MICROBIOLOGICAL CHARACTERISTICS OF BUDU, AN INDIGENOUS FERMENTED FISH SAUCE OF MALAYSIA

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Abstract

The study aimed to elucidate the microbial changes during fermentation of budu and to screen the isolates for some technological properties. Changes in microbial groups such as halophilic, proteolytic, Lactic Acid Bacteria (LAB), yeasts and enterobacteriaceae were monitored during the traditional fermentation process. The isolates were then phenotypically identified by Biolog Microlog Database System and the strains were further screened for their technological properties such as hydrolytic and probiotic activities. The initial microbial load of the fish substrate was 5.13 ± 0.01 Log CFU/g before decreased gradually to 3.20 ± 0.02 Log CFU/g after 12 months of fermentation. Similar trend was observed for both halophilic and proteolytic count throughout the budu fermentation. Micrococcus sp. was the predominant bacteria group to initiate the fermentation before replace by Staphylococcus arlettae that survived throughout the budu fermentation. Halophilic bacteria especially Micrococcus luteus BML1 and Staphylococcus xylosus exhibited good proteolytic and lipolytic activity compared to other bacterial strains. However, most of the identified strains showed weak amylolytic and pectinolytic activity. The results underlined the importance of the strains to further hydrolyze the budu during fermentation. Only Lactobacillus plantarum, L. plantarum BLP1, Candida glabrata and Saccharomyces cereviaseae strains were tolerant to acidic condition at pH 2.0 and survived 1.0 % bile salt. On the other hand, the L. plantarum, L. plantarum BLP1, Staph. arlettae, Staph. xylosus, Staph. cohnii, Staph. carnosus, Sacc. cereviaseae, Can. glabrata and C. parasilopsis strains exhibited antibacterial effect against Listeria monocytogenes L55, Salmonella tyhimurium S1000, Staphylococcus aureus S-277 and Escherichia coli O157:H7. The results demonstrate that budu fermentation is mixed cultures fermentation involving functional strains in hydrolyzing fish protein into solubilised liquid and hence, future study should be emphasized on the selection of technologically important strains as starter cultures for controllable budu fermentation process.

Introduction

Fish sauce is usually characterized by its unique flavour and aroma after a long period of fermentation. Thailand is the largest fish sauce exporter in the world with an annual export of more than USD $ 13 million to the US, Japan and Australia (Brillantes, 1999). This fermented product comes with many different names such as *nampla* (Thailand), *bakasang* (Indonesia), *yu-lu* (China), *patis* (Philippines), *ngapi* (Burma), *shottshuru* (Japan), *colombo-lumre* (India and Pakistan), *aekjeot* (Korea) and *budu* (Malaysia). The production of fish sauce is a time consuming process which relys on adventitious microorganisms to initiate the fermentation process. It is normally produced under backyard conditions without the use of starter cultures and the success of the process depends on the experience and skill of the producer rather than based on scientific and technological means. During fermentation, microbial succession occurred and resulted with complete solubilization of the fish protein into free amino acids and peptides for the development of its unique characteristic. Many different species of microorganisms are isolated from fish sauce produced in various regions including *nampla (*Tanasupawat and Komagata, 1995*), shottshuru* (Mura *et al*. 2000*)* and *bakasang* (Ijong and Ohta 1996).

The understanding on the types of dominant microorganisms involve in fish sauce fermentation is vital. According to Lopetcharat *et al*. (2001), dominant species of microorganisms that have been isolated in fish sauce usually are those produce proteolytic enzymes and tolerate to high salt content, such as *Bacillus*, *Pseudomonas*, *Micrococcus*, *Staphylococcus*, *Halobacterium* and *Halococcus sp*. In another study done by Lopetcharat and Park (2002), they found that *Staphylococcus*, *Bacillus* and *Micrococcus* were the predominant bacteria involved in fish sauce made from pacific whiting (*Merluccius productus*). Similar study by Fukami *et al*. (2004a) showed that *Staphylococcus xylosus* was the dominant strains that played found in fish sauce made from frigate mackerel. Besides, Anihouvi et al. (2007) also determined that *Bacillus* and *Staphylococcus* sp was the dominant microflora that play important role in the *Ianhouin* (fermented cassava fish) production.

The complexity of the fish sauce fermentation challenges scientists to study the traditional process with the aim to improve the fish sauce quality by a controllable fermentation process. Fukami et al. (2004b) found that the genotypic characterization through the DNA-DNA hybridization analysis of *Staphylococcus nepalensis* actually can improve the unpleasant odor of fish sauce. However, Jiang et al. (2007) claimed that yu-lu (Chinese fish sauce) is a mixed cultures fermentation and the process favors the growth of halotolerant and halophiles only. The fermentation process seems to increase the total soluble nitrogen, Trichloraacetic acid (TCA) soluble peptides and free amino acids that eventually improve the nutritional value of the product. Lipid oxidation that occurred during fermentation may cause bad taste and aroma to fish sauce that made from sardines (Kilinic *et al*., 2005). To a recent extend, Yongsawatdigul *et al*. (2007) even pointed out that the *nam-pla* (Thailand fish sauce) fermentation can be accelerated by using proteinases and bacterial starter cultures.

Budu, one of the Malaysian indigenous fermented food which is cloudy in color due to the sediment of fish bones and hydrolyzed fish meat and is popular in the east coast of peninsular Malaysia (Kelantan, Terengganu and Pahang). It is commonly consumed as condiment or flavouring agent in certain dishes. The production of budu is done by adding salt to anchovies (*Stolephorus* spp.) at the ratio of fish to salt 3:1 (w/w) and naturally ferment for at least 6 months and above. Current constrains that faced by budu industry include limited production scale with a long fermentation time which is not economically benefits the producer and limited technology or skill transferred which cause low quality control to the product. Therefore, this study is undertaken to elucidate the microbial and chemical changes during spontaneous budu fermentation as well as to screen for beneficial and functional properties of the isolates. The outcomes of this study may serve as preliminary selection criteria for developing a starter culture technology which may eventually solve the above problems.

MATERIALS AND METHODS

Collection of samples

The samples were obtained from a budu processing factory located in Tumpat, Kelantan, Malaysia. Fresh anchovies (30kg) were loaded to the mixing concrete base and 90kg of salt were mixed evenly with the fish. An approximately of 20kg of fresh anchovies mixtures were obtained from different sampling spots and transferred to sterilized porcelain pots or “tajau” and allow fermentation for 12 months at room temperature. Samples of budu were collected from the porcelain pots aseptically for the microbiological based on monthly basis.

Isolation, enumeration and identification of microorganisms

The total plate counts were determined using the standard plate count agar (Merck, Germany) with the incubation for 48 hours at 37°C. The determination of total halophilic counts were determined using the plate count agar supplemented with 10 % NaCl, and left incubated for 2-14 days at 37°C. Lactic acid bacteria (LAB) were isolated on MRS agar (HiMedia, India) plates supplemented with 0.01% cyclohexamide (Sigma, UK) and incubated under anaerobic condition at 30°C for 48-72 hours; yeasts and moulds were isolated on yeast extract-malt extract agar (HiMedia, India) and potato dextrose agar (Merck, Germany) respectively and incubated at 25°C for 72 hours; determination of total enterobacteriaceae count were done by spreading the inoculums (0.1ml) on Violet Red Bile agar (VRBA) (Oxoid, UK) and incubated at 37°C for 24 hour; Total proteolytic count were done by cultivated the inoculums (0.1ml) on nutrient agar (Merck, Germany) with 10 % NaCl (w/v), supplemented with 1% casein hydrolysate (w/v) (Merck, Germany) and incubated at 37°C for 48 hours (Tanasupawat *et al*. 1992).

Microbiological data were transformed into logarithms of the number of colony forming units (CFU/g). Colonies were selected from respective plates at the highest dilutions and the purity of the isolates were checked by streaking to freshly prepared agar plates of the isolation media, followed by microscopic examinations. Purified strains of isolates were preserved in nutrient broth using 15% (v/v) glycerol at -18°C prior to further identification.

Technological characteristics of Isolates

Screening for hydrolytic activity

The isolates were tested for their proteolytic, lipolytic, amylolytic (Harrigan and McCance, 1976) and pectinolytic (Speck, 1984) activity. Proteolytic activity was determined by using skim milk agar containing g/l distilled water: 100, skim milk and 20, nutrient agar (Merck). The plates were inoculated with single streaks, incubated at 30°C (bacteria) and 28°C (yeast) for 3 days and examined for clear zone around the colony. Lipolytic activity was studied by using fat agar which consists of nutrient agar (Merck) and sterile olive oil. The isolates were inoculated on the plates and incubated at 30°C (bacteria) and 28°C (yeast) for 3 days. The plate with a halo zone around the colony was further examined by flooding saturated cupric sulphate solution for 1 h. Apart from that, the purified bacteria and yeast cultures were streaked on nutrient agar (Merck) containing 2% of soluble starch for amylase production. The inoculated plates were incubated at 25-30°C for 5-7 days and then flooded with iodine solution. Production of amylase is indicated by the existence of a clear zone around the colonies, while the rest of the plate stains blue-black. The determination of pectinolytic activity of the isolates was done by streaking the colonies on the buffered solution agar (1.8% agar, 0.1M sodium acetate buffer, pH5.0) containing 1% pectin. The plates were then incubated at 37°C for 18h before flooded with 5N hydrochloric acid. The presence of pectinolytic activity is indicated by the opalescent halo zone surrounding the colony.

Screening for probiotic properties

The isolates were also tested for the pH, bile tolerance and antimicrobial activity in order to screen for their probiotic availabilities. Overnight cultures of LAB, bacteria and yeast were inoculated (1% w/v) respectively into MRS broth (MRSB), nutrient broth (NB) and yeast extract glucose peptone broth (YEGPB) tubes with pH adjusted at 1.5, 2.0, 3.0 and 5.0 (with 3N and N/10 HCl). The tubes were incubated at 37° and the grow was determined by visible turbidity. The tolerance of bile salt tests was done by inoculating an overnight culture (1% w/v) into MRSB, NB and YEGPB tubes which containing 0.1, 0.2, 0.3, 0.5 and 1.0 % of bile salts (Oxoid, UK) and incubated at 37°C for 48h, the grow of the cultures were determined by visible turbidity.

The agar spot method as described by Uhlman *et al*. (1992) with minor modifications was used to screen for antimicrobial activity of the isolates against selected indicator strains: *Salmonella typhimurium S1000*, *Listeria monocytogenes L55*, *Staphylococcus aureus S277* and *E-coli. O-157:H7.* Overnight cultures were grown in MRSB (LAB), NB (bacteria) and YEGPB (yeasts) at 37°C. The cultures were subsequently centrifuged at 2400 x g for 15 min, the supernatants were then neutralized with sterile 5M NaOH and then boiled for 5 min to inactivate residual viable cells. A sterilized paper disc (6mm) was immersed in this cell-free neutralized supernatants and imprint them on the agar's surface which containing selected indicator strains before incubated for 24h and the inhibition zone was determined.

RESULTS AND DISCUSSION

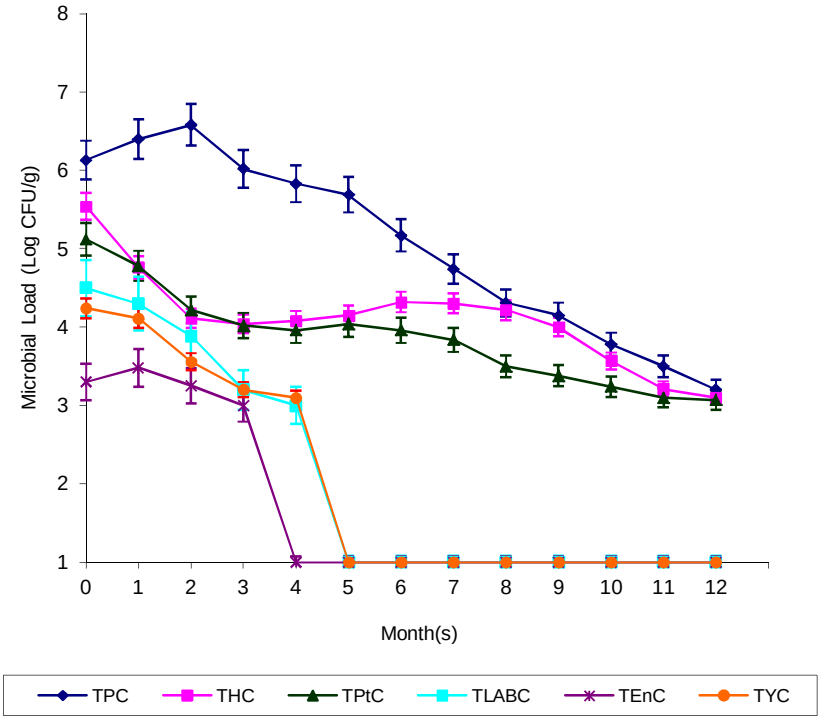
An increase of the microbial load for the total plate count (TPC) was observed from the initial 6.13 ± 0.15 Log CFU/g to 6.42 ± 0.10 Log CFU/g after 3 month of fermentation (Figure 1). It was then decreased significantly ($p<0.05$) to 3.20 ± 0.02 Log CFU/g after 12 months of fermentation. The increase in the TPC at the initial stage of fermentation could be due to the bacterial and autolytic spoilage that occurred once the anchovy's mixture was prepared while the reduction in count was because of the inhibition of natural flora by the high salt content (20-25% NaCl) in the budu mixture. The initial load for the halophilic count was 5.54 ± 0.10 Log CFU/g, the number was decreased subsequently, however it showed a minor increase from the 3rd (4.04±0.05 Log CFU/g) to 6th (4.32 ± 0.10 Log CFU/g) months of fermentation before the count started to decrease and reached 3.1±0.08 Log CFU/g at the 12th months of fermentation. This phenomenon occurs since most of the non-halophilic bacteria are unable to grow well in high salt medium (15-20% NaCl) at the early stage of fermentation and extreme condition at the later stage of fermentation allowed halophilic bacteria to flourish. The total Lactic Acid Bacteria count (TLABC) showed a steady reduction of growth before it was undetectable after 4 months of fermentation. Enterobacteriaceae count were very low (3.31 ± 0.10 Log CFU/g) at the initial stage, they increased at the first month of fermentation (3.48 ±0.05 Log CFU/g) but their number slowly decreased till to undetectable level after that. On the other hands, spoilage microorganisms such as moulds were not detected throughout the fermentation of budu.

A total of 134 isolates were found during budu fermentation (12 months), Gram-positive bacteria was the major group of microorganisms found in budu fermentation, consisted of 86.56% (116 isolates) followed by the yeast, 10.45% (14 isolates). Gram-negative bacteria were the least as they only consisted of 2.99% (4 isolates) which found at the early stage of fermentation. All strains were evenly distributed at the initial fermentation process, but only *Micrococcus* and *Staphylococci spp* were almost persisted up to the latter stage of fermentation (12 months). At the initial stage of fermentation, *Micrococcus luteus* and *M.luteus BML1* were predominant at the first six months of fermentation as they were believed to play fundamental role in microbial degradation of the fish mixture. However, they were reduced gradually after 6 months of fermentation and being replaced by *Staphylococci* due to microbial succession. It was observed that *Staphylococcus arlettae* and *S.arlettae BSA1* were dominant at the latter stage (6-12 months) of fermentation process, as strains of *S.cohnii*, *S.cohnii BSAC1*, *S. carnosus* and *S.carnosus BSCs1* strains were not dominant enough to overcome the *S.arlettae* strains (Table 1). All the *S. cohnii*, *S. cohnii BSC1*, *S. carnosus* and *S. carnosus BSCs1* strains were reduced progressively to undetectable limit after 8 months of fermentation. This indicated that they were not competent enough as compared to *S. arlettae* strains during the latter stage of budu fermentation. In this study, LAB was not frequently identified compared to other salt tolerant bacteria. Most of them only present at the initial stage of fermentation, however attention was given to *Lactobacillus plantarum* and *L.plantarum BLP1* as these strains tolerate well at the first few months of fermentation before

die off due to the bacteriostatic effect of high salt content in budu. Most of the LAB strains found in this study were not able to survive at the latter stage of fermentation as the environment was so selective for them to grow. Furthermore, the lack of nutrients especially carbohydrates in the fish mixture also discouraged them to proliferate.

All the *Micrococcus* and *Staphylococci* strains showed good proteolytic and lipolytic activity, compared to other tested strains. These two types of microorganisms played important role during fermentation as they might secrete exoenzymes (proteases or lipases) that can solubilized the fish mixture into desire attributes. In general, LAB will have weak proteolytic activity as the proteolytic system in most LAB might have wide range of peptidases, however in this study, LAB strains (except *Lactococcus lactis ssp lactis 1a* and *Pediococcus acidilactici*) showed moderate proteolytic and lipolytic activity. Only *L.plantarum*, *L.plantarum BLP1*, *Lactobacillus delbrueckii ssp delbrueckii*, *Saccharomyces cereviaseae*, *C. parasilopsis* and *C.fermata* had shown moderate to weak amylolytic capabilities compared to other strains tested. Until present, there's no report on the amylolytic producing bacteria or yeasts found in fermented fish or meat products. The amylolytic capabilities of the strains indicated that they might play minor role in the early stage of fermentation to break down carbohydrate (polysaccharide) into simple sugar in the presence of amylases, which were subsequently utilized by other fermenting microorganisms. In the contrary, all strains did not showed pectinolytic activity. This may due to the absence of possible pectinolytic enzymes especially depolymerases in the microorganisms tested.

Only *Lactobacillus plantarum, L.plantarum BLP1*, *Saccharomyces cereviseae* and *Candida glabrata* showed potential probiotic activity compared to other tested strains (Table 1). All of them were tolerant to low pH (pH1.5-2.0), bile salt (1.0%) and exhibited antimicrobial effect against selected food borne pathogens such as *Listeria monocytogenes L55*, *Staphylococcus aureus S-277*, *Salmonella typhimurium S1000* and *Escherichia coliO157:H7.* On the other hand, *Staphylococci* were sensitive to low pH condition (pH 1.5-2.0) even they were tolerance to bile salt (1.0%) and showed antimicrobial effect against the food borne pathogens. The information on survival ability of *Staphylococcus* in acidic condition is limited as more attention is given on their survival in salty environments as required by meat fermentation and fermented fish products. However, they were tolerance to bile salt (1.0%) because they were able to deconjugate the bile acid in the presence of bile salt hydrolase enzymes.



TPC- Total plate count, THC- Total halophilic count,TPtC- Total Proteolytic count, TLABC- Total LAB countTYC-Total Yeast Count, TMC- Total Mould Count

Figure 1: Microbial loads of budu during fermentation

Table 1: Screening on potential probiotic properties of tested strains

Strains tested	Growth at different temperature (°C)			pH tolerance[a]				Bile tolerance[a] (%)				Antimicrobial activity (inhibition zone, mm)[b]			
	25	37	42	1.5	2.0	3.0	5.0	0.1	0.3	0.5	1.0	*LM*	*SA*	*ST*	*EC*
Micrococcus luteus	+	+	-	-	-	-	-	-	-	-	-	11	10	8	7
M.luteus BML1	-	+	-	-	-	-	-	-	-	-	-	10	9	8	7
M.luteus (ATCC 9341)	+	+	-	-	-	-	-	-	-	-	-	10	11	8	x
Staphylococcus arlettae	+	-	-	-	-	-	+	+	+	+	+	16	13	12	9
S.arlattae BSA1	-	+	-	-	-	-	+	+	+	+	+	13	10	10	8
S.xylosus	+	-	-	-	-	-	-	+	+	+	+	15	14	12	11
S.cohnii	-	+	-	-	-	-	+	+	+	+	-	12	11	9	8
S.cohnii.BSCn1	+	-	-	-	-	-	+	+	+	-	-	11	9	8	7
S.carnosus	-	+	-	-	-	-	-	+	+	+	+	17	16	10	8
S.carnosus. BSCs1	-	+	-	-	-	-	-	+	+	-	-	14	11	9	7
Lactobacillus plantarum	+	+	+	-	+	+	+	+	+	+	+	12	11	8	9
L.plantarum. BLP1	-	+	+	+	+	+	+	+	+	+	+	11	9	10	8
L.delbrueckii ssp. delbrueckii	-	+	-	-	-	+	-	+	+	-	-	11	12	9	x
Lactococcus lactis ssp lactis 1	-	+	-	-	-	+	-	+	+	-	-	11	10	x	x
Pediococcus pentosaceus	+	+	-	-	-	+	-	+	-	-	-	12	10	x	9
P.acidilactici	+	+	-	-	-	+	-	+	-	-	-	x	9	x	x
Corynebacterium	-	+	-	-	-	-	-	-	-	-	-	x	x	x	x
Saccharomyces cereviaseae	+	+	-	-	+	+	+	+	+	+	+	14	12	11	10
Candida glabrata	+	+	-	-	+	+	-	+	+	+	+	12	10	8	9
C.parasilopsis	+	-	-	-	-	-	-	+	+	-	-	10	11	7	5
C.fermata	+	-	-	-	-	-	+	+	+	+	-	x	x	x	x

a- Growth on nutrient broth (bacteria), MRS broth (LAB), YEGPB(yeast) with respective acid (HCl) and bile salt concentrations

b- Inhibition zone formed after on Tryptic Soy Agar (TSA) plate after incubated for 24h.

+: Resistant strains, - : Sensitive strains

LM-Listeria monocytogenes L55, SA- *Staphylococcus aureus S-277*; ST-*Salmonella typhimurium S1000*; EC-*Escherichia coli O157:H7*

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