การเปลี่ยนแปลงประชากรจุลินทรีย์และทางเคมีระหว่างกระบวนการหมักปลาส้มที่เป็นผลิตภัณฑ์ปลาหมักของไทย

Microbial population and chemical changes during fermentation of *Plaa-som*, a Thai fermented fish product

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บทคัดย่อ

จากการสำรวจการเปลี่ยนแปลงประชากรจุลินทรีย์และทางเคมีระหว่างกระบวนการการหมักปลาส้ม พบว่าค่าความเป็นกรดด่างลดลงจากค่าเริ่มต้นที่ 6.47 ไปเป็น 5.24 ในวันที่ 3 ปริมาณกรดทั้งหมดค่อย ๆเพิ่มขึ้น จากค่าเริ่มต้นที่ 1.04% (w/w) จนถึงค่าสูงสุดในวันที่ 3 ที่ 4.76% (w/w) แล้วจึงลดลงจากวันที่ 3 จนถึง 2.88% (w/w) ในวันที่ 8 พบกรดแลคติกและ กรดน้ำส้มสายชูที่เพิ่มขึ้นอย่างต่อเนื่องจากวันที่ 0ไปเป็น 26.77 และ 16.21 mg/g ในวันที่ 3 ตามลำดับ ชี้ให้เห็นว่าได้มีการผลิตกรดทั้งสองชนิดนี้ได้ในระหว่างกระบวนการหมัก โดยพบว่าปริมาณของกรดทั้งสองชนิดนี้สอดคล้องกับปริมาณกรดทั้งหมดและมีความสัมพันธ์แบบผกผันกับค่าความเป็นกรดด่างระหว่าง 3 วันแรกของการหมัก จำนวนแบคทีเรียสร้างกรดมีความสัมพันธ์โดยตรงกับค่าความเป็นกรดด่างและปริมาณกรดทั้งหมด จำนวนแบคทีเรียสร้างกรดค่อย ๆเพิ่มขึ้นจาก 2.0 x 10^7 CFU/g ในวันที่ 0จนถึง 7.9 x 10^8 CFU/g ในวันที่ 3 และจะเริ่มลดลงจนเป็น 4.8 x 10^7 CFU/gในวันที่ 8 จำนวนแบคทีเรียทั้งหมดจากการบ่มเพาะในสภาวะมีอากาศ และในสภาวะอากาศน้อย ลดลงจากวันที่ 0 ที่ 5.5 x 10^8 และ 4.6 x 10^8 CFG/g จนมีจำนวน เป็น 1.9 x 10^8 และ 2.3 x 10^8 CFU/g ในวันที่ 3 ตามลำดับ หลังจากวันที่ 3 จำนวนแบคทีเรียทั้งหมดที่บ่มเพาะในสภาวะมีอากาศจะค่อย ๆเพิ่มขึ้นไปเป็น 4.4 x 10^8 CFU/g หลังจากวันที่ 4 จำนวนแบคทีเรียทั้งหมดที่บ่มเพาะในสภาวะมีอากาศน้อยจะค่อย ๆ ลดจำนวนลงจาก 3.9 x 10^8 CFU/g ไปเป็น 5.7 x 10^7 CFU/g ในวันที่ 8

Abstract

An investigation of microbial population and chemical changes during fermentation of *plaa-som* was conducted. The pH was found to decrease from 6.47 initially to 5.24 on day 3. Total acidity increased gradually from an initial value of 1.04% (w/w) reaching a peak on day 3 at 4.76 % (w/w). The total acidity then decreased from day 3 descending to 2.88 % (w/w) on day 8. Lactic acid and acetic acid were found to continually increase from day 0 to 26.77 and 16.21 mg/g on day 3, respectively. This indicates that both acids were produced during the fermentation. Their amounts correlate with total acidity and inversely with pH during the first 3 days of fermentation. The number of acid producing bacteria exhibit a direct relationship with pH and total acidity. The acid producing bacteria count increased gradually from 2.0 x 10^7 CFU/g on day 0 to 7.9 x 10^8 CFU/g on day 3. They then declined to 4.8 x 10^7 CFU/g on day 8. The number of total bacteria counts from both aerobic and microaerobic incubation conditions were found to decrease from 5.5 x 10^8 CFU/g and 4.6 x 10^8 CFU/g on day 0 to 1.9 x 10^8 and 2.3 x 10^8 CFU/g on day 3, respectively. After day 3 the total aerobic bacteria count gradually increased to 4.4 x 10^8 CFU/g. After day 4, the total microaerobic bacteria count gradually decreased from 3.9 x 10^8 CFU/g to 5.7 x 10^7 CFU/g on day 8.

คำสำคัญ: ปลาส้ม ปริมาณกรดทั้งหมด แบคทีเรียสร้างกรด

Keywords: *Plaa-som*, total acidity, acid producing bacteria

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Introduction

The Thai fermented fish product *plaa-som* is produced using traditional family recipes in the north-eastern and central regions of Thailand (Adams et al., 1985; Saisithi, 1987; Ishige, 1993). The purpose of the fermentation process is fish preservation which also imparts authentic flavors by its sour, spicy, and slightly salty taste (Valyasevi and Rolle, 2002). Nowadays, this fermented fish has received greater attention and is being marketed locally and nationwide. Therefore, improving its production to use a more controllable process has started to become a necessity. In order to improve its quality and safety, some research has been conducted to identify the microflora and their roles in contributing to the unique characteristics of *plaa-som*.

Plaa-som is commonly produced from a common freshwater silver barb fish species (*Barbodes gonionotus*) using either the whole fish or fillets (Adams, 1986). The major ingredients are garlic, ginger, chilli, pepper and spices with salt as a favoring agent, either raw, roasted, or steamed plain or sticky rice, and sugar (Pithakpol et al., 1995; Paludan-Müller et al., 1999, Valyasevi and Rolle, 2002). Lactic acid bacteria are well-known as the predominant microorganisms with their primary role to ferment the carbohydrate sources and convert these to a mixture of organic acids, with mainly lactic acid (Orillo and Pedersson, 1968; Saisithi et al., 1986; Olympia et al., 1992; Ostergaard et al., 1998; Paludan-Müller et al., 1999; Paludan-Müller et al., 2002). The lowering of the pH value by the organic acids, generally to below 4.5-5.0, with a slight saltiness are believed to be factors directly involved in preservation and safety of the product (Owens and Mendoza, 1985; Lee et al., 1994; Paludan-Müller et al., 2002). There are several *plaa-som* recipes, with various fish species as the raw materials, depending on family and local sensory preferences. This can cause a diversity in microbial population and results in many characteristics of the product as well as differences in taste and texture. Investigations on both microorganisms and chemical changes during *plaa-som* fermentation can provide useful background information on the mixed microbial community and their effects on *plaa-som* characteristics. This information can be used to enable a more controllable process for a consistent high degree of safety and quality. The microbiological and chemical changes during fermentation can be monitored in the production process of fermented food products to serve as product quality indices covering the sensory aspects of taste (sourness, saltiness), flavor (aroma), texture (firmness, springiness), and color, as well as safety aspects of the product (Valyasevi and Rolle, 2002). These changes can then be used to indicate process success or failure and also to analyze for critical control points which will lead to the practice of higher safety standards (Motarjemi, 2002). This study is aimed to determine the changes in microbial population and chemical changes during fermentation of *plaa-som* from available local production sources.

Materials and methods

1. Product selection and preparation of plaa-som sample

One *plaa-som* product was selected from the acceptable local recipes after receiving the highest sensory test score from sensory evaluation among 39 panelists (results not shown). The common fresh water barb fish, Tapian (*Barbodes gonionotus*) was

used as raw material. The size of each fish was selected to be in the range of 100-150 g. Sixteen whole fish were washed, scaled, gutted, and stripped then mixed thoroughly with all ingredients required from the selected recipe, including garlic, salt, and cooked jasmine rice. Two prepared fish were packed per plastic bag. The bags were left unsealed. The following day, eight bags of the *plaa-som* were brought to the laboratory on ice in a chilly bin and were then fermented at 30°C for 8 days. One bag of *plaa-som* was taken daily for both microbiological and chemical analyses.

2. Microbiological analyses

One fish from each plastic bag was aseptically weighed and chopped before mixing with sterile 0.1 % (w/v) peptone (Himedia, Mumbai, India) water in the ratio of 1:10 dilution (25g + 225 ml of 0.1% peptone water). The sample was homogenized in a stomacher 400 Lab Blender (A.J. Seward, Bury ST. Edmunds, UK) at high speed for 3 minutes. The homogenized sample was subsequently aseptically diluted using 10-times serial dilutions with 9.0 ml of 0.1% (w/v) peptone water as diluent per tube. Microbiological analyses for total bacteria count and acid producing bacteria count were conducted using a standard pour plate technique on duplicate agar plates from suitable dilution tubes according to Speck, 1984. Total bacteria count was performed on plate count agar (PCA, Merck, Darmstadt, Germany) and incubated in both aerobic and microaerobic conditions at 30°C for 48 hrs. The microaerobic incubation condition in this study was created in a sealed, thick plastic jar with a properly screwed lid and a lit candle inside. This exhausted most of the oxygen contained in the head space of the jar resulting in more CO_2 content and less O_2 content as the flame was gradually extinguished. The microaerobic incubation condition was assigned to favor the recovery of lactic acid producing bacteria from the fish fermentation which might not otherwise be recovered using only an aerobic incubation condition. Acid producing bacteria count was performed on de Man, Rogosa and Sharpe agar (MRS, Himedia, Mumbai, India) amended with 1.0% (w/v) $CaCO_3$ (MRS-$CaCO_3$ agar) and microaerobically incubated for 48 hrs. at 30°C. Acid producing colonies exhibited clear zones surrounding the colonies due to the dissolution of $CaCO_3$ by the acid. Ten acid producing bacterial colonies exhibiting clear zones around their colonies on MRS-$CaCO_3$ agar were randomly picked each day during fermentation and then re-purified on MRS-$CaCO_3$ agar. These acid producing isolates were subsequently either short term stored at 4°C in MRS agar tall tubes or long term stored at -80°C in a freezing medium containing 30% glycerol (Gibson and Khoury, 1986) for further study.

3. Chemical analyses

One fish from each plastic bag was weighed and blended with a food blender prior to conducting pH measurement, total acidity determination, and HPLC analysis. A portion of 20 g of blended sample was used for each analysis. pH value was measured in a 1:10 dilution (20 g of blended fish in 180 ml of distilled water) according to the A.O.A.C. method (A.O.A.C., 1990). After mixing the 1:10 dilution was filtered through a cloth. The filtrate was then equally divided into 3 flasks before measuring the pH in each flask. After pH measurement, the sample in each flask was subsequently analyzed for total acidity. Total acidity was determined as an equivalence to lactic acid using a titration against 0.01 N

NaOH containing a few drops of phenolphthalein to determine the end point. The sample for organic acid analysis using HPLC techniques was prepared in a 1:1 dilution (20 g of blended fish in 20 ml of distilled water). The dilution was filtered through a Whatman filter paper number 4. The filtrate was further subjected to protein and fat extractions prior to performing HPLC analysis. Five milliliters of the filtrate were subjected to protein extraction with 5 ml of 0.005 N perchloric acid, mixed, and kept at room temperature for 5 minutes. This was followed by an addition of 15 ml of petroleum ether for fat extraction. The supernatant left after protein and fat extractions was then filtered through a 0.45 μm filter membrane, and 10 μl of the filtrate was subsequently analyzed for organic acids using HPLC analytical techniques (Shimadzu RID-10A Detector, 0.005 M H_2SO_4 used as mobile phase, flow rate 0.6 ml/min, and temperature at 40 °C). Organic acids used as external standards in the analysis were acetic, butyric, lactic and propionic acids.

Results

1. Microbiological analyses

The number of acid producing bacteria exhibited a direct relationship with pH as well as the total acidity as illustrated in Figure 1. The acid producing bacteria count was found to increase gradually from 2.0 x 10^7 CFU/g at the initiation of the fermentation experiment (day 0) to 7.9 x 10^8 CFU/g on day 3 as shown in Figure 2. The number of acid producing bacteria then started to decrease from day 3 to 4.8 x 10^7 CFU/g on day 8. Figure 2 also shows the changes in the microbial populations found in *plaa-som* during fermentation and the relationships among them. The number of total bacteria counts from both aerobic and microaerobic incubation conditions were found to decrease from day 0 at 5.5 x 10^8 and 4.6 x 10^8 CFU/g to 1.9 x 10^8 and 2.3 x 10^8 CFU/g on day 3, respectively. After day 3 the total bacteria count from the aerobic condition was found to gradually increase to 4.4 x 10^8 CFU/g on day 8. The total bacteria count from the microaerobic condition showed a decreasing trend from 3.9 x 10^8 CFU/g on day 4 to 5.7 x 10^7 CFU/g on day 8.

2. Chemical analyses

Changes in pH and total acidity of *plaa-som* during the 8 days of fermentation at 30°C are shown in Figure 3. pH of the fish samples were between 6.47 and 5.24. The pH value was found to decrease from 6.47 initially to 5.24 on day 3. Thereafter pH value slightly increased to 5.50 on day 8. Total acidity increased gradually from an initial value of 1.04 reaching a peak on day 3 of 4.76 % (w/w). The total acidity then decreased from day 4 to day 8, from 4.10 to 2.88 % (w/w). Total acidity and pH exhibited an inverse relationship to each other during the first three days. Hence, the lowest pH and highest total acidity were found on day 3.

Lactic acid and acetic acid were found to continually increase from 12.82 and 6.43 mg/g on day 1 to 26.77 and 16.21 mg/g on day 3, respectively, as illustrated in Figure 4. Butyric and propionic acids were also analyzed but were not detected in the samples. This indicates that both lactic and acetic acids were present in *plaa-som* and their amounts were found to correlate with total acidity and pH during the first 3 days of fermentation.

Discussion

Plaa-som is categorized as a traditional Thai fermented fish product which contains salt at less than 8%, pH in the range of 3.9 to 6.1, and total aerobic bacteria count in the range of 2.4×10^5 to 6.0×10^{10} CFU/g (Tanasupawat and Komagata, 1995). The total bacteria count and pH found in *plaa-som* in this study corresponded to this categorization. The fermentation process is an open process and usually requires 3-4 days to provide a desirable sour taste. The duration of fermentation, however, also depends on local organoleptic preferences of the product and seasonal dependent factor (Valyasevi and Rolle, 2002). Hence, a shorter fermentation time is usually found in the warmer season. The eight-day experimentation period conducted in this study was designed to cover the duration of the entire fermentation process until the products exhibited undesirable appearances such as mushy texture, excessive water extrusion and odor. The pH was used as a simple scientific indicator of sour taste while total acidity was used as a quantitative indicator to confirm acid production during the fermentation. HPLC analysis was conducted to identify the acids produced. From days 3 to 8 pH of the *plaa-som* was stable while its acidity gradually decreased due to the pH buffering capacity of the samples (Figure 3). After day 8 the product was no longer suitable for consumption because of the undesirable appearances mentioned above. Similarly to other fermented fish products, the acid producing bacteria count is required to be at least 10^8 CFU/g to obtain an amount of acid to sufficiently reduce the pH in *plaa-som* (Olympia et al., 1992; Ostergaard et al., 1998). The maximum number of acid producing bacteria in this study was found when the pH was at its minimum on day 3, and this also related to the highest amount of total acidity (Figure 1). The number of aerobic and microaerobic bacteria was highest on day 0 from PCA agar plates incubated under aerobic and microaerobic conditions, respectively. This indicates that there were a number of bacteria from fish and ingredients present before the fermentation commenced and part of these bacteria exhibited growth under the microaerobic conditions. The acid producing bacteria counted from MRS agar plates amended with 0.1% (w/v) $CaCO_3$ (MRS-$CaCO_3$) were also grown under microaerobic conditions. The count of acid producing bacteria differed from the count of microaerobic bacteria which can be explained from the reason that MRS-$CaCO_3$ is considered a more suitable agar to support growth with acid production when compared to PCA, especially for lactic acid producing bacteria (de Man et al., 1960). The number of aerobically grown bacteria was higher than the number of microaerobically grown bacteria on day 0 and the number of acid producing bacteria was very low. The microaerobic bacteria count on day 0 was also much higher than that for the acid producing bacteria on day 0. As the fermentation progressed to day 3, however, the acid producing bacteria count increased substantially and microaerobic bacteria growth on PCA, a poor medium for growth with acid production, became suppressed. On commencement of fermentation, conditions began to favor the growth of acid producing bacteria and the populations of both aerobic and microaerobic bacteria decreased rapidly from days 1 to 3 (Figure 2). During the first three days of fermentation, there was an overall decrease in the numbers of aerobically and microaerobically grown bacteria as the number of

acid producing bacteria increased which can be explained by the pH drop over this period. Acid production from fermentation results in a decrease in pH and lower pH in the range of 4.5 to 5.5 with salt amendment inhibits growth of other bacteria including spoilage and pathogenic microorganisms (Owens and Mendoza, 1985, Tanasupawat and Komagata, 1995; Ostergaard et al., 1996). However, from days 3 to 8, the number of aerobic bacteria started to gradually increase and the product became no longer fit for consumption after day 8. This stage was further characterized by an increase in pH, decrease in acidity and a reduction in the number of acid producing bacteria. After day 4, both acid producing bacteria and microaerobic bacteria counts followed a declining trend as aerobic bacteria count increased. This indicated that conditions at this period were more suitable for aerobic bacteria growth. The chemical changes occurring in *plaa-som* during the initial fermentation period were as expected with acidity increase, pH reduction, and a mixture of organic acids produced resulting from the action of the microbial communities present in the process (Lee, 1989; Lee, 1997; Khieokhachee et al., 1997). A well-known microbial community involved in fermented food products is lactic acid bacteria (LAB). Their primary role is to convert fermentable carbohydrates contained in the raw material to organic acids (Orillo and Pedersson, 1968; Adams, 1986; Saisithi et al., 1986; Leisner, 1992; Olympia et al., 1992; Lee, 1993; Tanasupawat and Komagata, 1995; Ostergaard et al., 1998). The organic acids, mainly lactic and acetic acids, are produced by homofermentative and heterofermentative LAB found in fermented foods (Tanasupawat et al., 1993). Lactic acid is produced by both of these fermentation processes, whereas acetic acid is mainly produced as a by-product of the latter process only and therefore lactic acid generally predominates (Lee, 1989; Lee, 1997). These acids are considered to be effective anti-microbial agents for preservation of perishable fishes and impart distinctive organoleptic properties to the product (Adams and Hall, 1988; Lee et al., 1993; Lee et al., 1994). They have been detected in a variety of fermented fish products (Lee, 1989; Lee, 1993; Lee et al., 1993). Lactic acid and acetic acid contributing to the product's total acidity were quantified from *plaa-som* in this study. The amount of lactic acid and acetic acid produced also correlated to total acidity, which increased gradually reaching a peak on day 3 (Figures 3 and 4). The quantity of lactic acid predominated over acetic acid as expected in a fermented food resulting from LAB fermentation. Thus the HPLC analysis to determine the amount of organic acids produced during fermentation was conducted around the total acidity peak period from days 0 to 5. Lactic acid and acetic acid were found in significant amounts. Butyric and propionic acids, however, were not detected. This result closely conforms with other studies on fermented meat products showing significant amounts of lactic and acetic acids and very small amounts of butyric and propionic acids (Smitinont et al., 2000). The acid production suggests a relationship to pH (Adams and Hall, 1988). The pH measured from this *plaa-som* was found in the range 5.24 to 6.47 which is slightly higher than the pH of other fermented fish products such as *sikhae*, the Korean fermented fish product, and *som-fak*, the other type of Thai fermented fish product made from minced fish cake. The pH of *sikhae* is in the range of 4.4 to 6.6 (Lee, 1989; Um and Lee, 1996) and the pH of *som-fak* is below 5.0 (Saisithi et al., 1986).

This may be due to the variation of the fish species and recipes used in the selected samples in this study. However, the pH range is still close to that of other fermented fish products.

Conclusions

Microbial population and chemical changes during the fermentation of *plaa-som* have been investigated and found to correlate with the expected pattern. The results have provided useful information on microbial populations, pH, type of organic acids, acidity, and LAB isolates. Monitoring of microbiological and chemical changes during fermentation in a *plaa-som* production process can be compared with the values indicated in this study to serve as product quality indices to indicate process success or failure. Further analysis will enable critical control points to be established which will lead to higher safety standard practices.

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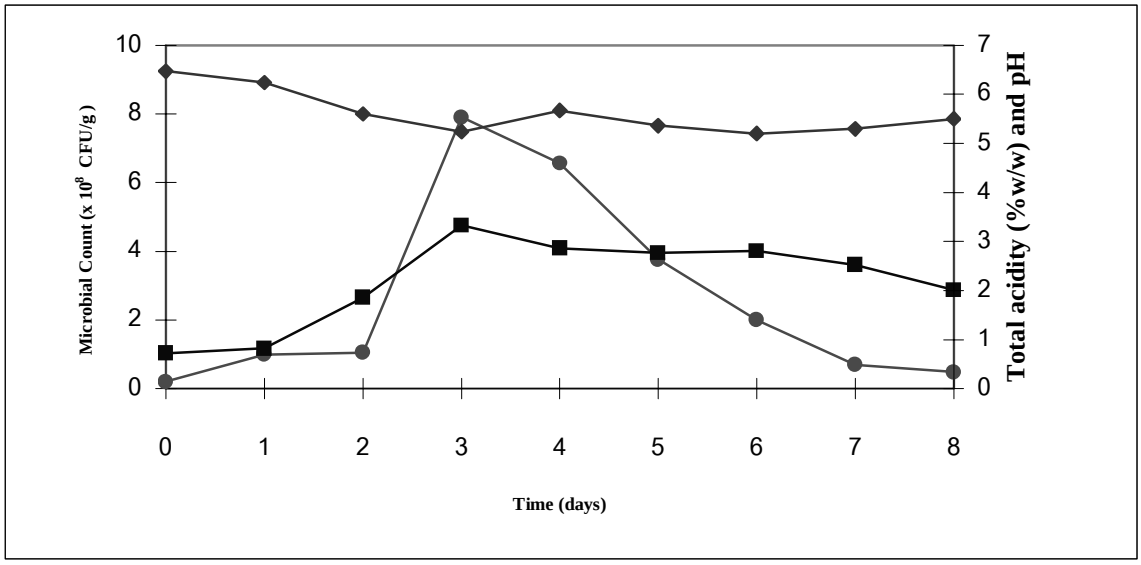

Figure 1 Acid producing bacteria count (●), pH (◆), and total acidity (■) of *plaa-som* measured during fermentation.

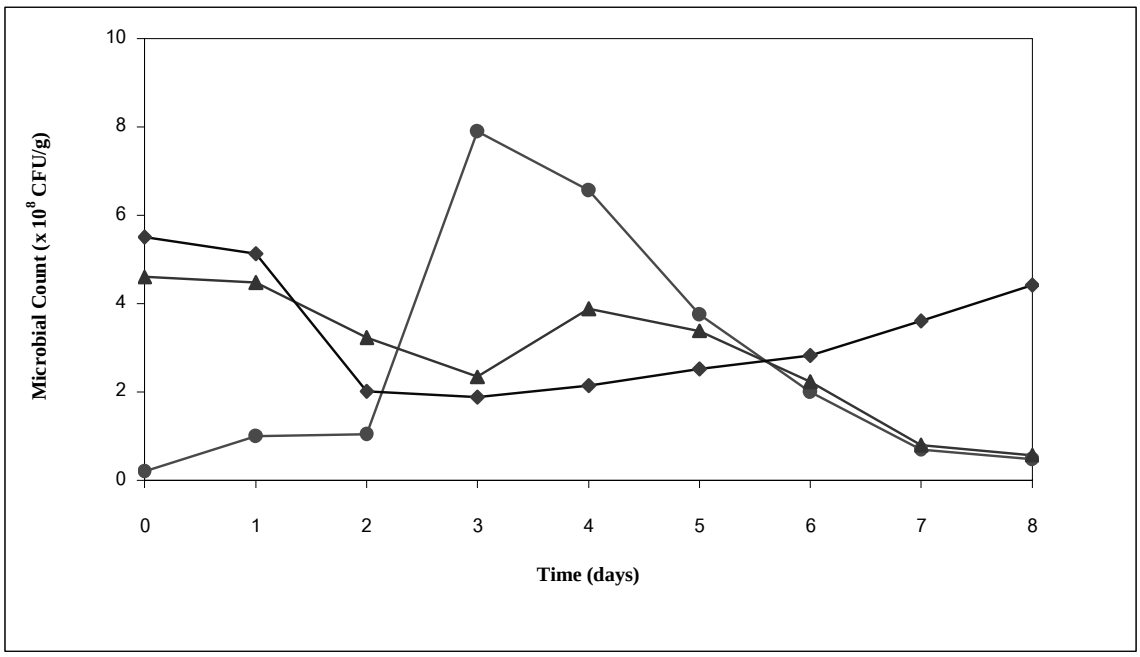

Figure 2 Changes and relationships between total bacteria counts incubated aerobically (◆) and microaerobically(▲) on PCA and the acid producing bacteria count incubated microaerobically on MRS-$CaCO_3$ agar (●).

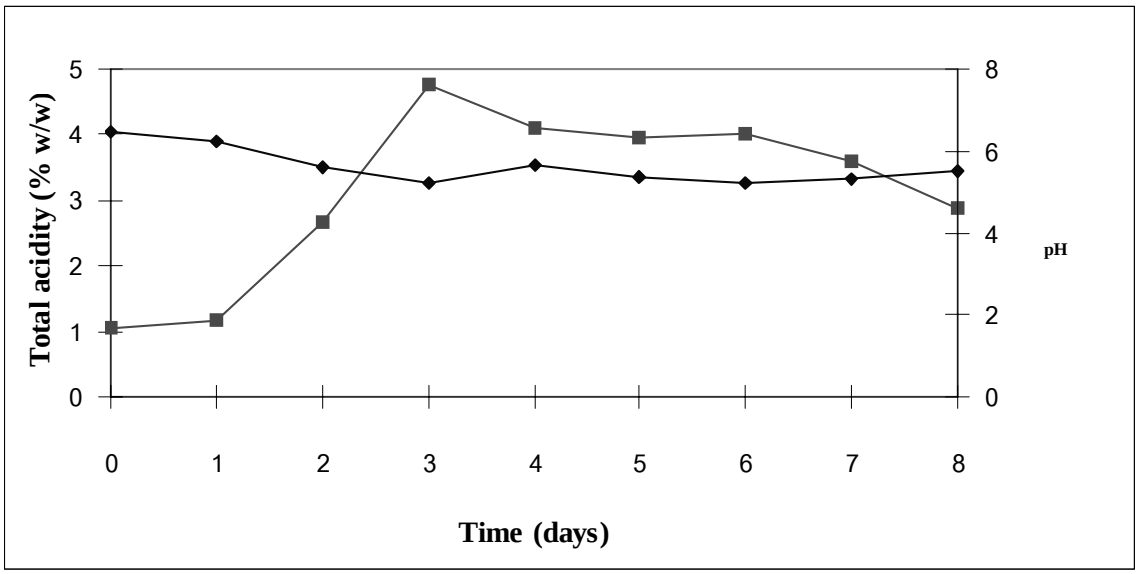

Figure 3 Relationship between total acidity (■) and pH (◆) during fermentation of *plaa-som*.

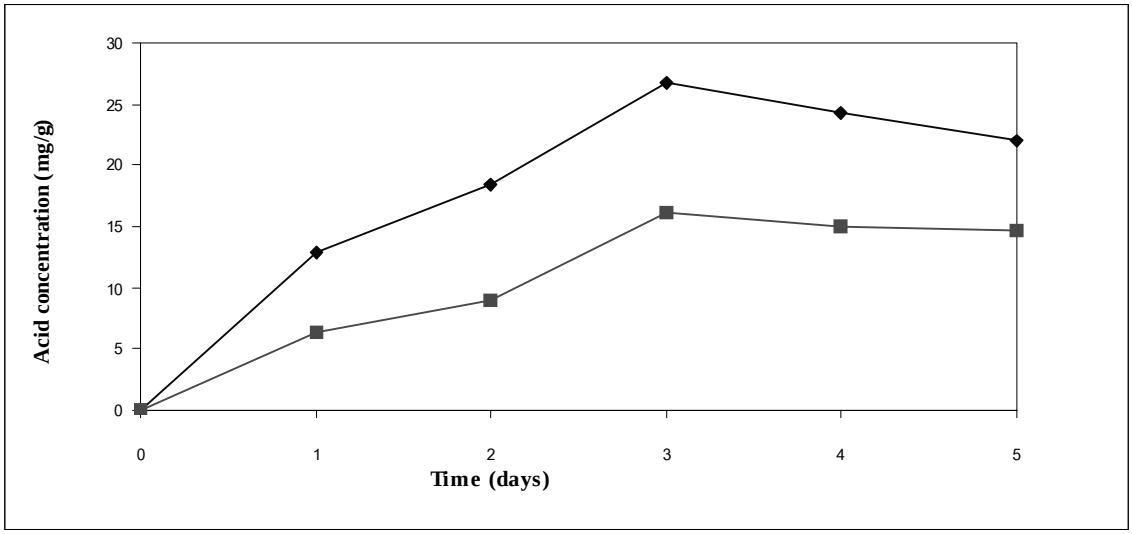

Figure 4 The concentration of lactic acid (◆) and acetic acid (■) found in *plaa-som* during fermentation.