Factors Influencing the Denitrification Rate of Red Beet Juice by the Bacteria *Paracoccus denitrificans*

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The aim of this work was to determine the factors influencing the reduction of nitrate ions in red beet juice by *Paracoccus denitrificans*. The investigations were carried out using juice containing nitrate ions in a range of 1−5 g/L. It was found that microbiological treatment of juice made possible the complete removal of nitrates. The rate of denitrification was affected by temperature, pH, and osmotic pressure of juice as well as its initial concentration and the cell density of bacteria. It was ascertained that the microbiological treatment of red beet juice changed only on a limited scale its flavor and color.

Keywords: *Denitrification; Paracoccus denitrificans; red beet juice; pigment; betanine; vulgaxanthine*

INTRODUCTION

Many vegetables, particularly root ones, tend to accumulate nitrates and nitrites which affects negatively their nutritive value. The WHO/FAO data indicate that an acceptable limit daily dose of nitrates in the adult diet should not exceed 3.65 mg/kg of body weight (WHO/FAO, 1978). This means that an adult can ingest no more than 0.2−0.3 g of nitrates/day. However, many foods contain large quantities of nitrates, and in extreme cases, raw vegetable materials can contain up to 5 g of nitrates/kg (Gierschner and Hammes, 1991).

Elimination of nitrates from food products is very difficult. Theoretically, only a few technological solutions are feasible: appropriate treatment during processing, nitrate separation on ionic exchangers, microbiological methods, or combinations of the above.

The most effective method of nitrate removal is microbiological treatment to convert nitrate ions into gaseous nitrogen by denitrifying bacteria (Mateju et al., 1992). So far, only a few papers have been published describing microbiological denitrification of food products (GPO, 1987; Emig et al., 1990; Gierschner and Hammes, 1991; Haug et al., 1990; Kerner et al., 1988; Zills, 1988). Most of them concern vegetable juices and drinking water. The method was used for processing carrot juice, red beet juice, and spinach puree. It should be pointed out that reduction of nitrates microbiologically is not simple and requires fulfillment of a number of conditions. Among the most important ones are the necessity of using microorganisms which do not produce toxins and allergens and conducting the process in such a way that the chemical composition, taste, and smell are maintained.

One of the best known denitrifying organisms is the Gram-negative bacteria *Paracoccus denitrificans*. The bacteria can grow autotrophically in the presence of hydrogen and carbon dioxide or heterotrophically in media containing organic carbon sources. They can reduce nitrate to nitrogen gas through the use of hydrogen as an electron donor. Under anaerobic conditions they can produce the nitrate and nitrite reductases which are induced by the presence of these ions in the medium. The utilization of hydrogen ions results in a rise of the pH value. The reaction can be written according to an overall reaction:

$$\text{organic matter} + \text{nitrate ions} + \text{hydrogen ions} = \text{organic matter}' + \text{gaseous nitrogen} + \text{carbon dioxide} + \text{water}$$

An excellent review on the stoichiometry of the heterotrophic biodenitrification using different organic carbon sources is given by Mateju et al. (1992).

The toxicological tests showed that *P. denitrificans* did not inhibit the SCP organisms growth (Kerner et al., 1988). The bacteria usually occur in some fermented sausages (e.g., salami type) and reduce nitrate ions used for product preservation. In this form they are consumed as a natural component of the human diet. *P. denitrificans* was also used for the denitrification of carrot juice and red beet juice (GPO, 1987; Gierschner and Hammes, 1991).

The aim of this paper is to determine the relationship between the conditions of the microbiological treatment of red beet juice and the rate of nitrate reduction by *P. denitrificans*.

MATERIALS AND METHODS

Red Beet Juice. Red beet juice was obtained by diluting juice concentrate obtained from the Proparex Cooperative in Nowy Tomysl, Poland, with distilled water at the ratio 1:4. The juice acidity was adjusted to pH 6.5−7.0 with 10% NaOH solution. Such prepared juice was sterilized by microfiltration through a 0.2 μm filter (Prostak, Millipore).

Microorganism. *P. denitrificans* ATCC 19367 was used in this study. It was grown anaerobically at 30 °C on agar slants with the following composition (g/L): nutrient broth (Difco) 15.0, glucose 10.0, KNO 3.2, agar-agar 15.0, pH 7.0. The cells were transferred from the agar slants to 100 mL of liquid medium of the above given composition, and after 48 h they were repassaged on the fresh liquid medium and used as an inoculum for experimental cultures.

Denitrification Method. The experimental fermentations were carried out anaerobically in Erlenmeyer flasks, 500 mL, containing 400 mL of the juice, at 30 °C for 24 h. The experiments on the denitrification kinetics were performed using a 5 L bioreactor from New Brunswick Sci. Co. (Edison, NJ) stirred at 100 rpm in the same culture conditions. The juice was seeded with 10% (v/v) inoculum consisting of 48 h submerged culture. Each experiment using Erlenmeyer flask

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cultures was carried out in 12 repetitions and in the bioreactor in three repetitions. The results are presented as mean values.

The studies on the effect of initial pH and temperature of juice on the denitrification process were carried out in Erlenmeyer flasks, and the fermentations were performed at a pH within the range from 4.0 to 8.0 and at a temperature from 10 to 30 °C for 24 h. The juice acidity was corrected by the use of 10% (w/v) NaOH and 10% (w/v) citric acid solutions. The fermentations of the juice using concentrates of different osmotic pressure were carried out for 48 h.

Analytical Methods. To determine nitrate and nitrite contents, the samples of red beet juice were centrifuged for 15 min at 12 000 rpm (Beckman 12), loaded onto a HPLC column (APS Hypersil 5 μm 200 × 4.6 mm, 79916−574), and determined using a Hewlett-Packard Series 1050 chromatograph equipped with a UV detector. The analyses were carried out using the eluent 15 mM phosphate buffer, pH 3.2, with flow 1 mL/min. at a temperature of 40 °C and a pressure of 80 bars.

Glucose, fructose, and saccharose were determined by HPLC analysis using a Hewlett-Packard Series 1050 chromatograph equipped with a RI detector, precolumn (silica gel Alltech), and column (Aminex HPX-87H, 300 mm × 7.8 mm, Biorad). The analyses were performed using 0.005 mM sulfuric acid as eluent, flow rate 0.6 mL/min, at detector sensitivity 8 × 10 RIU/full scale deflection at room temperature. The samples were previously passed through a 0.22 μm Millipore filter and loaded onto the column in a volume of 50 μL.

To determine the dry matter of juice, the bacteria cells were removed by centrifugation (12 000 rpm, 15 min), and afterwards the samples of juice were dried to constant weight at 105 °C. The bacterial sediments obtained in the centrifugation were used for determination of cell dry matter by drying at 105 °C to constant weight. The pH values of juice were determined using the pH meter Elmetron CP-215.

The pigment content was determined by a differential spectrophotometric method according to Nilsson (1970). Before measurement the juice was diluted with 0.1 M phosphate buffer at pH 6.5 and centrifuged at 12 000 rpm for 5 min (MPW 210 centrifuge). The red pigment content, calculated as betanine, was determined from extinction measured at 538 nm, while that of the yellow ones, calculated as vulgaxanthine I, at 476 nm. The dilution was selected in such a way so that the extinction values were within the range of 0.2−0.8.

Flavor evaluation of juice was carried out by seven panelists consisting of laboratory staff, all with prior experience in sensory evaluation. Flavor acceptability was measured using 5-point hedonic testing (1 = dislike extremely to 5 = like extremely). The pH value of juice was adjusted to 6.0, and juice was placed in covered cups (100 mL). The evaluation was executed after 1 h of equilibration to room temperature (20 °C). The individual flavor profiles were also determined using the following characteristics: red-beet-like, vinous, fruity, maltlike, moldy, and sour.

RESULTS AND DISCUSSION

In preliminary studies the strain *P. denitrificans* ATCC 19367 used in this study was selected from 12 bacteria for its high denitrifying activity. To define the optimal conditions for nitrate reduction, some culture parameters, namely, temperature, pH of juice, initial cell density, initial nitrate concentration in juice, and osmotic pressure of juice, were studied. The effects of these factors on the denitrification rate of juice are presented below.

Temperature. Temperature is one of the basic factors determining the denitrification rate of red beet juice. The results indicate that nitrate reduction in low temperature is very slow, and only at over 25 °C is a distinct acceleration of this process observed (Table 1). Due to 24 h fermentation carried out at 30 °C, almost all initial nitrate contents were eliminated, i.e., 2.0 g/L. On the basis of chromatographic analysis, it was shown that in the samples taken from fermented juice not even trace quantities of nitrites were found. A clear rise in the juice pH observed in fermented samples concomitant with total elimination of nitrates indicates that the denitrification process finished with transformation of nitrates into gaseous nitrogen with simultaneous production of OH^- ions. Reduction of nitrates was accompanied by a decrease in the juice dry matter contents up to 14.4% of the initial level. This indicates heterotrophic bacterial metabolism. At the same time it was noted that with the increase in cell densities in the juice, utilization of the juice dry matter recalculated per unit of bacterial mass was lowered from 2.5 to about 1.0 g/g.

Table 1. Influence of Temperature on the Denitrification of Red Beet Juice by *P. denitrificans* in the 24 h Fermentation[a]

T (°C)	nitrate reductn (%)	dry matter loss (%)	cell biomass conctn (g of DM/L)	final pH
10	5	5.2	2.8	7.2
15	11	8.5	3.4	7.3
20	26	9.2	5.8	7.4
25	98	12.8	6.2	7.6
30	100	14.4	6.6	7.8

[a] Initial nitrate concentration and pH were 2.0 g/L and 7.0, respectively.

Table 2. Influence of Initial pH of Juice on the Nitrate Reduction by *P. denitrificans* in the 24 h Fermentation[a]

pH		nitrate reductn	dry matter	biomass conctn
initial	final	(%)	loss (%)	(g/L)
4.0	4.2	24	1.5	3.4
5.0	5.3	43	3.4	4.1
6.0	6.5	96	7.8	5.8
6.5	7.0	98	7.7	6.0
7.0	7.8	100	7.9	6.2
7.2	7.9	100	8.4	6.4
7.5	8.0	100	8.4	6.5
7.8	8.0	100	8.5	6.5
8.0	8.1	100	8.9	6.4

[a] Initial nitrate concentration was 2.0 g/L; initial cell density was 3.0 g/L.

It should be stated that the slow course of the denitrification process at temperatures below 20 °C indicates that the juice obtained in industrial conditions will require heating.

pH Value of the Juice. Studies on the effect of initial pH of the juice on the course of denitrification showed that this factor plays an important role (Table 2). Rapid and total nitrate reduction was observed only at neutral and slightly alcalic pH. Below pH 5.0 strong inhibition of the denitrification was observed. For example, at pH 4.0 during 24 h fermentation, only 25% of the initial nitrate content was reduced, i.e., 0.5 g/L. Higher pH values of the juice also favored an increase in cellular biomass which reached its maximum at pH 7.0−7.5. It should be pointed out that the natural pH of the juice is about 6.0−6.5; hence, it is approximated to the optimal for the denitrification process. In the case of denitrification of the juice concentrates acidified to pH 4.5 with citric acid, the acid should be neutralized.

Initial Cell Densities. One of the basic factors determining the rate of each fermentation process is the initial cell concentration. This is also confirmed by the results given in Table 3. It was found that increasing initial bacterial cell concentration from 3.6 to 10.6 g of

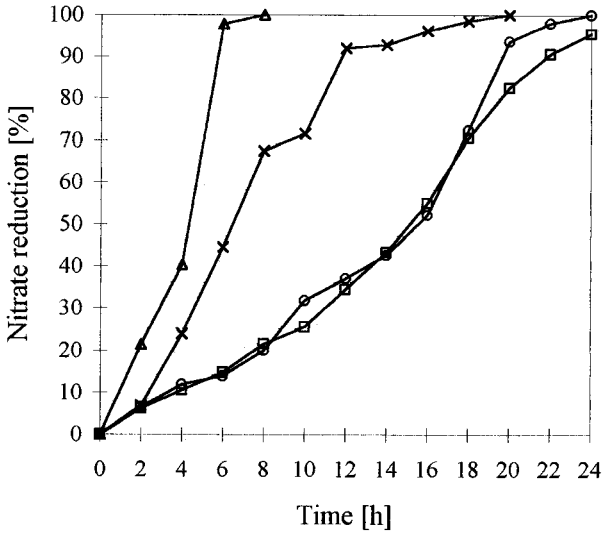

Figure 1. Percentage of nitrate reduction in juice by bacteria at different initial nitrate concentrations: (△) 0.8 g/L, (×) 2.3 g/L, (○) 3.0 g/L, and (□) 5.1 g/L.

Table 3. Denitrification Rate of Juice in Fermentations with Different Initial Cell Densities of *P. denitrificans*

t (h)	nitrate conctn (g/L)	nitrate reductn rate (g/L h)	biomass conctn (g of DM/L)	juice dry matter (g/L)	final pH
Initial Cell Density: 3.6 g of DM/L					
0	2.5	0.0	3.6	152.0	7.5
2	1.9	0.3	4.2	149.7	7.7
4	0.9	0.5	5.0	149.0	7.9
6	trace	0.4	5.3	148.2	8.0
8	0.0	0.2	5.5	147.1	8.1
Initial Cell Density: 10.6 g of DM/L					
0	2.5	0.0	10.6	151.6	7.5
2	1.7	0.4	11.8	150.6	7.9
4	trace	0.7	12.6	149.0	8.0
6	0.0	0.1	13.3	148.5	8.0
8	0.0	0.0	14.1	148.1	7.9

DM/L shortened the time needed for total juice denitrification from about 6−7 to 4−5 h. The nitrate reduction time during fermentation was variable and depended on bacteria concentration. However, in the conditions of the experiment it reached the highest values in the fourth hour of fermentation independently of the initial bacteria concentration. It is also worthwhile pointing out that at higher cell concentration lower losses of the juice dry matter were observed which is very significant for maintaining its original chemical composition. Gierschner and Hammes (1991) reported that at very high cell concentrations, of the order of 90−100 g/L, and their appropriate earlier feeding, it is possible to almost entirely limit losses in the dry matter of the processed juice. The data present in Table 1 indicate that at appropriately high cell concentration it is possible to reduce nitrate ions at the rate of 0.5−1.0 g/L h, which suggests the possibility of using this processing in industrial conditions.

Initial Nitrate Concentration. Results of fermenting the juice at various initial nitrate concentrations show that this factor has a considerable effect on the time necessary for total nitrate reduction (Figure 1). The fermentations were carried out at initial cell 2.0 g of DM/L and initial pH 6.8. It was found that even very high nitrate concentrations, up to 5.1 g of nitrate ions/L, can be totally reduced microbiologically. The rate of nitrate reduction, recalculated per juice volume unit, was positively correlated with the initial concentration of this component. Taking into account the time necessary to remove about 98% of the initial nitrate concentration, the nitrate reduction rates calculated for each initial nitrate concentration were very similar and varied between 0.13 and 0.14 g/L h. So, even at a nitrate concentration of 5.1 g/L the reaction proceeded without inhibition.

Table 4. Effect of Osmotic Pressure on the Nitrate Reduction in Red Beet Juice in 48 h Fermentations

dry matter of juice (g/L)	osmotic pressure (MPa)	initial nitrate conctn (g/L)	nitrate reduction (%)
295	−1.70	0.75	39
170	−1.16	0.80	100
125	−0.84	0.77	100
85	−0.71	0.77	100

Table 5. Fermentation Kinetics Carried Out in a Bioreactor with the Bacteria *P. denitrificans*

t (h)	nitrate (g/L)	nitrite (g/L)	dry matter (g/L)	cell biomass (g of DM/L)	pigments: yellow (g/L)	pigments: red (g/L)	pigments: ratio Y/R
0	1.50	0.0	155.0	3.78	0.622	0.987	0.63
1	1.38	0.0	153.1	4.54	0.645	0.961	0.67
2	1.16	0.0	150.8	5.41	0.657	0.947	0.68
3	0.15	0.0	148.3	6.31	0.679	0.922	0.64
4	0.07	0.0	146.7	6.97	0.695	0.896	0.77
5	0.06	0.0	146.1	7.23	0.726	0.879	0.83

Osmotic Pressure. In the industrial practice juice is often stored in the form of concentrate with dry matter contents of about 50% (w/v). Concentrated juices have high osmotic pressure which can inhibit growth and make the denitrification process impossible. Table 4 gives results of fermentation carried out using the juice with dry matter concentration within the range of 95−295 g/L. It was shown that inhibition of nitrate reduction occurs at the osmotic pressure level above −1.16 MPa. This means that the juice concentrates must be 2−3 times diluted prior to denitrification.

Denitrification Kinetics. The results of fermentation carried out in the 5 L bioreactor are presented in Tables 5 and 6. After a short time of adaptation the bacteria carried out the rapid reduction of nitrate ions which reached the maximal rate of about 1.0 g of nitrate/h between 2 and 3 h of fermentation. After 4 h of juice processing about 95% of the initial nitrate contents was reduced. Simultaneously with the denitrification process an insignificant loss of juice dry matter was detected which did not exceed 5.7% of the initial value. The main compounds utilized by bacteria were soluble sugars such as glucose, fructose, and saccharose which were consumed by 3.8%, 7.3%, and 5.2% of their initial contents, respectively. The data obtained in this study show an interesting observation. Recalculating the cell biomass yield from the dry matter consumed minus the nitrate weight reduced by bacteria results in a high yield coefficient reaching 60% in the hour of maximal denitrification rate. The energy requirements of bacteria usually obtained from organic matter fermentation were partially covered by energy gained from the nitrate reduction. This results in a reduction of the dry matter losses and therefore is a favorable factor in the juice treatment.

The cell density was double at the end of the process. The biomass yield calculated from dry matter utilized varied from 36.2% to 42.5%. The highest yield values were obtained just as the denitrification rate showed its maximum. In this time the volumetric nitrate ions

Table 6. Technological Characteristics of Microbiological Denitrification of Red Beet Juice with *P. denitrificans*

t (h)	nitrate reductn yield (g of DM/ g of NO_3)	specific denitrification rate (g of NO_3/ g of cell DM h)	vol nitrate reductn rate (g of NO_3/L h)	yield of biomass from juice DM (%)
1	15.8	0.026	0.12	40.6
2	10.4	0.041	0.22	38.1
3	2.5	0.160	1.01	36.2
4	20.1	0.011	0.08	41.4
5	30.0	0.003	0.02	42.5

reduction attained 1.0 g/L and the specific denitrification rate reached 0.16 g of nitrate/h g of dry matter of bacteria. These results are similar to the data reported (Gierschner and Hammes, 1991; Haug et al., 1990; Kerner et al., 1988). Haug et al. (1990) reported that the specific nitrate reduction rate in carrot-celery juice using two strains of *P. denitrificans* is between 0.138 and 0.341 g/h g. Kerner et al. (1988) have published that the specific nitrate reduction rate, obtained in the immobilized cell bioreactor and calculated on the fresh biomass unit, amounted to 31 mg/h g. Assuming the dry matter contents in the fresh bacteria biomass as 20% of their total weight, it appears that the adequate coefficient value is 0.155 g of nitrate/h g of cell dry matter. This value is similar to the result obtained in this study (i.e., 0.160 g/h g, Table 6).

The calculations show that the dry matter consumption related to the nitrate reduction range was depended on the denitrification rate. The lowest dry matter consumption was observed at the highest denitrification rate (Table 6).

Organoleptic Changes. The microbiological treatment of juice also caused some appreciable changes in its organoleptic properties. In this study two main features of fermented juice, the flavor and the pigment contents, were determined. It was observed that the fermentation process caused the characteristic changes in pigment contents manifested by decrease of red pigment and increase of yellow pigment concentrations (Table 5). The quantitative changes of pigment contents and their ratio coefficients did not affect the characteristic color of red beet juice defined as red-rose or red-violet. However, it was found that after 5 h of fermentation about 11% of red pigment disappeared whereas yellow pigment contents increased by 16.7%.

The sensoric analysis showed that the flavor of fermented juice was generally estimated as an agreeable although an untypical one. It was defined as a beet flavor with malt, caramel, or wine dominants.

It was noticed that the sensoric characteristics changed depending on the processing time as well as the temperature and the pH values of the fermentation process. Gierschner and Hammes (1991) have defined the organoleptic changes in vegetable juices treated by denitrifying bacteria as low.

CONCLUSIONS

Microbiological denitrification of red beet juice using *P. denitrificans* facilitates total reduction of this compound even at concentrations up to 5 g of NO_3/L. The rate of microbiological denitrification of red beet juice depends on such parameters as temperature, pH, and osmotic pressure of the juice and initial concentration of nitrates and bacterial cells. It was found that for reduction of nitrate contents in the juice the most favorable conditions are a temperature of 30 °C, pH of 7.0−7.5, osmotic pressure below −1.16 MPa, and possibly high initial biomass concentration of the bacterial cells. The highest volumetric denitrification rate amounted to 1.0 g of nitrate/h/L of bioreactor volume. The treatment of juice by *P. denitrificans* caused only insignificant changes in its chemical composition and organoleptic properties. The changes of juice flavor were more significant than the changes of juice color.

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