Bioconversion of grain-based starch into extracellular polymeric substances (EPSs) and anticancer efficacy evaluation

by

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B.S., Nanjing Tech University, 2016
M.S., Kansas State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

This project aims at exploring a novel anticancer compound (i.e., extracellular polymeric substances-EPSs) through biotransformation of crop starches by a marine protist (*Thraustochytrid striatum*) and using a novel 3D platform to evaluate the mimic *in vivo* anticancer efficacy of the EPSs in comparison with the traditional 2D platform. In addition to glucose as a control, five different types of starches from corn and wheat were studied as substrates for *T. striatum* fermentation to produce EPSs. The EPSs were fractionalized, purified, and characterized in structure and composition to identify its functioning components. The anticancer efficacy of the EPSs was assessed on both HepG2 (human hepatocellular carcinoma cells) and HeLa (human cervical cancer cells).

Raw starchy substrates were successfully converted into EPSs by *T. striatum* by using its own amylolytic enzymes. The starch-degrading enzyme complex was found to mainly include α-amylase and α-glucosidase, which was able to degrade corn and wheat starches (normal and waxy) into glucose and maltose in one-pot at room temperature without separate liquefaction and saccharification. The crude enzyme activities were dependent on the cell growth stage of *T. striatum*. The amylolytic enzymes of *T. striatum* could be a promising robust starch-degrading enzyme systems that has a potential commercial value for starch industry.

Among starchy substates examined, the EPSs yield varied with the growth of *T. striatum* cells. The composition analysis showed that the predominant carbohydrates of EPSs were mannose, glucose, and galactose. The monosaccharide compositions of EPSs derived from different starches were consistent, but the proportion of each monosaccharide differed significantly. In addition, the structure analysis of FTIR and NMR showed that the different-sourced EPSs also had different chemical structures. Overall, the main chain of EPSs consisted of

α-mannose and α-glucose. The sulfated-galactose was found in EPSs as the main structure of (1→3) linked 4-Osulfate-β-D-galactose, which was also found in red algae EPSs. The starch-based EPSs differed from the glucose-based counterpart. Such structural difference in EPSs could be because the EPSs synthesis involved substrate-regulated metabolism pathways.

The variation of starchy substrate affected the antiproliferative activity of EPSs against HepG2 and HeLa, i.e., EPSs' anticancer activity is starchy substrate related. Wheat-EPSs exhibited consistent and antiproliferation effect on HepG2, yet slightly inhibitory effect to HeLa cells. The inhibition of cancer cell proliferation could be attributed to the sulfated polysaccharides involving ROS-mediated apoptosis. However, more research will be needed to explore the mechanisms governing the EPSs' anticancer activity. A dosage over 200 μg/mL showed a significant antiproliferation effect on cancer cells. However, EPSs derived from other starchy substrates had worse performance of antiproliferation on cancer cells, which could be attributed to their different compositional and/or structural characteristics from wheat-EPSs.

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sourced EPSs also had different chemical structures. Overall, the main chain of EPSs consisted of a-mannose and a-glucose. The sulfated-galactose was found in EPSs as the main structure of (1→3) linked 4-Osulfate-β-D-galactose, which was also found in red algae EPSs[1]. The starch-based EPSs differed from the glucose-based counterpart. Such structural difference in EPSs could be because the EPSs synthesis involved substrate-regulated metabolism pathways.

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HepG2: human hepatocellular carcinoma cells;

HeLa: human cervical cancer cells;

AX: astaxanthin;

DCM: dry cell mass;

EPS: extracellular polysubstance;

HPLC: high -performance liquid chromatography;

FID: flame ionization detector;

NMR: nuclear magnetic resonance;

ROS: reactive oxygen species;

TFS: total fermentable sugar;

AHE: almond hull extract;

AP: aminopeptidase;

UF: ultrafiltration;

MWCO: molecular weight cutoff;

FT-IR: Fourier-transform infrared spectroscopy;

HSQC: heteronuclear single quantum coherence;

ANOVA: analysis of variance

PepGel: peptide hydrogel

DMEM: Dulbecco's Modified Eagle Medium;

AO/PI: acridine orange/propidium iodide assay;

TAC: total antioxidant capacity

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Chapter 1 - Literature Review

1. Introduction

Microbial biofilm constitute the typical environment for numerous microbial cells across both natural and artificial habitats, and present in a wide variety of ecosystems[2]. The presence of biofilm develops a sophisticated survival strategy for microorganisms, which enables biofilms to cooperate and enhances microorganism resilience in response to environmental challenges[3]. In natural ecosystem, the biofilm appeared in multiple marine microorganisms' extracellular secretions. Initially, the biofilm functions as a protection for microbials from environment and adhesion "gel" to assist the hosts to attach to surrounding surfaces[4,5]. The biofilm comprises secretion and degradation products from microbial cells and organic and inorganic materials from the surrounding environment[6]. Until 1978, the biofilm of bacterial *Pseudomonas* cells was identified as covered by a "glycocalyx" of fiber, by which cells are able to adhere to surfaces and other cells[7]. Later in 1982, the term "glycocalyx" was renamed as "extracellular polymeric substances (EPSs)"[8]. The term "EPSs" serves as a broad and inclusive designation encompassing various classes of macromolecules, including polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds[9]. These substances have been identified and found within the spaces of microbial aggregates, which are a groups of high molecular secretions from microorganisums[10,11]. Meanwhile, EPSs is an essential component forming the matrix of biofilms[5]. The EPSs governs the immediate living conditions of cells within their microenvironment by influencing factors, such as porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability[12].

The bioactivity of EPSs is another remarkable feature under attention, which was investigated as anticancer drugs from marine ecosystem[13]. These natural sources of anticancer

compounds have the advantages of low toxicity and side effects high safety, and great inhibitory effect on tumors[14]. A number of marine-derived metabolites were shown to possess anticancer activity, which inhibited tumor cell proliferation not only *in vitro* model studies, but also in clinical trials of cancer studies[13,15,16]. Several marine-derived anticancer agents were marketed and approved by the Food and Medication Administration (FDA), such as PRIALT (ziconotide) derived from *Conus magus*, *Plitidepsin* (also named as, Aplidine) derived from *Aplidium albicans*, Salinosporamides A (Marizomib) from the marine actinomycete Salinispora tropica, Nuceoside Ara-A (vidarabine) derived from the sponge *Tethya crypta*[17]. Upon the success in marine drug discovery, numerous secondary metabolites of a variety of marine species were developed into biomedicine products.

2. EPSs producing marine microbes

To explore and develop more natural marine drugs, plenty of antitumor EPSs producing marine microbes were investigated, including marine algae, marine bacteria, marine fungi, and marine protist[13,18]. The antitumor EPSs are vary with the type of microbes, and the gene and taxonomy predominantly regulate the EPSs formation and assembling[19]. Furthermore, the compositional and structural differences cause the functionality and bioactivity of EPSs to be different. Many studies have accomplished fundamental investigation of EPSs from different marine microbes, such as chemical composition and properties.

2.1 Marine bacteria

The EPSs matrix was first found in the extracellular biofilms of marine bacteria, *Pseudomonas* cells[7]. Later on, numerous marine bacteria were classified into Gram-negative and Gram-positive bacteria, based on the difference in assembling and transportation mechanisms of EPSs production[20]. For instance, the extracellular lipoprotein enzymes in the EPSs matrix of

Gram-positive bacteria are not observed in Gram-negative species[21]. Typical EPSs producing Gram-positive bacteria include *Leuconostoc*[22], *Bacillus*, and *Geobacillus* genera[23]. Comparing to Gram-positive bacteria, more EPSs producing marine Gram-negative bacteria have been studied, such as the *Halomonas*, *Planococcus*, *Enterobacter*, *Alteromonas*, *Pseudoalteromonas*, *Vibrio*, *Rhodococcus*, and others[24,25]. Besides, the compositions of Gram-negative bacterial polysaccharides often appear straightforward. The EPSs derived from Gram-negative bacteria are classified as homopolysaccharides (i.e., D-glucose) and heteropolysaccharides (i.e., with repeating units and comprise of 2–4 varieties of monosaccharides)[21,26]. Comparing to Gram-negative marine bacteria, the EPSs produced by Gram-positive marine bacteria are mostly composed of heteropolysaccharides with multiple monosaccharides (i.e., D-glucose, D-mannose, and rhamnose, and etc.)[27].

2.2 Marine algae

Marine algae are also a source of antitumor EPSs, which can be categorized into two main groups, macroalgae and microalgae[17]. Macroalgae, also known as seaweed, comprise green (e.g., *Gapsosiphone fulvescens*[28], *Codium isthmochladum*[29], *Monostroma nittidum*[30], *Udotea flabellum*[31], and *Ulva lactuca*[32]), brown algae (e.g., *Sargassum sp.*[33]), and red algae (e.g., *Gracilaria sp.* and *Prophyra sp.*)[17]. Microalgae mainly include blue-green algae, dinoflagellates, *Bacillariophyta*[34], and others[17].

Marine algae-derived polysaccharides are different in monomer units and structure. In red algae, the most found polysaccharides are agar, agarose, and carrageenan. In brown algae, the predominant polysaccharides are laminarin, fucoidan, and alginate. In green algae, the primary polysaccharides are ulvan, cellulose, mannan, and sulphated rhamnan[18,35]. Recent research indicated that marine algal polysaccharides primarily manifested its anticancer properties

through a range of actions: induction of apoptosis and cell cycle arrestment in cancer cells, triggering programmed cell death, scavenging reactive oxygen species (ROS), stimulating the immune system, and modulate the composition of the intestinal microbiota[18].

2.3 Marine fungi

Marine fungi are one of main bioactive EPSs producers existing in the ocean ecosystem. The mainly studied EPSs-producing marine fungi include *Phellinus linteus*[36], *Ganoderma lucidium*[37], *Fusarium sp.*[38,39], and *Inonotus obliquees*[40,41]. The EPSs produced from marine fungi are mostly heteropolysaccharides. The chemical composition consists of glucan, fructan, glucuronic acid, and rhamnose monomers[42]. Similar to EPSs derived for other marine microbes, the EPSs from marine fungi have various bioactivities, such as antibiotic, antioxidant, anti-mutant, anticoagulant, and immune-stimulant properties[42,43].

2.4 Marine protists

Compared to other EPSs-producing marine microbes, the marine protist are less studied. They are classified as nonfungal eukaryotes and widely exist in the ocean ecosystems[44] with plant microbial communities[45]. The *Thraustochytrid* is one of the most studied and well-known as a robust producer for biodiesel, polyunsaturated fatty acids, and carotenoids pigments[46], where the EPSs of *Thraustochytrid* strain were found to possess bioactivities and have been exploited as a source of future natural anticancer drugs. The mostly studied and documented *Thraustochytrid* strain includes *Aurantiochytrium* sp, *Schizochytrium*, *Thraustochytrium*, and *Ulkenia* genera [44,47]. The EPSs derived from *Thraustochytrid* strains are composed of proteins, lipids, and polysaccharides[5]. The EPSs protect the host cells against toxic compounds[4], prevent them from dehydration[48], and render the cell-cell and cell-surfaces adhesions[4]. Their polysaccharide chains undergo hydration upon contact with water, which is where the

dehydration properties of EPS matrix origin[4]. Later, the EPSs of *Thraustochytrid striatum* was reported to have significantly inhibitory effect on cancer cells at a concentration higher than 100 μg/mL, including HeLa cervical carcinoma and A549 lung carcinoma cells[49].

3. Composition of EPSs

To understand the anticancer efficacy of EPSs, the investigation of chemical composition, chemical structure, and properties were necessary. The chemical composition of EPSs varies among marine microbes in the range of polysaccharides (40%-95%), proteins (1%-60%), nucleic acids (1%-10%), and lipids (1%-40%)[9]. The predominant component of EPSs are carbohydrates and proteins[50]. The bioactivity (e.g., antitumor activity) of EPSs is correlated to the chemical composition and structure of EPSs, e.g., the molar ratio of monosaccharide, proteins, polypeptides, and molecular weight[51]. In this section, the main compositions and corresponding bioactivity of EPSs are summarized.

The EPSs matrix consists predominantly of polysaccharides and protein together with minor components, including lipids, extracellular DNA, humic substances, and minerals.[9]. The chemical composition of EPSs is correlated to the physiochemical properties, functionality, bioactivities. For marine bacteria, the backbone of EPSs is classified into homo-polysaccharides and heteropolysaccharides which are formed in different locations. The homopolysaccharides are synthesized extracellularly, but the heteropolysaccharides are assembled intracellularly at the location of cell membrane[52].

3.1 Carbohydrates

The predominant monosaccharides forming EPSs include pentose, hexose, and uronic acid (**Figure 1.1**)[53]. The predominant hexose present in EPSs are glucose, galactose, mannose,

fructose, rhamnose, and fructose[9], the pentose are arabinose, ribose, and xylose[24]. The uronic acid includes glucuronic acid, galacturonic acid, and mannuronic acid[9].

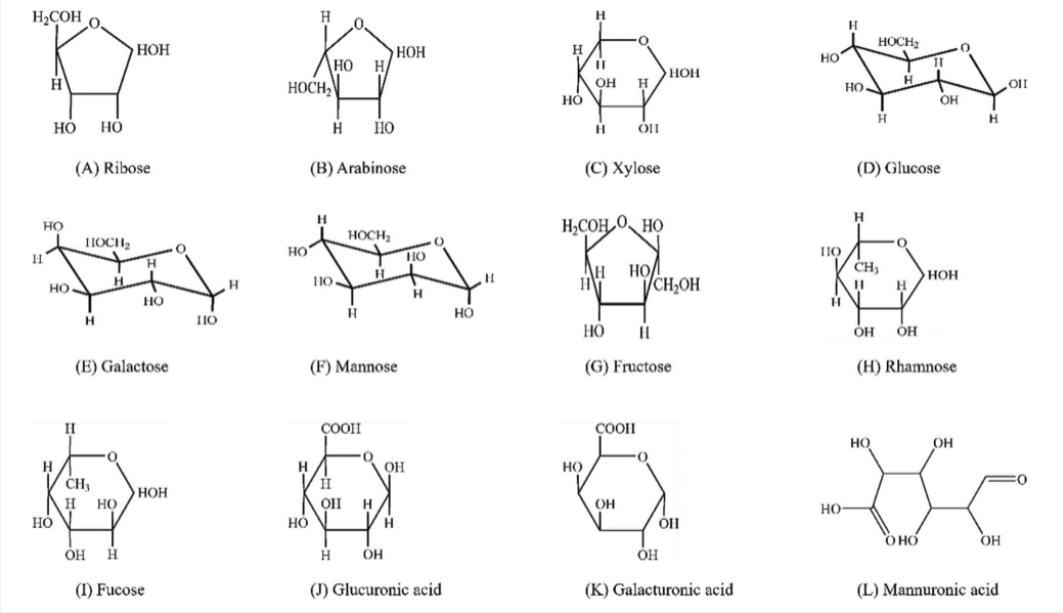

Figure 1.1 Main types of monosaccharides composed in polysaccharides[53]

In general, the polysaccharides in the EPSs are classified based on the construction and structural monomers (**Figure 1.2**). Homopolysaccharides consist of repeating of the same monosaccharide units, such as curdlan, cellulose, and salecan[26]. In detail, curdlan is a linear (1→3)-β-D-glucan with triple-stranded structure. It was widely found in Gram-negative marine bacteria and seaweed (e.g., *Gracilaria foliifera*)[54,55]. The triple helix structure makes curdlan as a thickener and/or a gelling agent in food additives[56]. Cellulose is (1→4)-β-D-glucan and exists in Gram-positive bacteria, alphaproteo-bacteria, and betaproteo-bacteria. It is capable of forming extracellular bacterial cellulose[56]. Salecan is an extracellular water-soluble secretion from *Agrobacterium sp. XZ09*. It has a backbone structure of "→3)-β-D-Glcp-(1→3-[β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)]3-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→", which are all linked by α-(1-3) and β-(1-3) glycosidic linkages[57,58].

Heteropolysaccharides are formed by various monosaccharides, such as gellan, wellan, and diutan. They are composed of a [4)-α-L-Rha*p*(1 → 3)-β-D-Glcp-(1 → 4)-β-D-Glc*p*A-(1 → 4)-β-D-Glc*p*-(1→]$_n$ backbone[59]. Gellan, the main backbone with L-glycerin ester at O-2 of the 3-linked D-glucose and acetic esters at O-6 of the same residue, is anionic polysaccharide found in the EPSs matrix of *Pseudomona elodea* and *Sphingomonas* [60,61]. It had been extensively explored as a food gelling agent, and widely used in food products[61]. Wellan and diutan share the same back as gellan, while the sidechain of wellan is α-L-Rha*p* and/or α-L-Man*p*, and diutan's sidechain is (1 → 4)-α-L-Rha*p*. Their similarity in backbone and sidechains cause their physicochemical properties to be similar[62].

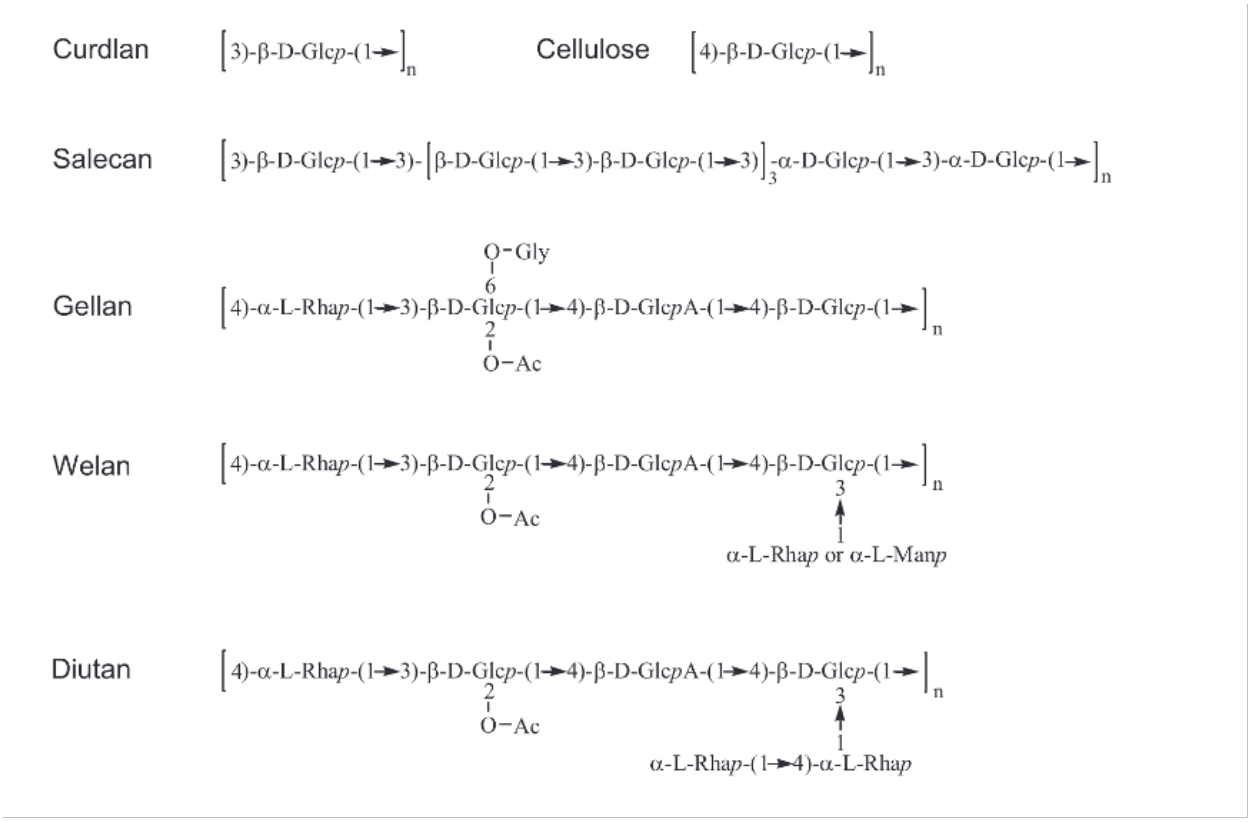

Figure 1.2 Chemical structure of examples polysaccharides and substituents of EPSs[26]

3.2 Protein

Protein is commonly found in the biofilm of marine microbes. It is predominantly enzymes and locates on the surface of marine bacterial cells[63]. Many of the enzymes catalyze the depolymerization of high-molecular-weight extracellular compounds, and assist nutrition uptake for cell growth[64,65]. The extracellular enzymes are versatile in functions, including protein-degrading enzymes, polysaccharides/oligosaccharide-degrading enzymes, lipid-degrading enzymes, phosphomonoesterases and oxidoreductases[66,67]. Enzymes can attach to the surface of the cell wall and/or are released into the environment. The hydrolytic enzymes convert the surrounding organic matter into soluble low-molecular organic matter and cause the formation of plumes in the ocean.

Protein-degrading enzymes including protease and peptidase are consistently found in the marine environment. They are important for marine bacteria to exquisite nitrogen rich organic compounds[68]. For instance, a widely found exocellular enzyme aminopeptidase (AP) in heterotrophic marine bacteria (e.g., *Photobacterium*, *Alteromonas*, *Ruegeria*, and *Sulfitobacter*)[69], can selectively hydrolyze proteins and peptides from the N-terminus and release amino acid residues[70]. The AP assists marine bacteria to uptake nitrogen including nutrients by broking down proteins and/or peptides into consumable amino acids, and shows a key role of degradation of dissolved organic matter for microbial nutrient absorption[69,71]. The AP production of marine bacteria *Ruegeria* was stimulated by high-peptone medium upon the exposure to high content of peptides[69].

The most identified and explored polysaccharides/oligosaccharides-degrading exoenzymes including α-amylase, α-/ β-glucosidase, amyloglucosidase, chitinase, chitobiosidase, β-glucuronidase, and β-xylosidase were found in plenty of biofilms of marine

bacteria[64]. In marine eukaryotes (i.e., *Thraustochytrids*), the polysaccharide-degrading enzymes were also detected, such as cellulase, chitinase, gelatinase, amylase, and α-glucosidase[72]. The polysaccharides/oligosaccharides-degrading enzymes degrade substrates for carbon nutrient acquisition and provide protection from environment. The first mentioned function of polysaccharides/oligosaccharides-degrading enzymes is to degrade carbohydrates and reduce the polymerization of high-molecular weight compounds (e.g., starch) in the aquatic environment. These enzymes are capable of degrading starchy substrates by hydrolyzing glycosidic bonds to release mono-/poly-saccharides to the environment[73]. As reported, marine protist *Thraustochytrids* can produce high level of β-D-glucosidase and β-D-galactosidase that contribute to the decomposition of organic matter in marine sediments[74]. Another functionality of the polysaccharides/oligosaccharides-degrading enzymes is to provide barrier protection to microorganisums[75]. The appearance of polysaccharides/oligosaccharides-degrading enzymes can destroy and deconstruct the EPSs in biofilms of other surrounding microbes[75]. For example, the EPSs isolated from marine bacteria *Pseudomonas fluorescens* showed a resistance to amylolytic enzymes secreted from its own rather than the exogenous amylolytic enzymes, which reflected the protection mechanism of enzymes for marine bacteria[75]. Besides, the cold-active, alkaline-resist, and salt-tolerant amylolytic enzymes derived from marine microorganisms is under research attention[76]. These properties have great potential values in exploring cold-active amylolytic enzymes in several industrial processes, such as starch liquefaction, textile, food, bakery, and brewing industries[76,77]. Recently, a piece of α-amylase encoding gene from marine bacterium *Zunongwangia profunda* (MCCC 1A01486) was expressed in *Escherichia coli*, and the expressed α-amylase was cold-active and salt-tolerant[76].

The exoenzymes produced by marine organisms show a great variety of functionality. The presence of exoenzymes promotes the nutrient transformation and uptake by the host cells from the surrounding marine environment. Besides, exoenzymes provide protection from from other microbes and toxicity from the surrounding environment[5]. The exoenzyme significantly influences the survival of marine microbes. However, the exploration of these exoenzymes is sophisticated due to the complexity of the extracellular matrix and disturbance of other coexisting compounds. Thus, the separation and purification processes are important. Several studies had successfully separated the exoenzymes by gene-editing and modification of marine bacteria rather than other marine microbes. The exploration of exoenzymes derived from marine microbes is still understudied, such as the cold-active amylolytic and proteolytic enzymes, which have a great potential in energy production and bioprocessing technology.

3.3 Lipid

Based on previous research, the lipid component in EPSs generally attaches to the polysaccharide chains and/or conjugates with protein structures [51]. So far, plenty of glycolipids, phospholipids, and rhamnolipids have been found in the EPSs matrix, and the lipopolysaccharides generally play a role as biosurfactants and assist microbial growth[28].

4. Structure-activity of EPSs

Cancer is known as excessive cell proliferation and spread of abnormal cells with increasingly aggressive behavior[78]. Cancer frequently originates in the epithelial cells of the lung, breast, liver, skin, and pancreas, whereas sarcomas arise from mesenchymal tissues, including fibroblasts, fat cells, bone-forming osteoblasts, and muscle cells[79]. In decades, the exploration and development of marine bioactive compounds have been in progress as natural cancer therapy agents. The marine bioactive compounds has shown that polysaccharides derived

from natural sources offer benefits such as safety, high therapeutic effectiveness, non-toxicity, affordability, and strong biocompatibility[14]. These marine bioactive compounds are categorized into various groups, such as alkaloids, carbohydrates, fatty acids, peptides, phenols, quinones, terpenes, and saponins[79]. Furthermore, plenty of studies proven that the chemical structure of these marine compounds is correlated to its bioactivity, such as anti-inflammatory activity, antioxidant activity, anti-tumor activity, antibacterial activity, and probiotic activity[1]. The EPSs' functionalities are affected by the microbial source that includes the microbial taxonomy and genome that regulate the production of EPSs. As an example, marine protist, *T. striatum* can produce protein-polysaccharide conjugates and extracellular polysaccharides with considerable antioxidant capacity. Its EPSs were reported to significantly inhibit A549 lung carcinoma cell, Hela cervical carcinoma cell, DU145 human prostate carcinoma, and B16-F0 mouse melanoma cell[49].

Chapter 2 - Characteristics of EPSs' chemical composition derived from starchy substrate by *Thraustochytrid striatum*

1. Introduction

Thraustochytrid striatum, a marine protist, is well known for its production of bioactive exocellular polymers and cellular fatty acids production[49,80]. The EPSs are observed as biofilms of marine protist, bacteria, and microalgal[12,81,82]. The EPSs are a complex matrix surrounding marine protist cell wall, which provide protection against desiccations[82], defend changes in physico-chemical conditions[4], and enhance cell survival[82]. Plenty of EPSs were found to have biological activities, such as anti-inflammatory and anti-proliferation specific cancer cells[83]. To explore the natural source of anticancer compounds, EPSs from multiple varieties of marine microbes were characterized for their chemical composition, structure and anticancer activity[84].

The anticancer functions of EPSs from marine microbes have been widely studied as a potential natural sourced drug[51]. The anticancer activity of EPSs are affected by their compositional and structural features, such as glycosidic linkage, molar ratio/composition of monosaccharides, molecular weight, branching, and protein[18,85]. To understand the anticancer efficacy of EPSs, the chemical compositions of EPSs were probed initially. The EPSs are a matrix mainly composed of polysaccharides, proteins, nucleic acids, and lipids[64]. Polysaccharides are one of the predominant compositions of EPSs, mainly including monosaccharides (e.g., D-glucose, D-galactose, D-mannose, L-fucose, and L-rhamnose) and uronic acid (e.g., D-galacturonic acid, L-guluronic acid, and D-mannuronic acid)[6,82]. These monomeric compositions of EPSs provide reducing capacity, which is likely because of the inherent reducing properties that enable the donation of electrons to reactive radicals, thus diminishing their reactivity[51]. Especially, the hydroxyl and carboxyl groups in uronic acid have

efficient free radical scavenging capacity through elevating negative charge of polysaccharides by donation of hydrogen atoms[51,86]. Another predominantly component in EPSs is protein which contains enzymes and negatively charged amino acids, which were confirmed to have certain antioxidant activity[87]. For instance, the protein-conjugated EPSs derived from *Oidiodendron truncatum* GW fungus demonstrated an inhibitory effect on lipid peroxidation and showed scavenging efficacy on DPPH and superoxide radicals[88]. Therefore, investigation of chemical composition and structure are important to elucidate the anticancer effect of EPSs.

Starch is the most abundant carbohydrate in nature. Its biocompatibility, sustainability, and low cost make starch an outstanding substrate for various applications. In addition to food and feed, crop starches have received many other value-added applications in starch derivatives, such as pharmaceutical encapsulation, bio-degradable drug packaging[89], and starch-based drug delivery hydrogels[90]. The alternative applications of starches in the pharmaceutical field would greatly benefit both the U.S. agriculture and the pharmaceutical industry. In the past decade, intensive studies have been focused on starch-derived pharmaceutical products. For example, starchy substrate-derived extracellular polysaccharides was studied as an anticancer reagent[49]. However, information regarding the functionality and the secretion pathway of starch-derived anticancer EPSs by marine protists is scarce such that further investigation is needed. The understanding of the characteristics and production of the anticancer EPSs could help manipulate the metabolism process toward maximizing EPSs production. Recent studies have shown that the EPSs (e.g., microbial polysaccharides) have anti-inflammatory and anticancer activities that possess high potential in pharmaceutical applications. Currently explored microbial EPSs (e.g., bacterium, microalga, and marine protist) are characterized as extracellular polysaccharides that were demonstrated to be anticancer compounds[24]. Among marine protists, *T. striatum* can

produce protein polysaccharide conjugates and extracellular polysaccharides with considerable antioxidant capacity. Its EPSs were reported to significantly inhibit A549 lung carcinoma cell, Hela cervical carcinoma cell, DU145 human prostate carcinoma, and B16-F0 mouse melanoma cell[49]. Besides anticancer compounds, *T. striatum* can secret amylolytic enzymes with activity as high as (750 U/L)[91]. Such amylolytic enzymes can degrade starchy substrates into saccharides to support cell growth and EPSs production. In addition, *T. striatum* can also accumulate intracellular polyunsaturated fatty acids (i.e., eicosatetraenoic acid and docosahexaenoic acid)[92,93] and carotenoids (e.g., astaxanthin)[94]. In our previous study, starch and yeast extract were the best carbon and nitrogen sources, respectively, and the production of fatty acids and carotenoid were cell-growth dependent[95]. As a result, *T. striatum* is expected to convert low-cost starch into high-value anticancer compounds and intracellular fatty acids and carotenoids.

This chapter studied the feasibility of extracellular EPSs production by *T. striatum* from starchy substrates, soluble starch, corn starch, wheat starch, waxy corn starch, and high amylose starch as well as glucose. The extracellular enzymes were examined, and the extracted EPSs were identified regarding their chemical composition of monosaccharides, sulfate concentration, protein concentration, and antioxidant capacity.

2. Materials and Methods

2.1 Materials

The starches used in this study included soluble starch (S516-500, Fisher Scientific, Fair Lawn, NJ, USA), corn starch (S4126, Sigma Chemical Co., St. Louis, MO, USA), waxy corn starch (S9679, Sigma-Aldrich), wheat starch (S1557, Spectrum Chemical Mfg. Corp., Gardena, CA, USA), and high-amylose starch (Hylon VII, National Starch and Chemical Company, NJ, USA).

2.2 Culture preparation

T. striatum ATCC24473 was purchased from the American Type Culture Collection (ATCC) and cultivated by following the vendor's guidelines. The seed culture medium contained 5 g/L of glucose, 1 g/L of yeast extract, and 1 g/L peptone in artificial sea water (ASW), and pH was adjusted to neutral. The ASW included (per liter): 30 g NaCl, 0.7 g KCl, 10.8 g $MgCl_2 \cdot 6H_2O$, 5.4 g $MgSO_4 \cdot 7H_2O$, and 1.0 g $CaCl_2 \cdot 2H2O$. The stock culture was incubated at 25℃ and 150 rpm in a shaking incubator (MAXQ 4000, Thermo Scientific, USA) for five days. The stock culture was then inoculated into fresh medium with inoculation size of 10% (v/v) to prepare inoculum for fermentation. The chemicals used in this study were purchased from Fisher Scientific (PA, USA) unless specified, otherwise.

2.3 Raw starch fermentation

To prepare cultivation medium, the ASW was mixed with 6 g/L yeast extract and 6 g/L of peptone as a nitrogen source and autoclaved; the mixture was then cooled down to ambient temperature followed by the addition of sterilized starch powder at 10% (w/v, dry basis) loading rate[95]. The inoculation size of *T. striatum* inoculum was 10% (v/v). The fermentation was carried out in 250-mL flasks with 50-mL working volume. The flasks were incubated at 25℃ and 150 rpm for 7 days. The α-glucosidase and α-amylase activities of the fermentation broth were analyzed at the end of fermentation.

2.4 EPS preparation

At the end of fermentation, the broth was centrifuged at 10,000 rpm and 4°C for 10 min. The supernatant was collected for further purification by ultrafiltration (UF) with continuous water (ultrapure water) washing. The UF was carried out in an Amicon® stirred cell through a PES membrane with a molecular weight cutoff (MWCO) of 10 kDa. The conductivity of the

filtrate was monitored until the conductivity change was negligible[48]. The recovered EPSs were lyophilized and stored under -20°C until used.

2.5 EPSs composition analysis

2.5.1 Monosaccharide analysis

Sulfuric acid hydrolysis method[96] was used with some modifications to measure monosaccharide compositions of EPSs. In brief, approximately 10 mg of lyophilized EPSs was dissolved in 2 mL of 2M H_2SO_4 solution and placed in an oven under 121°C for 5 h. After acid hydrolysis, the samples were cooled to ambient temperature, then neutralized by using $CaCO_3$ followed by filtration through a 0.22-μm filter into HPLC vials. The hydrolyzed EPSs samples were analyzed on a HPLC which was equipped with a reflective index detector (RID) (LC-20AB LC, Shimadzu Scientific Instruments, Somerset, NJ, USA) and an analytical column (Bio-Rad HPX-87P, Bio-Rad Laboratories, Hercules, CA). The mobile phase was HPLC grade ultrapure water, and the flow rate was set at 0.6 mL/min. The oven and RID temperatures were set at 80 and 35 ℃, respectively.

2.5.2 Sulfate content

Sulfate content of EPSs was determined by using colorimetric method. In detail, 20 mg of EPSs sample underwent hydrolysis in 3 mL of 60% formic acid (v/v) for 8 h at 100°C. The resulting hydrolysate was collected for sulfate content measurement. The color development occurred because of the reaction between sulfate and the gelatin-$BaCl_2$ reagent. To prepare the reagent, 2 g of gelatin was dissolved in 400 mL of DDI water at 60°C and kept at 4°C overnight. Subsequently, 2 g of $BaCl_2$ was dissolved into the gelatin solution, and the mixture was left for 3 h before used. For sulfate analysis, 500 μL of sulfate sample was mixed with 3.8 mL of trichloroacetic acid (4%, w/v) and 1 mL of gelatin-$BaCl_2$ reagent. This mixture was allowed to

develop color for 15 min, and the absorbance was measured at λ = 500 nm. Potassium sulfate served as a standard, with concentrations ranging from 0 to 100 μg SO_4^{2-}/mL[12, 13].

2.5.3 Protein content

The protein content of EPSs was determined by using P Pierce™ BCA Protein assay kit (Thermo Scientific, Rockford, IL) by following the vendor's instructions.

2.5.4 Antioxidant activity

The antioxidant activity was measured by using QuantiChrom™ Antioxidant Assay Kit (Bioassay Systems, CA, USA). The EPSs antioxidant capacity was expressed in Trolox equivalent. The operation process followed the vendor's instructions.

2.6 EPSs structural characteristic

The EPSs structure was investigated by using Fourier-transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR), the basic structure of EPSs was revealed.

2.6.1 FT-IR

A FT-IR spectrophotometer (Thermo Nicolet Nexus™ 670) equipped with a smart collector was used. A quantity of 2 mg lyophilized EPSs powder was prepared for the analysis on a FT-IR in the range of 400-4000 cm^{-1}, and the detector resolution was set at 4 cm^{-1} with 32 scans per sample. OMNIC 6.1a software (Thermo-Nicolet Corporation, Madison, WI, USA) was used to determine the peak positions and intensities.

2.6.2 NMR

The ^{1}H, ^{13}C, and 2D ^{13}C-^{1}H heteronuclear single quantum coherence (2D HSQC) spectra were recorded on a Varian Inova 400 MHz spectrometer (Varian NMR Systems, Palo Alto, CA, USA) using standard Varian pulse sequences. In general, about 40 mg EPSs was dissolved in ~2 mL D_2O (99.9%) for deuterium exchange for three successive freeze-drying cycles before

dissolution to reduce the intensity of the signal from HDO. The lyophilized sample was then dissolved in 0.5 mL D_2O (99.9%) for data acquisition in a 5-mm NMR tube at 27°C. The solution obtained was cloudy and left for 1 h to let undissolved particles precipitate. Around 20 μL acetone was added to the NMR tube as a reference. The spectra were acquired using the pulses and parameters according to the literature[98]. Additionally, a PRESAT experiment was conducted for the ^1H acquisition to suppress the water HDO peak, which may obscure the signals from the sugars of interest.

2.7 Advanced extracellular enzyme screening

The same amount (based on the protein content in enzyme broth) of STARGEN™ and crude wheat starch-derived amylolytic enzyme (WAE) were applied to 10% (w/v, dry basis) of raw normal corn starch slurry and incubated under room temperature at 150 rpm. The aliquots of hydrolysates were sampled at 0, 1, 2, 4, and 24h during enzymatic hydrolysis, and the released sugars were identified and quantified by using HPLC-RID.

2.8 Data analysis

The significant levels of different treatments were determined by analysis of variance (ANOVA) (α= 0.05). GraphPad Prism® version 10.1 (San Diego, CA) was used to perform experimental design, statistical analyses, and figure drawing.

3. Results

3.1 Raw starch fermentation

T. striatum can convert raw starches directly into EPSs. During fermentation process, no additional starch gelatinization, liquefaction, and saccharification was applied. In other words, external amylolytic enzymes were not required for starch degradation to support *T. striatum* fermentation. The *T. striatum* secreted extracellular amylolytic enzymes which were sufficient to

assist itself to degrade starchy substrate into monosaccharides and disaccharides. The released saccharides were functioned as carbon source for cell growth. Thus, the amylolytic enzymes secreted by *T. striatum* during starch fermentation were studied.

T. striatum was considered as starch degrading microbes of its capability of amylolytic enzyme production, where α-glucosidase and α-amylase were produced as exocellular enzymes. The produced amylolytic enzymes were confirmed through 18S RNA sequencing(GenBank, no. AB022112.1), and the expression of amylolytic enzyme were confirmed in Taoka's study[99]. During fermentation process, the crude amylolytic enzymes including α-glucosidase and α-amylase were examined and quantified at the end of fermentation[**Figure 2.1 (A) & (B**)]. Both of the amylolytic enzymes were found in the fermentation broth of each group of experiment. The enzyme activity of α-amylase was much stronger than α-glucosidase in each cases, the enzyme activity reflects the enzyme production quantity, starchy substrates seems more preferable by *T. striatum* for enzymes production. Similar results were reported by Xiao, comparing to glucose, starch supported *T. striatum* cell growth better and the enzyme accumulated faster[100]. Comparing with the crude enzymes fermented from glucose, α-amylase fermented from starchy substrates shown higher enzyme activity, while α-glucosidase shown lower enzyme activity. The α-amylase was the predominant enzymes produced, and its' higher secretion could be induced by the starchy substrate rather than glucose as carbon source. The starch/maltose regulated α-amylase secretion phenomena appeared in plenty of α-amylase producing microbes, for example, the *Bacillus sp*.[101,102]. Amongst all crude enzyme harvested from starch medium, the crude enzyme harvested from wheat starch fermentation broth shown the highest enzyme activity of both α-glucosidase and α-amylase, where the enzyme activity of α-amylase was 9.5 U/mL and α-glucosidase was 0.07 U/L. Different types of starchy substrate

directly affected the secretion of amylolytic enzyme by *T. striatum*. The structure and composition (i.e., amylose/amylopectin ratio), and particle size of starch granules could manipulate the production of amylolytic enzymes.

The time course of protein concentration indicated the exo-enzyme production during fermentation, as shown in **Figure 2.1 (C)**. Glucose medium resulted in the highest protein production of all tested substrates, while corn starch excreted the most protein among the starchy substrates. However, the protein concentrations and enzyme activities of different substrates did not have a consistent relationship, which could reveal that not all proteins were amylolytic enzymes and there may be other types of enzymes excreted by *T. striatum*. As reported by Taoka, amylolytic enzymes were not the sole type of exoenzyme produced by *T. striatum* (ATCC 24473), which also include agarose, proteinase, urease, lipase, gelatinase, phosphatase, and chitinase were also produced at the same time[99].

The antioxidant capacity reveals the capability of overcoming oxidative stress, which was crucial evidence of oxidative stress revealing drug. In this study, the antioxidant capacity was expressed as Trolox equivalent, and the antioxidant capacity of freeze-dried EPSs under certain concentration were presented in **Figure 2.1 (D)**. The antioxidant capacity of EPSs from each starch varieties had a positive correlation with EPSs concentration, where higher dosages presented higher antioxidant capacity. The EPSs derived from waxy corn starch had the strongest antioxidant activity followed by normal wheat, high amylose corn, normal corn, soluble starch, and glucose in that order. Of all EPS derived from starchy substrates shown higher antioxidant capacity than EPS derived from glucose. The result indicated that the EPS antioxidant capacity was highly related to the carbon source provided, where the substrate manipulates the bioactivity of EPS. As high levels of carbohydrate provided could elevate EPS quantity[48], the starchy

substrate whom induced the α-amylase secretion of *T. striatum* could facilitated starch degradation, with continuously sugar released. *T. striatum* was able to utilize the sugars for EPS production, as the EPS production elevated, the bioactivity of the lyophilized EPS increased.

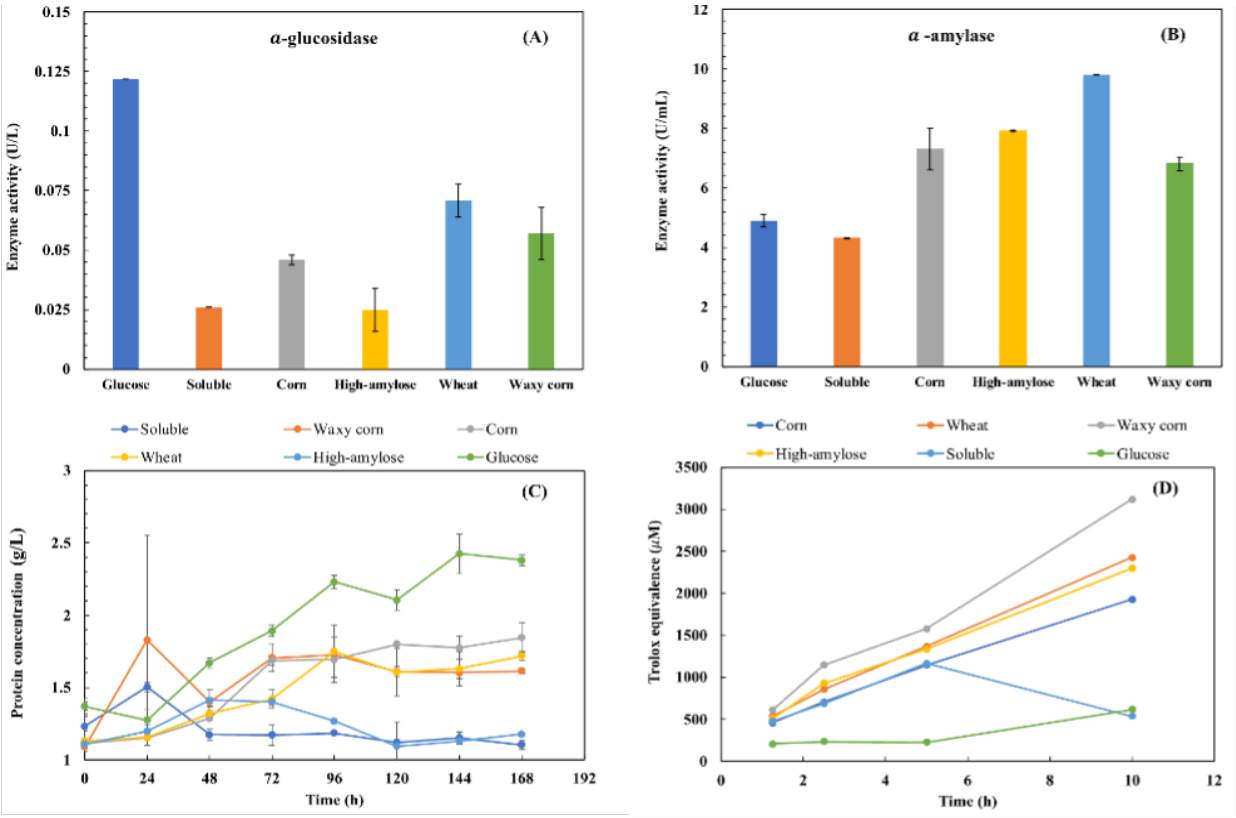

Figure 2.1 (A) α-glucosidase activity from different substrates, (B) α-amylase activity from different substrates, (C) protein concentration variation during fermentation, and (D) Trolox antioxidant activity of EPSs.

3.2 Amylolytic enzymes

The bioethanol industry has recently been seeking a cold process to produce bioethanol for cost reduction, the development of low temperature active enzyme was expected. Cold process means hydrolysis of uncooked raw starch into monosaccharides for bioethanol fermentation with the assistance of raw starch degrading enzymes[103]. Currently, two innovative enzyme product developed for cold starch-to-ethanol process are on market, one of the product is STARGEN™ developed by Genencor International Inc. (now DuPont), another enzyme product

is an acid fungal amylase and developed by Novozymes specific for Brolin's BPX™ ethanol production[104–107]. The development of no-cook enzyme saved energy from the ethanol producing process, hence the exploration of novel enzyme for cold process use enzyme were necessary.

In our research, amylolytic enzymes produced by *T. striatum* was detected, and capable to degrade raw starches under room temperature. Thus, the crude enzyme from *T. striatum* had a great potential to be utilize in cold process as a starch degrader. Previously, the wheat starch-derived crude amylolytic enzyme (WAE) had the highest amylolytic enzyme activity, hence it was selected for further raw starch hydrolysis examination in comparison with STARGEN™. As a result, with the detection by the WAE released both glucose and maltose simultaneously from raw normal corn starch slurry [**Figure 2.2 (A)**], whereas STARGEN™ released glucose only [**Figure 2.2 (B)**]. The difference in the produced sugars could be due to different amylolytic enzymes and/or different amylolytic activities in both enzyme sources, as crude WAE was found to mainly include α-amylase and α-glucosidase, while STARGEN™ includes glucoamylase from *Aspergillus niger* and α-amylase from *Aspergillus kawachi*[103,108]. In terms of hydrolysis rate, STARGEN™ was much faster than WAE in degrading raw corn starch. Overall, the WAE has a great potential to be explored as novel, robust raw starch-degrading enzymes comparable even superior to STARGEN™ while further treatments on the crude WAE will be needed, such as separation, purification, concentration, and characterization.

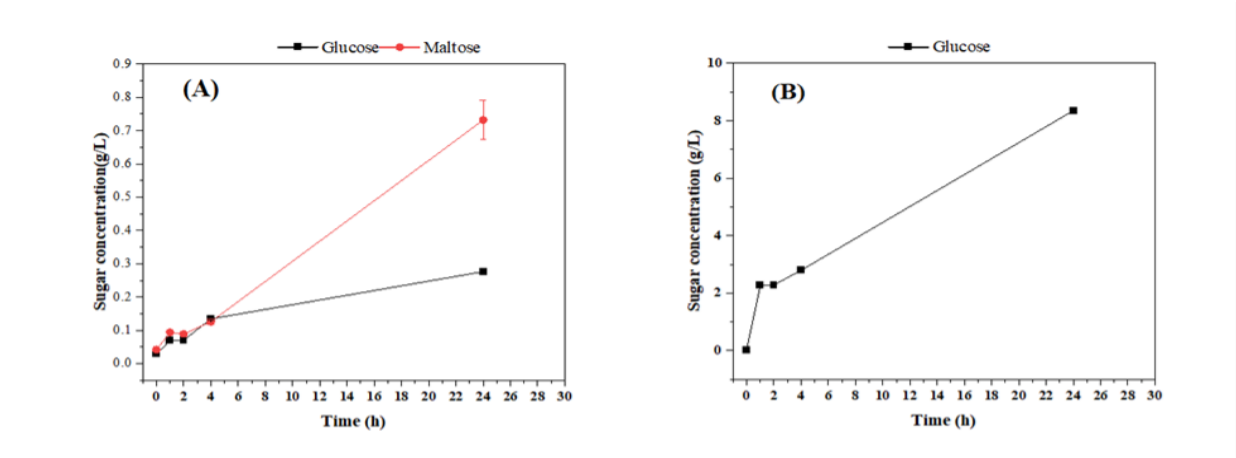

Figure 2.2 Sugar generated from raw normal corn starch during the hydrolysis by WAE (A) and STARGEN™ (B) in 24 h

3.3 EPSs characterization

The EPSs as a bioactive compound have been widely found in the marine environment, and the EPSs producing microorganisms, such as microalgal species, marine protists, marine bacteria, fungi were included[85]. To study EPSs bioactivities, identification and characterization of their chemical composition and structure was necessary. In this study, the chemical composition and structure of EPSs were investigated.

3.3.1 Chemical composition analysis

In the chemical composition analysis, the composition of EPSs was determined, including total carbohydrate, sugar monomers, sulfate. As shown in **Table 2.1**, the monosaccharides in EPSs were identified by HPLC-RID, the obtained peaks from HPLC spectra of each EPS variety were compared with monosaccharide standards to quantify and qualify the monosaccharides content. As presented, all EPSs contained three types of monosaccharides, which were glucose, mannose, and galactose. The major monosaccharide was glucose, which took up 57 ~ 86% of the total sugar content, followed by mannose (7 ~ 16% of the total sugar content). The galactose was in relatively lower content of 1~4% of the total sugar content. In

other word, the molar ratio of monosaccharides was different among each EPSs produced from different starchy substrates. In addition, comparing with the chemical composition analysis in our previous study, the monosaccharide profile of EPSs obtained in this research was the same, but the molar ratio of each monosaccharide was different[49]. The results indicated that the EPSs formation was affected by the carbon source and *T. striatum* had a preference of substrates for EPS production. The effect of carbon source on EPSs bio-formation was also found in in other microorganisms, such as *Lactobacillus delbruckii* which synthesized EPSs with different molar ratio of monosaccharides when cultured in glucose and fructose[109,110]. Furthermore, the molar ratio of monosaccharides in each EPSs could be related to the growth condition and status of *T. striatum*. The EPSs production occurs in the later stage of growth[110,111]. As reported by Jain et al,. the EPSs production and accumulation by *Thraustochytrids* SC-1 and CW-1 were growth related and achieved the maximum quantity at the stationary phase[48].

Table 2.1 Chemical compositions of EPSs from various starchy substrates

Starch varieties		Glucose	Soluble	Wheat	Corn	Waxy corn	High amylose
Sulfate content (μg $SO4^{2-}$/mg EPS)		0.16 ± 0	0.11 ± 0	0.05 ± 0	0.05 ± 0	0.15 ± 0	0.08 ± 0
Total sugar content (μg sugar/mg EPS)		4.04 ± 0.04	1.71 ± 0	3.51 ± 0.07	4.06 ± 0.06	2.84 ± 0.05	1.5 ± 0.03
Monosaccharides composition of EPSs	Glucose	3.58 ± 0.03	1.53 ± 0	3.18 ± 0.06	3.34 ± 0.03	2.05 ± 0.06	1.05 ± 0
	Galactose	0.06 ± 0.01	0.03 ± 0	0.07 ± 0	0.17 ± 0	0.26 ± 0	0.16 ± 0
	Mannose	0.39 ± 0.01	0.15 ± 0	0.25 ± 0.01	0.55 ± 0.03	0.53 ± 0	0.28 ± 0.02

The sulfate content was monitored in each variety of EPSs. Sulfate appeared in all EPS variety, from the content range of 0.05 ~ 0.16 μg SO_4^{2-}/ mg EPS. The sulfate content in glucose derived EPSs reached the highest sulfate, which was 0.15 μg SO_4^{2-}/ mg EPS. Meanwhile, the higher sulfate content reached 0.15 μg SO_4^{2-}/ mg EPS in waxy corn derived EPS, comparing amongst EPS derived from starchy substrates. As different type of starchy substrate provided to

T. striatum, under the same amount of sulfate was provided in the medium, herein the effect of medium to EPS sulfate content was negligible. The sulfate content in EPSs could be related to the cell growth and stages.

3.3.2 Structure characteristic

The FT-IR spectrum of each EPS is shown in **Figure 2.3**. Most peaks of EPSs were similar, indicating the similarity of chemical structure. However, the peak intensity of glucose-EPSs and high amylose- EPSs showed a weaker peak than that of other EPSs. The region of 3100-3300 cm^{-1} could be assigned to the stretching vibration of hydrogen-bonded O-H groups[112]. The weak absorption bands at about 3000-2850 cm^{-1} were attributed to the C-H stretching vibration of sugars[113]. Peaks between 1000 and 1200 cm^{-1} may reflect the presence of C–O–H and C–O–C of galactose. The strong absorption peaks at 1273 cm^{-1} and 875 cm^{-1} could be caused by S=O and C-O-S, respectively[1]. The selected spectral regions had contributions from amide I (1700-1600 cm^{-1}) and amide II (1565-1520 cm^{-1}) vibrational bands[114,115], indicating the presence of protein. Overall, the FTIR spectra revealed that the EPSs comprised a variety of components and functional groups, including carbohydrates, carbonyl, carboxyl, peptide, N-containing groups, etc.

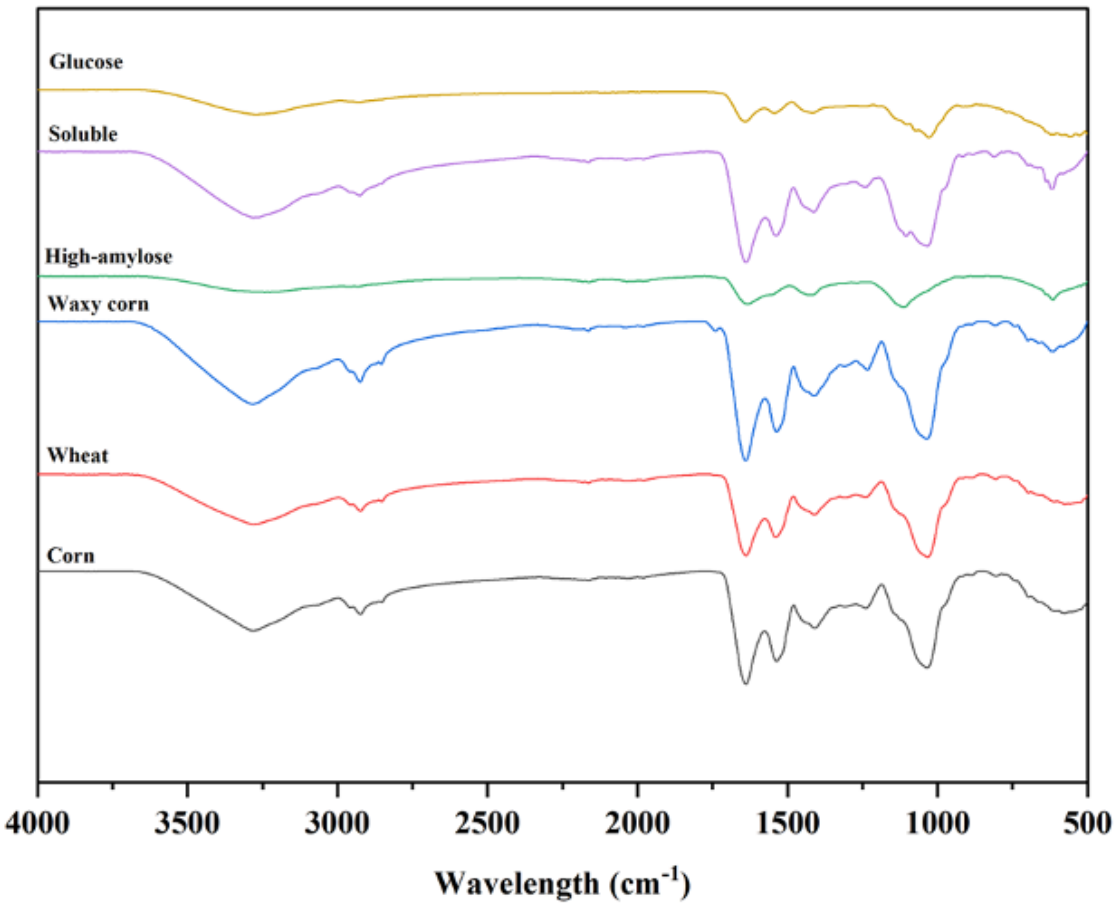

Figure 2.3 FT-IR spectrum of EPSs

NMR analysis including ^{1}H, ^{13}C, and 2D HSQC provided information about structural backbone and signature structures. The chemical shift (δ) was reported in parts per million (ppm) referenced to acetone (^{1}H = 2.225 ppm and ^{13}C = 30.50 ppm). After the spectra were calibrated to reference acetone at 2.225 ppm, and water HDO peak appeared at ~4.70 ppm. The anomeric centers of sugars were shown in the range of δ at 4.0 to 6.0 ppm. The ^{1}H spectra of all EPSs contained a typical dextran α-configuration chain-extending anomeric signal centered at 5.05 ppm (Data not given). The ^{13}C spectra of EPSs from different starchy substrates were shown in **Figures. 2.4**. The ^{13}C NMR spectra shown that EPSs consist of variety of component and functional groups, which includes carbohydrate, carbonyl, carboxyl, N-contain groups, and peptides. The peak intensities shown significant difference appear mainly at δ 30 to 80 ppm, where the other areas shown weaker.

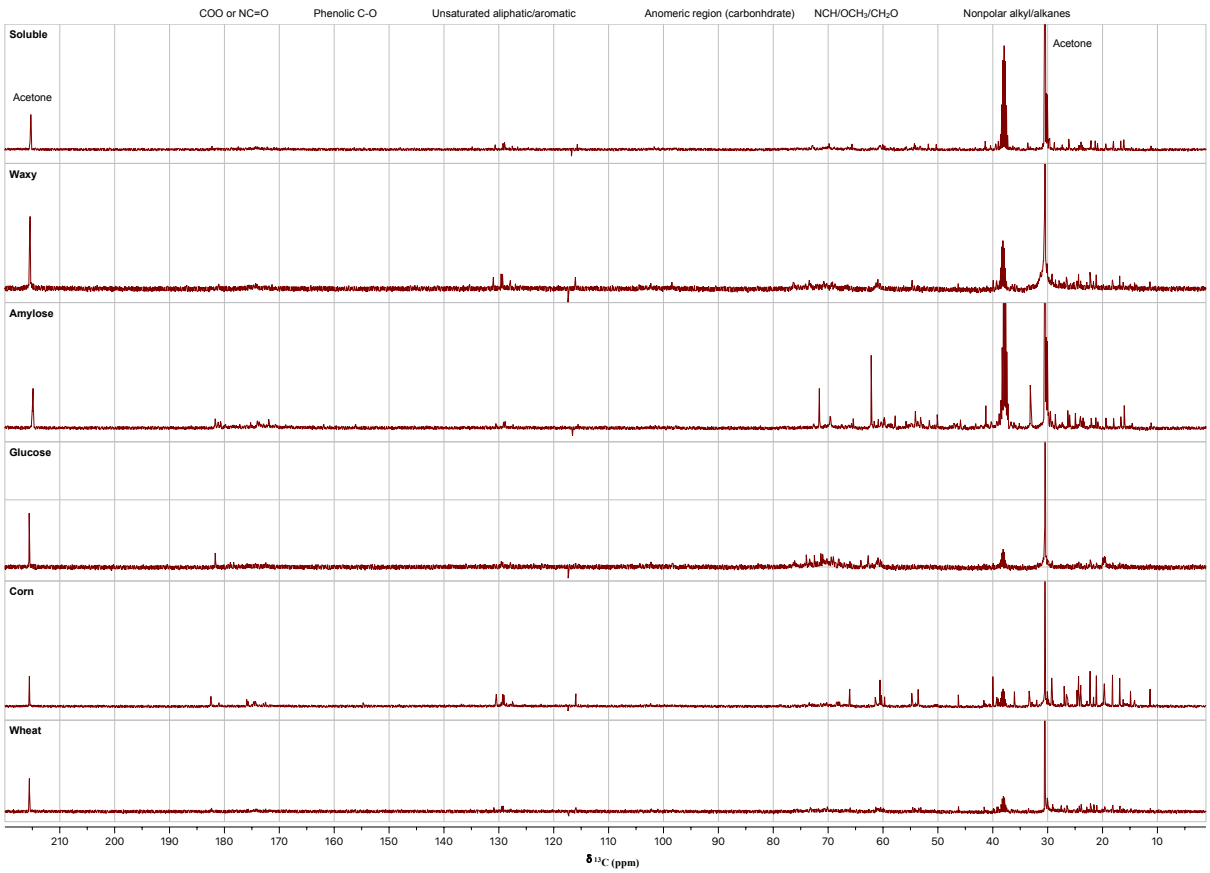

Figure 2.4 1D ^{13}C spectrum of EPS-derived from glucose and different starchy substrates recorded at 400 MHz in D_2O

Based on ^{13}C-^{1}H HSQC of EPSs (**Figure 2.5**), the predominant structure of each EPSs was heteropolysaccharides, which was the composite of multiple monosaccharides with different glycosidic bonds and various monosaccharides sidechains. The signature peak of sulfated galactose was found in most EPSs, except for soluble starch-EPSs. The δ_H 4.59/ δ_C102.4 were ascribed to C1-H1 of *(1→3) linked 4-Osulfate-β-D-galactose*[1,116], this compound was also found in the EPSs from red algae *Gracilariopsis lemaneiformis*[1] and *Gracilari caudate* [117]where sharing similar spectra. Moreover, the structure of EPSs predominantly consisted of *α-D-Manp*, which showed strong signals at δ_H 5.13 and δ_C 98.5 ~ 102.6 ppm. The major anomeric signals attributed to α-mannosyl residues, the most predominant peaks appeared strong signals and

correlated to higher content of component. However, due to the complexity of EPSs, lots of unassigned peaks still exist. The similar mannose backbone was one of the possible reasons of intensive overlapping peaks. Also, the impurity of EPSs could be another influencing factor. Further purification and characterization work will be needed in the future.

Notably, although the chemical monomers of EPSs showed similarities, their structures were entirely different among each starchy substrate. Similar results were observed in other studies. For instance, EPSs produced by *Lactococcus lactis* cultivated on both fructose and glucose showed different levels of fructosediphosphates expression, which resulted in an increment of EPSs expression when *L. lactis* was grown on fructose rather than glucose. The appearance of fructose functioned as a precursor of fructosediphosphates secretion and regulated the EPSs formation in *L. lactis*[118]. Thus, each starchy substrates and/or release sugars could function as a precursor for unique synthesis pathway of EPS, and further cause the difference in structures.

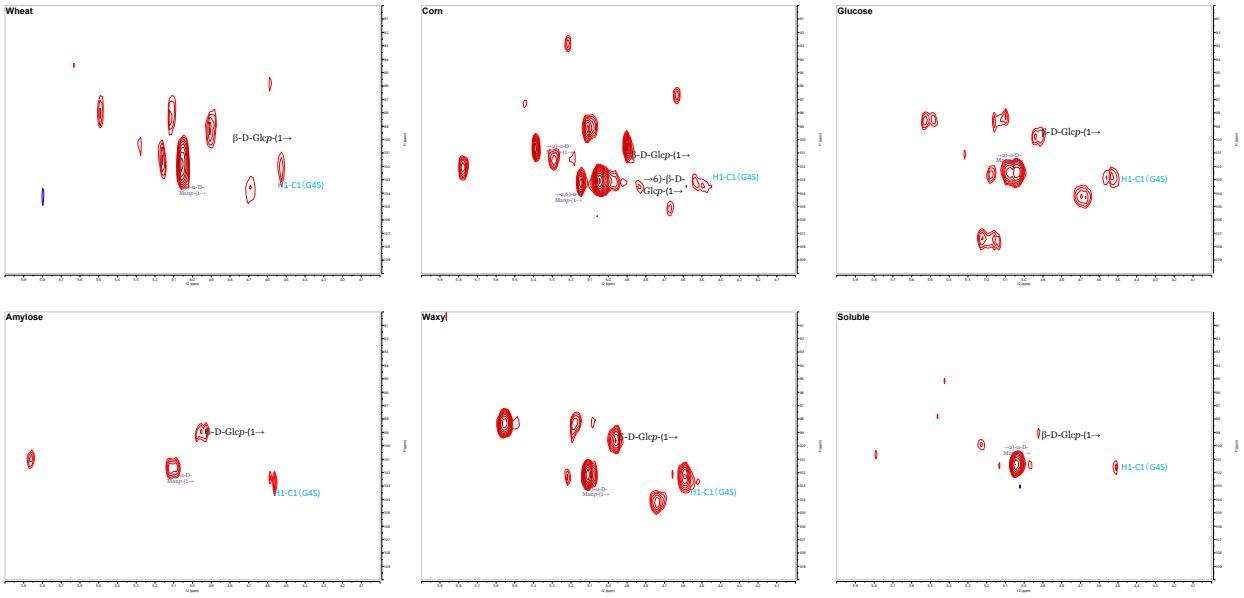

Figure 2.5 ^{13}C-^{1}H HSQC preliminary results of short-range spectra of the anomeric carbons for EPSs-derived from glucose and different starchy substrates recorded at 400 MHz in D_2O.

4. Conclusions

Raw starchy substrates were successfully converted into anticancer EPSs by *T. striatum* by using its own amylolytic enzymes. The starch-degrading enzyme complex was found to mainly include α-amylase and α-glucosidase, which was able to degrade corn and wheat starches (normal and waxy) into glucose and maltose in one-pot at room temperature without separate gelatinization, liquefaction or saccharification. The crude enzyme activities were dependent on the cell growth stage of *T. striatum*. The amylolytic enzymes of *T. striatum* could be a promising robust starch-degrading enzyme system that has a potential commercial value for industry.

Among starchy substates examined, the EPSs yield varied with the growth of *T. striatum* cells. The composition analysis showed that the predominant carbohydrates of EPSs were mannose, glucose, and galactose. The monosaccharide compositions of EPSs derived from different starches were consistent, but the proportion of each monosaccharide differed significantly. Moreover, the structure analysis of FT-IR and NMR showed that the EPSs were heteropolysaccharides, which consist of multiple monomers along with multiple sidechains. The EPSs showed different structures among the studied starchy substrates. The main chain of EPSs consisted of α-mannose and α-glucose. The sulfated-galactose was found in EPSs as the main structure of (*1→3*) *linked 4-Osulfate-β-D-galactose*. The starch-based EPSs differed from the glucose-based counterpart, and such structural difference in EPSs could be because the EPSs synthesis involved substrate-regulated metabolism pathways.

Chapter 3 - Anticancer efficacy of starch-derived EPSs on HepG2 and HeLa

1. Introduction

Extracellular polymeric substances (EPSs) have been considered as an essential component in biofilms of marine protist, bacterium, and microalge[12,81,82]. The biofilm was a complex matrix surrounding the microbial cell wall and provides protection for host cells against desiccations[82], defend changes in physico-chemical conditions[4], and enhance cell survival[82]. Plenty of EPS were found to have biological active, such as anti-inflammatory and anti-proliferation to specific cancer cells[83]. As a natural source of anticancer compound, EPS was characterized of chemical composition and structure, exploration and screening of anticancer effect, and anticancer mechanism study[84].

The EPS was found from multiple marine microbes in the ocean system, such as marine algae, bacteria, and protist[82]. Interestingly, some of the EPS were found to show anticancer and anti-inflammatory effects to cancer cells. To understand the anticancer efficacy of EPS, the chemical composition of EPS was probed initially. The composition of EPS varied from microbes, and versatile chemical composition and structures were explored and characterized. The EPS is a matrix mainly composed of polysaccharides, proteins, nucleic acids, and lipids[64]. The antioxidant activity of EPS is structure-activity corresponding. The glycosidic linkage, molar ratio and composition of monosaccharides, molecular weight, and sulfation influenced the antioxidant activity of EPS[18,85]. The polysaccharides composition of EPS was mostly and best discovered. The mostly found monomers were monosaccharides (i.e., D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose) and uronic acids (i.e., D-galacturonic acid, L-guluronic acid, D-mannuronic acid)[6,82]. These monomeric composition of EPS provides reducing capacity,

which is likely because of the inherent reducing properties that enable the donation of electrons to reactive radicals, thus diminishing their reactivity[51]. Especially, the hydroxyl and carboxyl group in uronic acid shown efficient scavenging of free radicals[119].

An effective drug delivery and cell culture system is important to evaluate and predict the efficacy of anticancer compounds. The traditional monolayer two-dimensional (2D) system has been mostly used for anticancer drug screening, which is simple and inexpensive[120]. However, the results from the 2D system often deviate from the outcome of those obtained from the animal models[121]. Moreover, the 2D system has some critical disadvantages that it misses the tissue-specific structural, mechanical, and biochemical cues and doesn't reflect the cell-to-cell and cell-to-matrix interactions[122,123]. Thus, the three-dimensional (3D) cell culture system has been explored in the past a few years to reveal a rather accurate prediction of *in vivo* drug screening results. Recently, a novel peptide hydrogel (PepGel) has been developed as an advanced 3D cell cultivation system, which enhances cell recovery and has less inhibition on drug treatment compared to the traditional 2D cultivation system[124]. This 3D extracellular matrix provides tissue mechanical properties and promotes the cell-to-cell interaction and communication[125].The PepGel can control the drug release efficiency through the inhibition of the diffusion rate of BSA-linked cisplatin such that the drug can be well delivered, even at low concentration (e.g., below 0.3wt%)[126]. The novel 3D PepGel system establishes a mimic *in vivo* environment for cell growth and reduces the susceptibility of the cells to the unfavorable growth conditions[124, 17]. Such a 3D system has been used for various cancer cell lines, such as HeLa cervical cancer cell[124], HepG2 hepatocellular carcinoma cells[13], SW480 colon adenocarcinoma[124], H9c2 rat cardiomyocytes[127], and normal cells.

This research was aimed at exploring starchy substrates effect to EPSs production by *T. striatum*, and the corresponding anticancer efficacy to HeLa and HepG2 cell in both 2D and 3D system. The morphology of HeLa and HepG2 cells after treatment by each EPS variety were observed. The EPSs effect to HeLa and HepG2 cells proliferation rate and viability were investigated, wherein the EPS anticancer efficacy was quantified.

2. Materials and Methods

2.1 EPS preparation

The EPS was used as treatment to HepG2 cells. A dosage of 10, 30,100, and 200 μg/ml of each EPS variety were prepared in DMEM medium solution. Each EPS treatment stock was sterilized by filtration through 0.22 μm sterilized filter before use.

2.2 2D culture screening system preparation and cell pellet collection

HepG2 and HeLa cells were inoculated at 2×10^4 cells/well, and were cultured with 1 mL DMEM medium supplemented by 10% FBS and 100 μg/mL of streptomycin/penicillin in 24-well plates at 37 °C. After one day of pre-culture for cell attachment, cells were treated with EPS of 10, 30,100, and 200 μg/ml for 72h treatment. Then, cells were harvested at the end of 72h treatment for cell morphology observation, cell viability and proliferation estimation. To harvest HepG2 and HeLa cells, the cells were rinsed with 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS)/well. The cells were then detached by rinsing by 0.05% trypsin-EDTA enzyme at 1 mL/well at 37 °C for 8 mins. Fresh DMEM medium at 1 mL/well was added to inhibit the trypsinization effect and the detached cell suspension was collected into centrifuged tubes. Cells were separated through centrifugation at 400g for 5 mins by centrifuge (Eppendorf 5702, Hamburg, Germany).

2.3 3D culture screening system preparation and cell pellet collection

HepG2 and HeLa cells at 2×10^4 cells/well were cultured with 1 mL DMEM medium supplemented by 10% FBS and 100 μg/mL of streptomycin/penicillin in 24-well plates at 37 °C. A PGmatrix-DMEM kit (PGD) commercially available on PepGel LLC (Manhattan, KS) were used for 3D culture. The PGD hydrogel were prepared based on the manufacturer's instructions. After 48h of pre-culture for cells to form physiological 3D colonies (spheroids)in 3D PGD hydrogel , cells were treated with EPS of 10, 30,100, and 200 μg/ml, respectivelyfor 72h. Then, cell spheroids were harvested at the end of 72h treatment for cell morphology observation, cell viability and proliferation analysis. The cell spheroids were then enzymatically dissociated by 0.05% trypsin-EDTA at 1 mL/well and incubated at 37 °C for 8 mins. Fresh media at 1 mL/well was then added to inhibit the trypsinization effect and the dissociated cells suspension was collected into centrifuge tubes and centrifuged at 600g for 6 mins using a centrifuge (Eppendorf 5702, Hamburg, Germany). . The upper supernatant was gently removed, and the cell pellet was collected and counted by a Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA) using AO/PI staining method[124]. Two replicates were made for each treatment.

2.4 Cell morphology and viability assay

The morphological characters were determined by using an inverted light microscope (Nicon Eclipse TE2000-u, Kanagawa, Japan). Morphologies of HepG2 cells cultured in 2D monolayer, or 3D hydrogel system were observed at the initial and end of EPS treatment. After treatment, viable cell number and cell viability were measured using acridine orange/propidium iodide (AO/PI) assay from Nexcelom Bioscience and counted by Cellometer Auto 2000 (Nexcelom Bioscience LLC, Lawrence, MA)[124].

2.5 Data analysis

The significant levels of different treatments were determined by analysis of variance (ANOVA) (α= 0.05). GraphPad Prism® version 10.1 (San Diego, CA) used to perform experimental design, statistical analyses, and figure delivery. A general linear model procedure followed by Tukey's multiple comparison was applied. All samples were assayed in duplicate in two to three different experiments. The results are present as means ± SD.

3. Results

3.1 EPS anticancer effect screening on HepG2 cell

3.1.1 Morphology

Cell morphology of HepG2 cell cultured in both 2D and 3D hydrogel were monitored (**Figure 3.1 & 3.2**). The morphologies of cells grown in both 2D, and 3D system were observed after 72h of treatment of each EPSs varieties under different dosages (i.e., 10 μg/mL, 30 μg/mL, 100 μg/mL, 200 μg/mL of EPSs). In 2D culture system (**Figure. 3.1**), grapelike, stellate, and rounded spheroids of HepG2 cells were observed. The distribution and density of HepG2 shown great differences, such as glucose-EPS treated HepG2 cells were widely spread with the increment of treatment, waxy corn-EPS treated HepG2 cells were widely spread with most of cells shown a grapelike clusters, the other EPSs treated HepG2 cells (i.e., wheat-EPS, corn-EPS, high-amylose EPS, and soluble-EPS)were individually rounded and stellate clusters with much smaller cluster diameters than glucose-EPS and waxy corn-EPS treated HepG2 cells. The results of each EPS variation involve the morphological changes of HepG2, indicating a potential of the functionalities of each EPS to HepG2 cells. In 3D hydrogel system (**Figure .3.2**), the cells were encapsulated in hydrogel, most of HepG2 cells were rounded spheroids with different spheroids diameter. The waxy corn-EPS treated HepG2 cells had larger spheroids diameter other than other

EPS varieties treated HepG2 cells. The morphologies of HepG2 cells were aligned with others studies, where HepG2 cells were grown in same Pepgel[128]. The distribution and density of spheroids had no distinctive differences. Comparing the result from 2D and 3D culture system, waxy corn-EPS treated HepG2 shown morphological differences against other experimental groups.

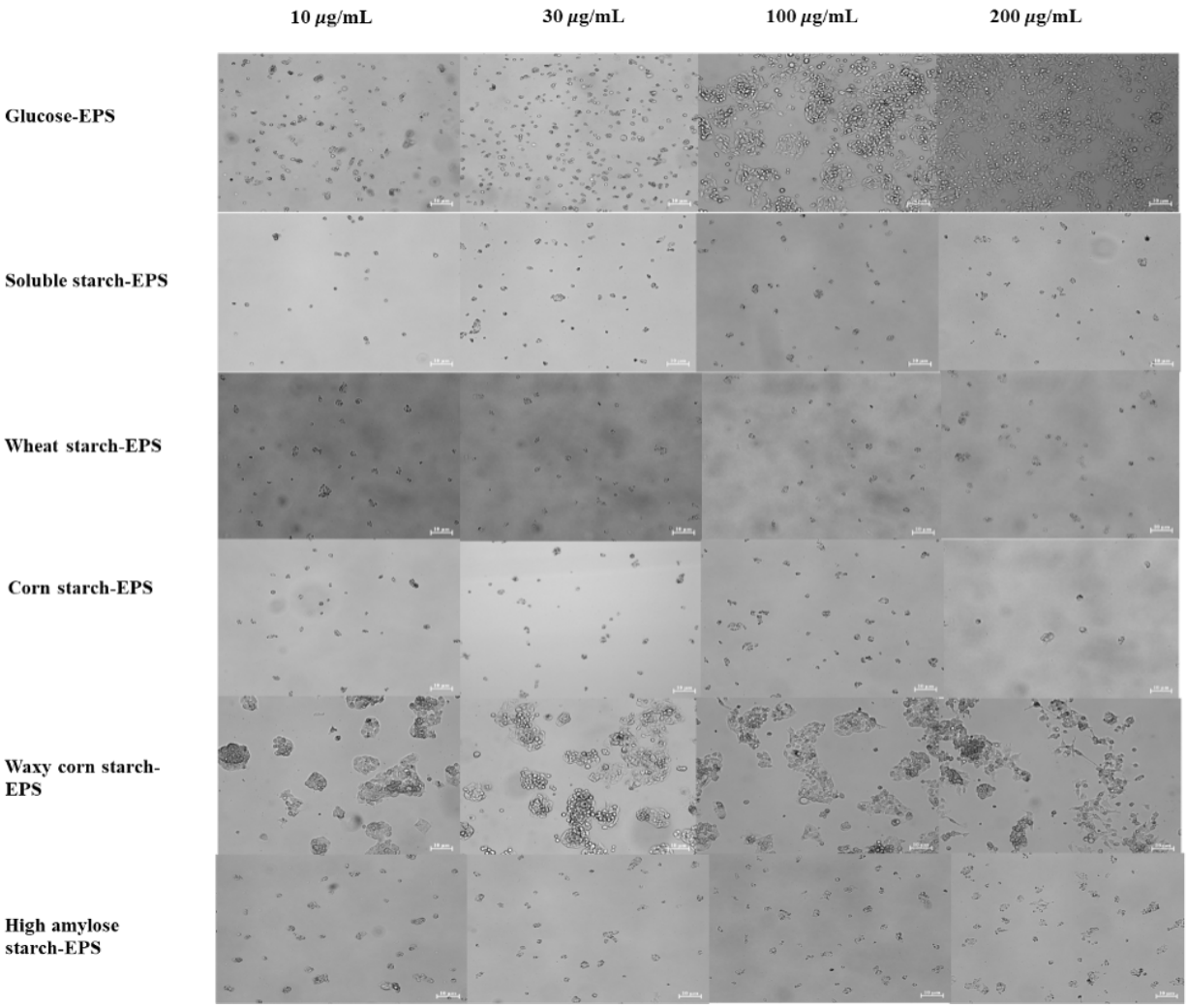

Figure 3.1 Morphologies of HepG2 cells culture in 2D monolayer system with treatment of EPSs from each starchy substrate variety at culturing 72h. Each image was shown with the scale bar of 10 μm.

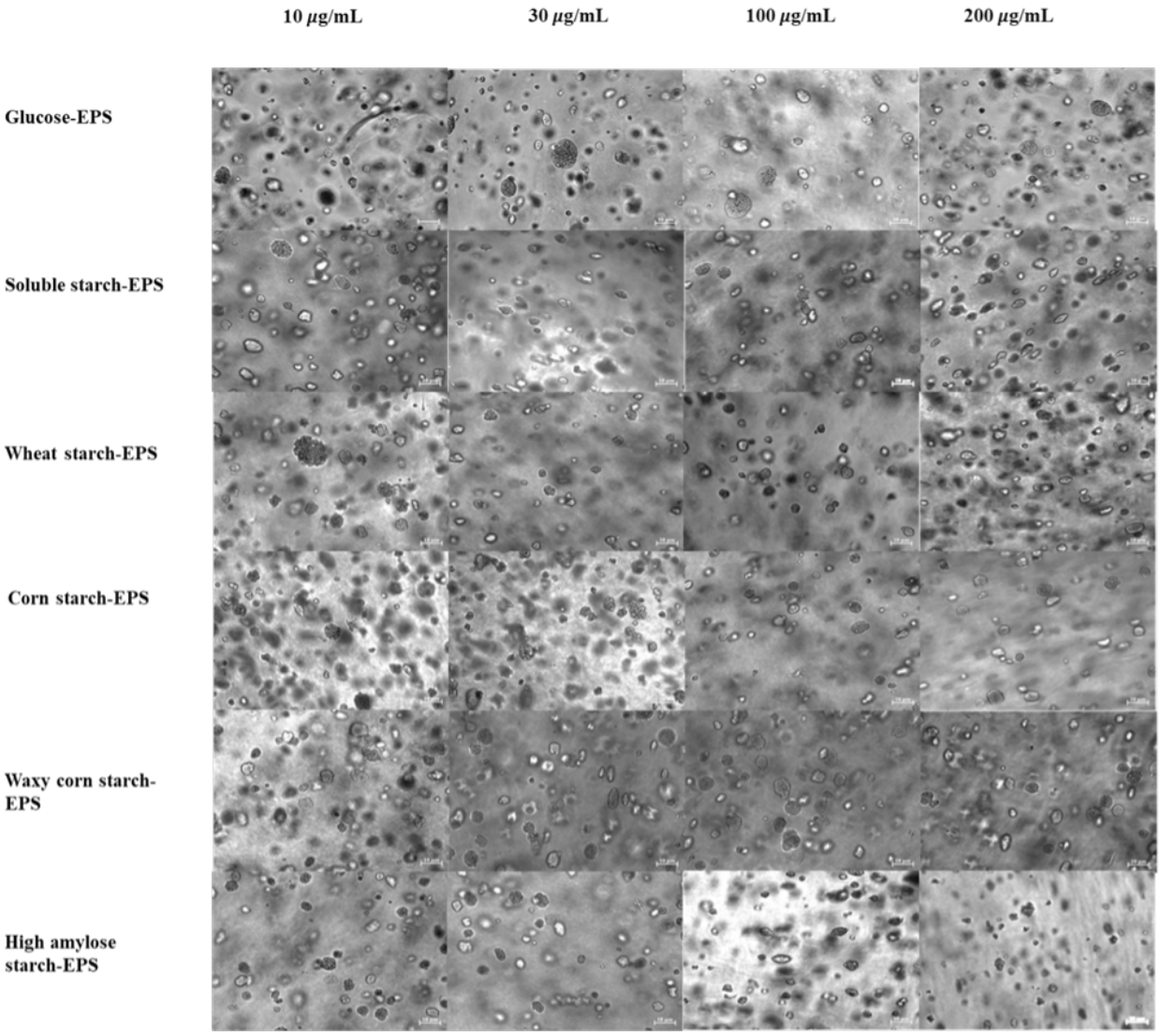

Figure 3.2 Morphologies of HepG2 cells culture in 3D monolayer system with treatment of EPSs from each starchy substrate variety at culturing 72h. Each image was shown with the scale bar of 10 μm.

3.1.2 Viability and proliferation of HepG2 cells

The effect of each starchy substrate derived EPS anticancer effect on HepG2 cell growth in both 2D and 3D culture were studied. The effect of EPS on HepG2 cells was investigated through observing antiproliferation effect. The antiproliferation effect of EPS was evaluated through viability (**Figure 3.3**) and proliferation rate (**Figure 3.4**) of HeLa cell. To interpretate, the viability of cells is calculated as the percentage ratio of viable cells to total number of

cells[129], it reveals the survival and growth of cultured cells in the cell culture. The proliferation rate was calculated as the ratio of density of viable cells at the end of treatment to initial inoculation (2×10^4 cells/mL), which characterizes and identifies the EPS anticancer efficacy to HepG2 cell growth.

The anticancer efficacy of EPS to HepG2 cell proliferation was shown differently from viability and proliferation rate. In **Figure 3.3,** the viability of each EPS screening under each dosage (i.e., 10, 30,100, and 200 μg/mL) in both 2D and 3D culture system were shown. The viability of HepG2 cells in 2D and 3D system were 96.8% and 97.8% respectively. The viability of each EPS variety shows a decreased tendency as EPS dosage increased, despite glucose-EPS treated HepG2. Amongst, the viability of wheat-EPS reached 57.9%, which was significantly lower than control and other groups. The results indicated that the inhibitory effect of each EPS varieties to HepG2 were different. On the other hand, the culture system significantly affects the viability of HepG2 cells under the same treatment applied. The viability of HepG2 cells culture in 3D hydrogel shown higher and/or parallel than in 2D culture. The results indicated that the culture system significantly affects the EPS anticancer efficacy. As reported by Xu et al., the viability of HepG2 and SW480 cells favored to grow in 3D hydrogel system compared to 2D monolayer system, wherein the cells viability and proliferation rate were higher in 2D system than 3D system[124]. The 3D hydrogel system provided a favorable cell growth circumstantial to HepG2, also the 3D hydrogel shown more resistance to EPS delivery. Furthermore, the 3D system gives more precision and accurate prediction of the anticancer efficacy of EPS than the 2D system. In **Figure 3.4**, the proliferation rates of each EPS screening under each dosage (i.e., 10, 30,100, and 200 μg/mL) in both 2D and 3D culture system were shown. The proliferation rate given the results of alive cells growth in both 2D and 3D system. The EPS dosage shows a

significant effect on the HepG2 cell proliferation rate. As in **Table 3.1**, the ANOVA analyzed the proliferation rate of each EPS in the same culture system compared to the relative control group. In the control group, the proliferation rate of 2D and 3D culture system shown non-significant difference. The dosage of EPS applied affects the proliferation rate of HepG2 in both 2D and 3D system. The dosage of 10 μg/mL shows a nonsignificant effect to cell proliferation. Thus, the 10 μg/mL was removed from the screening list in later HeLa cell screening. With the increment of EPS dosage, the proliferation rate was slightly decreased in corn-EPS and waxy corn-EPS groups, the others showed nonsignificant effects. Thus, the results indicated that the EPS had a slight inhibitory effect to HepG2 proliferation. The effectiveness of polysaccharides against cancer cells is associated with factors such as their structural characteristics, including chemical composition, molecular weight, and linkage patterns[130]. Thus, the inhibitory effect could be originated from sulfated polysaccharides. For instance, sulfated polysaccharides exhibited prospective apoptosis of HepG2. For example, a sulfated galactofucan characterized as [→1)-O-4-sulfonato-α-fucopyranose-(3→1)-α-fucopyranose-(3→] as the main branch with [→1)-6-O-acetyl-*β*-galactopyranose-(4→] as a side chain isolated from a marine macroalga *Turbinaria ornata* exhibited prospective apoptosis, reduction of cell viability on HepG2 [131]. The polysaccharides from *Phaeodactylum tricornutum* exhibited anticancer effects mainly through the induction of apoptosis without affecting the cycle and mitosis of HepG2 cells[132].

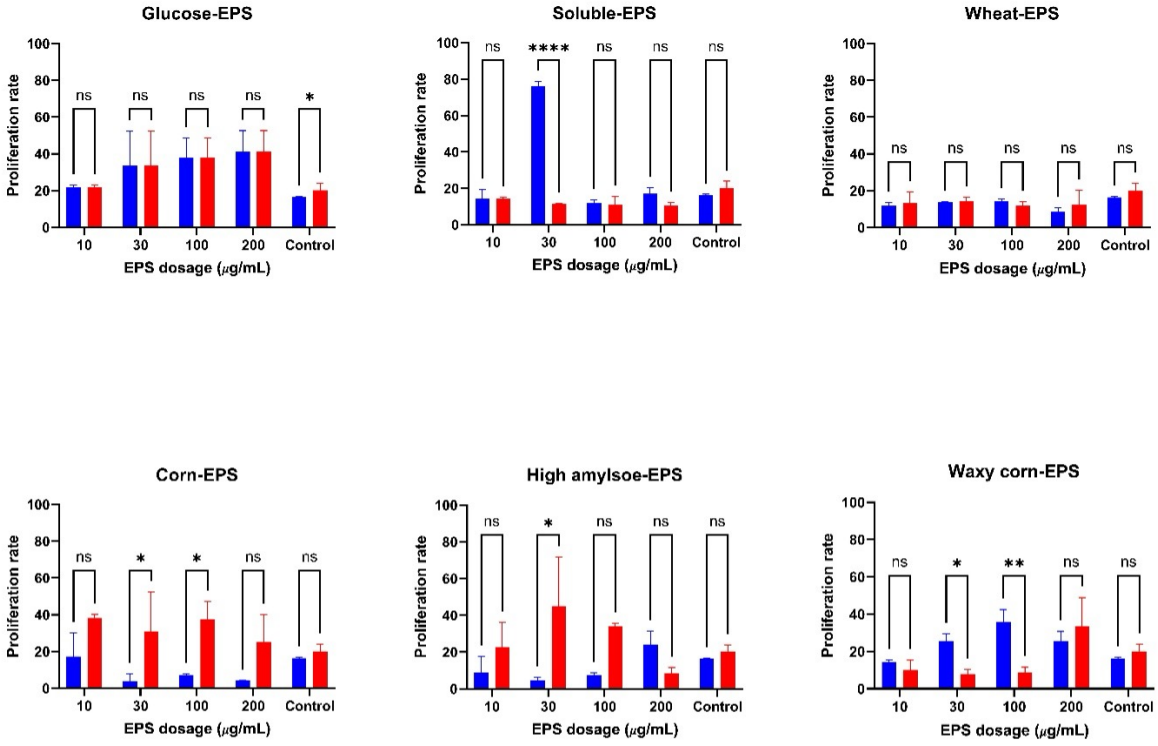

Figure 3.3 Effect of starch derived EPS to HepG2 cell viability in 2D(blue) and 3D(red) system. Viability of control group 2D=96.8% and 3D=97.8%.

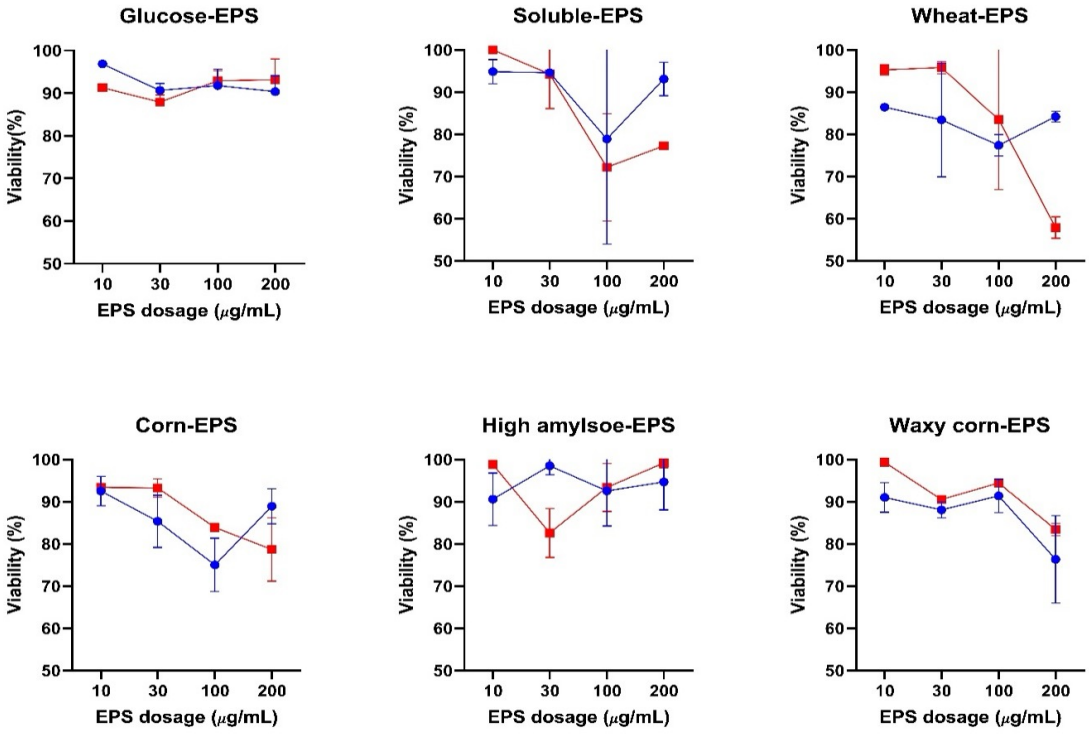

Figure 3.4 Effect of starch derived EPS to HepG2 cell proliferation rate in 2D(blue) and 3D(red) system. Proliferation rate of control 2D=16.18 and 3D=22.85.

Table 3.1 Two-way ANOVA of HepG2 cell proliferation rate to culture system results

EPS variety	EPS concentration (μg/mL)	2D-Control	3D-Control
Glucose-EPS	10	ns	ns
	30	***	**
	100	***	***
	200	***	***
Soluble-EPS	10	ns	ns
	30	ns	ns
	100	ns	ns
	200	ns	ns
Wheat-EPS	10	ns	ns
	30	ns	ns
	100	ns	ns
	200	ns	ns
Corn-EPS	10	ns	ns
	30	*	ns
	100	*	ns
	200	ns	ns
High amylose-EPS	10	ns	ns
	30	ns	ns
	100	ns	ns
	200	ns	ns
Waxy corn - EPS	10	ns	ns
	30	ns	ns
	100	ns	ns
	200	ns	ns

Note: Statistical differences were represented as ***p<0.001, **p<0.01, *p<0.05, and “ns”not significant.

3.2 EPS anticancer effect screening on HeLa cells

3.2.1 Morphology

Effects of EPS on HeLa cell morphologies in both 2D and 3D culture were monitored (**Figure 3.5 & 3.6**). The morphologies of cells grown in both 2D, and 3D system were observed after 72h of treatment of EPSs under different dosages. In 2D monolayer system (**Figure 3.5**), the cells morphologies were observed having a flat spreading shape, wherein from each EPS treatment shown different appearance. The cell shape was observed having no specific changes. The HeLa cell treated by waxy corn starch and wheat starch derived EPSs were observed shown more spare space and less cell clusters on the microplate, where the cells after treatment shown

morphological changes. The morphological changes included the reduction of cell density, loss of adhesion to microplate, and an EPS dosage to cell proliferation reduction tendency. However, the other groups had not shown distinct cell morphological changes and tendency. In the 3D hydrogel system (**Figure 3.6**), the cells were encapsulated in hydrogel and formed cell spheroids, the spheroids shaping as round, mass, and grape included. The similar shaping was also observed in others study of HeLa cell encapsulated in pepgel[133]. The morphologies and distribution of cell spheroids did not show a distinctive difference from control group. Compared to the observed result from 2D and 3D system, the groups (i.e., waxy corn starch and wheat starch derived EPS treated cells) observed having morphology changes in 2D system were not shown in 3D system, correspondingly. The difference in morphological properties attributed to different reasons, including the rate of EPS diffusion in hydrogel, 3D hydrogel affected cancer cell sensitivity to anti-cancer drug[134]. In addition, the morphological properties of HeLa cells could be responsible for cell proliferation, changes in morphology affected their function, intercellular organization of structures, and secretion and cell signaling[135,136].

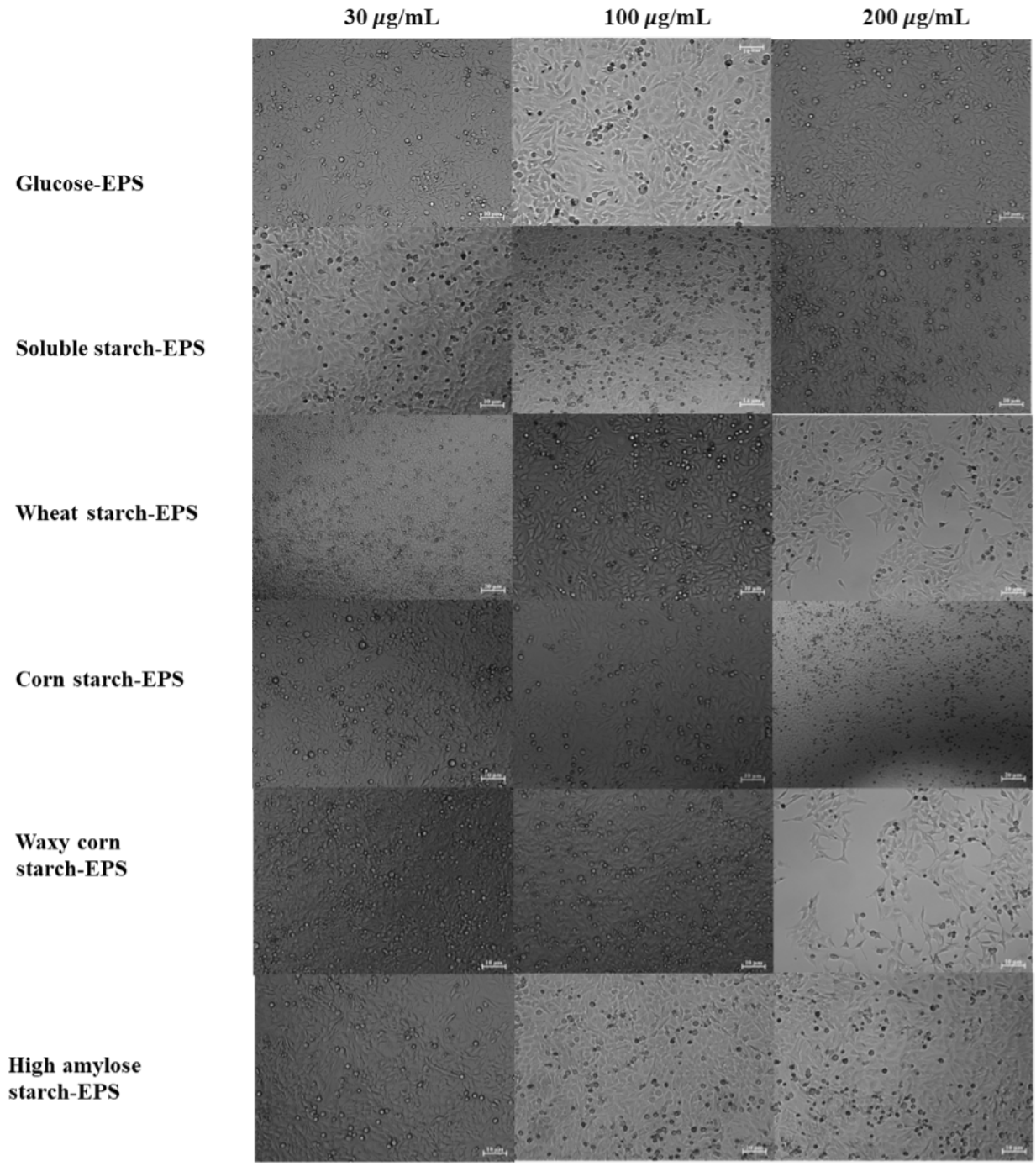

Figure 3.5 Morphologies of HeLa cells culture in 2D monolayer system with treatment of EPSs from each starchy substrate variety at culturing 72h. Each image was shown with the scale bar of 10 μm.

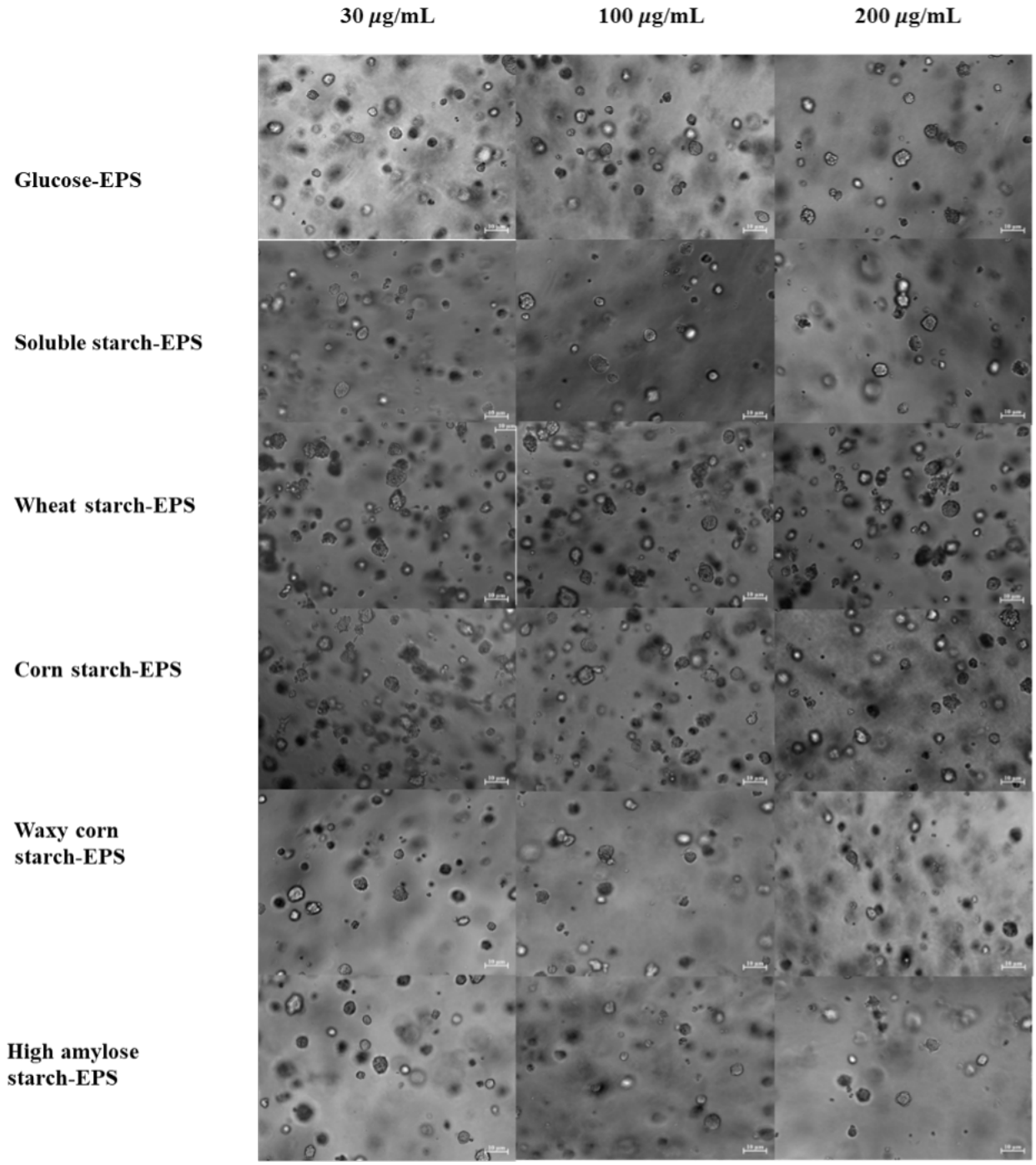

Figure 3.6 Morphologies of HeLa cells culture in 3D monolayer system with treatment of EPSs from each starchy substrate variety at culturing 72h. Each image was shown with the scale bar of 10 μm.

3.2.2 Viability and proliferation rate

The effect of each starchy substrate derived EPS anticancer effect on HeLa cell growth in both 2D and 3D culture was studied. The effect of EPS on HeLa cells was investigated through observing the morphology change and antiproliferation effect. The antiproliferation effect of EPS was evaluated through viability (**Figure 3.7**) and proliferation rate (**Figure 3.8**) of HeLa cell. To interpretate, the viability of cells is calculated as the percentage ratio of viable cells and total number of cells[129], it reveals the survival of cultured cells in the cell culture. The proliferation rate was calculated as the ratio of density of viable cells at the end of treatment and initial inoculation (2×10^4 cells/mL), which characterize and identify the EPS anticancer efficacy to HeLa cell survival.

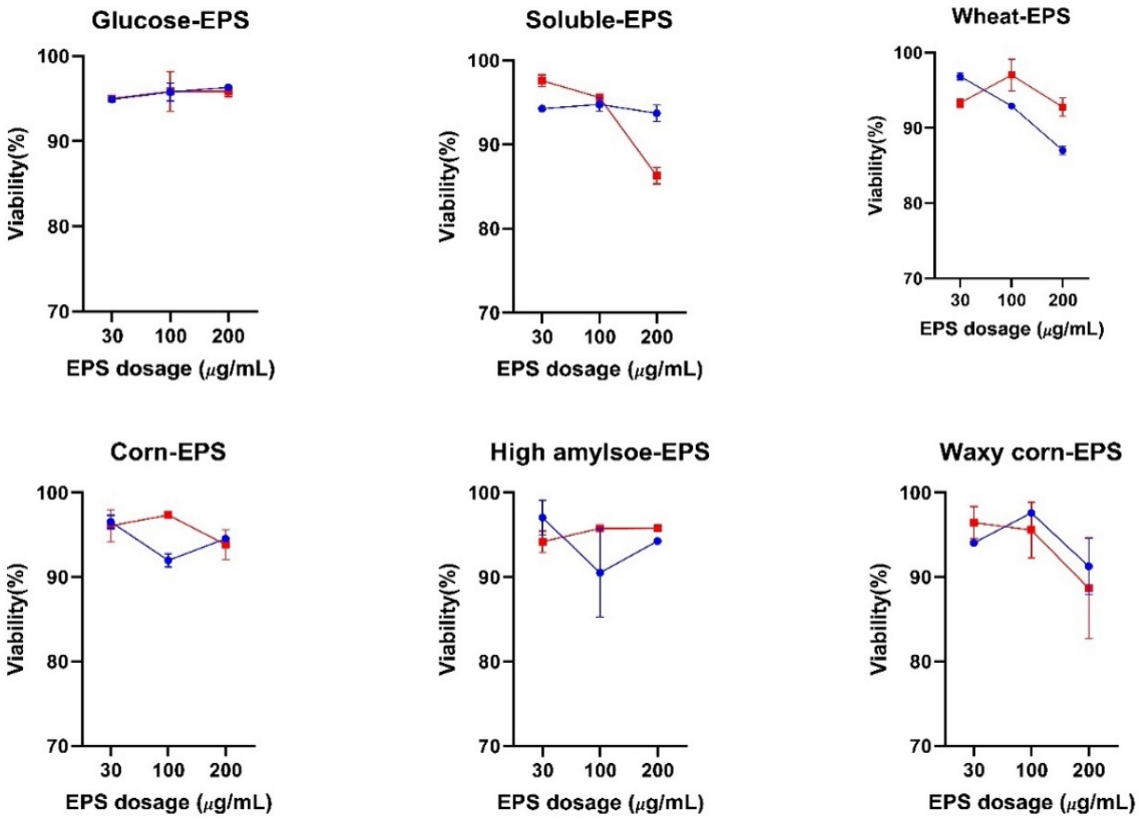

Figure 3.7 Effect of starch derived EPS to HeLa cell viability in 2D(blue) and 3D(red) system. Viability of control group 2D=94.9% and 3D=97.3%.

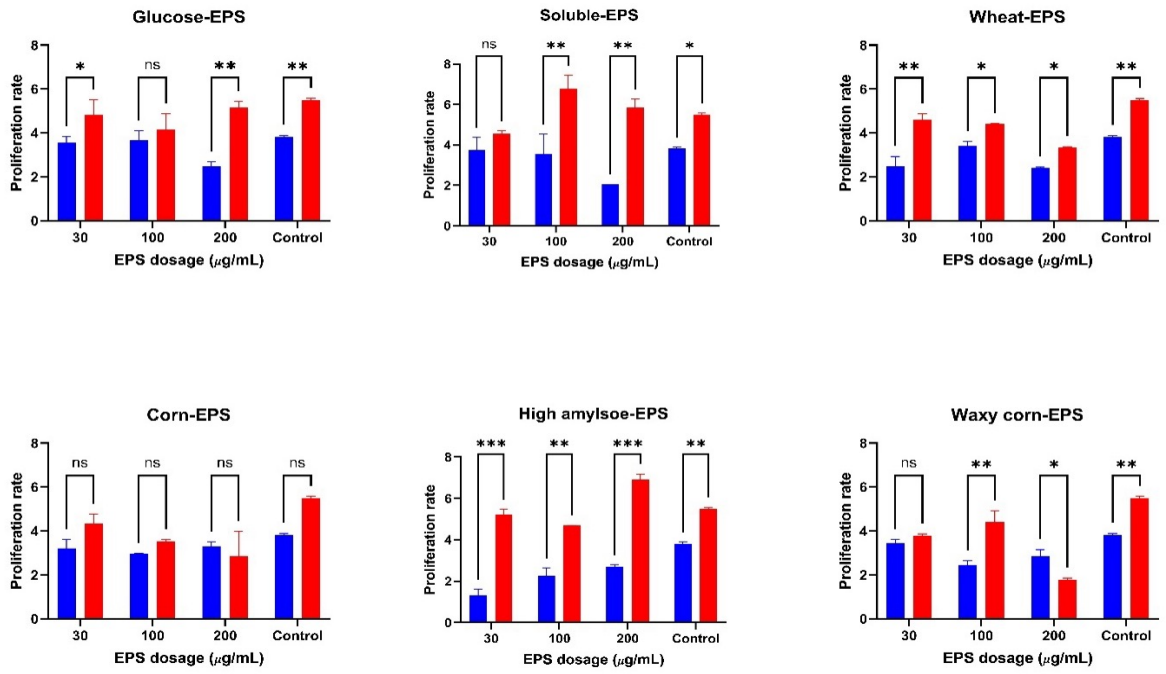

Figure 3.8 Effect of starch derived EPS to HeLa cell proliferation rate in 2D(blue) and 3D(red) system. Proliferation rate of control 2D=3.87 and 3D=5.45.

The anticancer efficacy of EPS to HeLa cell proliferation shown differently from chemical composition and properties difference, dosage of EPS treatment applied, and culture system. In **Fig. 3.7 & 3.8**, the EPS derived from each starchy substrate shown incompatible effect to HeLa cell proliferation. In details, the glucose-EPS shown slightly to negligible effect to HeLa cell proliferation, the viability and proliferation rate were in accordance with control with non-significant difference in both 2D and 3D culture system. Alternatively, the other EPSs, including soluble-EPS, waxy corn-EPS, high amylose-EPS, corn-EPS, and wheat-EPS, showed an inhibitory/facilitative effect to HeLa cell proliferation. On the other hand, the proliferation rate was inconsistent. Such as the soluble-EPS and high amylose-EPS show a facilitative effect to HeLa cell proliferation, on the contrary, the others (i.e., waxy corn-EPS, corn-EPS, and wheat-EPS) shown an inhibitory effect to HeLa cell proliferation. Moreover, the EPS performance to HeLa cell was influenced by the culture system. For instance, the proliferation rate of HeLa cell

cultured in 3D hydrogel was higher than in 2D culture. As shown in **Table 3.2**, the ANOVA analyzed the proliferation rate of each EPS in the same culture system compared to the relative control group.

Table 3.2 Two-way ANOVA of HepG2 cell proliferation rate to culture system results

EPS variety	EPS concentration (μg/mL)	2D-Control	3D-Control
Glucose-EPS	30	ns	ns
	100	ns	ns
	200	*	*
Soluble-EPS	30	ns	ns
	100	ns	ns
	200	ns	ns
Wheat-EPS	30	ns	ns
	100	ns	ns
	200	ns	*
Corn-EPS	30	ns	ns
	100	ns	ns
	200	ns	ns
High amylose-EPS	30	**	ns
	100	*	ns
	200	ns	*
Waxy corn-EPS	30	ns	*
	100	*	ns
	200	ns	**

Note: Statistical differences were represented as ***p<0.001, **p<0.01, *p<0.05, and “ns” not significant.

The EPS dosage was one of the reasons that caused viability and proliferation rate difference. As a result, the viability of HeLa cell followed an EPS dosage depended on decreasing and/or flat tendency. To point out, some viability curvature seems reaching the

maximum or minimum point at EPS dosage of 100 μg/mL, yet no significant difference was shown in neither in group nor to control comparison based on statistical analysis. In this case, Wheat-EPSs (200 μg/mL) treated HeLa reached a viability of 86.6% and 91.9% in 2D and 3D system, respectively. On the other hand, the proliferation rate was significantly affected by EPS dosage. In 2D system, 200 μg/mL of wheat-EPS and waxy corn-EPS shown significantly reduced the proliferation of HeLa. In 3D system, 200 μg/mL of wheat-EPS, corn-EPS, and waxy corn-EPS shown significantly reduced the proliferation of HeLa.

The composition variance of each EPS could be one of the reasons. In detail, the EPS composition and structure were regulated by the source of substrate provided to *T. striatum*, and future affect the chemical properties and composition of EPS, especially antioxidant activity. The structure of EPS is in relation to its antioxidant activity, where the features such as monosaccharide residues, functional group, protein content, glycosidic linkages, sulfate position and degree were likely involved in[51,137]. As results shown, the total antioxidant capacity (TAC) of each EPS followed a sequence of waxy corn-EPS, wheat-EPS, high amylose-EPS, corn-EPS, soluble-EPS, and glucose-EPS, from higher Trolox equivalence to lower. As mentioned above, glucose-EPS was observed having slightly and/or negligible affect to HeLa cell proliferation, in correspondence, the TAC of glucose-EPS was the lowest and slightly increased with EPS concentration, which could explain the observed phenomena. Alternatively, the rest of the EPS shown observable reduction of viability and/or proliferation rate. Therefore, the antiproliferation effect of EPS on the HeLa cell could be attributed to the TAC of EPS. In addition, the TAC measured the free radical scavenging capability of EPS, where the hydroxyl group and sulfated saccharides in EPS function as a hydrogen atom donor and possess strong free radical scavenging activity[137,138]. In our study, the chemical composition of EPS appeared of sulfated-

galactose and mannose backbone, which showed considerable TAC. Plenty of studies regard sulfated polysaccharides concentrated from microbes showing inhibition to HeLa cell proliferation. In example, the sulphated extracellular polysaccharide from *Alcaligenes faeclis* shown an inhibition effect to HeLa cell growth[139]. As reported in others study, the sulfated polysaccharides triggered apoptosis in HeLa cells via the ROS-mediated mitochondrial pathway, characterized by heightened ROS generation, mitochondrial membrane depolarization induction[130,132].

The EPSs were screening of its anticancer efficacy in both monolayer 2D culture and 3D hydrogel system. As comparing each EPS with same dosage in 2D and 3D culture systems, the proliferation rate and viability shown different significancy results from each EPS. Overall, when EPS dosage was equal and/or higher than 100 μg/mL, the proliferation rate of HeLa cells in 3D hydrogel system was significantly higher than 2D culture. The results indicate that 3D hydrogel system provides favorable growth environment to HeLa cell. Furthermore, the viability was insignificantly affected, except from wheat-EPS. Notably, wheat-EPS shown significantly difference in viability and proliferation rate, indicating the wheat-EPS was welly delivered to HeLa cells and inhibited HeLa cell proliferation. Our results shown was aligned with the result of HepG2 and SW480 treated with chlorogenic acid study, both HepG2 and SW480 were observed grown better and less susceptible to chlorogenic acid treatment[124]. The treatment delivery was different in 2D culture and 3D hydrogel system. In 2D culture, the cells were adhesively and flatly grown on microplate surface, which cause the cells were unable to fully submerge and attach to nutrient medium and treatment. This phenomena was widely reported and described as lose of cell-to-cell and cell-to-extracellular environment interaction and responses, alternatively, the 3D hydrogel system can mimic and provide favorable circumstance

for cell growth[120,136]. In addition, drug diffusion could be a potential involving factor to EPS performance in 3D hydrogel culture systems. The diffusion in 3D hydrogel of EPS was unknown, were needs future investigation.

To summary, the EPS anticancer efficacy to HeLa cell was investigated in 2D culture and 3D hydrogel system. The EPS dosage, chemical composition and properties of antioxidant activity, culture system was involved in the EPS anticancer efficacy performance. The EPS follows dose-dependent manner (≥ 100 μg/mL of EPS dosage). The 3D hydrogel system was a favorable growth environment to HeLa cells, both enhanced the nutrient delivery and EPS delivery to HeLa cells. However, the mechanism of EPS caused HeLa cells to need more research in the future, such as the apoptosis of HeLa and involving signaling pathways, especially the ROS-mediated mitochondrial pathway.

4. Conclusion

The marine protist, *T. striatum* can't only ferment simple sugars but also directly ferment raw starches (including normal, high amylose and waxy starches) without separate liquefaction and saccharification because it can generate robust amylolytic enzymes, such as α-amylase and α-glucosidase. *T. striatum* could be a potential amylolytic enzyme producer of industrial interest to support cold fermentation of starches. Fermentation of different starches by *T. striatum* can produce EPSs with different chemical composition, structure, and anticancer activities. The major component sugars of EPSs include glucose, mannose, and galactose but their proportion in the EPSs varies with the type of starches. Sulfated polysaccharide in EPSs is mainly sulfated galactose which could be the key component rendering the EPSs antioxidant and anticancer activities. The backbone of EPSs is a repeating chain of *α-D-Manp* with various monosaccharides sidechains. All EPSs derived from different starches were analyzed for their

anticancer activity against HepG2 and HeLa cells in both 2D and 3D systems. They demonstrated different anticancer activities while the anticancer efficacy of the EPSs exhibited dosage dependence in the range of 10~200 μg/mL. Of all EPSs, wheat starch-derived EPSs performed the best on the inhibition of both cancer cells. In addition, the anticancer activity of EPSs in the 3D system was weaker than that in the 2D system. More research is needed to (1) study the amylolytic enzyme system of *T. striatum* to produce promising starch degradation enzymes to support cold starch fermentation; (2) study how starch substrate regulates the EPSs production; (3) explore EPSs recovery, purification, and characterization (composition ad structure) technologies to identify functional components of EPSs; and (4) investigate the mechanisms governing EPSs anticancer performance.

Chapter 4 - Conclusion and Perspectives

The marine protist *Thraustochytrid striatum* is capable of fermenting not only simple sugars but also raw starches, including normal, high amylose, and waxy corn starches, and soluble starch without requiring separate liquefaction and saccharification steps. This is due to its ability to produce robust amylolytic enzymes such as α-amylase and α-glucosidase, making it a potential industrial producer of these enzymes for cold fermentation of starches. Fermentation of various starches by *T. striatum* results in the production of extracellular polymeric substances (EPSs) with distinct chemical compositions, structures, and anticancer activities. The primary sugars in these EPSs are glucose, mannose, and galactose, with their proportions varying depending on the starch type used. A significant component of these EPSs is sulfated galactose, which is believed to contribute to their antioxidant and anticancer properties. The backbone of the EPSs consists of repeating units of *α-D-Manp* with various monosaccharide side chains. Moreover, the examination for anticancer activity against HepG2 (human liver cancer cells) and HeLa (human cervical cancer cells) in both 2D and 3D systems were operated. The EPSs exhibited different levels of efficacy. The anticancer activity was dosage-dependent within the range of 10 to 200 μg/mL, with wheat starch-derived EPSs showing the strongest inhibitory effect on both cancer cell types. However, the anticancer efficacy in the 3D system was generally weaker compared to the 2D system.

The EPS derived from starchy substrates are known as macromolecules with biological activity. This research explored the starchy substrate usage for anticancer compound production, which given alternative consumption pathways for over-producing crop separated starches. Secondly, the elucidation of sugar compounds and structural investigation built up chemical component and bioactivity profile of EPS. Moreover, the most progress of EPS investigation was

its' biological activity was examined in not only 2D monolayer system, but also 3D Pepgel culture system. The 3D culture system mimic the complexity of *in vivo* environment, where the performance of EPS in 3D culture provides higher predictive data for inhibitory affect to cancer cells.

At present, the numerous polysaccharide drugs are utilized in clinical and adjuvant cancer treatments, showing promising application potential. However, the complex structures of some polysaccharides and the unclear mechanisms underlying their anticancer effects significantly limit their research and further clinical application. Therefore, it is important to elucidate the chemical structure and structure-activity of EPS and clarify the inhibitory effect mechanism of EPS. Also, the consistent production of EPS is expected, wherein the optimization of production process is vital. The current EPS production process is time consuming, especially ultra-purification process. The total time for EPS production was highly extended due to low efficiency of ultra-purification process. Induction of multi-stage separation and purification to EPS is reasonable, such as the combination of ultrafiltration with diafiltration[140], high-pressure appliance[141]. On the other hand, the process development needs to study the physic-chemical properties of EPS, such as solubility, molecular weight and size, charge and isoelectric point, thermal and pH stability.

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