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Locust bean gum hydrolysis:

Impact on molecular and functional properties

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Abstract

Locust bean gum (LBG) is a widely encountered additive in the food industry, with particular application in ice cream production, functioning as a viscosifier and stabilizer, where it reduces ice crystallization and slows down the melting rate. However, its incompatibility property with milk proteins, especially when mixed under high concentrations can lead to phase separation and wheying, often necessitating the use of additional stabilisers such as carrageenan, to mitigate this issue. Enzymatic hydrolysis was hypothesised as a potential method to modify the LBG structure, and thus its phase behaviour, by breaking native LBG chain into fractions with lower molecular weight. It was also anticipated that this would also reduce the LBG viscosity in solution.

In this study, the rheological behaviour of LBG during enzymatic hydrolysis was explored and the impact of hydrolysis on phase behaviour in model LBG/skim milk powder (SMP) solutions containing milk protein was studied. The effect of hydrolysis of LBG on its functional properties in ice cream was also investigated.

β-mannanase was chosen to hydrolyse the LBG. Rheological results indicated that the enzymatic hydrolysis could be used to progressively reduce relative viscosity of solution, noting that termination of the reaction was made challenging by the high heat stability observed for the enzyme. The results of Confocal Laser Scanning Microscope (CLSM) as well as the phase diagram plotted for LBG/SMP mixtures implied that hydrolysis of LBG slowed down the phase separation process. This was also evident when being applied in the ice cream making. Hydrolysis of LBG showed a more compatible result with protein during the aging stage. Interestingly, hydrolysis of LBG did not have significant effect on other properties in the ice cream, with a relatively low overrun value (73.3%) compared to the non-hydrolysed LBG (94.4%), and a slightly higher meltdown rate (0.26g/min) compared to the normal LBG (0.24g/min).

Overall, hydrolysis of LBG can improve the compatibility of polysaccharide/protein mixtures and has a promising prospect to be applied in the food industry.

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List of Abbreviations

LBG	Locust Bean Gum
SMP	Skim Milk Powder
IEP	Isoelectric Point
MW	Molecular Weight
CLSM	Confocal Laser Scanning Microscopy
ORD	Oxidative-Reductive Depolymerised
FBI	Flow Behaviour Index
PHGG	Partially Hydrolysed Guar Gum
E	Enzyme
E-BMACJ	endo-1,4-β-mannanase from *Cellvibrio japonicus* (*C. japonicus*)

Chapter 1 General introduction

Ice cream is one of the most important products in the dairy industry. The results of a survey indicated that the average American consumed about 5.5 kg of regular ice cream in 2019 (Wunsch, 2020). The total sales of ice cream in the US was reported to be 6.97 billion US dollars in 2020 (Wunsch, 2021). There was a 2.5% growth in the sales of ice cream in New Zealand which amounted to 435 million NZ dollars in 2018. The use of ice cream as a dessert was reported as the most popular among Kiwis (*New Zealand Ice Cream Market Analysis (2013 - 2023)*, 2019), noting that New Zealanders are the most avid consumers of ice cream in the world.

Commercial ice cream formulations typically contain stabilisers, which are able to provide multiple functional benefits. Locust bean gum is widely considered as the most important stabiliser in ice cream manufacturing. It is an additive (INS #410), with the physical appearance of white to cream coloured powder obtained after isolation from seed endospores of the fruit pod of carob tree (Barak & Mudgil, 2014). Its role in ice cream is to prevent the formation of large ice crystals during production and storage, as well as slow down the melting rate at the consumption stage (Guven et al., 2003) and can provide a positive contribution to mouthfeel.

Beyond ice cream, LBG has widespread use in many food products for both its technical functionality, and the fact that it represents a soluble fibre component of food products, and thus may contribute to the nutritional value of foods (Almeida et al., 2013a)

For dairy product incorporating LBG within the formulation, an important additional consideration is the thermodynamic incompatibility between the protein and polysaccharide, which, ice cream can lead to phase separation during aging of the mix. Additionally this can leading to wheying off on melting, in which differences in the appearance of the two phases make the product undesirable in its visual quality resulting in low acceptability for consumers (Barak & Mudgil, 2014). It is hypothesised that the hydrolysis of LBG, leading to a decrease in its molecular weight, may alter its phase behaviour in solution and allow for mixed protein-polysaccharide compositions that do not display phase separation (Dhawan & Kaur, 2007). The additional consequences of hydrolysis would be expected to be a reduction in viscosity, and a potential modification of LBG

functionality in regard to its inhibition of ice recrystallisation. Accordingly, the impact of hydrolysis on overall technical functionality in ice cream would need to be considered.

The objective of this study was to determine the validity of this hypothesis. Accordingly, the impact of hydrolysed LBG on LBG and skim milk powder (SMP) model solution was investigated. The results of LBG hydrolysis were also evaluated in ice cream production stage. This study aims to explore the behaviour of hydrolysed LBG in model solution and its functional properties in making ice cream.

Chapter 2 Review of literature

2.1 Locust Bean Gum

Locust bean gum (LBG), also known as carob gum or carob galactomannan, is a polysaccharide extracted from seed endosperm of carob trees (*Ceratonia siliqua* L.) which mostly grow in Mediterranean regions. It appears as a white to creamy white powder (Barak & Mudgil, 2014).

2.1.1 Structure

LBG is a linear galactomannan consisting of β-1,4 linked D-mannopyranosyl units which make up the backbone of the chain with 1,6-linked D-glucopyranosyl residues that branch outward in single units along the mannan chain, at a ratio of 3.5-4 : 1 (Figure 2-1) (Marita & Ana, 2012). An interesting feature of LBG is that hot water is required for solubilisation. This is due to intra-chain hydrogen bonding between the mannose backbone of neighbouring polysaccharide (noting that hydrogen bonding effects are weakened with increasing temperature). This is not observed for guar gum (which can be solubilised at ambient temperature) due to the higher degree of galactose branching which inhibits hydrogen bonding across the mannose backbone (Kontogiorgos, 2019; Maier et al., 1993; Mathur & Mathur, 2005).

LBG displays high, but broad molecular weights (MW) ranging from 50-1000 kDa (Dionísio & Grenha, 2012; Richardson et al., 1998), as measured by several methods, including gel permeation chromatography (Prajapati et al., 2013). The molecular size of LBG influences its rheological behaviour (Barak & Mudgil, 2014). Galactomannans have an extended rod shape confirmation when solubilized in water and display large volume of gyration. The extensive association of water within the radius of gyration is responsible to the a high viscosity of solutions (Mathur & Mathur, 2005). Therefore, the larger the molecular weight, the higher the viscosity. The use of LBG in food has multiple functions, including its use as a nutritional component. Not all of these functions necessarily require the viscosifying effects of the gum, and so interest in hydrolysing high-MW LBG molecules to reduce viscosity while maintaining (or creating new) functionality, have recently increased (Marita & Ana, 2012).

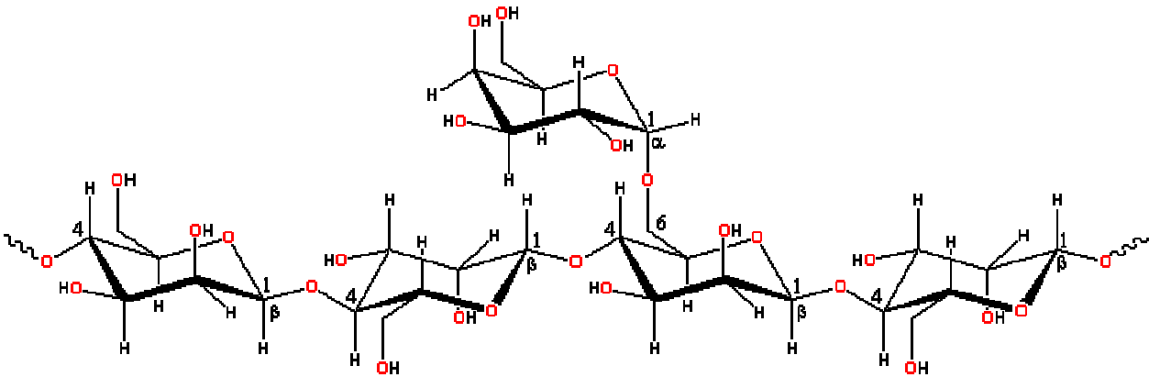


Figure 2-1 Locust bean gum structure. Adapted from El Batal et al. (2012)

2.1.2 Properties

As indicated, LBG can only be solubilized at high temperature but is partially soluble at ambient temperature (0.1% W/W LBG is 50% soluble after 1h agitation). When the temperature reaches 80°C, 70-85% of solubility can be obtained with 30 min agitation. Usually, the maximum solubility of LBG powder is 90%, as other factors, such as particle size and impurities, can influence solubilisation (Barak & Mudgil, 2014; Marita & Ana, 2012). This was demonstrated in a study conducted by Sébastien et al. (2014) who reported that 80°C solubilized LBG gives higher viscosity than 25°C.

While LBG requires heat for solubilisation, it has poor thermal stability when heated for extended periods which may result in the galactomannan backbone chain being oxidative-reductive depolymerised (ORD), and consequently lead to weaker interactions between molecules. As a result of this, the irreversible deduction of its viscosity in the final solution can be seen (Barak & Mudgil, 2014; Garcia-Ochoa & Casas, 1992). This effect was also supported by M. S. Kök et al. (1999), stating that LBG is susceptible to ORD and the addition of antioxidants can protect LBG from being degraded.

LBG is a neutral polysaccharide, its D-mannan main chain contains neither uronic acid groups nor other charged groups. Hence, its solubility and viscosity are hardly affected by ionic strength and pH in the range of 3-11 (Barak & Mudgil, 2014; Kontogiorgos, 2019; Prajapati et al., 2013). However, El Batal et al. (2012) observed the effect of pH on viscosity when the shear rate is low ($10s^{-1}$), but found no effect of pH on viscosity under relatively higher shear rate ($100s^{-1}$ and $1000s^{-1}$).

Since LBG has high-water binding capacity, it is able to form highly viscous solutions even at low concentrations (Daas et al., 2000). The rheological properties of LBG are highly controlled by the distribution of galactose side chains in mannose backbone. Specifically, a higher mannose to galactose ratio contributes to a greater thickening capability (El Batal et al., 2012), therefore LBG is thinner than guar gum and tara gum at the same concentration (Barak & Mudgil, 2014).

The viscosity of LBG can be influenced by the following external factors: a) Shear rate. LBG is a non-Newtonian fluid, behaving as a pseudoplastic material. Its apparent viscosity decreases with the increase of the shear rate (Garcia-Ochoa & Casas, 1992). b) Temperature. It is a thermo-dependent fluid. The viscosity of LBG decreases as the temperature rises (El Batal et al., 2012). c) Concentration. The apparent viscosity increases significantly, in a non-linear way, when gum concentration increases (Garcia-Ochoa & Casas, 1992).

Table 2-1 Factors affecting LBG viscosity

Intrinsic factors	**External factors**
Molecular weight Mannose to galactose ratio	Shear rate Temperature Concentration

LBG gives synergistic actions by forming more elastic and stronger gels when combined with other gums such as xanthan, agarose and carrageenan (Barak & Mudgil, 2014; Garcia-Ochoa & Casas, 1992). The interaction between galactomannas is greatly influenced by the distribution of the galactoslye group along the backbone chain. The galactomannan chain that contains less galactosyl substitutions turns out to be more interactive (Dea et al., 1986).

2.1.3 Functions and Applications in Foods

LBG, coded as E-410 in the European union, was recorded as the first used galactomannan applied in the food industry due to its ability of forming viscous solutions at low concentrations, stabilizing functions, its non-toxic property and minimal impact on taste/flavour (Barak & Mudgil, 2014; Meunier et al., 2014). It is a versatile food additive, mostly used as a thickener and a stabilizer, that can suit a wide range of pH, temperature and ionic strength (Garcia-Ochoa & Casas, 1992; Gonçalves et al., 2004; Urdiain et al., 2004).

In the food industry, LBG is commonly used as stabilising, thickening, and gelling agents in products such as: processed cheese, salad dressings, soups, gravies, bakery products, dairy products including infant formula and ice cream (Urdiain et al., 2004). The addition of LBG on its own or coupled with other gums provides desirable texture and chewiness, improves the smoothness of foods, as well as helps prevent the growth of ice crystals in frozen foods (Meunier et al., 2014). LBG also plays a role in other fields, such as pharmaceutical, textile, biomedical and cosmetic industries (Prajapati et al., 2013; Thombre & Gide, 2013),

The addition of LBG specifically in ice cream inhibits the formation of ice crystals, modifies the texture, and prevents the recrystallization of lactose during the frozen storage (Kök et al., 1999; Kontogiorgos, 2019). It contributes to the broader functions of stabilizers in ice cream, namely: increase the viscosity of the matrix; produce a smooth texture for eating; decrease the meltdown rate; prevent shrinkage and reduce moisture migration during storage; cover up the ice crystal detection in the mouth while eating; help easier pumping and more precise filling for processing; facilitate producing a stable foam and make the air incorporation more controllable (Clarke, 2004b). LBG, in particular, can provide a smooth texture in ice cream and slow down the meltdown rate. It can also slow down recrystallization by forming a barrier around the ice crystals due to its gel formation characteristic when freeze-concentrated, hence preventing accretion (Clarke, 2004c).

Polysaccharides, such as LBG have also been considered as nutritive food component, as they are soluble fibres (Yoon et al., 2008). Lack of dietary fibre intake has been associated with gastrointestinal tract and metabolic diseases (Almeida et al., 2013b). Consequently, there is a rising demand of dietary fibre from consumers, and food producers are making efforts to deliver high dietary fibre content foods with the addition of the ingredients that are high in dietary fibre (Almeida et al., 2013a). However, the utilization of soluble gums

especially in liquid products has a limit since the ideal dietary fibre that are added in food must be very low in viscosity, tasteless and should display clear solutions in beverages (Yoon et al., 2008). Due to the highly viscous nature of guar gum and LBG even in low concentrations (Buriti et al., 2014), partial hydrolysis of these gums has been considered as a tools which may allow the use of these polysaccharides specifically for their contribution as dietary fibre components in food products (Buriti et al., 2014).

2.2 Thermodynamic incompatibility of protein-polysaccharide mixtures

Protein and polysaccharide systems are widely utilized in food formulation, and their interactions can be a determining factor in the properties of food systems, such as structure, texture and stability. Ideally, one phase solution is desired in the food industry. However, it is always challenging in food formulation when dealing with protein-polysaccharide mixtures. Such biopolymers will tend to separate into two phases over time when mixed together, which can mainly be attributed to thermodynamic incompatibility (Harding et al., 1995; Hemar et al., 2001).

2.2.1 Mechanism

Biopolymer incompatibility was firstly discovered by Beijerinck in 1896 by mixing starch and gelatin in water (Grinberg & Tolstoguzov, 1997; Tolstoguzov, 2006; Tolstoguzov, 2017). Thermodynamic incompatibility is a characteristic of certain protein-polysaccharide systems, which mainly occurs when the ionic strength is high and/or the solution pH is higher than the protein isoelectric point (IEP), and which prevents the formation of inter biopolymer complexes (coacervation). (Grinberg & Tolstoguzov, 1997). The free energy of mixing, which correlates to the interaction between different polymer segments, determines if the separation occurs or not. In other words, the greater the interaction between the two polymers, the lower the concentrations are required for the separation (Albertsson, 1995).

2.2.2 Factors affecting phase behaviours

The interactions between protein and polysaccharides in the food system can be roughly described as two types— the repulsive and attractive, which result in two opposite phenomena—thermodynamic incompatibility and interbiopolymer complexing (Figure 2-2). It is reported that denatured proteins and polysaccharides are miscible at the molecular

level when they are in very dilute solutions. For most of formulated foods, the phase separates because they are not dilute aqueous solutions (Tolstoguzov, 2002).

Type of polysaccharide, protein-polysaccharide ratio, concentration of the biopolymers, and environmental factors, such as temperature, pH and ionic strength, can all influence the stability of the system (Cheng et al., 2015). Phase separation between protein and polysaccharides in a system with water as solvent is influenced by the polysaccharide's chemical structure (Grinberg & Tolstoguzov, 1997). The microstructure of the system changes along with the phase separation, and that is influenced by the phase volume ratio (Schorsch et al., 1999). Additionally, Hoskins et al. (1996) found out that the higher the MW, the lower concentration is needed for the separation to occur. (Walstra, 2003). Rate of phase separation is commonly observed to be greater at higher temperature due to decreased viscosity within the system.

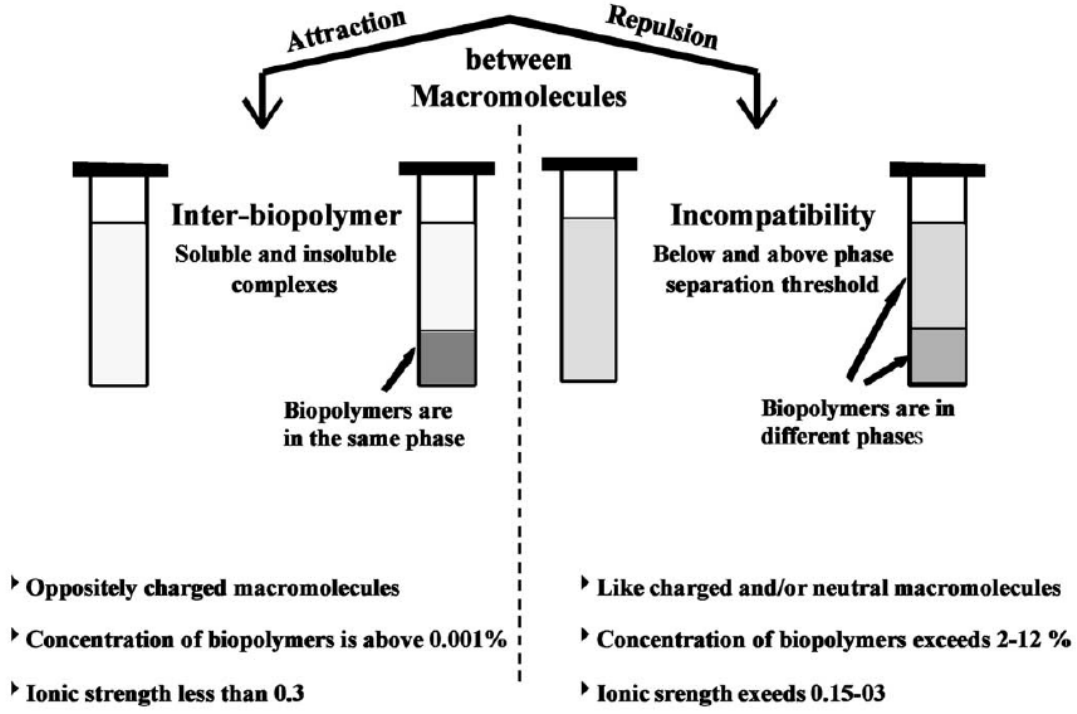


Figure 2-2 The behaviour of mixed biopolymer solutions. Adapted from Vladimir Tolstoguzov (2003)

The change of conditions, such as pH, ionic strengths and temperature will lead to the incompatibility of the two biopolymers, and it favours the association of same macromolecules and inhibits the attraction of different macromolecules. Repulsions of

different biopolymers result in each macromolecule preferring to be surrounded by its own type. Usually, the protein tends to self-associate more when the pH is at its IEP and this phenomenon enhances the incompatibility between proteins and polysaccharides. When the salt concentration is lower than a critical value, the two biopolymers are totally compatible (Tolstoguzov, 1999; Walstra, 2003). The critical salt concentration demanded for phase separation varies with the pH and salt composition. It is reported that the critical point increases in the following order: carboxyl-containing polysaccharides < neutral polysaccharides < sulphated polysaccharides. In general, when the salt concentration goes higher than 0.1M, protein and neutral polysaccharide, such as LBG, are not compatible (Grinberg & Tolstoguzov, 1997; Tolstoguzov, 1999).

Increased excluded volume (molecular weight) of polysaccharides and proteins lead to an increase in incompatibility. Tolstoguzov (1999) reported that partial hydrolysis of the macromolecules can decrease the biopolymer incompatibility. Enzymatic hydrolysis of protein has been explored and changes in the compatibility of the phases have been observed (Danilenko et al., 1992; Tolstoguzov, 1999).

2.2.3 Methodology of phase separation observation

There are several methodologies widely used to observe the phase separation. Phase-volume ratio (Agbenorhevi & Kontogiorgos, 2010) and visual observation of the phase separation (Chun et al., 2014; Hemar et al., 2001; Thaiudom & Goff, 2003) are two common methods for plotting the phase diagram.

Technics such as Confocal Laser Scanning Microscopy (CLSM) is frequently used and is a useful tool to help understand demixing process from the microstructure level (Sadeghi et al., 2018; Schorsch et al., 1999; Tolstoguzov, 2006).

2.2.4 Phase separation issues in ice cream products

As discussed, polysaccharides, as stabilizers, have very important purposes in the ice cream processing with only a very low level of addition, such as improving aeration, increasing the ice cream mix viscosity and controlling melt down (Bahramparvar & Mazaheri Tehrani, 2011). However, mixtures of polysaccharides and milk proteins (primarily casein micelles), can often display phase separation, depending on the specific polysaccharide in question. In the ice cream processing. Phase separation can be an issue at multiple stages of ice cream

production and utilisation. During manufacture it can lead to a portioning of ingredients during aging of the mix, which may lead to non-uniform freezing of the product. It is also the cause of wheying off. Wheying off is a phenomenon observed when the ice cream melts, and the liquid shows a water-like layer. For both these effects, carrageenan is normally added to inhibit the separation of the LBG and the casein micelles (Bahramparvar & Mazaheri Tehrani, 2011). Phase separation also needs to be taken into consideration in the manufacture of soft-serve ice cream mix. Since this commercially packed and distributed to retailers, the shelf life of the mix is usually 14-21 days (Vega et al., 2004), and this gives enough time period for the mix to exhibit extensive phase separation (Bahramparvar & Mazaheri Tehrani, 2011). Therefore, to study and guarantee the quality of the mix, especially the system stability, it is necessary for food scientists to investigate effective ways of preventing phase separation. While there may be various ways in which this could be achieved, this study focusses specifically on altering phase behaviour through enzymatic hydrolysis of the LBG.

2.3 Enzymatic hydrolysis of polysaccharides and its deactivation

2.3.1 Hydrolysis

Hydrolysis is the process of breaking down large molecules into small components in the presence of water (Obodovskiy, 2019). It usually involves the cleavage of chemical bonds to produce fragments of reduced molecular weight, and resulting in the release of energy (Speight, 2017). There are several types of hydrolysis such as acid, salt, base, thermal and enzymatic hydrolysis (Speight, 2018) .

Exploration of the application of modified biopolymers in the food industry has increased in recent years (DeLoid et al., 2018; Dhawan & Kaur, 2007). One particular type of hydrolysis used in the food industry is enzymatic hydrolysis, which can be applied to the modification of proteins, lipids and carbohydrates. This has also been found to reduce the molecular weights of long chain polysaccharides making them useful in further production of food products (Tello et al., 1994). For example, enzymatic hydrolysis has resulted in producing molecules with reduced molecular weight, low viscosity as well as improving the physicochemical properties of the food (Cheng et al., 2002). Several authors (Chen et al., 2018; Dhawan & Kaur, 2007; Xie et al., 2019) have reported that enzymatic hydrolysis is the

most effective way to hydrolyse LBG. Mannanases are a group of specific enzymes that can be used for cleaving galactomannan type polysaccharides, such LBG (a carob galactomannan), guar and fenugreek gums. Examples of mannanases include β-mannanases, β-mannosidases, α-galactosidases, β-glucosidases and acetyl-mannan esterases which can be produced from plants, animals and microorganisms (Dhawan & Kaur, 2007). It has been found that bacterial mannanases are the main source of mannanases (Dionísio & Grenha, 2012).

2.3.2 Enzymatic hydrolysis of LBG

Microbial mannanases have been largely studied and applied in many industries including the food industry. They are capable of hydrolysing galactomannan polysaccharides into oligosaccharides and even single sugar units (Dhawan & Kaur, 2007). Mannanses that are derived from microbes can tolerate a wide range of pH and temperature (Dhawan & Kaur, 2007). For example, endo-1,4-β-mannanase from *Cellvibrio japonicus* (*C. japonicus*) (E-BMACJ, produced from Megazyme) can be active under the pH range of 4 to 9 and can tolerate up to 80℃, whereas its optimum pH and reacting temperature are 7 and 50℃ respectively.

Several strains of fungi can produce mannan hydrolysis enzymes, and therefore the enzymes are named and characterised after these fungi (Moreira & Filho, 2008). *Aspergillus* is an example of fungi capable of producing mannan hydrolysis enzymes. The degradation of LBG by specific enzymes such as endo-β-mannanase catalyse the hydrolysis of β-1,4-mannosidic linkages. The two main factors which affect endo-β-mannanase's activity are the degree of polymerization and the M/G ratio (Tapie et al., 2008). During hydrolysis, endo-β-1,4-mannanase cleaves the β-1,4 mannopyranosyl linkages in the mannan backbone and therefore leads to oligosaccharides of varying lengths as shown in Figure 2-3. β-mannosidase is responsible for the hydrolysis of oligomannans while α-galactosidases, β-glucosidases and acetyl-mannan esterases are responsible for catalysing the removal of galactose, glucose and acetic acid respectively (Dhawan & Kaur, 2007)

Figure 2-3 Enzymatic actions on galactomannan. Reproduced from Dhawan and Kaur (2007)

The application of enzymatic hydrolysis of other galactomannan polysaccharides such as guar gum has been investigated. The results of the studies indicate the potential of enzymatic hydrolysis of other essential polysaccharides such as LBG to be used in the food industry.

In a study by Mudgil et al. (2012), it was shown that the enzymatic hydrolysis significantly decreased the intrinsic viscosity of guar gum. The value of flow behaviour index (FBI) below 1 represents that the fluid is shear-thinning material, and therefore the value above 1 means it obeys the rules of Newtonian behaviour. The depolymerisation changed guar gum solution from non-Newtonian fluid to Newtonian fluid, with the number of FBI changing from 0.31 to 1. However, the molecular structures of partially hydrolysed guar gum (PHGG) and native guar gum remain similar. Having no impact on the final food product , PHGG was

suggested to be added as a dietary fibre source (Mudgil et al., 2012).

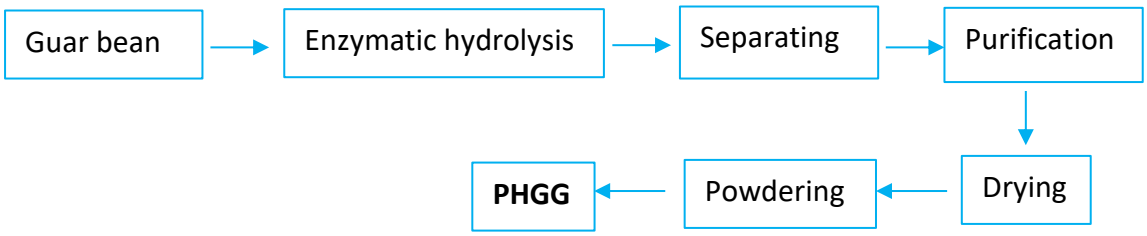


Figure 2-4 The processing of PHGG

PHGG is commercially named as Sunfiber®. It was found to be natural, safe and was a high source functional dietary fibre. It has been utilized in beverages, food, and medicinal foods around the world (Yoon et al., 2008). The molecule of PHGG is ten-fold smaller than the original one, ranging from 1000 to 100000 Daltons (Yoon et al., 2008). The viscosity of Sunfiber® has been dramatically decreased compared to the intact guar gum. Due to its high content of dietary fibre, PHGG has been primarily used as food material for nutrition purpose without changing the foods' original rheology (Yoon et al., 2008). They found that enzymatic hydrolysis of guar gum resulted in a product with increased qualities such as texture making it useful to be incorporated in food products (including application in animal nutrition) (Yoon, Chu, & Juneja, 2008). The successful hydrolysis of guar gum for its use in the food industry implies that further exploration of LBG hydrolysis is important since both guar gum and LBG are structurally compatible (Urdiain et al., 2004).

2.3.3 Deactivation of enzymatic hydrolysis

There are several ways to deactivate enzyme hydrolysis. Since enzymes are proteins, the use of heat and other chemicals to deactivate (via denaturation) them has been reported. Modification of the native structure of enzymes through denaturation reduces or ceases enzymatic activity and allows for control of extent of biochemical translations.

Non-denaturation routes, such as complexation or competitive binding to substrates can also be used to inhibit enzymatic activity. For example, a study conducted by Tejirian and Xu (2011) used phenolic compounds such as oligomeric phenols to inhibit cellulases. The ability of the oligomeric phenols to inhibit cellulases was due to their ability to reverse the complex. Other oligomeric phenols such as polyethylene glycol and tannase work by adsorbing cellulases onto cellulose and hence preventing enzyme activity.

The disadvantage of using phenols to inhibit or deactivate enzyme activity is its application in food. It is important to note that, not all phenols can be used in the food industry. This therefore calls for the use of other means such as heat and change in pH.

2.4 Ice cream processing

An example of food products that we can utilize the application of enzymatic hydrolysis is ice cream. Ice cream is a popular dessert consumed in almost every part of the world. It is a frozen product which consists of at least 5% fat and 7.5% milk solids other than fat (i.e., protein, sugars and minerals). Ice cream is made by heat treating and freezing of fat and milk solids as well as a sweetener (Clarke, 2004a). A schematic diagram about the processing of ice cream has been given in Figure 2-5. Basically, it involves mixing of ingredients, pasteurization, freezing and hardening.

The main use of the enzymatic hydrolysis of polysaccharide to improve upon final ice cream products come from its ability to produce refined ingredients which can serve as a source of dietary fibre to the ice cream(Buriti et al., 2014). This is important since people are choosing more healthy options in dietary requirement.

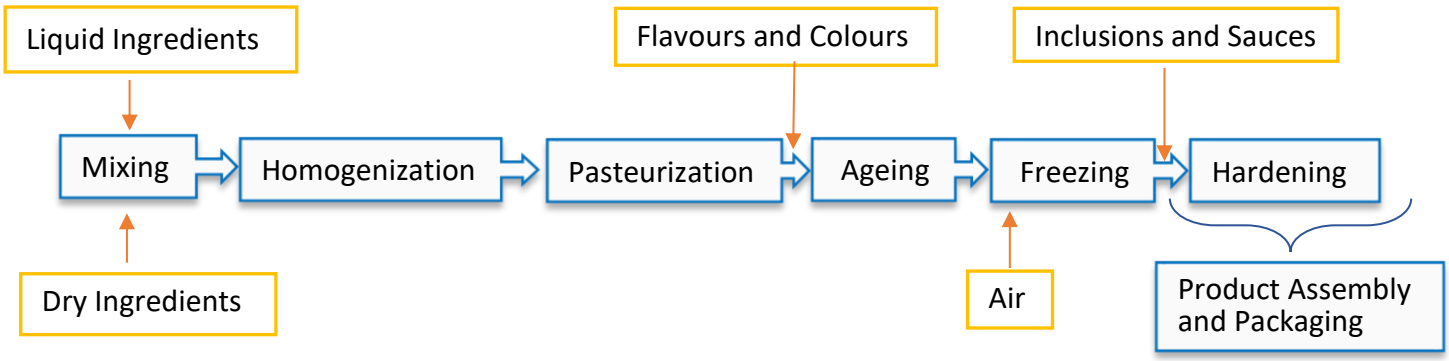


Figure 2-5 Flow diagram of ice cream manufacturing process. Reproduced from Clarke (2004)

2.5 Conclusion

The results of this literature review indicate the potential of LBG hydrolysis as an ingredient in making food products. A review of the literature indicates few prior studies investigating the potential of LBG hydrolysis and the products that can be obtained. The purpose of this study was therefore to evaluate LBG hydrolysis and identify the characteristics of the hydrolysed product. This will enable us to see if this can be applied in the food industry and whether it can add value to final food product. Another important objective is to observe the effect of hydrolysed LBG on phase separation, which is a common problem in the ice cream industry. It was hypothesized that phase separation can potentially be solved by partially hydrolysing LBG. This research explores the hydrolysis effect of LBG on phase separation. The results of this study will be useful in getting knowledge about we can add value to final food product.

Chapter 3 Materials and methods

3.1 Materials

Locust bean gum (LBG) and the skim milk powder (SMP) used in this study were kindly supplied from Fonterra Co-operative Group Limited. The endo-1,4-β-mannanase from *Cellvibrio japonicus* (*C. japonicus*) (E-BMACJ) (Lot 90901c) was purchased from Megazyme Ltd., Ireland. The analytical grade reagents used in this study were Bull Serum Albumin (BSA), sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot 2H_2O$), hydrochloric acid (HCl) and sodium hydroxide (NaOH). The materials used for making the ice cream include glucose syrup (Avon Glucose Syrup A2151, Penford, Auckland), sucrose (Pams, New Zealand), monodiglycerides (GRINDSTED PS217, Danisco, Australia), and Anhydrous Milk Fat (AMF) was provided by Fonterra, New Zealand.

3.2 Methods

3.2.1 Sample preparation

Locust bean gum solutions were prepared by carefully sprinkling the LBG powder into preheated 80°C reverse osmosis (RO) water with a continuous gentle stir for 40min in a water bath. A paddle mixer was used for agitation. The concentrations of LBG prepared were 0.5%, 1%, 2% (weight by weight). The solutions were centrifuged at 28000 g for 1h at 25°C to remove the insoluble residues. The other LBG concentrations needed for the study were diluted from the above three concentrations using RO water with appropriate heat by stirring.

40% (W/W) SMP solution was prepared by dissolving the precisely weighted skim milk powder into 50°C fixed amount RO water in a water bath and stirred for 30min with a stirrer and a magnetic plate. Other concentrations needed for the study were diluted from the above concentration using RO water with appropriate heat and stirring.

The phosphate buffer used for diluting the enzyme was prepared following the method below: 27g of $Na_2HPO_4 \cdot 12H_2O$ and 3.837g of $NaH_2PO_4 \cdot 2H_2O$ were added to 800mL distilled water and stirred for 30 min. The pH of the solution was adjusted to 7 using 1M HCl and NaOH.

The original E-BMACJ solution (5000U/mL) was diluted to 0.0625U/mL, 0.03125U/mL and 0.015625U/mL using the above phosphate buffer and stored in lightproof bottles.

All solutions were stored in the refrigerator at 4°C.

3.2.2 Measurement of LBG viscosity during hydrolysis

The viscosity of LBG during enzymatic hydrolysis was measured *in situ* in a Rheometer cup. This method was developed within the study, the larger the chain length, the greater the viscosity (as stated in 2.1.1).

The viscosity of 0.5%, 1% and 2% of LBG were measured respectively at both 20°C and 40°C by Modular Compact Rheometer MCR 302 (Paar Physica, Germany). The vane geometry ST22-4V-40 (Figure 3-2) was chosen for the system. The LBG solution was firstly added into the cylinder cup and equilibrated to the required temperature (20°C or 40°C). The enzyme was then injected into the LBG using a pipette, at a ratio of 1:100 (W/W) following the procedure below: weigh the LBG directly in the cylinder cup on the weighing balance. Calculate the required amount of the enzyme solution and pipette (the density of the enzyme solution is 1g/cm^3). The mixture was pre-sheared at 200/s for 30s to give a better interaction between the enzyme and LBG. The data was then recorded for 60 min at a constant shear rate of 0.1/s.

Figure 3-1 Rheometer MCR 301

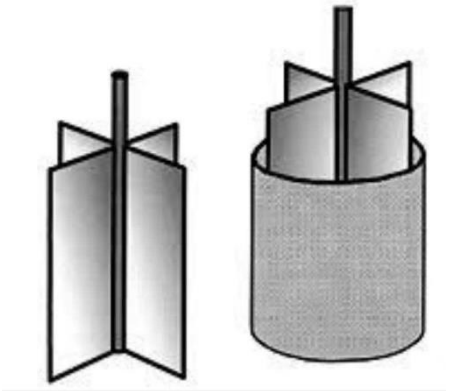


Figure 3-2 Geometry ST22-4V-40

3.2.3 Enzyme inhibition

Heat treatment, acid treatment and combination of heat and acid treatment were used to inhibit the enzyme activity after LBG hydrolysis, in order to find a suitable condition to deactivate the enzyme without destroying the material itself. 1% LBG was chosen as a standard concentration.

3.2.3.1 *Heat treatment*

0.03125U/mL enzyme was reacted with 1% LBG at a ratio of 1:100 (W/W) in several reagent bottles for 20min. Then the bottles were put into 85°C, 90°C, 100°C water bath for 20min,30min, 40min respectively.

3.2.3.2 *Acid treatment*

0.03125U/mL enzyme was reacted with 1% LBG at a ratio of 1:100 (W/W) in a beaker for 20 min. The pH of the substrate was adjusted to 3 using 1M HCl. The pH was brought back to 7 using 1M NaOH after 20min.

3.2.3.3 *Combination of acid and heat treatment*

0.03125U/mL enzyme was reacted with 1% LBG at a ratio of 1:100 (W/W) in several reagent bottles for 20 min. The pH was adjusted to 3 using HCl afterwards. The bottles were put into 85°C, 90°C, 100°C water baths for 20min,30min, 40min respectively. The bottles were then taken out and put into an ice bucket immediately until the temperature reached 20°C. The pH was adjusted back to 7 using 1M NaOH.

3.2.3.4 *Oil bath (thermalisation > 100 °C)*

1mL of the 0.03125U/mL was put into a sealed glass bottle and was oil bathed at 145°C for 3min and 5min. It was then injected into the 1% LBG solution to test the enzyme activity.

3.2.3.5 *Ultra-heat treatment*

2L of the LBG solution was prepared in a large metal beaker. A certain amount of the enzyme was added in the LBG solution at a proper ratio and reacted for 20min. The solution was then poured into the UHT tank. The temperature and time were set at 140°C for 24s. The sample was collected after the treatment.

3.2.3.6 *Testing the enzyme activity*

The enzyme activity was tested by putting the treated enzyme-LBG solution into the rheometer. The concentric cylinder system was chosen, and the shear rate was $0.1/s^{-}$. All the tests were carried out in duplicates overnight at 20°C. The activation or deactivation of the enzyme can be inferred from the viscosity change of the LBG solution. If the enzyme is successfully deactivated, the solution's viscosity should remain the same over time. if the viscosity keeps decreasing, it is an indication that the enzyme is not fully deactivated. The rate of decrease depends on the amount of active enzyme remaining. The faster it decreases the more active enzyme remains in the solution.

3.2.4 Observation of protein-polysaccharide phase separation

Experiments were conducted as described by Schorsch et al. (1999). In order to find the concentrations of the two polymers that are the closest to the critical points, preliminary experiments were conducted. SMP and LBG solutions were diluted in suitable beakers from the solutions that have been made in 3.2.1, following the concentrations in Table 3-1.

Table 3-1 Concentrations of SMP and LBG for observing phase separation

LBG% (W/W)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8						
SMP% (W/W)	1	2	3	4	5	6	7	8	9	10	15	20	25	30	40

The same weight of the two polymer solutions for each required concentration were mixed well and the mixture was poured into a suitable test tube and covered well. The tubes were then put on a rack at 25°C for 24h. The phase diagram was plotted according to the separation (the concentrations indicated in the axes are the original biopolymer concentrations).

0.03125U/mL enzyme was mixed with 1% LBG at a ratio of 1:100 (W/W) and reacted at ambient temperature (20°C) in a reagent bottle for 20min. The reaction was stopped by adjusting the solution pH to 3 using 1M HCl. Then the bottle was sealed and put into 90°C water bath for 20min. After cooling down and adjusting the pH back to 7 using 1M NaOH, the hydrolysed LBG solution was then diluted and mixed with the SMP solution following the same procedure as the non-hydrolysed LBG.

3.2.5 Confocal Laser Scanning Microscopy (CLSM)

The microstructure of the protein-polysaccharide solution was visualised using a Confocal Laser Scanning Microscope (Leica SP5 DM6000B, Leica Microsystems, Germany). 45µL of Fast Green dye was mixed well with 2.5mL of the protein-polysaccharide solution to stain the protein fractions. One droplet of the mixed sample was carefully placed onto the microscopic slide and was covered by a glass coverslip. The excitation wavelength of Fast Green is 530-630nm. The images were taken at certain time intervals to record the rapid change of the two biopolymer mixtures at different concentrations. The time was counted as soon as the stirring stopped.

3.2.6 Ice cream preparation

The ice cream mix was prepared following the recipe in Table 3-2

Table 3-2 Ice cream formulation

Ingredient	Zero control (W/W %)	Normal control (W/W %)
Fat	10	10
SMP	11	11
Sucrose	9	9
Glucose syrup	4	4
LBG	0	0.3
Monodiglycerides	0.3	0.3
Total Solid	34.3	34.6
Water	65.7	65.4

The work was carried out according to the method used by Clarke (2004d). Fat was firstly melted in the steam jacket until it turned into liquid. Blenders and hot water buckets were set up. The ingredients were weighed in advance and added in the hot water following the order of: SMP, sucrose (mixed with LBG for the normal control), glucose syrup, monodiglycerides and fat. After the ingredients were well mixed, the mixes were preheated to 50^{o}C and homogenized at 200/50 in turn and collected in the labelled buckets. After homogenization, the mixes were pasteurized at 83^{o}C for 15s. All samples were collected in sanitized labelled buckets. The buckets were put in a chiller at 5^{o}C for aging overnight. The mixes were then put through the ice cream machine (freezing) and immediately frozen at -30^{o}C (hardening).

The normal control sample was divided into two buckets after homogenization, and one was cooled down in an ice bucket until it reached 20^{o}C. The enzyme was then added at a ratio of 1:100 (W/W). The mix was continuously stirred for 5 min and half of it was poured into the UHT tank. The rest of the mix was kept for another 15 min (total hydrolysis time was 20min) with continuous stirring and was poured into the UHT tank afterwards.

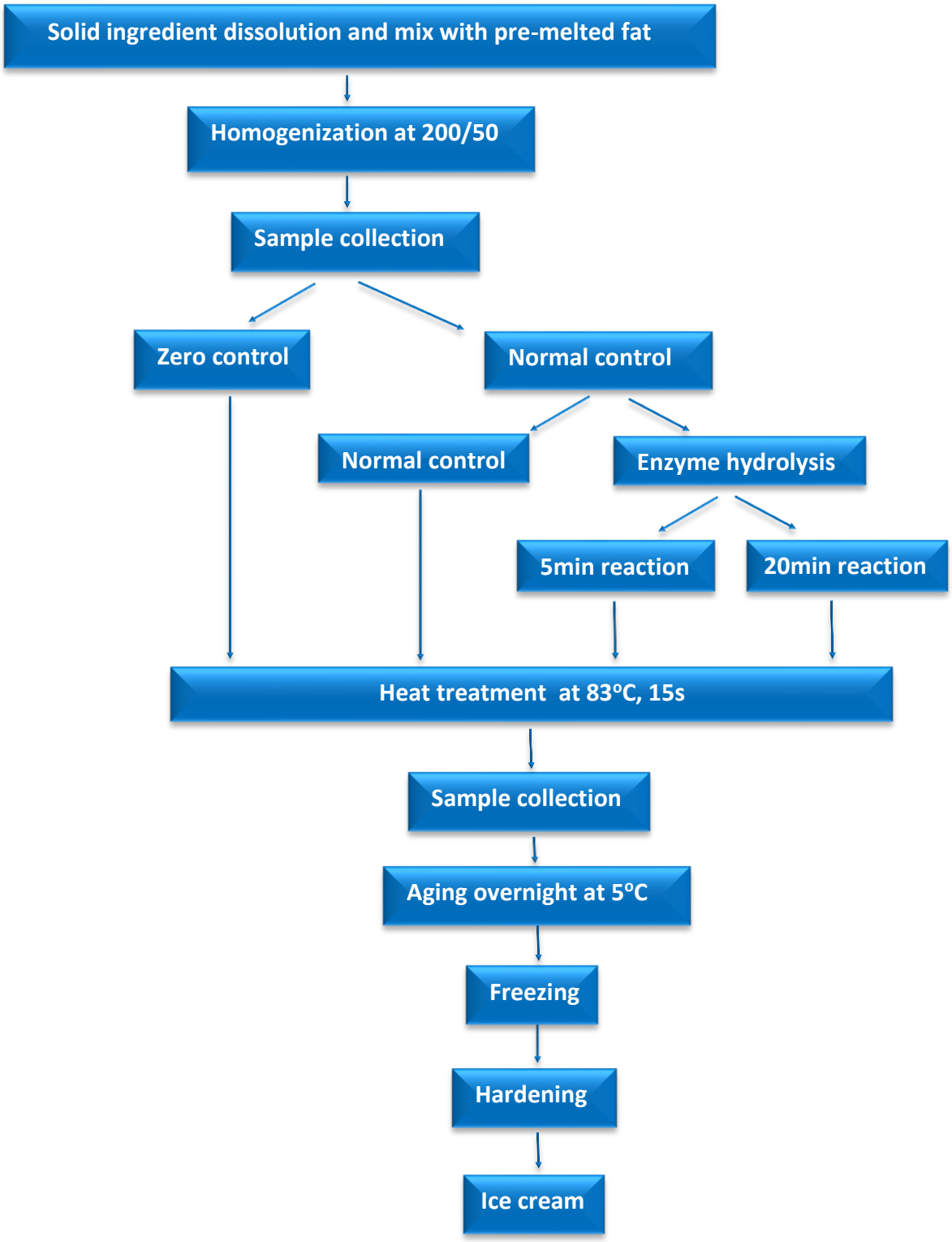


Figure 3-3 Ice cream making procedure

3.2.7 Ice cream characterisation

3.2.7.1 *Overrun in ice cream*

Ice cream overrun is the amount of air that can be incorporated in the ice cream during the freezing and aeration stage. This was calculated by comparing the weight of the ice cream mix and the ice cream at the same volume following the equation below:

Equation 1

$$Overrun\% = \frac{weight\ of\ mix(g) - weight\ of\ ice\ cream(g)}{weight\ of\ ice\ cream\ (g)} x100\%$$

3.2.7.2 *Meltdown rate of ice cream*

The meltdown rate of ice cream was analysed by putting the ice cream samples onto a mesh screen with weighing scales beneath each of them, as shown in Figure 3-4. The weight of the melted ice cream was then recorded every 10 min in 120 min period at controlled room temperature of 20°C.

Figure 3-4 Measurement of ice cream melt down rate

3.2.7.3 *Hardness tests of ice cream*

Hardness of ice cream is the resistance to ice cream distortion when applying an external force. The experiments were carried out at 20^{o}C using the texture analyser (TA.XT.Plus Texture Analyzer, UK). The 5mm stainless steel cylindrical probe was chosen for the system. Ice cream samples were kept in the freezer and transferred onto the TA stage immediately using an ice box to prevent deformation. The probe was penetrated to a depth of 10mm at a speed of 5mm/s into the sample. The pre-test speed and post-test speed was 3mm/s and 5mm/s respectively.

All the experiments were conducted in duplicate. While no statistical analyses were carried out, all the results are reproducible.

Chapter 4 Rheological behaviour of LBG during enzymatic hydrolysis

4.1 Results

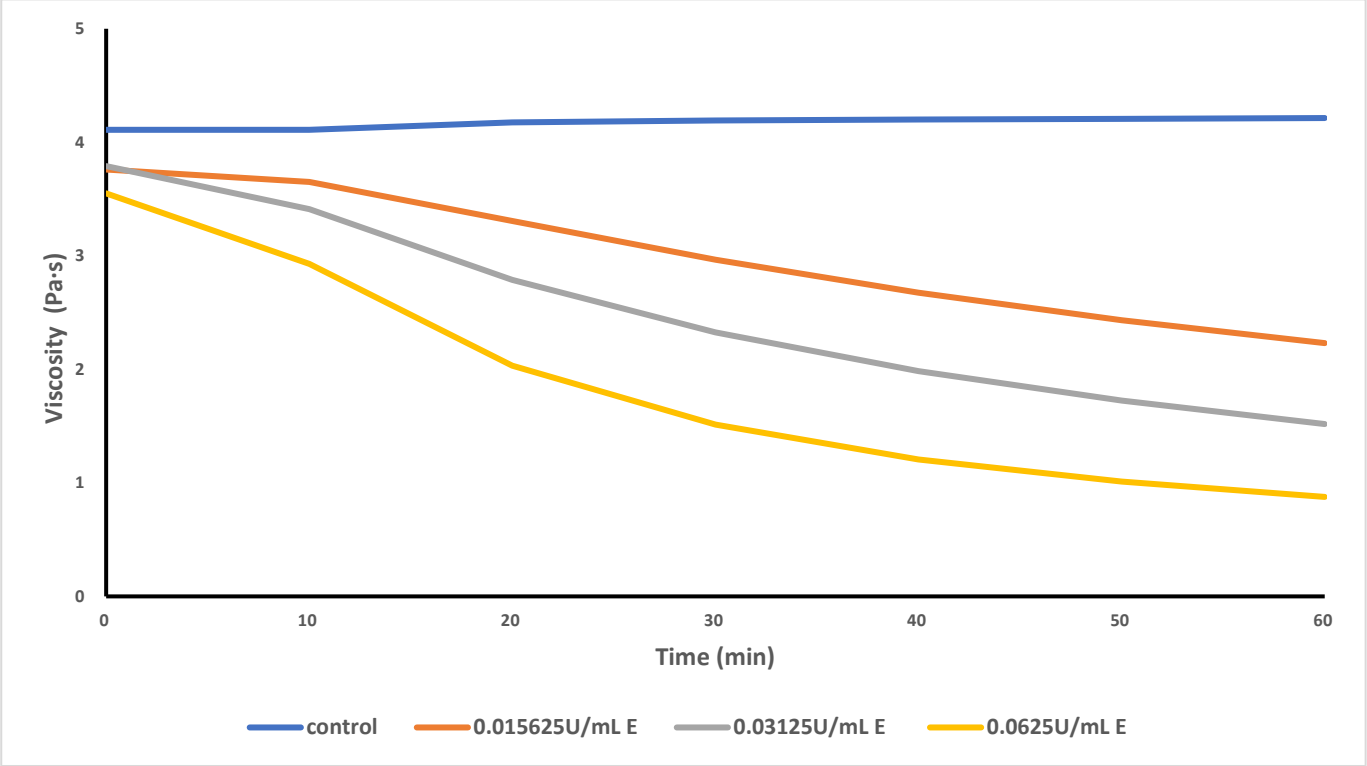


Figure 4-1 Effect of enzymatic hydrolysis on 1% LBG viscosity at 20℃

Figure 4-1 shows the effect of different concentrations of mannanase enzyme on the viscosities of 1% LBG concentration over a period of one hour (noting that the control solution did not contain any enzyme. The results highlight the enzyme concentration dependency in terms of decreasing LBG viscosity over time. The viscosity of 1 % LBG with the lowest enzyme concentration of 0.015625U/mL decreased from 3.76Pa·s at time t=0 to 2.23Pa·s at time t=60min and increasing concentration to 0.0625U/mL resulted in a decrease in viscosity to 0.88Pa·s viscosity after 60 minutes. The differences observed in the viscosity at time t=0min is due to the time it takes for complete mixing of LBG and the enzyme. The rheometer starts to read after the 30s mixing and therefore the rapid enzyme concentration reaction results in the differences at the starting point.

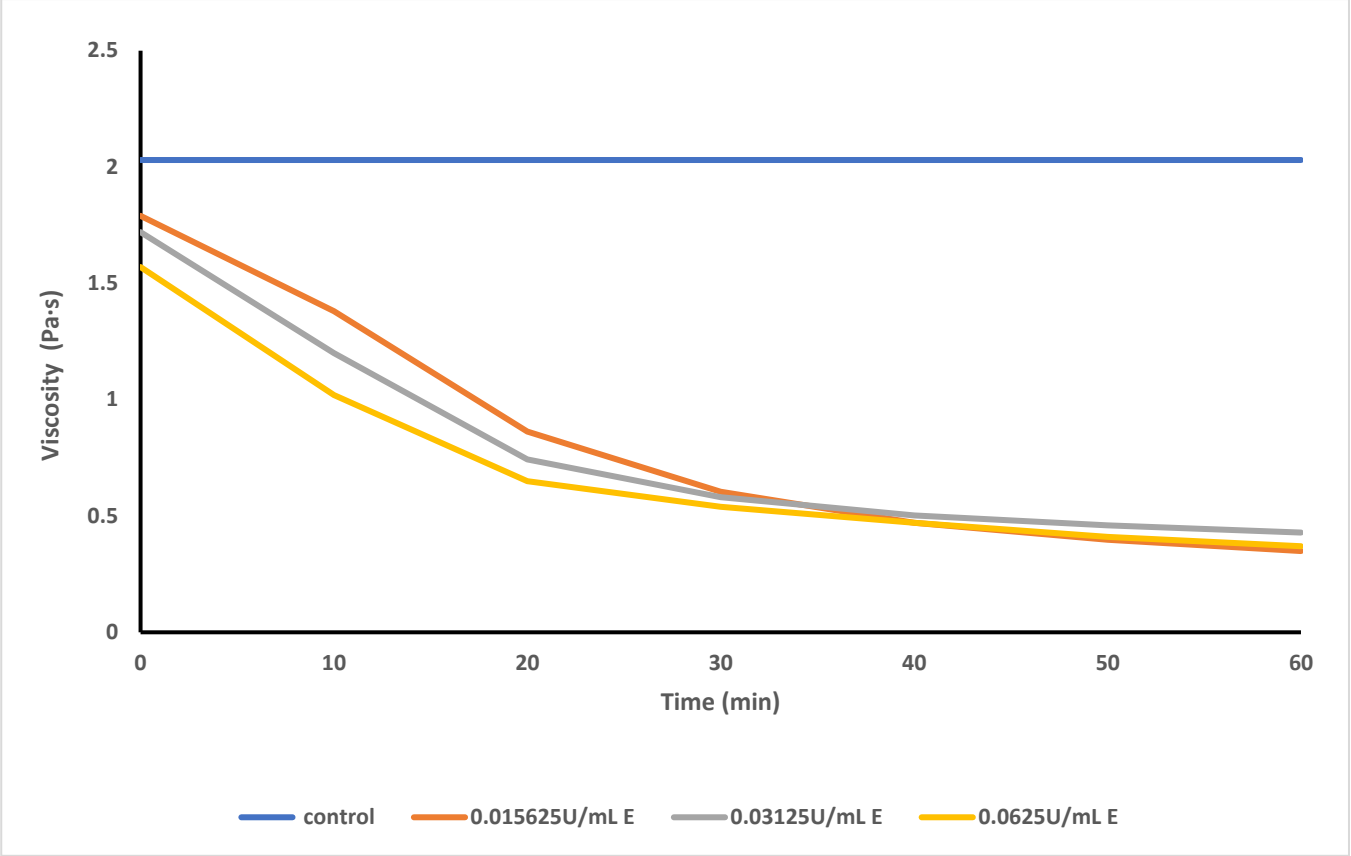


Figure 4-2 Effect of enzymatic hydrolysis on 1% LBG viscosity at 40℃

The effect of temperature on the enzymatic hydrolysis was also observed. This can be seen in the results outlined in Figure 4-2. Firstly, measurement at 40 ℃ reduced the viscosity of the LBG used as control from 4.11Pa·s (when measured at 20°C) to 2.03Pa·s. In terms of enzymatic addition, the rate of reaction with the different concentrations of enzymes was faster as can be seen in the sharp reduction observed. This occurred for all the three different enzyme concentrations used from time t=0 until t=20min, with a reduction in rate of viscosity decrease from t=20 - 30min and then appearing to level off over the remainder of the experiment. During this period the viscosity of all solutions was similar irrespective of enzyme concentration. It can also be noted that the starting viscosity of all samples containing enzymes was lower than that of the control, indicating that hydrolysis was occurring before the onset of measurements.

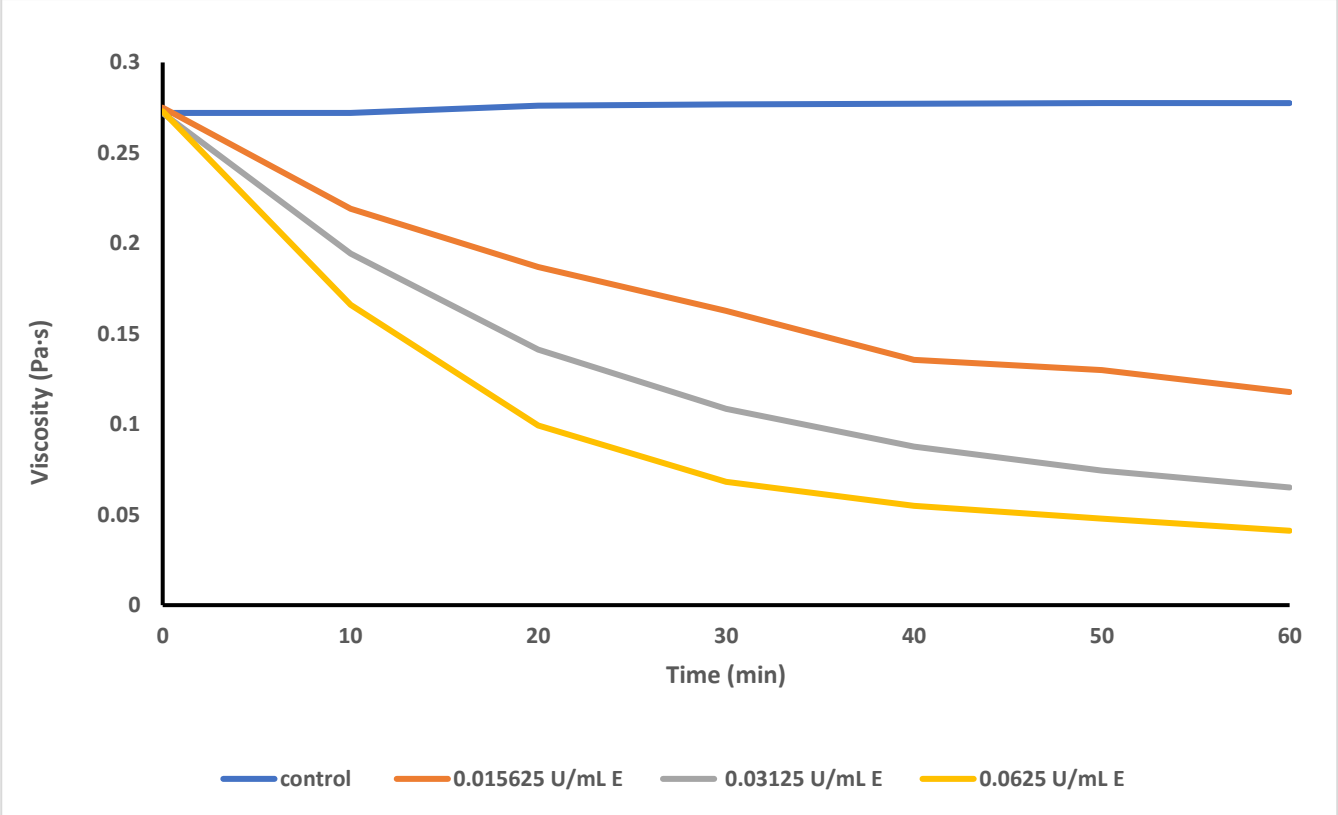


Figure 4-3 Effect of enzymatic hydrolysis on 0.5% LBG viscosity at 20˚C

The concentration of LBG was then reduced to 0.5% for hydrolysis to determine whether the LBG viscosity would affect the enzymatic hydrolysis as observed for the 1% LBG. The results affirmed the observation seen, which is higher concentrations of enzymes result in faster reductions in viscosity. This is an indication that, the enzymatic effect can still be applied to low concentrations of LBG and that, the concentration of LBG does not affect enzymatic hydrolysis.

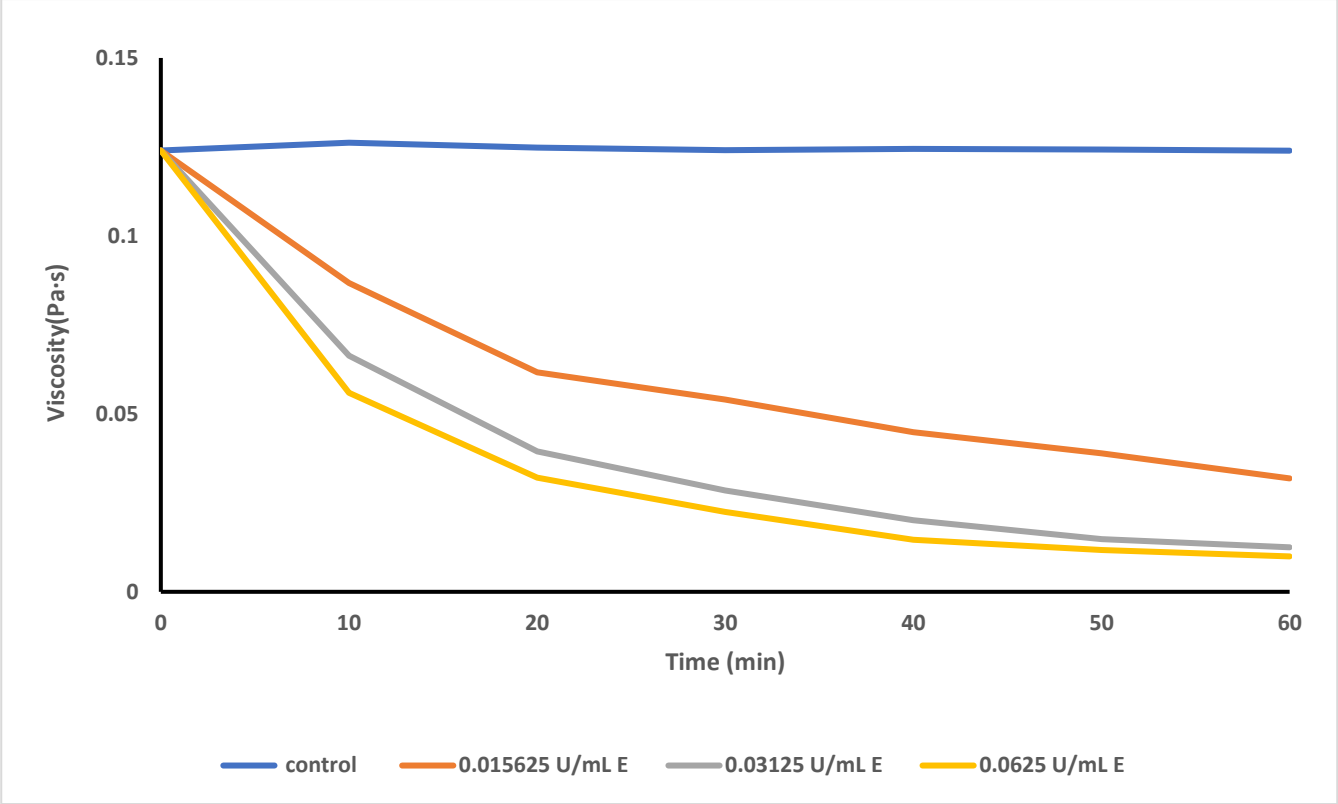


Figure 4-4 Effect of enzymatic hydrolysis on 0.5% LBG viscosity at 40℃

The temperature was then raised to 40℃ for 0.5% LBG to investigate if the enzyme activity would follow the same trend as observed at 1% LBG as at a relatively lower concentration (0.5%). The result in Figure 4-4 affirms the enzymatic activity on 0.5% LBG. Enzyme hydrolyzes more effectively at a higher temperature regardless the LBG concentration, although the rate and magnitude of change did appear to show more dependency on enzyme concentration compared to the 1% LBG solutions.

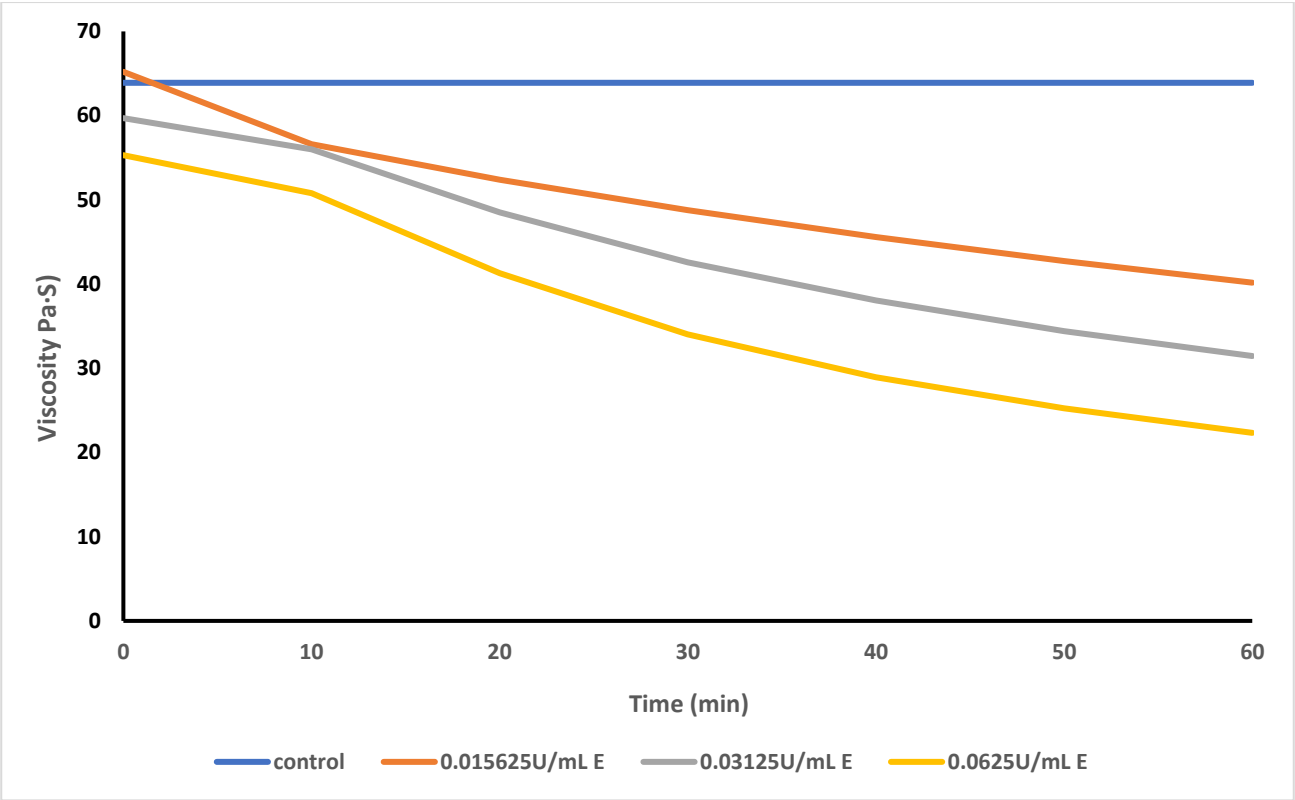


Figure 4-5 Effect of enzymatic hydrolysis on 2% LBG viscosity at 20 ˚C

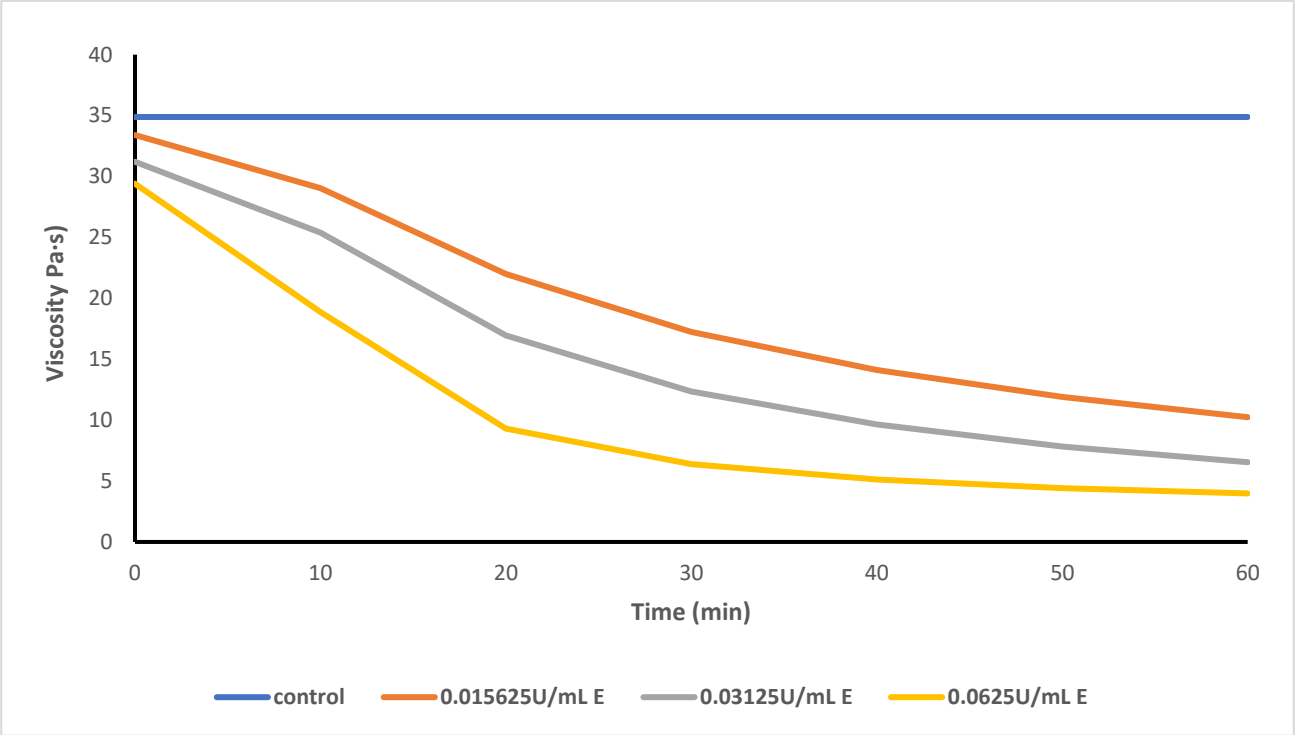


Figure 4-6 Effect of enzymatic hydrolysis on 2% LBG viscosity at 40˚C

Finally, hydrolysis on a higher concentration (2%) of LBG was conducted. The results in Figure 4-5 and Figure 4-6 are in line with the observations made for the other concentrations, that is, higher enzyme concentration gives a sharper reduction on the LBG viscosity at the same reaction temperature and higher temperature contributes to a faster enzymatic hydrolysis under the same enzyme concentration.

4.2 Discussion

The results of these experiments have shown some key points. It has revealed the success of enzymatic hydrolysis on an important rheological property of LBG, which is viscosity. The results are a clear indication that temperature and enzyme concentration are the most important factors affecting enzymatic hydrolysis of LBG. This is evident from the results as a change in these factors had significant effects on different concentrations of LBG's viscosity. It was revealed that the concentrations of LBG used in the study did not have effect on enzymatic hydrolysis. The ability of enzymatic hydrolysis to reduce the chain length and molecular weight of polysaccharides such as guar gum could also be a reason for the low viscosity in hydrolysed products (Mudgil et al., 2012). These results agree with what others have done with other polysaccharides. A study done by (Barak & Mudgil, 2014) reported that hydrolysis of guar gum showed a high impact on reducing viscosity at 30 ℃.The viscosity of guar gum decreased when temperature was increased from 4 ℃ to 37 ℃. This reduction was attributed to the high temperature decreasing inter molecular reactions in the gum and hence interfering with hydrodynamic domain (Srichamroen, 2007). The same effect has been reported by Mahammad et al., 2007 in guar gum who observed that high temperatures can decrease guar gum's viscosity. In addition, they reported that the rate of reduction in viscosity decreases with reaction time (2007).

Other researchers have also investigated the effect of different concentrations of enzymes on enzymatic hydrolysis (Mahammad et al., 2006). A decrease in the concentration of β-mannose from 1.2×10^{-5} to 1.2×10^{-6} U/mL resulted in an increase in reaction time for unentanglement of guar molecules. With such a decrease in enzymatic concentration, the reaction time increased from 62 minutes to 382 mins. These results agree with the results of this work.

In another study, enzymatic hydrolysis of LBG with Beta-D-mannanase at 50 degrees significantly decreased LBG's molecular weight from 5880010 to 3188 (Chen et al., 2018), noting that polysaccharides with low molecular weight tend to have low viscosity. One further point of consideration regarding the data, noting that the final viscosities hydrolysed 2% LBG solutions were higher than the final values at 1% and 0.5%. This observation can be interpreted in two ways: firstly, that the higher substrate:enzyme ratio reduces the extent of hydrolysis within the solution, leaving more intact LBG; or secondly, the rate of hydrolysis is independent of substrate:enzyme, and that higher viscosity is a consequence of a higher number density of hydrolysed fragment at the higher LBG concentration.

These finding provide insights into the rate and extent of hydrolysis in relation to enzyme and polymer concentration. To further control hydrolysis behaviours, the next objective was to determine the conditions for enzyme inactivation in order to terminate the hydrolysis reaction. These findings have been presented in the next chapter.

Chapter 5 Enzyme inhibition

To stop the hydrolysis processing at a given reaction time, a combination of acid and thermalisation was applied after 20 min of the enzyme reaction with 1% LBG. HCl was added before the heat treatment to adjust the pH to 3 in order to stop the enzymes' activity immediately, then it was heat treated under different temperature and time length. The pH was adjusted back to 7 after cooling down. . Any residual activity of the enzyme was determined by measuring the viscosity of LBG solutions with the treated enzyme over a 15 h. A concentration of 0.03125U/mL enzyme was selected. As discussed earlier, the reduction in viscosity of the pure LBG solution indicates the existence of active enzyme. Therefore, the viscosity-time curves of the LBG solution after specific acid-heat treatments are shown below.

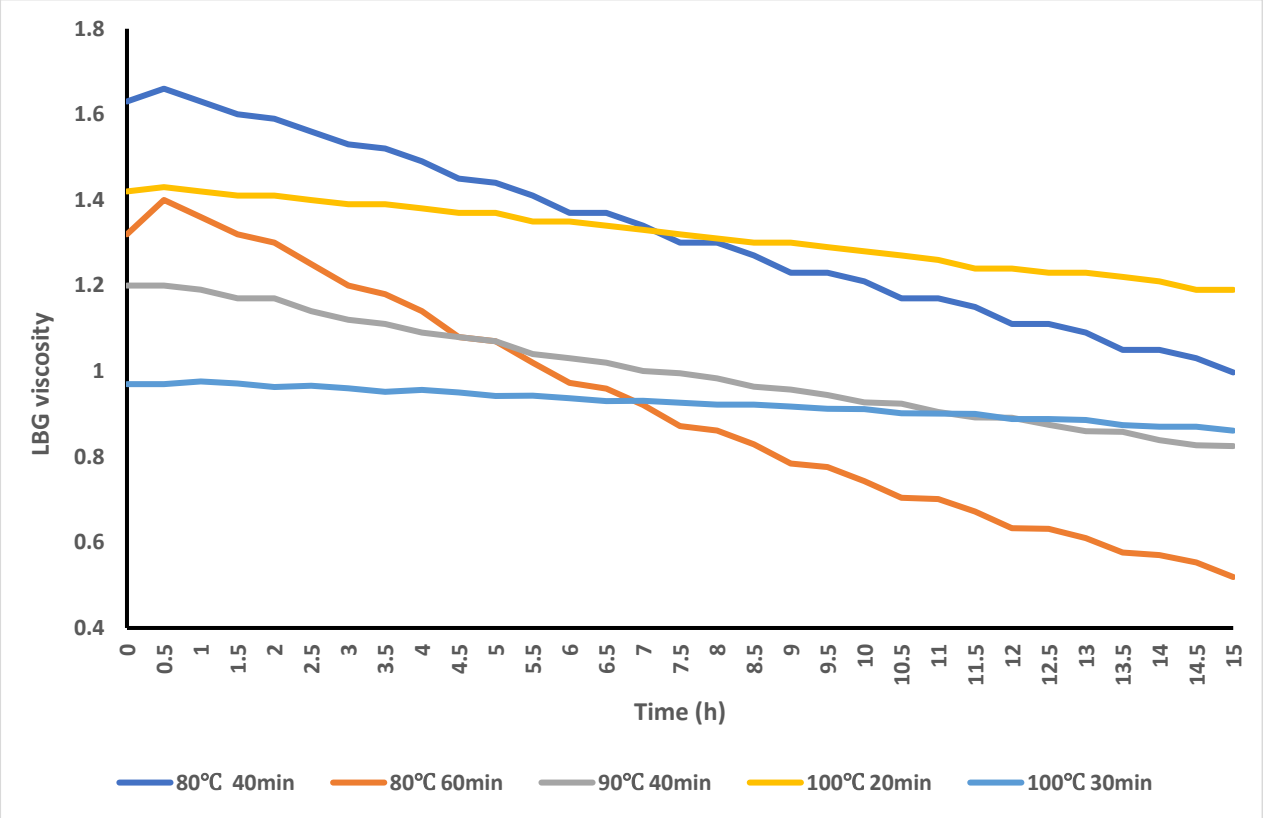


Figure 5-1 Enzyme activity with the substrate of 1% LBG after treatment by different temperature and time lengths. Measured at 20 °C under the shear rate of 0.1/s.

Three different temperatures in combination with 5 thermalisation times were used. These are 80℃ (t = 40 mins and 60 mins), 90℃ (t = 40 mins) and 100℃ (t = 20 and 30 mins). The differences in the viscosity of LBG at the starting point is due to high temperatures destroying the galactomannan backbone leading to reduction in viscosity. From the results, it is evident that both temperature and reaction times are important in effectively stopping hydrolysis. However, temperature is more prominent. The effect is also seen in a temperature of 100^{o}C with reaction times of 20 and 30min. The reaction time at 20min levelled off and the reduction in viscosity was relatively constant. The viscosity with a reaction time of 20min reduced from 1.42 at time t=0 to 1.19 at t= 15h. Also, the viscosity at a reaction time of 30min reduced from t=0.97 to t=0.86 after 15h. Even though the decrease in a relatively long-time range (15h) was low, the decrease in lower time range such as one hour was negligible. A similar observation was seen for the other treatments. It can also be deduced that, higher temperatures (e.g., 100℃) tend to inactivate enzyme activity but does not completely eliminate the activity. The lowest rate of reaction observed was 100 degrees at 20min.

These results imply that temperature and reaction time are necessary in enzyme inhibition. Higher temperature (100℃) works more effectively on enzyme deactivation than lower temperature (80℃), which can be seen by the relatively smooth decreasing rate of the lines, but destroys the LBG structure itself, which can be inferred from the relative lower starting point under the same treating time. Longer treatment time also gives more reduction on the LBG viscosity under the same temperature. Therefore, it is important to balance the treatment time and temperature. Enzymes are proteins and therefore have optimum temperatures in which they can work. Also, they can be inactivated at certain temperatures since relatively high temperatures can denature proteins (Wojcik & Miłek, 2016).

To try and determine appropriate conditions for full enzyme inactivation, higher temperature treatments were applied to mannanase enzyme solutions prior to addition to LBG solutions (on the assumption that full inactivation of the enzyme would not lead to any observable changes in LBG). Thermalisation of the enzyme prior to addition to the LBG also provides an indication of LBG activity without altering the viscosity of the LBG solutions by trying to heat treat the solutions during hydrolysis. Thus, 1mL 0.03125U/mL enzyme was carefully injected into a sealed container, then treated by 140°C oil bath for 5min. After cooling down, the enzyme was injected to the 1% LBG solution and the viscosity test was carried out for 15h.

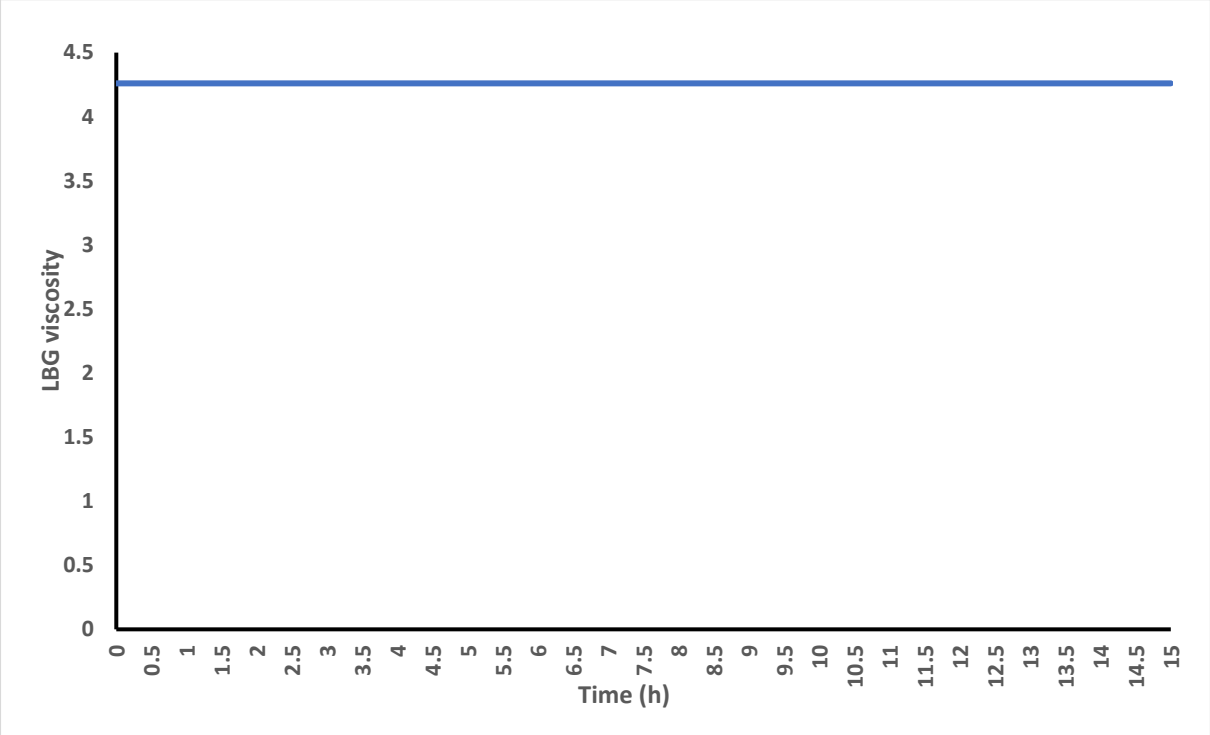


Figure 5-2 Effect of temperature (140℃) for 5min on the activity of enzymes in 1% LBG (non-heat treated) solution

Figure 5-2 shows no change in LBG viscosity over the measurement period. Therefore, it was observed that, a reaction temperature of 140℃ for 5 minutes completely suppressed the enzyme's activity since there was no change in viscosity after 15h.

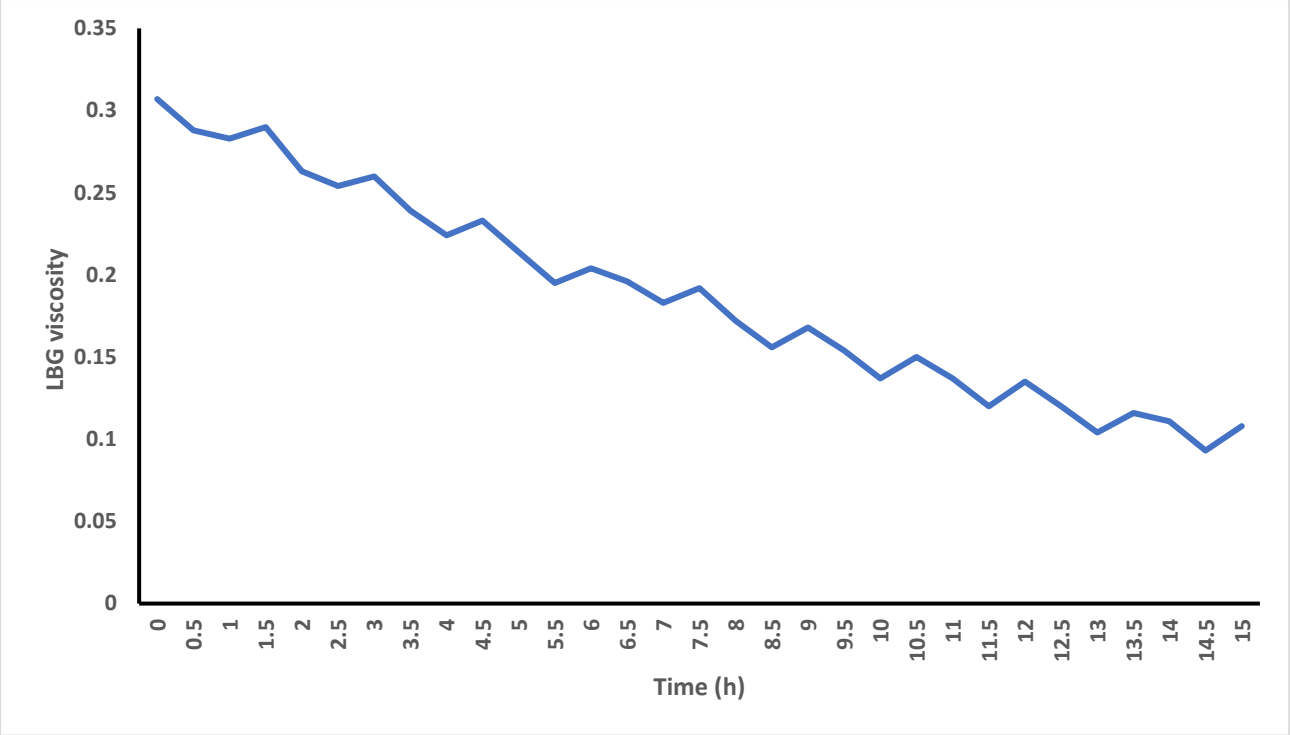


Figure 5-3 Effect of temperature (140˚C) for 24 s on the activity of enzymes in 1% LBG (heat treated) solution

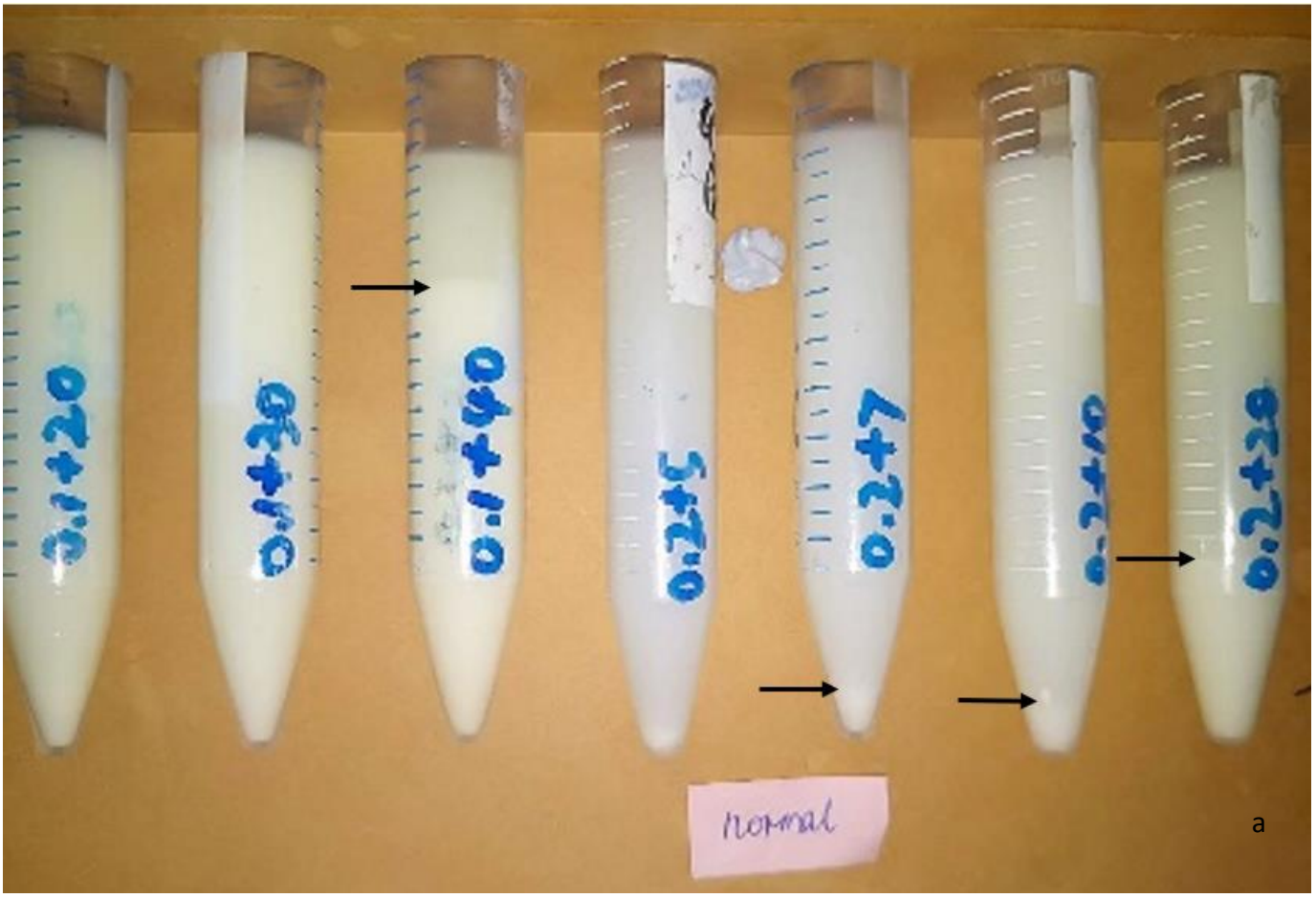
The activity of the enzyme was also tested in UHT treated LBG at 140°C with a reaction time of 24s, noting that high temperature short time conditions would likely have less impact on thermal degradation of the LBG. However, the results shown in Fig 5-3 indicate a reduction in LBG viscosity from 0.3Pa·s at time t=0 to about 0.15 at time t=15h. This means there might be a potential problem in the industry if this mechanism is followed since the enzyme was not inhibited. Moreover, with a viscosity value of 0.3 as the start point, it implies that the UHT temperature, even over shorter heating times, significantly reduced LBG viscosity. The results presented in this chapter indicate an important issue which must be addressed before LBG hydrolysis can be utilized in the food industry. The methods (temperature and heat treatment time) that were tried to inhibit enzymes activity were not adequate. The effect of high temperature (approximately 100℃) which has been observed to reduce the enzyme's activity cannot be used since the results have shown, as this level of thermalisation also has a direct effect on reducing LBG viscosity. This was also confirmed when UHT conditions were also employed. As a result of these setbacks, some researchers have used the normal reducing sugar test.

Results indicate a potential gap in the use of reducing sugars in testing for enzyme activity which most researchers do. This is because our results indicate that viscosity continues to decrease even with the ideal temperatures which have been reported in the literature. It is highly recommended that future studies should further explore methods which can completely inhibit enzyme's activity before it can be applied in the industry.

Considering all the factors, the combination of 90^{o}C for 40min heat with acid treatment was utilized for the enzymatic deactivation in the subsequent studies.

Chapter 6 Impact of hydrolysis on Phase behaviour in model system

To assess the impact of hydrolysis on the phase behaviour of LBG used in combination with other food products, different concentrations of skim milk powder and LBG solutions were combined as a model system. Skim milk consists primarily of protein and lactose and therefore different concentrations of LBG solutions was added to different concentrations of skim milk powder solutions. One major issue in the food industry is phase separation of ingredients during manufacture or storage of food products. Phase separation of polysaccharides and proteins can occur in aqueous systems due to the nature of their chemical bonds and molecular arrangements making them thermodynamically incompatible. In this study, it was hypothesised that hydrolysed LBG would help reduce the phase separation between LBG and skim milk powder by decreasing the relative radius of hydration of the LBG hydrolysates. This was investigated by visual observation of phase separation of mixed samples in solution, and also by the use of confocal microscopy. Full details of experimental design and evaluation are described in the materials and methods chapter. Visual observation of phase separation was used to prepare a phase diagram of SMP mixtures with native and hydrolysed LBG (treated with 0.03125U/mL E-BMACJ for 20min).



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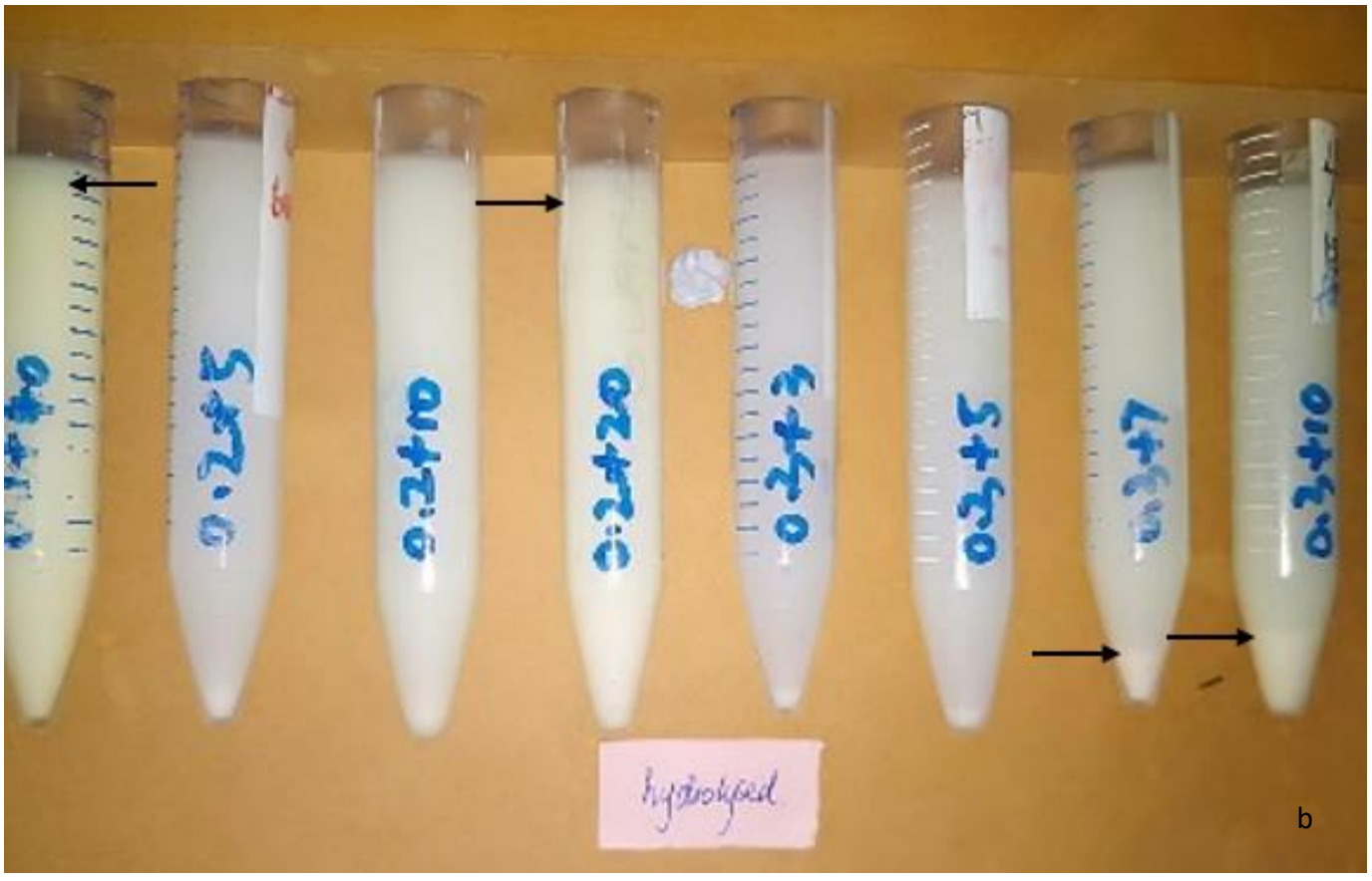

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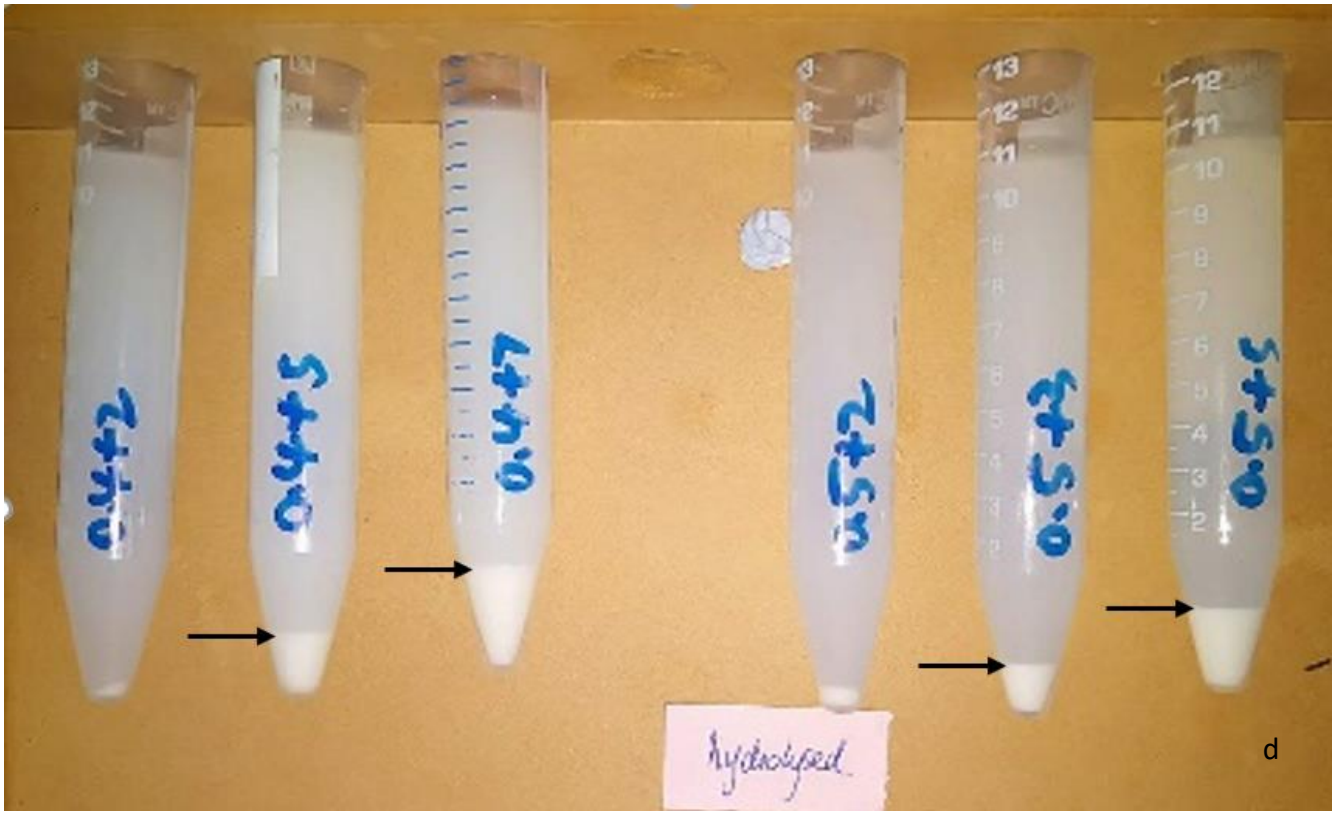

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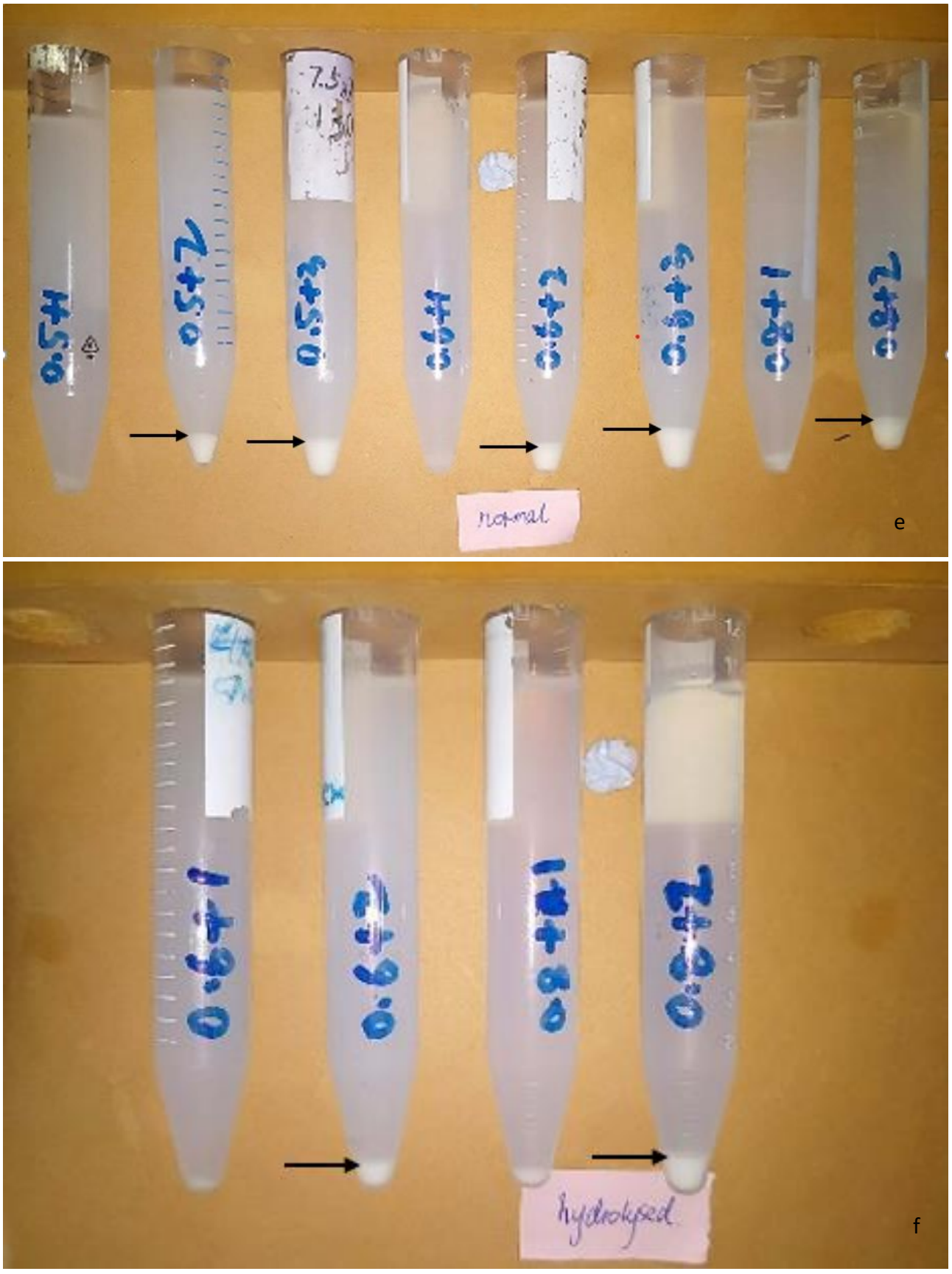

Figure 6-1 Comparison of phase separation of hydrolysed and non-hydrolysed LBG and SMP mixtures (24h after mixing). The first and second numbers on the tube indicate the concentrations of the LBG and SMP respectively. The black arrows refer to the phase separation boundary.

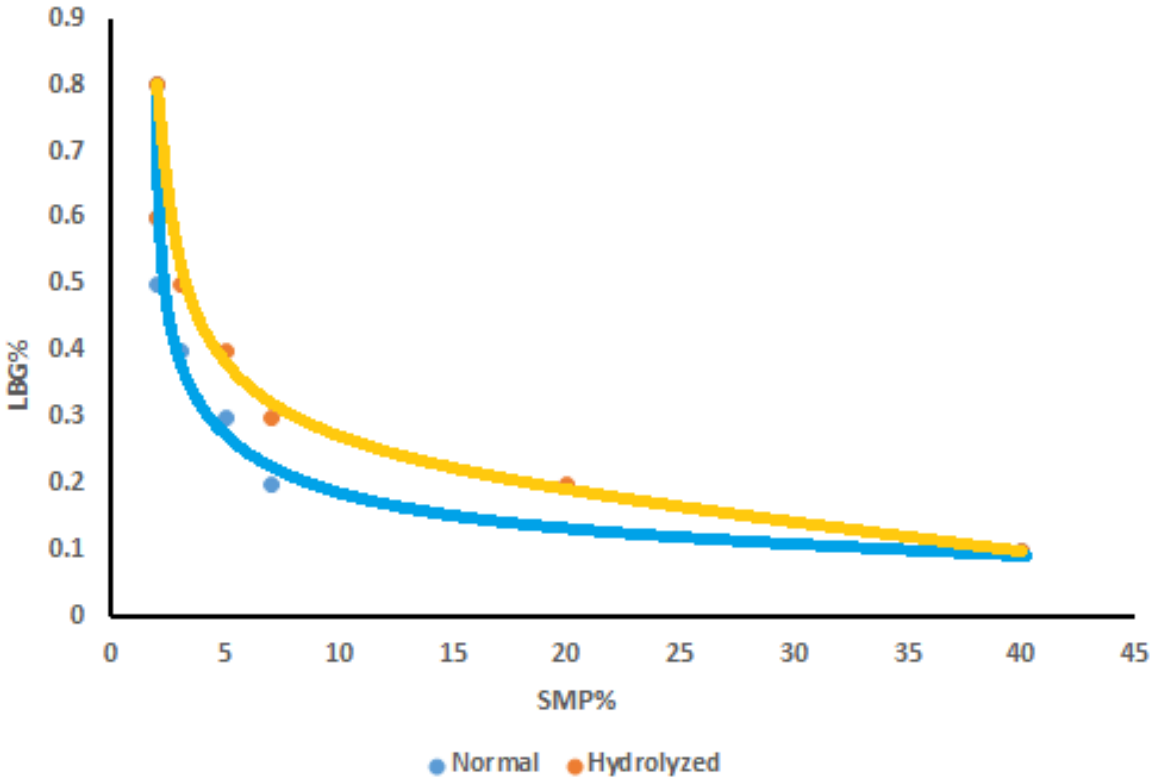

Figure 6-2 Phase diagram of LBG and SMP

From the results, because the use of hydrolysed LBG, the concentration of skim milk powder required for phase separation is higher compared to the concentration of skim milk powder required for normal LBG as can be seen in the phase diagram (Figure 6-2). This is an indication of the ability of hydrolysed LBG to be more compatible with the protein which leads to a delay in phase separation. This can be seen in the images (Figure 6-1) with the black arrows showing the phase separation point that is where we see clear differences between the two biopolymers.

It is important to note that the little dense white coloured debris at the bottom of some of the tubes, such as the tube for 0.4%LBG+2% SMP mixtures in Figure 6-1/c, are not as a result of phase separation but rather insoluble particles that could not be removed from LBG solution. It takes about 24h for the phase separation to be obviously observed visually, but it happens within minutes or even shorter in the microstructure. The confocal microscope helps us capture their changes in the microstructure of the complexes. This evidence can be inferred from the confocal microscope image as seen in Figure 0-5 (in the appendences), which indicates no separation occurred.

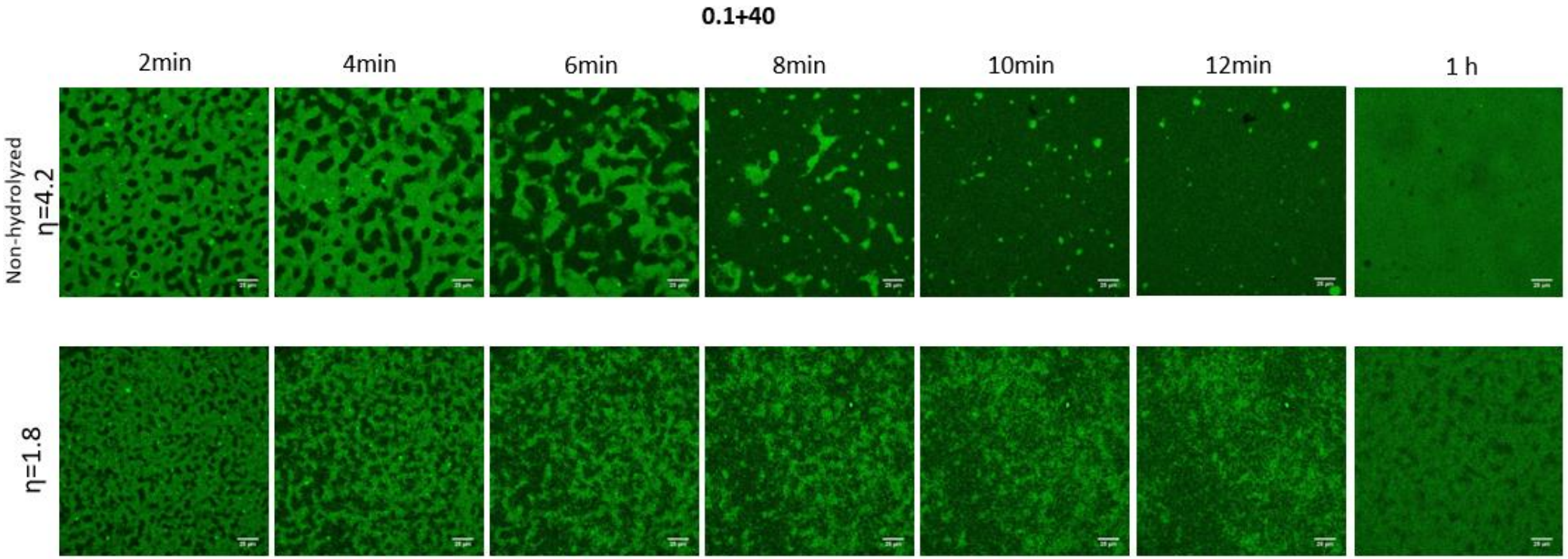

Figure 6-3 Microstructure of 0.1% LBG and 40% SMP mixtures

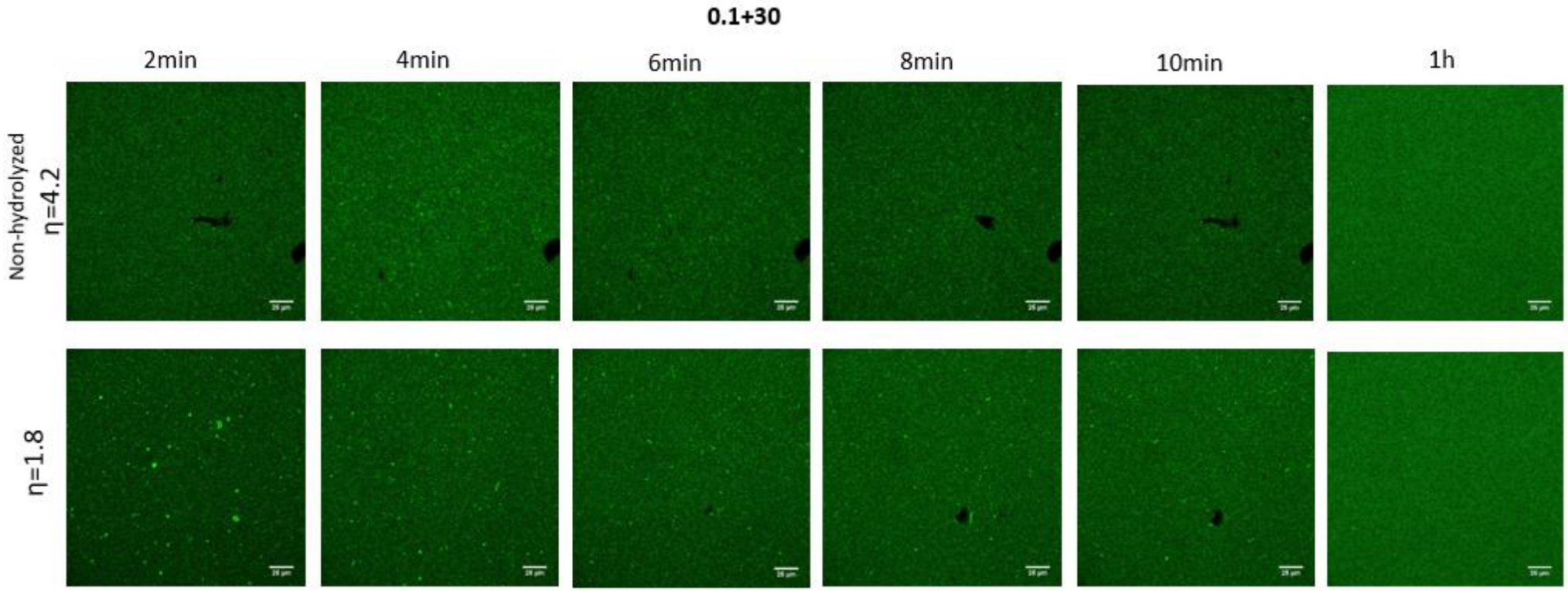

Figure 6-4 Microstructure of 0.1% LBG and 30% SMP mixtures

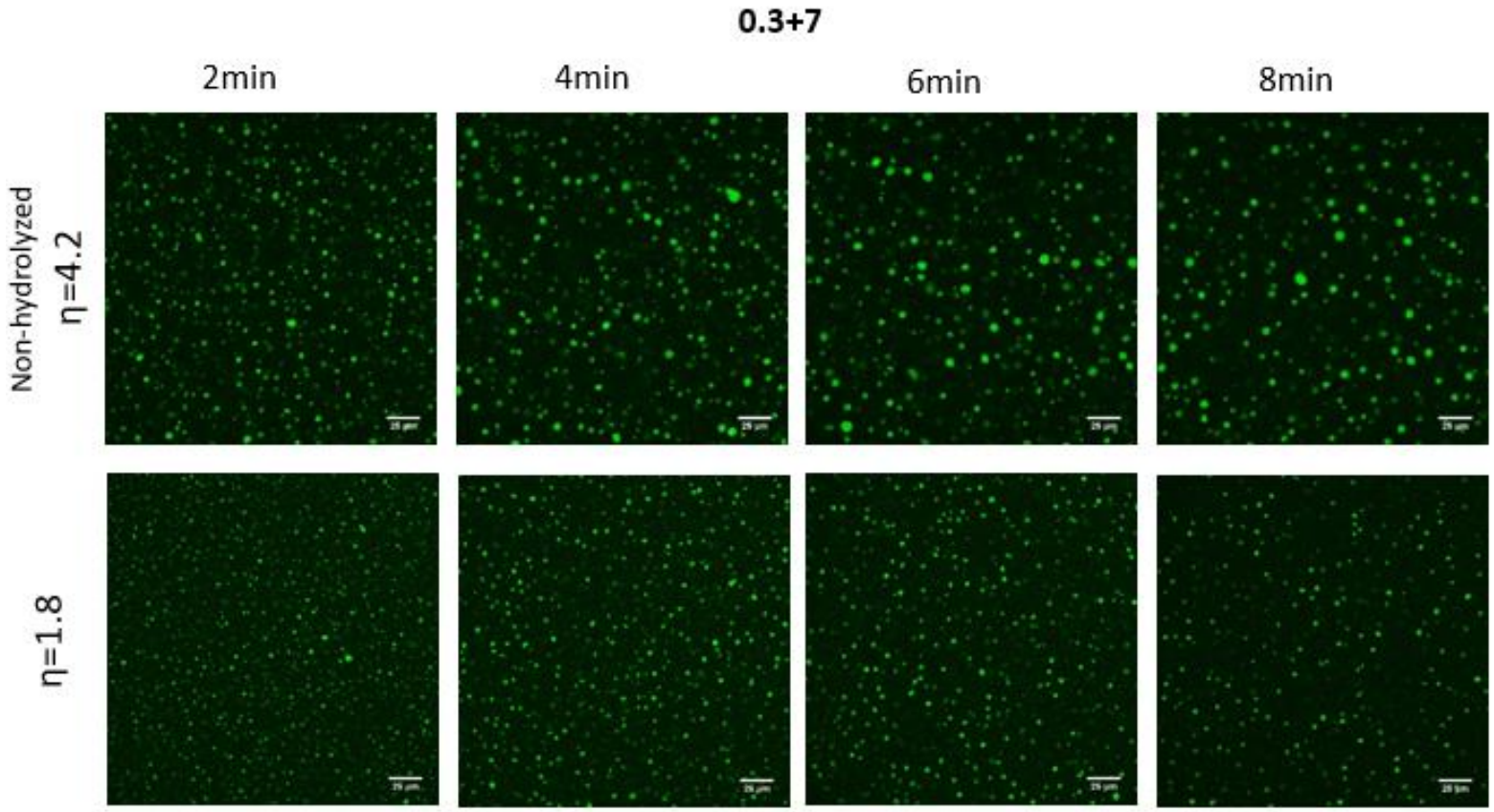

Figure 6-5 Microstructure of 0.3% LBG and 7% SMP mixtures

Confocal microscope was used to compare the microstructure of hydrolysed and non-hydrolysed LBG on phase separation. With the proteins been stained with fast-green and therefore appearing green, observation of a uniform image indicates that the two biopolymers are compatible as seen in Figure 6-4 (0.1% LBG with 30% skim milk powder solution). However, images appearing like dotted green (proteins) surrounded by black background (LBG) show incompatibility as both biopolymers are not able to mix uniformly (Figure 6-5). Also, their separation was rapid due to the high concentration of the two biopolymers as well as their incompatible nature. In terms of interpreting micrograph information for highly incompatible mixtures, it should be noted that the extent of phase separation can exceed the viewing boundaries of the micrograph, making the systems appear homogeneous.

Figure 6-3 showed the transient destabilization of non-hydrolysed and hydrolysed LBG. The images of the non-hydrolysed LBG showed that phase separation took a relatively short time to happen. From time t=2min (or before), the separation started as shown in the non-uniform mixture and the separation was directly proportional to time. By the 10^{th} minute, it had been completely separated with the few dotted green lines (protein) with a pronounced black background (LBG). However, for the hydrolysed LBG, the mixture was relatively uniform right from the beginning at time t=2min. Unlike the non-hydrolysed LBG, we could see a uniform and compatible mixture even at the 10^{th} and 12^{th} minute. These suggest that with the use of non-hydrolysed LBG, phase separation occurs rapidly, and separation continues to increase with time. On the other hand, with the use of hydrolysed LBG, phase separation occurs slower than the system with the intact LBG/protein. A further point to note here is that the viscosity of the hydrolysed mixture is lower than that of the non-hydrolysed sample, indicating that even with a degree of viscous resistance, phase separation is still more rapid in the non-hydrolysed mixture. The micrographs shown here are intended to represent regions of the phase diagram of stable, transitional and incompatible. Further examples of the micrograph data from the phase diagram are presented in the appendices.

High concentrations of SMP (30 - 40%) tended to provide more effective visualisation of the phase separating process with the combination of non-hydrolysed and hydrolysed LBG at concentration ranging from 0.1% to 0.8% (Figure 0-12 and Figure 0-13 in the appendences).

At LBG concentrations lower than 0.4%, the thermodynamic incompatibility is stronger at the non-hydrolysed LBG system than the hydrolysed LBG system, whereas at concentrations higher than 0.5%, the viscous forces begin to influence the rate of separation in the system. Accordingly, the phase separation becomes slower at the non-hydrolysed LBG mixture than the hydrolysed mixture; a phenomenon which has been reported previously by Erçelebi & Ibanoğlu (2009).

Many foods contain complex mixtures of proteins and polysaccharides. The interaction of these macromolecules and understanding their contributions in a food product is important. Several authors have reported about the study in phase separation phenomenon. An example can be found in a study conducted by Jara et al. (2010), whey protein and hydroxypropylmethylcellulose (a polysaccharide used in the food industry and has low viscosity) solutions are unstable at neutral pH which cause separation in a protein rich and a polysaccharide rich phase. This result was similar to the results obtained in our study.

Depletion flocculation is one of the reasons that causes the instability of the biopolymer mixtures. Addition of low concentrations of LBG in to the SMP solutions can result in flocculation (Harding et al., 1995). In the casein/LBG mixed systems, the osmotic pressure between the solvent in the space among the casein micelles and the bulk solvent containing the LBG results in the depletion flocculation (Asakura & Oosawa, 1958). This process speeds up the system to reach the thermodynamic incompatibility threshold, and therefore leads to the phase separation. A study done by Thaiudom and Goff (2003) affirmed this theory by comparing the phase separation behaviour of milk protein mixed with LBG, guar gum and xanthan gum respectively.

One of the factors which can affect phase separation is molecular weight. The hydrolysis of LBG resulted in reducing the molecular weight of LBG therefore making the molecular weight of the hydrolysed LBG to be lower than the non-hydrolysed LBG. Polymers with low molecular weight tends to mix more effectively with others in a solution. This has been confirmed in a study conducted by Thaiudom and Goff (2003). In a mixture of two biopolymers, the one with a higher molecular weight tends to concentrate more in one phase leading to fractionating (Frith, 2010; Vladimir Tolstoguzov, 2002). This may explain the results of this study where hydrolysed LBG had a more stable system than non-hydrolysed LBG. A similar study was conducted by Hussain et al., 2017. Enzyme hydrolysed

guar gum was implemented to reduce phase separation in yoghurt, their results showed that the microstructure stability had been improved by reducing the chain length of the complex. They had low osmotic potential and less viscosity compared to the normal crude and purified guar gum (Hussain et al., 2017)

Accordingly, LBG hydrolysis can be used to manipulate phase behaviour in mixed protein-polysaccharide solutions. While this may allow for improved physical stability in terms of reducing propensity towards phase separation, the hydrolysis will invariably impact on other properties of the system. The most obvious consequence, i.e., the progressive reduction in viscosity, has already been demonstrated within this thesis. However, reduction of molecular weight and chain length of native LBG may have consequence on functionality other than just viscosity. This is the focus of the next chapter, in which the influence of LBG hydrolysis on the properties of ice cream is investigated.

Chapter 7 Impact of hydrolysis of LBG on ice cream

In this chapter, the effect of hydrolysed LBG added as a stabiliser to ice cream was assessed. With the previous knowledge about hydrolysed LBG delaying phase separation, hydrolysed LBG was added incorporated into ice cream formulation and the properties of the resulting ice cream mixes and frozen ice creams compared to samples containing non-hydrolysed LBG.

LBG has been used in ice cream manufacture for many years, where it provides important contributions to mouthfeel, melt stability and (arguably most importantly) stability against ice recrystallisation during storage. However, one particular issue arising from the use of LBG is phase separation of the polysaccharide and milk protein components during mix aging and ice cream melting (so-called wheying off). It was therefore hypothesized that hydrolysed LBG would reduce the propensity for phase separation. The study conditions used to investigate the impact of LBG hydrolysis on the properties of ice creams are outlined in the materials and methods chapter

The impact of LBG hydrolysis on the phase stability of ice cream mixes is demonstrated in the confocal microscope images in Figure 7-1. A comparison of the microstructure of ice cream without LBG (zero control), with LBG (normal control) and with hydrolysed LBG shows that, the ice cream mix with 20min hydrolysed LBG has fewer and smaller voids (background), indicating increased compatibility. This is clearer in the image with a higher scale bar as shown. The image with no LBG only showed the microstructure of protein and fat molecule complex. Moreover, hydrolysis of LBG did not change the interaction between protein and fat droplets, which can also be seen in the image (fat was dyed to red colour using Nile Red, and protein was dyed to green using Fast Green).

This result indicates that hydrolysis of LBG can potentially be used in the ice cream making industry to slow down the phase separation during the aging stage or during ice cream melting. The application of hydrolysis in the ice cream affirms our hypothesis and the result is in line with the result showed in chapter 6 when it was conducted in the model system.

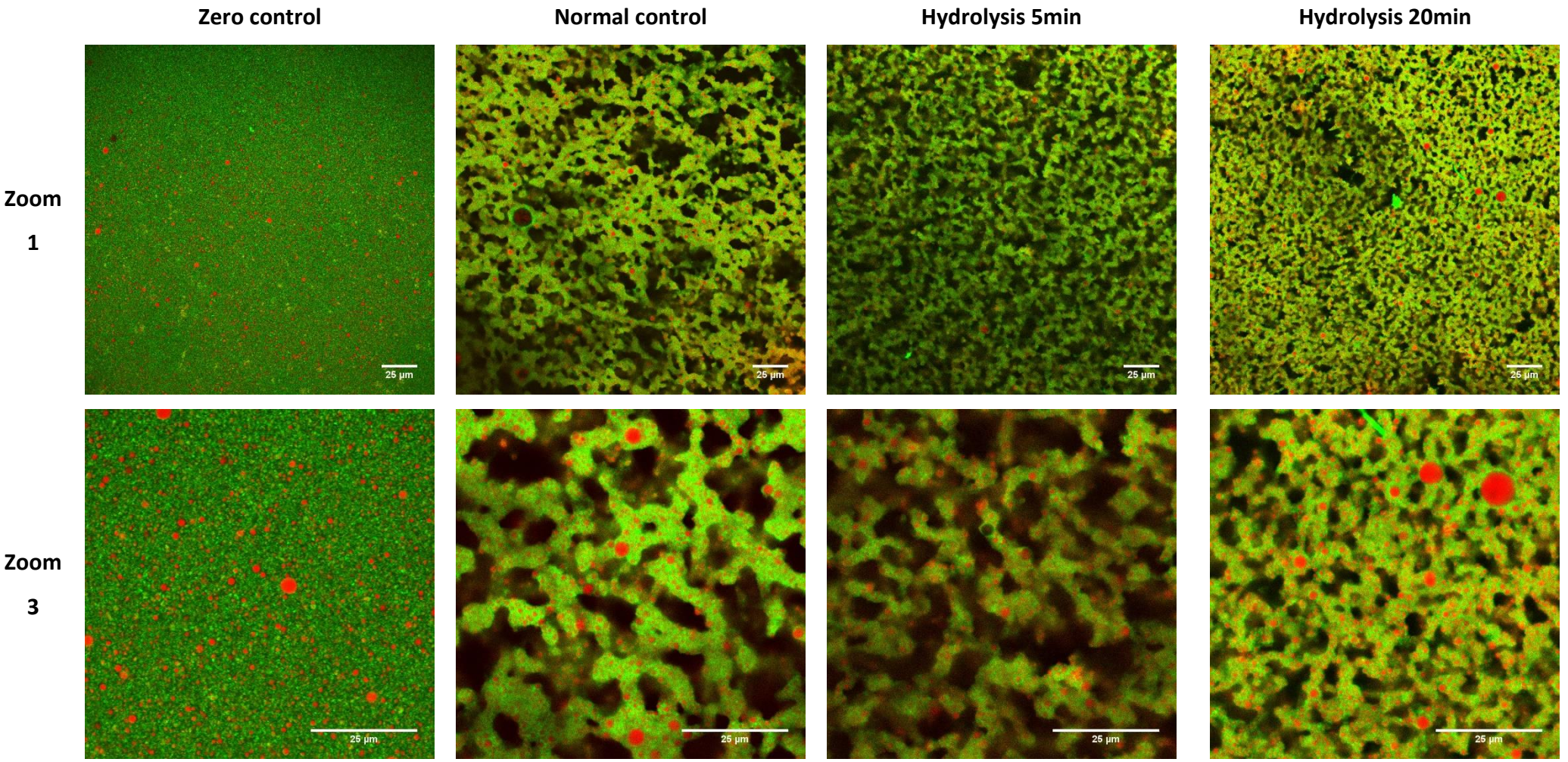

Figure 7-1 Microstructure of ice cream mix after aging

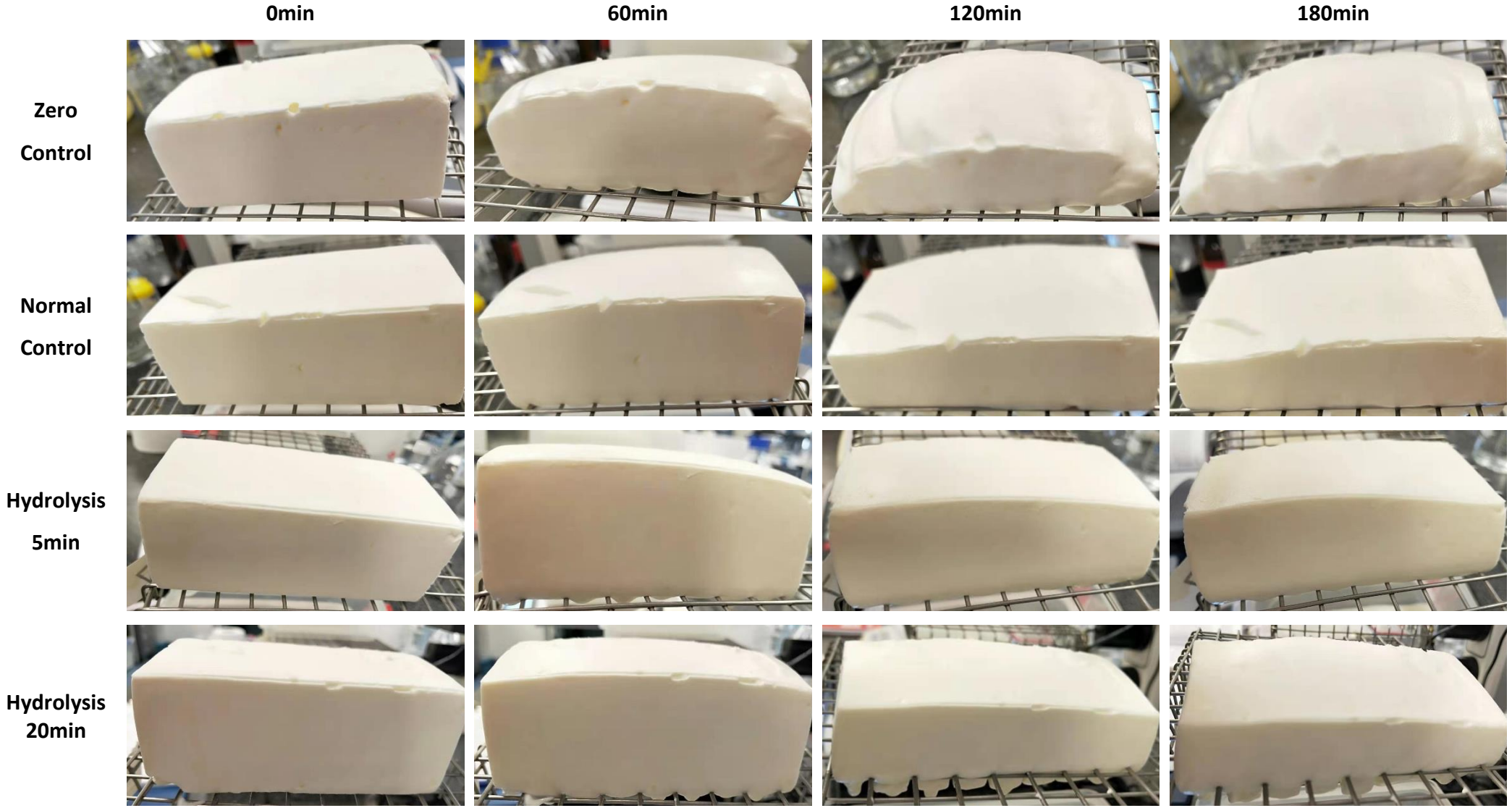

Figure 7-2 Images of ice cream meltdown

In addition, the effect of hydrolysed LBG on ice cream meltdown rate was studied. It should first be noted that all formulations contain added monoglyceride, which provides a greater impact on improving melt stability than the addition of stabilisers. However, the inclusion of stabiliser does typically provide an additive contribution to melt stability beyond the use of emulsifier alone. In this regard, it can be seen that the ice cream with no LBG added melted the fastest. Addition of LBG showed an incremental improvement in melt stability. Interestingly, hydrolysis of LBG did not appear to significantly affect rate of melt. From Table 7-1 we can see that the meltdown rate increases marginally with the increment of the hydrolysis time, which can be inferred that the more the LBG is hydrolysed, the faster the meltdown rate.

It also needs to be noted that the start-melting point of the four samples in Figure 7-3 are also close (started melting at 50min) and this is in line with a study done by Herrera et al. (2007), stating that the addition of hydrocolloids did not greatly affect the melting onset of the ice cream.

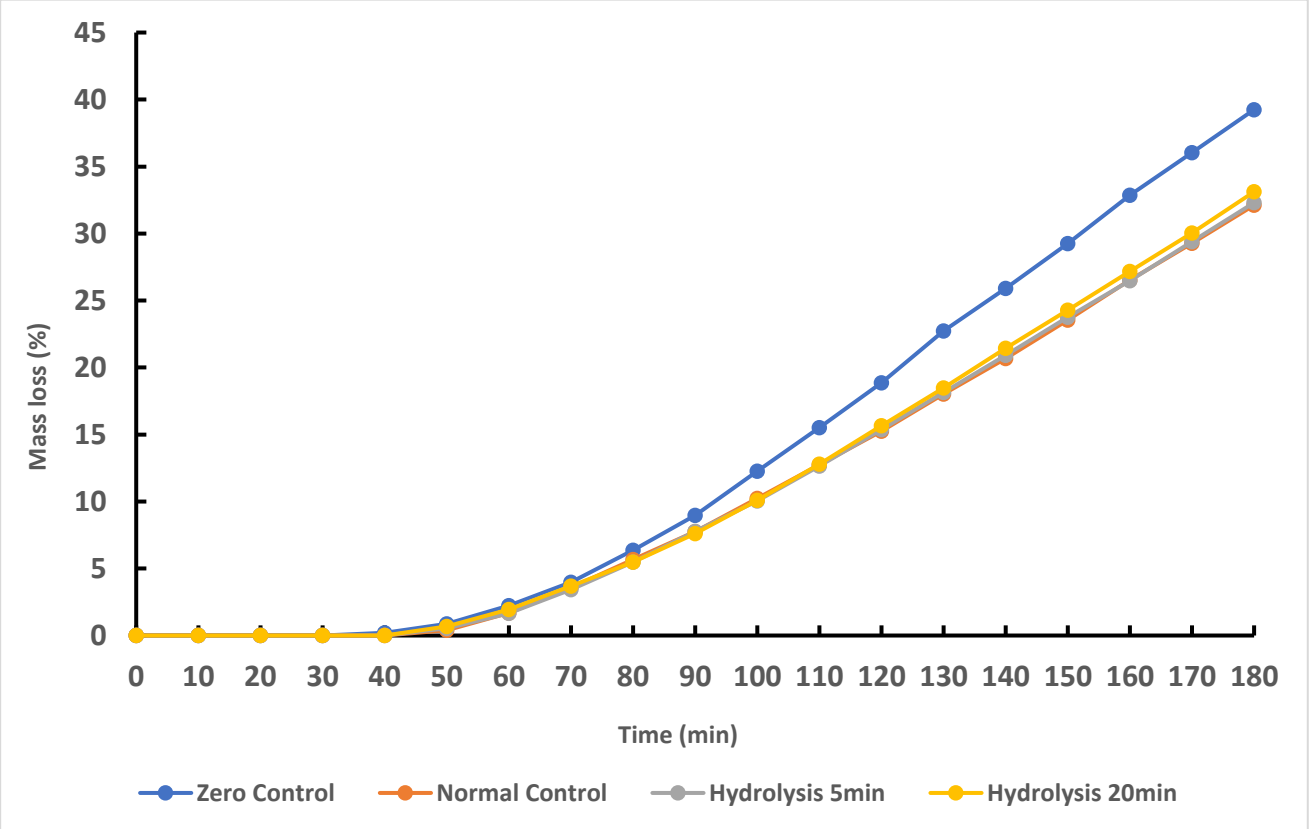

Figure 7-3 Ice cream meltdown curve

Table 7-1 Ice cream melt down rate

Sample name	Meltdown rate (g/min)
Zero Control	0.36
Normal Control	0.24
Hydrolysis 5min	0.26
Hydrolysis 20min	0.28

Table 7-2 Overrun (%) of zero control, normal control and ice cream with hydrolysed LBG

Sample name	Overrun %
Zero Control	44.4
Normal Control	94.4
Hydrolysis 5min	73.3
Hydrolysis 20min	67.0

The overrun value is an index of the air-trapping ability of the ice cream. It is calculated by Equation 1. The higher the overrun value indicates more air can be incorporated in the ice cream and *vice versa*. From Table 7-2, we can see that the zero control sample (no LBG added), has the lowest overrun value of 44.4%. The normal control sample (with the intact LBG added) recorded the highest overrun value, with a number of 94.4%. The overrun value decreases as the extent of LBG hydrolysis goes up, with 73.3% of overrun for 5 min hydrolysed LBG and 67.0% overrun for 20 min hydrolysed LBG. That means the more viscous the system is, or in another word, the longer the LBG chain, the higher the overrun value and hence the more air can be incorporated. This is in line with expectations, since one of the roles of stabiliser addition is to increase mix viscosity, which provides more effective whipping and air incorporation during freezing.

The amount of air trapped into the ice cream during freezing will also be expected to influence the size of the ice crystals - the larger the ice crystals, the lower the overrun value (Arbuckle, 1977). The amount of air that can be incorporated in the ice cream is important, as the structure of the air cell had been proved to be one of the main factors to affect meltdown rate and shape retention during the meltdown (Bahramparvar & Mazaheri Tehrani, 2011).

Slow meltdown, good shape retention and slower foam collapse are the desired factors by ice cream consumers (Wildmoser et al., 2005). Bahramparvar and Mazaheri Tehrani (2011) reported that the air cell structure affects the meltdown rate and the shape retention during meltdown. The smaller the air cells, the better the product quality, and the addition of stabilizers can reduce the air cell size.

A study done by Y. Chang and R. Hartel (2002) investigated the effects of stabilizer level on the development of air cells. Change of stabilizer (carrageenan, gaur gum and CMC) amount did not change the melting temperature and overrun. However, the existence of stabilizer can reduce the air cell size, and this can be attributed to the addition of stabilizers changed the rheological properties of the ice cream during freezing.

Ostwald ripening, coalescence and drainage are the three main mechanisms for the changes of air cells during the ice cream storage stage (Bahramparvar & Mazaheri Tehrani, 2011).

Addition of stabilizers can inhibit the air cell coarsening by increasing the ice cream mix

viscosity (Y. Chang & R. W. Hartel, 2002). Addition of stabilizers can also prevent disproportionation (a phenomenon happens owing to the differences Laplace pressure between air cells) by lifting up the viscosity of the serum phase and shaping a thick film at the air cell surface (Y. Chang & R. W. Hartel, 2002). Moreover, addition of stabilizer or decrease the storage temperature can potentially retard drainage, as drainage changes the film thickness between the air cell and therefore accelerate coalescence (Sofjan & Hartel, 2004).

Bahramparvar and Mazaheri Tehrani (2011) has also reported that stabilizers do not change the thermodynamic properties, such as glass transition and ice content, but help with the resistance of the ice cream's thermal deformation. The content of LBG presenting in the ice cream makes the ice cream to froze faster. In addition, the air cell changing rate depends on the storage temperature and the emulsifier and stabilizer content. Decrease in storage temperature can slow down the coarsening of the air cells (Bahramparvar & Mazaheri Tehrani, 2011).

Heat transfers from the surrounding environment into the ice cream to melt the ice crystals when the ice cream melts. LBG at this stage, functions to increase the melting resistance due to its ability of water-holding and micro viscosity enhancement (Marshall et al., 2003). From our results, it showed that the longer time hydrolysis treated LBG showed weaker ability of melting resistance. This can be attributes to the damage of the LBG backbone leading to its decrease in viscosity and water holding capacity.

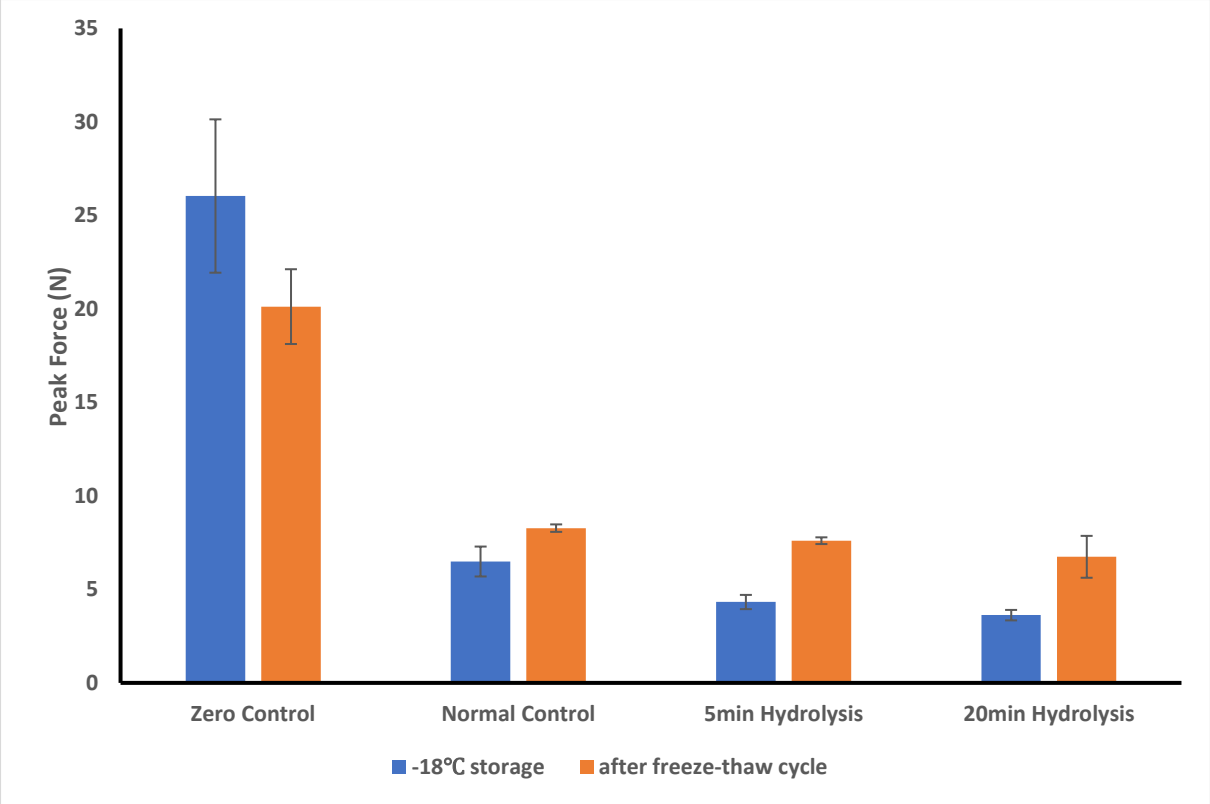

Figure 7-4 Firmness of ice cream stored at -18°C and firmness of ice cream after the freeze-thaw cycle

Figure 7-6 shows the firmness of the samples with non-LBG, intact LBG and hydrolysed LBG. Freeze-thaw cycle was compared to the constant storage. The figure indicates that the existence of LBG has a significant effect on preventing the formation of the ice crystals during storage. This is evident from the zero control and normal control samples. Zero control had a high number of peaking force whereas the normal control sample was significantly less firm. Slight difference of firmness between the samples with hydrolysed and non-hydrolysed were observed, with a number of 6.4N for the normal control, 4.3N for the 5min hydrolysed and 3.6N for the 20min hydrolysed sample respectively. This finding is interesting in that the initial hardness of these sample (prior to temperature cycling) is most likely to directly correlate with overrun. Certainly, the much higher firmness of the zero control sample is (at least partly) based on the fact that it has a much lower overrun compared to the normal control. However, relative to the normal control, overrun decreases for the samples containing hydrolysed LBG, and yet these samples produce a softer ice cream. This means that is the hydrolysed LBG is having a different effect on ice

cream structure, resulting in a softer product, although the findings do not provide any immediate alternative explanation for this effect.

However, it can also be observed that hydrolysis of LBG appeared less effective at preventing ice recrystallization during the freeze-thaw cycle. This can be seen in the yellow bars, the firmness of the samples with LBG are close, with a number of 8.2N for normal control, 7.6N for the 5min hydrolysis and 6.7N for the 20min hydrolysis. The magnitude of increase in firmness arising from temperature cycling therefore appears greater with increasing extent of hydrolysis.

Recrystallization happens under the mechanisms of accretion and migration. Accretion is coalescence of the two adjacent small ice crystals; migration occurs during the freeze-thaw cycle. When the ice cream begins to warm up, the smaller ice crystals melt and the liquid of the melted ice move to the surface of other larger ice crystals. When the temperature is lowered again, the liquid refreezes onto these remaining larger crystals, increasing their size. LBG can increase the serum phase viscosity in frozen ice creams and form a three-dimensional gel structure to prevent the growth of the ice crystals. The hydrating capacity and gelling ability of LBG makes it a good stabilizer to prevent the recrystallization (Adapa et al., 2000). Flores and Goff (1999) reported that stabilizers did not have effect of recrystallization when the ice cream was stored at constant temperatures (-30^{o}C and -16^{o}C) but played an important role when the temperature fluctuated. LBG in the ice cream can form cryogels under the heat shock during storage that can prevent the growth of the ice crystals (Bahramparvar & Mazaheri Tehrani, 2011). The gel structures can restrict water diffusion, and the firmness of the gel is related to the ice crystal change morphology (Muhr & Blanshard, 1986). In our study, the reason that the hydrolysed LBG showed relatively poorer crystallization-preventing effect than the non-hydrolysed LBG could be contributed to the decrease in viscosity and its gelling and hydrating ability due to hydrolysis.

Chapter 8 Conclusions

The development and optimization of novel polysaccharides which can be used in the manufacture of dairy food products is essential to the dairy industry. This study explored the modification of LBG, a polysaccharide with a high source of dietary fibre, and how it can be used in making ice cream. The main concern with LBG as a food ingredient is its incompatibility with proteins leading to phase separation which is common in the dairy industry. In addition, its high viscosity nature even at low concentrations makes it undesirable to be used in the food industry especially beverages that need clear solutions.

In an effort to solve this, the first part of the study was the use of an enzyme (β-mannanase) to hydrolyse or break down LBG. This is because LBG is composed of a long chain of galactomannan and therefore it was hypothesized that hydrolysis of LBG can reduce its intrinsic viscosity and help slow down phase separation. It was revealed that, the enzyme had effects on reducing the viscosity of LBG even when used in low concentrations, and the activity of the enzyme was only affected by the environment temperature and its concentration, the influence of other factors, such as LBG concentration, are neglectable.

The study continued with looking at the effect of the enzymatic hydrolysed LBG on the phase behaviour of LBG and SMP mixtures. The model system consists of different concentrations of skim milk powder. The results indicated that hydrolysed LBG/SMP were compatible at higher concentrations compared to the non-hydrolysed LBG/SMP complex. This was also confirmed with the result of confocal microscope by observing the microstructure which revealed that under the same concentrations, the hydrolysed LBG/SMP had a slower phase separation compared to the non-hydrolysed system.

The last part of the study investigated the application of hydrolysed LBG in ice cream. The results indicated that hydrolysis of LBG can potentially be used in making ice cream It was revealed that hydrolysed LBG can slow down phase separation during the aging stage. The application of hydrolysis in the ice cream affirmed our hypothesis and the result agreed with the result obtained in chapter 6 when it was conducted in the model system.

The results showed that the hydrolysis of LBG did not show a significant effect on the ice cream melt down rate. However, it was found that the further it was hydrolysed, the faster it melted. This implies that the viscosity of the intact LBG gave to the system was important

to control the air cell structure. Moreover, it was revealed that the overrun value decreased with the increment of the hydrolysis time. The hydrolysed LBG also showed a slightly poorer anti-recrystallization ability compared to the non-hydrolysed LBG.

In general, we can conclude that, hydrolysis of LBG could help with the phase separation problem during the aging stage and did not have significant effect on other properties in the ice cream. The result showed that LBG hydrolysis slightly negatively affected functions, such as gelling and hydrating capacity and anti-recrystallization ability of the LBG as a stabilizer compared to the intact LBG.

However, in some ways the most significant implication from this study is the resistance of the mannanase enzyme to inactivation. The enzyme proved to be remarkably stable to thermal inactivation, noting that temperature conditions leading to deactivation of the enzyme would as likely cause as significant a loss in LBG viscosity through thermal degradation as from enzymatic hydrolysis. This has presented challenges in ensuring that hydrolysis was fully halted as part of rheological and phase behaviour studies, as well as applied effects in a food system. Arguably the most important recommendation from this study would be to further explore mechanisms for effective enzyme inactivation, and particularly those that will not additionally independently impact on the properties of the LBG.

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Appendences

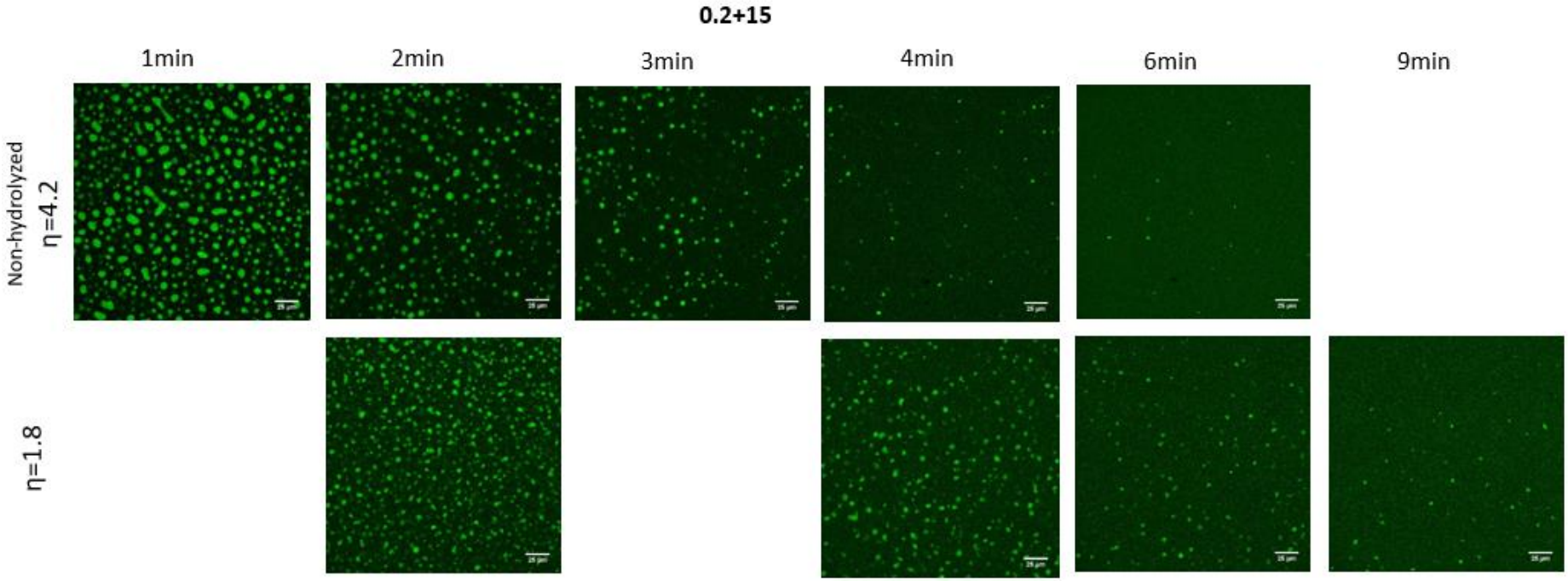

Figure 0-1 Microstructure of 0.2% LBG and 15% SMP mixtures

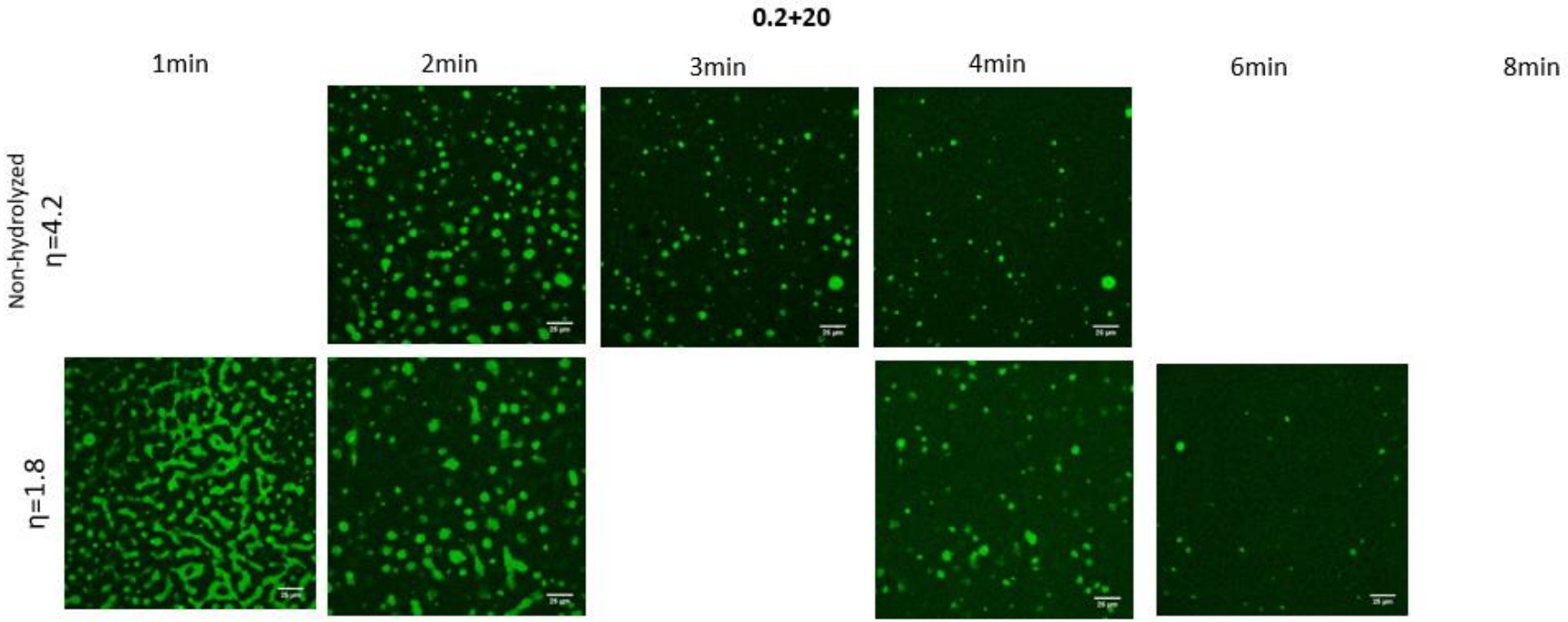

Figure 0-2 Microstructure of 0.2% LBG and 20% SMP mixtures

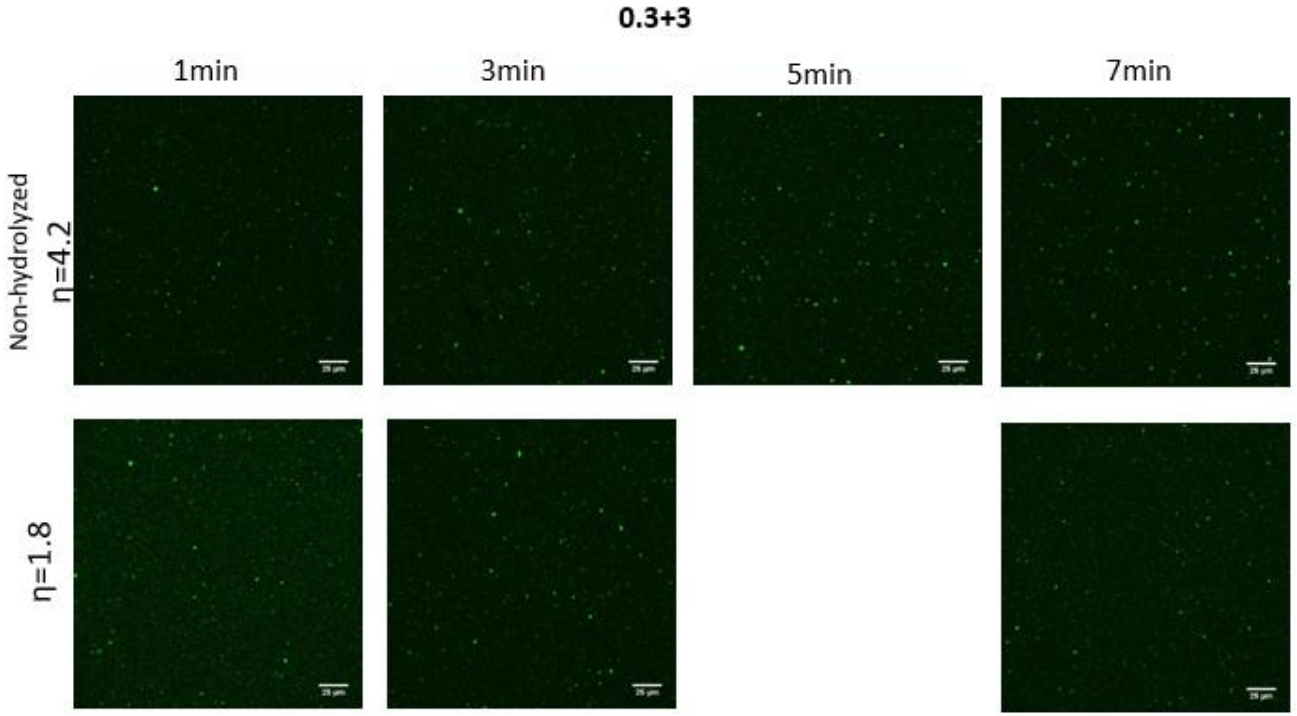

Figure 0-3 Microstructure of 0.3% LBG and 3% SMP mixtures

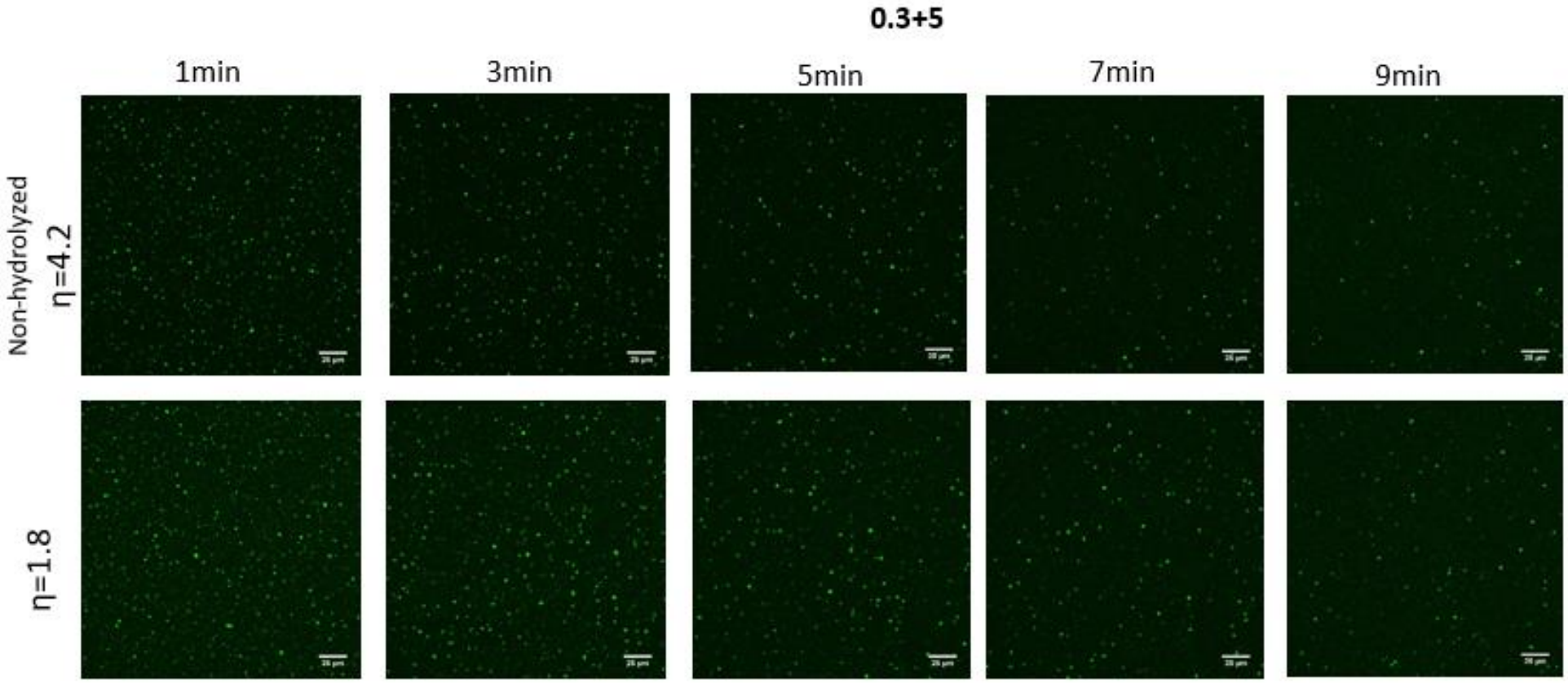

Figure 0-4 Microstructure of 0.3% LBG and 5% SMP mixtures

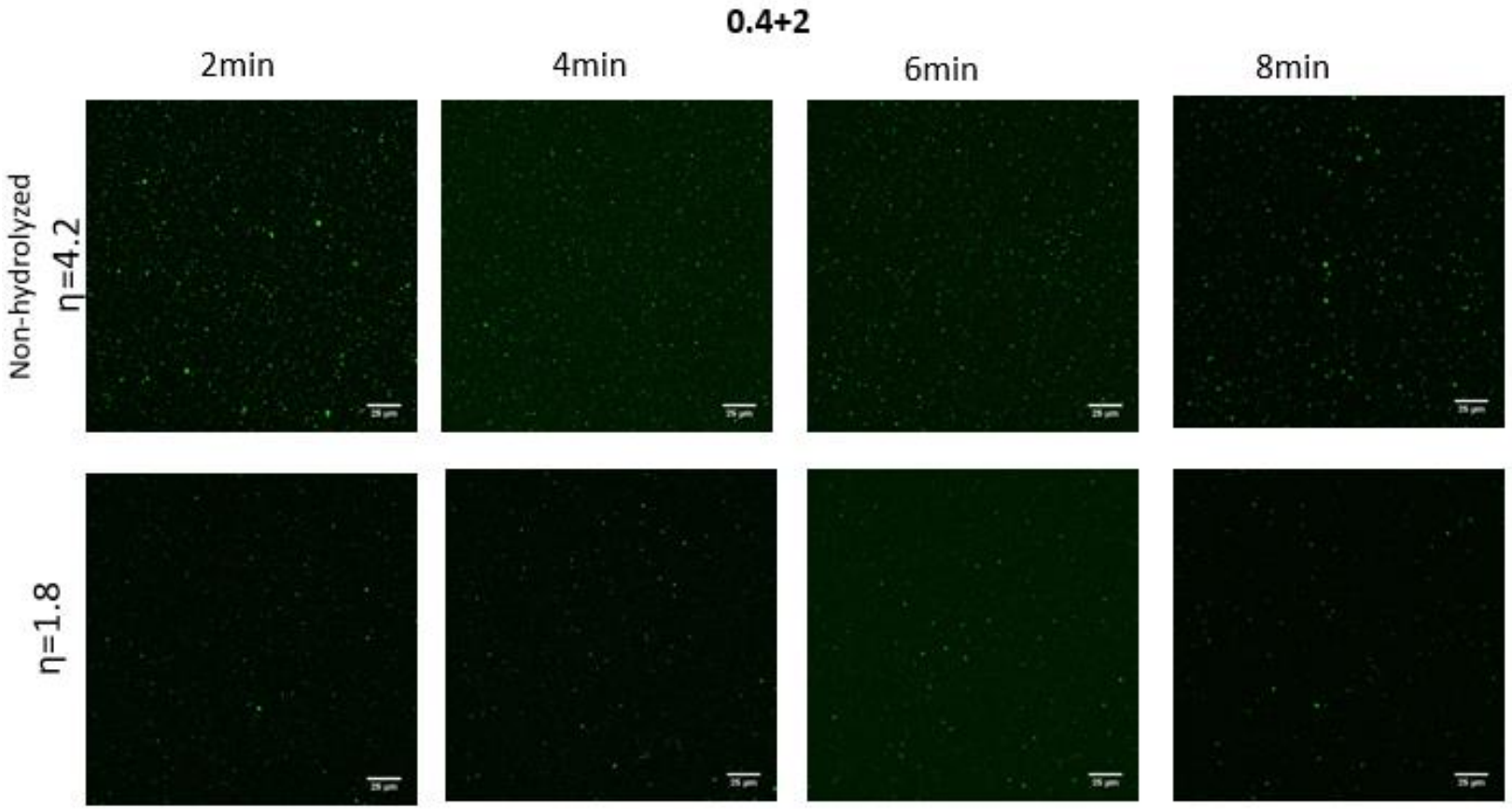

Figure 0-5 Microstructure of 0.4% LBG and 2% SMP mixtures

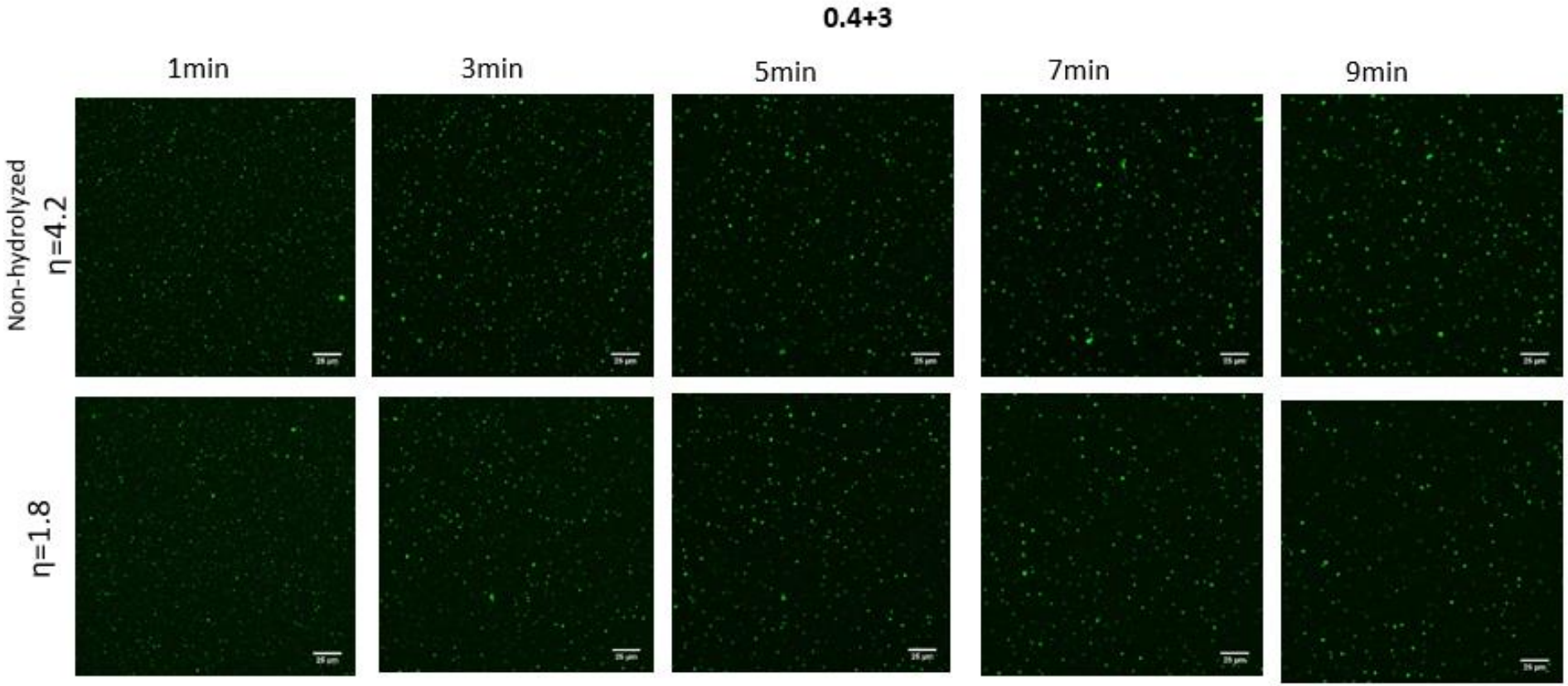

Figure 0-6 Microstructure of 0.4% LBG and 3% SMP mixtures

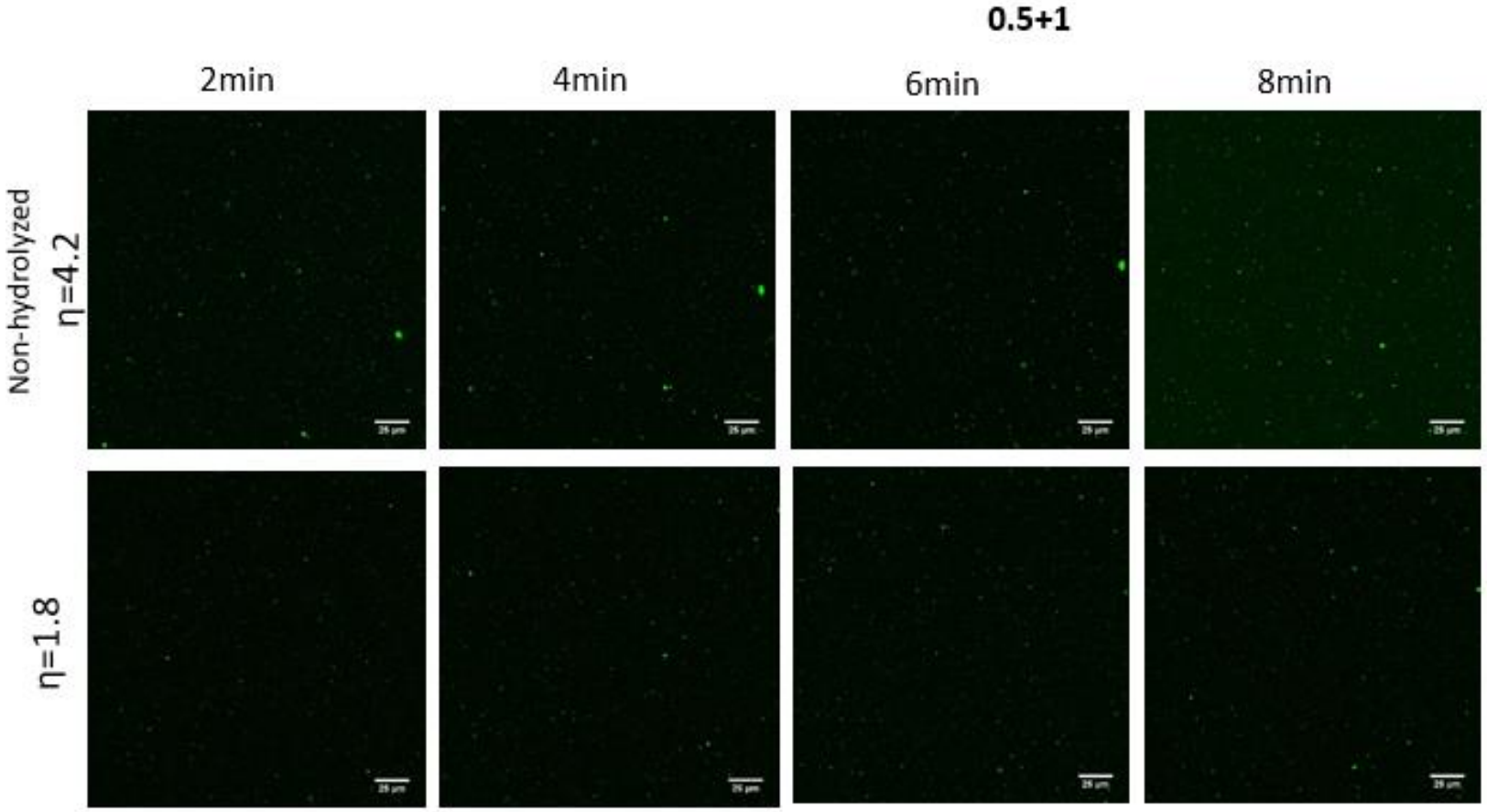

Figure 0-7 Microstructure of 0.5% LBG and 1% SMP mixtures

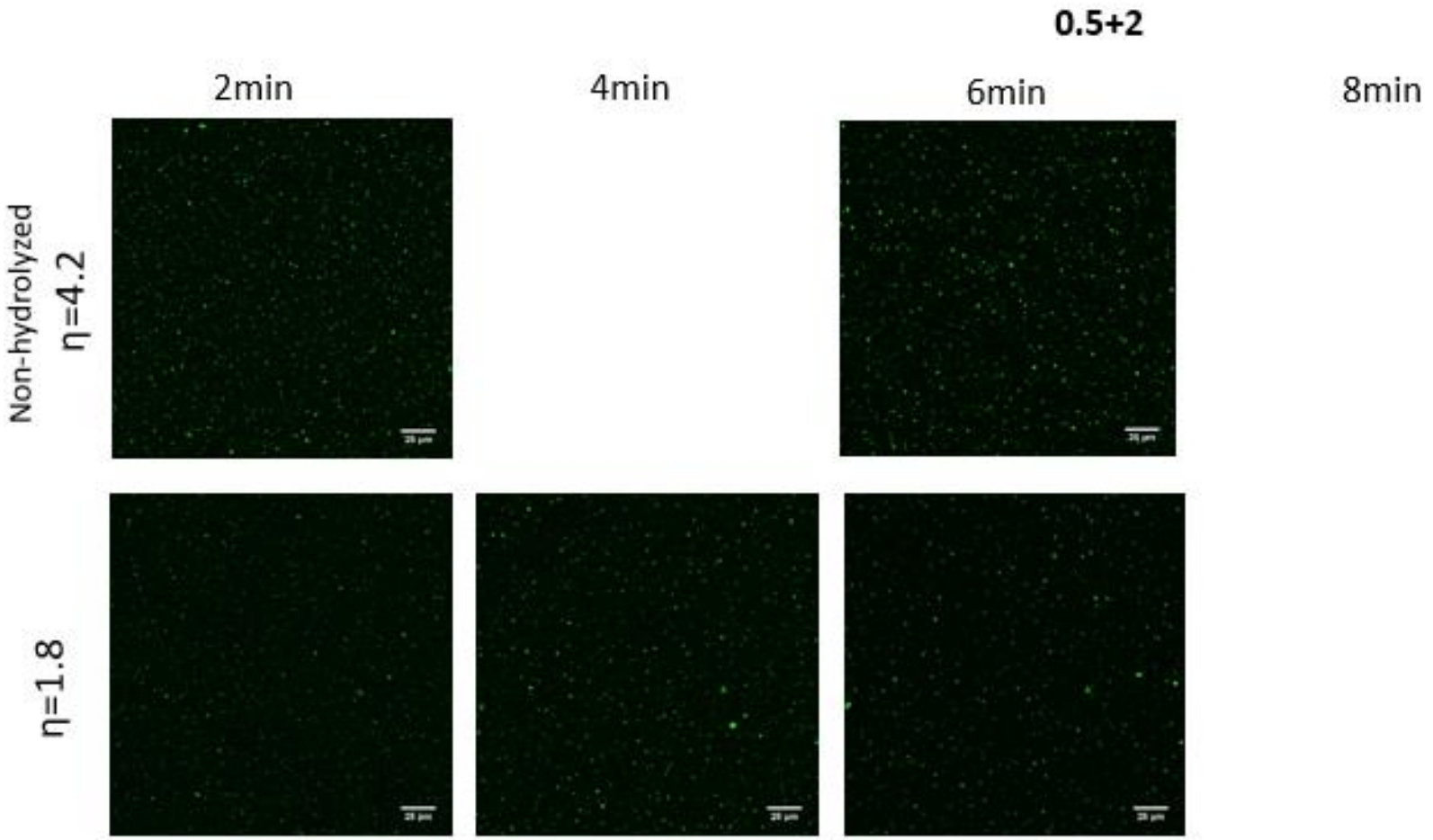

Figure 0-8 Microstructure of 0.5% LBG and 2% SMP mixtures

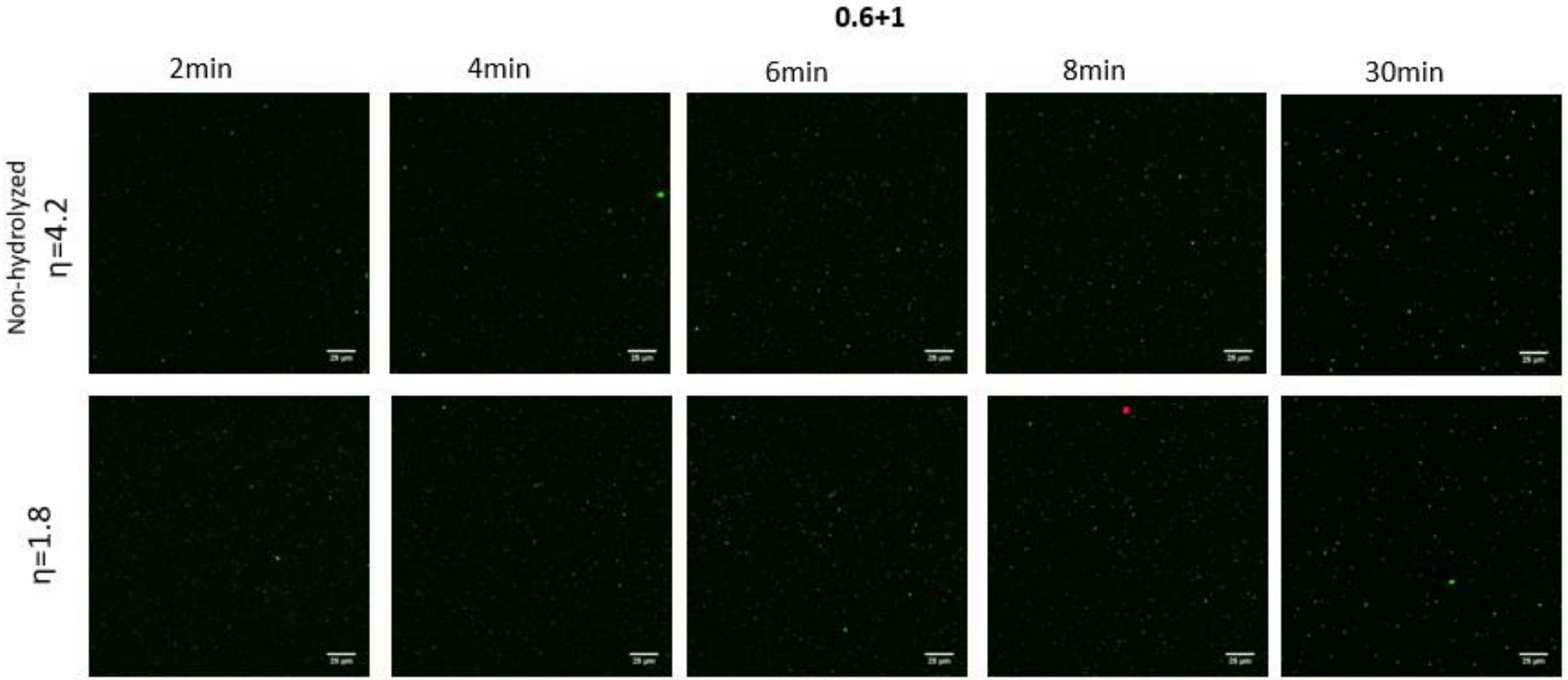

Figure 0-9 Microstructure of 0.6% LBG and 1% SMP mixtures

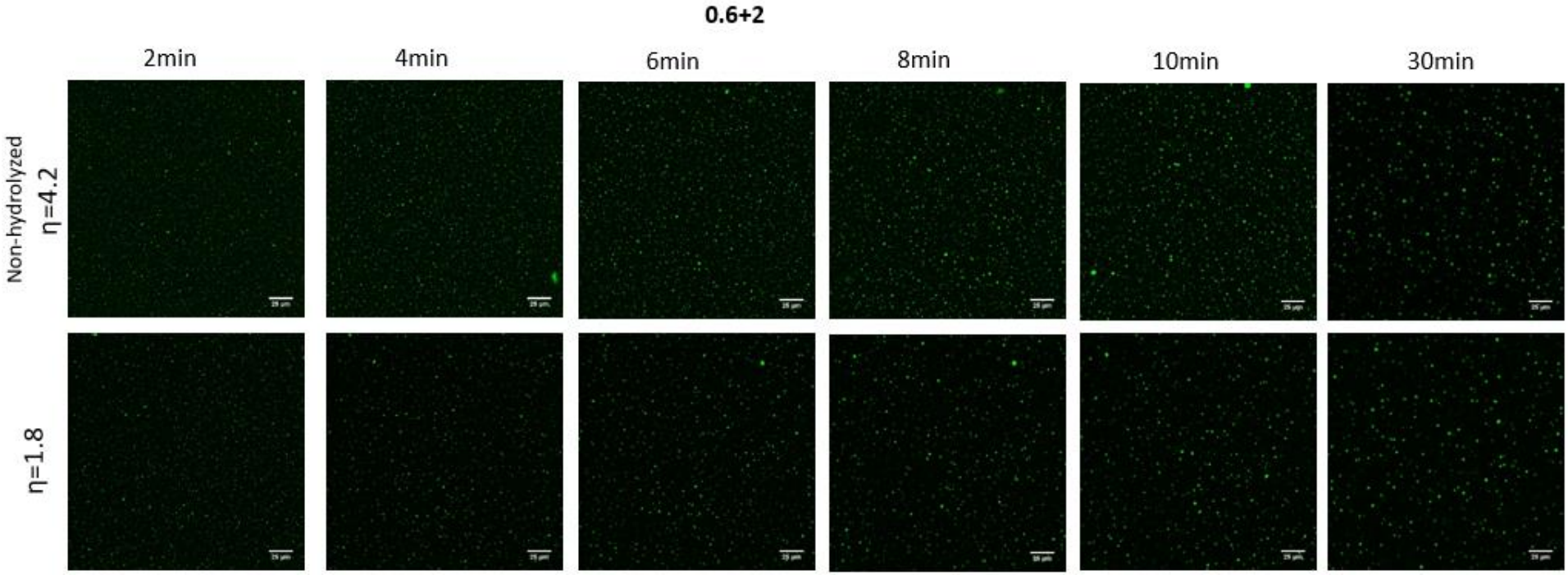

Figure 0-10 Microstructure of 0.6% LBG and 2% SMP mixtures

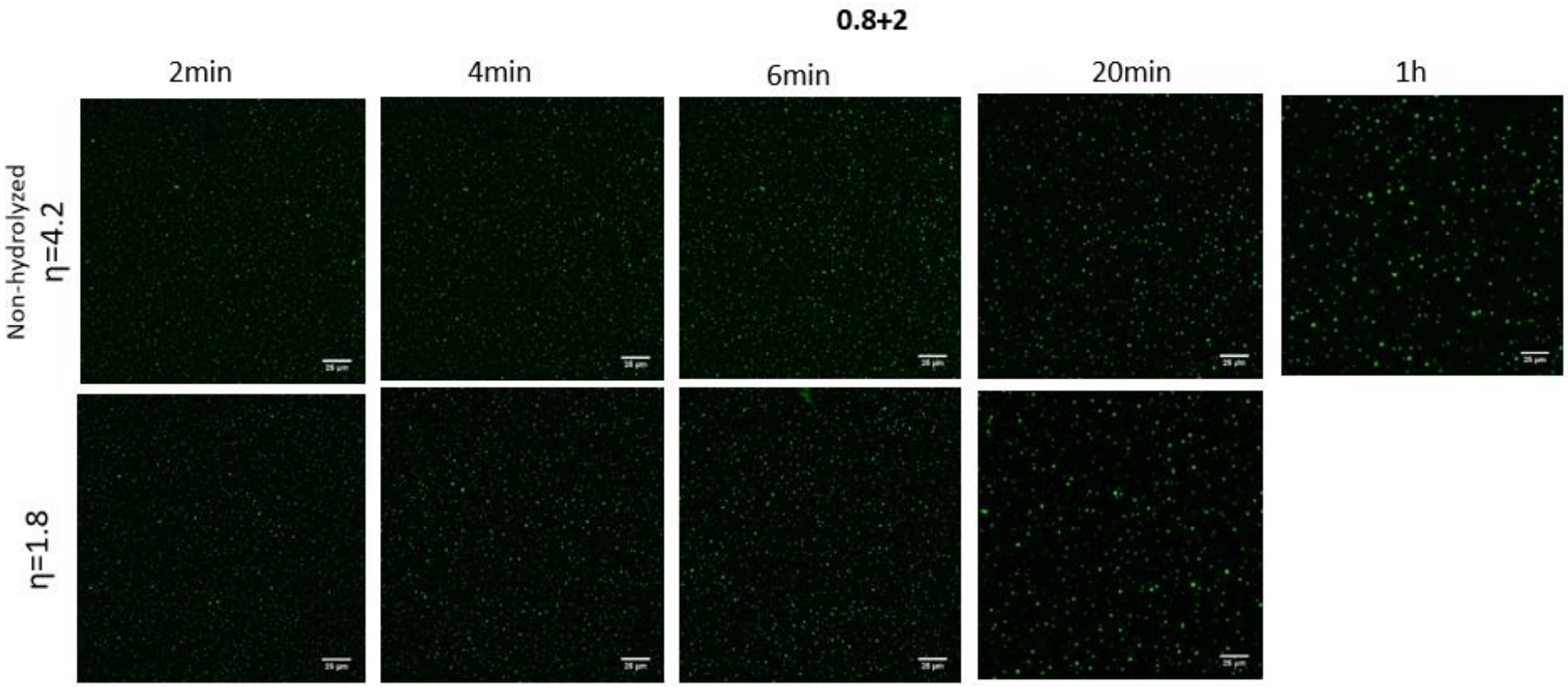

Figure 0-11 Microstructure of 0.8% LBG and 2% SMP mixtures

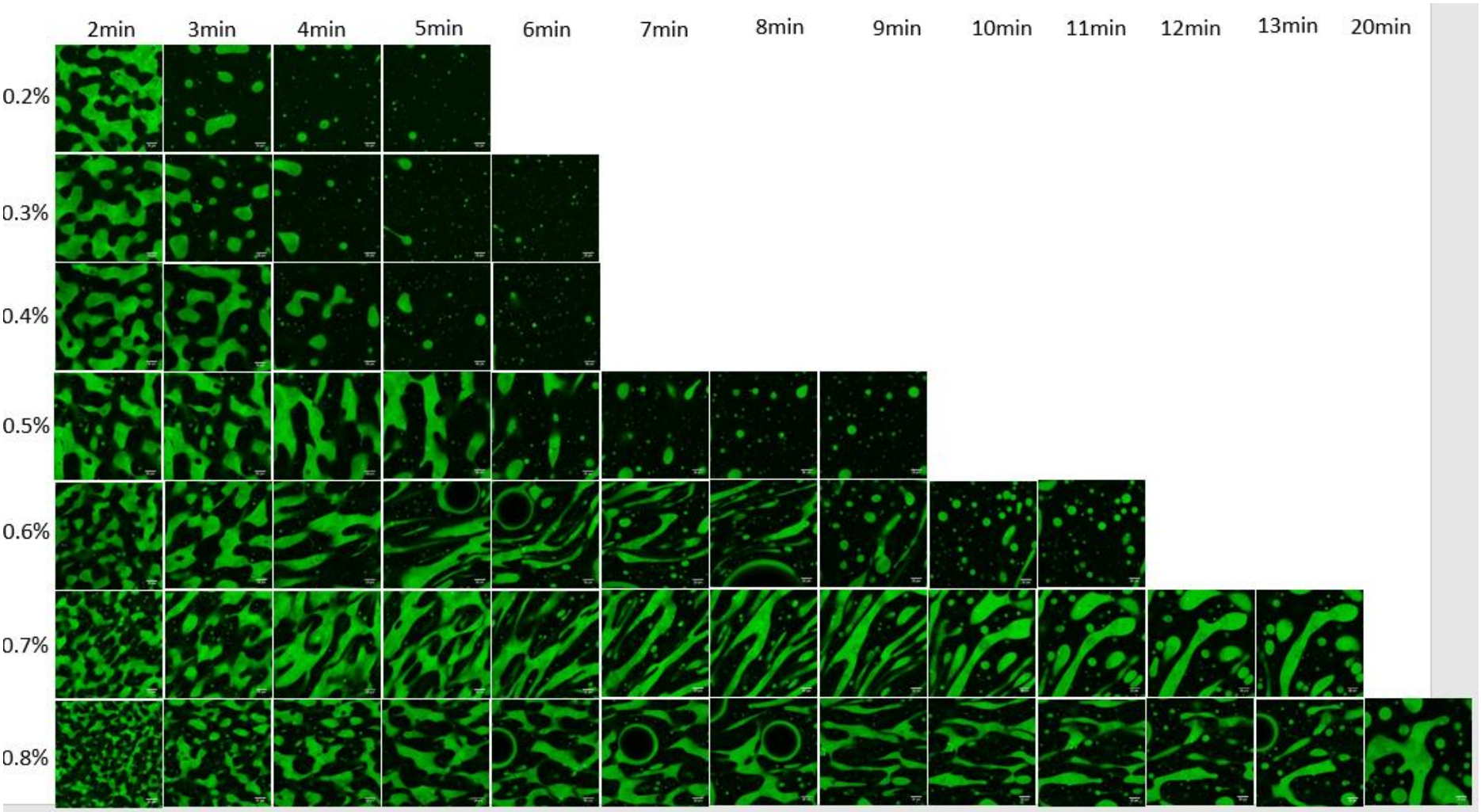

Figure 0-12 Confocal microscope images for non-hydrolysed LBG and 40% SMP mixtures

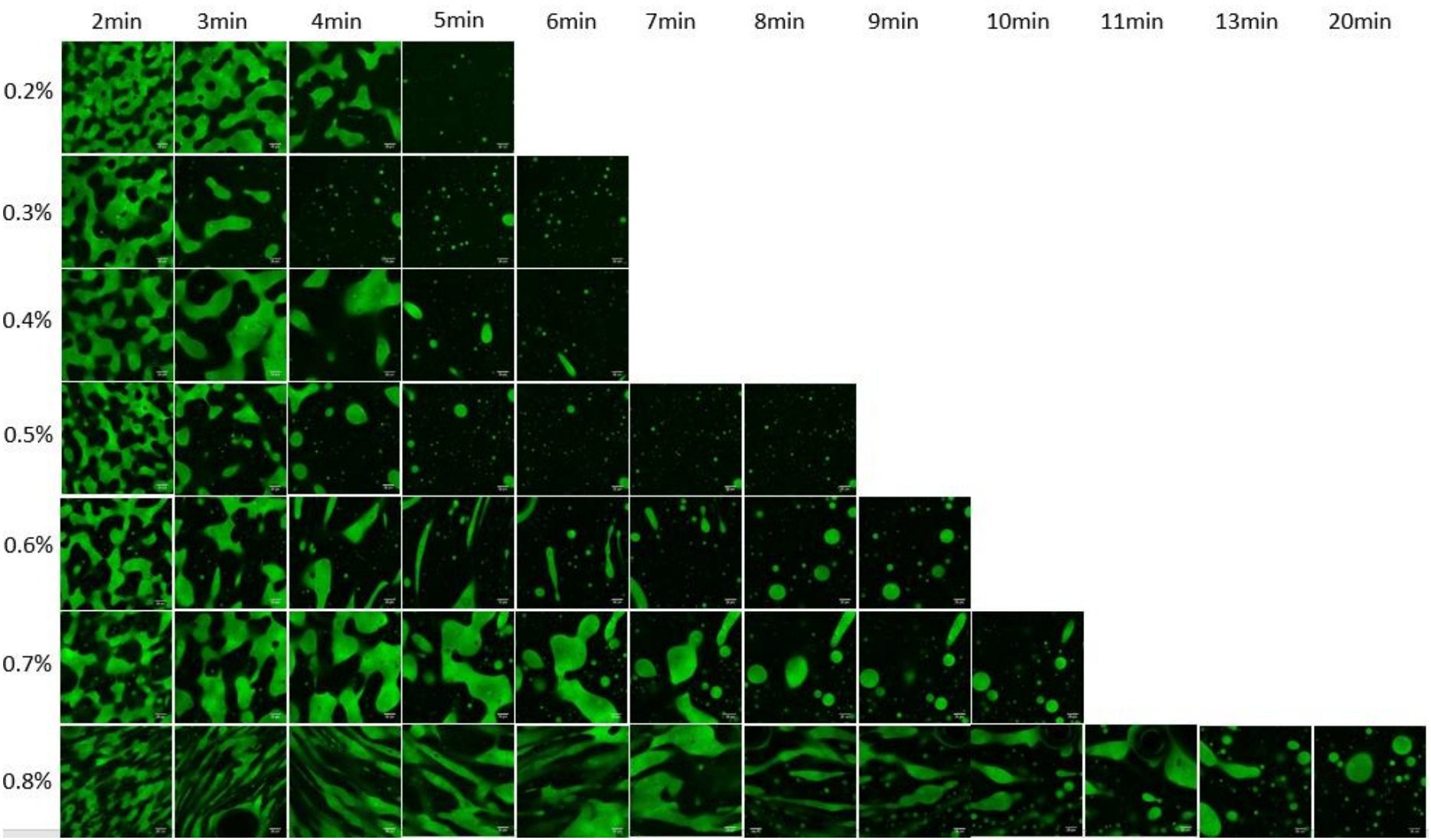

Figure 0-13 Confocal microscope images for hydrolysed LBG and 40% SMP mixtures