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Intact cell mass spectrometry as a progress tracking tool for batch and fed-batch fermentation processes

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ABSTRACT

Penicillin production during a fermentation process using industrial strains of *Penicillium chrysogenum* is a research topic permanently discussed since the accidental discovery of the antibiotic. Intact cell mass spectrometry (ICMS) can be a fast and novel monitoring tool for the fermentation progress during penicillin V production in a nearly real-time fashion. This method is already used for the characterization of microorganisms and the differentiation of fungal strains; therefore, the application of ICMS to samples directly harvested from a fermenter is a promising possibility to get fast information about the progress of fungal growth. After the optimization of the ICMS method to penicillin V fermentation broth samples, the obtained ICMS data were evaluated by hierarchical cluster analysis or an in-house software solution written especially for ICMS data comparison. Growth stages of a batch and fed-batch fermentation of *Penicillium chrysogenum* are differentiated by one of those statistical approaches. The application of two matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) instruments in the linear positive ion mode from different vendors demonstrated the universal applicability of the developed ICMS method. The base for a fast and easy-to-use method for monitoring the fermentation progress of *P. chrysogenum* is created with this ICMS method developed especially for fermentation broth samples.

The filamentous fungus *Penicillium chrysogenum* is a producer of β-lactam antibiotics such as penicillin. Penicillin is one of the most important antibiotics and, hence, is indispensable in human and veterinary medicine. A lot of different types of penicillin exist, for example, the naturally occurring penicillin G and the biosynthetically produced penicillin V. Penicillin V, also known as phenoxymethylpenicillin, is stable in acids and, therefore, is more convenient as oral drug than penicillin G, which is not stable under acidic conditions [1]. One possibility for a highly efficient penicillin V fermentation process benefits from *P. chrysogenum* biomass production during batch fermentation and a subsequent fed-batch fermentation for antibiotic production. Fast information of the fermentation progress will help to optimize secondary metabolite production by timely influencing specific fermentation parameters. Monitoring batch and fed-batch fermentation processes using intact cell mass spectrometry (ICMS)[1] can form a basis to achieve this progress information.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) has become a prominent technique in biological mass spectrometry, especially for analyzing intact cells, for example, microorganisms. The reason is that sample preparation is simple (dried solid samples are required), automated analysis is possible over a wide mass range, and with this method results can be obtained within a few minutes. ICMS provides a unique mass spectral fingerprint of surface-associated protein/peptide analytes, which allows the identification of microorganisms from different species as well as different strains [2]. After the implementation of ICMS to bacterial samples during the 1990s [3–7], the field was extended to fungi [8]. Since then, the discrimination between different microbial species or strains by means of ICMS does have a number of different applications [9,10], for exam-

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[1] *Abbreviations used:* ICMS, intact cell mass spectrometry; MALDI–TOF–MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ACN, acetonitrile; TFA, trifluoroacetic acid; vvm, volume of air, volume of medium, unit of time; PPG, polypropylene glycol; SEM, scanning electron microscope; S/N, signal/noise.

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ple, the rapid detection of emerging pathogens [11] and microorganism identification in bloodstream infections [12] or urine samples [13]. *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and wood rotting fungi are the focus of a review regarding filamentous fungal characterization with ICMS [14]. The characterization of intact *Penicillium* spores, or more precisely the comparison of different *Penicillium* species and strains by MALDI–MS, was published in 2005 [15]. A differentiation of six species—*P. expansum*, *P. chrysogenum*, *P. italicum*, *P. digitatum*, *P. citrinum*, and *P. pinophilum*—was achieved by the identification of specific marker ions for each species. One of the first published time-dependent ICMS studies was the monitoring of bacterial growth using *Escherichia coli* in 1999 [16]. Samples from shaking flask cultures were collected at different time points between 6 and 84 h and were analyzed. Significant variations of the peak pattern were observed, demonstrating that the growth stage of microorganisms needs to be considered by identifying bacterial strains by means of ICMS. Furthermore, ICMS was already used as a monitoring method for the biotechnological production of virus-like particles and viral proteins [17].

The implementation of an ICMS method for a batch fermentation and the more important fed-batch process has not been described so far and was the focus of this investigation. Next to the optimization of an ICMS method for fermentation broth samples of *Penicillium*, the application of the developed method on two different MALDI–TOF instruments, UltrafleXtreme (Bruker Daltonics, Bremen, Germany) and Axima CFR$^+$ (Shimadzu Kratos Analytical, Manchester, UK), was investigated. Hierarchical cluster analysis and a dedicated in-house software solution, written especially for ICMS data comparison, are the two approaches that were used for statistical analysis of the obtained mass spectra. This project forms the basis for monitoring the fermentation progress and should emphasize that ICMS is not limited to genus or strain assignment. Fast knowledge on the progress of fermentation on the protein/peptide level can help to timely influence process parameters, for example, to finally optimize secondary metabolite productivity.

Materials and methods

Chemicals

Ferulic acid and sinapinic acid were obtained from Fluka (Buchs, Germany). Analytical-grade acetonitrile (ACN, p.a., pro analysis) was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Riedel-de-Haën (Seelze, Germany). Water (18.2 MΩ cm) for all solutions was prepared by a Simplicity Millipore system (Billerica, MA, USA). Insulin, apomyoglobin, and cytochrome *c* were obtained as ProteoMass MALDI–MS standards and purchased from Sigma–Aldrich (Steinheim, Germany).

Strains and cultivation conditions

Sandoz (Kundl, Austria) provided the spore stock suspension of an industrial *P. chrysogenum* candidate strain for penicillin production. A batch fermentation and a following fed-batch process for penicillin production are used for the two-stage industrial-scale penicillin production process. For fed-batch cultivations A, B, and C, the spore stock solution was stored at 4 °C and experiments were performed over a time span of 2.5 months. After prolonged storage of the spore stock solution employed in cultivations A, B, and C, fed-batch cultivation D was inoculated with a newly produced spore stock solution. Similarly, batch cultivations I and II have been inoculated from one single spore stock suspension, and for inoculation of batch III a new stock suspension was employed. For all fed-batch fermentations, the complex batch medium consisted of sucrose (18 g/L), glucose (3 g/L), corn steep liquor (26 g/L), silicone oil (1 ml/L), and $CaCO_3$ (3.8 g/L). Effects of the complex medium component corn steep liquor on overall cultivation performance, as well as biological variation introduced by complex medium constituents, are discussed in Posch et al. [18]. In this context, one of the three batch-only fermentations (batch process II) was carried out on the complex medium described above, whereas the remaining two (batch processes I and III) were grown on defined medium. The composition of the defined medium was as follows: glucose (30 g L^{-1}), $(NH_4)_2SO_4$ (8.75 g L^{-1}), KH_2PO_4 (1.6 g L^{-1}), $NaNO_3$ (0.2 g L^{-1}), KCl (0.5 g L^{-1}), $CaCl_2\,2H_2O$ (0.067 g L^{-1}), $MgSO_4\,7H_2O$ (0.5 g L^{-1}), TES stock solution (10 ml L^{-1}), and silicone oil (1 ml L^{-1}). TES stock solution consisted of EDTA (14 g L^{-1}), $CuSO_4\,5H_2O$ (0.5 g L^{-1}), $ZnSO_4\,7H_2O$ (2 g L^{-1}), $MnSO_4\,H_2O$ (2 g L^{-1}), and $FeSO_4\,7H_2O$ (4 g L^{-1}).

For all three batch fermentations (batch processes I, II, and III), a 1.8-L stirred bioreactor (Applikon, The Netherlands) with an actual working volume of 1.5 L was used. Cultures were aerated through a standard single-port sparger that was located below the stirrer (constant aeration rate: 0.8 vvm [volume of air, volume of medium, unit of time — used for bioreactors]). PTFE (polytetrafluoroethylene) air filters of 0.2 μm pore size (Whatman, UK) were used to sterilize aeration air, and aeration flow was kept constant with the help of the mass flow controller system 2Proc (Aalborg, USA). The mode of aeration was switched from headspace to submerse to prevent blowout of spores (only after completed germination). Furthermore, the use of an off-gas condenser kept at a temperature of 8 °C by means of a cryostat prevented water stripping from the reactor. The external heat jacket was heated/cooled using a thermo circulator in order to keep the temperature of the culture at 25 °C. The addition of 2.5 M NaOH allowed keeping the pH of the culture at 6.5 ± 0.1. The pH value and the dissolved oxygen tension were measured with the help of a sterilizable pO_2 and pH probe, respectively (both from Mettler–Toledo, USA). A six-bladed Rushton turbine impeller (Applikon) with a separation distance of 25 mm was used to perform agitation. To guarantee sufficient dissolved oxygen tension, the rotation speed was controlled. Therefore, the dissolved oxygen tension was controlled at 40% by adjusting the agitator speed. The integrated process control and management system Lucullus (Biospectra, Switzerland) was used for all process control measures. Culture spores of rice were used to inoculate the processes. Therefore, different concentrations of viable spores for the cultivations were inoculated around the center point of 1.2×10^6 spores/ml by factors of 0.3 and 3. Serial dilution and counting of single colonies after plating were used to determine the viable spore concentration. The addition of PPG (polypropylene glycol) 2000 (up to 1 ml) should prevent foaming during the fermentation process.

The four used fed-batch cultivations (A, B, C, and D) were carried out in a 7.5-L stirred bioreactor (Infors, Switzerland) with the following medium composition: glucose (0.5 g/L), $(NH_4)_2SO_4$ (3.5 g/L), KH_2PO_4 (0.8 g/L), $FeCl_3{\cdot}6H_2O$ (0.01 g/L), $MgSO_4{\cdot}7H_2O$ (0.05 g/L), KCl (0.25 g/L), PPG 2000 (0.025 ml/L), $CuSO_4{\cdot}5H_2O$ (2.75 mg/L), $ZnSO_4{\cdot}7H_2O$ (17.8 mg/L), $MnSO_4{\cdot}H_2O$ (14.75 mg/L), and $CaCl_2{\cdot}2H_2O$ (32.5 mg/L). The feed concentration of glucose was 500 g/L with a flow of 1.017 ml/L/h. After 12 h, the feeding of a 100-g/L $(NH_4)_2SO_4$ solution was started at a rate of 1.35 ml/L/h and was subsequently set to 1.2 ml/L/h from 70 to 120 h, to 1.067 ml/L/h from 120 to 140 h, and to 0.9 ml/L/h from 140 h until process end (∼180 h). The used penicillin V precursor (sodium phenoxyacetate) was fed at 0.3 ml/L/h with a feed concentration of 160 g/L. Cultivation broth (80 ml/L) from batch fermentation (at the time of pH increase indicating carbohydrate exhaustion) was used for inoculation of the fed-batch processes. A standard multiport sparger located below the stirrer at a constant aeration rate of 1.35 vvm was used for aeration of the cultures. The pH of