MICROBIAL INTERACTION IN THE FERMENTATION OF THAI PORK SAUSAGE

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INTRODUCTION

The Thai fermented foods evolved in parallel with Thai culture enjoy a long history with different product varieties. Nham, the Thai fermented pork sausage, is produced from ground pork mixed with sliced pork skin, garlic, pepper, salt and a small amount of potassium nitrate. The mixture is wrapped with banana leaves or plastic bags and fermented for 3-4 days. It is usually served as a dish and consumed raw. At present, nham production has developed into a multimillion baht industry but the technology still remains indigenous relying on adventitious microorganism. There have been numerous studies in the last 20 years among the Thai scientist community trying to shed some light into the complex biochemical and microbiological interaction of the fermentation (Tanasupawat and Daengsubha, 1983; Suzuki et al., 1987; Wiriyacharee, 1992; Phitakpol et al. 1995; Tanasupawat and Komagata, 1995). However, the role of each microorganism during the course of fermentation is not well understood. Such information is fundamental to the better understanding of microbiology and biochemistry of the fermentation and how this has impact on product quality. The knowledge will provide us with the tool for developing technology for more reliable commercial production.

MATERIALS AND METHODS

Fermentation of Thai-sausage mix
The sausage was prepared by mixing ground pork (50%), pork skin (36%), sucrose (0.5%), garlic (5%), salt (2.4%), cooked glutinous rice (4%), pepper (0.08%), whole bird chilli (2%) and potassium nitrate (0.02%). The ingredients were thoroughly mixed and approximately 200 g packed tightly into a plastic casing and allowed to ferment at 30°C for 72-84 hours.

Sampling procedure
Samples were taken at 4 hour intervals from 0 to 84 hours for chemical and microbiological analysis. The microbiological analysis was also performed on the ingredients.

Chemical analysis
Samples were taken for pH measurement by introducing the electrode into the center of the sausage using pH meter. The organic acids were determined on samples up to 5 day using gas chromatograph. The samples were converted into methyl ester derivatives according to the method of Holdeman (1991).
Microbiological analyses
For microbiological analysis, samples of Nham (25 g) were aseptically suspended in 225 ml of 0.1% peptone water and macerated for 60 seconds using Stomacher. The homogenate was serially diluted in 0.1% peptone and the number of microorganisms were determined by spread or pour plate technique. The lactic acid bacteria were counted on MRS agar incubated at 30°C for 1-2 days. The Micrococcaceae were plated on mannitol salt agar (MSA) and nutrient agar (NA), incubated at 30°C for 2-3 days. Yeasts were numbered on malt extract agar (MEA) adjusting to pH 3.5 and incubated at 25°C for 3-4 days. Fungi were cultured on Dichoran Rose Bengal Chlorotetracycline agar (DRBC), incubated at 25°C for 3-4 days.

Identification of microbial isolates
Bacterial isolates were purified and some were identified by their genus using biochemical tests and morphological observation as described by Holt et al. (1994). Species were identified in selected isolates of Micrococcaceae using API Staph Identification kit (bioMerieux, France). Yeasts were identified by their genus according to the methods and classification keys of Kreger-van Rij (1984) and by their species by ID32C identification kit (bioMerieux, France).

Detection of Enzyme Production.
All isolates were examined for their production of proteases using an agar plate method on skim milk agar (Barrow and Feltham, 1993), lipases using tributyrin agar (Mourey and Khertvisi, 1976), gelatinases using gelatin agar (Barrow and Feltham, 1993) and amylases using soluble starch agar and detection method as described by Barrow and Feltham (1993). The plates were incubated at 30°C for Micrococcaceae and 25°C for yeasts and fungi. The positive reaction was recorded as a clear zone around the point inoculation. Micrococcaceae and lactic acid bacteria were examined for catalase activity using the method as described by Barrow and Feltham (1993). Nitrate reductase activity was determined by modified method of Costilow and Humphreys (1954).

RESULTS

Growth of microbial species and pH change during the fermentation.
The growth of microorganisms during fermentation is shown in Figure 1. At the beginning of the fermentation, Micrococcaceae, yeasts and lactic acid bacteria were found. Cells of micrococcaceae were at a constant number of 10^6 cfu/g during the first 16 hours of the fermentation. The pH decreased from 6 to 5.7. After 16 hours, no cells of Micrococcaceae was detected. The average number of yeasts during the first 24 hours of fermentation remained at 3.8x10^6 cfu/g. The final population at 84 hours decreased to 7.5x10^5 cfu/g. The number of lactic acid bacteria increased from about 3x10^7 cfu/g to about 1x10^9 cfu/g within 20 hours of the fermentation. Growth of lactic acid bacteria during this period was accompanied by a drastic decline in pH value from 6.27 to 5.3. After 20 h, no further growth of lactic acid bacteria was observed. The number of lactic acid bacteria remained at about 1x10^9 cfu/g throughout the fermentation. The pH of nham gradually decreased to the final value of 4.6 after 84 hours of fermentation.
Table). Number of microorganisms originated from raw materials.

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Bacteria (cfu/g)</th>
<th>Yeast (cfu/g)</th>
<th>Fungi (cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garlic skin</td>
<td>7.0 x 10^5-5.0 x 10^6</td>
<td>0</td>
<td>4.5 x 10^3-3.6 x 10^5</td>
</tr>
<tr>
<td>Rice</td>
<td>1.4-9.2 x 10^6</td>
<td>2.0 x 10^3-5.0 x 10^6</td>
<td>1.0 X 10^2</td>
</tr>
<tr>
<td>Lean Pork</td>
<td>7.4 x 10^5-1.4 x 10^6</td>
<td>2.0 x 10^2-1.7 x 10^3</td>
<td>7.0 X 10^2</td>
</tr>
<tr>
<td>Pork skin</td>
<td>1.1 x 10^6-1.9 x 10^6</td>
<td>1.9 x 10^3-7.1 x 10^3</td>
<td>0</td>
</tr>
<tr>
<td>Bird chilli</td>
<td>6.6 x 10^5-3.3 x 10^6</td>
<td>1.2 x 10^3-1.8 x 10^6</td>
<td>0</td>
</tr>
</tbody>
</table>

Types of microorganisms in nham fermentation

Lactic acid bacteria were classified based on positive gram reaction and negative catalase activity. Lactic acid bacteria found in nham were lactobacilli, pediococci and leuconostoc as shown in Table 2. Lactobacilli dominated early in the fermentation up to about 24 h. When the number started to drop off. The number of pediococci and leuconostoc
increased after 36 hours or once the pH reached 4.6 and constituted about 50% of the total lactic acid bacteria population.

Table 2. Percentages of lactic acid bacteria during the fermentation.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Percentages of lactic acid bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lactobacilli</td>
</tr>
<tr>
<td>12</td>
<td>99</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>36</td>
<td>93</td>
</tr>
<tr>
<td>48</td>
<td>70</td>
</tr>
<tr>
<td>72</td>
<td>63</td>
</tr>
<tr>
<td>84</td>
<td>50</td>
</tr>
</tbody>
</table>

Micrococcaceae were characterized by their coccal shape, positive gram reaction and positive catalase activity. Cells of Micrococcaceae were identified as *Staphylococcus xylosus*, *Staph. hominis*, *Staph. cohnii* and *Micrococcus kristinae*. However, only *Staph. cohnii* and *Staph. hominis* were at high number during the 16 hours fermentation.

Yeast were characterized by their genus based on their morphological, physiological and biochemical properties. Three distinctive types of yeasts were observed and identified as *Debaryomyces* spp., *Rhodotorula* spp. and *Candida* spp. The species dominated were *D. hansenii*, *R. rubra* and *C. parapsilosis*.

**Enzyme activities**

All lactic acid bacteria tested negative for true catalase (heme independent). Furthermore most lactic acid bacteria did not possess enzymes for proteolysis, lipolysis and amylolysis. Some of the lactic acid bacteria tested were able to reduce nitrate and nitrite in the presence of heme (data not shown). Both Micrococcaceae and yeasts exhibited proteolytic and lipolytic activities. Of the 132 strains of Micrococcaceae tested, 32% and 11% possessed the proteolytic and lipolytic activities respectively (Table 3). From a total of 86 yeast strains, 34% exhibited proteolytic and 12% lipolytic activities. A very low 4% of Micrococcaceae had amylolytic activity. All of Micrococcaceae cells were capable of producing true catalase and most were able to reduce nitrate and liquefy gelatin (Table 3). The fungi isolated from the sausage during the fermentation appeared to contribute to the amylolytic activity which hydrolyzed amylose from rice to fermentable sugar.

Organic acid production during nham fermentation

The main organic acids produced during nham fermentation were lactic acid and small amount of succinic acid is also detected (Figure 2). The concentration of lactic acid at the start of the fermentation was 0.1% (w/w). During the course of the fermentation, lactic acid was gradually produced to the final concentration of 0.6% (w/w) after 96 hours which is about 10 times higher than the initial amount. A small amount of succinic acid (0.01-0.03%) was also detected during the fermentation.
Table 3. Production of enzymes by different microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Number of Different isolates' Protease</th>
<th>Amylase</th>
<th>Lipase</th>
<th>Catalase</th>
<th>Nitrate Reductase</th>
<th>Gelatinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid bacteria Micrococcaceae</td>
<td>32</td>
<td>4</td>
<td>II</td>
<td>100</td>
<td>92</td>
<td>83</td>
</tr>
<tr>
<td>Yeast</td>
<td>86</td>
<td>34</td>
<td>0</td>
<td>12</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>Fungi</td>
<td>2</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>•</td>
<td>•</td>
</tr>
</tbody>
</table>

1 Not determined
2 Determined by morphology of colony
3 Total of 1,600 isolates tested

DISCUSSION
The microorganisms found during the course of fermentation are lactic acid bacteria, Micrococcaceae, yeasts and fungi. The number of lactic acid bacteria increased by about 2 log cycles during the fermentation. The growth of lactic acid bacteria was accompanied by a rapid drop in pH during the first 20 hours of the fermentation. This rapid change in pH explains a rapid decrease in number of acid-sensitive Micrococcaceae. Yeasts and fungi did not grow during the fermentation. It is likely that during the first 16 hours, when many of aerobic...
microorganisms were present, oxygen in the fermentation system was consumed rapidly resulting in a low availability of oxygen for growth of micrococcaceae, yeasts and fungi.

Lactic acid bacteria found in nham fermentation included lactobacilli, pediococci and leuconostoc. Lactobacilli were found during early stage of the fermentation, while pediococci and leuconostoc grew successively towards the end of the fermentation process after the pH has reached 4.6. The association of Micrococcaceae and Staphylococci with fermented red meat products is well documented (Adams, 1986). In this study, Micrococcaceae found were mostly Staphylococcus cohnii and Staphylococcus hominis. Others species, Staphylococcus xylosus and Micrococcus kristinae, were observed only during the early stage of the fermentation. The principal yeasts present in nham were Debaryomyces hansenii followed by Candida parapsilosis and Rhodotorula rubra. Yeasts from these groups have been reported in the studies on meat products by Suzuki et al (1987) and Grazia et al. (1989). Fungi dominated in nham was Penicillium spp. which appeared to originate from the garlic skin as described in Table 1.

Lactobacilli had a significant role in the production of lactic acid which caused a decrease in pH. The acidity resulted in protein coagulation and contributed to the firmness of the product. The lactic acid also gave the acidic taste and aroma. The proteolytic and lipolytic activities from Micrococcaceae and yeasts resulted in the hydrolyses of protein and fat on the pork skin. The proteolytic activity is likely to result in an increase in short peptides, amino acids and free fatty acids. Some of these compounds give specific taste and aroma and some can serve as precursors of other volatile compounds (Dwivedi, 1975; Verplaetse et al., 1989; Nagodawithana, 1994). The proteolytic and lipolytic activities of these two microbial groups therefore should involve in the production of characteristic taste and aroma of nham (Lucke, 1985). The catalase produced by Micrococcaceae cells degrades hydrogen peroxide which is formed particularly by aerobes and lactic acid bacteria and hence prevent discoloration of the product. The nitrate reductase of the Micrococcaceae reduces nitrate to nitrite. Nitrite can be further reduced by nitrite reductase or by chemically means at pH 5.4:5.5 (KrOckel, 1995) giving nitric oxide as the end product. Nitric oxide binds to metmyoglobin to form nitrosomyloglobin (nitric oxide-metmyoglobin) resulting in bright pink color. Therefore, Micrococcaceae is likely to play a major role in color development of nham. Gelatinase activity can hydrolyze gelatin, a major component of pork skin, to produce amino acids, which gives the taste and aroma characters of nham. Role of fungi in nham fermentation is not clear as the level of fungi found in nham was very low. However, the amyl-lytic activity of the fungi may involve hydrolysis of starch present in glutinous rice to produce nee sugars for acid production and growth of other microorganisms.

The studies suggested that the strains which produced important enzymes appeared only during the early stage of the fermentation. Hence if acid production during the fermentation occurred too rapidly, this may significantly change the characteristic taste and aroma of the product. The starter cultures of Micrococcaceae and yeast which possess high activity of important enzymes may also be used to assure and enhance the taste and aroma of the product.

One of the major barriers in the development of multi-strain starter culture is the ability to accurately and correctly identify the most suitable strains to use in the fermentation. The identification of the strains used in this study is the biochemical and physiological tests which have limitations in term of accuracy and reliability. With the rapid and profound developments of Random Amplified Polymorphic DNA (RAPD) fingerprinting, strains of
bacteria can be correctly and reliably identified. Identification of lactic acid bacteria, Micrococcaceae and yeasts using this technology is currently in progress.

Conclusions
The microbial interaction in nham fermentation are extremely complex involving activities of different enzymes. As the microorganisms found in nham originated from raw materials used for the production; the presence of some important microorganisms in some batches may be varied depending on levels of contamination of the raw materials. For a successful nham fermentation, starter cultures composed of selected strains of lactic acid bacteria, Micrococcaceae and yeasts should be used.

REFERENCES