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EFFECTS OF INCUBATION CONDITION AND DIFFERENT STARTER STRAINS FOR THE PRODUCTION OF NITRITES FROM NATURAL NITRATE SOURCES

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Abstract

Nitrates and nitrites are used in production of meat products and they play an important role as preservatives, but also in the formation of characteristic red color and taste of the meat. Vegetable products represent a significant potential as a natural source of nitrate for producing organic cooked meat products. The aim of this paper was to investigate the effect of temperature changes on the degree and speed of reduction of nitrates to nitrite salts, using different starter strains of microorganisms.

Staphylococcus carnosus was used as nitro-reducing starter culture for the first model, and *S. carnosus* in combination with *Lactobacillus sakei*, for the second model. Celery powder was used as a natural source of nitrate salts. Both models were incubated in the temperature range from 20 ^{0}C to 40 ^{0}C with a temperature gradient of 2 ^{0}C, for 24 and 48 hours. A method for determining nitrite is defined by the international standard ISO 2918:1999. Obtained values of nitrite concentrations were used to calculate the degree and speed of reduction of nitrates to nitrites.

The degree and speed of a chemical reaction increase with increasing temperature. The final concentration of nitrite salts after 24 hours of incubation for the first model was 85 ± 2 ppm at 40 ^{0}C and for the same model after 48 hours of incubation, 100 ± 10 ppm (40 ^{0}C). The second model showed similar patterns of increase, 81 ± 9 ppm (24h, 40 ^{0}C) and 83 ± 10 ppm (24h, 40 ^{0}C). The concentration of nitrite salts, the degree and speed of reduction of nitrate salts after 48 hours of incubation was increased, compared to the concentration of nitrite salts after incubation for 24 hours, in both models. The starter culture in which it was used only *S. carnosus*, proved to be more efficient when it comes to the reduction of nitrates.

***Key words**: Temperature, Reduction, Nitrate, Nitrite, Celery, Microorganisms.*

1. Introduction

Nitrates are crystalline substances, water soluble. They are obtained due to the reactions of nitric acid and metal or the reaction of oxygen, hydrogen and metal carbides. In nature, they can be found in the form of minerals. Nitrates are used as fertilizers, explosive materials, in the production of paints and in medicine. Nitrates have low toxicity, but entering into the body, they produce nitrites. Nitrites have direct toxicity, manifested by oxidation of hemoglobin to methemoglobin, or indirect toxicity due to their participation in the creation of nitrite nitrosamines. Together with the bacteria *Nitrobacter* are used in industry as food additives, especially as additives in meat and cheese products. They are also used as fertilizer and oxidizing agents in the chemical industry [1].

Nitrite ions can be found in groundwater, usually in minor amounts. Nitrites and nitrates are used in production of meat products and play an important role as preservatives in the formation of the characteristic red color and specific taste of the meat and inhibit the growth and development of pathogenic bacteria *Clostridium botulinum* [2, 3, 4]. In the reaction of nitrite with meat proteins, nitrosamines may be formed.

Nitrosamines are carcinogenic and toxic compounds. The adverse impact of nitrosamines has been demonstrated in many published papers over the last thirty years and therefore requires constant control of the presence of nitrosamines [5].

One mechanism by which nitrites may endanger human health is the oxidation of hemoglobin to methemoglobin. Methemoglobin cannot transport oxygen, and the effect is similar to a blood loss, or carbon monoxide poisoning. However, it is unlikely that a person can once ingest such a large amount of nitrites or nitrates to be life-threatening [6].

Concerns related to the use of nitrates and nitrites as additives during the curing of the meat are mainly related to the chemical toxicity and the formation of carcinogenic substances in the product during processing and subsequent formation of carcinogenic compounds in the human digestive system, after the food intake. These facts related to the use of nitrite salts, for the first time appeared in the seventies of the last century, when it was discovered that the carcinogenic nitrosoamines can be formed in the digestive tract after intake of nitrites from food [7].

Nitro-reducing strains of microorganisms provide nitrate reduction, giving the desired nitrite content, as well as desirable sensory properties of the final product. These strains were first discovered in the late nineteenth century and became commercially available within a few years. The first application of these strains was at dried fermented meat products, where it is desirable to have a stock of nitrites over a long period of drying and fermentation. For the development of a stable and desirable color during the incubation process, it is necessary to provide conditions of reduced pH values, catalase, protease and lipase activity, and avoidance of possible phenomena of discoloration through the production of peroxide [6].

One of the fundamental characteristics that should have every potential starter culture is the ability to dominate the microflora already present, to colonize the environment and to become a key, predominant microorganism in the process of incubation of meat products. The microorganisms which are used as starters, need to act as competitive microorganisms in relation to the microflora contained in the material in which they are added. They have to perform the desired metabolic activities expected of such culture, the rapid growth and reproduction in not so favorable conditions (anaerobic environment, increasing salt content, low temperature, reduced pH) [8].

Gram positive catalasa positive cocci (GCC+) such as *Kocuria* strains (previously *Micrococcus*), *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus lentus*, *Staphylococcus simulans*, and other, reduce nitrates to nitrites. These microorganisms can reduce nitrates at the temperatures from 15 ^{0}C, but they are much more effective at temperatures above 30 ^{0}C. Common optimum temperatures for these strains of microorganisms are from 38 to 42 ^{0}C. In this range of temperatures, time required to get the desired concentration of nitrites in the final product reduces to minimum [1, 9].

Staphylococci are facultative anaerobes that are able to metabolize a large number of sugars. Under anaerobic conditions, their main product is lactic acid and also can be formed acetates, pyruvates and acetone. The most commonly used nitro-reducing species in the production of various numbers of organic meat products are *S. xylose* and *S. carnosus*. These organisms have a high ability to survive and tolerate stress from the environment, caused by high levels of salt and low temperatures. Beside the reduction of nitrates, these strains also affect the texture and sensory properties of the final product. There are two main sources of nitrates in the human body:

- Preserved meat.
- Drinking water [4].

Nitrates are added to meat to prevent the growth of microorganisms, botulism and other diseases caused by poisoning by rotten meat, which is a greater danger than the toxicity of these supplements. Nitrates in drinking water originate from the fertilizers, which are used as additives in the soil, where they come in contact with water [4].

Nitrates can be reduced to nitrites still in the oral cavity of human. The reduction is continued further in the digestive tract, where the formed nitrites can react with the present amines and amides to form a group of carcinogenic compounds, nitrosoamines. The stomach is therefore exposed to the greatest risk caused by endogenous synthesis of nitrosoamines, since the acidic environment of gastric juice favors their formation [10].

The International Agency for Research on Cancer (IARC) in 2006 concluded that the intake of nitrites or nitrates can be potentially dangerous to humans. Studies have shown that from total daily intake of nitrites and nitrates, only 5% are nitrites, which are ingested by eating cured meat products. The rest of nitrites and nitrates are ingested by eating vegetables, fruits and fresh water [11].

The aim of this paper was to investigate the effect of temperature changes on the degree and speed of reduction of nitrates to nitrites, using different starter strains of microorganisms.

2. Materials and Methods

As a natural sources of nitrate salts was used celery powder, a commercial product of company "Sfinc Industry", Belgium. As a nitro reducing starter culture was used *Staphylococcus carnosus* purchased as a commercial product under the trademark "Sta carnosus - 100 Bn" and mix of *Staphylococcus carnosus - Lactobacillus sakei* (CTC 494) from the "THT" Belgium.

Analyses were carried out on two models in aqueous solutions, whose compositions are shown in Table 1 and Table 2.

Table 1. Composition of the substrate model with the culture of microorganisms (*Staphylococcus carnosus*)

Composition	Percentage (%)	Comments
Water	88%	
Sucrose	6%	Energy source
Staphylococcus carnosus	3%	Nitro-reducing strain
Celery	3%	Natural carrier of the nitrate salts

Table 2. Composition of the substrate model with the culture of microorganisms (*Staphylococcus carnosus* - *Lactobacillus sakei*)

Composition	Percentage (%)	Comments
Water	88%	
Sucrose	6%	Energy source
Staphylococcus carnosus - Lactobacillus sakei	3%	Nitro-reducing strain
Celery	3%	Natural carrier of the nitrate salts

Both models were incubated in the temperature range from 20 to 40 ^{0}C with a temperature gradient of 2 ^{0}C, for 24 and 48 hours. It was weighed 0.25 g of celery (natural carrier of the nitrate salts), followed by the addition of 0.25 g of bacterial cultures (*S. carnosus* and *L. sakei*), followed by 1 g of sucrose. After that, the entire content was transferred to the cuvette of 50 mL, and then filled with distilled water to the mark. The content of the cuvette was shaken to homogenize and then placed in a thermostat at a certain temperature and time interval. After the time is up, the sample was removed and heat treatment was carried out at 75 ^{0}C for a period of 20 min, after which the sample is cooled down under the water to stop the reduction. Method for determining nitrites is defined by the international standard ISO 2918:1999.

The degree of reduction of nitrate salts was determined according to the following equation:

$$\% = \frac{C_{NaNO2}\, C_{NaNO2}}{C_{NaNO3}\, C_{NaNO3}} * 100$$

Where:

$C_{NaNO2}\, C_{NaNO2}$ - Nitrite salts concentration after a certain time,

$C_{NaNO3}\, C_{NaNO3}$ - The initial concentration of nitrate salts (175 ppm).

The speed of reduction is calculated as:

$$V = \frac{C_{NaNO2}\, C_{NaNO2}}{t \quad t} \left[\frac{ppm\, ppm}{h \quad h}\right]$$

Where:

$C_{NaNO2}\, C_{NaNO2}$ - Nitrite salts concentration after a certain time,

t - Reaction time.

2.1 Statistical analysis of the obtained experimental data

All experiments were conducted on a model of at least three repetitions. Analysis of variance (ANOVA) was carried out over all the variables in the general linear model. A statistically significant difference between the mean values obtained in the experimental data is calculated using T - Tukey test with statistical significance of $p \leq 0.05$. In the tables, different letter codes a, b, c, d, etc., indicate the significance of that difference, and the same letter codes indicate that there are no differences.

3. Results and Discussion

The results of the incubation with *Staphylococcus carnosus* in one group of models and mix *Staphylococcus carnosus - Lactobacillus sakei* during 24 h are shown in the next Table 3 and Table 4.

As could be seen from the Table 3, results obtained using the nitro-reducing starter culture (*S. carnosus*), incubated for 24 h, showed that with increasing temperature, significantly ($p \leq 0.05$) increases the concentration of nitrite salts. At the temperature of 20 ^{0}C, the content of nitrite salts was 10 ± 1 ppm and after incubation at 40 ^{0}C, the content was 85 ± 2 ppm (Table 3). Also, there was an increase in the degree of reduction, from 5.71 to 48.57%. Speed of reduction followed this pattern as well, and increased more than 3 ppm/h, in a corresponding time and temperature range.

Second model, in addition to *S. carnosus,* included and *L. sakei*, during the same time of incubation (24 h) and the same temperature range, the significant increase

($p \leq 0.05$) is evident in all three parameters that were monitored (concentration of nitrite salts, degree and speed of reduction) (Table 4).

The results of the incubation with *Staphylococcus carnosus* in one group of models and mix *Staphylococcus carnosus - Lactobacillus sakei* in second one, during 48 hours are shown in the next Table 5 and Table 6.

Once increased incubation time, from the initial 24 h to 48 h, there was a rise in the concentration of nitrite salts and the degree of reduction, as compared to incubation time of 24 h (Table 5). Speed of reduction was reduced, from the value of 3.54 ppm/h (Table 3) to 2.08 ppm/h (Table 5).

Increment of incubation time affected similarly the second model, which uses nitro-reducing starter cultures *S. carnosus* and *L. sakei*. After the 48 h of incubation, concentration of nitrite salts and the degree of reduction had significantly ($p \leq 0.05$) higher values, contrary to 24 h of incubation. At the maximum temperature value (40 ^{0}C), speed of reduction was almost twice as lower as the one observed after incubation for 24 h, 1.73 ppm/h (Table 6) compared to 3.37 ppm/h (Table 4).

As a natural source of organic nitrites in cured meat products are used natural herbal preparations rich in nitrates. Present nitrates are reduced to nitrites by converting with the nitro-reducing starter cultures [6, 12]. This conversion takes place in a controlled environment under the optimum temperature and time. Organic meat products shall have all the sensory characteristics, color, texture, etc., which have products produced according to conventional method [2].

Permissible levels of nitrates and nitrites, which may be found in the finished product of meat in the European Union are currently regulated by Directive 2006/52/EC. According to this directive, the maximum allowed amount of nitrite salt is up to 150 ppm, and the amount of nitrate salts, depending on the product, can range up to 250 ppm [13].

Table 3. Concentration of nitrite salts, degree and speed of reduction of nitrates to nitrites, incubated for 24 h using nitro-reducing starter culture *Staphylococcus carnosus*

Staphylococcus carnosus				
Time (h)	**Temperature (ºC)**	**Concentration (ppm) ± Sd**	**Degree of reduction (%)**	**Speed of reduction (ppm/h)**
24	20	10a ± 1	5.71	0.42
24	22	14b ± 1.1	8	0.58
24	24	19c ± 2	10.85	0.79
24	26	21c ± 1	12	0.87
24	28	38d ± 5	21.71	1.58
24	30	46e ± 3	26.29	1.92
24	32	48e ± 9	27.43	2
24	34	59f ± 4	33.71	2.46
24	36	61f ± 2	34.86	2.54
24	38	73g ± 1	41.71	3.04
24	40	85h ± 2	48.57	3.54

Table 4. Concentration of nitrite salts, degree and speed of reduction of nitrates to nitrites, incubated for 24 h using nitro-reducing starter culture *Staphylococcus carnosus-Lactobacillus sakei*

Staphylococcus carnosus - Lactobacillus sakei				
Time (h)	**Temperature (ºC)**	**Concentration (ppm) ± Sd**	**Degree of reduction (%)**	**Speed of reduction (ppm/h)**
24	20	3a ± 0	1.71	0.12
24	22	4a ± 3	2.29	0.17
24	24	8b ± 4	4.57	0.33
24	26	37c ± 5	21.14	1.54
24	28	48d ± 11	27.43	2
24	30	53e ± 23	30.29	2.21
24	32	56e ± 4	32	2.33
24	34	62f ± 1	35.43	2.58
24	36	69g ± 11	39.43	2.87
24	38	71g ± 4	40.57	2.96
24	40	81h ± 9	46.29	3.37

Table 5. Concentration of nitrite salts, degree and speed of reduction of nitrates to nitrites, incubated for 48 h using nitro-reducing starter culture *Staphylococcus carnosus*

Staphylococcus carnosus				
Time (h)	**Temperature (°C)**	**Concentration (ppm) ± Sd**	**Degree of reduction (%)**	**Speed of reduction (ppm/h)**
48	20	12a ± 1.2	6.86	0.25
48	22	20b ± 2.4	11.43	0.42
48	24	25c ± 2.1	14.29	0.52
48	26	31d ± 3	17.71	0.65
48	28	33d ± 2	18.86	0.69
48	30	47e ± 2	26.86	0.98
48	32	58f ± 7	33.14	1.21
48	34	62g ± 4	35.43	1.29
48	36	63g ± 4	36	1.31
48	38	72h ± 12	41.14	1.5
48	40	100i ± 10	57.14	2.08

Table 6. Concentration of nitrite salts, degree and speed of reduction of nitrates to nitrites, incubated for 48 h using nitro-reducing starter culture *Staphylococcus carnosus-Lactobacillus sakei*

Staphylococcus carnosus - Lactobacillus sakei				
Time (h)	**Temperature (°C)**	**Concentration (ppm) ± Sd**	**Degree of reduction (%)**	**Speed of reduction (ppm/h)**
48	20	4a ± 0.1	2.29	0.08
48	22	10b ± 0.9	5.71	0.21
48	24	27c ± 6	15.43	0.56
48	26	40d ± 11	22.86	0.83
48	28	49e ± 13	28	1.02
48	30	53f ± 5	30.29	1.10
48	32	61g ± 1	34.86	1.27
48	34	66h ± 4	37.71	1.37
48	36	69h ± 2	39.43	1.44
48	38	76i ± 4	43.43	1.58
48	40	83j ± 10	47.43	1.73

The most studied and used natural supplement for the formation of color in steamed organic meat products is celery in the form of celery juice concentrate or dry powder celery. The most commonly used celery amounts ranging between 0.2% and 0.4% [1, 14]. In research conducted at the boiled sausages, prepared with celery powder and incubated at 38 °C for 30 min., or 120 min., it was noted that the incubation time is a key factor for the formation of the desired color of the final product. The amount of residual nitrites in these studies ranged between 24.5 ppm and 46.0 ppm [2, 15].

Also, it is noted that the amount of residual nitrates increases with the amount of added celery, and quantity of nitrites increases with prolongation of incubation time [14, 16, and 17].

From the presented study can be noticed that the results that were obtained in this study are in accordance with the present research. Incubation with nitro-reducing strains at various time intervals and temperatures, showed that the incubation time is a key factor in the concentration of nitrite salts in the finished product.

4. Conclusions

- In models, in which was used nitro-reducing starter culture *Staphylococcus carnosus*, over a period of 24 hours for the test temperature interval, there was recorded a significant ($p \leq 0.05$) increase in concentration of nitrite salts with increasing incubation temperature.

- With increasing temperature, there is a significant ($p \leq 0.05$) increase in concentration of nitrite salts, speed of reduction and the degree of reduction of nitrate salts, over 24 and 48 hours of incubation.

- If we compare the models observed at 24 and 48 hours, it can be noted that there has been a significant ($p \leq 0.05$) increase in the examined parameters for a given value of the test temperature.

- Similar to the models, where as nitro-reducing starter culture was used *Staphylococcus carnosus*, it can be noted that the nitro-reduction starter culture *Staphylococcus carnosus - Lactobacillus sakei*, have also shown a significant ($p \leq 0.05$) increase in concentration in a certain time interval.

- From the above, it can be concluded that the concentration of nitrite salts, the speed and the degree of reduction of nitrate salts during the incubation for 48 hours is greater than a certain value of these parameters during the 24 hours incubation.

- If we compare the concentration of nitrite salts using different starter cultures, the one with *Staphylococcus carnosus* and the other one with *Staphylococcus carnosus - Lactobacillus sakei*, it can be noted that the concentration of nitrites in the model with *Staphylococcus carnosus*is was greater than the concentration of nitrites in the model with *Staphylococcus carnosus - Lactobacillus sakei*.

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