Evaluation of enzyme addition on sucrose extraction during the sugar beet diffusion process

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Declaration

I, Thomas Kwun Long Cheung, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:_____Date:

Abstract

Due to removal of the EU sugar quota, the UK sugar industry is currently interested in maximising the efficiency of their existing process chains in order to remain competitive in global markets. One opportunity is improvement of the existing diffusion process to increase the yield of sucrose produced per beet, reducing the amount of sugar lost to pulp.

This thesis aims to investigate whether pectinases and cellulase have the potential to enhance sucrose extraction from sugar beets when applied to the existing diffusion process. To achieve this, this study focuses on the characterisation of several commercial enzymes in order to evaluate compatibility with the diffusion process, the development of a scale-down diffuser model to simulate industrial counter-current diffusion conditions, the expression of a novel, thermostable pectate lyase and the application of these enzymes in the diffusion process with the ultimate objective of enhancing sucrose extraction.

Initial characterisation studies were performed on seven commercially available enzyme preparations, evaluating invertase activity, temperature activity and thermostability. From these studies, and in combination with industry data and literature reviews, Rohament CL, a broad-range fungal cellulase and Rohapect SY+, a pectin lyase, were shortlisted for experiments within the sugar beet diffusion process. These two enzymes did not contain any measurable invertase activity and, of the enzymes investigated, displayed the best activity and stability under the diffusion conditions.

A counter-current, multi-stage diffusion model was then designed, built and tested to measure the effect of enzymes on the sugar beet diffusion process. This could accurately mimic performance of the standard diffusion process in terms of operating in a counter-current manner, however initial trials suggest no significant increase to the rate of sucrose extraction when Rohament CL, Rohapect SY+ or both enzymes were added to the diffusion process. Pre-treatment of sugar beet cossettes, via various processes, also did not enable any enzymatic enhancement to be observed.

Due to the poor thermostability of the commercial enzymes, a thermostable pectate lyase, TMA14, was subsequently expressed and characterised for application in the

diffusion process. This enzyme displays an optimum temperature activity profile at 90°C with significant activity at 70°C, and retains 99% stability over a 2 h duration at 70°C. Trials with TMA14 in the counter-current diffuser demonstrate no significant increase to rate of extraction, extraction ratio or pressability.

In conclusion, the potential for existing commercially available enzymes to enhance sucrose extraction appears to be limited due to the already high efficiency of the existing diffusion process. Future work needs to address the development of enzymes with the required specificities and thermostabilities to work under industrial diffusion process conditions.

Impact statement

This research has provided valuable insight regarding the application of enzymes on the sugar beet diffusion process with the objective of improving sucrose yield. This has been achieved by developing a scale-down diffusion model that mimics the industrial counter-current diffusion process whilst requiring a fraction of the resources and labour to do so. The outcomes of this research can have potential impact both within academia and outside of academia.

Within academia, this research has provided key research methods in which a particular industrial process can be simulated on the bench-scale. This information may serve as a useful point of reference for future projects attempting to conduct a similar objective for academic purposes. The results from this work can also be written for publication or contribute to teaching materials for industrial biotechnology modules.

Outside of academia, industry perspectives are shifting heavily towards improved sustainability and efficient use of resources. This research provides insight into alternative ways in which enzymes could be considered for improving the valorisation of other feedstocks and associated processing.

In the specific context of the sugar industry, this research could be used to guide choices regarding improved innovation, maximising existing processes for efficiency and, particularly in light of Brexit and the abolishment of the EU sugar quota, key decisions that may be critical for maintaining competitiveness on a national and international scale.

Acknowledgements

The number of people who have contributed towards my motivation, drive and ability to see this thesis through to completion are innumerous. Thus this section is by no means an exhaustive list and is merely a fraction of appreciation towards the people who have made this achievement possible in the hardest of times.

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I must also extend gratitude to my colleagues at British Sugar for the contribution of masses of beets. In particular, the knowledge and expertise from Mark Stewart was critical in my understanding of this research from an industrial perspective.

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To all the remaining staff within the Department of Biochemical Engineering at UCL, from the administrative staff, lab managers and supply managers, each of whom provide the means for the department to continue doing what it does, who provided the best possible environment to conduct research, I give my thanks.

I must also thank EPSRC, UKRI and British Sugar for the financial support that made this research and opportunity possible.

Finally, to my parents, Chi Leung Cheung and Sau Lan Wong. Words cannot express the gratitude I have for the years of sacrifice the two of you have made to enable me

to become the person I am today, and therefore this achievement is yours as much as it is mine. My siblings, Ray, Helen and Caren. The journey of a doctoral student is lonely and often littered with stressful obstacles, but I know I am never truly alone due to the unequivocal support of my family.

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Abbreviations

3,5-DNSA	3,5-Dinitrosalicylic acid assay
AF	Arabinofuranosidase
Ara	Arabinase
BMA	Braunschweigische Maschinenbauanstalt AG
CO_2	Carbon dioxide
D	Diffusion coefficient
DdS	De danske Sukkerfabrikker
DF	Dilution factor
EPSRC	Engineering and Physical Sciences Research Council
EU	European Union
FPU	Filter paper units
FT-NMR	Fourier-Transform Nuclear Magnetic Resonance
Gal	Galactanase
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
IPTG	Isopropyl-β-D-thiogalactopyranoside
KPI	Key performance indicator
LB	Lysogeny Broth
MWCO	Molecular weight cut off
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + Hydrogen
NIR	Near-Infrared Spectroscopy
OD	Optical density
PAE	Pectin acetyl esterase
PEF	Pulsed electric field
PG	Polygalacturonase
PGL	Pectate lyase
PL	Pectin lyase

PME	Pectin methyl esterase
Pol	Polarimetric reading
ppm	Parts per million
RGH	Rhamnogalacturonan hydrolase
RO	Reverse osmosis
rpm	Revolutions per minute
RT	Raffinerie Tirlemontoise
SBP	Sugar beet pulp
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	Set point
TDS	Total dissolved solids
UK	United Kingdom

Chapter 1

Introduction

1.1 Sugar from sugar beet

Table sugar, scientifically known as sucrose, is a staple component in the human diet primarily derived from two sources: sugar cane and sugar beet. Sugar cane, a tropical crop, is grown in warm temperate and tropical regions such as South Asia and the Caribbean and provides the majority of the global sugar supply. In comparison, sugar beet accounts for approximately 20-25% of the global sugar supply. It can be grown under a wide variety of conditions and with various substrates including organic soils or sands, rendering it a popular crop for countries that would otherwise be unable to produce their own sugar such as the USA and the majority of Europe. Despite health concerns regarding the consumption of excessive sugar in the diet, the demand for sugar continues to rise. In the 2022/2023 season, global consumption of sucrose was measured at 188m tonnes. By comparison, the global sugar production was 186m tonnes in the same year (Eastick, 2024). Additionally, sucrose has various applications outside of the food industry, such as in scientific and pharmaceutical research and agriculture (Queneau et al, 2007). As a result, sugar refineries must function more productively and efficiently to meet this increasing demand.

Since the discovery of sucrose in beets by Andreas Marggraf in 1747 and the introduction of the first sugar refinery utilising sugar beet as a cash crop in the 1800s, the industry has grown significantly from its origins in Europe, as depicted in Figure 1.1. In the 2022/23 period, industry figures report global sugar production was approximately 186 M tonnes. Of this, 41 M tonnes of sugar was produced by sugar beet (Eastick, 2024).

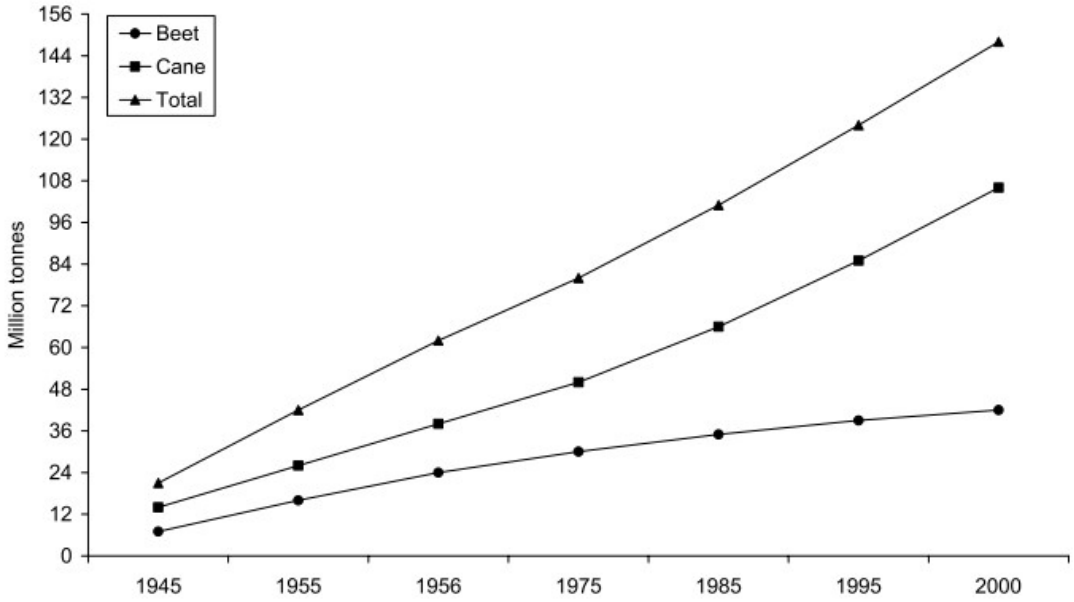

Figure 1.1: Global sugar production from sugar cane and sugar beet between 1945 and 2000. Sugar beet accounts for 25% of the total global sugar production of sucrose, the remainder of which is attributed to sugar cane (Draycott, 2006).

In October 2017 the EU, which supplies more than 50% of global of beet sugar, lifted the quota on sugar beet production in EU member states enabling each state to produce significantly more sugar than was previously permitted and to export it without limitations (European Commission, 2017). This has changed the sugar industry: previously, an 8% cap on the export of EU-produced sugar was enforced in order to support European farmers and processors. Member states are now no longer restricted by this cap in a bid to make European agriculture more market-oriented, providing the opportunity for significant expansion of the current market. EU production of sugar from sugar beet in 2016/17 was reported at 16.84M tonnes, rising to 17.62M tonnes and 18.05M tonnes in 2018/19 and 2019/20 respectively (Shahbandeh, 2021).

The changes to the sugar beet industry have had widespread implications for the UK as the sugar beet industry is extremely important for regional economies. In 2019/20, the UK was the fifth largest producer of sugar amongst European countries (Shahbandeh, 2021). Additionally, 60% of sugar consumed in the UK is produced from UK-grown sugar beet (Kenward, 2016).

The beet industry in the UK supports tens of thousands of diverse jobs, contributing to the sustainability of the country and economy. In addition, as a result of Brexit, the UK

must negotiate its own trade deals pertaining to the sugar industry to ensure that it is given ample opportunity to expand and maintain competitiveness in order to withstand changes in the sector. Due to these factors, considerable resources are currently being invested into the existing sugar beet industry to optimise the process in an attempt to solidify the UK's position in the dynamic sugar industry and satiate the demand for this fundamental product.

1.2 Sugar beet structure

To understand the mode of action by which sucrose is extracted from sugar beet (Section 1.3), it is important to first understand the structure and properties of the sugar beet plant itself. Sugar beet (*Beta vulgaris ssp. vulgaris*) is a temperate crop with each plant weighing up to 2 kg. It consists of a leafy crown and a conical taproot consisting of approximately 15-20% w/w sucrose (Van der Poel & Schiweck, 1998; Al-Jbawi et al, 2015) (Figure 1.2). The leafy crown is removed during harvesting as the remaining root contains the majority of the sucrose and is therefore the desirable part. Figure 1.3 presents the composition of the average sugar beet.

Figure 1.2: Photograph of a typical fresh sugar beet root as used in this work. The leafy crown has been removed and the remaining sucrose-rich taproot can be observed.

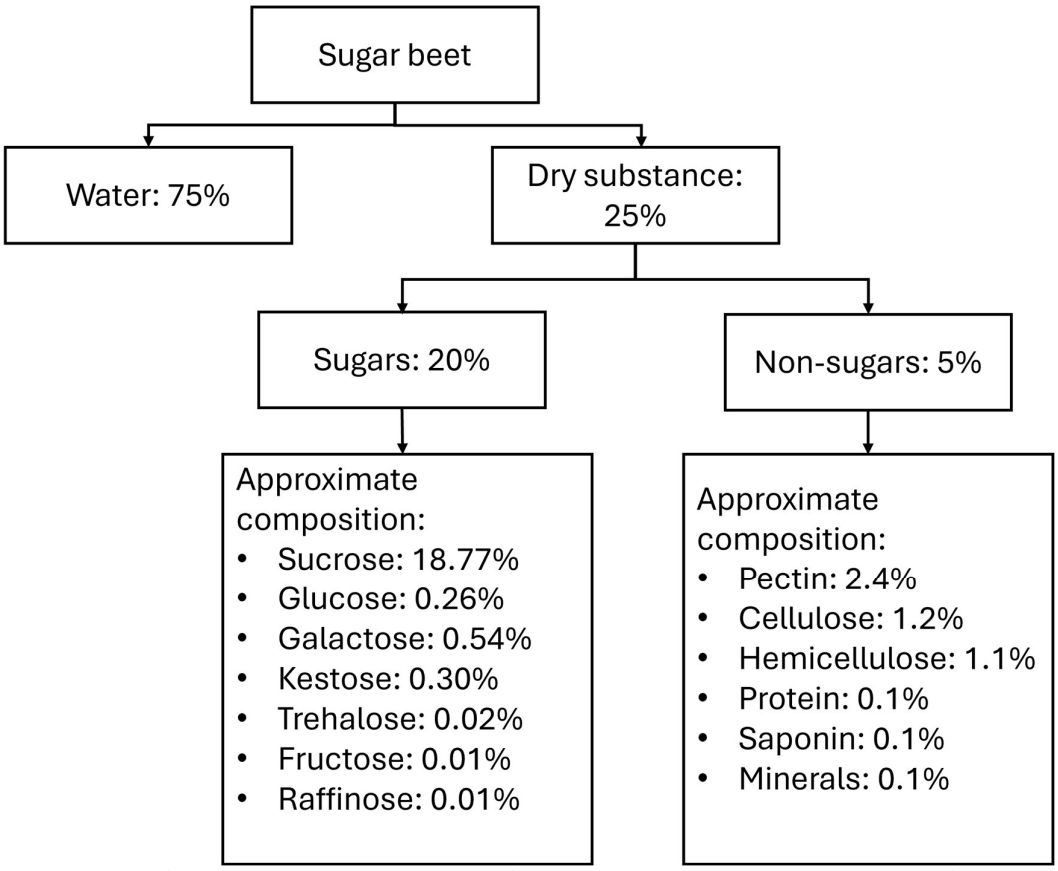

Figure 1.3: Sugar beet composition (% w/w). Average values obtained from Asadi (2006) and Gruska (2022).

Sucrose is synthesised in the leaves by photosynthesis before it is translocated into the root via the sieve tubes in the phloem (Doll and Hauer, 1981; Giaquinta, 1979). The root itself consists of numerous parenchymal cells loosely connected by the apoplastic network forming a vascular zone and a parenchymal zone (Richter & Ewald, 1983). Both zones consist of specialised parenchymal cells, although the cells within the vascular zone are smaller and more immature, whilst those within the parenchymal zone are more senescent. These cells are specially adapted for storage and possess a large pore, or vacuole, within the cytoplasm. It is within these vacuoles that soluble components such as proteins and sugars are stored in the aqueous sap.

As a result, in order for sucrose to diffuse out of the aqueous sap, it must first cross numerous barriers to mass transfer, the most significant of which is the primary cell wall (Dominguez & Munoz, 2017). This consists of three polysaccharides of approximately equal proportions: cellulose, hemicellulose and pectin (Foster et al., 2001). These polysaccharides interconnect to form a complex matrix that provides the structural integrity, rigidity and elasticity required for the cell wall to maintain its vital functions (Figure 1.4).

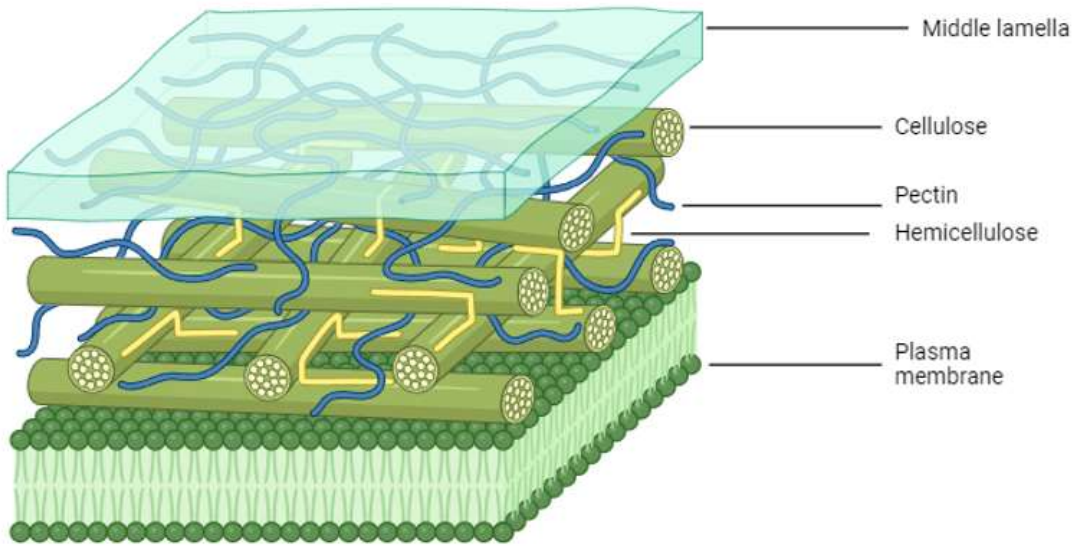

Figure 1.4: Schematic diagram depicting the arrangement of cellulose, hemicellulose and pectin in the primary cell wall between the middle lamella and the plasma membrane (Figure from Biorender).

The objective of the sugar beet refinery is therefore to disrupt the primary cell wall, enabling the sucrose to diffuse readily from the cell interior to the surrounding

extraction solvent from which it can be purified and crystallised. To achieve this, a highly efficient process consisting of numerous unit operations is required.

1.3 Sugar beet processing

British Sugar plc is the only sugar beet processor in the UK, possessing state-of-the-art biorefineries, the largest of which is the Wissington biorefinery. These inter-connected processes within these facilities are designed to maximise the productivity and efficiency of the sucrose extraction process. In addition to producing 400,000 tonnes of sucrose per annum at the Wissington site, less than 200 g of waste is generated per tonne of sucrose produced. This is achieved via industrial symbiosis i.e. by recovering waste process outputs and converting them into process inputs leading to the manufacture of additional co-products, a summary of how this is achieved in the Wissington biorefinery is depicted in Figure 1.5 and Table 1-1. The push for an integrated biorefinery stemmed from the initial limitations placed upon the core sugar business due to the now-redacted EU quota system: an integrated biorefinery has enabled British Sugar to remain globally competitive whilst also establishing a diverse variety of value-added co-product lines (Short et al, 2014).

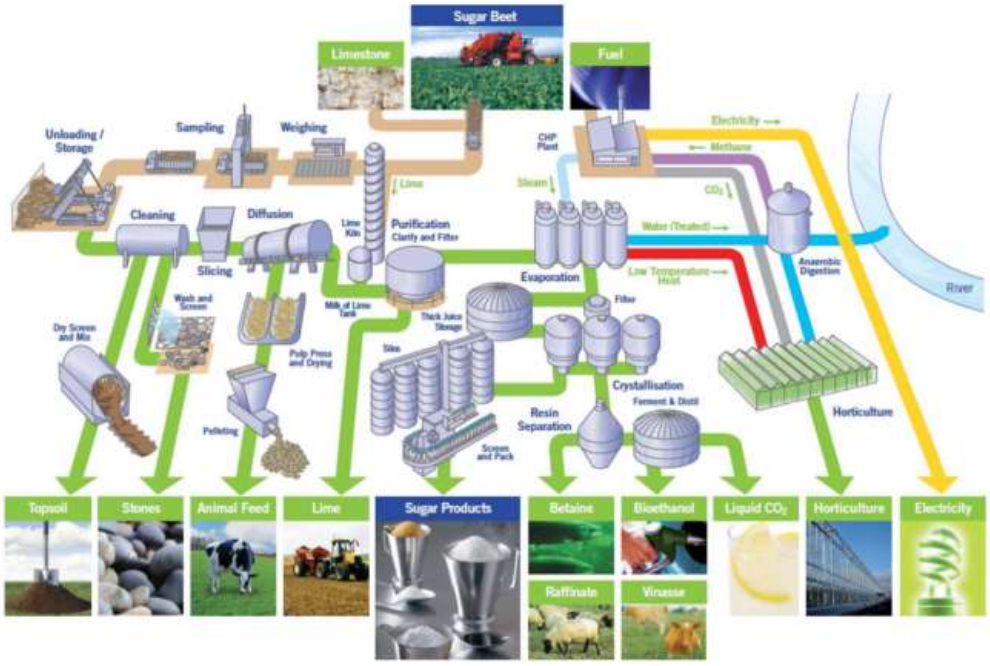

Figure 1.5: Schematic diagram of the British Sugar Wissington Biorefinery (Norfolk, UK) illustrating the key unit operations for sugar beet processing. Although sugar is

the primary product, there is a range of valuable by-products including animal feed, vinasse, bioethanol and electricity. Reproduced with permission from British Sugar.

Table 1-1: Summary of co-products produced by British Sugar at the Wissington biorefinery and their applications (Short et al, 2014).

Co-product	Amount per annum	Application
Topsoil	50,000 tonnes	Soil for landscaping and Brownfield land
Aggregate (i.e. stone)	500 tonnes	Construction, civil engineering
Sugar Beet Pulp (SBP)	140,000 tonnes	High energy animal feed
Raffinate and Vinasse	76,000 tonnes	Animal feed supplement
LimeX	120,000 tonnes	Sustainable calcium source for soil conditioning to correct soil acidification
Betaine	11,000 tonnes	Fish feed, cosmetics
Bioethanol	64,000 tonnes	Renewable fuel source
Heat and CO_2 for Horticulture	Tomatoes – 15,000 tonnes CO_2 – 40,000 tonnes	Previously used to produce tomatoes. Currently used to produce Epidiolex in partnership with GW Pharmaceuticals. Epidiolex is a FDA-approved medicinal cannabinoid candidate product effective against severe childhood epilepsy Excess CO_2 is sold to Air Liquide for a variety of applications including industrial refrigeration
Electricity	320GWh	The Combined Heat and Power (CHP) plant provides steam and electricity for the entire process. Excess electricity sold to the National Grid

The focus of this study will be on optimising the diffusion process in which the majority of sucrose is extracted from the sugar beet cossettes. However, it is first important to

understand the general processing of sugar beet from delivery of beet to the final preparation of sucrose.

1.3.1 Storage, cleaning and slicing

To manage sugar beet processing throughout the duration of the beet campaign, sugar beets are stored in large, outdoor piles upon delivery to the processing site. Storage piles are optimised to minimise spoilage by regulating the temperatures within the pile interior, which subsequently minimises sucrose losses due to sugar beet respiration and microbial activity (Van Eerd et al, 2011; Korobova et al, 2022).

they are cleaned using specialised equipment to remove stones and soil. These materials are collected and re-sold as topsoil or stones for the construction and landscaping industries. The beets are then washed using a beet-flume system to remove residual detritus. It is important that the wash water temperature does not exceed 20°C in order to minimise diffusion of sucrose at this stage into the wash water (Maarten de Bruijn, 2020c).

The beets are then sliced into V-shaped strips, known in the industry as cossettes. This is achieved using a specially designed blade to optimise the accessible surface area of the cossettes for maximum sucrose extraction efficiency. The ideal cossette has a width between 3 to 6 mm and a length between 30 to 60 mm. Cossette sizes are optimised to provide a large surface area to volume ratio to maximise diffusion efficiency. However, cossettes that are too thin may compress and form a compact mass that reduces juice circulation (Silin, 1964).

Additionally, the cossette quality can be evaluated by the Silin number, which is the length of 100 g of cossettes; the Swedish number, which is the mass ratio of cossettes longer than 50 mm to cossettes shorter than 10 mm; and the mush content which is the mass ratio of cossettes less than 10 mm long in 100g of cossettes. Ideal Silin numbers, Swedish numbers and mush content are 10 to 18 m, greater than 10 and less than 5% respectively (Asadi, 2006).

The cossettes are then transported into the diffuser via a conveyor belt, during which the cossettes are weighed. Beet sugar content is calculated by multiplying the total mass of cossettes processed in a certain period by the average sugar content of cossettes sampled regularly. This is critical as it provides an input reference for the

final mass balance of sucrose in order to calculate sucrose yield, defined as the percentage of sucrose extracted from the total amount of sucrose available.

1.3.2 Diffusion and pressing

Diffusion is the key stage in which sucrose is extracted from the cossettes. Cossettes enter the diffuser and lose approximately 98% sucrose to the diffusion water, retaining 2% sucrose and producing a raw juice containing 10-15% sucrose by weight. The sucrose purity of the raw juice is approximately 85-90%, with the remainder consisting of impurities, such as pectins, amino acids, soluble proteins and salts.

The diffusion process is entirely passive, relying on the concentration gradient between the sucrose-rich cossettes and the sucrose-poor water. In the past, diffusion was conducted in a series of discrete battery-style vessels. This system has the advantage over a single, large vessel as it enables the establishment of a counter-current gradient between the cossettes and the water. In a single vessel, sucrose equilibrium would be established very quickly and no further extraction would take place, resulting in inefficient extraction. The counter-current gradient ensures that the concentration gradient from sugar beet to diffusion water is maintained throughout the entire duration of the process to maximise extraction.

The battery-style system is no longer in use as it was highly laborious to operate and relatively inefficient. Instead, modern systems comprise a continuous counter-current system in which cossettes and water are fed into the diffuser at a constant rate. In the UK, there are three different types of diffuser designs: the Raffinerie Tirlemontoise (RT) diffuser, the De danske Sukkerfabrikker (DdS) diffuser and the BMA Tower diffuser (Information provided by Innovation Manager, British Sugar, Personal communication). These are discussed in detail in Section 1.3.2.1.

The diffusion process results in two product streams: the diffusion juice containing 10-15% sucrose, and the sugar beet pulp consisting of the exhausted cossettes with some residual sucrose. The pulp is most commonly sold as dried animal feed (Figure 1.5), and hence it is pressed for two reasons: (1) to remove as much water as mechanically possible prior to further drying via heat treatment, and (2) to extract the remaining sucrose in the form of pulp press water. For optimal pulp pressability, the pulp must contain an adequate amount of dry substance and be sufficiently rigid. This rigidity can be difficult to control due to parameters within the diffuser as described

earlier in this section. Consequently, pressing aids such as gypsum or calcium chloride, can be added to improve the dry content by binding to the pectin in the pulp (Asadi, 2006).

The pressing equipment used in industry comprises a twin-screw that tapers towards one end, decreasing the volume of the chamber and thus compressing the wet pulp with pressures greater than 10-12 bar. The pulp pressing stage also contributes to the overall efficiency and sustainability of the process as the pulp press water, i.e. liquid extracted from the sugar beet pulp after pressing, is returned to the diffuser to reduce fresh water requirements.

1.3.2.1 Diffuser designs and critical process parameters

The RT diffuser is the most similar to the battery-style system mentioned in Section 1.3.2. It is comprised of a large rotating drum divided into numerous discrete compartments by a screw attached within the drum. As the drum rotates, the diffusion water that was initially located at the bottom of the drum percolates through the cossettes within the compartments from top to bottom. Counter-current operation is maintained by utilising chutes that guide the cossettes into the adjacent cell as the drum rotates, such that the diffusion water and cossettes move in opposite directions. Initially, the fresh extraction solvent is located at the bottom of the drum whilst the fresh cossettes reside in the uppermost compartments. When the drum rotates, the extraction solvent moves toward the top of the drum and the fresh cossettes move toward the lower compartments. The solvent percolates from top to bottom, initially extracting sucrose from the more exhausted cossettes before continuing to extract sucrose from the fresh cossettes as the solvent sucrose concentration increases. Of the three diffusers, the RT diffuser provides the most efficient extraction of sucrose from sugar beet, but also results in the highest engineering cost (Information provided by Innovation Manager, British Sugar, Personal communication).

The DdS diffuser proposed in 1969 by Bruniche-Olsen for the Danish company DdS, is another continuous system designed to minimise energy usage and is currently the most energy efficient of the three diffusers used in the UK. This design consists of an inclined jacketed trough with a double-cylindrical bottom resembling two half-cylinders, illustrated in Figure 1.6. Two helical conveyer screws are located within the trough and rotate in opposite directions resulting in forwards and backwards movement but are

operated such that there is a net forwards movement to push the solids against the gradient to the top of the diffuser. The combined mechanical action of the twin screws promotes even solids mixing and prevents compaction, which would otherwise lead to impaired diffusion efficiency. The extraction solvent flows in the opposite direction down the incline gradient, resulting in counter-current operation.

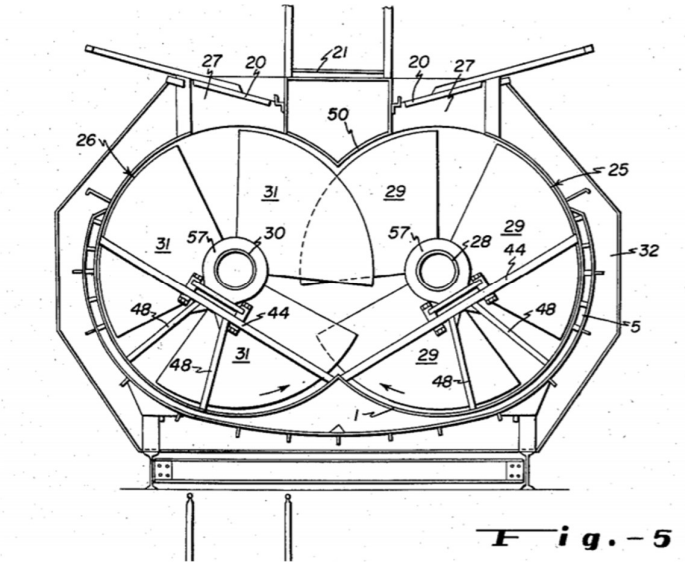

Figure 1.6: Initial design of the DdS diffuser illustrating the double-cylindrical base. The helical conveyer screws and their axes of rotations are also depicted, which provide the mechanical action that contributes to the movement of the solid phase (Bruniche-Olsen et al, 1957).

Although commonly employed in the sugar beet industry, British Sugar have identified several disadvantages with the use of the DdS diffuser, such as poor-quality diffusion juice. The variable temperature and residence time profiles also introduces the risk of issues such as microbial control and sucrose inversion to D-glucose and D-fructose.

The BMA Tower diffuser was developed by Braunschweigische Maschinenbauanstalt AG in Germany in the mid-1950s. As the name suggests, it differs from the DdS and RT diffusers as it consists of a vertical cylinder in which the cossettes move towards the top of the tower via a rotating-lifting mechanism, whilst the extraction solvent percolates downwards via gravity (Asadi, 2006). Due to the vertical design, BMA Tower diffusers are more space-efficient than the other designs. However, they require essential ancillary equipment such as cossette mixers and foam separators, considerably increasing the equipment costs (Figure 1.7). Compared to the RT and DdS diffuser, the BMA tower diffuser results in the lowest oxidation of cossettes due

to the mechanism of movement. This is advantageous as oxidation impairs the quality of the cossettes which may cause clarification issues in the subsequent processing stages.

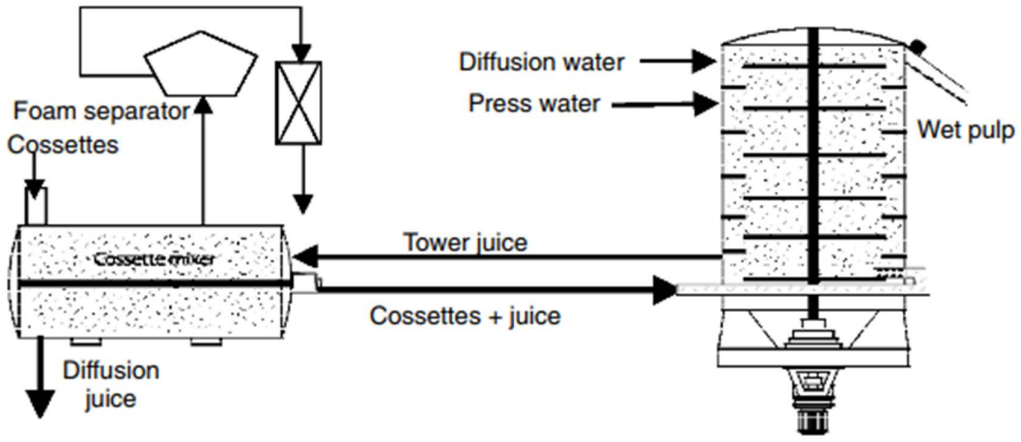

Figure 1.7: BMA Tower diffuser with the ancillary cossette mixer. The role of the cossette mixer is to recycle the heat from the hot tower juice produced after extraction by mixing it with the incoming cossettes to pre-heat them (Asadi, 2006).

There are several critical process variables to consider when operating the different extractor designs described above. These include the sucrose diffusion coefficient, temperature, pH, draft ratio and retention time each of which is described in detail in the following paragraphs.

The sucrose diffusion coefficient, *D*, is a key constant that describes the extent of diffusion and is affected by the absolute temperature. In the sugar beet industry, the temperature of the diffuser is typically maintained at 70-73°C (Asadi, 2006; Silver, 1956). If the temperature falls below 70°C, *D* decreases, whilst at greater temperatures, *D* increases but the sugar beet is more prone to rupture and likely to promote the release of non-sucrose components, which will impede further downstream processing and inhibit pulp pressability. Furthermore, a temperature range of 70-73°C is optimal for limiting microbial activity as common thermophilic microorganisms, such as *Leuconostoc spp*, are unable to survive at this temperature range. This genus is primarily responsible for root rot in sugar beet as it possesses invertase activity, hydrolysing sucrose into D-glucose and D-fructose for lactic acid and dextran production, therefore significantly compromising sucrose yield (Strausbaugh, 2015).

It should be noted that, although the optimum temperature range is between 70 and 73°C, the actual temperature often fluctuates significantly based on the type of diffuser and the location of the extractor. This is due to the insulation conditions of each diffuser and the mechanism by which heat is applied.

For example, a diffuser with an average operational temperature of 70 to 72°C may record temperatures of 65 to 68°C at the pulp end of the extractor (Maarten de Bruijn, 2020c). DdS diffusers are particularly subject to temperature changes and temperatures as low as 18°C have been reported towards the end of the diffuser (Information provided by Innovation Manager, British Sugar, Personal communication).

The pH at which the diffusion system is operated is also another important factor to consider. It impacts on the extent of sucrose extraction, invertase activity and pulp pressability, and therefore must be controlled. In addition to microbial invertase activity, the beet itself also possesses some native invertase activity. Acid invertase has a pH optimum of 5.0, whilst alkali invertase has a pH optimum of 8.0, hence the optimum pH of the diffusion juice is typically maintained at approximately 5.8-6.5 (Asadi, 2006; Haley, 1973; Mohammadnezhad et al, 2013; Joshi et al, 2006). Both microbial and beet invertase activity are at a minimum in this range, resulting in less sucrose inversion and higher sucrose yield. Additionally, acidic pH below 4.5 may result in acid hydrolysis of sucrose, whilst a pH above 6.5 will result in excessive solubilisation of pectins and other undesired components, which will disrupt latter processes (Maarten de Brujin, 2021a).

The importance of pH in the context of pulp pressability can be observed by the operation of sterile diffusion trials during the British Sugar campaign of 1975/76. To establish sterile diffusion, biocides were regularly applied to the system to ensure as little microbial activity as possible. However, it was noticed that the pH rose from the target pH of 5.5 to pH 6.0, suggesting that the microbial activity was contributing towards the maintenance of the optimal pH. This resulted in adverse effects to pulp pressability and limited the amount of sucrose that could be extracted from pressed pulp. To counteract this, British Sugar now maintains the pH at the desired range under sterile conditions by dosing the system with sulphuric acid as required (Information provided by Innovation Manager, British Sugar, Personal communication).

The draft ratio, i.e., the mass of diffusion juice leaving the diffuser relative to the mass of cossettes entering the diffuser, is a key factor as it directly determines the concentration gradient between the solid and liquid phases. An optimum draft ratio is within the range of 110-130%, indicating that 110-130 tonnes of diffusion juice should be produced for every 100 tonnes of beet cossettes (Asadi, 2006; Van der Poel & Schiweck, 1998). A reduction in draft may lead to incomplete exhaustion of the cossettes, resulting in sucrose losses in the sugar beet pulp. In addition, it may also promote clogging of the solids due to viscosity issues and impair the movement of the material in the diffuser. On the other hand, an increase in draft ratio will result in higher sucrose extraction and less sucrose lost in the pulp, but also requires excessive water usage which will negatively impact the overall efficiency of the process as greater energy will be required to heat the additional extraction solvent and separate the sucrose from the diffusion juice.

The retention time during which the beet cossettes are in contact with the extraction solvent is also important, as a longer retention time results in a greater extraction of sucrose. However, a compromise between retention time and acceptable sucrose loss must be made since it would be impractical and unrealistic to allow the system to reach total equilibrium as the residence time would approach infinity. Furthermore, although longer retention times increase sucrose extraction, it also increases the extraction of non-sugars and excessively degrades the beet structure, reducing sucrose purity and pulp pressability. Conversely, short retention times would result in incomplete exhaustion of the cossettes and an unacceptable amount of sugar loss (Asadi, 2006).

Traditionally, a retention time of 60-110 min has been identified as an optimum as it provides sufficient time for the disruption of the sugar beet cell wall and diffusion to occur (Asadi, 2006). However, this range varies depending on the specific type of diffuser involved. RT diffusers can be operated efficiently with a minimum retention time of 60 min as they are designed to operate under plug flow conditions, whereas DdS and BMA Tower diffusers require a longer retention time to achieve comparable extraction as they are subject to significant axial and radial dispersion, intermixing and dead zones within the diffuser (Information provided by Innovation Manager, British Sugar, Personal communication).

Some of the key performance indicators (KPI) and set-points (SP) in the cossette preparation, industrial diffusion and pressing process are outlined in Table 1-2.

Table 1-2: Key performance indicators (KPI) and set-points (SP) in the industrial cossette quality, diffusion and pulp pressing processes. RT and Tower diffusers have slightly different acceptable ranges due to the mode of operation. Data collated from Maarten de Bruijn (2021a), Asadi (2006).

Parameter		KPI or SP	Unit	Recommended		
Cossette quality				**Minimum**	**Target**	**Maximum**
Length	RT	KPI	Silin number (m/100g)		10-12	
	Tower				5-8	
Mush content		KPI	%		2-3	5
Diffusion						
pH		SP	pH	4.5	5.0	5.5
Average extraction temperature	RT	SP	°C	71	72	73
	Tower			68	70	72
Residence time		SP	min		60-120	250
Draft ratio		KPI	% (w/w)		110-130	
Pulp pressing						
Dry substance in pressed pulp		KPI	g/100g	22	28-30	35
Sugar in pressed pulp		KPI	g/100g		1.7-2.3	

1.3.3 Lime purification and carbonation

As discussed in Section 1.3.2.1, the raw juice obtained from the diffusion process is only 85-90% pure with regards to sucrose; therefore, the impurities must be removed via additional purification stages. Aside from enhancing sucrose purity, these

impurities must be removed because they will otherwise cause clogging, fouling or impair the performance of subsequent unit operations.

First, milk-of-lime is added to the diffusion juice to precipitate or decompose non-sucrose impurities such as proteins and colloids. Monosaccharides are also decomposed in this stage due to the alkalinity (Maarten de Bruijn, 2021a). This is followed by the addition of carbon dioxide to neutralise and precipitate out the calcium carbonate. The output of this process is known as thin juice and has a sucrose purity of 85 to 92% sucrose (Asadi, 2006).

1.3.4 Evaporation

Thin juice is relatively high in sucrose purity but also contains a large volume of water added during the diffusion process. The objective of the evaporation stage is to remove as much water as possible to concentrate the thin juice, with a dry substance of approximately 15% w/w, into a thick juice with a dry substance of approximately 60-75% w/w (Silin, 1964). This is achieved by performing the evaporation under vacuum, reducing the boiling point of the water in the thin juice. The condensate and heat are then collected and re-used in the sugar beet refinery.

1.3.5 Crystallisation

The final key step in the production of refined sugar from sugar beet is the crystallisation stage, during which the final product, crystalline sucrose, is obtained. The thick juice is supersaturated either by further evaporating water such that there is not enough water to dissolve the sucrose crystals, or by lowering the temperature of the thick juice so that the solubility of sucrose decreases and therefore crystallises out when seeding crystals are added. This produces extremely high purity sucrose as only sucrose crystallises, whilst the remaining impurities are retained in the thick juice, forming molasses (Asadi, 2006).

In summary, sugar beet processing comprises several key stages in order to extract and purify sucrose from sugar beets. Although sucrose is the primary product, biorefineries have been designed to maximise value from sugar beet and ensure minimal resources are wasted. The diffusion process is the primary stage in which sucrose is extracted and will therefore be the focus of this work.

1.4 Technological approaches for improving sugar yield

As previously mentioned in Section 1.1, in 2017, the European Commission voted to remove the 50-year old sugar quota with the intention of creating a situation in which the UK and EU sugar market could more readily adjust to the global market price of sugar. The de-regulation of the market meant that processors could produce and export as much sugar as possible. On the surface, this was a good opportunity for the UK sugar beet industry as, despite health-education campaigns and sugar taxes, the consumption of sugar in recent years has begun to increase; for instance, in 2019 the consumption of sugar foods increased by 3.4% compared to 2015 (PHE, 2020). Similarly, consumer spending on sugar, confectionary and ice cream in the UK has steadily risen since 2011 (Office for National Statistics, 2021).

However, with the removal of minimum price guarantees, the price of sugar has fallen significantly and UK and EU processors are struggling to compete with cheaper imports of beet and cane sugar. In 2019, the end of beet sugar quotas resulted in a 31% increase in sucrose production and a subsequent 24% decrease in EU sugar prices (Mattinson, 2019). This has led to an oversupply of sugar and a decrease in its value coupled with a falling demand. Furthermore, post-Brexit trade deals with larger sugar produces such as Australia stand to further disrupt the UK sugar beet economy.

Additionally, recent years have proved difficult for farmers growing sugar beet in the UK and the EU. Climate change has led to extreme weather situations including excessive rainfall and droughts that have severely disrupted the productivity of sugar beet farming. Furthermore, diseases such as beet yellows virus, beet chlorosis virus and beet mild yellowing virus are increasingly impacting sugar beet yields due to the growing pressures against the use of neonicotinoids and other pesticides (Friends of the Earth, 2017). As a result of these factors, the 2020-21 harvest in the UK reported a decrease in yields by approximately 25% against a 5-year average, with some farmers reporting losses of up to 80% of sugar beet (Warren, 2021).

Due to the uncertainty surrounding the future of the sugar beet market, sugar beet refineries must innovate in order to remain globally competitive. As sucrose is the primary output of the process, the main way in which competitiveness can be achieved is by further increasing the production yield of sucrose (Aguirre et al, 2015). Aside from mergers and acquisitions of other smaller companies (Burgess, 2015), there are two

main ways in which an increase in production yield can be achieved via biotechnological means: investment into plant breeding technologies or maximising the efficiency of the existing refinery processes. These are discussed in Sections 1.4.1 and 1.4.2 respectively.

1.4.1 Breeding technology

Selective breeding of sugar beets is not a novel approach, as they have been bred since the early 19th century to maximise sucrose yield (Knapp, 1958). Thus, the modern-day sugar beet is very different from the original sugar beet. Traditional breeding methods involve a rigorous procedure of selecting for optimal phenotypic traits such as disease resistance or sucrose yield, intercrossing the selected individuals, growing the seeds from these individuals and evaluating the adult-stage phenotype for successful heritability and expression of the desired traits (Bosemark, 1993; Hecker, 1967; Marlander et al, 2018). This method of breeding is highly successful as it is responsible for producing the high-sucrose sugar beets currently grown today. An analysis of beet variety between 2006 and 2016 in the Netherlands suggested that breeding was responsible for an average 1% yield increase per year (Hanse et al, 2018). However, selective breeding via the traditional method is a slow and arduous process and it is unable to compete with the fast-growing demand for further improved strains.

Hoffmann & Kenter (2018) suggest that the breeding of sugar beets for increased sugar content will eventually reach a ceiling as there is a natural limit to the extent that cell wall compounds can be sacrificed for sugar yield before fundamental structural integrity issues arise. In recent years, the sugar beet cell wall components, known in the industry as ‘marc’, has decreased from 4.5% to 4.0%. This correlates with an observed decrease in mechanical strength in the same period, although the precise relationship between these two factors is still being investigated (Maartern de Bruijn, 2020b). Sugar beets with poor mechanical strength below a certain threshold may result in lower sucrose yields due to losses from damage during processing. Therefore, selective breeding for increased sucrose content will eventually reach a ceiling as a compromise between sucrose content and essential mechanical strength must exist. As a result, future breeding success is likely to be due to improved pathogen resistance and reduced losses rather than further increases to sucrose content.

To meet the growing demands for improved strains, it is critical to integrate traditional sugar beet breeding technologies with modern molecular biology techniques. This will enable highly efficient selection using tools such as genetic linkage maps to identify and express genes encoding resistance to specific diseases (Hunger et al, 2003) or genes to maximise sucrose yield (Schneider et al, 2002; Stich et al, 2008; Reif et al, 2010; Würschum et al, 2013).

Compared to the conventional breeding process, molecular markers combined with next-generation sequencing technologies can be used to identify positive traits at very early developmental stages within the genotype prior to phenotypic presentation, accelerating the rate at which advances can be made (Richardson, 2010; De Lucchi et al, 2021). One of the next-generation advances in the molecular biology approach is gene-pyramiding, whereby multiple genes for disease-resistance or other biotic and abiotic stresses can be combined into a single genotype via recombinant DNA technology (Dormatey et al, 2020; Ramalingam et al, 2020). This can be achieved via traditional breeding, but it is difficult to identify the expression of multiple gene candidates in the phenotype. Combining multiple genes reinforces the resistance to disease that may otherwise be overcome given enough time. To validate these techniques, successful candidates can then be evaluated using traditional phenotypic selection methods and criteria.

1.4.2 Optimising the efficiency of existing processes

An alternative way of increasing the production yield of sucrose is by optimising the efficiency of the current sugar beet refinery process. The UK sugar beet refineries are already very efficient; in 2019/20, a yield of 12.5 tons of sugar per hectare of farmed land was obtained. By comparison, the EU (plus UK) average in 2019/20 was 11.7 tons per hectare. However, there is room for improvement, as countries including the Netherlands achieved a yield of 15 tons per hectare in the same year (European Commission, 2021).

As the diffusion process is the primary stage by which sucrose is extracted from the sugar beets, it is particularly susceptible to sucrose loss, second only to sucrose lost in molasses after the crystallisation process (Figure 1.8) (Maarten de Bruijn, 2020a). According to Asadi (2006), under optimal conditions, the cossettes lose approximately 98% of sucrose to the diffusion water and retain 2% as wet pulp. Similarly, industry

reports suggest diffusion losses of approximately 2 to 4% of sugar in whole beet. Although this is a relatively good yield, a 2% loss is amplified by the industrial scale of the process such that even a small loss can result in several hundred thousand tons of sugar loss per year, and subsequently significant losses in earnings per annum. Minimising losses at this stage will also reduce the losses in subsequent stages.

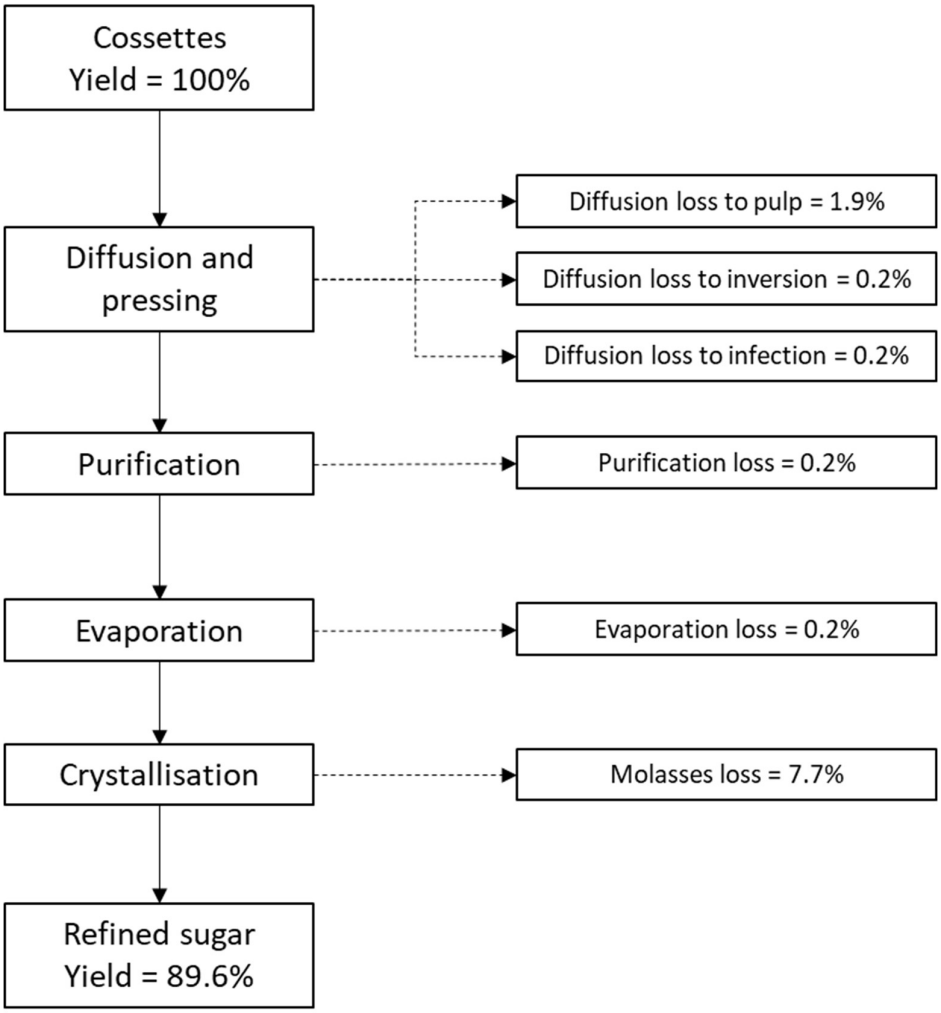

Figure 1.8: Summary of the key sugar beet processing stages and the respective average sucrose losses at each stage. Total sucrose losses from cossettes to final yield is approximately 10.4%, of which molasses loss comprises the majority. Diffusion losses can be categorised by loss to sucrose inversion, microbial infection and residual sucrose remaining in sugar beet pulp. Diffusion loss to the pulp is the second largest cause of sugar loss. Data and values obtained from Maarten de Bruijn (2020a). Solid arrows represent main processing stages. Dashed arrows represent causes of sucrose losses at each stage.

Compared to the selective breeding of sugar beets, the optimisation of sugar beet processes is not as well studied in academic literature. This is likely because these

processes are difficult to simulate in an academic environment without the support of industrial experience. Trials have been conducted within the sugar beet industry to optimise the refinery process, but as this data is not in the academic field, it is largely inaccessible. The following sections summarise the process-focused research currently described in the published literature.

1.4.2.1 Mathematical modelling

Several research groups have approached the optimisation problem from a mathematical modelling perspective coupled with industrial plant data for validation (Buttersack, 1997). For example, the optimisation of the diffusion process in the BMA tower diffuser (Sotudeh-Gharebagh et al, 2009; Mostoufi et al, 2010) and the RT diffuser (Merino-Gomez, 2001; Kiani et al, 2016). Given a solid dataset and the correct assumptions, these mathematical models are very useful for optimisation of sugar beet processes without investment into lab-scale practical models, and often demonstrate a very strong agreement between simulation outputs and actual plant data. However, certain intricacies within the diffusion process are difficult to model, such as sucrose inversion and microorganism contamination and are therefore based heavily on assumptions that may not reflect the practical reality (Both et al, 2013).

Furthermore, mathematical modelling is limited by the fact that it is underpinned by the data supplied by the existing process. In other words, this is a suitable technique for modifying existing parameters, equipment and process conditions, but it is unable to model factors that have no existing numerical data associated with it. Therefore, mathematical modelling is unable to test and trial the optimisation of sugar extraction via new technologies and techniques. In these cases, there is no substitute for conducting practical trials. Examples of these include electroporation and enzyme-assisted juice extraction.

1.4.2.2 Electroporation technology

Electroporation, or Pulsed Electric Field (PEF), is a physical technique first introduced by Stampfli in 1958 that involves exposing a biological membrane to a strong electrical field. This results in an increase in the electrical conductivity and permeability of the membrane. This effect can be reversible or irreversible depending on the properties of the applied electric field, such as the intensity, shape and number of pulses (Figure 1.9) (Kotnik et al, 2015; Kempkes and Munderville, 2017).

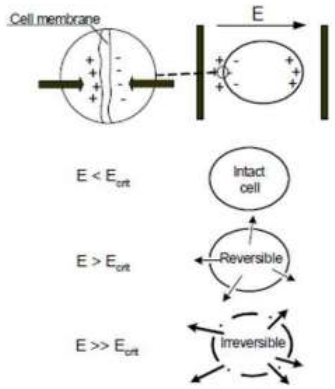

Figure 1.9: The effect of electroporation on the cell membrane can be reversible or irreversible, depending on the relationship between the applied electrical field, E, and the critical threshold for pore formation, denoted Ecrit. If E<Ecrit, then the cell membrane will become reversibly porous. If E >>Ecrit, then the membrane will become irreversibly disrupted, enabling the material to readily cross the cell membrane (Lindsey & Jones, 1987; Toepfl, 2006).

Due to the disruptive properties of electroporation it was initially used as an alternative to the traditional heat-shock or viral vector methods for the transformation and transfection of microbial and mammalian cells. Reported advantages include higher efficiency of DNA uptake rates and broad versatility, as it can be applied to any type of cell membrane (Lessard, 2013; Chicaybam et al, 2017).

The application of electroporation to transform plant cells was introduced later due to initial difficulties associated with the presence of the recalcitrant cell wall (Hiromichi et al, 1986). One example of the application of electroporation on sugar beet cells was by Lindsey & Jones (1987) whereby they investigated electroporation as an alternative to chemical permeabilisation of the sugar beet plasma membrane by monitoring the uptake and retention of phenosafranine.

The diverse applications of electroporation have since been explored further. Kotnik et al (2015) summarise the numerous applications of electroporation in biotechnology. Some of these capitalise on the destructive properties of electroporation ($E>E_{crit}$) as opposed to the transient membrane disruption ($E<E_{crit}$), such as wastewater treatment and non-thermal pasteurisation.

One of the applications of destructive electroporation is for the extraction of desirable solutes from cells; of particular interest to this research is the improved extraction of sucrose from sugar beet. The first recorded instances of this was by Zagorul'ko and

separately, Kartashev & Koval' in the 1950s. Kartashev & Koval' demonstrated that electroporated beet slices yielded more juice than untreated slices, especially at lower temperatures (Figure 1.10).

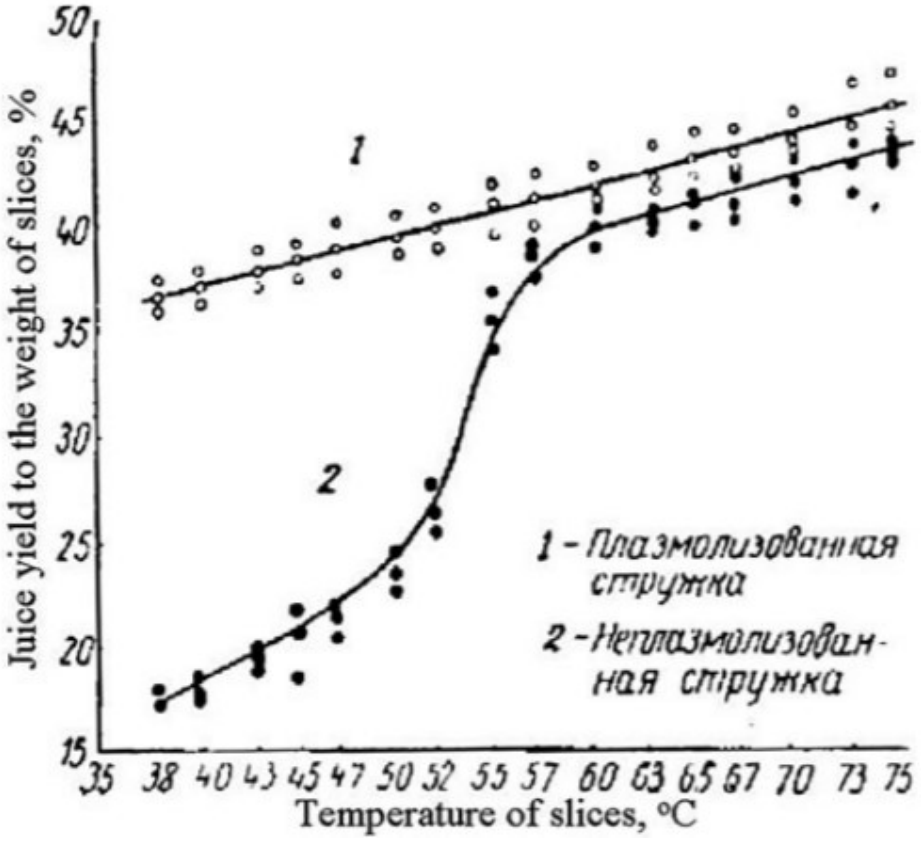

Figure 1.10: Kartashev & Koval' (1956) demonstrated an increase in juice yield when sugar beet slices were subjected to electroporation prior to diffusion extraction. A significant increase in juice extraction at lower temperatures could be obtained from electroporated sugar beet slices (open circles) compared to untreated beet (closed circles).

This idea was initially patented by a Russian group in 1989 (SU1521439A1). When sugar beet chips were subject to electroporation prior to diffusion, they observed an increase in juice quality and a 0.1% decrease in pulp loss. A similar idea was patented by Eshtiaghi & Knorr in 1999 (WO1999064634A1) with a focus on the electroporation of larger "blocks" of sugar beet to minimise excess rupturing of beet cells which may increase impurity leaching.

Eshtiaghi & Knorr (2002) demonstrated the application of electroporation as an alternative to conventional heat treatment by comparing the effects of these two treatments on the degree of sugar beet cell disintegration. They found that treatment

with 20 pulses of 2.4 kV cm^{-1} is approximately equal to the degree of disintegration achieved with incubating the beet at 72°C for 15min.

The evaluation of PEF-assisted sugar diffusivity kinetics in a batch system identified the optimal conditions of electroporation treatment as 250 pulses of 670 V/cm (Belghiti et al, 2004). These conditions were also shown to improve the purity of juice when compared to thermal pre-treatment, from 89% to 94%. This may be due to the moderate permeabilisation provided by the electroporation conditions in which the membrane is permeabilised but the cell wall remains intact, compared to the relatively harsh thermal pre-treatment method where the entire cell is denatured (Grimi et al, 2010).

Lebovka et al (2007) compared the sucrose diffusion coefficient *D* for untreated and PEF-treated sugar beet in a batch-style model. *D* was similar for untreated beet at 60°C and PEF-treated beet at 30°C, suggesting sucrose extraction at 30°C is as efficient as extraction at 60°C when PEF is used as a pre-treatment. This is particularly appealing as it opens up the possibility of ambient temperature extraction, which would reduce many of the disadvantages of traditional diffusion described in Section 1.3.2.1. Furthermore, the sucrose purity of the PEF-treated juice at 20°C is significantly higher than that of untreated juice and PEF-treated juice at 70°C.

Although electroporation appears to demonstrate clear benefits when applied to sucrose extraction, the previous work has been conducted on batch models in which there is typically a low quantity of beet and a relatively high volume of extraction water. To verify the benefits of electroporation and assess its application within an industrial context, Loginova et al (2011a) designed a PEF-assisted pilot scale extraction device (Figure 1.11).

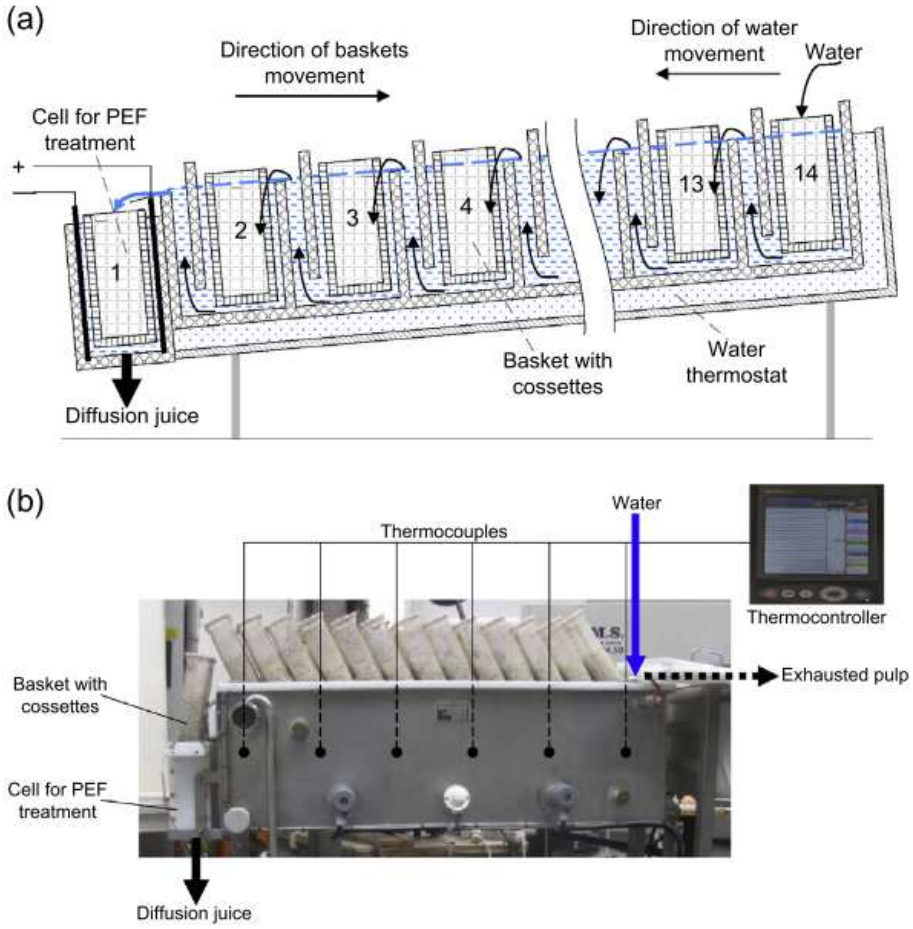

Figure 1.11: Schematic diagram of Pulse Electric Field-assisted diffusion model (Figure reproduced from Loginova et al, 2011a). The model consists of 14 cells each with a capacity for 500 g of cossettes and approximately 600 mL of juice. A) Schematic representation of the Pulse Electric Field-assisted diffusion model. B) Photograph of Pulse Electric Field-assisted diffusion model.

This model confirmed the benefits of electroporation, as sucrose concentration in the juice of PEF-treated beets at 30°C was statistically similar to the sucrose concentration in the juice of untreated beets at 70°C. An additional benefit was the significant reduction in colloids, proteins, pectins and colour in PEF-treated juice at 30°C but this was not reflected in the overall purity, potentially due to the relatively low quantity of these impurities compared to the sucrose concentration (Loginova et al, 2011b).

Outside of academia, there have also been some electroporation trials in collaboration with industry. Initial laboratory trials were conducted in 2004 in which electroporation applied to whole beets prior to slicing, resulting in improved cossette quality, although issues with electric field homogeneity were raised (Frenzel et al, 2004). In 2014, pilot-

scale trials results were successful in replicating laboratory scale data, reporting higher juice purity, lower color and lower colloidal content. Furthermore, the pressability of the pulp was improved when compared to non-PEF treated pulp (Vidal, 2014).

Following these trials, Südzucker AG in Germany developed a new process combining electroporation followed by alkaline extraction of sucrose. Extraction under alkaline conditions has its advantages as it reduces the risk of acid hydrolysis of sucrose and microbial invertase activity. This process resulted in a pressed pulp with dry matter 10% higher than the conventional method (Rudolph et al, 2016). However, when investigating the effects of electroporation on the entire process chain, electroporation has detrimental effects on the subsequent evaporation process as it increases the turbidity of the raw juice, affecting the final white sugar quality (Bagherzadeh et al, 2016).

The benefits of electroporation on sucrose extraction of sugar beets are evident, such as the increase in sucrose extraction and improved sucrose purity of juice, both of which have been demonstrated in a batch model and a pilot model resembling industrial diffusion. The most appealing benefit is the possibility of ambient temperature extraction, which would eliminate many of the disadvantages associated with traditional diffusion such as high impurity leaching and energy costs, however the effect of ambient extraction conditions on microbial contamination and invertase activity must be evaluated further.

Despite these benefits however, PEF has not been integrated into the sugar beet industry and there have been no successful attempts in establishing an industrial-scale PEF-assisted process to date. This is likely due to the need to redesign the entire processing chain in order to accommodate for an industrial sized electroporation system, the high capital investments that would be required to do so, and the reluctance to do so for a technology that is still relatively new. Therefore, despite successful laboratory and pilot trials, the application of electroporation on an industry scale is still a work in progress. Large-scale PEF generators have had numerous technical difficulties and reliability issues (Sitzmann et al, 2016). However, in the past few years there have been several companies specialising in commercial-scale PEF generators, such as Dutch-German company Pulsemaster; therefore there is still potential for the industrial-scale application of electroporation.

1.5 Enzymes used in fruit and vegetable processing

Macerating enzymes, particularly carbohydrases such as pectinases and cellulases, are used extensively for improving juice yield and quality from fruits and vegetables such as apples, watermelon and berries (Gerhartz, 1990; Srivastava & Kumar Tyagi, 2013; Saxena et al, 2014). Enzyme-assisted juice processing is an environmentally sustainable method of optimising the existing juicing process and therefore, the global enzyme market in fruit and vegetable processing is considered a high growth emerging opportunity, with market size projected to reach USD 41.39 billion by 2022 (Markets and Markets, 2017; Toy et al, 2020). The general principle of enzyme-enhanced juice extraction is to promote the solubilisation of the cell wall, enhancing solute diffusion into the surrounding extraction solvent (Rosenthal et al, 1996; Dominguez and Munoz, 2017).

As described in Section 1.2, the cell wall of sugar beets is predominantly composed of pectin, hemicellulose and cellulose. Therefore, pectinases and cellulases are the key enzymes that may be beneficial in enzyme-assisted extraction of sucrose from sugar beets.

1.5.1 Pectinases

Pectinases are the most commonly used enzymes for enhancing juice processing, accounting for approximately 25% of the global enzyme market (Oumer, 2017). Pectinases are responsible for degrading soluble pectins that may otherwise result in poor juice quality. Furthermore, pectin functions as a key structural component of plant cell walls that maintains the integrity of plant tissue; therefore hydrolysing the pectin chains will cause cell rupture and enable the release of the cellular components (Palin & Geitmann, 2012).

As pectin is an extremely varied polysaccharide, the term “pectinases” is a broad term for various different enzyme activities that act on the varied regions of pectin. Table 1-3 is a summary of pectinase classification based on the activity and the specific substrate the enzyme acts upon whilst Figure 1.12 is a schematic of where these enzymes act upon on a pectin chain. Pectinases commonly used in fruit and vegetable processing can be broadly divided into two categories: (i) esterases that eliminate methyl- and acetyl- residues from the pectin backbone, producing polygalacturonic acid and methanol or acetate or (ii) depolymerases that disrupt the pectin backbone

by a lyase-based transelimination or hydrolytic mode of action. These can further be classified as endo- or exo- enzymes depending on where the enzyme acts on the substrate (Toushik et al, 2017).

Table 1-3: Classification of pectinases according to their mode of action, substrate and product. The term pectic acid is interchangeable with polygalacturonic acid (Adapted from Sieiro et al, 2012).

Enzyme	EC Number	Mode of action	Substrate	Product
(Endo) Pectate lyase	4.2.2.2	Random endo-cleavage	Pectic acid	Unsaturated oligogalacturonates
(Exo) Pectate lyase	4.2.2.9	Cleavage of penultimate bonds from non-reducing end	Pectic acid	Unsaturated disaccharide
(Endo) Pectin lyase	4.2.2.10	Random endo-cleavage	Pectin	Unsaturated methyl-oligogalacturonates
(Endo) Polygalacturonase	3.2.1.15	Random endo-cleavage	Pectic acid	Oligogalacturonates
(Exo) Polygalacturonase	3.2.1.67	Terminal cleavage from non-reducing end	Pectic acid	Monogalacturonates
Pectin methyl esterase	3.1.1.11	Blockwise or random cleavage of C6-methyl ester	Pectin	Pectic acid and methanol
Pectin acetyl esterase	3.1.1.6	Random cleavage of acetyl ester	Pectin	Pectic acid and acetate

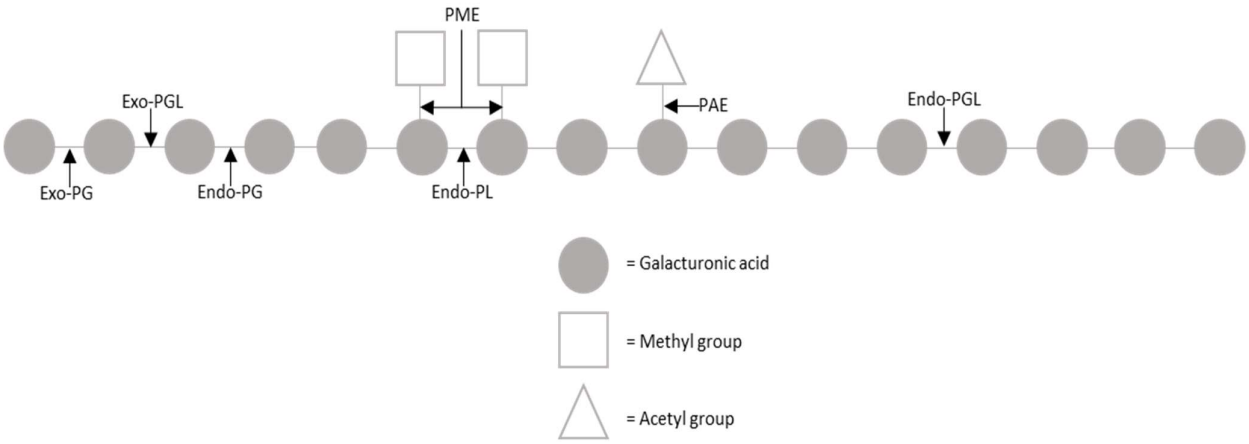

Figure 1.12: Schematic diagram of the mode of action of the pectinases listed in Table 1-3 on pectin. PG = Polygalacturonase, PGL = Pectate lyase, PME = Pectin methyl esterase, PAE = Pectin acetyl esterase, PL = Pectin lyase.

In addition to the pectinases listed in Table 1-3, there are also enzymes that act specifically on the highly branched regions of pectin that consist primarily of rhamnogalacturonan-I, such as arabanases and galactanases. These enzymes are sometimes designated hemicellulases as these side chains are present in both pectin and hemicellulose; as such, they are not strictly pectinases. Several of the enzymes used to degrade rhamnogalacturonan-I are detailed in Table 1-4.

Table 1-4: Classification of rhamnogalacturonan-I degrading enzymes according to their mode of action, substrate and product.

Enzyme	EC Number	Mode of action	Substrate	Product
(Endo) Rhamnogalacturonan hydrolase	3.2.1.171	Endo- cleavage of RG1 backbone	Rhamnogalacturonan-I	Oligosaccharides with GalA at the reducing end
(Endo) Arabanase	3.2.1.99	Endo- cleavage of (1,5)-α-arabinofuranosidic linkages	Arabinan sidechains, preferentially linear	Arabinose oligomers
α-arabinofuranosidase	3.2.1.55	Exo- cleavage of terminal non-reducing (1,2)-,(1,3)- or (1,5)-arabinose	Arabinan sidechains	Monomeric arabinose

(Endo)-1,4-β-galactanase	3.2.1.89	Endo- cleavage of (1,4)-galactan	Galactan sidechains	Galactose oligomers

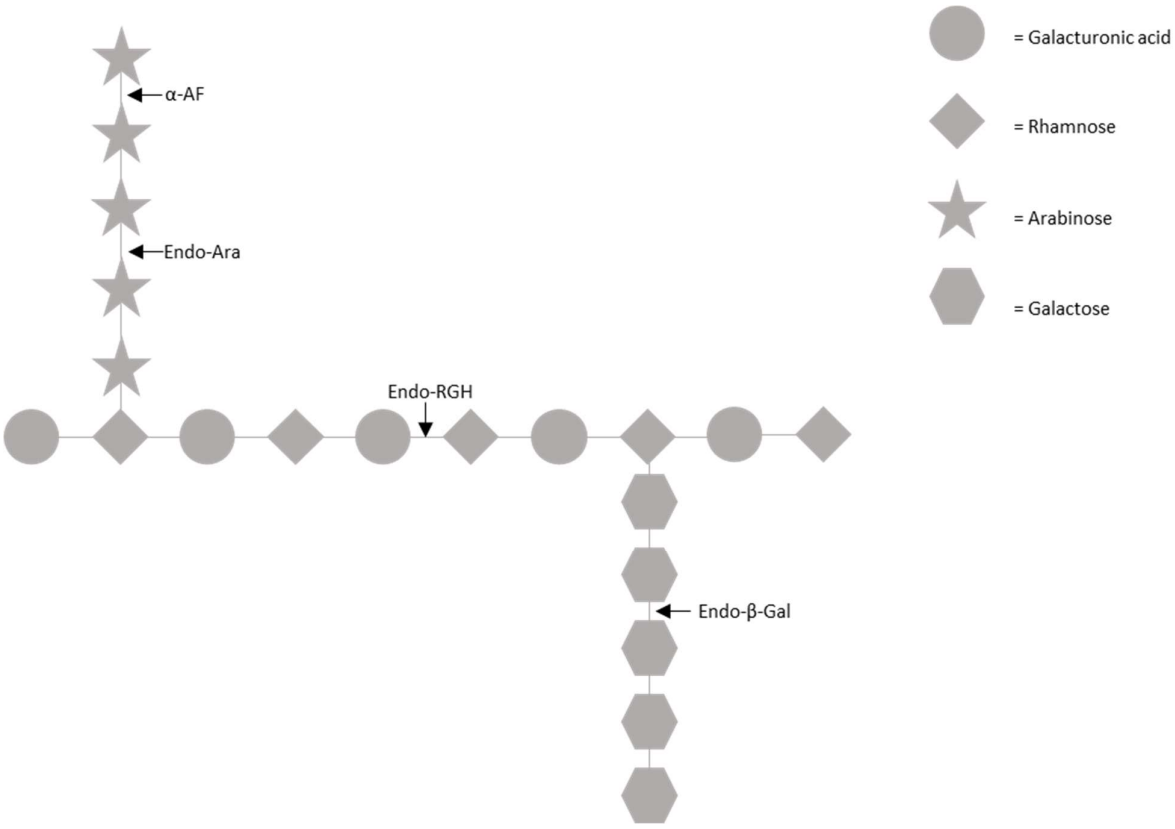

Figure 1.13: Schematic diagram of the mode of action of the rhamnogalacturonan-I degrading enzymes. AF = Arabinofuranosidase, Ara = Arabinase, RGH = Rhamnogalacturonan hydrolase, Gal = Galactanase.

In order to determine which pectinases are most suitable for a given application, it is important to understand the structure of the particular pectin. For example, highly methylated pectins will require significant pectin methyl esterase (PME) activity to enable the activity of polygalacturonases. Synergistic effects should also be considered as PME-mediated random demethylesterification releases protons that promote the activity of endopolygalacturonases (Moustacas et al, 1991; Kohli et al, 2015). Therefore, to achieve total solubilisation of pectin, a combination of the enzymes in Table 1-4 is required.

However, in situations where the total solubilisation of pectin is not ideal as it may interfere with subsequent processing, yet fractionation of pectin into oligopectin is

required, pectin lyase (4.2.2.10) is the only depolymerising pectinase that acts on methylated or acetylated pectin whilst also acting on the homogalacturonan pectin backbone. This is particularly appealing to applications in sugar beet pectin as its highly methylated and acetylated nature is resistant to pectate lyase or polygalacturonic acid activity without prior demethylation or deacetylation (Bonnin et al, 2003).

1.5.2 Cellulases

In addition to pectinases, cellulases have also been investigated for their benefits in aiding juice extraction from fruits and vegetables. Although cellulose is a homogenous polymer, the term cellulase refers to three key enzymes that are all necessary to convert cellulose into monomeric glucose (Table 1-5; Figure 1.14).

Endoglucanases (EC 3.2.1.4) randomly cleave the internal regions of amorphous cellulose fibres, producing smaller units with exposed reducing and non-reducing ends. Cellobiohydrolases (EC 3.2.1.91) cleave the penultimate (1,4)-β-glucosidic bonds of crystalline cellulose and smaller cellulose units, producing cellobiose. Finally, β-glucosidase (EC 3.2.1.21), although not strictly a cellulase as it does not act directly on cellulose, is responsible for cleaving the (1,4)-β-glucosidic bond in cellobiose, producing two glucose units (Toushik et al, 2017).

Table 1-5: Classification of cellulose degrading enzymes according to their type of activity, mode of action, substrate and product.

Enzyme	EC Number	Mode of action	Substrate	Product
β-1,4-Endoglucanase	3.2.1.4	Endo-hydrolysis of (1,4)-β-glucosidic bonds	Amorphous cellulose	Oligosaccharides and smaller polysaccharide units
Cellobiohydrolases	3.2.1.91	Cleavage of penultimate (1,4)-β-glucosidic bonds from reducing and non-reducing ends	Crystalline cellulose and smaller polysaccharide units with exposed reducing and non-reducing ends	Cellobiose

β-Glucosidase	3.2.1.21	Hydrolysis of terminal non-reducing β-glucosyl residues	Cellobiose	Glucose

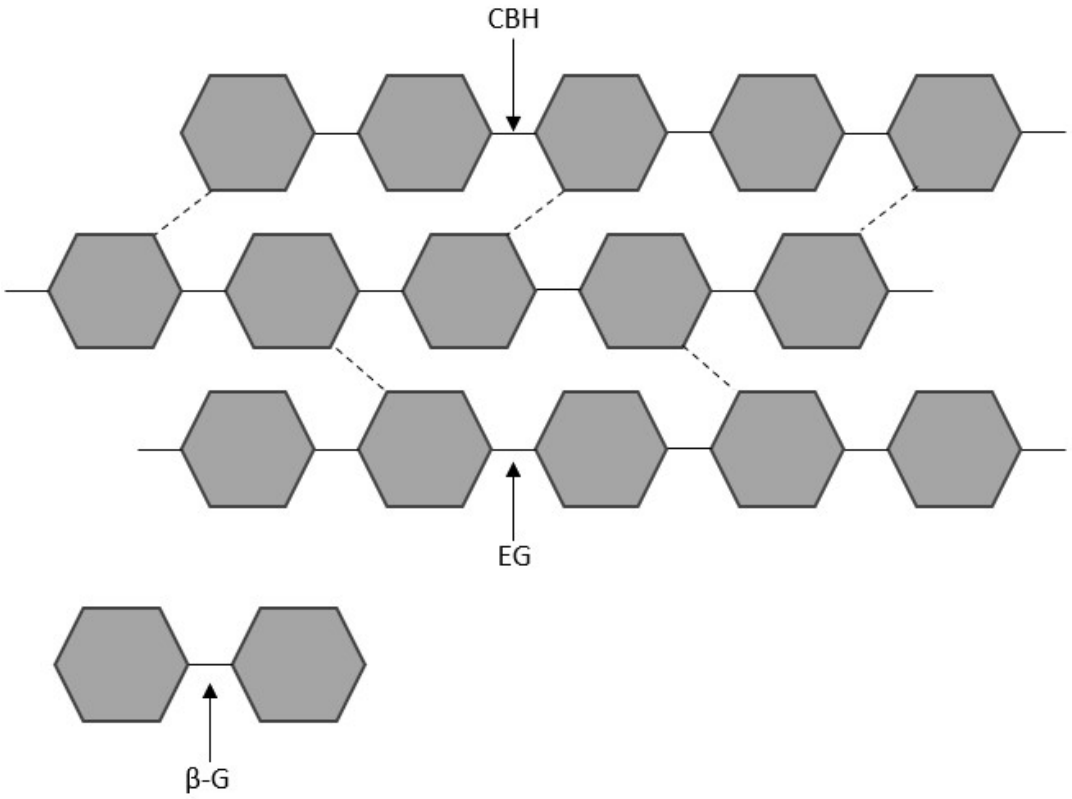

Figure 1.14: Schematic diagram of the mode of action of the cellulases listed in Table 1-5. EG = Endoglucanase, CBH = Cellobiohydrolase, β-G = β-Glucanase.

Although pectinases are more commonplace in the juice extraction industry, cellulases are sometimes used because they also contribute to the solubilisation of the cell wall and reducing viscosity thus potentially enhance juice extraction (Bhat, 2000). Furthermore, due to the interwoven nature of the cellulose microfibrils and pectin, incorporating cellulase activity may promote pectinase activity by enabling ready access to the pectin substrate. This is particularly effective when the objective is complete liquefaction of the biomass (Ejaz et al, 2021; Singh et al, 2021).

1.5.3 Application of enzymes in fruit and vegetable processing

Macerating enzymes can be applied at two different stages during processing for two distinct benefits. The first is as a pre- or co-treatment prior to or during juice extraction via pressing, filtration or diffusion, wherein the objective is to increase the juice yield, increase the total dissolved solids in the juice, or otherwise improve the efficiency of the juice extraction. The second option is adding the enzymes as a post-extraction treatment of the juice in order to clarify the juice and improve the final quality, for example, by eliminating indicators of poor juice quality such as colloidal pectin-induced haze and viscosity (Sharma et al, 2016). Patiar et al (2018) provides a comprehensive review of the benefits of pectinases for the purpose of juice clarification.

Juice clarification is typically an individual process step separate from extraction and further purification. As such, the conditions can be specifically optimised for the enzyme activity in this step. In contrast, the ideal use of enzymes as a pre- or co-treatment in industry would be as a supplementary step in combination with the pre-existing extraction step. However, it must be considered that the conditions for the extraction step may not be optimal for enzyme activity. The focus of enzyme application in this work will be for the purpose of aiding juice extraction, as the clarification of the sugar beet juice is provided by the subsequent liming and carbonation stages (Section 1.3.3).

There are several hypotheses as to why enzymes, particularly pectinases, improve juice yield from fruits and vegetables. The rigidity of unripe fruits is conferred by the binding of pectin to cellulosic microfibrils (Lara-Espinoza et al, 2018). During the ripening process, endogenous pectinases are produced, which modify the pectin components of the parenchymal cell walls and middle lamellae, resulting in fruit softening. As a consequence of this, the internal juice within the parenchymal vacuoles leaches out of the cells and into the external environment more readily (Paniagua et al, 2014; Ramadan, 2019). Therefore, incorporating pectinases into the extraction process to degrade the pectin further may result in more juice extraction (Danalache et al, 2018). Xiao et al (2017) attribute the increase in yield to the depolymerisation of pectin resulting in temperature-induced expulsion of air from plant tissues, simultaneously expelling the contents of the cell (Xiao et al, 2017; Bhatkar et al, 2021).

The degradation of pectin also reduces the water retention capacity of the cell such that free water is released in the system, increasing juice yield (Lee et al, 2006; Sharma, 2016). This also reduces the gelling capacity of pectin which decreases viscosity, improving filterability or pressability of the pulp (Toy et al, 2020). Although the benefit of cellulases is less well studied, it is believed that similar mechanisms are involved when cellulosic structures are degraded (Toy et al, 2020).

The enzyme-enhanced extraction process typically begins with the preparation of the biomass into an accessible pulp. The material is washed, peeled and pulped if necessary, followed by an initial homogenisation step. This is critical to reduce the inherent recalcitrance of the lignocellulosic biomass. Although homogenisation is a physical process often used for its efficiency and cost-effectiveness, it does require a significant energy input, therefore there are numerous alternative pre-treatment processes including acid or alkaline treatment, steam explosion and deep-eutectic solvent application, each of which has its own benefits and detriments that may be optimal for the particular biomass used (Galbe & Wallberg, 2019). At this stage, the homogenised biomass may be pasteurised briefly in order to inactivate innate enzymes that may interfere with the extraction process (Sreekantiah et al, 1971; Tapre & Jain, 2014).

The macerating enzymes are added to the homogenised biomass and incubated for a period of time under the optimal enzyme loading and reaction conditions. Optionally, water is added if the mixture is particularly viscous in order to produce a semi-aqueous phase to aid enzyme accessibility by facilitating homogenous activity on the substrate (Bhatkar et al, 2021). Concurrently, or subsequently, the juice is extracted by pressing, filtration or diffusion according to the material being processed and the equipment available. For post-extraction clarification treatment, the juice is incubated with the enzymes until the desired turbidity and viscosity is achieved (Cerreti et al, 2016; Patidar et al, 2018).

Following this procedure, enzymes have enhanced the juice recovery of various fruits and vegetables to varied success. Table 1-6 is a compilation of literature sources examining juice recovery with or without enzyme addition from various fruit and vegetable sources. The extent of juice yield improvement varies significantly, as the smallest increase in juice yield was 4.2% from soursop, reported by Makebe et al

(2020), whilst the largest reported increase was 111.9%, by Kaur et al (2008) on guava. However, direct comparisons of the juice yields between literature is difficult to assess due to the widely varied experimental conditions, for example, an incubation time of 67.5 min versus 436.2 min between Makebe et al and Kaur et al, as well as differing specific enzyme activities and concentrations, temperatures and general methodology.

Furthermore, there is an inherent difference between the cell wall compositions of these fruit and vegetables, for instance, soursop has a pectin content of 0.91% fresh weight basis (Badrie & Schauss, 2010) whilst guava has a pectin content of 2.5% fresh weight basis (Spiller et al, 2018); which is then further differentiated by the heterogeneous pectin composition. There are also factors to consider even when using the same biomass source, as they may vary significantly in cultivar, age and composition. Lozano et al. (2006) reported that the effect of enzymes on juice yield from pomme fruit is most significant with mature and cold storage pomme, whilst yield increases are not usually achieved from fresh, early season pomme fruits, therefore, comparisons of juice yield in literature can only be directly compared to control results reported by the same literature.

Following biomass preparation, the methods involved in the cited literature typically involve an incubation step with the enzyme followed by a solid-liquid separation step. Some authors opt for a passive filtration step through a fine mesh cloth whereas others actively press the material through a mesh cloth to extract the juice. In a few scenarios, the juice is separated from the residual solids via centrifugation. These differences may also explain why the juice recovery differs so greatly between different literature sources.

Nevertheless, some key commonalities can be obtained from these citations. Almost all of the literature cited utilise some significant pre-treatment of the biomass in the form of freeze-thawing, pasteurisation, homogenisation via a food processor or a combination of the above. As previously mentioned, this is to reduce the recalcitrance of the biomass and promote enzyme activity. The only exception to this is Jiao et al (2020) in which the only pre-extraction step involved is a manual slicing of the Chinese quince into 3-5 mm pieces, although this does not completely homogenise the quince pulp, the particle size reduction is sufficient for enzyme activity.

In addition to pectinase application, several of the citations use cellulase both with and without pectinase to determine whether cellulases can also improve juice yield. In particular, Mohanty et al. (2018) demonstrate the benefits of pectinases and cellulases on palm fruits (*Borassus flabellifer*). When only pectinase was added at a concentration of 0.33% (w/w), the juice yield was increased by 16.96%. Similarly, trials with cellulase only at a concentration of 0.30% (w/w) resulted in an increase in yield of 19.74%. When both pectinases and cellulases were mixed at a ratio of 1:0.75 (w/w; pectinase:cellulase) per 100 g of pulp, a maximum juice yield increase of 28.51% was achieved. This therefore demonstrates that the enhancement to juice yield is not contingent on the degradation of pectin only, but rather, hydrolysing the cellulose regions can achieve similar benefits. Furthermore, the combined action of both pectinases and cellulases may be required to achieve maximum benefits to juice extraction.

Another parameter to consider is the effect of enzyme addition in juice extraction on the total dissolved solids (TDS; °Brix). This is important to consider as although the juice yield may be increasing, if the TDS decreases, the net effect may be neutral or negative. This is particularly important to consider in sugar beet processing whereby the main consideration is not the volume of juice extracted but the concentration of sucrose within that juice. Table 1-7 is a summary of the studies in Table 1-6 that also measure the TDS of the extracted juice.

The effect of enzyme activity on TDS appears to vary as widely as the effect of enzyme activity on juice yield. In cashew apples, chironji and soursop, there is a significant increase in TDS when pectinases are added (Abdullah et al, 2021; Pradhan et al, 2020; Makebe et al, 2020). On the contrary, there is a decrease in TDS in kendu treated with enzymes (Panda et al, 2021).

In the trials where Mohanty et al (2018) treated kendu with cellulase or pectinase only, TDS decreases by 0.6 and 0.7 units respectively. However, when both enzymes were applied, TDS increased significantly from 10.90 to 12.80, suggesting that although each individual enzyme improved juice yield, the combined activity of cellulases and pectinases was required to increase TDS. A similar, albeit smaller, trend is observed by Handique et al (2019), although a non-enzymatic and separate pectinase and cellulase conditions were not examined. Instead, the most significant increase in TDS

is observed when maximum concentrations (0.3 g of cellulase, 0.3 g of pectinase per 100 g of banana pulp) are added. When pectinase or cellulase is dosed individually, the change to TDS varies from (+0.10) to (-0.20) respectively.

Sonawane et al. (2020) identified a negative linear relationship between TDS and pectinase concentration: although the juice yield was improved, the TDS decreased due to the overall dilution of the soluble solids. The majority of the citations attribute an increase in TDS to the increase in soluble products as a result of enzyme activity (Ghosh et al, 2016; Mohanty et al, 2018). This effect is particularly pronounced when the temperature exceeds the enzyme optimal range, as this is followed by a decrease in TDS (Handique et al, 2019). Thus, it is evident that external factors including temperature and incubation time must be considered in addition to enzyme addition. Panda et al. (2021) constructed a model via response surface methodology using a Box-Behnken design and concluded that enzyme concentration has less influence on TDS than temperature.

Table 1-6: Examples of enzyme addition to fruits and vegetables for improving juice recovery. (*) Indicates trials where there was no control for 0% enzyme concentration, instead the lowest concentration of enzyme is used as the control value. (**) Indicates trials where a range of enzyme concentrations were applied and the results from applying the maximum enzyme concentration are referenced here. Enzyme concentrations are presented as referenced from the source literature. Where both pectinases and cellulases have been applied, the enzyme concentration is expressed as a ratio, whereby P = pectinase and C = cellulase. Juice yield is reported as %(w/w) or %(v/w) as per the source literature.

Produce	Incubation time (min)	Incubation temperature (°C)	Enzyme concentration	Enzyme activity	Juice Yield (%)		Method of sample preparation and extraction	Water addition ratio (Water:Biomass; (v:w)	Reference
					Control	Enzyme-treated			
Bael (*Aegle marmelos correa)*	425	47	20mg/100g pulp	Pectinase 1.64 U/mg	69.01%	86.06%	Pulp homogenised, incubated, filtered through cheesecloth, heated to inactivate enzyme	2.5:1	Singh et al, 2012
	381	46.2	0.22g/100g pulp	Pectinase 8000-12000 U/g	32.00%	56.66%	Frozen, thawed, homogenised, incubated, passed through muslin cloth, cooled to deactivate enzyme	2:1	Sonawane et al, 2020
Bitter gourd (*Momordica charantia L.)*	140	48.8	10.2 mL/kg pulp	Pectinase	59.00%	82.00%	Pulp homogenised, incubated, heated to		Deshaware et al, 2017

							deactivate enzyme, cooled, centrifuge 1699 g 20 min		
Pineapple (*Ananas comosus)*	448	46	2.40mg/50g pulp		70.20%	88.00%	Pulp homogenised, incubated, filtered through cheesecloth, heated to deactivate enzyme		Kumar & Sharma, 2012
Sohiong (*Prunus nepalensis)*	80	45	0.01-0.05% (w/w)	Endo-PG 3.2.1.15 3.5-7 U/mg	28.00%*	49.00%**	Pulp homogenised, incubated, pressed through cheesecloth and nylon filter	1:2	Vivek et al, 2018
Cashew apple (*Anacardium occidentale l.)*	60	30	0.01-0.1% (w/w)	Endo-PG 3.2.1.15 8000-12000 U/mg	77.46%*	80.74%**	Frozen, thawed, homogenised, incubated, cooled to deactivate enzyme, filtered through cloth		Abdullah et al, 2021
Chironji (*Buchanania lanzan*)	118	48.7	0.09% (w/w)	Endo-PG 3.2.1.15	53.86%	72.52%	Frozen, thawed, homogenised, incubated,	1:1	Pradhan et al, 2020

				8000-12000 U/g			cooled to deactivate enzyme, filtered through cloth		
Guava (*Psidium guajava L.)*	90	40	0.01-0.2% (w/w)	Pectinase	61.00%*	78.00%**	Pulp homogenised, incubation, heated to deactivate enzyme, cooled, centrifuge 3000g 10min	1:2	Surajbhan et al, 2012
	436.2	43.3	0.70mg/100g pulp	Pectinase 3.5-7 U/mg	37.43%	79.30%	n.a		Kaur et al, 2008
Palm (*Borassus flabellifer)*	120_{P+C} 108.5_{C} 171.32_{P}	55_{P+C} 60_{C} 33.75_{P}	$1:0.75\ (w/w)_{P:C}$ $0.30\%\ (w/w)_{C}$ $0.33\%\ (w/w)_{P}$	Endo-PG 3.2.1.15 8000-12000 U/g Cellulase 3-10 U/mg	68.40%	$87.90\%_{P+C}$ $81.90\%_{C}$ $80.00\%_{P}$	Pulp extracted, pasteurised 90°C 10min, incubation, heated to deactivate enzyme, cooled, pressed	(0.7-1.2):1	Mohanty et al, 2018
Chinese quince (*Chaenomeles sinensis)*	90	50	0.05:0.08% (P:C)	Pectinase 10,000 PECTU/g Cellulase 100 FBG/g	74.20%	90.40%	Cut into 3-5mm pieces, incubated, heated to deactivate enzyme, cooled, pressed	3:1	Jiao et al, 2020

Custard apple (*Annona squamosal L.)*	268.2	47	2.21% (w/w)	Endo-PG 3.2.1.15	49.00%	88.00%	Pulp homogenised, incubated, heated to deactivate enzyme, cooled, pressed	1:1	Bhatkar et al, 2021
Apple (*Malus domestica cv. Shampion)*	60	20	10-50mL/100kg	PME 3.7 PEU/g PL 19,400 PECTU/g	68.30%	77.00%	Fruit homogenised, incubated with enzyme, hydraulic press		Oszmianski et al, 2009
Soursop (*Annona muricata L.)*	180	35-40	0.05%(v/w)	Endo-PG 3.2.1.15	47.70%	67.70%	Frozen, thawed, homogenised, pasteurised, incubated, heated to deactivate enzyme, centrifuged 3000 rpm 15min, vacuum filtered	1:1	Yusof & Ibrahim, 1994
	67.5	45	0.015-0.095% (w/w)	Endo-PG 3.2.1.15 1.11 U/mg	71.67%	74.67%	Homogenised, frozen, thawed, incubated, heated to deactivate enzyme,	1:1	Makebe et al, 2020

							cooled, centrifuged 6000g 15min		
Indian blackberry (*Syzygium cuminii)*	40	40	0.01-0.10% (w/v)	Endo-PG 8000-12000 U/g	68.45%*	72.55%**	Frozen, thawed, homogenised, incubated, cooled to deactivate enzyme, filtered through muslin cloth		Ghosh et al, 2016
Kendu (*Diospyros melanoxylon Roxb.*)	30	37.5	0.01-0.10% (w/w)	Endo-PG 8000-12000 U/g	70.00%*	73.00%**	Frozen, thawed, homogenised, incubated, cooled to deactivate enzyme, filtered through muslin cloth	1:1	Panda et al, 2021
Banana *(Musa sp.)*	30	35	(0.1-0.3)g:(0.1-0.3)g/100g (P:C)	Endo-PG 1.7 U/mg Cellulase 3 U/mg	50.00%$_{(0.1:0.1\ P:C)}$	56.67%$_{(0.3:0.1\ P:C)}$ 58.00%$_{(0.1:0.3\ P:C)}$ 69.33%$_{(0.3:0.3\ P:C)}$	Homogenised, incubated, filtered through muslin cloth, pasteurised to deactivate enzyme	0.5:1	Handique et al, 2019

Grape (*Vitis sp.)* Pomegranate *(Punica sp.)*	180	50	3.5mg/20g pulp	Pectinase	60.00% 52.00%	79.00% 74.00%	Homogenised, pasteurised 85°C 3min, incubated, heated to deactivate enzyme, centrifuged 2000 rpm 10min		Nisha, 2016

Table 1-7: Examples of the effect of enzyme addition during the juice extraction process on total dissolved solids (TDS; °Brix). Where both pectinases and cellulases have been applied, the enzyme concentration is expressed as a ratio, whereby P = pectinase and C = cellulase.

Fruit/vegetable	Juice yield (%)		Total dissolved solids (TDS; °Brix)		Reference
	Control	Enzyme-treated	Control	Enzyme-treated	
Cashew apple (*Anacardium occidentale I.)*	77.46%	80.74%	11.33 ± 0.12	12.11 ± 0.01	Abdullah et al, 2021
Chironji (*Buchanania lanzan*)	53.86%	72.52%	13.43 ± 0.115	14.47 ± 0.057	Pradhan et al, 2020
Palm (*Borassus flabellifer)*	68.40%	87.90%$_{P+C}$	10.90 ± 0.15	12.80 ± 0.05	Mohanty et al, 2018
		81.90%$_{C}$		10.30 ± 0.05	
		80.00%$_{P}$		10.20 ± 0.05	
Soursop (*Annona muricata L.)*	71.67%	74.67%	7.10 ± 0.14	7.40 ± 0.00	Makebe et al, 2020
Indian blackberry (*Syzygium cuminii)*	68.45%	72.55%	13.00	13.20	Ghosh et al, 2016
Kendu (*Diospyros melanoxylon Roxb*.)	70.00%	73.00%	10.30	9.40	Panda et al, 2021
Banana *(Musa sp.)*	50.00% $_{(0.1:0.1\ P:C)}$	56.67%$_{(0.3:0.1\ P:C)}$	11.10	11.20	Handique et al, 2019
		58.00%$_{(0.1:0.3\ P:C)}$		10.90	
		69.33%$_{(0.3:0.3\ P:C)}$		11.30	

Enzyme production and application is particularly attractive within an integrated biorefinery context. Food waste such as orange pomace and coffee pulp have been used for the production of pectinases in submerged and solid-state systems as the pectin in these streams induce pectinase production in species such as *Aspergillus,* with higher pectin content resulting in more pectinase production (Antier et al, 1993; Maldonado & De Saad, 1998). Mahmoodi et al. (2017) successfully produced pectinase from orange pomace and subsequently demonstrated that the pectinases could improve yield of extracted apple juice and its sugar concentration. As sugar beet pulp has a relatively high pectin content of 15-30% dry weight basis (Yapo et al, 2007), it has also been used to produce pectinases, cellulases and hemicellulases (El-Batal et al, 2013; Puligundla & Mok, 2021). Suhaili et al (2019) have also demonstrated the utilisation of sugar beet vinasse as an inexpensive feedstock for CV2025 ω-transaminase production in *E. coli* within an integrated biorefinery context.

1.5.4 Application of enzymes in sugar beet processing for sucrose extraction

To date, there is a wealth of knowledge regarding the application of enzymes on sugar beets and sugar beet pulp for a range of valorisation strategies, such as for bioethanol production, prebiotic potential, therapeutic applications and biopolymer formation (Zhang et al, 2016; Zicari, 2016; Cardenas-Fernandez et al, 2017; Tomaszewska et al, 2018; Puligundla & Mok, 2021; Usmani et al, 2022). However, literature on the application of enzymes to specifically improve the extraction of sucrose from sugar beets is very limited. Only Ovsyannikov et al. (2021) seem to have reported the use of enzymes in this context. Specifically, the authors propose a new sugar beet processing scheme whereby the crushed sugar beet is incubated with a commercial hemicellulase mixture (ViscoStar 150L; Dyadic International, Florida, USA) for 20 min at 60°C with a 5% w/w solution of enzyme followed by pressing, resulting in increased juice yields prior to a smaller-scale traditional diffusion extraction to extract the residual sucrose. The authors claim this process will enable reduction in heat and energy consumption, as the yields of the combined pre-pressing and small-scale diffusion are comparable to a single large-scale diffusion operation. However, the applicability of this method on an industrial scale is yet to be seen.

Although academic literature regarding enzyme addition in the sugar beet extraction process is sparse, there have been several industrial trials to investigate this approach. Novozymes A/S (Copenhagen, Denmark) recently submitted a patent

(WO2020002574A1) for the addition of pectin lyase to the sugar beet extraction process, claiming that this could improve the sugar yield, reduce the amount of sugar lost to molasses, or increase the amount of dry substance in the beet pulp, indicating more juice has been extracted.

Specifically, the patent holders measured this by exposing thawed cossettes to the enzymes and incubating the mixture in an orbital shaking incubator. After incubation, the mixture was filtered and the filtration rate and TDS of the filtrate was measured. As pectin lyase concentration increases, the filtration rate decreased significantly and the TDS of the filtrate increased marginally, which, according to the authors, suggests a higher sugar content in the filtrate.

As these trials are not conducted in the industrial diffusion process itself, the conditions evaluated are very different. For example, thawed cossettes are used as opposed to fresh cossettes, which would greatly affect the diffusion properties of the beet as freeze-thawing causes significant rupture of the cell wall and membrane prior to extraction (Pearce, 2001). Furthermore, the incubation step is conducted as a separate, optimised processing step to juice extraction. In an industrial diffuser, the enzyme incubation would be occurring concurrently with the juice extraction stage and is therefore subject to the conditions of the diffusion process. Despite this, there does appear to be some indication of a positive benefit when applying enzymes to aid sucrose extraction from sugar beet.

1.6 Methods for analysis of sugars from sugar beets

There are a multitude of techniques available for the quantification of sucrose, ranging from the relatively simple, such as drying and weighing of sugar solutions, to complex methods, such as Fourier Transform Nuclear Magnetic Resonance spectroscopy (FT-NMR). However, not all of these techniques are applicable to the industrial analysis of sucrose from sugar beet. This section reviews the current methods used in industry to analyse sucrose from sugar beets and the streams produced during sugar beet processing in order to determine the most suitable techniques for use in this research.

1.6.1 Drying and weighing of sugar solutions

Historically, drying of sugar solutions was used as a simple yet acceptable method of determining approximate sucrose concentrations in sugar beet juice (Browne, 1912). A known volume of a sugar solution is dried in a 100°C oven to constant weight and

the remaining solids are weighed to calculate the percent solids (w/w) in the solution. Although this does correlate with sucrose concentration, it is not a direct measure of sucrose due to the abundance of impurities that remain post-drying and as such, is no longer commonly used in the sugar industry.

1.6.2 Degrees Brix (°Brix)

In 1843, Czech chemist Carl Napoleon Balling devised a method of measuring the dry substance of a sugar solution using a hydrometer, called the Balling scale. German chemist Adolf Brix later corrected some errors in the Balling scale and modified it by establishing a series of tables correlating known concentrations of pure sucrose with the specific gravities of the solutions at a given temperature, hence the unit degrees Brix (ºBrix). As such, when ºBrix is used to measure sucrose, it is reported as a weight percentage, whereby 1 ºBrix is equivalent to 1g of sucrose in 100g solution. At the time, this was proposed for the measurement of wort sugars in beer fermentations but its applications have since expanded and it is now commonly used in many industries, and specifically in the sugar processing industry.

Traditionally, this was measured using a manual hydrometer or pycnometer, which compares the density of the sample solution to a standard reference. Although this is a perfectly valid, inexpensive way to measure ºBrix, it has several disadvantages including subjectivity, precise temperature control and relatively high volumes of sample required (25-600mL). Digital hydrometers and pycnometers are now available which overcome these issues.

An alternative method of measuring ºBrix is via Refractive Index (RI), i.e. the extent to which the dissolved solids in a solution refract a reference wavelength of the sodium D line (589.3nm) when the ray of light passes from the gas phase to the liquid phase. As with specific gravity, there is a direct relationship between the refractive index and ºBrix so either method can be used to measure sucrose concentration. As with hydrometers and pycnometers, digital refractometers are available which overcome subjectivity and temperature control issues. In summary, both specific density and refractometry are valid ways to measure ºBrix. When measuring a pure solution of sucrose both methods will report an identical ºBrix. In the sugar industry, refractometry is currently the most commonly used method for measuring ºBrix.

The main disadvantage of ºBrix, regardless of the method used to measure it, is that the non-sucrose dissolved solids, such as proteins, pectins and non-sucrose sugars, have contrasting effects on the output. If a solution of pure sucrose is analysed, then ºBrix is equivalent to the exact dry substance, that is, a 15 ºBrix solution contains 15% (w/w) sucrose. However, in an impure solution, the non-sucrose solids will have varying influences on the density or refractive index of the solution. For example, glucose and fructose will decrease refractive index and density, whilst raffinose will cause an increase (Ali et al, 2014). This is the case for sugar beet analysis, as the samples are never pure sucrose solutions. In this scenario, ºBrix is equivalent to an approximation of the dry substance. Despite this, ºBrix is still used over the drying method of determining dry substance (Section 1.6.1) due to the significant reduction in time and energy.

1.6.3 Polarimetry

Polarimetry is one of the most commonly used techniques in the sugar industry due to the chiral nature of sugars. When plane-polarised light passes through an optically-active solution it will rotate left (levorotation) or right (dextrorotation) based on the nature of the solutes. The extent of the rotation is the optical rotation (°), often referred to as degree of polarisation (pol). Every chiral molecule has a different degree of polarisation at a given temperature, referred to as the specific rotation ($[\alpha]_{\lambda}^{\theta}$) (deg dm^{-1} cm^3 g^{-1}), where λ refers to the wavelength of light (nm) and θ is the temperature (°C). Biot's law summarises the relationship between optical rotation and specific rotation, where additional variables l represents the length of the polarimeter tube (mm), and c represents the concentration of the sample (g mL^{-1}) (Equation 1.1).

$$\alpha = \frac{[\alpha]_{\lambda}^{\theta} \times l \times c}{100} \qquad \text{(Equation 1.1)}$$

In the particular example of sugars, specific rotation is often reported in literature as $[\alpha]_D^{20}$, where D refers to the sodium D line at 589nm, and 20 is the temperature in degrees Celsius (Haynes, 2014). Polarimetry may be considered similar to refractometry, but the main distinguishing factor is that the chiral property of sugars ensures that polarimetry is not affected by non-sugar, non-chiral components.

The International Commission for Uniform Methods of Sugar Analysis (ICUMSA) have standardised several methods in which the mass percentage of sucrose can be calculated from sugar beet via polarimetry. The °Z scale is a linear scale that defines the optical rotation of a normal solution of pure sucrose, where a normal solution of sucrose is equal to 26.016 g of pure sucrose dissolved in water at 20°C to a final volume of 100 mL. 100 °Z at 20°C, 589 nm and a 200 mm polarimeter tube corresponds to an optical rotation of 34.626 (OJEC, 1979). From this, it is possible to convert an optical rotation reading to a relative sucrose concentration by the following equation, where Pol is the direct optical rotation reading from the polarimeter:

$$°Z = \frac{100}{34.626} \times Pol \ = 2.889 \times Pol \qquad \text{(Equation 1.2)}$$

Some modern polarimeters designed specifically for use in the sugar industry and dubbed saccharimeters, report polarimetry directly as °Z and convert this into percent sucrose, reducing the need for further calculations. Furthermore, ICUMSA have developed several methods for the analysis of sucrose from various sugar beet sources, including the raw sugar beet root, raw juice, wet pulp and pressed pulp (Asadi, 2006). Polarimetry can therefore be used to measure the sugar content from root to refined sugar, providing a rapid means of assessing relative sucrose extraction at each processing stage.

In order to polarise a sample at 589nm the sample must be sufficiently transparent so that it does not absorb the incoming light beam. Raw sugar beet juice is typically a dark grey-black liquid and therefore needs to be clarified prior to analysis. This was traditionally achieved by mixing the sample with lead acetate followed by filtration (ICUMSA GS6-1). However, due to the environmental and health concerns with using lead, alternative clarifying reagents have been introduced, e.g. aluminium sulphate (ICUMSA GS6-3) and proprietary non-hazardous, biodegradable mixtures such as Octapol®. Recent developments in polarimeter technology enable the analysis of dark, unclarified samples using near-infrared light at 882nm (Player et al, 2000; van Staden & Mdlalose, 2000; ICUMSA GS1/2/3-2).

In addition to removing impurities responsible for the dark colouration of sugar beet juice samples, clarifying reagents have an additional purpose of removing interfering

optically active substances, such as non-sucrose sugars. Table 1-8 summarises the specific rotation of sugars that may be found in sugar beet. Given the levorotatory nature of fructose, an impure solution of sucrose with a significant concentration of fructose will present a lower optical rotation reading, resulting in inaccurate quantification of sucrose concentration as the ICUMSA method is calibrated to a solution of pure sucrose. Conversely, a solution of sucrose containing significant concentrations of raffinose will have a much higher optical rotation reading.

Table 1-8: Specific rotations of various sugars found in sugar beet (Asadi, 2006)

Sugar	**Specific rotation at 20°C $[\alpha]_D^{20}$ (deg dm^{-1} cm^3 g^{-1})**
Sucrose	+66.5 ± 1
D-Glucose	+52.7
D-Fructose	-92.4
D-Arabinose	+104.5
Raffinose	+123.2

Lead acetate is able to remove some, but not all, glucose and fructose (Englis & Tsang, 1922; Meade & Chen, 1977), but aluminium sulphate does not precipitate raffinose (Asadi, 2006). Similarly, Octapol® does not precipitate non-sucrose sugars as determine via HPAEC-PAD analysis. Due to this, sucrose concentrations calculated via polarimetry are often defined as approximate sucrose content.

A method to measure the exact sucrose content via polarimetry based upon the inversion of sucrose to invert sugars is available but this is only typically applied in molasses analysis (Asadi, 2006). This is because the inversion method takes significantly longer to prepare than the approximate sucrose method. Furthermore, the concentrations of glucose, fructose and raffinose relative to sucrose in the beet processing stages prior to evaporative concentration are extremely low so the effect of these non-sucrose sugars on the polarimetric output is insignificant. In later processing stages such as molasses preparation, where the sucrose purity is lower due to a lower volume and higher concentration of invert sugars, polarimetry may not be the most appropriate technique.

The speed and relative accuracy of polarimetry are the key reasons why this technique is still routinely used for sugar analysis in the sugar industry. However, it is susceptible to some inaccuracy due to the presence of non-sucrose sugars.

1.6.4 Enzyme-based assays

Enzyme-based assays are an alternative method of analysing sucrose based on the principle of enzymatically converting sucrose to a spectroscopically detectable compound, and these are often readily available from suppliers. For example, the sucrose assay kit available from Sigma-Aldrich uses invertase to convert sucrose to glucose and fructose, followed by conversion of glucose to 6-phosphogluconate by hexokinase and glucose-6-phosphate dehydrogenase. This reaction results in the reduction of NAD^+ to NADH, which can be measured at 340 nm. Due to the stoichiometry of this reaction, the increase in absorbance at 340 nm is directly proportional to the initial sucrose concentration when compared against a calibration curve (Bergmeyer & Bernt, 1974). Furthermore, unlike polarimetry, enzymatic controls for baseline glucose prior to invertase activity can be prepared, enabling the measurement of true sucrose concentration.

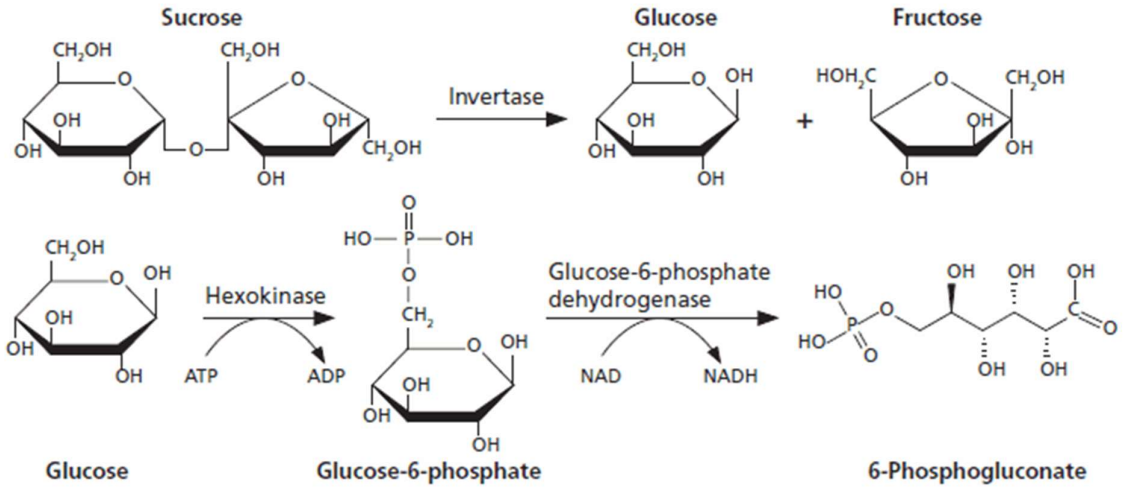

Figure 1.15: Enzymatic assay for sucrose concentration based on the conversion of sucrose to glucose and fructose via Invertase. Glucose is then phosphorylated to Glucose-6-phosphate via Hexokinase, followed by subsequent dehydrogenation to 6-Phosphogluconate by Glucose-6-phosphate dehydrogenase. The final reaction is facilitated by a reduction of NAD to NADH, which can be measured spectroscopy at 340 nm (Figure reproduced from Sigma-Aldrich, USA).

In comparison to refractometry and polarimetry, enzyme assays sacrifice time for potentially higher sensitivity and precision when identifying the exact sucrose concentration. To overcome this, some enzyme assays have been successfully optimised for microwell plate formats enabling high-throughput, parallel analysis of numerous samples, with the added potential of a fully automated assay platform (Campbell et al, 1999; Trebbi & McGrath, 2004; Vermeir et al, 2007).

Despite these benefits, enzyme-based assays for measuring sucrose have not been widely applied in the sugar beet industry. There are some concerns with regards to the accuracy of some enzyme-based assays in sugar beet roots with higher sucrose content, suggesting some potential biochemical interference that may need to be considered (Trebbi & McGrath, 2004). Furthermore, the skill required to prepare and perform enzyme-based assays in a robust, repeatable manner is significantly greater than that required for refractometry and polarimetry.

An additional factor as to why enzyme-based assays have not been widely adopted in industry may be due to the costs of enzyme assay kits. Although the initial costs of these kits are significantly less than equipment required for other techniques, they are expensive relative to the number of samples that can be analysed per kit. Finally, although enzyme-based assays for the determination of sucrose are relatively routine in a lab environment, there are no official documented methods supported by ICUMSA, hence the prevalence of alternative, standardised methods such as refractometry and polarimetry.

1.6.5 Chromatography

Analytical chromatography is a highly sensitive and specific technique based on the separation of a mixture according to the properties and interactions of the various constituents on the stationary phase. Gas chromatography and high-performance liquid chromatography have both been applied for the analysis of sucrose in the sugar industry.

Gas chromatography was the first chromatographic method routinely applied to the sugar beet industry. The initial step in gas chromatography requires the conversion of the sucrose mixture to a volatile derivative, often via trimethylsilylation of the hydroxyl moieties of sucrose (Walford et al, 2004). The sample is then carried through the stationary phase via an inert gas mobile phase and the components are separated

according to their interactions with the stationary phase. Detection of the separated compounds is mostly commonly achieved via flame ionisation, but mass spectrometry and thermal conductivity detectors have also been used (McGrath & Fugate, 2012).

Gas chromatography has been used in both the sugar beet and sugar cane industry with methods for both supported by ICUMSA (ICUMSA GS4/7/8/5-2; ICUMSA GS7/4/3-22) (Schaffler & Day-Lewis, 1983; Walford et al, 2004). Due to the specificity and sensitivity of gas chromatography, it is able to provide a true sucrose concentration unimpaired by impurities (Schoonees, 2003). Furthermore, this method can be used to quantify other sugars in parallel to sucrose quantification, which is useful for determining quality and purity of the sugar beet streams in a single assay (Long & Chism, 1987).

Limitations of gas chromatography include expensive initial capital and maintenance costs, long analysis time per sample, extensive method development and the requirement for derivatisation before analysis can be performed. Derivatisation reagents are often toxic and the addition of an additional step prior to analysis provides room for potential handling errors, which may affect the final quantification.

High-performance liquid chromatography and more specifically, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is an alternative chromatographic technique used to analyse and evaluate carbohydrates (Corradini et al, 2012). This technique is based on the principle that carbohydrates are weak acids, with pKa values in the range of 12-14. As such, these carbohydrates become oxyanions at high pH and are easily separated using an anion-exchange column without the need for prior sample derivatisation. Quantification can then be achieved by comparing the peak area with a prepared calibration standard.

As with gas chromatography, HPAEC-PAD is a highly sensitive and selective technique for the analysis of carbohydrates, requiring little volume of sample for analysis and relatively simple sample preparation. For these reasons, HPAEC-PAD has been commonly applied in the analysis of carbohydrate profiles in sugar beet pulp primarily within the research context of improving the valorisation of sugar beet pulp due to its relatively high arabinose content (Martinez et al, 2009; Zaidel & Norulfairuz, 2012; Cardenas-Fernandez et al, 2017). However, its use in the sugar industry for the

quantification of sucrose and other carbohydrates is not as widespread, despite methods supported by ICUMSA (GS7/8/4-24; GS1/2/3-4;)

Similarly to gas chromatography, this is largely due to the high costs and long analysis times associated with chromatographic equipment. An additional limitation of HPAEC-PAD for the analysis of sucrose in sugar beet juice is the need for large dilution factors which may result in significant quantification errors. This is particularly problematic when quantifying other sugars simultaneously with sucrose. Sugar beet is approximately 16.5% (w/w) sucrose, 0.1% (w/w) glucose and 0.05% (w/w) fructose, or approximately 0.3-0.6 g glucose and fructose for every 100 g of sucrose (Cooke & Scott, 2011; Schiweck et al, 2012). Diluting sucrose to appropriate levels for detection will also dilute glucose and fructose to trace levels, rendering it difficult to accurately quantify these sugars. As a result, HPLC/HPAEC-PAD may not be the most accurate method of measuring invert sugar content in sucrose-rich samples. Instead, ICUMSA recommends HPLC for the analysis of high impurity samples such as molasses, in which the concentration of non-sucrose sugars are within the same order of magnitude as sucrose (GS4/8-19; GS7/4/8-23)

1.6.6 Near-infrared (NIR) spectroscopy

NIR spectroscopy for the purpose of sucrose analysis from sugar beets is a comparatively recent innovation, using near-infrared wavelengths (780-2500 nm) combined with multivariate chemometric analysis to non-destructively predict polarisation, ºBrix and sucrose concentration from sugar beet (Pan et al, 2015a; Bahrami et al, 2019).

The principle of NIR spectroscopy is based on the tendency of hydrogen atoms to vibrate within this wavelength range (Bahrami et al, 2019). Sugars are particularly rich in O-H and C-H bonds, resulting in predictable NIR spectra. Chemometric analyses such as spectral pre-processing and regression analysis can then be applied to convert the spectral data into quantitative predictions in order to ascertain details on the sample attributes (Small, 2006).

The advantages of NIR spectroscopy include rapid multicomponent physical and chemical analysis in single samples (Alander et al, 2013). In comparison to polarimetry and chromatographic methods, NIR spectroscopy has the potential to provide more

information exceeding carbohydrate analysis, including fat, ash and protein content, moisture content and acidity (Alander et al, 2013; Wang et al, 2015).

Furthermore, samples do not require significant sample preparation. As such, NIR spectroscopy has been successfully applied to sugar beet roots (Pan et al, 2015a; Babaee et al, 2019) and sugar beet juices (Bahrami et al, 2019) with <1% standard error of predictions when compared to °Brix, polarimetry and HPLC. Pan et al (2015b) have further demonstrated the potential of combined Vis-NIR covering the 400-2500 nm wavelength to measure sucrose content from unsliced sugar beet roots with 1.02% standard error of prediction using a portable spectrometer. This has particularly attractive applications for the sugar beet industry as it enables farmers and factories to assess the critical quality attributes of sugar beets prior to further processing, potentially replacing polarimetry-based payment methods in the future (McGrath & Fugate, 2012).

The main limitation for NIR spectroscopy is the requirement of chemometric analyses in order to extract relevant, useful data. Given the multitude of chemometric analyses available and the relative novelty of NIR spectroscopy there is no standard method for sucrose analysis in sugar beet. As such, rigorous trial and error is the main way to determination which analysis is most suitable, which may offset the initial simplicity of NIR spectroscopy (Jamshidi et al, 2015; Bahrami et al, 2019).

1.6.7 Analytical method selection

Although numerous methods are available for the analysis of sucrose, a summary of which are detailed in Table 1-9, refractometry and polarimetry see widespread use within the sugar beet industry, with some instances of HPAEC-PAD and gas chromatography in some beet and cane factories (Schaffler and Day-Lewis, 1983; Asadi, 2006). This is largely attributed to the relative simplicity of refractometry and polarimetry whilst also providing very reliable estimations of sucrose content.

Although these estimations are not as precise when compared to advanced technologies such as chromatography, the rapidity, ease of handling and simple sample preparation of refractometry and polarimetry outweigh that of enzyme-based sucrose detection and chromatography.

Due to the high proportion of sucrose in beet samples prior to evaporation, polarimetry has been demonstrated to be an excellent technique for sucrose measurement. Bahrami et al (2019) analysed 130 samples of sugar beet raw juice and found that the average pol/sucrose ratio was 101.61%, this increase due to the contributing dextrorotation of glucose and other sugars.

NIR spectroscopy has great potential due to its non-destructive, portable and rapid capabilities, essentially combining the precision of chromatography with the simplicity of refractometry and polarimetry. Its potential for in-depth analyses of sugar beet parameters outside of carbohydrate analysis is particularly attractive as these are additional indications of sugar beet quality that should be monitored in order to further optimise sugar beet processing.

However, NIR spectroscopy is still far from becoming widely accepted within the sugar beet industry due to the need for complex chemometric analyses. These will need to be assessed and evaluated for robustness before widespread acceptance of NIR spectroscopy as a valid method of sucrose analysis in the sugar beet industry.

For these reasons, refractometry and polarimetry remain the most established techniques in the sugar beet industry, supported by ICUMSA with several detailed methods for the specific analysis of various sugar beet fractions. As such, refractometry and polarimetry will be the primary methods of analysis in this study, with supplementary HPAEC-PAD analysis where applicable.

Table 1-9: Summary of analytical techniques for sugar beet and sucrose analysis.

Technique	Advantages	Disadvantages
Refractometry	• Requires very little sample volume (µL-mL) • Accurate measurement of soluble solids	• Measures all soluble solids, including pectin, proteins and non-sucrose sugars
Polarimetry	• Highly accurate estimation of sucrose when measuring samples with high proportions of sucrose (e.g. pre-molasses beet samples) • Rapid analysis • New polarimeters can measure unclarified samples at 880nm • Supported and validated by ICUMSA	• Only provides an approximate sucrose concentration due to non-sucrose optically active components. Exact sucrose concentration can be measured via a time-consuming inversion method • Requires some sample preparation via clarifying reagent and subsequent filtering
Enzyme-based spectroscopy	• Sensitive method for quantifying sucrose • Adaptation of assays to microwell plate formats enable high-throughput and automation potential	• Relatively expensive if analysing large number of samples due to expense cost of enzyme kits • Requires technical expertise and knowledge of enzyme catalysis, may be less robust if not performed correctly
Chromatography	• Highly sensitive method for separation and quantification of sucrose and other sugars • Very low volumes of sample required (µL)	• Expensive equipment and maintenance costs • Long analysis times • Gas chromatography: requires derivatisation prior to analysis which uses hazardous chemicals • HPAEC-PAD: Significant dilution errors when diluting high concentrations of sucrose. May dilute non-sucrose sugars outside of detectable limit due to the proportion of sucrose to non-sucrose sugars in sugar beet • Extensive method development necessary
NIR Spectroscopy	• Minimal sample preparation required, potential for analysis of unsliced sugar beets • Predicts a wide range of parameters including physical and chemical features, e.g. sugars, moisture content, ash, fat, protein content with relatively low standard error of prediction • Rapid analysis, portable	• Requires extensive mathematical pre-processing and chemometric analysis to produce an empirical calibration model to transform spectral data into meaningful sugar beet analysis • Current methods of chemometric analysis involves a rigorous trial and error approach to identify appropriate analysis • Novelty of technique results in further validation necessary prior to acceptance in sugar beet industry

1.7 Critical evaluation of the present literature

Within the published literature, there is little research regarding optimisation of the sugar beet diffusion process. The most successful approach to date has been the application of electroporation (Section 1.4.2.2). Pilot-scale studies have been successfully conducted, demonstrating sucrose extraction and enabling low-temperature diffusion to be performed. For example, a similar level of sucrose extraction was obtained at 30°C with beet that had undergone electroporation versus at 60°C with untreated beet. This has the potential to significantly reduce the energy requirement for the diffusion process. However, the approach is hindered due to issues around homogenous electroporation quality when applied at full industrial scale. The effect of electroporation on the entire processing chain is also unclear. Furthermore, pilot trials have incorporated electroporation as a discrete processing step prior to diffusion. This will result in significant capital investment and an overhaul of the existing process to integrate this step.

As mentioned in Section 1.5.3, the use of macerating enzymes, such as cellulases and pectinases, to facilitate valorisation of sugar beet pulp to high-value products has also been described. However, the use of these enzymes for the specific purpose of enhancing sucrose extraction from sugar beet cossettes, without the implementation of a new processing step, has not been investigated. Similar enzymes have been used to enhance juice extraction from various fruit and vegetables, however this has primarily been applied as a separate enzyme incubation step prior to pressing of the biomass pulp. Ovsyannikov et al (2021) proposed a scheme in which crushed sugar beet was incubated with a commercial enzyme mixture prior to pressing and subsequent diffusion to extract residual sucrose. This has similar disadvantages to the application of electroporation as it requires an overhaul of the existing process chain.

The addition of enzymes directly to the diffusion process appears to have several advantages, of which the most significant is the simple integration into the existing refinery process. This would overcome the need for additional process steps and could be used within current diffusion processes with little modification to existing infrastructure. However, this has not been trialled in academia before and therefore there are numerous unknowns such as the enzyme activity or activities required, enzyme stability, optimum enzyme concentrations, point of addition to the diffuser, cossette recalcitrance etc.

A small number of industrial trials on enzyme-enhanced diffusion processes have been conducted with varying degrees of success. However, the trials have not been systematic and hence the results are inconclusive or difficult to interpret. This is due to restrictions around the modification of existing industrial equipment and the need to limit potential detrimental changes to product quality. For instance, enzymes may be added to one diffuser but not to another in a single processing facility. However, as established in Section 1.3.2.1, different types of diffusers operate very differently in terms of mixing, flow regime, retention time, microbial contamination and cossette oxidation. Therefore, positive benefits attributed to enzyme addition may be observed in one diffuser but may not result in the same effect when trialled in another type of diffuser. There is thus a need for a systematic and methodical study in which the addition of enzymes can be trialled in order to produce a conclusive understanding of the effect of enzymes on the diffusion process.

As highlighted in Section 1.3.2, the existing diffusion process is efficient, extracting up to 98% of sucrose from sugar beet cossettes. The addition of enzymes to the diffusion process could further optimise the process by increasing sucrose extraction or reducing the water required to extract the same amount of sucrose. This would need to offset the upfront cost of enzyme addition to be considered a benefit. However, this thesis focuses on investigating the potential for enhanced sucrose extraction by the addition of enzymes to the sugar beet diffusion process, establishing an experimental foundation for future economic analyses.

1.8 Aim and objectives

It is considered that enzymes, such as those described in Section 1.5, have the potential to enhance sucrose extraction during the sugar beet diffusion process (Section 1.5.4). Based on the critical analysis presented in Section 1.7, the **aim** of this thesis is to evaluate whether enzymes, particularly pectinases and cellulases, have the potential to enhance sucrose extraction from sugar beets when applied to existing diffusion processes. In order to achieve this aim, there are several key **objectives** as described below.

- A number of enzymes are available commercially in quantities appropriate for the scale of industrial sugar beet diffusion. However, these commercial enzymes commonly harbour a mixture of various activities and often have

optimum activities outside of diffusion process conditions. The first objective is thus to establish the necessary enzyme assays for activity analysis of several commercial pectinases and cellulases. These will be used to quantify the activities present in the commercial enzyme preparations, establish optimum operating conditions and hence evaluate their suitability for application within the sugar beet diffusion process. The results of these characterisation studies are presented in Chapter 3.

- Following the characterisation studies, the application of selected enzymes for enhanced sucrose release from sugar beet will be evaluated. In order to achieve this, the second objective will be to develop a scale-down diffuser model in which the diffusion process can be operated at a benchtop scale in a manner that better mimics industrial scale conditions. The initial model will be based on co-current operation to enable rapid assessment of the potential benefit of enzyme addition. Additionally, the effect of sugar beet recalcitrance will also be examined via a series of pre-treatment operations. This work is described in Chapter 4.
- The third objective will be to design and construct an improved diffuser model that more closely replicates operation of industrial scale diffusers. Specifically, this will allow operation in a counter-current manner and enable evaluation of key diffuser operating parameters including draft ratio, point of enzyme addition and pressing. The results from this work are also presented in Chapter 4 and will enable quantitative evaluation of any benefits arising from an enzyme-enhanced diffusion process.
- The final objective will be to explore the application of a novel, thermostable enzyme more suited for industrial diffuser operating conditions. This will involve the expression of a novel thermostable pectate lyase and characterisation of its activity based on the assays and methods established in Chapter 3. The novel enzyme will then be applied to the diffuser models constructed in Chapter 4 in order to quantify the potential benefits of using a thermostable enzyme adapted for industrial diffuser operating conditions. These results of this work are presented in Chapter 5.

In addition to the above chapters, Chapter 2 will describe the key experimental methods and processes used in this study and will provide detail on the design of the

final laboratory scale diffuser model and its operation. Finally, general conclusions arising from this research and suggestions for future work will be presented in Chapter 6.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this work were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated and were of the highest purity available. Milli-Q water was used for all experimental procedures.

2.1.2 Commercial enzymes

Viscozyme-L® (Novozymes A/S, Bagsværd, Denmark) was purchased from Sigma-Aldrich. P743L, C013L and Depol 793L were kindly provided by Biocatalysts Ltd (Cardiff, UK). Pectinex Smash XXL (Novozymes), Rohapect SY+ and Rohament CL (AB Enzymes, Darmstadt, Germany) were kindly provided by British Sugar Ltd (Peterborough, UK). Detailed information regarding declared enzyme activities is extracted from the data sheets provided by the suppliers and presented in Table 3-1.

2.1.3 Sugar beet

Sugar beet (*Beta vulgaris)* was provided by British Sugar Ltd (Peterborough, UK) on a regular basis between March 2018 and September 2021 to maintain a fresh supply of sugar beets. On receipt, sugar beets were washed with tap water to remove any superficial debris, dried and stored in a 4°C cold room to prolong shelf life prior to use. To minimise sucrose losses as described in Section 1.3.1, sugar beets were used within 2 months of receipt.

2.2 Enzyme assays

2.2.1 Polygalacturonase assay

The polygalacturonase assay is based on the 3,5-dinitrosalicylic acid (DNSA) assay for reducing sugars (Miller, 1959) as shown in Figure 2.1. The DNS reagent was prepared by dissolving 10 g of 3,5-dinitrosalicylic acid in 500 mL of Milli-Q water on a magnetic hot plate stirrer. 300 g of sodium potassium tartrate tetrahydrate was slowly added under continuous agitation until fully dissolved, after which 200 mL of 2 N sodium hydroxide was added. The solution was then diluted to a final volume of 1 L using additional Milli-Q water and stored in an amber glass bottle until use.

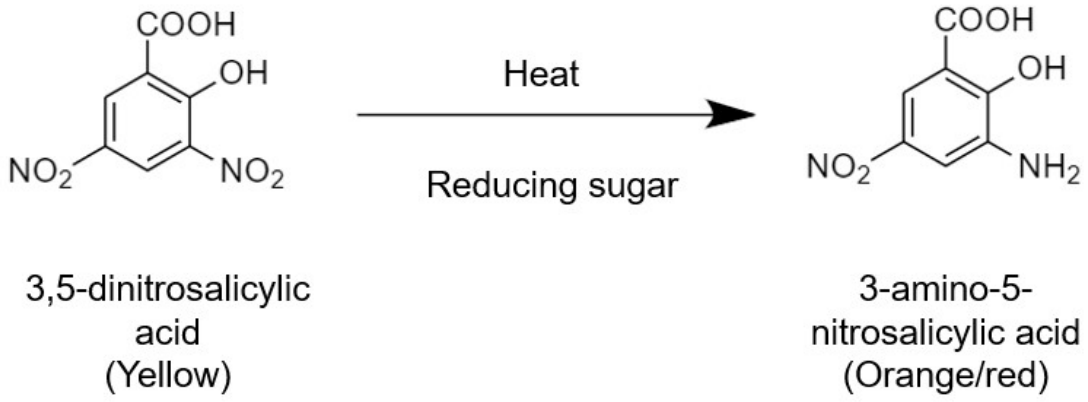

Figure 2.1: Reaction schematic for the 3,5-dinitrosalicyclic acid assay (DNSA). 3,5-DNS is yellow but upon heating in the presence of reducing sugars it reduces to 3-amino-5-nitrosalicylic acid. This can be measured via spectrophotometry at 540 nm and can be correlated to reducing sugar concentration via a standard curve.

Polygalacturonase activity was assessed using 1 g/L polygalacturonic acid as the standard substrate. 0.2 mL of diluted enzyme was added to 1.8 mL of polygalacturonic acid prepared in buffer according to the assay conditions. Samples were incubated for 10 min followed by the addition of 3 mL of the DNS reagent to terminate the enzyme activity and initiate colour development. Samples were boiled for 5 min then cooled to room temperature. Samples were then transferred into polystyrene cuvettes, diluted appropriately with Milli-Q water and the optical density was measured at 540 nm using a Jenway 7315 spectrophotometer (Cole-Parmer, Staffordshire, UK). Substrate blanks were prepared by substituting the enzyme with 0.2 mL of buffer. Enzyme blanks were prepared by replacing the substrate with 1.8 mL of buffer.

The enzymatic activity was calculated according to Equation 2.1, where D-galacturonic acid concentration was derived from a standard curve prepared with D-galacturonic acid (Figure A1.1), V_t and V_e represent the total reaction volume and enzyme volume respectively, DF is the dilution factor, t is the reaction time in min and MW_{GalAc} is the molecular weight of D-galacturonic acid, or 194.139 g/mol.

$$\frac{U}{ml} = \frac{(GalAc\ concentration)(V_t)(DF)}{(t)(V_e)(MW_{GalAc})} \times 1000$$

(Equation 2.1)

One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 μmol of D-galacturonic acid per minute under the reaction conditions.

2.2.2 Pectin and pectate lyase assay

The pectin/pectate lyase assay is based on the method proposed by Albersheim (1966). A 0.5% (w/v) citrus pectin (>74% galacturonic acid, dry basis) solution (Sigma-Aldrich) or sugar beet pectin (CP Kelco ApS, Denmark) was prepared by adding 5 mL of 99.5% ethanol to 1.25 g of pectin. This mixture was stirred with a magnetic stirrer until a homogenous mass was formed. Whilst stirring, 200 mL of pH 5.5 McIlvaine buffer was gradually added to the mixture over 10 min. The solution was continually stirred for at least 30 min taking care to avoid foam formation, after which the solution completed to total volume of 250 mL with buffer and refrigerated at 4 °C overnight. Following overnight incubation, the solution was centrifuged at 12,000 rpm for 20 min. The resulting supernatant was collected and refrigerated for up to 3 days or frozen at -20 °C.

The reaction mixture was prepared by adding 100 μL of enzyme diluted to a suitable concentration to 900 μL of 0.5% (w/v) pectin. The reaction mixture was incubated at temperatures between 20 °C and 90 °C at 800 rpm agitation (Thermomixer C, Eppendorf Ltd, Stevenage, UK) for 15 min. The reaction mixture was then incubated at 100 °C for 15 min to terminate enzyme activity. Enzyme activity was measured via spectrophotometry using a Jenway 7315 spectrophotometer due to the formation of 4,5 unsaturated galacturonide products which absorb light at 235 nm as a result of the conjugated double bond present at the C-6 carbonyl (Albersheim, 1966). Substrate blanks were prepared by substituting the enzyme with 100 μL of McIlvaine buffer. Similarly, enzyme blanks were prepared by substituting the pectin with 900 μL of McIlvaine buffer.

Lyase activity units were calculated according to Equation 2.2, where ΔAbs is the change in absorbance at 235nm, t is the reaction time, $\varepsilon_{product}$ is the extinction coefficient for the 4,5-unsaturated galacturonide product predetermined as 5500 M^{-1} cm^{-1} (Kester & Visser, 1994), d is the path length, V_t and V_e represent the total reaction volume and enzyme volume respectively and DF is the dilution factor.

$$\frac{U}{ml} = \frac{\Delta Abs}{t} \times \frac{1}{\varepsilon_{product} d} \times \frac{V_t}{V_e} \times \frac{1}{1000} \times DF$$

(Equation 2.2)

One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 μmol of unsaturated 4,5-galacturonide product per minute under the reaction conditions.

2.2.3 Cellulase assay

Cellulase activity was determined based on the traditional filter paper assay (Ghose, 1987). Whatman No.1 Qualitative Filter Paper Grade 1 (Whatman plc, Maidstone, UK) was cut into strips of 1 x 6cm, weighing approximately 50 mg each. Strips of filter paper were then coiled into 2 mL centrifuge tubes. 1 mL of McIlvaine buffer pH 5.5 was added to each tube and pre-heated at the required assay temperature in a Thermomixer C. After pre-heating, 500 μL of diluted enzyme was added to each centrifuge tube. Substrate controls were prepared by substituting the enzyme for 500 μL of buffer instead, whilst enzymatic controls were prepared by preparing samples without filter paper. Samples were then incubated for 1 h and 800 rpm at the assay temperature.

Following incubation, the supernatant was decanted and boiled for 15 min in order to terminate enzyme activity. The supernatant was then centrifuged at 12,000 rpm for 15 min. As opposed to traditional filter paper assays which utilises the 3,5-DNS assay to measure reducing sugars, glucose and cellobiose were measured via HPAEC-PAD according to the method by Chu et al, (2012). Cellulase activity is reported as the sum of glucose and cellobiose (g/L) (Chu et al, 2012).

2.3 Sugar beet processing

2.3.1 Preparation of sugar beet cossettes

Sugar beets were removed from the cold room and washed again with tap water prior to use. Sugar beets that exhibited significant signs of external deterioration, such as mould and other indications of microbial infection, were discarded. After washing and drying, the top and bottom of the sugar beet were sliced off to isolate the core of the beet, which was then peeled to remove surface impurities. The sugar beet core was sliced into cossettes using an industrial food processor (Magimix 3200XL, Burgundy, France) fitted with a 2 x 6 julienne blade. This resulted in 2 x 6mm (depth x width) cossettes of variable length. Cossettes were then cut to approximately 40 to 50 mm length (Figure 2.2). Cossettes were used immediately after slicing to limit oxidation and deterioration of the cossette quality. Cossettes that were bruised, discoloured,

rotten or otherwise impaired were discarded. Similarly, cossettes that were too fine, known as “mush” in industry, or cossettes that were improperly sliced to form “hands” were not used.

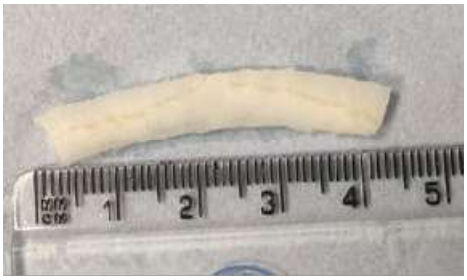

Figure 2.2: Average cossette dimensions and structure when prepared with the method used in this work. Dimensions of cossette are between 40-50 mm long, 2 mm deep and 6 mm wide.

Sugar beet quality is assessed by several methods, such as the Swedish number and mush content (Asadi, 2006). Swedish number is defined as the ratio of the mass of cossettes longer than 50 mm to those shorter than 10 mm in 100 g of cossettes, with an ideal Swedish number greater than 10. Similarly, the mush content is the proportion of cossettes less than 10 mm long in 100 g of cossettes, with an ideal mush content less than 5%. As the cossettes are processed manually and mush cossettes are not selected for use, the Swedish number and mush content are guaranteed to satisfy these ranges.

2.3.2 Cossette pre-treatment

2.3.2.1 Aqueous-autoclave pre-treatment

5 g of cossettes was added to 20 mL of Milli-Q water. The mixture was autoclave for 15 min at 121°C followed by centrifugation. A fraction of the supernatant was stored for sugar analysis via HPAEC-PAD (Section 2.8). The cossettes were washed by adding 30 mL of Milli-Q water and briefly centrifuging the samples at 5000 rpm for 5 min to remove residual sugars from the autoclaving process.

2.3.2.2 Alkaline pre-treatment

5 g of cossettes was added to 20 mL of 0.10 N NaOH at 25°C for 1h. After incubation, 1 N HCl was added to dilute the mixture to pH 5.5. Samples were then centrifuged and a fraction of the supernatant was stored for sugar analysis. The cossettes were washed as described in Section 2.3.2.1.

2.3.2.3 Diffusion conditions pre-treatment

A pre-treatment method designed to replicate the sugar beet diffusion conditions was also examined. 5 g of sugar beet cossettes were added to 20 mL of Milli-Q water and incubated at 70°C for 1h at 120 rpm in a shaking incubator (Climo-shaker ISF1-X, Kuhner, Switzerland). After incubation, samples were centrifuged and a fraction of the supernatant was stored for sugar analysis. Cossettes were washed as described in Section 2.3.2.1.

5 g of cossettes were added to 20 mL of Milli-Q water and incubated at 70°C for 1 h at 120 rpm in a shaking incubator. After incubation, samples were centrifuged and a fraction of the supernatant was stored for sugar analysis. Cossettes were washed as described in Section 2.3.2.1.

2.3.2.4 Physical pre-treatment

Frozen sugar beets were defrosted overnight at 4°C. Sugar beets were then peeled, sliced and milled using an industrial food processor (Magimix 3200XL Burgundy, France) fitted with a Sabatier blade, producing fine particles of sugar beet.

2.3.3 Single-stage co-current diffusion

Single-stage co-current diffusion was achieved by use of a pre-heated shaking incubator (Climo-shaker ISF1-X, Kuhner, Switzerland) to simulate heating and mixing. In this model, the required mass of cossettes were weighed and placed in a cylindrical, sealed plastic container. A pre-heated volume of pH 5.5 McIlvaine buffer was added to each container according to the selected diffusion ratio (Equation 4.4). Each container was then placed in a pre-heated shaking incubator at 250 rpm, 25 mm orbital shaking diameter. During the diffusion time, 500 µL samples were regularly taken for analysis. After diffusion, the pulp was separated from the diffusion juice and analysed separately.

2.3.4 Counter-current diffusion

Design of the counter-current diffusion model is detailed in Section 4.2.5. Following slicing, 250 g of cossettes were weighed and evenly distributed into five stainless steel cups approximately 9 cm in height with a diameter of 7 cm. The cups containing cossettes were then placed in an incubator whilst the subsequent materials were prepared. Concurrently, 275 mL of pH 5.5 McIlvaine buffer was heated to 78°C on a

laboratory hot plate. The buffer was then decanted into a vacuum flask (Zojirushi SM-TA36, Japan) to retain the temperature over the duration of the diffusion.

The initial diffuser model used in this work consists of five 320 mL stainless steel cups each filled with 50 g of cossettes (Figure 2.3). This results in a total of 250 g cossettes per diffusion run. These cups were placed in a 5 L water bath (Isotemp 2340 Digital Water Bath, Thermo Fisher Scientific, Massachusetts, USA) pre-heated to 70°C. A thermometer was used to measure the internal temperature of each cup, as well as to stir the cossettes and diffusion liquid every 2 min.

Figure 2.3: Image of initial diffuser model. Diffuser model consists of five stainless steel cups filled with cossettes. The cups are placed in a pre-heated water bath at the selected temperature. Buffer is then added to each cup in a stepwise process to maintain a counter-current gradient and mimic industrial extraction.

To initiate diffusion, 50-60 mL of pre-heated buffer is added to Cup 1 dependent on the selected diffusion ratio (Equation 4.4). After 10 min, the liquid from Cup 1 was poured into Cup 2 over a sieve, followed by the addition of fresh buffer into Cup 1. This process was repeated until each cup of cossettes had been treated five times. At 50 min, Cup 1 was removed and the exhausted wet pulp was stored in a container. Simultaneously, the diffusion juice from Cup 5 was decanted into a glass bottle. As a result, counter-current extraction was achieved as the concentration gradient between the cossettes and the extraction buffer was maintained throughout the process. After 90 min, each cup has been treated five times and the exhausted wet pulp was collected separately to the diffusion juice. The diffuser model evolved over the duration of the project. The

final version, which includes sealed cups and continuous agitation is depicted in Figure 2.4.

Figure 2.4: Image of the final diffuser model. A power generator enables the motor to rotate, causing the attached pullies to rotate. The pulley on the motor is connected to a pulley on the diffusion cup module via a belt, resulting in rotation of the diffusion cups as the motor rotates. This equipment was constructed in collaboration with the High Precision Design and Fabrication Facility (UCL Department of Biochemical Engineering).

2.3.5 Addition of enzyme to the diffusion process

Rohapect SY+ and Rohament CL used in the diffusion process were used undiluted. Enzyme loading was equivalent to 1 mL of enzyme per 250 g of sugar beet cossettes unless otherwise specified. Enzymes were added either at the beginning of the diffusion process or the midpoint of the diffusion process, described in detail in Chapter 4.

2.3.6 Wet pulp pressing

The press used in this study (Figure 2.5) is a modified manual fruit presser designed for pressing fruit for jam and wine production (“Torchietto Tommy”, Gruppo Palumbo, Italy). The press consists of a manual screw press with a stainless steel pressing basket within a cylindrical vessel with a spout, separating the pressed pulp and the

pressed pulp water enabling simple decanting of the pressed pulp water. The dimensions of the cylindrical vessel were 12 cm in height and 15 cm in diameter.

Two modifications were made to the press in order to ensure that the applied pressure is consistent between experiments. The first is a qualitative sensor in the form of a cable tie on the neck of the screw press that prevents the screw from pressing beyond a pre-defined limit. The second is a set of 4 x 50 kg load cells on the base of the press that function as a pressure sensor. These load cells form a full Wheatstone bridge arrangement capable of measuring up to 200 kg. The load cells are connected to a HX711 amplifier, which is in turn connected to an Arduino Uno module, enabling the monitoring of applied pressure via a laptop or display system.

Figure 2.5: Manual press used in this study. The press is modified to ensure consistent pressure is achieved between replicates by use of a cable tie to prevent excessive screwing. A pressure sensor was also constructed by modifying 4 x 50 kg load cells connected to an Arduino Uno with an accompanying HX711 amplifier.

After diffusion (Section 2.3.4), 100 g of total wet pulp was weighed and placed into a pre-wetted muslin pressing cloth and inserted into the pressing basket. The press was then manually lowered to the modified pressing indicators. The press was then lifted, and the pressed pulp water decanted into a sterile falcon tube. The pressed pulp was weighed and the volume of pressed pulp water recorded to determine the mass of pressed pulp and volume of pressed pulp water obtained from 100 g of wet pulp.

A summary of the counter-current bench-scale beet processing can be seen in Figure 2.6.

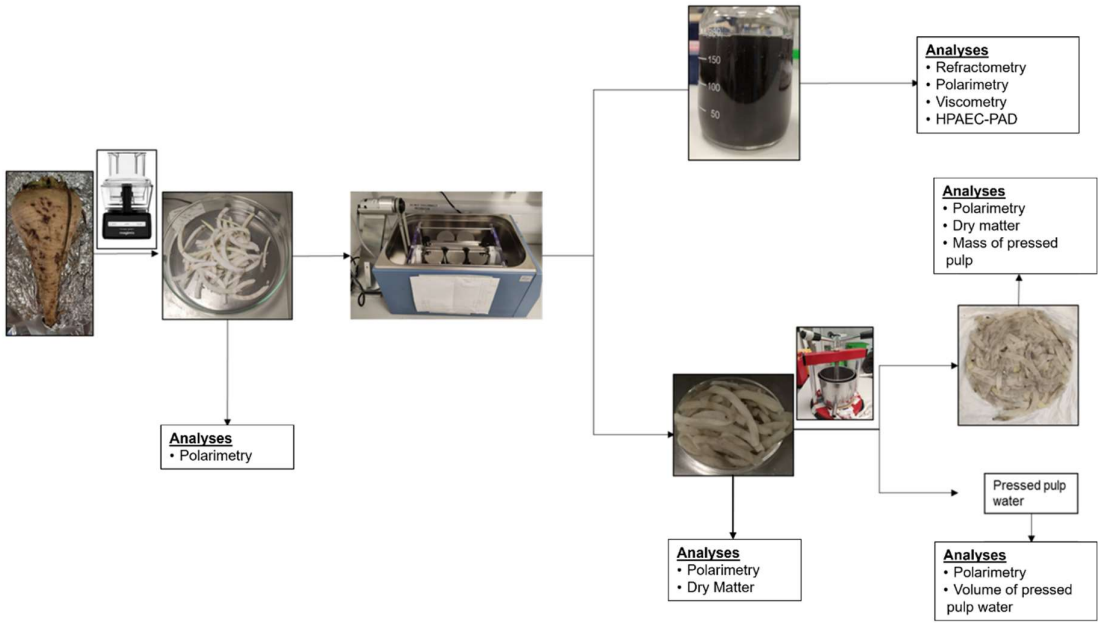

Figure 2.6: Summary of the counter-current bench-scale beet processing from whole beet to diffusion products. Whole sugar beets are washed, peeled, sliced manually and via an industrial food processor fitted with a julienne blade. A sample of cossettes are analysed for sucrose concentration via polarimetry. Cossettes are then weighed and processed in the counter-current diffuser model with or without the addition of enzymes resulting in a raw juice stream and a wet pulp stream. Raw juice is primarily analysed via refractometry and polarimetry for sugar content and purity. A sample of wet pulp is analysed for sugar content and dry matter content, and the remainder is pressed with the modified manual press model resulting in pressed pulp and pressed pulp water. Pressed pulp is analysed for the mass of pressed pulp from 100g of wet pulp and the sugar and dry matter content. Pressed pulp water is analysed for the volume of pressed pulp water obtained from 100g of wet pulp and its sugar content via polarimetry.

2.4 Refractometry

Refractometric analysis was performed using a Milwaukee MA871 digital refractometer (Milwaukee, North Carolina, USA). 200 uL of liquid sample was pipetted onto the glass prism. Calibration was achieved by blanking the instrument with Milli-Q water. After temperature stabilisation, the °Brix of the samples were read via the digital output.

2.5 Polarimetry

Polarimetry was selected as the primary method for the analyses of sugar beet and diffusion samples. Specifically an MCP 100 instrument (Anton Paar, Graz, Austria) coupled with a 100 mm polarisation tube using a 589 nm sodium lamp. It should be noted that this method requires approximately 5 mL of sample to fill the sample cell. Where this was not possible, samples were analysed for sucrose content via HPAEC-PAD instead (Section 2.8). Calibration of the polarimeter was achieved by using a quartz control plate in combination with the internal calibration feature.

2.5.1 Polarimetry of cossettes

The polarimetry analysis of cossettes prior to extraction was conducted according to the method described by Asadi (2006). Immediately after slicing (Section 2.3.1), 26 g of cossettes were added to 150 mL of Milli-Q water in a blender bottle. 5 g of Octapol (Baddley Chemicals Inc, Louisiana, USA) was added as a clarifying reagent. Cossettes were then blended with a standard kitchen blender (Breville Blend Active Personal Blender, Sydney) until smooth. Additional Milli-Q water was added to make the mixture up to a final volume of 200 mL. Samples were vacuum filtered to produce a clear filtrate, which could then be injected directly into the polarimeter module.

The output of polarimetry is the optical rotation of the sample. To convert this into percentage sucrose (w/w) the optical rotation is first converted into °Z according to the ICUMSA method (Section 1.4.3). The percentage of sucrose be calculated via polarimetry by applying Equation 2.3 (Asadi, 2006):

$$\%\, Sucrose\ (w/w) = {}^{\circ}Z \times \frac{26}{Mass_{sample}(g)} \times \frac{200}{Tube_{length}(mm)} \times \frac{Volume\ (mL)}{100} \qquad \text{(Equation 2.3)}$$

Given a mass of 26 g, a polarimetric tube length of 100 mm and a volume of 200 mL, the percentage of sucrose in cossettes can be calculated as:

$$\%\, Sucrose_{cossettes}\ (w/w) = \ ^{\circ}Z \times \frac{26}{26} \times \frac{200}{100} \times \frac{200}{100}$$

$$\%\, Sucrose_{cossettes}\ (w/w) = \ ^{\circ}Z \times 4 \qquad \text{(Equation 2.4)}$$

2.5.2 Polarimetry of juice

Following diffusion, 100 mL of raw diffusion juice was decanted into a separate vessel. 5 g of Octapol was added as a clarifying reagent. Samples were then vacuum filtered to produce a clear filtrate, which could then be injected directly into the polarimeter module.

Given that the juice is undiluted, the volume of juice becomes equal to the volume in a normal polarimetric sample, i.e. 26 g diluted to 100 mL (Section 1.4.3). Hence, the percentage of sucrose in the raw juice can be calculated as:

$$\%\, Sucrose_{juice}\ (w/w) = \ ^{\circ}Z \times \frac{26}{26} \times \frac{200}{100} \times \frac{26}{100}$$

$$\%\, Sucrose_{juice}\ (w/w) = \ ^{\circ}Z \times 0.52 \qquad \text{(Equation 2.5)}$$

2.5.3 Polarimetry of wet pulp and pressed pulp

Following diffusion, 20 g of wet pulp was added to 200 mL of Milli-Q water in a blender bottle. 2 g of Octapol was added as a clarifying reagent. The wet pulp was then blended with a standard kitchen blender until smooth. Additional Milli-Q water was added to constitute a final volume of 250 mL. Samples were vacuum filtered to produce a clear filtrate, which could then be injected directly into the polarimeter module. Pressed pulp was analysed with the same method except with 20 g of pressed pulp.

Due to the high moisture content and relatively low sucrose content of wet and pressed pulp, the moisture content must be factored into the percentage sucrose calculation (Asadi, 2006). Thus, the following correction is applied:

$$\%\, Sucrose_{pulp}\ (w/w) = (^{\circ}Z \times 0.52) \times \frac{250 - \%\, Moisture\ in\ sample}{Mass\ of\ sample} \qquad \text{(Equation 2.6)}$$

In industry, the moisture of the wet and pressed pulp is approximated as 85% and 75% respectively (Asadi, 2006). Where possible, the moisture used in Equation 2.6 was determined according to Section 2.7.

2.5.4 Polarimetry of pressed pulp water

0.5 g of Octapol was added to the pressed pulp water followed by vacuum filtration to produce a clear filtrate. Samples were then injected directly into the polarimeter module. As with juice analysis, the pressed pulp water is undiluted. Therefore, the percentage of sucrose of pressed pulp water can be calculated according to Equation 2.5.

2.6 Purity of juice

°Brix is a measurement of all dissolved solids (Section 1.6.2) whilst polarimetry with Octapol is a measurement of dissolved sugars (Section 1.6.3), the sucrose purity of the juice can be calculated from Equation 2.7:

$$Purity\ of\ juice\ (\%) = \frac{\%Sucrose_{juice}}{°Brix} \times 100$$

(Equation 2.7)

The non-sucrose sugars are not removed by the clarifying reagent and are therefore included in the polarimetric reading. However, given that the concentrations of these non-sucrose sugars are significantly lower than sucrose in the juice it is generally assumed that the purity is indicative of sucrose purity.

2.7 Dry matter and moisture content of pulp

The moisture content of the wet and pressed pulp was based on an oven-drying method measuring the loss of weight on drying. A clean and dried watch glass was weighed and the mass recorded. 30 g of wet or pressed pulp was then placed onto the watch glass and dried at 100 °C until no further changes in mass were observed. The dry matter was calculated from Equation 2.8, where $Pulp_{dry}$ refers to the mass of pulp post-drying and $Pulp_{wet}$ refers to the wet or pressed pulp prior to drying:

$$Dry\ matter\ (\%) = \frac{Pulp_{dry}}{Pulp_{wet}} \times 100$$

(Equation 2.8)

2.8 Quantification of individual monosaccharides (HPAEC-PAD)

The Dionex ICS-5000+ High Pressure Ion Chromatography (HPIC) system (Thermo Fisher Scientific Inc, Massachusetts, USA) was configured with an AminoPac PA10 anion exchange column, an injection loop of 10 µL, a pulsed amperometric detector system and an eluent generator fitted with a KOH 500 cartridge, coupled with the Chromeleon™ 7 Chromatography Data System (CDS) Software. Neutral sugars including sucrose, glucose and cellobiose were analysed using 5.0 mM KOH as the mobile phase with a flow rate of 0.25 mL/min at 30 °C for 30 min. Galacturonic acid was analysed using a mobile phase consisting of 5% v/v electrochemical-grade sodium acetate at 0.25 mL/min at 30°C for 15 min. Standard curves of each analyte were prepared prior to each analyses (Figure A1.2 to Figure A1.6) using Milli-Q water. The peak height or peak area was compared against the standard curves in order to produce quantitative analyses of each sample. Example chromatograms can be found in the appendix (Figure A1.7 to Figure A1.10).

Some samples required a pre-analysis hydrolysis treatment in order to analyse oligosaccharides or polysaccharides via HPAEC-PAD. 35 µL of 72% v/v H_2SO_4 was added to 1 mL of sample and autoclaved for 1 h at 121 °C to hydrolyse all oligosaccharides into constituent monosaccharides. Samples were then neutralised and centrifuged at 12,000 rpm for 15 min before analysis of the supernatant.

2.9 Protein purity and molecular weight analysis (SDS-PAGE)

The molecular weights of the enzymes used in this study were assessed via Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN Tetra Vertical Electrophoresis Cell with Mini-PROTEAN TGX Gels 10% acrylamide (Bio-Rad Laboratories Inc., California, USA). Enzymes were diluted appropriately and mixed with Laemmli 2x concentrated sample buffer (Sigma-Aldrich) at a 1:1 ratio, followed by incubation in a 100 °C heating block for 15 min. For the reference ladder, 10 µL of Spectra Multicolor Broad Range protein ladder (Thermo Fisher Scientific Inc., Massachusetts, USA) was added to the first well. 20 µL of each enzyme added to the subsequent wells. The gel was run at 150 V for approximately 1 h, then stained with InstantBlue Coomassie Protein Stain (Abcam, Cambridge, UK) overnight. After de-staining the gel with Milli-Q water, the gel was visualised and

analysed on a Gel-Doc-it imaging system using Labworks 4.5 software (Bioimaging systems, Cambridge, UK).

2.10 Viscosity analysis

The viscosity of the diffusion juice (with and without enzyme addition during the diffusion process (Section 2.3.4) was determined using a Kinexus Rheometer Lab+ fitted with a PU50 spindle (Malvern Instrument, Malvern, UK). The gap between the base and the spindle was set to 0.5 mm and the temperature of the system was maintained at 20 °C. Analysis was conducted via the rSpace software (Malvern Instrument, Malvern, UK) using a pre-programmed method to monitor the viscosity of the sample as a function of shear rate.

2.11 Novel thermostable pectate lyase expression

2.11.1 Microorganisms

Escherichia coli BL21 (DE3) containing the TMA14 gene from *Thermotoga maritima DSM 3109*, which codes for a thermostable pectate lyase was used throughout this study. Working stock cultures were stored in a 50% (v/v) glycerol solution at -80°C.

2.11.2 Media preparation and overnight culture

LB media was prepared by dissolving 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride in Milli-Q water. LB media was sterilised in an autoclave at 121°C for 20 min. An overnight culture was prepared by inoculating 10 mL of LB broth containing 0.05 g/L kanamycin with a glycerol stock containing the transformed *E. coli* BL21 (DE3) cells. The culture was incubated at 37°C, 250 rpm, 25 mm orbital shaking diameter for 16h.

2.11.3 Shake flask fermentation

3mL of the overnight culture was used to inoculate 300 mL of LB media containing 0.05 g/L kanamycin in a 2 L baffled shake flask. Fermentation was conducted at 37°C and 250 rpm in a shaking incubator. When OD 600 reached 0.3-0.5, cells were induced by adding Isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After induction, temperature was lowered to 25°C and fermentation continued up to 24 h post-induction.

2.11.4 Cell recovery and lysis

Following incubation, the cells were harvested via centrifugation at 8500 g, 4°C for 10 min. The cell pellet was resuspended in 12 mL of pH 5.5 McIlvaine buffer and lysed via sonication with 20 cycles of 10 seconds on and 15 seconds off at 12 µm amplitude. The lysed cells were then centrifuged at 18000 g, 4°C for 15 min to remove the cell debris. Clarified lysate was recovered for enzyme activity analysis and application in sugar beet extraction experiments. The clarified lysate was stored at 4°C for up to 6 months.

2.12 Statistical analysis

Statistical analysis was conducted using an unpaired Student's *t*-test to determine statistical significance between the independent control and experimental trial. p-values less than 0.05 were deemed statistically significant.

Chapter 3

Characterisation of commercial enzyme activity and stability for application in the sugar beet diffusion process

3.1 Introduction

Prior to incorporating enzymes into a process such as diffusional juice extraction from sugar beets, it is important to develop an understanding of the properties of the enzymes such as their activity and stability at conditions relevant to process operation; in this case, a temperature of 70 - 73°C, pH of 4.5 - 5.5 and a residence time of 60 - 110 min (Asadi et al, 2006). Commercial suppliers are the main source of enzymes for use in industrial applications due to the amount of enzyme required for the scale of operation and the work they will have performed on enhancing enzyme activity and stability. Suppliers often provide a range of conditions at which their enzymes work best, but information regarding their activity and stability under specific conditions is not usually available. A further drawback of commercially available enzymes is that the vast majority have been isolated from mesophilic microorganisms due to the disconnect between thermophilic microorganisms and the mesophilic expression systems commonly adopted in enzyme manufacture (Rigoldi et al, 2018). It is therefore important to understand how much of any commercial enzyme is active at the diffusion operation conditions.

Commercial enzymes are also often a blend of numerous enzyme activities prepared for use in a broad range of potential applications as advertised by the manufacturer. Macerating enzymes, for example, usually contain pectinases, cellulases, hemicellulases and amylases, and they may be applicable in the preparation of fermentation sugars from biomass as well as for juice extraction from fruits and vegetables. However, not all of the activities within these multi-enzyme mixtures are reported by the suppliers. There is therefore also a risk that some of these activities may be detrimental to the desired application of these enzymes. For example, some commercial enzyme complexes may contain invertases to convert sucrose into monomeric glucose to enhance the availability of sugars for fermentation. This would be detrimental to sucrose extraction from sugar beets as the primary objective here is to maximise sucrose concentration rather than glucose concentration. The aim of this

chapter is to develop a screening process for the selected commercial enzymes to assess the suitability for application within the sugar beet diffusion process.

3.2 Results

3.2.1 Evaluation of commercial enzymes according to supplier information

Several commercial macerating enzymes were considered for this work (Table 3-1). These enzymes can be broadly categorised into primarily pectinases and cellulases, although some of these enzyme preparations contain both activities plus additional activities. Suppliers often use different enzyme assays and experimental conditions when reporting enzyme activity; therefore, it is difficult to directly compare the properties of two different commercial enzymes even if they have the same declared activity (Biz et al, 2014). For example, Pectinex Smash XXL and Rohapect SY+ are both reported to be primarily pectin lyases, yet the activity is reported in undefined units of PECTU/g and PTF/g respectively. It is therefore important to standardise a set of assay conditions by which these enzymes can be directly compared to determine which are most suitable for application in this work.

As indicated in Table 3-1, the temperature and pH ranges provided by the technical data sheets are broad, which makes it particularly difficult to understand the optimum conditions for these enzymes. P743L has a declared temperature range of 10 to 60°C, but it is unclear where the optimum is within this range. Furthermore, information regarding activity outside of the temperature range, for example, at 70°C as per sugar beet diffusion conditions (Section 1.3.2.1), is not available. Rohapect SY+ and Cellulase 13L (C013L) are of particular interest as the suppliers declare that 70°C is within the temperature range of these enzymes, suggesting they may be active under diffusion conditions.

Table 3-1: Compilation of vendor-supplied information on the commercial enzymes used in this work and their respective declared activities, temperature range, pH range, recommended dosing and applications. All information is retrieved from the respective supplier's technical data sheets.

Enzyme	Supplier	Declared enzyme activities	Temperature range (°C)	pH range	Recommended dosing	Advertised application
Viscozyme-L	Novozymes A/S	Beta-glucanase (100 FBG/g) Xylanase Cellulase Hemicellulase Arabinase Polygalacturonase	25-55	3.3-5.5	200-400 mL/tonne of raw material	Increased olive oil extraction
P743L	Biocatalysts Ltd	Polygalacturonase (550 U/g) Cellulase	10-60	4.5-5.5	150-250 mL/tonne	Increased olive oil extraction
Cellulase 13L (C013L)	Biocatalysts Ltd	Cellulase (1500 U/g)	50-70	3.5-6.0	5% w/w	High-activity, complete cellulase preparation for liquefaction and maceration of fruit and vegetables
Depol 793L	Biocatalysts Ltd	Beta-glucanase (5500 U/g) Pectin lyase (500 U/g) Cellulase (1200 U/g)	30-60	4.0-7.0	100-500 ppm	Macerating enzyme for all fruit and vegetable applications
Pectinex Smash XXL	Novozymes A/S	Pectin lyase (19400 PECTU/g) Polygalacturonase	20-60	2.0-6.0	50-120 mL/tonne raw material	Improved juice yield
Rohapect SY+	AB Enzymes	Pectin lyase (100,000 PTF/g)	40-70	4.0-6.0	50-100 g/tonne raw material	Fruit and vegetable processing to improve extraction yield, pressing, clarification and filtration

Rohament CL	AB Enzymes	Cellulase	≤65	3.0-6.0	N/A	Hydrolysing non-starch polysaccharides for brewing and distilling

3.3.2 Evaluation of commercial enzymes for invertase activity

The ultimate objective of enzyme application within the sugar beet extraction process is to improve the yield of sucrose hence any invertase activity would be detrimental to the process. Therefore, an initial screen was performed to determine whether the commercial enzyme preparations in Table 3.1 contain invertase activity. A 1 g/L sucrose solution was prepared in pH 5.5 McIlvaine buffer. 0.1 mL of diluted commercial enzyme was added to 0.9 mL of 1 g/L sucrose and incubated at 50°C for 24 hr to enable time for any invertase activity. Substrate controls were prepared by incubating 0.9 mL of sucrose with 0.1 mL of buffer, and enzyme controls were prepared by incubating 0.9 mL of buffer with 0.1 mL of each diluted enzyme. After 24 hr, all samples were incubated at 100°C for 5 min to inactivate the enzymes. Glucose, sucrose and fructose concentration were measured via HPAEC-PAD as described in Section 2.8.

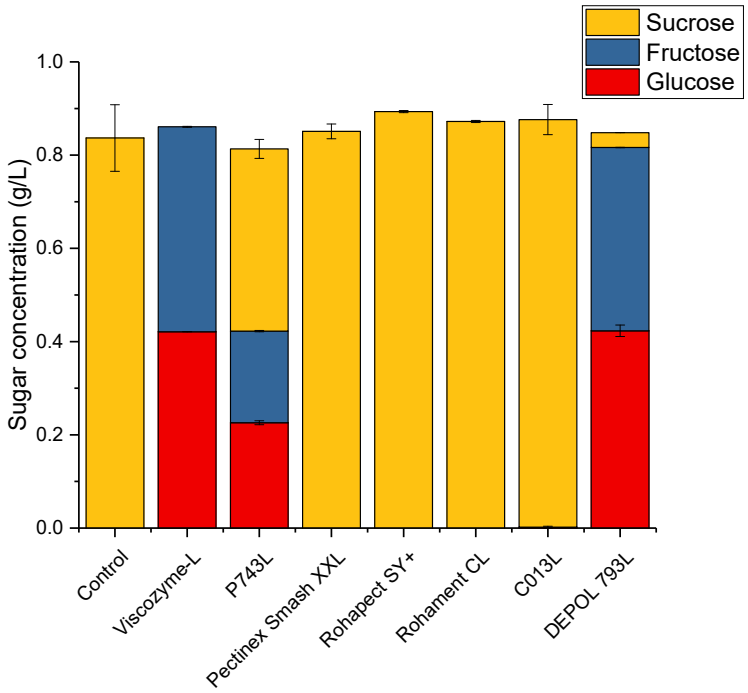

Figure 3.1: Evaluation of commercial enzymes for invertase activity. Assay for invertase activity conducted by incubating a diluted enzyme solution with 1 g/L of sucrose at 50ºC for 24 hr. Error bars for each sugar represent one standard deviation about the mean (n=3).

Figure 3.1 shows the concentration of glucose, sucrose and fructose of samples following enzyme incubation. Viscozyme-L and Depol 793L result in almost complete inversion of sucrose to glucose and fructose, as evidenced by the almost 1:1 ratio of glucose and fructose in these samples. P743L also contains some invertase activity, as only 48% w/w of the total sugar concentration in P743L samples is sucrose. Pectinex Smash XXL, Rohapect SY+, Rohament CL and Cellulase 13L do not contain any invertase activity as no glucose or fructose were detected in these samples.

According to Table 3-1, Viscozyme-L and Depol 793L are the most complex commercial enzymes, as the suppliers declare numerous side-activities including beta-glucanase, hemicellulase and ferulic acid esterase. Figure 3.1 therefore suggests that these complex mixtures may also contain undeclared invertase activity. Depol 793L is advertised as an all-purpose macerating enzyme so it makes sense that it contains many broad activities. Similarly, although Viscozyme-L is advertised primarily for improving oil yield from green olives, it has previously been used in research for the hydrolysis of starch despite not having declared starch-degrading activity (Mattusch et al, 2006; Guerra, 2017). Although the primary activity in P743L is polygalacturonase, the suppliers also note that it contains other side-activities such as cellulase, and likely some invertase activity.

By comparison, Rohapect SY+, Rohament CL and Cellulase 13L are relatively pure enzyme preparations, albeit the cellulases contain cellobiase, beta-glucosidase and other cellulose-degrading enzymes, as opposed to an enzyme cocktail of various activities. Pectinex Smash XXL and Rohapect SY+ are of particular interest as they contain only pectin lyase. Pectin lyase is able to act on innate methylated pectin without the need for additional enzyme activities, in contrast to other pectinase activities such as polygalacturonase, which requires demethylation in order to act optimally as it acts preferentially on homogalacturonan (Terefe, 2009). This is likely why pectin lyases have been identified as the key enzyme activity for improving sugar yield from sugar beets in the Novozymes A/S patent (WO2020002574A1).

3.3.3 Temperature activity assays

As the optimum operation temperature of the sugar beet diffuser is 70°C (Section 1.3.2.1), it is important to evaluate the activity of the commercial enzymes around this

temperature. The majority of the commercial enzymes in Table 3.1 declare activity up to 60°C but no information outside of this range is publicly available.

3.3.3.1 Polygalacturonase

Viscozyme-L and P743L were assayed for polygalacturonase activity according to the DNS assay detailed in Section 2.2.1. Although Viscozyme L degrades sucrose as confirmed in Figure 3.1, it is a well-studied food-grade polygalacturonase in literature (Berlowska et al, 2016; Zicari, 2016). Therefore, the polygalacturonase activity of Viscozyme L was also analysed as a comparison with P743L. Figure 3.2 shows the polygalacturonase activity for Viscozyme L and P743L between 20°C and 80°C, where one unit is defined as the amount of enzyme activity that produces 1µmol of galacturonic acid per minute under the specified reaction conditions.

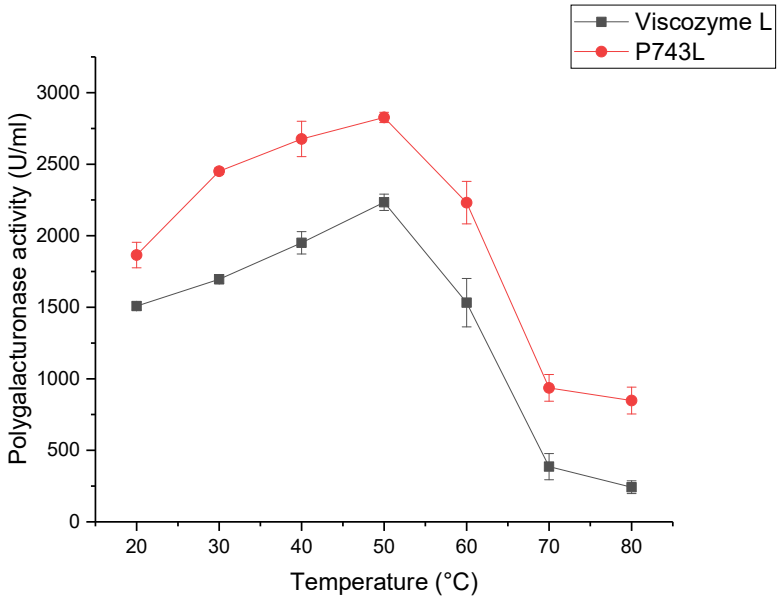

Figure 3.2: Temperature-activity profile for Viscozyme-L and P743L. Viscozyme-L and P743L exhibit optimum activity at 50°C but only have 17.3% and 33.1% relative activity at 70°C respectively. Polygalacturonase activity was measured using the DNS assay with 1g/L polygalacturonic acid at pH 5.5 as substrate, whereby one unit is defined as the enzyme activity that produces 1 µmol of galacturonic acid per minute (Section 2.2.1). Error bars represent one standard deviation about the mean (n=3).

P743L has a significantly higher polygalacturase activity than Viscozyme L across the 20 - 80°C temperature spectrum. Both Viscozyme L and P743L exhibit optimum activity at 50°C, at 2234 and 2827 U/mL respectively. Both enzymes also follow a similar curve with activity decreasing after 50°C, with a particularly sharp decline in

activity at 70°C as the relative activity at 70°C is 17% for Viscozyme L and 33% for P743L, suggesting that there would be low enzyme activity under the diffusion operating conditions.

3.3.3.2 Pectin lyase

Rohapect SY+ and Pectinex Smash XXL were assayed for pectin lyase activity according to the pectin lyase assay proposed by Albersheim (1966) detailed in Section 2.2.2. Figure 3.3 shows the pectin lyase activity for Rohapect SY+ and Pectinex Smash XXL between 20°C and 90°C, where one unit is defined as the amount of enzyme that releases 1 µmol of unsaturated product per minute under the stated reaction conditions.

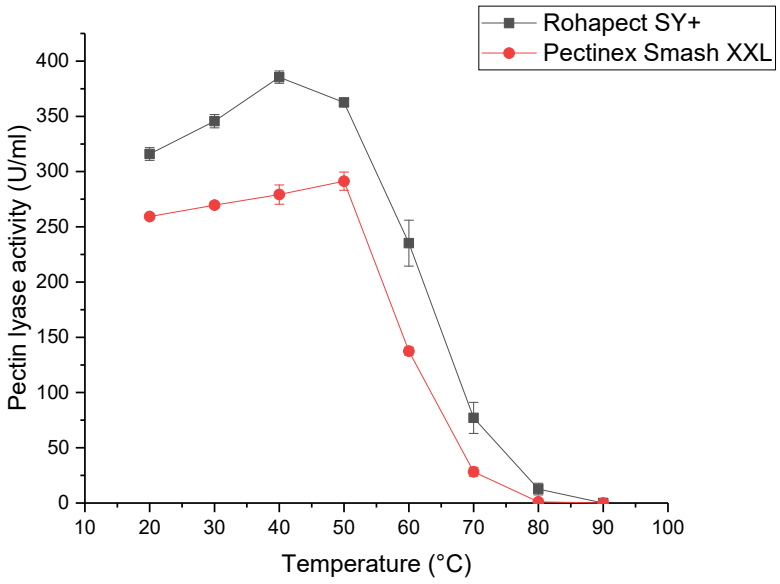

Figure 3.3: Temperature-activity curve for Rohapect SY+ and Pectinex Smash XXL. Pectin lyase activity was quantified by measuring the formation of unsaturated oligosaccharides from 0.5% w/w citrus pectin at pH 5.5 (Section 2.2.2). 1 U/mL is defined as the enzyme activity which produces 1 µmol of unsaturated product per minute. Rohapect SY+ and Pectinex Smash XXL have temperature optima at 40°C and 50°C respectively, but only have 20.0% and 9.7% relative activity at 70°C respectively. Error bars represent one standard deviation about the mean (n=3).

Rohapect SY+ is seen to have higher pectin lyase activity than Pectinex Smash XXL under the experimental conditions. Rohapect SY+ has an optimum working temperature of 40°C (385.45 U/mL) whilst Pectinex Smash XXL has a slightly higher optimum temperature of 50°C (291.31 U/mL), although the activity is still lower than

Rohapect SY+ at 50°C. According to the suppliers, Rohapect SY+ works best between 40 to 70°C. However, at 70°C the enzyme loses a significant amount of activity, as only 20.0% relative activity was recorded. A similar trend is observed with Pectinex Smash XXL, although the maximum advised working temperature for this enzyme is 60°C.

3.3.3.3 Cellulase

Rohament CL and Cellulase 13L were assayed for cellulase activity using the modified filter paper assay combined with HPAEC-PAD analysis method by Chu et al (2012) detailed in Section 2.2.3. According to Chu et al (2012), this method of analysis is high-throughput, more specific and less tedious compared to the traditional detection via the DNS assay, whilst also demonstrating a strong correlation between the filter paper units per ml (FPU/mL) and the sum of glucose and cellobiose (g/L) released. Furthermore, both the DNS assay and the HPAEC-PAD analysis primarily measure glucose and cellobiose, which represent the majority of the final products from cellulase activity. Therefore, although cellulase encompasses a range of activities, presenting the enzyme activity as the sum of glucose and cellobiose in g/L is an appropriate indication of general cellulase activity. In order to convert the sum of glucose and cellobiose into FPU/mL, Chu et al (2012) recommend the determination of an empirical calibration correlation for the specific enzymes being tested. However, given that the sum of glucose and cellobiose is sufficient for determining the general temperature activity profile of the enzymes, enzyme activity was retained as the sum of glucose and cellobiose in this work.

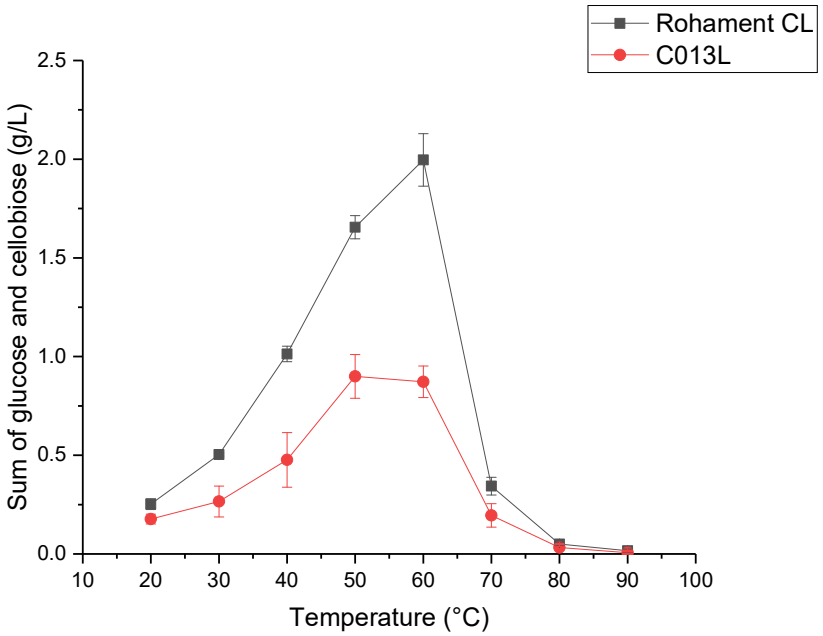

Figure 3.4: Temperature-activity curves for Rohament CL and Cellulase 13L (C013L) at pH 5.5. Activity was assayed via the filter paper-HPAEC-PAD method (Section 2.2.3). Rohament CL has an optimum temperature of 60°C whilst C013L has an optimal temperature of 50°C. Rohament CL has higher cellulase activity than C013L under the reaction conditions. Both enzymes exhibit a significant decrease in activity at 70°C, at 17.2% relative activity for Rohament CL and 21.7% relative activity for C013L. Error bars represent one standard deviation about the mean (n=3).

From Figure 3.4**,** it is evident that Rohament CL has a higher general cellulase activity than Cellulase 13L across all temperatures under the reaction conditions investigated. At 60°C, Rohament CL liberates 2.0 g/L of glucose and cellobiose, compared to 0.9 g/L for Cellulase 13L. Rohament CL has an advertised working temperature of up to 65°C whilst Cellulase 13L is advertised as functioning at 70°C. However, both enzymes lose considerable activity at 70°C as Rohament CL retains only 17.2% relative activity whilst Cellulase 13L retains 21.7% relative activity. This indicates that both enzymes would act poorly at 70°C in an industrial sugar beet diffusion process.

3.3.4 Temperature stability assays

To further assess the suitability of these commercial enzyme preparations for application within the sugar beet diffusion process, the thermal stability of these enzymes was assayed at 70°C in order to determine the residual activity levels of the enzymes following prolonged exposure to this elevated temperature. For example, although all of the commercial enzymes investigated exhibited relatively low activity at

70°C, the effective activity may be sufficient for application if the enzymes are stable across the whole duration of the diffusion process which has a residence time around 90 min (Section 1.3.2.1). Alternatively, the temperature stability information may also be useful to determine when and where the enzymes should be dosed in the diffusion process. A thermostable enzyme could be dosed at the beginning of the process, as its activity will persist throughout the entire process whereas a less stable enzyme may be dosed later in the process when the sugar beets are more degraded and thus potentially more susceptible to enzyme lysis.

3.3.4.1 Polygalacturonase

Polygalacturonase stability at 70°C was determined by incubating the enzymes at 70°C for 0-120 min prior to testing for enzyme activity at the optimum reaction temperature for each enzyme. The results are presented in Figure 3.5**.**

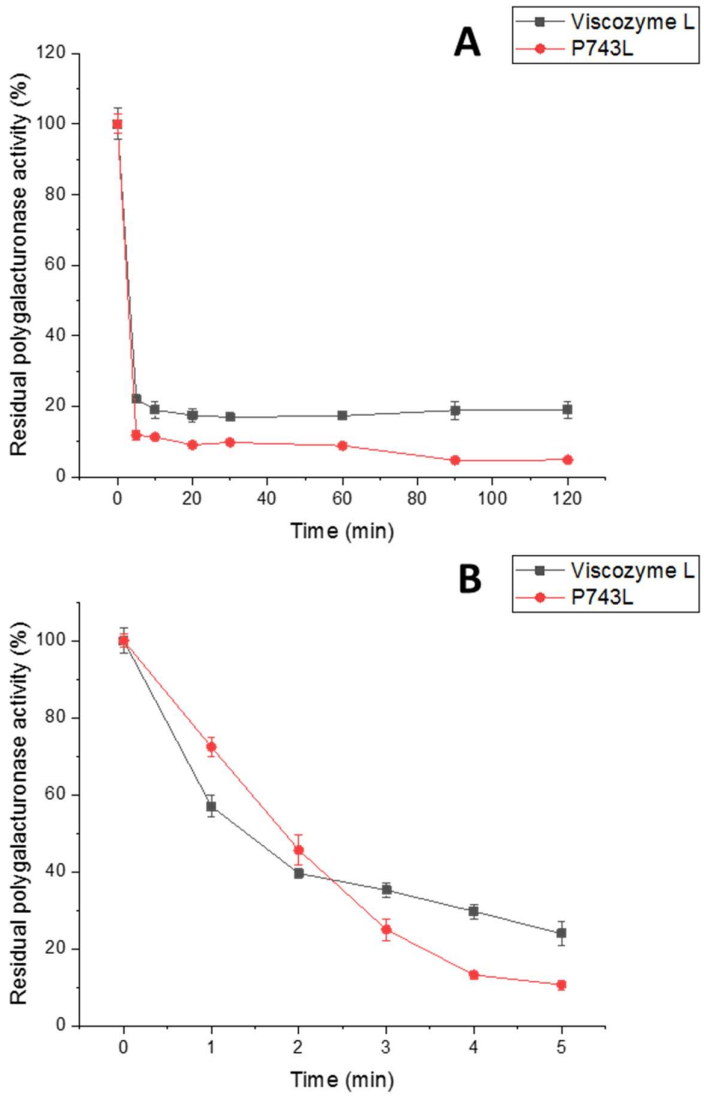

Figure 3.5: Temperature-stability profiles at 70°C over a period of 120min (A) and 5min (B) for Viscozyme-L and P743L at pH 5.5. The initial temperature stability assay was conducted over 120 min to correspond with the maximum duration expected in the diffuser (A). The assay was repeated over 5min with samples taken in 1min intervals (B). Viscozyme-L and P743L retain 24.0% and 10.6% residual activity after 5min of incubation at 70°C. Experiments performed as described in Section 2.2.1. Error bars represent one standard deviation about the mean (n=3). The 100% activity values at t=0 are equal to an activity of 2222.28 U/mL and 2528.70 U/mL for Viscozyme L and P743L respectively.

The initial temperature stability assay was conducted over an incubation period at 70°C of 120 min, with aliquots of enzyme removed for activity analysis at 0, 5, 10, 20, 30, 60, 90 and 120 min. This mimicked the maximum retention time in sugar beet diffusion process (Asadi, 2006). However, the stability of Viscozyme L and P743L were

lower than expected, with the vast majority of activity loss between 0 and 5 min with no significant further loss in activity between 10 and 120 min (Figure 3.5a). Therefore, the thermostability assay was repeated with a narrower period of 0 to 5 min with aliquots of enzyme removed at 0, 1, 2, 3, 4 and 5 min (Figure 3.5b).

The kinetics of the decrease in polygalacturonase activity is more clear in Fig 3.5B. Viscozyme L and P743L lose approximately 43% and 28% activity within the first minute of incubation at 70°C respectively. After 5 min of incubation, only 24.0% activity is retained for Viscozyme L and 10.6% activity is retained for P743L. Therefore, although P743L has a higher polygalacturonase activity than Viscozyme L under optimal conditions at approximately 2528.70 U/mL versus 2222.28 U/mL, Viscozyme L retains more polygalacturonase activity after incubation at 70°C for 5 min.

3.3.4.2 Pectin lyase

Pectin lyase thermostability was investigated by incubating the enzymes at 70°C for 0 to 5 min with aliquots of enzyme removed for activity analysis under optimum conditions in one minute intervals. The results are shown in Figure 3.6.

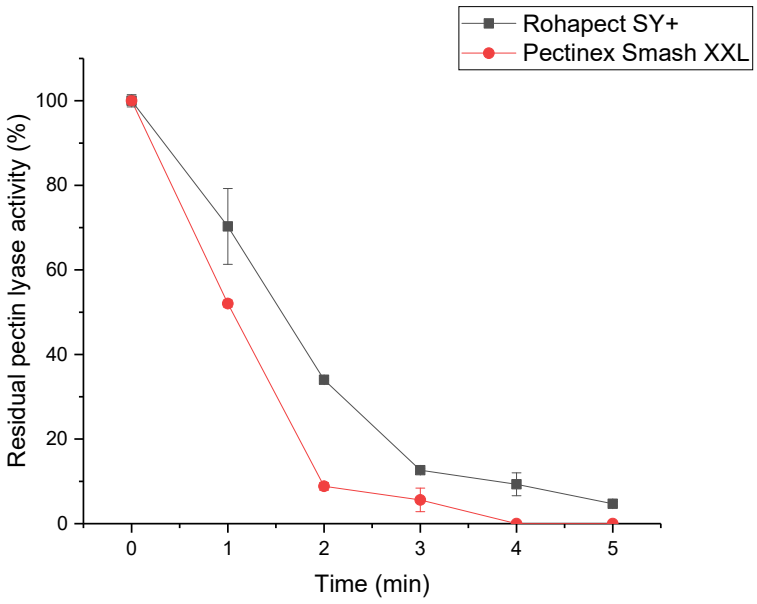

Figure 3.6: Temperature-stability profile at 70°C over a period of 5min for Rohapect SY+ and Pectinex Smash XXL at pH 5.5. Both enzymes lose significant amounts of activity after 5min at 70°C. Rohapect SY+ retains 4.7% activity whilst Pectinex Smash XXL has no detectable activity. Experiments performed as described in Section 2.2.2. Error bars represent one standard deviation about the mean (n=3). The 100% activity

values at t=0 are equal to an activity of 385.45 U/mL and 294.87 U/mL for Rohapect SY+ and Pectinex Smash XXL respectively.

Similarly to Viscozyme L and P743L (Figure 3.5b), Rohapect SY+ and Pectinex Smash XXL lose significant activity within the first 5 min of incubation at 70°C. Pectinex Smash XXL is particularly striking, as it retains no detectable pectin lyase activity after 4 min of incubation. Rohapect SY+ is slightly more stable as the decrease in pectin lyase activity is not seen to be as rapid, but after 5 min of incubation only 4.7% activity is retained. Although Rohapect SY+ is advertised for use within 40 to 70°C, Figure 3.6 suggests that Rohapect SY+ has very poor stability at 70°C.

3.3.4.3 Cellulase

Cellulase thermostability was measured by incubating the enzymes at 70°C for 0 to 5 min with aliquots removed in minute intervals for activity analysis under optimum conditions. The results are shown in Figure 3.7.

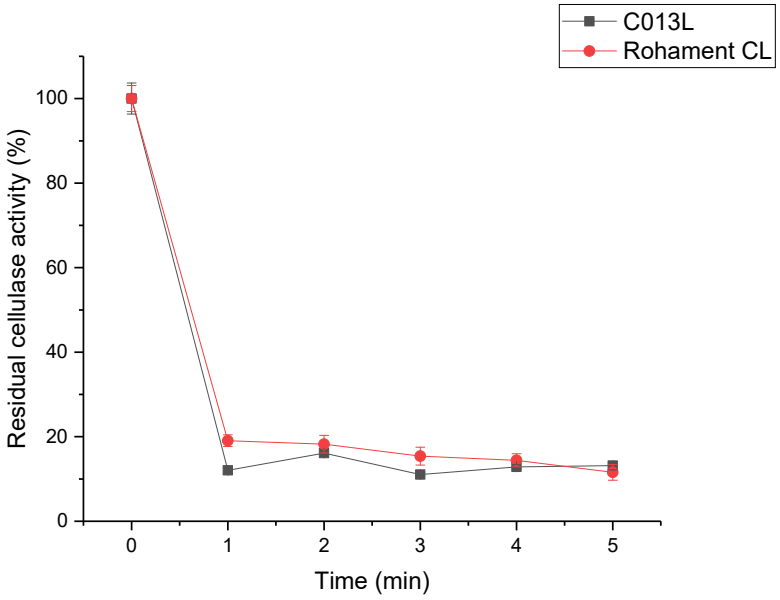

Figure 3.7: Temperature-stability profile at 70°C over a period of 5min for Cellulase 13L and Rohament CL at pH 5.5. Cellulase 13L and Rohament CL rapidly lose stability within the first minute of incubation, retaining only 13.2% and 11.6% residual activity respectively. Experiments performed as described in Section 2.2.3. Error bars represent one standard deviation about the mean (n=3). The 100% activity values at t=0 are equal to a sum of glucose and cellobiose activity of 1.17 g/L and 2.44 g/L for Cellulase 13L and Rohament CL respectively.

Of all the commercial enzymes studied in this work (Table 3.1), Cellulase 13L and Rohament CL exhibit the most dramatic loss in temperature stability, with approximately 13% and 19% residual activity after the first minute of incubation at 70°C, suggesting that these enzymes are highly unstable at this temperature and hence unsuitable for use in the sugar beet diffusion process.

3.3.5 Temperature stability with sucrose addition

Sucrose is commonly added to commercial enzyme and protein preparations as a stabilising agent as it increases the activation energy required for the enzyme to unfold, thus indirectly promoting stability by reducing the likelihood of unfolding (Lee & Timasheff, 1981; Arakawa & Timasheff, 1982; Taylor et al, 1995; Sola-Penna & Meyer-Fernandes, 1998). Therefore, there is a possibility that the sucrose within the sugar beet diffuser is able to stabilise the commercial enzymes such that the commercial enzymes are able to exhibit some activity at 70°C.

Furthermore, the sugar concentration in the diffuser changes over the course of the process, for example, there is relatively little extracted sucrose in the first 5 minutes compared to the end of the diffuser, where the diffusion juice contains approximately 10-15% w/w sucrose (Asadi, 2006). Therefore, the influence of sucrose concentration on enzyme stability may also help identify where and when the optimum enzyme addition point could be.

3.3.5.1 Pectin lyase

The method for determining the temperature stability of the commercial pectin lyases with sucrose is similar to the method in described previously, although the enzyme is instead diluted into a pre-heated 0/5/10/20% w/w sucrose solution at 70°C (Section 2.2.2). The enzyme is then incubated for 0 to 5 min with aliquots of enzyme removed for activity analysis under optimum conditions in minute intervals.

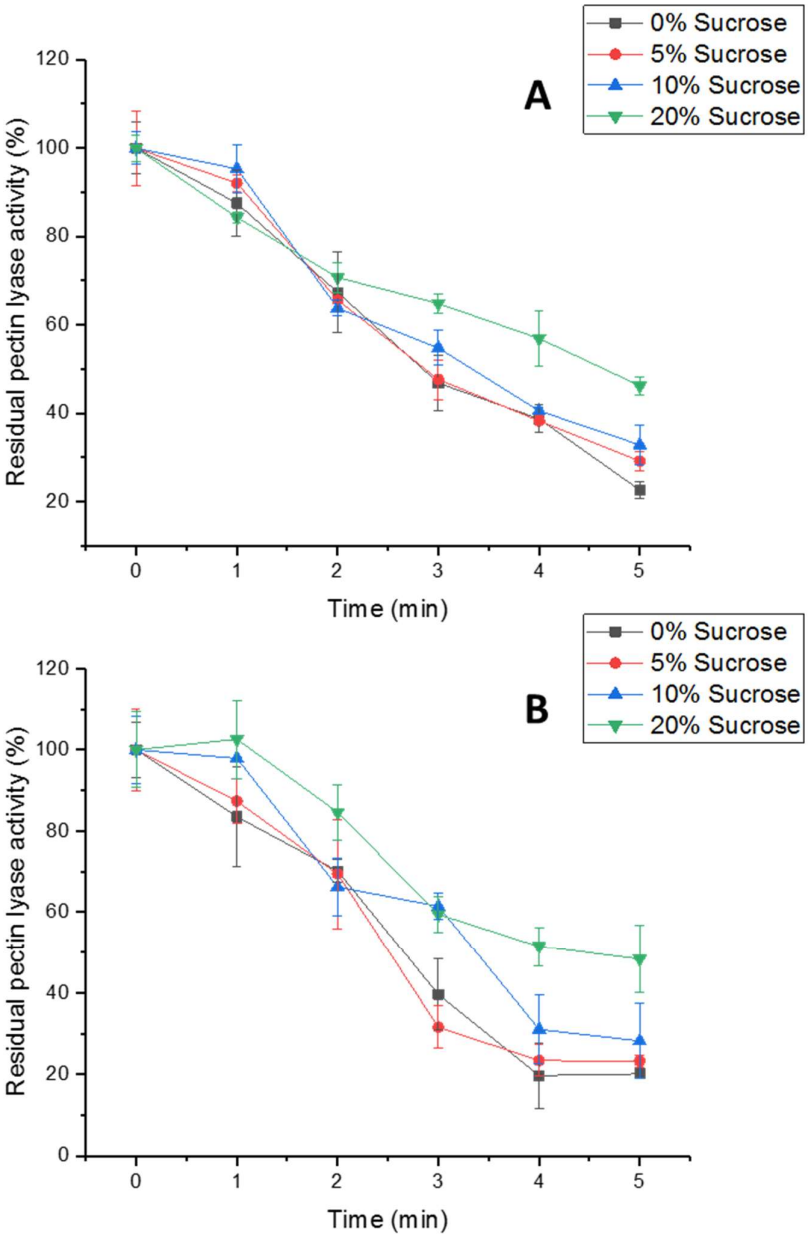

Figure 3.8: Temperature-stability profiles of Rohapect SY+ (A) and Pectinex Smash XXL (B) after 0-5 min of incubation at 70°C in sucrose solutions of 0%, 5%, 10% and 20% w/w sucrose at pH 5.5. Experiments performed as described in Section 2.2.2. Error bars represent one standard deviation about the mean (n=3). For Rohapect SY+, the 100% activity at t=0 is equal to an activity of 276.4, 273.0, 303.2 and 296.1 U/L for 0, 5, 10 and 20% sucrose respectively. For Pectinex Smash XXL, the 100% activity value at t=0 is equal to 179.4, 188.6, 202.3 and 192.3 U/mL for 0, 5, 10 and 20% sucrose respectively.

As shown in Figure 3.8, the temperature stability of Rohapect SY+ and Pectinex Smash XXL is slightly improved upon incubation in 5, 10 and 20% sucrose solutions.

This is most evident after incubation for 5 min, whereby both enzymes exhibit increasing residual pectin lyase activity as sucrose concentration increases. Rohapect SY+ retains 46.2% activity after 5 min of incubation at 20% sucrose, compared to 22.6% at 0% sucrose. Similarly, Pectinex Smash XXL retains 48.3% activity after 5 min of incubation at 20% sucrose compared to 20.1% at 0% sucrose. For both enzymes, the stabilising effect is less pronounced at lower sucrose concentrations. This may indicate that the optimum enzyme addition point is towards the end of the diffuser at which point the diffusion juice is highly saturated with sucrose.

3.3.5.2 Cellulase

As with pectin lyase stability in the presence of sucrose, the method of determining cellulase stability is similar to the method described previously in Section 2.2.3, except that the enzyme is diluted into a pre-heated 0/5/10/20% w/w sucrose solution at 70°C.

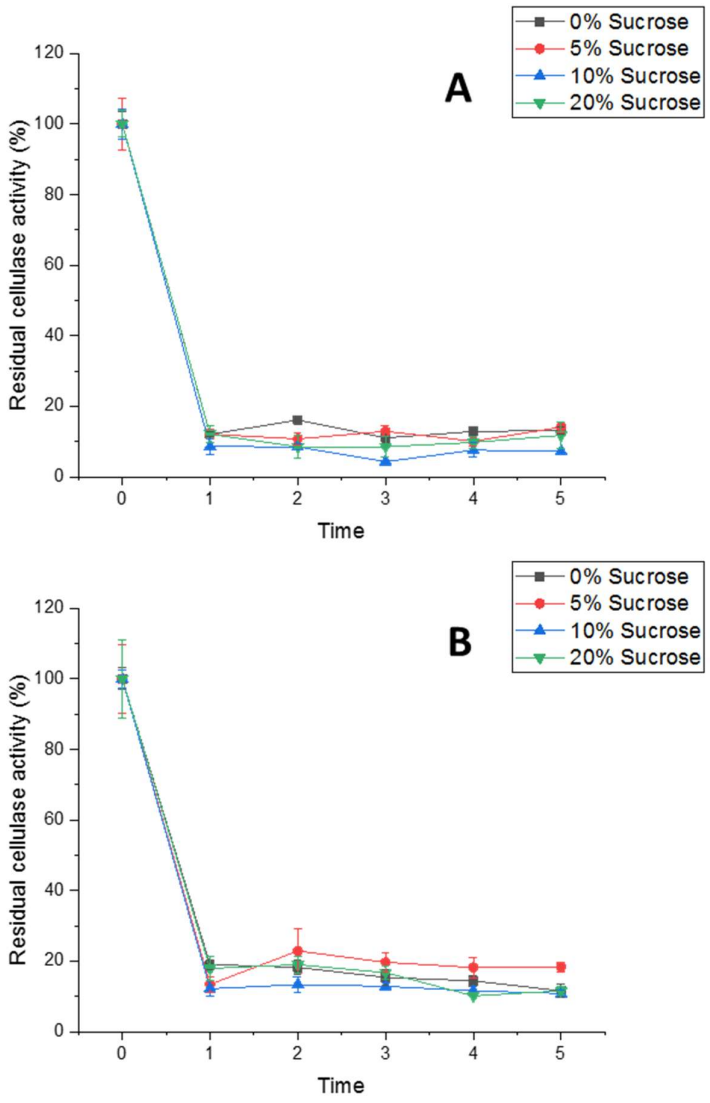

Figure 3.9: Temperature-stability profiles of Cellulase 13L (A) and Rohament CL (B) after 0-5 min of incubation at 70°C in sucrose solutions of 0%, 5%, 10% and 20% w/w sucrose at pH 5.5. Experiments performed as described in Section 2.2.3. Error bars represent one standard deviation about the mean (n=3). For Cellulase 13L, the 100% activity value at t=0 is equal to a sum of glucose and cellobiose of 1.17, 1.07, 1.10 and 1.09 g/L at 0, 5, 10 and 20% w/w sucrose respectively. For Rohament CL, the 100% activity value at t=0 is equal to a sum of glucose and cellobiose of 2.44, 2.16, 1.88 and 1.64 g/L at 0, 5, 10 and 20% w/w sucrose respectively.

Unlike pectin lyases, however, there is no clear improvement to thermal stability when the cellulases are incubated in a sucrose solution at 70°C as shown in Figure 3.9. Both enzymes lose the majority of their activity within the first minute and the addition of any level of sucrose does not appear to improve upon this.

3.4 Summary

Commercial enzymes are the main source of enzymes when considering industrial application due to the scale and quantity of enzyme required. Unfortunately, many of these commercial enzymes are derived from mesophilic microorganisms and are there not well-adapted for extreme temperatures, for example, 70°C in the sugar beet diffusion process.

As described in Section 3.1, the aim of this chapter was to screen the activities of commercial enzymes and evaluate the optimum conditions of these enzymes to assess the suitability for application within the sugar beet diffusion process.

In this work, seven commercial enzymes, Viscozyme L, P743L, Pectinex Smash XXL, Rohapect SY+, Rohament CL, Cellulase 13L and Depol 793L were studied as summarised in Table 3.1. These were categorised into polygalacturonases, pectin lyases and cellulases according to their primary activity. Commercial enzymes are often a mixture of enzyme activities in order to apply to a broad range of applications. Before determining the feasibility of these enzymes for improving sucrose extraction from sugar beets, it was first important to evaluate these enzymes for invertase activity that would contribute to the inversion of sucrose to glucose and fructose.

Of the seven enzymes investigated, Viscozyme L, P743L and Depol 793L demonstrated significant invertase activity. In particular, as shown in Figure 3.1, Viscozyme L fully converted sucrose to glucose and fructose after 24 h. These enzymes were therefore not compatible for application in the sugar beet diffusion process. Viscozyme L and P743L were still assayed for activity and stability data to enable comparison with the other investigated enzymes.

Following this, the activity profile of Viscozyme L, P743L and the remaining commercial enzymes at various temperatures was investigated. All of these enzymes had optimum temperature ranges between 40°C and 60°C whilst also exhibiting poor levels of activity 70°C at up to a 90.3% decrease in activity (Figure 3.3). Cellulase 13L and Rohapect SY+ were of particular concern as the information provided by the suppliers suggested that these enzymes could be used effectively at 70°C.

Following this, thermostability assays (Section 3.3.4) were conducted by incubating the enzymes at 70°C for a period of time before assaying for residual activity at the

temperature optimum. All of the commercial enzymes demonstrated poor thermostability at 70°C, losing up to 95.3% activity after incubation at 70°C for 5 min. Cellulase 13L demonstrated especially poor thermostability as only 11.6% residual activity was present after incubation at 70°C for 1 min (Figure 3.7).

Sucrose is often added as a stabilising agent to commercial preparations and given the sucrose-rich conditions of the diffusion process, it was important to determine the effect of sucrose on thermostability as this may improve enzyme stability and indicate the optimum point at which enzymes should be added to the diffuser (Section 3.3.5).

Rohapect SY+ and Pectinex Smash XXL showed some degree of improved thermostability when incubated with increasing sucrose concentrations, with maximum improved stability at 20% sucrose (Figure 3.8). Rohapect SY+ retains 46.2% activity after 5 min of incubation at 20% sucrose, whilst only retaining 22.6% activity at 0% sucrose. Although there is some significant improvement in stability, there is still a considerable loss in residual activity at 70°C. This data suggests that pectin lyase addition may be optimal towards the end of the diffusion process where the sucrose concentration is higher, enabling the enzymes to maintain activity to extract residual sucrose. Cellulase 13L and Rohament CL did not show significant improvements to thermostability when incubated with sucrose (Figure 3.9) as the majority of activity was still lost in the first minute of incubation at this temperature.

In summary, all the commercial enzymes investigated in this study have less-than optimal properties for application within the sugar beet diffusion process due to low activity at 70°C coupled with poor thermostability. However, there is the possibility that this low residual activity is sufficient to elicit a positive response in the diffusion process and therefore application trials on the diffusion process will be undertaken. In order to facilitate this, Chapter 4 describes the application of selected commercial enzymes on sucrose extraction from sugar beet under industrial conditions and the development of a scale-down diffuser model.

Chapter 4

Development of scale-down diffuser model and application of commercial enzymes to the sugar beet diffusion process

4.1 Introduction

The data from Chapter 3 suggests that the commercial enzymes are not particularly active under the conditions experience within the diffusion process (e.g. Figure 3.3) nor are they thermostable under the conditions experienced within the diffusion process (e.g. Figure 3.6). However, given the evidence that they can enhance sucrose extraction from the commercial scale diffusion process, there is the possibility that the residual enzyme activity present under industrial diffusion conditions is sufficient to enhance sucrose extraction in multi-stage extraction processes. It is therefore important to examine the application of these commercial enzymes for sucrose extraction from sugar beet cossettes and to do so under conditions that accurately reflect large scale diffuser operation. Given the data from this work and the information available from industry scale trials, it was decided that the focus of the work in this section would be with a commercial pectin lyase, Rohapect SY+ and a commercial cellulase, Rohament CL.

The aims of this chapter are two-fold: to explore the application of the selected enzymes for enhanced sucrose release from sugar beet cossettes, and to design and construct a counter-current diffuser model in which to evaluate the commercially available enzymes under conditions that mimic the industrial process.

The first step was to develop a proof-of-concept, single-stage process to assess the effect of these enzymes on sugar beet cossettes. The method is described in Section 2.3.3. Briefly, the process consists of a shaking incubator pre-heated at the required temperature. A suitable container containing a pre-determined volume of buffer is pre-heated in the shaking incubator. The cossettes are prepared according to Section 2.3.1 and placed into a suitable container. To initiate diffusion, the cossettes were added to the containers. This design is a single-stage, co-current system and does not

represent the counter-current diffusion system in industry, but is sufficient as a proof-of-concept model to determine the effect of these enzymes on sugar beet.

The studies on the co-current diffuser model were performed in parallel with the design of the counter-current diffuser model. The design and operation of this model are described in more detail in Section 4.2.6.

4.2 Results

4.2.1 Co-current diffuser model development and validation

An initial control experiment was conducted on the co-current diffuser model in order to investigate the effect of draft ratio, i.e. the volume of extraction solvent to the mass of sugar beet cossettes (Section 1.3.2.1, used interchangeably with the diffusion ratio as described in Section 4.2.6.1), on sucrose extraction at 70°C and pH 5.5 and a mixing speed of 250 rpm (Figure 4.1). As described in Section 1.3.2.1, a draft ratio of 100% is equivalent to the addition of 100 mL of extraction buffer for every 100 g of sugar beet cossettes, whilst a draft ratio of 120% represents the addition of 120 mL of extraction buffer for every 100 g of sugar beet cossettes.

The extraction of sucrose is measured by ºBrix (Section 1.6.2) and normalised against the sucrose concentration in the cossettes. This is to normalise the measured ºBrix values given the natural variation of sucrose concentration between individual sugar beet and between each experimental replicate. The initial percentage of sucrose in cossettes ranged between 17.21% and 18.71%. The ºBrix measured at 90 min ranged between 10.2° and 11.6°.

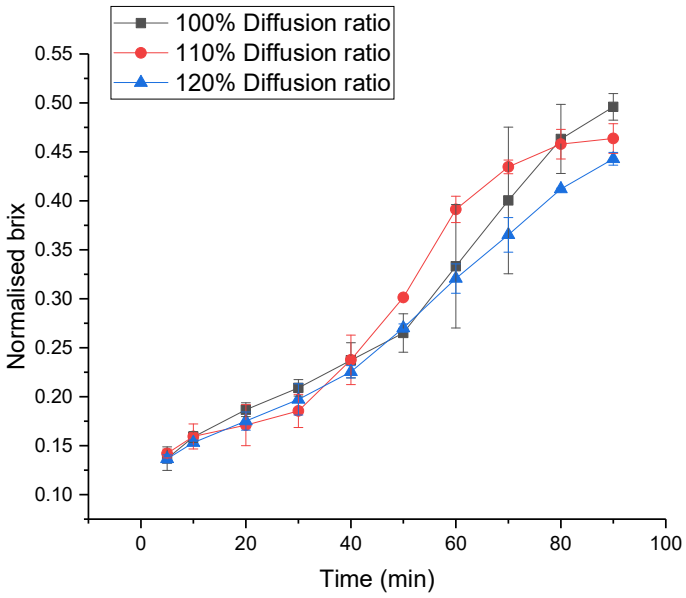

Figure 4.1: Change in normalised brix of the extracted juice against time at various diffusion ratios in the co-current single-stage model at 70ºC, pH 5.5, 250 rpm. ºBrix is normalised in order to account for the variation in cossette sucrose content in each replicate. Error bars represent one standard deviation about the mean (n=2). Experiments performed as described in Section 2.3.3.

The extraction of sucrose is observed by an initial rapid increase in sucrose concentration between 0 min and 5 min as the sucrose in the outer cells of the sliced cossettes is initially exposed to the extraction solvent. Following this, ºBrix values increase more slowly between 5 min and 30 min due to the recalcitrant nature of the sugar beet cossettes. The cell membrane of the cossette cells must be denatured in order to facilitate rapid extraction of sucrose. Silin (1958) determined that 90% of beet cell membrane is denatured after 8 min at 70°C with excess solvent to beet. In this single-stage model, an extension of the denaturation time may be because not all of the cossettes are fully submerged in extraction solvent at the diffusion ratios investigated, which will increase the time required for the internal temperature of the cossettes to reach 70°C.

Following denaturation of the sugar beet cell membrane, a rapid increase in ºBrix is observed after 30 min, followed by a plateau around 80 min, most notably at 110% diffusion ratio, as the system reaches equilibrium and diffusion of sucrose from the cossettes to the juice becomes limited.

The profiles of the extraction kinetics are very similar regardless of the diffusion ratio. Most importantly, the final ºBrix at 90 min is significantly more concentrated at 100% diffusion ratio compared to that at 120% diffusion ratio due to the reduced volume of extraction buffer.

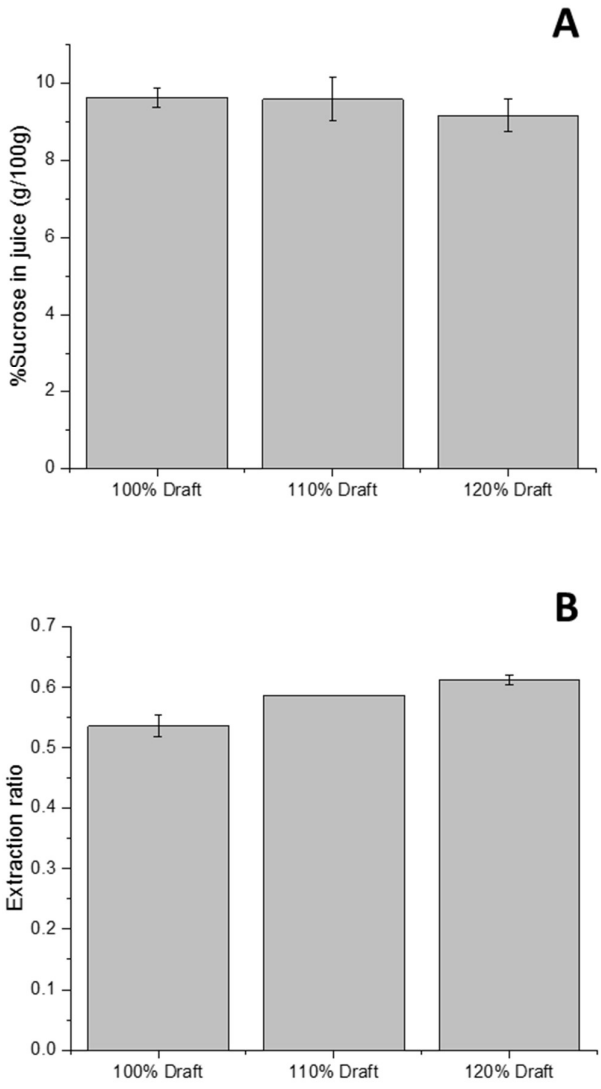

Figure 4.2: Sugar concentration in the extraction juice measured via polarimetry at varying diffusion ratios in the co-current single-stage model at 70ºC, pH 5.5, 250 rpm. There is no significant difference in the sucrose concentrations of the juice when directly comparing percentage of sucrose in juice (A). (B) is a comparison of the extraction ratios under each condition and a significant difference is observed between each condition, confirming that sucrose extraction is increased by higher diffusion ratio. Error bars represent one standard deviation about the mean (n=2). Experiments performed as described in Section 2.3.3.

Figure 4.2 is a comparison of sucrose extracted in the juice measured via polarimetry at varying draft ratios in the single-stage model. According to Figure 4.2A, there is no significant difference between the percentage sucrose in the juice at 100%, 110% or 120% draft ratio. However, directly comparing the percentage sucrose of the juices is not strictly correct, as this does not take into account the variation in sucrose of the cossettes, nor the volume of juice recovered. The latter is of particular importance when the diffusion ratio is varied between conditions, as this has a direct influence on the volume of juice recovered. Therefore, the standard yield calculation was modified as shown in Equation 4.1 to give the "extraction ratio" as a function of the sucrose yield whilst also factoring in the volume of juice produced and mass of cossettes used in order to better compare between extraction conditions.

$$Extraction\ ratio = \frac{\%Sucrose\ in\ juice \times Volume\ of\ juice\ recovered}{\%Sucrose\ in\ cossettes \times Mass\ of\ cossettes}$$

(Equation 4.1)

Figure 4.2B compares the measured extraction ratio at 100%, 110% and 120% diffusion ratio. After accounting for the additional factors, it is seen that there is a significant increase in sucrose extraction as diffusion ratio increases. This is as expected, since industrial beet refineries increase the draft ratio to increase sucrose extraction, albeit at the expense of higher water requirements and energy costs. Given that the average diffusion ratio used in industry is around 110% (Asadi, 2006), this diffusion ratio was selected for further investigation.

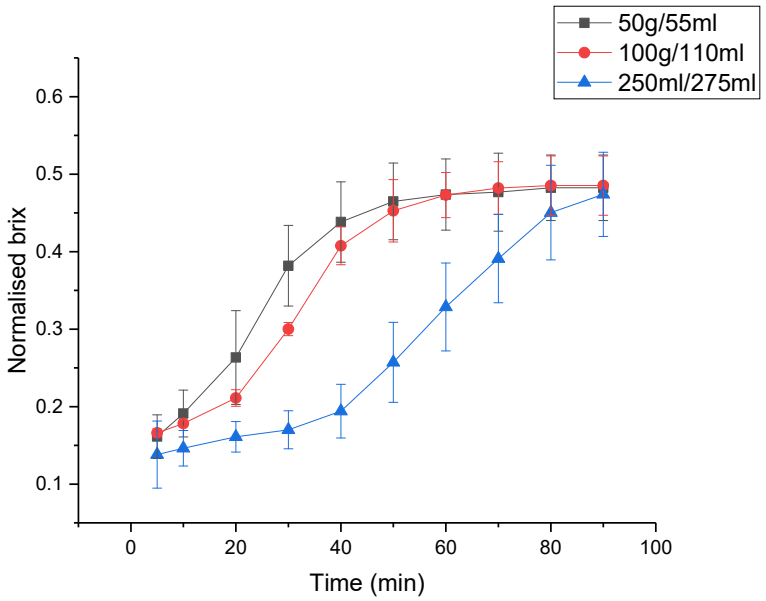

Figure 4.3: Evaluation of normalised brix) at 110% diffusion ratio at various scales in the single-stage co-current model at 70ºC, pH 5.5, 250 rpm. Error bars represent one standard deviation about the mean (n=2). Experiments performed as described in Section 2.3.3.

In addition to diffusion ratio, the scale of operation is another important factor to consider when designing the experimental model. One of the primary objectives of this project (Section 1.8) is to develop a bench-scale model of the diffusion process in which the changes and modifications to the industrial diffusion process can be trialled.

In Figure 4.3, the normalised ºBrix is measured at three different operating scales (mass of cossettes/volume of extracting solvent): 50g/55mL, 100g/110mL and 250/275mL. All have an equal diffusion ratio of 110% but represent different scales of benchtop operation and mass of material used. The size of the cossettes were uniform between scales of operation (Section 2.3.1). The extraction kinetics at 50g/55mL shows a rapid increase in extraction followed by a plateau at approximately 50 min. At the 100g/110mL scale, a similar trend is observed albeit at a lower rate. In comparison, the extraction at 250g/275mL is slower, with a significant lag phase followed by rapid extraction. Despite this, there is no significant difference in the final juice yield between the three different conditions.

The differences between the extraction rates is likely due to the physical characteristics of the single-stage model. The dimensions of the containers used in this work are 13.21 cm tall with a diameter of 11.43 cm. In each container, 50 g of cossettes are immediately submerged in 55 mL. Therefore, the cossettes denature faster as heat transfer to the cells within the sugar beet cossette is facilitated by the aqueous environment, resulting in the rapid increase in sucrose extraction. The majority of the cossettes are immediately submerged in the 100 g scale but not all, hence a similarly rapid extraction of sucrose is observed but slower than the previous scale. At the 250 g scale, the volume of cossettes is significantly greater than the volume of buffer, therefore a significantly smaller portion of the cossettes are immediately submerged. This results in a slower denaturation process and a subsequent slower rate of extraction.

Given the results of this experiment, it was determined that the 250g/275mL scale is most appropriate for future experiments. This is for several reasons. Firstly, the larger sample size provides a more accurate representation of the whole beet, whereas the smaller scales may be subject to significant sampling variation, abnormal cossettes or a disproportionate sample size isolated from a particularly sucrose rich or poor region of the beet. As such, it is most appropriate to use as large a scale possible for the bench-scale model.

Additionally, the slower rate of extraction enables greater scope for identifying any possible changes to extraction upon the addition of enzymes. In contrast, at the smaller scales, the rate of extraction is too fast and any potential benefit conferred by the enzymes may be masked by the rapid baseline rate of extraction. In summary, the optimal conditions for the single-stage model consists of a draft ratio of 110% and a scale of 250 g of cossettes with 275 mL of buffer.

4.2.2 Co-current diffuser trials with enzyme addition

Given the data in Chapter 3, it was determined that the pectin lyase Rohapect SY+ and the cellulase Rohament CL are the commercial enzyme candidates with the most likelihood of success for application in the sugar beet diffusion process. Therefore, an experiment was outlined to trial these enzymes using the single-stage model developed in the previous experiments.

The conditions of this experiment were 70°C at 250 rpm, pH 5.5 and a diffusion ratio of 110% at the 250g/275mL scale. In industry, enzymes have been trialled at concentrations of approximately 50 to 100 ppm on beet (Information provided by Innovation Manager, British Sugar, Personal communication). However, in order to maximise any potential benefit conferred by enzymatic activity, and given the stability data from Chapter 3, the enzyme concentration was increased to 1 mL of enzyme per 250 g of cossettes, or approximately 4640 ppm upon factoring an enzyme density of 1.16 g/mL in these initial trials.

Figure 4.4 summarises the data from the first trial in the single-stage model in which 1 mL of Rohapect SY+, 1 mL Rohament CL or 1 mL of each enzyme were dosed at t=0. A control experiment is included for comparison in which 1 mL of Milli-Q water is added instead of the enzyme solution. The rate of sucrose extraction is measured as normalised ºBrix. As can be seen in Figure 4.4A, there is no significant difference between the normalised ºBrix when Rohapect SY+ is added to the cossettes. Similarly in Figure 4.4B, there is no significant difference when Rohament CL is added. Finally, in Figure 4.4C, there is no significant difference when both enzymes are added to the cossettes compared to the control experiment.

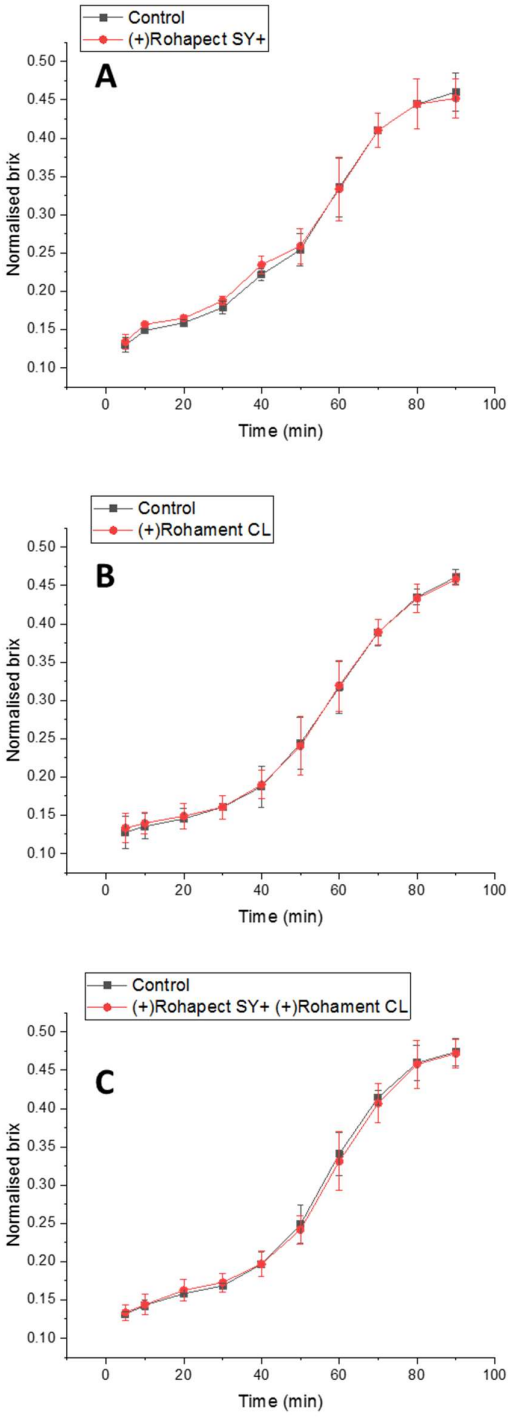

Figure 4.4: Rate of normalised brix increase when Rohapect SY+ (A), Rohament CL (B) or both enzymes are added (C) in the single-stage co-current model at t=0, 70ºC, pH 5.5, 250 rpm. In 4.4A and 4.4B, enzymes were added to a concentration of 4640 ppm. In 4.4C, a total of 9280 ppm of enzyme was added. There is no significant difference between the rate of sucrose extraction nor the final ºBrix when either

Rohapect SY+ or Rohament CL are added at t=0. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.3.

There are several reasons why this may have occurred. The enzyme activity data from Chapter 3 suggests that both Rohapect SY+ (Figure 3.3) and Rohament CL (Figure 3.4) have relatively poor activity at 70°C. Additionally, both enzymes exhibit poor thermal stability at 70°C, as both enzymes lose a significant amount of activity within 5 min of incubation at this temperature (Section 3.3.4). Therefore, these enzymes may only be active for a very short period under the diffuser conditions, after which they denature and are unable to function. To investigate this, an identical trial in which Rohapect SY+ and Rohament CL were added to the process at 50°C, the optimum temperature for these enzymes (Figure 4.5), was performed. Again, no significant improvement to the rate of extraction was measured.

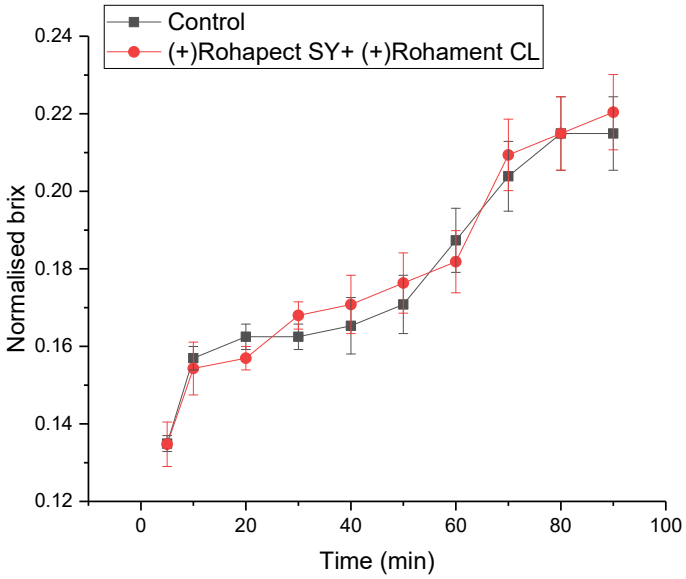

Figure 4.5: Rate of normalised brix increase when both Rohapect SY+ and Rohament CL are added in the single-stage co-current model at t=0, 50°C, pH 5.5, 250 rpm. No significant difference between the rate of extraction was observed when diffusion was operated at a lower temperature. Error bars represent one standard deviation about the mean (n = 2). Experiments performed as described in Section 2.3.3.

In addition to poor thermostability, the degree of recalcitrance within the sugar beet cossettes may be a factor limiting enzyme activity. Although the cell membrane is denatured under the diffusion conditions, the cell wall remains relatively intact. Recalcitrance is the property of plant cell walls to resist degradation by external forces

such as microbial, chemical or enzymatic activity (Zoghlami & Paës, 2019). Due to the heterogeneity of plant cell wall composition, the exact causes of biomass recalcitrance have been difficult to identify, but it is thought that properties such as lignin and hemicellulose content, cellulose crystallinity and degree of polymerisation confer significant recalcitrance (DeMartini et al, 2013, Zoghlami & Paës, 2019). Sugar beet has a relatively low content of lignin at approximately 0.15% (w/w) on whole, wet beet, suggesting that the recalcitrance may be due to other properties of the cell wall (Schiweck et al, 2007).

The poor activity and stability of these commercial enzymes, in combination with the inherent recalcitrance of sugar beet, is likely to be a significant factor to consider. To evaluate this, samples of juice were taken to analyse any detectable pectin lyase or cellulase activity as described in Section 2.2.2 and Section 2.2.3 respectively. However, no enzyme products were detected, suggesting that the recalcitrance of the sugar beet cossettes may be limiting enzyme activity.

Using this information, a secondary experiment was conducted in which Rohapect SY+ and Rohament CL were added to the cossettes at t=50 min, 70ºC, pH 5.5 and 250 rpm. This was to maximise the impact of any potential enzyme activity, since the cell membrane of the cossettes at 70ºC and 50 min are relatively denatured due to the diffusion conditions and the cell walls may be less recalcitrant than at t=0 min. Furthermore, as the enzymes exhibit poor thermal stability, adding them at the stage in which they are most likely to have an effect will maximise the short period in which the enzymes are stable. Figure 4.6 summarises the data from this experiment. Again, there is no significant difference in the normalised ºBrix between the control and the enzyme-added conditions. Therefore, it is likely that both poor thermostability and biomass recalcitrance are inhibiting significant enzyme activity and therefore the potential for improving sucrose extraction.

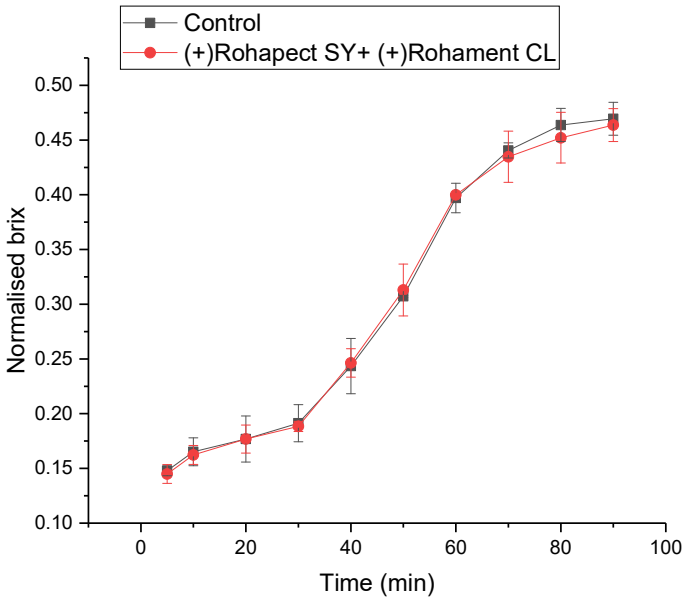

Figure 4.6: Rate of normalised brix increase when both Rohapect SY+ and Rohament CL are added in the single-stage co-current model at t=50min, 70ºC, pH 5.5, 250 rpm. Error bars represent one standard deviation about the mean (n=2). Experiments performed as described in Section 2.3.3.

4.2.3 Co-current diffuser trials with sugar beet pre-treatment prior to incubation with commercial enzymes

4.2.3.1 Aqueous pre-treatment methods

In order to overcome the recalcitrance of the sugar beet and improve the potential activity of the added enzymes, a pre-treatment stage may be necessary. These are routinely used in second-generation biofuel or platform chemical production from lignocellulosic biomass in order to improve enzyme accessibility (Kühnel et al, 2011; Baruah et al, 2018). A variety of pre-treatment techniques are available, including physical, chemical and biological methods.

To determine whether the recalcitrance of the sugar beet was inhibiting the activity of the enzymes in the previous single-stage model experiments, a pre-treatment experiment was devised to measure cellulase activity on sugar beet with or without a pre-treatment process. The three pre-treatment conditions are described in Section 2.3.2 and involved either 1) an aqueous-autoclave pre-treatment 2) an alkaline-based pre-treatment and 3) a condition designed to mimic the diffusion operation conditions at 70°C.

After each of the pre-treatment processes, the cossettes were then added to 19.5 mL of buffer and 0.5 mL of undiluted Rohament CL. A negative control was prepared by substituting the enzyme with McIlvaine buffer. Samples were then incubated in a shaking incubator at 50°C for 2 h at 120 rpm as this temperature was determined as the ideal temperature for cellulase activity in Section 3.3.3.3. Samples were taken hourly for analysis via HPAEC-PAD to determine the liberation of glucose when Rohament CL was added to each experimental condition following pre-treatment.

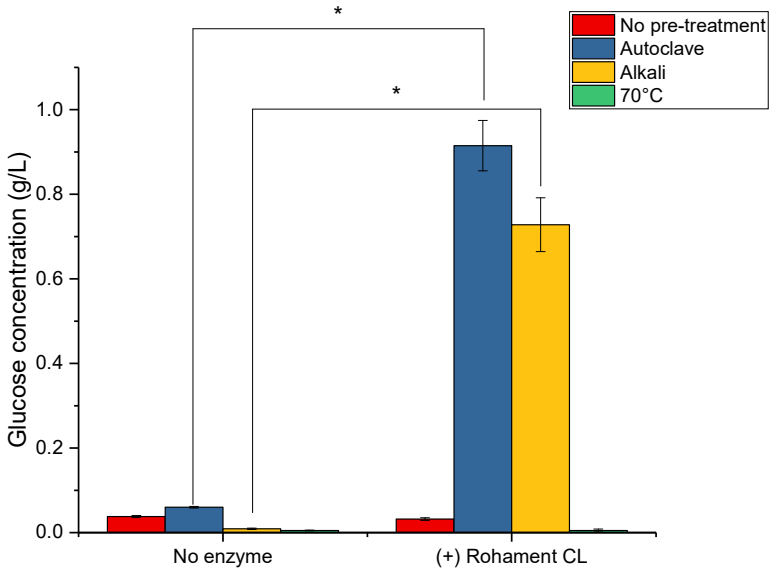

Figure 4.7: Comparison of glucose concentration in juice extracted from sugar beet cossettes at 50ºC, pH 5.5, 2 h, 120 rpm with the addition of 2.5% (v/v) Rohament CL after various aqueous pre-treatment processes. Pre-treating the cossettes via the autoclave or alkaline process significantly promotes the activity of Rohament CL, resulting in increased solubilisation of glucose (p=0.0016 and p=0.0026 respectively). Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.2.

A comparison of glucose concentration with the addition of Rohament CL to cossettes that have undergone the above pre-treatment processes can be seen in Figure 4.7. A significant increase in glucose concentration is only observed when the cossettes have been pre-treated by either the autoclave or alkaline processes. The 70°C pre-treatment process designed to replicate the standard industrial diffusion conditions does not cause an increase in glucose solubilisation.

The autoclave pre-treatment process can be considered a mild variation of the physicochemical steam explosion pre-treatment process commonly used to overcome recalcitrance and is often combined with acid or base to further enhance the processing step (Galbe & Wallberg, 2019; Obeng et al, 2019)

However, the solubilisation of glucose is not the primary objective of enzyme addition in this work and is simply an indication of positive enzyme activity post pre-treatment; therefore, it is also important to examine the effect of the enzymes in combination with the pre-treatment processes on sucrose concentration in the resultant juice (Figure 4.8).

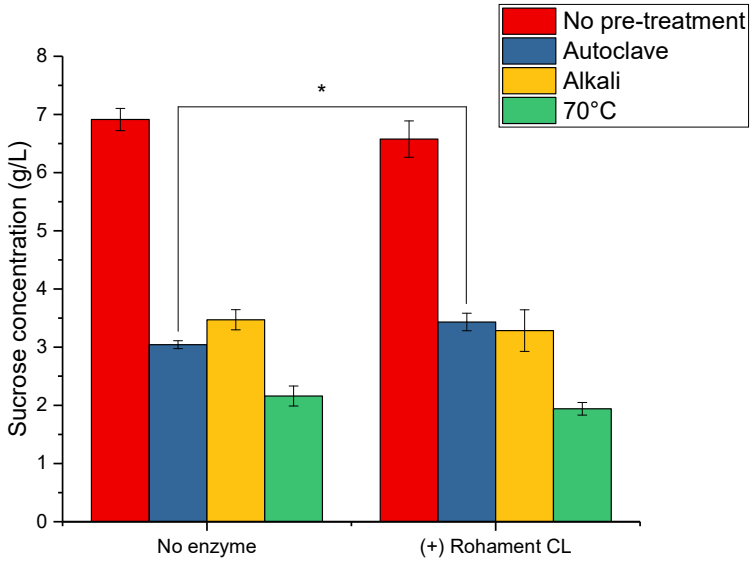

Figure 4.8: Comparison of sucrose concentration in juice extracted from sugar beet cossettes at 50ºC, pH 5.5, 2 h, 120 rpm with the addition of 2.5% (v/v) Rohament CL after various aqueous pre-treatment processes. A significant increase in sucrose concentration is observed when cossettes are autoclaved prior to incubation with Rohament CL (p=0.026). Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.2.

Figure 4.8 is a comparison between the extracted sucrose concentration with and without the addition of Rohament CL following the various pre-treatment conditions. The concentration of sucrose in each of the pre-treated samples is lower than that in the non-pre-treated samples due to the aqueous nature of the three pre-treatment methods investigated here. Each of these pre-treatment methods involve the addition of an aqueous medium to the cossettes, which results in leaching of sucrose prior to

the main diffusion process. Therefore, the comparison of sucrose concentration between pre-treatment conditions is inappropriate. However, comparisons of sucrose concentration with and without enzyme addition within the same pre-treatment methods are still valid.

There is no significant difference between the measured sucrose concentrations when Rohament CL is added under the no pre-treatment, alkaline and 70°C pre-treatment conditions. For the no pre-treatment and 70°C pre-treatment, this is expected as Figure 4.7 suggests that there is no significant cellulase activity present under these conditions as they are not robust enough to overcome the recalcitrance of the sugar beet cell wall matrix.

In contrast, the alkaline dataset is of particular interest as there is confirmed cellulase activity on alkaline-treated cossettes (Figure 4.7). Despite this, the results in Figure 4.8 still seem to suggest that cellulase activity does not improve sucrose extraction. However, a significant increase in extracted sucrose concentration is detected when Rohament CL is added to the autoclaved cossettes ($p = 0.026$). The difference between the autoclave and alkaline pre-treatment results may be due to the difference in the nature of the pre-treatment techniques, suggesting that an autoclave-based pre-treatment may sufficiently reduce the recalcitrance of the cossettes to enable enhanced sucrose extraction.

There is also the possibility that the aqueous pre-treatment methods in combination with the 50°C enzyme incubation period extracts 100% of the total sucrose available. Therefore, although positive cellulase activity is present, there may be no additional sucrose to extract. A mass balance that accounts for the maximum available sucrose for extraction, the sucrose present in the pre-treatment aqueous supernatant and the diffusion juice is required in order to determine whether 100% sucrose extraction is being achieved (Table 4-1).

Table 4-1: Mass balance between the maximum available sucrose, the sucrose in the pre-treatment aqueous supernatant and the sucrose in the diffusion juice for the cossettes treated with Rohament CL. A maximum average yield of 96.3% is achieved with the alkaline pre-treatment process. No significant improvement to total yield is achieved when Rohament CL is added to the diffusion process. Approximately 3.7% sucrose is still available for extraction post-diffusion.

Conditions	**Sucrose (g)**			**Total sucrose extracted (g)**	**Yield (%)**
	Theoretical maximum	**Pre-treatment aqueous supernatant**	**Diffusion juice**		
No pre-treatment	0.87*	N/A	0.13	0.13	15.12
Alkali (+) Rohament CL		0.78 ± 0.02**	0.07±0.01***	0.84± 0.02	96.30±2.45
Alkali (-) Rohament CL		0.78 ± 0.02**	0.07±0.003***	0.84±0.03	96.72± 2.81

* 0.87 g calculated from 5 g of cossettes with a sucrose content of 17.46% calculated via polarimetry
** 0.78 g calculated from an average concentration of 38.75 g/L via HPAEC-PAD with a total volume of 20 mL
*** 0.066 g and 0.069 g calculated from an average concentration of 3.29 g/L and 3.47 g/L respectively via HPAEC-PAD with a total volume of 20 mL

The yield of sucrose in cossettes that have not undergone a pre-treatment process is relatively low at 15.12%. This is because the diffusion process was carried out at 50°C in order to optimise enzyme activity, however this temperature is insufficient to denature the cossettes required for sufficient sucrose extraction. In comparison, cossettes that have been treated with any of the three pre-treatments have a higher yield as the pre-treatment stage causes further denaturation of the cell membrane.

A maximum average yield of 96.30% is achieved with the alkaline pre-treatment process coupled with the 50°C diffusion process. More specifically, 88.77% of the total available sucrose is extracted in the pre-treatment stage, with only 11.23% available for further extraction in the diffusion stage. Of this final 11.23%, approximately 7.5 to 7.9% is being extracted in the diffusion stage, leaving ~3% sucrose in the sugar beet pulp. No significant difference in yield is observed with or without the addition of Rohament CL.

Although the overall yield is not 100%, and therefore there is the potential for improved sucrose extraction, it may be that the small amount of sucrose available for extraction

when the enzymes are added to the diffusion stage makes it difficult to identify any significant benefit. Instead, significant differences may be observed when there is a larger concentration of sucrose available for extraction. Therefore, a non-aqueous method of pre-treatment would be more appropriate as this will overcome the recalcitrance, enabling enzymatic activity, whilst retaining the majority of the sucrose such that it is available for extraction in the primary diffusion stage.

4.2.3.2 Physical pre-treatment methods

Table 1-6 provided a compilation of numerous academic sources in which the primary objective was to optimise juice extraction from various fruit and vegetables by applying enzymes. In order to overcome recalcitrance of the starting biomass, the majority of these studies utilised a common approach in which the biomass was frozen, thawed and milled prior to incubation with the enzymes followed by pressing to isolate the juice from the pulp.

Freezing is a physical pre-treatment process that utilises the innate property of water to expand upon freezing (Jeong et al, 2016). Chang et al (2011) reported improved enzymatic hydrolysis yields on rice straw following freeze-thaw as the freezing process increases the surface area available for enzyme accessibility.

Additionally, milling is commonly used as a physical pre-treatment to reduce the particle size of the biomass and maximise the effective surface area, as well as deconstruct the bonds between lignocellulosic structures and reduce intermolecular crystallinity (Zoghlami & Paës, 2019). Although cossettes are sliced from sugar beets (Section 2.3.1), they are relatively large in comparison to the micrometer scale particles produced by milling.

Physical pre-treatment methods such as freezing and milling have particular benefits when applied within the fruit and vegetable industry as they do not produce strongly acidic, basic or toxic residues and will not pose a risk to the consumer. Additionally, as non-aqueous based treatments, no additional solvent is required to deconstruct the biomass. Therefore, the cossettes will retain the sucrose to be extracted in the primary diffusion stage.

As this method of pre-treating has successfully improved the juice yields from various fruit and vegetables in literature, an experiment was devised here to investigate whether enzyme activity can improve the sucrose extraction on physically pre-treated

cossettes. Section 2.3.2.4 describes the method for preparing physically pre-treated sugar beet.

To promote enzyme activity, a solution was prepared by adding Milli-Q water to 100 g of physically pre-treated sugar beet at a ratio of 1:1 (w/w). Enzymes were added to a concentration of 1% (v/w) enzyme to pulp. Four conditions were investigated: the separate addition of Rohapect SY+, Rohament CL and a combined addition of both enzymes. A non-enzymatic control was also prepared in which the enzyme was substituted with water.

Samples were incubated at 50°C and 100 rpm for 2 hr in a shaking incubator. At the end of each trial, the enzymes were inactivated by heating the mixture in a water bath at 90°C for 10 min. Samples were then rapidly cooled to room temperature using an ice water bath. The juice was recovered by pressing the pulp mixture, and juice yield was calculated as below:

$$Juice\ yield\ (\%) = \frac{Mass\ of\ juice\ recovered}{Mass\ of\ initial\ pulp + Mass\ of\ water\ added} \times 100 \quad \text{(Equation 4.2)}$$

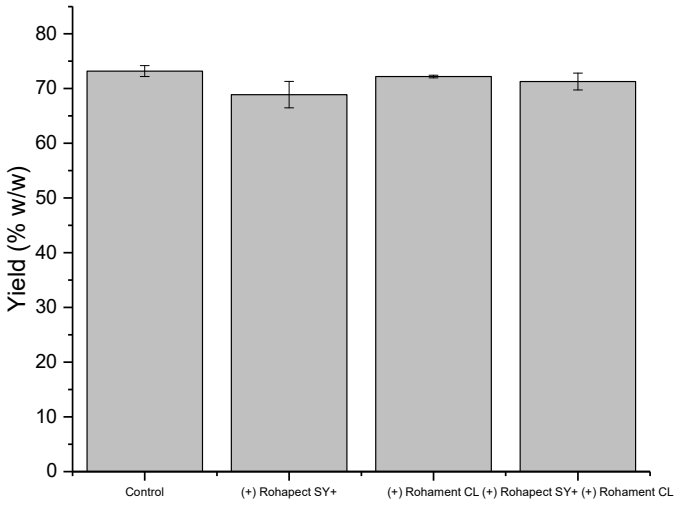

Figure 4.9: Yield of juice from freeze-thawed milled sugar beet following incubation with 1% (v/w) Rohapect SY+ and Rohament CL at 50ºC, pH 5.5, 2 h, 100 rpm followed by pressing. Error bars represent one standard deviation about the mean (n=3).

Figure 4.9 is a comparison between the juice yield recovered from freeze-thawed, milled sugar beet under the four conditions. When no enzyme is added to the mixture, an average juice yield of 73.18% is achieved. The enzymatic conditions trialled do not appear to significantly improve juice yield. This is in contrast with the literature in which significant improvements to juice yield from other fruit and vegetables are observed under similar conditions (Table 1-6).

However, juice yield is not the primary outcome of the enzyme application as an increase in sucrose concentration may be detected in a similar yield of juice. Therefore, it is important to measure the concentration of sugars in the resulting juice by ºBrix and polarimetry (Figure 4.10, 4.11).

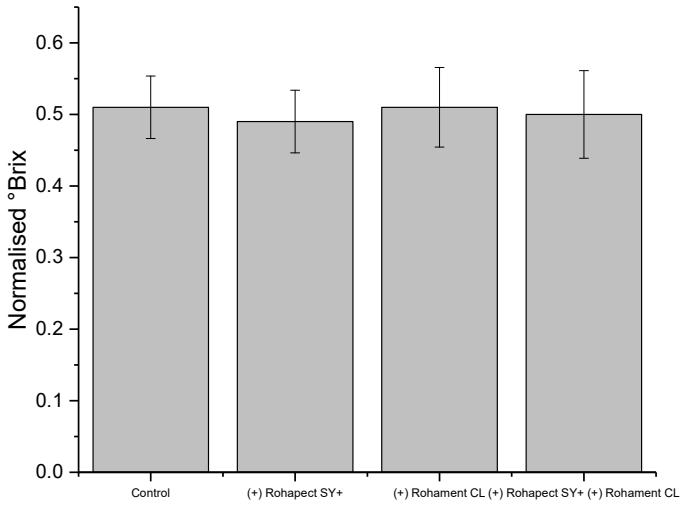

Figure 4.10: Normalised brix in the juice samples following incubation of freeze-thawed milled sugar beet with 1% (v/w) Rohapect SY+ and Rohament CL at 50ºC, pH 5.5, 2 h, 100 rpm. Error bars represent one standard deviation about the mean (n=3).

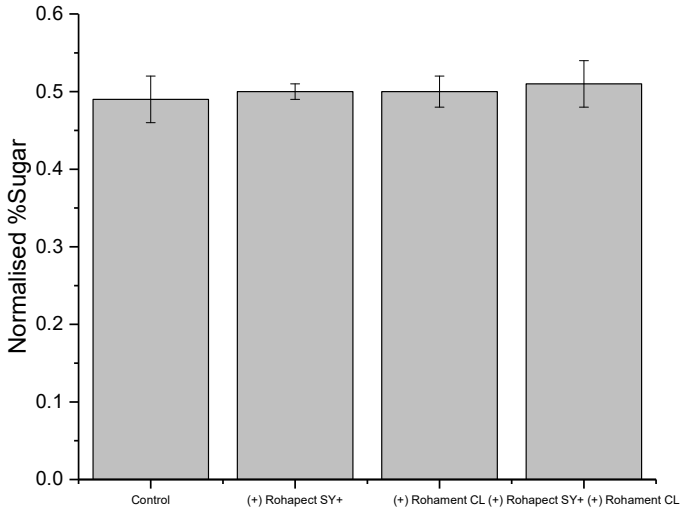

Figure 4.11: Concentration of sugars in the juice samples following incubation of freeze-thawed milled sugar beet with 1% (v/w) Rohapect SY+ and Rohament CL at 50ºC, pH 5.5, 2 h, 100 rpm expressed as Normalised % Sugar. Error bars represent one standard deviation about the mean (n=3).

Figure 4.10 is a comparison between the normalised brix in the juice samples following incubation of freeze-thawed milled sugar beet under each of the four conditions. To account for variation within the initial sucrose concentration between replicates, ºBrix was normalised against the percentage sucrose (w/w) in the cossettes determined by polarimetry. There is no significant difference between the normalised ºBrix when Rohapect SY+, Rohament CL or both enzymes are added to the mixture versus the control.

Although ºBrix is commonly used in industry as an indirect measure of sucrose concentration in juice samples, it also measures all soluble solids such as soluble proteins and salts. Therefore, Figure 4.11 measures the normalised percentage sugar (w/w) in the juice samples determined via polarimetry under each condition. There is no significant increase in the sugar concentration when treated with enzymes.

Once again, although polarimetry is a reliable method for sugar determination commonly used in industry, it also measures non-sucrose sugars such as glucose and fructose. The concentrations of non-sucrose sugars are very low in comparison to sucrose, but given that small differences in sucrose concentration may be masked by

the polarisation of non-sucrose sugar interference, HPAEC-PAD is a more accurate and precise method for directly measuring the sucrose concentration (Figure 4.12).

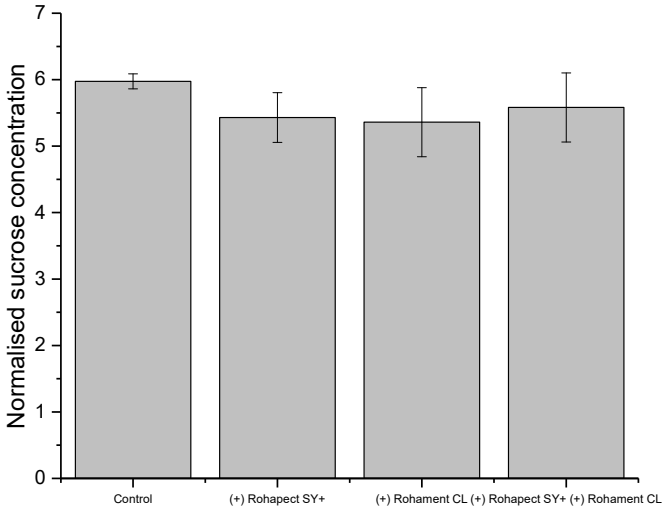

Figure 4.12: Normalised sucrose concentration in the juice samples following incubation of freeze-thawed milled sugar beet with 1% (w/v) Rohapect SY+ and Rohament CL at 50ºC, pH 5.5, 2 h, 100 rpm measured via HPAEC-PAD. Error bars represent one standard deviation about the mean (n=3).

In Figure 4.12, there is no significant difference between the sucrose concentrations in the control samples versus the enzymatically treated samples. It can therefore be concluded that Rohapect SY+ and Rohament CL are not exhibiting a positive benefit on sucrose extraction under the conditions investigated. There is the possibility that the pre-treatment process is extracting all of the sucrose, but a mass balance approach, similar to that shown in Table 4-1, suggests that approximately 12% of sucrose in the cossettes is not extracted. This is further supported by polarimetric analysis of the wet pulp produced from the extraction process.

Although there is no benefit to sucrose extraction, there is the issue of recalcitrance and whether these enzymes are able to act upon the pre-treated substrate. Therefore, HPAEC-PAD was also used to measure glucose and cellobiose concentrations for indications of cellulase activity, whilst measuring the optical density of the juice samples at 235 nm was used to measure pectin lyase activity.

HPAEC-PAD analysis suggested there was no significant difference between the glucose concentrations under the trialled conditions. Furthermore, there was no detection of cellobiose in any of the samples. This is particularly unusual for the Rohament CL conditions as this enzyme has confirmed cellobiohydrolase activity and is therefore capable of producing cellobiose from cellulose. Additionally, juice samples were acid hydrolysed and autoclaved prior to HPAEC-PAD analysis to analysis for larger glucose-derived oligo/polysaccharides produced via cellulase activity. There was no significant difference between the resulting glucose concentrations in the samples, suggesting that significant, detectable cellulase activity is not present on freeze-thawed, milled sugar beet.

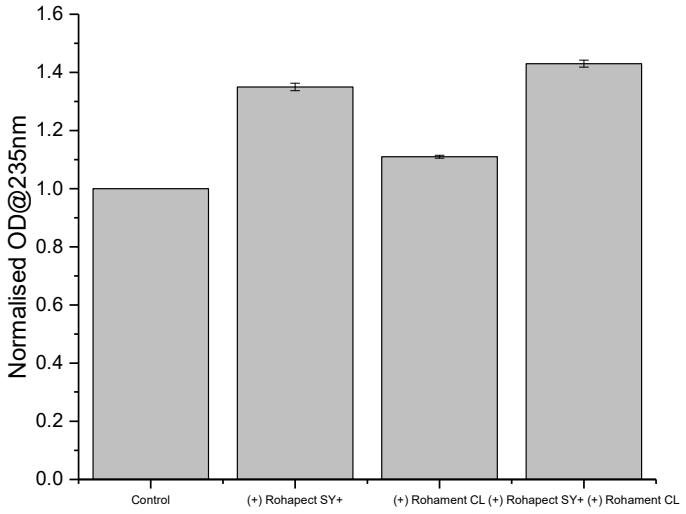

Figure 4.13: Detection of pectin lyase activity in the juice samples obtained from the incubation of freeze-thawed milled sugar beet with 1% (w/v) Rohapect SY+ and Rohament CL at 50ºC, pH 5.5, 2 h, 100 rpm. A statistically significant increase in optical density at 235 nm is observed under each of the enzymatic conditions ($p = 0.00046$, $p = 0.00069$ and $p = 0.000275$ for (+) Rohapect SY+, (+) Rohament CL and (+) Rohapect SY+ (+) Rohament CL respectively). Furthermore, a significant difference is observed between the addition of Rohapect SY+ independently versus the addition of Rohapect SY+ and Rohament CL together ($p = 0.00152$). Error bars represent one standard deviation about the mean (n=3).

Figure 4.13 presents the optical density of the juice samples at 235 nm under each of the four conditions. Optical density is normalised against the control in order to account for variation in initial cossette quality. A significant increase in optical density at 235

nm is observed under the (+) Rohapect SY+ and (+) Rohapect SY+, (+) Rohapect CL trials ($p = 0.00046$ and $p=0.000275$ respectively). This is indicative of positive pectin lyase activity, suggesting that Rohapect SY+ is able to act upon the freeze-thawed, milled sugar beet, forming the unsaturated oligogalacturonate product characteristic of lyase activity (Table 1-3).

There is also a significant increase in optical density at 235 nm when Rohament CL is added, compared to the control ($p = 0.00069$). This is unlikely due to primary cellulase activity as there was no detectable cellulase activity via HPAEC-PAD. Additionally, products of cellulase activity do not absorb strongly at 235 nm.

However, this may instead be indicative of some glucuronan lyase activity in the commercial mixture of Rohament CL (Kikuchi et al, 2019). Glucuronan lyases have been found in *Trichoderma reesei*, which is often used within the commercial enzyme industry for its high level of cellulase expression (Konno et al, 2009). Therefore, the positive increase in optical density at 235 nm may be attributed to secondary lyase activity within the commercial enzyme mixture.

A significant difference is also observed between the addition of Rohapect SY+ independently versus the addition of Rohapect SY+ and Rohament CL ($p = 0.00152$). This may suggest that the cellulase activity is able to promote pectin lyase activity, but it is more likely that this further increase in optical density at 235 nm is attributed to the aforementioned glucuronan lyase activity in Rohament CL.

In Section 1.3.2.2.3 the application of macerating enzymes for the enhancement of juice extraction from various fruit and vegetables was examined. In general, a significant increase in juice yield and °ºBrix was observed when biomass was exposed to conditions similar to those replicated in this work. Although the conditions were similar between this study and literature, no significant benefit was observed when macerating enzymes were applied to sugar beet. The only measurable difference was the detection of lyase activity, suggesting that Rohapect SY+ and Rohament CL are able to act upon the substrate, but unable to increase juice yield, °ºBrix or sucrose content.

The difference between these observations could potentially be due to differences in selected enzyme activity, although this is unlikely as several cited sources have demonstrated a positive increase in juice yield and °ºBrix with pectin lyase and

cellulase activity. The concentration of enzyme used is typically higher in this study than in literature at 1% (w/w), therefore it is also unlikely that lack of enzyme is the limiting factor. The difference may therefore be due to the structural or physiological properties of sugar beet, such as its particularly high sugar content.

4.2.4 Summary of co-current diffuser model trials

In summary, a single-stage co-current model was developed in order to investigate the effect of enzymes on various juice extraction parameters from sugar beet cossettes. No significant difference between the rate of juice extraction or the final sucrose concentration of the juice was observed when the commercial cellulase Rohament CL, or the commercial pectin lyase, Rohapect SY+, were added to the cossettes at the beginning of the diffusion process (Figure 4.4 and Figure 4.5).

As determined in Chapter 3**,** both Rohament CL and Rohapect SY+ denature relatively quickly at 70°C. Therefore, a second trial was conducted in which the enzymes were added at t = 50 min, at which point the cossettes have denatured significantly enough to rapidly lose sucrose (Figure 4.6). Again, no significant difference to rate of juice extraction or final concentration was observed. Furthermore, there were no indications of cellulase or pectin lyase activity as determined via optical density analysis at 235 nm or HPAEC-PAD.

In light of this observation, biomass recalcitrance may have been a major obstacle inhibiting enzymatic activity. Therefore, several biomass pre-treatment methods were applied to sugar beet cossettes in order to initially determine whether enzymatic activity could be detected, and subsequently whether this detectable activity resulted in an increase in sucrose concentration.

Three aqueous-based pre-treatment processes were investigated (Section 4.2.3.1). The alkaline and aqueous-autoclave pre-treatment methods were able to successfully enhance cellulase activity as significant levels of glucose were detected in the juice. A third method designed to simulate diffusion conditions by pre-incubating the cossettes at 70°C before incubating the cossettes at optimal enzyme activity temperature was unable to enhance cellulase activity, suggesting that this method is not robust enough to overcome biomass recalcitrance.

However, the data suggests that even with detectable cellulase activity under the alkaline pre-treatment, there was no increase in sucrose concentration in the diffusion

juice. In contrast, the aqueous-autoclave pre-treatment shows some indication of improved sucrose concentration. A mass balance approach (Table 4-1) confirmed that there is still remaining sucrose to be extracted, but this is lost in the wet pulp.

There are limitations to the alkaline and aqueous-autoclave pre-treatment method as they are aqueous-based and therefore extract the majority of the sucrose prior to the main diffusion process. Alternative physical pre-treatment methods such as freeze-thaw and milling are not subject to this limitation and have been successfully applied in literature, resulting in enzymatically enhanced juice extraction from various fruit and vegetable biomass. A physical pre-treatment method based on this principle was examined in Section 4.2.3.2.

No significant difference to juice yield, ºBrix, percentage sugar or sucrose concentration were observed when Rohament CL or Rohapect SY+ was added to freeze-thawed, milled sugar beet (Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12 respectively). There is also no detectable cellulase activity via HPAEC-PAD, suggesting these physical methods may not be able to disrupt the cellulose crystal matrix sufficiently to enable cellulase activity. However, positive pectin lyase, and potential glucuronan lyase, activity was detected in the juice via optical density analysis at 235 nm.

Ultimately, these experiments seem to suggest that even when residual enzyme activity is detected, there is no significant improvement to juice yield or sucrose concentration. However, given that these findings have been conducted on a co-current single-stage model, a counter-current diffusion model is required to understand whether these findings will be consistent in a model similar to one used in industry.

Although these results suggest that there is limited enzymatic activity on sugar beet without pre-treatment, trials in the counter-current diffuser will be conducted on non-pre-treated sugar beet cossettes. The purpose of the scale-down diffusion model is to mimic the industrial refinery process and the cossette quality is a critical feature to retain. This is also important as implementing a pre-treatment stage would require an additional unit operation and the objective of enzyme addition to the diffusion process is to enhance sucrose release without the addition of a discrete processing stage. These factors, in combination with the data that suggests that sucrose enhancement

on pre-treated beet with demonstrable enzyme activity is limited, are key reasons as to why non-modified cossettes will be used.

4.2.5 Scale-down counter-current diffuser design and construction

In order to evaluate the benefit of enzyme addition to the extraction of sucrose from sugar beets, it is important to develop a scale-down model that replicates the conditions of the industrial diffusion process (Section 1.3.2.1). Geometrically similar pilot scale models are already available but these are too large for the scope of this study, requiring approximately tens of kilograms of cossettes per diffusion run.

The scale-down model to be designed and constructed in this this study should be sized to fit on a normal laboratory bench. This requirement presents several constraints due to the nature of the diffusion process. Sugar beets are sliced into cossettes of a specific shape and dimension, typically V-shaped, 3-6mm wide and 30-60mm long (Asadi, 2006). Although there may be some small variations due to the differences between industrial slicing capabilities and the slicing used in this work, these dimensions are relatively fixed and impact on the surface area and diffusion kinetics.

Industrial diffusers, such as the DdS diffuser, operate with a pair of rotating screws running through the length of the diffuser (Section 1.3.2.1). Attempting to scale down the size of the diffuser and rotating screws, whilst maintaining the size of the cossettes would be incompatible as the size of the cossettes would be too large for the diffuser. Therefore, instead of developing a direct scale-down model of the counter-current screw diffuser, it was decided to opt for a more generic model that simulates the general counter-current extraction process.

Battery-style diffusers were commonly used in the sugar industry prior to the development of the modern diffusers. These function as a series of separate cells that each hold a certain mass of cossettes, essentially functioning as a sequence of extraction operations. Water is then introduced counter-current to the cossettes, transferring the water to the subsequent cell either manually or via a pump after a period of time to maintain the concentration gradient (Owens et al, 1951; Silin, 1967). These are no longer used in industry as they are laborious to operate and the single-stage-style system required numerous pauses during the process, limiting the productivity of the operation (Twitty, 2003). However, this system is more feasible to

conduct at the bench-top scale as continuous productivity is not a required factor and the size of the cells can be reduced without additional constraints on the cossette dimensions.

This type of model has also been used by Loginova et al (2011a) and Šereš et al (2017) to examine the effect of electroporation on sucrose extraction and biocide application on sucrose extraction respectively. The model by Loginova et al (2011a) is a pilot sized counter-current battery diffuser, capable of processing 6 kg of cossettes per hour (Figure 1.11). The model by Šereš et al (2017) was designed as a counter-current battery model, processing 500 g of sugar beet cossettes per diffusion run (Figure 4.14). This model was pivotal for the design of the model used in this work, as it is capable of simulating counter-current diffusion on a smaller scale.

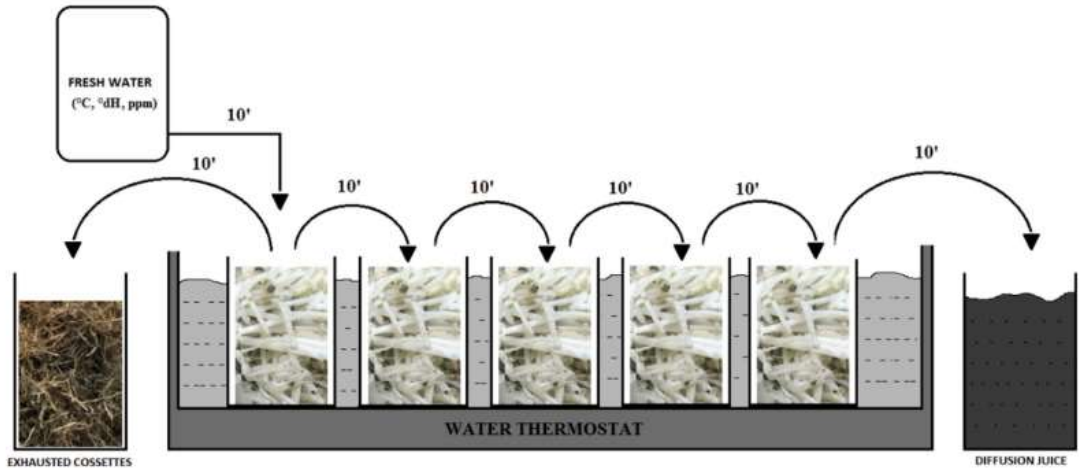

Figure 4.14: Counter-current battery model by Šereš et al (2017). Each cup holds 100g of cossettes, resulting in a throughput of 500g cossettes per run. Water is added counter-current to the movement of the cossettes in order to simulate counter-current extraction and maintain a diffusion gradient. Stirring was achieved by the addition of an IKA/CAT stirrer to mimic the movement conditions of the industrial process.

The design of the diffuser model used in this work is described and shown in Section 2.3.4. Figure 4.15 provides more detail of the actual operation of the diffuser in order to better simulate the counter-current, solid-liquid extraction process.

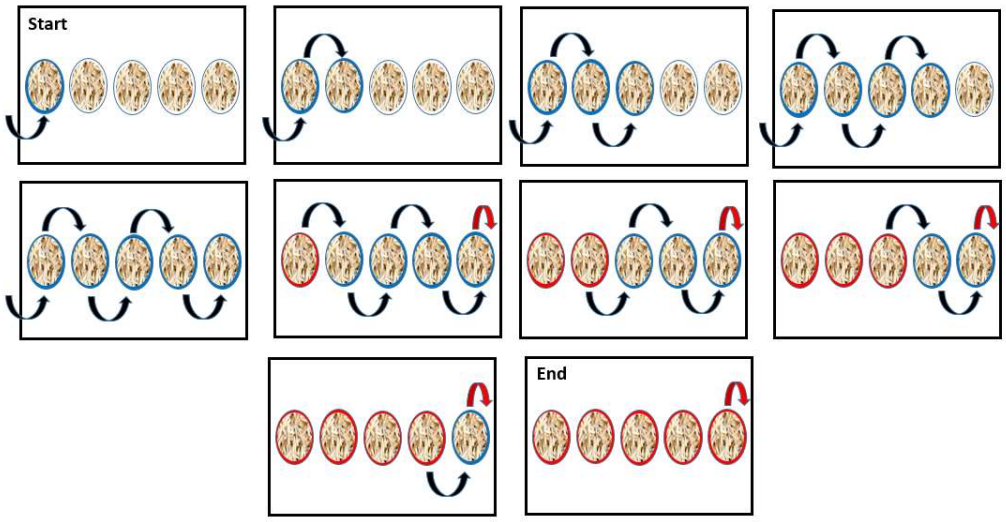

Figure 4.15: Illustration of diffuser model operation. Diffusion is initiated by the addition of 55 mL pre-heated buffer to Cup 1. Blue circles represent cups that presently contain buffer in them. After 10 min, the buffer is transferred to the next cup whilst Cup 1 is refilled with 55 mL of fresh, pre-heated buffer. This process continues up to 50 min after which the juice from Cup 5 is decanted into a glass bottle whilst the exhausted wet pulp from Cup 1 is stored in a separate container. Red circles represent Cups containing cossettes that have been processed and removed from the water bath. Similarly, red arrows represent the point at which the extraction buffer at each stage has exited the process. This process continues until each Cup has been treated five times, resulting in a total diffusion time of 90 min.

4.2.6 Scale-down counter-current diffuser operational considerations

The benchtop diffusion model can be compared with the industrial diffusion process by evaluating the critical process parameters of industrial diffusion and the impact on sucrose extraction in both instances.

4.2.6.1 Draft and diffusion ratio

As defined in Section 1.3.2.1, the draft ratio is defined as the weight ratio between raw juice exiting the diffuser and mass of cossettes entering diffuser (Asadi, 2006). The optimum draft ratio is typically between 100% and 120%. Draft ratios below this range will result in insufficient exhaustion of the cossettes and significant loss of sucrose in pulp, whilst draft ratios above this range will result in a decrease in juice purity, excessive amounts of water usage and significant strain on the subsequent evaporation process that may counter the improved sucrose extraction.

In industry, the mass of diffusion juice is not measured, and is instead calculated post-diffusion by measuring the cossette sugar content, diffusion juice sugar content, and sugar loss in pressed pulp and applying Equation 4.3.

$$Draft\ ratio\ (\%) = \frac{(Ws.c - Msl.pp)}{Ws.dif.j} \times 100$$

Where:
Ws.c = Cossette sugar content (%w/w)
Ws.dif.j = Diffusion juice sugar content (%w/w)
Msl.pp = Sugar loss in pressed pulp (%w/w) (Equation 4.3)

This approach is used in industry because the draft ratio is dictated by the diffusion loss, i.e. a desired diffusion loss in pressed pulp is pre-defined by the refinery and the draft ratio is selected to achieve that diffusion loss (Information provided by Innovation Manager, British Sugar, Personal communication). As the diffusion process is a continuous operation, this approach provides key flexibility that enables workers to implement changes to the system depending on the immediate conditions of the process. However, this is impractical to achieve in the benchtop model, as the benchtop model is not continuous and therefore this approach would be a retrospective calculation; it would also be counterintuitive to measure draft ratio as an output of the process when it is a key determinant of the other process outputs. In summary, the draft ratio is a key performance indicator rather than an adjustable set-point.

An alternative to draft ratio is the diffusion ratio, which is defined as the ratio of supply water to the ratio of cossettes entering the diffuser (British Sugar, internal communication). These are often used interchangeably in industry because it is assumed that the total masses entering and leaving the diffuser are approximately equal due to the closed nature of the system (Asadi, 2006). The diffusion ratio is more applicable to the benchtop model as the diffusion loss in pressed pulp is a flexible output rather than a fixed input (Equation 4.4).

$$Diffusion\ Ratio\ (\%) = \frac{Vol.in}{Mass.in} \times 100$$

Where:
Vol.in = Total volume of buffer added

(Equation 4.4)

Mass.in = Total mass of cossettes

As the diffusion ratio is a set-point value, the diffusion ratio can be controlled in the benchtop model by changing the volume of buffer added into cup 1 at each stage. For example, a diffusion ratio of 100% would require an addition of 50 mL buffer per stage, for a total of 250 mL, whilst a diffusion ratio of 120% would result in an addition of 60 mL buffer per stage, for a total of 300 mL

4.2.6.2 Temperature, pH and residence time

As discussed in Section 1.3.2.1, there are numerous factors to consider when determining optimum diffusion temperature. Advantages of a higher operating temperature include higher diffusion rate, enhanced cossette denaturation and lower levels of microbial activity. However, too high a temperature can result in enhanced impurity leaching and impaired pulp pressability. Conversely, lower temperatures may result in incomplete exhaustion of the cossettes and poses a risk of operating within the optimum temperature range for mesophilic microorganisms that degrade sucrose for lactic acid production. As a result, diffusion is typically operated at 70-73°C (Asadi, 2006). Temperature in the benchtop model designed here can be controlled by adjusting the temperature of the water bath in which each cup sits as required.

The pH of the process must be accurately controlled due to the activity of microbial and native sugar beet invertases, which facilitate the hydrolysis of sucrose to glucose and fructose. Given that sucrose is the primary product from diffusion, invertase activity should be reduced to a minimum to reduce product loss. Below pH 4.5, there is a risk of acidic hydrolysis of sucrose. Above pH 6.5, excessive solubilisation of pectin and other cell wall components occurs which results in purity complications and downstream purification issues (Asadi, 2006). As a result, the optimal diffusion pH is between pH 5.5 to 6.0. Diffusion juice in pure water has a natural pH of 6.0 to 6.5, so this is often reduced by dosing dilute sulphuric acid as required. In the benchtop model, pH is controlled by using McIlvaine buffer prepared at pH 5.5 as the extraction solvent as described in Section 2.3.4.

Finally, the residence time is critical because the efficiency of sucrose extraction is determined by the length of contact time between the cossettes and the water as this dictates the extent of equilibration between the two phases. In industry, this is typically between 50 and 110 min depending on the type of diffuser used (Asadi, 2006). Residence times outside of this range would result in either insufficient sucrose extraction, increased risk of invertase activity or a significant reduction in process productivity as less beet is processed per unit time. The residence time in the benchtop model can be controlled by adjusting the time allocated for each stage. 10 min/stage results in a total residence time of 50 min, with a total diffusion runtime of 90 min.

4.2.6.3 Cossette pre-heating

Cossette pre-heating is a critical step prior to diffusion, achieved in dedicated minglers or pre-scalder depending on the type of diffuser used. The purpose of this stage is to rapidly heat the cossettes up from ambient temperature to 70°C. This is for several reasons: pre-heating the cossettes accelerates the denaturation of cells, enhancing the rate at which sucrose is extracted as soon as the cossettes enter the diffuser. Pre-heating also minimises invertase activity by bypassing the temperature optima of these enzymes which are generally in the range of 30-50°C (Asadi, 2006). It is important to consider this in the benchtop model as the sucrose losses due to invertase are most prominent in the cold regions of the cossette mixer (Maarten de Bruijn, 2020c).

In industry, the pre-heating stage has an additional benefit of improving the energy efficiency of the sugar beet refinery as a whole. Raw juice leaving the diffuser needs to be cooled prior to subsequent processing. In order to recover the energy that would otherwise be lost, the raw juice is redirected to the minglers or pre-scalders. The heated raw juice mixes with the incoming fresh cossettes, heating the cossettes up to 70°C whilst cooling down to 25-30°C in exchange.

This method of pre-heating is not possible in the benchtop battery model, instead the buffer used is pre-heated to 78°C prior to diffusion. When the buffer is added to the fresh cossettes, the effect of the pre-heated buffer and the water bath cause the temperature to rapidly increase and equilibrate at 70°C within 1 to 2 min, avoiding the range of optimal invertase activity.

4.2.6.4 Dosing of additional components

In industry, the dosing of additional components including acids, bases, pressing aids and enzymes are dosed at various points in the diffuser, according to the feedback from the control system. Therefore, they can be dosed in the middle of the diffuser, in the press water returning to the diffuser or the fresh diffusion supply water. This is important to factor in when determining optimum enzyme dosage because the different addition points will result in different durations of exposure to the enzyme; for example, dosing the enzyme at the midpoint will have half the retention time of enzyme dosed in the diffusion supply water. Furthermore, dosing at the midpoint exposes the enzymes to cossettes that are already significantly exhausted, whilst dosing in the diffusion supply water results in contact with fresh cossettes.

In the scale-down model, the point at which enzymes are added will differ when compared to the industrial scale due to the difference between single-stage and continuous operation. In industry, enzymes are added continuously throughout the operation according to a designated final concentration across the entire diffuser. In the single-stage model, the addition of the enzyme must also be distributed to coincide with the final concentration. However, if the final concentration of enzyme is added at a single point, the concentration of enzyme throughout the remainder of the diffuser will not be equal. Therefore, the addition of the enzyme must be staggered to simulate continuous addition (Figure 4.16).

Additionally, although the continuous diffuser has a distinct midpoint where the cossettes are approximately 50% exhausted and the water is approximately 50% saturated, these correspond to two different midpoints in the scale-down diffuser: the water midpoint and the beet midpoint (Figure 4.17, Figure 4.18). In Figure 4.17, the extraction water is approximately 50% saturated as it has passed through 50% of the cossettes. However, the cossettes in Cup 3, where the enzyme is first added, is akin to fresh cossettes as they have not yet been in contact with water. In contrast, in Figure 4.18, the addition of the enzyme coincides with the point at which the cossettes are approximately 50% exhausted. These addition points should be considered as this, in combination with the stability of the enzymes, exposure time and integrity of the cossettes at point of entry, may affect potential enzyme activity.

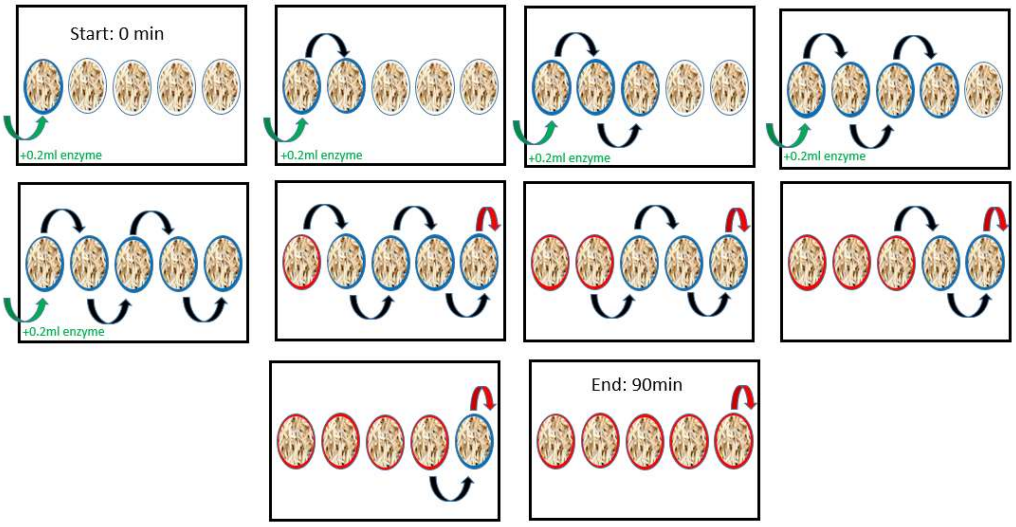

Figure 4.16: Schematic for Rohapect SY+ dosing at the beginning of diffusion in the counter-current diffuser model. 0.2mL of Rohapect SY+ is added to Cup 1 along with 55mL of buffer to initiate the diffusion. After 10min, the buffer is transferred to Cup 2 and 55 mL of fresh, pre-heated buffer and 0.2mL of Rohapect SY+ is re-added to Cup 1. This process is continued until a total of 1mL of Rohapect SY+ has been added to the total diffusion run, equal to 4640ppm of enzyme addition.

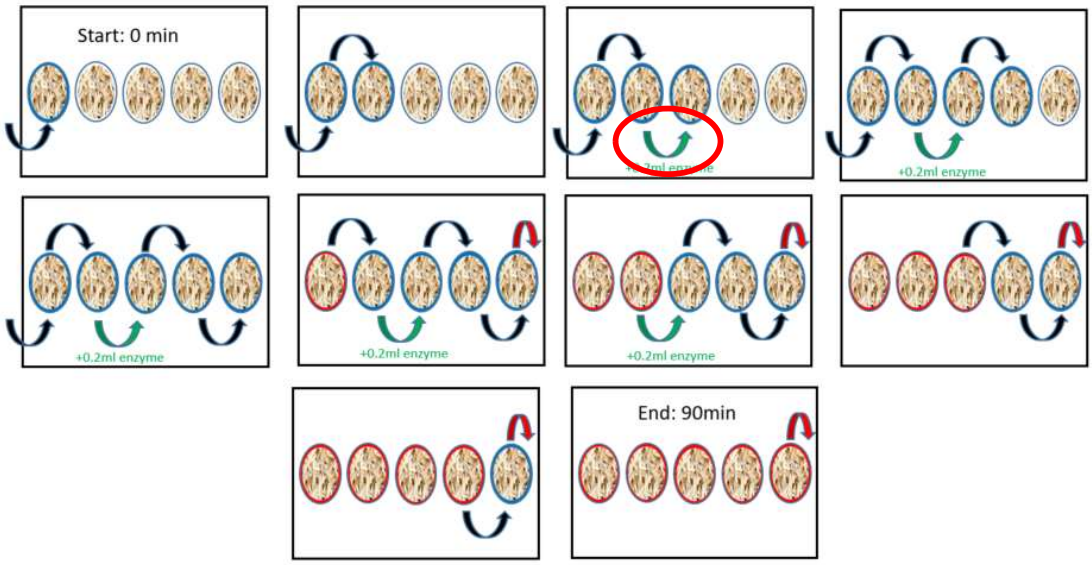

Figure 4.17: Water midpoint enzyme addition in counter-current diffuser model. Enzyme is dosed at the point where the water is midway saturated as it has been exposed to two cups of cossettes.

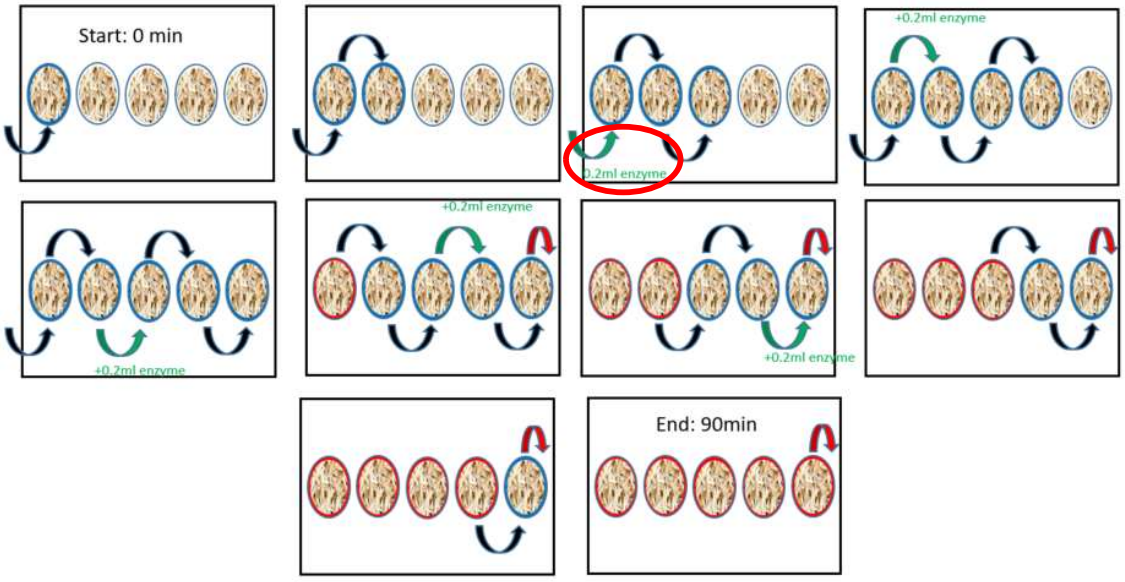

Figure 4.18: Beet midpoint enzyme addition in counter-current diffuser model. Enzyme is dosed at the midpoint where the beet is midway exhausted as it has been exposed to two passes of buffer.

4.2.6.5 Additional considerations

It is important to note that the diffuser model used in this work is an ideal system that operates at a fixed pH and fixed temperature according to the buffer used and the temperature of the water bath thermostat. In industry, the temperature, pH and residence time of the diffusion process are subject to variations due to numerous factors, including the amount of material being processed, the type of diffuser, the quality of the beet and the physical location within the diffuser at point of measurement (Section 1.3.2.1). As these variations are difficult to replicate and control, it was decided that the diffuser model would operate at fixed set points for consistency.

4.2.6.7 Limitations of initial diffuser model and development of final diffuser design

Although the initial diffuser model (Figure 2.3) is capable of conducting counter-current extraction of cossettes on the benchtop scale, there were some operational limitations with the design. Firstly, the cups containing the cossettes and extraction buffer are uncovered and therefore subject to excessive evaporation. This was difficult to control between replicates and could result in misleading differences in sucrose concentration due to the volume of juice produced. Additionally, the mixing of cossettes was a manual process in which the cossettes were stirred using a thermometer. Although care was

taken to ensure each cup was stirred equally at regular intervals, it is difficult to ensure that the mixing is standardised between replicates.

The final diffuser model depicted in Figure 2.4 alleviates these issues by use of a motor attached to a platform in which stainless steel cups containing the cossettes and buffer are attached. The rotation of the motor causes rotation of the platform, resulting in a consistent mixing of the cossettes that mimics the type of mixing observed in an RT diffuser whilst also limiting evaporative losses.

4.2.7 Scale-down counter-current diffuser validation without enzyme addition

In order to validate the counter-current diffusion model, samples were taken during each successive pass from each cup and analysed for sucrose concentration via HPAEC-PAD (Figure 4.19). Sucrose concentration consistently decreases with each successive pass, as it is highest in the first pass and lowest in the fifth pass. This is because the first pass represents the stage in the process at which the extraction buffer is in contact with fresh cossettes with the highest concentration of sucrose. The subsequent passes therefore contain less sucrose as the cossettes become progressively more exhausted.

Additionally, the sucrose concentration within the same pass increases with each cup as the extraction buffer is exposed to more cossettes. This is key to the nature of the counter-current extraction: as the extraction buffer becomes more saturated with sucrose, it remains exposed to cossettes that have a higher sucrose concentration and therefore maintains the diffusion gradient from cossettes to buffer. During the final pass, the extraction buffer contains the lowest sucrose concentration but the concentration is still higher within the relatively exhausted cossettes.

At the end of the diffusion process, the juice is collated into a single container. The concentration of the collated juice is also shown in Figure 4.19 and is 104 g/L, which is an approximate average of the juices from the five successive passes. The first and second pass have a higher concentration than the collated average as these passes were exposed to the freshest cossettes. The juice concentration is then diluted by the juice from the next three passes, forming the final diffusion juice.

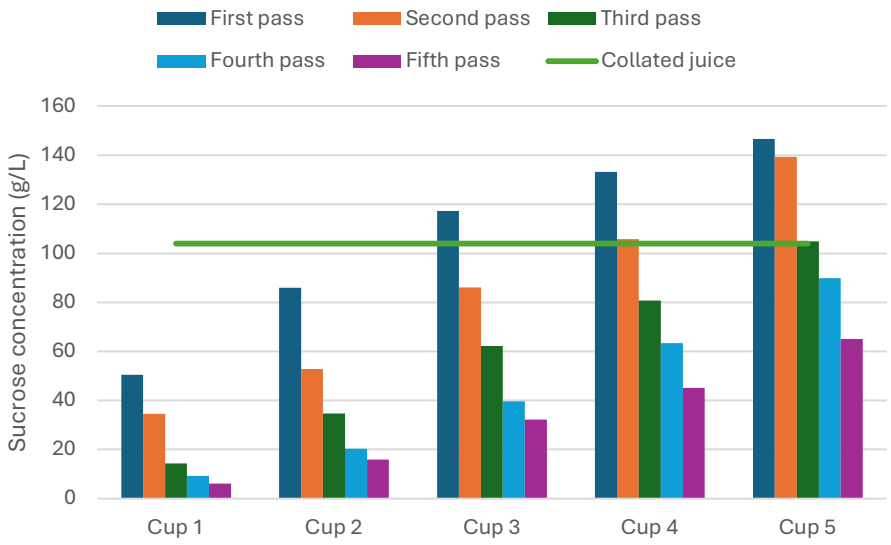

Figure 4.19: Sucrose concentration measured via HPAEC-PAD of samples taken at each stage of the diffusion process in the counter-current diffuser model. Sucrose concentration is highest in the early passes as this is when the cossettes have the highest sucrose concentration. As the process continues this sucrose content decreases as the sucrose is being leached out to the buffer, resulting in less sucrose being leached out to subsequent passes. The collated juice has a lower concentration than the maximum sucrose concentration because the collated juice is an average of the entire diffusion process. Experiment performed as described in Section 2.3.4.

To understand the effect of diffusion ratio on the counter-current diffusion model, the sucrose extraction ratio was determined at 100%, 110% and 120% diffusion ratio. This trial was similar to the diffusion ratio trials on the co-current model in Figure 4.2B. As expected, a higher volume of liquid results in a higher extraction ratio as there is more volume available for sugar extraction before an equilibrium between the cossettes and the extraction solvent is established.

By comparing Figure 4.2B and Figure 4.20 the key difference between the co-current single-stage model and the counter-current diffusion model can be observed. The extraction ratio in the counter-current diffusion model is consistently higher than the extraction ratio in the co-current diffusion model. For example, at 110% diffusion ratio, the extraction ratio in the co-current model was 0.587, whilst in the counter-current model the extraction ratio was 0.829. Similarly at 120% diffusion ratio, the extraction ratio was 0.613 and 0.871 in the co-current and counter-current model respectively.

This data suggests that the counter-current diffusion model is a more efficient method of sugar extraction from sugar beet cossettes in comparison to the co-current model.

This is due to the fundamentals of counter-current diffusion, as the concentration gradient is maintained between cossettes and juice such that the effective extraction time is longer. Therefore, this benchtop model is representative of the counter-current flow characteristics practiced in industry.

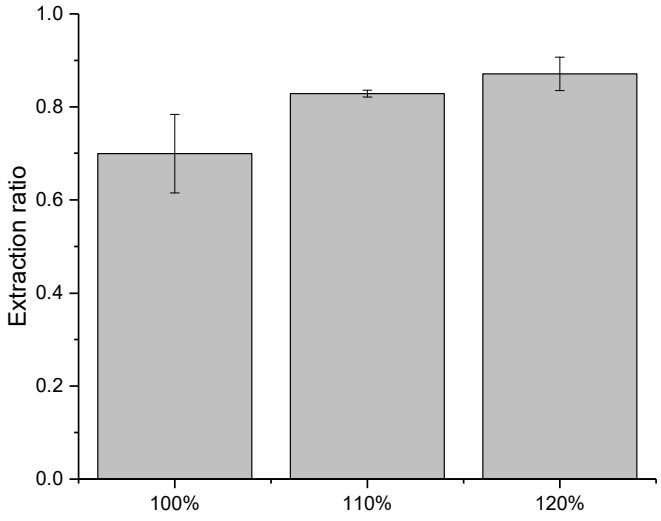

Figure 4.20: Extraction ratio at varying diffusion ratios in the counter-current diffusion model at 70ºC, pH 5.5, with an operation time of 90 min. Comparable data to the co-current single-stage model is presented in Figure 4.2B. Extraction ratio increases with higher diffusion ratio. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.4.

4.2.8 Initial scale-down counter-current diffuser model trials with Rohapect SY+ addition

Initial enzymatic trials using the counter-current diffuser model utilised Rohapect SY+ under similar conditions to the enzymatic trials in the co-current model described in Section 4.2.2, i.e. 1 mL of Rohapect SY+ for 250 g of cossettes, or approximately 4640 ppm. Figure 4.21 is a comparison of the extraction ratio between the control condition versus the addition of Rohapect SY+ at the beginning of the diffusion process, illustrated in Figure 4.16.

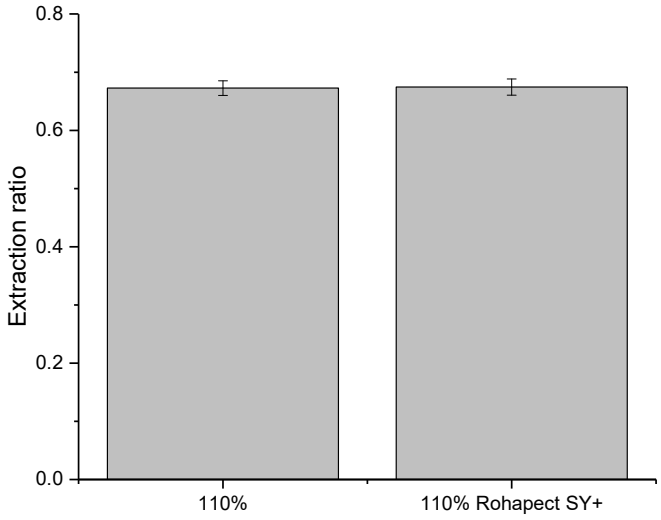

Figure 4.21: Comparison of the extraction ratio between the control condition versus Rohapect SY+ dosed at the beginning of the process in the counter-current diffuser model at 70ºC, pH 5.5, with an operation time of 90 min. There is no significant difference between the extraction ratio in the control versus when Rohapect SY+ is dosed at 4640ppm at the beginning of the diffuser. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.4.

Under these conditions in the counter-current diffusion model, no significant difference is observed between the extraction ratio of the control versus the addition of Rohapect SY+ at the beginning of the process. This is in line with the data from the co-current model (Section 4.2.2). In order to determine whether the point of enzyme addition within the counter-current diffuser plays an important role, a similar trial was conducted in which Rohapect SY+ was added at the two diffuser midpoints outlined in Figure 4.17 and Figure 4.18. The results for these further experiments are presented in Figure 4.22.

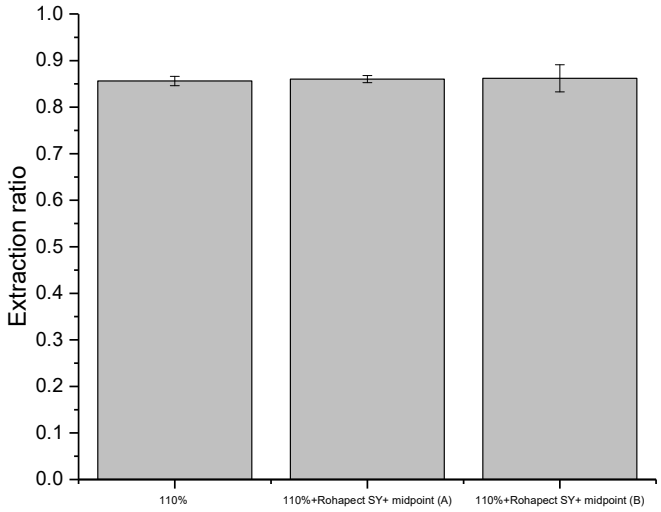

Figure 4.22: Comparison between the extraction ratio of the control condition versus the addition of Rohapect SY+ at the water midpoint (A) and the beet midpoint (B) in the counter-current diffusion model at 70ºC, pH 5.5, with an operation time of 90 min. There is no significant difference between the extraction ratio in the control versus when Rohapect SY+ is dosed at 4640ppm at the water or beet midpoints of the diffuser. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.4.

Regardless of which midpoint Rohapect SY+ was dosed at, no significant difference was measured between the extraction ratio compared to the control. Additionally, no evidence of pectin lyase activity was detected via optical density analysis at 235 nm for either addition point, further suggesting that the enzyme is not able to act upon the cossettes. This is supported by the pre-treatment experimental condition designed to mimic diffusion conditions in Figure 4.7, indicating that the enzyme is unable to overcome the recalcitrance of the cossettes under the diffusion conditions.

Increases to the efficiency of the extraction process may also be identified by measuring the residual sugar content within the wet pulp exiting the diffusion process. A positive effect of enzyme addition would result in a reduced sugar content within the wet pulp, in which case less sucrose is lost to wet pulp. However, no significant reduction in sugar content in the wet pulp was observed in these trials compared to the control scenario upon accounting for initial sugar variation.

These initial trials seem to suggest that Rohapect SY+ is unable to improve the extraction of sucrose from sugar beet cossettes in the counter-current diffuser model, regardless of which point the enzymes are added to the diffusion process. However, it is important to understand that the diffusion process is not a standalone process within the sugar beet refinery and is instead closely intertwined with the pressing process. Data from industrial trials suggests that the addition of enzymes may enhance the efficiency of the extraction process when measured as a reduction in pressed pulp polarisation, i.e. there is less sucrose remaining in the pressed pulp. This benefit would not be observed by measuring the extraction ratio or wet pulp polarisation, therefore consideration of pressing of the wet pulp should be incorporated into the counter-current diffusion model.

4.2.9 Scale-down counter-current diffuser model trials with Rohapect SY+ and Rohament CL addition

Although the data in Figure 4.2.2 indicates that there is no significant increase in the extraction ratio when Rohapect SY+ is added at the beet midpoint or the water midpoint, this experiment was repeated to validate these results. Additionally, the addition of the cellulase, Rohament CL, described in Section 3.2, was also investigated in order to determine whether cellulases can improve process efficiency in the counter-current diffusion model.

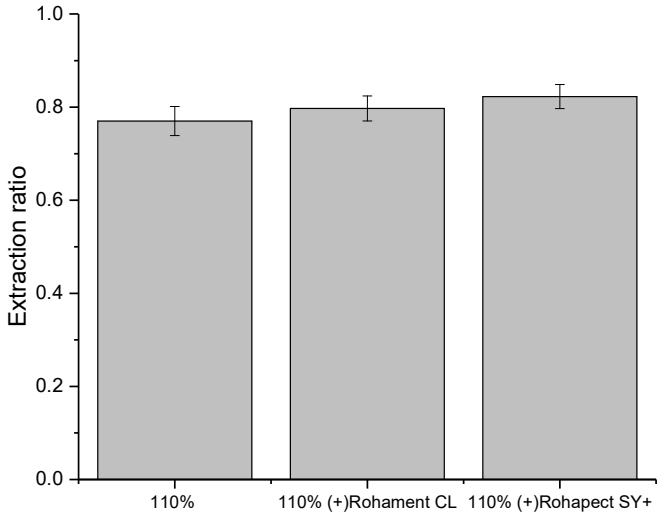

Figure 4.23: Comparison between the extraction ratio of the control condition versus the addition of Rohament CL and Rohapect SY+ at the beet midpoint at 70ºC, pH 5.5, with an operation time of 90 min. There is no significant difference between the measured extraction ratios for the control versus Rohament CL addition. However, there is a statistically significant difference upon addition of Rohapect SY+ when evaluated using an unpaired Student's t-test as described in Section 2.12 (p = 0.00999). Error bars represent one standard deviation about the mean (n=6).

According to the data presented in Figure 4.23, there is no significant difference between the extraction ratio in the control (0.770 ± 0.031) versus the addition of 1 mL Rohament CL at the beet midpoint (0.797 ± 0.027). However, there is a significant difference when Rohapect SY+ is added to the beet midpoint compared to the control (0.823 ± 0.026; p = 0.009989). The data for the Rohapect SY+ trial is in contrast to the data presented in Figure 4.22. However, the experiments were conducted identically albeit with n = 6 in Figure 4.23. An alternative reason for these differences may be due to differences in beet quality as the experiments were conducted on different single-stages of sugar beet. Furthermore, optical density analysis of the diffusion juice at 235 nm once again showed no positive lyase activity, therefore any increase in the extraction ratio is unlikely to be due to enzymatic activity.

Although the co-current trials with Rohament CL and Rohapect SY+ addition suggested that there was no significant benefit to the addition of both enzymes (Figure 4.4), a trial was conducted on the counter-current diffuser model in order to confirm whether this was also the case using the new diffuser model (Figure 4.24).

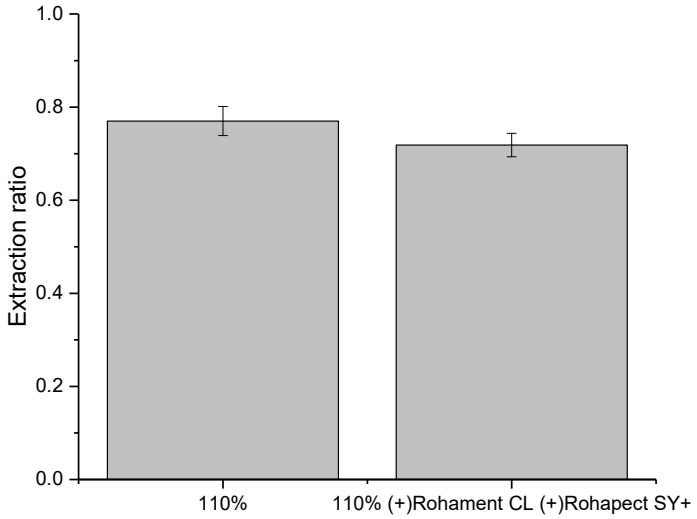

Figure 4.24: Comparison between the extraction ratio between the control condition versus the addition of both Rohament CL and Rohapect SY+ at the beet midpoint in the counter-current diffusion model at 70ºC, pH 5.5, with an operation time of 90 min. There is a statistically significant difference when evaluated using an unpaired Student's t-test as described in Section 2.12 between the extraction ratio for the control versus Rohament CL and Rohapect SY+ addition, suggesting that the addition of both enzymes dosed at 4640 ppm each may result in a decrease in extraction ratio (p = 0.01036). Error bars represent one standard deviation about the mean (n=6).

Comparison between the extraction ratio under the control (0.770 ± 0.031) and the trial in which Rohament CL and Rohapect SY+ were both added to the beet midpoint (0.719 ± 0.025) suggests that there is a statistically significant negative difference between the two conditions (p = 0.01036; n = 6). The implies that there is an overall negative effect on extraction ratio when both enzymes are added to the diffusion model. Therefore, the only positive scenario is the addition of Rohapect SY+.

In order to determine whether a further increase in Rohament SY+ concentration would facilitate greater increases to extraction ratio, the concentration of Rohapect SY+ was increased such that 2 mL of Rohapect SY+ was added to the beet midpoint, equal to a concentration of approximately 9280 ppm. This data is presented in Figure 4.25.

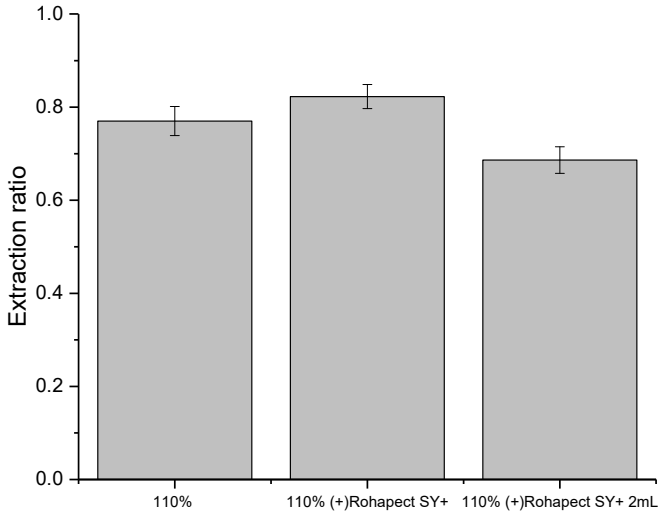

Figure 4.25: Extraction ratio upon addition of 2 mL of Rohapect SY+ at the beet midpoint in the counter-current diffusion model at 70ºC, pH 5.5, with an operation time of 90 min. In Figure 4.23, there was a statistically significant positive difference between the extraction ratio of the control condition and the addition of 1 mL of Rohapect SY+ at the beet midpoint. When the dosage of Rohapect SY+ is doubled there is a statistically significant negative difference between the control and the addition of 2 mL of Rohapect SY+ when evaluated using an unpaired Student's t-test as described in Section 2.12 (p = 0.01571). Error bars represent one standard deviation about the mean (110% and 110% (+)Rohapect SY+ n =6; 110% (+)Rohapect SY+ 2 mL n=3).

Although an increase to extraction ratio was observed when 1 mL of Rohapect SY+ was added to the beet midpoint, adding 2 mL of Rohapect SY+ to the same beet midpoint resulted in a significant decrease to extraction ratio when compared to the control (p = 0.01571). This may be indicative of some inhibitory effect when the enzyme is added at a higher concentration. However, given that neither condition resulted in detectable pectin lyase activity, it may be that these results, although repeated several times each, are due to the variations in beet quality.

In the sugar beet processing industry, an alternative measure of enzymatic benefit could be observed if the addition of enzyme resulted in a lower diffusion ratio necessary to extract the same amount of sucrose at a higher diffusion ratio without enzyme. This would result in a significant reduction in water required, which could yield environmental and economic benefits depending on the cost of enzyme required to achieve this. However, trials in the counter-current diffuser model indicate that there is

no significant improvement to extraction ratio at 100% draft, and neither Rohament CL nor Rohapect SY+ are able to increase extraction ratio to the level observed under the 110% control condition (Figure 4.26).

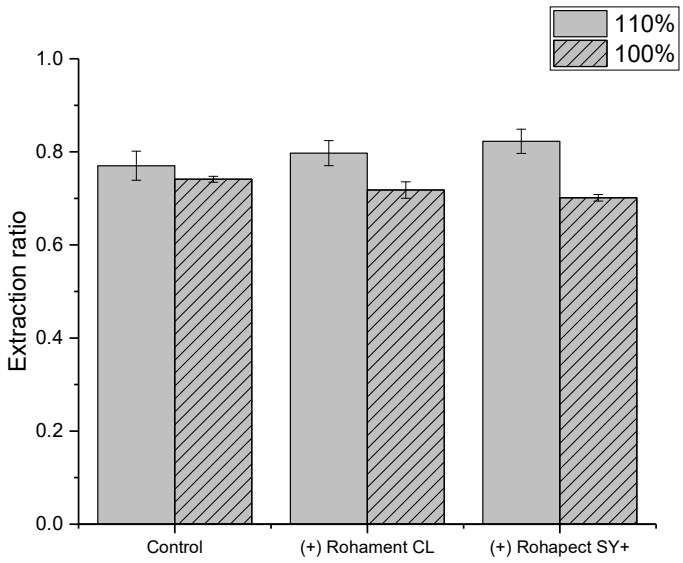

Figure 4.26: Extraction ratio at 100% and 110% diffusion ratio with Rohament CL, Rohapect SY+ or neither enzyme. Enzymes added to the counter-current diffusion model at the beet midpoint, experimental conditions of 70°C, pH 5.5, with an operation time of 90 min. No significant improvement to extraction ratio was observed at 100% diffusion ratio. Adding Rohapect CL or Rohament CL at a lower draft does not result in similar extraction ratios observed at 110% diffusion ratio. Error bars represent one standard deviation about the mean (110%, 110% (+) Rohament CL, 110% (+) Rohapect SY+ n = 3; 100%, 100% (+) Rohament CL, 100% (+) Rohapect SY+ n = 6).

The data thus far suggests that there is insufficient evidence to conclude that the addition of Rohapect SY+ or Rohament CL to the diffusion process improve the extraction of sucrose from sugar beet cossettes. However, it is important to understand that the diffusion process is not a standalone process within the sugar beet refinery and is instead closely intertwined with the pressing process. Data from industrial trials suggests that the addition of enzymes may enhance the efficiency of the extraction process when measured as a reduction in pressed pulp polarisation, i.e. there is less sucrose remaining in the pressed pulp. This benefit would not be observed by measuring the extraction ratio or wet pulp polarisation, therefore consideration of pressing of the wet pulp should be incorporated into the counter-current diffusion model.

4.2.10 Impact of enzyme addition on the pressing process

Potential improvements to the overall extraction efficiency of sucrose may be detected from the pressing process rather than the diffusion process. Although Rohament CL and Rohapect SY+ are unlikely to be exhibiting any significant enzymatic activity on the sugar beet substrate due to a combination of biomass recalcitrance and poor thermal stability, there is the possibility that these enzymes may be acting as a pressing aid, improving the pressability of the wet pulp and reducing residual sucrose in pressed pulp.

Typically, calcium derivatives such as gypsum or calcium chloride are used as pressing aids as these promote the formation of cross-links between the pectin chains, forming a pectin gel that improves the rigidity of the overall pulp structure (Buttersack et al, 1992; Asadi, 2006). However, Rohament CL and Rohapect SY+ do not contain calcium and therefore, any positive improvement to pressability would be due to an alternative mode of action.

In order to determine the effect of Rohament CL and Rohapect SY+ on the pressing process, 100 g of wet pulp was obtained immediately after the diffusion process at 110% diffusion ratio, with enzyme addition at the beet midpoint. The wet pulp was pressed using the manual press shown in Figure 2.5 in order to simulate the combined diffusion and pressing process in industry. After pressing, the measured parameters were the volume of pressed pulp water and the mass of residual pressed pulp (Figure 4.27).

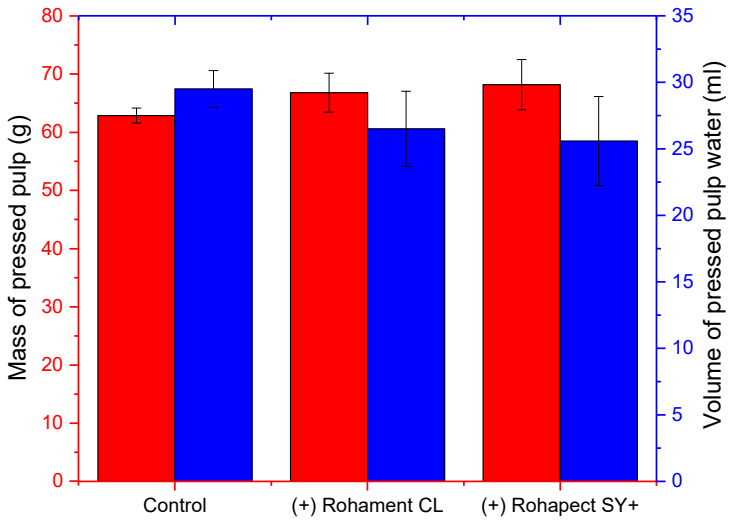

Figure 4.27: Mass of pressed pulp (in red) and volume of pressed pulp water (in blue) between the control, addition of Rohament CL and addition of Rohapect SY+. Enzymes added to the counter-current diffusion model at the beet midpoint, experimental conditions of 70°C, pH 5.5, with an operation time of 90 min. For the mass of pressed pulp, there is a significant increase when Rohament CL or Rohapect SY+ are added to the diffusion process ($p = 0.03653$ and $p = 0.02819$ respectively). For the volume of pressed pulp water, there is a significant decrease when Rohapect SY+ is added ($p = 0.03311$). Statistical analyses evaluated using an unpaired Student's t-test as described in Section 2.12. Error bars represent one standard deviation about the mean ($n = 6$). Experiments performed as described in Section 2.3.4 and Section 2.3.6.

There is a significant increase in the mass of pressed pulp when 1 mL of Rohament CL or Rohapect SY+ is added to the diffusion process ($p = 0.03653$ and $p = 0.02819$ respectively). This implies that both enzymes are affecting the pressing process significantly. However, this is not a positive benefit and is actually opposite to what would be desired: improvements to the pressing process would result in a lower mass of pressed pulp as more juice is pressed out, reducing the mass of the residual pulp. This is corroborated by the volume of pressed pulp water data, as there is a significant decrease in the volume of pressed pulp water when Rohapect SY+ is added ($p = 0.03311$).

Although the mass of pressed pulp does not reflect a positive benefit from the addition of Rohament CL or Rohapect SY+, the pressed pulp polarisation should also be assessed as this is how the effect of enzymes on pressing is measured in industry.

When normalised against cossette sugar content, the pressed pulp percentage sugar was 0.239 ± 0.0348, 0.254 ± 0.0536 and 0.267 ± 0.0692 for the control, Rohament CL trial and Rohapect SY+ trial respectively (n = 6) (Table 4-2). There was no significant reduction in pressed pulp sugar content, suggesting that neither Rohament CL nor Rohapect SY+ reduce the amount of sucrose remaining in the pressed pulp, and therefore do not improve the efficiency of the pressing process.

Table 4-2: Average sugar concentrations in the cossettes (%$Sugar_{coss}$) and pressed pulp (%$Sugar_{pp}$). %$Sugar_{pp}$ is normalised against %$Sugar_{coss}$ to compare the sugar content in the pressed pulp, accounting for variations in initial sucrose concentration. There is no significant difference between the normalised sugar content in the pressed pulp when 1 mL of Rohament CL or Rohapect SY+ is added to the diffusion process (n = 6).

	%$Sugar_{coss}$	**%$Sugar_{PP}$**	**Normalised %$Sugar_{PP}$/%$Sugar_{coss}$**
Control	14.55	3.47	0.239 ± 0.0348
(+) Rohament CL	16.16	4.10	0.254 ± 0.0536
(+) Rohapect SY+	15.24	4.03	0.267 ± 0.0692

One possible explanation for this is that the manual press does not exert enough force to press the wet pulp and therefore the data presented is within the experimental noise. Industrial presses utilise a compacting screw mechanism to achieve pressures in the region of 10-12 bar. This is difficult to achieve with a simple manual press. To investigate this, a separate trial was conducted in which calcium chloride, a standard pressing aid, was added to the diffusion process as a positive control. Calcium chloride was added at a concentration of 0.2% (w/w) which is within the dosing range in industry. However, there was no significant difference to the mass of pressed pulp or volume of pressed pulp water versus the control (data not shown).

Additionally, the pressing efficiency could be evaluated by comparing the dry substance of the wet and pressed pulp with those found in industry. Wet and pressed pulp have an average dry substance of 10% and 25% respectively. With pressing aids, pressed pulp dry substance can be increased to 35% (Asadi, 2006). However, using the control condition as an example, the average dry substance for wet and pressed pulp was 9.44% ± 0.69 and 10.94% ± 1.08 respectively (n = 6). The wet pulp dry substance is within the expected range of 10%; however, the dry substance of the pressed pulp is significantly lower than observed in industry. Therefore, these pressing

studies should ideally be conducted using a pneumatic or hydraulic press that is able to produce pressures similar to industry levels.

The effect of macerating enzymes on the pressing process is not well studied. The only previous work conducted on this was by Buchholz & Matalla (1987) and Gailing et al (2000). Bucchols & Matalla (1987) observed a significant increase in dry matter of pressed pulp when commercial enzymes containing pectin methylesterase, galacturonase, arabanase and glucosidase activity were added; however, this required high concentrations of enzyme and was therefore uneconomical. Gailing et al (2000) categorised these macerating enzyme activities into enzymes that had positive effects on pressing and enzymes that had negative effects on pressing. Pectin lyase, pectin methylesterase and cellulase had an overall negative effect on pulp pressability, whilst polygalacturonase, arabanase and xylanases increase the pressing efficiency.

Gailing et al (2000) therefore concluded that optimal sugar beet pressing requires a balance between pulp porosity and pulp rigidity. Polygalacturonases improve pressability at low concentrations by improving porosity, but at high concentrations a decrease in pressability is observed as the decrease in rigidity outweighs the increase in porosity. This could potentially explain why Rohament CL and Rohapect SY+ has a negative effect on pulp pressability, although given that there was no detectable cellulase or pectin lyase activity, this is unlikely.

However, it is important to note that both groups conducted their experiments on pressed pulp, i.e. sugar beet pulp that had already been pressed following diffusion, containing a dry matter of 23.5% (Buccholz & Matalla, 1987). Gailing et al (2000) do not specify the exact dry matter content, but it can be reasonably assumed that their material is either pressed pulp with a dry matter of 23.5%, or dried pulp with an approximate dry matter of 90%. This is in contrast to wet pulp that contains approximately 10% dry matter. The authors claim that an increase in pressed pulp dry matter can be achieved but this is inaccurate, as the starting material has already been pressed. Gailing et al (2000) rehydrate the pulp by pre-incubating the pressed pulp in water containing an excess of calcium sulphate, but this is not the same as raw wet pulp as it has been through the pressing process, which could be considered a

mechanical pre-treatment that decreases the pulp cell wall recalcitrance and improves its susceptibility to enzyme activity.

In contrast, the goal in this work was to determine whether these enzymes can affect the pressability of fresh, wet pulp. This is fundamentally different from the previous two studies as the starting material is structurally different. Additionally, the previous studies allude to a separate incubation stage in which the operating conditions can be optimised specifically for enzyme activity. In contrast, a one-pot process was attempted here whereby the enzymes are added directly to the diffusion process, eliminating the need for a separate processing stage.

4.3 Summary

As described in Section 4.1, the aims of this chapter were to explore the application of selected enzymes for enhanced sucrose release from sugar beet cossettes and to design and construct a counter-current diffuser model in which to further evaluate the commercially available enzymes.

In this chapter, two commercial enzymes, Rohapect SY+ and Rohament CL were trialled on the sugar beet diffusion process to determine whether sucrose extraction could be improved. Initial trials were conducted on a co-current, single-stage extraction model using a shaking incubator operated at 70°C and pH 5.5 for 90 min, similar to the conditions observed in industry. No improvements were found to either the rate of extraction or the final sucrose concentration when 4640 ppm of Rohament CL or Rohapect SY+ were added to the cossettes in the co-current model (Figure 4.4 and Figure 4.5). An additional trial was conducted where the enzymes were added at t = 50min, midway into the process for two reasons: firstly, the cossettes are more denatured and may have been more susceptible to enzymatic activity and secondly to maximise potential enzyme activity as the thermal stability of Rohament CL and Rohapect SY+ at 70°C is poor. However, this also did not have an effect (Figure 4.6) and no cellulase or pectin lyase activity was detected in the extracted juice.

One of the key reasons as to why there was no detectable enzyme activity could have been due to biomass recalcitrance. To investigate this, an aqueous-autoclave, alkaline pre-treatment and a 70°C incubation designed to mimic the industry conditions followed by diffusion at 50°C was conducted to determine whether these pre-treatment methods could facilitate enzyme activity (Section 4.2.3).

The aqueous-autoclave and alkaline pre-treatments were able to significantly increase Rohament CL activity (Figure 4.7), detected as a function of glucose concentration in the resulting juice. The 70°C incubation was unable to facilitate any enzyme activity as it was too mild to overcome the inherent recalcitrance.

Despite positive Rohament CL activity, significant improvements to sucrose concentration were not detected in the alkaline pre-treatment and only detected under the aqueous-autoclave condition (Figure 4.8). The alkaline pre-treatment control released a higher concentration of sucrose than the aqueous-autoclave condition, so it could be concluded that the alkaline pre-treatment was extracting 100% of the available sucrose and therefore no further improvement could be achieved by adding Rohament CL. However, a mass balance approach concluded there was approximately 3% sucrose remaining in the pulp so this is not the case (Table 4-1).

The aqueous-autoclave and alkaline pre-treatment are limited in their application by the aqueous-nature, resulting in a significant amount of sucrose extracted prior to the diffusion process. Therefore, a physical pre-treatment process involving freeze-thawing and milling was evaluated. This method of pre-treatment was commonly used in numerous literature sources for improving juice extraction from various fruit and vegetables.

No improvements to juice yield, ºBrix, % sugar or sucrose concentration were observed when Rohament CL, Rohapect SY+ or both enzymes were added to freeze-thawed milled sugar beets (Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12). Interestingly, lyase activity was detected under each of the enzymatic conditions (Figure 4.13). Lyase activity in Rohapect SY+ is attributed to pectin lyase activity, however the lyase activity in Rohament CL may have been indicative of an undeclared lyase activity, such as glucuronan lyase. This data seems to suggest that even with positive enzyme activity, there is no improvement to the extraction properties in sugar beet diffusion.

These studies were conducted in the co-current model. However, extraction kinetics and properties are considerably different in counter-current diffusion routinely used in industry. Therefore, a benchtop counter-current diffusion model was designed and constructed inspired by the battery-diffusion model for the purpose of investigating enzyme addition to the diffusion process without the need for industry-scale resources.

Several validation experiments confirmed that the model operates in a counter-current manner.

Initial enzymatic trials with Rohapect SY+ confirmed that there was no improvement to extraction ratio when Rohapect SY+ was added to the beginning, water midpoint or beet midpoint of the process (Figure 4.22). This experiment was later repeated whilst also examining the addition of Rohament CL at the beet midpoint in a separate trial. Interestingly, a significant increase to extraction ratio was observed when Rohapect SY+ was added to the beet midpoint (Figure 4.23; $p = 0.00999$; $n = 6$). This is in contrast to the preliminary trial (Figure 4.22) and could have been due to the differences in beet quality. It is important to note that no positive lyase or cellulase activity was detected in any of these trials. Furthermore, no significant improvements to extraction ratio were observed when both Rohament CL and Rohapect SY+ were added, nor when Rohapect SY+ concentration was doubled.

Thus far, there is no indication of positive benefit when Rohament CL or Rohapect SY+ was added to the diffusion process. As there was also no detectable enzyme activity, any positive benefits observed in industry could have been due to a non-enzymatic side effect of adding the enzymes rather than as a direct consequence of enzyme activity.

Given that in industry, the diffusion process is intertwined with the pressing process, it would be improper to consider the diffusion process as the only potential site of enzymatic enhancement. Industrial trials have observed a reduction in pressed pulp polarisation when enzymes are added to the diffusion process, indicating that less sucrose remains in the pulp following pressing. Therefore, there is some evidence to suggest that the enzymes may have been acting as a pressing aid to improve the pressing process. Therefore, a manual press was used to press the wet pulp immediately after diffusion in order to mimic the combined diffusion and pressing process.

When Rohament CL and Rohapect SY+ are added to the diffusion process, an increase to the mass of pressed pulp ($p = 0.03653$ and $p = 0.02819$ respectively, $n = 6$) and a significant decrease to the volume of pressed pulp water when Rohapect SY+ is added ($p = 0.03311$) is observed (Figure 4.27). However, this is the opposite of what

one would expect, as a positive benefit would be observed as a reduction in mass of pressed pulp and an increase to pressed pulp water.

This contradictory data may have been due to the type of press used in this work. The manual press was unable to exert similar pressures to those exerted in industry, and therefore the pressing data could have been noise. This is supported by the dry substance of the wet and pressed pulp, as the pressed pulp produced using the manual press contains considerably more moisture than the pressed pulp produced in industry.

To conclude, the two commercial enzymes evaluated in this chapter, Rohament CL and Rohapect SY+, do not appear to have any significant benefit on the extraction of sucrose from sugar beets. They are unable to act on fresh sugar beets, likely due to a combination of poor thermal stability and biomass recalcitrance. However, when these factors are overcome by various pre-treatment methods followed by diffusion operated at the optimum temperature for Rohament CL and Rohapect SY+ activity, no significant increases to sucrose extraction are observed. This, coupled with the fact that there is detectable enzymatic activity, suggests that the enzymes are unable to improve sucrose extraction even when enzyme activity is present under optimal conditions. Positive benefits of enzyme addition may be observed in the pressing process, however the manual press used in this work was unable to replicate industrial conditions and therefore the findings may not be corroborated in industry.

Given the low levels of residual enzyme activity in the commercial enzyme preparations under industrial diffusion conditions, subsequent work aimed to explored the production and application of a novel thermostable enzyme in the counter-current diffuser model.

Chapter 5

Expression, characterisation and application of a thermostable pectate lyase to enhance sucrose extraction

5.1 Introduction

In Chapter 4, the application of two commercial enzymes; Rohament CL, a broad-range cellulase and Rohapect SY+, a dedicated pectin lyase, was trialled in a novel benchtop diffuser model designed to replicate the conditions of the sucrose extraction process from sugar beet cossettes. The enzymes were unable to improve the extraction efficiency of the process when added directly to the diffuser (Section 4.2.9). Furthermore, the enzymes were unable to act on the substrate without an additional pre-treatment step, as indicated by HPAEC-PAD and optical density analysis (Section 4.2.3).

One potential explanation for this is the poor thermal stability of these commercial enzymes. Data from Chapter 3 confirms that both Rohament CL and Rohapect SY+ denature within the first few minutes of incubation at 70°C and therefore have little to no residual activity once added to the diffuser. Experiments at a lower operating temperature within the range of the commercial enzyme optima also resulted in no significant improvement, suggesting that thermal stability was not the only inhibitory factor and pre-treatment studies confirmed that biomass recalcitrance was a significant element to consider.

In order to confirm whether enzymes can improve the extraction process at 70°C, novel, thermostable enzymes that are able to maintain activity throughout the duration of the diffusion process were considered as a possible solution.

In this study, a novel thermostable pectate lyase was selected as the candidate enzyme of choice. Alternative available thermostable enzymes such as pectin methylesterases in combination with polygalacturonases were considered. However, these were not selected for further study due to concerns with the release of methanol and subsequent toxicity in a food context (Blumenthal et al, 2021).

The aim of this chapter is therefore to characterise the activity profile of a novel, thermostable pectate lyase and explore the application of a novel, thermostable pectate lyase on the diffusion process using the models developed in Chapter 4 to evaluate whether the thermostable pectate lyase can improve sucrose extraction from sugar beets. TMA14 is a novel thermostable pectate lyase developed by researchers in the UCL Department of Biochemical Engineering and originally isolated from *Thermotoga maritima* DSM 3109 found in anaerobic marine mud.

5.2 Results

5.2.1 TMA14 Expression

The gene encoding for TMA14 was first transformed into *E. coli BL21 (DE3)* for expression and characterisation studies. Expression materials and methods can be found in Section 2.11. To confirm enzyme expression, E. coli BL21 (DE3) was first grown in a 300mL shake flask and the protein fraction from the cell lysate was analysed by SDS-PAGE. Figure 5.1 shows the resulting SDS-PAGE gel which indicates overexpression of a protein at around 37 kDa. TMA14 has an approximate molecular weight of 37 kDa, which coincides with the predicted molecular weight determined via genome mining. These results confirm successful expression of the TMA14 enzyme.

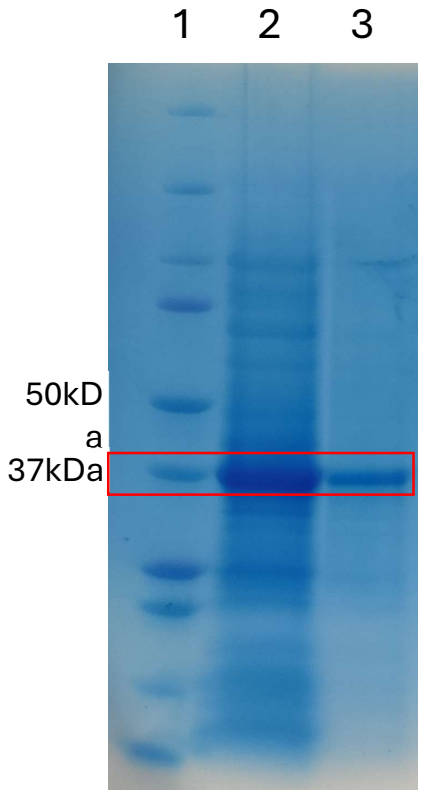

Figure 5.1: SDS-PAGE analysis of TMA14 expressed in E. coli BL21 (DE3). Lane 1: Precision Plus Protein Dual Color Standard (BioRad, USA). Lane 2: Crude lysate obtained directly from cell lysis. Lane 3: Crude lysate diluted 1 in 10 in Milli-Q water. TMA14 has an approximate molecular weight of 37kDa. Expression performed as described in Section 2.11.

5.2.2 TMA14 Characterisation

5.2.2.1 TMA14 activity profile on polygalacturonic acid

In order to determine the temperature-activity profile of TMA14, characterisation studies were first conducted using polygalacturonic acid as substrate (Figure 5.2). It is important to note that TMA14 is a pectate lyase and therefore requires the addition of calcium, as this is required for the binding of the substrate (Pickersgill et al, 1994). Preliminary characterisation assays without the addition of calcium resulted in no significant levels of activity, regardless of reaction conditions (data not shown).

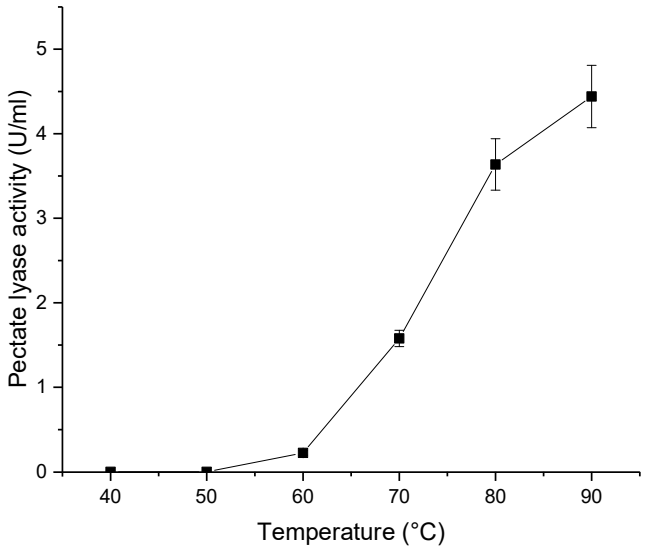

Figure 5.2: Temperature-activity profile for TMA14 on 0.5% (w/v) polygalacturonic acid with the addition of 1mM $CaCl_2$ at pH 5.5. Reaction time of 30 min. Optimum activity within the experimental conditions is achieved at 90°C, with 36% activity present at 70°C. No activity was detected at 40°C and 50°C. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.2.2.

TMA14 exhibits optimum activity on polygalacturonic acid at pH 5.5 with 1 mM $CaCl_2$ at 90°C. At 70°C, 36% activity is retained and minimal activity is present at temperatures less than 50°C. This suggests that TMA14 requires high temperatures for maximum activity.

As calcium is required for pectate lyase activity, it is also important to determine how much calcium should be added for optimal activity. Figure 5.3 summarises pectate lyase activity on polygalacturonic acid at 70°C at different calcium concentrations.

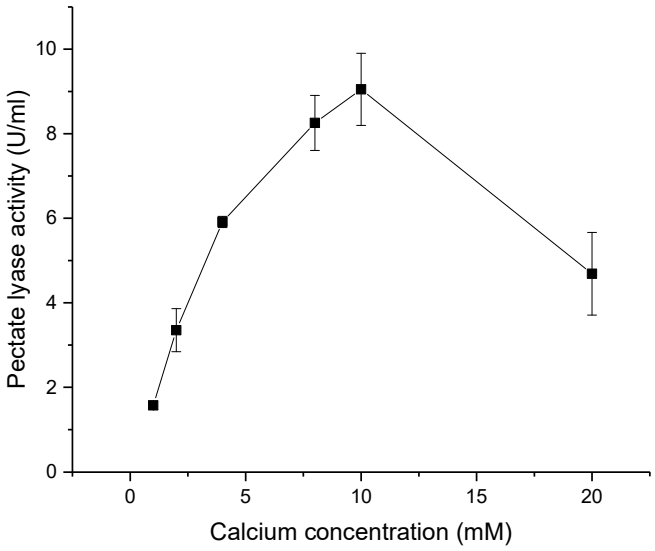

Figure 5.3: TMA14 pectate lyase activity increases with increasing calcium concentration up to an optimum activity at 10mM $CaCl_2$. Experiments conducted on 0.5% (w/v) polygalacturonic acid at 70ºC, pH 5.5 with a reaction time of 30 min. A significant decrease in activity is observed at 20mM CaCl2 due to the gelling of polygalacturonic acid at higher concentrations of calcium. Error bars represent one standard deviation about the mean (n=3).

TMA14 activity generally increases with an increase in calcium concentration, with a maximum activity of 9.05 U/mL at 10 mM $CaCl_2$. However, a significant decrease in activity is observed when calcium concentration is increased to 20 mM. This is likely due to the gelling of polygalacturonic acid at high concentrations of calcium via the shifted egg box mechanism (Braccini & Pérez, 2001; Cao et al, 2020). Additionally, the reaction mixture was visibly more viscous at 20 mM $CaCl_2$ than at lower concentrations. The gelling of the reaction mixture reduces the diffusive mobility of the enzyme and substrate, subsequently inhibiting the reaction kinetics and overall activity.

The stability of TMA14 at 70°C was determined by incubating the enzyme at 70°C for 6 h and removing an aliquot for activity assays hourly (Figure 5.4). TMA14 was found to exhibit very high stability at 70°C as it retains 84% residual activity after 6 h of incubation. The sugar beet diffusion process typically has a maximum residence time of 2 h (Table 1-2), therefore stability within this timeframe is particularly important.

TMA14 retains 99% activity after 2 h of incubation and is therefore likely to retain its activity throughout the duration of the diffusion process.

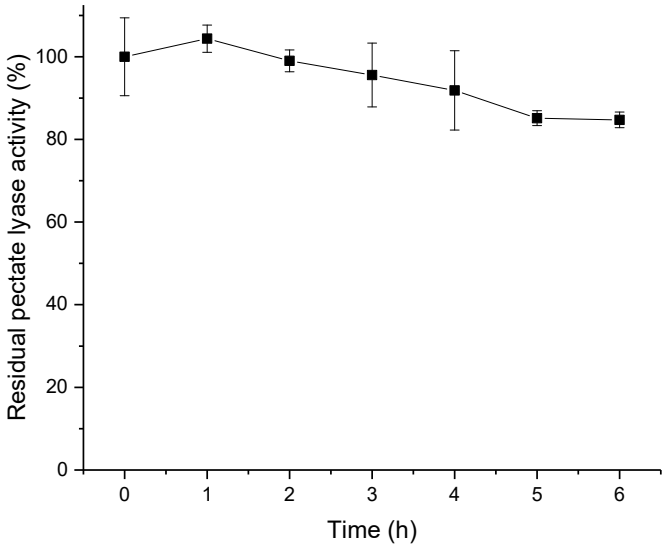

Figure 5.4: TMA14 thermal stability at 70°C and pH 5.5 on polygalacturonic acid with the addition of 10mM $CaCl_2$. Reaction time following thermostability incubation was 30 min. TMA14 is highly stable at 70°C as 84% activity is retained after 6h. For context within the sugar beet diffusion application, the diffusion process has a maximum residence time of 2h. TMA14 retains 99% activity after incubation at 70°C for 2h. Error bars represent one standard deviation about the mean (n=3).

In contrast to pectin lyases, pectate lyases, and in particular bacterial pectate lyases such as TMA14, usually have an optimum pH in the alkaline region, around pH 8.5 (Herron & Jurnak, 2003; Pedrolli et al, 2009; Atanasova et al, 2018). This is particularly significant as the sugar beet diffuser is optimised for operation in the acidic region between pH 5.5 to pH 6.0. The activity of TMA14 between pH 5.0 and pH 8.0 is presented in Figure 5.5.

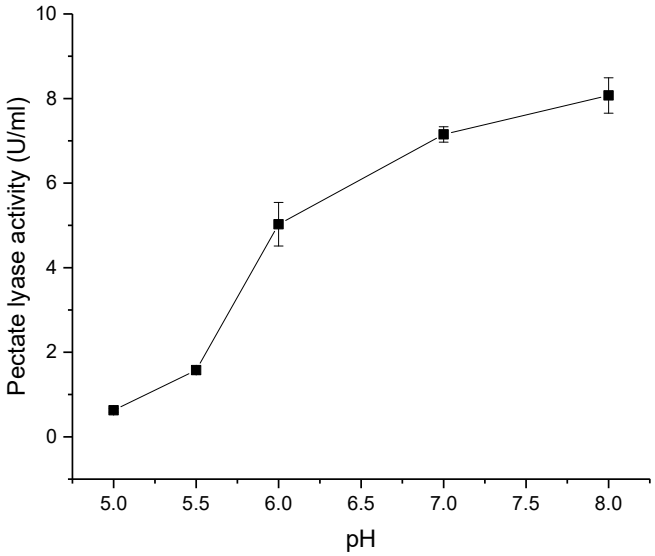

Figure 5.5: TMA14 pH assay on 0.5% (w/v) polygalacturonic acid at 70°C with the addition of 1mM $CaCl_2$. Reaction time of 30 min. Optimum activity within the experimental conditions is achieved at pH 8. 20% activity is retained at pH 5.5. Error bars represent one standard deviation about the mean (n=3).

TMA14 is seen to have the highest activity at pH 8.0, decreasing considerably as pH decreases. At pH 5.5, only 20% activity is retained. This is because key aspartate residues are protonated at low pH and therefore cannot bind the catalytic Ca^{2+} required for Michaelis complex formation (Ali et al, 2015). This is a concern as the enzyme is highly thermostable and therefore appropriate for application within the sugar beet diffuser, but its low activity at pH 5.5 is a significant limiting factor which would require large quantities of enzyme addition.

5.2.2.2 TMA14 activity profile on pectin

The previous characterisation studies were conducted on polygalacturonic acid, a standard substrate when assaying pectate lyase activity as pectate lyases favour non-esterified pectic acid. In order to understand how TMA14 may act on sugar beet cossettes, a substrate specificity assay at various calcium concentrations was conducted in order to compare activity of TMA14 on polygalacturonic acid versus sugar beet pectin (Figure 5.6).

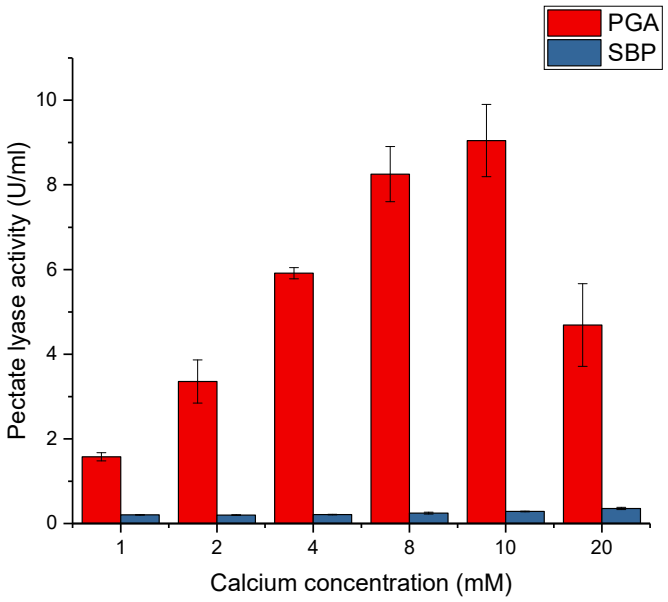

Figure 5.6: Substrate specificity assay comparing the activity of TMA14 on 0.5% (w/v) polygalacturonic acid (PGA) and 0.5% (w/v) commercially sourced sugar beet pectin (SBP) at 70°C, pH 5.5 and a reaction time of 30 min at varying calcium concentrations. The activity of TMA14 on SBP is significantly lower than the activity on PGA at the equivalent reaction conditions. The activity of TMA14 increases with calcium concentration on both substrates. However, a maximum activity on PGA is achieved at 10mM $CaCl_2$. In contrast, TMA14 activity on SBP continues to increase at 20mM $CaCl_2$, likely due to the poor gelling properties of sugar beet pectin. Error bars represent one standard deviation about the mean (n=3).

The activity of TMA14 on sugar beet pectin is seen to be significantly lower than the activity on polygalacturonic acid. At 10 mM Ca^{2+}, 70°C and pH 5.5, TMA14 has an activity of 9.05 ± 0.853 U/mL on polygalacturonic acid. In contrast, under the same conditions, TMA14 has an activity of 0.29 ± 0.008 U/mL on sugar beet pectin.

As previously mentioned, pectate lyases strongly favour non-esterified pectic acid with only limited activity on methylated galacturonate (Seyedarabi et al, 2009). However, sugar beet pectin has a high degree of methylation and acetylation. In particular, the sugar beet pectin used in this work provided by CP Kelco ApS, Denmark, has a reported degree of esterification of 55%, with 14-26% degree of acetylation.

The decrease in activity is therefore due to the inhibition of pectate lyase activity by the methyl and acetyl groups on sugar beet pectin. In particular, acetylation inhibits the binding of divalent cations, such as calcium (Ralet et al, 2003). This is also why

sugar beet pectin is particularly poor at forming gels despite a high degree of methylation.

Although the degree of acetylation on sugar beet pectin reduces TMA14 activity significantly, the data presented in Figure 5.7 suggests that the calcium concentration can be increased further than 10 mM as gel formation is not significant until 80 mM Ca^{2+}. A maximum activity of 0.54 ± 0.047 U/mL is obtained at 70°C, pH 5.5 and 50 mM Ca^{2+}.

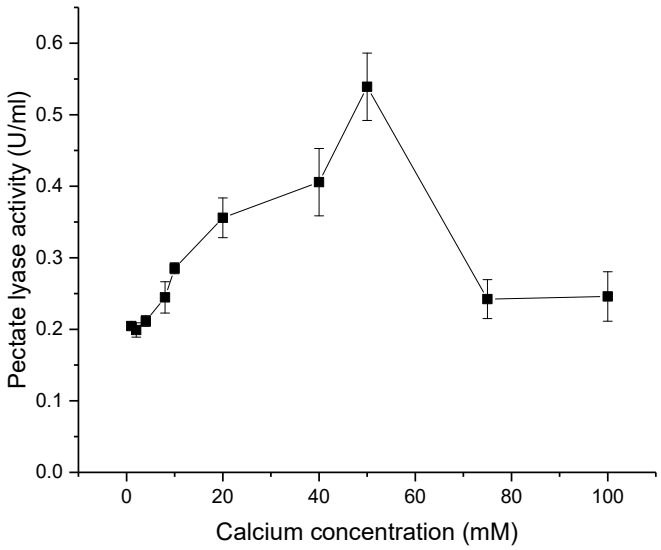

Figure 5.7: TMA14 activity assay on 0.5% (w/v) sugar beet pectin at 70°C, pH 5.5 and a reaction time of 30 min with increasing calcium concentration. Pectate lyase activity increases with an increase in calcium concentration up to a maximum at 50mM $CaCl_2$. Further increases in calcium concentration result in a reduction in activity. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.2.2.

Although TMA14 is not an ideal thermostable candidate for sugar beet diffusion application, it is still important to trial this enzyme in the process as the low activity in combination with the high thermal stability throughout the duration of the diffusion process may result in significant detectable pectate lyase activity and potential improvements to the sugar extraction process.

5.2.3 TMA14 application on co-current diffusion model

Prior to application in the sugar beet extraction models, the crude lysate containing TMA14 was first concentrated using a 10,000 MWCO Vivaspin® ultrafiltration unit (Sartorius AG, Germany) to maximise enzyme activity. To determine whether TMA14 could improve the rate of sucrose extraction when added to the diffusion process, 1 mL of TMA14 was added to 250 g of cossettes and 275 ml of pH 5.5 buffer at 70°C in the co-current single-stage model presented in Chapter 4. Given the thermostability of TMA14, the enzyme was added at the start of the diffuser (t = 0). 50 mM of $CaCl_2$ was also added to promote enzyme activity. The results are presented in Figure 5.8 and Figure 5.9.

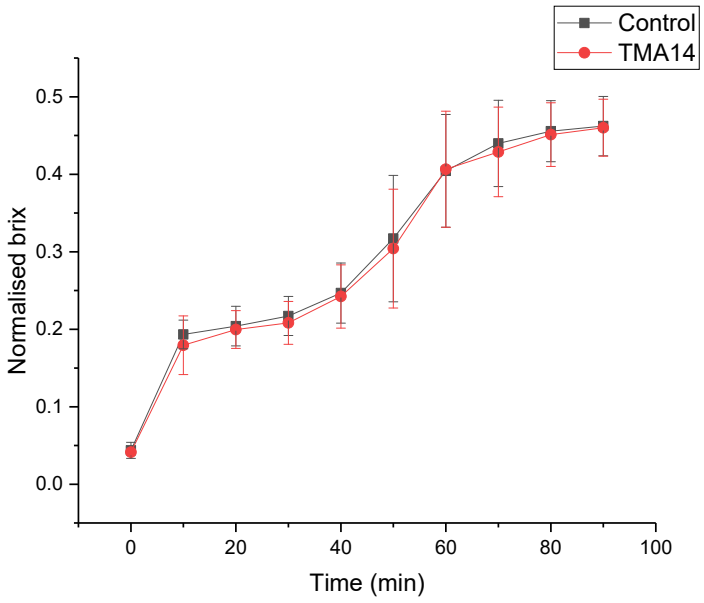

Figure 5.8: Comparison between the rate of normalised brix with and without TMA14 addition in the co-current diffusion model. Reaction conditions were 70°C, pH 5.5, 250 rpm, 50 mM $CaCl_2$, and a diffusion ratio of 110%. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.3.

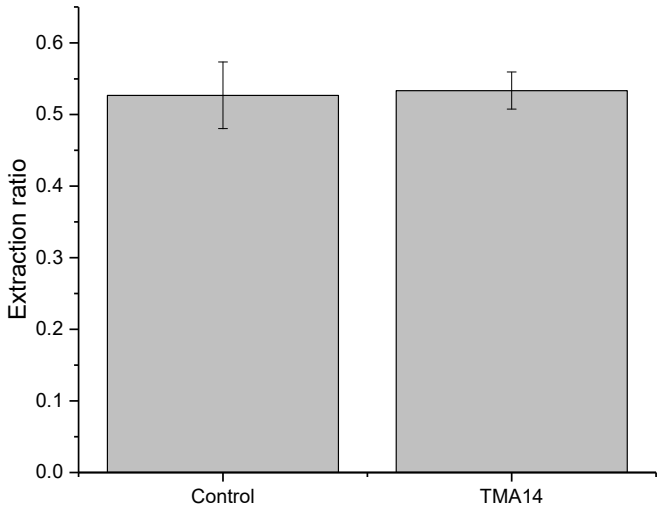

Figure 5.9: Comparison between the extraction ratios with and without TMA14 addition in the co-current diffusion model at 70ºC, pH 5.5, 250 rpm, 50 mM $CaCl_2$ and a diffusion ratio of 110%. No significant difference between the extraction ratio was observed upon addition of TMA14 to the diffusion process. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.3.

There was no significant difference between the normalised brix within the diffusion process nor the final extraction ratio under the specified conditions. Additionally, no significant pectate lyase activity was detected via optical density analysis at 235 nm. These results are similar to those presented in Chapter 4 with the addition of Rohament CL and Rohapect SY+ despite the thermostability of TMA14. This is likely due to a combination of factors including biomass recalcitrance (Section 4.2.3), low activity on esterified pectin (Figure 5.6) and low activity at pH 5.5 (Figure 5.5). In order to determine the impact of pH on TMA14 activity on sugar beet cossettes, these experiments were repeated at pH 6.5 (Figure 5.10, 5.11).

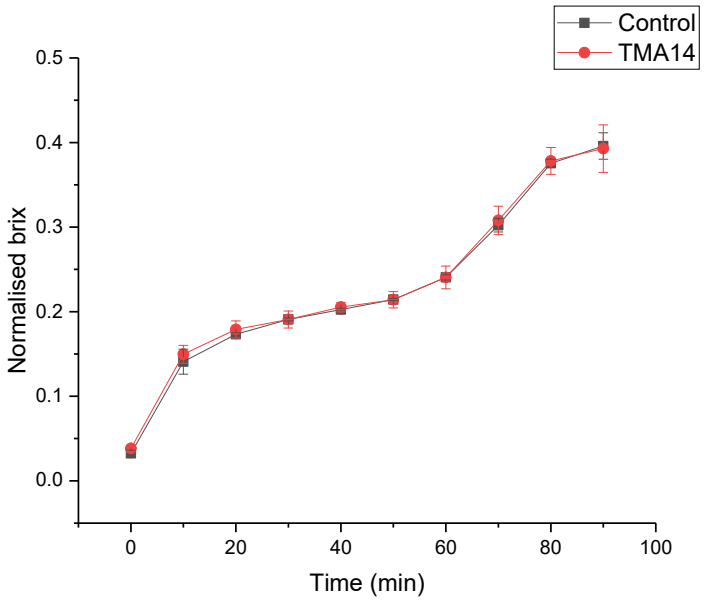

Figure 5.10: Comparison between the rate of normalised brix with and without TMA14 addition in the co-current diffusion model. Reaction conditions were 70°C, pH 6.5, 250 rpm, 50mM $CaCl_2$, and a diffusion ratio of 110%. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.3.

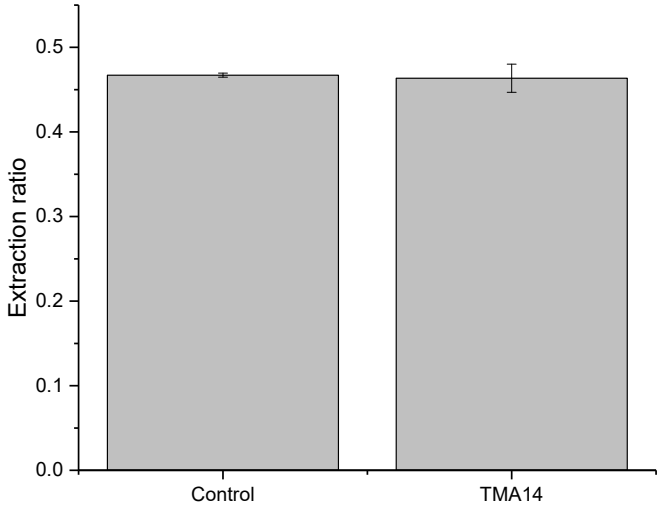

Figure 5.11: Comparison between the final extraction ratios with and without TMA14 addition in the co-current diffusion model at 70ºC, pH 6.5, 250 rpm, 50 mM $CaCl_2$ and a diffusion ratio of 110%. No significant difference was observed. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.3.

According to the characterisation studies, TMA14 retains 20% activity at pH 5.5 compared to the maximum activity available under the reaction conditions at pH 8.0. In comparison, at pH 6.5, TMA14 retains between 62% and 89% activity. Therefore, the pH of the diffusion process was increased to pH 6.5 to maximise potential activity. It should be noted that pH 6.5 is outside of the maximum operating pH for the diffusion process in industry, as a pH above 6.0 results in significant sucrose losses due to microbial degradation and impairments to the subsequent processing stages due to pectin solubilisation.

Despite the increase in activity compared to pH 5.5, there is again no significant improvement to the rate of extraction nor the final extraction ratio when TMA14 is added to the co-current diffusion model at pH 6.5. Furthermore, no detectable pectate lyase is observed via optical density analysis at 235 nm. This suggests that the biomass recalcitrance and poor pectate lyase activity on sugar beet pectin are significantly hindering activity and potential sucrose extraction benefits.

The extraction ratios obtained at pH 6.5 are considerably lower than those obtained at pH 5.5. For example, without the addition of TMA14, the extraction ratios at pH 5.5 and pH 6.5 are 0.537 ± 0.0468 and 0.467 ± 0.0025 respectively. This is likely due to the aforementioned disadvantages to the extraction process at pH greater than 6.0. Therefore, as there are no benefits to operating at pH 6.5 to the diffusion process nor to TMA14 activity, future experiments will be conducted at pH 5.5.

5.2.4 TMA14 application on pre-treated sugar beet

In Chapter 4, the commercial enzymes Rohapect SY+ and Rohament CL were unable to act on the sugar beet cossettes without a pre-treatment method being applied in order to overcome biomass recalcitrance. Therefore, in order to determine whether biomass recalcitrance was inhibiting TMA14-facilitated improvements to the extraction process, cossettes were pre-treated via freeze-thaw and milling as described in Section 4.2.3.2 prior to incubation at 70°C with 1% (v/w) TMA14.

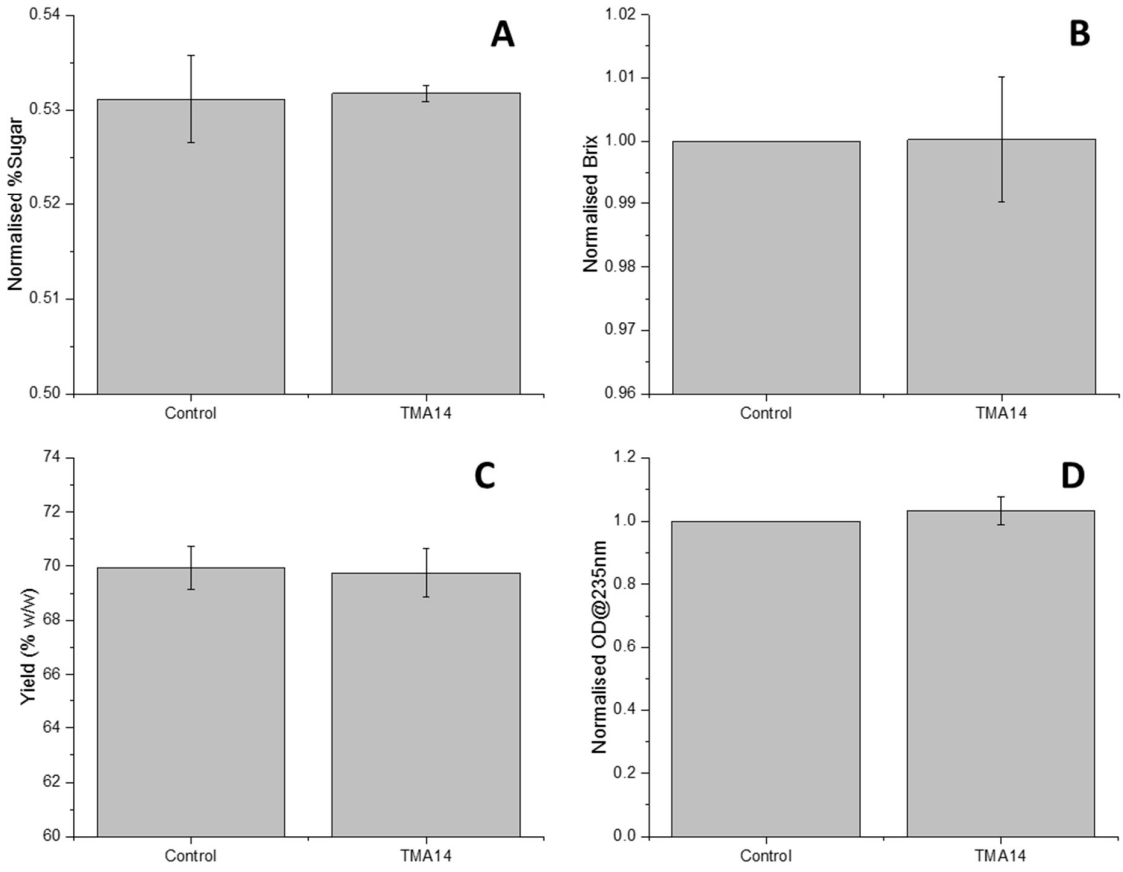

Figure 5.12: The effect of TMA14 addition to freeze-thawed milled sugar beet on (A) Percentage sugar measured via polarimetry, (B) °Brix measured via refractometry, (C) Yield of juice recovered following incubation of freeze-thawed milled sugar beets and pressing, (D) Pectate lyase activity detected via optical density analysis at 235 nm. No significant improvements to percentage sugar, °Brix or yield of juice were observed upon addition of TMA14 to the freeze-thawed milled sugar beet. No significant pectate lyase activity was detected in the juice samples. 50mM of $CaCl_2$. Error bars represent one standard deviation about the mean (n=3).

The effect of TMA14 on the pre-treated sugar beet was assessed by evaluating the percentage sugar in the juice measured by polarimetry (Figure 5.12A), the °Brix of the juice measured by refractometry (Figure 5.12B) and the yield of juice recovered after pressing the pre-treated sugar beet following incubation (Figure 5.12C). A non-enzymatic control was conducted simultaneously in which TMA14 was substituted with water. There was no significant difference in the normalised percentage sugar and the normalised °Brix between the control and the TMA14-added trials. Similarly, no significant difference in the yield of juice was observed.

Figure 5.12D is a comparison between the optical density at 235 nm in which the TMA14 data is normalised against the control condition to identify any indication of pectate lyase activity. No significant difference was observed, suggesting that no significant pectate lyase activity was present on the freeze-thawed milled sugar beet under the reaction conditions. This, coupled with the accompanying data, suggests that TMA14 is unable to act on the substrate nor is it able to improve the evaluated extraction parameters.

This is different to the similar data in Section 4.2.3.2 in which Rohapect SY+ was applied to freeze-thawed milled beet. Although no significant improvement to the extraction parameters were observed with the addition of either Rohapect SY+ or TMA14, there was evidence of positive pectin lyase activity when Rohapect SY+ was added, albeit at an optimal temperature of 50°C versus 70°C (Figure 4.13). The difference is likely due to the substrate specificity of TMA14 in combination with its relatively low activity profile, as Rohapect SY+, a pectin lyase, is able to act upon esterified pectin more readily than TMA14, a pectate lyase, despite the latter's temperature suitability.

5.2.5 Counter-current diffusion model trials with TMA14

Although there is insufficient evidence that TMA14 is able to act on sugar beet even under optimised pre-treatment conditions, it is necessary to examine this enzyme in the counter-current diffusion process combined with pressing in order to determine any potential value from the addition of this enzyme under industrial conditions. The counter-current diffuser is operated as described in Chapter 4. 1 ml of TMA14 was added to the diffusion process in stages at the beginning of the process as its thermostability renders it unlikely to inactivate over the diffusion period. The conditions of this experiment were 110% diffusion ratio, 70°C, pH 5.5 and an operation time of 90 min. Additionally, 50 mM of $CaCl_2$ was added in the same dosage pattern as TMA14 in order to provide necessary calcium for enzyme activity.

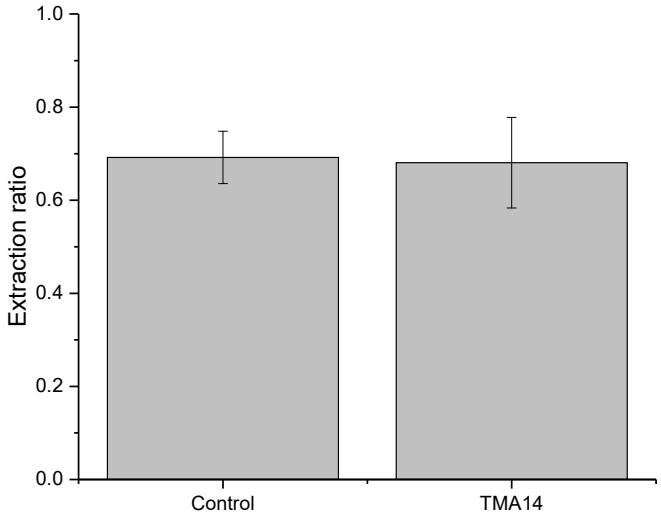

Figure 5.13: Comparison between the extraction ratios of the control versus TMA14 addition in the counter-current diffuser model at 70ºC, pH 5.5 and an operation time of 90 min. No significant difference between the extraction ratios were observed. Error bars represent one standard deviation about the mean (n=3). Experiments performed as described in Section 2.3.4.

In Figure 5.13, no significant difference in the extraction ratio was observed when TMA14 was added to the counter-current diffuser. There was also no detectable pectate lyase activity in the juice samples.

The mass of pressed pulp and the volume of pressed pulp water obtained from 100 g of wet pulp were also analysed to determine the effect of TMA14 on the pressing properties of the cossettes. According to Figure 5.14, there is no significant difference between these properties when TMA14 is added to the diffusion process. Similarly, no significant difference between the normalised pressed pulp sugar content was

observed: 0.242 ± 0.0554 versus 0.257 ± 0.0607 for the control and TMA14-added trial respectively.

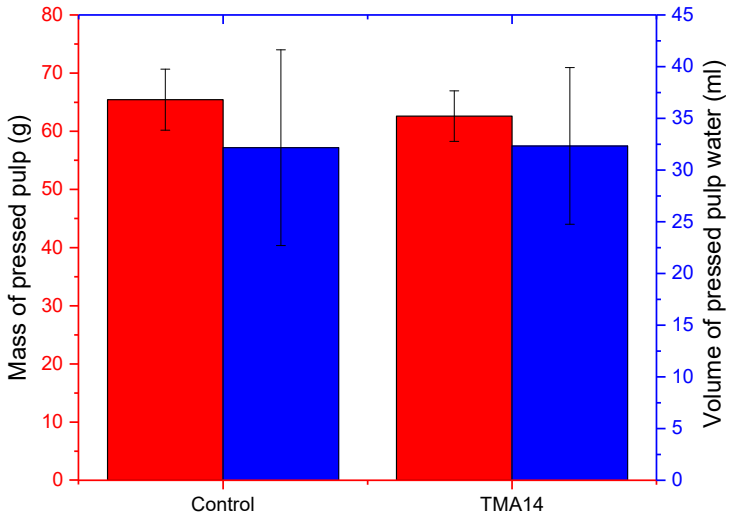

Figure 5.14: Mass of pressed pulp (in red) and volume of pressed pulp water (in blue) between the control condition and the addition of TMA14. TMA14 added to the counter-current diffusion model at the beet midpoint, experimental conditions of 70ºC, pH 5.5, with an operation time of 90 min. No significant difference between either of the two properties were observed when TMA14 was added to the diffusion process. Experiments performed as described in Section 2.3.4 and Section 2.3.6.

5.3 Summary

The aim of this chapter was to characterise the activity profile of a thermostable pectate lyase and explore the application of this enzyme on the diffusion models developed in Chapter 4 to determine if there is this enzyme confers a benefit to sucrose extraction when applied to the diffusion process.

To conclude, TMA14 is a novel thermostable pectate lyase developed by the UCL Department of Biochemical Engineering. It is able to demonstrate significant activity at 70°C and maintains 99% stability over a 2 h period at this temperature (Figure 5.2; Figure 5.4). However, as a bacterial pectate lyase it has a pH optimum towards the basic region at pH 8, with relatively poor activity at pH 5.5 (Figure 5.5).

Furthermore, pectate lyases strongly favour non-esterified pectin whereas pectin found in sugar beet is highly methylated and acetylated. Therefore, the activity on

sugar beet pectin is relatively low and is likely to be a concern when applied to the sugar beet diffusion process (Figure 5.6).

Trials with TMA14 in the co-current single-stage diffusion model resulted in no significant improvement to rate of sucrose extraction or final extraction ratio (Figure 5.8; Figure 5.9). Additional trials were also conducted at pH 6.5 as TMA14 exhibits an approximate 4-fold increase in activity compared to at pH 5.5. However, no positive improvement was measured (Figure 5.10; Figure 5.11). Furthermore, no significant pectate lyase activity was identified in either trial.

As TMA14 showed no significant activity on sugar beet cossettes, sugar beet cossettes were pre-treated via the freeze-thaw milling process described in Section 4.2.3.2 in order to determine the inhibitory extent of biomass recalcitrance. As described in Figure 5.12, no significant difference to °Brix, percentage sugar or juice yield were observed when TMA14 was added. Despite the pre-treatment process, no significant pectate lyase activity was observed.

In the counter-current diffuser model, there was no significant difference to extraction ratio (Figure 5.13) or the pressing process measured via mass of pressed pulp and volume of pressed pulp water obtained from 100g of wet pulp (Figure 5.14), suggesting that TMA14 provides no significant benefit when applied to a diffusion model that mimics industrial processing parameters.

By thermostability alone, TMA14 appears to be an excellent candidate for application in sugar beet processing. However, it is severely limited by its basic pH optima, generally low activity and substrate specificity. Novel thermostable enzymes with a higher activity at pH 5.5 and a substrate specificity that favours esterified pectin, such as pectin lyases, would be more appropriate for application in the sugar beet diffusion process.

Chapter 6

Conclusions and future work

6.1 Conclusions

The sugar industry in the UK operates highly efficient, sustainable processes and has been focusing heavily on transforming waste streams into profitable by- and co-products (Figure 1.5). Given the economic situation post-Brexit and the elimination of the EU quota for sugar pricing, focus has shifted back to maximising the amount of potential sucrose available for extraction in order to maintain the position of UK sugar on the global market. The application of enzymes to the sugar beet diffusion process is one such way that has been trialled in industry to varying degrees of success. As described in Section 1.8, the **aim** of this thesis was to evaluate whether enzymes, particularly pectinases and cellulases, have the ability to enhance sucrose extraction from sugar beets when applied to existing diffusion processes. The emphasis of the study was on understanding the mechanism of action of these enzymes in order to optimise the potential benefit of enzyme addition.

6.1.1 Commercial enzyme characterisation

The first objective outlined in Section 1.8 focused on assessing the potential of the commercial enzymes by characterising the types of activity and optimum activity profiles of various enzyme preparations as described in Section 3.2. The focus was on cellulases and pectinases, particularly pectin lyases, as these activities have previously been trialled in industry. All of the commercial enzymes exhibited poor activity and stability at 70°C (e.g. Figure 3.2), the optimum operating temperature for the sugar beet diffusion process (Section 1.3.2.1). Of the commercial enzymes investigated, Rohapect SY+, a pectin lyase (Section 3.3.3.2), and Rohament CL, a broad-range cellulase (Section 3.3.3.3), were highlighted as the most successful candidates due to a combination of the results from this work and industrial trials.

6.1.2 Diffuser model development and commercial enzyme application

The second objective was to develop a scale-down process in which the application of enzymes on the sugar beet diffusion process can be investigated without the need for considerable resource investment as would be required at pilot or industrial scale.

An initial co-current model was developed (Section 2.3.3) as a simple method to determine basic extraction parameters and initial improvements to sucrose diffusion upon addition of Rohapect SY+ and Rohament CL.

From this co-current model, no significant improvement to the rate of sucrose extraction nor the extraction ratio (Equation 4.1) was observed when 4640 ppm of Rohapect SY+ or Rohament CL was added to the process at 70°C, pH 5.5 with a retention time of 90 min (Figure 4.4). Subsequent experiments were designed in order to determine whether the enzymes were able to act on the substrate at all, as lignocellulosic biomass is typically recalcitrant towards enzyme activity without a prior pre-treatment process (Section 4.2.3). Initial pre-treatment experiments confirmed that significant enzyme activity was present when sugar beet had been treated with an aqueous-autoclave or alkaline pre-treatment process followed by co-current diffusion at 50°C (Figure 4.7). A third pre-treatment condition was also investigated in which the cossettes were incubated at 70°C prior to diffusive extraction with enzyme addition at 50°C. This pre-treatment was designed to simulate the conditions in the diffusion process to determine whether the diffusion conditions could overcome the recalcitrance of the beet. However, no significant enzyme activity was detected as shown in Figure 4.7.

Figure 4.8 depicts the extracted sucrose with and without the addition of Rohament CL following the aqueous pre-treatment methods. No significant improvement in sucrose extraction was observed under the alkaline pre-treatment condition despite significant enzyme activity (Figure 4.7). Interestingly there is an increase in sucrose extraction under the aqueous-autoclave condition ($p = 0.026$). This may be due to the aqueous nature of the pre-treatment methods investigated, as these could have extracted the majority of the sucrose in the pre-treatment stage. An alternative pre-treatment method is a physical pre-treatment involving freeze-thawing and milling of sugar beet into fine particle sizes (Section 4.2.3.2). This pre-treatment method has been used in literature (Table 1-6) in combination with pressing to successfully improve the °Brix and juice yield from various fruit and vegetable biomass. No cellulase activity was detected, and whilst there was positive lyase activity (Figure 4.13), there was no corresponding improvement to juice yield or sugar extraction when Rohapect SY+ or Rohament CL were added to freeze-thawed, milled sugar beet under conditions similar to literature (Figure 4.9; Figure 4.10; Figure 4.11; Figure 4.12).

These findings seem to suggest that even if Rohapect SY+ and Rohament CL are able to act upon sugar beet, it does not improve the extraction efficiency of the diffusion process. However, these experiments were conducted on the co-current model and therefore its similarities to the industrial process are limited. Subsequently, a counter-current diffusion model was developed (Figure 2.3; Section 4.2.5), inspired by the battery-style diffusion model (Section 1.3.2), to appropriately mimic the counter-current operation of the industrial process.

The counter-current diffusion model can be operated such that the operating conditions, i.e. pH, temperature, diffusion ratio, cossette pre-heating and residence time, can be adjusted as necessary (Section 4.2.6). In addition to this, critical elements such as the enzyme or pressing aid addition point can also be changed to simulate the various addition points in the industrial process.

In the initial trials, there was no significant improvement to extraction ratio when Rohapect SY+ was added to the diffusion model, regardless of where and when the enzyme was added (Figure 4.21; Figure 4.22). There was also no indication of any significant lyase activity, which coincides with the data from the co-current model suggesting that the enzymes are unable to act on non-pre-treated sugar beet.

Given the lack of enzyme activity in the diffusion process, it was hypothesised that any potential benefit could be seen in the pressing process (Section 4.2.10), as industrial data suggest a decrease in residual sucrose within the pressed pulp. A refined process was developed in which the diffusion process was followed by the manual pressing of wet pulp, producing pressed pulp, which could potentially reflect an improvement in sucrose extraction.

As previously mentioned, pressing is a critical component of the extraction process and can be considered a single processing step in combination with the diffusion process. The effect of Rohament CL and Rohapect SY+ was evaluated by pressing 100g of wet pulp immediately after diffusion and measuring the residual mass of pressed pulp and the volume of pressed pulp water obtained. Theoretically, an improvement to wet pulp pressability would result in a decrease in residual mass of pressed pulp and an increase in the volume of pressed pulp water obtained.

In practice, the opposite trends were observed (Figure 4.27): adding either Rohament CL or Rohapect SY+ to the diffusion process resulted in a statistically significant

increase in the mass of pressed pulp ($p = 0.03653$ and $p = 0.02819$ respectively, $n = 6$). Rohapect SY+ also caused a significant decrease in the volume of pressed pulp water ($p = 0.03311$, $n = 6$).

There were some limitations to the pressing experiments as the press used in this work was a manual press. The pressures produced by this press are relatively low compared to the pressures produced by an industrial screw press, which ranges between 10-12 bar. This was particularly evident when analysing the average dry substance of the pressed pulp: in one scenario, the average pressed pulp dry substance was 10.94% ± 1.08 ($n = 6$). In industry, pressed pulp has a typical dry substance of 25%. Therefore, it may be that this pressing process was unable to replicate the conditions of the industrial pressing process and therefore the effect of Rohament CL and Rohapect SY+ on the beet pressing is inconclusive.

6.1.3 TMA14 expression and application in sugar beet extraction

The final objective was to characterise a novel, thermostable pectate lyase and explore the application of this enzyme on the diffusion process, as it could better withstand the high temperature conditions of the process. TMA14 is a thermostable pectate lyase isolated from *Thermotoga maritima* DSM 3109 within the UCL Department of Biochemical Engineering. TMA14 is highly thermostable, with an optimum activity at 90°C (Figure 5.2) and retains 99% activity after 2 hr at 70°C (Figure 5.4). However, maximum activity was achieved at pH 8.0, with only 20% residual activity at pH 5.5 (Figure 5.5).

It should be emphasised that TMA14 is a pectate lyase and therefore requires the addition of calcium in order to function (Section 5.2.2.1). An optimal concentration of 10 mM and 50 mM Ca^{2+} was determined based on enzyme assays with polygalacturonic acid and sugar beet pectin as substrate respectively (Figure 5.3; Figure 5.7).

When 1 mL of TMA14 was applied to the co-current diffusion model at 70°C, pH 5.5 with 50 mM Ca^{2+}, no significant increase in the rate of extraction nor the final extraction ratio was observed (Figure 5.8; Figure 5.9). To determine whether the pH was inhibiting enzyme activity, a trial was conducted in which pH was increased to 6.5. However, no significant improvement was observed at pH 6.5 either (Figure 5.10; Figure 5.11).

There was also no significant difference in the amount of sugar extracted when TMA14 was added to freeze-thawed, milled beet (Figure 5.12). However, in contrast to the commercial data in the previous chapter, there was also no evidence of pectate lyase activity on the pre-treated beet. This is likely due to the relatively low activity of TMA14 due to pH and substrate specificity.

Similarly, in the counter-current diffuser model, no significant increase to extraction ratio was observed when 1 ml of TMA14 was added to the beet midpoint of the counter-current diffuser model at 110% diffusion ratio, 70°C, pH 5.5 and a retention time of 90 min (Figure 5.13). There was also no significant difference to the mass of pressed pulp, volume of pressed pulp water or pressed pulp polarisation, indicating that TMA14 has no significant effect on the combined counter-current diffusion and pressing process.

6.2 Summary

In summary, the results presented in this work provide an original approach to the examination of the potential benefit of pectinases and cellulases when applied to the sugar beet diffusion process for the purpose of improving the efficiency of sucrose extraction. A small-scale, counter-current diffuser model was designed that enabled evaluation of enzyme addition under industry relevant conditions. Using this model, it was found that the enzymes investigated in this study do not enhance the sugar beet extraction process. This is likely due to a combination of factors, including an already highly efficient extraction process, low enzyme activity and poor stability, and biomass recalcitrance. Although the primary focus of this study was the evaluation of enzyme potential, the diffusion models and protocols developed provide a standard by which future scale-down diffuser studies can be conducted for further optimisation of process parameters.

6.3 Future work

Future work comprises potential studies to clarify and expand on the findings reported in this thesis. A number of important areas of future work are described below.

- Expression, application and/or evolution of a variety of thermostable cellulases and pectinases that exhibit optimum activity at pH 5.5. Enzymes of particular interest include pectin lyases, as these typically have an optimum pH within the

diffusion condition range and are able to act on esterified pectin without additional enzyme activities.

- Additionally, if positive benefits using thermostable enzymes are identified on the benchtop scale-down diffuser model, experiments should be scaled to the pilot scale and following that, the industrial scale, in order to determine whether these findings are corroborated at larger scales.
- The diffuser model designed in this work could also be used to examine non-enzymatic modifications to the diffusion process, providing researchers an opportunity to make low-cost changes to the diffusion process and understanding the implications to the resulting outputs. Examples include concentration of pressing aids and other additives.
- Additional pressing experiments utilising a pneumatic press capable of generating industrial-scale pressures would be useful for conclusively determining the effect of enzymes on the pressing process.
- The effect of enzymes on subsequent processing stages after the diffusion and pressing process should also be investigated further, as the addition of enzymes may have additional benefits or detriments, such as the reduction of soluble pectin which would improve filtration, or the excessive solubilisation of sugar beet impurities, which would apply additional strain onto downstream purification.
- Once the positive benefits of any enzyme addition are identified, then a comprehensive economic analysis should be conducted to determine the value of adding enzymes to the diffusion process. This will involve a rigorous understanding of the costs associated with acquiring the enzyme and the value it contributes in terms of additional sucrose yield. Furthermore, an understanding of the entire process chain and the numerous by-products and co-products associated with the sugar beet process will be necessary to valorise enzyme addition.
    - A key factor to consider is that the successful application of enzymes for increased sucrose yield will result in a sugar beet pulp with a lower sucrose concentration, which may render it inappropriate for sale as animal feed. Future work may therefore involve collaboration with

research groups examining the improved valorisation of sugar beet pulp as this will ultimately improve valorisation over the entire process chain.

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Appendix

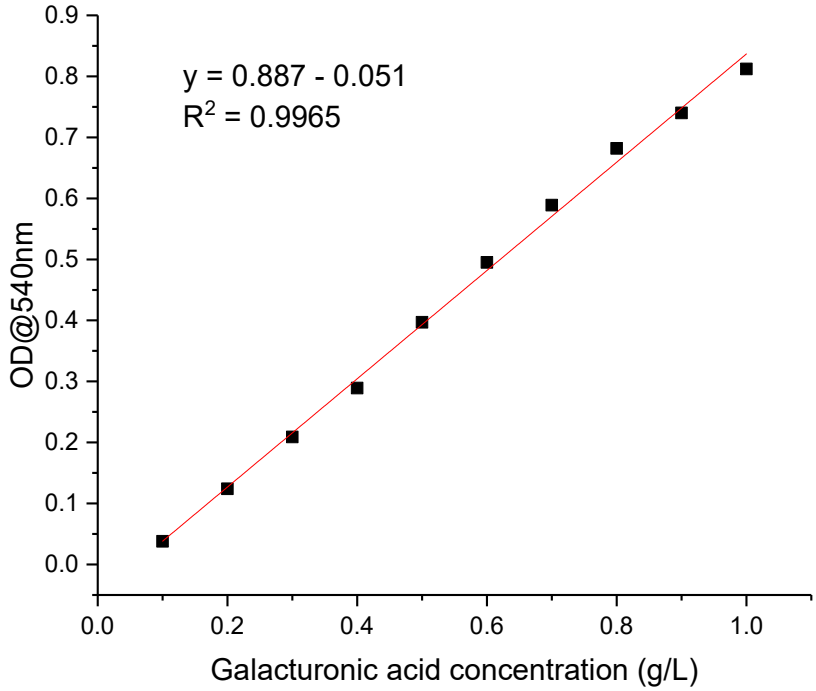

Figure A1.1: Galacturonic acid concentration (g/L) standard curve against optical density at 540 nm for the polygalacturonase assay (Section 2.2.1). Each point represents the mean value of triplicates. Solid line fitted by linear regression.

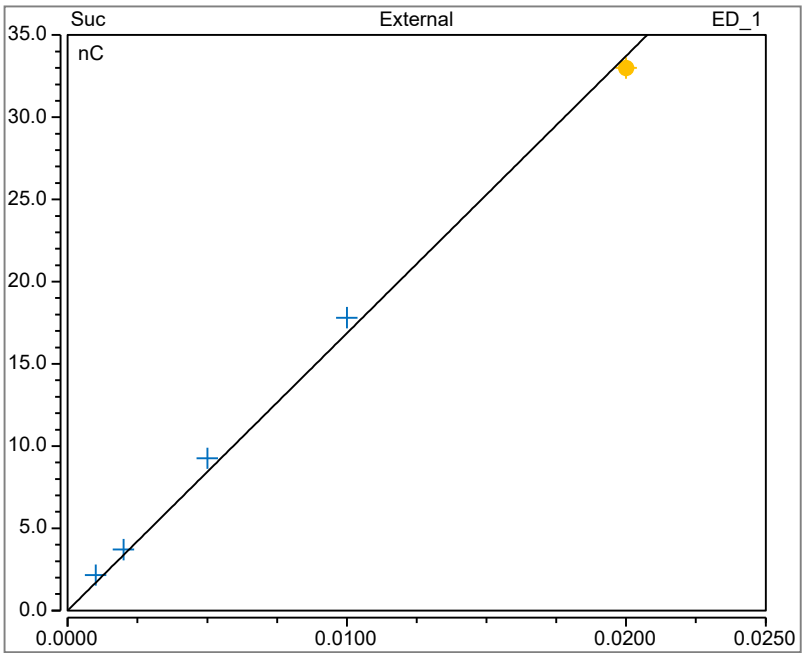

Figure A1.2: Sucrose concentration (g/L) standard curve via HPAEC-PAD as a function of peak height (Section 2.8). Standard curve concentrations prepared by serial dilution of a 1 g/L stock solution equal to 0.02 g/L, 0.01 g/L, 0.05 g/L, 0.002 g/L and 0.001 g/L. Each point represents the mean value of triplicates.

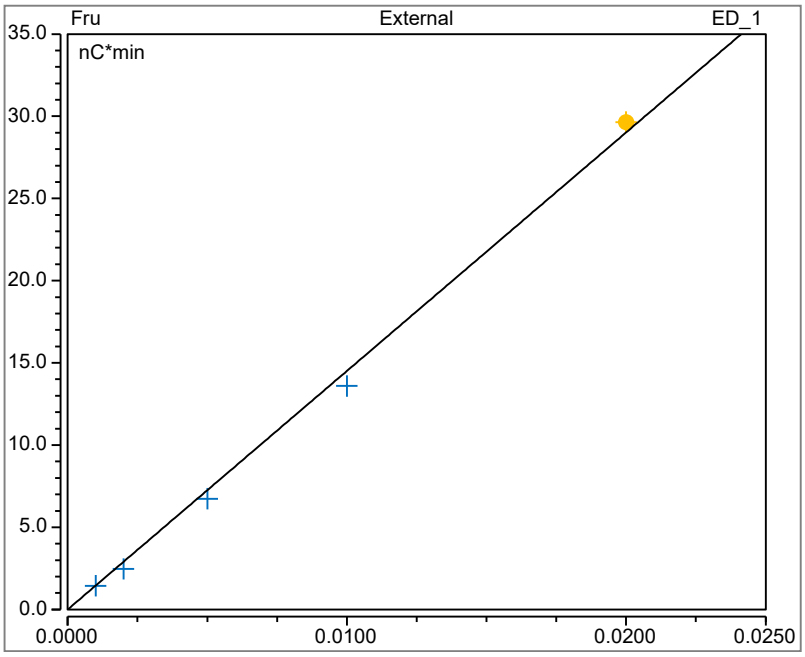

Figure A1.3: Fructose concentration (g/L) standard curve via HPAEC-PAD as a function of peak area (Section 2.8). Standard curve concentrations prepared by serial dilution of a 1 g/L stock solution equal to 0.02 g/L, 0.01 g/L, 0.05 g/L, 0.002 g/L and 0.001 g/L. Each point represents the mean point of triplicates.

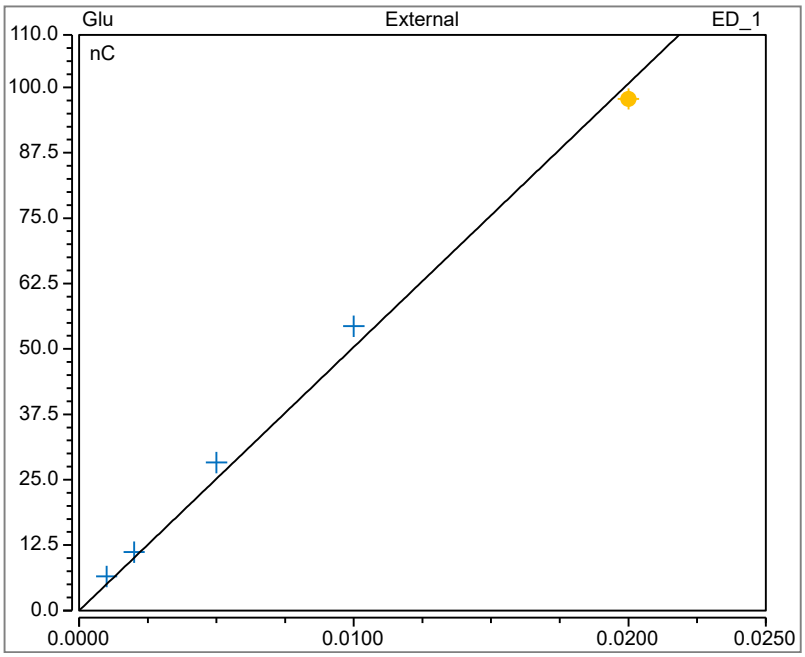

Figure A1.4: Glucose concentration (g/L) standard curve via HPAEC-PAD as a function of peak height (Section 2.8). Standard curve concentrations prepared by serial dilution of a 1 g/L stock solution equal to 0.02 g/L, 0.01 g/L, 0.05 g/L, 0.002 g/L and 0.001 g/L. Each point represents the mean point of triplicates.

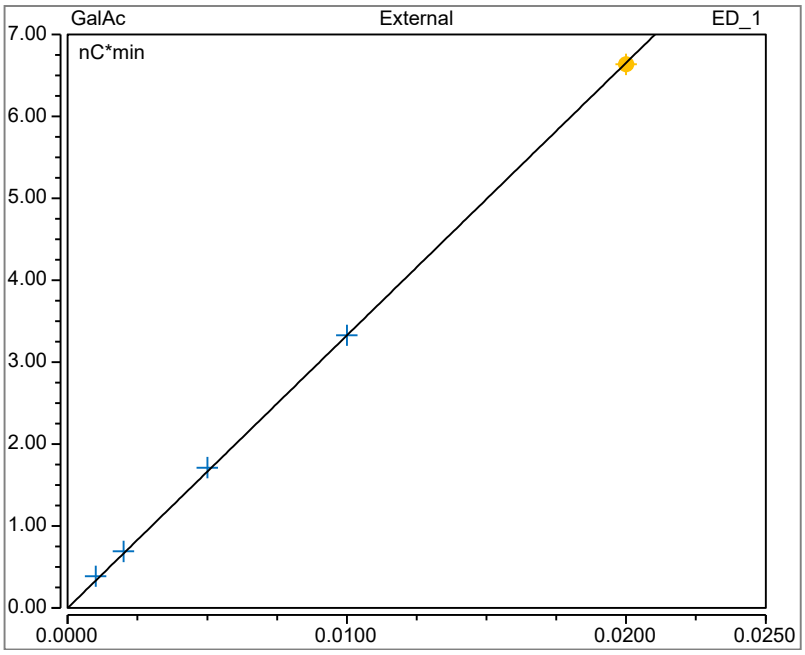

Figure A1.5: Galacturonic acid concentration (g/L) standard curve via HPAEC-PAD as a function of peak area (Section 2.8). Standard curve concentrations prepared by serial dilution of a 1 g/L stock solution equal to 0.02 g/L, 0.01 g/L, 0.05 g/L, 0.002 g/L and 0.001 g/L. Each point represents the mean point of triplicates.

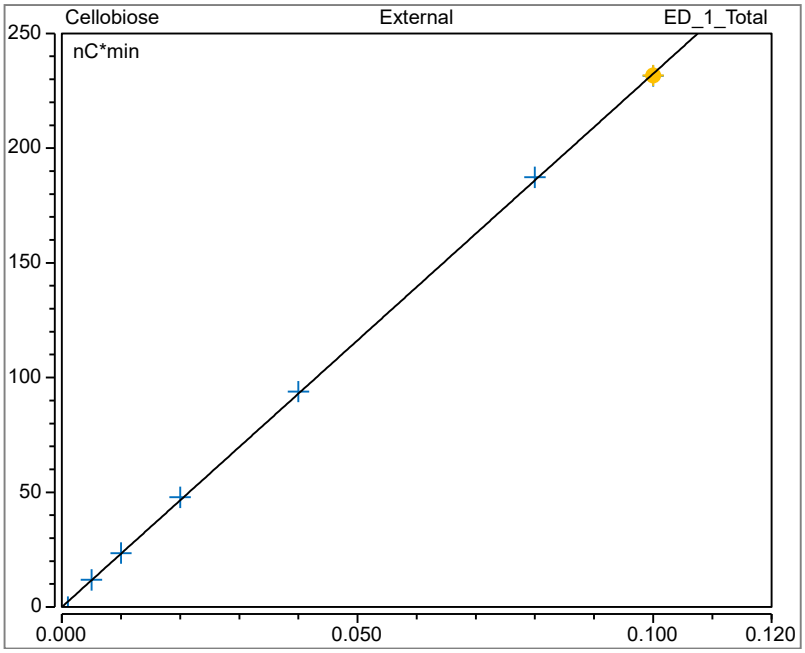

Figure A1.6: Cellobiose concentration (g/L) standard curve via HPAEC-PAD as a function of peak area (Section 2.8). Standard curve concentrations prepared by serial dilution of a 1 g/L stock solution equal to 0.1 g/L, 0.075 g/L, 0.0375 g/L, 0.02 g/L, 0.01 g/L and 0.005 g/L. Each point represents the mean point of triplicates.

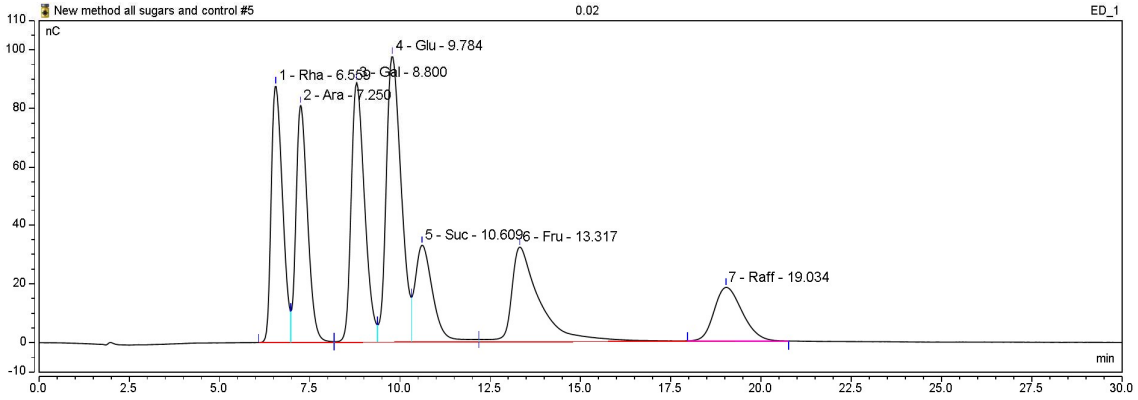

Figure A1.7: Chromatogram example of neutral sugars (Rhamnose, Arabinose, Galactose, Glucose, Sucrose, Fructose, Raffinose) analysis via HPAEC-PAD (Section 2.8). Mobile phase consisted of 5.0 mM of KOH with a flow rate of 0.25 mL/min at 30°C for 30 min.

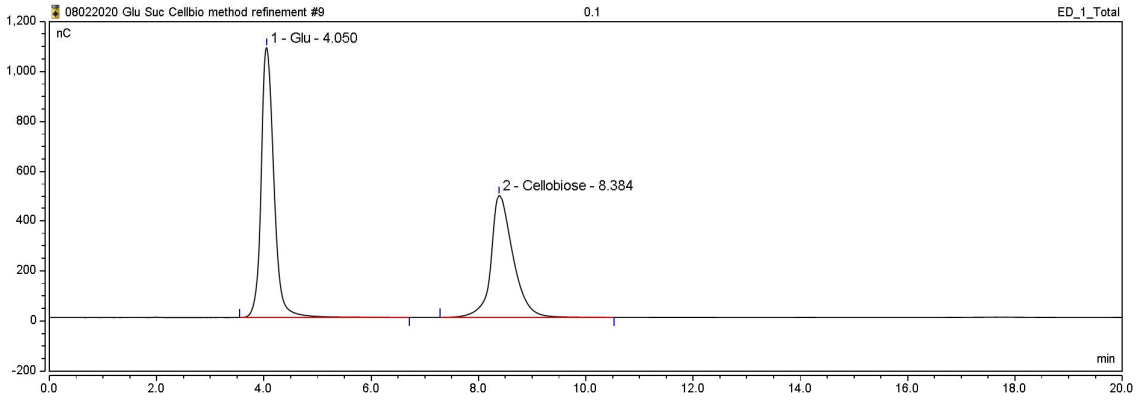

Figure A1.8: Chromatogram example of glucose and cellobiose analysis via HPAEC-PAD (Section 2.2.3 and Section 2.8). Mobile phase consisted of 5.0 mM of KOH with a flow rate of 0.25 mL/min at 30°C for 30 min.

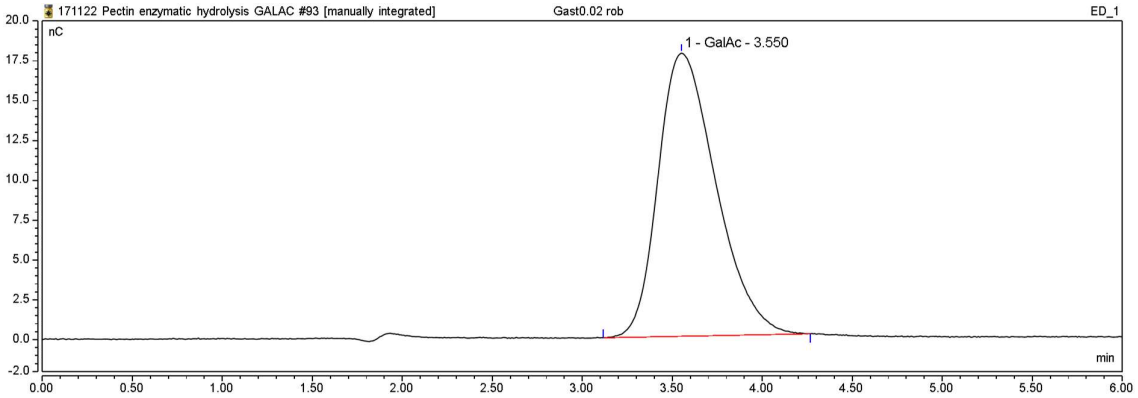

Figure A1.9: Chromatogram example of Galacturonic acid analysis via HPAEC-PAD (Section 2.8). Mobile phase consisted of 5% (v/v) electrochemical-grade sodium acetate at 0.25mL/min at 30°C for 15 min.

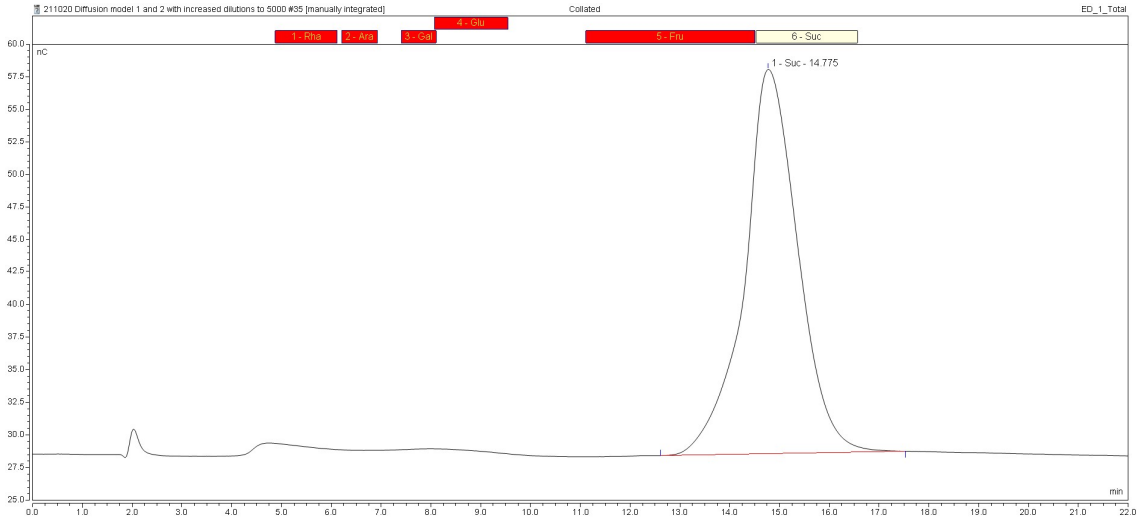

Figure A1.10: Example of a typical chromatogram from the analysis of diffusion juice via HPAEC-PAD (Section 2.8). Mobile phase consisted of 5.0 mM of KOH with a flow rate of 0.25 mL/min at 30°C for 30 min.