OPTIMIZATION OFFERMENTATION PARAMETERS FOR VEGETABLE JUICES WITH NITRATE CONTENT TO OBTAIN NATURAL NITRITES

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Abstract: *The aim of this study was to obtain a nitrites-containing juice by fermentation of plant materials that contain nitrates, and finding the best parameters of fermentation to obtain the highest concentration of nitrite.*

Celery and parsnip vegetable juices were obtained by manually pressing of chopped material plants. Staphylococcus carnosus and Staphylococcus xylosus used for vegetable juice fermentations were isolated and then identified from a starter culture. The nitrate and nitrite concentration (ppm) was determined before and after 20 hours and 30 hours of fermentation at different concentration of NaCl (0, 5.0, 7.5, 10.0, wt. %), different temperatures (4°C, 35°C, 45°C) and different pH value (5.0, 6.0, 7.0) for each composition. For nitrates and nitrites determination, colorimetric methods were used.

After 20 hours of incubation at 35°C, in the absence of NaCl and pH 7.0, it was obtained a concentration of 897.3±7.3 ppm nitrites in the presence of Staphylococcus xylosus, comparative with 799.3±6.7 ppm nitrites in the presence of Staphylococcus carnosus.

The highest nitrate concentration was determined for raw parsnip juice, while nitrites were not detected in fresh vegetable juices of celery and parsnip. Fermentation formulas without added salt showed the highest concentration of nitrites, optimum temperature for juices fermentation was 35°C, and optimum pH value for juices fermentation was 7.0.

Keywords: *vegetable juice, nitrate reductase, Staphylococcus carnosus, Staphylococcus xylosus, fermentation parameters.*

INTRODUCTION

Nitrate and nitrite are water soluble compounds containing nitrogen and oxygen. Nitrites and nitrates arise from the microbial degradation of nitrogen-containing materials, such as organic human, animal and vegetable wastes, but also fertilizers and pollutants. The ammonium ion, NH_4^+, generated from different types of decomposition, is oxidized to yield NO_2^- and NO_3^- (Scheurwater et al., 1997).

Nitrate is a major form of nitrogen available to plants in soil. Before the NO_3^- is taken up by the plant and assimilated into organic nitrogen, it must first be reduced to NO_2^- and then to NH_4^+. The reduction of NO_3^- to NO_2^- is catalyzed by nitrate reductase. Nitrite reductase catalyzes the reduction of NO_2^- to NH_4^+ (Scheurwater et al., 2002).

The nitrate ion, NO_3^-, is negatively charged, and, in general, is not absorbed by soil until it is taken up by plant roots. Nitrates are essential for the healthy growth of plants. High nitrate concentrations are found in vegetables especially if excessive amounts of nitrogen have been added in fertilizers as manures (Scheurwater et al., 1997).

Leafy vegetables, such as lettuce, spinach, silverbeet, have been found to accumulate nitrate at higher concentration than root or fruit vegetables. Plant variety is also a major consideration when assessing nitrate levels (Reuter and Robinson, 1997).

The aim of this study was to obtain a nitrites-containing juice by fermentation of plant materials containing nitrates, and finding better methods of fermentation to obtain the highest concentration of nitrite. Fermented juice containing nitrites can be used in food industry to obtain uncured products.

Fermented juice made from plants has been obtained using microorganisms (*Staphylococcus carnosus* and *Staphylococcus xylosus*) capable to produce nitrate reductase to reduce nitrate in the nitrite. The process of converting nitrate to nitrite using microorganisms requires time in order to find favorable conditions (pH, temperature, NaCl concentration) for optimal yield in regarding to the quantity of nitrite produced (Scheurwater et al., 2002). The study had several steps: (1) selecting a plant material that contains an important amount of nitrate, (2) choosing a bacterial microorganism used in the meat industry, able to produce the nitrate reductase in order to reduce nitrate to nitrite, and (3) choosing the best conditions (temperature, pH, concentration of NaCl) to get the best conversion efficiency of NO_3^- in NO_2^-. Most strains of *S. xylosus* and *S. carnosus*, like other meat-associated staphylococci, have a nitrate reductase activity (García-Varona et al., 2000; Mauriello et al., 2004). *S. xylosus* and *S. carnosus* are commonly used as starter culture in meat fermentation (Talon and Leroy, 2011).

MATERIALS AND METHODS

Vegetable materials. Selected plants were the celery root and parsnip root. Celery and parsnip were bought from a local market and then were washed. Plant materials were cut into pieces and chopped in a laboratory blender. Vegetable juices were obtained by manually pressing of chopped material plants.

Bacterial strains. *S. carnosus* and *S. xylosus* used for vegetable juice fermentations were isolated and then identified from a starter cultures. The two staphylococcus strains were determined by microbiological analysis. The isolates were confirmed as gram positive, catalase positive, non-motile, coagulase-negative, non-toxigenic, non-spore-forming, aerobic cocci, and were biochemically identified. All the isolates with that profile confirmed were subjected to further analyses.

Sample preparation. The working scheme used to determine the optimum fermentation conditions are presented in Table 1. Each composition contained celery or parsnip juice concentrate that was diluted 1: 10 in distillated water to which 0.3 wt. % yeast extract was added. The pH of each composition was adjusted with 1 M sodium hydroxide. The compositions were sterilized at 121°C for 15 minutes. Each composition was then inoculated with either *S. xylosus* or *S. carnosus*. The strains of *S. carnosus* and *S. xylosus* used for vegetable juice fermentations are coagulase-negative and non-toxigenic. The nitrate and nitrite concentration (ppm) was determined before and after 20 hours and 30 hours of fermentation for each composition.

Table 1. Sample preparation in order to find the optimal conditions for reduction of nitrates to nitrites

Sample	NaCl (wt. %)	Temperature (°C)	pH
1	0	35	7.0
2	5.0	35	7.0
3	7.5	35	7.0

4	10.0	35	7.0
5	0	4	7.0
6	0	45	7.0
7	0	35	5.0
8	0	35	6.0

Determination of nitrate in vegetable juice. For nirates determination, it was used a colorimetric method described by Cataldo et al. (1975). The complex formed by nitration of salicylic acid under highly acidic conditions absorbs maximally at 410 nm in basic ($pH>12$) solutions. Absorbance of the chromophore is directly proportional to the amount of nitrate-N present. Ammonium, nitrite and chloride ions do not interfere. It was pipetted an aliquot of 0.200 mL of extract or standard into a 50 mL Erlenmyer flask. It was added 0.8 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4 and the reaction mixture was gently homogenized. After 20 minutes at room temperature, it was added 19 mL of 2 N NaOH to raise the pH above 12, and the tubes were left to cool at room temperature. In parallel it was prepared a blank of 0.200 mL extractant (H_2O) with the same reagents. The absorbance was measured at 410 nm.

Determination of nitrite in fermented juice. Nitrite ions react with a Griess reagent (sulphanilamide and N-(1-naphltyl) ethylenediamine hydrochloride (NED)) for color formation and the purple color that developed after 20 min was read spectrophotometrically at 538 nm (AOAC, 1990). 10 mL of extracted solution were pipetted into a 50 mL volumetric flask and water was added to make up to 30 mL. 5 mL of sulphanilamide solution followed by 3 mL of concentrated HCl were then added and the solution was left in the dark for 5 min. After adding of 1 mL of NED solution, the mixture was left for 15 min. in the dark and then it was diluted to mark with water. The absorbance of the solution was measured in a 1 cm cell using a spectrophotometer at a wave length of about 538 nm (Giustarini et al. 2008). Absorbance values of the samples were compared with absorbance values for standard solutions containing 0.00, 0.20, 0.40, 0.60, and 0.80 ppm nitrite.

RESULTS AND DISCUSSIONS

Determination of nitrates and nitrites in vegetable juices. Nitrate concentration was determined for each vegetable juice, celery and parsnip. It can be observed that the highest nitrate concentration was determined for parsnip juice (Table 2). Also, nitrites were not detected in vegetable juices of celery and parsnip.

Table 2. Nitrate concentrations in vegetable juices of celery and parsnip

Vegetable juice	Nitrates (ppm)	Nitrites (ppm)
Celery	956.7± 10.1	0
Parsnip	1054±11.9	0

The nitrate concentrations in plants greatly differ depending on soil, fertilizers, growing conditions, etc. From the obtained results it can be noted that fresh untreated juices contain no nitrites. Similar results were obtained and Nabrzyski and Gajewska (1994).

Nitrate concentrations in vegetables depend on the biological properties of the plant culture, sun light intensity, type of soil, temperature, humidity, frequency of plants in the field, plant maturity, vegetation period, harvesting time, size of the vegetable unit, storage time and source of nitrogen (Tamme et al., 2006). Even among different samples of the same vegetable varieties, the nitrate concentration may vary in a wide range (Prasad and Chetty, 2008; Thomson et al., 2007). Environmentally, nitrite is formed from nitrate or ammonium ions by certain microorganisms that produce nitrate reductase.

Determination of nitrite in fermented juice

The effect of NaCl on nitrite concentrations. In Table 3, it can be observed that NaCl concentration shows an important influence on fermentation process of vegetable juices of celery and parsnip.

Table 3. NaCl effect on the fermentation process

Fermented juice	**NaCl (wt %)**	***Staphylococcus xylosus***		***Staphylococcus carnosus***	
		Nitrites (ppm) 20 h	**Nitrites (ppm) 30 h**	**Nitrites (ppm) 20 h**	**Nitrites (ppm) 30 h**
Celery	**0**	897.3±7.3	756.6±7.3	799.3±6.7	598.5±3.3
	5.0	647.2±6.1	435.4±3.4	547.2±4.5	323.5±7.4
	7.5	93.2±2.1	56.9±1.1	43.2±1.8	11.4±1.5
	10.0	0	0	0	0
Parsnip	**0**	987.6±10.2	867.4±8.1	898.3±10.2	765.5±8.5
	5.0	564.4±4.2	354.2±3.2	432.7±4.7	356.4±3.1
	7.5	90.5±1.2	34.9±0.9	65.7±1.5	23.3±1.1
	10.0	0	0	0	0

For both extracts, fermentation formulas without added salt showed the highest concentration of nitrites. It can be noticed that increasing salt concentration reduces the nitrate concentration obtained for both types of vegetable juices. Also, from the obtained results it can be noticed that a concentration of 10% salt inhibit the reducing reaction of nitrates to nitrites. Results indicate that after 20 hours of fermentation it is obtained the highest concentration of nitrites, while after 30 hours of fermentation, nitrite concentration starts to decrease. The results obtained indicate that after 30 hours of fermentation, nitrites begin to decompose.

Fermented juices in the presence of *S. xylosus* showed higher concentration of nitrites compared to the nitrite concentration obtained in the presence of *S. carnosus*.

The effect of temperature on nitrite concentrations. The obtained results indicate that the optimum temperature for fermentation of juices is 35° C.

On the other hand, at 35°C nitrite concentrations achieved after 20 hours of fermentation are higher than those measured after 30 hours of fermentation. Also, for both of vegetable juices, the reduction reaction of nitrite to nitrate is slow at 4°C. At 45°C it can be seen that the reaction stops at 30 hours (Table 4), probably due to distortion of nitrate reductase enzyme produced by the staphylococci. Fermented juices in the presence of *S. xylosus* achieved higher concentration of nitrites compared to the nitrite concentration obtained in the presence of *S. carnosus*.

Table 4. Temperature effect on fermentation process

Fermented juice	Temperature (°C)	*Staphylococcus xylosus*		*Staphylococcus carnosus*	
		Nitrites (ppm) 20 h	**Nitrites (ppm) 30 h**	**Nitrites (ppm) 20 h**	**Nitrites (ppm) 30 h**
Celery	**4**	39.2± 1.1	67.5±1.4	42.2± 0.9	54.7±1.6
	35	897.3±7.3	756.6±7.3	799.3±6.7	598.5±3.3
	45	9.3±0.4	0	10.2±0.7	0
Parsnip	**4**	45.2±1.2	72.2±1.3	37.5±0.7	66.3±1.1
	35	987.6±10.2	867.4±8.1	898.3±10.2	765.5±8.5
	45	11.3±1.0	0	12.7±1.3	0

The temperatures used for these cultures vary with the strain. Casaburi et al. (2005) demonstrated that staphylococcus strains were able to reduce nitrate to nitrite at temperatures as low as 15°C but the activity increased as temperature increased, with maximum activity at temperatures over 30°C. It is recommended that commercially available strains used for nitrate reduction should be used at temperatures of 38-42°C. Recent research has shown that time is a critical factor for the conversion of nitrate to nitrite.

Sindelar et al. (2007) concluded that incubation time (at 38°C) was more critical than the amount of vegetable juice powder to produce no nitrate/nitrite added emulsified cooked sausages that had characteristics similar to a nitrite cured control.

The effect of pH on nitrite concentrations. Table 5 shows the concentrations of nitrites obtained at different pH values. Optimum pH value is 7.0, after 20 hours of fermentation when the maximum concentration of nitrites is achieved. For the other two pH values, 5.0 and 6.0, the reaction happens more slowly.

Table 5. pH effect on fermentation process

Fermented juice	pH	*Staphylococcus xylosus*		*Staphylococcus carnosus*	
		Nitrites (ppm) 20 h	**Nitrites (ppm) 30 h**	**Nitrites (ppm) 20 h**	**Nitrites (ppm) 30 h**
Celery	**5,0**	585.2±3.2	433.4±4.5	478.6±4.3	378.3±4.5
	6,0	675.4±6.4	501.5±8.5	412.4±2.9	453.3±4.9
	7,0	897.3±7.3	756.6±7.3	799.3±6.7	598.5±3.3
Parsnip	**5,0**	603.6±8.6	573.6±6.3	678.3±7.8	557.2±3.5
	6,0	731.1±6.9	612.3±4.6	625.2±5.7	597.3±3.9
	7,0	987.6±10.2	867.4±8.1	898.3±10.2	765.5±8.5

In the presence of *S. xylosus,* fermented juices achieved higher concentration of nitrites compared to the nitrite concentration obtained in the presence of *S.carnosus*.

CONCLUSIONS

The highest nitrate concentration was determined for raw parsnip juice, while nitrites were not detected in fresh juices of celery and parsnip.

NaCl concentration had an important influence on the fermentation process of celery and parsnip vegetable juices. For both extracts, fermentation formulas without added salt showed the highest concentration of nitrites.

The optimum temperature for juices fermentation is 35°C. At 4°C, the reduction reaction of nitrite to nitrate is slow; at 45°C, the reaction stops after 30 hours.

The optimum pH value was determined as 7.0, after 20 hours of fermentation when the maximum concentration of nitrites was achieved.

In the presence of *Staphylococcus xylosus,* fermented juices achieved higher concentration of nitrites compared to the nitrite concentration obtained in the presence of *Staphylococcus carnosus*.

ACKNOWLEDGMENTS: This work was carried out through Partnerships in priority areas Program – PN II, implemented with the support of MEN – UEFISCDI (Romania), project nr. 149/2014.

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