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CHEMICAL COMPOSITION AND MICROBIAL DYNAMICS OF *BUDU* FERMENTATION, A TRADITIONAL MALAYSIAN FISH SAUCE

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The chemical and microbiological changes during spontaneous *budu* fermentation were elucidated on monthly basis (1–12 months). A significant increase ($P<0.05$) in pH, acidity, soluble protein, total protein, and moisture content was observed during *budu* fermentation, except for the fat content. The total microbial load decreased gradually from the initial of 6.13±0.01 to 3.45±0.13 log CFU g^{-1} after 12 months of fermentation. Overall, 150 isolates were identified, with a majority of bacteria (77%), followed by yeasts (12%) and 11% of unconfirmed species. *Micrococcus luteus* was the predominant strain that initiated the fermentation before it was replaced by *Staphylococcus arlettae* that exists throughout the fermentation. This study confirmed that lactic acid bacteria and yeasts often coexist with other microorganisms, even though a microbiological succession usually takes place both between and within species, which shaped the chemical and sensory characteristics of the final product. In addition, some of the isolates could be potentially valuable as starter cultures for further improved and controllable *budu* fermentation.

Keywords: *budu* fermentation, fish sauce, microbial succession, diversity, spontaneous fermentation

Fish sauce is a clear brown liquid seasoning produced via fermentation of heavily salted fish materials in most of the Southeast Asia countries. It is widely used as salt replacer or flavour enhancer in most prepared foods and sauces of Japanese and western markets, resulting from a balance of amino acids and high quantities of peptides (Fukami et al., 2004). However, due to its distinctive odour, the application is restricted to home cooked foods and prepared foods. It has various names depending on the country from which it is produced, such as *nampla* (Thailand), *bakasang* (Indonesia), *yu-lu* (China), *patis* (Philippines), *ngapi* (Burma), *shotshuru* (Japan), *aekjeot* (Korea), and *budu* (Malaysia). Many studies related to fish sauce fermentation and products were conducted, including the reduction of histamine in fish sauce using rice bran *nuka* (Kuda & Miyawaki, 2010); controlled fermentation of chum salmon sauce using starter cultures (Yoshikawa et al., 2010), and the production of *plaa-som* (Thai light fermented fish product) using lactic acid bacteria (LAB) starters (Saitong et al., 2010).

Fish proteins are gradually hydrolysed by both bacterial and fish proteinases during fermentation. This process relies on natural fortuitous microorganisms with strong degradation and hydrolysis capabilities, including *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, and halophilic lactic acid bacteria (Thongthai et al., 1992). These bacteria could play a significant role in the bioconversion of fish substrate into fish sauce with delighted colour, flavour, and aroma. However, the inconsistent product quality and extremely long fermentation time are among the factors that limit the growth of fish sauce industries. Many attempts have been made

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to improve the fermentation process, such as using selected starter cultures to accelerate the process (Alkolkar et al., 2010), reducing the formation of biogenic amines (Zaman et al., 2010), and improving the odour and flavour of the fish sauce (Udomsil et al., 2010).

Budu is a Malaysian indigenous fermented fish sauce that appears as a viscous brown liquid with grey colloidal fish separated from the indigestible suspended fish bones. It has a salty taste with intense odour and is commonly consumed as condiment or flavouring agent in dishes within the east coast of Peninsular Malaysia (Kelantan, Terengganu, and Pahang). The *budu* is processed traditionally by adding salt to the raw anchovies (*Stolephorus* spp.) at the ratio of fish to salt 2:1 or 3:1 and allow the fish substrate to ferment at ambient temperature (30–40 °C) for 6–12 months in large concrete tanks. During fermentation, a water-soluble protein rich *budu* is formed, due to the action of digestive and microbial enzymes.

Budu fermentation is regarded as indigenous process, which involves less technological input and requires long fermentation period to ensure the solubilisation of fish mixture. Furthermore, no control measure is applied during this spontaneous process, which usually associated with inconsistency of product quality and low production yield. Studies on indigenous fish sauces from various Asian countries have been carried out as an effort to elucidate the spontaneous process, which eventually led to product improvement and higher acceptability. However, limited scientific information is available on the microbial community of *budu* fermentation. Therefore, this study aimed to characterise the microbial diversity and chemical composition of *budu* during spontaneous fermentation, and the isolated microbial strains could be applied as potential starter cultures for a controllable fermentation.

1. Materials and methods

1.1. Sample preparation

The freshly prepared anchovy mixtures (fish to salt ratio of 3:1) were obtained from a *budu* processing factory located in Tumpat, Kelantan, Malaysia. The samples (approximately 30 kg) were transferred back to the Food Microbiology Research Laboratory, School of Food Science and Nutrition, and kept under similar fermentation conditions as at the producer. Samples were kept in sterilised porcelain pots (20 l) sheltered under zinc roof to allow fermentation (1–12 months). The fish mixture (approximately 100 g) was withdrawn aseptically from the pots for monthly microbiological and chemical analyses. All analyses were carried out in triplicate.

1.2. Enumeration and isolation of microorganisms during fermentation

Initially, 10 g of sample was transferred aseptically to a stomacher bag containing 90 ml of 0.1% (w/v) buffered peptone solution having 10% (w/v) NaCl and homogenised vigorously. Homogenates were serially diluted and inoculated (0.1–1.0 ml) to the respective agar plates. Microorganisms were isolated and enumerated using six different culture mediums: plate count agar (Merck, Germany) was incubated for 48 hours at 37 °C for the total viable counts; plate count agar supplemented with 10% NaCl and incubated for 2–14 days at 37 °C for halotolerant counts (Baross, 2001); MRS agar (Merck, Germany) containing 0.01% cyclohexamide (Sigma, UK) and incubated under anaerobic condition at 30 °C for 48–72 h for total lactic acid bacteria count; yeast extract-malt extract agar (HiMedia, India) was used for total yeast count; nutrient agar (Merck, Germany) supplemented with 10% NaCl (w/v) and 1% casein hydrolysate (w/v) for proteolytic count (Tanasupawat et al., 1992).

The microbiological data were transformed into logarithms of the number of colony forming units (CFU g^{-1}). Colonies were selected randomly from respective plates and were purified by streaking to freshly prepared agar plates, followed by microscopic examinations. Purified strains of isolates were maintained in nutrient broth using 15% (v/v) glycerol at –18 °C prior to further identification.

1.3. Biochemical characterization and identification of isolates

Five isolates from each typical colony with distinct morphological characteristics were selected and purified by streaking on selected agar in order to obtain pure cultures prior to the identification process. The isolates were then characterized based on biochemical and physiological characteristics. Eventually, a further characterization and identification of the isolates were performed based on the sugar utilization profiles via API Web Identification Software (Version 1.10, BioMerieux) before subjected to confirmation at species level by comparing with the established biochemical fingerprints using Biolog Microlog Microstation (Biolog, Hayward, CA).

1.4. Physicochemical and proximate analysis

1.4.1. Determination of total soluble solids, pH, salt content and soluble protein content. Approximately 1 ml of liquid fish sauce was placed evenly on the surface of a hand refractometer (Atago, Japan) and read against light. The total soluble solids were recorded in °Brix. The pH was measured with an auto cal pH meter on 5 g of *budu* sample homogenized with 5 ml of deionised water. The titratable acid of the sample was determined by multiplying the volume of alkali (ml) used for the neutralization of acids with the factor 0.09. The salt (NaCl) content was determined by the Volhard method, method number 953.43 (A.O.A.C., 2000), while the total soluble protein content was determined based on the Lowry method using bovine serum albumin (Sigma, Germany) as standard.

1.4.2. Determination of nutrient composition. The proximate compositions (moisture, ash, total nitrogen, fat, and crude fibre) of *budu* were determined according to A.O.A.C. (2000), method number 985.25.

1.5. Statistical analysis

The data were collected in triplicate and the results were displayed as the mean values and standard deviations. The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago). Cluster analysis was performed to observe the relationship (based on phenotypic characteristics) between the isolated species. The associations obtained were based on the Euclidian distances using Ward algorithm and dendrogram was then constructed to determine the cluster structure of the species involved.

2. Results and discussion

2.1. Microbiological changes during fermentation

The *budu* fermentation was characterised by an increase of the total plate count (TPC) from the initial 6.13±0.15 to 6.42±0.10 log CFU g^{-1} after 3 months of fermentation. However, it decreased significantly ($P<0.05$) to 3.20±0.02 log CFU g^{-1} after 12 months (Fig. 1). The

increase in the TPC at the early stage of fermentation could be due to the bacterial and autolytic spoilage once the anchovy's mixture was prepared. The reduction in TPC at the late fermentation (12 months) was associated with the inhibition of natural flora by the high salt content (20–25% NaCl). The result was in accordance to the finding of ANIHOUVI and co-workers (2007) who found that the aerobic mesophilic count of the *lanhouin* (Benin fermented cassava fish) decreased gradually, since less halophilic bacteria were eliminated throughout the fermentation, giving way for a salt tolerant group of microorganisms to become dominant.

The halophilic and proteolytic bacteria are the key microorganisms in most fermentations that degrade protein in nature. The growth of these bacteria groups was inconsistent as a decreasing trend was observed at the first 5 months of fermentation before a slight increase to 4.32±0.10 and 3.96±0.18 log CFU g^{-1}, respectively, at the 6th month of fermentation. This phenomenon could be explained by the proliferation of halophilic microorganisms once they are adapted to the fermentation environment. Similar result was reported by DISSARAPHONG and co-workers (2006) who discovered the total viable, proteolytic, and halophilic bacteria count of the fish sauce made from tuna viscera decreased in the extended fermentation period. The lactic acid bacteria (LAB) count exhibited a steady decreasing trend until the 5th month of fermentation and thereafter, it was undetectable at the latter stage of fermentation. According to PALUDAN-MULLER and co-workers (2002), the growth of the non-salt tolerant LAB species, such as *Lactobacillus*, *Pediococcus,* and *Lactococcus,* was easily inhibited by the presence of salt content greater than 7%.

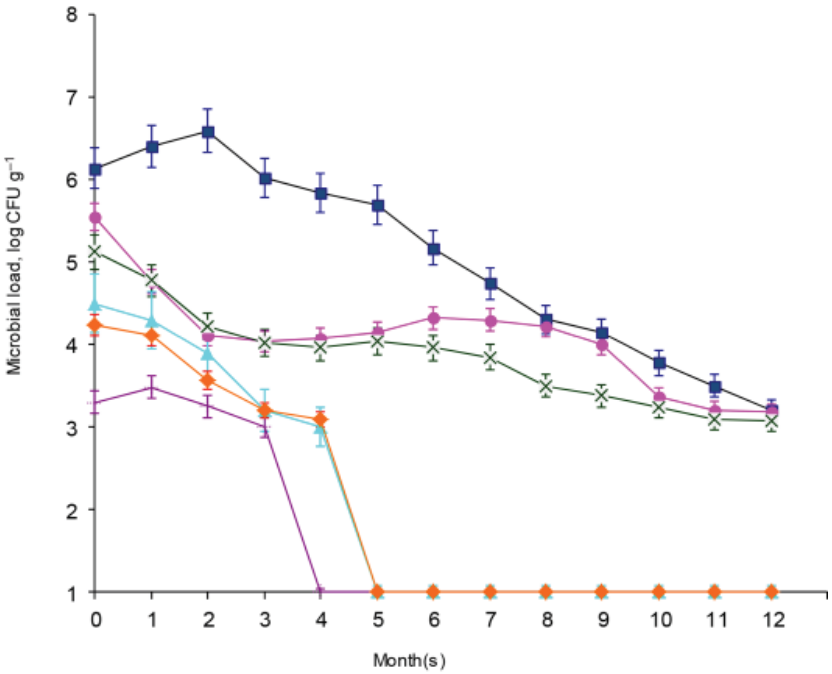

Fig. 1. Microbial changes during budu fermentation (■) TPC: Total plate count; (●) THC: Total halophilic count; (×) TPtC: Total proteolytic count; (▲) TLABC: Total lactic acid bacteria count; (○) TEnC: Total Enterobacteriacea count; (♦) TYC: Total yeast count

2.2. Phenotypic characterization and microbial succession in budu fermentation

A total of 150 isolates were identified in *budu* fermentation and the highest number of isolates corresponded to *Micrococcus* species (28.7% of total isolates), followed by *Staphylococcus* (26.7%), *Pediococcus* (9.4%), *Candida* (8.0%), *Lactobacillus* (6.7%), *Saccharomyces* (4.0%), and *Lactococcus* (2.7%). The species identified in this study are shown in Table 1 and their similarity index was at least over 0.5 based on Biolog identification databases (Biolog, Inc.). The isolates were then further classified and grouped into strains level using cluster analysis. For instance, LAB showed broad phenotypic diversity (10 clusters) although they were not the dominant species (Fig. 2). The cluster 7 (PA1, PA2, PA3) and cluster 9 (PP1, PP3) are the biggest LAB group as they outnumbered the other LAB strains found in this study. Nevertheless, the two clusters were distinguished by the ability to grow at different temperatures an in brine solution, arginine utilization, fermentation profiles on different sugar substrates, and hydrolytic properties. It was noted that cluster 7 (PA1, PA3), cluster 8 (PA2), cluster 9 (PP1, PP3), and cluster 10 (PP2) were biochemically identified as *Pediococcus acidilactici* and *P. pentosaceus*, which could not be differentiated at the rescaled distance of 5.

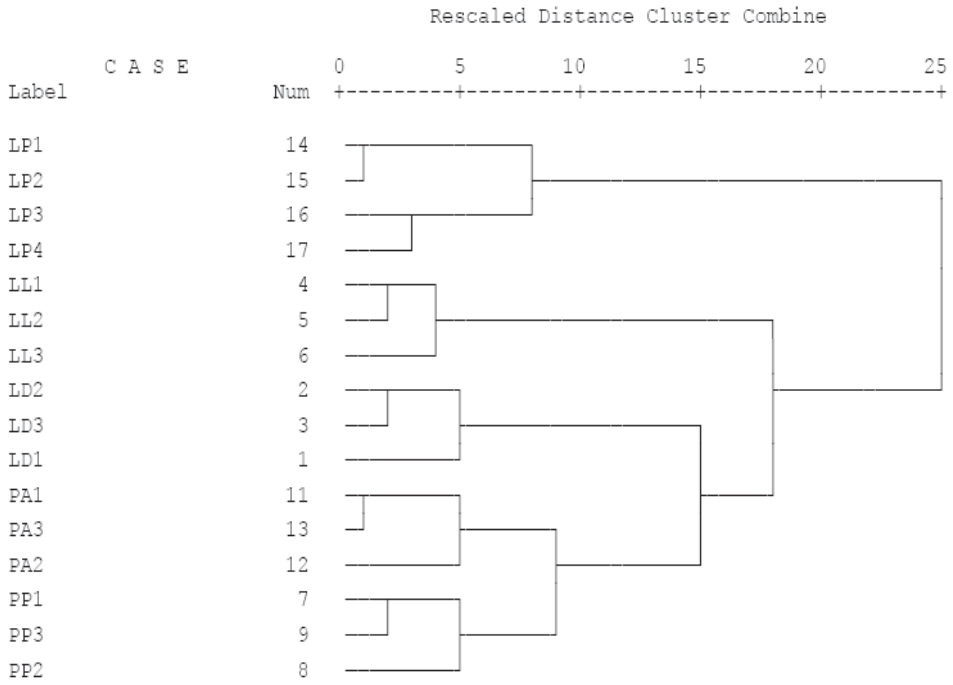

Fig. 2. Cluster analysis on the phenotypic characteristics of the LAB strains isolated from *budu* fermentation. The vertical line represents the bacteria strains and horizontal lines represent distances

LP 1 : *Lactobacillus plantarum* 1
LP 2 : *L. plantarum* 2
LP 3 : *L. plantarum* 3
LP 4 : *L. plantarum* 4
LL 1 : *Lactococcus lactis subs. lactis* 1
LL 2 : *Lc. lactis* subs. *lactis* 2
LL 3 : *Lc. lactis* subs. *lactis* 3
LD 1 : *Lactobacillus delbrueckii* 1
LD 2 : L. *delbrueckii* 2
LD 3 : L. *delbrueckii* 3
PA 1 : *Pediococcus acidilactici* 1
PA 2 : P. *acidilactici* 2
PP 1 : *P. pentosaceus* 1
PP 2 : *P. pentosaceus* 2
PP 3 : *P. pentosaceus*

Table 1. Distribution of microbial strains during *budu* fermentation

Species	Fermentation time (months)							Total isolates
	0	2	4	6	8	10	12	
Micrococcus spp.								43
Micrococcus luteus	4(16.0)[a]	4(16.0)	3(12.0)	2(8.0)	1(4.0)	NA	NA	25
M. luteus ATCC9341	3(16.7)	3(16.7)	2(11.1)	1(5.5)	NA	NA	NA	18
Staphylococcus spp.								40
Staphylococcus arlettae	2(14.3)	NA	1(7.2)	NA	3(21.4)	1(7.2)	1(7.2)	14
S. cohnii	4(33.3)	1(8.3)	1(8.3)	1(8.3)	1(8.3)	NA	NA	12
S. carnosus	2(28.3)	1(14.3)	1(14.3)	NA	NA	NA	NA	7
S. xylosus	1(14.3)	2(28.6)	1(14.3)	NA	NA	NA	NA	7
Lactobacillus spp.								10
Lactobacillus plantarum	2(33.3)	1(16.7)	2(33.3)	NA	NA	NA	NA	6
L. delbrueckii sp. *delbrueckii*	2(50.0)	1(25.0)	NA	NA	NA	NA	NA	4
Pediococcus spp.								14
Pediococcus pentosaceus	3(42.9)	2(28.6)	NA	NA	NA	NA	NA	7
P. acidilactici	2(28.6)	2(28.6)	NA	NA	NA	NA	NA	7
Lactococcus lactis ssp *lactis*1	2(50.0)	1(25.0)	NA	NA	NA	NA	NA	4
Corynebacterium afermentans ss. *lipophilum*	1(25.0)	1(25.0)	NA	NA	NA	NA	NA	4
Rahnella aquatilis	1(50.0)	NA	NA	NA	NA	NA	NA	2
Enterobacter agglomerans bgp7	1(50.0)	NA	NA	NA	NA	NA	NA	2
Saccharomyces cerevisiae	1(16.7)	1(16.7)	1(16.7)	NA	NA	NA	NA	6
Candida spp.								12
Candida famata	1(16.7)	1(16.7)	NA	1(16.7)	NA	NA	NA	6
C. parasilopsis	1(33.3)	1(33.3)	NA	NA	NA	NA	NA	3
C. glabrata	1(33.3)	1(33.3)	NA	NA	NA	NA	NA	3
Unidentified								13
Unidentified A	3(27.3)	2(18.2)	NA	NA	NA	NA	NA	11
Unidentified B	NA	NA	NA	NA	NA	NA	NA	2
Total	37	25	12	5	5	1	1	150

[a]: Figure without bracket indicates the number of strains; figure in bracket indicates the % of prevalence of the strains in that particular period of time; NA: Not available

The microbial dynamics of *budu* fermentation could be divided into the initial stage (0–6 months) with a diverse microflora and the latter stage (7–10 months) with less microbial diversity (Table 1). Both *Micrococcus luteus* and *M. luteus* ATCC 9341 pre-dominated the early stage of fermentation by facilitating microbial degradation on the fish mixture before

further hydrolysing the matrix into smaller components. This allowed the generation of amino acids that promoted other microorganisms to grow during fermentation or become the precursor for microbial metabolisms. Nevertheless, the predominant *M. luteus* strains gradually decreased during 6 months of fermentation, giving way to *Staphylococcus arlettae* that dominated at the latter stage of fermentation (7–10 months). The result is in accordance with the findings of Anihouvi and co-workers (2007), that the dominant organisms isolated from *lanhouin* (fermented cassava fish) were salt tolerant *Bacillus* and *Staphylococcus* spp. The presence of *Staphylococci* at the latter fermentation is vital as they might contribute to the sensory attributes of the *budu,* especially magnifying the typical meaty and ammonical flavour via complex biochemical reactions.

As one of the minority group, only 5 species of LAB (*Lactobacillus plantarum*, *L. delbrueckii* sp *delbrueckii*, *Pediococcus pentosaceus*, *P. acidilactici,* and *Lactococcus lactis* ssp *lactis 1*) and 4 species of yeasts (*Saccharomyces cerevisiae*, *Candida parasilopsis*, *Candida famata,* and *Candida glabrata*) were identified during *budu* fermentation. The study indicated that most of the LAB species grew well at the first few months of fermentation, owing to the richness in fermentable carbohydrate or protein that favoured their growth. On the other hand, *S. cerevisiae* and *C. farmata* were the only species that survived until middle stage (6th months) of the fermentation as compared to other yeasts found in this study. Yeasts are commonly responsible for the production of alcohol, organic acids, and other flavour compounds, which determine the desired organoleptic characteristics of the fermented product.

2.3. Physicochemical properties and proximate composition

2.3.1 Total soluble solids and pH. The initial total soluble solid (TSS) content was 31.50±0.50 before the significant increase ($P<0.05$) to 39.82±0.15 °Brix at the 12th month of fermentation (Table 2). This phenomenon could be due to the presence of free amino acids and small peptides that were released from hydrolysed fish protein. The pH for the fish mixture increased gradually to 6.72±0.12 during fermentation. The phenomenon was due to the formation of basic nitrogenous substances following putrefaction of the fish tissues in the fermentation.

2.3.2 Total protein, soluble protein, and fat content. The protein and soluble protein contents are the major parameters used to grade the quality of fish sauce and similar fermented fish products. For instance, the fish sauce in Thailand is rated by the total nitrogen content and the colour. Good quality fish sauce should have total nitrogen above 20 g l^{-1} (2.0%), while the grade 2 is between 15 and 20 g l^{-1} (1.5–2.0%) (Lopetcharat et al., 2001). The protein content (%) was in the range of 9.9–18.8% throughout *budu* fermentation (Table 2). The early microbial hydrolysis of the freshly prepared fish mixtures triggered the rapid release of protein nitrogen or non-protein nitrogen compounds (free amino acid, nucleotide, peptide, ammonia, urea, and trimethylamine oxide) that contributed to the increase of the protein content. The fat content of the fish sauce decreased significantly ($P<0.05$) from the initial 2.44±0.14 to 0.57±0.12% after fermentation (Table 2). This could be due to the fish enzymes (lipases) as well as the microbial exoenzymes that might decompose the triglyceride into free fatty acids during fermentation, which is associated with the flavour and characteristics of the product. Similar result was found by Kilinic and co-workers (2006).

Table 2. Chemical and proximate composition of *budu* during fermentation

Time (month)	Proximate						Chemical				
	MC (%)	Protein (%)	Ash (%)	Fat (%)	Fibre (%)	CHO (%)	TSS	pH	Acidity	Sol. protein (mg ml^{-1})	Salt (%)
0	46.10^{fg}±0.15	9.89^{u}±0.15	20.08^{f}±0.04	2.44^{ab}±0.14	2.20^{ab}±0.10	19.27^{d}±0.10	31.50^{r}±0.50	5.87st±0.03	0.62^{cd}±0.13	11.16^{v}±0.28	23.13^{b}±0.05
2	41.19^{ij}±0.53	12.88^{n}±0.07	19.77^{h}±0.03	20.46^{e}±0.10	1.82^{c}±0.08	22.09^{b}±0.26	36.82^{p}±0.02	5.46^{rs}±0.04	0.55^{efg}±0.05	18.75^{t}±0.07	23.53^{a}±0.26
4	47.96^{fg}±0.42	11.46^{r}±0.06	22.16^{b}±0.16	1.36^{ijk}±0.20	0.84^{e}±0.17	16.22^{e}±0.11	34.70^{o}±0.10	5.65^{no}±0.3	0.53^{gh}±0.10	27.86^{k}±0.02	22.66^{c}±0.12
6	59.88^{f}±0.18	12.74^{n}±0.05	23.56^{c}±0.16	1.03^{mn}±0.13	0.25^{g}±0.15	0.73^{g}±0.15	40.23^{gh}±0.06	6.15^{ij}±0.07	0.37^{mn}±0.16	32.54^{hij}±0.15	20.17^{n}±0.15
8	67.46^{d}±0.58	18.81^{a}±0.30	15.34^{p}±0.02	0.87^{op}±0.02	0.05^{hij}±0.03	0	43.22^{a}±0.04	6.40^{ef}±0.02	0.47^{ij}±0.15	39.96^{de}±0.13	19.67^{op}±0.04
10	70.53^{bc}±0.60	18.16^{b}±0.16	15.21^{pq}±0.15	0.83^{op}±0.16	0.02^{ij}±0.02	0	41.19^{d}±0.14	6.54^{bc}±0.01	0.38^{mn}±0.17	40.73^{b}±0.16	19.37^{p}±0.03
12	73.57^{a}±0.52	17.66^{d}±0.10	12.14^{u}±0.20	0.57st±0.12	0	0	39.82^{h}±0.15	6.72^{a}±0.12	0.35^{no}±0.12	39.89^{cd}±0.18	19.35^{p}±0.13

MC: Moisture content (%); TSS: Total soluble solid; Sol. protein: soluble protein (mg ml^{-1}); CHO: carbohydrate (%); means followed by different letters in the same column indicate a significant difference ($P<0.05$)

3. Conclusions

In conclusion, *budu* fermentation was driven by the conversion of fish mixture into liquids due to microbial degradation. The microbial action hydrolyses the fish protein to small peptides as well as free amino acids, which led to the increase of total soluble peptide content during fermentation. This microbial action eventually attributed to the unique sensory characteristics of the *budu*. *Micrococcus luteus* and *Staphylococcus arlettae* were the dominant strains that might possess desirable technological properties that assist in *budu* fermentation. Further studies are necessary to evaluate the feasibility of the dominant strains that possessed desirable technological properties in order to be applied as starter cultures for controllable *budu* fermentation.

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