# RESAERCH ARTICLES

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## CHANGES IN THE VIABLE BACTERIAL POPULATION, pH, AND CHLORIDE CONCENTRATION DURING THE FIRST MONTH OF NAM PLA (FISH SAUCE) FERMENTATION

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**Summary**

*Traditionally-fermented Nam Pla contained $6.5 \times 10^4$ viable haloduric bacteria per ml on the first day of fermentation. The organisms died steadily and reached 100 per ml after 11 days of fermentation. Halophilic bacteria which required 20% NaCl for growth were not detectable at the start of fermentation but gradually appeared and increased to $9.5 \times 10^5$ per ml after 14 days of fermentation. The chloride concentration and pH of one-day old Nam Pla were respectively 33.1% and 5.5. There was little change in the pH of fermenting Nam Pla. However, the chloride concentration decreased as much as 5% after four days of fermentation. This study showed that growth of halophilic bacteria was associated with the decrease in chloride concentration during Nam Pla fermentation. The roles of salinity and halophilic bacteria in the traditional fermentation of Nam Pla are discussed.*

### Introduction

Fish sauce is a cheap source of essential amino acids and vitamins[1] among rice consuming populations in Southeast Asia. The sauce provides each person with up to 7.5% of the proteinaceous nitrogen[2] and 1 $\mu$g of the vitamin $B_{12}$[3] in his daily dietary intake. To date, fish sauce production remains a traditional process. Small pelagic fishes are mixed with marine salt at a ratio of 1:1 to 3:1 by volume

Abbreviations: TSA, Trypticase Soy Agar; TGA, Thioglycollate Agar

and natural fermentation is allowed to proceed uninterrupted for up to twelve months. The clear brown resultant liquid is finally decanted for use[4,5]. The mechanism by which fishes are turned into the characteristic sauce remains to be elucidated. Generally fish visceral enzymes are believed to be responsible for early proteolysis of fish protein into peptides and amino acids[6,7], while microbial enzymes are held responsible for ripening with characteristic flavours and aromas[5,12]. The amino acid profiles[13] and the low carbon fatty acid constituents[5] for Nam Pla have been described, but their derivation remains unclear. The microorganisms isolated from random specimens of fish sauce have been identified as largely bacterial species and rarely fungi[14]. Both haloduric and halophilic bacteria have been isolated and implicated as playing respective roles in ripening[5,15,17] and in spoilage[18]. The present study was initiated to follow microbial succession and to determine the extent of microbial participation during the initial phase of Nam Pla fermentation. Concurrent changes in pH and salinity were also measured.

## Materials and Methods

Marine salt and small pelagic fish of the genus *Stolephorus* were mixed in a ratio of 1:3 by volume and packed into a 10 liter earthenware pot. The air space was driven off by the addition of saturated brine. The pot was covered and set in the shade for natural fermentation to proceed undisturbed. At two to three day intervals, liquid specimens were drained from a basal outlet, transported at ambient temperature, and assayed microbiologically within four hours.

Specimens were diluted with sterile saturated brine and plated on Trypticase Soy Agar (TSA, Difco, Detroit) and Thioglycollate Agar (TGA, Difco). Each medium was used unmodified or with NaCl (Sigma Grade, Sigma Chemical Co., Mo.) supplements of 10, 20 or 30%. To solidify TGA containing NaCl at 20 and 30%, addition of Agar Agar (Difco) to a final concentration of 2% was necessary. Duplicate sets of plates were incubated at 15 and 37°C. Anaerobic incubation of plates containing TGA media was carried out using the Gas Pak Anaerobic System (Bioquest, BBL, Baltimore). All plates were continuously examined for up to 20 days before being discarded. Sodium chloride concentration was determined according to the method in A.O.A.C.[19] and pH was measured using a Corning Model 5 pH meter (Corning Scientific Instruments, Mass.).

## Results

Based on cultural characteristics, two broadly distinguishable bacterial subpopulations were isolated from the serial samples of Nam Pla analyzed. Namely, the haloduric population, which tolerated up to 10% NaCl but was inhibited at 20% and 30% NaCl, and the halophilic population, which required 20% NaCl for growth. The halophilic organisms grew slowly and required up to 14 days for the colonies to become evident on agar media. As fermentation proceeded, the haloduric popula-

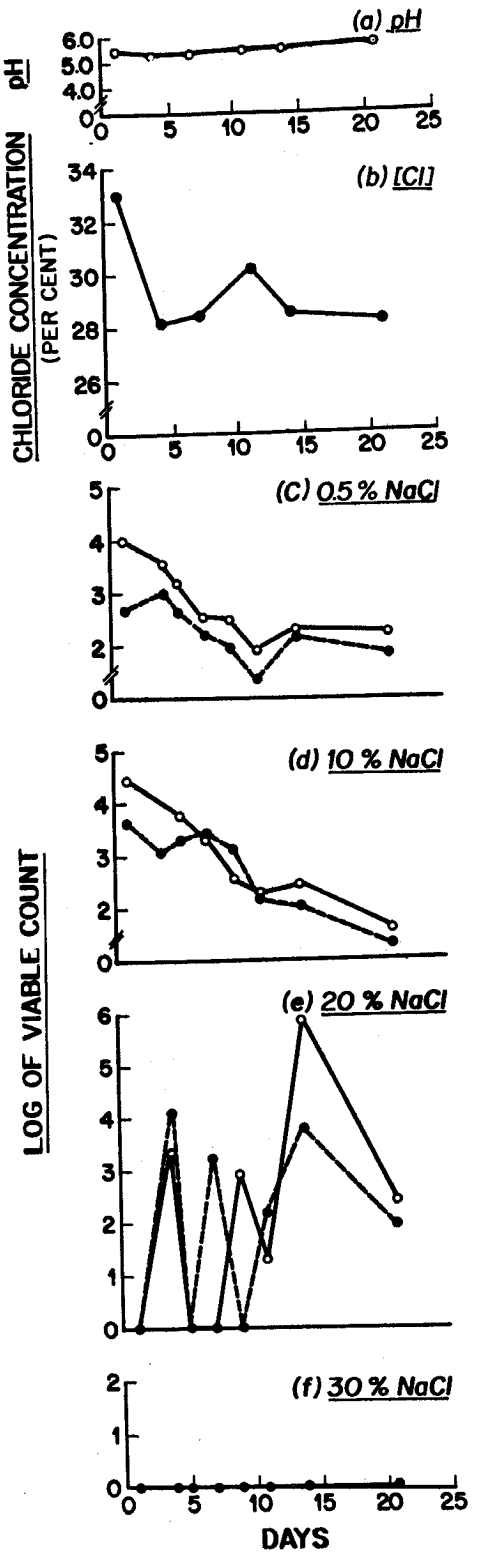


**Fig. 1.** Relationship of viable bacterial counts, pH (a), and chloride concentration (b) during Nam Pla fermentation. Viable bacterial counts were enumerated aerobically on TSA (solid lines) and anaerobically on TGA (broken lines) media at 0.5, (c); 10, (d); 20, (e); 30% NaCl, (f).

tion of samples decreased (Fig. 1 c and d) while the halophilic population increased (Fig. 1 e). The former started at $6.5 \times 10^4$ viable cells per ml, and gradually decreased to $10^2$ cells per ml by the end of two weeks. The latter were initially undetectable but increased to nearly $10^6$ cells per ml within the same period. These changes could be explained by the high NaCl concentration in the fermentation sauce (Fig. 1 b). The highest colony counts were obtained under aerobic conditions at 37°C. The parallel plates incubated anaerobically at 37°C, aerobiocally at 15°C and anaerobically at 15°C yielded lower counts in decreasing orders. Anaerobic growth at 15°C was almost completely inhibited by 10, 20 and 30 % NaCl but less so by 0.5% NaCl.

The overall change in pH during fermentation was slight. An initial drop from pH 5.5 on day one to 5.3 on day four was followed by a gradual increase as fermentation advanced (Fig. 1 a). The results indicated that haloduric bacterial growth had little influence on the pH of fermenting Nam Pla. The presence of an excess amount of NaCl together with the increasing quantities of free amino acids[20] presumably contributed to the buffering capacity in the sauce throughout fermentation. The chloride concentration decreased and then increased, starting with an initial drop of 5% by day 4 and followed by a 2% rise by day 11. These decreases in NaCl concentration, initiated primarily on day 1 and secondarily on day 11 (Fig. 1 b), coincided with increases in the halophilic bacterial population. Thus, subsequent peaks of cell concentration were detected on day 4 and day 14 (Fig. 1 e). The results suggested that salt concentrations as high as those present in fermenting Nam Pla may induce stasis in the growth of halophilic bacteria but may kill haloduric bacteria. This would mean that halophilic bacteria would multiply in fermenting Nam Pla only after the onset of salt dilution.

## Discussion

The low, i.e., $6.5 \times 10^4$, viable bacterial counts obtained from first day samples of Nam Pla fermentation liquor are comparable to those obtained by Takano[21] with day 2 samples of fermenting patis, the Philippine counterpart of Nam Pla. The decreasing number of haloduric bacteria explains the low viable number uniformly obtained by earlier investigators[5, 16, 17]. This study showed that high salt concentration, i.e., 28-33 % NaCl, was responsible for the selection of halophilic bacteria as the predominant bacterial population in the sauce as fermentation proceeded. The finding that the predominant bacterial population in fermenting Nam Pla is halophilic contrasts with the previous reports of haloduric bacteria predominance by Saisithi *et al*[5] and Liptasira[17]. However, this contradiction may be more apparent than real. The above authors used only four days and seven days incubation, respectively, while halophilic bacteria colonies require at least 14 days to become evident. Furthermore, we have demonstrated the adverse effect of low temperature on the majority of bacteria in fish sauce. If their specimens were chilled during transport, the practice might have contributed towards low viable bacterial counts.

A surprising result of this study is the fall and rise of NaCl concentration. We feel that this is a result of a dynamic interaction in a very heterogeneous fer-

mentation system. However, we feel that the many factors involved can be grouped into two types, those which work to reduce the salt concentration and those which work to increase it. Of the former there could be the outflow of water from the fish when they are first put into the broth[7] and the release of amino acids as a result of tissue digestion[20]. One would result in direct dilution of NaCl and the other in its decreased solubility. Of opposing factors, there could be resolubilization of salt crystals[7] always present in the fermenting container and amino acid uptake by the rising halophilic bacterial. The rate at which each of these factors is operating at any time during the fermentation would determine the resultant NaCl concentration.

Regarding the bacterial population, whether the simultaneous decrease in viable haloduric bacteria and increase in halophilic bacteria is a physiologically causal relationship remains to be seen. Meanwhile, taxonomic studies of the two subpopulations are underway. In addition we have initiated a series of on site investigation with large scale fermenters to determine whether the trend in this bench scale study are true in the industrial process.

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## บทคัดย่อ

น้ำปลาดิบซึ่งหมักจากปลากะตักและเกลือทะเลตามวิธีธรรมชาติ มีแบคทีเรียชนิดทนเกลือ (halodu-ric bacteria) อยู่ $6.5 \times 10^4$ ตัวต่อ ลบ.ซม. ในวันแรกของการหมัก เชื้อชนิดนี้จะตายลงอย่างรวดเร็วจนเหลือน้อยกว่า 1% ในวันที่ 11 เชื้อแบคทีเรียชนิดชอบเกลือ (halophilic bacteria) ซึ่งต้องการเกลือ 20% สำหรับเจริญพันธุ์ไม่ปรากฏตัวเมื่อแรกหมัก แต่จะพบจำนวนเพิ่มขึ้นเรื่อย ๆ จนถึง $9.5 \times 10^5$ ตัวต่อ ลบ.ซม. เมื่อหมักได้ 14 วัน น้ำปลาหมักวันแรกมีเกลือ NaCl 33.1% และมี pH ที่ 5.5 pH เปลี่ยนไปเล็กน้อยระหว่างการหมัก ส่วนเกลือ NaCl ลดลงถึง 5% เมื่อหมักได้ 4 ขั้น การเจริญพันธุ์ของแบคทีเรียชนิดที่ชอบเกลือสัมพันธ์กันกับการลดลงของคลอไรด์ในน้ำปลา ในบทอภิปรายได้กล่าวถึงบทบาทของเกลือและเชื้อแบคทีเรียในการหมักน้ำปลาตามวิธีธรรมชาติ