ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS ASSOCIATED WITH *BUDU* FERMENTATION

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I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS INVOLVED IN *BUDU* FERMENTATION

This study was carried out to determine the chemical and microbiological changes during *budu* fermentation (A1, A2 and A3 located in Tumpat, Kelantan). Samples from different producers were allowed to ferment at room temperature similar to that applied by producers. Samples were taken on monthly basis for the chemical (pH, acidity, total soluble solid, salt and soluble protein content), proximate compositions (moisture, crude fat, crude protein and fibre content) and microbiological analyses. Changes in microbial flora such as halophilic, proteolytic, lactic acid bacteria (LAB), yeasts and enterobacteriaceae were monitored and all the isolates were phenotypically identified by Biolog Microlog Database System before further characterized for hydrolytic (pectinolytic, lipolytic, proteolytic and amylolytic), enzymatic activity and probiotic properties. The moisture, protein and ash content of all samples were increased, while the fat content decreased significantly ($p<0.05$) during fermentation. Sample from producer A2 (Orkid) recorded the highest increase in total soluble protein, from the initial of 10.92±0.30 to 40.72±0.02 mg/ml after 12 months of fermentation. However, sample of producer A3 (Roslee) exhibited greatest decrease in fat content compared to other samples. The initial microbial load for all samples decreased significantly ($p<0.05$) during fermentation. The total proteolytic count for producer A1 (Ketereh) and A2 (Orkid) increased in the first few months before decrease at the end of fermentation. A total of 150 isolates were identified, with majority are bacteria (77%), followed by yeasts (12%) and 11% of unconfirmed identity. Only two species of *Micrococcus*, namely *Micrococcus luteus* and *Micrococcus luteus* ATCC 9341, while four species of *Staphylococcus* were identified as *Staphylococcus arlettae*, *Staphylococcus cohnii*, *Staphylococcus carnosus* and *Staphylococcus xylosus* were identified. *Saccharomyces cereviseae* and *Candida famata* were the major yeast species in all *budu* samples. The *M. luteus* ML2 was predominant strain to initiate *budu* fermentation before *Staphylococcus arlettae* SA strain took over the role. API ZYM test revealed that the *S. arlettae* SA1 and *L. plantarum* LP1 and LP2 were only strains to have strong lipolytic and proteolytic activity that associated with *budu* fermentation. However, none of the tested strains showed pectinolytic activity. *Lactobacillus plantarum* LP1 and LP2, *Staphylococcus arlettae* SA, *Saccharomyces cereviseae* SC3, *Candida glabrata* CG2 strains were identified as potential probiotics as they were tolerant to acid and bile salt as well as exhibited antimicrobial activity against selected foodborne pathogens. In conclusion, a consortium of microorganisms was involved in *budu* fermentation and led to the changes of sensory attributes of the *budu* during fermentation. Further studies are necessary to evaluate the feasibility of the selected strains as starter cultures in pilot processing for controllable *budu* fermentation.

ABSTRAK

Kajian ini dibuat bagi mengkaji perubahan kimia dan mikrobiologi semasa berlangsungnya fermentasi budu. Sampel budu dari pengusaha A1 (Ketereh), A2 (Orkid) dan A3 (Roslee) terletak di sekitar daerah Tumpat, Kelantan telah diperam dalam suhu persekitaran seumpama dengan tempat pemprosesan asal. Analisis bulanan terhadap kandungan kimia (pH, keasidan, jumlah bahan terlarut, garam dan protin terlarut), prokslmat (kelembapan, lemak, protln dan serat) serta mikrobiologi bagi sampel berlainan pengusaha telah dilakukan sepanjang fermentasi budu. Perubahan flora mikroorganisma jenis halofilik, proteolitik, lactik asid bakteria, kulat dan enterobakteria telah dikaji sepanjang fermentasi. Mikroorganisma yang terpencil telah dikenalpasti berdasarkan sistem pangkalan data Biolog Microlog dan pencirian lanjutan terhadap mikroflora tersebut turut dilakukan berdasarkan ciri-ciri hidrolitik (pektinolitik, lipolitik, proteolitik, amylolitik), aktiviti enzim serta ciri-ciri probiotik. Kandungan kelembapan, protin dan abu bagi semua sampel telah meningkat secara signifikan ($p<0.05$), manakala kandungan lemak mencatat penurunan signifikan ($p<0.05$) di sepanjang fermentasi budu. Sampel A2 mencatat peningkatan tertinggi bagi kandungan protin terlarut berbandingkan sampel yang lain, iaitu dari 10.92±0.30 kepada 40.72±0.02 mg/ml selepas 12 bulan, manakala sampel A3 mencatat penurunan kandungan lemak yang terbanyak. Jumlah kiraan mikroorganisma bagi sampel budu dari semua pengusaha menurun secara mendadak ($p<0.05$) sepanjang fermentasi. Namun, jumlah kiraan proteolitik bagi sampel dari pengusaha A1 dan A2 meningkat pada awal fermentasi sebelum ia menurun secara mendadak pada akhir fermentasi. Sejumlah 150 pencilan telah dikenalpasti dan majoritinya adalah bakteria (77%), diikuti oleh kulat (12%) dan sebanyak 11% isolat gagal dikenalpasti. Hanya dua spesis Micrococcus (Micrococcus luteus, Micrococcus luteus ATCC 9341) dan empat spesis Staphylococcus (Staphylococcus arlettae, Staphylococcus cohnii, Staphylococcus carnosus dan Staphylococcus xylosus) *telah dikenalpasti dalam kajian ini. Sehubungan itu, Saccharomyces cerevisiae dan Candida famata merupakan spesis yis yang kerap muncul dalam semua sampel yang dikaji. Keputusan juga menunjukkan bahawa* Micrococcus luteus ML2 *merupakan strain yang memulakan proses fermentasi sebelum diganti oleh* Staphylococcus arlettae SA *bagi melanjutkan fermentasi budu pada peringkat akhir. Keputusan API ZYM menunjukkan bahawa* S. arlettae SA *dan* L. plantarum LP1 *dan* LP2 *mempunyaiaktiviti lipolitik dan proteolitik yang kuat lalu memainkan peranan dalam fermentasi budu. Namun begitu, tiada sebarang strain yang menunjukkan aktiviti pektinolitik. Cuma* Lactobacillus plantarum LP1, LP2, Staphylococcus arlettae SA, Saccharomyces cerevisiae SC3 *dan* Candida glabrata CG2 *berpotensi sebagai strain probiotik memandangkan mereka adalah* rintang *kepada asid, garam hempedu serta menunjukkan aktiviti antimikrobial terhadap beberapa patogen bawaan makanan yang terpilih dalam kajian ini. Kesimpulannya, mikroorganisma yang dikenalpasti dalam kajian ini terlibat dalam penguraian budu yang membawa kepada perubahan sepanjang fermentasi berlangsung.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Food fermentation is one of the oldest forms of food biotechnology which provide 20-40% of our food supply. Typically, about one third of our food intakes are comprised of fermented foods such as yoghurts, cheese, yoghurt drinks, soy and fish sauce, soymilk, wines, breads, fermented sausages or related meat products and fermented soy bean products. Fermented foods that derived from plant or animal materials are usually acceptable and become essential part of the diet in most parts of the world. This is because a wide variety of raw materials are used and the technology of producing it basically ranging from the primitive to the most advanced in order to achieve an outstanding range of sensory and textural qualities of the final products (Paul-Ross *et al.*, 2002).

In some developing countries, food fermentation is still carried out as household or cottage industry level without any application of modern and scientific principle. The process normally initiated spontaneously and thus, retains the unique flavour and aroma characteristics of the end products compared to those fermented foods produced by controlled fermentation. However, the microbiological and biochemical aspects of this traditional process are complicated and not fully elucidated. In general, the process is carried out by means of minimum technological know-how with a low yields, and with variable quality, as in the production of *Hussuwa*, a traditional African fermented sorghum food (Yousif *et al.*, 2010). Furthermore, the poor hygienic conditions and improper handling during fermentation render the products susceptible to contamination. The inadequate knowledge on proper packaging or post fermentation treatments denies the fate of end product quality and eventually limits their acceptance by consumers. On contrary, controlled fermentation involving the use of starter cultures is already becoming a trend for most fermentation processes. It is believed that a large production scale can be met through the use of starter

cultures. Hence, the safety and quality control of fermented products are also well promised (Holzapfel, 1997).

The main reason for food fermentation can be ascribed to preservation. In addition, the nutritional properties of the fermented products can be improved through fermentation. In most developing countries, malnutrition become a major problems as the raw materials are so limited (Paredes-Lopez and Harry, 1988). Therefore, people from those regions depend on fermented vegetal starches or fermented root crops such as cassava to sustain their daily diets. Fermentation of a substrate have been diversified to the production of secondary metabolites such as biocatalysts (Yang *et al.*, 2005), starter cultures (Hugas and Monfort, 1997; Papamaloni *et al.*, 2002) and potential pharmaceutical metabolites (Aymerich *et al.*, 2000; Messi *et al.*, 2001). This indicates that the understanding of the fermentation process not only confined to the production of fermented products, but it also significantly contributes to the scientific knowledge and economic of human.

Indigenous fermented foods in Malaysia are mostly produced in small and backyard scales. The know-how in producing fermented foods is delivered from generation to generation within the locality and only small quantity of the product is distributed beyond a geographical area. Microbiological and biochemical changes during fermentation are partially understood, therefore the end products will always have low product yields with variable quality (Rolle and Satin, 2002). The processes is normally resulting from the competitive activities of a variety of contaminating microorganisms and those best adapted to the food substrate and to technical control parameters, eventually dominate the process (Holzapfel, 2002). This shows that there is a possibility the local producers may use the back-slopping technique to produce fermented foods. However, the fermentation is still confined to small-scale production due to inadequate financial supports as well as scientific understanding of the process.

Fish sauce is one of the fermented products that prepared by adding certain amount of salt to the fish species and allowed to ferment for certain periods of time. The final product will be in solubilized liquid forms due to

hydrolysis that aided by microorganisms. During fermentation, microbial succession encourages 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, 2001), shotturu (Mura *et al.* 2000) and bakasang (Ijong and Ohta 1996). An understanding on the types of dominant microorganisms involved in fish fermentation is vital. According to Lopetcharat *et al.* (2001), the 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 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*).

The complexity of the fish sauce fermentation is a challenge to scientists who interested to study the traditional process with the aim to improve the fish sauce quality through controlled fermentation process. Fukami *et al.* (2004b) found that the genotypic characterized through the DNA-DNA hybridization analysis of *Staphylococcus nepalensis* can actually be employed to improve the unpleasant odor of fish sauce. However, Jiang *et al.* (2007) claimed that yu-lu (Chinese fish sauce) is mixed cultures fermentation and the process favors the growth of halotolerant and halophiles only. The fermentation seems to increase the total soluble nitrogen, trichloraacetic acid (TCA) soluble peptides and free amino acids that eventually improve the nutritional value of the fermented product. Lipid oxidation that occurred during fermentation may cause bad taste and aroma to fish sauce that made from sardines (Kilinc *et al.*, 2005). According to 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, is popular in the east coast of peninsular Malaysia (Kelantan, Terengganu and Pahang). The

production of *budu* actually is a time consuming process as it required at least 8-12 months to ensure full solubilization of the fish mixture. There is less technological input in this traditional fermentation process as it is developed through trial and errors and the technique of production may depend on skill of producers. Thus, this uncontrolled process usually ends up with a small production scale and low quality products compared to fermented fishery products from neighboring countries like Thailand (nam-pla) and Vietnam (Nuoc-mam). In order to overcome inconsistent in the *budu* quality, the use of starter cultures derived from this spontaneous fermentation is appreciated. Even though *budu* has been consumed by local consumers due to its unique meaty flavours and aroma, but the microbial diversity as well as the chemical changes involved in *budu* fermentation are not well elucidated. Hence, this study is undertaken with the objectives:

1. To elucidate the microbiological and biochemical changes during fermentation.
2. To isolate and identify the microorganisms involved in *budu* fermentation.
3. To characterize the isolated microorganisms based on their biochemical profiles.
4. To screen for enzymatic and probiotic properties of isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 The *Budu* Industry

2.1.1 Small-and Medium Scale Food Processing Enterprises (SMEs)

Small and medium-scale enterprises (SMEs) played an important role in the Malaysian economy for the past few decades. A total of 548,307 or 99.2% were defined as SMEs which can be further classified into service sector based enterprise (86.6%), managing sector (7.2%) and agriculture sector (6.2%). However, statistic released by Department of Statistic, Malaysia (2005) showed that the largest number of establishments of SMEs is the textile and apparel sector which accounts for 23.4% of the total manufacturing sector. This is followed by food and beverages (15.0%), metal and metal products (13.0%) and eventually wood and wood products (14.1%).

There are more than 9000 food processing factories in Malaysia of which 95% are classified as small scale with the annual sale turnover is between RM250, 000 and less than RM10 million with a full time employees between 5 and 50 persons; while medium scale enterprise is the one with the annual sale turnover between RM 10 and RM25 million with a full time employees between 51 and 150 person. According to Chee (1986), small scale food processing enterprises may evolve if the enterprises expand which will eventually lead to the formation of a limited company. Another criteria that distinguish the small scale food enterprises from the large scale enterprises is the organizational structure which operated by a manager-owner assisted by a few workers. The products are generally cheap and rather low quality and the marketing strategy is done directly or through agents.

2.1.2 *Budu* Industry in Malaysia

Budu is one of fermented fishery products which are quite common among the local people in the East Coast of Peninsular Malaysia (Kelantan, Terengganu and Pahang). The production scale is usually very small with a minimum quality control

throughout the process, less technological inputs are implemented and most importantly the method of producing is a heritage from one generation to the next. Hence, the *budu* industry is considered as a small scale processing enterprise due to limited annual sale turnover and products are sold within local markets only.

The *budu* manufacturers in Kelantan are usually operating in small scale production to meet local market demands. This industry is not well expanded due to low consumers' acceptance on the unique flavour and aroma of *budu* as well as its appearances. On the other hand, the emergence of imported clarified fish sauce products from neighboring countries which is more acceptable in sensory attributes or appearance also affect the growth of the local industry. Besides, *budu* industry is also facing several problems, such as inconsistency in price as some of the producer sell their product directly to local customers based on the landing price of anchovies in different seasons, for instance, the price of *budu* during monsoon season is high compared to other non-monsoon seasons. Due to low quality of the end products cause the *budu* incompative with other imported fish sauces. Therefore, a strategic planning for improvement of *budu* production is needed in order to improve demand for the traditional fermented food.

2.2 Fermentation

Fermentation is defined as an energy yielding anaerobic metabolic breakdown of sugar containing compounds such as glucose into smaller compounds and eventually to yield desirable fermentation end products including acid, alcohol (ethanol) and other simple products without net oxidation. Fermentation can also be defined as a process of the bioconversion of organic substances by microorganisms and/or by enzymes of microbial, plant or animal origin into secondary metabolites. Fermentation is able to produce material in a way that would be difficult or very costly if ordinary chemical methods are chosen to synthesis it. For example, the production of enzymes that harvested from microorganisms through a solid state or submerged fermentation technology. Fermentation technology is currently applied in various fields such as food and

animal feed production, production of biopesticides, biofuel, pharmaceuticals and even waste treatments system.

Both submerged (SmF) and solid-state (SSF) fermentation have been applied in many fermentation process such as brewing, bread making industry, enzymes synthesis, biopulping industry, biodegradation of biomass from waste materials and production of pharmaceuticals or functional foods. Submerged (SmF) fermentation actually involves the cultivation of microorganisms in a liquid nutrient broth to produce expected end products, such as the production of enzymes (Ito *et al.*, 2001) and amino acids (Gomes and Kumar, 2005). However, solid-state (SSF) fermentation involving solids in absence (or near absence) of free water, and the substrate used must possess enough moisture to support the growth and metabolism of microorganisms (Pandey *et al.*, 2001). Therefore, in a SSF system, it actually stimulates the growth of microorganisms in nature on moist solids with a low energy requirement, produce lesser waste water and more environmentally friendly as they resolve the problem of solid waste disposal. Thus, there has been an increasing interest on SSF which has been applied in most bioprocesses such as bioleaching, biopulping, bioremediation and biobenefication (Classen *et al.*, 2000: Ogbonna *et al.*, 2001; Han and Rombouts, 2001; Haddadin *et al.*, 2001)

2.2.1 Industrial Fermentation

Industrial fermentation has great impact on human beings as most of the important primary metabolites such as amino acids, nucleotides, vitamins, enzymes, solvents, organic acids and vaccines have been produced through various fermentation processes. Microorganisms are efficiently utilized and optimized for the production of the above metabolites via large-scale industrial fermentation technology (Suryanarayan, 2003; Anderson, 2009). There are some advantages to apply microbial fermentation technique for the above purposes as compared to chemically synthesized process. At first, high rates of metabolism and biosynthesis can be achieved due to the high ratio of surface area to volume which facilitates the uptake of nutrients. Secondly, a tremendous variety of reactions which microorganisms are capable of carrying out also show the importance of their existence in most fermentation process. Then, the ability of the

microorganisms to adapt well in different environments as well as their capability to grow on most inexpensive carbon and nitrogen sources actually lower down the production cost in most fermentation industry. Apart from that, the ability to undergo genetic manipulation on the microorganisms either in vivo or in vitro to increase the production of the required products again show that the feasibility and benefits of manipulating microorganisms in any fermentation processes (Demain, 2000).

Fermentation usually consider as a low cost but high yield biological process because the raw materials such as corn starch, sugar cane waste, molasses or other carbohydrate rich substrates are utilized efficiently by microorganisms in order to produce essential primary and secondary metabolites as compared to chemical synthesis process (Schuster *et al.*, 2002; Rodriguez-Couto, 2008). The application of microorganisms in fermentation industry can also be much appreciated by the fact that even simple molecules such as L-glutamic acid and L-lysine are produced through fermentation rather than by chemical synthesis. Apart from that, most natural products able to undergo fermentation process since they are very complex and contain many centers of asymmetry that will never be made commercially by chemical synthesis. Below are several end products that being produced through industrial fermentation technology.

a. Production of Organic Acids

The production of organic acids seems vital as there is a high demands in food, pharmaceutical, leather, textile and feed stocks industries. The great expanding of food industry also sees the increased demand for the organic acids especially lactic and citric acids as more than 50% lactic acid produced is used to emulsify bakery products (Litchfield, 1996). Then, lactic acid also used as acidulant / flavouring agent as well as inhibitors to bacterial spoilage in processed foods such as candy, breads and bakery products, soft drinks, soups, dairy products, beers and processed eggs. Furthermore lactic acid is also used in cosmetic formulations, oilments and lotions due to their high water retaining capacity.