Brewery Spent Grain: Chemical Characteristics and utilization as an Enzyme Substrate

Khidzir, K. M. [1,], **Noorlidah Abdullah** [2] and **Agamuthu, P.** [3*]
[1] Solid Waste Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. [2] Division of Environmental Management, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. [3] Division of Biotechnology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia *agamuthu@um.edu.my (*corresponding author*)
Received on 21st May 2009, accepted in revised form 15th March 2010

ABSTRACT Grains in a brewery that have been removed of their sugar content is classified as a by-product known as spent grain. It is a low-cost material and is available year-round. In Malaysia, approximately 15% of brewery spent grain (BSG) that is produced is salvaged and sold as feedstock supplement, while the remainder is discarded in landfills. This is a waste of a potentially useful resource. The chemical composition of fresh BSG was studied. Nine species of fungi were individually inoculated onto sterile BSG and solid state fermentation occurred without chemical adjustment or supplementation. Activity of laccase, lignin peroxidase, xylanase, amylase, carboxymethyl cellulase, protease and tannase was assayed. BSG consists of lignin and cellulose. High enzyme activity of laccase was shown by *Pleurotus sajor-caju*, *Penicillium chrysogenum*, and *Aspergillus niger*. This study is possibly the first to report laccase activity by solid state fermentation of spent grain.

(**Keywords:** Solid state fermentation; waste reutilization; brewery spent grain; hydrolytic enzymes; fungi; sustainable management)

INTRODUCTION

Solid state fermentation (SSF) is the growth of microorganisms on a natural or man-made inert solid substrate [1] with sufficient moisture in the system to support the metabolism of the process microorganism [2]. Most of the moisture is absorbed within substrate particles while the space between substrate particles is filled with gas [3]. Due to its simplicity, low-cost, low-technical/technological demands and potential consumption of physically solid by-products, SSF is a promising alternative process in the value-addition of certain types of solid wastes, namely non-toxic, organic solid wastes from the agriculture and food and beverage industries.

Brewery spent grain (BSG) was studied as a substrate for SSF. BSG is of heterogeneous composition, consisting of malted barley [4], maize, wheat and rice [5]. Agamuthu [6] stated that 14kg of BSG was generated for every 100 liters of beer produced. The Carlsberg Group [7] stated 15.6kg of BSG was generated for every 100 liters of beer produced in both 2003 and 2004.

BSG is sold as a feedstock supplement [8] and was also reported to improve soil quality and yield of egg plant [9], to be suitable for production of xylitol [10] and alcohol [11], to remove heavy metals from industrial pollutants [12], to be suitable in the cultivation of oyster mushrooms [13], to be a source of fiber for consumption [14], to be able to increase the porosity and strength of bricks [15] and to be a suitable feed replacement in crayfish culture [16].

In Malaysia, BSG is under-utilized and is mostly used as cattle feed, in small amounts. This under-utilization is attributed to its high moisture content (causing transportation and storage difficulties), complex composition, the stigma of being labelled a waste material [17] and potential for rapid spoilage [18].

The objective of this study is to analyze the composition of BSG and assess its potential as a substrate for enzyme production by solid-state fermentation.

MATERIALS AND METHODS

All chemicals were obtained from Sigma, Aldrich, Fluka or BDH Analytical Supplies. All spectrophotometer readings were made using a Hach DR4000 Spectrophotometer (USA). Results are presented as the mean of four replicates with standard deviation.

1. Spent grain preparation

Fresh BSG was provided by Carlsberg Brewery (Malaysia) Ltd. A portion was immediately set aside for chemical analyses while 50g was delivered into 500ml conical flasks, sterilized at

121°C for 20 minutes (Arnold and Sons, Basildon Ltd. England) and left to cool.

2. Moisture content

The moisture content of fresh BSG was determined by drying fresh BSG in an oven (Memmert, Germany) at 50 ± 2°C for ten days. Once a day, the spent grain was removed, left at room temperature (27 ± 3°C) for ten minutes and weighed.

3. Acid Detergent Fiber, Lignin, Cellulose and Ash [19]

Acid Detergent Fiber (ADF)

A known mass of fresh BSG was refluxed (Witeg, Germany) at 90 ± 5°C for 60 minutes in a solution of cetyl tri-methylammonium dissolved in sulfuric acid (H_2SO_4). The solution was filtered (Advantech Qualitative Filter, Japan, 55mm) and the retained portion was rinsed with distilled water (90°C) and acetone (4°C) in tandem twice. The solid remnant was dried (Shellab 1375 FX, USA) at 105°C for 12 hours. Finally, the solid remnant was removed, left at room temperature for ten minutes and weighed. ADF (%) was calculated as the difference between the mass of treated and raw BSG.

Lignin

BSG similar in mass to the one used in ADF was delivered into a mixture of saturated potassium permanganate and buffer solution (ferric nitrate, argentum nitrate, potassium acetate, glacial acetic acid and tertiary butyl alcohol) and left at room temperature for 90 minutes. Next, the liquid portion was decanted and discarded. A de-mineralizing solution (dehydrate oxalic acid, ethanol, hydrochloric acid and distilled water) was poured onto the solid portion. After 10 minutes, the de-mineralizing solution was discarded while the solid portion was rinsed with 80% ethanol and acetone (4°C) in tandem twice. The solid remnant was weighed and dried in an oven at 105°C for 12 hours. Lignin (%) was calculated as the difference between the mass of the originally treated BSG and treated BSG after drying.

Cellulose

Cellulose was calculated the difference between the mass of lignin and ADF taken as a percentage of the original mass of BSG used.

Ash

The solid remnant from lignin analysis of lignin was baked (Wisetherm FH – 05, Korea) at 450°C for eight hours. After adequate cooling, the ash was removed from the furnace and weighed. Ash was calculated as the mass of the BSG after baking taken as a percentage of the original mass of BSG used.

4. Elemental analysis [20]

A known mass of fresh BSG was refluxed at 90 ± 5°C for 30 minutes in a mixture of 50% nitric acid (HNO_3) and 20% hydrochloric acid (HCl). After cooling, the mixture was poured into a 100ml volumetric flask, made to volume with distilled water and left overnight.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used to determine copper, cobalt, calcium, iron, manganese, magnesium, sodium, selenium, silica, phosphorus and sulphur in the mixture.

5. Total Carbon, Total Nitrogen, Total Protein, Starch and Lipids

Total Carbon [21]

A known mass of fresh BSG was weighed and baked in a furnace at 400°C for 12 hours. At the appropriate time, the remnants were removed from the oven, allowed to cool and weighed again. Total carbon was calculated as the difference between the mass of BSG before and after baking, taken as a percentage of the original mass of BSG used.

Total Nitrogen [22]

A known mass of fresh BSG was delivered into a mercuric sulfate solution. Potassium sulfate (0.5g), 1ml of concentrated sulfuric acid and four pieces of aluminum oxide granules were added. The mixture was refluxed at 90 ± 5°C for 30 minutes and then further refluxed at 60 ± 5°C for 60 minutes or until the mixture turned clear. Next, the mixture was refluxed again at 60 ± 5°C for 30 minutes and allowed to sufficiently cool.

The mixture was decanted into a distillation flask and 2ml of distilled water was used to rinse the inner walls of the flask. Next, 0.2g of zinc, 5ml of 10N sodium hydroxide (NaOH) and 10ml of 0.01428N H_2SO_4 was added and distillation took place. After distillation, 0.8ml of 0.1% alizarin red solution was added and mixed gently. The mixture was titrated with 0.01428N NaOH until the mixture became clear. The volume of 0.01428N NaOH used in titration of the mixture was noted.

A blank solution incorporating distilled water instead of BSG was also made and the method was repeated. Total nitrogen was calculated as a percentage of the mass of BSG used in the analysis.

Total Protein

Total protein was calculated based on formulas adapted from Hiller *et al*. [22] and AOAC International Methods 930.29 [23] and 991.20 [24].

Starch [25]

A known mass of fresh BSG was delivered into a test tube and mixed with 5ml of distilled water. The mixture was heated in a water bath (approximately 100°C) for 15 minutes, removed and allowed to cool. Next, 5ml of 60% perchloric acid ($HClO_4$) was rapidly mixed into the solution. The BSG was manually pressed against the inner part of the tube with a glass rod every three minutes for 30 minutes. The mixture was decanted into a volumetric flask filled with 100ml of distilled water and 3ml of 5% uranyl acetate solution was added. The mixture was centrifuged (Heraeus Christ, Germany) and 10ml of supernatant was removed and delivered into a clean test tube. The following was added: 0.1g of kieselguhr, 5ml of 20% NaCl solution and 2ml of iodine-potassium iodide solution. The mixture was thoroughly mixed and left for 12 hours.

Next, 5ml of an alcohol-sodium chloride solution was added. The mixture was centrifuged at 3000rpm for 15 minutes. The supernatant was gently removed and 2ml of an alcohol-sodium hydroxide solution was added on to the precipitate. The mixture was gently tapped and shaken until the precipitate lost its blue colour.

The inner walls of the test tube were washed with 5ml of the alcohol-NaCl solution. The mixture was centrifuged and 5ml of alcohol-NaCl and 2ml of 0.7M HCl solution was added to the solution to precipitate the starch. The mixture was placed in a water bath at approximately 100°C for 150 minutes and later allowed to cool.

After adequate cooling, the mixture was poured into a 25ml volumetric flask. Two drops each of phenol red, 1M NaOH and 0.05M oxalic acid were added. Distilled water was also added until the volume was made to 25ml. Five milliliters was removed and mixed with 5ml of a somogyi reagent. The mixture was heated in a water bath at approximately 100°C for 15 minutes. At the appropriate time, tubes were removed from the water bath and left to cool.

Next, 1 ml 2.5% iodine-potassium iodide solution and 3ml of 0.75M H_2SO_4 was mixed into the solution which was titrated with 0.0055M $Na_2S_2O_3$, with the starch indicator added just before the end point was reached.

Blank solution incorporating distilled water instead of BSG was also made and the method was repeated. Starch (%) was calculated as a percentage of the mass of BSG used in the analysis.

Lipids [26]

Fresh BSG was ground in an abrasive concrete pot and a known mass of ground BSG was weighed in a glass tube. An extraction solution was prepared by mixing propanol, hexane and distilled water and 5ml was added to the ground BSG sample. Next, the mixture was incubated at 60°C for 15 minutes and centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded. This procedure was repeated twice and both times, the precipitate was mixed with 5ml of the extraction solution. Lipid (%) was calculated as a percentage of the mass of BSG used in the analysis.

6. Fungi preparation and inoculation

In this study, nine fungi were studied: *Aspergillus niger*, *Penicillium chrysogenum*, *Rhizopus arrhizus*, *Trichoderma viridae*, *Trametes hirsuta*, *Pycnoporus sanguineus*, *Pleurotus sajor-caju*, *Schizophyllum commune* and *Ganoderma tsugae*.

Each fungus was maintained on malt extract agar (Oxoid, England) plates and incubated at 28 ± 2°C for seven days. Four agar plugs (each ± 8 mm) with its mycelia, were removed from the periphery of each seven-day old MEA plate and used as the inocula.

The inocula were delivered directly into each flask containing the cooled, sterile spent grain. Negative control was conducted by leaving sterile spent grain flasks un-inoculated. The mouth of each flask was covered with a cotton stopper. Four replicates were made. Incubation was done at 28 ± 2 °C for seven days.

7. Extraction

A volume of 400 ml of distilled water was poured into each flask. The flasks underwent mixing by mechanical shaking at 180 rpm for

60 minutes at 30°C (Series 25 Incubator Shaker, New Brunswick Scientific, USA). Further physical extraction was done by centrifugation (Heraeus Christ, Germany) at 4000 rpm for 20 minutes at 30°C. The supernatant was used as the enzyme extract.

8. Determination of enzyme activity (expressed in U/ml)

Laccase [27, 28]
First, 100 µl ml of the enzyme extract was mixed with 2.9 ml Syringaldazine (20 µM) and then with 50 µl of sodium phosphate (50 mM). Absorbance was read at 525 nm and enzyme activity was calculated.

Lignin peroxidase [27, 29]
A volume of 500 µl of the enzyme extract was mixed with 1 ml sodium tartarate buffer (100 mmol, pH 3), 500 µl of Veratryl Alcohol (2 mmol) and 500 µl of hydrogen peroxide (0.5 mmol). Absorbance was read at 310 nm. For standardization purposes, veratryl alcohol was used and the enzyme activity was calculated.

Xylanase [30]
After centrifugation, 200 µl of the enzyme extract was mixed with 1.8 ml of a 1% oat xylan solution (prepared in 0.05M sodium citrate buffer, pH 6.5). The mixture was incubated at 50°C for 5 minutes. Next, 3 ml of 3, 5 - dinitrosalicylic acid was added and the mixture placed in a water bath at 60°C for 15 minutes and later allowed to cool. Absorbance was read at 540 nm. For standardization purposes, xylose was used and the enzyme activity was calculated.

Amylase [31]
First, 150 µl of the enzyme extract was mixed with 200 µl of 1% soluble starch (prepared in 0.1M phosphate buffer). The mixture was incubated at 37°C for 25 minutes. Then, 400 µl of 3, 5 - dinitrosalicylic acid was mixed in and the mixture was placed in a water bath at 60°C for 5 minutes and later allowed to cool. Next, 8 ml of distilled water was added and absorbance was read at 489 nm. For standardization purposes, D-glucose was used and the enzyme activity was calculated.

Carboxymethyl cellulase (CMCase) [32, 33]
First, 400 µl ml of the enzyme extract was mixed with 1.6 ml of a 1% carboxymethylcellulose solution (prepared with a 40mM sodium acetate buffer solution). The mixture was incubated at 37°C for 25 minutes. Then, 400 µl of 3, 5 - dinitrosalicylic acid was added and the mixture placed in a water bath at 60°C for 5 minutes and later allowed to cool. Next, 8 ml distilled water was added and absorbance was read at 489 nm. For standardization purposes, D-glucose was also used and the enzyme activity was calculated.

Protease [34]
First, 30 µl of enzyme extract was mixed with 500 µl of casein and 470 µl of phosphate buffer (0.1M). The solution was incubated at 60°C for 10 minutes. A volume of 1 ml tricholoroacetic acid (10%) was added and the mixture was centrifuged for 15 minutes at 4°C. Next, 5 ml of sodium carbonate (0.44M) and 1 ml of Folin-Ciocalteau reagent is added to the supernatant. This mixture was incubated at 30°C for 30 minutes. Absorbance was read at 660 nm. For standardization purposes, L-Tyrosine was also used and the enzyme activity was calculated.

Tannase [35]
First, 50 µl ml of enzyme extract was mixed with 300 µl ml of a 0.5% tannic acid solution. Next, 3 ml of Bovine Serum Albumin solution (1mg/ml, prepared in 0.17M sodium chloride buffer solution) was added. The mixture was centrifuged at 7000 rpm for 10 minutes at 4°C. Then, 3 ml Sodium Dodecyl Sulfate (SDS, 1%) triethanolamine solution was added, as was 1 ml of Ferum Chloride ($FeCl_3$) solution. The mixture was left for 15 minutes. Absorbance was read at 530 nm. For standardization purposes, Gallic Acid was also used and the enzyme activity was calculated.

RESULTS AND DISCUSSION

The 73% moisture content of BSG (Table 1) is in accordance with the range stated by Mussatto *et al*. [36]. Due to its high moisture content, BSG can spoil within seven to ten days [37] in hot climates. In this study, un-sterilized BSG stored in sealed, thick polypropylene bags could only be stored at 4°C for approximately 14 days before putrifying.

Table 1: Moisture content of brewery spent grain

	Mean Moisture Content (%)	Standard Deviation
Spent Grain	72.616755	0.305080

BSG consists of approximately 57% lignin, 40% cellulose and 23% ADF (Table 2). There were high amounts of calcium, magnesium, silica and phosphorus (1038.5 ppm, 687.5 ppm, 242 ppm, 1977 ppm, respectively. See Table 3).

Table 2: Composition of fresh BSG

Parameter	Mean	Standard Deviation
Acid Detergent Fibre (% per gram of spent grain)	23.294280	0
Lignin (% per gram of spent grain)	56.744022	9.378141
Cellulose (% per gram of spent grain)	40.203778	14.709977
Ash (% per gram of spent grain)	2.269503	0.761340
Protein (% per gram of spent grain)	6.410000	0.311127
Lipids (% per gram of spent grain)	2.495000	0.106066
Starch (% per gram of spent grain)	0.280000	0.056569
Total Carbon (%)	35.610000	0.282843
Total Nitrogen (%)	1.025000	0.049497

Table 3: Elemental composition of fresh BSG

Element	Detection Limit	Mean Concentration	Standard Deviation
Copper (ppm)	0.01	2.5	0.7
Cobalt (ppm)	0.02	0	0
Calcium (ppm)	0.09	1038.5	3.5
Iron (ppm)	0.02	63.5	14.8
Manganese (ppm)	0.01	9.5	0.7
Magnesium (ppm)	0.01	687.5	108.2
Sodium (ppm)	0.03	60.5	3.5
Selenium (ppm)	0.01	6.0	0
Silica (ppm)	0.01	242.0	82.0
Phosphorus (ppm)	0.03	1977.0	66.5
Sulphur (ppm)	0.01	8.5	0.7

By virtue of being a by-product from a compendium of various grains, the exact formulation of BSG could vary according to the brewery and the production region. Flavour requirements of the beer and grain logistics often take precedence and affect grain composition, which affects BSG characteristics. Because of the variability, there is a value range for data of several BSG characteristics in published literature. Table 4 illustrates the differences of several major BSG characteristics.

Table 4: Value ranges of data reported for several BSG characteristics

Parameter	Characteristics (% of BSG weight)	References
Protein	6	Current study
	15	Mussatto and Roberto [38]
	22 – 27	Santos *et al*. [39]
	31	Prentice and Refsguard [40]
Lignin	16	Prentice and Refsguard [40]
	27	Mussatto and Roberto [38]
	57	Current study
Cellulose	9	Prentice and Refsguard [40]
	25	Mussatto *et al*. [36]
	40	Current study

Huige [41] attributed the differences in BSG characteristics to the time of grain harvest and the physical/chemical conditions of malting and mashing operations.

In a brewery, grains undergo steeping and germination to activate starch degrading enzymes which will hydrolyze the endosperm into maltose, dextrins and amino acids during the mashing process. The sugars and amino acids will be diverted away for alcohol production, leaving BSG to be disposed. These procedures could explain the starch content being detectable at only trace quantities [36]. Copper, manganese, selenium and sulphur were also detected in trace quantities.

There is high concentration of lignin in BSG. Lignin is insoluble and consists of carbon chains held together by carbon-carbon and ether bonds. The lignin polymer engulfs microfibrils of the secondary wall which consists of cellulose [42]. In BSG, due to the low starch concentration and lignin decreasing cellulose accessibility [43], lignin would have to temporarily assume the primary nutrient role for fungi.

The fungi in this study spanned three phyla: Ascomycota, Basidiomycota and Zygomycota. All the fungi studied could colonize sterile BSG. In general, fungal macrostructures were visible within the third day of incubation. Within five days, complete colonization of BSG was observed for all fungi. Taking *A. niger* and *R. arrhizus* as examples, the fermented BSG could not be shaken within the conical flasks and mycelia with black (*A. niger*) or green (*R. arrhizus*) sporangia were visible. This was also the case with *P. chrysogenum* and white filaments appearing similar to cotton completely engulfed the BSG.

Fungi are suitable process microorganisms for SSF as SSF closely resembles the natural environment of fungi [44] - higher temperatures, low water availability and high osmotic pressure [45]. SSF encourages deep contact between the substrate and fungi [46], allowing mycelia to rapidly colonize the solid substrate and reduces the need for absolute aseptic conditions.

All fungi showed positive enzyme activity (Table 5) but laccase was higher than its negative blank (by 100% - 1700%) and other enzymes studied (by 10^1 - 10^4). This observation was similar for every fungus studied.

Laccase is non-specific in its activity [47]. It allows fungi to completely degrade lignin by catalyzing the oxidation of aromatic hydrogen, with decarboxylation and demethylation of phenolic and methoxyphenolic acids, resulting in the reduction of oxygen to water [48]. Carbon dioxide [49] is also formed.
The Basidiomycota *P. sajor-caju* showed highest overall laccase activity (0.477 ± 0.222 U/ml). Both members of the Deutromycota, *A. niger* and *P. chrysogenum* showed similar activity (0.443190 ± 0.173757 U/ml and 0.456362 ± 0.134815 U/ml, respectively). Enzyme activities for the sole fungi of the Zygomycota, *R. arrhizus* were generally the lowest.

Fungi in this study appear to preferentially utilize laccase instead of lignin peroxidase (LiP). This is similar to the findings of Fenice *et al*. [50] who reported dominant activity of laccase compared MnP in strains of the Basidiomycota *Panus tigrinus* in both SSF and submerged fermentation; while Reddy *et al*. [51] also reported higher activity of laccase compared to LiP in SSF of banana wastes by *Pleurotus ostreatus* and *P. sajor-caju*.

Laccase would be utilized more as it is produced constitutively and a laccase-based oxidation system would require less induction compared to lignin peroxidase [52]. The fungi studied would secrete laccase extracellularly to hydrolyze lignin before absorbing the simpler materials as nutrients.

In BSG, the high percentage of lignin (57% per gram), coupled with low percentage of starch (<1) would explain the higher activity of laccase compared to other enzymes in this study. There were neither chemical inducers nor supplements added to the BSG cultures.