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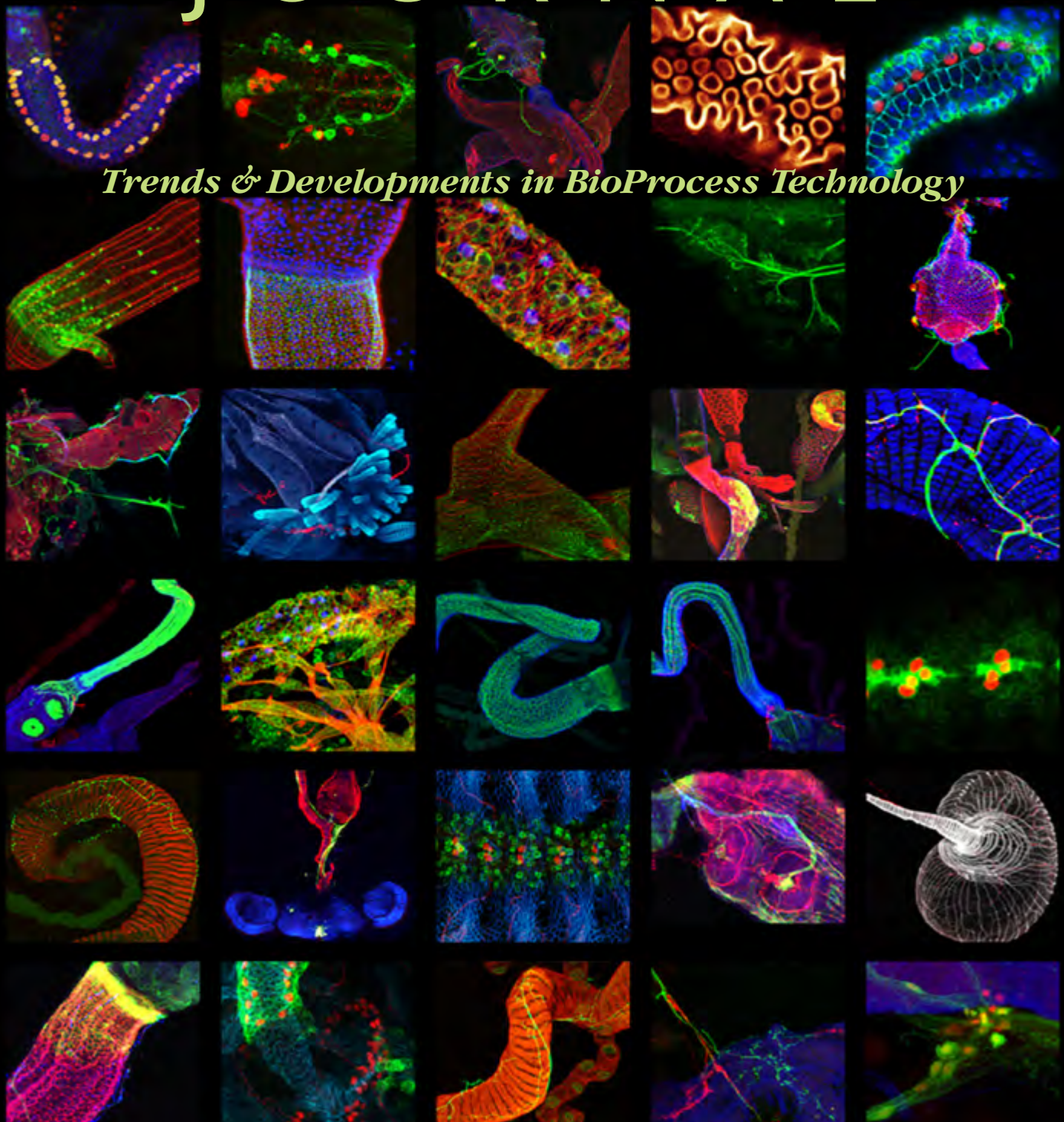

Trends & Developments in BioProcess Technology

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BioProcessing JOURNAL
TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Adapted from Application Note 20, Cambridge Isotope Laboratories, Inc.

Effective Site-Specific Isotopic Labeling: *Expression Optimization Using Specialized Media*

By KENNETH C. BONANNO

Introduction

For decades, recombinant genes have been expressed in a variety of cellular systems to generate protein reagents that are the potential targets for new small-molecule drugs. As these targets become more complex, researchers have developed innovative methods to study the structure of these proteins and their interactions with potential drugs.

BioExpress® 2000 media (Cambridge Isotope Laboratories, CIL) incorporates isotope-labeled amino acids into recombinant proteins expressed in baculovirus-infected insect cells to assist nuclear magnetic resonance (NMR) structural studies.[1] In order to use this labeled media most efficiently, expression conditions must be optimized at a small-scale prior to production.

Methods and Materials

The goal of this study was to maximize the expression of a histidine-tagged, human serine/threonine kinase in the *Spodoptera frugiperda* (SF9) cell line with site-specific isotopic labeling (^{15}N, ^{13}C-labeled glycine; ^{15}N, ^{13}C-labeled phenylalanine; and ^{15}N-labeled tryptophan) in CIL BioExpress® 2000 media. The target enzyme was expressed in baculovirus-infected insect cells cultured in small volumes (2 mL). Expression was optimized by adapting a previously designed high-throughput, small-scale expression protocol[2] and incorporating design of experiments (DOE).[3] Conditions most widely optimized for insect cell expression are: (1) multiplicity of infection (MOI); (2) harvest time (hours post-infection [HPI]); and (3) cell density at infection (cells/mL). A matrix of these variables was explored in this experiment to determine the maximum protein yield, measured in mg/L.

A Box-Behnken response surface model (RSM)[4, 5] was applied in the experimental design using three equally spaced factors (low, medium, high) for each of the three conditions (MOI, HPI, and cell density) in order to study the quantitative response/protein yield. This model provides 13 conditions including a center point with replicates. DOE allows the exploration of many variables via a limited number of experimental conditions using a statistically significant model produced by the experimental data.[6]

Cells were grown in 24-well block microplates (GE Healthcare/Whatman™) and infected with baculovirus at a cell density and MOI specified by the Box-Behnken design. Cell cultures were pelleted and frozen upon harvest. Cell lysates were solubilized and the target protein was purified using HIS-Select® nickel magnetic agarose beads (Sigma-Aldrich). The protein yield was determined using a dot blot assay with an anti-histidine primary antibody (BD BioSciences) and a goat anti-mouse secondary antibody that fluoresces at a 680 nm wavelength (LI-COR Biosciences) using the Odyssey® infrared imaging system

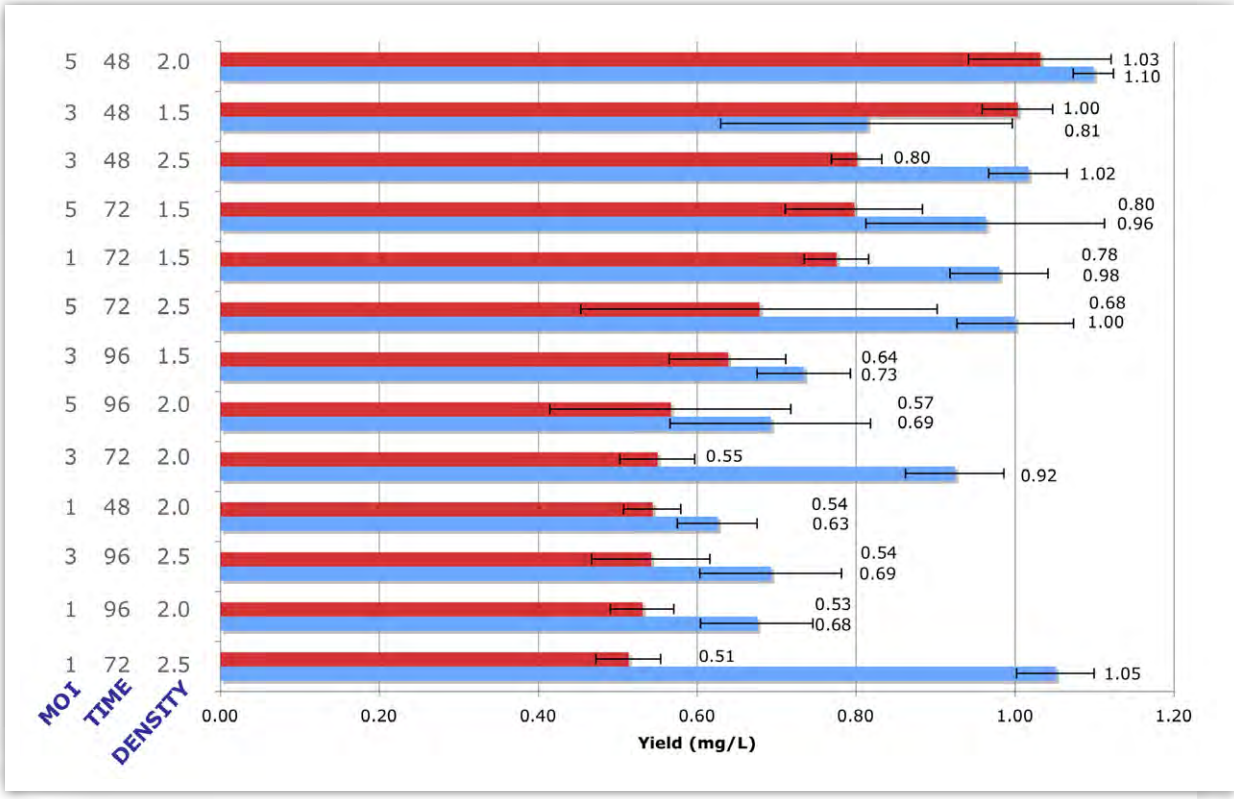

FIGURE 1. Site-specific isotopic labeling in BioExpress 2000 media (**red**) and unlabeled expression in a standard media (**blue**). Student's *t*-test indicates BioExpress 2000 media provides comparable expression yields to those of a standard media at 69 % of the conditions used in the DOE experiment.

A

Variables Explored	Low	Mid	High
Multiplicity of Infection	1	3	5
Cell Density (10^6 cells/mL)	1.5	2.0	2.5
Time of Infection (hrs)	48	72	96

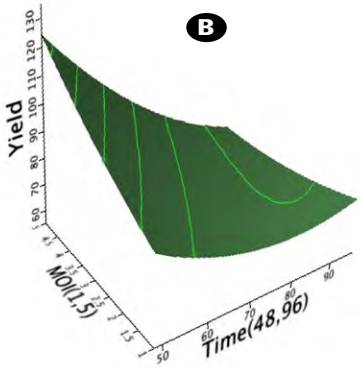

FIGURE 2. Insect cell expression of a recombinant human serine/threonine kinase with residue-specific $^{15}N/^{13}C$ labeling in SF9 cells. **(A)** A Box-Behnken design was used to maximize expression. **(B)** The contour profile represents the response surface model from the data showing that the optimal predicted yield at cell density 1.5×10^6 cells/mL would be achieved at high MOI and a relatively short amount of time.

and the analysis software provided (LI-COR Biosciences) (Figure 1).

The expression data was analyzed with statistical software (JMP™ v6.0) to determine if the data sufficiently fit the model (Figure 2). The Box-Behnken model was verified since yield data provided adequate fit ($R^2 = 80\,\%$) and there were significant regression factors in the model ($p_{yield} < 0.001$). Additionally, the *p*-value for the lack of fit test was not significant ($p > 0.05$). The data predicted optimal conditions for the large-scale expression to generate a yield of 1.3 ± 0.1 mg/L, which is a 20 % improvement over the best condition in the screen. As is the case with many experiments incorporating DOE, the optimal condition from the model (MOI = 5, HPI = 48, cell density = 1.5×10^6 cells/mL) corresponds to conditions not actually tested in the initial experiment. Before producing a large-scale bioreactor production batch, a 0.5 L scale expression experiment was carried out at the optimal conditions to validate the model. A 2.8 L Fernbach flask was used for the validation experiment since it has been found to provide comparable results to a bioreactor. Standard media was used for the validation since the small-scale study indicated that expression levels were comparable between the two media types at 69 % of the conditions, as indicated by the Student's *t*-test one-way analysis of variance (ANOVA) (Figure 1). The best experimental condition and the optimal condition predicted by

Variables Explored	Predicted Condition from RSD*	RSD* Optimal Condition
Multiplicity of Infection	5	5
Cell Density (10^6 cells/mL)	1.5	2.0
Time of Infection (hrs)	48	48
Small-Scale Yield (mg/L)	Not Tested	1.1
Validation Yield (mg/L)	2.2	1.6
*Response surface design		

FIGURE 3. Caliper LC90 quantitation and virtual SDS-PAGE representation of the validation expression experiment. The DOE-predicted condition produced a 40% greater yield than the best condition from the model, and a 100% improvement to the RSD optimal yield over the small-scale experiment.

Expression Scale	Volume (mL)	Vessel	Media	Yield (mg/L)	Analysis
DOE Screening	2	Deep Well Block	BioExpress 2000	0.5 – 1.0	Dot Blot **(A)**
Validation	500	Shake Flask	Standard Media	2.2	SDS-PAGE **(B)**
Production	2000	Wave Reactor	BioExpress 2000	1.3	SDS-PAGE **(C)**

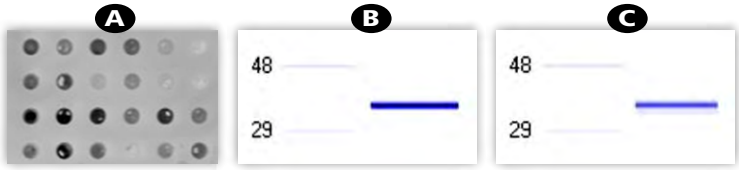

FIGURE 4. Expression summary. DOE screening occurred in a high-throughput format and was analyzed using infrared fluorescence Dot Blot with LI-COR quantitation. Validation and production yields were analyzed and quantified with Caliper LC90 and virtual SDS-PAGE. Validation confirmed the optimal condition defined by the DOE experiment using standard unlabeled medium. Large-scale expression was then performed using the optimal condition defined by the DOE experiment using labeled medium.

the model were expressed. The target protein was purified using nickel affinity resin, and the protein yield was quantified using LC90 Lab Chip® technology (PerkinElmer/Caliper) to validate the yields obtained in the small-scale screen. The model's predicted expression condition produced a 40% greater yield than the best experimental condition using a standard media (Figure 3).

The hypothesized optimal expression conditions (MOI = 5, HPI = 48, cell density = 1.5×10^6 cells/mL) were used for production in the BioExpress 2000 media using protocols recommended by CIL. One liter of cells was cultured in a 2 L Wave Cellbag (GE Healthcare) for each batch. The final yield of the affinity purified protein was 1.3 mg/L which was within the error of the model's prediction for labeled media. Figure 4 describes the process implemented from screening through validation into production.

Summary

It is important to connect laboratory techniques and procedures with statistical methods to maximize the experimental efficiency at every level of experimentation. Incorporating isotopically-labeled amino acids into insect cell media increases the cost of the media. Therefore, optimal expression conditions for the target protein are desired before advancing to this media. DOE and statistical analysis control the size of the experiment and allow efficient use of reagents, such as labeled media, for optimization purposes. This enables more of the labeled media to be used for the scale-up processes and ultimately, to supply material to generate NMR samples. Designing a screening experiment using high-throughput miniaturization and sound statistical models minimizes reagent consumption while maximizing the expression yields of protein production batches.

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