ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS ASSOCIATED WITH BUDU FERMENTATION

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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2010
DECLARATION

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.

November 2010

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<table>
<thead>
<tr>
<th><strong>NAME</strong></th>
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<tbody>
<tr>
<td><strong>MATRIC NO</strong></td>
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<tr>
<td><strong>TITLE</strong></td>
<td>ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS ASSOCIATED WITH <em>BUDU</em> FERMENTATION</td>
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IJAZAH: SARJANA SAINS (BIOTEKNOLOGI MAKANAN)

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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 amyloytic), 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 cerevisae 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 cerevisae 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), proksimat (kelembapan, lemak, protin 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 secara tritingi dan majoritinya adalah bakteria (77%), diikuti oleh kulat (12%) dan sebanyak 11% isolat gaga/ 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 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

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td>CERTIFICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF APPENDIX</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION

1

## CHAPTER 2: LITERATURE REVIEW

5

2.1 The Budu Industry 5
   2.1.1 Small and Medium Enterprises 5
   2.1.2 Budu Industry in Malaysia 5

2.2 Fermentation 6
   2.2.1 Industrial Fermentation 7
      a. Production of Organic Acid 8
      b. Enzyme Production 9
      c. Production of Bioactive Products 12
      d. Production of Biofuels 18

   2.2.2 Food Fermentation 19
      a. Classification of Fermented Food 23
      b. Traditional Fermented Foods 24
      c. Indigenous Fermented Foods in Malaysia 25

   2.2.3 Fish Sauce 26
<table>
<thead>
<tr>
<th>2.2.4 Budu Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Traditional <em>budu</em> Processing in Malaysia 35</td>
</tr>
<tr>
<td>b. Parameters Affecting Quality of <em>budu</em> 36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2.3 Microbial Interaction in Fermented Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.1 Bacterial and Yeast Interaction 39</td>
</tr>
<tr>
<td>2.3.2 Yeast and Yeast Interaction 41</td>
</tr>
<tr>
<td>2.3.3 Yeast and Filamentous Fungi Interaction 43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2.4 Safety Issues of Fermented Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.1 Presence of Biogenic Amines in Fermented Foods 44</td>
</tr>
<tr>
<td>2.4.2 Presence of Food Poisoning Microorganisms in Fermented Foods 47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2.5 Biotechnology Application in Food Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.1 Biotechnological Importance of Microorganisms 50</td>
</tr>
<tr>
<td>a. Production of Nutraceuticals 51</td>
</tr>
<tr>
<td>b. Production of Food Additives 53</td>
</tr>
<tr>
<td>2.5.2 Development of Starter Culture Technology 56</td>
</tr>
<tr>
<td>a. Spontaneous Fermentation and Back Slopping Technique in Food Fermentation 56</td>
</tr>
<tr>
<td>b. Approaches and Applications of Starter Cultures in Small-Scale Fermentation 58</td>
</tr>
<tr>
<td>c. Selection Criteria for Starter Cultures Development 59</td>
</tr>
<tr>
<td>d. Disadvantages of Using Starter Cultures 61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3: MATERIALS AND METHODS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Sample Preparation 63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3.2 Proximate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 Moisture and Ash Content 64</td>
</tr>
<tr>
<td>3.2.2 Crude Protein Content 64</td>
</tr>
<tr>
<td>3.2.3 Fat Content 65</td>
</tr>
<tr>
<td>3.2.4 Crude Fiber Content 65</td>
</tr>
<tr>
<td>3.2.5 Carbohydrate Content 66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3.3 Other Chemical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1 Total Soluble Solids 66</td>
</tr>
<tr>
<td>3.3.2 pH and Titratable Acid 66</td>
</tr>
<tr>
<td>3.3.3 Salt Content 66</td>
</tr>
<tr>
<td>3.3.4 Total Soluble Protein Content 67</td>
</tr>
</tbody>
</table>
3.4 Microbiological Analysis

3.4.1 Sample Preparation
3.4.2 Enumeration and Isolation
3.4.3 Phenotypic Characterization and Identification of Isolates
   a. Characterization of Non-LAB Microorganisms
   b. Characterization of LAB Microorganisms
   c. Characterization of Yeast
   d. Characterization of Mould

3.5 Identification of Microbial Groups Using the Biolog Microplates (GP2, GN2, AN, YT, FF)

3.6 Technological Characteristics of Isolate

3.6.1 Screening of hydrolytic activity
   a. Proteolytic Activity
   b. Lipolytic Activity
   c. Amylolytic Activity
   d. Pectinolytic Activity

3.6.2 Enzymatic Activities of Isolates By the API ZYM Method
3.6.3 Screening of Probiotic Activities

3.7 Statistical Analysis

CHAPTER 4 RESULT AND DISCUSSION

4.1 Chemical and Proximate Changes during Budu Fermentation
4.2 Microbiological Changes during Budu Fermentation
4.3 Phenotypic Characterization and Identification of Gram Positive and Catalase Positive Bacteria
4.4 Phenotypic Characterization and Identification of Gram Negative Bacteria
4.5 Phenotypic Characterization and Identification of LAB
4.6 Phenotypic Characterization and Identification of yeast
4.7 Cluster analysis of Identified strains
4.8 Microbial Succession Involved in Fermentation
4.9 Technological Properties of the Strains
   4.9.1 Screening of hydrolytic activity
   4.9.2 Screening of probiotic activity
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Production of enzymes through fermentation</td>
<td>11</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>List of bioactive peptide released from milk proteins by various microorganisms and microbial enzymes</td>
<td>15</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Classification of fermented foods</td>
<td>22</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Classification of fermentation</td>
<td>23</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Example of selected fermented foods around the world</td>
<td>24</td>
</tr>
<tr>
<td>Table 2.6</td>
<td>Indigenous fermented foods in Malaysia</td>
<td>26</td>
</tr>
<tr>
<td>Table 2.7</td>
<td>Fish sauce processing methods and different species of fish used in various countries</td>
<td>28</td>
</tr>
<tr>
<td>Table 2.8</td>
<td>Amino acid composition (mg/100mL) of various fish sauce</td>
<td>32</td>
</tr>
<tr>
<td>Table 2.9</td>
<td>Occurrence of biogenic amines in several fermented foods</td>
<td>46</td>
</tr>
<tr>
<td>Table 2.10</td>
<td>Incident of reported foodborne disease outbreak associated with fermented products</td>
<td>48</td>
</tr>
<tr>
<td>Table 2.11</td>
<td>Research priorities required for development of starter cultures</td>
<td>59</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Chemical and proximate analysis of sample from producer A1 (Ketereh) during budu fermentation</td>
<td>78</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Chemical and proximate analysis of sample from producer A2 (Orkid) during budu fermentation</td>
<td>79</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Chemical and proximate analysis of sample from producer A3 (Roslee) during budu fermentation</td>
<td>80</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Microbial load of sample from producer A1 (Ketereh) during budu fermentation</td>
<td>81</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Microbial load of sample from producer A2 (Orkid) during budu fermentation</td>
<td>84</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>Microbial load of sample from producer A3 (Roslee) during budu fermentation</td>
<td>85</td>
</tr>
<tr>
<td>Table 4.7</td>
<td>Physiological characteristics of Gram positive bacteria Isolated from budu</td>
<td>87</td>
</tr>
</tbody>
</table>
Table 4.8 Gram positive bacteria identified from budu samples

Table 4.9 Physiological characteristics of Gram negative bacteria isolated from budu

Table 4.10 Identification of Gram negative bacteria during fermentation

Table 4.11 Physiological characteristics of LAB found in budu fermentation

Table 4.12 LAB identified

Table 4.13 Physiological and biochemical characteristics of yeasts found in budu fermentation

Table 4.14 Identification of yeast during budu fermentation

Table 4.15 Microbial composition involved in budu fermentation

Table 4.16 Distribution of identified microbial strains during budu Fermentation

Table 4.17 Hydrolytic activities of the tested strains

Table 4.18 Enzymatic activities (API ZYM) of the corresponding strains

Table 4.19 Screening on the probiotic properties of the investigated strains
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Schematic flow sheet for the conversion of biomass to ethanol</td>
<td>19</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Production scheme of nampla (Thai fish sauce)</td>
<td>29</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Schematic <em>budu</em> processing in Malaysia</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Dendrogram of the Gram positive bacteria isolated from <em>budu</em> fermentation</td>
<td>100</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Dendrogram of the LAB isolated from <em>budu</em> fermentation</td>
<td>101</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Dendrogram of the yeasts isolated from <em>budu</em> fermentation</td>
<td>102</td>
</tr>
<tr>
<td>Appendix A:</td>
<td>Traditional <em>budu</em> processing</td>
<td>175</td>
</tr>
<tr>
<td>Appendix B:</td>
<td>Identification of isolates via Biolog Microlog System</td>
<td>176</td>
</tr>
<tr>
<td>Appendix C:</td>
<td>Substrate utilization of <em>Micrococcus luteus</em> in Biolog GP2 plate</td>
<td>177</td>
</tr>
<tr>
<td>Appendix D:</td>
<td>Substrate utilization of <em>M. luteus</em> ML1 in Biolog GP2 plate</td>
<td>178</td>
</tr>
<tr>
<td>Appendix E:</td>
<td>Substrate utilization of <em>M. luteus</em> ML2 in Biolog GP2 plate</td>
<td>179</td>
</tr>
<tr>
<td>Appendix F:</td>
<td>Substrate utilization of <em>M. luteus</em> ML3 in Biolog GP2 plate</td>
<td>180</td>
</tr>
<tr>
<td>Appendix G:</td>
<td>Substrate utilization of <em>M. luteus</em> ML4 in Biolog GP2 plate</td>
<td>181</td>
</tr>
<tr>
<td>Appendix H:</td>
<td>Substrate utilization of <em>M. luteus</em> ATCC9341 MLATCC in Biolog GP2 plate</td>
<td>182</td>
</tr>
<tr>
<td>Appendix I:</td>
<td>Substrate utilization of <em>M. luteus</em> ATCC9341 MLATCC1 in Biolog GP2 plate</td>
<td>183</td>
</tr>
<tr>
<td>Appendix J:</td>
<td>Substrate utilization of <em>M. luteus</em> ATCC9341 MLATCC2 in Biolog GP2 plate</td>
<td>184</td>
</tr>
<tr>
<td>Appendix K:</td>
<td>Substrate utilization of <em>Staphylococcus arlettae</em> in Biolog GP2 plate</td>
<td>185</td>
</tr>
<tr>
<td>Appendix L:</td>
<td>Substrate utilization of <em>S. carnosus</em> in Biolog GP2 plate</td>
<td>186</td>
</tr>
<tr>
<td>Appendix M:</td>
<td>Substrate utilization of <em>S. carnosus</em> SCN1 in Biolog GP2 plate</td>
<td>187</td>
</tr>
<tr>
<td>Appendix N:</td>
<td>Substrate utilization of <em>S. carnosus</em> SCN2 in Biolog GP2 plate</td>
<td>188</td>
</tr>
<tr>
<td>Appendix O:</td>
<td>Substrate utilization of <em>S. carnosus</em> SCN3 in Biolog GP2 plate</td>
<td>189</td>
</tr>
<tr>
<td>Appendix P:</td>
<td>Substrate utilization of <em>S. cohnii</em> in Biolog GP2 plate</td>
<td>190</td>
</tr>
<tr>
<td>Appendix Q:</td>
<td>Substrate utilization of <em>S. cohnii</em> SCH1 in Biolog GP2 plate</td>
<td>191</td>
</tr>
</tbody>
</table>
Appendix R: Substrate utilization of *S. cohnii* SCH2 in Biolog GP2 plate

Appendix S: Substrate utilization of *S. cohnii* SCH3 in Biolog GP2 plate

Appendix T: Substrate utilization of *S. xylosus* in Biolog GP2 plate

Appendix U: Substrate utilization of *S. xylosus* SX1 in Biolog GP2 plate

Appendix V: Substrate utilization of *S. xylosus* SX2 in Biolog GP2 plate

Appendix W: Substrate utilization of Corynebacterium afermentans ss lipophilum in Biolog GP2 plate

Appendix X: Substrate utilization of Rahnella aquatilis in Biolog GN2

Appendix Y: Substrate utilization of Enterobacter agglomerans bgp7 in Biolog GN2 plate

Appendix Z: Biochemical and technological characterization of isolates
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, trichloroacetic 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 iaming 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* incomparative 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 processes 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 lower 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, ointments and lotions due to their high water retaining capacity.
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