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Effect of Incubation Conditions on the Reduction of Nitrate to Nitrite by *Micrococcus roseus* and *Escherichia coli* O157: H7

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ABSTRACT

Nitrate reduction of *Escherichia coli* O157: H7 was compared with that of four microorganisms, namely, *Micrococcus roseus*, *Micrococcus luteus*, *Staphylococcus carnosus* and *Vibrio parahaemolyticus* in phosphate buffer solution containing 2053 mg/L nitrate, and the factors affecting nitrate reduction of *M. roseus* and *E. coli* O157: H7 were also studied. *M. roseus*, *E. coli* O157: H7 and *S. carnosus* showed similar but stronger reduction activities than the other test microorganisms, showing optimum activity in pH 7 buffer solution containing nitrate. Very little nitrite was formed when pH and temperature were below 5 and 15°C, respectively. Nitrate reduction was significantly increased with cell density and reached optimal level at 4 log CFU/mL. However, the reduction rate was significantly increased with increasing initial cell counts after 24 h incubation. About 80% nitrate was reduced in pH 7 buffer solution containing 503-2006 mg/L nitrate by *M. roseus* and *E. coli* O157: H7 at 37°C after 24h incubation, and the reduction reaction was inhibited at nitrate levels above 2006 mg/L. The reduction of nitrate to nitrite by *E. coli* O157: H7 in pH 7 buffer solutions containing 2098 mg/L nitrate at 37°C reached optimal level after 24 h. Results clearly show that *E. coli* O157: H7 was very active in the reduction of nitrate to nitrite, the number required to reduce nitrate would be extremely hazardous. Thus, nitrate reduction by *E. coli* O157: H7 would be inconsequential in real systems.

Key words: *Micrococcus roseus*; *Escherichia coli* O157: H7; Nitrate reduction; Nitrite

INTRODUCTION

Nitrate and nitrite are found in many foods[1,2], such as vegetables[3,4], dairy products[5], meats[6], drinking water and fruits[7]. The nitrate levels in most foods are higher than those for nitrite. It has shown that about 87% of dietary nitrate intake was from vegetable, while 39% from cured meat[8]. Nitrate in food is able to be transformed to nitrite in many ways, including storage conditions, processing methods[9], microorganism action[10] or digestion in the human gut[11]. Nitrite has long been employed to improve the color[12] and flavor[13] of cured meats and to inhibit the growth of *Clostridium botulinum*[14]. Being more toxic than nitrate, nitrite can further react with secondary amines in acidic conditions to form carcinogenic nitrosamines[15-17]. Many studies have shown that a direct relationship exists between nitrosamine and human cancers[18,19]. However, nitrite absorbed into the blood stream reacts with oxyhemoglobin to produce methemoglobin (metHb), which cannot transport oxygen and causes methemoglobinemia in infants[20]. Thus, minimization of nitrite concentration in the diet to prevent health risk is essential.

It has been reported that the reduction of nitrate to nitrite was greatly influenced by nitrate-reducing bacteria such as *Micrococcus* spp., *Vibrio* spp.[10], *Staphylococcus carnosus*[21] and *Escherichia coli*[22]. This was due to the production of nitrate reductase during the growth of these microorganisms. Therefore, *Micrococcus* spp. and *S. carnosus* were used to make some contribution to color and aroma formation in cured meat. However, they were also contaminants in many food processings[23-25]. *Escherichia coli* O157: H7 and *Vibrio parahaemolyticus* have been recognized as the cause of a number of worldwide food-borne outbreaks[26-28]. Items such as undercooked food[29], milk, yoghurt, apple juice, raw meats, salad dressings and vegetables have been implicated in high incidence of *E. coli* O157: H7[30,31]. The most vulnerable meat products are non-heated or fermented such as dry sausages, which have been the source for outbreaks of infection[32]. A study concerning the survival of *E. coli* O157: H7 in non-heated sausage have shown that high sodium chloride and sodium nitrite concentrations do not prevent the risk of *E. coli* O157: H7[33]. Certain dairy products[34] and ready-to-eat poultry products[35] contaminated with *E. coli* O157: H7 have been reported in Taiwan. *V. parahaemolyticus* was found to highly contaminate shellfish[36] and can grow in fermented squid in Japan[37]. *V. parahaemolyticus* was also the major food-borne pathogen in Taiwan. Recent studies in Taiwan have focused on detection method and growth factors of *E. coli* O157: H7[38,39]. However, there is no available information on nitrate reduction by this bacterium. In this study, we first compared the nitrate reduction activities of *E. coli* O157: H7 with *M. roseus*, *M. luteus*, *S. carnosus* and *V. parahaemolyticus*. Grajek and Walkowiak-Tomczak[40] found that temperature, pH and the cell density

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of bacteria affected the activity of nitrate reductase. Therefore, we then investigated the effect of incubation factors including pH values, temperature, initial cell count, nitrate concentration and incubation time on the reduction of nitrate to nitrite by nitrate-reducing bacteria.

MATERIALS AND METHODS

I. *Bacterial Strain*

Micrococcus roseus CCRC11577, *Micrococcus luteus* CCRC11543, *Staphylococcus carnosus* CCRC12922, *Escherichia coli* O157: H7 CCRC13084 and *Vibrio parahaemolyticus* CCRC10806 were obtained from Taiwan Culture Collection and Research Center (Hsinchu, Taiwan, R.O.C.). After subculture at least three times in tryptic soy broth (TSB), the culture was made on tryptic soy agar (TSA) slant, and maintained at 4°C in refrigerator. *V. parahaemolyticus* was inoculated on TSA (or TSB) with 3% NaCl.

II. *Culture Preparation*

M. roseus, *M. luteus*, *S. carnosus* and *E. coli* O157: H7 were individually transferred to TSB. *V. parahaemolyticus* was transferred to TSB with 3% NaCl. Inoculated cultures were incubated at 37°C for 18-24 h in incubator. The cells were harvested by centrifugation at 13,000×g for 15 min, and the pellets were washed twice with phosphate buffer (pH 7) and the final individual cell counts of approximately 10^5~10^6 CFU/mL were resuspended in pH7 phosphate buffer solution.

III. *Chemicals*

All bacteriological media were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Sodium nitrate, sodium nitrite and other chemicals were purchased from Riedel-de Haën (Seelze, Germany).

IV. *Preparation of Buffer Solution*

The phosphate buffer stock solution was prepared with 0.5 mol/L KH_2PO_4 and 500 mL distilled water, then adjusted pH to 7.2 with 1N NaOH. It was diluted to volume of 1 L with distilled water, sterilized in autoclave with pressure of 1.2 kg/cm^2 for 15 min at 121°C and stored under refrigerated conditions. The pH7 phosphate buffer was prepared with 1.25 mL of stock solution. After bringing to volume of 1 L with distilled water, it was sterilized in autoclave. The pH was adjusted to 7 by sterile 1N HCl. The pH 4-6 phosphate buffer solutions were prepared by adjusting pH from 7 to 6 (or lower) with sterile 1N HCl[41]. The buffer solution (pH 4~7) containing nitrate was prepared with 10mL phosphate buffer solution containing 2 g glucose and 0.2 g sodium nitrate, then filtered through a 0.2 μm sterile filter, and made up to 95 mL with sterile phosphate buffer. The glucose was used as a carbon source for bacterial growth.

V. *Assay for Nitrate Reduction by M. roseus, M. luteus, S. carnosus, V. parahaemolyticus and E. coli O157: H7*

An amount of 95 mL sterile phosphate buffer (pH 7) solution containing 2053 mg/L nitrate at pH 7 was individually inoculated with 5 mL bacterial culture in serum bottle and incubated in shaker bath (Firstek, model B602, Taipei, Taiwan) with shaking at 100 rpm and 37°C for 24 h. After incubation, pH value, nitrate and nitrite concentrations were measured.

pH value was determined by pH meter (Suntex, model SP-701, Taipei, Taiwan). Nitrate and nitrite were analyzed with a high performance liquid chromatography (Jasco, Model 980, Sectroscopic Co. Ltd, Japan) against external standards. Chromatographic separations were carried out using a Hypersil® SAX column (250 × 4.6 mm). The mobile phase was 0.05 M K_2HPO_4 (pH3.3) at a flow rate of 1.5 mL/min. Nitrate and nitrite were detected with UV detector (Jasco, Model UV970) at 210 nm wavelength[6]. A series standard concentrations of nitrate or nitrite were 0.5, 1, 5, 10, 25, 50 mg/L. Twenty μL of the sample solution and the standard nitrate or nitrite solution were injected separately into the HPLC, and standard was identified by comparing the retention time of peak in the sample with that of the standard. A standard curve graph was obtained by calculating linear regression of the peak area versus concentration. Determine the lowest concentration over the range that peak area versus concentration remains good linearity under linear regression. The detection limit was 0.5 mg/L for nitrate or nitrite.

VI. *The Effect of pH Values, Temperature, Nitrate Concentration and Initial Cell Count on the Reduction of Nitrate to Nitrite*

(I) pH. Ninety-five mL sterile phosphate buffer solution containing 2130 mg/L nitrate at pH 4-7 was individually inoculated with 5 mL *M. roseus* and *E. coli* O157: H7 culture in serum bottle and incubated in shaker bath with shaking at 100 rpm and 37°C for 24 h. (II) Temperature. Ninety-five mL sterile phosphate buffer solution containing 2020 mg/L nitrate at pH 7 was inoculated with 5 mL *M. roseus* and *E. coli* O157: H7 culture in serum bottle and incubated in shaker bath with shaking at 100 rpm for 24 h. Incubation temperatures were 7, 15, 25 and 37°C. (III) Nitrate concentration. An amount of 95 mL phosphate buffer solution containing 503, 1038, 1506, 2006 and 3227 mg/L nitrate at pH 7 were individually inoculated with 5 mL *M. roseus* and *E. coli* O157: H7 cultures in serum bottle and incubated in shaker bath with shaking at 100 rpm and 37°C. After incubation, pH value, nitrate and nitrite concentration were determined. (IV) Initial cell count. Cells of *M. roseus* and *E. coli* O157: H7 were harvested in the growth phase from TSB after 18~24 h

incubation in incubator, then separated by centrifugation at 13,000 Xg for 15 min. The pellet was suspended in pH 7 phosphate buffer solutions and the cell counts of approximately 4, 5, 6 and 7 log CFU/mL were made.

An amount of 95 mL phosphate buffer solution containing 2120 mg/L nitrate at pH7 was inoculated with 5 ml of *M. roseus* (or *E. coli* O157: H7) cultures in serum bottle and the initial cell counts in solutions were 2.3 log CFU/mL (2.6 log CFU/mL for *E. coli* O157: H7), 3.0 log CFU/mL (3.4 log CFU/mL for *E. coli* O157: H7), 4.0 log CFU/mL (4.0 log CFU/mL for *E. coli* O157: H7) and 5.0 log CFU/mL (4.8 log CFU/mL for *E. coli* O157: H7). They were all incubated with shaking at 100 rpm and 37°C. After 24h, pH value, final cell count, nitrate and nitrite concentrations were measured. Cell counts of each test microorganisms were enumerated on TSA plates following incubation at 37°C for 18~24 h.

VII. *Measure of Effect of Incubation Time on the Reduction of Nitrate to Nitrite*

An amount of 95 mL sterile phosphate buffer solution containing 2098 mg/L nitrate at pH7 was inoculated with 5 mL *E. coli* O157: H7 culture and incubated with shaking at 100 rpm and 37°C for 48 h. After 0, 4, 8, 12, 24, 36 and 48 h incubation intervals, nitrate and nitrite concentrations were measured.

VIII. *Statistical Analysis*

The experiments were carried out in triplicates. All data were expressed as means ± S.D. Data were analyzed by Analysis of Variance procedure of the Statistical Analysis System (SAS). The differences between treatment means were analyzed by Duncan's multiple range test. Significance was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Vibrio sp.[10], *M. roseus* and *S. carnosus*[21] are capable to reduce nitrate to nitrite. *M. roseus*, *M. luteus*, *S. carnosus*, *V. parahaemolyticus* and *E. coli* O157: H7 were chosen to determine the nitrate reduction activity in phosphate buffer containing 2053 mg/L nitrate. From Table 1, most tested bacteria possessed nitrate reduction activity except *M. luteus*. *M. roseus*, *E. coli* O157: H7 and *S. carnosus* had higher reduction levels. *V. parahaemolyticus* had lowest reduction level.

I. *Effect of pH Values on the Reduction of Nitrate to Nitrite*

As indicated in Table 1, *M. roseus* and *E. coli* O157: H7 had the higher nitrate reduction rate. Therefore, these two strains were used to study the factors that affect the nitrate reduction. The initial pH of substrate plays an important role in the reduction of nitrate to nitrite by microorganisms. As shown in Table 2, rapid nitrate reduction of both *M. roseus* and *E. coli* O157: H7 was observed at pH 7, and strong inhibition of nitrate reduction was obtained at or below pH 5. There was no nitrite formation in pH 4 and 5 buffer solutions containing *M. roseus*. A similar study shows that a strain of Paracoccus denitrificans had the maximum nitrate reduction in red beet

Table 1. Effect of *M. roseus*, *M. luteus*, *S. carnosus*, *V. parahaemolyticus* and *E. coli* O157: H7 on the change of pH and the nitrate reduction at phosphate buffer (pH 7) containing 2053 mg/L nitrate after 24 h incubation at 37°C.

Bacteria	Initial cell count (log CFU/mL)	Final pH	Final nitrate concentration (mg/L)	Nitrite concentration (mg/L)	Nitrate reduction (%)
Control		7.01 ± 0.01	2053 ± 45^{A}	—	—
M. roseus	4.8 ± 0.18	6.14 ± 0.01	508 ± 43^{D}	1424 ± 38^{A}	75.3
M. luteus	4.2 ± 0.40	6.48 ± 0.01	1967 ± 56^{A}	5 ± 1^{D}	4.2
S. carnosus	4.8 ± 0.11	6.44 ± 0.01	880 ± 29^{C}	1239 ± 26^{B}	57.2
V. parahaemolyticus	4.8 ± 0.25	6.10 ± 0.01	1577 ± 27^{B}	210 ± 15^{C}	23.2
E. coli O157: H7	4.3 ± 0.06	6.24 ± 0.02	305 ± 17^{E}	1375 ± 31^{A}	85.2

Five percent blank buffer solution was used as inoculum in the control group. All values are means ± S.D., and values bearing different uppercase superscripts in the same column are significantly different by Duncan's multiple range test ($p < 0.05$). — , not detected.

Table 2. Effect of pH value of the phosphate buffer containing 2130 mg/L nitrate on the changes of pH, nitrate and nitrite concentrations after 24 h incubation of *M. roseus* and *E. coli* O157: H7 at 37°C.

Initial pH	Final pH	Final nitrate concentration (mg/L)	Nitrite concentration (mg/L)	Final pH	Final nitrate concentration (mg/L)	Nitrite concentration (mg/L)
	M. roseus			*E. coli* O157: H7		
7	6.26 ± 0.02	508 ± 43^{C}	1300 ± 38^{A}	6.14 ± 0.01	305 ± 17^{C}	1375 ± 131^{A}
6	5.40 ± 0.02	1413 ± 84^{B}	598 ± 16^{B}	5.42 ± 0.01	1244 ± 37^{B}	735 ± 8^{B}
5	4.28 ± 0.01	1953 ± 45^{A}	—	4.52 ± 0.01	1901 ± 65^{A}	50 ± 3^{C}
4	3.99 ± 0.01	2027 ± 75^{A}	—	4.00 ± 0.01	2006 ± 76^{A}	—

Initial cell count: *M. roseus* 4.8 log CFU/mL, *E.coli* O157: H7 4.5 log CFU/mL. All values are means ± S.D., and values bearing different uppercase superscripts in the same column are significantly different by Duncan's multiple range test ($p < 0.05$). — , not detected.

juice at pH 7~8[40]. Reduction of nitrate in oral cavity by bacteria was markedly affected in an incubation system by pH with maximum activity at 6-6.4 and no activity beyond pH 4 and pH 9[42]. The result of this study shows that the reduction of nitrate to nitrite was greatly influenced by pH values of growth medium.

II. *Effect of Temperature on the Reduction of Nitrate to Nitrite*

Table 3 indicates that the pH values decreased with increasing incubation temperature after 24 h. Higher reduction of nitrate was observed at temperatures of 25 and 37°C. There was no significant difference ($p > 0.05$) in nitrate reduction activity at 25 and 37°C for *M. roseus*. Nitrate reduction became very slow as temperature was below 15°C. Pennington[8] reported that the improper storage of vegetables with high levels of nitrate at above 25°C not only encourages bacterial growth but also contributes to the accumulation of nitrite

III. *Effect of Initial Bacterial Cell Density on the Reduction of Nitrate to Nitrite*

Effects of different inoculums of *M. roseus* and *E. coli* O157: H7 on the change of pH value, final cell count, nitrate reduction rate in pH 7 phosphate buffer solution containing 2120 mg/L nitrate after 12h and 24h incubation are shown in Table 4. The pH values decreased and the final cell count increased with increasing inoculums of *M. roseus* or *E. coli* O157: H7 after 24 h incubation. After 12 h incubation, the final nitrate concentration decreased significantly ($p < 0.05$) with increasing initial counts. As the incubation extended to 24 h, more nitrates were reduced with increasing cell density and reached optimal level at 4 log CFU/mL. However, the reduction rate was significantly ($p < 0.05$) increased with increasing initial cell counts after 24 h incubation, similar reduction rate of *E. coli* O157: H7 was also found. These findings concur with the report of Grajek and Walkowiak-Tomczak[40] that increasing initial cell concentrations of *P. denitrificans* shorten the time needed for nitration reduction in red beet juice.

IV. Effect of Nitrate Concentration on the Reduction of Nitrate to Nitrite

Table 5 shows the changes of pH, nitrate and nitrite concentrations after 24 h growth of *M. roseus* and *E. coli* O157: H7 in pH7 buffer solution containing various amounts of nitrate. When the initial nitrate concentration increased from 503 to 2006 mg/L, the final nitrate concentration ranged from 85 to 299 mg/L for *E. coli* O157: H7

Table 3. Effect of temperature of pH 7 phosphate buffer solution containing 2020 mg/L nitrate on the changes of pH, nitrate and nitrite concentrations after 24 h incubation of *M. roseus* and *E. coli* O157: H7

Incubation temperature (°C)	Final pH	Final nitrate concentration (mg/L)	Nitrite concentration (mg/L)	Final pH	Final nitrate concentration (mg/L)	Nitrite concentration (mg/L)
	M. roseus			*E. coli* O157: H7		
37	6.36 ± 0.02	142 ± 4^{C}	1298 ± 23^{A}	6.20 ± 0.01	127 ± 5^{D}	1159 ± 66^{A}
25	6.45 ± 0.01	131 ± 23^{C}	1232 ± 21^{A}	6.56 ± 0.01	425 ± 28^{C}	1164 ± 11^{A}
15	6.65 ± 0.01	1425 ± 57^{B}	211 ± 1^{B}	6.61 ± 0.01	1484 ± 72^{B}	146 ± 11^{B}
4	6.80 ± 0.01	1966 ± 35^{A}	19 ± 0^{C}	6.82 ± 0.01	1874 ± 64^{A}	88 ± 8^{C}

Initial cell count: *M. roseus* 4.7 log CFU/mL, *E.coli* O157: H7 4.9 log CFU/mL. All values are means ± S.D., and values bearing different uppercase superscripts in the same column are significantly different by Duncan's multiple range test ($p < 0.05$).

Table 4. Effect of initial cell counts of *M. roseus* and *E. coli* O157: H7 on the changes of pH, final cell counts, nitrate concentration and reduction rate in pH 7 phosphate buffer solutions containing 2120 mg/L nitrate after 12 h and 24 h incubations at 37°C.

Microorganism used	Initial cell count (log CFU/mL)	Final nitrate concentration (mg/L)	Final pH	Final cell count (log CFU/mL)	Final nitrate concentration (mg/L)	Reduction rate (mg of nitrate reduced/CFU)
		12h incubation		24h incubation		
M. roseus	2.3	1995 ± 21^{A}	6.55 ± 0.02	5.3 ± 0.34	1665 ± 31^{A}	0.015 ± 0.001^{D}
	3.0	901 ± 35^{B}	6.38 ± 0.02	6.8 ± 0.23	821 ± 33^{B}	0.034 ± 0.001^{C}
	4.0	508 ± 27^{C}	6.28 ± 0.01	7.2 ± 0.22	498 ± 23^{C}	0.051 ± 0.001^{B}
	5.0	442 ± 24^{D}	6.25 ± 0.03	7.2 ± 0.13	501 ± 17^{C}	0.074 ± 0.001^{A}
E. coli O157: H7	2.6	2004 ± 51^{A}	6.53 ± 0.01	4.8 ± 0.31	1730 ± 85^{A}	0.018 ± 0.004^{D}
	3.4	1037 ± 35^{B}	6.34 ± 0.01	6.5 ± 0.42	512 ± 31^{B}	0.052 ± 0.001^{C}
	4.0	597 ± 32^{C}	6.25 ± 0.02	7.3 ± 0.34	454 ± 26^{B}	0.050 ± 0.001^{B}
	4.8	578 ± 18^{C}	6.21 ± 0.02	6.9 ± 0.22	470 ± 16B	0.079 ± 0.001^{A}

All values are means ± S.D., and values bearing different uppercase superscripts in the same column are significantly different by Duncan's multiple range test ($p < 0.05$).

Reduction rate (mg of nitrate reduced / CFU) = (Initial nitrate concentration – Final nitrate concentration at 24 h incubation) — (Final cell count at 24 h incubation — Initial cell count)

Table 5. Effect of nitrate concentrations in pH 7 phosphate buffer solutions on the changes of pH, nitrate and nitrite concentrations after 24h incubation of *M. roseus* and *E. coli* O157: H7 at 37°C.

Initial nitrate concentration (mg/L)	Final pH	Final nitrate concentration (mg/L)	Nitrate reduction (%)	Final pH	Final nitrate concentration (mg/L)	Nitrate reduction (%)
	M. roseus			*E. coli* O157: H7		
503	6.08 ± 0.02	132 ± 11	73.7^{C}	5.74 ± 0.02	85 ± 2	83.1^{A}
1038	6.11 ± 0.03	153 ± 12	85.3^{B}	5.92 ± 0.02	160 ± 3	84.0^{A}
1506	6.24 ± 0.01	180 ± 9	88.0^{A}	6.17 ± 0.02	220 ± 10	85.4^{A}
2006	6.31 ± 0.01	433 ± 25	78.4^{C}	6.24 ± 0.01	299 ± 3	85.1^{A}
3227	6.60 ± 0.02	2112 ± 210	34.5^{D}	6.44 ± 0.01	3140 ± 131	3.8^{B}

Cell counts of each culture of *M. roseus* and *E. coli* O157: H7 in substrate were 4.5 log CFU/mL and 4.3 log CFU/mL, respectively. All values are means ± S.D., and values bearing different uppercase superscripts in the same column are significantly different by Duncan's multiple range test ($p < 0.05$).

(132 to 433 mg/L for *M. roseus*), indicating that 83.1 to 85.4% (73.7 to 88.0% for *M. roseus*) nitrate was reduced. Beyond 2006 mg/L, nitrate reduction decreased significantly ($p < 0.05$), indicating that high level of nitrate strongly inhibited the activity of nitrate reduction caused by both *E. coli* O157: H7 and *M. roseus*. The results of this study show that the combination of nitrate-reducing bacteria and nitrate, up to a certain level, were responsible for the reduction of nitrate to nitrite.

Table 5 also shows that both *M. roseus* and *E. coli* O157: H7 had highest reduction activities in solution containing 1506 mg/L nitrate. However, there is no significant difference ($p > 0.05$) between 1506 mg/L and 2006 mg/L for nitrate reduction of *E. coli* O157: H7. Although *M. roseus* showed highest reduction activity in solution containing 1506 mg/L nitrate, the reduction rate in 2006 mg/L is about 89% of that in 1506 mg/L, indicating that our above results would not be influenced totally.

Reports indicate that plant foods are the primary sources of nitrate, while processed or cured meats are the primary sources of nitrite[8] in the human diet. Recent study reports[43] that lettuce, spinach, red beets, cabbage, celery and leeks are high in nitrate, even containing several hundred to thousands ppm (mg/L) of nitrate. Spiegelhalder et al.[44] and Eisenbrand et al.[18] have indicated that the extent of nitrite formation is related to the quantity of nitrate, to the concentration of the nitrate source and to the oral microflora.

V. *Effect of Incubation Time on the Reduction of Nitrate to Nitrite*

Changes of nitrate and nitrite concentrations in pH 7 phosphate buffer solution containing 2098 mg/L nitrate during incubation with *E. coli* O157: H7 at 37°C for 48 h were evaluated. From Figure 1, the nitrate residue significantly decreased ($p < 0.05$) during 4~24 h growth of *E. coli* O157: H7, and then remained steady thereafter. However, the nitrite concentration increased during 4~24 h incubation, then leveled off. The data clearly showed that the decrease of nitrate by *E. coli* O157: H7 resulted in the increase of nitrite during 24 h incubation.

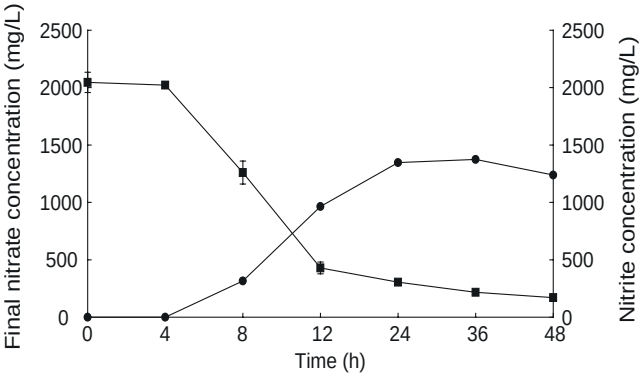

Figure 1. Changes of nitrate and nitrite concentrations in pH 7 phosphate buffer solutions containing 2098 mg/L nitrate during incubation with *E. coli* O157: H7 at 37°C for 48h. (Initial cell count: 4.7 log CFU/mL; ■, nitrate; ●, nitrite).

Reported studies in several foods show that nitrate concentrations decrease while nitrite accumulates, for example, during storage of ogi[45]. Grajek and Walkowiak-Tomczak[40] indicate that nitrite concentrations increase in carrot and red beet juice containing *P. denitrificants* during 24 h storage. If the storage time is prolonged, nitrite is subsequently converted into NO and N2[10]. Similarly, *S. carnosus*, which subsequently accumulates in the growth medium in the presence of nitrate, reduces nitrate to nitrite [21].

Therefore, the level of nitrite may increase in foods containing nitrate on the conditions that foods have been contaminated with *E. coli* O157: H7 or *M. roseus* and have endured improper prolonged storage.

CONCLUSION

M. roseus, *S. carnosus*, *V. parahaemolyticus* and *E. coli* O157: H7 were demonstrated to have the ability to reduce nitrate to nitrite. The present study clearly shows that maximum reduction of nitrate in buffer system containing nitrate was observed at temperature of 25~37°C, pH 7, high initial cell count and nitrate level of 2006 mg/L.

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Although *E. coli* O157: H7 was the more active of these bacteria tested, the number necessary to reduce nitrate would be an extreme hazard. Thus, nitrate reduction by this strain would be inconsequential in real systems. *Micrococcus* spp. are very common contaminants in many food systems during processing and storage, and their ability to reduce nitrate to nitrite would represent a larger potential public health concern.

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