Effect of Basil Seed Gum and Its Hydrolyzed Oligosaccharides on Yogurt Culture Growth and Yogurt Quality

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Abstract

Basil seeds are surrounded with a mucilage layer that has a remarkable swelling index in water and a high-water holding capacity. Basil seed gum (BSG) from this mucilage layer is a potential novel stabilizer for the food industry. The heterogeneous structure of BSG can affect digestibility in the body but it brings health benefits, such as balancing blood sugar levels, blood cholesterol and weight management, and is a good source of dietary fibre.

The primary objective of this study was to examine the effects of basil seed gum (BSG) extracted from RO water in yogurt production. Secondly, it aimed to investigate the influence of enzyme-hydrolyzed BSG oligosaccharides on yogurt cultures and yogurt quality. Additionally, the study explored the use of erythritol as a sucrose replacement in yogurt production and its potential effects. Overall, this research examines the potential of BSG and its oligosaccharides as functional ingredients for enhancing yogurt production.

The gum layer was successfully extracted in RO water instead of alkaline water, with a dry gum yield of 24.27 ± 0.64% at 50^{o}C, 1000 rpm for 2 h. BSG added at 0.06% to yogurt fermentation improved gel stability and water-holding capacity in yogurt samples but did not enhance the growth of YO-MIX® 726 yogurt cultures. BSG-oligosaccharides obtained through hydrolysis with xylanase in RO water under optimal conditions (E/S 2, 4 h, 65^{o}C) significantly improved the growth parameters of YO-MIX® 726 *Lactobacillus acidophilus*. The highest cell density (Max) of 1.02 OD_{595nm} was observed at 4% BSG-Oligosaccharides with maximum growth rate (μ_{Max}) at 0.10 h^{-1}, compared to the control with 0.29 OD_{595nm} and 0.06 h^{-1}, respectively.

However, the highest prebiotic score (0.53) was observed at 3% of BSG-Oligosaccharides obtained from xylanase hydrolysis in RO water. BSG-Oligosaccharides enhanced the growth of YO-MIX® 726 probiotics at a concentration 1.5% and strongly protected cell survival after 21-Day shelf-life storage at 4^{o}C. Meanwhile, erythritol had no significant effect on yogurt quality or the yogurt cultures at ≤ 8%.

A novel yogurt formulation containing 0.03% water-extracted BSG, 1.5% BSG-Oligosaccharides from xylanase-hydrolyzed BSG in water, and 4% erythritol as a sugar substitute demonstrated reduced whey separation, improved bacterial growth, and enhanced probiotic survival. This product shows promise as a low-glycaemic, probiotic functional food with potential prebiotic benefits.

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List of Abbreviations

Abbreviation	Terminology
μ_{Max}	maximum growth rate
0d	Day 0
1d	Day-1
21d	Day-21
ACS	American Chemical Society grade
AR	Analytical Reagent grade
BBD	Box-Behnken design
Blk	Enzyme blank without basil seed gum
BSG	Basil seed gum
BSG-Oligos	Oligosaccharides hydrolyzed from basil seed gum
CE-3.15	Oligosaccharides obtained from CE35 cellulase hydrolysis of basil seed gum in pH 3.15 buffer solution
CE	CE35 cellulase
CE-Blank	Residue of CE35 cellulase blank after hydrolysis and purification steps in pH 3.5 buffer solution
CE-H_2O	Oligosaccharides obtained from hydrolyzed by CE35 cellulase hydrolyzed basil seed gum in water
DP	degree of polymerization
DVS	Direct vat set
E/S	enzyme and substrate ratio
EC	*E. coli* NCTC 8196
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
FOSHU	Food for specified health uses
FSANZ	Food Standards Australia New Zealand
GI tract	Gastrointestinal tract
GOS	galactooligosaccharides
GuOS	glucooligosaccharides
KGM	Konjac glucomannans

LA	*Lactobacillus acidophilus*
LAB	Lactic acid bacteria
lag	lag phase
LB	*Lactobacillus delbrueckii subsp. bulgaricus*
Max	maximum cell density
MOS	maltooligosaccharides
MRS	Molten de Man Rogosa Sharpe
OD_{595nm}	Optical density (absorbance) at 595 nm
Prob	Probiotics
RDI	Recommended Dietary Intake
RO	Reverse osmosis
R-sq	The coefficient of determination
SCFA	short-chain fatty acid
SMP	Skimmed milk powder
SQRT	Square root
ST	*Streptococcus thermophilus*
TTA	total titratable acidity
XOS	xylooligosaccharides
Xs	SQZyme xylanase
Xs-3.5	Oligosaccharides obtained from SQZyme xylanase hydrolysis of basil seed gum in pH 3.5 buffer solution
Xs-Blank	Residue of SQZyme xylanase blank after hydrolysis and purification steps in pH 3.5 buffer solution
Xs-blank-H2O	Residue of SQZyme xylanase blank after hydrolysis and purification steps in water
Xs-H_2O	Oligosaccharides obtained from hydrolyzed by SQZyme xylanase hydrolyzed basil seed gum in water
ZAC	MagiZyme ZAC enzymes

Chapter 1 Introduction

Yogurt is widely accepted as a complementary functional food due to its high protein, low fat, essential minerals, and presence of probiotics, particularly in skim milk-based varieties (Sarkar, 2019; Thomas et al., 2004). Many consumers choose yogurt with probiotics for its health benefits (Martirosyan & Singh, 2015; Meybodi et al., 2020). Globally, probiotic foods account for approximately 65% of the functional food market (Burgain et al., 2011; Hadjimbei et al., 2022).

Yogurt serves as an effective and convenient means to deliver various nutrients to the body. For consumer acceptability yogurt requires some sweetness from added sweeteners. Commercial yogurt products typically contain sugars including lactose in a range of 4% – 19%, with an average of around 10% added cane sugar (Chandan & Kilara, 2013). Consumers are seeking to minimize or substitute high sugar content foods with low-carbohydrate, high-fibre, satiating meals that still offer a similar organoleptic experience as traditional food product (Primeau, 2020). Natural sweeteners, such as erythritol and stevia, have been proposed as alternatives to sucrose (Mooradian, Smith, & Tokuda, 2017; Neacsu & Madar, 2014).

Consuming probiotic yogurt daily is recommended if consumers are looking to supplement the probiotics in their gut (Drouin-Chartier, et al., 2016). For probiotic-enriched food to have therapeutic effects, it should contain at least 10^8 living cells per serving portion at the point of consumption (Meybodi et al., 2020; Ravula & Shah, 1998). However, the survival rate of probiotics in yogurt is impacted by the concentration of casein, sugar and metabolites but also affected by the yogurt cultures present especially if there are any antagonist effects between yogurt starter cultures and probiotics (de Vos et al., 2010; Ignatova et al., 2009; Lourens-Hattingh & Viljoen, 2001; Meybodi et al., 2020). The presence of prebiotic polysaccharides has the capability of enhancing and maintaining the growth of probiotic bacteria in yogurt (Karlton-Senaye & Ibrahim, 2013; Ranadheera et al., 2010).

Basil (*Ocimum basilicum)* is a native herb of hot tropical regions in Central Africa, Southeast Asia and the Middle East (Munir et al., 2017). Despite the therapeutic effects of basil seeds, basil seeds are used as a food ingredient in beverages and frozen desserts such as Indian sharbat and Malaysian falooda (Hosseini-Parvar et al., 2010). When soaked in water, the basil seed develops a thick, gel-like exterior layer, which has drawn interest for various applications (Hosseini-Parvar et al., 2010; Ghasempour et al., 2012). Basil seed gum (BSG), recovered from the gel/mucilage surrounding a basil seed once hydrated, is a good source of fibre and has been reported as a good source of prebiotics by Wongputtisin and Khanognuch (2015). Basil seed gum is made up of complex large molecular weight polysaccharides (Hosseini-Parvar et al., 2010; Ghasempour et al., 2012). This polysaccharide-rich gum could provide a good potential resource for enzyme hydrolysis and turn it to shorter chains and possibly act as a novel source of prebiotic to support probiotic growth and limit growth of pathogens and thus bring benefits to us.

1.1 Project Aim

The aim of this project was to use basil seed gum polysaccharides as a stabilizer for skim milk yogurt providing an acceptable yogurt with pre and probiotic benefits.

The following objectives were defined to achieve this aim:

1. To optimize the extraction conditions of basil seed gum (BSG) using water only
2. To optimize the yogurt formulation stabilized by BSG
    a. To evaluate the stability and potential of BSG as a prebiotic to enhance the growth of probiotics in yogurt
    b. To assess the combined effects of BSG and erythritol in a yogurt matrix
3. To optimize enzymatic hydrolysis conditions of BSG to produce prebiotic oligosaccharides
4. To modify and analyze yogurt formulations incorporating BSG, BSG-oligosaccharides, and erythritol to improve yogurt quality and functionality

Chapter 2 Literature Review

This literature review will first discuss the key ingredients needed for making yogurt especially using skim milk powder (SMP) as a base and the role of different components present in yogurt. It explores prebiotics as an ingredient to enhance the growth of probiotics and protect them during the storage shelf-life of yogurt. The review will also outline the primary prebiotics utilized in the dairy industry. Basil seed gum (BSG) is reviewed as a potential stabilizer and fat substitute in dairy products. BSG could also be a prebiotic that can promote the growth and survival of probiotics in yogurt. Lastly, the review covers information about basil seeds, the recovery of BSG, enzyme hydrolysis of polysaccharides, and the utilization of extracted polysaccharides in yogurt making.

2.1 Bovine milk as an ingredient in yogurt

Yogurt is generally defined as, "a cultured milk product made using *Streptococcus thermophilus* and *Lactobacillu delbrueckii* subsp. *bulgaricus*" (Savaiano & Hutkins, 2021), and these two bacteria are the primary microorganisms to influence the taste and texture of yogurt (Chandan, 2006a). In yogurt, milk is the main ingredient and yogurt starters cultures are essential to produce yogurt (Brisson & Singh, 2013; Chandan & O'Rell, 2007).

2.1.1 Milk Fat

Milk fats are present as globules and are packed inside the milk fat globule, which is surrounded by a membrane of glycolipids and membrane-specific proteins. The milk fat globule is present in a complex emulsion system in the aqueous phase of milk (Chai et al., 2022). Individual milk fat globules of homogenized bovine milk vary from 1 – 3.4 µm in diameter (Clark & Harte, 2022; Smith & Campbell, 2007). This arrangement in milk helps to minimize the thermodynamic interactions between water and hydrophobic fat molecules (Chai et al., 2022; Smith & Campbell, 2007). The viabilities of various yogurt cultures, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus acidophilus* was not affected by the fat levels in reconstituted milk (Obi et al., 2010). Although milk fat provides a rich and creamy mouthfeel to dairy products, the fat content in SMP is low at 1% (w/w)

and yogurts made with low fat milks or no fat generally exhibit poor sensory properties (Delikanli & Ozcan, 2017; Harte et al., 2007; Walstra et al., 2005).

2.1.2 Lactose in skim milk powder

Lactose is the most abundant sugar present in bovine milk (Walstra et al., 2005). The percentage of lactose in milk varies according to the species of animal and environmental effects. Lactose can be further classified into α- and β-lactose, based on the hydroxyl group on the anomeric carbon (C1) of the glucose unit (Walstra et al., 2005). β-lactose is more soluble in water and less hygroscopic than α-lactose (Walstra et al., 2005). Milk powders are highly susceptible to moisture uptake, softening, browning, compaction, collapse and caking (Pugliese et al., 2017). Lactose crystallization particularly, α-lactose crystals, is one of major issues in milk powder during storage which affects caking. This is due to the high lactose content of 50 – 56% (w/w) in the powder (Abbasi & Saeedabadian, 2015; Morgan et al., 2005; Pugliese et al., 2017). Large lactose crystals can be detected in the mouth and can result in a sandy texture in dairy products (Saqib et al., 2017; Szkolnicka et al., 2017). Lactose serves as the primary "food" source for probiotic bacteria in milk, as these microorganisms have limited or no proteolytic activity (Shihata & Shah, 2000) Lactic acid fermentation provides a freshness to the final fermented product and enhances the sensory profile of yogurts (Cheng, 2010; Spanier et al., 2001).

2.1.3 Proteins in milk

Milk proteins can be categorized into two major types, namely, caseins and whey proteins. The whey and casein ratio in milk proteins is approximately 20:80, which is 7% w/w whey, and 29% w/w casein are in SMP (Davoodi et al., 2016; Wood et al., 2021; Smith & Campbell, 2007).

2.1.3.1 Casein and casein micelles

Casein present in milk is comprised of α_{s1}-, α_{s2}-, β- and κ-casein in proportions of 4:1:4:1 (Chandan, 2006b; Chen et al., 2022). Casein exists as micelles in milk, and in many models postulated for the casein micelle, κ-casein is reportedly on the surface helping to stabilize the micelle in solution (Chen et al., 2022; Hussain et al., 2012). κ-casein acts as a protective

layer surrounding the micelles in which α_{s1}-, α_{s2}- and β-caseins constitute the inner hydrophobic core. Calcium and phosphate minerals are also present helping to stabilize and forming part of the casein micelle (Antuma et al., 2024; Datta & Deeth, 2001). The micelles are colloids of protein-calcium-phosphate complexes with an "open structure" where the casein micelle structure can be dissociated and re-associated (Hussain et al., 2012) Casein micelles help to stabilize fat globules in milk and provide valuable protein. The mechanism of milk gelation is due to proteins present in the milk, closely related to the surface of κ-caseins, colloidal protein-calcium-phosphate complexes, surface net charge (zeta potential), and steric stability (Datta & Deeth, 2001).

2.1.3.2 Physiochemical properties of casein micelles

Casein micelles are generally suspended in milk, at pH 6.7, due to steric repulsion between the micelles. The suspension stability of fermented milk also relies on the aggregation of the micelles. Caseins are heat and pressure-stable due to their naturally disordered structures. The caseins globular micelles formed have hydrophobicity against water and thus achieve maximum entropy and thermodynamic equilibrium, but casein micelles are pH sensitive (Chandan, 2006b). At the isoelectric point (pI: pH 4.6), casein micelles lose electrical charges and precipitate out of solution. The van der Waal and hydrophobic attractions overcome the electro-repulsion and steric stability between each of the casein micelles, and the micelles aggregate and precipitate to form a sediment (coagulum) (Chandan, 2006b). The aggregation can occur even before the pH reaches the pI point of casein, then large aggregates can be seen, and they will then sediment. This phenomenon occurs in reconstituted milk much earlier than fresh milk (Chandan, 2006b). Meanwhile, the failure of the native stabilization mechanism of casein micelles during the acidification of fermented milk is related to the collapse of κ-casein's protection with the glycol-hairy tail around the micelles (Shiby & Mishra, 2013).

2.1.3.3 Temperature effects on casein micelles

Important alterations occur to the casein micelles during the manufacture of skim milk powder; mainly due to heat treatment, but pH and salts can influence the solubility of casein-aggregated particles in reconstituted milk (Chandrapala et al., 2010). Commercial

skim milk powders are available as low-, medium-, and high-heat powders (Chandan, 2006b; Martin et al., 2007; Walstra et al., 1984). The heat-treatment temperature and time for low-, medium-, and high-heat dried SMPs are 72^{o}C for 15 s, 82^{o}C for 3 min, and 82^{o}C for 30 min, respectively (Rehman et al., 2003). Heating causes whey denaturation and denatured whey can bond to casein micelles. Water removal (especially during evaporation) also causes calcium ions in the remaining serum to become more associated with the micelles and results in much larger and denser casein micelle compared with the micelles in native milk (Martin et al., 2007). When the association between caseins and calcium ions, or caseins and denatured whey proteins takes place in SMP, re-equilibration of caseins, calcium ions and detaching denatured whey proteins from caseins are slowly in reconstituted skim milk; shear and temperature do not contribute to re-equilibration and detachment (Martin et al., 2007).

The large and dense packed caseins and denatured whey, and caseins and calcium ions can increase the viscosity of yogurt and lead to thicker yogurts (Cândido de Souza et al., 2021). However, theses associations of heat-treated milk proteins and calcium ions could cause rapid precipitation and a large amount of serum separation if SMP is used as the main milk base for yogurt fermentation (Glantz et al., 2010; Martin et al., 2007; Silva & O'Mahony, 2017). The particle size of SMP also increases with heat treatment temperature and time and with drying (Martin et al., 2007; Pugliese et al., 2017). Different heat-treated SMPs vary in their physical properties after reconstitution in water. The turbidity of reconstituted milk increases when the dehydration temperature rises for heated SMPs (Martin et al., 2007; Anema et al., 2022). This is possibly due to the attaching of denatured whey to casein micelles and increasing the casein micelle size. In addition, medium and high heat treated and dried SMP powders resulted in low viscosity milk, even less viscous than native liquid milk (Martin et al., 2007). More fluffy-hairy layers packed more closely on the casein micelles were observed in the reconstituted high-heat SMP under transmission electron microscopy (Martin et al., 2007) The fluffy layer is due to the attachment of denatured whey proteins (Figure 2.1). High-heat SMP gives a soft and fragile texture to yogurt gel and are not suitable for yogurt production (Martin et al., 2007; Pugliese et al., 2017; Bista et al., 2022).

Reconstituted milks tend to coagulate faster than fresh milk (Martin et al., 2007). After reconstitution of milk powder casein micelles are much closer together in water than the casein micelles in original liquid milk. This results from dehydration and compression as the micelles are drawn together (Martin et al., 2007). Coagulation and sedimentation of casein micelles are irreversible unless external forces modifying caseins are applied, for example, anion gum stabilizers to interact with casein micelle (Hege et al., 2020; Roach & Harte, 2008).

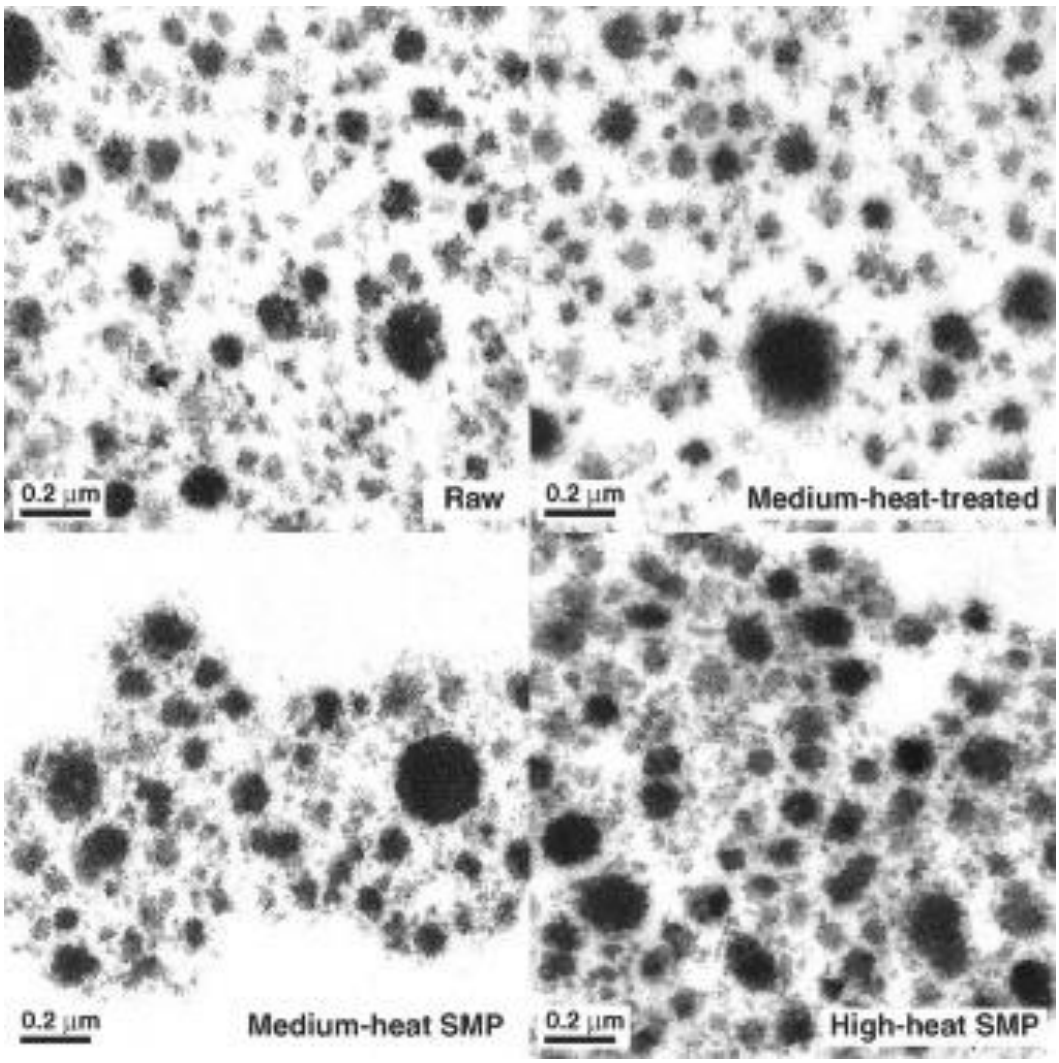


Figure 2.1 Transmission electron microscopy images of casein micelles in origin, medium-heat and high-heat skim milk powder after reconstitution in water (Martin et al., 2007).

2.1.3.4 Whey proteins

β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) are the two major whey proteins and exist as globular structures due to hydrogen and ionic bonding (Swaisgood, 2007). Whey

proteins remain soluble over a wider pH range than casein due to their higher proportion of charged amino acid residues, which enhance their solubility (Smith & Campbell, 2007). Whey proteins remain soluble even at their isoelectric point (pI) (pH 4.8-5.34) (Swaisgood, 2007). However, whey proteins are denatured easily under high temperature or high pressure compared with caseins, and heat or high pressure will alter the whey protein structure. In the whey protein thiol groups are exposed after heating which can form disulfide bonds with casein micelles (Daffner et al., 2021; Hill et al., 1982). Denatured whey proteins can form a gel at low pH or with the addition of salts to neutralize their charges (Tang, 1993). Denatured whey proteins have also been reported to enhance the gel firmness of yogurt due to their interaction with κ-casein in native milk-based yogurt (Chandan, 2006b; Kilara, 2015; Martin et al., 2007). However, like the coagulation of caseins, denatured whey proteins in SMP impact negatively on yogurt products, due to association with casein, increased particle size and therefore rapid sedimentation (Shiby & Mishra, 2013). Low temperature and short-time heat-dried milk powders are recommended to prevent whey-denature and sedimentation in yogurt (Bista et al, 2022; Shiby & Mishra, 2013).

2.2 Yogurt cultures

2.2.1 Yogurt starters

Streptococcus thermophilus and *Lactobacillus delbrueckii* subsp. *bulgaricus* are symbiotic cultures used to ferment lactose into lactic acid and produce medium-acid fermented milk through curdling of casein micelles, e.g. yogurt and acidophilus milk (Chandan and Kilara, 2013). During this fermentation volatile compounds such as acetaldehyde, diacetyl, and other minor volatile compounds contribute to the unique flavour of bovine yogurt (Aktar, 2022; Chandan et al., 2017; Surono & Hosono, 2011). In addition, the flavour and textural properties of yogurt can be enhanced by adding other ingredients, such as sweeteners, flavours and stabilizers (Aktar, 2022).

2.2.2 Probiotic bacteria – *Lactobacillus acidophilus* and *Bifidobacterium lactis*

The human gut microbiota provides metabolic, immunologic, and protective functions for the human body, primarily through their secreted metabolites (Peredo-Lovillo et al., 2020).

Changes in the surrounding environment, such as antibody uptake or the natural death of aging cells, can influence the gut intestinal (GI) tract microflora. For a healthy adult to maintain a gut microbiota level that is therapeutical, it is recommended to ingest no less than 10^8 cfu probiotic bacteria daily (Ouwehand, 2017). Yogurt and fermented foods are excellent vehicles for delivering probiotics and beneficial bacteria to the GI tract and contribute to maintaining health (Ouwehand, 2017). Probiotics usually face shelf-life stability issues in a low-pH environment. Prebiotic compounds can help protect probiotics to survive during refrigerated storage and are beneficial to probiotics and the existing human gut microflora (Carlson et al., 2018; Peredo-Lovillo et al., 2020).

2.2.2.1 Probiotic bacteria- definition

Probiotic bacteria or probiotics were initially defined as bacteria that had a positive impact on other microorganisms. However, with the emergence of new information, the definition has been updated to refer to, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Anukam & Reid, 2007; FAO/WHO, 2001). The health-related benefits of probiotics have appealed to many consumers. This has led to the current popularity of yogurt and other yogurt-related milk products. The popularity of the products is not only due to their perceived therapeutic functions but also to the expanding variety and sensory aspects (Kneifel et al., 1993; Lee & Salminen, 2009).

Traditional probiotics are a group of lactic acid bacteria, beneficial and naturally mutualistic and in symbiosis with the animal host body if present in adequate amounts (Fijan, 2014; Melara et al., 2022; Wang et al., 2021). Probiotics are specified in groups of the following genera, *Lactobacillus*, *Bifidobacterium*, *Enterococcus, Streptococcus*, *Pediococcus*, *Leuconostoc*, and *Bacillus* bacteria group; and yeast and fungus *Saccharomyces*, *Candia* and *Aspergillus* (Amara & Shibl, 2015; Fijan, 2014; Ouwehand et al., 2002).

Lactic acid bacteria (LAB) are gram-positive cocci or bacilli which are non-spore formers and are non-motile and convert lactose into lactic acids (Wood & Holzapfel, 1992). It was observed that children with diarrhoea had lower numbers of beneficial bacteria in their stool samples compared to healthy children in 1905 and the benefits of lactic acid bacteria (LAB) was first reported in 1916 (Anukam & Reid, 2007). More advantages of probiotic

bacteria were later explored by scientists such as the ability to improve insulin reproduction (Salles et al., 2020). It has been revealed that *L. acidophilus* was able to ameliorate sensitivities of the liver to glucose and thus improve insulin production in diabetes patients (Andreasen et al., 2010). Probiotics function inside the intestinal tract and can increase immunity as 70% of the immunoglobulin is produced inside the intestine of humans (Ibrahim et al., 2010). Humans can build resistance to many diseases, such as cold and influenza-like symptoms, bacterial infections like kidney failure, and pathogenic infections and cancers (Leyer et al., 2009; Reid, 2000). Lactic acid probiotics help by reducing the symptoms of lactose intolerance and alleviating hay fever for many teenagers (Montes et al., 1995; Ouwehand, 2017; Ouwehand et al., 2009). All these health benefits are a result of their capability to survive in the GI tract. In studies by Rajkumar et al., (2014) and Ghatani (2020), *Bifidobacterium* spp., *Lactobacillus plantarum* and *Lactobacillus paracasei* with bile salt hydrolase gene had a positive effect on the blood lipid profile and significantly reduced cholesterol, triacylglycerol and LDL levels in the blood.

The most common probiotic strains in yogurt or fermented milk products are those belonging to the genera *Lactobacillus* and *Bifidobacterium* where *Lactobacillus* spp. is used together with *Bifidobacterium* spp. or individually. To achieve the typical yogurt flavour, the yogurt starter cultures, *S. thermophilus* and *L. bulgaricus*, are added to enhance the flavour of fermented milk products. *Bifidobacterium* spp. has poor proteolytic activity and grow slowly or show no growth in yogurt products compared to *S. thermophilus* and *L. bulgaricus*, but to get the maximum health benefits from *Bifidobacterium*, it should be consumed on a regular base and yogurt makes it simple to incorporate into the diet (Prasanna et al., 2014).

There are various types of other probiotic microorganisms, including lactic acid spore-forming bacteria (e.g. *Bacillus subtilis* and *Bacillus coagulans*), acetic acid bacteria (*Gluconobacter* spp.), fungi and yeast for example *Saccharomyces boulardii* (Konuray & Erginkaya, 2018; Kumar et al., 2012; Manhar et al., 2016). These are emerging probiotics and are often found in food production (Konuray & Erginkaya, 2018). They can survive in harsh conditions and provide several health benefits to the body (Konuray & Erginkaya, 2018).

Examples of probiotic bacteria added to food products include *L. acidophilus* used in traditional acidophilus milk drink, *L. casei* used in Yakult® in Japan and Biofeel® in Korean fermented milk, *Bifidobacterium spp.*, and *Limosilactobacillus. reuteri* recently incorporated into probiotic daily supplements (Chandan et al., 2006b; Lee et al., 2023).

2.2.2.2 Dose level of probiotics

Lactic acid bacteria (LAB)are commonly used to ferment milk and other food products worldwide (Chandan & Kilara, 2013, Ozer, 2010; Widyastuti & Febrisiantosa, 2014). Among the most well-known and widely characterized LAB are the starter cultures used in yogurt production, namely *S. thermophilus* and *L. bulgaricus* (Chandan & Kilara, 2013, Ozer, 2010). There is still debate if on whether these yogurt starters can be defined as probiotic bacteria, and their survival rate after passage through the GI track is relatively low from an initial 10^8 cfu/g down to 10^4 cfu/g (Elli et al., 2006; Guarner et al., 2005; Mater et al., 2005). Therefore, probiotic bacteria as mentioned earlier are often added to yogurt and other fermented milk products (Chandan & Kilara, 2013; Hadjimbei et al., 2022). When probiotics are administered in sufficient amounts, they can survive passage through the upper digestive tract and deliver their benefits to the host in the intestinal tract (Anukam & Reid, 2007).

It is recommended to consume 100 g of yogurt daily with a probiotic dose of no less than 10^6 cells per gram or per ml of fermented dairy product (Ouwehand, 2017; Ravula & Shah, 1998). For common health issues such as diarrhoea, faecal recovery, and microbiota modulation, Ouwehand (2017) suggested doses of probiotics ranging from 10^9 - 10^{10} living cells daily. However, for chronic or severe illnesses, much higher doses are required, e.g. >10^{10} living cells for the treatment of antibiotic-associated diarrhoea and >10^{11} living cells for benefit to blood pressure control as reported by Laterza et al. (2018). For ulcer sufferers, the recovery dose level is between 10^7 cfu twice a day for *Helicobacter pylori* infection to 3.6 x 10^{12} cfu per day for ulcerative colitis (Laterza et al., 2018). A normal dose of around 10^{11} – 10^{12} living probiotic cells is commonly used to treat irritable bowel syndrome, constipation, antibiotic-associated diarrhoea, regular bowel movement in the elderly, and other chronic illnesses (Laterza et al., 2018).

2.2.2.3 *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis*

To aid in the treatment of chronic and severe illnesses, 75% of multi-strain probiotic formulations used in disease management include *Lactobacillus acidophilus*, while 68% include *Bifidobacterium lactis* (Laterza et al., 2018). These two probiotics have demonstrated superior therapeutic efficacy compared to other lactic acid-producing probiotics (Laterza et al., 2018).

Both probiotics (*L. acidophilus* and *B. lactis*) have shown strong microbiota modulation function with fecal detection increasing from 7% before intervention to 76% after yoghurt supplementation for *L. acidophilus*, and 56% to 100% for *B. lactis* (Savard et al., 2011). Each of the probiotics performs individual beneficial profiles to the host. *B. lactis* ferments lactose to L-(+)-lactic acid and this isomer of lactic acid is easily absorbed by humans, especially at an infant stage (McCartney, 2003). *B. lactis* is also able to facilitate calcium absorption, showing anticarcinogenic activity and a reduction in blood cholesterol (Prasanna et al., 2014). *B. lactis* can also produce indole-3-lactic acid from amino acids to provide unique anti-inflammatory activity and stimulate protective immune responses (Aragozzini et al., 1979). *B. lactis* treatment also reduced the expression of colonic tumor necrosis factors (oxide synthase and cyclo-oxygenase) (Philippe et al., 2011).

B. lactis was found to be abundant in the early stages of life, especially in infants and becoming less prevalent over time among the microflora in the GI tract (Saturio et al., 2021). *B. lactis* has been found to have better cell binding abilities than other *Bifidobacteria*, and thus more interference against pathogens attaching to host cells (Candela et al., 2007). *B. lactis* is more tolerant to oxygen than other *Bifidobacterium spp*., making it easier to store and maintain a stable shelf-life (Meile et al., 1997). Additionally, *B. lactis* has a higher survival rate than other commercial probiotics (Soni et al., 2020).

A synergistic effect was observed with *B. lactis* in a supplement with L. *acidophilus*. Most of the *Bifidobacteria* lack proteolysis capability and fail to grow in the food matrix and thus are dependent on the presence of other lactic acid bacteria (Lourens-Hattingh & Viljoen, 2001). Although some *B. lactis* strains have shown slight proteolysis capability and can slowly grow in milk, the growth rate and acid production of *B. lactis* were enhanced by the

presence of *L. acidophilus* (Gomes et al., 1998; Soni et al., 2020). *L. acidophilus* and *B. lactis* combination have also shown better levels of acid-tolerant and bile salt-tolerant levels than any other probiotic combination with *S. thermophilus* and *L. bulgaricus* or when used alone with *S. thermophilus* and *L. bulgaricus* (Prasanna et al., 2014; Soni et al., 2020). A synergistic effect was associated with the combination of *L. acidophilus* and *B. lactis* with about one log 10 higher viability than the other yogurt samples (Lourens-Hattingh & Viljoen, 2001; Soni et al., 2020; Viljoen, 2001).

L. *acidophilus* treatment resulted in reduced expression of colonic leukotriene B4 and cyclo-oxygenase productions, while *L*. casei lacks these functions (Peran et al., 2007). *L. acidophilus* can multiply and thus maintain high cell concentrations, allowing it to survive effectively in fermented food products. Meanwhile, it shows good health promotion and better disease treatments in multi-strain combinations (Akan, 2022; Laterza et al., 2018; Lourens-Hattingh & Viljoen, 2001; Soni et al., 2020).

Fermentation with additional *L. acidophilus* and *B. lactis* did not interfere with overall sensory acceptability (Prasanna et al., 2014; Soni et al., 2020). However, the survival of both probiotics is problematic during long refrigeration storage, especially with the present yogurt starter culture *L. bulgaricus* (Grosso & Fávaro-Trindade, 2004; Lourens-Hattingh & Viljoen, 2001). It appears that the presence of *L. bulgaricus* stimulates *L. acidophilus* to produce acidophilicin LA-1, and more hydrogen peroxide production by *L. bulgaricus* against *L. acidophilus* due to antagonism effects between them (Dave & Shah, 1997; Lourens-Hattingh & Viljoen, 2001).

Soni et al (2020) also mentioned that yogurt water holding capacity was disrupted more by the combination of *L. acidophilus* and *B. lactis* together with the yogurt starter cultures *S. thermophilus* and *L. bulgaricus* and thus serum separation was significant. Even some serum separation (syneresis) was observed when *L. acidophilus* was used as the sole probiotic in yogurt products (Olson & Aryana, 2008). Thus, gum stabilization could help compensate for this effect.

2.3 Other optional ingredients used for yogurt products

2.3.1 Sucrose

Sucrose, also known as saccharose or cane sugar, is abundant in plants and exists in many food products. Two forms of sucrose are used in the food industry, crystal and liquid sugar, where liquid sugar has 33 – 35% moisture, and crystalline sugar has less than 0.04% moisture (Chandan, 2006b; Tamime & Robinson, 2007). Sucrose can be converted into glucose and fructose by amylase in the mouth, stomach, and small intestine and quickly supply energy to the body (Chandan, 2006b; Tamime & Robinson, 2007). Sucrose is also used in food to enhance flavour and mask unpleasant tastes, for instance, the sourness in yogurts and bitterness in tea or coffee. Meanwhile, the addition of sugar in pre-heat treated milk is desirable due to its osmotic effects on the bacteria's cell membranes and this can lead to the destruction of any vegetative cells (Tamime & Robinson, 2007). However, high levels of sucrose content in any probiotic milk drink will influence the growth of probiotics but will supply nutrients for the growth of *S. thermophilus*. Chandan (2006b) reported that a sucrose concentration of 8% (w/w) or higher in yogurt products could decrease perception of flavour compounds such as acetaldehyde, while the addition of sugar (12 – 16 % w/w) can cause inhibition or delay in the fermentation period. A range of 8 – 10% w/w sucrose can give a better sensory perception as well as less impact on probiotic growth (Tamime & Robinson, 2007). A grain size ranging from about 149 – 840 µm (100 to 20 mesh) of cane sugar is preferred in yogurt manufacture to prevent caking or lumping during storage (Chandan, 2006b).

Sucrose present in a yogurt system not only contributes to flavour and aroma of the yogurt and also supplies food to *S. thermophilus*, but it can also support the gel matrix stability by interfering in the mobility of water around casein micelles via a preferential exclusion stabilization system (Figure 2.2) (Evers et al., 2011; Gomes et al., 2022; Liang et al., 2014; Panzica et al., 2012; Timasheff & Arakawa, 1988). Casein loses κ-casein tails during fermentation and caseins micelles coagulate due to loss of inter-micellar repulsive forces (De Kruif, 1999). When sugar is present, it interacts with water through weak co-solvent interactions, trapping non-tail caseins and reducing syneresis and micelle aggregation (Gomes et al., 2022).

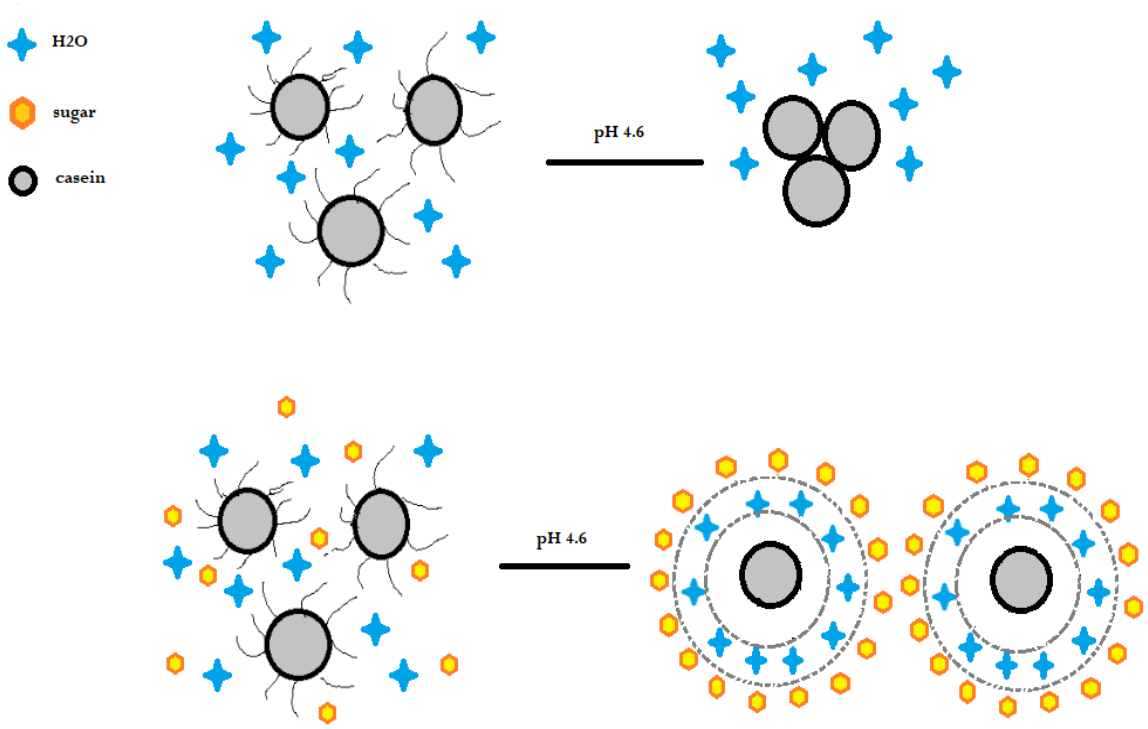


Figure 2.2 Illustrations of casein micelles coagulation in yogurt without the addition of sugar, top line; and preferential exclusion stabilization system of that weakly interacting cosolvents of water and sucrose to control protein stability in the acidic environment of yogurt, bottom line (Timasheff & Arakawa, 1988).

2.3.2 Erythritol – sugar substitute in yogurt

Sucrose can be replaced with sweeteners to reduce sugar levels, helping to mask acidic flavours, and suitable for consumers avoiding sugar (Chandan, 2006). Erythritol is used as a sweetener in sugar-zero food, candies, and bakery food products. Erythritol is a four-carbon alcohol sugar and only exists in one form. Compared to sucrose erythritol only imparts an equivalent of 60 – 80% sweetness (Grembecka, 2015). It is safe to consume below 533 mg/kg daily, has less of an aftertaste than other sweeteners and is sweeter than most other polyols (FDA, 2019; Grembecka, 2015). However, the survival of probiotics in the presence of erythritol has not been investigated (Palanivelu et al., 2022).

Erythritol was found naturally present in yeast or in a yeast-like fungi fermentation and then commercially exacted. It is a metabolite of osmophilic yeasts such as *Moniliella pollinis* or *Trichosporonoides megachiliensis* (Grembecka, 2015; Rice et al., 2020). It is also naturally synthesized by humans through the pentose phosphate pathway and is considered a safe ingredient in food products (FDA, 2019; Mazi & Stanhope, 2023). Some lactic acid bacteria

can produce erythritol e.g. *Oenococcus oeni*, *Leuconostoc mesenteroides,* and *Lactobacillus sanfranciscencis*, but these bacteria are less commonly used in commercial production of erythritol (Grembecka, 2015; Rice et al., 2020). Yeast or yeast-like fungi are fed with sugars such as glucose, fructose, xylose, sucrose or poly-sugars cellulose or triol glycerol (Rice et al., 2020). Glucose is commonly the substrate for enzyme transformation to erythritol, and a high-efficiency production rate of erythritol was found with glycerol as the substrate (Nakagawa et al., 2020; Rzechonek et al., 2018). After fermentation, the culture broth is heated and filtered to remove microorganisms, and erythritol is retrieved via chromatographic separation, crystalised and dried (Mazi & Stanhope, 2023; Rice et al., 2020; Rzechonek et al., 2018).

Erythritol is highly stable over a wide range of temperatures and pH environments and does not react with amino acids to form melanoidins at high temperatures (Grembecka, 2015). Erythritol does not absorb moisture and is a non-hygroscopic ingredient, and thus can be used as a bulking agent, anti-caking agent, and glazing agent with sweetness contribution to food products (FDA, 2019; Grembecka, 2015). It is also suitable to be blended with other sugar substitutes and protein powders due to its low chemical activity and non-moisture absorption properties (Grembecka, 2015; Nakagawa et al., 2020). Aspartame or acesulfame-K can increase the sweetness of erythritol by about 30% and erythritol can mask the intrinsic lingering bitter aftertaste of aspartame or acesulfame K for examples (de Cock, 2012).

Erythritol added to yogurt reduced serum release by 49.4% compared to the yogurt control, and the storage modulus (*G'*) of an erythritol-fortified yogurt was higher than that of a control yogurt sweetened with sucrose (Zhao & An, 2023). Sweeteners may interact with the casein micelle by interfering with casein micelle surface charges and increase hydrogen bonding with the casein micelles. This leads to an increase in the apparent viscosity of yogurt and reduced syneresis (Zhao & An, 2023).

2.3.3 Health benefits of erythritol

As erythritol is a polyol, it does not contribute to the glycaemic index when the food containing it is consumed, and it also does not contribute to tooth decay (Grembecka, 2015). It has been reported that erythritol can inhibit the growth of *Streptococci* and could reduce dental caries together with xylitol (Grembecka, 2015; Runnel et al., 2013; Söderling & Pienihäkkinen, 2020). Erythritol is zero calories per gram (Mazi & Stanhope, 2023). It is therefore commonly used in zero-sugar candy products, chewing gums, and ice cream zero-caloric beverages. It has been shown that erythritol can interfere with the absorption of fructose (Mazi & Stanhope, 2023). Despite zero calories, erythritol not only reduces blood sugar levels but it also increases satiety hormone excretion in the stomach and reduces hunger due to its higher osmolality than sucrose and other artificial sweeteners (Mazi & Stanhope, 2023; Sorrentino et al., 2020).

The antimicrobial function of erythritol is due to its high osmotic pressure. It has greater inhibition of bacterial growth than sorbitol and xylitol due to its ability to passively pass through a bacterial cell membrane (de Cock, 2012; de Cock et al., 2016; Mazi & Stanhope, 2023). Erythritol has radical scavenging properties and thus may help reduce oxidative stress by inhibiting the formation of reactive oxygen species in the body against hyperglycemia-induced vascular damage (den Hartog et al., 2010). Erythritol consumption leads to a reduction in arterial stiffness and improves endothelial function in type II diabetes sufferers who have consumed 26 g/day for 4 weeks (Flint et al., 2014). Erythritol at 5%, with a high-fat diet has shown to lower inflammation and liver fat accumulation in animal experiments (Mazi & Stanhope, 2023).

Erythritol has also been reported to be prebiotic. Its prebiotic function was found in case studies with *Lactobacillus casei* which was found to show improved survival of yogurt cultures when erythritol was added to yogurt samples (Costa et al., 2019; Gupta, 2023). Erythritol was used to inhibit *Streptococci* in a model oral microbiota system, and it was reported to have a potential oral prebiotic action (Grembecka, 2015; Runnel et al., 2013; Söderling & Pienihäkkinen, 2020). Erythritol is also able to ameliorate small intestinal inflammation by increasing the production of short-chain fatty acids fermented by bacteria in the lower GI tract (Calder, 2016; Kawano et al., 2021; Mazi & Stanhope, 2023).

2.4 Prebiotics used in dairy products

Prebiotics support the growth of probiotics (Gibson et al., 2017; Peredo-Lovillo et al., 2020). Some dietary fibres, e.g. inulin, β-glucan, and resistant starch, and oligosaccharides (fructo-, gluco- and galacto-oligosaccharides), are some of the proven prebiotics. There are also some emerging prebiotic sugars to be tested (Peredo-Lovillo et al., 2020; Rastall, 2010).

Prebiotics are substances that can be fermented by probiotics, helping them to grow and metabolize bioagents (Gibson et al., 2017; Peredo-Lovillo et al., 2020). This process leads to the production of short-chain fatty acids, which can affect the survival of harmful pathogens due to lowing pH in the gastrointestinal tract and also helps to the host's health through absorption of beneficial bio-compounds during probiotic fermentation in the body (Bamigbade et al., 2022; Gibson et al., 2017; Peredo-Lovillo et al., 2020). Symbiotic is the relationship between probiotics and prebiotics in favour of the host's health (Delgado-Fernandez et al., 2020). Prebiotics can be grouped into two categories, fermentable dietary fibres and short-chain oligosaccharides which have generally been derived from polysaccharides and fibres through various treatments (Gibson et al., 2017).

2.4.1 Fermentable dietary fibre

Dietary fibre is an edible carbohydrate polymer of three or more monomeric sugar units, which are resistant to gastric acid, bile salts, and endogenous digestive enzymes, and unable to be absorbed by the body in the lower GI tract (Holscher, 2017). Dietary fibre can be further classified into water-insoluble or water-soluble (Peredo-Lovillo et al., 2020). There is a lot of complexity involved in the effects of dietary fibre on human health. Some studies suggest that soluble fibre alters serum lipids and insoluble fibre was linked to the reduction in constipation (Dong et al., 2019). Although there are also reports showing that soluble fibres can help with constipation, like oat bran or psyllium soluble fibres increased stool weight and water retention (Jalanka et al., 2019; Jane et al., 2019). Table 2.1 provides the daily recommended intake of soluble fibres.

Table 2.1 Soluble dietary fibres considered as prebiotics and their profiles.

Prebiotic dietary fibre	Viscosity	Recommended Dose rate (daily)	Function	Reference
Inulin	Non-viscous fibre	15g	• Enhance calcium, magnesium and zinc absorption • Modulate gut microbiota • Improve in immune response • Colon cancer protection • Boosting SCFA synthesis • Anticarcinogenic • Lipid parameter regulation • Antioxidants • Triglycerides and serum cholesterol reduction • Intestinal disorder protection	de Almeida Gualtieri et al., 2013
Pectin	Increase the viscosity of a solution	5g	• Glycemic control • Bowel moving • Reducing glucose and cholesterol absorption • Increasing fecal mass • Thickening mucosa layer of intestine • Impacting cecal microbiota and SCFAs • Interfere with allergens	Blanco-Pérez et al., 2021
Locust bean gum **Guar gum** **Gum Arabic** **Xanthan gum**	Highly viscous in natural origin, lower viscosity after hydrolysis	10g (Partially hydrolysed such gum acted as potential prebiotics	• Like cellulose influences fecal volume • Partially hydrolysis may require performing prebiotic functions • Prebiotics for animals • Good source for microencapsulation of probiotics • Capability to enhance beneficial probiotic growth in the host	Al-Baadani et al., 2021; Calame et al., 2008; Kapoor et al., 2023; Khalid et al., 2022
β-glucan	Increase viscosity in solution Increase viscosity in solution	8mg/ml in research 0.75-3g	• By-product of yeast • Regulate microbiota • Similar probiotic activity to inulin • Cereal glucan • Modulate gut microbiota • Boosting SCFA synthesis • Improve the gut permeability flux • Anti-inflammation in intestine • Colon cancer protection • An immunomodulator	Wang et al., 2020 Shoukat & Sorrentino, 2021
Resident starch	Non-viscose fibre	5-8g	• Influence GI microbiota and SCFAs • Glycemic control • anti-colorectal cancer • impact on insulin response • Increasing fecal mass and decreasing cecal pH	Zaman & Sarbini, 2016

Additionally, resistant starch and inulin barely altered blood cholesterol levels in wild-type mice, but soluble fibres significantly increased short-chain fatty acids in the body (Mistry et al., 2018). Overall, dietary fibre can directly benefit humans by altering appetite, increasing satiety, and aiding in the absorption of lipids and fat globules in the lower GI tract (Holscher, 2017). Water-insoluble fibres are undigestible or mildly utilized by microbiota, therefore able to interfere with absorption of nutrients in the host (Holscher, 2017). Water-soluble fibres are more favorable for microbiota and the fibre can be enzymatically cleaved into smaller moieties and then absorbed by the bacteria as their food (Peredo-Lovillo et al., 2020). Currently, inulin, commercial plant gums, β-glucan, and resistant starch are being studied for their prebiotic functionality, with inulin being the most recognized prebiotic and often used together with probiotics as a health supplement (Table 2.1). However, inulin has little impact on the viscosity of the food product, limiting its ability to reduce syneresis (Khodear et al., 2018; Slavin, 2013; Zbikowska et al., 2020).

Although dietary fibres are not widely recognized as prebiotics, they do have various health benefits (Peredo-Lovillo et al., 2020). The fermentability of water-soluble fibres varies depending on the host's microbiota, which can influence the health benefits of the dietary fibres and the metabolism of probiotics (Wang et al., 2019). One potential solution is the combination of prebiotic dietary fibres and probiotics in supplements, which could gradually become more available in the market (O'Connor et al., 2021; Wang et al., 2023).

2.4.2 Prebiotic - Oligosaccharides

Oligosaccharides are a group of polysaccharide polymers which are generally composed of 2 – 10 monosaccharides, which can be either identical or different sugar molecules (Dai et al., 2018; Ochoa et al., 2014). The sugars can be categorized into two different groups based on their functions, the ordinary oligosaccharides and functional oligosaccharides (Wilson & Whelan, 2017). The functional oligosaccharides have indigestible and prebiotic properties and in contrast the ordinary oligosaccharides can be digested and absorbed by the body and thus provide energy (Mei et al., 2022; Wilson & Whelan, 2017). The functional oligosaccharides are generally derived from dietary fibres via different chemical treatments, enzyme hydrolysis or synthesis, and commonly used as growth enhancement sugars to support probiotics (Ahnen et al., 2020; Peredo-Lovillo et al., 2020).

There are a number of functional oligosaccharides, and they fall into the following classes: Fructooligosaccharides (FOS), glucooligosaccharides (GuOS), galactooligosaccharides (GOS), maltooligosaccharides (MOS), xylooligosaccharides (XOS), lactulose, lactosucrose, raffinose, stachyose, lactulosucrose, fructans, resistant dextrin. These are the key prebiotics and have been studied extensively for their prebiotic properties in vitro (Belorkar & Gupta, 2016; Rastall, 2010; Singh et al., 2017). The degree of polymerization (DP) is frequently referenced in health benefit claims of oligosaccharides and serves as a key factor in determining characteristics of oligosaccharides (Chen et al., 2020). The degree of polymerization (DP) is defined as the number of monomeric units in a polymer and, in this context, describes the properties of an oligomer molecule consisting of 2 – 10 monosaccharide units (Rudin & Phillip, 2013).

Oligosaccharides have been proven to stimulate GI probiotic growth and produce more health-beneficial bio-compounds, for example, butyric acid which is linked to a reduction of colonic inflammation and colon cancer (Ariestanti et al., 2019). Prebiotic oligosaccharides play the same role in the GI tract as dietary fibres (Singh et al., 2017). Prebiotics, such as polysaccharides and oligosaccharides, not only have symbiotic effects with probiotics that promote health but also enhance the overall quality of food products, such as reducing syneresis in yogurt (Dabija et al., 2018; Guven et al., 2005; Sahan et al., 2008; Zbikowska et al., 2020), acting as a fat substitute (Guven et al., 2005; Sahan et al., 2008), or stabilizing an emulsion system (Coorey et al., 2014).

2.4.2.1 Fructooligosaccharides (FOS)

Although FOS can be found naturally in chicory root (the main source of inulin), blue agave, garlic, and onion. Commercial FOS is generally derived from enzymatic hydrolysis of inulin (Belorkar & Gupta, 2016; Dou et al., 2022), but can be synthesized from sucrose by fungal fructosidase (Costa et al., 2022). Recently, a high concentration of FOS was found naturally in yacon root, at up to 70% of dry matter (Caetano et al., 2016; Paredes et al., 2018) and it has 30 – 50% the sweetness level of cane sugar without a glycemic effect (Niness, 1999). The recommended dose level of FOS is 7.5 – 15 g per day for more than four weeks of consumption (Dou et al., 2022). Side effects of FOS are flatulence and bloating, especially with shorter chain FOS (Liber & Szajewska, 2013). FOS has helped to reduce syneresis in

yogurts but a decrease in gel firmness a softer texture was observed (Pachekrepapol et al., 2021, Alatorre-Santamaría et al., 2022).

2.4.2.2 Glucooligosaccharides (GuOS)

Production methods of GuOS are restricted to acid, enzymatic hydrolysis, and transglycosylation synthesis from sucrose by dextransucrase (Chung & Day, 2002; Zeng et al., 2023). The bacteria *Leuconostoc* spp. can produce GuOS (Hughes & Rastall, 2007). GuOS are composed of α-1-2 linked β-D-glucose chains and are resistant to degradation against GI tract compounds and contribute to the modulation of microbiota (Hughes & Rastall, 2007). GuOS stimulated probiotics to excrete more antimicrobial compounds particularly from cocci bacteria, compared to FOS (Lee et al., 2020).

Different DP of GuOS can affect to the growth of probiotics in food. For example, DP 5 which has five monosaccharide units in the oligosaccharide, is good for probiotic *Candida* but *Lactobacillus* and *Bifidobacterium* can only use DP > 7 GuOS (Zeng et al., 2023). Therefore, GuOS are less favored for use in fermented food due to this limitation. Additionally, the growth of *L. acidophilus* was enhanced more by GuOS than by FOS (Chen et al., 2013; Rastall, 2010).

2.4.2.3 Galactooligosaccharides (GOS)

GOS occurs naturally in breast milk (Ben et al., 2008). Transferring a glycolyl from one D-galactosyl onto the D-galactose moiety of lactose, with the enzyme β-galactosidase is the only commercial way to produce GOS (Rastall, 2010). The production of GOS from buttermilk can produce a maximum of 70 g/kg using β-galactosidase, about a 7% production rate. This is used in infant formulas (Čurda et al., 2006). The addition of GOS at 0.24 g/ml enhanced *Bifidobacteria* and *Lactobacilli* growth in infants after three months of feeding (Mei et al., 2022). GOS (5 g) can lead to 13% more calcium absorption in an adolescent and modulation of microbiota, whereas 10 g resulted in only 6.6% more calcium absorption than the placenta group and similar modulation capability (Rastall, 2013). The other GOS in fermentable oligosaccharides, disaccharides, monosaccharides and polyols are α-linked galactosyl sucrose from stachyose and raffinose and produce more side effects of more suspected flatulence and bloating, as reported by Rastall (2013).

2.4.2.4 Shortage of FOS, GuOS and GOS as prebiotic ingredients

FOS and GuOS possibly cause side effects of flatulence and bloating when consumed (Liber & Szajewska, 2013; Rastall, 2013). There are also some drawbacks of FOS regarding the influence on the prebiotic score performance of *L. acidophilus* and *L. plantarum* growth against *E. coli* as reported by Huebner et al., (2007). FOS softens the gel structure in the yogurt products mentioned above (Alatorre-Santamaría et al., 2022). Its usage in fermented milk products then needs to be taken into consideration as well as GuOS which may not provide prebiotic function (Zeng et al., 2023). GOS derived from buttermilk is a good choice of prebiotic used in food products due to its much safer usage, especially in meals for children, and its effect at low doses as well as its stable physicochemical properties (Mei et al., 2022). However, its production rate is relatively low, and production remains a challenge (Čurda et al., 2006).

2.4.2.5 Mannooligosaccharides (MOS)

Mannooligosaccharides (MOS) are emerging potential prebiotics due to the abundance of naturally occurring mannan found in copra meal, guar beans, konjac tubes, palm kernel cake, and even coffee extraction waste, relatively cheap resources for deriving MOS via enzymatic hydrolysis (Jana et al., 2021). MOS is considered a potential prebiotic because of its relatively short chain, which is non-digestible by the host's gastrointestinal (GI) tract, allowing it to pass through the digestive system and to be utilized by probiotic cells. This characteristic enhances the growth of beneficial probiotics, particularly *Lactobacilli* and *Bifidobacteria*, which ferment MOS to produce short-chain fatty acids (Jana et al., 2021). MOS can enrich probiotics and deter pathogens as summarised in Table 2.2. MOS is a short chain with 3 – 10 mannose sugar units (Jana et al., 2021).

MOS with DP < 4, particularly mannobiose (DP2), a disaccharide consisting of two mannose units, have been demonstrated to have better growth-enhancing functions (Jana et al., 2021). MOS, DP 2 and DP 3, have shown significant growth promotion of *Lactobacilli* and growth suppression of entero-pathogens like *E. coli* and *Salmonella typhi* (Mary et al., 2019). It has been proved that small DP of MOS had better cellular diffusivity and molecular mobility and thus supported the growth and survival of probiotic cells. The much shorter-chain MOS contributes more therapeutic effects than the long-chain MOS (Endo et al., 2016;

Yang et al., 2008). Colorectal caco-cell growth is suppressed and reduced growth was observed in vitro with MOS present (Jana et al., 2021). Meanwhile, MOS is more resistant to enzymatic hydrolysis than FOS and GOS and there are possible to perform more prebiotic functions in the GI tract (Jana et al., 2021). Commercial MOS is derived from yeast or mannan-rich plant materials, e.g. the brands of SAF-mannan, Bio-Mos, and Active MOS which are widely employed in poultry feed as prebiotics (Jana et al., 2021). MOS can be added as a critical ingredient in functional foods (Ghosh et al., 2015; Jana et al., 2021).

Table 2.2 Effect of MOS on probiotic enrichment and pathogenic deterrence (Jana et al., 2021).

MOS source	Enriched probiotic	Deterred pathogen
Locust bean galactomannan **Palm kernel cake**	*Lactobacillus casei* *Lactobacillus brevis,* *Lactobacillus salivarius,* *Lactobacillus gallinarum*	*Salmonella enteric* -
Locust bean galactomannan and defatted copra meal **MOS with Metformin (MF)** **Palm kernel cake**	Lactobacilli*,* *Weissella confusa* JCM 1093 *Allobaculum* *Lactobacillus reuteri* C1 *Bifidobacterium infantis* *Lactobacillus acidophilus*	*E. coli* E010 - *Salmonella. typhi,* *Listeria monocytogenes* *E. aerogenes*
Copra meal	-	*Salmonella enteritidis* S003, *Staphylococcus aureus* TISTR 029, *E. coli* E010, *Shigella dysenteriae* DMST 1511

There are different methods for the preparation of short-chain MOS from parent mannan resources. Both acids and bases can liberate MOS from mannan, such as 0.1 N sulfuric acid, acetic anhydride, and 0.1 N sodium hydroxide (Jana et al., 2021). High pressure heat-treatment in water with catalysts at 180 – 240^{o}C for 3 – 60min duration can also liberate short-chain MOS (Jana et al., 2021; Miyazawa & Funazukuri, 2006). A variety of DP 2 – 8 MOS can be derived by these methods and neutralizing the pH is required for the acid and base preparation methods. Although the production of prebiotic compounds is maximized with chemical and physical degradation methods, unwanted by-products such as other oligomers, monosaccharides, acetic acid, furfural, hydroxymethylfurfural, formic acid, and levulinic acid are disadvantages of these preparation methods (Santibáñez et al., 2021). These unwanted by-products make the purification of prebiotics difficult.

Producing MOS by enzymatic hydrolysis results in less environmental issues, use of less energy and less equipment is required. It is easy to control the production of MOS to a smaller range of DP by enzymatic hydrolysis. β-mannanase from *Bacillus* spp. liberates MOS DP 3 – 5 from de-fatted copra meal and DP 5 – 8 of MOS from locust bean gum (Jana et al., 2021). For another example, β-mannanase from *Streptomyces* spp. and *Aspergillus terreus* released a high amount of MOS of DP 1 - 3 with the majority as DP 3 MOS from locust bean gum (Jana et al., 2021).

2.4.2.6 Xylooligosaccharides (XOS)

XOS has been considered as an alternative prebiotic choice in food production due to its stability in harsh environments and its relative abundance from plant sources (Mäkeläinen et al., 2009; Santibáñez et al., 2021). XOS had not been included and considered as a potential prebiotic before 2004 due to insufficient evidence of the prebiotic status (Gibson et al., 2004). It was then classified as a prebiotic in the latest updated prebiotic list of ISAPP (the International Scientific Association for Probiotics and Prebiotics) statement in 2017 (Gibson et al., 2017).

XOS promoted the growth of *Lactobacilli* and *Bifidobacteria* in the gut (Samanta, Jayapal, Jayaram, et al., 2015). XOS also has similar functions as other prebiotic oligosaccharides, including enhancing mineral absorption, modulating gut microbiota, improving immune response, anticarcinogenic, antioxidants, regulation of cholesterol and triglycerides, and boosting short chain fatty acids produced by bacteria in the GI tract (Akpinar et al., 2009; Samanta, Jayapal, Jayaram, et al., 2015). XOS has the lowest effective dose on the promotion of health at 2.8 g per day in comparison with FOS 7.5 – 15g per day and 0.4 g/kg per day of GOS (Dou et al., 2022; Mei et al., 2022; Na & Kim, 2007; Santibáñez et al., 2021). XOS is stable at 100^{o}C and between pH 2.5 – 8. XOS can be used as a sugar substitute to help regulate blood glucose and maintain glycemic levels like FOS (Santibáñez et al., 2021). A commercial XOS mixture is obtained from enzymatic hydrolysis of corncob and sugarcane fibre (Santibáñez et al., 2021). As XOS is an emerging prebiotic, the alkaline pre-treatment and enzymatic hydrolysis two-step method of XOS production from lignocellulosic biomass is still under research and development (Santibáñez et al., 2021).

Hemicellulose xylan is the main source of XOS and 20 – 40% is present in lignocellulosic biomass (Santibáñez et al., 2021). It includes homoxylans, arabinoglucuronoxylans, arabinoxylans, glucoronoxylans, glucoronoarabinoxylans, and heteroxylans. Seed exterior mucilage contains the most abundant heteroxylans, substituted with different types of monosaccharides and oligosaccharides with multiple branches (Santibáñez et al., 2021). XOS at DP 2 – 6 have been shown to have the highest prebiotic potential (Gullón et al., 2008).

There are two production methodologies for XOS, auto-hydrolysis or enzyme hydrolysis. Auto-hydrolysis which requires a large energy input, involves high temperature treatment at 145 – 190^{o}C for 7.5 h (Garrote et al., 1999; Qing et al., 2013). Auto-hydrolysis results in a mixture of unwanted residuals and XOS, and thus purification is required (Naidu et al., 2018; Qing et al., 2013; Vazquez et al., 2000). Before enzyme hydrolysis the raw material is pre-treated with a heat treatment in acid or base (Carrillo et al., 2018). Table 2.3 shows the different pre-treatments used to produce XOS from woody sources. Heat-treatment at 140^{o}C is used to loosen the lignin and cellulose fibrous structure of lignocellulosic biomass in acid or base solution to increase the yield of XOS (Hong et al., 2019). Lignin can be removed via alkaline or oxidizing pre-treatments. The enzymatic hydrolysis is then the next step to produce XOS from heteroxylans, especially exterior seed gums (Carrillo et al., 2018; Santibáñez et al., 2021).

Table 2.3 Pre-treatment methods for the preparation of XOS from woody resources (Santibáñez et al., 2021)

Preparation method	Condition/chemicals required	Advantages and disadvantages	Notes
Hydrothermal pre-treatment	140^{o}C	Easy to handle, but equipment is required	
Acidic pre-treatment	Inorganic acid - H_2SO_4, HNO_3, H_3PO_4 and HCl Organic acid – acetic acid, formic, propionic acid	Increased yield, but a large amount of xylose was produced with toxic residuals, and special handling and neutralizing are required.	Quite often used together with hydrothermal treatment to further enhance yield. KCl, $FeCl_2$, $ZnCl_2$ and $CuCl_2$ especially $ZnCl_2$ could be used as catalysts.
Alkaline pre-treatment	NaOH, Na_2CO_3, KOH, $Ca(OH)_2$ and NH_3	Same as acidic pre-treatment- Increased yield, but a large amount of xylose was produced with toxic residuals, and special handling and neutralizing are required. Acetyl groups could be removed, thus decreasing the solubility of hemicellulose and lowering the yield	To remove the lignin NaOH has a better function to recover hemicellulose than KOH.
Oxidizing pre-treatment	H_2O_2 Glacial acetic acid together with H_2O_2	H_2O_2 is unstable in alkali conditions and requested special operation conditions A large amount of xylose is produced from xylan	The main purpose for the removal of lignin
Physical pre-treatment	Hacking, grinding, milling, rolling, irradiation, sonication, pyrolysis, and spray drying with gamma irradiation	Equipment and energy required	Generally carried out with chemical pre-treatment

2.4.3 Enzymatic hydrolysis of oligosaccharides from basil seed gum (BSG)

Wongputtisin and Khanongnuch (2015) used *Thermoascus aurantiacus* endo-xylanase to hydrolyze basil seed gum and further investigated the hydrolyzed crude BSG derived oligosaccharides prebiotic properties on *Pediococcus acidilactici, Enterococcus faecium, Salmonella havana* and *Escherichia coli*. BSG has two main fractions glucomannan and xylan and has the potential to be hydrolysed by either β-mannanase or xylanases (Naji-Tabasi et al., 2016).

2.4.3.1 β-mannanase

β-mannanase (endo-1,4-β-D-mannan mannohydrolase, EC3.2.178), β-mannosidase (1,4-β-D mannopyranoside hydrolase, EC3.2.1.25), and β-glucosidase (1,4-β-D-glucoside glucohydrolase, EC 3.2.1.21) are the three major mannan-degrading enzymes, where β-mannanase is the only enzyme that works on the main mannan backbones (Moreira & Filho, 2008). Other mannan-degrading enzymes remove sidechains that attach to the main mannan backbones (Malgas et al., 2015; Moreira & Filho, 2008).

β-mannanase (also known as β-D-mannanase, endo-β-(1-4)-mannanase or endo-1,4-β-D-mannan mannohydrolase) can hydrolyze 1,4-β-D-mannopyranosyl linkages within mannans, glucomannans, galactomannans and galacto-glucomannans (Vu, 2012). It works at both β-(1,4)-linkage of glucose and mannose, and β-(1,4)-linkage of mannose and mannose (Malgas et al., 2015). MOS with normal DP 3 – 7 is generated by β-mannanase from locus bean gum, konjac, guar gum and copra meals by fungal enzymatic hydrolysis (Jana et al., 2021). Mannans and hetero-mannans are hemicelluloses and a part of a constituent in cell walls of plant and plant seeds, and thus β-mannanase has been used widely to shorten the polysaccharide chains and soften the fibre for further processing in food, animal feed, detergent, and paper and pulp industries especially in pulp bleaching and instant coffee extraction (Moreira & Filho, 2008; Vu, 2012).

The main component of BSG is β-(1-4)-glucomannans making up 43% of the mucilage. It provides a substrate for endo-β-(1-4)-mannanase to work on, which will cleave the links between glucose and mannose units. β-mannanase initiates the degradation of mannans by randomly cleaving the backbone of mannans into short β-(1-4)-mannooligomers (Malgas et al., 2015). β-mannanase, a main-chain-cleaving enzyme, randomly hydrolyses β-(1-4)-D-mannosidic linkages in the backbone glucomannan chains and releases linear and branched manno-oligosaccharides of various lengths (Chen et al., 2013; de Vries, 2003; Malgas et al., 2015), which had a predicted molar mass mainly between 38 – 45 kDa (Vu, 2012).

2.4.3.2 Xylanases

Xylan is the second most abundant polysaccharide component found in BSG at 24.29%. In general, hemicellulose xylan is heteropolysaccharide with DP 50 – 200 (Holtzapple, 2003). There are a variety of xylans and subclasses based on their degree of substitution. Cereal bran, seeds, and mucilage contain more complex heteroxylans deeply substituted with different monosaccharide and oligo-saccharide side chains (Dodd & Cann, 2009; Santibáñez et al., 2021). Xylans can be homo-xylans generally found in cell walls of red seaweeds, or heterogenous xylans which are commonly found in plant and plant seeds (Dodd & Cann, 2009). For example, Arabinoxylans are derived from cereal grains, glucuronoxylans from woody herbs and plants, and arabinoglucuronoxylans from grasses (Dodd & Cann, 2009). Highly heterogeneous xylans contain α-1,2-linked d-(4-O-methyl-) glucuronic acid and may have acetyl residues attached to O2 or O3 of D-xylose, or feruloyl or p-coumaroyl residues attached to O5 of the L-arabinose residues (de Vries, 2003). Acidic xylans in BSG containing D-xylose, L-arabinose, L-rhamnose, and D-galacturonic acid in a ratio of 15:9:7:12 (Wongputtisin & Khanongnuch, 2015). To extract XOS from BSG, enzymatic hydrolysis is the preferred way as it is eco-friendly, less by-products are produced, and a simple purification procedure can be used (Aachary & Prapulla, 2011; Qing et al., 2013).

The major xylan hydrolysis enzyme is endo-1,4-β-xylanase. Xylanases cleave off the glycosidic link between the xylose unit backbone and produce XOS in different steps, e.g. xylotriose, xylobiose and further xylose depending on the enzymes derived from different microorganisms (Santibáñez et al., 2021). Bacteria, archaea, fungi, yeast, marine algae, and protozoa can produce slightly different endo-1,4-β-xylanases, in which xylanases extracted from fungi and yeasts, especially filamentous fungi are commonly used in the food industry (Dhaver et al., 2022; Santibáñez et al., 2021). *Trichoderma*, *Aspergillus,* and *Fusarium spp*. are the major xylanase producers and are commonly used. *Bacillus* and *Streptomyces* produce xylanase and are less commonly used commercially (Santibanez et al., 2021). Bacteria-produced xylanases have a higher optimum pH than that of fungal xylanases and are thermostable (Santibáñez et al., 2021). Optimum working pH and temperature are two of the main factors influencing the efficiency of xylanase on xylan (Dhaver et al., 2022). The structure and composition of parent xylans should be considered when using xylanase to gain XOS (Rumpagaporn et al., 2015)

The major drawback of using xylanase to produce XOS is enzyme cost. Immobilised xylanase was introduced to solve the problem to allow for the recovery and reuse of xylanase (Illanes et al., 2016). Eudragit polymers, alginate-silica gel and calcium alginate beads were studied and used as immobilizing agents to trap xylanase via physical adsorption, entrapment and covalent binding (Rajagopalan et al., 2016).

The other cost-efficient way to produce XOS is by microbial fermentation in situ (Santibáñez et al., 2021). It is critical to select the right safe strains without sub-enzyme production of non-cellulolytic or non-xylanolytic enzymes. Genetically modified strains e.g. *Trichoderma reesei* containing Xyn2-xylanase mRNA gene and mutated *Streptomyces* would yield more xylanase, but it is a long way from being accepted in the industry to produce commercial xylanase and XOS (Amorim et al., 2019; Xiong et al., 2018). Auxiliary enzymes could facilitate endo-1,4-β-xylanase to hydrolyse different branching hemicelluloses and a synergy effect could be achieved via different combinations of enzymes (Santibanez et al., 2021). Beside endo-xylanases, cellulases are also commonly used to break down the backbone of heterogeneous xylans (de Vries, 2003).

2.4.3.3 Cellulase

Cellulases are produced by fungi, bacteria and animals. Cellulase hydrolyze the β-1,4 glucose linkages on cellulose (Wilson, 2009). The activity of cellulase on crystalline cellulose is very low due to the rigid structure of cellulose, but it is highly active in semi-cellulose sugar complex and very robust in extremely acidic conditions, for example, hydrolysis can be achieved in concentrated sulphuric acid at 125^{o}C (Wilson, 2009). There are two types of cellulases based on whether they attach or detach after hydrolysis on the cellulose chain, called processive and non-processive cellulases (Atalla & Isogai, 2010), in which it is more favourable to use processive cellulase in cellulose hydrolysis due to its high activity and capability to release cellobiose or other oligoglucans (Cruys-Bagger et al., 2013; Wu & Wu, 2020). There were also two subgroups of processive cellulases, cellobiohydrolases/exocellulases and endoglucanases (Cruys-Bagger et al., 2013; Wilson & Kostylev, 2012; Wu & Wu, 2020). Exocellulases have much more efficiency in turning cellulose into 93% reducing sugars, while endoglucanases only have the capacity of up to 40% production of reducing sugars from cellulose (Wilson & Kostylev, 2012). Endoglucanases are mainly produced by some bacteria

and fungi and could be potentially efficient enzymes to hydrolyze oligosaccharides from cellulose and hemicellulose (Wilson & Kostylev, 2012; Wu & Wu, 2020).

2.5 Basil seeds

Basil seeds (*Ocimum basilicum*) are black oval-shaped seeds, with the outside covered with polysaccharides. The seeds contain 10 – 22% protein, 10 – 29% fat, 47 – 63% carbohydrates, 5 – 7% minerals, and 5 – 9% moisture content with a reported total phenolic content of 64 mg GAE/g (Calderón Bravo et al., 2021; Munir et al., 2017). The seeds can be separated into two major components, the exterior mucilage cover where carbohydrates dominate with few protein moieties, and the center seed kernel containing the proteins and fats. Minerals are also stored in the kernel to support seed germination. The polysaccharide layer is only visible after soaking in water and coats the entire kernel. The mucilage layer, relative to the seed dimensions, is much thicker than the other seeds which also form a mucilage layer, such as flaxseed, fenugreek, and chia seeds (Bharati et al., 2022; Coorey et al., 2014; Segura-Campos et al., 2014). There are approximately 150 species in the *Ocimum* family (Srivastava et al., 2018). Basil seeds are normally grouped according to their cultivation areas (Naji-Tabasi & Razavi, 2017a). India and Iranian basil seeds vary slightly in physical and chemical composition possibly due to the soil components (Munir et al., 2017).

2.6 Basil seed gum

The exterior of basil seeds is covered with a thin and dry layer of polysaccharides which are not easily seen by the naked eye (Figure 3A). The typical size of basil seeds is 2.3 – 3.1 mm long, 1.3 – 1.8 mm wide and 0.1 – 1.3 mm thick (Calderón Bravo et al., 2021). After hydration in water the white gel layer can be 1.3 mm thick (Zhou et al., 2022). The dry seeds look slightly waxy on the surface. When the seeds are soaked in water, the polysaccharides form a mucilage, a white jelly-like coating (Figure 3B, C), which has good water-holding capacity. The seeds are, therefore, able to germinate well in hot and dried climates (Samateh et al., 2018). Basil seed gum (BSG) is able to absorb 34 – 35 ml of water per gram of whole seeds and therefore, the whole seed volume can be enlarged twice to the volume of the original dry seed (Figure 2.3c). BSG has potential dietary fibre properties (Guan et al., 2023; Nazir & Wani, 2021). The mucilage layer can be scraped from the seed kernel, and its

physicochemical properties have been extensively studied (Atik et al., 2020; Hosseini-Parvar et al., 2010; Kumar et al., 2012; Naji-Tabasi et al., 2017). Basil seed gum is a good source of fibre and has good physiochemical properties such as (emulsifying, foaming, thickening, gelling, binding, and fat-replacing stabilizing agent in foods (e.g. cheeses, ice cream, cakes) (Hosseini-Parvar et al., 2010; Kumar et al., 2012; Naji-Tabasi & Razavi, 2017a; Song et al., 2017). The gum is an effective disintegrant in tablet formulations, offering faster and more efficient swelling and leading to quicker breakdown of the tablet compared to starch (Naji-Tabasi & Razavi, 2017b). In addition, it can be used to create biodegradable edible films.

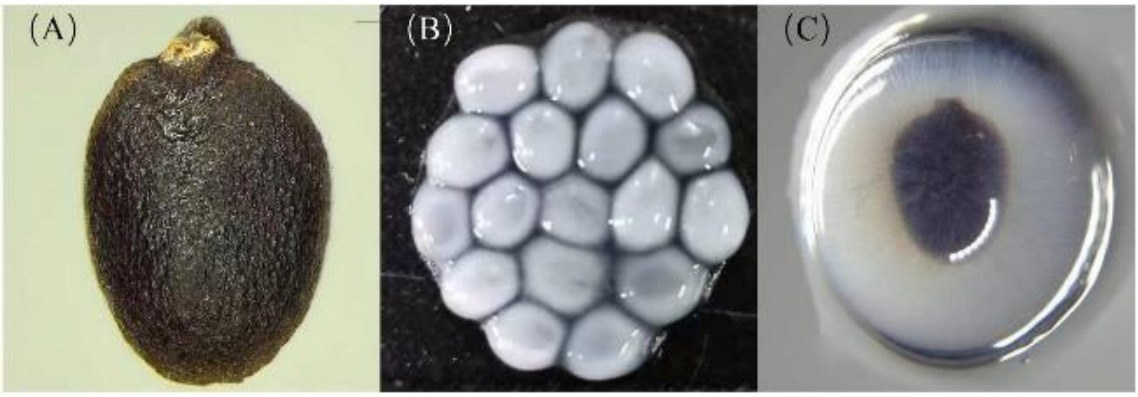


Figure 2.3 Basil seeds and the mucilage layer (A) dry basil seeds, (B) seeds soaked in water, (C) induvial entire seed with mucilage layer under stereomicroscope (Guan et al., 2023).

Basil seeds absorb about 84 times their weight of water (Naji-Tabasi & Razavi, 2017a). Chia seeds absorb about 27 times their weight in water (Atik et al., 2020). To form a gel with chia seed mucilage a concentration of 15% (w/v) is required whereas for BSG only 6 % (w/v) is required. This is due to polysaccharides present in BSG, which are more highly branched (Atik et al., 2020). Also, the yield of gum from basil seeds is more than double the yield from chia seeds, 21% and 10%, respectively (Samateh et al., 2018).

2.6.1 Constituents of basil seed gum (BSG)

BSG contains glucose, galactose, mannose, arabinose, xylose and rhamnose sugars. The sugar units constitute about 92.44% w/w of BSG, while uronic acid makes up 6.51% w/w and the remaining are a small portion of protein (Guan et al., 2023; Hosseini-Parvar et al., 2010; Naji-Tabasi et al., 2016). BSG is a typical hemicellulose based on the composition of

its sugar units (Guan et al., 2023; Moreira & Filho, 2008; Rafe et al., 2013). The sugar units form two major polysaccharides in the gum layer, β-glucomannans and xylans.

β-glucomannans have a random chain arrangement of β-(1,4)-linked D-mannose and β-(1,4)-linked D-glucose (Guan et al., 2023). The hydrophobic segment of this chain is comprised of glucose and mannose at a ratio of 10:2. This hydrophobic segment contributes to 43% of the polysaccharides present and form the acid-resistant core (Guan et al., 2023; Rafe et al., 2013). β-glucomannans normally have a high degree of polymerization, with greater than 200 monosaccharide residues (Moreira & Filho, 2008). β-(1-4)-linked xylans make up about 24.29% of BSG and are responsible for hydrophilic behavior (Guan et al., 2023; Hosseini-Parvar et al., 2010; Naji-Tabasi et al., 2016).

Uronic acid units are linked to a xylose unit at the C-2 or C-3 position and these two carbon positions are also linked to random glucomannan segments. Minor glucans (2.31%) are also present in the polysaccharide layer (Hosseini-Parvar et al., 2010; Hosseini-Parvar et al., 2015; Munir et al., 2017; Wongputtisin & Khanongnuch, 2015).

The xylan in BSG contains D-xylose, L-arabinose, L-rhamnose, and D-galacturonic acid in the following 15:9:7:12 ratio (Wongputtisin & Khanongnuch, 2015). Xylan is important for the hydrophilic tail of BSG. In an emulsion system, the uronic acid xylan tails provide good surface interaction properties for the gum together with the hydrophobic centre of glucomannan (Guan et al., 2023; Rafe et al., 2013).

Glucomannan present in BSG contains a high number of acetyl groups which provide hydrophobic sites that can interact with fats and lipids, while the xylan present has uronic acid hydrophilic tails, and thus BSG can be used as an emulsifier to stabilize an emulsion system in food (Naji-Tabasi et al., 2016; Rafe et al., 2013). BSG solution at 1% (w/w) appeared as fibril tails attached to the globular centre-like scattered cotton structure in Figure 2.4. The cotton-ball-like cord centres shown in Figure 2.4 are possibly formed via hydrophobic repulsion and van der Waals interaction of glucomannan (Wongputtisin & Khanongnuch, 2015). BSG can form micro-gel particles and has been reported to provide Pickering stabilization in emulsions (Hosseini-Parvar et al., 2016; Rafe et al., 2013).

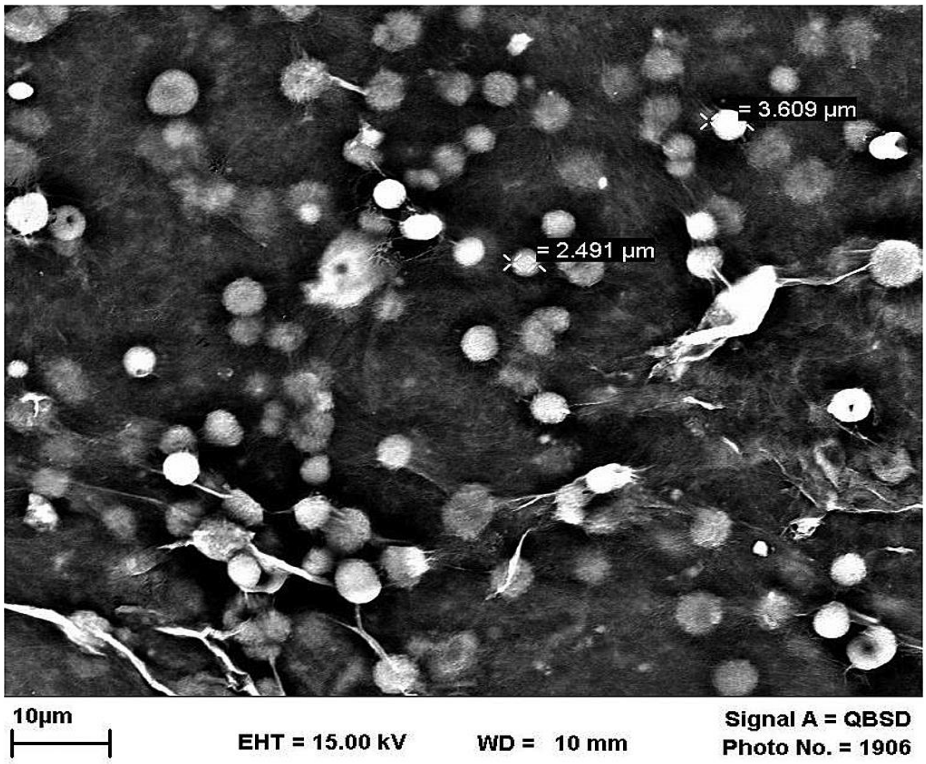


Figure 2.4 From top scanning electron microscope micrographs of BSG at 1% concentration (Rafe et al., 2013).

2.6.2 Extraction of basil seed gum

Basil seed gum extraction is carried with aqueous sodium hydroxide at pH 8 which results in dissolution of the polysaccharide layer (Hosseini-Parvar et al., 2010; Nazir et al., 2017; Razavi et al., 2009). This method results in a yield of gum of about 20.5% based on the whole seed weight (Guan et al., 2023). The seeds can be separated from the gum that forms on the surface of the seed using sieves or membrane filtration, followed by centrifugation (Table 2.4). BSG is then dried in either a hot-air oven or in a freeze dryer (Table 2.4).

Depending on the required usage for the BSG, it can be separated into two molecular weight ranges; Per-BSG with higher molecular weight (5980 kDa, yield 67%), and ethanol precipitated Super-BSG with more uronic acid content (MW 1045 kDa, 31% yield) (Guan et al., 2023; Naji-Tabasi et al., 2016). BSG can be purified with ethanol precipitation, this process also removes the water and protein present. However, the removal of protein from the gum reduces its surface activity and emulsifying ability (Hosseini-Parvar et al., 2016). A 92% yield of purified BSG can be achieved after 95%-ethanol purification leading to precipitation of the polysaccharides (Naji-Tabasi & Razavi, 2017a; Osano et al., 2014).

However, when used in a food matrix the BSG cannot be purified with any ethanol (BahramParvar & Goff, 2013; BahramParvar et al., 2012; Hosseini-Parvar et al., 2015; Naji-Tabasi & Razavi, 2017a; Song et al., 2017).

Extraction of BSG gum is affected by a few factors including temperature, pH, water:seed ratio, and soaking time (Table 2.4) (Guan et al., 2023; Naji-Tabasi & Razavi, 2017b; Nazir et al., 2017; Razavi et al., 2009).

To detach the gum from the seed, it is important to ensure minimal damage occurs during this “shredding” step. A variety of shredding methods include passing seeds between rotating rough plates or scraping the seeds through a mesh (Table 2.4). A juicer can also be used which can be an efficient and user-friendly method to separate the gum layer from the seeds (Guan et al., 2023).

After extraction and filtration, the BSG is dried using a number of methods, conventional hot air drying, freeze-drying, vacuum drying, and spray drying (Guan et al., 2023). Different drying methods can change the BSG gel hardness, stickiness, consistency, and adhesiveness, for example, freeze-drying contributes to the highest hardness and consistency of the BSG gel (Salehi & Kashaninejad, 2017). Hot air drying at 50^{o}C is more commonly used than freeze-drying (Table 2.4). Vacuum drying and spray drying have also been used for BSG drying (Guan et al., 2023). Long-time oven drying does not affect the color of BSG (Nazir et al., 2017; Razavi et al., 2009; Zameni et al., 2015). After drying, a dry sheet of BSG is formed and milled or ground for future use (Guan et al., 2023; Nazir et al., 2017; Razavi et al., 2009; Zameni et al., 2015).

Table 2.4 Extraction and drying methods of basil seed gum (BSG) in published research

pH	Temperature °C	Seed: water ratio	Shear rate	Extraction time (min)	Shredding method	Filtration method	Drying method	Reference
7.00	68 ± 1	1:20	Slowly	20	extractor	96% ethanol precipitation	Air force oven at 38°C	Naji-Tabasi et al., 2017
8.00	68 ± 1	1:65	1000 rpm	20	rotating extractor	20 µm filter and Centrifugation at 12,800 *g* for 30 min at 20°C	Freeze-dry	Hosseini-Parvar et al., 2010
8.09	68.71	1:65.98	1000 rpm	20	rotating extractor four times	cheesecloth filtration	50°C oven dry	Razavi et al., 2009
7.00	25	1:20	-	20	rotating extractor	25 mesh sieves (0.71 mm diameter)	oven drying freeze drying vacuum oven drying	Salehi & Kashaninejad, 2017
7.00	68	1:20	-	20	rotating extractor	Filtered and ethanol	Dehydrator at 38°C	Farahmandfar et al., 2019
7.00	50	1:20	-	20	rotating extractor	Filtered	Air force 50°C oven dry	Zameni et al., 2015
8.00	68	1:50	Stirring	20	rotating extractor	A cloth filter and Centrifugation at 12,800 *g* for 30 min at 20°C	Freeze-dry for 24 h	Kurd et al., 2017
Distilled water	35	1:10	Soak still for 8 h and then 1500 rpm for 10min	4810	-	Cheesecloth	-	Khazaei et al., 2014
8.00	56.71	1:66.84	Stirring	96	Mesh	Mesh size 10 (2 mm diameter)	50°C oven dry	Nazir et al., 2017

2.6.3 Physicochemical properties of BSG

BSG has shear-thinning and gel-like properties like most other gums. The shear-thinning property of BSG is dependent on concentration and temperature. In the temperature range of 5 – 85°C, the consistency coefficient for BSG viscosity at a shear rate of 1.0 s^{-1} decreased with increasing temperature at the same concentration (Naji-Tabasi & Razavi, 2017a). BSG has very high shear strength at very low or zero shear rate and exhibits stronger shear-thinning properties than xanthan, konjac, and guar gum at similar concentrations (Hosseini-Parvar et al., 2015; Naji-Tabasi & Razavi, 2017a). The shear-thinning behavior of BSG, provides liquid food fortified with BSG easy swallowing and pumping properties (Osano et al., 2014). The viscosity of BSG decreases as ionic strength increases and when the pH drops below pH 6 (Naji-Tabasi & Razavi, 2017a). However, when all the negative charges are dominated by positive ions such as Na^+ or Ca^{2+}, the viscosity does not change (Naji-Tabasi & Razavi, 2017a). Oil in water emulsion droplets stabilized with 0.3% BSG was affected by increasing ionic strength and decreasing pH (in the range of $4 \leq pH < 7$), but there was no phase separation in all emulsion samples after one month's storage and this could be due to BSG forming a gel-like structure at the interphase of the oil in water emulsion (Hosseini-Parvar et al., 2016).

2.6.3.1 BSG as a stabilizer

Hosseini-Parvar et al. (2010) have shown that the apparent viscosity of a 1% solution of BSG was about a thousand times higher than the apparent viscosity of guar gum or Konjac solution at the same concentration. In addition, when the concentration of BSG increased to 2%, the apparent viscosity was higher than observed with xanthan gum and matched that of carrageenan at the same concentration. BSG gum demonstrated shear thinning pseudoplastic behaviour at high shear stress and low shear rates. The gum was stable with temperature changes, making the gum a good stabilizer in food (Hosseini-Parvar et al., 2010; Hosseini-Parvar et al., 2016; Naji-Tabasi & Razavi, 2017a).

Stabilizers are often used in dairy products such as yogurt. In milk, casein micelles are suspended in an aqueous lay via electrostatic interaction and steric stabilization of κ-casein molecules (De Kruif & Zhulina, 1996). This balance is interrupted at the isoelectric point of the casein molecules (pH 4.6) at which point casein micelles disintegrate and casein

precipitates. At this point when casein aggregates, whey is expelled from the casein aggregates and released as 'serum'. Gelatin, guar gum, modified starch, and pectin are the common stabilizers used to reduce the visible release of serum in yogurt products (Kravtchenko et al., 1995; Williams & Phillips, 2003). BSG at a concentration of 0.2 – 0.5%, has been recommended for use as a stabilizer to reduce serum release (Williams & Phillips, 2003). Polysaccharide gums can interact with protein through covalent bonding, ionic and hydrogen bonding, hydrophobic and van der Waals attraction, and physical entanglement to form stable gel like structures (Adhav & Saikrishnan, 2023; Garti et al., 1999; Manab, 2017). They can also remain independent, and stabilization occurs through protein concentration and allowing the gum to stabilize the openings in the protein network via depletion stabilization when in sufficiently high concentration (Garti et al., 1999).

High uronic acid fractions in BSG contribute a polyelectrolyte character to the gum, where carboxylic acid groups of uronic acid in BSG can dissociate in water and release hydrogen ions and then leave a negative charge in the carboxylate groups (Matia-Merino et al., 2019). Uronic acid has a negative charge and can link to the positive charge of casein in acidic conditions to form a polyelectrolyte complex (Matia-Merino et al., 2019; Yang et al., 2021; Zahariev & Pilicheva, 2024). BSG can form carboxylic links with proteins and attach to water molecules via hydrogen bonding (Yang et al., 2021). BSG shows better water holding, oil holding capacity, and better swelling index than chia seed polysaccharides as shown in Table 2.5. BSG can interact with casein micelles and stabilize the water, thereby reducing serum release (Ghasempour et al., 2020; Guan et al., 2023; Nik et al., 2011; Rafe & Razavi, 2015).

Table 2.5 Water holding capacity, swelling index, and oil holding capacity of BSG, chia seed and other gums**.**

Source	Water holding capacity (g of water retained/g of sample)	Swelling index	Oil holding capacity (g of oil retained/g of sample)	References
Basil seed mucilage	97.5±2.4	462% at pH 6.0 950% in water	13.2±1.3	(Avlani, Ash, et al., 2019)
	32.82±0.93		8.37±1.02	(Norhayati et al., 2019)
		~725%		(Ziemichód et al., 2019)
Fatted Chia seed mucilage	103.2		25.79	(Segura-Campos et al., 2014)
Chia seed mucilage	266.55±6.12		58.61±0.56	(Coorey et al., 2014)
	110.5		11.67	(Segura-Campos et al., 2014)
	5.25		5.85	(Darwish & El-Sohaimy, 2018)
		~100%		(Fernandes et al., 2020)
		~580%		(Ziemichód et al., 2019)
Inulin	1.51±0.02 (from chicory)		0.97±0.02	(Rashid et al., 2018)
	4.95±1.13 (from Sunroot)		1.02±0.02	
Guar gum	24.83 ± 0.82		0.87 ± 0.06	(Coorey et al., 2014)
Gelatin	7.20 ± 0.31		1.05 ± 0.02	(Coorey et al., 2014)

2.6.3.2 BSG gelation

Hydrophilic homopolymers or copolymers can form three-dimensional water-swollen networks through covalent or ionic cross-linking (Peppas & Mikos, 2019). This polymeric network is defined as a hydrogel which can absorb large amounts of water or biological fluids (Peppas & Mikos, 2019). Hydrogels can be categorized into three different classes based on polymerization materials, polypeptide, polysaccharide and synthetic polymers like polyvinyl monomers hydrogels (Lapidot & Kost, 2001). Both chia seed gum and BSG gum can form a soft, porous gel known as a hydrogel in water and thus are able to hold water tighter to support the seed kernel to survive and germinate in harsh environments (Gutterman & Shem-Tov, 1997; Munir et al., 2017; Samateh et al., 2018). BSG can attract water molecules even in 40% ethanol to form gel whereas chia seed is unable to form a gel at ethanol solution of 10% or higher (Samateh et al, 2018). BSG at 0.5 – 3% forms a weak gel-like structure naturally (Rafe & Razavi, 2015). The gel network was not destroyed with

changes in temperature, neither at high nor in low temperatures (Rafe & Razavi, 2015). The BSG gel was stable at 121^{o}C for 20 min heat treatment and at freezing temperatures of -25^{o}C for 24 h (Rafe et al., 2013). BSG has been reported to have good freeze thaw stability and able to retain its full functionality after retorting at 121^{o}C for 20 min (Zameni et al., 2015). The elasticity of BSG hydrogel was improved by the presence of calcium ions especially in an acidic environment, and BSG has shown faster gelling capacity in a pH 4.5 solution (Bharati et al., 2022; Rafe & Razavi, 2013).

2.6.4 Application of BSG in dairy products

Hydrocolloids such as xanthan gum, guar gum, and modified cellulose, are used as fat substitutes due to their carbohydrate long chain structure, which can bind water molecules and interact with proteins (Kumar, 2021; Samakradhamrongthai et al., 2022). This similarity in structure could contribute to the similar mouth feeling of fat in processed food (Bourouis et al., 2023; Zhang et al., 2024). Therefore, many hydrocolloids have been studied and used as substitutes for fat in food products, that require high-fibre and low-fat content for a healthy diet. BSG has been used as a substitute for fat in pistachio butter, mayonnaise, ice cream, free-fat yogurt, and cheese (Hosseini-Parvar et al., 2015; Kim et al., 2020; Naji-Tabasi et al., 2017). BSG addition resulted in a reduction in the melting point of cheese samples and but also lead to increased hardness of the cheeses as concentrations of BSG increased (Hosseini-Parvar et al., 2015). BSG at 0.5% (w/w) resulted in a cream cheese with better properties than 0.5% xanthan gum as a fat substitute overall (Portaghi et al, 2023).

BSG was also studied as a fat-substitute in ice cream (BahramParvar et al., 2012; Javidi et al., 2016). It was found that at a concentration of 0.5%, BSG could be used alone as a fat substitute to reduce coarseness and coldness in low-fat ice cream and provide a similar level of creaminess as full-fat ice cream (Javidi et al., 2016). BSG at 0.3% (w/w) can be used as a novel stabilizer in ice cream production with 0.05% carboxymethyl cellulose (CMC) to increase the apparent viscosity, turbidity and air entrapment in ice cream mixes, also decreasing the draw temperature and melting rate compared to CMC or guar gum used alone or a combination of CMC and guar gum (BahramParvar et al., 2012).

BSG added to yogurt products has been reported to enhance the taste, and reduce serum release in yogurt products, especially at a 1% w/w concentration in skim milk yogurt (Kim et al., 2020). BSG is effective as the sole fat substitute in yogurt products at concentrations of 0.5 and 1% (Javed et al., 2022; Kim et al., 2020). Ribes et al (2021) reported that a higher concentration, 7.5% w/w chia seed gum, was required to produce a yoghurt with similar texture to full fat yogurt. Kim et al. (2020) added 1% BSG to low fat yogurt and found a similar viscosity to the full fat yogurt, 1953.33 ± 30.55 mPa.s and 2176.67 ± 150.44 mPa.s respectively. Chia seed gum does not reduce yogurt syneresis even at 7.5% concentration and 1% BSG stabilized the yogurt gel against any serum release during storage (Kim et al., 2020; Ribes et al., 2021). A lower concentration of BSG was studied at between 0.2 – 0.4% together with red beet extract (Ghasempour et al., 2020), and it was found that 0.4% BSG with additional red beet extract at 0.1% had the lowest pH value and the highest probiotic viability 10^8 cfu/g. The lowest concentration of BSG reported in yogurt was found to be effective at concentrations of 0.1 – 0.3% (Tan, 2019). Ghasempour et al. (2020) reported on the viability of *L. paracasei* yogurt cultures with 0.4% BSG and found 7 x 10^7 cfu/g compared to 0.2% BSG which resulted in 1 x 10^7 cfu/g. In comparison, Konjac and inulin added at 0.5% (w/v) and 1% w/v, respectively, were used as fat replacers and their addition was found to have no influence on yogurt properties (Dai et al., 2016; Gustaw et al., 2011). Inulin has been shown to reduce the viability of *L. acidophilus* after 21 days storage (Gustaw et al., 2011). BSG forms a web-like network with protein and assists with decreasing the amount of syneresis as well as increasing the firmness of the dairy products (Hosseini-Parvar et al., 2015; Nik et al., 2011). Greater than 0.3% up to 1% BSG were suggested to be used as the substitute for fat in dairy products in combination with other hydrocolloids to ensure consistency similar to full fat products (Hosseini-Parvar et al., 2015; Javidi et al., 2016; Kim et al., 2020; Nami et al., 2023).

2.6.5 Health benefits of BSG

BSG can slow the release of glucose into the bloodstream, as its hydrocolloid properties increase the viscosity of food and slow down interactions between digestive enzymes and nutrients. This slows down digestion and absorption of nutrients and increases feeling of satiety (Bharati et al., 2022; Giuntini et al., 2022; Samateh et al., 2018). BSG can therefore assist with reducing blood sugar levels and alleviating obesity and consequently help

manage type II diabetes and any complications (Bharati et al., 2022; Samateh et al., 2018). BSG has been found to inhibit the activity of the protein tyrosine phosphatase 1B which is a negative regulator of the leptin and insulin signaling pathways and thus influences secretion of leptin and insulin (Yili et al., 2014). BSG can facilitate the body's excretion of leptin and insulin and helps with managing diabetes and obesity health issues (Yili et al., 2014). BSG has also an ability to bind cholesterol and bile acid and thus can reduce cholesterol levels, improve blood lipid profile, and assist with the prevention of cardiovascular diseases (Nazir & Wani, 2021).

It is recommended to consume daily 30 – 35 g of dietary fibre for men and 25 – 32 g for women (Barber et al., 2020; Stephen et al., 2017). The fibre passes through the upper GI tract and is not broken down by the alimentary enzymes in the lower GI tract (Dhingra et al., 2012; Margină et al., 2020). However, the fibre, it can be fermented by beneficial bacteria in the lower GI tract (Margină et al., 2020). Sufficient uptake of dietary fibre (≥10g) has been reported to effectively reduce blood inflammatory markers, TNF-α, and C-reactive peptide in hemodialysis patients (Margina et al. 2020). Extracted BSG is a good source of daily fibre particularly soluble fibre, which supplies 25% of the RDI with about 7g fibre per 13 g seeds (McCulloch, 2019). Fibre is required to support gut health, help to balance blood sugar levels, lower blood cholesterol, and also contribute to satiety and thus help with weight management (Adam et al., 2016; Leelahagul et al., 1992; Melina et al., 2016; Naji-Tabasi & Razavi, 2017b; Tingirikari, 2018; Viseshakul et al., 1985). Consuming four grams of basil seeds can provide more dietary fibre than a whole lettuce bulb (Gajendiran et al., 2016). BSG can also indirectly promote health by providing phenolic and flavonoid compounds and increasing antioxidant capacity (Kim et al., 2020; Kim et al., 2021). BSG can also be used for encapsulation, to coat probiotics for delivery and release into the lower GI tract where they along with oligosaccharides can help to improve health. (Nami et al., 2023).

2.7 Conclusions

Yogurt is made from key ingredients varieties of milk, yogurt starter cultures and optional ingredients to improve the flavour, texture and thearuputic benefites. When skimmed milk powder is used as yogurt protein base, low and media heat SMP is recommended. Yogurt

starter cultures, *S. thermophilus* and *L. bulgaricus*, are responsible for the fermentation process and flavor development, while probiotics, for example *L*. *acidophilus* and *B. lactis*, offer therapeutic benefits to the body.

In yogurt production, sweeteners like sugar are added to enhance taste, and alternatives such as erythritol could potentially replace sugar without altering the product's overall quality. Prebiotics, including oligosaccharides, are important for gut health, as they stimulate the growth of beneficial microorganisms. Oligosaccharides like FOS, GOS, MOS and emerging XOS are particularly recommended for their beneficial properties in promoting probiotic growth. Enzyme hydrolysis is effective for breaking down larger polysaccharides into shorter chain oligosaccharides, and thus enhancing their prebiotic potential.

Basil seeds (BSG) produce a significant amount of gum, which can act as a stabilizer in yogurt, potentially reducing whey separation when used with SMP. BSG could be a promising ingredient to improve the texture and quality of yogurt. BSG is typically extracted from the seed using pH-adjusted water in the range of pH 7 – 8, although some extractions use distilled water. It is important to investigate whether BSG, when extracted in pure water, could enhance probiotic growth similarly to other prebiotics like inulin or resistant starch and their hydrolyzed fragments.

Chapter 3 Materials and Methodology

3.1 Materials

Low heat skimmed milk powder (SMP) was purchased from Fonterra Co-operative Group, New Zealand (June 2020). The SMP contained 32.91% protein, 0.93% fat, 54.50% carbohydrate, 7.90% ash and 3.79% moisture (w/w). The milk powder was packed into about 200 g portions in small foil pouches. Basil seeds were provided by Aotearoad, New Zealand (aotearoad.com), and then sub-packaged into 750 ml Whirl-Pak sample bags (Fisher, China). Food-grade enzymes were supplied by different manufacturers as shown in Table 3.1. Cane sugar (Chelsea, NZ Sugar, New Zealand) and erythritol (NatVia, Australia) were purchased from the local supermarket. SMP and basil seeds were stored a refrigerator for future use. The yogurt culture, YO-MIX® 726 (Danisco, France) commercial freeze-dried direct-vat-inoculate (DVI) for yogurt production contained *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* in the pack. YO-MIX® 726 powder was stored at -20 ± 1^{o}C freezer (Skope, New Zealand). All other chemicals used were analytical grade and purchased from Merck, New Zealand, or Thermo Fisher Scientific, New Zealand. Enzymes purchased for hydrolysis of basil seed gum are listed in Table 3.1.

Table 3.1 Supplier information for different enzymes used for hydrolysis of basil seed gum (BSG)

Name	Supplier	Enzyme concentration	Carrier	Source of enzyme
β-mannanase	Lyphar Biotech, CN	30,000 u/g	dextrin	*Aspergillus niger*
Magizyme ZAC mixture	Zymus, NZ	β-glucanase, Xylanase and minor Endo-1,3(4)-beta-D-glucanase	Not available	Not available
SQZyme xylanase	Suntaq, CN	80,000 u/g	Cornstarch and minor dextrin	*Trichoderma reesei*
CE35 cellulase	Sunson, CN	3,500 u/g	dextrin	*Trichoderma reesei*

3.2 Extraction of basil seed gum

3.2.1 Introduction

Most of the BSG extractions reported in the literature were conducted with pH adjustment, typically using 0.01M NaOH for this purpose (Hosseini-Parvar et al., 2010; Hosseini-Parvar et al., 2015; Hosseini-Parvar et al., 2016; Razavi et al., 2009). Sodium hydroxide solution can help large molecular weight polysaccharides to dissolve in water, but in the meantime can break down polysaccharide fibre at temperatures higher than 10^{o}C after more than two hours soaking (Shi, 2016). Sodium hydroxide residues can either retain the antimicrobial capability or inhibit microbial growth (Cytiva, 2020). Guan et al. (2023) have reported that the extraction yield of BSG was higher in an acidic environment than in a basic environment. Sodium ions can also bind with the negative charges of uronic acids in BSG to decrease the solubility of BSG in water and may lead to a weaker gel with time (Guan et al., 2023; Hosseini-Parvar et al., 2016). Therefore, extraction of basil seed gum using reverse osmosis (RO) water could be more favourable and lead to the potential of using the BSG as a food ingredient.

From previous studies, temperature, soaking time and water/seed ratio were the major variable factors that affected the efficiency of the gum extraction (Hosseini-Parvar et al., 2010; Nazir et al., 2017; Razavi et al., 2009). Nazir et al. (2017) and Hosseini-Parvar et al. (2010) had similar water/seed ratios, 66.84:1 and 65.98:1, respectively. The extraction water/seed ratio used in this study was then used at 66:1 water/seed ratio. However, shear rate (stirring speed) was not discussed in any of the studies. Hosseini-Parvar et al. (2010 and 2015) all used 1000 rpm and Nazir et al. (2017) did not mention shear rate. Shear rates were thus included as one of factors to be tested. The classical method of determining optimum conditions of the variable factors was set up by varying one parameter while maintaining other factors at a constant level.

3.2.2 Extraction experiments

Ten grams of basil seed were used for each preliminary screening experiment, and temperatures were used between 20 – 80^{o}C at 10^{o}C intervals. Extraction times between 20 min to 130 min were chosen with 10 min intervals for the bench-scale experiments (Table

3.2). A Box-Behnken design, one of the Response surface models, was then selected to assist in finding the optimum extraction condition for basil seed gum with RO water. The extraction flow chat is shown in Figure 3.1. A high shear rate was achieved using an overhead Silverson mixer model RW 20D S5 (IKA, Malaysia) and shear rate from 400 to 1600 rpm and varied every 200 rpm, were tested as the third factor in preliminary factor range screening for a Box-Behnken design of BSG extraction in RO water.

Table 3.2 Preliminary range screening for temperature, time and shear rate factors in the Box-Behnken design of BSG extraction.

Temperature (°C)		**Time (minute)**		**Shear rate (rpm)**	
Range	Fixed point	Range	Fixed point	Range	Fixed point
20-80°C with 10°C intervals	50°C	20-130 min	60 min	400-1600 rpm with 200 rpm interval changes	700 rpm

The basil seed gum mixture was cooled to room temperature and then most of the gum was recovered four times with a commercial centrifugal Fruit and Vegetable juicer (Avanti, Australia) (Razavi et al., 2009). The basil seed and mucilage that did not go through the juicer was collected and then centrifuged at 10375 x *g* for 10 min at 20 ± 0.5^{o}C (Sigma centrifuge 6-16KS, Germany). The supernatant mucous was combined with the mucilage collected from the juicer and then poured into a metal tray covered with baking paper for drying.

Higher shear rates were also tested at 1200 rpm, 1400 rpm and 1600 rpm, the maximum shear rate without major loss of rehydrated basil seeds. Apparent viscosity was also used to help with the selection of shear rate range in the preliminary test for screening optimum extraction conditions of BSG. Apparent viscosity of basil seed gum samples was measured at a shear rate of 20 rpm using a Brookfield DV II+ viscometer (England) with spindle No. LV2. BSG (250ml) in a tall form glass beaker was tested for each BSG sample at 28 ± 0.5^{o}C. The viscosity readings were taken at 60 s when the reading had stabilised. Analyses were carried out duplicate and results were in units of mPa.s.

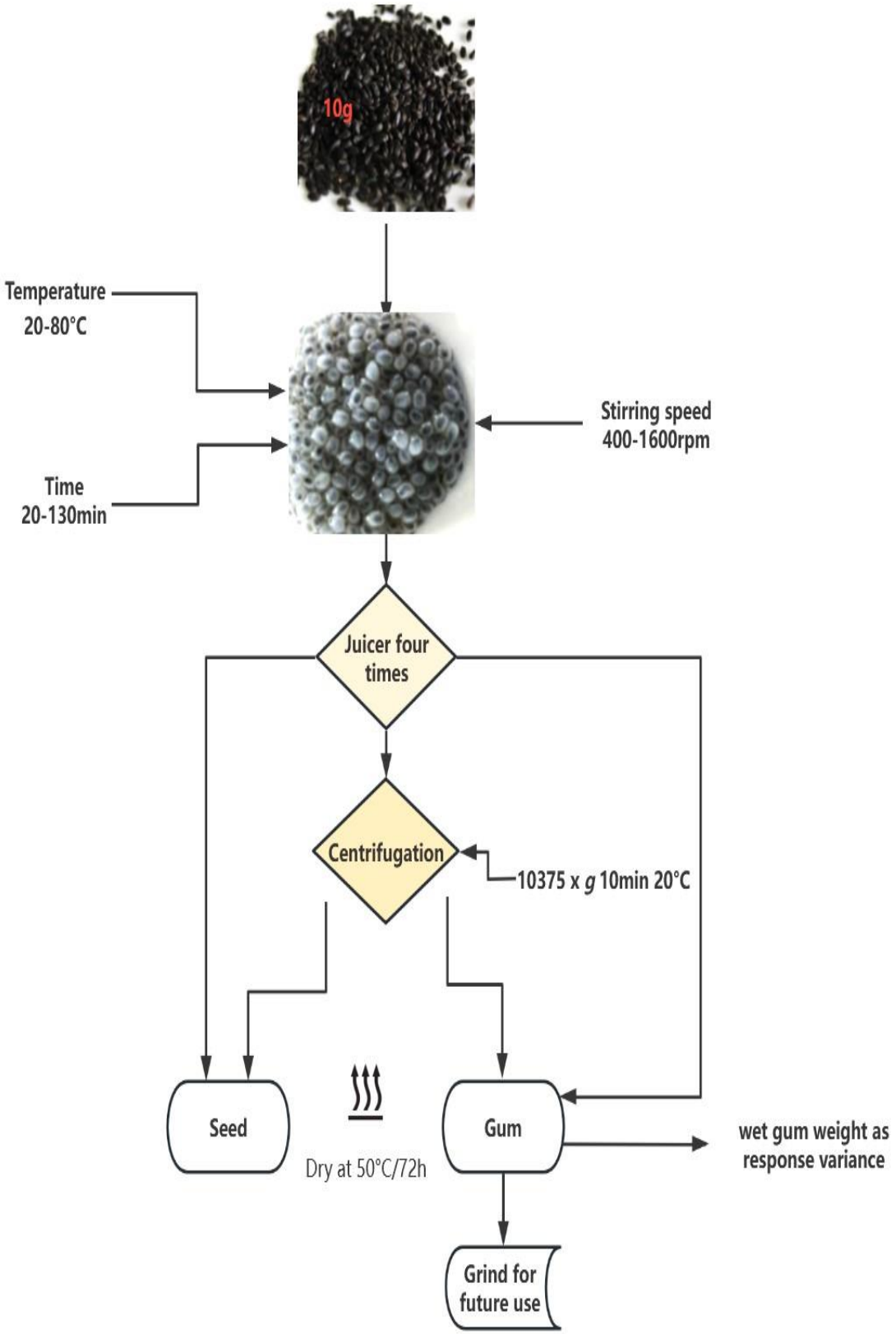


Figure 3.1 Diagram of BSG extraction from basil seeds in RO water

The optimum extraction condition of basil seed gum was obtained via the Box-Behnken experimental design (BBD) to achieve the highest yield of the gum. The response variables were the wet gum and separated wet seeds. The quadratic equation (Equation 3.1) was used to explain the behavior of this experimental design.

$$Y = b_o + \sum_{i=l}^{3} b_i X_i + \sum_{i=l}^{3} b_{ii} {X_i}^2 + \sum_{i=l}^{2} \sum_{j=i+1}^{3} b_{ij} X_i X_j \qquad (Equation\ 3.1)$$

In Equation 3.1, Y is the response variable (the weight of BSG wet gum or the weight of wet seeds), b_o, b_i, b_{ii} and b_{ij} are the regression coefficient estimated parameters by the model, where X_i and X_j represent the levels of independent variables (Araujo et al., 2017; Chen et al., 2013). The independent variables were shear rate, soaking temperature and time. There were three repeated central points to evaluate the experimental error and to adjust the model. The experimental design layout is in Table A2.3 (Appendix 2.1).

A commercial fan drying oven (Thermofisher Heratherm, America) at 50 ± 2°C was used for drying the gum for up to 72 hours (Nazir et al., 2017). Dried BSG was ground in a coffee grinder (Breville, Australia) stored in 750 ml resealable plastic bags and placed in a commercial freezer (Skope, New Zealand) at about -20 ± 1°C. The moisture content of the dried powder was consistently at around 6.0% w/w. BSG powder was used for all experiments and concentrations were calculated based on dry powder weight versus solution volume (w/v).

3.3 Set yogurt preparation

3.3.1 Preliminary ingredient screening

In preliminary tests, BSG, SMP, YO-MIX® 726 yogurt cultures, cane sugar and erythritol were weighed according to Table 3.3, dry-blended and then mixed with 400 ml of RO water. The ingredients were mixed well with water and split into four 100ml-sample containers for fermentation at 43 ± 0.5°C for 6 h. Yogurt processing steps were according to the steps in Figure 3.2.

Table 3.3 Preliminary range screening for factor BSG, SMP, YO-MIX® 726 cultures, cane sugar and erythritol for a Box-Behnken design.

Varied ingredient (factor)	Test range % (w/v)	A fixed concentration for other ingredients in each fermentation (fixed point factors) % (w/v)				
		BSG	**SMP**	**YO-MIX® 726 cultures**	**Cane sugar**	**Erythritol**
BSG	0 0.01 0.03 0.05 0.1 0.2 0.3 0.5 0.75 1.0	–	10.0	0.05	4	4
SMP	5.0 8.0 10.0 12.0 15.0 20.0	0.1	–	0.05	4	4
YO-MIX® 726 cultures	0.001 0.01 0.03 0.05 0.075 0.1 0.2	0.1	10.0	–	4	4
Cane sugar	0.1 1 2 4 6 8 10 12	0.1	10.0	0.05	–	4
Erythritol	0.0 2.0 4.0 6.0 8.0	0.1	10.0	0.05	4	–

3.3.2 Preparation of yogurt with BSG and erythritol for the BBD design

SMP at concentrations of 8 – 15% was dry blended with BSG at 0 – 0.2%, 8% sweetener (cane sugar/erythritol ratio), 0.001% DVI of YO-MIX® 726 yogurt culture. Combined well mixed powders were dissolved in RO water at 20 ± 2°C and then fermented in a 43 ± 0.5°C in a water bath for 6 h. Yogurt samples were tested after fermentation for firmness, serum separation (% Serum) and probiotic cell counts (Figure 3.2).

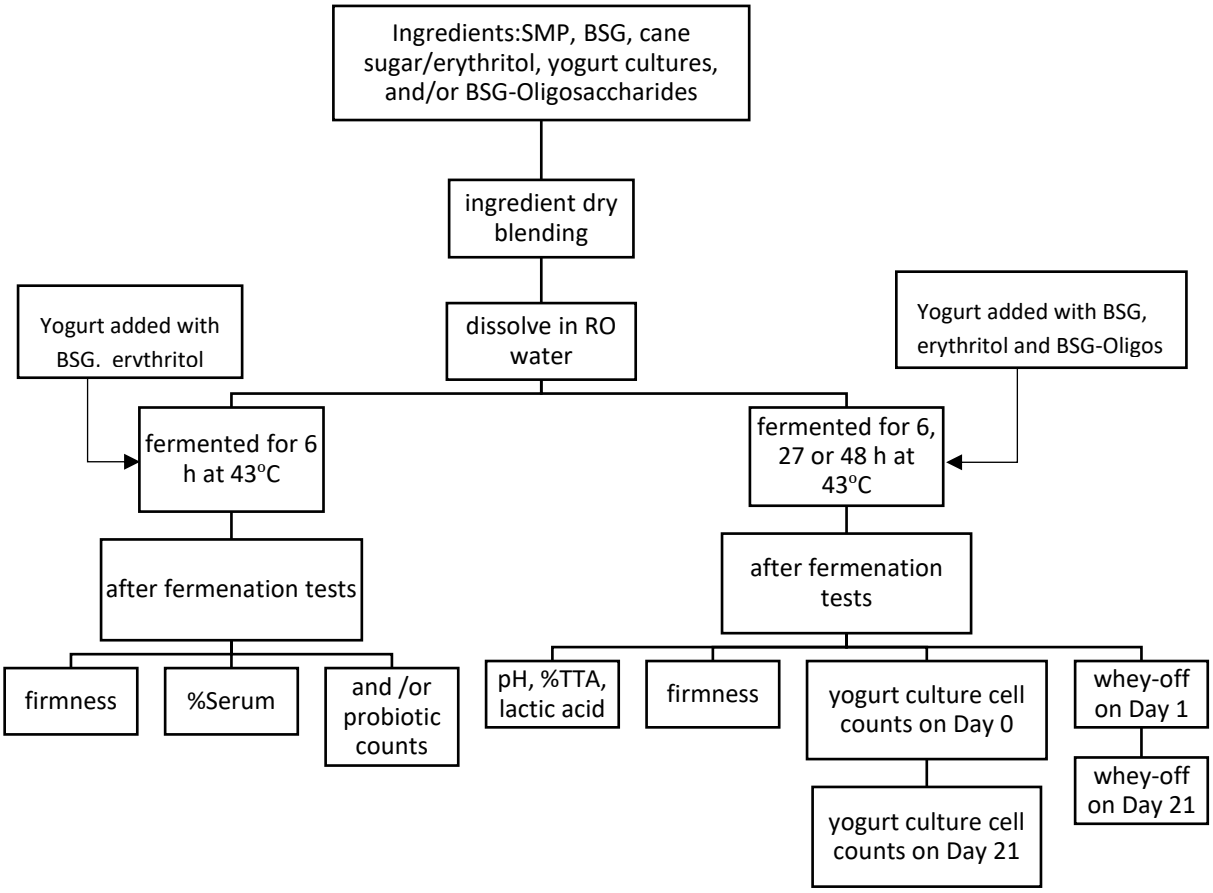


Figure 3.2 Processing charts of set yogurt containing BSG, erythritol, and/ or BSG-Oligosaccharides.

3.3.3 Preparation of yogurt with BSG, BSG-Oligos and erythritol for a BBD

For BSG-Oligo application in yogurt, SMP, BSG at 0 – 0.06%, BSG-Oligosaccharides at 0 – 3.0%, 8% sweetener with different sugar/erythritol ratios, 0.001% DVI of YO-MIX® 726 yogurt culture were used to ferment yogurt and the yogurt profile was analysed in Day 0 for firmness, yogurt bacteria cell count, pH, titratable acidity (%TTA) and lactic acid. Serum separation was tested on Day 1 and 21. Yogurt culture cell viability was also tested on Day 21 (Figure 3.2).

3.4 Acidity analysis of fermented milk

Yogurt bacteria metabolise lactose in bovine milk and then excrete lactic acid which lowers pH of the milk. The reduction in pH causes milk to form a curd due to casein coagulation

starting at pH 5 until pH 4.6, when the casein charges are completely neutralised at its isoelectric point (Laligant et al., 2003). Lactic acid production corresponds with the release of the typical yogurt aroma and flavour together with other chemical compounds from bacterial metabolism. Lactic acid contributes to the freshness sensation of fermented milk products and directly impacts the texture of yogurt (Cheng, 2010; Spanier et al., 2001). Understanding acidity changes during fermentation is therefore important. Direct electronic determination of pH is used to monitor the fermentation process (Robinson et al., 2007). pH was measured in the fermented milk to assist with formulation screening followed by determination of the total titratable acidity and quantifying lactic acid with HPLC in the final products.

3.4.1 pH measurement

A 10 g yogurt sample was mixed with 20 mL of RO water by vortexing for one minute before measuring pH using a Sartorius (Germany) pH meter equipped with a Thermo Fisher (USA) glass electrode at 20 ± 1°C (APHA, 2004; Song & Kim, 2019). The pH meter was standardised with pH 4.0, 7.0 and 10.0 standard buffer solutions (LabServ Pronalys, New Zealand), before each batch measurement. Samples were measured in duplicate.

3.4.2 Total titratable acidity as lactic acid (%)

Fermented dairy products should have 0.3% w/v or w/w lactic acid in the final product (FAO/WHO, 2003a). The AOAC method No. 16.023 for titratable acidity was used to determine acidity in fermented milk drinks (AOAC, 1990). Aqueous solutions of sodium hydroxide (NaOH) (AR, ThermoFisher, New Zealand), phenolphthalein (1%) indicator (AR, Ajax, Austrila) and potassium hydrogen phthalate (KHP) (AR, Ajax, Austrilia), were prepared for lactic acid titration (Appendix 1.2). Sodium hydroxide solution (about 0.1M NaOH) was standardized to confirm its concentration before conducting any titrations. Potassium hydrogen phthalate was used to standardize the 0.1M NaOH solution according to AOAC (1990). Three to four drops of phenolphthalein solution were used as an indicator to give a faint pink endpoint for KHP solution titrated against NaOH, and at the endpoint the solution remained a pink colour for 30 seconds. The titration was repeated until titres were within 0.05 accuracy, and the NaOH solution molarity was calculated using Equation 3.2. The molarity of sodium hydroxide (M_{NaOH}) (mol/L) was determined knowing the volume in litres

(V_{NaOH}) used in the standardisation titration and the molecular weight of KHP (MW_{KHP}) (204.23 g/mol).

$$M_{NaOH} = \frac{gram\ of\ KHP}{V_{NaOH} \times MW_{KHP}} \qquad (Equation\ 3.2)$$

In the experiment, lactic acid (%), expressed as 'titratable acidity', was calculated according to method AOAC (1990). For each sample, 10g was mixed thoroughly with 20g RO water before titration. Three to four drops of phenolphthalein indicator were added to the diluted sample and mixed well. The diluted samples were titrated against a standardized 0.1M NaOH solution until the first persistent pink colour appeared. Titratable acidity was expressed as % titratable acidity (w/w as lactic acid), using Equations 3.3, 3.4 and 3.5.

$$M_{Lactic\ acid} = C_{NaOH} \times V_{NaOH} \qquad (Equation\ 3.3)$$

$$m_{Lactic\ acid} = M_{Lactic\ acid} \times 90.08 g/mol \qquad (Equation\ 3.4)$$

$$\%Titrable\ acidity = \frac{m_{Lactic\ acid}}{m_{sample}} \times 100 \qquad (Equation\ 3.5)$$

Where $M_{Lactic\ acid}$ is the molarity of the lactic acid, C_{NaOH} is the confirmed concentration of sodium hydroxide solution (mol/mL) after standardisation, 'V_{NaOH}' is the volume of standardized sodium hydroxide solution (mL) required to neutralize the lactic acid in the sample. $m_{Lactic\ acid}$ is the molecular weight of lactic acid (90.08 g/mol). All samples were measured in duplicate.

3.4.3 Quantifying lactic acid by higher-performance liquid chromatography

3.4.3.1 Sample extraction

Yogurt samples were prepared using the following procedure. One gram of yogurt was homogenized with 5 ml of 45 mM H_2SO_4 (ACS, Fisher Scientific, America) for 1min in a VM-96B vortex mixer at 2500 rpm (Jeio Tech, Korea). The solution was then stirred for 30min in a MaxQ multi-purpose shaker (Thermo Scientific, America) at 240 rpm following an additional one-minute vortexing at 2500 rpm. The homogenised sample solutions were

centrifuged at 5500 x *g* for 20 min at 4 ± 0.5°C. The supernatant was collected and filtered through a #1 filter paper (Whatman, America) and then passed through a 0.45 µm pore size membrane (PES, Eco-sensa, America). The filtrates were then stored in 4 ± 1°C until analysis (da Costa et al., 2016).

3.4.3.2 Quantification of lactic acid in each sample by HPLC

Lactic acid was quantified by high-performance liquid chromatography (HPLC) (Shimadzu model LC-10AT, Japan) with a Rezex Ion Exclusion ROA-Orangic acid H+ (8% cross-linked resin) LC column, 300 mm x 7.8 mm diameter (Phenomenex, USA). The column temperature was maintained at 40°C. The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.5 ml/min (Costa et al., 2019; Kaminarides, Stamou, & Massouras, 2007; Kosasih, 2011). The sample injection volume was 20 µL (SIL-10A injector, Shimadzu Corp, Japan) and the detection was by SPD-10A UV-Vis detector at 210 nm (Shimadzu Corp, Japan). The integrator automatically calculated the peak area of lactic acid that occurred at a retention time of about 17.5 min (Appendix 1.7).

Lactic acid was quantified using an external standard. A standard curve for lactic acid was determined with known concentrations and determined peak areas, obtained by integration using Shimadzu LC Solutions Software (Shimadzu Prominence, Japan). The lactic acid linear regression equation was gained based on a regression line of lactic acid standards at concentrations of 0.1, 0.5, 1, 2, 5, and 10 mg/ml concentration of the lactic acid (>98%, Sigma, USA) (Costa et al., 2019; Kaminarides et al., 2007; Kosasih, 2011), and linear correlation calculation of lactic acid standards was repeated for each batch of lactic acid analysis for the samples in the Shimadzu HPLC instrument. An example of the lactic acid standard retention time and the regression line used for quantification is shown in Figure A1.3 (Appendix 1.7). Each yogurt sample was extracted and tested in duplicate.

3.5 Texture analysis-hardness

The TA-XT plus Texture Analyzer (Stable Micro Systems, Godalming, England) was used to analyse the firmness/hardness of the yogurt gels formed. A 100 ml sample cup (LabServ, New Zealand) was used for yogurt fermentation. The texture analyser was fitted with a 25 mm flat base test probe, which was used to penetrate the sample to a depth of 30 mm by

compression with a 5 kg load cell, samples were measured at 20 ± 2°C (Domagala, et al., 2006; Kosasih, 2011). The puncture test speed was set at 1 mm/s with pre and post-test speed at 5 mm/s. Results were recorded from the first significant peak on the texture curve profile plot from the texture analyser, a texture profile plot example is shown in Appendix 1.5. The value reported was force (g) (Bierzunska et al., 2019; Kosasih, 2011). All samples were tested according to either preliminary ingredient range screening in Table A2.5 (Appendix 2.2) or the Box-Behnken design layout of Table A2.30 (Appendix 2.4.2).

3.6 Syneresis measurement

3.6.1 Serum separation (% based on weight)

The syneresis index was measured as the weight in grams of drained serum per weight of yogurt product (Dannenberg & Kessler, 1988; Akalın et al., 2012; El Bouchikhi, Pagès, El Alaoui, Ibrahimi, & Bensouda, 2019). The syneresis analysis method was modified based on the methods reported by Akalin et al. (2012) and El Bouchikhi et al., (2019). A portion of BSG-SMP yogurt was removed from the container and the weight of the removed gel was measured immediately on a plastic plate. The weight (g) of serum drained off after 3 h at 10°C was measured and the syneresis index was calculated based on Equation 3.6 and reported as % Serum.

$$(\%)Serum = \frac{drained\ whey\ weight\ (g)}{weight\ of\ the\ tested\ gel\ (g)} \times 100 \qquad (Equation\ 3.6)$$

3.6.2 Serum separation (% based on height)

An alternative measure of syneresis index was also used and based on real-time serum separation height versus the whole gel height. This ratio was converted to a percentage serum of released (Equation 3.7) (Shirkhani et al., 2015; Viñarta et al.,, 2006).

$$(\%)Serum = \frac{serum\ (mm)}{height\ of\ the\ whole\ gel\ (mm)} \times 100 \qquad (Equation\ 3.7)$$

3.7 Microbiological analysis of yogurt cultures

The recommended level of probiotic bacteria cells for delivery of health benefits in yogurts is ≥ 10^6 cfu/g living probiotic bacteria (Lourens-Hattingh & Viljoen, 2001; FSANZ, 2015; Ouwehand, 2017; Ravula & Shah, 1998). To effectively deliver the therapeutic level of probiotic cells to consumers, viable cell counts must be assessed, and appropriate measures must be taken to ensure the survival of the probiotic bacteria (Allgeyer, et al., 2010).

3.7.1 Yogurt starter-*Streptococcus thermophilus* (ST)

M17 agar (Oxoid, England) was used for *S. thermophilus* plate counts, which is known to have limited the growth at high levels of lactobacilli (Micanel et al., 1997; Downes & Ito, 2001). Fifty millilitres of 10% lactose solution were added to 950 ml of M17 agar as a nutrient supplement for the growth of ST. The lactose was added after the media cooled down to 45^{o}C after autoclaving. The M17 culture plates were incubated at 37^{o}C for 48 hours aerobically.

3.7.2 Yogurt starter-*Lactobacillus delbrueckii* subsp. *bulgaricus* (LB)

MRS agar (Oxoid, England) was used for the total count enumeration of lactobacilli in the yogurts. The level of LB was obtained by subtracting the counts of probiotics from the total counts of the *lactobacilli* (IDF, 2006; APHA, 2004; Micanel, et al., 1997).

3.7.3 Probiotic bacteria- *Lactobacillus acidophilus* (LA) and *Bididobacterium animalis* Subsp. *lactics*

Several selective methods for probiotic bacterial enumeration are available such as the use of MRS agar with 0.2% ox-bile salts or with different sugar bases such as trehalose instead of lactose (Lima et al., 2009). However, the use of MRS agar with clindamycin is recommended by the International Dairy Federation (IDF, 2006). LA cannot survive in the MRS-clindamycin agar at any dilution at > 10^{-4} (Evans, 1998; Shah, 2000). Therefore, the true cell counts of *L. acidophilus* and *B. lactis* should be taken from any dilution of MRS-clindamycin agar plate higher than 10^{-4} dilution.

Clindamycin hydrochloride (C5269-10mg, Sigma Aldrich, USA) was dissolved in 100 mL sterile reverse osmosis water and then filtered through a 0.2 µm sterile syringe filter (FP point 2-S, Whatman, UK). The sterile solution was dispensed into 2 mL microcentrifuge tubes and immediately stored at -20 ± 1^{o}C until required for use. Clindamycin solution has a shelf-life of one year if stored frozen. For use, 0.5 mL of thawed clindamycin solution was added to 100 mL of MRS agar at 45^{o}C before pouring plates.

A summary of the media used for the enumeration of the individual cultures is listed in Table 3.4. Microbiological analyses were conducted on the fermented milk samples based on IDF standard methods (IDF, 2006; APHA 2004; Micanel, et al. 1997). A well-mixed one mL sample was aseptically pipetted into a 9mL 0.1% peptone water vial, giving a 10-fold (10^{-1}) dilution sample. The 10^{-1} diluted sample was further serially diluted up to 10^{-7}. One mL solution of each diluted sample was transferred into a sterile petri dish followed by pouring 15 – 18 mL of the respective agar at 45 ± 1°C into the Petri dish. The pour plate method is preferred due to the higher accuracy in contrast to the spread plate technique (Downs and Ito, 2001). The set MRS sample plates were placed into 7 L or 2.5 L AnaeroPack rectangular jars (Mitsubishi, Japan) and Oxoid AnaeroGen™ gas packs (Thermo scientific, USA) were used to burn the oxygen in the box to create anaerobic conditions for the growth of *L. bulgaricus*, *L. acidophilus* and *B. lactis*. The Oxoid anaerobic indicator (Oxoid, UK) indicated all oxygen was burnt when it turned from pink to white, and carbon dioxide replaced oxygen and created anaerobic conditions in the jar. The indicator remained a white colour until the end of the incubation period and an anaerobic environment was achieved for the *lactobacilli* and *B. lactic* growth in each of the anaerobic containers after 72 h incubation.

Colonies of *S. thermophilus* are circular and creamy white colour with smooth edges (IDF, 2003). To obtain better growth of the *lactobacilli* and *Bifidobacteria*, MRS plates were overlaid with extra MRS agar to improve the anaerobic conditions. Plates with 10 – 300 colonies were counted (IDF, 2006). The results from the microbial counts will be shown as cfu/g (colony forming unit per gram of fermented milk sample).

Table 3.4 Selective media and methods used to enumerate yogurt starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) and probiotics (*Lactobacillus acidophilus* and *Bididobacterium animalis* Subsp. *lactics*) in yogurt.

Medium	Culture	Incubation conditions	Enumeration method
M17 agar[1]	*S. thermophilus*	37°C for 48 h	Direct counting of typically developed colonies
MRS agar[2,a]	*L. delbrueckii* subsp. *bulgaricus*& *L. acidophilus* & *B.lactics*	37°C anaerobically for 72 hours (Oxoid Anaerogen gas pack and Mitsubishi AnaeroPack rectangular jar)	Direct counting of typically developed colonies
MRS-clindamycin agar[3,a]	*L. acidophilus* & *B.lactics*	37°C anaerobically for 72 hours (Oxoid Anaerogen gas pack and Mitsubishi AnaeroPack rectangular jar plus oxygen indicator)	Direct counting of typically developed colonies
MRS agar[2,a]	*L. delbrueckii* subsp. *bulgaricus*	Same as above	Estimation of LB = total count of *Lactobacilli* deducts count of *L. acidophilus*

[1]M17 – 50 mL of 10% sterile lactose solution (w/v) added to autoclaved of M17 agar at 45°C, pH 7.1 ± 0.2.
[2]MRS agar – molten de Man Rogosa Sharpe agar, pH 6.6 ± 0.1.
[3]MRS – clindamycin agar - molten de Man Rogosa Sharpe agar with 0.5 ppm clindamycin solution.
[a]Composition and full preparation steps for the culture media are found in Appendix 1.1.

3.8 Screening of optimum composition of BSG, SMP, cane sugar and erythritol

3.8.1 Preliminary screening for yogurt ingredients

Yogurt gel firmness and syneresis were measured to assess the individual impact of each ingredient on yogurt production, including BSG, SMP, yogurt culture, cane sugar and erythritol. SMP has been reported to have a positive effect on the growth and maintenance of yogurt culture cell concentrations in yogurt fermentation (Nor-Khaizura et al., 2012). While cane sugar has been reported to lower yogurt culture concentrations when the sugar level is above 12% w/v concentration (Chandan & Kilara, 2013). The impact of BSG and erythritol on yogurt production was unknown. Each ingredient was studied as one factor varied and the other four factors were fixed at a certain point during the screening step

(Ghasempour et al., 2020; Liu, 2013; Nor-Khaizura et al., 2012). The range of factors to be tested and its fixed point are listed in Table 3.3.

3.8.2 Optimum ingredient composition for yogurt production

The optimum level of each yogurt ingredient was chosen via a BBD with the aim to mimic the hardness of silken tofu, to achieve less serum separation and a high growth rate of yogurt cultures during fermentation. The response variables were firmness of the fermented yogurt samples, % serum and viability of the bacterial cells in the samples.

The quadratic equation (Equation 3.1) was used again to explain the behavior of this experimental design. Y is the response variables (the firmness, serum volumes or probiotic cell counts), b_o, b_i, b_{ii} and b_{ij} are the regression coefficient estimated parameters by the model where X_i and X_j are the level of each factor of interest (Araujo et al.,2017; Chen et al., 2013). The experimental design layout is shown in Table A2.30 (Appendix 2.4.2).

3.9 Enzyme hydrolysis of BSG

3.9.1 Verification of β-mannanase, xylanase or cellulase activity

One gram of β-mannanase was dissolved in 700 mL of 0.05M Na_2HPO_4-citric acid buffer solution at pH 6.0. The enzyme solution was stirred for 20 min at room temperature then followed by centrifugation at 1127 x *g* for 10 min (Sigma centrifuge 6-16KS, Germany) to remove any solid impurities which could cause false results in later determining enzyme activity by the Nelson-Somogyi method (Bailey et al., 1992; Chen et al., 2013). The supernatant was used for testing β-mannanase activity. Two millilitres of 1.0% (w/v) locust bean gum (LBG, Hawkins Watts, NZ) were added to 2 ml of the enzyme supernatant and then incubated at 40^{o}C for 20 min (Chen et al, 2013). The solutions were heated to boiling and held for 15 min to stop the enzyme activity.

The reducing sugars were quantified via the Nelson-Somogyi method using mannose as the standard reducing sugar (Krishnaveni, Balasubramanian, & Sadasivam, 1984; Somogyi, 1952) with a slight modification to the method by using the microtiter plate method (Shao & Lin, 2018). One unit of the enzyme activity was defined as one unit of enzyme hydrolyzing

the corresponding polysaccharide to produce 1.0 µg of mannose per minute (Bailey et al., 1992; Chen et al., 2013).

The same procedure described was used to verify xylanase and cellulase activity with different substrates and sugar standards, xylan and xylose for xylanase (Yadav et al., 2018), and CMC and glucose for the activity of cellulase (Lokapirnasari et al., 2015).

3.9.2 Experimental design for enzymatic hydrolysis of BSG

A preliminary screening was conducted to determine the optimal range of conditions for the factors by varying one parameter while keeping the others constant. This was followed by a Box-Behnken response surface design to identify the final optimal hydrolysis conditions for individual enzymes on the BSG substrate. The independent variables were the enzyme-to-substrate ratio (E/S), hydrolysis time, temperature and pH, with the following ranges: E/S of 0.0 – 5.0:1 (w/w), time from 0 to 8 h, temperature between 20 – 80^{o}C, and pH ranging from 3.0 to 8.0, with variations depending on the specific enzyme in Table 3.5 (Chen et al., 2013; Samanta Jayapal, Kolte, et al., 2015; Wongputtisin & Khanongnuch, 2015). One gram of BSG powder was dissolved into 100 ml 0.05 M citric phosphate buffer at different pH. Citrate phosphate buffer at 0.05M was made according to the formulation in Appendix 1.3 (AAT Bioquest, 2020; Chen et al., 2013).

Enzyme hydrolysis was stopped by boiling the reaction solution in a boiling pot for 15 min and sample solution was cooled in a water bath at 20 ± 2^{o}C before for further analysis. A better enzyme working range of each variable factor was then determined from the preliminary test and grouped into lower and upper levels with the central point of three levels to bring to a BBD to find the optimum hydrolysis condition for all enzymes in Table 3.5. Three levels of each factor were decided according to the top range of directly reducing sugar (DS), total reducing sugar (TS) and degree of polymerization (DP) results in each factor screening. The quadratic equation (equation 3.1) was used again to explain the behavior of this experimental design. An optimum hydrolysis condition was predicted based on well-fit models.

Table 3.5 Preliminary range screening of E/S, hydrolysis time, temperature and pH for determination of each factor level in enzyme hydrolysis in the Box-Behnken design.

	Enzyme/Substrate		**Hydrolysis time**		**Hydrolysis temperature**		**Buffer solution**	
Enzyme	**Mass of enzyme (g) to hydrolyze 1.0 g of BSG**	**Fixed point**	**Time (h)**	**Fixed point**	**Temperature (°C)**	**Fixed point**	**pH**	**Fixed point**
β-mannanase	0 0.05 0.1 0.2, 0.7, 1.0, 2.0	1:1	0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8	2h	20, 30, 40, 50, 60, 70, 80	40°C	2–10 (0.5 intervals)	6
ZAC mixture	0, 0.05, 0.1, 0.2, 0.7, 1.0, 2.0	1:1	0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8	2h	30, 40, 50, 60, 70	60°C	4.0–7.0 (0.5 intervals)	5.5
SQZyme xylanase	0, 0.05, 0.1, 0.2, 0.7, 1.0, 2.0	1:1	0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8	2h	35, 45, 50, 55, 65	50°C	3.5–7.0 (0.5 intervals)	4.5
CE35 cellulase	0, 0.05, 0.1, 0.2, 0.7, 1.0, 2.0	1:1	0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8	2h	20, 30, 40, 55, 60, 65, 75	60°C	3.0–7.0 (1.0 intervals)	4.5

3.9.3 Determination of direct reducing sugar (DS), total reducing sugar (TS) and degree of polymerization (DP)

Sugars that contain a free hemiacetal or hemiketal group have the reduction potential of metal ions and are referred to as reducing sugars (Shao & Lin, 2018). The amount of reducing sugar after enzymatic hydrolysis is often used to determine the functionality and efficiency of enzymes in the hydrolysis of polysaccharides and is defined as the direct reducing sugar (DS) (Chen et al., 2013; Shao & Lin, 2018). The total amount of reducing sugar of BSG after hydrolysis by chemicals is defined as the total reducing sugar (TS). The degree of polymerisation (DP) reflected the degree of degradation of BSG after enzymatic hydrolysis. The effect and termination of the enzymatic process can be monitored by determining DS, TS and DP (Chen et al., 2005; Chen et al., 2013; Farnet et al., 2010; Liu et al., 2015). TS was gained from enzyme hydrolyzed BSG digested in 8% sulfuric acid and then adjusted to alkali pH 8 – 10 (Chen et al., 2013; DuBois et al., 1956; Liu et al., 2015; Nielsen, 2010). Both the TS and DS were determined using the Nelson-Somogyi colourimetry test for reducing sugars (Breuil & Saddler, 1985; Chen et al., 2005; Chen et al., 2013; Farnet et al., 2010; Gusakov et al., 2011; Saqib & Whitney, 2011).

3.9.3.1 Standard curve of mannose, xylose and optimum absorbance wavelength

Glucose, mannose and xylose are the dominant sugar units in BSG, their standard solutions were prepared at concentrations of 0, 0.025, 0.05, 0.1, 0.2, and 0.4 mg/ml, and then the standard solutions were frozen in a -20 ± 1^{o}C freezer for further use. The absorbance of the standard solutions was determined over a wavelength scan between 540 – 620nm with 10nm intervals and the optimum absorbent wavelength was selected based on the highest r-squared value (R-Sq) in Minitab and an example of the standard curve for each sugar at their optimum absorbance listed in Appendix 1.6 (Shao & Lin, 2018; Somogyi, 1952).

3.9.3.2 Preparation of reducing sugar solution from hydrolysed BSG

Unhydrolyzed BSG debris and oligosaccharides can also take up copper and thus show a false reaction with arsenomolybdate (Shao & Lin, 2018). To avoid the false reaction, 100 mg BSG debris solution after enzymatic hydrolyzation was weighed and washed in 5 ml hot ethanol (~70^{o}C) twice. The supernatant was collected after centrifugation at 8000 x *g* at 20 ± 1^{o}C for 20 min (Shao & Lin, 2018; Wongputtisin & Khanongnuch, 2015). The supernatant

was transferred into a 100 mL beaker and heated in a UniTemp drier (LTE Scientific, England) at approximately 65^{o}C for 2 – 3 hours. The process was monitored frequently to ensure most of the ethanol evaporated from the sample solution while avoiding caramelization of the reducing sugars. Any caramelized samples were repeated to avoid the loss of reducing sugar in the experiment.

3.9.3.3 Direct reducing sugar assay – Nelson-Somogyi assay

The DS Nelson-Somogyi assay can detect reducing sugars at levels between 13 µg/ml and 10 mg/ml in water. This assay has the highest sensitivity compared to any other reducing sugar assays such as the sulfuric acid-phenol method, anthrone-sulfuric acid, Prussian blue method and DNS assay, especially in a much lower concentration of reducing sugar in water (Deng & Tabatabai, 1994). The Nelson-Somogyi assay with slight modifications was therefore carried out to quantify DS concentration in hydrolysed BSG solution in this study (Krishnaveni et al., 1984; Somogyi, 1952).

The Nelson-Somogyi assay was conducted in 96-well microtiter plates rather than traditional test tubes (Shao & Lin, 2018). One millilitre polypropylene deep 96-well plates (Nest, China) with silicon 96-well seals (Axygen, China) were used for the copper heating reaction and then further arsenomolybdate reaction. Each hydrolysed BSG solution (0.2 mL) and copper reagents were mixed in wells and were boiled in boiling water for 20 min and then cooled rapidly to room temperature in a 20 ± 2^{o}C water bath (Grant, UK). Arsenomolybdate reagent (0.2 mL) was then added to each well at room temperature. The mixture solutions were allowed to react for 15 min at room temperature (20 ± 1^{o}C) before being transferred to a Nunclon Delta surface polyethylene 96-well transparent microtiter plate (Thermofisher, Denmark). Absorbance was then measured using a BMG plate reader at 560 nm for xylose and 620 nm for mannose and glucose (Fluostar Optima BMG, Germany). The alkaline copper tartrate reagents were prepared freshly on the day they were used. Sodium potassium tartrate solution (reagent A) was heated in boiling water until crystals disappeared and then kept warm in a 35°C incubator (Conthern, New Zealand) before mixing with copper sulphate solution (reagent B) at a ratio of 96:4 to provide a 100 ml mixed solution for the assay on the day. Each sugar standard for different enzymes needed to be repeated for every 96-well plate reading and a new regression equation was

drawn based on each time reading of the sugar standards to calculate the sample sugar concentrations. If any absorbance readings for the samples were higher than the range of the sugar standards, a 10-times dilution was carried out on the samples and the Nelson-Somogyi Assay was repeated.

3.9.3.4 TS of BSG – Sulfuric acid digestion and DP

Five millilitres of non-hydrolyzed samples were hydrolysed with 5 ml of 8% sulfuric acid in a boiling water bath (Joanlab, China) for 2 h. The digested BSG was cooled to room temperature in a Grant water bath at 20 ± 2^{o}C and then adjusted to alkali pH (pH 8 – 10) with 1 M NaOH and then further diluted with deionized water to 25 ml in a volumetric flask (Chen et al., 2013). The TS of BSG was then quantified by the Nelson-Somogyi colourimetry reducing sugar assay as described in section 3.8.3.3, using a regression equation from the sugar standard curve. The DP was calculated using Equation 3.8.

$$\mathrm{DP} = \frac{\mathrm{TS}}{\mathrm{DS}} \qquad (Equation\ 3.8)$$

3.9.4 Recovery of oligosaccharides from hydrolyzed BSG (BSG-Oligos)

Enzyme-digested BSG solution was drained through a muslin cloth to recover the BSG oligosaccharides (BSG-Oligos). Any undigested BSG long fibres remained on the cloth. The cloudy solution was then centrifuged at 7669 x *g* for 20 min at 20 ± 2^{o}C to sediment large undigested BSG polymers and enzymes (Wongputtisin & Khanongnuch, 2015). The supernatant was then filtered through 25 µm Whatman filter paper # 4 followed by 6 µm Whatman filter paper # 3 (USA). After two filtrations, the clear BSG-Oligos solution was dried in a fan oven at 50 ± 2^{o}C for 48 h (Thermofisher, America). The dried BSG-Oligos were further purified with hot 95% ethanol to wash off single sugar units and centrifuged at 4000 x *g* for 20 min to collect the purified BSG-Oligos pellets (Chelliah et al., 2023). The ethanol-washed BSG-Oligos were placed in a hot water bath (~ 95°C) to evaporate any ethanol residue. The purified BSG-Oligos re-dissolved in the RO water again and the fan oven was used to dry at 50 ± 2^{o}C for 48 h and then packed in 118 ml Whirl-Pak sample bags (Fisher, China) stored in a lab freezer at -20 ± 2^{o}C (Fisher & Paykel, New Zealand) for further study (Liu et al., 2015). BSG-Oligos recovery steps are illustrated in Figure 3.3.

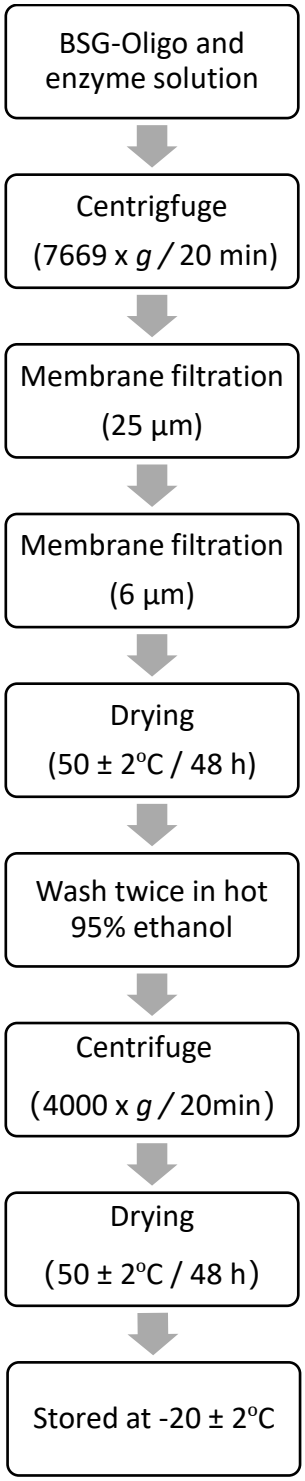


Figure 3.3 Flow chat of recovering BSG Oligosaccharides from the enzyme hydrolysis solution (Chelliah et al., 2023; Liu et al., 2015; Wongputtisin & Khanongnuch, 2015).

3.10 Kinetic growth studies of pure yogurt cultures with supplementary BSG, erythritol or BSG-Oligos

The growth of probiotics or the yogurt starter cultures can be affected by additional ingredients in the yogurt (Zare et al., 2012). Therefore, the effect of the addition of BSG, erythritol and BSG-Oligos on the growth of commercial yogurt cultures (*S. thermophilus*, *L. bulgaricus* and *L. acidophilus*) of YO-MIX® 726 (Danisco, France) in a yogurt were investigated. The growth of yogurt cultures was monitored for 24 or 48 hours while monitoring the absorbance at 595nm (OD_{595nm}), which corresponded to an increase in microbial cell population as a result of a change in turbidity (Zhao et al., 2012).

3.10.1 Purification of *S. thermophilus* (ST), *L. bulgaricus* (LB) and *L. acidophilus* (LA)

A yogurt sample was fermented using YO-MIX® 726 bacteria and serial dilutions were plated on different agars for single culture selection. M17 plates was used for ST at 37°C for 48 h aerobically.

Purification of LB from other YO-MIX® 726 bacteria were carried using several screening steps. A yogurt sample was diluted to 10^{-6} and serial dilutions were plated out onto acidified MRS agar at pH 4.8 (Tharmaraj & Shah, 2003). The plates were then incubated at 45 ± 0.5°C for three days under anaerobic conditions. A few individual colonies of LB were selected and then cultivated in MRS broth without any modification. This MRS broth was then diluted following a serial dilution on MRS-clindamycin to further confirm that the chosen colonies were LB (Robinson, 2002). LB was inhibited with clindamycin antibiotic present in the MRS broths at dilutions greater than 10^{-4} (Sohrabvandi et al., 2012).

The cultures grown anaerobically on MRS-clindamycin plates at higher than 10^{-4} dilution would be LA or *B. lactis* at 37°C for 72 h. All isolated cells were also confirmed with Gram staining. The cells of *B. lactis* have pointed ends, irregular shape with branched morphology and thin in diameter rather than LA (Zhou et al., 2020). Although some strains of *B. lactis* are tolerant to air oxygen levels, it needs stricter anaerobic conditions for growth than LA (Ruiz et al., 2011; Zhou et al., 2020). LA was selected for the probiotic growth curve assay to evaluate the effect of each ingredient using a plate reader.

3.10.2 Growth curve assay for studying growth parameters

3.10.2.1 Preparation of diluted single culture solution

A single colony from each of the plates was then inoculated in M17 broth for ST and MRS broth for LA and LB for 18 h at 37^{o}C. After incubation, turbid broths were centrifuged (Heraeus Biofuge Primo R, Germany) at 3250 x *g* for 10 min at about 4^{o}C (Zhao et al., 2012). The supernatant was carefully decanted, and the cells were washed with 10 ml of 0.1% peptone saline solution twice. The harvested bacterial cells were further diluted to give an absorbance at 595 nm between 0.06 – 0.08 at OD_{595nm} using a Nova III spectrophotometer (Biochrom Ltd., England) (Kosasih, 2011; Zhao et al., 2012).

3.10.2.2 Preparation of microtitre plates for growth curve assay

One hundred microliters (100 µL) double strength BSG, erythritol and BSG-Oligos in different concentrations were separately pipetted into a 96-well micro-titre plate (Nunclon Delta, Thermofisher, Denmark). Additional 2.5 times strength or double strength MRS broth (Merck, Germany) or M17 broth (Oxoid, the UK) were pipetted to support the yogurt culture growth, followed by 20 µL of the adjusted concentration of ST, LB or LA added to each well. The single strength of MRS or M17 broth medium fortified with different concentration of BSG or erythritol or BSG-Oligos was prepared as the Table 3.6 below.

The 96-well micro-titre plate was placed into the chamber of the BMG plate reader and the wells were scanned at absorbance 595nm and 37 ± 0.5^{o}C for 24 or 48 hours with 30 seconds of orbital 2-mm width shaking before each hour reading (Kosasih, 2011; Kotikalapudi et al., 2010; Q. Zhao et al., 2012). A FLUOstar Optima BMG Plate Reader (Labtech, Germany) was used to monitor changes in optical density for each yogurt culture.

Table 3.6 The microtiter plate preparation for single dose mixture media in the yoghurt bacterial kinetic growths.

96-well microtiter plate preparation						
sugars final conc. in microplate %	**10% sugars to be added**	**Double strength of the sugar in first column**	**Double strength MRS/M17**	**2.5 strength MRS/M17**	**H_2O**	**Culture**
0.01	-	100µl	-	80µl	-	20µl
0.05	-	100µl	-	80µl	-	20µl
0.1	-	100µl	-	80µl	-	20µl
0.2	-	100µl	-	80µl	-	20µl
0.5	-	100µl	-	80µl	-	20µl
1	20µl	-	100µl	-	60µl	20µl
2	40µl	-	100µl	-	40µl	20µl
3	60µl	-	100µl	-	20µl	20µl
4	80µl	-	100µl	-	-	20µl
5	100µl	-	-	80µl	-	20µl
Single strength MRS broth – 52g/L; Single strength M17 broth – 37.25g/950ml						

3.10.2.3 Growth parameter analysis of YO-MIX® 726 yogurt bacteria with added BSG-Oligos at different concentrations

The absorbance at 595 nm was recorded against time to determine growth curve parameters. Maximum specific growth rate (μ_{Max}), maximum cell concentration (Max), and lag time (lag) were found from the growth curve. The primary growth model, Modified Logistic model was fitted to the growth curve data (Equation 3.9) using Sigma plot 14.5 (Faisal et al., 2022; Fujikawa et al., 2003; Purgar et al., 2022). The results for μ_{Max}, Max and lag were used for comparing effect of different concentrations of BSG-Oligos which may have a positive effect on the growth of the yogurt starters and probiotics (Fujikawa et al., 2003; Ortiz-Moreno et al., 2020).

$$y(t) = \frac{D}{1 + exp\left[\frac{4\mu_{Max}}{D}(\lambda - t) + 2\right]} \qquad (Equation\ 3.9)$$

The y(t) was the cell density of the yogurt cultures at different times t ($OD_{595\ nm}$). D is a constant value of the difference between upper and lower asymptotic values of y. λ is the lag phase duration.

3.11 Prebiotic activity assay to facilitate further concentration selection of BSG-Oligos used in yogurt production

Prebiotics are utilized by beneficial probiotic bacteria which helps to promote their growth, more than glucose (Huebner et al., 2007). Prebiotic sugar substrates have much lower utilization rate than any other non-beneficial fibres and oligosaccharides by intestinal flora except probiotics (Huebner et al., 2007; Roberfroid, 2007). Huebner et al (2007) have used an assay to quantify the prebiotic activity of 1% prebiotics against 1% glucose based on the effect of living cell number changes of one probiotic bacterium against normal intestinal bacteria e.g., *E. coli*. Zhang (2018) has simplified the assay to compare between one probiotic and one *E. coli* strain. The prebiotic activity assay in this study was slightly modified based on Huebner et al (2017) and Zhang (2018) studies.

LA from YO-MIX® 726 (Danisco, France) was isolated and purified as described in Section 3.10.1. The culture was stored at -80^{o}C before use. Modified MRS base broth was supplemented with different concentrations of glucose or BSG-Oligos as the carbon source. *Escherichia coli* NCTC 8196 (*E. coli*) was cultivated in M9 broth with slight modification based on the recipe of VWR M9 broth and supplied with the same amount of glucose and BSG-Oligos. Modified formulations of MRS and M9 broth are listed in Appendix 1.8. *E. coli* was revived on nutrient agar plates (Oxoid, UK) at 37^{o}C for 24 h from the frozen stock culture and then re-freshed in nutrient broths (Oxoid, UK) at the same conditions overnight, and also for LA on MRS plates and then in MRS broths anaerobically, before the prebiotic activity assay. Overnight cultivated *E. coli* and LA cultures about 100µl was inoculated into M9 broths and MRS broths with either glucose or BSG-Oligos and were incubated at 37 ± 1^{o}C for 12 h, 24 h and 48 h. *E. coli* was plated on nutrient agar and Oxoid MRS agar (UK) was used for the enumeration of *L. acidophilus*. Equation 3.10 was used to calculate the prebiotic activity score of the BSG-Oligos at a particular concentration (3% or 4% w/v) (Huebner et al., 2007; Zhang et al., 2018). LA was *L. acidophilus* in the equation. The assay was duplicated, and the results were averaged.

$$\textbf{Prebiotic activity score} =$$

$$\left[\frac{log_{10}\, LA\ in\ MRS - BSG - Oligo\ broth\ at\ 12h, 24h\ and\ 48h - \ log_{10}\, LA\ at\ 0h}{log_{10}\, LA\ in\ MRS - glucose\ broth\ \ at\ 12h, 24h\ and\ 48h\ - \ log_{10}\, LA\ at\ 0h}\right]$$

$$-\left[\frac{log_{10}\, E.coli\ in\ MRS - BSG - Oligo\ broth\ at\ 12h, 24h\ and\ 48h\ - \ log_{10}\, E.coli\ at\ 0h}{log_{10}\, E.coli\ in\ MRS - glucose\ broth\ \ at\ 12h, 24h\ and\ 48h\ - \ log_{10}\, E.coli\ at\ 0h}\right]$$

$$(Equation\ 3.10)$$

3.12 Statistical analysis

Data was evaluated in Minitab 21 (Minitab, Inc., America). Normality and homogeneity of the data were tested before any analysis performance in the Minitab. Unevenly distributed data were transformed into either square root or logarithm and then normality and homogeneity were retested (Norris & Aroian, 2004). Logarithmic transformation a general transformation method was used especially for bacterial enumeration and then data analysis (Manikandan, 2010).

There were multiple factors with unknown optimum working ranges, and thus the Box-Behnken response surface design was introduced to this study to find the optimum reaction condition for each test. The predicted models were retested in a real-time experiment with predicted optimal factor values in the model, and the response variable should be in the predicted response variable range (Box & Behnken, 1960; Min, 2010; Peng et al., 2020). A factorial design was also introduced in this study to screen major factors that impacted final test results as well as the interaction between factors. Optimization was based on major factors to screen the best formulation according to different requirements.

A statistically significant level was set out at 95%. P-value < 0.05 was considered a significant difference and vice versa. The properties of the kinetic growth curve of yogurt bacteria were analysed by SigmaPlot 14.5 (Systat Software, Inc. America) via the Modified Logistic model (Fujikawa et al., 2003; Ortiz-Moreno et al., 2020). One-way ANOVA was used to differentiate different growth properties and cell survival rates in different experiments in this study via Tukey's multiple comparison test. Experimental data was collected in replicates unless otherwise specified.

Chapter 4 Water extraction of basil seed gum and its application in yogurt

4.1 Introduction

A pH adjusted reverse osmosis (RO) water at pH 7.0 – 8.0 is commonly used for basil seed gum extraction referred to Table 2.4. The objectives for this chapter were to extract basil seed gum using only reverse osmosis (RO) water and to study BSG effects on yogurt profiles with erythritol partially replacing cane sugar. To enhance yogurt quality, preliminary screening of yogurt ingredients included studies on the levels of basil seed gum (BSG) and erythritol, and their effects on yogurt cultures (YO-MIX® 726). The impact of ingredient concentrations on yogurt gel firmness and serum separation was also evaluated. A Box-Behnken design (BBD) was applied to optimize the concentrations of yogurt ingredients for improved quality

4.2 Water extraction of basil seed gum

Basil seed gum (BSG) has been extracted in slightly alkaline reverse osmosis (RO) water at pH 8.0 by Razavi et.al (2009) and Hosseini-Parvar et.al (2010). Sodium ions from sodium hydroxide used to adjust the pH can form a bridge link between uronic acid moieties on the backbone of the gum and therefore, reduce the solubility of the gum (Grönberg & Hjorth, 2018; Lv et al., 2020). The addition of sodium hydroxide to adjust pH can also inhibit the growth of yogurt cultures (Grönberg & Hjorth, 2018; Lv et al., 2020). To eliminate these negative impacts with alkali pH adjustment, basil seeds were extracted in RO water with no pH adjustment.

BSG extraction yield was influenced by three factors, temperature, ratio of water and basil seeds, and time for rehydration of basil seeds in the studies above. The best ratio of water and seed for gum extraction was 66:1. During extraction the shear rate (stirring speed) was fixed at 1000 rpm (Razavi et al., 2009; Hosseini-Parvar et al., 2010; Hosseini-Parvar et al., 2015; Hosseini-Parvar et al., 2016). The factors of temperature, time and shear rate were investigated to find out whether these factors influenced gum extraction. Preliminary screening was conducted to determine the range for each factor. Based on these ranges,

optimal gum extraction conditions were estimated using a Box-Behnken factorial design (BBD).

4.2.1 Preliminary screening of different factors

Preliminary screening was carried out to determine the range of conditions to use for temperature, time and shear rate during BSG extraction. Extraction temperature was set between 20 – 80^{o}C and time was fixed at 60 min and shear rate at 700 rpm. The time of extraction was between 20 – 130 min, and temperature was fixed at 50^{o}C and shear rate at 700 rpm. For shear rate, the range was set between 400 – 1600 rpm, and temperature was fixed at 50^{o}C, and extraction time was for 60 min.

The three factors were evaluated based on wet seed weight and wet gum weight. The results are shown in Figure 4.1 for each factor and the actual data collected available in Table A2.1 (Appendix 2.1). Wet seed weight was selected as the response variable for the range selection as weight of wet gum did not show a large variation for all the conditions tested for temperature, time and shear rate (Figure 4.1). There was a sharp drop in wet seed weight at 50^{o}C then levelling off between 60 – 80^{o}C as shown in Figure 4.1a-1. For all the extraction times evaluated the weight of wet seeds remained relatively constant between 60 – 90 g (Figure 4.1b-1) and there was also no significant impact on either wet seed weights or wet gum weights at different gum extraction times ($p > 0.05$). Wet seed weight declined gradually as shear rate increased from 600 rpm to 1200 rpm and then there was no further increase in wet seed weight beyond a shear rate of 1200 rpm rate (Figure 4.1c-1).

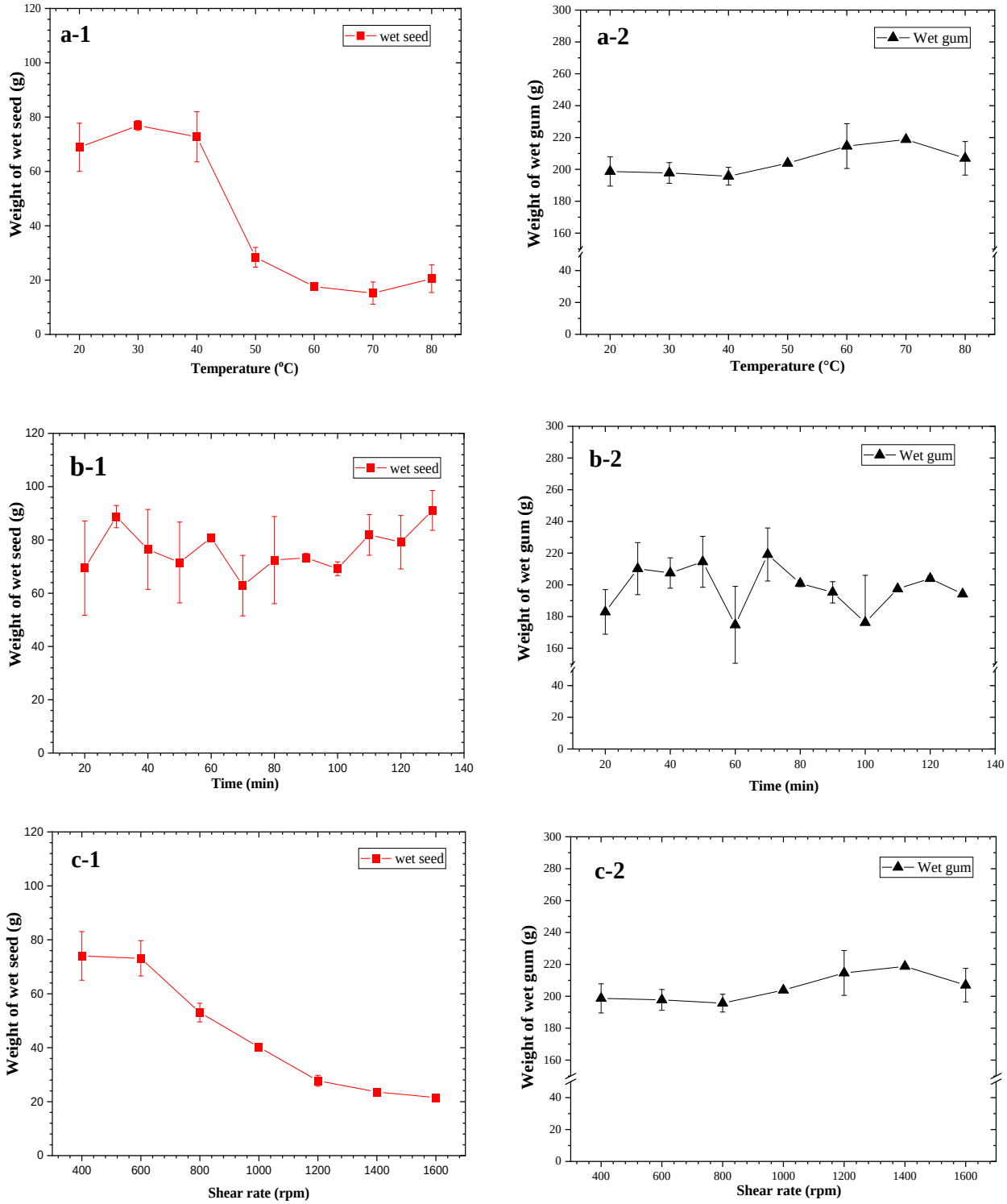


Figure 4.1 Effect of (a) temperature, (b) time and (c) shear rate on weight of wet seed (■) (a-1, b-1, c-1) and weight of wet gum (▲) (a-2, b-2, c-2) extracted from basil seed in RO water. Mean values ± standard deviation for n=2.

The apparent viscosity of the BSG solution increased with an increasing shear rate until 1000 rpm (1485.5 ± 21.9 mPa.s) (Figure 4.2) and the actual data collected available in Table A2.2 (Appendix 2.1). The apparent viscosity declined below this maximum value at a shear rate of 1200 rpm and then did not change with increasing shear rates up to 1600 rpm (Figure 4.2). Razavi et.al (2009), and Hosseini-Parvar et.al (2010, 2015 & 2016) used a constant shear rate of 1000 rpm to extract basil seed gum. The mixing speed range of shear rate was chosen for the BBD design between 400 – 1000 rpm.

For the temperature factor the temperature range chosen for the BBD design was between 50 – 70^{o}C with 60^{o}C as the centre point to determine the optimum temperature for BSG extraction in a BBD design. Therefore, the range and centre point values for each factor are listed in Table 4.1.

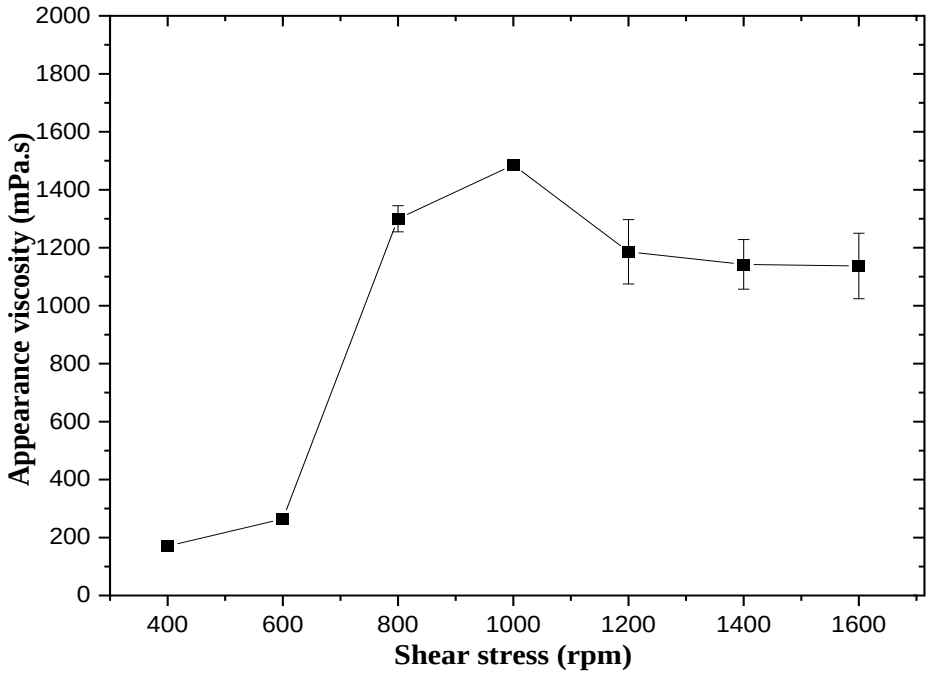


Figure 4.2 The apparent viscosity of BSG determined at a shear rate of 20 rpm at different mixing speeds (shear rate) for further screening the range of shear rate. Mean values± standard deviation for n=2

Table 4.1 Range of factors derived from the preliminary study and used in the Box-Behnken design.

Factors	Level		
	-1 (lower level)	0 (Centre point)	1 (upper level)
Temperature (°C)	50	60	70
Extraction time (minute)	60	90	120
Shear rate (rpm)	400	700	1000

4.2.2 Optimization of BSG extraction conditions

A 15-run BBD for BSG extraction in RO water was used to optimize the extraction conditions with three levels (Table 4.2). Four response variables were obtained from the BBD, the wet seed weight, dried seed weight, wet gum weight and dried gum weight in grams. The centre values were repeated three times to investigate the variability in the results (Box & Behnken, 1960; Min, 2010; Peng et al., 2020). The results from the BBD are plotted in Figure 4.3 and listed in Table A2.3 (Appendix 2.1). The seed swelling capacity was the ratio of the wet seed and dried seed. The percentage of dried gum yield was calculated the weight of dried gum divided by 30 g for original dry seed weight.

Table 4.2 Box-Behnken experimental design for estimating optimum BSG extraction conditions in RO water

Run Number and Conditions Temperature:Time:Shear rate	Factors		
	Temperature (°C)	Time (min)	Shear rate (rpm)
1-50°C-60min-700rpm	50	60	700
2-50°C-90min-400rpm	50	90	400
3-50°C-90min-1000rpm	50	90	1000
4-50°C-120min-700rpm	50	120	700
5-60°C-60min-400rpm	60	60	400
6-60°C-60min-1000rpm	60	60	1000
7-60°C-90min-700rpm	60	90	700
8-60°C-90min-700rpm	60	90	700
9-60°C-90min-700rpm	60	90	700
10-60°C-120min-400rpm	60	120	400
11-60°C-120min-1000rpm	60	120	1000
12-70°C-60min-700rpm	70	60	700
13-70°C-90min-400rpm	70	90	400
14-70°C-90min-1000rpm	70	90	1000
15-70°C-120min-700rpm	70	120	700

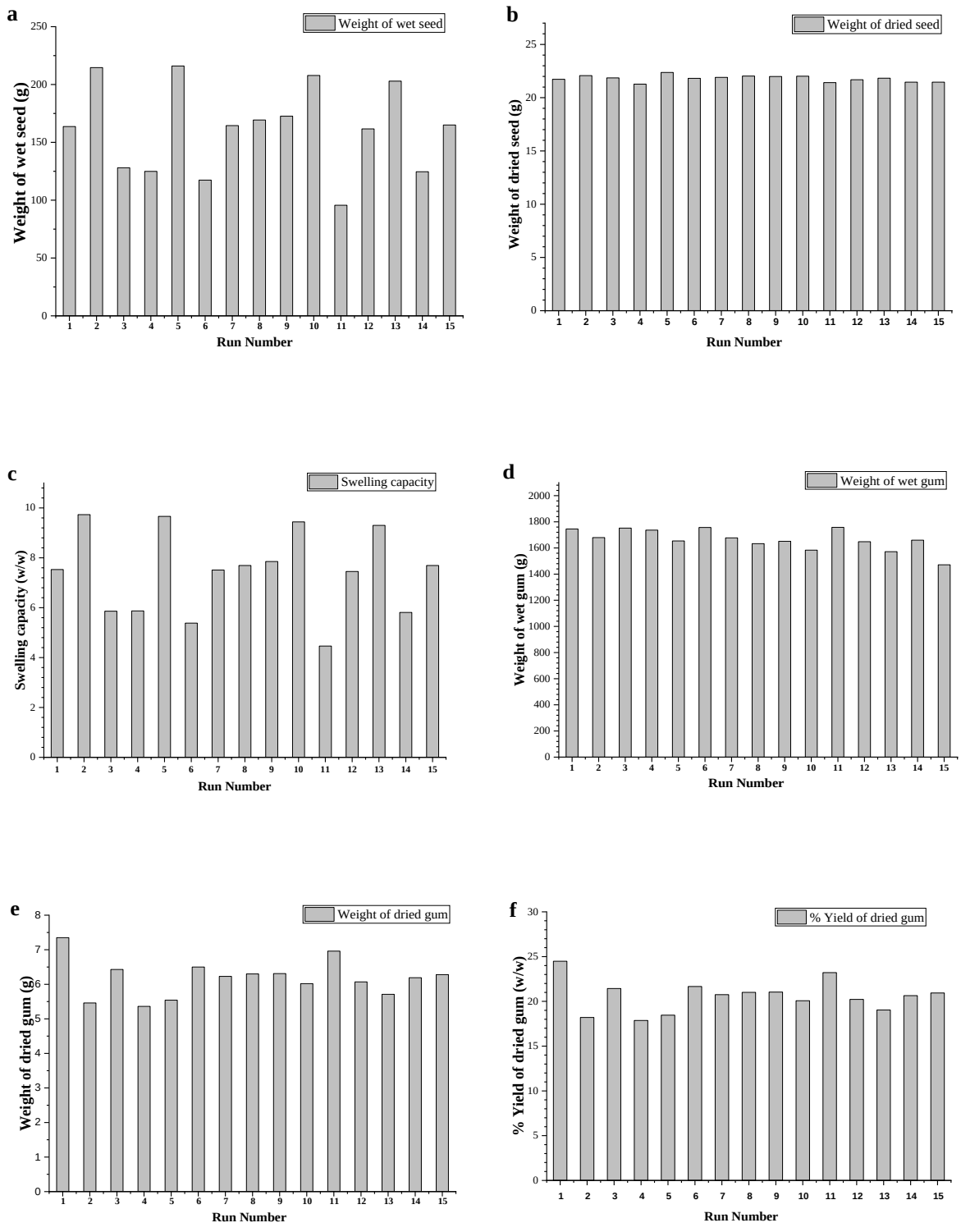


Figure 4.3 Histograms of results from the Box-Behnken design of BSG extraction in RO water experiments, for (a) wet seed weight, (b) dried seed weight, (c) seed swelling capacity (g wet set seed/g dried seed), (d) wet gum weight, (e) dried gum weight and (f) % yield of dried gum ((g dried gum/g original seed) %).

The results of basil seed gum (BSG) extraction in RO water, as analyzed using the BBD design, are presented in Figure 4.3. In Figure 4.3b, the weights of the dried seeds showed minimal variation, whereas the weight of the wet seeds varied with changes in extraction conditions, (Figure 4.3a). This observation also confirms that shear rate was possibly a significant factor that influenced BSG extraction. Conversely, the weights of both the wet gum and the dried gum exhibited relatively low variability compared to the weight of the wet seeds (Figure 4.3d and 4.3e). The ANOVA analysis supporting these observations is provided in Table A2.4 (Appendix 2.1).

The swelling capacity of whole basil seeds, which reflects the increase in weight as the seeds absorbed water, followed the same trend as the plot of wet seed weight, as shown in Figure 4.3c. The percentage yield of the dried BSG varied between approximately 18% – 25%, (Figure 4.3f). The wet seed weight could be a more representative indicator in these analyses, as demonstrated in Figure 4.3.

However, from the ANOVA analysis of the BBD, weights of wet gum and wet seeds were more reliable predictive models compared to the other response variables. Shear rate (rpm) was the dominant factor that affected the water extraction of BSG against both response values of wet gum and wet seed weight in this experiment ($p < 0.05$). Time was the second effective factor in retrieving wet seed kernel from the original seed ($p < 0.05$). All three factors had an impact on the wet gum extraction yield ($p < 0.05$).

From the ANOVA Analysis of the BBD, the results of the response optimization are listed in Table 4.3. The predictive optimum temperature for the extraction in this BBD model (50^{o}C) was different from Razavi et al (2009) at 68.71^{o}C and Naji-Tabasi & Razavi (2017b) at 69^{o}C and was close to Nazir et al. (2017) at 56.7^{o}C. This optimum temperature of 50^{o}C was the lowest temperature required for BSG extraction. Extraction time was a significant factor impacting on both wet seed and wet gum weights, which was different from the observations in the preliminary study that time did not influence BSG extractions (Figure 4.1 b-1 and b-2). BSG extraction time was 96 min in the report of Nazir et al. (2017). Razavi et al. (2009) and Hosseini-Parvar et al. (2010) have used 20 min for BSG extraction in their studies. The model was validated by an additional experiment under the predicted optimal

conditions of BSG extraction, and the model validation test was repeated twice (Table 4.3). The observed values of wet seed weight and wet gum weight in the model validation test were 90.3 ± 3.5 g and 1771.0 ± 25.8 g, respectively. Both values sit in the 95% confidence range of the predictive values in Table A2.4 (Appendix 2.1). Both wet seed weight and wet gum weight models were validated. BSG gum extraction was carried out under these conditions to achieve the optimum yield of dry BSG gum for future study (Table 4.3).

Table 4.3 Validation of optimum BSG extraction conditions

Variable factors	Optimum conditions		Wet seed weight (g)	Wet gum weight (g)	Dried seed weight (g)	Dried gum weight (g)
Temperature	50°C	**Predictive value**	87.94	1800.5	-	-
Time	120min					
Shear rate	1000rpm	**Observed value**	90.30±3.5	1771.00±25.8	21.45±0.16	7.28±0.19

4.2.3 Yield of dried BSG

The wet gum from the water extraction BBD design was dried and the yields of dry gum were calculated. The dried gum had a yield at the lowest 17.84% (g dried basil bum /30 g of original dry basil seed) and highest 24.48% at different extraction conditions from this BBD design, and the validation tests gave % yield of 23.63 – 24.93%. The yield of dry BSG showed less variation compared to the range of 7.86 – 20.5% of Nazir's findings (2017). The dried gum yields were 9% higher than Razavi's results (2009) and comparable to the 20 – 25% yields found by Avlani, Agarwal, et al. (2019). In addition, the same research used a 1500 rpm shear rate, lower temperature and longer extraction times at 40^{o}C and 4 h, respectively. The results from this study were also similar to what Samateh et al (2018) reported a 21% yield of BSG extraction in water. The yield of BSG was much higher than that of mucilage from chia seed (7 – 10%) (Samateh et al., 2018; Muñoz, et al., 2012). These results showed that BSG can be extracted with RO water.

4.3 Preliminary screening of yogurt ingredients

BSG has been studied as an emulsifier stabilizer, and fat substitute in food products (Hosseini-Parvar et al., 2015; Hosseini-Parvar et al., 2016; Song et al., 2017). Dietary fibres can stimulate probiotic multiplication (Bharati et al., 2022; Singh et al., 2017; Wongputtisin

& Khanongnuch, 2015). BSG is also a rich source of dietary fibre, containing 98.5% polysaccharides (Bharati et al., 2022; Nazir & Wani, 2021). BSG also has antimicrobial activity and can also be used as a water-absorbing ingredient in wound dressing (Tantiwatcharothai & Prachayawarakorn, 2019). The effect of different concentrations of BSG on the growth of *Streptococcus thermophilus (*ST*)*, *Lactobacillus delbruekii* subsp*. bulgaricus* (LB) and *Lactobacillus acidophilus* (LA) present in YO-MIX® 726 yogurt cultures were investigated. Erythritol is a natural plant base sweetener and was used to partially replace cane sugar. The effect of erythritol on the growth of the yogurt cultures was also evaluated. Selected concentrations of BSG and erythritol were further studied in yogurt to determine their effect on yogurt quality.

4.3.1 Effect of different concentrations of BSG on the yogurt cultures

BSG as a potential dietary fibre may facilitate yogurt culture growth, but it is also known to have antimicrobial effects. The effect of different concentrations, 0.01 – 0.2% BSG powder (w/v) on the growth of YO-MIX® 726 yogurt cultures was studied by monitoring growth via absorbance at 595 nm (OD_{595nm}) using an BMG-Optima plate reader. The growth curves for each culture are shown in Figure 4.4.

No inhibition of ST growth was found at 0.01%, 0.1% and 0.2% BSG concentrations, but at 0.05% added BSG the growth was enhanced after 11 h incubation at 37^{o}C in M17 broth compared to the ST-control (Figure 4.4a). BSG did not inhibit the growth of LB at lower concentrations 0.01% and 0.05% but was found to accelerate growth after 10 h (Figure 4.4b). LB growth was inhibited when 0.1 – 0.2% BSG was added to the MRS broth at 37^{o}C (Figure 4.4b). For the growth of LA, 0.01% added BSG showed growth enhancement compared to LA-control. When higher concentrations of BSG were added > 0.05% LA growth was inhibited compared to LA control (Figure 4.4c). In summary there was growth inhibition of all three yogurt cultures when the concentration of BSG increased from 0.1% to 0.2%, especially on both *Lactobacilli.*

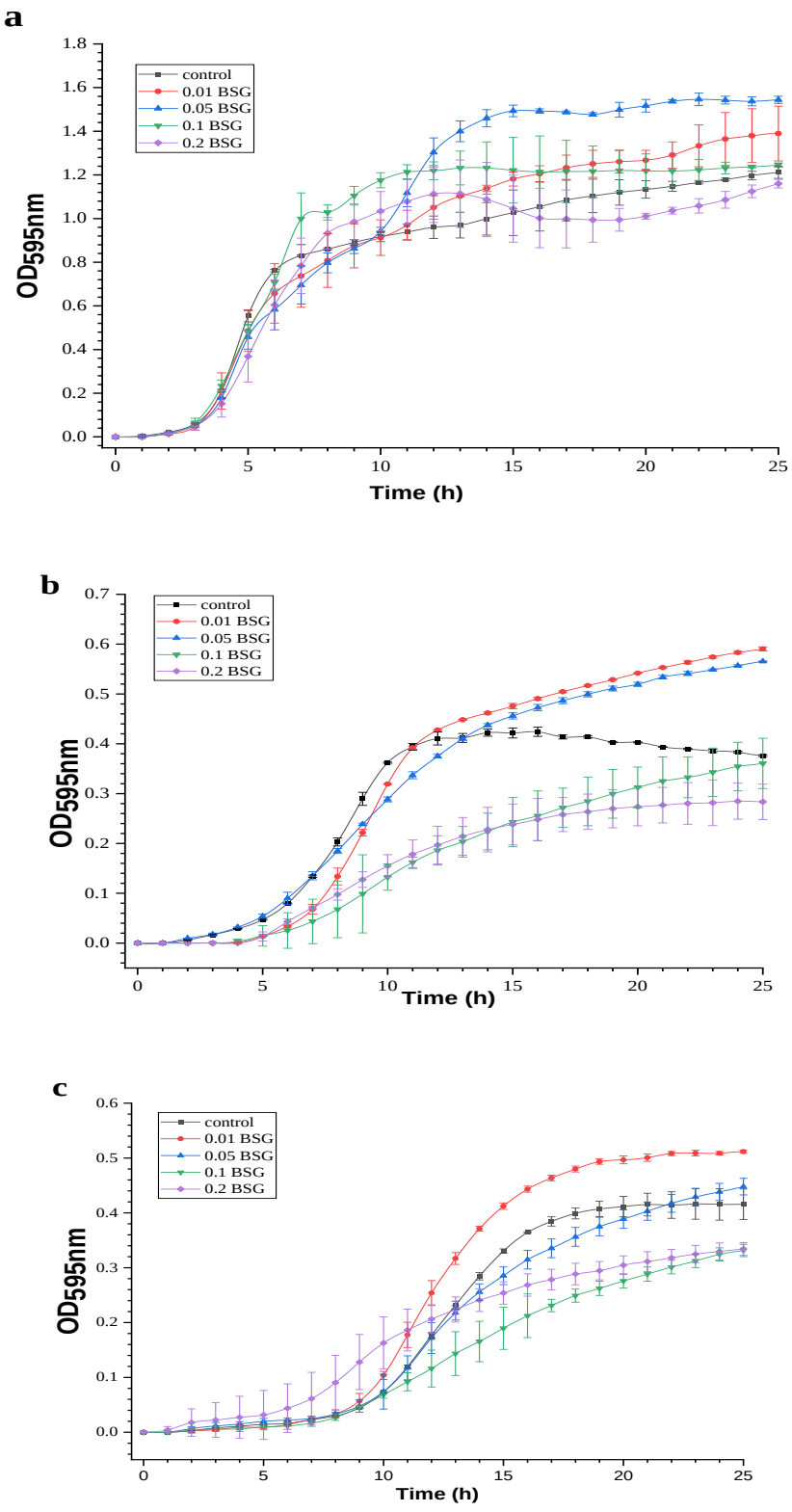


Figure 4.4 Growth curves of YO-MIX®726 cultures over time for different concentrations of basil seed gum (BSG), (a) *Streptococcus thermophilus* (ST), (b) *Lactobacillus delbrueckii subsp. bulgaricus* (LB), and (c) *Lactobacillus acidophilus* (LA), growth determined by absorbance at 595nm (OD_{595nm}) over 24 h at 37°C. Mean values ± Standard deviation for n=4.

Ghasempour et al. (2020) demonstrated that adding 0.4% basil seed gum (BSG) enhanced the growth of *Lactobacillus paracasei* in yogurt products. Furthermore, in the same study, a lower concentration of 0.2% BSG further promoted post-fermentation growth during storage. Konjac gum (0.05%) enhanced the growth of *L. acidophilus* in yogurt products (Wang et al., 2008). Basil seed gum (Ghasempour et al., 2020), chia seed gum (Kwon et al., 2019), and locust bean gum (Yilmaz-Ersan et al., 2018) have been shown positive effects on growth promotion of yogurt cultures in food products.

4.3.2 Effect of different concentrations of erythritol on the growth of the yogurt cultures

When yogurt is consumed, the sugar content of 125 g of yogurt can be up to 28 g of total sugar and about 12 g of added sugar (Coyle et al., 2019). The daily recommended added sugar intake is six teaspoons (about 36 g) or less for a healthy 70 kg adult, equivalent to about 5% daily energy intake (CDC, 2022; Johnson et al., 2009; McIntyre & Dutton, 2015). Sweeteners were studied to replace cane sugar in yogurt with the aim of keeping the same level of sweetness in yogurt. There is limited knowledge about the effects of sweeteners on yogurt cultures, particularly on probiotics. Erythritol was added at different concentrations to determine its effect on the growth curves of the YO-MIX® 726 yogurt cultures. The corresponding growth curves are shown in Figure 4.5.

ST growth did not appear to be affected by the addition of erythritol at all concentrations except at 10% erythritol there was a slight reduction in ST growth compared to all other concentrations (Figure 4.5a). LB growth was significantly impacted by the addition of erythritol. From concentrations of erythritol $\geq$ 1% the growth of LB was inhibited and at 10% no growth was observed (Figure 4.5b). Erythritol (4% to 10%) stimulated early cell growth of LA, reducing the start point of the exponential growth phase to six hours, compared to eight hours in the control (Figure 4.5c). Overall, erythritol suppressed the overall growth of LA compared to the control after 10 h incubation (Figure 4.5c).

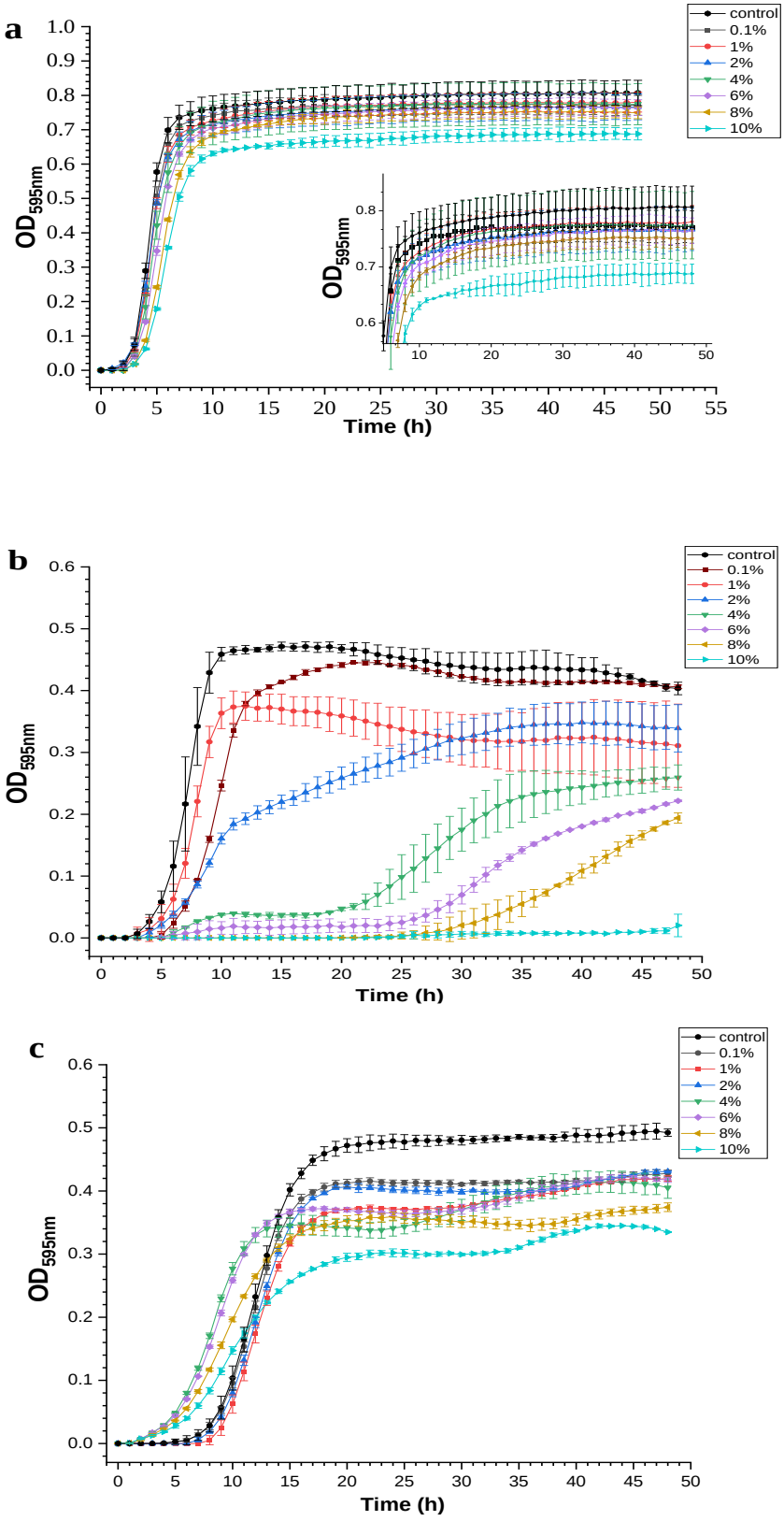


Figure 4.5 Growth curves of (a). *Streptococcus thermophilus* (ST) (insert is an expansion of data from 5 hours), (b) *Lactobacillus delbrueckii subsp. bulgaricus* (LB), and *Lactobacillus acidophilus* (LA) with added different concentrations of erythritol, and growth determined by absorbance at 595 nm over 48 h at 37°C. Mean values ± standard deviation for n=4.

Published research has shown that the growth rates for *Lactobacillus casei* and *Bifidobacterium bifidum* BB-12 were affected when a high level of erythritol was added to the yogurt products, at 12 – 24% w/v and 8.4 – 16.8% w/v, respectively (Costa et al., 2019; Kalicka et al, 2019). Although erythritol had a negative effect on yogurt culture growth in this study, the inclusion of other ingredients in foods can lead to positive effects with erythritol. Tao et al (2021) reported that erythritol and curdlan gum formed a porous structural gel which enhanced the water-holding capacity of curdlan gel. But conversely a porous structure can provide a protective barrier for bacteria to survive in harsh environments, e.g. encapsulation, cryo-structuration, and immobilisation onto polymers and biofilms (Berillo et al., 2021; Shahmoradi et al., 2023). It is still unknown whether the complexed milk gel structure with additional BSG and erythritol could help reduce the negative effects of erythritol on cell growth. Therefore, the next step was to investigate the impact of erythritol and BSG on yogurt gel structure, serum separation and culture survival.

4.3.3 Ingredient range screening – yogurt gel firmness and serum parameters

Although BSG at ≤ 0.05% did not show a negative impact on the growth of all three YO-MIX® 726 yogurt cultures, there was a limitation to these results as they were carried out in pure media broth with added BSG. Addition of BSG at higher than 0.2% resulted in the formation of a gel within the media.

BSG was tested at higher concentrations in yogurt between 0 – 1% (w/v). The concentration of skim milk powder (SMP) added to the yogurt formulation was tested between 5 – 20% (w/v). These concentrations were screened to find the optimum concentration range for SMP and BSG. Cane sugar concentration 0.1 – 12% and erythritol concentration 0 – 8% were analyzed and the optimum concentration range selected based on the results. The concentration of YO-MIX® 726 yogurt cultures was also tested over the range of addition rates from 0.001 to 0.2%. These five factors were varied between the ranges outlined to find the optimal concentrations using a BBD design. Yogurt gel firmness and serum separation were used to monitor these factors and data is shown in Table A2.5 (Appendix 2.2). The impact of each variable on gel firmness and % serum release is shown in Figure 4.6.

The concentrations of BSG and SMP significantly impacted yogurt gel firmness and serum amount (water-holding capacity) ($p < 0.05$), whereas the cultures, cane sugar and erythritol had minimal impact on either the gel firmness or % serum ($p > 0.05$) (Figure 4.6).

The yogurt gel with BSG between 0.01% and 0.2% had the firmest gel (firmness 130.09g) when included in yogurt (Figure 4.6a-1). As the BSG concentration increased beyond 0.2%, the firmness of the BSG-yogurt gel began to decline dropping down to the lowest firmness at 26.19 g with 1% BSG added to yogurt. The yogurt without any BSG had similar firmness to 0.3% BSG. As the concentration of basil seed gum (BSG) increased, serum release from yogurt gel decreased by approximately 10% for concentrations of ≥0.3% BSG (Figure 4.6a-2). However, at concentrations higher than 0.3%, the gel formed visible layers. Similarly, Yang et al. (2021) observed that BSG at 0.25 – 1% improved arachin gel strength and water-holding capacity, though significant phase separation occurred at 0.75% and 1% BSG. Nik et al. (2011) found that 0.1% and 0.2% BSG improved yogurt microstructure, with 0.2% being optimal for enhancing water-holding capacity. Additionally, Li et al. (2012) reported that flaxseed gum improved the casein gel strength at 0.1 – 0.5%. BSG can interact with casein to improve cheese microstructure (Hosseini-Parvar et al., 2015). Based on these findings, future studies will aim to optimize gel firmness and water-holding capacity using BSG concentrations between 0.01 – 0.2%.

When the concentration of SMP was increased the gel strength also increased until 15% SMP, but at 20% SMP the gel firmness decreased to a value close to 12% SMP (Figure 4.6b-1). There was a corresponding decrease in serum release with an increase in SMP concentration (Figure 4.6b-2). Serum amount reduced (increasing water holding capacity) when SMP concentration increased (Arab, et al., 2023; Wacher-Rodarte et al., 1993). Yogurt samples with 14.5% or higher SMP had an unpleasant mouthfeel, even though increasing the SMP content decreased syneresis (Tamime and Robinson, 2007; Wacher-Rodarte et al., 1993). It was therefore concluded that a range for SMP would be between 8 – 15% for the next stage of optimization.

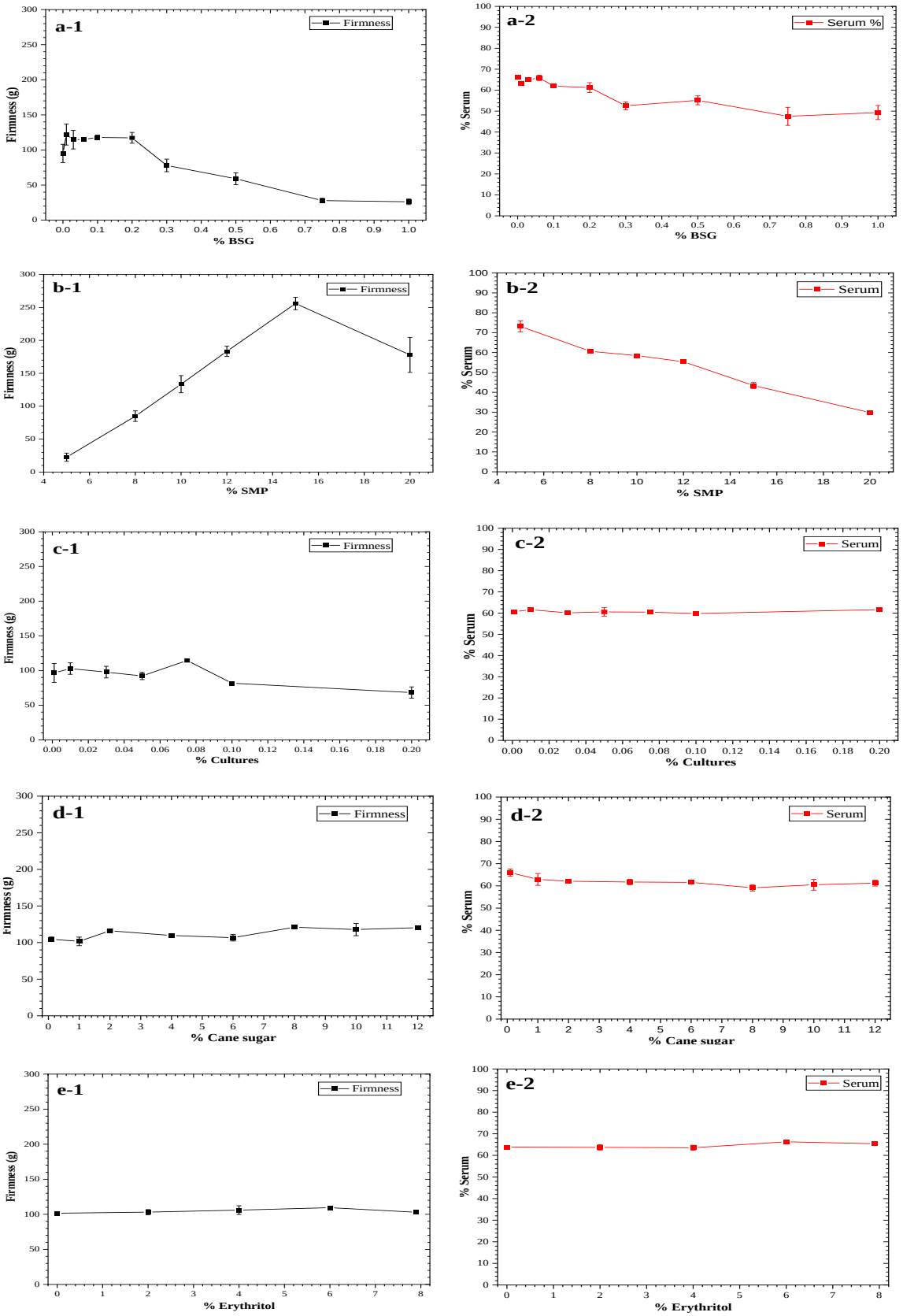


Figure 4.6 Effect of (a) basil seed gum (%w/v BSG), (b) skimmed milk powder (%w/v SMP) (c) % YO-MIX®726 culture, (d) % w/v cane sugar and (e) % w/v erythritol on yogurt (a-1, b-1, c-1, d-1, e-1) gel firmness (■) and (a-2, b-2, c-2, d-2, e-2) serum separation (■) for the factor range selection in yogurt Box-Behnken design. Mean values ± standard deviation, for n=2.

The YO-MIX® 726 culture concentration and cane sugar concentration did not affect gel firmness and serum ($p > 0.05$) (Figure 12c-1, c-2, d-1, d-2). Growth of yogurt cultures have been found to be inhibited if more than 12% sugar is added to the milk before fermentation and concentrations higher than 8% sugar lead to reduced acetaldehyde product and therefore, there was not the typical yogurt flavour (Chandan & Kilara, 2013). Tamime and Robinson (2007) recommended to use of 8 – 10% sugar in yogurt products for better taste and mouthfeel. The concentration of cane sugar was therefore used at ≤ 8% throughout the study and the culture level was kept at the lowest level of 0.001%. Erythritol at 0 – 8% also did not affect gel firmness and serum ($p > 0.05$) (Figure 12e-1, and 12e-2). The concentration of erythritol was increased with cane sugar decreased to have a total sugar concentration of 8%.

The final ranges of each factor chosen for the BBD were a range of 0.01 – 0.2% BSG, 8 – 15% SMP, and between 0 – 7.9% erythritol and 0 – 8% cane sugar (Table 4.4). This design was used to determine the optimum concentrations of each ingredient to form a yogurt with better water-holding and less serum separation. Any effect on the growth of YO-MIX® 726 yogurt cultures in yogurt were investigated after six-hour incubation at 43°C.

Table 4.4 Range of factors selected for the Box-Behnken design for yogurt making with BSG, SMP and sugar/erythritol ratio.

Factors	Level		
	-1 (lower level)	**0 (Centre point)**	**1 (upper level)**
% BSG (w/v)	0.01	0.11	0.20
% SMP (w/v)	8.0	11.50	15.0
% Sugars/% erythritol (w/v)	0.1/7.9	4.05/3.95	8.0/0.0

Note: For sugar/erythritol ratio as sugar % increased, erythritol % decreased

4.4 Yogurt fortified with BSG and erythritol – a BBD design

Using the results from the primary ingredient screenings, a 30-run BBD with three factors and three levels for each factor, including three replicates at the centre point, was used to fit a second-order response surface model. The experimental design details are listed below (Table 4.5). Sweetener was the third factor, where the percentage of cane sugar increased while percentage of erythritol decreased, maintaining a final sweetener concentration of 8% in each yogurt sample.

An optimum composition of ingredients, BSG, SMP and sweeteners, were estimated in the BBD and any effects of BSG, SMP and sweeteners on serum, firmness and numbers of probiotic bacteria present in the yogurt after 6 h fermentation was investigated. The result of each response variable was plotted (Figure 4.7).

The yogurt pH showed minimal variation despite the ingredient components varying (Figure 4.7a). A wave-like trend of serum separation was observed across all sample runs with a decreasing pattern overall (Figure 4.7b). Increasing SMP concentration appeared to reduce serum separation from the yogurt gel. Based on the run number and conditions in Table 4.5 and Figure 4.7b, an increase in BSG slightly reduced serum release. SMP possibly contributed the most to the water holding capacity consistent with results from the preliminary ingredient screening tests (Figure 4.6b-2).

Contrary to the preliminary test, cane sugar appeared to positively influence serum retention in the yogurt gel as the cane sugar concentration increased the serum amount decreased based on run information in Table 4.5 and Figure 4.7b. The firmness of yogurt samples was increased primarily with higher SMP concentrations and likely cane sugar contributed to the yogurt firmness (Figure 4.7c). The other ingredient concentration changes had little effect on yogurt cultures. ST, LB and probiotics showed minor variations (Figure 4.7d, 4.7e, and 4.7f).

The results from the BBD were analyzed using response surface methodology (RSM) and analysis of variance (ANOVA) in Minitab 21 to further justify the findings above. The response factors were firmness of gel, % serum release, and plate counts of YO-MIX® 726 cultures (cfu/g) after 6 h fermentation. The ANOVA analysis for the BBD is summarized in Table A2.7 (Appendix 2.2).

Table 4.5 Box-Behnken experimental design for determining ingredient concentrations to produce yogurt with addition of BSG and erythritol.

	Factors			
Run Number and Conditions			**Sugar**	
BSG:SMP:Cane sugar:Erythritol	**BSG %**	**SMP %**	**% Cane sugar**	**% Erythritol**
1-0.01-8-4.05-3.95	0.01	8.0	4.05	3.95
2-0.01-8-4.05-3.95	0.01	8.0	4.05	3.95
3-0.01-11.5-0.1-7.9	0.01	11.5	0.1	7.9
4-0.01-11.5-0.1-7.9	0.01	11.5	0.1	7.9
5-0.01-11.5-8-0	0.01	11.5	8.0	0
6-0.01-11.5-8-0	0.01	11.5	8.0	0
7-0.01-15-4.05-3.95	0.01	15.0	4.05	3.95
8-0.01-15-4.05-3.95	0.01	15.0	4.05	3.95
9-0.11-8-0.1-7.9	0.11	8.0	0.1	7.9
10-0.11-8-0.1-7.9	0.11	8.0	0.1	7.9
11-0.11-8-8-0	0.11	8.0	8.0	0
12-0.11-8-8-0	0.11	8.0	8.0	0
13-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
14-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
15-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
16-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
17-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
18-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95
19-0.11-15-0.1-7.9	0.11	15.0	0.1	7.9
20-0.11-15-0.1-7.9	0.11	15.0	0.1	7.9
21-0.11-15-8-0	0.11	15.0	8.0	0
22-0.11-15-8-0	0.11	15.0	8.0	0
23-0.2-8-4.05-3.95	0.2	8.0	4.05	3.95
24-0.2-8-4.05-3.95	0.2	8.0	4.05	3.95
25-0.2-11.5-0.1-7.9	0.2	11.5	0.1	7.9
26-0.2-11.5-0.1-7.9	0.2	11.5	0.1	7.9
27-0.2-11.5-8-0	0.2	11.5	8.0	0
28-0.2-11.5-8-0	0.2	11.5	8.0	0
29-0.2-15-4.05-3.95	0.2	15.0	4.05	3.95
30-0.2-15-4.05-3.95	0.2	15.0	4.05	3.95

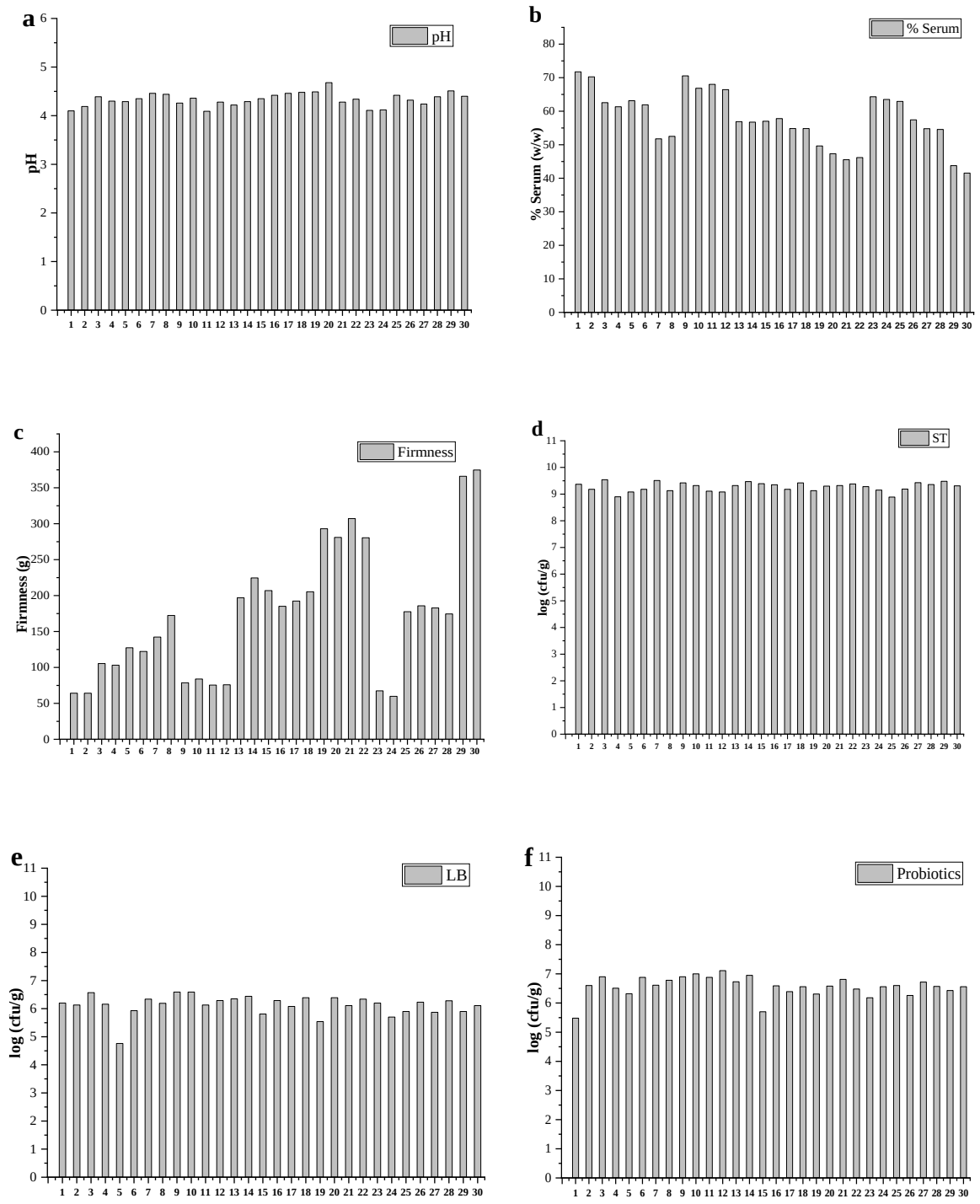


Figure 4.7 Histograms of results from Box-Behnken design experiments for added BSG and erythritol to yogurt (a) pH, (b) % serum, (c) Firmness, (d) *Streptococcus thermophilus* (ST), (e) *Lactobacillus delbrueckii subsp. bulgaricus* (LB), and (f) probiotics including *Lactobacillus acidophilus* and *Bifidobacterium lactis*.

4.4.1 Firmness of yogurt samples

The ANOVA analysis of the gel firmness showed a Lack-of-Fit of the firmness model ($p < 0.05$). The firmness model did not fit the data well. Therefore, the firmness data was transformed into log (Firmness). The Lack-of-Fit of log (Firmness) had a p-value higher than 0.05 and there was no evidence that the model did not fit the data in Table A2.7(Appendix 2.2). Analysis of log (Firmness) was used for further statistical analysis in optimizing yogurt ingredient composition study.

Both BSG and SMP had a significant impact on the gel firmness ($p < 0.05$) in contrast to Erythritol/sugar where Erythritol and cane sugar did not have any effect on the gel firmness in the Firmness model ($p > 0.05$). The results agree with the results in the primary screening of both ingredients and to the findings reported by Zhao et al (2021). Other researchers have found that sugarcane molasses results in a reduction in yogurt gel firmness (Noureldin, et al., 2020) and negative impact of adding cane sugar to yogurt gel firmness (Dias et al., 2020). The combination of BSG and cane sugar however had a significant impact on the gel firmness ($p < 0.05$), especially at lower concentration of SMP (≤ 11.5%) (Figure 4.8a and 4.8b).

BSG addition resulted in the firmest yogurt gel from around 0.09% to 0.13% and beyond these points, yogurt gel was found to be weaker when SMP was at 8% (Figure 4.8a). As SMP increased, the BSG content associated with the firmest gel shifted slightly from 0.09% to 0.10% and extended up to approximately 0.18% BSG (Figure 4.8b). In SMP 15% samples, the lowest effective BSG concentration was around 0.11%, with no defined upper limit (Figure 4.8c). The impact of the combination of erythritol and cane sugar was less important in the contribution of yogurt gel firmness as SMP concentration increased (Figure 4.8c). If reducing sugar levels and increasing erythritol concentration to enhance health benefits is desired, the hardest gels can be achieved by slightly increasing the BSG concentration to approximately 0.10% and raising the SMP concentration to reach the target firmness.

Erythritol influenced the firmness of the yogurt only when added together with BSG and the centre level of about 4 – 5% cane sugar plus 4 – 3% erythritol to achieve a total of 8% sweeteners producing the highest yogurt gel firmness in this study. The impact on gel

firmness of erythritol was associated with BSG in yogurt. Costa et al (2019) found erythritol-enhanced yogurt gel firmness peaking at 5% of erythritol.

Despite the interactions among BSG, cane sugar, and erythritol affecting gel firmness, the combination of BSG and SMP significantly enhanced yogurt gel firmness, with firmness increasing as the concentrations of both ingredients increased to a certain point. This was supported by the significance of the square terms and the curvature observed in the model in Table A2.7 (Appendix 2.2). Without BSG, SMP could only contribute to the yogurt gel firmness at 2.2 log (Firmness) (158 g), and with an additional 0.15% BSG, the yogurt gel firmness was about 2.55 log (Firmness) (354 g). As the concentration of SMP decreased to 11.5%, log (Firmness) increased by about 0.2 from 2.1 at 0% BSG to about 2.3 at between 0.10 – 0.18% BSG (Figure 4.8b). When 8% SMP was used for yogurt fermentation, the firmness of the yogurt gel decreased with a peak area of firmness at about 1.9 log (Firmness) with about 0.10% BSG (Figure 4.8a). These findings suggest that BSG interacted with SMP to affect yogurt gel firmness, as evidenced by the ANOVA, which indicates a significant interaction between BSG and SMP ($p < 0.05$). Guggisberg et al. (2009) tested inulin and at 4% inulin an increase in yogurt gel firmness from 155 g to 196 g was reported. BSG could increase yogurt gel firmness at lower concentrations, but there was an upper limitation of the highest firmness that BSG could facilitate with SMP to obtain, which was reached at 0.13% BSG in this study (Figure 4.8c).

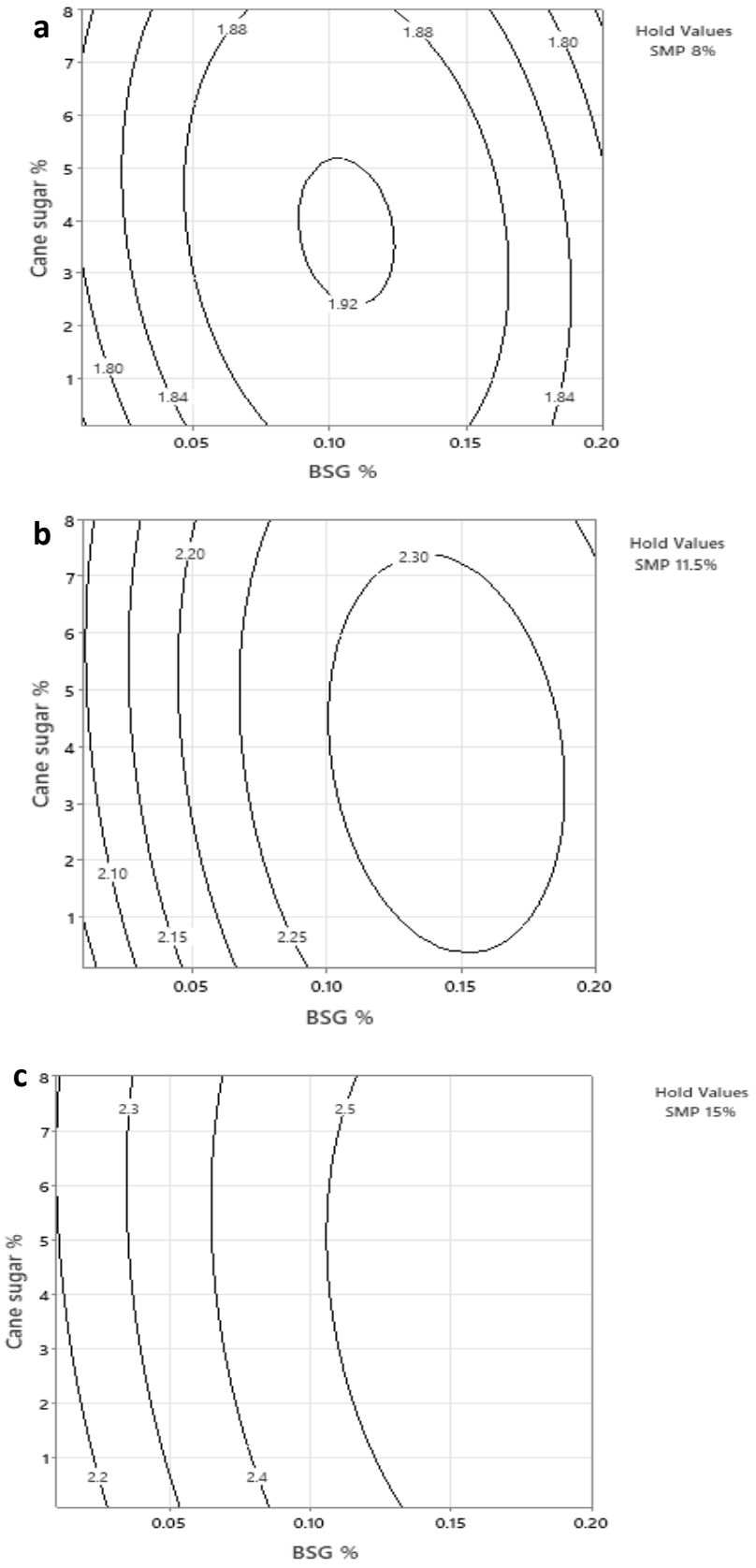


Figure 4.8 Contour plots showing the effect of BSG, cane sugar and erythritol on yogurt gel firmness at (a) 8, (b)11.5 and (c) 15% SMP-based yogurt samples.

4.4.2 Effects of serum separation with BSG, SMP, cane sugar and erythritol

As found with the contribution to the yogurt gel firmness, BSG and SMP affected positively the water-holding capacity of the gel and reduced the serum released from the yogurt gels ($p < 0.05$). A surface response analysis shown in Figure 4.9, as the SMP concentration was increased by 3.5%, the percentage of serum released from the gel was decreased by around 10%. Both BSG or SMP independently, had a positive impact on reducing serum release from the gel. BSG and SMP however did not have synergistic effects on the water-holding capacity of the yogurt gels, unlike their effect on yogurt gel firmness. There was interaction between BSG and cane sugars which affected the gel water holding capacity ($p < 0.05$).

Tao et al. (2021) reported a positive effect of 5% erythritol (w/v) on water holding capacity of curdlan gel. Gomes et al. (2022) observed corn fibre and polydextrose reduced the amount of whey released from the yogurt gel. Cane sugar can increase the yogurt gel water holding capacity and reduce the amount of serum release from the gel ($p < 0.05$), which was compatible with the findings of Gomes et al., (2022). They further confirmed that any porous structures formed with additional erythritol, or cane sugar acted like foam and were able to absorb more water and thus positively influenced the gel water holding capacity. Gomes et al (2022) claimed that lowering the cane sugar concentration would reduce the pore size in the yogurt causing it to shrink thus more serum is released into the yogurt. In this serum model, cane sugar had a curvature effect on the serum amount released from the yogurt gels.

More cane sugar content could enhance the water-holding capacity of the yogurt gels together with increasing the concentration of BSG, especially at a higher SMP level. The cane sugar at 4.2% (erythritol at 3.8%) with 0.16% BSG or higher for both ingredients would result in the lowest concentration of serum release and highest water-holding capacity at a high SMP concentration (≥11.5%) (Figure 4.9). It is not fully known what erythritol does but it may attach to polysaccharide chains possibly through Van Der Waals interactions and hydrogen bonds, and hindered the crystalline sheet-like structure formed and more porous foam-like structures are formed to facilitate the serum leaking from the gel structure (Tao et al., 2021).

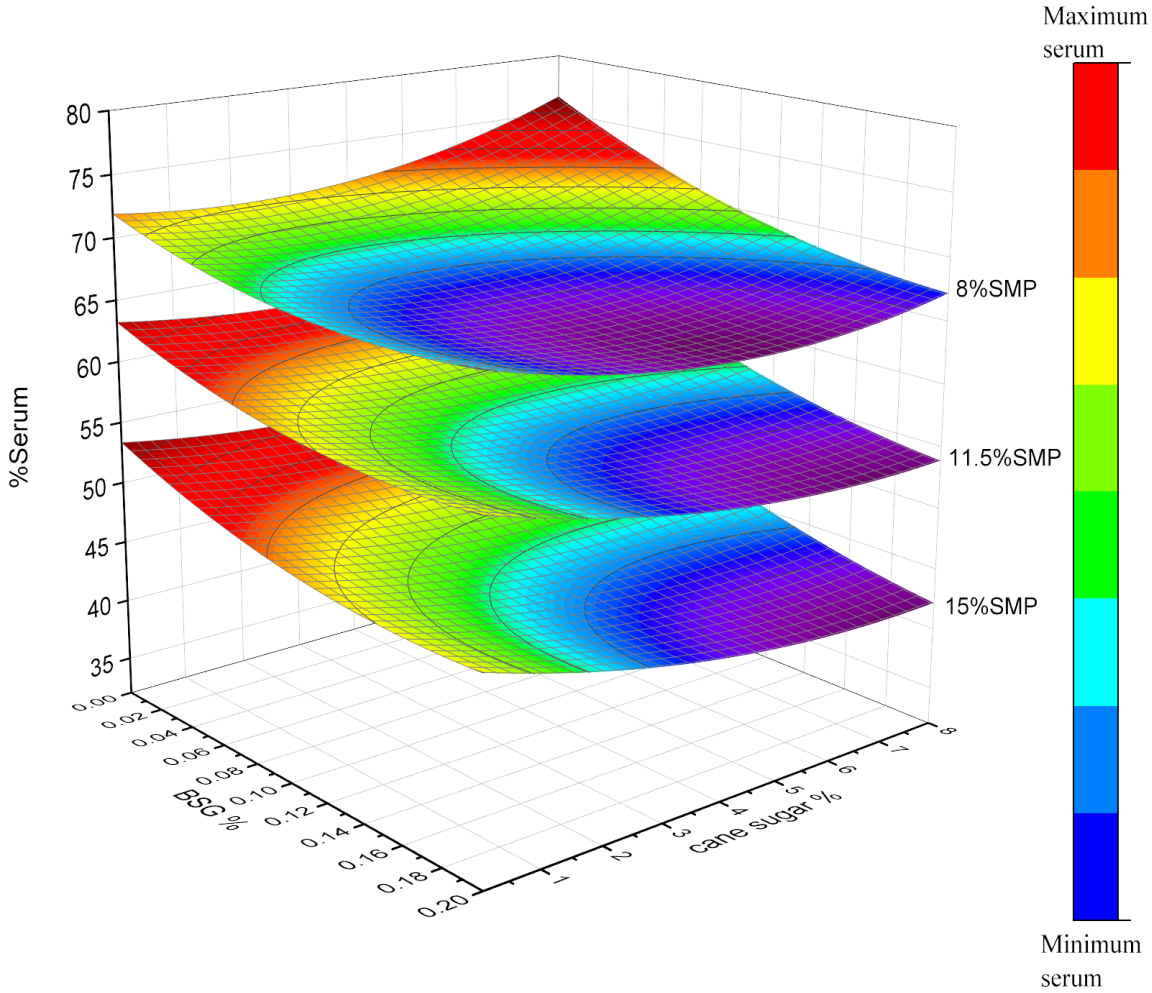


Figure 4.9 Response surface 3-D plots showing the effect of the combination of SMP, BSG and sugars on serum separation of yogurt gel.

4.4.3 Effects of BSG, SMP, cane sugar and erythritol on pH

Despite the addition of BSG, the pH decreased with increasing cane sugar concentration. In contrast, increasing erythritol or SMP content slowed down acid secretion by the yogurt cultures and thus the yogurt samples had higher pH values. Additionally, casein protein would slow down acidification in milk fermentation (Karam, et al., 2013). It could explain the reason for with increasing concentration of SMP, the yogurt gel had a higher pH value than those of lower SMP content, which enhanced the buffer system of calcium casein in yogurt fermentation (Karam et al., 2013). Additional polysaccharides had no impact on the pH value of yogurt products and therefore it is implied that BSG also had no impact (Gomes et al., 2022). The pH value increased with increasing erythritol amount. The suppression of yogurt cultures during fermentation, as demonstrated in the initial microbial growth curves with the addition of erythritol (Figure 4.5), could be a contributing factor.

4.4.4 BSG, SMP and cane sugar effect on growth of ST, LB and LA

In contrast to the findings in Section 4.3.1, where YO-MIX cultures were growth in media broth, in the yogurt samples, there were no significant effects of BSG, SMP, cane sugar, or erythritol on probiotic bacterial counts ($p > 0.05$). Similarly, these additives had no impact on the survival of ST and LB ($p > 0.05$). In the range of 0.01 and 0.2% BSG, 8 and 15% SMP and 0.1 and 8% sugar (7.9 and 0% erythritol), there was no impact on YO-MIX® 726 yogurt cultures. It was different from the preliminary growth curve study of the yogurt cultures versus different concentrations of BSG, and erythritol in M17 or MRS broth (Section 4.3.1 and 4.3.2).

4.4.5 Optimization of BSG, SMP and E/S concentration to form a target yogurt texture

BSG and SMP interacted and formed yogurt gels with different textures compared to traditional yogurt. It was firmer than yogurt and gave a silken tofu-like mouthfeel. To mimic tofu-like texture, designed models were created to optimize the concentrations of BSG and SMP together with erythritol partially replacing sugar to lower calorie intake daily which was a recommended level of 533 mg/kg (FDA, 2019; Grembecka, 2015; Johnson et al., 2009). Silken tofu samples were purchased locally, which had log (Firmness) at 2.31 ± 0.04. Log (Firmness) 2.31 was then set up as the target and water holding capacity (% serum) was chosen as the minimum goal. Although, there was no effect of yogurt ingredients on the number of probiotic bacteria in the BBD model, a maximum probiotic goal was also set up to gain a maximum number of probiotic cells and thus check the fermented milk would match the health benefit level >10^6 cfu/g (FAO/WHO, 2003; FSANZ, 2015). Therefore, the optimized yogurt compositions were predicted using the models, and the observed values were obtained experimentally with the optimized ingredient composition to validate the models.

Table 4.6 Parameters, predictive value and observed value based on Log firmness, % Serum and probiotic number.

Response	Goal	BSG	SMP	Erythritol	sugar	Predictive value	Observed value
Log (Firmness)	2.31					2.31	2.30±0.02
%serum	Minimum	0.0565g	15g	7.9g	0.1g	50.40	49.23±3.00
Log Probiotics	Maximum					6.77	6.39±0.11

The observed values fell within the 95% prediction interval range Table A2.8 (Appendix 2.2). This further demonstrated that the models were validated for predicting the concentrations of BSG, SMP, erythritol, and cane sugar on the firmness and water holding capacity of different fermented milk gels. Additionally, the probiotics fell within the 95% prediction interval range, achieving the recommended therapeutic levels. In comparison to silken tofu, fermented milk gel had double the water loss of the silken tofu (Fan et al., 2021; Yang & James, 2013). Meanwhile, erythritol had no effects on the probiotic number. In contrast, BSG did not show any prebiotic function on the growth of probiotics in yogurt. From these models, the number of probiotics remained at 10^6 cfu/g which just achieved the minimum level of probiotic requirement for the promotion of health in fermented dairy products (FAO/WHO, 2003; FSANZ, 2015).

4.5 Conclusions

During BSG extraction from basil seeds, shear rate and soaking time were the two factors that affected the weight of wet basil seeds. Increased temperature, soaking time and shear rate increased the weight of the wet gum. Both wet seed and gum models were used for prediction of optimum gum extraction conditions and 50^{o}C at a shear rate 1000 rpm for 120 min soaking time, selected for gum extraction. The yield of dried basil seed gum (BSG) was 17.8 g to 24.5 g per 100 g original basil seeds.

BSG showed potential growth enhancement at 0.01 – 0.05% in the bacterial media broths in this study, but there was no significant effect on the yogurt culture population in yogurt fermentation. The log (Firmness) and serum models could be used for the prediction of optimum ingredient composition for making different firm yogurts with maximum water holding capacity and thus less serum separation. Cane sugar level could be reduced to 0.1% with the addition of 7.9% erythritol to mimic a sugar-free fermented milk gel, with tofu like texture and which maintained the therapeutic probiotics level >10^6 cfu/g. Serum separation from the gel could however only be lowered to 49.2%.

Chapter 5 Enzymatic hydrolysis of BSG

5.1 Introduction

BSG has shown potential growth enhancement of YO-MIX® 726 yogurt cultures at 0.01 – 0.05% in previous results. In general, gums exhibit limited prebiotic function in promoting probiotic growth in vitro. Prebiotics that promote probiotic growth are generally shorter chain oligosaccharides derived from gums by chemical or enzymatic hydrolysis and sometimes synthesis via physical processes such as high hydrostatic pressure treatment (Davani-Davari et al., 2019; Yue et al., 2022; Wongputtisin & Khanongnuch, 2015). If erythritol was used to replace cane sugar, it is suggested to fortify dairy products with prebiotics to support growth and survival of probiotics (Costa et al., 2019; Kalicka et al., 2019). As a result, enzymatic hydrolysis was considered as a method to break down BSG sugar units into shorter chains and then to evaluate their potential in promoting probiotic growth. The activity of four different enzymes was evaluated to optimize the hydrolysis of BSG, MagiZyme® ZAC, SQZyme xylanase, CE35 cellulase and β-mannanase.

5.2 MagiZyme® ZAC hydrolysis of BSG

MagiZyme® ZAC (Zymus, Auckland, New Zealand) contains: β-glucanase, xylanase and minor endo-1,3(4)-beta-D-glucanase, hemicellulose, endo-1,4-beta-D-xylan, xylohydrolase and other cellulases derived from a fungal strain *Trichoderma reesei*. The manufacturer's recommended working range of pH is between 4.0 – 6.5 with an optimum performance at pH 5.5. The effective temperature for the activity of ZAC is from 50 – 70^{o}C, and the optimum temperature is at 60^{o}C. The starting point of the enzyme dosage was 0.06 – 0.09% w/w recommended by Zymus International. However, the optimum hydrolysis conditions of ZAC are varied when substrates are changed and process parameters also influence each other, e.g. changing pH, ratio of substrate and enzyme, hydrolysis time and temperature (Chen et al., 2013; Wahba et al., 2024; Wongputtisin & Khanongnuch, 2015). All four parameters, enzyme-to-substrate ratio (E/S), time, pH, and temperature, were evaluated based on their effects on direct reducing sugar (DS), total reducing sugar (TS), and degree of polymerization (DP) of hydrolyzed BSG. The range for each factor was determined through

data analysis and incorporated into a Box-Behnken design (BBD) to optimize ZAC hydrolysis conditions for basil seed gum.

5.2.1 Preliminary screening of different factors for ZAC hydrolysis of BSG

Preliminary ZAC hydrolysis experiments were set up for a range selection of factors; E/S, time, pH of citrate phosphate buffer and temperature for ZAC hydrolysis of BSG. Each factor was set at a fixed condition (1:1, 2 h, pH 5.5 or 60^{o}C) with only one factor changed for each hydrolysis reaction to determine the best enzymatic hydrolysis conditions. A single-factor-at -a-time approach was used. The E/S was set at 0, 0.05, 0.1, 0.2, 0.7, 1 and 5 grams of ZAC to one gram of BSG powder (Chen et al., 2013; Singh, et al., 2018; Wongputtisin & Khanongnuch, 2015). Time was chosen as 0, 0.5 and 1 – 8 h with one hour increments (Chen et al., 2013; Singh, et al., 2018; Wongputtisin & Khanongnuch, 2015). The pH range was set between pH 4.0 – 7.0 with 0.5 increments, with the manufacturer recommending pH 5.5 as the constant point. The temperature range chosen was based on the manufacturer's suggestion between 50 – 70^{o}C and then extended to a lower temperature of 30^{o}C. Therefore, temperature was evaluated between 30 – 70^{o}C with 10^{o}C increments.

The four factors were evaluated by monitoring direct reducing sugar (DS), total reducing sugar (TS) and degree of polymerization (DP). The results are shown in Figure 5.1, and the actual data collected available in Table A2.9 (Appendix 2.3.1). For the DP there was a sharp drop from E/S 0.0 - 0.7, and then DP levelled off between E/S of 1 – 5, which was also observed in the DS and TS concentrations (Figure 5.1a). Before E/S 1, an increasing concentration of DS was observed (Figure 5.1a). DS and DP of ZAC hydrolyzed BSG between E/S 0.7 – 5.0 range were found to be statistically similar, as determined by Tukey's method in the ANOVA analysis in Table A2.10 (Appendix 2.3.1). Therefore, the range of E/S 0.7 – 2.0 was selected to use for ZAC hydrolysis of BSG in a BBD.

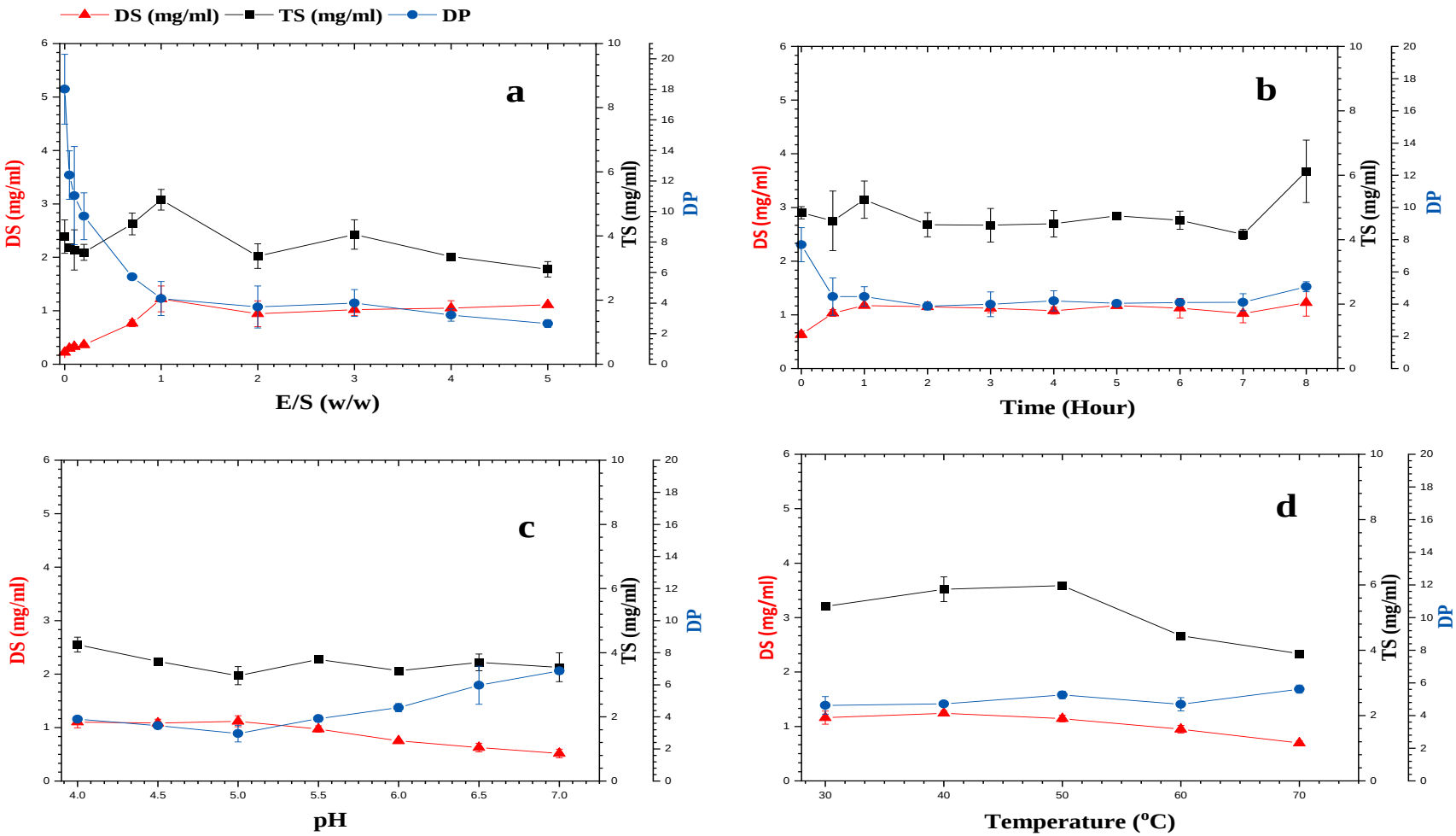


Figure 5.1 Effects of different factors on experiments to hydrolyse BSG with MagiZyme ZAC enzyme, (a) Ratio of MagiZyme ZAC and basil seed gum (E/S), (b) Time, (c) pH and (d) Temperature of the enzyme hydrolysis, evaluated based on directly reducing sugar (DS) (▲), total reducing sugar (TS) (■) and degree of polymerisation (DP=TS/DS) (●). Mean values ± standard deviation, n=2

In Figure 5.1b, except for times 0 and 8 h, all other hydrolysis times showed time had no effect on DS, TS or DP, giving similar results for three assays and confirmed by ANOVA analysis in Table A2.10 (Appendix 2.3.1). Therefore, the time range chosen for ZAC hydrolysis for the BBD design was between 0.5 – 7.0 h.

The pH of the sodium buffer solution significantly influenced the DS and DP values during the ZAC hydrolysis of BSG. ZAC enzymes exhibited optimal activity in slightly acidic conditions in this preliminary test, with pH levels between 4.0 – 5.5 (Figure 5.1c). The highest DS and lowest DP were observed at pH 5.0. Above pH 5.5, DS decreased as pH increased, with similar DS levels recorded at pH 5.5 and 6.0. According to Tukey grouping analysis in Table A2.10 (Appendix 2.3.1), no significant differences in DP were detected across the pH range of 4.0 – 6.0. Based on these results, the selected pH range for ZAC hydrolysis of BSG in the BBD experiment was set between 4.0 – 6.0.

The DP of ZAC hydrolyzed BSG remained consistent across all tested temperatures (Figure 5.1d). DS and TS of the hydrolyzed BSG varied with hydrolysis temperature. DS concentration decreased as temperature increased beyond 50^{o}C and TS concentration dropped significantly ($p < 0.05$) to temperatures above 50^{o}C. These trends were further confirmed by Tukey grouping analysis, which showed that hydrolysis temperatures between 30 – 60^{o}C resulted in similar DS levels, while the TS mean values were consistent for temperatures between 30 – 50^{o}C. Based on these findings, hydrolysis temperatures from 30 – 50^{o}C were then chosen for the BBD experiment. The variable factor levels used in the BBD are presented in Table 5.1.

Table 5.1 Factors and their levels used in the BBD for optimizing ZAC hydrolysis condition of basil seed gum.

	Level		
Factors	**Lower (-1)**	**Center (0)**	**Upper (-1)**
E/S	0.7	1.35	2
Time (h)	0.5	3.75	7
pH	4	5	6
Temperature (°C)	30	40	50

5.2.2 Optimization of ZAC hydrolysis conditions

Based on the results of the preliminary screening tests, a 27-run Box-Behnken Design (BBD) was employed to optimize the hydrolysis conditions of BSG using ZAC enzymes. Four factors were evaluated, E/S, time, pH and temperature (Table 5.3). The response variables, DS, TS and DP of hydrolyzed BSG are present in Figure 5.2 and data is listed in Table A2.11 (Appendix 2.3.1).

Table 5.2 A Box-Behnken design for optimizing ZAC hydrolysis conditions for basil seed gum

Run number and conditions	Factors			
E/S:Time:pH: Temperature	E/S	Time (h)	pH	Temperature (°C)
1-E/S0.7-0.5h-pH5-40°C	1.35	7	5	50
2-E/S0.7-3.75h-pH4-40°C	1.35	3.75	6	30
3-E/S0.7-3.75h-pH5-30°C	2	3.75	4	40
4-E/S0.7-3.75h-pH5-50°C	0.7	0.5	5	40
5-E/S0.7-3.75h-pH6-40°C	1.35	0.5	5	50
6-E/S0.7-7h-pH5-40°C	1.35	3.75	5	40
7-E/S1.35-0.5h-pH4-40°C	1.35	3.75	4	30
8-E/S1.35-0.5h-pH5-30°C	1.35	0.5	5	30
9-E/S1.35-0.5h-pH5-50°C	1.35	3.75	4	50
10-E/S1.35-0.5h-pH6-40°C	2	3.75	6	40
11-E/S1.35-3.75h-pH4-30°C	2	7	5	40
12-E/S1.35-3.75h-pH4-50°C	2	3.75	5	50
13-E/S1.35-3.75h-pH5-40°C	1.35	7	6	40
14-E/S1.35-3.75h-pH5-40°C	2	0.5	5	40
15-E/S1.35-3.75h-pH5-40°C	1.35	3.75	5	40
16-E/S1.35-3.75h-pH6-30°C	1.35	7	4	40
17-E/S1.35-3.75h-pH6-50°C	1.35	0.5	4	40
18-E/S1.35-7h-pH4-40°C	0.7	3.75	6	40
19-E/S1.35-7h-pH5-30°C	0.7	3.75	5	50
20-E/S1.35-7h-pH5-50°C	1.35	3.75	6	50
21-E/S1.35-7h-pH6-40°C	1.35	0.5	6	40
22-E/S2-0.5h-pH5-40°C	0.7	7	5	40
23-E/S2-3.75h-pH4-40°C	0.7	3.75	4	40
24-E/S2-3.75h-pH5-30°C	2	3.75	5	30
25-E/S2-3.75h-pH5-50°C	0.7	3.75	5	30
26-E/S2-3.75h-pH6-40°C	1.35	7	5	30
27-E/S2-7h-pH5-40°C	1.35	3.75	5	40

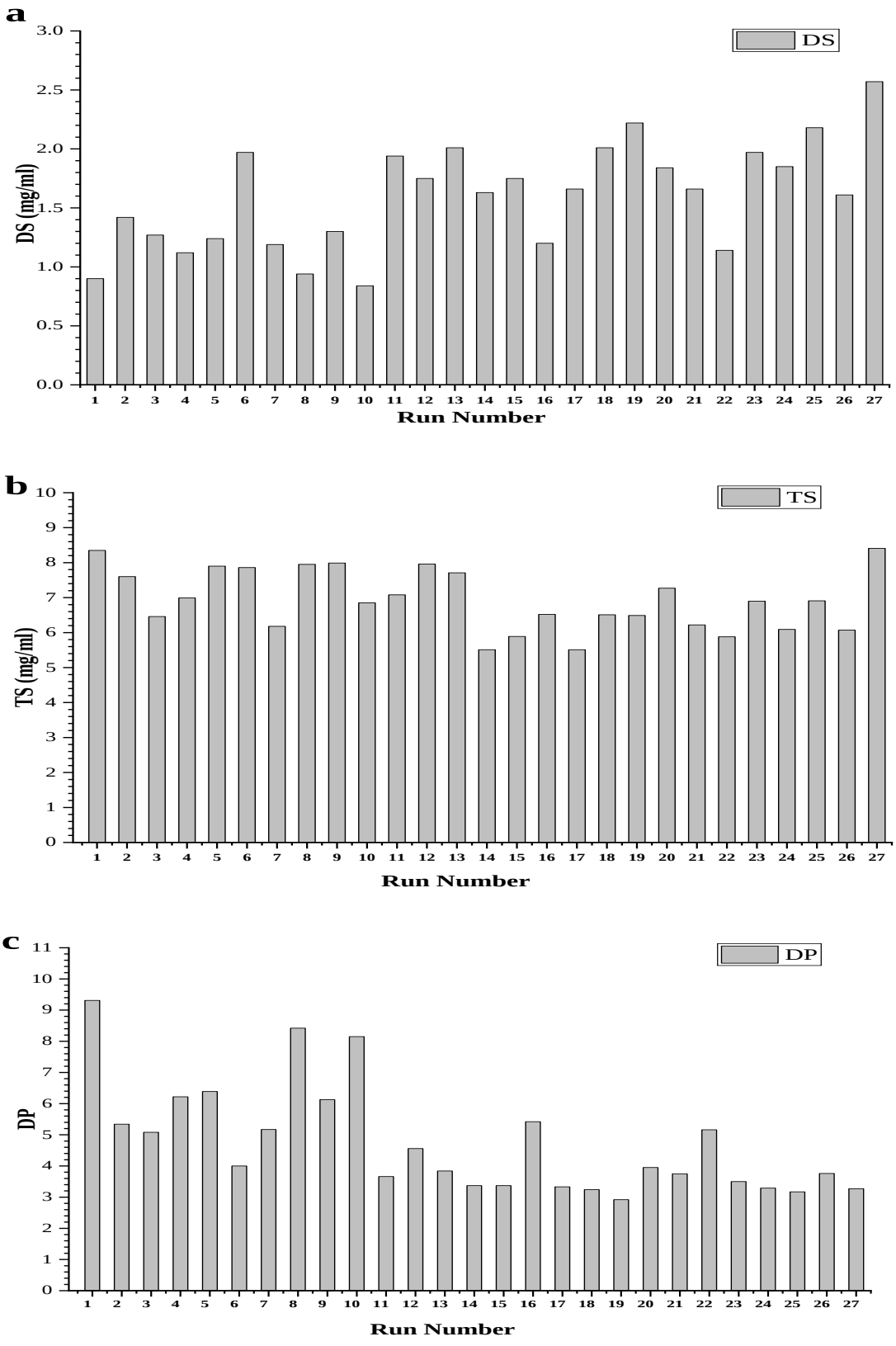


Figure 5.2 Histograms of results from hydrolysis experiments of BSG with ZAC enzyme of (a) direct reducing sugar (DS), (b) total reducing sugar (TS), (c) degree of polymerization (DP).

As the enzyme concentration and time increased, DS increased with a possible optimum pH at 5 (Figure 5.2a). TS did not show very much difference among the samples (Figure 5.2b). DP decreased with increasing concentration of enzyme (Figure 5.2c). Time could be another factor that could have influenced the DP values, as a duration of 0.5 hours yielded the highest DP value across all runs conducted with the same enzyme concentration. These variables were analysed individually against the four factors in the BBD to determine the optimal ZAC hydrolysis conditions in a citrate phosphate buffer solution. The ANOVA analysis supporting these findings, are provided in Table A2.12 (Appendix 2.3.1).

E/S, time and pH had significant effects on the hydrolysis of BSG with ZAC enzyme, especially on DS and DP values ($p < 0.05$). The temperature of reaction was not significant ($p > 0.05$) between 30 – 50^{o}C and the optimum hydrolysis temperature was estimated using the BBD to be 30^{o}C. The temperature was lower than the optimum working temperature 60^{o}C for ZAC enzymes recommended by Zymus International and was outside the working temperature range of 50 – 70^{o}C as recommended (Zymus, 2018). The experimental data for DS, DP, and TS were analyzed using a multivariate quadratic regression model (Box-Behnken design) to capture nonlinear trends, as shown in Table A2.12 (Appendix 2.3.1). The DS and DP models were both significant and demonstrated a better fit to the collected BBD data for ZAC-hydrolyzed BSG compared to the TS model. Additionally, the DS and DP models exhibited high R^2 values (>0.9) and a non-significant lack of fit ($p > 0.05$), indicating a strong fit and robust predictive capability.

Optimum hydrolysis conditions of ZAC were then predicted based on the DS and DP models listed in Table 5.3. Both models were validated by the repeated experiments under optimal enzymatic hydrolysis conditions, and observed values were in the 95% prediction interval of the DS and DP model.

Table 5.3 Validation experiment results for ZAC hydrolysis of BSG

Factors	Optimum conditions	Response variable	Predictive value	Observed value
E/S	1.79	DS	2.57 mg/ml	2.34±0.16 mg/ml
Time	7h			
pH	4.08	DP	2.92	3.10±0.08
Temperature	30ºC			

5.3 SQZyme xylanase hydrolysis of BSG

SQZyme xylanase (Xs) (Suntaq, Guangzhou, China) has the main component of β-Xylanase extracted from a fungal strain *Trichoderma reesei*, with corn starch and dextrin present as carriers. The manufacturer's recommended working ranges for pH, temperature and E/S were pH 3.5 – 7.0, 35 – 65^{o}C and 20 – 25g/1000kg, respectively. The product specification for SQZyme-xylanase reported the optimum working pH range from 4.5 – 5.5 and optimum working temperatures 45 – 55^{o}C. Preliminary screening tests were used to select factor ranges for a BBD.

5.3.1 Preliminary screening of factors for SQZyme xylanase hydrolysis of BSG

Preliminary tests for xylanase (Xs) hydrolysis were conducted to select the range levels of the factors, E/S, time, pH and temperature. The Xs hydrolysis reaction of BSG was evaluated at a fixed point for three factors, at 2 h, pH 4.5, 50^{o}C or E/S 1:1, and one factor was varied at a time to determine the optimal range for enzymatic hydrolysis conditions for that factor. The range for E/S was set at 0, 0.05, 0.1, 0.2, 0.7 and 1 – 5 (with increments of one gram of Xs per gram of BSG). Time was set at 0, 0.5, 1, 2, 3, 4 and 7 h. The pH was evaluated at pH 3.5, 4.5, 5.0, 5.5 and 7 based on manufacturer recommendations, and optimum pH ranged between 4.5 – 5.5. The temperature selection was based on the manufacturer's recommendation and the temperatures used were 35, 45, 50, 55, and 65^{o}C. Between 45 – 55^{o}C there was a 5^{o}C incremental increase in temperature. The four factors of E/S, time, pH and temperature were evaluated based on DS and TS concentrations and DP. The results of preliminary hydrolysis screening for Xs are shown in Figure 5.3, and the actual data collected is available in Table A2.13 (Appendix 2.3.2).

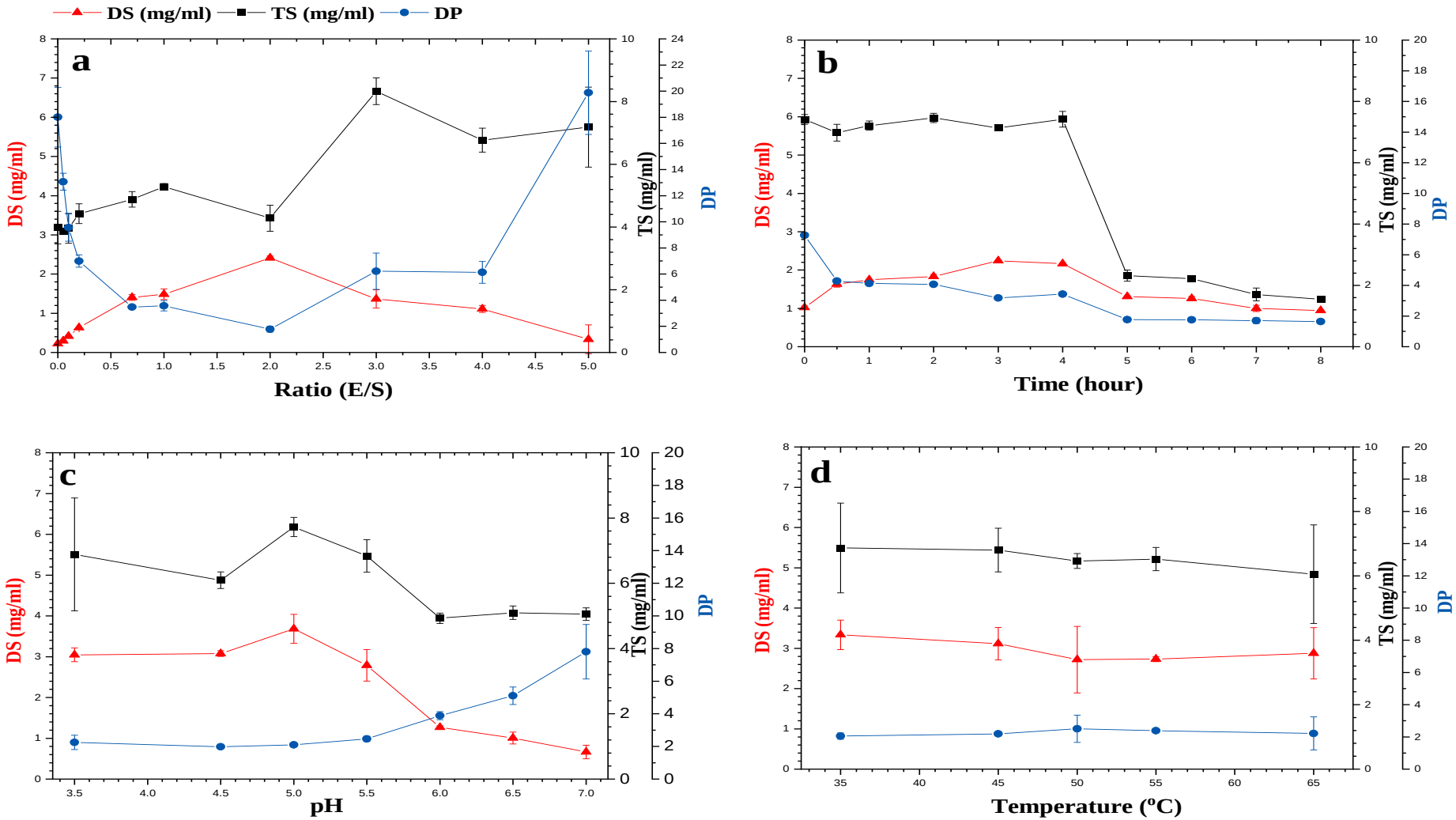


Figure 5.3 Effects of different factors on experiments to hydrolyse BSG with SQZyme xylanase enzyme, (a) Ratio of SQZyme xylanase and basil seed gum (E/S), (b) Time, (c) pH and (d) Temperature on hydrolysis of basil seed gum, based on directly reducing sugar (DS) (▲), total reducing sugar (TS) (■) and degree of polymerisation (DP) (●) of hydrolysed BSG. Mean values ± standard deviation, n=2

DS concentration increased with increasing xylanase concentration until E/S 2 then the DS concentration dropped from E/S 3 onwards (Figure 5.3a). DP declined between an E/S 0 – 2 then increased from E/S 3 up to 5 (Figure 5.3a). The ANOVA analysis showed that all three response variables (DS, TS and DP) were significantly different at different E/S ratios ($p < 0.05$). E/S 2 had the highest DS value. Consequently, an E/S range of 0.7 – 2 was chosen for analysis of optimum hydrolysis condition in a BBD using Minitab.

Figure 5.3b shows the impact of time on the hydrolysis of BSG with xylanase. DS concentration increased after 0.5 h and remained at the same concentration until after 5 h when the DS concentration declined back to the initial concentration at 0 h. The DP values were high at approximately 16 and dropped to 3, remaining at this level from 5 – 8 h. Based on the grouping information from the ANOVA analysis, DS values of 3 h and 4 h enzyme hydrolysis were the highest compared to the other groups, and TS and DP values were similar after 0.5 to 4 h hydrolysis time (Table A2.14, Appendix 2.3.2). Time had an impact on the DS, TS and DP values of Xs-hydrolyzed BSG ($p < 0.05$). The range selected for the hydrolysis time was between 0.5 – 4 h for the BBD analysis with 2.25 h as the centre time.

When evaluating the effect of pH on xylanase hydrolysis the DS concentration increased from pH 3.5 – 5 then from pH 5.5 declined steadily to pH 7 (Figure 5.3c) Under acidic conditions Xs hydrolyzed BSG produced more DS and TS, indicating higher enzyme activity, and this matched the manufacturers recommended optimum pH working range. TS results showed similar trends as DS and DP remained unchanged before pH 5.5 and increased from pH 6.0 to 7.0 (Figure 5.3c). From lignin xylan, more reducing sugars, xylose, xylobiose, xylotriose and xylotetraose, were produced after hydrolysis with xylanase at a lower pH 4.0 (Singh et al., 2018). In addition, relatively higher pH levels (5.5 – 7.0) resulted in less release of reducing sugar from beechwood xylan (Díaz-Arenas et al., 2022). The ANOVA analysis for results showed significant differences in DS, TS or DP at different pH hydrolysis conditions ($p < 0.05$). Over the range of pH 3.5 – 5.5 the DS, TS and DP values remained at similar mean levels in all response variables. Therefore, the pHs 3.5, 4.5 and 5.5 were studied further in a BBD to find the optimum pH condition.

Minimal changes were observed in the DS, TS, and DP trends with increasing temperature (Figure 5.3d), and the ANOVA analysis indicated no significant differences in these response variables ($p > 0.05$). For lignin xylan hydrolysis, the upper temperature of 50^{o}C was reported as the optimum temperature for xylanase activity (Singh et al., 2018). In contrast, Díaz-Arenas et al. (2022) achieved higher reducing sugar release from beechwood xylan at a lower temperature of 25^{o}C using xylanase. Temperature selection depends largely on substrate properties, and real-time experiments are essential to refine the experimental temperature range. However, in this study, the preliminary tests indicated that temperature was unlikely to be a significant influencing factor. The finding in this study was also different from Wongputtisin and Khanongnuch (2015) who reported 55^{o}C was the optimum hydrolysis temperature for the xylanase with continuously increasing in reducing sugar release from 0 to 6 h and then levelled off onwards. To confirm this finding, the temperature range was maintained between 35 – 65^{o}C, with 50^{o}C as the central point for further testing. Therefore, the variable factor levels used for the xylanase BBD (Xs-BBD) for BSG are shown in Table 5.4.

In addition, the variance in TS results were possibly due to the composition of BSG and its branching structures (Kurzyna-Szklarek et al., 2022; Guan et al., 2023). The other reason caused the variation was possibly the hydrolysis conditions, particularly duration time which would influence the yield of monosaccharides (Kurzyna-Szklarek et al., 2022). Meantime, Xs contains corn starch and minor dextrin as carriers and can be hydrolyzed by 8% sulphuric acid to release reducing sugar and caused variation of TS as enzyme concentration increased (Li et al, 2020).

Table 5.4 Factors and their levels in the BBD.

Factors	**Level**		
	Lower (-1)	**Center (0)**	**Upper (1)**
E/S	0.7	1.35	2
Time (hour)	0.5	2.25	4
pH	3.5	4.5	5.5
Temperature (°C)	35	50	65

5.3.2 Optimization of SQZyme xylanase hydrolysis conditions

Based on preliminary screening tests outlines in Section 5.3.1, a 27-run BBD of BSG hydrolyzed by SQZyme xylanase was used to optimize the hydrolysis conditions (Table 5.5). The same four factors, E/S, time, pH and temperature with three levels, were evaluated by the response variables DS, TS and DP of Xs hydrolyzed BSG in the BBD (Table 5.5). Optimum SQZyme xylanase hydrolysis conditions on BSG were predicted using the best fit models of the response variables based on the coefficient R-sq (Chen et al., 2013). DS, TS and DP results of Xs-hydrolysed BSG are presented in Figure 5.4 and data is available in Table A2.15 (Appendix 2.3.2).

Table 5.5 A Box-Behnken design for optimizing SQZyme xylanase (Xs) hydrolysis conditions for BSG.

Run Number and conditions	Factors			
E/S: Time:pH:Temperature	E/S	Time (h)	pH	Temperature (°C)
1-E/S0.7-0.5h-pH4.5-50°C	0.7	0.5	4.5	50
2-E/S0.7-2.25h-pH3.5-50°C	0.7	2.25	3.5	50
3-E/S0.7-2.25h-pH4.5-35°C	0.7	2.25	4.5	35
4-E/S0.7-2.25h-pH4.5-65°C	0.7	2.25	4.5	65
5-E/S0.7-2.25h-pH5.5-50°C	0.7	2.25	5.5	50
6-E/S0.7-4h-pH4.5-50°C	0.7	4	4.5	50
7-E/S1.35-0.5h-pH3.5-50°C	1.35	0.5	3.5	50
8-E/S1.35-0.5h-pH4.5-35°C	1.35	0.5	4.5	35
9-E/S1.35-0.5h-pH4.5-65°C	1.35	0.5	4.5	65
10-E/S1.35-0.5h-pH5.5-50°C	1.35	0.5	5.5	50
11-E/S1.35-2.25h-pH3.5-35°C	1.35	2.25	3.5	35
12-E/S1.35-2.25h-pH3.5-65°C	1.35	2.25	3.5	65
13-E/S1.35-2.25h-pH4.5-50°C	1.35	2.25	4.5	50
14-E/S1.35-2.25h-pH4.5-50°C	1.35	2.25	4.5	50
15-E/S1.35-2.25h-pH4.5-50°C	1.35	2.25	4.5	50
16-E/S1.35-2.25h-pH5.5-35°C	1.35	2.25	5.5	35
17-E/S1.35-2.25h-pH5.5-65°C	1.35	2.25	5.5	65
18-E/S1.35-4h-pH3.5-50°C	1.35	4	3.5	50
19-E/S1.35-4h-pH4.5-35°C	1.35	4	4.5	35
20-E/S1.35-4h-pH4.5-65°C	1.35	4	4.5	65
21-E/S1.35-4h-pH5.5-50°C	1.35	4	5.5	50
22-E/S2-0.5h-pH4.5-50°C	2	0.5	4.5	50
23-E/S2-2.25h-pH3.5-50°C	2	2.25	3.5	50
24-E/S2-2.25h-pH4.5-35°C	2	2.25	4.5	35
25-E/S2-2.25h-pH4.5-65°C	2	2.25	4.5	65
26-E/S2-2.25h-pH5.5-50°C	2	2.25	5.5	50
27-E/S2-4h-pH4.5-50°C	2	4	4.5	50

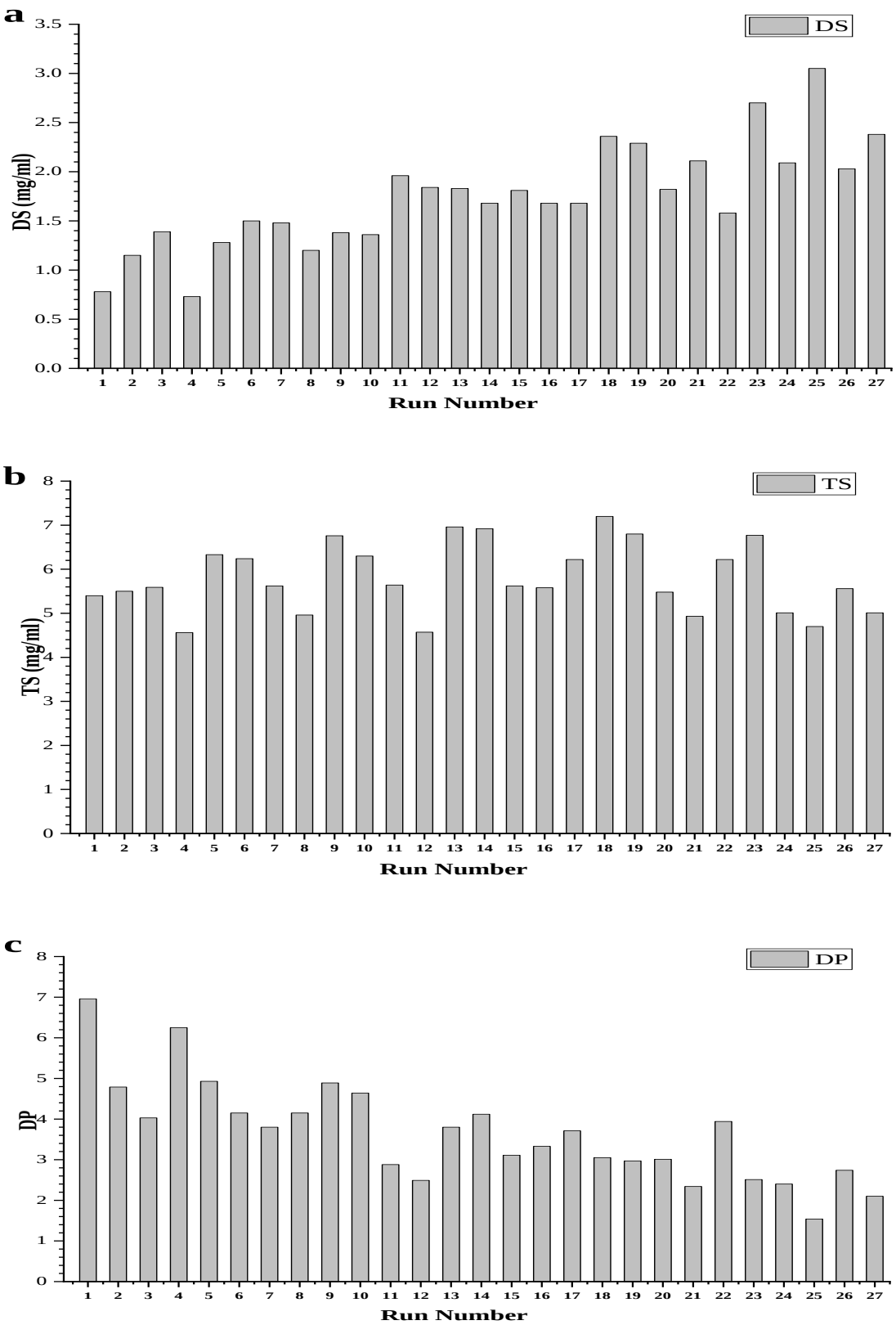


Figure 5.4 Histograms of results from hydrolysis experiments of BSG with SQZyme xylanase enzyme (a) directly reducing sugar (DS), (b) total reducing sugar (TS), (c) degree of polymerization (DP).

In Figure 5.4a an increase in DS concentration was found with increasing Xs concentration. Xs appeared to be more effective at lower pH levels, releasing more DS from BSG, as observed in samples 18, 19, 25, and 27. These samples had pH values of 3.5 or 4.5, and temperature impacted on DS release, with these four samples spanning a temperature range of 35 – 65^{o}C. In contrast, time appeared to slightly influence the TS value across all samples, but there was minimal variation in TS (Figure 5.4b). Meanwhile, DP decreased as the concentration of Xs increased (Figure 5.4c). It was not very clear which factor would significantly impact DS, TS, and DP values of Xs hydrolysed BSG from the histogram plots, except E/S. Results were analysed in ANOVA to support the observations above Table A2.16 (Appendix 2.3.2).

In the BBD, the ratio of SQZyme xylanase versus BSG and time were the two main factors that influenced DS and DP of hydrolyzed BSG ($p < 0.05$). pH had a small but significant influence on the DS of Xs-hydrolyzed BSG ($p < 0.05$) but had no effect on DP ($p > 0.05$). These findings agree with the preliminary screening test for pH range (Figure 5.4). The optimum pH 3.5 was comparable to Samanta Jayapal, Kolte, et al (2015) finding an optimum at pH 3.55 and lower than the optimum pH 4.0 of xylanase hydrolysis on lignin xylan (Singh et al., 2018).

Temperature had no effect on the hydrolysis of BSG by Xs which was comparable to the previous results for the temperature range selection. Again, this varied from Samanta Jayapal, Kolte, et al (2015) finding that the temperature had a significant effect on xylanase hydrolysis results. Optimum hydrolysis temperature of xylanase was reported to be 55^{o}C by Wongputtisin and Khanongnuch (2015), and 50^{o}C reported by Singh et al. (2018). Increasing temperature increased the amount of reducing sugar released in their studies. Although temperature did not individually influence DS and DP values in this study, it interacted with E/S to influence these two values ($p < 0.05$). Increased E/S or temperature would increase DS and reduce DP. A temperature at 65^{o}C was estimated by the BBD to maximize DS values of Xs hydrolysed BSG. Wongputtish and Khanonguch (2015) have found that the amount of reducing sugar was about 1.80 mg/ml the highest amount and DP was around 5 – 6. In this research from the prediction model SQZyme Xs could release 3.25 mg/ml reducing sugar from the hydrolysis of BSG and the calculated value of DP was 0.88.

Singh et al. (2018) and Díaz-Arenas et al. (2022) have shown yield of DS at 372 and 379 mg/g of xylanase hydrolyzed lignin and beechwood xylans, respectively.

Table 5.6 Results of validation experiment

Variable factors	Optimum conditions		Predictive value	Observed value
E/S	2:1	DS	3.25mg/ml	3.18±0.15mg/ml
Time	4 h			
pH	pH 3.5	DP	0.88	1.11±0.05
Temperature	65°C			

Optimum hydrolysis conditions of SQZyme xylanase were predicted based on the DS and DP models and listed in Table 5.6. Both models were validated by the repeated experiments under the optimal enzymatic hydrolysis conditions and observed values of DS and DP fell within the 95% prediction interval of the respective models.

5.4 Sunson-CE35 cellulase hydrolysis of BSG

CE35 (Sunson, Guangzhou, China) is a highly efficient cellulase also derived from *Trichoderma reesei*, with dextrin as the carrier. Cellulase hydrolyses β-1,4 glucose linkages of cellulose or hemicellulose. Sunson CE35 cellulase's recommended working temperature range is from 40 – 75°C with optimum temperature between 55 – 65°C, and pH working range 3 – 7 with optimum pH between pH 4 – 6. The recommended cellulase enzyme dosage and working time to soften fibre is 0.1 – 1% (w/w) for 1 – 3 h. Time and doses used in gum hydrolysis were not specified by the manufacturer. The preliminary range screening tests were undertaken as previously described in Sections 5.2.1 and 5.3.1. The experimentally determined ranges for each factor were subsequently brought into a BBD to estimate the optimal hydrolysis conditions for cellulase on BSG.

5.4.1 Preliminary screening of factors for CE35 cellulase hydrolysis of BSG

Preliminary experiments were conducted to determine suitable ranges influencing the hydrolysis of BSG by CE35 cellulase. The four factors of E/S, time, pH, and temperature were evaluated by determining DS, TS and DP. To isolate the effect of each factor, a single-factor-at-a-time approach was employed. While varying one factor, the other three were held constant at fixed conditions, an E/S 1, a time of 2 hours, pH 4.5 and 60°C.

The E/S ratio was tested at 0, 0.05, 0.1, 0.2, 0.7g, and between 1 – 5 g at one-gram intervals (grams of CE per gram of BSG). CE concentration was increased up to 5 g/g BSG to assess whether higher enzyme concentrations would result in a significant increase in reducing sugar released from BSG. Hydrolysis time was evaluated at intervals of 0, 0.5 h and for 1 – 8 h with one-hour increments. The pH range was evaluated between 4.0 – 7.0 with increments of one pH unit. Temperature selection was initially based on the manufacturer's recommendation of 40 – 75^{o}C. This range was extended to include a lower temperature of 20^{o}C (Mudgil et al, 2014), with 10^{o}C increments between 20 – 40^{o}C and with 5^{o}C increments between 55 – 65^{o}C to gain a more comprehensive understanding of CE hydrolysis temperature activity. The results of preliminary screening test are shown in Figure 5.5, and collected data is also available in Table A2.17 (Appendix 2.3.3).

As the concentration of CE increased, the amount of reducing sugar released (DS) from BSG increased (Figure 5.5a). A sharp rise in DS was observed between E/S 0 – 0.7, followed by an increase from E/S 1 – 5. Although there was a slight increase in DS between E/S 1 – 5, the mean comparison for these results were not significantly different ($p > 0.05$). The carrier for CE was dextrin which contains maltose and maltotriose reducing sugars, the increasing in DS could be due to the increase in concentration of these reducing sugars rather than the reducing sugar units released from BSG by CE (Blanco & Blanco, 2017). There were also no differences in DP values between E/S 2 – 5 according to the grouping information in the ANOVA analysis in Table A2.18 (Appendix 2.3.3). A sharp drop of DP was observed between E/S 0 – 0.7, compared to a sharp increase in DS and TS over the same range (Figure 5.5a). E/S was found to significantly influence DS, TS and DP of CE hydrolysed BSG ($p < 0.05$), but the impact was more significant between E/S 0.7 – 2. Therefore, E/S 0.7 – 2 range was selected to optimize CE hydrolysis conditions on BSG in the BBD.

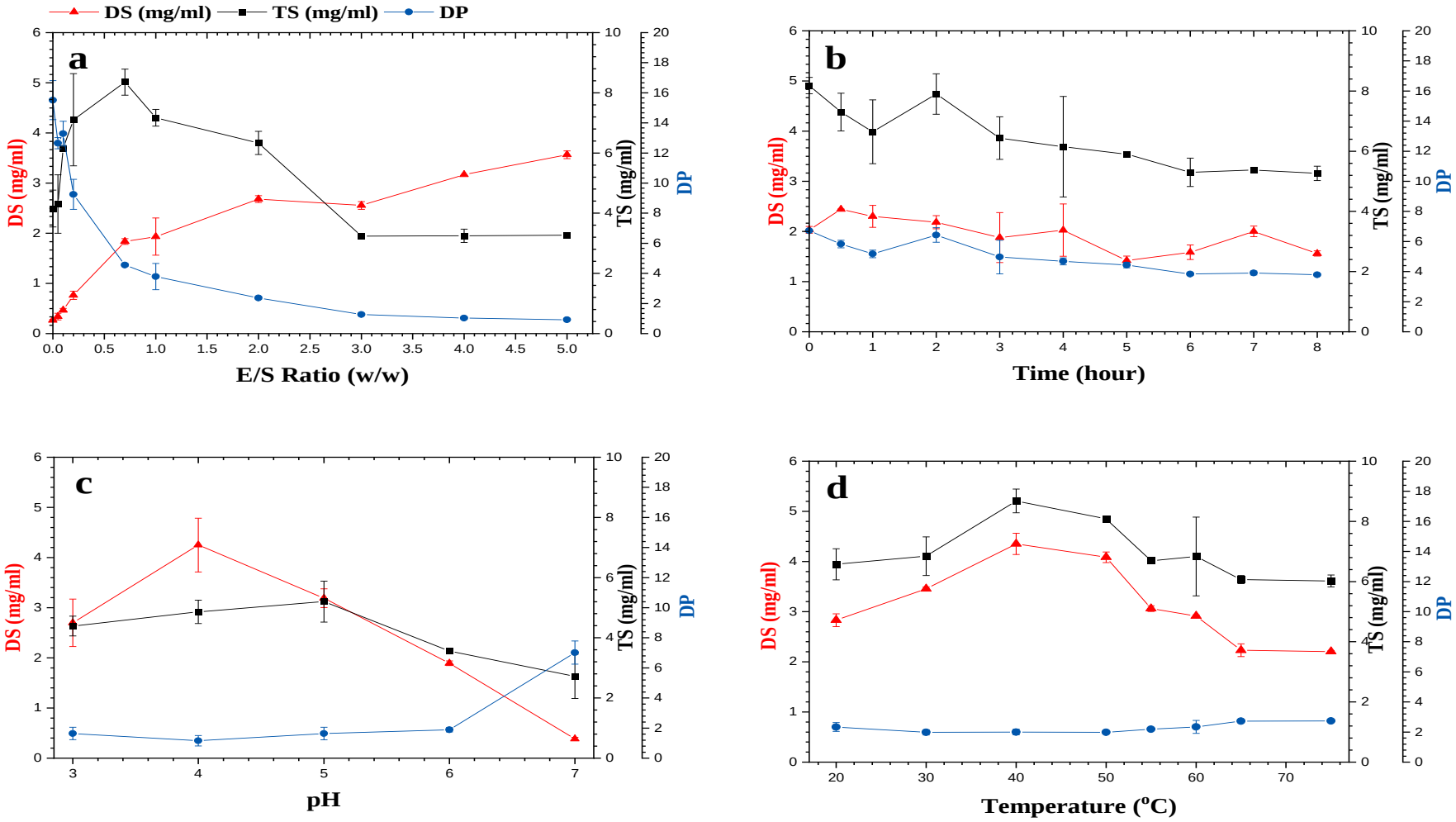


Figure 5.5 Effects of different factors on experiments to hydrolyse BSG with CE35 cellulase enzyme, (a) Ratio of the enzyme and basil seed gum (E/S), (b) Time, (c) pH and (d) Temperature on hydrolysis of CE35 cellulase on basil seed gum, based on directly reducing sugar (DS) (▲), total reducing sugar (TS) (■) and degree of polymerisation (DP) (●) . Mean values ± standard deviation, n=2.

DS, TS and DP all decreased with increasing hydrolysis time with CE (Figure 5.5b). The grouping information using Tukey comparison did not show many differences across all hydrolysis times in Table A2.18 (Appendix 2.3.3). There was a slight increase in DS concentration at 7 h while TS and DP values remained relatively stable between 6 – 8 h. To get a more clear understanding of the optimal enzyme hydrolysis time, the range of hydrolysis times chosen were from 0.5 – 7 h and then brought into the BBD for further analysis.

The TS results also varied in CE hydrolysis. The variation was possible due to the composition of BSG and its branching structures (Kurzyna-Szklarek et al., 2022; Guan et al., 2023). Meanwhile, dextrin as the carrier for CE can be hydrolyzed to monosaccharides by strong acids like sulphuric acid (Arai and Ogiwara, 1983; Mason, 2009). As a result, increasing the enzyme concentration led to a higher release of reducing sugars, contributing to the variation in TS.

Like SQZyme xylanase, CE35 cellulase was found to work better in acidic conditions (Figure 5.5c). A peak for DS concentration was observed at pH 4 and then with an increasing pH the DS concentrations continuously decreased. This finding differs from the optimal pH of cellulase hydrolysis on guar gum, previously reported at pH 6.0 (Mudgil et al., 2014). There was little change for DP between pH 3 – 6. TS up to pH 5 was constant then declined after pH 5. The DS values of pH 3, 5 and 6 shared the same ANOVA analyis letters in the grouping which indicated no significant difference among treatments. Therefore, pH 3 – 6 were brought into the BBD for optimising CE hydrolysis conditions.

Different hydrolysis temperatures did not have an affect on the DP values of hydrolyzed BSG ($p > 0.05$). Temperature was the main factor to have an impact on DS of hydrolysed BSG ($p < 0.05$) and had a peak concentration between 40 – 50^{o}C, which was slightly lower than the manufacturer's recommended optimum working temperature between 55 – 65^{o}C. The same maxima was for TS between 40 – 50^{o}C (Figure 5.5d). Hydrolysis temperature of CE was selected over a range of 30 – 60^{o}C. The levels of each factor were chosen and brought into the BBD to optimize CE hydrolysis conditions for BSG as shown in Table 5.10.

Table 5.7 Factors and their levels used in the BBD for CE hydrolysis of BSG.

Factors	Level		
	Lower (-1)	Center (0)	Upper (1)
E/S	0.7	1.35	2
Time (hour)	0.5	3.75	7
pH	3	4.5	6
Temperature (°C)	30	45	60

5.4.2 Optimum hydrolysis condition of CE35 cellulase in the BBD

Using the results from the preliminary screening tests, a 27-run Box-Behnken Design (BBD) was designed by Minitab to optimize the hydrolysis conditions for BSG using CE. Four factors were evaluated, the enzyme to BSG ratio (E/S), time, pH and temperature (Table 5.8). The response variables, DS, TS and DP of hydrolyzed BSG are present in Table 5.8 and plotted in Figure 5.6 with corresponding data available in Table A2.19 (Appendix 2.3.3).

Table 5.8 Optimizing CE hydrolysis conditions for BSG using a BBD

Run number and conditions	Factors			
E/S:Time:pH:Temperature	E/S	Time (h)	pH	Temperature (°C)
1-E/S0.7-0.5h-pH4.5-45°C	0.7	0.5	4.5	45
2-E/S0.7-3.75h-pH3-45°C	0.7	3.75	3	45
3-E/S0.7-3.75h-pH4.5-30°C	0.7	3.75	4.5	30
4-E/S0.7-3.75h-pH4.5-60°C	0.7	3.75	4.5	60
5-E/S0.7-3.75h-pH6-45°C	0.7	3.75	6	45
6-E/S0.7-7h-pH4.5-45°C	0.7	7	4.5	45
7-E/S1.35-0.5h-pH3-45°C	1.35	0.5	3	45
8-E/S1.35-0.5h-pH4.5-30°C	1.35	0.5	4.5	30
9-E/S1.35-0.5h-pH4.5-60°C	1.35	0.5	4.5	60
10-E/S1.35-0.5h-pH6-45°C	1.35	0.5	6	45
11-E/S1.35-3.75h-pH3-30°C	1.35	3.75	3	30
12-E/S1.35-3.75h-pH3-60°C	1.35	3.75	3	60
13-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45
14-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45
15-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45
16-E/S1.35-3.75h-pH6-30°C	1.35	3.75	6	30
17-E/S1.35-3.75h-pH6-60°C	1.35	3.75	6	60
18-E/S1.35-7h-pH3-45°C	1.35	7	3	45
19-E/S1.35-7h-pH4.5-30°C	1.35	7	4.5	30
20-E/S1.35-7h-pH4.5-60°C	1.35	7	4.5	60
21-E/S1.35-7h-pH6-45°C	1.35	7	6	45
22-E/S2-0.5h-pH4.5-45°C	2	0.5	4.5	45
23-E/S2-3.75h-pH3-45°C	2	3.75	3	45
24-E/S2-3.75h-pH4.5-30°C	2	3.75	4.5	30
25-E/S2-3.75h-pH4.5-60°C	2	3.75	4.5	60
26-E/S2-3.75h-pH6-45°C	2	3.75	6	45
27-E/S2-7h-pH4.5-45°C	2	7	4.5	45

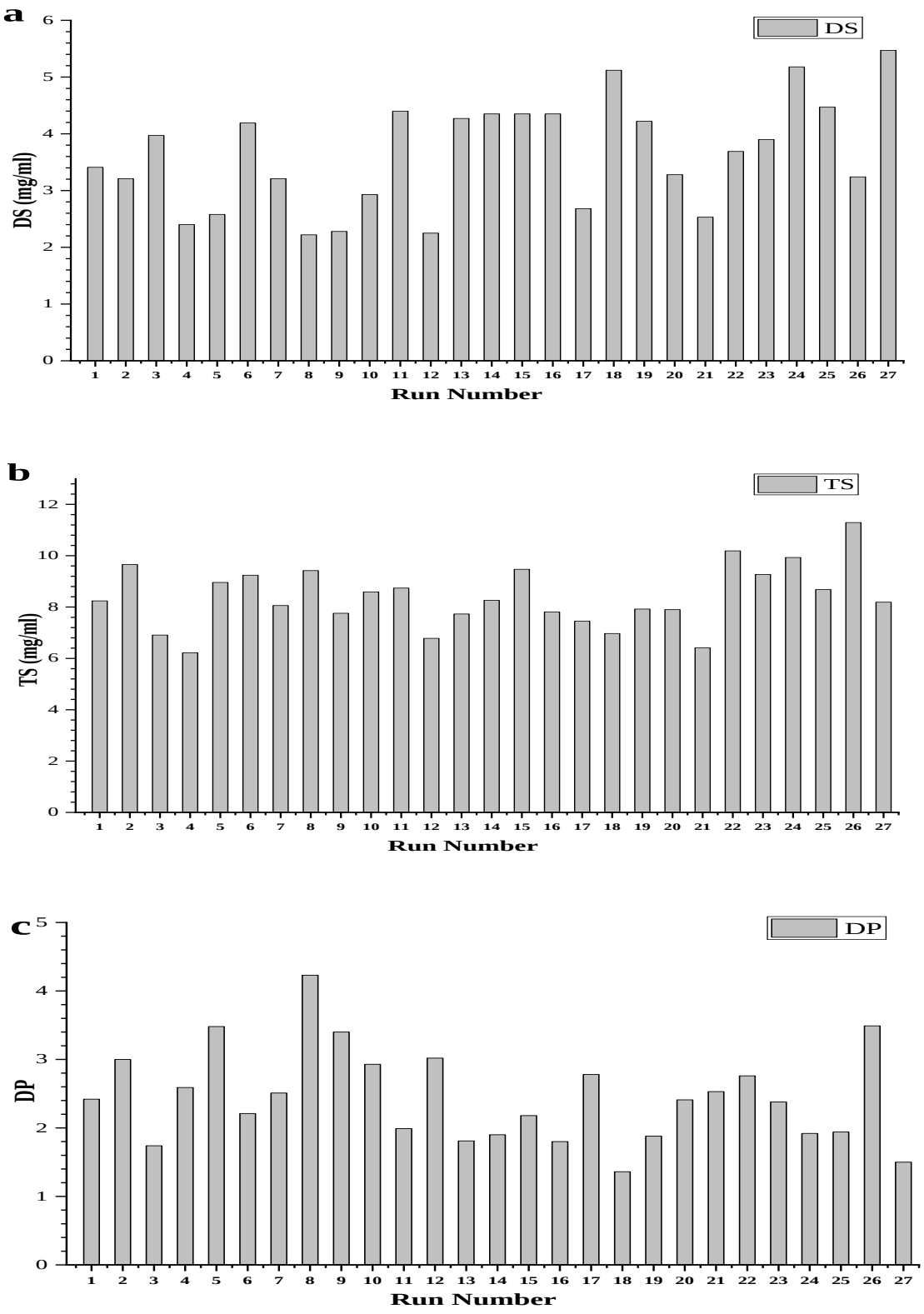


Figure 5.6 Histograms of results from hydrolysis experiments of BSG with CE35 cellulase enzyme (a) directly reducing sugar (DS), (b) total reducing sugar (TS), (c) degree of polymerization (DP).

As the enzyme concentration and time increased, DS showed an increasing trend with an optimum at pH 4.5 and 45^{o}C hydrolysis condition based on run information in Table 5.8 and Figure 5.6a. The highest DS concentrations were observed for Runs 3, 6, 11, 18 and 27. These samples had increasing concentration of CE, and hydrolysis time was between 3.75 – 7 h. The majority of these samples had pH 4.5 and 45^{o}C hydrolysis conditions. TS did not show variation between all the runs compared to DS (Figure 5.6b). DP slightly decreased with increasing concentration of the enzyme (Figure 5.6c). Hydrolysis time could still be the effective factor to influence the DP values, as a duration of 0.5 hours yielded the highest DP value and 7 h resulted in a relatively low DP, e.g. sample run 8 (E/S1.35-0.5h-pH4.5-30^{o}C) and run 19 (E/S1.35-7h-pH4.5-30^{o}C) (Figure 5.6c). The data was analysed using ANOVA to determine if there were significant differences in DS, TS, and DP values across the treatment groups. The ANOVA analysis supporting these findings, are provided in Table A2.20 (Appendix 2.3.3).

All four factors, E/S, time, pH and temperature significantly influenced CE enzymatic activity to release DS from BSG ($p < 0.05$). Different pH and time, or pH and temperature conditions have interacted to influence CE enzymatic activity for releasing DS from BSG ($p < 0.05$). Mudgil et al. (2014) reported that E/S, time, pH or temperature did not cooperatively influence cellulase activity rather than worked individually to affect enzyme activity. However, there were no significant differences observed for TS ($p > 0.05$). E/S did not influence DP values, whereas time was the primary factor significantly influencing DP values in this BBD ($p < 0.05$). Acidic conditions were preferred for optimal BSG hydrolysis by CE, particularly at a pH close to the lowest recommended level of 3. In contrast, cellulase worked on hydrolysis of guar gum much better at pH 6.0 (Mudgil et al., 2014). Rice straw was used as a substrate to test the optimal working pH at pH 4, but at pH 7.0 onwards, cellulase from *Trichoderma reesei* lost its activity (Kogo et al., 2017). A relative low reaction temperature of 37^{o}C was estimated in the BBD (Table 5.9), whereas previous studies reported higher optimal temperatures for cellulase activity at 50^{o}C and 60^{o}C, respectively (Mudgil et al., 2014; Kogo et al., 2017).

From ANOVA analysis, it was found that the DS model had a higher R-sq (88%) compared to the DP model (62%). The predicted DS concentration (7.27 mg/mL) was higher than the prediction from combining both DS and DP models (6.76 mg/mL). As a result, the optimum conditions predicted by DS model only are listed below (Table 5.9). ANOVA analysis details are available in Table A2.20 (Appendix 2.3.3). The model was validated by repeated experiments under the optimal enzymatic hydrolysis conditions, and observed values were in the 95% prediction interval of the DS.

Table 5.9 Validation experiment results for CE

Variable factors	Optimum conditions	Predictive value (DS)	Observed value (DS)
E/S	2:1		
Time	7h	7.27mg/ml	6.81 ±0.05 mg/ml
pH	3.15		
Temperature	37^{o}C		

5.5 β-mannanase hydrolysis of BSG

There was minimal release of reducing sugar from hydrolyzed BSG by β-mannanase. This could be attributed to the composition of BSG, which contains glucomannan in a 10:2 ratio of glucose to mannose (Hosseini-Parvar et al., 2010). β-Mannanase specifically targets glucose and mannose linkages but its activity can be ceased at non-reducing end of galactose ends where BSG contained about 25% of galactose sugar units in the mucilage (Hosseini-Parvar et al., 2010; Malgas et al., 2015). These structural features limit the enzyme's ability to cleave glucomannan into shorter glucomannan-oligomers. Furthermore, Rafe et al. (2013) reported that cotton thread-like structures present in BSG. It may contribute to its rigid structure, making it more resistant to enzymatic action. As a result, β-mannanase showed limited activity in breaking down BSG.

5.6 Conclusions

The optimum hydrolysis conditions for ZAC enzymes were determined to be an enzyme-to-substrate (E/S) ratio of 1.79:1, with hydrolysis carried out for 7 hours at pH 4.08 and 30^{o}C. For SQZyme xylanase, the optimal conditions were an E/S ratio of 2:1, 4 hours of hydrolysis,

pH 3.5, and 65^{o}C. Similarly, CE35 cellulase showed optimum hydrolysis at an E/S ratio of 2:1, for 7 hours, at pH 3.15 and 37^{o}C.

ZAC enzymes had lower observed reducing sugar content (DS) at 2.34 mg/ml compared to 3.18 mg/ml using the SQZyme Xs and 6.81 mg/ml of CE35 cellulase of hydrolyzed BSG. SQZyme xylanase was recommended to be used for further hydrolysis experiments as the results for DS and DP show good yields. The CE35 cellulase released twice the amount of DS from BSG compared to other enzymes and was therefore also selected for future studies to optimize BSG hydrolysis.

Chapter 6 Impact of BSG-oligosaccharides on yogurt cultures and yogurt quality profiles

6.1 Introduction

Prebiotics are generally short-chain oligosaccharides, fructo- and gluco-oligosaccharides, and xylooligosaccharides which are typically derived from plants or plant seeds via enzyme hydrolysis, acid/base cleaving or physical pretreatments (Allgeyer et al., 2010; Brownawell et al., 2012). BSG-oligosaccharides were derived from basil seed gum using SQZyme xylanase (Xs) and CE35 cellulase (CE).

The first objective of this chapter was to determine the impact of the BSG-oligosaccharides on the growth of YO-MIX® 726 cultures: *Streptococcus thermophilus* (ST), *Lactobacillus delbrukii* subsp. *bulgaricus* (LB) and *Lactobacillus acidophilus* (LA). The second objective was to determine the impact of the BSG-oligosaccharides on yogurt quality.

6.2 Effect of BSG-oligosaccharides on the growth of YO-MIX®726 yogurt cultures

Two enzymes, Xs and CE were selected to hydrolyze BSG. The optimum hydrolysis of BSG by Xs was at an E/S ratio of 2, pH 3.5, 65°C for 4 h determined based on direct reducing sugar (DS) and degree of polymerization (DP) models. The optimum hydrolysis conditions for CE on BSG was an E/S ratio of 2, pH 3.15, 37°C for 7 h determined based on DS model. BSG-oligosaccharides (BSG-Oligos) were recovered from the buffer solution and dried to a powder for further study, referred to the procedure in Figure 3.3.

It was hypothesised that small amounts of dextrin and/or soluble starch present as enzyme carriers may have remained in BSG-Oligos dried powder. Dextrin and soluble starch are considered prebiotics and can enhance the growth of yogurt cultures in vitro (Śliżewska et al., 2012). In addition, citrate phosphate residuals were likely to be present in the BSG-Oligos, and it was unclear whether these salts would have any impact on the yogurt bacteria growth. To investigate the effect of reducing sugars in the carriers and also sodium salts from the buffer on the growth of cultures extra enzyme blank solutions and hydrolysis

of BSG in water were included in the experimental plan. Firstly, a blank BSG-Oligos recovered from a hydrolysis reaction with enzyme and buffer only (Xs-Blank and CE-Blank), no BSG substrate added. Secondly, instead of the citrate phosphate buffer, RO water was used for the hydrolysis reaction with BSG substrate and enzyme, and the BSG-oligos were labelled Xs-H_2O and CE-H_2O. YO-MIX 726 yogurt cultures, *Streptococcus thermophilus* (ST), *Lactobacillus delbrukii subsp. bulgaricus* (LB) and *Lactobacillus acidophilus* (LA), supplemented with different BSG-Oligos from various hydrolysis treatment information is listed in Figure 6.1.

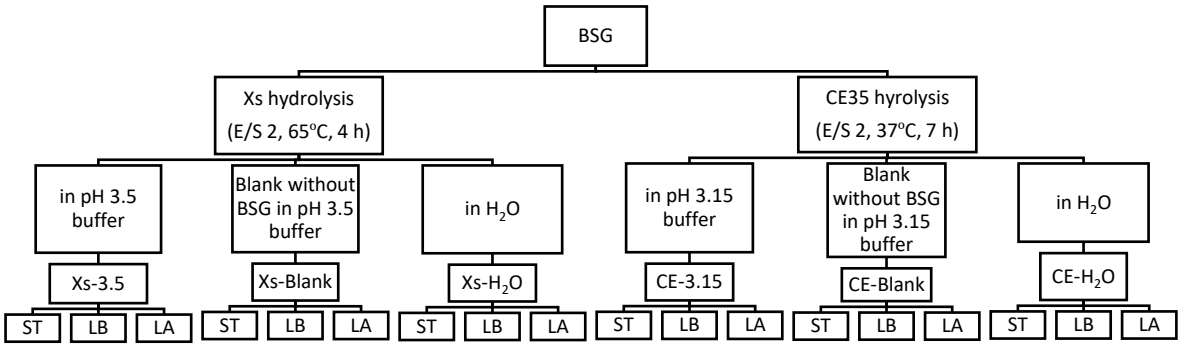


Figure 6.1 Diagram of YO-MIX 726 yogurt cultures supplemented with different BSG-Oligosaccharides from various hydrolysis treatments.

6.2.1 *Streptococcus thermophilus* (ST)

ST was isolated from YO-MIX®726 on M17 agar and subsequently cultured in M17 broth supplemented with different concentrations of BSG-Oligos powder obtained from Xs or CE hydrolysis of BSG. Three BSG-Oligos powders were tested for each enzyme: pH 3.5, Blank and RO water (H_2O). The BSG-Oligos powder concentrations used for the bacterial cell growth curve test were 0.01, 0.05, 0.1, 0.2, 0.5, then 1 – 5% (w/v) with one percentage increments. The analyzed data from the growth curves are presented in Figure 6.2. The maximum growth rate (μ_{Max}), maximum cell density (Max) and lag time (lag) of ST were determined using a modified logistic growth model determined from the absorbance readings for ST cell growth after 24 h incubation.

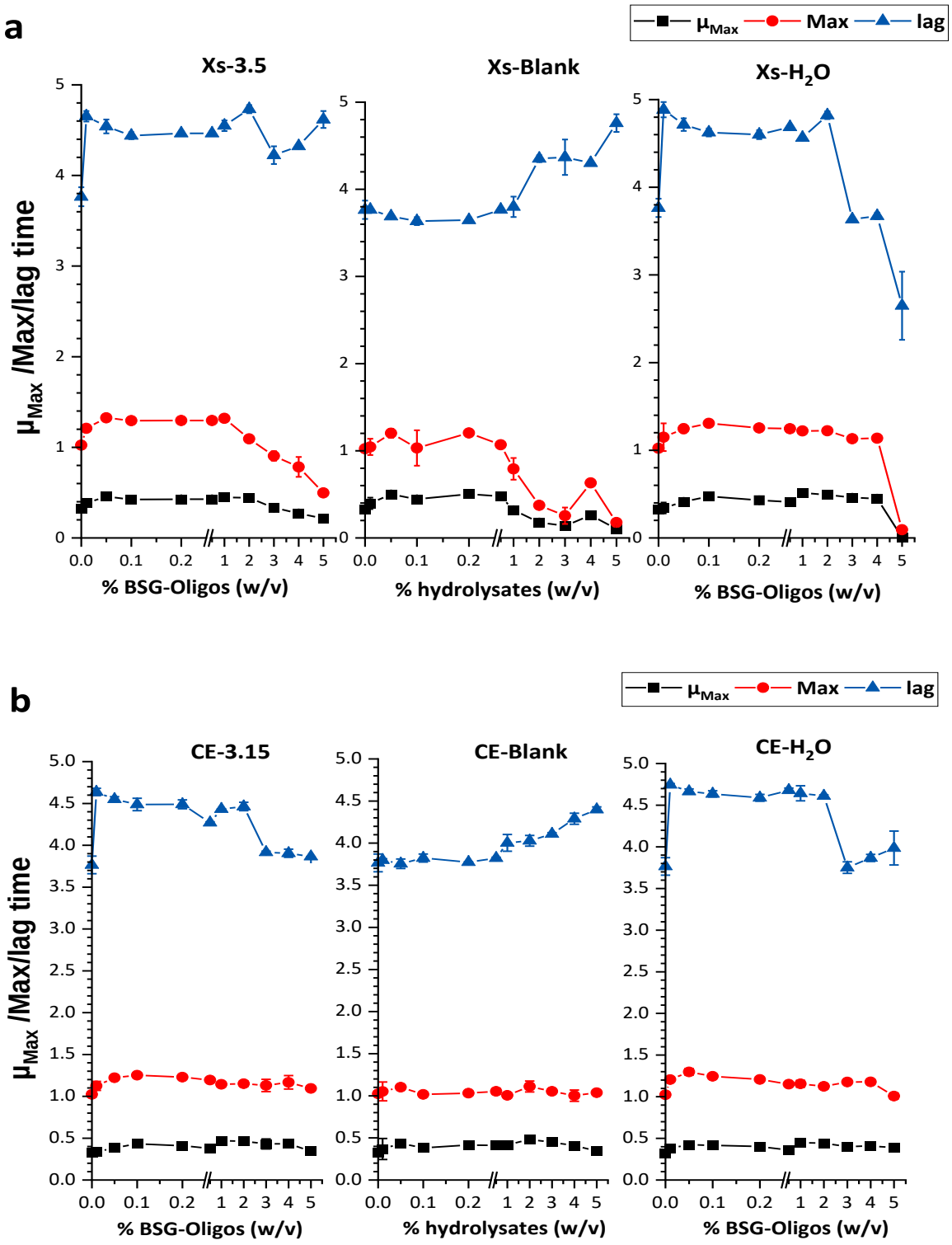


Figure 6.2 Maximum growth rate (μ_{Max}, h^{-1}), maximum cell density (Max, OD_{595nm}) and lag time (h) of YO-MIX®726 *Streptococcus thermpophilus* (ST) in M17 broth with additional BSG-oligosaccharides or hydrolysates of enzyme carrier retrieved from (a) Xylanase hydrolysis of BSG in pH 3.5 citrate phosphate buffer, xylanase blank (Xs-Blank) and in RO water (Xs-H_2O); (b) CE35 cellulase hydrolysis of BSG in pH3.15 citrate phosphate buffer (CE-3.15), in RO water (CE-H_2O) and CE35 blank (CE-Blank) after 24 h incubation at 37°C. Mean values ± standard deviation for n=2.

Decreasing values for Max and μ_{Max} for ST were observed in Xs-3.5 samples from 2% BSG-Oligos onwards therefore higher concentrations of Xs-3.5 up to 2% supported ST culture growth (Figure 6.2a). When Xs-H_2O was added to the ST culture, Max and μ_{Max} was reasonably constant for all concentrations except for a sharp drop observed at 5% Xs-H2O samples. The results with Xs-H_2O indicate that the salts present in the buffer may have had a negative impact on the growth of ST. High Max values (enhanced cell growth) were observed at $\leq$1% Xs-3.5 or $\leq$3% Xs-H_2O.

Increasing the Xs-Blank concentration led to a decrease in the maximum cell density (Max) (Figure 6.2a) and a reduction in the μ_{Max} of ST cells from 0.5% Xs-Blank onwards. Higher concentrations of Xs-Blank oligosaccharides inhibited the growth of ST indicating that any soluble starch, dextrin or salts from the enzyme or buffer did not support ST growth. The increasing osmotic pressure caused by residual dextrin and sodium salt, along with the rise in cell viscosity due to the diffusion of intracellular fluid against osmotic pressure changes, may contribute to this effect (Lievens et al., 2015).

ST growth was relatively constant for CE-3.15 for all concentrations tested (Figure 6.2b). CE-Blank samples had slightly lower Max values, similar to the control but they were also constant for all concentrations tested. CE-H_2O showed very similar results to CE-3.15 indicating that the hydrolysis reaction can be carried out in water.

CE used dextrin as the enzyme carrier, which can partially dissolve in ethanol during the ethanol extraction process of BSG-Oligos (Prosky, 2000; Gonçalves et al., 2016). Consequently, dextrin could still be present in the BSG-Oligos powder or the CE-Blank after purification. Dextrin is a good carbon source to support ST growth, and enhances yogurt culture growth in yogurt fermentation with the optimum concentration at 1.5% (Slizewska et al., 2012; Peerkhan & Nair, 2021). Dextrin is the only carrier in CE, whereas Xs had minimal dextrin. It is postulated that ST cells were able to overcome the negative impact of the buffer salts when dextrin was present in the BSG-Oligos samples. The potential presence of dextrin in the CE oligosaccharides could explain why Max for CE remained high compared to Xs. (Figure 6.2). In summary, CE-3.15 showed slightly better growth than the CE-Blank samples and ST-control (Figure 6.2b).

Despite the negative impact of buffer salts on Xs-BSG-Oligos, slightly higher Max values were found compared to CE-BSG-Oligos at concentrations ≤ 0.5%. Xs-H2O samples had slightly higher μ_{Max}, although no significant differences were observed across all μ_{Max} values for both enzymes (Figure 6.2).

For both Xs and CE enzymes, Xs-3.5, Xs-H2O, CE3.15 and CE-H_2O, a longer lag time was observed at concentrations up to 2% compared to Xs-Blank and CE-Blank (Figure 6.2). The results were analysed using the Tukey method at 95% confidence. The results for μ_{Max} and Max for the following concentrations were significantly higher than other concentrations: Xs-H2O at concentrations 0.1, 1, 2 and 3%, CE-3.15 at 1% and 2% ($p < 0.05$). The highest Max was found at concentrations ≤ 0.5% for Xs-3.5, Xs-H_2O, CE-3.15 and CE-H_2O (Appendix 2.4.1).

6.2.2 *Lactobacillus delbrukii* subsp. *bulgaricus* (LB)

LB was isolated from YO-MIX®726 on MRS agar and subsequently cultured in MRS broth supplemented with different concentrations of BSG-Oligos powder obtained from Xs or CE hydrolyzed BSG.

When monitoring LB growth, the 0.5% Xs-3.5 samples had the highest Max which then decreased dramatically as the Xs-3.5 BSG-Oligos concentration increased (Figure 6.3a). The maximum growth rate (μ_{Max}) was observed at 1 – 3% Xs-3.5. In contrast, LBs Max increased significantly for Xs-H_2O from 0.01% up to 5%. The growth of LB declined after 1% Xs-3.5 to much lower than the Xs-blank and LB control samples (Figure 6.3a). BSG-Oligos of Xs-H_2O resulted in higher growth rates and maximum growth. These findings were comparable to the findings 2% (w/v) galacto-oligosaccharides or fructo-oligosaccharides to support the growth of *L. bulgaricus* strains reported by Ignatova et al. (2009).

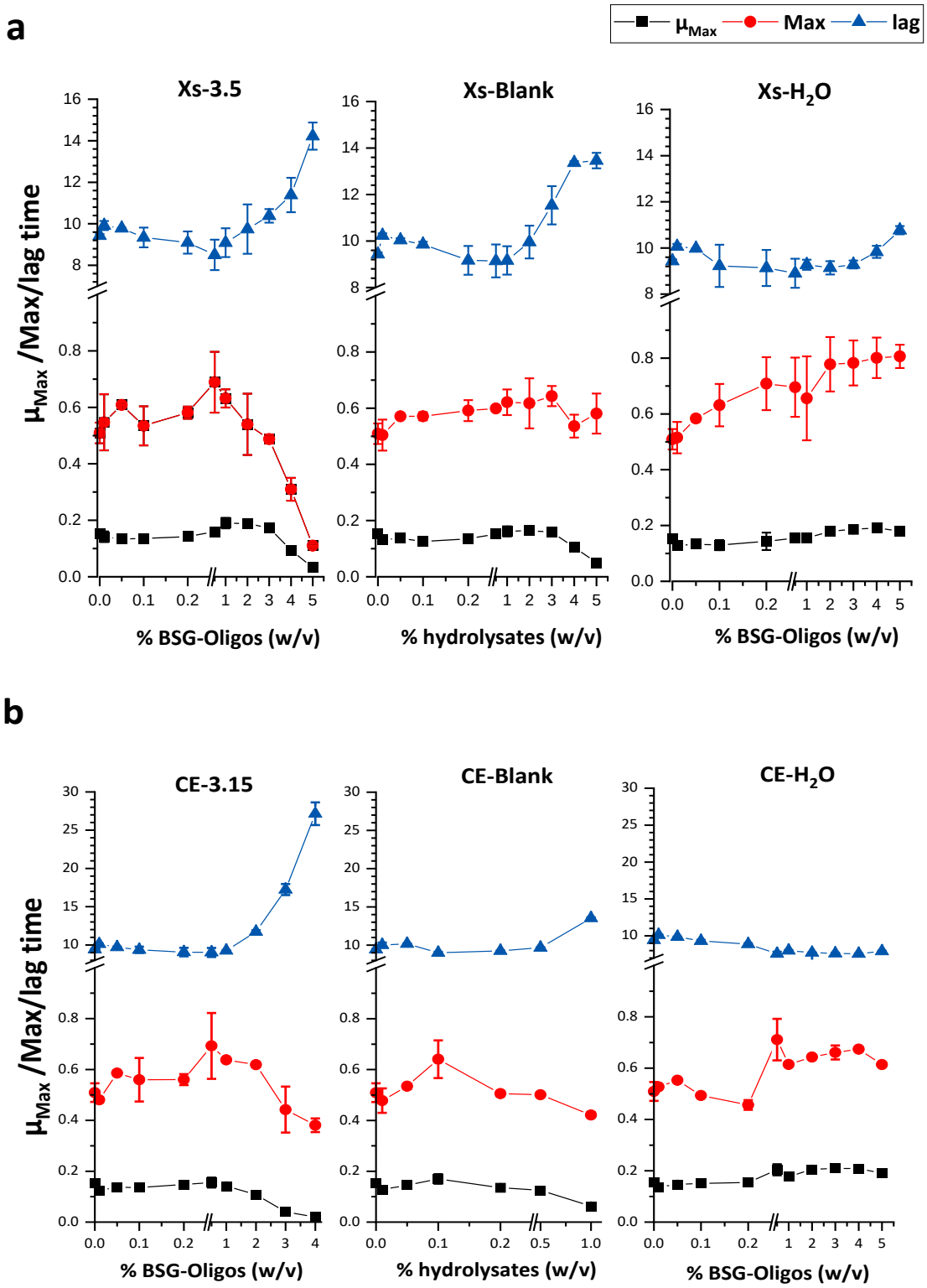


Figure 6.3 Figure18. Maximum growth rate (μ_{Max}, h^{-1}), maximum cell density (Max, OD_{595nm}) and lag time (h) of YO-MIX® 726 *Lactobacillus delbrukii* subsp. *bulgaricus* (LB) in MRS broth with additional BSG-oligosaccharides or hydrolysates of enzyme carrier retrieved from (a) Xylanase hydrolysis of BSG in pH 3.5 citrate phosphate buffer solution, xylanase blank (Xs-Blank) and in RO water (Xs-H_2O); (b) CE35 cellulase hydrolysis of BSG in pH 3.15 citrate phosphate buffer solution(CE-3.15), CE35 blank (CE-Blank) and in RO water (CE-H_2O) for 48 h at 37°C. Mean values ± standard deviation for n=2.

At 3% CE-3.15 addition LB growth dramatically reduced and continued to decline with increasing BSG-Oligos up to 5% (Figure 6.3b). LB exhibited high Max values and μ_{Max} for CE-H_2O especially from 0.5 to 5% (Figure 6.3b). The exclusion of any buffer salts appears to support LB growth. LB growth in CE-Blank increased up to 0.1% then decreased at higher concentrations up to 1% and no growth appeared after 1%. The dextrin content in the CE cellulase powder carried over into the BSG-Oligos may be the cause of the reduced growth by LB. It has been reported that *L. bulgaricus* strains are unable to utilize dextrin (Dewi & Purnamayati, 2021; Li et al., 2012; Wheater, 1955). Undigested dextrin could increase the osmosis pressure in the liquid layer and thus reducing the viability of LB (Nguyen et al., 2020; Tymczyszyn, Gómez-Zavaglia, & Disalvo, 2005).

The lag phases observed for 0.5-5% CE-H_2O were shorter compared to all other samples. LB strains are reported to be sodium intolerant and dextrin non-fermenter's compared to other yogurt cultures (Chun et al., 2012; Sherman & Hodge, 1940; Wheater, 1955). This could explain why LB exhibited significantly better growth with CE-H_2O than CE-3.15. Samples of CE-H_2O at 0.5, 2, 3, and 4% had the highest μ_{Max}, but Xs-H_2O at 2, 3, 4 and 5% had the highest maximum cell density (Max), significantly higher than the mean values of LB-control ($p<0.5$), details were listed in Table A2.25 (Appendix 2.4.1).

6.2.3 *Lactobacillus acidophilus* (LA)

LA was isolated from YO-MIX®726 on MRS agar and subsequently cultured in MRS broth supplemented with different concentrations of BSG-Oligos powder obtained from Xs or CE35 hydrolysis of BSG.

The μ_{Max} and Max for LA increased as the concentration of BSG-Oligos increased in Xs-H_2O, CE-H_2O and CE-3.15 samples with the most significant increase observed in Xs-H_2O (Figure 6.4). In contrast, on XS-3.5, Xs-Blank and CE-Blank there was no increase in μ_{Max} or Max with increasing concentration of BSG-Oligos for LA, whereas a reduction from 1% addition for Xs-3.5 and Xs-Blank, and CE-Blank growth rates did not decline. Xs-3.5 samples had longer lag times in comparison to Xs-H_2O samples, and Xs-H_2O samples had the shortest lag times over all samples (Figure 6.4).

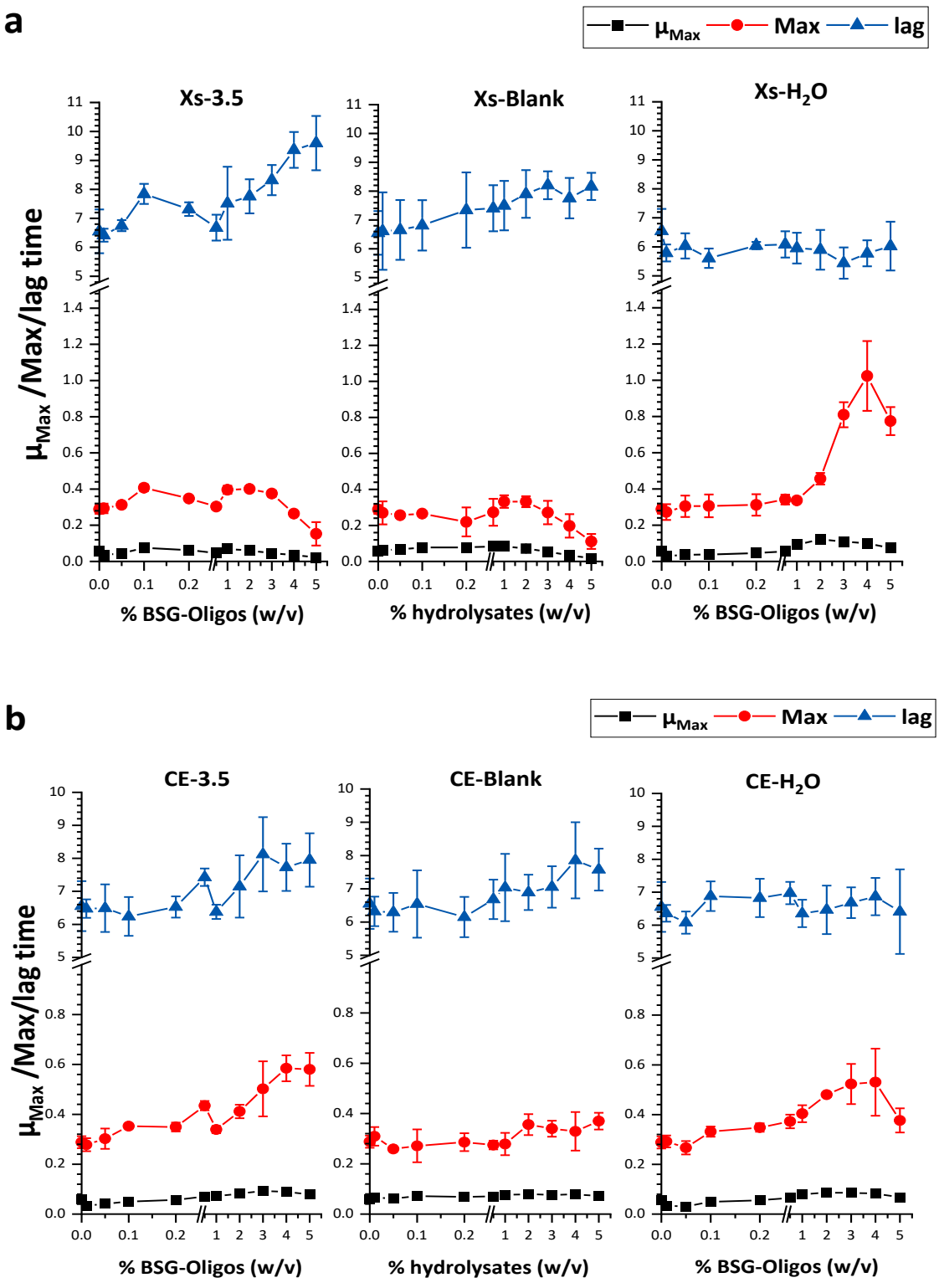


Figure 6.4 Maximum growth (μ_{Max}, h^{-1}), maximum cell density (Max, OD_{595nm}) and lag time (h) at OD_{595nm} of YO-MIX® 726 *Lactobacillus acidophilus* (LA) in MRS broth with additional BSG-oligosaccharides or hydrolysates of enzyme carrier retrieved from (a) Xylanase hydrolysis of BSG in pH 3.5 citrate phosphate buffer solution, xylanase blank (Xs-Blank) and in RO water (Xs-H_2O); (b) CE35 cellulase hydrolysis of BSG in pH 3.15 citrate phosphate buffer solution(CE-3.15), CE35 blank (CE-Blank) and in RO water (CE-H_2O) for 24 h at 37°C. Mean values ± standard deviation for n=4.

Unlike Xs-3.5 and Xs-Blank, both CE-3.15 and CE-H_2O samples increased in Max with concentration for LA (Figure 6.4b). CE-3.15 exhibited a slightly higher Max than CE-H2O at concentrations between 0.5% and 3% in Figure 6.4b. However, at these same concentrations, CE-H2O displayed higher Max values compared to CE-3.15. For instance, 2% CE-H2O produced a Max of 0.5 OD_{595nm}, while CE-3.15 reached 0.4 OD_{595nm}.

Xs-H2O samples have very high cell density readings for LA and the highest Max compared to any other LA samples, especially between 2 – 5% (Figure 6.4a). This indicated that removal of buffer salts at the higher concentrations of BSG-Oligos promotes LA growth.

6.3 Selection of the optimum concentrations of BSG-oligosaccharide

Table 6.1 provides a summary of values (μ_{Max}, Max and lag) from Figures 6.1 – 6.3 which showed significant differences to ST, LB or LA control. These values were selected after ANOVA analysis by Turkey's method (Appendix 2.4.1). Relatively lower concentrations (0.05 – 0.5%) of BSG-Oligos showed higher growth of ST compared to the control and Blank samples (Table 6.1).

Although, CE-H_2O showed beneficial effects on the Max for LB in Figure 6.2b and the lag phases for concentrations > 0.5% were shorter than those of the LB control, there was no significant difference in Max values between CE-H_2O samples and the LB control ($p > 0.05$), with similar results observed for μ_{Max} (Table 6.1). In contrast, Xs-H_2O (≥ 2%) had the greatest increase in Max for LB across all groups with no difference of μ_{Max} and lag compared to those of the LB control (Table 6.1).

Table 6.1 Samples from Figures 6.1 – 6.3 with significant difference to the control sample for μ_{Max}, Max and lag time for *S. thermophilus (ST)*, *L. bulgaricus (LB)* and *L. acidophilus (LA)*

		ST			LB			LA		
	%	μ_{Max} (h^{-1})	Max (OD_{595nm})	Lag time (h)	μ_{Max} (h^{-1})	Max (OD_{595nm})	Lag time (h)	μ_{Max} (h^{-1})	Max (OD_{595nm})	Lag time (h)
Xs-3.5	0.01	-	-	4.65	-	-	-	-	-	-
	0.05	0.46	1.33	4.54	-	-	-	-	-	-
	0.1	-	1.29	4.44	-	-	-	-	-	-
	0.2	-	1.30	4.47	-	-	-	-	-	-
	0.5	-	1.32	4.55	-	-	-	-	-	-
	1	-	-	4.73	-	-	-	-	-	-
	2	-	-	5.08	-	-	-	-	-	-
	3	-	-		-	-	-	-	-	10.04
	4	-	-	4.32	-	-	-	-	-	9.35
	5	-	-	4.62	-	-	14.21	-	-	-
Xs-H2O	0.01	-	-	4.89	-	-	-	-	-	-
	0.05	-	1.25	4.72	-	-	-	-	-	-
	0.1	0.47	1.31	4.63	-	-	-	-	-	-
	0.2	-	1.25	4.60	-	-	-	-	-	-
	0.5	-	1.25	4.69	-	-	-	-	-	-
	1	0.51	-	4.57	-	-	-	0.09	-	-
	2	0.49	-	4.82	-	0.78	-	0.12	0.46	-
	3	0.46	-	-	-	0.78	-	0.10	0.81	-
	4	-	-	-	-	0.80	-	0.10	1.02	-
	5	-	-	-	-	0.81	-	-	0.77	-
	0.01									
	0.05	0.50	-	-	-	-	-	-	-	-
	0.2	0.50	-	-	-	-	-	-	-	-
	0.5	0.48	-	-	-	-	-	0.09	-	-
Xs-Blank	1	-	-	-	-	-	-	0.09	-	-
	2	-	-	4.35	-	-	-	-	-	-
	3	-	-	4.38	-	-	11.52	-	-	-
	4	-	-	4.30	-	-	13.37	-	-	-
	5	-	-	4.76	-	-	13.46	-	-	-
CE-3.15	0.01	-	-	4.64	-	-	-	-	-	-
	0.05	-	-	4.55	-	-	-	-	-	-
	0.1	-	1.25	4.49	-	-	-	-	-	-
	0.2	-	1.23	4.49	-	-	-	-	-	-
	0.5	-	-	4.27	-	-	-	-	-	-
	1	0.47	-	4.43	-	-	-	-	-	-
	2	0.47	-	4.64	-	-	11.72	0.08	-	-
	3	-	-	-	-	-	-	0.09	0.50	-
	4	-	-	-	-	-	-	0.09	0.58	-
	5	-	-	-	-	-	-		0.58	-
CE-H2O	0.01	-	-	4.75	-	-	-	-	-	-
	0.05	-	1.30	4.67	-	-	-	-	-	-
	0.1	-	1.25	4.64	-	-	-	-	-	-
	0.2	-	-	4.59	-	-	-	-	-	-
	0.5	-	-	4.68	-	-	7.56	-	-	-
	1	-	-	4.65	-	-	-	-	-	-
	2	-	-	4.61	-	-	7.72	0.09	0.48	-
	3	-	-	-	-	-	7.63	0.09	0.52	-
	4	-	-	-	0.21	-	7.57	0.08	0.53	-
	5	-	-	-		-	-	-	-	-
CE-Blank	0.01	-	-	-	-	-	-	-	-	-
	0.05	-	-	-	-	-	-	-	-	-
	0.1	-	-	-	-	-	-	-	-	-
	0.5	-	-	-	-	-	-	-	-	-
	1	-	-	4.27	-	-	13.53	-	-	-
	2	0.49	-	4.26	-	-	-	-	-	-
	3	-	-		-	-	-	-	-	-
	4	-	-	4.29	-	-	-	-	-	-
	5	-	-	4.40	-	-	-	-	-	-
Control	0	0.33	1.02	3.77	0.15	0.51	9.43	0.06	0.29	6.52

Xs-H_2O had much higher Max for LA at concentrations of 3 – 5% across all groups, with a significant peak observed (1.02 OD_{595nm}) at 4% (Table 6.1). CE-H_2O of LA at 2 – 4% had similar μ_{Max}, but their Max values were only comparable to the Xs-H_2O at 2% and a statistical comparison was listed in Table A2.28 (Appendix 2.4.1). Although CE-3.15 had similar growth enhancement in the Max and μ_{Max} of LA at concentration of 3 – 5%, its negative effects on the growth of LB could not be overlooked and thus CE-3.15 was not included in future study.

BSG-Oligos derived from Xs-H_2O and CE-H_2O have shown better growth enhancement on LB and LA and no significant effects on the growth of ST compared to their controls, except for 5% Xs-H_2O, which appeared significant negative effects on the growth of ST (Figure 6.2a).

Therefore, Xs-H_2O and CE-H_2O at concentrations of 2 – 4% were selected for further analysis. Comparisons of these BSG-Oligos at 2 – 4% with ST, LB and LA cultures are presented in Table 6.2, contributing to the selection of optimum concentration of BSG-Oligos.

Xs-H_2O at 2 – 3% had greater ST growth compared to the others, the μ_{Max} was significantly different (Table 6.2). Although Max for ST growth with 2% Xs-H_2O was found to be 0.1 - 0.2 OD_{595nm} different from other samples, there was no difference across all ST samples in Table 6.2. In addition, Xs-H_2O at 3 – 4% had similar lag times compared to ST control, whereas 2% Xs-H_2O had one hour longer lag time than the control.

LB growth was promoted between 2 – 4% Xs-H_2O (Table 6.2). Xs-H_2O samples at 2 – 4% showed significantly higher maximum growth (Max) than the control and had comparable growth rates to CE-H_2O samples. Although CE-H_2O samples had shorter lag times for the concentration range of 2 – 4% compared to the others, their Max readings were about 0.10 – 0.16 OD_{595nm} lower than the same concentrations for the Xs-H_2O samples in Table 6.2.

Table 6.2 Comparation of growth parameters (μMax, Max and lag time) of three YO-MIX® 726 yogurt cultures at 2-4% BSG-Oligosaccharide levels

YO-MIX® 726 yogurt bacteria	BSG-Oligos-%	μ_{Max} (h^{-1})	Max (OD_{595nm})	Lag time (h)
Streptococcus thermophilus (ST)	Xs-H2O-2	0.49^{a}	1.22^{a}	4.82^{b}
	Xs-H2O-3	0.46^{a}	1.13^{a}	3.63^{a}
	Xs-H2O-4	0.45^{b}	1.14^{a}	3.67^{a}
	CE-H2O-2	0.44^{b}	1.12^{a}	4.61^{b}
	CE-H2O-3	0.40^{b}	1.18^{a}	3.75^{a}
	CE-H2O-4	0.41^{b}	1.18^{a}	3.87^{a}
	Control	0.33^{b}	1.02^{a}	3.77^{a}
Lactobacillus bulgaricus (LB)	Xs-H2O-2	0.18^{ab}	0.78^{a}	9.14^{b}
	Xs-H2O-3	0.19^{ab}	0.78^{a}	9.28^{b}
	Xs-H2O-4	0.19^{ab}	0.80^{a}	9.83^{b}
	CE-H2O-2	0.21^{a}	0.67^{ab}	7.72^{a}
	CE-H2O-3	0.21^{a}	0.66^{ab}	7.63^{a}
	CE-H2O-4	0.21^{a}	0.64^{ab}	7.57^{a}
	Control	0.15^{b}	0.51^{b}	9.43^{b}
Lactobacillus acidophilus (LA)	Xs-H2O-2	0.12^{a}	0.46^{c}	5.87^{a}
	Xs-H2O-3	0.10^{ab}	0.81^{b}	5.42^{a}
	Xs-H2O-4	0.10^{b}	1.02^{a}	5.77^{a}
	CE-H2O-2	0.09^{b}	0.48^{c}	6.43^{a}
	CE-H2O-3	0.09^{b}	0.52^{c}	6.68^{a}
	CE-H2O-4	0.08^{b}	0.43^{c}	6.85^{a}
	Control	0.06^{c}	0.29^{d}	6.52^{a}

Different letter comparisons within each group column indicate a significant difference from each other ($p < 0.05$).

Xs-H_2O samples showed pronounced growth of LA, in which Xs-H_2O had significantly higher μ_{Max} at 2% and Max at 3 – 4% across all samples ($p < 0.05$). In addition, Xs-H_2O at 4% supported LA achieving the highest Max at 1.02 OD_{595nm} followed by 3% at 0.81 OD_{595nm}. At these concentrations μ_{Max} was comparable in all other samples and different to the LA control.

Xs-H_2O at concentrations of 3 – 4% resulted in enhanced growth of LA and LB. Additionally, ST also had significantly higher ($p < 0.05$) maximum growth rates (μ_{Max}) and comparable maximum cell growth density (Max) and lag times across all samples (Table 6.2). The Xs-H_2O BSG-Oligos concentrations of 3 – 4% were therefore selected for further study.

6.4 Growth of YO-MIX® 726 cultures with BSG-Oligosaccharides versus xylanase blank in water

To determine if the BSG-Oligos contributed to culture growth a blank Xs using water instead of citrate phosphate buffer was used to compare the growth of YO-MIX® 726 cultures. This would also determine if the carriers on the enzyme contributed to culture growth which has been found by others (Slizewska, et al., 2012). Treatment information of YO-MIX® 726 yogurt cultures, ST, LB and LA, supplemented with BSG-Oligosaccharides from Xs hydrolysis and hydrolysates of Xs carriers is shown in Figure 6.5.

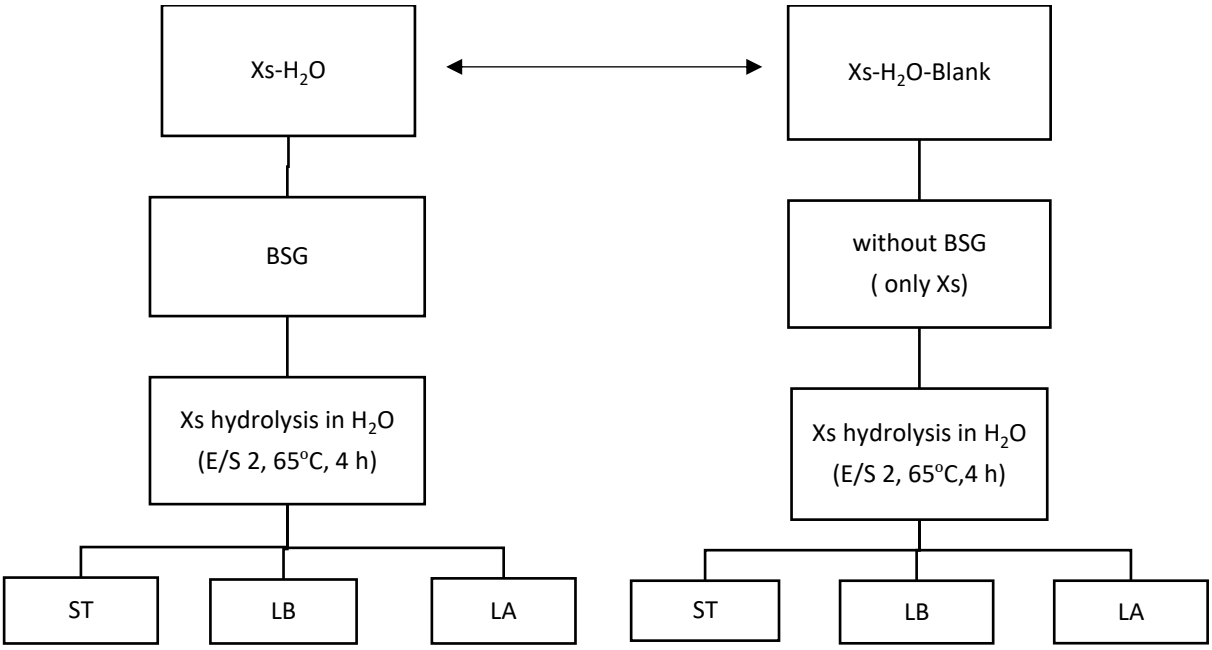


Figure 6.5 Treatment diagram of YO-MIX® 726 yogurt cultures supplemented with BSG-Oligosaccharides from SQZyme-xylanase hydrolyzed BSG in water (Xs-H_2O) and hydrolysates from the xylananse blank in water (Xs-H_2O-Blank).

The results for ST, LB and LA for Xs-H_2O and Xs-H_2O-Blank are shown in Figure 6.6. For ST, the presence of BSG-Oligos showed no improvement in ST growth cross all concentrations except 5% Xs-H_2O (Figure 6.6a). In contrast, BSG-Oligos enhanced the growth of LB with approximately 0.20 Max and 0.05 μ_{Max} absorbance differences observed between the peaks of the blank and Xs-H_2O of LB samples (Figure 6.6b).

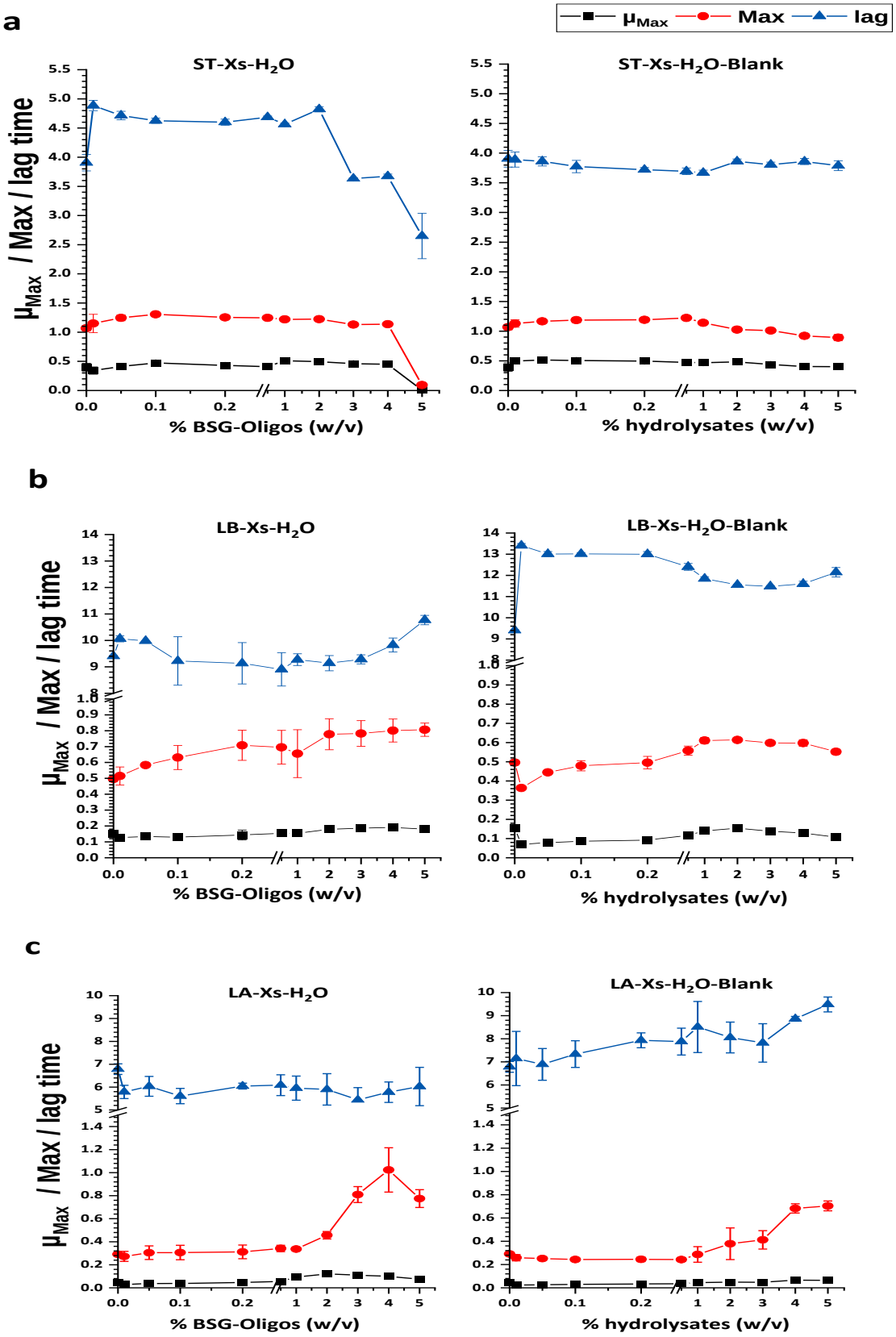


Figure 6.6 Comparation of growth parameters (μ_{Max} (h^{-1}), Max (OD_{595nm}), and lag time (h)) of YO-MIX® 726 cultures (a) *S. thermophilus*, (b) *L. bulgaricus*, (c) *L. acidophilus*—with Xs-BSG-Oligosaccharides and xylanase blank in water. Mean values ± standard deviation for n=2 or 4.

BSG-Oligos also resulted in better growth enhancement of LA (Figure 6.6c). There were approximately 0.40 and 0.30 absorbance differences between Xs-blank-H_2O and Xs-H_2O at the same concentration of 3 and 4%, respectively (Figure 6.6c). The Xs-blank-H_2O lag time for LB or LA was much longer than that of the controls and treatments with BSG-Oligos (Figures Table 6.6b and 6.6c)

The treatments with 3% and 4% Xs-H2O showed better growth profiles of both LB and LA with relatively high growth rates, higher maximum cell density and relatively shorter lag times compared to the controls and other samples. Therefore, 3% and 4% Xs-H_2O BSG-Oligos were finally selected for further testing.

The BSG-Oligos were only tested on YO-MIX® 726 yogurt cultures. It was unknown whether these BSG-Oligos could have any capability to enhance enteric bacteria numbers. The prebiotic score offers background information on the effect of oligosaccharides on enteric bacteria and assesses whether the BSG-Oligos can selectively stimulate and promote the growth of selected YO-MIX® 726 *Lactobacillus acidophilus*.

6.5 Prebiotic activity assay of 3% and 4% (w/v) BSG-Oligosaccharides

The prebiotic score was introduced first by Gibson and Roberfroid (1995) to check the selective stimulation from probiotic bacteria rather than any other intestinal microflora, especially pathogenic bacteria and then simplified by Huebner et al. (2007). Single strains of *E. coli* were selected as the enteric bacterium to calculate the prebiotic activity score between candidate prebiotics and probiotics (Huebner et al., 2007; Ismail, et al., 2023; Zhang, et al., 2018).

The prebiotic activity assay was carried out between *E. coli* NCTC 8196 and YO-MIX® 726 *L. acidophilus* in M9 broth and MRS-base broth respectively fortified with either Xs-H2O-BSG-Oligos or glucose at 3% and 4%. In this study, prebiotic activity was monitored at 12, 24 and 48 h. Cell counts at these times were subtracted from 0 h and the prebiotic activity score was determined using Equation 3.11. The microbial counts at 0 h and the difference

for 12, 24 and 48 h from 0 h are presented in Table 6.3. Prebiotic scores for LA each time with BSG-Oligos 3% and 4% are shown in Figure 6.7.

Table 6.3 The effect of BSG-Oligos on the growth of YO-MIX® 726 *Lactobacillus acidophilus* (LA) and enteric *E. coli* NCTC 8196 at different incubation times (hour).

	Log cfu /ml			
Time	**0h**	**12h**	**24h**	**48h**
LA-BSG-Oligo-3	3.25 ± 0.37^{A}	2.48 ±0.19 ^{A}	5.0±0.21^{A}	5.12±0.08 ^{A}
LA-BSG-Oligo-4	3.32 ±0.37 ^{A}	2.52 ±0.43 ^{A}	4.69 ±0.65 ^{A}	4.63±0.33^{AB}
LA-Glu-3	3.26 ±0.43 ^{A}	1.94 ±0.18 ^{A}	4.39±0.66 ^{A}	3.77±0.35^{B}
LA-Glu-4	3.19 ±0.40 ^{A}	2.58 ±0.06 ^{A}	4.42±0.68 ^{A}	3.98±0.04^{B}
EC-BSG-Oligo-3	2.90±0.62^{a}	4.05±0.30^{bc}	6.19±0.61^{a}	6.19± 0.68^{a}
EC-BSG-Oligo-4	3.05±0.55^{a}	3.55±0.08^{c}	5.69±0.15 ^{a}	5.60± 0.59 ^{a}
EC-Glu-3	2.97±0.54^{a}	5.41±0.25^{a}	5.59±0.03 ^{a}	5.71± 0.53 ^{a}
EC-Glu-4	2.97±0.58^{a}	5.27±0.46^{ab}	5.90±0.19 ^{a}	5.56± 0.62 ^{a}

The results at 12, 24, and 48 hours are presented as changes in bacterial counts over time, calculated by subtracting the initial count at 0 hours from the counts at each respective time point and expressed in log cfu/ml. Significant differences are indicated by superscript letters, where different letters express a statistically significant difference ($p < 0.05$). Capital letters represent differences in *Lactobacillus acidophilus* (LA), while lowercase letters indicate differences in *E. coli* NCTC 8196 within the same group.

E. coli growth was enhanced by glucose at 12 h, showing a one to two log increase compared to *E. coli* with added BSG-Oligos. BSG-Oligos appeared to slow down *E. coli* growth at 12 h. Meanwhile, BSG-Oligos helped to enhance the growth of *L. acidophilus*, especially at 3% concentration. At 12 h incubation, the highest prebiotic scores were found with 3% BSG-Oligos of 0.53 ± 0.01 compared to 4% BSG-Oligos (0.35 ± 0.15). The maximum score obtained was comparable to the functionality of the Raftilose P95 fructo-oligosaccharides (0.58) and slightly lower than purified galacto-oligosaccharides on the *L. acidophilus* NCFM (0.66) (Huebner et al., 2007; Olson & Aryana, 2012).

BSG-Oligos prebiotic scores were also higher than found by Ismail et al., (2023) in which they used xylo-oligosaccharides with a prebiotic activity score of 0.12. The score was also higher than the prebiotic score of the pectin-oligosaccharide 0.41 (Zhang et al., 2018). *E. coli* NCTC 8196 remained more viable with glucose than with YO-MIX® 726 *L. acidophilus* on the last day of incubation.

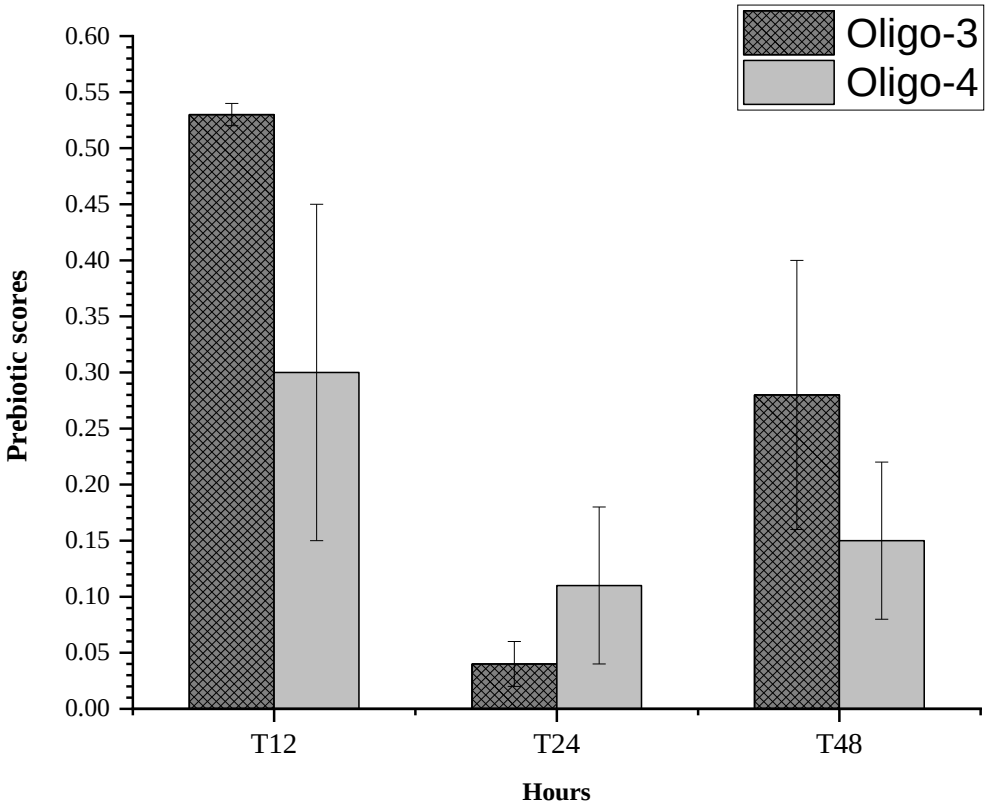


Figure 6.7 Prebiotic activity scores of YO-MIX® 726 *Lactobacillus acidophilus* on BSG-Oligosaccharides at concentration of 3% and 4% measured at time 12 h, 24 h and 48 h. Mean values ± standard deviation for n=2.

BSG-Oligos supported *L. acidophilus* cells to result in the highest viable cell number at 3% with a slight increase in viable cell numbers approximately 0.12 log at 48 h incubation compared to the prebiotic score at 24 h (Table 6.3). BSG-Oligos produced with 3% Xs-H2O were then added to yogurt production to further investigate their effects on the growth of YO-MIX® 726 yogurt cultures and yogurt quality. The analysis included yogurt profiles, pH, total titratable acidity (%TTA), lactic acid, serum separation, hardness and cell viabilities of the yogurt cultures after fermentation and after refrigerated storage.

6.6 Yogurt fortified with BSG-Oligosaccharides, BSG and erythritol – profile study

A fractional factorial design was used to analyze the effects of different ingredients in yogurt products. The set yogurt process flow chart is presented in Figure 3.2 (Chapter section 3.3). BSG at 0, 0.03 and 0.06% with BSG-Oligos at 0, 1.5 and 3% were included with 7.5, 11.25 and 15% SMP (w/v). Erythritol was increased with cane sugar(sucrose) decreasing to maintain total sweetener level at 8%. As YO-MIX® 726 *L. acidophilus* grew slowly in previous tests, fermentation time was introduced as a factor to determine its effect on yogurt profiles over 6, 27 and 48 hours of fermentation time. A fractional factorial design was used to investigate the effects of ingredients and fermentation time on yogurt quality (Table 6.4).

Table 6.4 Fractional factorial design for investigating the effects of ingredients and fermentation time on yogurt profiles

Run Number and Conditions SMP:BSG:BSG-Oligos:Sucrose:Erythritol:Time		**Independent variables (factors)**				
	SMP (%)	**BSG (%)**	**BSG-Oligos (%)**	**Sucrose (%)**	**Erythritol (%)**	**Time (h)**
1-7.5-0-0-8-0-48	7.5	0	0	8	0	48
2-7.5-0-3-8-0-6	7.5	0	3	8	0	6
3-7.5-0.06-0-0-8-48	7.5	0.06	0	0	8	48
4-7.5-0.06-3-0-8-6	7.5	0.06	3	0	8	6
5-11.25-0.03-1.5-4-4-27	11.25	0.03	1.5	4	4	27
6-11.25-0.03-1.5-4-4-27	11.25	0.03	1.5	4	4	27
7-15-0-0-0-8-6	15	0	0	0	8	6
8-15-0-3-0-8-48	15	0	3	0	8	48
9-15-0.06-0-4-4-6	15	0.06	0	4	4	6
10-15-0.06-3-4-4-48	15	0.06	3	4	4	48

After fermentation, samples were analyzed for pH, %TTA, lactic acid, firmness and bacterial cell counts of YO-MIX® 726 yogurt culture ST, LB and probiotics for Day 0. The percentage of serum separation based on serum heights was measured on Day 1 and Day 21. The yogurt culture viability was also analyzed on Day 21. After fermentation, yogurt samples were stored at 4 ± 1°C for yogurt profile analysis after 21 days. The factors to be monitored were SMP, BSG, BSG-Oligos, sugars (cane sugar and erythritol) and fermentation time. The results for each run were shown in Figure 6.8.

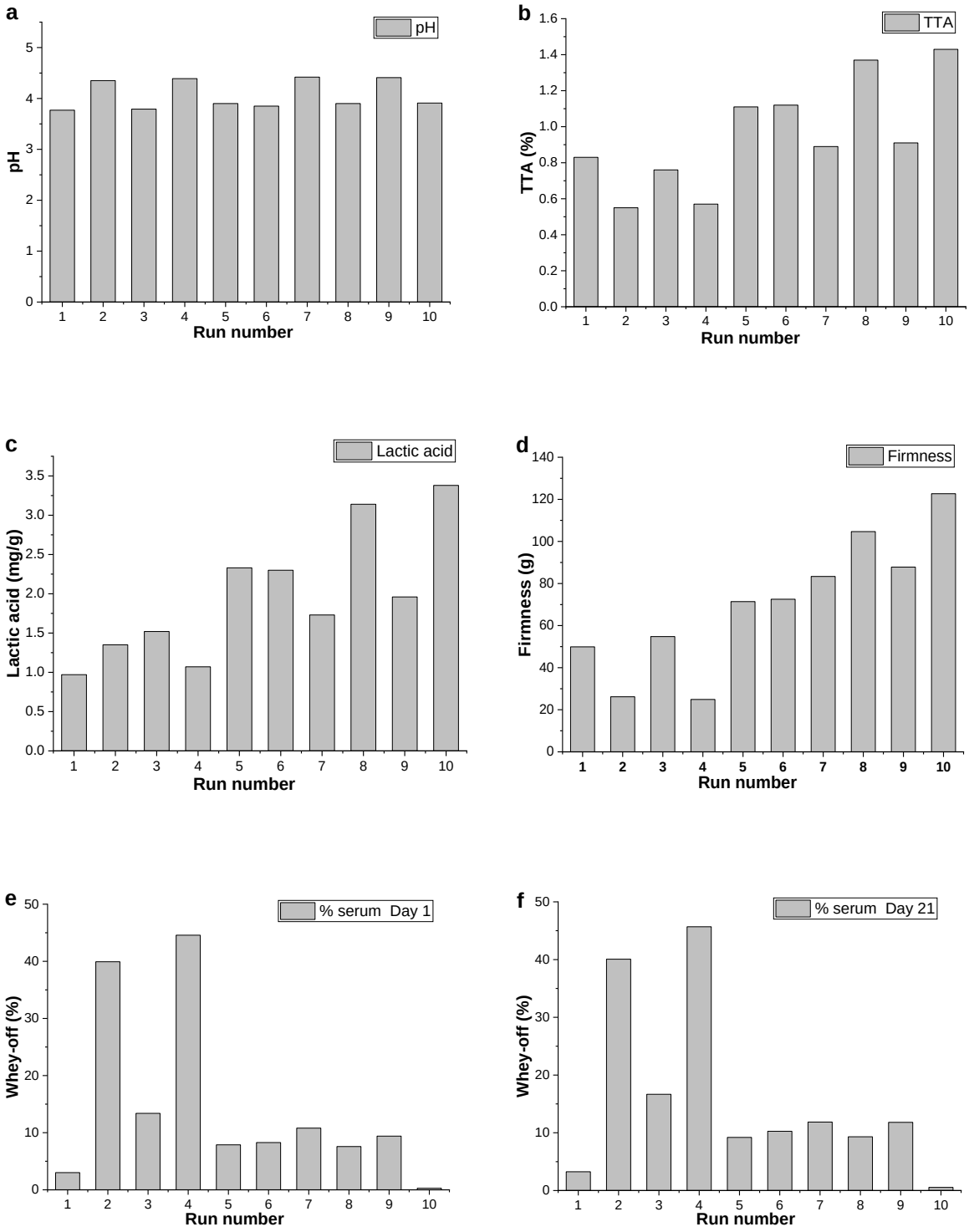


Figure 6.8 Histograms of results from hydrolysis experiments of yogurts fermented with different ingredients and fermentation times(a) pH (Day 0), (b) total titratable acidity (%TTA, Day 0), (c) lactic acid (Day 0), (d) firmness (Day 0), (e) %serum on Day 1 and (f) %serum on Day 21.

Fermentation time appears to be a major factor influencing the variability in pH observed. After 6 h fermentation, the pH of yogurt samples was above pH 4 but dropped to about pH 3.8 when the fermentation was extended to 27 and 48 h (Figure 6.8a). Total titratable acidity (%TTA) showed an increasing trend with increasing concentration of SMP concentration (Figure 6.8b). The lactic acid concentration in all yogurt samples also increased with increasing SMP concentration (Figure 6.8c). With stimulated growth with added BSG-Oligos, more lactic acid was produced, increasing the %TTA. Time appears to have an influence on lactic acid production particularly in Runs 5, 6, 8 and 10 (Table 6.4). Yogurt gel firmness also increased with SMP concentration (Figure 6.8d). Serum separation was not different between days 1 and 21. Both SMP and time appeared to be the two key factors in reducing serum separation in the yogurt gel (Figure 6.8e and 6.8f).

The effect of the different factors on ST, LB and LA growth after fermentation is shown in Figure 6.9. The longer fermentation time affected ST survival, as all 48 h fermented yogurt samples had ST cell counts around 6.5 – 7 logs and all other runs were above 8.0 logs (Figure 6.9a). After 21 days ST cell counts were not very different to day 0 except for run numbers 1 and 8 (Figure 6.9b). The ingredients did not appear to influence the growth of ST.

For LB, most of the runs on day 0 were between 5 – 6 logs cfu/g, except Runs one and seven (Figure 6.9c). The difference between Run one and seven was the SMP content (7.5% and 15%), cane sugar (8% and 0%), and fermentation time (48 h and 6 h), respectively. These three factors may have influenced the cell count of LB at Day 0. In Figure 6.9d, after 21 days, LB in Runs three and eight had significant reductions in viability, with Run eight showing a decline of 4 logs compared at Day 0. LB in Run seven also showed a 2-log reduction after 21 days. The common missing ingredient in these three runs was cane sugar, which was replaced by erythritol.

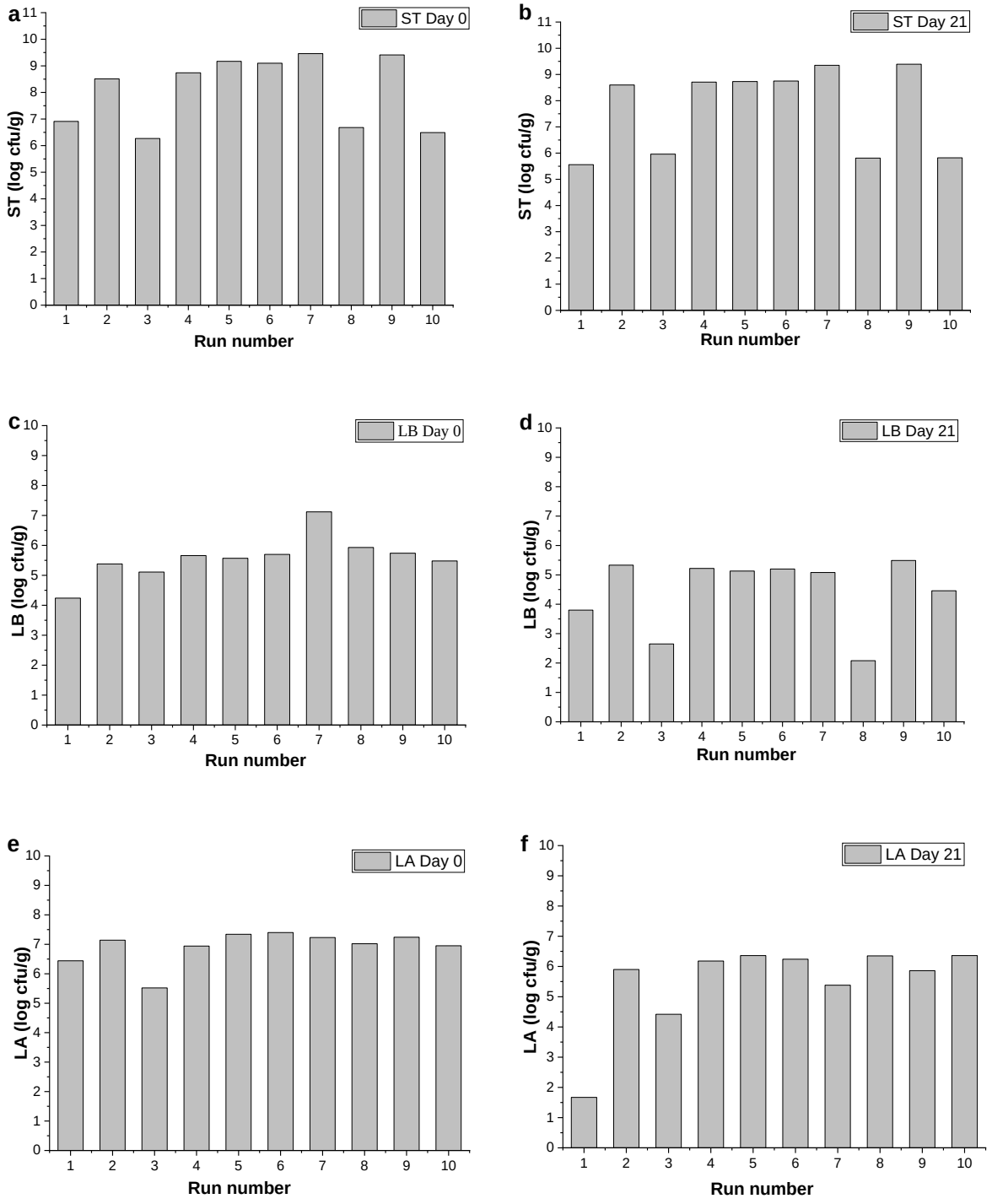


Figure 6.9 Histograms of YO-MIX® 726 yogurt culture $\log_{10}$ cell count in yogurt products for (a) *Streptococcus thermophilus* (ST) on Day 0, (b) ST on Day 21, (c) *Lactobacillus delbrueckii subsp. bulgaricus* (LB) on Day 0, (d) LB on Day 21, (e) *Lactobacillus acidophilus* (LA) on Day 0 and (f) LA on Day 21.

For LA, at Day 0 Runs one and three showed slightly lower probiotic counts (*L. acidophilus* and *B. lactic*) compared to the other samples, while other samples which were around 7 logs (Figure 6.9e). Run one sample exhibited a large reduction in LA after Day 21 storage and Run three remained at a similar cell count compared to Day 0. BSG-Oligos in Runs two, four, eight and ten supported LA during storage compared to Run one, three and seven samples without 3% BSG-Oligos, but there was also no cane sugar in Runs four and eight which was replaced by erythritol. Erythritol has shown minimal or no effect on the survival of the probiotics, particularly LA, aligning with the growth curve findings in Section 4.3.2 and yogurt fermentation in section 4.4.4. However, the finding contrasted with that reported by Yao et al. (2015), who found erythritol showed inhibition against LA at the concentration of 1 – 8% in formula milk.

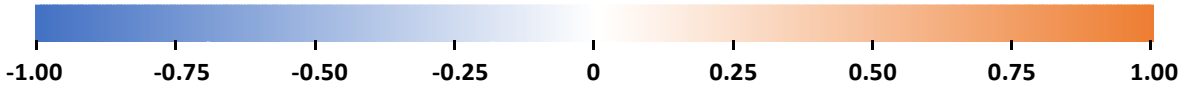
The results from the above experimental design were then analyzed using a quarter-fraction factorial method with analysis of variance (ANOVA) in Minitab 21 to further justify the findings above. A summary of the ANOVA results for the factorial design is provided in Table A2.30 (Appendix 2.4.2), and more detailed discussions based on the ANOVA results are presented below.

6.6.1 Effects of SMP on yogurt profiles

A Pearson correlation was carried out to determine the strength of the linear correlation between ingredients, response variables and fermentation condition. The outcome of this analysis is shown in Table 6.5. A correlation (r) of 0.3 – 0.5 (-0.3 – -0.5) is a low positive (negative) correlation, 0.5 – 0.7 (-0.5 – -0.7) is a moderate positive (negative), 0.7 to 0.9 (−0.7 to −0.9) is a high positive (negative) correlation, and any correlation above 0.9 or less than -0.9 are considered to be very high correlations (Hinkle et al., 2003). ANOVA was used to test the factor's significant effect on response variable.

Table 6.5 Pearson correlation matrix for evaluation of each two variables in yogurt samples, values in the table are correlation coefficients (r).

	SMP	BSG	Oligo	Sucrose	Erythritol	Time	pH	%TTA	Lactic acid	Firmness	%serum	serum-21d	ST	LB	LA	ST-21d	LB-21d	LA-21d
SKM	1																	
BSG	0	1																
Oligo	0	0	1															
Sucrose	0	0	0	1														
Erythritol	0	0	0	-1	1													
Time	0	0	0	0	0	1												
pH	0.45	0.1	0.14	-0.06	0.06	-0.8	1											
%TTA	0.78	0.08	0.16	0.08	-0.08	0.47	-0.12	1										
Lactic acid	0.78	0.16	0.31	0	0	0.31	0.06	0.9	1									
firmness	0.93	0.08	0	0.08	-0.08	0.31	0.16	0.88	0.86	1								
serum	-0.47	0.16	0	-0.39	0.39	-0.7	0.43	-0.79	-0.56	-0.64	1							
%serum-21d	-0.43	0.12	0.04	-0.43	0.43	-0.66	0.43	-0.77	-0.55	-0.59	0.99	1						
ST	0.31	-0.16	-0.31	0	0	-0.78	0.61	-0.04	-0.04	0.01	0.25	0.19	1					
LB	0.74	-0.12	0.04	-0.43	0.43	-0.43	0.63	0.39	0.43	0.52	0.08	0.13	0.61	1				
LA	0.39	-0.16	0	0.08	-0.08	-0.55	0.35	0.29	0.36	0.2	0.06	0.01	0.79	0.6	1			
ST-21d	0.31	0.23	-0.31	-0.08	0.08	-0.78	0.63	-0.04	0.08	0.08	0.44	0.38	0.86	0.59	0.76	1		
LB-21d	-0.08	0.23	0.08	0.39	-0.39	-0.86	0.58	-0.33	-0.21	-0.29	0.5	0.44	0.64	0.16	0.58	0.71	1	
LA-21d	0.39	0.16	0.7	0	0	0.08	0.09	0.62	0.77	0.38	-0.32	-0.35	0.02	0.27	0.43	0.04	0.07	1



SMP was the most effective ingredient in this yogurt profile study. As SMP concentration increased, there was a significant increase in the firmness, total titratable acidity, lactic acid production and decreased serum separation ($p < 0.05$). SMP was the primary protein resource, and this influenced the acidity profiles of these samples although pH changes were more strongly associated with fermentation time ($p < 0.05$) than SMP concentration ($p > 0.05$). Lactic acid, %TTA and firmness of yogurt samples increased with higher protein content and conversely, syneresis decreased (Donato et al., 2007; Pakseresht et al., 2017).

Serum separation was correlated to the reduction of SMP ($r < -0.47$) according to the Pearson correlation matrix (Table 6.5). SMP and BSG may be interacting to form stable structures that stabilize the yogurt gel matrix (Ghasempour et al., 2020; Razmkhah et al, 2010). These structures effectively trapped more water, thereby reducing the amount of whey separation. However, in this study, individually SMP positively impacted on the yogurt profiles rather than working together with other ingredients. This contrasted with Hosseini-Parvar et al. (2015), and Razavi et al. (2010) who found strong interaction between SMP and other ingredients. SMP is normally added to reduce serum separation (whey-off) and improve the organoleptic properties of yogurt (Pakseresht et al., 2017). It is worth noting that fat content has also contributed to yogurt gel firmness and serum release, but in this study, SMP was the main protein source with a very low level of fat content and thus BSG gum could be functioning as a partial fat-substitute on stabilizing the protein water matrix (Lucey et al., 1998).

SMP showed a positive correlation with all yogurt cultures, especially LB after fermentation ($r = 0.74$) according to the Pearson correlation matrix (Table 6.5). However, SMP did not show a protective effect during refrigerated storage for LB ($p > 0.05$). The correlation between SMP and LB count at Day 21 was only -0.08 (Table 6.5). In contrast, increased concentration of SMP was positively linked to the survival of ST ($r = 0.31$) and LA ($r = 0.39$) at Day 21.

6.6.2 BSG gum and yogurt profiles

BSG had a significant effect on lactic acid production ($p = 0.039$), but did not have a significant effect on the %TTA and pH of yogurt samples ($p > 0.05$). Tan (2019) found

that %TTA increased significantly from 0.90% to 1.02% ($p < 0.05$) with the addition of BSG at concentrations of 0.1 – 0.3%. In this study only up to 0.06% BSG was used. BSG gum present resulted in more lactic acid 0.18 mg/g especially at longer fermentation times, > 6h in Figure A2.1 (Appendix 2.4.2).

Even though used at lower concentrations 0.03% – 0.06%, BSG gum had a positive impact on gel firmness ($p < 0.05$). Other polysaccharide gums need much higher concentrations to influence in yogurt gel firmness, at least 0.5% or higher concentrations, for example, gum Arabic and xanthan gum, guar gum at 1%, gelatin and modified starch at 1.5%, and 0.5% inulin (Jackson et al., 2023; Nguyen et al., 2017; Rafiq et al., 2020; Srisuvor et al., 2013). BSG addition resulted in a firmness of 0.72 N at 0.06% in this study, similar to 0.73 N with 0.08% carrageenan (Nguyen et al., 2017).

BSG has functionality similar to other gums, by increasing the viscosity of the solution and thus maintaining the stability of food matrices during processing or storage (Hosseini-Parvar et al., 2010; Razavi et al., 2012; Razavi & Naji-Tabasi, 2017). BSG can work with milk protein to improve yogurt structure by the filling pores of the gel matrix and smoothing the matrix structure, thereby holding more water in the yogurt protein matrix (Kim et al., 2020; Rafe et al., 2013). Firmness was inversely correlated to serum separation (% whey-off) ($r = -0.64$) for Day 1 or -0.59 for Day 21 (Table 6.5). Although BSG did not have a statistically significant effect on serum separation, if BSG-Oligos concentrations were increased this could result in a firmer yogurt gel and thus serum would be retained in the gel matrix. Figure 6.10 shows the interaction plots for BSG and BSG-Oligos.

The interaction between BSG and BSG-Oligos had a positive impact on reducing the amount of whey-off with serum% reduced from 20.61% (4.54^2) to 12.96% (3.60^2) for Day 0 (Figure 6.10a). The centre combination of 1.5% BSG-Oligos and 0.03% BSG gave the lowest serum 8.06% (2.84^2) (Figure 6.10a). Serum release (%) was about 7.93% lower in the yogurt sample with 3% BSG-Oligos and 0.06% BSG (3.75^2) compared to the sample without BSG (4.69^2) on Day 21 (Figure 3.10b). The serum release (%) in the sample with 1.5% BSG-Oligos and 0.03% BSG (the centre point) was about 9.73% (3.12^2), the lowest serum separation observed for Day 21 (Figure 6.10b). In an earlier experiment, 0.3% or higher concentrations of BSG

reduced %serum, but the firmness of the gel decreased from this point onwards (Figure 4.6). Excessive inclusion of gums can soften yogurt gel and influence serum release by increasing the solution viscosity rather than bridging with proteins (Bharati et al., 2022; Hong, et al., 2024; Ghasempour et al., 2020; Kim et al., 2020).

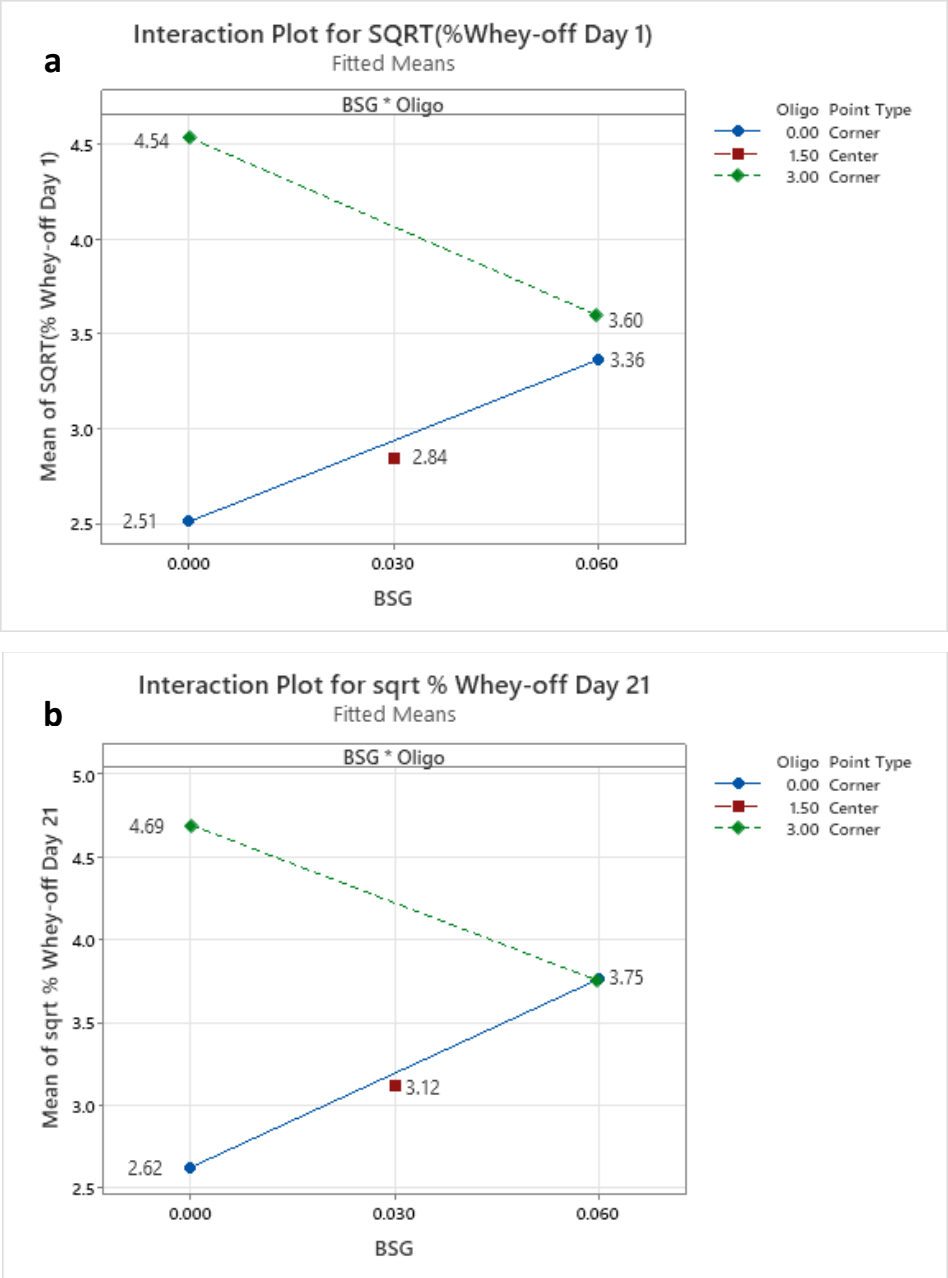


Figure 6.10 Interaction plot of % serum (%whey-off) between BSG and BSG-Oligos at (a) Day 1 and (b) Day 21.

In previous experiments, only 0.01 to 0.05% BSG concentration showed positive enhancement on the growth of all yogurt cultures. In a mixed environment, BSG did not enhance the growth of ST during storage but maintained its growth ($p < 0.05$). Others have reported that polysaccharide gums do not have any effect on the growth of yogurt cultures (Park et al., 2019; Ghasempour et al., 2012). Inulin is reported to be protective for yogurt cultures (Aryana & McGrew, 2007; Donkor et al., 2007). BSG gum exhibited a growth enhancing function similar to inulin, which is recognized as a full fibre-type prebiotic, and both fibres did not show significant interaction with yogurt cultures within the yogurt gel matrix (Jackson et al., 2023; Liu, 2013). BSG could interact with BSG-Oligos to support ST growth ($p < 0.05$). In addition, the viability of probiotics and LB was positively related to the presence of BSG gum, even though this correlation was not significant there was some protection to LB from BSG (Table 6.5).

6.6.3 BSG-oligosaccharides and yogurt profiles

The addition of BSG-Oligos had a significant impact ($p < 0.05$) on yogurt fermentation, as its concentration increased so did the yogurt %TTA and lactic acid concentration, increasing by 0.13% and 0.68 mg/g, respectively (Figure A2.1, Appendix 2.4.2). There was a negative correlation between %TTA and %serum, and lactic acid versus %serum ($r = -0.79$ and $r = -0.56$, respectively), and a positive correlation between % serum and pH in which serum decreased with decreasing pH ($r = 0.43$). Lactic acid and %TTA are directly linked to the metabolism of the yogurt cultures.

BSG-Oligos are shorter chain sugars. Their addition resulted in increasing in serum separation for Day 1 and 21 ($p < 0.05$). This result was comparable to short chain oligosaccharides found to release more whey water (Brennan & Tudorica, 2008; Jackson et al., 2023; Khalid et al., 2022; Pimentel et al., 2012). However, BSG-Oligos had minimal influence on the gel structure and on serum separation in samples with added BSG gum (Figure 6.10). The interaction between BSG and BSG-Oligos was observed consistently on both Day 0 and Day 21 of storage.

BSG-Oligos was not associated with growth and survival of LB ($p > 0.05$) and had negative effects on ST ($p < 0.05$) compared to SMP. BSG-Oligos had a slightly reduced survival of ST

by 0.7 log from 8.78 to 8.08 log between 0% – 3% BSG-Oligos on Day 21, but the cell number remained above 10^8 cfu/g. In addition, 1.5% BSG-Oligos had no effect on the survival of ST, 8.74 log compared to 8.78 log of 0% BSG-Oligos on Day 21. It was comparable with what was found for FOS, GOS and lactulose on *S. thermophilus* (Delgado-Fernández, et al., 2019). Lactulose at 4% was the only prebiotic which reduced the viability of *S. thermophilus* and FOS, GOS and 2% lactulose had no impact on either *L. bulgaricus* or *S. thermophilus* (Delgado-Fernández, et al., 2019).

The factorial design based on results obtained was used to predict the growth of probiotic cultures with the addition of BSG-Oligos, growth was predicted for Day 0 and Day 21 (after storage) (Figure 6.11). Short-chain BSG oligosaccharides stimulated metabolite excretion from yogurt cultures but also the growth of probiotics was reported for other prebiotics (Pranckutė, et al., 2016). BSG-Oligos has shown positive effects on the growth of probiotics ($p < 0.05$) and strongly supported their survival after 21 days storage at 4°C ($p < 0.05$, $r = 0.70$) (Figure 6.11). These findings align with the results of the prebiotic score assay tested above and provide additional evidence that enzyme hydrolyzed gums have protective prebiotic function on probiotics (Khalid et al., 2022).

BSG-Oligos supported the survival of yogurt cultures in an acidic environment. It also stimulated their metabolism, leading to the production of acids, which significantly impacted firmness, %TTA, and lactic acid in this factorial experiment. In contrast to the finding that XOS and other prebiotic oligosaccharides positively impacted yogurt gel firmness (Li et al., 2023), BSG-Oligos did not have a significant effect on yogurt gel firmness and it was similar to inulin, pectin, GOS and β-glucan (Ng et al., 2018; Jackson et al., 2023).

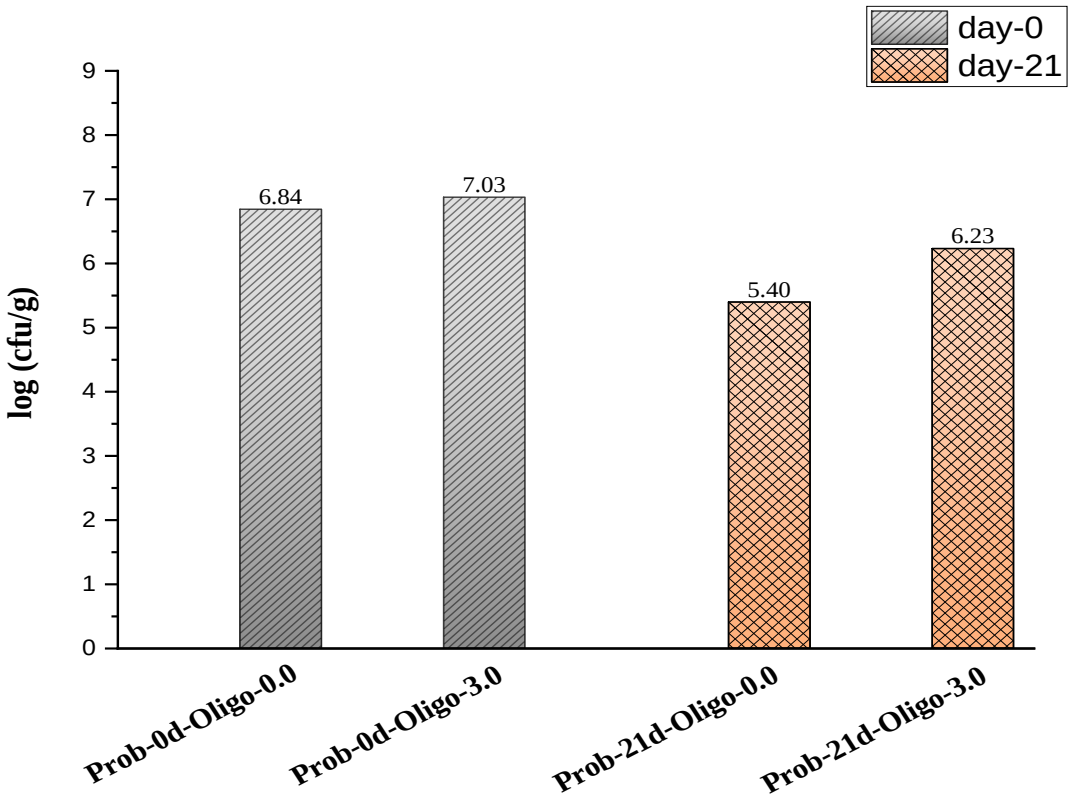


Figure 6.11 Predicted Cell counts of BSG-Oligos on YO-MIX® 726 probiotics at 0% and 3% on Day 0 and Day 21 using a quarter factorial design.

6.6.4 Sugars and yogurt profiles

Sugar is another important ingredient in yogurt making. It is used to mask the unpleasant sour taste as well as supplying glucose as food to yogurt cultures (Miele et al., 2017; Vedamuthu, 2013). The cane sugar concentration at 8% or lower did not have an impact on the pH and lactic acid concentration but slightly influenced %TTA results ($p < 0.05$). This founding was different to that observed by Linke and Birmeier (2000) who found cane sugar increased lactic acid and %TTA and decreased pH at ≤ 15% sucrose addition.

Sugars had a significant impact on serum separation ($p < 0.05$). Cane sugar, as a hydrophilic solute, can have an impact on the water structure and influences casein-casein interactions in an acidic environment. The casein micelle network is highly linked to the syneresis of yogurt products (De Kruif, 1999; Gomes et al., 2022). Cane sugar stabilizes casein micelles, enhances casein-casein associations in water, and increases the consistency of the liquid layer. Additionally, cane sugar functions like a glue, clustering casein micelles more tightly

and forming a stronger matrix to trap water effectively (Haque & Aryana, 2002; Sabadini et al., 2006). It was also found in this study that cane sugar was the major factor to reduce serum release and thus assisted in reducing syneresis (whey-off) in the yogurt products ($p < 0.05$). Cane sugar also has the capability to stabilize casein micelles through a preferential exclusion stabilization system, in which cane sugar and water can form cosolvents which surround and pack tightly around casein and stabilize both native and denatured states (Haque & Aryana, 2002; Kendrick et al., 1997; Timasheff & Arakawa, 1988). Cane sugar can protect proteins from denaturing and stabilize their structure (Hao et al., 2016; Nastaj et al., 2020).

It is generally believed that *S. thermophilus* can cleave cane sugar into its two sugar base units and thus able to feed itself and other yogurt cultures with glucose to support cell multiplication (Thomas et al., 2011; Vedamuthu, 2013). No direct link between cane sugar and ST ($p > 0.05$) was observed and LB reduced in cell numbers with increasing concentration of cane sugar ($p < 0.05$, $r = -0.43$). Researchers have claimed that most LB strains are unable to utilize cane sugar (Amoroso et al., 1989; Wheater, 1955) and commercial LB strains are also unable to utilize cane sugar for fermentation (Ashraf & Shah, 2011). Increasing cane sugar above 4% significantly impacted on the viability of LB in yogurt products ($p < 0.05$). However, there was a significant positive relationship between cane sugar content and LB survival at Day 21 ($r = 0.39$) possibly due to the protection of casein from unfolding and denature by cane sugar. Meanwhile, the positive effect of a 4% sugar level (4% cane sugar and 4% erythritol) on LB was compatible with that of 8% cane sugar, with counts of 5.16 log and 5.14 log, respectively (Figure A2.1, Appendix 2.4.2).

Cane sugar at 4% is also recommended by many researchers to maintain better yogurt flavor and assist with the stabilization of yogurt gel structure (Al-Fayez, 2000; Fernández-Garía et al., 1998; Haque & Aryana, 2002; Sobowale et al., 2011; Temesgen & Yetneberk, 2015). It has also supported the finding in this study that the yogurt samples with added 4% cane sugar content performed better across all the response variables.

In contrast, increasing the concentration of erythritol interfered with the interaction of casein micelles and water and thus increased the serum release. The stability of protein

influenced by of different sweeteners falls in this order with best to least beneficial, sucrose > maltitol > xylitol > erythritol (Hao et al., 2016; Nastaj et al., 2020). This order corresponds to their molecular composition. Erythritol, a four-carbon single sugar alcohol, had decreased the viscosity in the aqueous phase and could neither trap water nor interact with water to protect the inner core of the protein (Hao et al., 2016; Nastaj et al., 2020). Erythritol could not stop the protein from unfolding and denaturing (Hao et al., 2016; Kendrick et al, 2017; Nastaj et al., 2020). In addition, erythritol increased the total soluble solid content by 5^{o}Brix than sucrose alone. More caseins unfold and lose their spherical micelle structure due to the loss of the protective layer of cosolvent water and sucrose, thinning the aqueous phase viscosity, less water is attracted, and thus erythritol as the major factor that has influenced the increase in serum release at day 0 and day 21 ($p < 0.05$).

6.6.5 Fermentation time and yogurt profiles

All response variables were highly related to fermentation time, including pH, %TTA, lactic acid, % serum, firmness, and viability of ST, LB and LA ($p < 0.05$), except viability of LA at the Day 21 storage ($p > 0.05$). Longer fermentation time allowed the yogurt cultures to produce more lactic acid and thus positively increased the total titratable acidity and lowered the pH of the yogurt products ($r = -0.80$). Among the fermentation durations, the central point 27 h performed significantly better compared to the other two fermentation times and showed the highest results or comparable values to the highest results overall (Table 6.6). The findings indicated that metabolites required a longer fermentation time to reach the maximum level, also demonstrated by Akan (2022). Time also had a positive correlation with the yogurt gel firmness ($r = 0.31$) and highly impacted the gel serum separation ($r = -0.70$) as a result of acidification (Akan, 2022; Damin et al., 2006; Damin et al., 2008).

Table 6.6 Fitted mean values of each response variables at different fermentation times, estimated from the factorial design in Minitab, based on the original data in Table A 2.30 (Appendix 2.4.2).

Fermentation Time (h)	Response variables											
	pH	%TTA	Lactic acid (mg/g)	Firmness (g)	Serum (%)	Serum-21d (%)	ST-0d	ST-21d	LB-0d	LB-21d	LA-0d	LA-21d
6	4.39	0.73	1.53	55.55	23.43	24.9	9.03	9.08	5.97	5.31	7.16	5.91
27	3.88	1.12	2.31	71.97	8.07	9.73	9.13	8.74	5.63	5.16	7.37	6.30
48	3.84	1.09	2.25	83.00	4.71	5.86	6.59	5.80	5.21	3.95	6.75	6.05

Longer fermentation time had a negative impact on the survival of ST where it reduced by 2.5 log, between 6 h and 48 h on Day 0 (Figure 6.12a). The central point at 27 h-fermentation yielded the highest viable number of ST and LA, while LB showed slightly lower cell count, approximately 0.34 log less than that of 6 h. Increasing fermentation time to 48 h, all yogurt cultures have some degree of reduction compared to the other two fermentation times (Figure 6.12).

ST growth depended on fermentation time. For ST, the longer the fermentation time the less the cells were able to survive in the harsh acidic environment in yogurt (Figure 6.12a). When fermentation was extended for a longer duration, a greater reduction in cell numbers was observed, ranging from 0 to 0.8 log (Figure 6.12a). During storage, there was no change in the viability of ST between day 0 and day 21 for yogurt samples with the same fermentation time.

LB survival during storage was affected by the fermentation time, paralleling with ST (Figure 6.12b). Results for these two cultures was comparable at shorter fermentation times, ST and LB maintained similar levels during storage (Kosasih, 2011; Liu, 2013; Mani-López et al., 2014). However, this result was in contrast to the results of ST in yogurt sample fermented at 42^{o}C for 24 h, had a 1.8 to 3.5 log reduction and LB had 30 – 50% reduction of initial cell count at last day of storage as found by Mani-López et al. (2014). In addition, the 27 h fermented samples maintained similar levels for both yogurt starters as found between Day 0 and Day 21, with no more than 0.5-log reduction.

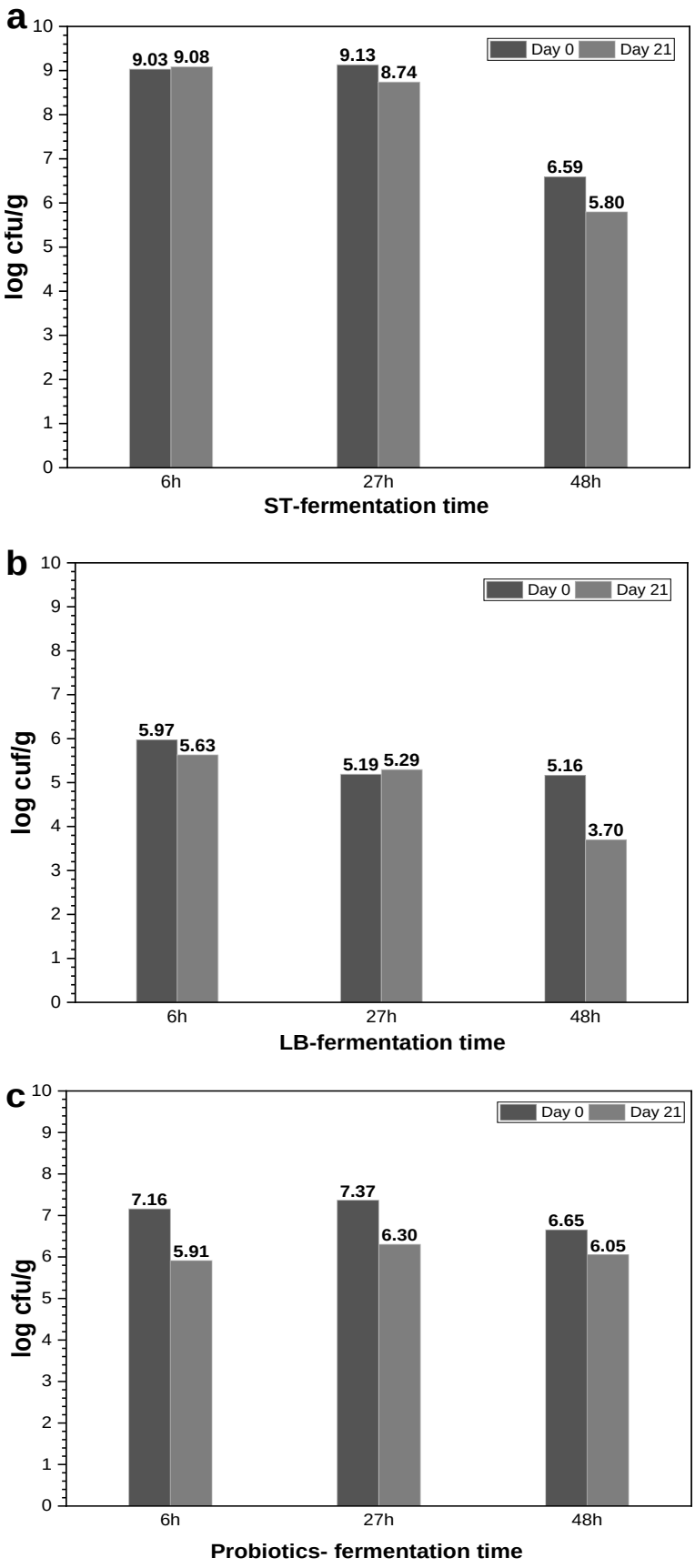


Figure 6.12 Predicted mean viable cell counts of (a) *S. thermophilus*, (b) *L. bulgaricus* and (c) the probiotics *L. acidophilus* and *B. lactis* at different fermentation times on Day 1 and Day 21.

The factorial design based on results obtained was used to predict the growth of probiotic cultures at the different fermentation times and predict log cfu/ml Day 0 and Day 21 (after storage) (Figure 6.12c). In contrast to the yogurt starters, the probiotics maintained better viability by reducing the difference between Day 0 and Day 21 count for different fermentation times, with log reductions of 1.25, 0.93 and 0.60 for 6 h, 27 h and 48 h fermentation, respectively (Figure 6.12c).

It is also shown that 27 h fermented yogurt samples could maintain better viability of probiotics at both Day 0 and Day 21 compared to the other two samples (Figure 6.12c). In addition, the 6 h fermented samples had relatively low survival rates at Day 21 with a predicted mean level below 10^6 cfu/g, below the minimum therapeutic recommended level. Longer fermentation times are also recommended to optimize probiotic cell viability (Akan, 2022; Sukma et al., 2021).

6.7 Conclusions

BSG-Oligos at 3 and 4% significantly enhanced the growth of LB and particularly LA, with minimal effects on ST growth in the growth curve study. BSG-Oligos at 3% achieved a prebiotic score of 0.53, comparable to the fructo-oligosaccharides Raftilose P95 FOS. As a result, BSG-Oligos at 3% were selected for yogurt fermentation due to their high prebiotic score and better protection compared to the 4% concentration.

In yogurt fermentations, while SMP was the most significant factor influencing all response variables, BSG primarily supported the survival of ST during storage, with minimal effects on yogurt gel firmness and lactic acid production, and had no observed impact on serum separation

The addition of BSG-oligos softened the yogurt gel, stimulated lactic acid production, and slightly enhanced the probiotic growth. It also provided protection to the probiotics under harsh storage conditions, ensuring their viability throughout the storage period. The central fermentation duration of 27 hours yielded the highest results for most response variables. Furthermore, the combination of 4% cane sugar and 4% erythritol was recommended to

reduce serum release, improving yogurt quality. These findings highlight the potential of BSG-oligosaccharides as an additive to yogurt, enhancing probiotic survival and stimulating metabolite production to create yogurt products.

Chapter 7 Overall discussion

7.1 BSG extraction in RO water

BSG was extracted in RO water to eliminate any carry over of alkali or acids as a result of pH justification. All factors, temperature, time and shear stress had a significant influence on the gum extraction ($p < 0.05$). The optimal conditions for BSG recovery were soaking at 50^{o}C for 120 min with shear rate at 1000 rpm, based on the conditions tested in this study.

The soaking temperature was lower than other reported temperatures in literature, where others used temperature ranging from 56.7^{o}C to 69^{o}C (Nazir et al, 2017; Razavi et al., 2009; Naji-Tabasi and Razavi, 2017). As the temperature used was lower than others, this meant the soaking time had to be longer than others used (Nazir et al, 2017; Razavi et al., 2009; Hosseini-Parva et al., 2010). Shear stress was the same as used by Razavi et al., (2009) and Hosseini-Parva et al., (2010). The yield of dried BSG was at 17.84 – 24.48%, higher than other previously reported studies between 7.9 – 21% (Nazir et al., 2017; Razavi et al., 2009; Samateh et al., 2018). A lower temperature and slightly longer soaking time improved the yield of BSG extracted.

The predicted optimal extraction conditions were validated through experiments, and the observed response values fell within the 95% prediction range. This confirmed that the models for wet seed and wet gum weights can be reliably used for further BSG extraction. However, a higher predicted yield could be achieved by utilizing a prediction model that incorporates % yield and the weight of dried gum, along with wet seed and wet gum. These models predicted that a soak time of 60 minutes at 50^{o}C and a shear rate of 1000 rpm would result in an optimum yield of 33.4%. A composite desirability value of 0.93 from Minitab indicates a highly favourable combination of these extraction conditions for achieving the desired 33.4% dry BSG yield. Therefore, further refinement of extraction conditions can be pursued based on the prediction model.

7.2 Preliminary screening of yogurt ingredients and yogurt production

Both SMP and BSG were found to be the main factors influencing yogurt gel firmness and %serum in the preliminary ingredient screening. In the growth curve studies, the growth of yogurt cultures ST, LB, and LA was found to be optimal at low concentrations of BSG between 0.01% and 0.05% but inhibited at concentrations higher than 0.1%. When BSG was added at concentrations above 0.2%, a gel formed in the media broth, which affected the absorbance test. Ghasempour et al. (2020) used 0.2% BSG to support the survival of yogurt bacteria during refrigerated storage of yogurt and used 0.4% BSG to enhance the growth of yogurt bacteria in yogurt products, rather than media broth. Therefore, the concentration of BSG that maximizes yogurt culture growth should be used, but additional BSG is needed to maintain the bacteria viability during storage or further enhance their growth during fermentation in the food matrix. As a result, the effect of BSG on yogurt cultures should be re-tested in yogurt production and storage, and plating enumeration methods should be used for counting yogurt cultures when higher concentrations of BSG are desired.

When investigating the use of the alternative sweetener, erythritol, all cultures, ST, LB and LA decreased in cell density as the concentration of erythritol increased in M17 or MRS media broth. This is because erythritol cannot be utilized as a nutrient to support yogurt culture growth, and increasing its concentration may raise osmotic pressure, leading to a reduction in yogurt culture viability (Runnel et al., 2013; Mazi & Stanhope, 2023). Both Costa et al. (2019) and Kalicka et al. (2019) also found for probiotics *Lactobacillus casei* and *Bifidobacterium bifidum* BB-12, a reduction in growth with increased concentrations of added erythritol.

Firmness and %serum were two response variables used to screen the ranges for each ingredient (SMP, sweeteners (cane sugar, erythritol), BSG, YOMIX® 726 cultures) for yogurt making. BSG and SMP were found to be the two main factors that affected the yogurt gel firmness and %serum released in preliminary screening tests, and this was further confirmed in the BBD design. For the growth of probiotic culture (LA), BSG and erythritol did not affect their growth contrary to the findings observed in the growth curve studies in

media broths above. Erythritol at 3% and cane sugar at 5% resulted in the lowest %serum. Tao et al. (2021) also observed a positive effect of 5% erythritol (w/v) on water holding capacity of curdlan gel. From these findings the addition of erythritol will help with yogurt texture but too high a concentration will affect yogurt culture growth.

The log (firmness) and % serum models, along with the probiotic model, were used to predict the optimal yogurt ingredient composition to mimic the hardness of silken tofu while minimizing serum release. A Minitab desirable composite value of 0.82 indicates an optimal combination of 0.06% BSG, 15% SMP and 0.1% cane sugar and 7.9% erythritol can achieve targeted desired outcomes of the response variables: 6.77 log probiotics, 50.40% serum release and 2.31 log firmness. Additionally, validation experiments further confirmed that the models accurately fit the collected data. These models can be utilized for future production of silken-tofu-like yogurt products with consistent firmness and reduced serum release, especially when a specific target firmness is required.

7.3 Enzymatic hydrolysis of BSG

Enzyme hydrolysis of BSG to smaller molecular weight oligosaccharides and sugars will provide desirable prebiotics for probiotic growth. BSG contains 43% glucomannans and 24% xylans, which could be potential sources of the oligosaccharides MOS and XOS. The enzyme ZAC is predominantly a mixture of β-glucanase and xylanase, with some minor activity from other enzymes present. The enzyme Xs contained xylanase only and the enzyme CE contained cellulase only. The maximum concentration of direct sugars (DS) from BSG was achieved using 1.79% ZAC (w/v) for 2.57 mg/ml DS released, and 2% Xs and CE for 3.25 mg/ml and 7.27 mg/ml, respectively. High concentrations of enzymes will result in high cost therefore the higher release of DS may be of no benefit.

The enzymes ZAC and CE had low optimum temperatures, at 30^{o}C and 37^{o}C, respectively and Xs worked better at 65^{o}C. The manufacturers recommended that all enzymes operate optimally under relatively less acidic conditions, within a pH range of 4.5 – 5.5, but it was found they produced more DS from BSG at pH 3.0 – 4.0. Lignin xylan can be hydrolysed by xylanase at pH 4.0 and beechwood xylan is hydrolysed by xylanase at pH 5.5 or higher (Díaz-

Arenas et al, 2022; Singh et al., 2018). Each enzyme required specific optimization to determine the optimum hydrolysis conditions for the substrate material. Xylanase was able to hydrolyze the exterior hairy-like BSG fibre but may have left the central core of glucomannan unhydrolyzed (Figure 2.4). β-Mannanase, in turn, could hydrolyze the core after the removal of the hairy layer. Therefore, it could be one of reasons if β-mannanase as a hydrolysis enzyme was used alone without the xylanase present, there were fewer reducing sugars released as β-mannanase could not reach the central core.

The results indicated that E/S ratio, time, and pH significantly influenced the hydrolysis activity of Xs. Additionally, the interaction between E/S ratio and pH or temperature also affected this enzyme activity. Temperature was another key factor impacting CE activity, along with E/S ratio, time, and pH. However, the predictive model for DS was slightly less effective in determining the optimal hydrolysis conditions for CE, as it exhibited lower observed DS values and there was a lack of DP prediction.

Enzyme hydrolysis can enable precise cleavage of specific glycosidic bonds, facilitating the selective production of BSG-derived oligosaccharides with a targeted degree of polymerization (DP). The reaction conditions can be optimized using response surface design to achieve high purity under mild conditions (≤ 65^{o}C, pH 3 – 4) within a hydrolysis duration of ≤ 7 hours. Previous studies have reported that enzymatic hydrolysis is more energy-efficient than acid or high-pressure treatments (Garrote et al., 1999; Naidu et al., 2018; Qing et al., 2013; Vázquez et al., 2000). Meanwhile, enzymatic hydrolysis has been shown to better preserve the bioactivity of oligosaccharides, significantly reduce the formation of toxic compounds and toxic residuals during production, minimize waste generation, and enhance sustainability compared to conventional acid and high-pressure methods. Although a direct comparison is not conducted in this study, these reported benefits suggest that enzymatic hydrolysis holds significant potential for the sustainable production of BSG-derived oligosaccharides. The derived model can be utilized to identify optimal parameter combinations, making it valuable for large-scale enzyme hydrolysis of BSG, particularly for xylanase hydrolysis of BSG.

7.4 Impact of BSG-Oligosaccharides on yogurt cultures and probiotic score assay

BSG-Oligos obtained from Xs and CE hydrolyzed BSG in citrate phosphate buffer solutions showed slight growth enhancement of ST, LB and LA at the concentration of ≤ 1%. However, at concentrations >1%, they negatively impacted the growth of these yogurt cultures compared to the control and enzyme blank samples, which did not contain BSG as a substrate. The enzyme blank samples maintained relatively high culture growth up to 4% for most cases, suggesting that the enzyme powder from the manufacturer contained residual sugars or oligosaccharides after hydrolysis. These residual sugars or oligosaccharides were the hydrolysis products of the adjuvants corn starch and/or dextrin in the enzyme powder, so when the enzyme was at high enough concentration it also provided extra nutrients for the bacteria growth. Meantime, citrate phosphate buffer residuals could have negatively impacted on the yogurt culture growth.

Instead of using the buffer solution the BSG was hydrolysed with the enzyme Xs in water. The BSG-oligos produced in this solution significantly stimulated the growth of LB and LA when 3 – 4% such BSG-Oligos was used. In addition, the BSG-Oligos produced in RO water showed better growth enhancement than Raftilose P95 (FOS). The maximum growth (Max) was 1.02 ± 0.19 OD_{595nm} with BSG-oligos from water, an increase in 0.74 OD_{595nm} compared to increase of 0.11 OD_{600nm} of LA and 0.18 OD_{600nm}, for Raftilose P95 and inulin, respectively (Huebner et al., 2007; Olson & Aryana, 2012). BSG-Oligos from water resulted in a high μ_{Max} for LA. The μ_{Max} was 0.12 ± 0.01 OD_{595nm} h^{-1} compared to the LA control of 0.06 ± 0.001 OD_{595nm} h^{-1} in this study. Although there was not significant difference in the lag times for LA supplied with BSG-Oligos from water, the lag time was shorter at 5.4 – 5.8 h for enzyme concentrations of 2 – 4%, compared to the LA control which had a lag time of 6.5 h. BSG-Oligos hydrolyzed in water demonstrated significant prebiotic function, enhancing the growth of yogurt cultures, especially LA.

Enzyme hydrolysis of polysaccharides is normally carried in a citrate phosphate buffer solution at acidic condition to obtain maximum oligosaccharides from gums (Chen et al, 2013; Dhaver et al., 2023; Geetanjali & Singh, 2022; Wongputtisin & Khanongnuch, 2015).

However, the residual buffer chemicals influenced the prebiotic function of BSG oligosaccharides and affected yogurt culture growth in this study. Further investigation is needed to quantify DS and TS, enabling the calculation of DP for Xs-hydrolyzed BSG in water, and the development of a predictive model to optimize hydrolysis conditions. The choice of prebiotic source and its concentration significantly impact results; therefore, careful selection is essential.

BSG-Oligos at 3% and 4% from Xs hydrolyzed BSG in water, were further investigated using the prebiotic score assay. The 3% Xs BSG-Oligos had the highest prebiotic activity score (0.53) after 12 h (Figure 6.5). The scores were comparable to the prebiotic activity of the Raftilose P95 FOS (0.58) and slightly lower than purified GOS (0.66) in supporting LA growth (Huebner et al., 2007). The scores were higher than XOS (0.12) (Ismail et al., 2023) and pectin-oligosaccharide (0.41) (Zhang et al., 2018). Although there were no significant differences between the scores of 3% and 4% BSG-Oligos at the end of incubation period (48 h), 3% BSG-Oligos provided better protection of LA, doubling the prebiotic scores (Figure 6.5).

Simply adding sugar to yogurt cultures can provide an immediate energy source, but using BSG-Oligos can offer additional benefits beyond just sweetness. The presence of the low molecular weight oligosaccharides is highly beneficial for the growth of yogurt cultures, particularly the *Lactobacilli*. BSG-derived oligosaccharides have been shown to selectively promote the growth of *L. acidophilus*, with prebiotic scores comparable to commercial oligosaccharides. As a result, they can effectively support the growth of *L. acidophilus* during yogurt fermentation and enhance their survival rate to have a positive effect on health. The BSG oligos produced using 3% BSG-Oligos from Xs hydrolysed BSG in water was taken into yogurt making.

7.5 Profile study of yogurt fortified with BSG-Oligosaccharides, BSG, and erythritol

BSG up to 0.06% and BSG-Oligos up to 3% were used for the next step of the study to produce yoghurt with 7.5 – 15% SMP. Erythritol concentration was increased with

reduction in cane sugar to maintain a total sweetener level at 8%. SMP was the main factor to have an impact on the yogurt quality profile (Donato et al., 2007; Pakseresht et al., 2017). The yogurt acid profile: pH, TTA and lactic acid were significantly correlated with the SMP concentration ($p < 0.05$). Yogurt culture growth and survival were highly correlated to SMP concentration ($p < 0.05$), except for LB at Day 21.

BSG at ≤ 0.06% did not significantly influence the overall yogurt profile. However, in this study, a slight increase in lactic acid content was observed with increasing BSG levels (p,0.039; r, 0.1). BSG concentration influenced the yogurt gel firmness (p, 0.041) but not the serum release ($p > 0.05$). BSG did not reduce serum released from the gel. These findings contrasted with the findings by Tan (2019), where total titratable acidity (%TTA) increased significantly from 0.90% to 1.02% with the addition of BSG at concentrations of 0.1 – 0.3%. In the earlier preliminary study reported in this thesis (Chapter 4) when BSG was used but no BSG-Oligos the gel firmness increased and serum separation was reduced, with interaction with SMP to improve these two yogurt profiles (Section 4.4). In these subsequent trials (Section 6.6) BSG in combination with BSG-Oligos significantly reduced serum release at Day-1 and day-21 of storage ($p < 0.05$).

BSG-Oligos were added to yogurt as a new ingredient. Acidification of yogurt was positively linked to its content, increasing BSG-Oligos increased %TTA and lactic acid content. However, it was positively correlated with serum release, significantly increasing the amount of serum produced ($p < 0.05$). It has been reported that short chain oligosaccharides depleted the gel stability, released serum and increased in serum volume when using prebiotic oligosaccharides (Brennan & Tudorica, 2008; Jackson et al., 2023; Khalid et al., 2022; Pimentel et al., 2012). If BSG-Oligos are used to increase acid production in yogurt, BSG is suggested to be added with BSG-Oligos to compensate for the gel structure depletion by BSG-Oligos and reduce the amount of serum released.

Addition of BSG-Oligos affected the growth and survival of ST during storage of yogurt at 4 ± 1^{o}C. This was also observed with 2 – 4% lactulose impacted on *S. thermophilus* (Delgado-Fernández et al., 2019). FOS and GOS do not have an impact on *S. thermophilus* growth, but one log reduction of *S. thermophilus* at the end of storage was observed in the research

of Delgado-Fernández, et al. (2019). FOS, GOS and lactulose had no impact on *L. bulgaricus* (Delgado-Fernández et al., 2019), and BSG-oligos also did not affect the growth and survival of LB. BSG-Oligos in this study also affected the growth of probiotics particularly *L. acidophilus* by enhancing the growth of the probiotics in yogurt (p,0.041). BSG-Oligos helped to support their survival on storage for 21-days at 4 ± 1^{o}C ($p < 0.05$, $r = 0.70$) and increased their survival by about 0.83 log between 0% and 3% BSG-Oligos at Day-21 (Figure 6.8). The results are comparable to enzyme hydrolyzed xanthan gum's protective effect on probiotics (Khalid et al., 2022). BSG-Oligos can stimulate metabolism and protect probiotic cells in harsh environments. These findings align with the results of the prebiotic score assay and provide additional evidence that enzyme hydrolyzed gums have protective prebiotic function on probiotics.

The yield of BSG-Oligos produced was relatively low in this study, which suggests a need for process improvements. Ultra-filtration and nanofiltration could be used to concentrate the BSG-oligos and could enable selection of specific BSG-Oligos based on molecular weight compared to using the conventional filter paper method (Qing et al., 2013; Wen et al., 2024). The other concern is enzyme usage, as it is not reused. A fixed bed immobile enzyme microreactor could be a solution for reuse of xylanase in future research (Illanes et al., 2016; Nagy et al., 2022; Rajagopalan et al., 2016).

Increased cane sugar concentrations significantly decreased the amount of serum released on storage ($p<0.05$). LB is unable to ferment cane sugar, cane sugar increases the osmotic pressure in the yogurt environment and causes lower viable cell counts of LB (Amoroso et al., 1989; Ashraf & Shah, 2011; Carvalho et al., 2003; Wheater, 1955). However, the level of cane sugar did not influence ST and LA growth and survival at ≤ 8%. Despite the negative effects of erythritol such as increased serum release, the combination of 4% erythritol and 4% cane sugar exhibited the best results across all response variables.

Fermentation time was important. Increasing fermentation time, decreased in pH and serum release ($p < 0.05$, $r < -0.4$) and increased lactic acid amount and gel firmness ($p < 0.05$, $r > 0.4$). All YO-MIX® 726 yogurt cultures at Day 0, and ST and LB at Day 21, were highly negatively correlated to fermentation time ($r < -0.6$). The survival of probiotics was not

linked to fermentation time, and after 27 h fermentation of yogurt samples, the probiotics showed better predicted growth and survival than 6 h and 48 h fermentation times. It was also suggested that *L. acidophilus* had a better growth rate with longer fermentation times (Akan, 2022).

The centre ingredient composite design showed better predicted mean values for all response variables. The curvature effects were significant ($p < 0.05$). It suggested that a simple linear model was not sufficient (Minitab, 2024). A factorial design is effective for identifying significant factors in linear relation experiments, but a response surface method, such as the Box-Behnken Design (BBD) or Central Composite Design (CCD), are more suitable for a curved relationships between factors and response variables and more accurate and precise to optimize ingredient compositions for future research (Myers et al., 2016; Narukulla et al., 2024). Furthermore, BSG-derived oligosaccharides have the potential to serve as a selective symbiotic ingredient to promote the growth of probiotics for yogurt production and support their survival through shelf-life.

Chapter 8 Conclusions and Recommendations

8.1 Conclusions

The Box-Behnken design successfully optimized the water extraction of BSG, with yield of wet gum (1771.0 ± 25.8g) and wet seeds (90.3 ± 3.5g) per 30g of dry original basil seed. BSG can be efficiently extracted on a commercial scale using RO water under optimal conditions of 50^{o}C for 2 h at a 1000 rpm to achieve maximum wet gum yield and minimize wet seed weight. The water-extracted gum has shown gel stabilization at 0.06%, a relatively low concentration without additional BSG-Oligos in yogurt. However, at this concentration 0.06% BSG in yogurt did not enhance growth of the yogurt cultures.

SQZyme xylanse (Xs) in water, at a high enzyme and BSG ratio of 2:1, was able to hydrolyze BSG into BSG-Oligos and significantly enhanced the growth rate, maximum growth density and reduce the lag time for *L. acidophilus* in MRS broth. BSG-oligos at 3% exhibited a higher prebiotic score (0.53) compared to 4% (0.30) after 12 h incubation.

BSG-oligos supported the growth and protected YO-MIX® 726 probiotics during 21 days of storage at 4 ± 1^{o}C, with effective concentration range from as low as 0.15% up to 3%.

Similar to other oligosaccharides, BSG-Oligos addition resulted in softened yogurt gel, as their presence affected the protein network. However, BSG can interact with BSG-Oligos to reduce serum release.

The yogurt formulation to provide the best overall response variables was SMP (11.5%), BSG (0.03), BSG-Oligos (1.5%), cane sugar (4%) and erythritol (4%) fermented for 27 h. BSG can be extracted in water without losing gum functionality while increasing dry gum yield. Its oligosaccharides serve as functional prebiotics, enhancing probiotic growth and survival in yogurt products. The combination of BSG and BSG-oligos is essential for maintaining a relatively stable yogurt gel.

8.2 Recommendations

There was a lack of physiochemical properties studies on water-extracted BSG gum, this could be carried out compared to the pH adjusted water extracted BSG.

A chemical structure study would supply more information about enzyme hydrolyzed BSG-Oligosaccharide via HPAEC-PAD and help to know the degree of hydrolysis and what is present in the BSG-oligos added to the yogurt. This could also help establish a correlation between the prebiotic function of BSG-oligos and the method of enzymatic hydrolysis, whether performed in buffer or water.

Artificial GI tract studies would help to understand the digestibility of BSG-Oligosaccharides by GI enzymes and chemicals to further check its prebiotic potential. Furthermore, the atherogenic index and thrombogenic index can be also worked through different compositions of fatty acids in BSG-Oligosaccharides fortified yoghurt, analyzed by GC.

There was still large amount of left-over fibre moieties after enzyme hydrolysis, and it could be the core of glucomannan which may be further hydrolyzed by β-mannanase. A fixed bed immobile enzyme microreactor could give a solution to reuse xylanase. Also, ultrafiltration and nanofiltration could be used to help with the production of BSG-Oligosaccharides.

As BSG-Oligosaccharides have shown cell protection, further studies on encapsulation could enhance probiotic cell stability, thereby extending shelf life and maintaining therapeutic efficacy in probiotic food products.

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Appendices

Appendix 1 Material and Methods

1.1 Microbiological media preparation

- Methods used according to IDF (2006)
- MRS broth and agar were prepared as the preparation instruction on the bottles as well as M17 broth and agar.
- Lactose solution (Ajax, USA) for M17 broth and agar

  Dissolve 10 g of lactose in 100 ml RO (reverse osmosis) water and autoclave the solution at 121°C for 15minutes. 50 ml of 10% lactose is added into 950 ml M17 agar at 45 ± 1°C or broth at room temperature.
- MRS-clindamycin agar

It is the selective agar for the growth of probiotic in this study with addition of 0.5 ppm clindamycin.

Preparation of clindamycin solution

10 mg of clindamycin powder (Sigma Aldrich, USA) was dissolved in 10 ml RO water, and then was further diluted in 100 ml volumetric flask to the mark after the powder was completely dissolved into water. This gave 100ppm clindamycin concentrated solution and the concentrated solution was pipetted into sterile 2 ml microcentrifuge tube (Eppendorf, Germany) for storage at -18°C. The frozen stock solution has a shelf-life up to a year.

Fresh MRS-clindamycin agar was prepared on the day for the microbial analysis, and frozen concentrated clindamycin was used as 1 ml/200 ml of MRS ratio at 45 ± 1°C. The media were kept in dark without exposure to direct sunlight.

Fresh agar plates of M17 and MRS-plain were prepared and used in same day. Agar plates were dried in conventional oven at 50°C for 10 min before use. 10 µl of each dilution samples were pipetted on the corresponded agar plate section and air dried until water drops were fully diffused in agar plate about 5 min. MRS drop plates were placed in anaerobic condition soonest as the sample drops were dried within one hour.

1.2 Titration preparation for TA (%lactic acid)

- Preparation of reagents
  - 1% phenolphthalein indicator solution

    1g of phenolphthalein powder (Ajax, Austrila) was dissolved in 100 ml ethanol (95%) and mixed thoroughly. The solution was kept in an amber reagent bottle for use.
  - ~0.1M (0.1N) sodium hydroxide solution was standardized against KHP for accuracy.
  - Potassium hydrogen phthalate (KHP)

    A small quantity of Potassium hydrogen phthalate was dried at 120^{o}C for 2 hours and then cooled in air-tight desiccators with fresh desiccant for at least 60 minutes. The thoroughly dried KHP was transferred quickly to a clean, dry air-tight jar and tightened the lid firmly. 0.2000g oven dried KHP was dissolved into 25 ml hot water and allowed to cool down before use.

1.3 Citrate phosphate buffer – 0.05M Na2HPO4-citric acid

Table A1.1 Citrate phosphate buffer

pH	Sodium Phosphate Dibasic Dihydrate (g/L) 177.99 g/mol	Citric Acid (g/L) 192.12 g/mol
3.0	4.2612	3.1125
3.5	4.2655	3.1128
4.0	4.8275	3.1210
4.5	4.8261	3.1121
5.0	4.8312	3.1151
5.5	4.8372	3.1185
6.0	4.8324	3.1271
6.5	4.8265	3.1293
7.0	4.8402	3.1815

- Prepare 800 ml of RO water in a suitable container.
- Add Sodium Phosphate Dibasic Dihydrate and citric acid to 800 ml RO water.
- Adjust solution to final desired pH using about 0.1M HCl or 0.1M NaOH.
- Add distilled water until volume is 1 L.

1.4 Quantification of reducing sugar by Nelson-Somogyi method

1.4.1 Reagents

Alkaline Copper tartrate

- Reagent A: Dissolve 2.54 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 ml water and make up to 100 ml. (at cool room, solution likely turns to solidified when pipetting, need to be warmed up just before use e.g 55^{o}C for a few minutes until solid fully dissolved)
- Reagent B: Dissolve 15 g copper sulphate in a small volume of distilled water. Add one drop of sulfuric acid and make up to 100 ml. Mix 4 ml of B and 96 ml of solution A just before use.

Arseno-molybdate Reagent:

- Dissolve 2.5 g ammonium molybdate in 45 ml water. Add 2.5 ml sulphuric acid and mix well. Then add 0.3 g disodium hydrogen arsenate dissolved in 25 ml water. Mix well and incubate at 37^{o}C for 24 to 48 h.
- Working Standards: 0, 0.05, 1, 2, 3, 4 and 6 mg/ml.

1.4.2 Procedure

- Weigh 100 mg of the sample and extract the sugars with the hot 80% ethanol twice (5 ml each time)
- Collect the supernatant and evaporate it - use boiling pan to evaporate the 80% ethanol, roughly about 12 min. Need to check regularly to make sure the sample is dried completely but not caramelized.
- Add 10 ml water and dissolve the sugars.
- Pipette out aliquots (sample and standards solutions) of 0.2 ml to 1 ml dilution 96-deep-well plates.
- Add 0.2 ml of alkaline copper tartrate reagent to each tube.
- Place the tubes in a boiling water for 10 min.
- Cool the plate to room temperature and add 0.2 ml of arsenomolybolic acid reagent and wait for 15 min.
- Transfer sample solution into 96-well titer plate for reading in the BMG plate reader.

- Read absorbance at 620nm.

1.4.3 Calculation

- Absorbance corresponds to 0.1 ml of test = ‘x’ mg of mannose, glucose or xylose
- 10 ml contains = (‘x’ ÷ 0.1) × 10mg of mannose, glucose or xylose = % of reducing sugars

1.5 An example texture profile plot of a yogurt sample

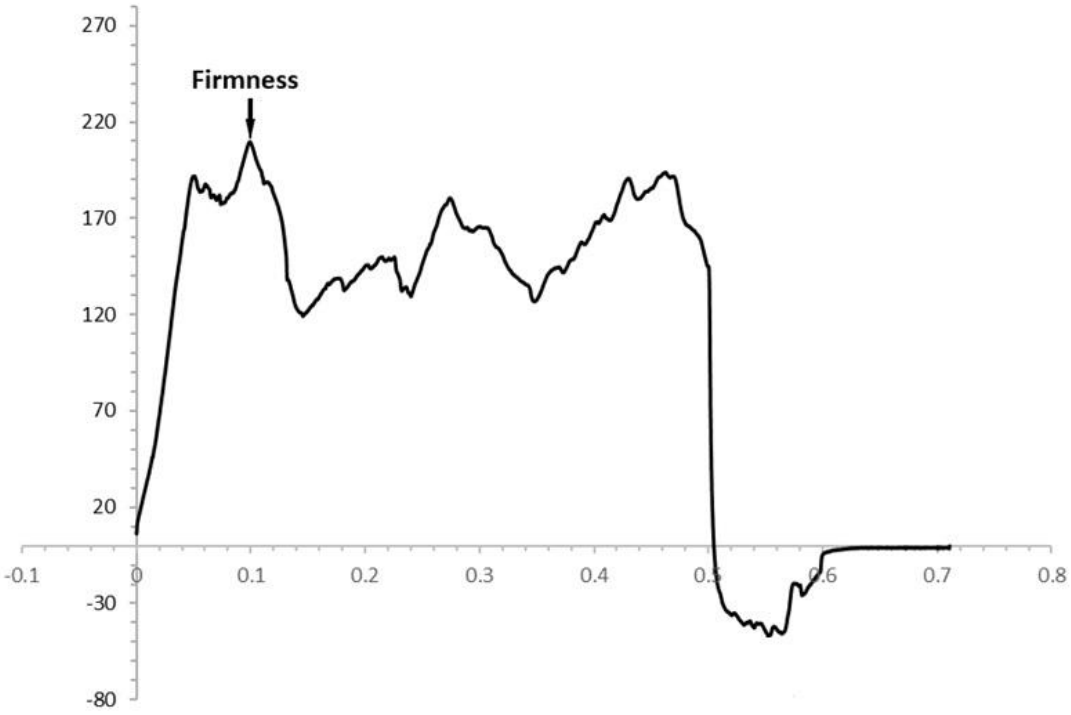


Figure A1.1 Texture profile of a yogurt sample in the Box-Behnken design

1.6 Standard curve for optimum absorbance wavelength of glucose, mannose, and xylose standards

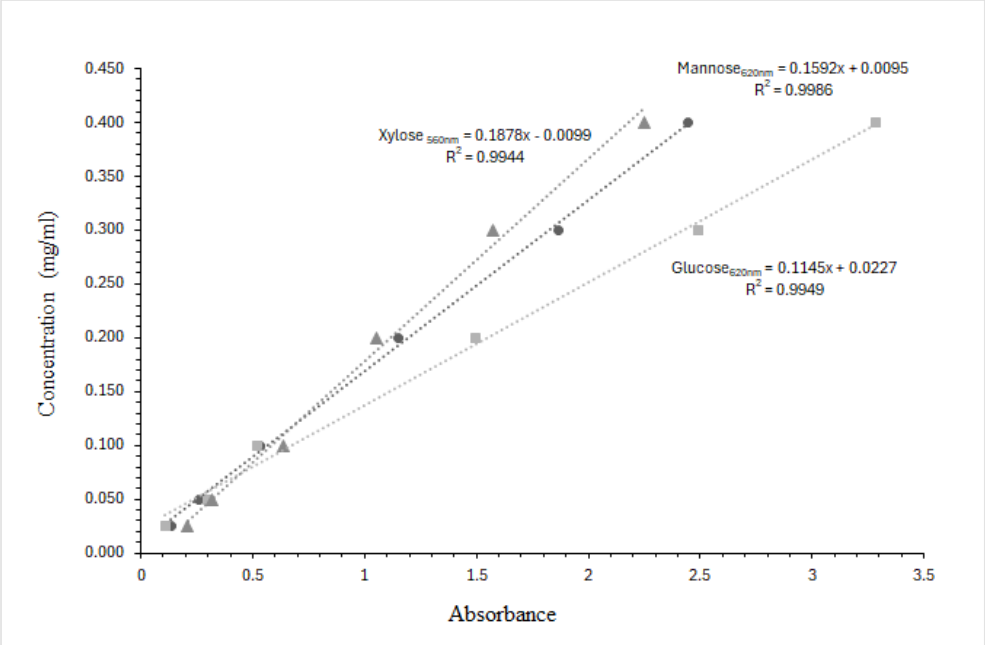


Figure A1.2 Standard curve examples for Xylose at the optimum wavelength of 560nm, Mannose and Glucose at 620nm using Nelson-Somogyi reducing sugar method.

1.7 HPLC – standard retention time and regression line for quantifying lactic acid

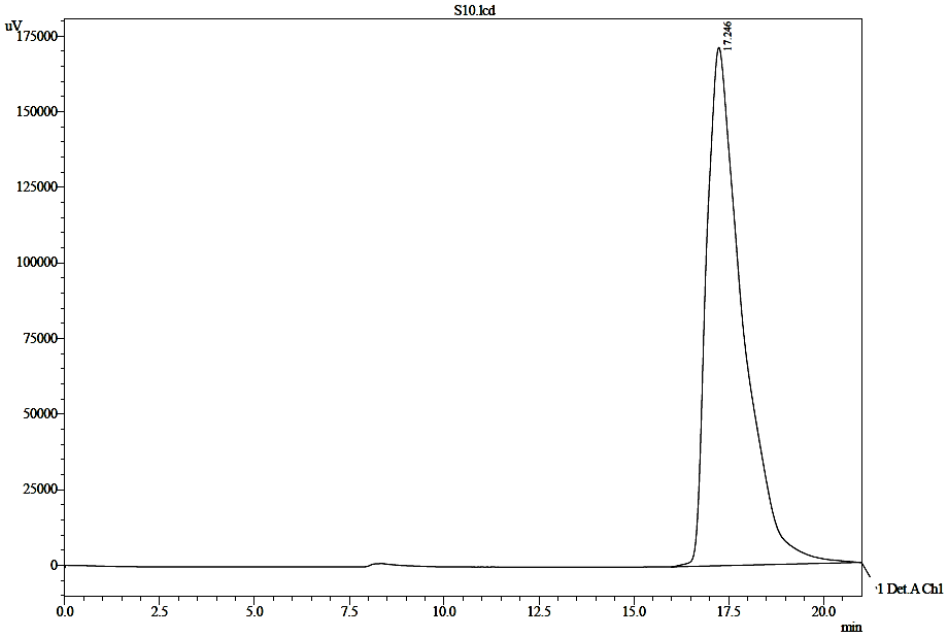


Figure A1.3 The chromatogram of lactic acid standard at concentration of 10mg/ml

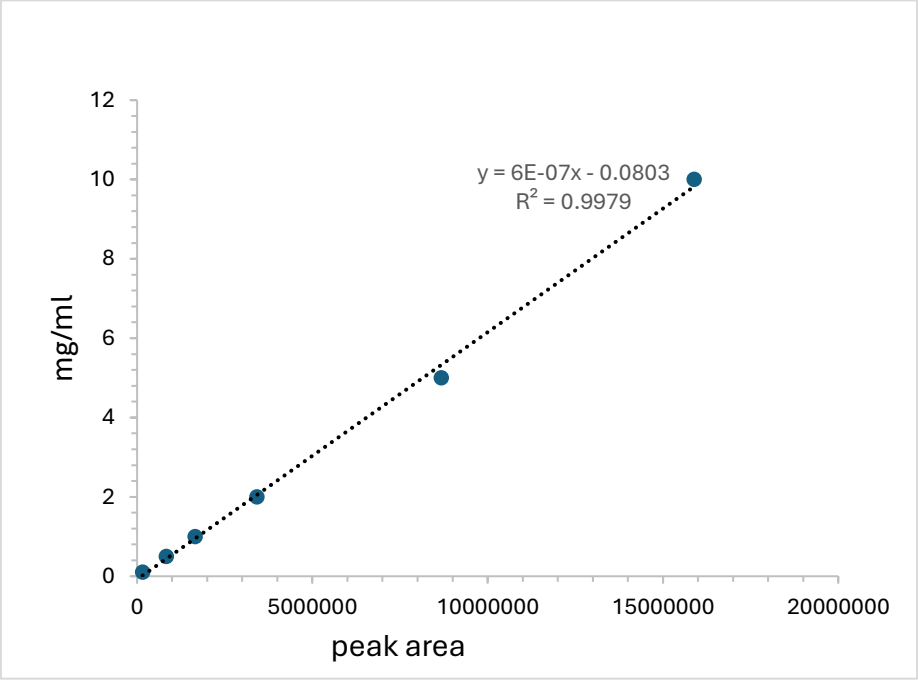


Figure A1.4 The fitted line plot of lactic acid standards and their regression equation for calculation of lactic acid concentration in yoghurt samples.

1.8 Media for Prebiotic Score Assay

Table A1.2 M9 broth

Slightly modified based on VWR-M9 broth formulation	
Sodium phosphate dibasic	6g
Potassium phosphate monobasic	3g
Sodium chloride	0.5g
Ammonium chloride	1g
RO Water	700/600ml
pH 7.4	
Autoclave at 121°C for 15min	
Ingredients to be added separately after autoclaving	
1M magnesium sulfate	2ml
1M calcium chloride	0.1ml
Glucose/BSG-Oligo	10%

Table A1.3 MRS broth without lactose

modified based on Thermofisher MRS broth formulation	
Peptone	10g
Yeast extract	5g
Beef extract	10g
Dipotassium phosphate	2g
Ammonium acetate	5g
Magnesium sulfate	0.2g
Manganese sulfate	0.05g
Tween 80	1.08g
Sodium citrate	2g
RO Water	700/600ml
Autoclave at 121°C for 15min	
Ingredients to be added separately after autoclaving	
Glucose/BSG-Oligo	10%

Appendix 2 Data and statistical analysis

2.1 Water extraction of BSG

Table A2.1 Preliminary range screening for temperature, time and shear rate based on wet seed weight and wet gum weight.

Factor	Factor range	Response variable		Factor	Factor range	Response variable		Factor	Factor range	Response variable	
		Wet seed (g)	Wet gum (g)			Wet seed (g)	Wet gum (g)			Wet seed (g)	Wet gum (g)
Temperature °C	20	62.61	131.98	Time (min)	20	58.97	168.86	Shear rate (rpm)	400	67.66	620.87
	20	75.19	133.21		20	67.31	196.98		400	80.38	589.41
	30	78.18	150.71		30	68.61	193.81		600	77.75	612.74
	30	75.62	145.89		30	70.57	226.63		600	68.50	569.13
	40	79.28	153.75		40	69.01	197.88		800	55.47	576.40
	40	66.23	178.53		40	61.94	217.03		800	50.60	603.87
	50	30.96	142.40		50	60.27	198.50		1000	39.80	611.11
	50	25.82	194.38		50	67.42	230.59		1000	40.59	607.37
	60	18.49	182.23		60	67.17	150.50		1200	26.31	683.02
	60	16.71	182.23		60	66.75	199.05		1200	29.18	604.71
	70	12.31	155.52		70	66.62	202.43		1400	23.23	658.84
	70	18.10	177.13		70	55.27	235.89		1400	23.98	656.73
	80	16.92	174.00		80	68.00	202.83		1600	21.56	650.98
	80	24.11	161.34		80	60.29	198.87		1600	21.45	585.43
					90	64.82	201.99				
					90	64.04	188.6				
					100	62.45	146.48				
					100	63.67	206.01				
					110	69.1	197.02				
					110	65.5	197.99				
					120	64.01	202.29				
					120	68.74	205.66				
					130	72.13	194.51				
					130	68.6	193.86				
	p-value	0.000	0.220		p-value	0.571	0.536		p-value	0.000	0.359

Table A2.2 Apparent viscosity of basil seed gum at different shear rate.

Shear rate (rpm)	Appearance viscosity (mPa.s)
400	165
400	176
600	250
600	279
800	1255
800	1345
1000	1501
1000	1470
1200	1075
1200	1297
1400	1057
1400	1228
1600	1024
1600	1250
p-value	0.000

Table A2.3 Box-Behnken experimental design for estimating optimum BSG extraction conditions in RO water.

Run Number and Conditions Temperature:Time:Shear rate	Independent variables (factors)			Response variables					
	Temperature (°C)	Time (min)	Shear rate (rpm)	Dried seed kernel weight (g)	Wet seed kernel weight (g)	Dried gum weight (g)	Wet gum weight (g)	Swelling capacity (g water/g dried seed)	% Yield dried gum (g dried gum /g original starting seed)
1-50°C-60min-700rpm	50	60	700	21.73	163.69	7.35	1745.08	7.53	24.48
2-50°C-90min-400rpm	50	90	400	22.07	214.61	5.46	1679.58	9.73	18.20
3-50°C-90min-1000rpm	50	90	1000	21.86	128.04	6.43	1752.18	5.86	21.44
4-50°C-120min-700rpm	50	120	700	21.27	124.90	5.36	1736.59	5.87	17.87
5-60°C-60min-400rpm	60	60	400	22.37	216.04	5.54	1653.90	9.66	18.46
6-60°C-60min-1000rpm	60	60	1000	21.82	117.32	6.50	1756.87	5.38	21.66
7-60°C-90min-700rpm	60	90	700	21.91	164.51	6.23	1677.34	7.51	20.75
8-60°C-90min-700rpm	60	90	700	22.03	169.34	6.30	1633.23	7.69	21.01
9-60°C-90min-700rpm	60	90	700	21.99	172.60	6.31	1651.24	7.85	21.04
10-60°C-120min-400rpm	60	120	400	22.02	207.80	6.02	1583.25	9.44	20.07
11-60°C-120min-1000rpm	60	120	1000	21.42	95.60	6.96	1757.93	4.46	23.21
12-70°C-60min-700rpm	70	60	700	21.69	161.60	6.07	1648.22	7.45	20.22
13-70°C-90min-400rpm	70	90	400	21.83	202.94	5.71	1571.86	9.30	19.04
14-70°C-90min-1000rpm	70	90	1000	21.46	124.60	6.19	1659.93	5.81	20.64
15-70°C-120min-700rpm	70	120	700	21.46	165.00	6.28	1471.37	7.69	20.94

Table A2.4 Analysis of the three factors using BBD design for BSG extraction in water based on wet seed weight and wet gum weight

	Wet seed weight analysis	Wet gum weight analysis
Analysis of Variance	**Source** / **P-Value** Model 0.000 Linear 0.000 Temp 0.406 Time 0.035 Shear rate 0.000 temp*time 0.049 Lack-of-Fit 0.141	**Source** / **P-Value** Model 0.000 Linear 0.000 temp 0.000 time 0.028 shear rate 0.001 temp*time 0.038 Lack-of-Fit 0.283
Model Summary	**R-sq** 96.11% **R-sq(adj)** 93.95% **R-sq(pred)** 85.28%	**R-sq** 86.44% **R-sq(adj)** 81.02% **R-sq(pred)** 60.67%
Regression Equation	wet seed = 370.5 - 2.88 temp - 0.06 time - 0.1566 shear rate- 0.01289 time*time + 0.0352 temp*time	wet gum=1297 + 5.60 temp + 7.36 time + 0.1826 shear rate - 0.1403 temp*time

Solution

Solution	temp	time	shear rate	wet gum Fit	wet seed Fit	Composite Desirability
1	50	120	1000	1800.51	87.9363	1

Multiple Response Prediction (chosen models)

Variable	Setting
temp	50
time	120
shear rate	1000

Response	Fit	SE Fit	95% CI	95% PI
wet gum	1800.5	29.2	(1735.4, 1865.6)	(1698.7, 1902.3)
wet seed	87.94	8.05	(69.73, 106.14)	(60.12, 115.75)

Solution

Solution	temp	time	shear rate	% yield Fit	wet seed Fit	wet gum Fit	dried gum Fit	Composite Desirability
1	50	60	1000	33.3917	123.096	1780.06	7.33681	0.925601

Multiple Response Prediction (models to repeat in future)

Variable	Setting
temp	50
time	60
shear rate	1000

Response	Fit	SE Fit	95% CI	95% PI
% yield	33.39	1.43	(30.20, 36.59)	(28.40, 38.39)
wet seed	123.10	6.87	(108.12, 138.07)	(92.56, 153.63)
wet gum	1780.1	29.2	(1715.0, 1845.2)	(1678.2, 1881.9)
dried gum	7.337	0.520	(6.000, 8.673)	(5.586, 9.088)

Application of BSG in yogurt with erythritol to partially replace cane sugar

Table A2.5 Preliminary range screening for factors basil seed gum (BSG), skimmed milk powder (SMP), Yomix-726 yogurt cultures, cane sugar and erythritol based on Firmness and % serum of yogurt gel.

Factor	Factor range	Response variable		Factor	Factor range	Response variable		Factor	Factor range	Response variable		Factor	Factor range	Response variable		Factor	Factor range	Response variable	
		Firmness (g)	Serum % (w/v)			Firmness (g)	Serum % (w/v)			Firmness (g)	serum % (w/v)			firmness (g)	serum % (w/v)			Firmness (g)	serum % (w/v)
BSG % (w/v)	0	84.27	66.15	SMP % (w/v)	5	16.27	75.91	culture % (w/v)	0.001	110.18	59.96	Cane sugar % (w/v)	0.1	107.6	64.4	Erythritol % (w/v)	0	103.54	64.52
	0	85.93	66.09		5	28.78	70.39		0.001	82.89	61.38		0.1	101.49	67.55		0	99.63	63.18
	0.01	127.47	63.85		8	76.86	60.32		0.01	111.1	61.71		1	95.75	65.56		2	99.46	62.46
	0.01	132.71	62.9		8	92.89	60.92		0.01	94.52	61.49		1	107.46	60.25		2	106.73	64.96
	0.03	124.02	65.44		10	146.51	57.67		0.03	89.25	59.74		2	113.46	62.53		4	99.84	62.37
	0.03	115.3	64.57		10	120.43	59.22		0.03	106.09	60.49		2	118.72	61.65		4	112.15	64.69
	0.05	113.56	64.96		12	191.14	55.84		0.05	97.4	58.57		4	109.94	62.93		6	107.78	66.16
	0.05	116.74	66.75		12	175.56	54.92		0.05	86.91	62.55		4	109.43	60.54		6	111.17	66.43
	0.1	115.39	62.51		15	265.33	41.89		0.075	112.58	60.82		6	111.22	61.67		7.9	102.62	65.23
	0.1	120.31	61.4		15	246.58	44.93		0.075	116.41	60.07		6	102.17	61.5		7.9	103.3	65.58
	0.2	122.73	59.59		20	204.56	30.59		0.1	83.71	59.12		8	123.06	60.56				
	0.2	111.88	62.89		20	151.35	28.99		0.1	79.76	60.51		8	118.89	57.68				
	0.3	84.31	51.22						0.2	60.01	61.38		10	126.27	58.07				
	0.3	71.69	53.89						0.2	76.42	61.89		10	109.36	62.93				
	0.5	53.09	56.67										12	120.43	62.54				
	0.5	64.98	55.64										12	120.28	59.92				
	0.75	30.23	48.46																
	0.75	25.63	50.56																
	1	23.41	50.95																
	1	28.96	51.7																
	p-value	0.000	0.000		p-value	0.000	0.000		p-value	0.058	0.701		p-value	0.068	0.281		p-value	0.546	0.204

Table A2.6 Box-Behnken experimental design for determining ingredient concentrations to produce yogurt with addition of BSG and erythritol after 6 h fermentation.

Number of run and information BSG:SMP:Cane sugar:Erythritol	BSG %	SMP %	Sugars		Response variables					
								YO-MIX® 726 Cultures (cfu/g of yogurt)		
			Cane sugar %	Erythritol %	pH	% Serum	Firmness (g)	ST	LB	LA
1-0.01-8-4.05-3.95	0.01	8.0	4.05	3.95	4.10	71.73	64.18	9.37	6.2	5.48
2-0.01-8-4.05-3.95	0.01	8.0	4.05	3.95	4.19	70.25	64.11	9.18	6.13	6.6
3-0.01-11.5-0.1-7.9	0.01	11.5	0.1	7.9	4.39	62.55	105.4	9.54	6.57	6.9
4-0.01-11.5-0.1-7.9	0.01	11.5	0.1	7.9	4.30	61.32	103.09	8.9	6.16	6.51
5-0.01-11.5-8-0	0.01	11.5	8.0	0	4.29	63.12	127.36	9.08	4.76	6.32
6-0.01-11.5-8-0	0.01	11.5	8.0	0	4.35	61.9	122.29	9.18	5.93	6.88
7-0.01-15-4.05-3.95	0.01	15.0	4.05	3.95	4.46	51.76	142.35	9.51	6.34	6.61
8-0.01-15-4.05-3.95	0.01	15.0	4.05	3.95	4.44	52.5	172.37	9.13	6.19	6.78
9-0.11-8-0.1-7.9	0.11	8.0	0.1	7.9	4.26	70.54	78.64	9.42	6.59	6.9
10-0.11-8-0.1-7.9	0.11	8.0	0.1	7.9	4.36	66.85	83.88	9.32	6.59	7
11-0.11-8-8-0	0.11	8.0	8.0	0	4.09	68.01	75.44	9.11	6.13	6.88
12-0.11-8-8-0	0.11	8.0	8.0	0	4.28	66.42	75.91	9.08	6.29	7.11
13-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.22	56.88	196.95	9.32	6.35	6.73
14-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.29	56.74	224.59	9.47	6.44	6.95
15-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.35	57	206.93	9.39	5.81	5.7
16-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.42	57.79	185.14	9.35	6.29	6.59
17-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.46	54.82	192.37	9.18	6.08	6.39
18-0.11-11.5-4.05-3.95	0.11	11.5	4.05	3.95	4.48	54.82	205.31	9.42	6.39	6.56
19-0.11-15-0.1-7.9	0.11	15.0	0.1	7.9	4.49	49.64	293.05	9.13	5.54	6.31
20-0.11-15-0.1-7.9	0.11	15.0	0.1	7.9	4.68	47.31	280.99	9.3	6.39	6.58
21-0.11-15-8-0	0.11	15.0	8.0	0	4.28	45.52	307.13	9.32	6.11	6.81
22-0.11-15-8-0	0.11	15.0	8.0	0	4.34	46.18	280.5	9.38	6.34	6.48
23-0.2-8-4.05-3.95	0.2	8.0	4.05	3.95	4.11	64.3	67.47	9.28	6.2	6.18
24-0.2-8-4.05-3.95	0.2	8.0	4.05	3.95	4.12	63.51	59.87	9.15	5.7	6.56
25-0.2-11.5-0.1-7.9	0.2	11.5	0.1	7.9	4.42	62.91	177.55	8.89	5.9	6.6
26-0.2-11.5-0.1-7.9	0.2	11.5	0.1	7.9	4.32	57.42	185.84	9.19	6.23	6.26
27-0.2-11.5-8-0	0.2	11.5	8.0	0	4.24	54.79	182.82	9.43	5.87	6.72
28-0.2-11.5-8-0	0.2	11.5	8.0	0	4.39	54.56	174.46	9.36	6.28	6.57
29-0.2-15-4.05-3.95	0.2	15.0	4.05	3.95	4.51	43.77	366.08	9.48	5.9	6.43
30-0.2-15-4.05-3.95	0.2	15.0	4.05	3.95	4.40	41.55	374.78	9.31	6.11	6.56

Table A2.7 Data analysis of response variables in the BBD for optimal ingredients selection in yogurt production.

	Firmness (g)	Log (Firmness)	% Serum	pH	ST	LB	Probiotic (cfu/g)
Source	P-Value	P-Value	P-Value	P-Value	P-Value	P-Value	P-Value
Model	0.000	0.000	0.000	0.002	0.225	0.438	0.750
Linear	0.000	0.000	0.000	0.000	0.725	0.355	0.968
BSG	0.000	0.000	0.000	0.979	0.763	0.216	0.887
SMP	0.000	0.000	0.000	0.000	0.312	0.392	0.924
Sugar	0.508	0.347	0.011	0.017	0.696	0.380	0.644
Square	0.000	0.000	0.004	0.436	0.304	0.606	0.286
BSG*BSG	0.000	0.000	0.011	0.204	0.318	0.205	0.366
SMP*SMP	0.932	0.000	0.332	0.321	0.959	0.983	0.781
Sugar*Sugar	0.003	0.004	0.006	0.729	0.098	0.813	0.105
2-Way Interaction	0.000	0.000	0.066	0.695	0.075	0.282	0.697
BSG*SMP	0.000	0.000	0.309	0.790	0.534	0.911	0.329
BSG*Sugar	0.243	0.030	0.015	0.820	0.053	0.342	0.553
SMP*Sugar	0.535	0.282	0.620	0.261	0.079	0.092	0.770
Lack-of-Fit	0.009	0.338	0.177	0.180	0.638	0.453	0.163
R-sq	0.9838	0.9921	0.9737	0.6853	0.3858	0.3326	0.2237

Table A2.8 Regression modelling and response optimization for yogurt production based on target log (Firmness), minimizing % Serum, and maximizing probiotic levels

Log (Firmness)

Model Summary

R-sq	R-sq(adj)	R-sq(pred)
99.21%	98.86%	98.26%

Regression Equation

Log (Firmness) = 0.411 + 0.684 BSG
+ 0.2261 SKM + 0.01547 Sugar
- 12.91 BSG*BSG
- 0.007699 SKM*SKM - 0.002016 Sugar*Sugar
+ 0.2840 BSG*SKM - 0.0570 BSG*Sugar
+ 0.000733 SKM*Sugar

Parameters for optimization

Response	Goal	Lower	Target	Upper	Weight	Importance
Serum	Minimum		41.5545	71.7308	1	1
log Firmness	Target	1.77721	2.3100	2.5738	1	1
Probiotics	Maximum	5.47712	7.1139		1	1

Optimization solution

Solution	BSG	SKM	Sugar	Serum Fit	log Firmness Fit	Probiotics Fit	Composite Desirability
1	0.0565032	15	0.1	50.3983	2.31000	6.76963	0.823383

Multiple response prediction

Response	Fit	SE Fit	95% CI	95% PI
Serum	50.40	1.06	(48.18, 52.61)	(46.37, 54.43)
log Firmness	2.3100	0.0170	(2.2744, 2.3456)	(2.2453, 2.3747)
Probiotics	6.770	0.188	(6.380, 7.160)	(5.932, 7.607)

% serum

R-sq	R-sq(adj)	R-sq(pred)
97.37%	96.19%	93.70%

% Serum = 87.38 - 36.4 BSG - 1.54 SKM
- 0.561 Sugar + 185.2 BSG*BSG
- 0.0482 SKM*SKM
+ 0.1159 Sugar*Sugar - 1.79 BSG*SKM
- 4.04 BSG*Sugar - 0.0208 SKM*Sugar

2.3 Enzyme hydrolysis of BSG

2.3.1 Magizyme ZAC

Table A2.9 Factor range screening of ratio of Magizyme ZAC versus basil seed gum (E/S), time, pH and temperature for optimizing enzymatic hydrolysis conditions of ZAC on basil seed gum.

Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable		
		DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP
E/S	0.00	0.22	3.61	16.39	Time (hour)	0	0.68	4.70	6.94	pH	4	1.18	4.42	3.75	Temperature (°C)	30	1.25	5.29	4.24
	0.00	0.22	4.35	19.63		0	0.59	4.97	8.44		4	1.03	4.09	3.97		30	1.08	5.39	5.01
	0.05	0.32	3.58	11.26		0.5	0.99	5.24	5.29		4.5	1.14	3.76	3.31		40	1.28	6.14	4.81
	0.05	0.27	3.68	13.51		0.5	1.08	3.93	3.65		4.5	1.03	3.70	3.59		40	1.21	5.60	4.63
	0.10	0.36	3.12	8.77		1	1.19	4.83	4.04		5	1.19	3.09	2.59		50	1.24	6.00	4.85
	0.10	0.30	4.01	13.31		1	1.15	5.65	4.90		5	1.05	3.49	3.33		50	1.15	5.96	5.18
	0.20	0.39	3.31	8.61		2	1.17	4.73	4.06		5.5	1.02	3.80	3.74		60	0.90	4.49	4.98
	0.20	0.34	3.66	10.77		2	1.13	4.20	3.70		5.5	0.93	3.78	4.05		60	1.00	4.40	4.40
	0.70	0.72	4.14	5.74		3	1.06	4.82	4.53		6	0.77	3.37	4.40		70	0.66	3.85	5.80
	0.70	0.81	4.61	5.70		3	1.18	4.08	3.45		6	0.74	3.51	4.76		70	0.72	3.94	5.44
	1.00	1.39	4.90	3.51		4	1.03	4.78	4.64		6.5	0.68	3.51	5.14					
	1.00	1.05	5.36	5.09		4	1.12	4.21	3.76		6.5	0.57	3.88	6.81					
	2.00	0.77	3.64	4.72		5	1.17	4.78	4.09		7	0.46	3.23	7.01					
	2.00	1.12	3.10	2.78		5	1.17	4.70	4.01		7	0.57	3.87	6.72					
	3.00	1.10	3.72	3.38		6	1.20	4.80	3.99										
	3.00	0.94	4.37	4.63		6	1.05	4.41	4.21										
	4.00	0.95	3.34	3.51		7	1.14	4.28	3.74										
	4.00	1.15	3.37	2.94		7	0.90	4.05	4.49										
	5.00	1.12	2.79	2.49		8	1.05	5.94	5.66										
	5.00	1.10	3.13	2.84		8	1.40	7.31	5.21										
						24	0.86	4.18	4.87										
						24	1.24	4.61	3.71										
	p-value	0.000	0.006	0.000		p-value	0.030	0.029	0.005		p-value	0.000	0.076	0.001		p-value	0.003	0.000	0.139

Table A2.10 Grouping information of Magizyme ZAC activity using the Tukey method.

Factor	Factor range	Response variable									Factor	Factor range	Response variable							Factor	Factor range	Response variable							Factor	Factor range	Response variable			
		DS (mg/ml)			TS (mg/ml)		DP						DS (mg/ml)		TS (mg/ml)		DP					DS (mg/ml)		DP						DS (mg/ml)		TS (mg/ml)	
E/S	0			C	A	B	A				Time (hour)	0		B	A	B	A		pH	4	A					C	Temperature (°C)	30	A		A	
	0.05		B	C		B	A	B				0.5	A	B	A	B		B		4.5	A					C		40	A		A	
	0.1		B	C		B		B	C			1	A	B	A	B		B		5	A					C		50	A		A	
	0.2		B	C		B		B	C	D		2	A	B		B		B		5.5	A	B				C		60	A	B		B
	0.7	A	B		A	B			C	D		3	A	B		B		B		6		B	C		B	C		70		B		B
	1	A			A					D		4	A	B		B		B		6.5			C	A	B							
	2	A				B				D		5	A		A	B		B		7			C	A								
	3	A			A	B				D		6	A	B	A	B		B														
	4	A				B						7	A	B		B		B														
	5	A				B						8	A		A		A	B														
												24	A	B		B		B														

Means that do not share a letter are significantly different.

Table A2.11 Information of sample runs, and data collected for estimating optimum hydrolysis condition of ZAC enzymes on BSG in a BBD.

Run number and conditions E/S:time:pH:Temprature	Level of factors				Response variables		
	E/S	Time (h)	pH	Temp (°C)	DS (mg/ml)	TS (mg/ml)	DP
1-E/S0.7-0.5h-pH5-40°C	1.35	7.00	5	50	1.84	7.27	3.95
2-E/S0.7-3.75h-pH4-40°C	1.35	3.75	6	30	1.20	6.52	5.42
3-E/S0.7-3.75h-pH5-30°C	2.00	3.75	4	40	1.97	6.90	3.50
4-E/S0.7-3.75h-pH5-50°C	0.70	0.50	5	40	0.90	8.35	9.31
5-E/S0.7-3.75h-pH6-40°C	1.35	0.50	5	50	1.30	7.99	6.13
6-E/S0.7-7h-pH5-40°C	1.35	3.75	5	40	2.01	7.71	3.84
7-E/S1.35-0.5h-pH4-40°C	1.35	3.75	4	30	1.94	7.08	3.66
8-E/S1.35-0.5h-pH5-30°C	1.35	0.50	5	30	0.94	7.95	8.42
9-E/S1.35-0.5h-pH5-50°C	1.35	3.75	4	50	1.75	7.96	4.56
10-E/S1.35-0.5h-pH6-40°C	2.00	3.75	6	40	1.61	6.07	3.76
11-E/S1.35-3.75h-pH4-30°C	2.00	7.00	5	40	2.57	8.41	3.27
12-E/S1.35-3.75h-pH4-50°C	2.00	3.75	5	50	2.18	6.91	3.17
13-E/S1.35-3.75h-pH5-40°C	1.35	7.00	6	40	1.66	6.22	3.75
14-E/S1.35-3.75h-pH5-40°C	2.00	0.50	5	40	1.14	5.88	5.16
15-E/S1.35-3.75h-pH5-40°C	1.35	3.75	5	40	1.63	5.51	3.37
16-E/S1.35-3.75h-pH6-30°C	1.35	7.00	4	40	2.01	6.51	3.24
17-E/S1.35-3.75h-pH6-50°C	1.35	0.50	4	40	1.19	6.18	5.17
18-E/S1.35-7h-pH4-40°C	0.70	3.75	6	40	1.24	7.90	6.39
19-E/S1.35-7h-pH5-30°C	0.70	3.75	5	50	1.12	6.99	6.22
20-E/S1.35-7h-pH5-50°C	1.35	3.75	6	50	1.66	5.51	3.33
21-E/S1.35-7h-pH6-40°C	1.35	0.50	6	40	0.84	6.85	8.15
22-E/S2-0.5h-pH5-40°C	0.70	7.00	5	40	1.97	7.86	4.00
23-E/S2-3.75h-pH4-40°C	0.70	3.75	4	40	1.42	7.60	5.34
24-E/S2-3.75h-pH5-30°C	2.00	3.75	5	30	1.85	6.09	3.29
25-E/S2-3.75h-pH5-50°C	0.70	3.75	5	30	1.27	6.46	5.08
26-E/S2-3.75h-pH6-40°C	1.35	7.00	5	30	2.22	6.49	2.92
27-E/S2-7h-pH5-40°C	1.35	3.75	5	40	1.75	5.89	3.37

Table A2.12 ANOVA data analysis for the DS and DP response variables in the ZAC hydrolysis of BSG using the BBD

Analysis of Variance

DS

Source	F-Value	P-Value
Model	11.74	0.000
Linear	36.55	0.000
Ratio	32.93	0.000
Time	100.50	0.000
pH	12.26	0.004
Temperature	0.51	0.487
Square	1.66	0.223
Ratio*Ratio	0.84	0.379
Time*Time	4.05	0.067
pH*pH	4.72	0.051
Temperature*Temperature	0.73	0.409
2-Way Interaction	1.91	0.160
Ratio*Time	1.12	0.311
Ratio*pH	0.25	0.624
Ratio*Temperature	1.94	0.189
Time*pH	0.00	1.000
Time*Temperature	4.67	0.052
pH*Temperature	3.49	0.086
Lack-of-Fit	0.76	0.690

DP

Source	F-Value	P-Value
Model	23.49	0.000
Linear	61.39	0.000
Ratio	72.30	0.000
Time	162.23	0.000
pH	10.25	0.008
Temperature	0.76	0.402
Square	10.03	0.001
Ratio*Ratio	10.16	0.008
Time*Time	38.13	0.000
pH*pH	3.39	0.091
Temperature*Temperature	3.17	0.100
2-Way Interaction	7.20	0.002
Ratio*Time	12.67	0.004
Ratio*pH	0.67	0.428
Ratio*Temperature	1.72	0.214
Time*pH	6.54	0.025
Time*Temperature	11.92	0.005
pH*Temperature	9.68	0.009
Lack-of-Fit	3.61	0.236

Model Summary

DS

S	R-sq	R-sq(adj)	R-sq(pred)
0.171503	93.19%	85.25%	65.78%

DP

S	R-sq	R-sq(adj)	R-sq(pred)
0.481273	96.48%	92.37%	80.37%

Regression Equation

DS

DS = -1.07 + 0.307 Ratio + 0.429 Time
+ 0.889 pH - 0.0291 Temperature
- 0.161 Ratio*Ratio
- 0.01415 Time*Time - 0.1613 pH*pH
- 0.000636 Temperature*Temperature
+ 0.0429 Ratio*Time - 0.066 Ratio*pH
+ 0.0184 Ratio*Temperature - 0.0000 Time*pH
- 0.00570 Time*Temperature
+ 0.01602 pH*Temperature

DP

DP = 9.08 - 4.12 Ratio - 2.081 Time + 0.72 pH
+ 0.035 Temperature + 1.573 Ratio*Ratio
+ 0.1218 Time*Time + 0.383 pH*pH
+ 0.00371 Temperature*Temperature
+ 0.406 Ratio*Time
- 0.303 Ratio*pH - 0.0486 Ratio*Temperature
- 0.1894 Time*pH + 0.02556 Time*Temperature
- 0.0749 pH*Temperature

Multiple Response Prediction

Variable	Setting
Ratio	2
Time	7
pH	4
Temperature	30

Response	Fit	SE Fit	95% CI	95% PI
DS	2.632	0.262	(2.061, 3.203)	(1.950, 3.314)

Multiple Response Prediction

Variable	Setting
Ratio	1.7899
Time	7
pH	4.08081
Temperature	30

Response	Fit	SE Fit	95% CI	95% PI
DS	2.568	0.214	(2.103, 3.034)	(1.971, 3.165)
DP	2.922	0.600	(1.615, 4.229)	(1.246, 4.597)

2.3.2 SQZyme-xylanase

Table A2.13 Factor range screening of SQZyme-xylanase versus basil seed gum ratio (E/S), time, pH and temperature for optimizing the enzyme hydrolysis conditions on basil seed gum.

Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable		
		DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP
E/S	0.00	0.22	3.61	16.39	Time (hour)	0	1.06	7.57	7.12	pH	3.5	3.17	8.11	2.56	Temperature (°C)	35	3.59	7.85	2.18
	0.00	0.22	4.35	19.63		0	0.97	7.25	7.44		3.5	2.93	5.66	1.93		35	3.08	5.88	1.91
	0.05	0.29	3.93	13.67		0.5	1.55	6.70	4.32		4.5	3.13	6.27	2.01		45	2.83	6.32	2.23
	0.05	0.26	3.79	14.81		0.5	1.71	7.25	4.25		4.5	3.03	5.91	1.95		45	3.40	7.28	2.14
	0.10	0.36	3.62	10.01		1	1.75	7.36	4.21		5	3.93	7.93	2.02		50	3.30	6.30	1.91
	0.10	0.37	4.30	11.65		1	1.75	7.06	4.04		5	3.43	7.52	2.19		50	2.13	6.62	3.10
	0.20	0.53	4.20	7.88		2	1.88	7.61	4.04		5.5	3.06	7.19	2.35		55	2.69	6.27	2.33
	0.20	0.54	4.65	8.66		2	1.78	7.31	4.10		5.5	2.52	6.48	2.58		55	2.77	6.78	2.44
	0.70	1.12	5.06	4.53		3	2.24	7.22	3.23		6.0	1.30	4.82	3.71		65	3.33	4.97	1.49
	0.70	1.01	4.71	4.66		3	2.25	7.04	3.14		6.0	1.24	5.04	4.06		65	2.43	7.13	2.94
	1.00	1.10	5.22	4.75		4	2.15	7.16	3.33		6.5	0.90	4.95	5.48					
	1.00	0.90	5.36	5.94		4	2.18	7.67	3.52		6.5	1.11	5.24	4.73					
	2.00	1.47	3.99	2.71		5	1.66	8.38	5.05		7	0.78	5.19	6.62					
	2.00	1.40	4.58	3.26		5	1.69	8.01	4.74		7	0.55	4.92	8.98					
	3.00	1.20	8.63	7.20		6	1.68	8.15	4.85										
	3.00	1.53	8.03	5.24		6	1.57	8.04	5.12										
	4.00	1.17	6.50	5.54		7	1.45	7.80	5.39										
	4.00	1.05	7.04	6.72		7	1.28	7.37	5.74										
	5.00	0.08	8.09	29.20		8	1.33	7.34	5.54										
	5.00	0.22	6.29	10.55		8	1.29	7.50	5.83										
	p-value	0.000	0.000	0.026		p-value	0.000	0.010	0.000		p-value	0.000	0.029	0.000		p-value	0.735	0.920	0.941

Table A2.14 Grouping information of SQZyme-xylanase activity using the Tukey method

Factor	Factor range	Response variable											Factor	Factor range	Response variable																Factor	Factor range	Response variable					
		DS (mg/ml)				TS (mg/ml)				DP					DS (mg/ml)				TS (mg/ml)			DP											DS (mg/ml)		TS (mg/ml)		DP	
E/S	0				D				D	A			Time (hour)	0				D	A	B	C	A								pH	3.5	A		A			C
	0.05				D				D	A	B			0.5		B					C					E	F				4.5	A		A			C
	0.1				D				D	A	B	C		1		B				B	C						F				5	A		A			C
	0.2				D			C	D		B	C		2		B			A	B	C						F	G			5.5	A		A		B	C
	0.7		B	C				C	D			C		3	A					B	C								H		6		B	A		B	C
	1			C				C			B	C		4	A				A	B	C							G	H		6.5		B	A	A	B	
	2	A						C	D			C		5		B			A						D	E					7		B	A	A		
	3	A	B			A					B	C		6		B			A	B				C	D												
	4	A	B	C			B				B	C		7			C		A	B	C		B	C													
	5				D	A	B			A				8			C		A	B	C		B														

Means that do not share a letter are significantly different.

Table A2.15 Information of sample runs, and data collected for estimating optimum hydrolysis condition of SQZyme-xylanase on BSG in a BBD.

Run Number and conditions E/S:Time:pH:Temperature	Levels of variable				DS (mg/ml)	TS (mg/ml)	DP
	Ratio	Time (h)	pH	Temperature (°C)			
1-E/S0.7-0.5h-pH4.5-50°C	1.35	2.25	4.5	50	1.83	6.96	3.80
2-E/S0.7-2.25h-pH3.5-50°C	1.35	0.5	4.5	65	1.38	6.76	4.89
3-E/S0.7-2.25h-pH4.5-35°C	0.7	2.25	5.5	50	1.28	6.33	4.93
4-E/S0.7-2.25h-pH4.5-65°C	1.35	0.5	5.5	50	1.36	6.30	4.64
5-E/S0.7-2.25h-pH5.5-50°C	1.35	2.25	3.5	65	1.84	4.57	2.49
6-E/S0.7-4h-pH4.5-50°C	2	2.25	5.5	50	2.03	5.56	2.74
7-E/S1.35-0.5h-pH3.5-50°C	1.35	2.25	5.5	65	1.68	6.22	3.71
8-E/S1.35-0.5h-pH4.5-35°C	1.35	2.25	4.5	50	1.68	6.92	4.12
9-E/S1.35-0.5h-pH4.5-65°C	2	4	4.5	50	2.38	5.01	2.10
10-E/S1.35-0.5h-pH5.5-50°C	1.35	4	3.5	50	2.36	7.20	3.05
11-E/S1.35-2.25h-pH3.5-35°C	0.7	0.5	4.5	50	0.78	5.40	6.96
12-E/S1.35-2.25h-pH3.5-65°C	1.35	2.25	3.5	35	1.96	5.64	2.88
13-E/S1.35-2.25h-pH4.5-50°C	0.7	2.25	3.5	50	1.15	5.50	4.79
14-E/S1.35-2.25h-pH4.5-50°C	1.35	4	4.5	65	1.82	5.48	3.01
15-E/S1.35-2.25h-pH4.5-50°C	1.35	2.25	5.5	35	1.68	5.58	3.33
16-E/S1.35-2.25h-pH5.5-35°C	1.35	4	5.5	50	2.11	4.93	2.34
17-E/S1.35-2.25h-pH5.5-65°C	2	0.5	4.5	50	1.58	6.22	3.94
18-E/S1.35-4h-pH3.5-50°C	1.35	0.5	3.5	50	1.48	5.62	3.80
19-E/S1.35-4h-pH4.5-35°C	1.35	4	4.5	35	2.29	6.80	2.97
20-E/S1.35-4h-pH4.5-65°C	1.35	0.5	4.5	35	1.20	4.96	4.15
21-E/S1.35-4h-pH5.5-50°C	2	2.25	3.5	50	2.70	6.77	2.51
22-E/S2-0.5h-pH4.5-50°C	0.7	2.25	4.5	65	0.73	4.56	6.25
23-E/S2-2.25h-pH3.5-50°C	1.35	2.25	4.5	50	1.81	5.62	3.11
24-E/S2-2.25h-pH4.5-35°C	0.7	2.25	4.5	35	1.39	5.59	4.03
25-E/S2-2.25h-pH4.5-65°C	2	2.25	4.5	65	3.05	4.70	1.54
26-E/S2-2.25h-pH5.5-50°C	0.7	4	4.5	50	1.50	6.24	4.15
27-E/S2-4h-pH4.5-50°C	2	2.25	4.5	35	2.09	5.01	2.40

Table A2.16 ANOVA data analysis for the DS and DP response variables in the SQZyme-xylanase hydrolysis of BSG using the BBD

DS

Analysis of Variance

Source	F-Value	P-Value
Model	16.30	0.000
Linear	48.52	0.000
Ratio	130.71	0.000
Time	58.49	0.000
pH	4.84	0.048
Temperature	0.03	0.868
Square	1.08	0.409
Ratio*Ratio	0.36	0.558
Time*Time	1.72	0.215
pH*pH	0.91	0.359
Temperature*Temperature	0.01	0.921
2-Way Interaction	4.96	0.009
Ratio*Time	0.05	0.826
Ratio*pH	5.21	0.041
Ratio*Temperature	20.81	0.001
Time*pH	0.13	0.724
Time*Temperature	3.45	0.088
pH*Temperature	0.12	0.737
Lack-of-Fit	5.45	0.165

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.176880	95.00%	89.17%	71.84%

Regression Equation

DS = 2.32 + 0.483 Ratio + 0.742 Time
- 0.411 pH - 0.0549 Temperature
- 0.109 Ratio*Ratio
- 0.0328 Time*Time + 0.0731 pH*pH
+ 0.000034 Temperature*Temperature
+ 0.0175 Ratio*Time
- 0.311 Ratio*pH
+ 0.04138 Ratio*Temperature
- 0.0183 Time*pH
- 0.00626 Time*Temperature
+ 0.00203 pH*Temperature

Multiple Response Prediction

Variable	Setting
Ratio	2
Time	4
pH	3.5
Temperature	65

Response	Fit	SE Fit	95% CI	95% PI
DS	3.249	0.270	(2.660, 3.838)	(2.545, 3.952)

DP

Analysis of Variance

Source	F-Value	P-Value
Model	14.41	0.000
Linear	41.99	0.000
Ratio	112.38	0.000
Time	51.44	0.000
pH	2.10	0.173
Temperature	2.04	0.179
Square	3.81	0.032
Ratio*Ratio	2.67	0.128
Time*Time	1.99	0.184
pH*pH	3.23	0.097
Temperature*Temperature	2.27	0.158
2-Way Interaction	3.09	0.046
Ratio*Time	1.24	0.287
Ratio*pH	0.01	0.920
Ratio*Temperature	12.60	0.004
Time*pH	3.23	0.097
Time*Temperature	0.66	0.434
pH*Temperature	0.79	0.392
Lack-of-Fit	0.65	0.740

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.432337	94.39%	87.83%	72.31%

Regression Equation

DP = -6.61 - 0.68 Ratio + 0.148 Time + 3.02 pH
+ 0.201 Temperature + 0.724 Ratio*Ratio
+ 0.0862 Time*Time - 0.337 pH*pH
- 0.001253 Temperature*Temperature
+ 0.212 Ratio*Time
+ 0.034 Ratio*pH - 0.0787 Ratio*Temperature
- 0.222 Time*pH - 0.00667 Time*Temperature
+ 0.0128 pH*Temperature

Multiple Response Prediction

Variable	Setting
Ratio	2
Time	4
pH	3.5
Temperature	65

Response	Fit	SE Fit	95% CI	95% PI
DS	3.249	0.270	(2.660, 3.838)	(2.545, 3.952)
DP	0.878	0.660	(-0.561, 2.317)	(-0.842, 2.598)

2.3.3 CE35 cellulase

Table A2.17 Factor range screening of CE35 cellulase versus basil seed gum ratio (E/S), Time, pH and Temperature for optimization enzymatic hydrolysis conditions on basil seed gum.

Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable			Factor	Factor range	Response variable		
		DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP			DS (mg/ml)	TS (mg/ml)	DP
E/S	0	0.28	4.59	16.43	Time (hour)	0	1.98	6.52	3.29	pH	3	2.57	6.09	2.37	Temperature (°C)	20	2.92	6.21	2.13
	0	0.25	3.72	14.59		0	2.08	6.91	3.32		3	1.90	5.63	2.95		20	2.74	6.94	2.54
	0.05	0.39	4.99	12.92		0.5	2.45	6.27	2.56		4	4.17	6.06	1.45		30	3.48	7.30	2.10
	0.05	0.29	3.62	12.39		0.5	2.44	5.39	2.21		4	3.41	6.61	1.94		30	3.43	6.39	1.86
	0.1	0.44	6.09	13.87		1	2.45	5.92	2.41		5	2.86	6.19	2.16		40	4.50	8.40	1.87
	0.1	0.49	6.22	12.70		1	2.15	4.43	2.06		5	2.60	7.15	2.75		40	4.20	8.96	2.13
	0.2	0.82	8.18	9.96		2	2.09	6.91	3.30		6	1.46	5.00	3.42		50	4.16	8.10	1.95
	0.2	0.71	6.03	8.54		2	2.28	5.96	2.62		6	1.40	5.06	3.62		50	4.01	8.06	2.01
	0.7	1.80	8.05	4.48		3	1.52	5.47	3.58		7	0.75	4.71	6.30		55	3.02	6.65	2.20
	0.7	1.87	8.66	4.62		3	2.23	4.46	2.00		7	0.70	3.67	5.21		55	3.10	6.73	2.17
	1	1.67	7.37	4.41		4	2.40	5.86	2.45							60	2.90	5.91	2.04
	1	2.20	6.97	3.17		4	1.66	3.50	2.11							60	2.93	7.76	2.65
	2	2.63	6.06	2.30		5	1.36	4.42	3.26							65	2.32	6.16	2.66
	2	2.73	6.61	2.42		5	1.48	4.45	3.00							65	2.14	5.97	2.79
	3	2.50	3.24	1.30		6	1.48	3.50	2.36							75	2.22	6.16	2.78
	3	2.61	3.24	1.24		6	1.69	4.16	2.47							75	2.19	5.88	2.68
	4	3.17	3.41	1.07		7	1.93	3.95	2.05										
	4	3.17	3.09	0.98		7	2.08	3.86	1.86										
	5	3.51	3.21	0.91		8	1.60	3.96	2.48										
	5	3.62	3.32	0.92		8	1.52	3.62	2.38										
	p-value	0.000	0.000	0.000		p-value	0.031	0.020	0.120		p-value	0.002	0.021	0.002		p-value	0.000	0.017	0.051

Table A2.18 Grouping information of CE35 cellulase activity using the Tukey method

Factor	Factor range	Response variable														Factor	Factor range	Response variable			Factor	Factor range	Response variable								Factor	Factor range	Response variable					
		DS (mg/ml)					TS (mg/ml)			DP								DS (mg/ml)	TS (mg/ml)				DS (mg/ml)			TS (mg/ml)		DP					DS (mg/ml)		TS (mg/ml)		
E/S	0					E		B	C	A						Time (h)	0	A	B	A	pH	3		B		A	B		B	Temperature (°C)	20			C		A	B
	0.05					E		B	C		B						0.5	A		A		4	A			A			B		30		B			A	B
	0.1					E	A	B		A	B						1	A	B	A		5	A	B		A			B		40	A				A	
	0.2					E	A					C					2	A	B	A		6		B	C	A	B		B		55	A	B			A	
	0.7				D		A						D				3	A	B	A		7			C		B	A			60			C		A	B
	1				D		A						D	E			4	A	B	A											65				D		B
	2		B	C			A	B					D	E	F		5		B	A											75				D		B
	3			C					C					E	F		6	A	B	A																	
	4	A	B						C						F		7	A	B	A																	
	5	A							C						F		8	A	B	A																	

Means that do not share a letter are significantly different.

Table A2.19 Information of sample runs, and data collected for estimating optimum hydrolysis condition of CE35 cellulase on BSG in a BBD.

Run number and conditions E/S:Time:pH:Temperature	Levels of variable				DS (mg/ml)	TS (mg/ml)	DP
	E/S	Time	pH	Temper-ature			
1-E/S0.7-0.5h-pH4.5-45°C	0.70	0.50	4.5	45	3.41	8.24	2.42
2-E/S0.7-3.75h-pH3-45°C	0.70	3.75	3.0	45	3.21	9.66	3.00
3-E/S0.7-3.75h-pH4.5-30°C	0.70	3.75	4.5	30	3.97	6.91	1.74
4-E/S0.7-3.75h-pH4.5-60°C	0.70	3.75	4.5	60	2.4	6.22	2.59
5-E/S0.7-3.75h-pH6-45°C	0.70	3.75	6.0	45	2.58	8.96	3.48
6-E/S0.7-7h-pH4.5-45°C	0.70	7.00	4.5	45	4.19	9.24	2.21
7-E/S1.35-0.5h-pH3-45°C	1.35	0.50	3.0	45	3.21	8.06	2.51
8-E/S1.35-0.5h-pH4.5-30°C	1.35	0.50	4.5	30	2.22	9.42	4.23
9-E/S1.35-0.5h-pH4.5-60°C	1.35	0.50	4.5	60	2.28	7.76	3.40
10-E/S1.35-0.5h-pH6-45°C	1.35	0.50	6.0	45	2.93	8.59	2.93
11-E/S1.35-3.75h-pH3-30°C	1.35	3.75	3.0	30	4.40	8.74	1.99
12-E/S1.35-3.75h-pH3-60°C	1.35	3.75	3.0	60	2.25	6.78	3.02
13-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45	4.27	7.73	1.81
14-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45	4.35	8.26	1.9
15-E/S1.35-3.75h-pH4.5-45°C	1.35	3.75	4.5	45	4.35	9.47	2.18
16-E/S1.35-3.75h-pH6-30°C	1.35	3.75	6.0	30	4.35	7.81	1.8
17-E/S1.35-3.75h-pH6-60°C	1.35	3.75	6.0	60	2.68	7.45	2.78
18-E/S1.35-7h-pH3-45°C	1.35	7.00	3.0	45	5.12	6.97	1.36
19-E/S1.35-7h-pH4.5-30°C	1.35	7.00	4.5	30	4.22	7.92	1.88
20-E/S1.35-7h-pH4.5-60°C	1.35	7.00	4.5	60	3.28	7.90	2.41
21-E/S1.35-7h-pH6-45°C	1.35	7.00	6.0	45	2.53	6.41	2.53
22-E/S2-0.5h-pH4.5-45°C	2.00	0.50	4.5	45	3.69	10.19	2.76
23-E/S2-3.75h-pH3-45°C	2.00	3.75	3.0	45	3.90	9.27	2.38
24-E/S2-3.75h-pH4.5-30°C	2.00	3.75	4.5	30	5.18	9.93	1.92
25-E/S2-3.75h-pH4.5-60°C	2.00	3.75	4.5	60	4.47	8.68	1.94
26-E/S2-3.75h-pH6-45°C	2.00	3.75	6.0	45	3.24	11.29	3.49
27-E/S2-7h-pH4.5-45°C	2.00	7.00	4.5	45	5.47	8.19	1.50

Table A2.20 ANOVA data analysis for the DS and DP response variables in the CE35 cellulase hydrolysis of BSG using the BBD

DS

Analysis of Variance

Source	F-Value	P-Value
Model	14.50	0.000
Linear	18.76	0.000
Ratio	19.85	0.000
Time	13.93	0.002
pH	14.80	0.001
Temperature	15.60	0.001
Square	13.82	0.000
pH*pH	25.50	0.000
Temperature*Temperature	7.96	0.012
2-Way Interaction	7.33	0.005
Time*pH	8.31	0.011
pH*Temperature	6.35	0.023
Lack-of-Fit	82.30	0.012

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.400590	87.88%	81.82%	70.70%

Regression Equation

DS = -2.80 + 0.793 Ratio + 0.674 Time + 2.288 pH
+ 0.0366 Temperature - 0.3836 pH*pH
- 0.002232 Temperature*Temperature
- 0.1184 Time*pH + 0.0285 pH*Temperature

Multiple Response Prediction

Variable	Setting
Ratio	2
Time	7
pH	3.15152
Temperature	37.2727

Response	Fit	SE Fit	95% CI	95% PI
DS	7.273	0.293	(6.645, 7.902)	(6.379, 8.168)

DP

Analysis of Variance

Source	F-Value	P-Value
Model	4.49	0.004
Linear	6.00	0.003
Ratio	1.68	0.211
Time	12.26	0.002
pH	5.35	0.032
Temperature	4.74	0.042
Square	4.47	0.048
pH*pH	4.47	0.048
2-Way Interaction	1.48	0.253
Ratio*Time	1.39	0.253
Ratio*Temperature	1.57	0.226
Lack-of-Fit	4.97	0.180

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.339487	62.33%	48.46%	31.78%

Regression Equation

DP = 2.29 + 1.140 Ratio + 0.022 Time - 0.961 pH
+ 0.0436 Temperature + 0.1235 pH*pH
- 0.0948 Ratio*Time
- 0.0218 Ratio*Temperature

Multiple Response Prediction

Variable	Setting
Ratio	2
Time	7
pH	3.72852
Temperature	30

Response	Fit	SE Fit	95% CI	95% PI
DP	1.523	0.194	(1.119, 1.928)	(0.696, 2.351)
DS	6.759	0.273	(6.142, 7.376)	(6.025, 7.494)

2.4 BSG-Oligosaccharides and yogurt

2.4.1 BSG-Oligos and YO-MIX® 726 cultures

Table A2.21 Grouping information of exp (antilog (ST-μ_{Max})) using the Tukey Method and 95% Confidence

ST	N	Mean	Grouping															
Xs-H2O-1	2	25.85	A															
Xs-Blk-0.2	2	24.33	A	B														
Xs-Blk-0.05	2	23.68	A	B	C													
Xs-H2O-2	2	22.604	A	B	C	D												
CE-Blk-2	2	21.74	A	B	C	D	E											
Xs-Blk-0.5	2	20.0180	A	B	C	D	E	F										
Xs-H2O-0.1	2	19.613	A	B	C	D	E	F										
CE-3.15-1	2	19.00	A	B	C	D	E	F										
CE-3.15-2	2	18.510	A	B	C	D	E	F	G									
Xs-H2O-3	2	17.918	A	B	C	D	E	F	G	H								
Xs-3.5-0.05	2	17.661	A	B	C	D	E	F	G	H	I							
CE-H2O-1	2	16.863	A	B	C	D	E	F	G	H	I	J						
CE-Blk-3	2	16.81		B	C	D	E	F	G	H	I	J						
Xs-3.5-0.5	2	16.70		B	C	D	E	F	G	H	I	J						
Xs-H2O-4	2	16.665		B	C	D	E	F	G	H	I	J						
Xs-Blk-0.1	2	16.32		B	C	D	E	F	G	H	I	J						
Xs-3.5-1	2	16.08		B	C	D	E	F	G	H	I	J						
CE-Blk-1	2	15.859		B	C	D	E	F	G	H	I	J						
CE-H2O-2	2	15.82		B	C	D	E	F	G	H	I	J						
CE-3.15-3	2	15.55		B	C	D	E	F	G	H	I	J	K					
CE-Blk-0.05	2	15.34		B	C	D	E	F	G	H	I	J	K					
CE-3.15-4	2	15.14			C	D	E	F	G	H	I	J	K	L				
CE-3.15-0.1	2	15.058			C	D	E	F	G	H	I	J	K	L				
Xs-H2O-0.2	2	14.87			C	D	E	F	G	H	I	J	K	L				
Xs-3.5-0.2	2	14.518				D	E	F	G	H	I	J	K	L				
Xs-3.5-0.1	2	14.197				D	E	F	G	H	I	J	K	L				
CE-H2O-0.05	2	14.141				D	E	F	G	H	I	J	K	L	M			
Xs-3.5-2	2	13.79				D	E	F	G	H	I	J	K	L	M			
CE-Blk-0.2	2	13.70				D	E	F	G	H	I	J	K	L	M			
CE-H2O-0.1	2	13.667				D	E	F	G	H	I	J	K	L	M			
Xs-H2O-0.05	2	13.47					E	F	G	H	I	J	K	L	M	N		
CE-Blk-0.5	2	13.33					E	F	G	H	I	J	K	L	M	N		
Xs-H2O-0.5	2	13.090					E	F	G	H	I	J	K	L	M	N		
CE-H2O-4	2	13.08					E	F	G	H	I	J	K	L	M	N		
CE-Blk-4	2	13.06					E	F	G	H	I	J	K	L	M	N		
CE-3.15-0.2	2	13.0025					E	F	G	H	I	J	K	L	M	N	O	
Xs-Blk-0.01	2	12.74					E	F	G	H	I	J	K	L	M	N	O	
CE-H2O-0.2	2	12.575						F	G	H	I	J	K	L	M	N	O	P
CE-H2O-3	2	12.493						F	G	H	I	J	K	L	M	N	O	P
CE-Blk-0.01	2	12.11						F	G	H	I	J	K	L	M	N	O	P
CE-H2O-5	2	11.6220						F	G	H	I	J	K	L	M	N	O	P
CE-3.15-0.05	2	11.267						F	G	H	I	J	K	L	M	N	O	P
CE-Blk-0.1	2	11.27						F	G	H	I	J	K	L	M	N	O	P
Xs-3.5-0.01	2	11.251						F	G	H	I	J	K	L	M	N	O	P
CE-H2O-0.01	2	11.249						F	G	H	I	J	K	L	M	N	O	P
CE-3.15-0.5	2	11.18						F	G	H	I	J	K	L	M	N	O	P
CE-H2O-0.5	2	9.784							G	H	I	J	K	L	M	N	O	P
Xs-H2O-0.01	2	9.34								H	I	J	K	L	M	N	O	P
CE-Blk-5	2	9.133								H	I	J	K	L	M	N	O	P
CE-3.15-5	2	9.1206								H	I	J	K	L	M	N	O	P
CE-3.15-0.01	2	8.79									I	J	K	L	M	N	O	P
Xs-3.5-3	2	8.587										J	K	L	M	N	O	P
ST-C	2	8.37										J	K	L	M	N	O	P
Xs-Blk-1	2	8.18										J	K	L	M	N	O	P
Xs-3.5-4	2	6.536											K	L	M	N	O	P
Xs-Blk-4	2	6.171												L	M	N	O	P
Xs-3.5-5	2	5.1405													M	N	O	P
Xs-Blk-2	2	4.52322														N	O	P
Xs-Blk-3	2	3.982															O	P
Xs-Blk-5	2	3.5489																P
Xs-H2O-5	2	2.75381																

Means that do not share a letter are significantly different.

Table A2.22 Grouping information of antilog (Max) of ST Using the Tukey Method and 95% Confidence

ST	N	Mean	Grouping																
Xs-3.5-0.05	2	21.184	A																
Xs-3.5-0.5	2	20.94	A	B															
Xs-H2O-0.1	2	20.294	A	B	C														
CE-H2O-0.05	2	19.90	A	B	C	D													
Xs-3.5-0.2	2	19.754	A	B	C	D	E												
Xs-3.5-0.1	2	19.681	A	B	C	D	E												
Xs-H2O-0.2	2	17.963	A	B	C	D	E	F											
CE-3.15-0.1	2	17.876	A	B	C	D	E	F	G										
Xs-H2O-0.05	2	17.66	A	B	C	D	E	F	G	H									
Xs-H2O-0.5	2	17.629	A	B	C	D	E	F	G	H	I								
CE-H2O-0.1	2	17.616	A	B	C	D	E	F	G	H	I								
CE-3.15-0.2	2	16.949	A	B	C	D	E	F	G	H	I	J							
Xs-H2O-2	2	16.700	A	B	C	D	E	F	G	H	I	J	K						
CE-3.15-0.05	2	16.671	A	B	C	D	E	F	G	H	I	J	K						
Xs-H2O-1	2	16.6212	A	B	C	D	E	F	G	H	I	J	K	L					
Xs-3.5-0.01	2	16.207	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-H2O-0.2	2	16.1258	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-H2O-0.01	2	16.089	A	B	C	D	E	F	G	H	I	J	K	L	M				
Xs-Blk-0.2	2	16.027	A	B	C	D	E	F	G	H	I	J	K	L	M				
Xs-Blk-0.05	2	15.94	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-3.15-0.5	2	15.678	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-H2O-4	2	15.077	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-H2O-3	2	15.016	A	B	C	D	E	F	G	H	I	J	K	L	M				
CE-3.15-4	2	14.82		B	C	D	E	F	G	H	I	J	K	L	M				
Xs-H2O-0.01	2	14.57			C	D	E	F	G	H	I	J	K	L	M				
CE-H2O-1	2	14.334			C	D	E	F	G	H	I	J	K	L	M	N			
CE-3.15-2	2	14.172			C	D	E	F	G	H	I	J	K	L	M	N			
CE-H2O-0.5	2	14.127			C	D	E	F	G	H	I	J	K	L	M	N			
CE-Blk-2	2	14.111			C	D	E	F	G	H	I	J	K	L	M	N			
CE-3.15-1	2	13.914				D	E	F	G	H	I	J	K	L	M	N			
Xs-H2O-4	2	13.717				D	E	F	G	H	I	J	K	L	M	N			
CE-3.15-3	2	13.58					E	F	G	H	I	J	K	L	M	N			
Xs-H2O-3	2	13.526					E	F	G	H	I	J	K	L	M	N			
CE-3.15-0.01	2	13.39						F	G	H	I	J	K	L	M	N			
CE-H2O-2	2	13.320						F	G	H	I	J	K	L	M	N			
CE-Blk-0.05	2	12.647						F	G	H	I	J	K	L	M	N			
CE-3.15-5	2	12.4176						F	G	H	I	J	K	L	M	N	O		
Xs-3.5-1	2	12.385						F	G	H	I	J	K	L	M	N	O		
CE-Blk-1	2	11.77						F	G	H	I	J	K	L	M	N	O		
Xs-Blk-0.5	2	11.739						F	G	H	I	J	K	L	M	N	O		
Xs-3.5-2	2	11.599							G	H	I	J	K	L	M	N	O		
CE-Blk-0.01	2	11.45								H	I	J	K	L	M	N	O		
Xs-Blk-0.1	2	11.37									I	J	K	L	M	N	O		
CE-Blk-0.5	2	11.316										J	K	L	M	N	O		
CE-Blk-3	2	11.300										J	K	L	M	N	O		
Xs-Blk-0.01	2	11.21										J	K	L	M	N	O		
CE-Blk-5	2	10.891										J	K	L	M	N	O		
CE-Blk-0.2	2	10.751										J	K	L	M	N	O		
ST-C	2	10.566											K	L	M	N	O	P	
CE-Blk-0.1	2	10.380												L	M	N	O	P	
CE-H2O-5	2	10.190													M	N	O	P	
CE-Blk-4	2	10.11													M	N	O	P	
Xs-3.5-3	2	8.053														N	O	P	Q
Xs-Blk-1	2	6.33															O	P	Q
Xs-3.5-4	2	6.17															O	P	Q
Xs-Blk-4	2	4.2878																P	Q
Xs-3.5-5	2	3.142																	Q
Xs-Blk-2	2	2.3626																	Q
Xs-Blk-3	2	1.819																	Q
Xs-Blk-5	2	1.5038																	
Xs-H2O-5	2	1.2420																	

Means that do not share a letter are significantly different.

Table A2.23 Grouping information of exp (lag) of ST using the Tukey method and 95% confidence

ST	N	Mean	Grouping
Xs-3.5-2	2	160.49	A
Xs-H2O-0.01	2	132.51	B
Xs-H2O-2	2	124.26	B C
Xs-Blk-5	2	117.05	B C D
CE-H2O-0.01	2	115.01	B C D E
Xs-3.5-1	2	113.61	B C D E F
Xs-H2O-0.05	2	111.73	B C D E F G
Xs-H2O-0.5	2	108.48	B C D E F G H
CE-H2O-0.5	2	107.65	B C D E F G H
CE-H2O-0.05	2	106.21	B C D E F G H I
Xs-3.5-0.01	2	104.94	C D E F G H I J
CE-H2O-1	2	104.08	C D E F G H I J
CE-3.15-0.01	2	103.29	C D E F G H I J
CE-H2O-0.1	2	103.18	C D E F G H I J
Xs-H2O-0.1	2	102.14	C D E F G H I J K
Xs-3.5-5	2	101.48	C D E F G H I J K
CE-H2O-2	2	100.505	C D E F G H I J K L
Xs-H2O-0.2	2	99.57	C D E F G H I J K L
CE-H2O-0.2	2	98.42	C D E F G H I J K L M
Xs-H2O-1	2	96.07	D E F G H I J K L M
CE-3.15-0.05	2	94.71	D E F G H I J K L M N
Xs-3.5-0.5	2	94.67	D E F G H I J K L M N
Xs-3.5-0.05	2	93.97	D E F G H I J K L M N
CE-3.15-0.2	2	89.15	E F G H I J K L M N
CE-3.15-0.1	2	89.06	E F G H I J K L M N
Xs-3.5-0.2	2	86.97	F G H I J K L M N O
CE-3.15-2	2	86.85	F G H I J K L M N O
Xs-3.5-0.1	2	84.69	G H I J K L M N O
CE-3.15-1	2	84.1960	H I J K L M N O
CE-Blk-5	2	81.40	H I J K L M N O P
Xs-Blk-3	2	79.8	I J K L M N O P
Xs-Blk-2	2	77.65	J K L M N O P Q
Xs-3.5-4	2	75.416	K L M N O P Q R
Xs-Blk-4	2	73.912	L M N O P Q R S
CE-Blk-4	2	73.02	L M N O P Q R S T
CE-3.15-0.5	2	71.519	M N O P Q R S T U
CE-Blk-1	2	71.4	M N O P Q R S T U
CE-Blk-2	2	71.0	M N O P Q R S T U V
Xs-3.5-3	2	68.46	N O P Q R S T U V W
CE-Blk-3	2	60.903	O P Q R S T U V W X
CE-H2O-5	2	54.36	P Q R S T U V W X
CE-3.15-3	2	50.172	Q R S T U V W X
CE-3.15-4	2	49.66	R S T U V W X
CE-H2O-4	2	47.82	S T U V W X
CE-3.15-5	2	47.6940	S T U V W X
CE-Blk-0.1	2	45.79	T U V W X
CE-Blk-0.5	2	45.661	T U V W X
Xs-Blk-1	2	44.84	U V W X
CE-Blk-0.01	2	44.643	U V W X
CE-Blk-0.2	2	43.543	V W X
ST-C	2	43.29	W X
Xs-Blk-0.01	2	43.229	W X
Xs-Blk-0.5	2	43.212	W X
CE-Blk-0.05	2	42.78	W X
CE-H2O-3	2	42.60	W X
Xs-Blk-0.05	2	40.007	X Y
Xs-H2O-4	2	39.38	X Y
Xs-Blk-0.2	2	38.380	X Y
Xs-Blk-0.1	2	37.93	X Y
Xs-H2O-3	2	37.868	X Y
Xs-H2O-5	2	14.67	Y

Means that do not share a letter are significantly different.

Table A2.24 Grouping information of μ_{Max} of LB using the Tukey method and 95% confidence

Time (h)	N	Mean	Grouping														
CE-H2O-3	2	0.20970	A														
CE-H2O-4	2	0.208479	A	B													
CE-H2O-2	2	0.20567	A	B	C												
CE-H2O-0.5	2	0.2041	A	B	C												
Xs-H2O-4	2	0.19125	A	B	C	D											
CE-H2O-5	2	0.190459	A	B	C	D	E										
Xs-3.5-1	2	0.1901	A	B	C	D	E										
Xs-3.5-2	2	0.188661	A	B	C	D	E	F									
Xs-H2O-3	2	0.18634	A	B	C	D	E	F	G								
Xs-H2O-5	2	0.17977	A	B	C	D	E	F	G	H							
Xs-H2O-2	2	0.17925	A	B	C	D	E	F	G	H							
CE-H2O-1	2	0.17714	A	B	C	D	E	F	G	H							
CE-H2O-0.2	3	0.1760	A	B	C	D	E	F	G	H							
Xs-3.5-3	2	0.17270	A	B	C	D	E	F	G	H							
CE-H2O-0.1	3	0.1704	A	B	C	D	E	F	G	H							
CE-Blk-0.1	2	0.1698	A	B	C	D	E	F	G	H							
Xs-blk-2	2	0.16427	A	B	C	D	E	F	G	H							
Xs-blk-1	2	0.1616	A	B	C	D	E	F	G	H	I						
Xs-3.5-0.5	2	0.15958	A	B	C	D	E	F	G	H	I	J					
Xs-blk-3	2	0.15882	A	B	C	D	E	F	G	H	I	J					
CE-3.15-0.5	2	0.1555	A	B	C	D	E	F	G	H	I	J					
Xs-H2O-1	2	0.15434	A	B	C	D	E	F	G	H	I	J					
Xs-H2O-0.5	2	0.15417	A	B	C	D	E	F	G	H	I	J					
LB-C	2	0.15373		B	C	D	E	F	G	H	I	J					
Xs-blk-0.5	2	0.152063			C	D	E	F	G	H	I	J					
CE-3.15-0.2	2	0.14746				D	E	F	G	H	I	J	K				
CE-H2O-0.05	2	0.14681				D	E	F	G	H	I	J	K				
CE-Blk-0.05	2	0.14535				D	E	F	G	H	I	J	K				
Xs-H2O-0.2	2	0.1435				D	E	F	G	H	I	J	K				
Xs-3.5-0.2	2	0.142321				D	E	F	G	H	I	J	K				
Xs-3.5-0.01	2	0.1418				D	E	F	G	H	I	J	K				
CE-3.15-1	2	0.141523				D	E	F	G	H	I	J	K				
Xs-blk-0.05	2	0.13731				D	E	F	G	H	I	J	K				
CE-H2O-0.01	2	0.13688				D	E	F	G	H	I	J	K				
CE-3.15-0.05	2	0.136628				D	E	F	G	H	I	J	K				
CE-3.15-0.1	2	0.13641				D	E	F	G	H	I	J	K				
Xs-3.5-0.1	2	0.13564				D	E	F	G	H	I	J	K				
CE-Blk-0.2	2	0.135434				D	E	F	G	H	I	J	K				
Xs-blk-0.2	2	0.13499					E	F	G	H	I	J	K				
Xs-3.5-0.05	2	0.13383						F	G	H	I	J	K				
Xs-H2O-0.05	2	0.13316						F	G	H	I	J	K				
Xs-blk-0.01	2	0.13162							G	H	I	J	K				
Xs-H2O-0.1	2	0.1300								H	I	J	K				
CE-Blk-0.01	2	0.12789								H	I	J	K				
Xs-H2O-0.01	2	0.12759								H	I	J	K				
Xs-blk-0.1	2	0.125361								H	I	J	K				
CE-Blk-0.5	2	0.1251								H	I	J	K				
CE-3.15-0.01	2	0.12506								H	I	J	K				
CE-3.15-2	2	0.108271									I	J	K	L			
Xs-blk-4	2	0.10484										J	K	L	M		
Xs-3.5-4	2	0.09419											K	L	M	N	
CE-Blk-1	2	0.062551												L	M	N	O
Xs-blk-5	2	0.050197													M	N	O
CE-3.15-3	2	0.0403														N	O
Xs-3.5-5	2	0.03269															O
CE-3.15-4	2	0.019898															O

Means that do not share a letter are significantly different.

Table A2.25 Grouping information of Max of LB using the Tukey method and 95% confidence

Time (h)_2	N	Mean	Grouping								
Xs-H2O-5	2	0.8064	A								
Xs-H2O-4	2	0.8011	A	B							
Xs-H2O-3	2	0.7826	A	B	C						
Xs-H2O-2	2	0.7779	A	B	C	D					
CE-H2O-0.5	2	0.7113	A	B	C	D	E				
Xs-H2O-0.2	2	0.7083	A	B	C	D	E	F			
Xs-H2O-0.5	2	0.6955	A	B	C	D	E	F			
CE-3.15-0.5	2	0.6927	A	B	C	D	E	F	G		
Xs-3.5-0.5	2	0.6890	A	B	C	D	E	F	G		
CE-H2O-4	2	0.67390	A	B	C	D	E	F	G		
CE-H2O-3	2	0.6612	A	B	C	D	E	F	G	H	
Xs-H2O-1	2	0.656	A	B	C	D	E	F	G	H	
CE-H2O-2	2	0.64349	A	B	C	D	E	F	G	H	
Xs-blk-3	2	0.6429	A	B	C	D	E	F	G	H	
CE-Blk-0.1	2	0.6408	A	B	C	D	E	F	G	H	
CE-3.15-1	2	0.63779	A	B	C	D	E	F	G	H	
Xs-3.5-1	2	0.6318	A	B	C	D	E	F	G	H	I
Xs-H2O-0.1	2	0.6314	A	B	C	D	E	F	G	H	I
Xs-blk-1	2	0.6212	A	B	C	D	E	F	G	H	I
CE-3.15-2	2	0.618681	A	B	C	D	E	F	G	H	I
Xs-blk-2	2	0.6174	A	B	C	D	E	F	G	H	I
CE-H2O-1	2	0.61429	A	B	C	D	E	F	G	H	I
CE-H2O-5	2	0.61407	A	B	C	D	E	F	G	H	I
Xs-3.5-0.05	2	0.608425	A	B	C	D	E	F	G	H	I
Xs-blk-0.5	2	0.59886	A	B	C	D	E	F	G	H	I
Xs-blk-0.2	2	0.5913	A	B	C	D	E	F	G	H	I
CE-3.15-0.05	2	0.58577	A	B	C	D	E	F	G	H	I
Xs-H2O-0.05	2	0.58372	A	B	C	D	E	F	G	H	I
Xs-3.5-0.2	2	0.5813	A	B	C	D	E	F	G	H	I
Xs-blk-5	2	0.5809	A	B	C	D	E	F	G	H	I
Xs-blk-0.1	2	0.5712	A	B	C	D	E	F	G	H	I
Xs-blk-0.05	2	0.57112	A	B	C	D	E	F	G	H	I
CE-3.15-0.2	2	0.5603	A	B	C	D	E	F	G	H	I J
CE-3.15-0.1	2	0.5597	A	B	C	D	E	F	G	H	I J
CE-H2O-0.05	2	0.553109		B	C	D	E	F	G	H	I J
Xs-3.5-0.01	2	0.5471			C	D	E	F	G	H	I J
Xs-3.5-2	2	0.5398			C	D	E	F	G	H	I J
Xs-blk-4	2	0.5360			C	D	E	F	G	H	I J
Xs-3.5-0.1	2	0.5347			C	D	E	F	G	H	I J
CE-Blk-0.05	2	0.53448			C	D	E	F	G	H	I J
CE-H2O-0.01	2	0.527273				D	E	F	G	H	I J
Xs-H2O-0.01	2	0.5150					E	F	G	H	I J
LB-C	2	0.5092					E	F	G	H	I J
CE-Blk-0.2	2	0.50556					E	F	G	H	I J
Xs-blk-0.01	2	0.5041					E	F	G	H	I J
CE-Blk-0.5	2	0.4998					E	F	G	H	I J
CE-H2O-0.1	2	0.49364					E	F	G	H	I J
Xs-3.5-3	2	0.4876					E	F	G	H	I J
CE-3.15-0.01	2	0.48080					E	F	G	H	I J
CE-Blk-0.01	2	0.4780					E	F	G	H	I J
CE-H2O-0.2	2	0.4567						F	G	H	I J
CE-3.15-3	2	0.4422							G	H	I J
CE-Blk-1	2	0.42139								H	I J
CE-3.15-4	2	0.3808									I J
Xs-3.5-4	2	0.3100									J

Means that do not share a letter are significantly different.

Table A2.26 Grouping information of log (lag) of LB using the Tukey method and 95% confidence

Time (h)_2	N	Mean	Grouping										
Xs-3.5-5	2	1.1527	A										
CE-Blk-1	2	1.13119	A	B									
Xs-blk-5	2	1.12906	A	B									
Xs-blk-4	2	1.12615	A	B									
CE-3.15-2	2	1.06897	A	B	C								
Xs-blk-3	2	1.0615		B	C								
Xs-3.5-4	2	1.0558		B	C	D							
Xs-H2O-5	2	1.03214			C	D	E						
Xs-3.5-3	2	1.01626			C	D	E						
Xs-blk-0.01	2	1.00999			C	D	E	F					
CE-Blk-0.05	2	1.00747			C	D	E	F					
CE-H2O-0.01	2	1.00540			C	D	E	F					
CE-3.15-0.01	2	1.00514			C	D	E	F					
Xs-H2O-0.01	2	1.00266			C	D	E	F					
Xs-blk-0.05	2	1.00176			C	D	E	F					
CE-Blk-0.01	2	1.00068			C	D	E	F					
Xs-H2O-0.05	2	0.999101			C	D	E	F					
Xs-blk-2	2	0.9975			C	D	E	F					
Xs-3.5-0.01	2	0.99622			C	D	E	F					
CE-H2O-0.05	2	0.993934			C	D	E	F					
Xs-blk-0.1	2	0.99382			C	D	E	F					
Xs-H2O-4	2	0.99239			C	D	E	F					
Xs-3.5-0.05	2	0.990539			C	D	E	F					
Xs-3.5-2	2	0.9872			C	D	E	F	G				
CE-3.15-0.05	2	0.98692			C	D	E	F	G				
CE-Blk-0.5	2	0.9835			C	D	E	F	G	H			
LB-C	2	0.97451				D	E	F	G	H			
CE-3.15-0.1	2	0.9715				D	E	F	G	H	I		
Xs-3.5-0.1	2	0.9701				D	E	F	G	H	I		
Xs-H2O-3	2	0.96765					E	F	G	H	I	J	
Xs-H2O-1	2	0.96712					E	F	G	H	I	J	
CE-3.15-1	2	0.966840					E	F	G	H	I	J	
CE-Blk-0.2	2	0.96577					E	F	G	H	I	J	
Xs-H2O-0.1	2	0.9638					E	F	G	H	I	J	K
Xs-blk-0.2	2	0.9617					E	F	G	H	I	J	K
Xs-blk-1	2	0.9616					E	F	G	H	I	J	K
Xs-H2O-2	2	0.96086					E	F	G	H	I	J	K
Xs-blk-0.5	2	0.9606					E	F	G	H	I	J	K
Xs-H2O-0.2	2	0.9599					E	F	G	H	I	J	K
Xs-3.5-0.2	2	0.9586					E	F	G	H	I	J	K
Xs-3.5-1	2	0.9580					E	F	G	H	I	J	K
CE-3.15-0.2	2	0.9572					E	F	G	H	I	J	K
CE-H2O-0.1	3	0.9561					E	F	G	H	I	J	K
CE-3.15-0.5	2	0.9549					E	F	G	H	I	J	K
CE-Blk-0.1	2	0.95425					E	F	G	H	I	J	K
Xs-H2O-0.5	2	0.9491					E	F	G	H	I	J	K
CE-H2O-0.2	3	0.9320						F	G	H	I	J	K
Xs-3.5-0.5	2	0.9287						F	G	H	I	J	K
CE-H2O-1	2	0.90309							G	H	I	J	K
CE-H2O-5	2	0.89823								H	I	J	K
CE-H2O-2	2	0.887604									I	J	K
CE-H2O-3	2	0.88235										J	K
CE-H2O-4	2	0.87898											K
CE-H2O-0.5	2	0.8787											K

Means that do not share a letter are significantly different.

Table A2.27 Grouping information of μ_{Max} of LA using the Tukey method and 95% confidence

prob	N	Mean	Grouping
Xs-H2O-2	4	0.12139	A
Xs-H2O-3	3	0.10271	A B
Xs-H2O-4	4	0.09951	B C
CE-3.15-3	4	0.09294	B C D
Xs-H2O-1	4	0.09289	B C D
CE-3.15-4	4	0.08885	B C D E
Xs-Blk-1	6	0.0861	B C D E F
CE-H2O-2	4	0.08606	B C D E F G H
CE-H2O-3	4	0.08513	B C D E F G H I J K
Xs-Blk-0.5	8	0.085	B C D E F
CE-H2O-4	4	0.08259	B C D E F G H I J K L
CE-3.15-2	4	0.08226	B C D E F G H I J K L
CE-H2O-1	4	0.07976	C D E F G H I J K L M N
CE-Blk-2	6	0.07962	D E F G H I J K L M
CE-3.15-5	4	0.07911	D E F G H I J K L M N
CE-Blk-4	4	0.07831	D E F G H I J K L M N
Xs-Blk-0.1	4	0.07796	D E F G H I J K L M N
CE-Blk-1	6	0.07793	D E F G H I J K L M N
Xs-Blk-0.2	4	0.07758	D E F G H I J K L M N O
CE-Blk-3	4	0.07648	D E F G H I J K L M N O P
Xs-3.5-0.1	2	0.07566	C D E F G H I J K L M N O P Q R S T U V W X
Xs-Blk-2	6	0.0753	D E F G H I J K L M N O P
CE-3.15-0.5	2	0.074454	D E F G H I J K L M N O P Q R S T U V W X Y Z AA
Xs-H2O-5	4	0.07402	D E F G H I J K L M N O P S V
CE-Blk-0.5	6	0.07297	E F G H I J K L M N O P S V
CE-Blk-5	4	0.0721	E F G H I J K L M N O P Q R S T U V W X
CE-H2O-0.5	2	0.071903	D E F G H I J K L M N O P Q R S T U V W X Y Z AA AB AC AD AE
CE-Blk-0.1	4	0.07174	E F G H I J K L M N O P Q R S T U V W X
CE-3.15-1	4	0.07136	E F G H I J K L M N O P Q R S T U V W X Y Z AA
Xs-3.5-1	4	0.0711	E F G H I J K L M N O P Q R S T U V W X Y Z AA
CE-Blk-0.2	4	0.06857	F G H I J K L M N O P Q R S T U V W X Y Z AA AB AC AD AE

CE-H2O-5	4	0.06738				H			K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE												
Xs-Blk-0.05	4	0.06677			G	H		J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE												
Xs-Blk-0.01	2	0.06634	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH									
CE-Blk-0.01	4	0.06587					I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE												
Xs-3.5-0.2	2	0.065713	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH									
Xs-H2O-0.5	2	0.06487	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH									
CE-Blk-0.05	4	0.06191									M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH									
CE-3.15-0.2	2	0.0602								L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK						
Xs-3.5-2	4	0.06002										N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH									
CE-H2O-0.2	2	0.058809								L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN			
Xs-H2O-0.2	2	0.05872								L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN			
Control	4	0.05801											O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH		AJ							
CE-3.15-0.1	2	0.053089												P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO		
Xs-Blk-3	2	0.05191																		V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO		
Xs-3.5-0.5	2	0.050889															S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO		
Xs-H2O-0.1	2	0.04918														R			U			X			AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
CE-3.15-0.05	2	0.048161													Q	R		T	U		W	X		Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
Xs-3.5-0.05	2	0.046983																					Y	Z	AA			AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
Xs-H2O-0.05	2	0.046208																									AC		AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
CE-H2O-0.1	2	0.04588																								AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
Xs-3.5-3	4	0.045718																														AH	AI	AJ	AK	AL	AM	AN	AO	AP	
Xs-3.5-0.01	2	0.039223																													AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
Xs-Blk-4	2	0.03866																												AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ
Xs-3.5-4	4	0.03544																																				AN	AO	AP	AQ
CE-3.15-0.01	2	0.03464																																AJ	AK	AL	AM	AN	AO	AP	AQ
CE-H2O-0.01	2	0.03249																															AI		AK		AM	AN	AO	AP	AQ
Xs-H2O-0.01	2	0.03149																																		AL	AM	AN	AO	AP	AQ
CE-H2O-0.05	2	0.026089																																					AO	AP	AQ
Xs-3.5-5	3	0.02484																																						AP	AQ
Xs-Blk-5	2	0.02118																																							AQ

Table A2.28 Grouping information of Max of LA using the Tukey method and 95% confidence

prob	N	Mean	Grouping												
Xs-H2O-4	4	1.0238	A												
Xs-H2O-3	4	0.8098		B											
Xs-H2O-5	4	0.7748		B											
CE-3.15-4	4	0.5846			C										
CE-3.15-5	4	0.5803			C										
CE-H2O-4	4	0.5303			C	D									
CE-H2O-3	4	0.5229			C	D	E								
CE-3.15-3	4	0.5021			C	D	E	F							
CE-H2O-2	4	0.48014			C	D	E	F	G						
Xs-H2O-2	4	0.4565			C	D	E	F	G	H					
CE-3.15-0.5	2	0.45067			C	D	E	F	G	H	I				
Xs-3.5-0.1	2	0.41565			C	D	E	F	G	H	I	J	K	L	
CE-3.15-2	4	0.4115				D	E	F	G	H	I				
CE-H2O-1	4	0.4037				D	E	F	G	H	I		K		
Xs-3.5-1	4	0.40223				D	E	F	G	H	I		K		
Xs-3.5-2	4	0.40057				D	E	F	G	H	I		K		
CE-H2O-0.5	2	0.39581			C	D	E	F	G	H	I	J	K	L	
CE-H2O-5	4	0.3766				D	E	F	G	H	I	J	K	L	
Xs-3.5-3	4	0.37534				D	E	F	G	H	I	J	K	L	
CE-Blk-5	4	0.3698				D	E	F	G	H	I	J	K	L	
Xs-H2O-0.5	2	0.36433				D	E	F	G	H	I	J	K	L	M
CE-H2O-0.2	2	0.362437				D	E	F	G	H	I	J	K	L	M
Xs-H2O-0.1	2	0.3604				D	E	F	G	H	I	J	K	L	M
CE-3.15-0.2	2	0.359034				D	E	F	G	H	I	J	K	L	M
Xs-H2O-0.2	2	0.3559				D	E	F	G	H	I	J	K	L	M
Xs-3.5-0.2	2	0.35464				D	E	F	G	H	I	J	K	L	M
CE-3.15-0.1	2	0.35292				D	E	F	G	H	I	J	K	L	M
Xs-Blk-2	6	0.3492							G	H	I	J	K	L	
Xs-H2O-0.05	2	0.34803				D	E	F	G	H	I	J	K	L	M
CE-Blk-3	4	0.3397						F	G	H	I	J	K	L	M
CE-3.15-1	4	0.33893						F	G	H	I	J	K	L	M
CE-3.15-0.05	2	0.33730				D	E	F	G	H	I	J	K	L	M
Xs-H2O-1	4	0.33645							G	H	I	J	K	L	M
CE-Blk-2	6	0.3336							G	H	I	J	K	L	M
CE-Blk-4	4	0.3293							G	H	I	J	K	L	M
Xs-Blk-1	6	0.3268								H	I	J	K	L	M
CE-H2O-0.1	2	0.3265					E	F	G	H	I	J	K	L	M
Xs-3.5-0.5	2	0.31389						F	G	H	I	J	K	L	M
Xs-3.5-0.05	2	0.3129						F	G	H	I	J	K	L	M
Xs-Blk-3	2	0.3098						F	G	H	I	J	K	L	M
CE-Blk-0.01	4	0.3094								H	I	J	K	L	M
Xs-3.5-0.01	2	0.30924						F	G	H	I	J	K	L	M
CE-H2O-0.01	2	0.2911							G	H	I	J	K	L	M
Control	4	0.2881									I	J	K	L	M
Xs-H2O-0.01	2	0.2864							G	H	I	J	K	L	M
CE-Blk-0.2	4	0.2860									I	J	K	L	M
CE-Blk-1	6	0.2838									I	J	K	L	M
CE-3.15-0.01	2	0.28327							G	H	I	J	K	L	M
CE-Blk-0.5	6	0.27687									I	J	K	L	M
CE-Blk-0.1	4	0.2715									I	J	K	L	M
Xs-Blk-0.01	4	0.2698									I	J	K	L	M
Xs-Blk-0.1	4	0.26586									I	J	K	L	M
Xs-Blk-0.5	8	0.2655											K	L	M
Xs-3.5-4	4	0.26430									I	J	K	L	M
CE-Blk-0.05	4	0.25914									I	J	K	L	M
Xs-Blk-0.05	4	0.25629									I	J	K	L	M
CE-H2O-0.05	2	0.24585									I	J	K	L	M
Xs-Blk-4	2	0.2455									I	J	K	L	M
Xs-Blk-0.2	4	0.2195										J		L	M
Xs-3.5-5	4	0.1528													N
Xs-Blk-5	2	0.14626													M

Means that do not share a letter are significantly different.

Table A2.29 Grouping information of log (lag) of LA using the Tukey method and 95% confidence

prob	N	Mean	Grouping					
Xs-3.5-5	3	1.00187	A					
Xs-3.5-4	4	0.9706	A	B				
Xs-3.5-3	4	0.9196	A	B	C			
CE-3.15-3	4	0.9066	A	B	C	D		
CE-3.15-5	4	0.8987	A	B	C	D	E	
Xs-Blk-5	2	0.89363	A	B	C	D	E	F	G
Xs-Blk-3	2	0.8925	A	B	C	D	E	F	G
CE-Blk-4	4	0.8918	A	B	C	D	E	F	
Xs-3.5-2	4	0.8890	A	B	C	D	E	F	
CE-3.15-4	4	0.8867	A	B	C	D	E	F	
Xs-3.5-0.1	2	0.878407	A	B	C	D	E	F	G
CE-Blk-5	4	0.8783	A	B	C	D	E	F	
Xs-Blk-2	6	0.8725	A	B	C	D	E	F	
CE-H2O-0.2	2	0.86463	A	B	C	D	E	F	G
Xs-Blk-0.2	4	0.8607	A	B	C	D	E	F	G
CE-H2O-0.1	2	0.86035	A	B	C	D	E	F	G
CE-3.15-0.5	2	0.8596	A	B	C	D	E	F	G
Xs-3.5-0.2	2	0.85784	A	B	C	D	E	F	G
CE-H2O-0.5	2	0.85703	A	B	C	D	E	F	G
Xs-Blk-0.5	8	0.8550			C	D	E	F	
Xs-Blk-4	2	0.85451	A	B	C	D	E	F	G
Xs-3.5-1	4	0.8542		B	C	D	E	F	G
CE-3.15-0.05	2	0.85175	A	B	C	D	E	F	G
CE-3.15-2	4	0.8515		B	C	D	E	F	G
Xs-Blk-1	6	0.8492		B	C	D	E	F	G
CE-Blk-3	4	0.8472		B	C	D	E	F	G
CE-H2O-5	3	0.8457		B	C	D	E	F	G
CE-Blk-2	6	0.8450			C	D	E	F	G
CE-H2O-4	4	0.8357			C	D	E	F	G
Xs-Blk-0.1	4	0.8306			C	D	E	F	G
CE-3.15-0.2	2	0.8276		B	C	D	E	F	G
CE-3.15-0.1	2	0.82727		B	C	D	E	F	G
CE-H2O-3	4	0.8241			C	D	E	F	G
Xs-3.5-0.05	2	0.82367		B	C	D	E	F	G
CE-Blk-1	6	0.8219			C	D	E	F	G
Xs-Blk-0.05	4	0.8191			C	D	E	F	G
Control	4	0.8142			C	D	E	F	G
Xs-3.5-0.01	2	0.81389		B	C	D	E	F	G
Xs-Blk-0.01	4	0.8137			C	D	E	F	G
CE-H2O-0.01	2	0.81371		B	C	D	E	F	G
CE-3.15-0.01	2	0.81281		B	C	D	E	F	G
CE-Blk-0.1	4	0.8120			C	D	E	F	G
CE-H2O-2	4	0.8085			C	D	E	F	G
Xs-3.5-0.5	2	0.807232			C	D	E	F	G
CE-3.15-1	4	0.80463			C	D	E	F	G
CE-Blk-0.5	6	0.8040			C	D	E	F	G
CE-H2O-1	4	0.8024			C	D	E	F	G
CE-Blk-0.01	4	0.8000			C	D	E	F	G
Xs-H2O-0.05	2	0.79996			C	D	E	F	G
CE-H2O-0.05	2	0.79873			C	D	E	F	G
CE-Blk-0.05	4	0.7975			C	D	E	F	G
Xs-H2O-0.2	2	0.78910			C	D	E	F	G
CE-Blk-0.2	4	0.7874			C	D	E	F	G
Xs-H2O-5	4	0.7770				D	E	F	G
Xs-H2O-1	4	0.7736				D	E	F	G
Xs-H2O-0.01	2	0.7719			C	D	E	F	G
Xs-H2O-0.1	2	0.76922			C	D	E	F	G
Xs-H2O-2	4	0.7686					E	F	G
Xs-H2O-0.5	2	0.76632			C	D	E	F	G
Xs-H2O-4	4	0.7608						F	G
Xs-H2O-3	4	0.7341							G

Means that do not share a letter are significantly different.

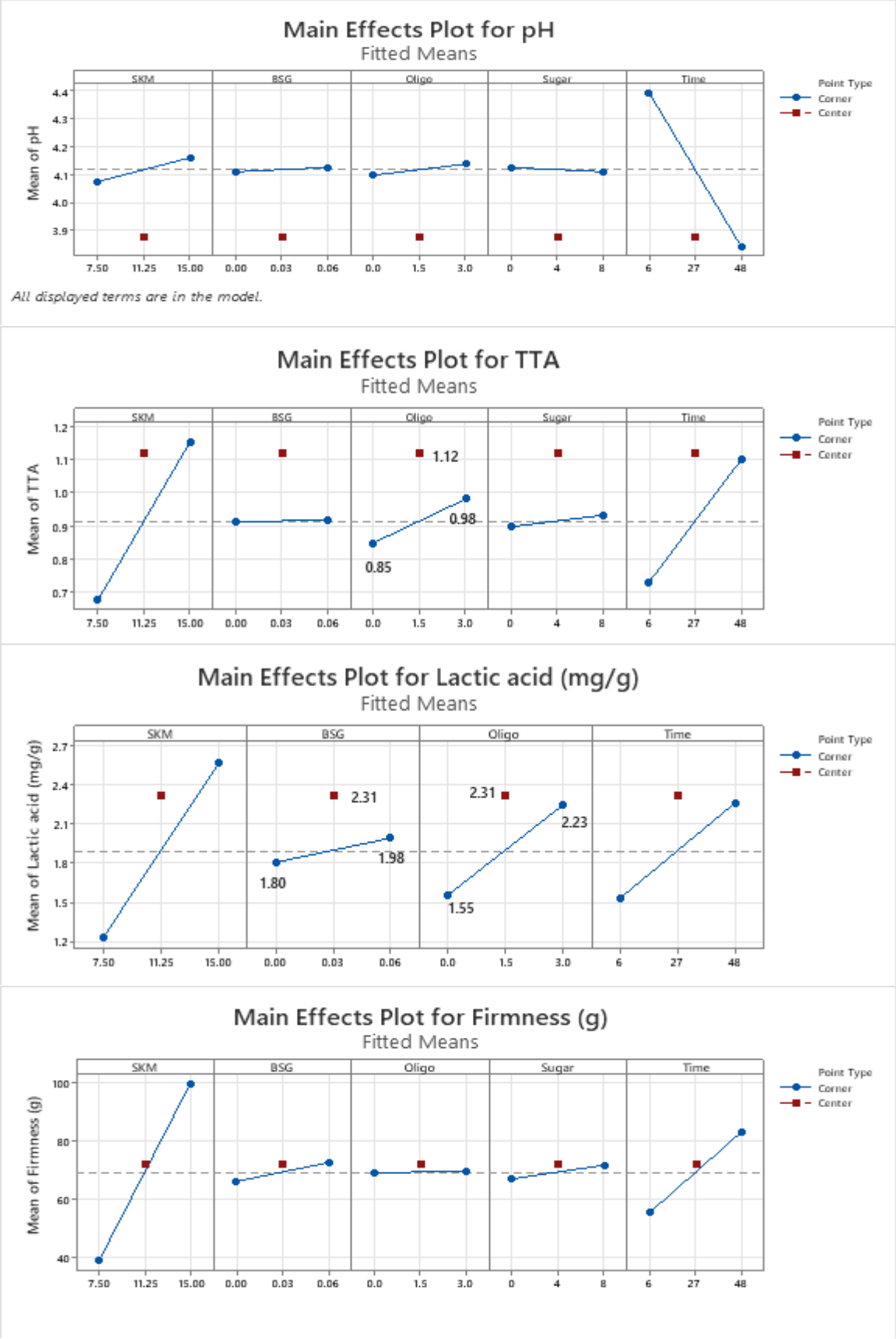
2.4.2 Yogurt fortified with BSG-Oligosaccharides, BSG and erythritol – profile study

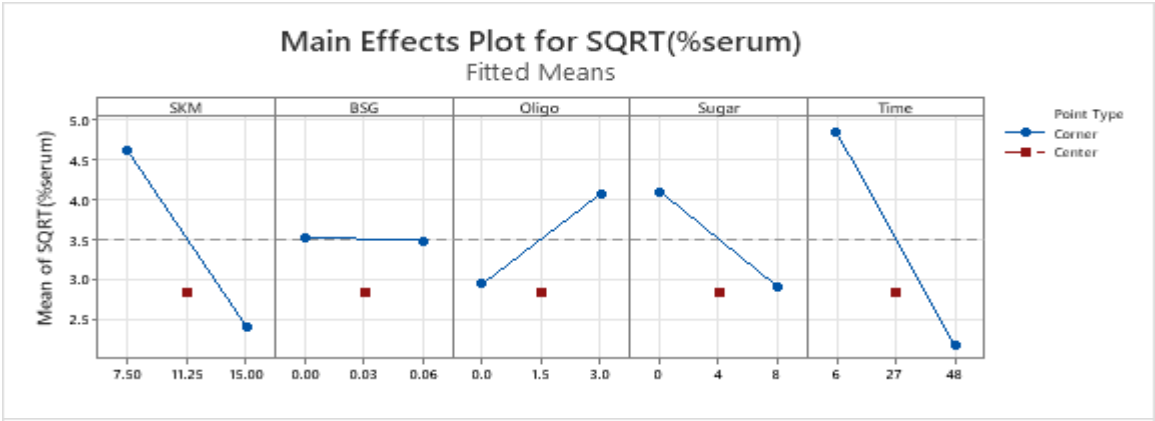
Table A2.30 Information of sample runs, and data collected for yogurt making in a quarter factorial design.

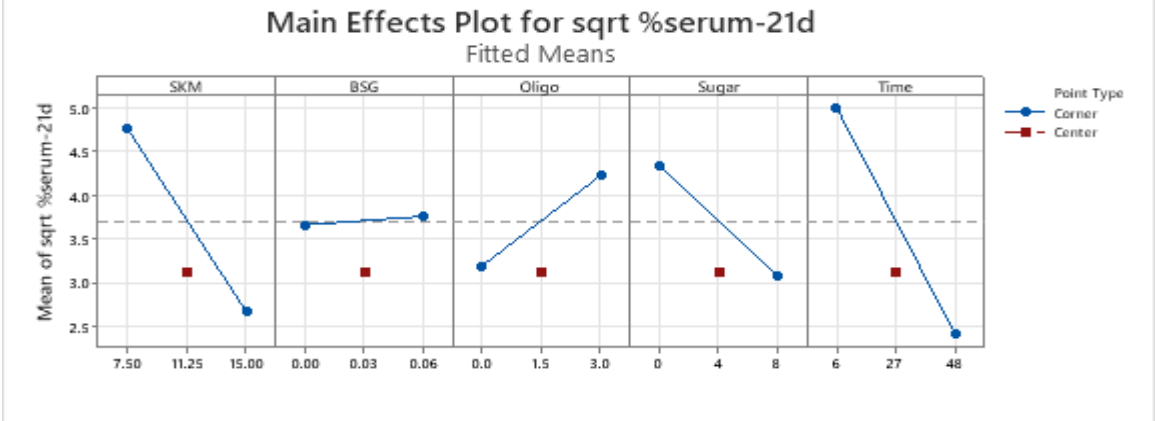
Run number and information SMP:BSG:Oligo:cane sugar:erythritol:time	Factors						Response variables											
	SKM % (w/v)	BSG % (w/v)	Oligo % (w/v)	Cane sugar % (w/v)	Erythritol % (w/v)	Time (h)	pH	TTA%	Lactic acid (mg/g)	firmness(g)	%serum	% serum -21d log(cfu/g)	ST log(cfu/g)	ST-21d log(cfu/g)	LB log(cfu/g)	LB-21d log(cfu/g)	LA log(cfu/g)	LA-21d log(cfu/g)
1-7.5-0-0-8-0-48	7.5	0	0	8	0	48	3.77	0.83	0.97	49.91	3.01	3.25	6.91	5.56	4.24	3.8	6.44	1.67
2-7.5-0-3-8-0-6	7.5	0	3	8	0	6	4.35	0.55	1.35	26.17	39.93	40.07	8.51	8.6	5.38	5.33	7.14	5.9
3-7.5-0.06-0-0-8-48	7.5	0.06	0	0	8	48	3.79	0.76	1.52	54.77	13.39	16.67	6.27	5.96	5.11	2.65	5.52	4.42
4-7.5-0.06-3-0-8-6	7.5	0.06	3	0	8	6	4.39	0.57	1.07	24.9	44.58	45.68	8.74	8.71	5.66	5.22	6.94	6.18
5-11.25-0.03-1.5-4-4-27	11.25	0.03	1.5	4	4	27	3.9	1.11	2.33	71.41	7.87	9.2	9.17	8.73	5.57	5.13	7.34	6.36
6-11.25-0.03-1.5-4-4-27	11.25	0.03	1.5	4	4	27	3.85	1.12	2.3	72.52	8.27	10.27	9.1	8.75	5.7	5.2	7.4	6.24
7-15-0-0-0-8-6	15	0	0	0	8	6	4.42	0.89	1.73	83.34	10.8	11.86	9.46	9.35	7.12	5.08	7.23	5.38
8-15-0-3-0-8-48	15	0	3	0	8	48	3.9	1.37	3.14	104.69	7.58	9.31	6.68	5.81	5.93	2.08	7.02	6.35
9-15-0.06-0-8-0-6	15	0.06	0	8	0	6	4.41	0.91	1.96	87.79	9.4	11.81	9.41	9.39	5.74	5.49	7.24	5.86
10-15-0.06-3-8-0-48	15	0.06	3	8	0	48	3.91	1.43	3.38	122.66	0.27	0.55	6.49	5.82	5.48	4.46	6.95	6.36

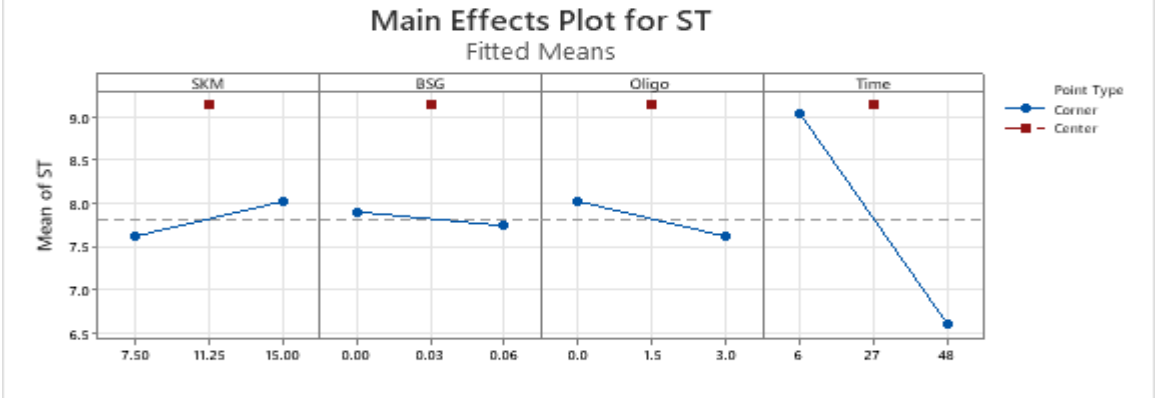
Table A2.31 Data analysis of response variables in the fractional factorial design for yogurt making.

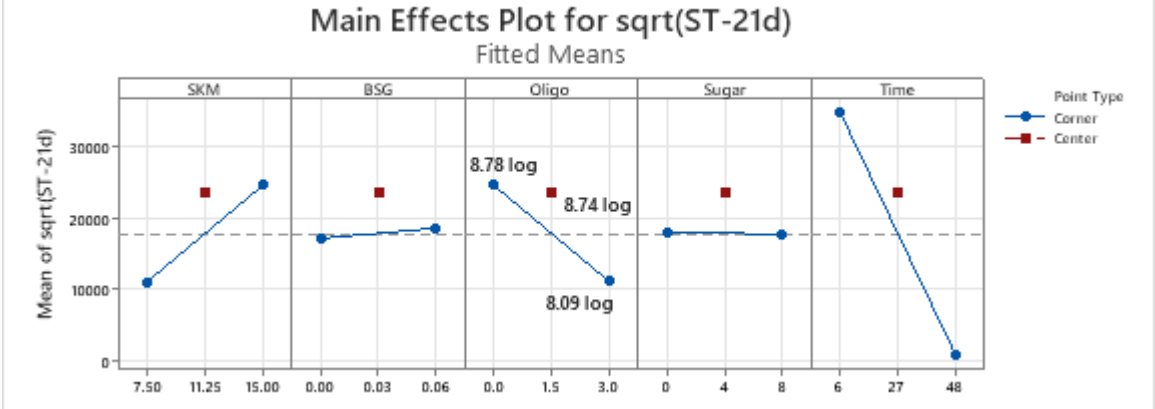
	pH	TTA	Lactic acid	Firmness	Serum-1d	Serum-21d	ST-0d	ST-21d	LB-0d	LB-21d	LA-0d	LA-21d
Source	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**	**P-Value**
Model	0.006	0.001	0.003	0.003	0.003	0.005	0.001	0.000	0.002	0.019	0.015	0.014
Linear	0.005	0.001	0.002	0.002	0.002	0.004	0.001	0.000	0.001	0.013	0.016	0.012
SMP	0.041	0.000	0.001	0.001	0.001	0.002	0.009	0.000	0.001	-	0.009	0.020
BSG	0.486	0.356	0.039	0.041	0.653	0.436	0.051	0.011	0.097	0.147	0.073	0.162
Oligo	0.152	0.003	0.003	0.680	0.005	0.009	0.008	0.000	-	0.759	0.041	0.003
Sugar	0.486	0.048	-	0.075	0.005	0.006	-	0.325	0.002	0.048	0.114	-
Time	0.001	0.000	0.003	0.002	0.001	0.002	0.000	0.000	0.002	0.003	0.006	0.165
2-Way Interactions	0.629	0.072	0.032	0.069	0.009	0.009	0.028	0.014	0.012	0.095	0.264	0.199
BSG*Time	-	-	0.032	0.069	-	-	0.022	0.014	0.012	-	0.264	0.199
BSG*Oligo	0.629	0.072	0.031	-	0.009	0.009	0.040	-	-	0.095	-	-
Curvature	0.007	0.002	0.010	0.220	0.019	0.035	0.001	0.001	0.553	0.190	0.006	0.012
Lack-of-Fit	1.000	0.410	0.200	0.186	0.189	0.414	0.418	0.937	0.539	0.317	0.738	0.967
R-sq	99.83%	99.97%	99.91%	99.92%	99.91%	99.86%	99.96%	100.00%	99.36%	97.25%	99.58%	97.77%


Main Effects Plot for pH
Fitted Means
SKM
BSG
Oligo
Sugar
Time
Mean of pH
4.4
4.3
4.2
4.1
4.0
3.9
7.50
11.25
15.00
0.00
0.03
0.06
0.0
1.5
3.0
0
4
8
6
27
48
Point Type
Corner
Center
All displayed terms are in the model.
Main Effects Plot for TTA
Fitted Means
Mean of TTA
1.2
1.1
1.0
0.9
0.8
0.7
1.12
0.98
0.85
Main Effects Plot for Lactic acid (mg/g)
Fitted Means
Mean of Lactic acid (mg/g)
2.7
2.4
2.1
1.8
1.5
1.2
2.31
1.98
1.80
2.31
2.23
1.55
Main Effects Plot for Firmness (g)
Fitted Means
Mean of Firmness (g)
100
80
60
40


Main Effects Plot for SQRT(%serum)
Fitted Means
SKM
BSG
Oligo
Sugar
Time
Mean of SQRT(%serum)
5.0
4.5
4.0
3.5
3.0
2.5
7.50
11.25
15.00
0.00
0.03
0.06
0.0
1.5
3.0
0
4
8
6
27
48
Point Type
Corner
Center


Main Effects Plot for sqrt %serum-21d
Fitted Means
SKM
BSG
Oligo
Sugar
Time
Mean of sqrt %serum-21d
5.0
4.5
4.0
3.5
3.0
2.5
7.50
11.25
15.00
0.00
0.03
0.06
0.0
1.5
3.0
0
4
8
6
27
48
Point Type
Corner
Center


Main Effects Plot for ST
Fitted Means
SKM
BSG
Oligo
Time
Mean of ST
9.0
8.5
8.0
7.5
7.0
6.5
7.50
11.25
15.00
0.00
0.03
0.06
0.0
1.5
3.0
6
27
48
Point Type
Corner
Center


Main Effects Plot for sqrt(ST-21d)
Fitted Means
SKM
BSG
Oligo
Sugar
Time
Mean of sqrt(ST-21d)
30000
20000
10000
0
8.78 log
8.74 log
8.09 log
7.50
11.25
15.00
0.00
0.03
0.06
0.0
1.5
3.0
0
4
8
6
27
48
Point Type
Corner
Center

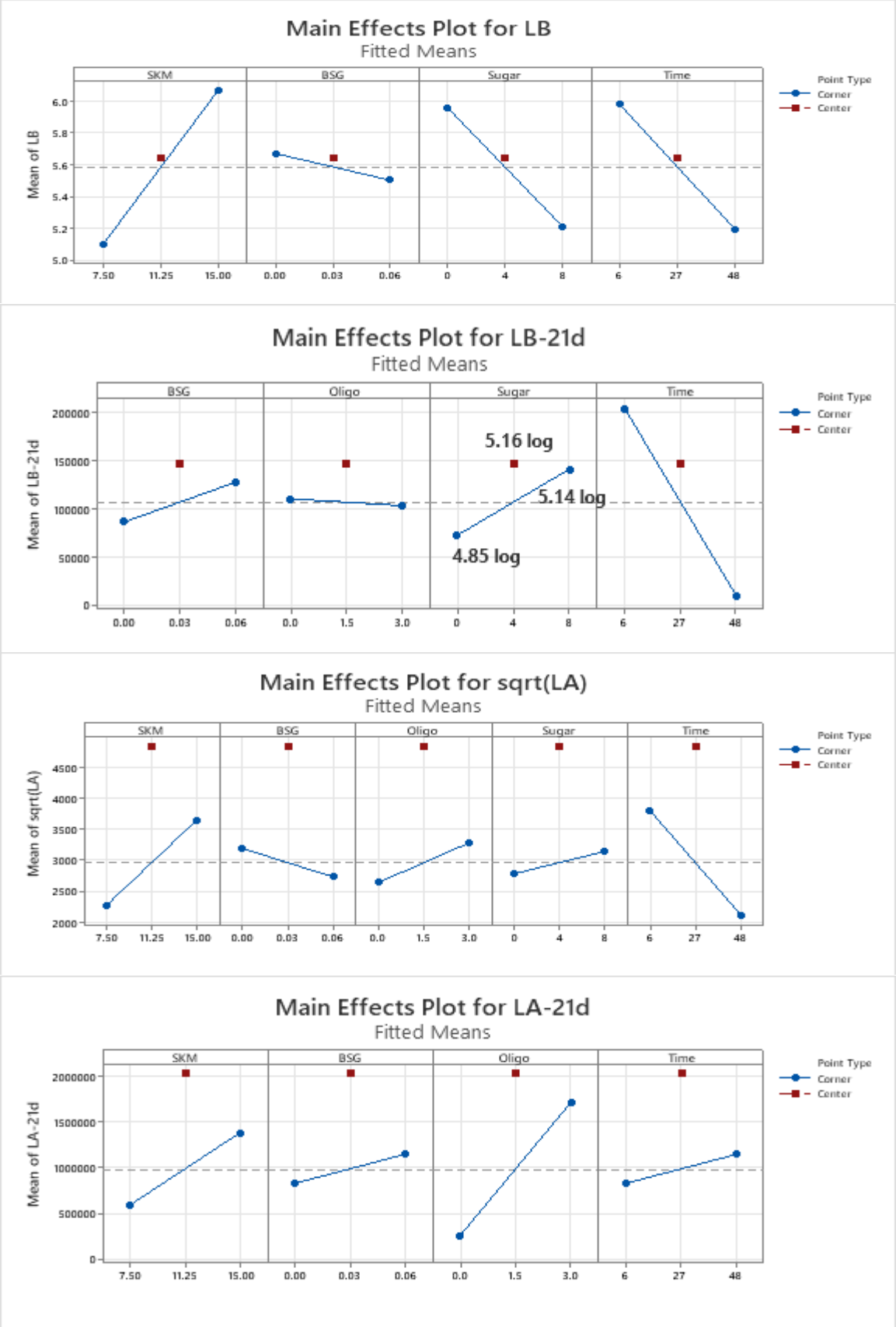


Figure A2.1 Main effect plots of all response variables in the fractional factorial design for yogurt making.