Valorization of brewer's spent grain using fermentation : potential for food sustainability

Cooray, Sachindra Thilomini

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**NANYANG
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SINGAPORE

**VALORIZATION OF BREWER'S SPENT GRAIN USING
FERMENTATION: POTENTIAL FOR FOOD
SUSTAINABILITY**

SACHINDRA THILOMINI COORAY

**INTERDISCIPLINARY GRADUATE SCHOOL
NANYANG ENVIRONMENT & WATER RESEARCH INSTITUTE**

2018

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FERMENTATION: POTENTIAL FOR FOOD
SUSTAINABILITY**

SACHINDRA THILOMINI COORAY

Interdisciplinary Graduate School

Nanyang Environment & Water Research Institute (NEWRI)

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Dedicated to Ammi and Udara

In memory of baby Thyaga Perera

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Abstract

Brewer's spent grain (BSG) is a by-product generated in large quantities during beer manufacturing. Even if most of the starch content in BSG is removed during brewing, there are significant amounts of proteins, fibres and other nutrients that remain. This material is mostly disposed in landfills or given away as animal feed, without being utilized in a significant manner. Breweries are burdened due to the massive cost incurred in its disposal and the environmental implications arising therewith. Despite many studies carried out to use BSG for other valuable applications, there lies more room for further development. Furthermore, there is limited knowledge on the metabolomics variation observed when spent grain is subjected to fermentation.

This study hypothesised that fermentation can be used in value addition of underutilised BSG. It is also hypothesised that an untargeted metabolomics approach can be used in monitoring the fermentation and final product. The overall aims were to (1) establish a workflow for a metabolomics study of biological processes; (2) enhance the economic value of BSG using fermentation and follow a metabolomics approach to investigate the changes taking place in this material; and (3) develop a process to extract nutrients from fermented BSG to be used in preparing a novel growth media.

For the metabolomics analysis, methanol was selected to extract extracellular metabolites from samples, as it was able to extract more compounds compared to other solvents and silylation was selected as the derivatization method prior to gas chromatography separation. This developed metabolomics workflow was useful in identifying up-regulating compounds during fermentation. According to our findings, fungi fermentation improved the content of amino acids, citric acid, some vitamins and antioxidant compounds in spent grain, enhancing its nutritional value. Next the study was focused towards extracting the nutrients from the fermentation product. This extract was used in growing *R. toruloides* and *S. cerevisiae*, as a substitute for conventional growth

media. The new media was able to fulfil the complete nitrogen requirement for growth, while sustaining the carotenoid and fatty acid production by *R. toruloides* in comparable levels to conventional growth media. According to the metabolome of the novel media, the prior fermentation step was able to increase the amino acid content of the new media.

In conclusion, our hypothesis was supported and the objectives were achieved. An optimized metabolomics workflow was developed to analyse the fermentation progress. In addition, value added compounds were generated by the bioprocess to enhance the economic value of BSG. Finally, using this fermented spent grain, a novel nutrient media for yeast growth was produced. The study further demonstrates how a metabolomics investigation is important to provide information to shed light on microbial activities in biological processes, such as fermentation. Overall, fermentation can be used to add value to underutilized food waste material such as BSG. Furthermore, it facilitates the extraction of nutrients and useful bioactive compounds in lignocellulosic material. Such waste valorization methods are helpful in producing foodstuff and food ingredients as an approach to achieve food sustainability.

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List of Abbreviations

Acetyl-CoA	Acetyl coenzyme A
ANOVA	Analysis of variance
BF ₃	Boron trifluoride
BOD	Biological oxygen demand
BSG	Brewer's spent grain
CE	Capillary electrophoresis
CSTR	Continuously stirred tank reactor
D-glucose-6P	D-glucose-6-phosphate
DMF	Dimethylsilyl
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organisation
GBF	Germinated barley foodstuff
GC-MS	Gas chromatography Mass spectrometry
GMP	Good Manufacturing Practices
HILIC	Hydrophilic interaction chromatography
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
HSD	Honest Significant Difference
IS	Internal Standard
LCYC	Lignocellulosic yeast carriers
MTBSTFA	<i>N</i> - <i>tert</i> -Butyldimethylsilyl- <i>N</i> -methyltrifluoroacetamide
MSI	Metabolomics Standards Institute
MSTFA	<i>N</i> -methyl- <i>N</i> -(trimethylsilyl)trifluoroacetamide
NaCl	Sodium chloride

NIST	National Institute of Standards and Technology
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PCOS	Polycystic ovary syndrome
PHB	Polyhydroxybutyrate
PPP	Pentose phosphate pathway
RP	Reverse phase
SSF	Solid-state fermentation
TBDMSCI	<i>tert</i> -Butyldimethylchlorosilane
TCA	Tricarboxylic acid cycle
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
QTOF-MS	Quadrupole time-of-flight Mass spectrometer
VFAs	Volatile fatty acids
UPLC	Ultra performance liquid chromatography
YP media	Yeast extract – Peptone media

Chapter 1. Introduction

This introductory chapter presents an outline of the background related to the research and the research problem, and states the aims of the study, its significance and importance. Section 1.1 introduces the background of the research topic, and section 1.2 describes the research problem. Section 1.3 outlines the aims and objectives of the study while section 1.4 discusses about the significance and contribution of this research. Lastly, section 1.5 presents the overview of this thesis and section 1.6 summarises the framework of this research thesis.

1.1. Research background

Nearly one in ten people in the world suffer from severe food insecurity, especially in Africa and Asia, and on the other hand, there is an increasing trend in the number of countries dealing with food over consumption [1]. In addition food related diseases and food waste production are two more common issues prevailing throughout the world. A large amount of food waste could be reused as foodstuff or used for the production of goods with value. This food waste is associated with usage of valuable resources such as land, water and fertilizer in the production process. This is also a cause for environmental problems, such as emission of green-house gases as a result of the natural decomposition in landfills [2]. There are several main concepts in defining food waste. Initially Food and Agriculture Organisation (FAO) of the United Nations defined food waste as nutritious edible material that is supposed to be used for human consumption generated at any point of the food supply network, but lost, discarded or spoilt [3]. Stuart proposed that in addition to the FAO definition, food waste should also include edible material purposely used instead to feed animals or used as by-products of food manufacturing diverted from the food chain [4]. Thus, according to these two definitions, side stream by-products such as brewer's spent grain and rapeseed cake are

considered as examples of food waste. “Cradle to cradle” and “circular economy” are two of the popular industrial ecology concepts targeting towards a *zero waste economy*, where wastes are used as starting material in novel products and applications [5].

Figure 1. 1: Food waste hierarchy

The competition between crops (considering land and all other resources) for food and biomass feedstock has directed the attention of many researchers to investigate the use of waste by-products and residues as resources for raw material and energy. Moreover, if it is possible for these novel applications from waste by-products to generate food ingredients, or help in enhancing food production it will help in improving food sustainability. According to the waste hierarchy (Figure 1.1), the most appropriate action in respect of waste would be prevention and the least favourable action will be disposal [6, 7]. Therefore, approaches such as reuse, recycle and recovery are considered as better options of tackling the problem of waste.

Waste valorization has been triggered by the massive quantities of waste generated and the depletion of natural resources worldwide. This approach has captured the attention of scientists and industries as an initiative to process waste in an environmentally friendly manner. Environmentally friendly approaches, such as waste valorization are important in steering sustainable development. The usual technologies, i.e. landfilling, incineration and composting, in handling waste have difficulties in

treating those with high organic loads due to the production of methane gas. In addition, no value added products could be produced if a decomposition method was used to handle waste. However, waste valorization has the ability to produce chemicals, fuel and material in a sustainable manner.

Fermentation is a simple bioprocess where microorganisms are grown on a substrate material. This is a commonly used processing technique especially in food related applications. Fermentation is an appealing methodology for the biological conversion of biomass and its residue into high-value material. There are reports of how fermentation is used to improve nutritional content of food in transforming into a balanced diet [8].

Proteins and other micronutrients present in lignocellulosic waste material, such as brewer's spent grain is strongly linked to the cellulose and hemicellulose in the outer layer of the grain. As a result, the availability of these nutrients is hindered. Using microorganisms during their metabolic activity is a widespread method used to increase the nutrient availability of such material. Solid-state fermentation (SSF) is a popular technique adopted in biotechnology to produce secondary metabolites and aggregate value to underutilized material such as agro industrial by-products and biomass (e.g. wheat bran, rice bran, spent grain and wheat straw). In SSF these by-products are used as substrates in bioprocesses and their properties are changed for nutrient enrichment. SSF requires less space for fermenters and does not require strict control of conditions during processing. Due to these advantages SSF is considered a promising technology in producing value added industrial applications.

The worldwide food demand is forecasted to increase by 40% by the next 10 to 20 years [9]. One seventh of the present global population is malnourished and has insufficient protein sources [10]. Thus, increasing the supply of nutritious food, especially in the developing regions, is a timely action that needs to be initiated. In addition to

increasing food production, methods of enhancing the nutritional value of food need to be investigated. Sustainability can be described as using resources at a rate lower than which Earth can replace those resources. Waste to food conversion approaches will be able to reduce the pressure on food producing resources, which are currently available in limited supply and will ensure that more nutritious food is available in the future. Hence, transforming waste bio resources to food sources is one approach of how food sustainability can be achieved.

1.2. Research problem

Waste material generated as side streams in production plants are increasing in volume with time. However, majority of these by-products are not utilized to their fullest capacity, but treated as undesirable waste. Brewer's spent grain (hereinafter referred to as BSG) is such an underutilized waste material produced in massive quantities during beer manufacturing throughout the world. In 2014, the worldwide beer production was reported at 193 billion litres [11]. Approximately 31% of the original malt weight is converted to BSG. In other words, 20kg of BSG is generated for every 100 litres of beer produced [12]. BSG accounts for about 85% of the by-products generated in a brewery [12]. This by-product accounts for increased levels of suspended solids and biological oxygen demand (BOD) generated by a typical brewery [13]. At the moment, industries try to manage the waste BSG by using it as animal feed or dumping in landfills. But these methods do not take a significant use of the valuable components present in BSG. Such a disposal approach cannot be considered a sustainable method to handle the brewery by-product.

The availability of BSG generated is growing with the rising levels in beer production. Therefore, methods to manage spent grain effectively are highly sought-after due to the increasing cost of disposal faced by brewers. Being available year around at

low or no cost makes BSG an appealing starting material for other applications. Yet, the high moisture and sugar content present in BSG speeds up the natural deterioration, making this biomass an unstable material. Drying can effectively lengthen the useable period of the BSG, while reducing the transport cost involved therein [14].

1.3. Aims and objectives

This thesis investigates the value addition to waste BSG using fermentation techniques. We have examined how solid-state fermentation can be used in enhancing the nutritional value of spent grain with the use of food grade fungi. Moreover, we examine how fermented BSG can be used in producing a nitrogen rich growth media for microorganisms.

Furthermore, the thesis explores how a metabolomics approach can be used to detect and evaluate changes taking place in the metabolome during the biological process. Environmental bioprocesses are complex in nature. A deep understanding of these processes would enable better control and maximization of the potential uses. Many of the previous studies carried out on this subject discuss about changes in the classes of analytes within biological processes, but seldom on a global untargeted approach to investigate a broad range of analytes. We aim to analyse metabolomic changes in an untargeted approach to obtain a holistic view about the variations. Metabolomics is a powerful tool that can be used in such a study due to its ability to individually monitor compound changes while processing. This approach would enable us to understand in depth about the microbial activity, and also to investigate value added material produced during the process. Information regarding such generated valuable compounds will be useful in identifying other applications where it could be used in. Extensive studies using metabolomics can further shed light on the mechanisms of environmental bioprocesses as well as novel and potential applications as a result of bioprocesses. This study only covers

the metabolomics variations in the fermented products, and does not extend to protein analysis.

1.4. Significance and contribution

Lignocellulose materials are currently being investigated as a raw material to produce valuable chemicals and biofuels using bioconversion means. Bioconversion of these materials is gaining the interest of industries and scientists around the world as a result of inadequate fuel resources, environmental pollution issues and sustainability initiatives taken to address these issues [15]. Such an approach also helps in moving towards natural, biodegradable and environmentally favourable products that are non-competing with the existing food chain.

In this study, alternative uses for spent grain targeting food related applications are evaluated by the means of adding value to the biomaterial rather than considering it as a troublesome waste by-product. Our study shows that fungi fermentation can increase the nutritional value of BSG. The study identified that the amount of amino acids, vitamins, citric acid and antioxidants present in the original BSG increased after the fermentation. Moreover, the study has investigated how fermented BSG extracts can be used as a growth media to provide the required nutrition to sustain yeast production.

Fermentation, along with other such biological processes have reported to alter the present concentration levels in compounds, such as proteins, carbohydrates, amino acids and fatty acids as a result of the microbial activity. The traditional method to analyse concentrations of compounds of interest during bioprocesses has been to use assays such as Bradford dye-binding assay and phenol-sulphuric assay. However, these methods require large sample volumes and long run times for the complete analysis. Furthermore, these analyses are only able to investigate selected groups of compounds at once. Using a

metabolomics study can eliminate all these drawbacks by allowing simultaneous detection and quantification of known as well as unknown metabolites.

Overall, valorization of BSG by enhancing its nutritional content will help in producing food sources to expand the food supply globally. Findings in this study will also help in reducing the demand for food crops to be used for industrial manufacturing. The key strategy behind this research was to add value to waste BSG to improve its nutritional content using solid-state fermentation, which can be useful in converting the waste material into a food source.

1.5. Thesis outline

A brief outline of this thesis is presented under this section.

- Chapter 1 provides an introduction to the thesis. This section begins by discussing about the background and the context of the research, which is followed by aims and objectives, significance and the contribution. Finally an overview of the sections of the thesis is provided.
- Chapter 2 comprises of two main sections; (1) Introduction to the topic in general and summary of research carried out in the area of valorization of biomass, in particularly for BSG, and (2) metabolomics related to microorganisms and biological processes, together with the general workflow for a typical metabolomics study.
- Chapter 3 investigates the changes taking place in metabolomics during the fermentation process using BSG. We have applied an untargeted qualitative metabolomics approach using GC-MS to detect these changes. The analysis allowed identifying valuable bio-compounds produced as a result of fungal growth on the food waste. Furthermore, such an analysis will be useful in finding potential uses for BSG.

- Chapter 4 reports the method we developed to extract the biologically interesting compounds generated in the fungal fermentation. This extract is evaluated as a novel growth media for yeast. In this section, we compare the cell yield of *S. cerevisiae* and *R. toruloides* grown in the new media against the yield obtained using conventional Yeast-Peptone (YP) media. In addition, metabolome of the different media are assessed to unravel the similarities between the two growth media.
- Lastly, in Chapter 5 a summary of the thesis is presented. Further developments for the current research involving BSG is also discussed along with future perspectives of metabolomics.

1.6. Framework of the research

Chapter 2. Literature Review

Part I – Valorization of brewer's spent grain

2.1. How brewer's spent grain is produced

A typical beer manufacturing process comprises of two main steps (1) brewing process and (2) fermentation process. Malted barley is the commonly used starting material in this process. Mussatto et al. describes the spent grain generation process as demonstrated in Figure 2.1, which refers to the important steps followed in beer manufacturing during the brewing process [12].

Figure 2. 1: BSG generation as a by-product of beer production process

Initially the raw barley is kept for 4–6 weeks as a dormancy period prior to processing. Malted barley is produced by controlled germination, which enhances the grain's enzymatic content. Next, steeping is carried out by placing the cleaned barley in water tanks (5–18°C) for about 2 days. During steeping the moisture present in the grain reaches 42–48%, and this hydration triggers germination and activates aleurone metabolism. Afterwards, the barley is transferred to a germination vessel (15–21°C) and held for about a week. During germination the structure of the starchy endosperm is modified using enzymes such as amylases, proteases and β -glucanases. Afterwards, the malted barley is dried (40–60°C) till 4–5% moisture content is achieved. The drying helps to develop flavour components as well as to reduce microbial contamination. The homogeneity and equilibrium in the malted product are achieved by storing the barley for 3–4 weeks. The steps followed up to this point are referred to as the malting process. Next follows the brewing process in which the barley malt is converted to wort and spent grain. Sometimes the malted barley is mixed with other grains such as crushed corn or rice and milled. Afterwards, this mixture is treated with hot water (70–74°C) to enzymatically hydrolyse the malt, which is referred to as mashing. During mashing, amylase from the malted barley converts the grain starch into fermentable and non-fermentable sugars, while proteins are degraded partially to amino acids and polypeptides. The mixture's filtrate is referred to as wort. This sugar-rich liquid is further processed to produce beer. The solid leftover solid fraction containing most of the proteins and fibres is referred to as brewer's spent grain (BSG).

2.2. BSG production volume and its environmental impact

BSG accounts for about 85% of the by-products generated in the brewery industry [12]. Approximately, 31% of the original malt weight is converted to BSG. World beer production was reported at 193 billion litres in 2014 and China, United States and Brazil

are reported as global leaders in beer production [11, 16]. In Singapore alone, it was reported that about 115 million litres of beer were consumed in 2013 [17], and the production volumes keep steadily increasing each year. It is also reported that for every 100 litres of beer produced, 20kg of BSG is generated [12]. Therefore, a massive quantity of BSG is generated and this quantity will keep increasing every year. Industries are burdened with disposal methods for the generated BSG and one of the most common ways is to dump it in landfills. BSG accounts for 30-60% of BOD and suspended solids generated by a typical brewery [13]. Therefore, direct disposal in landfills can lead to environmental problems as a result of the organic waste. Moreover, BSG in its life cycle involves environment impacts similar to food crops such as, deforestation, watering, pest control and erosion, resulting in a substantial carbon footprint. However, if it is not used till the fullest potential and discarded along with the left over materials, the resources used in the production life cycle are lost.

2.3. BSG structure and content

Major constituents of BSG are the walls of husk, pericarp and the grain's seed coat that are abundant in lignin, cellulose and non-cellulosic polysaccharides. BSG may also consist of proteins and lipids. The starch content present is insignificant, as most of it will be used during wort production. The content in BSG varies according to factors such as, type of barley, time of harvest and processing conditions. Overall, BSG is a type of biomass abundant in protein and fibre (cellulose, arabinoxylan and lignin) accounting for approximately 20% and 70% respectively [12]. Table 2.1 summarises the composition of BSG reported by various authors. Fibres comprise of hemicellulose and cellulose (Table 2.1). Hemicellulose, a branched heteropolymer, primarily consists of arabinoxylan (non-cellulose polysaccharide). Lignin, which is useful in maintaining structural rigidity in

plant cell walls, is a poly-phenolic macromolecule. Hemicellulose and lignin are responsible for the recalcitrant structure in biomass.

Table 2. 1: Typical composition of BSG reported by Lynch et al. [18] in g/100g dry material (%w/w) collected from Russ et al. [19], Meneses et al. [20], Mussatto and Roberto [21] and Robertson et al. [22]

Components	Quantity present (% dry weight)
Cellulose	16.8 – 33
Hemicellulose	28 – 33
Lignin	7 – 27.8
Proteins	15.3 – 24
Ashes	2.4 – 4.6
Lipids	10.6

Spent grain is reported to contain vitamins (biotin, choline, folic acid, niacin, pantothenic acid, pyroxidine riboflavin and thiamine) and minerals such as calcium, sodium, phosphorus, cobalt, iron, copper, magnesium, manganese, potassium, selenium, and sulphur [14]. Essential amino acids (e.g. lysine, histidine, methionine, phenylalanine and tryptophan) and non-essential amino acids (e.g. alanine, serine, glycine and proline) are also reported to be present in BSG [12, 22]. BSG also contains phenolic compounds such as ferulic acid and *p*-coumaric acid [23] along with poly- and oligo- saccharides. Indications of dietary compounds preventing progression of cancer, inflammations and oxidation activities have been found recently in this biomaterial [24, 25].

2.4. Current uses of BSG

With the massive quantity of spent grain produced, brewers are looking into ways to dispose this waste by-product in the most economic manner ensuring minimal environmental damage. Due to the political and social pressure laid upon industries, measures have been taken by many companies to alter their processes to reduce and reuse

the generated residues. Moreover, residues generated are not considered waste but seen as starting material for other processes. Thus, researchers are keenly looking into the challenge of finding alternative ways to enhance and reap economic value for spent grain. Similar to other lignocellulose materials, BSG is reported to contain various materials of value.

One of the main reasons hindering biomass possessing is the recalcitrance of plant cell walls. In the outer layer of the grain, proteins and nutrients are tightly connected to cellulose and hemicellulose. Therefore, physical, chemical or biological conversion needs to be performed on the BSG. The high moisture content (approximately 75–80%) makes BSG unstable and prone to fast microbial deterioration. Drying is proposed as an effective solution to extend its shelf life, which in turn will also reduce the volume and overall weight, lowering transport and storage cost [26]. Main complications related to the current use of BSG as animal feed for layers and fish are due to the high fibre content and low protein digestibility [27]. However, being available through out the year at low or no cost at all, makes spent grain a favourable raw material for any potential application.

To date the main usage of BSG has been as animal feed, mainly for cattle and it is further reported to increase milk production without affecting fertility [12]. Along with its inherent high protein content, BSG has been able to deliver all essential amino acids in animal feed when combined with other cheap nitrogen sources (e.g. urea). More recent studies are being carried out for BSG to be used as feed for poultry, pigs and fish as well. In one such study, fish feed partially supplemented with BSG was able to increase the body weight of the fish compared to those fed only with rice bran [28]. Recently there have been developments to regulate and incorporate good manufacturing practices (GMPs) to ensure the quality of BSG used in animal feed by the US Food and Drug Administration [29]. If BSG is not used as animal feed, it is dumped in landfills without using to its fullest potential.

Investigations are carried out to incorporate spent grain in bakery products for human consumption [12, 30]. Hossona reports an increase of 50% and 10% in proteins and essential amino acids respectively, 7% reduction in calories and double the amount of fibre present, by the addition of 10% BSG to the products [31]. But, BSG is too granular to directly add as a food ingredient. Therefore, it must be converted to flour. And there is a limited amount that could be used in these foods due to its distinct flavour and colour [32]. Due to the high protein content present, BSG is ideal to be used in baby food, cereal fortification, amino acid isolation and supplementation of protein in biotechnological processes such as in antibiotic production.

Other alternative uses of BSG are for energy production through direct combustion or biogas [33] and bioethanol production [34]. There are many reports of using spent grain for charcoal production [35], activated carbon production [36, 37], paper manufacturing [19, 38], use as a brick component [19], mushrooms cultivation [30, 39] and composting to be used as fertilizer for agriculture [30]. Studies have demonstrated the use of BSG to produce lignocellulosic yeast carriers (LCYC) by immobilized cell for applications such as whey fermentation using kefir [40], alcohol production from molasses [41, 42], brewing beer/winemaking [43, 44], and immobilized enzymes such as laccases [45].

2.5. Valorization of waste biomass

In early 1990s, more attention was given to waste remediation, which later turned into waste prevention followed by the current focus, waste exploitation. The thinking on waste management simply to comply with environmental regulations should be diverted into seeking potential value added products from waste. From such diversion, the industry will benefit from being economical in waste disposal and the consumer will benefit by increasing value added compounds. Even though these waste material originate from

diverse sources, at the end it comprises mainly of polysaccharides, lignin, triglycerides and proteins [46]. Current waste valorization approaches are targeted at three main areas; (1) fuel production and replacement of fossil fuels; (2) production of high value chemicals, and (3) production of useful material [47]. The general known method of producing carbon-based compounds in chemical industries is from petroleum products. However due to the decreasing supply and increasing cost, more attention is focused on using biomass in generating chemicals instead. Department of Energy of the United States published a report stating 12 top value added chemicals that have the potential to be generated using biomass that includes chemicals such as, aspartic acid, xylitol, glutamic acid and glycerol [48].

Biomass residue, which mainly includes polysaccharides and lignin, is an ideal candidate for bio renewable chemical production without requiring additional land to grow specific crops such as corn [46]. Significant quantities of protein-rich waste are generated in food manufacturing. For example, brewer's/distiller's spent grain, press cakes from rapeseed and palm, fish silage, feathers from poultry slaughterhouses and shells from shrimp and prawn industry. A potential scenario would be to use the essential amino acids in this protein waste for food and nutrition related applications and the non-essential amino acids as chemical feedstock [49]. This would also avoid the food versus fuel problem. In addition, there lies opportunities in converting organic wastes, such as wastewater sludge, food waste and municipal/industrial waste, into volatile fatty acids (VFAs) that can be used in biodegradable plastic production and bioenergy generation with the use of bioprocesses [50, 51] and emerging technologies [52]. Several studies have proposed a bio-refinery concept for value added material production from waste bio-products, where the biomass will be processed into several products such as biomolecules, bioenergy, biomaterial and biofuels in an integrative manner [53]. Such an approach will be cost effective compared to standalone manufacturing in terms of energy

usage and production yield. Dávila et al. investigates the production of xylitol, ethanol and polyhydroxybutyrate (PHB) simultaneously in a bio-refinery using BSG and demonstrates that it is economically more viable than standalone production [54].

2.6. Improving BSG by producing value added products

The lignocellulosic material in BSG can be processed into value added compounds such as α -amylase, xylitol, ethanol, activated carbon and lactic acid, and enzymes such as xylanase, feruloyl esterase, amylase, laccases by white-rot fungi [55]. BSG hydrosylates have demonstrated potential as functional food components to treat health conditions such as, type-2 diabetes [56], immunomodulatory effects [57] and hypertension [56]. Moreover, value added products such as precursors for food grade chemicals (e.g. glucose, galactose, mannose, arabinose, xylose, acetic acid, ferulic and p-coumaric acids), oligosaccharides, xylitol and hydrolysates with antioxidant properties [58], are found to be produced using BSG by utilising different hydrolysis processes [12].

BSG appears feasible to be used as a raw material for acetone, butanol and ethanol fermentation [34, 59]. There are instances where pure chemical treatments using phosphoric acid [60], treatments using mixtures of enzymes [61] and even combined chemical and enzymatic treatments [62] were used to pre-treat BSG in ethanol production. The combined chemical and enzymatic treatment on BSG has been able to generate protein hydrolysates with improved technofunctional (colour, solubility and foaming properties) properties [63]. Instances of using a mixture of enzymes to treat BSG in ethanol production has been reported and fed-batch solid-state fermentation was shown to be more efficient than a batch process [61]. There are reports on two approaches used; (1) sequential enzymatic hydrolysis (saccharification) and fermentation, and (2) simultaneous hydrolysis and fermentation. The latter is supposed to in theory, produce better yield and reduce the investment cost since it requires only one reactor. On the

contrary, one study related to BSG demonstrated better yields in sequential processing than in simultaneous processing [60]. *Aspergillus oryzae*, *Aspergillus niger* and *Humicola insolens* with *Saccharomyces cerevisiae* has been reported as some microorganisms used in ethanol production from spent grain.

Traditionally, in lactic acid fermentation starchy material such as corn or potatoes were used. However, waste and other lignocellulose materials are considered to be used presently as substrates to reduce the competition for food sources. BSG is evaluated to be used as an alternative nutrient source to grow these lactic acid producing bacteria [64]. But prior to the fermentation, Jelena et al. conditioned the BSG to hydrolyse using a mix of commercial enzymes; amylase, amyloglucosidase and cellulase. In this study lactic acid yield increased when spiked with glucose and yeast extract during fermentation operated under fed-batch conditions [64]. Other studies showed that the addition of calcium carbonate and yeast extract to hydrolysed BSG [65, 66] and optimizing factors such as pH and reducing sugar content [67] can increase the lactic acid yield in fermentation. Thus, fermentation yield/efficiency can be improved when the composition of the substrate used in fermentation is matched with the nutritional requirement of the strains employed. If the levels are not adequate, yeast extract, peptone, De Man, Rogosa and Sharpe (MRS) broth or corn steep liquor can be added to the substrate.

Spent grain has the potential to be used for human consumption due to the presence of elevated dietary fibre therein. Bioprocessed spent grain can be considered as a suitable material for food and feed to replace the traditionally used wheat and barn. Terrasan et al. reports of growing *Penicillium janczewskii* on BSG by SSF by which the spent grain will increase its nutritional value due to the production of xylanolytic enzymes [68]. This processed BSG had improved digestibility, increased amino acid content and no mycotoxins. Another study investigating the incorporation of BSG to bread reported increased phenolic content and antioxidant capacity, and improved sensory

and aromatic characteristics of bread due to sourdough and xylanase fermentation [69]. A study confirmed the prebiotic potential of arabinoxylans extracted from BSG by short chain fatty-acid production and gut microbiota modulation [70].

Valorization in this context refers to enhancing the economic value of some material to take use of its fullest potential by producing bio-based products rather than considering those as troublesome waste. In doing so, it is important to improve biomass digestibility using pre-treatment methods and hydrolysis.

2.7. Pre-treatment of biomass

As a starting point for all bioconversion processes using biomass, it is important to follow a prior treatment to breakdown lignin and cellulose crystalline structures. Pre-treatment can be physical, chemical, biological or a combined process. Physical pre-treatments focus towards size reduction and structure deformation using methodologies such as extrusion, comminution or ultrasound. The size reduction is effective in improving the enzyme entry into lignocelluloses. But overall, physical pre-treatment helps in increasing available surface area and lessening the crystalline structure of cellulose [71]. A study using physical pre-treatment reports that the protein content can be increased from 24% to 46% (w/w) by converting the BSG to GBF (germinated barley foodstuff). [72] GBF is a mix of protein and dietary fibre produced by soaking the BSG, pressing/grinding, straining the protein and dietary fibre-rich fraction and finally centrifuging and drying. However, physical methods are reported to be less efficient and costly.

Chemical treatment is another approach that has been considered to treat BSG and other biomass. Oxidants, acids, salts and alkalines are few chemicals that can be used in such treatments. A recent report shows enhanced bio hydrogen production from BSG after the pre-treatment with calcined-red mud [73]. But the main disadvantage in using

chemical treatments is the generation of toxic residues in the downstream process. Therefore, high concentration acids should be avoided due to its corrosiveness and high operating cost. Recently, methods such as steam explosion, wet oxidation and CO₂ explosion have been developed to combine physical and chemical pre-treatment [71].

In addition to physical and chemical approaches, researchers have looked at biological approaches in treating biomaterial. In biological treatment commercial enzymes or microorganisms are used in achieving similar results from the above-mentioned physical and chemical pre-treatments. Enzymatic treatment has advantages of not generating toxic compounds and being more environmentally friendly over chemical treatment [74]. But using commercial enzymes are costly especially in large scale processing. A less costly alternative for commercial enzymes is using microbial fermentation for pre-processing. Other advantages of biological treatment are low energy requirement for the process and generation of fewer inhibitors as a result of mild processing conditions [75]. Some examples of using biological pre-treatment are as follows.

Bekatorou et al. reports that BSG treated with *Aspergillus* species has shown 20-36% increase in protein content and proposed to be used as protein enhanced animal feed [76]. Treimo et al. reports acatase as the most effective peptidase for the solubilisation of proteins, with an ability to release up to 77% of total protein [77]. A study using *Rhizopus oligosporus* has been able to increase the crude protein and soluble protein content significantly while exhibiting a small level of increase in crude fibre, ether content and ash [78]. A research conducted by Niemi et al. shows that the pre-treatment of BSG with carbohydrase mix from *Humicola insolens* considerably improved the protein solubilisation in the residual biomass [79]. A study using Effective Microorganisms (EM) has shown to increase crude protein in BSG by 24.48%, where a mix of bacteria

producing lactic acid, photosynthetic bacteria, yeast, actinomycetes and fungi was used in the fermentation [80].

2.8. Fermentation

Fermentation is a widespread biological process used in biomass valorization. Fermentation is an energy deriving metabolomics process using organic matter, without the need for external oxidizing agents. This is an important bioprocess proven to be useful in many applications such as bioethanol production [81, 82], hydrogen production using agro-industrial residues by dark fermentation [83] and lactic acid production [84, 85]. Fermentation is a popular method used in the production of various enzymes such as, lipase [86] and cellulase [85]. Out of all its applications, the most popular use of fermentation is in food processing, as a method to prolong shelf life and to enhance the quality of food. Solid-state fermentation (SSF) and submerged fermentation (SmF) are the broad types as to how a typical fermentation will be carried out.

In SSF microorganisms are grown in an environment with low or no content of water. The substrate used in SSF is not only the solid support for the growth of microorganisms, but also the nutrient source. This is an ancient technology that has been used mainly in food processing. In addition to food industry, current applications in SSF are in pharmaceuticals, biochemistry, biofuels and clothing. Almost all fermentation processes in ancient days were based on SSF, before SmF took over in popularity in mid 1900s with the development of penicillin. Once again around 1960s SSF gained the interest of industries as the widespread of agricultural and industrial waste by-product protein enrichment aimed towards producing feed for cows [87]. It has been reported that SSF has higher product yield compared to SmF due to high productivity levels for enzymes and lowered substrate inhibition vulnerability. SSF requires less energy in sterilization and is able to mimic the natural habitat for most microorganisms, while

generating less final waste [88]. Other benefits include the reduced costs in SSF due to minimal setting up and operating costs and ease in downstream processing [89]. The challenges in SSF are in the control and instrumentation, scaling up, separating used-up biomass, mixing and aeration.

Bourdichon et al. reports a full list of microorganisms used in food fermentation with an inventory covering 195 bacterial species and 69 yeasts and moulds [90]. Generally recognized as safe (GRAS) state microorganisms have demonstrated to be safe under the parameters of its proposed use [91]. Probiotics are a special class of food that consist of living microorganisms which provide positive effects such as fighting gastrointestinal infections, improving lactose metabolism, reducing serum cholesterol levels, stimulating the immune system and improving inflammatory bowel disease [92]. Even though traditionally, probiotics referred to fermented dairy products, today it spans to other fermented non-dairy products such as fruits [93, 94], vegetables [95-97], cereals [98, 99] and meat [100, 101].

Fermentation can be used in the process of creating value added material. Some examples of using waste biomaterial to create better value are as follows. Kupaski reports an increase in the ash and a decrease in polysaccharides present in rice bran during SSF using *Rhizopus oryzae* [102]. This phenomenon is due to the use of nutrients from substrates for the fungi cell wall development in mycelial synthesis as indicated by the rise in fibre content in the biomass compared to unfermented rice bran. There have been other reports on the conversion of carbohydrates into proteins using fungal species in SSF. *Aspergillus niger* in corn cob, *Trichoderma reesei* in beet pulp and white rot fungi in sugarcane bagasse have reported such increases in protein content [103, 104]. SSF with *Rhizopus oligosporus* grown on roast buckwheat groats was reported to enhance water soluble vitamins (thiamine, pyridoxine and L-ascorbic acid) as well as α -, δ - and γ -tocopherols content [105]. In another study SSF with *Aspergillus niger* on rapeseed cake

was reported to show 76.89% reduction in glucosinolates, 44.6% reduction in phytic acid, 9.12% reduction in neutral detergent fibre, 103.71% increase in trichloroacetic acid soluble protein, 23.02% increase in crude protein and 23.54% increase in ether extract contents while significantly improving amino acids in-vitro digestibility. Activity of enzymes endoglucanase, phytase, acid protease and xylanase were increased as well [106]. You et al. has developed a simultaneous enzymatic transformation and microbial fermentation process to transform non-food biomass to starch used in producing ethanol, amylose and single cell proteins in a single bioreactor using a mix of enzymes derived from bacteria, fungi and plant sources [107]. Lactic acid is an important chemical due to its applications covering diverse fields. One of the recently popular use of lactic acid is in manufacturing the biopolymer poly-lactic acid (PLA), by sugar fermentation [84, 85]. Even if there are chemical synthesis methods of producing lactic acid, optically pure lactic acid can only be generated by fermentation [108]. *Lactobacilli* and lactic acid producing bacteria require large amounts of nutrients, which are generally provided by yeast extract. Therefore, fermentation can be considered as a widely used bioprocess in many applications providing favourable outcomes.

2.9. Monitoring performance in bioprocesses

Process monitoring is an integral part in any bioprocess. Successful system monitoring helps in developing, optimizing and maintaining such systems in maximum productivity levels. In addition, continuous monitoring will help to enhance the performance, reduce operating cost and improve quality control [109]. Temperature, pH level, pressure, redox, flow measurement, biosensors, viscosity, artificial intelligence and biomass are some of the bioprocess instruments used for monitoring purposes. It is important that these monitoring methods are cost effective and able to provide an extensive range of parameters to better understand and control processes.

Chromatography and mass spectrometry usage is currently gaining popularity in monitoring bioprocesses as it can be used for both off-line and on-line. It also has the advantage of being fast, specific, accurate and stable in monitoring applications. In many of the process monitoring situations a handful of parameters are selected and analysed, but measuring additional biochemical and metabolic parameters can provide in depth information, which is useful for optimisation. Metabolomics investigation for bioprocess monitoring has found useful in areas such as wastewater treatment [110], brewing [111] and fermentation [112].

Part II –Metabolomics as a monitoring tool

2.10. What are metabolites?

Metabolism involves the growth, maintenance and normal function in all organisms. This process is a collection of many chemical reactions, where low molecular mass compounds are formed in-between or as final products. Thus, metabolites are the final outcomes of the upstream consumption of compounds from the environment and cell regulatory activities [113]. Metabolomics aims to uncover deviations in biological systems in terms of metabolic characteristics by profiling small molecules (<1500Da). Compared to genomic, transcriptomics and proteomics, which are the other segments of the omics platform, metabolomics is located at the final omics level of biological systems [114]. Therefore, studies in the research area of metabolomics can provide functional information and response of these systems to environmental or genetic changes effectively. In contrast to other omics fields (genomics, transcriptomics and proteomics), metabolomics is able to provide a systematic approach in studying biological systems and networks. Metabolome reflects changes in phenotype and function due to its close

connection to the physiology of the cells [115]. This approach is able to monitor the global response of all influences to an event without considering the effect of a single contributor. Metabolomics studies have shown broad impact and vast growth in the fields of human diseases [116-118], drug discovery [119-121], plant research [122], food & nutrition and environmental monitoring [123]. This recently emerged omics field deals with the identification and characterization of small molecular weight compounds. Metabolites comprise of different groups of small molecule compounds including amino acids, fatty acids, carbohydrates and organic acids [124]. Therefore, the physical and chemical properties of metabolites differ considerably. As a result, the optimum extraction and detection conditions for different types of compounds vary and the methods that need to be used will be complicated. Primary metabolites are simple compounds having direct connections to metabolic pathways of microorganisms, while secondary metabolites are complex compounds that do not have a direct relationship to cell metabolism. Thus, only primary metabolites have direct relationships between growth rate and their production rates. Ethanol, acetic acid, citric acid and amino acids are examples of primary metabolites, while antibiotics and vitamins are examples of secondary metabolites [125].

2.11. Microbial metabolomics

Microbial metabolomics applications span a wide range of fields from metabolic engineering to novel drug discovery programs. Microorganisms are extensively used in producing value added bio-products using industrial biotechnological methods. It is essential to obtain both information regarding intracellular metabolites and extracellular metabolites in order to obtain a holistic understanding on microbial metabolism. Rapid sampling methods, inhibition of metabolic activity and deactivation of enzymatic activity using quenching techniques, extraction of metabolites and quantification are important

parts in analysing intracellular microbial metabolomics. Filtration and centrifugation of cell free supernatant are the most important steps when extracellular microbial metabolomics studies are conducted. Insight on microbial metabolomics can help in identifying issues in metabolic reactions [126]. Method validation (especially in sample preparation and metabolite measurement) is an integral part in microbial metabolomics as the technique involves high-resolution analysis [115].

2.12. Approaches of metabolomics study

Several types of analysis methods exist in metabolomics studies. *Targeted analysis* is useful for screening purposes, where exclusive substrate and product compounds are analysed. This analysis is important in assessing behaviour of those compounds under determined conditions. This approach requires superior purification to remove any interference and selective extraction of the chosen metabolites [127]. *Untargeted analysis* is a comprehensive method in which all metabolites in the system are identified and quantitated where necessary. *Metabolite profiling* is a hypothesis driven method where a class of metabolites related to a group of compounds or related to some pathway are analysed. In contrast, *metabolic fingerprinting* is a hypothesis generating approach, where selected metabolites of interest are analysed by developing specific analytical methods with an objective of classification. Maintaining high throughput levels is one of the main objectives in metabolic fingerprinting [113, 121].

Studies involving metabolomics can again be categorised as (1) discriminative, (2) informative or (3) predictive. Discriminative studies focus on identifying the difference between samples, without building statistical models or discovering possible pathways causing such changes. These studies use multivariate data analysis (MVDA) methods to achieve maximized classification while commonly using principal component analysis (PCA). Informative metabolomics aims to obtain intrinsic information regarding samples

by identifying and quantifying both targeted and untargeted metabolites, in order to develop and continuously update metabolite databases. Informative studies are used to discover possible pathways, new bioactive compounds and biomarkers, to build specialized metabolite databases and to carry out metabolite functionality studies. Predictive metabolomics use statistical models generated using metabolite profiles and abundance in order to predict variables, which are difficult to quantify using other methods. Partial least square (PLS) regression is usually used to produce such models in predictive analysis.

2.13. Steps in a typical metabolomics study

Figure 2. 2: Diagram representing the steps in metabolomics workflow [127]
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The important steps to follow in a metabolomics analysis are, (1) sample preparation, (2) metabolites extraction, (3) derivatization, (4) separation of metabolites, (5) metabolite detection and (6) data analysis (Figure 2.2). Out of these steps, the

detection and the data analysis steps are the most essential according to reported metabolomics studies. The actual workflow will depend on factors such as metabolomics approach used, sample type and detection instrument used.

2.13.1. Sampling and sample preparation

Sampling should be considered during experiment design. Multiple samples should be acquired sufficiently to reduce the biological variability and to ensure statistical validity. Other factors such as storing, preserving and transporting of the collected samples should be considered as well, as to not alter the original conditions of the samples [128]. It is also important to include quality control samples, replicates and blank samples. After sampling degradation needs to be attended especially for biological samples. Methods such as liquid nitrogen freezing, freeze-clamping, cold methanol treatment and acidic treatment have been used by studies to stop metabolism [121].

The important objectives in sample preparation are, (1) to extract the analytes and remove any interferences by matrix, (2) to stop damages to columns and analysis instruments and (3) to make the samples compatible with the analytical method selected [128]. Without proper sample preparation techniques there is no use of having state-of-art instruments for detection, as the data collected will poorly represent the actual results. However, any kind of sample preparation will cause losses in some degree. Therefore, *just enough* sample preparation is recommended, where the focus is on acceptable results rather than on optimum results [128]. This concept depends on the enhanced sensitivity and selectivity achievable by tandem mass spectrometry and chromatographic separation. An untargeted analysis intends to detect a variety of metabolites. Thus, the sample preparation for such an analysis should ideally be non-selective, fast and simple. In contrast sample preparation in a targeted analysis should be selective as much as possible since metabolite nature is known beforehand.

Quenching is generally the first step in handling biological samples such as cells, to prevent possible degradation and oxidation reaction. Solid samples are generally freeze-dried and subsequently ground to improve the release of metabolites during extraction. Steps such as centrifugation, filtration and concentration adjustment are commonly followed when handling liquid samples. Concentration methods such as lyophilization and solid phase extraction (SPE) help to maximize the quantity of analyte information collected. Preparation techniques without using solvents are preferred when volatile metabolites are considered to eliminate problems such as incomplete extraction and losses in sample concentration [121].

2.13.2. Extraction

The extraction step aims to maximize compound concentration that is subjected to analysis. The method utilised should not modify the target metabolites physically or chemically during the extraction process [126]. The main problem with primary and secondary metabolite extraction is the complexity in the cultivation media. As a result, several by-products will be extracted along with the desired products. Reactive extraction and aqueous 2-phase extraction methods can be used to improve product recovery [129].

In an untargeted metabolomics analysis several extraction solvents and extraction methods will need to be tested since the compounds of interest are initially unknown. Factors such as the compounds hydrophilicity or hydrophobicity should be considered in selecting extraction solvents. Extraction solution with combination of methanol-water-chloroform could be advantageous to be used in untargeted studies due to its capability extract both hydrophilic and hydrophobic compounds [127]. The progressive selective extraction followed by analysis of extracts would also be a successful approach to use in untargeted metabolomics. Extraction of compounds in targeted analysis depends greatly on prior knowledge about the nature of the analyte. The solvents used for extraction may

depend on factors such as, (1) polarity of the solvent and molecules of interest as well as the polarity of other impurities present in the solution [130], (2) nature of the target molecule on pH and heat, and (3) application of the extraction such as commercial use, lab use or research. In general, polar solvents such as, methanol, ethanol or water can be used to extract polar metabolites, while chloroform can be used to extract non-polar (lipophilic) compounds. Methanol is widely used as an extraction solvent due to its ability to extract a wide range of compounds, from water-soluble compounds to those that are non-polar. The nature of the solvent (organic or aqueous), volumes, solvent ratio and solvent pH may affect the number of metabolites extracted as well as reproducibility. Solvents with relatively low boiling points (acetone, dichloromethane, ethyl acetate and hexane) are preferred over water and *n*-butanol, since they can be easily concentrated by evaporation. As a rule of thumb, when extracting known compounds the solvent used should have a similar polarity to the compounds. On the contrary, when the compounds of interest are unknown it is best to familiarize with the compound classes, which are to be expected. Moreover, several trial extractions using different solvents and techniques can be performed, and the metabolites yield can be compared to draw conclusions on the method [130]. Disruption techniques, such as ultra sonication, can be used to facilitate extraction in both targeted and untargeted analysis. Extraction always makes a compromise between efficiency and speed.

2.13.3. Derivatization

Derivatization changes the functionality of an analyte in order to facilitate chromatographic separation [131]. Volatility is an important requirement for analysis by gas chromatography (GC). Thus, derivatization is an essential step before GC separation to help improve the volatility of the compounds of interest since many of the metabolites are non-volatile. In GC studies, compounds with functional groups $-OH$, $-SH$, $-NH$ and

–COOH are of prime concern because these functional groups tend to make intermolecular hydrogen bonds. These bonds influence the compound volatility, thermal stability as well as their ability to interact with column packing material. Hence, a derivatization step will allow compounds carrying such functional groups to be detected by GC-MS [131]. However, it should be noted that it is usually difficult to control the derivatization process and water present in the derivatization samples hinders the accurate chemical conversion of some cases leading some derivatizing reagents to hydrolyse. Therefore, anhydrous conditions are crucial in these reactions. Alkylation, acylation and silylation are the three general derivatization reactions used in GC.

2.13.3.1. Alkylation

Alkylation in most situations is used as a starting point of further derivatization or as a method to protect the active hydrogens in compounds. In the alkylation reaction the active hydrogen is replaced by an alkyl group, which results in a less polar product. This process is also known as esterification (Equation 1) [131].

Derivatization by alkylation is primarily used in analyzing polyfunctional amines and organic acids. Boron trifluoride (BF_3) in methanol or *n*-Butanol ($F_3B: \text{HOC}_n\text{H}_{2n+1}$; $n = 1$ or 4) is an easy to use and cheap reagent that can be used for alkylation. BF_3 reacting with acids is commonly used to form methyl or butyl esters. A drawback with alkylation is its inability to derivatize sugars and sugar alcohols [132].

2.13.3.2. Silylation

Silylation is the standard and commonly used derivatization method in metabolomics analysis by GC-MS, where sugars and their derivatives (sugar alcohols, amino sugars, etc.), phenols, mono- and di- carboxylic acids and fatty acids are efficiently derivatized [133]. In silylation the active hydrogen is replaced with a silyl group

$[-\text{Si}(\text{CH}_3)_3-]$. The reaction is favoured by a leaving group with low basicity that can stabilize a negative charge in the intermediate state [131]. Silylation is comparatively a safe derivatization method and covers a broad spectrum of metabolites. Silylation needs reaction conditions without the presence of water, thus, samples need to be completely anhydrous. It should be noted that the silylation reaction mixture is not removed from the derivative agents before injecting to the GC. Thus, injected samples could contain derivatives, residual agents as well as non-derivatized non-volatile compounds. The choice of reagent used for derivatization involves a balance between maximising derivatized metabolites while minimizing the amount of unanticipated products.

N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) is considered as the most volatile from the trimethylsilyl acetamides [131]. In derivatization using MSTFA, the N-methyltrifluoroacetamide group is replaced by the analyte. Adding a TMS group, which forms TMS ethers, TMS sulfides or TMS amines is the common method to derivatize polar compounds having $-\text{OH}$, $-\text{SH}$ or $-\text{NH}$ functional groups [124]. TMS derivatization of monosaccharides usually forms several peaks, because sugars present as mixtures of different anomers in solution. Thus, TMS derivatization of reducing sugars result in 5 tautomeric forms (one open chain, two pyranose and two furanose forms). However, the amount of tautomerism formed can be reduced by transforming aldehydes and keto groups into oximes using hydroxyamines or alkoxyamines before forming TMS ethers. The oximation limits the rotation along the $\text{C}=\text{N}$ bond and as a result only *syn* and *anti* forms are produced [124]. In general derivatization is initiated by oximation (convert aldehydes and ketones to oximes), followed by silylation (reduce hydrophilicity of functional groups, hydroxides $-\text{OH}$, $-\text{SH}$ or $-\text{NH}$ to increase volatility). Methoxamine hydrochloride in pyridine and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) are reported as the most suitable reagents for oximation and silylation respectively.

2.13.3.3. Acylation

In derivation by acylation, an acyl group is presented to an organic compound. In addition to producing volatile derivatives, acylation improves the stability of thermally labile molecules. An example of acylation is the reaction between acetic anhydride and alcohol to create acetic acid and acetate ester (Equation 2) [131]. However, it is difficult to prepare acylation derivatives as they interfere with other reaction products such as acid by-products. Therefore, these by-products need to be removed before using in the GC. The challenges regarding the acylation reagents are its high sensitivity to moisture while being hazardous and odorous.

Out of these three methods of derivatization alkylation and silylation are the most widely used types in metabolomics analysis and metabolomics profiling [132, 133].

2.13.4. Separation and detection

Separation and detection are key steps in metabolomics investigation. Gas chromatography (GC), high performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC) and capillary electrophoresis (CE) are some frequently used separation methods in metabolomics analysis work. Chromatography is used to separate a mixture of compounds based on the interactions between the analyte and a matrix. Mass spectrometry (MS), nuclear resonance (NMR) and fourier transform-infrared spectroscopy (FTIR) are some detection methods that have been extensively used in metabolomics work.

Reverse phase (RP) columns are widely used in metabolomics studies, for the separation of a wide variety of bioanalytical solutes such as, organic acids, carnitines and lyso-phospholipids (lyso-PLs). Hydrophilic interaction chromatography (HILIC) columns

are used in separating lipids (e.g. phospholipids (PLs), triglycerides, cholesterol esters and cardiolipids). Two-dimensional (2D) separation approach combines different separation mechanisms to achieve improved resolution leading to high-throughput omics methods. As an example, HILIC and RP are used together to effectively separate central carbon metabolites and lipids in a single run [134, 135].

Unlike in other omics fields where a single instrument can perform all measurements, metabolomics require variety of instruments depending on the subject compounds. But recently NMR, GC-MS and LC-MS have been the popular choices for metabolomics studies. Generally NMR is used to detect hydrogen atoms in metabolites. As long as the concentrations are higher than the limit of detection, all hydrogen containing metabolites give a ^1H NMR spectrum [136]. NMR methods are able to thoroughly elucidate structures of the analytes and have the ability for absolute quantification of metabolites with minimum sample preparation in a non-destructive nature. Thus, biological samples will not be required for any chemical or physical pre-treatment or a separation step in NMR. But the detector sensitivity is considerably less compared to MS methods. Therefore, only metabolites with high abundance can be detected [137]. Moreover, the identification based on chemical shift signals makes the data analysis challenging in complex mixtures [121]. NMR is able to detect most organic compounds but not salts and inorganics [138].

MS methods are able to perform quantitative analysis with high sensitivity and selectivity. In this technique molecules are broken down into ions using a high-energy source. Afterwards the charged ions are separated based on their mass/charge (m/z) ratio using magnetic separation. An MS instrument typically consists of an electronic bombardment source, an ion separation section and a detector. MS methods require prior sample preparation, where there could be some losses and discrimination for some metabolite classes depending on the ionization methodology and sample introduction

system. Thus, for a comprehensive metabolomics analysis a combined GC-MS and LC-MS approach will be more suitable [121]. LC-MS has unparalleled dynamic range and excellent sensitivity compared to other detection methods. It also possesses the ability to accurately analyse complex mixtures with thousands of analytes [137]. However, LC-MS is not compatible with gaseous samples and majority of spectral features are not yet identified. GC-MS enables the detection of volatile and uncharged metabolites that cannot be detected using LC-MS. GC-MS is a mature technology with good sensitivity and excellent separation reproducibility. In addition there are many databases and software for metabolite identification when a GC-MS platform is used. GC-MS is known to provide good reproducibility in retention time, gives on ion suppression effects and a relatively cheap MS instrument compared to the others available. Longer run times and difficulties in identifying novel compounds are the drawbacks for GC-MS technique [139, 140].

2.13.5. Data analysis

A typical omics study acquires a massive amount of data that is overwhelming and complex to understand by only using simple methods. Therefore, bioinformatics tools are extensively used to find significant changes acquired from the samples analysed by processing the massive amount of data generated. This stage is a crucial phase in a metabolomics study. The steps followed in a typical data analysis pipeline are (1) pre-processing, (2) statistical analysis, (3) annotation, and (4) interpretation and pathway/network analysis [141]. The strategy followed in data analysis can be subdivided as targeted approach and untargeted approach.

A common workflow for data analysis is depicted in Figure 2.3. The initial point is to find significant metabolites from all detected metabolites. First features from the raw data files are extracted and aligned accordingly. Afterwards, the data is converted to a suitable form (normalization, centering, scaling, etc.) for comparable purposes.

Afterwards, multivariable analysis can be performed to filter metabolites appearing to be distinctly altered among the samples in the data matrix. These selected variables are then subjected to other statistical analysis to reduce false positive and false negative results.

Figure 2. 3: Data analysis workflow [142] (Reproduced with permission from Elsevier)

2.13.5.1. Pre-processing and pre-treatment

Feature picking, also known as deconvolution, is an important pre-processing step in MS-based metabolomics. Software such as XCMS (The Script Research Institute), MetAlign (Plant Research International), MassHunter (Agilent Technologies, USA) and MarkerLynx™ (Waters Corporation, UK) can be used in feature detection. Readily available data processing software can be helpful in detecting and aligning peaks. These software packages reduce time and effort spent to construct data matrices and help in identifying peaks, which are tiresome tasks if performed manually. Then, a 2-dimensional (2D) data matrix consisting of metabolites and their respective quantities is constructed, which is referred to as aligning. Aligning corrects retention time deviation within samples during instrumental analysis and facilitates in constructing the 2D data matrix.

After features have been extracted from the chromatography and aligned, the pre-treatment steps are followed, commencing with normalizing the data across all samples.

Normalization is used to distinguish and remove systematic variations in peak intensities between samples caused without a relationship to the biological processes (e.g. sample dilution or instrument detector sensitivity variations) [143]. This step also helps in stabilizing the variance within a dataset. Normalization ensures that spectra are comparable across runs and sample sets. Missing value imputation is carried out as the next step. According to the sample at hand the estimation can be performed using different approaches such as small value estimation, mean/median replacement, KNN (k-nearest neighbour) and Bayesian PCA [143]. *Transformation* (log transformation and power transformation) is used to handle heteroscedasticity and to improve skewed distributions to result in symmetry in data [143, 144]. Log transformation is usually used in metabolomics, but a drawback with the log transformation is its inability to process zero values, which is a common abundance level found in a metabolite matrix. In correcting this problem either the *zero* values need to be converted to *one* values, or use power transformation. Afterwards these features can be pre-processed using *scaling* (Pareto scaling, unit variance (UV) scaling and vast scaling) and *centering*. The choice of method to be selected will depend on the data set properties, objective of the study and the selected analysis method [144]. In scaling methods, a scaling factor dedicated to each variable, is used to divide all variables. Unit variance scaling uses standard deviation and Pareto scaling uses square root of standard deviation as the scaling factors. In Pareto scaling high abundance metabolites and those with greater fold changes are decreased more compared to ones with low abundances. Thus, high abundance variables in the sample will be less dominant. As a result, Pareto scaling is widely used in metabolomics studies compared to other pre-processing methods. Centering changes all concentrations to fluctuate around zero instead of the mean concentration. Thereby, centering focuses on the fluctuating part of the dataset. As all variables are treated equally in scaling, this method tends to be bias towards variables with high abundance. Once the normalization,

missing value imputation, transformation and scaling have been performed the data can be processed further by statistical analysis.

2.13.5.2. Statistical data processing

Data collected using the MS instruments cannot be considered useful unless it is processed to convert to reliable and predictive information. Therefore, methods need to be developed to extract information from this data while ignoring random variations from noise and background. One such method that could be used in processing data is multivariate data analysis (MVDA). MVDA resolves the peak intensities captured in the spectrum into coordinates in multidimensional plots of metabolic behaviours. This analytical method helps in handling the huge quantity of complex data captured in metabolomics. It allows identifying and visualizing distinctive patterns in the data set. In general the main objectives of metabolomics data analysis can be broken down as (1) overview, (2) classification, (3) discrimination and (4) regression. Principal component analysis (PCA) is a non-supervised multivariate method used in discriminative metabolomics to obtain an overview of the collected data. PCA forms principal components using linear combinations of detected metabolites while increasing variation between samples. Based on the variance within data PCA divide the data evaluated into several parts as score matrix, loading matrix and residual matrix [142]. Information regarding trends, outliers, sample diversity and quality control can be gathered by this method. Partial least squares (PLS) is a supervised MVDA model used to acquire knowledge regarding sample discrimination by reducing dimensionality while maximizing the correlation between variables. PLS is greatly useful in predictive metabolomics studies.

2.13.6. Annotation

Metabolomic data obtained using the sections mentioned previously are finally submitted to compound identification. Identification is done by either database matching or comparing with pure standards, which are run under the same conditions.

There are broad categories of databases useful in metabolomics research. Metabolic pathway databases such as, KEGG [145], BioCyc [146] and Reactome [147] provide information about pathways of different organisms with summarized metabolite information. Lipid map [148], KEGG glycan [149], ChEBI [150], PubChem [151] and DrugBank (<http://www.drugbank.ca>) [152] are compound specific databases that provide details about names, structure and physiochemical details on specific compound classes such as, lipids, carbohydrates, drugs, toxins etc. Spectral databases contain reference spectra (for NMR, GCMS and LCMS) for metabolites with software to identify compounds using spectral matching. Examples of such databases are MassBank [153], MMCD [154], Metline [155], Madison Metabolomics Consortium Database (BMRB) [156] and Golm Metabolome Database [157]. Examples for organism specific metabolomics databases are the Human Metabolome Database (HMDB) (www.hmdb.ca) [158-160], BIGG [161] and SYSTONOMAS [162].

2.14. Summary and research gaps

According to the literature review the following conclusions can be drawn;

- Brewer's spent grain is an underutilised waste biomaterial rich in fibre, nitrogen and other micronutrients generated in beer manufacturing process. BSG is generated in massive volumes throughout the world, but it is not being used in any significant application other than as animal feed.
- Waste valorization is gaining the interest of the scientific community as a solution for waste management problems as well as to find alternative methods to develop products without consuming the currently depleting environmental resources such as land, water, petroleum etc. As a result of inherent richness in nutrients, BSG is appealing to be used as a raw material to produce food and food ingredients.
- The BSG lignin and cellulose crystalline structure obstructs the accessibility to nutrients and other interesting compounds to be used in bioprocessing. Physical, chemical or biological methods are useful in pre-treating the BSG before further use. Out of these methods, biological treatment has the added advantage of being less costly, more environmentally friendly and not generating any toxic residues.
- Microbial fermentation is a less costly alternative for using commercial enzymes for biological pre-treatment. High production yield, low energy requirement and ability to mimic natural microbial habitats makes solid-state fermentation superior compared to other fermentation techniques.
- Close monitoring of a bioprocess is useful for the effective performance in the process. Metabolomics is the study of small molecular weight compounds, which can provide functional information about biological systems. An untargeted metabolomics analysis has the advantage of detecting a wide range of metabolites compared to the targeted approach, in which even the unexpected changes in the small compounds will be captured.

- Sample preparation, extraction, derivatization, separation, detection and data analysis are important steps in a typical metabolomics workflow. Chromatographic separation and mass spectrometry detection are used in metabolomics to achieve high accuracy, high selectivity and high sensitivity.
- Derivatization is important in a GC-MS analysis to improve the detectability of compounds. Alkylation and silylation are the two derivatization methods mostly used when metabolites are analysed through GC-MS.

Therefore according to the literature review, there are opportunities to produce industrially relevant uses for BSG due to the dearth of applications at present. Further, BSG shows high potential of being able to be used as a raw material for such applications as it is available throughout the year with low or no cost. Special attention can be drawn towards food related applications from BSG as a result of the high content of proteins and fibres present. In this manner, the conversion of waste to food will be a useful approach for achieving food sustainability.

CHAPTER 3. Valorization of Brewer's Spent Grain using Fungi Solid-State Fermentation

Abstract

This chapter describes how fermentation methods can be employed to add value to waste brewer's spent grain. As the literature review suggests, brewer's spent grain is the main by-product generated during the production of beer in massive quantities throughout the world. In this Chapter, we report our study based on an untargeted metabolomics approach to investigate the value added compounds generated in brewer's spent grain as a result of solid-state fermentation using food grade fungi. The results show that the metabolites attributing to significant changes between unfermented BSG and fermented BSG were increased levels in amino acids, citric acid, vitamin and antioxidants. This data provided insight on the change in concentration in the metabolites observed during fermentation, proving that fermentation enhanced BSG's nutrient content. In conclusion, this study reveals the importance of a metabolomics study to provide information to shed light on valorization in fermentation. Furthermore, data from metabolomics studies can be used to find novel applications for food wastes and innovative processing methods for valorization of waste materials.

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3.1. Introduction

Producing high value added bio-products, including amino acids, antibiotics, vitamins, enzymes and bulk chemicals, using microorganisms has been a current topic in the biotechnological industry. Using biomass from agro-industrial wastes to exploit opportunities in biotechnological applications in producing value added products is beneficial, as these material do not compete with world food supply [163]. Brewer's spent grain (BSG) is rich in fibre and nitrogen, but is an underutilized by-product of beer manufacturing process. In 2014, the worldwide beer production was reported to be 193 billion litres, and for every 100 litres of beer produced approximately 20kg of BSG was generated [12]. This waste material is known to hold substantial amounts of valuable compounds that remain untouched during the brewery process. However, majority of the spent grain is used for landfills or as animal feed [164, 165]. Alternative uses for BSG are sought after because its cost of disposal is high. A main factor hindering the processing of BSG is the recalcitrance of plant cell walls. Therefore, physical, chemical or biological treatments need to be performed for this purpose.

Previous studies have been conducted to evaluate important compounds present in BSG. BSG essentially comprises of lignocellulose matter (cellulose and non-cellulose carbohydrates), protein and lignin [12, 22]. Major portion of total lipids present in BSG are triacylglycerols, followed by fatty acids (mainly linoleic, palmitic, and oleic acids), and minor amounts of diacylglycerols and monoacylglycerols have also been reported [166-168]. In addition, BSG has shown to contain many biologically active compounds such as polyphenols, flavonoids and antioxidants [168, 169]. Since BSG contains many interesting nutritional compounds as mentioned, studies have been conducted to incorporate BSG in human diet [170, 171]. This material has also been considered to be used as a substrate along with other agro-wastes to produce microbial cell mass without the need for extra nutrients [172] as well as to grow fungal strains [173].

In addition to the readily available valuable material in BSG, studies have been conducted to use chemical processes and/or enzymatic hydrolysis to further obtain value added compounds. However, the enzymatic treatment has added advantages of not generating toxins and being more environmentally friendly. Commercial enzymes have been used to extract sugars [174] and to release hydroxycinnamic acids (*p*-coumaric and ferulic acids) from BSG [175]. Nevertheless, the use of commercial or crude enzymes is costly and less economical. Thus, using microbial fermentation to hydrolyse biomass will be cost effective.

Microbial fermentation of BSG has reported to increase significantly the protein (crude and soluble) content [76, 78], to release sugars [176] and to produce lactic acid [177]. Enzymes produced for the degradation of biomass during microbial fermentation helps to release nutrients from BSG. Concurrently those microorganisms will produce primary and secondary metabolites, which in return can further add value to the original BSG. Filamentous fungi have been used in producing fermented food and beverages since ancient times. Today the usage of these fungi has expanded into biotechnological fields to produce chemicals to be used in manufacturing food ingredients, medicine, enzymes and proteins [178]. There are reports indicating that secondary metabolites and extracellular enzymes produced by these filamentous fungi comprise of bioactive compounds that are useful as antibiotics, immunosuppressive drugs, cholesterol reducing agents and antitumour drugs [179].

An untargeted global investigation of fermented products is useful to find any potentially valuable chemicals produced as a result of fermentation. Thus, an approach to use a metabolomics study is beneficial to evaluate the possibility of producing interesting compounds by BSG fermentation. Metabolomics helps to simultaneously explore hundreds of individual compounds in a single run rather than as compound groups. The metabolite extraction is an integral part in a metabolome investigation and solvent

extraction is the most widely used metabolite extraction method. The most suitable solvent for a particular extraction depends on its application. According to previous scholars, hexane, methanol, ethyl acetate, dichloromethane and water are some commonly used solvents employed for metabolite extraction [180].

Gas chromatography – Mass spectrometry (GC-MS) is a well-developed technology permitting the analysis of many metabolites in a single run [181]. The main advantage of using GC-MS for the analysis is the availability of comprehensive commercial and in-house MS libraries, making it easier to identify metabolites. An essential requirement for a GC-MS analysis is that all analytes need to be volatile to separate in gas phase [181]. Derivatization modifies the functionality of an analyte to enable chromatographic separation [131, 182]. In the derivatization process chemical groups are introduced to convert analytes to increase volatility and improve thermal stability, to decrease polarity and to enhance sensitivity of the mass detector. Alkylation, acylation and silylation are the three general derivatization reactions used in GC. In the alkylation reaction, an alkyl group replaces the active hydrogen, where a less polar product is generated. This process is also known as esterification. Silylation involves the replacement of active hydrogen with a trimethylsilyl (TMS) group. Acylation introduces an acyl group to the organic compounds by replacing a hydroxyl group. Out of these three methods of derivatization, alkylation and silylation are considered to be the widely used methods in metabolomics analysis and metabolomics profiling [132, 133].

In this study our objective was to investigate the use of fermentation on underutilized BSG using food grade fungi to produce value added products, which in turn is analysed using a metabolomics based approach. Moreover, we explore metabolomics sample preparation strategies for metabolite extraction and derivatization for GC, to be used in the untargeted metabolomics analysis to evaluate the changes taking place during fermentation. The study provides important information regarding BSG fermentation and

how this bioprocess is being able to increase the nutritional quality of the waste biomaterial. Furthermore, we aim to show how a metabolomics study is able to evaluate the changes taking place in the fermentation process.

3.2. Materials and methods

3.2.1. Chemicals

Type-1 ultrapure water was prepared using Elga PURELAB® Pulse. Solvents for extraction; hexane, methanol, ethyl acetate and dichloromethane, were purchased from Sigma-Aldrich (St. Louis MO, USA). Hydrochloric acid (HCl) and amino acid standard (AAS18) were purchased from Sigma-Aldrich (St. Louis MO, USA). All derivatizing agents, dimethylformamide (DMF), *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCI), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), 10% BF₃-Methanol (w/w) and methoxyamine hydrochloride were purchased from Sigma-Aldrich (St. Louis MO, USA).

3.2.2. Brewer's spent grain and fermentation

BSG was kindly provided by Asia Pacific Breweries (Singapore) Pte, Ltd. and was stored in airtight plastic containers at -80°C until used. Fermentation of BSG was performed according to a method proposed with minor modifications [183]. For the analysis, 10g of BSG was inoculated with 1ml spore suspension (10⁷ CFU/ml) of fungi and incubated at 37°C for three days. The experiment was conducted with multiple replicate samples. Samples were collected at different time periods; at 0 hours, 24 hours, 48 hours and 72 hours. The BSG samples were freeze-dried to remove all moisture present within and stored at -20°C until the metabolites were extracted. The freeze-drying helps to remove the excess water and to avoid any problems due to dilution effects.

3.2.3. Composition analysis of BSG before and after fermentation

Analysis of the composition of BSG before and after fermentation was outsourced to ALS (Australian Laboratory Services), Singapore. Fat content was measured using solvent extraction, total dietary fibre using enzymatic-Gravimetric method, total sugars (as invert sugar) using Lane-Eynon method, starch content using enzymatic methods and HPLC and ash content by incinerating at 550°C.

Amino acid analysis was performed according to a previous study [184]. Freeze-dried BSG samples were used for the analysis. 200µl of 6M HCl was added to samples of approximately 0.4mg. These samples were baked in a 105°C oven for 18 hours in sealed tubes. Then the hydrolysate was dried at 95°C in a heat block until completely dried. 20µl of dimethylformamide (DMF) was added and the dried hydrolysate was resuspended in DMF. This DMF was transferred into a new 2ml tube. 20µl of *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCI) was added to the DMF solution and sealed well. The sample was incubated in a heat block at 85°C for 1 hour. The derivatized samples were injected to the GC-MS. Amino acid standard AAS18 was used for quantitation.

Chromatographic separation was performed using 7890A GC-5975C inert MS system (Agilent Technologies, CA, USA). 1µl samples were injected into the HP-5MS capillary column by the auto-injector operated under splitless mode. Carrier gas (helium) was flown at 1.5ml/min flow rate. The inlet source temperature was maintained at 230°C and MS source temperature at 250°C. The oven temperature was held at 160°C for 1min and raised to 290°C at a rate of 20°C/min and held for 1 min. Then once again it was ramped to 310°C at a rate of 20°C/min and held for 1min. Data were acquired in full scan mode from 180-550m/z with a solvent delay of 2.7mins.

3.2.4. Selection of extraction solvent and extracellular metabolites extraction

Suitability of an appropriate solvent for metabolite extraction was investigated using ultrapure water, hexane, methanol, ethyl acetate and dichloromethane. Metabolites were extracted using a method suggested by Rauf et al. with slight modifications [185]. 100ml of the solvent was added to 30g of the fermented substrate. The mixture was placed in a rotary shaker for 30mins at 180rpm in order to homogenize the flasks. Afterwards the broth was centrifuged (9000g, 10mins, 4°C). The supernatant containing the metabolites was filtered through a 0.22µm filter.

3.2.5. GC-MS sample preparation and analysis

1.5ml supernatant of the sample media prepared as mentioned in the previous step was spiked with 10µL internal standard (IS) (ribitol, 2 mg/mL dissolved in water). The sample that used ultrapure water as the extraction solvent was freeze-dried, but the other samples were left in a heat block at 30°C to dry overnight.

3.2.5.1 Silylation as derivatization

For Silylation, the lyophilized/dried samples were derivatized according to Wang et al. before the GC-MS analysis [186]. Methoximation was performed by dissolving the samples in 50µL of methoxyamine hydrochloride (20mg/mL in pyridine) to protect the carbonyls and incubating at 37°C for 60min. Afterwards, silylation was carried out by adding 100 µL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) to each sample and incubating at 70 °C for 30min. Subsequently the samples were mixed for 60min at room temperature and then analysed in GC-MS within 24 hours in a random order.

3.2.5.2 Alkylation as derivatization

For Alkylation, the lyophilized samples were derivatized for analysis according to Horak [187]. The dried residue was re-dissolved in 500 μ L of 10% BF₃-Methanol (w/w) and incubated in a sealed vial in a heat block at 95°C for 20min. Then the tube was cooled down to near room temperature. Afterwards, 300 μ L of saturated NaCl in water and 300 μ L of n-hexane were added. Then the samples were centrifuged (14,000rpm, 10min, 20-25°C). The hexane layer was removed to GC-MS vials for analysis.

3.2.5.3. GC-MS analysis

Metabolites were analysed using Agilent 7890A GC–5975C inert MSD (with Triple Axis Detector) system (Agilent Technologies, CA, USA) equipped with a HP-5MS, 5% Phenyl-Methyl-Silox capillary column (30m $\times$ 250 μ m $\times$ 0.25 μ m, Agilent J&W Scientific, Folsom, CA, USA). Samples of 1 μ L were injected to the system by the auto-sampler in splitless mode. The solvent cut-off was set to 5mins. Carrier gas (helium) was set to flow at 1.1ml/min. The inlet and ion source temperatures were adjusted at 250°C and 230°C. The oven temperature was retained at 75°C for 4min and increased at a gradient of 4°C/min till 280°C and remained for 2mins. Data was acquired in full scan mode from 35–600m/z.

3.2.6. Data visualization and statistical analysis

Data extraction was performed by deconvolution algorithm using Agilent MassHunter Qualitative Analysis software (B.07.00). The extracted compounds were aligned, normalized (according to the IS, ribitol) and filtered using Agilent Mass Profiler Professional software (B.02.01). Multivariate data analysis was performed using MetaboAnalyst 3.0 [188, 189]. Data scaling was performed using Pareto scaling prior to principal component analysis (PCA) and orthogonal partial least squares discriminant

analysis (OPLS-DA). PCA and OPLS-DA were used to identify significantly changed features in the samples. Metabolites displaying significant changes between unfermented BSG and fermented BSG samples were determined and their accurate masses were compared with spectra in the National Institute of Standards and Technology (NIST) mass spectral library with similarity above 75% for feature identification. Statistical analysis of the data was performed using one-way ANOVA by the post hoc Tukey's Honest Significant Difference (HSD) to determine significant metabolic changes; wherein $p < 0.001$ was considered significant. Clustering heatmaps were constructed to obtain an overview of the fermentation process, which was created using Ward clustering algorithm and Euclidean distance calculation. Finally, a list of metabolites with tentative assignments was created.

3.3. Results and discussion

3.3.1. Constituent variation in BSG with the fermentation

After 3 days of fermentation the content of BSG was changed as reported in Table 3.1. A noticeable decrease was sighted in the fat content (5.57%), dietary fibres (7.82%), sugar content (>1.04%) and starch content (1.74%), but ash content was not altered significantly.

With reference to Table 3.1, the reduction in fat content could be due to the hydrolysis of fat by lipases. According to previous studies fungi are reported to produce lipases during fermentation [190]. The significant decrease in dietary fibres could be as a result of degradation by pectinase and cellulase enzymes produced during fermentation. Similarly the invertase and amylase produced as the fermentation progressed could have broken down the sugars and starch molecules. Proteases produced during fermentation can breakdown complex proteins into simpler amino acids [191]. Even if there was a reduction in the level of dietary fibre, the final amount of fibre is significant. Thus, the

fermented BSG is still favourable as a source of dietary fibre for food and other related applications.

Table 3. 1: Composition of BSG before fermentation and after fermentation of 3 days reported based on dry matter content (g/100g) [192]

	Analysis method	Unfermented BSG	Fermented BSG
Fat content (g/100g dry)	QWi FD/FC 22/Acid Hydrolysis/Solvent extraction	10.09	4.52
Total dietary fibre (g/100g dry)	AOAC 985.29 Sec 45.4.07 (2000)	45.65	37.83
Total sugar content (g/100g dry)	QWI FD/FC 36/Acid hydrolysis/Lane & Eynon titration	3.04	<2
Starch content (g/100g dry)	QWI FD/FC 125/Enzymatic hydrolysis/HPAEC	2.17	0.43
Ash content (g/100g dry)	QW1 FD/FC 05/Ashing at 550°C	3.35	3.39

Filamentous fungi demonstrate mycelium growth in which it branches out to form a network of hyphae. This growth pattern enables the fungi a greater surface area for nutrient uptake. Fungi are known to efficiently degrade plant biomass, which is comprised mainly of polysaccharides and lignin. However, fungi cannot take up complex polymeric compounds. Therefore, extracellular enzymes are produced to degrade the polymers to mono- or short oligomers [193, 194].

Figure 3. 1: Fold change of amino acids present in unfermented BSG and fermented BSG after three days. Key: fermented BSG amino acid level (red) and unfermented BSG amino acid level (blue) [192]

Amino acid content of the BSG was increased as a result of the fermentation by the fungi (Table 3.2 and Figure 3.1). The analysis method was able to detect essential amino acids, valine, leucine, threonine, phenylalanine and lysine, together with other amino acids, alanine, glycine, proline, serine, aspartic acid, glutamic acid and tyrosine. All detected amino acids showed increased levels after the fermentation period (Table 3.2). Considering the fold changes observed (Figure 3.1) threonine (6.018 fold) and serine (3.597 fold) showed the maximum increase. Total amino acid levels changed from 3.807mg/g in unfermented BSG to 7.772mg/g in fermented BSG after three days of fermentation.

Table 3. 2: Amino acid (mg/g BSG) changes in absolute values for the unfermented and fermented BSG after 3 day, as *mean $\pm$ standard deviation* (three replicates) [192]

	Unfermented BSG	Fermented BSG
Alanine	0.100 $\pm$ 0.001	0.223 $\pm$ 0.090
Glycine	0.100 $\pm$ 0.001	0.131 $\pm$ 0.063
Valine	0.233 $\pm$ 0.087	0.396 $\pm$ 0.137
Leucine	0.311 $\pm$ 0.122	0.496 $\pm$ 0.169
Proline	1.907 $\pm$ 0.389	4.253 $\pm$ 0.862
Serine	0.024 $\pm$ 0.001	0.087 $\pm$ 0.048
Threonine	0.056 $\pm$ 0.001	0.337 $\pm$ 0.216
Phenylalanine	0.158 $\pm$ 0.068	0.362 $\pm$ 0.148
Aspartic acid	0.316 $\pm$ 0.199	0.481 $\pm$ 0.331
Glutamic acid	0.402 $\pm$ 0.261	0.743 $\pm$ 0.532
Lysine	0.100 $\pm$ 0.001	0.144 $\pm$ 0.045
Tyrosine	0.100 $\pm$ 0.001	0.119 $\pm$ 0.037
Total Amino acids	3.807 $\pm$ 0.154	7.772 $\pm$ 0.325

3.3.2. Selection of extraction solvent and derivatization method

One of the aims of this study was to establish a sample preparation methodology to analyse the fermentation products of BSG by GC-MS. The goal in the metabolomics study was to analyse as many as possible metabolites in a single detection run. Therefore, selection of both the extraction solvent and derivatization method was based on the number of compounds detected after analysing the chromatographs of the samples, once the background noise and solvent ions were removed.

Figure 3. 2: Number of metabolites extracted by Agilent MassHunter Qualitative Analysis software when different extraction solvents (water, hexane, methanol, ethyl acetate (EA) and dichloromethane (DCM)) were used and when the two derivatization methods, silylation and alkylation, were used. Key: Alkylation of unfermented BSG (light blue), alkylation of fermented BSG (dark blue), silylation of unfermented BSG (yellow) and silylation of fermented BSG (orange) [192]

The number of metabolites extracted in Agilent MassHunter Qualitative Analysis software varied according to the solvent used and the derivatization method employed (Figure 3.2). According to the results, methanol was able to extract more extracellular metabolites, compared to water, hexane, dichloromethane and ethyl acetate. Furthermore an organic solvent, such as methanol, is easier to use in down processing compared to water. Methanol being a mild nonpolar solvent helps in maximising the metabolome coverage as well [195]. Moreover, methanol is known to effectively precipitate proteins in solutions [196], which is advantageous in the GC-MS analysis. Therefore methanol was selected for the extracellular metabolite extraction for this analysis.

Metabolites have diverse properties. Thus, to conduct a comprehensive untargeted analysis it is best to use multiple separation methods, such as to separate polar and nonpolar compounds. Even though separate methods provide better coverage, it is time

consuming and high in terms of manpower usage and cost. Therefore, our approach in this study was to use a single method for metabolite extraction.

Derivatization is essential in GC to improve the chromatographic characteristics of analytes. Derivatization is able to decrease analytes' polarity and/or increase detector sensitivity of the targets. According to our results, when alkylation and silylation derivatization methods were compared, the latter provided a greater number of hits over alkylation. The aim of this study was to conduct a global analysis on metabolomics. Hence silylation was selected, as it was the derivatization method that showed more peaks in the chromatograph (Figure 3.2). Silylation is one of the classical and broadly used derivatization methods in metabolomics analysis by GC-MS. Sugars, sugar alcohols and amino sugars, phenols, simple carboxylic acids and fatty acids are efficiently derivatized by silylation [133]. In the reaction the active hydrogen is replaced with a silyl group $[-Si(CH_3)_3-]$. It should be noted that the silylation reaction mixture is not separated from the derivatives before injecting to the GC. Thus, injected samples could contain derivatives, residual agents as well as non-derivatized non-volatile compounds. However in silylation, samples need to be completely free of water, as anhydrous reaction conditions are required.

3.3.3. Metabolite variation during the fermentation process

Fermentation metabolome was inspected to gain insight about the changes that had taken for the four time points using GC-MS together with multivariate data analysis. Figure 3.3 depicts the overlay spectra obtained by GC-MS from samples collected every 24 hours during fermentation, commencing from day zero. As the fermentation progressed, the chromatograph pattern changed and the number of peaks detected increased. This peak pattern indicates that more and different metabolites were produced with time as the fungi grew.

Figure 3. 3: Chromatogram of the spectra observed by GC-MS when the fermentation progressed on the BSG from day 0 (black), day 1 (red), day 2 (green) to day 3 (blue). The changes in the peak patterns indicate the changes in metabolite content [192].

Even if the spectra differences depict a change in the metabolites, a more reliable method such as multivariate analysis is essential to find further details about the exact changes that have taken place. PCA and OPLS-DA are two examples of such multivariate analysis that can be used for reducing dimensionality. PCA is able to create two- and three-dimensional ordination plots after dimensionality reduction using linearly uncorrelated principal components. PCA score plot generated in our study, clearly displayed metabolic discrimination between the BSG fermentation samples (Figure 3.4A), in which separation took place between principal components (PCs) one and two accounting for 80.1% of the variance. It can be clearly seen that there are distinguishable differences among the samples when the fermentation was progressed, except for the variation between days 2 and day 3 that are not significant.

Figure 3. 4: (A) PCA score plot and (B) PCA loading plot, for extracted metabolites as the fermentation progressed from day 0 to day 3. Component 1 (65%) vs. component 2 (15.1%) of Day 0 (red), Day 1 (green), Day 2 (dark blue) and Day 3 (light blue) [192].

Citric acid and alanine were the dominant differently expressed compounds according to the PCA loading plot (Figure 3.4B), showing a differentiation in the fermentation samples along the first principal component. Therefore, it can be deduced that production levels in citric acids and alanine increased with fermentation time when the unfermented and fermented BSG samples were compared. Moreover according to this loading plot, glucose, D-fructose, D-gluconic acid, galactose, D-manitol and D-(+)-talose displayed higher correlation to the unfermented BSG over the other samples. Thus, we can deduce that microorganisms must have consumed these compounds for their growth, and have accumulated citric acid and alanine.

PCA, model being an unsupervised model, in some instances may tend to divert the focus due to systematic variations, such as instrument drift as a result of temperature variations, contaminants present and other experimental changes as a result of sample collection and metabolite extraction [197]. In contrast OPLS-DA, a supervised multivariate analysis, has the ability to eliminate discriminatory patterns due to external factors.

Figure 3. 5: (A) OPLS-DA score plot and (B) S-plot loadings for extracted metabolites when the fermentation progressed from day 0 to day 3. Key: metabolites captured in day zero (red), day 1 (green), day 2 (dark blue) and day 3 (light blue) [192].

The OPLS-DA model demonstrated better separation for the analysed samples, with the total explained variance R^2 (cumulative) of 95.5% and the cross validation predictive ability Q^2 (cumulative) of 91.5% (Figure 3.5A). A better separation of the sample classes was seen in the OPLS-DA score plot compared to the PCA model. S-plot represents the covariance and correlation loadings generated using the OPLD-DA model. Compounds contributing most for the separation between the unfermented BSG samples and the day 3 fermented BSG samples according to the S plot (Figure 3.5B) are summarized in Table 3.3.

According to the results, the most significant change in terms of reported abundances were seen in citric acid, L-norvaline and the carbohydrates. The intensity variation of the above-mentioned metabolites during the fermentation can be visualized as illustrated in Figure 3.6A. The generated heat map depicts metabolites accountable for discrimination patterns observed (Figure 3.6B). Details about the time point of the changes taken place during the fermentation can also be understood using the heat map. According to the heat maps, the available carbohydrate levels started dropping from

fermentation day 1 and the other interesting metabolites commenced accumulating from fermentation day 2.

Table 3. 3: List of tentatively identified metabolites with significant changes when the fermentation progressed through the three days. The intensity changes were calculated based on the unfermented BSG samples against the fermented samples. One-way ANOVA followed by Turkey's post hoc test was performed to find the significantly changing metabolites (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Intensity change represented as fold change ($\text{abundance}_{\text{Day},3} : \text{abundance}_{\text{Day},0}$) [192].

Annotated Compound Name	Class	Fold change in intensity	Regulation
Citric acid	Tricarboxylic acid	36.51	↑***
Alanine	Amino acid	2.233	↑***
L-Isoleucine		1.600	↑**
L-Tyrosine		1.190	↑**
3-Hydroxyanthranilic Acid	Aminophenol	5.0192	↑***
L-Norvaline	Amino fatty acids	56.439	↑***
Myo-inositol	Carbohydrates	6.8123	↑***
Pantothenic Acid	Vitamin	1.8389	↑***
Glucose	Carbohydrates	0.0068	↓***
D-Fructose		0.0104	↓***
D-Galactose		0.0036	↓*
D-(+)-Talose		0.1051	↓***
D-Mannitol		0.0242	↓*

The OPLS-DA loading plot and heat map correlation was able to show the significantly changing metabolites in BSG during the fermentation process (Figure 3.5 and Figure 3.6). In summary, carbohydrates such as glucose, fructose, galactose and mannitol were reduced in the concentrations observed after the fermentation and other nutrient components such as, citric acid, alanine, L-tyrosine, L-isoleucine, pantothenic

acid, 3-hydroxyanthranilic acid, myo-inositol and L-norvaline were increased in the fermented biomass.

Figure 3. 6: Heatmap analysis correlating from Day 0 (left) to Day 3 (right) as the fermentation progress in BSG. Metabolites indicated in red are up regulated and the ones in blue are down regulated. Key: metabolites captured in day 0 (red), day 1 (green), day 2 (dark blue) and day 3 (light blue) [192].

3.3.4. Metabolomics response for BSG fermentation

Decreased levels of D-glucose, D-galactose and D-fructose in the fermented BSG indicates the possible utilisation of these carbohydrates in glycolysis. In carbohydrate metabolism D-glucose is initially converted to D-glucose-6-phosphate (D-glucose-6P). D-glucose-6P can either be converted to D-fructose-6P or enter pentose phosphate pathway (PPP) to proceed with glycolysis. D-fructose is converted to D-fructose-6P and enters glycolysis. D-galactose is converted to D-glucose-6P (Leloir pathway) and follows the above mentioned route [194]. Therefore, according to the observed results, glycolysis can be considered a dominant catabolism occurring during the fungi growth. Mannitol is reported to function as a reserve for carbohydrate and a protective agent against osmotic and oxidative stress for fungi in coenzyme regulation, formation of spores and storing reducing power [198]. According to our study reducing levels in mannitol were sighted. Thus, it appears that the fungi grown in our study has utilized mannitol as a carbon source as well.

Pyruvate, a key intermediate in glycolysis, is useful in the biosynthesis of amino acids such as tyrosine, tryptophan and dihydroxyphenylalanine. Complex B vitamins, such as thiamine, pyridoxine hydrochloride, calcium D-pantothenate and nicotinic acid, are constituents of pyruvate dehydrogenase coenzyme, which help to transform pyruvate to acetyl-CoA. Acetyl-CoA, may then enter the citric acid (TCA) cycle to perform cell growth and metabolism [199]. As indicated in our study, the reported increase levels of pantothenic acid, could be acting as a precursor for CoA production.

Our results indicate a clear increase in the levels of citric acid after 3-days of fermentation. Filamentous fungi are known producers of organic acids such as citric acid [200, 201]. Glucose is catabolized by glycolysis to produce pyruvate in the cytosol. A fraction of this pyruvate is converted to citric acid by citrate synthesis in mitochondria. The other fraction is converted to oxaloacetate, which is then converted to malate and

finally to citrate (TCA cycle) [202, 203]. Out of the worldwide citric acid production of 1.6 million tonnes in 2007 [204], 70% is used in food and beverage industry (as an acidifier or antioxidant to preserve and enhance the flavour and aromas) and 20% in pharmaceuticals (as antioxidant) [201]. Its global production is still reported to be increasing [204].

The metabolomics analysis shows a significant increase in alanine and the amino acid analysis performed a clear increase in many other essential and non-essential amino acids (Table 3.2). Amino acids act as building blocks of proteins and they are important in regulating key metabolic pathways [205]. The increase in amino acid levels indicates the potential use of fermented BSG as a food resource with enhanced nutrition. From the global amino acid market of US\$7 billion, 56% comprise of animal feed supplements segments [206]. Thus, the fermented BSG shows a potential suppletion in such sector.

3-Hydroxyanthranilic Acid is a product of tryptophan catabolism in the nicotinamide pathway. This compound is an antioxidant, which acts as anti-inflammatory and neuro-protective [207]. A study reports that 3-hydroxyanthranilic acid is the key compound responsible for the antioxidant activity in tempeh [208]. This antioxidant has also been effective for preventing autoxidation in soybean oil and soybean powder. Myo-inositol, which has shown a noteworthy level of production in the fermented BSG is biosynthesised from glucose-6P, derivative of glucose. This compound is regarded important in terms of promoting female fertility and treating polycystic ovary syndrome (PCOS) [209].

Similar to many metabolomics studies only some of the peaks extracted were putatively annotated to a metabolite with a good score, while many could not be identified. Annotations were done at level 2 in the requirements by the Metabolomics Standards Initiative (MSI) [210]. Metabolomics has been found to be useful in comprehensive understanding and quantitation of fermentation effect in terms of

fermentative capacity of microorganisms and benefits of probiotics and functional food [211]. Our study is an extension of metabolomics analysis to explore interesting compounds generated by fungi fermentation on BSG.

Rhizopus species have been known to produce amylase, galactosidase and glucosidase during fermentation, which help to digest oligosaccharides and starch [212]. Many studies have looked into how this fungi species can be used in producing lipases to be used in food and biotechnology related applications [213-215]. Fermentation of these fungi has the potential to increase the phenolic content, isoflavone aglycone content and antioxidant activity [216-218]. Moreover, *Rhizopus* species have been investigated in producing protein rich fungal biomass production for aquaculture feed applications [219]. Due to its ability to convert the original substrate to a protein rich material and to produce other interesting nutritional ingredients, we selected this filamentous fungi for the BSG fermentation. And as expected conferring previous studies *Rhizopus* was able to enhance functional properties of our substrate according to the metabolomics results obtained.

3.4. Conclusions

This study provides evidence of valorization in BSG, an underutilized waste material, using fungi fermentation. We observed increased levels of amino acids, citric acid, vitamin and antioxidant level in fermented BSG when compared with unfermented BSG, while reducing levels of carbohydrates, fats and dietary fibres. According to the results, the fermentation seems to enhance the nutritional value of BSG and its potential to be used in food and nutrition related applications as a value added source. The data provided insight on the changes in metabolites observed during fermentation, proving that fermentation enhanced spent grain's nutrient content. In conclusion, our study reveals the importance of a metabolomics study to provide information to shed light on valorization in fermentation. Furthermore, data from metabolomics studies can be used to find novel

applications for food wastes and innovative processing methods for valorization of waste materials to be used for other food applications.

CHAPTER 4. Evaluation of Brewer's Spent Grain as a Novel Growth Media for Yeast

Abstract

Brewer's spent grain (BSG) is a by-product generated in the beer manufacturing industry, which is extremely rich in protein and fibre. In this study we use low cost BSG as the raw material for the production of a novel growth media, through a bioconversion process utilizing food grade fungi to hydrolyse BSG. The novel fermentation media was tested on the yeast *Rhodosporidium toruloides*, a natural yeast producing carotenoid. The yeast growth was analysed using the growth curve and the production of intracellular fatty acids and carotenoids. Untargeted GC-MS based metabolomics was used to analyse the constituents of the different growth media. Growth media prepared using fermented BSG was found to be able to support the growth in *R. toruloides* (21.4mg/ml) in comparable levels to YP media (24.7mg/ml). Therefore, the fermented BSG media was able to fulfil the complete nitrogen requirement for *R. toruloides* growth. This media was able to sustain normal metabolomics activity in yeast, as indicated by the level of fatty acid and carotenoid production. This can be explained by the fact that, in the fermented BSG media metabolites and amino acids were found to be higher than in the unfermented media, and close to the levels in YP media.

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4.1. Introduction

4.1.1. Brewer's spent grain and its current status

BSG (Brewer's spent grain) is the protein and fibre rich residue produced during the process of beer manufacturing. The availability of spent grain increases with the increase in the beer production levels. Alternative uses for BSG are highly sought-after due to the increasing cost of disposal. The majority of the produced BSG is currently disposed in landfills or used as feed for animals such as cattle, pig and chicken [12]. BSG is a lignocellulosic material abundant in protein and fibre (cellulose, arabinoxylan and lignin) accounting for approximately 20% and 70% respectively. BSG is reported to contain vitamins and minerals as well. Both essential and non-essential amino acids are also reported to be present in BSG [12]. BSG is rich in phenolic compounds along with oligo-saccharides and polysaccharides. Similar to other lignocellulose material, BSG is reported to contain various valuable materials. One of the main reasons hindering the possessing of these materials is the recalcitrance of plant cell walls. Therefore, physical, chemical or biological conversion needs to be performed on the BSG. Being available through out the year at low or no cost at all, makes spent grain a favourable raw material for any potential application.

4.1.2. Yeast industrial uses and yeast nutrition

Yeast possesses the ability to convert sugars into ethyl alcohol and carbon dioxide in the absence of oxygen. Furthermore, yeast can produce some useful compounds, especially related to flavour enhancement. As a result yeasts have found to be useful in many applications related to food production, such as baking, alcoholic beverage production (beer, wine, sake and spirits) and other fermented food production (cheese and sausages). These species are also used in the production of industrial ethanol, enzymes and single cell protein (biomass) manufacturing [220]. Some strains are even used in

bioremediation applications and fine chemical production [221]. Oxygen availability and the provided carbon source type greatly influence the metabolism of this species.

Yeast species acquire essential nutrients from their growth environment using simple food sources. Carbon, hydrogen, oxygen, phosphorus, potassium, magnesium and sulphur need to be available in macronutrient levels, while trace elements are only need to be available in micronutrient levels. Yeast obtains carbon and energy most commonly from sugars. Hydrogen ions are important on growth and metabolism of yeast cells in order to control both extracellular and intracellular pH. Even though yeast cannot fix molecular nitrogen, simple inorganic nitrogen sources are utilized to fulfil this requirement. Sulphur is used in biosynthesis of sulphur containing amino acids. Phosphorus is needed for the nucleic acids and phospholipids, while potassium and magnesium are important in maintaining the main metallic cationic environment in the cell.

4.1.3. Yeast extract and peptone media

Yeast extract – Peptone (YP) is the conventional growth media used for fungi species, in producing a range of bio products such as succinic acid [222], microbial oil [223] and valuable metabolic products [224].

Yeast extract is made through autolysis [225]. Initially yeast is grown at 30°C with sufficient oxygen supply and sugar to provide required nutrients. Then, the culture is centrifuged to concentrate the cells by removing the residual sugar. Next the autolysis is carried out at 45–55°C. Yeast stop growing around 40°C. During this stage, yeasts' endogenous digestive enzymes denature proteins present in the cells to simpler amino acids and peptides, and at the same time making the cell walls permeable. Thereby, the small compounds that are generated are able to be transported out of the cell and mix with the aqueous solution. Afterwards, the liquid solution is separated from the yeast cells.

This liquid is enriched with proteins, amino acids, vitamins (B1, B2, B6, niacin, folic acid, pantothenic acid and biotin) and minerals (potassium, sodium, calcium, magnesium, iron and zinc) from the original yeast. Finally this extract is converted to a paste or concentrated liquid using an evaporation process at around 60°C or converted to powder form using spray drying.

Peptones are a widely used nitrogen source for microbial media. Most peptones are produced by incubating milk or meat with trypsin, pepsin or other proteolytic enzymes, to digest proteins into amino acids, peptides and polypeptides. Sometimes peptones are produced by cooking milk or meat in acid. There are varieties of peptones, such as plant-based peptones (made using potatoes, soybeans and wheat), meat peptones (made from a porcine, bovine or poultry origin), casein peptone (made from animal sources) and gelatin peptone (made by boiling collagen, isolated from animal skin, bones and tissues, and subsequent pancreatic digestion) [226].

However, it is expensive to use YP media in large quantities industrially. Thus, development of a novel cost effective media to support yeast growth will be favourable for many industries. Meanwhile, introducing a substitute for YP media helps to reduce the competition with food sources and natural resources in the YP production process.

The objective of this study was to develop a novel application for BSG, to be used as a growth media for yeast strain, *Rhodosporidium toruloides*. Possibility of introducing an inexpensive substitute for YP media from BSG would allow reducing the cost related to using expensive growth media. Alternatively, such novel media would enable to reduce the competition for food sources and natural resources, while establishing alternative uses for food waste material such as BSG.

4.2. Materials and methods

4.2.1. Brewer's spent grain

BSG was kindly provided by Asia Pacific Breweries (Singapore) Pte. Ltd. and was stored in airtight plastic containers at -80°C till used.

4.2.2. Chemicals

All growth media and solutions were prepared with ultrapure water. Agar, D-dextrose, acetic acid, heptadecanoic acid, chloroform, methanol, pyridine, n-hexane, fatty acid methyl ester mix and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract and peptone were purchased from Biobasic Inc. (Toronto, Canada). Potato dextrose agar was purchased from Merck (Darmstadt, Germany). Carotenoid standards were purchased from CaroteNature (Ostermundigen, Switzerland). Derivatizing agents 10% BF₃-methanol, methoxyamine hydrochloride and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.3. Strains and cultivation

The food grade fungi used to ferment BSG was initially grown on Potato Dextrose Agar plates. The yeast strains *Saccharomyces cerevisiae* (BY4741 wild type strain, European *Saccharomyces Cerevisiae* Archive for Functional Analysis (EUROSCARF)) and *R. toruloides* (CBS 5490, Central Bureau voor Schimmelcultures, Utrecht, The Netherlands) were maintained on agar plates containing 20g of agar, 10g of yeast extract, 20g of peptone and 20g of D-dextrose (per litre).

4.2.4. Medium and culture conditions

All growth media were prepared with deionized water. The fermentation and the extraction of nutrient for the preparation of BSG growth media was performed according to a method proposed by Kiran et al. with minor modification [227]. The unfermented as well as the fermented BSG media were prepared using the same method for the nutrient extraction. The extracts were centrifuged (14500rpm, 20mins, 4°C), filtered (0.45µm) and autoclaved (121°C, 20mins). These solutions were used as the unfermented BSG media and the fermented BSG media to grow *R. toruloides* and *S. cerevisiae*. YPD fermentation media was prepared with 20g of peptone, 10g of yeast extract and 20g of dextrose (per litre).

The BSG fermentation media and BSG unfermented media were prepared with 20g of dextrose per litre of the solution made as described previously. *S. cerevisiae* and *R. toruloides* strains used were grown on YPD agar plates (2% glucose), made of 10g of yeast extract, 20g of peptone, 20g of agar and 20g of dextrose dissolved in 1 litre of ultrapure water. The inoculum was grown in the same YPD media mentioned previously. The inocula (50ml) were grown overnight at 30°C in 250ml Erlenmeyer flasks. These cultures of *S. cerevisiae* and *R. toruloides* were used to inoculate 50ml of the fermentation media with an optical density (OD₆₀₀) of 0.2. Fermentation was carried out at 30°C in 250ml Erlenmeyer flasks incubator for 5 days shaken at 200rpm. Three biological replicates were carried out during the experiments.

4.2.5. Growth analysis

The growth of the yeast cultures was found out using the optical density (OD₆₀₀) of the culture media. Samples were collected every 24 hours for 5 days and OD₆₀₀ was measured using NanoDrop 2000UV spectrophotometer (Thermo Scientific, Waltham,

MA, USA). The OD₆₀₀ values were correlated to dry weight to construct the growth curve for the yeast. Samples with varying concentrations of cells were centrifuged (10,000g, 5mins) and washed twice with ultrapure water, and were analysed using an infrared moisture analyser (Sartorius MA37) to find the dry cell weight. These data was used in constructing a calibration curve for OD₆₀₀ and yeast cell dry weight.

4.2.6. Fatty acid measurement of *R. toruloides*

Lipids were extracted from the yeast cells using the 2:1 chloroform-methanol method adopted by Chen L. W. [228]. 1ml of the culture medium was extracted from the fermentation flask. The cell pellets were washed twice using ultrapure water. These washed cells were centrifuged (10mins, 10,000rpm, 4°C) and separated. The cell pellets were resuspended in 1ml of NaCl (0.9% w/w) and acidified with 200µl of acetic acid. 10µl of heptadecanoic acid (10mg/ml in ethanol) was added as the internal standard (IS) to account for the fatty acid loss during processing. Approximately 300µl of glass beads were added and the cells were disrupted in the FastPrep[®]-24 instrument (30 sec, for 4 times).

3ml of a 2:1 chloroform-methanol mixture was added to the samples. This solution mix was inverted several times, vigorously vortexed, and centrifuged (10,000g, 10min at 4°C). The chloroform layer at the bottom was transferred to another tube. This step was repeated once again. The collected chloroform was dried overnight to dryness on a heatblock at 30°C.

Fatty acid derivatization was performed according to a method used by Horak et al. [187]. The dried lipids were redissolved in 500µl of 10% BF₃-methanol and incubated in sealed tubes in a heatblock at 95°C for 20 min. Afterwards, the tubes were cooled down to near room temperature. 300µl saturated NaCl in water and 300µl n-hexane were

added. Samples were centrifuged (14,000rpm, 10mins, at room temperature). The upper hexane layer containing the extracted fatty acid methyl esters (FAMES) were transferred to glass vials for GC-MS analysis. FAME mix C8-C24 was used as the standard. FAME standard curves were obtained by running varying concentrations (0.125-1 mg/ml) of the FAME mix C8–C24 and internal standard.

Separation and detection of chromatograph was performed using Agilent Technologies 7890A GC- 5975 MSD system. 1µl samples were injected into the HP-5MS (5% Phenyl-Methyl-Silox, 325C: 30m × 250µm × 0.25µm Agilent J&W Scientific, Folsom, CA, USA) capillary column by splitless mode using an auto-injector. The solvent cut-off was set to 3.5mins. Carrier gas (helium) was set to flow at a rate of 1.1 ml/min. The inlets temperature was maintained at 250°C and MS source temperature at 230°C. The oven was maintained at a temperature of 80°C for 1 min, then elevated to 250°C at a rate of 7°C/min, and finally held at 250°C for 10 min. Data were collected in a full scan from 35–600 m/z. The detected peaks were integrated after noise reduction and baseline correction.

4.2.7. Carotenoid measurement

1ml of the culture was used to measure the intracellular carotenoid production by *R. toruloides*. The samples were centrifuged for 10mins at 10,000rpm, 4°C and washed with ultrapure water. The cell pallettes were broken down using glass beads in the Fast Prep Grinder (MP Biomedicals, Solon, OH, USA). Carotenoids were extracted with 1ml of acetone until the cell pallets were colourless.

Agilent 1100 high-performance liquid chromatography (HPLC) equipped with a photodiode array detector was used to identify and quantitate the carotenoids extracted. Carotenoids, (1) torularhodin, (2) torulene, and (3) β-carotene were identified using the

standards. Li-Chrospher 100 RP-18 column (250mm×4.6mm id, 5µm) and a guard column (4mm×4mm id) of the same material (Merck, Rahway, NJ, USA) were used for the separation. Acetone and ultrapure water were used as the mobile phases with a gradient from 70% to 100% (acetone in ultrapure water) at a 0.5ml/min flow. Detection was performed at 450nm, and the UV-vis absorption spectra were recorded online using the photodiode array detection system [229].

4.2.8. GC-MS sample preparation and analysis for media metabolites

1.5ml of the original sample media was spiked with 10µL internal standard (ribitol, 2 mg/mL dissolved in water) and freeze dried. The freeze dried samples were derivatized for GC-MS analysis according to Wang et al. [186]. Methoximation was performed by dissolving the samples in 50µL of methoxyamine hydrochloride (20 mg/mL in pyridine) to protect the carbonyls and incubating at 37°C for 60min. Afterwards, silylation was performed by adding 100µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) to each sample and incubating at 70°C for 30 min. Subsequently the samples were shaken for 60mins at room temperature and then analysed in GC-MS. All samples were analysed within few hours after preparation.

Metabolites were analysed using Agilent 7890A GC-5975C inert MSD (with Triple Axis Detector) system (Agilent Technologies, CA, USA) equipped with a HP-5MS, 5% Phenyl-Methyl-Silox capillary column (30m×250µm×0.25µm Agilent J&W Scientific, Folsom, CA, USA). 1µl samples were injected to the system by the autosampler in splitless mode. The solvent cut-off was set to 5mins and helium at a flow rate of 1.1ml/min was used as a carrier gas. The inlet temperature and ion source temperature were maintained at 250°C and 230°C respectively. The oven temperature

was kept at 75°C for 4 min and increased at 4°C/min to 280°C and remained for 2mins.

Data was acquired in full scan mode from 35 – 600 m/z .

4.2.9. Data processing

Chromatographic peak deconvolution and identification were processed using Agilent MassHunter Qualitative Analysis software (B.06.00). The list of compounds extracted was subjected to alignment, normalization (according to the IS, ribitol) and filtering in Mass Profiler Professional (B.02.01). Afterwards, PCA was performed on the obtained data to eliminate any outliers present in the data. Clustering heatmap was created using K-Means algorithm and Euclidean distance calculation. Statistical analysis of the data was performed by one-way ANOVA followed by the post hoc Tukey's Honest Significant Difference (HSD) to the determined significant metabolic changes; $p < 0.05$ was considered significant. The multiple testing was adjusted by Benjamin Hochberg false discovery rate (FDR). Accurate masses of the compounds were searched against NIST mass spectral library with similarity above 75% for feature identification. Statistical analysis was performed on these identified compounds to find out the importance of change.

4.3. Results and discussion

4.3.1. Growth of *R. toruloides* in unfermented BSG media

This study investigated the suitability of a nutrient media produced by BSG to grow *R. toruloides* in. The study explored the potential of BSG media to be the sole carbon and nitrogen source for the yeast. Initially, nutrients were extracted from original unfermented BSG to produce the growth media as the starting point. *R. toruloides* was cultured in the media under controlled parameters and monitored daily over a period of 5 days using the optical density of the fermentation medium. The hypothesis was that the

nutrient extract was able to provide all growth nutrients for yeast, being the sole carbon source and the nitrogen source. No growth was observed in the yeast when compared with the growth in conventional YP media (2% w/w glucose) (Figure 4.1). Afterwards, another trial was carried out, to use the BSG nutrient extract only as a nitrogen source, where dextrose (2% w/w) was added externally as the carbon source. But the observation was that in comparison to the YP growth media (2% w/w glucose) the growth was remarkably low (Figure 4.1). Hence, media from unfermented BSG was unsatisfactory to be used as a growth media.

From this trial it can be concluded that a pre-treatment on the BSG was required to enhance the extraction process. A biological treatment was more suitable for this application, as the final usage was to grow an organism.

Figure 4. 1: Growth curves for *R. toruloides* when grown in YPD, Unfermented BSG and Unfermented BSG (2%w/w glucose) media [230].

4.3.2. Growth of *R. toruloides* in fermented BSG media

Another trial was carried out using fermented BSG to produce the growth media. The BSG was fermented using food grade fungi. Initially, the BSG media was used as the sole nutrient source (to supply both carbon and nitrogen requirements) for the yeast to grow. But, the media was unsuccessful as no growth was observed (Figure 4.2). Similar to the previous trial using unfermented BSG, afterwards an additional carbon source (2% w/w glucose) was added and the experiment was repeated. It was seen that the growth was improved in *R. toruloides* of the fermented media (Figure 4.2). Moreover, it was observed that the yeast strains were able to grow at a level similar to YPD media. The fermented BSG growth media showed exceptionally better growth in *R. toruloides* compared to unfermented BSG growth media, and showed similar growth patterns when compared with YPD media. However, according to the trend in the growth curve, fermented BSG media is only able to fulfil the nitrogen requirement for yeast growth and an external carbon source (2% w/w glucose) had to be supplied. Thus, a prior fermentation step needs to be performed since the growth media produced from the original BSG was unable to sustain the yeast growth.

Figure 4. 2: Growth curves for *R. toruloides* when grown in YPD, Fermented BSG and Fermented BSG (2%w/w glucose) media [230]

4.3.3. Fatty acid and carotenoids production

R. toruloides, being an oleaginous microorganism is reported to accumulate lipids. When the yeast cells were analysed it was observed that methyl esters of oleic acid (C18.1) and linoleic acid (C18.2) were present. The total amount of fatty acids produced from *R. toruloides* in 1ml of different media can be summarized as shown below (Table 4.1). According to the results, the YPD media showed the highest level of fatty acid production (8.69g/L) by yeast, but the yeast grown in fermented BSG media were able to produce fatty acids in levels (7.032g/L) comparable to the YPD media. However, *R. toruloides* produced significantly low amount of fatty acids in the unfermented BSG media (1.69g/L). Lipid content (% g/g biomass) variation showed a similar descending pattern by *R. toruloides* grown in YPD, fermented BSG and unfermented BSG media respectively.

Table 4. 1: Production of fatty acids by *R. toruloides* in the three different culture media at the end of the fermentation, indicated as *mean ± standard deviation* [230].

Culture media	Fatty acid content (%) (g lipid/g biomass)	Fatty acid yield (g lipid/L)
YPD media	44.36 ± 3.29	8.69 ± 0.23
Fermented BSG media	35.31 ± 2.64	7.03 ± 0.32
Unfermented BSG media	22.18 ± 1.19	1.69 ± 0.17

The total fatty acid production by *R. toruloides* in the fermented BSG (7.03mg/ml) was close to the levels produced by the yeasts grown in YPD (8.69mg/ml), unlike the low levels produced by the yeasts grown in unfermented BSG media (1.69mg/ml). The yeasts grown in fermented BSG media also demonstrate an improvement in fatty acid content (35.31% g/g cell dry mass) when compared to those in unfermented BSG media (22.18% g/g cell dry mass).

It is known that oleaginous yeasts begin accumulating lipids when the nitrogen source is exhausted. Therefore, there exists an inverse correlation between the cell mass production, which depends on the initial nitrogen content, and the fatty acid accumulation by the cells, depending on the C/N ratio of the media. In oleaginous yeasts, lipid accumulation could occur via two different pathways. (1) *de novo* synthesis, acetyl-CoA and malonyl-CoA building blocks are used for the lipid synthesis, and (2) *ex novo* lipid accumulation pathway, in which fatty-acids, oils and tricarboxylic acids from culture media are involved. Huang et al. reports that the organic nitrogen source used in fermentation affects the lipid production [231]. Moreover, organic nitrogen sources tend to increase lipid production, while inorganic nitrogen sources favour cell growth [232]. When sugars are used as the carbon source, oleaginous yeasts are reported to produce unsaturated fatty acids, such as, oleic acid (C18.1) and linoleic acid (C18.2), which is of more value in terms of biological function and economic value [233]. It is being reported that the main requirement for high lipid production rate is the excess of the carbon source contained in the medium, while under nutrient conditions for the nitrogen source [234], maintaining a high C/N ratio. Trace metal ions such as Mn^{2+} , Mg^{2+} , Cu^{2+} , Zn^{2+} and Ca^{2+} are reported to influence the biomass and lipid accumulation [235]. This observation in the study stated that the fermented BSG is able to provide the adequate nutrient requirement for *R. toruloides*' normal metabolomics activities.

Carotenoids are an important type of metabolites produced by *R. toruloides*. The HPLC analysis of this yeast confirmed the production of 3 types of carotenoids; torularhodin, torulene and β -carotene. A qualitative analysis of these 3-types suggests similar level of carotenoids production even if the nitrogen source of the fermentation media was changed. Yeasts in YPD media was able to produce the highest amount of torularhodin closely followed by yeasts grown in fermented BSG media. An opposite pattern was observed in torulene, where *R. toruloides* grown in unfermented BSG

produced the highest quantity. The level of β -carotene production was quite close within all three-test subjects (Figure 4.3).

Figure 4. 3: Relative production of carotenoids; torularhodin, torulene and β -carotene by *R. toruloides* when grown in YPD, Fermented BSG (2%w/w glucose) and Unfermented BSG (2%w/w glucose) media [230].

In the carotenoid synthesis pathway Lycopene is the branch point of β -carotene and torulene/torularhodin pathways, and torularhodin is produced by further oxidation of torulene. According to Figure 4.3, the YPD media and fermented BSG media seem to be promoting further oxidation of torulene, unlike the unfermented BSG media. Carotenoids, being a secondary metabolite, in most yeasts initiates production during the late logarithmic phase and continues till the end of stationary phase. Media with a high C/N ratio tends to promote lipid production rather than carotenoids. However, as growth progresses the C/N ration changes promoting production of secondary metabolite such as carotenoids [236]. Higher quantities of carotenoids produced when inorganic nitrogen salts are used. A study reports the highest level of β -carotene production when ammonium nitrate (NH_4NO_3) is used as the nitrogen source and highest level of torulene production when peptone is used [237]. The supplementation of the growth media with

amino acids (alanine and threonine) tends to increase the carotenoid production and this could be as a result of amino acids influencing the pigment formation [238].

The fatty acid production and the carotenoid production by *R. toruloides* in the fermented BSG was close to the levels produced by the yeasts grown in YPD, unlike the low levels produced by the yeasts grown in unfermented BSG media. Hence, from the observations from fatty acid production and carotenoid production in the study it is clear that the fermented BSG is able to provide the adequate nutrient requirement for the normal metabolomics activities of *R. toruloides*.

4.3.4. Growth of *S. cerevisiae* in BSG media

Since the new fermented BSG media was able to sustain the growth of *R. toruloides*, the same was investigated afresh using another yeast strain. The experiment was repeated using *S. cerevisiae* and the three types of media. The results obtained were similar to the trials with *R. toruloides* (Figure 4.4). The fermented BSG media showed similar growth patterns to the YPD media only when an external glucose source was supplied. The unfermented BSG media was not able to sustain the *S. cerevisiae* growth.

A previous study reports how BSG together with discarded oranges is used in cell mass production [172]. The solid-state fermentation was based on *K. marxianus*, kefir and *S. cerevisiae*. Using BSG, these yeast species were able to generate maximum levels of $0.21 \pm 0.05 \text{ g/g}_s$, $0.10 \pm 0.03 \text{ g/g}_s$ and $0.26 \pm 0.02 \text{ g/g}_s$ of cell biomass respectively. Whey, molasses, potato, orange and malt spent rootless in different quantities were also used for the fermentation along with BSG. Our study indicates a growth of $0.71 \pm 0.1 \text{ g/g}_s$ of *R. toruloides* cells and $0.023 \pm 0.001 \text{ g/g}_s$ of *S. cerevisiae* cells using submerged fermentation. Yet, even when the conventional YPD media was used to grow yeast it showed similar levels to the results obtained using the media derived from BSG. The

lower cell mass levels could be due to the differences in the initial inoculum levels and the changes in growth conditioned supplied to the yeasts.

Figure 4. 4: Growth curve for *S. cerevisiae* [230].

4.3.5. Metabolite profiling of growth media

Figure 4.5: Spectra observed by GC-MS of Unfermented BSG media (top), Fermented BSG media (middle) and YP media (bottom) [230].

Metabolome of the YP, fermented BSG media and unfermented BSG media was examined to gain insight into their variation. The studies were established using GC-MS results and multivariate data analysis. Figure 4.5 illustrates the spectra of the three different media overlaid together. Even if the visual inspections show that there are changes among the media a reliable method, multivariate analysis, was used to accurately describe the variations. Principal component analysis (PCA) is a commonly used method to reduce the raw data dimensionality into few principal components describing the maximum possible variation in the data. PCA scores plot clearly depicted that the three media considered in this study inherited varying properties among them (Figure 4.6).

Figure 4. 6: PCA plot for extracted metabolites from the different media. Component 1 vs. Component 2 of YP media (brown circle), Fermented BSG media (red square) and Unfermented BSG media (blue triangle) [230].

All significantly differentiating metabolites determined were visualised using hierarchical cluster analysis (HCA) (Figure 4.7). The HCA diagram validates the increased metabolite concentrations in the fermented BSG media after the fermentation process and its relative proximity to YP media. The profiling data from the metabolomics analysis demonstrated a complex variation of metabolites in the fermented BSG media compared to the unfermented. This fact was further validated by the clustering analysis. This clustering analysis also demonstrated close similarity in the fermented BSG media and YP media, which partially explained why *R. toruloides* exhibited similar growth patterns in both media.

Figure 4. 7: Heatmap correlation of YP media, Unfermented BSG media and Fermented BSG media metabolite extract GS-MS spectra. Metabolites indicated in red are up regulated and the ones in blue are down regulated [230].

Out of 129 metabolites aligned from the analysis only 38 were identified through the library search. The identified differential metabolites found in fermented and unfermented BSG media and YP media are represented in Table 4.2.

Table 4. 2: List of identified metabolites extracted from the growth media with their relative abundances measured as signal values by the MS detector [230].

Compound Name	Fermented BSG media	Unfermented BSG media	YP media
2-butenedioic acid	47,556	0	7,495
2-hydroxyisocaproic acid	428,585	703,628	0
Gluconic acid	42,443,796	65	5
Adenine	5	0	4,528,553
Alanine	92,608,400	47,046	79,485,704
Asparagine	232,784	950	27,334,972
Butanedioic acid	3,847,280		30
Glycine	267,426	466,725	448,586
L-5-oxoproline	29,703,624	363	80,928
L-cysteine	739,977	0	0
L-glutamic acid	82,777,448	0	70,706,632
L-leucine	6,023	0	44,094,996
L-lysine	646	0	656,699
L-ornithine	6,495,508	2,539	77,047,232
L-threonine	43,247,852	96	85,696,200
L-tyrosine	0	0	65,736
L-valine	753,659	0	608,750
Maltose	0	47	4,080,956
Phenylalanine	4,407,764	0	58,384,488
Phosphonic acid	2,996	0	20,095,596
Phosphoric acid	69	703,758	30,840,496
Propylene glycol	95,308	293,640	0
Putrescine	32,753,962	6,894	380,827
Serine	691	0	92,337,480
Stearic acid	9,795,037	4,565,880	775,570
Uracil	9,385,660	2,289	4,053,908

According to the significant analysis performed on the identified metabolite profiling data, fermented BSG media reported a higher concentration of essential amino

acids (L-threonine, L-valine, phenylalanine, L-leucine, L-lysine), non-essential amino acids (alanine, asparagine, L-cysteine, L-glutamic acid, serine), gluconic acid, butanedioic acid, L-5-oxoproline, L-ornithine, phosphonic acid, putrescine, stearic acid and uracil when compared to unfermented BSG media. According to this data it is clear that the fermentation has increased the availability of essential and non-essential amino acids along with other compounds. Meanwhile, the fermented BSG media demonstrated similar or higher levels of concentrations in L-valine (essential amino acid), non-essential amino acids (alanine, L-cysteine, L-glutamic acid), 2-butenedioic acid, 2-hydroxyisocaproic acid, Gluconic acid, butanedioic acid, L-5-oxoproline, propylene glycol, putrescine, stearic acid and uracil when compared with those identified in YP media. According to the metabolomics profiling data, it further confirms that the fermentation step and the autolysis during the nutrient extraction has increased the availability of simpler nitrogenous compounds, especially amino acids, present in the media. When the fermented and the unfermented BSG media are compared, it can be deduced that the intermediate fermentation step has helped to break down the BSG proteins. This could be due to proteases secreted by the microbial during solid-state fermentation.

From a cost perspective, to produce 1 litre of conventional growth media it will cost approximately SGD25 for peptone and SGD5 for yeast extract. Whereas, to produce 1 litre of the novel media it will only cost roughly SGD3.

4.4. Conclusion

In this study, we aim to extract nutrients present in the BSG and develop a novel growth media for yeasts. The prepared growth media was compared against conventional YPD media. None of the BSG media was able to fulfil the requirement of a carbon source. Fermented BSG media successfully fulfilled the complete nitrogen requirement

for yeast growth. The biological pre-treatment and autolysis enhanced the novel media by improving the accessibility of nitrogen sources. Fatty acid production and carotenoid production of *R. toruloides* cells in fermented BSG media were reported in comparable levels to those grown in YPD media. Development of this novel media from BSG will be a more sustainable option while offering a cost-effective alternative for expensive nitrogen sources for yeast cultivation.

CHAPTER 5. Conclusions and future perspective

5.1. Research summary

This thesis investigated how the value of waste BSG can be increased using fermentation methods focused towards improving food applications to facilitate in achieving food sustainability. The importance of this study is two fold; (1) Investigation of how fermentation can be used to convert BSG into an economically viable value added material, and (2) Use metabolomics as the analytical methodology to investigate the fermentation products and sample constituents. Moreover, we have discussed about a process to develop a novel media in which yeast could be grown. Conclusions from this research can be summarized as follows.

The driving force behind our study was the high volume of BSG produced, which are not being used to its fullest potential. Currently, BSG is being treated as a troublesome waste material. This is a grave problem for the brewery industry due to the massive cost that needs to be incurred in disposal of waste. The current practice is to use the generated spent grain for dumping in landfills or for feeding animals, such as cattle, pigs and poultry. As most of the starch content in the original barley is used up during beer production, the major portion of the leftover substances are fibres and proteins in spent grain. Even though this material is a rich source of fibres and proteins, no substantial usage has been taken. Therefore, we used fungal fermentation to improve the nutrient content of BSG and to produce value added compounds from the fermentation metabolites produced. Manufacturing industries are working towards achieving sustainable practices in manufacturing and operation as a result of the stringent regulations on environmental practices and waste handling methods. Therefore, valorization techniques such as the study we have conducted are helpful for commercial industries in meeting sustainable manufacturing.

In Chapter 3 we investigated the value added compounds produced during the BSG fermentation process. For this study a global metabolomics approach was more suitable as it can provide a broader understanding on the changes observed in molecular level. Compared to conventional analysis methods or targeted metabolomic approaches, the untargeted metabolomics approach was able to provide a comprehensive analysis. In this part, a pipeline workflow for the metabolomics study was developed. We optimized the metabolite extraction process and the sample preparation for the GC-MS analysis.

Figure 5. 1: Summary of the BSG fungi fermentation and the resulting value addition

According to the metabolomics analysis we found out that the fermented BSG was enriched with higher content of amino acids, citric acid, vitamins and antioxidants (Figure 5.1). Therefore, the fermented BSG will be enriched with functionally important substances appealing as a raw material for other food applications.

In Chapter 4 we developed a method to produce a growth media to sustain the normal yeast activity using fermented BSG. This novel growth media is helpful in lowering the cost associated with yeast growth when conventional media is used (Figure 5.2). The main task in this section was to design the process to extract fermented

metabolites from the solid material. When the new BSG media was compared against the conventionally used YP media, it was noted that the new media was able to provide the complete nitrogen requirement for yeast to sustain its normal activity.

Figure 5. 2: Summary depicting the usefulness of the new media produced from BSG

Conclusively, this work has shown that fermentation can be used in enhancing the economic value of BSG. Furthermore, metabolomics is a valuable analytical method in investigating the composition of fermented products. The importance in this thesis study is the investigation on how an industrial waste biomaterial such as BSG can be further used to increase the global food and nutrition supply. The overall outline and the main findings are summarised as depicted in Figure 5.3.

Figure 5. 3: Overall workflow and main findings of the thesis

5.2. Limitations

This thesis aims at valorising underutilized spent grain and finding new applications to use BSG in a more meaningful way. However, some limitations were identified as mentioned below.

In this study the cell mass was measured as an indication of growth for both the yeast species *R. toruloides* and *S. cerevisiae*. However, the cell mass measured using optical density does not only measure viable cells. Moreover the media developed in this study was only able to provide the required nitrogen content for yeast growth. Therefore, an external source of glucose was required to be supplied.

Study of metabolomics uses sophisticated analytical techniques and advanced computational methods to characterize complicated biological samples. Yet, two of the major drawbacks of metabolomics are, (1) the complex nature of the collected analytical data and difficulties in interpretation, and (2) the inability in predicting the number of metabolites that will be produced in a bio-system. In addition, this study was aimed towards conducting an untargeted metabolomics analysis for qualitative purposes. Therefore, in order to perform quantitative measurements a targeted analysis needs to be performed. Moreover, in this thesis metabolite identification was done exclusively by comparing the accurate masses of the compounds to available libraries. Accuracy of the identification can be enhanced by running standards of the shortlisted compounds of interest.

5.3. Future perspective

In this research project we have attempted to investigate how fermentation can be used in producing important fermentation products and developing novel applications for BSG aiming towards future food applications. Nevertheless, to overcome the limitations

discussed in the previous section and to improve the overall study some suggestions can be proposed.

The experiments for this study were conducted in small lab scale quantities. Therefore, the next stage in the project will be to scale up the production volumes. Fermentation being a simple technology still attracts the attention of many researchers and industries worldwide, due to its potential and diverse applications. However, there still exists space for development in fermentation techniques to find solutions for typical drawbacks. Main considerations in the scaling-up of solid-state fermentation lies in heat generation and sample heterogeneity, which can cause severe problems. Selection of microorganisms, substrates and bioreactors are the most important parameters to be considered in a typical fermentation scale-up [89]. In addition to processing conditions in scaling up in a scenario of commercialization, downstream processing and waste treatment need to be considered as well.

Extraction is an important factor to consider in developing economically viable industrial applications after BSG is fermented using the techniques mentioned in this research project. The principal parameter affecting the extraction process is reported to be the matrix properties and composition of the sample [239]. Non-conventional extraction methods such as, ultrasound-assisted extraction, microwave extraction, enzyme based extraction, pressurised liquid extraction and supercritical fluid extraction, have shown to be advantages as a result of lessened extraction times, environmental friendliness by not generating any waste solvents, high extraction selectivity and low temperature extractions [240]. Supercritical fluid extraction of decaffeinated coffee/tea, defatted cocoa and extracts from natural material have shown promising results in previous studies [241]. Extrusion is another method used in food production. The recently developed supercritical fluid extrusion demonstrates to be a promising technology in extracting

compounds from material without the effects of high temperature and high shear forces, as seen in traditional extrusion techniques [242].

In this study, we have only used the GC-MS for metabolite detection. However, with the advancement of separation and chromatographic techniques there are more methods to detect metabolites with greater accuracy, sensitivity and resolution. In addition to GC using an additional platform such as liquid chromatography can enhance the coverage of metabolite detected in an analysis. Therefore, a platform such as UPLC-QTOF will be able to provide additional information to the study we have performed.

The metabolites in this study are tentatively identified by comparing their reported accurate masses to available libraries. If the matching score is high, it is assumed to be a good match. However, to accurately confirm the compounds they have to be compared to the peaks from pure standards of the same compounds as stated by the Metabolomics Standards Initiative (MSI) [210]. MS/MS mode, also known as tandem mass spectrometry, can be used to further identify the metabolites. In this mode the molecules are fragmented using collision induced dissociation (CID). Triple quadrupole (QQQ) and quadrupole time of flight (Q-TOF) are the commonly used mass spectrometers in such metabolomics analysis work.

While an untargeted analysis can provide the basic details for the metabolomics analysis, the next stage of this study will be to conduct a targeted analysis for the tentatively identified important fermentation products. A targeted analysis can be performed using the accurate mass and the retention time of the compound's peak when similar operating conditions and similar methods are used in the separation and detection steps. Accurate quantitation can be performed using such targeted analysis and pure standards of the interested compounds. Quantitative results are useful in measuring the performance in bioprocesses.

The traditional system of linear economy uses environmental resources to manufacture beneficial products, and finally dispose these products after use. Considering the scarcity of natural resources such a take, make and dispose approach is unsustainable even if it seems as an efficient way to operate. Circular economy model is a solution for Earth's emerging crisis of resource shortage. In this model the loop is closed and the resources are used for as long as possible. Here the maximum use is extracted while consumed, and afterwards all possible materials are recovered and regenerated when the product life ends.

Figure 5. 4: Conversion of a linear economy to a circular economy involving BSG.

In this thesis we have investigated how to valorize food waste using solid-state fermentation to produce commercially valuable metabolites and how to recover/extract parts of the value-added waste to be used for other applications. This up cycling step is a starting point for zero waste food processing in the food industry to achieve circular economy. Therefore, to close the cycle in this project, after extracting valuable compounds from the fermented BSG the remaining fibrous biomass can be further utilized for other applications (Figure 5.4). One proposed function can be for the manufacturing of polymer films using microbial fermentation to produce packaging material with special characteristics such as biodegradability [243].

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List of Publications

1. **Cooray, S.T.** & Chen, W.N., (2018). Valorization of brewer's spent grain using fungi solid-state fermentation, *Journal of Functional Foods*, 42, 85-94
2. **Cooray, S.T.**, Lee J.J.L.& Chen, W.N., (2017). Evaluation of brewer's spent grain as a novel media for yeast growth, *AMB Express*, 7:117

Conferences attended

1. **Cooray, S.T.** & Chen, W.N., (2017). Metabolomics approach to investigate food waste valorisation through fermentation, *Metabolomics 2017: 13th Annual Conference of the Metabolomics Society*, Brisbane, Australia