CHAPTER 2

Literature reviews

2.1 Soy and soy germ isoflavones

Soy isoflavones are naturally presented in soybean and have similar structure as human estrogen hormone. Thus, they are referred to as a phytoestrogen. In soybean, isoflavones are present as glucosides. Fermentation or digestion of soybean results in the release of sugar molecules from the isoflavone glucosides, turning them to isoflavone aglycones. Major soy isoflavone glucosides are called daidzein, genistein and glycitein, while their isoflavone aglycones are called genistein, daidzein and glycitein, respectively. Human body can absorb isoflavones in fermented soy products faster than non-fermented soy products (Setchell *et al*., 2002). Examples of fermented soy products high isoflavone aglycones include Tua nou (Thailand), Natto (Japan), Chungkookjang (Korean) Dawadawa (Nigeria) and Kinema (Napal) (Steinkraus 1995).

Setchell *et al*. (2002) reported that isoflavone glucosides, when ingested, were not absorbed by enterocytes of adults and suggested that they required hydrolysis of the sugar moiety by intestinal β-glucosidases to increase bioavailability. This suggests that consumption of isoflavone aglycone-rich soya foods might be more effective in preventing chronic diseases than consuming the glucoside-rich products. However, Richelle *et al*. (2002) discovered that the enzymatic hydrolysis of isoflavone glucosides into aglycones in a soya drink (made from soy germ) before consumption did not enhance the absorption of isoflavones in postmenopausal women after a single dose. Their plasma and urine isoflavones pharmacokinetics were similar for both aglycones-rich and glucosides-rich soya drinks. In contrast, Hutchins *et al.* (1995) and Slavin *et al*. (1998) reported that the fermentation of cooked soybeans by *Rhizopus oligosporus* (tempeh) enhanced the bioavailability of daidzein and genistein in both men and women during 9 days of consumption (112 g tempeh/day) when compared with the ingestion of non-fermented cooked soybeans under the identical condition. The chemical forms of

these isoflavones affect their biological activities (Izumi *et al*., 2000). Among them, isoflavone aglycones, especially the genistein and daidzein, exhibit higher biological activity than isoflavone glucoside (Kuo *et al*., 2006). However, due to the fact that raw soybean contains low quantity of aglycones (2-5%) (Song *et al*., 2008 ; Jackson *et al*., 2002), it is difficult to obtain high concentration of isoflavone aglycones from soybean. Fortunately, isoflavone aglycones can be converted through the hydrolysis of isoflavone glucosides, which are abundantly found (95-98%) in soybean (Ismail *et al*., 2005). Usually, acid can be used for the conversion of isoflavone glucosides into isoflavone aglycones. For example, when isoflavone glucosides were hydrolyzed by hydrochloric acid (Lee *et al*., 2011), almost all isoflavone glucosides were completely transformed and the total content of isoflavone aglycones arrived at 1.3 mg/g. Nowadays, enzymatic hydrolysis (such as using β-glucosidase) has become the common method to perform the conversion since it is more moderate and specific than chemical hydrolysis. For instance, Song *et al.* (2011) used β-glucosidase as catalyst to hydrolyze isoflavone glucosides resulting in the highest reaction rate.

Intestinal bacteria have an important role in increasing bioactivitiy of isoflavones (Hendrich 2002; Turner *et al*., 2003). Unabsorb isoflavones are excreted in urine and found predominantly as glucuronide and sulphate conjugates. Owing to their phenolic nature, isoflavones undergo biotransformation at hydroxyl groups by mammalian UDP glucuronosyltransferases and sulphotransferases in the intestinal mucosa and liver (Hendrich 2002). Nowadays, studies on the pharmacokinetics of isoflavones in human subjects have not yet investigated the effects of fermenting soya foods with bifidobacteria. Bifidobacteria constitute a major part of natural micro flora in human intestine. Its highest populations are found in the ileum and colon (Orrhage and Nord, 2000). It was discovered that β-glucosidase produced by *Bifidobacterium animalis* Bb-12 hydrolyzes isoflavone glucosides into bioactive and bioavailable aglycones when grew in soya milk (Tsangalis *et al*., 2002, 2003, 2004). In clinical studies, *B. animalis* Bb-12 could effectively modulate intestinal microbial balance (Playne, 2002). Hence, the enrichment of isoflavone aglycones in soya milk by fermentation prior to consumption and the modulation of intestinal microflora via the ingestion of viable bifidobacteria may enhance the bioavailability of isoflavones consumed on a daily basis. Suggested dosage of isoflavone consumption is

approximately 18-20 mg one serving size (4 time to 80 mg/day) (Barnes *et al.,* 1995). But, Marjorie *et al.* (2002) suggested that isoflavones consumption administered to humans in single doses exceeding normal dietary intake many fold resulted in minimal clinical toxicity. Genistein and daidzein (free and total) were rapidly cleared from plasma and excreted in urine.

The highest amount of isoflavones is found in soy germ, while only 2% is available in soybean. Isoflavones in soybean are 5-6 times higher in soy germ than the other parts; seed coat and cotyledon (Nahas *et al.*, 2004). Soy germ contains 25-40% daizein, 25-40% glycitein and 5-20% genistein with total isoflavones of 45-60%. Yue *et al.* (2009) investigated the amount of isoflavone and antioxidant activity in other parts of soybean including cotyledon, seed coat and soy germ in four species. It was found that total isoflavones in cotyledon, seed coat and soy germ (Figure 2.1) were 2.73-9.71, 5.56-16.94 and 27.76-81.43 mg per 100 g soy germ, respectively. So, this study, soy germ will be used as a source for isoflavone glucosides to be substrate of isoflavone aglycones production.

Figure 2.1 Soy germ

Typical isoflavone extraction uses organic solvents such as acetronitile, acetone, methanol and ethanol. Wiriyacharee *et al*. (2012) study isoflavones extraction by five

kinds of solvents were used in isoflavones extraction: water, ethanol, methanol, acetone and acetonitrile. Acetone extraction had the highest content of isoflavones in form of glucosides (daidzin, genistin and glycitin) and aglycones (daidzein, genistein and glycitein) of 0.56 and 18.46 mg/100g wet weight, respectively. But organic solvents are toxic to human at high concentrate and have high extraction cost. So, mechanical extraction combined with solvents extraction is a preferred method for isoflavone extraction. Supercritical fluid extraction is a one of the mechanical processed of separating one component (the extractant) from another (the matrix).

Isoflavones or phytoestrogens are organic compounds of flavonoids found in many plants, especially in soybeans. It is structurally and functionally similar to estrogen, which is a female hormone. This has the effect of reducing hot flushes from menopausal symptoms and it has the ability to help prevent some types of cancer, cardiovascular disease and osteoporosis (Murphy *et al*., 2002; Messina, 1999). Isoflavone in soybean and fermented soybeans has many chemical structures compose with aglycones are daidzein, genistein and glycitein and the structure of glucosides such as daidzin, genistin and glycitin are shown in Figure 2.2.

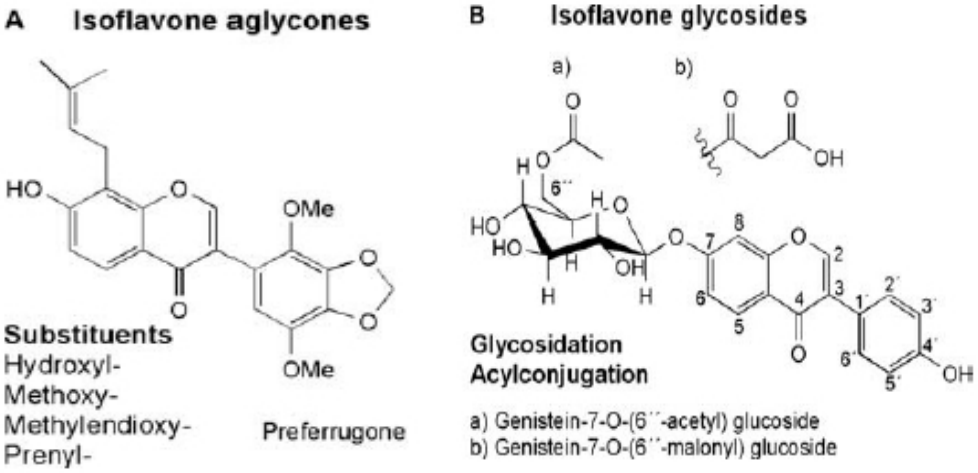

Figure 2.2 Structural characteristics of isoflavones

Source: Shimoni, (2004)

The main structure of the isoflavones was composed of 2 benzene rings with carbon ring type heterocyclic pyrane for the isoflavones metabolism found that the part of glycosidic conjugates, which is water soluble well it must be digested with an

enzyme to cut off sugar by the enzyme glucosidases. In the process of absorption isoflavones into the body. Diadzein is metabolized to isoflavones equol, dihydrodaidzein and O-desmethylangolensin. Genistein is metabolised to dihydrogenistein and 6-hydroxy-O-desmethylangolensin. Isoflavones is similar to estrogen. It can catch with estrogen receptors, it is also known as phytoestrogen (Sarkar *et al*., 2002)

The quantity and type of isoflavones in the product depends on the variety of soybean. The study found that isoflavone aglycones structure is rapidly absorbed through the gastrointestinal (Setchelle, 2000). Isoflavones in fermented soybean showed that daidzein, genistein and glycitein. It has a lot of structure. There are more interested in other types of structures.

2.2 Isoflavones extraction

Isoflavone is a flavonoid substance that is soluble in various polar solvents. Luciana *et al*. (2014) studied the extraction of isoflavone from different solvents. The purpose of this study was to determine the optimal conditions for the extraction of isoflavones in all structure from soybean that has been extracted. Then, the 4 types of solvent were varied: acetone, acetone and acetone. It was found that the characteristics of isoflavones from variation solvent is different. Isoflavone form glycosidic was extracted well with polar solvents from aqueous acetone and acetonitrile. Isoflavones form malonyl-glycosidic and total isoflavones are well extracted in aqueous acetone and ethanol-water mixed. The isoflavone aglycones is well extracted in water and acetone. It can be seen that isoflavones is well soluble on the polarity (Luthria *et al*., 2007).

- **Isoflavones extraction using ultrasonic technique**

It is a technique of diffusion of sound energy into intermediates. It can be divided into2 major categories. 1.) The use of low power and high frequencies (low power and high frequencies) frequency range is 2-10 MHz, for used analysis (dianostic ultrasound). 2.) Using high power and low frequencies (power ultrasound) is about 20-100 kHz at the level of 10-1000 W / cm^2 applied to food processing. Ultrasound will cause cativation, which is a process that occurs in the middle or ultrasound sound wave solution by chemical and physical changes due to the bubbles that are produced by the

compression and decompression in the liquid structure of the ultrasound wave and in the food industry such as emulsion process, fermentation process, drying process, freezing process sterilization process in food and the extraction process.

The principle of ultrasound waves to destroy the surface of the cell and cell walls allow the solvent to penetrate the material to be extracted easily. Including the substance to extract it easier. Reduce the time to extract. Include extra yield in extraction if using this method to extract. It is used to extract plant extracts such as extract isoflavone. Rostagno *et al*. (2003) investigated the effect of extraction of isoflavones from frozen soybeans compared to conventional extraction and extraction using ultrasonic. It was found that the optimal condition for extraction of isoflavones was the use of 50% ethanol solution at 60 ° C ultrasonics for 20 minutes can extract the isoflavone from soybean.

- **Isoflavones extraction using supercritical fluid extraction**

Supercritical fluid extraction can be used as a sample preparation step for analytical purposes, or on a larger scale to either strip unwanted material from a product (e.g. decaffeination) or to collect desired products (e.g. essential oils). Carbon dioxide (CO_2) is the most used supercritical fluid, which sometimes is used in combination with other co-solvents (all modifier) such as ethanol or methanol. Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31°C and critical pressure of 74 bar. Addition of modifiers may slightly alter this. The following discussion will mainly refer to extraction with CO_2, except where specified. Zuo *et al*. (2008) studied effect of supercritical carbon dioxide (SC-CO_2) with methanol to extract isoflavone from soy meal was undertaken at various conditions. The influence of modifier composition in terms of methanol content in water was studied to identify optimum modifier strength. It was found that aqueous methanol was an effective solvent and the optimal modifier composition was 80% methanol in water. The highest isoflavones recovery was 87.3%, at 40 °C and 50 MPa, and CO_2 flow rate of 9.80 kg/h. Isoflavones recovery increased with the addition of the modifier up to 10.2%. However, the studied of Rostagno *et al*. (2002) was found extraction genistin and genistein by supercritical carbon dioxide at 70 °C at 200 bar using a mixture of carbon dioxide (55.2 g) and modifier (10 mol), resulted in lower values than conventional methods.

Therefore, this study aims to optimize the isoflavone extraction by supercritical fluid extraction from soy germ. Super critical fluid has chemical properties is between gas and liquid. The density of the supercritical fluid is like with liquid. When used as a solvent, it is surrounded by a supercritical fluid molecule and interact to reduce energy until it dissolves well at the same time, the superficial fluid has viscosity and diffusion coefficient like to gas, it can penetrate the structure of the solid with these features It is recommended to use the super critical fluid as a solvent. because there are more advantages than liquid. In liquid state mass transfer rate is faster and has better solubility.

2.3 The role of isoflavones on health

Research has shown that isoflavone can reduce the risk of heart disease, stroke, reduce the risk of breast cancer by inhibiting the action of estrogen in humans and prevent osteoporosis by stimulating bone formation and inhibiting the breakdown of bone tissue and reducing the adverse effects of postmenopausal women and reduce the risk of prostate cancer in older men (Murphy *et al*. 2002; Messina, 1999).

- **Prevention of heart disease**

Women of reproductive age have a lower risk of heart disease than men. After menopause, the risk of both sexes is nearly the same. (American Heart Association, 2007). Hormone replacement therapy (HRT) helps older women control their LDL levels. Reduce coronary heart disease risk. Research of Anderson *et al*. (1995) supports that consumption of soy protein results in lowered cholesterol levels. There are also other researches found that genistein in soybean is beneficial to heart health. Moreover isoflavones is an antioxidant that protects the blood vessels from the oxidation of LDL. It helps reduce the amount of cholesterol at the same time, HDL (High density lipo protein). This is a great source of energy. Soybean estrogen inhibits the oxidation of LDL cholesterol. Preventing blood clotting by the artery wall, which causes heart disease. In addition, the substance in soy also helps to make blood vessels flexible.

- **Control hormone system in menopausal women**

In the premenstrual period, women have elevated blood levels of estrogen. In addition to increasing the risk of heart disease and osteoporosis in women (American Heart Association, 2007), it also causes menopausal symptoms such as hot flush, sweating, sleepless nights, headaches and vaginal dryness.

Phytoestrogens in soybeans in genistin and daidzin were weak estrogen (Knight *et al*., 1996). It was interest to researchers looking for alternatives to reduce health problems in postmenopausal women. Research has shown that the consumption of soy isoflavones in women before menopause help slow down menstruation and have effect. Anti-estrogen a comparative study of gynecologic women found that isoflavone in diets could be augmented and weakly inhibited by estrogen. It depends on the amount of estrogen receptor in the cell.

Dalais *et al*. (1998) studied the use of phytoestrogens from soy germ by dividing the group of menopausal women with hot flushes into 2 groups. First group get soy germ and other groups do not get soy germ (control group). It showed that 44% of subjects receiving soy sauce had hot flushes. However, while the control group had hot flushes only 10% lost. According to Han *et al*. (2002) reported that postmenopausal women who received Isoflavone capsule 100 mg daily. Hot flushes decrease within 4 months.

- **Osteoporosis**

Osteoporosis is found in menopausal women. Menopause at the age of 50 and older are at risk for osteoporosis cause estrogen was decreased. Reduce the bone mass rapidly and gradually decreases with age. Some women who menstruate faster than normal. Bone loss may increase by 1-3% per year, especially during the first year of menopause and at age, the bone loss is 0.7-1.0 percent per year. The use of estrogen, it is a good way to prevent the loss of bone. Osteoporosis in women and spine protection. Including women after menopause. Reduce bone loss by 50%. Isoflavone daidzein used in the treatment of osteoporosis in postmenopausal women was ipriflavones, which are metabolized to daidzein is effective in suppressing the breakdown of osteoporosis. Teruyoshi *et al*. (2017) reported that dietary kudzu vine extracts and a diet consisting ofpuerarin, the major isoflavone, improved glucose metabolism, weight gain

andosteoporosis independent of the estrogen receptor-mediated pathway in OVX mice (female rat whose ovaries have been removed). As population ages, health problems associated with age and menopause in women are becoming increasingly notable. The findings in this study suggestthat dietary kudzu vine isoflavones and puerarin present a promising approach to prevent life style related diseases like obesity, diabetes and osteoporosis

- **Cancer Prevention**

Studies in asian populations that consume more soy products than westerners. Similarly, asian men with prostate cancer are 20 times less likely to develop prostate cancer than men in the west. Researchers believe that isoflavones in soybean to reduce the risk of prostate cancer by interfering with the hormone testosterone. In men, or inhibition of cancer cells by reducing the production of testosterone hormones. In addition, research (Messina, 2007) studies that soy consumption reduces the risk of thyroid cancer.

A study of the safety of isoflavones research from 28 women in the reproductive age to treat isoflavones at 45 mg per day for 14 consecutive days, soy protein intake was no side effects (Hargreaves *et al*., 1999). In addition, 177 patients with metastatic breast cancer consumed soy isoflavones 150 mg per day for 4 weeks had no side effects on the endocrine system and mammary gland tissues (Quella *et al*., 2000). In addition Takehito *et al*. (2002) conducted a study on the production of alfalfa isoflavones from an extract from local fermented soybean products to combat capillary abscess formation. When using genistein in combination with polysaccharide, there is a better anti-capillary effect than using only soybean extract. This study shows that the consumption of isoflavones products is beneficial and safe for consumers. In addition, the consumption of soybean products is beneficial to the body, such as reducing the risk of stroke and helps keep bones strong in the elderly. However dietary reference intakes (DRIs) are not currently available, but research by Setchell and Cassidy (1999) recommends consumer should consumed isoflavones in range 20-50 mg per day.

2.4 The researched of isoflavone aglycones production

Based on research on the production of isoflavone aglycones in the past, it was found that the most of isoflavones are in the soy germ. Production of isoflavone aglycones was produce by fermenting soy germ with *B. coagulans* PR03 for 120 hours and extraction with ethanol at 80% concentration for 2 hours. Then the isoflavone aglycones solution was extracted through evaporation for ethanol removed to be crude extracted of isoflavone aglycones. Then, isoflavone aglycones purification using column containing Amberlite XAD-4 resin and it was found that the efficiency of isoflavone aglycones purification as compared to other resins such as diaion HP20, Amberlite XAD-7HP and Amberlite XAD-16HP (Wiriyacharee, 2012). There is also research for the purification of isoflavone aglycones using amberlite XAD-4 resin at different flow times of the resin solution also affected the best purity of isoflavone aglycones at 40 minutes , purity is 42.70% isoflavone. (Wiriyacharee, 2013)

2.5 Isoflavone aglycones purification

Normally, there are 2 methods of isoflavones purification compose of liquid-liquid extraction and column chromatography. The purification by column chromatography using resin as absorbent. Resin is a polymer that is durable had polar and non-polar types both types have high adsorption. Price is low and easy to regenerate (Scordino *et al*., 2004) and it is widely used in the separation and absorption (Di Mauro *et al*., 2002) . Resins have a wide range of resin types. Polystyrene-dinbenzene such as amberlite XAD-4, XAD-16HP and polylmethacrylate such as amberlite XAD-7HP (Wu and Lai, 2007)

Amberlite XAD-2 are also used in volatile extraction in glycoside form (Crouzet and Chassagne, 1999), amberlite XAD-2 resin is hydrophobic, absorbing non-polar glycoside.

Cho *et al*. (2009) conducted a study on the purification of isoflavones from germinated soybean seed using amberlite XAD-2 and Diaion HP-20 as isoflavone adsorbent found that diaion HP-20 was better than amberlite XAD-2 and reported in Chang *et al*. (2007). It was found that amberlite XAD 16-HP were able to produce 37% pure isoflavones and 48.9% of total isoflavone content can not be absorbed

Hua *et al*. (2012) studied separation and genistein production is purified from soybeans by the adsorption of resins in the AB-8 resin and gradually diluted with gradient solvent from deionized water, 20% ethanol and 70% ethanol in the first process. In second process using 40% and 70% ethanol was used in the sequestration. It is found that genistein is 90% purity.

Wei *et al*. (2008) conducted a preliminary study on increasing and the separation of genistein and apigenin from root of the pigeon pea, using in 11 different types of resins, both physical and chemical. Resin type ADS-5 is effective in isolation genistein and apigenin increased to 9.36 and 11.09 times, respectively, and the yields were 89.78 and 93.41 respectively.

Benny *et al*. (2007) adapt process that combines conceptual design. Isoflavone isolation and purification system from soy germ study of the structure, process and chemical characteristics of the relevant substances. Based on basic information for example, the balance of solids and liquids in the chromatographic separation to isolate the isoflavones in the mixture using the chromatography or precipitation principle of the substance to obtain the pure substance compose of genistein and daidzein were isolated by isolation and purification (Figure 2.3).

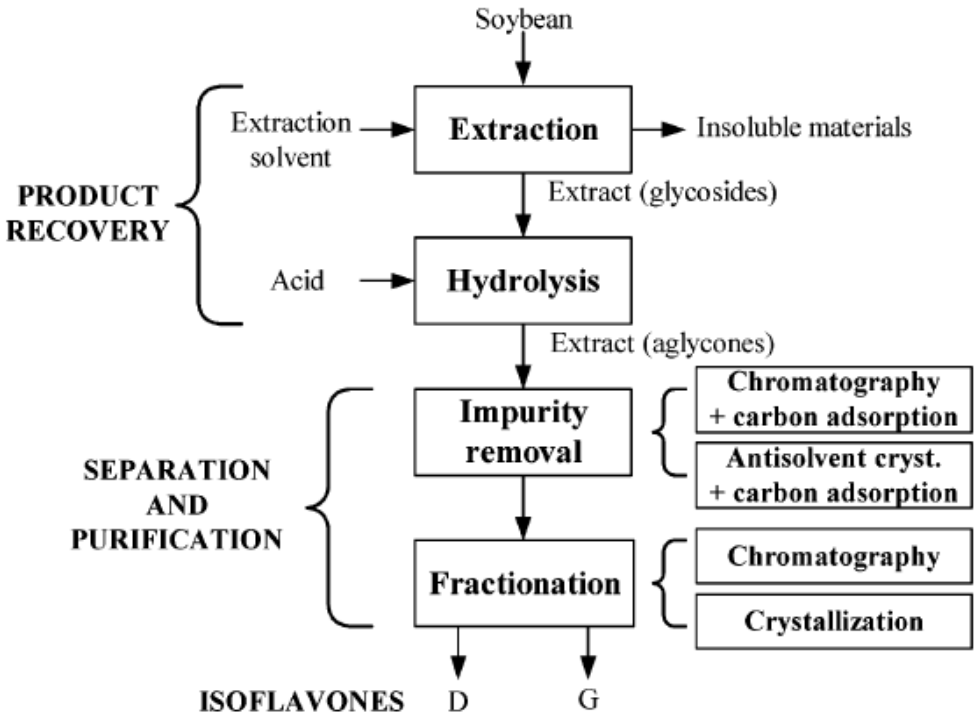

Figure 2.3 Isoflavones purification

Source: Benny *et al*. (2007)

2.6 Bacteria associated with soy fermentation and β-glucosidase production

Bacteria that produce β-glucosidase are endospore-forming bacteria. Endospore is a term that occurs when bacteria are found in an unsuitable environment is gram positive bacteria by creating vegetative cell in form of sporangium, which responds in a food shortage and it can live without the mechanism of metabolism or cryptobiosis. This special structure is more durable than normal cells to conditions that are limited, such as heat, dry air, cold weather, radiation and harmful chemicals. Especially, *Bacillus* spp. is the most suitable bacteria for food production.

B. subtilis is a safe species for consumers and not considered a human pathogen. Moreover, it has health benefits. It is classified in super kingdom prokaryota, kingdom eubacteria, family Bacillaceae, genus bacillus. general characteristics single-celled and size 0.6 - 0.7 x 2.0 - 3.0 μm with endospore ovary in the middle of the cell or toward the end of the cell. The colonies on the media are curcular, uneven rim, opaque surface, surface may be wrinkled cream or brown. If media is very moist, the colonies will spread much. On the media containing 10% glucose, the colonies are thick brown. Growing on liquid media the infection is on the surface of the media. The turbidity is slightly turbid. Aerobic or Facultative anaerobic bacteria produce catalase is gently added to pH 5.5 - 8.5. Hydrolytic enzymes break down polysaccharides, nucleic acids and lipids by using them as a carbon source and have oxygen. The important role of this microorganism in fermentation is to release the protease enzyme (Nongnuch, 2004) affected on fermented soy bean had a characteristic flavor

Fermented soybean by *Bacillus* spp. is a variety of types, but they may vary in product characteristics. Native fermented foods from soybeans are similar with production of tua-nao. However, the nature of the product may vary such as Natto, Kinema and Chungkookjang. Reported that Natto, a native Japanese fermented soybean meal, is used as a feedstock and uses *B. subtilis* var. natto to ferment the natto with a distinctive smell and taste. There is the production of mucilage, which is a polymer. Natto lumps the mucilage on the surrounding skin. *Bacillus* spp. produces β-glucosidase. The glycoconjugal degradation in the structure of isoflavone glucosides cause transform to isoflavone aglycones by daidzien and genistein which are isomer in high doses and the proportion of total isoflavone aglycones volume (Anderson *et al*. 1995).

The traditional fermented soybean was produced by fermentation by various strains from natural. The isolation and identification from Thai native fermented soybean was characterized at each time of fermentation. The amount and type of bacteria varies. *Bacillus* spp. is the most abundant bacteria in the genus and lactic acid bacteria. It can also be found after fermentation for 24 hours (Leejeerajumnean *et al*. 2003). According to Phongphisutthinant *et al*., (2015) reported that *B. coagulans* as predominant bacteria to enrich isoflavone aglycones for tua-nao production by *B. coagulans* PR03.

Table 2.1 *Bacillus* spp. detected in Thai native fermented soybean.

Fermented time (hours)	Bacteria strain
0	*B. subtilis*, *B. megaterium*
24	*B. subtilis*, *B. megaterium*
48	*B. subtilis*, *B. megaterium*
72	*B. subtilis*, *B. megaterium*, *B. cereus*
Final product	*B. subtilis*, *B. megaterium*

ที่มา : Leejeerajumnean *et al.* (2001)

- ***B. coagulans***

B. coagulans also called *B. thermoacidurans* is a rod shape, a gram-positive bacterium that produces spores. It grows at high temperatures (thermophilic bacteria) and spores of *B. coagulans* are suitable for growing at 35-50 ° C and pH 5.5 - 6.5. Additionally, they are probiotic and safe (Office of the Food and Drug Administration, 2008)

B. coagulans are useful bacteria are known as *Lactobacillus sporogenes*, *B. coagulans* are probiotics to live in acid in the stomach and be transported to the small intestine without loss. Most probiotics can not survive because they can not withstand the temperature and pH of the stomach.

- **Factors affecting the growth of *Bacillus* spp.**

Most of the bacteria in *Bacillus* spp. grow well at medium temperatures (Mesophilic bacteria). The temperature ranges from 30 to 45 °C but some species grow well at high temperatures (thermophilic bacteria) which is responsible for canned food

spoilage and also some of the species grow well at low temperatures (psychrophilic bacteria) and grow in pH range 2-11, depending on the species. Results from laboratory experiments showed that *Bacillus* was suitable for several cultivars generation time is about 25 minutes.

2.7 β-glucosidase

β-glucosidases are a group of enzymes that hydrolyze glycosidic bonds releasing the non-reducing terminal glucosyl residues from glucosides and oligossaccharides. These natural occurring enzymes can be synthetized by plants, animals and microorganisms. Soy contains glycosidic isoflavones (daidzin, genistin and glycitin), which can be hydrolyzed by β-glucosidases turning them to the aglycones (daidzein, genistein and glycitein). Isoflavone aglycones generally exhibit higher biological activity and can be absorbed faster in the body than isoflavone glucosides (Izumi *et al.*, 2000)

Park *et al.* (2003) characterized β -glucosidase from *Paenibacillus xylanilyticus* KJ-03 capable of hydrolyzing isoflavone daidzin and genistin. They found that the β -glucosidase has optimal activity at low temperatures (around 10 °C) indicating that it is a cold-active enzyme. The substrate specificity showed that the purified enzyme hydrolyzed aryl β- glucoside substrates and isoflavones such as daidzin and genistin.

Microorganisms such as *B.subtilis* can produce β-glucosidase enzyme to hydrolyse the glucose moiety of isoflavones conjugates, releasing the aglycones with higher biological activity. This transformation also happens during fermentation of soy. Wiriyacharee *et al.* (2011) studied microorganism from Tou nao of local Thailand northern and identified forty-one isolates of *B. subtilis, B. coagulans, B. licheniformis, B. pumilus* and *B. megaterium* from fermented soybean. Each isolate was called the pure starter culture for soybean fermentation. All of the isolates were divided into nine groups according to the sample sources. Then isoflavone content was determined from these fermented soybean samples in order to select the best performing strain for isoflavone aglycones production. It was found that *B. subtilis* CR01, *B. subtilis* NATTOA02, *B. coagulans* PR03 and *B. megaterium* PY03 produced the highest amount of daidzein, genistein, glycitein and total isoflavones in each group. Moreover, found that *B. coagulans* PR03 could produce isoflavone aglycones from soy germ

fermented more than other microorganisms (Wiriyacharee *et al.,* 2013). In this study, we will extract β-glucosidase from *B. coagulans* PR03 and may use immobilized enzyme to produce isoflavone aglycones.

Purification of isoflavone has 2 approaches: 1.) liquid-liquid extraction and 2.) column chromatography by Polystyrene-dinylbenzene absorption (such as Amberlite XAD-4 resins, XAD-16HP resins) and Polylmethacrylate absorption (such as Amberlite XAD-7HP resins) (Wu and Lai, 2007). On this aspect, Wiriyacharee *et al*. (2013) found Amberlite XAD-4 to be the optimal resin for isoflavone purification with the yield and purification percentages of 53.66% and 31.00%, respectively. Amberlite XAD-4 is a macroporous styrene-divinylbenzenecopolymer. This resin is best for the removal of soluble phenolic compounds (Li *et al.,* 2001).

2.7 Sensory Evaluation (Wiriyacharee ,2002)

Sensory evaluation is a scientific principle that analyses and measures human responses to the product derived from human senses in term of sight, smell, touch, taste and hearing. The aim of sensory evaluation is to determine the food quality characteristics and the degree of compliance with the legal requirements and consumer habits.

Panel Management

Sensory evaluation composed of two general types which are descriptive panel and consumer panel. A descriptive panel is commonly used to determine differences between food samples. The descriptive panelist is experienced in the type of food being tested and receives extensive training prior to the testing. A consumer panel is selected from the public according to the demographics necessary to taste test product. The number of panelists may range from 200 to 500 people. Consumer panelists can be screened on test criteria; for example demographics or potential use of product. The questions asked of consumer panels should be answerable by untrained panelists.

Sensory Method

There are several methods of sensory testing however in this research is just discussed the 7 point hedonic scale.

Over the last half century a number of scales have been developed and utilized to measure hedonic response in both basic psychophysical and applied research. The 7-point hedonic scale is scale for testing consumer preference and acceptability of foods. The 1-4 samples are presented simultaneously and panelist must taste and record the preference and acceptability according the preference scale (7=like very much, 6=like moderately, 5=like slightly, 4=neither like nor dislike, 3=dislike slightly, 2=dislike moderately, 1=dislike very much). The results will be reliable if the panelist makes a decision on preference right away. Duncan's New Multiple Range Test (DMRT) used to analyze the variance of 95%.