Synergistic Effect of a Mixed Culture in Solid-state Fermentation

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ABSTRACT

Okara and brewer's spent grain (BSG) are manufacturing side streams produced in massive amounts from the soy and beer industries, respectively, and contributes significantly to food waste. However, these materials are still nutritious and can be repurposed for farming and food applications through nutritional profile enhancement using biovalorisation. In this study, solid-state fermentation (SSF) was performed using okara and BSG as substrates to investigate the effects of fermentation using pure and mixed cultures of *Aspergillus oryzae* and *Bacillus subtilis*. The synergistic effect of cooperative metabolism in mixed cultures was also studied. The total phenolic content (TPC), crude protein content, and total dietary fibre (TDF) of the fermented substrates were determined after fermentation to evaluate their nutritional profiles. Okara fermented with a mixed culture under specific conditions produced the highest TPC of 1100 mg/100g dry matter, which was more than 13 times that of the control. Its TDF profile was also the most ideal, striking a good balance between IDF and SDF reduction. These results reflected the presence of cooperative metabolism between *Aspergillus oryzae* and *Bacillus subtilis* during fermentation, which afforded greater enhancement in the nutritional profile of substrates compared to that of pure cultures. However, the crude protein content of the samples could not be accurately determined due to the formation of volatile nitrogenous compounds and loss of dry matter during fermentation. Competition between *Aspergillus oryzae* and *Bacillus subtilis* in samples containing BSG also impeded the progress of fermentation. More specific methods of protein quantification and optimisation of sample preparation for BSG in future work is recommended. In this study, mixed cultures in SSF has shown great promise, and further research of this methodology will facilitate its use and development in future applications.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 Background

Food wastage is a global problem of growing concern, where approximately 1.3 billion tonnes of food is lost or wasted. This comes up to roughly one-third of the annual food produced globally for human consumption.[1] In Singapore, food waste accounts for approximately one-tenth of the total waste generated. However, less than 20 % of food waste is recycled, while the remainder is disposed of and incinerated. These numbers are expected to increase in the coming years owing to the growing population and economy.[2] Food manufacturing side streams also contribute greatly to this food waste problem, in which some still possess nutritional value. Such manufacturing side streams include okara, which is the soy pulp produced from the manufacturing of soymilk and bean curd. The annual global production of okara amounts to 14 million tonnes, with approximately 30 tonnes disposed of on a daily basis in Singapore.[3,4] Brewer's spent grain (BSG), a side stream of beer production, has an estimated annual global output of approximately 38.6 million tonnes.[5] Despite attempts to reuse these side streams, much of these are still disposed of due to their short shelf life and exorbitant production.[6,7] However, these side streams still possess abundant nutritional value, as shown in Table 1, which can still be further enhanced through methods such as fermentation.

Table 1: Nutrient Composition of Okara and BSG [6,7]

Components	Okara (g/100 g dry matter)	BSG (g/100 g dry matter)
Protein	15.2	18.0
Lipids	8.3	6.6
Dietary Fibre	42.4	41.3
Carbohydrates	4.1	26.2

In order to reduce the wastage of these manufacturing side streams, they can be converted into nutritious material suitable for farming and food applications via various methods. This would thus place them back into the loop of their production life cycles and allow for greater sustainability.

1.2 Solid-state Fermentation

Solid-state fermentation (SSF) involves the fermentation of solids where free water is absent. However, the substrate must still contain moisture levels necessary to support microbial growth and metabolism.[8] It is a simple and economical approach to improving the nutritional value and functional properties of these side streams, in which many biochemical processes occur. For example, protein hydrolysis during fermentation produces amino acids and short-chain low molecular weight compounds which improves the nutritional and physicochemical properties of the substrates. Furthermore, antioxidant peptides are also generated, which helps prevent oxidative damage and cardiovascular diseases when ingested.[9] Phenolic compounds, which are known to possess antioxidant properties, can also be synthesised by SSF. They are able to absorb and neutralise free radicals as well as decompose peroxides, which may be associated with lower mortality in cancer patients.[10] Hence, through the use of appropriate microorganisms, SSF is an efficient and effective solution to improving the nutritional profile of the side streams. They can then be repurposed in various applications such as fertilisers, animal feed formulation, and human nutrition.

1.3 *Aspergillus oryzae*

Aspergillus oryzae is an aerobic filamentous fungus suitable for SSF. It plays an imperative role in Asian food culture and industry, in which it has been utilised for several millennia in the production of fermented food products such as soy sauce. Other species belonging to the same *Aspergillus* subgenus, namely *A. flavus* and *A. parasiticus*, are known to produce potent carcinogenic substances known as aflatoxins. Despite belonging to the same subgenus, previous studies have shown that *A. oryzae* does not produce aflatoxins. Hence, *A. oryzae* has been regarded as a microorganism bearing a "safe" status and has been used extensively throughout history.[11] *A. oryzae* is known to produce various proteolytic enzymes which are able to drive and facilitate the mass degradation and biochemical processes that occur during SSF. Some of these enzymes include peptidase, protease, glycoside hydrolases, and amidohydrolase.[12]

1.4 *Bacillus subtilis*

Bacillus subtilis var. Natto is a Gram-positive probiotic bacterium and is another suitable microorganism for SSF. It has long been used as a starter culture for various fermented food products such as natto and doenjang, and hence been regarded as safe.[3] When used in SSF, *B.*

subtilis secretes a spectrum of extracellular enzymes, such as amylase and cellulase, that efficiently degrade macromolecules in substrates. This facilitates the release of various nutrients and the production of antioxidants such as phenols, thereby improving the nutritional value and increasing the antioxidant activity of substrates.[5]

1.5 Mixed Culture Fermentation

Mixed culture fermentation refers to a fermentation process in which the inoculum consists of two or more organisms, with any combination of bacteria, fungi, and yeast. This technique offers numerous advantages, such as protection against contamination and increased yields.[13] Through a mixed culture, it is also possible for biochemical modifications characteristic of each microorganism to be achieved simultaneously. Most importantly, cooperative metabolism is possible in a mixed culture where one species could produce specific metabolites that aid the growth of the other. This thereby facilitates better growth of the microorganisms during fermentation, leading to higher yields. Mixed cultures can also facilitate biochemical changes that are required for the production of certain foods, which would not be possible in a pure culture. For example, in the ripening of cheese, *Brevibacterium linens* secretes methanethiol, which is then modified by *Kluyveromyces lactis.* The aromatic profile of volatile sulfur compounds thus produces the distinct flavours of cheese.[14] For this study, *A. oryzae* secretes significant amounts of glycoside hydrolases where enzymatic hydrolysis releases glucose units during fermentation.[12] This increase in glucose levels is advantageous to the growth of *B. subtilis* as glucose is its preferred carbon source.[15] Hence, by leveraging the synergistic effect through cooperative metabolism, achieving greater nutritional profile enhancement by mixed culture fermentation may be possible.

From the literature, SSF using pure cultures on okara and BSG have been reported to successfully enhance their nutritional profiles.[3,16] However, studies on SSF using mixed cultures are limited, with neither its potential nor its effects having been thoroughly studied.

1.6 Objectives

The aims of this study are to (1) investigate the changes in the nutritional profile of okara and BSG after a mixed culture solid-state fermentation, and (2) make comparisons with that of pure cultures.

1.7 Hypothesis

It is hypothesised that solid-state fermentation using *B. subtilis* and *A. oryzae* in a mixed culture will exhibit cooperative metabolism and invoke a greater improvement in the nutritional profile of the fermentation substrates compared to that of a pure culture, encompassing total phenolic content (TPC), crude protein content, and total dietary fibre (TDF).

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Acetone (Technical Grade) and ethanol (Technical Grade) were purchased from Aik Moh Singapore (Singapore). Boric acid was purchased from Merck (New Jersey, USA). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), bovin albumin (BSA), bromocresol green, DL-Dithiothreitol, Folin & Ciocalteu's phenol (FC) reagent, gallic acid, 70 % isopropanol, 2-(N-Morpholino)ethanesulfonic acid (MES), 2-mercaptoethanol, methyl red, phenylmethanesulfonyl fluoride, 85 % phosphoric acid, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Missouri, USA). Coomassie Brilliant blue G-250 was purchased from AppliChem GmbH (Darmstadt, Germany). 37 % hydrochloric acid and methanol (HPLC grade) were purchased from VWR International (Rue Carnot, France). Kjeltabs (3.5 g K_2SO_4, 0.4 g $CuSO_4 \bullet 5H_2O$) were purchased from FOSS (Hillerød, Denmark). Purified Milli-Q water of 18 mΩ-cm resistivity was used for the preparation for all buffers and solutions. Sodium carbonate anhydrous (Na_2CO_3) was purchased from QREC (Selangor, Malaysia). Sodium chloride (NaCl) was purchased from Goodrich Chemical Enterprise (Singapore). Sodium hydroxide (NaOH) pellets were purchased from VWR International (Leuven, Belgium). 98 % sulfuric acid was purchased from Kanto Chemical (Tokyo, Japan).

2.2 Materials

Fresh okara and BSG were kindly provided by Sing Ghee Beancurd Manufacturer (Singapore) and Brewerkz Brewing Co. (Singapore) Pte Ltd, respectively. Both fresh okara and BSG were stored in double airtight Ziploc bags at -20 ℃ until use. The *koji* starter, M-1, containing the spores of *Aspergillus oryzae*, was purchased from Nihon Jyozo Kogyo (Tokyo, Japan). The *Natto* starter, NAT, containing the endospores of *Bacillus subtilis var. natto*, was purchased from Nattomoto Yuzo Takahashi Laboratory Co. (Yamagata, Japan).

2.3 Pre-treatment of Substrates

Frozen okara was thawed and autoclaved (Sanyo Labo Autoclave, Osaka, Japan) twice at 121 ℃ for 15 minutes. Frozen BSG were thawed and blended (Waring Commercial Blender BB300K, Connecticut, USA) to reduce grain size before being autoclaved twice at 121 ℃ for 15 minutes.

2.4 Solid-state Fermentation

The controls and fermented okara and BSG samples were prepared as described.

2.4.1 Controls

30 g of Okara, 50 % okara/BSG (by weight), and BSG were each plated into sterile petri dishes. 1 mL of sterile deionised water was added to replicate the increase in moisture from the inoculant of the samples to be fermented. These controls were subsequently placed in conditions identical to the fermented samples.

2.4.2 Fermentation with Pure Cultures

In the preparation of the inoculants, M-1 and NAT spore powders were individually weighed and suspended in sterile deionised water at a concentration of 10^7 spores/gram of dry matter. 1 mL of M-1 and NAT inoculant were individually added to 30 g of okara, 50 % okara/BSG, and BSG in petri dishes. Following inoculation, M-1 samples were fermented in the dark at 22.5 ℃ and 52 % humidity for 96 hours, while NAT samples were fermented in an incubator (Innova 4230 Refrigerated Incubator Shaker, New Brunswick Scientific, New Jersey USA) at 38 ℃ and 52 % humidity for 96 hours. Upon completion of fermentation, the samples were dehydrated overnight at 67 ℃ in a food dehydrator (Excalibur Food Dehydrator, California, USA) and ground using a food grinder (Gewürz & Kaffee Mühle, Rommelsbacher, Germany) before being stored in Ziploc bags at 4 ℃.

2.4.3 Fermentation with Mixed Culture

To prepare the mixed culture inoculant containing M-1 and NAT, the respective spore powders were weighed and suspended together in sterile deionised water at an individual concentration of 10^7 spores/gram of dry matter, and this inoculant was added to 30 g of okara, 50 % okara/BSG, and BSG in petri dishes. Following inoculation, one set of samples was placed in the dark to ferment at 22.5 ℃ and 52 % humidity, while another set was placed in an incubator to ferment at 38 ℃ and 52 % humidity for 48 hours. The fermentation temperatures are summarised in Table 2 below. After 48 hours, the fermentation conditions were swapped between the two sets, and were left to ferment for another 48 hours. Upon completion of fermentation, the samples were dehydrated overnight at 67 ℃ in a food dehydrator and ground using a food grinder before being stored in Ziploc bags at 4 ℃.

Table 2: Fermentation Temperatures for Different Cultures

Fermentation Culture	1^{st} – 48^{th} Hour	49^{th} – 96^{th} Hour
M-1	22.5 ℃	22.5 ℃
NAT	38.0 ℃	38.0 ℃
M-1/NAT Condition A	22.5 ℃	38.0 ℃
M-1/NAT Condition B	38.0 ℃	22.5 ℃

2.5 Total Phenolic Content (TPC) Analysis

The TPC of the samples was determined via the Folin-Ciocalteu assay. This method yields a coloured product that absorbs at 760 nm when phenolic compounds oxidise in an alkaline (carbonate) solution with a molybdotungstophosphate heteropolyanion (FC) reagent.[17] 1 mL of 50 % methanol (v/v) was added to 100 mg of the samples, and the mixtures were then vortexed before centrifugation at 15,000 rpm for 10 minutes. The supernatants obtained were diluted as required, while the FC Reagent underwent a 10-times dilution. 100 µL of the diluted supernatants, 1 mL of the diluted FC reagent, and 800 µL of 1M Na_2CO_3 solution were then added sequentially to individual 4 mL glass vials. The vials were subsequently incubated (Ecotherm, Torrey Pines Scientific Inc., CA, USA) for 15 minutes at 45 ℃, and 200 µL of the incubated mixtures were pipetted into a 96-well microplate (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate, Thermo Fisher Scientific, Massachusetts, USA). The absorbance values were then measured at 760 nm using a microplate reader (Infinite 200, Tecan Austria, Grödig, Austria), and their respective TPC values were determined via a gallic acid standard calibration curve (refer to Annex A), where the values obtained were expressed in milligrams of gallic acid equivalents (GAE) per 100 grams of dried matter (mg GAE/100 g).

2.6 Crude Protein Analysis

Crude protein content was determined via the Kjeldahl method using the Kjeltec auto-analyser (Kjeltec^{TM} 8400, FOSS, Hillerød, Denmark).[18] 200 (±1) mg of samples, 2 Kjeltabs catalyst, and 12 mL of 98 % concentrated sulfuric acid were added into 250 mL Kjeldahl digestion tubes. The mixtures were digested (FOSS Tecator Digestor 8, FOSS, Hillerød, Denmark) at 420 ℃ for 60 minutes before being cooled to room temperature and loaded into the analyser unit. The unit then performed titration using 0.1 N HCl to a colourimetric endpoint and the crude protein content was calculated with a conversion factor of 6.25 on the total nitrogen content. The equation for this calculation is shown in Equation 1 below.

$$Crude\ Protein\ (\%) = \frac{(V_S - V_B)\ \text{x}\ N\ \text{x}\ 14.01}{W\ \text{x}\ 10}\ \text{x}\ 6.25$$

Equation 1: Percent Crude Protein[18]

Where:

V_S = Volume of HCl used for titration of sample (mL)

V_B = Volume of HCl used for titration of reagent blank (mL)

N = Normality of HCl used (0.1 N)

14.01 = Atomic weight of nitrogen (g/mol)

W = Mass of sample used (g)

10 = Conversion factor to percentage

6.25 = Conversion factor from percent Kjeldahl nitrogen to percent protein

2.7 Total Dietary Fibre (TDF) Determination

The soluble and insoluble dietary fibre content of the samples were determined using Megazyme's Total Dietary Fiber assay kit (Megazyme, Ireland), which referenced AACC method 32-07.01 and AOAC method 991.43. Firstly, 1 (±0.005) g duplicates of each sample were enzymatically treated via incubation using heat-stable α-amylase, protease, and amyloglucosidase sequentially. Minor modifications to the procedure were made to separate the insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). The sample mixtures were centrifuged at 4000 rpm for 40 minutes to separate the IDF residue from the supernatant consisting of the SDF, and SDF was subsequently precipitated from the supernatant using 95 % ethanol (v/v). One duplicate of each sample was analysed for ash content, while the other was analysed for protein content via the Kjeldahl method. The IDF and SDF values were calculated using equation 2 as shown below.

$$Dietary\ Fibre\ (\%) = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \text{ x } 100$$

Equation 2: Percent Dietary Fibre[19]

Where:

R_1 = Weight of residue from m_1

R_2 = Weight of residue from m_2

p = Protein weight from R_2

A = Ash weight from R_1

$$B = \frac{BR_1 + BR_2}{2} - BP - BA$$

BR = Blank residue

BP = Blank protein from BR_1

BA = Blank ash from BR_2

m_1 = Sample weight of duplicate 1

m_2 = Sample weight of duplicate 2

100 = Conversion factor to percentage

2.8 Statistical Analysis

All experiments and measurements were performed in triplicates, and data in the following sections are presented as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Solid-state Fermentation with Pure Cultures

The fermentation of substrates using M-1 and NAT proceeded as expected under their respective fermentation conditions. The extent of fermentation in substrates inoculated with M-1 could be observed through the degree of mycelium growth on the substrates, while that of substrates inoculated with NAT could be observed through the darkening of substrates. This is illustrated in Fig. 1 below.

Fig. 1: Visual Changes in Substrates After Fermentation

3.2 Solid-state Fermentation with Mixed Culture

There were two fermentation conditions for the samples inoculated with the mixed culture; Conditions A and B. The former condition was kept at 22.5 ℃ for the first 48 hours, then 38 ℃ for the subsequent 48 hours, while the inverse was carried out for the latter condition. Although the optimal growth temperatures for M-1 are between 32 – 36 ℃ and at 37 ℃ for NAT, previous experimentation showed that M-1 was still able to grow at ambient temperature while NAT could not.[11,20] Hence, 22.5 ℃ was chosen as the fermentation temperature for M-1 and 38 ℃ was chosen for NAT to avoid overlap with their respective optimal growth temperatures. By varying the fermentation temperature, Conditions A and B would be able to facilitate targeted growth of the individual microbes. Hence, the effects of the mixed culture could be objectively compared with that of pure cultures, allowing cooperative metabolism to be thoroughly studied. Samples fermented under Conditions A and B both exhibited characteristics of M-1 and NAT growth, hence allowing the conclusion that both microbes did grow during the period of fermentation. The visual outcome of fermentation in all of the samples is illustrated in Annex B.

3.3 Total Phenolic Content

Phenolics are aromatic compounds containing at least one hydroxyl substituent, and possess potent antioxidant activity. They have also been reported to effectively prevent diseases associated with oxidative stress such as cancer, liver, kidney, and cardiac diseases.[21] From previous studies, it was observed that the extent of fermentation is proportionate to TPC. Thus, a substantial increase in TPC after fermentation is highly desirable.

From the data obtained from the TPC assay shown in Fig. 2, it was observed that mixed culture samples containing okara under Condition A gave the highest TPC after fermentation, reaching 1100 mg GAE/100 g dry matter from an initial 82 mg GAE/100 g, which corresponded to an increase of over 13-folds. Comparing the TPC of M-1 and NAT in pure cultures, it was evident that NAT was largely responsible for the increase in TPC, and should thus share a proportionate relationship. Condition A facilitated M-1 growth in the initial 48 hours, giving increased glucose levels. This supplemented the carbon source for the growth of NAT in the subsequent 48 hours, leading to the most significant increase in TPC, thus exhibiting cooperative metabolism. This is supported by the TPC for Condition B, in which samples were not expected to exhibit cooperative metabolism, and a smaller increase in TPC was observed since NAT did not receive glucose supplementation.

However, the same trend was not observed in BSG samples. Furthermore, M-1 also exhibited suboptimal levels of TPC enhancement in BSG samples. These observations could be attributed to the lack of accessible nutrients in BSG. Shown in Fig. 3 is the cross-section of a barley grain, which is a main component of BSG. The bulk of its nutritious material is found in the endosperm, which lies under the husk of the grain.[22] Prior to fermentation, BSG was blended to reduce the grain size such that the husks could be broken, thereby releasing the nutrients for microbial fermentation and growth. However, this process might not have been effective, resulting in the husks remaining intact after blending. Due to this, M-1 might not have been able to gain access to the endosperm, thus impeding its growth. With the lack of nutrients and the presence of NAT, competition between the microorganisms might have arisen. This thus may have impeded the growth of M-1 and resulted in little to no glucose supplementation. Hence, for BSG samples under Condition A, the growth of NAT was suboptimal due to the decreased glucose production by M-1, leading to a significantly smaller increase in TPC.

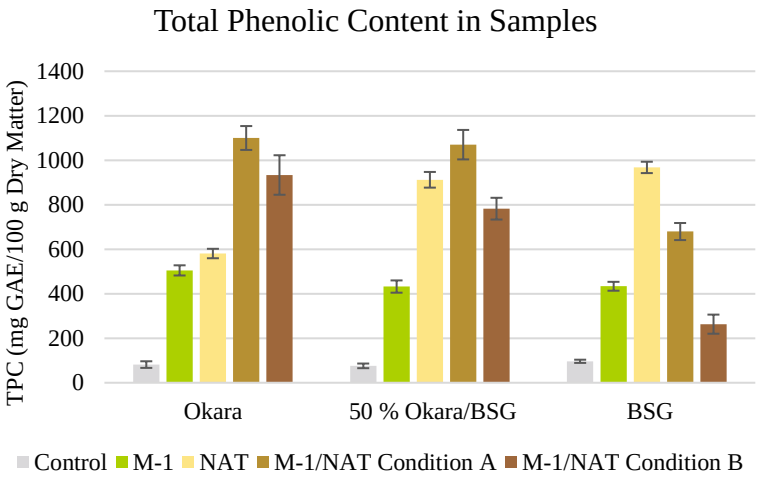

Fig. 2: Total Phenolic Content in SSF Samples

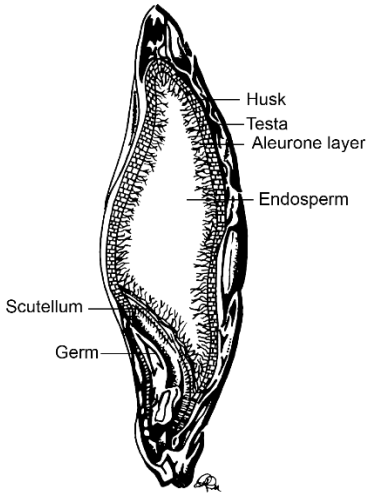

Fig. 3: Cross-section of Barley Grain[23]

3.4 Crude Protein Content

During fermentation, microorganisms release a spectrum of enzymes to facilitate the degradation of substrates and the release of nutrients. Proteolytic enzymes such as proteases and peptidases are examples of such enzymes that can modify the protein composition during the fermentation of substrates. From the crude protein data shown in Fig. 4, the greatest increase and decrease came from okara substrates with pure M-1 and NAT cultures, respectively. Pure M-1 reached 30.7 % (w/w) protein from an initial 23.6 % (w/w), while pure NAT fell to the lowest value of 13.7 % (w/w). Substrates with mixed cultures exhibited sub-optimal levels of crude protein, with the lowest at 15.7 % (w/w) in okara Condition B and the highest being a meagre increase of under 2 % (w/w) in BSG Condition B. The data for substrates with M-1 pure cultures aligned with those from preliminary studies, but this was not the case for substrates in NAT pure cultures. In SSF performed on chickpeas using *Bacillus subtilis* by Li and Wang[9], the authors observed a 7.1 % (w/w) increase in soluble proteins after 48 hours of fermentation. This suggested that the Kjeldahl method might not be suitable for an accurate determination of protein levels in NAT-fermented substrates.

Crude protein determination via the Kjeldahl method is based on an estimation of total nitrogen content using a conversion factor of 6.25. Hence, the values obtained might not be representative of the actual functional protein levels present within the samples, since the protein composition might have been altered during the fermentation process. Furthermore, a study performed by Owens *et al.*[24] on volatile compound formation during fermentation of soya beans using *Bacillus subtilis* showed that several pyrazine analogues, which are nitrogenous

compounds, were produced in significant amounts and gave off strong odours. The total nitrogen content in NAT-fermented substrates is thus expected to decrease due to loss of nitrogen from volatile nitrogenous compounds during fermentation. This hence may lead to a decrease in crude protein content when analysed by the Kjeldahl method. Lastly, the increase in crude protein in M-1-fermented substrates may not be representive of absolute changes, but rather, relative changes. During fermentation, dry matter is usually lost due to the metabolic processes of microorganisms, where carbohydrates and fats are hydrolysed and metabolised as energy sources.[25] Therefore, the increase in crude protein content in M-1-fermented substrates could be a relative change caused by a reduction in dry matter. To mediate these issues, a more specific method of analysis could prove to be useful in providing more representative results. One such method would be the Bradford assay for soluble proteins, which involves specific intermolecular interactions between proteins and the Bradford reagent.[26] Studies have shown that soluble protein is more digestible when ingested compared to insoluble protein, and hence possess greater nutritional benefit.[27] Therefore, testing for soluble protein levels will be more practical compared to other assays, such as total protein. Quantification of dry matter loss could also be performed to facilitate the determination of absolute changes in protein content.

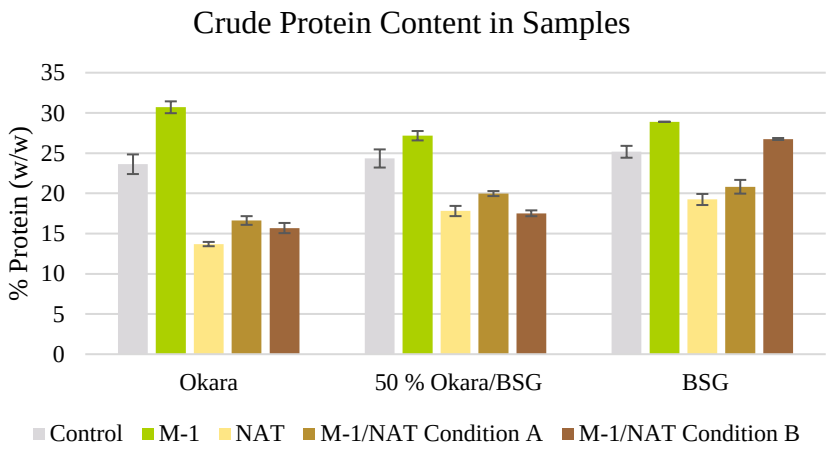

Fig. 4: Crude Protein Content in SSF Samples

3.5 Total Dietary Fibre

According to the European Food Safety Authority (EFSA), dietary fibre is defined as non-digestible carbohydrates and lignin, which typically refers to polysaccharides arising from plant-based food. Dietary fibres are also known as non-starchy polysaccharides (NSPs), and are classified as either soluble dietary fibre (SDF) or insoluble dietary fibre (IDF) depending on their water solubility.[28] When incorporated into diets, IDF plays a key role in gastrointestinal

function, while SDF possesses important effects on the metabolism of glucose and lipids, aiding in the regulation of blood glucose and cholesterol levels.[19] Hence, high-quality dietary fibre is regarded to compose of more than 10 % SDF.[29] In contrast, higher levels of IDF has been reported to result in increased feed rate of passage, as well as reduced contact between digestive enzymes and dietary nutrients, thus leading to reduced nutrient digestibility.[30] In addition, the inclusion of fermentable fibres in diets may enhance microbial nitrogen production due to higher energy availability during subsequent fermentation in the colon. Thus, fermentable fibres can promote microbial growth, possibly leading to increased protein levels from fermentation.[30] Since SDF is known to be significantly more fermentable than IDF, fermented substrates possessing higher levels of SDF are likely to exhibit greater nutritional benefit upon ingestion. Hence, an increase in SDF and decrease in IDF after SSF are highly desirable.

From the data obtained in the determination of TDF shown in Fig. 5, the fermentation of okara in mixed culture under Condition A gave the most ideal results. Although M-1 fermented okara had the greatest reduction in IDF of 16.1 % (w/w), there was also a corresponding reduction in SDF of 6.4 % (w/w). In contrast, although okara fermented with a mixed culture under Condition A also exhibited SDF reduction, this was only a minute reduction of 0.7 % (w/w). It also exhibited a 4.8 % (w/w) reduction in IDF, thus striking a good balance between IDF and SDF reduction. Okara fermented with a mixed culture under Condition B also produced similarly positive results, with an 8.9 % (w/w) and 0.9 % (w/w) reduction in IDF and SDF, respectively. Based on the data collected on the TDF content in substrates containing BSG shown in Fig 6 and 7, the effect of the various fermentation parameters on these substrates was not as significant. This was possibly due to competition between NAT and M-1 in BSG as mentioned in Section 3.3, as well as the variations in the nutritional profile of substrates introduced by BSG. Variation in substrate composition can alter the profile of enzymes secreted by microorganisms during fermentation to optimise nutrient uptake.[31] Hence, this could have led to M-1 and NAT possessing similar NSP degradation activity in the presence of BSG, resulting in less significant changes to the TDF content.

These reductions in IDF and SDF observed in okara samples could be attributed to the secretion and action of several enzymes such as β-glucosidase, cellulase, and xylanase by M-1 and NAT.[12,32] These enzymes break down the complex structures of the polysaccharides found in dietary fibre into smaller carbohydrates such that they can serve as energy and carbon sources

for the metabolic processes occurring within the microorganisms.[12,32] Despite the observed reduction in SDF being undesirable, it is ultimately unavoidable since they serve as important nutrients in microbial growth. Hence, by leveraging on cooperative metabolism to supplement the nutritional requirements of NAT, a good balance was struck between IDF and SDF reduction under Condition A which furnished acceptable results.

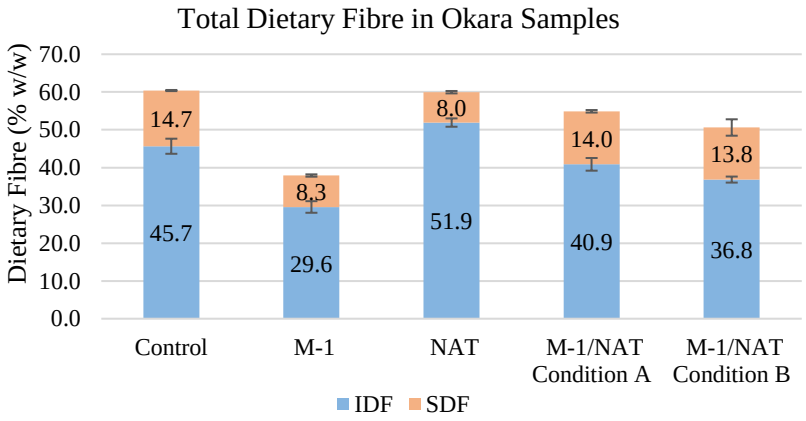

Fig. 5: TDF in Okara Samples

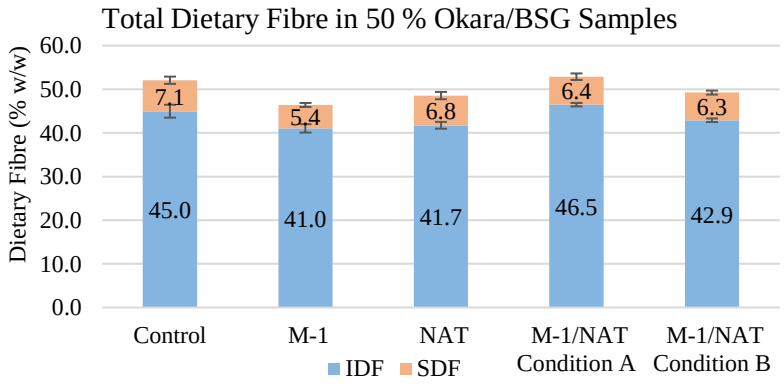

Fig. 6: TDF in 50 % Okara/BSG Samples

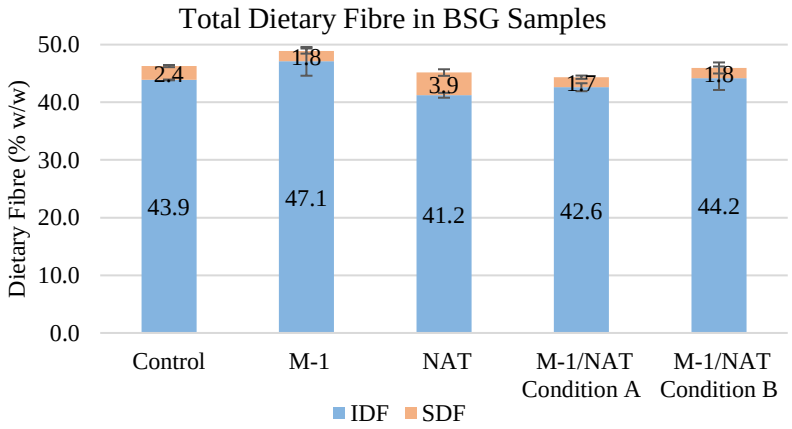

Fig. 7: TDF in BSG Samples

4. CONCLUSION AND FUTURE WORK

Solid-state fermentation of okara and BSG using pure and mixed cultures of M-1 and NAT under various conditions were successfully performed, in which their effects were investigated and compared. Okara fermented with M-1 and NAT in a mixed culture under Condition A afforded the largest increase in TPC and the most balanced TDF profile, proving that cooperative metabolism was present, and a mixed culture was effective in producing greater enhancements in nutritional profile. However, the crude protein content of fermented substrates could not be accurately determined via the Kjeldahl method due to the production of volatile nitrogenous compounds and loss of dry matter. In addition, the fermentation of BSG left more to be desired, owing to competition between M-1 and NAT caused by the lack of accessible nutrients. Hence, future work could include more specific methods of protein quantification such as the determination of soluble protein via the Bradford assay, while quantification of dry matter loss could be performed to determine absolute changes in protein levels. Pretreatment of BSG to reduce grain size could also be explored such that nutrients are effectively released, and competition between M-1 and NAT during solid-state fermentation is prevented. Nevertheless, this study has demonstrated the immense potential of mixed cultures in solid-state fermentation, and greater efforts invested will allow for this potential to be fully harnessed.

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6. ANNEX A

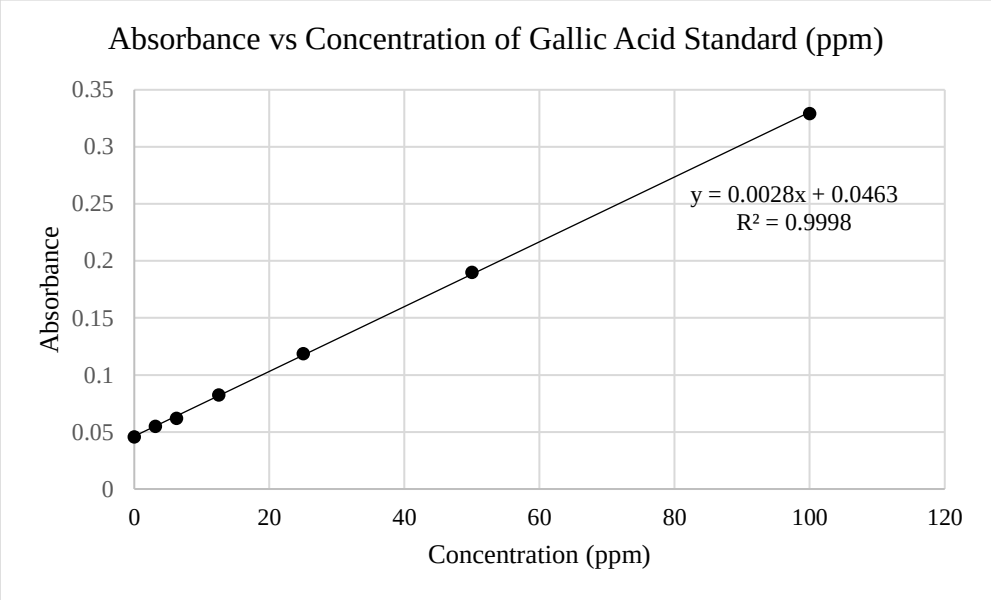

Fig. 8: Gallic Acid Standard Calibration Curve

7. ANNEX B

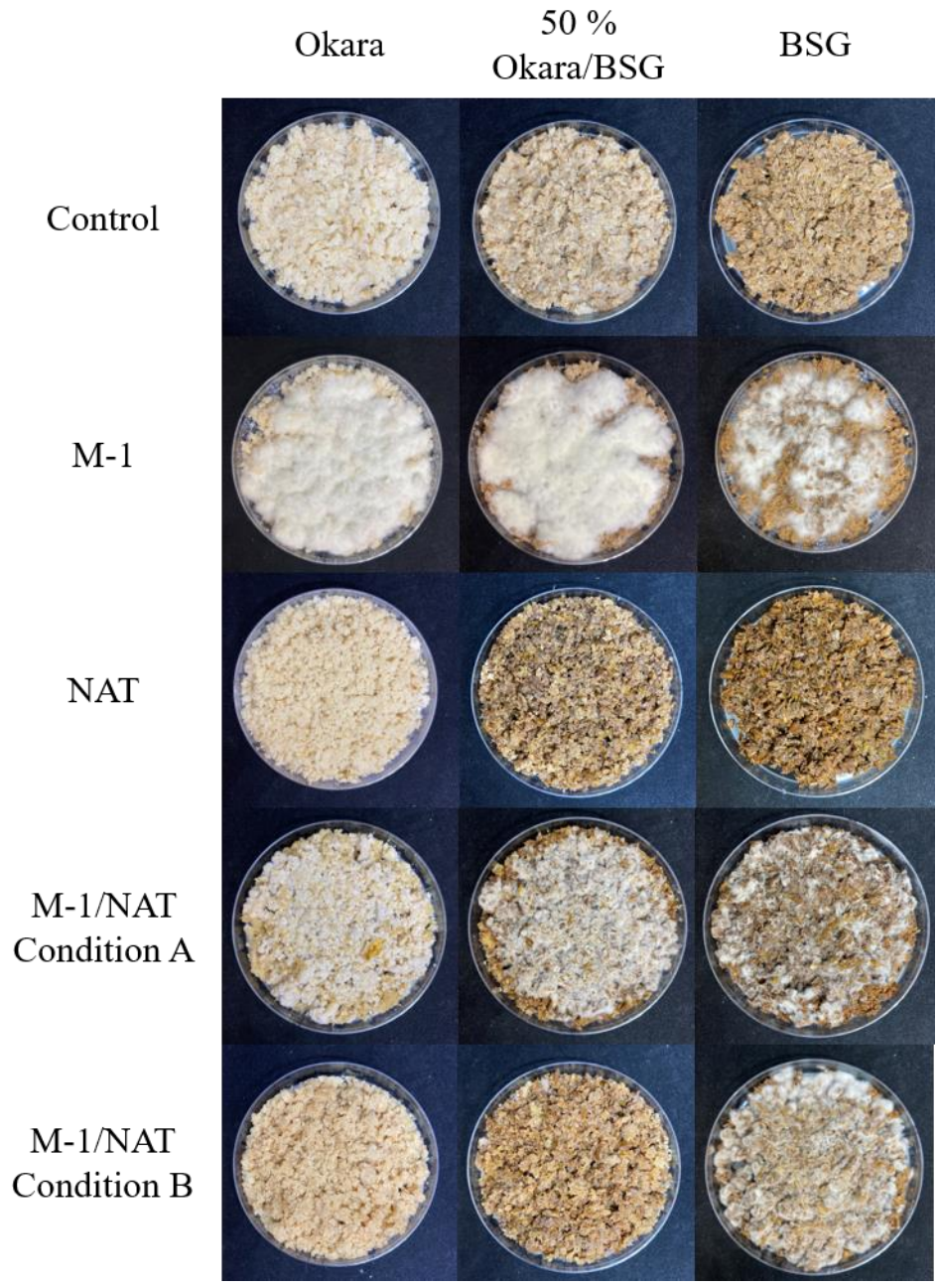

Fig. 9: Controls, and Samples After Fermentation