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Article

Isolation and identification of blacken spoilage inducing bacteria from Korean dried persimmon

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Abstract

Blacken spoilage is one of the common problems during the cold storage of dried persimmon in Korea. We collected the spoiled dried persimmon in the refrigerator and classified them to 4 types depending on their appearances. Furthermore we isolated blacken spoilage inducing bacteria from type D of dried persimmons. Among the isolates we identified the seven blacken spoilage inducing bacteria. They are *Aeromonas hydrophila* DP1, *Cedecea davisae* DP2, *Ewingella americana* DP3, *Flavimonas oryzihabitans* DP4, *Providencia rettgeri* DP5, *Providencia rustigianii* DP6 and *Serratia plymuthica* DP7. Strains were identified based on their morphological, cultural and physiological properties. We also found that *Ewingella americana* DP3, *Flavimonas oryzihabitans* DP4 were the major blacken spoilage inducing bacteria during dried persimmon storage.

Keywords : Dried persimmon, blacken spoilage bacteria, *Ewingella Americana, Flavimonas oryzihabitans,*

Introduction

Persimmon is one of the major fruits grown mostly in Far East Asian countries including Korea, China and Japan. Fresh persimmon fruit needs to be consumed shortly after harvest because of its perishability. Therefore, more than 50% of persimmon used to be processed to dried persimmon by sun-drying at the harvesting area in Korea. Amount of dried persimmon production has increased about 10% annually (Kim *et. al*, 1995; Cho, 2007). Dried persimmon is called Gotgam in Korea, Hoshigaki in japan and Shi-bing in China. Most of dried persimmon processing, such as peeling and dehydration, is carried out in the open field, which causes the contamination of diverse microbe including harmful bacteria. Bacterial contamination, stiffening or blackening of dried persimmon spoilage are common problems during long term storage (Kim *et. al*, 1993). In order to improve the quality of dried persimmon for prolonged storage sulfur fumigation treatment (Lee *et. al*, 1994) and improved packaging material (Lee *et. al*, 1995) have been studied. Because the use of sulfur additives onto the agricultural produce have been more and more regulated currently, the use of natural products for the prevention of spoilage have been studied onto several agricultural produce (Cleveland et al, 2001). In order to study the microbial contamination of the dried persimmon, the changes of total bacterial cell, yeast and fungi wereinvestigated during air drying (Hong *et. al*, 2001). In this study, among various microbe found in dried persimmon we isolated and identified seven blacken spoilage inducing bacteria during long term storage.

Materials and Methods

Collection and classification of spoiled dried persimmon

Ny/PE packaged Sangju Dungsi (A variation of Diospyros kaki THUNB from Sangnju, Korea) dried persimmons were purchased from the local company in Sanju, Korea. Degree of Dried persimmon spoilage was classified into 4 groups by the spoiled appearance. Classification criteria are as follows; A; slight softening, similar to original condition, B; softening, slight white powder, C; slight blackness, deep softening, D; deep blackness, deep softening. Microbes from each sample were grown in 4 different rich media (Table 1).

Isolation and cultivation of spoilage inducing bacteria

Nutrient agar plates used in this experiment were shown in Table 1. In order to isolate bacteria from dried persimmon, the blacken part of dried persimmon were isolated and vortexed

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Table 1. Media for microflora determination of the spoiled dried persimmon

Media	Composition	(g/L)
Plate count agar	digest of casein	5
	yeast extract	2.5
	Dextrose	1
Lactobaccilli MRS	proteose peptone NO.3	10
	beef extract	10
	yeast extract	5
	Peptone	20
	Dextrose	1
	Tween 80	2
	sodium acetate	5
	magnesium sulfate	0.1
	manganese sulfate	0.05
	dipotassium phosphate	2
Acetobactor medium	Glucose	30
	yeast extract	5
	Ethanol	30
	$CaCO_3$	10
Potato dextrose agar	Potato	200
	Dextrose	20

vigorously in distilled water. Resuspended samples were serially diluted and plated in 4 respective agar plates shown in Table 1.Microbe was initially classified by colony morphology. These colonies were streaked a few times to obtain a pure colony. The isolated colonies were cultured in liquid media (Table 2) for 24 to 48 hours at 37℃ for subsequent experiments.

In order to observe the effect of isolated bacteria on dried persimmon, each strain was grown in overnight then and resuspended in the same amount of PBS after centrifugation. Prior to the experiment classification A grade dried persimmon is illuminated on 500μW/cm^2 UV for 20 minutes. One ml of each resuspended culture was inoculated to UV treated dried persimmons. Blackened spoilage of dried persimmon was monitored by appearance, color and smell at 30℃. When blacken spoilage was observed bacteria was re-isolated and compared to originally inoculated bacteria by observing microscopic morphology and gram staining.

Identification of isolated bacteria

In order to identify the isolated bacteria color and shape of colonies were first observed (Gerhardt *et. al*, 1981). The isolated bacteria were also observed under microscope and tested for Gram staining. Biochemical characteristics of the bacteria are tested with API 20E (API System S.A., BioMerieux SA, France) kit. Identification with API 20E kit was followed by manufacturer's instruction. Analytical Index provided by API System S.A was served as a reference (Stanek, *et. al*, 1981; Ekwall, *et. al*, 1982; Aldridge, *et. al*, 1981).

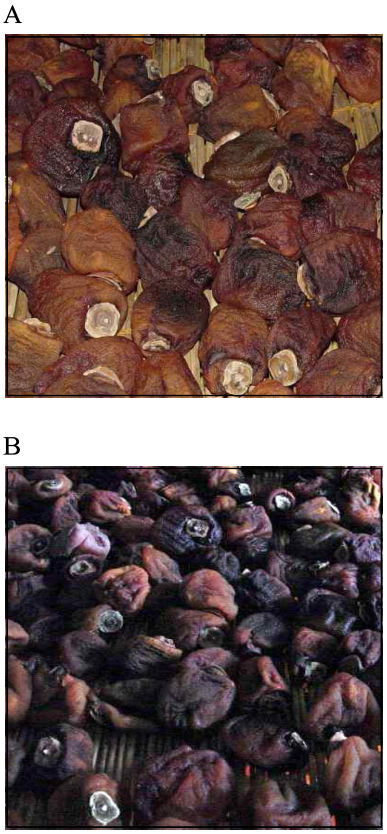

Fig. 1. The photos of the spoiled dried persimmon.
A: the pool of the spoiled gotgam, B: the deep blackckened gotgam (D in Table 1.).

Selection of major blacken spoilage bacteria by color measurement

Either the seven bacteria grown overnight or PBS was inoculated on the dried persimmon. The inoculated dried persimmons were stored at 37℃ and their surface changes were monitored. And the color values ofbacteria inoculated dried persimmons were measured for 40 days at 30 ℃. Hunter colorimeter (Colorimeter, Model CR-200, Minolta Co., Japan) was used to measure whiteness (L), redness (a) and yellowness (b) and ΔE values were calculated (Simon *et. al*, 1996). L, a and b values of the standard plate are 97.79, -0.38, and 2.03, respectively.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Table 2. Liquid media for growth of microorganism isolated from the spoiled dried persimmon

Media	Composition	(g/L)
Nutrient broth	beef extract	3
	peptone	5
ctobaccilli MRS broth	Proteosepeptone NO.3	10
	beef extract	10
	yeast extract	5
	peptone	20
	dextrose	1
	Tween 80	2
	sodium acetate	5
	magnesium sulfate	0.1
	manganese sulfate	0.05
	dipotassium phosphate	2
Acetobacter liquid medium	glucose	5
	$MgSO_4 \cdot 7H_2O$	0.2
	acetic acid	10
	ethanol	50
	glycerin	10
M broth	yeast extract	3
	malt extract	3
	peptone	5
	dextrose	10
Potato dextrose broth	potato	200
	dextrose	20

Results and Discussions

Microflora of spoiled dried persimmon

Commercially available dried persimmons are collected and classified into 4 groups depending on their spoilage pattern and degree. Microbe from each sample was grown in 4 corresponding media shown in table 2. The total microbial counts on bacteria, lactic acid bacteria, acetic acid bacteria, yeast, and mold from respective classified sample were shown in Fig. 2. As the spoilage progresses the number of total microbes increased on all media plates except mold. The number of general bacteria from nutrient media (NM) and yeast from YM broth media (YM) showed highest colony forming unit (CFU) in all groups. Additionally CFU detected in NM and YM from group D is more than 2 orders of magnitude compared to that of non-spoiled group A. This suggests that these general bacteria and yeast may play a major role in the spoilage. CFU of mold does not exceed 10^3 CFU/g regardless of spoilage progress but mold became visual after long term storage. Number of lactobacillus and acetic acid bacteria also increase 2 orders of magnitude as spoilage progresses. There are more *Lactobacilli* on lactobaccilli MRS media than acetic acid bacteria on acetobacter medium in the spoiled group of dried persimmon. It was reported that the growth of acetic acid bacteria on the spoiled persimmon occurred with the softening of texture after alcohol production by yeast (Adams *et. al.*, 1985).

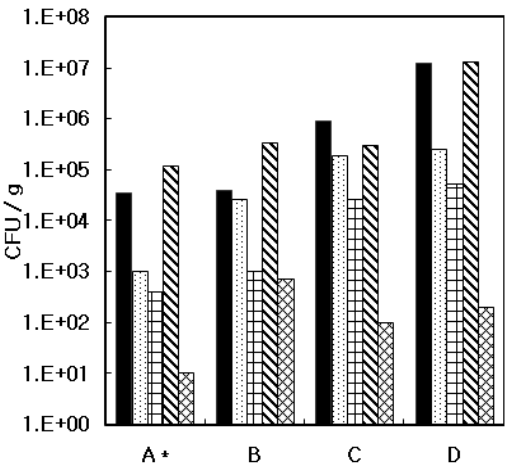

Fig. 2. Total microorganism count of the spoiled dried persimmon.

■ : Bacteria , ⬚ : Lactic acid bacteria, ▦ : Acetic acid bacteria, ▧ : Yeast, ⊠ : Mold (The same as Table 1.).

Isolation and identification of blacken spoilage inducing bacteria

Seven bacteria isolated from spoiled dried persimmons were inoculated on UV treated dried persimmon and then incubated for 48 hour at 30℃. All isolated bacteria showed growthand induced spoilage involved blacken and odor. The isolated bacteria grownon dried persimmons are compared with originally inoculated strains by microscopic morphology and Gram staining. Isolated bacteria were identified by API system. Physiological and biochemistry charactersof the isolated strains were shown in Table 3. *Aeromonas hydrophila, Cedecea davisae, Ewingella americana, Flavimonas oryzihabitans, Providencia rettgeri, Providencia rusting, Serratia plymuthica* were identified as a blacken inducing bacteria on the dried persimmons. These identified bacteria were termed as *Aeromonas hydrophila* DP1, *Cedecea davisae* DP2, *Ewingella americana* DP3, *Flavimonas oryzihabitans* DP4, *Providencia rettgeri* DP5, *Providencia rustiginii* DP6, *Serratia plymuthica* DP7, respectively. DP1 is *Vibrionaceae* family and D2~D7 are *Enterobacteriaceae* family. All of them are Gram (-)rod shaped facultative aerobe.

Table 3. Biochemical properties of each microorganism from the spoiled dried persimmon

	DP1	DP2	DP3	DP4	DP5	DP6	DP7
Colony color (NA)	W1	C	C	W	W	W	I
Gram stain	-	-	-	-	-	-	-
Motility	+	+	-	D	+[1)]	+	d[2)]
Aerobic	d	d	d	D	d	d	d
Oxidase	+	-	-	-	-	-	-[3)]
β-Galactosidase	+	+	+	-	-	-	+
Arginine dihydrolase	-	+	-	-	-	-	-
Lysine decarboxylase	+	-	-	-	-	-	-
Ornithine decarboxylase	-	+	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	d
H2S production	-	-	-	-	-	-	+
Urease production	-	-	-	+	+	-	-
Tryptophane deaminase	+	d	d	-	+	+	+
Indole production	-	-	-	-	+	+	-
Gelatinase	+	-	+	-	-	-	d
Acid from :							
Glucose	+	+	+	+	+	d	+
Mannitol	+	+	+	-	+	-	+
Inositol	-	-	-	-	+	-	d
Sorbitol	-	-	-	-	-	-	d
Rhamnose	-	-	-	-	d	-	-
Sucrose	+	+	-	-	-	-	+
Melibiosese	-	-	-	-	-	-	+
Amygdalin	-	d	-	-	-	-	+
Arabinose	+	-	-	+	-	-	-

1 W : white, C : creamy, I : iridescent
[1)]positive for 90～100% of strain. [2)]positive for 26～74% of strain.
[3)]positive for 0～10% of strain

Selection of major blackenspoilage inducing bacteria

In order to find out major spoilage causing bacteria, 1 ml of each strain was inoculated on UV treated dried persimmon then ΔE value was monitored over 40 days (Fig. 2). ΔE value represents color changes including blacken spoilage so high ΔE value means deeply progressed blacken spoilage. In general, as storage period gets longer L value decreased whereas a value was increased. No significant difference of b value was observed (Data not shown).

All the tested strains contributed to the increase of ΔE values which mean the degrees of browness during storage period. Either *Ewingella americana* DP3 or *Flavimonas oryzihabitans* DP4 treated persimmon showed the highest ΔE at the end of the 40 day storage. Both *Ewingella americana* DP3 or *Flavimonas oryzihabitans* DP4 treated persimmon showed not only high initial ΔE value but also steep increase of ΔE value. This suggested that *Ewingella americana* DP3 and *Flavimonas oryzihabitans* DP4 were the major blacken spoilage inducing bacteria. *Aeromonas hydrophila* DP1 and *Serratia plymuthica* DP7 were also significantly affect blacken spoilage. *Flavimonas oryzihabitans* DP4 was also known as *Psusedomonas oryzihabitans* and it was known to be a yellow pigment producing bacteria (Freney *et. al.*, 1988). Even though no significant b value was observe during spoilage process it would be interesting to find out if *Flavimonas oryzihabitans* can cause yellowness on dried persimmon in a certain condition. When persimmon is stored without bacterial inoculation ΔE value decreasedfor the first 15 days because dehydration driven white power formation contribute to the increase of whiteness represented by L value. As storage period extend L value of non-inoculated group increased but not as much as bacteria inoculated groups.

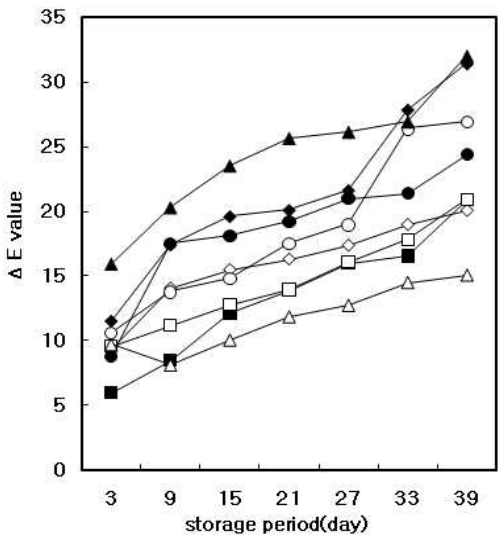

Fig. 2. Changes of Hunter color values of dried persimmon inoculated with the isolated strains during storage at 30℃.
(△-△ : non-treated, ○-○ : DP1, ◇-◇ : DP2, ▲-▲ : DP3, ◆-◆ : DP4, □-□ : DP5, ■-■ : DP6, ●-● : DP7 strain)

Acknowledgments

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