**The use of aqueous two-phase separation for the *in situ* production and purification of lipopeptides from *Bacillus amyloliquefaciens***

Robert W.M. Pott1

*1 Department of Process Engineering, University of Stellenbosch, South Africa, rpott@sun.ac.za*

**Highlights**

* *B. amyloliquefaciens* can be cultivated under two-phase conditions
* Lipopeptides preferentially report to the PEG-rich phase
* Differing PEG molecular weights can selectively partition different lipopeptides

**1. Introduction**

Significant quantities of fruit and vegetables are lost due to postharvest diseases(1). Disease control using synthetic chemicals are being phased out of use, particularly in the EU. Biomolecules, such as lipopeptides (LPs), that possess antifungal activity, biodegradability and digestibility have attracted interest as potential biocontrol agents as replacements(2). Particular focus has been placed on lipopeptides produced by *Bacillus* spp., as they provide a promising alternative in reducing postharvest spoilage due to fungal phytopathogens. The industrial production of these compounds is a developing field, with some LP products being commercially available(3), while antifungal LPs are not yet produced on an industrial scale. In order to develop a process which can produce these compounds at large scale, significant work in separating and purifying the different homologues of LP is required. In particular, in batch culture, as soon as the bacterial cells enter their stationary growth phase, they begin to metabolize the already produced LPs resulting in reduced product yields(4,5). Current concentration, recovery and purification methods employed such as acid precipitation, solvent extraction and membrane filtration take place after fermentation has been stopped and do not address this problem.

The primary aim of this study was therefore to explore the potential of PEG-salt ATPS as an *in situ* extractive fermentation process to reduce the metabolization of the LPs produced by *Bacillus amyloliquefaciens* DSM 23117. The effect of salt type (tartrate, sulphate, phosphate and citrate) and concentration (Standard media 0.2, 0.4 and 0.8 M) as well as the PEG molecular weight (6000 and 8000) on the growth of the microorganism and LP production were examined and compared against standard growth conditions.

**2. Methods**

ATPS partitioning in pure component systems was conducted using the appropriate salt, buffer and PEG wt. % determined from binodal curves of ATP systems used for protein purification in literature(6). ATP systems were prepared using known concentrations of salt, PEG, and LP mixtures (surfactin, fengycin). ATP systems were agitated in an incubator at 150 rpm for 15 min at 30°C. After mixing, the systems separated into phases. LP concentrations in top and bottom phases were determined using HPLC (Phenomenex Luna 3 μm C18 column, Dionex Ultimate 3000 Diode-array detector, trifluoroacetic acid mobile phase). LP homologue partitioning was visualized using thin layer chromatography (as described in (7)). For culture experiments, shake flasks experiments, at 30oC, 100 rpm, in baffled 250ml flasks, were conducted growth rate, substrate utilization and lipopeptide production were determined using cell dry weight and HPLC for the quantification of glucose utilized and lipopeptides produced, respectively.

**3. Results and discussion**

Some sample results are presented here. Further details will be presented in the full conference presentation.

Surfactin and fengycin were successfully separated into top and bottom phases of several ATPS systems, an example of which is shown below in Figure 1. This is the first instance where comparatively simple separation of lipopeptide homologues has been shown. The implication of this is that fengycin, the compound desired in antifungal applications, is separated from the co-produced but undesired surfactin. Fengycin partitions to the top phase, and can thereafter be precipitated using acid precipitation and used as a purified product. The incidence of product consumption is thus minimized at the same time as purification of the product away from the fermentation broth is enacted.



**Figure 1.** TLC image of lipopeptides in 40% (w/w) PEG 6000 and 49% (w/w) K2HPO4. T refers to top (PEG) phase, B refers to the bottom (salt) phase, S refers to the surfactin standard and F refers to the fengycin standard

The organism was found to be able to survive, and even thrive, under increased salt concentrations. This increased salt concentration is a necessary part of operating the culture under *in situ* extraction using ATPS, since 2-phase formation only occurs under sufficient salt concentrations. Following the demonstration of salt tolerance, experiments were conducted using PEG addition to the system. Very similar results were obtained, demonstrating that the organism can viably grow under ATPS conditions. Under the ATPS growth conditions lipopeptide concentration in the top PEG phase was monitored, and found to follow the same trend as standard growth media LP production

**4. Conclusions**

The production, *in situ* purification and recovery of LPs from *B amyloliquefaciens* was demonstrated using PEG-salt ATPS systems. This work opens the opportunity for the use of ATP systems in the continuous production of LPs, or for a simple purification method to separate LP homologues.

**5. Acknowledgements**

With thanks to Sebenzile Mazibuko and Dineo Baloyi for their hard work in the laboratory. Generous financial support was provided by Hortgro.

**References**

1. James A, Zikankuba V. Postharvest management of fruits and vegetable: A potential for reducing poverty, hidden hunger and malnutrition in sub-Sahara Africa. Yildiz F, editor. Cogent Food Agric [Internet]. 2017 Apr 1 [cited 2019 Feb 28];3(1). Available from: https://www.cogentoa.com/article/10.1080/23311932.2017.1312052

2. Pretorius D, van Rooyen J, Clarke KG. Enhanced production of antifungal lipopeptides by Bacillus amyloliquefaciens for biocontrol of postharvest disease. N Biotechnol [Internet]. 2015 Mar 25 [cited 2017 Jun 21];32(2):243–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25541516

3. Kaneka. Kaneka surfactin [Internet]. [cited 2019 Feb 28]. Available from: http://www.kaneka.co.jp/en/branch/nb\_development/surfactin\_catalogue\_eng.pdf

4. Mazibuko S. Purification of Bacillus amyloliquefaciens lipopeptides for postharvest disease control. Stellenbosch University; 2018.

5. Rangarajan V, Herbst WJ, Mazibuko S, Clarke KG. Bacillus lipopeptides for a novel postharvest disease control technology. In: 4th international symposium on postharvest pathology. 2017.

6. Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: Phase separation and applications. J Chromatogr A [Internet]. Elsevier; 2012 May 18 [cited 2018 Jun 20];1238:1–10. Available from: https://www.sciencedirect.com/science/article/pii/S0021967312004621

7. Dlamini B. Downstream purification of surfactin produced by Bacillus subtilis ATCC 21332. Stellenbosch University; 2017.