TOWARDS CREATING A SUSTAINABLE CELLULAR DAIRY INDUSTRY IN THE FRASER VALLEY

Report in Collaboration with the NSERC-CREATE SynBioApps Program and the University of Fraser Valley

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Introduction

The increasing need for land area due to the rising human population requires agricultural land area to shrink yet keep up with the growing demand of animal-derived products, which presents major challenges in sustainable land use. The traditional dairy sector is also a major contributor of greenhouse gases[1–3], and uses large areas of land[4]. A possible alternative, termed cellular agriculture[5], is the production of animal products such as meat and dairy products in laboratories using recombinant DNA technologies in microbial host organisms.

Cellular dairy, the process of using microorganisms such as yeast, bacteria or fungi to produce milk proteins through fermentation is an emerging industry globally. These milk proteins can be used in powdered form in many food products, or combined further with water, sugar, fats and salts to form an animal free milk equivalent. The milk produced is molecularly identical to natural dairy but eliminates the need for animal agriculture. This has exciting advantages from health, economic and environmental standpoints. A Berkley start-up company, Perfect Day, has been able to commercialize their animal-free milk in North America, both as milk and ice cream. Their US patent details their process of producing casein and whey proteins in the filamentous fungi *Trichoderma reesei*[6]. These cells have been engineered to include the appropriate genes to produce each protein, with consideration taken to produce them in the appropriate relative concentration[6]. However, with economic and environmental factors being driving forces for this product, little consideration is taken for where the feedstock is sourced, or if more environmentally friendly feedstocks can be used.

Forestry is the largest manufacturing sector in British Columbia's economy. Forest feedstock refers to the by-products of any industrial forest process, this includes all parts of the tree and is often used by many other industries (pulp and paper production). B.C Hydro estimates that by 2035 there will be a surplus (up to 400 oven dry tonnes) of feedstock across the province in the form of sawmill waste, roadside residue pulp log[7]. Forestry waste is characterized by being very rich in carbon biomass, nevertheless, this material is complex (due to high quantities of lignin) and fiber-rich which makes it challenging to use in downstream processes. For this reason, transformations such as fermentation, physicochemical and thermochemical processes are needed. Biogas and

biorefineries are the most developed industries using forestry as feedstock, including smaller industries such as enzymes, surfactants, and plastics, among others[8].

Agricultural waste is defined as the non-product plant matter associated with growing fruits, vegetables, grains, and other food crops. It is non-usable in an edibility sense but can be utilized for other uses such as conversion to biochar, compost, supplementation in animal feed, and direct combustion[9]. The Fraser Valley region of BC is the largest agricultural region in the province, boasting numerous field crops (corn, wheat, soybeans, etc.), fruit crops, and vegetable crops[10]. The agricultural waste from this large sector has a potential to become a robust source of sugars for a cellular dairy industry. The berry crops are big players in the BC economy since they are produced for local consumption, export, and processing markets[11]. These include blueberries, cranberries, raspberries, blackberries, and strawberries. Additionally, it's estimated that 95% of the berry production in B.C occurs in the Fraser Valley[11], making this market a possible target to become a source of feedstock, and the crops consistently generate significant amount pruning and unused/perished waste (Table 1).

Type of vegetal waste generated	**Amount of waste generated**	**Reference**
Pruning waste	3000-7500 kg per hectare (Up to ~64000 tones in BC)	[12,13]
Spoiled product	Up to 20% of total production depending on weather events, economy, market, etc. (Up to ~69000 tones in BC 2019)	[14,15]

Table1. Summary of types of waste generated by blueberry crops

Filamentous fungi are known for their ability to degrade lignocellulosic matter[16], are commercially used to produce homologous and more importantly, heterologous proteins[17], and are genetically well-characterized[18]. They are extremely versatile organisms able to grow on a wide variety of substrates, which can be attributed to the different enzymes they produce. Among filamentous fungi, *Aspergillus* spp., *Fusarium* spp., *Trichoderma* spp. are widely used.

We partnered with Robert Newell, Lenore Newman, and Evan Bowness from the University of the Fraser Valley in British Columbia, the authors of an excellent paper that explores agricultural land use, alternative dairy, and sustainability of feedstock sources through a telecoupling lens[5], to investigate the feasibility and sustainability of various possible local sources of feedstock for an emerging cellular dairy industry. We also propose a host organism development strategy that can utilize agricultural waste as feedstock to produce milk proteins.

Methods

Selection of feedstock

Consideration of sustainability is an important aspect behind the rationale of a cellular dairy industry, and hence is an important selection criterion. Growing sugar crops that occupy large amounts of cultivable or habitable land to produce the feedstock for a cellular dairy industry also does not perfectly align with cellular agriculture's land-sparing agenda. Forestry waste is mostly composed of recalcitrant lignin-rich polymers which require extremely harsh pre-treatment steps, which have adverse environmental effects, before being able to be used as a viable feedstock[19]. We propose agricultural waste streams as a possible feedstock for a cellular industry in the Fraser Valley. Berry crops are extremely popular crops in BC which also consistently produce large amounts of waste in terms of prune and spoilage waste (Table 1). The use of such a waste stream for the industry not only provides a sustainable, cleaner source of feedstock, but also helps to reduce environmental pollution that results from agricultural waste disposal.

Selection of the host organism for protein production

The requirement of multi-step processing of agricultural waste before it can be channeled as feed may significantly increase costs and have environmental impacts. Our search for an organism to utilize these waste streams as feedstock was guided by our goal to implement consolidated bioprocessing (CBP) in the production of milk proteins. CBP in this context refers to performing all the biological events required to breakdown the waste matter and utilize the breakdown products to produce our proteins of interest, all by a singular organism[20]. This may significantly reduce costs as the physical process will, in theory, be essentially single step without requiring separate waste processing and fermentation. Although we do not expect to completely forego pretreatment, we expect to

be able to utilize much milder levels of pretreatment by adopting CBP. To that end, we screened for organisms following a few criteria:

i) Ability to breakdown plant biomass

ii) History of being used as host to produce protein products commercially

iii) Resources available for genetic engineering

Within the category of filamentous fungi, multiple stood out as potential hosts to harbour our milk production pathway. Our team chose *Aspergillus niger* as our production organism due to its long history of producing commercial proteins[21] among the other organisms of interest as well as avoiding re-treading work done by Perfect Day in *Trichoderma reesei*.

Degradation of Lignocellulose

The lignocellulose degradation pathway within *Aspergillus niger* is not yet fully characterized, but it is known to be a complicated process assisted by a wide variety of proteins. Chiefly among them are cellulases and hemicellulase that are responsible for breaking apart the cellulose and hemicellulose polymers into their composite sugars. Among these enzymes are a host of assisting proteins including lipases, esterases, surface interacting proteins, carbon/nitrogen metabolism proteins, and transporters[22]. This degradation pathway confers *A. niger* the ability to thrive on a variety of lignocellulose sources including wheat straw[23], rice straw, wheat bran[24], and sugarcane bagasse[25] as sole carbon sources. Research is progressing into the degradation of wood waste by *A. niger* but due to its complex lignin composition, it is not easily degraded and it can take up to 4 months for significant degradation to occur[26]. Due to *A. niger*'s inability to effectively degrade or utilize lignin, we sought to confirm that pruning waste from blueberry bushes in the Fraser Valley would have a lignin composition that would not be inhibitory to the growth of *A. niger*. Pacheco et al. (2018)[27] noted that the composition of Chilean blueberry bush stems and trunks had a similar lignin composition to that of wheat straw, which *A. niger* has been shown to grow on in previous studies. With this in mind, we reasoned that BC blueberry bush trunks and stems would be suitable as a carbon feedstock for our milk-producing *A. niger*.

Strain development

To allow selection with the *pyrG* selectable marker[28], uracil dependent auxotrophs of *A. niger* will be developed as described previously[29]. Briefly, fungal conidia will be grown on media containing 5-fluoroorotic acid (5-FOA) and uracil. Wildtype *A. niger* converts 5-FOA to toxic compounds that kill these cells. However, *pyrG* mutants are able to grow, but are dependent on exogenous uracil.

Construction of a synthetic polycistronic expression cassette

Instead of attempting to produce all the milk proteins, lipids, and carbohydrates in milk in a single host organism, we selected 4 different milk proteins- β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin[30] to incorporate in our synthesis construct, which we think is going to give us a good approximation of the final liquid. To get around the Perfect
Day patent limitations, we selected milk protein sequences from the white-tailed deer (*Odocoileus virginianus*). Complete protein sequences of bovine β-casein (https://www.uniprot.org/uniprot/T1T0C1), κ-casein (https://www.uniprot.org/uniprot/P02668), α-lactalbumin (https://www.uniprot.org/uniprot/B6V3I5), and β-lactoglobulin (https://www.uniprot.org/uniprot/P02754) were obtained from UniProt, and sequences from the white-tailed deer were obtained from NCBI protein blast (blastp). Construction of the expression cassette was done in benchling (https://www.benchling.com/). The protein sequences were linked together using 2A peptides (Figure 1), (Table 2), which allow production of equimolar levels of multiple proteins from polycistronic constructs. The 2A peptides are thought to work via the ribosome skipping synthesis of a peptide bond between the C-terminal glycine and proline residues[31], and were shown to be able to express at least three genes in a polycistron in *A. niger*[31]. To allow excretion of the milk proteins into the medium, 250 bp of glucoamylase A gene from *A. niger* was fused to each of the milk protein genes[32]. The *pyrG* selectable marker[28] sequence was obtained from NCBI. Sequences for the highly active constitutive promoter in *A. niger*, TEF-1[33], and CYC1 terminator were obtained from the pMST-1313 plasmid submitted to addgene (https://www.addgene.org/) by the Michael Sauer lab. For all translations, codon usage was optimized for fungus, constructs were ensured to be in frame, and stop codons were added. All the sequences, constructs, and plasmid files are available on GitHub (https://github.com/Chemotactic/Cellular-Dairy-Final-Project-Milk-Build).

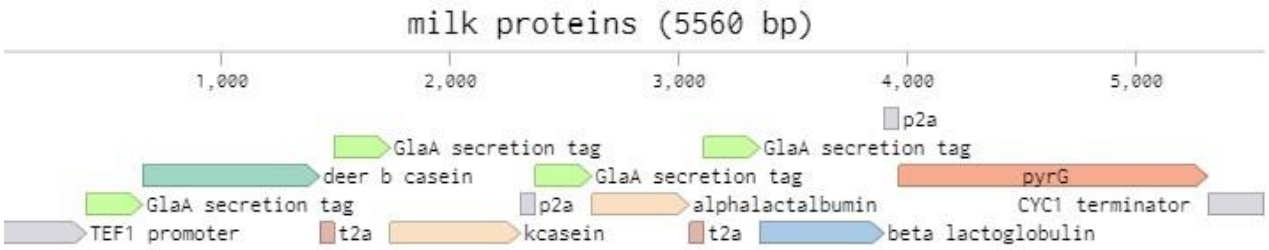

Figure 1: Polycistronic synthetic construct containing the *glaA* secretion tag-fused milk proteins linked together with 2A peptides under the control of the constitutive TEF1 promoter.

Name	**Sequence**	**T_m**
T2A	5'-ggttctggtgaaggtagaggttctttattaacttgtggtgatgttgaagaaaatccaggtcca-3'	
P2A	5'-ggatctggggctaccaacttcagcctcttgaaacaggcaggcgatgtagaggagaaccctggccct-3'	
Fragment-F	5'-ctgcaggtcgactctagaggcacacaccatagcttcaaaatgtttctact-3'	60 ℃
Fragment-R	5'-ccatgattacgaattcgagcgcaaattaaagccttcgagcgt-3'	60 ℃
Vector-F	5'-gctcgaaggctttaatttgcgctcgaattcgtaatcatggtcatagct-3'	60 ℃
Vector-R	5'-ttttgaagctatggtgtgtgcctctagagtcgacctgcag-3'	60 ℃

Table 2: Oligonucleotides used in this project (for *in silico* simulations)

Construction of plasmid for bacterial propagation

The simple pUC18 backbone (https://www.addgene.org/50004/) was selected for propagation in *E. coli*. For convenient restriction site flanks around the fragment, the fragment was inserted into the plasmid via Gibson assembly (Figure 2). Primers were designed to allow linearization of the plasmid leaving *Xba*I and *EcoR*I digestion sites on the ends, and to produce compatible ends on the milk protein producing fragment via PCR (Table 2). The PCR products were then ligated to re-circularize the plasmid with the fragment perfectly flanked by *Xba*I and *EcoR*I sites (Figure 3).

Transformation of A. niger

Transformation of *A. niger* would be performed as described previously[34]. Briefly, siliconized flasks containing minimal medium and L-tryptophan will be inoculated with conidia and shaken at room temperature overnight. Mycelium will be harvested by filtration, washed with 0.6 M $MgSO_4$, and dried. The cells will be suspended in buffered osmotic medium by thorough mixing, and cooled. B-glucuronidase and Novozyme 234 will be

added and incubated in ice for 5 mins. BSA will be added and the cells will be shaken at 30°C for 90 min. The suspension will then be centrifuged to harvest the protoplasts. PEG 4000 and $CaCl_2$ will be added to induce competence and introduce DNA into the protoplasts.

Selection of transformants

Transformants will be plated on minimal media with sorbitol + 1.5% agar[34] lacking uracil. Transformants producing the *pyrG* gene products together with all the milk proteins in the synthetic construct should be selected in the media, since the *pyrG* gene is at the end of the construct without a promoter of its own.

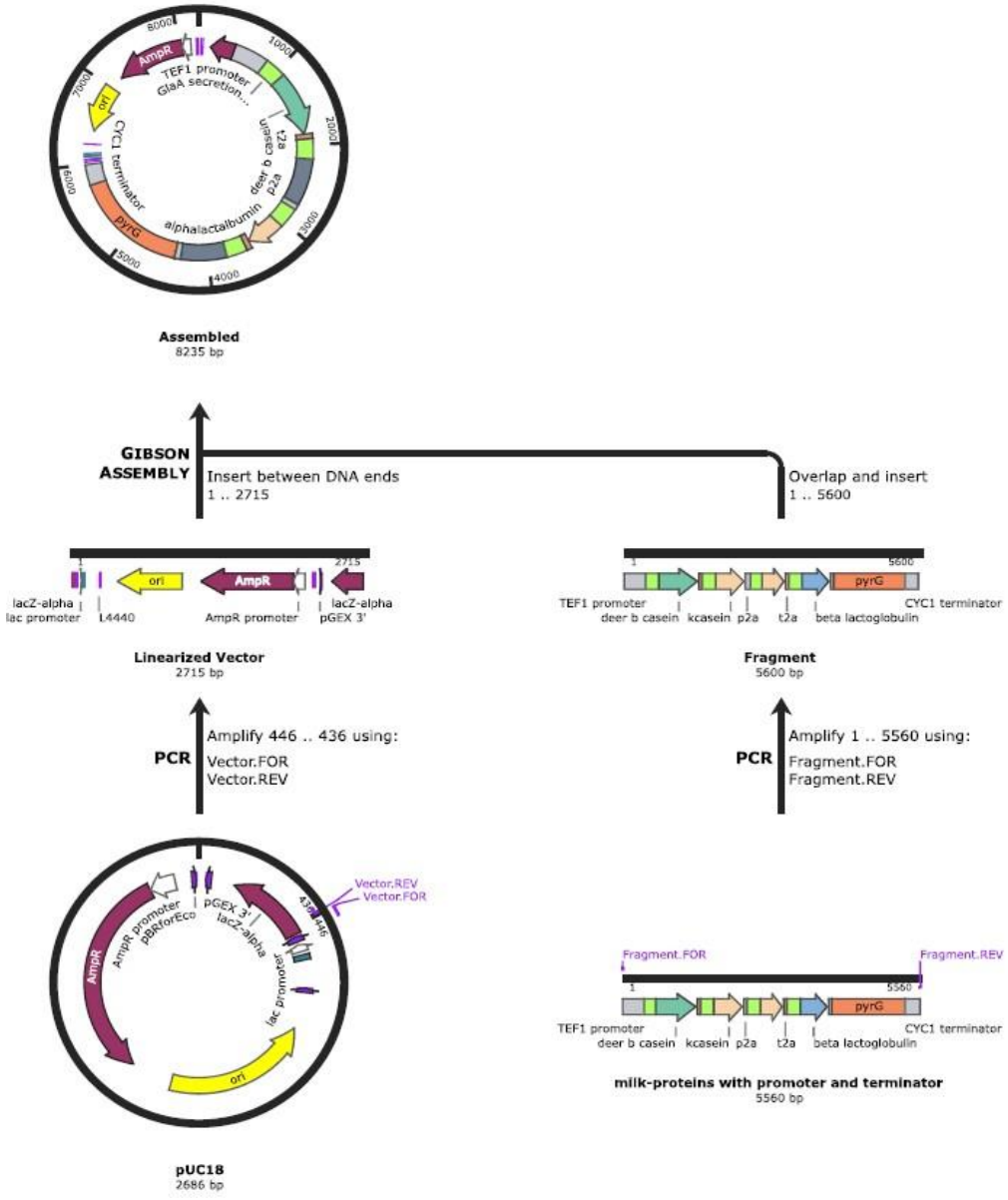

Figure 2: Insertion of the expression cassette into the pUC18 plasmid for bacterial propagation via Gibson assembly

Pretreatment of Feedstock

With *A. niger* engineered, we turned our attention to the production of proteins at large scale. Lignocellulosic biomass is commonly pretreated when used as a sugar source for bioethanol production[35]. This pre-treatment is often necessary for two key reasons. Firstly, it acts as a way to reduce the particle size of the biomass. Secondly, it acts as a way to

destabilize the lignocellulosic complex, separating the components for easier access. Pre-treatment methods can be broadly separated into physical, chemical, physio-chemical, and thermo-chemical processes. Two pre-treatment options that we investigated are ball-milling and steam explosion. Ball-milling is the simpler of two and relies on the physical grinding of biomass to reduce the crystallinity of the biomass and reduce it to a more accessible particle size. Steam explosion is the more robust pretreatment option and is often combined with a physical particle reduction method. In steam explosion, biomass is subjected to steam under high pressure and temperature for seconds to minutes. The pressure is then rapidly released which causes the water inside the biomass to quickly escape. This causes the biomass to explode into fibers and causes hydrolysis of glycosidic and hemicellulose-lignin bonds within the biomass, rendering it more available for enzymatic degradation. While steam explosion has the advantage over ball-milling by "pre-opening" the cellulose and hemicellulose polymers, it has the disadvantage of also releasing inhibitory compounds from the biomass which may interfere with *A. niger* or the secreted milk proteins. As *A. niger* has been shown to grow on lignocellulosic biomass that has only undergone physical degradation, we chose ball-milling as our pretreatment option to forgo the creation of inhibitory molecules[35]. Specifically, we propose a milling time of 120h which was shown to reduce wheat straw to an acceptable particle size for enzymatic degradation[36].

Production of Proteins

After the biomass has been pretreated, fermentation of *A. niger* will occur to produce our four milk proteins. Fermentation of *A. niger* is historically done via Solid-State Fermentation (SSF) or by Submerged Fermentation (SmF)[37]. While SmF allows the secretion of proteins into a liquid media for easier purification and processing, SSF has been shown to allow better growth of filamentous fungi as well as higher production of proteins[38]. To have both the culture conditions of SSF and the solubilization of proteins of SmF, we propose a sequential fermentation approach. This sequential fermentation relies primarily on SSF with *A. niger* growth on pretreated biomass and secretion of milk proteins into the dry environment. Once fermentation is concluded, the fermentation vessel will be flooded with sterile deionized water[6], solubilizing the proteins akin to SmF.

This wash process will be done gently so as to not disturb the majority of the *A. niger* due to the potential of toxic compounds being released from lysed *A. niger*.

A SSF vessel will be seeded with pretreated lignocellulose, adjusted to 70% (w/w) moisture content with sterile water. Moisture content within the vessel will be maintained using

sterilized spray nozzles situated above each solid-state bed. Once seeded, spores of our milk-producing *A. niger* will be added at a concentration of $4x10^6$ spores/g of lignocellulosic material[39]. Once inoculated, the fermentation will be maintained at 30℃ and will continue for seven days. Once fermentation is concluded, the vessel will be flooded with sterile, deionized water, mixed gently, and drained to continue with purification of the milk proteins.

Protein extraction, analysis and, purification

Using the SmF or the sequential fermentation method described above, protein will be found solubilised in the liquid media of the bioreactor, and no extraction is necessary. This is because of the strategic use of the *glaA* tags to signal each milk protein for secretion outside of the fungal cell. Alternatively, if SSF is the fermentation method employed, previously described methods can be used to extract the protein from the solid media[40]. An extraction into the liquid phase would accomplish this by adding sufficient water to the fermented substrate to pull out the proteins. Since they are all water soluble, they should dissolve into the surrounding liquid. It is worth noting that β-casein is not inherently hydrophilic. However, in the presence of κ-casein the two proteins form a micelle which will be soluble due to the amphiphilic nature of κ-casein. Since the formation of casein micelles is essential for an accurate milk mimic, ion concentration may need to be adjusted at this stage to induce their interaction[6]. Once we have the solubilised proteins, the next step is a primary filtration, to remove excess biomass leftover from the feedstock and any cellular material. Depending on the scale of the operation, two approaches can be taken. For benchtop scale lab experiments, a centrifuge is a fast and cost-effective way to remove this material. By spinning at 3200 rpm the solution will separate by density, leaving the protein in solution and collecting the waste as a solid at the bottom. Alternatively, for a large-scale ferment, a more robust filter system is needed, one option being the plate and frame filter press, to filter down to the size of 0.2 μm to remove down to the cellular size.

The aim of the project is not to produce a singular product in high purity, but is instead to formulate a palatable milk alternative. Therefore, purification of the proteins is not to a target purity, but is instead aimed at passing safety assessment. As this is a consumable product, this analysis is paramount, however since this is an emerging industry, policy is not yet in place to govern cellular dairy production and will have to be written alongside the development of this procedure. As a baseline, current conventions for industrial enzyme production can be used to evaluate the degree of testing that will be needed[41]. For example, chemical composition analysis will need to be done, HPLC-MS being most suitable for this application. The No Observed Adverse Effect Level (NOAEL) of any impurities will need to

be determined to assess whether they must be removed[42]. If there are no potentially harmful contaminants, the process can proceed to the final formulation, however further purification may be needed.

Final purification will need to be gauged based on the needs in order to be cost effective. One highly efficient method that can accommodate large volumes is tangential flow filtration[43], which separates based on size but requires less maintenance than conventional filtration systems. A cut off size can be selected based on filter pore size, which will need to fall between contaminant size and the size of the milk proteins. This method also allows for the control of protein concentration in solution. The potential remains that continents will be of similar size to the proteins, in which case other separation techniques will need to be used. One method that can be easily scaled-up and has previously been used for milk proteins is leveraging the isoelectric point of the proteins. The pH of solution can be adjusted until the ionic charges are neutralized, causing the proteins to precipitate out where they can be collected[6].

Milk Formulation

The final formulation of milk is formulated based on a previously described serial addition of components[6]. To buffer the pH of the solution and adjust salinity, potassium phosphate, sodium citrate and calcium chloride are added at appropriate concentrations. Next, a lipid mixture simulating the fatty acid composition of bovine milk, which can be obtained from plant-based sources, is homogenized and then added. Optionally lipid soluble flavor compounds and vitamins can be added at this stage. Finally, the solution is mixed while adding maltose until the desired emulsion is obtained[6].

Future directions and Conclusion

At this point we only rely on agricultural waste as feedstocks. To reach mass commercialization, more feedstocks from the Fraser Valley need to be explored, and additional research is needed to improve carbohydrate degradation pathways in *A. niger* in order to have wider feedstock availability. In particular, improving *A. niger*'s inherent lignocellulolytic ability holds a lot of value. Because the lignocellulose degradation pathway is starvation-dependent in *A. niger*[23], circumventing the pathway's downregulation in the presence of glucose could increase the overall amount of lignocellulases produced and confer better saccharification of the feedstock. The transcription of many of the CAZY (carbohydrate-active enzyme) genes in the lignocellulose degradation pathway are regulated by the transcriptional activator *xlnR*. When *A. niger* lacks an easily-accessible

carbon source, scouting proteins are transcribed and translated, mediated by the alleviation of the transcriptional repressor *creA*. When these scouting proteins encounter a lignocellulose source of carbon, *xlnR* is subsequently activated and induces the expression of a wide variety of cellulases and hemicellulases[44]. When easily-accessible carbon becomes available (often in the form of glucose), *xlnR* and the CAZYs regulated by it are downregulated. Introduction of a point mutation within *xlnR* to confer constitutive activity that is not downregulated by the presence of glucose could be an exciting approach to explore. A CRISPR/Cas9 system could be utilized to mediate this mutation, as was described by Kun et al (2020)[45]. Using the ANEp8-Cas9 plasmid with an *amdS* selection marker instead of *pyrG*, we could replicate the valine-756-phenylalanine mutation in the C-terminal region of *xlnR*. The *amdS* selection marker allows growth of *A. niger* on media with acetamide as a sole nitrogen source which wild-type *A. niger* is not able to utilize effectively[46].

We cannot predict how much milk protein is produced, so there is much room to learn about the factors controlling the expression of proteins. Here we only targeted the protein; lipid production is totally unexplored.

The current bioprocess design that we proposed mitigates some of the technical risks and capital costs associated with scale-up. Clearly, there is a lot more to innovate in bioprocess design to meet the unique needs of the alternative dairy.

To better mimic cow's milk, the biologically relevant concentrations of individual milk protein will need to be replicated. At this time, the proposed model simultaneously produces all four proteins at the same rate. This means that the final formulation will have similar concentrations of the β and κ caseins, and α and β lactalbumins compared to cattle dairy which typically has a molar casein to whey ratio of 82:18[47]. There are two feasible approaches to rectify this, the first being to use regulation and control to induce production of each protein at different rates. The next is through separating the protein gene inserts into different strains of *A. niger*, allowing for the production of each protein in different bioreactors, where complete purification can be performed and later combined in the appropriate ratios.

Figure 3: Milk protein producing fragment inserted in pUC18, flanked by *XbaI* and *EcoRI* sites.

Establishing an industry like a cellular dairy industry that looks to commercialize new, cutting-edge scientific techniques is both exciting and daunting. Among a plethora of challenges that such an industry will have to face while setting up in British Columbia, we try to tackle the feedstock problem. The main selling points of animal-free milk products come from its animal-free nature. The production procedures are free from ethical issues, farming related environmental problems and land use. Given the fact that its products will probably cater mostly to consumers conscious about the ethical and environmental implications of animal product consumption, considering the use of a sustainable, environment-friendly feedstock may be an interesting option. The use of traditional sugar crops for feedstock may be more practical, but competing with the land-use of animal farms

of traditional dairies with the land requirements of growing sugar crops does not go well with cellular dairy's 'land-sparing' agenda. Utilizing agricultural waste as feedstock not only solves the feedstock problem, it may also alleviate issues of waste disposal by closing the loop to a circular economy. In this report, we lay down the groundwork that introduces the cellular dairy industry, and define the waste streams and their scope in relation to the industry. In this report, we analyzed the feasibility of agricultural waste as a feedstock for a cellular dairy industry, proposed *Aspergillus niger* as our organism of choice for the production of milk proteins using this feedstock, and suggested synthetic biological and genetic engineering workflows to develop and optimize this system.

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