Recovery of Geldanamycin using
Liquid-Core Microcapsules as a Novel Approach

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Introduction:

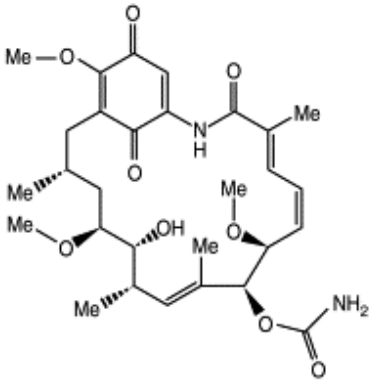

Fig. 1. Structure of geldananmycin

The benzoquinone ansamycin antibiotic geldananmycin (GM) (Fig. 1), which is produced as a secondary metabolite by *Streptomyces (S.) hygroscopicus* var. *geldanus var. nova* was discovered in 1970 (Deboer, 1970) and is a naturally occurring antibiotic. It is a broad-spectrum antibiotic which exhibits activity against gram positive and gram negative bacteria. Interest in the molecule increased upon the discovery of its antitumor properties. In 1994, Whitesell et al showed that the principal cellular target of GM is Heat shock protein 90 (Hsp90), a ubiquitous and abundant protein chaperone of mammalian cells. This chaperone is necessary for the folding, assembly and activity of multiple mutated and over expressed signalling proteins that promote the growth and/or survival of tumour cells. GM binds to Hsp90 with high affinity, resulting in the loss of its chaperone ability towards its client proteins which are left malformed, unstable and readily degradable by the cells own mechanisms resulting in the destruction of the tumour.

The accumulation of the produced product in a bioprocess operation can inflict numerous problems, such as product degradation, and feedback inhibition, which affect the overall efficiency, resulting in lower productivities and yields (Freeman, 2003). This is the main reason why certain bioprocesses fail to become commercially successful. These problems can be overcome by in situ product recovery (ISPR), which involves immediate removal of the product from the vicinity of its production environment as soon as it is formed using various techniques.

Casey (2006) used adsorbent resins as an ISPR-methodology for GM recovery and showed that the incorporation of these resins into *S. hygroscopicus* fermentations results in a 3-fold increase in GM production in shake-flask fermentations. Unfortunately this type of ISPR process was susceptible to many problems, such as low capacity of resins for GM when contacted with whole broth, non-specific adsorption of nutrients and by-products and the inability to recycle resins for future experiments. These problems reduced the overall efficiency of the ISPR process but emphasised the ability of ISPR to increase the production of GM.

Previously a novel approach to successfully recover 2-Phenylethanol (PEA) from fermentation media using dibutyl sebacate liquid-core microcapsules (Stark, 2001) as an ISPR procedure has been reported. Immediate removal of the PEA from the vicinity of its producing cell eliminated the inhibitory affect of PEA on the organism and resulted in nearly a 50% increase in production yields for the compound of interest. This methodology, termed capsular perstraction involves the encapsulation of an organic solvent within an alginate hydrogel. Similar types of capsules have also

been used to successfully extract a range of herbicides and pesticides (Wyss, 2004) and pharmaceuticals from water (Whelehan, 2007).

In this study, liquid-core microcapsules having a diameter of 737.36 μm were used as a novel approach to recover GM in-situ from its fermentation environment. Initially, work focused on establishing the problems associated with GM accumulation in the broth. Subsequent experiments involved the encapsulation of a suitable solvent for GM extraction inside a porous hydrogel membrane composed of alginate. These capsules were then used to recover GM from broth to alleviate the problems associated with product accumulation. This study examines the feasibility of using liquid-core microcapsules as a novel approach for ISPR of GM from fermentation broths.

Materials and Methods:

Preparation of Liquid-Core Microcapsules: Full details of the procedure used to make liquid-core microcapsules has been described previously (Stark, 2001). Briefly, mononuclear microcapsules were produced using the prilling technique with an Inotech encapsulator (IE-50-R Basel, Switzerland). The encapsulator was fitted with a concentric nozzle with internal diameter of 200 μm and external diameter of 300 μm. Spherical microcapsules were obtained by the application of a set vibrational frequency with defined amplitude to the co-extruded liquid and collected in a gelling bath consisting of $CaCl_2$. Size distribution of capsules was determined using a video camera attached to a light microscope interfaced to a PC operating with Cell F image analysis software. Samples containing up to 200 capsules where taken and the mean size and standard deviation was determined. Capsules themselves can be described as having an external diameter (d_{ext}) which consists of alginate and the liquid-core and an internal diameter (d_{int}) which consists solely of the liquid-core.

Antibiotic Fermentation: Full details of antibiotic production and recovery in *S. hygroscopicus* fermentations have been described elsewhere (Casey, 2006). Briefly, a spore inoculum of *S. hygroscopicus* was used to inoculate fermentations. This was prepared by culturing the organism on static cultures of Bennett's medium agar at 30 ^{0}C. Spores were then recovered and inoculated at 1% (v/v) in Bennett's medium and incubated at 30 ^{0}C at an agitation speed of 150 rpm.

Capsular Perstraction: All capsular perstraction experiments were carried out under the fermentation conditions. A constant volume of capsules (measured using graduated tubes) was used for all experiments, so results could be accurately compared. The measurement of GM in the liquid-core of capsules required capsules to be burst open to liberate the oil or back-extraction into another organic phase.

Analytical Methods: Reverse phase high performance liquid chromatography (Agilent, 1100 Series) was used to determine GM concentrations in media and oleic acid. The column used was a 5 μm Kingsorb, C-18 HPLC column with associated security guard cartridge system (Phenomenex Cheshire, UK). Isocratic conditions were implied and the GM was passed through the column using either a mobile phase of 50%:50% (v/v) acetonitrile:H_2O or 80%:20% (v/v) methanol: H_2O. Samples were analyzed at 308 nm.

Results and Discussion:

Capsular Perstraction of GM from Fermentation Media: Choice of Organic Phase Extractant:

In order to chose the organic phase extractant for the liquid-core of microcapsules, different concentrations of GM in fermentation media were extracted using capsules consisting of several different liquid-cores and the best was chosen for further encapsulation and extraction. Oleic acid (OA) was selected for encapsulation (Figure 2) due to (1) its ability to extract large quantities of the antibiotic (partition co-efficient value of 220 for GM between OA and the media); (2) its very high hydrophobicity ($logP_{oct}$ = 7.7), which ensures a negligible diffusion of OA through the capsule wall into the aqueous media phase, when equilibrium is obtained; and (3) the fact that extracted GM could be removed from capsules for detection and purification using a method which was not detrimental to the capsules, thus allowing them to be re-cycled for future use. Figure 3 shows the extraction of GM from fermentation media using OA liquid-core microcapsules. The GM concentration in the media rapidly declined to less than 80% of its initial value after around 10 min with equilibrium reached at around 25 min. This rapid extraction of GM can be attributed to its fairly hydrophobic nature ($logP_{oct}$ = 1.71) and limited solubility in media which makes the hydrophobic phase of capsules a more suitable environment for this compound.

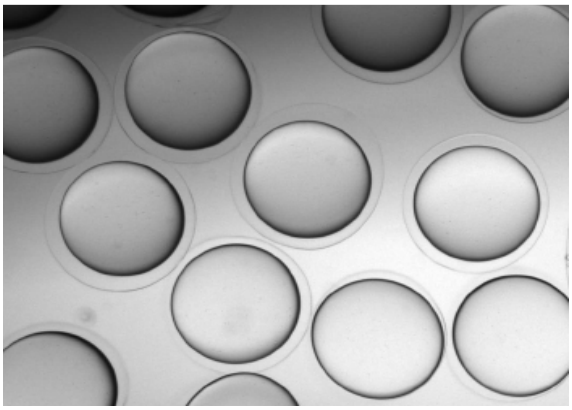

Figure 2: Light microscope image of mono-dispersed OA liquid-core microcapsules used to extract GM from media. Capsules have a d_{ext} of 737.36µm ± 2.59% and an d_{int} of 570µm ± 2.85%.

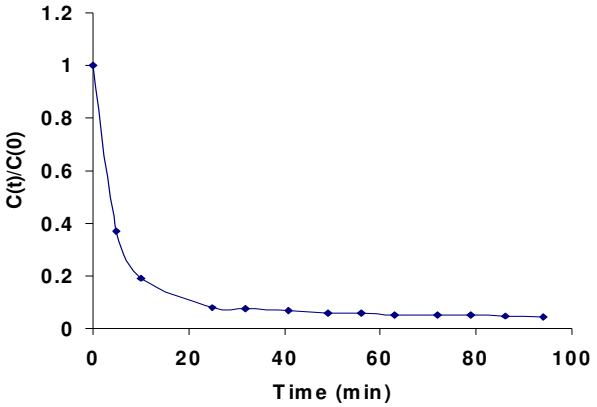

Figure 3: Extraction of GM from media using OA liquid-core microcapsules. Symbols: C(t)/C(0): Ratio of concentration at time t to initial concentration.

GM Degradation in Fermentation Broth: Using Microcapsules to Overcome this Problem

From the results obtained, Casey (2006) hypothesized that GM production yields increased in fermentations containing adsorbent resins due to the ability of the resins to remove GM from the hostile fermentation environment, which was causing the subsequent degradation of the antibiotic. In order to test this hypothesis experiments were undertaken involving the addition of a known amount of GM to the fermentation broth (cells were removed by filtration through a 0.20 µm filter) containing spent media and by-products produced by the cells. Figure 4 highlights the degradation of GM in the fermentation broth over the time period outlined. This result proves Casey's hypothesis and additional experiments (results not shown) allowed the conclusion to be made that by-products produced by the cells are responsible for the degradation. To overcome this problem, microcapsules containing OA were then added to similar fermentation broth just after the addition of the known amount of GM to verify if the capsules could remove the GM and prevent this degradation. Figure 4 displays how microcapsules added to the broth absorbed all the added GM present and hence prevented it from breaking down. It is hypothesized that the added GM was selectively extracted rapidly from the broth, as seen in Figure 3. This extraction resulted in reduced degradation of the

GM, since it was protected from the action of compounds which were not extracted within the core of the microcapsules.

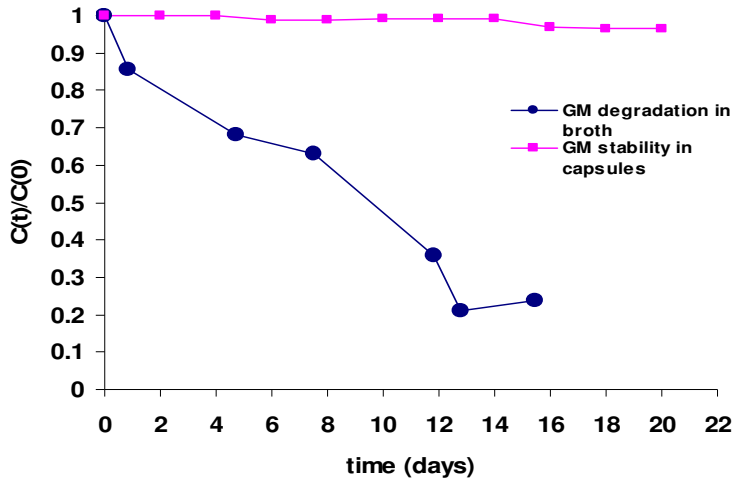

Figure 4: Graph showing the degradation of GM in fermentation broth over a 16 day period and using microcapsules to recover GM in-situ from the broth to alleviate this problem. Symbols: C(t)/C(0): Ratio of concentration at time t to initial concentration.

Conclusion:

The goal of this study was to assess the feasibility of using OA liquid-core microcapsules as a novel ISPR methodology to recover GM from fermentation broths. Results to date indicate that these capsules could be employed as an ISPR procedure as they were shown to remove GM from the detrimental fermentation environment. Capsules have many advantages over other ISPR processes like adsorbent resins, such as their ability to extract higher quantities of product (encapsulation of liquid-core with a high affinity for GM), prevention of non-specific adsorption of unwanted products enabling capsules to pre-concentrate and purify the product before downstream processing and the ability to completely back-extract the product from capsules to enable their future use.

References:

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