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# RPPR Final Report

as of 02-Jun-2021

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**Final Report** for Period Beginning 17-May-2019 and Ending 16-Feb-2020

**Title:** Breaking the yield bottleneck of fibrous squid protein

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## STEM Degrees:

## STEM Participants:

**Major Goals:** Motifs from structural proteins such as silk, elastin, collagen, keratin, resilin, and recently Squid Ring Tooth (SRT) protein, have been used to create multifunctional biomaterials for diverse applications. The beak and SRT have attracted the interest of materials engineers due to their unique composite structure and extreme toughness. In particular,

SRT are composed of a highly stiff, naturally occurring biomaterial with an elastic modulus (E) in the range of a 4-8 GPa. We are exploring both native and recombinant SRT proteins and their biosynthetic variants in order to fabricate materials with tunable properties such as extensibility, stiffness, tensile strength, toughness, conductivity, optical transparency and self-healing abilities. These materials are highly desirable for wound dressing, electronic devices, adhesives, optics, sensors and many more high-tech applications. As a potential biomaterial with unusual properties, new methods must be developed for the cost-effective production and purification of these materials in order to make them accessible for potential military and civilian applications. In particular, since SRT is made up of long and highly specific chains of amino acids, it will require recombinant DNA expression and purification from a tractable host cell in order to be produced. To achieve this overall objective, we are focusing on the recombinant production of SRT with a purification process based on highly scalable and inexpensive liquid extraction methods.

A key requirement for the successful development of SRT as a practical biomaterial is the ability to produce and purify the basic protein matrices at very large scale and at low cost. Obviously the harvesting of SRT directly from squid is unrealistic, and further undesired since it is impossible to modify the protein sequence easily in the native host. For this reason, we will develop an optimized expression and purification method based on an Escherichia coli expression host. Although expression of recombinant structural proteins at industrial scale has proven challenging, particularly with proteins such as biosilk, we think that success is possible for SRT due to its thermoplastic nature and low molecular weight. Accordingly, we have produced preliminary results that suggest the unique properties of SRT will allow highly efficient production and purification, at low cost. We therefore propose here to continue this work, using a variety of inexpensive raw materials and conventional optimization approaches, to develop a highly effective production and purification method for engineered SRT. In particular, we will focus on the identification of optimal carbon and nitrogen sources for commercial fermentation that are significantly less expensive than the conventional laboratory reagents used in our preliminary results. The fermentation process will then be optimized using established

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experimental design methods, where the optimized process will finally be demonstrated at full pilot scale. In parallel to the fermentation studies we will also be identifying and optimizing purification methods for the expressed protein. In this case, the mechanical properties of the expressed proteins make it intractable for chromatographic processes, but suggest that conventional polymer processing unit operations may be successful. Our preliminary results in this area suggest that chemical extraction and precipitation may be especially effective, but inexpensive and environmentally friendly reagents must be identified for large-scale processing. The results of this work will be enabling for the commercial development and realization of the full potential impact of this material in the applications mentioned above.

These goals will be met through the following independent specific aims:

**Aim 1. Nitrogen source and fermentation optimization for increased fermentation yield of SRT:** Our initial goal will be to examine the impacts of inexpensive waste products as alternate nitrogen sources. Perhaps the best known of these is corn steep liquor, but several additional possibilities have been described in the literature. In our work experiment, we will substitute molar nitrogen equivalents for each nitrogen source and evaluate SRT expression in shake-flasks as has been done in our previous work. We anticipate that some nitrogen sources may exhibit toxicity or have otherwise negative impacts on cell growth, which will also be examined by using different concentrations of each source to calibrate the effects on cell growth and viability against effects on protein expression. The use of shake-flasks is preferred since it will allow a higher throughput screening process. Our goal is to identify an inexpensive nitrogen source that can perform as well as our current growth medium (based on tryptone and yeast extract). Given that our initial hypothesis, that the expressed proteins are expressed as highly stable inclusion bodies during fermentation, is correct, we expect that any nitrogen source that supports growth and low-level expression over time should be sufficient.

**Aim 2. Investigation of purification methods for SRT as a model for self-aggregating proteins:** Although our previously developed DMSO extraction method was highly successful, we have significant cost, toxicity and disposal concerns about the use of DMSO at very large scale. Although it may be possible to produce a purification process where the DMSO is recycled and redistilled, it is clear that there may be better options for immediate development at minimal cost. Thus our initial goal was to determine which basic methods can be used at large scale to provide reasonable improvements in purity. We will approach this goal through the initial investigation of three classes of chemical processes: (1) centrifugation and washing of the SRT inclusion bodies; (2) extraction (dissolution) and precipitation of the SRT protein; and (3) thermal processes to melt the TR42 protein and allow more effective extraction. Our premise is that the unique properties of the TR42 protein make it behave more like a conventional polymer than a highly structured recombinant protein, and we should therefore be able to develop a relatively scalable process based on conventional polymer processing to separate it from more “biological” contaminant proteins.

**Accomplishments:** See uploaded PDF (with the following summary):

In summary, we have developed eco-friendly processing and production technology based on industrial wet waste (i.e., corn steep liquor, molasses, and soybean extract) enabled growth that lowers the environmental impact of SRT based protein production. Synthetic tandem repeat proteins inspired by SRT proteins are easier to produce, with 1 g/L yield (i.e., 100x increase compared to previously reported expression) and high purity (>80%), compared to other bioengineered proteins due to their low molecular weights, and offer novel physical properties compared to other natural and synthetic fibers such as self-healing and thermal switching. We envision sustainable materials that are formed by protein-based materials as the ideal choice due to their natural biodegradability, programmable physical and chemical properties, and reduced waste and energy demand compared to currently available fibers, if they can be scaled to industrial production using various waste sources (e.g., commercial, and residential sources or organic-rich wastewaters from industrial and commercial operations).

**Training Opportunities:** This contract supported one postdoc (Tarek Mazeed) during the course of the work, where he was able to expand his ability in experimental design and his understanding of polymer biology and physics while developing a viable production process for SRT. In addition, Tarek worked with two undergraduate students at Ohio State (Jim Bradcovich and Joel Francis) during different parts of the project, who gained some experience in shake-flask fermentation, experimental design and data analysis. These two students are not listed in the participants section since they were not financially supported during their work in the lab.

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## as of 02-Jun-2021

**Results Dissemination:** Results of the work are being prepared for publication with the following citation:

Tarek El-Sayed Mazeed, Burcu Dursun, Oguzhan Colak, Huihun Jung, Yusuke Kikuchi, Benjamin D. Allen, David W. Wood, Melik C. Demirel, "Enhancing Sustainability and Elasticity of Fibers by Tandem Repeat Proteins", in preparation for ACS Sustainable Chemistry & Engineering, 2021

Because of the narrow scope and short period of the study we did not do much outreach, but the work is continuing in the laboratory of Melik Demirel. Dr. Demirel is commercializing the technology and will be promoting this work as part of this effort.

**Honors and Awards:** Nothing to Report

**Protocol Activity Status:**

**Technology Transfer:** The methods and data developed under this project have been transferred to the laboratory of Melik Demirel at Penn State University. No independent intellectual property was developed at Ohio State University and not patent disclosures or applications were filed.

### PARTICIPANTS:

**Participant Type:** Postdoctoral (scholar, fellow or other postdoctoral position)

**Participant:** Tarek Mazeed Hassanin

**Person Months Worked:** 9.00

**Funding Support:**

Project Contribution:

National Academy Member: N

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**Article Title:** Enhancing Sustainability and Elasticity of Fibers by Tandem Repeat Proteins

**Authors:** Tarek El-Sayed Mazeed, Burcu Dursun, Oguzhan Colak, Huihun Jung, Yusuke Kikuchi, Benjamin D. All

**Keywords:** Sustainability, Squid, Protein-fibers, Self-assembly, synthetic proteins

**Abstract:** Protein-based fibers are lightweight, biodegradable, have excellent moisture and temperature regulation, and exceptional mechanical properties, but they are limited in production capacity. Biosynthetic protein-based fibers have the potential to overcome these concerns, but large-scale production with high yield and purity, as well as inexpensive feedstock, must be achieved. Here we developed an optimized expression and purification method for biosynthetic tandem repeat proteins, that are inspired from squid ring tooth (SRT) protein using three wet-waste feedstock, corn steep liquor, molasses, and soybean extract. SRT is composed of a highly stiff, naturally occurring bioplastic and these properties arise from the molecular architecture of the constituent proteins, which are segmented co-polymers with alternating semicrystalline and amorphous domains similar to silk. We have developed protocols to use liquid industrial and agricultural waste as feedstock for SRT production, which has th

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as of 02-Jun-2021

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I certify that the information in the report is complete and accurate:

Signature: David W. Wood

Signature Date: 6/1/21 6:26PM

## Introduction

Natural protein-based fibers are biodegradable,<sup>1</sup> but expression of recombinant proteins at industrial scale has proven challenging. Peptide motifs from structural proteins such as silk<sup>2</sup>, elastin<sup>3</sup>, collagen<sup>4</sup>, keratin<sup>5</sup>, resilin<sup>6</sup>, and recently Squid Ring Teeth (SRT) protein,<sup>7, 8</sup> have been used to create multifunctional materials for diverse applications. Our focus has been on the study and modification of SRT in particular, for diverse applications in a variety of sectors. Various squid species have developed several features that allow them to be successful predators, which include sharp and rigid beaks, strong tentacles, and numerous SRT that allow these tentacles to grip prey. In particular, SRT are composed of a highly stiff, naturally occurring bioplastic with an elastic modulus (E) in the range of a 4-8 GPa and 100 MPa strength.<sup>9</sup> These properties arise from the molecular architecture of the constituent proteins, which are segmented co-polymers with alternating semicrystalline and amorphous domains. Although the proteins that compose SRT tissues were only discovered recently, they have quickly gained the attention of several research groups due to their unique behavior. We have been exploring both native and recombinant SRT proteins and their biosynthetic variants in order to fabricate materials with tunable properties such as extensibility<sup>10</sup>, biocompatibility<sup>11</sup>, thermal conductivity<sup>12</sup>, optical transparency<sup>13</sup> and self-healing abilities<sup>14</sup>. SRT can be precisely tuned since a defined amino-acid sequence is genetically encoded in the DNA. This allows absolute control over stereochemistry, sequence, and chain length for tunable physical properties as well as environmentally friendly manufacturing. Here we developed an optimized expression and purification method for biosynthetic tandem repeat SRT proteins using industrial and agricultural waste as a feedstock. We showed hundred fold increase in protein yield compared to previously reported expression<sup>15</sup>.

## Results

SRT is a high-strength thermoplastic protein that could be a green alternative to conventional plastics. However, in order to be industrially feasible, the expression levels of the SRT protein in well-established production hosts should be high (e.g., 1-10 g/L of fermentation volume), and it should be produced using an inexpensive medium (e.g., industrial waste). Further, the purification of the expressed SRT should also be simple and inexpensive, where chromatographic steps should be avoided if possible. Towards this goal, we focused on the production of three sizes of the SRT tandem repeat (TR) protein, TR-n4, TR-n7, and TR-n11, referring to the sizes of the tandem repeat protein produced (molecular weights of 15, 25, and 42 kDa, respectively). Earlier, we developed a rolling-circle amplification method<sup>15</sup> to produce tandem-repeat coding sequences in a single cloning step (**Figure 1a**). The designed building block for the tandem repeat proteins was based on the cross-linked crystal-forming sequence PAAASVSTVHHP and amorphous sequence YGYGGLYGGLYGGGLGY observed in native SRT. We demonstrated earlier that the resulting polypeptides have similar semicrystalline  $\beta$ -sheet domains and amorphous tie-chains to the native squid proteins. As previously reported, the material properties of these proteins depend on their molecular weights, and we hypothesized that sequences of different lengths might require distinct conditions for optimal production.

To produce these proteins, we worked with *E. coli* strain BL21(DE3) as well as BLR(DE3) as shown in **Figure 1b** and **1c** respectively. Because our protein-coding sequences are highly repetitive, we hypothesized that the recombination-deficient strain BLR(DE3) could enable more robust production. Since all of the proteins studied in this work are intrinsically insoluble under physiological conditions, our chosen production strategy was to allow slow accumulation of the

TR proteins in inclusion bodies using the low-level uninduced expression provided by the pET14b plasmid in our chosen strains. The smallest of the proteins, TR15, shows early expression from the uninduced T7 promoter, and appears to reach its maximum production level by SDS-PAGE within 24 hours as shown in **Figure 2**. The larger TR-n7 and TR-n11 proteins are expressed more slowly and appear to reach their maximum production levels by 48 hours of fermentation. All three proteins remain stable during expression, with no loss of yield observed during 96 hours of incubation. Contrary to our expectations, strain BLR(DE3) produced uniformly less protein by SDS-PAGE across all conditions when compared to the standard strain BL21(DE3). This observation could be due to less efficient growth in the *recA*-deficient BLR strain, or perhaps protein production was affected by wider genomic differences between these strains that were not appreciated until recently.<sup>16</sup>

It is known that transient anoxic conditions can cause limitations in amino acid production and plasmid stability. Hence we studied the effect of oxygen on TR-n11 production using a bioreactor with incubation at 37 °C and 500 rpm. The maximum production of TR-n11 was reached after 96 hours with agitation speeds of 500 rpm as shown in **Figure 3**. The significant increase of TR-n11 expression by batch fermentation compared to the shake flask culture could be explained by improved oxygen transfer in the bioreactor (Blibech et al., 2011). Qualitatively, the biomass in shake flask was lower than bioreactor production. (i.e., OD<sub>600</sub> 21.7 in flask compared to OD<sub>600</sub> 77.3 in bioreactor).

The adoption of SRT proteins for materials applications requires high-yield, high-purity production from inexpensive feedstocks. Hence, we screened alternative nitrogen sources using four (**Figure 4a**) selected agri-industrial residues at varying dilutions for their ability to serve as nutrients for TR-n11 production. Corn steep liquor (CSL) is a cheap potential source of nitrogen in fermentation, which is a major side-product in the corn starch industry. It is also a cheap source of proteins, amino acids, minerals, vitamins, and trace elements. Cane molasses is a by-product of sugar refineries, which contains saccharides and nitrogenous compounds, vitamins, and trace metal elements. The bagasse is constituted of lignin, hemicellulose, and cellulose. Three wet-waste sources, corn steep liquor (CSL), molasses, and soybean extract (SBE) enabled growth of our production strain and yielded TR-n11 protein, whereas growth in green juice extract was unsuccessful. **Figure 4b** and **4c** shows the protein gel (cell lysate and purified respectively) of four feedstock with varying dilutions. CSL feedstock growth medium reached the greatest production after 96 h of incubation.

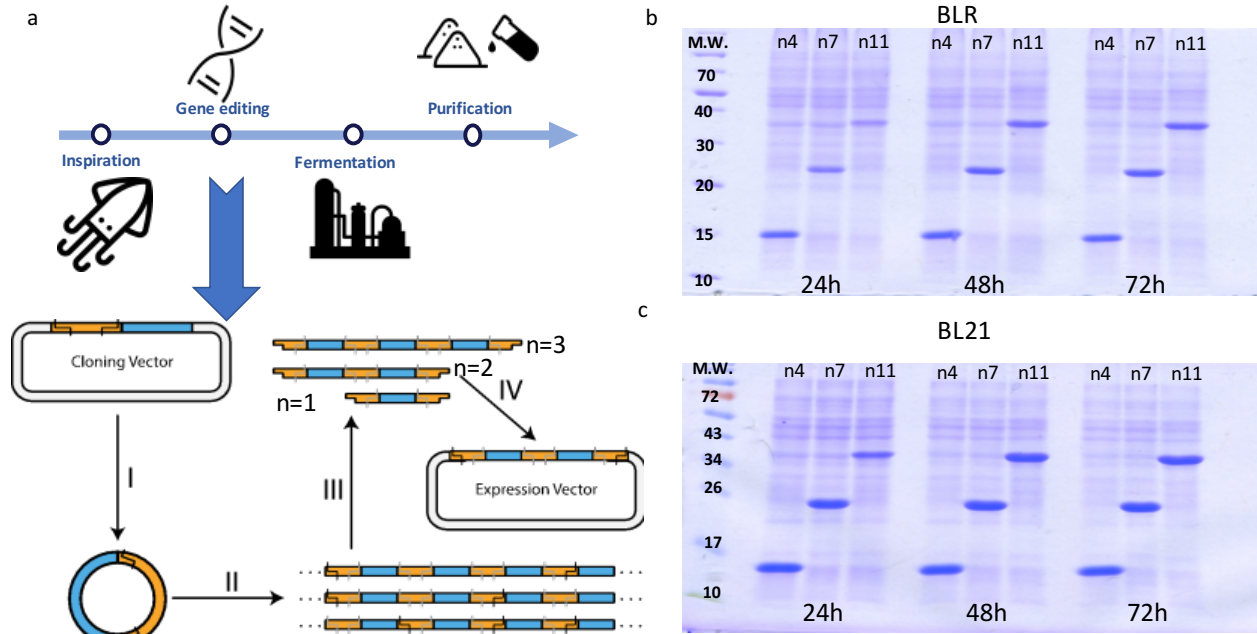
Five assays were tested for production and purification of the recombinant TR-n11 protein as shown in **Figure 5**. Our initial approach to purify recombinant TR proteins was to simply wash bacterial inclusion bodies using a variety of detergents and solvents. Although this led to modest increases in purity over cell lysate pellets, the purity was still less than 70% as judged by SDS-PAGE. We therefore attempted an organic extraction method where the inclusion body pellet was dissolved in DMSO and then re-precipitated using purified water as a counter solvent. This approach is based on the observation that TR proteins are highly soluble in pure DMSO, but become highly insoluble upon the addition of small amounts of water. Smaller protein domains, typical of the major contaminating proteins in the cell lysate, are expected to remain soluble in DMSO up to fairly high concentrations of water (up to 200 mg/ml), which would afford a significant purification. As expected, this approach led to a substantial increase in purity,

exceeding 70% (**Figure 5b**), which is also confirmed with Maldi (**Figure 5c**). The fact that this purity can be achieved in a single step using a highly scalable and simple method suggests that the unique properties of the SRT protein can be exploited to develop highly innovative and effective purification methods.

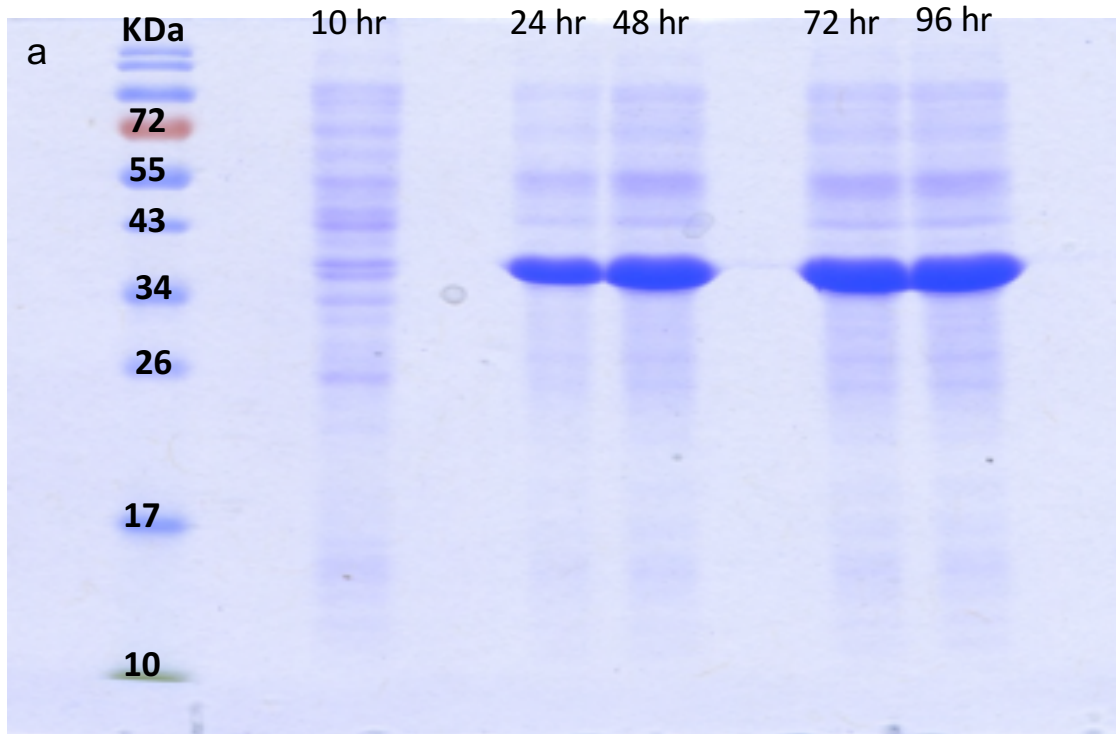
### **Conclusion**

In summary, we have developed eco-friendly processing and production technology based on industrial wet waste (i.e., corn steep liquor, molasses, and soybean extract) enabled growth that lowers the environmental impact of SRT based protein production. Synthetic tandem repeat proteins inspired by SRT proteins are easier to produce, with 1 g/L yield (i.e., 100x increase compared to previously reported expression<sup>15</sup>) and high purity (>80%), compared to other bioengineered proteins due to their low molecular weights, and offer novel physical properties compared to other natural and synthetic fibers such as self-healing<sup>17</sup> and thermal switching<sup>12</sup>. We envision sustainable materials that are formed by protein-based materials as the ideal choice due to their natural biodegradability, programmable physical and chemical properties, and reduced waste and energy demand compared to currently available fibers, if they can be scaled to industrial production using various waste sources (e.g., commercial, and residential sources or organic-rich wastewaters from industrial and commercial operations).

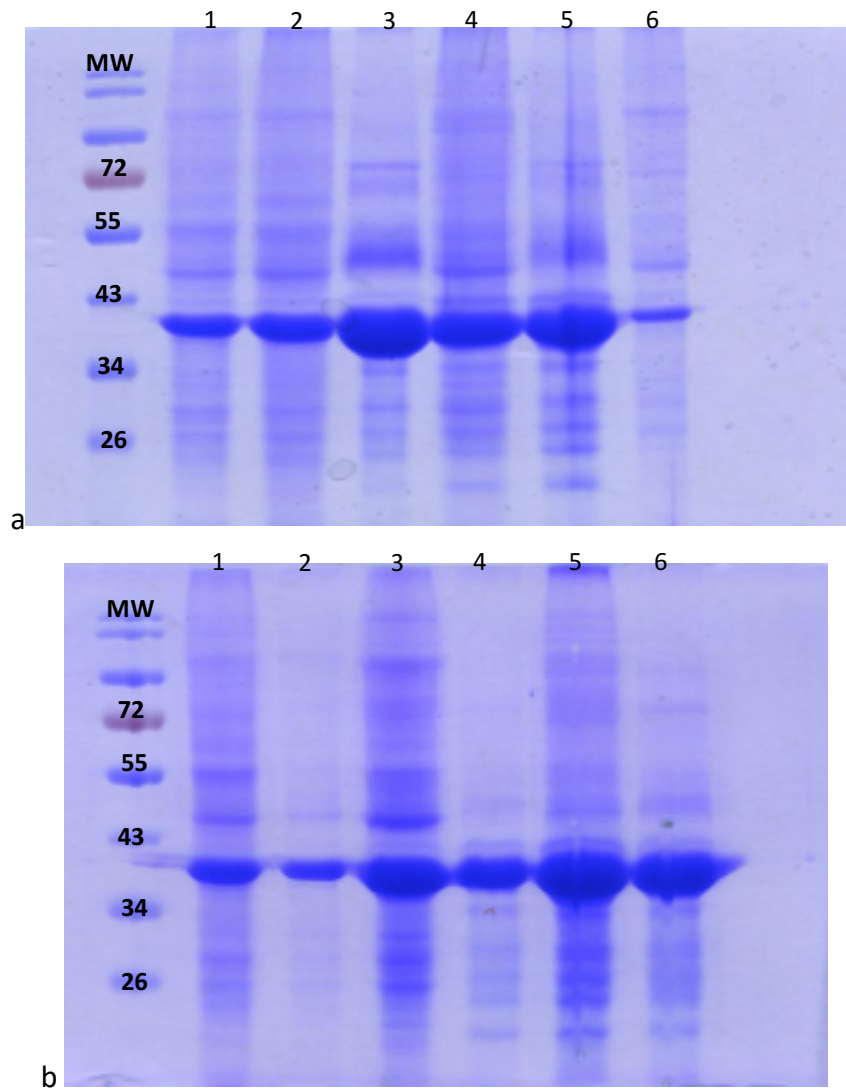
## FIGURES



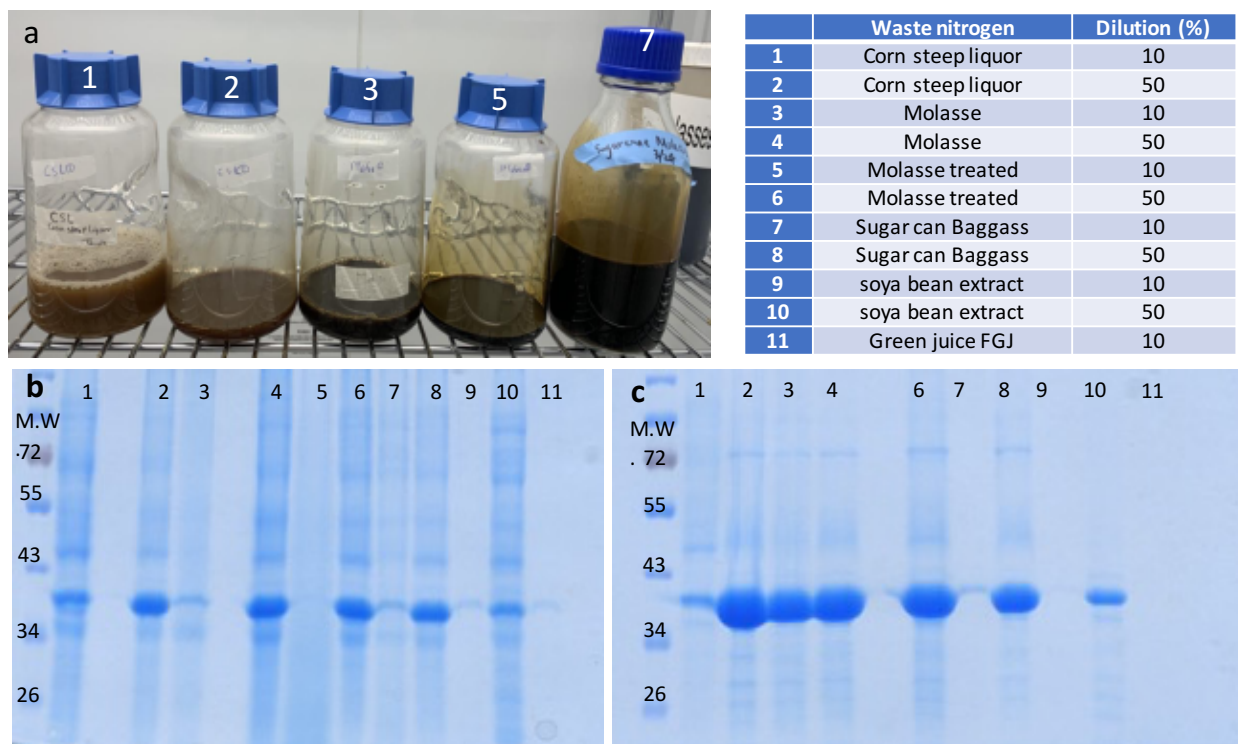
**Figure 1:** a) Overview of the design and preparation of tandem repeat polypeptides inspired by squid ring teeth. Rolling circle amplification is used to assemble repetitive genes. SDS-PAGE of cell-lysate samples taken at several time points for strains (a) BLR(DE3), and (b) BL21(DE3) bearing plasmid pET14b with inserts TR-n4, TR-n7, and TR-n11 incubated in 4X LB media at 37°C.



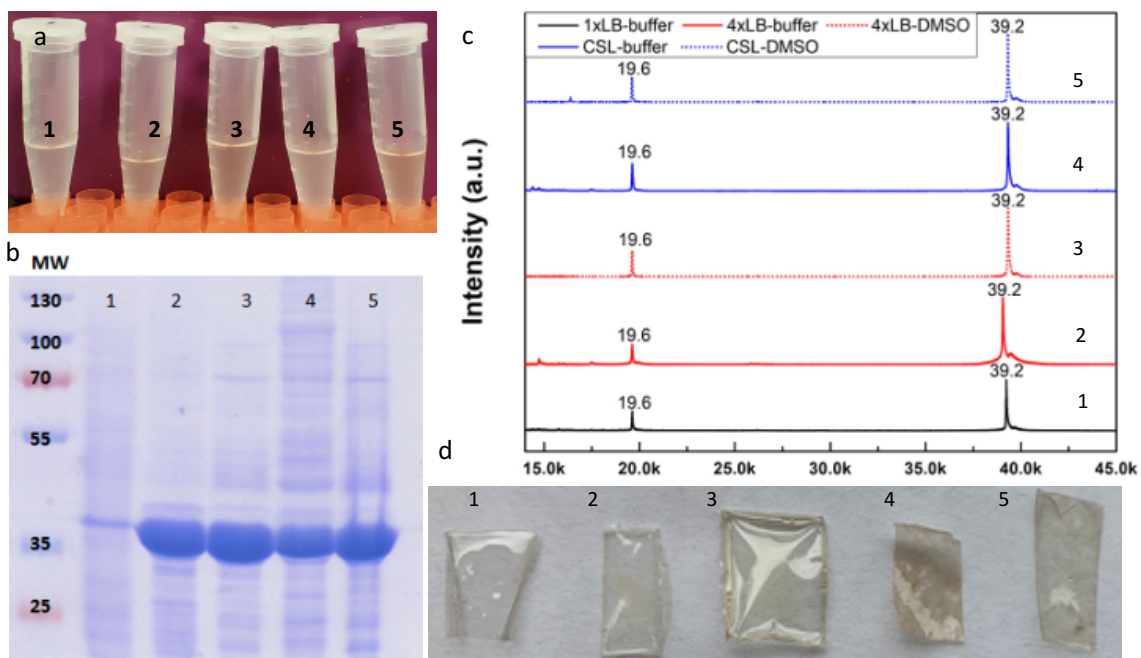
**Figure 2.** Time evolution of TR-n11 protein characterized by SDS page



**Figure 3.** a) TR-n11 expressed from BL21/PET14/TR42 with no induction and purified using Buffer protocol and DMSO. All samples were loaded with 20 mg purified TR-n11 dissolved in 1 ml 15 M Urea under 6 assay conditions: 1) 1xLB feedstock and Buffer purification, 2) 4xLB feedstock and Buffer purification, 3) 4xLB feedstock and DMSO purification, 4) CSL feedstock and Buffer purification, 5) CSL feedstock and DMSO purification, 6) CSL feedstock with residue and buffer purification). b) TR-n11 expressed from BL21/PET14/TR42 with induction and purified using buffer protocol and DMSO purification. All samples were loaded with 20 mg purified TR-n11 dissolved in 1 ml 15 M Urea under 6 assay conditions: 1) 1xLB feedstock and Buffer purification, 2) 1xLB feedstock and DMSO purification, 3) 4xLB feedstock and buffer purification, 4) 4xLB feedstock and DMSO purification, 5) CSL feedstock and buffer purification, 6) CSL feedstock and DMSO purification.



**Figure 4:** a) Pictures of corn steep liquor, molasse, sugar can baggass and soya bean extract are shown. Cell-lysate (b) and buffer purified (c) samples of SDS-PAGE of TRn-11 (~40kDa) protein produced in BL21 (DE3) under un-optimized conditions from 11 different feedstock listed in the table.



**Figure 5:** Five assays are tested for production and purification of the recombinant TR-n11 protein: 1) 1xLB feedstock and Buffer purification, 2) 4xLB feedstock and Buffer purification, 3) 4xLB feedstock and DMSO purification, 4) Corn syrup liquid (CSL) feedstock and buffer purification and 5) CSL feedstock and DMSO purification. a) All five production yield good solubility in HFIP solvent. SDS-PAGE of cell lysates shows high yield production of TRn-11 (~40kDa) for all except 1<sup>st</sup> method. c) MalDI spectra also confirms the high purity of these samples. d) All five protein films were succesfully casted to form thin films that are mechanically robust.

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