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# NITRATE REDUCTASE ACTIVITY OF MICROCOCCACEAE ISOLATED FROM BULGARIAN RAW-DRIED SAUSAGE

R.N.BORPUZARI and K.BOSCHKOVA

Higher Institute of Food and Flavour Industries, Department of Technology of Meat and Fishes, Plovdiv, Bulgaria

## INTRODUCTION

The *micrococcaceae* are important microorganisms in the starter cultures used for European-style sausage fermentations. These microorganims are predominantly *micrococci* and *staphylococci* with the crucial ability to reduce nitrate/nitrite. The nitric oxide that is formed by bacterial activities combines with the myoglobin of meat to give nitrosomyoglobin, which imparts the desired colour to the fermented meat products. Nitrate is ultimately transformed, via two different pathways, either to dinitrogen monoxide and then to elementary nitrogen or to ammonia (Folett and Ratcliff, 1963; Lucke,1985). The *micrococcaceae* thus remove added or accumulated nitrates by the action of their nitrate- and nitrite reductases. In sausage fermentations, the nitrate reductase activity of these microorganisms needs, to be coordinated with the decreasing pH of the sausage, the temperature of the fermentation and the anaerobic conditions of growth (Christiansen *et al.*, 1975; Liepe, 1982; Selgas *et al.*, 1988). It is therefore important, from a practical perspective, that these microorganisms be most active during the early stages of sausage fermentation, i.e., before the lactic acid bacteria can cause an appreciable fall in the pH of the sausage through the depletion of sugars, and grow optimally at the fermentation temperature.

Earlier reports indicated that the bacterial reduction of nitrates occurs during the first 24 to 48 hours of the fermentation process (Zaika *et al.*, 1976; Palumbo and Smith, 1977). Bacus (1984) also reported that the micrococci are more active during the first four days of ripening of sausage. In this present work, an attempt was made to investigate the nitrate reductase activities of *micrococcaceae* isolated during different stages of the production of "Lukanka" a typical Bulgarian raw-dried sausage, with the objectives of identifying the stage(s) at which these microorganisms are most active and the influences of incubation temperature, the fermentation time and the viable cell counts on the nitrate reduction ability of *micrococci*. The data would allow the selection of the strains possessing superior nitrate reductase activity for possible use in starter cultures.

## MATERIALS AND METHODS

### Test organisms

A total of 354 strains of *micrococcaceae* (213 *micrococci* and 141 *staphylococci*) that had been isolated from Lukanka during different stages of its production, were examined. The production stages were after mixing (A), after the initial fermentation phase (B), and on the 3rd (C), 6th (D), 13th (E), and 28th (final) day of ripening (F). The isolation and characterization procedures used have been previously described (Borpuzari and Boschkova - submitted for publication).

### Determination of nitrate reductase activity

The nitrate reductase activities of the test organisms were determined by the spectrophotometric method of Kuusela *et al.* (1978) modified by the substitution of naphthyl-1-amine for N(1-naphthyl-)ethylenediammonium chloride.

The test organisms were inoculated onto slants of a medium containing (%w/v) - yeast extract (0.1), peptone (1.0), glucose (0.2), disodium phosphate (0.25), and NaCl (2.5), pH 7.2 (Selgas *et al.*,1988), and incubated at 30°C for 24 hours. Bacteria from these cultures, were suspended in 1% NaCl solution to give suspensions of approximately $2x10^8$cfu/ml.

The influence of incubation temperature and time on the nitrate reductase activities of the six *micrococca*l strains that possessed the highest nitrate reductase activities were studied. These strains were incubated for periods of 2, 6, 12, 24, 48, 72, 144, or 312 hours at temperatures of 12 or 25°C. The numbers of viable cells in the cultures of two of the six strains were counted, by plating suitable dilutions, to determine the effect of cell concentration on their nitrate reduction activities.

## RESULTS AND DISCUSSION

The *micrococcaceae* were classified as `strong', `moderate', `weak', or `non-reducers' depending on the quantity (g) of nitrate they reduced. Strains reducing >15g of nitrate were classified as strong, those reducing >7.5 but <15g were classified as moderate, and the strains reducing <7.5g were classified as weak.

Table 1 shows the classification of *micrococcal* and *staphylococcal* strains on the basis of the above criteria, and their percent distributions during different stages of Lukanka production. Out of the 213 *micrococcal* strains, 21 (9.86%) were strong, 27 (12.68%) were moderate, 146 (68.54%) were weak and remaining 19 (8.92%) were non-reducers of nitrate. Similarly, of the 141 strains of *staphylococci*, 17 (12.06%) were strong, 30 (21.28%) were moderate, 85 (60.28%) were weak and nine (6.38%) were non-reducers of nitrate. It was found that the maximum number of *micrococcal* strains (nine) possessing strong nitrate reductase activity were isolated on the 3rd day of ripening, whereas, the maximum number of strongly nitrate reducing *staphylococci* (four) were isolated from the sausage mix. The percent incidence of strongly reducing *micrococci* diminished thereafter, and in the finished product no *micrococci* possessing strong nitrate reductase activity could be isolated. Also, the percent incidence of strongly reducing *staphylococci* declined from a maximum in the sausage mix, and the finished product contained only 1.42% of strongly reducing *staphylococci*. Maximum fractions of moderately and weak by reducing *micrococci*, 3.75% and 20.19% respectively, were isolated on the 13th day of ripening. The maximum per cent incidences of moderately (10.64%) and weakly (16.31%) reducing strains of *staphylococci* were isolated from the sausage mix. Fractions of non-reducing *micrococci* (3.75%) and *staphylococci* (4.25%) were maximal in the finished product.

From the results it is evident that the 3rd day of ripening of Lukanka is the most appropriate stage for isolating *micrococci* that possess strong nitrate reductase activity. This may be due to the fact that up to the 3rd day of ripening the conditions are conducive to the optimal growth and activity of the *micrococci*. After this stage, the pH of the sausage

mass decreased because of the action of the lactic acid bacteria. The activity of the nitrate reductase enzyme is then retarded as it is sensitive to decreasing pH. The drop in pH also accelerates the drying of the sausage, and thereby reduced the $a_w$ of the sausage, which affects the growth and activity of the *micrococci* (Bacus, 1984; Incze, 1992).

The effects of temperature the period of incubation and the viable cell counts on the nitrate reductase activity of six *micrococcal* strains are presented in Table 2. In general, maximum activity of the strains were observed after six hours of incubation at both the temperatures. Activities then ranged from 21.44 to 24.71g at 12°C and 19.21 to 26.76g at 25°C. However, their activities were slightly higher at 25 than at 12°C, the maximum value being 26.76g and 24.71g at 25 and 12°C, respectively. It was observed that the nitrate reductase activity of the strains increased rapidly from the initial activity at two hours of incubation till the sixth hour followed by a gradual decrease to the 72nd hour, and thereafter the activity remained virtually constant until the end of the incubation period at 144 hours. A similar pattern was observed when the period of incubation was extended up to 312 hours for strains M56 and M59.This may be due to the test microorganisms possessing both the nitrate- and nitrite reductase enzyme systems, as a result of which the added nitrate would first be reduced to nitrite, during the early part of incubation, followed by the breakdown of nitrite during the subsequent period of incubation.

The viable cell counts of the two *micrococcal* strains increased from $1.0x10^8$cfu/ml to reach maximum numbers after six hours of $1.8x10^8$ and $2.1x10^8$cfu/ml for strain M56 and $1.6x10^8$ and $2.6x10^8$cfu/ml for strain M59 at 12 and 25°C, respectively. Thereafter, the counts decreased gradually to the end of the incubation period at 312 hours. This may be another reason for the maximum nitrate reductase activity observed at six hours at both the temperatures. However, during the later part of incubation, the number of the viable cells did not seem to influence directly the nitrate reductase activity of the *micrococcal* strains as the quantity of nitrate reduced was not proportional to the decrease in the viable cell counts. Kuusela *et al.* (1978) also reported that the number of cells did not directly reflect the activity of the culture.

## CONCLUSIONS

The 3rd day of ripening of Lukanka is the most appropriate stage for isolating maximal numbers of *micrococci* possessing strong nitrate reductase activity. The nitrate reductase activity of *micrococci* was influenced by the temperature and period of incubation. The greatest activity was observed after six hours of incubation at both 12 and 25°C. The viable cell counts also influenced the nitrate reductase activities of the *micrococcal* strains which were, however, not proportional to the quantity of nitrate reduced.

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Table 1. Classification of micrococcaceae on the basis of their nitrate reductase activity and percent distribution during different stages of Lukanka production.*

| Category Type of organism | Stage of isolation A | B | C |
|---|---|---|---|
| Strong | | | |
| Micrococci | 0.94 | 2.82 | 4.22 |
| Staphylococci | 2.83 | 2.13 | 2.13 |
| Moderate | | | |
| Micrococci | 3.29 | 0.00 | 0.94 |
| Staphylococci | 10.64 | 0.71 | 0.00 |
| Weak | | | |
| Micrococci | 11.74 | 13.61 | 4.22 |
| Staphylococci | 16.31 | 5.67 | 2.84 |
| Non-reducers | | | |
| Micrococci | 1.41 | 0.00 | 0.94 |
| Staphylococci | 1.42 | 0.00 | 0.00 |

| Category Type of organism | Stage of isolation D | E | F |
|---|---|---|---|
| Strong | | | |
| Micrococci | 0.47 | 1.41 | 0.00 |
| Staphylococci | 2.13 | 1.42 | 1.42 |
| Moderate | | | |
| Micrococci | 3.29 | 3.75 | 1.41 |
| Staphylococci | 0.71 | 7.09 | 2.13 |
| Weak | | | |
| Micrococci | 5.16 | 20.19 | 13.61 |
| Staphylococci | 5.67 | 15.60 | 14.18 |
| Non-reducers | | | |
| Micrococci | 0.00 | 2.82 | 3.75 |
| Staphylococci | 0.00 | 0.71 | 4.25 |

* Percent distribution calculated on the basis of 213 micrococcal and 141 staphylococcal strains respectively.

Table 2. Effect of incubation temperature, incubation time and viable cell count on the nitrate reductase activity of selected micrococcal strains.*

| | Incubation period (h) | | | |
|---|---|---|---|---|
| | 2 | 6 | 12 | 24 |
| M56 | | | | |
| NR 12°C | 17.41 | 24.00 | 17.54 | 17.35 |
| 25°C | 18.69 | 25.22 | 18.94 | 17.35 |
| VC 12°C | 15.0±1.3 | 18.0±0.7 | 11.0±0.5 | 7.5±0.8 |
| 25°C | 16.0±1.3 | 21.0±1.5 | 18.0±1.5 | 14.0±1.5 |
| M59 | | | | |
| NR 12°C | 16.96 | 21.57 | 16.13 | 16.13 |
| 25°C | 17.98 | 26.76 | 19.07 | 17.73 |
| VC 12°C | 10.0±0.2 | 16.0±1.3 | 7.0±0.7 | 7.0±0.7 |
| 25°C | 13.0±1.5 | 26.0±1.4 | 20.0±0.6 | 14.0±1.3 |
| M224 | | | | |
| NR 12°C | 16.90 | 23.88 | 19.58 | 18.90 |
| 25°C | 19.84 | 26.18 | 20.10 | 18.83 |
| M319 | | | | |
| NR 12°C | 23.02 | 24.71 | 16.00 | 16.00 |
| 25°C | 18.56 | 25.79 | 19.46 | 18.82 |
| M321 | | | | |
| NR 12°C | 16.77 | 21.44 | 18.18 | 16.51 |
| 25°C | 18.63 | 19.21 | 18.94 | 17.41 |
| M325 | | | | |
| NR 12°C | 20.35 | 22.53 | 19.46 | 17.34 |
| 25°C | 19.84 | 24.32 | 19.20 | 18.43 |

Table 2 (cont). Effect of incubation temperature, incubation time and viable cell count on the nitrate reductase activity of selected micrococcal strains.*

| | Incubation period (h) | | | |
|---|---|---|---|---|
| | 48 | 82 | 144 | 312 |
| M56 | | | | |
| NR 12°C | 16.00 | 14.47 | 14.27 | 14.21 |
| 25°C | 16.64 | 15.81 | 15.62 | 15.62 |
| VC 12°C | 5.6±0.2 | 4.1±0.2 | 1.3±0.2 | 0.8±0.05 |
| 25°C | 6.0±0.9 | 4.0±0.3 | 1.8±0.2 | 1.1±0.06 |
| M59 | | | | |
| NR 12°C | 15.10 | 14.34 | 14.15 | 14.15 |
| 25°C | 16.71 | 16.03 | 15.87 | 15.78 |
| VC 12°C | 5.0±0.2 | 3.0±0.2 | 1.0±0.1 | 0.7±0.03 |
| 25°C | 7.0±0.6 | 4.0±0.2 | 2.0±0.1 | 1.6±0.13 |
| M224 | | | | |
| NR 12°C | 18.37 | 18.43 | 18.43 | ND |
| 25°C | 18.50 | 17.15 | 18.50 | ND |
| M319 | | | | |
| NR 12°C | 15.62 | 15.04 | 15.62 | ND |
| 25°C | 17.35 | 15.17 | 15.74 | ND |
| M321 | | | | |
| NR 12°C | 16.00 | 16.00 | 16.00 | ND |
| 25°C | 16.38 | 15.19 | 16.26 | ND |
| M325 | | | | |
| NR 12°C | 16.90 | 16.58 | 16.77 | ND |
| 25°C | 17.60 | 16.77 | 16.77 | ND |

* Means value of two independent trails; NR=Quantity of nitrate reduced (μm); VC=Means±SE of viable counts (cfu/ml x $10^7$); ND=Not determined.