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Development of Preservation Prediction Chart for Long Term Storage of Fermented Cucumber

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Off-flavors and odors in fermented cucumbers result from the growth of undesirable microorganisms during the secondary fermentation. Under laboratory conditions using a sterile fermented cucumber slurry medium, the spoilage fermentations were reproduced. Using this system the salt and pH conditions that allow the spoilage to occur were determined by varying the NaCl concentration and pH of the slurry medium. At pH 3, no spoilage was observed, regardless of the salt concentration, while at pH 3.5, pH 4, and pH 4.5, spoilage occurred in the 0 and 2% NaCl samples. For pH 5.0 samples, spoilage products were seen for all NaCl treatments. Based on these results the Preservation Prediction Chart was developed. The Chart may be used for selection of proper pH value and salt concentration for long term storage of fermented cucumber.

Key words : Preservation Prediction Chart, fermented cucumbers, secondary fermentation, salt and pH conditions

Introduction

Most commercial cucumber fermentations are the result of naturally occurring microorganisms and the environmental conditions such as salt concentration, pH, and temperature of brine [1]. During fermentation the microbial activities occur in various stages [2]. In an initiation stage various Gram-positive and Gram-negative bacteria compete for predominance, however, an acidification in controlled fermentation influences the types of bacteria that grow during this stage [10]. During primary stage of fermentation, carbohydrates are converted to acids and other end products by lactic acid bacteria and yeasts. A secondary fermentation stage could occur if residual sugars remain and lactic acid bacteria have been inhibited by low pH. Fermentative yeasts may grow and cause gaseous spoilage in this condition. If pH rises in low salt concentration, spoilage bacteria could grow in the secondary fermentation stage [5]. Off-flavors and odors in fermented cucumbers result from the growth of undesirable such microorganisms during the secondary fermentation.

The major function of salt in the brining of cucumbers, in addition of inhibition of softening enzyme activity, is to control of microbial growth. The commercial use of salt concentrations has been about 5% to 8% for fermentation and 8% to 16% for storage [7]. The fermentation salt concentration(6%) has evolved as a compromise to achieve a desirable fermentation and maintain a desirably textured product. Much of the salt used for storage of fermented cucumbers must be leached prior to further processing for human consumption. However, the current increasing health concerns of consumers about excessive intake of salt in the diet and the tightening environmental regulations for salt disposal have pushed the pickle industry to develop other fermentation procedure assuring reduced salt consumption [3]. However, low salt fermentations may contribute to spoliage. When cucumbers were brined at 2.3% NaCl, they underwent normal lactic acid fermentation, resulting in less than 1% lactic acid and pH 3.7. Subsequently, however, the cucumbers spoiled due to production of butyric acid and other products were formed by undesirable bacteria while the level of lactic acid was reduced . *Clostridium tertium* grew and contributed to the butyric spoilage of fermented cucumbers [4].

The objective of our research was to determine the salt and pH conditions that allow the secondary spoilage fermentation to occur in the low(0% to 5%) salt brine pickles. We were able to reproduce the low salt secondary fermentation under laboratory conditions using sterile fermented cucumber slurry as a growth medium. With this model system for the spoilage fermentation we determined the

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salt and pH conditions that allow spoilage to occur. Based on these results the Preservation Prediction Chart was developed. The Chart may be used for selection of proper pH value and salt concentration for long term storage of fermented cucumber.

Materials and Methods

Growth medium

A fermented cucumber slurry (FCS) broth was used as growth medium and sterile FCS broth was prepared as follows: size 3a and 3b cucumbers (diameter, 2.5 to 3.0 cm) were brined in a 20L plastic pail using calcium acetate cover brine [9]. The brined cucumbers were inoculated to a cell density of 10^6 cfu/ml with *Lactobacillus plantarum* MOP3 [11]. The fermentation was allowed to go to complete utilization of sugar over the course of two weeks, at which time the pH was around 3.4. The fermented cucumbers were then blended into the course slurry in a Warring blender and filtered through cheesecloth. The homogeneous slurry were distributed into 1 L bottles and stored at -20°C. When need, the slurry was removed from freezer and thawed. After centrifugation at 10,000 x g for 20 min, the supernatant was collected and autoclaved. The NaCl concentration and pH of the slurry was adjusted aseptically as required with the addition of 5M NaCl and 6N NaOH. Prior to inoculation the slurry was dispensed into sterile screw cap vials.

Source of the inoculum

The source of the inoculum for the reproduction of spoilage secondary fermentation was brine from a 0% NaCl cucumber fermentation, carried out in sealed quart jars with rubber syringe septa in the jar caps. This jar had been brined approximately 1 year prior to sampling for inoculation and demonstrated spoilage fermentation features of opaque, dull color and high pressure. Three types of spoilage were identified by the principal acid or alcohol produced: butyric acid (A type), propionic acid/propanol (B type), and propanol (C type).

Reproduction procedures

All procedures for isolation and cultivation were done anaerobically within a glove chamber (Coy Laboratory, Grass Lakes, Mich.) containing a 10% CO_2 - 10% H_2 - 80% N_2 atmosphere. Anaerobiosis was monitored with Oxygen-Hydrogen Gas Analyzer (Coy Laboratory, Grass Lakes, Mich.). The spoilage fermentation inoculants were removed with a sterile 5 ml syringe through the rubber septa in the jar caps. 2 ml of the spoilage fermentation brine was used to inoculate 18 ml of FCS broth in screw cap tubes, which had been preincubated in the anaerobic chamber with the caps loose to allow gas equilibration. Following inoculation the tubes were returned to the anaerobic chamber and incubated at 30°C for 3 months. The reproduction experiments were carried out in triplicate for each type of spoilage fermentation.

HPLC analysis

Slurry samples were prepared for HPLC analysis by filtration through a 0.22 micron filter (Millipore Corporation, Bedford, MA)to remove suspended particulate matter. Samples were analyzed using an anion-exchange column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA) with a 0.8 mL/min flow rate of 0.03 N H_2SO_4 at 75°C. A UV detector (UV-6000, Thermo Separation Products Inc., San Jose, CA) and a differential refractometer (Waters 410, Waters, Milford, MA) were connected in series for detection of organic acids (at 210 nm) and ethanol. Sugars were separated by a Carbopac PA1 column (Dionex Corp., Sunnyvale, CA) with a 0.8 mL/min flow rate of 0.12 N NaOH at room temperature, and detected by a pulsed amperometric detector (PAD-2; Dionex Corp., Sunnyvale, CA).

For each treatment in the experimental protocol a control that was inoculated but not allowed to ferment (frozen) was analyzed for the presence of carry-over organic acids/alcohols from the initial inoculums. The values obtained from the carry-over controls were subtracted in each case from experimental samples and the corrected value was reported in the tables.

Results

Reproduction of spoilage secondary fermentations

Table 1 shows the concentration of contents in each of three types of spoilage fermentation inoculants prior to the inoculation to the FCS broth tubes for reproduction of fermentation. Type A inoculum contained butyric acid, propionic acid, and propanol with increased pH. Type B inoculum contained propionic acid and propanol, and Type C inoculum contained propanol with a trace of propionic acid. Compare to the no spoilage control sample, all

Table 1. Three types of inoculant for reproduction of the spoilage fermentations

Type	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Propanol	pH
Control*	113.52	65.83	0	0	0	4.4
A	0	109.66	7.98	27.38	38.44	6.5
B	10.1	131.4	28.89	0	27.43	4.3
C	9.67	140	2.78	0	44.75	4.1

Control*: no spoilage fermentation sample.

three spoilage types had completely deplete the lactic acid and contained increased amounts of acetic acid along the spoilage products. Table 2 shows the reproduced fermentation results of each type of the spoilage fermentation after 3 months of incubation at 30°C. The pHs at 3 months of incubation time point are also shown. The reproduction of spoilage fermentations were carried out in triplicate. The control was inoculated with sterile slurry samples but not incubated. Each type of spoilage fermentation reproduced the type of spoilage seen in the original spoilage sample. Butyric acid production was shown in fermentations A1-A3, propionic acid/propanol fermentation products were seen in fermentations BI-B3, and a primarily propanol fermentation products was seen in fermentations Cl-C3. Some propanol was observed in the unincubated control, and it may be produced during the primary fermentation, or may naturally present compound that was eluted with propanol on the HPLC column. Figure 1 shows the mean of the concentrations of products from triplet fermentation for the three types of reproduced spoilage fermentations.

Table 2. Reproduction of three types of spoilage fermentations

a) Reproduction of type A(Butyric acid) spoilage.

Type	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Propanol	pH
Control*	114.72	58.73	0	0	7.02	4.5
A1	0.68	43.77	3.81	87.75	7.63	6.2
A2	1.54	42.36	3.92	87.46	7.32	6.6
A3	0.57	43.73	3.69	88.54	8.25	6.4
A average	0.93	43.29	3.81	87.92	7.73	6.4

Control* : no incubation

b) Reproduction of type B(Propionic/Propanol) spoilage.

Type*	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Propanol	pH
control	114.72	58.73	0	0	7.02	4.5
B1	49.59	109.14	19.59	0	12.06	5.0
B2	49.25	108.15	18.89	0	14.84	4.9
B3	47.77	104.72	18.64	0	12.5	5.0
B average	48.87	107.34	19.04	0	13.13	5.0

Control* : no incubation

c) Reproduction of type C(Propanol) spoilage.

Type*	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Propanol	pH
control	114.72	58.73	0	0	7.02	4.5
C1	40.96	105.3	2.44	0	15.71	5.0
C2	44.39	110.13	3.41	0	18.31	5.1
C3	41.82	105.44	2.23	0	13.44	5.1
C average	42.39	106.96	2.69	0	15.82	5.1

Control* : no incubation

Salt and pH matrix

To determine the salt and pH conditions that allow the spoilage to occur, spoilage fermentations were carried out with 0, 2, and 4% NaCI, with a pH range of 3 to 5. All samples were inoculated in triplicate, using sterile slurry as the growth medium described in Materials and Methods. HPLC analysis of the products of fermentations at 3 months post inoculation are given in table 3. The initial inoculum for these experiments came from a spoilage fermentation that had demonstrated all three of the major spoilage fermentation products: butyric acid, propionic acid, and propanol. The HPLC data presented represent the average values for three identical fermentations. As shown in Fig. 2, the progress of the fermentation could be determined by the amount of residual lactic acid and the generation spoilage products. Compared to 4% salt (Fig.2, c), the 0% salt (Fig.2, a) samples had a reduced lactic acid concentration, and showed an increase in the production of

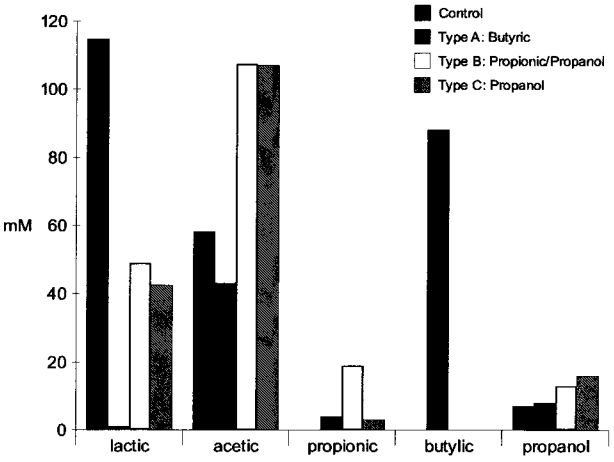
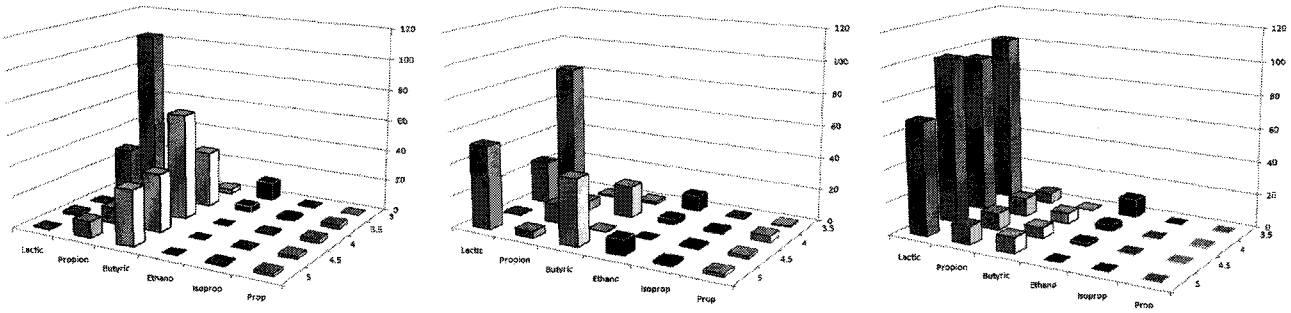


Fig. 1. Products in three types of reproduced spoilage fermentation

Table 3. Comparison of products and spoilage occurrence with different pH and salt concentration

Sample		Condition		Average value for HPLC products from triplicate samples								Spoilage occured
From	To	pH	NaCl	Succ	Lactic	Acetic	Propion	Butyric	Ethano	Isoprop	Prop	
1	3	3	0%	3.8	102.97	53.86	0.66	3	12.25	t	t	No
4	6	3.5	0%	0.95	28.75	50.94	7.37	35.83	4.27	1.43	2.66	Yes
7	9	4	0%	0.36	0	42.28	4.51	67.47	0	0.33	2.59	Yes
10	12	4.5	0%	0.13	1.6	44.3	9.98	37.13	0	t	1.84	Yes
13	15	5	0%	0.3	0.07	50.01	10.42	35.45	0	0.81	1.79	Yes
16	18	3.5	2%	2.17	81.9	59.51	0.83	1.51	9.94	0.35	0.7	Y/N
19	21	4	2%	1.24	26.38	36.89	5.46	19.66	3.8	1.34	4.19	Yes
22	24	4.5	2%	0	0.52	43.89	11.75	51.06	0	1.14	1.8	Yes
25	27	5	2%	0.12	51.86	53.39	4.11	41.31	10	1.64	1.88	Yes
28	30	3.5	4%	3.53	101.94	48.92	6.18	ud	11.29	t	t	No
31	33	4	4%	3.75	95.16	52.4	11	6.44	3.97	0.54	1.01	Y/N
34	36	4.5	4%	3.69	100.04	62.14	10.15	6.43	2.12	0.59	0.6	Y/N
37	39	5	4%	0.05	68.41	48.97	10.95	9.51	ud	0.25	0	Y/N

ud=undetermined, t=trace



1) 0% salt 2) 2% salt 3) 4% salt

Fig. 2. Secondary fermentation products at the different salt concentration and pH condition

spoilage acids and alcohol for all pH values except pH 3. The results are summarized in the preservation prediction table presented in Fig. 3. At pH 3 no spoilage was observed, regardless of the salt concentration, while at pH 3.5, pH4, and pH 4.5, spoilage occurred in the 0 and 2% NaCl samples. For pH 5.0 samples, spoilage products were seen for all NaCl treatments.

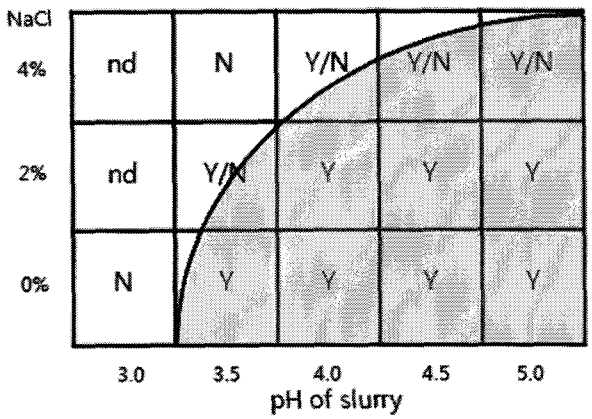


Fig. 3. Preservation Chart

Discussion

Environmental concerns are increasingly forcing pickle manufacturers to consider the problem of disposal of waste brine. Brine recycling and other strategies have significantly reduced the waste disposal problem [5]. Closed cucumber fermentation tanks offer the potential of reducing the amount of salt used during commercial fermentation and storage of cucumbers [8]. It was observed, however, that lower salt concentrations can lead to the spoilage of fermented cucumbers during long term storage. The spoilage of fermented cucumbers is characterized by butyric acid, propionic acid and propanol production under anaerobic conditions. By sequencing the 16S rRNA gene, and a BLAST (Basic Alignment Search Tool) search of the RDP, we could identified bacterial eight species from spoilage fermentations which belong to three genera: *Clostridium,*

Lactobacillus and *Bacillus* from low salt spoiled brine [9]. We have also experimented to determine the salt and pH conditions that allow the spoilage to occur. The results could be summarized into a chart and we report on this paper.

Initially we were able to reproduce the spoilage fermentations under laboratory conditions using a sterile fermented cucumber slurry medium. The ability to reproduce the different types of spoilage after 3 months of fermentation is shown in Table 2. The fermentations were all marked by an increase in pH from the initial value (pH 3.4) of the sterile slurry medium of pH 4.4, and a decreasing in the concentration of lactic acid. Virtually all of the lactic acid was consumed in the butyric acid fermentation, and a production of acetic acid was also seen.

By varying the NaCI concentration and pH of the slurry medium we have determined the conditions allowing the spoilage to occur. A 3 month experiment inoculated with a butyric, propionic, and propanol spoilage samples showed conditions which would or would not allow the spoilage fermentation to occur. The final pH values of each slurry, as well as HPLC analysis of organic acids after the 3 month incubation were indicators of spoilage fermentations. It is possible that with longer term incubation spoilage would occur in the some of the non-spoiled samples, and continuous culture experiments are currently underway to answer that question.

Acknowledgment

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초록 : 발효오이의 산패예견표의 개발

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발효된 오이의 산패는 원하지 않는 미생물의 2차적 생장에 의한 결과이며 장기보관을 원하는 발효오이 식품은 일반적으로 고농도의 염을 사용한다. 염의 농도를 최소한으로 하면서 산패를 방지할 수 있는 pH의 범위를 모색하기 위하여 다양한 조합의 pH와 NaCl 농도를 갖는 발효오이즙(FCS) 배양액에 3가지 종류의 산패액을 각각 접종하여 발효오이의 산패여부를 조사하였다. pH3에서는 NaCl의 첨가가 없더라도 산패는 일어나지 않는데 비하여 pH 5.0에서는 4%의 NaCl에서도 모두 산패가 진행되었다. pH 3.5, pH 4, pH 4.5 에서는 0%와 2% NaCl의 범위 내에서 다양한 결과를 보였다. 이 결과를 바탕으로 발효오이의 산패를 예견할 수 있는 조견표를 작성하였다. 조견표의 사용은 발효오이의 장기보관을 위한 적절한 산도와 염의 농도의 선택을 가능하게 할 것이다.