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UROP application  
deadline: April 14th

## Proposed Summer UROP Project

### Production of isotopically-enriched serum albumin for biomedical research using *Pichia pastoris* as a biosynthetic host

#### Overall Objective:

The project focuses on the development and optimization of the substrate yield of a novel process that uses [ $^{13}\text{C}_6$ ]glucose and [ $^{15}\text{N}_2$ ]ammonium sulfate to produce isotopically-labeled serum albumin from the yeast *Pichia pastoris*, with robust and consistent quantity and enrichment for biomedical research applications.

#### Background:

Stable isotope molecules are used routinely in biotechnology<sup>1,2</sup> and biomedical sciences<sup>3-5</sup> as a tool for enabling the direct measurement of metabolic behavior and molecular properties. Their enrichment of atomic weights above naturally abundant levels provides a readily detectible means of tracking atomic transitions or molecular structure via mass spectrometry (MS) or nuclear magnetic resonance (NMR). In contrast to radioisotopes, their stability facilitates their safe and routine use without special precautions. Additionally, since MS and NMR are able to detect a multitude of signals corresponding to different isotopic labeling extents or positions (i.e. mass fragments or chemical shifts), stable isotopes are capable of providing a much richer spectrum of molecular information than radioisotopes, which enable determination of only specific activity<sup>6</sup>.

However, a major limitation of using such stable isotope tracers is their availability and affordability. While these isotopes are present in nature, their limited abundance necessitates the use of complex and expensive separation processes that are capable by only a handful of commercial plants. Likewise, the scale and sophistication of these processes leads to a vastly higher per-unit cost than these isotopes' non-labeled analogs (e.g. \$191 - \$750 per g D- [ $^{13}\text{C}_6$ ]glucose vs. \$0.01 - \$0.21 per g cell-culture grade unlabeled D-glucose; Sigma-Aldrich product listings). While these compounds, though relatively costly, are still nonetheless commercially available to research labs, many molecules of scientific interest are not. Vendors such as Cambridge Isotope Laboratories, Sigma-Aldrich, and Omicron Biochemicals sell a wide array of isotopically-labeled small and/or commonly used organic compounds, but not equivalent forms of macromolecules or more uncommon organic compounds, which leaves custom orders as the only potential, even more expensive, purchasing option.

An alternative approach to obtaining substantial quantities of more specialized stable isotope molecules is to purchase enriched substrates and perform chemical and/or biological reactions to transform them into desired products, a strategy that has been successfully used in the past to make labeled forms of desired compounds for research applications<sup>7,8</sup>. In particular for complex organic macromolecules, using biological organisms as a metabolic factory is appealing due to the robust and specific nature of their innate enzymatic capacity and the many control knobs available to enable and improve synthesis. For instance, metabolic engineering can be used to allow production of heterologous proteins in microbial hosts. Further, bioreactors enable scale-up for larger quantities, and culture parameters can be adjusted for further optimization of yield, productivity, and titer.

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Serum albumin is one such molecule of scientific interest for which a stable-isotope-labeled form is not currently commercially available. Human serum albumin (HSA), a 66.5 kDa single polypeptide, is the most abundant protein in blood plasma<sup>9</sup>. It is crucial for maintaining the proper osmolarity of blood and transporting a variety of compounds through the body. Therapeutically, it is administered to patients for treatment of hypoalbuminemia or traumatic shock to restore fluid retention<sup>10</sup>. In addition to its traditionally-recognized roles, it has more recently been identified as a potential nutrient source for rapidly-growing tumors through the mechanism of micropinocytosis, and the use of [<sup>13</sup>C]- and/or [<sup>15</sup>N]-labeled forms of serum albumin in cell culture or mouse infusion systems could help give validation to this hypothesis.

The yeast *Pichia pastoris* provides an ideal platform for the production of isotopically-labeled serum albumin. *P. pastoris* has been shown to be readily capable of producing appreciable quantities of properly-folded recombinant HSA, and no adverse responses were observed when the recombinant protein was administered in clinical trials (with administration of non-recombinant HSA as a reference)<sup>10</sup>. In contrast to *E. coli*, *P. pastoris* can readily secrete expressed protein products, which greatly facilitates purification.

While *P. pastoris* has been previously used to generate [<sup>13</sup>C]- and [<sup>15</sup>N]-labeled forms of protein, there do not appear to be any published accounts in which the extent of carbon enrichment was able to be controlled. Prior researchers have used a two-substrate system in which labeled glucose was first added to build the culture density and unlabeled methanol was subsequently used to induce protein expression (via a methanol-responsive promoter)<sup>11</sup>. In contrast, if serum albumin is placed under control of a constitutively-active glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter, as through the pGAPZ vector from Invitrogen, a single carbon source such as glucose can be used both for growth and protein production, enabling isotopic enrichment to be directly determined by the substrate composition.

Logistically, the HSA can be produced by following a template protocol for protein expression in *P. pastoris* using a Sartorius Biostat A Plus fermentor (Fig. 0). Either carbon- or nitrogen-labeled protein can be generated, depending on whether [<sup>13</sup>C<sub>6</sub>]glucose or [<sup>15</sup>N<sub>2</sub>]ammonium sulfate is used as the source of enrichment. The secreted protein can be purified simply from centrifuging the harvested supernatant (Fig. 1), concentration via membrane filtration, and affinity chromatography with a Cibacron Blue resin<sup>12</sup>. Isotopic enrichment can subsequently be validated through MS of the purified protein (Fig. 2).

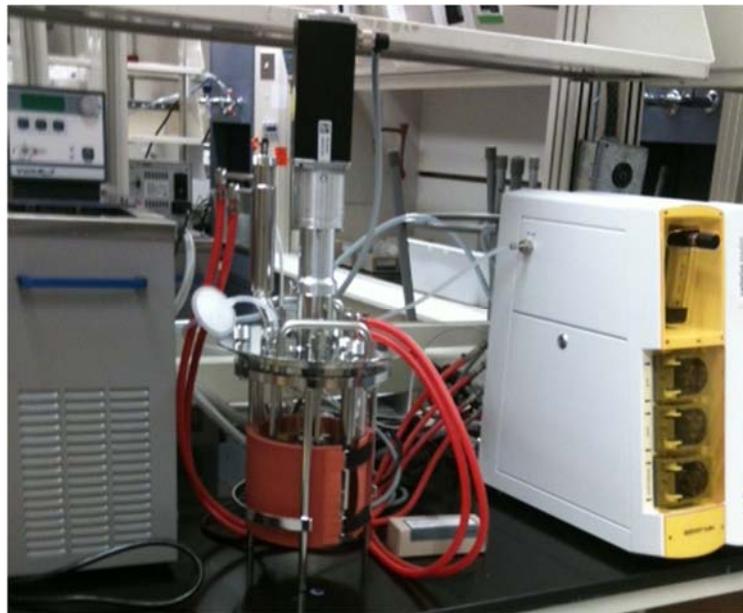
More fundamentally, however, the project explores the challenges present when using a bioprocess to maximize the conversion of a limited precursor (the labeled substrates) into a valuable product (the labeled albumin). Mass balances are central to this entire mission, as every carbon or nitrogen atom that is incorporated into biomass or catabolized (i.e. to maintain bioenergetic or redox homeostasis) is no longer available to be incorporated into the desired protein (Fig. 3). In monitoring the progress of shake flask and bioreactor experiments, the corresponding major substrates and products will be able to be comprehensibly quantified – glucose via high-performance liquid chromatography (HPLC) (or YSI Biochemistry Analyzer), ammonium via colorimetric assay (or YSI Biochemistry Analyzer), protein via Bradford assay, biomass via optical density or dry cell weight measurements, CO<sub>2</sub> via micro-gas chromatography, and non-volatile side-products (e.g. ethanol, acetate) via HPLC – enabling closure of carbon and nitrogen balances. Over the course of the project, the determination of associated consumption and

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production metabolic fluxes over a range of varying conditions will demonstrate the tradeoffs and limitations of these reactions.

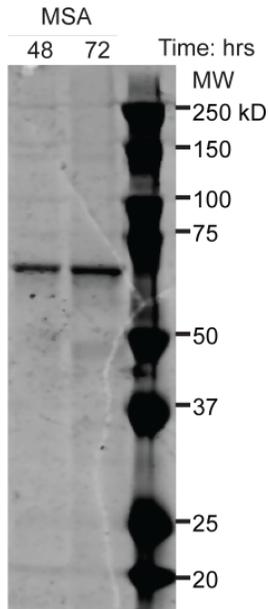
### Student Work Plan

- 1) Transform *P. pastoris* with basic and protease-deficient serum albumin expression plasmids, and screen colonies for production using Bradford assay or Bioanalyzer.
- 2) Optimize small-scale protein yield on glucose and ammonium through varying culture parameters (e.g. temperature, media composition) in shake flasks.
- 3) Use 2 L bioreactor to scale up culture using optimized conditions from shake flasks and develop production protocol with unlabeled substrates.
- 4) Perform bioreactor culture using developed protocol using [ $^{15}\text{N}_2$ ]ammonium sulfate and [ $^{13}\text{C}_6$ ]glucose to produce labeled serum albumin.
- 5) (Time permitting) Concentrate labeled protein using membrane filtration and purify using affinity chromatography.

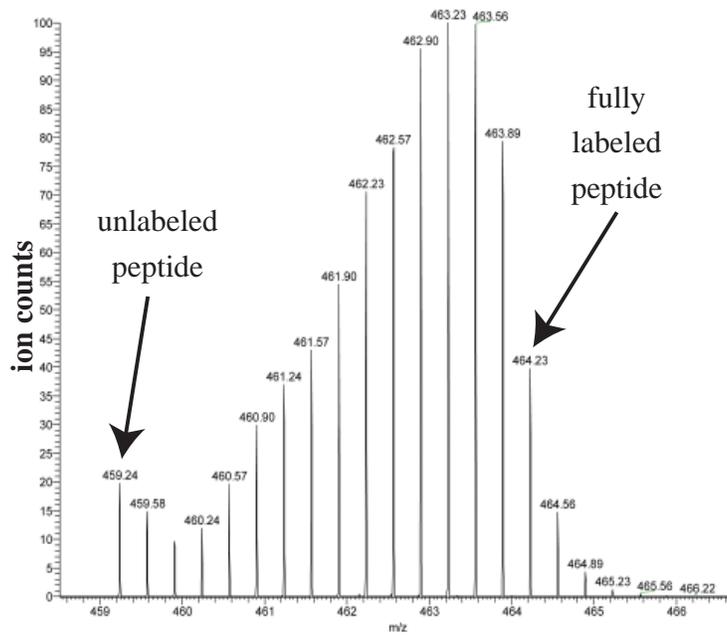


**Figure 0.** Sartorius Biostat A Plus top-driven fermentor unit, including the 2-L glass vessel (center), the temperature-control unit (left), and the computer-controlled tower with pumps and flow meters (right), for the study of isotopically-labelled albumin from *P. pastoris*.

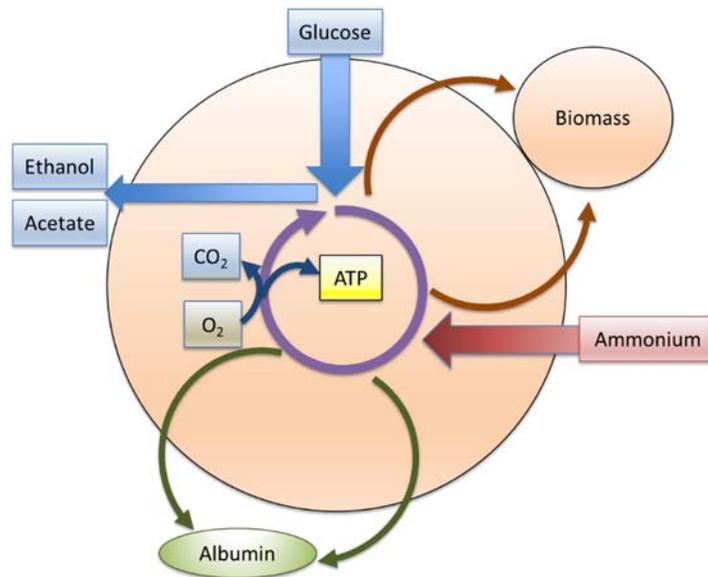
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**Figure 1.** SDS PAGE gel of media supernatant from shake-flask cultures of *P. pastoris* expressing albumin (66 kDa) at indicated time points.



**Figure 2.** Mass spectrum of a peptide derived from partially-labeled HSA from engineered *P. pastoris* grown on a mixture of unlabeled and [<sup>15</sup>N<sub>2</sub>]ammonium sulfate.



**Figure 3.** Cartoon schematic of the major metabolic processes in the proposed *P. pastoris* protein production system.

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