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Integrated systems for biosurfactant synthesis

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Abstract: Biosurfactants are difficult to produce in an economic manner for several reasons:
1. Overproducing strains of bacteria are rare and those found generally display a very low productivity. In addition complex media need to be applied.
2. The regulation of biosurfactant synthesis is hardly understood, seemingly it represents a "secondary metabolite" regulation. Among others O_2-limitation has been described as an essential parameter to govern biosurfactant production.
3. An improvement of the production yield is hampered by the strong foam formation. Consequently diluted media have to be applied and only immobilized systems provided an increased productivity of about 3 g l^{-1} h^{-1}.
In the following improved cultivation methods with developed defined media are described leading to enhanced productivities with the potential of 5 to 10 g l^{-1} h^{-1} upon optimization of the bleeding rate of an integrated continuous process. Instead of indirect product analytics by physical methods (CMC, surface tension) HPLC analysis for a new biosurfactant has been developed.

INTRODUCTION

Due to their biodegradability biosurfactants were originally ment to replace chemically synthesized surface active molecules (1, 2). However it has to be stated that the expected breakthrough in terms of applications of these biosurfactants has not yet occured. Among the reasons for this finding are the high production costs of biosurfactants, the limitation in the use of diluted media, technical difficulties encountered in the production process, in particular the strong formation of foam. In addition, the strains used often suffer from a bad public acceptance, such as *P. aeruginosa*. Fundamental improvements are therefore necessary as the required performance is not yet guaranteed.

In the following the results achieved using direct analytics (HPCL) instead of indirect product determination (CMC, surface tension) with improved, yet simple and effective culture systems for preliminary testings are presented. As a model application this system was successfully applied to the design of defined media to replace expensive complex media which are generally far more difficult to handle. In addition efforts were made to replace the harmful strain *P. aeruginosa* by *P. fluorescens*.

In a second step an integrated system for a high performance continuous production process was developed. By applying a highly effective membrane technology for cell retention a dramatic increase in potential product yield was achieved.

RESULTS

In order to improve the analytics of biosurfactants the production of these surface active molecules by *Bacillus licheniformis* in batch culture was investigated. *B. licheniformis* produces biosurfactants with a basic structure of a cyclic peptide of 7 amino acids with different ß-OH fatty acid side chains (6, 8). For the product determination no easy and

fast method had so far been known and indirect measurements (CMC, surface tension determination) had to be applied. By simplifying the probe preparation a direct product concentration measurement by HPLC was developed to allow for routine determination of biosurfactant concentrations in the culture medium (Fig. 1). Thus, irrespective of the solubility of the respective biosurfactant a fast and reliable analytical method was developed (3).

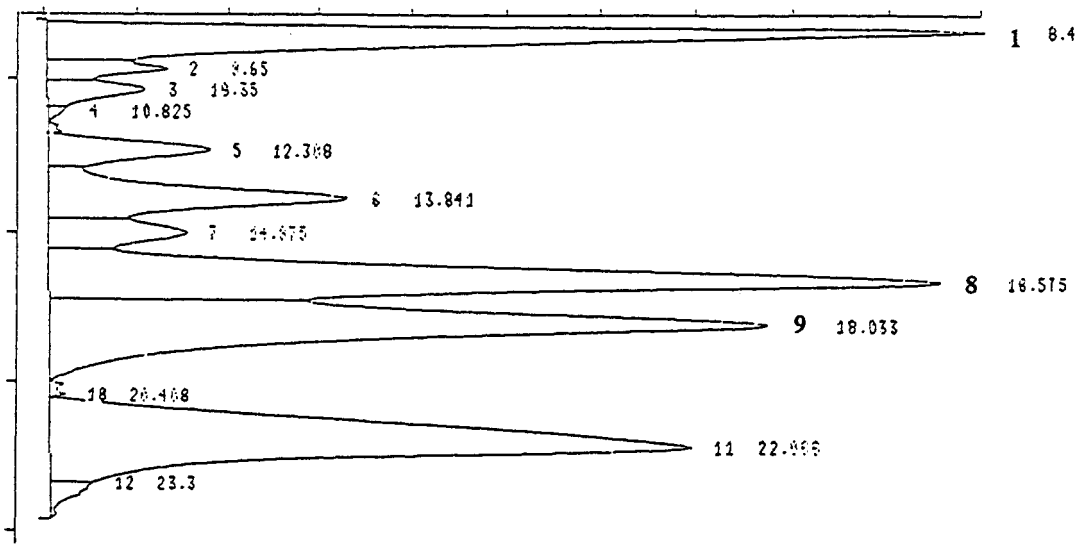
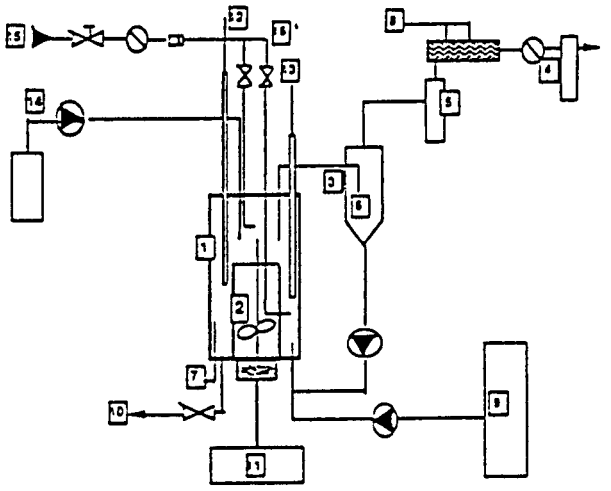


Fig. 1: HPLC chromatogram of a purified biosurfactant fraction (8 g l^{-1}) in CH_3OH:H_2O (95:5, v/v). The sample was withdrawn from the bioreactor, centrifuged and the biosurfactants precipitated. After washing the pellet the biosurfactants were dissolved at a 10 fold concentration compared to the actual concentration in the culture medium. HPLC was performed on a Nucleosil rp-C_{18} column with CH_3CN:H_2O:$C_2HF_3O_2$ (80:19.9:0.1, v/v) as a liquid phase. Peak 1 represents the internal standard phenylalanine, peak 8 and peak 9 represent two different biosurfactants of known structure and peak 11 a biosurfactant of unknown structure (Realini P., 1991)

The newly developed HPLC analytics were in the following applied to the design of a defined medium for *B. licheniformis*. As a C-source glucose or saccharose (3%) was used, 5 different salts, 7 trace elements and 9 vitamins were added. The highest product yields were obtained in batch culture on saccharose and under O_2-limitation (120 F_{cmc}). As growth is competing with product formation the first phase of the batch culture is not O_2-limited to allow a high biomass production and only after a shift to O_2-limited conditions the biosurfactant synthesis is induced. It has to be stressed that only due to the fast HPLC analytics these culture conditions could be elaborated in such a straight forward manner.

Regarding the culture system improvements were made to design a bioprocess fit for preliminary testings of biosurfactant production without the need for sophisticated high performance systems. As shown in Fig. 2 a simple chemostat (compact loop reactor) was used which could also be operated in a batch mode.

Under these circumstances it was possible to address a second major problem related to biosurfactant production, namely to try to swith from the pathogenic production strain *P. aeruginosa* to *P. fluorescens* (4) which is accepted as a harmless strain. The biosurfactant produced by *P. fluorescens* ATCC15453 was identified as an extracellular, surface active rhamnolipid comigrating with the rhamnolipid RL2 of *P. aeruginosa* in TLC (9). A defined medium for *P. fluorescens* containing 8 salts including $NaNO_3$ or



1. culture containment,
2. propeller and steel cylinder,
3. air out,
4. exhaust analysis,
5. safe lock,
6. foam reversion,
7. temperature control,
8. cooling system,
9. sterile medium supply,
10. drain,
11. propeller motor,
12. pO_2 control,
13. pH control,
14. NaOH supply,
15. sterile air in,
16. switchable airation (submerse, surface).

Fig. 2: Schematic representation of the bioreactor for the production of biosurfactants with *B. licheniformis* (Realini P., 1991).

$(NH_4)_2SO_4$ as N_2-source and 7 trace elements was developed. Again the cell growth phase was seperated from the biosurfactant synthesis phase by shifting the temperatur from 30°C to 25°C. In addition glucose was used as a C-source during the growth phase, whereas glyerol as C-source was pulsed to stimulate biosurfactant production at 25°C. By repetitive glycerol pulses the rhamnolipid concentration was increased to 1600 mg l^{-1}. This number represents a 40 fold increase as compared to a simple repetitive batch culture.

An additional improvement in product yield could be obtained by introducing a cell retention system leading to a constantly high product formation rate for a period of over 300 h. During this time 2350 mg l^{-1} of the rhamnolipid RL2 could be produced which corresponds to a product formation rate r_p of 7.7 mg $l^{-1}h^{-1}$. This impressive product formation rate is expected to increase even more when using a system with continuous cell retention under conditions that avoid O_2-limitation. Thus by applying such simple but already integrated systems to the production of biosurfactants, these high product concentrations could be obtained without the need for any special measurements to be taken.

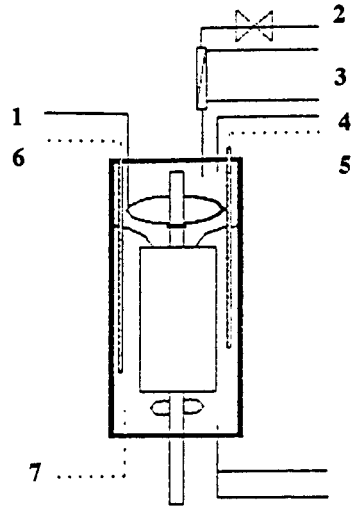


Fig. 3:
Bioreactor equipped with surface airation for the production of biosurfactants (Perren R., 1991).

1. air inlet,
2. exhaust air,
3. cooling system,
4. medium inlet,
5. pH control,
6. pO_2 control,
7. medium outlet, drain.

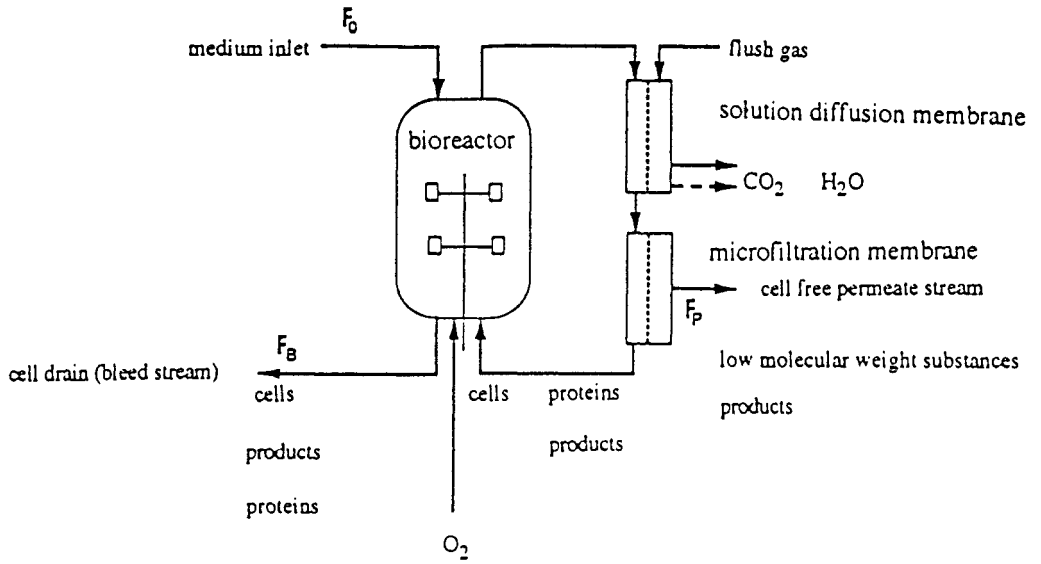


Fig. 4: Schematic drawing of an integrated production process using a specialized membrane system for the exchange of O_2 and CO_2.

In an effort to develop an integrated high performance system for the production of biosurfactants a specialized membrane technology was applied (5). Following the general trend for high performance systems with modular architecture (7) two membrane modules were connected via an external loop to the chemostat culture as can be seen in Fig. 4. The reactor outlet is diveded into the bleed stream which regulates the specific growth rate of the cells and the cell free permeate stream through the first membrane system where low molecular weight substances and the products leave the reactor. Cells and high molecular weight substances are carried back into the bioreactor. This allows to decrease the time which the product remains in the medium. The second membrane module is used for the diffusion of O_2 into the medium solution and the removal of CO_2 which avoids the formation of foam. For a period of over 1200 h no significant decrease of gas permeation fluxes could be detected.

As shown in Fig. 5 the biosurfactant product yield of a *P. aeruginosa* culture could be increased more than four fold by increasing the bleeding rate from 0.01 to 0.07 h^{-1}. This led to a poductivity of 545 mg h^{-1} which was achieved already in a first attempt without any further optimizations. It is therefore safe to state that it is possible to still

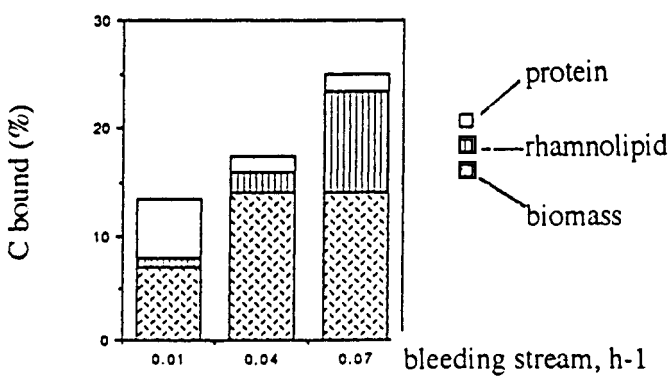


Fig. 5: C-recovery for all organic products of a *P. aeruginosa* production process. Increasing the bleeding rate from 0.04 h^{-1} to 0.07 h^{-1} led to a four fold increase in rhamnolipid production while the biomass remained constant.

increase the biosurfactant productivity up to 12 fold. Among the parameters that need further optimization to reach this value are the improvement of the defined medium for *P. aeruginosa*, the cell retention system or the optimization of pO_2. A strong pressure on the productivity originates from the competition between cell growth and product synthesis. Systems like the one presented above where a bleeding stream determines the cell growth rate, which is therefore uncoupled from the product retraction in the cell free permeate, help to decrease this pressure. Although detailed studies have not yet been carried out it seems realistic to speak about possible productivities of 5 to 10 g l^{-1} h^{-1}.

CONCLUSIONS

By developing a fast and reliable direct analysis system for biosurfactants using HPLC the laborious indirect determination methods could be replaced and the doors to an on-line analysis of product concentration are opened. In combination with improvements of the bioprocess itself, in particular attempts to avoid the massive formation of foam, a relatively simple system was used to test a new strain, *P. fluorescens*, for its capacity of biosurfactant production and to design a defined medium. This rather simple system can be used for the screening of other strains and enables the investigator to obtain informative data in a short time. For the design of a high performance integrated production systems a specialized membrane technology was developed and successfully applied which gives the potential to increase the biosurfactant productivity up to 10 g $l^{-1}h^{-1}$.

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