OPTIMIZATION AND BIOACTIVITIES OF BETA GLUCAN EXTRACTED FROM *Pleurotus ostreatus* CULTIVATED ON GERMINATED RICEBERRY RICE (*Oryza sativa*)

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Jutamat Nacha

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ABSTRACT

Beta-glucan (β-glucan), a polysaccharide recognized for its prebiotic effects and potential to inhibit colon cancer cell proliferation, is derived from sources like grains (β-1,3/1,4-glucan) and fungi (β-1,3/1,6-glucan). This study focused on optimizing the co-culture of germinated Riceberry rice with *Pleurotus ostreatus* mycelium to enhance the yield of this valuable bioactive compound. Optimal conditions for beta-glucan production were achieved after 9 days of co-cultivation, resulting in a substantial yield of 222.94 ± 2.33 mg/g of dried Riceberry rice. The extracted β-glucan was confirmed as a β-1,3/1,6-glucan using FTIR spectroscopy. Biological activity assessment demonstrated the extract's promising prebiotic properties, significantly promoting the growth of *Lacticaseibacillus rhamnosus* and *Bacillus coagulans*, as indicated by their respective Prebiotic Indices (6.36±0.72 and 115.70±10.19) and Prebiotic Activity Scores (0.56±0.03 and 1.39±0.06). Notably, the extract exhibited potent anti-colorectal cancer activity in vitro, inhibiting 66.23% of SW480 cell growth at a concentration of 1 mg/mL, a more pronounced effect than observed with standard beta-glucan and 5-fluorouracil. Additionally, the extract induced a significant level of apoptosis (28.91%) in these cancer cells. The formulated beta-glucan extract shows strong nutritional value, reinforcing its role as a promising health-promoting and anticancer nutraceutical.

Keywords: Beta-glucan, *Pleurotus ostreatus*, Riceberry Rice, Prebiotic, Anti-cancer, Mushroom Mycelium

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ABBREVIATIONS AND SYMBOLS

FTIR	Fourier Transform Infrared
NMR	Nuclear Magnetic Resonance Spectroscopy
TGA	Thermogravimetric Analysis
δ	Chemical Shifts
β-D-glucan	Beta-glucan
α-D-glucan	Alpha-glucan
GOS	Galacto-oligosaccharides
FOS	Fructo-oligosaccharides
GABA	Gamma-aminobutyric Acid
BSA	Bovine Serum Albumin
DMSO-d6	Dimethyl Sulfoxide-d 6
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
RO water	A water purification process that uses a semi-permeable membrane to separate water molecules from other substances
LCMS	Liquid Chromatography Mass Spectrometry
Symbiotic	A relationship is one in which organisms, people, or things exist together in a way that benefits them all
RR	Riceberry Rice
GR	Germinated Riceberry Rice
MTT assay	3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide Assay
(CCD 841 CoN)	Cell line name of colon cell
SW480	Cell cancer name of colon cancer cell
ATCC	American Type Culture Collection
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum

ABBREVIATIONS AND SYMBOLS

PI	Prebiotic Index
PAS	Prebiotic Activity Score
Beta-glucan STD	Standard Beta-glucan
P. ostreatus	*Pleurotus ostreatus*
L. rhamnosus	*Lacticaseibacillus rhamnosus*
B. coagulans	*Bacillus coagulans*
B. longum	*Bifidobacterium longum*
E. coli	*Escherichia coli*

CHAPTER 1

INTRODUCTION

Oyster mushrooms of the genus *Pleurotus* are highly popular globally and in Thailand due to their ease of cultivation, high nutritional value, and potential in the food and pharmaceutical industries (Hasan & Abdulhadi, 2022). Several species are found in Thailand, including *Pleurotus ostreatus*, *P. angustatus*, *P. cornucopiae*, *P. cystidiosus*, and *P. tuber-regium*, contributing significantly to the agricultural economy and offering substantial market value (Carrasco-González et al., 2017). From a health perspective, beta-glucans in *Pleurotus* enhance immunity via gut microbiota modulation and possess anti-inflammatory, antioxidant, and antitumor properties (Wang et al., 2022). Additionally, phenolic compounds in these mushrooms provide genoprotective effects against oxidative stress and genotoxins, while also exhibiting prebiotic activity by supporting beneficial gut bacteria (Boulaka et al., 2020).

Among the *Pleurotus* species found in Thailand, the white oyster mushroom (*P. ostreatus*) is one of particular interest for the production of biologically active compounds, especially beta-glucan, which is present in high amounts and exhibits more remarkable properties compared to other species. One reason why *P. ostreatus* is more attractive than other species due to its ease of cultivation, high yield, and the fact that the beta-glucan extracted from this mushroom demonstrates a wide range of bioactive effects (Lopez & Bhaktikul, 2018; Soodpakdee et al., 2022).

Beta-glucan is a polysaccharide that is found in the cell walls of bacteria, plants, mushrooms, and yeast (Markowiak & Ślizewska, 2017). The structure of beta-glucans is made up of β-D-glucose monomer units joined together by glycosidic connections at various positions (1,3), (1,4), or (1,6). beta-glucans from various sources will have varying impacts or functions. The immunomodulatory function of the beta-glucans is generally referred to as microbiota-independent. According to Bai et al. (2019) reported benefits of beta-glucan in healthy humans have many parts including reducing glycemia, cancer prevention and serum cholesterol, anti-inflammation, as well as improving immunity (Bai et al., 2019).

In terms of prebiotic properties, beta-glucan from *P. ostreatus* can stimulate the growth of beneficial gut bacteria such as *Lacticaseibacillus* and *Bifidobacterium*, which helps to balance the gut microbiota and promotes overall health (Soodpakdee et al., 2022). Regarding anticancer activity, beta-glucan from *P. ostreatus* stimulates the immune system, particularly the functions of macrophages, T-cells, and NK cells, enabling it to inhibit cancer cell growth and induce apoptosis in cancer cells. Additionally, there are reports that it can reduce side effects from chemotherapy and enhance the effectiveness of cancer treatment (Lavi et al., 2006; Susanti et al., 2018).

P. ostreatus cultivation uses lignocellulosic substrates like rice straw or sawdust (Plahlevanloo et al., 2023). However, germinated riceberry rice—a Thai purple rice variety rich in anthocyanins, gamma-aminobutyric acid (GABA), and antioxidants—has emerged as a superior substrate. Germination enhances nutrient bioavailability, potentially boosting mycelial growth and beta-glucan synthesis (Laokuldilok et al., 2011). In a solid-state (containing glutinous rice and rice bran) fermentation experiment, Sawangwan and Saman showed that rice fermented with Aspergillus oryzae had prebiotic characteristics (Sawangwan & Saman, 2016). These prebiotic properties of fungi could be due to their high concentration of short-chain polysaccharides, especially beta-glucan. The mycelium of edible mushrooms, including *Pleurotus ostreatus*, *Lentinus edodes*, *Auricularia auricula-judae*, and *Ganoderma lucidum,* also serves as an excellent source of prebiotic compounds. Research from Gaia Herbs et al. (2024) explains that mycelium can be cultured faster than the fruiting body (Gaia Herbs et al., 2024). Mycelium cultivation offers advantages over fruiting body culture for beta-glucan production due to controllable growth conditions, higher scalability, and optimized extraction efficiency (Brandon Rich, 2023). In this work, Riceberry rice will be fermented with *P. ostreatus* mycelium, and the optimal cultivation time for achieving the highest beta-glucan content will be investigated. Furthermore, the prebiotic and anti-colorectal cancer properties of the Riceberry rice will also be studied in comparison with non-fermented Riceberry rice.

1.1 Objectives

1.1.1 To optimize the cultivation of *Pleurotus ostreatus* on germinated Riceberry rice for β-glucan production.

1.1.2 To evaluate the prebiotic potential of extracted β-glucan

1.1.3 To determine the cytotoxic and pro-apoptotic effects of extracted β-glucan on colorectal cancer cell lines.

1.2 Scope of Research

This study aims to optimize the cultivation time of *Pleurotus ostreatus* on germinated Riceberry rice for high β-glucan production, measured via β-glucan assay, with consideration for upscaling. During *Pleurotus* cultivation on Riceberry rice, ligninolytic enzymes break down the rice cell walls, resulting in significant changes in bioactive compounds, including increased protein content, shifts in phenolic profiles, and alterations in fatty acid and amino acid composition. So, in this experiment, GABA and amino acid content must be measured during this cultivation. The β-glucan will then be extracted, and its quality assessed based on yield, purity, structural characteristics, and protein content. Furthermore, the prebiotic potential of this extract will be evaluated by examining its effect on the growth of *Lacticaseibacillus rhamnosus*, *Bacillus coagulans*, and *Bifidobacterium longum*, using a commercial β-glucan as a standard. The final part of this work is to investigate the anti-colorectal cancer activity *in vitro*, determining the extract's impact on SW480 (Human colon adenocarcinoma) and CCD 841 CoN (normal colon) cells through MTT and cell apoptosis assays

CHAPTER 2

LITERATURE REVIEW

2.1 *Pleurotus ostreatus*

Pleurotus ostreatus is an edible mushroom. The 40 species of genus *Pleurotus*, which are often known as "oyster mushrooms," occur abundantly in tropical and subtropical climates and are readily artificially farmed. This mushroom has been reported to have high bioactive compound content, such as protein, lipid, and polyphenol, but the main bioactive compound is short-chain polysaccharides (especially β-glucan) that are found in the cell wall of the mushroom, around 80% (Sawangwan et al., 2018). Recently has been much research, as reported in the many medicinal properties (figure 2.1) related to the bioactive compounds that are contained in *P. ostreatus*.

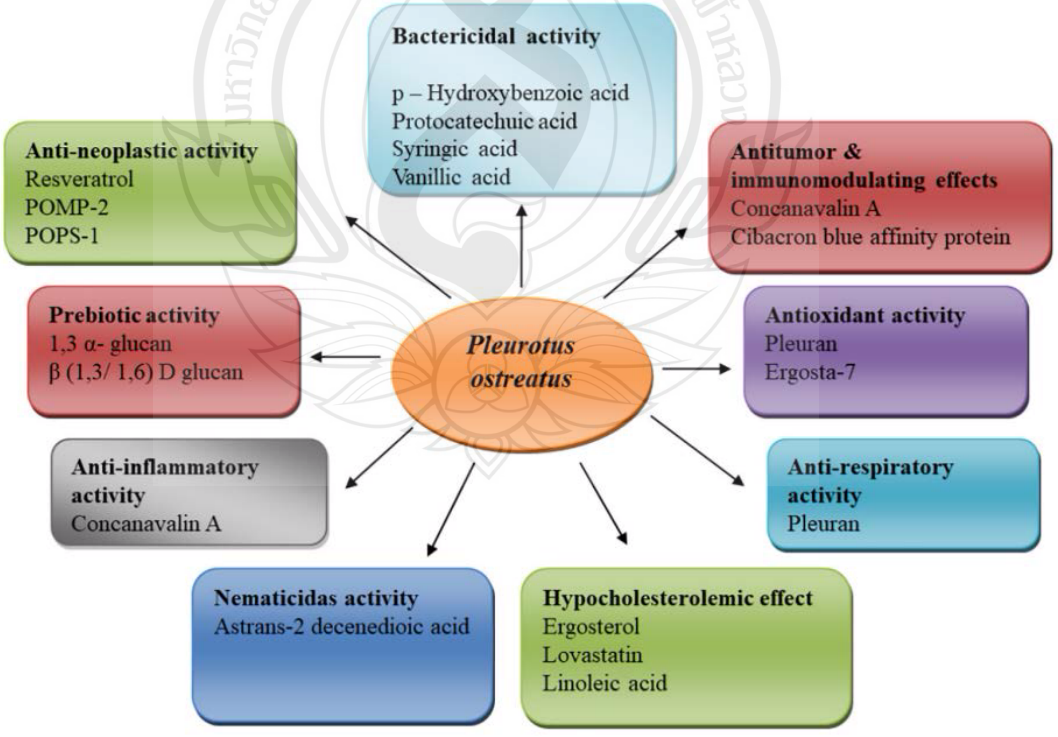


Figure 2.1 Bioactivities of the bioactive compounds found in *P. ostreatus*

2.2 Riceberry Rice (*Oryza sativa* L.)

Thailand has many rice varieties that are sold in the market. More research reported that the color rice has high bioactive compounds, including phenolic, GABA, vitamins, and antioxidants, more than white rice (Cho & Lim, 2018). Riceberry rice is a hybrid rice bred from a Thai white rice variety named "Hom Dok Mali 105" and Thai purple rice named " Hom Nin". This hybrid rice, called "Riceberry rice" for its higher nutrients, higher antioxidants, and softer texture than the Hom Nin rice. Riceberry rice and black rice have higher phenolic compounds than whole brown rice (B Krishna Veni, 2019) (Table 2.1) Riceberry rice is high in antioxidants, particularly anthocyanins, the molecules that give it its dark purple color. Antioxidants help the cell neutralize damaging free radicals, reducing oxidative stress and inflammation (Kammapana, 2023). However, vitamin E complex (α-, γ-, σ-tocotrienols, and tocopherols) and phenolic compounds have been reported to have the potential anti-cancer activities (Goufo & Trindade, 2014).

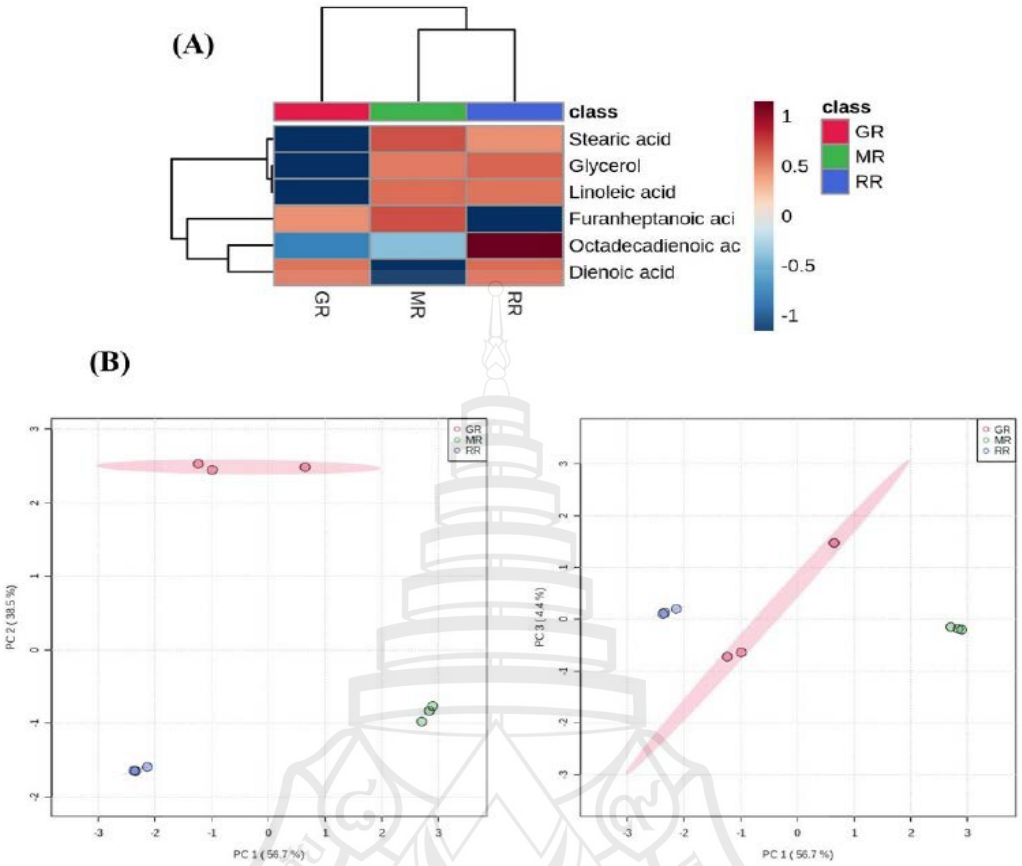
Table 2.1 Nutrition in whole rice of different colored rice

Composition	**Black rice**	**Whole brown rice**	**Riceberry rice**
Lipids (%)	3.87	0.9	-
Ash (%)	1.98	0.46	1.53
Protein (%)	11.9	2.58	10.5
Dietary Fiber (%)	5.67	1.8	2.71
Carbohydrates (%)	63.45	72.96	70.19
Phenolic compounds (mg/g)	23.78	2.45	20.79
Energy (kcal)	362	349	341

Source B Krishna Veni (2019)

2.3 Riceberry Rice (*Oryza sativa*) Cultivated with Mycelium of *Pleurotus ostreatus*

Fermentation is one of the classic techniques for converting biomass into value-added goods by employing microbes (Omarini et al., 2019). Rice contains high nutrients, including proteins, fibers, and minerals (Fe, P, and Mg), and antioxidant chemicals (Alauddin et al., 2017). And a source for microorganisms. *Pleurotus* species produce ligninolytic enzymes such as laccases, manganese peroxidases, and versatile peroxidases, which play a crucial role in breaking down lignocellulosic materials like rice cell walls (Enshasy et al., 2019). These enzymes degrade lignin, allowing access to cellulose and hemicellulose for further enzymatic hydrolysis (Asgher et al., 2016). When *Pleurotus* species digest rice cell walls using ligninolytic enzymes, several bioactive compounds undergo significant changes, including increased protein, modification of phenolic profiles, and changes in fatty acid composition. More research studies about the bioactive compounds of rice fermentation with *P. ostreatus*. This shows that the bioactive compound of the fatty acid group has a high level after Rice fermented with *P. ostreatus*. (Table 2.2) It fermented rice with mushrooms, increasing the content of fatty acids (Nacha et al., 2023). (Figure 2.2) Table 2.2 Fatty acid composition of *P. sapidus*, rice bran (unfermented), and fermented rice bran at different cultivation times (4 and 6 days) (Omarini et al., 2019).



Source Nacha et al. (2023)

Figure 2.2 Fatty acid levels in Riceberry rice (RR), Germinated Riceberry rice (GR), and Germinated Riceberry rice with mycelium (MR) (A) Different levels of fatty acid in samples from blue to red; blue shade indicates the lowest abundance in samples, while red shade denotes the highest amount. (B) Principal component analysis (PCA) of RR, GR, and MR was performed by comparing PC1 with PC2 and PC1 with PC3

Table 2.2 Fatty acid composition of *P. sapidus*, rice bran (unfermented), and fermented rice bran at different cultivation times (4 and 6 days)

		Fatty Acid Profile (%)			
Fatty Acid	**Nº Carbons**	***P. sapidus***	**Rice Bran**	**FRB day 4**	**FRB day 6**
Undecanoic acid	C11:0	2.28 ± 0.00 [a]	nd [b]	nd [b]	nd [b]
Lauric acid	C12:0	5.16 ± 0.28 [a]	0.02 ± 0.02 [b]	0.05 ± 0.02 [b]	0.06 ± 0.03 [b]
Myristic acid	C14:0	1.28 ± 1.11 [a]	nd [b]	nd [b]	nd [b]
Pentadecanoic acid	C15:0	2.02 ± 1.67 [a]	nd [b]	nd [b]	nd [b]
Palmitic acid	C16:0	16.03 ± 0.85 [a]	21.27 ± 0.14 [b]	22.09 ± 1.96 [b,c]	21.08 ± 0.62 [b]
Palmitoleic acid	C16:1	0.83 ± 0.26 [a]	0.22 ± 0.02 [b]	0.19 ± 0.01 [b]	0.17 ± 0.03 [b]
Margaric acid	C17:0	0.72 ± 0.00 [a]	nd [b]	nd	nd
Stearic acid	C18:0	8.50 ± 0.38 [a]	1.76 ± 0.53 [b]	2.04 ± 0.21 [b]	1.99 ± 0.10 [b]
Oleic acid	C18:1	19.62 ± 4.29 [a]	39.93 ± 0.35 [b]	40.26 ± 1.04 [b]	40.36 ± 0.28 [b]
Linoleic acid	C18:2	39.07 ± 4.52 [a]	33.94 ± 0.18 [b]	32.79 ± 0.87 [c]	34.07 ± 0.82 [b]

Table 2.2 (continued)

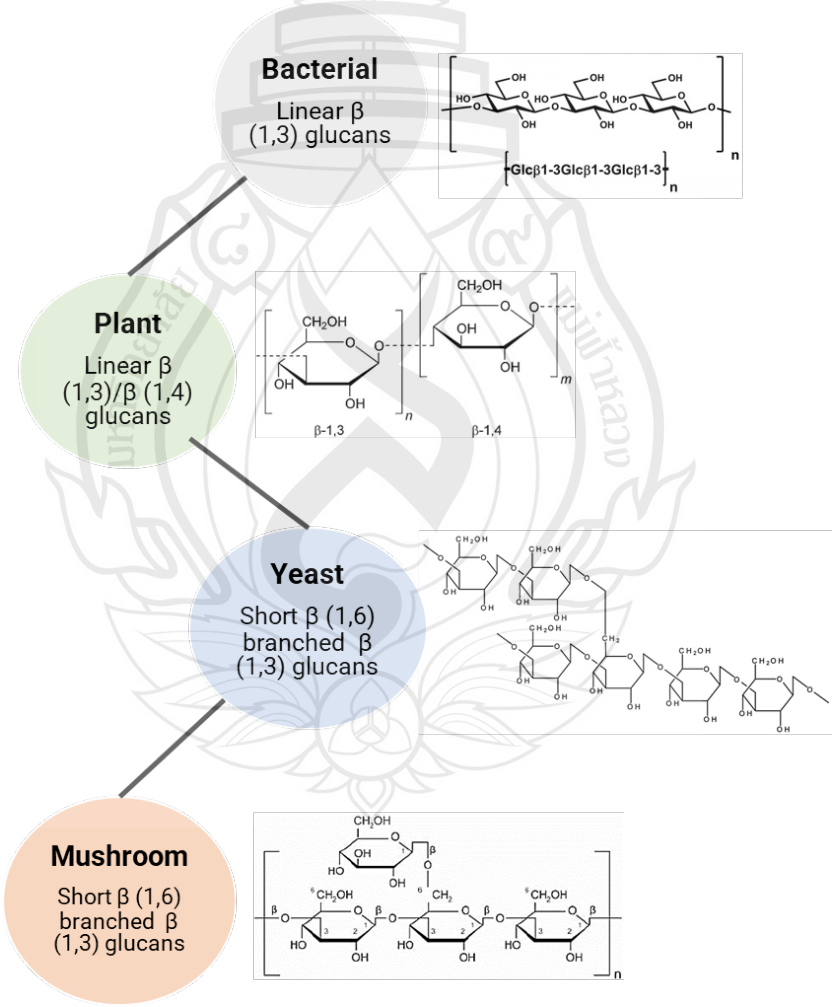
Fatty Acid	Nº Carbons	Fatty Acid Profile (%)			
		P. sapidus	Rice Bran	FRB day 4	FRB day 6
Linolenic acid	C18:3	0.42 ± 0.00 [a]	1.50 ± 0.01 [b]	1.35 ± 0.03 [c]	1.50 ± 0.24 [b,c]
Arachidic acid	C20:0	nd [a]	0.85 _ 0.01 [b]	0.83 _ 0.09 [b]	nd [a]
Eicosenoic acid	C20.1	0.67 ± 0.00 [a]	0.66 ± 0.18 [a,b]	0.69 ± 0.19 [a,b]	0.80 ± 0.06 [b]
Behenic acid	C22:0	1.05 ± 0.47 [a]	0.37 ± 0.00 [b]	0.05 ± 0.01 c	nd [d]
Lignoceric acid	C24:0	2.81 ± 0.95 [a]	nd [b]	nd [b]	nd [b]

Note nd = not detected; FRB = fermented rice bran at day 4 and day 6; the results are presented as mean ± standard deviation (n = 3); a–d: in each line different letters indicates that the mean value of at least one sample is significantly different, $p < 0.05$ (significant differences are represented by different letters).

Source Omarini et al. (2019)

2.4 Beta-glucan

Beta-glucan is a polysaccharide that is found in the cell walls of bacteria, plants, mushrooms, and yeast (Markowiak & Ślizewska, 2017). The structure of β-glucans is composed of β-D-glucose monomer units, which are held together by glycosidic linkages at differing positions (1,3), (1,4), or (1,6) (Figure 2.3). β-glucans from different sources will have different effects or functions (Bae et al., 2013). In bacteria cell wall has a linkage in position (1,3), plants have that linkage in positions (1,3) & (1,4), mushrooms and yeast have that linkage in the same position is (1,6) & (1,3), but its different lengths of structure (Jimmy Fu, 2020).

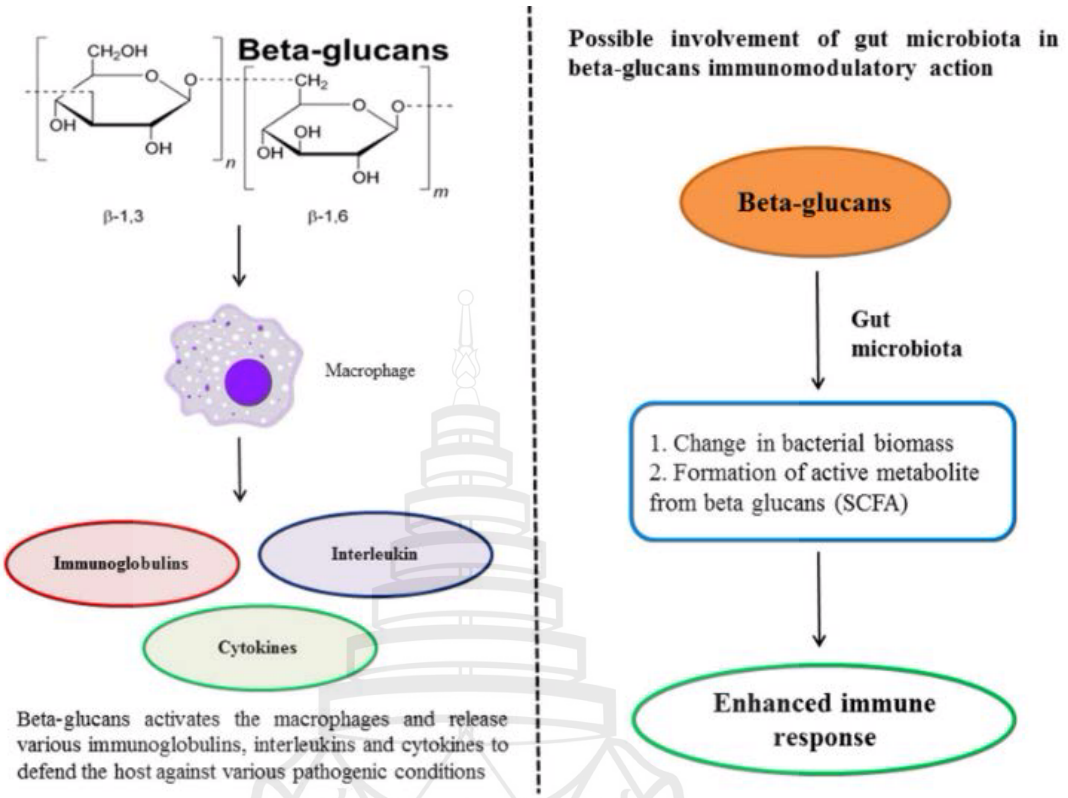


Source Murphy et al. (2020)

Figure 2.3 Structure of beta-glucan in different sources

2.4.1 Biological Activity of Beta-glucan

Beta-glucan has high potential as a prebiotic and an important factor in protecting the body against colon cancer (Qi et al., 2019). The immunomodulatory function of the beta-glucans is generally referred to as microbiota-independent because of their ability to bind to the immune cells directly. The other, beta-glucans, are recognized for their anti-cancer capabilities through their direct impact on p53 expression in SW480 cells, which is not well proven in the search results. However, they may have an indirect impact on p53 activity via processes like as apoptosis and altered metabolism. The p53 status (wild-type or mutant) can also play an important role in influencing the responsiveness to therapies (Solomon et al., 2018). Since beta-glucans are digested and fermented by various bacterial flora in the gut. The part of the immune system, beta-glucan, can't attack cancer cells directly, but it comes to binds with receptors on macrophages to activate macrophages to release immunoglobulins, interleukins, and cytokines to defend the host against various pathogenic conditions. For part of the gut microbe, they will break down beta-glucan to short-chain fatty acids, which inhibit inflammatory reactions and enhance immune response (Jayachandran et al., 2018) (Figure 2.4).



Source Jayachandran et al. (2018)

Figure 2.4 Possible mechanism of action of beta-glucan immunomodulatory activity via gut microbiota

2.4.2 Impact of Beta-glucan

The impact of beta-glucan was shown in 2 parts. The first part is the modulation of gut microbiota (Chaikliang et al., 2015; Mikkelsen et al., 2017; Velikonja et al., 2019) form information in Table 2.3 explained that beta-glucan from different sources promoted the growth of microorganisms differently. The second part is colon cancer protection. Beta-glucan from cereal and mushroom extract has an apoptotic effect on tumor cells and human colon cancer cells (Dong et al., 2019; Shamekhi et al., 2020).

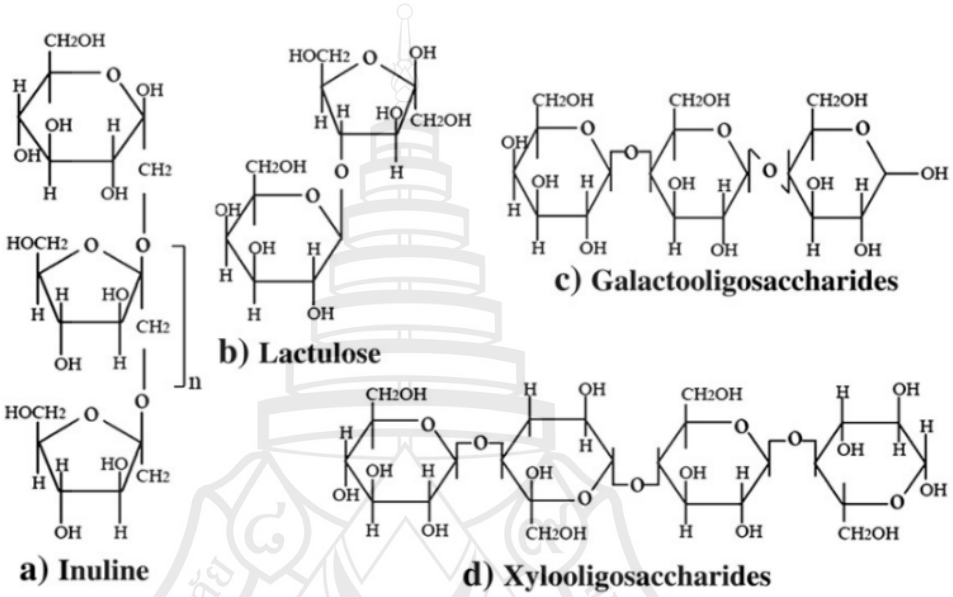
Table 2.3 Impact of beta-glucan on the nodulation of gut microbiota and colon cancer protection

Impact of Beta-glucan	Source	Benefits	References
Modulation of Gut Microbiota	Cereal	*Bifidobacterium spp.* and *Akkermansia municiphila*	Velikonja et al. (2019)
Modulation of Gut Microbiota	Cereal	*Bifidobacterium*	Mikkelsen et al. (2017)
Colon cancer protection	Mushroom extract *(A. auricula* Judae)	*Bifidobacteria / Lactobacillus*	Chaikliang et al. (2015)
	Cereal	Apoptosis of tumor cells	Dong et al. (2019)
Colon cancer protection	Mushroom extract	Apoptosis of Colorectal Cancer (SW480) in *P. ostreatus* and *V. volvacea*	Shamekhi et al. (2020)

2.5 Prebiotic

Prebiotics are substrates, nondigestible carbohydrates that humans cannot digest. This substrate can be digested by using microorganisms in the gastrointestinal system or probiotics. Which are live helpful microbes, are basically "fuel" for these good bacteria. They promote the growth or activity of probiotics (Kolida et al., 2002). Nowadays, sources of Prebiotics that occur naturally in many foods can be separated into 4 groups: vegetables, fruit, whole grains, and others. The research of Aida et al. (2009) stated that mushrooms have potential sources of prebiotics because the structure of the cell wall that contains chitin, hemicellulose, β- and α-glucans, mannans, and Xylans galactans (Aida et al., 2009). All of these have fiber, which is a property of

prebiotics. Several carbohydrates function as prebiotics, including Fructooligosaccharides (FOS), Inulin, Galactooligosaccharides (GOS), Xylooligosaccharides (XOS), and Lactulose (Huebner et al., 2007). Sources are different that affect to promotion of the growth of probiotics differ because the structure of prebiotics differs. (Figure 2.5) (Choque Delgado et al., 2011)



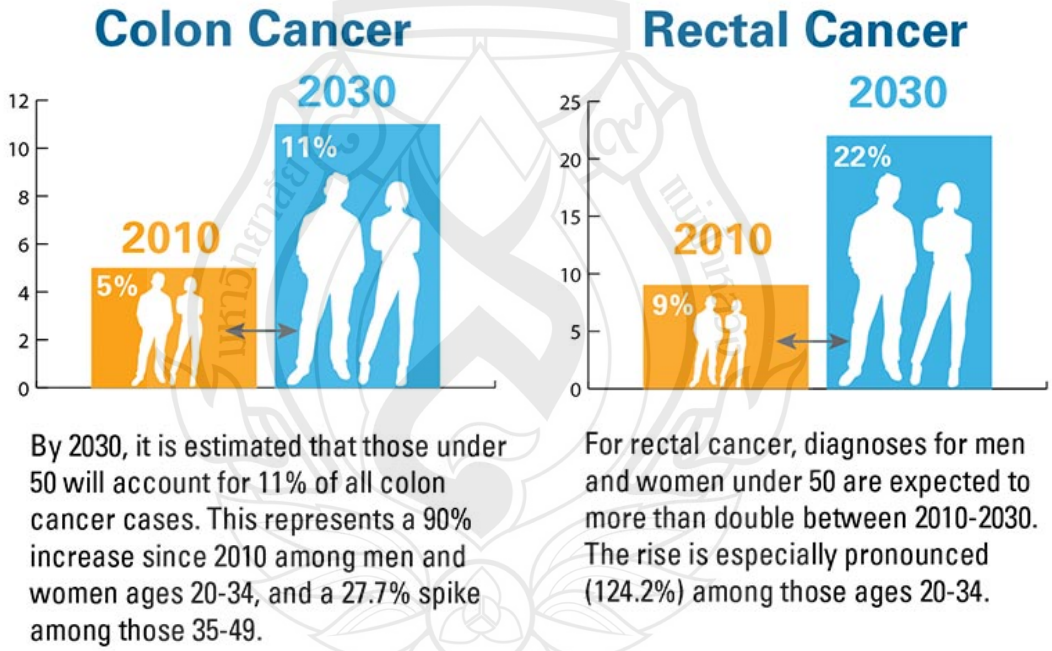
Source Choque et al. (2011)

Figure 2.5 The structural formulas of prebiotics

2.6 Colorectal Cancer

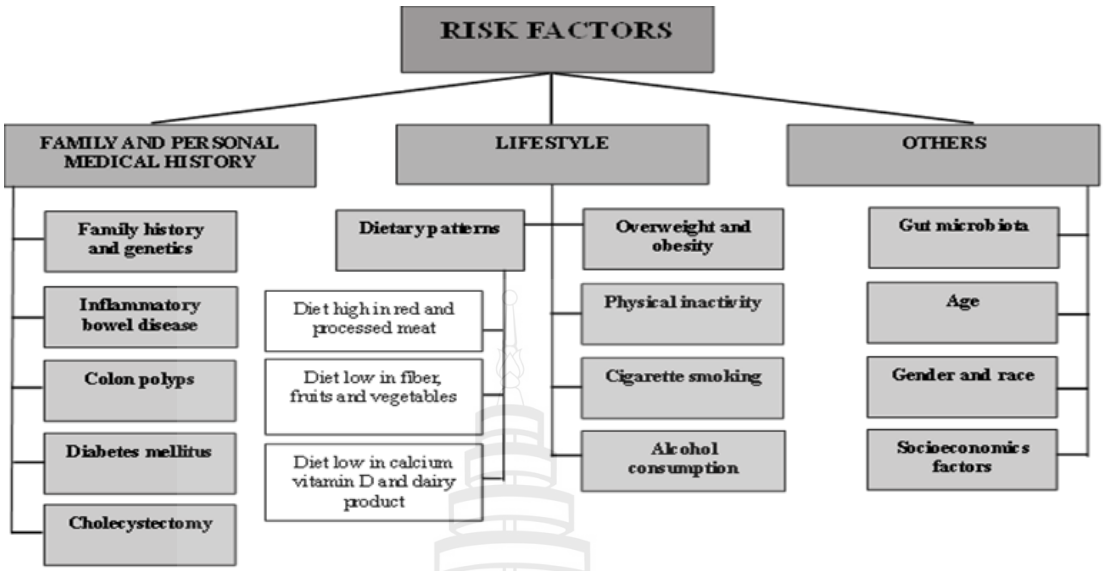
According to data from Colorectal Cancer Statistics in 2023 found that CRC is the second most common cause of cancer death in the country (Siegel et al., 2023). Colorectal cancer is a cancer that starts in the colon or rectum. The colon and rectum are digestive system components that constitute the large intestine. This cancer develops in the colon or the rectum. The colon and rectum are parts of the digestive system that make up the large intestine. Colorectal cancer commonly begins as a small polyp or growth on the inner lining of the colon or rectum, which can develop into malignant cells (Sawicki et al., 2021). According to data from Dana-Farber Cancer Institute, 2030, colon cancer rates in young people will increase rates more than 124% (Figure 2.6). According to research by Sawicki et al. (2021), if a family member or

relative has ever had cancer, there is an increased risk. However, other variables contribute to the incidence of cancer. A critical role in the genesis of CRC, Obesity, and overweight is linked, according to research. Physical inactivity, smoking, alcohol drinking, and poor eating habits (low fiber diet, fruits and vegetables, calcium and dietary supplements, red foods, and highly processed meats) all raise the risk of CRC (Sawicki et al., 2021). (Figure 2.7) The causes of colorectal cancer include consumption, which is the accumulation of toxins in the colon. That affects abnormal excretion, poor absorption of nutrients, leading to an imbalance in the intestines occurs through consumption, resulting in the accumulation of toxins in the colon. This results in abnormal excretion, poor absorption of nutrients, leading to an imbalance in the intestines.



Source June Leslie (2021)

Figure 2.6 Expectation of colorectal cancer statistics in 2030



Source Sawicki et al. (2021)

Figure 2.7 The main risk factors associated with colorectal cancer

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Sample Preparation

3.1.1 Rice Strain and Culture Conditions

The Riceberry rice (*Oryza sativa* L.) used in this research was sourced from a farmer cooperative located in Chiang Rai province, Thailand. To activate the grains and promote nutrient release through germination, an initial step involved rinsing the rice twice. Following this, the rice was soaked in RO water using a 1:1 rice-to-water ratio for 16 hours. Subsequently, sterilization was achieved through autoclaving at 121 °C under 15 psi of pressure for 15 minutes.

3.1.2 Fungal Strain and Maintenance

Pleurotus ostreatus, the fungal strain utilized in this study, was obtained from the local mushroom farm in Chiang Rai province, Thailand. The fungal mycelium was cultured on potato dextrose agar (PDA) at 30 °C for 10 days and subsequently stored at 4 °C until experimentation.

3.1.3 Mycelium Cultivation on Rice

Fungal mycelium, cultured on PDA at 30 °C for 10 days, was inoculated into potato dextrose broth (PDB) at a density of 15 plugs per 300 mL and incubated at 30 °C with shaking at 125 rpm for 7 days. The resulting mycelial biomass was then used to inoculate Riceberry rice at a 10% w/v ratio and incubated at 30 °C for a time course of 3, 5, 7, 9, 11, 13, and 15 days to determine the optimal cultivation time for beta-glucan yield. Samples were collected and dried at 60 °C for 1-2 days. The dried samples were processed into powder using a drum drying method before analysis of beta-glucan, GABA, and amino acid content. Beta-glucan content was determined using the K-YBGL β-glucan assay kit.

3.2 Determination of β-glucan using K-YBGL β-glucan Assay Kit

K-EBHLG β-glucan Assay Kit (Yeast and Mushrooms) (Megazyme, Ireland) was used to determine β-glucan content. Enzymatic Yeast Beta-Glucan test kit, an enzymatic procedure for the measurement and analysis of 1,3:1,6-β-glucan in yeast. 20 mg of sample powder was added to 0.4 ml of 2 M Potassium Hydroxide on ice-cold conditions and vortexed. Then, it was cooled on an ice bath for 30 min. 1.6 ml of 1.2 M sodium acetate buffer (pH 3.8) was added and vortexed. After 40 µl of Glucamix enzyme was added and cap the tubes were capped. Continue mixing in the ice water bath for 2 min before transferring into a water bath set at 40 °C and incubating (without stirring) for 16 hours. After 16 hrs., add 10 mL of distilled water. 1 mL of solution was centrifuged at 3000 rpm for 10 minutes and filtered with Whatman Type I. 0.1 mL of solution was transferred into a new test tube, and 4 mL of GOPOD reagent was added, vortexed well, and incubated at 40 °C for 20 minutes before measuring the blank at OD at 510 nm. β-Glucan (% w/w) was calculated from equation 1.

$$\beta\text{-Glucan (\% w/w)} = \Delta A \times F \times \frac{12.04}{0.1} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180} \qquad (1)$$

ΔA = Absorbance read against reagent blank.

F = Conversion from absorbance to µg (150 µg of D-glucose standard divided by the GOPOD absorbance of this 150 µg)

W = Weight of sample analyzed in mg.

3.3 Determination of Gamma-aminobutyric Acid (GABA) and Amino Acid by using LC-MS/MS Analysis

3.3.1 Sample Extraction

The *P. ostreatus* mycelium cultivated on germinated Riceberry rice powder is analyzed amount of GABA and amino acids. 1 g of crude is extracted in 15 mL of

deionized water at 90 ℃ for 5 min. The solution is centrifuged at 8000 rpm for 15 min. The supernatant is filtered through a 0.45-μm filter membrane (Gökmen et al., 2012).

3.3.2 Gamma-Aminobutyric Acid (GABA) Determination

The GABA content in crude extract was analyzed following the method previously described by Qui et al. (2020), using 1 mg/mL of standard GABA. (Sigma, USA) are prepared as stock solutions. The serial dilution of the stock solution (0.76, 1.56, 3.12, 6.25, 12.5, 25, and 50 μg/mL) will be analyzed by using LC-MS/MS analysis. One microliter of crude extracts will be analyzed by reversed-phase ACE Excel 3 column (Water, USA) (HILIC silica (100 mm×2.1 mm) (120 HILC-Z)) at 30 ℃ and a flow rate of 0.4 mL/min. The condition of the mobile phase is (0.1% formic acid in water (A): 10 nM ammonium formate (B)) (Qiu X, 2020).

3.3.3 Amino Acid Determination

The amino acid content in the crude extract was analyzed following the method previously described by Kennedy et al. (2021). Standard of amino acid (Sigma, USA) are prepared within the range 0.01-100 (nmol/mL) was analyzed by using LC-MS/MS analysis. 1μl of crude extracts will be analyzed by reversed-phase ACE Excel 3 column (Water, USA) (HILIC silica (100 mm×2.1 mm) (120 HILC-Z)) at 30 ℃ and a flow rate of 0.2 mL/min. A linear gradient system was used with mobile phase A (: 0.1% Formic acid in Water) and mobile phase B (0.1% Formic acid in acetonitrile) as follows: 1% solvent B for 2 min (0–2 min), 10% B for 2 min (2–4 min), 90% B held for 6 min, then to 90% and back to 1% B for 0.2 min (10–10.2 min). The column was re-equilibrated for 4.8 min (10.2–15 min) under the initial conditions (0% B). Total run time was 15 min, including column re-equilibration (Kennedy & Bivens, 2021).

3.4 Determination of Protein

3.4.1 Bradford Assay

The Bradford assay is a widely used method for protein quantification due to its simplicity, speed, and sensitivity. The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. The method was adopted from Kruger et al. (2004). In brief, the crude beta-glucan extract was dissolved in 2 mg in 100 μl of DI water and

centrifuged at 6000 rpm for 10 min to collect supernatant. The supernatant was added into 96 wells (5 µl) and 250 µl of 1x dye reagent (Coomassie Brilliant Blue G-250). The reaction was incubated in the dark at 25 ℃ for 5 min. After that, measure protein concentration by using a microplate reader at 595 nm of absorbance (Kruger, 2004).

3.4.2 Kjeldahl Method

The Kjeldahl method is a widely used technique for determining the nitrogen content in organic and inorganic substances, particularly in food, fertilizers, and environmental samples. Developed by Johan Kjeldahl in 1883, this method is essential for estimating protein content based on nitrogen concentration (Sáez-Plaza et al., 2013). The Kjeldahl method consists of three main steps: digestion, distillation, and titration. First step, the sample is heated with concentrated sulfuric acid (H_2SO_4), breaking down organic matter and converting nitrogen into ammonium sulfate $(NH_4)_2SO_4$. Catalysts like copper, selenium, or mercury are often added to accelerate the reaction. Next step, Sodium hydroxide (NaOH) is added to neutralize the acid and convert ammonium ions into ammonia (NH_3). The ammonia is then distilled and captured in a boric acid (H_3BO_3) solution. Last step, the trapped ammonia is quantified using acid-base titration, typically with hydrochloric acid (HCl) or sulfuric acid (H_2SO_4). The amount of acid required to neutralize the ammonia determines the nitrogen content in the sample.

3.5 Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) is a technique used to measure changes in the mass of a material as a function of temperature or time. The thermogravimetric analysis (TGA) of isolated and standard β-glucan from Saccharomyces cerevisiae (Sigma-Aldrich, USA) was conducted using a modified version of a previously established method. The thermal properties were assessed with a Pyris 1 TGA analyzer (Perkin Elmer, USA) under a nitrogen flow of 25 mL/min. A precisely measured 10 mg sample was placed in open alumina crucibles and heated from 50 °C to 800 °C at a rate of 10 °C/min. The resulting thermogravimetric curve, displaying percent weight loss versus temperature, was used to evaluate the thermal stability of the polysaccharides (Suraiya et al., 2024).

3.6 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical technique used to identify and characterize materials based on their infrared absorption patterns. Crude beta-glucan extract was analyzed using FTIR spectroscopy within the frequency range of 4000–400 cm^{-1}, employing a Spectrum X instrument (Perkin Elmer, USA) based on the method by Ul Ashraf et al. Beta-glucan was individually placed on the attenuated total reflection (ATR) crystal, with pressure applied to eliminate air from the powder particles. The final spectrum was obtained by averaging 128 scans at 4 different resolutions (Suraiya et al., 2024).

3.7 Probiotic Growth Stimulation

L. plantarum, *B. coagulans*, and *B. longum* were probiotics that were used in to prebiotic test and compared with *E. coli* (negative control). The microbial strains were inoculated at a 1% v/v concentration into prebiotic media and incubated at 37 °C with 200 rpm for 48 hrs. The growth of the microbial strains was collected every four hours, and the number of colony-forming units (CFU) was determined by counting colonies on MRS (DEMAN, ROGOSA, SHARPE) plates for the probiotic strains and nutrient agar plates for *E. coli*. The plates were incubated at 37 °C for 24-48 hrs. Probiotic growth stimulation (Figueroa-González et al., 2019).

3.8 Prebiotic Index and Prebiotic Activity Score

Prebiotic index and prebiotic activity score were used to determine the potential activity of prebiotics that promote the growth of probiotics. The prebiotic index (PI) value was determined from equation (2). If PI value close to 1 suggests low effectiveness of the evaluated carbohydrate, whereas a value higher than 1 indicates a positive impact on probiotic growth. Prebiotic activity scores (PA) were determined from equation (3). When compared to lactose, a high prebiotic activity score (PA) implies that probiotic bacteria can survive well. When grown on prebiotics rather than

lactose, *E. coli* should develop at a slower pace. As a consequence, the prebiotic activity score may be calculated using equation (3) in connection with a given probiotic strain (Figueroa-González et al., 2019).

$$PI = \frac{\text{CFU of probiotic in prebiotic carbohydrate}}{\text{CFU of probiotic in control carbohydrate}} \qquad (2)$$

$$PA = \frac{\text{(LogP24-LogP0) prebiotic}}{\text{(LogP24-LogP0) lactose}} - \frac{\text{(LogE24-LogE0) prebiotic}}{\text{(LogE24-LogE0) lactose}} \qquad (3)$$

Where PA is a prebiotic activity score, LogP is the log of growth (CFU/mL) of the probiotic bacteria at 24 hours (P24) and 0 hours (P0) of culture on prebiotic and lactose, LogE is the log of growth (CFU/mL) of E. coli at 24 hours (E24) and 0 hours (E0) of culture on prebiotic and lactose (Figueroa-González et al., 2019).

3.9 Beta-glucan Preparation for Cell Culture Testing

The beta-glucan extraction method was developed from Saffa et al. (2020). In brief, the sample powder was dissolved with DI water in a ratio of 1:20 (w/v). The solution was adjusted to pH 7.0 with 20% Na_2CO_3. Boiling at 90 °C for 6 hrs. with shaking (100 rpm). After boiling, the solution was vortexed and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was transferred to a new container, and the pH was adjusted to 4.5 with 2 M of HCl. The solution was centrifuged at 8000 rpm for 30 min at 4 °C. The supernatant was transferred and mixed with absolute ethanol in a ratio of 1:1 for 12 h at 4 °C to precipitate the beta-glucan. The mixture was separated by using a centrifuge at 3000 rpm for 10 min at 4 °C. Lastly, the pellet was evaporated with absolute ethanol by using a Hot Air Oven-dried at 60 °C (Al-Saffar et al., 2020).

3.10 Anti-cancer Activities

3.10.1 Cell Culture Conditions

Colon adenocarcinoma cell lines (SW480) and normal human colon tissue (CCD 841 CoN) were purchased from the American Type Culture Collection (ATCC, USA). Cell line was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (100 units/mL), was incubated at 37 ˚C, 5% CO_2, 95% atmospheric air (McCool et al., 1994).

3.10.2 Cell Cytotoxicity Assay

According to Liu et al. 2023 reported β-glucan *Lentinus edodes* has potential for anticancer cells (Liu et al., 2023). The effects of β-glucan on SW480 cell proliferation were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The method was adapted from Liu et al. 2023. In brief, SW480 cells were seeded at a concentration of 5 x 10^3 cells/well in a 96-well plate for 12 hours. Then the media was removed and β-glucan in different concentrations was added, 200 µl (range 25 to 400 µg/mL). The negative control is well well-treated cell. The cells will then be treated with the various β-glucan concentrations and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. After 24 h, 10 µL of MTT (Sigma Aldrich, USA) was added to each well and incubated again for 4 hrs. The solution was discarded, and 100 µl of DMSO was added to each well, and the absorbance was measured at 540 nm using a microplate reader. Cell proliferation rate was calculated using equation (4). Cytotoxicity of each sample was expressed as an IC_{50} value.

$$\% \text{ Viability} = \frac{\text{absorbance of control - absorbance of tested sample}}{\text{absorbance of control}} \times 100 \qquad (4)$$

3.10.3 Cell Apoptosis Assay

Annexin V–FITC Apoptosis Detection Kit was used to determine cell apoptosis by the manufacturer’s protocol and according to Liu et al. 2023 (Liu et al., 2023). SW480 cells (6×10^5 cells/well) were seeded into 6-well plates and incubated for

24 hours. After that, the existing media was discarded, and new media having varied β-glucan concentrations were added and incubated for 48 hours. The cells were collected and resuspended in 200 µl binding buffer. The samples were incubated for 15 minutes at room temperature in the dark after being mixed with 2 µl of Annexin V-FITC and 2 µl of PI. The apoptosis will be examined on a FACS can flow cytometer (1×10^6 cells were counted for each measurement).

3.11 Statistical Analysis

The SPSS statistical software was used to analyze. All experiments were performed in triplicate (n=3), and data are presented as mean±SD. The experimental design was used factorial design. *p*-values of < 0.05 will be considered to be significantly different.

CHAPTER 4

RESULT

4.1 Optimization of Cultivation Time for Commercial-scale β-glucan Production

The cultivation of *P. ostreatus* mycelium on Riceberry rice lasted for 15 days, with the mycelium beginning to grow on the rice from day 3 (as shown in Figure 4.1). Samples were collected to measure the beta-glucan content and various bioactive compounds from the product before scaling up production, conducted every 2 days from day 3 to day 15.

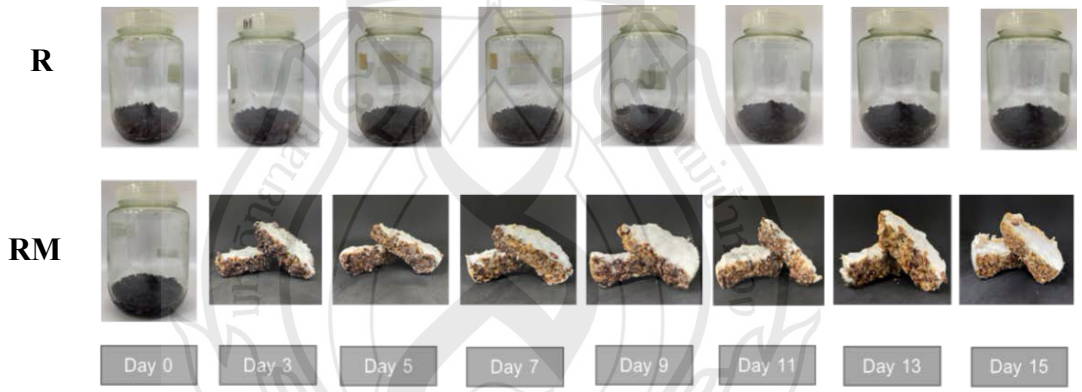


Figure 4.1 *P. ostreatus* mycelium cultivated on germinated Riceberry rice (RM) compared with Riceberry rice (R) from day 0 to day 15

4.2 Examination of Bioactive Compounds in *P. ostreatus* Mycelium Cultivated on Riceberry Rice before Scaling Up Production

From the analysis of beta-glucan content in samples of oyster mushroom mycelium grown on Riceberry rice, it was found that the highest amount of beta-glucan occurred on day 9, with a concentration of 148.04±7.68 mg/g of dry sample. In contrast, the beta-glucan content in samples of Riceberry rice that were not cultivated with oyster mushroom mycelium was only 0.48±0.10 mg/g of dry sample. Furthermore, the beta-glucan content significantly decreased when cultivation continued until day 15, as shown in Figure 4.2. Therefore, the oyster mushroom mycelium on Riceberry rice at the 9-day mark is deemed suitable for scaling up beta-glucan production.

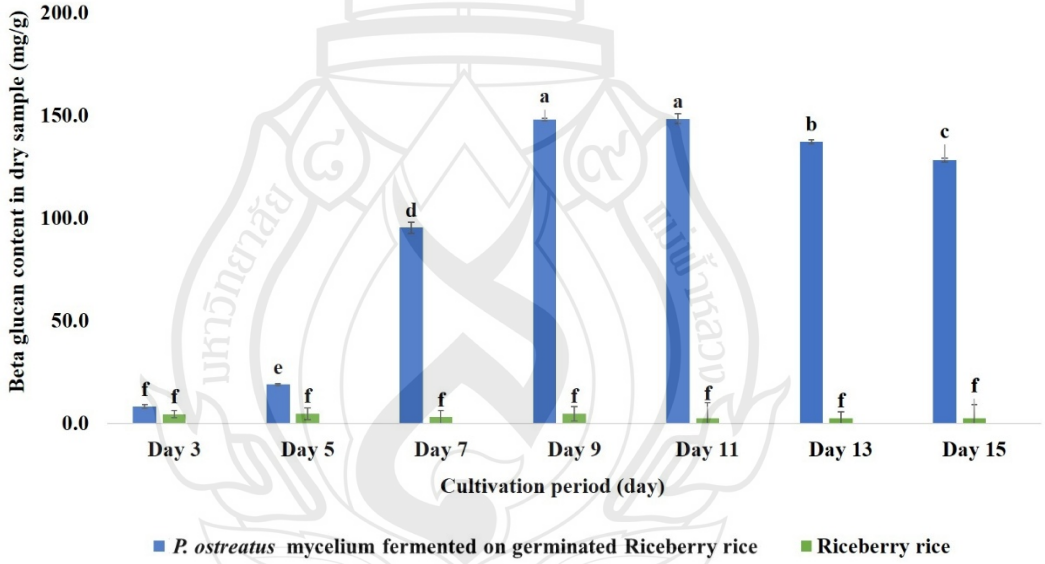


Figure 4.2 Comparison of beta-glucan levels in *P. ostreatus* mycelium cultivated on Riceberry rice and Riceberry rice samples

In addition to measuring beta-glucan levels, other important compounds such as GABA and various essential amino acids were also present in the samples. Therefore, an analysis of GABA and essential amino acid content in the mycelium samples grown on Riceberry rice was conducted. It was found that GABA levels in sprouted Riceberry rice significantly increased after cultivating the mycelium for 9 days, reaching a value of 0.88 ± 0.05 mg/g of dry sample, as shown in Figure 4.3. Regarding the amounts of amino acids and short peptides in the samples, it was observed that amino acids were produced during the cultivation of mycelium on Riceberry rice compared to plain Riceberry rice. The amino acids identified during the first day of cultivating oyster mushroom mycelium on Riceberry rice included aspartic acid, isoleucine, glycine, alanine, leucine, methionine, cysteine, glutamine, and tryptophan. Additionally, amino acids produced after cultivating the mycelium from day 3 onward included arginine, serine, threonine, lysine, glutamic acid, tyrosine, and histidine, as shown in Figure 4.4.

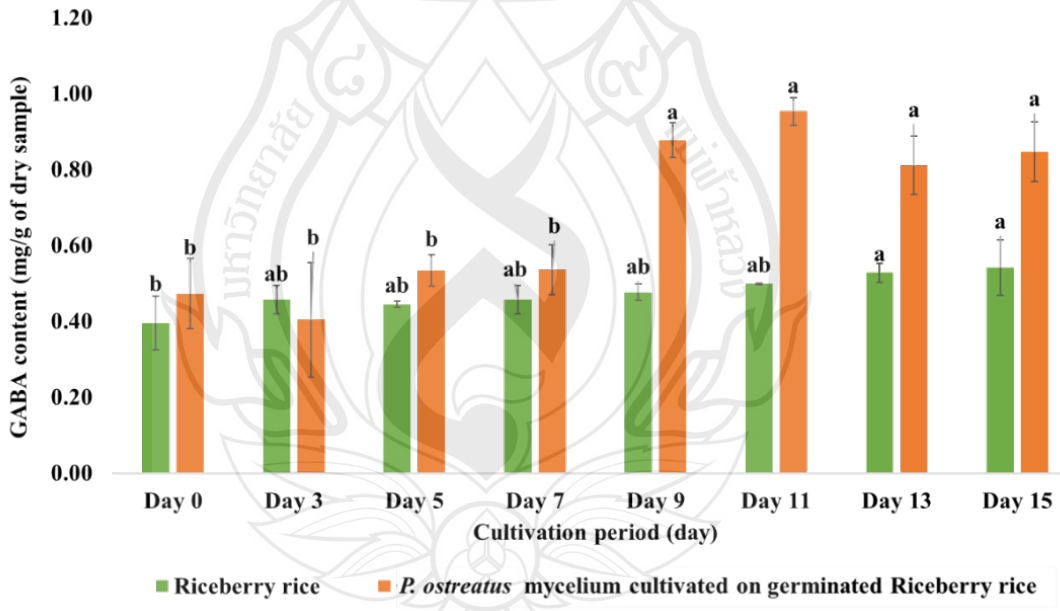


Figure 4.3 Comparison of GABA levels in Riceberry rice samples and *P. ostreatus* mycelium grown on Riceberry rice over different periods

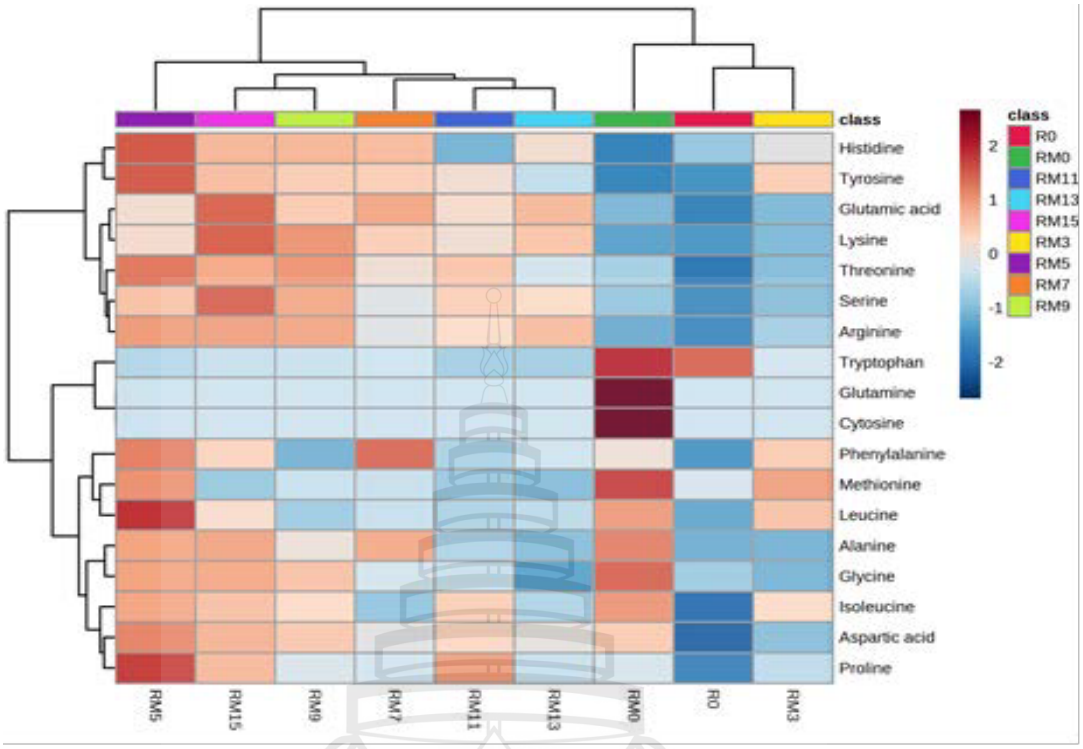


Figure 4.4 Levels of amino acids and short peptides in Riceberry rice samples and mycelium grown on Riceberry rice (RM) over different periods from day 0 to day 15 (RM0 to RM15) compared to plain Riceberry rice (R)

4.3 Upscaling Beta-glucan Production from *P. ostreatus* Mycelium Cultivated on Riceberry Rice at a Scale of 60 Kilograms

The results of the beta-glucan production experiment involved comparing the use of 10% and 20% w/w mycelium inoculum, as shown in Figure 4.5. During the period from day 0 to day 9, the beta-glucan content was analyzed, revealing that the concentrations from the 10% and 20% inoculate did not show significant differences. On day 9, the measured beta-glucan content in samples using a 10% inoculum was 131.09±9.22 mg/g of dry sample, while the samples with a 20% inoculum had a beta-glucan content of 148.03±7.86 mg/g of dry sample, as illustrated in Figure 4.6.

This study indicates that an inoculum amount of 10% w/v is sufficient and suitable for the production of beta-glucan from mushroom mycelium.

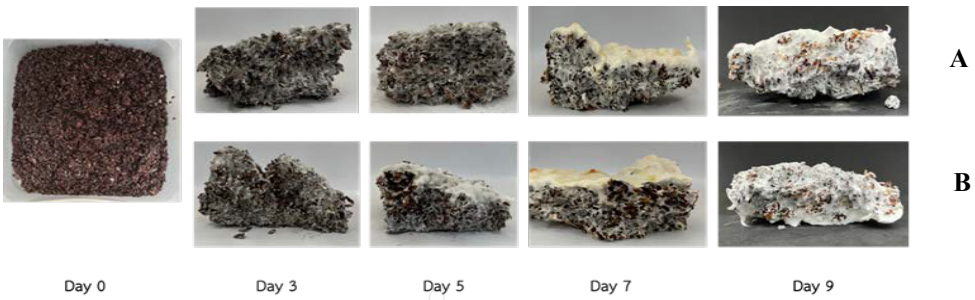


Figure 4.5 Comparison of mycelium grown on rice at different inoculum percentages (10% (A) and 20% (B)) from day 0 to day 9

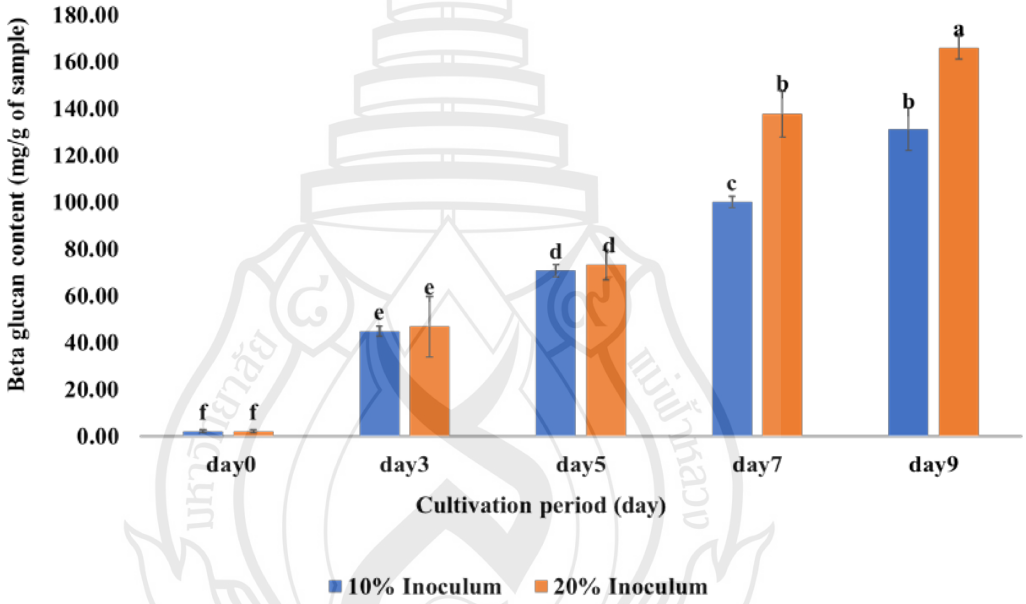


Figure 4.6 Comparison of beta-glucan levels in Riceberry rice samples and mycelium grown on Riceberry rice at different inoculum amounts and periods

4.4 Determination of Beta-glucan from Upscaling of *P. ostreatus* Mycelium Cultivated on Riceberry Rice

From the upscaling of production in the process of cultivating mycelium on Riceberry rice, it was found that increased production capacity still allowed beta-glucan equivalent to that before expansion, while reducing the inoculum amount from 20% to 10%. However, the amount of beta-glucan obtained remained comparable. In this experiment, samples of mycelium grown on Riceberry rice were extracted to obtain

beta-glucan extract, yielding a beta-glucan extraction rate of 54.95±0.63%. The beta-glucan content in this extract was measured at 222.94±2.33 mg per gram of beta-glucan extract, which is equivalent to the beta-glucan content in the powdered mycelium grown on Riceberry rice before extraction. Additionally, the extract contained GABA at a concentration of 6.60±0.53 mg per gram of beta-glucan extract, along with essential amino acids, primarily glutamic acid and arginine, which were found at concentrations of 906.21±83.93 mg and 992.68±28.11 mg per gram of beta-glucan extract, respectively, as shown in Table 4.1.

Table 4.1 Amounts of various important compounds found in beta-glucan extract

Various important compounds	**Amounts of compounds (mg/g of dry sample)**
Beta glucan	222.94±2.33
GABA	6.60±0.53
Aspartic acid	256.09±30.94
Serine	299.31±13.20
Alanine	212.50±27.37
Glycine	70.81±9.69
Glutamine	55.03±3.92
Threonine	183.84±19.52
Glutamic acid	906.21±83.93
Proline	216.61±252.44
Lysine	56.38±4.05
Arginine	992.68±28.11
Valine	96.28±8.17
Tyrosine	55.43±5.36
Phenylalanine	82.84±6.03

4.5 Examination of the Physical Properties of Beta-glucan Extracted from *P. ostreatus* Mycelium Cultivated on Germinated Riceberry Rice

4.5.1 Protein Determination in Crude Extract

The results of this experiment showed that the mycelium samples grown on Riceberry rice contained a higher amount of water-soluble protein compared to the extracted beta-glucan. The residual mycelium after beta-glucan extraction predominantly consisted of insoluble proteins. The total protein content in the extracted beta-glucan was found to be 9.05±0.29%, as illustrated in Figure 4.7. In the initial beta-glucan extraction process, water is used as the extraction solvent, which may not be able to dissolve all the proteins. As a result, a significant portion of the proteins remains in the residual material after extraction. In some cases, proteins may be retained or bound to the cellular structure rather than existing in a water-soluble form, leading to a substantial amount of protein remaining in the residue after extraction.

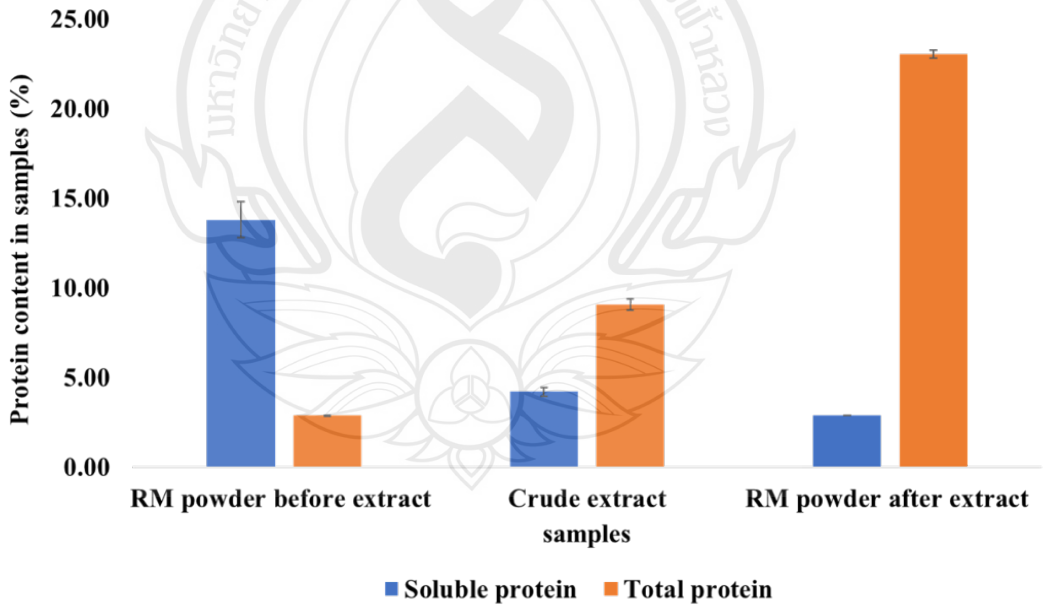


Figure 4.7 Comparison of water-soluble protein and total protein obtained from powdered mycelium grown on rice before extraction, beta-glucan extract powder, and mycelium powder after extraction on day 9

4.5.2 Physical characterization using Fourier Transform Infrared Spectroscopy (FTIR)

The structural analysis of the beta-glucan extract using FTIR revealed that the beta-glucan extracted from powdered oyster mushroom mycelium grown on Riceberry rice had a structure similar to that of standard beta-glucan, as shown in Figure 4.8. The sample exhibited distinct infrared absorption characteristics of polysaccharides, with a prominent peak at 3298 cm^{-1} and a narrow peak range of 2880-2923 cm^{-1}, indicating the presence of O-H and C-H functional groups associated with stretching vibrations of polysaccharides, respectively. The absorption peak in the range of 1631-1647 cm^{-1} corresponds to interactions between water and protein. The extracted beta-glucan contains associated proteins when compared to standard beta-glucan, with absorption peaks in the spectrum range of 1000-1200 cm^{-1} indicating characteristics related to sugars (pyranose rings) involving stretching vibrations of C-O-C and C-OH functional groups in (1→3)-β-D-glucan at absorption values of 1072-1078 cm^{-1} and peaks at frequencies 993-994 cm^{-1} corresponding to (1→6)-β-D-glucan. Additionally, small peaks in the range of 885-891 cm^{-1} were observed, indicating the presence of α- and β-glycosidic linkages, suggesting that both α- and β-glucans are present in the beta-glucan extract.

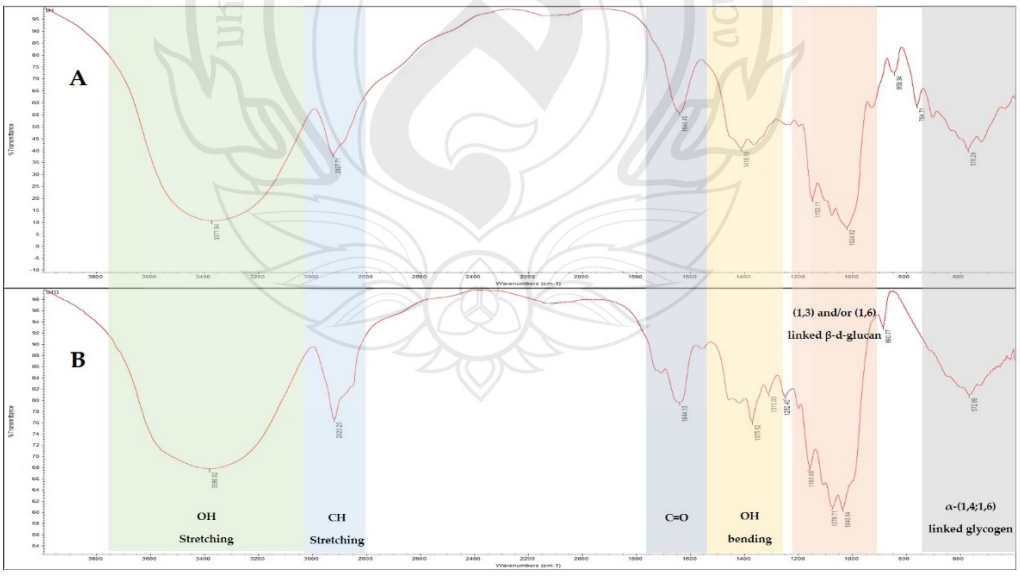


Figure 4.8 FTIR spectrum from 4000-400 cm^{-1} of beta-glucan extract from mycelium grown on Riceberry on day 9 (A) compared to standard beta-glucan (1,3;1,6) (B)

4.5.3 Physical Characterization using Thermogravimetric Analysis (TGA)

The results from the thermogravimetric analysis (TGA) indicated that the purity of the extracted beta-glucan was 82.83% when compared to standard beta-glucan (Sigma-Aldrich), which had a purity level of 78%, as shown in Table 4.2.

Table 4.2 Percentage of weight of beta-glucan extract from *P. ostreatus* mycelium grown on Riceberry rice on day 9 compared to standard beta-glucan (1,3), (1,6)

Sample	Percentage of weight (%)			Normalize (%)	% Yield
	Moisture	Beta-glucan	Residue mass	Beta-glucan	
Beta-glucan extract	14.24	68.59	17.17	82.83	45.56
Standard of bata-glucan	8.79	70.17	21.07	78.93	-

4.6 Prebiotic Properties of Beta-glucan Extract

The results of the prebiotic property testing of the beta-glucan extract obtained from *P. ostreatus* mycelium grown on Riceberry rice, when tested with *Lacticaseibacillus rhamnosus*, indicated that after 24 hours of cultivation, the beta-glucan extract promoted the growth of *L. rhamnosus* more effectively than other growth media. After 36 hours, it was found that the extract continued to support the growth of the bacteria comparably to inulin, which is a standard prebiotic, as shown in Figure 4.9. When comparing the growth promotion of *B. coagulans* and *B. longum*, the beta-glucan extract from *P. ostreatus* mycelium grown on Riceberry rice effectively promoted the growth of *B. coagulans* at 24 hours, similar to inulin and commercially available beta-glucans, as illustrated in Figure 4.10. However, for *B. longum*, the beta-glucan extract promoted growth less effectively than inulin and commercially available beta-glucans, starting to show growth promotion at 24 hours, as shown in Figure 4.11. Therefore, the

Log CFU values at 24 hours were selected for calculating the Prebiotic Index and Prebiotic Activity Score to confirm which group of bacteria was better supported by the beta-glucan extract from *P. ostreatus* mycelium grown on Riceberry rice. The calculation of the prebiotic index was conducted to confirm which strains of probiotics were better promoted by the beta-glucan extract obtained from *P. ostreatus* (oyster mushrooms) cultivated on Riceberry rice. The experimental results indicated that the beta-glucan extract from oyster mushroom mycelium grown on Riceberry rice significantly promoted the growth of *L. rhamnosus* and *B. coagulans* more effectively than *B. longum*, with prebiotic index values of 6.36±0.72, 115.70±10.19, and 1.43±0.72, respectively. It was found that the prebiotic index of the beta-glucan extract promoted the growth of *L. rhamnosus* better than inulin and standard beta-glucan substances, with values of 0.41±0.09 and 1.84±0.15, respectively. Regarding the promotion of *B. coagulans* growth, the beta-glucan extract showed comparable effectiveness to inulin and standard beta-glucan substances. Prebiotic activity score was a quantity that evaluates the value of different carbohydrates as prebiotics according to especially their capacity to promote the development of helpful probiotic bacteria while suppressing pathogenic strains. Which was used to compare in this experiment is *E. coli* showed that the values of the prebiotic activity score aligned with the prebiotic index. It was found to promote the growth of *B. coagulans* more effectively than *L. rhamnosus* and *B. longum*, with values of 1.39±0.06, 0.56±0.03, and 0.21±0.10, respectively, as shown in Table 2. Conversely, inulin was found to promote the growth of *B. longum* more effectively than the beta-glucan extract, with a prebiotic activity score of 1.22±0.22, as shown in Table 4.3.

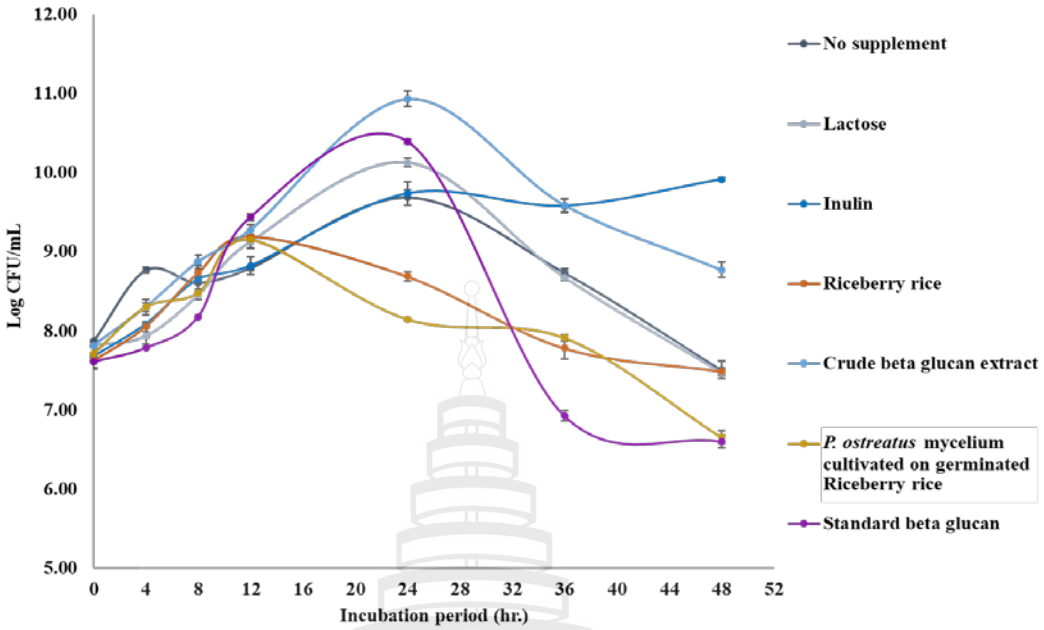


Figure 4.9 Growth of *L. rhamnosus* in media supplemented with different samples

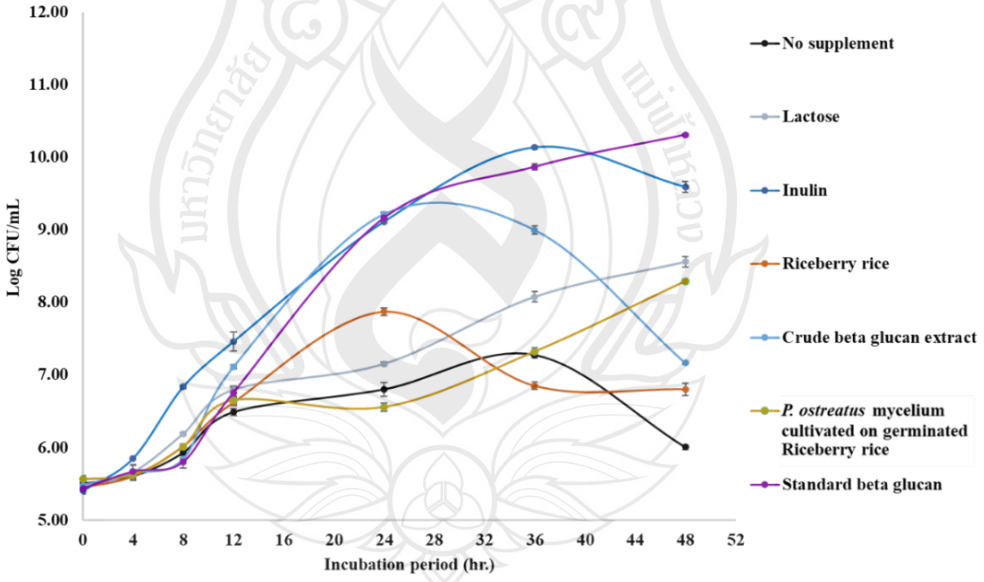


Figure 4.10 Growth of *B. coagulans* in media supplemented with different samples

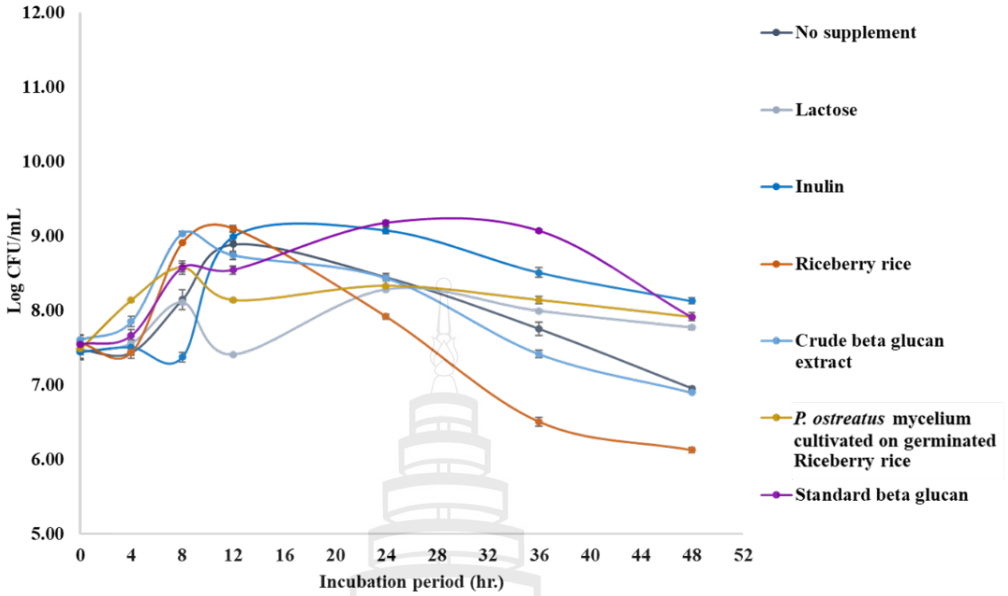


Figure 4.11 Growth of *B. longum* in media supplemented with different samples

Table 4.3 Prebiotic Index and Prebiotic Activity Score for each strain in media supplemented with different samples

Samples	Prebiotic index			Prebiotic activity score		
	L. rhamnosus	***B. coagulans***	***B. longum***	***L. rhamnosus***	***B. coagulans***	***B. longum***
No supplement	0.37 ± 0.06^{c}	0.45 ± 0.07^{d}	1.48 ± 0.07^{c}	0.09 ± 0.04^{C}	0.06 ± 0.07^{D}	0.48 ± 0.10^{C}
Inulin	0.41 ± 0.09^{c}	90.53 ± 2.28^{c}	6.23 ± 0.32^{b}	0.14 ± 0.02^{B}	0.87 ± 0.05^{C}	1.22 ± 0.22^{A}
Riceberry rice (R)	0.04 ± 0.00^{c}	5.23 ± 0.36^{d}	0.43 ± 0.01^{d}	-0.09 ± 0.02^{D}	0.14 ± 0.03^{D}	-0.12 ± 0.06^{D}
Crude beta-glucan extract	6.36 ± 0.72^{a}	115.70 ± 10.19^{a}	1.43 ± 0.02^{c}	0.56 ± 0.03^{A}	1.39 ± 0.06^{A}	0.21 ± 0.10^{C}
P. ostreatus mycelium cultivated on Riceberry rice (RM)	0.02 ± 0.01^{c}	0.17 ± 0.14^{d}	0.85 ± 0.59^{d}	-0.54 ± 0.07^{D}	-0.15 ± 0.08^{D}	0.29 ± 0.08^{C}
Beta-glucan from yeast	1.84 ± 0.15^{b}	102.49 ± 2.17^{b}	7.84 ± 0.19^{a}	0.16 ± 0.03^{B}	1.13 ± 0.06^{B}	0.91 ± 0.12^{B}

Note In this experiment, measurements were performed in triplicate, and the results were used to calculate statistical differences. Each bacterial strain was analyzed independently of the other strains in separate values for prebiotic index and prebiotic activity score. Different superscript letters of prebiotic index (a–d) and prebiotic activity score (A-D) indicate significant differences ($p< 0.05$).

4.7 Anti-colorectal Cancer Properties of Crude Beta-glucan

4.7.1 Cell Toxicity Testing using MTT Assay

The inhibition of colon cancer cells (SW480 cells) after treatment for 24 hours showed that the beta-glucan extract (crude extract) at a concentration of 1 mg per mL effectively inhibited SW480 colon cancer cells comparably to standard beta-glucan (Beta glucan STD), as illustrated in Figure 4.12. The cell survival rates for the cancer cells were 44.06±1.86 and 47.54±1.72, respectively. In contrast, the cancer-inhibiting drug 5-FU only managed to inhibit the cancer cells by 19.13±3.04%. When the cancer cells were cultured with various test substances for 48 hours, it was found that both the beta-glucan extract and the standard beta-glucan could further inhibit cancer cell growth, as shown in Figure 4.13, with values of 33.77±2.42 and 34.43±7.20, respectively, which were close to the values obtained with the cancer-inhibiting drug.

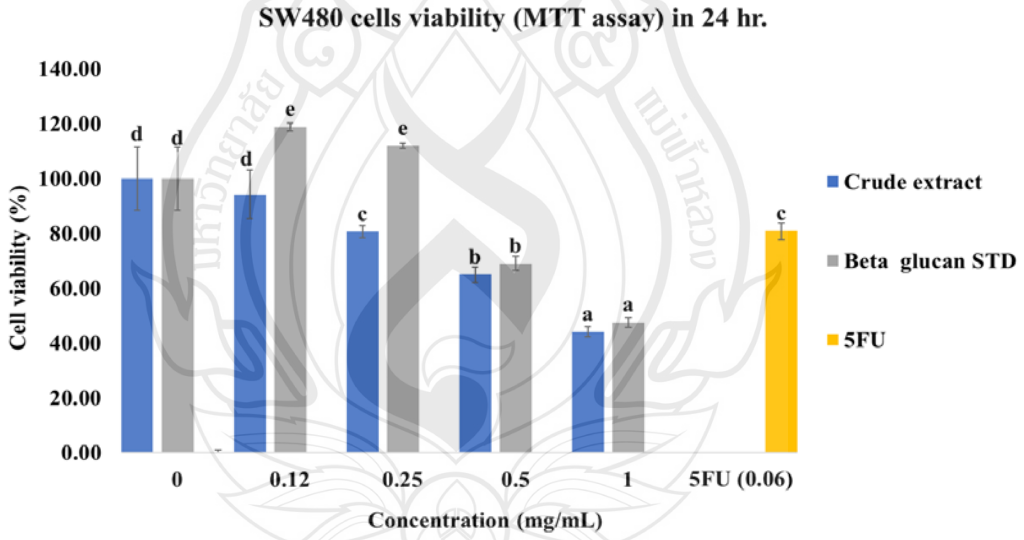


Figure 4.12 Cell toxicity values against SW480 colon cancer cells at 24 hours for beta-glucan extract compared to the standard substances

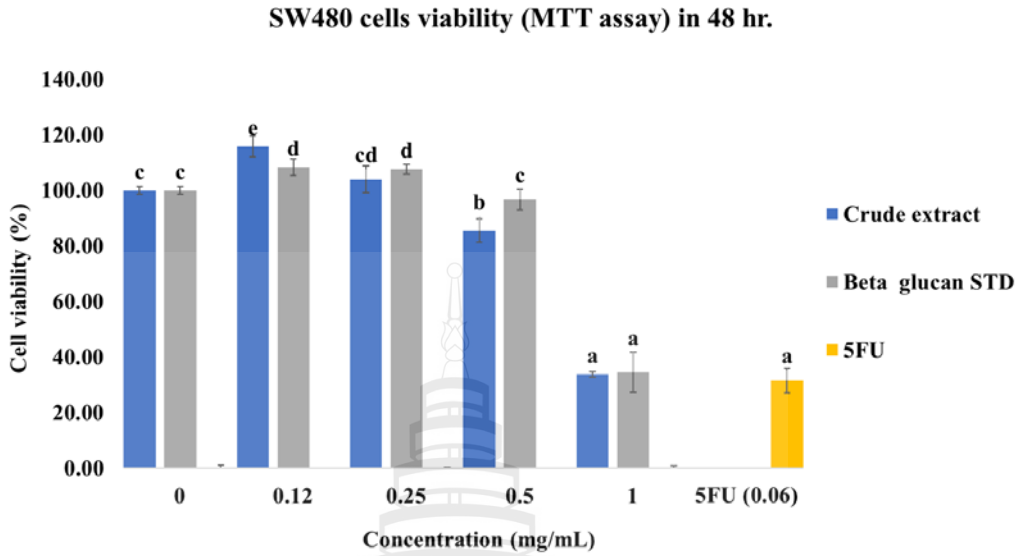


Figure 4.13 Cell toxicity values against SW480 colon cancer cells at 48 hours for beta-glucan extract compared to the standard substances

From this experiment, it can be concluded that the beta-glucan extract from oyster mushroom mycelium can inhibit cancer cells when tested at 24 hours, with values comparable to standard beta-glucan from yeast. The speed of action in inhibiting colon cancer cells by the beta-glucan extract was better than that of the colon cancer inhibitor 5-FU, as this drug began to take effect only after 48 hours of treatment. So, the next experiment was used to measure the time of reaction at 48 hours. It was found that beta-glucan extract and standard beta-glucan at a concentration of 1 mg/mL could effectively inhibit cell growth, comparable to the drug 5FU. Therefore, in this experiment, beta-glucan extract at a concentration of 1 mg/mL was tested on intestinal cells (CCD 841) to evaluate its cytotoxicity. The results showed that both the beta-glucan extract and standard beta-glucan at 1 mg/mL were not toxic to the intestinal cells (CCD 841), as the percentage of cell viability was not significantly different from the untreated control. In contrast, 5FU at a concentration of 500 µM was toxic to the cells, with cell viability at 26.44% (Figure 4.14).

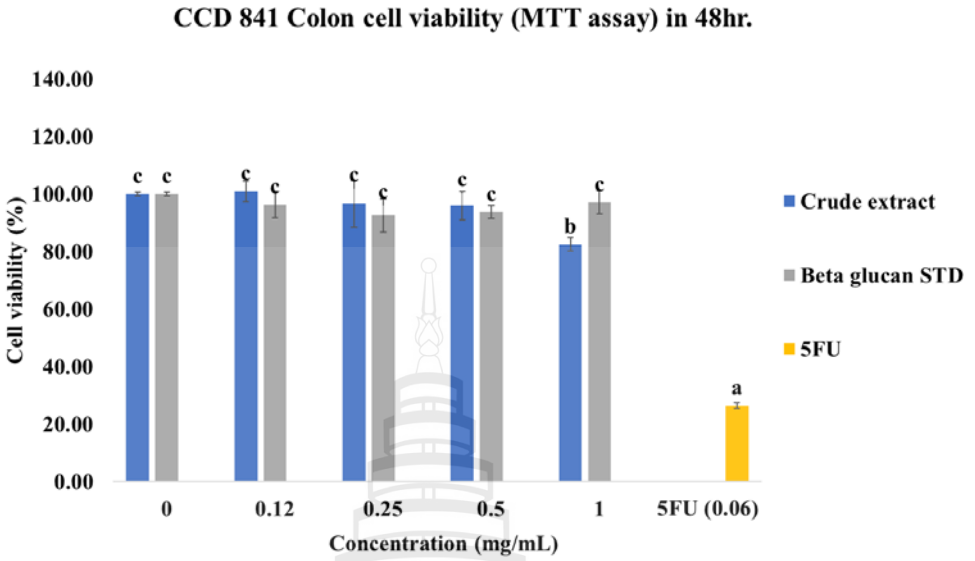


Figure 4.14 Cell toxicity values against CCD841 colon cells at 48 hours for beta-glucan extract compared to the standard substances

4.7.2 Testing for Apoptosis and Necrosis in SW480 Colon Cancer Cells

The assessment results for apoptosis and necrosis in SW480 colon cancer cells indicated that the beta-glucan extract at a concentration of 1 milligram per milliliter could inhibit the growth of SW480 colon cancer cells compared to untreated cancer cells. As shown in Figure 4.15, the beta-glucan extract in Figure B exhibited an increased percentage of apoptosis and necrosis compared to untreated cancer cells in Figure A. The percentage of apoptosis in SW480 colon cancer cells treated with the beta-glucan extract was 23.01±0.89%, compared to 10.02±0.63% in untreated cancer cells, as shown in Table 4.4. Conversely, crude beta-glucan at a concentration of 1 milligram per milliliter did not promote an increase in apoptosis level in normal colon cells when compared to 5-fluorouracil-induced apoptosis up to 73.90±1.19% in normal colon cells, as shown in Table 4.5.

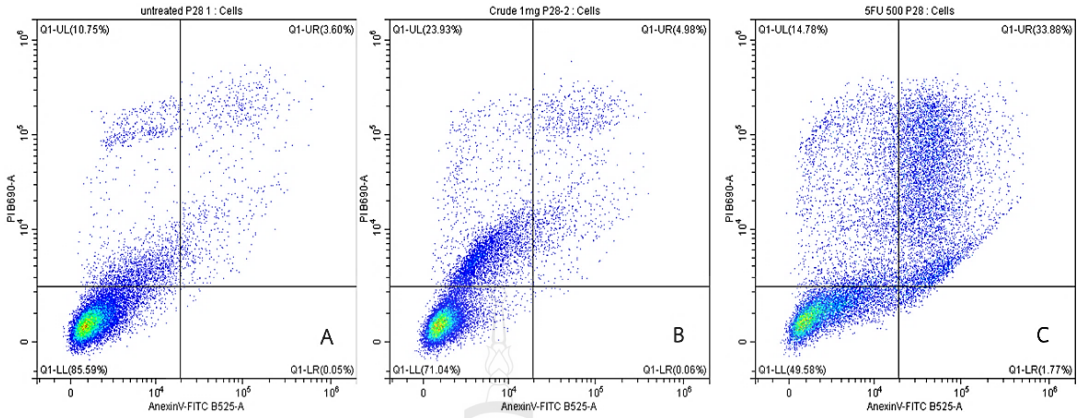


Figure 4.15 Analysis of apoptosis in SW480 colon cancer cells using Flow Cytometry, showing images of cells stained with Annexin V-FITC and PI. Image A represents untreated cells, Image B represents cells treated with beta-glucan extract (Crude), and Image C represents cells treated with the drug 5-fluorouracil (5FU)

Table 4.4 Percentage of apoptosis in SW480 colon cancer cells using Flow Cytometry

Treatment	Cell confluence (%)		
	Live cell	Early apoptosis cell	Late apoptosis/ Necrosis cell
Untreated	86.20±0.71[a]	0.04±0.01[b]	10.02±0.63[c]
Crude 1 mg/mL	71.80±0.66[b]	0.07±0.02[b]	23.01±0.89[a]
5FU 0.06 mg/mL	49.73±1.07[c]	1.92±0.39[a]	13.30±1.35[b]

Table 4.5 Percentage of apoptosis in CCD841 colon cancer cells using Flow Cytometry

Treatment	Cell confluence (%)		
	Live cell	Early apoptosis cell	Late apoptosis/ Necrosis cell
Untreated	80.54±0.90[a]	0.10±0.05[b]	18.70±0.62[b]
Crude 1 mg/mL	83.85±1.81[a]	0.05±0.01[b]	15.87±2.23[b]
5FU 0.06 mg/mL	25.44±1.23[b]	0.66±0.08[a]	73.90±1.19[a]

CHAPTER 5

DISCUSSION AND CONCLUSION

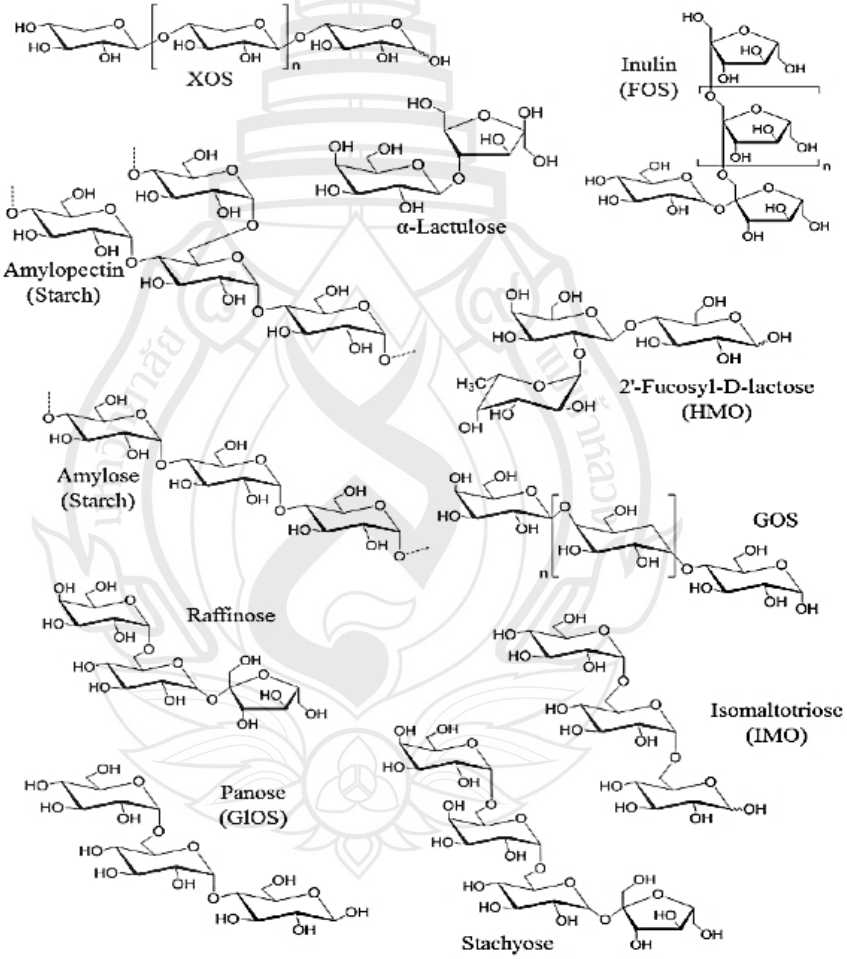
5.1 Discussion

The study on the expansion of beta-glucan production from cultivating *P. ostreatus* mycelium on Riceberry rice indicates its suitability for scaling up beta-glucan production. Research by Krishna Veni (2019) states that colored rice is high in protein and fiber, and contains numerous antioxidants found in the rice's color, known as anthocyanins (B Krishna Veni, 2019). These nutrients promote the growth of mushroom mycelium. In this experiment, a maximum beta-glucan yield of 148.04 ± 7.68 mg per gram of dry sample was achieved with a cultivation period of 9 days. In comparison, research by Bobade et al. (2021), which used sawdust mixed with rice straw and cultivated for 30 days to produce mushroom fruiting bodies, yielded only 55.17 mg per gram (Bobade et al., 2021), which is less than one-third of the yield in this study. In addition to beta-glucan, rice also contains important compounds such as GABA and essential amino acids. Research by Kamjijam et al. (2021) examined the levels of GABA and amino acids in germinated Riceberry rice and found that germinated rice had increased levels of both GABA and essential amino acids (Kamjijam et al., 2021). The process of cultivating *P. ostreatus* mycelium on Riceberry rice further enhances the already present essential amino acids. Thus, this study expanded the production scale of mycelium grown on Riceberry rice from 25 grams to 750 grams, achieving a production capacity of up to 60 kilograms per cycle. After scaling up production, beta-glucan was extracted from the mycelium grown on Riceberry. The extracted beta-glucan was then analyzed using FTIR techniques to confirm its structure as beta-glucan. The results indicated that the beta-glucan extracted from powdered *P. ostreatus* mycelium grown on Riceberry rice had a structure similar to standard beta-glucan, characterized by functional groups such as C-O-C and C-OH in (1→3)-β-D-glucan with absorption values at 1072-1078 cm^{-1} and peaks at frequencies of 993-994 cm^{-1} corresponding to (1→6)-β-D-glucan. The findings are

consistent with research by Jantaramanant et al. (2014) and Sermwittayawong et al. (2018), which reported (1→3)-β-D-glucan at frequencies of 1073-1078 cm^{-1} and (1→6)-β-D-glucan at 993-994 cm^{-1} in polysaccharides from oyster mushrooms (Jantaramanant et al., 2014; Sermwittayawong et al., 2018). Additionally, small peaks in the range of 885-891 cm^{-1} indicated the presence of α- and β-glycosidic linkages, suggesting both α- and β-glucans in the extracted beta-glucan. The analysis showed consistency with FTIR results, indicating that the beta-glucan from mushrooms consists mainly of two types: branched (1→3,1→6)-β-D-glucan and linear (1→3)-α-D-glucan (Synytsya & Novak, 2014). After confirming that the extract was indeed beta-glucan, its quantity was measured, yielding a concentration of 54.95±0.63%, with a beta-glucan content of 222.94±2.33 mg per gram of extract, equivalent to that found in powdered mycelium before extraction. The extract also contained GABA at a concentration of 6.60±0.53 mg per gram and essential amino acids such as glutamic acid and arginine at concentrations of 906.21±83.93 mg and 992.68±28.11 mg per gram of extract. Purity analysis using TGA indicated a purity level of 80%. Previous reports have shown that beta-glucan possesses prebiotic properties and can inhibit colon cancer cell growth (Qi et al., 2019). This study selected *L. rhamnosus*, *B. coagulans*, and *B. longum* for testing the prebiotic properties of the beta-glucan extract because Figueroa-González (2019) found that commercial prebiotics significantly promoted the growth of *L. rhamnosus* over other probiotic strains, with a prebiotic index value of 7.22 (Figueroa-González et al., 2019). Research by Huebner et al. (2007) studied the prebiotic properties of commercial prebiotics on two groups of bacteria, namely *Lacticaseibacillus* and *Bifidobacterium*. It was found that the prebiotic substances available in the market promoted the growth of both bacterial groups differently (Huebner et al., 2007) due to the varying structures of each prebiotic substance. As shown in Figure 5.1, this resulted in different growth promotion effects on probiotic bacteria, as each strain possesses distinct enzymes for breaking down these substances, leading to varied utilization of prebiotics for promoting growth (Fernández et al., 2015). Prebiotics such as beta-glucan, inulin, and fructo-oligosaccharides (FOS) help maintain gut health by influencing the gut microbiota and encouraging the synthesis of healthy short-chain fatty acids (SCFAs). The synthesis of SCFA and the gut microbiota are affected differently by each of these fibers (Carlson et al., 2017; Fehlbaum et al., 2018). In this

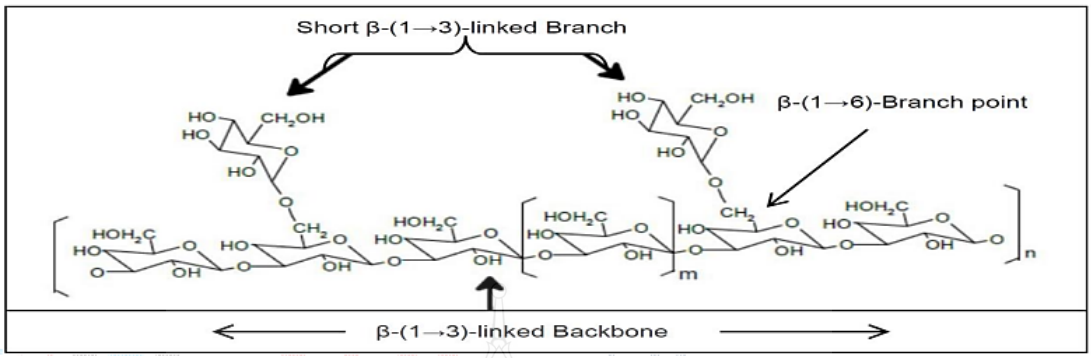
experiment, inulin was selected as a prebiotic to compare against the beta-glucan extract. The results indicated that the beta-glucan extract from oyster mushroom mycelium grown on Riceberry exhibited prebiotic effects similar to those found in the study by Figueroa-González (2019), which reported that beta-glucan extracts promoted the growth of *L. rhamnosus* better than inulin, with a prebiotic index and prebiotic activity score of 6.36±0.72 and 0.56±0.03, respectively (Figueroa-González et al., 2019). Additionally, the extract also effectively promoted the growth of *B. coagulans*, yielding a prebiotic index and activity score of 115.70±10.19 and 1.39±0.06. Conversely, inulin was found to promote the growth of *B. longum* more effectively than the beta-glucan extract, which aligns with data from Optibacprobiotics stating that prebiotics such as FOS and inulin can effectively stimulate the growth of *Bifidobacterium* strains due to their enzymes capable of digesting FOS and inulin (Prebiotics: A look at FOS & Inulin, 2023). Bacillus coagulans has specific enzymes like beta-glucanase that can efficiently break down beta-glucan into simpler sugars, which it can then use for growth and energy. The presence of beta-glucan increases Bacillus coagulans's growth and activity because of its more responsive cell wall structure, which facilitates improved adhesion and utilization (Fath El-Bab et al., 2022). *Bifidobacterium* and *Lacticaseibacillus*, while they can also metabolize beta-glucan, may not do so as effectively due to differences in their metabolic pathways The structure of beta-glucan is a complex polysaccharide with intricate branching patterns, as shown in Figure 5.2, which may hinder certain probiotic strains from utilizing it effectively due to a lack of necessary enzymes for digestion. In tests for inhibiting colon cancer cells using the MTT assay, Liu (2023) and Shamekhi et al. (2020) demonstrated that extracts from mushrooms could inhibit SW480 colon cancer cells effectively (Liu et al., 2023b; Shamekhi et al., 2020). This study corroborated Liu's findings that a beta-glucan extract concentration of 400 µg/mL could inhibit SW480 cells by up to 40%. In comparison, the extract from this study at a concentration of 1000 µg/mL was able to inhibit SW480 cells by up to 36% after 48 hours of treatment, showing efficacy comparable to standard beta-glucan and 5-fluorouracil at a concentration of 500 µM. Furthermore, when the beta-glucan extract at 1000 µg/mL was tested on intestinal CCD841 cells, the extract showed no cytotoxicity to CCD841 cells. Related research has studied the cytotoxic effects of mushroom extracts on both normal and cancer cells

by selecting articles reporting IC_{50} values and using standard methods such as MTT or LDH assays for evaluation. It was found that extracts from mushroom fruiting bodies and mycelia exhibit selective cytotoxicity against cancer cells with minimal effects on normal cells (Nowotarska et al., 2024) The extract tested at a concentration of 1000 µg/mL induced apoptosis in cancer cells by up to 28.91%, compared to a control treatment with 5-fluorouracil at a concentration of 500 µM showing an inhibition rate of only 48.66%. Conversely, crude beta glucan did not promote increase in apoptosis level in normal colon cell when compare 5-fluorouracil induced apoptosis up to 72.89% in normal colon cell.



Source Fernández et al. (2015)

Figure 5.1 Structure of prebiotics in each group



Source Waszkiewicz-Robak. (2013)

Figure 5.2 Structure of beta-glucan

5.1 Conclusion

In summary, *P. ostreatus* was successfully cultivated on Riceberry rice under optimal conditions of 30 °C, 60% humidity, and 20% inoculum. This study demonstrated that a 9-day cultivation period maximized beta-glucan production (29.54%) and enhanced GABA levels (2.25%) and essential amino acids. Initial scale-up attempts to 60 kg based on this 9-day period were promising, and the finding that reducing inoculum concentration to 10% showed minimal impact on beta-glucan yield suggests the feasibility of even larger-scale production with optimized resource utilization. The scaled-up extraction of beta-glucan yielded a comparable concentration (22.29%) and extraction rate (54.95%), with LC-MS/MS confirming the retention of GABA and essential amino acids. While protein content was reduced, FTIR and TGA analyses confirmed the desired (1→3,1→6)-β-glucan structure and a purity of 80%. Further research should indeed explore additional metabolites like polyphenols and organic acids to fully understand the bioactivity of the extract and ensure the consistent retention of all health-benefiting compounds throughout cultivation and extraction.

The beta-glucan extract exhibited prebiotic properties comparable to inulin in vitro, promoting the growth of *L. rhamnosus* and *B. coagulans*. While these findings offer valuable insights for bioinformatics in predicting potential probiotic synergies for specific populations, the translation to real-world physiological effects necessitates in vivo studies. Therefore, future work should focus on investigating the crude beta-glucan

as a postbiotic in animal models or clinical trials to determine its bioavailability, metabolic impacts, and ability to improve gut health in different demographic groups.

The in vitro anti-colorectal cancer activity of the beta-glucan extract, with a 66.23% inhibition of SW480 cell growth and a 28.91% induction of apoptosis, warrants further investigation into the underlying mechanisms, including assays for p53, Bcl-2, BAX, and Caspase-3 activity. Understanding these pathways could facilitate the targeted therapeutic applications. Furthermore, to support practical applications, future research should address the scale-up of the entire production process beyond the initial 60 kg trial, including a thorough economic feasibility analysis. Evaluating the shelf-life stability of the extracted beta-glucan under various storage conditions is also crucial to ensure product viability. Finally, exploring suitable encapsulation methods for targeted delivery and enhanced stability of the beta-glucan in food or pharmaceutical formulations would be a valuable direction for future studies.

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APPENDIX A

ANALYSIS OF VARIANCE OF DATA

Table A1 Analysis of variance of beta-glucan content in Riceberry rice samples and *P. ostreatus* mycelium cultivated on Riceberry rice at different cultivation times

Source	df	Mean Square	F	Sig.
Corrected Model	8	20873.742	18.978	.000
Intercept	1	128571.271	116.897	.000
Time	7	8235.481	7.488	.000
Treatment	1	109341.566	99.413	.000
Error	55	1099.870		
Total	64			

Table A2 Analysis of variance of beta-glucan content in upscaling of *P. ostreatus* mycelium cultivated on Riceberry rice at different inoculum amounts and periods

Source	df	Mean Square	F	Sig.
Corrected Model	5	18259.087	37.191	.000
Intercept	1	229682.676	467.825	.000
Time	4	22482.526	45.793	.000
Treatment	1	1365.332	2.781	.105
Error	34	490.959		
Total	40			

Table A3 Analysis of variance of prebiotic index for *L. rhamnosus* in media supplemented with different samples

PI	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	91.507	5	18.301	197.829	.000
Within Groups	1.110	12	.093		
Total	92.617	17			

Table A4 Analysis of variance of prebiotic index for *B. coagulans* in media supplemented with different samples

PI	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	46865.826	5	9373.165	493.898	.000
Within Groups	227.735	12	18.978		
Total	47093.561	17			

Table A5 Analysis of variance of prebiotic index for *B. longum* in media supplemented with different samples.

PI	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	149.521	5	29.904	365.502	0.000
Within Groups	0.982	12	0.082		
Total	150.503	17			

Table A6 Analysis of variance of prebiotic activity score for *L. rhamnosus* in media supplemented with different samples

PAS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.950	5	0.390	162.149	0.000
Within Groups	0.029	12	0.002		
Total	1.979	17			

Table A7 Analysis of variance of prebiotic activity score for *B. coagulans* in media supplemented with different samples

PAS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.814	5	1.363	351.434	0.000
Within Groups	0.047	12	0.004		
Total	6.860	17			

Table A8 Analysis of variance of Prebiotic activity score for *B. longum* in media supplemented with different samples

PAS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.580	5	0.716	46.953	0.000
Within Groups	0.183	12	0.015		
Total	3.763	17			

Table A9 Analysis of variance of cell toxicity values against SW480 colon cancer cells at 24 hours for beta-glucan extract compared to the standard substances

Source	df	Mean Square	F	Sig.
Corrected Model	10	1838.941	38.180	0.000
Intercept	1	202841.586	4211.400	0.000
Treatment * Conc	4	303.617	6.304	0.002
Treatment	2	1834.532	38.089	0.000
Conc	4	3988.129	82.802	0.000
Error	22	48.165		
Total	33			

Table A10 Analysis of variance of cell toxicity values against SW480 colon cancer cells at 48 hours for beta-glucan extract compared to the standard substances

Source	df	Mean Square	F	Sig.
Corrected Model	10	3288.445	296.011	.000
Intercept	1	186765.721	16811.826	.000
Treatment * Conc	4	68.192	6.138	.002
Treatment	2	16.236	1.461	.254
Conc	4	5929.688	533.764	.000
Error	22	11.109		
Total	33			

Table A11 Analysis of variance of cell toxicity values against CCD841 colon cells at 48 hours for beta-glucan extract compared to the standard substances

Source	df	Mean Square	F	Sig.
Corrected Model	10	1380.702	59.890	0.000
Intercept	1	176437.710	7653.287	0.000
Treatment * Conc	4	94.740	4.110	0.012
Treatment	2	4019.962	174.373	0.000
Conc	4	95.133	4.127	0.012
Error	22	23.054		
Total	33			

APPENDIX B

STANDARD GRAPH OF SOLUTION USED IN THE TEST OF QUALITY AND QUANTITY OF CRUDE BETA-GLUCAN EXTRACTION

The amount of soluble protein in water was determined by comparing the absorbance of the sample with the standard curve of Bovine Serum Albumin (BSA) solution at concentrations ranging from 0, 0.125, 0.25, 0.5, 0.75, 1, and 1.5 mg BSA/mL at the absorbance at 765 nm wavelength as shown in Figure B1.

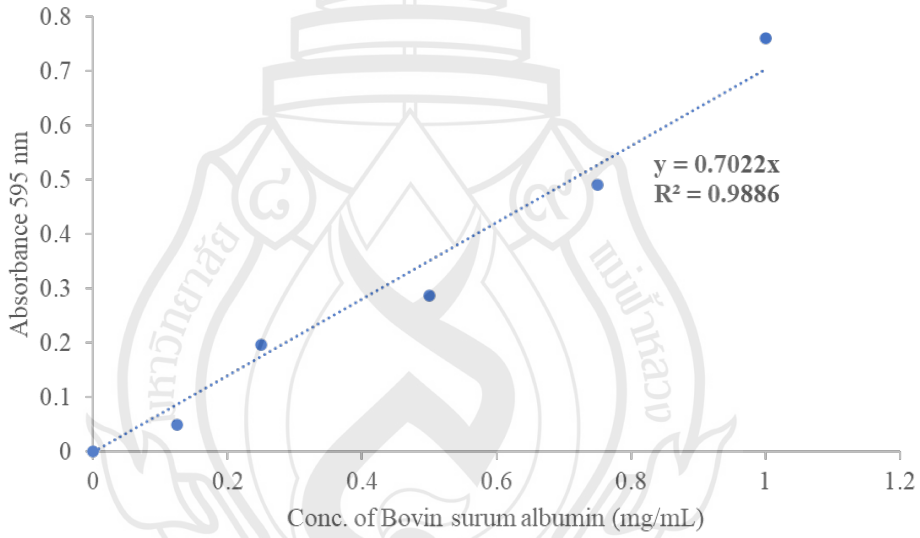
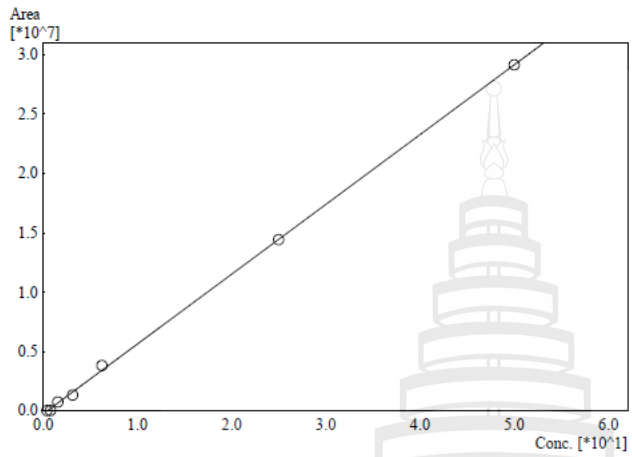


Figure B1 Soluble protein determination by using Bradford assay

Calibration Curve

ID# : 1 m/z : 104.10>87.05
Name : GABA
Quantitative Method : External Standard
Function : f(x)=588418*x-258220
Rr1=0.9998271 Rr2=0.9996543
MeanRF: 3.818215e+005 RF SD: 2.644877e+005 RF %RSD: 69.269993
FitType : Linear
ZeroThrough : Not Through
Weighted Regression : None



#	Conc.(Ratio)	MeanArea	Area
1	0.38	3442	3442
2	0.76	3634	3634
3	1.56	727297	727297
4	3.12	1325871	1325871
5	6.25	3800781	3800781
7	25	14415480	14415480
8	50	29149520	29149520

Figure B2 GABA and amino acid analysis by using LCMS/MS

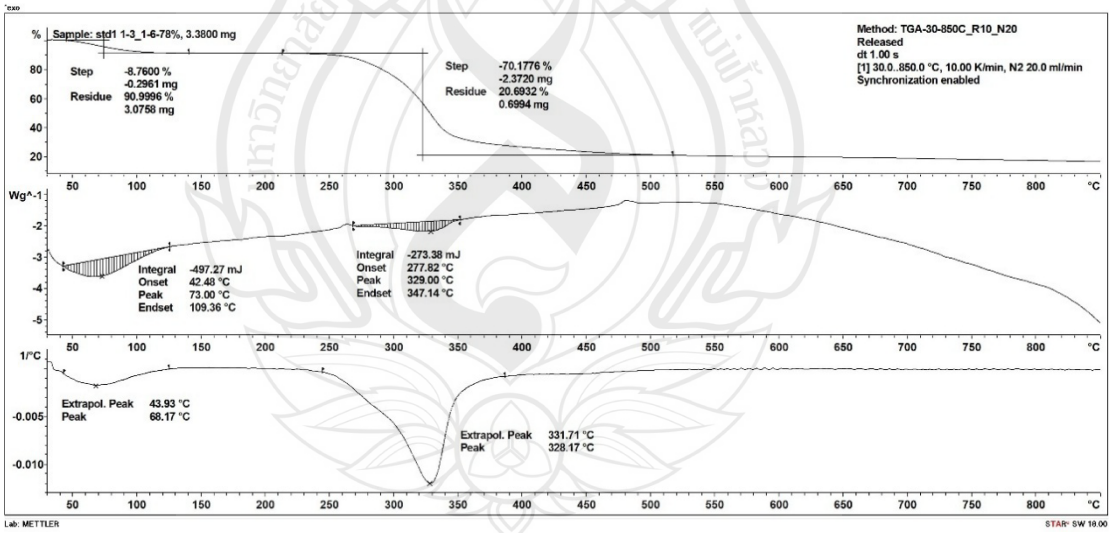


Figure B3 % Purity of beta glucan standard analysis by using TGA

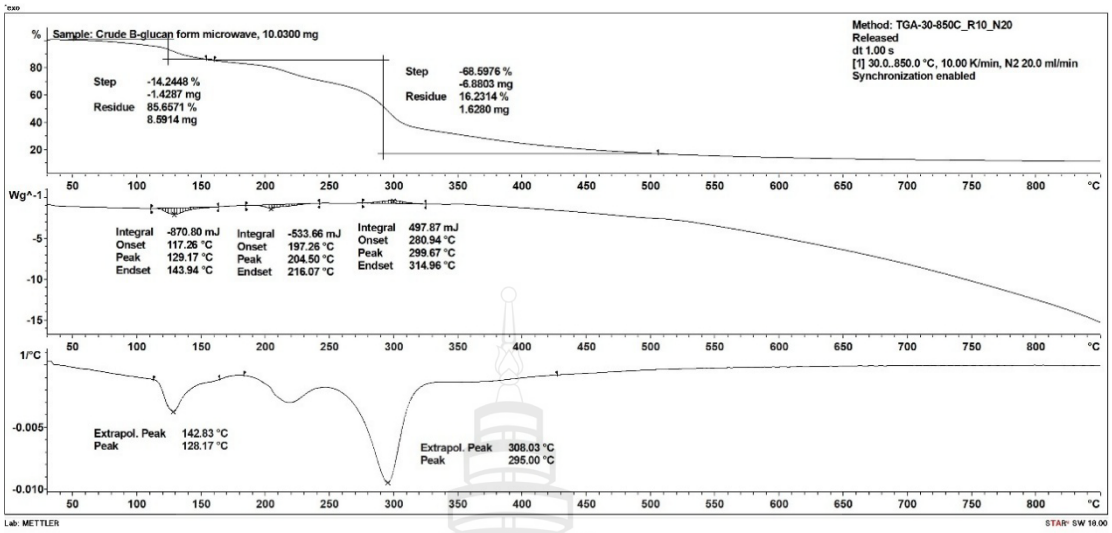


Figure B4 % Purity of crude beta glucan extraction analysis by using TGA

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