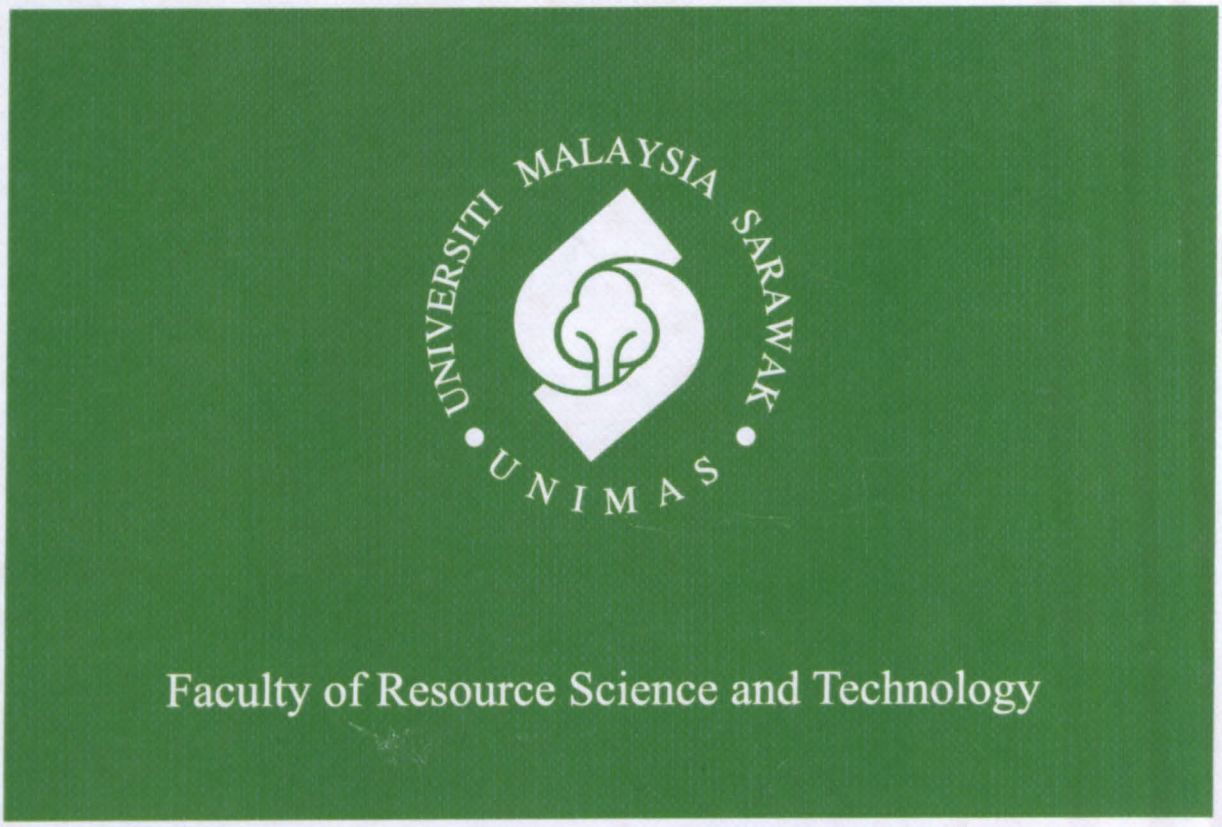

PRODUCTION OF BIO-POLYMER BY *ENTEROCOCCUS FAECIUM* NO. 78 (PNCM-BIOTECH 10375)

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Bachelor of Science with Honours
(Resource Biotechnology)
2015

Production of Bio-polymer by *Enterococcus faecium* no. 78 (PNCM-BIOTECH 10375)

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A final year project report in partial fulfilment of the requirement for the degree of Bachelor Science with Honours (Resource Biotechnology)

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2015

ACKNOWLEDGEMENT

I would like to express my appreciation and thank you to my supervisor Associate Professor Dr. Cirilo Nolasco Hipolito for his patience guidance, supervision and time spent during this project. His guidance and advice has been a great help for me to complete my final year project. I would also like to express my greatest gratitude to all of my lab mates for their generous help throughout the whole project planning and execution.

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List of Abbreviations

HSS	Hydrolysed Sago Starch
LSS	Liquefied Sago Starch
EPS	Exopolysaccharide
CPS	Capsular Polysaccharide
RPM	Revolutions per minute
YE	Yeast Extract
MM	Molecular Mass
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth

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ABSTRACT

The purpose of this project is to identify the viability of the microorganism *Enterococcus faecium* no. 78 (PNCM-BIOTECH 10375) of synthesize biopolymer under fermentation. It is reported that *E. faecium* CRL201 is capable of producing capsular polysaccharide (CPS). Therefore, *E. faecium* was grown under different fermentation conditions using Liquefied Sago Starch (LSS) at the concentrations of 20, 40, 60, 80 and 100 g/l. The fermentation conditions for the bacteria were fixed at a pH of 6.5, temperature of 30°C and agitation speed of 250 rpm. The results showed that *E. faecium* is able to produce CPS, however,it was associatted with the concentration of substrate. The exopolysaccharide (EPS) produced was around 20% of the substrate feed. It was clear that the lactic acid produced is a product apart of lactic acid. In the case of lactic acid only 31.89 g was produced from 55 g of substrated provided. It was demonstrated that *E. faecium* produced EPS at any concentration of substrate tested. Therefore, it could be worthy to evaluate the overall costing of LA production as well as its polymeric composition in order to have a clear panorama of the potential of this strain for industrial application.

ABSTRAK

Tujuan projek ini adalah untuk mengenalpasti kebolehan mikroorganisma Enterococcus faecium no. 78 (PNCM-BIOTECH 10375) untuk mensintesiskan biopolymer semasa proses fermentasi. Didapati bahawa E. faecium CRL201 berkebolehan menghasilkan capsular polisakarida (CPS). Dalam kajian ini, E. faecium dikultur dalam beberapa keadaan fermentasi yang berbeza-beza kepekatan substratnya iaitu 20, 40, 60, 80 dan 100 g/L. Parameter fermentasi yang seperti pH 6.5, suhu 30 °C dan kelajuan kegolakan 250 rpm dikekalkan. Didapati bahawa E. faecium boleh menghasilkan CPS tetapi ianya bergantung kepada kepekatan substrat. Kuantiti EPS yang dihasil ialah 20% daripada jumlah substrat. Oleh itu, CPS yang dihasilkan ialah satu produk lain selain asid laktik. Daripada 55 g substrat yang dibekalkan, hanya 31.89 g asid laktik yang dihasilkan. Ini menunjukkan bahawa E. faecium berkebolehan menghasilkan EPS pada semua kepekatan substrat yang diuji. Oleh itu, kos keseluruhan menghasilkan LA dan polimer diteliti lebih mendalam untuk mendapatkan gambaran sebenar potensi kajian ini diaplikasi di peringkat industri.

Keywords: Enterococcus faecium, biopolymer, exopolysaccharide, capsular polysaccharide, EPS

1.0 Introduction

There is an ever increasing demand and dependence on these synthetic polymers which, as a result, raises the concern towards the environment as well as human health. In order to cater this need with the focus of an eco-friendly design and towards a more sustainable future, this has been the recent philosophy behind the designing of such new materials. Current technology utilized involves the use of petroleum-based polymers which are of non-renewable origin and are not biodegradable. Besides that, the disposals of these materials are made even more difficult they are resistant to microbial degradation which results in accumulation in these polymers (Ghanbarzadeh & Almasi, 2013). Often, they will have various additives incorporated in high concentrations such as non-stick compounds, colorants and UV stabilizers, which are harmful when disaggregated (Moore, 2008). As a result, concerns towards a sustainable and eco-friendly future results in the development and focus towards renewable resource-based products.

One such example is the development of biopolymers such as polysaccharides and polylactides. Biopolymers are materials that are of biological origin that have several differences compared synthetic polymers such as structure and physical properties. Biopolymers are developed with the concept of the environment in mind such as biodegradability which can be used for short-term packaging of products or biocompatibility of biopolymers to be used in biomedical devices (Avérous, 2008). Due to the different properties that biopolymers have compared to their synthetic counterparts, they also might have the potential to substitute their production methods in various applications. Biopolymers could also open up new opportunities and substitute petroleum-based composite materials in various applications with new benefits for all parties (Mohanty, Misra & Drzal, 2002).

However, in reality, biopolymers at the moment are only used in a selected few specialized applications such as in the biomedical field (Avérous, 2008). At the moment, the overall consumption of biodegradable polymers worldwide have had an increase from 14 million kg in 1996 up to 68 million kg in just 5 years (Gross & Kalra, 2002). This production is just a coverage of only 5 to 10% of the current plastics market (Ghanbarzadeh & Almasi, 2013). In the long run, it was hoped that the development of biopolymers will come to a stage where it can displace the more common fossil fuel-based polymers as they improve to be more cost-effective and performance-efficient (Lasprilla, Martinez & Hoss, 2011).

Biopolymers can also be synthesized by microorganisms such as in the case of lactic acid bacteria (LAB). With such an enormous amount of microorganisms present in the environment, the range of which biopolymers synthesized is equally as vast. Such EPS are synthesized for a wide variety of reasons. One is of the interest of these microorganisms is to serve as protection from the engulfment from other protozoa or phagocytes, desiccation and even attacks from antimicrobial agents (Nwodo *et al.*, 2012).

If biopolymers can be used on a full industrial scale, the future prospect for this product can be useful towards human health in the application of probiotics (Nwodo *et al.*, 2012). The consumption of live microbial food ingredients could bring about benefits to the human health (Nwodo *et al.*, 2012). It was found that some of the health-promoting effects can be partially due to EPS such as antiulcer, cholesterol lowering, antitumor and also antiviral properties (Nwodo *et al.*, 2012). Therefore, it is in this project that we hope to achieve two objectives:

a) To produce exopolysaccharides (EPS) by *Enterococcus faecium* through the process of fermentation
b) Identification of the conversion rate of glucose utilized by *E. faecium* for the synthesis of EPS

2.0 Literature Review

2.1 Biopolymer

Biopolymers are macromolecules biologically synthesized through various processes such as enzymatic, chemical and bacterial polymerizations of specific molecular structures (Belgacem & Gandini, 2011). The existence of such polymers have been around for eons and has played a role in the sustainability and constituents of life itself (Belgacem & Gandini, 2011). Over time, humanity has managed to make use of such polymers for their exploitation for development and technological advancements (Belgacem & Gandini, 2011). At the start of the twentieth century, a novel class of material was made which is synthetic polymers which consisted of monomers from fossil fuels (Belgacem & Gandini, 2011). In the end, the total quantity of these synthetic polymers produced worldwide have exceeded 140 million tons per annum (Nampoothiri, Nair & John, 2010). Despite the overwhelming benefits of synthetic polymers towards development, soon concerns arose over their disposal and effects towards the environment and thus, humanity begins to look back to biopolymer as an alternative.

Biopolymers can also be called as renewable polymers because they are derived from renewable sources that can aid in developing a sustainable supply chain and that they are also biodegradable. Biopolymers can be classified into two classes which are agro-polymers and biopolyesters (Avérous, 2008). The application that was first introduced was in the medical field because of the property of in vivo degradation, which opened up the possibility of interactions with the human body such as tissue replacement and controlled drug release (Nwodo *et al.*, 2012).

Besides the addition of benefits that biopolymer has towards the biomedical field, they also have an even wider range of applications such as medical biotechnology where they can be

used as a microsphere vectors for more efficient drug delivery as well as dental impression making in dentistry and in absorbent dressings as an active ingredient (Nwodo *et al.*, 2012). Besides that, an example such as dextran has the benefits as a very efficient plasma substitute for patients at shock and bleeding (Nwodo *et al.*, 2012). Biopolymers such as Xanthan gum have the property as a stabilizer for emulsions and suspensions (Nwodo *et al.*, 2012). As a result, it has been used widely in the field of agriculture as an addition to fungicides, insecticides and herbicides as it is able to uniformly suspend the solid components found in each formulations (Nwodo *et al.*, 2012). On the other end of the spectrum, Xanthan gum is also used in the petroleum industry for pipeline cleaning, oil drilling and fracturing (Nwodo *et al.*, 2012). It is use as an additive to drilling fluid due to its thermal resistance towards degradation and compatibility with salt (Nwodo *et al.*, 2012).

As for potential applications of biopolymers, it was found that certain EPS can act as a potent somnogen which can potentially replace xenobiotics for sleep induction in the future due to its lack of side effects to the human body (Nwodo *et al.*, 2012). With such a wide variety of novel properties not found in its oil-based counterparts, biopolymers have an immense potential in opening up new opportunities towards further development in a more environmental-friendly way. However, such potential has been slowed by high-production costs and lack of understanding towards their properties.

2.2 Exopolysaccharide

Exopolysaccharides (EPS) are polymers composed of polysaccharides extruded by microorganisms into the external environment such as lactic acid bacteria (LAB). They are a long-chain of polysaccharides which are repeating units for sugar and its derivatives which are branched (Welman & Maddox, 2003). EPS also consists of a combination and mixture of various polysaccharides as well as other non-sugar molecules including glycoproteins, nucleic acids, sulphates and even metal ions (Edward *et al.*, 2011). With such an enormous amount of microorganisms present in the environment, the range of which biopolymers synthesized is equally as vast. Therefore, they are separated into four main classes which are polysaccharides, polyesters, polyanhydrides and polyamides (Nwodo, Green & Okoh, 2012). These four classes are grouped under the name of extracellular polymeric substances. For example, the polymer that falls under the class of polysaccharides are called capsular polysaccharides (CPS) or slime EPS which are synthesized and extruded into the external environment (Mozzi *et al.*, 2006). These EPS can also be further divided into the composition of its monomers on whether it consists of only one type of monomer as a homopolysaccharide or a heretopolysaccharide where the repeating units are two or more types of monomers (Mozzi *et al.*, 2006).

Commonly, EPS are synthesized for the interest of the microorganisms themselves. At the end the polymers will be vital in the increase in the virulence factors of the bacteria (Huebner *et al.*, 1999). Among these functions of EPS include protection from the engulfment from other protozoa or phagocytes, desiccation and even attacks from antimicrobial agents (Nwodo *et al.*, 2012). The protection of the microorganism not only refers to the external factors but also internally where the EPS helps to protect the cell against bursting due to the high osmolarity of the cytoplasm compared to the external media of the cell (Delcour, Ferain, Deghorain, Palumbo & Hols, 1999). As for several

microorganisms, they rely solely on the tensile strength of these EPS for their survival as its structural integrity has to be maintained at all times and under all circumstances (Delcour *et al.*, 1999).

At the moment with such an immense diversity on the architectures and structural design of the EPS of various microorganisms, there is no universal mechanism that can explain the formation of these EPS for all microbes (Delcour *et al.*, 1999). There is a need for constant maintenance of the EPS throughout the life-cycle of the microorganism, even during cell growth which requires EPS expansion which requires mesh cleaving of the polymers for the insertion of new materials (Delcour *et al.*, 1999). Current prevailing principles of the EPS formation revolves around two which are "make before break" and "inside to outside" (Delcour *et al.*, 1999). The first principle explains that the integration of new EPS material occurs prior to the cleaving of cross-links at the insertion site through the formation of cross-links into a previously existing, covalently closed EPS sheet first (Delcour *et al.*, 1999). This ensures that the cross-link cleaving can then be anticipatively be repaired immediately (Delcour *et al.*, 1999). The second principle implies that new EPS is first synthesized at the internal side of the cell wall which is between the cell wall and the cell membrane (Delcour *et al.*, 1999). The new EPS will then be pushed out radially to the external layer of the cell wall by more recent EPS layers (Delcour *et al.*, 1999).

These capsules which are made of EPS are also often used as an adherent towards surfaces. Usually, there is the possibility of over synthesis when abundant sugar is found in the extracellular environment, such as in the case of dextran (Nwodo *et al.*, 2012). Even the CPS can have a more conserved function among certain species in the field of cryoprotection and of high salinity (Nwodo *et al.*, 2012). This is more on the microbial community that can be found in the sea ice and also other marine environments (Nwodo *et*

al., 2012). It was also found in some bacterial species where the EPS production is regulated by mobile elements in plasmids (Edward *et al.*, 2011).

Current applications of EPS has been extensively studied for its various applications in multiple fields such as pharmaceutical, cosmetic and even the food industry (Tavares *et al.*, 2005). There have also been reports on the capabilities of EPS in their medicinal properties such as anti-tumour, hypolipidaemic and immunostimulating activities (Tavares *et al.*, 2005). Other applications of EPS includes the removal of heavy metal ions from the environment. This is due to one of their properties of flocculation as they are able to bind to heavy metal ions found in solutions (Vu *et al.*, 2009).

2.3 Enterococcus faecium

The term of Enterococcus was first coined by a scholar of the name Thiercelin to describe a diplococcus organism which is Gram-positive and found in the gastrointestinal tract (Vu & Carvalho, 2011). The initial organism that was first found was eventually given another name by Andrewes and Horder with the name of *Streptococcus faecalis* which was isolated from a patient (Vu & Carvalho, 2011). Another organism was then given *Streptococcus faecium* which has similar physiogenomies to *Streptococcus faecalis* (Vu & Carvalho, 2011). The streptococci was then divided into four distinct groups which are pyogenic, lactic, viridans and enterococcus (Vu & Carvalho, 2011). Prior to 1984, enterococci was placed under the genus of Streptococcus until it was then separated into their own genus of Enterococcus (Vu & Carvalho, 2011). This change in classification was first proposed by Kalina and was then confirmed when there were significant differences between enterococci and streptococci through DNA-DNA and DNA-RNA hybridization (Vu & Carvalho, 2011).

In humans, it was found through molecular analysis that approximately 1% of the total microbiotic population which only accounts for a small fraction is contributed by the genus of Enterococcus (Lammers, 2009). Initially, *Enterococcus faecium* is seen as a non-pathogenic microorganism, but it is recently emerged as a pathogen as a result of its high-level resistance to antibiotics (Lammers, 2009). Although there are about 38 different species under the Enterococcus genus, the most important and that have the potential as human pathogens are *Enterococcus faecalis* and *Enterococcus faecium* (Vu & Carvalho, 2011). It was found that *E. faecium* frequently causes clinical infections in the elderly as well as immunocompromised individuals (Lammers, 2009). Among these clinical infections include urinary tract infection and peritonitis where the latter has seen to show a relationship with the increase in mortality rates (Leendertse *et al.*, 2009).

A common physiology of Enterococci are that they are tough non-spore-forming microorganisms with the ability to proliferate in a wide variety of hostile conditions (Vu & Carvalho, 2011). As they can switch between respiration and fermentation readily due to their facultative anaerobic nature, they can thrive in either oxygen or non-oxygen environments (Vu & Carvalho, 2011). Environments having a high salt content are also able to sustain the life of Enterococci with a salt concentration of up to 6.5% NaCl where other microorganisms such as the Gram-positive Streptococci will not be able to grow at all (Vu & Carvalho, 2011). Although, they are able to proliferate at an optimal temperature of 37 to 42.7°C, depending on the culture media that they are growing in, they are also able to withstand extreme temperatures ranging from 5°C up to 50°C, or even at the upper limit of 60°C for 30 minutes (Vu & Carvalho, 2011). In terms of pH, survival ranges from 4.8 to 9.6 or even up to 10 in some strains, although the optimum growing pH is at 7.5 (Vu & Carvalho, 2011). This hardiness of Enterococci is due to their physical characteristics such as membrane impermeability and stability towards alkali and acids resulted in their resistance to a wide range of pH (Vu & Carvalho, 2011). Another example is the presence of membrane lipids or fatty acids which contributed to their proliferative ability at such a wide spectrum of temperatures (Vu & Carvalho, 2011).

3.0 Materials and Methods

3.1 Materials

Raw Sago Starch (Herdsen Sdn. Bhd., Pusa, Sarawak)

Enzyme 1, 4-α-D-glucan glucanohydrolase (Novozyme, Denmark)

3.2 Methods

3.2.1 Preparation of Liquefaction of Sago Starch

Liquefied Sago Starch (LSS) was prepared by suspending 400 g of starch (dry basis) in 6000 mL of distilled water. The suspension was agitated and allowed to settle for 24 hours for the washing of starch. The distilled water was then removed and replaced with 1000 mL of distilled water. The suspension was then filtered using a mesh cloth with a pore size of 300 μm for impurities removal. The pH of the suspension was then adjusted to 4.5. α-amylase with the concentration of 0.5 μL/g of starch was then added to the suspension in order to liquefy the starch for 2 hours at 98°C. The suspension (liquefied starch) was then cooled to room temperature and then centrifuged at 1000 g for 10 minutes. The pellet was then removed to produce the final LSS product.

3.2.2 Culturing of Enterococcus

The stock culture of *E. faecium* was first maintained at -84°C within a Potato Dextrose Agar (PDA) media. The strain was then thawed and inoculated in 250 mL glucose media containing glucose (40 g/L) and yeast extract (YE) (5 g/L). After 24 hours, the culture are then separated into 10 universal tubes containing 25 mL of culture respectively. The cultures are then stored at 4°C for subsequent use. Prior to the start of the fermentation process, 25 mL of the culture was then added into 115 mL of glucose media having the same concentration of glucose (40 g/L) and YE (5 g/L). The 140 mL culture was then incubated for another 24 hours.

3.2.3 Preparation of Batch Fermentation

A fermenter jar with a working volume of 700 mL was used for the fermentation process. Before fermentation, the prepared LSS was added at the specified concentrations together with the corresponding volume of distilled water. YE (5 g/L) was also prepared separately with an adjusted volume of 100 mL. Both the fermenter and YE was sterilized for 15 minutes at 121°C. Once cooled down to room temperature, the YE and culture media was added into the fermenter inside a laminar flow hood and sealed. The temperature of the fermentation process was set at 30°C and agitated at 250 rpm. The concentration of the LSS used was from the range of 20, 40, 60, 80 and 100 g/L.

3.2.4 *E. faecium* Growth Analysis

10 mL of sample is taken from the fermenter at a 12 hour interval for all fermentation processes. Only for the fermentation with the concentration of 20 g/L that samples are taken every 6 hours instead. 4 mL of the sample was taken and centrifuged for 10 minutes at 6000 g. This is used as a blank for the spectrophotometric analysis of *E. faecium* at 650 nm. The remaining 6 mL of sample are then separated into 3 different cuvettes for analysis. Optical Density (OD) measurements are taken in triplicates and an average was then calculated.

3.2.5 Exopolysaccharide Analysis

3.2.5.1 EPS Isolation

The fermentation media was then centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was removed for the DNS Reagent test. The pellet obtained was then dissolved overnight in 70% ethanol. Centrifuge was repeated at 10,000 g for 30 minutes at 4°C. The

supernatant was mixed with 1:1 volume ratio of 95% ethanol for EPS precipitation. The precipitate was then centrifuged again for 10,000 g for 30 minutes at 4°C. After that, the pellet was the agitated and further centrifuged at 10,000 g for two hours at 4°C. The EPS was then be subjected to desiccation and stored at room temperature.

3.2.5.2 EPS Analysis

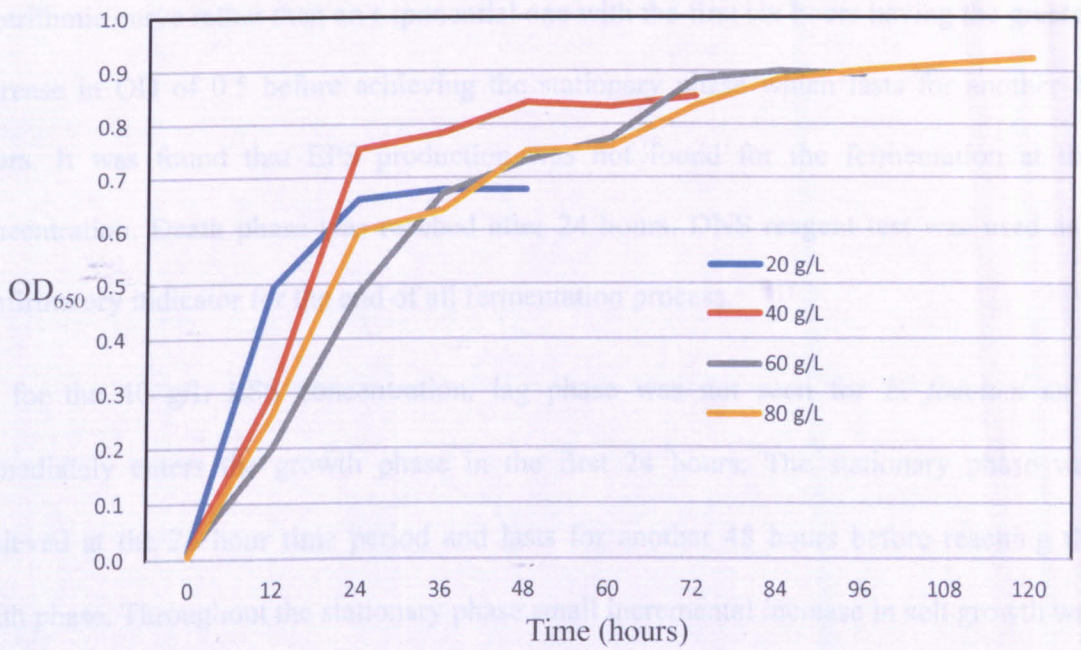
The mass of the EPS was measured and then measured against the loss of glucose and NaOH throughout the fermentation process. Glucose analysis was done through the DNS reagent test for the fermentation supernatant (Gusakov, Kondratyeva & Sinitsyn, 2011). DNS solution and potassium sodium tartrate solution (Rochelle salt) was first prepared. A glucose standard of 1% concentration was prepared. Glucose of different concentrations was prepared with a range of 0 % to 100 % was prepared. 3 mL of DNS reagent and 3 mL of the supernatant was added into a test tube. The test tube was then covered with aluminium foil to prevent liquid loss during evaporation. This step was then repeated with the glucose solutions of different concentrations. The mixture was then heated at 90° C for 15 minutes in a water bath to develop the red-brown colour. After 15 minutes, 1 mL of Rochelle salt was then added into the solution to stabilize the colour. Once the solution has cooled to room temperature, its absorbance was then recorded with a spectrophotometer at 575 nm against the glucose standard as the blank. This step was also repeated using LSS replacing the supernatant solution. The quantification of mass of EPS was also measured against the biomass of *E. faecium* at the end of the fermentation process.

4.0 Results

The production of EPS was found to be closely related to the concentration of LSS used for the batch fermentation process. The overall growth rate of *E. faecium* was measured with absorbance reading from the fermentation process at a time interval of 12 hours. A six hour

time interval for sampling was used instead for the fermentation with a concentration of 20 g/L of LSS due to the low glucose content and duration of fermentation. It was found that the duration of fermentation follows a linear increase as the concentration of LSS increases. The discrepancies found are the increase in LSS concentration from 20 to 40 g/L where the fermentation duration increases from one to three days rather than the linear increase of 1 day as compared to other fermentation concentrations. The duration for fermentation of 100 g/L of LSS lasted for 492 hours (20 days 12 hours).

4.1 Growth Curve of *E. faecium*



Graph 1.0: The growth curve of *E. faecium* with OD_{650} against time in hours for different fermentation conditions (20, 40, 60, and 80 g/L).

The duration of fermentation of each experiment was done based on the concentration of LSS used as a longer time needed for the full utilization of glucose present in the fermentation media. Complete use of glucose in the fermentation media was identified through the consumption of NaOH, spectrophotometric analysis and DNS reagent test.

With a regular time interval, the sample was aseptically withdrawn from the fermenter for spectrophotometric analysis to determine the optical density at 650 nm (OD_{650}). A graph for each LSS concentration was plotted with OD_{650} versus time. The graph indicates that the cellular growth for all fermentation was according to the initial exponential growth curve for the first 24 hours, with the 20 g/L LSS concentration as the exception as the growth phase was seen for the first six hours before reaching the stationary phase. A small incremental increase in OD was seen in the fermentation process of all concentrations.

The growth phase of the fermentation process with the concentration of 20 g/L follows a logarithmic curve rather than an exponential one with the first six hours having the greatest increase in OD of 0.5 before achieving the stationary phase which lasts for another 18 hours. It was found that EPS production was not found for the fermentation at this concentration. Death phase was reached after 24 hours. DNS reagent test was used as a confirmatory indicator for the end of all fermentation process.

As for the 40 g/L LSS concentration, lag phase was not seen for *E. faecium* as it immediately enters the growth phase in the first 24 hours. The stationary phase was achieved at the 24 hour time period and lasts for another 48 hours before reaching the death phase. Throughout the stationary phase small incremental increase in cell growth was seen.