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High-performance thin-layer chromatography (HPTLC) for the simultaneous quantification of the cyclic lipopeptides Surfactin, Iturin A and Fengycin in culture samples of *Bacillus* species

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ABSTRACT

A high-performance thin-layer chromatography method has been established for the identification and simultaneous quantification of the cyclic lipopeptides Surfactin, Iturin A and Fengycin in *Bacillus* culture samples. *B. subtilis* DSM 10^T, *B. amyloliquefaciens* DSM 7^T and *B. methylotrophicus* DSM 23117 were used as model strains. Culture samples indicated that a sample pretreatment is necessary in order to run HPTLC analyses. A threefold extraction of the cell-free broth with the solvent chloroform/methanol (2:1, v/v) gave best results, when all three lipopeptides were included in the analysis. For the mobile phase, a two-step development was considered most suitable. The first development is conducted with chloroform/methanol/water (65:25:4, v/v/v) over a migration distance of 60 mm and the second development using butanol/ethanol/0.1% acetic acid (1:4:1, v/v/v) over a migration distance of 60 mm, as well. The method was validated according to Validation of Analytical Procedures: Methodology (FDA Guidance) with respect to the parameters linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and recovery rate. A linear range with $R^2 > 0.99$ was obtained for all samples from 30 ng/zone up to 600 ng/zone. The results indicated that quantification of Surfactin has to be performed after the first development ($hR_F = 44$), while Fengycin is quantified after the second development ($hR_F = 36$, hR_F range = 20–40). For Iturin A, the results demonstrated that quantification is in favor after the first ($hR_F = 19$) development, but also possible after the second ($hR_F = 59$) development. LOD and LOQ for Surfactin and Iturin A after the first development, and Fengycin after the second development were determined to be 16 ng/zone and 47 ng/zone, 13 ng/zone and 39 ng/zone, and 27 ng/zone and 82 ng/zone, respectively. Results further revealed the highly accurate and precise character of the developed method with a good inter- and intraday reproducibility. For the precision and accuracy, expressed as % recovery and relative standard deviation, respectively, the determined values did not exceed ±15% as specified by the FDA Guidance. The recovery assay conducted for samples obtained from two strains with the solvent chloroform/methanol (2:1, v/v), which was determined to be most suitable if all three lipopeptides are of interest, gave recoveries of 96.5% and 99.6%, 68.6% and 71.6%, and 102.5% and 95.2% for Surfactin, Iturin A and Fengycin, respectively. Overall, a suitable and reliable method for the simultaneous quantification of the lipopeptides Surfactin, Iturin A and Fengycin in biological samples using HPTLC was successfully developed and validated.

1. Introduction

Surfactants have a high impact in many industrial fields. Due to their surface-active properties, they find their application in health and personal care products, food products, crop protection or therapeutic products [1]. Research focusing on the production of bio-based surfactants has increased in order to address customers demand for products based on renewable resources.

Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LOD, limit of detection; LOQ, limit of quantification; MSM, mineral salt medium.

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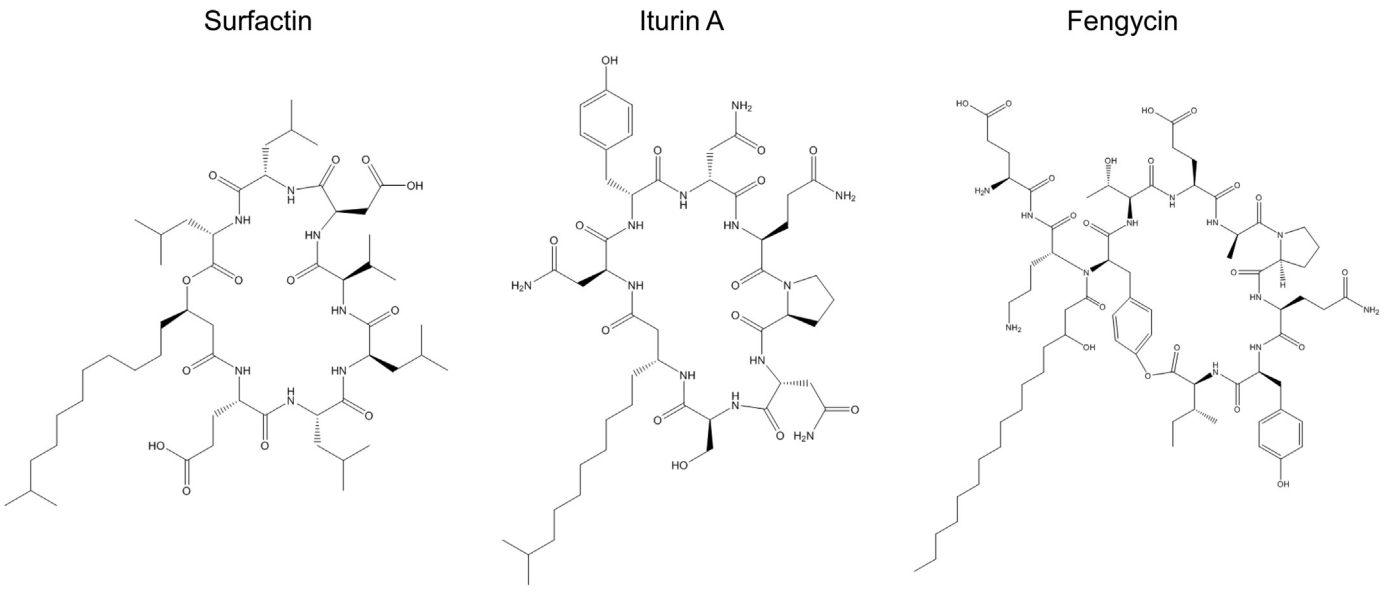

Fig. 1. Main congener structures of the cyclic lipopeptides Surfactin, Iturin A and Fengycin.

Amongst others, biosurfactants benefit from their biodegradability and generally low toxicity [2]. Due to their promising interfacial properties, applications in the food industry, pharmaceutical industry, as cleaning agents and for agricultural uses are conceivable, as well as in environmental applications such as microbial enhanced oil recovery [3]. Chemically produced surfactants are favored due to their overall availability in huge quantities and low production costs. The microbial production of many biosurfactants lacks high yields and much research needs to be conducted to make them available for industrial applications. In addition, the complexity of the cultivation media often makes analytics and downstream processes very demanding [4].

Biosurfactants are often grouped according to their structure such as being glycolipids, lipopeptides, phospholipids or polymeric surfactants [5,6]. Amongst these biosurfactants, the cyclic lipopeptides belonging to the Surfactin, Iturin and Fengycin family (Fig. 1) display highly promising physicochemical properties such as reduction of surface tension and emulsifying properties. Next to these surface-active characteristics, these biosurfactants stand out due to their strong bioactive properties. The cyclic lipopeptides are produced by different strains belonging to the genus *Bacillus*, e.g., *Bacillus subtilis* [7,8], *Bacillus amyloliquefaciens* [9,10] and *Bacillus circulans* [9,11]. As each family of lipopeptides consists of several congeners, which differ either in the length of the fatty acid chain or in the amino acid sequence of the peptide moiety [12], the strains typically produce a complex mixture of lipopeptides [10,13]. These structural variances cause different surface-active and bioactive attributes. Also with respect to their bioactive properties, each family of lipopeptides has different characteristics. Surfactin, for example, shows both antiviral, antibacterial and antitumor activities, while Iturin has mostly antifungal with limited antibacterial properties [14]. In a study conducted by Malfanova [15], Fengycin showed the highest antifungal activity.

The broad spectrum of molecular structures allows developing tailor made products. For appropriate analysis, quantification methods that allow distinguishing between different lipopeptide profiles of different strains and the produced amounts are needed. However, the presence of congeners in each family, as well as the chemical similarities in between the lipopeptide families constitute the main challenge in developing a suitable and reliable quantification method with an appropriate resolution. In order to be able to compare the lipopeptide profiles and the amounts produced by the respective strain, high-performance liquid chromatography (HPLC) is a common tool [16,17]. Quantification using HPLC is highly reliable and analyses are favored by a good resolution, but are also very time-consuming. High-performance thin-layer chromatography (HPTLC) is an interesting alternative to HPLC, as this analytical tool is favored by its higher throughput and allows quantifying many samples in a short time. Furthermore, as a new plate is used for each analysis, cross-contaminations are reduced and a more convenient handling in terms of sample preparation can be performed. In addition, HPTLC allows a higher flexibility as a broad range of solvents can be employed and the selection is not restricted by e.g. UV transparency or viscosity. Hence, this study is aimed at introducing a reliable method for the simultaneous quantification of the cyclic lipopeptides Surfactin, Iturin A and Fengycin in *Bacillus* culture samples using HPTLC.

2. Material and methods

2.1. Chemicals and materials

Surfactin (≥98%) and Iturin A (≥95%) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Fengycin (≥90%) was ordered by Lipofabrik (Villeneuve d'Ascq, France). All chemicals used for the cultivation medium, the mobile phases and the extraction experiments were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and were of analytical grade. HPTLC silica gel 60 plates from Merck (Darmstadt, Germany) were pre-washed with methanol and dried in a drying oven (UV 110, Memmert GmbH & Co. KG, Staufen, Germany) at 60 °C for 1 h and were stored dust-free until use.

2.2. Preparation of standard solutions

Individual standard solutions with a concentration of 0.5 mg/mL were prepared in methanol for Surfactin, Iturin A and Fengycin. These individual standards were pooled and further diluted with methanol to obtain a standard solution ($SIF_{0.1}$) with a concentration of 0.1 mg lipopeptide/mL. In addition, a Surfactin stock solution in methanol was prepared with a concentration of 1 mg/mL. This stock standard was used to prepare a total of five standards in