CHARACTERIZATION AND NUTRITIVE VALUE OF FERMENTED SAGO BY *BACILLUS AMYLOLIQUEFACIENS* FOR AQUACULTURE AND LIVESTOCK FEED.

Sitti Nur Janna Binti Jalil

(28360)

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</tr>
<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
</tr>
<tr>
<td>SHB</td>
<td>Sago Hampas Broth</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Potassium hydrogen phosphate</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>Magnesium Sulphate</td>
</tr>
<tr>
<td>SSSF</td>
<td>Solid State Fermentation</td>
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<td>SLF</td>
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</tr>
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Characterization and nutritive value of fermented sago by *Bacillus amyloliquefaciens*

for agriculture and livestock feed.

SITTI NUR JANNA BINTI JALIL (28360)

Resource Biotechnology Programme
Faculty Resource Science and Technology
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

ABSTRACT

Fermentation of sago pith residue ('hampas') by using cellulolytic bacterium has high chances in creating marketable value-added products. This includes product for aquaculture and livestock feed by producing enzyme that able to breakdown or utilize fibre content in sago hampas residue. An experiment was conducted to improve the nutrient content of sago hampas through fermentation by using *Bacillus amyloliquefaciens* UMAS1002 as inoculum. The characteristic and nutritive value of fermented sago hampas was determined in this study based on optimum fermentation condition on three different parameters which are dosage of inoculums (10%, 20% and 30%), fermentation period (5 days, 10 days and 15 days) and Nitrogen sources (from yeast extract, peptone, malt extract). Results of the study showed that optimum conditions of the fermentation of sago hampas by *B. amyloliquefaciens* UMAS1002 was at 20% (v/w) dosage of inoculums, 10 days of fermentation period and yeast extract as nitrogen sources. These conditions increase nutritional values of the product thus become promising for use and application to aquaculture and livestock feed.

**Keywords:** Fermentation, Sago pith residue ('hampas'), *Bacillus amyloliquefaciens*, optimum condition, parameter.
Characterization and nutritive value of fermented sago by *Bacillus amyloliquefaciens* for agriculture and livestock feed.

SITTI NUR JANNA BINTI JALIL (28360)

Resource Biotechnology Programme
Faculty Resource Science and Technology
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

ABSTRAK

Penapaian hampas sago menggunakan bakteria selulolitik iaitu *Bacillus amylo liquefaciens*UMAS1002 bertujuan menghasilkan produk nilai tambah di pasaran, termasuklah produk produk perikanan dan makanan ternak dengan menghasilkan enzim yang dapat memecahkan atau mendegradasikan kandungan serat dalam sisa hampas sago. Dalam kajian ini, ciri-ciri dan nilai nutrisi hampas sago yang telah difermentasi ditentukan berdasarkan keadaan penapaian secara optimum pada tiga parameter berbeza termasuklah dos inokulum, tempoh penapaian dan sumber nitrogen. Hasil kajian mendapati keadaan optimum bagi penapaian hampas sago adalah pada 20% (v/w) dos inokulun, 10 hari tempoh penapaian dan sumber nitrogen daripada extrak yis. Keadaan optimum dapat meningkatkan nilai nutrisi pada produk yang telah ditapai sehingga itu berpotensi untuk digunakan dalam akuakultur dan juga sebagai makanan untuk hewan ternak.

Kata kunci: penapaian, hampas sago, *Bacillus amylo liquefaciens*, keadaan optimum, parameter
1.0 INTRODUCTION

The sago palm is known as economic species and nowadays, Malaysia, Thailand, Indonesia, Philippines and New Guinea and pacific island has grown commercially these species. In India, they have grown this sago palm at some parts of South India (Kerala and Tamilnadu) and Assam. Sago palms are those species of the genus Metroxylon belonging to palmace family. The word of Metroxylon was derived from the Greek words, ‘metra’ meaning ‘pith or heart’ and xylon meaning ‘xylem or wood’. Sago palm mainly found between longitude 900-1900 east and between latitude 100 north and south up to an altitude of 1000 m above sea level. It is an extremely hardy plant; thriving in swampy, acidic peat soils, grow best on mineral soil which contain high organic matter with pH higher than 4.5. Although sago palms grow best, mature earlier and produce better yield on mineral soils rich in organic matter, they also can adapt naturally on peat and swampy soils where other crops cannot survive.

New developments in sago cultivation technology and increasing productivity of modern sago processing factories in sago industry are now facing with waste disposal problems. There are three major types of by-products produce during the processing of sago which are sago bark, sago pith residues or commonly known as ‘hampas’ and wastewater. Bark and ‘hampas’ are classified as a solid residue whereas wastewater is liquid residue. Figure 1 shows the flowchart of sago residues production during sago processing. As stated by Bujang et al. (1996), it has been estimated that approximately 7 tons of sago hampas residues of Metroxylon saga are produced daily from single sago starch processing mill. This residue commonly will become a waste and currently washed off into nearby stream resulting in pollution and environment nuisance (Awg-Adeni et al., 2010; Auldrey et al., 2009). Hampas is the fibrous pith residue after starch extraction and it is the number one contributor to water pollution. Dealing this waste is difficult as it is not easily dried due to its high
moisture, fiber and lignocellulose content. Initial studies by Chew and Shim (1993), showed that hampas has significant amounts of about 66% starch and 15% lignocellulose on a dry weight basis. Besides that, sago hampas also contained high level of organic material, Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD), which contravened the standard limit discharge enacted in the Environmental Quality Act, 1974 (sewage and industrial effluents regulation, 1979). However, biotechnology studies has been investigated that, this residue are able to be transformed into value-added products and additionally, its utilization able to reduce the polluting effect from the sago processing mills.

![Flowchart of sago residue production during sago processing](image)

**Figure 1:** Flowchart of sago residue production during sago processing (Yean & Lan, 1993).

Plants particularly in the forms of grains and roots are the main ingredients in livestock feed for example poultry and fish. Nowadays, the uses of these materials as ingredient for livestock feed are limited because it competes with human needs. The use of wastes like sago hampas was an alternative to overcome this problem (Wizna et al., 2008).
However, the uses of these residues are limited due to their starchy lignocellulosic and fibrous component. Poultry cannot digest fiber because it does not have cellulase to degrade it. Same goes with fish or shrimp; they generally cannot digest or utilize dietary fiber because of the lack of certain enzymes and appropriate fiber-utilizing microorganisms in the digestive system. Abd Aziz (2002) stated that sago pith residue contained 14.8% crude fiber and only 1% crude protein. This data showed that sago pith residue does not have sufficient nutritional value, since the crude fiber was quite high whereas, crude protein content was too low. To overcome this problem, it is important to reduce crude fiber content and increase other nutritional values.

Many kinds of processing method on high-fiber animal feed, such as physical, chemical, biological and fermentation process has been carried out to improve their nutritive value. Microbial conversion of these wastes seems to be a practical and promising alternative for increasing their nutritional value, transforming them into animal feed and thus producing a value-added product. Until today, numerous studies have been carried out in order to degrade this residue. Biotechnology approach is an attractive and proficient means in utilizing this waste. Microbial strains such as fungi and bacteria were employed to degrade this waste due to their ability in degrading the cellulose components (Coughlan, 1985). Cellulose is the most abundant renewable organic molecule in the world, making it a huge resource of renewable energy. However, cellulose, along with lignin and hemicellulose, the other components of lignocellulosic materials, is very resistant to biological degradation. Cellulases, a family of enzyme is able to digest cellulose into glucose units. Genus Bacillus was an important group in secreting extracellular commercial enzymes such as proteolytic, amylolytic and also cellulolytic enzymes especially when they are provided with suitable medium and growth condition. Besides that, they have been known to produce many kinds of enzymes e.g. alpha-amylase, alphacetolactate, decarboxylase, beta-glucanase, hemicellulase,
maltogenic amylase, protease and xylanase that have been produced commercially. Based on previous study by Wizna et al. (2008), *B. amyloliquefaciens* as inoculum tend to decrease the fiber content in cassava waste by 32%. This scenario based on the ability of *B. amyloliquefaciens* in producing enzymes that able to break down fibre content in the sago hampas before undergoing fermentation process.

Considering the lack of study, the objective of this research study was firstly to investigate the optimum fermentation condition of fermented sago hampas residue based on combination of different parameters which are dosage of inoculums, fermentation period and nitrogen sources and secondly was to analyse the nutritive value including dry matter, ash and organic matter, crude fibre and crude protein of the sago hampas residue after undergoing fermentation. Thirdly was to improve nutrient content of sago hampas residue through fermentation with cellulolytic bacteria.
2.0 LITERATURE REVIEW

2.1 The sago palm industry in Malaysia

In Malaysia, sago palm has been exploited since 19th century in Sarawak and along the coastal belts of Johor in Batu Pahat and Muar district. However, Sarawak was the largest sago growing area (Awo-Adeni et al., 2010). It is estimated that more than 90% of all sago planting areas are found in the state of Sarawak in East Malaysia. The largest (75%) sago planting area is in Mukah and Sibu where over 50% of the sago starch is produced (Tie et al., 1991). The natural adaptation of sago palms on peat and swampy soils (Bala et al., 2011) where other crops cannot survive make the sago palm particular significance to Sarawak because about 1.5 million hectares or 12% of Sarawak’s land area is under peat. In some case studies, it had been reported that about 62% of sago in Sarawak is grown in peat. Naturally, peat contain lack of nutrients, high in acidity and possess a rather low nutrient buffering capacity, on which crops other than sago palm difficult to grow. Sago reaches maximum height of 25 m and the diameter of 40 cm, grows in clumps, and has pinnate leaves and very thick stems. The stem of full-grown sago is about 20 m long and the fruits in clusters and takes about 24 months to mature. The leaflets are linear-elliptic, up to 1.5 m in length.

Virtually, the whole parts of the palm have their own local uses. The leaves are used as roofing material and made into attap hats, baskets and blow-pipe darts. The sticky saps are used for making gum and resins. Bark and rhachis are used as firewood. Ripe fruits and the uncooked palm heart or cabbage are used as food. Even, the sago worms which obtained from the stumps of felled sago have high market price. Last but not least, the most important product is the starch. Starch was obtained from the trunks of sago palms after undergoes long gestation of about 9 to 14 years, or when they are about to flower. According to Mary Margaret (Mar 25, 2012), sago palm was probably one of the first plants used by man in
South-East Asia and Ocean. For example, the Melanau and Penan community in Sarawak harvested large trunks of this palm for its starchy pith, which is an important carbohydrate source for this traditionally nomadic people. In Mukah, the modern sago processing factories has been developing in order to isolate good quality and quantity of sago starch. The world’s first large-scale commercial plantation of 7700 ha near Mukah, was developed by the Sarawak land development agency (Awg-Adeni et al., 2010). According to Yean & Lan (1993), isolation of sago starch involves debarking, pulping, starch extraction, settling, washing and drying. Once the starchy pith is processed, it can be used to make sago biscuits and other food products, including white bread and also for industrial uses such as in production of textile and adhesives.

2.2 Sago processing and by-product production

Almost all the sago factories in Sibu division are located along the coastal belt of Mukah, Oya, Dalat and Ilgan. Previous study showed that there are about 10 multimillion dollar factories in the Sibu Division with a production capacity of 200 to 500 tonnes dry flour/factory per month. With the promotion of sago cultivation and sophistication of its processing, large quantities of solid and liquid wastes are generated creating serious environmental problems. Proper strategies for the treatment of these wastes are required prior to safe and environmentally sound disposal.

The mature sago palm which may reach 9-12 m are cut into 75-90 cm log sections and transported by Lorries or linked up into rafts and floated by river to the factory. Debarking, to separate the pith from the bark is the first stage of sago processing. Debarking usually was done in crude manner with an axe. According to previous studies done by researcher, a conventional factory may generate about 8-15 tonnes of bark per day for every 750-1200 log section processed. After debarking, the debarked pith was split lengthwise into batons and fed
into a rasper or hammer mill where it is milled while water is added. Rotating plates then render the pith into a fine pulp which passes through several rotating drums with sieves or vibrator sieves for starch extraction. Large amount of treated river water is mixed with the pulp to separate and wash the starch granules out of the rotary sieves. At this stage, ‘hampas’ which is the leftover fibrous pith residue is produced.

According to previous research, it is estimated that about 50 to 110 tonnes of ‘hampas’ and 300 to 1000 m$^3$ of wastewater are generated. This high quantities of ‘hampas’ and residual starch in the wastewater contribute to high Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and particularly high suspended solid in the combined effluent which disobeyed the standard limit discharge enacted in the Environmental Quality Act, 1974 (sewage and industrial effluents regulation, 1979).

2.3 Composition of sago hampas

Large amount of ‘hampas’ was generated after starch extraction and considered as one of the major pollutants in rivers and streams near sago processing factory. ‘Hampas’ can also be termed as ‘sago refuse’, ‘pith residue’, ‘squeezed fibres’, ‘sago pith meal’, ‘waste of pulverized sago pith’ or ‘ampas’.

Studied by Shahrim et al. (2008) showed that the major constituents of ‘hampas’ included 66% starch, 15% crude fibre, 1% crude protein on a dry weight basis. However, studies by different research workers showed varying results of sago hampas composition (Table 1). The variation in the values is due to the differences in the processing technique, physiology of the sampled palms and variation in assay methods.

Based on physico-chemical properties of hampas, several enzymatic and microbial treatment strategies have been proposed in order to utilize this waste to produce several products that have marketable value subsequently reduce the pollution effect.
Table 1: The approximate composition of sago ‘Hampas’ (% dry weight).

<table>
<thead>
<tr>
<th>References</th>
<th>Apparent starch</th>
<th>Crude fibre</th>
<th>Crude protein</th>
<th>Ash</th>
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<td>15.9</td>
<td>3.8</td>
<td>6.4</td>
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<td>12.4</td>
<td>0.7</td>
<td>2.1</td>
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<td>Horigome et al. (1991)</td>
<td>72.5</td>
<td>13.4</td>
<td>0.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Shim (1992)</td>
<td>81.7</td>
<td>3.2</td>
<td>0.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Vickineswary &amp; Shim, (1996)</td>
<td>65.7</td>
<td>14.8</td>
<td>1.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

2.4 Possible utilization of sago hampas

Development of suitable methods for utilization of sago hampas not only reduce waterways pollution, but also contribute to additional income in industry.

According to Bintoro (1995), sago pith residue decomposed faster when mixed with soil, organic matter or inorganic fertilizer. The decomposed sago pith residue could be used as organic fertilizer to improve crop growth and this type of decomposer able to decrease negative effect of over dosage of inorganic fertilizer such as Sodium Nitrate, Ammonium Nitrate, Rock Phosphate and etc. sago hampas, an underutilized agro-industrial residue could be used as a substrate for growing edible mushrooms which are in great demand over the world. The high starch content of the ‘hampas’ could serve as a co-substrate for the basidiomycete to derive energy from the lignin degradation (Eriksson & Kirk, 1985).
Others potential use of sago ‘hampas’ is as glue extender/filler in the adhesive mix used in plywood manufacturing and for hydrolysis to reducing sugars by enzymes like α-amylase and amyloglucosidase. The study by Apun et al. (2000) indicates that the Bacillus amyloliquesciens UMAS 1002 has the ability to hydrolyze sago ‘hampas’ into reducing sugar. The ‘hampas’ which is in the form of dried-powder has possibility to be converted into fermentable sugar through acid and enzymatic hydrolysis (Kumoro et al., 2008). Besides that, hampas may be used for hydrolysis to confectioners’ syrup (Phang et al., 2000) and as animal feed.

2.5 Limitation of sago hampas waste for aquaculture and livestock feed

Most of the important animals in south-east are cattle, pigs, poultry, goats and sheep and much of the traditional animal feed resources come from plant which become limited because it competes with human need (FAO, 1983). At the first Sago Symposium held in 1976, it was discussed that sago waste itself are showed potential use for livestock feed. However, the nutrition contents in sago hampas are insignificant. It has higher ratio of amylase to amyllopectin which may prove unsuitable for young animals with underdeveloped enzymatic system.

Ozawa et al. (1996) stated that sago pith contained 23% cellulose, 9.2% hemicelluloses, 5.8% pectin and 3.9% lignin. Studies by Chanjula & Ngamponsai (2009) supported that sago hampas give potential feed for ruminants because they able to produce enzyme that able to degrade it. Toharmat (2002) stated that, rumen takes about 72 hours to end up the digestion of sago hampas inside the rumen due to high fibre content. However, non-ruminant animal are not able digest properly this waste since they do not have cellulase to degrade it. Various pre-treatment and upgrading option have been done in order to make sago ‘hampas’ become more digestible and palatable. Hydrothermal and chemical pre-
treatment using dilute acids (H$_2$SO$_4$), alkalis (NaOH), ammonia, urea, peroxides (H$_2$O$_2$) and organosolvents (ethylene diamine) that disrupt both the macro- and microscopic structures of lignocellulosic material which then increasing the solubilising of lignin and hemicellulose (Weil et al., 1994; Mosier et al., 2005).

Besides that, bio-fermentation of this waste with the present of microbial strains such as fungi and bacteria able to degrade this waste due to their ability in degrading the cellulose components (Coughlan, 1985). Cellulose is a polymer of D-glucose units linked by β-1, 4 glucosidic bonds that form large crystalline fibrils. In lay terms, cellulose is a long chain of carbon rings connected through an oxygen atom between each glucose molecule. When these chains are laid down next to each other, they form hydrogen bonds which hold the chains together to form large fibres. Cellulose is produced by a variety of plants and bacteria, plus a few animals (Ross et al., 1991). It is found both as a homopolymer and in lignocellulosic material, a crystalline glucose polymer in a matrix with lignin and hemicellulose in an approximate mass ratio of 2:1:1 (OTA, 1995). Combination of cellulose, lignin and hemicellulose is very resistant to enzymatic degradation (Wood & Kellogg, 1988).

2.6 Lignocellulose and fiber degradation by *B. amyloliquefaciens*

Celluloses commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms including fungi, actinomycetes and bacteria (Immanuel et al., 2006). According to Sonia et al. (2013), bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. The cellulolytic property of some bacterial genera such as *Cellulomonas* sp., *Cellvibrio* sp., *Pseudomonas* sp., *Bacillus* sp., and *Micrococcus* sp. was also reported. Enzyme production is closely controlled in microorganisms and for improving its productivity these controls can be optimize. Cellulase yields appear to depend
upon a complex relationship involving a variety of factors like inoculums size, pH value, and 
temperature, presence of inducers, medium additives, and aeration and growth time 
(Immanuel et al., 2006; Sonia et al., 2013).

Cellulose degradation occurs in three general steps (Bisaria et al., 1989; Klyosov, 
1990). First, the long chain polymer is degraded into random lengths of 4 to 6 glucose units 
by an endoglucanase, which is often exported to the environment outside the cell. Second, an 
exoglucanase cleaves the shortened chains into dimers. This enzyme is exported or held in 
close association with the outer membrane. The final step is cleavage of the dimer into 
glucose by a β-glucosidase, most commonly located within the cell.

A bacterium which has high growth rate as compared to fungi has good potential to be 
used in cellulase production. According to Faisal & Awang (2006), microbe from genus 
Bacillus represent one of the important groups of bacteria in secretting extracellular 
commercial enzymes such as proteolytic, amylolytic and also cellulolytic enzymes. These 
enzymes are expected to be able to transform complex molecules particularly lignocelluloses, 
which become the limiting factor in animal feed into simpler components.

According to Wizna et al., (2008), fermentation of sago hampas residue with B. 
amyloliquafaciens as an inoculum able to decrease the lignocelluloses and fibrous content of 
the product since they are enzyme that able to degrade the fibre and provide protein value as 
well.

Fermentation is an energy yielding metabolism as the process whereby mass 
cultivation of cells or its products are carried out in bioreactors. The vessel was used just for 
source in providing optimum conditions for the microorganisms to grow. Solid state 
fermentation (SSF) with batch mode was applied in doing this research. SSF can be defined 
as insoluble substrate fermented with adequate moisture which has numerous importance 
compared to submerged liquid fermentation (SLF) (Doelle et al., 1992) due to simple
technique applied with lower capital investment, lower level of catabolite repressions, low waste water output, better product recovery and high quality production. Table 2 below shows further comparison of solid state fermentation over submerged state fermentation.

Table 2: The differences between solid state and submerged liquid fermentation (Manpreet et al, 2005).

<table>
<thead>
<tr>
<th></th>
<th>Solid state fermentation</th>
<th>Submerged state fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less chance of contamination because of low availability of water</td>
<td>Higher water activity becomes major cause of contamination</td>
<td></td>
</tr>
<tr>
<td>Organism required less water for growth</td>
<td>Media concentration is much lower compared to water content</td>
<td></td>
</tr>
<tr>
<td>Downstream processing is easy, cheaper and less time consuming</td>
<td>Downstream process is difficult and very expensive</td>
<td></td>
</tr>
<tr>
<td>Liquid waste is not produced</td>
<td>High quantity of liquid waste produced</td>
<td></td>
</tr>
<tr>
<td>Less energy consuming for aeration and gas transfer</td>
<td>High air pressure required consumes more power</td>
<td></td>
</tr>
</tbody>
</table>
3.0 MATERIALS AND METHODS

3.1 Substrate

Sago hampas was obtained from sago processing factory in the district of Mukah, Sarawak. It was dried at 60°C and ground using grinder into 40 mesh size before added into the medium. The medium was autoclaved before any further analysis to ensure all microorganisms are dead except for only newly introduced \textit{B. amyloliquefaciens} UMAS1002 inoculum.

3.2 Microorganism

\textit{B. amyloliquefaciens} UMAS 1002 was obtained from bacterial culture that has been identified and isolated by Universiti Malaysia Sarawak (UNIMAS) researcher and preserved in UNIMAS laboratory.

3.3 Inoculum preparation

The bacterial culture firstly was incubated at 37°C for about 17 hours. Then, 100µL of culture was grown in 10ml Luria Broth (LB) for overnight. After that, 100µL was transfer on nutrient agar and incubated again at 37°C for overnight and was subcultured every 2 weeks. After overnight incubation, the colony was inoculated by using inoculum loop and put into 10ml of LB. Luria Broth (LB) was used to create a suspension of \textit{B. amyloliquefaciens} from the original colony growing on a nutrient agar plate. It provides the necessary nutrients and environment for optimal growth. The concentration of inoculum suspension was determined by using spectrophotometer under absorbance at \textit{D}_{600} with range between 0.8-1.0 A to each fermentation flask. 2ml of this inoculum was added to optimize different parameters which is inoculum dosage, nitrogen sources and fermentation period in order for improving sago hampas nutritive value.
3.4 Method of fermentation

The fermentation was carried out in 250mL Erlenmeyer flasks (Figure 2). Sago kampas broth (SHB) will be used as fermentation medium. 8ml of SHB was added to the 10g of sago kampas in the flask. The SHB contained in g/L of the following: 1g of KH₂PO₄, 5g of MgSO₄, 5g of CaCl₂.2H₂O, 2g yeast extracts or malt extracts or peptone. The pH of medium was adjusted to pH 7 by using 1M NaOH or 1M HCl. Then, they were autoclaved for about 1 hour at 121°C and inoculated with B. *amyloliquofaciens*, followed by incubation at 37°C. At regular intervals, sterile distilled water was added to each flask to maintain the initial moisture content during incubation (Chandra *et al.*, 2007).

Figure 2: Sago kampas used as substrate for fermentation with *B. amyloliquofaciens* as inoculum.