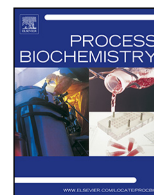
Process Biochemistry 49 (2014) 130–139

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Brown drug substance color investigation in cell culture manufacturing using chemically defined media: A case study

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A R T I C L E I N F O

Article history:
Received 8 September 2013
Received in revised form 19 October 2013
Accepted 22 October 2013
Available online 30 October 2013

Keywords:
Protein drug substance color
N-formylkynurenine
Kynurenine
CHO cell culture
Chemically defined media
Iron

A B S T R A C T

Drug substance (DS) color is an important quality attribute for release, stability and comparability studies of biologics. With the increase of DS concentrations and biologics pipelines made in chemically defined media, atypical DS color other than colorless or pale yellow has been recently reported in the biopharmaceutical industry. We recently observed a brown DS color in manufacturing. Although analytical characterization data indicated that the brown color DS had no major quality issue, it is necessary to find the root cause and reduce DS color to ease placebo design for clinical use. It was demonstrated that the brown color was caused by the chemically defined basal medium containing high levels of iron and vitamin B12 (VB12) regardless of cell lines. Iron caused tryptophan oxidation in the protein to form N-formylkynurenine and kynurenine products, which likely contributed to a yellow DS color. A pink DS color was caused by the residual VB12 bound to DS. The brown color was the result of the combinatory effect of yellow and pink colors. Finally a modified basal medium was developed to produce a pale yellow DS in manufacturing.

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1. Introduction

Drug substance (DS) and drug product color is one of the quality attributes required to be measured for release, and is also evaluated during the stability and comparability studies of monoclonal antibodies (mAbs) and fusion proteins, which constitute a large and growing portion of biologics pipelines mainly produced using Chinese hamster ovary (CHO) cells. Atypical DS color is probably most apparent for manufacturer, health care provider and patient. Although color alone does not necessarily indicate drug safety or efficacy issues, inconsistent or atypical product coloration suggests lack of process understanding and control, and raises potential concerns over product impurity levels, as well as product biophysical/biochemical stability during cell culture production, purification, formulation, transportation and/or storage [1,2]. Therefore, it is imperative to gain a thorough understanding of process and molecule related factors that impact product coloration, and the linkage between color and other critical quality attributes that ultimately impact clinical trials.

Some proteins naturally have color due to chromophore content [3,4], whose distinctive spectrophotometric absorption profile can be readily captured by spectrophotometer as a signature of the protein. The absorption of mAbs and fusion proteins is limited and largely similar in the visible spectrum range. Color testing by visual assessment is currently the general practice in the biopharmaceutical industry. It is described in European Pharmacopoeia that the drug product liquids for clinical trials are required to be colorless or slightly colored [5]. A narrow color range from colorless to pale yellow is typical for mAbs and fusion proteins. In recent years, as high concentration formulation becomes more attractive to enable subcutaneous administration [6], slight variation in product coloration becomes more noticeable. Thus, there are increased interests in understanding the origins and impact of color change in biologics. However, published reports in this area are still limited. Qi et al. [2] investigated DS discoloration associated with high concentration liquid formulation during stability studies. Photodegradation, specifically oxidation of a tryptophan (Trp) residue located in the light chain and its photodegraded byproducts, contributed to the observed color change. The same group also evaluated effects of environmental and formulation parameters on discoloration and product degradation, and identified multiple degradation pathways including aggregation, fragmentation,

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http://dx.doi.org/10.1016/j.procbio.2013.10.015

Table 1
Comparison of the upstream processes including clone, basal and feed media, and heavy metal concentrations (μM) in final DS for the 500-L bioreactor runs.

Upstream process	Cell line	Basal medium			Feed medium			DS		
Process1	C1		B1			F1			LotA	
		Iron	Copper	VB12	Iron	Copper	VB12	Iron	Copper	Cobalt
		1×	1×	1×	1.6×	0.9×	3.3×	0.03	ND[a]	ND
Process2	C2		B2			F2			LotB	
		Iron	Copper	VB12	Iron	Copper	VB12	Iron	Copper	Cobalt
		11×	85×	12×	1.6×	0.9×	3.3×	1.60	0.88	0.38

[a] ND: not detected.

oxidation and deamidation that correlated with loss of bioactivity [2].

As cell culture and downstream purification processes strive toward higher productivity, it presents both opportunities and challenges to understand and control process parameters affecting product quality attributes, including color. One example is the upstream medium optimization. Due to safety concerns related to raw materials derived from animal sources or lot-to-lot variations of undefined hydrolysates, fully chemically defined media are being adapted for cell culture platform process development [7,8], which enables the effect of individual medium components and their interactions on cell culture productivity and product quality to be more readily studied. Different concentrations of iron [9–14] and copper [15–17] in serum free and chemically defined media were studied for their effects on growth of different cell lines, productivity of different proteins and different quality attributes. There is very limited literature on the effect of vitamin B12 available. Although a lot of progress has been made to improve cell growth and productivity, functions and optimum concentrations of many medium components and their interactions are still not fully understood. For example, a few reports have just been published to study the effect of cell culture media on DS color. Of which, high levels of iron and a mixture of vitamins in cell culture media caused more intensive DS color using 2-L bioreactors [14]. Furthermore, the mechanism of pink DS color caused by vitamin B12 was identified via in vitro incubation experiments [18]

Here, it is reported a case study of an investigational approach to addressing the atypical DS color issue during the manufacturing of a fusion protein. Similar challenges as other researchers in measuring DS color were encountered. The total color method and the NIFTY fluorescence method were used for quantitative measurement of DS color, but both methods cannot measure different hues [14]. It was found that the visual assessment of color intensity and hues by putting 5 mL DS in a glass vial was straightforward and sufficient for this study. DS color change from pale yellow to light brown was initially observed in 500-L bioreactors. A detailed DS characterization was performed to evaluate the impact of the DS color change on product quality attributes. The root cause of this color change was investigated. Experiments were designed to evaluate the effects of cell line, upstream medium compositions, and other upstream and downstream process parameters. The mechanisms of action were studied. The effects of selected medium components on cell culture performance were also evaluated. Finally, a modified basal medium was developed that resolved the DS color issue in manufacturing.

2. Materials and methods

2.1. Cell lines, media and cell culture processes

Two CHO DG44 cell lines, C1 and C2, were used for expression of a proprietary fusion protein using a vector with a DHFR selectable marker.

Three chemically defined basal media, B1, B2 and B3, were used. B1 was a proprietary platform basal medium containing low levels of VB12 (1×), iron in the form of $FeSO_4$ (1×) and copper in the form of $CuSO_4$ (1×) (Table 1). After performing design of experiment (DOE) based on B1, B2 was developed with enhanced nutrients including amino acids, mineral salts, lipids and vitamins. The concentrations of VB12, iron and copper in B2 were more than 10-fold of those in B1 (Table 1), while the concentrations of all other components were within 2-fold difference. To reduce DS color, B3 was modified based on B2 after reducing VB12, iron and copper to the low concentrations (1×), the same as those in B1. Two chemically defined feed media, F1 and F2, were used for fed batch culture process. F1 was a proprietary platform feed medium. After DOE design based on F1, F2 was developed with enhanced nutrients including amino acids and lipids, while maintaining the same concentrations of VB12 (3.3×), $FeSO_4$ (1.6×), and $CuSO_4$ (0.9×). Seed media were prepared with basal media plus 1 μM methotrexate.

Two lead lab upstream processes were scaled up in 500-L bioreactors with an initial working volume of 300 L. For LotA DS manufacturing, the bioreactors were operated in fed-batch mode using the upstream process1 including cell line C1, B1 basal medium and F1 feed (Table 1). The bioreactors were initially operated at 37 °C and shifted to a lower temperature when the culture reached a proprietary level. Feeding was started when appropriate viable cell density (VCD) was achieved and fed daily thereafter. Additional glucose was applied to maintain its concentration at a proprietary level. The pH and DO were maintained at pH 7.1 and 60% of air saturation respectively. The bioreactor pH was controlled by addition of CO_2 gas to decrease pH and addition of 1 M Na_2CO_3 base to increase pH as needed. The inoculation density was 0.3×10^6 cells/mL for cell line C1. For LotB DS manufacturing, the 500-L bioreactors were operated in fed-batch mode using the upstream process2 including cell line C2, B2 basal medium and F2 feed (Table 1). The bioreactors were operated at 37 °C for the entire run. The inoculation density was 0.6×10^6 cells/mL, while DO was maintained at 30% of air saturation. Other operation controls for LotB were similar to those for LotA DS manufacturing.

Fed batch shake flask experiments with different basal media and F2 feed were performed for the root cause investigation of atypical DS color and their impact on cell culture performance. The cultures were maintained on a shaker at 37 °C in an incubator with 6% CO_2. One molar Na_2CO_3 base was used to increase pH as needed. The same seeding densities and feeding strategies of the upstream process2 were applied to either 250-mL shake flasks containing an initial volume of 80 mL or 1-L shake flasks containing an initial volume of 400 mL.

In order to reduce the light brown DS color, 20-L bioreactor runs using the process2 with F2 feed were performed to test the impact of different basal media on cell culture performance and DS color.

2.2. In-process assays

VCD and cell viability were measured off-line using a Cedex automated cell counter (Innovatis AG). VCD was reported as normalized values, which are the actual values divided by the average of peak VCD of two 500-L runs for the process2. Culture samples were also analyzed off-line using a BioProfile 400 Analyzer to monitor pH, pCO_2, pO_2, glucose, glutamine, glutamate, lactate, and ammonium (Nova Biomedical Corporation). A Protein A HPLC method was used to measure protein titer [19]. Titers were reported as normalized values, which are the actual values divided by the average of two 500-L run titers for the process2.

2.3. Harvest and purification process

Only one harvest and purification process was used in this study. Cell culture fluid was clarified using centrifugation, depth filtration followed by sterile filtration through 0.2 μm filters. Clarified harvest was processed through a three column process, consisting of MabSelect Protein A capture, viral inactivation, cation exchange chromatography (CEX), anion exchange chromatography (AEX), and ultrafiltration/diafiltration (UF/DF) steps to generate formulated bulk drug substance at a target concentration of 50 g/L, unless otherwise specified. During the lab scale downstream processing for color evaluation, the depth filtration was not used for the harvest step and UF/DF was performed using 30 kDa spin columns (Cat# UFC 903008, EMD Millipore). For DS abbreviations, the manufacturing lots at 500-L bioreactor scale were named as LotA and LotB, while the lab lots at 1-L shake flask scale were named as DS1, DS2, DS3, etc.

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