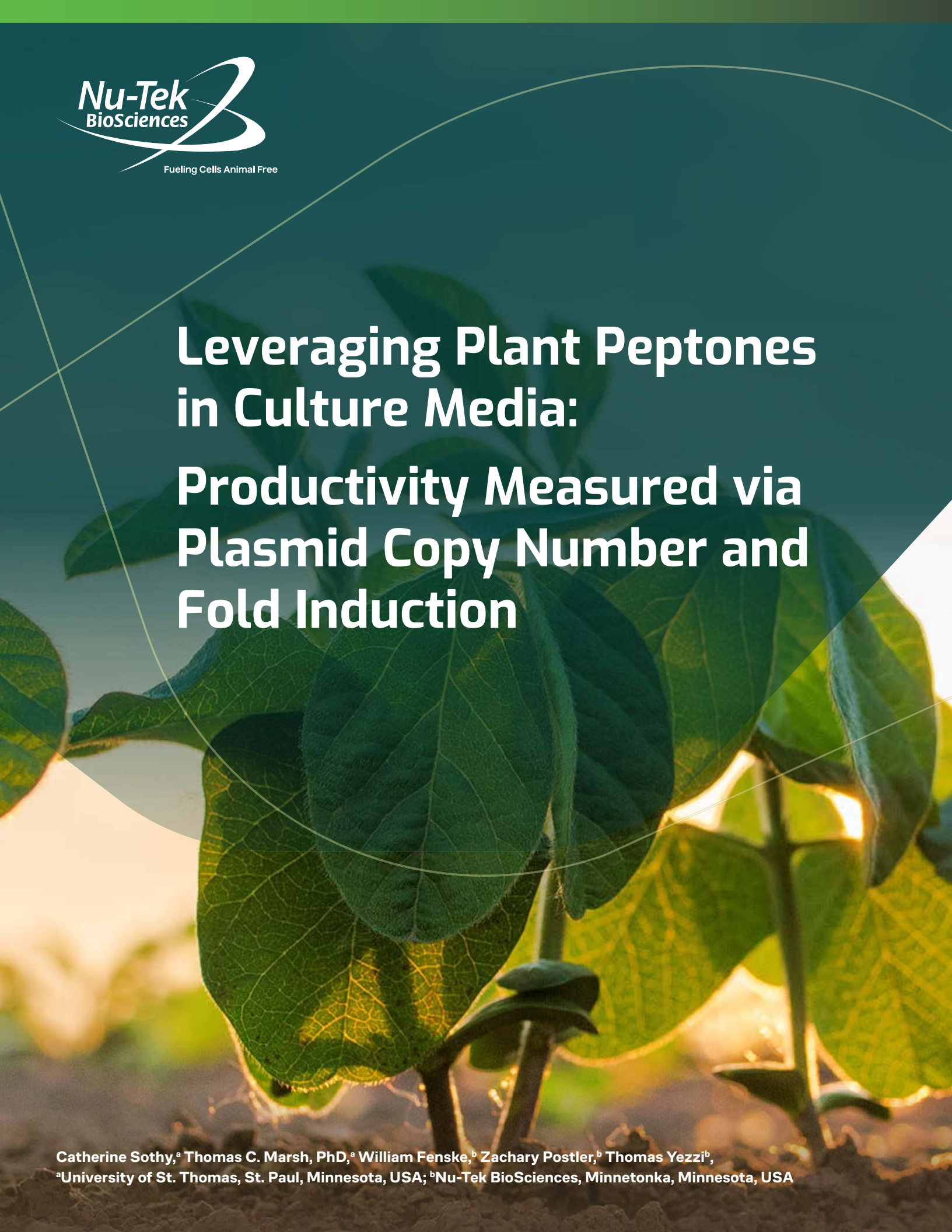
Nu-Tek BioSciences

Fueling Cells Animal Free

Leveraging Plant Peptones in Culture Media:

Productivity Measured via Plasmid Copy Number and Fold Induction



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Key Points

1. **Biopharmaceutical manufacturers often utilize organisms to produce breakthrough therapies;** measuring success of that cultivation through the assessment of plasmid copy number and expressed (or unexpressed) genes is a common proxy for the control manufacturers have over their production process.

2. **Expression of genes (either induced or uninduced) in organisms such as E. coli can provide manufacturers with important insights** into the utility of certain culture media formations for their unique production requirements.

3. **Nu-Tek's plant-based products provide consistent cell growth performance (compared to animal-origin media),** decreases risk of contamination, improves sustainability, and provides additional supply chain options for manufacturers who are looking to meet customer demand.

Overview

Cultured mammalian, bacterial, and insect cells have become the cornerstone of biotechnology and pharmaceutical manufacturing. Billions of dollars depend on these miniature production powerhouses, and the utility of living cell cultures continues to expand as new unmet medical needs are identified.[1] There are several challenges that arise in the cultivation process (referred to as "industrial fermentation"); namely, culture growth, cell productivity, and supply chain security of the nutrient base necessary to maintain healthy cell populations.[2] The use of plant-based, rather than animal-derived, cell culture media to support cell line growth and function has increased in utility in the industry. Peptones address key challenges in supporting productive cultures, from scalability, to contamination concerns, to supply chain support.

The switch to plant-based media, particularly if cell lines have been previously adapted to animal media, can be challenging. Diligent review of critical data to support media adaptation from animal-based to plant-based media is required; one method of assessment, in the form of plasmid copy number and fold induction, can provide assurance that plant-based culture media is appropriately supporting these valuable cell lines. Use of the model organism E. coli EPI300 can provide such insights when evaluating a culture media; **using peptone sources like those from the Nu-Tek BioSciences' portfolio, have the potential to support the growth of these valuable cell lines without reliance on animal-derived ingredients**.

Industrial Fermentation: Mass Production at a Microscopic Scale

Pharmaceutical manufacturers are focused on developing cell lines and cultures with higher yields of target proteins, optimizing cell culture media for increased production volumes, and implementing bioreactors for enhanced scalability. Advancements in gene editing technologies, such as CRISPR-Cas systems, have further improved production efficiency and improved product quality by allowing modifications to host cell genomes.[3] In addition to manipulating cells directly to enhance efficiency, research has shown that the consistency and quality of raw materials provided by suppliers are essential in controlling variability and maintaining product quality.[3]

To address the growing demand, pharmaceutical companies are investing in high-throughput bioreactor systems and automated processes to generate cell culture data more quickly and efficiently.[4] These systems allow for better prediction of product quality and volumetric productivity while reducing manual labor and costs. Furthermore, the industry is adopting quality by design (QbD) initiatives, which require a deeper understanding of cell culture processes and their impact on final product quality.[4] As the field continues to evolve, maintaining robust and scalable cell cultures will remain a critical factor in meeting the increasing demand for biopharmaceutical products and advancing drug discovery and development efforts.

Growth Media: Current Practices and Challenges

Animal-origin based growth media, such as those containing beef extracts, blood (serum), and casein, are widely used in industrial fermentation. These media have been in use since the 1950's and provide a undefined mixture of growth factors, hormones, proteins, and other components that support cell viability and proliferation.[6] While they remain effective for cultivating a variety of cell types, the use of animal-derived components in cell culture media is becoming less prevalent, with serum-free formulations growing in popularity industrial settings.[8]

Animal-based products in cell culture media present several challenges. First, these components introduce variability and unpredictability in the cell culture process, as their composition can vary depending on the source, batch, and storage conditions.[2] This variability can affect cell performance and behavior, leading to inconsistent results. Second, animal-derived components increase the risk of cross-contamination, potentially introducing adventitious agents such as bacteria, fungi, viruses, and prions. These risks have driven increasing pressure from regulatory bodies, such as the US FDA, for manufacturers to pursue non-animal-based products as part of their manufacturing process.[8] Third, the use of animal-derived materials raises ethical concerns and may conflict with religious, cultural, or personal values of some stakeholders. Finally, these components are costly, limited in availability, and can hinder the scalability and sustainability of cell culture processes.

An alternative source of nutrients that does not require animal-derived components is of vital strategic importance to companies as production demand grows and new products are developed. Plant- and yeast-based products (such as peptones and extracts, respectively) can provide the necessary carbon and nitrogen nutrients for cells to grow in culture and provide additional assurances against supply chain challenges and contamination.

Plasmid Copies and Inducible Expression: Proxy Measurements for Drug Substance Production

Cell cultures that are well-maintained can produce critical drugs at a scale required to service commercial demand. Production must have a consistent quality profile and meet the volume required for projected need; without this consistency, pharmaceutical manufacturers would not achieve their promised patient support.

Each pharmaceutical developer uses proprietary processes and ingredients to cultivate their cell lines. It is not possible to fully replicate each production method; however, a proxy measurement of plasmid yield and control of plasmid copy number can be used to show cell growth and survival behavior in a new or augmented media. Plasmid copy number plays a crucial role in pharmaceutical cell culture, particularly in the production of biologic therapies. The available plasmid copy numbers (PCN) per cell significantly impacts protein expression levels, metabolic burden on host cells, and overall product yield.[5] High PCN can lead to higher yield of plasmid per cell, or increased protein production but may also result in reduced cell growth rates and metabolic stress. Conversely, low copy number plasmids may offer more stable expression but with potentially lower yields. Recent advancements have led to the development of systems with tunable copy numbers, allowing researchers to optimize plasmid replication for specific applications and cellular conditions.[5]

The ability to control the timing of plasmid and protein production during the growth of the host organism is equally critical in the production of biopharmaceuticals, as it affects the functionality and viability of the culture system depending on the desired product characteristics. For example, a variety of expression control systems are used for E. coli, the most common including those induced by the presence of specific carbohydrates. Achieving precise control over the induction stimuli that regulates gene expression or plasmid copy number is a major consideration in media formulation and composition. The ability to pivot from established media to alternatives is ensuring that expression of inductive systems does not get inadvertently triggered, especially when the final product is toxic. However, if the cellular product is non-toxic, the so called ‘leaky expression’ may even be beneficial to specific yield. These advancements in understanding plasmid copy numbers and fold induction mechanisms are driving innovations in the pharmaceutical industry, enabling the development of more efficient and effective biologic therapies.

CASE STUDY

Nu-Tek Products
Plasmid Production and Fold Induction in E. coli EPI300

Nu-Tek BioSciences produces animal-origin free hydrolysates and peptones in its state-of-the-art, purpose-built facility in Austin, Minnesota (United States). The company's products are used by pharmaceutical and biotechnology manufacturers to generate blockbuster drugs – either as the sole source of nutrients for cell cultures, or as a critical tool for augmenting existing fermentation batches. Companies that use Nu-Tek products often require data to initiate research and development programs; of particular interest is the ability of plant-based products to perform similarly (or better) than animal-origin products.

In partnership with the University of St. Thomas (Marsh Lab), Nu-Tek's HSP-A (soy peptone), HSP-I (soy protein isolate) and HPP-A (pea protein isolates) were evaluated using two commonly used strains of E. coli (DH5a and epi300) as the model organism for plasmid DNA production, and evaluation of "leaky" expression by the cells when exposed to a different nutrient media of varying peptone composition. **This study demonstrates the performance – plasmid yield and impact on copy number control– as metrics of the plant-based (soy and pea) media compared to Tryptone-containing (animal-based) media.** This is significant, as Tryptone, an animal-derived casein protein hydrolysate, is commonly used in microbiology to culture bacteria, fungi, and protozoa and is less prone to trigger leaky expression in E. coli production platforms using carbohydrate-based induction systems.

Methodology

Cultures of E. coli strains DH5a, carrying pUC19, and EPI300 carrying a construct of pCC1Fos were maintained and propagated in LB media (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl). Media included 50 mg/L ampicillin for maintaining pUC19 and 12.5 mg/L chloramphenicol for maintaining pCC1Fos. The different peptone sources used in the LB formulation included pancreatic digest of casein (Tryptone, Sigma-Aldrich), HSP-A (soy), HSP-I (soy isolate), and HPP-A (pea) (Nu-Tek Biosciences). Blends of plant peptones were also prepared in varying proportions, resulting in a final volume of 10 g/L of peptone. Resulting culture cell densities were measured using a Cary 300 UV-Visible Spectrophotometer at 600 nm wavelength. All cultures used to assess growth performance were inoculated to an initial cell density of 0.05 OD600 and incubated at 37°C with 250 RPM shaking for 12 hours.

A QIAprep Spin miniprep kit was used to purify pUC19 and pCC1Fos. The yield of plasmid DNA was measured by spectrophotometry using a NanoDrop2000 (ThermoFisher). Purification of plasmid DNA from DH5a, a common strain of E. coli known maintain and amplify plasmid DNA. DNA isolation to measure relative plasmid copy number of the pCC1Fos construct was performed using the QIAGEN DNeasy Blood and Tissue kit. Quantitative real time PCR (qPCR) was used to measure the amount of plasmid copy number relative to the amount of host chromosomal DNA. The primers used for qPCR targeted the Chloramphenicol resistance (CMR) gene (fosmid DNA) and the dnaB gene (E. coli chromosomal DNA). This ratio was collected as an indicator of plasmid copies per genome. A two-stage cycle was used for a total of 40 cycles, each with a 30 second phase of alternating 94 and 60°C. Known quantities of pCC1Fos construct and EPI300 genomic DNA were used to produce standard curves from five 10-fold dilutions, ranging from 0.001-10 ng of dnaB and 0.0001-1 ng of ChlR. Successful substitutions of media components for alternatives, such as plant-derived for animal-derived, should show similar - if not better - performance than the standard components, especially as it relates to cell proliferation.

Results: Impact of the inclusion of plant-based peptone on the yield of high copy number plasmid DNA.

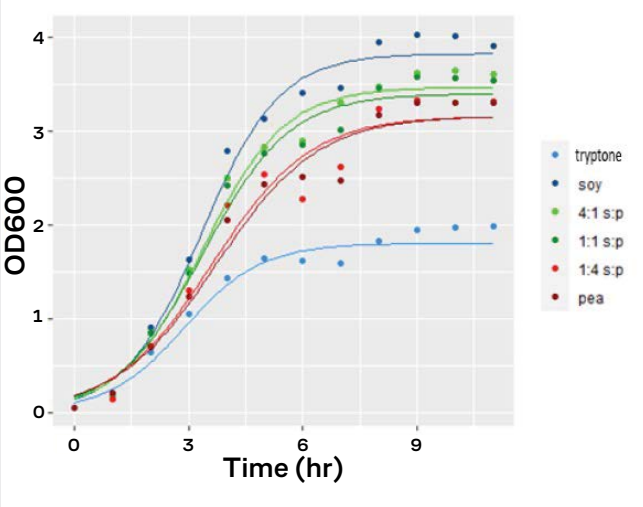

Figure 1. Growth performance and plasmid yield as a function of peptone source. The plot of OD600 vs time shows the growth of E. coli strain DH5a:pUC19 propagated in LB with peptone source indicated by the legend. Ratios indicate proportion of soy to pea protein isolate in the total of 10 g/L peptone in the LB formulation.

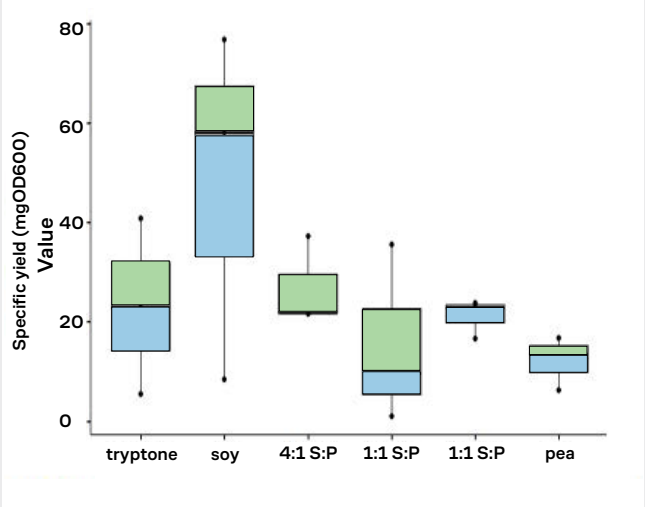

Figure 2. Specific yields of plasmid DNA as a function of media composition indicate similar specific yield of pDNA.

Figure 1 shows the change in cell density (OD600) over the culture duration when using HSP-A and HPP-A. All alternatives to Tryptone show increased carrying capacity and specific growth rates.

The second consideration of changing media components observed is the similarity of plasmid copies numbers per cell (typically 500-700 copies per cell for pUC19). The specific yield of pUC19 (mg/OD600), which is proportional to copy number per cell, is also shown in Figure 1. No statistically significant difference in the copy number of pUC19 per cell was measured when using animal-free media components compared to Tryptone. **Taken together, the data in Figures 1 and 2 show an increase in plasmid yield in HSP-A test groups.** This yield demonstrates the positive response of cell production using a plant-based media.

Impact of peptone composition on the of control plasmid copy number

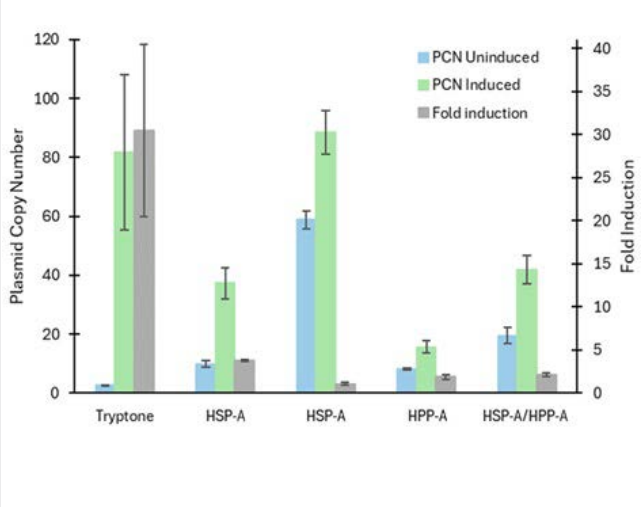

Figure 3. **Comparing plasmid copy number for uninduced and induced cultures and fold induction.**

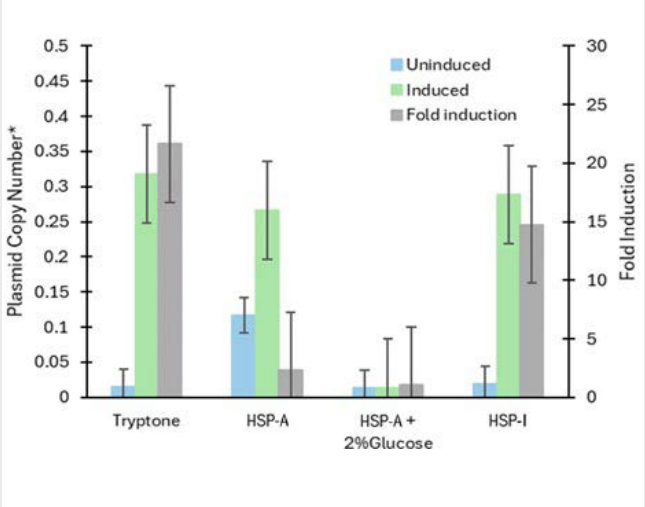

Figure 4. **Relative change in plasmid quantity proportional to PCN for induced v. uninduced cultures**

The E. coli strain EPI300 is engineered to control the Plasmid Copy Number (PCN) of pCC1Fos using an AraC pBAD induction system. Fosmids are typically low copy number plasmids and, in this case, pCC1Fos maintained in EPI300 can have as much as 30-fold increase in PCN when the induction system is activated to increase pCC1Fos PCN by the uptake of arabinose (the inducer). The presence of arabinose and similar carbohydrates in plant-derived peptones is an important consideration when using a carbohydrate system to induce gene expression in a controlled manner.

The qPCR data in Figures 3 and 4 show comparable yields for HSP-A and Tryptone; however, the control of PCN in epi300 by the addition of the arabinose inducer is decreased by the presence of carbohydrate components of the animal-free peptones. Compared to the tryptone induction, the organisms show instances of “leaky expression” in soy and pea blends due to trace carbohydrates. While “leaky expression” may be acceptable or even advantageous depending on the expression product, often rigorous control is preferred. However, using peptones prepared from HSP-I does not cause “leaky expression,” as it lacks carbohydrate and achieves a similar yield and fold induction compared to tryptone. **Reduction of leaky expression through catabolite repression of the pBAD promoter was observed when 2% w/v glucose was included with the peptone HSP-A (Figure 4)**.

The Future of Animal-Origin Free Cell Culture

Cultured cells, and their resulting cellular products, are the cornerstone of biotechnology innovation. Pharmaceutical companies are actively leveraging cells and their ability to produce life-saving products; as such, companies are also pursuing ways to limit risks to the development and production of their product portfolios. With multiple risks and rising regulatory pressures in the use of animal-derived products, researchers are exploring plant-derived options to support their cell culture needs.

With the shifting needs of cell culture nutrient sources, Nu-Tek BioSciences offers several options that developers could utilize. From the development work demonstrated above, several data points have been highlighted:

- **The plant protein hydrolysates manufactured by Nu-Tek BioSciences successfully supported growth of E. coli and production of plasmids.**
- **Cells grown with HSP-I behave similarly to those grown with Tryptone in that they do not demonstrate "leaky expression," providing evidence supporting the use of plant-based peptones in place of animal-derived products for cell culture that requires "leaky expression" to be controlled; this can be important to certain. Alternatively, cells grown in HSP-A demonstrated "leaky expression," which may be critical to some biotechnology production processes.**
- **Combined with cost savings, reduced contamination risk, and stable supply, plant-based media additives are well-positioned for use as an alternative to animal-based products.**

Nu-Tek's products fill a growing need in the pharmaceutical and biotechnology industry; as the demand for fermentation grows, animal-based culture media may not meet the demand and production risk mitigation strategies. Model organisms, cultivated using plant-based peptones and expressing the behaviors required by pharmaceutical demands, provide a strong indication of the utility of peptones to meet cell culture and fermentation needs. More information about Nu-Tek BioSciences's plant-based peptones and culture media can be found at www.nu-tekbiosciences.com.

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