Table 1) showed that sodium dehydroacetate inhibited the growth of four microorganisms, and *K.oxytocawas* was more vulnerable to dehydroacetate than other three spoilage microorganisms. Dehydroacetate is a food preservative (Food Chemicals Codex 2004) widely used in oriental foods such as wet noodles, pickled vegetables and also in fungal decay control in postharvest fruits and vegetables, in addition, it is safe [10]. To our knowledge, its effectiveness in controlling *K.oxytoca*, *S.marcescens*, *K.ozena* and *S.plymuthica* isolated from soybean milk is the first time being reported here. Compared with sodium dehydroacetate, nipagin complex esters showed stronger inhibition against *K.oxytoca* (S5), *S.marcescens* (S10) and *S.plymuthica* (S21), among which, *K.oxytoca* (S5) was more sensitive to nipagin complex esters. As the diameter of the inhibition zone towards these three microorganisms formed by nipagin complex esters ranged from 12 to 24 mm and were all larger than that by dehydroacetate. This suggested that the inhibition effects of complex esters against these three spoilage microorganisms were stronger than that of dehydroacetate. However, its antimicrobial effects towards *K.ozena* could not be detected. Another antiseptic possessing high inhibition effects on these four spoilage bacteria was nisin, inhibition effects of which ranged from 10 to 26 mm. It had been proven that nisin could prevent the outgrowth of gram-positive bacteria and spores of many *Clostridium* and *Bacillus* spp. [11], and the discovery of its inhibitive effects on *K.oxytoca*, *S.marcescens*, *K.ozena* and *S.plymuthica* enlarged its inhibitive spectrum. So far, nisin is the only bacteriocin licensed in Europe as a food preservative (E234) [6]. Since nisin was generally considered safe, it was frequently used as hurdles to inhibit growth of various bacteria and to preserve foods in the last decade. Calcium propionate, potassium sorbate,  $\xi$ -polylysine, sodium diacetate and chitosan (470) demonstrated no inhibition effects on *K.oxytoca*, *S.marcescens*, *K.ozena* and *Serratia plymuthica* (S21) when concentrations of these three organics were below 1.00%. Previous researchers reported that Chitosan (224) could inhibit the growth of *K.oxytoca* at 0.10% concentration with clear zone more than 14 mm, and generally showed stronger bactericidal effects on gram-positive bacteria than gram negative bacteria [9], which suggested that polymers of different molecular weight could have different inhibition effects. Potassium sorbate is a considerable antimicrobial weak acid,

owing to its desirable physiological properties, neutral flavor and its effectiveness against bacteria and fungi. But in our experiment, it didn't show desirable inhibition effects on neither of the bacteria.

Table 1. Inhibition test of 3 antiseptics to spoilage microorganisms isolated from spoilage soybean milk

Concentration (%)		<i>Klebsiella oxytoca</i>	<i>S.marcescens</i>	<i>K.ozena</i>	<i>S.plymuthica</i>
sodium dehydroacetate	0.010	-	- <sup>3</sup>	-	-
	0.100	14.0b <sup>1</sup> ±3.2 <sup>2</sup>	8.0a±2.3	8.0a±1.6	9.0a±1.5
	1.000	22.1c±3.8	12.3b±4.9	12.7b±5.0	16.0b±8.7
nipagin complex esters	0.010	12.0a±4.5	9.0a±1.8	-	11.2a±1.0
	0.100	19.3b±6.5	13.3a±6.6	-	15.0b±2.6
	1.000	24.0c±1.1	18.1b±1.3	-	22.4c±5.1
nisin	0.010	10.1a±2.9	12.0a±3.9	12.2a±6.4	12.7a±2.7
	0.10	12.0a±0.64	13.2a±4.5	13.3a±5.2	14.1b±3.1
	1.000	26.4c±2.2	18.2c±3.7	18.0c±3.2	17.0c±4.3

<sup>1</sup> The number is used to measure inhibitive ability of corresponding antiseptics, and it was recorded as diameter of inhibitive zone in millimeter. Values are average of 3 replicates <sup>2</sup> inhibit effects were not detected. T-test was used for statistics in this inhibition test.

The MIC values for inhibiting these four species of spoilage microorganisms, ranged from 0.004% to >1%, and differed with isolates and antiseptics (Table 2).

Table 2. The minimum inhibitory concentration (%) of different organics on growth of the 4 spoilage microorganisms after incubation for 48 hours at 37 °C

isolate	Organics							
	sodium dehydroacetate	nipagin complex esters	potassium sorbate	ξ-polylysine	nisin	calcium propionate	chitosan (470)	sodium diacetate
<i>Klebsiella oxytoca</i>	0.01	0.007	>1.0	>1.0	0.008	>1.0	>1.0	>1.0
<i>Serratia marcescens</i>	0.05	0.006	>1.0	>1.0	0.004	>1.0	>1.0	>1.0
<i>Klebsiella ozena</i>	0.05	0.008	>1.0	>1.0	0.006	>1.0	>1.0	>1.0
<i>Serratia plymuthica</i>	0.05	0.006	>1.0	>1.0	0.007	>1.0	>1.0	>1.0
threshold <sup>1</sup>	<0.3g/kg	<0.1 g/kg	<1g/kg	<0.05g/kg	<5g/kg	<2.5g/kg	<1.0g/kg	<1g/kg

<sup>1</sup>In this table, threshold refers to the upper limit set by the Chinese food industry regulation GB2760-2007 "general standard for food additives"

As for *K.oxytoca*, MIC vales ranged from 0.007 to >1%. Its growth could be totally inhibited within 48 hours by anticeptics. *Serratia marcescens* (10), *Serratia plymuthica* (21) could be inhibited by corresponding organics under their minimum inhibitive concentrations within 48 hours as well. *Klebsiella ozena* (16) could be inhibited by nisin (0.006%) within 36 hours, and some growth still could be detected after 48 hours when using sodium dehydroacetate to inhIBUTE the growth of *Klebsiella ozena* (16).

### 3.3 Results of Orthogonal Test and Spoilage Control of Soybean Milk

The soybean milk inoculated by these four spoilage bacteria with initial isolation ratio was treated in orthogonal experiment, which employed L9 (34) design and the level of each factor and arrangement were showed in Table 3.

Table 3. ORTHOGONAL TESTS DETAILS

Experiment	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9
Sterilization time(min)	5	10	15	15	5	10	10	15	5
Sodium dehydroacetate(%)	0.010	0.020	0.030	0.020	0.030	0.010	0.030	0.010	0.020
nipagin complex esters(%)	0.002	0.006	0.010	0.002	0.006	0.010	0.002	0.006	0.010
Nisin(%)	0.002	0.004	0.006	0.002	0.004	0.006	0.002	0.004	0.006

Results showed that the optimal antiseptic condition was determined as A2B3C1D2 when all levels of the four factors were considered. The highest inhibitive ability was achieved by using heat treatment at 100 °C for 10 minutes, and then adding nisin 0.004%, nipagin complex esters 0.008%, and dehydroacetate 0.002%. Under optimal conditions, soybean milk could be maintained for 5.5 days. Orthogonal experimental results showed that increase of the heat treatment time may not cause the improvement of effective lethal effects. It had been reported that spores produced by sporulating cells subjected to a heat treatment were more resistant to heat than were spores produced by untreated cells, which might help explain this phenomenon [12].

The best formula was finally used to preserve soymilk that was wrapped in sterile commercialized containers, such products could be well accepted and maintained under upper limit of microbial count(100 CFU/ml greatly (DB44/425-2007 General technical specifications for soy products in Guangdong Province) for more than 6 days at 25 °C, and 11 days at 4 °C.

#### 4. Conclusions

*K.oxytoca* (S5, S7, S8), *S.marcescens* (S9, S10, S14, S15, S17), *K.ozena* (S16, S18), and *S.plymuthica* (S21, S25) were isolated from samples of spoilage soybean milk. The optimum formula gained from orthogonal tests could maintain soybean milk under upper limit of the microbial count 100 CFU/mL, circumscribed by Chinese food regulation, for more than 6 days stored at 25 °C, and 11 days at 4 °C.

Further research is needed to replace organics adopted in this experiment with natural reagents, such as bacteriocin, essential oil and other active components of plants, because modern food industry and consumers ask for additive free, fresher and more natural taste food products [13].

#### 5. References

- [1] Wang H, Murphy PA. Isoflavone content in commercial soybean foods. *J Agric Food Chem.* 1994, 42:1666–1673
- [2] J W Erdman Jr and E J Fordyce. Soy products and the human diet. *Am J Clin Nutr.* 1998, 49:725–737
- [3] Liao FH, Shieh MJ, Yang SC, Lin SH, Chien YW. Effectiveness of a soy-based compared with a traditional low-calorie diet on weight loss and lipid levels in overweight adults. *Nutrition.* 2007, 23:551–556
- [4] Lawlor KA, Schuman JD, Simpson PG, Taormina PJ. Microbiological Spoilage of Beverages. *Dordrecht Heidelberg, London.* 2010, pp 245–284
- [5] Leistner L, Gould GW. Hurdle technologies: combination treatments for food stability, safety, and quality. Springer, New York. 2002.
- [6] A. Kobilinskya, A.I. Nazerb, F. Dubois-Brissonnet. Modeling the inhibition of Salmonella typhimurium growth by combination of food antimicrobials. *Int J Food Microbiol.* 2007, 115(1):95– 109
- [7] Zhang H, Wei H, Cui Y, Zhao GQ, Geng FQ. Antibacterial interactions of Monolaurin with commonly used antimicrobials and food components. *J Food Sci.* 2009, 74: 418- 421
- [8] Wang LP, Zhang QH, Zhao Y. Separation and Preliminary identification of spoilage Organisms in transmutative soy milk. *Microbiology (CHN).* 2007, 34:621–624
- [9] No HK, Park NY, Lee SH, Hwang HJ, SP. Antimicrobial activities of chitosns and chitosan oligomers with different molecular weight on spoilage bacteria isolated from tofu. *J Food Sci.* 2002, 67(4):1511–1514
- [10] Ye NZ. Use of sodium dehydroacetate in food. *China Food Additives.* 2002, 3:64–66 (in Chinese with English abstract)
- [11] J. Delves-Broughton, P. Blackburn, R. J. Evans, J. Hugenholtz. Applications of the bacteriocin, nisin. *Antonie Leeuwenhoek.* 1996, 69(2):193–202
- [12] Faille C, Membre JM, Kubaczka M, Gavini F. Altered Ability of Bacillus cereus Spores To Grow under Unfavorable Conditions (Presence of Nisin, Low Temperature, Acidic pH, Presence of NaCl) following Heat Treatment during Sporulation. *J Food Protect.* 2002, 65(12):1930–1936
- [13] Valero M, France's E. Synergistic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth. *Food Microbiol.* 2006, 23:68–73.