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Prediction of filamentous process performance attributes by CSL quality assessment using mid-infrared spectroscopy and chemometrics

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ABSTRACT

Every biopharmaceutical production process aims for control strategies to achieve process robustness in order to ensure consistent product quality. Process variability can origin from process parameters, the biological nature as well as from high lot-to-lot variability of raw materials. In filamentous processes raw materials with very complex matrices, such as corn steep liquor (CSL), are used, which are especially challenging to characterize.

In this study, CSL was characterized in detail for its ingredients presenting an overall composition of its matrix of 50 analyzed components (19 amino acids, 5 organic acids, 8 reducing sugars, 7 water-soluble vitamins and 11 trace elements/minerals) in order to facilitate analytical reduction to fingerprinting methods FT-MIR was evaluated as fast and non-destructive spectroscopic fingerprinting method for adequate assessment of CSL quality. Feasibility of this method was shown by the correlation of certain bands in the spectra to substance groups present in CSL, such as the Amide I and II band and amino acids, respectively. Additionally, applicability of FT-MIR could be shown for classification of different CSL lots differing in provider and corn quality as well as for predictability of process performance attributes. The latter was demonstrated on a fed-batch filamentous fungi process for the production of antibiotics. By multivariate data analysis, it could be shown that CSL quality assessment via FT-MIR can be used for the prediction of maximal biomass generated in the process, with a correlation coefficient R^2 of 0.964, as well as for the prediction of an unwanted impurity. The combination of a fast and easy method for CSL quality assessment and correlations of this quality with process performance attributes may facilitate the establishment of a risk-based acceptance criteria for raw material quality release of CSL. As CSL is a frequent used raw material, we believe that this method will also be useful for other processes and that CSL quality assessment is of high relevance in academia and industry.

1. Introduction

Quality assessment of raw materials that are used for media preparation for bioprocesses is a highly discussed issue. Especially within the production of pharmaceutical products, the influence of raw materials on the complete production process has to be defined and specified, in order to assure controlled quality (ICH, 2017). Particularly, in mammalian cell culture tools for the quality analysis of raw materials are already established (Jose et al., 2011; Kirdar et al., 2010; Li et al., 2010). The variability of raw material quality increases with biological origin and therefore also with the complexity of the analyzed material.

Corn steep liquor (CSL) is a byproduct of the wet-milling process and is commonly used as N-, C- or vitamin source in diverse bioprocesses. The most prominent example of CSL application is the penicillin production process with *Penicillium chrysogenum*. Since 1941 CSL has been used as a media supplement in this process, as it yielded 5-fold

Abbreviations: ATR, attenuated total reflection; CSL, Corn Steep Liquor; DTGS, deuterated triglycine sulfate; FT-MIR, Fourier transform - mid-infrared spectroscopy; HPLC, high performance liquid chromatography; IC, ion chromatography; ICP-OES, inductively coupled plasma - optical emission spectrometry; LV, latent variable; NIR, near-infrared spectroscopy; PCA, principal component analysis; PC, principal component; PLS, partial least squares

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higher productivity. The reason is that its complex composition arbitrates as a substrate, which enhances spore germination of filamentous fungi in the early stages of the cultivation process (Posch et al., 2012). The exact mechanisms of this phenomenon have not been disclosed in detail yet mainly because of the complexity of CSL but also due the complex metabolic system of the fungi, which metabolizes not only the C-substrates available in CSL but also amino acids and vitamins.

Additionally to its complex structure, CSL consists of two phases, liquid and solid phase. These characteristics complicate on the one hand the analytical fractionation of its detailed composition and on the other hand the prediction of bioavailability of the various components present in CSL for the cells. The uncertainty of the bioavailable compounds in CSL leads to a lack of knowledge of how CSL is actually influencing the product quality and overall process performance.

In terms of risk assessment it is crucial to establish a method which facilitates release or rejection of raw material in quality control, especially for complex matrices such as CSL. Tools for quality assessment of CSL have not properly been investigated so far. Most studies have focused on the detailed determination of the composition of the liquid phase in CSL. High-performance liquid chromatography (HPLC) was used to determine amino acids and vitamins (Christianson et al., 1965; Hofer and Herwig, 2017; Xiao et al., 2013; Zhang et al., 2011). Atomic absorption spectrometry (AAS) and inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used for the detailed analysis of metal element composition (Zhang et al., 2011). Corn steep water during steeping was investigated for carbohydrates, lactic acid, glycolic acid and fatty acids by gas chromatography- mass spectrometry (GC–MS) (Hull et al., 1996). The successful classification of different CSL origins was reported, as well as correlations between CSL quality attributes, such as total nitrogen, and NIR spectra (Hofer and Herwig, 2017; Xiao et al., 2012; Xiao et al., 2013). However, only Gao et al. (Gao and Yuan, 2011) reported any relationships between CSL quality and process performance. They focused on a bacterial process for the production of 2-Keto-L-gulonic acid (2-KLG) and were able to show the influence of specific components in the supernatant of CSL on the 2-KLG concentration in the harvest. CSL was analyzed by gas chromatography with time-of-flight mass spectrometry (GC-TOFMS) and ICP-AES. However, no practicable tool has been presented so far for quality assessment of CSL. Furthermore, the impact on the more complex biological system of filamentous fungi has not been investigated up to now. Additionally, the complexity of CSL composition and bioavailability was disregarded so far, as only the liquid phase of CSL was investigated in order to obtain classification and correlations with e.g. 2-KLG concentrations.

The aim of this study was to establish a fast and easy tool for direct analysis of raw material attributes within CSL, which will allow a reliable prediction of related process performance attributes, such as filamentous growth. Acceptance criteria for the quality control release or rejection of the raw material can be deviated of this method and may facilitate a reasonable risk-based raw material management program at the earliest possible time point, i.e. after delivery of the raw material.

In order to reach this goal, understanding of the CSL matrix is necessary, which further on allows a sound analytical reduction by e.g. fingerprinting techniques, which is the focus of this contribution. Therefore, CSL was analyzed in detail for specific components such as amino acids, organic acids, reducing sugars, water-soluble vitamins and trace elements. Most likely, the nutrient effect of CSL on process performance is not triggered by a single ingredient but a nutrient mix. The identification of this mix might be possible but not feasible, as – concerning incoming quality control of the raw material – it would take different methods to analyze all critical substances, including time-consuming sample preparation procedures. Hence, the CSL samples were additionally analyzed for their fingerprinting via near infrared spectroscopy (NIR) and Fourier transform – mid infrared spectroscopy (FT-MIR). Both methods allow rapid and immediate analysis of the homogenized sample without any previous preparation steps. Furthermore, spectroscopic measurements are accepted methods in the field of process analytical technology (PAT) (Bakeev, 2010). In order to achieve the goal of improved process robustness through CSL quality assessment the following steps were performed:

i) Identification of major substance classes in total CSL as reference for the fingerprinting analysis
ii) Evaluation of a suitable non- destructive fingerprinting method for CSL analysis
iii) Investigation of correlation between specific substances and the fingerprinting spectra via partial least squares (PLS) regression
iv) Classification of CSL samples by spectroscopic fingerprinting
v) Investigation of correlations between CSL quality (i.e. spectra) and process performance via PLS

Finally, a conclusive tool for assessment of raw material quality and process performance could be presented for the investigated bioprocess for antibiotic production via filamentous fungi. This method may be implemented for establishment of an acceptance criteria for release or rejection of raw material directly after delivery, hence, it may increase process robustness. We believe that the proposed method for quality assessment of CSL is of high relevance to further research activities for academia and industry.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and substances were of analytical grade and purchased from either Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (St. Louis, MO, USA). For analytical methods ultra-pure water was used, derived from a Milli-Q system from Merck Millipore (Billerica, MA, USA).

2.2. Analytical methods

2.2.1. HPLC methods for the analysis of vitamins, amino acids and organic acids

Vitamins were analyzed according to (Hofer and Herwig, 2017).

Amino acids were digested from dried CSL following a two-step protocol: first enzymatically by protease from *Streptomyces griseus* for 72 h at 37 °C and then chemically by methansulfonic acid solution containing 0.2% tryptamine for 1 h at 160 °C. Chromatographic separation was achieved with a reversed phase column (Agilent Eclipse AAA, 3 × 150 mm, 3.5 µm), a guard column (Agilent Eclipse AAA, 4.6 × 12.5 mm, 5 µm) and a gradient using eluent (A) 40 mM NaH_2PO_4 monohydrat pH 7.8 and eluent (B) MeOH/ACN/MQ (45/45/10, v/v/v). The protocol was run with a flowrate of 1.2 mL min-1, the column oven temperature was set to 40 °C and the injection volume was 10 µL. As most amino acids have no fluorophore in their structure, an in-needle derivatization step was performed using *ortho*-phthaldialdehyd (OPA) containing 1% of 3-MPA and 9-Fluormethylencarbonylchlorid (FMOC). In order to guarantee sample quantification despite the derivatization step, every sample and the standards were spiked with sarcosine and norvaline as internal standards. Primary amines and norvaline were detected at Ex 340 nm/Em 450 nm and secondary amines and sarcosine were detected at Ex 266 nm/Em 305 nm.

Organic acids could be extracted from dried CSL with a method adapted from (Suh et al., 1997). 20 µL of sample were injected on an Aminex HPX-87H (Bio-Rad) column and guard column Aminex HPX 87-H. The method was run isocratically with 0.1% TFA in MQ at 50 °C for 60 min with a flowrate of 0.6 mL min^{-1}. Organic acids were detected at 210 nm.

For all HPLC methods an Ultimate 3000 (Thermo Fisher Scientific, USA) equipped with a pump (LPG-3400SD), a split-loop autosampler (WPS-3000 SplitLoop), a column oven (Col.Comp. TCC-3000SD) a