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Identification, Characteristics, and Growth Inhibition of the Strain Isolated from Spoiled Wet Noodle

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Abstract To determine the cause of wet noodle spoilage, microorganisms isolated from wet noodles were identified and characterized. In addition, the growth inhibitory effects of organic acid mixture (OA: lactic acid 27.8%, acetic acid 12.0%, succinic acid 1.0%) and sodium dehydroacetate (SD) on the isolated strain were estimated in nutrient broth medium. The isolated strain was Gram-positive, rod shaped, motile, and spore forming. Based on physiological characteristics and the API 50 CHB-kit test results for the assimilation of 49 carbohydrates, the isolated strain was identified as *Bacillus amyloliquefaciens* (92.6%), which is able to degrade starch. Decimal reduction times (D-values) at 100, 105, and 110°C for spores of *B. amyloliquefaciens* were 8.5, 5.1, and 2.5 min, respectively, and the z-value was 12.8°C. We estimated that *B. amylo-liquefaciens* isolated from spoiled wet noodles was a thermophilic species having high heat-resistance. Viable cell numbers in wet noodles and broth medium inoculated with *B. amyloliquefaciens* were decreased by 2-4 log cycles by combined treatment with 0.03 or 0.05% OA and 0.3% SD. These results revealed that OA combined with SD could be used as a potential agent to inhibit *B. amyloliquefaciens* in wet noodles.

Key words: *Bacillus amyloliquefaciens,* wet noodle, organic acid, growth inhibitory effect, sodium dehydroacetate

Introduction

Noodles and cooked rice have been played an important role in Korean diet. Noodles in Korea are classified as cut fresh, stretched dry, and extruded on the basis of noodle-making method, and are also classified as dry, wet, boiled, instant, and pasta according to the type of processing and distribution after noodle-making (1, 2).

After the 1990s, production of wet noodles by small restaurants was replaced by mass production by larger firms, and the supply has been dominated by the larger distributors. With increasing income in recent years, the consumption of wet noodles instead of dry noodles has been rapidly expanding in the Korean market (3, 4). In addition, the consumption of wet noodles increased because the changes of nutrients, flavor, taste, and texture by drying and heating were minimized by the generalization of the cold chain system, and cooking time was reduced.

However, wet noodles, which have good elasticity and texture, are susceptible to microbial spoilage due to high moisture content. It is known that *Bacillus* can proliferate on wet noodles with high moisture content (5).

The quality of noodles was evaluated by microbiological and sensory factors such as color, taste, and texture (6). Mesophilic bacterial counts in sterilized wet noodles were standardized at less than 1.0×10^6 CFU/g and *Escherichia coli* negative in the current Korean Food Code (7). Therefore, growth inhibition of microorganisms is needed for the shelf-life extension of wet noodles.

Many studies have predicted the shelf-life of noodles by bacterial count (5), shelf-life extension of noodles by plantain (8), dandelion (9), *Lycii fructus* (10), *Prunus mume* extract (11), *Opuntia ficus-indica* (12), chitosan (13), organic acids (14), propylene glycol (2), and condensed phosphate (15).

Currently, 15 synthetic preservatives, such as dehydroacetic acid, sorbic acid, benzoic acid, and *p*-hydroxybenzoic acid and their salts, are used to inhibit the growth of microorganisms in food (7). Although these synthetic preservatives are effective, they can be detrimental to human health, and the demand for food products which are preservative free or contain only trace amounts is increasing (16).

In this study, the microorganisms from spoiled wet noodles were isolated and identified through examination of the morphological and physiological characteristics to verify the cause of spoilage. In addition, we investigated the synergistic effects of an organic acid mixture (OA: lactic acid 27.8%, acetic acid 12.0%, succinic acid 1.0%) and sodium dehydroacetate (SD) in wet noodles and broth medium to inhibit the strain isolated from spoiled wet noodles.

Materials and Methods

Isolation and identification of a saprogenic strain The strain was purely isolated from spoiled wet noodle product (1 kg, packed by nylon and polyethylene) produced between Mar, 2002 and Feb, 2003 by Youngwoo Frozen Foods Co., Ltd. (Namwon, Korea). One loopful was taken from the spoiled section of the product and diluted in 10 mL of 0.1% peptone water, followed by incubation at 45°C for 24 hr and heat treatment in a water bath at 100°C for 2 min. The diluted strain was streaked on a nutrient agar (Oxoid, Basingstoke, England) and cultivated in an

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incubator at 45°C for 24 hr. The dominant six colonies were taken from the agar and subjected to several isolation processes. Based on the shape and color of the colonies, and on microscopic observation, the isolated colonies were judged to be of the same species. The isolated strain was place in 50% of glycerol stock, freeze dried, and stored at -60°C.

The properties of the isolated strain (SWN-1) were investigated by testing with Gram staining and microscopic observation. Bergey's Manual of Systematic Bacteriology (17) was used to examine the shape and characteristics of the strain. The API 50CHB-kit (BioMerieux Co., Marcy L'Etoile, France), an identification system for microorganisms, was used to investigate and identify the assimilation of the 49 carbohydrates (18).

Degradation of starch (19) Bacterial pre-culture (0.1 mL) was transferred to a new broth medium and grown for 18 hr. The culture was then incubated for 24 hr at 45°C on six points of the agar plate containing 0.2% soluble starch. Degradation of starch by the isolated strains was tested based on the formation of a transparent ring using Gram's iodine (Hayashi Pure Chemical Ind. Ltd., Osaka, Japan) solution.

Preparation of spore suspension and spore dyeing Spores were produced by storage in a freezer at 4 °C for 3-4 days after the cultivation of SWN-1 at 30°C for 24 hr in the nutrient broth (20). The frozen stored spore suspension underwent centrifugation (Beckman J2-21, CA, USA) for 5 min at 10,750 × g and was washed with 0.85% saline solution 2-3 times. Subsequently, the residuals were diluted with 10 mL of 0.85% saline solution and dispensed in 1 mL volumes to the TDT tube for the culture media with a strain density of about 10^7-10^8/mL, and flame-sealed (18).

The spores were dyed using the Schaeffer-Fulton method (21). Briefly, the prepared smear was steamed by heating, while covered with malachite green on asbestos. After evaporation, additional dye was applied and maintained for 5 min without drying. After washing with water for 30 sec, the smear was contrast-dyed with safranin for 20 sec. Subsequently, the slide was inspected under oil immersion. Germination of spores was verified using an optical microscope (SELOPT SBH, Seoul Optical Instrument Inc., Seoul, Korea) with a malachite green oxide (Showa Chemical Inc., Tokyo, Japan) indicator.

Test of heat resistance Samples of the spore suspension were heat-treated for 5, 15, and 25 min at 100, 105, and 110°C in an oil bath (Samheung, Uijeongbu, Korea). They were then cooled by flowing water and inoculated on to the nutrient agar. Viable strains were cultivated for 48 hr at 30°C for spore counting. The D-value (decimal-reduction time) and z-value were calculated based on the survival curves of the spores (18, 22).

Assessment of viable cell number OA, which is used by a wet noodle manufacturer, and SD at different concentrations were added into 10 mL of nutrient broth medium containing 10^5-10^6 CFU/mL bacteria and incubated for 72 hr at 30°C. The viable cell numbers were counted at 24-hr-intervals by the agar plate count method. During incubation, the pH of the broth medium was measured by using a pH meter (Model 520A, Orion Research Inc., Beverly, MA, USA). In addition, 1 mL of culture (10^5-10^6 CFU/mL) of *Bacillus amyloliquefaciens* isolated from spoiled wet noodles was inoculated to 50 g of wet noodles, and the viable cell numbers were counted at 24-hr-intervals by the agar plate count method.

Results and Discussion

Morphological and physiological characteristics of the isolated strain Table 1 presents the morphological and physiological characteristics of the strains (SWN-1) isolated from spoiled wet noodles. The isolated strains were Gram positive, rod-shaped, motile, and spore forming. The test of physiological characteristics indicated catalase positive, Voges-Proskauer response negative, casein degradation positive, and starch dissolving. The isolated strains didn't utilize propionate and citrate.

Table 2 shows the results of the microorganism identification system (API 50 CHB-kit) test for the assimilation of the 49 carbohydrates. The isolated strains utilized L-arabinose, glucose, fructose, maltose and glycogen, but not erythritol, D-xylose, L-sorbose, sorbitol, xylitol, and fucose, as a carbon source.

The strains isolated from spoiled wet noodles were identified as *Bacillus amyloliquefaciens* (92.6%) according to their morphological and physiological characteristics and the results of the API 50 CHB-kit.

Degradation of starch The agar section inoculated with the isolated strain didn't present an iodine phase showing a violet color by the reaction with starch (Fig. 1). This indicates that the isolated stains had the dissolvability of starch. This result corresponded to the identification results from the API 50 CHB-kit as presented in Table 1.

Heat resistance of spores Damage and loss of cells or spores of the microorganism was caused by the treatment of heat, cooling, UV-radiation, and chemicals (23). Therefore, the heat resistance of a spore is a very

Table 1. Morphological and physiological characteristics of the strain isolated from spoiled wet noodles

Characteristics	Results
Morphological characteristics	
Shape	rod
Gram stain	+[1)]
Mobility	+
Spore formation	+
Physiological characteristics	
Catalase	+
Voges-Proskauer reaction	-[2)]
Utilization of propionate	-
Utilization of citrate	-
Degradation of casein	+
Hydrolysis of starch	+

[1)] 90% or more of strains are positive.
[2)] 90% or more of strains are negative.

Table 2. Carbohydrate assimilation of the isolated strain by using the API 50CHB-kit

Carbohydrates	Results	Carbohydrates	Results
Glycerol	-[1)]	Salicine	+
Erythritol	-	Cellobiose	+
D-Arabinose	-	Maltose	+
L-Arabinose	+[2)]	Lactose	-
Ribose	+	Melibiose	-
D-Xylose	-	Saccharose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β-Methyl-D-xyloside	-	Melezitose	-
Galactose	-	D-Raffinose	-
D-Glucose	+	Amidon	+
D-Fructose	+	Glycogen	+
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	+
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
Mannitol	+	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α -Methyl-D-glucoside	+	L-Arabitol	-
N-Acetyl glucosamine	-	Gluconate	-
Amygdalin	+	2-Keto-gluconate	-
Arbutine	+	5-Keto-gluconate	-
Esculine	+		

[1)] Negative
[2)] Positive

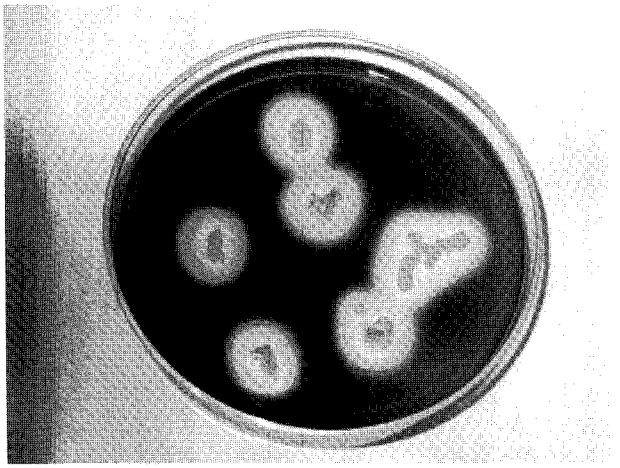

Fig. 1. Starch degradation by *Bacillus amyloliquefaciens* isolated from spoiled wet noodle.

important criterion in the preservation of foods by thermal sterilization.

As shown in Fig. 2, the survival rates of the isolated strains or spoores decreased rapidly with increasing heating temperature and longer time at the same temperature. The D-value times, indicating the period required to reduce the microorganism density to 1/10 at a certain temperature, were 8.5, 5.1, and 2.5 min at 100, 105, and 110°C, respectively. The linear regressive equations for the thermal death of the spores at 100, 105, and 110°C were Y = -0.7337X + 6.9778 (R^2 = 0.9606), Y = -0.9390X + 6.6617 (R^2 = 0.9842), and Y = -1.5357X + 7.150 (R^2 = 0.6548), respectively. These D-values of *B. amyloliquefaciens* were lower than that of *B. stearothermophilus* which was 4-5 min at 121°C (24). It is known that the heat resistance of *B. stearothermophilus* spore is about 20 times higher than that of *Clostridium botulinum* spore (25).

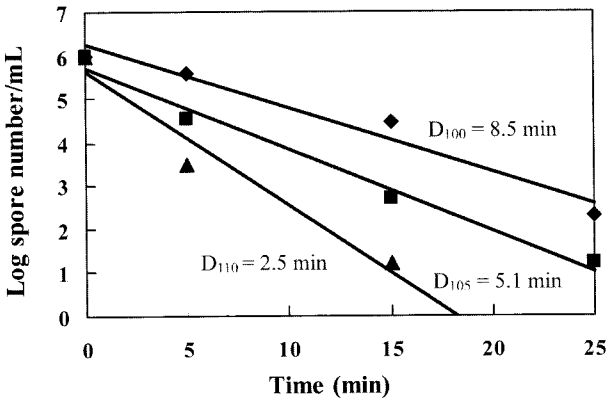

Fig. 2. Survival curves for *Bacillus amyloliquefaciens* spores isolated from spoiled wet noodle at 100, 105, and 110°C. - ◆ -: 100°C, - ■ -: 105°C, - ▲ -: 110°C.

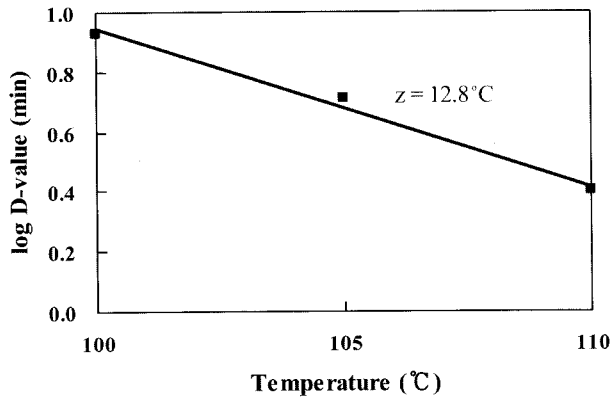

Fig. 3. Thermal death time curve of *Bacillus amyloliquefaciens* isolated from spoiled wet noodle.

Fig. 3 shows the changes of the D-value over the temperatures to verify the z-value by using the results presented in Fig. 2. The linear regressive equation for the z-value was Y = -3.0X + 11.367 (R^2 = 0.9941). The z-value temperature required to change the D-value by 1 log cycle from the linear equation was 12.8°C. This value was higher than the range of 7.8-12.2°C previously reported for *B. stearothermophilus* (24, 26-28).

In conclusion, *B. amyloliquefaciens* isolated from spoiled wet noodles had high heat resistance.

Growth inhibitory effects by organic acid mixture (OA) and sodium dehydroacetate (SD) Sodium propionate (0.3-1.0%, w/w) did not show a growth inhibitory effect on *B. amyloliquefaciens* isolated from spoiled wet noodles according to absorbency measurement at 600 nm. However, SD did inhibit the growth of *B.*

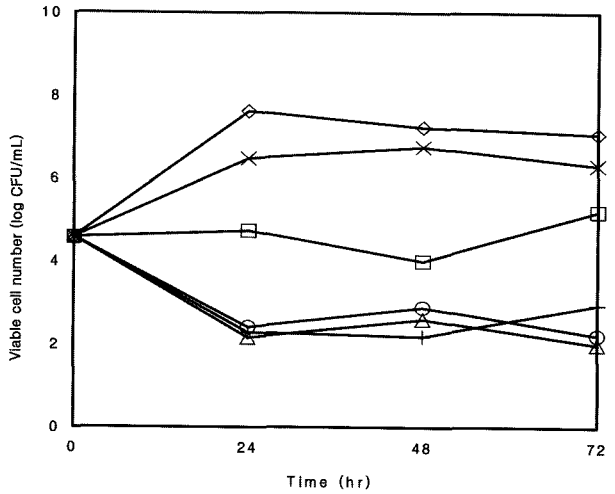

Fig. 4. Changes of viable cell number by treatment of organic acid mixture (OA) and sodium dehydroacetate (SD) on *Bacillus amyloliquefaciens* isolated from spoiled wet noodle. - ◇ -: Control, - □ -: 0.03% OA, - △ -: 0.05% OA, -x-: 0.3% SD, - ○ -: 0.03% OA + 0.3% SD, -+-: 0.05% OA + 0.3% SD.

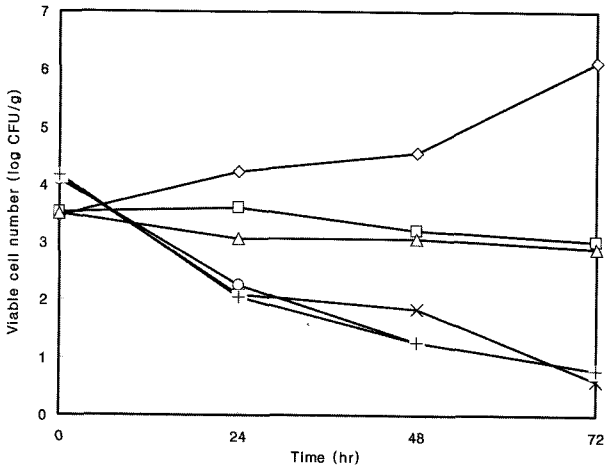

Fig. 5. Changes of viable cell number by treatment of organic acid mixture (OA) and sodium dehydroacetate (SD) on wet noodle inoculated with *Bacillus amyloliquefaciens* isolated from spoiled wet noodle. See Fig. 4 for symbols.

amyloliquefaciens at over 0.3% (w/w) (data not shown).

The growth inhibitory effects by OA, which is used by a wet noodle manufacturer, with or without 0.3% of SD, are shown in Fig. 4. The growth inhibitory effect of 0.3% SD on *B. amyloliquefaciens* was low and the viable cell numbers of *B. amyloliquefaciens* were maintained by treatment with 0.03% OA for 72 hr. However, the viable cell numbers of *B. amyloliquefaciens* were decreased by 2-4 log cycles by combined treatment with 0.03 or 0.05% OA and 0.3% SD.

The growth inhibitory effects of OA with or without 0.3% of SD on wet noodles inoculated with *B. amyloliquefaciens* (10^5-10^6 log CFU/mL) isolated from spoiled wet noodles are shown in Fig. 5. The viable cell numbers of *B. amyloliquefaciens* were decreased by 2-5 log cycles by treatment with 0.3% SD, and by treatment with 0.03 or 0.05% OA in combination with 0.3% SD. SD (0.3%), which showed very weak inhibitory effects in broth medium, showed a strong inhibitory effect similar to combined treatment with OA (0.03, 0.05%) and 0.3% SD in wet noodles. We assumed that the difference of the growth inhibitory effect of SD whether in broth medium or in wet noodles was based on difference of components.

Table 3. Changes of pH in nutrient broth medium with added organic acid mixture[1)] (OA) and sodium dehydroacetate (SD) for 72 hr at 30°C

Treatments	Time (hr)			
	0	24	48	72
Control	7.20	6.62	6.84	7.41
0.03% OA	4.19	4.12	4.43	4.87
0.05% OA	4.01	3.77	4.17	4.17
0.3% SD	6.99	6.39	6.65	6.72
0.03% OA + 0.3% SD	5.14	5.01	5.44	5.43
0.05% OA + 0.3% SD	5.04	4.73	5.15	5.12

[1)]Composed of lactic acid 27.8%, acetic acid 12.0%, and succinic acid 1.0%.

Table 3 shows that the pH of the nutrient broth medium treated with 0.3% SD or OA (0.03%, 0.05%) was 6.99 or 4.01-4.19 at the initial stage, respectively. However, the pH of the medium treated by SD in combination with OA was 5.04-5.14. Our data showed that the combination of OA and SD exerts an antimicrobial activity more efficiently than the medium pH. These results were in agreement with previous reports that the efficacy of preservatives can be increased by adding organic acids such as propionic, acetic, citric, tartaric, lactic, and malic acids, either alone or in combination (29, 30).

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