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Use of Microorganisms for the Enrichment of Zn and Se by using Solid-state Fermentation

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Use of Microorganisms for the Enrichment of Zn and Se by using Solid-state Fermentation

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ABSTRACT

Zinc (Zn) and selenium (Se) are essential minerals for human health and naturally found in many food sources. However, the risk of Zn and Se deficiency has been recognized in several countries. As most people throughout the world consume rice, grains, and cereals as staple foods in their daily diet, which contain a certain amount of phytic acid (antinutrients), the phytic acid content inhibits mineral absorption (Zn and Se) by the human body. Moreover, the low levels of Zn and Se content found in the crop's soil also reduce the amount of minerals in the food sources. Therefore, it is crucial to develop an effective method of naturally supplementing Zn and Se that promotes their absorption. There is yeast, bacteria, fungi, or a combination of these fermented products, enriched with Zn and Se, available in the market. In addition, the fermentation process can reduce the phytic acid content. However, there is no research or data on the application of solid-state fermentation (SSF) for the enhancement of Zn and Se. Therefore, in our study, SSF was applied to determine the amount of Zn and Se uptake by *Aspergillus oryzae*, *Bacillus subtilis*, and co-culture. The objectives of the study were to [1] determine the amount of Zn and Se uptake by *A. oryzae, B. subtilis*, and co-culture in sterile coarsely ground and whole sorghum grain by using solid-state fermentation and [2] measure the amount of phytic acid reduction (antinutrients) in the fermented coarsely ground biomass and whole grain samples containing Zn and Se. The sorghum grain (substrate) was treated with these organisms and supplemented with different concentrations of Zn or Se. After SSF, the samples were analyzed with Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The dried substrate was used to analyze the amount of phytic acid present in the grain sorghum after the SSF. The quantification of phytic acid in the substrate was determined using UV/VIS spectroscopy. The results showed that the biological efficiency of the organisms was affected by the addition of different concentrations of Zn and Se. Considering all the concentrations, the highest level of Zn absorption was obtained by

adding 50 μg/g of zinc acetate. In the Se absorption, 3.2 μg/g of selenium aspartate was efficient. The obtained data show that *A. oryzae, B. subtilis* and Co-culture can grow in the Zn or Se-containing substrate. SSF process with coarsely ground sorghum grain containing *A. oryzae* and co-culture significantly reduced the phytic acid content. The results showed that the SSF process with *A.oryzae* and/or Co-culture positively reduced the phytic acid content, which could help in the proper absorption of Zn and Se by the human body. The fermented biomass could be used as a Zn and Se-enriched ingredient for functional food products.

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INTRODUCTION

The trace minerals zinc (Zn) and selenium (Se) are two essential micronutrients for human health, which are involved in numerous aspects of cellular metabolism, the catalytic activity of enzymes, and plays a role in immune function, protein synthesis, wound healing, DNA synthesis, and cell division. These essential minerals are naturally found in a wide variety of food sources such as dairy, beans, nuts, grains, poultry, and seafood. Additionally, Zn and Se are also available in dietary supplements, fortified breakfast cereals, bread, and dairy products. According to the Food and Nutrition Board (Institute of Medicine, Food and Nutrition Board, NIH), the recommended daily allowance (RDA) of Zn is 5 mg/day for children, 11 mg/day for adult males, and 8 mg/day for adult females. As for Se, children are recommended to consume 30 µg/day, while adults should consume 55 µg/day. The lack of consumption of Zn leads to growth retardation, loss of appetite, impaired immune function, and lack of Se intake leading to poor immune response, cognitive decline, white muscle disease, skeletal muscle disorders, and KashineBeck disease (Rayman, 2012). However, the accessibility of these minerals from food and supplements falls short of meeting the recommended daily intake in certain parts of the world. Se deficiency is observed in North China, North Europe, Siberia, and New Zealand, and 33 % of the world population is affected by Zn deficiency (Singh et al., 2017; Ermakov et al., 2010; Fang et al., 2015).

One reason could be due to reduced or low amount of Zn and Se in the environment. The amount of Zn and Se in each type of plant-based food depends on the amount of these minerals in the soil and whether they are in a form that is amenable to plant uptake. As a result, Zn and Se concentrations in plant-based foods vary widely by geographic location. Another reason could be due to the presence of antinutrient (phytic acid) in whole grain bread, cereals, legumes, and other foods that bind Zn and Se and inhibit its absorption by the human body. Thus, the bioavailability

of Zn and Se from grains and plant foods is lower than from animal foods, although many grains and plant-based foods are reliable sources of Zn and Se. This issue can be reduced by subjecting grains and cereals to the fermentation process, which is solid-state fermentation technology that helps reduce the amount of phytic acid in grains and cereals and helps in proper absorption of minerals.

Recognizing the inadequacy of Zn and Se, there is an increased need for a rapid and efficient approach to enhance the environment with these minerals. Studies have attempted to enrich different food products with Zn and Se, as well as innovative ideas for increasing the level of this element in various organisms (Haug et al., 2007), such as Zn-enriched yeast, Se-enriched yeast, and Lactobacilli Zn biosorption. The fungal isolate, *Aspergillus oryzae* showed promising efficiency for the absorption of Zn ions (Aftab et al., 2017), yogurt fermentation (Alzate et al., 2010), and the genus *Pleurotus* and *Agaricus* (edible mushrooms) are macro-fungi of the Basidiomycetes family, research showed that they absorb Se into their mycelia and translocate these elements to their fruiting bodies (Thomet et al., 1999; Werner and Beelman 2002; da Silva et al., 2012). A study was carried out to assess the significance of solid-state fermentation of peanut oil cakes (POC) by *A. oryzae* on in vitro bioavailability of minerals (Fe, Zn, and Ca) through Caco-2 cells (Sadh et al., 2017) which showed significant increase in the Fe, Zn and Ca absorption.

In these studies, Zn and Se-enriched microorganisms were grown in a liquid medium, solid substrate, and mushroom cultivation. However, there are no studies on the enrichment of Zn and Se by *A. oryzae* and *B. subtilis* using sorghum grain as a substrate using solid-state fermentation technology. The work conducted in Dr. Ruben Morawicki's research team (University of Arkansas, Fayetteville, AR) on solid-state fermentation with *A. oryzae* and *B.*

subtilis improved the nutritional quality of grain sorghum. Results from this study showed a significant improvement in crude protein content after using the co-culture (*A. oryzae* and *B. subtilis*) (Diaz-Gonzalez and Morawicki, ICEF13 - 13th International Congress on Engineering and Food, 2019). Based on this experience with SSF using these microorganisms, and the fact that these two microorganisms are used in traditional food fermentations, we proposed to use *A. oryzae* and *B. subtilis* for the enrichment of Zn and Se. Also, there is ample proof from various research studies that fungi can absorb minerals in their mycelia, suggesting a similar capability can be anticipated in *A. oryzae*. A few studies exhibited that *Bacillus* species were capable of mineral uptake (Alzate et al., 2010; Pophaly et al., 2014; Aftab et al., 2017). The idea of using *B. subtilis* in combination with the *A. oryzae* is because they both grow synergistically (Bader et al., 2010). SSF technology supports the enrichment of Zn and Se through the fermentation process by using fungi, yeast, and bacteria. When Zn and Se are bound to an organic substrate and are efficiently absorbed by organisms, has a high biological activity and low toxicity. Therefore, we hypothesized that the SSF process helps to uptake the added Zn and Se by microorganisms, may enhance the substrate's Zn and Se content, and may positively affect reducing phytic acid in the fermented sorghum grain. The objectives of the present study were to [1] determine the amount of Zn and Se uptake by *A. oryzae, B. subtilis,* and co-culture in sterile coarsely ground sorghum grain, and whole sorghum grain by using solid-state fermentation, and [2] measure the amount of phytic acid reduction (antinutrients) in the fermented coarsely ground biomass and whole grain samples containing Zn and Se.

CHAPTER 1: LITERATURE REVIEW

1.1 Importance and demand of Zinc (Zn) and Selenium (Se)'s as essential micronutrients

The deficiencies of Zn and Se micronutrients have caused serious health problems (Mayer et al., 2008). Since Zn and Se have a significant impact on many metabolic processes and many countries have a deficiency of these minerals in the soil, much attention was given to research to develop effective methods of supplementing the environment with Zn and Se, which is an enrichment of fertilizers with Zn and Se compounds (Arthur et al., 2003). Various methods of supplementing these micronutrients have been developed, including pharmaceuticals, dietary supplements, fermented food products, and dry yeast biomass containing Zn and Se. To prevent micronutrient malnutrition, it is essential to increase the micronutrient content of food through food processing techniques such as soaking, fermentation, germination, debranning, and autoclaving, which are traditional methods used in food consumption (Ertop et al., 2018). Fermentation technology can be employed to enhance the Zn and Se content of food using fungi, yeast, and bacteria.

Zinc (Zn) enriched fermented foods by microorganisms

Zn is an essential nutrient that plays an important role in several biological processes of living organisms. Numerous studies have been undertaken to enrich Zn content in different food products using microorganisms (Dumont et al., 2006; Prasanna et al., 2016; Verma et al., 2021). Mineral accumulation by microorganisms is a complex process that depends on various factors. One crucial characteristic of a mineral-accumulating microorganism is its ability to resist the toxic effects of the mineral while maintaining its metabolic activities. Metal-resistant strains are crucial steps in identifying a metal-accumulating organism (Malik et al., 2004). Previous studies

have shown that some strains of microorganisms, including yeast, fungi, and *Lactobacillus* species, have the potential to accumulate significant amounts of trace elements such as Se or Zn and integrate them into organic compounds under appropriate conditions (Thomet et al., 1999; Suhajda et al., 2000; da Silva et al., 2012; Roepcke et al., 2011; Chen et al., 2013; Aftab et al., 2017).

Saccharomyces cerevisiae is a well-researched organism and a model system for accumulating minerals in high concentrations. It can absorb Zn from the liquid medium, and the enriched biomass is a potential source of microelements for animal and human nutrition. Additionally, it is used in various industries, such as biosorption and as a high-quality protein source in feed and food (Wang and Chen, 2006; Swanson and Fahey, 2004). The culture conditions used in these studies were based on other studies (Nicola et al., 2009; Chen and Wang, 2007; Stehlik-Tomas et al., 2004), which used similar conditions and mineral concentrations. In these studies, stock solutions of Zn were prepared in 25, 50, 100, 200, and 300 mg in 100 mL yeast extract peptone dextrose (YPD) medium. Results showed that the highest Zn accumulation in yeast cells was achieved at a concentration of 200 mg/100 mL YPD medium. Another study by Ren et al., 2011 investigated the biotransformation of Zn with different concentrations, ranging from 0 to 400 μg/mL fermentative medium. The biotransformation of Zn was affected not only by the increasing amount of Zn but also by yeast growth. Low concentrations of Zn promoted probiotic growth, while high concentrations inhibited growth, with the highest observed concentration of 150 μg/mL.

Furthermore, Roepcke et al. (2011) identified the Zn biosorption of fungal isolates, and out of 25 isolates, *A. oryzae* SV/09, *A. flavus* NA9, and *Paecilomyces formosus* DTO 63f4 showed promising efficiency for the biosorption of Zn ions.

Thomet et al. (1999) determined the accumulation of Zn by mycelia and fruiting bodies of edible mushrooms *Agaricus macrosporus* and *Agaricus silvicola*, using optical emission spectroscopy.

Selenium (Se) enriched fermented foods by microorganisms

Food industries use *S. cerevisiae*, described them either as baker's or brewer's yeast. The medium is usually beet or cane molasses which are added nutritional salts, and other growth factors to ensure maximal biomass and measured amounts of Se salts (for example, sodium selenite) as the Se source. Aerobic fermentation was followed by controlling the pH, temperature, Se feeding profile, and proper aeration to allow optimal growth of the yeast strain and maximum biomass production. Se-enriched yeast is produced that is then pasteurized, thereby killing the yeast, and dried, (frequently by spray drying) and the final product is used to incorporate in various food products. One company (Pharma Nord, Vejle, Denmark) does not use molasses as a growth medium instead grows the yeast on pure glucose syrup with appropriate pharmacopeia-grade additives to produce a pharmaceutical-grade Se-enriched yeast. As a result of the fermentation in the Se-enriched medium, the Se becomes organically bound to the yeast. The amount bound was greater than 90 %, typically 94 % (percentage of complexed organic Se found in three lots of LAMIN Se-yeast by KABS Laboratories, St-Hubert, QC, Canada) (Rahman et al., 2011).

Six strains of *Lactobacillus* species (LB1, LB2, LB3, LB4, LB5, and LB7) were used to evaluate strain capability to uptake Se (Andreoni et al., 2000). All these bacteria, when cultivated in the presence of 2 mg Se in MRS (De Man, Rogosa, and Sharpe) broth, showed a facility to absorb selenite, mostly during the exponential phase of growth. The LB7, LB1, and LB3 strains presented the highest specific accumulation, corresponding to 387, 347, and 284 μg/g dry weight, respectively. The three closely related strains LB3, LB4, and LB5, identified as *L. rhamnosus*, showed different selenite sensitivity and comparable Se accumulation capability.

The production of Se-enriched fermented milk (Alzate et al., 2010) was done by using Se-enriched biomass of non-pathogenic microorganisms (lactic acid bacteria, yeast propagated in a culture medium containing inorganic sources of Se). The results showed that microorganisms survived in the Se concentration ranging from 0.5 to 2.5 µg/g during the fermentation process.

Studies were conducted on *Pleurotus ostreatus* fungus which is an edible mushroom that possesses important nutritional and medicinal properties (da Silva et al., 2012; Werner and Beelman 2002; Thomet et al., 1999). *P. ostreatus* was grown in coffee husks enriched with various concentrations of sodium selenite (0, 3.2, 6.4, 12.8, 25.4, 51, 76.4, and 102 mg/kg). The results from these studies showed the maximum Se absorption by *P. ostreatus* mushrooms when coffee husks were enriched with 51 mg/kg of Se. At 76.4 and 102 mg/kg, Se absorption was inhibited, possibly due to the excess sodium selenite present in the substrate. At concentrations of 3.2 and 12.8 mg/kg of Se, 34% of added Se was absorbed, while in the substrate with 51 mg/kg only 16% was absorbed. Considering the obtained results, the consumption of 1.0 g of dried mushrooms grown on a substrate with 3.2 mg/kg of Se is enough to supply the amount of Se recommended for adults, 55µg/day (IOM, 2000). These results demonstrate the great potential of coffee husks in the production of Se-enriched mushrooms and show the ability of this fungus to absorb Se.

Studies of bread fermentation with Se-enriched bacteria and starter cultures indicated the ability to accumulate microorganisms and biotransform Se into the products (Alfthan et al. 1991; Arthur et al., 1992). The results of these studies indicated that people consuming bread enriched with Se showed changes in Se content in blood and plasma and changes in glutathione peroxidase activity were demonstrated. In summary, the addition of Se to biomass causes an increase of this element in both animal and plant products. Consumption of given products by

humans leads to an increase in the level of Se and thus improves the effectiveness of many healthy metabolic processes.

The results from the above study, which demonstrated the accumulation of Se and bioconversion abilities through research materials (transformation of inorganic forms into organic forms by way of enzymatic transformation), allowed for the development of effective methods of obtaining a plant or microbial Se-enriched biomass, which can be used in the production of food products such as beer, yogurt, or cheese formed through the fermentation process (Alfthan et al. 1991; Alzate et al., 2010).

Zn and Se-enriched foods by microorganisms

Previous studies have shown that certain strains such as yeast and *Lactobacillus* can accumulate trace elements such as Se and Zn and incorporate them into organic compounds under suitable conditions. The studies conducted by Ren et al. (2011) showed that the yeast and *Lactobacillus* strains could coexist and incorporate inorganic Se and Zn into organic form under optimal culture conditions using a special culture medium. The initial concentrations of Zn and Se were 5 and 150 μg/mL, the inoculation volume was 5%, and the liquid volume of the medium was 50 mL in a flask. The medium was then incubated at 32°C for 36 h, resulting in final product biomass of 26.83 g/L, an organic Se concentration of 173.35 μg/g, and an organic Zn concentration of 4.38 mg/g.

According to Zieba et al. (2020) the effectiveness of *P. eryngii* mycelia from *in vitro* cultures and fruiting bodies in terms of Zn and Se accumulation was investigated. The mycelium of *P. eryngii* could effectively absorb Zn and Se from growth media, making it possible to biofortify with trace elements that have functional activity in the human body. Substrates were supplemented with sodium selenite at a concentration of 50 mg/L, Zn sulfate, and Zn hydro-aspartate at a concentration of 87.2 and 100.0 mg/L, respectively, to increase the Se and Zn

content in *P. eryngii* fruiting bodies and mycelia. The results demonstrated that both fruiting bodies and mycelium of *P. eryngii* could be considered functional food or raw material enriched in essential nutrients and could be used in food supplementation.

1.2 Detection methods for Zn and Se

Samples were analyzed for total Zn and Se content using graphite furnace atomic absorption spectrophotometry (SIMAA 6000, Perkin Elmer, USA) by EPA Method 7740 (USEPA, 1986). Narayana et al. (2003) used the spectrophotometric method to determine Zn and Se amounts using potassium iodide and starch as reagents. The amount of Zn and Se content of the nutrient broth, and the dried yeast samples were determined by the Inductive Coupled Plasma Atomic Emission System (ICP-AES). The dried yeast samples were prepared for analysis as per the procedure by Suhajda et al., 2000. According to Selvakumar et al. (1998) Se content was also determined in MRS broth by atomic absorption spectrometry (Perkin-Elmer, Mod. AS-60).

Se contained in the skimmed milk was quantified by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). Anion-pair reversed-phase column was used with a mobile phase consisting of 0.05% tri-fluoro acetic acid (TFA), and 0.1% heptafluorobutyric acid (HFBA) as ion pairing, and 1% MeOH (v/v) at pH 2.6. The analytical peaks were evaluated in terms of peak area measurements with standard addition calibration for both m/z 78 and 82. In the preliminary separation of the seleno compounds by injecting 2 mL of the above-mentioned aqueous extract into a Superdex HiLoad 16/60 peptide column. A 5 mM HPO24/H2PO4 (pH 7.5) buffer solution at 1 mL/min was used as the mobile phase. The detectors used were a DAD and an ICP-MS (Alzate et al., 2010).

1.3 Importance of sorghum grain and its nutritional profile

Sorghum (Kingdom: Plantae and Family: Poaceae) one of the most vital cereal crops worldwide, is also the fifth highest-produced crop globally, with an annual output of 3.5 million tons in 2021, as reported by (FAO, 2021). Its adaptability to different environmental conditions has made it a critical source of energy and nutrition, particularly in semiarid regions and underdeveloped countries. Sorghum's exceptional agronomic performance contributes to its popularity, as it can grow in various conditions, including heat, drought, high altitudes, and even saline-alkaline and barren soil (Smith et al., 2000).

The nutritional content of sorghum varies between different types, each with unique genotypes, color, and phenolic profiles. These types include white, yellow, red, brown, and black sorghum (Xiong et al., 2019). Sorghum grains consist of three main components, namely pericarp, embryo, and endosperm. The main constituents of sorghum grain are carbohydrates, which encompass starch and non-starch polysaccharides, as well as proteins and lipids (Hill et al., 2019).

Starch is the primary carbohydrate stored in the endosperm of sorghum grains, with significant variations in content ranging from 32.1 to 72.5/ 100 g of grain depending on the variety (Udachan et al., 2012). Sorghum is also a rich source of fiber, with non-starch carbohydrates consisting of both soluble and insoluble fibers ranging from 10% to 25% and 75% to 90%, respectively. The fiber content in sorghum ranges from 6 to 15 g/ 100 g of grain and is primarily found in the pericarp and the endosperm cell walls. The endosperm of sorghum grain contains nearly 80% of both starch and proteins. Compared to other cereal grains, sorghum's traditional nutritional value is moderately reduced because it has lower digestibility of proteins

and starch, leading to a decrease in metabolizable energy (Taylor and Schüssler 1986; Taylor and Emmambux 2010; Stefoska-Needham et al., 2015).

Studies have shown that sorghum lipids are mainly composed of unsaturated fatty acids with a high content of polyunsaturated fatty acids, like maize but highly unsaturated. The primary fatty acids found in sorghum include oleic, linoleic, linolenic, stearic, and palmitic acids (USDA, 2019). Sorghum is also a rich source of vitamins and minerals. It contains a complex of vitamin B, including pyridoxine, thiamine, and riboflavin, as well as fat-soluble vitamins such as A, D, E, and K. The main minerals present in sorghum include potassium, phosphorus, magnesium, and zinc (USDA, 2019). These findings suggest that sorghum can be a valuable source of essential nutrients, especially for those with dietary restrictions or deficiencies.

Food Industrial Application

Sorghum has been a staple food crop in developing regions such as Asia and Africa, feeding millions of people globally. Traditionally, sorghum has been utilized to make various foods. Recently, the food industry's core innovation has been to develop new, functional, and healthy foods and beverages using sorghum, resulting in several food products made and investigated (Xiong et al., 2019). Sorghum is an ideal alternative food source for people with celiac disease as it is gluten-free, unlike other major gluten-containing cereal crops like wheat and barley. Sorghum can be used to make healthy snacks such as cookies and biscuits with high phenolic content and antioxidant activity. Sorghum can also be utilized to create functional staple foods, promoting human health, and providing an excellent option for gluten-intolerant people who rely heavily on gluten-based or wheat-based foods such as noodles, bread, and cereals (DeMesa-Stonestreet et al., 2010).

In addition to its agronomic benefits, sorghum possesses high nutritional value, is gluten-free, rich in resistant starch, and offers a wide range of bioactive phenolic compounds (Awika et

al., 2004). While compared to the other major cereal crops, sorghum is rich and has various phenolic compounds such as simple phenolic acids, flavonoids, and tannins (Dykes et al., 2009; Singh et al., 2014). Sorghum grain diverse phenolic compounds offer multiple health benefits, including cancer prevention, antioxidant and anti-inflammatory activities, prevention of diabetes, obesity, dyslipidemia, and cardiovascular disease (Yang et al., 2009; Xiong et al., 2019).

1.4 Antinutritional content in Sorghum grain

Sorghum contains antinutritional factors such as phytic acid, trypsin inhibitors, and tannins. However, the amount of phytic acid content in sorghum grain is excessive compared to other antinutrients (Thakur et al., 2019). The phytic acid compounds are known to usually interfere with carbohydrate and protein digestion and the bioavailability of minerals. As phytate binds to carbohydrates, proteins, and minerals, it consequently minimizes the digestibility of these nutrients (Haug and Lantzsch 1983). For sorghum to be efficiently employed to the utmost potential as human food and to increase its nutritional quality, these undesirable components present in sorghum must be eliminated or reduced. For this purpose, several methods have been practiced but researchers have presented germination and fermentation as means. In cereals, fermentation is known for reducing the antinutritional content such as phytic acid and mobilizing nutrient contents (Osman et al., 2004).

Phytic acid, present in grains as metal cation complexes with Zn, Fe, Ca, and proteins, can be enzymatically degraded to increase the number of soluble minerals. Natural fermentation, such as sourdough fermentation, provides the optimum pH (generally below pH 4.5) required for phytic acid degradation (Ertop et al., 2018). Fermentation can effectively reduce food inhibitors, phytic acid, and tannins, as demonstrated by a study on millet grain fermentation for 12 h and 24 h (Coulibaly et al., 2011). However, a combination of fermentation with other treatments, such

as germination, can be more effective in enhancing the nutritional value of grains. A study reported an 88.3% reduction in phytate content when germinated pearl millet was fermented with mixed pure cultures of *S. cerevisiae*, *L. brevis*, and *L. ermentumat* at 30°C for 72 h, resulting in significant changes in chemical composition and elimination of anti-nutritional factors (Abdelrahaman et al., 2005). Furthermore, a study conducted by Rahman et al. (2011) showed that the phytic acid content of three varieties of grains varied significantly, ranging from 267.3 mg/100 g to 369.1 mg/100 g. The effect of fermentation on phytic acid content resulted in a progressive and significant decrease of over 50% in phytic acid contents of the Fetarita, Safra, and Ahmer varieties. Therefore, fermentation can effectively reduce the phytic acid content in grains, and a combination of fermentation with other treatments can further enhance nutritional value.

1.5 Solid-state fermentation

Solid-state fermentation (SSF) is a fermentation process that utilizes non-soluble materials as physical support and nutrients, in the absence of free-flowing liquid. It has been employed successfully to produce enzymes, antibiotics, and flavorings and is currently used on a commercial scale in the food industry and waste treatment. Microorganisms performing SSF have various physiological functions beyond their nutritional properties, and they break down complex organic compounds into smaller molecules during fermentation. Fermenting soybean meal with different microorganisms improved its bio-functional properties, leading to an increase in phenols, flavonoids, peptides, and other active ingredients. *Bacillus* sp. reduced antinutrients and improved the nutritional value of soybean products (Chi et al., 2016; Teng et al., 2012; Dai et al., 2017). Solid substrates provide a conducive environment for the growth of bacteria, yeasts, and fungi, which are utilized in SSF processes. Among them, filamentous fungi exhibit the

highest level of adaptation to SSF and are prominent (Kumar et al., 2021). Due to their physiological, enzymological, and biochemical properties, filamentous fungi hold great significance as the primary group of microorganisms employed in SSF processes (Manan and Webb 2017). The hyphal mode of growth provides significant advantages to filamentous fungi over unicellular microorganisms in terms of colonizing solid substrates and utilizing available nutrients. This growth mode involves the apical extension of hyphal tips and the generation of new hyphal tips through branching. Unlike a linear extension, the exponential growth pattern of biomass is mainly achieved through frequent branching during the vegetative stage. This growth pattern enables filamentous fungi to penetrate solid substrates effectively. The firm and solid structure of the mycelium, supported by the cell wall structure and branching, allows for efficient secretion of hydrolytic enzymes at the hyphal tips. This concentrated enzyme action facilitates penetration into various solid substrates and enhances access to the full range of available nutrients within the particles (Walker and White 2017).

Factors that influence SSF

The success of SSF is dependent on several factors, including the microorganisms used, the solid substrate, and the bioreactor. The efficiency and effectiveness of SSF can be improved by controlling and optimizing these factors. Factors that influence the performance of SSF can be categorized into three major categories: biological factors, physicochemical factors, and mechanical factors.

The biological factors that influence SSF include the type of microorganism used, the inoculum, the substrate, the media, and the inert carrier. The selection of the appropriate microorganism is crucial for the success of SSF, and it is essential to choose a microorganism that can survive and thrive in solid-state fermentation conditions. The inoculum size and quality can also affect the fermentation performance, and it is crucial to use a high-quality inoculum to

ensure consistent and efficient fermentation. The substrate and media used in SSF should provide adequate nutrients to support the growth and metabolism of the microorganism. In contrast, the physicochemical factors include moisture content, pH, temperature, gaseous environment, aeration, and particle size. Mechanical factors include mixing or agitation and particle size of the bioreactor (Manan, 2014).

Advantages of SSF

Fermentation has been widely used to produce a variety of substances that are highly beneficial to individuals and industries. Over the years, fermentation techniques have gained immense importance due to their economic and environmental advantages. Ancient techniques have been further modified and refined to maximize productivity. Fermentation is a process by which bacteria, yeasts, and molds convert sugars and carbohydrates into less complex products such as carbon dioxide and alcohol. It involves the conversion of large molecules to small molecules or molecular oxidation/reduction mechanisms mediated by selected microorganisms. Some studies have demonstrated that the fermentation of cereals and legumes enhances their nutritive value and antioxidant properties, reduces some anti-nutritional endogenous compounds such as phytic acid, and exerts beneficial effects on protein digestibility and biological value (Kuala & Sharma 2018; Ertop et al., 2018; Ijeoma et al., 2020).

Numerous research studies have provided evidence of the efficacy of utilizing food-grade microorganisms in solid-state fermentation (SSF) to release bound phenolic compounds in cereal and legume products. These studies have explored the impact of SSF on cereal and legume grains, as well as their by-products, by investigating the involvement of microorganisms, their hydrolytic enzymes, the fermentability of agri-food substrates, and the potential health advantages derived from the enhanced bioactive compounds produced through SSF (Liu et al., 2022). SSF with fungi (*Aspergillus* spp. and *Rhizopus* spp.), bacteria (*B. subtilis* and lactic acid

bacteria spp.), and yeast (*Saccharomyces cerevisiae*) significantly increased the bioactive phenolics and antioxidant capacities in cereal and legume grains and by-products (De Villa et al., 2021).

The use of spore-forming microbes such as fungi and yeasts as the inoculum in SSF offers advantages in having extended storage capacity, while optimal pH control serves a similar purpose in the selective growth of microbes in SSF. For example, GRAS (Generally recognized as safe) filamentous fungi and yeast optimally grow around pH 5.5 to 6, while food bacteria, such as *Bacillus* sp., have optimal growth at neutral or alkaline pH (Kumar et al., 2021). Aeration has a considerable effect on SSF since it facilitates oxygen intake and the release of carbon dioxide, volatile products, and heat from the system.

Bioactive enhancement by microorganisms in SSF, filamentous fungi have been the most widely used microorganisms due to their natural growth in SSF conditions, increased enzyme production, and efficient production of high-value compounds (yPostigo et al., 2021; Šelo et al., 2021). Their growth is effectively supported by the mycelial system that penetrates the substrate particles, and their inherent ability to break down insoluble cell wall components through hydrolytic enzyme secretions at the hyphal tips. The highly interconnected branches of mycelia facilitate nutrient and gas exchange, particularly between the aerobic and anaerobic regions (Saharan et al., 2017). Lignocellulolytic enzymes including amylases, xylanases, pectinases, cellulases, phenolic esterases, and laccase have been associated with the effective digestion of grain substrates and release of bioactive, most dominantly phenolic compounds (Lizardi-Jiménez and Hernández-Martínez 2017; Sadh et al., 2017).

Fermentation offers a range of benefits, including the destruction of undesirable components, enhancement of nutritional value and appearance, reduction in cooking time and energy usage, and the assurance of food safety (Simango, 1997). This process also improves the

texture, taste, aroma, and shelf life of the final product (Chavan et al., 1979). Maize and other crops can benefit from fermentation to enhance their organoleptic properties and to increase nutrient bioavailability and bioaccessibility (Hotz and Gibson 2007). Fermentation also provides protection against harmful bacteria through the antimicrobial activity of lactic acid and can reduce aflatoxins through detoxification (Chaves-López et al., 2014; Li et al., 2007). In countries where cereals and pulses are consumed as a staple food, fermentation can effectively reduce mineral deficiency among the population, owing to its unique benefits (Kumar et al., 2010).

Application of solid-state fermentation

The recent trends in SSF have mainly focused on the development of biotransformation of crop residues and crops for the enrichment of nutrition, biological detoxification of the agro-industrial residue, bio pulping, and manufacturing or the production of value-added products, such as enzymes, organic acids, secondary metabolites, antibiotics, vitamins, biofuel, and bio-control agents, etc. (Christensen et al., 1988; Machida et al., 2008; Kuala and Sharma 2018; De Villa et al., 2021).

1.6 Role of microorganisms in solid-state fermentation

Microorganisms are the most important factors during the development of an SSF and the choice of substrates. Microorganisms that are particularly suitable for SSF are filamentous fungi since the technique simulates their natural habitat. In this condition, they can synthesize considerable amounts of enzymes and other metabolites (Farinas, 2015). Although filamentous fungi are considered the most appropriate microorganisms for SSF, and secondly the yeasts, which are also able to grow in a low water activity environment, there are also some species of bacteria (e.g., *B. subtilis*, *B. thuringiensis*, and *Lactobacillus* spp.) which have been reported to successfully produce enzymes in solid-state condition (Singhania et al., 2009).

Fermentation can be done both naturally and by using a starter culture (Nkhata et al., 2018). In cereals and legumes, the microorganisms that are involved are the surface flora of seeds for natural fermentation and their complex microflora is due to the nature of cereal grain. The nature and the type of microorganisms that are nurtured by these grains mainly depend on various factors like the soil conditions and the climatic conditions in which these plants were grown, the biological conditions, the weather condition after harvesting, and the storage condition and duration (Soccol et al., 2017). Generally, fermentation is natural, involving mixed cultures of yeast, bacteria, or both. The most common mold involved is *Aspergillus* even though *Fusarium, Paecilomyces, Trichothecium, Cladosporium,* and *Penicillium* are utilized for some products. Whereas the most utilized bacteria for fermentation are the species *Bacillus*, *Lactobacillus*, *Pediococcus*, *Leuconstoc*, *Streptococcus*, and *Micrococcus* (Chavan et al., 1989).

In the present study we are using *A. oryzae* and *B. subtilis* for solid-state fermentation. The role played by these two organisms are mentioned below,

Aspergillus oryzae

Aerobic filamentous fungus *A. oryzae* is commonly found in soils and plants, especially rice, and is often used in traditional fermentation in Japan, China, and Korea due to its optimal growth temperature of 25-30°C (±1°C) and unique solid-state cultivation technique that is considered to have emerged more than 2000 years ago (Kobayashi et al., 2007). This fungus is widely employed in the manufacturing of soy sauce, bean curd seasoning, and vinegar in many traditional fermentation industries. Generally, filamentous fungi can produce diverse and extensive amounts of enzymes in a secretory manner. That is *A. oryzae* is highly capable of the production of different enzymes (Christensen et al., 1988). Furthermore, the ability to produce proteins is highly enhanced in solid-state cultivation as nearly 50 g of α–amylase is produced by *A. oryzae* from 1 kg of wheat bran (Machida et al., 2008). For the manufacturing of various

chemicals and diverse enzymes, SSF is utilizing *A. oryzae* and *A. sojae* in the industries (Yu et al., 2004). Chen et al. (2013) reported that after 36 h of fermentation, the protein content increased from 50.47 to 58.93% (w/w) and significant improvement was observed in the amino acid contents. The research thoroughly studied the *A. oryzae* solid-state fermentation of soybean, and the resulting premium product could provide a better protein source for monogastric animals. Overall, SSF with *A. oryzae* exhibited better bioactive enhancement potential than other *Aspergillus* species, especially in the fermentation of cereal products.

Bacillus subtilis

Bacillus species are aerobes gram-positive, rod-shaped bacterium that forms heat-resistant, dormant, and can grow in the temperature range from 25 to 37°C and at pH values from 5 to 9 (Wang et al., 2015). *Bacillus* species have been traditionally considered bio-safe microorganisms with the ability to produce abundant enzymes and generate a diverse variety of enzymes that have good amylase, esterase, protease, lipase, and some cellulase and xylanase activity (Yao et al.,2013). *B. subtilis* also has excellent fermentation properties which contribute to the degradation of macromolecular substrates, conversion of ingredients, as well as the production of new compounds, leading to improvement in nutritional and functional properties of the substrate after fermentation. As a probiotic, *B. subtilis* is often used as a feed additive to promote growth performance, natural immunity, and disease resistance in aquatic species, such as flounder (*Paralichthys olivaceus*), Nile tilapia (*Oreochromis niloticus*), and white leg shrimp (*Penaeus vannamei*) (van Dijl et al., 2013).

B. subtilis is popularly used in the fermentation of soybean food products, such as Korean soybean paste doenjang and Japanese fermented soybean natto (De Villa et al., 2021). SSF with *B. subtilis* alone or in combination with other microorganisms not only led to a decrease in allergenic proteins but increased protein digestibility and available amino acids and peptides of

whole soybean or soybean meal (Zhang et al., 2021). Similarly, soybean meal fermented with *B. subtilis*, *Lactobacillus casei*, and yeast co-culture reduced both allergenicity and intestinal damage in mice, which was attributed to the hydrolysis of allergen sequences in the soy protein into low-molecular-weight polypeptides and amino acids (Gopikrishna et al., 2021). These studies provide the base for the involvement of enzymes during *B. subtilis* SSF of foods or agri-processing by-products; however, further research is still required for unlocking the roles of the specific enzymes and substrates. Studies demonstrated the ability of *B. subtilis* alone or in combination with other microorganisms to improve digestibility, reducing allergenicity, and increasing bioactive peptides in sorghum food products (De Villa et al., 2021).

1.7 Use of co-culture (*A. oryzae* and *B. subtilis*) in solid-state fermentation

Co-culture fermentations can be utilized in the production of foods, food additives, pharmaceuticals, enzymes, bulk and fine chemicals, bioremediation, and degradation of lignocelluloses. They offer the opportunity to use cheap substrates and increase yields and product quality. The further potential of co-culture rests in the discovery of new substances with industrial or pharmaceutical interest such as fine chemicals or antibacterial active substances and other secondary metabolites that are produced in cocultivation only (Bader et al., 2010).

There are many instances where the utilization of co-culture appears to be advantageous over a single microorganism because of the potential for synergistic utilization of the metabolic pathways of all involved strains in a co-culture situation. In nature, most biotransformation takes place by the combination of metabolic pathways from different microorganisms. Some examples of the coexistence of different microorganisms are the forest soils, compost piles, the aerobic and the anaerobic zones of water, spontaneous fermentations of sugar-containing saps, and human skin (Bader et al., 2010).

Furthermore, cocultivation processes can help find new substances of industrial interest, because several secondary metabolites are produced during cocultivation (Oh et al., 2007). The controlled cultivation of cocultures enables the synergistic utilization of the metabolic pathways of the participating microorganisms under industrial, reproducible, and controlled conditions. The optimal values of process parameters (pH, temperature, and oxygen demand) and the acceptable ranges of the substrate and product concentrations must be known and considered to achieve controlled fermentation, as in pure culture cultivation.

A fermentation study involving *A. oryzae* and *B. subtilis* showed a consistent increase in the proportion of soluble protein increased by 19.4% and 63.11%, after co-culture fermentation due to stronger hydrolysis by *B. subtilis* under fermentation conditions (Teng et al., 2012).

1.8 References

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CHAPTER 2: OBJECTIVE 1

Zinc and Selenium uptake by *Aspergillus oryzae, Bacillus subtilis,* and co-culture in sterile coarsely ground, and whole sorghum grain by using solid-state fermentation.

2.1 Introduction

Zinc (Zn) and Selenium (Se) are essential trace minerals that play crucial roles in maintaining overall health and well-being. The lack of availability of Zn and Se can have detrimental effects on human health. In the case of Zn, several factors contribute to its limited availability in natural sources. One factor is the depletion of Zn content in agricultural soils due to intensive farming practices, leading to reduced crop Zn levels. Furthermore, food processing and refining procedures can further decrease Zn content. Additionally, plant-based diets are rich in phytates, such as whole grains and legumes, and can inhibit zinc absorption, potentially resulting in inadequate Zn intake. Similarly, Se availability in its natural form can be limited. The Se content in soil largely determines its presence in crops. Se deficiency in soil is common in certain regions, making it challenging for plants to absorb enough Se. Consequently, individuals relying on locally grown food from these regions may experience inadequate selenium intake. Addressing the lack of availability of Zn and Se requires a different approach. One of the approaches is the sustainable production of fermented products enriched with essential minerals such as Zn and Se, which has become increasingly important due to their limited availability from food sources. Therefore, it is crucial to find alternative ways of utilizing food and agro-industrial residues to produce mineral rich food products. Solid-state fermentation (SSF) has emerged as a promising biotechnological technique for the sustainable production of ingredients. In SSF, microorganisms play a crucial role in synthesizing enzymes, especially extracellular enzymes. These enzymes can break down complex compounds such as

carbohydrates, proteins, fats, and polysaccharides, among others, into smaller molecules that can be assimilated by cells. *Aspergillus oryzae* and *Bacillus subtilis* are generally recognized as safe as per Food and Drug Administration (FDA) for human consumption and are the most widely used industrial microorganisms for fermentation technology. Additionally, *A. oryzae* is a filamentous fungus and as a crucial microorganism in SSF persists due to their mycelial growth mode (Mitchell et al., 2011). They are capable of penetrating inside the substrate and contribute to the nutritional enhancement of the substrate after fermentation, improving the texture, aroma, taste, and color of the final product. Utilizing *B. subtilis* along with *A. oryzae* has the advantage of improving the rate of substrate utilization which exhibits symbiotic behavior and mutual growth of microbial communities, mixed culture processes are common in most natural habitats (Nigam and Pandey, 2009). In the present study, sorghum grain was used as a substrate because it possesses several desirable qualities that make it suitable for SSF. Its nutritional composition, moisture retention capacity, physical structure, sterilizability, availability, and compatibility with microorganisms contribute to its effectiveness as a substrate in SSF processes. We investigated the potential of coarsely ground sorghum grain and whole sorghum grain as a substrate for SSF to enrich Zn and Se using the microorganisms *A. oryzae* and *B. subtilis*, as well as a co-culture approach.

2.2 Materials and Method

2.2.1 Culture Propagation, Inoculum, and Spore Suspension Preparation

A. oryzae and *B. subtilis* propagation

Cultures of *A. oryzae* NRRL 692 and *B. subtilis* NRRL 744 were obtained from the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) and was revived as per the recommended procedure by ARS and were maintained frozen in potato dextrose broth (PDB)

and nutrient broth (Difco VWR, USA) with 50% v/v of an 80-86% sterile glycerol solution in 2 mL aliquots at -80°C.

Inoculum and Spore Suspension Preparation of *A. oryzae*

A 2 mL *A. oryzae* culture from a frozen vial was transferred into 50 mL of sterilized potato dextrose broth (PDB). The composition (g/L) of the PDB is potato starch (from the infusion) 4.0 g/L and dextrose 20.0 g/L and incubated at 30°C and 200 rpm for 48 h. After the complete incubation period, 10 mL of fully-grown culture was transferred to a new 50 mL sterilized PDB. Incubation was repeated at 30°C and 200 rpm for 48 h in a MaxQ 4450 orbital shaker (ThermoScientific, Waltham, MA, USA). After the complete incubation period, the fully-grown culture was centrifuged at 1450 g for 10 minutes. The supernatant was discarded and a loop full of the centrifuged pellet was taken to do a T streak on agar plates.

After the activation, fungal spores from the above medium were cultured on potato dextrose agar (PDA) petri dishes at 30°C for 7 days. Fungal spores grown for seven days in PDA were harvested from the petri dishes by gently washing them with 0.1% Tween 80 (polyethylene sorbitol ester, 100 µL of 0.1% tween 80 was dissolved in 100 mL of distilled water and sterilized by autoclaving at 121°C for 15 min) and then filtered through a Miracloth to remove all fragments of mycelium, spore suspension was collected in a sterile bottle, and cells were counted with a hemocytometer under the microscope to select the volume of inoculum needed for fermentation (10^7 spores/mL). The spore suspension was stored at 4°C until further use.

Inoculum and Spore Suspension Preparation of *B. subtilis*

For the activation, 2 mL of *B. subtilis* culture from a frozen vial (-80°C) were transferred into 50 mL of sterilized difco sporulation media (DSM). The composition (g/L) of the DSM was: 8.0 g nutrient broth (Difco), 1.0 g potassium chloride (KCl), 1 mL of magnesium sulfate (1M

solution), 1 mL of manganese chloride ($MnCl_2$, 10 mM solution), 0.5 mL calcium chloride ($CaCl_2$) 1M solution, and 1 mL of iron II sulfate ($FeSO_4$, 1 mM solution), completed to 1 L with deionized water, the prepared growth media was sterilized as per the instructions given by the manufacturer, the prepared media plates were incubated at room temperature for 48 hours to check the sterility, after the pre-incubation of sterile broth was used for the testing and to inoculate the culture into the broth and incubated at 37°C and 150 rpm for 24 h in a MaxQ 4450 orbital shaker. After the complete incubation period, 10 mL of fully-grown culture was transferred into a new 50 mL sterilized DSM. Incubation was repeated at 37°C and 150 rpm for 24 h.

To prepare the spore suspension, *B. subtilis* was propagated using DSM, cultured at 200 rpm and 37 ˚C for 48 h. The *B. subtilis* inoculum was prepared 48 hours before inoculation of the substrate by transferring a stock culture (2 mL) onto a 125 mL flask containing 50 mL of sterile DSM broth and incubated at 37°C and 200 rpm for 24 hours in a MaxQ 4450 orbital shaker. At least two pre-cultures were cultivated before inoculation. Next, the inoculum was transferred to centrifuge tubes and centrifuged at 1450 g for 10 minutes. The supernatant was discarded, the cell pellets were re-suspended in distilled water (5 mL) and cells were counted with a hemocytometer under the microscope to select the right volume of inoculum needed for fermentation (5×10^7 cells/mL) to be used as the inoculum.

Zn and Se Concentrations - Coarsely Ground Sorghum Grain

Zinc acetate and selenium aspartate were sourced from Lifeplus International (Batesville, AR, USA). For the enrichment of Zn and Se, different concentrations were added into coarsely ground sorghum grains. Zinc acetate (0, 50, 100, 150, 200, 300, and 400 µg/g) and selenium aspartate (0, 3.2, 6.4, 12.8, 25.4, 51, 76.4, and 102 µg/g) were added.

2.2.2 Solid-State Fermentation Process: Coarsely Ground Sorghum Grain

Substrate Preparation

White grain sorghum (food-grade) from Anthony's premium sorghum grain (gluten-free, non-GMO, and whole-grain) was used in this experiment. Cleaned sorghum grains (15g) were coarsely ground by pestle and mortar, mixed with water (hydration) at a 2:1 ratio in a 250 mL Erlenmeyer flask, and sterilized at 121°C for 20 minutes. After sterilization, mineral supplementation with Zn or Se of the required concentration was added to the samples before inoculation.

***Aspergillus oryzae* Fermentation**

Sterilized coarsely ground samples were inoculated with *A. oryzae*. Inoculation consisted of adding 2.5 mL of *A. oryzae* spore suspension (10^7 spores/mL) (Chen et al., 2013), and different concentrations of Zn and Se were added into the substrate and incubated in a stationary state at 30˚C for seven days. After the completion of fermentation, the samples were oven-dried at 60˚C in a VWR gravity convection oven, re-ground in a Hamilton Beach 80335 coffee grinder, and stored at 4˚C for mineral (Zn and Se), moisture, and phytic acid analysis (**Figure 2.1.a**).

***Bacillus subtilis* Fermentation**

Sterilized coarsely ground samples were inoculated with *B. subtilis.* Inoculation consisted of adding 3.0 mL of *B. subtilis* spore suspension (5×10^7 spores/mL) (Delmy Diaz, Rubén Morawicki, 2019), different concentrations of Zn and Se added into the substrate and incubated in a stationary state at 37˚C for two days. After the completion of fermentation, the samples were oven-dried at 60˚C in a gravity convection oven, re-ground in a coffee grinder, and stored at 4˚C for mineral (Zn and Se), moisture, and phytic analysis (**Figure 2.1. a**).

Co-culture Fermentation

Sterilized coarsely ground sorghum samples were inoculated with *A. oryzae*, 2.5 mL of spore suspension (10^7 spores/mL) was inoculated and incubated at 30 ˚C for seven days followed by inoculation of 3.0 mL of *B. subtilis* spore suspension (5 x 10^7 spores/mL) incubated at 37 ˚C for two days. After the completion of fermentation, the fermented samples were oven-dried at 60˚C in a VWR gravity convection oven, re-ground in a coffee grinder, and stored at 4˚C for mineral (Zn and Se), moisture, and phytic analysis (**Figure 2.1. b**).

Zn and Se Analysis

The oven-dried substrates at 60°C for 24 h (fermented and nonfermented) were ground in an electric grinder to obtain a powder form and stored at 4˚C for further analysis. The stored samples were sent to inductively coupled plasma-atomic emission spectrometry (ICP-MS) for the estimation of Zn and Se (**Figure 2.2**).

2.2.3 Solid-State Fermentation Process: Whole Sorghum Grain

In the coarsely ground sorghum grain, the exact amount of Zn and Se was challenging due to the complex entanglement of microorganisms and solid particles, particularly with filamentous fungi that penetrate deeply into the substrate. To solve this issue, sterile whole sorghum grain was used for the SSF.

Substrate Preparation

White grain sorghum (food-grade) from Anthony's premium sorghum grain (gluten-free, non-GMO, and whole-grain) was used in this experiment. Sterile whole grain sorghum grain (15 g) was mixed with water (hydration) at a 2:1 ratio in a 250 mL Erlenmeyer flask and sterilized at 121°C for 20 minutes. After sterilization, mineral supplementation with Zn or Se of the required concentration was added to the samples before inoculation.

Zn and Se Concentrations - Whole Sorghum Grain

For the enrichment of Zn and Se, different concentrations were added into whole sorghum grains. Zinc acetate (0, 10, 25, 50, 100, 250, and 400 µg/g) and Selenium aspartate (0, 1, 2, and 3.2 µg/g) were added.

Fermentation and Extraction: *Aspergillus oryzae*

Sterilized whole grains were inoculated with *A. oryzae* of 10^7 spores/mL (Chen et al., 2013), and different concentrations of Zn (0, 10, 25, 50,100, 250, and 400 µg/g**)** and Se (0, 1, 2, 3.2 µg/g**)** were added into the substrate and incubated in a stationary state at 30˚C for seven days. After the completion of fermentation with *A. oryzae*, the mycelia formation on the whole grain was observed. The whole grain covered with mycelium was broken down into small cubes using a sterile spatula. Then, the fermented mycelia containing whole grains were transferred into a 250 mL screwcap bottle using sterile forceps under aseptic conditions, and then 50 mL of sterile distilled water was added into the fermented substrate, and the bottle was placed in the shaker incubator for continuous agitation at 200 rpm for 2 hrs. Firstly, to extract the mycelium from the whole sorghum grain, the mixture is filtered into a sterile beaker. Secondly, to extract the complete mycelium from the whole grain another 50 mL of sterile distilled water was added and followed by stirring at 200 rpm for 2 hrs. Finally, after complete agitation, the extracted mycelium was collected in the beaker. The sample mixture collected was subjected to freeze drying for 5 to 6 days. The freeze-dried mycelium was obtained and ground well using a pestle and mortar to get a fine powder. The powdered mycelium was sent for Zn and Se analysis (**Figure 2.3**).

Fermentation and Extraction: *Bacillus subtilis*

The fermentation of whole sorghum grain containing the inoculum of *B. subtilis* alone was not performed because this microorganism does not form the mycelium on the whole

sorghum grain which in turn forms a slimy layer that is different from *A. oryzae*. Due to its morphology, it was not possible to extract the required amount of samples for the Zn and Se analysis.

Fermentation and Extraction: *Co-culture*

For the co-culture fermentation, after the completion of fermentation with *A. oryzae* (7 days). The samples were inoculated with *B. subtilis* (5 x 10^7 cells/mL) and incubated for another 48 hours at 37°C in a Thermo Scientific MaxQ 4450 incubator. After the completion of the incubation period, the same procedure (2.2.2) as above was used for the extraction of mycelium from the whole grain (**Figure 2.3**).

Zn and Se Analysis

For the chemical analysis, the extracted fermented freeze-dried mycelium was ground into a fine powder using a pestle and mortar and then the samples were sent to an external analytical laboratory for ICP-MS (**Figure 2.4**).

2.2.4 Moisture content analysis

Moisture determinations were done in triplicates using a calibrated Omnimark uWave Microwave Moisture/Solid Analyzer (Omnimark Instrument Corporation, Long Island, NY, USA). A 1g of dried fermented sorghum grain sample was placed on a moisture analyzer membrane and weighed using a balance. The sample was transferred into a sample cell of the Moisture/Solid Analyzer. The analyzer was set to the appropriate moisture measurement program for sorghum grain. Necessary information was entered, and the balance was set to start the measurement program and the analyzer was allowed to complete the measurement. The analyzer took 2 to 3 min to dry the sample and to determine the moisture content. Moisture content reading was recorded, and the sample was removed from the analyzer and a similar procedure was followed for other samples.

2.2.5 Statistical Analysis

All statistical analyses were performed by using Statistical Analysis System (SAS) software 9.4 (SAS Institute Inc., Cary, NC) using a one-way analysis of variance test (ANOVA). Data were expressed as mean ± Standard deviation, at least in triplicate. A difference of $p<0.05$ was considered significant.

2.3 Results and Discussion

Zinc and selenium-enriched solid-state fermentation by using coarsely ground sorghum grain as a substrate

This study aimed to evaluate the effect of SSF on Zn and Se uptake from the coarsely ground sorghum grain with *A. oryzae, B. subtilis,* and Co-culture. The substrate was inoculated with the desired amount of spore suspension of *A. oryzae, and B. subtilis*, and different concentrations of added zinc acetate and selenium aspartate. Sorghum grains are known to be a good source of various minerals, including iron, zinc, magnesium, selenium, and phosphorus. The exact mineral content of sorghum grains can vary depending on the variety and growing conditions, but they are generally considered to be nutrient-dense food. In this study, the Zn and Se content in the raw sorghum grain was determined by using ICP-MS. The results showed that there was 13-14 µg/g of Zn and 0.2 µg/g of Se amount in the raw sorghum grain. Zinc acetate and selenium aspartate salts were supplemented. Zinc acetate is an inorganic form of zinc as it is derived from the reaction of a metal (zinc) with an acid (acetic acid). On the other hand, selenium aspartate is an organic form of selenium, aspartate is an amino acid, and organic forms of minerals are typically bound to organic molecules such as amino acids or proteins. The amount of Zn and Se present in zinc acetate and selenium aspartate was determined by using ICP-MS. After the analysis, 6,994 µg of Zn was present in 1 gram of zinc acetate and 2,340 µg of Se was in one gram selenium aspartate. Having this information helped to know the exact amount of pristine Zn and Se present in different concentrations of zinc acetate and selenium aspartate.

The coarsely ground fermented samples were analyzed by ICP-MS to determine the amount of Zn and Se increment after the SSF. Similarly, the extracted mycelium was used to

determine the increase of Zn and Se after whole-grain SSF. After the ICP-MS analysis, the obtained Zn and Se amount was used to know the increment by deducting the amount of Zn and Se present in the sorghum grain as well as in the zinc acetate and selenium aspartate which resulted in the total amount of Zn and Se increment after SSF.

2.3.1 Zinc-enriched fermentation of coarsely ground sorghum grain using *A. oryzae*, *B. subtilis*, and Co-culture

The increment of Zn content after the supplementation of the substrate with zinc acetate and SSF was presented in **Figure 2.5**. The variations in uptake of different concentrations of zinc acetate by *A. oryzae*, *B. subtilis*, and co-culture were measured. The results indicated that the biomass yield was affected by concentrations of 0, 50, 100, 150, 200, 300, and 400 µg/g of zinc acetate. We obtained a range of 65.7-220.3 µg/g of Zn from *A. oryzae* fermented biomass, 67.0-224.5 µg/g of Zn from *B. subtilis* fermented biomass, and 75.0-379.5 µg/g of Zn from co-culture fermented biomass. However, higher concentrations of zinc acetate (100 to 400 µg/g) led to low zinc content in the biomass but no variation in the growth rate.

It is worth noting that in 50 µg/g of zinc acetate concentration, *A. oryzae* showed an 8-fold increment in the Zn content after SSF, *B. subtilis* showed a 9-fold increase, and with co-culture 18-fold increment was observed. The lower concentration, 50µg /g of zinc acetate, was more efficient than higher concentrations. Overall, co-culture fermentation was more effective compared to single-organism inoculation. In the co-culture fermentation, there was a synergetic effect between both organisms (*A. oryzae* and *B. subtilis*) which helps to complement each other and in turn increased the amount of Zn in the substrate after fermentation. Therefore, co-culture fermentation is the optimal cultivation condition for Zn enrichment in coarsely ground sorghum grain.

2.3.2 Selenium-enriched fermentation of coarsely ground sorghum grain using *A. oryzae, B. subtilis,* and Co-culture

The increment of Se content after the supplementation of the substrate with selenium aspartate and SSF is shown in **Figure 2.6**. The data obtained after the absorption of Se using *A. oryzae*, *B. subtilis*, and co-culture fermentation, reveals an interesting pattern in the effect of supplemented selenium aspartate content. The seven concentrations of selenium aspartate showed no variation in microbial growth, but the level of Se uptake from the substrate showed a decrease in the higher concentration compared to low concentrations. *A. oryzae* showed 0.47-1.20 µg/g of Se, 0.35-0.92 µg/g of Se from *B. subtilis* fermented biomass, and co-culture fermented biomass showed 0.60-1.92 µg/g of Se.

When selenium aspartate was added at concentrations of 3.2 µg/g and 6.4 µg/g, there was a significant 2.6-fold and 2-fold increase in the Se content after SSF using co-culture. Interestingly, when *A. oryzae* and *B. subtilis* were individually used for SSF, the Se content did not show any significant increase compared to the co-culture fermentation. This highlights the importance of the co-culture fermentation approach, indicating that the presence and interaction of both microorganisms are necessary for the observed increase in Se content in coarsely ground grain. It is likely that the co-culture system provides complementary enzymatic activities or metabolic pathways that enable efficient Se uptake and utilization (Yao and Nokes 2013).

However, when a higher concentration of Se (12.8-102 µg/g) was supplemented, there was no significant increase in the Se content after co-culture fermentation. This suggests that it is possible that the microorganisms' metabolic capacity or the availability of selenium-binding sites becomes saturated at this higher concentration, limiting their ability to incorporate additional Se (Ferreira et al., 2021).

2.3.3 Zinc-enriched fermentation of whole sorghum grain using *A. oryzae* and Co-culture

In the coarsely ground grain SSF (**Figure 2.5**), it was observed that a concentration of 50 μg/g of zinc acetate yielded the highest increase in Zn content after fermentation. This indicates that a low concentration is more effective in promoting Zn uptake and accumulation during fermentation when using coarsely ground grain.

The whole grain study aimed to investigate the effects of lower zinc acetate concentrations (10 and 25 μg/g) on SSF. The results showed that 10 μg/g of zinc acetate resulted in the highest increment in Zn content compared to the higher concentrations (**Figure 2.7**). To add on, there was a significant difference observed between the concentrations. Compared to the *A. oryzae* fermentation alone, the co-culture showed a slightly higher increment in Zn content at the 10 μg/g concentration. This indicates that the presence of *B. subtilis* in the fermentation process may have a synergistic effect with *A. oryzae*, leading to enhanced Zn uptake and accumulation. The low concentration of Zn in whole grain fermentation is more efficient. This could be due to the specific characteristics of the whole-grain matrix. The availability and accessibility of Zn within the whole grain may lead to differential responses in Zn uptake and utilization by *A. oryzae* and *B. subtilis*.

The findings indicate that the optimal zinc acetate concentration and the effects of co-culture fermentation vary depending on the substrate used. The presence of *B. subtilis* in co-culture fermentation shows a slight advantage in terms of Zn increment, suggesting potential synergistic effects. Additionally, the preference for a low concentration of Zn in whole-grain fermentation suggests unique characteristics of the whole-grain matrix that influence Zn metabolism.

To the best of our knowledge, there are no existing studies or research that directly examine the specific effect of concentration by SSF using *A. oryzae* and *B. subtlis* with different concentrations of zinc acetate and selenium aspartate. However, there are few studies conducted using *S. cerevisiae* to examine the amount or concentration of Zn absorption after fermentation. The study conducted by Šillerová et al. (2021) aimed to assess the impact of zinc on the yield of yeast biomass, the medium supplemented with 250 mg/g $ZnSO_4$, produced yeast biomass enriched with 3820 μg Zn/g. For *S. cerevisiae*, previous research by De Nicola et al. (2009) suggested that a concentration range of 0.25–0.50 μg/mL was optimal for cell growth. According to Azad et al. (2014), the yeast culture exhibited the lowest concentration of Zn in its biomass. The relationship between the Zn content in cells and the supplementation of Zn in the culture medium indicated that the cells were unable to restrict the accumulation of zinc, even at toxic levels. However, with further increases in $ZnSO_4$ concentration, the concentration of accumulated Zn slightly decreased. Previous studies reported the production of zinc-enriched yeast biomass. Stehlik-Thomas et al. (2004) achieved a total Zn concentration of 700 μg/of dry biomass by adding 100 mg/g $ZnSO_4$ to the medium. Supplementation of Zn salts in the medium for yeast cultivation, specifically *S. cerevisiae*, promotes the production of organic zinc (Azad et al, 2014). This enhancement is more pronounced when the $ZnSO_4$ concentration reaches 30 mg/L, while the highest level of zinc sulfate (60 mg/L) hinders yeast growth and Zn uptake. Consequently, adding $ZnSO_4$ to the cultivation medium at concentrations exceeding 30 mg/L should be avoided.

The bioaccumulation of Zn by the cells is dependent on the cellular volumes (de nicola et al., 2009b). The potential of cells to accumulate higher concentrations of Zn may be influenced by various factors, including the presence of intra-vacuolar components capable of binding Zn, such as polyphosphate bodies (Jones and Gadd, 1990).

2.3.4 Selenium-enriched fermentation of whole sorghum grain using *A. oryzae* and Co-culture

After the SSF, it was observed that there was a significant increase in Se content in the whole sorghum grain (**Figure 2.8**). Specifically, a concentration of 3.2 μg/g of selenium aspartate resulted in a 10-fold increase in Se content compared to the initial concentration. This indicated that *A. oryzae* fermentation using whole grain effectively enhances Se uptake and accumulation. Furthermore, when the co-culture of *A. oryzae* and *B. subtilis* was inoculated, a similar trend was observed. The Se content showed an 8-fold increase after SSF using 3.2 μg/g of selenium aspartate in the co-culture system.

The higher concentrations of selenium aspartate did not exhibit any significant increase in Se content after SSF. This implies that there may be an optimal concentration of selenium aspartate that promotes its uptake and utilization by *A. oryzae* and *B. subtilis*. Beyond this optimal concentration, further increases in selenium aspartate concentration did not lead to a proportional increase in selenium aspartate content in the mycelium. It is possible that higher concentrations of selenium aspartate could have toxic or inhibitory effects on the mycelium, negatively impacting its growth and metabolic activity.

It was also observed that lower concentrations of selenium aspartate (1 μg/g and 2 μg/g) did not show any increment in the Se content after fermentation (data is not shown). The lack of significant change at these lower concentrations suggests that they may be below the threshold required to induce a noticeable increase in Se uptake by the mycelium.

The effectiveness of the 3.2 μg/g concentration of selenium aspartate differed between the whole grain and coarsely ground grain fermentations. While it was effective in enhancing Se content in the mycelium during whole grain fermentation, its effectiveness was not observed in

coarsely ground grain fermentation (**Figure 2.6**). The reasons for this difference could be attributed to variations in the availability and accessibility of selenium aspartate in the two different grain substrates, as well as differences in the metabolic requirements and responses of *A. oryzae* and *B. subtilis* to selenium aspartate.

da Silva et al. (2012) used the coffee husks, a valuable agro-industrial residue to determine the ability of *P. ostreatus* mushrooms to absorb and accumulate selenium (Se) when enriched with selenite. The Se content in the mushrooms corresponded to the amount of sodium selenite added to the substrate. The lowest tested concentration of Se (3.2 µg/g) resulted in the maximum absorption (57.6 µg/g) by *P. ostreatus* mushrooms when the coffee husks were enriched with Se. However, at higher concentrations, the absorption of Se was inhibited, potentially due to an excess of sodium selenite in the substrate. In substrates with Se concentrations of 3.2 µg/g, 34% of the added Se was absorbed, whereas in the substrate with 51 µg/g, only 16% was absorbed. Based on these findings, consuming 1.0 g of dried mushrooms cultivated on a substrate with 3.2 µg/g of Se would be sufficient to meet the recommended daily intake of Se for adults, which is 55 µg per day.

2.3.5 Moisture content analysis of unfermented and fermented coarsely ground sorghum grain

Moisture content is a critical parameter to assess the quality and stability of fermented products (Manpreet et al., 2005). Maintaining an appropriate moisture level is important for the optimal growth and metabolic activity of the microorganisms involved in fermentation (Sharma et al., 2020). High moisture levels can lead to microbial growth, spoilage, and reduced shelf life. We can ensure that the fermented sorghum grain meets the desired quality standards by analyzing the moisture content. Excess moisture in fermented products can create a favorable

environment for the growth of harmful microorganisms such as molds and bacteria (Gichau et al., 2020). Therefore, analyzing the moisture content of the fermented grain is important for quality control, safety, process optimization, product consistency, and nutritional assessment. It allows producers to ensure product quality, safety, and desirable characteristics while providing valuable information for process optimization and nutritional labeling. In this study, the moisture content of the unfermented and fermented coarsely ground sorghum grain was presented in **Tables 2.1 and 2.2**.

The results indicate that there is a significant difference in moisture content between the Zn and Se fermented and unfermented sorghum grain samples of *A. oryzae, B. subtilis*, and Co-culture. The moisture content of unfermented sorghum grain falls within the range of 8.4-9.4%, while the fermented Zn and Se samples have a lower moisture content ranging from 4.7-5.8% for Zn and 4.9-5.8% for Se. Overall, there is no significant difference between the fermented Zn and Se samples.

In general, the lower moisture content in fermented sorghum grain is favorable from a safety perspective. The lower moisture content makes it less likely for spoilage microorganisms to proliferate, ensuring the safety of the product (Adebo, 2020). Based on our data, there is a high moisture content in the fermented samples which can be prone to contamination but during our study we never encountered any contamination issue with the dried biomass.

It is important to note that moisture content alone cannot determine the overall safety of a product. Other factors such as pH, water activity, and the presence of specific microorganisms or toxins should also be considered for a comprehensive safety assessment. Additionally, proper handling, storage, and processing practices should be implemented to maintain the safety of the fermented sorghum grain throughout its shelf life. Furthermore, it is recommended to conduct

further microbial testing and analysis to confirm the absence of harmful microorganisms and ensure the overall safety of the product (Institute of Food Technologists 2020).

Tables 2.3 and 2.4. showed the moisture content of whole grain samples focusing on the influence of fermentation and the presence of Zn and Se. The whole grain unfermented samples exhibited a higher mean moisture content, while the fermented samples had a lower mean moisture content. Furthermore, the fermentation process, particularly with the addition of zinc acetate and selenium aspartate, had a significant impact on the moisture content of the samples. The study showed significant differences in moisture content between the unfermented and fermented sorghum grain samples. However, there was no significant difference in moisture content between the fermented Zn and Se samples. The comparison between coarsely ground and whole grain samples revealed that the fermented samples, particularly those treated with zinc acetate and selenium aspartate additives, had a lower mean moisture content compared to the unfermented samples. This suggests that these additives may have influenced moisture absorption or retention during fermentation. These findings have implications for the processing and storage of both coarsely ground and whole-grain samples. Further testing is essential for maintaining the safety of the fermented sorghum grain.

2.4 Conclusion

Our study investigated the effects of zinc acetate on biomass yield and zinc uptake by *A. oryzae*, *B. subtilis*, and Co-culture. The results from the coarsely ground sorghum grain revealed that concentrations up to 400 µg/g of zinc acetate had no significant impact on biomass yield. Higher concentrations (100 to 400 µg/g) resulted in reduced zinc content in the biomass, while 50 µg/g led to increased zinc content in *A. oryzae* and Co-culture after solid-state fermentation (SSF). Co-culture fermentation demonstrated higher zinc uptake compared to single-organism fermentation. Similarly, selenium aspartate supplementation did not affect microbial growth but influenced Se uptake. Co-culture fermentation of *A. oryzae* and *B. subtilis* exhibited increased Se content when supplemented with 3.2 µg/g of selenium aspartate. However, higher concentrations did not yield significant increases, indicating metabolic saturation or selenium-binding sites. The findings from the whole grain fermentation showed that the lower concentrations of zinc acetate (10 µg/g) resulted in higher Zn content during fermentation, with a slight advantage observed in co-culture fermentation. Regarding selenium aspartate, both *A. oryzae* fermentation and co-culture fermentation showed significant increases in Se content at 3.2 µg/g supplementation. These findings offer insights into cultivation conditions and substrate compositions for enriching Zn and Se in microbial biomass. The moisture content of fermented dried sorghum grain showed significant differences between Zn and Se fermented and unfermented sorghum grain samples of *A. oryzae*, *B. subtilis*, and co-culture. Overall, the unfermented samples had high moisture content when compared to fermented samples. Further research is needed to understand underlying mechanisms and assess the bioavailability of fermented biomass for nutrient enrichment in food products.

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Table 2.1. Moisture content analysis of unfermented and fermented coarsely ground sorghum samples containing Zinc (Zn)

Samples (Zn Concentration)	Moisture Content (%)		
	Aspergillus oryzae	***Bacillus subtilis***	**Co-culture**
Control (Unfermented)	8.4 ± 0.5 [a]	8.8 ± 0.2 [a]	9.4 ± 0.2 [a]
Zn, 0 µg	5.7 ± 0.0 [b]	5.5 ± 0.2 [b]	5.7 ± 0.1 [b]
Zn, 50 µg	4.8 ± 0.4 [b]	5.0 ± 0.1 [b]	4.7± 0.1 [b]
Zn, 100 µg	4.8 ± 0.5 [b]	5.3 ± 0.0 [b]	5.4 ± 0.2 [b]
Zn, 150 µg	5.0± 0.1 [b]	5.0 ± 0.1 [b]	5.1 ± 0.0 [b]
Zn, 200 µg	5.2 ± 0.3 [b]	5.4 ± 0.2 [b]	5.5 ± 0.1 [b]
Zn, 300 µg	5.2 ± 0.0 [b]	5.3 ± 0.1 [b]	5.8 ± 0.1 [b]
Zn, 400 µg	5.5 ± 0.0 [b]	5.1 ± 0.1 [b]	5.3 ± 0.5 [b]

The data are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae, B. subtilis* and Co-culture. Control (unfermented) contains only sterile coarsely ground sorghum grain (Without inoculum and Zn), and fermented samples contains different concentrations of Zn (0 µg/g to 400 µg/g), sterile coarsely ground sorghum grain and inoculum.

Table 2.2. Moisture content analysis of unfermented and fermented coarsely ground sorghum samples containing Selenium (Se)

Samples (Se Concentration)	Moisture Content (%)		
	Aspergillus oryzae	***Bacillus subtilis***	**Co-culture**
Control (Unfermented)	9.2 ± 0.2 [a]	8.8 ± 0.1 [a]	8.0 ± 0.2 [a]
Se, 0 µg	5.7 ± 0.1 [b]	5.6 ± 0.2 [b]	5.3 ± 0.0 [b]
Se, 3.2 µg	5.5 ± 0.4 [b]	5.2 ± 0.0 [b]	5.4 ± 0.1 [b]
Se, 6.4 µg	5.8 ± 0.1 [b]	5.8 ± 0.1 [b]	5.5 ± 0.2 [b]
Se, 12.8 µg	5.0 ± 0.1 [b]	4.9 ± 0.2 [b]	5.4 ± 0.3 [b]
Se, 25.4 µg	5.1 ± 0.1 [b]	5.4 ± 0.3 [b]	5.1 ± 0.0 [b]
Se, 51 µg	5.3 ± 0.1 [b]	5.7 ± 0.2 [b]	5.8 ± 0.2 [b]
Se, 76.4 µg	5.1 ± 0.1 [b]	5.5 ± 0.2 [b]	5.0 ± 0.1 [b]
Se, 102 µg	5.3 ± 0.1 [b]	5.4 ± 0.2 [b]	5.4 ± 0.1 [b]

The data are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae, B. subtilis* and Co-culture. Control (unfermented) contains only sterile coarsely ground sorghum grain (Without inoculum and Se), and fermented samples contains different concentrations of Se (0 µg/g to 102 µg/g), sterile coarsely ground sorghum grain and inoculum.

Table 2.3. Moisture content analysis of unfermented and fermented whole grain sorghum samples containing Zinc (Zn)

Samples (Zn Concentration)	Moisture Content (%)	
	Aspergillus oryzae	**Co-culture**
Control (Unfermented)	8.2 ± 0.0 [a]	7.4 ± 0.2 [a]
Zn, 0 µg	5.3 ± 0.6 [b]	5.2 ± 0.0 [b]
Zn, 10 µg	5.3 ± 0.6 [b]	5.0 ± 0.1 [b]
Zn, 25 µg	4.9 ± 0.0 [b]	4.8 ± 0.0 [b]
Zn, 50 µg	5.8 ± 0.1 [b]	5.5 ± 0.0 [b]
Zn, 100 µg	5.5 ± 0.0 [b]	5.5 ± 0.3 [b]
Zn, 250 µg	5.8 ± 0.2 [b]	5.4 ± 0.1 [b]
Zn, 400 µg	5.8 ± 0.1 [b]	5.5 ± 0.0 [b]

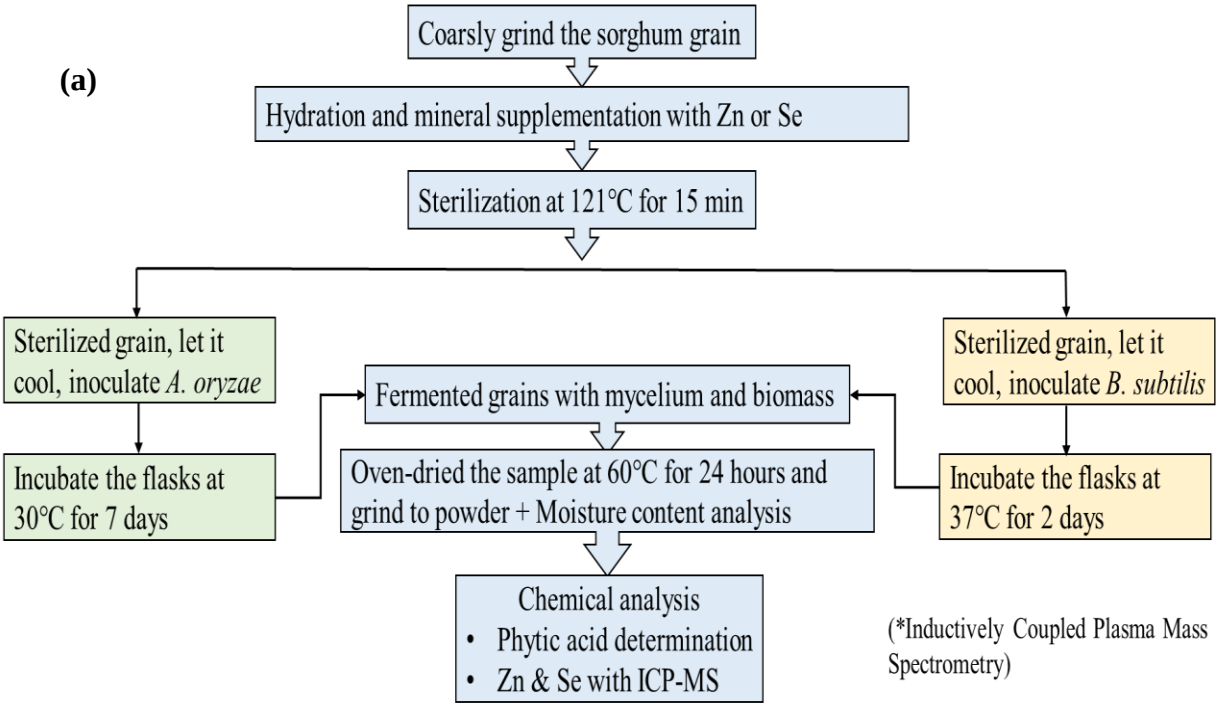
The data are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Zn), and fermented samples contains different concentrations of Zn (0 µg/g to 400 µg/g), sterile whole sorghum grain and inoculum.

Table 2.4. Moisture content analysis of unfermented and fermented whole grain sorghum samples containing Selenium (Se)

Samples (Se Concentration)	Moisture Content (%)	
	Aspergillus oryzae	**Co-culture**
Control (Unfermented)	8.4 ± 0.1 [a]	8.1 ± 0.1 [a]
Se, 0 µg	5.9 ± 0.0 [b]	5.1 ± 0.0 [b]
Se, 3.2 µg	5.2 ± 0.0 [b]	5.3 ± 0.6 [b]
Se, 25.4 µg	5.6 ± 0.5 [b]	5.8 ± 0.1 [b]
Se, 102 µg	5.2 ± 0.2 [b]	5.4 ± 0.2 [b]

The data are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Se), and fermented samples contains different concentrations of Se (0 µg/g to 102 µg/g), sterile whole sorghum grain and inoculum.

(a)



(b)

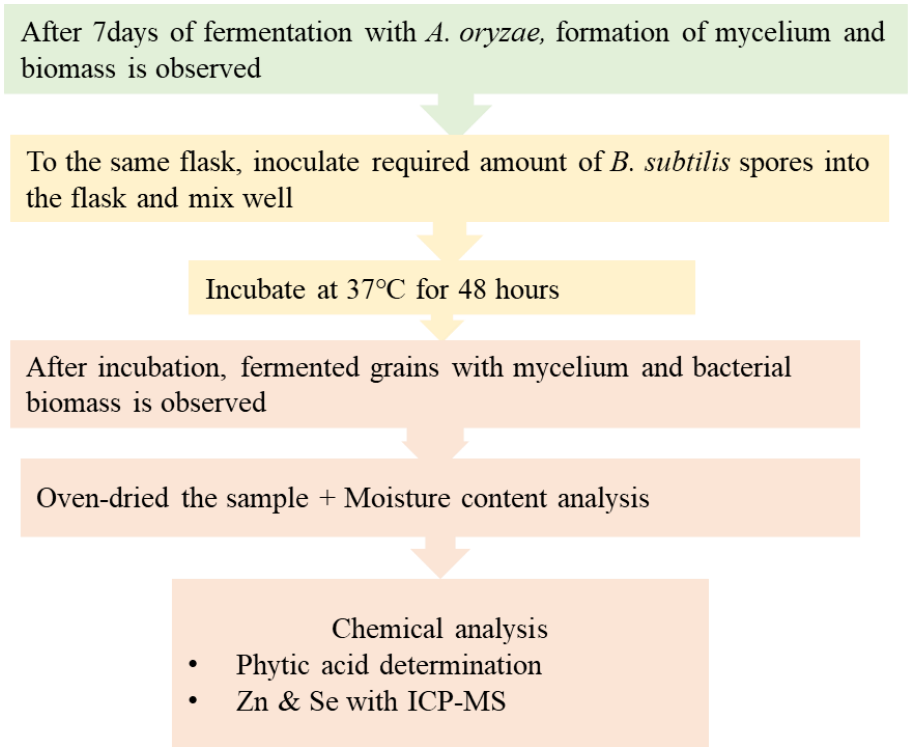
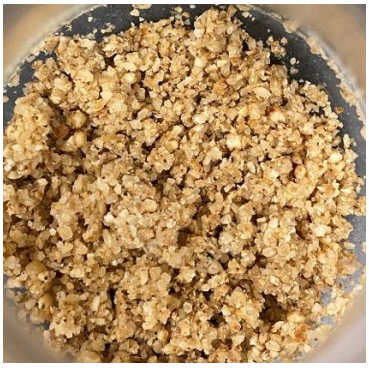
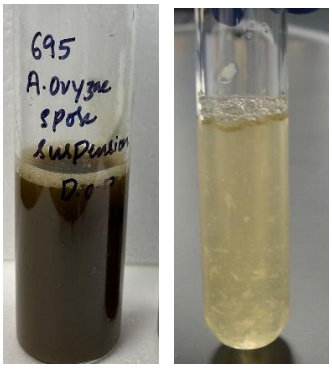


Figure 2.1.(a) Coarsely ground sorghum grain fermentation process (*Aspergillus oryzae* and *Bacillus subtilis*) and (b) Coarsely ground sorghum grain fermentation process (Co-culture)



Coarse ground sorghum grain



Spore suspension of *A. oryzae* and *B. subtilis*

Fermented grains with mycelium and biomass

Oven-dried at 60℃ for 48 hours

Fermented fine ground powder

Figure 2.2. Photos of coarsely ground sorghum grain after fermentation process

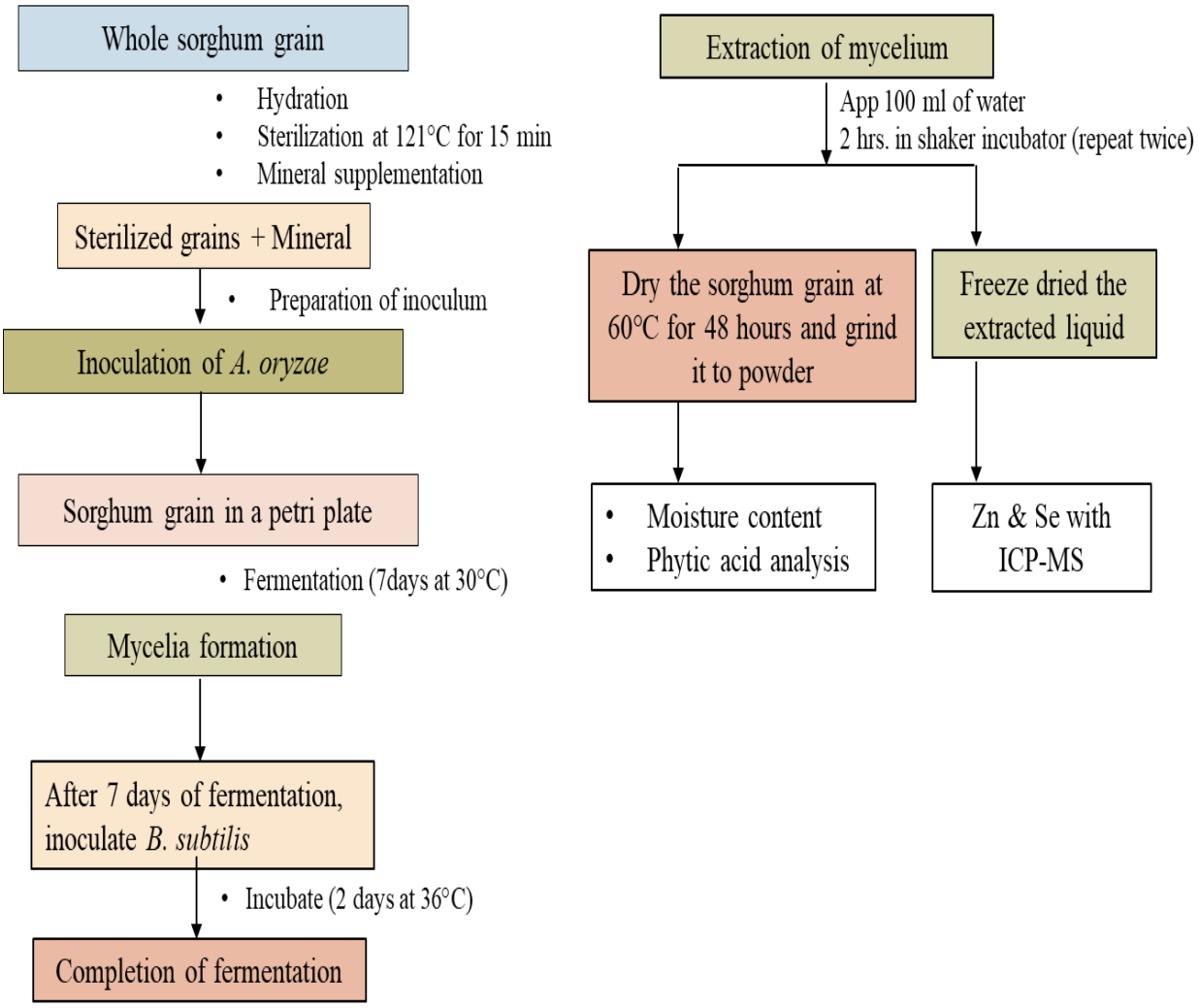
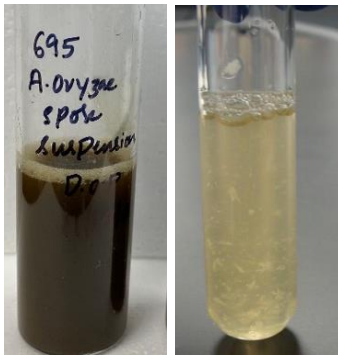
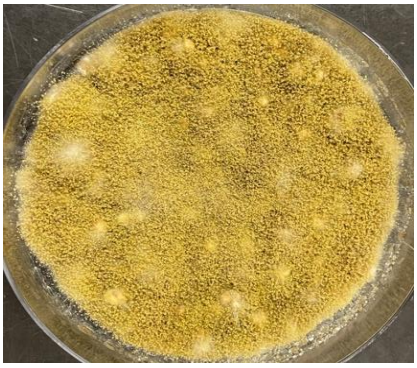


Figure 2.3. Whole sorghum grain fermentation process

Sterile whole sorghum grain

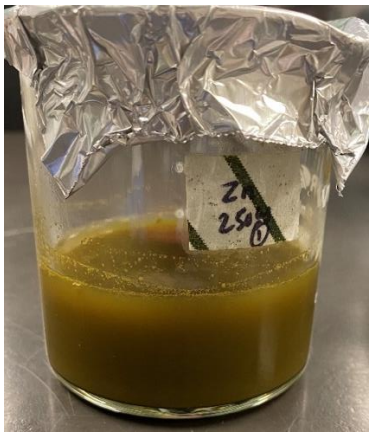


Spore suspension of *A. oryzae* and *B. subtilis*



Fermented grains with mycelium

Extraction of mycelium



Extracted mycelium

After separation of mycelium

Dried sorghum grain

Freeze dried mycelium

Fine powdered mycelium

Figure 2.4. Photos of whole sorghum grain after fermentation process

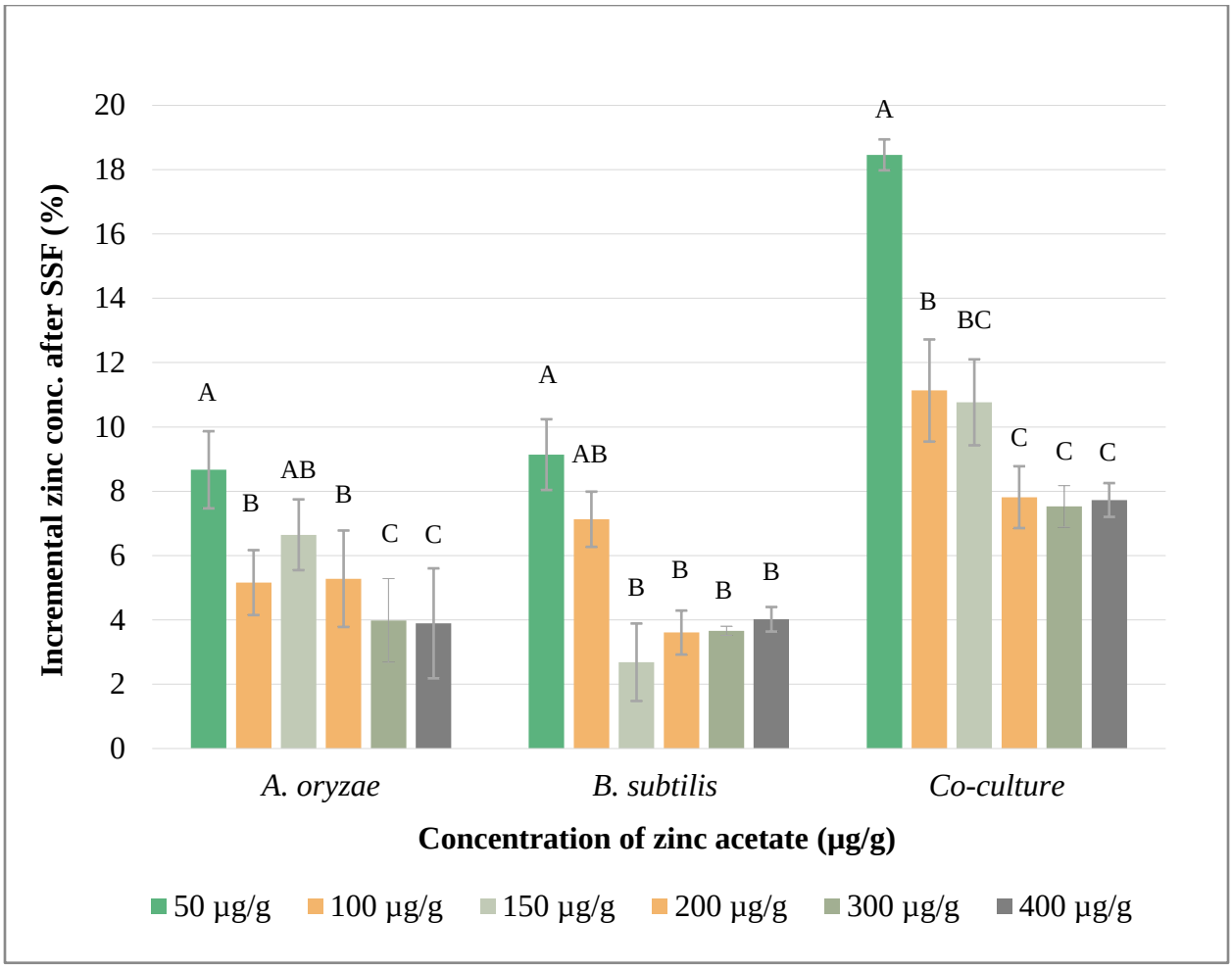


Figure 2.5. Incremental Zinc (Zn) content in coarsely ground sorghum grain fermented with *Aspergillus oryzae*, *Bacillus subtilis*, and Co-culture. The data of fermented sorghum grain with different concentrations of zinc acetate during solid state fermentation (SSF) are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae*, *B. subtilis* and Co-culture. The fermented samples contain different concentrations of Zn (50 µg/g to 400 µg/g), sterile coarsely ground sorghum grain and inoculum.

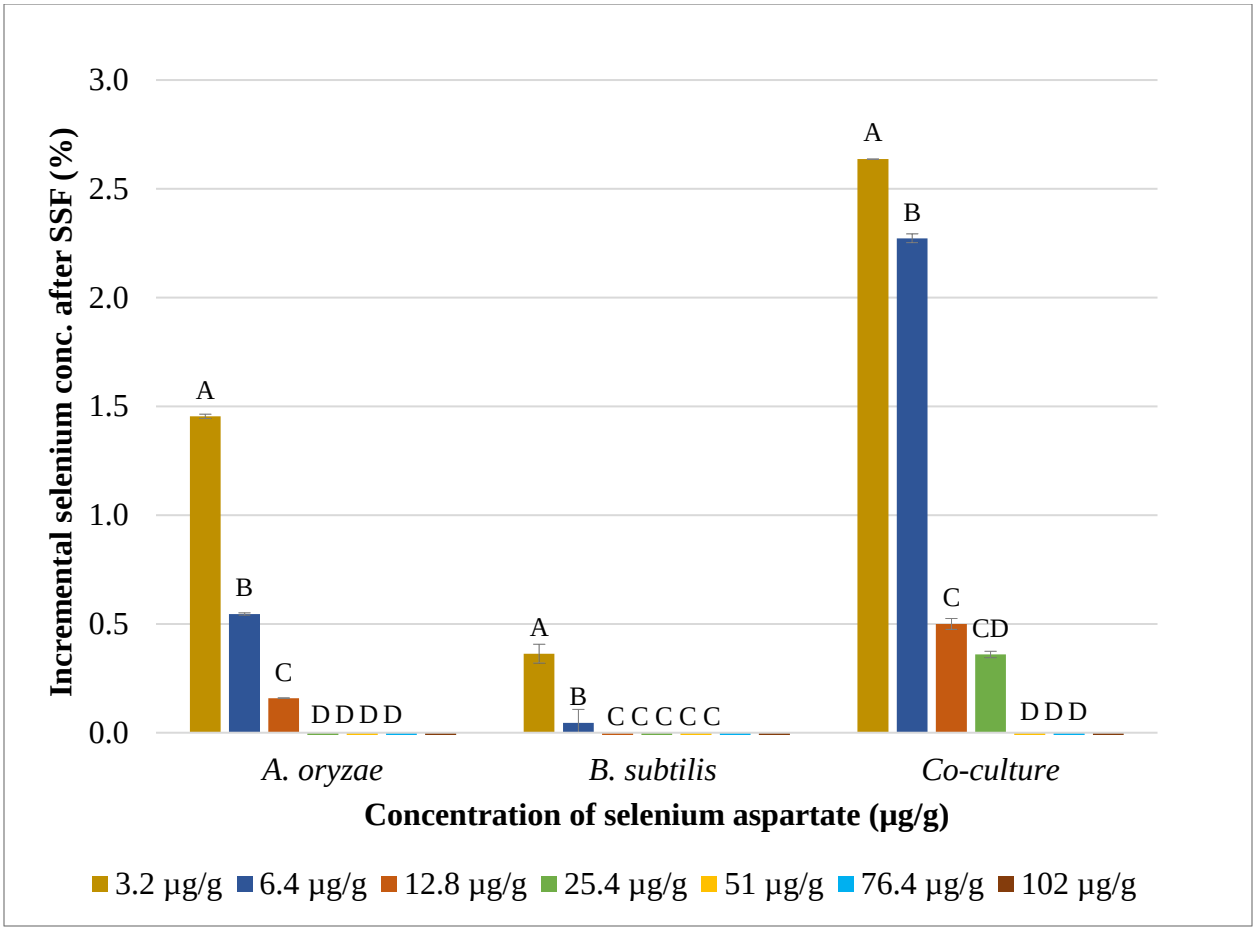


Figure 2.6. Incremental Selenium (Se) content in coarsely ground sorghum grain fermented with *A. oryzae*, *B. subtilis*, and Co-culture. The data of fermented sorghum grain with different concentrations of selenium aspartate during solid state fermentation (SSF) are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae*, *B. subtilis* and Co-culture. The fermented samples contain different concentrations of Se (3.2 µg/g to 102 µg/g), sterile coarsely ground sorghum grain and inoculum.

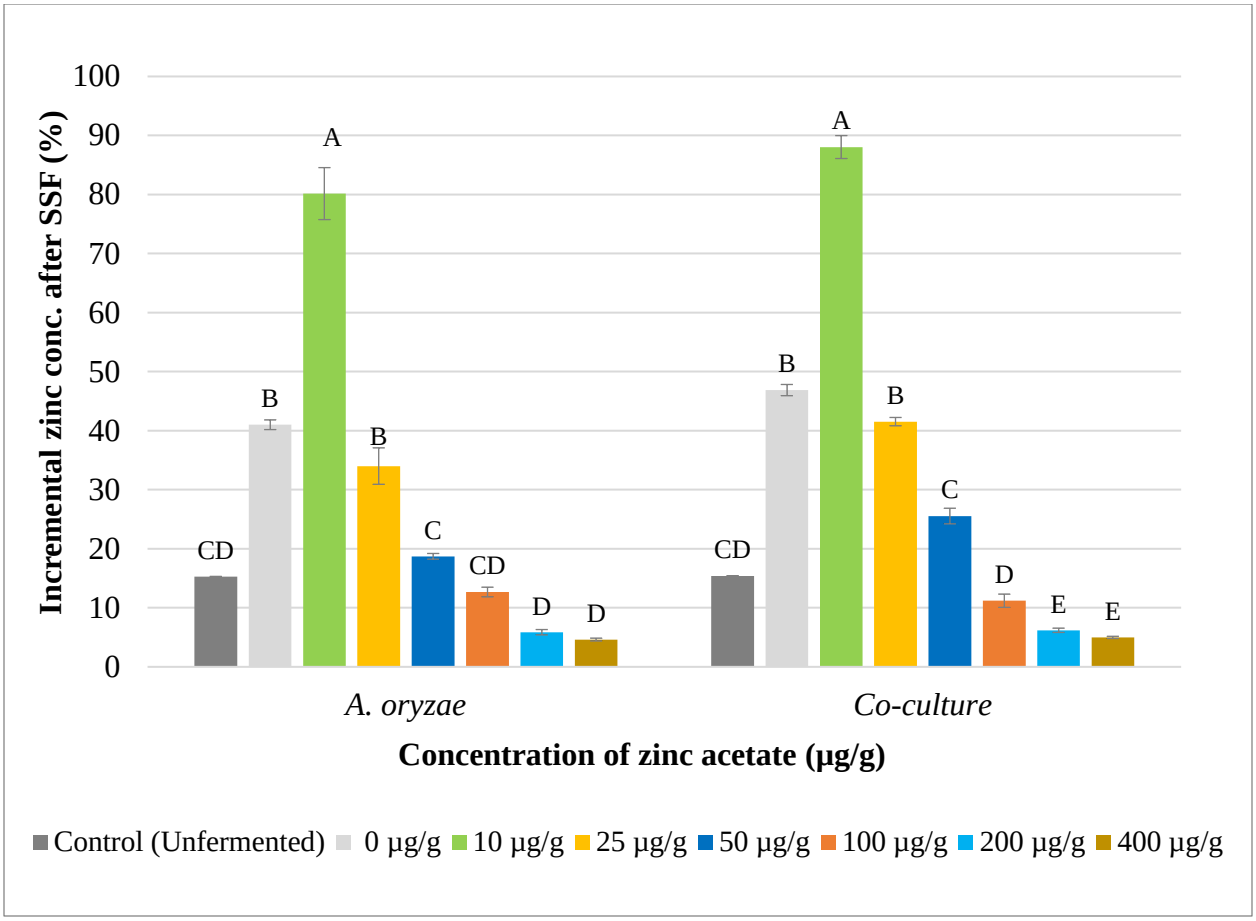


Figure 2.7. Incremental Zinc (Zn) content in whole sorghum grain fermented with *A. oryzae* and Co-culture. The data of fermented sorghum grain with different concentrations of zinc acetate during solid state fermentation (SSF) are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Zn), the fermented samples contain different concentrations of Zn (0 µg/g to 400 µg/g), sterile whole sorghum grain and inoculum.

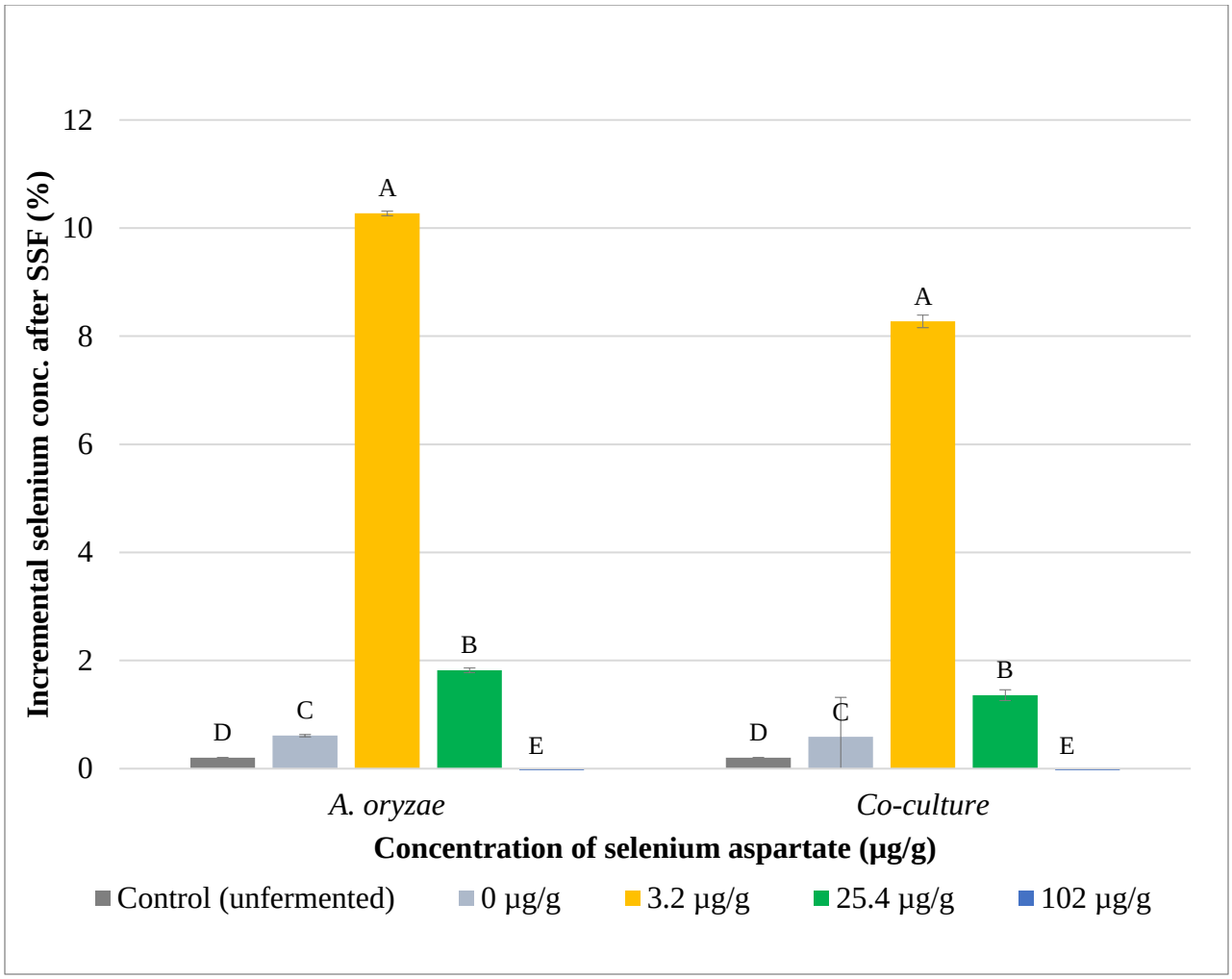


Figure 2.8. Incremental selenium (Se) content in whole sorghum grain fermented with *A. oryzae* and Co-culture. The data of fermented sorghum grain with different concentrations of selenium aspartate during solid state fermentation (SSF) are presented as mean ± standard deviation of the mean (n = 6). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Se), the fermented samples contain different concentrations of Se (0µg/g to 102 µg/g), sterile coarsely ground sorghum grain and inoculum.

CHAPTER 3: OBJECTIVE 2

Phytic acid analysis (antinutrients) in fermented coarsely ground biomass and whole grain samples containing Zn and Se

3.1 Introduction

Phytic acid is a naturally occurring substance found in sorghum grain. It is a form of stored phosphorus that plants use for their growth and development. However, phytic acid has a downside for human nutrition as it binds to essential minerals such as iron (Fe), zinc (Zn), selenium (Se), and calcium (Ca), reducing their bioavailability and absorption in the digestive tract. This can lead to mineral deficiencies and related health problems, particularly in populations with diets low in animal protein and high in phytate-rich foods.

Various processing techniques have been developed to reduce the negative effects of phytic acid in sorghum grain. For example, traditional methods such as soaking, germination, and fermentation have been shown to decrease phytate levels by activating enzymes (Gibson et al., 2010). These techniques also enhance sorghum's nutrient content and digestibility by breaking down complex carbohydrates and proteins into more accessible forms.

A study conducted by Ertop et al. (2018) showed how fermentation plays a crucial role in reducing antinutrient levels and enhancing mineral extractability, *in-vitro* protein digestibility, and overall nutritional value of food grains. This decrease in phytic acid levels during fermentation is attributed to the action of phytase enzymes released by microorganisms. Thus, it is recommended that cereals and other grain-based foods be consumed after undergoing fermentation treatment, which not only decreases phytic acid levels but also reduces the amounts of tannins and polyphenols while increasing mineral bioavailability and digestibility (Gupta et

al., 2015). Badau et al. (2005) has also shown that the fermentation of food grains can improve the bioavailability of minerals and proteins.

In the preset study, we have determined the amount of phytic acid in the fermented coarsely ground, whole sorghum grain and unfermented sorghum grain.

3.2 Materials and Method

Reagents

The phytic acid assay kit (Cat. No. K-PHYT) from Megazyme International (Bray, County Wicklow, Ireland) provided the following components: alkaline phosphatase (ALP) (Cat. No. E-ALPEC; EC 3.1.3.1; 80 U/mL), phytase (12,000 U/mL), ALP assay buffer (pH 10.4) containing 400 mM glycine, 4 mM magnesium chloride, and 0.4 mM zinc sulfate, and phytase assay buffer (pH 5.5) containing 200 mM sodium acetate (McKie et al., 2016). Ammonium molybdate, ascorbic acid, phytic acid dipotassium salt, sulfuric acid, trichloroacetic acid were purchased from Sigma Aldrich (St. Louis, MO 68178), and zinc sulfate heptahydrate, hydrochloric acid, sodium hydroxide pellets were obtained from Merck Millipore (Burlington, MA 01803).

Preparation of Color Reagent for Phosphorus Determination

Solution A was prepared by dissolving 10 g of ascorbic acid by stirring in approximately 90 mL of distilled water, and then 5.35 mL of concentrated sulfuric acid was added. The final volume was adjusted to 100 mL with distilled water. Then solution B was prepared by dissolving 1.25 g of ammonium molybdate by stirring in approximately 20 mL of distilled water, and the final volume was adjusted to 25 mL with distilled water. To obtain a final solution, one part of solution B was added to five parts of solution A (e.g., 25 mL of solution B was added to 100 mL

of solution A) to obtain the color reagent. The color reagent was prepared on the day of use (McKie et al., 2016).

Extraction of Phytic Acid from Fermented Samples

The fermented sample (0.04 g) was added to 20 mL of hydrochloric acid (0.66 M) and mixed vigorously in a shaker (Thermoscientific MaxQ4450) overnight at room temperature (23°C). The extract (1 mL) was transferred to a 1.5 mL microfuge tube and centrifuged at 11000 × g for 10 min. The supernatant (0.5 mL) was transferred to a microfuge tube and neutralized by the addition of 0.5 mL of sodium hydroxide (0.75 M) and used in the enzymatic dephosphorylation of the phytic acid reaction.

The enzymatic dephosphorylation of phytic acid was performed by adding phytase and ALP. The total phosphorus reaction was prepared by combining distilled water (0.60 mL), phytase assay buffer (0.20 mL), sample extract (0.05 mL), and phytase at 12,000 U/mL (0.02 mL). The reaction mixture was thoroughly mixed and incubated at 40°C for 10 min. After incubation, ALP assay buffer (0.20 mL) and ALP at 80 U/mL (0.02 mL) were added, and the reaction mixture was again mixed thoroughly and incubated at 40°C for 15 min. To stop the reactions, 0.3 mL trichloroacetic acid (50%, w/v) was added, and the mixture was thoroughly mixed using a vortex mixer, followed by centrifugation at 11,000 × g for 10 min. The supernatant (1 mL) was transferred to a microfuge tube for use in the colorimetric determination of phytic acid.

To prepare a phosphorus calibration curve, phosphorus standard solutions (STD0, STD1, STD2, STD3, and STD4) were prepared in distilled water at concentrations of 0, 0.1, 0.5, 1, and 1.5 mg phosphorus/L, respectively, using a traceable phosphorus standard solution. Each standard solution was used in the colorimetric determination of phosphorus assay, and the absorbance values at 655 nm were measured and used to calculate the phytic acid concentrations.

To determine the phosphorus concentration, the supernatants obtained from the enzymatic dephosphorylation of phytic acid procedure (described above) were subjected to a colorimetric assay. Specifically, 1 mL of supernatant was mixed with 0.5 mL of color reagent in a 1.5 mL microfuge tube, and the mixture was incubated in a water bath at 40°C for 1 hour. After incubation, the reaction solutions were mixed thoroughly and approximately 1 mL was transferred to a 1 cm path-length microcuvette, and the absorbance at 655 nm of each solution was recorded using a UV spectrophotometer. The absorbance values of samples and phosphorus standard solutions were then used to calculate the amount of phytic acid content.

3.3 Phytic Acid Content in Fermented Coarsely Ground and Whole Sorghum Grain

The coarsely ground sorghum grain supplemented with different concentrations of Zn and Se. (0, 50, 100, 150, 200, 300, and 400 µg/g for Zn) and (0, 3.2, 6.4, 12.8, 25.4, 51, 76.4, and 102 µg/g for Se) were fermented using *A. oryzae*, *B. subtilis*, and Co-culture. The dried fermented samples were used for the analysis.

The whole sorghum grain supplemented with different concentrations of Zn and Se. (0, 10, 25, 50, 100, 250, and 400 µg/g for Zn) and (0, 1, 2, and 3.2 µg/g for Se) were fermented using *A. oryzae* and co-culture. The dried fermented samples were used for the analysis.

3.4 Statistical Analysis

All statistical analyses were performed by using Statistical analysis system (SAS) software 9.4 (SAS Institute Inc., Cary, NC) using a one-way analysis of variance test (ANOVA). Data were expressed as mean ± Standard deviation, in duplicates. A difference of $p<0.05$ is considered significant.

3.5 Results and Discussion

Solid-state fermentation (SSF) has been recognized as an effective technique for improving the digestibility of sorghum grain by eliminating phytate compounds. Phytates are naturally occurring compounds found in sorghum grain that bind to essential minerals such as iron, zinc, selenium, and calcium, rendering them less bioavailable to the human body. The presence of phytates can lead to mineral deficiencies and hinder nutrient absorption. SSF involves the use of microorganisms such as *A. oryzae* or *B. subtilis* that possess phytase enzymes. These enzymes can break down phytates into inositol and inorganic phosphorus, making them more easily digestible. Through fermentation, the phytate content in sorghum grain can be significantly reduced, enhancing its nutritional value. By degrading phytates, SSF enhances the bioavailability of essential minerals present in sorghum grain.

Doherty et al. (1982) examined the phytic phosphorus content in various types of sorghum. The levels of phytic phosphorus in the whole grain varied between 170 to 380 mg/100 g. The study also noted that phytic phosphorus constituted more than 85% of the total phosphorus present in the whole grain. The concentration of phytic acid in sorghum ranged from 875.1 to 2211.9 mg/100 g. These high levels of phytate content in sorghum grain can hinder the absorption of essential minerals such as iron, zinc, selenium, and calcium, making them less available in plant-based complementary foods (Ratnavathi and Elangovan, 2009; Das et al.,2020). Microorganisms could utilize phytases to break down phytates in plant matter and acquire phosphorus (Sreeramulu et al., 1996; Türk, Carlsson, and Sandberg, 1996). This process of fermentation mechanism can lead to a decrease in phytate levels in plant materials. A study conducted by Mamiro et al. (2001) reported a significant reduction in phytic acid content in both kidney beans and finger millet throughout the processing stages. In the case of finger millet samples, the most substantial reductions were observed following germination and fermentation,

resulting in reductions of 49.2% and 66.5%, respectively. Overall, the processing of finger millet led to a 94.7% decrease in phytic acid content. Similarly, in kidney beans, the overall decrease was 86.5%, with the most significant reduction occurring after fermentation (73.1%).

3.5.1 Phytic acid determination in a coarsely ground fermented sample containing Zn and Se

The results of the solid-state fermentation of the coarsely ground sample using microorganisms in the presence of Zn and Se demonstrated a significant reduction in phytic acid levels (**Figures 3.1 and 3.2**). The analysis revealed a reduction in phytic acid content after the fermentation process in *A. oryzae* and co-culture samples. The amount of phytic acid content was significantly lower in the fermented samples containing Zn and Se compared to the control ($P<0.05$) and samples containing no Zn or Se (0 μg/g). This reduction was likely due to the enzymatic action of phytases released by the microorganisms during fermentation. The presence of minerals in the fermentation medium may have further facilitated the reduction in phytic acid levels by creating a favorable environment for phytase activity. Overall, the results indicate that solid-state fermentation using microorganisms in the presence of minerals is an effective strategy for reducing phytic acid levels in the sample. However, *B. subtilis* fermented samples did not reduce the phytic acid content. This could be due to *B. subtilis* not being able to reduce the phytase content alone, and a lack of synergistic effect (absence of *A. oryzae* in the samples).

3.5.2 Phytic acid determination in whole grain fermented sample containing Zn and Se

The results of the solid-state fermentation of the whole-grain sample showed a slight reduction in fermented samples containing Zn and Se compared to control ($P<0.05$) and samples containing no Zn or Se (0 μg/g) (**Figures 3.3 and 3.4**). As observed, the coarsely ground fermented samples showed a significant reduction in phytic acid levels. The findings suggest that

the surface area of the grain plays a crucial role in the effectiveness of the fermentation process. The increased surface area of the coarsely ground grain may have allowed for greater contact between the grain and the microorganisms, facilitating the enzymatic action of phytases and resulting in the observed reduction in phytic acid levels. Overall, the results indicate that solid-state fermentation of coarsely ground grains is an effective strategy for reducing phytic acid levels in the sample.

3.6 Conclusion

Solid-state fermentation has been shown to effectively reduce the levels of phytic acid in various grains and legumes. Our study also found a significant reduction in phytic acid content after SSF. Specifically, the phytic acid content was reduced in coarsely ground sorghum grain by 85 to 90% in *A. oryzae* and co-culture fermented samples compared to the unfermented control group. Whereas the whole sorghum grain fermented samples showed 25 to 40% reduction in the phytic acid compared to unfermented samples. These results suggest that solid-state fermentation could reduce the phytic acid content in grains and legumes, potentially improving their nutritional value and bioavailability of essential minerals. Further research is needed to optimize the fermentation process and evaluate its effects on other nutritional components of the fermented products.

3.7 References

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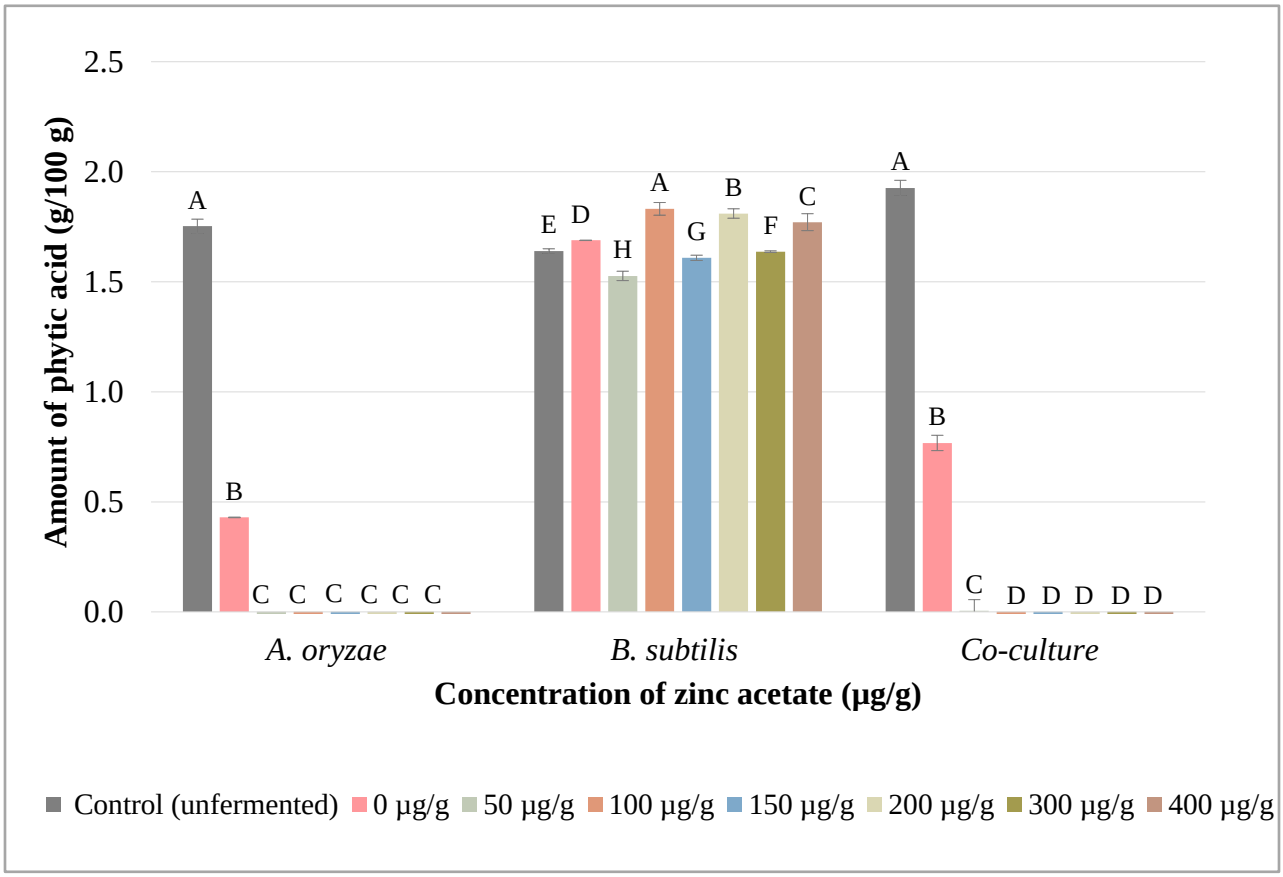

Figure 3.1. Total amount of phytic acid in Zinc (Zn) containing coarsely ground fermented samples after Solid state fermentation. The data are presented as mean ± standard deviation of the mean (n = 4). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae*, *B. subtilis* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Zn), the fermented samples contain different concentrations of Zn (0 µg/g to 400 µg/g), sterile coarsely ground sorghum grain and inoculum.

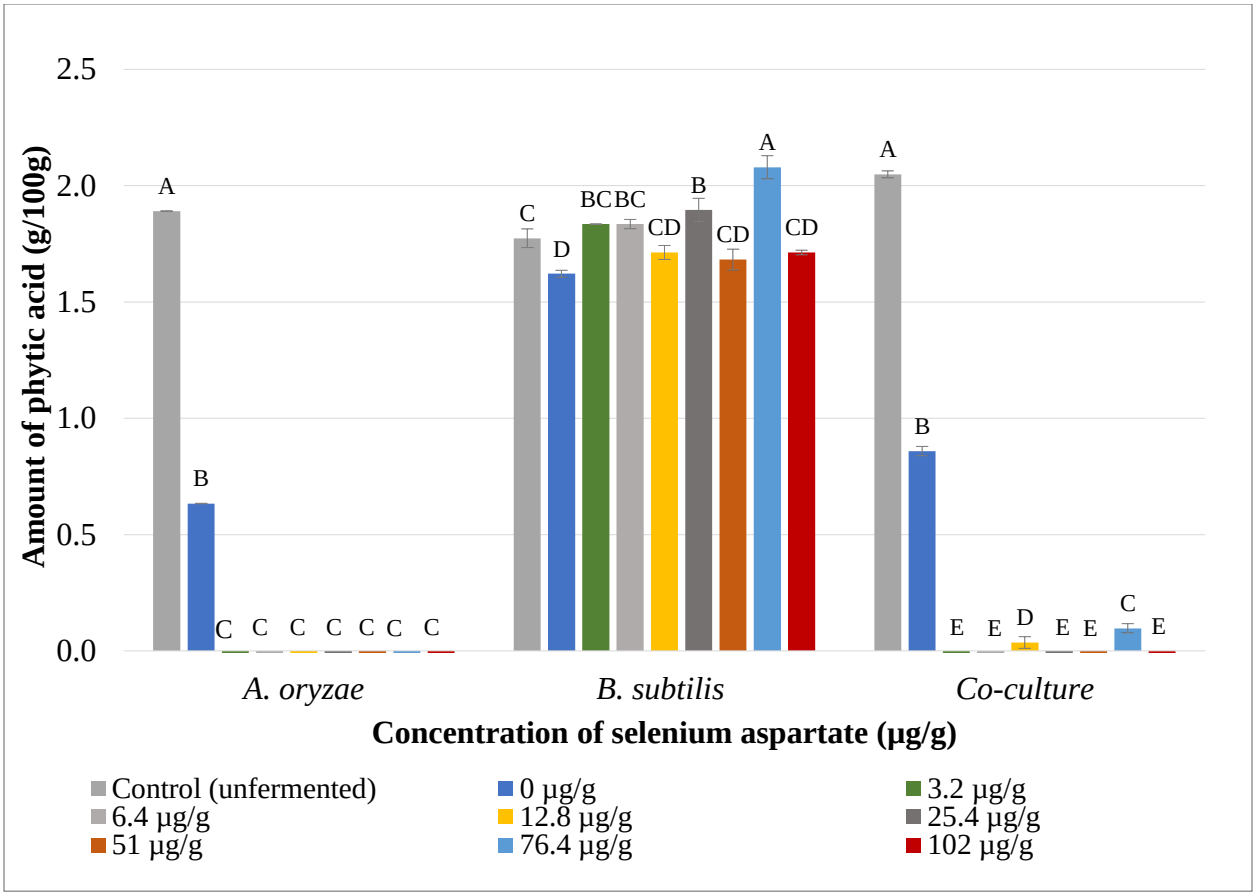

Figure 3.2. Total amount of phytic acid in Selenium (Se) containing coarsely ground fermented samples after Solid state fermentation. The data are presented as mean ± standard deviation of the mean (n = 4). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae*, *B. subtilis* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Se), the fermented samples contain different concentrations of Se (0 µg/g to 102 µg/g), sterile coarsely ground sorghum grain and inoculum.

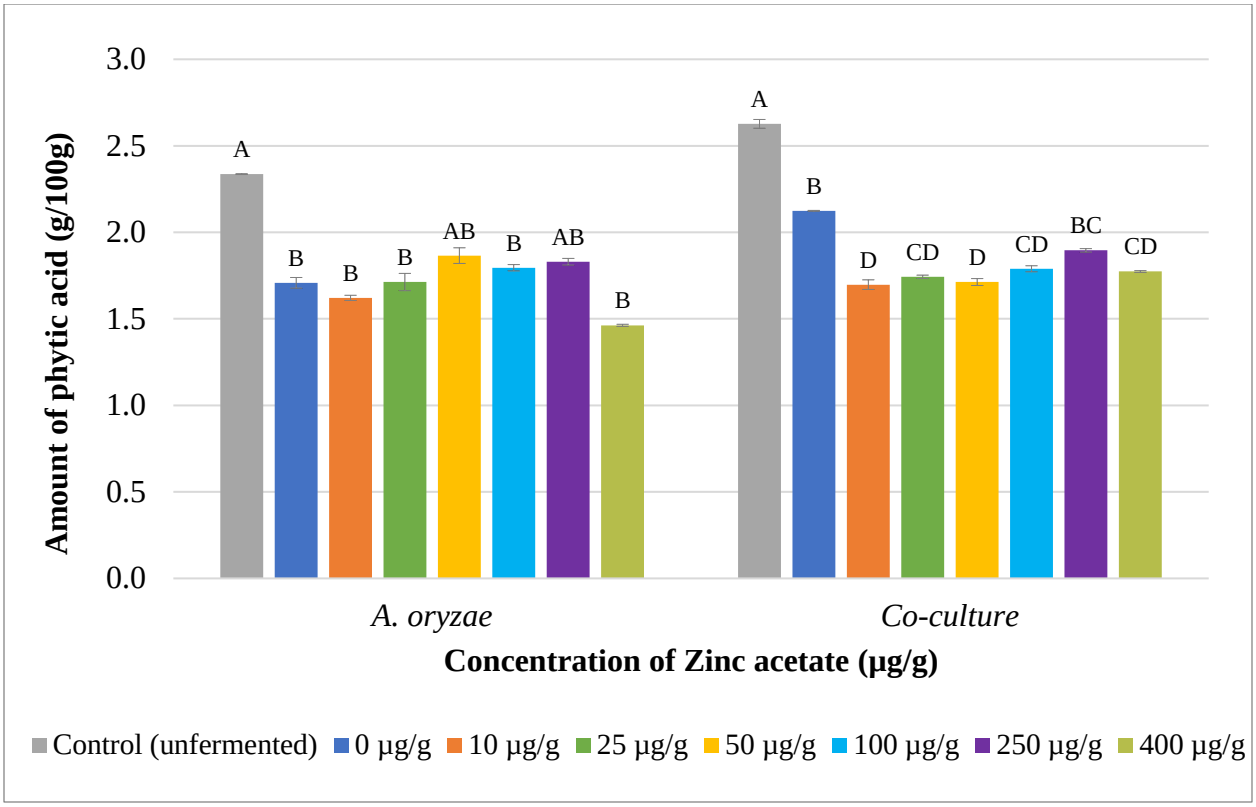

Figure 3.3. Total amount of phytic acid in Zinc (Zn) containing whole grain fermented samples after Solid state fermentation. The data are presented as mean ± standard deviation of the mean (n = 4). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Zn), the fermented samples contain different concentrations of Zn (0 µg/g to 400 µg/g), sterile whole sorghum grain and inoculum.

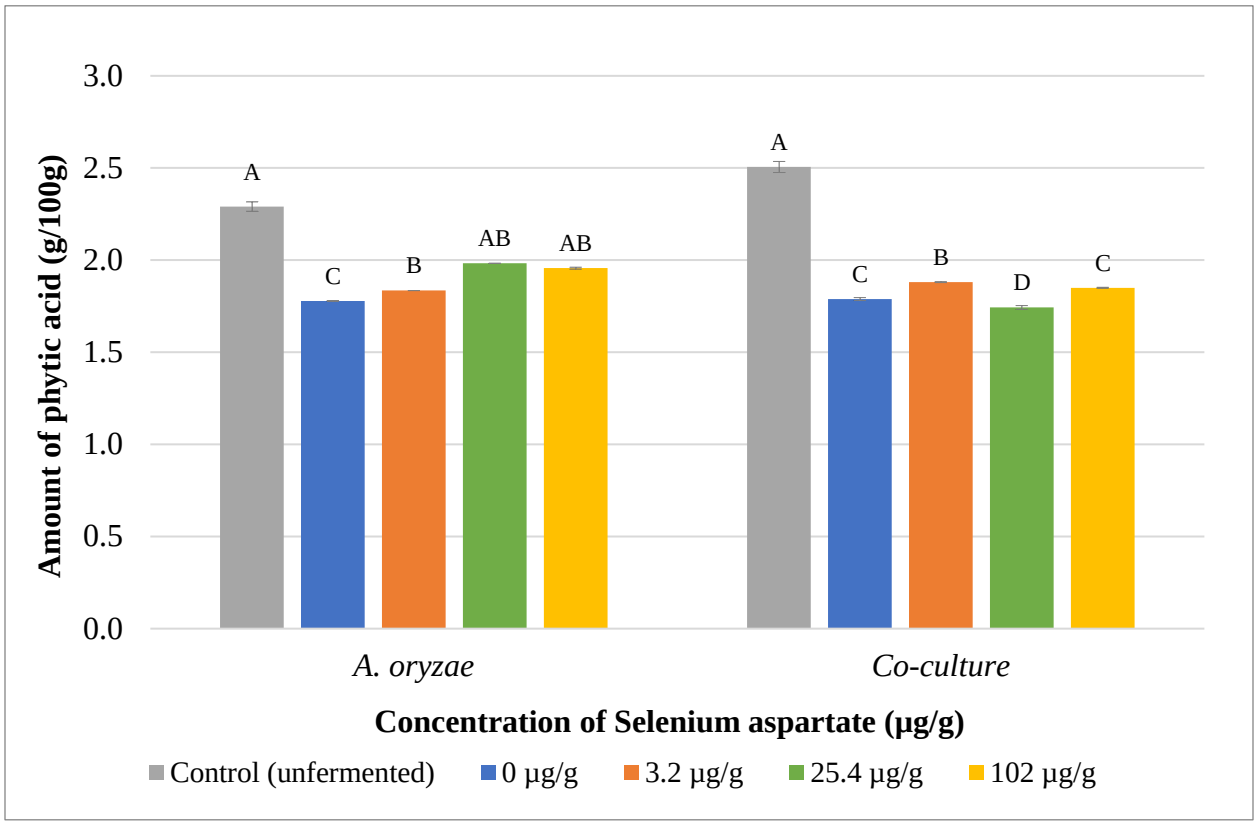

Figure 3.4. Total amount of phytic acid in Selenium (Se) containing whole grain fermented samples after Solid state fermentation. The data are presented as mean ± standard deviation of the mean (n = 4). Means with different letters are significantly different ($P < 0.05$) from each other within *A. oryzae* and Co-culture. Control (unfermented) contains only sterile whole sorghum grain (Without inoculum and Se), the fermented samples contain different concentrations of Se (0 µg/g to 102 µg/g), sterile whole sorghum grain and inoculum.

CHAPTER 4: OVERALL CONCLUSION

The present study highlights the effectiveness of SSF with *A. oryzae, B. subtilis,* and Co-culture in enhancing the uptake and accumulation of Zn and Se in sorghum grain. The supplementation of zinc acetate and selenium aspartate during fermentation influenced the uptake of these nutrients by microorganisms. The study also observed that the effectiveness of the supplementation and Co-culture fermentation varied depending on the substrate used, with coarse ground grain and whole grain showing different responses. This result suggests that the grain matrix plays a role in nutrient metabolism during fermentation. Moisture content analysis revealed that fermented sorghum grain had lower moisture content compared to unfermented samples, which is advantageous for product safety and stability by reducing the risk of microbial growth and spoilage. In addition, the study found that SSF effectively reduced the levels of phytic acid in sorghum grain. Coarsely ground sorghum grain fermented with *A. oryzae* and Co-culture exhibited a significant reduction in phytic acid content, indicating the potential for SSF to enhance the nutritional quality of grains and legumes. These findings have implications for developing nutrient-enriched food products and contribute to our understanding of microbial nutrient metabolism in SSF processes. Further research is needed to explore the bioavailability and potential health benefits of fermented biomass for human consumption.