JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3843-3848

Study on Characteristics and Nitrate Reduction of Kluyvera Isolated from Ginger

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(Received: 11 June 2014; accepted: 12 July 2014)

A high nitrite content ginger paste was found during the 26th Universiade. This study aimed to explore the reason behind this phenomenon. *Kluyvera cryocrescens* and *Kluyvera ascorbata* were isolated from the ginger paste. Both the two strains increased steadily if the pH was 5.0, 6.0 or 7.0. Under pH 4.0 conditions, strains reduced sharply. Compared these data we can find that the best pH for *K.cryocrescens* propagation was pH 6.0, and as for *K.ascorbata* was pH 5.0. Also *Kluyvera* strains can grow well if the NaCl concentration weas 0.5%, 1.0% or 2.0%, they not survival if the NaCl concentration reached 5%.The ginger paste provided suitable pH and temperature for Kluyvera propagation when it was stored at 8°C. Nitrite concentration in most of ginger slices sample reached a peak value when the bacterial concentration of *Kluyvera* reached 10^8 CFU/g. In this study, it is confirmed that *Kluyvera* played a major role in reducing nitrate to nitrite in the ginger paste.

Key words: Kluyvera cryocrescens, Kluyvera ascorbata, nitrite, ginger.

Awareness on Food safety is of increasing importance in China, the point was stressed during the 26th Summer Universiade in 2011. A high nitrite concentration ginger sample had caused attention. All ginger was from the local market in Shenzhen during the Universiade. Ginger paste is an ingredient for salads. After washed, raw ginger was mashed, packaged in food containers and saved in refrigerator at 8°C. Ginger paste will be consumed within 48 h. There are totally fourteen ginger samples and eighteen ginger pastes had been tested at the same time. Before package, the nitrite content of all ginger samples was below the detection limit (1.0 mg/Kg, counted by NaNO2). After 30 h, ten of eighteen ginger paste was detected nitrite concentration below 50 mg/Kg, seven of eighteen ginger paste were detected nitrite concentration between 50 to 100 mg/Kg, one of the eighteen ginger paste was detected nitrite concentration achieved 789 mg/Kg. The nitrite concentration of whole ginger was below the detection limit on the whole detection period.

High levels of nitrate in vegetables are frequently reported[1-2]. The total daily intake of nitrate is contributed by vegetables[3]. The potential hazard of vegetable-borne nitrate comes from the fact that it can be converted to methaemoglobin-producing nitrite[4]. Methaemoglobin cannot bind oxygen and the competition effect produces a leftward shift in the oxygen-dissociation curve, causing hypoxaemia[5]. Infants and children are particularly susceptible to methaemoglobinaemia[4, 6-7]. Generally, for adult, 0.3-0.5 g nitrite concentration intake will cause toxic reactionÿand the lethal doses of nitrite was estimated 3 g. Moreover, nitrite may also combine with the

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secondary amine which intake from other foods, and then induce the digestive system cancer [8]. With freshly harvested vegetables, the nitrite is usually low[9]. However, under adverse post-harvest storage conditions, nitrite contents in vegetables increase as a result of bacterial contamination and endogenous nitrate reductase action[10].

In this case, the content nitrite of the ginger paste reached 789 mg/kg. However, according to the ADI of WHO/FAO (1994), human daily intake of nitrite is 0.2 mg/kg, and if a person is 60 kg that the allow intake should be 12 mg. So, eat more than 15.2 g of the ginger paste will over the value of ADI. The reasons of high nitrite content in the ginger paste must be explored. According to EFSA (2008)[10], the nitrate converted to nitrite mainly due to bacterial contamination and endogenous nitrate reductase action. The level of nitrite of the ginger paste sample has reached 789 mg/Kg within 30 h at 8°C, which is doubtable that not only the endogenous nitrate reductase action took place, but also bacteria contaminations were also contributed. Therefore, it is necessary to isolate bacteria in the ginger paste sample and analyze the bacteria growth characteristics.

MATERIALS AND METHODS

Culture medium, chemicals, and equipments

Plate count Agar (PCA), Nutrient broth (Beijing Land Bridge Technology Co., LTD). Nitrate and Nitrite (national institute of metrology,China), VITEK 2 Compact (Biomerieux France). Spectrophotometer (Agilent-8453, Agilent Technologies Ltd, USA);

Identification of the selected strain

10 g ginger paste which nitrite concentration achieved 789 mg/Kg was diluted with 90 mL normal saline (NS), and 100 µL of the diluted solution was spread on Plate count Agar (PCA). PCA was cultured at 37°C around 3 to 6 d, at aerobic conditions. The aerobic plate count was counted by plate counting method. Meanwhile, ten randomly selected colonies-forming units were purified, gram stained, and analyzed with VITEK 2 Compact and manual biomedical test analyzation.

Growth characteristics of *Kluyvera*

Growth characteristics *of Kluyvera* from the ginger pastes was observed in nutrient broth. The broths were cultured separately at different temperatures (1, 4 and 8°C). Different pH values (4.0, 5.0, 6.0 and 7.0) and Sodium Chloride concentration (0.5, 1, 2, and 5%) were designed for each temperature experiment group.

Simulation experiment

Eight different ginger species were from different regions in China. Gingers were mashed and sterilized 30 min under 100°C (three Parallel samples had been made for each ginger specie; each sample was weight of 400 g). The nitrate content of each sample was tested after sterilization. *Kluyvera cryocrescens* and *Kluyvera ascorbata* were added separately in eight different species ginger pastes. 4 mL of the *K.cryocrescens* bacterial suspension (2.2×10^7 CFU/mL) were added in eight different sterilized ginger paste samples, similarly, 4 mL *K.ascorbata* bacterial suspension (1.2×10^8 CFU/mL) were added separately in eight different sterilized ginger paste sample. Control groups were stored in the same conditions without *Kluyvera* contaminate. All ginger pastes were stored in the 8°C condition. Nitrite value and bacterial concentration were measured daily.

Determination of nitrate and nitrite

5.00 g samples were added into12.5 mL saturated borax solution, mixture was set in boiling water bath for 15 min. After cooled down, potassium ferrocyanide and zinc acetate was added for protein precipitation. The mixture then was placed for 30 min to remove fat and filtrated. 2 mL p-aminobenzene sulfonic acid was added into the filtrate, and was placed for 3 min, 1 mL n-(1-naphthyl)-ethylenediamine dihydrochloride was added. The mixture was shaken well and placed for 15 min. The spectrophotometer was set up, zero adjustment, then absorbance was measured at wavelength of 538 nm, and the standard curve method was used to derive nitrite content. The extracted solution was then percolated into a Cadmium column, after reducing nitrate to nitrite, using a colorimetric method to count total nitrite content. After that, minus the nitrite contents, and then multiplied by the conversion factor 1.232, nitrate content in the sample was derived.

RESULTS AND DISCUSSION

Identification of bacteria

Ten candidate strains were selected and purified, four of those ten strains showed nitrate

reduction negative features, rest six strains showed nitrate reduction positive features (data not shown). The VITEK 2 compact identification result showed that six facultative anaerobic candidates belonged to the genus of *Kluyvera* (3 strains are *Kluyvera cryocrescens* and the rest are *Kluyvera ascorbata*, Table 1). *Kluyvera* were discovered by Farmer in 1981[11], clearly belongs to family

Table 1. Biochemical reaction of *Kluyvera cryocrescens* and *Kluyvera ascorbata*

	Kluyvera cryocrescens	*Kluyvera ascorbata*		*Kluyvera cryocrescens*	*Kluyvera ascorbata*
APPA	-	-	SAC	+	+
ADO	-	-	dTAG	-	-
PyrA	-	-	dTRE	+	+
lARL	-	-	CIT	+	+
dCEL	+	+	MNT	+	+
BGAL	-	+	5KG	+	+
H2S	-	-	lLATk	+	+
BNAG	-	-	AGLU	-	+
AGLTp	-	-	SUCT	+	+
dGLU	+	+	NAGA	-	-
GGT	-	+	AGAL	+	+
OFF	+	+	PHOS	-	+
BGLU	+	+	GlyA	-	-
dMAL	+	+	ODC	+	+
dMAN	+	+	LDC	+	+
dMNE	+	+	lHISa	-	-
BXYL	+	+	CMT	-	-
Balap	-	-	BGUR	-	-
ProA	-	+	O129R	+	+
LIP	-	-	GGAA	-	-
PLE	+	-	lMLTa	-	-
TyrA	+	+	ELLM	+	+
URE	-	-	lLATa	-	-
dSOR	+	-			

+ reaction showed positive; - reaction showed negative; all the reagents were expressed as acronym

Table 2. The growth of *Kluyvera ascorbata* in different conditions

Time (d)	Bacterial concentration (CFU/mL) pH[a]			Temperature (8°C)				Concentration of NaCl[b] (%)			
	4.0	5.0	6.0	7.0	1	4	8	0.5	1.0	2.0	5.0
0	1.5×10^2	2.6×10^2	1.1×10^2	4.0×10^2	2.4×10^4	2.8×10^4	4.0×10^4	4.5×10^2	4.6×10^2	3.3×10^2	3.8×10^2
1	1.1×10^2	5.7×10^2	6.0×10^2	4.3×10^2	8.0×10^3	8.6×10^3	6.2×10^5	5.0×10^2	9.8×10^2	1.5×10^3	22
2	90	7.6×10^2	1.3×10^3	4.5×10^2	3.3×10^3	3.5×10^3	2.8×10^5	5.3×10^2	3.7×10^3	3.6×10^3	4
3	80	1.1×10^3	8.4×10^3	4.7×10^2	8.0×10^2	1.3×10^3	7.7×10^5	5.5×10^2	9.5×10^3	7.6×10^3	<1
4	50	2.0×10^3	9.5×10^3	5.0×10^2	1.8×10^2	1.4×10^2	3.4×10^5	6.2×10^2	3.7×10^4	4.2×10^4	-
5	45	4.5×10^3	4.5×10^4	5.6×10^2	1.4×10^2	40	1.2×10^5	7.6×10^2	5.8×10^4	6.6×10^4	-
6	34	1.6×10^4	1.2×10^5	6.1×10^2	80	5	3.1×10^5	8.1×10^2	5.7×10^4	1.1×10^5	-
7	10	2.7×10^4	6.2×10^5	6.5×10^2	<1	<1	8.9×10^5	8.5×10^2	1.7×10^5	3.0×10^5	-
8	<1	1.8×10^5	6.6×10^6	1.9×10^3	-	-	4.5×10^6	1.3×10^3	4.4×10^5	6.7×10^5	-
22	-	2.0×10^8	-	5.9×10^7	-	-	4.0×10^7	3.1×10^7	1.9×10^7	-	-

a,b The bacterial of *Kluyvera ascorbata* growed at 8°C; - stand for no survival.

Enterobacteriaceae, contains *Kluyvera cryocrescens, Kluyvera ascorbata, Kluyvera cochleae* and *Kluyvera georgiana*, normally exists in food, solid and pollutant water. Biochemical tests showed that those six strains were gram negative, single cell was approximately 0.5-1.0 um in diameter, formed straight rod, catalase positive, oxidase negative, indole production positive, methyl-red positive, voges-proskauer negative, citrate positive, motility positive and KCN growth positive. All biochemical test results confirmed that those candidates belong to *Kluyvera*.

Growth characteristic of *K.cryocrescens* and *K.ascorbata*

K.ascorbata cannot propagate at 1°C and 4°C. At 8°C, it showed a stable increase (Table 2). Although *K.cryocrescens* was no obvious growth at 1°C and grew slowly at 4°C, it always kept alive. However, at 8°C, the counts increased rapidly, after incubated 5 d the bacterial concentration reached 1.3×10^8 CFU/mL (Table 3). From that, we can see the resistance of low temperature of *K.cryocrescens* was better than *K.ascorbata*. Both *K.cryocrescens* and *K.ascorbata* increased steadily if the pH was 5.0, 6.0 or 7.0. Under pH 4.0 conditions, number of cells reduced sharply (Table 2 and 3). Compared these data we can find that the best pH for *K.cryocrescens* propagation was pH 6.0 , and the best pH for *K.ascorbata* was pH 5.0. Both *Kluyvera* strains can grow when the sodium chloride concentrations were 0.5%, 1.0% and 2.0%. *Kluyvera* could not grow when the NaCl concentration reached 5%.

The simulation test of ginger paste contaminated by *K.cryocrescens* and *K. ascorbata*

Nitrate concentration of those eight ginger samples were 1093.4±3.8 to 2914.8±4.1 mg/Kg at starting point (Table 4). *K.cryocrescens* and *K.ascorbata* had been added to the eight different ginger samples separately. Nitrite concentrations of those eight ginger pastes which contaminated with *K.cryocrescens* have reached a peak value of 74.9±4.1-780.3±8.6 mg/kg after stored 6 to 10 d, and all the bacterial concentration achieved 10^8 CFU/g. For the *K.ascorbata* groups, the nitrite concentration reached a peak value around 5 to 10 d, and the peak value was 104.7±7.2-782.4±8.6 mg/kg. Meanwhile, bacterial concentrations were 10^7-10^8 CUF/g.

The aim of simulating test was to figure out the correlation between bacteria propagation and nitrite concentration increase. In most of ginger pastes samples, nitrite concentration start rapidly increasing when the bacterial concentration reached 10^7 CFU/g. While bacterial concentration of *Kluyvera* was from 10^7 CFU/g to 10^8 CFU/g, nitrite concentration reached the peak value that was 74.9±4.1-782.4±8.6 mg/Kg. The test showed the similar result with the ginger paste sample that was collected from Universide. During the simulation test, the nitrite concentration peak value of ginger pastes which contaminated by *K.cryocrescens* was higher than *K.ascorbata*. It might suggest that *K.cryocrescens* had higher nitrate reduction ability. The nitrite concentration of the control groups kept under the detection limit

Table 3. The growth of *Kluyvera cryocrescens* in different conditions

Time (d)	Bacterial concentration (CFU/mL)										
	pH[a]			Temperature (8°C)				Concentration of NaCl[b] (%)			
	4.0	5.0	6.0	7.0	1	4	8	0.5	1.0	2.0	5.0
0	1.0×10^3	1.0×10^3	1.0×10^3	1.0×10^3	4.0×10^4	4.0×10^4	4.0×10^4	1.0×10^3	1.0×10^3	1.0×10^3	1.0×10^3
1	1.7×10^2	1.0×10^3	1.0×10^3	2.9×10^3	6.0×10^4	6.2×10^4	1.6×10^5	6.0×10^3	6.0×10^3	3.8×10^3	70
2	1.6×10^2	1.3×10^3	1.6×10^3	6.2×10^4	4.0×10^4	6.3×10^4	1.4×10^6	1.8×10^4	1.7×10^4	1.5×10^4	50
3	1.5×10^2	2.7×10^4	1.0×10^5	1.1×10^5	4.1×10^4	7.5×10^4	4.1×10^6	1.1×10^5	1.0×10^5	7.6×10^4	40
4	1.3×10^2	1.1×10^5	1.9×10^6	3.5×10^5	4.0×10^4	7.6×10^4	1.4×10^7	2.5×10^5	2.4×10^5	1.7×10^5	-
5	1.1×10^2	3.8×10^5	1.0×10^7	1.2×10^7	4.0×10^4	7.7×10^4	1.3×10^8	3.5×10^5	3.8×10^5	3.5×10^5	-
6	70	3.7×10^6	1.5×10^8	1.5×10^7	4.2×10^4	1.6×10^5	2.1×10^7	8.5×10^5	1.0×10^6	6.5×10^5	-
7	51	1.2×10^7	1.9×10^8	1.0×10^8	2.6×10^4	2.0×10^5	1.8×10^7	1.9×10^6	2.5×10^6	1.4×10^6	-
8	40	1.1×10^8	1.8×10^8	8.7×10^7	2.6×10^4	2.4×10^5		2.2×10^6	1.9×10^7	2.0×10^6	-
22	-	8.8×10^7			2.1×10^4	5.4×10^6		1.0×10^8	1.0×10^8	2.1×10^7	-

a,b The bacterial of *Kluyvera cryocrescens* growed at 8°C; - stand for no survival.

Table 4 . The simulation test of ginger paste contaminated by *K.cryocrescens* and *K. ascorbata*

Name	Produce place	Beginning nitrate concentration (mg/Kg,$NaNO_3$)	*Kluyvera cryocrescens*			*Kluyvera ascorbata*		
			Peak value of nitrite (mg/Kg) $NaNO_2$	Bacterial concentration (log CFU/g)	Time[a] (d)	Peak value of nitrite (mg/Kg) $NaNO_2$	Bacterial concentration (logCFU/g)	Time[a] (d)
Bai Ginger	Yuxi,Yunnan	1205.1±3.5	296.4±7.4	1.9×10^8	6	196.3±4.5	4.9×10^7	5
Huang Ginger	Sanmin, Fujian	1180.4±4.7	590.6±6.4	2.6×10^8	6	782.4±8.6	1.0×10^8	6
Xirou Ginger	Haikou,Hainan	1190.6±5.1	303.9±5.2	3.3×10^8	8	407.6±5.3	8.0×10^7	6
Jin Ginger	Guangdong	1093.4±3.8	402.8±4.4	1.9×10^8	6	148.4±5.9	1.9×10^7	9
Guangdong Da ginger	Guangdong	2032.4±2.5	201.6±6.7	1.8×10^8	10	158.7±8.7	1.8×10^8	10
Changyi Da Ginger	Changyi,Shandong	2914.8±4.1	594.5±7.2	2.3×10^8	7	387.9±8.5	2.1×10^7	8
Huangzhua Ginger	Wuxue,Hubei	1788.3±4.3	74.9±4.1	3.5×10^8	10	104.7±7.2	1.2×10^8	9
Red sprout Ginger	Shangrao,Jiangxi	1140.2±5.7	780.3±8.6	3.2×10^8	7	286.8±5.6	2.1×10^8	9

a: time of nitrite concentration reached peak value; bacterial concentration : indicator bacterial concentration when the nitrite concentration reached the peak value; n=3.

(1.0 mg/Kg, counted by $NaNO_2$) during the experiment period, meanwhile, bacterial concentration maintained below 10 CFU/g. Furthermore, there was no relation between the nitrate content at beginning point and peak value of nitrite shown in this experiment. As a conclusion, these results proved that the *K.cryocrescens* and *K. ascorbata* propagation was the key factor of nitrite concentration increase in ginger paste.

In this study, results showed that *K. cryocrescens* and *K. ascorbata* could grow at 8°C, while the pH value of ginger pastes samples were 5.0-7.0, which can provided suitable pH and temperature for the growth of *Kluyvera*. Furthermore, the usual pH value of vegetables is pH 4.0-7.0, and which is indicated pH value of most vegetables are suitable for the growth of *Kluyvera*. Moreover, *K.cryocrescens* and *K.ascorbata* die when NaCl concentration reached 5%. Consequently, the high NaCl concentration is believed as an effective measure to control bacterial growth in pickled vegetables. Lower storage temperature (which according to this experiment should below 4°C) would also be an effective way to control the growing of *Kluyvera* in vegetables.

It is also worth to mention that *Kluyvera* is considered as a potentially dangerous pathogen as report by Sarria *et al.*[12]. Many previous papers in early 1980s infrequently associated this organism with clinical infection. Most of the reports showed that Kluyvera involved in gastrointestinal or urinary tract and the soft tissues[13]. Moreover, *K. ascorbata* was isolated from patient whom had a finger abscess[14]. In addition, it is able of causing infection in immunocompetent individuals[15]. Presently, clinical improvements are focusing on *Kluyvera* infections. However, there is limit information about the mechanism of infections. This report might provide an idea for studies on *Kluyvera* species; the next step of our research will continually focus on the relation between vegetable (especially pickle and ready to eat food) and *Kluyvera* propagate.

CONCLUSIONS

As conclude, *Kluyvera* had significant contribution to the nitrite concentration increase in the ginger paste sample. Results showed that *K. cryocrescens* and *K. ascorbata* could grow at 8°C,

while the pH value of ginger pastes samples were 5.0-7.0. The ginger paste (when storage at 8°C) provided suitable pH and temperature for the growth of *Kluyvera*. Furthermore, the high NaCl concentration is believed as an effective measure to control bacterial growth in pickled vegetables. Moreover this study indicated that lower storage temperature would be an effective way to control the growing of *Kluyvera* in vegetables. As *Kluyvera* is a conditioned pathogen as report by Sarria *et al.*,[12] and the pathogenesis is unclear, this experiment might provide a new direction for the further research on *Kluyvera*.

ACKNOWLEDGMENTS

We wish to thank Ling-bing Meng and Yao-yu Wang for help with determination of nitrite.

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