# DEGRADATION OF PHYTIC ACID CONTENT IN SOY PULP BY *Bacillus thuringiensis* SP4 THROUGH SOLID-STATE FERMENTATION

By

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## ABSTRACT

### DEGRADATION OF PHYTIC ACID CONTENT IN SOY PULP BY *Bacillus thuringiensis* SP4 THROUGH SOLID-STATE FERMENTATION

**CHAN ONN KEI**

Fresh soy pulp is a loose material consisting good source of nutrients and was given merit to use as an excellent protein source in the animal feed industry. The high phytate content in the soy pulp has become one of the prime concerns for livestock consumptions, as the digestion of phytic acid is unfavorable for monogastric and agastric aquatic animals. The undigested phytate is believed to inhibit the bioavailability of micronutrients and act as an anti-nutritional agent. Present study was intended to produce low-phytate soy pulp through solid-state fermentation (SSF) with locally isolated *Bacillus thuringiensis* SP4. A total of 10 phytic acid degrading bacteria were isolated using rice bran extract (RBE) and soy pulp extract (SPE) agars. Isolate SP4, which was identified as *Bacillus thuringiensis* SP4, was distinguished one among others by decreasing 62.65% of the phytic acid content in soy pulp after 72 hours SSF. Through 2-level full factorial design, the significant factors affecting the reduction of phytic acid were inoculum size and initial moisture content. Subsequently, 3-level factorial design was employed to determine the optimal conditions of the screened factors and maximal phytic acid reduction was achieved 86.40% at run no. 20. The linear regression model was then validated

through confirmatory runs and less than 6% error rates reflected the model was adequate for predicting the total phytic acid reduction in SSF. The fermented soy pulp was analyzed by scanning electron microscopy (SEM) to observe the morphology and fourier transform infrared (FTIR) analysis to predict the changes of functional groups. Conclusively, the presented results suggested soy pulp could be used as *in situ* source of phosphohydrolases, where high nutritional values can be expected from the fermented low-phytate soy pulp. The prospects of using soy pulp as low-cost substrate in bioprocess also serve as an alternative for soybean waste management.

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**APPROVAL SHEET**

This dissertation entitled "**DEGRADATION OF PHYTIC ACID CONTENT IN SOY PULP BY *Bacillus thuringiensis* SP4 THROUGH SOLID-STATE FERMENTATION**" was prepared by CHAN ONN KEI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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I understand that the university will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may to be made accessible to UTAR community and public.

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CHAN ONN KEI

## DECLARATION

I, Chan Onn Kei hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any or other institution.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| pKa | Acid dissociation constant |
| BLAST | Basic Local Alignment Search Tool |
| df | Degree of freedom |
| DNA | Deoxyribonucleic acid |
| DCW | Dry cell weight |
| EDTA | Ethylenediaminetetraacetic acid |
| FSP | Fermented soy pulp |
| FOC | Flaxseed oil cake |
| LB | Luria-Bertani |
| PBS | Phosphate buffer saline |
| PCR | Polymerase Chain Reaction |
| rDNA | Ribosomal deoxyribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| RBE | Rice bran extract |
| SSF | Solid-state fermentation |
| SPE | Soy pulp extract |
| sp. | Species |
| SD | Standard deviation |
| SmF | Submerged fermentation |
| TBE | Trisborate EDTA |
| USP | Unfermented soy pulp |
| WBE | Wheat bran extract |

# CHAPTER 1

# INTRODUCTION

With the boost in the world's population and improved living standards, there is an increased demand for food, as well as growing competition for limited resources e.g. water, energy, land. Besides, challenges such as climate change, pest densities, and toxic contaminants are always threatening our food productivity, especially in crop production. Therefore, ongoing efforts from different sectors have been made to ensure global food security where one of the present strategies is to systematically recycle agricultural wastes. Balancing of crop production with a series of well-developed waste management systems is indeed a promising solution in the long-run that include several biological treatments and advanced valorization technologies.

The bioconversion of soy pulp into value-added product by novel bacteria isolates through solid-state fermentation (SSF) technique was addressed in this study. The United States Department of Agriculture (USDA) had reported a global soybean (*Glycine max*) output of 345 million metric tons by July 2017, whereby this widely consumed legume had covered an estimated 6% of the Earth's arable land (McFarlane and O'Connor, 2014; FAS, 2017). Despite the fact that Western countries e.g. United States, Brazil, and Canada, are the top soybean producers worldwide, soybean has been largely consumed by diverse cultures and has a remarkably long history of consumption in Asian countries. For instance, Hong Kong and Singapore are the top soymilk-consuming

regions among the Asian countries, followed by Thailand, China, and Malaysia (Starling, 2011). With the increasing consumption of soybean worldwide, large quantities of by-products such as soy pulp, soybean hull, and soybean meal, are derived from the manufacturing process.

Every kilogram of soybeans that are processed can harness approximately 1.1 – 1.2 kg of fresh soy pulp which annually equals to around 0.7 million tons of soy pulp from tofu manufacturers in Japan alone where a majority of it is being dumped in landfills or burnt due to its high perishability (Khare et al., 1995; Mizumoto et al., 2006). Meanwhile, the disposal of soy pulp is an arising environmental concern requiring high waste management cost. For example, the cost of soy pulp disposal in Japan is about 16 billion yen per annum (Muroyama et al., 2006). Despite the massive generation of soy pulp worldwide, only a small scale of it is used for home-based traditional food productions, such as Japanese soups, tempeh, *idli* (Indian steamed cake), *Meitauza* (fermented Chinese food), vegetables dishes, and salads (Soy20/20, 2005; Vong and Liu, 2016).

In the past 20 years, the applications of soy pulp in the bioprocess industry have been attracting considerable attention from researchers due to its widely recognized high protein content and exceptional nutritional quality, which is particularly useful for animal feed production (O'Toole, 1991; Ohno, Ano and Shoda, 1996; Li et al., 2013). However, the commercial-scale applications of soy pulp are still underutilized because it is susceptible to putrefaction, and the direct incorporation of soy pulp into feed manufacturing is limited by the

presence of indigestible oligosaccharides by non-ruminants, enzyme inhibitors, and anti-nutritional factors, for instance saponins, lectins, and phytic acid (Anderson and Wolf, 1995; Barnes et al., 2012; Mondala et al., 2015). As such, the presence of phytic acid is of prime concern for human nutrition and animal health management, whereby its negative aspects will be discussed further in following chapters.

As far as Malaysia is concerned, there is a significant amount of soybean curd and soymilk manufacturing industries located in both urban and peri-urban areas. According to the report of Foreign Agricultural Service (FAS), about 15% of the 60,000 tons of soybean imports contributes to the food production while the remainder is used as feed for livestock and poultry (FAS, 2017). Meanwhile, majority of the soy pulp produces is generally discarded as domestic or industrial waste where only a small portion is regarded as feed for ruminants (Rahman et al., 2014). Besides, soy pulp generated by small-scale manufacturers, such as the few that are that located in rural areas (i.e. Bentong, Pahang and Kampar, Perak), are often picked up by nearby farmers and are directly farmed on the fish or poultry without knowing the adverse effects brought about by the raw soy pulp. In fact, soy pulp is a relatively inexpensive source of protein and the removal of limiting factors are prerequisite before implementing a proper and reliable usage of soy pulp, especially in the development of feed resources. As such, the focal aim of this study is to degrade the phytic acid content in soy pulp and produce an economical-attractive biomass through SSF. The removal or reduction of certain

aforementioned barriers in the soy pulp can also be expected in coupled with the fermentation process.

The scope of this study focuses on reducing the anti-nutritional factors especially phytic acid content in soy pulp through SSF, thereby significantly exploiting the usage of soy pulp in the food and feed industry. A novel screening medium for isolating phytic acid degrading bacteria from environmental samples has been developed and the kinetic method for assaying phytic acid content in soy pulp has also been modified. Besides, the use of statistical optimization strategies has resulted in increasing the amount of reduced phytic acid content in soy pulp. Efforts in analyzing the physical properties of soy pulp before and after the fermentation process have also been carried out. In fact, this study has demonstrated a model for researchers in dealing with phytic acid in other agricultural biomass, especially cereal-based feed and food. It may serve as a strategic method for better food and feed quality with environmental protection, as well as lowering the production costs.

The objectives of this study were to:

a. isolate and screen for phytic acid degrading bacteria from soil samples;
b. identify the best phytic acid degrading bacteria obtained from SSF based on its morphology, biochemical properties and 16S rDNA sequencing;
c. optimize the fermentation parameters using response surface methodology with Design-Expert® 7.0; and

d. compare the characteristics of fermented and unfermented soy pulp using scanning electron microscope and fourier-transform infrared spectroscopy.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Soy Pulp

Agricultural and forestry residues are often regarded as the most promising sustainable biomass in the fermentation industry. As the costing of substrates occupies 30 – 40% of total production costs, biovalorization of agricultural waste is economically favorable and effective in reducing the operational costs of solid-state fermentation (SSF) (Yazid et al., 2017). The agro wastes that are commonly used in SSF are wheat straw, rice straw, oat bran, sugarcane bagasse, corn cobs, cassava bagasse, rice husk, coffee husks and pulp, corn stover, sesame oil cake, lemon peel, and so on (Kumar and Kanwar, 2012; Subramaniyam and Vimala, 2012; Soccol et al., 2017). Other than serving as a solid support for microbial growth and nutrient absorption, these agro wastes also act as important carbon sources that require some necessary macro and micronutrient supplementation. Nevertheless, the availability and feasibility of these solid substrates from local supplies are also the critical aspect to be considered for SSF production.

Efforts have been made to produce a wide variety of products from soy pulp fermentation in recent years, including bioactive compounds, prebiotics, and foodstuff (Li et al., 2013). Soy pulp is a loose material that is suitable for microbial fermentation and processing requirements and its general nutritional compositions are shown in Table 2.1. Fresh soy pulp contains around 70 – 80%

of moisture and is rich in water-insoluble components, which include high protein content, carbohydrates, dietary fibers, and minerals, whereby their composition may vary based on soybean cultivars, country of origin, processing techniques and sequences (Teng et al., 2012; Li et al., 2013). For instance, in the traditional Chinese way of soymilk processing, soybeans are first ground before being extracted, filtered and heated; while in the Japanese way, the soaked soybeans are cooked, followed by grinding and filtering (O'Toole, 1991).

**Table 2.1:** General compositions of soy pulp (Vong and Liu, 2016).

| **Macronutrients** | **Amount (g $100g^{-1}$ of dry matter)** |
|---|---|
| Carbohydrate | 3.8 – 5.3 |
| Protein | 15.2 – 33.4 |
| Fat | 8.3 – 10.9 |
| Dietary fiber | 42.4 – 58.1 |
| Insoluble dietary fiber | 40.2 – 50.8 |
| Soluble dietary fiber | 4.2 – 14.6 |
| Ash | 3.0 – 4.5 |
| **Micronutrients** | **Amount (g $100g^{-1}$ of dry matter)** |
| Thiamine ($B_1$) | 0.48 – 0.59 |
| Riboflavin ($B_2$) | 0.03 – 0.04 |
| Niacin ($B_3$) | 0.82 – 1.04 |
| Potassium | 936 – 1350 |
| Sodium | 16 – 96 |
| Calcium | 260 – 428 |
| Magnesium | 130 – 165 |
| Iron | 0.6 – 11 |
| Copper | 0.1 – 1.2 |
| Manganese | 0.2 – 3.1 |
| Zinc | 0.3 – 3.5 |
| **Phytochemicals** | **Amount (g $100g^{-1}$ of dry matter)** |
| Isoflavone aglycones | 5.41 |
| Isoflavone glucosides | 10.3 |
| Malonyl glucosides | 19.7 |
| Acetyl glucosides | 0.32 |
| Phytic acid | 0.5 – 1.2 |
| Saponins | 0.1 |

Although no documentations are available for the direct incorporation of soy pulp into animal feed, recent studies have reported the supplementation of soybean meal (another common soybean by-product) into broilers' diets (Xu et al., 2017), as well as served as a potential replacement of fish meal in the aqua feeds, including tiger groupers (Shapawi et al., 2013), rainbow trouts (Barnes et al., 2012), and Nile tilapias (Mahmoud et al., 2014). Moreover, it is reported that soy pulp offers a better protein quality than other soy products; for instance, the protein efficiency ratio of soy pulp is 2.71 as compared with 2.11 for soymilk (Li et al., 2013). As such, its superior nutritional quality has given merits for soy pulp to be used as an excellent protein source in the feed industry.

Undoubtedly, soy pulp is an inexpensive source of protein with high availability in Malaysia, as well as in Southeast Asia. However, efforts are yet to be made to convert them into usable biomass and fermentation with microorganism is a major step forward.

### 2.1.1 Phytic Acid

As mentioned in Chapter 1, the presence of several barriers has limited the usage of soy pulp as renewable biomass, while the anti-nutritional phytic acid in soy pulp was the focal subject in this study due to its significant negative impacts to the environment and diet intake. Phytic acid is present in a considerable amount in plant-derived foods and directly consumed by humans, such as wheat bran, soybean, oat meal, barley flour, sorghum, cowpea, and sunflower meal (Sreedevi and Reddy, 2013). Besides, the by-products or

residues from these staple foods are also used as the major protein ingredients for poultry and livestock feeds. For instance, the average daily intake of phytic acid for humans on vegetarian diets and inhabitants of rural areas on mixed diets is 2000 – 2600 mg and 150 – 1400 mg, respectively (Reddy, 2002).

In 1855 – 1856, phytic acid was firstly isolated by Hartig as small, non-starch particles from the seeds of various plants, which is now notably known as the principle storage form of phosphorus in nature (Tran, 2010). Three terminologies, namely phytic acid, phytate, and phytin are commonly used to describe the different forms of these phosphorus storage compounds, where the terms phytic acid and phytate have been used interchangeably in the previous literatures, as well as in this study (Reddy et al., 1989). To be specific, phytic acid is used to describe the free form of acid (completely deprotonated) while phytate is referring to the salt of phytic acid when mixed with cations. Meanwhile, the term phytin, is specifically refers to the complex salt of phytic acid with potassium, calcium, and magnesium (Selle and Ravindran, 2007).

As the primary storage form of both phosphate and inositol in nature, a molecule of phytic acid contains approximately 28.2% of phosphorus (Angel et al., 2002; Jorquera et al., 2008). They are natural compounds that are synthesized by plants and accumulated in seed during plant seed maturation, which is responsible for around 60 – 90% of the total phosphorus in dormant seeds (Loewus, 2002). For instance, phytic acid is mostly present in cereal grains and legumes, including soy beans, oil seeds, rice bran, wheat bran, nuts, and corn (Canan et al., 2011). Undoubtedly, they represent a vital class of

organic phosphorus and essential phosphorus sources for plant growth and seed germination.

Indeed, the form of phytic acid present and its location vary among plants, where phytate has been observed in roots, tubers, fruits, vegetables, and nuts. The main phytate in the legumes and cereal grains are potassium-magnesium phytines, which can be found in wheat, rice, broad beans (*Vicia faba*), and sesame seeds; whereas calcium-magnesium-potassium phytines are commonly found in pollen grains, soybeans, as well as Great Northern beans (Scott and Loewus, 1986; Marschner, 1995; Tran, 2010). In other words, phytates also could be considered as the storage sites of different metal ions, especially potassium, magnesium, calcium, zinc and iron. According to the reviews by Balaban and co-researchers (2017), phytic acid makes up to 30% of all phosphorus fractions in roots, while its fraction boosts up to 80% in cereal grains and seeds, where lastly it accumulates at the final stages of the plant life cycle and is returned to the soil with seeds. For instance, the embryo of maize accounts up to 80% of the total phytate present in plants (Reddy et al., 1989).

Upon the discovery of this widespread compound, phytic acid or its official nomenclature, *myo*-inositol 1,2,3,4,5,6-hexakis (dihyodrogen) phosphate (official abbreviation: $InsP_6$ or IP6), has been the subject of investigation for scientists from different fields over the last century. The molecular formula of phytic acid is $C_6H_{18}O_{24}P_6$ with a molecular mass of 660.04 g $mol^{-1}$. With the advent of technologies including X-ray crystallography, elemental analysis, nuclear magnetic resonance (NMR), and titration of sodium phytate, several

studies have supported the two most accepted structures of phytic acid that were proposed by Anderson (1914) and Neuberg (1980), respectively. After all, the structure proposed by Anderson in 1914 (Figure 2.1) has been concluded as the pre-dominant form of phytic acid found in plant materials and is generally used in the literature. Figure 2.1 illustrates a fully phosphorylated alcohol-inositol phosphate molecule, where all six hydroxyl groups are replaced by phosphate residues. As such, different degrees of *myo*-inositol phosphate can be formed, depending on the number of phosphate substitutions (Balaban et al., 2017).

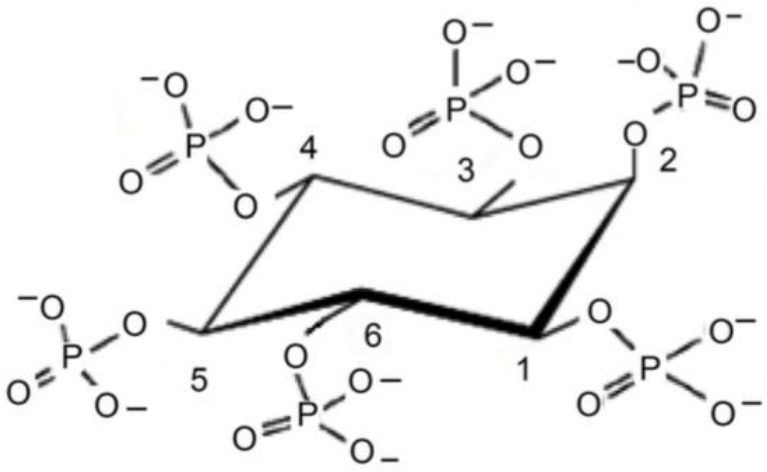


**Figure 2.1:** *Myo*-inositol 1,2,3,4,5,6-hexakis (dihyodrogen) phosphate. Adapted from Balaban et al. (2017).

Among the nine possible inositol conformations with stereoisomers of hydroxyl groups, one axial and five equatorial oriented hydroxyl groups were recognized as the most stable *myo*-inositol conformation (Balaban et al., 2017). As for the conformational state of phytic acid, Blank and co-researchers (1971) had proposed that the phosphate group at C-2 is in the equatorial position while the phosphate groups at C-1, C-3, C-4, C-5, and C-6 are in the axial position.

On the contrary, Johnson and Tate (1969) suggested that the phosphate group at C-2 is in the axial position while the other groups of phosphate are in the equatorial position. The controversy remained unclear until 1980, where Isbrandt and Oertel (1980) established that the phytic acid conformational preferences depended greatly on the pH of aqueous solution through the analysis of $^{13}C$ NMR, $^{31}P$ NMR, and Raman spectroscopy. The works have demonstrated that phytic acid exists in the conformation of one axial, five equatorials in acidic solution and under strong alkaline condition, the conformation of five axials, one equatorial is dominant. Subsequently, several studies have been conducted revealing that the conformation of phytic acid was also affected by the ionic medium and ionic strength of the aqueous solution (Brigando et al., 1995; Bauman et al., 1999).

In an aqueous solution, phytic acid exhibits several levels of negative charge over a wide range of pH due to the presence of 12 ionizable hydrogen atoms with different dissociation constants (Tran, 2010). Among the reactive sites, six groups have pKa 1.1 to 2.1 (strongly acidic), two have pKa 6.0 to 6.3 (weakly acidic) and the remaining four groups have pKa 9.0 to 11.0 (very weakly acidic) (Costello et al., 1976; Reddy et al., 1982). Due to the high density of negatively charged properties, phytic acid has become a very strong chelating agent and has been identified as a stronger chelating agent than ethylenediaminetetraacetic acid (EDTA) (Chung et al., 2006).

Several crucial physiological functions of phytic acid have been discovered and thoroughly reported in the literatures. Other than regarded as the main

storage form of both inositol and phosphorus in nature, phytic acid has also been reported for its hypolipidemic, anti-neoplastic or anti-carcinogenic, anti-oxidant properties, as well as its function in the prevention of Parkinson's disease, diabetes and kidney stone formation (Crea et al., 2008; Al-Fatlawi et al., 2014). Phytic acid is also known for its ability to modify the bioavailability of metal ions and marked as components of cell signaling and phosphorus transfer systems present in animal and plant tissues (Kumar et al., 2010; Tran, 2010).

### 2.1.2 Negative Aspects of Phytic Acid

Although phytic acid has excellent health-beneficial roles, it has been shown to have anti-nutritional effects in the diets of humans and animals, which has been reported intensively in previous studies (Martin and Evans, 1989; Liu, Cheng and Zhang, 2005). With a complete dissociation in aqueous solutions, such as biological fluids, natural water, wastewaters and soil solutions, the six negatively charged phosphate groups of phytic acid are strongly chelates with metal cations through electrostatic interactions, forming insoluble phytate-metal complexes, including calcium, magnesium, zinc, iron, copper, manganese, and aluminum (Kerovuo, 2000; Crea et al., 2008; Kumar et al., 2010). Moreover, a single phosphate group can form one or more hydrogen bonds with the metal ions and the bond formations dependent on the cation ionic radius (Balaban et al., 2017). For instance, calcium is associated with two adjacent phosphate groups of phytic acid because it has a longer ionic radius than zinc and magnesium (Figure 2.2). Subsequently, the formation of these stable complexes had adversely affected the bioavailability of essential

minerals in diets and eventually caused mineral deficiency. For example, nutritional rickets has been reported in the populations feeding mainly on *chapatti*, which is a type of unleavened bread that has high phytate content (Tran, 2010). Indirectly, phytic acid also impairs the function ability of a number of digestive enzymes, as metal ions are essential for enzymatic reactions and stability. For instance, the chelating complexes of phytate-copper have been reported to reduce more than 95% enzymatic activity of carboxypeptidase A and phytic acid has shown similar levels of inhibition with EDTA on α-amylase (Jacobsen and Slotfeldt, 1983; Martin and Evans, 1989).

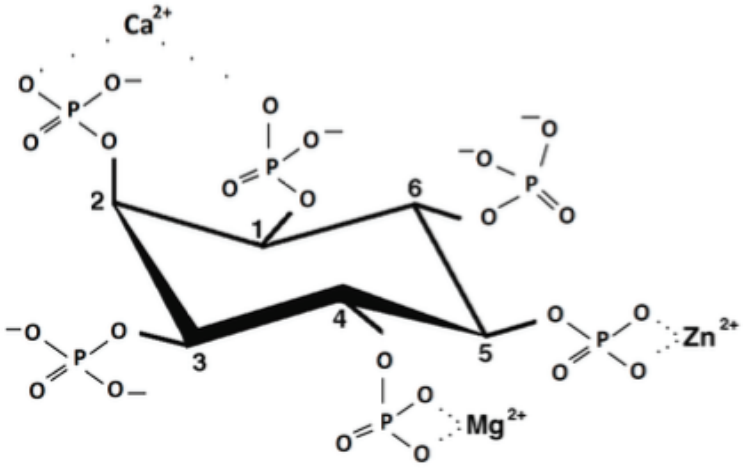


**Figure 2.2:** Phytate complex. Adapted from Balaban et al. (2017).

Besides, phytic acid can interact with positively charged dietary proteins over a wide range of pH and resist proteolysis, and also tends to form phytate carbohydrate and lipophytin complexes, leading to poor macromolecules digestibility (Kumar et al., 2010). Conclusively, the presence of high phytic acid content has severely affected the solubility and bioavailability of these valuable minerals and proteins, thereby consequently reducing the nutritive quality in the human diet and feedstuffs.

Despite the widespread occurrence of phytic acid in nature, it is actually poorly metabolized by monogastric or agastric aquatic animals, that include swine, poultry, fish, and humans, either due to the absentce or insufficient amount of intestinal phytases along the digestive tracts (Sasirekha et al., 2012). Therefore, other than acting as an anti-nutritional agent, the indigestible phytic acid also had limiting the uptake of phosphorus by consumers.

From an environmental perspective, the undigested phytates that end up in the excrements will lead to run-off of phosphorus into aquatic ecosystems, which consequently contributes to severe pollution risks (Brinch-Pedersen et al., 2014). Problems with high levels of phytic acid in manure disposals are now widely recognized in association with intensive livestock farming. For instance, excessive phosphorus will give rise to cyanobacteria blooms and eutrophication of surface waters, forming anaerobic aquatic conditions, coupled with the released of unpleasant odor into the environment (Mallin, 2000; Salman et al., 2014).

## 2.2 Degradation of Phytic Acid

### 2.2.1 Physical Treatment

Dephosphorylation of phytic acid is a prerequisite for overcoming the aforementioned adverse effects. Phytase (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate phosphohydrolase) is a general term used to describe phosphohydrolase enzyme. As the phosphate groups are removed in a stepwise manner from the inositol ring and release inorganic phosphate, the mineral binding strength of the phytate will be decreased. For instance, only phytic acid

and inositol pentaphosphate have negative effects on the bioavailability of minerals, whereas other lower inositol phosphates (e.g. inositol triphosphate) have poor binding capacity or the complexes formed are more readily soluble (Sandberg et al., 1989; Kumar et al., 2010). As such, phosphatidylinositols will be generated as intermediates, or in some cases, as end products, which represent different degrees of dephosphorylation from phytic acid (Liu et al., 1998).

Various physical treatments that are used in food/feed processing and preparation such as milling, soaking, cooking, and germination are common efforts made to reduce the amount of phytic acid in cereal grains and legumes, where these treatments could activate the naturally present phytases in plants (Kumar et al., 2010). Soaking is a better method compared with milling because major parts of dietary fibers and minerals are simultaneously being removed during the milling process (Gupta et al., 2015). The effectiveness of soaking and germination in removing phytic acid has been reported by several studies and the combination of cooking and soaking has shown a significant reduction of phytic acid content in grains (Pearlas and Gibson, 2002; Greiner and Konietzny, 2006; Coulibaly et al., 2011). However, the capability of dephosphorylation through these methods depends greatly on the plant species, cultivation conditions and environment, due to the differences in their intrinsic phytase activities (Egli et al., 2002; Brinch-Pedersen et al., 2014).

### 2.2.2 Biological Treatment

In order to increase mineral bioavailability, phytic acid content must be reduced to very low levels and it is almost impossible to fully hydrolyze the phytic acid by endogenous phytases during food/feed processing or preparation (Hurrell, 2003). Therefore, supplementation of exogenous phytases is desired and considered as the most versatile solution to achieve maximum removal of phytic acid content, especially in the sector of feed manufacturing.

In the food industry, phytase has been used to produce low-phytate content food and improve the quality of human diets. For instance, the addition of phytase in bread making (e.g. whole meal bread) has improved the nutritional value and promoted the activation of endogenous α-amylase by increasing the bioavailability of calcium (Haros et al., 2001). Furthermore, phytase can also be added in the production of phytate-free soybean milk, tarhana (i.e. traditional Turkish fermented cereal food), and *chapatti* (Kumar et al., 2010).

Ever since the first commercial phytase, Natuphos® was released in 1991, phytases were often included as feed additives coupled with the supplementation of dietary phosphorus or used in feed pre-treatment in order to ensure phosphorus uptake by livestock, especially swine, poultry, and fish (Kumar et al., 2010; Gupta et al., 2015). For instance, using phytase in fish feed has been used in common aquaculture species such as salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), striped bass (*Morone saxatilis*), and channel catfish (*Ictalurus punctatus*) via spraying the phytase onto the pellets, or pre-treating the fish feed before being pelleted, which consequently

improves the bioavailability of phosphorus by 22 – 25% in the past decade (Cain and Garling, 1995; Cao et al., 2007).

However, the inclusion of these phytase additives and dietary phosphorus has directly increased the cost for feed production due to the high costs of enzymatic protein isolation and purification. According to Bogar et al. (2003), the cost of commercial phytase supplementation is around 2 – 3 USD per metric ton of feed. Furthermore, around 70% of the total phosphorus in the feeds are released in manure due to uptake inefficiency, and the excess dietary phosphorus is washed into waterways may further contribute to significant environmental pollution (Jorquera et al., 2008; Salman et al., 2014). Besides, some minor enzymatic side activities caused by the extreme inclusion of phytases in animal diets can also be expected, independently affecting the nutrient uptake (Salman et al.,2014). As such, Selle and Ravindran (2007) had suggested that high liberation of phosphorus caused by the phytase hydrolysis may have prompted calcium and phosphorus imbalances in the gastrointestinal tract, as well as altering the effective dietary electrolyte balance (DEB) since both phytic acid and phytase affect the secretion of sodium into the gut lumen.

The present study establishes low-phytate content soy pulp through SSF *in situ*, which acts as the key strategy to improve the nutritional quality of feedstuff in the long-run, as well as reducing the production costs by replacing the need of phytase and dietary phosphorus supplementation in feed production. In fact, recent studies have shown the improvement of nutritional value of soybean via SSF by increasing the bioavailability of nutrients and reducing the levels of

anti-nutritional factors (Teng et al., 2012). Nevertheless, the specific information on the *in situ* reduction of phytic acid content in the soy pulp using SSF is relatively limited.

## 2.3 Solid-state Fermentation

Fermentation technique is the biological conversion of complex substrates into simple compounds by various microorganisms, including bacteria and fungi (Subramaniyam and Vimala, 2012). Three fermentation techniques – submerged, semi-solid, and SSF are commonly used in industrial production.

In recent years, SSF has been presented as a promising technology for biomass valorization with the aid of microorganisms by converting them into value-added end products. SSF is defined as a fermentation process that involves a solid matrix that contains enough moisture to support microbial activities (Gottumukkala et al., 2012). The solid matrix could be either the source of nutrients or a support matrix impregnated with proper nutrients that are required for the development of microorganisms (Singhania et al., 2009). This technique is widely used in the production of biopesticides (Jisha and Benjamin, 2014), enzymes (Soleimaninanadegani et al., 2014), antibiotics (Awad et al., 2011), aroma and phenolic compounds (Dey and Kuhad, 2014), bioethanol (Rodriguez et al., 2010), composting and animal feed (Shi et al., 2017).

The advantages of SSF have been well-described in a number of recent review articles (Bhargav et al., 2008; Kumar and Kanwar, 2012; Subramaniyam and

Vimala, 2012; Yazid et al., 2017). In comparison with submerged fermentation (SmF), SSF could have achieved higher productivity in bioconversion of low-cost solid substrates because broth rheology caused by the low-cost solid substrate might influence the mass transfer and transport in the SmF reactors (Yazid et al., 2017). Besides, minimal liquid phase in SSF also can reduce wastewater output and decrease the contamination risk of microorganisms. As such, this fermentation technique allows the usage of unsterilized solid substrates and creates non-strict sterile environments for the process (De la Cruz et al., 2015). For instance, studies have reported that the production of lipase and protease have been successfully scaled-up under non-sterilized conditions and resulting in stable and high enzymatic activity (Yazid et al., 2017). As agitation and sterilization are not always needed, SSF only requires minimal energy consumption and low capital investment (Wang and Yang, 2007; Yazid et al., 2017). As an example, the capital investment for lipase production by *Penicillium restrictum* in SmF was 78% higher than SSF, resulting in 47% less price of SSF products (Kumar and Kanwar, 2012).

### 2.3.1 Factors Influencing the SSF

Fungi has been widely recognized as the most adaptable organism in SSF due to the growth of prolonged hyphae on particle surfaces and their penetrability into inter-particle spaces and thereby colonizing the solid matrix (Santos et al., 2014). Until recent years, studies have consistently reported satisfactory outcomes of SSF production using bacteria as the host cultures (Jorquera et al., 2008; Kumar and Kanwar, 2012; De la Cruz et al., 2015). Regardless of the types of microbial and fermentation techniques used, the fermentation process

and production are highly affected by a wide range of factors, such as moisture content, aeration, and particle size.

#### 2.3.1.1. Moisture Content

In SSF, microorganisms are grown under low moisture levels within the substrate and may experience limited metabolism when compared to SmF. Moisture content can vary during the fermentation period due to the evaporation of water throughout the metabolic heat transfer, water consumption as well as liberation throughout microbial metabolism (Kumar and Kanwar, 2012). As such, water activity of substrates will strongly affect the microbial activity, thus determining the types of microbes that can grow in SSF, as well as those that can modify microbial metabolic production and its excretion by controlling this factor (Pandey et al., 1999; Bhargav et al., 2008). It has been reported that bacteria require high water content of 60 – 85%, which is higher than the requirement of fungi that ranges between 40 – 60% (Yazid et al., 2017).

#### 2.3.1.2. Aeration

The roles of aeration in SSF are to meet the oxygen demand in the aerobic bioprocess by maintaining high oxygen levels and low carbon dioxide levels among the inter-particle space, and mass and heat transport in a heterogeneous system (Manan and Webb, 2017). Oxygen requirement varies according to the microbes and it usually can be achieved in SSF with relatively low aeration levels. Meanwhile, oxygen uptake is affected by several parameters, such as moisture content, bed depth, forced aeration, mixing, and perforations in the

culture vessel (Singhania et al., 2016). Although aeration rate was reported to benefit microbial growth and production, static fermenter is often used (especially when agitation is not possible) in small-scale SSF without forced aeration and agitation, which offers easy operation (Krishania et al., 2018).

#### 2.3.1.3. Particle Size

An optimal substrate particle size is necessary to facilitate microbial fermentation in SSF, where the optimum sizes of biomass are usually achieved by chopping, grinding, or rasping. Smaller sizes of substrates are capable of providing a larger surface area for microbial attachment. On the contrary, a particle size too small forms a dense and firm matrix, which may interfere with substrate agglomeration, liquid mass transfer and gaseous exchange, resulting in poor microbial growth (Nigam et al., 2009; Kumar and Kanwar, 2012). In other words, larger particle substrate sizes have larger inter-particle space and provide better aeration efficiency (Kumar and Kanwar, 2012). Besides, during the process of fermentation, the particle size of the substrate may change accordingly, which may have affected the microbial growth, substrate consumption, water content, and conductivity of heat plus yield of products (Manan and Webb, 2017).

## 2.4 Process Optimization

Optimization of the fermentation process is a critical aspect because medium composition, physical and chemical growing conditions for microbial development, and strain selection can significantly affect the yield of the desired products. From a commercial production aspect, an ideal fermentation

process is usually dictated by the need to convey desired outcomes in high productivity of product formation coupled with low impurities, low production cost, and ease of the downstream processing.

According to Kennedy and Krouse (1999), the fermentation improvement strategies are categorized into ‘closed strategy’ and ‘open strategy’. Closed strategy refers to a fixed number and type of components used while the open strategy puts no assumptions on component selection. Researchers often modify the closed strategy due to cost and time effectiveness, alongside with sufficient information from previous experiences or literature.

### 2.4.1 One-Factor-At-A-Time

The principle of one-factor-at-a-time (OFAT) strategy is varying only one variable at one time over a desired range while keeping other components constant (Kennedy and Krouse, 1999). This procedure is repeated with other variables as well, until an optimum condition is achieved. OFAT is a classical strategy that is relatively easy to be conducted, and the individual effects of the factors on the response can be clearly observed. However, this approach fails to depict the interactive effects among the factors and it requires large amounts of resources for the amount of information gained, in terms of costing, time consumption, materials, labor, and so on (Czitrom, 1999; Nair et al., 2014).

### 2.4.2 Factorial Design

Design of experiments (DOE) utilizes the advancement of statistical techniques to overcome the limitations of classical OFAT in the process of optimization.

DOE is a series of strategically planned experiments that are executed to obtain a mass amount of information about the effect of more than one factor at a time of the outcome (Singh et al., 2017). In this case, full factorial designs were employed to optimize the phytic acid reduction of soy pulp in SSF. Full factorial designs have been the most commonly preferred optimization technique when several parameters are involved in the fermentation process. In full factorial design, every combination of factor levels and their interaction effects on the response are investigated. As a result, this technique is efficient when chemical parameters predominate the effectiveness of the physical parameters (Lundstedta et al., 1998; Ashok and Kumar, 2017). In contrast, fractional factorial design only tests on the well-reported combinations of factors and it is usually employed when full factorial design is impractical or a very little knowledge is available about the interactions of factors (Singh el al., 2017). In other words, full factorial design is the preferred DOE used in SSF.

### 2.4.3 Response Surface Methodology

Response surface methodology (RSM) was previously known as Box-Wilson methodology, which is an optimization technique to design an experiment, build models, evaluate the effects of factors and search the optimum conditions for the bioprocess with a limited number of experiments (Box and Wilson, 1951; Kumari et al., 2016). Nowadays, RSM has been effectively applied in many fields, including life sciences, aerospace, electronics, process industries, automotive, and agricultural settings (Nair et al., 2014). The advantage of RSM is less number of experiment trials needed when evaluating multiple parameters, which will directly reduce the time needed and save experimental

cost. According to Gupte and Kulkarni (2003), RSM can be performed in three steps: screening of factors following the path of steepest descent/ascent, followed by fitting of the quadratic regression model, and lastly, optimized by using the canonical regression analysis method. In short, RSM is capable of determining the effects of different factor levels with a specific response, while achieving quantitative understanding of the system behavior over the tested region (Singh et al., 2017).

# CHAPTER 3

# RESEARCH METHODOLOGY

## 3.1 Overall Experimental Design

Figure 3.1 shows the experimental design of this study. The methods used were described in the following sections and the formulation of media, buffers, and solutions were listed in Appendix A.

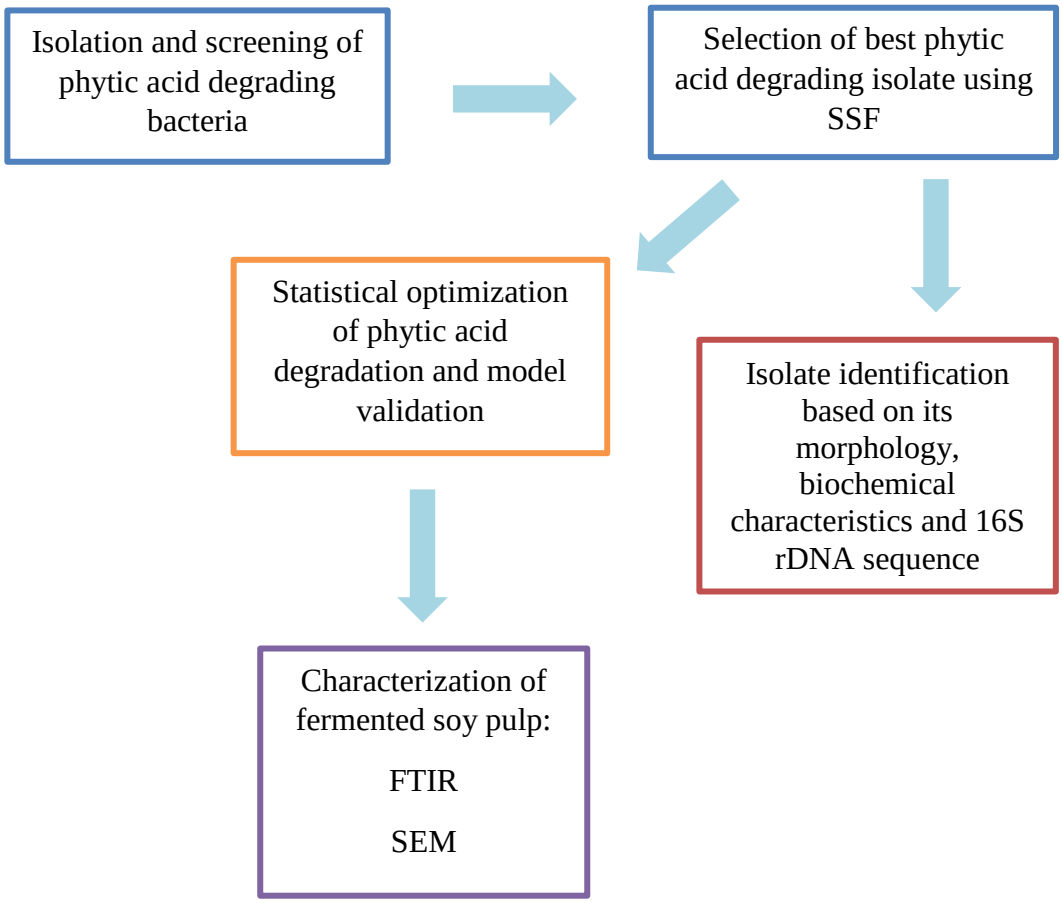


**Figure 3.1:** The overall experimental design for the phytic acid degradation by locally isolated bacteria.

## 3.2 Soy Pulp Preparation

Soy pulp was used as the sole carbon source in this study and it was consistently obtained in bulk from small-scale tofu producing stalls in local wet markets around Bentong, Pahang. The soy pulp was sun-dried continuously for a few days until it reached constant weight. Subsequently, the dried soy pulp was ground and sieved to particle size ranging between 500 – 850 μm and kept at room temperature before being used as solid substrate.

## 3.3 Preparation of Rice Bran Extract and Soy Pulp Extract Agars

The composition of rice bran extract (RBE) agar and soy pulp extract (SPE) agar were modified from Powar and Jagannathan (1982). The rice bran was kindly provided by Padiberas Nasional Berhad (Kompleks BERNAS Sg. Manik, Chikus, Perak, Malaysia), while soy pulp with fine particles size less than 500 μm, was a by-product of the substrate preparation as mentioned in Section 3.2. RBE was prepared by autoclaving 100 g of rice bran in 1000 mL of distilled water. Then, the residues were filtered and the filtrate was used as the RBE. Around 0.04% ammonium sulfate, 0.02% magnesium sulfate heptahydrate, 0.1% casein, 0.05% monopotassium phosphate, 0.04% dipotassium phosphate, and 2% agar were dissolved in RBE before being sterilized by autoclaving. Identical preparation procedures were carried out to prepare SPE agar by replacing the RBE with SPE.

## 3.4 Isolation and Screening of Phytic Acid Degrading Bacteria

### 3.4.1 Bacteria Isolation

Soil samples of rhizospheres and poultry farms were collected from Kampar areas and processed within 24 hours in the laboratory. Soil clods were broken into small soil particles and mixed with the remaining sample. Approximately 10 g of soil sample was suspended into 90 mL of 0.8% saline solution and then subject to 10-fold dilutions. Suspensions from $10^{-5}$ to $10^{-8}$ dilutions of each sample (0.1 mL) were spread onto RBE and SPE agars. The plates were incubated at 37 °C for 72 hours with daily observation. Any developed colonies that showed clear zones of hydrolysis were sub-cultured onto RBE and SPE agars for single-colony isolation and incubated.

### 3.4.2 Screening of the Isolates with Counterstaining Technique

Counterstaining technique was conducted as delineated by Bae et al. (1999). Following the growth of bacteria isolates on RBE and SPE agars, the colonies were washed from the agar surface with distilled water. Then, the agar was flooded with 2% aqueous cobalt chloride solution and incubated at room temperature for 5 minutes. Next, the cobalt chloride solution was discarded and replaced with freshly prepared coloring reagent containing equal volumes of 6.25% ammonium molybdate tetrahydrate solution and 0.42% ammonium monovanadate solution. The solution was removed upon 5 minutes of incubation and the clear zones of hydrolysis were re-examined. The remaining of clear zones after the counterstaining treatment indicated that the isolate was able to degrade phytic acid.

## 3.5 Identification of Phytic Acid Degrading Isolate

### 3.5.1 Phenotypic Analysis

Phenotypic analysis was subdivided into morphological and biochemical characterization. Morphological characterization, which included colony size, pigmentation, optical properties, and form of colonies, was conducted by visually observing the overnight culture of the isolate on LB agar. Besides, Gram staining, Coomassie brilliant blue (CBB) staining and biochemical identification using Analytical Profile Index (API) test and VITEK® 2 Compact analyses were carried out. For Gram staining, a loopful of bacteria cells on LB agar was firstly smeared on a clean glass slide and heat fixed. The fixed smear was allowed to cool before being flooded with crystal violet solution for 1 minute. After that, the crystal violet solution was rinsed off with distilled water and replaced with iodine solution. After 1 minute, the iodine solution was rinsed off and flooded with decolorizer for 5 seconds. Lastly, the decolorizer was rinsed off and safranin was added and soaked for 30 seconds before rinsing (Smith and Hussey, 2005). For CBB staining, a loopful of selected isolate which had been cultivated on LB agar for 72 hours was smeared on a clean glass slide and heat fixed. The fixed smear was stained with CBB solution for 3 minutes and washed with distilled water (Muniady et al., 2011; Jisha and Benjamin, 2014). Both stained specimens were allowed to dry and observed under a compound microscope. API test and VITEK® 2 Compact analysis of isolate were conducted according to the manufacturer's protocol (bioMéroeix, United States). API® 50CHB strip (Appendix B) and VITEK® BCL card (Appendix C) were used based on the preliminary results of the above-mentioned phenotypic analysis. The API result was interpreted with

APIweb™(https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin).

### 3.5.2 16S rDNA Identification

Genomic DNA extraction of isolate was performed using HiYield™ Genomic DNA Mini Kit (Real Biotech Corporation, Taiwan) according to the protocol recommended by the manufacturer (Appendix D). The purities of the genomic DNA extract were measured using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, United States) and used as the templates for PCR. A primer pair was used to amplify the 16S rDNA of the isolate, as listed in Table 3.1 (Hongoh et al., 2003).

**Table 3.1:** Set of primers used in PCR.

| Primer Set | Sequences |
|---|---|
| 63F | 5'-CAG GCC TAA CAC ATG CAA GTC-3' |
| 1387R | 5'-GGG CGG WGT GTA CAA GGC-3' |

The PCR reaction for the isolate was performed in a total volume of 25 μL containing the compositions as shown in Table 3.2.

**Table 3.2:** Composition of PCR reaction mixture.

| Reagent | Manufacturer | Final Concentration |
|---|---|---|
| MyTaq™ Red Reaction Buffer (with 5 mM dNTPs and 15 mM $MgCl_2$) | Bioline, Canada | 1× |
| MyTaq Red DNA Polymerase | Bioline, Canada | 2.5 U |
| Forward primer | BioSune, China | 0.4 μM |
| Reverse primer | BioSune, China | 0.4 μM |
| Genomic DNA | - | 5.63 ng/μL |
| Deionized distilled water | - | Top up to 25 μL |

The PCR was performed in SC200 SuperCycler Thermal Cycler (Kyratec, Australia), with the following amplification protocol: 4 minutes of initial denaturation at 94 °C; followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds with an extension at 72 °C for 45 seconds; and one cycle of final extension at 72 °C for 8 minutes. The PCR products (1 μL) were assayed with 0.8% agarose gel that was pre-stained with SYBR Safe™ DNA (Thermo Scientific, United States), using 1× Trisborate EDTA (TBE) buffer for approximately 30 minutes at 100 V (constant). The sizes of amplified fragments were estimated by referring to a HighRanger 1 kb DNA ladder (Norgen, Canada). The gels were then visualized under a UV trans-illuminator and the gel images were captured and analyzed by using Image Lab™ Software (Bio-Rad, United States). These PCR products were purified with Xprep PCR Purification Kit (Phil Korea Technology, Korea) according to the manufacturer's protocol (Appendix E) and out-sourced to MyTACG Bioscience Enterprise for DNA sequencing. The consensus sequence of the isolate was aligned and compared with the database using Basic Alignment Search Tool (BLAST), https://blast.ncbi.nlm.nih.gov/Blast.cgi, from the National Center for Biotechnology Information (NCBI) to perform species identification.

### 3.5.3 Phylogenetic Analysis

The phylogenetic tree of isolate was constructed based on the 16S rDNA sequences with Mega 5.7 software (Pennsylvania State University, United States) using the neighbor-joining (NJ) method. The statistical significance of

the branching in the tree was determined using bootstrap analysis with 1,000 replicates.

### 3.5.4 Cry Protein Analysis via Scanning Electron Microscopy

Pure isolate was firstly cultivated in LB broth for at least 72 hours at 37 °C with agitation at 200 rpm. 1 mL aliquot was sampled from the culture to a 1.5 mL Eppendorf tube and underwent the preparation scheme as shown in Table 3.3. Each step was suspended with the respective reagent and centrifuged at 10,000 × *g* for 10 minutes at 4 °C after incubation.

**Table 3.3:** SEM specimen preparation protocols.

| **Step** | **Reagents** | **Application Time** |
|---|---|---|
| Fixation | 4% paraformaldehyde | Overnight |
| Wash | 0.01 M PBS | 10 minutes with 3 changes |
| Dehydration | Ethanol: | |
| | 25% | 5 minutes |
| | 50% | 10 minutes |
| | 75% | 10 minutes |
| | 95% | 10 minutes |
| | 100% | 10 minutes with 3 changes |
| Critical point drying | - | - |

The freeze-dried sample was stuck on a disc with scotch tape and coated with a thin layer of platinum in a JFC-1600 auto fine coater (JEOL, United States). The sample was then degassed in a chamber and imaged in a JSM-6701F FE-SEM (JEOL, United States) under 4.0 kV with working distance of 6.0 mm.

## 3.6 Degradation of Phytic Acid in Soy Pulp Through Solid-state Fermentation

### 3.6.1 Inoculum Preparation

For seed culture preparation, the stock culture of the isolate was inoculated into a sterilized 50 mL LB broth in a 250 mL Erlenmeyer flask. The inoculated medium was incubated overnight at 37 °C and 200 rpm. The bacterial density of the overnight culture was measured at 600 nm and adjusted to $OD_{600}$ 1.20±0.05, at which point it was used as the inoculum for SSF.

### 3.6.2 Solid-state Fermentation

In an Erlenmeyer flask (250 mL) containing 10 g of the soy pulp was supplemented with basal mineral solution containing 0.5% ammonium sulfate, 0.1% magnesium sulfate heptahydrate, and 0.1% sodium chloride at pH 7.5 (Roopesh et al., 2006; Lee et al., 2014). The moisture : substrate ratio for the SSF was 2.5 mL : 1 g. After autoclaving, 10% inoculum was inoculated to the Erlenmeyer flask and mixed thoroughly with a glass rod under sterile condition. The inoculated flask was incubated at 37 °C.

### 3.6.3 Optimization of Process Parameters on Phytic Acid Degradation

#### 3.6.3.1. Effect of Nitrogen Sources

The effect of organic and inorganic nitrogen sources on phytic acid reduction was evaluated by replacing 0.5% ammonium sulfate in the basal mineral solution with sodium nitrate, yeast extract and urea at the same concentration. The other fermentation parameters used were as described in Section 3.6.2. Five days of SSF of the selected isolate was conducted in triplicate for each

nitrogen source and analyzed with phytic acid assay, which thus determined the optimum nitrogen source used in the subsequent fermentation process.

#### 3.6.3.2. Screening of the Significant Parameters by Using Two-Level Full Factorial Design

The experimental designs were modeled and statistically analyzed using Design-Expert® 7.0 (Stat-Ease Inc. Minneapolis, United States), including multiple regression analysis and analysis of variance (ANOVA). Each run of fermentation was carried out for three days and phytic acid assay was used to analyze the fermented content of Day 0 and Day 3, respectively. The total phytic acid reduction was served as the dependent response variable in the experimental designs.

Screening of the significant factors that influenced the phytic acid degradation was conducted using two-level factorial design. Table 3.4 lists the factors that have been included in this screening and their respective levels. Each independent variable was investigated at two levels, and the $2^4$ experimental design resulted in a total of 48 randomized runs with three replicates.

**Table 3.4:** Independent variables studied in two-level full factorial design.

| | **Factors** | **Unit** | **Level** | |
|---|---|---|---|---|
| | | | **-1** | **+1** |
| *A* | Inoculum | % | 5 | 20 |
| *B* | Nitrogen concentration | % | 0.2 | 1.5 |
| *C* | Substrate | g | 5 | 15 |
| *D* | Initial moisture content | mL $g_{sp}^{-1}$ | 2 | 4 |

### 3.6.3.3. Respond Surface Optimization with Three-Level Factorial Design

Two significant factors that were determined from the two-level full factorial design were further optimized using three-level factorial design as the respond surface optimization, as listed in Table 3.5. Each factor was investigated at three levels, and 27 randomized runs were employed given by the $3^2$ experimental design with triplicates.

**Table 3.5:** Independent variables studied in $3^2$ factorial design.

| | **Factor** | **Unit** | **Level** | | |
|---|---|---|---|---|---|
| $X_1$ | Inoculum | % | 5 | 12 | 25 |
| $X_2$ | Initial moisture content | mL $g_{sp}^{-1}$ | 2.5 | 3.6 | 5 |

The response data were modeled by multiple regression analysis and fitted to the following first-order equation:

$$\mathcal{Y} = \beta_0 + \beta_1 \mathcal{X}_1 + \beta_2 \mathcal{X}_2 \ldots \text{Equation 3.1}$$

In which $\mathcal{Y}$ is the response function; $\mathcal{X}_1$ and $\mathcal{X}_2$ are the independent coded variables; $\beta_0$ is the intercept; $\beta_1$, and $\beta_2$ are the regression coefficients.

### 3.6.3.4. Confirmatory of the Model

The fitted model was used to determine the relative sensitivity of the response to the variables. Five confirmatory experiments were conducted to validate the generated model and each experiment was repeated at least three times.

## 3.7 Phytic Acid Extraction

The phytic acid extraction was carried out with slight modification and altered based on the protocols of Saad et al. (2011) and Ahmad et al. (2013). Approximately 10 g of the fermented content was oven-dried at 70 °C until it

reached constant weight. The dried fermented soy pulp (1 g) was crushed and extracted using 10 mL of 5% sulfuric acid (pH 0.6) at 200 rpm and 37 °C for 30 minutes. Next, the mixture was centrifuged at 4 °C and 9,000 rpm for 40 minutes. The supernatant was collected and used for phytic acid assay.

## 3.8 Sample Analysis

### 3.8.1 Phytic Acid Assay

The method of Haug and Lantzsch (1983) as delineated by Mahmood et al. (2010) and Ahmad et al. (2013) with some modification was used to quantify the amount of phytic acid content in the fermented soy pulp. For the phytic acid extract, 0.5 mL was added with 1 mL of ferric solution and heated at 105 °C for 30 minutes. The reaction mixture was allowed to cool at room temperature and 2 mL of 1% 2,2-bipyridine solution was added. The light pinkish color that resulted was measured within 4 minutes at 510 nm with GENESYS 20 spectrophotometer (Thermo Scientific, United States). Standard curve of phytic acid was calibrated using phytic acid sodium salt (Sigma-Aldrich, United States). The percentage of phytic acid was calculated based on the following equation,

$$\text{Phytic acid (\%)} = \frac{\mathcal{Y} \times \mathcal{V}}{\mathcal{W}} \times 100\% \quad \text{…Equation 3.2}$$

Where, $\mathcal{Y}$ = concentration of phytic acid in sample (g/mL); $\mathcal{V}$ = extraction volume (mL); $\mathcal{W}$ = weight of sample (g)

### 3.8.2 Morphology and Chemical Composition Analysis of Soy Pulp

#### 3.8.2.1. Scanning Electron Microscopy

The surface morphological images of unfermented soy pulp (USP) and fermented soy pulp (FSP) were observed by SEM. A small portion of wet substrates at Day 0 and Day 3 was retrieved during the extraction procedures (Section 3.7) and dried at 70 °C in a convection oven until constant weights were achieved. They were used as the samples for USP and FSP imaging, respectively. The dried samples were stuck on a disc with scotch tape and coated with a thin layer of platinum in a JFC-1600 auto fine coater (JEOL, United States). The samples were then degassed in a chamber and imaged in a JSM-6701F FE-SEM (JEOL, United States) under 4.0 kV with working distance of 11.8 mm.

#### 3.8.2.2. Fourier-transform Infrared Spectroscopy

The chemical compositions of soy pulp were recorded on a Spectrum RX 1 Fourier-transform infrared (FTIR) spectrometer (PerkinElmer, United States) in the spectral range of 400 – 4000 $cm^{-1}$. The dried samples of USP and FSP were ground with potassium bromide (KBr) powder and pressed into pellets. A pellet of phytic acid sodium salt (Sigma-Aldrich, United States) was also prepared as the reference for data analysis.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Isolation and Screening of Phytic Acid Degrading Bacteria

### 4.1.1 Bacteria Isolation and Screening in Solid Agar

Rice bran extract (RBE) and soy pulp extract (SPE) agars were used to isolate phytic acid degrading bacteria from the soil sample. The formation of clear zones (Figures 4.1 A and C) around the bacteria colonies on the solid media after 24 hours of incubation were considered as potential phytic acid degrading isolates. After the counterstaining treatment, the remaining of clear zones on the agars (Figures 4.1 B and D) indicated the existence of phytic acid degrading activities. Therefore, a total of 10 phytic acid degrading isolates were successfully obtained, which were designated as Isolate PF1, PF2, PF5, SP2, SP3, SP4, SP6, SP7, SP12, and SP15, where PF indicated the isolation from poultry farm soils and SP signified the isolation from rhizosphere soils.

Based on the morphology and biochemical characteristics listed in Table 4.1, Isolate PF1 was presumptively identified as *Corynebacterium* microbe, while the other isolates were thought to belong to *Bacillus* group. The *Bacillus* sp. was found to have single cellular or paired arrangements with rounded square ends. Furthermore, Isolate PF1 was non-mobile and exhibited non-sporulating rods. Figure 4.2 illustrates Gram staining of the isolate and reveals the presence of ellipsoidal spores at the central or terminal regions that do not distend the sporangium.

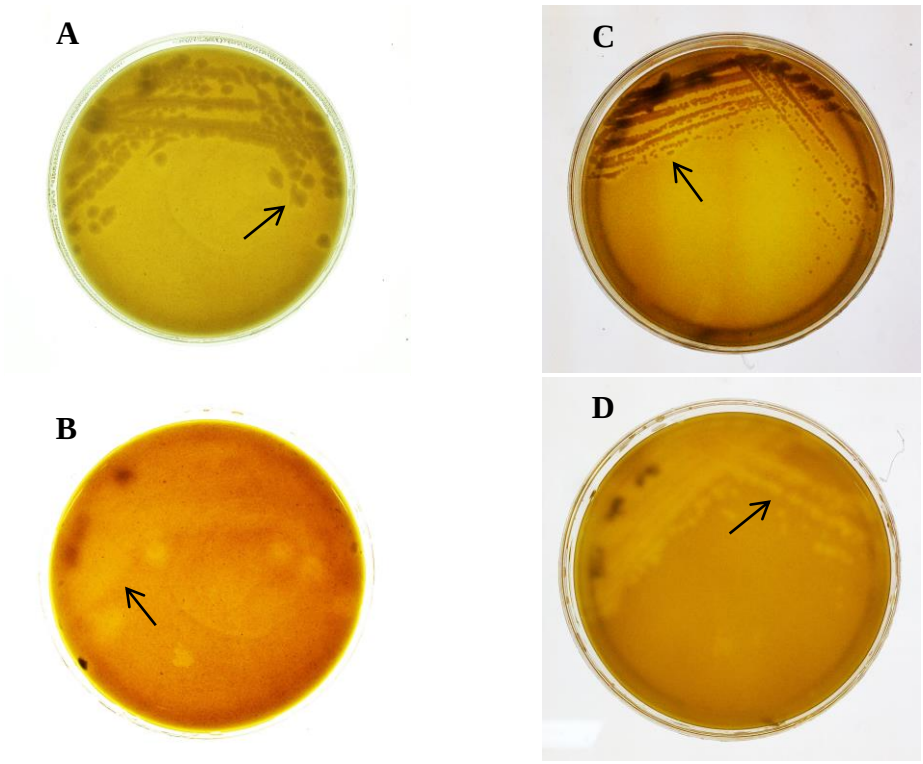


**Figure 4.1:** Screening of phytic acid degrading bacteria. A = RBE agar, before counterstained; B = RBE agar, after counterstained; C = SPE agar, before counterstained; D = SPE agar, after counterstained.

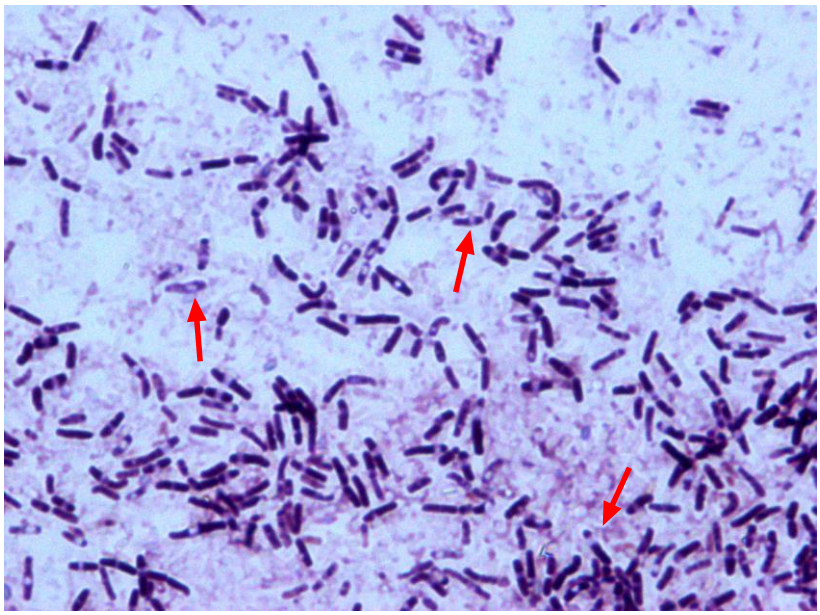

**Figure 4.2:** Isolate SP4: gram-positive bacilli of with spores (red arrow). Magnification:400×

**Table 4.1:** Morphological and biochemical characteristics of phytic acid degrading isolates.

| Isolate | Source | Colony Morphology | Gram Staining | Cellular Morphology | Spore Formation | Motility | Catalase | Indole Production | Hydrogen Sulfide Production |
|---|---|---|---|---|---|---|---|---|---|
| PF1 | Poultry farm | Pinpoint; smooth | + | Thin rod-shaped; singular | - | - | + | - | - |
| PF2 | Poultry farm | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |
| PF5 | Poultry farm | Small; rough | + | Thick and long rod-shaped; singular / pair | + | + | + | - | - |
| SP2 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular / pair | + | + | + | - | - |
| SP3 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular / pair | + | + | + | - | - |
| SP4 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |
| SP6 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |
| SP7 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |
| SP12 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |
| SP15 | Rhizosphere | Moderate; rough | + | Thick and long rod-shaped; singular | + | + | + | - | - |

Rhizosphere and poultry farm soils were used in this study and both possessed noticeable phytic acid degrading bacteria. According to the study reported by Mittal and co-researchers (2011), soil samples from cattle shed and poultry farm were found to be the most preferred source for the isolation of phytic acid degrading bacteria when compared with compost and garden soils. This can be justified by the important roles of soil microorganism in regulating the high total phosphorus content in animal wastes, whereby phytic acid appeared to be one of the major components of phosphate monoesters species. The relationship of phosphorus dynamics and occurrence of phytic acid degrading bacteria was highlighted in the decomposition of dairy cattle dung by the study of Fuentes et al. (2009). Similar characteristics were also acquired by rhizobacteria as plants may poorly possess an innate ability to directly uptake phosphorus from soil phytate. In fact, several studies have reported the capability of phytate utilizing bacteria from rhizosphere soils and their potential to serve as plant growth promoting rhizobacteria in conjunction with other plant growth promoting activities (Jorquera et al., 2008; Singh et al., 2014).

Among the 10 isolates obtained from soil samples, 90% of them were preliminarily identified as *Bacillus* microbes. In fact, bacteria from the *Bacillus* genus are widely used as bio-insecticides in agricultural practices and act as biological control agents to prevent excessive usage of pesticides (Huguette and Donald, 2012; Priyadarshini et al., 2013). This has justified the predominant occurrence of *Bacillus* microbes among soil microbial communities, as well as the rhizosphere and poultry farm soil samples

collected in this study. For instance, in the study conducted by Mariam-Aisha and co-researchers (2017), five out of six bacteria isolated from cow dung and termite gut were identified as *Bacillus* sp., including *B. pumilus*, *B. thuringiensis*, and *B. vireti*.

Both RBE and SPE agars appeared more intense in color after the counterstaining treatment (Figure 4.1 B and D). The counterstaining technique has been used in previous studies in order to eliminate false positive results caused by microbial acid production, which shift the of pH towards acidic, thus forming clear peripheral zones on the solid media (Bae et al., 1999; Mittal et al., 2011; Sumengen et al., 2012). In general, synthetic semi-opaque phytase screening medium (PSM) that are involved in the addition of commercially available phytic acid (e.g. sodium phytate) are often used in the isolation of phytic acid degrading bacteria. Since phytic acid mainly deposits in plants, the agricultural by-products or residues such as wheat bran, oat hulls, sesame bran and peanut pods may offer an alternative in replacing commercial phytic acid used in the screening medium, depending on their accessibility. For instance, wheat bran extract (WBE) agar has been used to screen phytase producing microbial in the study of Joseph and Raj (2007), Mittal et al. (2011), and Sreedevi and Reddy (2013). However, WBE agar is not feasible in Malaysia as wheat is almost entirely an imported commodity. Instead, RBE and SPE agars were used in this study as an alternative low-cost screening medium for the isolation of phytic acid degrading bacteria, where these two materials were readily available in Malaysia.

Rice bran and soy pulp contained phytic acid concentration ranging from 5.94 – 6.09 g 100 $g^{-1}$ and 0.5 – 1.2 g 100 $g^{-1}$ in dried materials, respectively, which varied according to the local cultivation conditions (Van der Riet et al., 1989; Liu et al., 2005). Similar with WBE agar, these two agars appeared to be bright yellow-brownish in color and had higher opaqueness than the commercial PSM. Hence, the clear zones formed were more readily observed. Although soy pulp has lower phytic acid concentration, SPE agar was sufficient as the qualitative screening medium (Figure 4.1) and since rice bran has a very high commercial values, soy pulp was expected to be a more economically-friendly resource than rice bran. For instance, rice bran contains hypoallergenic proteins that are applicable in infant formulation and it reportedly has dipeptodyl peptidase IV inhibitory and anti-cancer activities (Sheflin et al., 2017; Wang et al., 2017). The optimization of phytic acid extraction yield from defatted rice bran has been demonstrated in a recent study in order to develop value-added products and in turn fully utilize rice bran (Sha et al., 2017). In addition, SPE agar has never been reported in the literatures.

### 4.1.2 Quantitative Screening of Phytic Acid Degrading Ability Through Solid-state Fermentation

Plate screening on solid media is a classical way to determine the phytic acid degrading ability of isolates. Other than acting as a qualitative method, this technique also serves as an indicator in identifying the strength of phytic acid reduction by the respective isolates. For instance, only the isolates that showed larger hydrolytic zones on PSM were chosen for further evaluation in the study of Sreedevi and Reddy (2013) and Demirkan et al. (2014). However, it was

reported that the isolate showed that the highest enzyme activity might exhibit smaller clear zones than the other isolates studied (Shamna et al., 2012). Hence, the ability of 10 isolates to reduce the phytate content in soy pulp were evaluated quantitatively through solid-state fermentation (SSF) in this study.

Phytic acid assay was used to determine the phytic acid degrading ability of 10 isolates. The phytic acid concentration of the extract was firstly obtained using the formula generated from the standard curve of phytic acid ($R^2$ = 0.99777), which is $y = -0.03x + 1.084$ (Appendix F). The maximum phytic acid reduction of soy pulp by each isolate was obtained by comparing the lowest phytic acid content of the fermented soy pulp with the phytic acid content at Day 0, using the Equation 4.1:

$$\text{Maximum phytic acid reduction (\%)} = \frac{\text{\% of phytic acid at Day 0} - \text{\% of phytic acid (lowest)}}{\text{\% of phytic acid at Day 0}} \times 100\% \quad \ldots\text{Equation 4.1}$$

Figure 4.3 shows the maximum phytic acid reduction in soy pulp by isolates throughout 10 days of SSF. The result showed that around 80% of the isolates have successfully reduced more than half of the initial phytic acid content in soy pulp, 173.46±23.5 µg/mL, which was equivalent to around 0.173±0.02 g 100 $g^{-1}$ of dried soy pulp. Isolate SP4 was more distinguished than others since it decreased 62.65% of the phytic acid content in soy pulp after 3 days of SSF. Due to its capability to degrade the phytic acid content within a short period of time, therefore it was selected for the subsequent study.

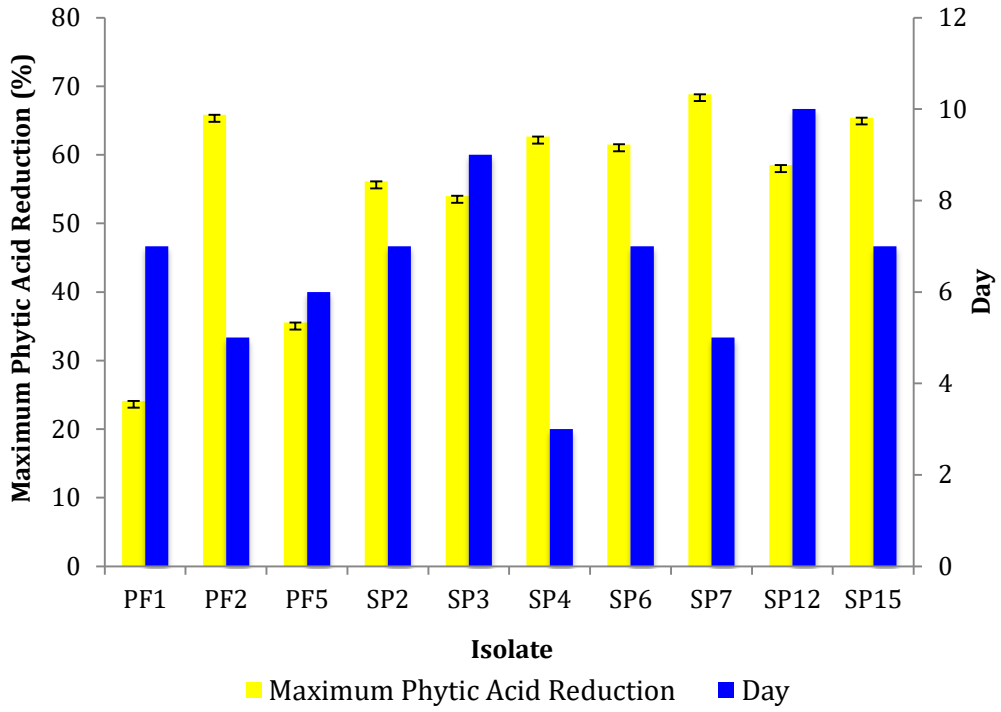


**Figure 4.3:** Maximum phytic acid reduction in soy pulp subjected to 10 days SSF with isolates. Each data point was the means of triplicate with SD.

The initial phytic acid content of soy pulp used in this study was around 0.173±0.02 g 100 $g^{-1}$ of dried soy pulp, which was lower than 0.5 – 1.2 g 100 $g^{-1}$ as reported by Van der Riet et al. (1989). The variation in concentration may differ depending on the soybean cultivars, as well as the soybean processing techniques and sequences, such as soaking, malting, and cooking (Kumar et al., 2010; Vong and Liu, 2016). For instance, it was reported that soaking had increased the phytic acid content in soybeans whereas malting had been proven to considerably reduce the amount of phytic acid in millet under optimal conditions (Egounlety and Aworh, 2003; Gupta et al., 2015). Recently, high phytic acid concentration 3.67 – 3.93±0.03 g 100 $g^{-1}$ of dried soy pulp had been reported in the study by Vong et al. (2017).

The 10 isolates degraded ranges of phytic acid content in soy pulp throughout the 10 days of SSF, and Isolate SP4 was found to be the best phytic acid degrading isolate by decreasing 62.65% of the phytic acid after 72 hours of fermentation (Figure 4.3).

Although there is no comprehensive study available on the *in situ* reduction of phytic acid in soy pulp by bacteria, fungus fermentation has been known to directly degrade the phytic acid in soy meal (SM) and be able to achieve good levels of degradation, including *Aspergillus usami*, *A. oryzae*, and *A. ficuum* (Ilyas et al., 1995; Chen et al., 2013; Chen et al., 2016). Nevertheless, fungus fermentation usually requires a long-lasting fermentation period and production of spores may not be favorable to the end-products (e.g. feedstuff). A similar concept in a recent study conducted by Mikulski and Klosowski (2017) had reported that the *Saccharomyces cerevisiae* strains were able to degrade less than 45% of phytic acid content in submerged fermentation of model media supplemented with an initial phytic acid concentration of 103.0±2.1 µg/mL. Besides, another study that involved the SSF of flaxseed oil cake (FOC) by two *Rhizopus oligosporus* strains had reportedly decreased 48% and 33% phytate content after 96 hours of fermentation, respectively (Dulinski et al., 2017). In addition, the fermentation of soy pulp with *Yarrowia lipolytica* in the study by Vong et al. (2017) had reported no reduction of phytic acid content in the substrate observed as this fungus did not exhibit phytase activity. It can be concluded that Isolate SP4 had shown higher phytic acid degradation efficiency than the reported strains of the aforementioned studies and it was selected for the subsequent study.

## 4.2 Identification of Isolate SP4

Coomassie brilliant blue (CBB) staining of the isolate is illustrated in Figure 4.4, which had revealed the production of endospores by Isolate SP4. The image of CBB-stained specimen shows dark blue structures, which may have been attributed to the presence of crystal proteins. In short, Isolate SP4 was preliminary identified as *Bacillus* sp. through morphological and biochemical analyses, with the probability of being either *B. cereus* or *B. thuringiensis*. From the API® 50 CHB test result, Isolate SP4 has 96.4% identity with *B. cereus* while VITEK® BCL analysis showed that it has 92% probability belongs to *B. thuringiensis*.

As reviewed by Jain et al. (2016), phytic acid degrading *Bacillus* sp. has been detected from a wide range of aquatic and terrestrial environments, which include *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, and *B. nealsonii*. Of the API® 50 CHB gallery and VITEK® 2 system BCL card test, Isolate SP4 was regarded as a member of *B. cereus* group. *B. cereus* group is also referred to as *B. cereus* sensu lato that comprises of eight closely related bacterial species, comprising *B. thuringiensis*, *B. anthracis*, *B. pseudomycoides*, and *B. mycoides*, while the specific *B. cereus* is known as *B. cereus* sensu stricto (Kovac et al., 2016). The identification performance of VITEK® BCL card has been evaluated in previous studies and among the members of *B. cereus* group, *B. cereus* and *B. thuringiensis* were reportedly indistinguishable thus requiring supplementary testing for precise identification (Halket et al., 2010).

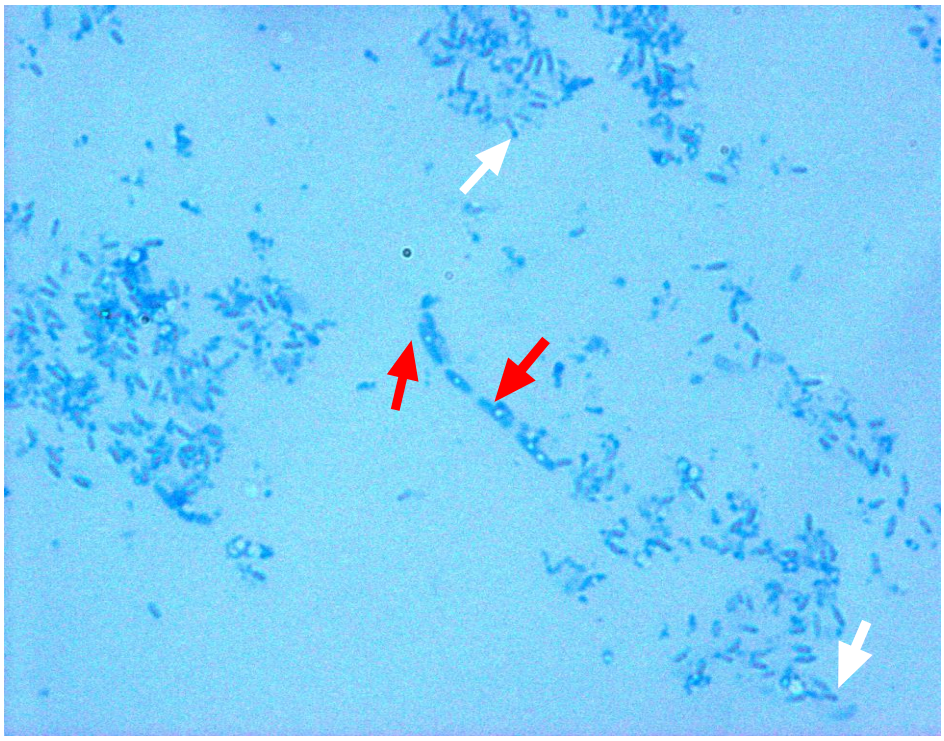

**Figure 4.4:** CBB-stained spore-crystal of Isolate SP4. Spores were unstained (red arrows) and dark blue structures were protein crystals (white arrows). Magnification: 100×.

For molecular analysis, th4 63F and 1387R primer pair were used to amplify the 16S rDNA fragment of Isolate SP4 to confirm the species level of the isolate. The amplified products were successfully obtained at the expected size of 1300 bp (Figure 4.5).

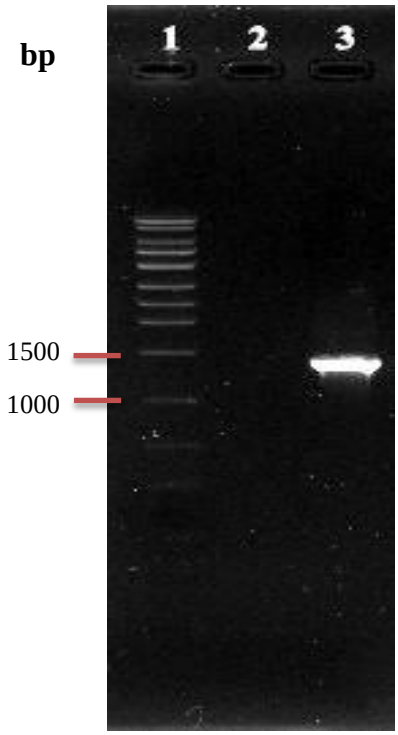


**Figure 4.5:** PCR-amplified and purified 16S rDNA fragment of Isolate SP4 by primer set 63F and 1387R. Lane: 1 = 1 kb DNA ladder; 2 = Negative control; 3 = PCR product.

The 16S rDNA consensus region of Isolate SP4 (Appendix G) was analyzed with NCBI BLAST. For the phylogenetic study, a total of 1557 bp of 16S rRNA gene were included in the Neighbor-joining phylogenetic analysis (Figure 4.6). The sequence of Isolate SP4 was compared with the nucleotide sequences *B. thuringiensis and B. cereus* extracted from the database, while sequences of *B. licheniformis*, *B. pumilus*, and *B. subtilis* were used as the remote control for the analysis. The phylogenetic tree shows that Isolate SP4 formed a cluster with six reference sequences of *B. thuringiensis* and *B. cereus*, supported by 100% bootstrap value, whereas the other three *Bacillus* sp. formed a single clade separately. As such, the attribution of Isolate SP4 to the genus *Bacillus* had been verified and the analysis had strongly validated that Isolate SP4 belonged to the *B. cereus* group. The evolutionary distances were computed using Maximum Composite Likelihood method and were in the units

of the number of base substitutions per site. There was a total of 369 positions included in the final dataset and the positions containing gaps and missing data were eliminated.

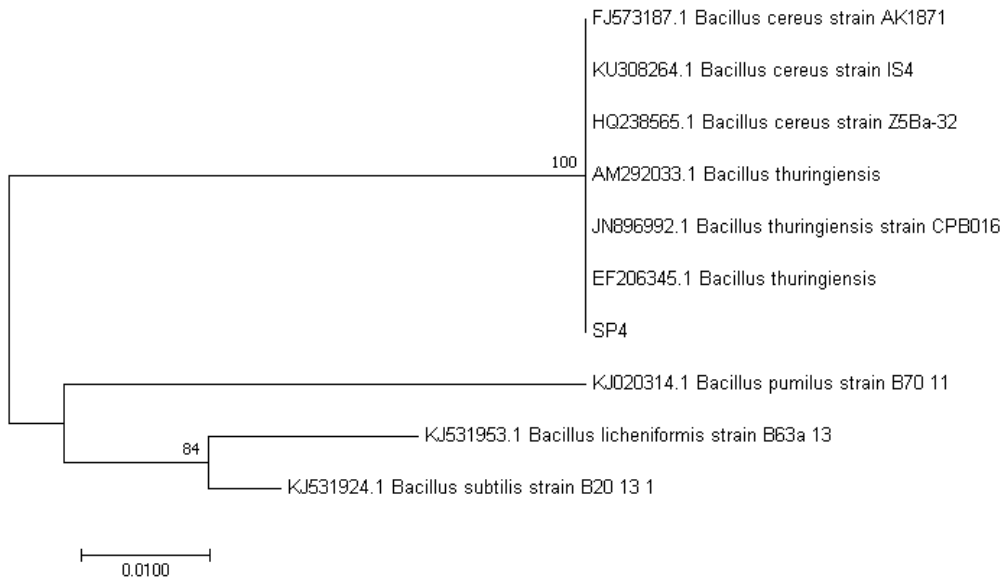


**Figure 4.6:** 16S rRNA gene unrooted neighbour-joining phylogenetic tree of Isolate SP4. The bootstrap confidence values were generated with 1,000 permutations and values lower than 50% are not shown.

Additionally, the attribution of Isolate SP4 to genus *Bacillus* was verified with the 16S rDNA sequencing. However, the highly conserved 16S rDNA with 99.7 – 100% homology had failed to distinguish the specific species of Isolate SP4 within the *B. cereus* group (Punina et al., 2013). In fact, it has been reported that even with most of the molecular typing methods, such as DNA sequencing of *rpoB*, and multiple-locus sequence typing (MLST) loci, classification of *B. cereus* group at the species level can be challenging (Cardazzo et al., 2008; Caamano-Antelo et al., 2015). The neighbor-joining phylogenetic tree (Figure 4.6) illustrates the distribution of Isolate SP4 among the *Bacillus* sp. and the topology was similar to the phylogenetic structures reported in literatures (Punina et al., 2013; Perez et al., 2017). Consistent with

the findings, whole genome sequencing of 22 *B. cereus* group diary isolates performed by Kovac and co-researchers (2016) have once again confirmed high genomic similarity among the isolates, especially of *B. thuringiensis*, *B. anthracis*, and *B. cereus*.

In order to differentiate Isolate SP4 in between *B. thuringiensis* and *B. cereus*, the presence of parasporal crystals, which is a characteristic feature of *B. thuringiensis*, was visualized using scanning electron microscopy (SEM) analysis. Through SEM images as seen in Figure 4.7, the formation of bi-pyramidal shape protein crystals by Isolate SP4 were revealed and readily recognizable as compared with the light microscopic study of CBB-stained specimen (Figure 4.4) These crystalline parasporal inclusions are commonly known as Cry proteins and are exclusively produced by *B. thuringiensis* during the stationary growth phase, along with sporulation (Lakxmy et al., 2011; Palma, 2015). Cry proteins are encoded by *cry* genes; to date, there are around 300 *cry* genes which have been identified and a combination of multiple *cry* genes in a *B. thuringiensis* strain has frequently been reported (Khojand et al., 2013; Dagga et al., 2016). Indeed, the bi-pyramidal shapes of the protein crystals produced by Isolate SP4 were often encoded by *cry1* gene (Iatsenko et al., 2014). With these, the identity of Isolate SP4 was confirmed as *B. thuringiensis* and tentatively named as *B. thuringiensis* SP4.

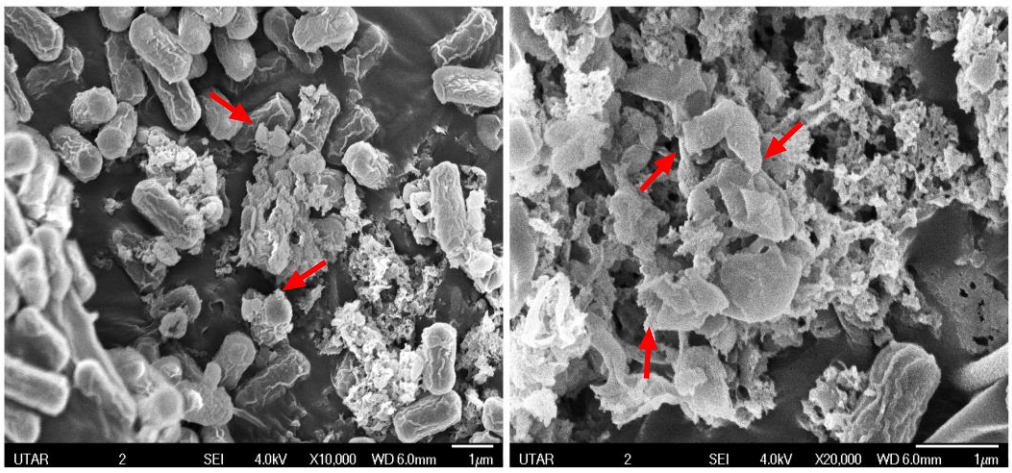


**Figure 4.7:** SEM images of bi-pyramidal crystal proteins (red arrows) produced by Isolate SP4 after 72 hours.

## 4.3 Statistical Optimization of Phytic Acid Degradation

One-factor-at-a-time (OFAT) was used to study the effect of different nitrogen sources on phytic acid degradation. As for the process parameters optimization, two-level full factorial was used to screen for the significant variables. Subsequently, the Respond Surface Methodology (RSM) using a three-level factorial was implemented to determine the optimum conditions that produced the highest phytic acid degradation by *B. thuringiensis* SP4.

### 4.3.1 Effect of Nitrogen Sources

Four different types of organic and inorganic nitrogen sources were investigated. The effect of nitrogen sources was investigated because they are considered the secondary energy sources of microorganism development. Hence, the supplementation of solid substrate with an additional nitrogen source could result in small changes of carbon/nitrogen ratio, which subsequently leads to great variations in enzyme activities and enhances microbial growth (Kumar and Kanwar, 2012; Shamna et al., 2012). The growth

of *B. thuringiensis* SP4 in this study was measured with dry cell weight (DCW). Conclusively, the results (Figure 4.8) show that there is no significant correlation between the performance and bacterial growth of *B. thuringiensis* SP4, with an $R^2$ value of 0.0608. This result was in accordance with the study of Mikulski and Klosowski (2017), who observed that *S. cerevisiae* Sano fodder strain was capable of hydrolyzing phytic acid effectively compared to other strains, with a relatively low biomass concentration.

Supplementation of ammonium sulfate in the basal medium shown the highest reduction of phytic acid content in soy pulp compared to the rest (Figure 4.9). This may be attributed to the ability of organic nitrogen sources to stimulate the production of proteases and thus influence the phytase production negatively, resulting in low total phytic acid degradation in soy pulp (Awad et al., 2013). Various organic nitrogen sources have been reported to significantly enhance the production of proteases by *Bacillus* sp., *Pseudomonas fluorescens*, *Prevotella ruminicolo*, and *Escherichia coli* (Wang and Hsu, 2005; Kalaiarasi and Sunitha, 2009; Palsaniya et al., 2012). Therefore, a simple inorganic nitrogen source may be more suitable for use in this study.

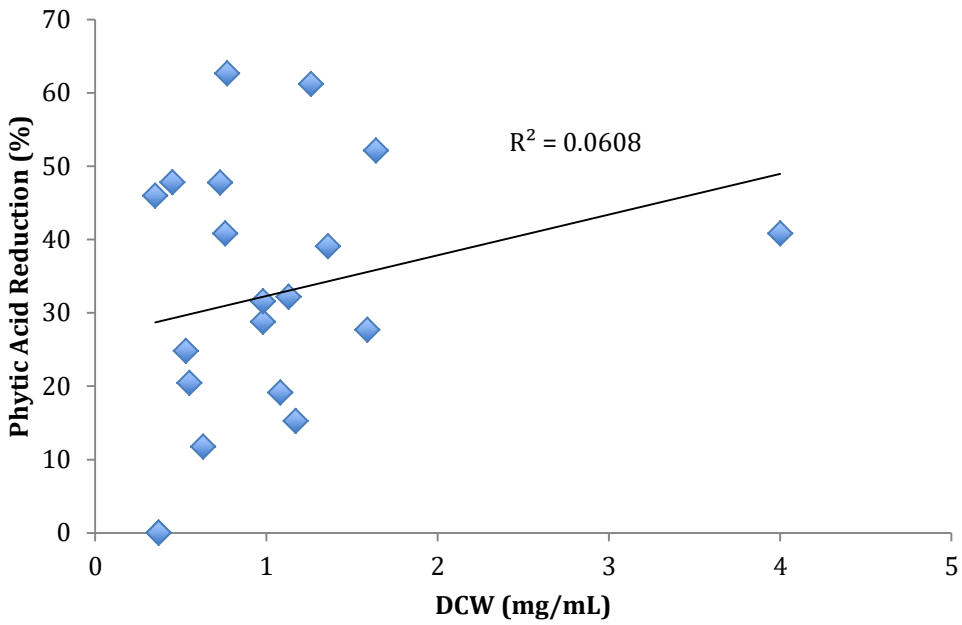


**Figure 4.8:** Correlation of phytic acid reduction with dry cell weight of *B. thuringiensis* SP4 in SSF.

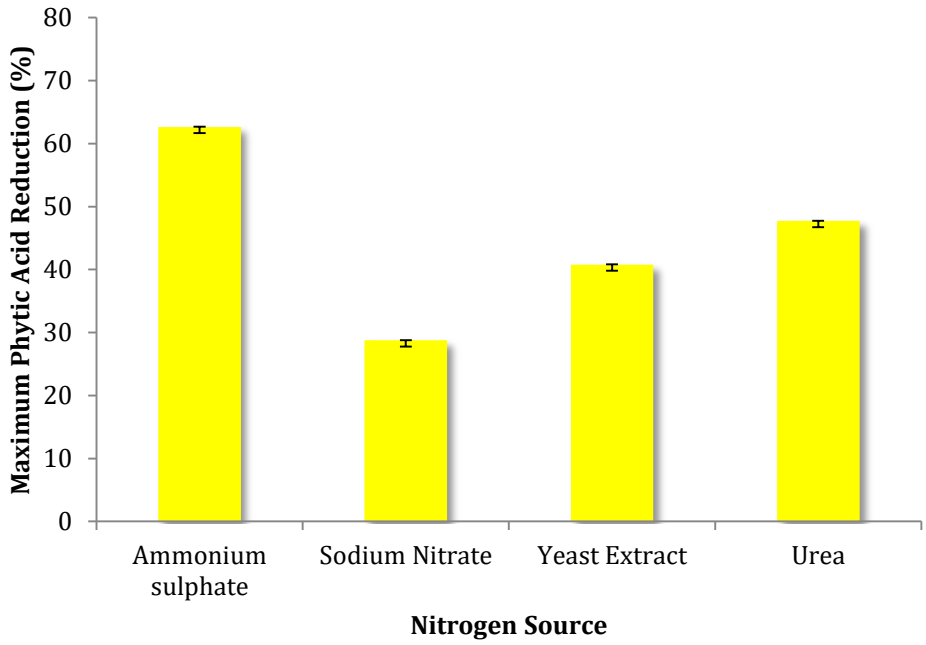


**Figure 4.9:** Effect of different nitrogen sources on phytic acid reduction by *B. thuringiensis* SP4. Each data point was the means of triplicate with SD.

### 4.3.2 Screening of the Significant Parameters on Phytic Acid Degradation

Fermentation parameters were statistically optimized in a stepwise manner. The SSF of soy pulp by *B. thuringiensis* SP4 was performed and the reduction of phytic acid served as the response variable in experimental designs.

Four factors, as seen in Table 3.4, were investigated with two-level factorial design. Table 4.2 depicts the experimental design of factors studied in coded levels, respectively, with phytic acid reduction as the response. All the model terms were evaluated with analysis of variance (ANOVA) as shown in Table 4.3. The degradation of phytic acid in soy pulp by *B. thuringiensis* SP4 was found significantly affected by Factor A and Factor D, with the *p* value of 0.0293 and < 0.0001, respectively. Model term with lower *p* value ($p < 0.0001$) indicated a critical effect in the model and the Factor D located far away from the line in the half normal probability plot (Figure 4.10) implied a stronger signal as compared with Factor A. Next, the model terms that lied along the line were negligible and no significant interaction among the factors was observed, as indicated by *p* values larger than 0.05. Therefore, only the factor of the inoculum size and initial moisture content were selected for further optimization. Table 4.4 shows the ANOVA of the model. The model *F* value of 7.24 implied the model was significant with insignificant lack of fit.

The regression model generated from the two-level factorial design has successfully described the linearity of phytic acid reduction by *B. thuringiensis* SP4 and Factor A and Factor D, with positive coefficient respectively. In other

words, increasing the inoculum and initial moisture content resulted in the increased of phytic acid reduction (Figure 4.11 and Figure 4.12).

**Table 4.2:** Two-level factorial design matrix of variables and the corresponding experimental and predicted phytic acid reduction.

| Run | Factors | | | | Phytic Acid Reduction (%) | |
|---|---|---|---|---|---|---|
| | ***A*** | **B** | **C** | **D** | **Experimental** | **Predicted** |
| 1 | +1 | -1 | -1 | -1 | 15.54 | 17.72 |
| 2 | -1 | +1 | +1 | +1 | 91.35 | 43.95 |
| 3 | -1 | +1 | -1 | -1 | 12.97 | 6.90 |
| 4 | +1 | +1 | +1 | +1 | 45.49 | 54.77 |
| 5 | -1 | +1 | +1 | +1 | 40.09 | 43.95 |
| 6 | +1 | -1 | -1 | +1 | 46.68 | 54.77 |
| 7 | -1 | +1 | +1 | -1 | 6.49 | 6.90 |
| 8 | +1 | +1 | -1 | +1 | 72.92 | 54.77 |
| 9 | +1 | +1 | -1 | -1 | 41.10 | 17.72 |
| 10 | -1 | -1 | +1 | +1 | 52.49 | 43.95 |
| 11 | -1 | -1 | -1 | -1 | 14.72 | 6.90 |
| 12 | +1 | +1 | -1 | +1 | 50.47 | 54.77 |
| 13 | -1 | -1 | -1 | +1 | 47.87 | 43.95 |
| 14 | +1 | -1 | +1 | -1 | 11.74 | 17.72 |
| 15 | +1 | -1 | +1 | +1 | 49.32 | 54.77 |
| 16 | -1 | +1 | +1 | -1 | 3.97 | 6.90 |
| 17 | +1 | -1 | +1 | +1 | 93.80 | 54.77 |
| 18 | +1 | +1 | +1 | +1 | 25.42 | 54.77 |
| 19 | +1 | -1 | +1 | -1 | 25.22 | 17.72 |
| 20 | -1 | +1 | +1 | -1 | 2.22 | 6.90 |
| 21 | +1 | -1 | -1 | +1 | 51.66 | 54.77 |
| 22 | -1 | +1 | -1 | -1 | 12.77 | 6.90 |
| 23 | -1 | +1 | -1 | +1 | 25.82 | 43.95 |
| 24 | +1 | +1 | -1 | -1 | 0 | 17.72 |
| 25 | -1 | +1 | -1 | +1 | 38.10 | 43.95 |
| 26 | +1 | -1 | +1 | +1 | 58.65 | 54.77 |
| 27 | -1 | -1 | +1 | -1 | 5.81 | 6.90 |
| 28 | -1 | +1 | -1 | -1 | 0.2 | 6.90 |
| 29 | -1 | -1 | -1 | +1 | 63.43 | 43.95 |
| 30 | -1 | -1 | +1 | +1 | 48.70 | 43.95 |
| 31 | +1 | -1 | -1 | +1 | 49.24 | 54.77 |
| 32 | +1 | -1 | -1 | -1 | 25.65 | 17.72 |
| 33 | +1 | +1 | +1 | +1 | 61.05 | 54.77 |
| 34 | -1 | -1 | +1 | -1 | 0 | 6.90 |
| 35 | -1 | -1 | -1 | -1 | 0 | 6.90 |
| 36 | -1 | +1 | -1 | +1 | 0.43 | 43.95 |
| 37 | -1 | -1 | -1 | +1 | 46.35 | 43.95 |
| 38 | +1 | +1 | +1 | -1 | 24.02 | 17.72 |
| 39 | -1 | -1 | +1 | -1 | 0 | 6.90 |
| 40 | +1 | +1 | -1 | +1 | 28.84 | 54.77 |
| 41 | -1 | -1 | -1 | -1 | 0 | 6.90 |
| 42 | +1 | +1 | +1 | -1 | 48.91 | 17.72 |
| 43 | -1 | -1 | +1 | +1 | 47.05 | 43.95 |
| 44 | +1 | +1 | +1 | -1 | 12.07 | 17.72 |
| 45 | -1 | +1 | +1 | +1 | 49.32 | 43.95 |
| 46 | +1 | +1 | -1 | -1 | 0 | 17.72 |
| 47 | +1 | -1 | +1 | -1 | 32.07 | 17.72 |
| 48 | +1 | -1 | -1 | -1 | 0 | 17.72 |

*A* = Inoculum; *B* = Nitrogen concentration; *C* = Substrate; *D* = Initial moisture content.

**Table 4.3:** Estimated model coefficient on phytic acid reduction.

| Factor[a] | Coefficient Estimate | Standard Error | df[b] | *F* value | *p* value[c] |
|---|---|---|---|---|---|
| Intercept | 30.83 | 2.39 | 1 | 26.03 | |
| *A* | 5.41 | 2.39 | 1 | 5.14 | 0.0293 |
| *B* | -1.92 | 2.39 | 1 | 0.64 | 0.4272 |
| *C* | 3.97 | 2.39 | 1 | 2.77 | 0.1048 |
| *D* | 18.52 | 2.39 | 1 | 60.24 | < 0.0001 |
| *AB* | -0.14 | 2.39 | 1 | $3.31\times10^{-3}$ | 0.9544 |
| *AC* | 0.43 | 2.39 | 1 | 0.034 | 0.8567 |
| *AD* | -1.97 | 2.39 | 1 | 0.68 | 0.4140 |
| *BC* | 1.31 | 2.39 | 1 | 0.30 | 0.5852 |
| *BD* | -3.33 | 2.39 | 1 | 1.95 | 0.1710 |
| *CD* | 1.9 | 2.39 | 1 | 0.64 | 0.4303 |

[a]*A* = Inoculum; *B* = Nitrogen concentration; *C* = Substrate; *D* = Initial moisture content.
[b]Degree of freedom.
[c]Statistically significant at 95% confidence level, with $p < 0.05$.

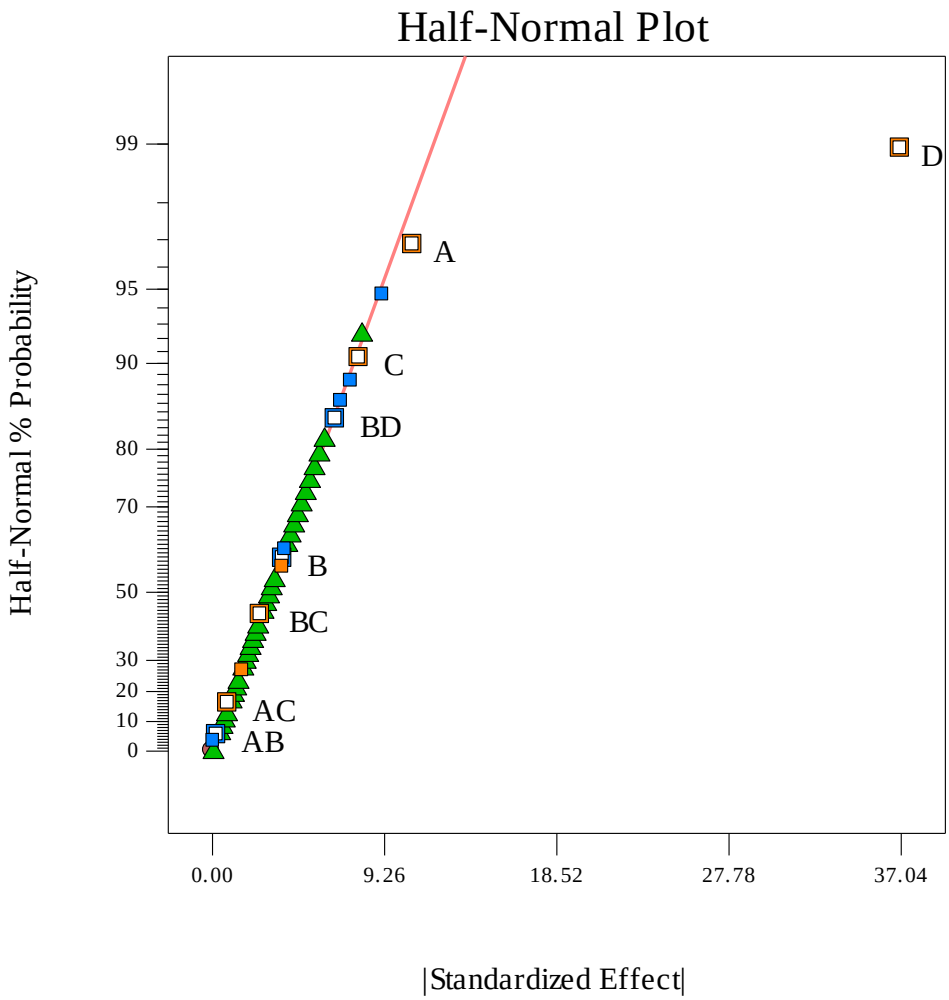


**Figure 4.10:** Half-normal probability plot of phytic acid degradation by *B. thuringiensis* SP4.

**Table 4.4:** ANOVA of phytic acid reduction model from two-level factorial design.

| Source | Sum of Squares | df[a] | Mean Square | *F* value | *p* value[b] |
|---|---|---|---|---|---|
| Model | 19790.95 | 10 | 1979.10 | 7.24 | < 0.0001 |
| Residual | 10114.26 | 37 | 273.36 | | |
| Lack of Fit | 2302.50 | 5 | 460.50 | 1.89 | 0.1244 |
| Pure Error | 7811.77 | 32 | 244.12 | | |
| Correlation Total | 29905.22 | 47 | | | |

[a]Degree of freedom.
[b]Statistically significant at 95% confidence level, with $p < 0.05$.

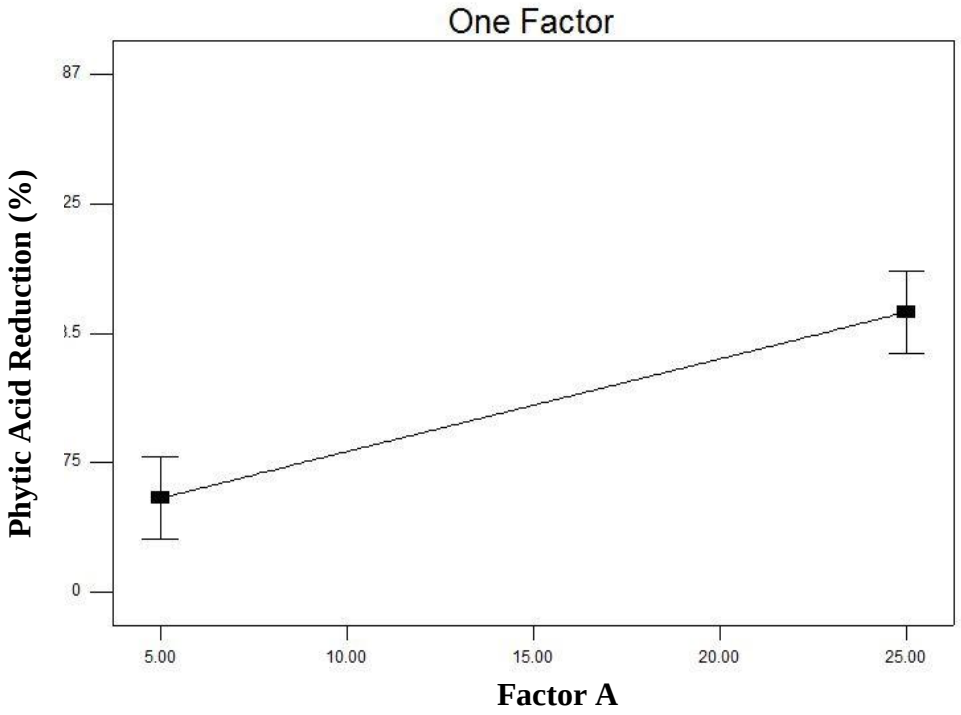


**Figure 4.11:** Main effect plot of Factor A in phytic acid degradation by *B. thuringiensis* SP4.

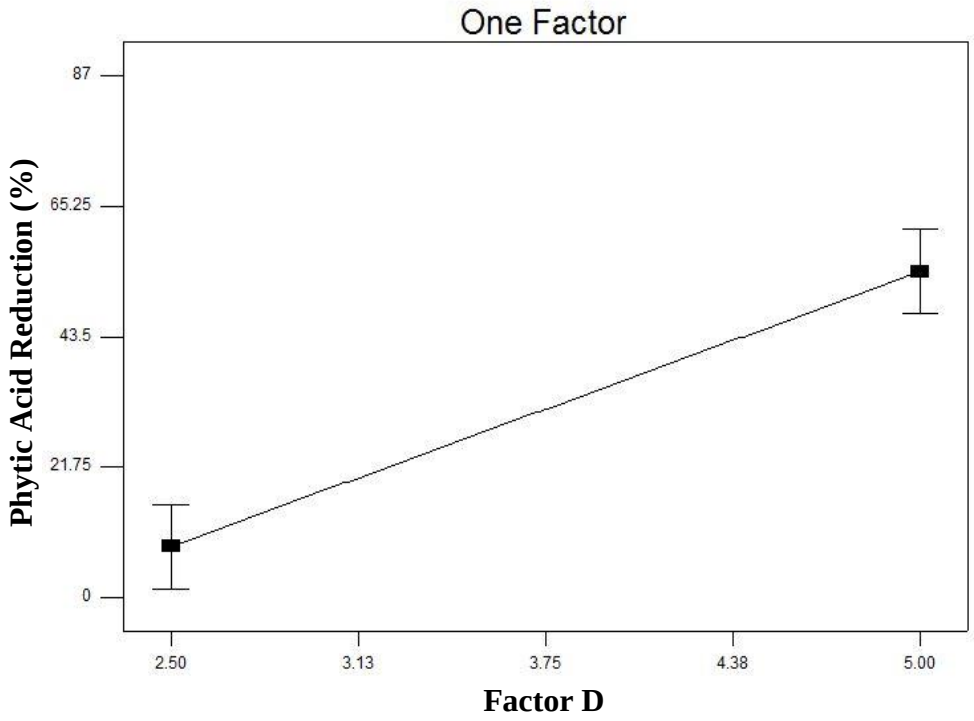


**Figure 4.12:** Main effect plot of Factor D in phytic acid degradation by *B. thuringiensis* SP4.

In comparison to filamentous fungi and yeasts, bacteria often require higher water activity, whereby low moisture within the substrate may limit the growth and metabolism of microorganisms (Bhargav et al., 2008). Although the significance of inoculum size was a notch below the initial moisture content, a minimal level of inoculum may not be adequate to initiate bacterial growth and the reduction of phytic acid was found to increase gradually with a higher level of inoculum. Notwithstanding this direct relationship, high inoculum level could cause competitive inhibition and lead to exhaustion of nutrients (Awad et al., 2011; Yasmeen et al., 2013). Thus, it is crucial to determine the true relationship of response and significant factors in order to achieve desirable outcomes.

### 4.3.3 Respond Surface Methodology

Subsequently, three-level factorial design was employed to determine the optimal conditions of the significant factors that influenced the degradation of phytic acid. Table 4.5 shows the design matrix of factors with their respective levels, with phytic acid reduction as the response. The maximum experimental phytic acid reduction by *B. thuringiensis* SP4 was achieved 86.40% at the run number 20, by using 25% of inoculum and 5 mL $g_{sp}^{-1}$ of initial moisture content. Figure 4.13 illustrates the fitting of predicted values of phytic acid reduction along with experimental data.

**Table 4.5:** Three-level factorial design matrix of variables and the corresponding experimental and predicted phytic acid reduction.

| Run | Factors | | Phytic Acid Reduction ($Y$; %) | |
|---|---|---|---|---|
| | $X_1$ | $X_2$ | Experimental | Predicted |
| 1 | 5 | 2.5 | 0 | -7.13 |
| 2 | 5 | 5 | 0 | 38.69 |
| 3 | 5 | 5 | 45.30 | 38.69 |
| 4 | 5 | 3.6 | 0 | 13.03 |
| 5 | 5 | 3.6 | 0 | 13.03 |
| 6 | 12 | 5 | 55.90 | 49.63 |
| 7 | 12 | 5 | 60.47 | 49.63 |
| 8 | 25 | 2.5 | 26.58 | 24.12 |
| 9 | 5 | 2.5 | 5.14 | -7.13 |
| 10 | 12 | 2.5 | 0 | 3.81 |
| 11 | 25 | 5 | 59.86 | 69.94 |
| 12 | 25 | 5 | 58.09 | 69.94 |
| 13 | 25 | 2.5 | 0 | 24.12 |
| 14 | 12 | 2.5 | 0 | 3.81 |
| 15 | 12 | 2.5 | 0 | 3.81 |
| 16 | 12 | 3.6 | 0 | 23.97 |
| 17 | 12 | 3.6 | 33.40 | 23.97 |
| 18 | 12 | 5 | 70.21 | 49.63 |
| 19 | 12 | 3.6 | 61.93 | 23.97 |
| 20 | 25 | 5 | 86.40 | 69.94 |
| 21 | 25 | 3.6 | 47.86 | 44.28 |
| 22 | 5 | 5 | 33.86 | 38.69 |
| 23 | 5 | 3.6 | 17.20 | 13.03 |
| 24 | 25 | 3.6 | 49.43 | 44.28 |
| 25 | 25 | 2.5 | 24.71 | 24.12 |
| 26 | 5 | 2.5 | 0 | -7.13 |
| 27 | 25 | 3.6 | 44.70 | 44.28 |

$X_1$ = Inoculum; $X_2$ = Initial moisture content.

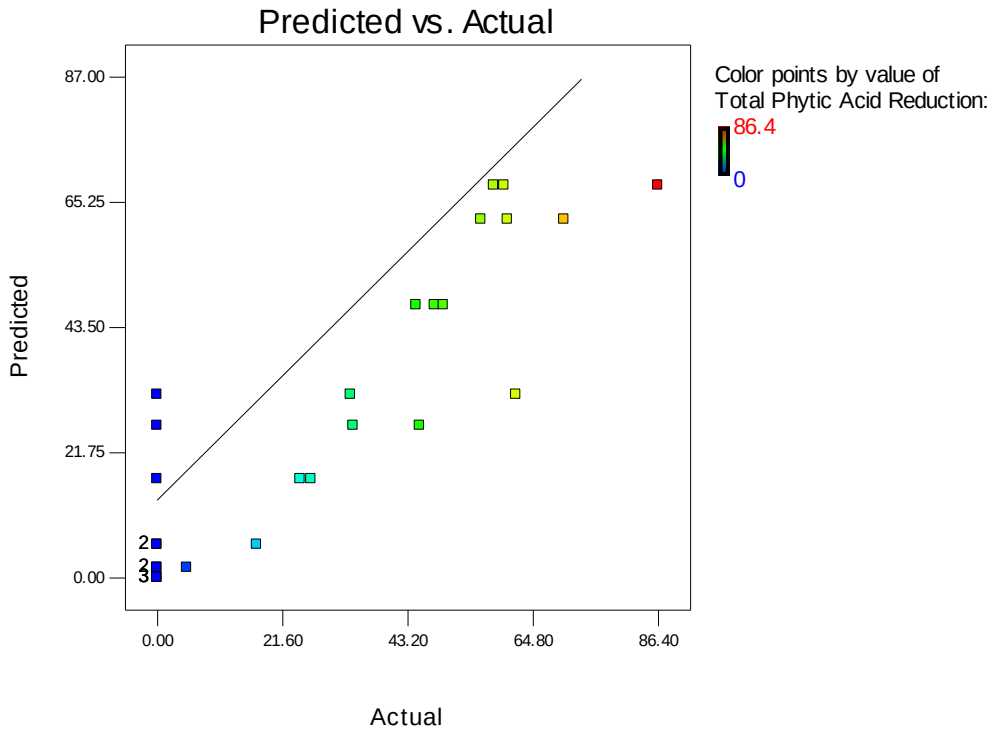


**Figure 4.13:** Plot of predicted values versus experimental data of phytic acid reduction.

The fitting of the phytic acid reduction model was analyzed by the coefficient of determination of $R^2$. The ANOVA test shown in Table 4.6 implies that the model had $R^2$ value of 0.7139 for the response, indicating that this model could explain 71.4% of data variability, showing adequate representation of the process by the model. The predicted $R^2$ value of 0.6058 was in reasonable agreement with the adjusted $R^2$ value of 0.6766. No significant interaction in between the two factors was observed, which was in accordance with the result of the two-level factorial design during the screening of factors (Table 4.3). The model *F* value of 27.84 implied the mode was significant and there was only a 0.01% chance that a model *F* value this large could occur due to noise. The regression model for phytic acid reduction was written as:

$$y = -60.75915 + 1.56236x_1 + 18.32799x_2 \quad \text{…Equation 4.2}$$

In which $\mathcal{Y}$ = phytic acid reduction (%); $\mathcal{X}_1$ = Inoculum (%); and $\mathcal{X}_2$ = Initial moisture content (mL $g_{sp}^{-1}$). Figure 4.14 shows the response surface plots of phytic acid reduction. The total phytic acid reduction varied significantly upon different inoculum sizes and initial moisture content. The optimum fermentation condition was indicated by the hump in the three dimensional contour plot (Figure 4.14).

**Table 4.6:** ANOVA of phytic acid reduction model from $3^2$ factorial design.

| Source | Sum of Squares | df[a] | Mean Square | *F* value | *p* value[b] | |
|---|---|---|---|---|---|---|
| Model | 14018.50 | 2 | 7009.25 | 27.84 | < 0.0001 | Significant |
| $X_1$ | 4525.54 | 1 | 4525.54 | 17.98 | 0.0003 | |
| $X_2$ | 9492.96 | 1 | 9492.96 | 37.71 | < 0.0001 | |
| Residual | 6042.35 | 24 | 251.76 | | | |
| Lack of Fit | 1734.44 | 6 | 289.07 | 1.21 | 0.3470 | Not significant |
| Pure Error | 4307.91 | 18 | 239.33 | | | |
| Correlation Total | 20060.85 | 26 | | | | |

[a]Degree of freedom.
[b]Statistically significant at 95% confidence level, with $p < 0.05$.

The optimized phytic acid reduction was about 1.38-fold compared to the non-optimized condition. Based on the response surface plot (Figure 4.14), reduction of phytic acid varied gradually upon changes in the inoculum size and initial moisture content, at which the hump indicated the optimum conditions of SSF. Although the maximal point of the plot was at 25% inoculum and 5 mL $g_{sp}^{-1}$ moisture content, the linear model suggested a possibility of increasing the maximal response relative to phytic acid reduction. Therefore, it can be concluded that *B. thuringiensis* SP4 could reduce more than 86.40% of phytic acid in soy pulp under aforementioned optimal conditions.

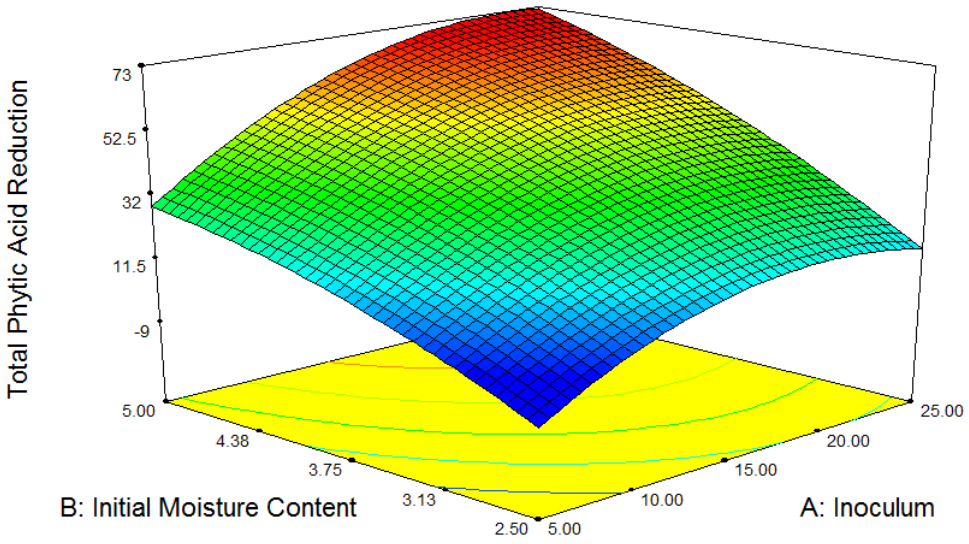


**Figure 4.14:** Response surface plot of phytic acid reduction by *B. thuringiensis* SP4 showing the interaction between inoculum sizes and initial moisture content.

### 4.3.4 Confirmatory of the Model

The linear regression model of phytic acid reduction generated from the three-level factorial design was verified with confirmatory runs. Table 4.7 shows the values of each parameter tested, and each point was conducted in triplicate. The results of experimental runs were in close agreement with the predicted values of phytic acid reduction. Less than 6% error rates between experimental and predicted results reflected that the model was adequate for predicting the phytic acid reduction in SSF by *B. thuringiensis* SP4. Figure 4.15 illustrates the model for phytic acid reduction and shows that the validation points fitted well in the linear regression model generated from the general factorial design.

From the validation runs, the maximum phytic acid reduction of 71.42±2.6% was obtained, which was slightly higher than the predicted value. In short, from

the optimization study, a significant amount of phytic acid degradation in soy pulp by *B. thuringiensis* SP4 was successfully achieved using factorial designs.

**Table 4.7:** Experimental validation of the model. Experimental values were the means of triplicate with SD.

| Point | $X_1$ | $X_2$ | Phytic Acid Reduction ($Y$; %) | |
|---|---|---|---|---|
| | | | **Experimental*** | **Predicted** |
| 1 | 15 | 3.75 | 33.76 ± 2.3 | 31.41 |
| 2 | 17.76 | 3.31 | 27.26 ± 1.1 | 27.65 |
| 3 | 23.31 | 3.72 | 48.48 ± 4.5 | 43.84 |
| 4 | 24.82 | 4.51 | 53.44 ± 0.4 | 60.68 |
| 5 | 24.49 | 4.92 | 71.42 ± 2.6 | 67.68 |

*Means of triplicate with SD.

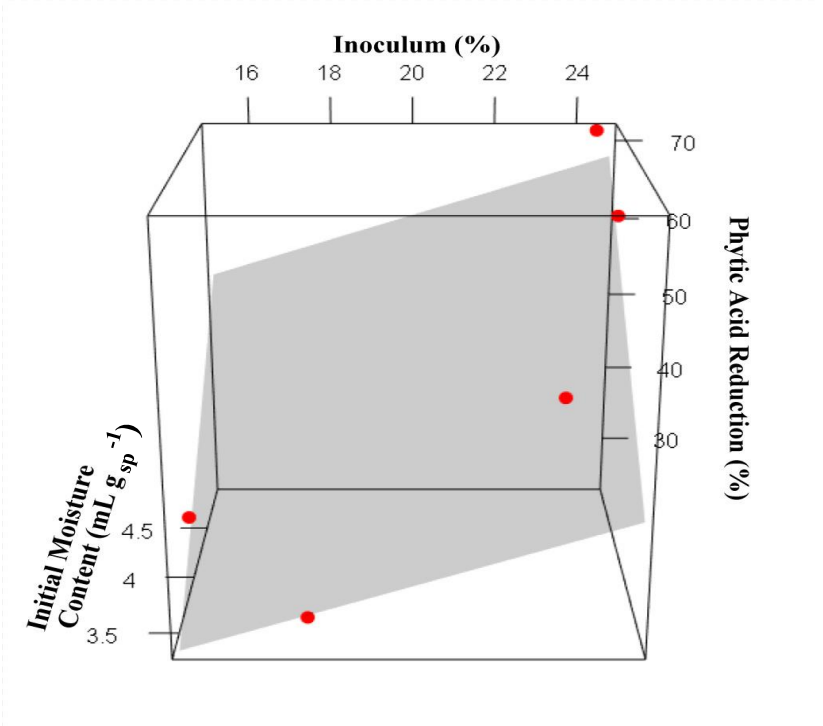


**Figure 4.15:** Linear regression model of phytic acid reduction in soy pulp by *B. thuringiensis* SP4 in function of inoculum and initial moisture content. Red dots represented the distribution of validation points on the model.

## 4.4 Morphology and Chemical Composition of Soy Pulp

To compare morphology and composition of dried unfermented soy pulp (USP) and fermented soy pup (FSP), they were analyzed with SEM and fourier-transform infrared spectroscopy (FTIR), respectively.

### 4.4.1 Scanning Electron Microscopy

A great variation of the surface microstructure between USP and FSP was obviously shown by the SEM images. The USP exhibited firm, rigid and highly ordered internal structures at Day 0 (Figure 4.16 A and B). After fermentation, those microstructures appeared collapsed and the destruction may be attributed to the efficiency of *B. thuringiensis* SP4 in hydrolyzing the soy pulp biomass in SSF (Figure 4.16 C and D).

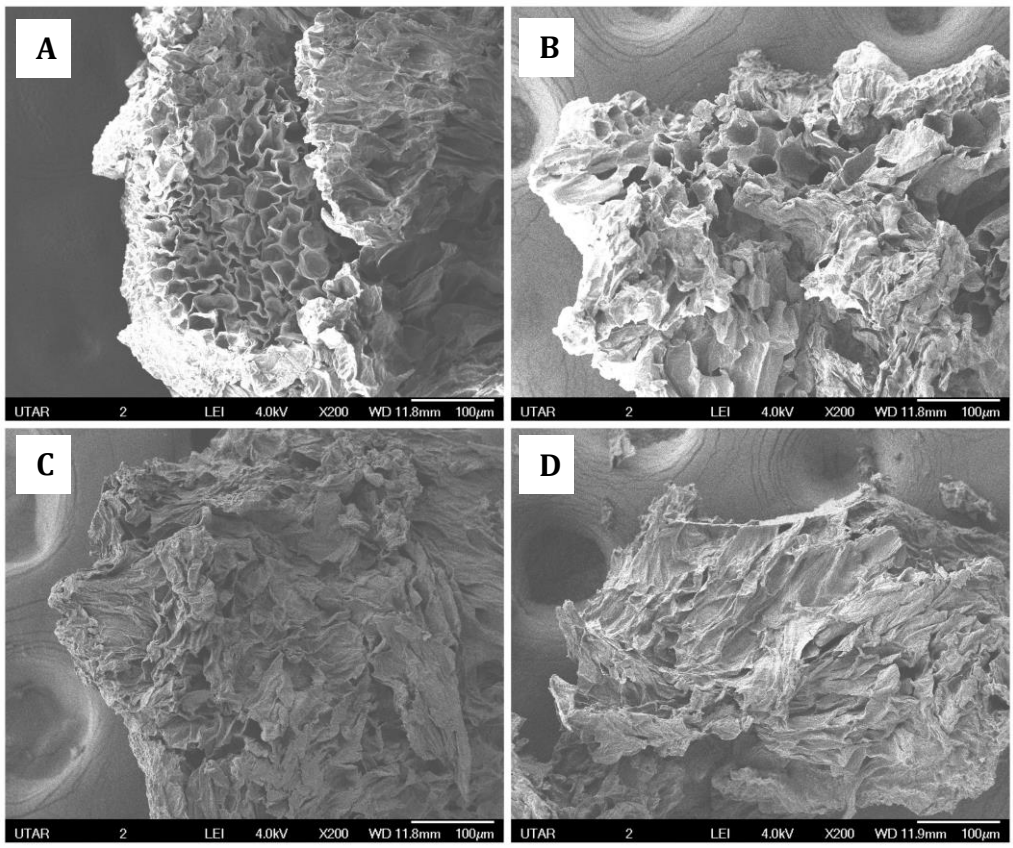


**Figure 4.16:** The morphology of dried soy pulp under SEM. USP exhibited rigid internal structure (A and B) while those structure appeared collapsed (C and D) after fermented by *B. thuringiensis* SP4.

The distinct morphological changes may be attributed to the changes or losses of soy pulp constituents brought about by the fermentation process (Li et al., 2016). According to Vong and Liu (2016), dried soy pulp contains 40 – 60% of insoluble fibers mainly in the form of cellulose and hemicellulose, and the lack of fermentable carbohydrates. However, *B. thuringiensis* SP4 showed efficient growth on this biomass and the destruction of the structures in FSP may be attributed to the ability of *B. thuringiensis* SP4 to utilize the carbohydrates of hemicellulose, cellulose, and lignin due to the presence of cellulase and xylanase. In fact, recent studies have been focused on cellulase and xylanase production from several bacteria genera (e.g. *Bacillus*, *Streptomyces Microbacteria*) for commercial enzyme production and studies

have reported that *Bacillus* sp. recovered from natural resources appeared to be hemicellulase producers (Maki et al., 2011; Bajaj et al., 2012; Adhyaru et al., 2014). For instance, in the study by Nkohla et al. (2017), Isolate PO2 *B. cereus* reportedly exhibited both cellulase and xylanase activities out of the 52 bacterial isolates recovered from decaying wood shavings. Besides, *Bacillus* sp. is also capable of adhering to substrate particles and producing filamentous cells for efficient penetration and degradation (Alshelmani et al., 2014). Indeed, the ability of *Bacillus* sp. to saccharify lignocellulosic biomass in bioprocessing has been extensively reported in several plants, including wheat straw, wheat bran, rice husk, sugarcane bagasse, paddy husk and corn stover (Kawaguchi et al., 2016; Ahmed et al., 2017; Soccol et al., 2017).

### 4.4.2 Fourier-transform Infrared Spectroscopy

The reduction of phytic acid in fermented soy pulp was further supported by FTIR analysis. By analyzing the USP spectrum in Figure 4.17, phytic acid characteristic bands were found at 519 $cm^{-1}$ and 1066 $cm^{-1}$, 1652 $cm^{-1}$, 3430 $cm^{-1}$, which corresponded to $PO_4^{3-}$, $HPO_4^{2-}$, $OH^-$, respectively; while 1066 $cm^{-1}$ was attributed to the stretching vibration of P=O in phosphate ester group, and 519 $cm^{-1}$ was attributed to the deformation vibration of $PO_4$. As such, the presence of phytic acid in USP and FSP were verified qualitatively by showing the same peaks as the spectrum of the reference material – phytic acid sodium salt hydrate. Next, the aforementioned characteristic bands of phytic acid in FSP appeared weakened; especially at the peaks of $PO_4^{3-}$ which were unobvious after SSF, that may indicate the degradation of $PO_4^{3-}$ into free

phosphate ions. Besides, a new peak also formed in the spectrum of FSP at 618 $cm^{-1}$, which has not been found in the spectra of pure phytic acid and UFP.

In the recent study by Dave and Modi (2018), FTIR was used to estimate the phytic acid content during the bread making process and they claimed that FTIR was able to provide a fairly definitive identification of phytic acid compared to the chemical method, where the latter is more time-consuming and laborious. Besides, the spectra analysis by Dave and colleague (2018) on pure phytic acid, phytase-supplemented bread and non-supplemented bread were highly consistent with the spectra obtained in this study (Figure 4.17) whereby the weakened peaks suggest the significant decrease in phytic acid content. Accordingly, Gao et al. (2009) reported that the hydrogen ions were reduced simultaneously during the phytic acid conversion and this can justify the weakening of the $OH^-$ band in the FSP spectrum. Besides, the formation of a new peak at 618 $cm^{-1}$ suggested the degradation of fibers and minor changes in the molecular structures of the oligosaccharides caused by the fermentation of *B. thuringiensis* SP4 (Li et al., 2016). This result also further justifies the morphological changes of soy pulp after SSF, as shown by the SEM images in Figure 4.16.

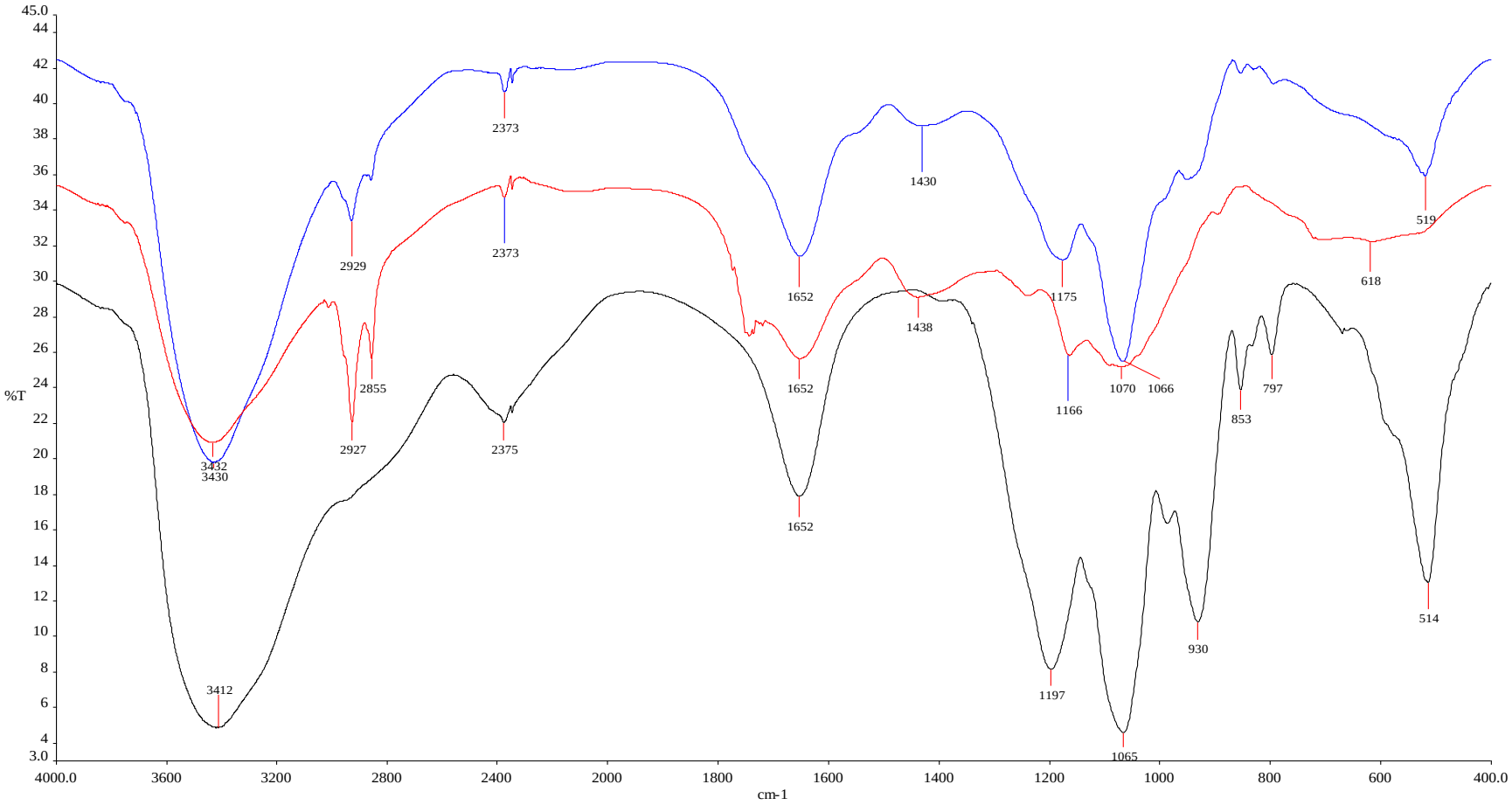


**Figure 4.17:** FTIR spectra of dried soy pulps. ( ▬ Pure phytic acid; ▬ Unfermented soy pulp; ▬ Fermented soy pulp)

## 4.5 Future Perspectives

The current study has demonstrated a great possibility of soy pulp biovalorization for commercial use. Following fermentation, the anti-nutritional factor of phytic acid was reduced considerably and a wide variety of macromolecules and essential minerals were expected to increase in their bioavailability. A recent study by Dulinkski and co-researchers (2017) had provided strong evidence of enhancing the *in vitro* bioavailability of minerals upon the efficient hydrolysis of phytic acid content in flaxseed oil cake (FOC), where the concentrations of calcium, magnesium and phosphorus were found improved by 1.81, 1.23, and 1.87-folds, respectively. Studies have also been to evaluate the potential and feasibility of fermented soy pulp via microbial fermentation. Certain favorable properties, such as production of flavor-active peptides, single-cell proteins, and porous structure of FSP had increased the specific surface area that facilitated the extraction yield of bioactive substances, as well as their potential applicability as structural materials, insulators, absorbents, so on and so forth, were embraced in the aforementioned research (Li et al., 2016; Vong and Liu, 2016).

As such, fermentation of soy pulp by *B. thuringiensis* SP4 in this study has produced a desirable biomass that can be used as a functional ingredient or feedstuff directly. Pre-fermentation processing steps include chemical, physical and enzymatic treatment that may be applied on soy pulp prior to fermentation to increase the amount of fermentable oligosaccharides thereby enhancing microbial growth. Comprehensive nutritional compositions and healthy functions of fermented soy pulp as well as its physical and biochemical

characteristics also need to be evaluated thoroughly in order to reveal its potentiality for industrial use. Besides, bacterial fermentation of soy pulp had shown to effectively degraded the oligosaccharides and made them more readily available for non-ruminants' uptake. It is also worth stressing that low-phytate soy pulp may be a promising source for feed formulation as the inclusion of phytases and inorganic phosphate additives could be reduced, which in turn offers a strategic cost reduction in the feed industry.

# CHAPTER 5

# CONCLUSIONS

The present study demonstrated that soy pulp could be used as *in situ* source of phosphohydrolases in solid-state fermentation (SSF), where this approach can produce an environmentally friendly biomass with great economical values. Bacterial isolates with phytic acid reducing ability were successfully isolated from soil and isolate *Bacillus thuringiensis* SP4 that was distinguished among others by decreasing 62.65% of phytic acid content in the soy pulp after 72 hours of fermentation. Statistically optimized of fermentation parameters using factorial designs have been demonstrated to be effective and revealed an increase of phytic acid reduction by 1.38-fold. Although the current results of soy pulp fermentation were at bench-scale, the linear regression model of phytic acid reduction suggested a good scope for scale-up SSF. Moreover, works on instrumental analysis further proved the degradation of phytic acid in fermented soy pulp.

In conclusion, the prospects of biotransformation of soy pulp into low-phytate content biomass offered a brand new commercial potential in soy pulp biovalorization. This approach may lead to the decrease of manufacturing costs, concurrently suggesting an alternative for waste management and method of overcoming pollution caused by the disposal of soy pulp. As for industrial applications, further examination and exploration are definitely essential to provide well-documented evidence that low-phytate soy pulp with

improved nutritional quality may be an outstanding biomass waiting to be exploited, which may be a particularly valuable source for animal feed production. More importantly, this study proposed a sophisticated solution for the adverse effects of phytic acid content in cereal grains and legumes through fermentation directly, especially in exploiting the lignocellulosic by-products, such as wheat bran, rice husk, rice straw, corncob, sesame oil cake and groundnut husk. It is also vital to note that this study marks the first study in the production of low-phytate soy pulp by *B. thuringiensis* through SSF.

## REFERENCES


Adhyaru D.N., Bhatt N.S. and Modi H.A., 2014. Enhanced production of cellulose-free, thermo-alkali-solvent-stable xylanase from Bacillus altitudinis DHN8, its characterization and application in sorghum straw saccharification. *Biocatalysis and Agricultural Biotechnology*, 3(2), pp. 182-190.

Ahmad, I. et al., 2013. Determination and inheritance of phytic acid as marker in diverse genetic group of bread wheat. *American Journal of Molecular Biology*, 3(3), pp. 158-164.

Ahmed, A.A.Q., Babalola, O.O. and McKay, T., 2017. Cellulase- and xylanase-producing bacterial isolates with the ability to saccharify wheat straw and their potential use in the production of pharmaceuticals and chemicals from lignocellulosic materials. *Waste and Biomass Valorization*, 9(5), pp. 765-775.

Al-Fatlawi, A.A. et al., 2014. Rice bran phytic acid induced apoptosis through regulation of Bcl-2/Bax and p53 genes in HepG2 human hepatocellular carcinoma cells. *Asian Pacific Journal of Cancer Prevention,* 15(8), pp. 3731-3736.

Alshelmani, M.I., Loh, T.C., Foo, H.L., Lau, W.H. and Sazili, A.Q., 2014. Biodegradation of palm kernel cake by cellulolytic and hemicellulolytic bacterial cultures through solid state fermentation. *The Scientific World Journal*, 2014(729852), pp.1-8.

Anderson, R.J., 1914. A contribution to the chemistry of phytin: I. composition of barium phytate and phytic acid. II. a study of the properties of phytic acid and its decomposition products. Eight paper on phytin. *Journal of Biological Chemistry,* 17, pp. 171-190.

Anderson, R.L. and Wolf, W.J., 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *Journal of Nutrition,* 125(3)**,** pp. 581-588.

Angel, R., Tamim, N.M., Applegate, T.J., Dhandu, A.S. and Ellestad, L.E., 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. *The Journal of Applied Poultry Research*, 11(4), pp. 471-480.

Ashok, A. and Kumar, D.S., 2017. Different methodologies for sustainability of optimization techniques used in submerged and solid state fermentation. *3 Biotech,* 7(5)**,** pp. 301-313.

Awad, G.E.A., Elnashar, M.M.M. and Danial, E.N., 2011. Optimization of phytase production by *Penicillium funiculosum* NRC467 under solid state fermentation by using full factorial design. *World Applied Sciences Journal,* 15 (11)**,** pp. 1635-1644.

Awad, G., Helal, M., Danial, E.N. and Esawy, M.A.T., 2013. Optimization of phytase production by *Penicillium purpurogenum* GE1 under solid state fermentation by using Box-behnken design. *Saudi Journal of Biological Sciences,* 21(1)**,** pp. 81-88.

Bae, H.D., Yanke, L.J., Cheng, K.J. and Selinger, L.B., 1999. A novel staining method for detecting phytase activity. *Journal of Microbiological Methods,* 39(1)**,** pp. 17-22.

Bajaj B.K., Khajuria Y.P. and Singh V.P., 2012. Agricultural residues as potential substrates for production of xylanase from alkali-thermotolerant bacterial isolate. *Biocatalysis and Agricultural Biotechnology,* 1(4), pp. 314-320.

Balaban, N.P. et al., 2017. Microbial phytases and phytate: exploring opportunities for sustainable phosphorus management in agriculture. *American Journal of Molecular Biology,* 7(1), pp. 11-29.

Barnes, M.E., Brown, M.L., Rosentrater, K.A. and Sewell, J.R., 2012. An initial investigation replacing fish meal with a commercial fermented soybean meal product in the diets of juvenile rainbow trout. *Open Journal of Animal Sciences,* 2(4)**,** pp. 234-243.

Bauman, A.T., Chateauneuf, G.M., Boyd, B.R., Brown, R.E. and Murthy, P.P.N., 1999. Conformational inversion processes in phytic acid: NMR spectroscopic and molecular modeling studies. *Tetrahedron Letters*, 40(24), pp. 4489-4492.

Bhargav, S., Panda, B.P., Ali, M. and Javed, S., 2008. Solid-state fermentation: an overview. *Chemical and Biochemical Engineering Quarterly,* 22(1)**,** pp. 49-70.

Blank, G.E., Pletcher, J. and Sax, M., 1971. The structure of *myo*-inositol hexaphosphate, dodecasodium salt octama-contahydrate: a single crystal X-ray analysis. *Biochemical and Biophysical Research Communications,* 44, pp. 319-325.

Bogar, B., Szakacs, G., Linden, J.C., Pandey, A. and Tengerdy, R.P., 2003. Optimization of phytase production by solid substrate fermentation. *Journal of Industrial Microbiology and Biotechnology*, 30(3), pp. 183-189.

Box, G.E.P. and Wilson, K.B., 1951. On the Experimental Attainment of Optimum Conditions. *Journal of the Royal Statistical Society. Series B (Methodological)*, 13(1), pp. 1-45.

Brigando, C., Mossoyan, J.C., Favier, F. and Benlian, D., 1995. Conformational preferences and protonation sequence of *myo*-inositol hexaphosphate in aqueous solution; potentiometric and multinuclear magnetic resonance studies. *Journal of the Chemical Society, Dalton Transactions,* 0(4)**,** pp. 575-578.

Brinch-Pedersen, H., Madsen, C.K., Holme, I.B. and Dionisio, G., 2014. Increased understanding of the cereal phytase complement for better mineral bio-availability and resource management. *Journal of Cereal Science*, 59(3), pp. 373-381.

Caamano-Antelo, S. et al, 2015. Genetic discrimination of foodborne pathogenic and spoilage *Bacillus* spp. based on three housekeeping genes. *Food Microbiology*, 46, pp. 288-298.

Cain, K. and Garling, D., 1995. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. *The Progressive Fish-Culturist,* 57(2), pp. 114-119.

Canan, C. et al., 2011. Studies on the extraction and purification of phytic acid from rice bran. *Journal of Food Composition and Analysis,* 24(7), pp. 1057-1063.

Cao, L. et al., 2007. Application of microbial phytase in fish feed. *Enzyme and Microbial Technology,* 40(4), pp. 497-507.

Cardazzo, B. et al., 2008. Multiple-locus sequence typing and analysis of toxin genes in *Bacillus cereus* food-borne isolates. *Applied and Environmental Microbiology,* 74(3), pp. 850-860.

Chen, L., Madl, R.L. and Vadlani, P.V., 2013. Nutritional enhancement of soy meal via *Aspergillus oryzae* solid-state fermentation. *Cereal Chemistry*, 90(6), pp. 529-534.

Chen, L., Vadlani, P.V., Madl, R.L. and Gibbons, W., 2016. Degradation of phytic acid and soy protein in soy meal via co-fermentation of *Aspergillus oryzae* and *Aspergillus ficuum*. *Journal of the American Oil Chemists's Society*, 93(1), pp. 45-50.

Chung, Y.H. et al., 2006. Effects of phytic acid on viability of *Vibrio vulnificus* and on septicemia-induced mice. *Journal of the Korean Society for Applied Biological Chemistry,* 49(1)**,** pp. 15-20.

Costello, A.J., Glonek, T. and Myers, T.C., 1976. 31P nuclear magnetic resonance-pH titrations of *myo*-inositol hexaphosphate. *Carbohydrate Research,* 46(2)**,** pp. 159-171.

Coulibaly, A., Kouakou, B. and Chen, J., 2011. Phytic acid in cereal grains: healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. *American Journal of Plant Nutrition and Fertilization Technology,* 1(1), pp. 1-22.

Crea, F., Stefano, C.D., Milea, D. and Sammartano, S., 2008. Formation and stability of phytate complexes in solution. *Coordination Chemistry Reviews,* 252(10-11), pp. 1108-1120.

Czitrom, V., 1999. One-factor-at-a-time versus designed experiments.*The American Statistician*, 53(2), pp. 126-131.

Dagga, A., Aziz, M.A., Amnama, A.A.A., Al-Sharif, M. and Hindi, M.E., 2016. Isolation and molecular characterization of *cry* gene for *Bacillus thuringiensis* isolated from soil of gaza strip. *International Journal of Current Microbiology and Applied Sciences,* 5(4)**,** pp. 659-666.

Dave G. and Modi H., 2018. FT-IR method for estimation of phytic acid content during bread-making process. *Journal of Food Measurement and Characterization*, 2018, pp.1-7.

De Barjac, H. and Sutherland, D.J., 2012. *Bacterial control of mosquitoes and black flies: biochemistry, genetics and applications of Bacillus thuringiensis israelensis and Bacillus sphaericus.* Netherlands: Springer.

De la Cruz, Q.R. et al., 2015. Challenges and opportunities of the bio-pesticides production by solid-state fermentation: *filamentous fungi* as a model. *Critical Reviews in Biotechnology,* 35(3)**,** pp. 326-333.

Demirkan, E., Baygin, E. and Usta, A., 2014. Screening of phytate hydrolysis *Bacillus* sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. *Turkish Journal of Biology,* 39(2)**,** pp. 206-214.

Dey, T.B. and Kuhad, R.C., 2014. Enhanced production and extraction of phenolic compounds from wheat by solid-state fermentation with *Rhizopus oryzae* RCK2012. *Biotechnology Reports*, 4, pp. 120-127.

Dulinski, R. et al., 2017. Solid-state fermentation reduces phytic acid level, improves the profile of *Myo*-inositol phosphates and enhances the availability of selected minerals in flaxseed oil cake. *Food Technology and Biotechnology,* 55(3)**,** pp. 1-17.

Egli, I., Davidsson, L., Juillerat, M.A., Barclay, D. and Hurrell, R.F., 2002. The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *Journal of Food Science,* 67(9), pp. 3483-3488.

Egounlety, M. and Aworh, O.C., 2003. Effect of soaking, dehulling, cooking and fermentation with *Rhizopus oligosporus* on the oligosaccharides, trypsin inhibitor, phytic acid and tannins of soybean (*Glycine max* Merr.), cowpea (*Vigna unguiculata* L. Walp) and groundbean (*Macrotyloma geocarpa* Harms). *Journal of Food Engineering,* 56(2-3)**,** pp. 249-254.

FAS. 2017. *Oilseeds: world markets and trade*. *United States Department of Agriculture* [Online]. Available at: https://www.fas.usda.gov/data/oilseeds-world-markets-and-trade [Accessed: 10 December 2017]

Fuentes, B., Jorquera, M. and Mora, M.D.I.L., 2009. Dynamics of phosphorus and phytate-utilizing bacteria during aerobic degradation of diary cattle dung. *Chemosphere,* 74(2)**,** pp. 325-331.

Gao, L., Zhang, C., Zhang, M., Huang, X. and Jiang, X., 2009. Phytic acid conversion coating on Mg-Li alloy. *JOurnals of Alloys and Compounds,* 485(1), pp. 789-793.

Gottumukkala, L.D., Rajasree, K., Singhania, R.R., Soccol, C.R. and Pandey, A., 2012. Solid-state fermentation: current trends and future prospects. In: El-Mansi, E.M.T. et al. (eds.). *Fermentation Microbiology and Biotechnology.* 3rd ed. London: Taylor & Francis Group, pp. 403-416.

Greiner, R. and Konietzny, U., 2006. Phytase for food application. *Food Technology and Biotechnology,* 44(2), pp. 125-140.

Gupte, M. and Kulkarni, P., 2003. A study of antifungal antibiotic production by *Thermomonospora* sp MTCC 3340 using full factorial design. *Journal of Chemical Technology and Biotechnology*, 35(1), pp. 22-26.

Gupta, R.K., Gangoliya, S.S. and Singh, N.K., 2015. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *Journal of Food Science and Technology,* 52(5)**,** pp. 676-684.

Halket, G., Dinsdale, A.E. and Logan, N.A., 2010. Evaluation of the VITEK2 BCL card for identification of *Bacillus* species and other aerobic endosporeformers. *Letters in Applied Microbiology,* 50(1)**,** pp. 120-126.

Haros, M., Rosell, C.M. and Benedito, C., 2001. Fungal phytase as a potential breadmaking additive. *European Food Research and Technology,* 213(4-5)**,** pp. 317-322.

Haug, W. and Lantzsch, H.-J., 1983. Sensitive method for the rapid determination of phytate in cereals and cereal products. *Journal of the Science of Food and Agriculture,* 34(12)**,** pp. 1423-1426.

Hongoh, Y., Yuzawa, H., Ohkuma, M. and Kudo, T., 2003. Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiology Letters*, 221(2), pp. 299-304.

Huguette, D.B. and Donald, J.S., 2012. Bacterial control of mosquitoes and black flies: biochemistry, genetics and applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Netherlands; Springer, pp. 348.

Hurrell, R.F., 2003. Influence of vegetable protein sources on trace element and mineral bioavailability. *Journal of Nutrition,* 133(9), pp. 2973-2977.

Iatsenko, I., Nikolov, A. and Sommer, R.J., 2014. Identification of distinct *Bacillus thuringiensis* 4A4 nematicidal factors using the model nematodes *Pristionchus pacificus* and *Caenorhabditis elegans*. *Toxins,* 6(7), pp. 2050-2063.

Ilyas, A. et al., 1995. A note on the removal of phytate in soybean meal using *Aspergillus usami*. *Asian-Australasian Journal of Animal Sciences,* 8(2), pp. 135-138.

Isbrandt, L.R. and Oertel, R.P., 1980. Conformational state of *myo*-inositol hexakis(phosphate) in aqueous solution. A 13C NMR, 31P NMR, and raman spectroscopic investigation. *Journal of the American Chemical Society,* 102(9)**,** pp. 3144-3148.

Jacobsen, T. and Slotfeldt, D.E., 1983. Phytic acid and metal availability: a study of Ca and Cu binding. *Cereal Chemistry,* 60, pp. 392-395.

Jain, J., Sapna and Singh, B., 2016. Characteristics and biotechnological applications of bacterial phytases. *Process Biochemistry,* 51(2)**,** pp. 159-169.

Jisha, V.N. and Benjamin, S., 2014. Solid-state fermentation for the concomitant production of δ-endotoxin and endospore from *Bacillus thuringiensis* subsp. *kurstaki*. *Advances in Bioscience and Biotechnology,* 5(10)**,** pp. 797-804.

Johnson, L. and Tate, M., 1969. The structure of *myo*-inositol pentaphosphates. *Annals of the New York Academy of Sciences,* 165, pp. 526-535.

Jorquera, M., Martinez, O., Maruyama, F., Marschner, P. and de la Luz Mora, M., 2008. Current and future biotechnological applications of bacterial phytases and phytase-producing bacteria. *Microbes and Environments,* 23(3)**,** pp. 182-191.

Joseph, I. and Raj, R.P., 2007. Isolation and Characterization of Phytase Producing *Bacillus* Strains from Mangrove Ecosystem. *Journal of the Marine Biological Association of India,* 49(2)**,** pp. 177-182.

Kalaiarasi, K. and Sunitha, P.U., 2009. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. *African Journal of Biotechnology,* 8(24)**,** pp. 7035-7041.

Kawaguchi, H., Hasunuma, T., Ogino, C. and Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. *Current Opinion in Biotechnology,* 2016(42), pp. 30-39.

Kennedy, M. and Krouse, D., 1999. Strategies for improving fermentation medium performance: a review. *Journal of Industrial Microbiology and Biotechnology,* 23(6)**,** pp. 456-475.

Kerovuo, J., 2000. *A novel phytase from Bacillus.: characterization and production of the enzyme.* PhD thesis, University of Helsinki, Kantvik, Finland.

Khan, A.W., Zohora, U.S., Rahman, M.S., Okanami, M. and Ano, T., 2012. Production of iturin A through glass column reactor (GCR) from soybean curd residue (okara) by *Bacillus subtilis* RB14-CS under solid state fermentation. *Advances in Bioscience and Biotechnology,* 3(2)**,** pp. 143-148.

Khare, S.K., Jha, K. and Gandhi, A.P., 1995. Citric acid production from Okara (soy-residue) by solid-state fermentation. *Bioresource Technology,* 54(3)**,** pp. 323-325.

Khojand, S., Keshavarzi, M., Zargari, K., Abdolahi, H. and Rouzbeh, F., 2013. Presence of multiple *cry* genes in *Bacillus thuringiensis* isolated from dead cotton bollworm *Heliothis armigera*. *Journal of Agricultural Science and Technology,* 15(6)**,** pp. 1285-1292.

Kim, D., Chung, S., Lee, S. and Choi, J., 2012. Relation of microbial biomass to counting units for *Pseudomonas aeruginosa*. *African Journal of Microbiology Research,* 6 (21)**,** pp. 4620-4622.

Kovac, J. et al., 2016. Production of hemolysin BL by *Bacillus cereus* group isolates of dairy origin is associated with while-genome phylogenetic clade. *BMC Genomics,* 17, pp. 581-596.

Krishania, M. et al., 2018. Design of bioreactors in solid-state fermentation. In: Pandey, A., Larroche, C. and Soccol, C.R. (eds). *Current Dvelopments in Biotechnology and Bioengineering: Current Advances in Solid-state Fermentation*. United States: Elsevier, pp. 83-96.

Kumar, V., Sinha, A.K., Makkar, H.P.S. and Becker, K., 2010. Dietary roles of phytate and phytase in human nutrition: a review. *Food Chemistry,* 120(4), pp. 945-959.

Kumar, A. and Kanwar, S.S., 2012. Lipase production in solid-state fermentation (SSF): recent developments and biotechnological applications. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology,* 6(1)**,** pp. 13-27.

Kumari, V., Kumar, V., Chauhan, R., Asif, M. and Bhalla, T.C., 2016. Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from *Aspergillus* sp. RL2Ct. *3 Biotech,* 6(2), pp. 1-10.

Lakxmy, A.P. et al., 2011. Mosquitocidal activity of a native *Bacillus thuringiensis* isolate Bt ReX02 from Gunung Jerai Forest, Malaysia against *Culex quinquefasciatus* and *Aedes albopictus*. *European Review for Medical and Pharmacological Sciences,* 15(2)**,** pp. 149-155.

Lee, J., Park, I. and Cho, J., 2014. Extracellular phytase production by *Bacillus* sp. T4 using solid state fermentation. *The Journal of Animal & Plant Sciences,* 24(4)**,** pp. 1116-1122.

Li, E. and Orduna, R.M.D., 2010. A rapid method for the determination of microbial biomass by dry weight using a moisture analyser with an infrared heating source and an analytical balance. *Letters in Applied Microbiology,* 50(3)**,** pp. 282-288.

Li, S. et al., 2013. Soybean curd residue: composition, utilization, and related limiting factors. *ISRN Industrial Engineering,* 2013(423590), pp. 1-8.

Li, S., Chen, Y., Li, K., Lei, Z. and Zhang, Z., 2016. Characterization of physiochemical properties of fermented soybean curd residue by *Morchella esculenta*. *International Biodeterioration & Biodegradation,* 109, pp. 113-118.

Liu, B.L., Rafiq, A., Tzeng, Y.M. and Rob, A., 1998. The induction and characterization of phytase and beyond. *Enzyme and Microbial Technology,* 22(5), pp. 415-424.

Liu, Z., Cheng, F. and Zhang, G., 2005. Grain phytic acid content in japoica rice as affected by cultivar and environment and its relation to protein contetn. *Food Chemistry,* 89(1)**,** pp. 49-52.

Loewus, F. 2002. Biosynthesis of phytate in food grains and seeds. In: Reddy, N.R. and Sathe, S.K. (eds). *Food Phytates*. Boca Raton, Florida: Taylor & Francis Group, pp. 53-63.

Lundstedt, T. et al., 1998. Experimental design and optimization. *Chemometrics and Intelligent Laboratory Systems,* 42(1-2)**,** pp. 3-40.

Mahmood, T. et al., 2010. Effect of environmental changes on phytic acid content of wheat (*Triticum aestivum*). *Pakistan Journal of Nutrition,* 9(5)**,** pp. 447-451.

Mahmoud, M.M.A., Kilany, O.E. and Dessouki, A.A., 2014. Effects of fish meal replacement with soybean meal and use of exogenous enzymes in diets of Nile Tilapia (*Oreochromisniloticus*) on growth, feed utilization, hispopathological changes and blood parameters. *Life Science Journal,* 11(2)**,** pp. 6-18.

Maki M.L., Broere M., Leung K.T. and Qin W., 2011. Characterization of some efficient cellulose producing bacteria isolated from paper mill sludges and organic fertilizers. *International Journal of Biochemistry and Molecular Biology*, 2(2), pp. 146-154.

Mallin, M.A., 2000. Impacts of industrial animal production on rivers and estuaries. *American Scientist,* 88(1), pp. 26-37.

Manan, M.A. and Webb, C. 2017. Design aspects of solid state fermentation as applied to microbial bioprocessing. *Journal of Applied Biotechnology & Bioengineering*, 4(1), pp. 1-25.

Mariam-Aisha, F., Foo, Y.H., Neelam, S. and Mohd Fairuzuddin Faizan, M.Y., 2017. 16S rRNA identification of lignocellulose degrading bacteria from cow dung and termite gut revealed pathogenic bacteria strains. *Journal of Engineering and Applied Sciences,* 12(7), pp. 1777-1781.

Marschner, H. 1995. *Mineral nutrition of higher plants*, 2nd ed. Great Britain: Elsevier Science Ltd.

Martin, C.J. and Evans, W.J., 1989. Phytic acid-enhanced metal ion exchange reactions: the effect on carboxypeptidase A. *Journal of Inorganic Biochemistry*, 35(4), pp. 267-288.

McFarlane, I. and O'Connor, E.A., 2014. World soybean trade: growth and sustainability. *Modern Economy,* 5(5), pp. 580-588.

Mikulski, D. and Klosowski, G., 2017. Evaluation of phytic acid utilization by *S. cerevisiae* strains used in fermentation processes and biomass production. *Journal of Basic Microbiology,* 57(1), pp. 87-91.

Mittal, A. et al., 2011. Isolation and biochemical characterization of acido-thermophilic extracellular phytase producing bacterial strain for potential application in poultry feed. *Jundishapur Journal of Microbiology,* 4(4)**,** pp. 273-282.

Mizumoto, S., Hirai, M. and Shoda, M., 2006. Production of lipopeptide antibiotic iturin A using soybean curd residue cultivated with *Bacillus subtilis* in solid-state fermentation. *Applied Microbiology and Biotechnology,* 72(5)**,** pp. 869-875.

Mondala, A., et al., 2015. Direct solid-state fermentation of soybean processing residues for the production of fungal chitosan by *Mucor rouxii*. *Journal of Materials Science and Chemical Engineering,* 3(2)**,** pp. 11-21.

Muniady, S., Rathinam, X. and Subramaniam, S., 2011. Quick isolation and characterization for the confirmation of a novel *Bacillus thuringiensis* strains from chicken manure samples. *African Journal of Microbiology Research,* 5(20)**,** pp. 3131-3137.

Nair, A.T., Makwana, A.R. and Ahammed, M.M., 2014. The use of response surface methodology for modeling and analysis of water and wastewater treatment processes: a review. *Water Science & Technology*, 69(3), pp. 464-478.

Neuberg, C.A., 1980. An analysis of the constitution of phytin. *Biochemische Zeitschrift,* 9, pp. 557-560.

Nigam, P.S., Gupta, N. and Anthwal, A., 2009. Pre-treatment of agro-industrial rsidues. In: Nigam, P.S. and Pandey, A. (eds). *Biotechnology for Agro-Industrial Residues*. *Utilization.* 1st ed. Netherlands: Springer, pp. 13-33.

Nkohla A. et al., 2017. Optimization of growth parameters for cellulose and xylanase production by *Bacillus* species isolated from decaying biomass. *Journal of Biotech Research*, 8, pp. 33-47.

Ohno, A., Ano, T. and Shoda, M., 1996. Use of soybean curd residue, okara, for the solid state substrate in the production of a lipopeptide antibiotic, iturin A, by *Bacillus subtilis* NB22. *Process Biochemistry*, 31(8), pp. 801-806.

O'Toole, D.K., 1991. Characteristics and use of okara, the soybean residue from soy milk production - a review. *Journal of Agricultural and Food Chemistry,* 47(2)**,** pp. 363-371.

Palma, L., 2015. Protocol for the fast isolation and identification of insecticidal *Bacillus thuringiensis* strains from the soil. *Bt Research,* 6(7)**,** pp. 1-3.

Palsaniya, P., Mishra, R., Beejawat, N., Sethi, S. and Gupta, B.L., 2012. Optimization of alkaline protease production from bacteria isolates from soil. *Journal of Microbiology and Biotechnology Research,* 2(6)**,** pp. 858-865.

Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P., 1999. Solid state fermentation for the production of industrial enzymes. *Current Science*, 77(1), pp. 149-162.

Pearlas, L.A. and Gibson, R.S., 2002. Use of soaking to enhance the bioavailability of iron and zinc from rice-based complementary foods used in the Philippines. *Journal of the Science of Food and Agriculture,* 82(10), pp. 1115-1121.

Perez, K.J., et al., 2017. *Bacillus* spp. isolated from Puba as a source of biosurfactants and antimicrobial lipopeptides. *Frontiers in Microbiology,* 8(61)**,** pp. 1-14.

Powar, V.K. and Jagannathan, V., 1982. Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. *Journal of Bacteriology,* 151(3)**,** pp. 1102-1108.

Priyadarshini, S., Gopinath, V., Priyadharsshini, N.M., MubarakAli, D. and Velusamy, P., 2013. Synthesis of anisotropic silver nanoparticles using novel strain, *Bacillus flexus* and its biomedical application. *Colloids and Surfaces B: Biointerfaces*, 102, pp. 232-237.

Punina, N.V., Zotov, V.S., Parkhomenko, A.L., Parkhomenko, T.U. and Topunov, A.F., 2013. Genetic diversity of *Bacillus thuringiensis* from different geo-ecological regions of Ukraine by analyzing the 16S rRNA and *gyr*B genes and by AP-PCR and saAFLP. *Acta Naturae,* 5(1)**,** pp. 1-16.

Rahman, M.M., Nakagawa, T., Abdullah, R.B., Embong, W.K.W. and Akashi, R., 2014. Feed intake and growth performance of goats supplemented with soy waste. *Pesquisa Agropecuaria Brasileira,* 49(7)**,** pp. 554-558.

Reddy, N.R., Sathe, S.K. and Salunkhe, D.K., 1982. Phytates in legumes and cereals. *Advances in Food Research,* 28, pp. 1-92.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, D.K. 1989. *Phytates in cereals and legumes*. Boca Raton, Florida: Taylor & Francis Group.

Reddy, N.R. 2002. Occurrence, distribution, content, and dietary intake of phytate. In: Reddy, N.R. and Sathe, S.K. (eds). *Food Phytates*. Boca Raton, Florida: Taylor & Francis Group, pp. 25-52.

Rodriguez, L.A. et al., 2010. Bioethanol production from grape and sugar beet pomaces by sollid-state fermentation. *International Journal of Hydrogen Energy,* 35(2010), pp. 5914-5917.

Roopesh, K., Ramachandran, S., Nampoothiri, K.M., Sazkacs, G. and Pandey, A., 2006. Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*. *Bioresource Technology,* 97(3)**,** pp. 506-511.

Saad, N., Esa, N.M., Ithnin, H. and Shafie, N.H., 2011. Optimization of optimum condition for phytic acid extraction from rice bran. *African Journal of Plant Sciece,* 5(3)**,** pp. 168-176.

Salman, H.A., Arjunan, S. and Senthilkumar, R., 2014. Cloning of phytase-phyl gene isolated from *Bacillus licheniformis* (ATCC 14580) into *E. coli* DH5A host. *International Journal of Pharma and Bio Sciences,* 5(1), pp. 1000-1004.

Sandberg, A.S., Carisson, N.G. and Svanberg, U., 1989. Effects of inositol tri-, tetra-, penta-, hexaphosphates on *in vitro* estimation of iron availability. *Journal of Food Science,* 54(1), pp. 159-186.

Santos, R.C.A. et al., 2014. Microbial lipase obtained from the fermentation of pumpkin seeds: immobilization potential of in hydrophobic matrics. *Acta Scientiarum, Technology*, 36(2), pp. 193-201.

Sasirekha, B., Bedashree, T. and Champa, K.L., 2012. Optimization and partial purification of extracellular phytase from *Pseudomonas aeruginosa* P6. *European Journal of Experimental Biology*, 2(1)**,** pp. 95-104.

Scott, J.J. and Loewus, F.A. 1986. Phytate metabolism in plants. In: Graf, E. (ed.). *Phytic acid: chemistry and application.* Minneapolis: Pilatus Press.

Selle, P.H. and Ravindran, V., 2007. Microbial phytase in poultry nutrition. *Animal Feed Science and Technology,* 135, pp. 1-14.

Sha, R. et al., 2017. Application of Plackett-burman design in screening the significant parameters in extraction of phytic acid from defatted rice bran by acetic acid. *Waste and Biomass Valorization*, pp. 1-9.

Shamna, K.S., Rajamanikandan, K.C.P., Kumar, D.J.M., Balakumaran, M.D. and Kalaichelvan, P.T., 2012. Extracellular prodcution of phytases by a native *Bacillus subtilis* strain. *Annals of Biological Research,* 3(2)**,** pp. 979-987.

Shapawi, R., Ebi, I. and Yong, A., 2013. Soybean meal as a source of protein in formulated diets for Tiger Grouper, *Epinephelus fuscoguttatus* juvenile. Part 1: Effects on growth, survival, feed utilization and body compositions. *Agricultural Sciences,* 4(7)**,** pp. 317-323.

Sheflin, A.M. et al., 2017. Dietary supplementation with rice bran or navy bean alters gut bacterial metabolism in colorectal cancer survivors. *Molecular Nutrition & Food Research,* 61(1), pp. 1-23.

Shi, C., Zhang, Y., Lu, Z. and Wang, Y., 2017. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. *Journal of Animal Science and Biotechnology,* 8(50), pp. 1-9.

Singh, P., Kumar, V. and Agrawal, S., 2014. Evaluation of phytase producing bacteria for their plant growth promoting activities. *International Journal of Microbiology,* 2014(426483), pp. 1-7.

Singh, V. et al., 2017. Strategies for fermentation medium optimization: an in-depth review. *Frontiers in Microbiology*, 7(2087), pp. 1-16.

Singhania, R.R., Patel, A.K., Soccol, C.R. and Pandey, A., 2009. Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, 44(1), pp. 13-18.

Singhania, R.R., Patel, A.K., Thomas, L. and Pandey, A., 2016. Solid-state fermentation. In: Wittmann, C. and Liao, J.C. (eds). *Industrial Biotechnology: Products and Processes*. 4th edn. Germany: Wiley-VCH, pp. 193-196.

Smith, A.C. and Hussey, M.A., 2005. *Gram stain protocols*, United States.

Soccol, C.R., et al., 2017. Recent developments and innovations in solid state fermentation. *Biotechnology Research and Innovation,* 1(1), pp. 52-71.

Soleimaninanadegani, M., Madihah, M.S. and Ang, S.K., 2014. Factors affecting cellulase production by *Aspergillus fumigatus* SK1 from solid state fermentation of oil palm empty fruit bunches using application of 2-level factorial design. *Bulletin of Environmental and Scientific Research*, 3(2-3), pp. 16-24.

Soy20/20. 2005. *Okara: overview of current utilisation* [Online]. Available at: http://www.soy2020.ca/pdfs/overviewofokarautilisation.pdf [Accessed 19 December 2017].

Sreedevi, S. and Reddy, B.N., 2013. Screening for efficient phytase producing bacterial strains from different soils. *International Journal of Biosciences,* 3(1)**,** pp. 76-85.

Starling, S. 2011. *Asia countries to dominate soy milk consumption* [Online]. Available at: https://www.foodnavigator.com/Article/2011/04/20/Asia-continues-to-dominate-soy-milk-consumption [Accessed 19 December 2017].

Subramaniyam, R. and Vimala, R., 2012. Solid state and submerged fermentation for the production of bioactive substances: a comparative study. *International Journal of Science and Nature,* 3(3)**,** pp. 480-486.

Sumengen, M., Dincer, S. and Kaya, A., 2012. Phytase production from *Lactobacillus brevis*. *Turkish Journal of Biology,* 36(5)**,** pp. 533-541.

Teng, D. et al., 2012. Bio-modification of soybean meal with *Bacillus subtilis* or *Aspergillus oryzae*. *Biocatalysis and Agricultural Biotechnology,* 1(1)**,** pp. 32-38.

Tran, T.T. 2010. *Thermostable phytase from a Bacillus sp.: Heterologous production, mutation, characterization and assay development.* PhD thesis, Lund University, Sweden.

Van der Riet, W.B., Wight, A.W., Cilliers, J.J.L. and Datel, J.M., 1989. Food chemical investigation of tofu and byproducts okara. *Food Chemistry,* 34(3), pp. 193-202.

Vong, W.C. and Liu, S., 2016. Biovalorisation of okara (soybean residue) for food and nutrition. *Trends in Food Science & Technology*, 52, pp. 139-147.

Vong, W.C., Lim, X.Y. and Liu, S., 2017. Biotransformation with cellulase, hemicellulase and *Yarrowia lipolytica* boosts health benefits of okara. *Applied Microbiology and Biotechnology*, 101(19), pp. 7129-7140.

Wang, H.T. and Hsu, J.T., 2005. Optimal protease production condition for *Prevotella ruminicola* 23 and characterization of its extracellular crude protease. *Anaerobe,* 11(3)**,** pp. 155-162.

Wang, L. and Yang, S.T. 2007. Solid state fermentation and its application. In: Yang, S.T. (ed.). *Bioprocessing for value-added products for renewable resources.* USA: Elsevier, pp. 465-489.

Wang, X., Chen, H., Fu, X., Li, S. and Wei, J., 2017. A novel antioxidant and ACE inhibitory peptide from rice bran protein: biochemical characterization and molecular docking study. *LWT – Food Science and Technology*, 75(2017), pp. 93-99.

Xu, X., et al., 2017. Effects of coated proteases on the performance, nutrient retention, gut morphology and carcass traits of broilers fed corn or sorghum based diets supplemented with soybean meal. *Animal Feed Science and Technology,* 223(2017), pp. 119-127.

Yasmeen, Q., Asgher, M., Sheikh, M.A. and Nawaz, H., 2013. Optimization of ligninolytic enzymes production through response surface methodology. *Bioresources,* 8(1)**,** pp. 944-968.

Yazid, N.A., Barrena, R., Komilis, D. and Sanchez, A., 2017. Solid-state fermentation as a novel paradigm for organic waste valorization: a review. *Substainability,* 9(2)**,** pp. 224-252.

## APPENDIX A

**Table A.1:** The formulation of medium, buffers, and solutions.

| Medium/ Buffer/ Solution | Compositions |
|---|---|
| LB agar | 10 g Tryptone<br>5 g Yeast extract<br>10 g NaCl<br>20 g Agar<br>Top up to 1000 mL with distilled water<br>Autoclave |
| LB broth | 10 g Tryptone<br>5 g Yeast extract<br>10 g NaCl<br>Top up to 1000 mL with distilled water<br>Autoclave |
| Ferric solution | 0.2 g Ammonium iron (III) sulphate .12$H_2O$<br>100 mL 2 N HCl<br>Top up to 1000 mL with distilled water |
| 1% 2,2-bipyridine solution | 10 g 2,2-bipyridine<br>10 mL Thioglycolic acid<br>Top up to 1000 mL with distilled water |
| Coomassie brilliant blue staining solution | 0.25 g Brilliant Blue G<br>400 mL Methanol<br>70 mL Acetic acid<br>Top up to 1000 mL with distilled water |
| 10 × TBE | 54 g Tris base<br>27.5 g Boric acid<br>4.65 g EDTA<br>Top up to 500 mL with deionized distilled water |

## APPENDIX B

**Table A.2:** Test Substrates on API® 50CHB strip.

| Well | Test | Well | Test |
|---|---|---|---|
| 1 | Control | 26 | Esculine |
| 2 | Glycerol | 27 | Salicine |
| 3 | Erythritol | 28 | Cellobiose |
| 4 | D-Arabinose | 29 | Maltose |
| 5 | L-Arabinose | 30 | Lactose |
| 6 | Ribose | 31 | Melibiose |
| 7 | D-Xylose | 32 | Saccharose |
| 8 | L-Xylose | 33 | Trehalose |
| 9 | Adonitol | 34 | Inuline |
| 10 | *β*-Methyl-xyloside | 35 | Melezitose |
| 11 | Galactose | 36 | D-Raffinose |
| 12 | D-Glucose | 37 | Starch |
| 13 | D-Fructose | 38 | Glycogene |
| 14 | D-Mannose | 39 | Xylitol |
| 15 | L-Sorbose | 40 | *β*-Gentiobiose |
| 16 | Rhamnose | 41 | D-Turanose |
| 17 | Dulcitol | 42 | D-Lyxose |
| 18 | Inositol | 43 | D-Tagatose |
| 19 | Mannitol | 44 | D-Fucose |
| 20 | Sorbitol | 45 | L-Fucose |
| 21 | *α*-Methyl-D-mannoside | 46 | D-Arabitol |
| 22 | *α*-Methyl-D-glucoside | 47 | L-Arabitol |
| 23 | *N*-acetyle glucosamine | 48 | Gluconate |
| 24 | Amygdaline | 49 | 2-ceto-gluconate |
| 25 | Arbutine | 50 | 5-ceto-gluconate |

## APPENDIX C

**Table A.3:** Test Substrates on VITEK® BCL card.

| Well | Test | Well | Test |
|---|---|---|---|
| 1 | *β*-Xylosidase | 34 | D-Melezitose |
| 3 | L-Lysine-Arylamidase | 36 | N-Acetyle-D-Glucosamine |
| 4 | L-Aspartate Arylamidase | 37 | Palatinose |
| 5 | Leucine- Arylamidase | 39 | L-Rhamnose |
| 7 | Phenylalanine Arylamidase | 41 | *β*-Glucosidase |
| 8 | L-Proline Arylamidase | 43 | *β*-Mannosidase |
| 9 | *β*-Galactosidase | 44 | Phosphoryl Choline |
| 10 | L-Pyrrolydonyl-Arylamidase | 45 | Pyruvate |
| 11 | *α*-Galactosidase | 46 | *α*-Glucosidase |
| 12 | Alanine Arylamidase | 47 | D-Tagatose |
| 13 | Tyrosine Arylamidase | 48 | D-Trehalose |
| 14 | *β*-N-Acetyle-Glucosaminidase | 50 | Inulin |
| 15 | Ala-Phe-Pro Arylamidase | 53 | D-Glucose |
| 18 | Cyclodextrin | 54 | D-Ribose |
| 19 | D-Galactose | 56 | Putrescine assimilation |
| 21 | Glycogen | 58 | Growth in 6.5% NaCl |
| 22 | myo-Inositol | 59 | Kanamycin Resistance |
| 24 | Methyl-A-D-Glucopyranoside acidification | 60 | Oleandomycin Resistance |
| 25 | Ellman | 61 | Esculin hydrolysis |
| 26 | Methyl-D-Xyloside | 62 | Tetrazolium Red |
| 27 | *α*-Mannosidase | 63 | Polymixin_B Resistance |
| 29 | Maltotriose | | |
| 30 | Glycine Arylamidase | | |
| 31 | D-Mannitol | | |
| 32 | D-Mannose | | |

*Product technical documentation of VITEK®2 System.

**Some may not be disclosed.

## APPENDIX D

### HiYield™ Genomic DNA Mini Kit (Bacterial Protocol)

**DNA Lysate**

1. Transfer bacterial culture ($<10^9$) to a microcentrifuge tube. Centrifuge for 1 min at 10,000 × g and discard the supernatant.
2. Add 200 µL of Lysozyme Buffer (freshly prepared; 20mg/ml lysozyme; 20mM Tris-HCl; 2mM EDTA; 1% Triton X-100; pH 8.0) to the tube and vortex or pipette to resuspend the cell pellet. Incubate at room temperature for 10 minutes, and the tube every 2-3 minutes.
3. Add 200 µL GB Buffer to the sample. Vortex for 5 seconds to mix sample.
4. Incubate at 70℃ for 10 minutes until the sample lysate is clear. During incubation, invert the tube every 3 minutes. At the same time, preheat required Elution Buffer (200 µL per sample) at 70℃.

**DNA Binding**

1. Add 200 µL of ethanol (96 – 100%) to the sample lysate and vortex immediately for 10 seconds to mix sample. If precipitate appears, break up by pipetting.
2. Place a GD Column on a 2 mL Collection Tube.
3. Apply the total mixture (including any precipitate) from previous step to the GD Column.
4. Close the cap and centrifuge at 10,000 × g for 5 minutes.
5. Discard the flow-through and return the GD column to the 2 mL Collection Tube.

**Wash**

1. Add 400 µL of W1 Buffer to the GD Column. Centrifuge at 10,000 × g for 30 seconds.
2. Discard the flow-through and return the GD Column to the 2 mL Collection Tube.

3. Add 600 µL of Wash Buffer (ethanol added) in the GD Column. Centrifuge at 10,000 × g for 30 seconds.

4. Discard the flow-through and return the GD Column to the 2 mL Collection Tube. Centrifuge at 10,000 × g for 3 minutes to dry the column matrix.

**DNA Elution**

1. Transfer dried GD column into a clear 1.5 mL microcentrifuge tube.

2. Add 100 µL of preheated Elution Buffer to the centre of the column matrix.

3. Allow to stand for 3-5 minutes until Elution Buffer is absorbed by the matrix.

4. Centrifuge at 10,000 × g for 30 seconds to elute purified DNA.

## APPENDIX E

**Xprep PCR Purification Kit**

1. Add 5 volumes of PB Buffer to 1 volume of PCR reaction and mix.
2. Place a Binding column tube in a 2 mL tube.
3. Apply the sample to the Binding column tube to bind DNA.
4. Centrifuge at 10,000 × g for 30-60 seconds to make the sample pass through the Binding column tube. Discard the flow-through.
5. Add 500 µL of WB Buffer to the Binding column tube and centrifuge at 10,000 × g for 30-60 seconds to wash. Discard the flow-through.
6. Repeat the washing procedure using 500 µL of WB Buffer.
7. Centrifuge the Binding column tube at 10,000 × g for an additional 2 minutes for drying. Placed the Binding column tube in a clean 1.5 mL microcentrifuge tube.
8. Add 30 µL of EL Buffer to the center of the Binding column filter, and let the column stand for 1 minute.
9. Centrifuge at 10,000 × g for 1 minute to elute purified PCR products.

## APPENDIX F

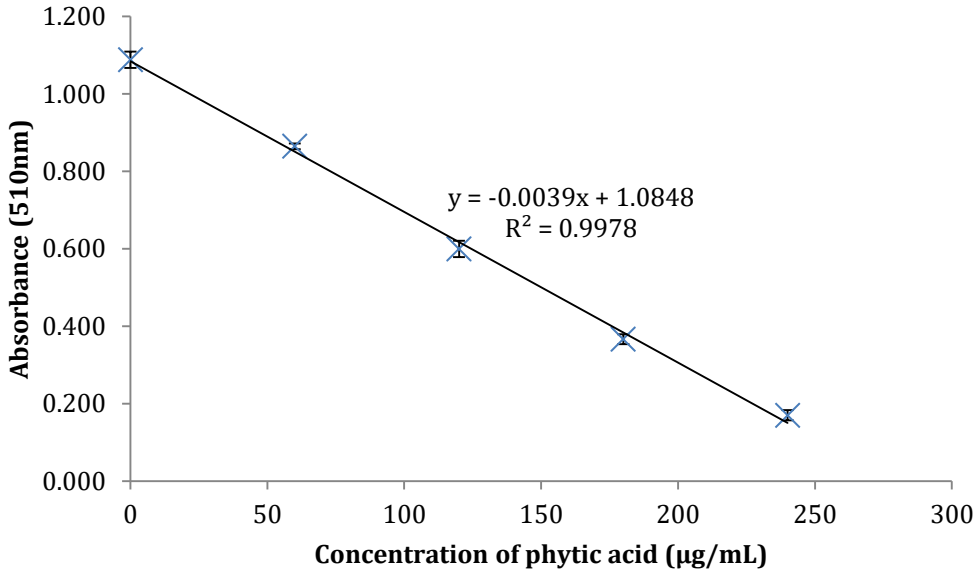


**Figure A.1:** Phytic acid standard curve.

Where the percentage of phytic acid in the sample material was calculated as follow:

$$\text{Phytic acid (\%)} = \frac{[(y\ \mu g/mL \times 10\ mL) \div 1000000]}{1\ g\ \text{of soy pulp}} \times 100\%$$

## APPENDIX G

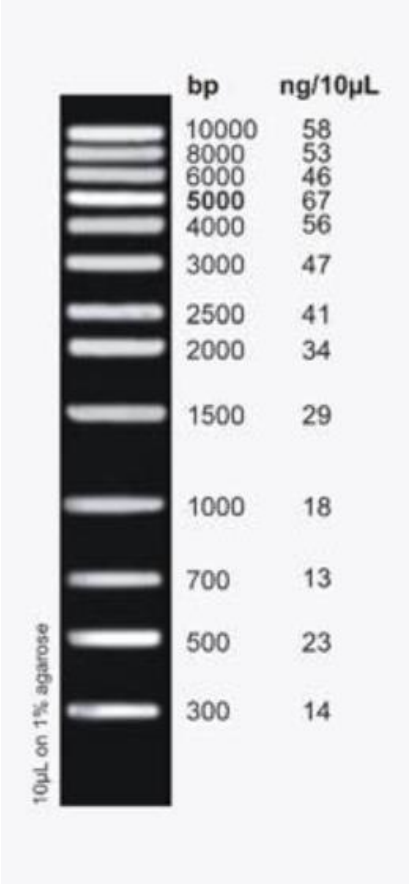


**Figure A.2:** HighRanger 1 kb DNA ladder.

## APPENDIX H

**Consensus sequence of *Bacillus thuringiensis* SP4**

Accession number: MG270578

TTGCTCTTAT GAGTTAGCGG CGGACGGGTG AGTAACACGT GGGTAACCTG
GGGTAACCTG CCCATAAGAC TGGGATAACT CCGGGAAACC GGGGCTAATA
CCGGATAACA TTTTGAACCG CATGGTTCGA AATTGAAAGG CGGCTTCGGC
TGTCACTTAT GGATGGACCC GCGTCGCATT AGCTAGTTGG TGAGGTAACG
GCTCACCAAG GCAACGATGC GTAGCCGACC TGAGAGGGTG ATCGGCCACA
CTGGGACTGA GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTAGGGAAT
CTTCCGCAAT GGACGAAAGT CTGACGGAGC AACGCCGCGT GAGTGATGAA
GGCTTTCGGG TCGTAAAACT CTGTTGTTAG GGAAGAACAA GTGCTAGTTG
AATAAGCTGG CACCTTGACG GTACCTAACC AGAAAGCCAC GGCTAACTAC
GTGCCAGCAG CCGCGGTAAT ACGTAGGTGG CAAGCGTTAT CCGGAATTAT
TGGGCGTAAA GCGCGCGCAG GTGGTTTCTT AAGTCTGATG TGAAAGCCCA
CGGCTCAACC GTGGAGGGTC ATTGGAAACT GGGAGACTTG AGTGCAGAAG
AGGAAAGTGG AATTCCATGT GTAGCGGTGA AATGCGTAGA GATATGGAGG
AACACCAGTG GCGAAGGCGA CTTTCTGGTC TGTAACTGAC ACTGAGGCGC
GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
AACGATGAGT GCTAAGTGTT AGAGGGTTTC CGCCCTTTAG TGCTGAAGTT
AACGCATTAA GCACTCCGCC TGGGGAGTAC GGCCGCAAGG CTGAAACTCA
AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTCTG ACAACCCTAG
AGATAGGGCT TCTCCTTCGG GAGCAGAGTG ACAGGTGGTG CATGGTTGTC
GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
CTTGATCTTA GTTGCCATCA TTCAGTTGGG CACTCTAAGG TGACTGCCGG
TGACAAACCG GAGGAAGGTG GGGATGACGT CAAATCATCA TGCCCCTTAT
GACCTGGGCT ACACACGTGC TACAATGGAC GGTACAAAGA GCTGCAAGAC
CGCGAGGTGG AGCTAATCTC ATAAAACCGT TCTCAGTTCG GATTGTAGGC
TGCAACTCGC CTACATGAAG CTGGAATCGC TAGTAATCGC GGATCAGCAT
GCCG