NITROSYLMYOGLOBIN FORMATION IN RAW PORK BATTERS WITHOUT NITRITE ADDITION: ROLE OF *STAPHYLOCOCCUS XYLOSUS* FERMENTATION

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Abstract – *Staphylococcus xylosus* and *Pediococcus pentosaceus* isolated from Chinese dried sausage were assessed for their ability to convert metmyoglobin into nitrosylmyoglobin in Mann-Rogosa-Sharp broth model systems and raw pork meat batters without the addition of nitrite. The results showed that samples in model systems with *S. xylosus* cultures had an absorption spectra that is typical of nitrosylmyoglobin, an obvious pink colour (judged by visual inspection) and a significantly higher *a-value than the control samples or samples inoculated with *P. pentosaceus*. In raw meat batters, the *a**-values of the *S. xylosus* samples were almost the same as those for the meat with nitrite added. The complementary analysis of meat batter samples by photochemical information from UV-Vis, electron spin resonance and resonance Raman spectroscopy revealed that the existing status of the myoglobin in meat batters inoculated with *S. xylosu*s was mainly pentacoordinate nitrosylmyoglobin. This study provides a potential solution for nitrite substitute in meat products.**

Key Words – Metmyoglobin, Pentacoordinate nitrosylmyoglobin, Replacement of nitrite

I. INTRODUCTION

Nitrite is widely used during meat curing because it can react with myoglobin (Mb) to form a characteristic pink colour [1]. However, carcinogenic, teratogenic and mutagenic N-nitrosamines and secondary or tertiary amines may form from nitrite [2]. Therefore, substitutes for nitrite have been exploited in meat industry to maintain the color of nitrite-cured meat products. Therefore, substitutes for nitrite have been explored by the meat industry, mainly in order to maintain the colour characteristics of nitrite-cured meat products. Natural or synthesised alternatives to nitrite have been developed. However, none of these alternatives has been used widely during meat processing until recently.

Another approach is the microbial conversion of metmyoglobin ($MbFe^{III}$) into nitrosylmyoglobin ($MbFe^{II}NO$, the cured-meat pigment). Several bacteria have been proved capable of converting $MbFe^{III}$ into $MbFe^{II}NO$ in culture medium model systems [3]. Moreover, some of the strains have been applied in meat products to evaluate the "reddening" ability to replace nitrite and some of these strains have produced interesting results. [4]. Nearly all of the meat samples used in the previous research was fermented sausages or hams. The red pigments in these samples could not be determined to result from microbial conversion, because the fermentation of meat products is complex, with many factors affecting the redox potential of the iron atoms within the products. [4]. Thus, it is important to simplify the experimental system to clarify whether the conversion process is truly attributable to microbial fermentation.

The objective of this study was to evaluate the suitability of two coccobacteria (*S. xylosus* and *P. pentosaceus*) as meat starter cultures to form a cured meat colour in Mann-Rogosa-Sharp (MRS) broth model systems and raw pork meat batters without the addition of nitrite or nitrate.

II. MATERIALS AND METHODS

2.1. Chemicals and Bacterial Cultures

Myoglobin lyophilized powder was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of biological or analytical grade. Two coccobacteria strains, *S. xylosus* A1 and *P. pentosaceus* R1 were isolated from Harbin dried sausage, a type of traditional of Chinese traditional naturally fermented meat product.

2.2. $MbFe^{III}$ Conversion in MRS Broth Model Systems

The capacity of the two cocci in MRS broth to convert $MbFe^{III}$ into red derivatives was assessed as described by Arihara *et al.* [3]. After 12 h of cultivation at 30 °C, the media culture was centrifuged to precipitate the bacterial cells, and the supernatant of MRS broth was used for the measurement of colour and for the UV-Vis spectral analysis.

2.3. Colour formation assay in raw meat batters

Fresh pork meat was bought from a local supermarket. The meat was trimmed of fat and connective tissue and then was ground through 4 mm plates of meat grinder in a cold room. All raw meat batters (100 g each) were prepared from minced pork containing sodium chloride (3.0%). Four meat batter groups with triplicate were each formulated as: control meat batter with only sodium chloride added, nitrite-cured meat batter treated with 0.1 g/kg sodium nitrite, the other two model meat batters inoculated with *S. xylosus* or *P. pentosaceus* starter cultures at levels of 10^6 CFU/g meat. All the samples were vacuum packaged in sterilised plastic bags and incubated at 30 °C for 8 h and then held at 4 °C for 16 h [4].

2.4. Colour Measurement

The colour of the samples was determined using a ZE-6000 colourimeter (Nippon Denshoku, Kogyo Co., Tokyo, Japan). The results are shown as *L** (lightness), *a** (redness) and *b** (yellowness). The instrument was set in the reflectance mode.

2.5. UV–VIS Spectral Analysis

Spectral analysis was conducted according to the method of Arihara *et al.* [3], with minor adjustments. Absorption scans were made from 350 to 700 nm at 1 nm intervals using a UV–Vis spectrophotometer (UV-6000PC, Shanghai Metash Instruments Co., Ltd., China).

2.6. Electron Spin Resonance (ESR) Spectroscopy

Each meat batter sample (about 0.3 g) was submitted directly to ESR spectroscopy using the method described by Gøtterup *et al.* [5].

2.7. Resonance Raman (RR) Spectroscopy

RR spectra were employed here to probe Mb selectively in pork batter samples as described by Wackerbarth *et al.* [6], with the 457.9-nm excitation line of an Ar^+-laser, using a confocal Raman spectrometer (HR-800, Horiba Jobin Yvon Co., Ltd., France) equipped with an electronically cooled CCD camera.

2.8. Statistical analysis

Data were analyzed using the General Linear Models procedure of the Statistix 8.1 software package. All specific experiments were carried out in triplicate. Analysis of variance (ANOVA) was done to determine the significance. Significant differences ($P<0.05$) between means were identified using LSD procedures.

III. RESULTS AND DISCUSSION

3.1. Capacity for $MbFe^{III}$ Conversion to $Fe^{II}NO$ in MRS Broth Model Systems

After removing the cultured cells by centrifugation at 4 °C, the supernatant was immediately subjected to spectrophotometric measurement at 350-700 nm in order to avoid oxidation of the red pigment by atmospheric oxygen. It was evident that samples with *S. xylosus* cultures showed absorption peaks at approximately 420, 540 and 580 nm (Fig. 1), which was a typical absorption spectra of $MbFe^{II}NO$ [7]. On the other hand, control samples or samples with *P. pentosaceus* cultures exhibited absorbance bands at approximately 410, 505 nm and 635 nm, respectively, which was characteristic spectra of aqueous $MbFe^{III}$ [7]. It supported the conclusion that *S. xylosus* A1 fermentation contributed to the formation of $MbFe^{II}NO$ without addition of nitrite. The mechanism of microbial conversion of $MbFe^{III}$ to $MbFe^{II}NO$ by *S. xylosus* is not clear, but it is believed that "reddening"

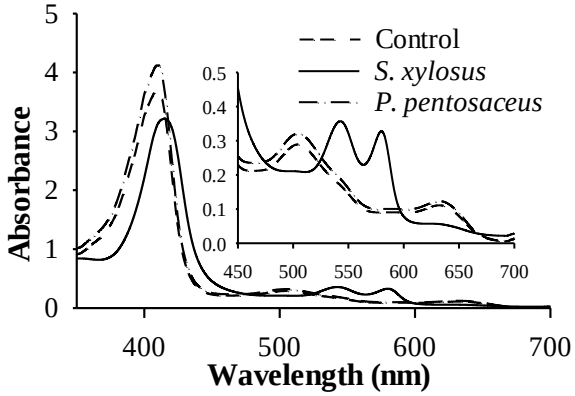

Figure 1. Absorption spectra of metmyoglobin-containing MRS broth inoculated either with *S. xylosus* A1 or *P. pentosaceus* R1 and incubation at 35 °C for 12 h, after removing cells by centrifuge.

LAB contains an enzyme called nitric oxide synthase (NOS) that could synthesize NO from L-arginine [8]. Indeed, NOS has been found and purified from *S. aureus* [9]. These considerations may be relevant for strains of *S. xylosus* as well.

3.2. Colour Formation Assay in Raw Meat Batters

Meat batter samples were examined to determine whether and what kind of Mb derivatives *S. xylosus* A1 can generate from $MbFe^{III}$ in a meat matrix. The *a**-values of vacuum packaged batters inoculated with *S. xylosus* showed a significant increase ($P < 0.05$), but there was no increase in samples treated with *P. pentosaceus* compared with the control samples (Table 1). The *a**-values of the *S. xylosus* samples were almost the same as those of the meat with added nitrite ($P>0.05$), which indicates that red Mb derivatives were also formed in meat samples, similar to the model system. However, there was no difference ($P > 0.05$) in the *L**- and *b**-value among the control, nitrite and *S. xylosus* treatment meat samples.

Table 1 Colorimetry of meat batters treated with different cultures

Samples	*L**-value	*a**-value	*b**-value
Control	44.9 ± 1.0^{ab}	9.7 ± 0.6^{b}	14.8 ± 1.1^{a}
P. pentosaceus	42.3 ± 1.2^{b}	9.6 ± 0.7^{b}	15.0 ± 0.8^{a}
S. xylosus	42.8 ± 1.0^{ab}	12.8 ± 0.7^{a}	15.1 ± 0.6^{a}
Nitrite	46.4 ± 0.8^{a}	13.0 ± 0.7^{a}	15.4 ± 0.8^{a}

Values are given as means ± SD from triplicate determinations; [a-b] Different letters in the same line indicate significant differences ($P < 0.05$).

To characterise the kind of Mb derivatives that were converted from $MbFe^{III}$ by *S. xylosu*s A1 in meat batters, the pigment was extracted using 75% acetone and then the spectra were measured. Almost no pigment was extracted out from the *P. pentosaceus* samples or control samples, while pink pigments obtained from the other two samples (nitrite and *S. xylosus* treatment) were used for analysis of UV–Vis spectroscopy. Because reduced $MbFe^{II}$, $MbFe^{II}O_2$ and $MbFe^{III}$ are reported to be poorly soluble in a 75-80% acetone/water solution [8], the pink color pigment was recognized as NO-Mb, the cured-meat pigments from both the ESR two samples showed maxima at 480, 542 and 565 nm (Fig. 2), which was another evidence for NO-Mb formation [8]. Spectroscopy was applied to meat samples in this experiment in order to detect $MbFe^{II}NO$ and investigate the coordination states of iron. As shown in Fig. 3, ESR signal was absent in the samples inoculated with *P. pentosaceus* and the control samples, indicating that no $MbFe^{II}NO$ was formed in these two samples. However, the ESR spectra of the other two samples showed three *g* factors with a rhombic symmetry, which suggests that the pink color pigment is pentacoordinate $MbFe^{II}NO$ [5]. It was further suggested that pentacoordinate $MbFe^{II}NO$ was formed in meat batters by *S. xylosus* fermentation.

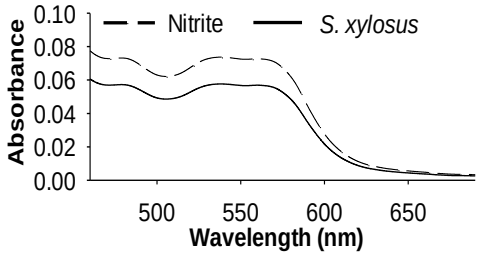

Figure 2. Absorption spectra of haem pigment extracted from different meat batters.

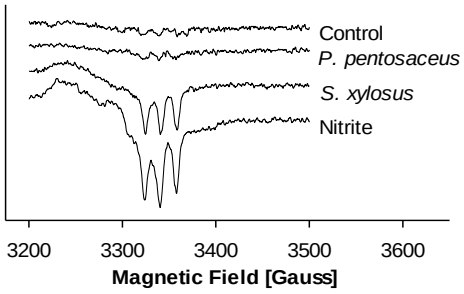

Figure 3. ESR spectra of meat batters prepared with 100 mg/kg of nitrite, or inoculated either with *S. xylosus* or *P. pentosaceus*.

However, the ESR signal for the three *g* factors of the samples inoculated with *S. xylosu*s was weaker than those treated with nitrite, indicating that not all of the Mb in the meat batters formed $MbFe^{II}NO$ during fermentation. Therefore, RR spectra were used to clarify the type of Mb derivatives existed in the meat batters. It is particularly suitable for probing Mb in tissues [6]. It was proved that $MbFe^{II}NO$ was formed in the meat batters treated with *S. xylosu*s and nitrite by UV-Vis and ESR spectra analysis above. This configuration was also reflected in RR spectra (Fig. 4) by characteristic frequencies of the marker bands at1375 (υ_4) and 1585 cm^{-1} (υ_2) albeit with a low signal-to-noise ratio [10]. It was also observed the

marker band at 1632 cm^{-1} (υ_{10}) from the *S. xylosu*s sample, which was considered to be the characteristics of ferric haem [11]. However, this band was not obvious in the nitrite sample. It meant that there was still some $MbFe^{III}$ existing in the *S. xylosu*s sample. This was the answer why the ESR signal was weaker than the nitrite sample as mentioned above.

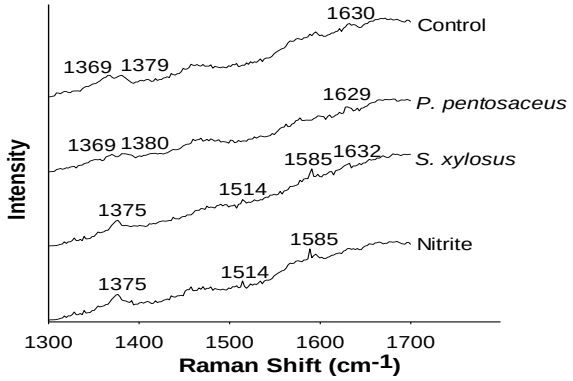

Figure 4. Resonance Raman (RR) spectra of meat batters prepared with 100 mg/kg of nitrite, or inoculated either with *S. xylosus* or *P. pentosaceus*.

The photochemical information from UV-Vis, ESR and RR spectra were complementary to each other in these experiments. According to the results discussed above, the existing status of Mb in meat batters inoculated with *S. xylosu*s is primarily $MbFe^{II}NO$.

IV. CONCLUSION

In MRS broth, *S. xylosus* A1 inoculation converted $MbFe^{III}$ into $MbFe^{II}NO$ while *P. pentosaceus* R1 could not. Pentacoordinate $MbFe^{II}NO$ was generated in pork batters by *S. xylosus* fermentation without the addition of nitrite as assessed by UV-Vis, ESR and RR spectra complementary analyses. These findings demonstrate a potential solution for colourising cured meat products and producing natural/organic foods without the addition of nitrite/nitrate.

ACKNOWLEDGEMENTS

This study was supported by Program for Innovative Research Team of Northeast Agricultural University (Grant No: CXZ011) and the Science Foundation of National Public Beneficial Vocation (Grant No: 200903012-02).

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