Solid State Fermentation of Brewers' Spent Grains for Improved Nutritional Profile Using *Bacillus subtilis* WX-17

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Abstract: Brewers' spent grains (BSG) are underutilized food waste materials produced in large quantities from the brewing industry. In this study, solid state fermentation of BSG using *Bacillus subtilis* WX-17 was carried out to improve the nutritional value of BSG. Fermenting BSG with the strain WX-17, isolated from commercial natto, significantly enhanced the nutritional content in BSG compared to unfermented BSG, as determined by the marked difference in the level of metabolites. In total, 35 metabolites showed significant difference, which could be categorized into amino acids, fatty acids, carbohydrates, and tricarboxylic acid cycle intermediates. Pathway analysis revealed that glycolysis was upregulated, as indicated by the drop in the level of carbohydrate compounds. This shifted the metabolic flux particularly towards the amino acid pathway, leading to a 2-fold increase in the total amount of amino acid from 0.859 ± 0.05 to 1.894 ± 0.1 mg per g of BSG after fermentation. Also, the total amount of unsaturated fatty acid increased by 1.7 times and the total antioxidant quantity remarkably increased by 5.8 times after fermentation. This study demonstrates that novel fermentation processes can value-add food by-products, and valorized food waste could potentially be used for food-related applications. In addition, the study revealed the metabolic changes and mechanisms behind the microbial solid state fermentation of BSG.

Keywords: solid state fermentation; brewers spent grain; metabolomics; nutrition; pathway analysis

1. Introduction

An estimated one third of the food produced globally is lost during processing or wasted. Global food production is expected to rise due to the expanding population in the world. This would lead to huge amounts of food wastages annually, which cause significant environmental, economic, and climate issues. The nutritional contents remaining in these food processing residues can be harnessed to potentially become materials for other processes. Utilizing these residues would be a viable and cost-effective solution to the issues caused by massive amounts of food wastes generated worldwide [1].

In the beer industry, large quantities of by-products are generated. In particular, brewers' spent grain (BSG) represents 85% of the total by-products [2]. The remaining by-products includes spent hops and surplus yeast. The annual global production of BSG is estimated to be 38.6×10^6 tons [3]. It was reported that approximately 14–20 kg of BSG could be generated from 100 L of beer [4].

Currently, most of the BSG is used as animal feed, which contribute to methane gas production, while the minority are disposed in landfills [5]. Hence, other more economical and environmental friendly uses for BSG are needed.

The components of BSG consist of the barley malt grain husks, pericarp, and seed coat layers of the grains [6,7]. BSG are lignocellulosic materials that contain cellulose, non-cellulosic polysaccharides, and lignin [6]. Also, BSG were found to be abundant in proteins, essential amino acids, fiber, and phenolic compounds [3,8]. Nutrients such as lipids, fatty acids, and polyphenols were also found to be present in BSG [9,10]. The predominant lipids identified were triglyceride and the fatty acids were linoleic, palmitic, oleic, α-linoleic, and stearic acids. Other fatty acids, including myristic and vaccenic acids, were present in lower amounts [9]. One of the main challenges in the utilization and extraction of useful components in BSG is that the proteins and nutrients are bound to the cellulose and hemicellulose. Hence, in order to extract these compounds, physical, biological, chemical pre-treatments, or a combined pre-treatment are required [11]. Biological pre-treatment would be a more environmentally friendly option as it does not require chemicals or solvents, and has the added advantage of not generating toxic compounds [12]. For agricultural by-products, solid state fermentation (SSF) commonly serves as a biological pre-treatment method, which is also convenient and economical. This is because SSF requires less energy, produces less wastewater, and hence is overall more environmentally friendly [13]. The amount of polysaccharides, along with the other nutritional contents in BSG, also makes it a suitable substrate for SSF [10]. This has been successfully carried out with fungi and some bacteria on food such as soybean waste, rice, cassava, corn cobs, and bagasse [14,15]. Some of the targeted products generated by SSF studies were antioxidants, protein content, and lipids [16,17]. Until now, some microorganisms, such as *Trametes versicolor* and *Streptomyces* sp. strain AMT-3, have also proven to be able to grow using BSG as the carbon and nitrogen sources for production of xylanase, laccases, and polyphenols under SSF conditions [18,19]. These studies showed the potential of using microorganisms to valorize BSG, and hence generating products that can be used in food-related processes.

Bacillus are generally regarded as safe (GRAS) species, which are well known to secrete abundant amounts of extracellular enzymes [20]. In this study, we used *Bacillus subtilis (B. subtilis)* WX-17, which was previously isolated from natto (traditional food in Japan, where cooked soybeans are fermented using *B. subtilis*, giving beneficial effects in health) [21] as the host for SSF to enrich the nutritional content of BSG. Gas chromatography-mass spectrophotometry (GC-MS) was applied for metabolomics analysis to provide important insights into the mechanism behind BSG fermentation with *B. subtilis* WX-17. To date, few studies have been carried out on metabolic profiling of fermented BSG. This study would help to shed light on the metabolic changes and mechanisms behind the microbial SSF of BSG. With enrichment of nutritional content in BSG, fermented BSG could possibly be used in food-related applications, such as culture medium, functional food ingredients in human diet, and value-added animal feed.

2. Materials and Methods

2.1. Microorganism for Fermentation

B. subtilis WX-17 previously isolated from Marumiya Kyushu Ichiban natto (Japan) (accession number NCIMB 15204) was maintained on nutrient agar plates at 37 °C.

2.2. Brewer's Spent Grains and Fermentation Conditions

BSG were taken from Asia Pacific Breweries (Singapore) Pte, Ltd. (Singapore) and stored in sealed polyethylene bags at −80 °C until used. *B. subtilis* WX-17 was cultured in sterile falcon tube with 5 mL of nutrient broth for 24 h at 37 °C.

Ten g of BSG was placed in boiling water for 20 min and cooled. The BSG were then inoculated with *B. subtilis* WX-17 (1×10^6 CFU/g) and 5 mL of sterile water. The plates of BSG were incubated at

37 °C for two days. The inoculated samples were wrapped with two layers of cling wrap. Both cling wrap layers were punctured with tiny holes using a sterile needle. Samples were collected at 0 h and 48 h to represent unfermented and fermented BSG, respectively. The collected BSG was then lyophilized and stored at –20 °C until further analysis.

2.3. Gas Chromatography-Mass Spectrophotometry (GC-MS) Conditions

Amino acids, fatty acids, carbohydrates, and tricarboxylic acid (TCA) cycle intermediates were detected via GC-MS. The tests were done by the GC-MS system (Agilent Technologies 7890A-5975C Inert MSD) equipped with a HP-5MS capillary column (30 m × 0.250 mm i.d, 0.25 μm film thickness). Samples of 1 μL were injected into the system by the auto-sampler in splitless mode. Carrier gas (helium) was set to flow at 1.1 mL/min.

2.4. Analysis of Amino Acids Using GC-MS

Based on [22], 4 mg of samples were re-suspended in 200 μL of 6 M HCl. Twenty μL of γ–aminobutyric acid (10 mg/mL) were added as the internal standard. The tubes were sealed and incubated for 12–24 h in an oven at 105 °C. The cell hydrolysates were then dried in a heat block set at 95 °C. After drying, 20 μL of dimethylformamide (DMF) and 20 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane purchased from Sigma-Aldrich were added. The tubes were then sealed and incubated at 85 °C for 1 h. Samples were then centrifuged at 14,800 rpm for 5 min and supernatants were transferred to glass vials. Forty μL of DMF were added into the glass vials and the solution was well mixed being sent for GC-MS analysis. Each sample was measured in triplicate.

For amino acid detection, the solvent delay was set to 2.5 min. The ion source temperature and injector temperature were set at 230 and 280 °C, respectively. The oven temperature set to hold at 160 °C for 1 min then ramped to 310 °C at the rate of 20 °C/min, and finally held at 310 °C for 0.5 min. Data were obtained in full scan mode from 180 to 550 *m/z* with a 2–4 scan per sec. The identification of amino acids was carried out accordingly by using a NIST08 mass spectral library. Samples were normalized using γ–aminobutyric acid before comparison.

2.5. Derivatization for Metabolomics Study

Three g of fermented BSG were weighed and 10 mL of methanol was added. The samples were homogenized 6 times, for 30 s each run, using FastPrep-24 MP homogenizer. In between the homogenization, the samples were placed in ice bath to cool the sample. The samples were then centrifuged at 9000× *g* for 10 min at 4 °C. The supernatant containing the metabolites, both carbohydrates, and tricarboxylic acid (TCA) cycle intermediates were pressed through a syringe attached with a 0.22 μm filter. Then, 1.5 mL of filtered supernatant was added with 10 μL of 2mg/mL ribitol dissolved in ultrapure water as the internal standard. Samples were dried in a heat block at 30 °C overnight. According to the method in a previous study [23], the dried samples were then derivatized with 50 μL of methoxamine (MOX) hydrochloride in 20 mg/mL pyridine and incubated at 37 °C for 1 h. Then, silylation was carried out by adding 100 μL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) to the precipitate and incubated at 70 °C for 30 min. Each sample was measured in triplicate.

The ion source temperature and injector temperature were set at 230 and 250 °C, respectively. The oven temperature was as follows: 75 °C for 4 min, ramped to 280 °C at the rate of 4 °C/min, and held at 280 °C for 2 min. Data were acquired in full scan mode from 35 to 600 *m/z* with a 0.3-s scan time. The identification of carbohydrates was carried out accordingly by using the NIST08 mass spectral library. Normalization was done using ribitol before comparison.

2.6. Analysis of Fatty Acids Using GC-MS

Ten mg of fermented BSG and 10 mg of fresh BSG (control) were weighed and placed into Eppendorf tubes following a method from a previous study [23]. Then, 1 mL of 0.9% NaCl solution and 200 µL of acetic acid were added to each sample. Then, 10 µL of 10 mg/mL heptadecanoic acid dissolved in ethanol was added as internal standard. The samples were sonicated for 30 s each. Then, 3 mL of a chloroform-methanol 2:1 solution was added and the samples were inverted several times, vortexed vigorously, and centrifuged at $10,000 \times g$ for 10 min at 4 °C. Then, 1 mL of the chloroform layer, which is the bottom layer, was collected and dried overnight at 30 °C. The dried lipid residue was re-dissolved in 500 µL BF3-methanol 10% (FLUKA, 15716) and incubated in a sealed vial in a heat block set at 95 °C for 20 min. Fatty acid methyl esters (FAMEs) were extracted with the addition of 300 µL saturated NaCl in ultrapure water, then an addition of 300 µL n-hexane. Samples were vortexed for 5 min and centrifuged at 14,800 rpm for 5 min. Then, 200 µL of samples (top layer) were transferred into glass vials for GC-MS analysis. Each sample was measured in triplicate.

The injector temperature was set at 250 °C and MS source temperature was set at 230 °C. The oven was held at a temperature of 80 °C for 1 min, then elevated to 250 °C at a rate of 7 °C/min, and finally maintained at 250 °C for 10 min. Data were acquired in full scan from 35–600 *m/z*. The identification of fatty acids was carried out by using the NIST08 mass spectral library. Normalization was done by using heptadecanoic acid before comparison.

2.7. Antioxidant Assay

Free radical-scavenging activity of the samples was investigated by 1,1-dipheny-2-picryl-hydrazil (DPPH) with some modifications [24]. Then, 0.6 mM of DPPH solution was prepared in ethanol. Samples for the test were prepared by adding 100mg of fermented BSG after 2 days to 300 µL of ethanol. Then, 150 ul of each sample was drawn and added to a solution of 250 µL of ethanol and 100 µL of DPPH. The tubes were then incubated in a dark place for 30 min at room temperature. The absorbance of each sample was measured at 515 nm against ethanol as the blank using Thermo Fisher NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA.). The antioxidant activity was quantified in signal inhibition percentage. A standard curve was generated by Trolox standards to correlate the weight of Trolox to the signal inhibition (%). The signal inhibition % obtained from each sample was then converted into the weight of Trolox.

2.8. Statistical Analysis

MetaboAnalyst 4.0 (Montreal, Canada) was utilized to construct the clustering heatmap and partial least square-discriminant analysis (PLS-DA) for statistical analysis [25]. Heatmap was clustered with calculated Euclidean distance and ward clustering algorithm. All experiments were conducted in triplicate and the standard deviation was calculated.

3. Results

3.1. Analysis on Untargeted Extracellular Metabolic Profiling

An untargeted metabolomics study was carried out using GC-MS. This was to provide insights into the changes that occurred during fermentation. A total of 35 differential metabolites were identified, which could be categorized into carbohydrates, TCA cycle intermediates, fatty acids, and amino acids. To make sense of the metabolomic changes, the metabolites were mapped on biochemical pathways. Also, to gain an overall view of the changes in metabolite abundance, analysis was carried out using a heatmap and partial least square-discriminant analysis (PLS-DA) [26]. Based on the heatmap (Figure 1), the carbohydrate levels were shown to have decreased and the amino acid, fatty acid, and TCA cycle intermediate levels were found to increase after fermentation for 48 hrs. In addition, the respective changes in the metabolites over 3 days of fermentation are shown in the heatmap in the Supplementary Materials (Figure S1). In general, after 2 days of fermentation, amino and fatty acids were upregulated,

while carbohydrates were downregulated. However, after 3 days of fermentation, fatty acids were downregulated dramatically, which might be due to the lack of carbon source for *B. subtilis* WX-17 leading to the beta-oxidation of fatty acids to generate an additional carbon source. Hence, optimum valorization was achieved after 2 days of fermentation and the analyses of results were based on 2-days data, accordingly.
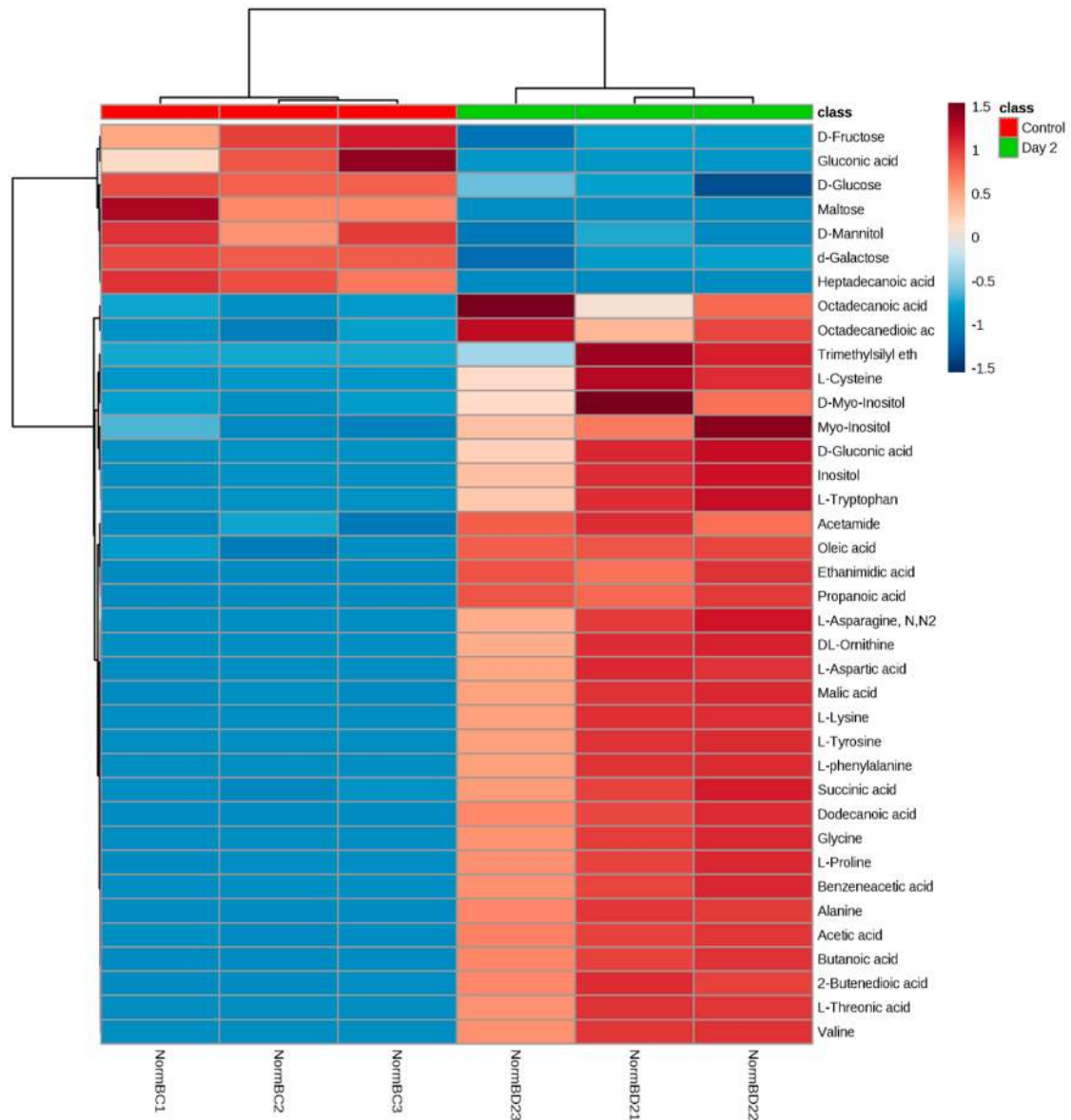

Figure 1. A heatmap analysis of metabolites variations. Fermentation was carried out for 2 days and metabolites were analyzed by gas chromatography-mass spectrophotometry (GC-MS). The unfermented brewers' spent grains (BSG) samples, in triplicate, are displayed from the left, followed by the fermented BSG samples, in triplicate. The areas shaded in red indicate a higher amount of the specific metabolites, whereas the areas shaded in blue indicate a lower amount of the specific metabolites.

Similarly, a clear difference in the metabolites between fermented and unfermented BSG was observed using PLS-DA, as shown in Figure 2. The principal components, PC1 and PC2, displayed a 95.3% and 3.3% variance, respectively. The PLS-DA plot had a variance R^2 value of 0.99, which is considered to be extremely substantial [27]. The observed trend of having overall significant changes in the metabolites based on PLS-DA analysis was congruent and correlated with the trend observed from the clustering heatmap. The metabolic profiles were further investigated by the use of

respective component tests and biochemical pathways triggered during the fermentation of BSG with *B. subtilis* WX-17.
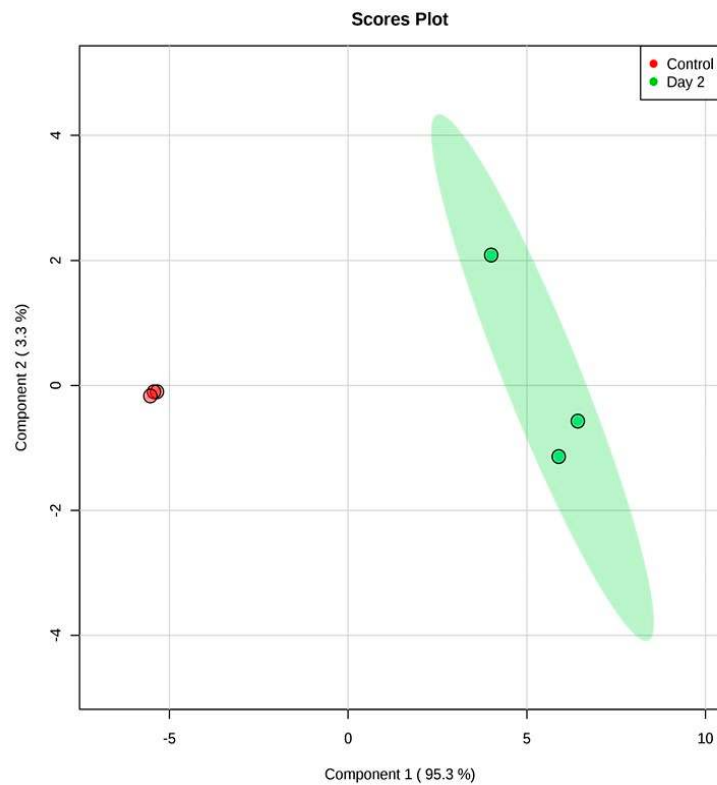

Figure 2. A partial least square-discriminant analysis (PLS-DA) score plot for metabolites analyzed by GC-MS for unfermented BSG (red) and fermented BSG samples (green), with a 95% confidence interval. Each dot represents all of the metabolites detected in each replicate.

3.2. Amino Acids Analysis

The amino acid metabolites were found to increase in the fermented BSG sample as compared to unfermented BSG (Table 1). Several amino acids metabolites were detected, including leucine, phenylalanine, lysine, threonine, serine, proline, glutamic acid, aspartic acid, and tyrosine. The increases in the respective amino acids were calculated and expressed as fold changes (Table 1). Among the amino acids, proline was found to have the highest increase, at 3.5 times. In terms of total amino acids, the amount increased from 0.859 mg/g in unfermented BSG to 1.894 mg/g in fermented BSG after 2 days of fermentation.

Table 1. Amino acids results (mg/g) before and after fermentation.

Amino Acids	Unfermented Brewers' Spent Grains (BSG)	Fermented Brewers' Spent Grains (BSG) (Day 2)	Fold Change
Leucine	0.113 ± 0.031	0.134 ± 0.098	1.185
Serine	0.015 ± 0.005	0.017 ± 0.009	1.812
Aspartic Acid	0.024 ± 0.003	0.034 ± 0.007	1.542
Threonine	0.015 ± 0.005	0.026 ± 0.016	2.092
Phenylalanine	0.021 ± 0.003	0.027 ± 0.015	2.133
Proline	0.349 ± 0.182	1.230 ± 0.568	3.527
Glutamic Acid	0.304 ± 0.20	0.407 ± 0.376	1.625
Lysine	0.012 ± 0.006	0.011 ± 0.010	1.200
Tyrosine	0.006 ± 0.001	0.009 ± 0.001	1.560
Total amino acids	0.859 ± 0.049	1.894 ± 0.125	2.204

3.3. Fatty Acids Analysis

It was shown that the total fatty acid content of BSG increased in the fermented BSG sample as compared to unfermented BSG sample (Table 2). A total of four fatty acids were detected by GC-MS analysis. The fatty acids were hexadecenoic acid (palmitic acid), 9,12-octadecanoic acid (linoleic acid), 9-Octadecanoic acid (oleic acid), and octadecanoic acid (stearic acid). These could be categorized into saturated or unsaturated fatty acids. The essential unsaturated fatty acids were linoleic acid and oleic acid, whereas palmitic acid and stearic acid were the saturated fatty acids. Oleic acid was found to have the highest increase with a 2.366-fold increase, whereas palmitic acid was found to decrease by 0.844-fold in the fermented BSG as compared to unfermented BSG (Table 2). Overall, increasing levels of essential unsaturated fatty acids, and decreasing or unchanged levels of saturated fatty acids after 2 days of fermentation, were observed. Unsaturated fatty acids, such as linoleic acid or oleic acid, would slightly increase the level high density lipoprotein (HDL) cholesterol, also known as good cholesterol, in humans. HDL is reported to aid in the removal of triacylglycerols from the bloodstream. Hence, the increase in unsaturated fatty acids could be interpreted as a higher nutritional value in fermented BSG [28].

Table 2. Fatty acids results (mg/g) in BSG before and after fermentation.

Fatty Acids	Unfermented BSG	Fermented BSG (Day 2)	Fold Change
Palmitic Acid	1.805 ± 0.003	1.523 ± 0.205	0.844
Linoleic Acid	0.445 ± 0.100	0.731 ± 0.220	1.643
Oleic Acid	0.041 ± 0.006	0.097 ± 0.053	2.366
Stearic Acid	4.596 ± 0.091	4.734 ± 0.131	1.03
Total Fatty Acids	6.89 ± 0.055	7.085 ± 0.152	1.028

3.4. Carbohydrates Analysis

In total, 5 carbohydrates were detected. The carbohydrates detected from GC-MS were D-fructose, D-mannitol, D-glucose, D-galactose, and maltose. After 2 days of fermentation, it was observed that D-fructose, D-mannitol, D-glucose, D-galactose, and maltose decreased by 64.9%, 68.9%, 62.2%, 66.5%, and 86.2%, respectively (Figure 3).
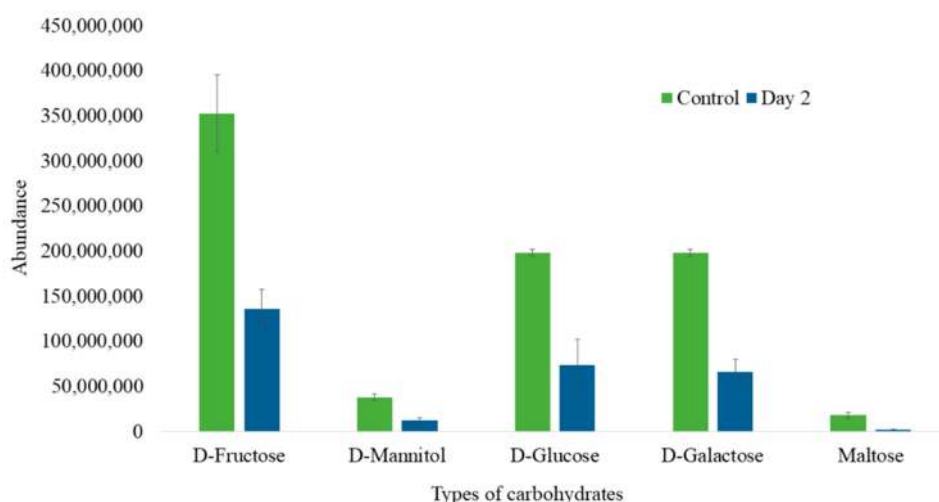

Figure 3. Metabolites belonging to the class of carbohydrate, as analyzed by GC-MS. Green bars represent the levels of the unfermented BSG samples, whereas the blue bars represent the levels of the fermented BSG samples.

3.5. Antioxidant Test

The antioxidant properties of the samples were evaluated through DPPH radical scavenging activity and quantified by Trolox equivalent (Table 3). The method used DPPH assay, which is a test of the amount of electron donation of antioxidants to neutralize the deep purple DPPH radical. The respective samples displayed a visual discoloration, which is reflected in the signal inhibition %. The conversion of signal inhibition % to weight of Trolox was done via calibration curve and it was observed that fermented BSG showed an increase in Trolox equivalent from 1.2 ± 0.03 μg/g BSG to 6.94 ± 0.21 μg/g BSG, an approximate 5.8-times increase in antioxidant activity.

Table 3. Trolox quantification for antioxidant activity before and after fermentation.

Samples	Signal Inhibition %	Weight of Trolox (μg/g BSG)
Unfermented BSG (Control)	4.72	1.2 ± 0.03
Fermented BSG (Day 2)	28.27	6.94 ± 0.21

4. Discussion

The regulations in the metabolites after fermentation were hypothesized by analyzing them using various biochemical pathways. The respective biochemical pathways could have possibly triggered the changes in the metabolites during the fermentation.

4.1. Carbohydrate Pathway Analysis

In the carbohydrate analysis, it was observed that ᴅ-fructose, ᴅ-mannitol, ᴅ-glucose, ᴅ-galactose, and maltose obviously decreased after fermentation (Figure 3). This result indicates that the various sugars were being used by *B. subtilis* WX-17 for growth. It is speculated that various microbial enzymes, such as invertase, amylase, cellulose, and pectinase, are first produced as a bacterial fermentation progress to hydrolyze long-chain polymeric sugars and starch molecules into simpler carbohydrates during fermentation by *B. subtilis* WX-17 [29–32]. For the inter-conversion among the carbohydrates, maltose can be catalyzed by maltose-6′-phosphate glucosidase to produce ᴅ-glucose (starch and sucrose metabolism pathway). ᴅ-glucose can be converted into alpha-ᴅ-glucose-1P and then converted into uridine diphosphate (UDP-glucose) with the aid of the enzyme, UDP glucose pyrophosphorylase [33]. UDP-glucose produces sucrose, which in turn is converted into fructose through the enzyme sucrose phosphorylase (starch and sucrose metabolism); fructose can be converted back into ᴅ-glucose or alpha-ᴅ-glucose to produce galactose (galactose metabolism pathway). In the following step, glycolysis converts glucose into pyruvate to supply adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH), which triggers the generation of other primary or secondary metabolites that are important to cell growth. Besides, it has been reported that BSG contains phytic acid and fermentation reduces the level of phytic acid [7]. This leads to an increase in the product myo-inositol from phytic acid hydrolysis. Myo-inositol was also detected to have increased based on the GC-MS analysis, which reflected the reduction of the antinutrient phytic acid.

4.2. Amino Acids Metabolism Analysis

It was observed that the amount of amino acids increased in the fermented BSG sample, as compared to unfermented BSG. This could be attributed to the enzymes, such as proteases, which are produced by *B. subtilis*. This hydrolyzes the complex proteins present in BSG into simple amino acids [34]. The production of proteases from microorganisms for proteolysis during fermentation and release of amino acids was also observed in another fermented food study [35]. In addition, it is also possible that the amino acids were synthesized from, or associated with, the carbohydrate metabolic pathways.

For instance, serine can be synthesized through glycolysis. In turn, this can aid the synthesis of glycine and threonine, as reactions are reversible. The series of correlated serine, glycine, and threonine

pathways can be seen in Figure 4. The production of serine yields cysteine (cysteine and methionine metabolism pathway). The reversible reactions in the alanine pathway produce pyruvate, valine, and leucine. Mainly, glycolysis and breakdown of amino acids contribute to the production of pyruvate. A decrease in the precursors for glycolysis, such as D-fructose and D-mannitol, could possibly be due to the upregulation of glycolysis. The upregulation of glycolysis increased the production of amino acids (Figure 4). The product of glycolysis, pyruvate, is then converted into acetyl-CoA, which enters the TCA cycle to produce energy, and also aspartic acid. Valine, alanine, and asparagine are produced by aspartic acid (alanine, aspartate, and glutamate metabolism pathway). Also, lysine can be produced from aspartic acid (lysine biosynthesis pathway). Lysine is also produced from 2-oxoglutarate, which is an intermediate of the TCA cycle. The increasing amount of intermediate after fermentation drives the metabolic flux towards the production of lysine, which explains its increase. Glutamate can also be produced when pyruvate enters the TCA cycle. Proline is produced from glutamate (alanine, aspartate, and glutamate metabolism pathway). Proline can also be produced by arginine (arginine and proline metabolism pathway). Through the urea cycle, aspartic acid can be converted into arginine. The production of phenylalanine, tyrosine, and tyroptophan mainly stems from the compound phosphoenolpyruvate (PEP). PEP is converted into shikimate with the aid of the enzyme shikimate dehydrogenase, which then forms chorismate (shikimate pathway). Chorismate synthesizes either tryptophan or L-arogenate to produce phenylalanine and tyrosine (phenylalanine, tyrosine, and tryptophan biosynthesis pathway). Most of the amino acids identified were found to be glucogenic amino acids. Such amino acids could be converted into glucose. On the other hand, a few exclusively ketogenic amino acids, including lysine and leucine, were detected as well.
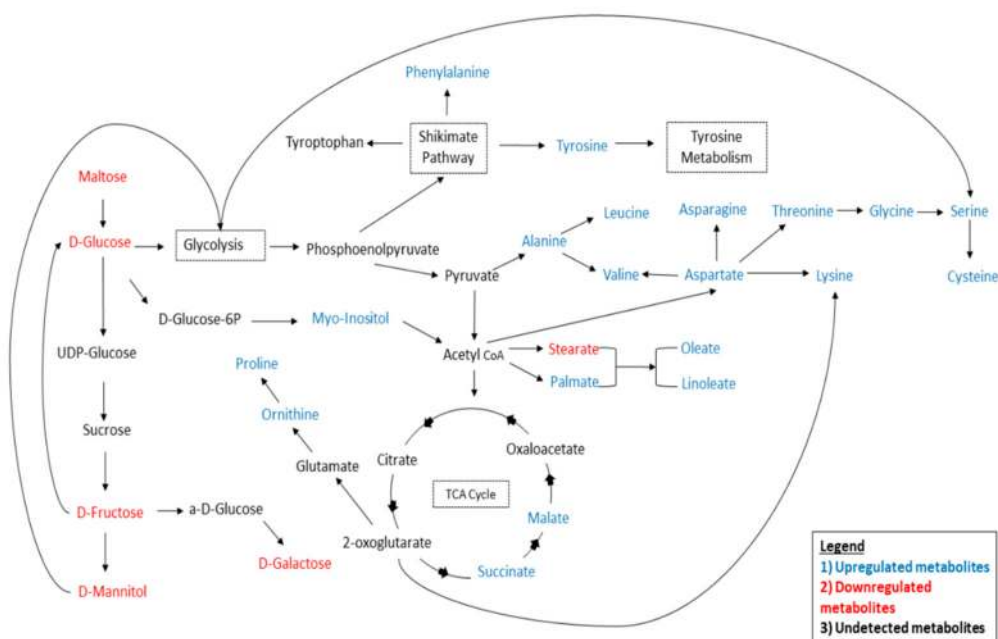

Figure 4. Metabolic changes mapped on metabolic pathways during fermentation of BSG by *B. Subtilis* WX-17. Colored labels indicate the respective increase, decrease, or undetected levels of metabolites at each given time.

Inferring from the results and hypothesized pathway analyses, SSF using *B. subtilis* WX-17 on BSG as a substrate was able to utilize the respective useful components in BSG. The increase in production of amino acids is similar to the observation in another SSF study of cassava using *Trichoderma pseudokoningii*, where protein content was increased from 8.4% to 12.5% [36]. It could be deduced that SSF produced useful enzymes and compounds that improved the nutritional content of the food by-product. The increase in amino acid production from the results was also in-line with an increase in amino acids observed in SSF of soybeans with *B. subtilis* [37]. In terms of amino acids

production, SSF has been evaluated on different food by-products and results have shown that it leads to an upregulation of amino acids.

4.3. TCA Cycle Metabolism Analysis

The TCA cycle is an important sequence of enzyme-catalyzed reactions that aerobic microorganisms use to generate energy. Metabolites belonging to the TCA cycle were found to increase in the fermented BSG sample, as compared to unfermented BSG. As fermentation progresses, the synthesis of intermediates in the TCA cycle increase as well [38]. Based on the upregulation of glycolysis, this leads to an increase in pyruvate, which is then converted into acetyl coenzyme A (acetyl-CoA) and enters the TCA cycle. The increased amounts of precursor, acetyl-CoA, into the TCA cycle subsequently leads to an increase in the TCA cycle intermediates (Figure 4). The analysis showed increased levels of succinic acid and malic acid, which are key components in the TCA cycle. The synthesis of these components are catalyzed by the enzymes succinate dehydrogenase and malate dehydrogenase, respectively [33].

4.4. Fatty Acid Metabolism Analysis

Fatty acids were suggested to be produced from acetyl-CoA and facilitated by the enzyme fatty acid synthase (fatty acid biosynthesis pathway). Also, *B. subtilis* WX-17 could have possibly produced lipases, which help to hydrolyze lipids present in BSG into fatty acids [39,40]. In the presence of fatty acid synthase, various reactions are catalyzed and saturated fatty acids, such as stearic acid and palmitic acid, are produced through elongation and hydrolysis. Unsaturation would cause the saturated fatty acids to be converted into unsaturated fatty acids, such as oleic acid and linoleic acid. With the increase in the precursor acetyl-CoA due to upregulation of glycolysis observed after fermentation, it was postulated that saturated fatty acids would increase. However, this was not the case, as saturated fatty acids were downregulated, while unsaturated fatty acids were upregulated. This is possibly due to the rate of unsaturation being higher than the rate of production of unsaturated fatty acids. As can be seen from the results, the results after fermentation gave an increase of 1.643 and 2.366 times for unsaturated fatty acids, which are linoleic acid, and oleic acid, respectively. The increase in the fatty acids trend was also observed in another study with soybean fermentation using *B. subtilis*, where increase in unsaturated fatty acids and decrease in saturated fatty acids were observed [41]. Also, the results and pathway analysis suggested a similar trend compared to a study that worked on SSF of rice bran using *Mucor rouxiiI*, which showed an increase in unsaturated fatty acids, such as gamma-linolenic acid [42]. Overall, microbial SSF across these food by-products have displayed a trend of increased unsaturated fatty acids.

4.5. Antioxidant Test Analysis

BSG has been reported to be a source of antioxidant phenolic compounds, which could be present in the husk and cell walls [43,44]. Particularly for phenolic acids content in BSG, ferulic acid and *p*-coumaric acid were found to be present in relatively high concentrations [43]. Other phenolic compounds present include flavonoids, proanthocyanidins, and amino phenolic compounds. There are various methods such as solid to liquid extraction, acid hydrolysis, and saponification to extract phenolic compounds from BSG fermentation [43,44]. It was reported that bioactive phenolic compounds can also be successfully extracted from natural sources using solid state fermentation [45]. In particular, it has been reported that fermentation with *B. subtilis* could produce nattokinase, which is a polypeptide that has antioxidant activity [46,47]. Hence, both solid state fermentation process and production of nattokinase could account for the increase in antioxidant properties and phenolic content in fermented BSG shown in the results.

5. Conclusions

This work demonstrated valorizing BSG through solid state bacterial fermentation using *B. subtilis* WX-17. The increased amount of amino acids, unsaturated fatty acids, and antioxidants after

fermentation showed the capabilities of *B. Subtilis* WX-17 to degrade the complex macronutrients in BSG into useful components. The increase in the respective useful components suggested that microbial SSF on BSG were able to produce other useful compounds in addition to the other products from SSF, such as the enzymes and polyphenols from other studies. The in-depth investigation using GC-MS, statistical analysis, and pathway analysis provided the metabolic changes at different time points during fermentation. With the enhanced nutritional content of fermented BSG, future work could involve food-related applications or examine more novel fermentation methods for effective valorization of BSG.

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