Optimisation of *Proteus Mirabilis* Strain PI18 l β-Glucan Extraction

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled Extraction of Microbial β-glucans Exopolysaccharides from PI18 Strain Isolated from Soil Sample of Agropark, University Malaysia Kelantan by Lee Chun Eng, matric number F15A0067 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Bioindustrial Technology) with Honours, Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan.

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LIST OF ABBREVIATIONS AND SYMBOLS

16S rRNA	16S ribosomal RNA
α	Alpha
ATCC	American Type Culture Collection
β	Beta
CPS	Capsular polysaccharide
$COCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
ccm	Cubic centimetres per minute
˚C	Degree celcius
$(NH_4)2HPO_4$	Diammonium hydrogen phosphate
DMSO	Dimethyl sulfoxide
dH_2O	Distilled water
EPS	Exopolysaccharide
FTIR	Fourier-transform infrared spectroscopy
g	Gram
g/L	Gram per litre
HPLC	High Performance Liquid Chromatography
HA	Hyaluronic acid
$FeSO_4.7H_2O$	Iron (II) sulphate heptahydrate
LPS	Lipopolysaccharides
L	Litre
LB	Luria-bertani
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$MnSO_4.H_2O$	Manganese (II) sulfate monohydrate

PS	Microbial polysaccharide
μm	Micrometer
mg	Milligram
mg/mL	Milligram per mililitre
mL	Millilitre
M	Molarity
nm	Nanometer
N	Normality
NMR	Nuclear magnetic resonance
g−1	Per gram
%	Percentage
PI	Positive Isolate
KH_2PO_4	Potassium dihydrogen phosphate
r	Radius
r.p.m.	Rotation per minute
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
TA	Teichoic acids
T	Temperature
UMK	Universiti Malaysia Kelantan
UDP	Uridine diphosphate glucose
w/v	Weight per volume
$ZnCl_2$	Zinc chloride

Optimisation of *Proteus Mirabilis* Strain PI18 β-Glucan Extraction

ABSTRACT

Biopolymer, Microbial Polysaccharide (EPS) has showed promising benefit in various industries throughout last decade of study. However, the novelty and the complexity of the microbial EPS lead to drawback in exploiting this area. Previously, PI18 strain has shown promising β-glucan production through Scleroglucan base extraction method. In order to optimise further the β-glucan productivity, understanding the biosynthesis of β-glucan from PI18 strain by subject the bacteria in nutrient stress and modifying previous extraction strategy to be time and cost efficient. This study has found that PI18 can biosynthesis β-glucan effectively in Minimal medium (Production media) and pH 7.00 in controlled environment (Bioreactor). Current study has achieved two days peak β-glucan production on Day 2-3 (1.412-1.424 mg/mL) by cultivation fresh seed culture. As compare with previous study only show peak β-glucan on Day 4 (2.752 mg/mL). Through modification of Scleroglucan base extraction strategy, the homogenization and three times dilution step can be eliminated to one-time dilution with distilled water can achieved better yield in shorter time.

Keywords: exopolysaccharide, β-glucan, extraction, PI18, Scleroglucan.

Pengoptimuman Proteus Mirabilis Strain PI18 β-Glukan Ekstraksi

ABSTRAK

Biopolimer, Polisakarida Mikrob (EPS) telah menunjukkan manfaat yang menjanjikan dalam pelbagai industri sepanjang dekad yang lalu. Walau bagaimanapun, kebaharuan dan kerumitan mikrob EPS menyebabkan kelemahan dalam mengeksploitasi kawasan ini. Sebelum ini, ketegangan PI18 telah menunjukkan pengeluaran β-glukan yang menjanjikan melalui kaedah pengekstrakan asas "Scleroglucan". Untuk optimasasikan produktiviti β-glukan, kita perlu mengkaji biosintesis β-glukan dari strain PI18 dengan membedakan bakteria dalam tekanan nutriensi dan mengubah strategi pengekstrakan sebelumnya menjadi masa dan kos. Melalui kajian ini telah mendapati bahawa PI18 boleh biosintesis β-glukan berkesan dalam Minimal medan dan pH 7.00 dalam persekitaran terkawal (Bioreactor). Dalam kajian semasa telah mencapai dua hari puncak pengeluaran β-glukan pada Hari ke 2-3 (1.412-1.424 mg / mL) dengan penanaman kultur segar. Seperti yang dibandingkan dengan kajian terdahulu hanya menunjukkan puncak β-glukan pada Hari 4 (2.752 mg / mL). Melalui pengubahsuaian strategi pengekstrakan asas Scleroglucan, homogenisasi dan langkah pencairan tiga kali dapat dihapuskan untuk pencairan satu kali dengan air suling dapat mencapai hasil yang lebih baik dalam masa singkat.

Kata kunci: eksopolisakarida, β-glukan, pengekstrasi, PI18 scleroglucan

CHAPTER 1

INTRODUCTION

1.1 Research Background

Polysaccharides are generated universally by living organisms. Polysaccharide has sugar monosaccharide unit that link together by glyosidic linkage. These polysaccharides can be heteropolysaccharide, that contain different monomer or homopolysaccharide consist of same sugar. These abundant polysaccharides produced universally has numerous studies done and different variety of complex chemical structure. Polysaccharide consists high potential application in various industrial area as biodegradable plastic, cosmetics, food engineering, agronomy, biofuel and others (Laroche & Michaud, 2007). In growing industry, polysaccharide from plant source are abundant, this source is a concerning problem for industry as the crop plant has depleting resource and climate change in production bottlenecks (Van Beilen & Poirier, 2008). Microbial polysaccharide (PSs) still remains favourable source as compare with plant due to the controllable and enhance microbial activities for economic production efficiency (Rehm, 2010).

PSs can be mainly consisting of peptidoglycans, lipopolysaccharides (LPSs), teichoic acids (TAs), exopolysaccharides (EPSs), and capsular polysaccharides (CPSs) (Jennings, 1983). Peptidoglycans has linear repeating disaccharide or polysaccharide with cross-linkage with oligopeptide fragments. LPSs has an O-antigenic polysaccharide build up with acrylate disaccharide namely Lipid. TAs are polyphosphate and various amide related group. CPSs elucidated out as heteropolysaccharides at current studies, EPSs can be hetero- and homopolysaccharides (Kamerling & Gerwig, 2007). In some microbial has capsular exopolysaccharides that can give them distinct feature such as resistance toward desiccation and pathogenicity (Ghosh & Maiti, 2016).

PSs can be subdivided into EPSs (xanthan, dextran, alginate, cellulose, colonic acid and hyaluronic acid (HA)). Wide range of bacteria and archaea can produce EPS (Parolis et al., 1996). EPSs properties is depending on the subunit composition, structure and molecular mass. EPS has high commercial value as all the polysaccharide. 2 types of glyosidic-linkage are bond between 2 subunits in ESPs which is α- and β- linkage depending on the structural of the composition (Rehm, 2010).

β-glucan is one of the ESPs that can be extracted from bacterial. β- (1.3)-D-glucan and β-(1,3)(1,6) –D- glucan are mostly studied in papers and patents. These β-glucans have several conformations, linear, breached and cyclic. Microbial β-glucan are much linear conformation as compare to plant β-glucan such as cereal grain and mushroom. Numerous studies have demonstrated wide range of utility in numerous field (Laroche & Michaud, 2007). The application includes gelling properties, film properties, non-fat products in food industry and hypercholesterolemia, diabetes, glycaemia, immunopotentiator, anti-tumorigenic effects, AIDS and prebiotic in both pharmaceutical and nutraceutical industries (Rahar, Swami, Nagpal, Nagpal, & Singh, 2011).

In this study, optimisation on extraction and understanding biosynthesis of microbial β-glucan from P118 strain that previously done by (Tee, 2018) P118 strain was identified known as *Proteus Mirabilis* via 16rRNA sequencing analysis (Tan, 2017) is main focus of the study. However according to Tee (2018) this strain has high EPS production.

Curdlan extraction strategy depend on the physiochemical of the linear β-1,3-glucan. The insolubility of Curdlan in water is attributed by the existence of extensive intra- and inter- molecular hydrogen-bond crystalline. Unbranched β-(1,3)-glucan are soluble in both alkaline and Dimethyl sulfoxide (DMSO) (Gidley & Nishinari, 2009). The precipitation is done by neutralising with acid that is adopt from alkaline-acid method by Hunter et al., (2002) and Williams et al., (1991).

Scleroglucan extraction strategy focuses extraction of class of (1,3;1,6)- β-glucan with branch-on-branch structure. This strategy is dependent on the Scleroglucan structure that has similarity with Schizophyllan type of β-glucan that extracted through homogenisation, dilution, heating, precipitation, centrifuge that help in liberating this type of β-glucan from the microbial EPS and induce higher yield of β-glucan (Zhang, Xu, & Zhang, 2008). The purification is later done with ethanol for removal of protein (Shokri, Asadi, & Khosravi, 2008).

Gellan gum extraction strategy focus on β-D-glucose (D-Glc), L-rhamnose (L-Rha) and D-glucuronic acid (D-GlcA).The recovery is mainly adopted from Kang and Veeder (1982), that comprises heating, centrifugation, solvent precipitation and oven drying. These step generally is used for separating the gellan-gam from the EPSs microbial and recovery through solvent precipitation and removal of protein (Shokri et al., 2008).

Since β-glucan has wide range of identifying the structure and different bacteria will produce different EPS (β-glucan) in term of degree of branching and physiochemical properties. The optimum extraction method is still under investigation and aim to get higher yield than previous extraction strategies. As for understanding the biosynthesis of EPS from PI18 strain is study through modifying several parameters.

1.2 Problem Statement

Previously, Tee (2018) successfully extracted β-glucan from PI18 strain through Scleroglucan base extraction strategy. However, the extraction strategy is time consuming and inefficient. Besides that, optimum condition for production of β-glucan from PI18 strain is under investigation.

1.3 Objectives

The objectives of this study are:

1 To optimisation of the scleroglucan base extraction strategy for PI18 strain.

2 To investigate the optimal condition for biosynthesis of β-glucan from PI18 strain through modification of nutrient.

1.4 Scope of Study

The scope of study is stressed on optimising the Scleroglucan base extraction strategy by shorten the time taken for the extraction. The study of biosynthesis of EPS from PI18 is done through modifying nutrient content by subject the cultivation in rich and minimal medium.

1.5 Significant of Study

The result of this study by optimizing the extraction and understand biosynthesis of EPS from PI18 strain can accomplish a superior yield, purity and cost-effective method. This reduces expensive down-streaming process that can give an entrance for nutraceutical and pharmaceutical industry to avoid competition against β-glucan from agriculture base. This study will give an insight to the novelty of β-glucan for future research purpose. β-glucan endeavor conveys a more feasible market for pharmaceutical or nutraceutical industry to promote more study on β-glucan for beneficial effect for preeminent accomplishment in near future.

CHAPTER 2

LITERATURE REVIEW

2.1 Polysaccharides

Polysaccharides are simple carbohydrate or sugar polymer molecules that made up into a long chain of monosaccharide by repeating the units. Recent study suggested 40 monosaccharide have been described, most of the polysaccharide are formed from a limited range of carbohydrate usually they exhibit in pentose and hexoses (Laroche & Michaud, 2007). These polysaccharides can be both heteropolysaccharides and homopolysaccharides. The bonding between them is linked by either α- or β- glyosidic bond in linear or branched backbone through elimination of water. Different degree of branching and subunit of the pentose sugar will result different polysaccharide and contribute different properties.

By study the complexity of the polysaccharide leads to understanding the high value of industrial application. Biopolymer are composed of organic polysaccharide that is non-toxic constituent and considered to be biocompatible. These biocompatibility has been contributed in numerous medical application such as tissue engineering, drug

delivery, nanomedicine and wound dressing (Doh & Yeo, 2012; Rehm, 2010). Bacterial, fungal and vegetable are major source of polysaccharide that contribute universally (Laroche & Michaud, 2007).

2.2 Microbial Polysaccharide

Microbial polysaccharides are generally polysaccharides produced by microorganism. It gives microorganism specific structural characteristic depending the polymeric design by the cell for distinct function. Lipopolysaccharide, a polysaccharide with lipid group attach give a pathogenic property for the bacteria as cellular defence mechanism (Serrato, 2014). Microbial polysaccharides are water soluble biopolymer produced by bacteria has a rheological characteristic that is used as binders, coagulants, film former, gelling agent, emulsifier, stabilizer and thickening as well as suspension agents (Huang & Tang, 2007; Pace, 1981).

2.3 Exopolysaccharide

Exopolysaccharides (EPSs) are carbohydrate polymer that synthase and excreted by some bacteria or fungi onto their cell walls. EPSs also known as extracellular polymeric substances that have high molecular weight of natural polymers (Staudt, Horn, Hempel, & Neu, 2004). The structure and composition of varied depending on the homo- or heteropolysaccharides that contain different organic and inorganic constituent (Andhare, Chauhan, Dave, & Pathak, 2014). EPS produced by microbe are immerse and

diverse nature. 4 major classes of polysaccharides are slime and microcapsular polysaccharide, inorganic polyanhydrides (polyphosphates), polyamides and polyesters are collective termed EPSs (Nwodo, Green, & Okoh, 2012).

EPSs can be either homopolysaccharides that made up of single monomer bond with glycosidic link or heteropolysaccharide that has more than 2 monomeric units joint with glycosidic bond. Besides that, EPSs contain different organic moieties like amino acid, inorganic and organic acid (Nanjani & Soni, 2012). Homopolysaccharides clustered into four groups; α-D-glucan, β-D-glucan, fructans and polygalactan (Stewart-Tull, 1980). While heteropolysaccharides composition included repeating unit of D-glucose, D-galactose, L-rhamnose in some cases, *N*-acetylglucosamine (GlcNAc), gluconic acid (GlcA) or *N*-acetylgalactosamine (GalNAc) (Ruas-Madiedo, Hugenholtz, & Zoon, 2002).

2.4 β-Glucan

β-glucan are polymer of β-(1,3)-D-glucose with or without any side chain of β-(1,6)-D-glucose. These compounds can be found in many cell walls of bacteria, yeast and plant. For instance, some major cell walls component of *Saccharomyces cerevisiae (S. cerevisiae),* that found out to have enhancing immune function (Shokri et al., 2008). Simplest form of β-glucan are considered pseudo-helical chain formation of β-(1,3)-D-glucan (Burton & Brant, 1983). β-(1,3)-D-glucan physiochemical properties dependent on the nature and stability of ordered conformation present under hydrated conditions. Besides that, it exhibited range of solution, network and gel properties depending on the chemistry and molecular size of the polysaccharide (Gidley & Nishinari, 2009). Recent

studies suggested β-glucan demonstrated exhibit anti-tumour, antimicrobial and radioprotective activities (Zhang, Guo, & Wang, 2008).

2.5 Microbial Exopolysaccharides

Microbial EPS are polymer base on the principle of carbohydrate with varying structure and composition. Certain microbial responsible for producing different type of cell-surface polysaccharide that comprise capsular polysaccharide (CPS), lipopolysaccharide (LPS), cyclic beta glucan (CG), K-antigen polysaccharide (KPS), gel-forming-polysaccharide (GPS), neutral polysaccharide (NP), cellulose fibrils and extracellular polysaccharide (EPS) (Ghosh & Maiti, 2016).

EPS are mainly 2 types, Succinoglycan (EPS I) and Galactoglucan (EPS II) by rhizobial strain (Reinhold et al., 1994). EPS-I is octasaccharide repeating units consist one galactose and seven glucose residue bonded by β-1,3; β-1,4 and β-1,6 glycosidic linkage, while EPS-II is disaccharide repeating unit linked by α-1,3 and β-1,3 glycosidic bond (Her, Glazebrook, Walker, & Reinhold, 1990). Some marine microbial produce different type of EPS, soluble (Sol-EPS), loosely bound (LB-EPS) and tightly bound extracellular polymeric substances (TB-EPS) (Brian-Jaisson et al., 2016).

2.5.1 Biosynthesis of Microbial Exopolysaccharide

Most microbial EPSs are synthesized intracellularly either throughout growth or during late logarithmic phase and export to the extracellular environment as macromolecules (Rehm, 2009). However, there is some exception (e.g. levans and dextrans) where the synthesis and polymerization occurred outside the cell by action of enzyme secretion to convert substrate into polymer in the extracellular environment Rehm, 2009).

Microbial exoplysaccahride are synthesized in 4 steps with the aid of 4 group of enzyme (Suresh Kumar, Mody, & Jha, 2007). First step is the uptake of subtrate, bacterial cell utilize glucose molecule through active transport, simple diffusion or translocation. Second step invovled phophorylation of glucose to glucose-6-phosphate through hexokinase and continuously isomerization to glucose-1-phosphate by phosphoglucomutase. Enzyme group II "UDP-glucose pyrophosphorylase" convert glucose-1-phosphate to UDP-glucose (uridine diphosphate group). At this stage UDP-glucose is intermediate subtance to interconvert to other catabolic or anabolic pathway. Thridly, UDP-gluose undergo polymerization through anabolic parthway depending the type of polysaccharide to be produced. Fourth step modification of polysaccharides by acrylation, acetylation, sulphation and methylation. The modified polysaccahried is excreted to cell surface in the form of slim or a capsule (Mishra & Jha, 2013).

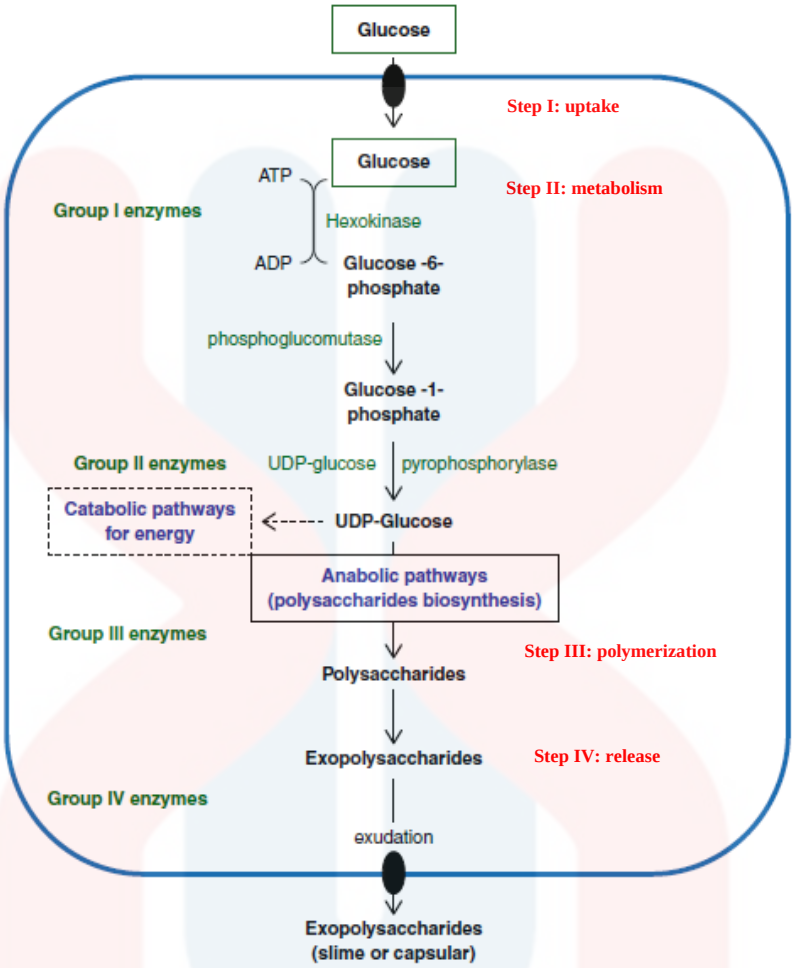


Figure 2.1: Microbial exopolysaccharides biosynthesis.

(Source: Mishra & Jha, 2013)

2.6 Commercial Microbial Exopolysaccharide

Microbial EPSs has been applied in different industrial due to the rheological characteristic that give benefit in various industries. According to Freitas, Alves, and Reis (2011), Xanthan gum, Gellan, Alginate, Hyaluronan, Succinoglycan and Levan are commercially exploited bacterial EPSs.

2.6.1 Xanthan gum

Xanthan gum is one of the largest microbial polysaccharide markets due to rheological feature over a wide range of pH and temperatures. It is used for salad dressing, syrups, beverages, texturized coating, abrasives and enhance oil recovery (Chaitali, Kapadi, Suraishkumar, & Gudi, 2003). Its first industrial produced biopolymer and widely accepted as commercial polymer (Rottava et al., 2009). It is heteropolysaccharide secreted by genus *Xanthomonas*, with glucose backbone with trisaccharide side chain containing glucuronic acid, mannose, acetyl and pyruvil residue (Freitas et al., 2011). The structure of Xanthan is shown in Figure 2.2.

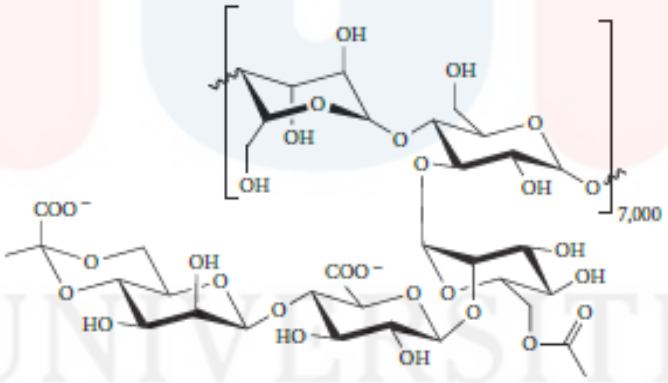


Figure 2.2: Structure of Xanthan.

(Source: Rehm, 2010)

2.6.2 Gellan gum

Gellan gum is heteropolysaccharides produce by genus *Sphingomonas*, has common backbone of tetrasaccharides that has rhamnose, glucose and glucuronic acid. Gellan, Welan, Rhamsan and Diutan are differentiated by variation in composition and

linkage of the side chain (Coleman, Patel, & Harding, 2008). Figure 2.3 shows Gellan-gum structure.

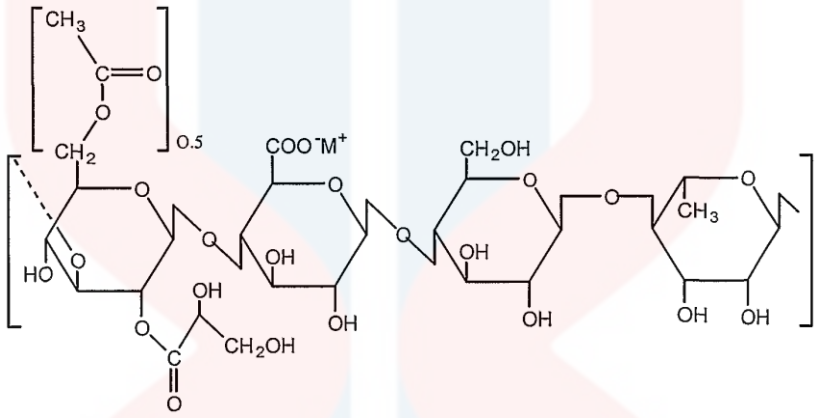


Figure 2.3: Structure of Gellan-gum.

(Source: Andhare et al., 2014)

2.6.3 Alginate

Alginate is linear polysaccharide that compose of mannuronic and guluronic acid that form a block of poly-guluronic and poly-mannuronic. These group of polysaccharides are secreted by genera *Azotobater* and *Pseudomonas* spp. Figure 2.4 shows Structure of Alginate.

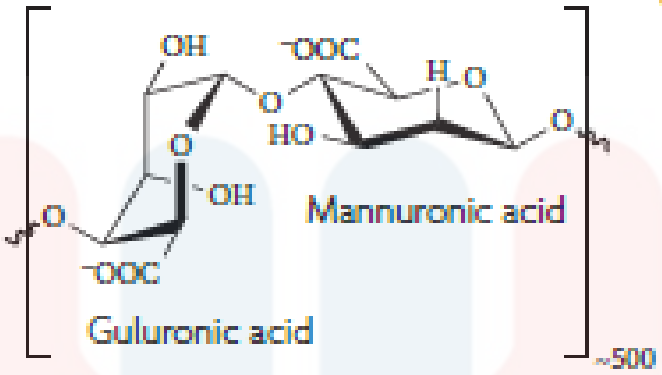


Figure 2.4: Structure of Alginate.

(Source: Rehm, 2010)

2.6.4 Hyaluronan

Hyaluronan are linear polymer of disaccharide unit compose of gluconic acid and *N*-acetylglucosamine produced by *Pseudomonas aeruginosa* and group of A and C *Streptococci* attenuate strain (Andhare et al., 2014; Rehm, 2009). Figure 2.5 shows Structure of Hyaluronan.

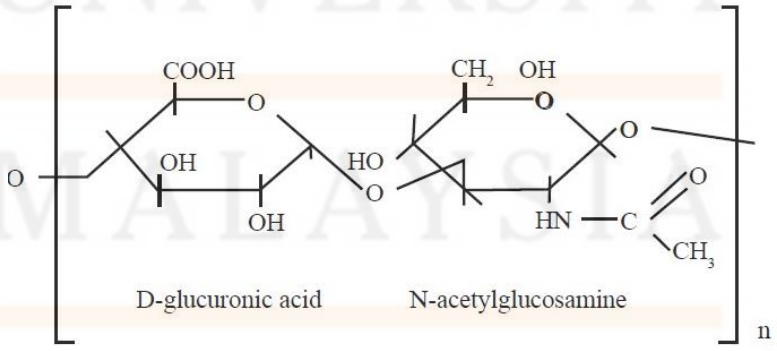


Figure 2.5: Structure of Hyaluronan.

(Source: Andhare et al., 2014)

2.6.5 Succinoglycan

Succinoglycan are branched EPS with galactose and glucose backbone and tetrasaccharide side chain with glucose residue. Common non-saccharide substituents are succinate, pyruvate and acetate. It can be produce by *Rhizobium, Alcaligenes, Pseudomonas* and *Agrobacterium* (Rehm, 2009).Figure 2.6 shows structure of Succinoglycan.

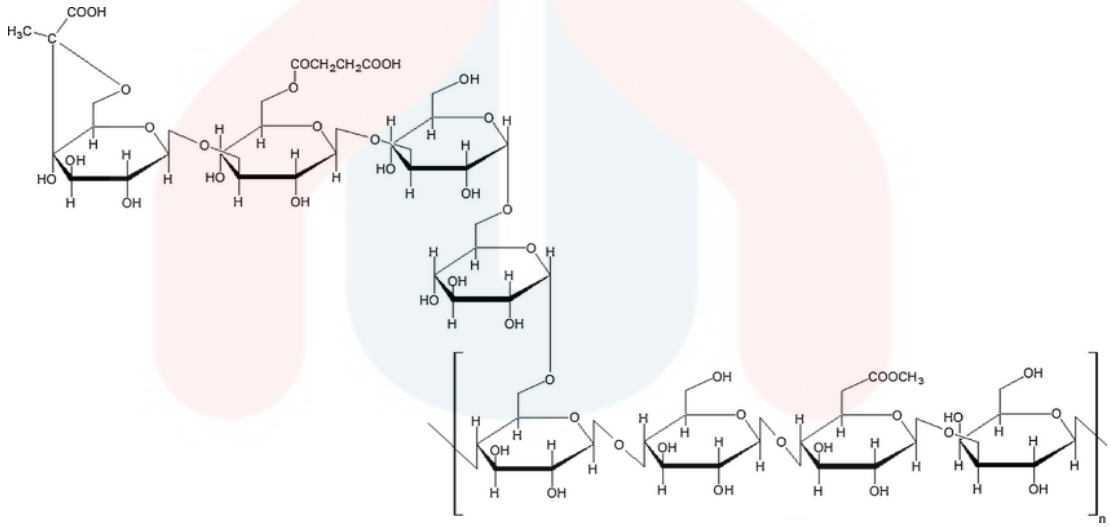


Figure 2.6: Structure of Succinoglycan.

(Source: Andhare et al., 2014)

2.6.6 Levan

Levan is a highly branched fructose homopolysaccharides synthesized sucrose by levansucrase enzyme, levansucrase are extracellular enzyme synthesize by bacteria genera *Bacillus*, *Rahnella*, *Aerobacter*, *Erwinia*, *Streptoccoccus*, *Pseudomonas* and *Zymomonas* (Ayala-Hernández, Hassan, Goff, Mira de Orduña, & Corredig, 2008). Figure 2.7 shows structure of Levan.

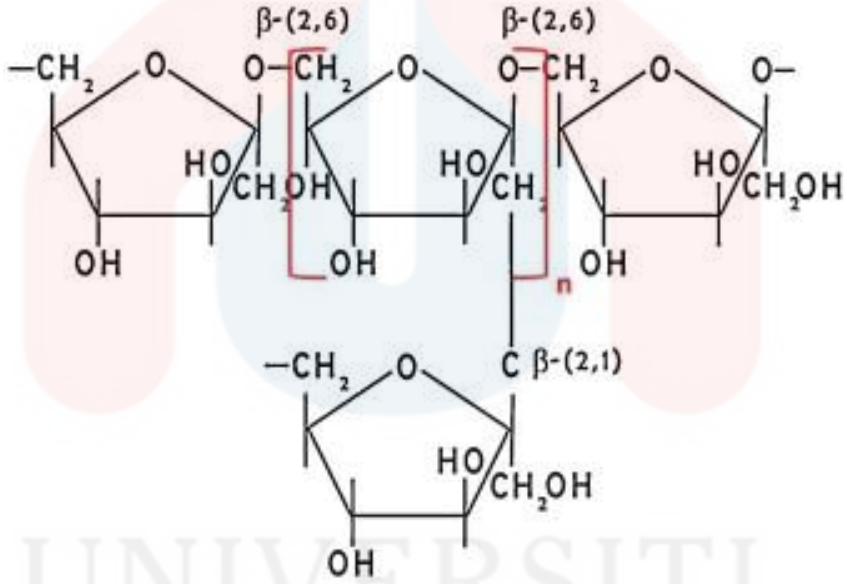


Figure 2.7: Structure of Levan.

(Source: Andhare et al., 2014)

2.7 Extraction Methods of Exopolysaccharide

There are numerous ways of extracting exopolysaccharide. Extraction of EPS is divided into Physical and Chemical method. Physical method included centrifugation (Laroche & Michaud, 2007), sonication (Many & Vizhi, 2014; Shokri et al., 2008) and heating (Hunter et al., 2002). Chemical method included Sodium hydroxide, Hydrochloric acid, acetic acid, citric acid lyses at high temperature (Hunter et al., 2002; Many & Vizhi, 2014).

2.7.1 Physical Methods

Physical method involves centrifugation, sonication and heating in aids of separating the EPS from cell surface.

I. Centrifugation

Centrifugation help in separate out soluble EPS from cell surface. According to Miao et al. (2013) 1700 g centrifuged for 10 minutes at 20 ^{o}C to precipitate.

II. Sonication

The cell fractionation is done by sonication with keep in ice bath to prevent overheating. Cell wall disruption is carry our 60 % amplitude for 48 minute (2:4 min Pulse on; off basic) to accomplish maximum cell disruption (Shokri et al., 2008).

III. Heating

According to Laroche and Michaud (2007) heating can produce different form of Curdlan gel. Heat treatment also can lower stability incorporate β-glucan gum. Some EPS can be soluble upon heating in water solvent (Gidley & Nishinari, 2009).

2.7.2 Chemical Methods

Chemical Method involves Alkaline and Acid chemical or solvent in aid of separation the EPS from cell surface by promoting solubility for EPS.

I. Sodium Hydroxide (NaOH) & alkali chemical

NaOH and dimethyl sulfoxide (DMSO) can be used in removal bacterial cell by dissolving Curdlan (Gidley & Nishinari, 2009; Laroche & Michaud, 2007).

II. Acidic (Hydrochloric acid, acetic acid & citric acid)

According to Hunter et al. (2002) Acetic acid is used for insoluble solids drawn off from the solution during EPS extraction.

2.7.3 Combination of Physical and Chemical Methods

Combination of physical and chemical extraction method in order to promote yield. Polysaccharide are recovered by addition of NaOH to remove the bacterial cell. Precipitation is achieved by neutralize with formic acid further collected by centrifuged (Laroche & Michaud, 2007).

2.7.4 Curdlan Extraction Strategy

Curdlan extraction strategy rely on the pH solubilisation method. Since β-1,3-glucan of Curdlan is soluble in alkaline and DMSO solution (Gidley & Nishinari, 2009). The culture broth is centrifuged at 8,000 r.p.m in 4 ^{o}C for 30 minute (Yu et al., 2011). The pellet is added with NaOH solution. Curdlan is a helical conformation will change to pseudo-helical upon the alkaline environment. DMSO also can be substitute NaOH as the DMSO has similar function as alkaline (Gidley & Nishinari, 2009). The suspension is then re-centrifuge at 8,000 r.p.m for 30 minutes. By adding HCl, Curdlan will salt out and precipitate under a change of pH environment (Yu et al., 2011). The crude Curdlan is then washed and dried at 80 ^{o}C.

2.7.5 Scleroglucan Extraction Strategy

Scleroglucan extraction is adopted from Fariña, Siñeriz, Molina, and Perotti, (2001) and Viñarta, Yossen, Vega, Figueroa, and Fariña, (2013). The culture is homogenised at 8000 r.p.m for 1 hour. The homogenized broth will be diluted with 3 times volume of distilled water and neutralized with NaOH. The mixture is heated at 80 ^{o}C for 30 minutes. 95 % ethanol and isopropanol are used to precipitate the EPS from supernatant.

2.7.6 Gellan gum Extraction Strategy

Gellan gum extraction strategy is adopted from Freitas et al. (2011). Culture broth is heated at 90 ^{o}C to 95 ^{o}C, heat treatment will reduce the viscosity and inactivate enzyme while lyses bacteria cell. Dilution of the mixture to allow crude EPS dissolved before subject to centrifugation. Cold isopropyl alcohol is added into the mixture and keep for 4 ^{o}C for precipitation. The crude gellan is centrifuge and dried in over at 55 ^{o}C for an hour.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

P118 excessive producing strain, previously isolated from Agropark of Universiti Malaysia Kelantan, Malaysia. This strain is known as *Protues Mirabilis* via 16S rRNA sequencing analysis (Tan, 2017).

3.2 Inoculation of β-Glucan Producing Strains

P118 strain was streak on Luria-Bertani (LB) agar (NaCl (10 g/L), Tryptone (10 g/L), Yeast extract (5 g/L) and agar powder (16 g/L) at pH 7) and incubated for 2 days at 30 ^{o}C.

3.3 Verification of β-Glucan Producing Strain

P118 strain was streak on LB agar supplemented with 0.005 % (w/v) aniline blue and sterile sucrose 5 % (w/v). The plate was incubated at 30 ^{o}C for one day. Formation of blue colony indicates the bacterium has β-glucan producing potential strain (Tan, 2017).

3.4 Seed Culture

5 mL of LB broth was prepared for seed culture of PI18 strain. A single colony of LB agar medium was selected and transfered into LB broth using inoculation loop. The broth was cultured and incubated overnight in Lab Companion SL-600R Benchtop Shaker at 30 ^{o}C at 150 r.p.m. Cell growth was measured using spectrophotometer Genesis 20 (Thermo Scientific) at 600 nm.

3.5 Producing of β-Glucans

3.5.1 Preparation of Production Media

Bacterial seed culture was inoculated into production media in both culture and bioreactor. The production media was prepared according Ruffing and Chen (2010) with modification done by Tee (2018). The content of the production media included 0.23 % $(NH_4)2HPO_4$, 0.1 % KH_2PO_4, 0.04 % $MgSO_4.7H_2O$ and 1 % trace element (5 g/l

$FeSO_4.7H_2O$, 2 g/l $MnSO_4.H_2O$, 1 g/l $COCl_2.6H_2O$, 1 g/l $ZnCl_2$) with 20 % (w/v) sucrose as carbon source and 0.4 % (w/v) sodium citrate as buffer. The pH of production media will be maintained at 7.

3.5.2 Cultivation of β-Glucan in Flask Culture

5mL of seed culture was transferred aseptically into 200 mL of production media. The culture incubated in incubator shaker at 150 r.p.m and 30 ^oC for 1 week. The cell growth was measured at 600 nm by using UV-VIS Spectrophotometer Genesis 20 (Thermo Scientific) every day.

3.5.3 Cultivation of β-Glucan in Bioreactor

40 mL of seed culture was transferred into 1.5 L of production media. The culture incubated in bioreactor (BIOSTAT®A) at 300 r.p.m., 30 ^oC, 1000 ccm of air and maintain at pH 7. The pH control was maintained by 1 M NaOH or 1 M of acetic acid incubated for one week. EPS was extracted once the bacterial cell reached stationary growth phase.

3.6 Extraction of β-Glucans

β-glucan was extracted via Curdlan, Scleroglucan, Gellan Gum base strategy.

3.6.1 Curdlan Extraction Strategy

The Curdlan extraction method is based on Yu et al. (2011) with slight modification. Culture broth was centrifuged at 9500 r.p.m, 4 ^{o}C for 30 minutes. The pallet dissolved in 1 M NaOH and placed in incubator shaker overnight at 150 r.p.m to dissolved completely. The suspension was centrifuged at 9500 r.p.m, 4 ^{o}C for 30 minutes. The supernatant precipitated by neutralizing with 15 % of acetic acid. The suspension centrifuged at 13,000 r.p.m, 4 ^{o}C for 20 minutes. Crude β-glucan dried in oven at 60 ^{o}C.

3.6.2 Scleroglucan Extraction Strategy

Broth was diluted with three-time volume of deionised water and shaked overnight in incubator shaker at 150 r.p.m and neutralized with 1 M NaOH. The mixture was heated at 80 ^{o}C for 30 minutes proceeded with centrifuge at 12,000 r.p.m, 25 ^{o}C for 25 minutes. Cold 95 % ethanol added into mixture keep overnight at 4^{o}C for overnight. Pallet was precipitated by centrifuge at 12,000 r.p.m, 25 ^{o}C for 15 minutes. Crude β-glucan was dry in oven at 60 ^{o}C.

3.6.3 Gellan-gum Extraction Strategy

Culture broth subjected to heat treatment at 95 ^{o}C for 15 minutes proceeded with one-time dilution with deionised water and leaved it in incubator shaker with 150 r.p.m overnight. The mixture was centrifuged at 10,000 r.p.m, 4 ^{o}C for 25 minutes. Equal amount of 95 % ethanol adds into the mixture kept 4 ^{o}C overnight. Crude β-glucan was obtained when centrifuge at 12,000 r.p.m, 4 ^{o}C for 20 minutes. Crude β-glucan was dried in oven at 60 ^{o}C.

3.7 Quantification of β-Glucan

The extracted β-Glucan is quantify using dry weight. The dry weight is determined using Equation 3.1.

$$Dry\ weight\ of\ \beta - glucan\ \left(\frac{mg}{mL}\right) = \frac{Weight\ of\ extracted\ EPS\ with\ microcentrifuge\ tube - Weight\ of\ microcentrifuge\ tube}{Volume\ of\ sample} \quad (3.1)$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 Screening of β-Glucans Producing Strain on LB Plate Supplement with Aniline Blue

The sub-culture of PI18 strain (*Proteus mirabilis*) and MR-1 strain (*Shewanella oneidensis*) on LB supplemented with Aniline Blue (0.005%) enable identification on β-glucan producer bacteria. According to Koenig, Rühmann, Sieber and Schmid (2017) the fluorescence properties of aniline blue bind to β-(1,3)-glucan, β-(1,4)-glucan and β-(1,3)-β-(1,6)-glucan will exploit the fluorescence properties of aniline blue. Based on Figure 4.1 PI18 strain exhibits positive result after 48 hours of incubation this suggest PI18 strain is an β-glucan producer. While MR-1 strain showed negative result where there is no blue pigment formation after 4 Days of incubation. Furthermore, PI18 strain start showing blue pigment after 24 hours incubation this suggest the strain require 24 hours of adaptive stage before producing β-glucan that can be exploit the florescence properties.

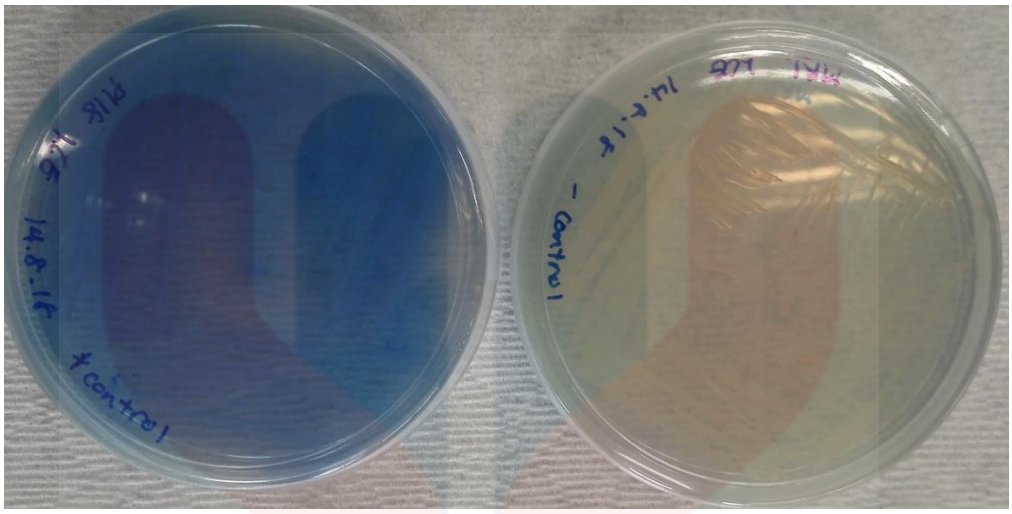


Figure 4.1: Blue staining formation on PI18 strain shown β-glucan producer (Left), while MR-1 shown no blue staining formation suggested not a β-glucan producer (Right), after incubated for 4 Days.

During the incubation period several findings have discovered that potentially related to optimising the β-Glucan produced by PI18 strain. In order to control enhance the microbial β-Glucan producer productivity. The better understanding the biosynthesis of EPS is required which lead to understand PI18 strain growth pattern on LB supplemented with Anillin Blue. Base on Figure 4.2, it shows the blue pigment exhibit in some certain location where the bacteria grew and produce metabolite accumulate at the location this occurrence is known as localisation, Where the certain group or location of bacteria producing secondary metabolite accumulate at a location for various uses (Paul, de Nys, & Steinberg, 2006). This illustrated PI18 strain produce β-Glucan in group that adapted the environment within 48 hours β-Glucan is producing and accumulated in certain spot of the agar.

Figure 4.2: PI18 strain on LB supplemented with Aniline Blue after incubated for 48 Hours.

After 2 weeks of incubation, the growing PI18 strain bacteria cell result higher blue intensity in the bacteria colony where almost all the bacteria exhibit blue pigment. Base on Figure 4.3, where the blue pigment is no longer accumulate in the agar instead all concentrated on the bacterial cell. This indicated the β-Glucan are stored or accumulate in the vicinity of the bacterial instead released into surrounding. According to Sanchez-Garcia, Lagaron and Hoa (2010) EPS producing bacteria use these biopolymers as a storage materials in response to particular environment stress. Since EPS play a pivotal role in cell protection, adhesive ability of bacteria to solid surface and participating in cell to cell interaction (Nicolaus, Kambourova, & Oner, 2010). The environment stress correlated to the role EPS, after long period of incubation nutrient in LB agar is greatly reduced thus PI18 Strain most likely react to nutrient stress that behaved this way.

Figure 4.3: PI18 strain on LB supplemented with Aniline Blue after incubated for 2 weeks and stored in -4 ^{o}C for 3 weeks.

Figure 4.4 is PI18 strain Sub-Culture from Figure 4.3 after 1-week incubation and 1-week refrigerator store. After 2 Days of incubation, the characteristic of the PI18 cell changes drastically. Where the bacterial cell is growing in small even isolated colony and have consistent distance between colony and less white milky semi-solid observed around them as compare to Figure 4.2 and certainly no Blue pigment localisation. This suggested the β-Glucan is produce by PI18 however its evenly diffuse throughout the agar media.

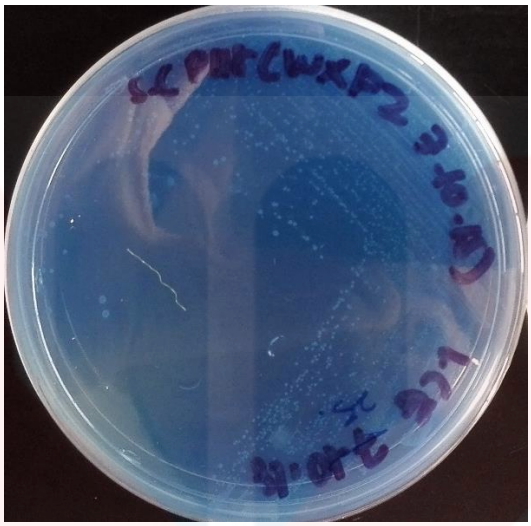


Figure 4.4: PI18 strain Sub-Culture from PI18 FZ (WX) 10.07.2018 on LB agar supplement with Aniline Blue after incubated 2 Days

4.2 Cultivation and Extraction of β-glucan in Production Media Supplement with LB (Nutrient-Rich Media)

In order to culture in large scale, seed culture (2.5 %) is necessary in scaling up in large quantity by transferring a colony of PI18 strains. PI18 strain is described as a fast growing and excessive β-glucan producer can grow effectively in large amount with single colony (Tee, 2018). Previous studies done by Tan (2017) and Tee (2018), culturing PI18 strain in production media show least significant β-glucan product or extracted through various strategies method during cultivation in uncontrolled environment. Where the Production Media has minimal nutrient media. Thus, current study is done cultivated PI18 in Production media (Minimal medium) additional with LB broth which rich media given the Tryptone and Yeast Extract provide various nutrient and amino acid for bacterial culture. This cultivation is done in both controlled (Bioreactor) and uncontrolled (Flask) environment.

The extraction of β-glucan was carried out using Scleroglucan base extraction strategy as previously study done by Tee (2018) showed this strategy is most compatible and effective in extracting from PI18 strain. Base on Figure 4.5, the highest extracted β-glucan is Day 4 in non-controlled environment (0.82 mg/mL) while in controlled environment is Day 6 (0.26 mg/mL). However, both controlled and non-controlled environment in Rich-medium show an inconsistent β-glucan produce or extracted. This revealed the PI18 during the cultivation do not produce β-glucan consistently. Besides that, the Flask culture showed relatively higher β-glucan production, this is due to PI18 strain subjected different stresses such as pH, oxygenation or temperature. However, definite stress can't be determined at this stage.

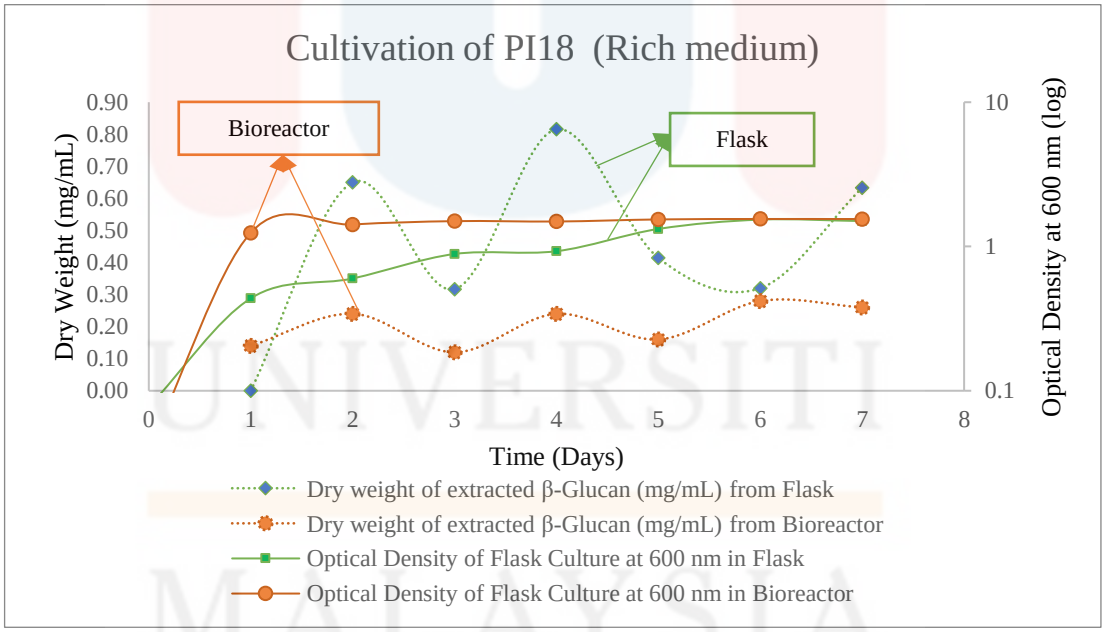


Figure 4.5: The comparison of Bacteria Growth and Extraction β-glucan between Controlled (bioreactor) and non-controlled environment (Flask) cultivation of PI18 strain in 7 Days.

The optical density (OD) at 600 nm is directly corelated to bacteria density show the bacteria growth in bioreactor is much faster which only take 1 Day to reach stationary phase while Flask culture take 6 Days to reach stationary phase. This indicates the PI18 strain grow optimally in 30 ^{o}C. However, there is no correlation between β-glucan produces related to growth of bacteria.

In order to understand the novelty of PI18 strain, we study the growth of this bacteria strain and the pH in the culture. During the rapid growth Day 1 (Figure 4.6), the culture broth has drastic pH drop (4.39 pH) at this stage culture broth suggested the rapid growing of PI18 strain will result acidity in the production broth. During the cultivation, the broth pH remained in the range of 4.39-5.09 pH while the bacteria is growing throughout this period. It indicates the minimum growth pH is within this range of pH. While the optimum pH for PI18 is 7.00 from Figure 4.6 indicated controlled environment at pH 7.00 can achieved optimum growth within 24 hours of cultivation.

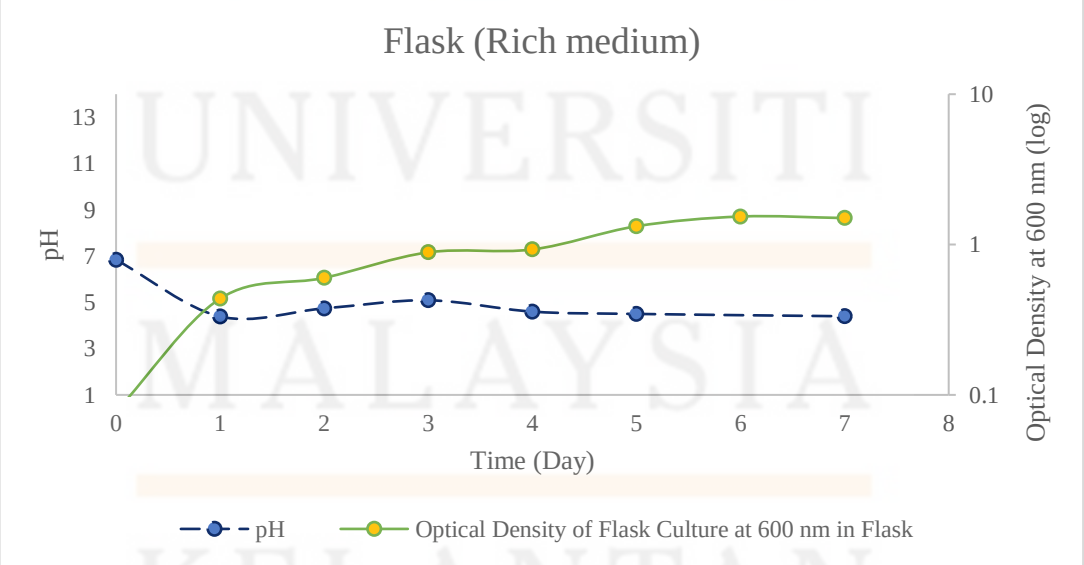


Figure 4.6: The comparison of pH and growth of the PI18 strain in Rich-media and non-controlled environment in 7 Days.

4.3 Cultivation and Extraction of β-glucan in Production Media (Minimal Media)

Through Scleroglucan base extraction strategy, the highest extracted β-glucan Minimal Media is between 1.412-1.424 mg/mL on Day 2-3 in controlled environment (Bioreactor) while non-controlled environment (Flask) is 0.365 mg/mL on Day 2. The OD between control and non-controlled different are very drastic this is due to the non-controlled environment temperature was too low in the lab during the cultivation. This affected the bacteria unable to grow effective as compare to controlled environment which station on 30 ^{o}C throughout the cultivation period. In this study, there is a significant in relationship between growth and β-glucan produced. The β-glucan produced by PI18 occurred only in the Pre-Stationary phase or Post-Log phase (Figure 4.7).

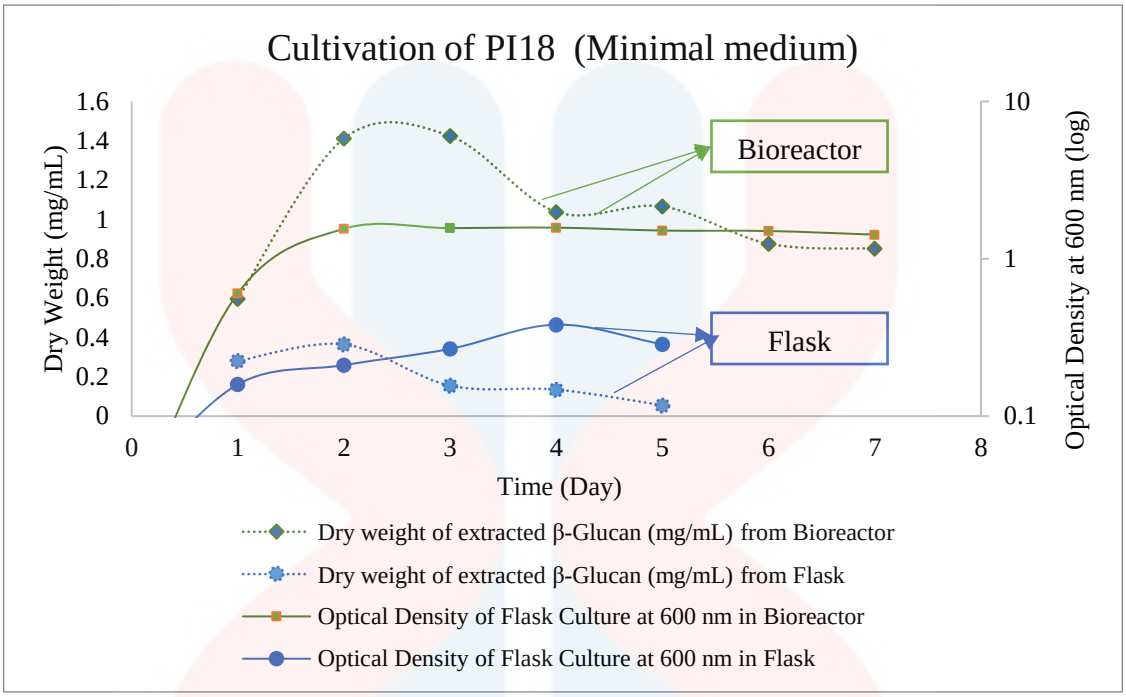


Figure 4.7: The comparison of Bacteria Growth and Extraction β-glucan between Controlled (bioreactor) and non-controlled environment (Flask) cultivation of PI18 strain in Production Media (Minimal media) for 7 Days.

Figure 4.7 showed the trend where the bacteria start producing β-glucan after exponentially and the extracted β-glucan in the broth reduce after the peak. This illustrated the bacteria secreted produce EPS during the Post-Log phase and gradually decrease or stop its β-glucan production. Even the non-controlled cultivation showed similar trend. This hypothesis can be confirmed by referring Figure 4.5 where there is relative higher β-glucan extracted in the Post-Log phase.

The pH study between pH and growth has illustrated relevant relationship. Figure 4.8 is the growth of PI18 in non-controlled environment however its different than previous Figure 4.6 where the environment temperature is relative warmer and cell growth are much faster. In Figure 4.8, bacteria growth is slower, and the pH drop is gradually inversely relation to the bacteria growth which gradually increasing.

Theoretically, when PI18 strain grows, it produces chemical or metabolite into surrounding that affect the cultivation broth turn acidic.

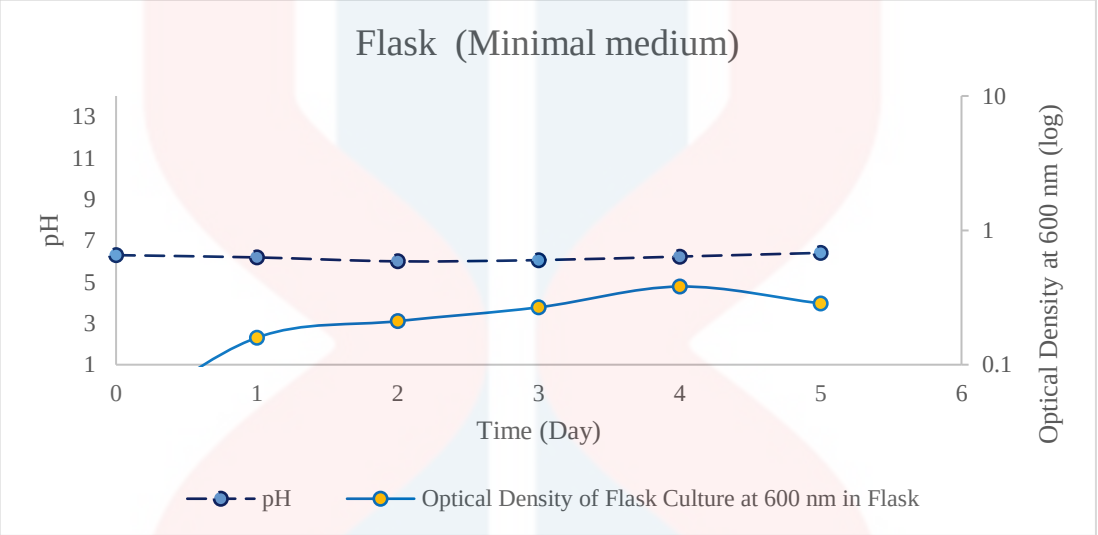


Figure 4.8: The comparison between pH and growth of the PI18 strain in Minimal media and non-controlled environment in 7 Days.

4.4 Factors influencing the β-glucan production of PI18 strain.

According to Ates (2015) the EPS production can be affected by environmental factors and specific culture condition such as temperature, pH, Carbon-to-Nitrogen (C/N) ratio, oxygenation rate and carbon source will impact the EPS production. Besides that, under different stresses condition such as biotic stress competition and abiotic stress EPS is produce for the purpose of self-protection for cell from predation, desiccation, effect of antibiotics, antimicrobial substances and adhere to other bacteria, animal and plant tissue (Ates, 2015).

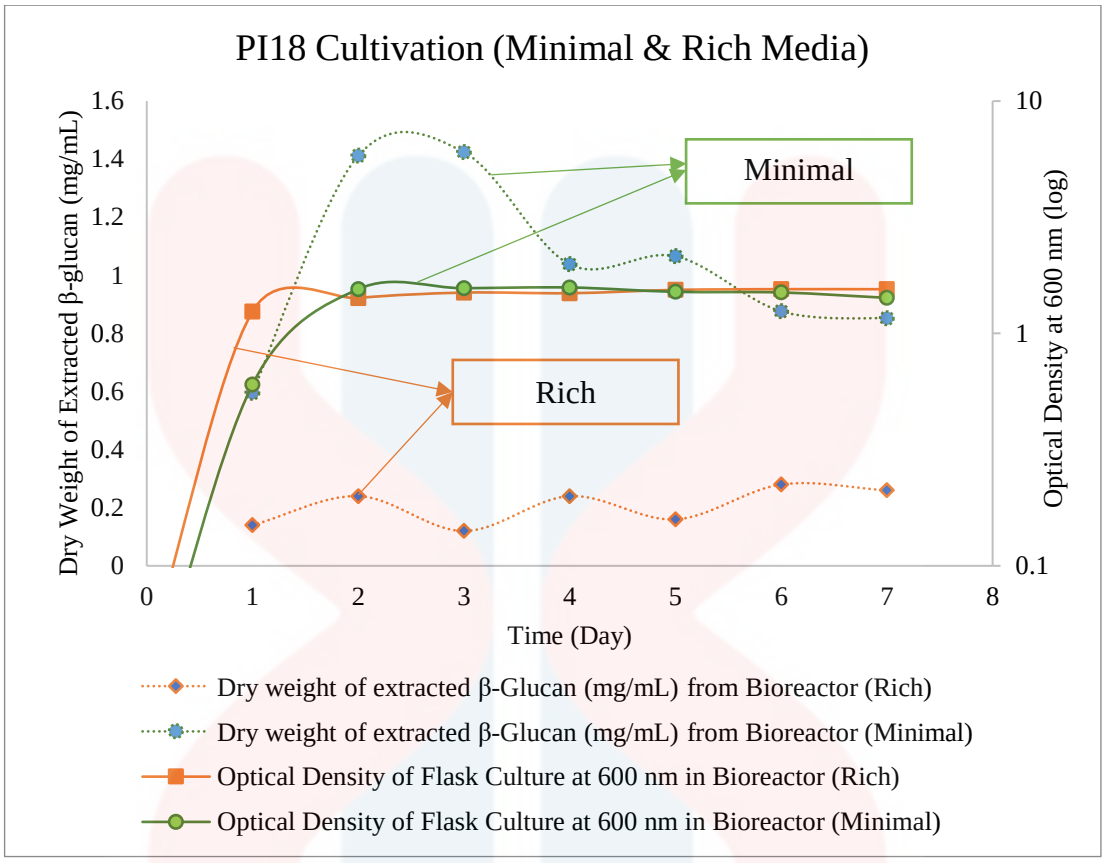


Figure 4.9: Comparison of the cell growth of PI18 strain and production of β-glucan in controlled environment (Bioreactor) in both Minimal and Rich media.

Base on Figure 4.9, the comparison Minimal and Rich Media indicate the β-glucan produces more significant in Minimal media instead of Rich media. Both bioreactor cultivation condition similar in term pH, stirring speed (r.p.m), temperature and carbon source. However, oxygenation rate, (C/N) ratio and nutrient content are most likely the factor affecting the β-glucan productivity. With current finding can conclude that addition of LB is one of the factors hinder the β-glucan produce by PI18 strain.

To support this hypothesis, understanding the biosynthesis of EPS producing bacteria is required. A curdlan (β-glucan) producer *Agrobacterium* sp. ATCC 31749 transcriptome analysis is done by Ruffing and Chen (2012) to understand the regulation of EPS biosynthesis. The study of transcriptome has shown polyP in acidocalcisome is

enhances under stationary phase and stress condition. Curdlan biosynthesis is an energy-intensive process that require high-energy molecule for ascent glycosidic bond. Energy is one of the limiting factor of curdlan biosynthesis from ATCC 31749 (Zheng et al., 2007).

In *E. coli* the level of polyP is low in exponential phase and increase up to 1000-fold during amino acid starvation (Kuroda, Murphy, Cashel, & Kornberg, 1997). This concluded the Rich media contain Tryptone and Yeast Extract that supply amino acid to the culture resulted lesser β-glucan produce in rich media.

Nitrogen limitation show high level accumulation of polyP (Ault-Riché, Fraley, Tzeng, & Kornberg, 1998). Both nitrogen depletion, amino acid and stationary phase are factor affecting the Curdlan production. This explained previous study done by Tee (2018) when extracting EPS through Curdlan strategy by culturing in non-controlled environment (Flask) and the β-glucan extracted is increasing as the culture nitrogen and nutrient upon depletion resulted more β-glucan is produce during stationary phase of ATCC 31749.

The biosynthesis of curdlan (β-glucan) producer *Agrobacterium* sp. ATCC 31749 showed the novel regulation mechanism including RpoN-independent NtrC regulation and intracellular pH regulation by acidocalcisomes in related to EPS biosynthesis. However, PI18 strain may show some of relevancy in related to ATCC 31749 such as the stringent response (stress) due to nutrient limitation will trigger the β-glucan production. But the β-glucan productivity only produced drastically on first 3 days of cultivation in minimal medium (Figure 4.9). This elucidates PI18 strain has regulatory gene that coded for β-glucan biosynthesis expressed during or after Day 3. Yet, our understanding of PI18 is limited on how their biosynthesis is regulated at molecular level.

Besides that, the cell growth rate and β-glucan produces are not dependent but relevant since higher cell concentration more β-glucan can produce. Despite the Rich media reached stationary phase on Day 1 while Minimal media on Day 2 the β-glucan produce from Minimal is much higher. A study of phosphate concentration in nitrogen-limiting and optimisation of Curdlan producer through two-step culture method done by Kim et al (2000) showed Ammonium is necessary for cell growth but has inhibitory effect on curdlan productivity and Phosphate is necessary for cell growth but least need for curdlan production minimum 0.1-0.3 gL^{-1} for optimum productivity.

On the other hand, phosphate depleted will result drop of curdlan production and foam are produced (Kim et al., 2000). Since both our Minimal media are similar composition this explained the foam formation in bioreactor during cultivation of PI18 strain (Figure 4.10). However, during the cultivation the foam are affected by the oxygenation rate thus study of the chemical composition during cultivation is required to further clarify and proved this finding.

Figure 4.10: Visual Observation 48 Hours (Left) and 72 Hours (Right) of cultivation of PI18 strain in Minimal Media in controlled environment (Bioreactor).

During this experiment, the cultivation of PI18 is done maintained pH at 7.0. Based on Figure 4.11 shows the supply of NaOH is directly related to the β-glucan produced by PI18 strain and Figure 4.13 show high aerobic activity during peak of β-glucan. while the acid is used to maintain the broth pH at 7.0. Since pH plays a pivotal role in β-glucan synthesis, pH 5.5 is optimal for curdlan synthesis while at 7.0 pH is optimum for growth in ATCC 31749 strain (Lee, Lee, Kim, & Park, 1999). It is potentially to trigger β-glucan production by manipulate the pH in the broth.

Since PI18 strain are aerobic bacteria, this suggested during the late-Log Phase and Pre-stationary Phase has high aerobic activity that result the drop of pH that causes the addition of NaOH to maintain the broth pH. During Log Phase the bacteria grow rapidly that require high amount of oxygen where the respiration releases carbon dioxide, cause increase in dissolved carbon dioxide that result formation of carbonic acid in media.

At late-Log phase bacteria respirate to produce high energy compound polyP that result biosynthesis of β-glucan in PI18. According to Ates (2015) oxygenation rate can be one of the factor that affect the biosynthesis.

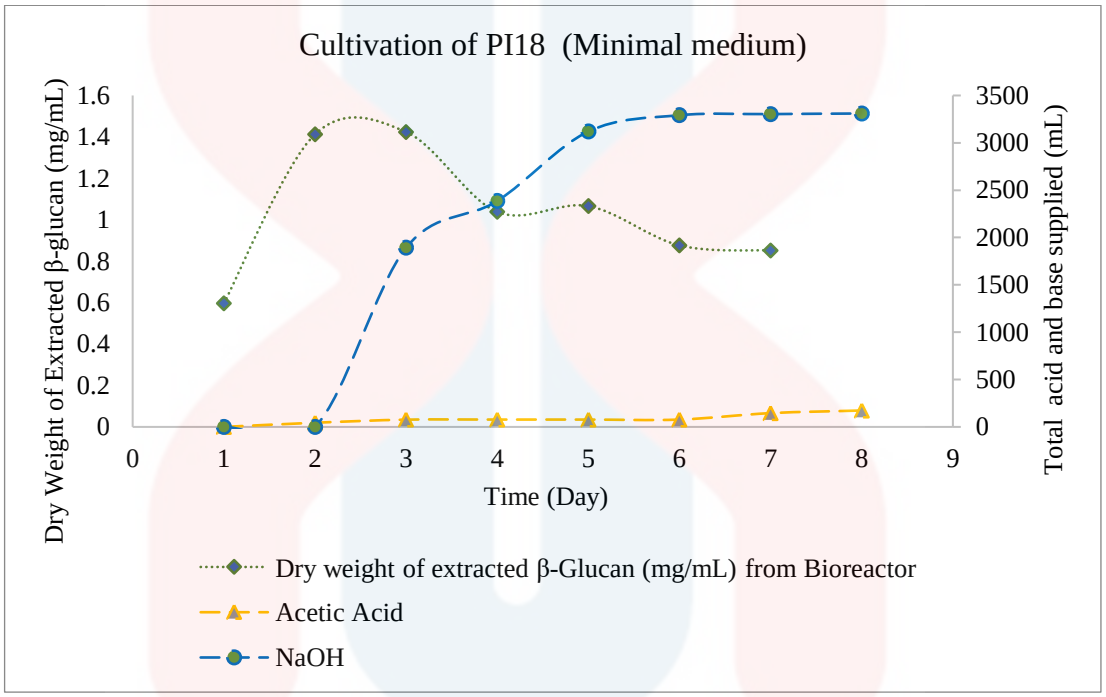


Figure 4.11: The total acid and base supplied to the culture (Minimal media) in bioreactor during the cultivation of PI18 maintained at pH 7.0.

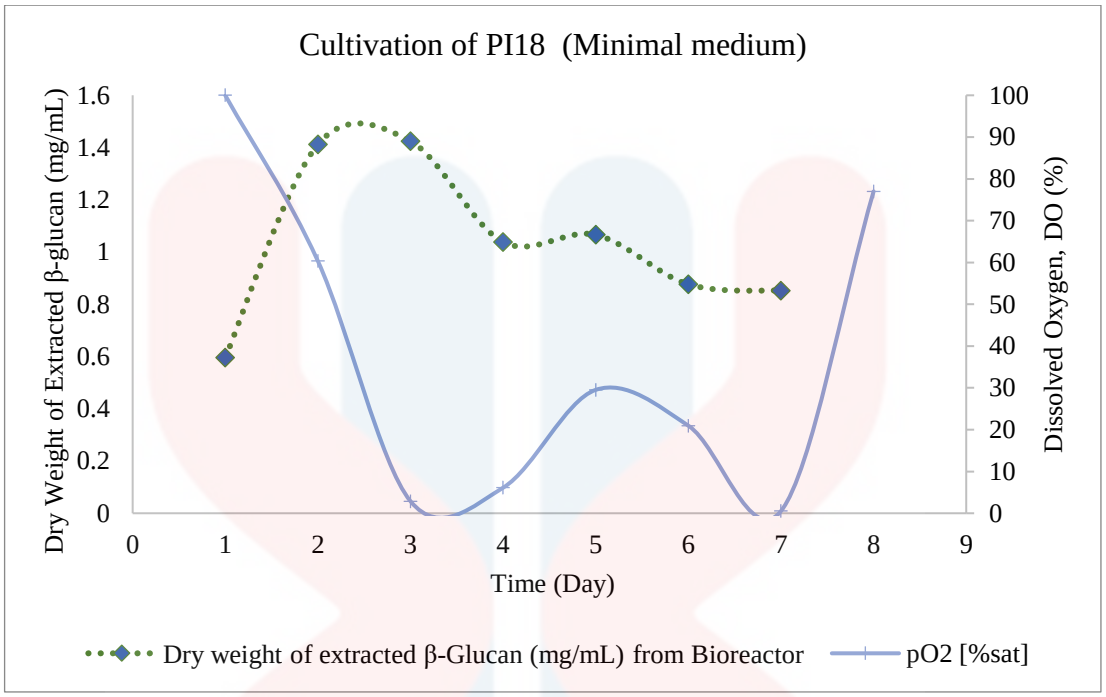
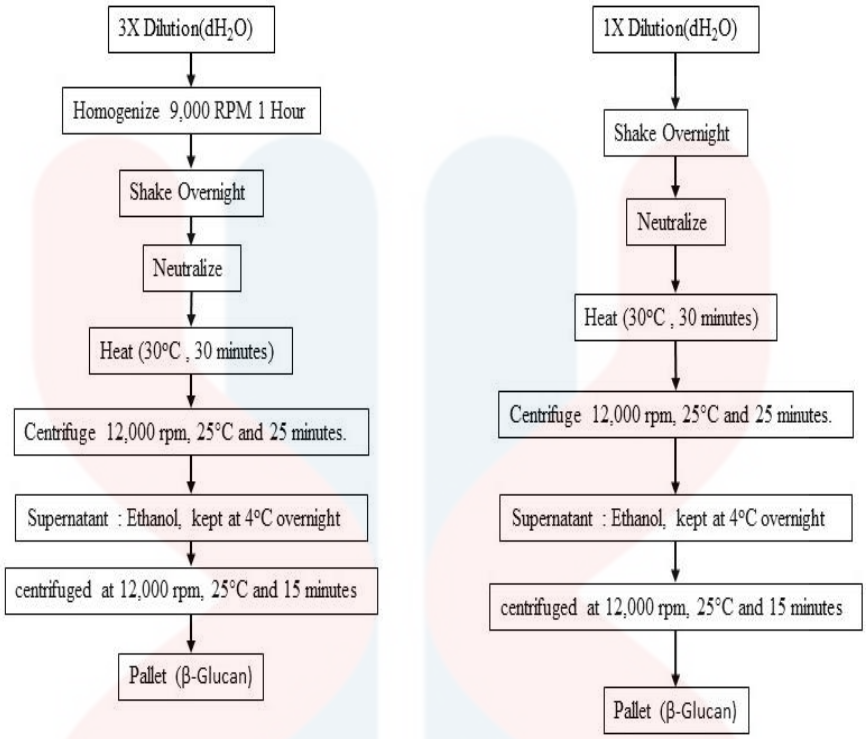


Figure 4.12: The dissolved oxygen, DO (%) in (Minimal media) during the cultivation of PI18.

4.5 Scleroglucan extraction base strategy optimisation.

Scleroglucan base extraction strategy was modified according Fariña et al. (2001) and Viñarta et al. (2013). This strategy was used for Scleroglucan extraction from *Slcerotium rolfsii* fungi in fermenter. Unlike fungi, bacteria such as PI18 are much smaller and less viscos. Three-time dilution and homogenization steps are unnecessary.

In this study EPS extraction was followed on Scleroglucan base extraction strategy modified by Tee (2018) however 3 times dilution substitute to equal amount dilution and homogenization is eliminated, hence time and resource are shorten (Figure 4.16 (b)). Nevertheless, overnight shaking was performance since Scleroglucan is a water-soluble polysaccharide that attach on cell (EPS).



(a) (b)

Figure 4.13: A Schematic of Scleroglucan base extraction strategy. (a) Protocol by Tee (2018), (b) Optimised extraction method.

The optimised extraction strategy, Table 4.1 shows higher yield as compare with previous developed scleroglucan strategy done by Tee (2018), this is mainly due to the lost transfer during final centrifugation where the more the volume is required to transfer while discarding the supernatant in the final step.

Table 4.1: Comparison of β-glucan extracted through scleroglucan base strategy.

β-glucan extracted (mg/mL)	
Previous scleroglucan strategy	Optimised scleroglucan strategy
0.9	2

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Excessive β-glucan producer strain, PI18 produce β-glucan excessively during late log-phase and early stationary phase peak between Day 2-3 (1.412-1.424 mg/mL). The optimal conditions for β-glucan production in PI18 is Minimal media (Production media), while Rich media (Production media + LB) resulted adverse β-glucan production but promote cell growth. The current finding of extracted β-glucan was optimised and studied as the peak productivity achieved 2 Days (Day 2-3). Yet, our understanding is limited about the novelty regulatory mechanism and strigent response of PI18 in related to β-glucan biosynthesis at molecular level post a major setback in optimising the β-glucan producivity. Besides that, extraction strategy is optimised by excluding homogenization and dilution volume is reduced. However, futher optimisation is required to reduce its cost and time consuming method through better equiment such as fine sieve recovery downstream process.

5.2 Recommendation

We have successfully able to conclude and gain slight knowledge in PI18 strain of its biosynthesis of β-glucan through minipulate the nutrient. However, a better understanding the β-glucan regulatory gene and stress response able to enhance the producitivity by manipulate the factors. To achieved this, transcriptome profilling can be done understand the β-glucan expression and regulatory mechanism. Besides that, further analysis the nutrient concentration such as phosphate, ammonium and nitrogen during the cultivation can lead to a better understanding behaviour of PI18 in biosynthesis of β-glucan. Besides that, detail characterization of the newly extracted β-glucan through Gas chromatography-mass spectrometry (GC-MS), fourier-transform infrared spectroscopy (FTIT) and nuclear magnetic resonance (NMR) can obtain the properties and characteristics of β-glucan produce by PI18 strain.

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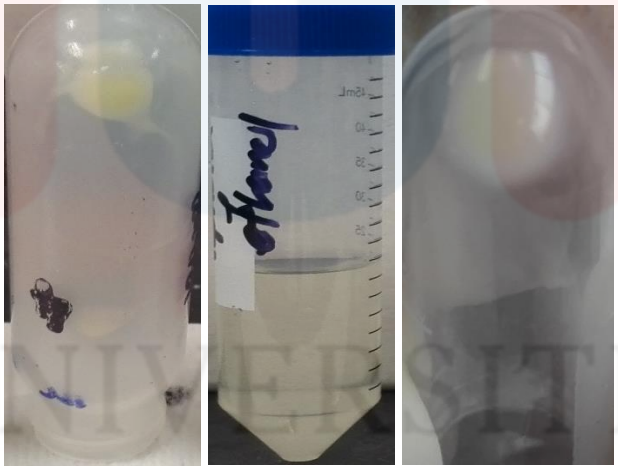
APPENDIX

(a) (b) (c) (d)

Figure A.1: Cultivation of PI18 strain in large scale, (a) cultivation in rich media flask, (b) cultivation in rich media bioreactor, (c) cultivation in minimal media flask and (d) cultivation in minimal media bioreactor



(a) (b) (c)

Figure A.2: Optimise Scleroglucan base extraction strategy (a) pallet of culture broth after centrifuged at 12,000 r.p.m, 25 ℃ for 25 minutes. (b) Equal amount of 95% ethanol is transferred into the supernatant allowed for precipitation. (c) crude β-glucan is extracted by centrifuged at 12,000 r.p.m, 25 °C for 15 minutes discarded the supernatant.