MICROBIAL DIVERSITY OF *SASAD* – A TRADITIONAL STARTER FOR ALCOHOLIC FERMENTED BEVERAGE IN SABAH

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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ABSTRACT

This study was carried out to isolate and identify the microflora present in *sasad*, a traditional fermentation starter for alcoholic beverages in Sabah. Proximate analysis and physicochemical was done using AOAC method. The microorganisms isolated were identified based on their biochemical and physicochemical profiles. Moisture content in the *sasad* ranged from 13.32% to 15.51%, ash content from 0.71% to 0.81%, protein content from 0.42% to 0.65%, fat content from 0.14% to 0.51%,fiber content from 1.15% to 1.48% and carbohydrate composition from 81.05% to 83.95%. The pH of all the three samples ranged from 4.32 to 5.12 and the total titratable acidity which ranged 0.76% to 1.12%. The total count of aerobic mesophilic microflora ranged from 7.94 log CFU/g to 8.41 log CFU/g, lactic acid bacteria count ranged from 5.65 log CFU/g to 7.53 log CFU/g, yeasts and moulds count ranged from 7.60 log CFU/g to 8.29 log CFU/g and total amylolytic microorganisms ranged from 7.79 log CFU/g to 8.27 log CFU/g. *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Lactobacillus casei*, *Pediococcus pentosaceus*, *Pediococcus acidi lactici* and *Leuconostoc mesenteroides* were the lactic acid bacteria identified. On the other hand, 6 strains of yeasts were identified as *Hanseniaspora valbyensis*, *Saccharomyces bayanus*, *Saccharomyces fibuligera*, *Candida crusei*, *Saccharomyces cerivisiae* and *Saccharomyces unispora*. *Candida spp.* and *Sm. Cerevisiae*. In conclusion, *sasad* from different location and raw materials showed the difference of microflora found in each type of *sasad*.

ABSTRAK

MIKROFLORA DALAM SASAD – PEMULA TRADISIONAL BAGI PENGHASILAN MINUMAN BERALKOHOL DI SABAH

Kajian ini dijalankan untuk memencilkan dan mengenalpasti mikroflora dalam sasad iaitu pemula tradisional bagi penghasilan minuman beralkohol di Sabah. Dalam kajian ini, sebanyak 3 sampel telah diambil untuk menjalankan analisis proksimat, pH, keasidan titrat, menggunakan kaedah AOAC. Kaedah fisikokimia dan biokimia digunakan untuk mengenalpasti spesies mikroflora. Kandungan kelembapan sasad adalah dalam lingkungan 13.32% hingga 15.51%, abu dalam lingkungan 0.71% hingga 0.81%, protein dalam lingkungan 0.42% hingga 0.65%, lemak dalam lingkungan 0.14% hingga 0.51%, serabut kasar 1.15% hingga 1.48% dan kandungan karbohidrat 81.05% hingga 83.95% . pH adalah dalam julat 4.32 dan 5.12 manakala peratus keasidan titrat adalah antara 0.76% hingga 1.12%. Didapati jumlah aerobik mesofilik flora adalah dalam julat 7.94 log CFU/g antara 8.41 log CFU/g, jumlah bakteria asid laktik 5.65 log CFU/g antara 7.53 log CFU/g, jumlah yis dan kulat dalam lingkungan 7.60 log CFU/g antara 8.29 log CFU/g dan jumlah mikroorganisma amilolitik dalam julat 7.79 log CFU/g dan 8.27 log CFU/g. Sebanyak 6 strain bakteria laktik acid telah dikenalpasti sebagai Lactobacillus plantarum, Lactobacillus curvatus, Lactobacillus casei, Pediococcus pentosaceus, Pediococcus acidi lactici and Leuconostoc mesenteroides. Sebanyak 6 strain yis juga telah dikenalpasti Hanseniaspora valbyensis, Saccharomyces bayanus, Saccharomyces fibuligera, Candida crusei, Saccharomyces cerivisiae and Saccharomyces unispora. Candida spp. and Sm. Cerevisiae. Secara kesimpulan, sasad dari kawasan berlainan dan menggunakan bahan berlainan menunjukkan perbezaan dari segi mikroflora.

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CHAPTER 1

INTRODUCTION

Food fermentation is the oldest biotechnology where it is responsible for many properties of fermented foods such as flavour, shelf life, texture and health benefits (Giraffa, 2004). Microorganisms have been consumed for thousands of years in fermented dairy foods, fermented beverages, and traditional oriental foods and sauces (Steinkraus, 1995). Indigenous, also referred to as traditional, fermented foods are those popular products that since early history have formed an integral part of the diet and that can be prepared in the household or in cottage industry using relatively simple techniques and equipment (Aidoo *et al.*, 2005). The knowledge to prepare those foods was transferred from generation to generation, very often with improvements without changing the original consistency, organoleptics, and the nutritive value of the product.

In Oriental countries, microbial inocula, in the form of dry powders or hard balls made from starchy cereals, are used to prepare food and alcohol fermentations (Tsuyosi *et al.*, 2005). These starters which also known as amylolytic starters are used in the form of starchy tablets containing mixed cultures of starch-degrading moulds and fermenting yeasts and they are used for the manufacture of beers, wines and pasty snacks from various kinds of cereals and starchy crops, such as rice, sorghum, millet and cassava (Aidoo, Nout & Sarkar, 2005). For example, *look-pang*, or also known as "Chinese yeast cake" to the Western people, is a Thai term for dry form of fermentation starter. It used for production of traditional fermented products from starchy raw materials, like *kao-mag* (alcoholic sweetened rice), *lao* (rice wine) and *num som sai chu* (vinegar) (Lotong, 1998). These starters may contain fungi, yeasts and/or lactic acid bacteria and are commonly referred by different names in each area; *ragi tapé* in Indonesia, *look-pang* in Thailand, *chin-hueh* in China, *bubod* in the Philippines, *nuruk* in Korea and *murcha* in Himalayan regions (Abe *et al.*, 2004).

In East Malaysia, a similar kind of traditional fermentation starter known as *sasad* made by the Kadazandusun ethnic are also used to produce traditional fermented products from starchy raw material like fermented cassava, *tempe*, and alcoholic beverages like rice wine. Various types of *sasad* can be found in local markets of Sabah and each varies in their application due to the difference in the preparation methods and ingredient used. There are various methods to prepare the fermentation starter and it is commonly prepared as follows; rice flour mixed with grounded spices such as garlic (*Allium sativum*), white pepper (*Piper retrofractum*), black pepper (*Piper nigrum),* red chillies *(Capsicum frutescens),* cinnamon *(Cinamon burmani),* clove (*Syzygium aromaticum)* and ginger (*Zingiber officinale*) are blended together and water is added to the mixture to make a thick dough which is then molded into small circular flat cakes. In the villages the cakes are placed on bamboo trays which are lined with banana leaves and on top covered with cloth. The trays are kept in a wind free place or room for 2-3 days. This is the natural "fermentation" incubator. Then the rather dry cakes are sun dried and turned over several times until they are really dry. This drying process may take about 3-4 days depending on the weather.

Yeasts, bacteria and filamentous fungi all contribute to the microbial ecology of alcoholic beverages production and the chemical composition of these beverages. Studies on the microflora of starters such as Indonesian ragi and Chinese chiu-chu have been reported as early as the end of the 19th century (Aidoo *et al.*, 2005). The principal amylolytic moulds are *Amylomyces rouxii, Rhizopus spp., Mucor spp.* and *Aspergillus spp.* Common yeasts in many starter tablets are *Hansenula spp., Saccharomycopsis fibuligera, Candida spp.* and *Saccaharomyces cerevisiae* (Saono *et al.*, 1996). It is generally held that moulds are the major producers of amylases that degrade starch into fermentable sugars, and then the yeasts convert the sugars to alcohol (Dung *et al.*, 2005). The moulds in the *ragi tapai* are strong amylolytic and degrade mainly the carbohydrate of the rice or glutinous rice into simple sugars which are then further decomposed by the yeasts into alcoholic compounds. Alcoholic production presents such an ecosystem where, in relation to yeasts, there is the potential for yeast–yeast interactions, yeast–filamentous fungi interactions, and yeast–bacteria interactions (Fleet, 2003).

Sasad has wide application on local traditional fermented foods such as starter for *tapai* and traditional alcoholic beverages. It is a dry, round-to-flattened, creamy white to dusty white, solid ball-like starter ranging from 1.9 to 11.8 cm in diameter and from 2.3 to 21.2 g in weight. Each locality has its own way of reproducing starch-based rice wine starter, depending on available ingredients and preferences. Many studies have been done on similar type of starter elsewhere to determine the microflora and the characteristic of these starters (Assanvo *et al.*, 2006; Dung, Rombouts & Nout, 2005; Sujaya *et al.*, 2001; Tsuyoshi *et al.*, 2005).

However, little information is available for the production of a standardised starter. Thus, this study was carried out to determine the microbial diversity of these starters in order to improve the quality and functional properties of the fermented products. It would be a big advantage to producers to have a standardised product quality and improved manufacturing process.

Specific objectives of the studies were:

1. To determine the physico-chemical properties and proximate composition of various type of *sasad*.

2. To enumerate the numbers of different microbial groups present in various *sasad* at various stage of storage.

3. To characterize and identify the microorganisms isolated based on their biochemical and physicochemical profiles.

CHAPTER 2

LITERATURE REVIEW

2.1 Fermented Foods

Fermentation has been used for several thousand years as an effective and low cost means to preserve the quality and safety of foods. It is a process in which microorganisms, in the absence of oxygen, generate energy by oxidizing carbohydrates and related compounds (Josephen & Jespersen, 2004). Animal and plant tissues subjected to the action of microorganisms and/or enzymes to give desirable biochemical changes and significant modification of food quality are referred to as fermented food (Parveen & Hafiz, 2003). Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, improving protein and fiber digestibility, enhancing micronutrient bioavailability, and by degrading anti-nutritional factors. Food fermentation covers a wide range of microbial and enzymatic processing of food and ingredients to achieve desirable characteristics such as prolonged shelf-life, improved safety, attractive flavour, nutritional enrichment, and promotion of health (Giraffa, 2004).

According to Steinkraus (1995), the traditional fermented foods contain high nutritive value and developed a diversity of flavours, aromas, and textures in food substrates. The raw materials traditionally used for fermentation are diverse and include fruits, cereals, honey, vegetables, milk, meat, and fish. There a wide variety of fermented products, and are not limited to wine, beer, vinegar, bread, soy sauce, sauerkraut, *kimchi*, pickled olives, different fermented milk products, a large number of cheeses, and a variety of sausages (Hansen, 2004).

Fermented foods are produced world-wide using various manufacturing techniques, raw materials and microorganisms. However, there are only four main fermentation processes: alcoholic, lactic acid, acetic acid and alkali fermentation (Soni & Sandhu, 1990). Alcohol fermentation results in the production of ethanol, and yeasts are the predominant organisms such as in wines and beers. Lactic acid fermentation like fermented milks and cereals is mainly carried out by lactic acid bacteria. A second group of bacteria of importance in food fermentations are the acetic acid producers from the *Acetobacter* species which convert alcohol to acetic acid in the presence of excess oxygen. Alkali fermentation often takes place during the fermentation of fish and seeds, popularly used as condiment (McKay & Baldwin, 1990).

Indigenous fermented foods constitute a group of foods that are produced in homes, villages and small scale cottage industries which are sold to the rural populace who buy them for food and social ceremonies (Iwuoha & Eke, 1996). Some of these products have undergone industrial development and are now manufactured on a large scale (Aidoo *et al.,* 2005). The fermented foods are derived from substrates like roots, legumes, cereals, oilseeds, nuts, meat, fish, milk, palm tree, sap and so forth. Some of the most commonly available traditional fermented foods include fermented dairy products, fermented meats, fermented soy products, fermented vegetables, fermented cereal foods and beverages as shown in Table 2.1.

2.1.1. Fermented Dairy Products

Fermented dairy products are commonly produced in milk-producing countries and by nomadic people (Nip, 2004). Fermented milks originated in the near-east and spread through central and Eastern Europe. The earliest example of fermented milk was warm, raw milk from cows, sheep, goats, camels or horses of the nomads roaming the area. It can be classified into semisolid and solid cultured dairy products. Fermented semisolid dairy foods have a long history of consumption worldwide. This category of dairy foods, which includes yogurt, sour cream, fromage frais and many variations, continues to show increased sales because of the healthful reputation of these products and their convenience (Patel & Walker, 2004).

Some examples of indigenous fermented dairy products produced includes *dahi*, *kefir* and *roub*. Fermented dairy products containing probiotic lactic acid bacteria and *Bifidobacteria* are currently the best-known examples of functional foods. *Dahi* is the most popular and oldest fermented milk product in the Indian Subcontinent (Yadav, Jain & Sinha, 2006). It is prepared by lactic acid fermentation of buffalo milk, in a similar fashion to yogurt, and is considered as a functional food due to its nutritional and therapeutical values (Yadav *et al.*, 2007).

Table 2.1. Traditional fermented foods and beverages in Asia.

Name of the food	Country	Major ingredients	Functional microflora	Fermentation contribute to:
Pancakes and leavened low-salt bread				
Idli	India, Sri Lanka	Rice and blackgram dal	*LAB, Saccharomyces cerevisiae*	Flavour, texture, nutritional value
Dhokla	India	Rice and Bengalgram	*LAB, Pichia silvicola*	Flavour, texture, nutritional value
Nan, kulcha and bhatura	India, Pakistan, Afghanistan, Iran	Wheat flour	*LAB, Saccharomyces cerevisiae, and other yeasts*	Texture, flavour
Amyloytic fermentation starters				
Ragi	Indonesia	Rice	*Amylomyces rouxii, Hansenula spp., Saccharomycopsis fibuligera*	Starch degradation, alcoholic fermentation
Murcha/marcha	India, Nepal	Rice	*Mucor spp., Rhizopus spp., Pichia burtonii, Saccharomyces cerevisiae*	Starch degradation, alcoholic fermentation
Loog-pang	Thailand	Rice	*Saccharomyces fibuligera, Pichia anomala, and other yeasts*	Starch degradation, alcoholic fermentation
Tane koji	Japan	Rice	*Aspergillus oryzae, Aspergillus usamii*	Starch and protein degrading enzymes for wine making
Men	Vietnam	Rice	*Amylomyces rouxii, Saccharomyces cerevisiae*	Starch degradation, alcoholic fermentation

Table 2.1. Continued.

Name of the food	Country	Major ingredients	Functional microflora	Fermentation contribute to:
Sweet – low alcoholic snacks fermented with amylolytic starters				
Tape ketan, Tape ketella/peujeum	Indonesia	Rice, cassava tubers	*Amylomyces rouxii, Mucor spp., Rhizopus spp., Saccharomycopsis fibuligera, Pichia burtonii, Pichia anomala*	Starch degradation, glucose formation, alcoholic fermentation, flavour
Kao-mark	Thailand	Rice		
Wines brewed with amylolytic starters				
Sake	Japan	Rice	*Aspergillus oryzae, Saccharomyces sake, Hansenula anomala*	Saccharification, alcohol, flavour
Yakju and takju	Korea	Rice, wheat, barley, maize, millet	*Aspergillus oryzae, Aspergillus sojae, Rhizopus spp., Saccharomyces cerivisiae, Hansenula anomala, Hansenula subpellicusa, Candida sake*	Saccharification, alcohol, flavour
Tapuy	The Philippines	Rice	*Saccharomycopsis fibuligera, Rhodoturola glutinis,Debaryomyces hansenii, Candida parapsilosis, Trichosporon fennicum*	Saccharification, alcohol, flavour
Ruou nep than	Vietnam	Purple glutinous rice	*Amylomyces rouxii, Saccharomyces cerevisieae*	Saccharification, alcohol, flavour

Table 2.1. Continued.

Name of the food	Country	Major ingredients	Functional microflora	Fermentation contribute to:
Jnard/jaanr/thumba	Nepal, India, Bhutan	Finger millet/ rice/ maize/ wheat	*Mucor spp., Rhizopus spp., Saccharomycopsis fibuligera, Pichia anomala, Saccharomyces cerevisiae, LAB*	Saccharification, alcohol, flavour
Beverages fermented from sugary juices				
Palm wines (Toddy/ tari, Tuack, Tuba)	India, Bangladesh, Sri Lanka, Thailand, Malaysia, Indonesia the Philippines	Sap of coconut, date of palmyra palm	*LAB, AAB, Saccharomyces cerevisiae, Kodamaea ohmeri and other yeasts*	Alcohol and flavour production
Kombucha/ tea fungus	Japan, Indonesia, China, Russia	Tea liquor, sugar	*AAB, Brettanomyces spp., Zygosaccharomyces kombuchaensis, Saccharomyces spp.*	Organic acids, vitamins, health beverage
Wadi	India, Pakistan	Blackgram dal	*Candida krusei, LAB*	Acidification, leavening, nutritional value
Papad/papadam	India	Blackgram dal	*Candida krusei, Saccharomyces cerevisiae*	Texture, flavour

Table 2.1. Continued.

Name of the food	Country	Major ingredients	Functional microflora	Fermentation contribute to:
Condiments				
Soy sauces	Japan, China	Soybeans and wheat	*Aspergillus oryzae, Aspergillus sojae, Zygosaccharomyces rouxii, Candida spp.*	Enzymic degradation of starch and proteins, formation of flavour
Miso	Japan	Rice and soybeans	*Aspergillus oryzae, Zygosaccharomyces rouxii,* LAB	Flavour

AAB, acetic acid bacteria; LAB, lactic acid bacteria
Source: Aidoo *et al.* (2005)

Kefir is an acid-alcoholic fermented milk, originally made in the Balkans, Eastern Europe and the Caucasus (Fontán *et al.*, 2006). It is a refreshing, naturally carbonated fermented milk beverage with a slightly acidic taste, yeasty flavour and creamy consistency where agitated, the beverage foams and fizzes, a characteristic that led to kefir being named "the champagne of cultured dairy products" (Powell *et al.*, 2007). *Kefir* grains are employed as starters and these grains are white or lightly yellowish irregular masses in which bacteria and yeast are contained in a matrix of proteins and polysaccharides (Bosch *et al.*, 2006). Lactobacilli are present as the largest portion (65–80%) of the microbial population, with lactococci and yeasts making up the remaining portion of the microbes present in the *Kefir* grain (Witthuhn *et al.,* 2005). The population composition may differ if the grains have different origins, or if the grains are cultured using different methods and substrates.

Roub is the most important indigenous fermented milk product of a considerable economic and dietary importance to the people of Sudan especially in the west and east of Sudan (Zuber *et al.,* 2005). The housewife makes *roub* during rainy season to turn as much milk into *roub* each evening (Abdelgadir *et al.,* 1998). *Roub* is fermented in a traditional way on household levels. Milksurplus to the consumption of the family is collected in a container, inoculated with a starter from the fermentation of the previous day, and left to ferment overnight (Abdelgadir *et al.*, 2001).

Dairy products represent a specific environment for the growth and selection of different yeast and bacteria species. As part of a microbial community, together with bacteria, yeasts may contribute to the sensory characteristics of kefyr, koumiss and

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