



## Research article

Potential novel nutritional beverage using submerged fermentation with *Bacillus subtilis* WX-17 on brewers' spent grainsYong Xing Tan<sup>a,b,1</sup>, Wai Kit Mok<sup>c,1</sup>, Wei Ning Chen<sup>c,\*</sup><sup>a</sup> Interdisciplinary Graduate School, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore<sup>b</sup> Advanced Environmental Biotechnology Centre, Nanyang Environment and Water Research Institute, Nanyang Technological University, 1 CleanTech Loop, 1 CleanTech One #06-08, Singapore 637141, Singapore<sup>c</sup> School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, N1.2-B1-35, Singapore 637459, Singapore

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## ABSTRACT

Food processing generates side streams that are not fully utilized and typically treated as waste materials. One of such food by-product, brewers' spent grains (BSG) are disposed in huge quantities from the beer industry annually. Submerged fermentation of BSG using *Bacillus subtilis* WX-17, without supplementary components, is herein employed. The fermentation products were extracted in the liquid phase, resulting in a potential novel nutritional beverage containing *Bacillus subtilis* WX-17. *Bacillus subtilis* WX-17, was still viable after a period of 6 weeks with a final cell count of 9.86 log CFU/mL. Gas chromatography-mass spectrophotometry (GC-MS) was employed for identification of the metabolites produced from the growth of *Bacillus subtilis* WX-17. Seven essential amino acids and citric acid cycle (TCA) intermediates were found to have increased significantly ( $p < 0.05$ ) whereas all carbohydrates decreased significantly ( $p < 0.05$ ) in the beverage after submerged fermentation. Additionally, antioxidant activity quantified using DPPH radical scavenging activity, increased by 2.08-fold while total phenolic content increased from  $125.7 \pm 0.74 \mu\text{g/mL}$  to  $446.74 \pm 1.26 \mu\text{g/mL}$ . The results proved the potential of employing submerged fermentation of BSG using *Bacillus subtilis* WX-17 to produce a novel and highly nutritious beverage.

## 1. Introduction

Brewers' spent grains (BSG) are generated as waste side streams during beer production process, where global yield is estimated at up to  $38.6 \times 10^6$  tonnes per year [1]. Currently, they are commonly used as either animal feed (e.g. for cattle) [2, 3] or disposed in landfills [4]. In parallel, BSG have been reported to contain proteins, fibres and phenolic compounds in abundant amounts [1, 5]. It is therefore worth exploring the development of high-value BSG-based products by harvesting their remaining nutrients, especially from the perspective of food security, economics as well as environment sustainability. Fermentation is a promising approach to increase nutrient content, thereby achieving a high-value BSG-based products. At the same time, this technique would enhance the utilization of BSG.

Microbial fermentation provides a feasible way to re-utilize or add value to bio-waste/food-products. *Bacillus subtilis* (*B. subtilis*) is one of the most noticeable bacterial workhorse for fermentation, since it can

produce many health benefits and anti-microbial compounds [6]. Specifically in food processing, *B. subtilis* has been used for the production of traditional soya-based natto for centuries [7]. Although fermentation using BSG has been proven to be viable for the cultivation of several microorganisms [8, 9], studies are rare on submerged fermenting of *B. subtilis* without additional supplementation. It is well established that *B. subtilis* can secrete abundant amounts of extracellular enzymes, including amylases and cellulases [10]. As such, *B. subtilis* is able to release the nutrients of BSG by breaking down the material, which is rich in protein and cellulose, in turn releasing or producing antioxidants such as phenols. Considering that no submerged fermentation of *B. subtilis* with BSG and *B. subtilis* drink have been reported till date, development of a nutritional *B. subtilis* beverage from BSG will be intriguing and worth exploring. According to [11], *B. subtilis* is a common species present in health beneficial products that is widely used as supplements and improving human health.

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This work studies the possibility of utilizing BSG as a substrate in submerged fermentation to enable the growth of *Bacillus subtilis* WX-17 (*B. subtilis* WX-17) without additional supplements yet producing nutritional components in the liquid. The microorganism involved is *B. subtilis* WX-17, which was isolated previously from natto. Antioxidant assays and metabolomics using gas chromatography-mass spectrophotometry (GC-MS), coupled with testing of *B. subtilis* WX-17 viability across time would be employed to evaluate the fermented liquid product as a nutritional beverage.

## 2. Methods and materials

### 2.1. Submerged fermentation of *B. subtilis* WX-17 with BSG

*B. subtilis* WX-17 was previously isolated from natto according to [12] and subsequently cultured in nutrient broth for a day at 37 °C. BSG were provided by Asia Pacific Breweries (Singapore) Pte. Ltd. and stored at -80 °C. With minor modifications from [13], BSG were grinded into 5 mm mesh size using ceramic mortar and pestle. Ten grams of autoclaved, grinded BSG were inoculated with *B. subtilis* WX-17 (10<sup>6</sup> CFU/g) and 50 mL sterile water in an Erlenmeyer flask. Submerged fermentation was carried out for 72 h at 37 °C, 200 rpm. Samplings of both unfermented and fermented BSG were carried out at 0 h and 72 h. The samples were filtered using vacuum filtration with filter paper of 40 µm particle retention size. The supernatant collected was stored in -20 °C until further analysis.

### 2.2. Metabolomics analysis

Metabolomics analysis was conducted according to the method in [14] with some minor modifications. 100 µL of Methoxamine (MOX) hydrochloride in pyridine (20 mg/mL) was added for derivatizing 1 mL of freeze dried samples and incubated for 1 h at 37 °C. Next, 200 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) silylation reagent was added to the samples and incubated at 70 °C for 30 min. 100 µL was drawn from each sample and measured thrice. The column specifications and GC-MS method were used according to [14].

### 2.3. Phenolic content analysis

According to a protocol in [15], total phenolic content was analysed and expressed in terms of gallic acids equivalent (GAE). 1 mL of sample was added to 5 mL of purified water and 0.5 mL of Folin-Ciocalteu reagent before being vortexed. Samples were left to sit for 5 min. 1.5 mL of 20% sodium carbonate was added to each sample and tubes were filled up to 10 mL using purified water. The samples were incubated at 20 °C in dark condition for 2 h. Subsequently, the optical density was measured using Thermo Fisher NanoDrop 2000c spectrophotometer at wavelength of 750 nm. Deionized water was used as blank.

### 2.4. Antioxidant activity analysis

1,1-diphenyl-2-picryl-hydrazil (DPPH) free radical-scavenging capacity was investigated according to [16] with some modifications. 600 µL of sample was added to 600 µL of 0.1 mM DPPH solution prepared in ethanol. The samples in tubes were afterwards incubated for 20 min in the dark condition at 20 °C. Samples were measured at a wavelength of 515 nm using spectrophotometer. The antioxidant activity was obtained from the conversion of absorbance readings and quantification in terms of weight of Trolox.

### 2.5. Evaluation of *B. subtilis* WX-17 viability

Ten times dilution was carried out by adding liquid sample to sterile water in an Eppendorf tube. The dilution process was performed 11 times

consecutively. 100 µL from each tube was plated onto nutrient agar petri dishes and incubated for 24 h at 37 °C. Thereafter, the cell counts in colonies forming units (CFU) were recorded. This is repeated weekly for a duration of 6 weeks.

### 2.6. Statistical analysis

Samples were statistically analyzed by partial least square-discriminant analysis (PLS-DA) and heatmap using MetaboAnalyst 4.0 [17]. Heatmap was generated with Euclidean distance calculation together with ward clustering algorithm. Statistical differences between samples were deduced based on Student's t-test with  $p < 0.05$  as the benchmark for statistical significance.

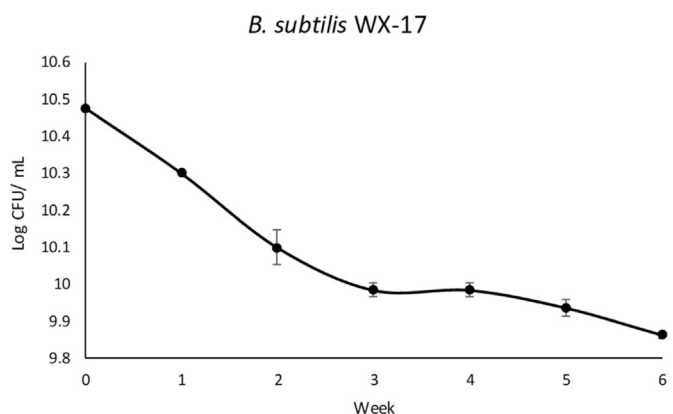
## 3. Results & discussion

### 3.1. Evaluation of *B. subtilis* WX-17 viability

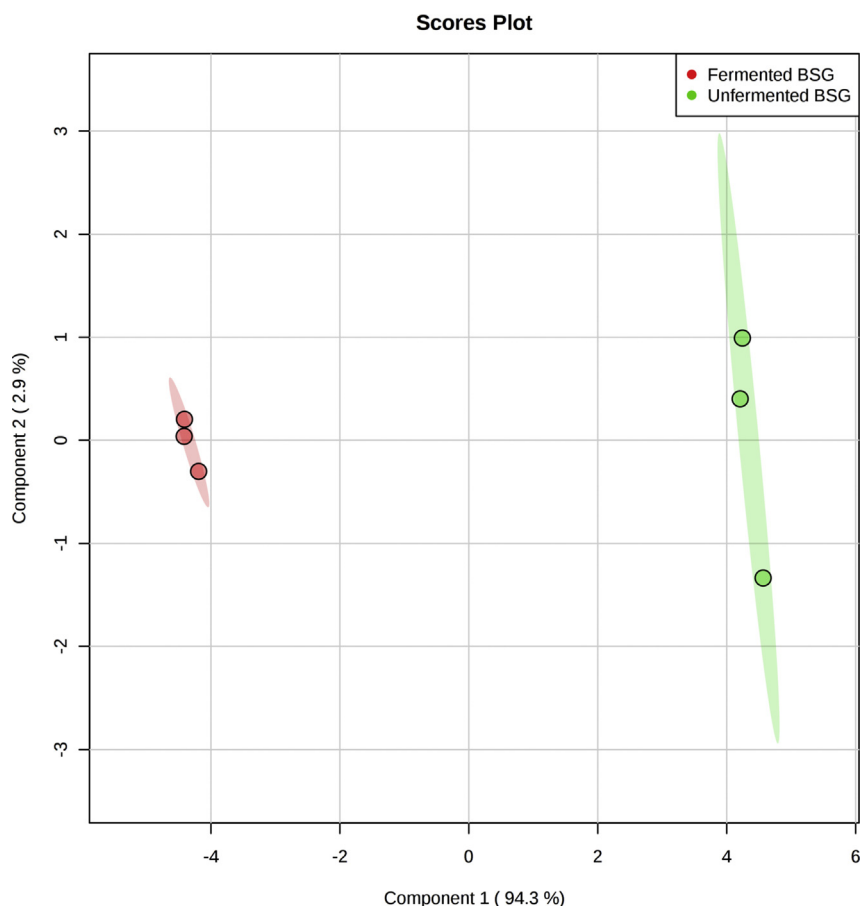
The changes in *B. subtilis* WX-17 cell count in colonies forming units/mL (CFU/mL) were recorded over 6 weeks at 4 °C (Figure 1). The count decreased slightly across 6 weeks without much variations, with an initial cell count of 10.48 log CFU/mL in week 0 to a final cell count of 9.86 log CFU/mL in week 6 (Figure 1). The relatively high survival rate of *B. subtilis* WX-17 after 6 weeks could be due to the formation of endospores by *Bacillus* species, which have been found to be metabolically dormant life forms and extremely resistant to various external conditions such as gamma radiation, UV as well as desiccation [18, 19]. The viability cell count amount of *B. subtilis* WX-17 obtained is relatively higher or similar to other common beneficial microorganisms in beverages such as *Bifidobacterium adolescentis* and *Lactobacillus casei*, which are reported to be around 9 log CFU/mL and 8.72 log CFU/mL respectively [20]. *B. subtilis* was evaluated to be potentially beneficial for humans and biotherapeutic [21, 22]. Hence, it can be inferred that the viability of *B. subtilis* WX-17 in the fermented beverage after 6 weeks would provide beneficial properties. In addition, the fermented liquid with *B. subtilis* WX-17 can possibly serve as a feed supplement for domestic animals. According to [23], the presence of *B. subtilis* tends to have a positive effect on feed efficiency of Holstein calves.

### 3.2. Statistical analysis on detected metabolites

An untargeted metabolomics analysis was employed to gain insights into the submerged fermentation process. A total of 23 significantly different metabolites ( $p < 0.05$ ) were observed. Using a statistical analysis, PLS-DA, a distinct separation between the metabolites in fermented and unfermented BSG was obtained (Figure 2). The variances of the principal components namely, PC1 and PC2 on the axes were 94.3% and



**Figure 1.** Changes in cell count of *B. subtilis* WX-17 grown in BSG media stored at 4 °C over 6 weeks.



**Figure 2.** PLS-DA plot for different metabolites detected for control (unfermented BSG) samples in green and fermented BSG samples in red as shown in the legend. Each cluster was presented with 95% confidence interval and explained variances are indicated in the brackets. Each dot represents metabolites in each replicate.

2.9% respectively. The clear difference between the samples is mainly due to the first principal component. The PLS-DA plot can be evaluated using  $R^2$  value and  $Q^2$  value, which represents the explained variance and the predictive capability of the model respectively [24]. With a  $R^2$  value and  $Q^2$  value of 0.99, it can be considered to be significantly substantial [25].

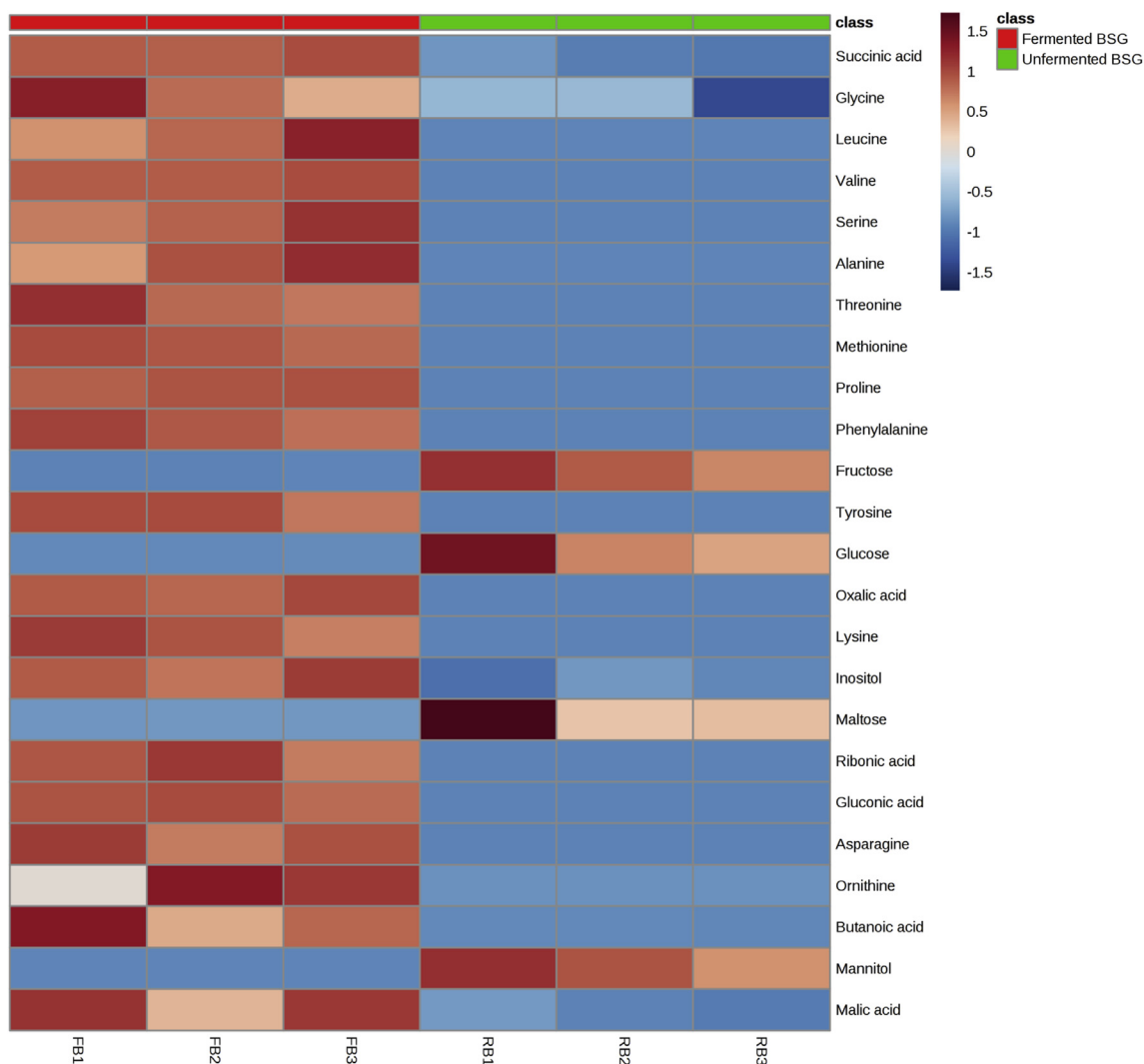
The detected metabolites were mapped onto a clustering heatmap and can be mainly classified into types of amino acids, carbohydrates, and the citric acid (TCA) cycle intermediates (Figure 3). Heatmap provides a visual overview and uses a color gradient to indicate the changes in the metabolites after fermentation [26]. There were a total of 13 amino acids detected by GC-MS that were upregulated after fermentation of BSG. Increased levels of amino acids would enhance the nutritional value of the fermented beverage. During the fermentation process, *B. subtilis* could possibly produce enzymes such as proteases, which allows proteolysis to take place and produce various amino acids. This hydrolysis of protein complexes present in BSG into simple amino acids is in line with an observation in another fermented food study [27].

Amino acids are required nutrients for human functions, growth and health [28]. As proteins are made up of amino acids, the increased levels of amino acids would also provide better health benefits such as improved skeletal muscle protein synthesis [29]. The various amino acids can be further categorized into essential and non-essential amino acids. Essential amino acids are non-producible by the human body and only can be obtained from dietary intake [30]. The essential amino acids detected in this study include lysine, threonine, methionine, valine, leucine, tyrosine, phenylalanine [31]. The increase in the concentrations of 7 essential amino acids would improve the nutritional value of the beverage as a supplement for humans (Table 1).

Based on the heatmap, various carbohydrates detected substantially decreased after submerged fermentation (Figure 3). It can be inferred that remaining carbohydrates present in BSG served as carbon sources for *B. subtilis* WX-17 growth. It is postulated that the fermentation using *B. subtilis* WX-17 produced various types of microbial enzymes that hydrolyze polysaccharides into simpler carbohydrates [32, 33, 34]. Metabolites categorized under the TCA cycle intermediates, which are succinic acid and malic acid, were observed to have increased after submerged fermentation (Figure 3). In the growth of microorganisms, the TCA cycle is a series of chemical reactions that generate energy to aerobic microorganisms. The upregulation in TCA cycle intermediates is in line with previous studies [35, 36], which showed that increase in TCA cycle metabolites stimulate growth of microorganisms. Overall, in both heatmap and PLS-DA, several significantly different metabolites ( $p < 0.05$ ) were obtained after submerged fermentation.

### 3.3. Antioxidant and phenolic content assay

Based on DPPH radical scavenging activity results from this study, submerged fermented BSG produced approximately 2.08 times more antioxidants in terms of Trolox equivalent compared to the unfermented BSG (Table 2). A small portion of inhaled oxygen in humans would naturally transform into free radicals, which might cause damage to the body. As long as there is a balance between free radicals transformation and eradication, these free radicals will not cause any harm [37]. However, external conditions such as stress and smoking could lead to an increase in levels of free radicals and might result in several human diseases [38]. Adequate amounts of antioxidant intake would be able to control the excessive amounts of free radical production [39]. With an



**Figure 3.** A heatmap analysis of metabolites detected in both control and submerged fermented BSG. The three columns from the left denotes the metabolites obtained from submerged fermented BSG and subsequently the remaining three columns representing the metabolites in unfermented BSG samples. Higher amounts of specific metabolites were shown in red, whereas blue indicated lower amounts of the specific metabolites.

**Table 1.** Essential Amino acids results (µg/mL) for control and fermented BSG.

Essential amino acids	Unfermented BSG	Fermented BSG
Leucine	N.D.	4.11 ± 0.74
Valine	N.D.	0.82 ± 0.02
Threonine	N.D.	0.69 ± 0.09
Phenylalanine	N.D.	31.2 ± 2.87
Methionine	N.D.	5.21 ± 0.27
Lysine	0.01 ± 2E-04	44.92 ± 4.28
Tyrosine	N.D.	28.11 ± 1.89

N.D. – Not detected.

**Table 2.** Antioxidant activity based on weight of Trolox and GAE (µg/mL).

Samples	Weight of Trolox (µg/mL)	GAE (µg/mL)
Unfermented BSG (Control)	3.51 ± 0.15	125.7 ± 0.74
Fermented BSG (Day 3)	7.31 ± 0.11	446.74 ± 1.26

increase in DPPH radical scavenging activity after submerged fermentation, the antioxidant compounds present would further enhance the nutritional value of the beverage.

In this study, there was an increase in phenolic content from  $125.7 \pm 0.74 \mu\text{g GAE/mL}$  to  $446.74 \pm 1.26 \mu\text{g GAE/mL}$  after submerged fermentation, which is in agreement with a study that reported microbial fermentation serving as a powerful method for extracting phenolic compounds (Table 2) [40]. BSG had been found to contain phenolic compounds that are bounded to the cell wall [41, 42]. Cell wall degrading enzymes would be required to extract the components [40]. Hence, it is postulated that *B. subtilis* WX-17 was able to produce enzymes such as cellulases to degrade the cell wall and extract the phenolic compounds through submerged fermentation [43].

#### 4. Conclusion

This work demonstrates that submerged fermentation with BSG as the sole substrate was feasible to grow *B. subtilis* WX-17 on BSG. Growth of *B. subtilis* WX-17, in the beverage had a final cell count of  $9.86 \log \text{CFU/mL}$  after a period of 6 weeks when stored at  $4^\circ\text{C}$ . It produced a nutritious beverage, tested to contain an increased amount of amino acids, total phenolic content and antioxidant activity. The combination of higher nutritional content together with presence of viable *B. subtilis* in the liquid would justify the potential of using the liquid as a novel nutritional beverage for human health. Further investigations on consumer acceptance, flavour of the beverage or different nutritional component analysis can be executed.

#### Declarations

##### Author contribution statement

Yong X. Tan, Wai K. Mok: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Wei N. Chen: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

No additional information is available for this paper.

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