EXPLORING BIODIVERSITY FOR THE PRODUCTION OF EXOPOLYSACCHARIDES FROM SELECTED *BACILLUS* SPECIES AND CHARACTERIZATION OF THEIR STRUCTURAL PROPERTIES

By

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ABSTRACT

Selected bacilli, including *Bacillus licheniformis* (14580), *Bacillus amyloliquefaciens* (23350; OB6), *Bacillus subtilis* (B26), and *Geobacillus stearothermophilus* Donk (12980), were explored as microbial sources for the production of exopolysaccharides (EPS). Three media with different nutrient compositions were investigated as culture media: (i) mineral base-medium with added yeast extract (M1), (ii) succinate-containing mineral base-medium with added yeast extract (M2) and (iii) tryptone and yeast extract-containing base-medium (M3) as both semi-solid and liquid media. Sucrose was utilized as the inducer for the production of EPS. The EPS production was not only dependent on the type of strain, but also on the media and the fermentation conditions. *Gb. stearothermophilus* Donk did not produce significant EPS in all investigated media. While *B. amyloliquefaciens* (OB6), produced the greatest yield of <5 kDa sized EPS (54.14 g/L, M2 medium) over the fermentation time course of 144 hours; this EPS was composed primarily of glucose (63.4–93.8%), xylose (5.4–18.6%) and fructose (0.4–15.9%). The second most significant yield of 5-30 kDa EPS (48.57 g/L, M2 medium) was obtained with *B. licheniformis* (14580) after 48 hours of fermentation, with an exceptionally heterogeneous monosaccharide profile containing greater quantities of galactose (1.5–87.5%), fructose (5.1–60.7%) and glucose (1.9–32.9%). For *B. amyloliquefaciens* (23350) and *B. subtilis* (B26), the yielded EPS quantities were 6.74 and 6.59 g/L in M1 and M3 media at 11 and 72 hours, respectively. The EPS produced by both of these bacteria were low molecular weight (<5 kDa) and dominantly composed of glucose (70.3–96.3%). The lowest yields were observed with *B. licheniformis* (14580) in M1 medium at a concentration of 3.50 g/L after 144 hours. These EPS were higher molecular weight heteropolymers (30-100 kDa) primarily composed of galactose (44.7–51.0%) and glucose (33.0–40.0%). The effects of the concentration of selected nutrients (yeast extract of 10–200 g/L; sodium succinate of 0–100 g/L) and the inducer, sucrose (100–400 g/L) on the EPS production by *B. licheniformis* (14580) were studied using response surface methodology. The sodium succinate/sucrose concentration interaction had the greatest effect on the biomass yield from 48–96 hours. At 120 hours, the interactions between the concentration of yeast extract and sodium succinate became the most significant one. Greater yields of EPS (g/L culture) were obtained upon maximizing sodium succinate and sucrose concentrations and minimizing yeast extract content in the mineral media, and greater content of contaminating proteins (g/g biomass) were observed in the presence of high concentrations of yeast extract.

RÉSUMÉ

Des bacilles, *Bacillus licheniformis* (14580), *Bacillus amyloliquefaciens* (23350, OB6), *Bacillus subtilis* (B26) et *Geobacillus stearothermophilus Donk* (12980) sont explorées comme sources microbiennes pour la production des exopolysaccharides (EPS) dans trois milieux de culture de composition différentes: (i) un milieu de base minéral additionné d'extrait de levure (M1), (ii) un milieu de base au succinate additionné d'extrait de levure (M2), et (iii) un milieu au tryptone et à l'extrait de levure (M3). Ces milieux ont été utilisés sous forme solide et sous forme liquide et contiennent tous du saccharose comme un précurseur pour la production d'EPS. Des résultats ont montré que la production d'EPS dépend non seulement de l'espèce bactérienne, mais aussi du milieu de culture et des paramètres physico-chimiques de fermentation. Parmi les souches étudiées, *Gb. stearothermophilus* Donk n'a pas produit de quantités significatives d'EPS dans tous les milieux de culture étudiés. En revanche, *B. amyloliquefaciens* (OB6) a produit le plus haut rendement d'EPS de <5 kDa (54.14 g/L) après 144 heures de fermentation dans le milieu de culture M2. Les EPS produits sont composé composés principalement de glucose (63.4–93.8%), de xylose (5.4–18.6%) et de fructose (0.4–15.9%). Le deuxième plus haut rendement en EPS de 5-30 kDa (48.57 g/L) était obtenu avec *B. licheniformis* (14580) après 48 heures de fermentation dans le milieu de culture M2, et avec un profil de monosaccharides particulièrement hétérogène avec le galactose (1.5–87.5%), le fructose (5.1–60.7%) et le glucose (1.9–32.9%) comme constituants majeurs. Pour *B. amyloliquefaciens* (23350) et *B. subtilis* (B26) le rendement en EPS était de 6.74 et 6.59 g/L dans les milieux de culture M1 et M3 après 11 et 72 heures de fermentation, respectivement. Les EPS synthétisés par ces bactéries étaient de faibles poids moléculaires (<5 kDa) et principalement composés de glucose (70.3–96.3%). Le rendement le plus bas était obtenu avec *B. licheniformis* (14580) dans le milieu M1, à la concentration de 3.5 g/L après 144 heures de fermentation. Les EPS obtenus étaient de haut poids moléculaires (30–100 kDa) et principalement composés de galactose (44.7–51.0%) et de glucose (33.0 - 40.0%). Les effets de la concentration en certains nutriments (extrait de levure à 10 - 200g/L, succinate de sodium à 0–100g/L, en plus du précurseur, le saccharose, à 100–400g/L) sur la production d'EPS par *B. licheniformis* (14580) ont été étudiés par la méthode des surfaces de réponses (Box et Wilson). L'interaction entre les concentrations de succinate de sodium et du saccharose avait un effet prépondérant sur les rendements en biomasse entre 48 et 96 heures de fermentation. Cependant, à 120 heures, l'interaction entre la concentration de l'extrait de levure et du succinate

de sodium est devenue plus significative. Un rendement supérieur était obtenu en maximisant la concentration du succinate de sodium et la concentration du saccharose, et en minimisant celle de l'extrait de levure dans les milieux minéraux de base. Par contre, la quantité en protéines produite (g/g biomasse) a augmenté en fonction de la concentration en extrait de levure dans les milieux de cultures.

CONTRIBUTION OF AUTHORS

This thesis consists of the three following chapters:

Chapter I providing a comprehensive literature review on the topic representing a scientific support of the objectives of the study. The literature review presents state-of-the-art knowledge on exopolysaccharide (EPS) production by lactic acid bacteria and bacilli under different growth and environmental conditions, including culture media, pH, and temperature and duration of fermentation. The main techniques used to identify and characterize the EPS produced; their structure, composition and yields were also reviewed.

Chapter II elucidates the methodologies and experimental procedures used to achieve the objectives of the study; and Chapter III presents the results obtained and provides an extensive discussion in order to draw the main conclusions and to foresee practical applications and future studies to be conducted for better knowledge of the topic.

Connecting statements are also included to provide a succinct summary of each chapter and to introduce the next one.

Afshan Malick, the author, was responsible for the experimental work and the writing of the thesis.

Dr. Salwa Karboune, The MSc student's supervisor, guided all the research and critically revised the thesis prior to its submission.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance
ATCC: American Type Culture Collections
CCRD: Central composite rotatable design
CHO: Carbohydrates
CPS: Capsular polysaccharide
DNS: 3,5-Dinitrosalicylic acid
DP: Degree of polymerization
EPS: Exopolysaccharides
ESM: EPS Selection Media
FAO: Food and Agricultural Organization of the United Nations
FOS: Fructooligosacccharides
GIT: Gastrointestinal Tract
GRAS: Generally regarded as safe
HPAEC-PAD: High-pressure anion exchange chromatography with pulsed amperometric detection
HPLC: High performance liquid chromatography
Hrs/h: Hours
IUPAC: International Union of Pure and Applied Chemistry
LPS: Lipopolysaccharides
MALLS: Multi-angle laser-light scattering
MS: Mass spectroscopy
MRS: Man Rogosa Sharpe
MW: Molecular weight
NMR: Nuclear magnetic resonance
ppm: parts per million
QPS: Qualified Presumption of Safety
RI: Refractive index
RSM: Response surface methodology
scFA: Short chain fatty acids
SEC: Size exclusion chromatography
SEM: Scanning electron microscopy
TEM: Transmission electron microscopy
TFA: Trifluoroacetic acid
WHO: World Health Organization

INTRODUCTION

Polysaccharides are of great interest to the food industry as multifunctional food ingredients that can be easily incorporated into a wide range of processed food products. In addition to their techno-functional properties, such as texture-modifying, stabilizing and emulsifying properties, some polysaccharides (i.e. fructans and glucans) are recently gaining more attention as health-promoting components owing to their biological functions. For instance, fructans can selectively support the gastrointestinal health by increasing the beneficial microflora, leading to the production of short chain fatty acids. The beneficial health effects of glucans in stimulating the immune system and lowering plasma cholesterol have also been highlighted in many studies (Agriculture & Agri-Food Canada, 2008). However, because of low extraction yields of these ingredients from natural sources and their limited chemical structures, microbial production is an attractive approach for the production of well-defined polysaccharides. Many bacterial species, in particular the lactic acid bacteria are known to excrete exopolysaccharides (EPS). These extracellularly produced polysaccharides have attracted substantial attention as techno-functional ingredients as they show great diversity in structural and functional properties.

Exopolysaccharides (EPS) are extracellularly produced biopolymers composed of sugar residues that are arranged in different ways to yield various macromolecules (e.g. xanthan gum and curdlan) of different structural and functional properties (Nobre, et al., 2015; Oguzhan & Yangilar, 2013). Many bacteria are known for their ability to synthesize and excrete EPS as extracellular polymeric substances; however, the quantities yielded depend on the species and growth conditions (Mishra & Jha, 2013; Chen, et al., 2006; Pawar, et al., 2013). EPS can have a wide range of applications within the food and pharmaceutical industries owing to its diverse functional properties, including emulsifying, stabilizing, binding, gelling, coagulating, lubricating, biofilm forming, thickening and suspending properties.

In the food industry, EPS have stimulated increased research interest, during the last two decades, especially for their application as prebiotics in the formulation of health-promoting functional foods (Grimoud, et al., 2010; Hongpattarakere, et al., 2012). Also, recent studies have demonstrated their potential to be used as food stabilizers and thickeners. Yet, such applications remain hampered by the fact that most the known producers of this prebiotic material are non-food grade bacteria. Therefore, current studies are mainly focused on the EPS produced by lactic acid

bacteria (LAB) which have been studied in significant detail in this respect; due to their Generally Recognized as Safe (GRAS) status and the possibility of their direct incorporation into fermented dairy products to generate EPS *in situ* at physiological levels. Studies have previously demonstrated the large quantities of EPS that can be produced by certain *Bacillus* spp., and the types of polysaccharides produced by these bacteria have notably varied within the same species (Lee, et al., 1997; Shih, et al., 2010). Therefore, it was hypothesized that *Bacillus* spp. would produce greater quantities and a wider range of EPS as compared to LAB depending on the nutrient source. The production and characterization of EPS produced by *Bacillus* bacteria is of strong interest, as these bacteria have indeed shown potential to produce significantly higher yields of EPS than LAB.

The overall objective of the study was to investigate the EPS production by selected bacterial strains of *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus amyloliquefaciens* OB6, *Bacillus subtilis* B26 and *Geobacillus stearothermophilus* Donk, and to characterize their structural properties. This was explored by achieving four specific objectives:

(1) Screening of selected microbial sources in terms of their ability to produce EPS on three types of mineral media.
(2) Determining biomass and EPS production of the selected *Bacillus spp.* and determination of optimum fermentation time.
(3) Characterizing the EPS structures by the determination of the molecular weight distribution of the polymers and their monosaccharide profile.
(4) Investigating the effects of fermentation parameters on the EPS production by a selected *Bacillus* strain among the five studied ones by using response surface methodology.

CHAPTER I. LITERATURE REVIEW

1.1 Definitions & Concepts

1.1.1 Probiotics

Probiotics are defined as live, beneficial microorganisms that are capable of sustained proliferation and colonization of the digestive tract, and provide health benefits for the host when administered in adequate quantities (FAO; WHO, 2002; Saad, et al., 2013). Consequently, these microorganisms resist degradation by digestive enzymes, acid and bile, which allow their survival and unhindered passage through the gastrointestinal tract (GIT). The most commonly used probiotic microorganisms currently approved for incorporation into foods are the heterogeneous group of LAB in particular, *Lactobacillus* spp (e.g., *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei*), *Streptococcus thermophilus* and bifidobacteria (e.g., *Bifidiobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis, Bifidobacterium animalis, Bifidobacterium longum*, *Bifidobacterium lactic*) (Saad, et al., 2013). Once established in the GIT, these microorganisms can inhibit the growth of enteric pathogens in various ways; they can antagonize pathogens directly by producing bactericidal peptides/proteins, immunomodulate by activating immune cells (T-cells, dendritic cells), and/or improve the epithelial barrier function with competitive exclusion by blocking epithelial binding receptors, or by strengthening the tight epithelial junctions by increasing expression of tight junction proteins (**Figure 1.1**) (Corr, et al., 2009). Stable colonic microbiota provide hosts with a vital barrier that limits pathogen contact with the intestinal epithelium (Holzapfel & Schillinger, 2002). Meanwhile, metabolites derived from probiotic microorganisms, such as short-chain fatty acids (scFAs), can stimulate immunity and in tandem, inhibit the growth of harmful bacteria such as pathogenic serotypes of *Escherichia coli* and *Clostridium perfringens* (Sanders & Gibson, 2006). However, screening of probiotic bacterial strains for industrial purposes is a tedious process, as they are known to require strict environmental conditions for growth and stability; in addition to difficulties finding compatible strains that can be incorporated in functional foods and health supplements (Lee & Salminen, 2009).

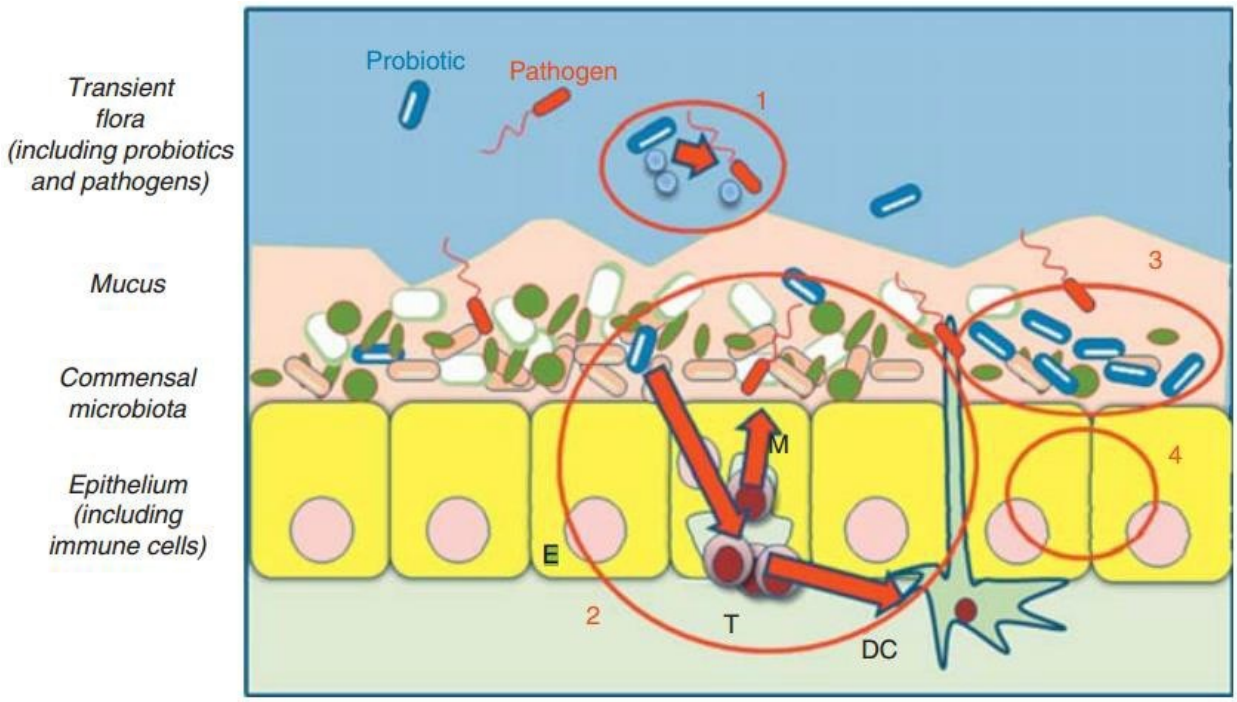


Figure 1.1. Schematic representation of mechanisms used by probiotic bacteria to protect hosts against infections by pathogens

(1) Direct antagonism— bacteriocin production, (2) immunomodulation (T-cell, Dendritic cell activation), (3) enhance epithelial barrier function via blocking binding receptors (4) strengthening of epithelial tight junctions via increased expression of tight junction proteins. From Corr et al., (2009).

1.1.2 Prebiotics

Presently, prebiotics are defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity in the gastrointestinal microbiota, thus conferring benefit(s) upon the host's health" (Bindels, et al., 2015; Gibson, 2010). The main properties of prebiotics are: (i) resistance to hydrolysis caused by gastric acidity, and mammalian enzymes, (ii) resistance to gastrointestinal absorption, (iii) resistance to fermentation by the intestinal microflora, and (iv) the ability to stimulate the growth and/or activity of selective intestinal microorganisms that are associated with health benefits and wellbeing (Gibson, 2010). Today, non-digestible oligosaccharides (particularly inulin-type fructans such as oligofructose, (trans)-galacto-oligosaccharides, glucooligosaccharides, and lactosucrose), fully achieve these criteria for prebiotic classification (de Vrese & Schrezenmeir, 2008; Bindels, et al., 2015; Gibson, 2010). Prebiotics are thought to be a more practical means of manipulating the colonic microflora than probiotics, as prebiotics selectively stimulate the growth and activity of specific bacterial species that are naturally occurring in the intestines.

1.1.3 Synbiotics

The term "synbiotic" is used when a product contains both probiotics and prebiotics, in which the prebiotic compound will selectively favor the probiotic component. Some examples of synergistic relationships that are known are *Bif. lactis* Lafti B94, which is able to utilize a range of prebiotics including inulin, fructo-, galacto-, soybean- and xylo-oligosaccharides and resistant starch in order to grow (Scholz-Ahrens, et al., 2007). While synergism between the probiotics and prebiotics has been substantiated, the health implications they may have remain to be established. A possible benefit to synergism, however, is the ability of some probiotic bacteria, such as *Bif. lactis* Lafti B94, to enhance mineral absorption in bones when their proliferation is selectively favored in the presence of prebiotics (Crittenden, et al., 2001). This was exemplified by demonstrating the increase of folate production by folate-producing probiotic strains of *S. thermophilus* and bifidobacteria (Crittenden, et al., 2001). Since folate content of erythrocytes is associated with enhanced bone mineral density and bone mineral content in postmenopausal women, the latter findings indicate that some probiotics may improve mineral absorption in bones via the provision of folate, and hence have a potential to improve the health of bones (Scholz-Ahrens, et al., 2007).

1.1.4 Dietary Carbohydrates – Polysaccharides

Dietary carbohydrates are carbohydrates that resist gastric digestion; and they represent a complex family of active compounds that can be classified in different ways. The simplest one of these classifications is based on the degree of polymerization. The International Union of Pure and Applied Chemistry (IUPAC) has designed nomenclature (IUPAC nomenclature) stating that simple sugars are composed of 1 or 2 saccharide units, oligosaccharides are composed of a few saccharide units (3 to 10 units) and polysaccharides are composed of more than 10 units of mono- or disaccharides linked in sequence by glycosidic bonds.

Polysaccharides are a class of high molecular weight carbohydrates defined as complex sugars that can be soluble or insoluble in water, and they are generally tasteless. Polysaccharides are known as glycans which have a basic compositional nomenclature formula set as $(CH_2O)_n$, where "n" varies between 3 and 6 for alternating monosaccharides; or $C_x(H_2O)_n$ for dietary polysaccharides such as cellulose or β-glucans that possess only one kind of monosaccharide, and where "x" is generally a large number between 200-500 (Song, et al., 2012; Venugopal, 2011). Polysaccharides can be classified as homopolysaccharides (homoglycans) if they are composed of only one kind of monosaccharide, and if, however, they possess more than one kind of monosaccharide, they will be known as heteropolysaccharides (heteroglycans) (Song, et al., 2012). There are two types of polysaccharides that are currently attracting wide interest for food and pharmaceutical applications, which are the homopolymers known as glucans and fructans.

1.1.4.1 Glucans

Glucans are long-chain glucose polymers present in α or β form. The α form encompasses digestible starchy glycans such as amylose and amylopectin found in the starch granules of plants. In these glycans, the glucose molecules are linked with α(1,4)-glycosidic bonds, and are hydrolyzed by digestive enzymes in humans. Amylose is a linear chain of repeating glucose units with glycosidic bonds, and amylopectin is a highly branched structure with α(1,6) bonds every 15-30 glucose units (Song, et al., 2012; Brown, 2011). Other examples of α-glucans are microbial dextran and pullulan. Dextran possesses α(1,6) glucosyl units with α(1,2), α(1,3) and α(1,4) branching, and is typically produced by *Leuconostoc mesenteroides* (Bhavani & Nisha, 2010). Pullulan, commonly produced by *Aureobasidium pullulans,* consists of repeating maltotriose units with α(1,4) linked glucose, with each maltotriose unit linked with α(1,6) glycosidic bonds

(Oguzhan & Yangilar, 2013).

Cellulose and curdlan are examples of dietary fibers in β-glucan formation. These polysaccharides are non-digestible due to their β-linkage nature and are hence, considered to be functional fibers. Cellulose is composed of β(1,4) glucosyl units in a linear chain of several hundred to over nine thousand β(1,4) D-glucose units. It is known as the 1,4-β-D anomer of starch and does not contain branching structures (Walter, 1998; Brown, 2011). Curdlan, a linear chain of β(1,3) glucosyl units, is produced by non-pathogenic bacteria including *Agrobacterium biobar* and used as a water-holding agent in meat products such as sausages, hams and hamburgers (Miwa, et al., 1994).

1.1.4.2 Fructans

Fructose based polysaccharides known as fructans are naturally occurring storage carbohydrates in a variety of vegetable products of the *Lilialiaceae* and *Compositae* families (Schaafsma & Slavin, 2015). Currently, the two fructans of interest to the food and pharmaceutical industries are inulin and levan.

Inulin compounds consist of β(2,1)-fructofuranosyl units with a terminal D-glucose unit linked to the main chain by α(1,2) bonds. In fact, inulin represents a family of fructose-rich carbohydrates with degrees of polymerization (DP) of 3-10 which are referred to as oligofructoses and those with DP of 10-65 are known as inulin (Kolida & Gibson, 2007). Inulin is primarily found in roots and tubers such as chicory from which it is commercially extracted, and the Jerusalem artichoke (Brown, 2011). Inulin and fructooligosaccharides (FOS) have proved effective in conferring health benefits when added to the diet. Animal studies have demonstrated their effectiveness in reducing (i) tumors in the large intestine of rats and (ii) breast cancer in mice; and in preventing the development of metastases in rats' lungs when 5-15% inulin or FOS is added to their diet. This effect was even greater with pre- and probiotic combinations (de Vrese & Schrezenmeir, 2008).

Levan is another dietary fiber consisting of a linear fructose polymer composed solely of β-(2,6) linked fructose units. Many bacteria possess enzymes (e.g. levanases and levansucrases) which are able to produce levan, including *Acetobacter pasteurianus, Aerobacter levanicum*, *Bacillus amyloliquefaciens, Bacillus polymyxa, Bacillus subtilis, Leuconostoc mesenteroides,*

Microbacterium laevaniformans and *Streptococcus salivarius* (Han, 1990). This polysaccharide is of great interest to the food and pharmaceutical industries. In addition to its actual use in the production of gums, sweeteners, emulsifiers, stabilizers and encapsulating agents, it also has been reported to possess health benefits owing to its anti-tumor, blood plasma volume extending and cholesterol lowering activities (Tian, et al., 2011; Han, 1990).

1.1.5 Properties of Polysaccharides

Polysaccharides possess techno-functional properties and health benefits that make them suitable candidates for industrial applications. Particularly for food applications, polysaccharides can act as texture modifiers, stabilizing agents, biofilm-formers, gel-formers, emulsifiers, and they are also used as water-binders to improve water retention (Shih, et al., 2005). Polysaccharides that are capable of modifying texture through viscosity production are generally gums (e.g. xanthan gum) secreted by the bacterium *Xanthamonas campestris*. As for medical and pharmaceutical applications, polysaccharides such as levan have been used as blood plasma extender, hypocholesterolemic agents, modifiers of tumor cells and tablet binders (Shih, et al., 2005; Ghaly, et al., 2007). Moreover, polysaccharides can be used as fillers and bulking agents, thickening agents, encapsulating agents, and carriers for flavors and fragrances (Ghaly, et al., 2007; Ben Ammar, 2002).

1.2 Methods of Polysaccharide Production

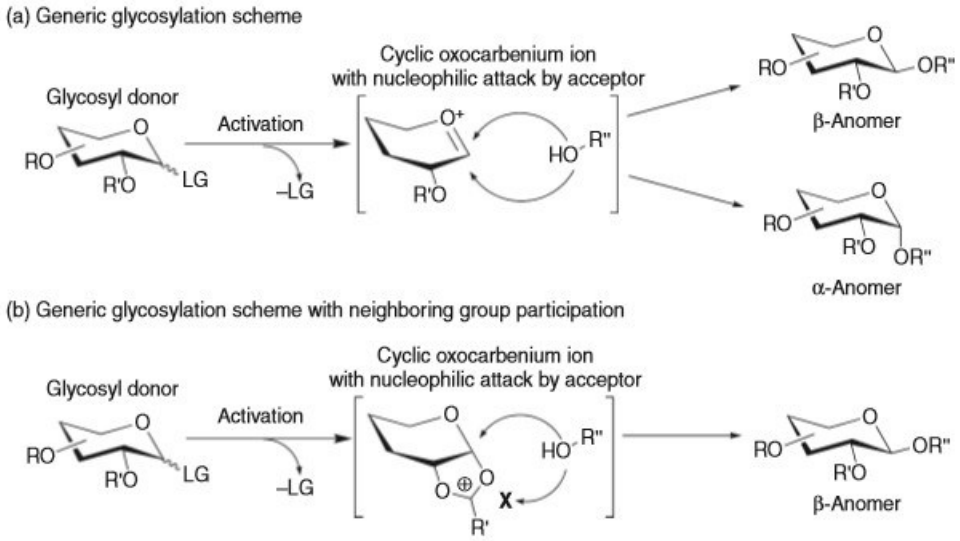
Currently, polysaccharide production is of wide interest due to their multi-functionality, health benefits and industrial applications, and hence there is always a need for novel polysaccharides adapted for specific applications. Therefore, it is important to develop methods for the production of polysaccharides, which are safe and can be produced at high yields either naturally or by chemical synthesis.

1.2.1 Chemical Synthesis of Polysaccharides

The chemical synthesis of polysaccharides proceeds according to the reaction below, providing that the water formed is removed (Micheel, 1961):

$$n\mathrm{C_6H_{12}O_6} - (n-1)\mathrm{H_2O} \leftrightarrow (\mathrm{C_6H_{10}O_5})_n \quad (n \geq 1) \tag{1}$$

This chemical reaction generates a variety of polysaccharides, many of which can be novel. At present, it is considered to be one of the most important sources of pure and structurally defined polymers used for biomedical research (Song, et al., 2012). However, the generation of polysaccharides will often require tedious experimentation to control the stereochemistry of the glycosidic bonds. In order to differentiate the hydroxyl group conformation and to control the branching of the linkages, several protection and de-protection steps are required (Song, et al., 2012). While this method of production allows to obtain polysaccharide of high purity and at high yields, it does not guarantee an absolute stereo-chemical control over the glycosylation process, and certain unfavorable coupling reactions can lead to significant losses in yields and result in side products or anomeric mixtures, which must be carefully purified before the synthesis can proceed (**Scheme 1.1**) (Song, et al., 2012). For example, the yields obtained from an experiment conducted by Okada *et al*., (1983) demonstrated that the polymerization of 3(a),4(a)-bis((benzyloxy)bicycle(3.2.1)octane, conducted with varying concentrations of different initiators resulted in yields that varied from 0-94%. In particular, at varying temperatures of 0-78°C, using the same concentration of an initiator antimony pentachloride, resulted in yields ranging between 26-94%. To attain these yields, the 3(a),4(a)-bis((benzyloxy)bicycle(3.2.1)octane had to be treated with dichloromethane as a stabilizer (Okada, et al., 1983). This demonstrates that beyond the tedious experimentation required for polysaccharide generation, chemical synthesis will often require the use of highly hazardous and expensive chemicals such as antimony pentachloride which is considered to be the most toxic of all antimony chlorides (CDC, 1994). Thus, if experimentations are not carefully executed, health risks are of a great concern; as well as the effects on yields obtained after purification, which will likely be far too low to hold significance for industrial feasibility.



Scheme 1.1. Chemical glycosylation reaction leading to varying anomer formation

The chemical glycosylation reaction often proceeds in a cyclic oxocarbenium ion that can produce either the α or β anomeric products. Stereochemical control of the glycosylation reaction as illustrated (a) without neighboring group participation (R' =benzyl, etc) and (b) with neighboring group participation (R' = acetyl, benzoyl, etc) LG, leaving group. From Song et al., (2012).

1.2.2 Enzymatic Synthesis of Polysaccharides

Enzymatic synthesis of polysaccharides is strongly recognized as a desirable and efficient method for the production of complex carbohydrate structures. In contrast to the chemical method, the enzymatic synthesis can utilize the specificity of natural glycosyl hydrolases/glycosidases (EC 3.2) and glycosyltransferases (EC 2.4) to regulate the cleavage of glycosidic linkages with highly specific regio- and stereo-selectivity (Song, et al., 2012). Glycosidases are used to specifically break and form glycosides through reverse hydrolysis or transglycosylation. This enzyme is incapable of naturally catalyzing the transglycosylation reaction but by modulating its environment, glycosidases can be adapted to synthesize polysaccharides while glycosyl-transferases can naturally catalyze transglycosylation reactions (Crout & Vic, 1998). Glycosidases are inexpensive to use and have high enzyme availability in nature; however the polysaccharides are produced at low yields, as glycosidases produce polysaccharides naturally only when the reaction is thermodynamically favored. Yields with this enzyme can also decrease as a result of substrate inhibition. Glycosyl-transferases, on the other hand, provide higher yields and have greater regio- and stereo-selectivity, but the enzyme is less available in nature and requires expensive chromogenic substrates as activated donors (Crout & Vic, 1998).

1.2.3 Naturally Synthesized Polysaccharides

Natural polysaccharides are synthesized by plant, animal or microbial sources and typically possess bioactive properties. As such, they have long been used for food, feed, medicine and pharmaceutical applications. Natural polysaccharides are produced to reinforce cellular membranes as protective means against pathogenic invaders and stress, as immunostimulators or as storage for consumption under adverse environmental conditions. Naturally occurring polysaccharides can have significant compositional heterogeneity, and can be readily isolated and extracted without the use of harsh chemicals.

1.2.3.1 Plant Based Polysaccharides

Polysaccharides of plant origin are typically located in the plant cell walls, and are of nutritional interest as dietary fibers due to their β linkages (Song, et al., 2012). Nevertheless, extracting and studying these polysaccharides uses isolation techniques that can result in limited yields and significant compositional heterogeneity with persistent impurities, such as fatty acids, peptides and/or proteins, which can exist in both electrically neutral and charged forms

(Albersheim, et al., 2011). The most difficult aspect in extracting polysaccharides from plant materials lies in the fact that they are all produced in the primary (20-30% cellulose, 25-30% hemicellulose, 30-35% pectin, 25-30% xyloglucans), and secondary cell walls (37-57% cellulose, 17-30% lignin, <10% pectin, 20-37% hemicellulose). This particularly complicates the separation of the different types of polysaccharides formed in the cells (Albersheim, et al., 2011).

The variability of the types of glycopolymers produced by plants are also limited, since cellulose, amylose, chitin, hemicellulosic polysaccharides (xyloglucans and arabinoxylans), pectic polysaccharides (rhamnogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II) and certain glycosylaminoglycans are the most naturally occurring glycans. These polymers are found as bioconjugates that must be further cleaved from lipids or peptides by enzymatic or chemical means (Song, et al., 2012).

Natural polysaccharides can be extracted for plant materials by enzymatic or chemical methods. With enzymatic extraction, the whole process is substantially less expensive and when these polysaccharides are incorporated into foods, they can be labelled as “natural” additives. On the other hand, the process of chemical extraction is faster at yielding polysaccharides. However, in both cases multi-step separation techniques such as chromatographic separation and mass spectrometric analysis are required for both isolation and purification and the resultant yields are significantly low (Song, et al., 2012).

1.2.3.2 Microbiologically Synthesized Polysaccharides

Many microbial strains are known to produce important amounts of biopolymers, including polysaccharides of different chemical properties by utilizing simple or complex substrates. In microorganisms, these biopolymers can be either intracellular or extracellular, with the intracellular being limited in use and diversity. Conversely, extracellular biopolymers have a vast structural diversity and are grouped in four distinct classes: (i) polysaccharides, (ii) inorganic polyanhydrides, (iii) polyesters and (iv) polyamides, which have been collectively designated as extracellular polymeric substances, microcapsular polysaccharides and slime polysaccharides (Nwodo, et al., 2012).

The use of bacterial cultures to produce polysaccharides appears to be the most cost effective approach, as the bacteria can be kept under continuous growth conditions and produce

polysaccharides at high yields. Cultivating bacteria under fermentation conditions and using appropriate carbon and nitrogen sources yield various polysaccharides at the necessary quantities for further treatments, such as purification, testing, etc. Among all the methods used for polysaccharide production discussed above, microbial polysaccharides have the advantage of being produced at the highest yields while still being labelled as natural additives when incorporated into foods (**Figure 1.2-1.3**). The type of polysaccharides produced vary depending on the bacterial species and strains utlized; however, while many bacterial species have been thoroughly studied for polysaccharide production characteristics in the perspective of their application in the food and pharmaceutical industries; further research is still needed in this area to take the best practical advantage of microbial polysaccharides.

1.3 Types of Microbial Polysaccharides

1.3.1 Endopolysaccharides

Endopolysaccharides are the lipopolysaccharides (LPS) and capsular polysaccharides (CPS) that form part of the membrane of some bacteria. Lipopolysaccharides (LPS) are present only on the surface of gram-negative bacteria and consist of three parts: (i) the *O*-polysaccharide (*O*-antigen), (ii) a core oligosaccharide (short chain of sugar residues within gram-negative LPS), and (iii) a lipid A anchor (lipid component of an endotoxin held responsible for toxicity in gram-negative bacteria) (Song, et al., 2012). The *O*-antigen is composed of repeating units of five to eight monosaccharides and their characteristics vary depending on the bacterial species. LPS are important for the sustainability of all gram-negative bacteria as they are responsible for the structural integrity of the bacterial cell and protect the outer membrane from external attacks. LPS also act as immunostimulators as they possess pattern recognizing receptors in the innate immune system, which has driven their wide use in the development of vaccines against various pathogens (Song, et al., 2012; Oner, 2013).

Capsular polysaccharides surround the microbial cell structures forming a cohesive layer referred to as glycocalyx which are formed by polysaccharides and glycoproteins that are covalently linked to the cell surface (Polak-Berecka, et al., 2013). These polysaccharides are present in both gram-negative and gram-positive bacteria, and have the ability to elicit immune responses and, as such, have also been used in the development of vaccines to prevent microbial infections (Song, et al., 2012). While there is no reports on the prebiotic activity of CPS, they have

been shown to increase the longevity of probiotic bacteria such as *Lactobacillus rhamnosus* E/N (Polak- Berecka, et al., 2013). Like LPS, CPS are typically regarded as important virulence factors for pathogenic organisms as they allow the bacterium to survive under adverse environmental conditions. However, their presence in probiotic microorganisms is considered ideal, as they increase their survivability in the stomach and aid in their attachment to the GIT walls (Patel, et al., 2010).

1.3.2 Exopolysaccharides

Exopolysaccharides (EPS) are a protective exocellular layer of polysaccharides that prolongs the survival of probiotic microorganisms in their host's GIT; through cellular adhesion, they create an environment that captures nutrients and maintains adequate pH and water activity (Patel, et al., 2010). These polysaccharides can be either loosely attached to the cell membrane or are freely excreted from the cells, and thus they do not normally contribute to the cellular composition of the microorganism, which implies that the other structural components of the cell are not altered regardless of the presence or absence of EPS (Sutherland, 1990).

Exopolysaccharides are extremely sensitive to the growth conditions which can drastically alter their composition, physical properties and organization on the bacterial surface. Microbial EPS are extremely diverse in nature since they are primarily composed of carbohydrates and other additional constituents that may be of organic or inorganic nature. Monosaccharides, such as D-glucose, D-mannose and D-galactose in pyranose forms, are frequently found in EPS, in addition to 6-deoxyhexoses, L-fucose and L-rhamnose. The difference between eukaryotic and prokaryotic EPS lies in the presence of pentoses such as D-ribose or D-xylose in the former, while they are unlikely to occur in the EPS derived from prokaryotic sources (Sutherland, 1990). Additionally, some polysaccharides have been reported to contain one or more uncommon sugars such as L-hexoses or furanose forms of hexose, glucose and galactose (Sutherland, 1990).

While the EPS are primarily composed of carbohydrates, other non-carbohydrate constituents are found in the EPS produced by various bacterial strains. Phosphates are one of these non-carbohydrate constituents, which are widespread and frequently found in bacterial polysaccharides of immunological significance. For example, LAB that produce bioactive molecules, such as peptidoglycans and lipoteichoic acids, are released as pro-interferon cytokines to reduce food-borne allergens (Sutherland, 1990; Jadhav, et al., 2015). Phosphorylated EPS

resemble the teichoic acids found in the cell wall of gram-positive bacteria, although many bacteria produce extracellular polysaccharide materials. It is worth mentioning, however, that many gram-negative bacterial polysaccharides that have been studied thus far are devoid of phosphates. Such studied bacteria include *Klebsiella, Rhizobium, Xanthomonas* and *Pseudomonas*, but phosphorylated-EPS have been discovered for *E. coli* strains. Acetate and pyruvate are other frequently found organic acid substituents of EPS-producing bacteria, while phosphates remain the most common inorganic acid substituent. Glycerate, hydroxylbutanoate, propionate, succinate, L- glutamate, serine and sulphate are also among the substituents found in the EPS of various strains of microorganisms (Sutherland, 1990).

1.4 Exopolysaccharide Production by Bacteria

Bacteria produce EPS to maintain their cellular integrity and to compete with other pathogenic microorganisms living in the same environment; however, EPS possibility meditate the bacterial reaction to starvation under limited nutrient conditions (Holden, 2011). Although it is widely accepted that bacteria cannot use EPS as a direct nutrient source, certain bacterial species such as *Pseudomonas aeruginosa* have been reported to produce lysases that specifically hydrolyze EPS releasing fermentable carbohydrates (Sutherland, 1990; Boyd & Chakrabarty, 1994). The presence of these enzymes suggest that EPS are used as a nutrient sources under starvation circumstances; however, no such evidence has yet been provided. In addition, EPS have also been reported to contribute to biofilm formation, which aids in the maintenance of the bacterial community (Jefferson, 2004). Thus it is possible that bacteria do not hydrolyze their own EPS to cope with starvation in low-nutrient environments, but rather to maintain the bacterial community (Holden, 2011). This potential would support the synbiotic properties of a bacterial species, which would produce EPS as prebiotics to sustain the growth of other communal GIT probiotic bacteria rather than a source for its own nutritional requirements.

In this regard, current studies have suggested that LAB and bifidobacteria, in addition to some *Bacillus* spp., are the most suitable candidates to be included in probiotic supplement pills (Patel, et al., 2010). Due to their ubiquitous nature, LAB and *Bacillus* spp. are attracting increased interest, and have an undeniable potential for exopolysaccharide production.

1.4.1 Lactic Acid Bacteria

These bacteria form a heterogeneous group of non-spore-forming, facultative anaerobic or aerotolerant, catalase negative, gram-positive bacteria; they are usually non-motile cocci- or rod-shaped and produce lactic acid as their main carbohydrate fermentation end product. According to current taxonomic classifications, these microorganisms belong to the *Bacilli* class of the *Lactobacillales* order. Most LAB benefit from the GRAS status due to their safe use in food fermentation for centuries, and the most commonly used among them as probiotics are: *Lactobacillus* spp., *Streptococcus thermophilus* (the only streptococcal species currently considered to have probiotic properties), *Bifidobacterium* spp. and *Leuconostoc* spp.

Over the years, LAB have been extensively studied for their EPS production capabilities, and most studies reported yields typically ranging between 0.1 to 2.0 g/L of culture (**Figure 1.2**). The production of EPS is considered to be significant when a lactic acid bacterium produces more than 0.8 g/L of culture. In one particular article, however, EPS yields as high as 28.85 g/L were obtained after 24 hrs of fermentation by *Lactobacillus fermentum* CFR 2195 (Yadav, et al., 2011), the highest yields ever reportedly obtained under such a short period of time. Higher yields of dextran (54-55 g/L) were reported to be produced by *Leuconostoc mesenteroides* under optimal growth conditions in molasses-containing medium after nine days of fermentation (Vedyashkina, *et al.,* 2005).

1.4.2 *Bacillus* spp.

The genus *Bacillus* comprises gram-positive, rod-shaped, obligate aerobic bacteria that are equally as ubiquitous in nature as LAB. Except for a few species (e.g., *Bacillus anthracis* and *Bacillus cereus* sensu stricto), most *Bacillus* species are non-pathogenic, non-parasitic and have not been associated with human or animal diseases. *Bacillus* spp. have proven to be of particular interest to medical, agricultural, pharmaceutical and nutritional applications. Species of this genus are notably known for their capability of producing antibiotics such as bacitracin produced by *B. licheniformis* and polymyxin produced by *B. polymyxa* (Schallmey, et al., 2004). *Bacillus* species are also known for their ability to produce different types of enzymes with various industrial applications, as selected strains of this genus can profile significant yields (20-25 g/L) of extracellular enzymes (Schallmey, et al., 2004).

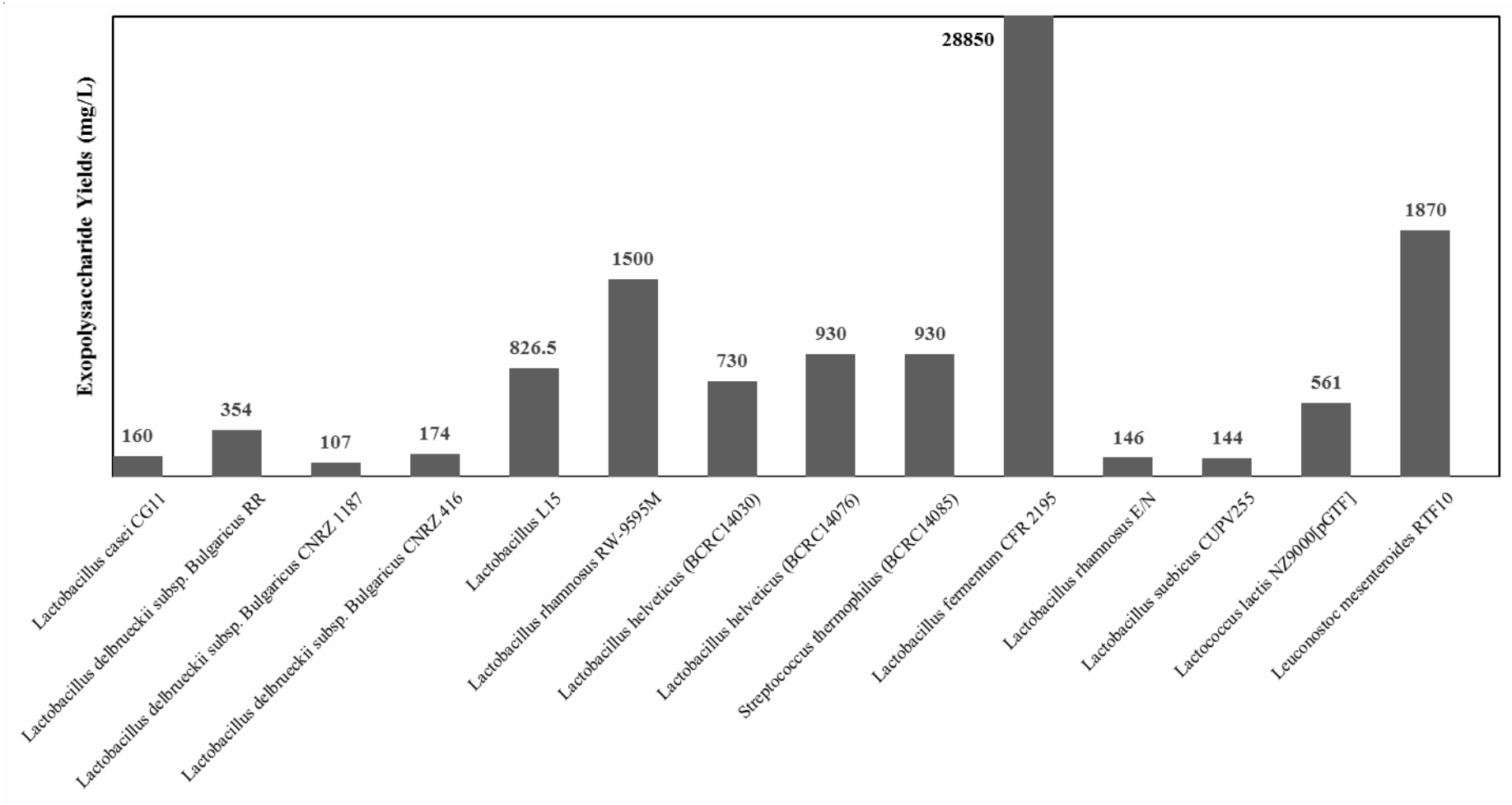


Figure 1.2. Exopolysaccharide yields (mg/L) produced by different strains of lactic acid bacteria

Data compiled from: Cerning *et al.*, (1994); Kimmel *et al.,* (1998); Petry *et al.*, (2000); Chen *et al.*, (2006); Cinquin *et al.*, (2006); Lin & Chien, (2007); Yadav *et al.*, (2011); Polak Berecka *et al.*, (2013); Notararigo *et al.*, (2013)

From the regulatory standpoint, for probiotic bacteria to be used in foodstuff, they first must obtain the GRAS status. Certain LAB already had this status and are used in probiotic supplement pills. These include *Lactobacillus johnsonii* La1/Lj1/NCC 533 and *Lactobacillus rhamnosus* GG (Health Canada, 2015); as well as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (FDA, 2015). While no *Bacillus* strain has yet been granted this status by the Food and Drug Administration (FDA) of the USA or Health Canada as probiotics, the FDA has approved the incorporation of by-products of non-pathogenic *B. subtilis* and *B. licheniformis*. In addition, *Bacillus coagulans* have been added by the European Food Safety Authority (EFSA) to their Qualified Presumption of Safety (QPS) (Hong, et al., 2005; Sanders, et al., 2003). Most of the recent studies on EPS production by *Bacillus* species have used cocktails of multiple strains isolated from natural sources such as soil and food wastes (Patel, et al., 2009). On the contrary, individual strains of *Bacillus* spp. with substantial capabilities of producing EPS have been overlooked so far with regards to yields and monomeric composition of the polysaccharides they produce. As compared to LAB, most of the reported EPS yields by *Bacillus* spp. were equal or greater than those recorded for the highest EPS-producing LAB. **Figure 1.3** shows the few reported EPS yields obtained from *Bacillus* species.

1.5 Microbial Production Process of EPS

Biosynthesis of EPS occurs both intra and extracellularly. Production of intracellular EPS requires a glycotransferase enzyme (EC 2.4) which can link sugars of nucleotides to a lipid carrier molecule within the cytosol. Availability of nucleotide sugars greatly affects the biosynthesis of certain EPS such as alginate and gellan which are formed through the inter-conversion of glucose-6-phosphate (glycolysis intermediate) into glucose-1-phosphate (sugar nucleotide precursor) catalyzed by phosphoglucomutase (PGM) (EC 5.4.2.2); a key step in sugar nucleotide biosynthesis (Patel, et al., 2010). In contrast, production of EPS such as levan and mutan are catalyzed extracellularly by levansucrase (EC 2.4.1.10) and mutansucrase (EC 2.4.1.5) respectively, from sucrose. This mechanism requires enzymatic hydrolysis of sucrose to release glucose and transfer the D-glucose moiety of sucrose to acceptor monosaccharides and oligosaccharides.

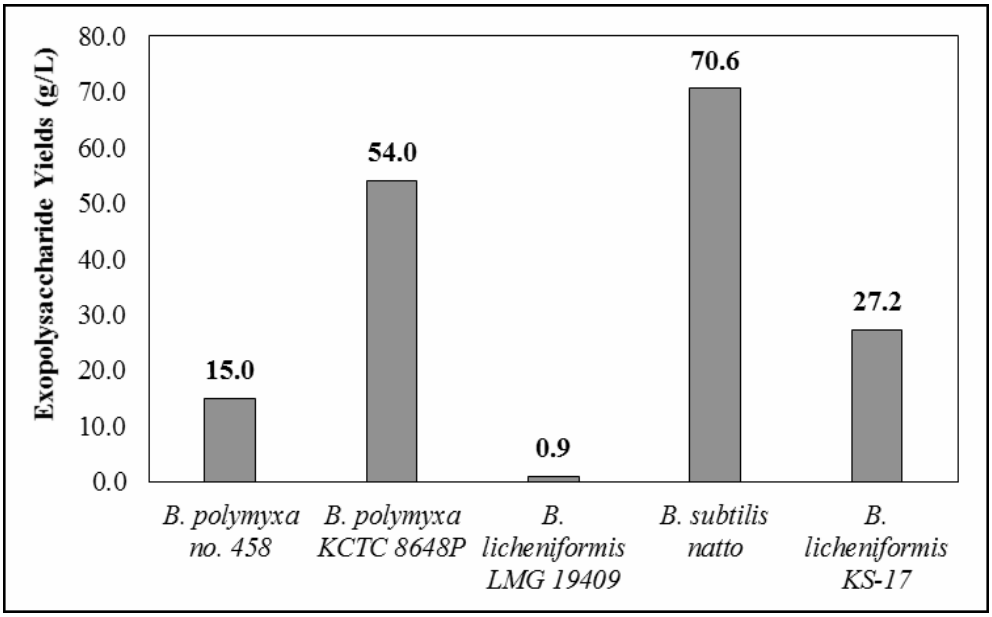


Figure 1.3. Exopolysaccharide yields (g/L) produced by *Bacillus* species

Data compiled from: Mitsuda *et al*., (1981); Lee *et al*., (1997); Larpin *et al*., (2002); Shih *et al.,* (2010); Song *et al*., (2013)

The quantity of EPS produced depends on the bacterial species and its growth conditions (Patel, et al., 2010). For example, LAB that produce EPS in milk-based media tend to produce EPS composed of linear and branched repeating units varying in size from tetra- to hepta-saccharides with molecular masses ranging between $1x10^6$ and $2x10^6$ Da. However, the resultant yields are quite low and vary between 50-425 mg/L (Petry, et al., 2000).

1.6 Isolation & Structural Characterization of EPS

Isolation of EPS is achieved by removing the bacterial cells from the fermentation media by centrifugation or ultrafiltration techniques, followed by ethanol precipitation of the cell-free supernatant at 4°C for 24 hrs. The resulting precipitate is primarily composed of EPS and some denatured proteins. The methods used to isolate EPS should not affect the chemical and physical properties of the extracted polysaccharides. However, it is well known that such methods may add contaminating polysaccharides originating from the culture media (e.g. mannans from yeast extract), or from the bacterial cell wall as a result of untimely cell lysis. According to Patel *et al*., (2010), ultra-filtration is the most reliable method to avoid EPS contamination as it removes low molecular weight polysaccharides of the culture media, and as such, it partially purifies the EPS. Another method of interest is gel permeation chromatography (GPC) that requires high pressure liquid chromatography (HPLC) and a refractive index (RI) detector to separate polysaccharides based on their molecular weight distribution. This method can also be utilized to determine EPS concentrations of less than 20 mg/L in cell-free supernatant cultures, which when measured against a standard curve of known polysaccharides of varying molecular weights, could help determine the extent of external contaminations (e.g., monosaccharides, disaccharides and proteins) present in a given samples. This method also provides an effective evaluation of EPS contamination (Ruas-Madiedo & Reyes-Gavilan, 2005; Patel, et al., 2010).

After the isolation step, EPS can be characterized by determining the molecular weight, monosaccharide composition and linkage patterns, among other structural properties. This step is necessary to distinguish EPS of microbial origin from that of non-microbial sources, as these EPS types differ markedly in their molecular characteristics and, as a consequence, they have different effects when incorporated into foods. The monomers that constitute the EPS are linked with ‘stiff’ linkages with varying degrees of flexibility. Generally, the flexibilities of different linkages are reported as follows: β(1,4) are stiffer than β(1,3) and β(1,2). In addition, α linkages

are more flexible than β linkages, and thus EPS with alternating linkages of α(1,2) and α(1,3) tend to be more flexible (Patel, et al., 2010).

Further characterization of EPS, proceeds by the determination of their molecular mass using different techniques depending on the polysaccharide retention time. Size exclusion chromatography coupled with refractometric detectors can be used for both characterization and separation of EPS on the basis of their molecular weights. Chromatography techniques (see § 1.9.3) are used to determine the monomer composition of EPS after total acid hydrolysis or by methanolysis and pertrimethylsilation (Patel, et al., 2010; Torino, et al., 2015).

1.7 Optimization of EPS Production by Bacteria

Among LAB, *Lactobacillus* spp*., Leuconostoc* spp., *Lactococcus* spp*., and S. thermophilus* produce various homopolysaccharides and heteropolysaccharides with different molecular masses, types of monomer linkages, solubility and degrees of branching. Most of these LAB produce polysaccharides extracellularly from sucrose by utilizing glycansucrases or intracellularly using glycosyltransferases from sugar nucleotide precursors. *Lactobacillus* spp*., Leuconostoc* spp*.,* and *Streptococcus* spp*.* synthesize different α-glucans depending on the position of the glycosidic bounds. For example, *Leuconostoc* spp. produce dextran with α(1,6) linkages, *Streptococcus* spp. produces α(1,3) mutan, *Leuconostoc mesenteroides* produces alternan with alternating α(1,3) and α(1,6) linkages and α(1,2) glycosidic bonds. Based on glucansucrase specificity, elongated oligosaccharides can be formed with varying glycosidic linkages at their non-reducing ends (Patel, et al., 2010). *Leuconostoc* bacteria produce more biomass in the MRS medium than in the EPS medium, however the EPS quantity is higher when produced in the EPS medium (where it is 22.5 g/L vs. 14 g/L in MRS broth) (Patel, et al., 2010).

A wide variety of bacterial species of LAB, propionibacteria, streptococci and bacilli have been used as biological material to produce EPS. To this end, specific adjustments of the fermentation conditions, depending on the bacterium used, are crucial for maximum EPS production. Key environmental conditions (e.g., temperature, pH) and composition of culture media (e.g., carbon and nitrogen sources) are strain-dependent. This is particularly challenging in view of the wide diversity of the strains and type of EPS they produce, each having specific requirements to produce a given type of EPS. Optimal production of EPS has been reported to

parallel optimal growth temperatures of the producing strain (Petry, et al., 2000; Vijayendra, et al., 2008). For mesophilic bacteria such as *Lactobacillus rhamnosus, Propionibacterium* spp. *Leuconostoc* spp. and *Lactococcus lactis*, temperatures in the range of 20-25°C were shown to be the most favorable to EPS production (Fauquant, et al., 1988; Cerning, et al., 1992; Gamar, et al., 1997; Vijayendra, et al., 2008). Similarly, temperatures between 40-45°C were reported to allow maximum production of EPS by thermophilic LAB such as *S. thermophilus* and *L. bulgaricus* (Vaningelgem, et al., 2004).

In addition to temperature, the pH and composition of the culture medium are essential factors that affect the nature and amount of EPS production. Although optimum pH values for EPS production vary from species to another and among strains of the same species, they generally range between 6.0 and 8.0 for LAB, but may be lower (e.g. 5.0) for some bacteria (**Table 1.1**). However, control of pH throughout the fermentation process appears to be necessary to maintain it at optimal values. In the case of *S. thermophilus*, for example, EPS production under pH-controlled conditions was increased by at least fivefold as compared to fermentation under uncontrolled pH conditions (Vaningelgem, et al., 2004). The same authors demonstrated that some nutrients of the cultivation media such as whey protein hydrolysate and ammonium enhance EPS production. Additional specific requirements should be taken into account when designing a fermentation protocol; for example, probiotic bacilli require the availability of oxygen and a high oxidoreduction potential to produce EPS due to their strict aerobic nature (Lee, et al., 1997). The impact of specific ingredients of the culture media, among other fermentation parameters, in relation to the yield and type of EPS are discussed below (Gamar, et al., 1997).

1.8 Effects of Growth Parameters on the Yield and the MW Distributions of PS

1.8.1 Effects of Carbon/Nitrogen Sources

Carbon and nitrogen sources are of major importance to the production of EPS during the fermentation of bacterial strains. Although they are both required for EPS production, the ratio of carbon to nitrogen should be adjusted for each species or strain to achieve maximum yields. Complex media with high carbon to nitrogen ratio were shown to have a favorable effect on the production of EPS, indicating the necessity to use nutrient-rich media's containing peptones, yeast extract and beef extract as nitrogen sources for optimal growth and EPS production, beside a suitable carbohydrate as the carbon source. Despite the extensively demonstrated influence of

carbohydrate sources on the yields of the EPS, these ingredients of culture media do not influence the chemical structure of EPS (Patel, et al., 2010). **Table 1.1** summarizes optimal conditions for EPS production by species and strains of LAB and some bacilli under different pH and temperature conditions, with different carbohydrates and nitrogen source.

The nature and amount of the carbon source was reported to impact EPS production as well as the bacterial growth (Lee, et al., 1997). These authors demonstrated that sucrose is the most suitable substrate for EPS production by *B. polymyxa* KCTC 8648P, and that its presence in excess positively impacts the final EPS yields. As shown in **Figure 1.4**, increasing sucrose concentration in the culture media to as high as 100 g/L caused the EPS yields to exceed 40 g/L. In fact, it has been demonstrated that for LAB, the sugars of preference are in the order of sucrose > lactose > glucose (Pawar, et al., 2013). Most *Bacillus* spp are known to prefer sucrose, as was demonstrated in the study by Lee et al., (1997) who observed that the carbon source preference for *B. polymyxa* KCTC 8648P was sucrose > glucose > soluble starches > lactose. Findings of this study strongly suggest that EPS production is closely related to the carbon source provided to the producing bacterium. Polak-Berecka *et al*., (2013) showed that *L. rhamnosus* E/N produced high amount of EPS under optimal growth conditions in sucrose-containing medium; however, the bacterium started to hydrolyze the EPS produced after the depletion of sucrose from the culture medium. They, therefore, suggested studying the time course for bacterial growth in order to determine the exact stage where the EPS yield is highest.

Two forms of nitrogen sources, mineral and non-mineral, are used in culture media for EPS production, although the non-mineral nitrogen sources tend to result in lower EPS yields (Lee, et al., 1997). This phenomenon was explained by the fact that non-mineral nitrogen compounds such as ammonium sulfate and urea disassociate in distilled and deionized solutions, and form ammonium acids resulting in a pH decrease to unfavorable ranges, thereby lowering EPS yields.

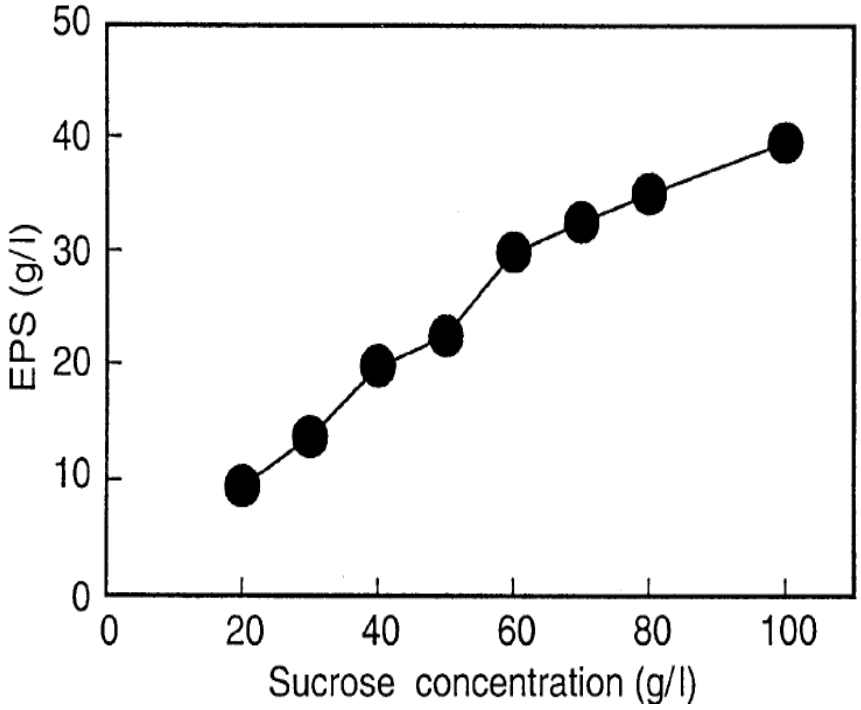


Figure 1.4 Exopolysaccharide yields (g/L) by *Bacillus polymyxa* KCTC 8648P with increasing sucrose concentration. From Lee *et al.,* (1997).

Table 1.1. Growth Conditions for Varying Bacterial Species and Exopolysaccharide (EPS) Production

Bacteria	Substrate	Conditions	EPS (g/L)	Nitrogen Source (g/L)	References
L. casei CG11	Glucose	25°C, pH 6.0	0.16	Undefined Amino acids	Cerning *et al.,* (1994)
B. polymyxa KCTC 8648P	Sucrose	30°C, pH 7.0	54.00	KNO_3	Lee *et al.*, (1997)
L. delbrueckii subsp. *bulgaricus* RR	Glucose	38°C, pH 5	0.35	Bacto-Casitone (30)	Kimmel *et al.*, (1998)
L. delbrueckii subsp. *bulgaricus* CNRZ 1187	Glucose	42°C, pH 6	0.11	Cysteine (0.4), Aspartic acid-0.3, Glutamic acid (0.3), Alanine (0.2), arginine (0.2),	Petry *et al.*, (2000)
L. delbrueckii subsp. *bulgaricus* CNRZ 416	Glucose	42°C, pH 6	0.17	glycine (0.2), histidine (0.2) isoleucine (0.2), leucine (0.2), lysine (0.2), methionine (0.2), phenylalanine (0.2), proline (0.2), serine (0.2), threonine (0.2), tryptophane (0.2), tyrosine (0.2), valine (0.2)	
B. licheniformis LMG 19409	Sucrose	30°C, pH 6.6	0.88	MRS medium amino acids	Larpin *et al.*, (2002)
Lactobacillus rhamnosus RW-9595M	Glucose	37°C, pH 6.5	1.50	amino acids in Basal Mineral Media	Cinquin *et al.*, (2006)
L. helveticus (BCRC14030)	Lactose	37°C, pH 5	0.25-0.73	Undefined	Lin & Chien., (2007)
L. helveticus (BCRC14076)	Lactose	37°C, pH 5	0.63-0.93		
S. thermophilus (BCRC14085)	Lactose	37°C, pH 5	0.73-0.93		
L. fermentum CFR 2195	Sucrose	37°C pH 6.7	28.85	N/A	Yadav *et al.*, (2011)
Weissella cibaria	Sucrose	37°C pH 6.9	14.00	Peptone water, yeast extract, Hemin	Hongpattarakere *et al.*, (2012)
Weissella confuse	Sucrose	37°C pH 6.9	7.60		
Lactobacillus plantarum	Sucrose	37°C pH 6.9	4.90		
Pediococcus pentosaceus	Sucrose	37°C pH 6.9	5.00		
B. licheniformis KS-17	Sucrose	37°C, pH 7.1	27.20	Ammonium sulfate	Song *et al.*, (2012)
L. rhamnosus E/N	Lactose	37°C, pH 6.3	0.15	MRS medium amino acids	Polak-Berecka *et al.*, (2013)
L. suebicus CUPV255	Glucose, Sucrose	28°C, pH 5.5	0.14	Undefined	Notararigo *et al.*, (2013)
L. lactis NZ9000[pGTF]	Glucose	30°C	0.56		
L. mesenteroides RTF10	Sucrose	30°C	1.87		

1.8.2 Effect of pH and Temperature on EPS Production

Extensive studies have been conducted to determine the optimal pH and temperatures for optimal EPS production by various bacterial species. For example, Kimmel *et al.*, (1998) studied *L. bulgaricus* RR in this respect and found that it was capable to produce 354 mg/L of EPS when grown at 38°C and pH 5 (Kimmel, et al., 1998). However, one of the key problems with the study, and even at a larger scope for the development of probiotic supplements, lies in the fact that each strain requires specific conditions to produce EPS optimally. With *Propionibacterium*, high EPS yields were obtained at incubation temperatures of 23°C and optimal pH of 6.0 in skimmed milk–based media supplemented with 3.0 g/L of yeast extract (Fauquant, et al., 1988). Optimal temperatures for EPS by different bacteria are presented in **Table 1.1**.

It is well established that EPS production is pH-dependent, and optimum EPS production generally occurs around the optimum growth pH of the bacteria and EPS yields demonstrate steep declines when the optimum pH is crossed. As previously mentioned, the use of non-mineral nitrogen sources in culture media may contribute to the decrease of pH. Therefore, it would be advantageous to use complex culture media containing mineral nitrogen and buffers to stabilize the pH when batch fermentation without pH control is to be used. In this regard, Grobben *et al*., (1998) showed that *L. bulgaricus* grew better and produced more EPS in complex media than in simplified media; EPS production doubled in complex media with added vitamins. pH stability is of greater concern to LAB which acidify their growth medium via lactic acid production. Therefore, the use of strong buffers at high quantities or conducting pH-controlled fermentations are necessary measures to ensure optimal growth and EPS production.

While it would seem reasonable that EPS yields would be greatest under non-optimal growth conditions, as EPS production tends to increase as a stress response, studies have shown that this assumption does not hold true. According to Chen *et al.*, (2006), EPS production by *Lactobacillus* L15 was significantly decreased when the bacterium was incubated at temperatures that differ from its optimal growth temperature; at 25°C, this bacterium produced only 10% of the EPS it produced at the optimal growth temperature of 30°C. Conversely, at 37°C, the maximum EPS yield was achieved faster than at optimal growth temperature (30°C) suggesting that 37°C induced an overexcitement of cells and overstimulation causing a shift in the time required for maximum EPS yield (**Table 1.1**) (Cerning, et al., 1992).

1.9 Structural Characterization of Polysaccharides

1.9.1 Visualization of Bacterial Polysaccharides

The external surface of bacterial species can be visualized by light microscopy after appropriate staining. Negative staining allows the distinction between capsular polysaccharides and extracellular slime. Scanning electron microscopy (SEM) can provide strong visual evidence for the presence of EPS. Transmission electron microscopy (TEM) is another modern technique that can reveal significant detail about the surface structures of bacteria, including constitutive polysaccharides. However, the latter technique is unable to distinguish between different chemotypes, and does not allow sufficient resolution to provide substantial information on the microstructure of the polysaccharides. Furthermore, the high water content of polysaccharides, usually exceeding 99%, hinders sample preparation and the achievement of high resolution, which accounts for another drawback of this technique (Sutherland, 1990). To overcome this limitation, sample preparation was improved by pre-treatment with anticapsular IgG and freeze-etching to show EPS in an un-collapsed state and allows for appropriate visualization of the bacterial capsules' long fibrous strands extending out of the bacterial cell surface (Sutherland, 1990).

1.9.2 Colorimetric Analysis of Polymeric Compounds

1.9.2.1 Phenol-Sulfuric Acid Assay

Phenol-Sulfuric Acid Assay (Phenol Assay) is a widely used colorimetric method for carbohydrate analysis, which can be applied for EPS quantitation. This method uses 400 μL sample, 200 μL of 5% phenol (w/v) and 1 mL sulfuric acid. The reaction is highly exothermic and the hot acidic medium breaks down polymeric substances into monomers such as glucose, which is dehydrated to 5-hydroxymethylfurfural that forms a yellow-like color when reacting with phenol that has maximum absorbance at 480 nm. The solution is then allowed to cool for 10 to 20 minutes before measuring the absorbance with a spectrophotometer at 480 nm in glass cuvettes (Dubois, et al., 1956). Carbohydrate concentrations are then determined by using a standard curve obtained with 10 mM glucose solution.

1.9.2.2 3,5-Dinitrosalicylic Acid Assay

This assay utilizes 3,5-dinitrosalicylic acid, an aromatic compound that reacts with reducing carbohydrates and other reducing molecules to form 3-amino-5-nitrosalicylic acid which produces

a yellow-like color whose darkness increases with the concentration of the reducing ended carbohydrates. The assay requires 250 μL of samples 375 μL of DNS to boil for 5 minutes before stabilizing the reaction with 125 μL potassium sodium tartrate (PST). The absorbance of the solutions is then measured at 540 nm in a spectrophotometer (Miller, 1959). A standard curve obtained with 100 mM glucose solution is used to determine the concentrations.

1.9.3 Chromatographic Techniques

1.9.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is used to separate non-volatile mixtures. For solutions of unknown carbohydrate, this technique aids in the separation and detection of monosaccharides, disaccharides, oligosaccharides and polysaccharides by separating them on the basis of their molecular weights. TLC requires a sheet of glass, plastic, or aluminum foil to be coated with adsorbent materials such as silica gel acting as the stationary phase. Silica gel plates are advantageous to use as they are chemically stable against almost all solvents, strong acids and other corrosive reagents that may be used (Maloney, 2003). Samples and standards are loaded onto the plate which is then placed in an adequate solvent mixture in a developing chamber saturated with the mobile phase of the solvent mixture and allowed to develop undisturbed. Different solvents are used depending on the type of carbohydrates analyzed. In general, solvent systems contain three components combined in various proportions so as to give a homogeneous solution: water, a water-soluble component and a water-insoluble component. However, simple solutions of organic solvents are extensively used, among which acetonitrile-water solution (85:15, v/v) is the simplest and most widely used, especially in the separation of mono-, di- and tri-saccharides. More complex mixtures of carbohydrates can be separated by using different mixtures of other organic solvents and acids. For example, a solution of butanol, acetic acid and water (5:4:1) has been used successfully to separate carbohydrate polymers (Tian, et al., 2011). After development, the plate is dried (mild heat) and treated for visual detection of the carbohydrates under UV or visible light.

Detection systems of carbohydrates use specific reagent solutions that are applied to the dry plate which is further heat-dried at temperatures exceeding 100°C for few minutes to 2 h (Tian, et al., 2011; Robyt, 2000). Different detection systems have been developed differing in their sensitivity and specificities, with N-(1-naphthyl)ethylenediamine, concentrated sulfuric acid

and methanol solution being considered as the most sensitive (Robyt, 2000). Recently, Tian *et al*., (2011), developed a spray system designed specifically for the detection of fructose-containing carbohydrate polymers. After elution and drying, the plate is successively sprayed by a resorcinol solution in acetic acid (0.1% (w/v) resorcinol and 0.25% (w/v) thiourea) and sulfuric acid solution (5%) in methanol before being heat-dried (100°C for 2 h) which allows visualization of the separated compounds under visible light.

1.9.3.2 High Performance Size Exclusion

Size exclusion chromatography (SEC) is a generic name of liquid chromatography technique that separates macromolecules on the basis of their sizes. In addition to the separation of many types of EPS, this technique provides valuable information on their molecular weight distribution (Wu, 1995). Apparent average molecular weight (MW) of polysaccharides is determined after SEC fractionation by using a standard curve previously set-up with different standards such as Dextran Blue, T70, T10, and Vitamin B12. A variant of this technique is the high pressure SEC (HPSEC) which uses rigid or semi rigid supports allowing rapid separations, typically lasting for less than 1 h. It also presents another major advantage over the SEC, in that it is equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors to determine the molecular weight and z-average radius of gyration (R_x) of the EPS without need for standards (Werning, et al., 2012).

1.9.3.3 High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

This is a highly sensitive and selective technique commonly used to determine carbohydrates, including mono-, di-, tri-, oligo- and polysaccharides, sugar alcohols, and amino sugars. Unlike other chromatography techniques, no analyte derivatization is required owing to its pulsed amperometric detection (PAD). This technique is especially useful to determine the monosaccharide profile of EPS after acid hydrolysis to release their constitutive monomers prior to injection.

Apart from the convenience that no sample derivatization is required for separation or detection, this technique has other advantages including: (i) high sensitivity, (ii) high resolution separation, (iii) high capacity: can detect a minute amount of one carbohydrate in the presence of

a greater concentration of another, and (iv) the ability to determine a wide range of carbohydrates in one analysis (Corradini, et al., 2012).

1.9.3.4 Mass Spectroscopy

The basic principle of mass spectrometry (MS) is to generate ions from inorganic or organic compounds by any suitable method desired in order to separate and analyze the released ions qualitatively and quantitatively with respect to the mass-to-charge ratios and abundance (Gross, 2011). This technique can be used to determine the type of bonds between residues of an EPS molecule. In this technique, polysaccharides are methylated, e.g., as described by Ciucanu & Kerek, (1984) and hydrolyzed with 3M trifluoroacetic acid (TFA). The resulting products are reduced with sodium borodeuteride ($NaBD_4$), acetylated and analyzed by gas chromatography mass spectroscopy (GC-MS) (Leal, et al., 2008). Identification of the peaks obtained by the chromatogram can be determined by both their retention time and mass spectra that help quantify the monomers obtained (Ciucanu & Kerek, 1984; Werning, et al., 2012).

1.9.4 Nuclear Magnetic Resonance-Linkage

Nuclear Magnetic Resonance (NMR) is a technique that has been largely used in the analysis of EPS due to its ability to clarify and identify the structures of oligosaccharides (Prapulla, et al., 2000). To resolve the three-dimensional structure of EPS molecules, both the ring size (pyranose/furanose) of monosaccharide residues and the relative orientations of the adjacent monosaccharides have to be determined. NMR provides details on the conformation of polysaccharides and allows elucidation of the types of glycosidic linkages and the structure of the repeating units that constitute the EPS molecules (Duus, et al., 2000). Prior to NMR analysis, purified EPS is first dissolved in D_2O to allow deuteration, i.e., replacement of protons by deuterium. An H-NMR spectrum for the EPS provides information about the number of monosaccharides present in the repeating unit by counting the resonances found in the anomeric region (4.4-5.5 ppm). Resonances downfield of 1 ppm in the H-NMR spectrum indicate a methyl group that can be related to the presence of a methyl pentose (e.g., fucose or rhamnose) residue. Resonances close to 2 ppm reveal the presence of *N*-acetyl and/or *O*-acetyl functionalities. Common hexoses can also be identified in C-NMR spectra of 95-110 ppm (Werning, et al., 2012).

For maximum benefit from NMR analysis, it is recommended to first determine the monomer composition of polymers by acid hydrolysis followed by HPAEC-PAD as described above. Once the monomers are known, samples can undergo methylation, and the linkage pattern of the monomers can be determined for all free hydroxyl groups in the hydrolyzed polysaccharides using GC-MS. Partially methylated alditol acetate is separated by GC and fragmented by MS in order to give specific primary fragment ions. This analysis is completed using H and C-NMR spectroscopy.

1.10 Industrial Applications: Viscosity & Emulsifying Abilities of Microbial Polysaccharides

Microbial polysaccharides such as secreted EPS are known to play a significant role in improving appearance, stability and rheological properties of novel food products and, hence, they can be used as additives to enhance the texture of food products. However, the addition of synthetic texturizing agents in food is prohibited in various countries in the European Union and in the United States. Therefore, further studies are needed, aiming to replace additives as thickening and gelling agents by EPS-producing bacteria (Patel, et al., 2010). The thickening and gelling properties are due to the ropy nature that the EPS possess, and is known to increase viscous properties and the fatty mouthfeel sensation within the food products in which they are incorporated or produced. This application is actually widely used in dairy products fermented with EPS-producing starter cultures. Notably, ropy yogurt is increasingly available in the market, and its ropiness is the result of *in situ* production of polysaccharides the starter culture (*S. thermophilus* and *L. bulgaricus*) used for its fermentation. It is worth mentioning that the incorporation of EPS-producing bacteria in foods as an alternative to pure EPS can be a practical and feasible way to by-pass regulatory restrictions. However, while such an alternative appears to be attractive for most LAB, it is not always possible for other EPS-producing bacteria that do not benefit from the GRAS status, as is the case for many *Bacillus* spp.

According to Ruas-Madiedo & Reyes-Gavilan, (2005) not all mucoid or slime-producing strains are ropy. Characteristically, mucoid colonies possess a glistening and slimy appearance on appropriate agar media. However, some mucoid colonies are unable to produce strands when they are extended using an inoculation loop (Dierksen, et al., 1997). Typically, ropy characteristics of bacteria can be detected by using the EPS selection media (ESM) developed by Van den Berg *et al.,* (1993) to isolate EPS-producing LAB from sour-dough, sausages, table olives, and dairy products. On this medium, ropiness of strains was determined after 24 h growth at 30°C by testing

resistance of their colonies to flow through a pipette. This test revealed that only 30 out of 607 suspected colonies prove to be EPS producers, and this characteristic was confirmed in even less (11) colonies when grown on ESM containing 50 g/L of glucose instead of 10 g/L (Ludbrook, et al., 1997). Therefore, as stated above, the determination of ropiness in bacteria by using an inoculation loop to stretch the EPS strands and measuring the length in millimeters remains the simplest and most reliable technique (Ruas-Madiedo & Reyes-Gavilan, 2005).

Physiochemical properties of EPS, including viscosity depend on their molecular mass, monosaccharide composition, primary structure and interaction with milk constituents, mainly ions and proteins. Two rheological characteristics can be distinguished: viscosity and elasticity. Viscosity is the property of a material to resist deformation and, hence, in the context of fermented dairy products, this attribute can be described as slimy and fluid (Patel, et al., 2010). Elasticity, on the other hand, is the property of a material to recover its initial conformation subsequent to a deformation. These attributes correspond to a firm body and gum-like fermented milk products. Both of these rheological characteristics are important to develop desirable organoleptic qualities of products and to improve their appealing appearance and pleasant mouth-feel.

Viscosity is a significant characteristic of EPS solutions. Emulsification and viscosity are desirable features of a biopolymer for its application under extreme conditions of pH, temperature and salinity. Viscosity of EPS solutions depends on various structural properties of EPS such as the composition of the polysaccharide, the chain stiffness, branches and side groups in the polysaccharide chain. Two kinds of viscosities can be distinguished and they both can be used to study EPS: intrinsic viscosity and apparent viscosity. Intrinsic viscosity is an essential property of a polysaccharide, which helps its isolation from culture solutions. Polysaccharides that exhibit high intrinsic viscosity tend to possess high molecular mass and a rigid structure (Vaningelgem, et al., 2004).

CONNECTING STATEMENT 1

A literature review on exopolysaccharide (EPS) production by bacterial species was presented in Chapter I. It detailed media composition for the stimulation of EPS production, compared characteristics of EPS production between LAB and *Bacillus* species, and explored different techniques that can be used to determine structural characteristics of the yielded EPS. Chapter II details the methodology that was utilized in order to (1) screen selected *Bacillus* spp. for their EPS production abilities on three types of mineral media; (2) determine biomass and EPS production of the selected *Bacillus* spp. and determine each one's optimum fermentation time; (3) structurally characterize the yielded EPS by determining the molecular weight distribution of the polymers and their monosaccharide profile; and (4) investigate the effects of fermentation parameters on the EPS produced by a selected *Bacillus* strain using response surface methodology.

CHAPTER II. MATERIALS & METHODS

2.1 Chemicals and Media

Chemical reagents used for fermentations were of laboratory grade, and those used for structural analysis were of high-performance liquid chromatography (HPLC) grade. L (+) arabinose was purchased from Acros (Fairlawn, NJ). Potassium phosphate dibasic (K_2HPO_4) anhydrous, magnesium sulfate ($MgSO_4$), calcium phosphate ($CaPO_4$), manganese sulphate ($MnSO_4$) crystalline, potassium phosphate monobasic (KH_2PO_4) crystalline, agar, sodium hydroxide (NaOH), yeast extract and sodium chloride (NaCl) crystalline were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium succinate dibasic anhydrous and tryptone were purchased from Fluka (St. Louis, MO). Sucrose, 3,5-dinitrosalicylic acid, ferrous sulfate ($FeSO_4$), sodium phosphate dibasic (Na_2HPO_4), potassium sodium tartrate, D-fructose, α-D-glucose, D (+) xylose and sodium molybdate (Na_2MoO_4) from Sigma Aldrich (St. Louis, MO). Nutrient broth and potato dextrose agar (PDA) were purchased from Difco (Sparks, MD). Deionized water (Millipore) was used in all experiments.

2.2 Bacterial Strains and Growth Conditions

Four mesophilic strains of *Bacillus* genus and a thermophilic strain of *Geobacillus stearothermophilus* were used to study exopolysaccharide (EPS) production. The origin and culture conditions of these strains are summarized in **Table 2.1**. Working cultures, maintained as stocks on PDA slants at refrigerator temperature (4°C), were activated before transferring to fresh PDA slants and grown overnight (12-24 h) at their respective optimum temperatures (**Table 2.1**).

Table 2.1. Selected Investigated Strains, their Origins and Culture Conditions

Bacterial species	Strain	Origin	Culture conditions[1]	
			Medium	Temperature of incubation (°C)
Bacillus amyloliquefaciens	ATCC 23350	ATCC[2]	PDA[3] or NB[4]	35
Bacillus licheniformis	ATCC 14580	ATCC	PDA or NB	35
Bacillus amyloliquefaciens	OB6		PDA or NB	30
Bacillus subtilis	B26	Leaf blades and seeds of *Panicum virgatum* L[5]	PDA or NB	30
Geobacillus stearothermophilus (Donk)	ATCC 12980	ATCC	PDA or NB	55

[1] The incubation was carried out for 12 to 15 hrs for all strains

[2] American Type Collection Culture

[3] Potato dextrose agar was used as an agar medium for the storage of the strains

[4] NB: Nutrient broth was used as pre-culture media

[5] Refer to Gagne *et al*., (2015)

2.3 Exopolysaccharide Production on Agar Media

Each of the five strains (**Table 2.1**) were grown on three different media supplemented with sucrose (14.7 g/L) as a starting substrate for EPS production, (i) mineral base-medium with added yeast extract (M1), (ii) succinate-containing mineral base-medium with added yeast extract (M2) and (iii) tryptone and yeast extract-containing base medium (M3) as described previously (Ghaly, et al., 2007; Tian, et al., 2011). The mineral/yeast extract-based media (M1) was composed of (g/L): $Na_2HPO_4 \cdot 2H_2O$-0.267, KH_2PO_4-0.136, $(NH_4)_2SO_4$-0.05, $FeSO_4 \cdot 7H_2O$-0.0005, $MnSO_4 \cdot H_2O$-0.00018, $Na_2MoO_4 \cdot 2H_2O$-0.00025, $CaPO_4 \cdot 2H_2O$-0.001, $MgSO_4 \cdot 7H_2O$-0.02, Yeast Extract-10. While the succinate containing mineral/yeast extract-based media (M2) was composed of (g/L): Sodium Succinate-100, K_2HPO_4-7, KH_2PO_4-3, $(NH_4)_2SO_4$-0.5, $FeSO_4 \cdot 7H_2O$-0.005, $MnSO_4 \cdot H_2O$-0.0018, $Mn_2MoO_4 \cdot 2H_2O$-0.0025, $CaPO_4 \cdot 2H_2O$-0.01, $MgSO_4 \cdot H_2O$-0.25, Yeast Extract-100. The tryptone/yeast extract-based media (M3) comprised of (g/L): bacto tryptone-5, bacto yeast extract-2.5, bacto dextrose-1 and bacto agar-20.

To test the strains for EPS production on agar media, bacto agar (1.5-2.0%, w/v) and sucrose (ca. 14.7%, w/v) were added to each of these media after autoclaving at 121°C for 30 min. Molten and tempered media (ca. 50°C) and sucrose solution were gently mixed and poured into sterile petri-dishes. After solidification, the plates were inoculated with the strains to be tested by streaking and were incubated at the respective optimum growth temperatures of the inoculated strains (**Table 2.1**) for 48 to 160 hrs. The incubation was ceased when colonies were large enough to be harvested for further testing. Ropy colonies were then recovered with sterile plastic hockey sticks, suspended in 1 mL of sterile deionized water into 2 mL Eppendorf tubes and centrifuged (4862 *g*, 6 min) in a Eppendorf™ Minispin™ microcentrifuge (Eppendorf, Mississauga, Canada). The cell-free supernatants were recovered as crude-EPS and analyzed for the contents of total carbohydrates and total reducing ends, by using Phenol-Sulfuric acid (Phenol Assay) and 3,5-Dinitrosalicylic acid (DNS Assay) assays, respectively. Thin Layer Chromatography (TLC) was used to detect the presence of polysaccharides and characterize the molecular weight (MW) distribution of yielded carbohydrates.

The supernatant was then precipitated in absolute (>99.5%) ethanol (1:1) at 4°C overnight and centrifuged at 13,614 *g* for 4 min. The resulting polysaccharide-containing pellets were acid hydrolyzed, neutralized and then injected in a High Performance Anionic Exchange Column with

Pulsed Amperometic Detector (HPAEC-PAD) equipped with a separatory column (PA20) in the Dionex system (ICS-3000) to determine their constitutive monomers. These tests were conducted to determine the most favorable medium for EPS production by each of the strains studied.

2.4 Analytical Methods

2.4.1 Phenol-Sulfuric Acid Assay

This technique was used as described by Dubois *et al*., (1956). Briefly, to 400 µL sample of ethanol-precipitated polysaccharides, 200 µL of phenol (5%, w/v) and 1 mL of concentrated sulfuric acid were consecutively added. The mixture was thoroughly homogenized by using a vortex mixer and allowed to cool for 20 min at room temperature. The absorbance was then measured in a DU 800 spectrophotometer (Beckman Coulter, San Ramon, USA) at 480 nm in glass cuvettes (Dubois, et al., 1956). A standard curve of glucose was constructed to quantify the total sugars.

2.4.2 3,5-Dinitrosalicylic Acid Assay

The reducing end content of the EPS extracts was studied by using DNS assay as described by Miller (1959). A volume of 375 µL of DNS was added to 250-µL of sample and the mixture was boiled for 5 min. After boiling, the reaction was stabilized by the addition of 125 µL potassium sodium tartrate (PST). The carbohydrate reducing end content was then determined by measuring the absorbance at 540 nm in a DU 800 spectrophotometer (Beckman Coulter, San Raman, CA) against a glucose standard curve.

2.4.3 Protein Determination

The protein content was determined according to the AOAC official analytical method DUMAS (AOAC, 1995) on a Leco® TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, USA) (Waglay, et al., 2014). The protein content was estimated by multiplying the total nitrogen content by a factor of 6.25.

2.4.4 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to detect the presence of monosaccharides, disaccharides and polysaccharides in the EPS produced by each of the five studied strains grown on agar media M1, M2 and M3. Carbohydrate solutions of inulin (1%, w/v), sucrose (30 mM), and

fructose (30 mM) were used as standards. A 5 μL sample of each of the standard solutions and EPS was mounted onto silica gel plates. The plates were placed in solvent chambers containing butanol, acetic acid and water (5:4:1), and allowed to elute undisturbed until the solvent front reached the finish line. Then, they were taken out of the chambers and sprayed with a 5% (v/v) sulfuric acid in methanol solution, dried and heated in a Napco Dryer (Model 322, South Haven, USA) at 70°C for 2-3 h until spots of separated carbohydrates could be visually analyzed under visible light.

2.5 EPS Production by Fermentation in Agitated Flask Cultures

The time course for the production of EPS during fermentation in agitated flask cultures of liquid media, was investigated using the most suitable medium (M1, M2 or M3) for EPS production by each of the species studied.

2.5.1 Inoculum Preparation

To ensure consistency of the results, all strains were activated to the mid-exponential phase of growth prior to conducting EPS production by fermentation. This was done by transferring a loop-full from the slant culture of each strain into 50 mL of sterile nutrient broth in a 250 mL Erlenmeyer flask, and incubating with continuous shaking at 150 rpm in an orbital agitator (N-BioTEK Shaking Incubator, Gyeonggi-do, Korea) at optimum growth temperature (**Table 2.1**) for 12 to 15 h. During incubation, the absorbance (600 nm) was measured regularly until a value of 0.7, corresponding to the mid-exponential phase, was reached.

2.5.2 Flask Fermentation

Production of EPS by the selected *Bacillus* strains was monitored by flask fermentation with best selected media (M1, M2 and M3) to compare their yields and determine the optimal medium composition for these strains to produce maximum EPS. Before inoculation, the media's were supplemented with filtered sucrose solution (50%, w/v) by aseptically adding 166.6 mL to 400 mL media (i.e. 14.7% sucrose in culture media). The sucrose stock solution was sterilized by filtration through a 22-μm filter membrane.

Fermentation experiments were conducted in 1 L baffled Erlenmeyer flasks containing 400 mL of M1, M2 or M3 supplemented with sucrose. For each medium, duplicate flasks were

prepared and inoculated with a volume of the previously prepared inoculum to give an initial absorbance of 0.013 AU/ml of media. Inoculated flasks were placed in an orbital agitator (New Brunswick Scientific™, Excella E10 model, Pittsburg, USA) shaking at 150 rpm, and were incubated at the optimum growth temperature of each strain. At regular intervals, 30 mL samples were aseptically withdrawn from the flasks over a time course ranging from 4 to 144 hrs. The samples were collected in sterile centrifuge tubes (Corning, Ultident, St. Laurent, Canada) and their OD (600 nm) was measured before centrifugation (11295 g for 25 min). The supernatants were subjected to ethanol precipitation (1:1) at 4°C for at least 24 hrs and centrifuged at 16260 g for 25 min (Zentrifuge Heraeus Centrifuge 190000 rpm/min Rotor 3334 6x, Thermo Fisher Scientific, Waltham, USA) to recover the biomass in the pellets. These EPS extracts were freeze-dried in a CHRIST Shelved-Lyophilization Chamber (CHRIST, Gamma 1-16S LSC, Osterode am Harz, Germany) and stored at refrigeration temperatures until need for the analyzation of the carbohydrate (EPS) and reducing-ended contents, molecular weight distribution, and monosaccharide profile, as described below (§ 2.5.1 and 2.5.2).

2.6 Structural Characterization of EPS

Polysaccharide analysis was conducted using chromatographic techniques, presently recognized to be the most powerful techniques for polysaccharides analysis. MilliQ distilled and deionized water (Millipore) was filter-sterilized by passage through a 0.22-µm filter membrane (Millipak 20 Express Filter, Millipore, Etobicoke, Canada) to be used in all of the chromatographic techniques of this study.

2.6.1 Molecular Weight Distribution

The molecular weight distribution in EPS samples was analyzed by high pressure size exclusion chromatography (HPSEC) using a Waters HPLC system (Model 25P, Waters Corp. Milford, MA, USA) equipped with a refractive index detector (Model 2414). For size exclusion analysis, three TSKgel PWxl columns composed of spherical, hydrophilic polymethacrylate beads were used in series at 30°C (TSK G3000 PWxl, TSK G4000 PWxl, TSK5000 PWxl) as described by Khodaei *et al*., (2016). A solution of 0.1 M NaCl used as the mobile phase was isocratically eluted through the columns at a flow rate of 0.4 ml/min and the eluate was analyzed by the refractive index detector. Freshly prepared solutions (1 mg/mL) of dextran standards of varying molecular weights (50 to 670 kDa) were used to construct the calibration curve by plotting the log

of the molecular weights versus the elution time. In order to inject into the HPLC, ethanol-precipitated EPS samples were centrifuged at 13614 g for 4 min to remove contaminants, and were injected into the HPLC to the capacity of the loop of 20 µL.

2.6.2 Monosaccharide Profile

EPS extracts were chemically hydrolyzed according to the method described by Khodaei *et al*., (2016). Freeze-dried EPS samples were suspended (0.6% w/v) in HCl/Methanol (1:4, v/v) and incubated at 60°C for 24 h. Then, the mixture was boiled for 1 h in trifluoroacetic (TFA) acid at a ratio of 1:8 (v/v). The resulting hydrolysates were neutralized with 1M NaOH solution and centrifuged (13614 g, 4 min) to remove impurities, and the monosaccharide composition was determined by using HPAEC-PAD Dionex system (ICS-3000) equipped with a CarboPac PA 20 column (3 x 150 mm) operating at 30°C, and Chromeleon Software. Isocratic elution was performed with 10 mM NaOH (0.4 ml/min). Seven monosaccharides, rhamnose, arabinose, glucosamine, galactose, glucose, xylose and fructose, were used as internal/external standards for the identification and calibration of the peaks.

2.7 Optimization of EPS Production

2.7.1 Experimental Design

The optimization of EPS was conducted with *B. licheniformis* ATCC 14580 in the medium M2, as it showed the greatest and most interesting EPS composition. To optimize EPS production, the composition of the medium M2 was investigated. The effects of the concentrations (g/L) of the main nitrogen source (yeast extract), the main mineral source (sodium succinate) and the inducer (sucrose) were studied using a five-level, three-variable central composite design (CCD) consisting of 20 points (8 factorial (± 1), 6 axial (± α), 6 central). Each run was conducted in duplicate for four different durations (48, 72, 96 and 120 h). Fermentation batches were sampled (30 mL) in duplicates from each duplicated fermentation and centrifuged at 11,295 g, for 25 min to remove bacterial cells. After recovering the EPS by ethanol precipitation as described above, the biomass yield (g/L culture, w/v), the EPS (total carbohydrate) yield (g/L culture, w/v) and the total protein content (g/g biomass) were determined. Their corresponding models were constructed by using the Design Expert software version 9.0.6 (Stat-Ease Inc., Minneapolis, MN).

2.7.2 Statistical Analysis

Regression analysis was performed on the basis of the experimental results obtained in this study, and was fitted to an empirical quadratic polynomial equation using the response surface regression (RSREG) procedure of SAS system software 9.2 and the software Design Expert 9.0.6 (Seo, et al., 2012).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (2)$$

Where Y is the response, β_0 the constant, β_i the coefficient for the linear effect, β_{ij} the coefficient for the interaction effect, β_{ii} the coefficient for the quadratic effect, X_i and X_j the coded level of variables. Generated contour plots were obtained using the fitted model, and by keeping the least effective independent variables at a constant value, while changing the other to independent variables (Seo, et al., 2012).

CONNECTING STATEMENT 2

Chapter II outlines the methodology followed to study exopolysaccharide (EPS) production by various *Bacillus* strains as well as the methodology employed to characterize the yielded EPS. Chapter III illustrates the results obtained and provides a detailed discussion on the yields from each of the studied *Bacillus* strains. At the end of this chapter, a brief conclusion is given along with recommendations for future research on the topic.

CHAPTER III. RESULTS & DISCUSSION

3.1 Screening of Selected Strains for the Carbohydrate Production on Agar Media

The carbohydrate production by selected bacilli was investigated using a screening technique on agar media. The species of interest were two strains of *Bacillus amyloliquefaciens* (23350-35°C, OB6-30°C), *Bacillus licheniformis* 14580 (35°C), *Bacillus subtilis* B26 (30°C), and *Geobacillus stearothermophilus* Donk 12980 (55°C). The bacterial species have been reported to produce varying types of EPS, such as levan (Ghaly, et al., 2007; Santos, et al., 2013; Srikanth, et al., 2015). *B. licheniformis* LMG 19409 studied by Larpin *et al.,* (2002) has been reported to produce heteropolymers composed of 80.5% mannose, and 6.5% glucose; and *Gb. stearothermophilus,* which had not been substantially studied for EPS production, has been found to produce galacto-oligosaccharides in the presence of lactose (Park & Oh, 2010). Three different agar media were selected and will henceforth be referred to as mineral based-medium with added yeast extract (M1), succinate-containing mineral based-medium with added yeast extract (M2) and tryptone/yeast extract-containing medium (M3). These media were supplemented with 14.7% (w/v) sucrose as an inducer. Different incubation times (48, 112, 96, 120, 160 hrs) were assessed for screening purposes and to determine the optimal duration of incubation for carbohydrate production (**Table 3.1)**.

The trends of carbohydrate production varied depending on the strain used. As compared to other strains, *Gb. stearothermophilus* Donk (12980) showed no significant carbohydrate production on all of the three media (**Table 3.1**). This was corroborated by TLC results showing faint presence of EPS and weak spots of mono, di- and oligosaccharides on M1 medium. These results may be attributed to: (1) the low expression of enzymes involved in carbohydrate synthesis; (2) the effect of the metabolites and/or temperature on the enzyme activity and (3) the effect of temperature on the agar media. *Gb. stearothermophilus* Donk (12980) is known to produce numerous thermostable extracellular enzymes in culture supernatants, such as xylanases, β-xylosidases, α-arabinofuranosidases, and α-glucuronidases (Horikoshi, et al., 2011).

Table 3.1. Screening of Selected *Bacillus* spp. for EPS Producing Abilities in Selected Agar Media at Optimal Incubation Periods

Strains	Time (hr)	Medium	Carbohydrates (mmol/plate)	Reducing Ends (mmol/plate)	TLC Band		
					Monosaccharides	Disaccharides/ Oligosaccharides	EPS
B. licheniformis (14580)	160	M1	0.254 (±0.125)	0.014 (±0.001)	0[5]	0	+++[3]
	160	M2	0.127 (±0.013)	0.037 (±0.002)	±[4]	±	++[2]
	160	M3	0.449 (±0.000)	0.417 (±0.007)	0	++	±
B. amyloliquefaciens (23350)	48	M1	0.270 (±0.000)	0.285 (±0.009)	++	++	+++
	160	M2	0.102 (±0.008)	0.040 (±0.006)	±	0	+[1]
	160	M3	0.211 (±0.001)	0.043 (±0.002)	+[1]	+	++
Gb. stearothermophilus Donk (12980)	160	M1	0.008 (±0.000)	0.002 (±0.000)	+	+	±
	160	M2	0.000 (±0.000)	0.002 (±0.000)	0	±	0
	160	M3	0.002 (±0.000)	0.002 (±0.000)	0	+	0
B. subtilis (B26)	96	M1	0.199 (±0.006)	0.077 (±0.000)	++	0	++
	120	M2	0.010 (±0.000)	0.116 (±0.004)	+	0	+
	96	M3	0.177 (±0.008)	0.110 (±0.001)	+++	0	+++
	120		0.270 (±0.000)	0.205 (±0.047)	+++	0	+++
B. amyloliquefaciens (OB6)	96	M1	0.360 (±0.000)	0.313 (±0.004)	+	++	++
	96	M2	0.270 (±0.000)	0.087 (±0.001)	+	0	+++
	96	M3	0.185 (±0.006)	0.085 (±0.000)	+	0	+

[1] (+++) Strong Presence, [2](++) Presence, [3](+) Weak Presence, [4](±) Faint Presence, [5](0) No presence

Each of these enzymes possesses different optimum temperatures, some of which are lower or higher than the optimum growth temperature of the bacterium (55°C). For example, α-glucuronidase AguA produced by *Gb. stearothermophilus* 236 has an optimum temperature of 40°C with a half-life of 50 min at 50°C (Choi, et al., 2000); and an α-arabinofuranosidase AbfA, produced by *Gb. stearothermophilus* T-6 demonstrated an optimum temperature of 70°C, with a half-life of 53 h at 60°C (Gilead & Shoham, 1995). Therefore, certain enzymes may have been produced during the growth of *Gb. stearothermophilus* Donk (12980) but exhibited only a weak enzymatic activity either due to the inadequate temperature of incubation or to the thermal inactivation during the fermentation course. The effect of metabolites/side-products generated may have also contributed to the low yield of EPS produced by *Gb. stearothermophilus*. Welker & Campbell, (1963) showed that the poorer the carbon source (i.e. Complex carbohydrates requiring multiple enzymes to oxidize the environmental carbon sources), the greater is the amount of enzyme produced. A yield of 109 units/ml of α-amylase was obtained from *Bacillus stearothermophilus* with glycerol as the carbon source, while this bacterium produced only 45 units/ml of the enzyme with sucrose as the carbon source. This result is believed to be due to the fact that the fructose, when released from sucrose, acts as a carbon-metabolite repressor for α-amylase formation in *B. stearothermophilus*. As compared to other bacteria, a higher growth temperature was required for *Gb. stearothermophilus,* which resulted in the dehydration of the mineral agar media during the whole period of incubation (160 hours).

For the other four *Bacillus* strains, the mineral base-medium with added yeast extract (M1) resulted in a carbohydrate production of 0.199 to 0.360 mmol/plate, with *B. amyloliquefaciens* (23350) and *B. subtilis* (B26) yielding the highest and lowest amounts, respectively. The TLC profile of carbohydrates produced in M1 medium showed the largest amounts of EPS as compared to the other media (**Table 3.1**). These results are congruent with those obtained by Inthanavong *et al.,* (2013) who demonstrated that the mineral-based medium M1 stimulates more efficiently the expression of transglycosidase in *Gb. stearothermophilus* Donk than does the M2 succinate-containing mineral-based medium. Indeed, the use of succinate-containing mineral-based medium with added yeast extract (M2) decreased the carbohydrate production as compared to M1 medium, where the most significant decrease was observed with the *B. subtilis* (B26) strain. Such a decrease on M2 medium could be explained by either the formation of sodium carbonate, a highly alkaline salt produced from the oxidation of sodium succinate, or by the expression of protease and sucrose,

which may have hydrolyzed the sucrose inducer (Kampen, 2001; Inthanavong, et al., 2013). TLC profile of carbohydrates indicated less production of EPS on M2 medium as compared to M1 (**Table 3.1**).

Contrary to other culture media used in this study, the carbohydrate production in the tryptone/yeast extract-containing medium (M3) was not consistent from one strain to another one. The highest carbohydrate productions by *B. licheniformis* (14580) and *B. subtilis* (B26) were recorded on the M3 medium, which also allowed the highest production of EPS by *B. subtilis* (B26). Conversely, *B. licheniformis* (14580) produced the lowest amount of EPS on this medium, out of the three tested. Results of **Table 3.1** also indicate that the M3 medium allowed the highest production of di/oligosaccharides and monosaccharides by *B. licheniformis* (14580) and *B. subtilis* (B26), respectively. As for *B. amyloliquefaciens* (23350) and *B. amyloliquefaciens* (OB6), M3 favored the production of EPS and monosaccharides, although, the latter strain did not produce significant amounts of these components (**Table 3.1**). The content of reducing ends as an indicator of the presence of short-chain carbohydrates, such as mono, di- and oligo-saccharides, was also dependent on the type of culture media. The concentration of carbohydrates was compared to the content of reducing ends, and the overall results show that the content of reducing ended carbohydrates is inversely related to the amounts of EPS in the analyzed samples. As can be observed from the results in **Table 3.1,** *B. licheniformis* (14580) produced greater amounts of EPS on M1 and M2 culture media compared to M3; a finding that was confirmed by the TLC profile of the carbohydrate extracts (**Table 3.1**). For *B. amyloliquefaciens* (23350), greater content of reducing ends was observed with M1, and the TLC profile revealed greater EPS presence on M1 and M3 (**Table 3.1**). The higher production of disaccharides/oligomers on M1 medium may explain such results. *B. subtilis* (B26) demonstrated greater potential for the production of carbohydrates with a high content of reducing ends on all of the investigated media. These results can be attributed to the release of high amounts of monosaccharides as shown by the TLC profiles. For *B. amyloliquefaciens* (OB6), the high contents of reducing ends with relatively low presence of monosaccharides revealed the predominance short chain in the EPS produced.

Based on the experimental results, the most favorable media were selected to be used in subsequent experiments on EPS production by each of the strains studied as summarized in **Table 3.2**. For *B. licheniformis* (14580), M1 and M2 media were selected, since they both demonstrated

high carbohydrate production with the presence of small amounts of reducing ends and greater presence of EPS. Likewise, M1 and M2 media were selected for further studies on *B. amyloliquefaciens* (OB6), as both of them allowed for higher carbohydrate production at shorter incubation times. Notably, while greater carbohydrate concentrations and lower amounts of reducing ended carbohydrates were obtained on M2 medium with *B. amyloliquefaciens* (OB6) and as such was retained for further studies; on the other hand, M1 medium was strictly retained for comparison purposes (**Table 3.2**), since it showed stronger presence of EPS as revealed by TLC analysis (**Table 3.1**).

For *B. amyloliquefaciens* (23350), the greatest concentration of carbohydrate was produced on M1 and M3 culture media, while, greater content of reducing end carbohydrates was obtained on M1. Since the TLC profile of carbohydrate produced by *B. amyloliquefaciens* (23350) demonstrated stronger presence of EPS on M1 than M3, the former medium (M1) was selected for further studies. Moreover, both M1 and M3 media demonstrated the highest concentrations of carbohydrate produced by *B. subtilis* (B26), and the TLC profiles indicated that the EPS was produced at higher amounts on M3 medium as compared to M1 (**Table 3.1**). M3 medium also demonstrated a greater ability to maintain and increase the production of carbohydrates than M1 did, where the content of these compounds declined drastically after 96 hrs of incubation. Therefore, M3 was retained for subsequent studies on *B. subtilis* (B26).

3.2 Time Course for EPS Production

The time courses of bacterial growth (OD_{600}) and production of biomass (EPS, protein and other polymers), and EPS were investigated over 144 hrs of fermentation. In M1 medium, the results showed a steady increase in the OD_{600} at the exponential growth phase of *B. licheniformis* (14580) up to 24 hrs followed by a sharp decline for the next 96 hrs to reach the stationary phase of growth where the OD_{600} remained constant (**Figure 3.1, A**). Exponential phase of bacterial growth is generally short and followed by either constant bacterial counts (stationary phase) or their decrease (death phase) as the nutrient availability depletes and production of toxic waste increases. Stationary phase is caused by the net growth of new cells being equal to the decline in old cells and if monitored for a longer period of time, they taper into a death phase (Srivastava & Srivastava, 2003). The death phase is where the carbon sources are depleted, the nutrient media becomes enriched with toxic levels of nitrogen from cellular debris, intracellular enzymes and co-factors; and

inhibition of transcription and translation of bacterial DNA occurs (Moulton, 2014). A parallel increase of the biomass and the EPS production was recorded during the first 4 hrs of fermentation to reach 2.95 and 1.62 g/L, respectively. Afterwards, the biomass kept increasing globally while demonstrating a trend of faint dips and spikes. This fluctuation in the biomass may be attributed to the fact that when the fermentation proceeds for many hours, some reversible DNA rearrangements can occur and lead to the production of different cell variants that differ in their EPS production capabilities (Gancel & Novel, 1994). However, in spite of the fact that some bacteria possess specific EPS hydrolytic enzymes (e.g., lyases produced by *Pseudomonas aeruginosa*), EPS-producing bacteria typically do not consume the EPS they produce. Therefore, -the dips and spikes cannot be explained by the hydrolytic enzymes of the EPS-producing strains (Sutherland, 1990; Boyd & Chakrabarty, 1994; Sutherland, 2004). The EPS production, on the other hand, increased to a lower extent as compared to the total biomass; the highest amounts of biomass and EPS of 5.84 and 3.50 g/L, respectively, were obtained after 144 hrs of fermentation (**Figure 3.1, A**).

The growth curve of *B. licheniformis* (14580) in M2 medium showed a longer exponential phase in which the bacterial growth rates varied and were lower when compared to those observed on M1 medium (**Figure 3.1, B**). The exponential phase is the phase in which primary metabolites are produced. Typically, a long-lasting exponential phase indicates that the production of primary metabolites that enhance growth of bacterial cells are maintaining positive production against secondary metabolites (inhibitory to the growth of bacterial cells) over a longer period of time.

Table 3.2. Highest Performances of *Bacillus* Strains in the Production of Total Carbohydrates (CHO) and Exopolysaccharides (EPS) on Different Culture Media to be Selected for Further Studies

Bacillus strain	Highest CHO Production[1]	Most Abundant Polysaccharide Production[2]	Selected Media
B. licheniformis (14580)	M1, M3	M1, M2	M1, M2
B. amyloliquefaciens (23350)	M1, M3	M1	M1
B. subtilis (B26)	M1, M3	M3	M3
B. amyloliquefaciens (OB6)	M1, M2	M1, M2	M1, M2

[1] Determined as mM/plate

[2] As a relative proportion

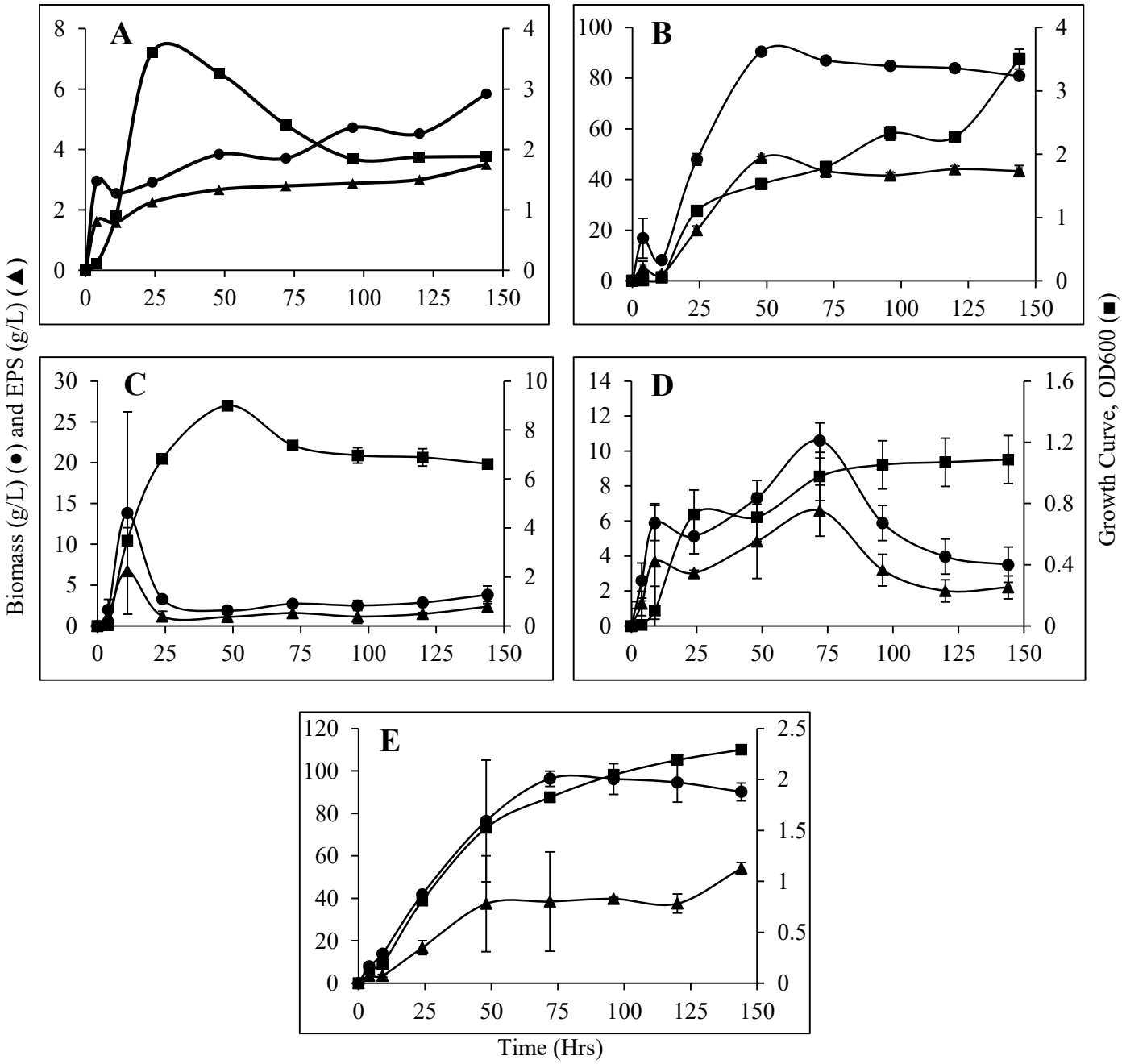


Figure 3.1. Growth (OD600) (■), biomass (g/L) (●) and EPS (g/L) (▲) production by *Bacillus* species studied over time in selected liquid media

Where *B. licheniformis* (14580), M1 **(A)**[1], *B. licheniformis* (14580), M2 **(B)**, *B. amyloliquefaciens* (23350), M1 **(C)**, *B. subtilis* (B26), M3 **(D)**, *B. amyloliquefaciens* (OB6), M2 **(E)**

[1]Data shown for one flask fermentation

One possible explanation of the reduced growth rates of *B. licheniformis* (14580) in M2 medium compared with M1 may be the presence of the higher concentration of yeast extract in M2 medium as well as sodium succinate. These two nutrients, when metabolized, can lead to the production of toxic by-products that hinder the propagation of bacterial cells (Srivastava & Srivastava, 2003). Furthermore, increases in EPS production while the growth of the bacterium is declining has also been observed in *Lactobacillus rhamnosus* by Pham *et al*., (2000). However, such behavior was not observed with the same bacterium in the presence of lactose as the carbon source.

In M2 medium, the biomass and the EPS production by *B. licheniformis* (14580) increased with the bacterial growth up to 48 hrs to reach a maximum of 90.44 g/L and 48.57 g/L, respectively (**Figure 3.1, A**). Although the bacterial growth continued to increase after 48 hrs, no significant changes in the biomass and the EPS production was observed over the remaining fermentation time course (**Figure 3.1, B**). This trend may reveal the occurrence of carbon catabolite repression, whereby EPS production can be suppressed by un-metabolized carbon sources to be subsequently induced once the carbon sources are depleted; this has been demonstrated by Rajagopalan & Krishnan, (2008).

The results showed that *B. licheniformis* (14580) produced 15.5 and 14.9 times more biomass and EPS, respectively, in M2 than in M1; although the OD_{600} was more or less similar in both culture media. Additionally, the highest yields of EPS were obtained earlier on M2 (at 48 hrs of fermentation) than on M1 (at 144 hrs of fermentation) (**Figure 3.1, A-B**). The differences between the M1 and M2 media are that (1) the buffering capacity of M2 medium which contains higher concentrations of phosphate and succinate buffers, (2) the concentration ratio (1:1) of sodium succinate to yeast extract in M2, and (3) the higher concentration (100 g/L) of yeast extract in M2, compared with M1 containing only 10 g/L.

Additionally, the pH of the medium limits EPS production when it falls out of the range of 6.0-8.0 (Sutherland, 1990); in our study, the pH of the media was only monitored during 96 hrs of the fermentation with *B. amyloliquefaciens* (OB6) and *B. licheniformis* (14580) in M1 and M2, as these strains showed the greatest EPS production capability. The pH varied from 7.3-6.2 and 6.8-7.1 in M1 and M2 media with *B. amyloliquefaciens* (OB6) and *B. licheniformis* (14580), respectively (data not shown). Thus, it can be hypothesized that the low EPS produced in M1

may be due to the decrease in the pH. Lee *et al*., (1997) and Pawar *et al*., (2013) have investigated the pH effects using a strain of *B. polymyxa* and unknown bacteria in saline soil respectively, and found that the optimum pH for EPS production was approximately 7.0-7.5 for both.

The OD_{600}, biomass and EPS production of *B. amyloliquefaciens* (23350) grown on M1 medium are shown in **Figure 3.1, C**. The growth of *B. amyloliquefaciens* (23350) peaked at 48 hrs and then began to decrease and entered the stationary phase between 72 and 120 hrs of fermentation before reaching the death phase at 144 hrs (**Figure 3.1, C**). The results also demonstrate that during the exponential phase of growth, *B. amyloliquefaciens* (23350) produced the greatest biomass (13.84 g/L) and EPS amount (6.74 g/L) at 11 hrs, which decreased significantly thereafter. This decrease supported the rapid growth of *B. amyloliquefaciens* (23350) at higher rate (0.15 OD_{600}/hr). *B. amyloliquefaciens* (23350) possesses desirable characteristics such as its ability to produce high amounts of EPS within a short period of fermentation; however, the rapid metabolism of the EPS by *B. amyloliquefaciens* (23350) may limit its application. Many studies have demonstrated decreases in total EPS yields over time, of which enzymatic degradation or a change in physical parameters of the culture may be the main cause (Cerning, et al., 1992; Cerning, 1990; Gancel & Novel, 1994).

B. subtilis (B26) exhibited a different growth pattern in M3 medium where it grew exponentially for 24 hrs before reaching the stationary phase, which was sustained until 48 hrs of fermentation, after which the exponential growth was re-initiated in a typical bi-phasic growth pattern. Then, the strain started a second stationary phase that continued from 72 to 144 hrs (**Figure 3.1, D**). The biomass and the EPS production also showed two peak points, both of which were achieved during the two exponential growth phases. The highest biomass and EPS yields of 10.61 g/L and 6.59 g/L were recorded after 72 hrs of fermentation. From 72 to 144 hrs, *B. subtilis* (B26) entered a second stationary phase of growth, during which the EPS production underwent a sharp decline. This decrease may reveal the depletion of EPS as a result of its hydrolysis by the producer strain (i.e., *B. subtilis* (B26)). At the last stage, the gap between the biomass and EPS yields became significantly smaller. When a bacterium reaches its death phase, the carbon sources (e.g., sucrose and yeast extract) are depleted so it is then possible that the gap between the biomass and EPS yields change as a result of the consumption of the produced proteins by

B. subtilis (B26), in order to maintain the second stationary growth phase (Moulton, 2014) (**Figure 3.1, D**).

Time course for *B. amyloliquefaciens* (OB6) growth in M2 medium began with a 4 hrs lag phase before starting the exponential phase from 9-48 hrs at a rate of 0.03 OD_{600}/hr; after which the growth rate changed to $8x10^{-3}$ OD_{600}/hr at a second exponential phase from 48-144 hrs. The growth curve showed no obvious signs of reaching a stationary phase within the 144 hrs fermentation course. Meanwhile, the trends for biomass and EPS production followed a different pattern from that of the other three *Bacillus* strains, where the highest yield of biomass did not correspond to the time at which the bacterium produced the highest amounts of EPS. The biomass and EPS yields paralleled the initial exponential growth phase until 48 hrs of fermentation where the biomass was 37.44 g/L and remained roughly constant until 96 hrs. The biomass yield continued to increase steadily and reached a maximum of 96.38 g/L at 72 hrs. At this time, the EPS production was 38.50 g/L, but as the second exponential phase began the biomass yield started declining slowly to attain 83.50 g/L at 144 hrs. The steady decline of the biomass was accompanied by a steady increase of EPS production for the remaining period of fermentation until it reached its highest level of 54.14 g/L at 144 hrs (**Figure 3.1, E**).

The growth of *B. amyloliquefaciens* (OB6) was also monitored in M1; however, as previously mentioned this media was selected for comparative purposes as the highest amount of carbohydrate was produced by this strain during the screening step, but the TLC results demonstrated greater EPS presence with M2 (**Table 3.1**). This bacterium was grown in M1 in duplicate flasks at 30°C and the growth was monitored from 48 to 96 hrs. The results showed that *B. amyloliquefaciens* (OB6) produced approximately 7.2 times less EPS and 9.9 times less biomass in M1 medium than it did in M2 (data not shown). The higher performances of M2 compared to M1 may be explained by its richer composition in nutrients and better buffering capacity; the concentrations of the common buffering ingredients were at least tenfold higher in M2 than in M1, in addition to the presence of sodium succinate which is lacking in M1. These results are consistent with those obtained with *B. licheniformis* regarding EPS production in the same media. Thus, M2 was appropriately determined as the most suitable medium for optimal EPS production by *B. amyloliquefaciens* (OB6).

The highest amounts of EPS were produced in M2 broth by *B. amyloliquefaciens* (OB6) and *B. licheniformis* (14580), which yielded 54.14 g/L and 48.57 g/L, respectively. To our knowledge, the amount of EPS produced by *B. amyloliquefaciens* (OB6) and *B. licheniformis* (14580) are the highest reported to date, for *Bacillus amyloliquefaciens* and *Bacillus licheniformis* strains. Comparatively, the highest yield reported thus far by a *Bacillus* spp was 70.6 g/L of levan produced by *B. subtilis* natto by Shih *et al*., (2010) and second highest EPS yield (54 g/L) was reported to be produced by *B. polymyxa* KCTC 8648P (Lee, et al., 1997). *B. licheniformis* strains were shown to produce significantly lower amounts of EPS as Song *et al*., (2012) reported that *B. licheniformis* KS-17 produced 27.20 g/L of EPS (e.g. twofold less than achieved by this study), while the amount of EPS produced by *B. licheniformis* LMG 19409 strain was as low as 0.88 g/L (Larpin, et al., 2002). **Table 1.1** shows that the amount of EPS produced by various bacterial strains ranges between 0.14 and 54.0 g/L, with the lowest yields being produced by LAB. In fact, most LAB strains produce between 0.1-2.0 g/L of EPS (**Figure 1.2**). According to Badel *et al*., (2011) lactobacilli are the weakest EPS producers, as they typically produce less than 1 g/L for homopolysaccharides; a yield that may be even lower when the culture conditions are not optimal. Such low yields were attributed to the fact that LAB acidify the culture media to below pH 5.0 causing glycosyl-hydrolase activation which results in enzymatic digestion/hydrolysis of EPS; thereby decreasing their yields (Badel, et al., 2011).

A limited number of studies have investigated the kinetics of EPS production by *Bacillus* spp. The highest EPS yield of 70.6 g/L was reported by Shih *et al*., (2010), and **Table 1.1** allows comparison of the reported results on EPS production by different bacterial strains. It can be seen from this table that the production of EPS by *Bacillus* spp. is favorable at pH of 7-7.1, lower nitrogen sources and in the presence of sucrose as a substrate. All these criteria are met and observed with the results obtained from the four bacterial species studied herein.

3.3 Structural Characterization of EPS

Structural properties of the EPS produced by selected *Bacillus* spp. were characterized, particularly, their monosaccharide composition and the molecular weight distribution. The results, reported in **Figure 3.2, A** show that the EPS produced by *B. licheniformis* (14580) in both M1 and M2 media had a highly heterogeneous monosaccharide profile. At the exponential phase of growth, this bacterium metabolized the nutrients present in M1 to form EPS primarily

composed of glucose and trace amounts of xylose, fructose, glucosamine and rhamnose. After 96 hrs of fermentation in M1, galactose became the dominant monosaccharide representing 44.7% of the polymers, followed by glucose with 40.0% of the polymers; after 120 hrs of fermentation, glucose and galactose content of EPS represented 51.0% and 33.0%, respectively (**Figure 3.2, A**). The polymers produced in this medium had molecular weights (MW) dominantly ranging between 30-100 kDa, by 96 hrs of fermentation, 6.4% of the tested sample was composed of polymers of more than 500 kDa, and the remaining molecules had molecular weights ranging between 5 and 30 kDa (**Figure 3.3**). In this case, the source of the glucose comes from the inducer, sucrose, however, for galactose to be synthesized UDP-glucose pyrophosphorylase must be expressed in order to favor UDP-glucose synthesis, which would then convert to UDP-galactose using a UDP galactose 4'epimerase (Das, 2005).

The heterogeneity of the monosaccharide profile of the EPS produced by *B. licheniformis* (14580) was more pronounced in M2 medium than in M1. Contrary to M1 medium, the galactose was found to be the main monosaccharide of EPS at the beginning of the fermentation. Glucose, xylose and fructose monosaccharides, which were present in the EPS during the first 4 hrs of fermentation, were not detected afterwards at the exponential phase. At the stationary phase, the molar proportion of glucose, xylose and fructose monosaccharides in the EPS were constantly maintained at 27, 18 and 54%, respectively (**Figure 3.2, B**). The heterogeneity detected with this distribution has been observed with other studied *Bacillus* spp as well. Lee *et al*., (1997) reported the production of heteropolymers by *B. polymyxa* composed of glucose, galactose, glucuronic acid, mannose and fucose. Larpin *et al*., (2002) showed that mannose and glucose represented 80.5% and 6.5%, respectively of the EPS produced by *B. licheniformis,* while Kekez *et al*., (2015) and Ghaly *et al*., (2007) reported that *B. licheniformis* is mainly a levan-producing bacterium. Therefore, the type of culture media, the temperature and the pH greatly influence the type of EPS produced. Accordingly to Gancel & Novel, (1994), reversible DNA arrangements lead to the formation of a variety of cell types that differ in exopolymer production. While this may hold true for the yields of biomass or EPS, it is also possible that the variety of cell types formed may produce different types of polysaccharides, which may also have different molecular weights.

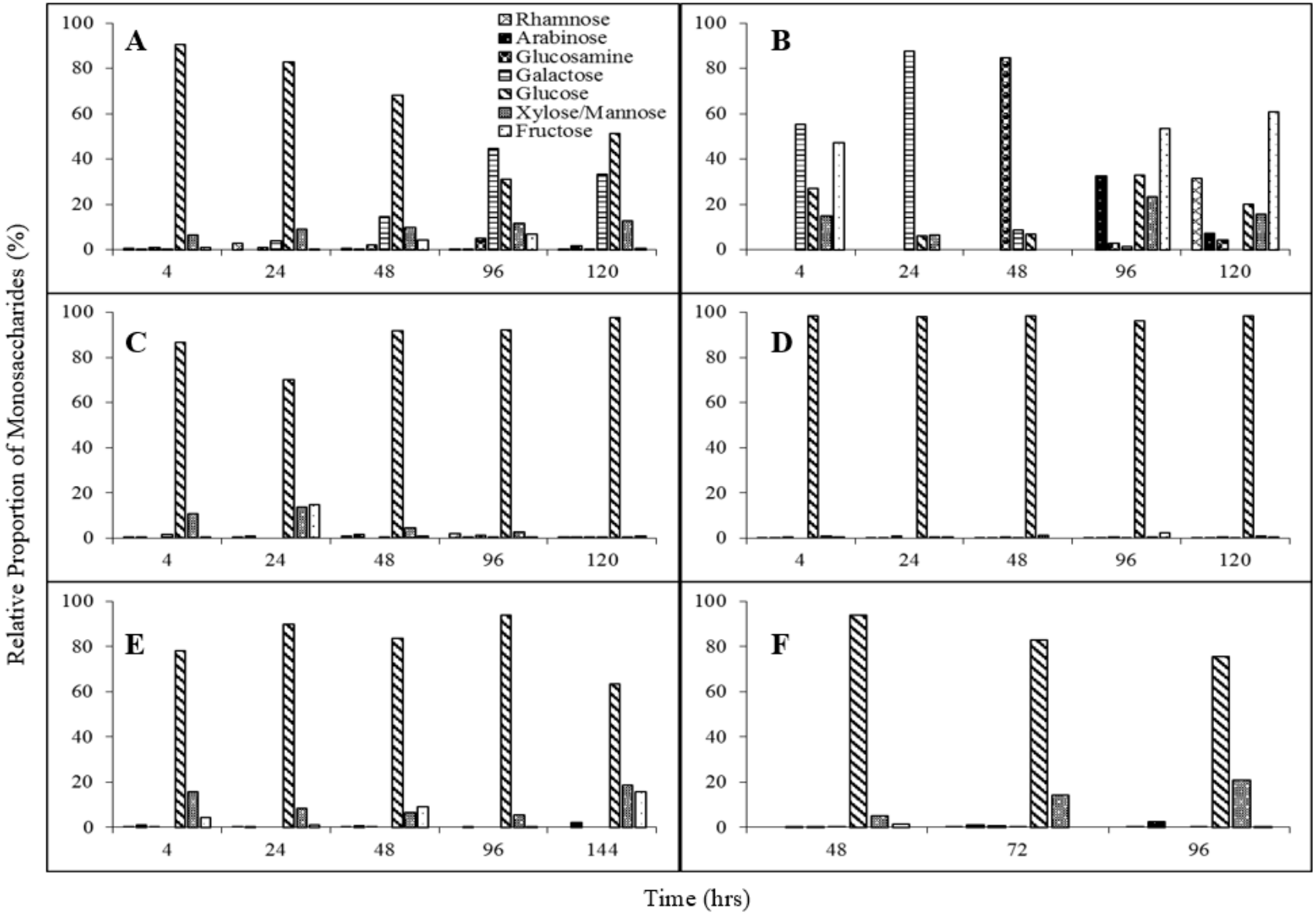


Figure 3.2. Monosaccharide profile of EPS secreted by selected *Bacillus* spp. in best identified media

B. licheniformis (M1) – **A**; *B. licheniformis* (M2) – **B**; *B. amyloliquefaciens* 23350 (M1) – **C**; *B. subtilis* B26 (M3) – **D**; *B. amyloliquefaciens* OB6 (M2) – **E**; *B. amyloliquefaciens* OB6 (M1) – **F**

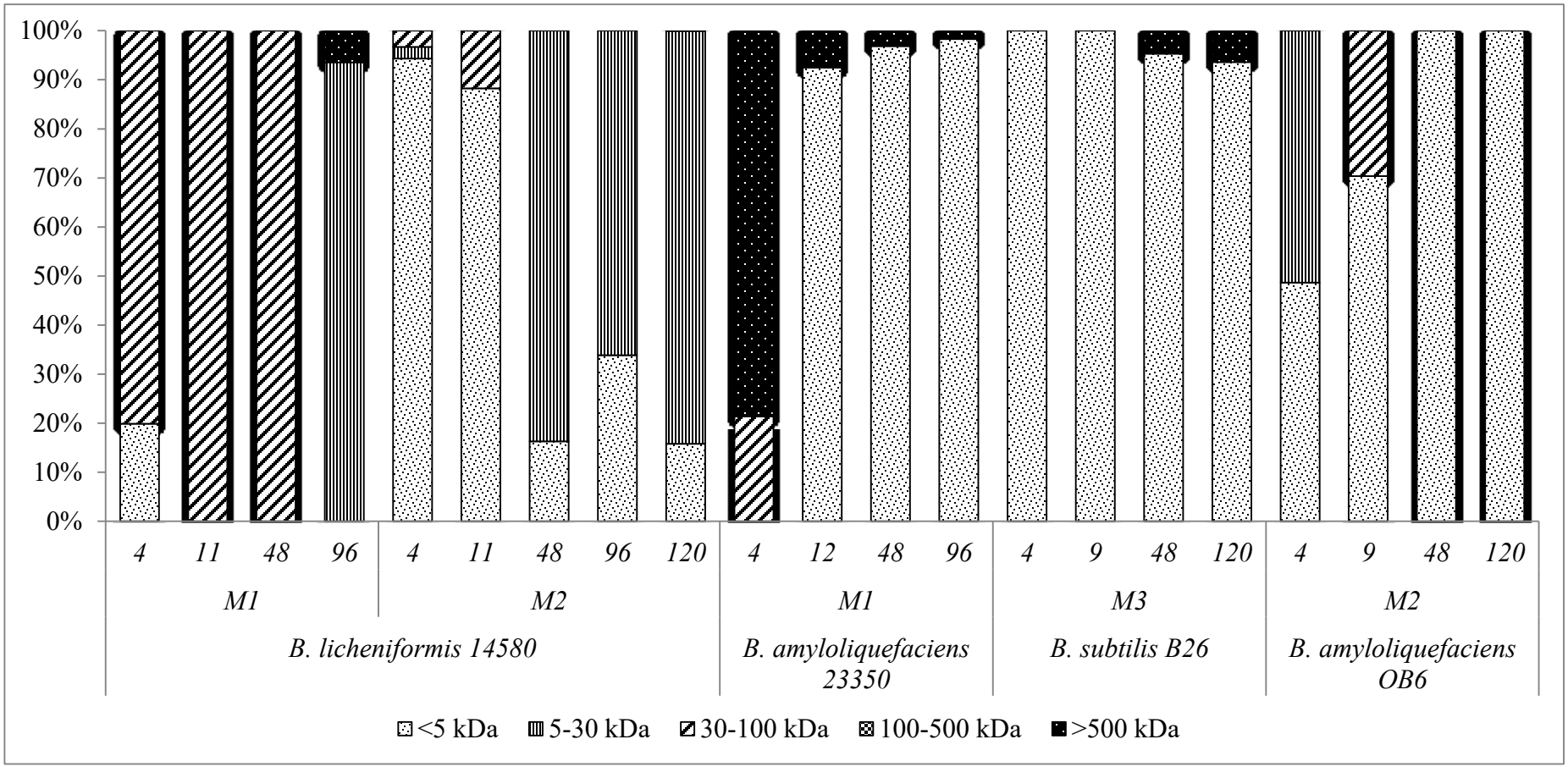


Figure 3.3. Molecular weight distribution of EPS secreted by selected *Bacillus* spp in the most favorable media

Where *B. licheniformis* (14580) is grown in M1 medium, *B. amyloliquefaciens* (23350) is grown in M1 medium, *B. subtilis* (B26) is grown in M3 medium and *B. amyloliquefaciens* (OB6) is grown in M2 medium.

Comparisons of the molecular weight distribution for the EPS produced by *B. licheniformis* (14580) in M1 and M2 demonstrated that higher MW polymers were yielded over extended periods of fermentation, but greater sized polymers were generally produced by this strain in M1 (**Figure 3.3**). In this medium, polymers with molecular weights ranging between 30-100 kDa were the main EPS produced (80.1-100%) within 4-48 hrs of fermentation; at 96 hrs, EPS with MW higher than 500 kDa was released and represented 6.4% of the total EPS produced, while the remaining 93.6% polymers had MW ranging between 5 and 30 kDa. In the M2 medium, 3.3-11.7% of the polymers produced at 4-11 hrs of fermentation had larger MW (30-100 kDa) detected while those having <5 kDa composed 88.3-94.3% of the EPS released in the same time frame. From 48-120 hrs of fermentation, the largest polymers produced (5-30 kDa) composed 66.1 to 84.2% of the polymers released.

Conversely, *B. amyloliquefaciens* (23350) produced EPS with a monosaccharide profile primarily composed of glucose when the bacterium was cultured in M1. At 24 hrs, *B. amyloliquefaciens* (23350) produced EPS composed of slightly higher amounts of fructose (14.8%) and xylose (13.6%) which were detected along with glucose (70.3%) (**Figure 3.2, C**). Due to the high proportion of glucose production, it is likely that the backbone of the polysaccharide structure is composed of glucans with branches containing other monosaccharides. The results also revealed a shift from an abundant high-MW distribution (>500 kDa) at the early stage to the low-MW (<5 kDa) distribution at the later stage of the fermentation (**Figure 3.3**). These results may be attributed to the hydrolysis of EPS and their use as a carbon source as denoted by the fermentation time course. Very little information about EPS production by *B. amyloliquefaciens* strains is available. In a recent study, Han *et al*., (2015) reported that *B. amyloliquefaciens* LPL061 produced EPS primarily composed of mannose (65.3-96.9%), followed by glucose (3.1-34.7%). *B. amyloliquefaciens* (23350) was expected to produce levan due to the presence of intra and extracellular levansucrase (Tian, et al., 2011). These results may reveal the inhibition of levansucrase enzyme in the presence of greater concentrations of sucrose. Therefore the abundance of glucose and the formation of glucans would be directly related to the high concentration of glucose present with the substrate that was utilized to form the polymers.

As was the case for *B. amyloliquefaciens* (23350), *B. subtilis* (B26) produced EPS containing primarily glucose as monosaccharide units throughout the whole period of fermentation, with the

lowest molar proportion of 96.3% recorded at 96 hrs. While conclusions cannot be drawn without the characterization of glyosidic linkages involved in this polysaccharide, it is likely to be a glucan (**Figure 3.2, D**). The MW distribution of the EPS produced by *B. subtilis* (B26) over the time course of fermentation showed a tendency to become higher MW as the time course progressed. From 4 to 9 hrs of fermentation, the EPS extracts were exclusively composed of polysaccharides with <5 kDa MW, while polysaccharides with >500 kDa MW appeared at proportions of 4.7 to 6.4% from 48 to 120 hrs of fermentation (**Figure 3.3**). Like *B. amyloliquefaciens,* some strains of *B. subtilis* such as *B. subtilis* natto have been studied and shown to produce low MW (<50.000 – 568.000 Da) levan (Santos, et al., 2013).

Our results also show that *B. amyloliquefaciens* (OB6) produced EPS primarily composed of glucose (63.4-93.8%) molecules with the presence of xylose (5.4-18.6%) and fructose (0.4-15.9%). Notably, the greatest presence of xylose and fructose was observed at 144 hrs, where the polysaccharides were predominated by glucose (63.4%), xylose (18.6%) and fructose (15.9%) (**Figure 3.2, E**). *B. amyloliquefaciens* (OB6) produced EPS composed of 51.3% of 5-30 kDa MW polysaccharides and 48.7% of <5 kDa MW. After 9 hrs of fermentation, the MW distribution of EPS became 29.6% of 30-100 kDa and 70.4% of <5 kDa EPS. From 48-120 hrs, all the polysaccharides produced were low MW sized at less than 5 kDa (**Figure 3.3**).

The variability in the types of EPSs produced during fermentation can be attributed to the availability of nutrients, the rate of their metabolism, the levels of the precursor produced and the expression of various enzymes by the bacterium (Srivastava & Srivastava, 2003; Donot, et al., 2012). While certain bacterial species are capable of producing more than one type of polysaccharide, simultaneous secretion of two or more EPS types is unusual (Sutherland, 2004). For example, some *Pseudomonas* spp. can produce either alginates or levan; while *Azotobacter chroococcum* produces both alginate and some type-specific EPS. *E. agglomerans* 1.15 can produce colonic acid type polysaccharides with one or more sugar nucleotide precursors shared, and depending on its environment, can also produce glucomannan and/or cellulose (Sutherland, 1990; Sutherland, 2004).

With the lack of information on the carbon linkages between the types of monosaccharides for each EPS sample produced, it may be speculated that the strains studied herein would produce novel polysaccharides. This holds particularly for *B. licheniformis* (14580), *B. amyloliquefaciens*

(23350) and *B. amyloliquefaciens* (OB6), as the partial characterization of the polysaccharides produced by these bacteria revealed that they were more heterogeneous than the other strains (Ghaly, et al., 2007; Larpin, et al., 2002). In the case of *B. amyloliquefaciens* (23350), it was observed that the use of the same strain in the same growth media with a higher concentration of substrate resulted in the inhibition of levansucrase enzyme which would normally have resulted in the production of levan, the possible yielded glucan with traces of xylose and fructose would lead to the discovery of a different type of polysaccharide produced by the same bacterial strain under slightly different conditions.

Out of the four *Bacillus* strains studied, *B. licheniformis* (14580) was chosen for further studies with the optimization of EPS production in M2. This choice was based on the high EPS yield (48.57 g/L); the second highest yield after that of *B. amyloliquefaciens* (OB6) (54.14 g/L) and the heterogeneous monosaccharide profile of the EPS extracts that this strain produces, suggesting a possible discovery of a novel polysaccharide. In addition, *B. licheniformis* (14580) grown with M2 is also of interest for its potential to have an industrial application. The results of the physical and structural characteristics of the polysaccharides produced in liquid media are summarized in **Table 3.3**.

Table 3.3. A Summary of the Efficiency of Selected *Bacillus* Species to Produce EPS and Structural Properties of their Produced EPS

Bacteria	Media	Time (h)	Biomass (g/L culture)	EPS (g/L culture)	Molecular Weight (kDa)	Monosaccharide Profile Dominant Monosaccharide
B. licheniformis (14580)	M1	144	5.84	3.50	30-100	Heteropolymers Glucose/ Galactose
	M2	96	90.44	48.57	5-30	Heteropolymers Glucose/Galactose/ Fructose
B. amyloliquefaciens (23350)	M1	11	13.84	6.74	<5	Glucose
B. subtilis (B26)	M3	72	10.60	6.59	<5	Glucose
B. amyloliquefaciens (OB6)	M1	48	9.72	7.46	N/A	Heteropolymers Glucose/Xylose
	M2	144	96.38[a] 83.50[b]	54.14	<5	Heteropolymers Glucose/Xylose/ Fructose

[a] obtained at 72 hrs
[b] obtained at 144 hrs

3.4 Optimization of *B. licheniformis's* Exopolysaccharide (EPS) Production

Response surface methodology (RSM) was used to investigate the EPS production by *B. licheniformis* in culture media of varying compositions. Indeed, attempts to optimize the culture medium were made by varying three major nutrient components: X_1 - sodium succinate (g/L), X_2 - yeast extract (g/L) and X_3 - sucrose (g/L). The most significant differences between M1 and M2 were the concentration difference between yeast extract as the nitrogen source (10 g/L – M1; 100 g/L – M2) and the presence of sodium succinate (100 g/L) in M2 at equal proportion with yeast extract. Based on the previous results, it was hypothesized that these two ingredients are critical to EPS production. Certainly, sodium succinate is an effective buffering agent when it disassociates into a weak acid and is known to prevent drastic drops in the pH in laboratory media (Matsumoto, 1994). Sucrose is routinely used at a concentration exceeding 100 g/L as an inducer for EPS production, as it is the best known substrate to stimulate EPS production (Lee, et al., 1997; Badel, et al., 2011; Donot, et al., 2012).

The experiments were designed using the Stat-Ease, Inc software Design Expert. Twenty runs were set based on the central composite rotatable design (CCRD). For each run, sodium succinate and yeast extract contents varied from 0-100 g/L and from 10-200 g/L, respectively, whereas sucrose concentration varied from 100-400 g/L (**Table 3.4**). *B. licheniformis* was grown at 35°C in M2 medium with all its other ingredients remaining unchanged. The biomass yield (g/L culture), the EPS yield (g/L culture) and the protein content (g/g biomass) were the three investigated responses that were determined for each run at 48, 72, 96 and 120 hours. The biomass was estimated after ethanol precipitation and lyophilization, whereas the EPS yield was quantified by measuring the total carbohydrate content. The protein content was determined using DUMAS. **Table 3.4** summarizes the experimental conditions and the corresponding responses.

Table 3.4. CCRD Design for the Optimization of the Exopolysaccharide Production by *B. licheniformis* (14580) and the Experimental Results

Runs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sodium Succinate (g/L)		50.00	79.73	79.73	100.00	79.73	50.00	20.27	50.00	50.00	20.27	50.00	50.00	50.00	79.73	50.00	50.00	20.27	50.00	0.00	20.27
Yeast Extract (g/L)		200.00	48.51	161.49	105.00	161.49	105.00	161.49	105.00	105.00	161.49	105.00	10.00	105.00	48.51	105.00	105.00	48.51	105.00	105.00	48.51
Sucrose (g/L)		250.00	160.81	160.81	250.00	339.19	400.00	339.19	250.00	250.00	160.81	250.00	250 .00	250.00	339.19	100.00	250.00	339.19	250.00	250.00	160.81
Biomass (g/L culture)	**48**	34.35	35.44	36.55	38.56	37.62	36.70	34.28	35.02	34.99	34.05	37.35	36.71	37.12	39.49	34.78	37.13	33.63	36.90	33.60	33.93
	72	36.00	35.60	36.42	38.04	39.56	38.34	34.54	36.05	36.24	34.37	36.93	36.53	36.92	38.85	34.75	36.94	34.02	36.94	33.70	33.97
	96	36.98	34.95	36.85	38.15	39.85	38.62	34.81	36.50	36.67	34.53	37.07	35.76	37.03	38.40	34.68	37.02	34.30	36.92	33.72	33.91
	120	36.48	34.61	36.20	37.71	39.39	38.65	34.82	36.34	34.49	26.65	36.55	36.36	36.66	38.34	33.13	36.85	34.32	36.67	33.71	33.88
EPS (g/L culture)	**48**	17.63	30.49	23.44	31.05	28.56	34.07	24.15	32.09	26.79	17.24	37.35	36.71	34.69	38.64	31.94	34.94	29.65	33.75	21.18	31.90
	72	28.77	32.24	27.58	30.86	36.10	36.40	28.94	33.79	32.17	9.81	34.75	36.53	36.69	38.04	28.71	32.17	32.67	34.98	23.76	26.50
	96	23.82	27.42	26.94	28.75	29.24	35.86	27.03	33.40	30.12	8.53	33.77	35.76	33.88	37.30	26.74	33.13	32.38	33.99	17.87	18.94
	120	27.94	26.18	25.99	31.30	33.73	37.96	26.63	33.57	34.16	7.97	25.97	36.36	33.65	37.67	26.45	39.98	32.17	33.93	16.30	19.14
Protein (g/g biomass)	**48**	0.15	0.01	0.08	0.02	0.09	0.07	0.12	0.05	0.06	0.20	0.00	0.00	0.07	0.02	0.08	0.07	0.07	0.09	0.13	0.04
	72	0.12	0.01	0.09	0.05	0.09	0.06	0.12	0.06	0.06	0.29	0.07	0.00	0.07	0.02	0.06	0.07	0.05	0.06	0.19	0.13
	96	0.13	0.02	0.10	0.04	0.10	0.07	0.15	0.06	0.04	0.30	0.09	0.00	0.09	0.03	0.08	0.07	0.06	0.08	0.23	0.17
	120	0.15	0.05	0.13	0.07	0.10	0.06	0.16	0.08	0.06	0.33	0.10	0.00	0.08	0.02	0.11	0.08	0.07	0.08	0.26	0.25

3.4.1 Analysis of Variance and Model Fitting

Mathematical models were fitted to the experimental data using Design-Expert software version 9.0.6, and the significance of the models were tested with ANOVA (**Table 3.5**). The significance of each coefficient was determined by using the *F* and *p* values as shown in **Table 3.5.** The variables are deemed more significant if the *F*-value is larger and the *p*-value is smaller. Each response was investigated at four separate hours of 48, 72, 96 and 120. The biomass (g/L culture) response was determined to follow a linear model at 48 hrs, a quadratic model from 72-96 hrs and followed a linear model once more at 120 hrs. The EPS (g/L of culture) response followed a quadratic relationship from 48-96 hrs before becoming a linear model at 120 hrs. Finally, the third response, protein content (g/g biomass) followed a linear model at 48 hrs before following a quadratic model from 72-120 hrs. The lack of fit was not significant relative to pure error with *F* values of 5.55 to 29.20 and *p*-values of >0.0001 to 0.0065. These results indicate the good quality of the fit and its ability to predict within a range of the variables employed. In addition, the coefficient of determination (R^2) values were 0.91-0.98 at the intermediate stage of fermentation (72-96 hrs), revealing the appropriateness of the models to predict the responses at this stage.

During the fermentation (48 to 120 hrs), the variables with the most significant effects on the biomass yield were the linear terms of sodium succinate concentration (X_1, *F*-value of 24.64-239.58, *p*-value of < 0.0001-0.0003) and the sucrose concentration (X_3, *F*-value of 5.33-132.76, *p*-value of <0.0001-0.0437). As expected, these variables were more significant at the intermediate stage of fermentation (72-96 hrs) than at later (120 hrs) stages. In addition to the linear terms of sodium succinate (X_1, *F*-value of 9.06-22.65, *p*-value of 0.0008-0.0131) and sucrose (X_3, *F*-value of 3.47-29.23, *p*-value of 0.0003-0.0922) concentrations, the yeast extract concentration (X_2, *F*-value of 14.52-36.03, *p*-value of 0.0001-0.0034) had a significant linear effect on the EPS yield at the intermediate stages of the fermentation (48 to 96 hours). Contrary to the biomass and EPS yields, the protein content was significantly affected by the linear terms of all variables (*F*- value of 0.33-61.56, *p*-value of <0.0001-0.5769), at all investigated fermentation times, with sucrose concentration being the most significant at the early stages of reaction while the impact of yeast extract concentration was more significant at later stages. Among all interactive effects, the most important was the one between the sodium succinate and the sucrose concentrations (X_1X_3) for the EPS yield model (*F*-value of 24.7-34.4, *p*-value of 0.0002-0.0006) at 72 to 96 fermentation hours,

for the protein content model (F-value of 10.65-13.68, p-value of 0.0041-0.0085) at 72 to 120 hrs and for the EPS yield model only at 96 hrs (F-value of 5.52, p-value of 0.0407). These results demonstrated a possible synergistic action between the carbon sources. The interaction between the sodium succinate and yeast extract concentrations (X_1X_2) was significant only at the advanced stage of the fermentation (96 to 120 hrs) in the biomass yield model (F-value of 5.06-5.72, p-value of 0.0326-0.0482) (**Table 3.5**).

It is known that nitrogen limiting conditions tend to enhance EPS production, as well as the fact that sucrose is usually the most appropriate substrate for EPS production (Cerning, 1990; Sutherland, 1990; Kimmel, et al., 1998). In M2 medium, yeast extract acts both as limited nitrogen and carbon source, the interaction therefore between higher sucrose and lower amounts of yeast extract resulting in higher yields of biomass and EPS is theoretically valid. Sodium succinate in M2 medium acts as a buffer and, therefore, the presence of succinate along with phosphates in the base media, enhance EPS production due to higher internal regulation of the pH, which may explain the performances of M2 (Sutherland, 2004).

The fitted models for biomass yield, EPS yield and protein content in terms of coded factors at selected fermentation times are given by Equations 3 to 14.

$$Biomass\ \left(\frac{g}{L}culture\right)_{48\ h} = 35.91 + 1.58X_1 + 0.61X_3 \quad (3)$$

$$Biomass\ \left(\frac{g}{L}culture\right)_{72\ h} = 36.48 + 1.53X_1 + 0.93X_3 + 0.76(X_1X_3) - 0.34X_1^2 \quad (4)$$

$$Biomass\ \left(\frac{g}{L}culture\right)_{96\ h} = 36.69 + 1.49X_1 + 0.48X_2 + 1.04X_3 + 0.28(X_1X_2) + 0.72(X_1X_3) - 0.36X_1^2 - 0.20X_2^2 \quad (5)$$

$$Biomass\ \left(\frac{g}{L}culture\right)_{120\ h} = 35.56 + 1.84X_1 - 0.31X_2 + 1.79X_3 + 1.22(X_1X_2) \quad (6)$$

$$Crude\ EPS\ yields\ \left(\frac{g}{L}culture\right)_{48\ h} = 33.12 + 2.55X_1 - 5.08X_2 - 2.61X_1^2 - 2.24X_2^2 \quad (7)$$

$$Crude\ EPS\ yields\ \left(\frac{g}{L}culture\right)_{72\ h} = 32.59 + 3.82X_1 - 3.24X_2 + 4.16X_3 + 2.25(X_1X_2) + 2.49(X_2X_3) - 2.63X_1^2 \quad (8)$$

$$Crude\ EPS\ yields\ \left(\frac{g}{L}culture\right)_{96\ h} = 31.24 + 3.83X_1 - 3.25X_2 + 4.07X_3 - 2.47(X_1X_3) - 3.62X_1^2 \quad (9)$$

$$Crude\ EPS\ yields \left(\frac{g}{L}culture\right)_{120\ h} = 31.79 + 4.70X_1 - 2.46X_2 + 5.25X_3 - 3.81X_1^2 \quad (10)$$

$$Protein\ content\ \left(\frac{g}{g}biomass\right)_{48\ h} = 0.071 - 0.030X_1 + 0.043X_2 \quad (11)$$

$$Protein\ content\ \left(\frac{g}{g}biomass\right)_{72\ h} = 0.069 - 0.049X_1 + 0.045X_2 - 0.020X_3 + 0.038(X_1X_3) + 0.024X_1^2 \quad (12)$$

$$Protein\ content\ \left(\frac{g}{g}\ biomass\right)_{96\ h} = 0.076 - 0.054X_1 + 0.044X_2 - 0.017X_3 + 0.032(X_1X_3) + 0.027X_1^2 \quad (13)$$

$$Protein\ content\ \left(\frac{g}{g}biomass\right)_{120\ h} = 0.090 - 0.062X_1 + 0.042X_2 - 0.037X_3 + 0.040(X_1X_3) + 0.034X_1^2 \quad (14)$$

Table 3.5. Analysis of Variance for Response Surface Model of Biomass Yield (g/L culture), EPS Yield (g/L Culture) and Protein Yield (g/g biomass)

	Biomass Yield (g/L culture)								EPS Yield (g/L culture)								Protein Yield (g/g biomass)							
	48 h		72 h		96 h		120 h		48 h		72 h		96 h		120 h		48 h		72 h		96 h		120 h	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value	Value
Model	5.55	0.0065	29.20	< 0.0001	9.32	0.0004	9.32	0.0004	7.37	0.0022	12.43	0.0002	7.03	0.0027	7.03	0.0027	7.38	0.0022	15.82	< 0.0001	12.55	0.0002	12.55	0.0002
$^{\Delta}X_1$	36.03	0.0001	160 .55	< 0.0001	239.58	< 0.0001	24.64	0.0003	9.06	0.0131	20.38	0.0011	22.65	0.0008	17.73	0.0018	18.51	0.0016	39.52	< 0.0001	61.56	< 0.0001	45.05	< 0.0001
$^{\circ}X_2$	1.21	0.2971	0.89	0.3686	25.58	0.0005	0.55	0.4729	36.03	0.0001	14.52	0.0034	16.28	0.0024	5.13	0.0469	39.60	< 0.0001	33.95	0.0002	39.55	< 0.0001	21.20	0.0010
$^{\square}X_3$	5.33	0.0437	59.37	< 0.0001	132.76	< 0.0001	23.16	0.0003	3.47	0.0922	24.22	0.0006	29.23	0.0003	22.11	0.0008	0.33	0.5769	6.05	0.0337	6.98	0.0246	15.96	0.0025
X_1X_2	0.31	0.5896	0.24	0.6315	5.06	0.0482	5.72	0.0326	0.12	0.7391	3.81	0.0793	0.74	0.4105	1.08	0.3233	1.20	0.2997	2.18	0.1708	0.96	0.3504	0.017	0.8983
X_1X_3	3.59	0.0873	24.69	0.0006	34.45	0.0002	0.21	0.6570	0.95	0.3534	2.61	0.1371	5.52	0.0407	1.35	0.2717	0.87	0.3720	13.68	0.0041	12.67	0.0052	10.65	0.0085
X_2X_3	0.79	0.3963	3.921E-004	0.9846	0.31	0.5881	3.34	0.0906	0.48	0.5039	4.72	0.0548	0.089	0.7712	9.642E-003	0.9237	2.90	0.1192	2.30	0.1605	0.70	0.4212	0.11	0.7505
X_1^2	0.32	0.5843	9.89	0.0104	17.27	0.0020	-	-	10.20	0.0096	11.41	0.0070	24.04	0.0006	13.11	0.0047	1.36	0.2712	10.34	0.0093	16.11	0.0025	14.26	0.0036
X_2^2	1.77	0.2127	4.03	0.0725	6.36	0.0303	-	-	7.51	0.0208	1.26	0.2875	4.16	0.0686	1.01	0.3385	1.00	0.3403	0.14	0.7123	0.23	0.6414	0.25	0.6262
X_3^2	1.07	0.3253	1.43	0.2595	2.88	0.1205	-	-	0.056	0.8174	1.36	0.2709	0.85	0.3772	0.98	0.3462	1.27	0.2867	0.11	0.7500	0.12	0.7383	0.66	0.4341
Lack of Fit	0.55	0.7376	4.36	0.0658	4.09	0.0741	3.84	0.0774	0.50	0.7662	4.94	0.0521	3.92	0.0801	0.65	0.6782	0.41	0.8234	2.70	0.1499	3.30	0.1081	1.51	0.3319
R^2	0.83		0.96		0.98		0.81		0.87		0.92		0.91		0.86		0.87		0.93		0.93		0.92	

P < 0.05 indicates statistical significance
$^{\Delta}X_1$ – Sodium Succinate (g/L)
$^{\circ}X_2$ – Yeast Extract (g/L)
$^{\square}X_3$ – Sucrose (g/L)

3.4.2 Effects of Culture Medium Composition on Biomass Production

At 48 hrs, the biomass (all polymers) production followed a linear relationship before the model of best fit became quadratic. The results show that, at 48 hrs of fermentation, maximizing sucrose (g/L) and sodium succinate (g/L) concentrations in the culture medium allows for greater biomass production (**Figure 3.4**). Media containing a maximum concentration of either sucrose or sodium succinate favored the biomass production, revealing the synergistic interaction of these two carbon sources. However, at 72 hrs of fermentation, the addition of yeast extract at concentrations lower than 161.49 g/L had no significant effect on the biomass production, while at 161.49 g/L, with increased concentrations of sucrose and sodium succinate, the interaction between these nutrients increased the biomass production (**Figure 3.4**). At 96 hrs of fermentation, the interaction between sodium succinate and sucrose had the most significant effect showing the same trend as that observed at 72 hrs. However, varying concentrations of yeast extract (g/L) exhibited more significant effect on the biomass yield at this fermentation time, where the addition of 161.49 g/L of yeast extract has resulted in the production of a higher amount of biomass (36.48 g/L culture), providing that the sucrose and sodium succinate concentrations are maximized. At 120 hrs, the interaction of sodium succinate (g/L) and the yeast extract (g/L) concentrations had the most significant effect, as the highest yield of biomass was produced when the concentrations of sodium succinate and yeast extract were maximized in the presence of 339.19 g/L of sucrose (**Figure 3.4**). The lowest biomass yields were obtained when the yeast extract concentration was maximized in the presence of low concentration of sodium succinate.

3.4.3 Effects of Culture Medium Composition on EPS Production

The behavior of EPS yields (g/L culture) over time showed that the fermentations followed a quadratic model from 48-96 hrs to become linear at 120 hrs, while at 48 hours no interaction between the linear terms was considered significant (**Equations 7-10**). At 72 hrs, the most significant interaction in the EPS yield model was that of yeast extract and sucrose concentrations (X_2X_3), which showed different degrees of significance depending on the sodium succinate concentration. At low sodium succinate concentration of 20.27 g/L, and yeast extract concentration at its lowest level of 48.51 g/L EPS yields were maximized. It could, therefore, be concluded that the increase of yeast extract concentration decreases the yield of EPS, revealing the negative effect of yeast extract on EPS production.

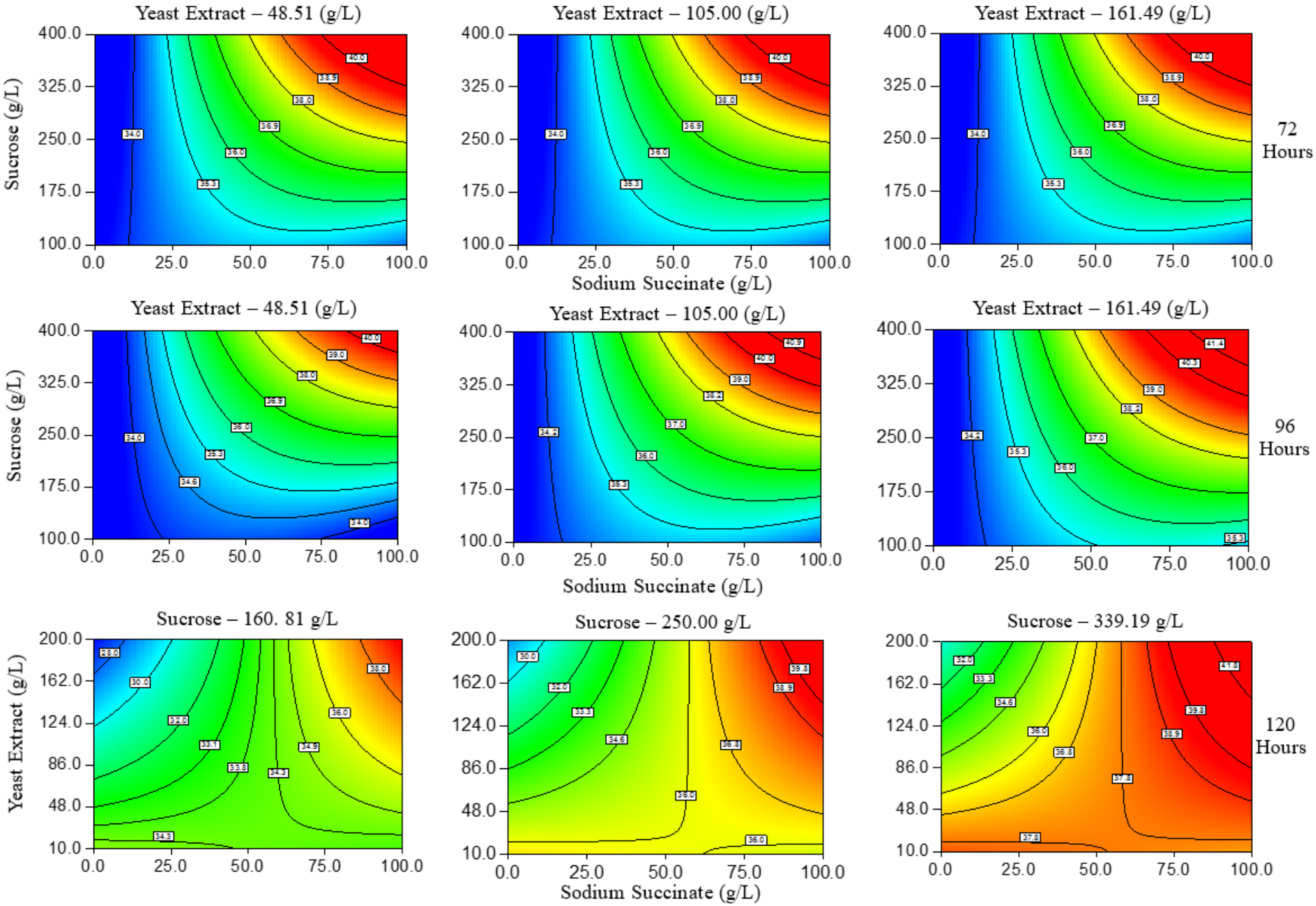


Figure 3.4. Contour plots demonstrating biomass yields (g/L culture) obtained by *B. licheniformis* (14580) with M2 optimization over time

The negative effect of yeast extract on the EPS yields seem to be mitigated by the high concentrations of sucrose and sodium succinate. Indeed, at the high sodium succinate concentration of 79.73 g/L, the yeast extract concentration has no significant effect on the EPS yield when the sucrose concentration was higher than 250 g/L (**Figure 3.5**). This is congruent with findings of Lee *et al.*, (1997) and Sutherland, (1990, 2004) demonstrating the role of high carbon and low nitrogen sources in media in the stimulation of EPS production. The relationship between sucrose and sodium succinate (X_1X_3) demonstrated similar results at 96 hrs to those observed at 72 hrs. The interaction between these media ingredients (X_1X_3) was such that by maximizing sodium succinate and sucrose concentrations and minimizing yeast extract to 48.51 g/L, yielded greater amounts of EPS (g/L culture). These yields did not vary significantly when yeast extract concentrations were 48.51 g/L and 105 g/L (yielding 36.29 g/L and 33.04 g/L, respectively) but were exceptionally low when yeast extract concentration was as high as 161.49 g/L.

3.4.4 Effects of Culture Medium Composition on Protein Content

Protein yields (g/g biomass) were the third most important parameter investigated in view of the optimization of EPS production. This response was studied to determine the culture condition that would produce the least contaminated EPS with proteins. For further purification of the optimized EPS yielded, it is important to know the protein content in the EPS to determine the extent of purification required to obtain the purest possible samples of EPS for a more detailed study.

The contour plots indicate that the protein content of the EPS produced varied from 0.01 to 0.45 g/g biomass; representing 1–45% of total EPS depending on the nutrient concentration of the culture medium. At 72 to 120 hrs, the interaction that most significantly impacted the protein content (g/g biomass) was sucrose and sodium succinate, which demonstrated greater protein yields in the biomass when sucrose and sodium succinate concentrations are minimal. Increasing yeast extract concentration to 161.5 g/L and fermentation time to 120 hours increased the content of protein contaminants. These results indicate clearly that in order to obtain protein-free EPS, maximum (+1) amounts of sucrose and sodium succinate should be added, while the addition of yeast extract must be carefully monitored (**Figure 3.6**).

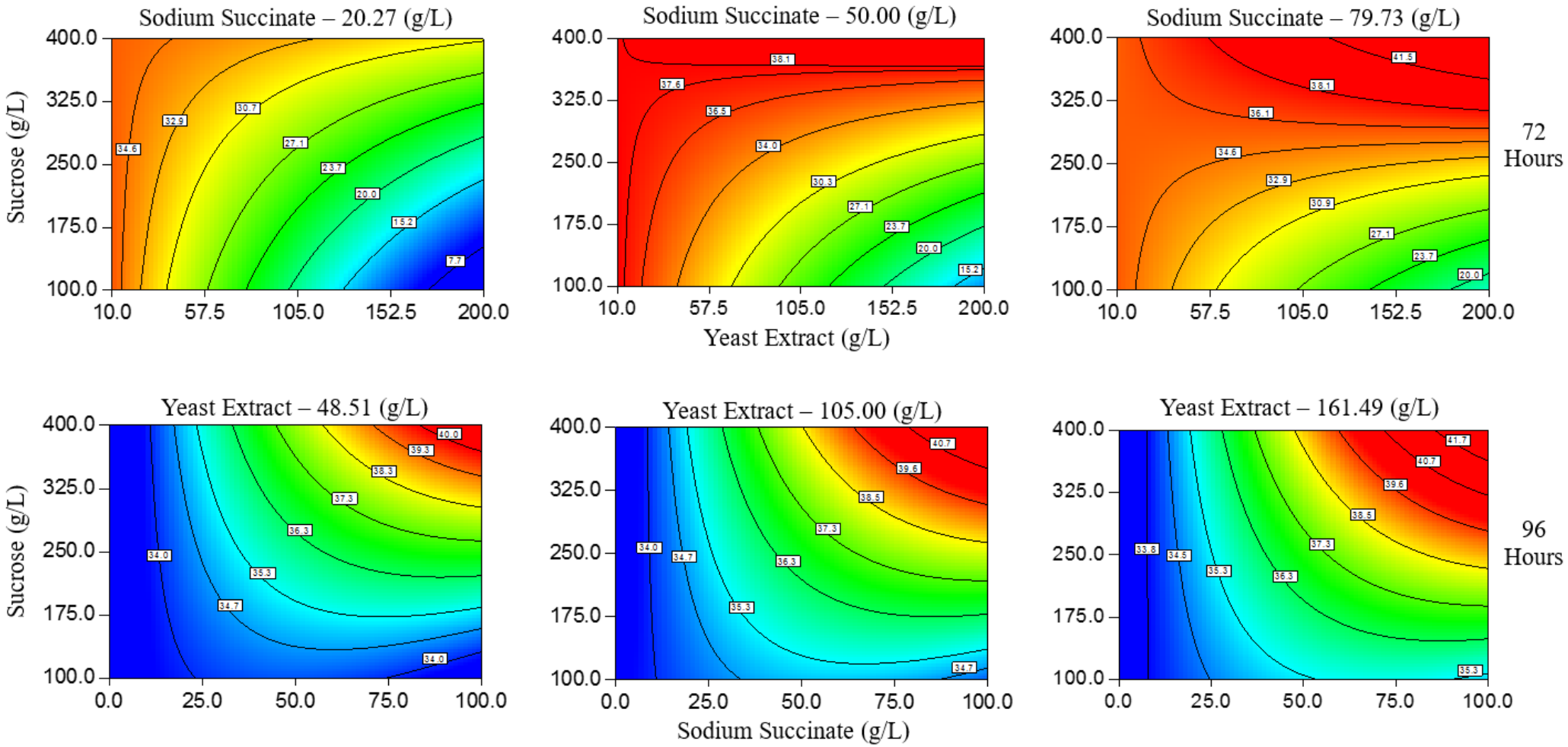


Figure 3.5. Contour plots of EPS yields (g/L culture) obtained by growing *B. licheniformis* (14580) in optimized M2 over time

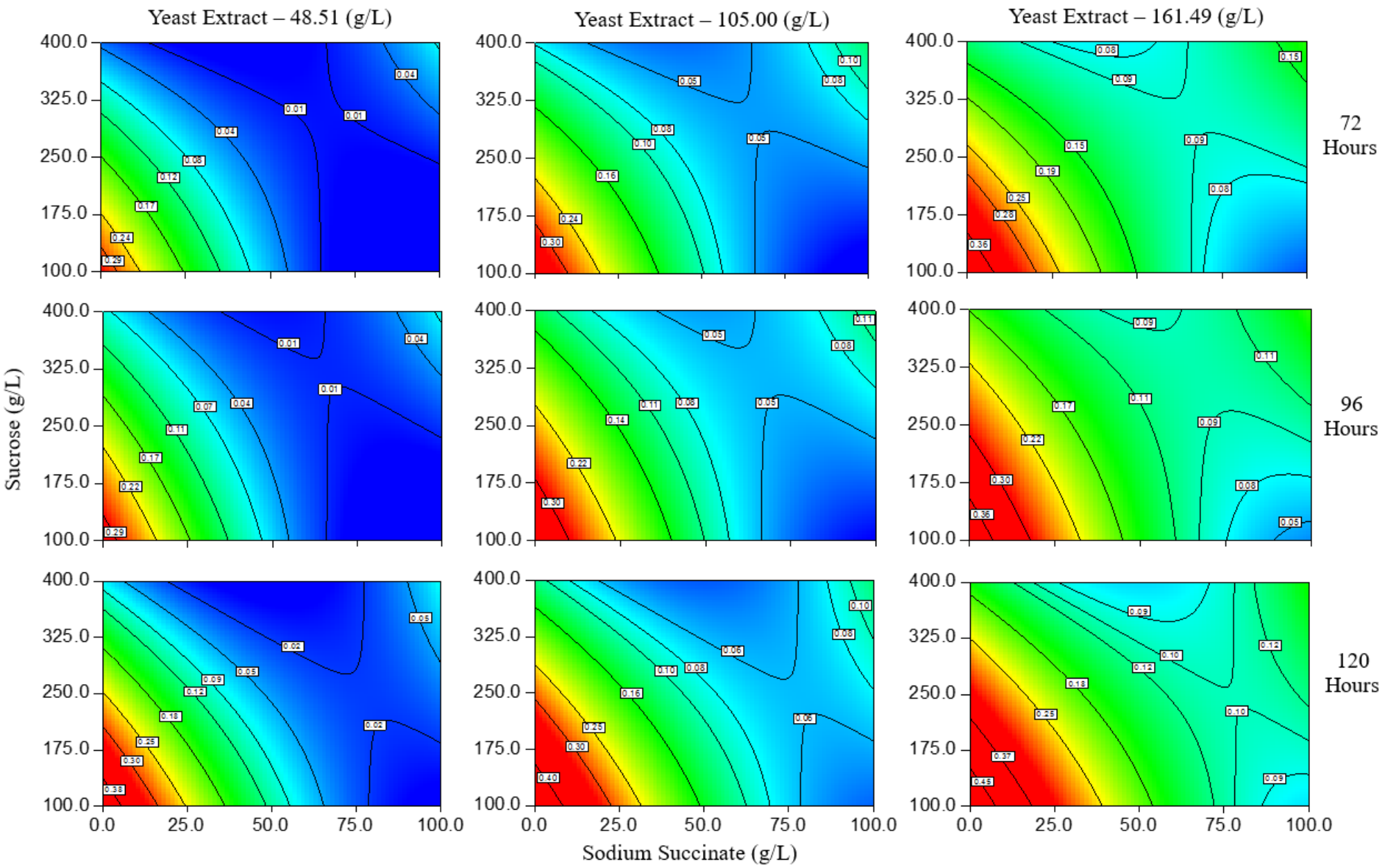


Figure 3.6. Contour plots for protein content (g/g biomass) obtained by growing *B. licheniformis* (14580) in optimized M2 over time

4. Conclusions

Microbial EPS production by *Bacillus* spp has been shown to be a very promising approach. The investigated strains were found to produce high amounts of EPS with yields (3.50 - 54.14 g/L) greatly exceeding those reported for LAB (0.100 - 28.85 g/L) (Petry, et al., 2000; Yadav, et al., 2011) and more/less within the same range as those reported for *Bacillus spp* (0.88 - 70.6 g/L) (Larpin, et al., 2002; Shih, et al., 2010). The EPS production appears to be not only dependent on the type of strain, but also on the media and fermentation conditions. The use of mineral base-medium with added yeast extract (M1) favored the production of low molecular weight polymers (<5 kDa) composed primarily of glucose by *B. amyloliquefaciens* (23350); and higher molecular weight heteropolymers (30-100 kDa) composed mainly of galactose and glucose by *B. licheniformis* (14580). Succinate-containing mineral base-medium with added yeast extract (M2) yielded the highest amounts of EPS, producing heteropolymers with *B. amyloliquefaciens* (OB6) composed of low molecular weight polysaccharides (<5 kDa) containing glucose, xylose and fructose molecules. With *B. licheniformis* (14580), M2 medium allowed for the production of great yields of heteropolymeric EPS that dominantly produced polymers of 5-30 kDa MW and composed of glucose, galactose and fructose. With tryptone and yeast extract-containing base medium (M3), *B. subtilis* produced low molecular weight (<5 kDa) polymers primarily composed of glucose.

The interactive effects of carbon/nitrogen sources and the inducer concentrations were investigated by response surface methodology (RSM) on *B. licheniformis* (14580) with M2 medium. The obtained results demonstrated that maximizing concentrations of sodium succinate and sucrose had the greatest effects on biomass yields from 72-96 hrs. By 120 hrs, the interaction of interest switched to the concentrations of yeast extract and sodium succinate, where the greatest EPS yields were obtained at higher concentrations of sucrose. EPS (g/L of culture) was influenced at 72 hrs by the interaction between yeast extract and sucrose, where it was observed that the highest EPS yields were obtained when greater concentrations of sodium succinate and sucrose were added to the media with lower concentrations of yeast extract. The protein content (g/g biomass) observed in the obtained samples showed that the interaction of significance was between sucrose and sodium succinate from 72 to 96 hrs of fermentation and greater amounts of contaminating proteins were observed when sucrose and sodium succinate concentrations were minimized and yeast extract concentration were maximized.

Overall, studying the production of EPS by *Bacillus* spp possibly led to the production of novel polysaccharides and revealed that modulating the organisms' microenvironment and the composition of the nutrients in culture media can lead to a variety of polysaccharides with different properties.

Recommendations for Future Work:

(1) Determination of the type of linkages between the monosaccharide to determine structural conformation of the yielded EPS
(2) Investigation of the functional properties of produced EPS to determine how best they can be incorporated into food products and the health benefits that they can confer.
(3) Identification of the putative novel polysaccharides

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