Toward the production of milk-identical proteins using precision fermentation on agricultural waste substrates

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Abstract

Animal farming is responsible for a considerable portion of global greenhouse gas emissions. Decreasing carbon production from this source would thus slow the progress of global warming. In addition, treaties such as The Paris Agreement stipulate that there must be a global drive toward carbon neutrality. Novel food production methods must be explored to decrease carbon emissions associated with animal farming, ranging from milk production to meat production. Recombinant protein expression and precision fermentation are gaining popularity as potential options for producing food proteins such as bovine milk proteins. Bovine milk constitutes a staple part of global diets, and demand is increasing. However, the current method of dairy farming is a resource-heavy and environmentally damaging process. Precision fermentation to produce bovine identical milk proteins could usher in a new era of food production that steers the dairy industry towards an economical and environmentally friendly future.

In this study, we explore the production of bovine milk proteins from microbial hosts as a potential source of consumable proteins. We use a protease-deficient strain of *Escherichia coli* (*E. coli*) BL21 (De3) as a microbial host to express casein, the dominant protein in cow's milk. Additionally, we investigate common agricultural waste substrates as food sources for host organisms. We aim to determine the most efficient carbon source for maximum microbial growth sourced from an existing industry, redirecting waste from landfills.

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Table of contents

List of figures

List of tables

Abbreviations

BCN	beta-casein native signal
BCN-HA	beta-casein native signal with a HA tag
BLG	beta-lactoglobulin
BNO	beta-casein no signal
BNO-HA	beta-casein no signal with a HA tag
BCN-alpha	beta-casein alpha-amylase signal
CSN2	gene encoding beta-casein
CSN3	gene encoding kappa-casein
GRAS	generally regarded as safe
HA tag	human influenza hemagglutinin tag
KCN	kappa-casein native signal
KCN-HA	kappa-casein native signal with a HA tag
KNO	kappa-casein no signal
KNO-HA	kappa-casein no signal with a HA tag
KCN-alpha	kappa-casein alpha-amylase signal
OD	optical density
PCR	polymerase chain reaction
RPM	revolutions per minute

Chapter 1: Introduction

1.1 Alternative food sources are required in the future

The dire effects of climate change, which is caused primarily by the human-driven emission of greenhouse gases, requires that we mitigate the process as much as possible. The internationally binding UNFCCC (United Nations Framework Convention on Climate Change) Paris Agreement between 196 parties is committed to limiting global warming to 1.5 °C compared to pre-industrial levels (*The Paris Agreement to the United Nations Framework Convention on Climate Change*, 2015). The agreement requires international carbon neutrality by mid-century (i.e. no net production of carbon dioxide). In New Zealand, the dairy industry is responsible for over 20% of all greenhouse gases, which is more than any other economic sector (Ministry for the Environment , 2021b). Given New Zealand's commitment to the Paris Agreement, there is a need for the dairy sector to reduce its greenhouse gas emissions (Ministry for the Environment, 2022a; *The Paris Agreement to the United Nations Framework Convention on Climate Change*, 2015). The New Zealand government released its first emissions reduction plan in April 2022, which targets mitigation programs rather than investment into alternative clean technologies (Ministry for the Environment, 2022b). Whilst essential, the word mitigation only implies the reduction of serious or severe reality. Global leaders must support the movement away from traditional polluters to novel, clean technologies to meet their obligations.

Further intensifying the need for reduced carbon production in agriculture is population growth. With the world population set to approach 10 billion by 2050, the strain on our planet's resources for food production will be unprecedented (United Nations , 2019). Over 37% of the world's land area is committed to agriculture, and this number continues to rise (Food and Agriculture

Organization of the Unites Nations (FAO), 2020). Nevertheless, while the demand for food increases, agricultural intensification is bound by land and resource availability.

Finally, consumer sentiment about animal farming is changing (Whitton et al., 2021). More than ever, veganism is rising in popularity, with consumers choosing animal-free food, deeming traditional methods of animal agriculture unacceptable (Alonso et al., 2020; Bolton & von Keyserlingk, 2021; Gheihman, 2021; Schiano et al., 2020; Sexton et al., 2022). According to Fonterra (2020), the New Zealand dairy industry practices complete pasture-based grazing which is considered more ethical than barn raised, as the cows spend the majority of their lives outside free-roaming. However, globally, only 10-15% of dairy is produced this way (Moscovici Joubran et al., 2021). Despite this, New Zealand dairy farming practices are still considered unethical by many consumers - vegan and non-vegan alike (Alonso et al., 2020; Bolton & von Keyserlingk, 2021; Schiano et al., 2020). Surplus calves born into the New Zealand dairy industry are marked as “bobby calves” and are slaughtered within the first weeks of life. These calves are viewed as an unnecessary by-product of their mother’s pregnancies, and have very little value to the industry (Bolton & von Keyserlingk, 2021). Heightened awareness around this practice has facilitated a shift away from the consumption of cow's milk towards other alternatives such as soy, coconut, almond and oat milk (Clark & Bogdan, 2019; Schiano et al., 2020).

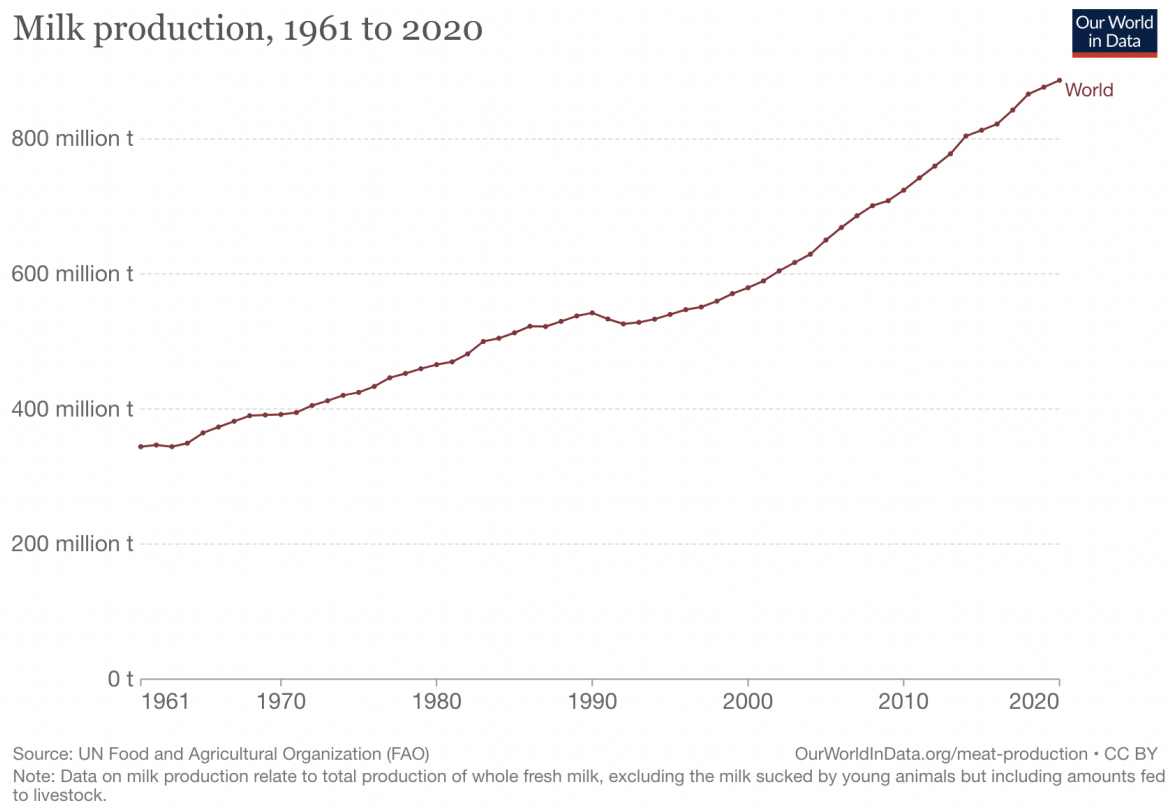


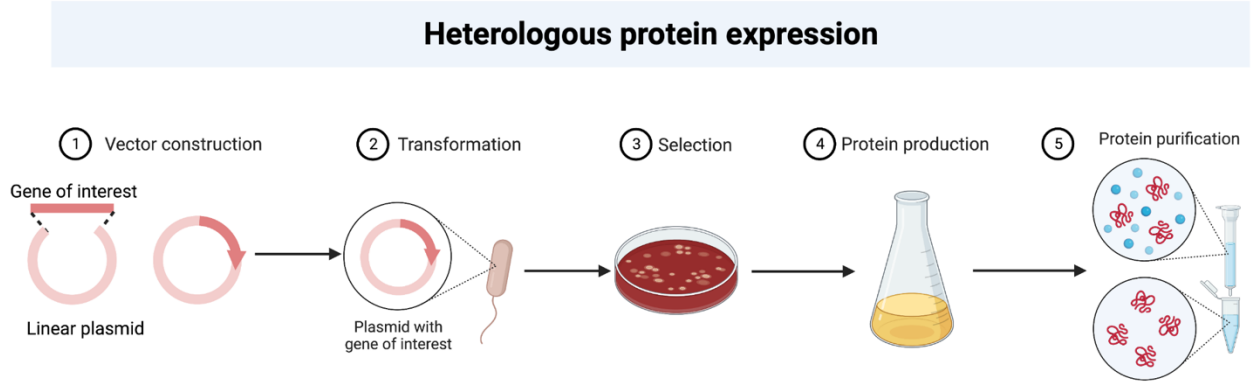
Figure 1.1: Global milk production continues to increase. Milk production has increased from 344.18 million tonnes in 1961 to 886.86 million tonnes in 2020. From Our World in Data, 2020 (https://ourworldindata.org/grapher/milk-production-tonnes). CC-BY.

However, overall global milk demand continues to outpace the effects of veganism on consumption, with milk production rising from 344 million tonnes in 1961 to over 880 million tonnes in 2020 (Ritchie & Roser, 2017) (**Fig. 1.1**). This is primarily attributed to increasing population and incomes, allowing people to consume more meat, eggs and dairy (Roser & Ritchie, 2017). Meeting the increased demand for dairy proteins while upholding sustainable and ethical practices presents a challenge.

1.1.1 Precision fermentation

One prominent area of research to meet such demand is using microorganisms as cell factories to produce specific functional ingredients, often proteins (K. F Chai et al., 2022; S. Singh et al., 2022; Teng et al., 2021; Terefe, 2022). This technology has been termed “precision fermentation” and it is used to produce heterologous proteins of which the coding sequence has been introduced into host organisms via genetic engineering (S. Singh et al., 2022; Teng et al., 2021; Terefe, 2022; K. F. Chai et al., 2022) (**Fig. 1.2**). The resultant protein is then extracted and purified for downstream applications. Heterologous protein expression to produce functional food ingredients is a well-established technology and is commonly used to produce a range of substances, including enzymes, insulin, riboflavin (vitamin B2), vanilla, and stevia (Hanlon & Sewalt, 2021). Modelling of greenhouse gas production from precision fermentation supports the viability of microbially produced proteins as an efficient, economical, and sustainable method of protein production (Hanlon & Sewalt, 2021; Vestergaard et al., 2016). Significant benefits, such as minimising land use for crops, reducing production costs, and reducing wasteful agricultural processes, have resulted in this technology being explored as a source for an expanding number of food ingredients (Hanlon & Sewalt, 2021). In particular, precision fermentation presents an opportunity to source milk proteins, a widely consumed and demanded product (Vestergaard et al., 2016).

Figure 1.2: Schematic of plasmid-based heterologous protein expression.



A linearised plasmid and the gene of interest, encoding a protein, are assembled to form a vector. The host organism is transformed with the DNA vector and expresses the protein of interest, which is purified for downstream applications. Created with BioRender.com

1.2 Casein proteins

The mammalian characteristic of producing milk for young functions to provide the neonate with its complete nutritional needs. Milk contains a vast range of minerals and vitamins alongside carbohydrates, lipids and proteins (Park, 2009). Humans are the only species that drink the milk of other mammals and whose consumption persists into adulthood (Montgomery et al., 2007). Bovine milk for humans is considered nutritionally beneficial, particularly for young children as a rich source of calcium (Black et al., 2002; Goulding et al., 2004; Rumbold et al., 2022; Teegarden et al., 1999). The ability of milk to be fractionated into a range of functional ingredients, for example the constituent fats and proteins, provides the dairy industry with multiple markets into which it can sell whole milk products or individual milk ingredients (Bansal & Bhandari, 2016; Carr & Golding, 2016). While New Zealand is the largest global dairy exporter, the primary export is not whole milk. Instead, the largest export is milk powder. In 2019, $5.73 billion worth of dried milk

powder was exported from New Zealand, primarily to the Chinese market (Observatory of Economic Complex (OCE) , 2020).

The milk protein casein is one of the main ingredients of milk powder and is a marketable commodity in its own right. Casein is a family of heterogeneous phosphoproteins found in all mammalian milk, though the composition and protein sequences differ between mammals (Bijl et al., 2020). Casein makes up 80% of the total protein content in bovine milk and consists of four subtypes, alpha-s1, alpha-s2, beta and kappa. Each subtype differs in its amino acid sequence, primary structure and post-translational modifications (Bijl et al., 2020). All four casein proteins contain the nine essential amino acids for human function and are thus a complete protein source (Hoffman & Falvo, 2004; Thorn et al., 2015).

1.2.1 The casein micelle

Casein proteins resemble unfolded yet functional proteins and tend to aggregate to form a critical colloidal structure, the casein micelle (Thorn et al., 2015). These micelles are composed of thousands of casein molecules that form thermodynamically stable aggregates (Holt et al., 2013). The casein micelle sequesters calcium, and much like other secretory calcium-binding phosphoproteins (SCPP), caseins use a similar mechanism to regulate the calcium phosphate of their environment (Bijl et al., 2020). The formation of the casein micelle also interferes with the propensity of individual casein proteins to form amyloid fibrils, which are insoluble aggregates that have been shown to damage mammary glands and impact milk secretion (Brooker, 1978; Claudon et al., 1998; Thorn et al., 2009). This is particularly true of kappa-casein and alpha-s2-casein. Unlike beta-casein and alpha-s1-casein, kappa-casein and alpha-s2-casein both contain two cysteine residues that form disulphide bonds (J. Liu et al., 2016; Thorn et al., 2009). At

physiological pH and temperature, disulphide bond reduction can cause the formation of amyloid fibrils (Thorn et al., 2008). Through chaperone action, beta-casein and alpha-s1-casein inhibit amyloid fibril formation, facilitating the formation of the micelle (Thorn et al., 2008).

Not only is casein a key ingredient in milk powder and other traditional dairy products, but it is an important additive in nutritional bars, as a thickener and emulsifier. It is also included in non-edible goods such as paint, paper and cosmetics (Costa et al., 2021). For the human consumer, the most noticeable function of the casein micelle in cheese is to provide the characteristic stretch and melt (Bijl et al., 2020).

Given casein's fundamental role in nutrition deliverance, dairy food and products, the casein micelle is well studied. However, the exact structure of the casein micelle is yet to be fully elucidated and remains under debate (Bijl et al., 2020; Horne, 2006). Casein proteins contain sticky proline-glutamine (P-Q) rich sequences that inspire protein-protein interactions. The P-Q rich sequences contribute to their ability to adhere to themselves and each other (Thorn et al., 2015). The same disulphide bonds responsible for forming amyloid fibrils are thought to contribute to the formation of the casein micelle. However, the nature and extent are unclear (Thorn et al., 2015). Several models have been put forward to suggest the micelle's exact structure and the general consensus is that calcium phosphate and casein proteins associate to form a matrix surrounded by kappa-caseins which confer stability (Adams et al., 2019; de Kruif et al., 2012; G. N. Smith et al., 2020) (**Fig. 1.3**). However, the exact mechanism by which this occurs is under debate (Carver et al., 2017; Horne & Lucey, 2017). Where Horne and Lucey (2017) strongly believe in the involvement of casein-casein hydrophobic interactions, Holt et al. (2013) dispute

this, citing main chain interactions through hydrogen bonding as the mechanism for micelle formation (Holt et al., 2013; Horne, 2017; Thorn et al., 2015).

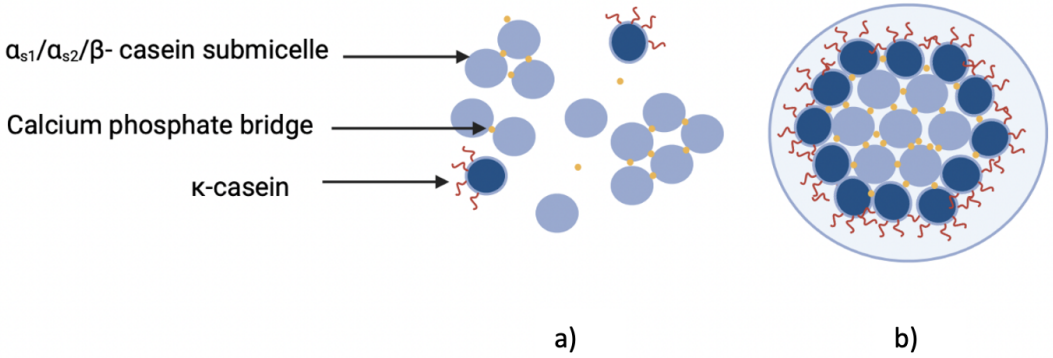


Figure 1.3: Schematic of the casein micelle. a) Individual casein sub-micelles interact via the formation of calcium phosphate bridges. **b**) Casein micelle formation. Figure adapted from (Middendorf et al., 2021)**.** CC-BY**.** Made using Biorender.com.

1.2.2 Alpha-casein

Bovine milk contains two alpha-casein subtypes, alpha-s1 and alpha-s2, which are the most calcium sensitive of the caseins (Bhat et al., 2016). Alpha-s1-casein is 214 amino acids long, including a 15 amino acid signal sequence (The UniProt Consortium, 2021). There are ten known allelic variants for alpha-s1-casein (A, B, C, D, E, F, G, H, I, and J). Alpha-s2-casein is 222 amino acids long, also including a 15 amino acid signal sequence (The UniProt Consortium, 2021). Alpha-s2-casein has only five known allele variants (A, B, C, D and E) (Meier et al., 2019). Alpha-s1-casein can be phosphorylated at up to 9 sites, whereas alpha-s2-casein has between 10 and 13 phosphorylation sites (Fang et al., 2016). Due to this, alpha-s2-casein is the most hydrophilic of the caseins (Kalyankar et al., 2016; Meier et al., 2019). Variant C of alpha-s1-casein is associated with a smaller net charge when compared to the B variant, leading to a stronger self-association (Schmidt, 1970). The result is a firmer curd in cheesemaking (Sadler et al.,1968).

Variant A differs significantly with residues 14-16 deleted. The loss of hydrophobic residues in this region results in micelles containing the A variant being packed more densely than micelles with the B variant (Day et al., 2015; Farrell et al., 2004). Additionally, the missing residues are involved in the cleavage by chymosin during cheese curdling. Thus, the deletion results in a much softer curd owing to those two factors (Farrell et al., 2004). Variant D of alpha-s2-casein has residues 51-59 deleted as a result of exon skipping (Bouniol et al., 1993). Compared to other variants, it has reduced calcium sensitivity (Gai et al., 2021).

1.2.3 Kappa-casein

Kappa-casein is 190 amino acids long, 21 of which encode a signal sequence. The mature protein has a molecular weight of approximately 19 kDa (Bhat et al., 2016). There are 12 known kappa-casein allelic variants, A and B being the most common (Bijl et al., 2020; Caroli et al., 2009). In terms of cheese-making properties, it is commonly accepted that cows homozygous for the B allele have a much faster coagulation time and increased curd firmness (Di Gregorio et al., 2017; Nilsson et al., 2020; Pazzola et al., 2020; Poulsen et al., 2013; Tyulkin et al., 2018). It has been suggested that the B allele has a reduced micelle size, resulting in a more compact arrangement and increased inter micelle bonds per unit of surface area (Bonfatti et al., 2010).

Post-translational modifications of the casein proteins influence their stability (Nilsson et al., 2020). Kappa-caseins can be phosphorylated at three available sites and glycosylated at up to 6 sites (Holland et al., 2006). While several isoforms exist, research is inconclusive as to what the effects are on cheese coagulation time and curd firmness. Some studies have indicated that a higher proportion of glycosylated kappa-casein improves cheese formation by stabilising the casein micelle (Bonfatti et al., 2014; Jensen, Poulsen, et al., 2012). However, Bonfatti et al. (2014)

found that samples with a higher frequency of glycosylation exhibited decreased coagulation times. Bilj et al. (2014) found that the only significant positive impact of coagulation of milk was attributed to the increase in the relative concentration of phosphorylated kappa-casein. In addition, Bijl et al. (2014) found a high proportion of glycosylated casein in milk with the B allele. Conversely, it has been theorised that a higher portion of glycosylation adds to the steric hindrance and electrostatic repulsion, preventing the micelles from coagulating (Jensen, Holland, et al., 2012; Jensen, Poulsen, et al., 2012).

1.2.4 Beta-casein

Beta-casein is 224 amino acids long, including a 15 amino acid signal sequence. The mature protein is approximately 24 kDa (The UniProt Consortium, 2021). There are 12 recorded allelic variants for the beta-casein gene. The most common, A1 and A2, differ at position 67. A2 encodes a proline, whereas A1 has a histidine (Sebastiani et al., 2020) (**Figure 1.4**). The A2 allele has been found to form smaller micelles than A1 due to decreased hydrophobicity (Raynes et al., 2015). However, some research suggests that the B allele, followed by A1, is the most favourable for coagulation time and curd firmness (Comin et al., 2008; Poulsen et al., 2013). The consequence of the amino acid change at position 67 in the A1 variant is the production of a seven amino acid long protein, beta-casomorphin-7 (BCM-7). BCM-7 is produced in the intestine by the elastase enzyme that targets the histidine at position 67, cleaving it from beta-casein (Sebastiani et al., 2020). BCM-7 has been linked to several chronic diseases such as type 1 diabetes, heart disease, infant death, autism and gastrointestinal disorders (Brooke-Taylor et al., 2017; Chia et al., 2017; Kamiński et al., 2007). However, the lack of proven causation prevents conclusions from being drawn around the health impacts of A1 milk (Brooke-Taylor et al., 2017; Chia et al., 2017; Kaskous, 2020; Küllenberg de Gaudry et al., 2019). The B allele of beta-casein

also has the histidine at position 67, indicating that it may influence human chronic diseases. Interestingly, many studies have found that the A2 allele can also produce BCM-7, although at lower levels than A1. Lambers et al. (2021) simulated gastrointestinal digestion and found BCM-7 produced from both A1 and A2 milk. Their results support the claims of several other researchers who have suggested that A2 is not exempt from proteolysis resulting in BCM-7 (Asledottir et al., 2017, 2018; Cieślińska et al., 2007).

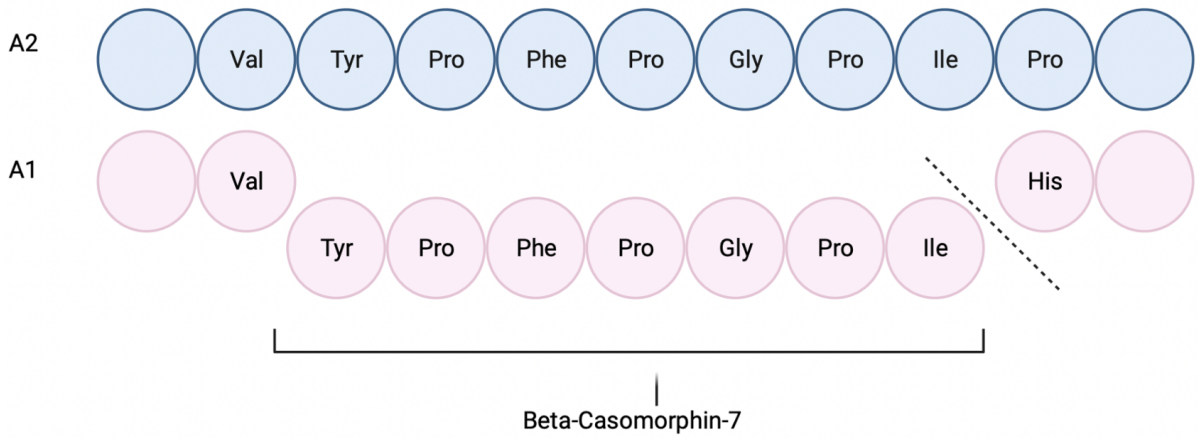


Figure 1.4: Beta-casomorphin-7 is the result of A2 beta-casein cleavage in the intestine. An amino acid change at position 67 from proline to histidine in A2 beta-casein results in an elastase recognition site. Cleavage at this location causes the release of BCM-7, which has been linked to various human diseases. Made using Biorender.com.

Regardless of the scientific support for or against the disease-causing mechanism of BCM-7, extensive marketing has gone into promoting A2 milk products (Fortune Business Insights, 2022; The A2 Milk Company, 2022). Thus, when considering allele selection for heterologous beta-casein expression, the final consumer optics must be kept in mind.

1.3 Whey proteins

Whey proteins are water-soluble proteins that make up the remaining 20% of bovine milk proteins (Bijl et al., 2020). Of that, 50% is beta-lactoglobulin, 20% is alpha-lactalbumin, and the remaining 30% comprises blood serum albumin (BSA), immunoglobulins, lactoferrin, transferrin and other enzymes (Madureira et al., 2007). Whey was initially considered a liquid by-product of cheese making. However, increased interest and research into whey proteins have revealed some nutritional benefits, and it is now broadly used in dairy products (H. C. Liu et al., 2007; Mann et al., 2019).

1.3.1 Beta-lactoglobulin

Beta-lactoglobulin is 162 amino acids long, with a 16 amino acid signal sequence and a molecular weight of 18.2 kDa (Crowther et al., 2016). It makes up over 50% of the total whey content in milk and significantly influences the behaviour of the whey fraction. Under physiological conditions, beta-lactoglobulin exists as a dimer (Mercadante et al., 2012). At temperatures above 60 °C, reversible dissociation and conformational changes occur. Above 70 °C, unfolded monomers can aggregate to form oligomers and complexes with other milk proteins, namely kappa-casein, via disulphide interactions (Asaduzzaman et al., 2021). Beta-lactoglobulin denaturation and subsequent aggregation at higher temperatures facilitate its ability to form gels (Donato & Guyomarc'H, 2009). These gels form the basis of yoghurts, ice cream, cream cheese and other soft dairy products (Lee & Lucey, 2010). X-ray crystallography investigations of beta-lactoglobulin demonstrate a close similarity in structure to a plasma-retinol binding protein, a member of the lipocalin family (Papiz et al., 1986). Thus, beta-lactoglobulin is classed as a lipocalin (Pervaiz & Brew, 1987). Lipocalins share a structural similarity, comprising nine antiparallel beta-sheets (strands A-I), of which eight fold back on themselves to form an internal cavity called the calyx.

The internal calyx acts as a binding site for various substrates such as vitamin A, vitamin D, fatty acids and minerals, which is essential in facilitating nutrient transfer from mother to calf (Al-Shabib et al., 2018; Sahihi, 2016; Yang et al., 2009; Yi & Wambo, 2015; J. Zhang et al., 2014). There are at least 11 allelic variants of beta-lactoglobulin, with the A and B alleles being the most common. Variant B differs from variant A by two amino acid substitutions: D64G and V118A (Farrell et al., 2004). The B variant has been associated with a higher yield, fat and protein content (Czerniawska-Piatkowska et al., 2011; Tsiaras et al., 2005). Meza-Nieto et al. (2013) demonstrated that the properties of the B variant and its increased association with casein micelles influence the coagulation properties resulting in higher cheese yield. Conversely, some studies have reported the A allele to have a shorter curdle time and increased firmness due to decreased solubility, resulting in better oligomerisation and gelation (Glantz et al., 2011; Qin et al., 1999).

1.3.2 Alpha-lactalbumin

Alpha-lactalbumin is the second most abundant whey protein in milk, at 22% in human milk and 3.5% in cow's milk. It is 142 amino acids long, including a 19 amino acid signal sequence with a molecular weight of 16.2 kDa (The UniProt Consortium, 2021). There are four known allele variants (A, B, C, and D) (Visker et al., 2012). During milk production, alpha-lactalbumin is produced in the mammary epithelial gland and combines with the enzyme beta-1-4-galactosyltransferase to form lactose synthase, which converts glucose and galactose into lactose (Layman et al., 2018). Thus lactose concentration in milk is proportional to the alpha-lactalbumin concentration (H. Deeth & Bansal, 2019). Alpha-lactalbumin has a bilobal structure similar to that of a lysosome. One lobe comprises α-helices and β-sheets, while the other is unordered. Between the lobes is a cleft which is the predominant calcium-binding site (Brew, 2013).

Importantly, while milk composition studies dominate the world of traditional dairy research, during precision fermentation the heterologous proteins are expressed without the native combination of proteins, fats, sugars and minerals. Thus, the characteristics of the individual allele become more important.

1.4 Heterologous expression of dairy proteins

Heterologous gene expression of casein or other milk proteins, presents an opportunity to produce dairy-identical proteins for consumption to avoid the associated ethical and environmental consequences of the traditional dairy and agricultural industries (Vestergaard et al., 2016).

1.4.1 Expression of dairy proteins in *Escherichia coli*

Previous studies of casein expression in the bacterial host *Escherichia coli* (*E. coli*) have shown that proteins with the same molecular weight as the natural bovine casein are produced (C. Kang, 1988). However, *E. coli* cannot perform post-translational modifications such as phosphorylation and glycosylation required by many eukaryotic proteins (Kaur et al., 2018). Human beta-casein has been expressed from *E. coli* with protein yields of 300-500 mg/l (Hansson et al., 1993). However, the beta-casein expressed from *E. coli* was unphosphorylated. Although expression of beta-casein in the eukaryote *Saccharomyces cerevisiae (S. cerevisiae)* yielded only 30-50 mg/l, it showed the same phosphorylation profiles as the native beta-casein from human milk (Hansson et al., 1993).

Alpha casein can also be expressed at high levels from *E. coli,* with expression of codon-optimised casein sequences consisting of up to 18% of total cell protein compared to less than 7.6% for native bovine sequence (Goda et al., 2000). However, as with beta-casein when produced in *E. coli*, the proper post-translational modifications do not occur. For proper modification and folding, casein expression requires a eukaryotic host.

Beta-lactoglobulin has been successfully expressed in *E. coli*, with yields of up to 160 mg/l (Ariyaratne et al., 2002; Batt et al., 1990; Keppler et al., 2021). Given the lack of post-translational modifications that the native beta-lactoglobulin possesses, the expressed protein also correctly folds and appears functionally similar to the native protein (Loch et al., 2016; Ponniah et al., 2010) The ability to perform post-translational modifications with a mammalian pattern is important when considering host organisms.

1.4.2 Expression of dairy proteins in *Saccharomyces cerevisiae*

Fungal hosts are often favoured for the expression of recombinant mammalian proteins due to their ability to perform post-translational modifications (Quartley et al., 2009). *S. cerevisiae* has been used to express both casein proteins and beta-lactoglobulin with the expected phosphorylation modifications. However, Hansson et al. (1993) achieved only 10% protein yield compared to the expression achieved in *E. coli.* Yields of up to 10 mg/l have been achieved for beta-casein, with phosphorylation profiles analogous to native bovine beta-casein (Jimenez-Flores et al., 1990). However, the protein molecules also exhibited a higher molecular weight, determined to be due to additional glycosylation (Jimenez-Flores et al., 1990). While the functionality or coagulation ability was not tested in any of these studies, the ability of *S. cerevisiae* to perform post-translational modifications holds promise that these caseins may be able to

perform with similar functionality to native bovine milk proteins. Beta-lactoglobulin has been expressed by *S. cerevisiae*, with a yield of 1.1 mg/l (Totsuka et al., 1990). One issue with *S. cerevisiae* is the tendency to hyperglycosylate (Tang et al., 2016). Furthermore, compared to other expression systems, it has a low protein yield, which has encouraged researchers to look into alternative hosts for more efficient protein production (Baghban et al., 2019).

1.4.3 Expression of dairy proteins in *Pichia pastoris*

Yeasts such as *Pichia pastoris* (*P. pastoris*) are widely favoured expression hosts (Ahmad et al., 2014). Pichia is a non-fermentative crabtree-negative yeast. As a result, glucose is not converted to ethanol or other organic acids under anaerobic conditions, facilitating the formation of higher biomass and protein yields (Ahmad et al., 2014). However, it can store a high amount of toxic methanol, and its endogenous proteases can impact the heterologous proteins (Baghban et al., 2019). Advantageously, *P. pastoris* has well-characterised inducible promoters for expression such as AOX1, which is repressed by glucose, glycerol or ethanol and activated with methanol (Ahmad et al., 2014; Rozanov et al., 2020; Vogl & Glieder, 2013). Heterologous proteins expressed under the control of the AOX1 promoter have resulted in yields of up to 22 g/l (Hasslacher et al., 1997). *P. pastoris* is generally regarded as safe (GRAS), making it an appropriate host for the production of consumable proteins (Ahmad et al., 2014). Beta-casein has been expressed successfully in *P. pastoris* with yields of 0.7-1.0 g/l with proper post-translational modifications and no hyperglycosylation (Choi & Jiménez-Flores, 2001; Choi & Jiménez-Flores, 1996). Beta-lactoglobulin has been expressed in *P. pastoris,* also resulting in high yields of more than 1 g/l and identical physical characteristics to the native bovine beta-lactoglobulin (Kim et al., 1997).

1.4.4 Limitations of current expression systems for expressing dairy-identical proteins

There are very few recent studies detailing heterologous expression of casein and whey proteins. However, the past few years have seen significant advances in the technology surrounding heterologous gene expression and the techniques by which protein yield can be increased (Baghban et al., 2019; Gonzalez et al., 2019; Kaur et al., 2018; Rantasalo et al., 2019; Zarzhitsky et al., 2020). With only a small number of published studies on heterologous expression of dairy proteins, it is hard to predict when proper post-translational modification will occur, or what the expression level and functionality of the protein will be in different expression systems. Finally, there is currently no evidence to suggest that the casein proteins can be produced with the structure and modifications that would allow them to carry out their function as the coagulation factor in milk.

1.4.5 *Trichoderma reesei* as a host organism

Trichoderma reesei (*T. reesei*) was initially discovered during World War II on the rotting canvas of United States Army soldiers in the Solomon Islands (Schuster & Schmoll, 2010). Since its discovery over 70 years ago, the filamentous fungi has become increasingly popular for its ability to secrete high levels of cellulase enzymes (Schuster & Schmoll, 2010). Now a well-studied and genetically characterised microorganism, *T. reesei* is often exploited as a host for heterologous protein expression (Nogueira-Lopez et al., 2019; Rantasalo et al., 2019; Y. Sun et al., 2022; G. Zhang et al., 2014; J. Zhang et al., 2018). Most gene expression systems utilise the cellulosic gene promoter to engage high expression and secretory pathways allowing heterologous expression at high yields (S. Chai et al., 2022; Jørgensen et al., 2014; Nogueira-Lopez et al., 2019; Peterson & Nevalainen, 2012; Schuster & Schmoll, 2010; Y. Sun et al., 2022; G. Zhang et al., 2014; J. Zhang et al., 2018). Importantly, *Trichoderma spp*. are a GRAS organism, making

them suitable for the production of heterologous proteins for food (Blumenthal, 2004). *Trichoderma spp.* have previously been used to produce pet food, fruit juice, livestock feed, pharmaceuticals and textiles (Harkki et al., 1989). They possess eukaryotic machinery to facilitate post-translational modifications and do not hyperglycosylate like *S. cerevisiae* (Nevalainen et al., 2005; T. Zhang et al., 2020). However, *T. reesei* grows very slowly compared to other host organisms such as *E. coli* (Harkki et al., 1989; Nevalainen et al., 2005). Furthermore, *T. reesei* express a high level of endogenous enzymes such as cellulase, which may negatively impact heterologous protein expression.

1.4.6 *Kluyveromyces lactis* as a host organism

Kluyveromyces lactis (*K. lactis*) is another common host for precision fermentation (Spohner et al., 2016). Like *P. pastoris*, *K. lactis* is crabtree-negative and can produce a high biomass (Spohner et al., 2016). *K. lactis* has been used to produce over 100 proteins and enzymes, many for commercial use, such as human serum albumin, chymosin, glucoamylase, interferons, and insulin (Almeida et al., 2015; Lodi et al., 2005; Paciello et al., 2010; Spohner et al., 2016). *K. lactis also* has GRAS status, making it permissible to use for food products (van Ooyen et al., 2006). Popular promoters include the phosphoglycerate kinase (PGK) promoter and acid phosphatase (PHO5) promoter from *S. cerevisiae* or *K. lactis* lactase promoter (LAC4; beta-galactosidase) (van Ooyen et al., 2006). Furthermore, successful cellular export of proteins has been shown using the alpha-mating factor from *S. cerevisiae* and cellobiohydrolase I (*cbhI*) promoter from *T. reesei* (Madhavan & Sukumaran, 2015), achieving heterologous protein expression in the order of g/l (Spohner et al., 2016).

1.5 Methods for increasing heterologous protein expression

1.5.1 Codon-optimisation

Each organism has a unique codon bias, where certain codons are favoured over others encoding the same amino acid (Gustafsson et al., 2004) (**Table 1.1**). Codon optimisation is the process of altering the coding sequence of a recombinant gene in accordance with the codon bias of the host organism (Mauro & Chappell, 2014). The amino acid sequence of the final gene remains unchanged, however codon optimisation can improve mRNA stability, translation rate, protein folding and post-translational processing, thus improving protein expression (Alexaki et al., 2019; Burgess-Brown et al., 2008; Gustafsson et al., 2004; Hale & Thompson, 1998; Hia & Takeuchi, 2020). However, codon optimisation may have unpredictable negative effects as synonymous changes can impact the protein's folding, structure, and function (Lipinszki et al., 2018; Nilsson et al., 2020).

Table 1.1: Comparison of arginine codon usage between *E. coli* and *Bos taurus* (Cow)

		E. coli		*Bos taurus* (Cow)	
Triplet	**Amino acid**	**Fraction**	**Frequency/ Thousand**	**Fraction**	**Frequency/ Thousand**
CGU	Arginine	0.36	20.2	0.08	4.6
CGC	Arginine	0.37	20.8	0.20	11.1
CGA	Arginine	0.07	3.8	0.11	6.4
CGG	Arginine	0.11	6.2	0.22	12.5
AGA	Arginine	0.05	2.9	0.19	10.7
AGG	Arginine	0.03	1.8	0.20	11.4

Codon frequencies obtained from http://www.kazusa.or.jp/codon/.

1.5.2 Protein purification

Successful expression of a heterologous protein such as casein for food, depends on the ability to purify it. Protein fusion tag technology has been developed to promote efficient purification of proteins, encouraging high yield and purity (Lichty et al., 2005). There are several different options for protein tags, depending on the host, protein characteristics and purpose (Lipinszki et al., 2018). The six histidine (6-His) tag is a frequently used and effective tag (Lichty et al., 2005). With the addition of histidines, the tagged protein can be purified through immobilised metal affinity

chromatography (Saraswat et al., 2013). Small in size and charge, the 6-His tag is often thought to have minimal impact on the protein's structure, although it can impact function (Booth et al., 2018; D. K. Yadav et al., 2016; D. Zhao & Huang, 2016). However, to mitigate effects on function for downstream applications, typically a protease cleavage site is included for easy removal of the tag (Goh et al., 2017). Epitope tags bound to target proteins are captured with immobile antibodies. The elution is often required to be at low or high pH, which irreversibly affects the properties of the target protein (Kimple et al., 2013). Similarly, the maltose binding tag which binds cross-linked amylase can significantly increase protein yield however it also impedes translational efficiency, reducing expression levels (D. K. Yadav et al., 2016). The glutathione S-transferase (GST) tag and calmodulin tag are only appropriate for use in prokaryotes, as eukaryotes possess these two proteins endogenously (Harper & Speicher, 2011; Malhotra, 2009; X. Zhao et al., 2013). When considering tags for the expression of casein as a food source, minimal impact on the protein is essential.

1.5.3 Signal peptides and chaperones

Secretion signals and molecular chaperones can be used to maximise the efficiency of protein isolation and production, respectively. A secretion signal is encoded by a peptide at the N-terminus of a protein, and facilitates the translocation of proteins out of the host cell. This means cell lysis is no longer necessary for protein isolation (Owji et al., 2018). Cell lysis is unfavourable during protein isolation due to the contamination by host endogenous proteins. These may pose a toxicity risk which should be minimised when producing proteins such as casein, for consumption (Han et al., 2017). The bacterial host *E. coli* is a poor secretor, and signal peptides, particularly in the Sec and Tat pathways, have been investigated to encourage the translocation of heterologous peptides across the cell membrane (Crane & Randall, 2017; Han et al., 2017;

Ismail et al., 2011; Low et al., 2012; Natale et al., 2008; Velaithan et al., 2014). In fungal hosts such as *P. pastoris*, *K. lactis* and *T. reesei*, signal peptides originating from fungi are often used, as they are excellent extracellular protein producers (Madhavan & Sukumaran, 2015). One of the most commonly used signal sequences, particularly for heterologous expression in *P. pastoris,* is the alpha-mating factor from *S. cerevisiae* (Ahn et al., 2016; Karim & Tasnim, 2022; Lin-Cereghino et al., 2013). The importance of efficient protein secretion for heterologous protein production has led to the identification of many signal sequences, and the development of novel or improved sequences through mutation (Aza et al., 2021; Barrero et al., 2018; Chahal et al., 2017; Lin-Cereghino et al., 2013). The glucoamylase and alpha-amylase secretion signals from *Aspergillus awamori* are also proven signal sequences for improved expression of heterologous proteins (S. Chai et al., 2022; Rantasalo et al., 2019; W. Smith et al., 2014; Zhong et al., 2011). Finally, *T. reesei* is already an efficient secretor of proteins containing cellobiohydrolase I (*cbhI*) signal peptides (Joutsjoki et al., 1993; J. Zhang et al., 2018; Zhong et al., 2011).

Molecular chaperones facilitate the folding and translocation of the protein across the cellular membrane by binding hydrophobic protein pockets and preventing aggregation (Camberg et al., 2013; Hartl et al., 2011). This is particularly important in *E. coli* hosts, where proteins readily form inclusion bodies (Palmer & Wingfield, 2004). In *E. coli*, the two predominant chaperones are the ATP-dependent DnaK-DnaJ-GrpE and GroEL-GroES systems (Baneyx & Palumbo, 2003). Co-overexpression of chaperones and the protein of interest in *E. coli* can enhance the solubility of heterologous proteins (Baneyx & Palumbo, 2003). Other molecular chaperones, such as Spy, can improve the solubility of proteins when expressed in fusion with the target protein (Ruan et al., 2020).

In eukaryotes, the heat shock protein (HSP) family makes up the most common and versatile eukaryotic chaperone network (Buchner, 2019). Amongst others, PDI (protein disulphide isomerase), Ero1 (endoplasmic reticulum oxidoreduction 1) and BiP are chaperones that have all been used with varying success for protein expression in both *P. pastoris* and *K. lactis* (González Siso & Cerdán, 2012; Iwata et al., 2004; Lodi et al., 2005; Yu et al., 2020; W. Zhang et al., 2006). While several chaperones have been shown to aid in the expression of heterologous proteins, evidence suggests that a bespoke chaperone co-expression strategy designed for each different protein is optimal. This is often done through trial and error, as chaperon-protein interactions differ between different proteins (Damasceno et al., 2007; Mamipour et al., 2017; Samuel et al., 2013; van Ooyen et al., 2006). In the context of secretion of heterologous casein proteins, alpha-s1-casein, alpha-s2-casein and beta-casein are all relatively hydrophobic and may require assistance to cross the cell membrane (Bhat et al., 2016). Furthermore, the propensity of casein proteins to form amyloid fibrils suggests that they may form inclusion bodies inside the cell, resulting in insufficient secretion (Thorn et al., 2009). Given that casein proteins act as chaperones to one another in the micelle, it is thought that heterologous expression of casein may be improved with the co-expression of a chaperone (Thorn et al., 2009).

1.6 Agricultural waste substrates can be used to grow host organisms

Each year, New Zealand produces 17.49 million tonnes of waste, of which 14.7% is food and green waste from industrial and household sources (Ministry for the Environment, 2021a). Reynolds et al. (2016) estimated that the industrial food waste in 2011 from the manufacture and production of food amounted to 103,000 tonnes. Fruit, vegetable and cereal waste contributed roughly 17,000 tonnes, with organic waste contributing an additional 52,000 tonnes. Up to 60% was still fresh or edible food (Reynolds et al., 2016). While it is estimated that food and green

waste has declined since 2011, food waste contributes significantly to greenhouse gases and landfill (Ministry for the Environment, 2004, 2021a). Redirecting waste towards composting, recycling, or value-added pathways has been a central focus of government agencies striving to meet global climate targets under the Kyoto Protocol and Paris Agreement (Bioresource Processing Alliance, 2022; *Kyoto Protocol to the United Nations Framework Convention on Climate Change*, 1997; Ministry for the Environment, 2004, 2022a, 2022b; *The Paris Agreement to the United Nations Framework Convention on Climate Change* , 2015). Promising uses for agricultural food waste include the preparation of media cultures for the growth of microorganisms (Lad et al., 2022). Using waste in precision fermentation has the potential to reduce operational and environmental costs, reduce landfill, and would support a circular bioeconomy.

1.6.1 Growth of *Trichoderma spp.* on agricultural-waste

As saprotrophs and mycoparasites, *Trichoderma spp.* secrete large amounts of enzymes that facilitate the decomposition of plant material and can flourish on a broad range of nutrient sources (Mukherjee et al., 2022). The most common growth source for lab strains of *T. reesei* is potato dextrose agar (PDA). PDA is nutrient-rich and can be made from pre-prepared commercially available powder or through the boiling and straining of potatoes (U.S Food and Drug Administration (FDA) , 2020). *Trichoderma spp*. can grow robustly on various agricultural waste such as maise husk, rice husk, wheat bran, sawdust, broom sorghum grain, rice straw, vermicompost, areca nut leaf, banana leaf, coconut leaf, and cow manure (Boblina et al., 2019; Chakrabarty et al., 2014; Nabi et al., 2017; Naeimi et al., 2020; Saju et al., 2002; R. S. Singh et al., 2001; L. S. Yadav, 2020; Yparraguirre & Galliani-Pinillos, 2020), although optimal cultivation conditions vary between strains (Rossi-Rodrigues et al., 2009). *Trichoderma lixii* (*T. lixii*) has been cultured on vegetable waste, spent tea levels, cow manure, vermicompost, and sugarcane

bagasse under varying temperatures and moisture content, with spore production highest on sugarcane (Sachdev et al., 2018).

Many of these substrates have high cellulose content, and *Trichoderma spp.* are heralded for their ability to secrete high yields of cellulases. Cellulases have industrial interest due to their multifaceted role in biofuel production, textile polishing, paper and pulp manufacture, and agriculture (Jayasekara & Ratnayake, 2019). Increased market price of cellulase has directed efforts towards obtaining high volumes cheaply and efficiently.

However, few studies have investigated whether growth on agricultural waste affects cellulase production or hydrolytic performance. Sipos et al. (2010) found a correlation between the amount of xylan in the carbon source and the amount of xylanase and mannanase enzymes produced. However, growth rate needs to be considered as well. *T. reesei* grows faster on glucose than wheat straw and lactose, although evidence suggests protein secretion is maximised when *T. reesei* was grown on lactose (Bischof et al., 2013). Proteins are generally secreted at the tip of the growing hyphae, thus, a greater number of active hyphal tips should result in higher protein yield. Interestingly, glycerol, not lactose, is known to increase the length and number of hyphal tips. Conversely, glucose and Cellulosec™ media result in a higher number of spores (da Silva Delabona et al., 2016).

A related but separate topic is the expression of heterologous enzymes from *T. reesei*, which has been demonstrated on wheat bran, potato, orange, banana, kiwi fruit peels, and barley straw (Long et al., 2018; Shenouda et al., 2022; Siamphan et al., 2022; Tao et al., 2008). However, in all cases, the heterologous proteins have been from other fungal organisms. No research has

been done to test how growth medium influences the expression of non-fungal recombinant proteins from *T. reesei*. Given its increasing popularity as a host for heterologous protein expression, this is an important area to explore. Maximising the efficiency of production of valuable proteins and enzymes from *T. reesei* on waste products would fully optimise a circular model of economic operation in which sustainability is at the core.

1.6.2 Growth of *Pichia spp.* on agricultural-waste

The use of agricultural waste as a substrate for *Pichia spp.* growth is comparatively understudied. Unlike *Trichoderma spp., Pichia spp.* are not included in the array of cellulolytic fungi (Andlar et al., 2018). However, given the ability of *P. pastoris* to metabolise methanol and produce ethanol, the feasibility of bio-ethanol production using waste as a substrate has been studied (Dong et al., 2020; Krainer et al., 2012; Shin et al., 2015). To improve *P. Pastoris'* ability to degrade cellulose waste materials, Sun et al. (2015) engineered a recombinant strain to produce xylanase and metabolise xylose, a significant cellulose component (T. Sun et al., 2020). *Pichia stipitis (P. stipitis)*, has been successfully grown on hydrolysed sugar beet pulp to produce ethanol (Günan Yücel & Aksu, 2015). While the study demonstrated the potential for sugar beet pulp as a growth substrate, it comes with some heavy caveats when applied to the context of recombinant protein expression for food. *P. stipitis,* while advantageous in xylose degradation, is not favoured over *P. pastoris* for heterologous protein production (Caspeta et al., 2012; Juturu & Wu, 2018). Additionally, the heavy processing of sugar beet reduces its attraction as a cost-effective and straightforward source for the expression of recombinant proteins. While *P. pastoris* is unable to degrade cellulose, common agricultural-waste such as fruit and vegetables contain easy-to-ferment simple sugars such as glucose (Prielhofer et al., 2015). There remains a gap in the

knowledge surrounding the ability of *P. pastoris* to efficiently grow on and express heterologous proteins on common agricultural waste.

1.6.3 Growth of *Kluyveromyces spp.* on agricultural-waste

While *K. lactis* is the favoured *Kluyveromyces* strain for heterologous protein production, *Kluyveromyces marxianuns (K. marxianuns)* has recently seen increased industrial interest. *K. marxianuns* can metabolise an extensive range of sugars to produce ethanol, which is important in the development of biofuels (Lane & Morrissey, 2010; Varela et al., 2017). However, ethanol yields from *K. marxianuns* have been found to be lower on corncob and soybean waste compared to when cultured on synthetic sugars (M. Zhang et al., 2010). The reduction in ethanol yields was attributed to the presence of inhibitory compounds produced during substrate hydrolysis (M. Zhang et al., 2010). Conversely, fermentation on taro peel waste resulted in higher ethanol yields than when grown on the base media with glucose (Wu et al., 2016). The significant barrier in these studies is the requirement of a pre-treatment step to break down the cellulose into fermentable sugars. These steps often require expensive enzymes or acids, increasing both the cost and effort of the process. To mitigate this, recombinant strains of *K. marxanius* have been designed to express hemicelluloses and xyloses for simultaneous saccharification and fermentation of lignocellulosic waste to produce ethanol (Wang et al., 2013; M. Zhang et al., 2010).

K. lactis can produce ethanol from the lactose present in whey, the by-product of cheese manufacturing (Ariyanti & Hadiyanto, 2013; Ferreira et al., 2015; Oda & Nakamura, 2009; Sadik & Halema, 2014). A recombinant strain of *K. lactis* containing the alpha-galactosidase gene from *S. cerevisiae* showed promising growth and protein expression when cultured on whey and sugar

beet molasses (Álvarez-Cao et al., 2018). There has been limited research on *Kluyveromyces spp.* growth on agricultural-waste. Most studies have focussed on the fermentation of *K. lactis* on lactose and the ability to produce ethanol as a biofuel source (de Freitas et al., 2020; Mansour et al., 1993; Marcus et al., 2021; Oda & Nakamura, 2009; Sampaio et al., 2020; Veteikytė et al., 2017). To our knowledge, only one study has investigated fermentation of waste substrates for the production of recombinant proteins with waste utilisation (Álvarez-Cao et al., 2018).

1.7 Research hypothesis and objectives

We aim to provide proof-of-principle results showing that dairy-identical proteins can be obtained via precision fermentation and thus outside the traditional dairy industry, and the repurposing of agricultural waste for precision fermentation can further minimise its environmental impact.

In order to confirm our hypothesis we will address four objectives:

1. **Construct protein expression systems for bovine beta-casein and kappa-casein.** We will codon optimise the bovine beta-casein and kappa-casein open reading frames for the chosen host organism and clone them into an expression vector.

2. **Stably transform host strains with expression vectors.**

We will transform expression vectors into our chosen host and analyse successful transformants with colony PCR to confirm the presence of the beta-casein or kappa-casein open reading frame.

3. **Express beta-casein and kappa-casein proteins from a microbial host.**

We will induce expression of the proteins of interest and detect their presence using SDS-PAGE and western blot.

4. **Explore the growth of potential host organisms on waste media.**

We will perform growth assays with commonly used host organisms on a range of agricultural-waste to determine the viability of waste as a cultivation media.

Chapter 2: Materials and Methods

2.1 Strains used in this study

Expression of recombinant bovine casein was tested in both fungal and bacterial hosts (**Table 2.1**).

Table 2.1: Strains used in this study

Strain	Description	Source
QM6a *Trichoderma reesei*	ATCC 16331	Artemio Mendoza, Lincoln University
Top10 *Escherichia coli*	One Shot ™ TOP10 chemically competent *E. coli* cells.	Invitrogen™ Catalogue number: C404010
BL21 (DE3) *Escherichia coli*	One Shot™ BL21 Star™ (DE3) Chemically Competent *E. coli* *lon* and *ompT* protease deficient	Invitrogen™ Catalogue number: C601003

2.2 Gene inserts

Beta-casein and kappa-casein alleles (**Table 2.2**) were chosen based on their reported benefits to human health or cheese coagulating properties. Nucleotide sequences were obtained from the National Center for Biotechnology Information (NCBI). Open reading frames were cloned with

either the native signal sequence, no signal sequence or the alpha-amylase signal sequence from *Aspergillus awamori* (**Table 2.3**). The nucleotide sequences were ordered from Twist Biosciences.

Table 2.2: Genes used in this study

Gene	Protein	Allele	NCBI Accession
CSN2	Beta-casein	A2	M16645
CSN3	Kappa-casein	B	AY380229

Table 2.3: Gene insert nomenclature

Gene	Protein	Signal	Tag	Abbreviation	Length (bp)
CSN2	Beta-casein	Native signal	None	BCN	675
CSN2	Beta-casein	No signal	None	BNO	630
CSN2	Beta-casein	alpha-amylase*	None	BCN-alpha	714
CSN2	Beta-casein	Native signal	HA	BCN-HA	702
CSN2	Beta-casein	No signal	HA	BNO-HA	657
CSN3	Kappa-casein	Native signal	None	KCN	573
CSN3	Kappa-casein	No signal	None	KNO	510
CSN3	Kappa-casein	alpha-amylase*	None	KCN-alpha	594
CSN3	Kappa-casein	Native signal	HA	KCN-HA	600
CSN3	Kappa-casein	No signal	HA	KNO-HA	537

*alpha-amylase signal is the signal sequence from the alpha-amylase gene in *Aspergillus awamori.*

2.3 Plasmids

Plasmids used for expression in *E. coli* (**Table 2.4**) were constructed by Gibson assembly into a pBAD/myc-His-B backbone (**Fig. 2.1**). Plasmids used for expression in *T. reesei* (**Table 2.5**) were constructed by assembling the gene into a pUOE8 backbone (**Fig. 2.2**).

Table 2.4: Plasmids used in this study for expression in *E. coli*

Name	Insert	Host	Backbone	Resistance	Source
pBAD/myc-His-B		*E. coli*	pBAD/myc-His-B	Ampicillin	Invitrogen™ Catalogue numbers V430-01, V440-01
pEM07E	BNO	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM08E	BCN	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM09E	KNO	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM10E	KCN	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM11E	BNO-HA	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM12E	BCN-HA	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM13E	KNO-HA	*E. coli*	pBAD/myc-His-B	Ampicillin	
pEM14E	KCN-HA	*E. coli*	pBAD/myc-His-B	Ampicillin	

Table 2.5: Plasmids used in this study for expression in *T. reesei*

Name	Gene Insert	Organism for expression	Backbone	Resistance	Source
pUOE8		*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	Artemio Mendoza of Lincoln University (Nogueira-Lopez et al., 2019)
pEM01T	BCN-alpha	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	
pEM02T	BNO	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	
pEM03T	BCN	*T. reesei*	pUOE8	Chloramphenicol (*E. coli)* Hygromycin (*T. reesei*)	
pEM04T	KCN-alpha	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	
pEM05T	KNO	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	
pEM06T	KCN	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	
pEM07T	BLG	*T. reesei*	pUOE8	Chloramphenicol (*E. coli*) Hygromycin (*T. reesei*)	Daniel La Grange, Daisy Lab

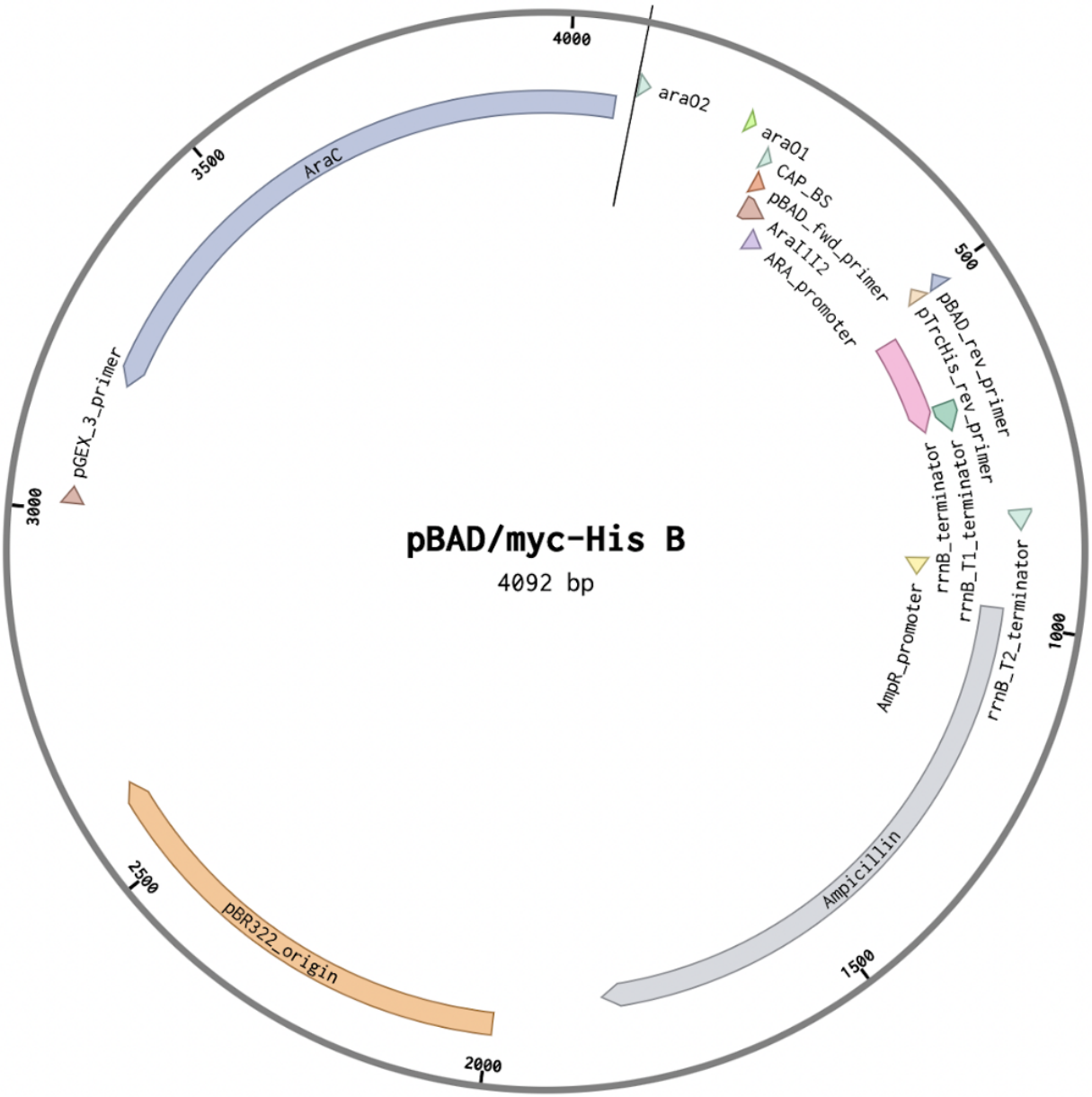


Figure 2.1: Plasmid map of pBAD/myc-His-B. pBAD has a tightly regulated araBAD promoter, inducible with arabinose, and an rrnB terminator. pBAD confers ampicillin resistance to bacterial cells.

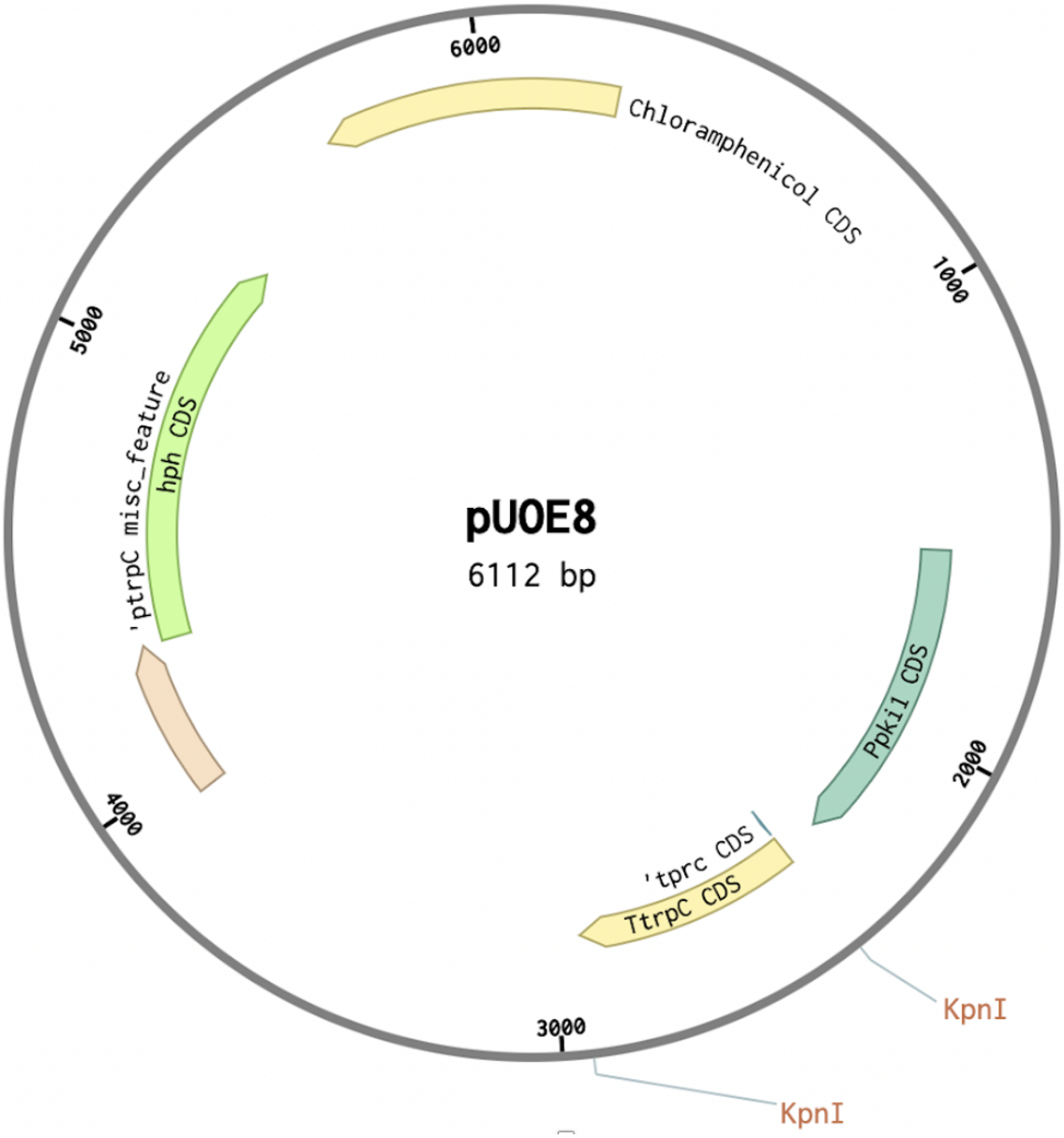


Figure 2.2: Plasmid map of pUOE8. pUOE8 has a strong pyruvate kinase promoter (Ppki1) and cbh1 terminator, both from *T. reesei*. The plasmid confers chloramphenicol resistance to bacterial hosts and hygromycin resistance to *Trichoderma spp.* hosts. There are two Kpn1 cut sites, one downstream from the promoter and the other upstream from the terminator, for easy integration of the gene of interest.

2.4 Media

All media and solutions, unless otherwise stated, were prepared using double deionised water and autoclaved for 15 minutes at 121 °C. All measurements are per 1000 ml unless otherwise stated.

Luria Bertani (LB) Broth

25 g Luria Bertani Broth Base (Miller's LB Broth Base™) (Invitrogen)

Luria Bertani (LB) Agar

25 g Luria Bertani Broth Base (Miller's LB Broth Base™) (Invitrogen)

15 g agar

Potato Dextrose Broth (PDB)

4 g potato infusion powder for microbiology (Millipore®)

20 g dextrose

Potato Dextrose Agar (PDA)

4 g potato infusion powder for microbiology (Millipore®)

20 g dextrose

20 g agar

Yeast Peptone Dextrose (YPD)

10 g yeast extract

20 g peptone

20 g dextrose

Buffered complex glycerol medium (BMGY)

10 g yeast extract

20 g peptone

Autoclave at 121 °C for 15 minutes

Add:

100 ml 1M potassium phosphate buffer (pH 6)

100 ml 10× yeast nitrogen base

2 ml 500× biotin

100 ml 10× glycerol

10× Phosphate-Buffered Saline (PBS) stock solution (ph 6.8):

For 100 ml of 10× PBS:

1.78 g Na_2HPO_4

0.24 g K_3PO_4

8 g NaCl

0.2 g KCl

1× PBS solution (pH 7.4):

For 100 ml:

10 ml 10× PBS stock solution

90 ml ddH_2O

2.5 Antibiotics

25 mg/ml chloramphenicol stock solution

25 mg of chloramphenicol was dissolved in 1 ml of 100% ethanol and filter sterilised with a 0.22 μm filter and syringe, then stored at -20 °C.

100 mg/ml ampicillin stock solution

100 mg of ampicillin was dissolved in 1 ml of ddH_2O and filter sterilised with a 0.22 μm filter and syringe, then stored at -20 °C.

2.6 Permanent Storage of *E. coli* cultures

Bacterial strains were streaked on LB agar media and grown overnight at 37 °C for 16 hours. Single colonies were used to inoculate LB media which was grown again overnight for 16 hours at 37 °C with shaking at 250 RPM. Strains that contained antibiotic-resistance genes were grown in the presence of the antibiotic at a concentration of 1 μl/ml. 750 μl overnight culture was added to 250 μl of 50% glycerol in a 1.5 ml screw cap vial and stored at -80 °C for long-term storage.

2.7 Permanent Storage of *T. reesei* cultures

Fungal strains were inoculated on PDA and grown at room temperature for several days. A sterile swab was used to collect spores from the plate for inoculation in PBD. The cultures were grown again at room temperature for several days. 750 µl of culture was added to 250 µl of 50% glycerol in a 1.5 ml screw cap vial and stored at -80 °C for long-term storage.

2.8 Preparation of electrocompetent *E. coli* cells.

Single *E. coli* colonies grown on an LB agar plate were picked and grown overnight in LB broth at 37 °C with constant shaking at 250 RPM. 1 ml of the culture was used to inoculate 100 ml of LB broth which was then incubated at 37 °C with shaking at 250 RPM. Once the culture reached an OD of 0.5-0.6, it was immediately submerged and swirled in ice for 10 minutes. The culture was left to sit on ice for an hour and then centrifuged at 4200 g for 10 minutes at 4 °C. The pellet was resuspended with 10% glycerol, left on ice for 10 minutes then re-centrifuged at 4500 g. Resuspension in glycerol and centrifugation at 4500 g were repeated as a second wash. After removing the supernatant with a 25 ml serological pipette, the remaining cells were pooled and dispensed into 70 µl aliquots in 1.5 ml screw cap vial and stored at -80 °C for long-term storage.

2.9 Transformation of electrocompetent *E. coli*

Glycerol stocks of electrocompetent *E. coli* stored at -80 °C were defrosted on ice. 1 µl of plasmid and 70 µl of electrocompetent *E. coli* cells were added to a 1.5 ml microcentrifuge tube, vortexed, and spun down. The total volume was then transferred to a pre-chilled electroporation cuvette and shocked in an electroporator at 1850 V. Immediately, 500 µl of warm SOC media was added to the cuvette. The solution was then transferred to 5 ml tubes and incubated at 37 °C for 1 hour

with shaking at 250 RPM. The cells were then placed on selective LB agar plates in the following dilutions: 1:10, 1:100 and undiluted. Plates were incubated at 37 °C upside down overnight.

2.10 Preparation and transformation of competent BL21(De3) Star *E. coli* using divalent-cation mediated (TSS) transformation

A single BL21 *E. coli* colony was used to inoculate 1 ml of LB and left to grow overnight at 37 °C with shaking at 250 RPM. 100 ml LB in a 1 L flask was autoclaved and inoculated with 100 μl overnight culture. The culture was incubated at 37 °C with shaking at 250 RPM until slightly turbid. Once the culture reached OD 0.2-0.5, it was halved into two 50 ml tubes pre-chilled at -20 °C and incubated on ice for 15 minutes. The cells were harvested by centrifugation at 4200 g for 10 minutes at 4 °C. The supernatant was poured off, and the excess was removed with a pipette. The pellet was resuspended in 1 ml ice-cold 1× TSS. Aliquots of 75 μl were pipetted into pre-frozen 1.5 ml microcentrifuge tubes. Cells were either stored at -80 °C or used for transformation straight away. For transformation, 20 μl competent cells were added to a 1.5 ml microcentrifuge tube with 2 μl of plasmid DNA. The cells were left on ice for 5 minutes and then heated at 42 °C for 30 seconds. 80 μl of SOC media was added, and tubes were inverted to mix. The cells were incubated at 37 °C with shaking for 45 minutes. Cells were plated on selective LB agar plates and left overnight at 37 °C to grow.

2× Transformation and Storage Solution (TSS)

0.8 g bacto-tryptone

0.5 g yeast extract

0.5 g NaCl

20 g polyethylene glycol (PEG) 8000

20 ml ddH_2O

10 ml 1M $MgSO_4$

10 ml dimethyl sulfoxide

Adjust pH to 6.5 and add ddH_2O to 100 ml.

Filter sterilise through a 0.22 µm filter and store at 4 °C.

2.11 Gibson Assembly®

2.11.1 Primer Design

All primers were designed using the assembly tool in Benchling (*Benchling [Biology Software]*, 2022). Primers were ordered from Integrated DNA technologies. Primer details are shown in **Table 2.6.**

Table 2.6: Individual primer sequences and melting temperatures

Name	Primer Description	Tm (°C)	Primer Sequence
EM1TF	KCN-alpha FWD for *T. reesei* pUOE8	75.6	CTCGAGGGGGGGCCCGGTACATGCG CGTGTCGACGAGCAG
EM1TR	KCN-alpha REV for *T. reesei* pUOE8	67.8	CTATAGGGCGAATTGGGTACTTAGACC GCAGTCGACGTGACT
EM2TF	KNO FWD for *T. reesei* pUOE8	76.8	CTCGAGGGGGGGCCCGGTACATGGG CGCCCAGGAGCAAAACCA
EM2TR	KNO REV for *T. reesei* pUOE8	68.0	CTATAGGGCGAATTGGGTACTTAGACC GCAGTGCTCGTGACT
EM3TF	KCN FWD for *T. reesei* pUOE8	71.0	CTCGAGGGGGGGCCCGGTACATGATG AAGTCCTTTTTCCTCGTG
EM3TR	KCN REV for *T. reesei* pUOE8	68.0	CTATAGGGCGAATTGGGTACTTAGACC GCAGTGCTCGTGACT
EM4TF	BCN FWD for *T. reesei* pUOE8	72.5	CTCGAGGGGGGGCCCGGTACATGAA GGTCCTCATCCTTGC
EM4TR	BCN REV for *T. reesei* pUOE8	65.1	CTATAGGGCGAATTGGGTACTTAGACA ATAATCGGGAAGGGG
EM5TF	BNO FWD for *T. reesei* pUOE8	73.9	CTCGAGGGGGGGCCCGGTACATGCG AG AGCTGGAAGAGCTCAA
EM5TR	BNO REV for *T. reesei* pUOE8	65.1	CTATAGGGCGAATTGGGTACTTAGACA ATAATCGGGAAGGGG
EM6TF	BCN-alpha FWD for *T. reesei* pUOE8	74.6	CTCGAGGGGGGGCCCGGTACATGCG CGTGTCGACTTCCAG
EM6TR	BCN-alpha REV for *T. reesei* pUOE8	66.9	CTATAGGGCGAATTGGGTACTTAGACA ATAATCGGGAAGGGGCC
EM7EF	KNO FWD for *E. coli* pBAD	70.4	AACAGGAGGAATTAACCATGGGTGCC CAGGAGCAGAACCA
EM7ER	KCN REV for *E. coli* pBAD	70.4	GCAGATCTCGAGCTCGGATCTTAGAC CGCCGTTGAAGTAACTTGG

EM8EF	KCN FWD for *E. coli* pBAD	61.9	AACAGGAGGAATTAACCATGATGAAAA GTTTTTTCCTAGTTG
EM8ER	KCN REV for *E. coli* pBAD	69	GCAGATCTCGAGCTCGGATCTTAGAC CGCCGTTGAAGTAA
EM9EF	BNO FWD for *E. coli* pBAD	67.2	AACAGGAGGAATTAACCATGCGTGAG CTGGAAGAACTCAA
EM9ER	BNO REV for *E. coli* pBAD	67.5	GCAGATCTCGAGCTCGGATCTTAGAC AATAATTGGGAAGGGAC
EM10EF	BCN FWD for *E. coli* pBAD	64.2	AACAGGAGGAATTAACCATGAAGGTTC TCATCCTTGC
EM10ER	BCN REV for *E. coli* pBAD	67.2	GCAGATCTCGAGCTCGGATCTTAGAC AATAATTGGGAAGGGA
EM11EF	*E. coli* pBAD for KNO FWD	69	TTACTTCAACGGCGGTCTAAGATCCGA GCTCGAGATCTGC
EM11EF	*E. coli* pBAD for KNO REV	71.8	TGGTTCTGCTCCTGGGCACCCATGGT TAATTCCTCCTGTTAGCC
EM12EF	*E. coli* pBAD for KCN FWD	69	TTACTTCAACGGCGGTCTAAGATCCGA GCTCGAGATCTGC
EM12ER	*E. coli* pBAD for KCN REV	63.3	AGGAAAAAACTTTTCATCATGGTTAATT CCTCCTGTTAGCC
EM13EF	*E. coli* pBAD for BNO FWD	65.9	CCTTCCCAATTATTGTCTAAGATCCGA GCTCGAGATCTGC
EM13ER	*E. coli* pBAD for BNO REV	68.8	TTGAGTTCTTCCAGCTCACGCATGGTT AATTCCTCCTGTTAGCC
EM14EF	*E. coli* pBAD for BCN FWD	65.9	CCTTCCCAATTATTGTCTAAGATCCGA GCTCGAGATCTGC
EM14ER	*E. coli* pBAD for BCN REV	66.2	GCAAGGATGAGAACCTTCATGGTTAAT TCCTCCTGTTAGCC
EM19EF	*E. coli* pBAD for BCN-HA FWD	68.6	ATATGATGTGCCGGACTATGCGTAAGA TCCGAGCTCGAGATCTGCA

EM20EF	*E. coli* pBAD for KCN-HA FWD	68.9	CGGTCTACCCATACGATGTTCCAGATTACGCTTAAGATCCGAGCTCGAGATCTGC
EM21ER	BCN-HA REV for *E. coli* pBAD	69.6	AGCTCGGATCTTACGCATAGTCCGGCACATCATATGGGTAGACAATAATTGGGAAGG GAC
EM22ER	KCN-HA REV for *E. coli* pBAD	69.5	AGCTCGGATCTTAAGCGTAATCTGGAACATCGTATGGGTAGACCGCCGTTGAAGTAACTT

2.11.2 PCR amplification of sequences

PCR was performed using the New England Biolabs™ inc. Phusion® High Fidelity kit. Each PCR reaction was made up to a total of 98 μl to provide enough mixture for both a reaction and negative control for each amplification (**Table 2.7**).

Table 2.7: Reagent volumes for PCR reaction

Reagent	Volume for two PCR reactions (μl)
ddH_2O	65
NEB™ Phusion® 5× Hi-Fidelity buffer	20
Forward primer	5
Reverse primer	5
dNTPs	2
NEB™ Phusion® Polymerase	1

49 μl of the reaction mixture was aliquoted into each of two tubes. 1 μl of target DNA was added to one tube and 1 μl of ddH_2O was added as a negative control to the second tube. PCR was done for 25 cycles at the annealing temperatures of each primer pair listed in **Table 2.8.** The annealing temperature was calculated using the NEB Tm calculator https://tmcalculator.neb.com/#!/main.

Table 2.8: Primer pairs and their annealing temperature

Primer pair and description	Annealing temperature (°C)
EM1TF - KCN-alpha FWD for *T. reesei* pUOE8 EM1TR - KCN-alpha REV for *T. reesei* pUOE8	68
EM2TF - KNO FWD for *T. reesei* pUOE8 EM2TR - KNO REV for *T. reesei* pUOE8	69
EM3TF - KCN FWD for *T. reesei* pUOE8 EM3TR - KCN REV for *T. reesei* pUOE8	69
EM4TF - BCN FWD for *T. reesei* pUOE8 EM4TR - BCN REV for *T. reesei* pUOE8	62
EM5TF - BNO FWD for *T. reesei* pUOE8 EM5TR - BNO REV for *T. reesei* pUOE8	62
EM6TF - BCN-alpha FWD for *T. reesei* pUOE8 EM6TR - BCN-alpha REV for *T. reesei* pUOE8	67
EM7EF - KNO for *E. coli* pBAD FWD EM7ER - KNO for *E. coli* pBAD REV	66
EM8EF - KCN for *E. coli* pBAD FWD EM8ER - KCN for *E. coli* pBAD REV	56

EM9EF - BNO for *E. coli* pBAD FWD EM9ER - BNO for *E. coli* pBAD REV	64
EM10EF - BCN for *E. coli* pBAD FWD EM10ER - BCN for *E. coli* pBAD REV	59
EM11EF - *E. coli* pBAD for KNO FWD EM11EF - *E. coli* pBAD for KNO REV	64
EM12EF - *E. coli* pBAD for KCN FWD EM12ER - *E. coli* pBAD for KCN REV	64
EM13EF - *E. coli* pBAD for BNO FWD EM13ER - *E. coli* pBAD for BNO REV	64
EM14EF - *E. coli* pBAD for BCN FWD EM14ER - *E. coli* pBAD for BCN REV	64
EM7EF - KNO for *E. coli* pBAD FWD EM22ER - KCN-HA for *E. coli* pBAD REV	63
EM8EF - KCN for *E. coli* pBAD FWD EM22ER - KCN-HA for *E. coli* pBAD REV	60
EM9EF - BNO for *E. coli* pBAD FWD EM21ER - BCN-HA for *E. coli* pBAD REV	59
EM10EF - BCN for *E. coli* pBAD FWD EM21ER - BCN-HA for *E. coli* pBAD REV	59
EM19EF - *E. coli* pBAD for BCN-HA FWD EM13EF - *E. coli* pBAD for BNO REV	63
EM19EF - *E. coli* pBAD for BCN-HA FWD EM14EF - *E. coli* pBAD for BCN REV	62

EM20EF - *E. coli* pBAD for KCN-HA FWD EM11ER - *E. coli* pBAD for KNO REV	64
EM20EF - *E. coli* pBAD for KCN-HA FWD EM12ER - *E. coli* pBAD for KCN REV	64

2.11.3 Restriction digest of pUOE8

Plasmid pUOE8 was linearised using restriction enzyme KpnI-HF® from New England Biolabs™. pUOE8 was incubated with KpnI-HF for at least one hour at 37 °C with Cutsmart® buffer (**Table 2.9**). The digest introduced two cuts into the pUOE8 backbone at the sequence recognition sites downstream of the promoter and upstream of the terminator (**Fig. 2.3**).

Figure 2.3: KpnI-HF restriction site. Source:

New England Biolabs https://international.neb.com/products/r3142-kpni-hf#Product%20Information.

Table 2.9: Reagent volumes for KpnI-HF restriction digest

Reagent	**Volume**
DNA	1 μg
Kpn1-HF	1 μl (20 units)
10× rCutSmart buffer	5 μl (1×)
ddH_2O	To 50 μl

2.14.4 Linearisation of pBAD/myc-His-B with PCR

pBAD/myc-His-B was linearised in preparation for Gibson Assembly with PCR primers. Primers were designed to amplify the plasmid and introduce complementary overlaps to the gene inserts. PCR was run for 25 cycles at the annealing temperatures for each primer pair listed in **Table 2.8.**

2.11.5 Quantification of DNA

Plasmid DNA was quantified using Qubit fluorometric quantification. The Qubit was standardised with Qubit broad range reagent standards 1 and 2. 1 μl of dye was dissolved in 199 μl buffer to make a total of 200 μl per sample of DNA. 195 μl of the dye-buffer solution was added to 5 μl of DNA sample. DNA quantification was tested under the dsDNA broad range setting, and results were recorded in ng/μl.

2.11.6 Quality check of PCR products

To confirm successful PCR amplification, 5 μl of PCR reaction was mixed with 1 μl of 6$\times$ loading dye (Thermofisher Scientific). The sample was loaded on a 1% agarose gel which was prepared by dissolving 0.5 g of agarose in 50 ml of 1$\times$ Tris-acetate EDTA (TAE). 5 μl of SYBR™ Safe DNA Gel Stain (Invitrogen™) was added to the flask. The liquid gel was poured into a casting tray and left to solidify. Gels were run at 60 volts for 35-45 minutes and then visualised under ultraviolet light. DNA bands were compared to a 1 kb DNA ladder.

2.11.7 Gibson Assembly

Gibson Assembly was used to ligate the gene insert into the plasmid backbone. NEBuilder® HiFi DNA Assembly Master Mix was used to ligate the fragments together. The reaction volumes were

calculated using the NEB ligation calculator http://nebiocalculator.neb.com/#!/ligation with an insert: plasmid ratio of 2:1. Reaction mixtures were set up as per **Table 2.10.**

Table 2.10: Reagent volumes for Gibson Assembly reaction

Reaction volumes (μl)	
Recommended DNA molar ratio	Vector: Insert 1:2
NEBuilder® HiFi DNA Assembly Master Mix	10
Vector backbone	2
Insert fragment	As determined by NEB ligation calculator
ddH_2O	10 - insert volume
Total volume	20

2.11.8 Confirming the presence of inserts

Colony PCR was performed to confirm that transformed *E. coli* colonies contained the plasmid with the correct insert. Selected colonies were first streaked on a selective media plate with the appropriate antibiotic, and the same pipette tip was used to inoculate 100 μl of ddH_2O in a PCR tube. The mixture was heated to 95 °C for 5 minutes. PCR was then performed using the appropriate primers to amplify the gene insert.

2.12 Purification of plasmid DNA

Single colonies of *E. coli* containing plasmid DNA were picked and grown in 3 ml LB media overnight at 37 °C with shaking at 250 RPM. 1 μl of selective antibiotic per 1 ml of LB was added

depending on the plasmid's selective resistance marker. Plasmid extraction was performed per the Strataprep Plasmid Miniprep Kit (Agilent Technologies) protocol https://www.agilent.com/cs/library/usermanuals/public/400761.pdf.

2.13 Oxford Nanopore sequencing of plasmids

Plasmids to be sequenced were isolated from overnight cultures of *E. coli* using the Stratprep Miniprep Plasmid Kit (Agilent) as per the manufacturer's instructions set out in https://www.agilent.com/cs/library/usermanuals/public/400761.pdf. Concentrations were determined using the Qubit and diluted to a final concentration of no more than 50 ng of plasmid DNA per 9 μl. Sequencing was performed using Oxford Nanopore MinION following the rapid barcoding as per https://nanoporetech.com/sites/default/files/s3/posters/pdf/p17012-barcoding-web.pdf. Sequencing was set for 1.5 hours. The data was basecalled using Guppy v6.0.1, and Medaka was used to produce de novo plasmid assemblies. Alignments were done in benchling (*Benchling [Biology Software]*, 2022).

2.14 Protein expression in *E. coli*

Single *E. coli* colonies containing the gene of interest were grown overnight in 3 ml LB with the addition of 3 μl of 50 μg/ml ampicillin. Cultures were incubated at 37 °C with shaking at 250 RPM until they reached an OD of between 1-2. The following day, 10 ml of LB with the addition of 5 μl of 50 μg/ml ampicillin were inoculated with 100 μl of overnight culture. Cultures were left at 37 °C with shaking at 250 RPM until the culture reached an OD of 0.5. 1 ml of each sample was removed and centrifuged for one minute at 18,000 g. The supernatant was discarded, and the pellet was frozen at -20 °C as a time-zero sample. L-arabinose was added to the remaining culture at a

concentration of 0.2% and left at 37 °C with shaking at 250 RPM. After four hours, 1 ml aliquots were centrifuged at 18,000 g for 1 minute. The supernatant was discarded, and the pellet was frozen at -20 °C until required for analysis.

2.15 Determination of protein expression and abundance using SDS-PAGE

Pellets were resuspended in 100 μl of sample buffer and heated at 95 °C for 10 minutes. Samples were centrifuged at 4,000 g for 1 minute. 5 μl of protein marker and 10 μl of each sample were loaded into a pre-cast stacking gel. The gels used were 4–15% Mini-PROTEAN (Bio-Rad). Electrophoresis was run at 200 V for 35 minutes. The gel was removed from the chamber and carefully extracted from plastic plates. The gels were stained with coomassie blue and left at 22 °C (room temperature) for an hour with gentle shaking at 100 RPM. The coomassie blue was poured out, and the gel was washed with water twice to remove the excess stain solution. The gel was covered with de-stain solution and left at 22 °C for 15 minutes with gentle shaking. A second wash was done with fresh de-stain solution and left at room temperature with shaking overnight until the background stain was satisfactorily removed.

Sample Buffer (SDS reducing buffer)

3.55 ml ddH_2O

1.25 ml 0.5M Tris-HCL pH 6.8

2.5 ml Glycerol

2.0 ml 10% (w/v) SDS

0.2 ml 0.5% (w/v) bromophenol blue

Total volume = 9.5 ml

Add 50 µl beta-mercaptoethanol to 950 µl sample buffer prior to use.

10× Electrode (Running) Buffer, pH 8.3

For 1000 ml:

30.3 g Tris Base

144.0 g Glycine

10.0 g SDS

Dissolve and bring the total volume up to 1000 ml with ddH_2O. Dilute 50 ml stock with 450 ml deionised water for each electrophoresis run. Mix thoroughly before use.

Coomassie blue stain

For 1000 ml:

1.25 g Coomassie brilliant blue R250 (0.125%)

500 ml Methanol (50%)

100 ml Acetic acid (10%)

400 ml ddH_2O

De-stain solution

For 1000 ml:

300 ml Methanol (30%)

100 ml Acetic acid (10%)

600 ml ddH_2O (60%)

Positive control

1 mg of lyophilised beta-casein from bovine milk (Sigma) and 1 mg kappa-casein from bovine milk (Sigma) were each dissolved in 1 ml of water. 1 μl of the solution was added to 99 μl Sample Buffer to give a final concentration of 10 μg/ml.

2.16 Determination of protein expression and abundance using western blotting

The protein samples were first run on an SDS page gel. The gel was then rinsed in ddH_2O and equilibrated in 200 ml of transfer buffer for 15 minutes. The nitrocellulose membrane (Bio-Rad Nitrocellulose Membrane, Precut, 0.45 μm, 7 $\times$ 8.5 cm #1620145) was also equilibrated in 200 ml of transfer buffer for 15 minutes. Equilibrated gel, membrane and filter paper were placed in a transfer cassette. The transfer cassette was placed in the chamber and the chamber was topped up with transfer buffer. An ice pack and magnetic stir bar was placed inside the chamber, and the chamber was placed on a stir plate. The transfer was run at 100 V for 60 minutes. The membrane was removed and washed in TBS for 10 minutes with gentle shaking. The membrane was then blocked in 5% skim milk in TBST for an hour with gentle shaking. The membrane was then washed another two times with TBST for 10 minutes each, with gentle shaking. The membrane was then incubated in the antibody solution for up to 2 hours with gentle shaking. The membrane was then washed another 3 times in TBST for 10 minutes each, with gentle shaking. Finally, a TBS wash was done for 10 minutes with shaking to remove any tween from the membrane. Tetramethylbenzidine (TMB) blotting substrate solution (Thermofisher Scientific) was added with a sufficient volume to cover the membrane, and left until a colour change was observed. The reaction was stopped by rinsing the membrane with water.

1× Transfer Buffer

For 1000 ml:

3.03 g Tris base

14.4 g glycine

500 ml ddH_2O

200 ml MeOH

Adjust volume to 1000 ml with ddH_2O

TBS

For 1000 ml:

2.42 g Tris base

29.29 g NaCl

1.5L ddH_2O

Adjust pH to 7.5 with HCl and add ddH_2O to 1000 ml.

TBST

For 150 ml:

75 µl Tween20

150 ml TBS

Blocking Solution

5 g powdered trim milk

100 ml TBST

Antibody Solution

For 4% BSA, 5 μg/ml antibody concentration:

35 μl of 1 mg/ml abcam HRP Anti-HA tag antibody (ab1190)

280 mg Bovine serum albumin (BSA)

7 ml TBST

2.17 Transformation of *T. reesei*

2.17.1 Media and reagents

Sorbitol osmoticum solution

50 mM $CaCl_2$

700 mM sorbitol

50 mM MES (4-Morpholineethanesulfonic acid)

Use NaOH to adjust to pH 5.5. Filter sterilise using a 0.22 μm cellulose acetate membrane.

Regeneration medium

4 g Potato infusion

20 g Glucose

171 g Sucrose

8 g Agar

1000 ml ddH_2O

Autoclave at 121 °C for 15 minutes to sterilise.

PEG 40% solution

40 g PEG-3350

100 ml mannitol Osmoticum solution

Heat to dissolve if needed. Autoclave at 121 °C for 15 minutes to sterilise.

Hygromycin B

1 mg Hygromycin B

100 ml ddH_2O

Filter sterilise using 0.22 µm cellulose acetate membrane.

PDA plates with hygromycin

39 g PDA

1000 ml ddH_2O

Autoclave at 121 °C for 15 minutes to sterilise. Cool to less than 50 °C and add Hygromycin B.

PDA plates with Hygromycin plus Triton X-100 0.1%

1 ml Triton X-100

39 g PDA

1000 ml ddH_2O

Autoclave at 121 °C for 15 minutes to sterilise. Cool to less than 50 °C and add Hygromycin B.

Enzyme solution

50 mg Glucanex® (Sigma-Aldrich CAS: L1412)

5 ml sorbitol osmoticum solution

Vortex to dissolve then filter sterilise with a 0.22 µm cellulose acetate membrane.

Glass wool tubes

A hole was made in the bottom of a 1.5 ml microcentrifuge tube with a hot needle and steel wool packed inside with tweezers. Tubes were autoclaved for 15 minutes at 121 °C.

2.17.2 Plasmid linearisation

Plasmids were digested and linearised with SacI-HF (New England Biolabs®) to facilitate chromosomal integration (**Fig. 2.4**) (**Table 2.11**).

Figure 2.4: SacI-HF restriction site. Source: New England Biolabs https://international.neb.com/products/r3156-saci-hf#Product%20Information.

Table 2.11: Reagent volumes for restriction digest with SacI

Reagent	Volume
DNA	5 µg
SacI	1 µl (20 units)
10× rCutSmart buffer	10 µl (1×)
ddH_2O	To 100 µl

Reactions were left overnight (17 hours) at room temperature. Digestions were cleaned up using OMEGA E.Z.N.A® Cycle Pure Kit as per the manufacturer's instructions

https://www.omegabiotek.com/product/pcr-clean-up-kit-e-z-n-a-cycle-pure//?utm_source=google&utm_medium=ppc&utm_campaign=130133683644&utm_term=ultra clean%20pcr%20clean%20up%20kit&utm_content=b&gclid=CjwKCAjwm8WZBhBUEiwA178UnJbbRNXvC1GeIhrPbc4QylEY8IlMc4cE9yWaYk6AQKZT5q83KMTY0hoC6swQAvD_BwE
Products were then quantified using Qubit as previously described.

2.17.3 Preparation of protoplasts and washing spores

T. reesei was plated on PDA plates and left to grow at 25 °C for 5-7 days. 5 ml of ddH_2O was added to the PDA plate containing *T. reesei* spores. A sterile swab was used to dislodge the spores from the plate, which were then pipetted through the glass wool tube to remove mycelia. A haemocytometer was used to count the number of spores under a microscope and then diluted to 1×10^8 concentration with PDB. Spores were incubated for 15 hours at 28 °C with shaking at 250 RPM. A microscope was used to visualise the *T. reesei* and confirm that germ tubes had not yet started to branch.

2.17.4 Germ tube preparation

Germ tubes were centrifuged at room temperature at 10,500 g for 10 minutes. The supernatant was decanted into a 50 ml tube, and the pellet was centrifuged again to remove as much medium as possible. The pellet was then washed with 20 ml sorbitol osmoticum and centrifuged at room temperature at 10,500 g for 10 minutes. The supernatant was removed carefully. The enzymatic solution was added, and the tube gently vortexed to mix. Germ tubes in enzyme solution were incubated at 28 °C with 100 RPM shaking for 2-3 hours. The tube was fixed horizontally to ensure good contact between cells and the enzymatic solution. Protoplasts were counted with the haemocytometer counting chamber to ensure there were at least 1×10^8 for each transformation.

Protoplasts were suspended in 30 ml osmoticum and then centrifuged at 5,800 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 10 ml sorbitol osmoticum before centrifuging again at 5,800 g for 10 minutes. The supernatant was discarded, and the pellet was gently resuspended with 500 μl - 2 ml sorbitol osmoticum, depending on protoplast concentration. Protoplasts were incubated on ice. Regeneration media was melted and then kept at 50 °C until needed.

2.17.5 Transformation of protoplasts

1 μg of linearised plasmid DNA for transformation was diluted in 20 μl ddH_2O and mixed with 240 μl protoplasts. Transformation reactions were incubated on ice for 20 minutes and then returned to room temperature. 260 μl PEG solution was added to each transformation reaction and incubated for 30 minutes at room temperature. Protoplasts were mixed with regeneration media, plated on Petri dishes, and incubated for 5-7 days at 25 °C. The hyphal tips of successful transformations were cut out of the agar and placed on new selection plates.

2.17.6 Isolation of transformants

Colonies that grew were transferred to 25 ml PDA with hygromycin B and then incubated for 1-2 days. The hyphae tips were cut using a sterile scalpel, and the cuts were transferred to a new PDA plate with hygromycin B. The plate was incubated for 1-2 days. The hyphae tips were again transferred to a new PDA plate with no hygromycin B. The plates were incubated for five days at 25-28 °C. The spores were washed from the plate with sterile ddH_2O, and 40 μl of spore suspension was transferred to the border of a PDA plate with hygromycin B and Triton X-100 0.1%. The spores were separated with a sterile loop and then incubated for 3-5 days until single colonies started to form. Single colonies were transferred to PDA with hygromycin B plates. They

were incubated for two days at 28 °C. This process, starting with cutting the hyphae tips, was repeated twice.

2.17.7 Induction of expression

The protocol for the induction media was followed from Rantasalo et al. (2019).

Glucose media

70 g glucose

20 g yeast extract

36.7 mM KH_2PO_4

37.8 mM $(NH_4)2SO_4$

2.4 mM $MgSO_4$

4.1 mM $CaCl_2$

1000 ml ddH_2O

(pH 4.8)

10 ml of glucose media was added to a 125 ml flask. A 1×1 cm square of PDA with a colony of *T. reesei* for induction of expression was passed through a syringe into the media. 1 ml of glucose media was added each morning and afternoon. Culture samples were taken once a day for analysis on an SDS-PAGE gel.

2.18 Waste substrate growth assays

2.18.1 Growth media

For each agricultural-waste growth media (**Table 2.12**) 50 g of cut, unpeeled vegetables were weighed out and boiled for 30 minutes in 300 ml ddH_2O until soft. The liquid and boiled vegetables were strained through a muslin cloth, and the extract was collected. ddH_2O was topped up to 500 ml. For media containing dextrose, 5 g of dextrose was added to the 500 ml media and dissolved. All media was then autoclaved at 121 °C for 15 minutes.

Table 2.12: Types of agricultural-waste growth media used in this study

Agricultural-waste growth media
Apple
Apple dextrose
Broccoli
Broccoli dextrose
Carrot
Carrot dextrose
Cabbage
Cabbage dextrose
Potato
Potato dextrose

Vegetables sourced for this media were purchased locally. The condition of the vegetables had slightly deteriorated beyond their best eating as a proxy for agricultural waste. Agricultural waste is inclusive of several streams of refuse. Fresh fruit and vegetables vulnerable to microbial decay

and injury during harvesting, processing, storage or transport are considered unsuitable for sale and become waste. During value-added processes such as winemaking or juice production, residues not included in the final product also become waste (Schieber, 2017; Torres-León et al., 2018).

2.18.2 Growth assay of *T. reesei*

Growth of *T. reesei* was measured using a BioTek ® Synergy 2 plate reader. *T. reesei* was grown on a PDA plate for five days. 3 ml of Polysorbate 80 (Tween80) with NaCl was added to the plate, and a sterile swab was used to scrape the spores from the plate. Using a pipette, the mixture was passed through a glass wool tube. The spore solution was collected in a plastic test tube. 3 μl of spore solution was loaded onto a haemocytometer, and spores were counted. Dilutions were done to achieve 1×10^6 – 1×10^7 spores per ml with the respective growth media. 150 μl of inoculated growth media was added to each well and covered with 50 μl mineral oil to prevent condensation, which can impact OD readings. Plates were read for 48 hours at 28 °C. Readings were taken every 5 minutes. Growth in five replicates of each media was tested, and three replicates of each media without any inoculation. Data from replicates were averaged, and the negative control averages were subtracted to account for any influence the media colour had on the OD.

2.18.3 Growth assay of *P. pastoris*, *K. lactis* and *E. coli* on waste media

To first test growth on waste media, a single colony of each strain was resuspended in 500 μl of ddH_2O. 3 μl of the suspension was inoculated into 3 ml media and incubated at 28 °C for 48 hours. Growth was observed in both strains, with none in the negative controls. For the plate reader assay, 3 ml of standard lab growth media (LB for *E. coli,* YPD for *K. lactis*, and BMGY for *P.*

pastoris) was inoculated with a colony and left to grow at 28 °C overnight with shaking for *E. coli* and 48 hours with shaking for *P. pastoris* and *K. lactis* to increase cell density. 1 ml of culture was spun down, and the supernatant was removed. The pellet was then re-suspended with PBS and spun down again. This step was repeated another two times to ensure the removal of all residual growth media. 2 μl of the suspension was used to inoculate 148 μl of media in each well. 50 μl of mineral oil was used to cover each well to reduce the impact that the formation of condensation may have on the OD readings. A BioTrek® Synergy 2 plate reader was used to perform the growth assay. Plates were read every 5 minutes for 48 hours at 28 °C with shaking 20 seconds before each reading to avoid cells settling on the bottom of the plate. Growth in five replicates of each media was tested, and three replicates of each media without any inoculation as negative controls. Data from replicates were averaged, and the negative control averages were subtracted to account for any influence the media colour had on the OD.

2.18.4 Calculation of the maximum specific growth rate

The maximum specific growth rate (μ_{max}) was calculated as the gradient of the slope that represents exponential growth with https://mdphan.shinyapps.io/GrowthCurvesAnalysis/.

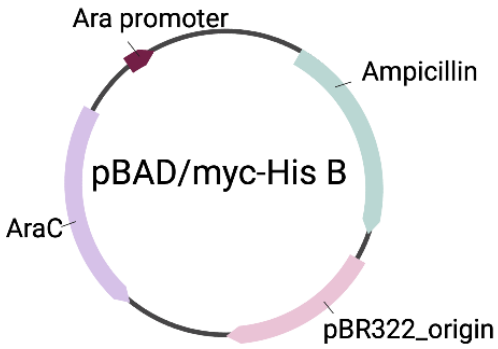
Chapter 3: Results

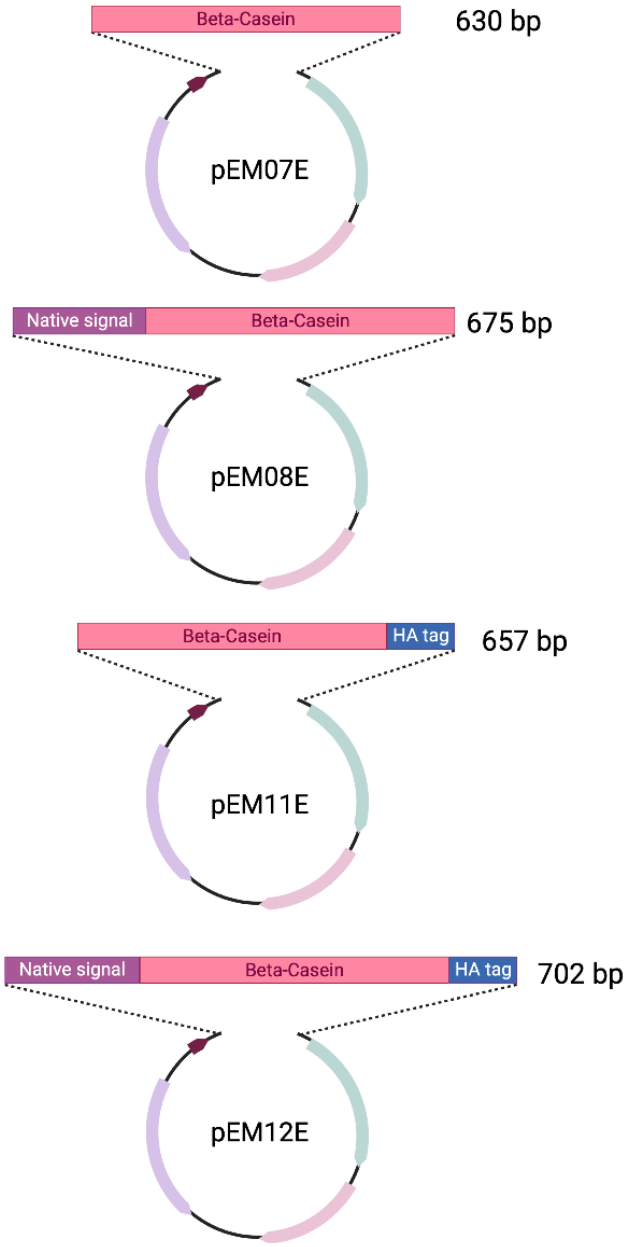
3.1 Construction of protein expression systems for bovine beta-casein and kappa-casein expression in *E. coli*

Top10 *E. coli* was chosen as the first host organism for the cloning and expression of heterologous bovine casein. *E. coli* is a well-characterised, fast-growing host with simple and established methods of transformation (Kaur et al., 2018). mRNA sequences encoding the A2 allele for bovine beta-casein (accession number M16645) and the B allele for bovine kappa-casein (accession number AY380229) were downloaded from the National Center for Biotechnology Information (NCBI). The coding sequences were codon optimised for expression in *E. coli* using the codon optimisation tool in Benchling (*Benchling [Biology Software]*, 2022). Parameters were set so that no regions were protected or cut sites preserved. GC content was permitted to vary between 0 and 1. The codon-optimised sequences were aligned with the native sequences to compare base changes (**Sup. Fig. 5.1**). To confirm that no amino acid changes occurred, the sequences were translated and aligned with the native amino acid sequences using benchling's alignment tool (**Sup. Fig. 5.2**) (*Benchling [Biology Software]*, 2022). Four different gene inserts were designed for the expression of casein: beta-casein with the native bovine signal sequence (BCN), beta-casein with no signal sequence (BNO), kappa-casein with the native bovine signal sequence (KCN) and kappa-casein with no signal sequence (KNO) (**Fig. 3.1a** and **b**). Each of the four casein gene inserts were obtained via DNA synthesis from Twist Biosciences (see methods). PCR was used to introduce complementary overlaps on the inserts for the pBAD/myc-His-B plasmid (**Sup. Fig. 5.3**). PCR was used to linearise the plasmid pBAD/myc-His-B and introduce overlaps complementary to each casein gene insert (**Sup. Fig. 5.4**). Given the PCR products of pBAD for KCN and KNO inserts were larger than expected, we decided to

proceed with the assembly of pEM07E (BNO insert) and pEM08E (BCN insert) initially. Gibson assembly was performed to assemble fragments, before transformation into electrocompetent Top10 *E. coli* cells. Colony PCR of successful transformants confirmed that the genes of interest had been successfully integrated into the plasmid backbone (**Fig. 3.2**).

a)


Ara promoter
Ampicillin
pBAD/myc-His B
AraC
pBR322_origin


Beta-Casein
630 bp
pEM07E
Native signal
Beta-Casein
675 bp
pEM08E
Beta-Casein
HA tag
657 bp
pEM11E
Native signal
Beta-Casein
HA tag
702 bp
pEM12E

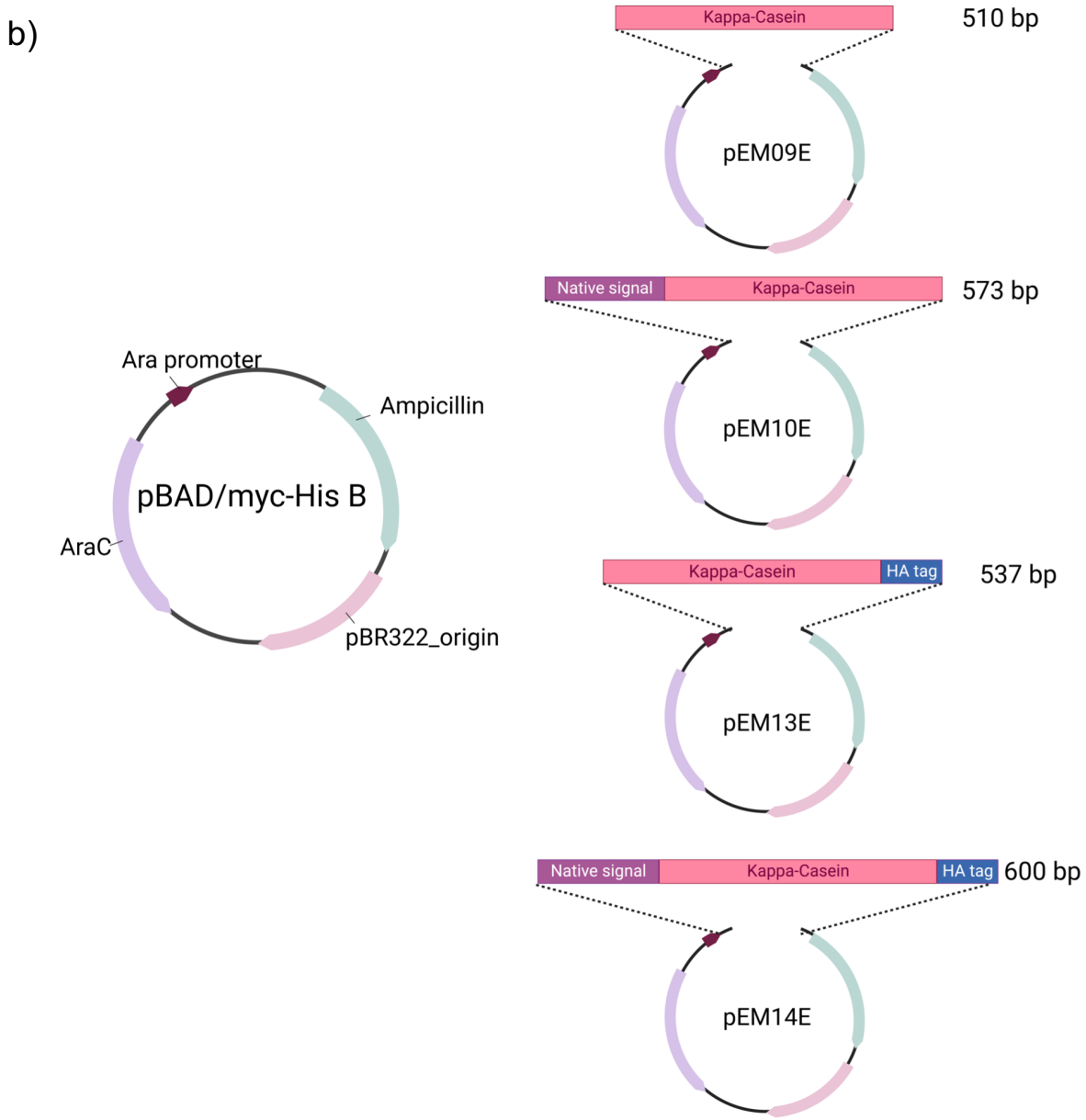


Figure 3.1: Schematic of plasmid constructs used for expression in *E. coli*. a) Four variations of the beta-casein open reading frame were cloned into the pBAD plasmid: beta-casein with no signal (BNO), beta-casein with the native signal (BCN), beta-casein with no signal and an HA tag (BNO-HA), and beta-casein with the native signal and an HA tag (BCN-HA). **b)** Four variations of the kappa-casein open reading frame were cloned into the pBAD plasmid: kappa-casein with no signal (KNO), kappa-casein with the native signal (KCN), kappa-casein with no signal and an HA tag (KNO-HA), kappa-casein with the native signal and an HA tag (KCN-HA).

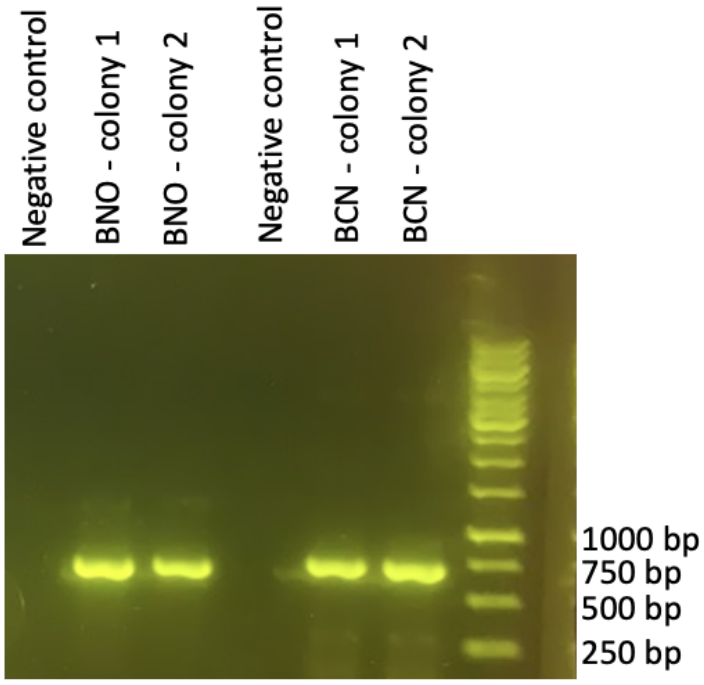


Figure 3.2: Colony PCR to confirm the presence of casein inserts in pBAD plasmid in transformed Top10 *E. coli*. *E. coli* transformed with pEM07E (BNO insert) and pEM08E (BCN insert) underwent colony PCR to confirm the presence of the gene insert. Two colonies of each transformant were amplified with primers targeting the insert. The results show that each transformed colony contains the correct size insert.

3.2 Expression of bovine beta-casein and kappa-casein in *E. coli*

To begin with, the expression of beta-casein with the native bovine signal (BCN) was tested. Expression was induced with 0.2% L-arabinose in *E. coli* transformed with pEM08E once the culture had reached an OD of 0.6. 1 ml samples were collected 4 hours after induction. The SDS-PAGE gel showed no evident expression of casein from Top10 *E. coli* when compared to the beta-casein positive control, nor around the location on the gel where we would expect to see a protein of the right size (24 kDa) (**Fig. 3.3**). We hypothesised that *E. coli* proteases might be degrading any recombinant casein that is being expressed. We then transformed BL21 DE3, a protease-deficient lab strain of *E. coli* with pEM08E. Expression was repeated, and samples were run on an SDS-PAGE gel. Visualising the gel showed a faint band of the expected size, correlating

with the positive control (**Fig. 3.4a**). ImageJ software was used to analyse the intensity of the bands. It verified increased intensity at that location compared with the negative controls (**Sup. Table. 5.1**). The results indicated an increased intensity in the location where beta-casein would be expected on the gel. However, given that ImageJ results are non-quantitative and depend on the user's technique, it does not allow us to conclude that expression has occurred. To see if the expression was improved beyond 4 hours, we induced expression in *E. coli* transformed with pEM07E and pEM08E. Samples were taken at 5, 6, and 20 hours. **Figure 3.4b** shows the appearance of a band in the 5 and 6 hour samples taken of *E. coli* containing pEM07E, indicating expression of beta-casein with no signal (BNO). However, these results were hard to elucidate given the high background of endogenous *E. coli* proteins.

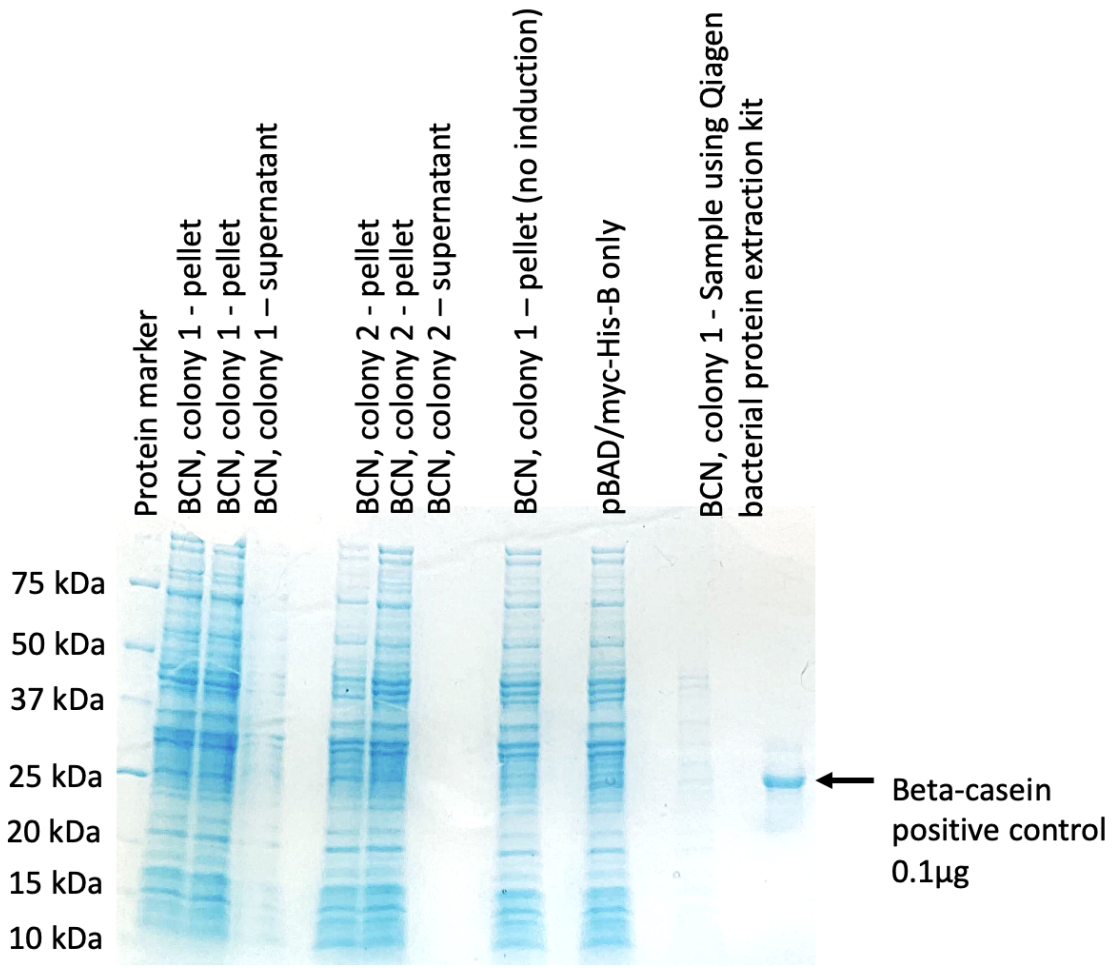


Figure 3.3: SDS-PAGE analysis of recombinant proteins expressed in Top 10 *E. coli* transformed with pEM08E. Two colonies that showed the successful transformation of pEM08E (BCN insert) were induced for expression with 0.2% L-arabinose. Samples were run on an SDS-PAGE gel and compared to a beta-casein positive control. There are no clear bands of the size we would expect if there had been protein expression.

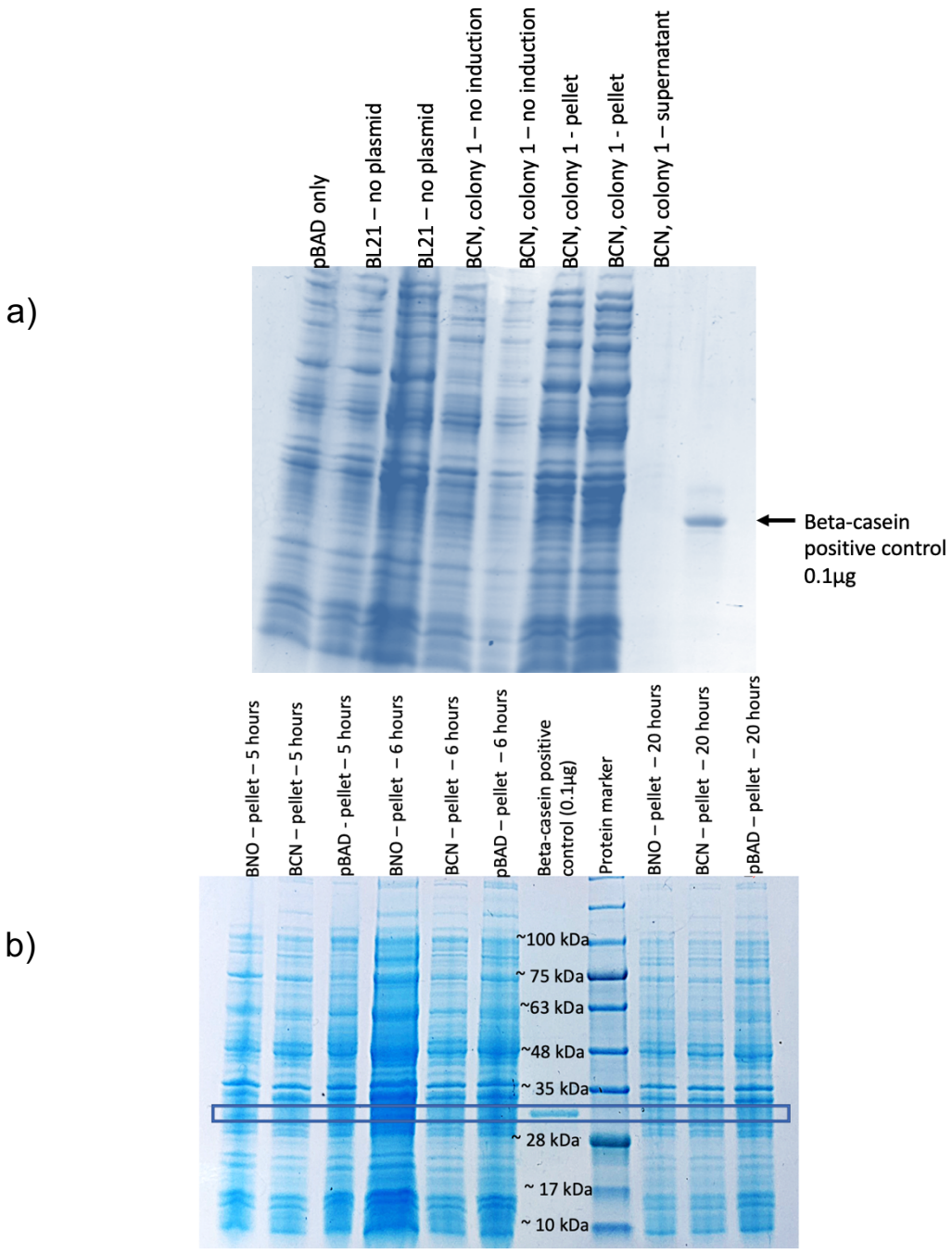
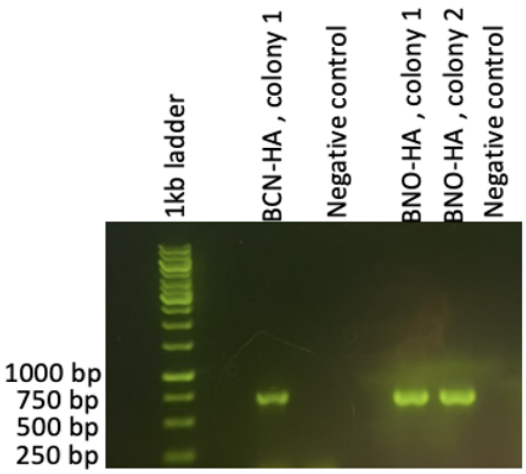


Figure 3.4: SDS-PAGE analysis of recombinant proteins expressed in BL21 *E. coli* transformed with pEM07E and pEM08E a) Expression of proteins was induced in an *E. coli* colony transformed with pEM08E (BCN insert). Samples induced with 0.2% L-arabinose, and samples with no induction, show a darker band in the location of beta-casein relative to the positive control. **b)** Time trial expression of *E. coli* transformed with pEM08E (BCN insert) and pEM07E (BNO) insert. Bands corresponding to the location of the beta-casein positive control can be seen in the 5 and 6 hour samples of pEM07E (BNO insert) but not in any of the other lanes.

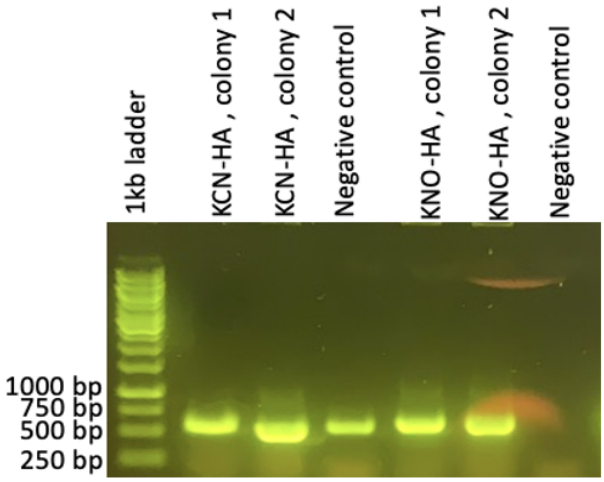
3.3 Beta-casein and kappa-casein are expressed at a higher molecular weight in *E. coli*

In order to increase the sensitivity and specificity of protein expression detection, we used PCR to introduce a single human influenza hemagglutinin (HA) tag onto the end of each of the four casein inserts for antibody detection (**Fig. 3.1**, **Sup. Fig. 5.5** and **Sup. Fig. 5.6**). Inserts were assembled into the pBAD/myc-His-B backbone and were used to transform chemically competent *E. coli.* Transformants with pEM11E, pEM13E and pEM14E returned several colonies; however, transformations with pEM12E only resulted in one successful transformant. Successful transformants underwent colony PCR to confirm the HA-tagged inserts were correctly assembled into the plasmid (**Fig. 3.5**).

a)



b)



c)

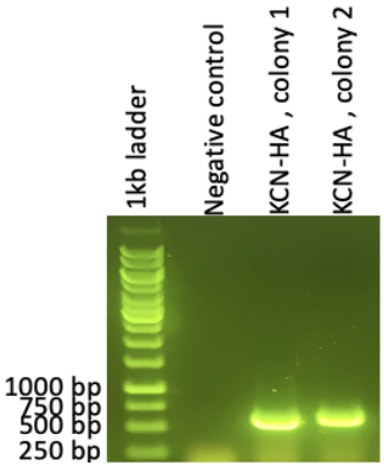


Figure 3.5: Colony PCR to confirm the presence of HA-tagged casein inserts in pBAD plasmid in transformed BL21 *E. coli*. PCR products were run on a 1% agarose gel. **a)** successful amplification of the BCN-HA insert and BNO-HA inserts. **b)** Successful amplification of the KNO-HA inserts. Amplification of KCN-HA appeared successful; however, the negative control also indicated a positive result. **c)** Repeating the KCN-HA PCR proved successful with no signal in the negative control lane.

PCR results showed that all colonies tested had successfully been transformed with the gene insert. Induction of expression was repeated as previously described, and samples were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane for western blot antibody detection (**Fig. 3.6b** and **b**). The membrane was blocked with 5% skim milk, as is standard for performing western blots. To detect the HA-tagged caseins, the membrane was incubated with 5 *μ*g/ml HA-tag horseradish peroxidase (HRP) conjugated antibodies diluted in 4% bovine serum albumin (BSA). The addition of tetramethylbenzidine (TMB) substrate revealed bands in the lanes where expression of KCN-HA, KNO-HA, and BNO-HA would be expected. There was no expression identified in the BCN-HA lane. The negative pBAD/myc-His-B plasmid-only lane showed no bands upon the addition of TMB.

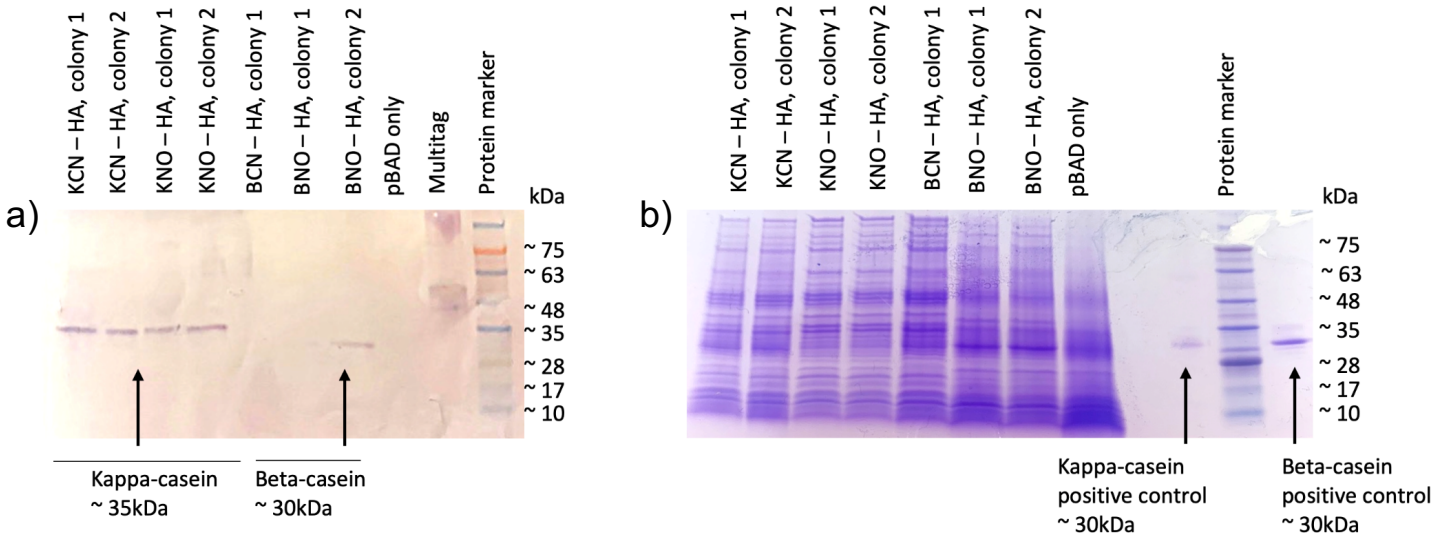


Figure 3.6: Western Blot and SDS-PAGE analysis of recombinant proteins expressed in BL21 *E. coli*. *E. coli* containing the genes of interest were cultured to an OD of 0.5 then expression was induced by adding 0.2% L-arabinose. **a**) A western blot was performed to detect samples containing HA-tagged bovine kappa-casein or beta-casein. Kappa-casein with and without the native signal and beta-casein without the native signal were all observed on the western blot. **b**) SDS-PAGE gel shows the location of the positive kappa-casein and beta-casein controls relative to the ladder and *E. coli* proteins.

The western blot revealed that kappa-casein was expressed at a much higher molecular weight than expected. In nature, the mature kappa-casein protein is approximately 19 kDa (Bhat et al., 2016), while we observed a protein mass of approximately 38 kDa. To precisely calculate the expected protein size for each gene insert, https://www.genscript.com/sms2/protein_mw.html was used. The expression of KCN-HA should result in a kappa-casein protein with a molecular weight of 22.3 kDa while KNO-HA should produce a kappa-casein protein of 20 kDa.[1] Our results indicated that kappa-casein, with and without the native signal, had a mass almost twice the expected size. It is possible that this was due to the protein ladder being inaccurate. To better quantify ladder accuracy, a lyophilised kappa-casein positive control (Sigma - Aldrich, CAS number: 9000-71-9) was run alongside. A side-by-side comparison of the SDS-PAGE and western blot revealed the kappa-casein to be closer in size to the positive control than the initial molecular weight estimation would suggest (**Fig. 3.6**). Despite this, the recombinant kappa-casein was still larger than the positive control, and larger than the recombinant beta-casein. The recombinant beta-casein also appeared to be expressed at a higher molecular weight than expected. Given its amino acid sequence, the mature beta-casein protein should have a mass of approximately 24 kDa (Bhat et al., 2016). We expected the expression of BCN-HA and BNO-HA to produce proteins of approximately 26.2 kDa, and 24.7 kDa, respectively. [2] While no expression of BCN-HA was seen, the expression of BNO-HA resulted in a protein approximately 30 kDa, 5 kDa heavier than expected. However, when using lyophilised beta-casein (Sigma - Aldrich, CAS number: 22086-1G-F) as a positive control, the location on the SDS-PAGE gel, relative to the protein marker, was higher than we expect from a 24 kDa protein (**Fig. 3.6**). A comparison

[1] https://www.genscript.com/sms2/protein_mw.html
[2] Ibid

between the location of the recombinant beta-casein, and the positive control beta-casein, in relation to the protein marker, indicated they are a very similar size.

3.4 Oxford Nanopore sequencing of plasmids

To investigate why beta-casein and kappa-casein were expressed at higher molecular weights than anticipated, we sequenced the plasmids using Oxford Nanopore sequencing to confirm that the sequence of the insert was correct.

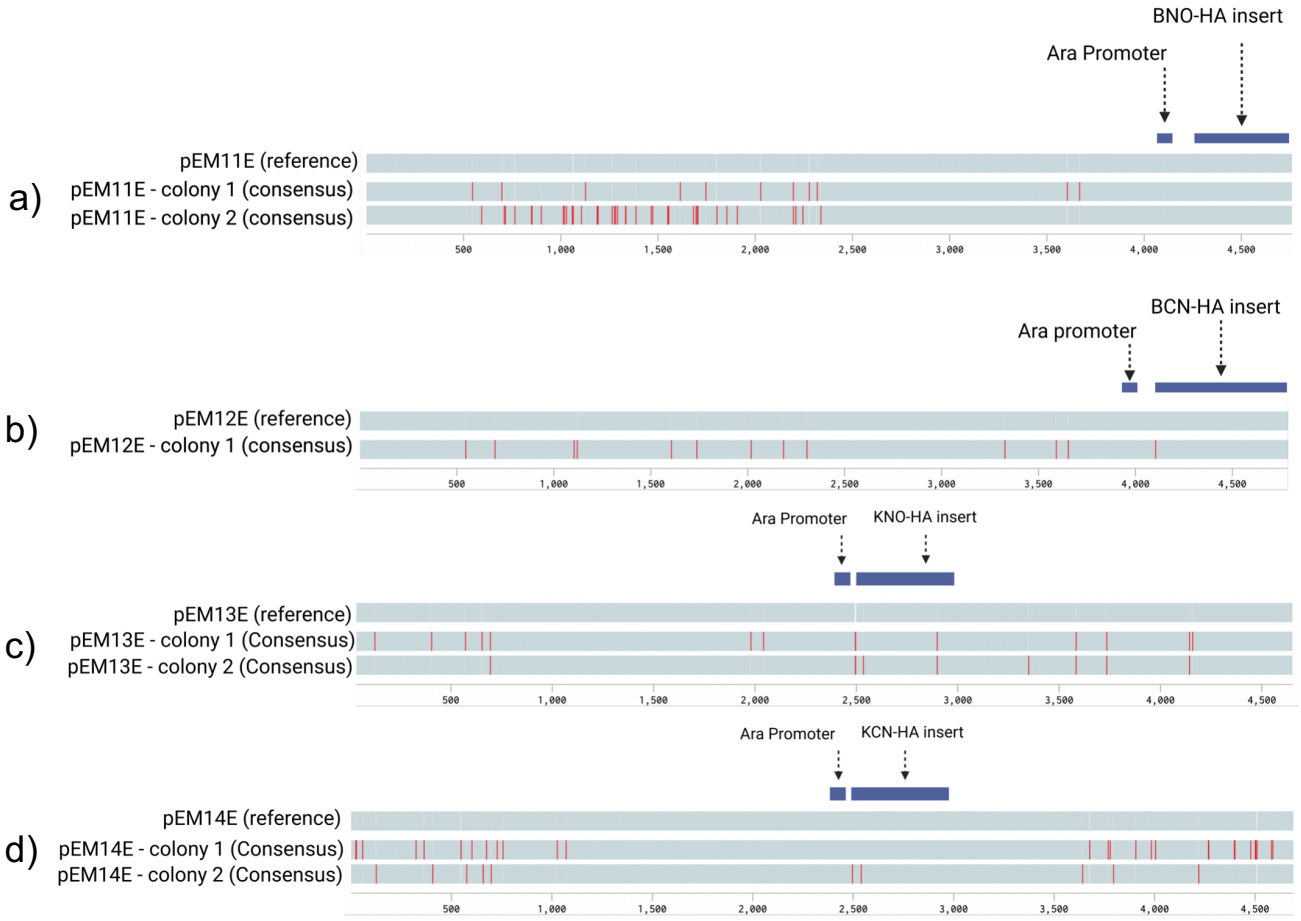


Figure 3.7: Alignments of Oxford Nanopore sequencing results with reference plasmids. a) pEM11E (BNO-HA insert). **b)** pEM12E (BCN-HA insert). **c)** pEM13E (KNO-HA insert). **d)** pEM14E (KCN-HA insert).

Oxford Nanopore sequencing revealed that all plasmids were the correct size compared to their respective reference plasmid (**Fig. 3.7**). None of the plasmids had multiple copies of inserts, and each plasmid had a start and stop codon upstream and downstream of the insert, respectively. Neither of the *E. coli* colonies transformed with pEM11E harboured any nucleotide changes within the promoter or insert region (**Sup. Fig. 5.7**). The single colony transformed with pEM12E had a 3-base pair deletion, causing the absence of a methionine (**Sup. Fig. 5.8**). However, the following codon also encoded a methionine, so translation initiation should not have been impacted. Despite this, no expression of BCN was seen. Both colonies containing pEM13E, had an additional six nucleotides downstream of the start codon (**Sup. Fig. 5.9**). The nucleotides encoded glycine and alanine. Revisiting the primers used to amplify the casein sequence revealed that the last two amino acids of the signal sequence had been included in the primer design (**Table 2.5**). Therefore, during PCR, the kappa-casein gene fragment had been amplified to include extra glycine and alanine. Sequencing results of colony 2 also indicated an insertion of adenine at position 2519. However, given that the sequence is a string of five adenines, this is very likely an artefact of the sequencing method, as indels are commonly called in Oxford Nanopore sequencing. The same occurred at position 2882 for both colonies, where an additional cytosine had been inserted at the start of a series of five cytosines. Sequencing results of pEM14E colonies 1 and 2 show discrepancies between the two (**Fig. 5.10**). Colony 1 has no changes compared to the reference plasmid. However, colony 2 has a methionine deletion upstream of the gene insert. This should not influence the initiation of translation as two methionines follow. However, it is interesting that this change is only observed in one colony and not the other, given that they are both the result of transformation with plasmids harbouring inserts from a single PCR run. Given the lack of errors seen among the other sequencing results, the deletion of an entire codon is unlikely to be a sequencing error. Instead, this could be an error that occurred in the upstream

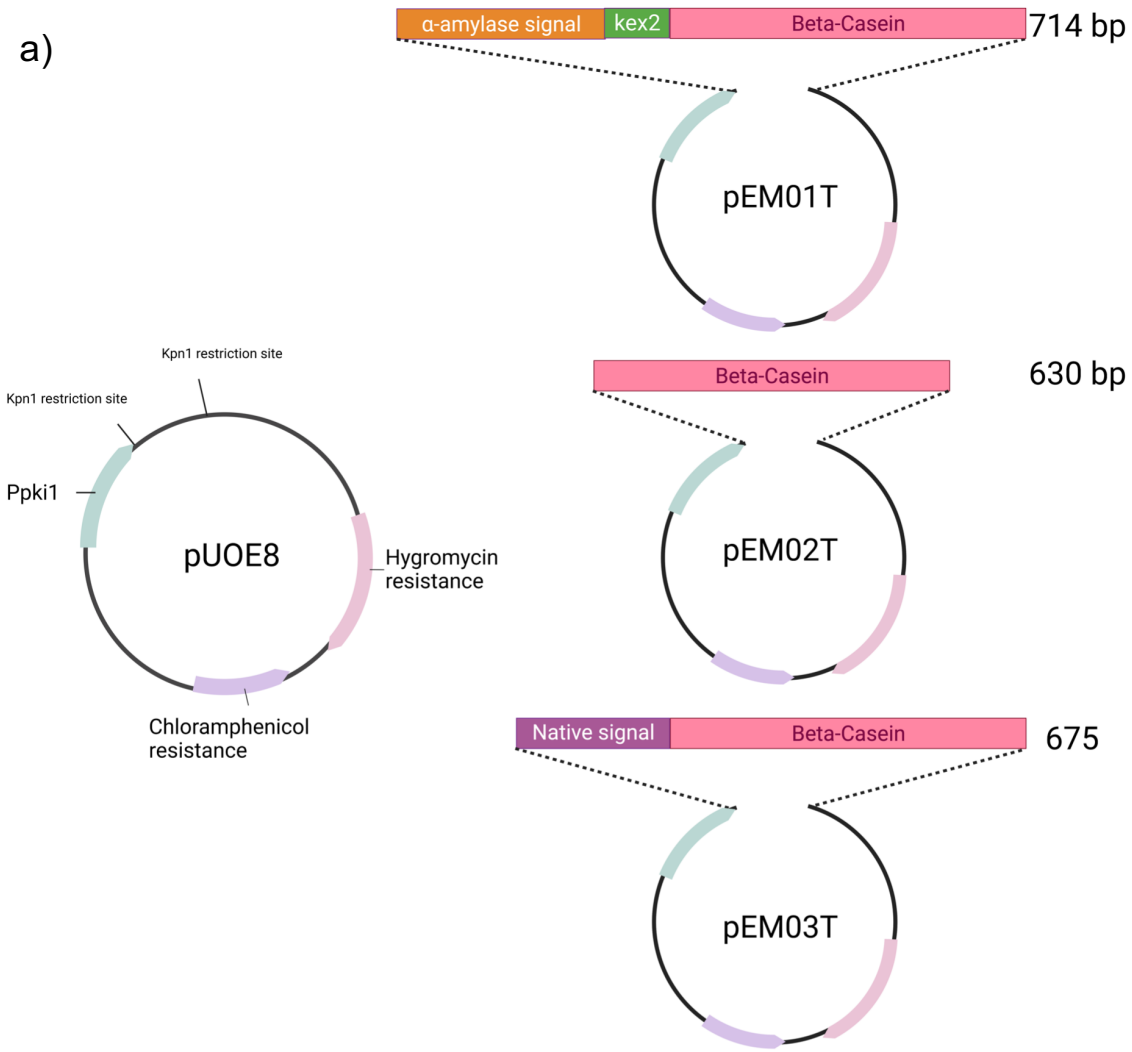
stages of insert PCR or Gibson assembly. Colony 2 also has a base change at position 2526 within the gene insert, from adenine to guanine, which does not cause an amino acid change.

3.5 Construction of protein expression systems for bovine beta-casein and kappa-casein for expression in *T. reesei*

Having successfully expressed casein in *E. coli*, we tested expression in *T. reesei* to see if the proteins could be secreted extracellularly or produced at a higher yield. Codon usage for *T. reesei* was taken from the codon usage database (Nakamura et al., 2000). Beta-casein and kappa-casein sequences were codon optimised for *T. reesei* using Geneious 2021.0.3.[3] Benchling was used to align the nucleotide and translated amino acid sequences to confirm that no amino acid changes had occurred (**Sup. Fig. 5.11 - 5.18**) (*Benchling [Biology Software]*, 2022). The alpha-amylase secretion signal from *Aspergillus awamori*, proven to be effective at facilitating secretion of recombinant proteins in *T. reesei* (Rantasalo et al., 2019), was codon optimised for *T. reesei* and added to the N terminal of the gene sequence with a Kex2 protease cleavage site allowing cleavage of the signal sequence. Six gene inserts were designed for the expression of casein in *T. reesei*: beta-casein with the alpha-amylase signal (BCN-alpha), beta-casein with the native bovine signal (BCN), beta-casein with no signal sequence (BNO), kappa-casein with the alpha-amylase signal (KCN-alpha), kappa-casein with the native bovine signal (KCN), kappa-casein with no signal sequence (KNO) (**Fig. 3.8a** and **b**). Gene inserts were amplified using PCR (**Table 2.8**) to introduce overlaps complementary to the insertion site in the plasmid pUOE8 (**Sup. Fig. 5.19**). Plasmid pUOE8 was linearised using the restriction enzyme KpnI-HF which produced two cuts in the backbone (**Sup. Fig. 5.20**). The linearised plasmid and fragment were assembled

[3] https://www.geneious.com

using Gibson assembly before transforming into electrocompetent Top10 *E. coli* cells. Plasmid DNA from successful transformants underwent PCR to ensure inserts had the expected size (**Fig. 3.9**). Plasmid DNA was extracted from *E. coli* and used to transform *T. reesei.* Successful transformation was indicated by resistance to hygromycin.


a)
α-amylase signal
kex2
Beta-Casein
714 bp
pEM01T
Kpn1 restriction site
Kpn1 restriction site
Ppki1
pUOE8
Hygromycin resistance
Chloramphenicol resistance
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Native signal
Beta-Casein
675
pEM03T

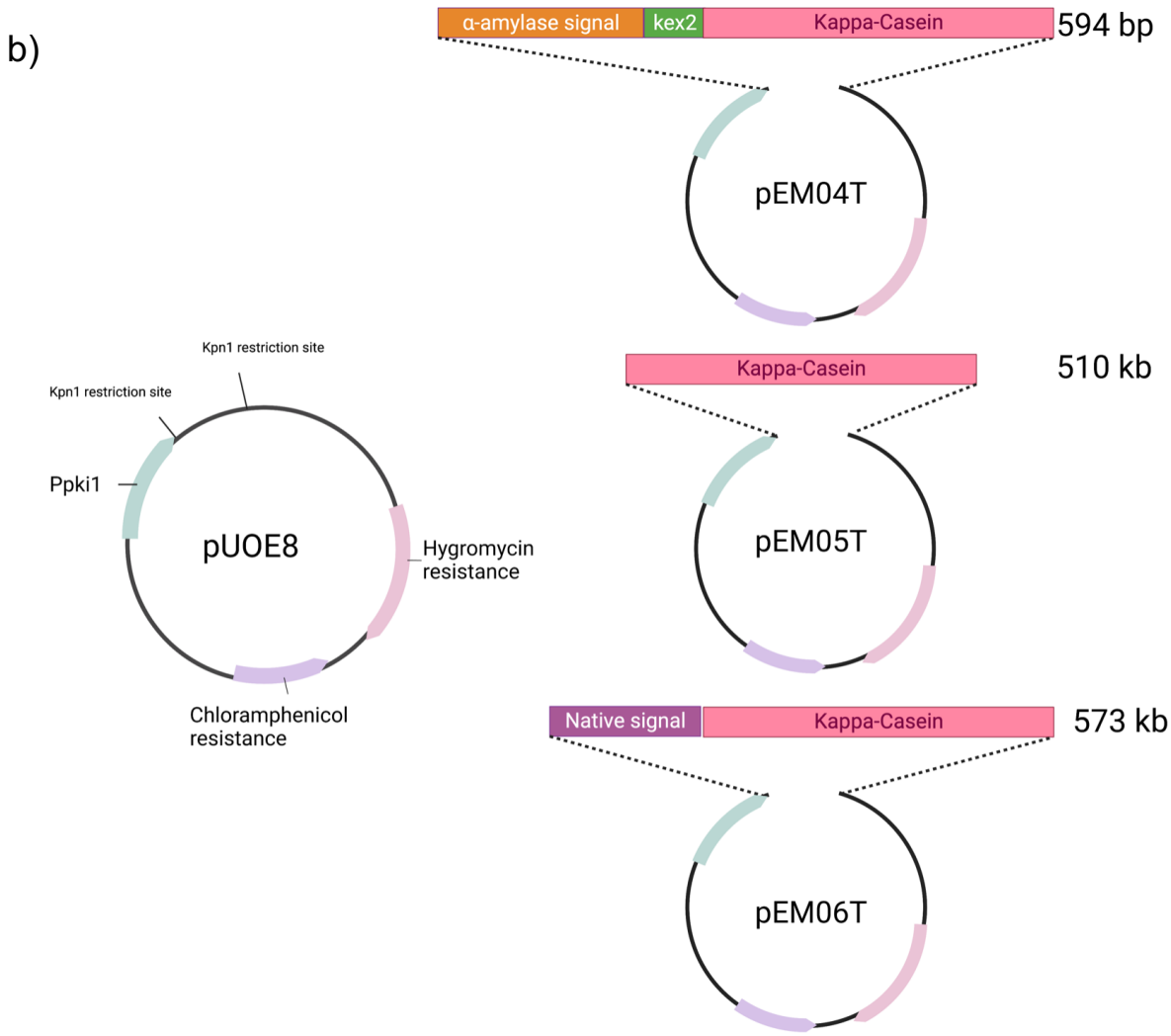

Figure 3.8: Schematic of plasmid constructs used for expression in *T. reesei*. a) Three variations of the beta-casein gene were cloned into the pBAD plasmid: beta-casein with alpha-amylase secretion signal from *Aspergillus awamori*, beta-casein with no signal, beta-casein with native signal **b)** Three variations of the kappa-casein gene were cloned into the pBAD plasmid: kappa-casein with alpha-amylase secretion signal from *Aspergillus awamori*, kappa-casein with no signal, kappa-casein with the native signal.

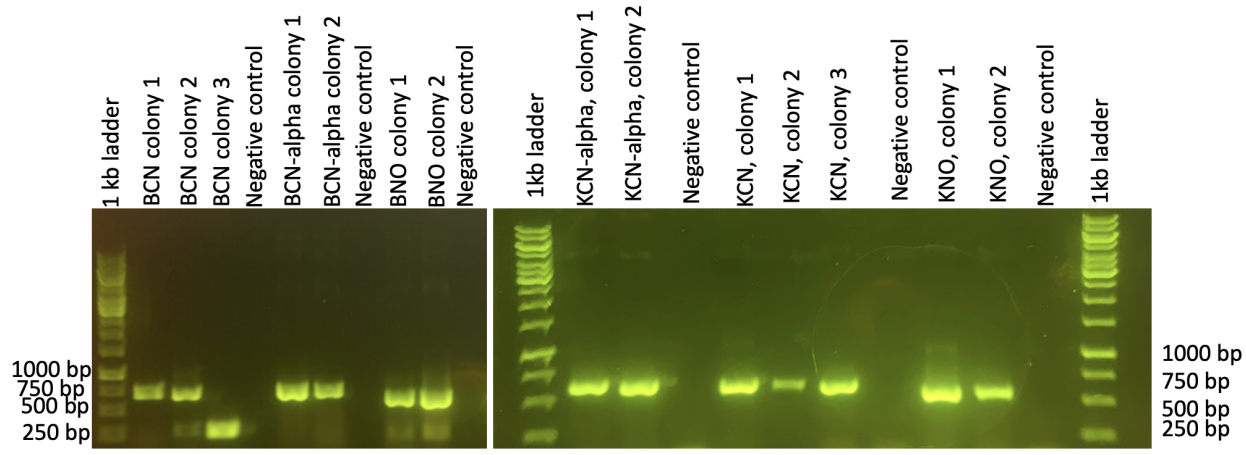

Figure 3.9: Colony PCR to confirm the presence of casein inserts in pUOE8 backbone in transformed *E. coli*. Results of PCR reactions with primers to amplify each gene insert ran on a 1% agarose gel. All colonies showed successful amplification of the gene inserts at the expected size, except for pEM03T (BCN insert) colony 3. The PCR product was 250bp, 425 bp too small.

Transformed *T. reesei* was inoculated into induction media and samples were taken after 48 hours. Samples were run on an SDS-PAGE gel and no expression of beta-casein, kappa-casein or beta-lactoglobulin was seen in *T. reesei.* The protein samples run on the SDS-PAGE gel were relatively clear with no proteins observed in any of the lanes (**Fig. 3.10**). This included the plasmid only pUOE8 sample, indicating that *T. reesei* was not growing robustly and thus not producing proteins as expected. While we were able to successfully transform *T. reesei* with plasmids containing the coding sequences for bovine beta-casein and kappa-casein, we were unsuccessful at expressing the proteins of interest. We believe that optimising media conditions could improve the expression of these proteins.

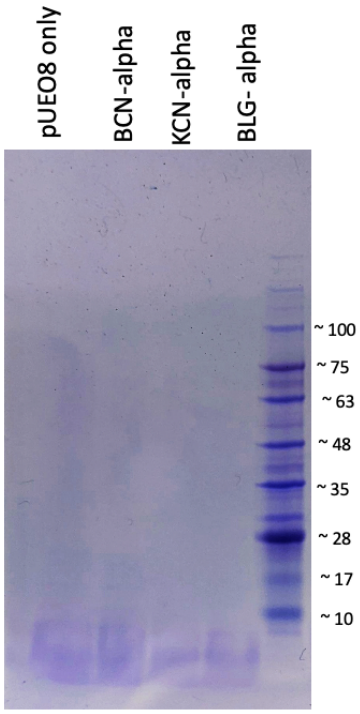

Figure 3.10: SDS-PAGE analysis of recombinant proteins expressed in *T. reesei*. Samples were taken from *T. reesei* transformed with pUOE8 (lane 1), pEM01T with BCN-alpha insert (lane 2), pEM04T with KCN-alpha insert (lane 3), pEM07T with BLG-alpha insert (lane 4). The protein standard's molecular weights are on the gel's right. No expression was seen.

Having tested the efficiency of protein expression in these hosts, we then evaluated whether the same hosts could be robustly grown on agricultural waste substrates, with the goal of producing these proteins via precision fermentation on waste products and optimising the circular economy.

3.6 *T. reesei* grows robustly on common agricultural waste

We tested the growth of wild type QM6a *T. reesei* on ten different types of agricultural-waste media (**Table 2.12**). Potato dextrose broth (PDB) from powder was used as a positive control for *T. reesei* growth. *T. reesei* grew on all agricultural-waste media (**Fig. 3.11**)

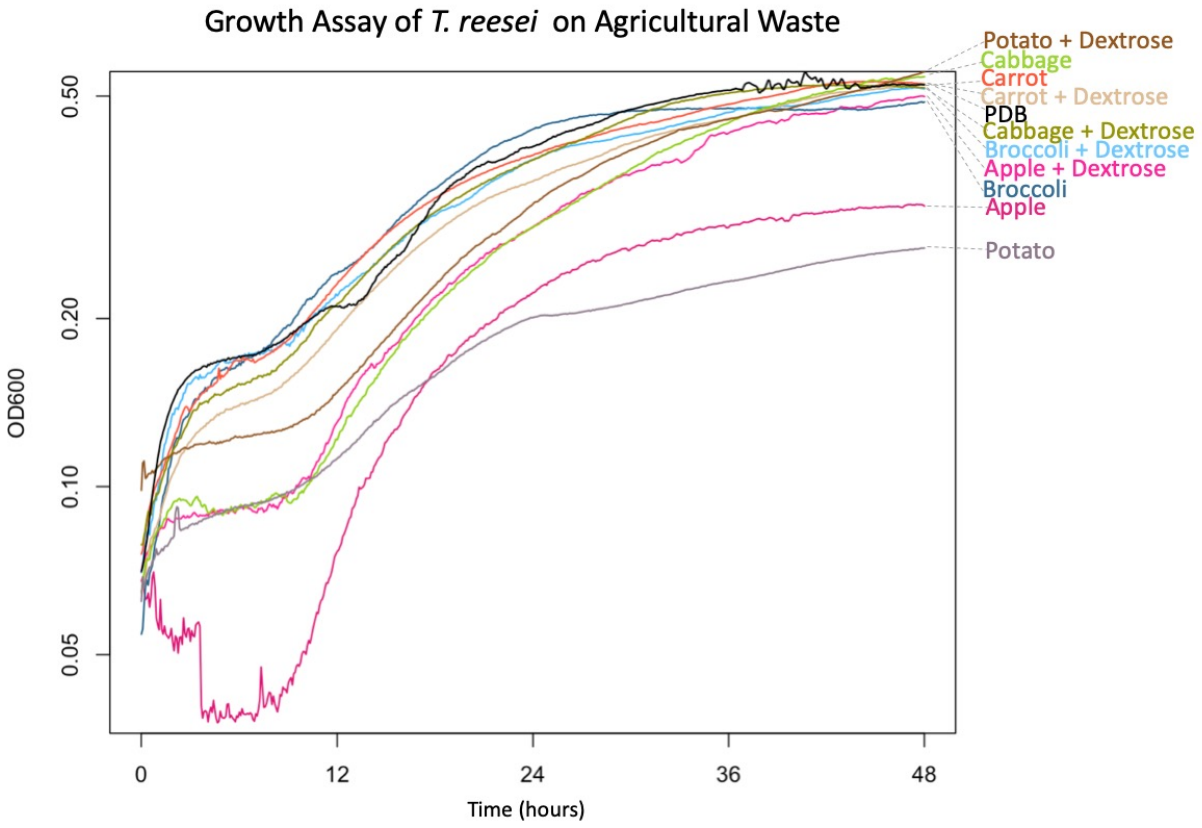

Figure 3.11: Growth curves of *T. reesei* grown on different agricultural-waste substrates. OD readings were normalised by subtracting the background OD of the media at each respective time point. The Y axis shows the log of the optical densities at absorbance 600nm.

The growth of filamentous fungi in submerged culture typically follows the same sigmoidal curve kinetics of a bacterial culture such as *E. coli* as is observed in **Supplementary Figure 5.21**. (Moore et al., 2020). However, *T. reesei* grown on agricultural-waste, demonstrated two distinct growth phases across most media. The first phase, occurring in the first 12 hours of growth, was

characterised by an initial short period of exponential growth followed by a plateau. The second phase was characterised by re-entry into the exponential growth phase for an extended period relative to the first growth stage (**Fig. 3.11**). After 36 hours, growth typically decelerated, entering the stationary phase again. The dual growth stages are seen for all agricultural-waste media except for apple, which starts off with an initial considerable decline in OD. The graph for growth on apple is much noisier than any of the media tested. All media tested, besides apple and potato, reached a similar final OD of approximately 0.5. This was the same as the PDB (potato dextrose broth) positive control commonly used as a lab nutrient source for *T. reesei*. Growth on apple media reached a final OD of 0.320. Interestingly, *T. reesei* grew the worst when cultured on potato media with a final OD of 0.267. It is important to note that at the end of the 48-hour growth assay, growth on several of the media had not yet reached the stationary phase, suggesting the nutrients had not yet been exhausted. This included apple dextrose, broccoli dextrose, carrot, carrot dextrose, cabbage, potato, and potato dextrose. However, growth on apple, broccoli, cabbage dextrose and PDB had decelerated to the stationary phase by the end of the 48 hours.

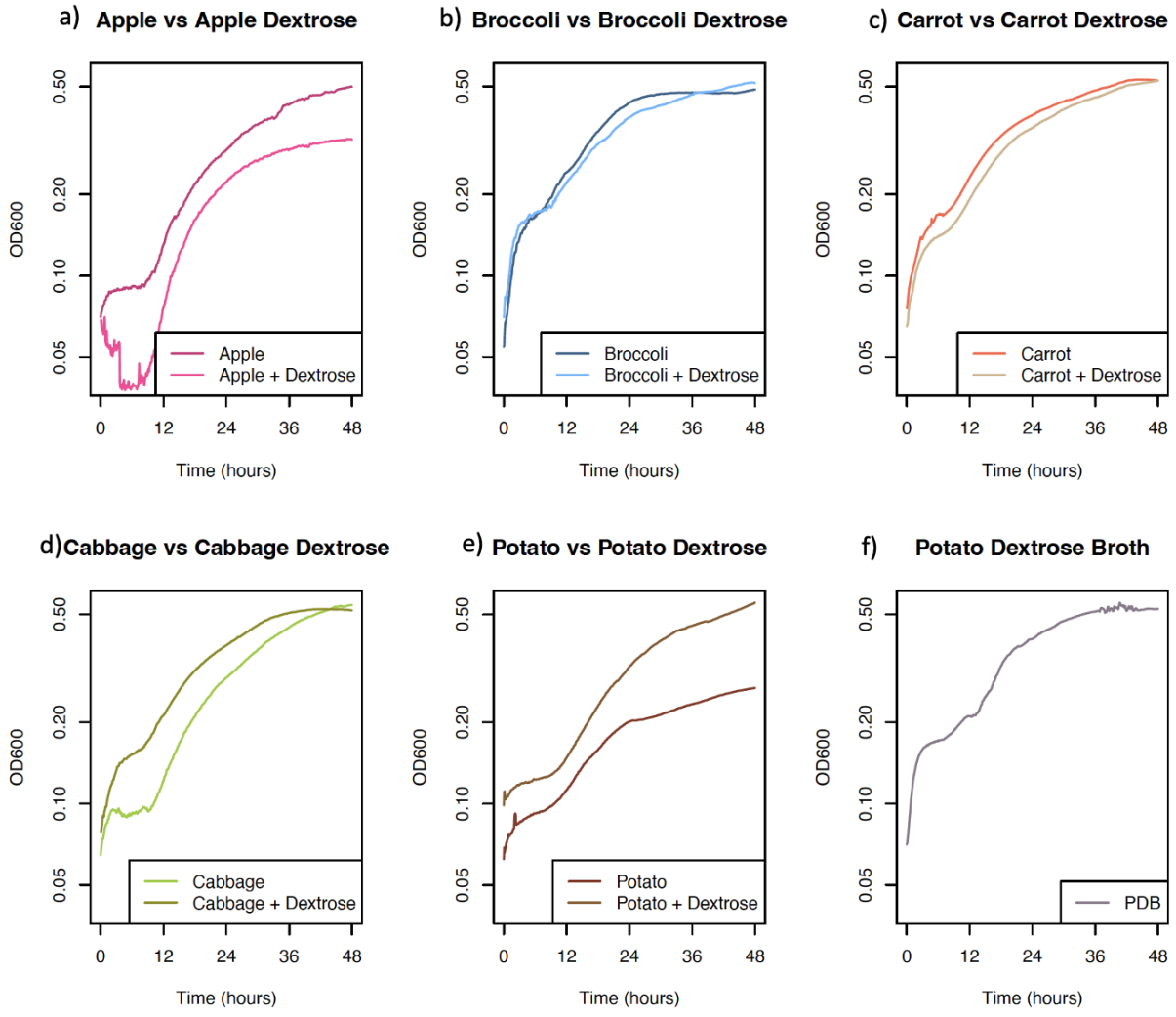

Figure 3.12: Comparison of *T. reesei* growth on agricultural-waste media with and without dextrose supplementation. a) apple vs apple dextrose **b)** broccoli vs broccoli dextrose **c)** carrot vs carrot dextrose **d)** cabbage vs cabbage dextrose **e)** potato vs potato dextrose **f)** potato dextrose broth. The Y axis shows the log of the optical densities at absorbance 600nm.

The final OD of broccoli, carrot and cabbage media supplemented with dextrose differed minimally from the respective media without (**Fig. 3.13**). In the case of the apple and potato-based media, the addition of dextrose improved the growth of *T. reesei* and resulted in a higher final OD. Growth on apple dextrose resulted in an OD of 0.5, similar to that of the best-performing growth substrates and the positive PDB control. Apple alone, however, only reached an OD of 0.320. The difference in OD for the potato-based media was larger. At the end of the 48-hour assay, potato dextrose

had yet to reach the stationary phase, and the OD was 0.554. Conversely, the OD of *T. reesei* grown in potato only media was 0.267. These results show that the addition of dextrose is typically not required for agricultural-waste to be an effective substrate for the growth of *T. reesei*.

The maximum specific growth rate (μ_{max}) is the rate of increase of biomass of a cell population per unit of biomass concentration. The specific growth rate is an important parameter in the optimisation of fermentation. It is representative of the dynamic behaviour of organisms as well as an indication of sufficient substrate (Srivastava & Gupta, 2011).

Table 3.1: Maximum specific growth rate (μ_{max}) and maximum OD of *T. reesei* grown on agricultural-waste

Media	μ_{max} (hr^{-1})	Max OD
Apple	0.045	0.320
Apple dextrose	0.034	0.500
Broccoli	0.110	0.488
Broccoli dextrose	0.101	0.518
Carrot	0.035	0.531
Carrot dextrose	0.032	0.526
Cabbage	0.095	0.541
Cabbage dextrose	0.140	0.523
Potato	0.022	0.267
Potato dextrose	0.076	0.554
Potato dextrose broth	0.051	0.545

Growth on potato media resulted in both the lowest μ_{max} (0.022 hr^{-1}) and lowest maximum OD (0.267) (**Table 3.1**). However, μ_{max} and maximum OD did not appear to be correlated. The highest maximum OD resulted from growth on potato dextrose at 0.554, however it did not achieve a particularly high μ_{max} at only 0.076 hr^{-1}. Growth on cabbage dextrose and broccoli resulted in the highest μ_{max} at 0.140 hr^{-1} and 0.110 hr^{-1} respectively. *T. reesei* growth on both these media also resulted in maximum OD of just over 0.5, indicating that the higher μ_{max} does not necessarily mean a higher final OD.

3.7 *P. pastoris* grows robustly on common agricultural waste

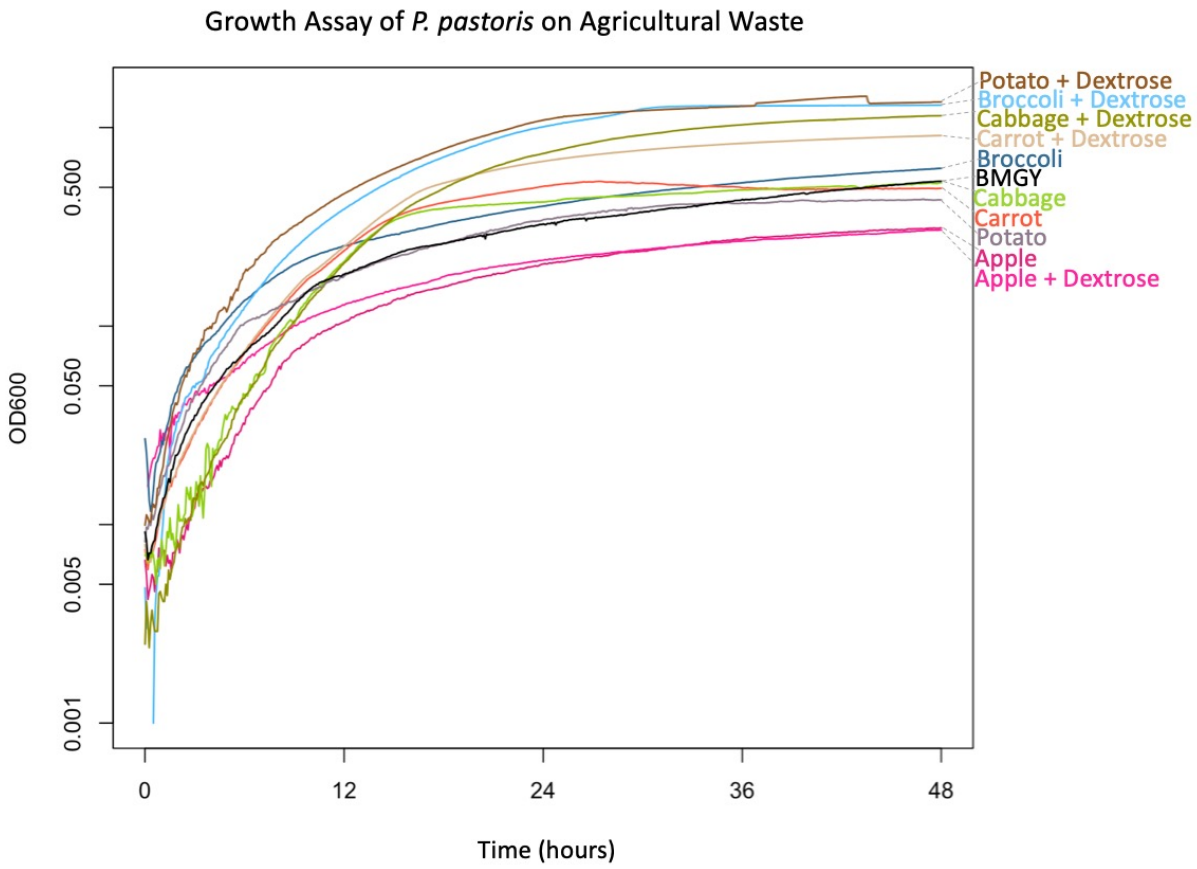

Figure 3.13: Growth curves of *P. pastoris* grown on different agricultural-waste substrates. OD values were normalised by subtracting the background OD of the media at all time points. Y axis shows the log of the optical densities at an absorbance of 600 nm.

Given the robust growth on agricultural-waste we observed with *T. reesei*, we next trialled the growth of another potential host organism, *P. pastoris,* on the same agricultural-waste substrates. However, we hypothesised that *P. pastoris* would not be able to grow as efficiently on agricultural-waste when compared to *T. reesei* due to its lack of cellulase production. Interestingly, *P. pastoris* grew successfully on all agricultural-waste media tested (**Fig. 3.14**). Buffered glycerol complex medium (BMGY) was used as a positive control to establish the standard growth pattern for *P. pastoris*. BMGY is the typical complex medium for *P. pastoris* cultivation and contains peptone,

yeast extract, yeast nitrogen base, and a phosphate buffer. Both peptone and yeast extract are nutrient-rich substrates formed through protein hydrolysis and yeast cell autolysis, respectively. Yeast nitrogen base contains trace elements, vitamins and inorganic salts (Matthews et al., 2018). The growth curves of all media tested follow a uniform pattern with no observed lag phase, a generally short exponential phase and a plateau in the graph suggesting resource depletion. By 12 hours, growth on all media had decelerated except for carrot dextrose and cabbage dextrose. Exponential growth on both of these media persisted for approximately 18 hours. Comparatively, exponential growth on BMGY only lasted 12 hours before decelerating. Although we had expected that the growth on BMGY would outperform that of all other agricultural-waste media, given its nutrient-dense profile and favoured use for *P. pastoris* cultivation, our results showed that BMGY was not the optimal media to maximise either the growth rate or biomass of *P. pastoris*. Rather *P. pastoris* growing on potato dextrose, broccoli dextrose, cabbage dextrose, carrot dextrose and broccoli results in a higher maximum OD (**Table 3.2**). Growth on apples and apple dextrose was the lowest, followed by potato. Potato dextrose appeared to be the best growth media, with the fastest initial rise in OD and the highest maximum OD of 1.438. These results are very similar to that obtained from growth on broccoli dextrose, which had the second-highest maximum OD of 1.297. Growth on cabbage dextrose resulted in a final OD of 1.148. However, initial growth was slower. Interestingly, all media supplemented with dextrose, with the exception of apple dextrose, outperformed the positive control BMGY. The OD measurements of *P. pastoris* growing on broccoli dextrose had an initial decrease in the first half an hour, but increased from that point onwards. This also occurred for growth on broccoli but to a lesser extent.

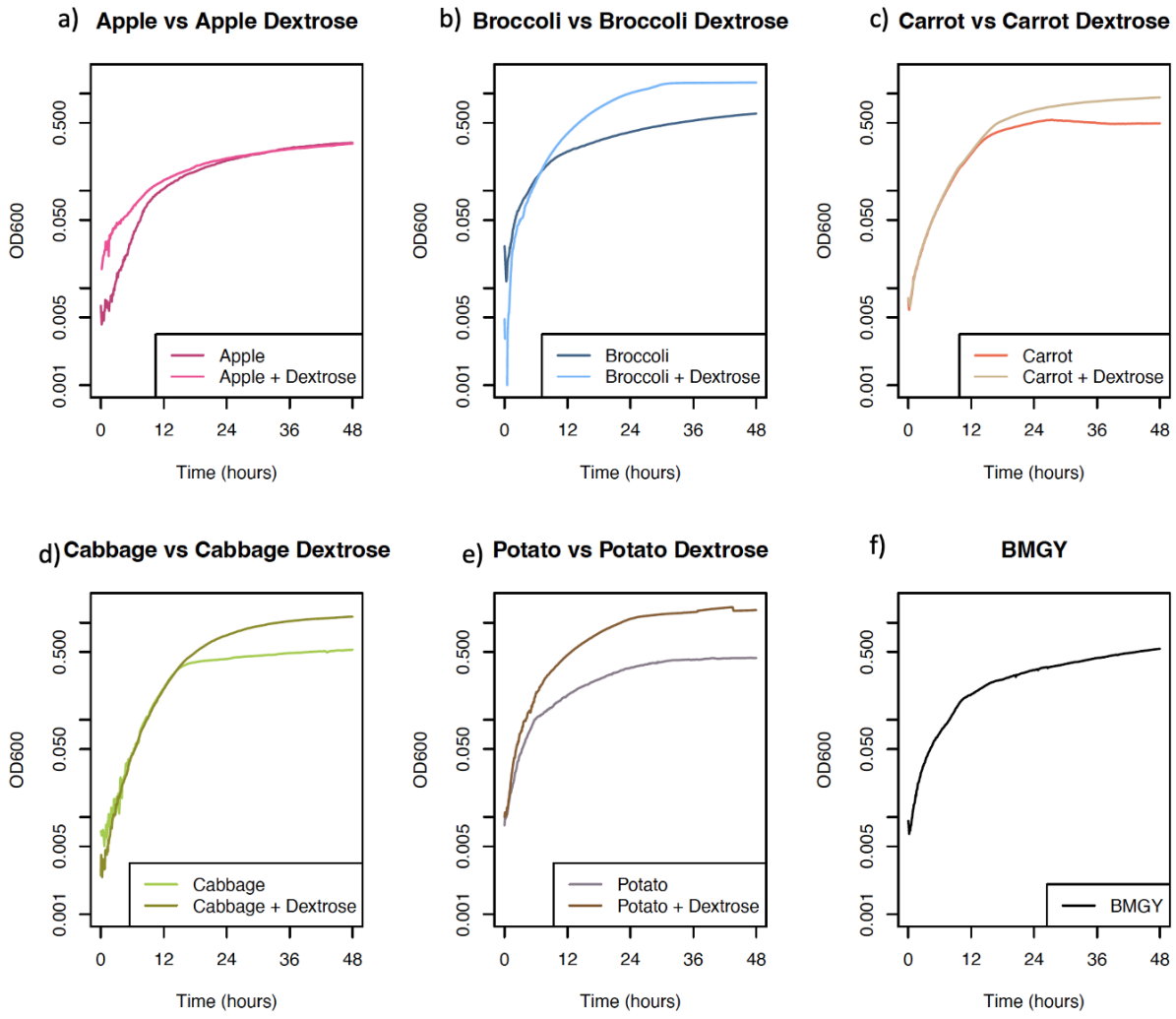

Figure 3.14: Comparison of *P. pastoris* growth on agricultural-waste media with and without dextrose supplementation. a) apple vs apple dextrose **b)** broccoli vs broccoli dextrose **c)** carrot vs carrot dextrose **d)** cabbage vs cabbage dextrose **e)** potato vs potato dextrose **f)** BMGY. The Y axis shows the log of the optical densities at absorbance 600nm.

Growth on media supplemented with dextrose was generally improved compared to growth on the dextrose-free counterpart (**Fig. 3.15**). While the slope of the exponential phase of each media pair tended to be similar, the media not supplemented with dextrose often reached the stationary phase first, save for growth on apple and apple dextrose media. While *P. pastoris* initially grew faster on apple dextrose, reaching a higher OD quicker, by 24 hours, the final OD was almost the

same between the two media (0.312 for apple and 0.306 for apple dextrose). From 24 hours onwards, growth on apple dextrose was the same as on apple.

Table 3.2: Maximum specific growth rate (μ_{max}) and maximum OD of *P. pastoris* grown on agricultural-waste

Media	μ_{max} (hr^{-1})	Maximum OD
Apple	0.024	0.312
Apple dextrose	0.370	0.306
Broccoli	0.032	0.623
Broccoli dextrose	0.061	1.297
Carrot	0.045	0.536
Carrot dextrose	0.054	0.912
Cabbage	0.160	0.528
Cabbage dextrose	0.337	1.148
Potato	0.027	0.436
Potato dextrose	0.111	1.438
BMGY	0.027	0.537

Growth on apple dextrose resulted in the highest μ_{max} at 0.370 hr^{-1}, closely followed by growth on cabbage dextrose at 0.337 hr^{-1}. Interestingly, the μ_{max} of *P. pastoris* growing on broccoli dextrose or potato dextrose was substantially smaller, despite both achieving high final ODs. While *P. pastoris* growth on potato dextrose had the highest maximum OD (1.446), the μ_{max} was only 0.111 hr^{-1}. The μ_{max} of growth on BMGY at 0.27 hr^{-1} was bigger than only apple and the same as

what was seen for growth on potato. Our results show that a high μ_{max} is not an effective indicator of a high final OD.

3.8 *K. lactis* grows robustly on common agricultural waste

Given the robust growth on agricultural-waste we observed with *T. reesei*, we next trialled the growth of another potential host organism, *K. lactis,* on the same agricultural-waste substrates. However, we hypothesised that *K. lactis* would not be able to grow as efficiently on agricultural-waste when compared to *T. reesei* due to its lack of cellulase production. Interestingly, *K. lactis* grew successfully on all agricultural-waste media (**Fig. 3.16**). The growth curves tended to follow one of two distinct patterns. The first, characterised by a short steep exponential phase and early deceleration into a stationary phase, was observed for growth on apple, apple dextrose and broccoli. The second pattern exhibited a respectively shallower and much longer exponential phase resulting in higher final OD values. This was seen for growth on broccoli dextrose, cabbage dextrose, yeast peptone dextrose (YPD), carrot dextrose, carrot and, to a lesser extent, cabbage. Following neither of those trends was growth on potato and potato dextrose. Growth of *K. lactis* on potato had an initial gradual increase in OD, maintained until 36 hours before a plateau. Growth on potato dextrose also began with an initial gradual increase in OD. The graph increases in steepness from about 24 hours and does not reach a plateau before the 48-hour experiment concludes.

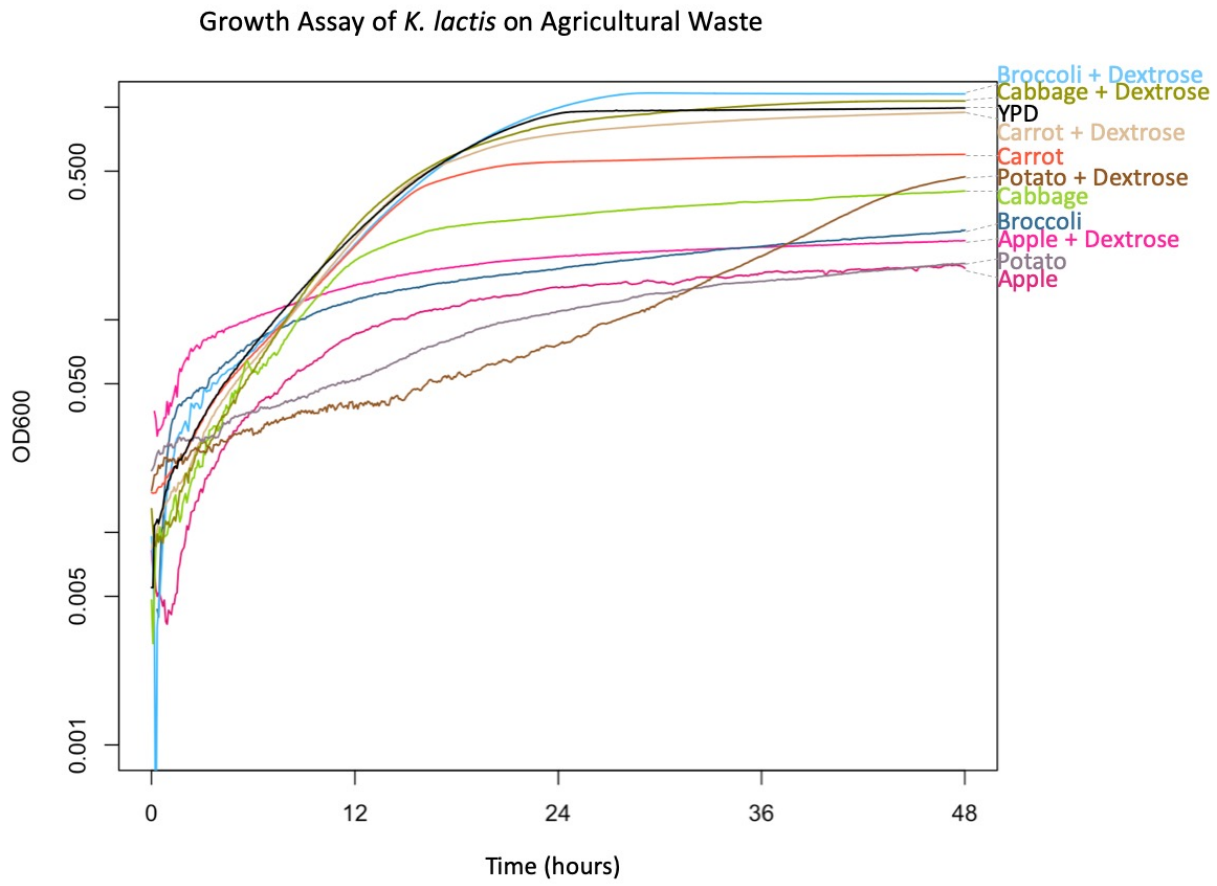

Figure 3.15: Growth curves of *K. lactis* grown on different agricultural-waste substrates. ODs were normalised by subtracting the background OD of the media at all time points. Y axis shows the log of the optical densities at an absorbance of 600 nm.

K. lactis grew the most on broccoli dextrose with a maximum OD of 1.165, outperforming the positive control and standard *K. lactis* growth media of YPD. *K. lactis* grown on cabbage dextrose, and carrot dextrose, also reached a similar maximum OD of 1.07 and 0.945, respectively. Growth on apple resulted in the lowest OD maximum at 0.181. While the maximum OD of *K. lactis* on carrot dextrose, broccoli dextrose, and cabbage dextrose were all similar, ranging between (0.945-1.165), the maximum OD of *K. lactis* grown on the respective un-supplemented media varied. Growth on carrot reached a maximum OD of 0.599, while cabbage was 0.403 and broccoli

even less so at 0.264. These results help to elucidate the variations in media nutrients, irrespective of dextrose.

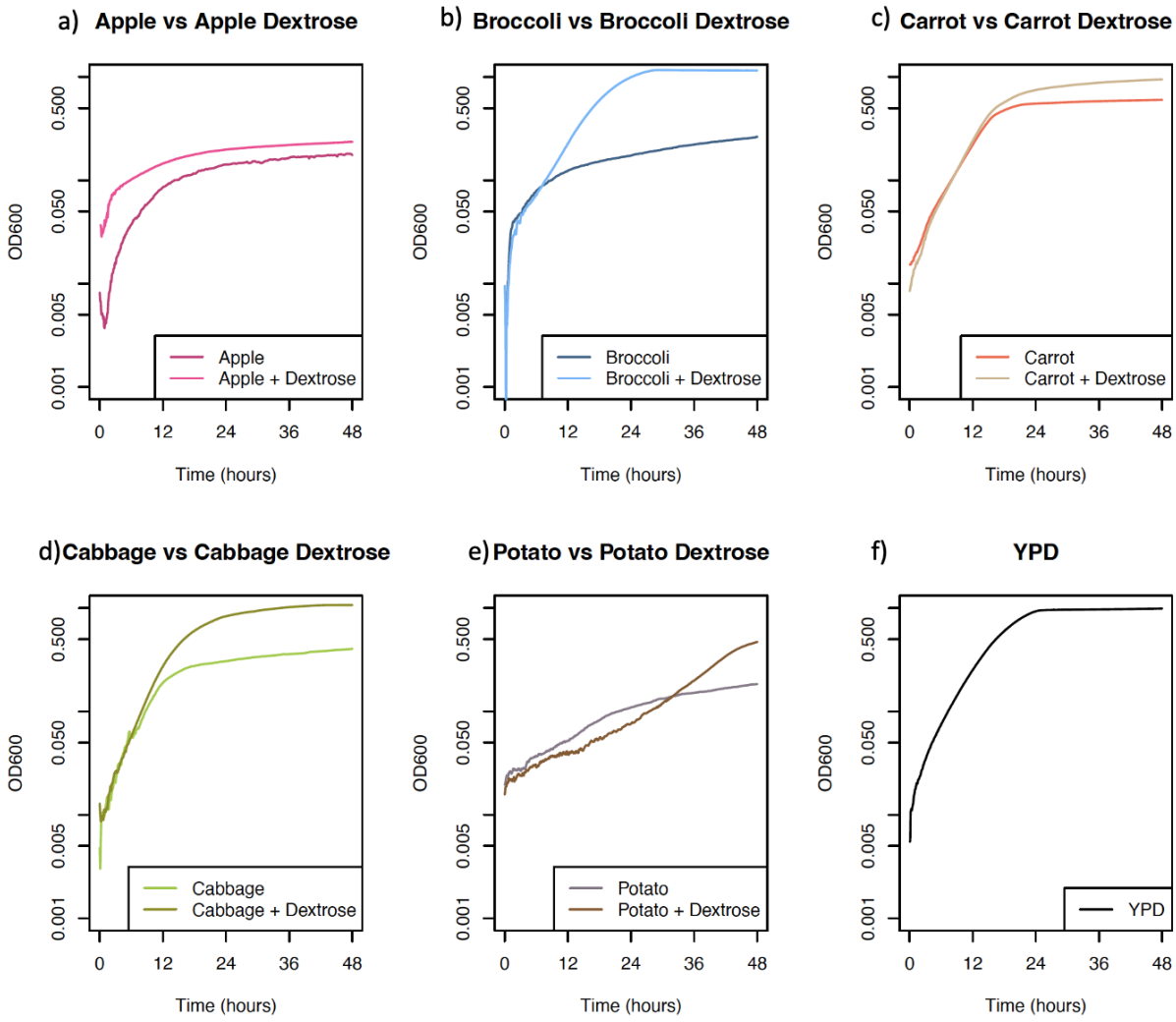

Figure 3.16: Comparison of *K. lactis* growth on agricultural-waste media with and without dextrose supplementation a) apple vs apple and dextrose b) broccoli vs broccoli and dextrose c) carrot vs carrot and dextrose d) cabbage vs cabbage and dextrose e) potato vs potato dextrose f) YPD. The Y axis shows the log of the optical densities at absorbance 600nm.

K. lactis growth on agricultural-waste media supplemented with dextrose was improved compared to agricultural-waste media without (**Fig. 3.17**). This was true for all media tested, with the greatest

difference occurring between growth on broccoli and broccoli dextrose. The difference between apple and apple dextrose was difficult to elucidate due to the large difference in starting OD. Growth on all media resembled a standard three phase growth curve consisting of lag, exponential, and stationary phase (**Fig. 3.12**), except for potato and potato and dextrose. Neither demonstrates a distinctive exponential or stationary phase but both continue to increase in OD over the entire 48 hours. In the first 30 minutes the OD readings of *K. lactis* on broccoli declined. However, after 60 minutes an increase in growth was observed. This also occurred with growth on apple, but to a much smaller extent. The OD readings of growth on broccoli dextrose had a large dip in the first few readings however then continued to increase, demonstrating a distinctive exponential phase and stationary phase. The initial decline may be an error in readings.

Table 3.3: Maximum specific growth rate (μ_{max}) and maximum OD of *K. lactis* grown on agricultural-waste

Media	μ_{max} (hr^{-1})	Max OD
Apple	0.018	0.181
Apple dextrose	0.495	0.235
Broccoli	0.186	0.264
Broccoli dextrose	0.074	1.165
Carrot	0.055	0.599
Carrot dextrose	0.066	0.945
Cabbage	0.037	0.403
Cabbage dextrose	0.082	1.07
Potato	0.013	0.184

Potato dextrose	0.033	0.471
YPD	0.066	0.991

Growth on apple dextrose had the highest μ_{max} by a factor of more than 2.5, yet had the third lowest maximum OD at only 0.235 (**Table 3.3**). Broccoli dextrose had the highest maximum OD at 1.165 and had a μ_{max} of 0.074 hr^{-1}, which is more than six times less than the μ_{max} for growth on apple dextrose. This leads us to believe that apple dextrose may be the most preferred media for the growth of *K. lactis*, but the nutrient availability limits biomass accumulation.

3.9 *E. coli* grows robustly on common agricultural waste

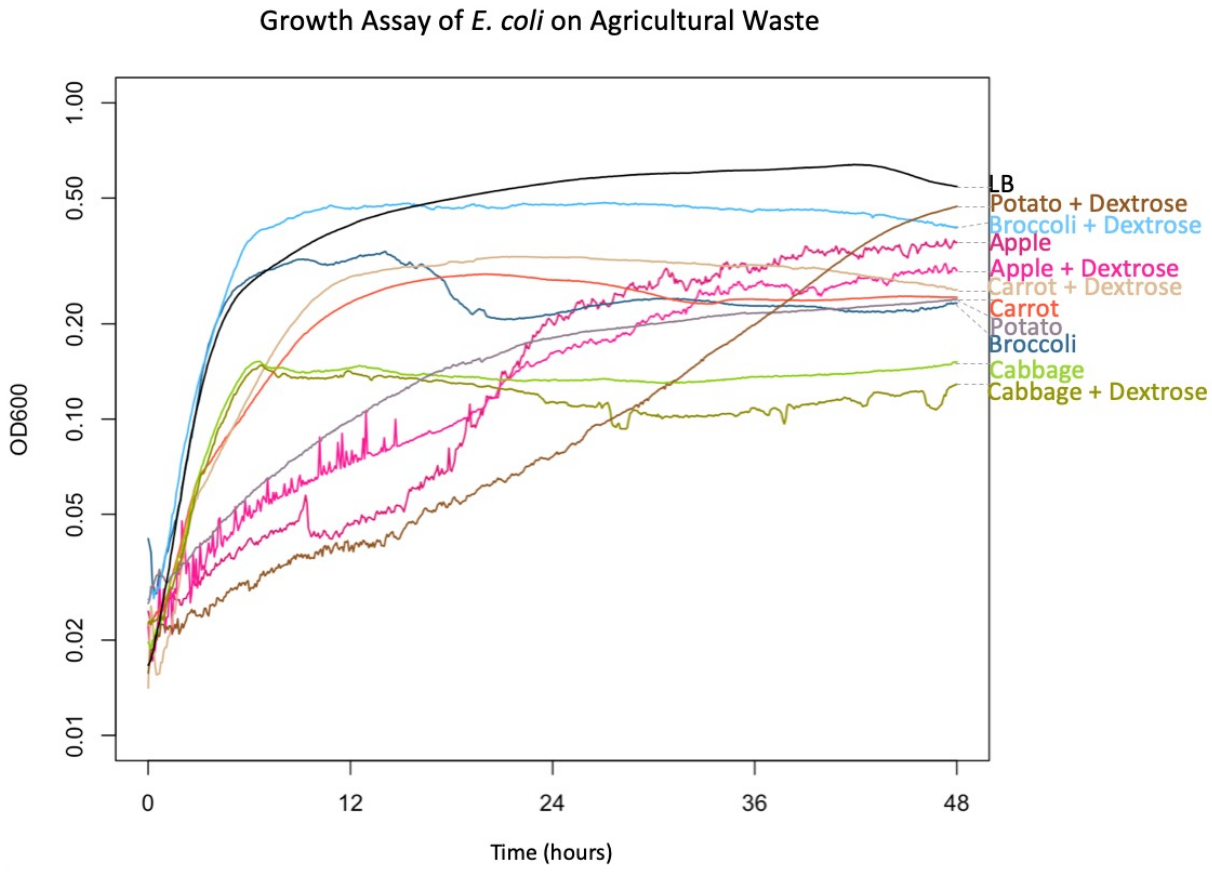

Figure 3.17: Growth curves of *E. coli* grown on different agricultural-waste substrates. ODs were normalised by subtracting the background OD of the media at all time points. Y axis shows the log of the optical densities at an absorbance of 600nm.

Given the successful growth of *P. pastoris and K. lactis* on agricultural-waste substrates, we tested the growth of *E. coli* on the same substrates (**Fig. 3.18**). In the lab, *E. coli* is typically grown on LB broth, a nutritionally rich media. In nature, however, *E. coli* has been found growing on a wide variety of fruit and vegetables (Abadias et al., 2009; Abdul-Raouf et al., 1993; Dingman, 2000; Islam et al., 2005; Janisiewicz et al., 1999). However, these studies focussed more on the presence and growth of *E. coli* rather than waste valorisation. Given this, we hypothesised that *E. coli* would grow on agricultural-waste media. Growth in LB media was used as the positive control

and resulted in the highest OD of all media tested, with a maximum OD of 0.637 (**Table 3.4**). Interestingly, *E. coli* growth did not seem to be improved on any of the media supplemented with dextrose, compared to the media without (**Fig. 3.19**). Each pair of agricultural-waste substrates tended to follow the same pattern, with *E. coli* reaching a similar final OD irrespective of dextrose supplementation. This was particularly true for growth on apple and apple dextrose, cabbage and cabbage dextrose, and carrot and carrot dextrose. *E. coli* grown on broccoli and broccoli dextrose had an initial exponential growth phase of the same slope; however, growth on broccoli decelerated earlier and experienced an unusual decline at 24 hours before a plateau. Growth on potato dextrose does follow the characteristic *E. coli* growth curve with distinct exponential growth, deceleration and stationary phases. *E. coli* growing on potato dextrose maintained a steady, nearly linear progression for the entire duration of the experiment and finishes with a final OD of 0.266.

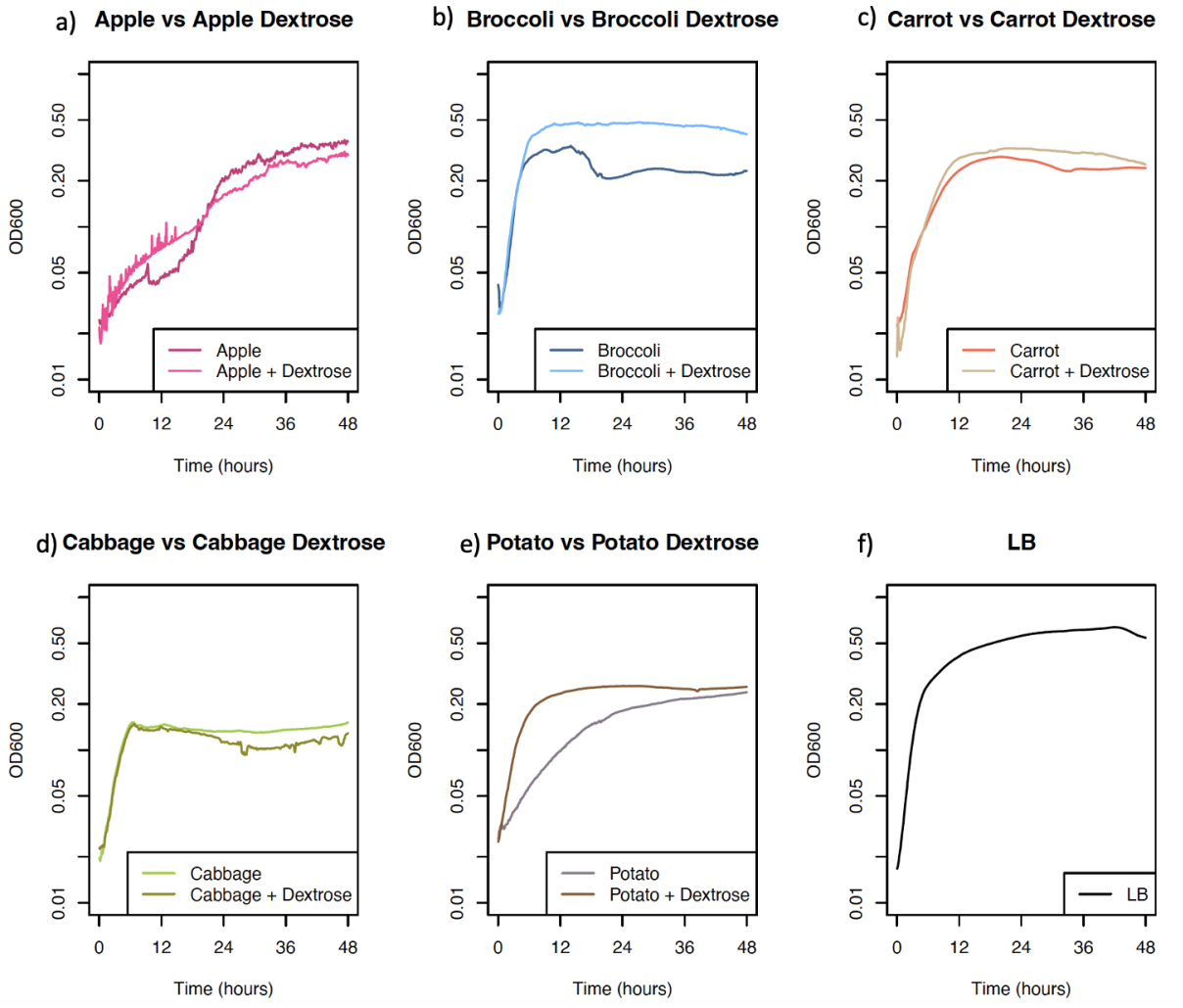

Figure 3.18: Comparison of *E. coli* growth on agricultural-waste media with and without dextrose supplementation a) apple vs apple and dextrose b) broccoli vs broccoli and dextrose c) carrot vs carrot and dextrose d) cabbage vs cabbage and dextrose e) potato vs potato dextrose f) LB. The Y axis shows the log of the optical densities at absorbance 600nm.

Broccoli dextrose is the only media that improves the growth of *E. coli* when compared to the dextrose free counterpart (**Fig. 3.19**). For the first 6 hours of the experiment, the rate at which the OD increased was almost identical between broccoli and broccoli dextrose. After which, growth on broccoli started to decelerate, and growth on broccoli dextrose continued to increase. The unusual decline in growth that *E. coli* experienced when grown on broccoli, exacerbates the

difference in final OD. The maximum OD on broccoli dextrose was 0.484 compared to 0.338 on plain broccoli. The final OD of *E. coli* on all the other media tested was not substantially improved with the addition of dextrose. Growth during the exponential phase on cabbage based media and carrot based media is nearly identical between the respective media pairs, finding that adding dextrose did not increase the rate at which the OD increases. Overall, we found that *E. coli* grew on all agricultural-waste substrates tested. While growth on the common lab media, LB, reached the highest final OD, these results suggest that agricultural-waste is a viable nutrient source for the growth of *E. coli.*

Table 3.4: Maximum specific growth rate (μ_{max}) and maximum OD of *E. coli* grown on agricultural-waste

Media	μ_{max} (hr^{-1})	Maximum OD
Apple	0.519	0.371
Apple dextrose	0.403	0.310
Broccoli	0.092	0.338
Broccoli dextrose	0.098	0.484
Carrot	0.027	0.287
Carrot dextrose	0.117	0.327
Cabbage	0.049	0.152
Cabbage dextrose	0.047	0.148
Potato	0.012	0.239
Potato dextrose	0.051	0.266
LB	0.065	0.637

Growth on apple and apple dextrose resulted in the highest μ_{max} at 0.519 hr^{-1} and 0.403 hr^{-1} respectively (**Table 3.4**). Growth on these media also resulted in two of the highest maximum OD values at 0.371 and 0.310 respectively. Despite this, the μ_{max} of *E. coli* grown on LB is nearly eight times smaller than apple but still results in a higher maximum OD. These results suggest that apple based media may be the best media for fast *E. coli* growth.

Chapter 4: Discussion

4.1 Recombinant bovine beta-casein and kappa-casein expressed in *E. coli* exhibit a higher molecular weight than expected

Our experiments demonstrate that bovine beta-casein and kappa-casein can be produced using *E. coli* as the host organism. We found that expression was first visible in BL21 *E. coli*, likely due to the reduction in proteases compared to Top10 *E. coli*. Expression was initially suspected based on an SDS-PAGE gel; however, high levels of endogenous *E. coli* proteins and low heterologous protein expression clouded results. By performing a western blot using HA tags for antigen binding, we could conclude that both beta-casein and kappa-casein had been expressed. However, the location of the bands relative to the protein ladder indicated that kappa-casein is substantially larger than expected. We hypothesise that the protein ladder is not an entirely reliable resource as the manufacturer's guide suggests band locations based on a 4-20% gel, and in our experiment, a 4-15% gel was used (Abcam Pre-stained protein Ladder - Mid-range molecular weight (10-180 kDA) (ab11602)). Irrespective of the protein ladder, the kappa-casein protein expressed was larger than the beta-casein protein, which contrasts with expectations from the known coding sequences (Bijl et al., 2020). We estimate that the kappa-casein expressed is roughly double the expected size. This is true of both kappa-casein with and without the native signal, suggesting that the native bovine signal is not recognised or cleaved by *E. coli.* Kappa-casein has previously been expressed in *E. coli* at the correct molecular weight (C. Kang, 1988). *E. coli* does not perform post-translational modifications such as phosphorylation and glycosylation as prokaryotic hosts can, therefore, the increased molecular weight is unlikely to be attributed to this (Kaur et al., 2018). Upon comparing the beta-casein and kappa-casein positive control, we observed that the recombinant beta-casein was the same size as the native bovine

beta-casein positive control, and the recombinant kappa-casein is only slightly larger than the native bovine kappa-casein positive control. The manufacturer of the positive controls (Sigma-Aldrich) states that beta-casein can exist with five phosphate groups, and kappa-casein can have many isoforms depending on the glycans attached. Therefore, the molecular weight of the positive control should be larger than what *E. coli* can express. Oxford Nanopore sequencing of the plasmids did not suggest any unexpected sequences were present, only one copy of the insert appearing to have been cloned into the pBAD plasmid, and each having a start codon upstream and a stop codon downstream of the gene insert. While the KNO-HA inserts did have two additional amino acids, this does not account for the molecular weight being double than expected. Considering the unreliable protein ladder and positive control, with the sequencing results, it is hard to precisely quantify the molecular weight of the casein proteins expressed by *E. coli*, and further experiments, such as LC-MS, proteolysis, or mass spectrophotometry, are required.

Our experiment demonstrates the first steps in developing a host organism capable of producing dairy-identical proteins. We successfully expressed both beta-casein and kappa-casein; however, each has a higher molecular weight than expected. Further research is needed to determine why this might be and, thus, the steps that need to be taken to ensure beta-casein and kappa-casein can be produced at the correct molecular weight.

4.2 Expression of recombinant bovine beta-casein and kappa-casein has not been achieved in *T. reesei*

We did not succeed in expressing bovine casein in *T. reesei*. There are likely several reasons for this. Our *T. reesei* strains did not grow well in the glucose induction media. Although the protocol was followed from Rantasalo et al. (2019), who demonstrated successful expression in *T. reesei*, the precise growth conditions may not have been identical. One possibility is that there were differences in the pH of the media. *Trichoderma spp.* are known to prefer acidic environments with a pH of between 4-6 with an optimum of pH 4.8 (Rantasalo et al., 2019). However, our media was unbuffered and exhibited a pH of below pH 4. The unbuffered nature of the media may have allowed pH changes throughout the experiment, which may also cause poor growth. While pH 4 is not necessarily damaging to *T. reesei,* casein proteins denature and aggregate at low pH; protein aggregation is known to cause severe growth defects in some cases (Gasser, 2008).

We also hypothesise that the casein may not have been expressed extracellularly. Previous work in our lab has revealed that fungal host organisms can efficiently secrete beta-lactoglobulin but not casein proteins. Casein proteins are insoluble, and it is not uncommon for insoluble recombinant proteins to form aggregate bodies or experience difficulties being expressed from the cell (Ferrer-Miralles et al., 2022). However, if this were the case, we would expect to see at least expression of soluble beta-lactoglobulin on our SDS-PAGE gel from *T. reesei* transformed with pEM07T. In future work we will refine the media composition to ensure it is an appropriate environment for *T. reesei* growth. If extracellular casein export is still not observed, we will investigate alternative signal sequences or chaperone methods to facilitate protein expression in *T. reesei.*

4.3 Common agricultural waste is a suitable substrate for the fermentation of *T. reesei*, *P. pastoris*, *K. lacti*s and *E. coli*

The future of food production via precision fermentation is poised to be an ethical and sustainable alternative to current food production (Vestergaard et al., 2016). To ensure maximum efficiency of precision fermentation, we investigated the feasibility of using agricultural-waste as a growth media for commonly used host organisms such as *T. reesei, K. lactis, P. pastoris and E. coli.* Previous research has investigated *Trichoderma spp*. growth on commonly found agricultural products and achieved success in mass multiplication and bioactivity as a bioagent.

We tested five different agricultural-waste substrate pairs: apple and apple dextrose, broccoli and broccoli dextrose, carrot and carrot dextrose, cabbage and cabbage dextrose, potato and potato dextrose. All four organisms tested grew on all media types. The growth of *P. pastoris* and *K. lactis* was generally improved on media supplemented with dextrose. *T. reesei* and *E. coli* showed little distinction between growth on media with or without dextrose. These results are seen especially in media prepared from apples where the sugar content is naturally high. In media such as cabbage or potato, where the sugar content is lower than in apple media, there is more of an improvement with the addition of dextrose. This indicates that while dextrose improves the growth of some organisms, it is not an essential addition to achieving growth.

These findings support the hypothesis that raw agricultural-waste is sufficient to use as a culture media for the growth of microorganisms. All four organisms demonstrated efficient growth on broccoli-based media. One explanation may be due to the higher protein content in broccoli when compared to all other waste substrates used (FDA, 2020). Given that supplementation with

dextrose does not significantly improve growth across the media types, sugar availability is unlikely to become the limiting nutrient resource. Instead, other components, such as nitrogen, are likely to become a limiting nutrient source given the poor protein content typically in fruit and vegetables. Therefore, broccoli media, with a higher protein content, may outperform other growth media due to the additional protein content.

The growth curves of apple across the growth assays were all relatively noisier than the curves of all other media tested. This may be a result of the "edge effect" that can influence the results of the wells closest to the edge of a 96-well plate (Mansoury et al., 2021). The apple media was placed in the first well in all experiments, followed by apple dextrose. Repeating the growth assays with apple media in a more central row would be beneficial to gain a more accurate understanding of growth on apple media. In some instances, OD readings initially decline before increasing again. These erroneous readings are likely errors occurring at the beginning as the cell culture establishes itself in the media and the plate reader reaches the correct incubation temperature.

4.4 Agricultural waste may suit continuous or fed-batch fermentation

We calculated the μ_{max} to evaluate the rate of increase in biomass concentration. Across the results of all four organisms we found that a high final OD was not always indicative of the best growth media as quantified by maximum growth rate. The yield of proteins expressed from *K. lactis* and *P. pastoris* highly depends on the biomass reached (Vieira Gomes et al., 2018). Substrates that result in a high μ_{max} indicate a quick rate of increased biomass. In the case of *K. lactis*, apple had the highest μ_{max}, yet one of the lowest maximum OD. This indicates that *K. lactis* rapidly accumulates biomass but exhausts the nutrients from the apple dextrose media. Similar results were also found for *E. coli* and *P. pastoris*, where apple dextrose gave high μ_{max} values

but not consistently high maximum OD's. Apple dextrose media appears to be depleted of nutrient resources early on. With continuous or fed-batch fermentation, the regular addition of new media would prevent the deceleration of growth and facilitate the continued accumulation of biomass and, therefore, high protein yield. Further investigations should be performed to investigate this hypothesis, simulating batch-fed fermentation.

There may have been other factors affecting growth that we did not consider or quantify, such as the waste being at varying degrees of decay. Additionally, the open boiling method of preparation reduced accuracy in terms of concentration, as different amounts of water had boiled off for each preparation, although this could have been mitigated through re-weighing the water (and media).

In the future, the combined traits of protein producer and waste utiliser should be considered. In addition, other common New Zealand agricultural-waste sources would be interesting to consider, such as kiwifruit, spent grain from breweries and grapes from the wine industry. This has the potential to simultaneously increase both industries' efficiency and environmental circulatory.

4.5 Conclusion and future perspectives

The economics of heterologous production of proteins depends on several factors: host efficiency, the scale of production, ease of downstream processing, availability and cost of inputs. In order for the microbial production of bovine milk proteins to be a sustainable and viable option as an alternative food source, all factors must be optimised. Here we have demonstrated that a simple *E. coli* expression system can produce bovine milk proteins, and this system, along with other common host organisms, can be effectively cultivated on readily available agricultural waste. In future work we will combine the two processes to establish a truly sustainable bioeconomy of waste valorisation and valuable food protein production.

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Chapter 5: Appendix

a)

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        1                                                                    82
BCN     atgaaggtcctcatccttgcctgcctggtggctctggcccttgcaagagagctggaagaactcaatgtaccgggtgagattg
BCN opt atgaaggttctcatccttgcttgcctggtggcgctggcccttgcgcgtgagctggaagaactcaacgtaccgggcgaaattg

.....

        83                                                                   164
BCN     tggaaagcctttcaagcagtgaggaatctattacacgcatcaataagaaaattgagaagtttcagagtgaggaacagcagca
BCN opt tggaatcgctgtcaagcagcgaggaatcaattactcgcatcaataagaaaattgagaaatttcaaagtgaggaacaacaaca

.....

        165                                                                  246
BCN     aacagaggatgaactccaggataaaatccacccctttgcccagacacagtctctagtctatcccttccctgggcccatccct
BCN opt aacagaagacgaattacaggataaaatccacccctttgcgcagacccagtcgctagtctacccgttccctgggccgataccg

.....

        247                                                                  328
BCN     aacagcctcccacaaaacatccctcctcttactcaaacccctgtggtggtgccgcctttccttcagcctgaagtactgggag
BCN opt aacagcttaccacaaaacatcccgccgcttaccaaacccctgtggtggtgccgccgttcttgcagccggaagtaatgggcg

.....

        329                                                                  410
BCN     tctccaaagtgaaggaggctatggctcctaagcacaaagaaatgccctttcctaaatatccagttgagccctttactgaaag
BCN opt tctccaaagtgaaggaggctatggccctaaacataaagaaatgcccttcctaaatatccagttgaaccctttaccgaaag

.....

        411                                                                  492
BCN     ccagagcttgactttgactgatgttgaaaatcttcatttgcctcctctcttgctccagtcttggatgcatcagcctcaccag
BCN opt tcagagcctgactttaacggatgttgaaaatctgcacttgccgctgccgctgttgcagtcttggatgcatcagccgcatcag

.....

        493                                                                  574
BCN     cctcttcctccaactgtcatgtttcctcctcagtccgtgctgtccctttctcagtccaaagtcctgcctgttccccagaaag
BCN opt ccgttaccgccaacggttatgtttccgcctcagtccgtgctgtccctgtctcagagtaaagtcctgccggttccacagaaag

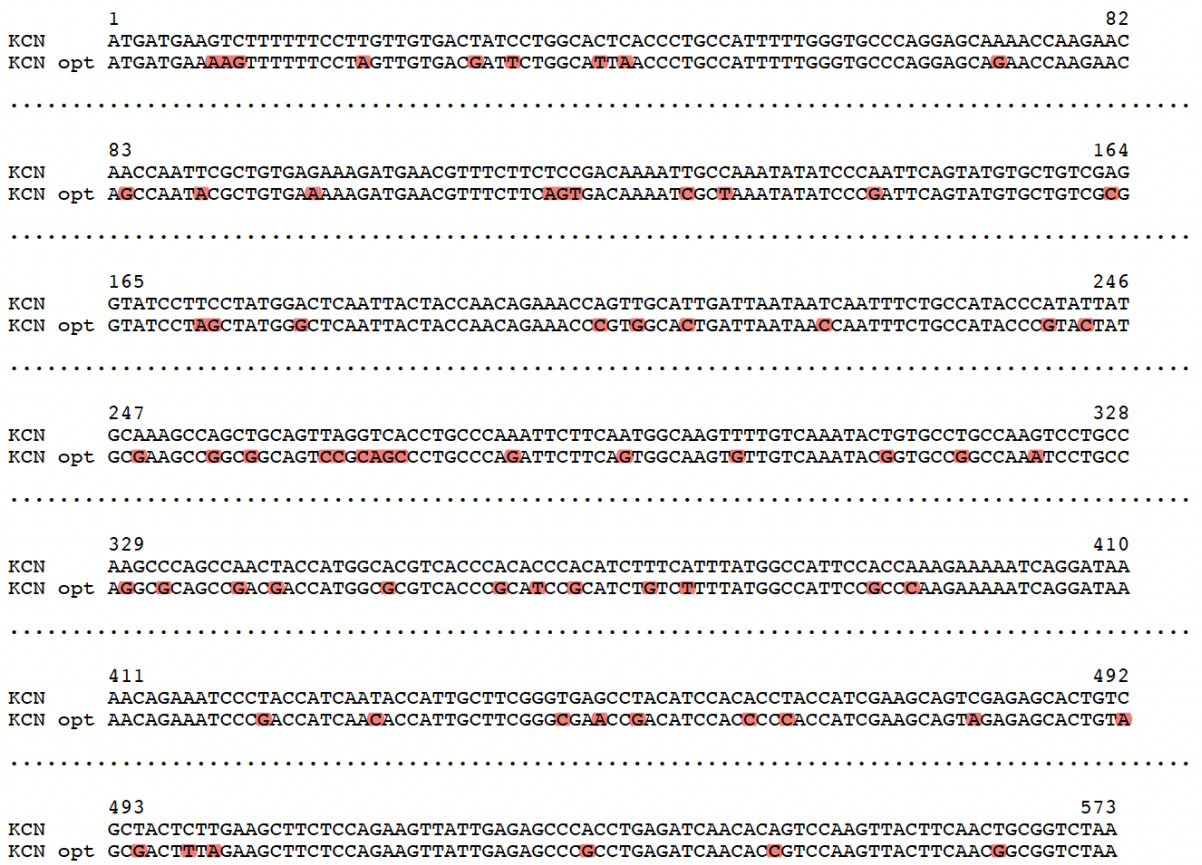
.....

        575                                                                  656
BCN     cagtgccctatccccagagagatatgcccattcaggcctttctgctgtaccagcagcctgtactcggtcctgtccggggacc
BCN opt cagttccgtatccacaacggggatatgccaattcaggcatttctgctgtaccaggagccggtactcggtcctgtggcgccggtcc

.....

        657             675
BCN     tttccctattattgtctaa
BCN opt cttcccaattattgtctaa
```

b)



Supplementary Figure 5.1: Nucleotide alignment of the native bovine casein open reading frames with the codon-optimised versions for *E. coli.* a) Bovine beta-casein A2 allele open reading frame (top), aligned with the *E. coli* codon optimised version (bottom). Nucleotide changes are highlighted in red. **b)** Bovine kappa-casein B allele open reading frame (top), aligned with the *E. coli* codon optimised version (bottom). Nucleotide changes are highlighted in red.

a)

```
        1                                                                                82
BCN     MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIP
BCN opt MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIP

.....................................................................................

        83                                                                              164
BCN     NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQ
BCN opt NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQ

.....................................................................................

        165                                                   224
BCN     PLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
BCN opt PLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

.....................................................................................
```

b)

```
        1                                                                               82
KCN     MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYY
KCN opt MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYY

.....................................................................................

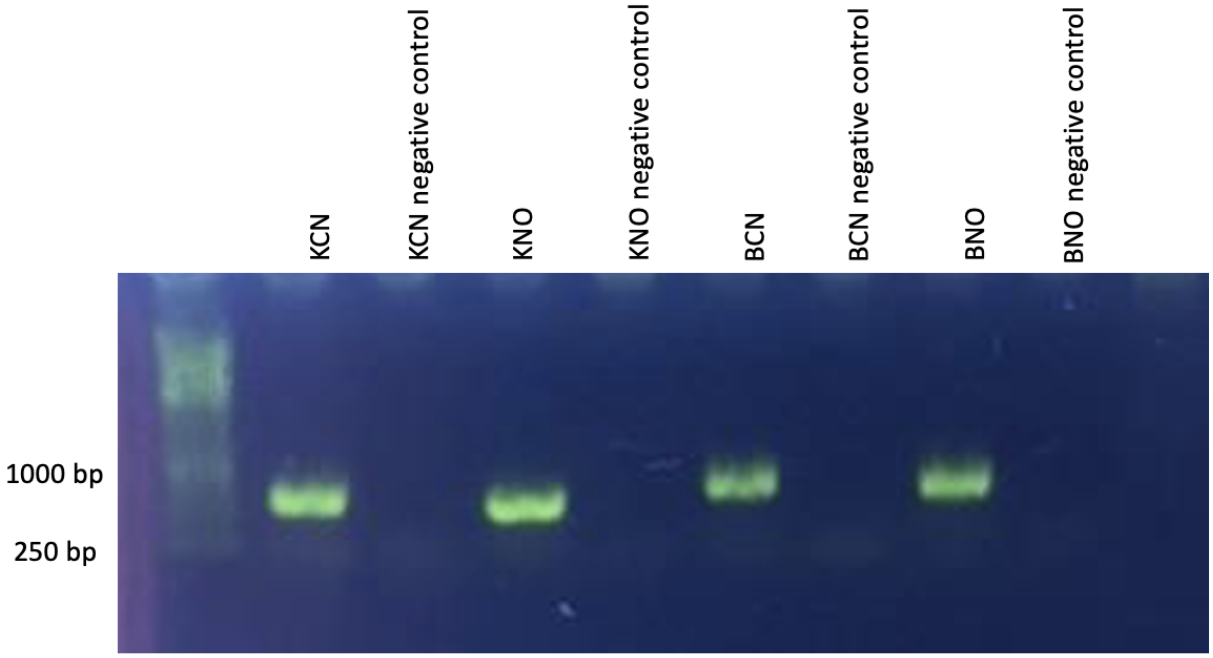
        83                                                                              164
KCN     AKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTV
KCN opt AKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTV

.....................................................................................

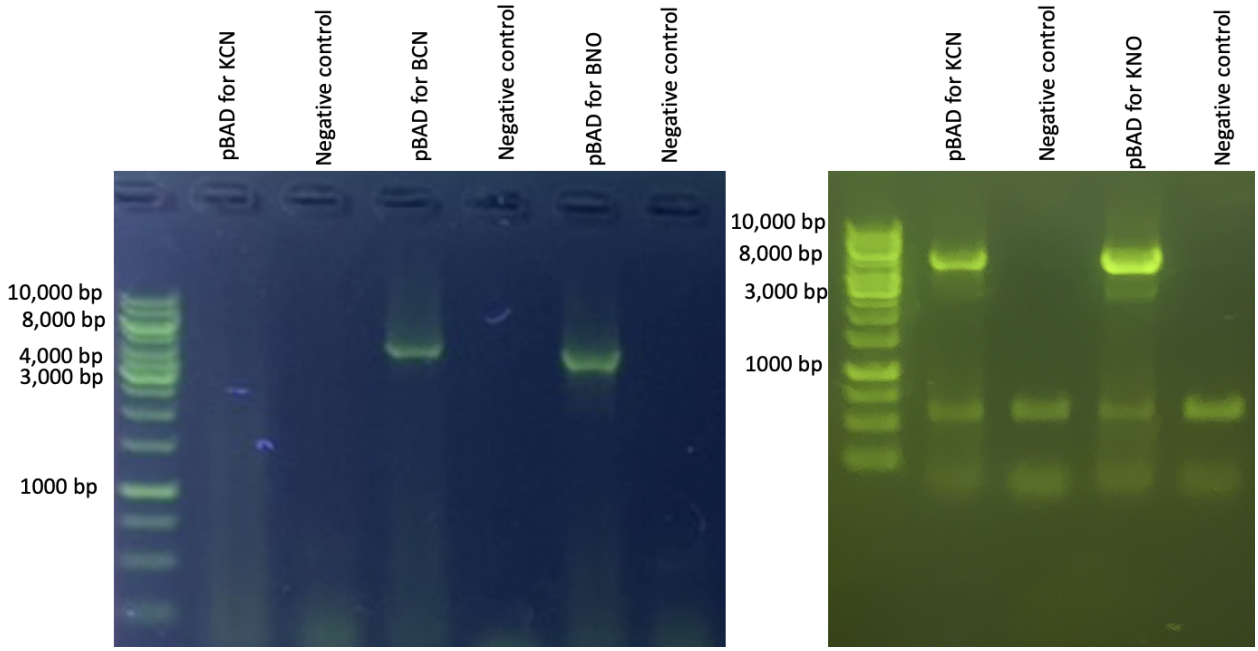
        165                   190
KCN     ATLEASPEVIESPPEINTVQVTSTAV
KCN opt ATLEASPEVIESPPEINTVQVTSTAV

.....................................................................................
```

Supplementary Figure 5.2: Alignment of casein amino acid sequences for expression in *E. coli*. a) Native bovine amino acid sequence for the beta-casein A2 allele (top), aligned with the translated codon optimised amino acid sequence for expression in *E. coli* (bottom). No amino acid changes are seen. **b)** Native bovine amino acid sequence for the kappa-casein B allele (top), aligned with the translated codon-optimised amino acid sequence for expression in *E. coli* (bottom). No amino acid changes are seen.



Supplementary Figure 5.3: Confirmation of successful PCR amplification of casein inserts for expression in *E. coli*. Results of PCR reactions run on a 1% agarose gel. All four inserts were amplified and are the correct size.



Supplementary Figure 5.4: Confirmation of successful PCR of pBAD plasmid for assembly with casein inserts. Results of PCR reactions run on a 1% agarose gel. Amplification of pBAD for BCN was successful, resulting in a PCR product of approximately the correct size (~4100bp). The PCR product of pBAD for the BNO insert appeared slightly smaller. The initial PCR of pBAD for KCN yielded no result so this was repeated. The results of amplification of pBAD for KNO and KCN inserts appeared to be about 6000bp (~1900bp too large.) Additionally, all lanes, including the negative controls, gave bands of approximately 500bp.

Supplementary Table 5.1: ImageJ analysis of SDS-PAGE gel band intensity from proteins expressed by *E. coli* transformed with pEM08E (Fig. 3.3)

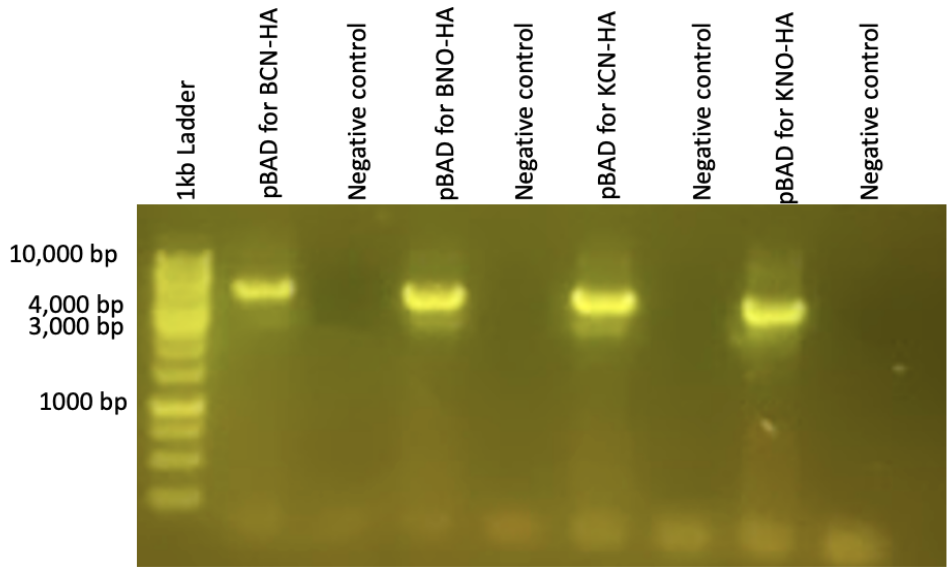
Lane	Area under the graph
pBAD only	112.263
BL21 only - no plasmid	66.364
BL21 only - no plasmids	84.435
BCN, colony 1 - no induction (pellet)	1313.134
BCN, colony 1 - no induction (pellet)	969.184
BCN, colony 1 - induction (pellet)	2211.004
BCN, colony 1 - induction (pellet)	1728.447
BCN, colony 1 - induction (supernatant)	0

ImageJ software was used to calculate the intensity of the bands in the location where expression of the beta-casein protein was expected to be seen. The area under the curve correlates to the intensity of the band. A zero indicates no intensity detected at the expected location and, thus, no resultant curve.

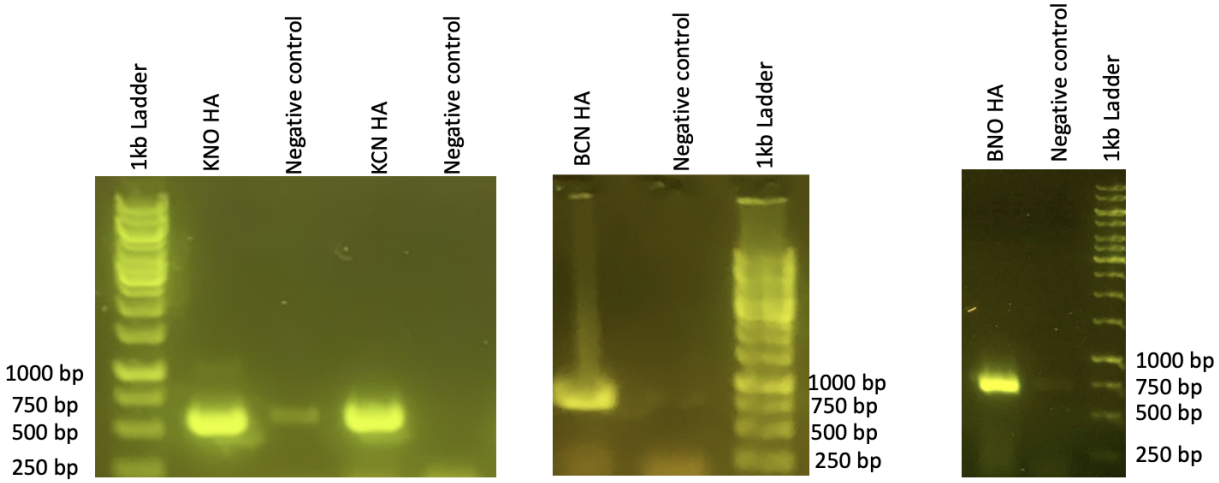
Supplementary Table 5.2: ImageJ analysis of SDS-PAGE gel band intensity from proteins expressed by *E. coli* transformed with pEM08E and pEM07E (Fig. 3.4b)

Lane	Area under the curve
BNO - 5 hours	927.255
BCN - 5 hours	66.021
pBAD - hours	0
BNO - 6 hours	251.678
BCN - 6 hours	0
pBAD - 6 hours	0
BNO - 20 hours	0
BCN - 20 hours	0
pBAD - 20 hours	0

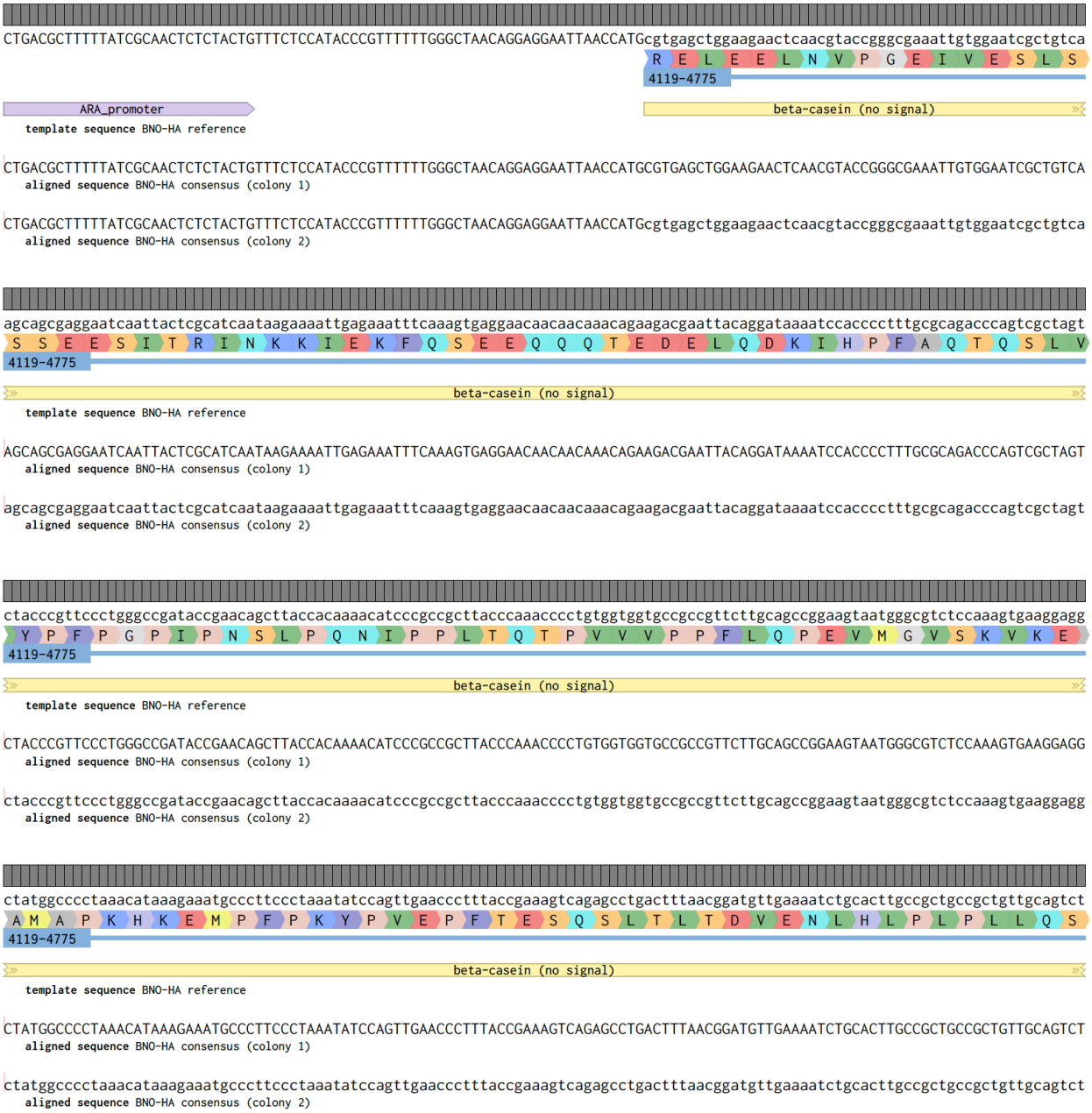
ImageJ software was used to calculate the intensity of the bands in the location where the beta-casein protein was expected. The area under the curve correlates to the intensity of the band. A zero indicates no intensity detected at the expected location and, thus, no resultant curve.



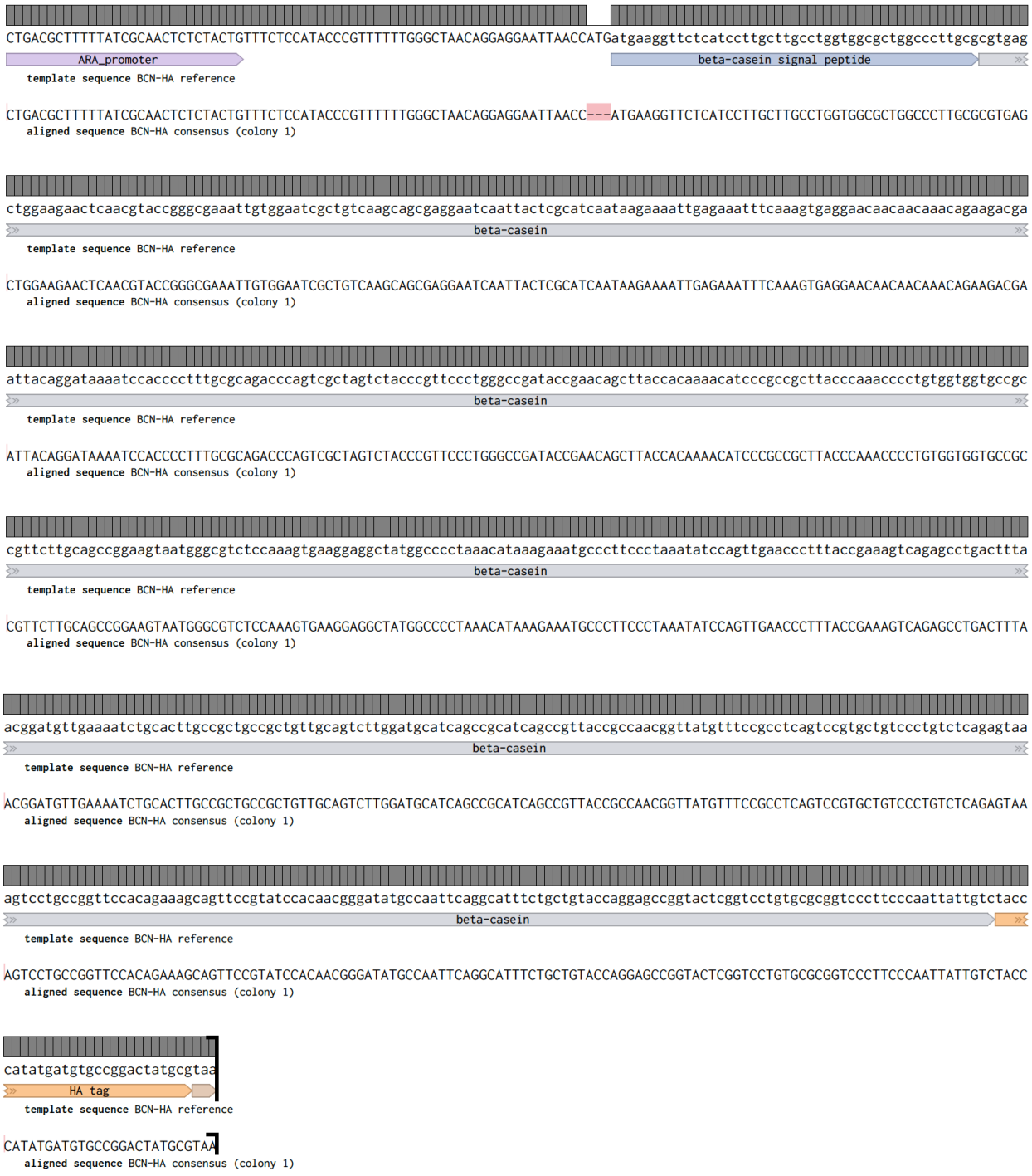
Supplementary Figure 5.5: Confirmation of successful PCR amplification of pBAD plasmid for assembly with HA-tagged casein inserts. Results of PCR reactions run on a 1% agarose gel. Successful amplification of pBAD plasmid with complimentary overlaps for each of the four HA-tagged casein inserts.



Supplementary Figure 5.6: Confirmation of successful amplification of gene inserts with the addition of an HA tag. Results of PCR run on a 1% agarose gel. Successful amplification of all inserts was seen. There was a faint band in the negative control lane for KCN-HA, which was likely a result of contamination during loading. The rest of the PCR products returned no bands in the negative control lanes. All bands are of the expected size.


CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGcgtgagctggaagaactcaacgtaccgggcgaaattgtggaatcgctgtca
R E L E E L N V P G E I V E S L S
4119-4775
ARA_promoter
beta-casein (no signal)
template sequence BNO-HA reference
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGCGTGAGCTGGAAGAACTCAACGTACCGGGCGAAATTGTGGAATCGCTGTCA
aligned sequence BNO-HA consensus (colony 1)
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGcgtgagctggaagaactcaacgtaccgggcgaaattgtggaatcgctgtca
aligned sequence BNO-HA consensus (colony 2)
agcagcgaggaatcaattactcgcatcaataagaaaattgagaaatttcaaagtgaggaacaacaacaaacagaagacgaattacaggataaaatccacccctttgcgcagacccagtcgctagt
S S E E S I T R I N K K I E K F Q S E E Q Q Q T E D E L Q D K I H P F A Q T Q S L V
4119-4775
beta-casein (no signal)
template sequence BNO-HA reference
AGCAGCGAGGAATCAATTACTCGCATCAATAAGAAAATTGAGAAATTTCAAAGTGAGGAACAACAACAAACAGAAGACGAATTACAGGATAAAATCCACCCCTTTGCGCAGACCCAGTCGCTAGT
aligned sequence BNO-HA consensus (colony 1)
agcagcgaggaatcaattactcgcatcaataagaaaattgagaaatttcaaagtgaggaacaacaacaaacagaagacgaattacaggataaaatccacccctttgcgcagacccagtcgctagt
aligned sequence BNO-HA consensus (colony 2)
ctacccgttccctgggccgataccgaacagcttaccacaaaacatcccgccgcttacccaaaccccctgtggtggtgccgccgttcttgcagccggaagtaatgggcgtctccaaagtgaaggagg
Y P F P G P I P N S L P Q N I P P L T Q T P V V V P P F L Q P E V M G V S K V K E
4119-4775
beta-casein (no signal)
template sequence BNO-HA reference
CTACCCGTTCCCTGGGCCGATACCGAACAGCTTACCACAAAACATCCCGCCGCTTACCCAAACCCCTGTGGTGGTGCCGCCGTTCTTGCAGCCGGAAGTAATGGGCGTCTCCAAAGTGAAGGAGG
aligned sequence BNO-HA consensus (colony 1)
ctacccgttccctgggccgataccgaacagcttaccacaaaacatcccgccgcttacccaaaccccctgtggtggtgccgccgttcttgcagccggaagtaatgggcgtctccaaagtgaaggagg
aligned sequence BNO-HA consensus (colony 2)
ctatggcccctaaacataaagaaatgcccttccctaaatatccagttgaaccctttaccgaaagtcagagcctgactttaacggatgttgaaaatctgcacttgccgctgccgctgttgcagtct
A M A P K H K E M P F P K Y P V E P F T E S Q S L T L T D V E N L H L P L P L L Q S
4119-4775
beta-casein (no signal)
template sequence BNO-HA reference
CTATGGCCCCTAAACATAAAGAAATGCCCTTCCCTAAATATCCAGTTGAACCCTTTACCGAAAGTCAGAGCCTGACTTTAACGGATGTTGAAAATCTGCACTTGCCGCTGCCGCTGTTGCAGTCT
aligned sequence BNO-HA consensus (colony 1)
ctatggcccctaaacataaagaaatgcccttccctaaatatccagttgaaccctttaccgaaagtcagagcctgactttaacggatgttgaaaatctgcacttgccgctgccgctgttgcagtct
aligned sequence BNO-HA consensus (colony 2)

Supplementary Figure 5.7: Sequencing results of pEM11E and alignment with reference BNO-HA insert. Reference sequence for BNO-HA insert (top) was aligned with the results of Oxford Nanopore sequencing of the pEM11E plasmid extracted from two colonies. The section shown includes the ARA promoter and BNO-HA insert. No base changes are seen in this region.



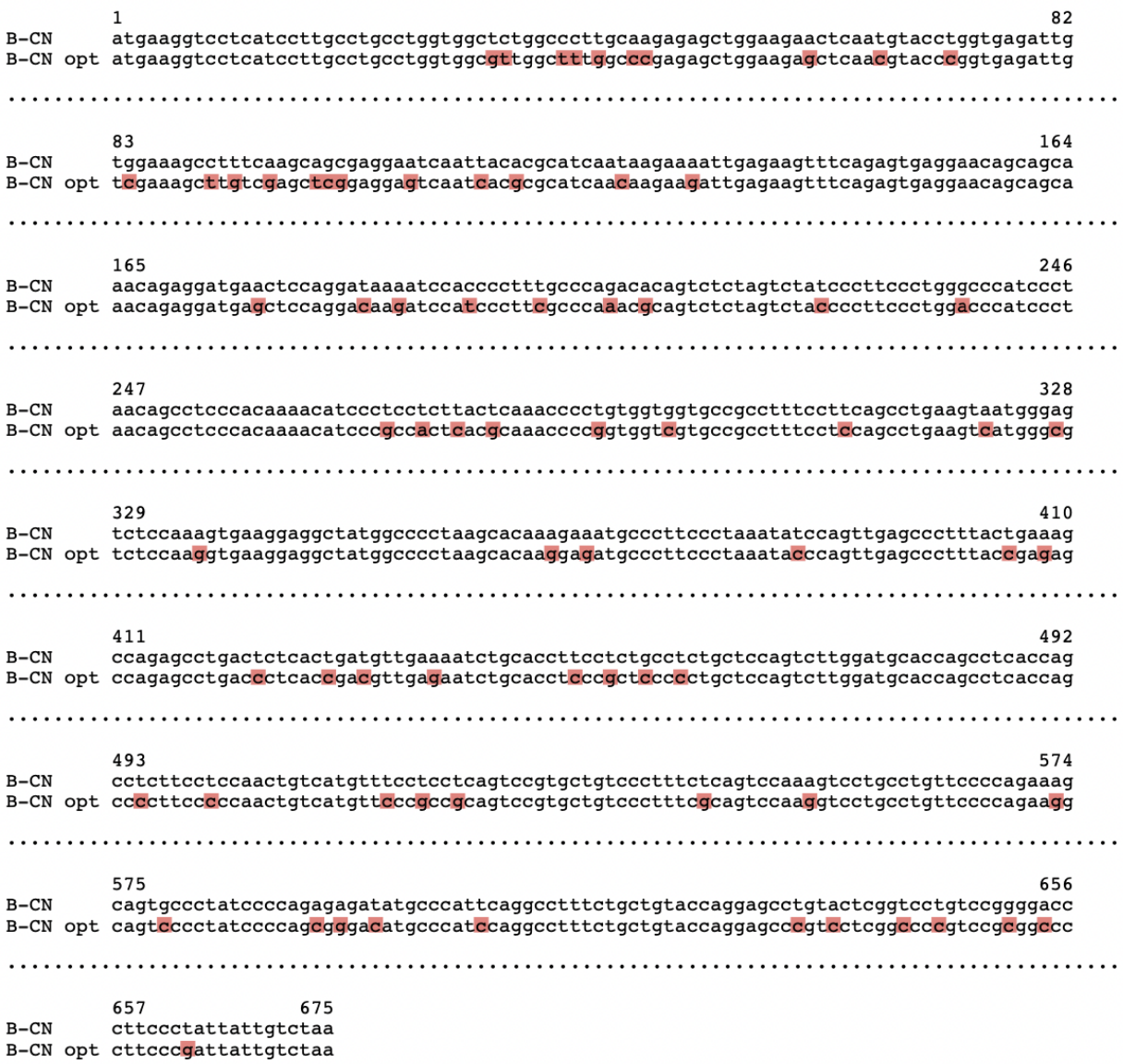
Supplementary Figure 5.8: Sequencing results of pEM12E and alignment with reference BCN-HA insert. The reference sequence for the BCN-HA insert (top) was aligned with the Oxford Nanopore sequencing results of the pEM12E plasmid extracted from a single transformed colony. The section shown includes the ARA promoter and BCN-HA insert. A deletion of a methionine residue is observed upstream of the insert. Given that a second methionine follows, translation initiation should not be impacted. There are no other base changes observed within this region.


CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATG------CAGGAGCAGAACCAAGAACAGCCAATACGCTGTG-AAAAAGATGA
Q E Q N Q E Q P I R C E K D E
2499-3153
ARA_promoter
Kappa-casein
template sequence KNO-HA reference
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGGGTGCCCAGGAGCAGAACCAAGAACAGCCAATACGCTGTGAAAAAAGATGA
aligned sequence KNO-HA consensus (colony 1)
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGGGTGCCCAGGAGCAGAACCAAGAACAGCCAATACGCTGTG-AAAAAGATGA
aligned sequence KNO-HA consensus (colony 2)
ACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCACTGATTAATAACCAATTTCTGC
R F F S D K I A K Y I P I Q Y V L S R Y P S Y G L N Y Y Q Q K P V A L I N N Q F L
2499-3153
Kappa-casein
template sequence KNO-HA reference
ACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCACTGATTAATAACCAATTTCTGC
aligned sequence KNO-HA consensus (colony 1)
ACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCACTGATTAATAACCAATTTCTGC
aligned sequence KNO-HA consensus (colony 2)
CATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGCGTCAC
P Y P Y Y A K P A A V R S P A Q I L Q W Q V L S N T V P A K S C Q A Q P T T M A R H
2499-3153
Kappa-casein
template sequence KNO-HA reference
CATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGCGTCAC
aligned sequence KNO-HA consensus (colony 1)
CATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGCGTCAC
aligned sequence KNO-HA consensus (colony 2)
CCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCA-CCCCCACCATCGAAGCAGTAG
P H P H L S F M A I P P K K N Q D K T E I P T I N T I A S G E P T S T P T I E A V
2499-3153
Kappa-casein
template sequence KNO-HA reference
CCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCACCCCCCACCATCGAAGCAGTAG
aligned sequence KNO-HA consensus (colony 1)
CCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCACCCCCCACCATCGAAGCAGTAG
aligned sequence KNO-HA consensus (colony 2)
AGAGCACTGTAGCGACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
E S T V A T L E A S P E V I E S P P E I N T V Q V T S T A V Y P Y D V P D Y A *
2499-3153
Kappa-casein
HA tag
template sequence KNO-HA reference
AGAGCACTGTAGCGACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
aligned sequence KNO-HA consensus (colony 1)
AGAGCACTGTAGCGACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
aligned sequence KNO-HA consensus (colony 2)

Supplementary Figure 5.9: Sequencing results of pEM13E and alignment with reference KNO-HA insert. The reference sequence for KNO-HA insert (top) was aligned with the Oxford Nanopore sequencing results of the pEM13E plasmid extracted from two colonies. The section shown includes the ARA promoter and KNO-HA insert. Both plasmids sequenced have an insertion of two residues, a glycine and an alanine. This is due to incorrect primer design resulting in the last two amino acids of the signal sequence being retained upstream of the intended insert. Colony 1 also has an insertion of an adenine base upstream of a series of adenines, and both colonies have an insertion of a cytosine base upstream of a series of cytosines (highlighted). The insertions are likely a sequencing error resulting from the repeated base pairs.


CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGATGATGAAAAGTTTTTTCCTAGTTGTGACGATTCTGGCATTAACCCTGCCATTTTTGGGTGCCCAGGA
M M K S F F L V V T I L A L T L P F L G A Q E
2497-3751
ARA_promoter
signal sequence
Ka...n
template sequence KCN-HA reference
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGATGATGAAAAGTTTTTTCCTAGTTGTGACGATTCTGGCATTAACCCTGCCATTTTTGGGTGCCCAGGA
aligned sequence KCN-HA consensus (colony 1)
CTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACC---ATGATGAAAAGTTTTTTCCTAGTTGTGACGATTCTGGCATTGACCCTGCCATTTTTGGGTGCCCAGGA
aligned sequence KCN-HA consensus (colony 1)
GCAGAACCAAGAACAGCCAATACGCTGTGAAAAAGATGAACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCA
Q N Q E Q P I R C E K D E R F F S D K I A K Y I P I Q Y V L S R Y P S Y G L N Y Y Q Q K P V A
2497-3751
Kappa-casein
Kappa-casein
template sequence KCN-HA reference
GCAGAACCAAGAACAGCCAATACGCTGTGAAAAAGATGAACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCA
aligned sequence KCN-HA consensus (colony 1)
GCAGAACCAAGAACAGCCAATACGCTGTGAAAAAGATGAACGTTTCTTCAGTGACAAAATCGCTAAATATATCCCGATTCAGTATGTGCTGTCGCGGTATCCTAGCTATGGGCTCAATTACTACCAACAGAAACCCGTGGCA
aligned sequence KCN-HA consensus (colony 1)
CTGATTAATAACCAATTTCTGCCATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGC
L I N N Q F L P Y P Y Y A K P A A V R S P A Q I L Q W Q V L S N T V P A K S C Q A Q P T T M A
2497-3751
Kappa-casein
template sequence KCN-HA reference
CTGATTAATAACCAATTTCTGCCATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGC
aligned sequence KCN-HA consensus (colony 1)
CTGATTAATAACCAATTTCTGCCATACCCGTACTATGCGAAGCCGGCGGCAGTCCGCAGCCCTGCCCAGATTCTTCAGTGGCAAGTGTTGTCAAATACGGTGCCGGCCAAATCCTGCCAGGCGCAGCCGACGACCATGGCGC
aligned sequence KCN-HA consensus (colony 1)
GTCACCCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCACCCCCACCATCGAAGCAGTAGAGAGCACTGTAGC
R H P H P H L S F M A I P P K K N Q D K T E I P T I N T I A S G E P T S T P T I E A V E S T V A
2497-3751
Kappa-casein
template sequence KCN-HA reference
GTCACCCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCACCCCCACCATCGAAGCAGTAGAGAGCACTGTAGC
aligned sequence KCN-HA consensus (colony 1)
GTCACCCGCATCCGCATCTGTCTTTTATGGCCATTCCGCCCAAGAAAAATCAGGATAAAACAGAAATCCCGACCATCAACACCATTGCTTCGGGCGAACCGACATCCACCCCCACCATCGAAGCAGTAGAGAGCACTGTAGC
aligned sequence KCN-HA consensus (colony 1)
GACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
T L E A S P E V I E S P P E I N T V Q V T S T A V Y P Y D V P D Y A *
2497-3751
Kappa-casein
HA tag
template sequence KCN-HA reference
GACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
aligned sequence KCN-HA consensus (colony 1)
GACTTTAGAAGCTTCTCCAGAAGTTATTGAGAGCCCGCCTGAGATCAACACCGTCCAAGTTACTTCAACGGCGGTCTACCCATACGATGTTCCAGATTACGCTTAA
aligned sequence KCN-HA consensus (colony 1)

Supplementary Figure 5.10: Sequencing results of pEM14E and alignment with reference KCN-HA insert. The reference for the KCN-HA insert (top) was aligned with the Oxford Nanopore sequencing results of the pEM14E plasmid from two colonies. The section shown includes the ARA promoter and KNO-HA insert. Base changes are highlighted in red. The second colony containing pEM14E, shows a deletion of a methionine residue upstream of the casein insert. As another methionine follows, this should not impact the initiation of translation. The second colony also has a base change occurring from A to G within the signal sequence (highlighted).



Supplementary Figure 5.11: Nucleotide alignment of beta-casein A2 allele with the codon optimised version for *T. reesei*. The native bovine beta-casein A2 allele open reading frame (top) was aligned with *T. reesei* codon optimised version to observe base changes. Highlighted bases indicate nucleotide changes.

```
        1                                                                                82
BCN     MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIP
BCN opt MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIP

.....................................................................................

        83                                                                              164
BCN     NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQ
BCN opt NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQ

.....................................................................................

        165                                                    224
BCN     PLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
BCN opt PLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

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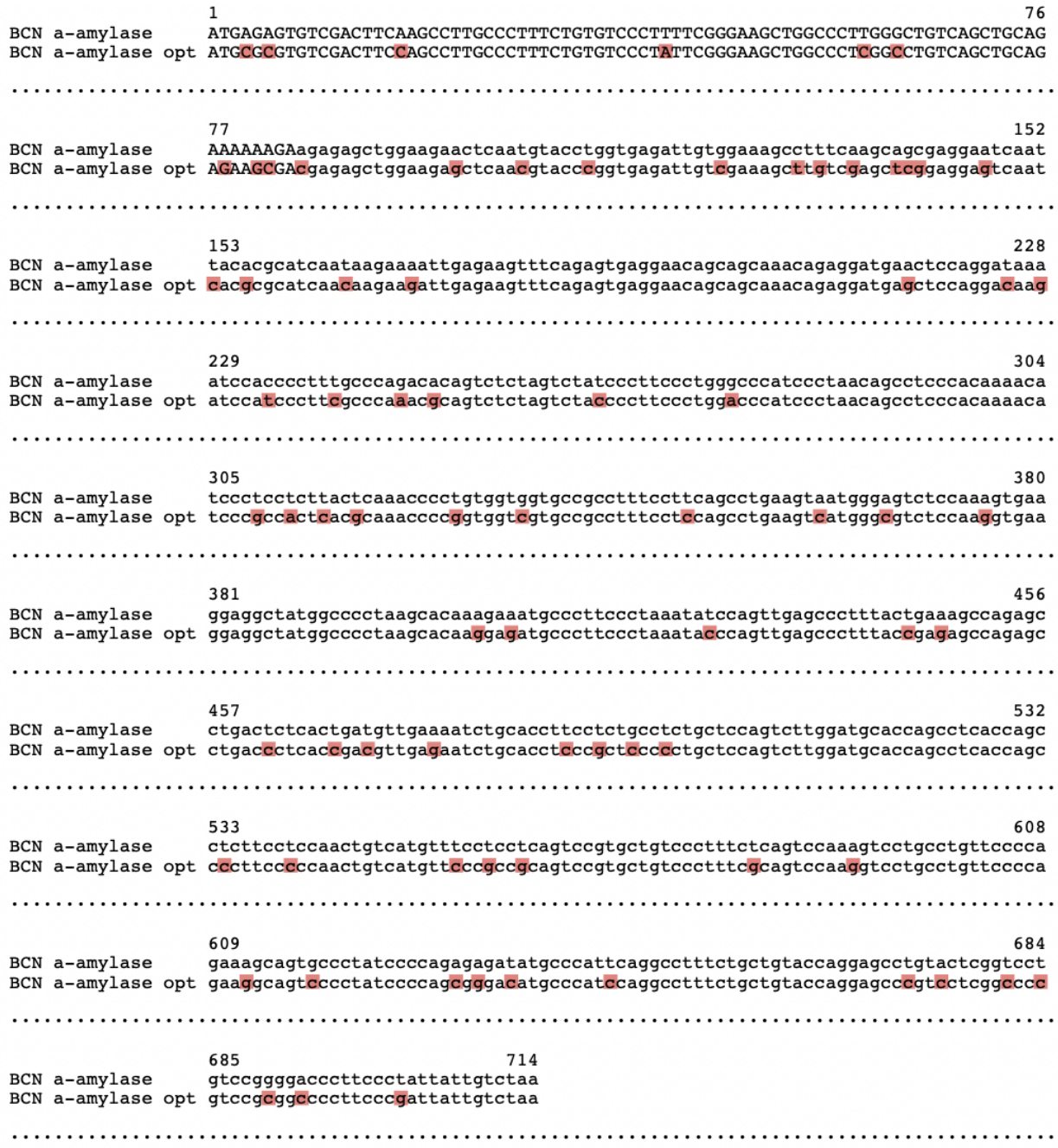
Supplementary Figure 5.12: Alignment of beta-casein A2 allele with alpha-amylase signal amino acid sequences for expression in *T. reesei*. The native bovine beta-casein A2 allele with alpha-amylase signal amino acid sequence (top) was aligned with the translated codon optimised version for expression in *T. reesei*. Highlighted bases indicate nucleotide changes of which there are none.



Supplementary Figure 5.13: Nucleotide alignment of beta-casein A2 allele and alpha-amylase signal, with the codon optimised version for *T. reesei*. Beta-casein A2 allele with the alpha amylase signal and kex cleavage site (capitalised) in replacement of the native secretion signal (top) with the codon optimised version for *T. reesei*. Highlighted bases indicate nucleotide changes.

```
                      1                                                                          72
BCN a-amylase, kex2,   MRVSTSSLALSVSLFGKLALGLSAAEKRRELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDE
BCN a-amylase kex2 opt MRVSTSSLALSVSLFGKLALGLSAAEKRRELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDE

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                      73                                                                         144
BCN a-amylase, kex2,   LQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPV
BCN a-amylase kex2 opt LQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPV

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                      145                                                                        216
BCN a-amylase, kex2,   EPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQ
BCN a-amylase kex2 opt EPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQ

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                      217               237
BCN a-amylase, kex2,   AFLLYQEPVLGPVRGPFPIIV
BCN a-amylase kex2 opt AFLLYQEPVLGPVRGPFPIIV

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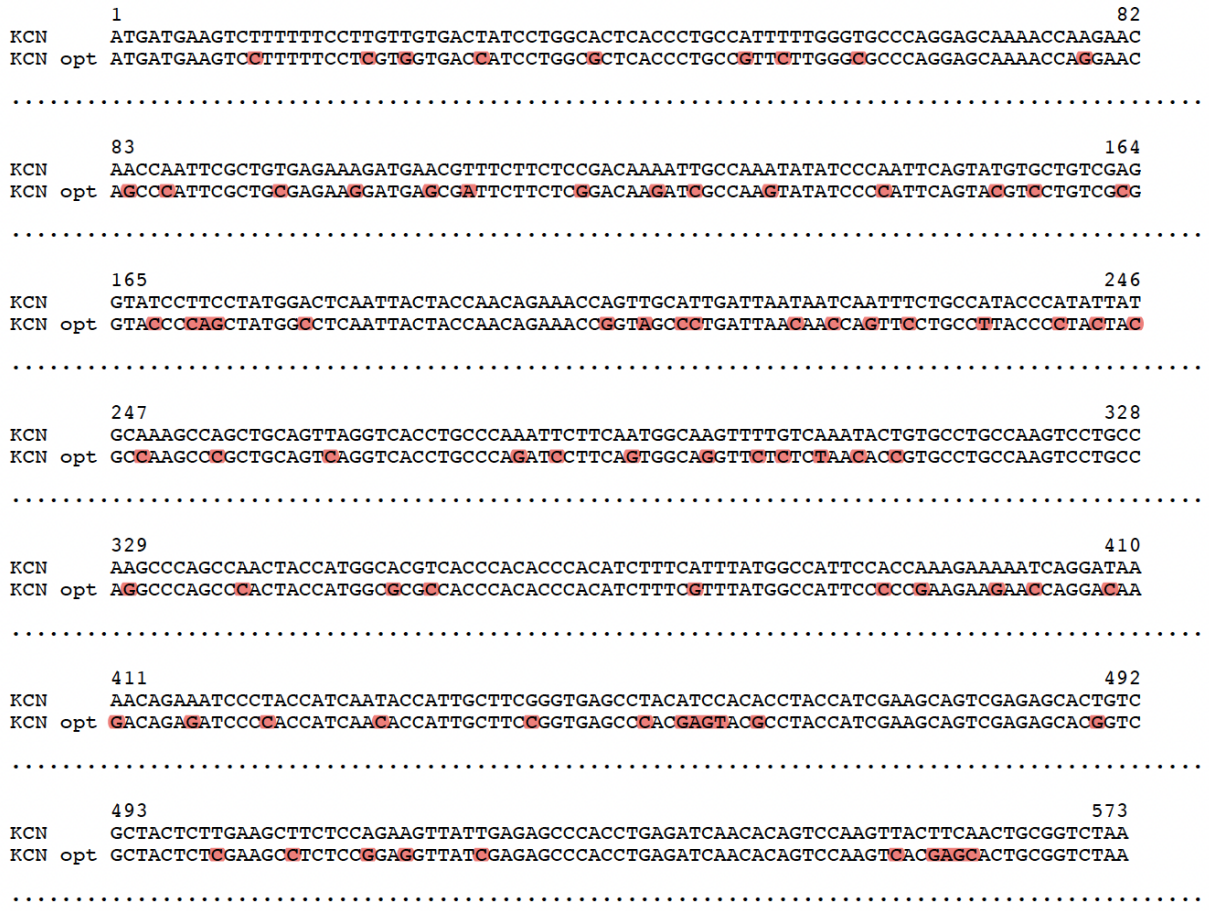
Supplementary Figure 5.14: Alignment of beta-casein A2 allele amino acids with the translated codon optimised version for *T. reesei*. The native bovine beta-casein A2 allele (top) was aligned with the translation of the codon optimised beta-casein sequence for *T. reesei*. Highlighted bases indicate nucleotide changes of which there are none.



Supplementary Figure 5.15: Nucleotide alignment of kappa-casein B allele with the codon optimised version for *T. reesei*. The native bovine kappa-casein B allele nucleotide sequence (top) was aligned with the codon optimised version for *T. reesei*. Highlighted bases indicate nucleotide changes.

```
        1                                                                                82
KCN     MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYY
KCN opt MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYY

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        83                                                                              164
KCN     AKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTV
KCN opt AKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTIEAVESTV

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        165                     190
KCN     ATLEASPEVIESPPEINTVQVTSTAV
KCN opt ATLEASPEVIESPPEINTVQVTSTAV

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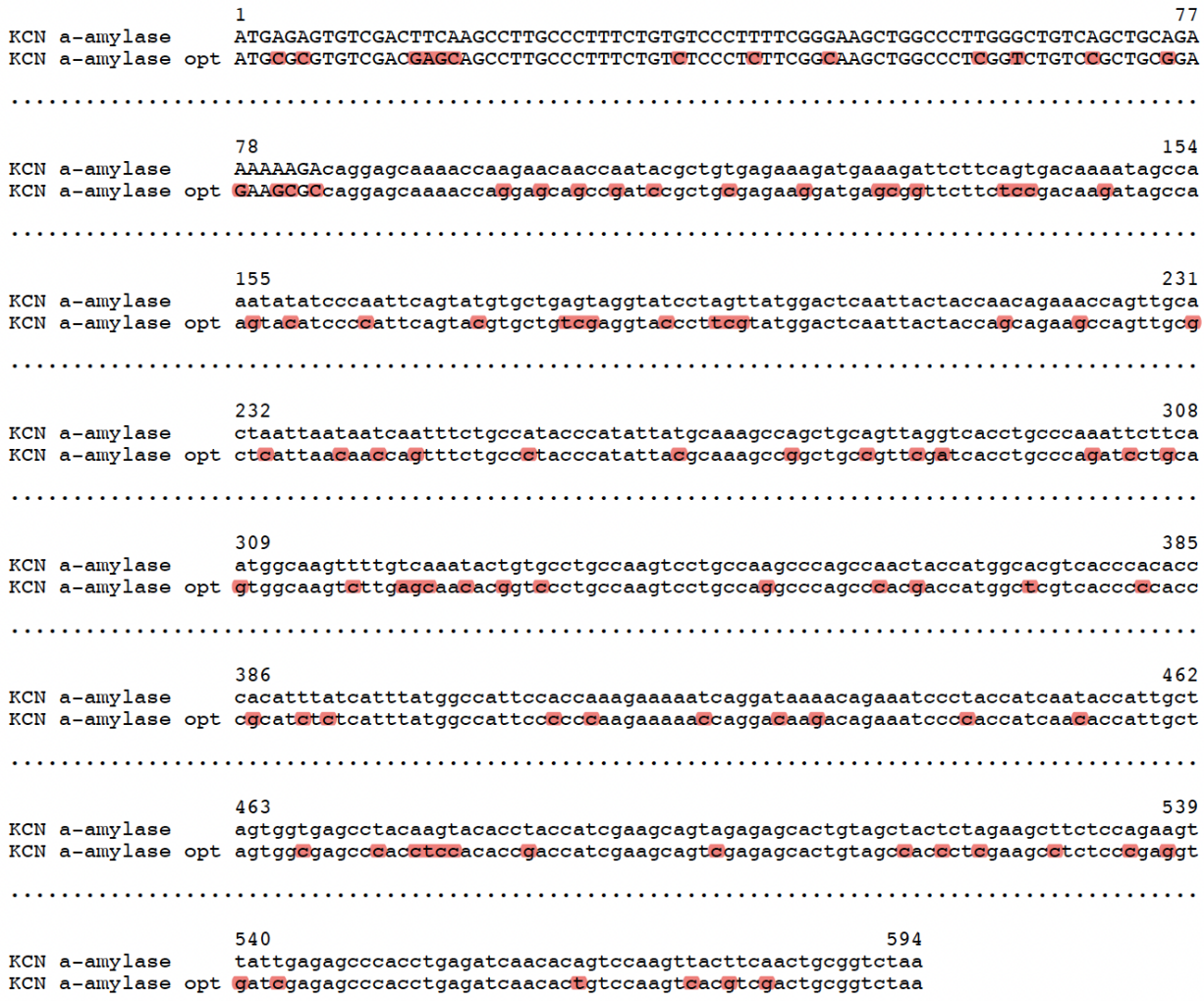
Supplementary Figure 5.16: Alignment of kappa-casein B allele amino acid sequence with the translated codon optimised version for *T. reesei*. The native bovine kappa-casein B allele amino acid sequence (top) was aligned with the translation of the codon optimised kappa-casein sequence for *T. reesei*. Highlighted bases indicate nucleotide changes of which there are none.



Supplementary Figure 5.17: Nucleotide alignment of kappa-casein B allele with alpha-amylase secretion signal and the codon optimised version for *T. reesei*. The native bovine kappa-casein B allele with alpha-amylase secretion signal nucleotide sequence (top) was aligned with the codon optimised version for *T. reesei*. Highlighted bases indicate nucleotide changes.

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                        1                                                                   71
KCN a-amylase, kex2,... MRVSTSSLALSVSLFGKLALGLSAAEKRQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYY
KCN a-amylase kex2 opt  MRVSTSSLALSVSLFGKLALGLSAAEKRQEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYY

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                        72                                                                 142
KCN a-amylase, kex2,... QQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQ
KCN a-amylase kex2 opt  QQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQ

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                        143                                                 197
KCN a-amylase, kex2,... DKTEIPTINTIASGEPTSTPTIEAVESTVATLEASPEVIESPPEINTVQVTSTAV
KCN a-amylase kex2 opt  DKTEIPTINTIASGEPTSTPTIEAVESTVATLEASPEVIESPPEINTVQVTSTAV

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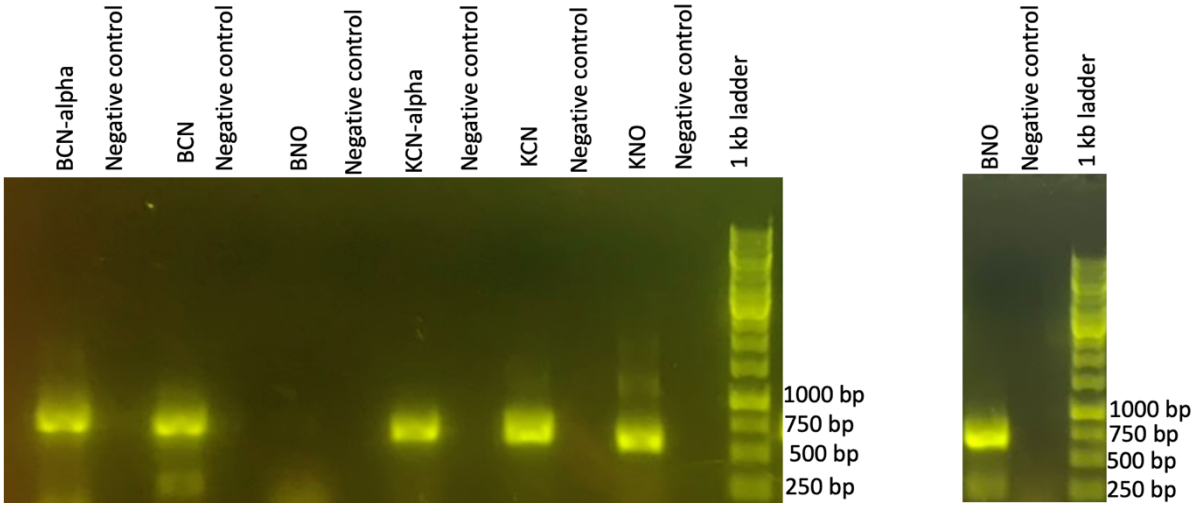
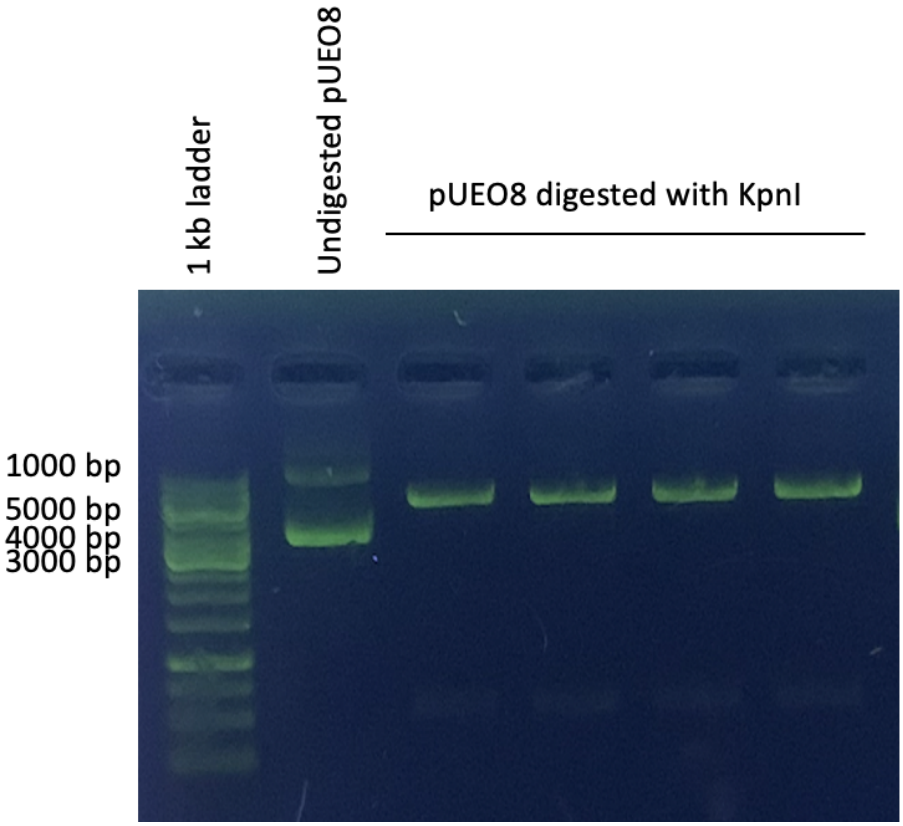
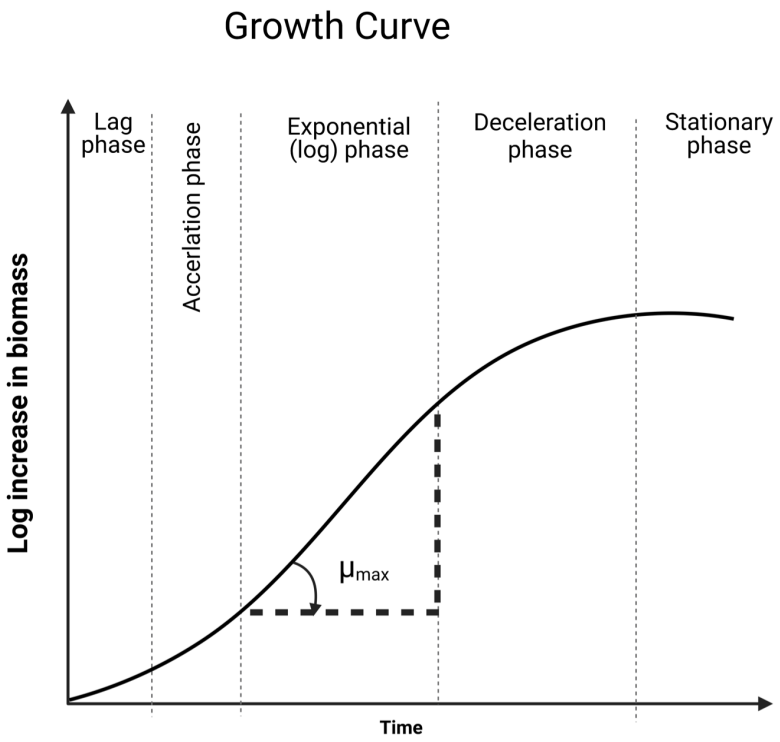
Supplementary Figure 5.18: Alignment of kappa-casein B allele and alpha-amylase signal sequence amino acids with the translated codon optimised version for *T. reesei*. The native bovine kappa-casein B allele with alpha-amylase secretion signal (top) was aligned with the translation of the codon optimised kappa-casein sequence for *T. reesei*. Highlighted bases indicate nucleotide changes of which there are none.



Supplementary Figure 5.19: Confirmation of successful amplification of gene inserts for assembly into pUOE8. Results of pCR run on a 1% agarose gel. Successful amplification of all inserts was seen with no bands in the negative control lanes. The initial PCR amplification of BNO insert was unsuccessful so was repeated with success. All bands are of the expected sizes.



Supplementary Figure 5.20: Confirmation of restriction digest of pUOE8 with KpnI-HF. Results of the restriction digest were run on a 1% agarose gel. The undigested plasmid shows two bands at approximately 10,000 bp and 4,000 bp which are unusual sizes given the intact plasmid is 6112 bp long. The digested plasmid is the correct size at approximately 5500 bp. There are also bands at 500 bp, the size of the fragment removed during the digest. However, these appear incredibly faint and are hard to see in the above photo.



Supplementary Figure 5.21: Expected growth curve for a cell population submerged in liquid culture. Vertical dotted lines indicate the parameters of each growth stage: lag phase, acceleration phase, exponential (log) phase, deceleration phase and stationary phase. Fungi typically follow these same growth kinetics. μ_{max} is indicated as the gradient of the exponential phase and represents the maximum specific growth rate. Graph adapted from (Moore et al., 2020) and made using biorender.com.