Centrifugal Partition Chromatography as a potential method of isolation and purification of amphiphilic substances from solid state fermentation process

M. Bochynek[1], P. Hodurek[2,3] A. Lewińska[4], M. Łukaszewicz[1,3]

[1] Faculty of Biotechnology, University of Wrocław, Wrocław, Joliot-Curie 14a, 50-383, Poland
[2]Faculty of Medicine, Wroclaw Medical University, Wrocław Chałubińskiego 10, 50-368, Poland
[3] InventionBio S. A, Bydgoszcz, Jakóba Hechlińskiego 6, 85-825, Poland
[4] Faculty of Chemistry, University of Wrocław, Wrocław, Joliot-Curie 14, 50-383, Poland
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Presenting author email: michal.bochynek@uwr.edu.pl

Introduction

Solid state fermentation (SSF) can be a source of many valuable active compounds and a method of waste biomass utilization. [1] However, isolating pure natural products from such a matrix is a demanding process. Typically used silica-packed columns in contact with complex samples of natural origin are problematic due to the possibility of blockage, damage or irreversible adsorption. The solution to these problems is the centrifugal partition chromatography (CPC), method based on the use of two immiscible liquids system as a mobile and stationary phases. Specially selected system of two phases can be introduced into the rotor - disk containing hundreds of small vessels connected in series. Its rotational move produces centrifugal force stabilizing the stationary phase inside. The separation of analytes takes place on the basis of the differences in the Nernst partition coefficients values between the biphasic system components. [2] This method allows the injection of any type of sample without risk of damaging the equipment, and also consumes much less solvents than typical preparative chromatography. However, in case of amphiphilic analytes, it has limitations. As a result of the emulsification of the biphasic system, leakage of the stationary phase can be observed, which results in lack of separation. This phenomenon is called an emulsification plug and is reported to be a significant problem in CPC. [3] In our research, surfactin a group of cyclic lipopeptides produced by *Bacillus subtilis* was used as a model biosurfactant with strong amphiphilic properties to show the solution to this problem. In addition, the finally optimized method was tested for the isolation of pure surfactin from extract obtained after SSF process and for the separation of structural analogues. The aim of the study is to show the solution to the problem of stationary phase leakage and to show the applicability of CPC in the purification of biosurfactants.

Materials and methods

Biomass extraction

The biomass from SSF process was extracted with 0.1 M $NaHCO_{3(aq)}$ and centrifuged. Then supernatant was collected and extraction step was repeated. Combined extracts were acidified with 6 M hydrochloric acid to pH=2 and left in 4°C overnight to precipitate all surfactin. Next day the solid residue was isolated after centrifugation and freeze-dried. Such obtained solid, brown crude product was used for surfactin content measurement (HPLC-UV) and purification with CPC.

CPC procedures

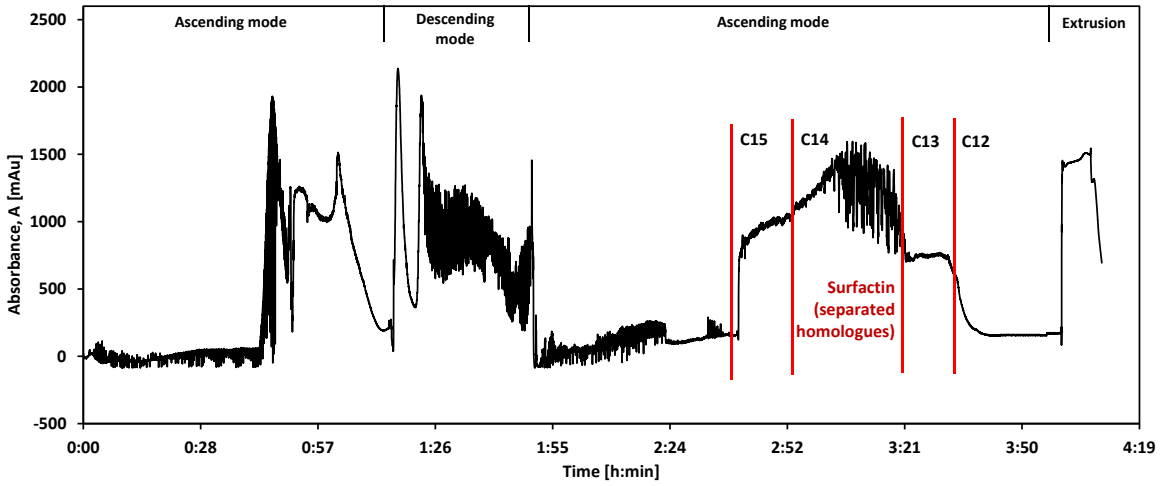
The separations were performed on Gilson Glider CPC system composed of 250 ml rotor and chromatographic station PLC2250 equipped with injection loop, UV detector and automatic sample collector. During the procedures eluted surfactin was detected with 207 nm and collected as 20 ml fractions. Collected fractions and entire purified product were analyzed then with HPLC-UV. During the method optimization pressure was monitored as the rotor's stability indicator.

Surfactin quantitative and qualitative analysis

Biomass extracts, isolated products and fractions from CPC separations were analyzed for surfactin content with reversed phase HPLC-UV system (AcquityArc, Waters). The samples were dissolved in methanol and filtered before analysis. The mobile phase consisted of acetonitrile and water. Separation method was 10 minutes long with increasing acetonitrile gradient. The detection was conducted with UV lamp set to 207 nm.

Results and discussion

The biomass is reservoir of multiple valuable products but their isolation and purification is very demanding task. For this issue easy scalable and low solvent consuming method based on CPC was developed. The proper solvent system was found to be a composition of *n*-heptane/*n*-butanol/methanol/aqueous buffer (20 mM disodium phosphate with 50 mM NaCl) at a ratio of 2:3:2:3. To describe its behavior inside the rotor pseudoternary diagram was prepared. Then after confirming the applicability of this system surfactin homologues' partition coefficients were determined with use of HPLC-UV. They were not ideal (slightly lower than 1.5) so dual-mode was applied during elution procedure to improve the separation. In first experiments typically used parameters in CPC (flow rate 5-8 ml/min and rotational speed 800 rpm) resulted in total rotor content leakage. In the next attempts the flow rate, rotational speed and injection volume were optimized with observation of pressure as the rotor stability indicator. Reduction of flow rate to 2 ml/min and elevation of rotational speed to 2000 rpm turned out to be the solution of emulsification plug problem. But with use of single Asc/Desc switching (single dual-mode) separation of surfactin into particular homologues was not possible. Therefore the double dual-mode was applied giving satisfying results but it made the method time consuming. Due to this fact the flow rate gradient from 2 ml/min to 3,5 ml/min was used. Finally optimized method was tested with use of surfactin containing extract obtained from fermented biomass. Resulting chromatogram is shown on graph. 1. Isolated surfactin had 80% purity what compared to its content in extract (20% by mass) is satisfying value. Moreover isolated fraction contained evet 70% of particular surfactin homologue what proved possibility to separate them with CPC.



Graph 1. CPC chromatogram of surfactin containing SSF extract.

Conclusions

Our research has demonstrated that difficulties connected with samples complexity and presence of amphiphilic substances in CPC use can be solved. We used surfactin – a biosurfactant naturally produced by *Bacillus subtilis*, having classic amphiphilic properties as a model. The designed and tested method turned out to be effective tool to isolate surfactin from complicated mixtures of natural origin and separate its homologues with satisfactory purity. The injected extract contained 20% of surfactin while isolated product had more than 80% purity. Moreover collection of the single fractions allows to obtain C13, C14 and C15 homologs with purity about 70% (compared to the other homologues) what makes it possible for further research or purification with preparative HPLC. Our results show first use of CPC in biosurfactant isolation after SSF process and give a solution to the problem of emulsification of the phases involved in the separation.

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