1. INTRODUCTION

1.1. Background of research

Natto is the fermented soybean product that was created in Northern Japan 1000 years ago (Shih *et al*., 2009). There are 3 kinds of natto such as *itohiki* natto, *hamanatto,* and *daitokuji*. *Itohiki natto* made by inoculated with bacteria, incubated for 24 hours, and without added salt. *Hamanatto* and *daitokuji* made by inoculated with mold, incubated for 3-6 months, and added with salt (Shurtleff and Aoyagi, 2011). Several countries in Asia also have a similar product of natto such as "Shi (China), Thua-nao (Thailand), Chung-kook-jong (Korea), and Tao-si (Philippines). In general, natto has 59,5% moisture; 16,5% protein; and 10% lipid (Wei *et al*., 2001). Natto was made from steamed soybeans and inoculated with *Bacillus subtilis* (Weng *et al*., 2010). Fermentation of soybean by *Bacillus subtilis* will produce a specific odor, musty flavor, and slimy appearance caused by viscous and sticky polymers (Shih *et al*., 2009). The sticky polymer contains several substances such as glutamic acid, amino acid, and fructan (Weng *et al*., 2010). In Japan, natto was mixed to the thinly slice of green onion, mustard, seaweed, and a small amount of soy sauce and it usually served as a side dish with steamed rice. Moreover, natto also used as a flavoring agent in meat, seafood, and vegetables (Wei *et al*., 2001).

Fermentation is one of the methods to increase bioactive compound through breakdown the cell walls and induce the synthesis variety of bioactive compound (Hur *et al*., 2014). Fermentation of soybean will generate proteolysis activity, where it was enhanced nutritional value as well the taste, and removed undesirable flavor (Zhu *et al*., 2008). Fermentation of soybean has an antidiabetic effect due to the increase of isoflavones aglycone.

Nowadays, the utilization of black soybean could be use only for making soy sauce. Black soybean also has been used for medical interest such as detoxification and anti-inflammation to improved the red blood cell (Sumardi *et*

al., 2017). In the previous study, Black soybean developed as healthy food like black soybean tofu, black soybean sprout, and black soybean okara natto. Black soybean has higher anthocyanins compared with soybean because of seed coats of black soybean darker than soybean (Shih *et al*., 2009).

In this research, we focused on raw material substitution of natto and adding Co-culture *Saccharomyces cerevisiae* with different fermentation time and ratio. Different time usage to find out whether long fermentation time will affect on antioxidant activity and total phenolic content. Moreover, different ratio usage to find out whether additions of *Saccharomyces cerevisiae* will affect on pH and sensory properties of black soybean natto. Fermentation time have to be done until 48 hours to avoid *Saccharomyces cerevisiae* generate alcohol. *Saccharomyces cerevisiae* was expected to provide sweet flavor as well as to reduce the ammonia smell in natto. Yao *et al*., (2010) has studied the effect of fermentation using various microorganisms in black soybean. The results showed that yeast could increase antioxidant activity (DPPH and FRAP method) and Total Phenolic Content. Yeast can also be used for legumes fermentation. Santos *et al*., (2018) explained that yeast could increase isoflavone aglycone as a biotransformation product of β-glucoside isoflavones in soybean. *Saccharomyces* also has enzymatic activities to breakdown starch, oligosaccharides, and protein. Whereas, fermentation will produce sweet flavor and compounds that easily digestible (Omemu *et al*., 2007).

Table 1. Main microorganism and enzymatic activity used in food fermentation

Microorganisms	Species	Active enzymes
Bacteria	*Bacillus subtilis*	Amylase, Cellulases, hydrolases, peptidase, and proteases
Yeast	*Saccharomyces cerevisiae*	Alcohol dehydrogenase, amylase, β-glucosidase, maltase, and proteases

(Hur, 2014)

1.2.Literature review

1.2.1.Black Soybean

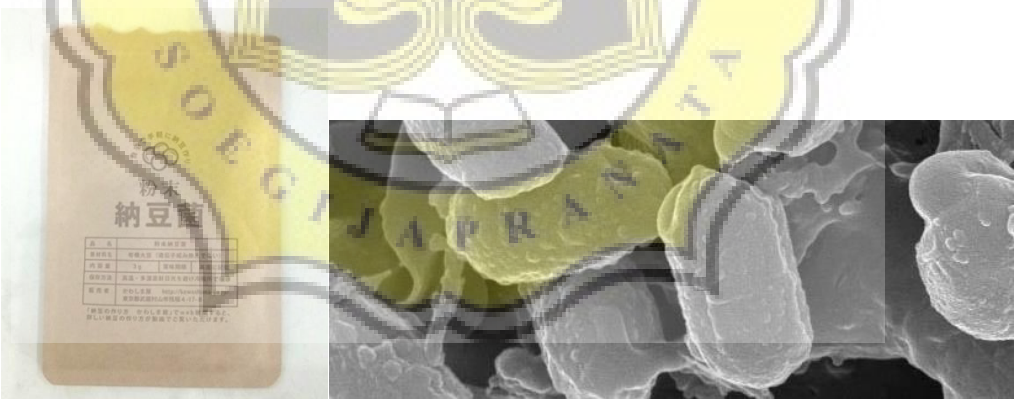
Black soybean (*Glycine max* (L.) Merr.) is classified in legume and include in soybean variety. Black soybean typically grows in the tropical region of Southeast Asia (Fetriyuna, 2015). The nutritional composition of black soybean that are protein (32–43.6%), carbohydrates (31.7–31.85%), lipids (15.5–24.7%), water (5.6–11.5%), minerals (calcium, phosphorous, magnesium, and potassium), and vitamins (Vitamin E and B) (Ganesan *et al*., 2017). Even though black soybean is included in soybean category, it has a darker seed coat due to higher anthocyanin content in the seed coat (Shih *et al*., 2009). Black soybean contains phytochemical active such as γ-tocopherol, isoflavones, flavonoids and anthocyanins as antioxidant components (Dajanta *et al*., 2013). The form isoflavones which exists in black soybean natto are daidzein and genistein (Sumardi *et al*., 2017). There are two types of black soybean: green and yellow cotyledon-black soybeans. Green cotyledon has higher genistein, anthocyanin, and glutathione than yellow cotyledon. Moreover both of them have a higher antioxidant activity than soybean .(Shih *et al*., 2009).

Black soybean has many bioactive compounds such as isoflavone, vitamin E, saponin, anthocyanin, and carotenoid (Hu *et al*., 2010). Isoflavone can suppress lipoprotein oxidation, reduce DNA injury caused by cyclophosphamide, and reduce risk cardiovascular disease (Hasim *et al*., 2015). Moreover, consumption of black soybean can reduce risk of contracting breast cancer in women (Zilic *et al*., 2015). (Ganesan *et al*., 2017) studied that high anthocyanins in black soybean have a health benefit such as reduce blood pressure, reduce cardiovascular complication, diabetes and cancer prevention, and reduce body weight. In recent years, black soybean has been used for soy sauce production and *In-shi*, Chinese traditional fermented food (Juan *et al*., 2010). There are some research said that black soybean can be developed as healthy food like black soybean tofu, black soybean sprout, and black soybean okara natto.

1.2.2.*Bacillus subtilis*

Bacillus spp. is classified in gram-positive bacteria, heat resistance, spore-forming bacteria, rod shape, non-pathogenic bacteria, and have ability to growth in inexpensive media. *Bacillus subtilis* has already been recognized by FDA as GRAS (Generally Regarded As Safe) (Shrestha *et al.*, 2010). *Bacillus subtilis* is an ancient culture which has already been used for soybean product (Zhang *et al.*, 2014).

During fermentation process, *Bacillus spp.* can produce various kind of enzyme such as polysaccharide and nucleic acid hydrolyzing enzyme (Shrestha *et al.*, 2010). *Bacillus spp.* is often use for fermentation soybean because this bacteria belongs to hydrolytic bacteria, so it can reduce the oligosaccharides and indigestible polysaccharides (Shrestha *et al.*, 2010). *Bacillus subtilis* will generate nattokinase during fermentation and cause fibrinolytic activity in natto (Juan *et al.*, 2010). *Bacillus subtilis* will also produce protease enzyme that will breakdown protein into amino acid and peptide during fermentation (Weng *et al.*, 2012).



(source: https://thejapanstore.jp/collections/natto/products/natto_001)

(a) (b)

Figure 1. *Bacillus subtilis* var. natto

"*Kawashima-ya*" starter (a), under the microscope (b)

1.2.3.*Saccharomyces cerevisiae*

Saccharomyces cerevisiae has an optimum pH of 4.5 – 6.5 and grows well at temperature of 20°C - 30°C. During fermentation *Saccharomyces cerevisiae* also needs some oxygen at the beginning of fermentation processes (Walker *et al*., 2016). Yeast can be also used for fermentation in legumes. Santos *et al*., (2018) explained that yeast can increase isoflavone aglycone because there is biotransformation β-glucoside isoflavones into isoflavone aglycones in soybean. Whereas, isoflavone aglycone has a function to suppressing lipoprotein oxidation, reduce DNA injury caused by cyclophosphamide, and reduce risk cardiovascular disease (Hasim *et al*., 2015).

In the previous study, *Saccharomyces cerevisiae* used for making tempeh. Which, it expected to improve the aroma of tempeh and to cover the unpleasant aroma from the bean (Kustyawati *et al*., 2017). Moreover, yeast also has an enzymatic activity that can breakdown many complex substances such as starch, oligosaccharides, and protein. Where, it will produce sweet flavor and easily digestible (Omemu *et al*., 2007). Fermentation uses yeast to produce ethanol and carbon dioxide (Lin *et al*, 2012).

1.2.4.Natto Fermentation

Fermentation is a method to enhance the nutritional and organoleptic of food. Besides that, fermentation can also increase bioactive compound by breaking down the cell wall so it can induce the synthesis variety of bioactive compound (Hur *et al*., 2014). The main process of making a natto product is a fermentation process. (Wei *et al*., 2010) mentioned that the step for making natto in manufacture scale consist of soaking, steaming/cooking, inoculating, and incubation. The best fermentation time which produces the good quality natto product was 18 h to 20 that result in complete fermentation. Maruo *et al*., (1989) in Wei *et al*., (2001). (Maruo *et al*., (1989) in Wei *et al*., (2001) also present that fermentation time 16 h to 18 h result good quality of natto. Further (Ikeda *et al*., (1985) in Wei *et al*., (2001) present that fermentation time 14 h result a good quality soybean natto. In this study fermentation processes were done by using

inoculum *Bacillus subtilis* and *Saccharomyces cerevisiae*. In fermentation processes, there are many changes both physically and chemically. Before fermentation processes, soybean must be soaked and steamed. The steaming processes were carried out to ease inoculum bacteria penetrate into beans by changing the microstructure of beans, so it affected the texture of natto (Wei *et al*., 2001).

During fermentation processes, *Bacillus subtilis* will produce protease enzyme. Protease enzyme will breakdown the protein contained in beans into amino acid and peptide.

$$RCH(NH_3^+)COO^- + nO_2 \rightarrow nCO_2 + nH_2O + NH_4^+ + OH^-$$

(Allagheny *et al*, 1995).

Secondary fermentation will happen if natto is not store in good condition. Natto is usually stored in low temperature to prevent secondary fermentation. Secondary fermentation will produce ammonia smell resulting from oxidation of amino acid. Ammonia plays a role in an increase in pH value (Leejeerajumnean *et al*., 2000). Peptides resulting from protein breakdown will generate higher amount of antioxidant (Hur *et al*., 2014). During fermentation, glycosidase and glucuronidase will increase and release antioxidant substance called as flavonoid (Hu *et al*., 2014). Total phenolic content also increases during fermentation. This happens because enzyme β-glucosidase activity produced by *Bacillus subtilis* will hydrolyze phenolic puchoside and generate the free phenolic like aglycones (Hu *et al*., 2010). Other than that, fermentation also result a physical change. *Bacillus subtilis* which produce stickiness as a final result in natto characteristic. Stickiness in natto caused by fibrinolytic enzyme in *Bacillus natto* and usually called as nattokinase (Zhao *et al*., 2013). (Obeid *et al*., 2015) studied that nattokinase indicate that there is strong fibrinolytic activity. It was contributed to a healthy heart and cardiovascular. Viscosity also increases during the fermentation processes. *Bacillus natto* also produced γ-PGA that cause a viscous texture in natto product (Kada *et al*., 2008).

1.2.5.Natto characteristic

Characteristic of a good natto are: having a white color viscous substance, a slimy appearance, unique flavor, and a soft bean texture (Hu *et al*., 2010). Statements from Hu *et al*., (2010) and Shih *et al*., (2009) state that natto must have a silky and sticky appearance while stirred with a chopstick. Silky appearance in natto appears when natto is stirred with chopstick, where the appearance is like fibrin, it caused by fibrinolytic enzyme produced by *Bacillus subtilis* (Zhao *et al*., 2013). (Wei *et al*., 2001) states that silky and sticky texture are the important criteria for good natto. The viscous substance in natto caused by γ-PGA produced by *Bacillus subtilis*. γ-PGA is biodegradable because it synthesized by glutamic dependent bacteria such as *Bacillus subtilis* (Zhang *et al*., 2014).

Ammonia smell will appear in natto if natto is not stored in good condition after fermentation. Ammonia smell will deteriorate natto product because it creates an unpleasant smell and this smell is not acceptable by consumer. So, natto is strongly not recommended to be stored for longer time in inappropriate conditions (Weng *et al*., 2012). pH value of natto is also increased by production of ammonia. Ammonia plays a role in increase pH value from 8 into 9 (Leejeerajumnean *et al*., 2000). According to USDA (United States Department of Agriculture) natto has a 55.02% water content, 19.40% protein, 12.68% carbohydrates, 23.1 µg vitamin K (Phylloquinone), and total isoflavones 82.28 mg.

1.3.Purpose of research

The purpose of this research is to identify the effect of different fermentation time and different ratio of *Bacillus subtilis* var. natto and *Saccharomyces cerevisiae* on antioxidant activity, total phenolic content, color, pH, and sensory properties of Black Soybean Natto.