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Evaluation of the feasibility of applying Liquid-core Capsules for In-Situ Product Recovery of Geldanamycin in a Streptomyces hygroscopicus fermentation

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Titre / *Titel*:

The use of liquid-core microcapsules for the in-situ recovery of liposoluble compounds

Description / *Beschreibung*:

The project involves the following:

- Preparation of liquid core microcapsules from a range of polyelectrolyte complexes, containing a range of organic phases.
- Installation and calibration of HPLC techniques for the determination of geldanamycin and a range of drugs.
- The extraction of geldanmycin from microbial cultures using liquid-liquid and capsular perstraction. Comparison of extraction (mass transfer) rates and capacities.
- Extraction of a range of drugs having poor water solubilities from different water environments using liquid-liquid and capsular perstraction. Comparison of kinetics.
- If time permits, to carry out a series of fermentations in the presence and absence of liquid-core microcapsules to recover geldanamycin and thereby show that in-situ product recovery can overcome product inhibition and/ or increase product stability thereby leading to increased process volumetric productivity.

Objectifs / *Ziele:*

- To develop quantitative analytical methods for the determination of geldanmycin and other drugs.
- To prepare monodisperse liquid-core microcapsules
- To quantitatively characterize the extraction of geldamycin using microcapsules
- To show that microcapsules can be used for in-situ product recovery from bioprocesses
- To show that microcapsules can overcome fermentation product inhibition
- To show that microcapsules can extract certain compounds at significantly higher rates than liquid-liquid extraction
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Rapport reçu le / *Schlussbericht erhalten am* ..... Visa du secrétariat / *Visum des Sekretariats:* .....

Evaluation of the feasibility of applying Liquid-Core capsules for In-Situ Product Recovery of Geldanamycin in a *Streptomyces hygroscopicus* fermentation

Objective

The aim of this work was to produce Liquid-Core capsules for the In-Situ Product Removal (ISPR) of Geldanamycin (GM) from a Streptomyces (S.) hygroscopicus culture. These capsules were characterized for their size distribution and mass transfer characteristics. Then the feasibility of the ISPR of GM through Liquid-Core capsules was evaluated and tested. Therefore the physical-chemical properties of GM, such as solubility, stability, partition coefficient in organic-aqueous 2-phase systems were investigated. The production of GM in S. hygroscopicus var. geldanus cultures was then monitored in order to investigate if there was a product inhibitory effect of GM and to test the application of the Liquid-Core capsules as a tool for ISPR of GM in a culture.

Results

Monodispersed and homogenous Liquid-Core capsules, with Dibutyl sebacate (DBS) as the capsule core, were produced with an average capsule diameter of 0.712 ± 0.040 mm and an average core diameter of 0.554 ± 0.030 mm. An extractive capacity of 3.48 mg_{GM}/g_{DBS} could be measured for the capsular perstraction of GM with the produced capsules. This was a much higher capacity than for non-encapsulated DBS, where only a capacity of 2.2 mg_{GM}/g_{DBS} could be achieved. The capsules showed an initial adsorption rate of 843.7 $mg_{GM}/(l*h)$ at 28℃ and 405.61 $mg_{GM}/(l*h)$ at 20℃, which indicates that the main mass transfer resistance is due to the diffusion through the capsule wall.

Solubility tests showed that the solubility of GM in water is very low, but rather high in different solvents like Dimethyl sulfoxide, Acetonitrile and Dibutyl sebacate. The stability tests showed that GM was unstable in aqueous solutions under culture conditions in the presence of O_2 and at low pH values. However in Dibutyl sebacate a good stability of GM could be shown.

Through monitoring of GM production during the cultivation of S. hygroscopicus a strong degradation of GM could be observed at the end of the cultivation, possibly due to proteins/ enzymes liberated by the cells. Investigations of the product inhibition of GM in a S. hygroscopicus culture showed that the addition of GM in a culture doesn't inhibit the GM production, but actually increases GM production.

It could be shown that it is feasible to apply Liquid-Core capsules for the ISPR of GM in a S. hygroscopicus fermentation, as GM is more stable in the organic solvent used for the liquid core of the capsules and also because GM degrades spontaneously under culture conditions and as a result of proteins liberated by cells during cultivation. Consequently Liquid-Core capsules as a form of ISPR would be a possibility to overcome these problems and increase the overall yield of geldanamycin.

Keywords

Liquid-Core capsules, Dibutyl sebacate, Geldanamycin, Streptomyces hygroscopicus

Untersuchung der Machbarkeit, Alginatkapseln zur integrierten Produktisolation von Geldanamycin in einer *Streptomyces hygroscopicus* Fermentation anzuwenden

Ziel

Das Ziel dieser Arbeit war, Mikrokapseln mit einem flüssigen Kern, die zum integrierten Aufarbeitungsprozess von Geldanamycin (GM) in einer Streptomyces (S.) hygroscopicus Kultur dienen sollen, herzustellen und aufgrund ihrer Grössenverteilung und Massentransfer zu charakterisieren. Anschliessend sollte die integrierte Aufarbeitung von GM mittels Mikrokapseln untersucht und ausgetestet werden. Dazu wurden vorerst die physiko-chemischen Eigenschaften von GM, wie Löslichkeit, Stabilität, Verteilungskoeffizient in organisch-wässrigen 2-Phasen Systemen untersucht und die Produktion von GM in einer S. hygroscopicus var. geldanus Kultur verfolgt. Dabei wurde die Produktinhibition von GM untersucht und die Zugabe von Mikrokapseln in eine Kultur ausgetestet.

Resultate

Mikrokapseln mit Dibutyl sebacate (DBS) als flüssigen Kern konnten mit einer guten Monodispersion und Homogenität hergestellt werden. Ein durchschnittlicher Kapseldurchmesser von 0.712 ± 0.040 mm mit einem durchschnittlichen Kerndurchmesser von 0.554 ± 0.030 mm konnten dabei hergestellt werden. Die Kapseln zeigten für GM eine extraktive Kapazität von 3.48 mg_{GM}/g_{DBS}, was eine grössere Kapazität darstellte als bei nicht-verkapseltem DBS, wo eine Kapazität von nur 2.2 mg_{GM}/g_{DBS} ermittelt werden konnte. Die Kapseln zeigten Anfangs-adsorptionsgeschwindigkeiten von 843.7 $mg_{GM}/(l*h)$ bei 28℃ und 405.61 $mg_{GM}/(l*h)$ bei 20℃, was darauf hinwies, dass die Resistenz des Massentransfers von GM in die Kapseln hauptsächlich durch die Diffusion durch die Kapselwand aus Alginat verursacht wird.

Löslichkeitsuntersuchungen von GM haben gezeigt, dass GM eine sehr schwache Löslichkeit in Wasser besitzt, hingegen eine relativ gut löslich in Lösungsmitteln wie Dimethylsulfoxid, Acetonitril und DBS. Die Stabilitätstests zeigten, dass GM instabil in wässrigen Lösungen bei Kultivationsbedingungen ist in Gegenwart von O_2 und bei tiefen pH-Werten. Hingegen zeigte GM in Dibutyl sebacate eine sehr gute Stbilität.

Bei der Produktion von GM in S. hygroscopicus Kulturen konnte ein starker Abbau von GM am Ende der Kultivierung beobachtet werden. Es konnte gezeigt werden, dass GM im Kulturmedium aufgrund freigesetzter Proteine durch die Zellen instabil ist und zerfällt.

Die Untersuchungen der Produktinhibition von GM in S. hygroscopicus Kulturen haben gezeigt, dass die Anwesenheit von GM in einer S. hygroscopicus Kultur keine Produktinhibition aufzeigt, sondern sogar eine erhöhte Produktion von GM erzeugt, wenn GM zu Beginn der Kultivierung dazugegeben wurde.

Es konnte aufgezeigt werden, dass es machbar ist, Alginatkapseln mit DBS als flüssigen Kern zur integrierten Produktaufarbeitung von GM in einer S. hygroscopicus Fermentation anzuwenden, und die Ausbeute an GM zu verbessern.

Schlüsselwörter

Alginatkapseln, Dibutyl sebacate, Geldanamycin, Streptomyces hygroscopicus

Evaluation de la faisabilité d'appliquer des capsules d'alginate pour la récolte intégrée de Geldanamycin dans une fermentation de *Streptomyces hygroscopicus*

Objectif

L'objectif de ce travail était de produire des capsules d'alginate contenant un noyau liquide d'une phase organique, et de caractériser celles-ci par rapport à leur distribution de taille et par rapport au transfert de masse. Ensuite la faisabilité d'intégrer la récolte de Geldanamycin (GM) d'une culture Streptomyces (S.) hygroscopicus avec ces capsules était établie. Les caractéristiques physico-chimiques de la récolte intégrée de GM, comme la solubilité, la stabilité, le coefficient de partage dans un système 2 phases aqueux-organique, étaient évaluées. La production de GM dans une culture de S. hygroscopicus var. geldanus était suivie afin de déterminer si le GM avait un effet inhibiteur et de définir l'efficacité de l'ajout des capsules dans une culture.

Résultats

Les Capsules d'alginate contenant un core de Dibutyl sebacate (DBS) comme phase organique étaient produites avec un diamètre moyen des capsules de 0.712 ± 0.040 mm et un diamètre moyen des noyaux de 0.554 ± 0.030 mm. Une capacité d'extraction de 3,48 mg_{GM}/g_{DBS} pourrait être mesurée pour GM avec les capsules produites. Ce qui est plus élevé que la capacité d'extraction de DBS non-encapsulé qui était de 2.2 mg_{GM}/g_{DBS}. Les capsules avaient montré un taux d'adsorption initial de 843.7 $mg_{GM}/(l*h)$ à 28°C et de 405.61 $mg_{GM}/(l*h)$ à 20°, ce qui indique que le transfert de masse est limité à cause de la diffusion à travers de la membrane d'alginate des capsules.
Les expériences ont montré que la solubilité de GM dans l'eau était très faible, par contre elle était relativement élevée dans différents solvants comme dimethylsulfoxide, acétonitrile et DBS. Il avait aussi été montré que GM était instable dans les solutions aqueuses aux conditions de culture en présence de O_2 et à de faibles valeurs de pH. Par contre GM était stable dans DBS.
En suivant la concentration de GM pendant la culture de S. hygroscopicus, une dégradation de GM a été observe à la fin de la culture. L'étude de l'inhibition du produit par GM dans une culture de S. hygroscopicus a montré que la présence de GM ne causait pas d'inhibition pour la production de GM. Par contre, un ajout de GM au début de la culture augmentait la production de GM. Par conséquent, il est possible d'utiliser des capsules d'alginate, contenant un noyau liquide de DBS, pour la récolte intégrée de GM dans une fermentation de S. hygroscopicus. Car GM a montré une meilleure stabilité dans DBS que dans le milieu de culture.

Mots-clés

Capsules d'alginate, Dibutyl sebacate, Geldanamycin, Streptomyces hygroscopicus

Contents

1 Introduction

1.1 Geldanamycin

Geldanamycin (GM) is a polyketide belonging to the family of benzoquinone ansamycin antibiotics. It is a naturally occurring compound that was discovered in the culture filtrates of *Streptomyces (S.) hygroscopicus* var. *geldanus* by DeBoer et al. and was published in 1970 [1]. Although GM was originally identified as a result of screening for antibiotic activity, current interest in it is based primarily on its cytotoxicity towards tumour cells and therefore it's potential as an anticancer agent. It was shown that GM was moderately active in vitro against protozoa, bacteria and fungi, and also against L-1210 and KB cells growing in cultures and against the parasite Syphacia oblevata, in vivo [2].

GM induces a cytotoxic effect onto tumour cells, by inhibiting Heat Shock Protein-90 ("Hsp90"), a molecular chaperone protein that's involved in the folding, activation and assembly of multiple mutated and over expressed signalling proteins ("client proteins") that promote the growth and/or survival of tumour cells (see image 1). Hsp90 client proteins play important roles in the regulation of the cell cycle, cell growth, cell survival, apoptosis, angiogenesis and oncogenesis [3]. GM binds with high affinity to the ATP binding pocket of Hsp90 and alters its function. It induces the destabilisation and degradation of its client proteins which include mutated v-Src, Bcr-Abl and p53. Despite its antitumour potential, GM has several major disadvantages as a drug candidate, like hepatotoxicity, that has led to the development of GM analogues, in particular analogues containing a substitution on the 17 position: 17-AAG, 17-DMAG and some others. [4]

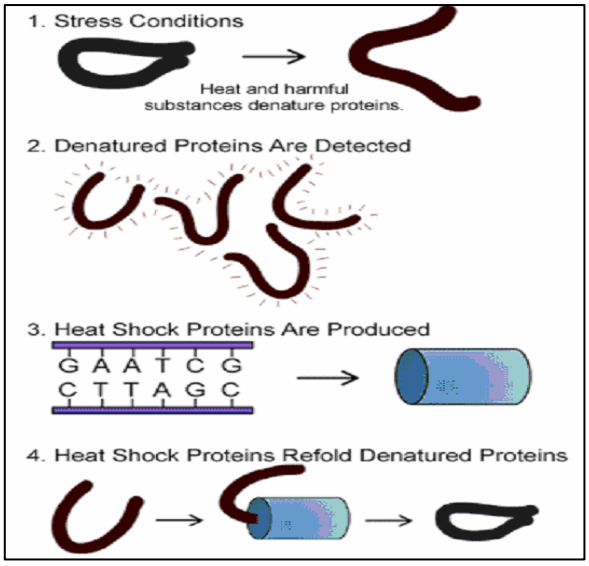


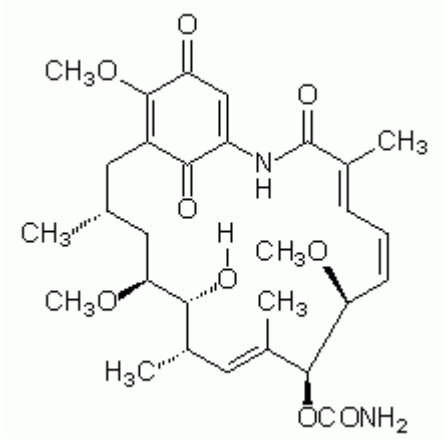
Image 1: *The role of Heat Shock Proteins [4]*

1.1.1 Physical and chemical properties of GM

On the basis of the physical and chemical properties (see table 1), GM is a complex molecule consisting of an unsaturated moiety attached to a quinone.

GM is soluble in alcohols, aliphatic chlorinated solvents, particularly chloroform, and to a lesser extend, acetone, benzene and ethyl acetate, but only very slightly in water. In solution, it decomposes rather readily with acid, base or heat, in the presence of oxygen and exposed to light; but as a dry material it is stable. [2]

Table 1: *Pharmacological profile of Geldanamycin*

Physico-chemical properties	
Molecular Structure:	
Molecular Formula:	$C_{29}H_{40}N_2O_9$
Molecular weight:	560.64 g/mol
Boiling Point:	Not Available
Melting Point:	252-255℃ [1]
Solubility:	DMSO (10 mg/ml), DCM (10 mg/ml), Water insoluble
Appearance:	yellow fine crystalline powder
Storage:	In a tightly closed container protected from light at -18℃ (solid) for max. 1 year; at -18℃ (in solution) for max. 3 mont hs.
Hazards Identification:	Geldanamycin: not classified as hazardous material Warnings: The usual precautions taken when handling chemicals should be observed. Label precautionary statements: avoid contact and inhalation
Classification	
	CAS: 30562-34-6
Sources	
	Steptomyces hygroscopicus var. *geldanus*
Price	
	820 €/g [5]

1.2 Streptomyces

S. hygroscopicus var. *geldanus* is the organism which produces GM. It also produces other compounds like nigercin, nocardamine and a libanamycin-like activity when cultivated in preferential media [2].

Streptomyces are Gram positive bacteria of the genus Actinobacteria. With more than 500 different species *Streptomyces* is the largest genus of the Actinobacteria. They are found predominantly in soil and in decaying vegetation and produce spores [6]. Geosmin, a volatile metabolite of *Streptomyces,* is the reason for the distinct “earthy” odor of *Streptomyces*.

Streptomyces is the largest antibiotic producing genus [7] producing both antibacterials and antifungals and also a wide range of other bioactive compounds such as immunosuppressants. [8]

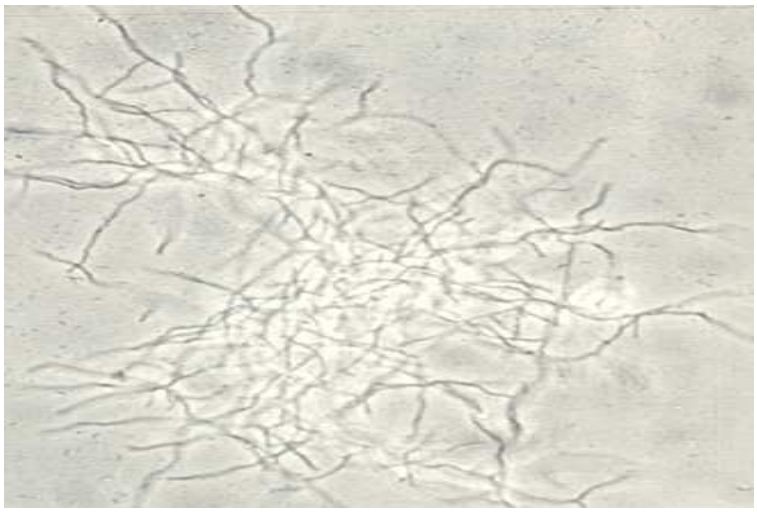


Image 2: *Steptomyces hygroscopicus* var. *geldanus filaments (400x)*

A complex secondary metabolism is characteristic for *Streptomyces* [9]. They produce a large number of antibiotics that are used clinically (Neomycin, Chloramphenicol, Streptomycin). *Streptomyces* are infrequent pathogens, whereas *S. hygroscopicus* var. *geldanus* is non-pathogenic. [10]

Streptomyces spp. have been used in recent years for production of recombinant human proteins. As they have the ability to secrete correctly folded proteins. Traditionally *Escherichia (E.) coli* was the species of choice to host eukaryotic genes since it was well understood and easy to work with. However *E. coli* introduces problems such as incorrect or lack of glycosylation and incorrect protein folding resulting in insolubility and loss of bioactivity of the product [11].

1.3 Production of Geldanamycin

GM is a natural product produced by *S. hygroscopicus* var. *geldanus* NRRL 3602 during a submerged fermentation in Bennett's Media at 28℃ [12]. InvivoGen produce GM from a mutant strain of *S. hygroscopicus*, inactivated for the synthesis of Nigericin, a common contaminant of GM. [13] Fermentek uses no mutants, but native unmodified strains only, as a matter of company policy. [14]

GM is produced as a secondary metabolite, which are also called "idiolites" because they are formed during the "idiophase" (production phase) of batch cultures, whereas the growing phase is termed the "trophophase". These special metabolites are not essential for growth of the producing organism, although they probably have survival functions in nature.

Idiolites have a large chemical diversity and unusual structures. Their production is dependent on genetical and environmental factors, apparently because of the low specifity of the enzymes involved in the secondary metabolism. In contrast, in primary metabolisms, biosynthetic processes are carried out with great specifity and generally only one substrate is needed to form a product.

An important characteristic of secondary metabolites is that they are usually produced only at low specific growth rates of the producing culture. This type of regulation affects a whole range of biosynthetic processes. Individual biosynthetic pathways are also affected by regulatory mechanisms, such as induction, catabolite regulation and end product regulation. [15] [16]

1.4 In-Situ Product Removal

In-Situ Product Removal (ISPR) is an approach to increase the productivity of a biotechnological process. It consists of removing an inhibitory or unstable product from it's producing environment as soon as it is formed [17]. It can increase the productivity or yield of a given biological process by any of the following means:

a) overcoming inhibitory or toxic effects of the produced product to allow continuous formation at maximal production level [18]

b) minimizing product losses due to degradation or uncontrolled release (e.g. by evaporation) [19]

c) reducing the total number of downstream-processing steps [20]

ISPR is restricted to extra cellular products, since it is very difficult to release intracellular products without affecting cell viability. Intracellular products from microbial cells are separated after the cell mass is destroyed. Furthermore, ISPR is also applied to remove by-products such as ethanol or lactic acid that lower the performance of a fermentation process.

ISPR, also called “extractive” fermentation or bioconversion, is part of the general idea of integrated bio processing, which represents the general coordination of upstream-, reaction- and downstream technologies. [21] [22]

In previous assessments of the recovery of GM from fermentation broths, adsorbent resins were used in a solid phase extraction. The extractions were batch adsorptions between the liquid phase fermentation broth, containing the GM and the solid phase adsorbent resins. The resins assessed were Amberlite XAD-4, 7, 16, 1180 and 1600, Sepabeads SP-850 and Diaion HP-20. All resins assessed were capable of recovery of in excess of 90% of GM at a resin concentration of 15 g/l, although resins XAD-16, XAD-1600, XAD-1180 and Diaion HP-20 showed a higher capacity at lower resin concentrations than the others resins. All resins displayed a level of positive selectivity for adsorption of GM over that of contaminants. It was found that the rate of adsorption were dependant on temperature, where longer adsorption times were required at lower temperatures [23]. Although adsorbent resins also have disadvantages like the toxicity of resins to cells [24], the low capacity of absorbents, especially when contacted with whole broth, and the non-specific adsorption of nutrients, cells and by-products [25].

1.5 Liquid-Core Capsules

In this work a novel perstraction system (derived from permeation and extraction) to remove GM from a *Streptomyces hygroscopicus* var. *geldanus* culture broth was investigated. This consists of the use of Liquid-Core microcapsules having an average diameter of 0.7 ± 0.04 mm. Similar types of microcapsules have already been used as extraction aids in ISPR-fermentation processes and for the extraction of herbicides and pesticides from water. [26] [27]

An appropriate method for the removal of GM from a *S. hygroscopicus* var. *geldanus* culture would be a liquid-liquid extraction, as GM is soluble in several organic solvents, and only very slightly soluble in water. Liquid-liquid extraction is a physical separation process that separates compounds based on their relative solubilities in two immiscible liquids, usually an aqueous solution and an organic solvent. The principle behind liquid-liquid extraction involves the contacting of the two liquids, mixing, where the desired compound is transferred from one phase to the other due to a higher solubility or affinity, and settling of the phases due to a different density of the phases. This mass transfer operation results with an extract, which is the solvent with the extracted compound, and the raffinate, the aqueous solution with a poor concentration of the extracted compound. An important factor for a successful extraction beside others is the solvent selection. But unfortunately a liquid-liquid extraction is

adherent to many problems: The direct contact of an organic phase with the cells could cause the formation of stable emulsions during an extractive fermentation. In addition, the choice of an extractive phase is limited to solvents that don't cause molecular or phase toxicity. These problems could be avoided through encapsulation of the organic phase in a hydrogel membrane like alginate. As alginate allows penetration of the desired compound through the capsule wall into the organic phase and doesn't allow the organic phase to interact directly with the cells in the broth. Thereby the cells are protected from the solvent and no stable emulsions are formed. The small dimensions of the micro capsules provide a large interfacial surface, favourable for a faster extraction process. This also means that considerably less solvent is required compared to liquid-liquid extraction processes. Furthermore, it enables continious operation. The capsules can be used directly in the fermenter, what simplifies the clarification of the reaction suspension, since the capsules float to the surface when there is no agitation. Another advantage is the possibility to concentrate the product in the capsules.

Dibutyl sebacate, Oleic acid and Miglyol were chosen to be tested on their suitability for encapsulation. These oils were chosen because of their high $\log K_{OW}$ values. The relatively high degrees of hydrophobicity of these oils ensure that the equilibrium concentration of the oils in an aqueous solution, through exodiffusion from capsules, is very low. [26] [27]

Through liquid-liquid extractions of GM from aqueous solutions with these oils, the partition coefficients of GM for the different oils were investigated. From this result the optimal oil for encapsulation was chosen.

Table 2: *Properties of the solvents that were used for the experimental determination of the Partition Coefficient of GM (K_{GM})* [26]

Solvents	Food processing aid	m.p. (℃)	b.p. (℃)	Flash p. (℃)	Density (kg/l)	Viscosity (mPa*s)	$\log K_{OW}$ (-)	Price (CHF/l) (Fluka 1L)
Oleic acid (fatty acid)	o.k.	16	360	189	0.89	50	7.7	19
Dibutyl sebacate (fatty acid ester)	o.k.	-11	349	178	0.94	9	6.2	78
Miglyol	o.k.	n.a.	n.a.	250	n.a.	30	n.a.	8

Alginate was used in this work as the shell material to encapsulate the water-immiscible solvents. These membranes of natural polysaccharides show very little diffusion limitations towards small uncharged molecules and are therefore ideal for the extraction of GM. In addition, alginate has GRAS status which means that it's Generally Recognized As Safe. The prilling technique was applied in this work to produce capsules with a narrow size

distribution. Perfectly monodispersed capsules enable the mass transfer characteristics of the capsule system to be determined.

The encapsulation process was discovered and developed in the 1940's and 1950's by Barrett K. Green of the National Cash Register Corporation (NRC). The interest in cell, enzyme and biomolecule immobilisation is large and therefore a fast growing area. For this reason numerous techniques for bead and capsule production have been developed, like dripping, coacervation, rotating disc atomization, air jet, atomization, electrostatic dripping, mechanical cutting and the vibrating nozzle technique. In this work the vibrating nozzle technique was applied. This technique applies a sinusoidal frequency to the nozzle which causes the break-up of the extruded liquid jet.

The encapsulation device IE-50 R [Inotech Labor AG, Basel, Switzerland] which was used in this work is displayed in the image 3 below. [28]

Image 3: *Encapsulator IE-50 R*

To produce Liquid-Core capsules, a Concentric Nozzle System was used, which is an additional Kit to the Single Nozzle Unit of the Inotech Encapsulator IE-50 R. The image 4 below represents a schematic figure of the concentric nozzle.

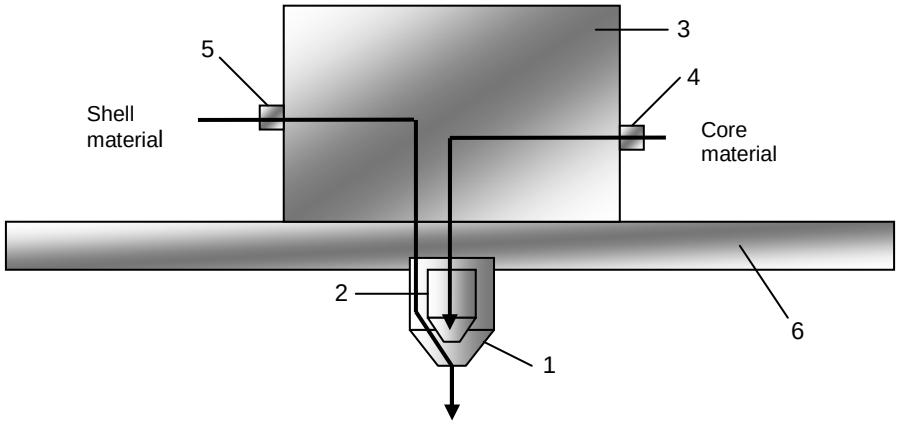


Image 4: *Schematic representation of the concentric nozzle unit*

Legend:

1 Shell nozzel
2 Core nozzel
3 Concentric Pulsation body
4 Luer connection, female
5 Luer connection, female
6 Cover plate

The Encapsulator IE-50 R produces round, uniform beads from 0.1 mm to 2 mm and capsules from 0.5 mm to 2 mm. The encapsulation can be processed in sterile and non-sterile conditions. Scale-up is possible [28], operation is easy, and methods assistance is available. Productivity is 50 to 3000 beads per second with a 4% coefficient of variation. The characteristics of the produced beads and capsules are controlled by several key parameters that include the vibration frequency, voltage, nozzle size, flow rate and the physical properties of the polymer solution. Settings were regulated in order to obtain monodispersed and spherical capsules. The capsule diameter can be estimated as twice the nozzle diameter. In contradiction to this, the diameter of the core capsule can be varied in a much broader range of 1x to 2.5x the core nozzle diameter.

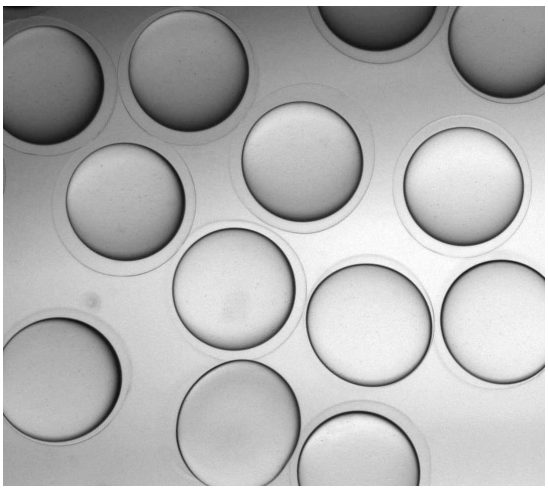


Image 5: *Light microscope picture of liquid-core capsules with a 40x magnification.*

1.6 Mass transfer of GM into Liquid-Core capsules

The mass transfer of GM into the produced alginate microcapsules, containing a core of DBS, was studied with respect to the following points:

- Evaluation of the mass transfer, i.e. partition coefficient K
- Determination of the main resistance to mass transfer of GM into the capsules.

This information enables the possibility of planning of the use of capsules as extraction aids in a fermentation process.

In liquid-core capsules 3 resistances to mass transfer of a target compound into the capsule exist (see image 6):

1. the stagnant aqueous film around the capsule
2. the diffusion across the alginate membrane
3. the stagnant organic film inside the capsule

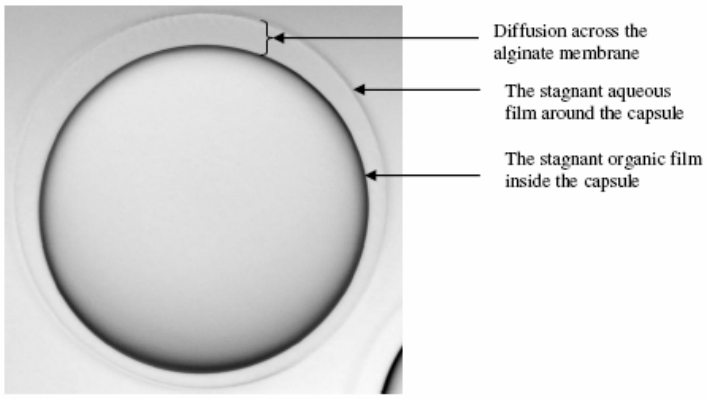
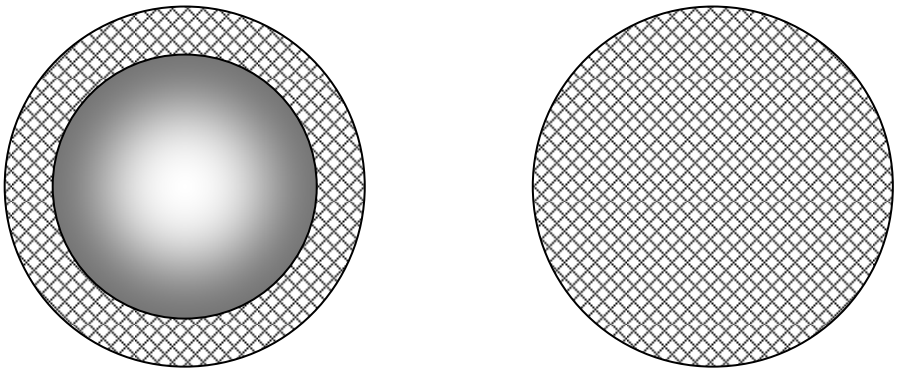


Image 6: *The 3 resistances to mass transfer of a Liquid-Core microcapsule.*

A bead model (see image 7) was used to simplify the description of the capsule system, since it only consists of two phases: an aqueous phase and one imaginary phase of DBS and alginate.



Liquid-Core Microcapsule Modell Bead Modell

Image 7: *Schematic drawing of the bead model, which is used for the determination of the mass transfer.*

The transfer of the target compound from the aqueous into the bead phase can be described by simple mass transfer kinetics (equation 1):

$$-\frac{dc_{aq}}{dt} = ka(c_{aq} - Kc_b) \qquad \textit{Equation 1}$$

With the partition coefficient K (equation 2):

$$K = \frac{c_b^e}{c_{aq}^e} \qquad \textit{Equation 2}$$

k: overall mass transfer coefficient
K: partition coefficient
c_b: GM concentration in the bead at equilibrium
c_{aq}: GM concentration in the aqueous phase at equilibrium

The mass balance can be described by following equation 3:

$$V_{aq}^0 c_{aq}^0 - V_{aq} c_{aq} = V_b (c_b - c_b^0) + m_{spl} \qquad \textit{Equation 3}$$

Considering the mass of GM withdrawn by the sampling procedure (equation 4):

$$m_{spl} = V_{spl} \cdot \sum_{nsamples} c_{aq}[t = sample] \qquad \textit{Equation 4}$$

V_{aq}: aqueous volume
V_b: bead volume
m_{spl}: mass of GM withdrawn by sampling
V_{spl}: sample volume

The perstraction of GM with Liquid-Core capsules can be compared with an adsorption process, where the target compound is accumulated on the adsorbing material through attractive force in short distance. Adsorption is a physical process where the accumulated material doesn't react chemically with the adsorbing material. This process is generally reversible, where the reversed process is named desorption. [29]
Adsorption is a unit operation with the aim to separate substances. It enables the recovery of molecules with a certain selectivity. Thereby the soluble compound is transferred to the

surface of the adsorbing particle. According to the chemical characteristic of the particle surface, the compound can be recovered through its charge, hydrophobicity or affinity for a certain functional group. Adsorption has the advantage that it can concentrate the product. Adsorption is applied in several areas like chromatography, purification in industrial scale of different molecules, and also for the decontamination of diluted waste waters. At present the most used adsorbent materials in the industry are activated carbon and synthetic resins.

An adsorption is divided into four steps:

1. Contact of the solution with the adsorbent material, adsorption of the product
2. Rinsing the adsorbent material
3. Desorption, release of the product
4. Regeneration of the adsorbent material

The adsorption can be implemented as a batch process in shake flasks or in a column such as a fixed-bed or fluidised-bed. The adsorbent material can be characterized through the evaluation of the capacity (thermodynamics of the adsorption) of the adsorption material and also through the measurement of the speed of the adsorption (kinetic aspect of the adsorption). [30]

In this work the Liquid-Core capsules were characterized by their capacity and kinetics. To evaluate the kinetics, following empirical kinetic models for the mass transfer of GM through the capsules were compared:

Pseudo first-order (Lagergren, 1898):

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \qquad \textit{Equation 5}$$

q_e and q_t: adsorption capacity at equilibrium e and at time t [mg*g^{-1}]

k_1: constant of the pseudo first-order adsorption [l*min^{-1}]

$$\log(q_e - q_t) = \log(q_e) - \frac{k_1}{2.303} \cdot t \qquad \textit{Equation 6}$$

Equation 6 is obtained after integration of equation 5. When setting $\log(q_e-q_t)$ as a function of time t, a linear regression is obtained. k_1 and q_e can be determined from the slope and the intercept.

Pseudo second-order (Ho et al., 2000):

$$\frac{dq_t}{dt} = k_2 (q_e - q_t)^2 \qquad \textit{Equation 7}$$

k_2: constant of the pseudo second-order adsorption [g*mg^{-1}*min^{-1}]

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_e^{\,2}} + \frac{1}{q_e} \cdot t \qquad \textit{Equation 8}$$

After integration and linearisation of equation 7, equation 8 is obtained. Through setting t/q_t into the function of time t, q_e and k_2 can be determined from the slope and the intercept.

Elovich (Chien and Clayton, 1980; Sparks, 1986):

$$\frac{dq_t}{dt} = \alpha \cdot \exp(-\beta \cdot q_t) \qquad \textit{Equation 9}$$

α: initial adsorption rate [mg*g^{-1}*min^{-1}]

β: desorption constant [g*mg^{-1}]

$$q_t = \frac{1}{\beta} \cdot \ln(\alpha \cdot \beta) + \frac{1}{\beta} \cdot \ln(t) \qquad \textit{Equation 10}$$

To simplify the Elovich equation 9, it is hypothesized that $\alpha\beta t >> t$. After integration, equation 10 is obtained. Through setting q_t in function of ln(t), α and β can be obtained from the slope and the intercept.

Intraparticle diffusion model (Weber and Morris, 1963; Srivastava et al., 1989):

$$R = k_{id} \cdot t^a \qquad \textit{Equation 11}$$

R: percentage of adsorbed material [%]

t: contact time [h]

a: describes the adsorption mechanism

k_{id}: intraparticular diffusion constant [h-1]

$$\log(R) = \log k_{id} + a \cdot \log(t) \qquad \textit{Equation 12}$$

Through linearisation of equation 11, equation 12 was obtained. Through setting log(R) in function of log(t), a and k_{id} could be obtained with the slope and the intercept. [31]

2 Materials

2.1 Instruments

The instruments used in this project are listed in alphabetic order:

- Autoclave Tomy SS-325, No. 60569, Tomy Seko Co. Ltd., Tokyo, Japan
- Centrifuge 5810, Eppendorf – Netheler – Hinz GmbH, 22331 Hamburg, Germany
- Duran bottles 100 ml
- Encapsulator IE-50 R, Inotech AG, Dietikon, Switzerland
- Erlenmeyer culture flasks 250 ml without baffles
- Filter Units Steritop-GP PES 500mL (0.22 µm, 33mm neck), SCGPS05RE, Millipore Ireland B.V., Carrigtwohill, Co. Cork, Ireland
- High pressure liquid chromatography, Aligent 1100, Agilent Technologies Ireland Ltd., Dublin, Ireland
    - G1322A Degasser, No. JP62354350
    - G1312A Bin Pump, No. DE43618836
    - G1313A ALS, No. DE43631814
    - G1314A VWD, No. DE60400291
- Hypodermic Syringe CC Lick Tip 20 ml, 5157, Socorex Isba S.A., 1024 Ecublens, Switzerland
- Image analysis software cell^F, Olympus UK Ltd, Hertfordshire, WD24 4JL, England
- Incubator (25℃), Sanyo MIR-153, 00201227, AGB Sci entific LTD, Dublin 2, Ireland
- Incubator (28℃), Room X1C4, Dublin City Universit y, Dublin, Ireland
- Kingsorb C18 (octadecyl) bonded silica column (4.6 x 150 mm), particle diameter 5 µm, Phenomenex, Cheshire SK102BN, England
- Laminar Flow Workstation, X181B, Environmental Monitoring Services Ltd., Dublin, Ireland
- Light microscope, Olympus BX51, Olympus UK Ltd, Hertfordshire, WD24 4JL, England
- Luer-Lok Syringe 60 ml, 309653, BD, NJ 07417 USA
- Melting Point Apparatus, Griffin MFB.590.010T, Gallenkamp, Loughborough LE11 3GE, England
- Membrane filters (Cellulose acetate) 0.2µm, 100 mm, 10404 121, Whatman Schleicher & Schüll GmbH, 37586 Dassel, Germany
- Needles 0.8 mm x 40 mm, 304432, BD Microlance^{TM}, Becton Dickinson S.A., 22520 Fraga, Spain

- Needles 0.8 mm x 80 mm, 0641, Erosa, Rose GmbH, 54294 Trier, Germany
- Nylon membrane filters 0.2 µm, 25 mm, PALL Nyaflor ®, P/N 66601, Michigan, USA
- Orbital shaker, SANYO MIR-S100, AGB Scientific LTD, Dublin 2, Ireland
- pH-meter WTW pH522, Joe Walsh scientific, Dublin, Ireland
- Rotator RZR1, No. 50111, Heidolph, GB Scientific Ltd, Dublin, Ireland
- Security Guard, AJO-4287, Phenomenex, Cheshire SK102BN, England
- Sonicator, 5510E-MT, Branson Ultrasonics, Berkshire, UK
- Stainless Steel Filtration device, BL0417-1, Schleicher & Schüll GmbH, 37586 Dassel, Germany
- Syringe filters 0.2 µm, non-pyrogenic, sterile-r, No. 83.1826.001, Sarsted, 51588 Nümbrecht, Germany
- Syringes 2 ml, Omnifix®, 4616022V, B. Braun Melsungen AG, 34209 Melsungen, Germany
- Syringe pump IER-560 with control unit IER-565, Inotech Encapsulation AG, Basel, Switzerland
- Syringe pump model 200, Ismatec S.A., Labortechnik-Analytik, 8152 Glattbrugg-Zürich, Switzerland
- Texture Analyser, model Ta-ST2I, Stable Micro Systems, Godalming, England
- Thermocouple thermometer 2038T, Digitron, Devon TQ2 7AY, England
- UV Cabinet, CN-6, Vilber Lourmat, 77202 Marne-la-Vallée Cedex 1, France
- Video camera, DP30 BW, Olympus UK Ltd, Hertfordshire, WD24 4JL, England
- Vortex mixer SA3, Stuart®, Barloworld Scientific, Staffordshire ST15 0SA, England
- Water bath, Clifton, Berg X181, Shaw Scientific limited, Dublin, Ireland
- Water bath shaker, Aquatron No. 890243, HT Infors AG, 4103 Bottmingen, Switzerland

2.2 Reagents

The reagents used in this project are listed in alphabetic order:

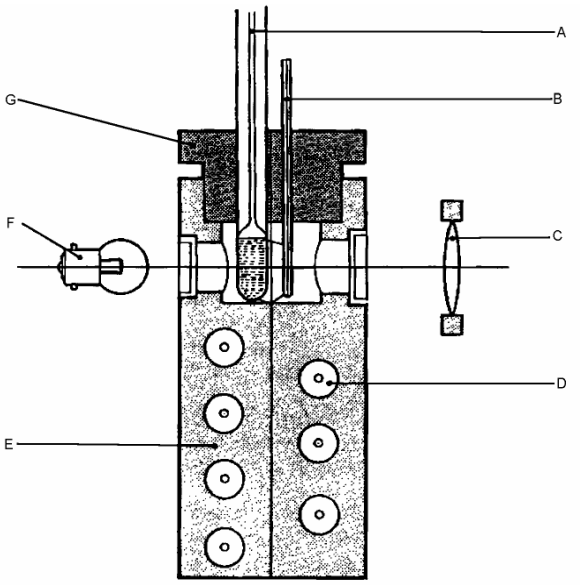
- 1-Octanol, 74850 Fluka, Sigma-Aldrich Chemie GmbH, 9471 Buchs, Switzerland
- Acetonitrile 230 (Methyl Cyanide), H047, ROMIL LDT, CB5 9QT Cambridge, GB
- Bacteriological Peptone, Oxoid LTD., Basingstoke, Hampshire, England
- Beef Extract, LP 0029, Oxoid LTD., Basingstoke, Hampshire, England
- Calcium chloride Dihydrate, 21101, Fluka, Fluka Chemie GmbH, 9471 Buchs, Switzerland

- D(+)-Glucose monohydrate puriss., 16301, Riedel de Haën, Sigma-Aldrich Laborchemikalien GmbH, 30926 Seelze, Germany
- Dibutyl sebacate tech., D49504-1L, Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany
- Dimethyl sulfoxide minimum 99.5% GC, D4540-500ML, Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany
- Geldanamycin >99%, LC Laboratories, MA 01801, USA
- Magnesium sulfate 7-hydrate, 101514Y, AnalaR® BDH Laboratory Supplies Pool, BH15 1TD, England
- Methanol Chromasolv® for HPLC, 3480 Sigma-Aldrich Chemie GmbH, 9471 Buchs, Switzerland
- Miglyol® 812 (glycerol tricaprylat/caprat, glycerol trioctanoate/decanoate) Neutralöl, Chg. 060404, Hänseler AG, 9101 Herisau, Switzerland
- MOPS, >99.5%, M3183-100G, Sigma-Aldrich Chemie GmbH, 89552 Steinheim Germany
- Na-Alginate Powder, IE-1105, Inotech, Basel, Switzerland
- N-Z Amine A® from bovine milk, C0626-500G, Sigma-Aldrich Chemie GmbH, 89552 Steinheim Germany
- Oleic Acid ultra pure (72%), Riedel de Haën, 30926 Seelze, Germany
- Sodium chloride, 71379, Fluka, Sigma-Aldrich Chemie GmbH, 89552 Steinheim Germany
- Sodium hydroxide white, pellets, purified, 06213, Riedel de Haën, 30926 Seelze, Germany
- Sodium phosphate dibasic heptahydrate puriss. p.a., Reag. ACS, 30413, Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH, 30926 Seelze, Germany
- Sodium phosphate monobasic monohydrate, reagent ACS, crystals, 424395000, Acros Organics, Geel, Belgium
- *Streptomyces hygroscopicus var. geldanus,* Strain NRRL 3602, ARS Patent Culture Collection, Peoria, Illinois, USA
- Tween PBS, ABG, DCU, Dublin, Ireland
- Yeast Extract, LP 0021, Oxoid LTD., Basingstoke, Hampshire, England

3 Methods

3.1 Determination of the melting point of GM

The melting point of GM was determined using an apparatus with a capillary tube in a metal block described in the OECD guideline for testing of chemicals [32]. The apparatus used for this experiment was a Griffin Melting Point Apparatus (MFB.590.010T) from Gallenkamp. A thermocouple thermometer 2038T from Digitron was used to monitor the temperature in the apparatus. A sample of commercially available GM powder was placed into one end of a glass capillary tube and inserted into the apparatus. When the temperature was increased, the effect it had on the sample could be observed through an eye piece. As soon as the sample melted, the temperature was noted. This measurement was carried out in duplicate.



A. Thermometer
B. Capillary tube
C. Eye-piece
D. Electrical resistance
E. Metal heating block
F. Lamp
G. Metal plug

Image 8: *Apparatus with capillary tube in a metal block for visual observation of the melting point*

3.2 Solubility of GM

3.2.1 Solubility of GM in pure water

The solubility of GM in water was investigated with a simple shake flask method described in the OECD guideline for testing of chemicals [33]. The experiment was carried out in 100 ml Duran bottles at 28°C on an orbital shaker at 200 rpm in the dark. The bottles contained 50 ml of a 50 mg/l GM solution in water. Samples were taken after 24, 48 and 72 hours and equilibrated at 20°C in the dark for 24 hours without shaking. The samples were then filtered with a nylon filter (0.22 µm), diluted with ACN 1:1 and injected onto the HPLC.

3.2.2 Solubility of GM in water with Dimethylsulfoxide (DMSO) as a co-solvent

The solubility of GM in water with DMSO as a co-solvent was investigated with a GM concentration of 20 mg/l in water with 2%, 4% and 20% DMSO. The experiment was carried out at 20℃.

As it was known from the supplier, GM is soluble in DMSO to a concentration of 10 g/l. As DMSO is miscible with water, DMSO was used as a co-solvent to get GM into an aqueous solution, as the previous experiment showed that GM on its own is not soluble in water at the chosen conditions.

A stock solution of 1 g/l GM in DMSO was prepared. 1 ml of this stock solution was added to 49 ml of water to obtain 20 mg/l GM in an aqueous solution with 2% DMSO. The GM stock solution was diluted 1:1 and 1:10 with DMSO to obtain GM stock solutions of 0.5 g/l GM and 0.1 g/l GM in DMSO. From the 0.5 g/l GM stock solution 2 ml were added to 48 ml water to obtain 20 mg/l GM in an aqueous solution with 4% DMSO. From the 0.1 g/l GM stock solution 10 ml were added to 40 ml water to obtain 20 mg/l GM in an aqueous solution with 20% DMSO. After vortexing the solutions for 10 seconds the solutions were filtered with a nylon filter (0.22 µm) and analysed with HPLC.

To ensure the solubility of the stock solution with 1 g/l GM in DMSO, a sample of this solution was also filtered and injected on the HPLC column.

3.2.3 Solubility of GM in Bennett's Media at culture conditions

50 mg/l GM was added to sterile Bennett's fermentation media at 3 different pH's (5; 6; 7,3) and incubated for 72 hours at 28℃ and 150 rpm on a n orbital shaker in the dark. The media contained 3 g/l Yeast Extract, 3 g/l Beef Extract, 2 g/l N-Z Amine A, 0.5 g/l $MgSO_4.7H_2O$ and 50 g/l D-Glucose monohydrate. The experiments were carried out in 250 ml Erlenmeyer flasks with a working volume of 100 ml. For each pH, 3 flasks were prepared. After 24, 48 and 72 hours one flask of each pH was taken off the orbital shaker and let equilibrate at room temperature (20 ± 0.5℃) for 24 hours. Samples were then filtered with a nylon filter (0.22 µm) to remove insoluble GM, then diluted 50:50 with ACN and finally injected on the HPLC column. Samples were taken in triplicate and run on the HPLC to quantify the amount of soluble GM present.

3.3 Stability of GM

3.3.1 Stability of GM in solvents: DMSO and ACN

A 1 g/l GM solution in DMSO was stored for 5 days at 4℃. The solution froze, as the freezing point of DMSO is at 18℃. Before this solution could be sampled, it had to be defrosted for 4 h at room temperature. A 1 g/l GM solution in DMSO was also stored for 5 days at room temperature (20 ± 0.5℃). A further GM solution with a concentration of 0.1 g/l GM in ACN was stored for 3 months at 4℃.

The solutions were sampled at the beginning and after 5 days. The solutions with DMSO were diluted 1:10 with ACN. All the samples were then filtered with a nylon filter (0.22 µm) and injected on the HPLC column to investigate the stability of GM in these solutions at the mentioned conditions.

3.3.2 Stability of GM in water with 2% DMSO in the presence of O_2

To observe if GM degrades spontaneously as a solubilised compound in an aqueous solution at culture conditions (28℃, 150 rpm), an experiment was carried out in sterile water with 10 mg/l GM (using 2% DMSO as a co-solvent). The solution was incubated with a working volume of 10 ml in 250 ml shake flasks with a cotton wool stopper to let oxygen penetrate into the solution. As it was reported by De Boer et al. (1970) [1], GM degrades in the presence of oxygen.

The solution was sampled and analysed at the following times: 0, 1, 2, 3, 4, 5, 6, 7 and 14 days. The samples were diluted with ACN (50:50), filtered with a nylon filter (0.22 µm) and then analysed using HPLC.

3.4 Determination of the Octanol/Water Partition Coefficient (K_{OW}) of GM

To determine the $LogK_{OW}$ value of GM in a *n*-Octanol/water mixture, a shake flask method was used, which is described in the EPA Product Property Test Guidelines [34].

For the water phase a phosphate buffer (PB) pH 7 was used and 1-Octanol was used as the *n*-Octanol phase. Before the experiment was started, the PB and 1-Octanol were pre-saturated with each other, as the solubility of water in octanol at 25℃ is 0.275 mole fraction and the solubility of octanol in water is $7.5*10^{-5}$ [35]. To saturate the 1-Octanol solution, 10 ml of PB were added to 100 ml of 1-Octanol and mixed on a magnetic stirrer at room temperature (20 ± 0.5℃) for 1 hour. To saturate the PB, 10 ml of 1-Octanol were added to

800 ml of PB and also mixed on a magnetic stirrer at room temperature for 1 hour. Both solutions were then equilibrated at room temperature for 20 hours.

GM was added to the presaturated PB with MeOH as a co-solvent to help solubilise GM. Then 2 ml of a 250 mg/l GM solution in MeOH were added to 48 ml PB. The final GM concentration in the pre-saturated PB was therefore 10 mg/l. 1-Octanol was then added to the GM solution. The experiment was carried out three times, each time with a different volume of 1-Octanol: 0.3 ml, 0.6 ml and 1 ml. Each experiment was carried out in triplicate, and with each experiment a control solution was run, which contained no 1-Octanol.

50 ml Duran bottles were used for this experiment. The bottles were incubated at room temperature (20 ± 0.5℃) and mixed on a rotator wit h a rotation through 360° about their transverse axis at 28 rpm during 15 min. The contents of the bottles were then transferred into glass universals and centrifuged at 2.9 G for 20 min. The aqueous phase was then sampled with a needle and syringe, diluted 1:1 with ACN and was run on the HPLC to measure the GM concentration in the aqueous phase.

The Partition Coefficient K of GM in the 1-Octanol/water mixture could be determined using the following equation:

$$K = \frac{c_b}{c_{aq}} \qquad \textit{Equation 13}$$

Where:

c_b is the organic concentration at time t (calculated by equation 2)

c_{aq} is the aqueous concentration at time t

The mass balance can be described with following equation:

$$V_{aq}^0 c_{aq}^0 - V_{aq} c_{aq} = V_b \, (c_b - c_b^0) \qquad \textit{Equation 14}$$

Where:

V_{aq}^0 is the initial aqueous phase volume

V_{aq} is the aqueous phase volume when the sample has been taken

c_{aq}^0 is the initial aqueous concentration

V_b is the organic phase volume

c_b^0 is the initial organic concentration

3.5 Determination of the Partition Coefficients (K) of GM in aqueous solutions with 3 different oils

The partition coefficients (K) of GM in water/oil systems were determined with liquid-liquid extractions. Oleic Acid (OA), Dibutyl sebacate (DBS) and Miglyol (MG) were chosen as organic phases. An aqueous 20 mg/l GM solution with 4% DMSO was used as the aqueous phase. The liquid-liquid extractions were carried out in 50 ml Duran bottles at 28℃ and 150

rpm with an aqueous volume of 20 ml and 0.05 ml oil. Samples were taken after 17 and 23 hours from the aqueous and the organic phase with a syringe and needle. The aqueous phase was diluted 50:50 with ACN and the organic phase was diluted 1:10 with MeOH. The sample of the aqueous phase was injected onto HPLC with a mobile phase containing ACN:Water (50:50 v/v %) and the sample of the organic phase was run on the HPLC with a mobile phase containing MeOH:Water (80:20 v/v %). The analytical procedures are explained in more details in chapter 3.10. The partition coefficients of GM in water with different oils were calculated using equations 13 and 14.

3.6 Stability of GM in DBS and OA

The stability of GM in DBS and OA was determined using a simple shake flask method. Solutions containing 0.2 g/l GM in DBS and 0.2 g/l GM in OA were incubated at 28℃ and 200 rpm. 25 ml glass universals were used for these experiments with a working volume of 25 ml.

Samples were taken after 1, 2, 6, 7, 8, 9, 12, 13, 14, 15, 16 and 19 days. The samples were diluted 1:10 with MeOH and injected onto HPLC with a mobile phase containing MeOH:Water (80:20 v/v %).

3.7 Production of Liquid-Core Microcapsules

Capsules composed of a hydrophobic liquid core and a hydro gel membrane were produced using the co-extrusion jet break-up technique. The encapsulator (Inotech Encapsulator IE-50 R) was fitted with a concentric nozzle with an inner diameter of 200 µm and an external diameter of 300 µm. Two syringe pumps were connected to the encapsulator to supply DBS through the central nozzle and alginate solution through the external nozzle. Spherical capsules were obtained by applying a defined amplitude to the co-extruded jet. They were collected in a gelling bath placed below the nozzle, which was agitated by a magnetic stirrer. The resulting capsules were incubated in the gelling bath for 45 min, filtered and washed intensively with de-ionised water to remove any un-reacted reagents.

A stock solution of sodium alginate (2.5 %) was prepared in MOPS buffer (10 mM), pH 7 and filtered through a 0.22 µm filter under a pressure of 2-2.5 bar. The capsule-size distribution was measured and the mass transfer of GM into the capsules was determined. The parameters used to manufacture capsules are outlined in table 3.

Table 3: *Parameters for the production of Liquid-Core Microcapsules*

Parameters:	
Alginate solution:	2.5% w/v in MOPS (10mM), pH 7
Polymerization solution:	32 g/l $CaCl_2$ in MOPS (10 mM) and 0.1% Tween 80, pH 7
Oil:	Dibutyl Sebacate
Nozzle internal diameter:	200 µm
Nozzle external diameter:	300 µm
Reaction Vessel diameter:	ø 14 cm (Foss)
Stirring bar length:	6 cm
Impact height:	18 cm
Volume of polymerization solution:	1 L
Volume of Alginate:	50 ml
Volume of oil:	20 ml
Alginate flow rate:	8.75 ml/min (323)
Oil flow rate:	3.5 ml/min
Frequency:	678 Hz
Amplitude:	7 %
Stirring Speed (on stirring plate):	2 (keep as low as possible and make sure impact is between vortex and wall)
Voltage:	1.7 kV
Polymerization time:	45 min

3.8 Characterisation of the Liquid-Core Microcapsules

3.8.1 Measurement of the Capsule Size Distribution

The outer diameter of the capsules and the inner diameter of the liquid cores were measured with a video camera (DP30 BW) attached to a light microscope (Olympus BX51) with a magnification of 40x. The size distribution was determined by measuring the internal and external diameters of 63 capsules using the image analysis software Cell^F (see image 9). The measured values were noted on a result sheet (see appendix 10.1).

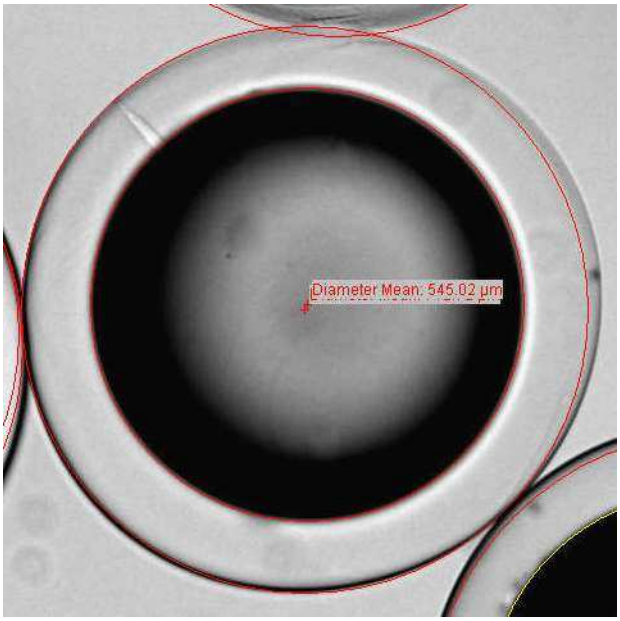


Image 9: *Measurement of the capsule size using a light microscope with a 100x magnification.*

3.8.2 Mass transfer characteristics and capacity at 20 and 28℃:

The mass transfer of GM into Liquid-Core capsules was investigated at two different temperatures. The aim was to observe if the mass transfer limitation was due to the diffusion across the capsule wall.

The experiment at 20℃ was carried out at room temperature which was at 20 ± 1℃. The experiment at 28℃ was carried out in a temperature regulated incubation room. The experiments were carried out in 25 ml glass universals on a rotator at 28 rpm, while the flasks were wrapped in aluminium to protect the GM from light. A working volume of 10 ml was used and an initial GM concentration of 0.1 g/l was used. The solution was prepared through addition of 0.1 ml of a 10 g/l GM in DMSO stock solution to 9.9 ml deionised water. For the experiment at 20℃, 0.846 g capsules and for the experiment at 28℃, 0.733 g (dry weight) were added to the GM solutions at t=0. The solutions were sampled every 5-10 minutes until equilibrium was reached, diluted 50:50 with ACN and analysed using HPLC.

An other experiment was carried out to investigate the adsorption kinetics and to compare the kinetics with different kinetic models: “Pseudo first order”, “Pseudo second order”, “Elovich” and “Intraparticle diffusion model”.

This experiment was carried out at 20℃ in 100 ml shake flasks with a working volume of 50 ml at 150 rpm. The GM concentration in the solution was 0.1 g/l. The solution was prepared through addition of 0.5 ml of a 10 g/l GM in DMSO stock solution to 49.5 ml deionised water. 4.656 g capsules (dry weight) were added to the GM solution at t=0. Samples were taken in short time intervals of 4-6 min with 1-ml syringes and diluted 50:50 with ACN and analyzed using HPLC.

3.8.3 Sterilisation of Liquid-Core capsules using UVC light at 254 nm

Liquid-Core capsules were produced in non-sterile conditions and then sterilised through UVC radiation at 254 nm for 48 hours before adding them to a *S. hygroscopicus* var. *geldanus* culture (cultivation is described in chapter 3.9.5). Therefore 10 ml capsules (dry) were placed in an open Petri dish with 10 ml deionised water, to prevent the capsules from drying out. The capsules in the Petri dish (without lid) were then exposed to UVC radiation in a closed cabinet, which was sterilised beforehand with EtOH and was placed in a Laminar Flow. To assure the sterility of the capsules after the UVC treatment, a sample of 5 ml capsules were dissolved in 25 ml of a sterile 20 g/l sodium citrate solution and mixed for on a magnetic stirrer. 1 ml of this suspension of dissolved capsules was then plated out on agar plates. This was carried out in triplicate. The agar plates contained Bennett's media (described in chapter 3.9) with 15 g/l Oxoid Nutrient Agar No. 3. The agar plates were incubated at 28℃ for 1 week and then inspected for any growth of colonies.

3.8.4 Investigation of the influence of UV radiation of Alginate beads through strength and elasticity measurements

In order to investigate, if UV radiation of Liquid-Core capsules has a negative effect on the capsule strength and elasticity, the mechanical characteristics were analyzed by a Texture Analyser. This apparatus measures the force, applied via a piston that is necessary to compress a capsule until it bursts. This burst force (given in gramm by the apparatus) is used as an experimental parameter for the strength of the examined capsule. In addition, the compression distance (in mm) is determined from the point, where the piston touches the capsule up to its bursting. Measurements were done bead by bead to determine a mean value from 10 beads. The applied compression speed was 0.30 mm/s at a compression distance of 3 mm.

This experiment was carried out with simple Alginate beads instead of Liquid-Core capsules, as it was assumed that UV light would have the same effect on both types of spheres. Therefore Alginate beads made of 2.5% (w/v) Sodium-Alginate in MOPS buffer at pH 7.0 were extruded into a gelling bath containing 32 g/l $CaCl_2$, 10 mM MOPS and 0.1 % Tween 80. For sterilisation, 10 ml of these beads with a size distribution of 1.5 mm ± 1% were then placed in a Petri dish with 10 ml of deionised water and exposed to UV light (as described in chapter 3.8.3) for 65 hours. After 0.5, 1.13, 1.65, 19 and 65 hours the mechanical resistance of the beads was tested. A control was treated the same as the experimental sample but wasn't placed under the UV light and was also tested. 10 random beads were taken from the Petri dish from the control and experimental sample and the mechanical resistance of each was measured to see if it had changed.

3.9 Cultivation of S. hygroscopicus with monitoring of the GM production

S. hygroscopicus var. geldanus NRRL 3602 was used throughout this assessment. Spores were produced on Bennett's medium agar containing: 20 g/l technical agar No. 3; 1 g/l yeast extract; 1 g/l beef extract; 2 g/l N-Z-amine A; 10 g/l Dextrose monohydrate. Spores were recovered using resuspension solution containing: 3 g/l yeast extract, 5 g/l bacteriological peptone and 1 g/l $MgSO_4$ $7H_2O$. The cultivation medium was Bennett's liquid medium containing: 1 g/l yeast extract, 1 g/l 'Lab-lembco' beef extract, 2 g/l N-Z Amine A, 50 g/l dextrose monohydrate. A working volume of 100 ml was used for the cultivations.

A spore inoculum of *S. hygroscopicus* was used to inoculate cultivations. This was prepared by culturing the organism on static cultures of Bennett's Medium agar, in 5 L Erlenmeyer flasks, for 21 days at 28℃. Spores were recovered by washing the agar with resuspension solution at 100 rpm for 1 h at 4℃. Bennett's mediu m was then inoculated at 1% (v/v) using a spore suspension of $6.7*10^9$ spores/ml and incubated at 28℃ at an agitation of 150 rpm. [36]

3.9.1 Investigation of the stability of the produced GM during cultivation

In a first cultivation a sample was taken each day from the culture broth and the GM concentration was determined. The method for extracting GM from the culture broth was the following: 0.5 ml of the culture broth was added to 0.5 ml ACN and vortexed for 5 min, filtered with a 0.22 µm nylon filter and injected onto the HPLC. With this method all of the GM could be detected, that was solubilised, precipitated or intracellular.

Through plotting the GM peak area and the peak area of unknown products with time, a relationship between the amount of GM and the amount of unknown products can be established to show if GM degrades during cultivation.

3.9.2 Investigation of the solubility of the produced GM during cultivation

In further cultivations of *S. hygroscopicus* samples were taken and the GM concentration was detected using different sampling methods to investigate in which form (soluble, precipitated or intracellular) the produced GM was present in the broth.

To measure the amount of GM that is solubilised in the broth, a sample was taken and filtered with a 0.22 µm filter to remove cells and precipitated GM. The filtrate that contained solubilised GM was then diluted 50:50 with ACN and injected onto HPLC.

The total GM amount in the broth was measured by mixing 0.5 ml broth with 0.5 ml ACN for 5 minutes on the vortex, to solubilise the precipitated GM and release intracellular GM. Then the sample was filtered with a 0.22 µm nylon filter and injected on the HPLC column.

To measure intracellular GM, 6 ml of the fermentation broth was centrifuged and the cells were washed three times with a PB pH 7. Then the cells were resuspended in 3 ml PB pH 7 and sonicated for 20 minutes at a frequency of 40 kHz to lyse the cells and release intracellular GM. This suspension was then mixed with 3 ml ACN for 5 minutes using a vortex, filtered with a 0.22 µm nylon filter and analysed using HPLC. As a control, pure GM in deionised water (20 mg/l with 4% DMSO) was also sonicated for 20 minutes at 40 kHz, to ensure that sonication doesn't degrade GM.

Two cultivations were carried out. With the first one an altered media composition was used: Instead of 1 g/l yeast extract and 1 g/l beef extract, 3 g/l of each were used. In the second fermentation the Bennett's liquid media was used which is described above (chapter 3.9).

3.9.3 Investigation of GM degradation in cultures

A fermentation broth of a culture which was cultivated for 24 days and in which the GM was degraded at the end of the fermentation, was harvested. The cells of this culture were separated through centrifugation. 20 ml of fermentation broth without cells were acidified with 0.5 M HCl to a pH 2, and then heated to 70°C in a water bath for 90 min to denature all proteins. The broth was then filtered with a 0.22 µm filter to remove precipitates. 100 mg/l GM was then added to the broth through addition of 1 ml of a 2 g/l GM solution in DMSO to 19 ml denatured fermentation broth. The solutions were then incubated at 30°C, 150 rpm for 9 days. Samples were taken each day, diluted 50:50 with ACN and filtered with a 0.22 µm filter and injected on the HPLC. The same experiment was repeated with neutralisation of the broth to pH 7 before adding GM.

The aim was to show if added GM stays stable after denaturing proteins through heat and acid which possibly caused the degradation of GM during fermentations.

3.9.4 Investigation of a product inhibitory effect of GM

In further cultivations the aim was to monitor the GM concentrations during the cultivations and investigate if there was a product inhibitory effect of GM when different concentrations of GM stock solutions were added to the cultures.

4 cultures were carried out in parallel. In two of them sterile filtered GM stock stolutions in DMSO were added at the beginning of the fermentations: 1 ml of a 2.5 g/l GM stock solution and 1 ml of a 5 g/l GM stock solution were added what corresponded to GM concentrations

of 25 mg/l and 50 mg/l. Two control fermentations one with addition of 1 ml DMSO and one without any addition were run to observe if DMSO has an influence.

During the cultivation the total GM was measured. Samples were taken after 18, 21 and 24 days. 0.5 ml of the culture broth was mixed with 0.5 ml ACN for 5 min on the vortex, then filtered with a 0.22 µm nylon filter and analysed on the HPLC.

3.9.5 Cultivation of *S. hygroscopicus* with addition of Liquid-Core Capsules

The cultivation of *S. hygroscopicus* was also carried out with addition of sterile Liquid-Core capsules. These were produced in non-sterile conditions and then sterilised through UVC radiation at 254 nm for 48 hours before adding them to the culture (as described in chapter 3.8.3). 10 ml of sterile capsules were added at t=0 to the culture. A control culture which contained no capsules was run at the same time. The cultures were sampled at t=0, 18, 21 and 24 days. Samples were taken by mixing 0.5 ml of the culture broth (without capsules) with 0.5 ml ACN for 5 min on the vortex, then the sample was filtered with a 0.22 µm nylon filter and analysed using HPLC.

3.10 Analytical Procedures

The experiments were carried out with commercially available pure GM (99%). GM was analysed with RP-HPLC with a C18 (octadecyl) bonded silica column with security guard, with dimensions of 4.6 x 150 mm and a particle diameter of 5 µm. An ACN:Water mixture (50:50) was used as the mobile phase with a flow rate of 1 ml/min and an injection volume of 0.05 ml. GM was detected with a UV detector at a wavelength of 308 nm at a retention time of 9.97 min.

A standard curve of GM in ACN was established in a concentration range of 10 mg/l to 100 mg/l. The standard curve was obtained through plotting the GM concentration in function of the obtained peak area of each concentration (see appendix 10.2). From the slope of this curve the GM concentration of any other sample in this concentration range could be calculated. The standard curve showed a very good correlation coefficient of 0.9995. A standard curve of GM in ACN was also established in a concentration range of 1 mg/l to 10 mg/l. It could be shown that the absorbance of GM at such low concentrations was still proportional to the concentration, as a good correlation coefficient of 0.9982 was obtained (see appendix 10.2).

Aqueous samples containing GM were diluted 50:50 with ACN and filtered with a nylon filter (0.22 µm) before injecting them onto HPLC.

To detect GM in DBS and OA, an appropriate HPLC method had to be developed. A method found in literature [37] which was used to determine plasticizers like DBS in different solvents was adapted. A mobile phase consisting of MeOH:H_2O (80:20 v/v %) was used with a flow rate of 1 ml/min and an injection volume of 0.05 ml. The samples containing GM in DBS or OA where diluted 1:10 with methanol and analysed with a C18 bonded silica column with security guard, with dimensions of 4.6 x 150 mm and a particle diameter of 5 µm. GM could be detected at a retention time of 4.15 minutes with a UV detector at a wavelength of 308 nm.

Standard curves of GM in OA and DBS were established in a concentration range of 10 mg/l to 100 mg/l. They showed very good correlation coefficients of 0.9995 and 0.9978 (see appendix 10.3 and 10.4).

4 Results and Discussion

4.1 Determination of the melting point of GM

To ensure the purity of the GM which was used for all the experiments, the melting point was determined. Although the providers of this GM powder stated it to be 99% pure, the experiment was still carried out to ensure that the GM didn't take any contaminations while it was used for other experiments stored at -80℃ over 13 months. The product had an expiration date of July 4, 2016 when stored at or below -20℃, so no modification of the product was expected due to the expiration of the product.

The experiment was carried out twice, while in both cases a melting point of 525.15 K respectively 252℃ was obtained. This value corresp onded to a value found in literature [1]. As a sharp temperature range was observed during the melting of the GM powder in the glass capillary, it can be assumed that the purity of the GM is very high. According to the used method an estimated accuracy of ± 0.5 K can be taken into consideration [29].

4.2 Solubility of GM

4.2.1 Solubility of GM in water

The HPLC results showed no peaks at the retention time of GM. This implies, that none of the GM added to the water was solubilised at the chosen conditions (28℃, 200 rpm, 72 h). For this reason a co-solvent was used in the next experiment to solubilise GM in water.

4.2.2 Solubility of GM in water with DMSO as a co-solvent

The HPLC results showed that GM didn't solubilise completely in the aqueous GM solution with 2% DMSO; only 34% of the GM could be detected and therefore solubilised. As there

where no other peaks visible, it could be assumed, that no degradation of GM occurred, that could be detected at the same wavelength.

The HPLC results of the stock solution (1 g/l GM in DMSO) showed that GM was fully soluble in this solution. So it could be concluded, that GM unsolubilises as soon as a certain amount of water is added. It could be shown, that already a volumetric fraction of 4% DMSO is enough to solubilise 85% of 20 mg/l GM. With 20% DMSO 99% of the GM in the sample could be detected and therefore solubilised.

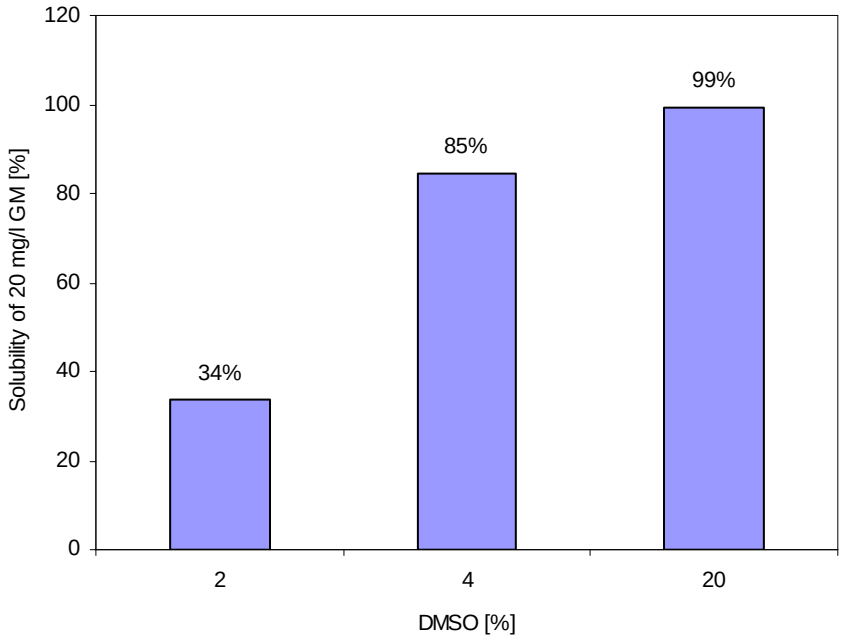


***Figure 1**: Solubility of GM [%] as a function of the volumetric fraction of DMSO [%] in the aqueous solution.*

4.2.3 Solubility of GM in Bennett's Media at culture conditions

As GM is produced by *S. hygroscopicus* in a submerged fermentation, it was expected that GM is soluble in an aqueous solution. As previous results showed that GM wasn't soluble in pure water, this experiment was repeated in Bennett's Media at culture conditions. The experiments were carried out at different pH's to see if media components and pH influence the solubility of GM in an aqueous solution.

The results showed that none of the added 50 mg/l GM was solubilised. This indicates that the solubility of GM is not influenced by compounds in Bennett's Media and pH also hasn't got an influence on the solubility of GM.

4.3 Stability of GM

4.3.1 Stability of GM in DMSO and ACN

The HPLC chromatograms (see appendix 10.6) showed that after 5 days at 4℃ the 1 g/l GM solution in DMSO contained only 38% of the initially present GM. It could also be observed that large new peaks appeared at retention times 1.99 and 3.38 min. These were not visible in the fresh GM solution in DMSO and neither in the GM solution in ACN. As DMSO has a very high freezing point of 18℃, it freezes when it's stored in the fridge at 4℃, whereas ACN has a very low freezing point of -65℃ and can be stored in the freezer at -20℃.

These results are consistent with GM being stable in ACN at -20℃ for at least 3 months and in a 1 g/l DMSO solution at room temperature for at least 5 days. It could also be concluded that freezing of GM in a solubilised condition causes degradation of the molecule.

Degradation wasn't observed with a 1 g/l GM solution in DMSO at room temperature, and neither with a 0.1 g/l GM solution in ACN stored at 4℃ for 5 days.

4.3.2 Stability of GM in water with 2% DMSO

The results showed that GM, solubilised in an aqueous solution with 2% DMSO as a co-solvent, stays stable over 7 days. After 14 days degradation of 49% was observed (see figure 2). This indicates that GM degrades spontaneously with the presence of oxygen at culture conditions. This could be a problem in the fermentation of GM, which takes 21 days. GM that's produced in the first 7-14 days would then possibly be degraded by the end of the fermentation. For this reason the ISPR of GM in a *S. hygroscopicus* fermentation would be a possibility to overcome this problematic aspect.

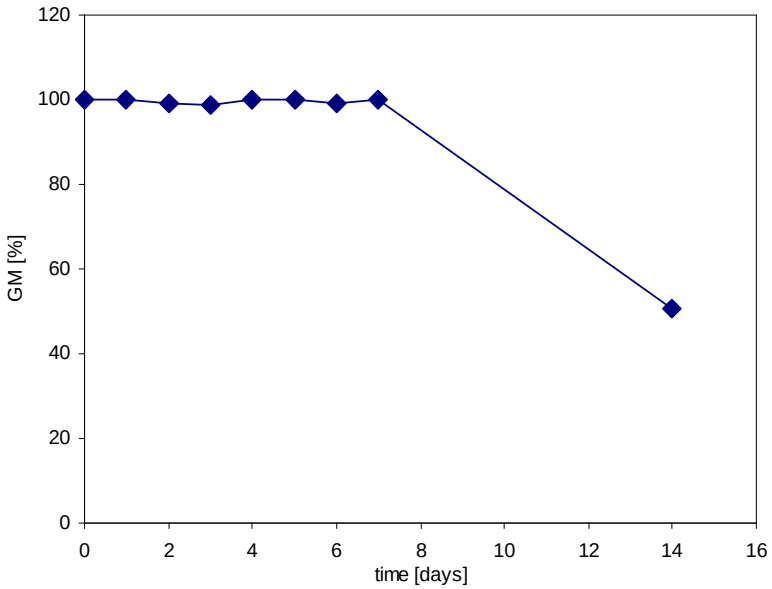


Figure 2: *Stability of 10 mg/l GM in H_2O at culture conditions over 14 days.*

4.4 Determination of the Partition Coefficient K_{OW} (1-Octanol/Water) of GM

The $logK_{OW}$ is a criterion which describes the hydrophobicity of compounds with respect to an octanol/water mixture. For GM a Partition Coefficient in a 1-Octanol/water mixture of 51.20 with a standard deviation of ± 2.33 was determined, and a $logK_{OW}$ value of 1.71 with a standard deviation of ± 0.02 was calculated.

Table 4: *Determined values of $logK_{OW}$ and P of GM in an Octanol/water mixture*

$logK_{OW}$ = 1.71 ± 0.02 (0.03; standard; 3)
K_{OW} = 51.20 ± 2.33 (4.03; standard; 3)

This represents a relatively high hydrophobicity of GM, which might explain the low solubility in water. At the same time this information indicates that GM tends to accumulate in hydrophobic spheres like cells. This also reveals that GM could be accumulated in a sphere like a liquid-core capsule with a permeable capsule wall and a core with a hydrophobic organic solvent.

As K_{OW} can be implicated in diverse areas as bioaccumulation and drug design, relationships between physiochemical phenomena and biological/biochemical and environmental processes are present. K_{OW}, in thermodynamics called a free energy function, is directly concerned with the energetics of transfer of substances between phases. The connection with biological activity can be presented with the following model (see figure 10), where the interaction of a chemical compound with an organism to produce a biological response is illustrated.

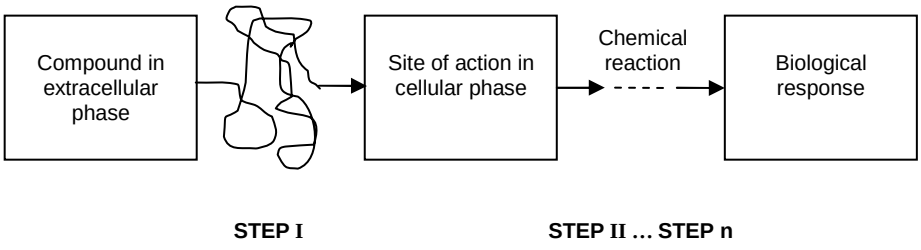


Image 10*: Simplified model for the interaction of a chemical compound and a biological system* [38]

Step 1 represents a diffusional process where the molecule in a dilute solution arrives at a particulate site on a cell. This step is slow and therefore the rate controlling step which depends on the molecular structure of the compound. The following steps represent the chemical reaction to achieve the biological response.

Hydrophobic compounds tend to accumulate in organisms because they are metabolised only slowly and are effectively stored in tissue. The thermodynamic tendency to partition into organic phases (represented by K_{OW}) continues to operate, and the organism continues to absorb the chemicals as long as they are present in the environment. The partition coefficients for other solvent-water pairs are directly related to those for the octanol-water pair.

4.5 Determination of Partition Co-efficients (K) of GM in aqueous solutions with 3 different oils

The partition co-efficient is a measure of the solvents capacity for the product, and is defined as the ratio of the product concentration in the solvent to the product concentration in the aqueous phase, at equilibrium. Oleic Acid (OA), Dibutyl sebacate (DBS) and Miglyol (MG) were chosen as the organic solvents, based on their high degree of hydrophobicity, with DBS and OA possessing $logK_{OW}$ values of 6.2 and 7.7 respectively [1]. This high level of hydrophobicity ensures that the mutual solubility between the oils and water at equilibrium is very low.

To evaluate the phase behaviour of GM in a two-phase aqueous-organic system, liquid-liquid extractions with these oils as the organic phases were carried out. For each oil the partition coefficient (K) of GM was calculated. The measurements of the amount of GM in the aqueous and organic phases showed after 17 and 23 hours the same amounts of GM. Therefore the equilibrium was reached within 17 hours. The carried out liquid-liquid extractions of GM showed following K and logK values for the 3 tested oils:

Table 5: *Partition coefficients (K) and logK values of GM between water and Oleic acid (OA), Dibutyl sebacate (DBS) or Miglyol (MG)*

	K	logK
OA	219.50	2.34
DBS	458.00	2.66
MG	159.59	2.20

DBS showed the highest level of extraction for GM with a partition co-efficient of 219.50 and a logK value of 2.66. OA and MG also showed high extraction levels for GM with logK values of 2.34 and 2.20 respectively. From these results it was decided to encapsulate DBS within an alginate membrane for further extraction experiments.

In consideration of the density of OA, which is 0.89 kg/m^3 at 20℃ [39], the extractive capacity of OA at these conditions can be expressed as 2.71 mg_{GM}/g_{oil}. For DBS, with a density of 0.9 kg/m^3 at 20℃ [39], an extractive capacity of 2.20 mg_{GM}/g_{oil} was calculated and

for MG, which has a density that's less than 1 kg/m^3, an extractive capacity of 1.96 mg_{GM}/ml_{oil} could be calculated. The capacity was investigated in a bulk volume of 20 ml with a GM concentration of 20 mg/l and with an oil volume of 0.05 ml. Therefore with the same conditions for a 1-L fermentation an oil volume of 2.5 ml would be needed.

Stable emulsions were observed when the flasks were incubated on a carousel with 360° rotation through the transfer axis of the flasks. Also higher rpm values lead to the formation of stable emulsions. The phases didn't separate spontaneously after they were allowed to settle for 18 hours at 28℃. Therefore the usage of free oils for the liquid-liquid extraction of GM isn't a convenient method. Additionally the toxicity of the oil towards the cells is a possible risk.

4.6 Stability of GM in DBS and OA

It could be shown that GM stays stable in DBS for 6 days. Then a degradation of 20% after 19 days could be observed. In OA the GM remained stable for 8 days. Then also a slight degradation of 16 % could be observed after 19 days. The experiment shows that GM is more stable in DBS and OA than in an aqueous environment at culture conditions, as shown in chapter 4.4, where GM degraded to 49% after 14 days.

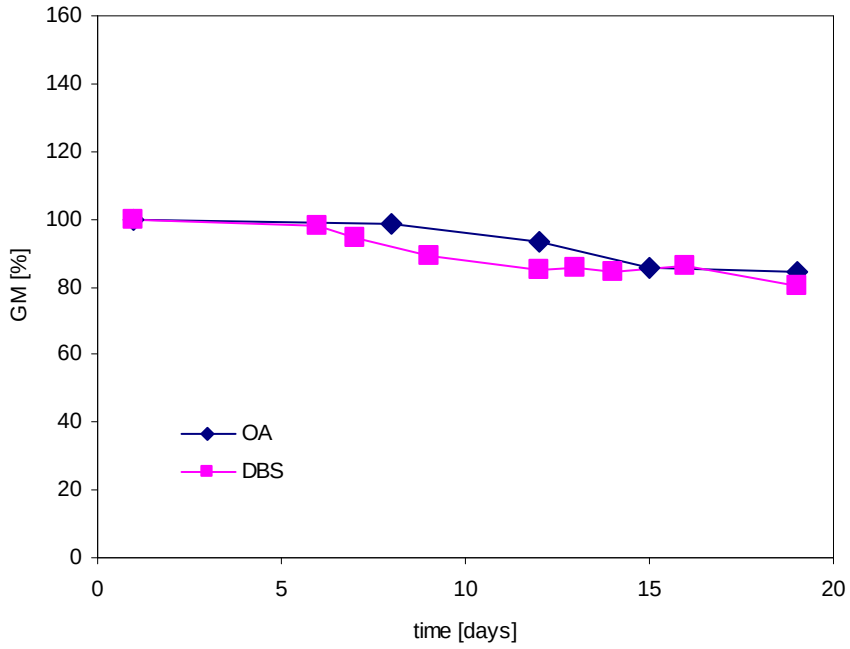


***Figure 3**: Stability of 0.4 g/l GM in OA and 0.2 g/l GM in DBS over 19 days at 28℃ and 200 rpm.*

As it could be shown that GM is unstable in an aqueous environment at cultivation conditions over 14 days and stable in DBS and OA over 19 days, an ISPR method to recover GM from the fermentation broth is required.

4.7 Production of Liquid-Core capsules

As GM had the highest partition co-efficient in an aqueous-organic system with DBS, this oil was chosen to be encapsulated. Another advantage of this oil for the encapsulation was that it's not so viscous as OA or MG, what facilitated the encapsulation process.

The aim was to obtain uniform monodispersed capsules, which means that capsules are formed regularly spherical and contain only one core of oil. A high frequency and flow rate enable the production of small capsules but on the other hand it's more difficult to separate the stream into droplets and in the polymerization bath they tend to collide with each other what causes fusions of several oil drops in one capsule. If the stirring speed is increased, this problem can be solved, but then the capsules tend to get egg-shaped instead of spherically because of the increased shear forces.

The capsule production can also vary with the properties of the Alginate solution. Slight variations of the Alginate solution can cause large variations in the capsule production. Therefore a large batch of Alginate solution should be prepared and the capsules should be produced all at once with the same Alginate solution.

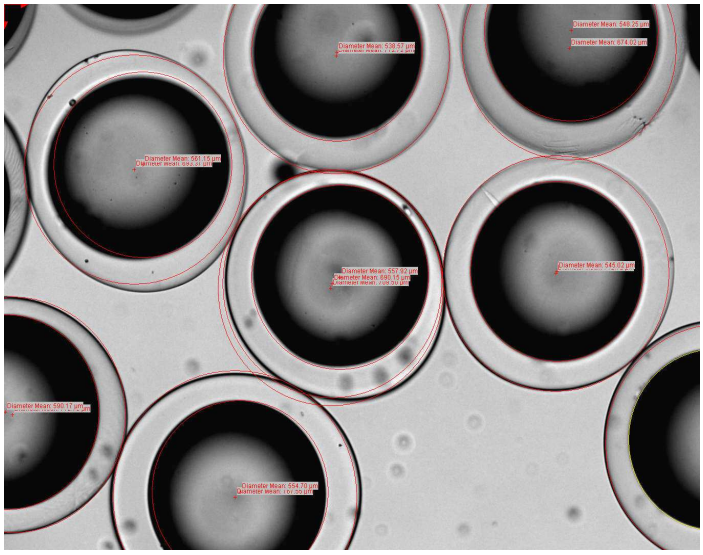


***Image 11**: Produced Liquid-Core Microcapsules (40x)*

4.8 Characterisation of the Liquid-Core capsules

4.8.1 Measurement of the Capsule Size Distribution

An average capsule diameter of 712.31±39.51 µm and an average core diameter of 554.30±30.34 µm were measured for the produced capsules. A capsule volume of $1.9*10^{-10}$ m^3 and core volume of $8.9*10^{-11}$ m^3 was calculated. The oil volume in the capsules was herewith at 47% of the total volume of the capsules. Following table gives an overview of the average capsule size, core size and alginate shell measured:

***Table 6**: Measured capsule, core and alginate shell sizes of the produced Liquid-Core capsules*

	Capsule	Liquid-Core	Alginate shell
Average diameter [µm]	712.31±39.51	554.30±30.34	158.01 ±32.21
Average Volume [µm^3]	1.9*10^8	8.9*10^7	1.0*10^8
Volume [%]	100	47	53

4.8.2 Mass transfer and capacity analysis

The mass transfer of GM across Liquid-Core capsules was investigated at two different temperatures. The aim was to observe if the mass transfer limitation was due to the diffusion across the capsule wall.

The results presented on figure 4 showed that at 20℃ the equilibrium was reached after 135 min and at 28℃ the equilibrium was reached after 1 10 min.

A capacity of 1.47 $mg_{GM}/g_{capsules}$ and a partition coefficient of 18.71 could be calculated. Considering that 47% of the capsule volume is oil, the capsule capacity can also be illustrated as 3.48 $mg_{GM}/g_{oil.}$ In chapter 4.5 it could be shown that DBS has an extractive capacity of 2.2 $mg_{GM}/g_{oil,}$ when it's not encapsulated. This shows, that through encapsulation of DBS, the extractive capacity can be increased. The main reason for this is the larger interfacial surface of the oil when it's encapsulated.

With the same conditions for a 1-L fermentation, 70-80 g capsules would be sufficient to perstract 0.1 g/l GM from the fermentation broth. These capsules could be added at the beginning of the fermentation. As the GM production in a *S. hygroscopicus* fermentation can vary a lot, the measurement of the GM concentration in the broth would indicate, as soon the capacity of the capsules is reached, as then no GM would be adsorbed anymore by the capsules. Then new capsules could be added to the fermentation broth.

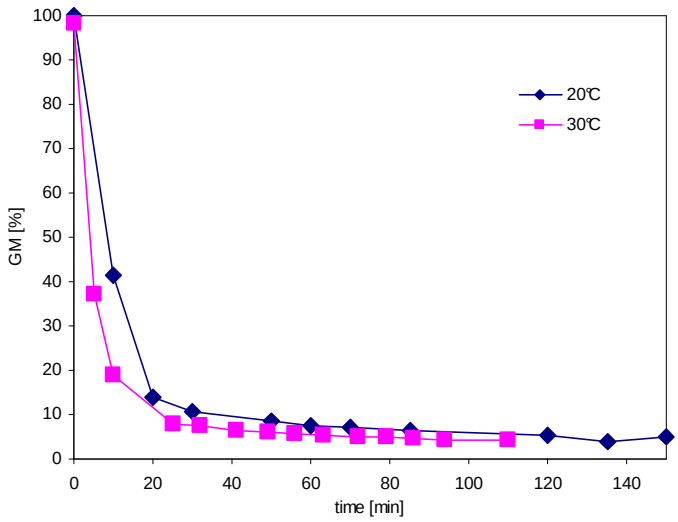


***Figure 4:** Perstraction of 1 mg GM (10 ml of 0.1 g/l GM sol.) with 0.733 g liquid-core capsules at 20 and 28℃.*

Figure 4 shows the perstraction of GM at two different temperatures. At 28℃ an initial adsorption rate of 843.7 mg/(l*h) and at 20℃ an in itial adsorption rate of 405.61 mg/(l*h) could be calculated. This shows that the temperature has a great influence on the mass transfer. Through a temperature increase of 8℃ the initial adsorption rate increased in double. This shows that the mass transfer resistance is due to the diffusion across the alginate membrane.

As the cultivation temperature of *S. hygroscopicus* is 28℃, this is an advantage for the mass transfer of GM through the capsules. Another possibility to increase the mass transfer across the capsule wall besides heat would be producing capsules with a thinner capsule wall. This could be achieved through decreasing the alginate pump speed or the diameter of the external nozzle. Another possibility to increase the mass transfer across the capsule wall could also be achieved through decreasing the Alginate concentration for the capsule wall. This would lead to a decreased cross-linking in the capsule wall. Although these provisions could also cause the capsules to break during a 21-day fermentation.

Figure 5 shows the measurements of remaining GM in a 0.1 g/l GM solution during the adsorption through 4.656 g Liquid-Core capsules. These measurements were used to determine the kinetic adsorption model of the Liquid-Core capsules. The results showed that the adsorption through liquid-core capsules follows the kinetic adsorption model of the "pseudo second order model". A correlation of R^2=1 was obtained for this model. Other tested models like "pseudo first order", "Elovich" and "the intraparticle diffusion model" showed correlations which were not as good (see appendix 10.5).

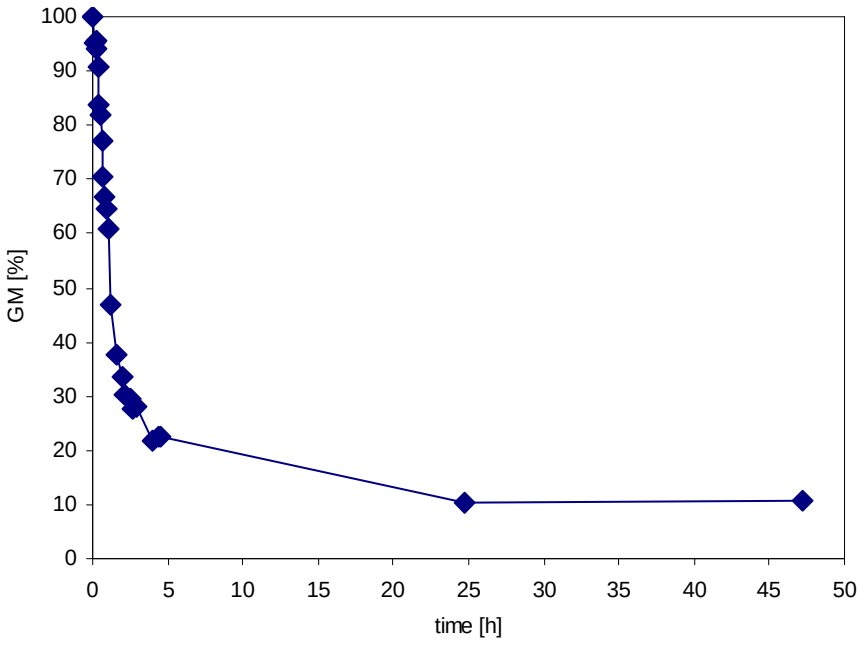


Figure 5: *Adsorption of 5 mg GM (50 ml of 0.1 g/l GM solution) with 4.656 g liquid-core capsules at 20℃.*

4.8.3 Mechanical stability of Alginate beads after sterilisation through UV radiation

To use Liquid-Core capsules as a tool for the ISPR of GM in a *S. hygroscopicus* culture, the sterility of the capsules had to be guaranteed. UVC radiation at 254 nm for 48 h was therefore chosen, what was shown to be an efficient method for sterilisation, as an exposure of the capsules to UVC light at 254 nm for 48 hours showed no growth of colonies when these were plated out on Bennett's medium agar (as described in chapter 3.8.3). Through measurement of the mechanical characteristics after different intervals of UVC radiation, also the influence on the mechanical stability of the capsules was investigated. It was shown by Daniel Stark [26] that sterilisation of Liquid-Core capsules through autoclaving had a very negative effect on the mechanical stability, as heat causes the extrusion of the water out of the Alginate wall and also breaks the bonds of the calcium and alginate cross-linked membrane. The burst force and the elasticity of the capsules were measured by the Texture Analyser as described in chapter 3.8.4. The burst force is the key parameter for the stability of the capsules, and consequently, the influence of the different exposure times of UV radiation to the burst force was investigated. The burst forces for different times of exposure to UVC radiation are displayed in figure 6 below.

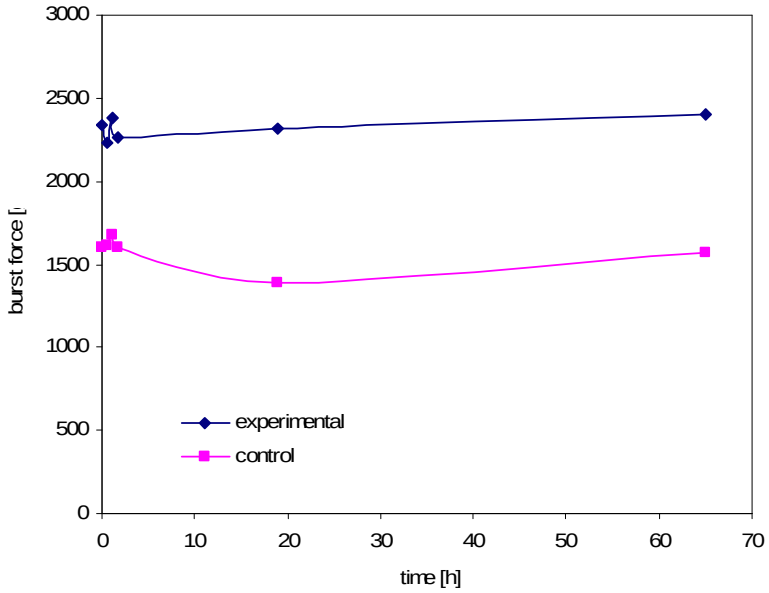


Figure 6: *Burst force of alginate beads as a function of time exposed to UVC light (experimental) and without exposure to UVC light (control).*

It could be shown that the UVC radiation over 65 hours didn't have a negative effect on the mechanical stability of the capsules. Also the control samples which were not exposed to UVC light showed a stable mechanical stability over 65 hours. As it could be shown by Daniel Stark [26] the burst force can be very heterogeneous with standard deviations up to

40%, although the capsules have a good monodispersity. Therefore absolute values of the burst force have to be used cautiously.

4.9 Cultivation of S. hygroscopicus and monitoring of the GM production

Different cultivation experiments were carried out in order to investigate the stability and the solubility of GM during cultivation, and also if there was a product inhibitory effect of GM and to establish what causes the degradation of GM at the end of cultivation.

4.9.1 Investigation of the stability of the produced GM during cultivation

The stability of GM during cultivation was investigated through monitoring of the GM concentration and the appearance of degradation products through HPLC during the cultivation. Figure 7 shows the GM production during 12.5 days of a *S. hygroscopicus* cultivation and the peak area of unknown compounds which were also detected at the same wavelength as GM with the HPLC. After 12.5 days a GM concentration of 450 mg/l could be obtained. At the same time as the GM concentration increased, the peak area of the unknown compounds also increased. These unknown compounds with retention times of 1.99 and 3.38 min were already discovered in the stability experiment of GM described in chapter 4.3.1. Therefore these results are consistent with GM possibly degrading during the cultivation of *S. hygroscopicus* at the same time as it's produced. This means that higher GM production could be achieved if GM was removed from the culture broth as soon as it's produced and transferred in to an environment in which it's more stable.

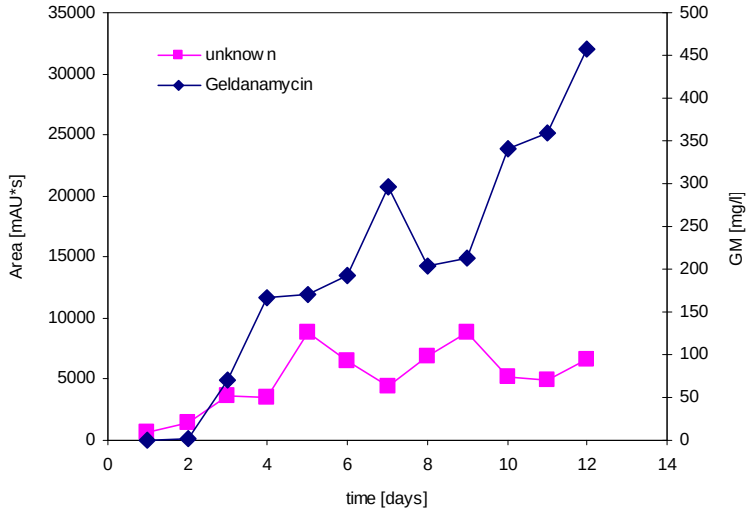


Figure 7: *GM concentration [mg/l] and Peak Area [mAU*s] of GM and other unknown compounds during the fermentation of S. hygroscopicus*

4.9.2 Investigation of the solubility of the produced GM during cultivations

To investigate the solubility of GM when it's produced during the cultivation, the soluble, not-soluble and intracellular GM was measured with different sampling methods. The aim was to indicate where GM is located during fermentation and if GM accumulates in the cells as soon a high level of GM is reached in the broth.

It was assumed that the GM is secreted from the cells in a soluble form and as soon as the solubility limit is reached GM precipitates. Another possibility that was taken into consideration was, that GM would accumulate inside the cells when a certain level in the broth was reached, or that product inhibition could occur, what would mean that GM production would stop as soon as a certain concentration in the bulk was reached.

Figure 8 shows the lapse of the GM production with time in a first cultivation. The measurements showed that a maximum soluble GM concentration of 49 mg/l was reached after 15 days. The total GM concentration in the broth was measured at 72 mg/l. After 16 days the GM concentration decreased to nearly half, and after 20 days no GM could be detected in the broth anymore. 4 % GM could be measured in the cells after 13 days, but no GM was detected in the cells after 15, 16 and 20 days.

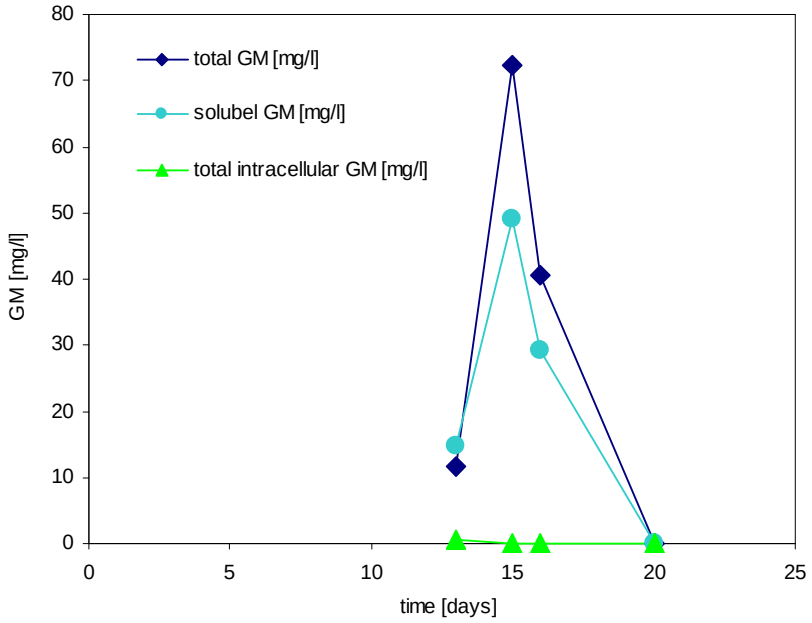


Figure 8: *GM production during a S. hygroscopicus fermentation*

The results show that GM is produced in a soluble form at the beginning and precipitates as soon the solubility limit is reached. In this fermentation the highest soluble GM concentration was measured at 49 mg/l after 15 days while at the same time the total GM concentration was measured at 72 mg/l. Herewith a solubility of GM could be measured at 49 mg/l.

As there was only 4% GM measured in the cells after 12 days and none after 15, 16 and 20 days, it was assumed that the cells secrete all the GM and don't store it intracellular as inclusion bodies or in other form.

Figure 9 shows the decline of the GM production with time in a second cultivaiton, which was carried out with the same conditions as the first cultivation, except for a different (optimised) media composition. The measurements showed that a maximum soluble GM concentration of 86 mg/l was reached after 11 days. At the same time the total GM concentration in the broth was measured at 157 mg/l. After 18 days the GM concentration (total and soluble) decreased to nearly half. But after19 days again an increase of GM was observed. After 21 days a total GM concentration of 154 mg/l and a soluble GM concentration of 52 mg/l were reached. After that the GM concentration decreased again. After 25 days the soluble GM concentration was 5 mg/l and the total GM concentration was 80 mg/l.

A phenomenon like this wasn't observed in the previous fermentation, as it wasn't monitored for longer than 20 days.

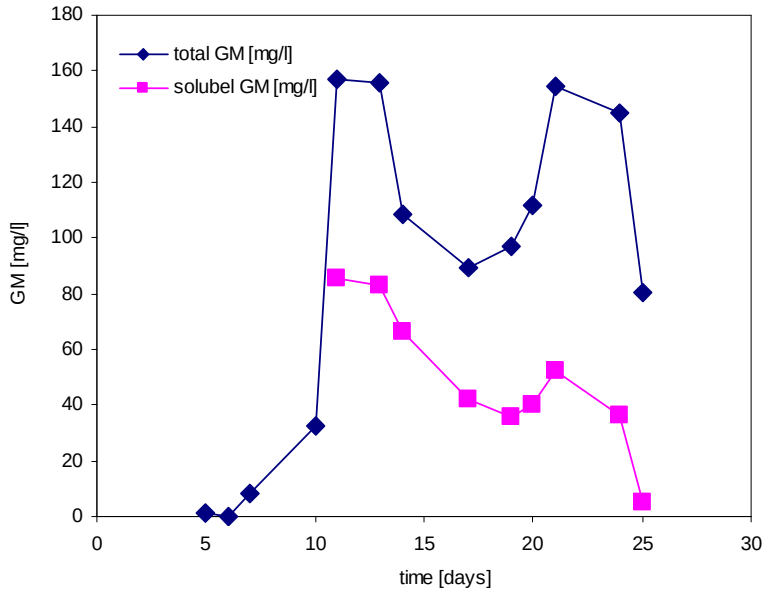


Figure 9: *GM production during a S. hygroscopicus fermentation*

The de- and increase of GM during the fermentation could be due to changing conditions in the cultivation broth like compounds which are secreted by the cells like proteins, changing pH and changing media composition. These factors could cause degradation of GM.

The differences in the produced amount of GM compared to the first cultivation could be due to the different composition of the Bennett's media used for both cultivations. The composition was altered because it was shown that the GM production could be increased with the new media composition. [36]

4.9.3 Investigation of the degrading affect of GM during cultivation

Interesting for the in-situ product recovery (ISPR) with liquid-core capsules is the fact that GM disappears towards the end of the cultivation as observed in chapter 4.9.2. This fact is a good reason to apply ISPR for GM recovery. To find out if liquid-core capsules are a good method for the ISPR in this case, it's interesting to know, why the GM disappears in the broth. It's assumed that the organism is either consuming the GM and uses it as a nutrient and then eventually produces it again. An other possibility could be that liberated enzymes from the organism degrade GM, what causes the decrease of the GM concentration. These enzymes could be liberated through cell death or be produced at a certain growth phase. If either of these assumptions are true, liquid-core capsules could be a good method to protect the GM from the cells or enzymes, as enzymes require H_2O for their activity and therefore would probably not enter the oil core of the capsules. If GM breaks down spontaneously at culture conditions, ISPR could only be efficient, if GM would be more stable in the oil phase than in the aqueous phase. The results of these experiments were presented in chapter 4.6 and showed that GM is more stable in the oil phase than in the aqueous phase.

The results presented in figure 10 show the GM concentration plotted with time in 3 different cultivation broths (without cells); in a denatured culture broth at pH 2, in denatured culture broth at pH 7 and in a not denatured culture broth. It shows a slight degradation of GM in a cultivation broth at pH 2 which was treated with heat and acid to denaturise all present proteins in the broth before adding GM to the broth. After 7 days the GM concentration decreased 27%. As this degradation can't be due to proteins or enzymes present in the broth, because these were denatured, it was assumed that this degradation could be due to the low pH in the broth that could destabilise the GM and cause breakdown of the molecule. This result also shows that GM is unstable at very low pH values like pH 2.

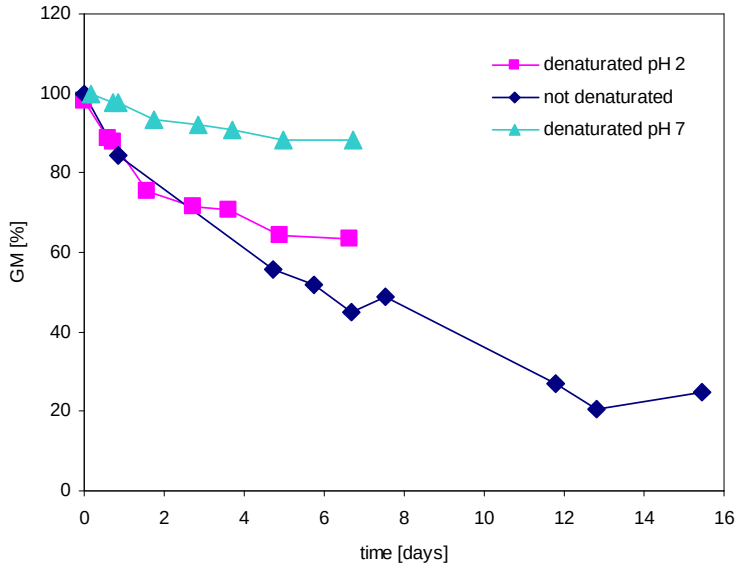


Figure 10: *Degradation of GM in denaturized culture broth at pH 2 and pH 7 and not denaturized culture broth after a 24-day cultivation.*

A relatively stable condition of GM could be observed in the denatured cultivation broth, which was neutralised to pH 7 before adding GM to the broth. The GM concentration after 7 days was 88%.

A strong degradation could be observed in the cultivation broth, which was not denatured. A GM concentration was measured after 7 and 25 days what showed a degradation of 55% after 7 days and a degradation of 75% after 25 days. This confirms that cells didn't consume the GM, as these were removed, but that cellular compounds secreted by the cells degraded the GM.

As the previously produced GM in the culture disappeared towards the end of the fermentation, it was assumed that either the cells or cellular compounds like proteins which were secreted by the cells degraded the produced GM. These results are consistent with GM degrading at the end of a cultivation as soon as certain proteins are liberated or produced by the cells. An other degrading cause is an acid pH. As at the end of a cultivaiton the pH decreases through production of acids by the bacteria, this could also reinforce the degradation of the produced GM.

To ensure that the degradation of GM is caused by a specific protein and to identify this, samples of the culture could be analysed with SDS-PAGE, what separates proteins by molecular weight. The appearance of a new protein during the degradation of GM at the end of the cultivation would then be expected.

4.9.4 Investigation of a product inhibitory effect of GM

The aim of this experiment was to investigate if GM which is present in the cultivation broth inhibits the GM production of the cells, as product inhibitory is a major reason to apply ISPR. Therefore cultures were prepared with addition of different GM concentrations at the beginning of the cultivation and compared with the GM production in cultures without addition of GM. If product inhibitory was a problematic affect, a lower GM production would be expected in cultures where GM was added. The results obtained were contradictory to this.

The figure 11 shows that the cultures with added GM had much higher GM production after 21 days compared to the control fermentations (without addition of GM). It could be observed that with all cultures the GM production started to increase after 18 days. With both control cultures the GM production stagnated after 21 days. The culture with initial addition of 25 mg/l GM showed after 24 days a strong decrease of the GM production. This phenomenon was also observed in previous fermentations. The cultivations with initial addition of 50 mg/l GM showed after 24 days only a slight decrease in the GM concentration.

For organisational reasons more samples couldn't be taken and the fermentations couldn't be prolonged.

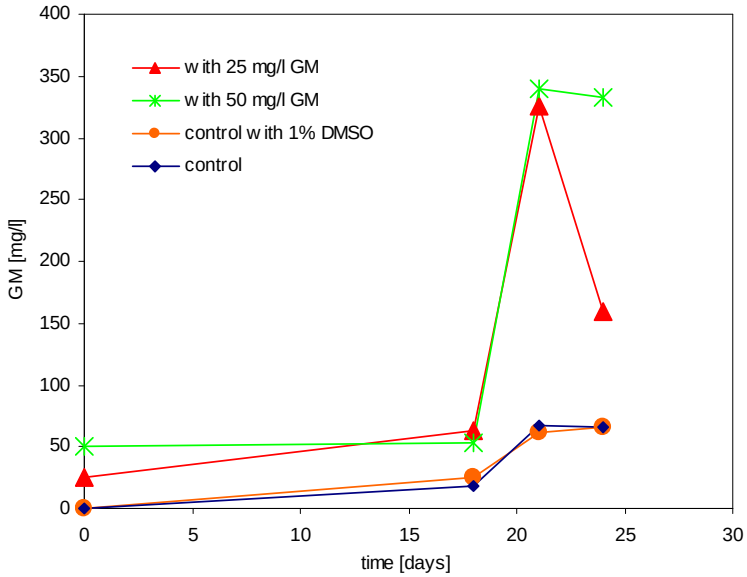


***Figure 11**: GM production during a S. hygroscopicus fermentation with addition of GM.*

These results prove that GM is not inhibitory for the cells. Rather the opposite could be shown: a higher GM production could be observed with addition of GM at the beginning, although the GM production didn't start earlier than in the control fermentations. Therefore this phenomenon can't be described as an induction of the GM production, but the addition of GM caused an enhancement of the GM production. It should also be taken into consideration that while GM is produced, it also could be degraded at the same time, as during the fermentation cells die and liberate enzymes and proteins which degrade GM as it was shown

in chapter 4.9.3 of this report. Besides this the pH is a critical factor for GM, as it could also be shown in chapter 4.9.3 that GM degrades at low pH values. This is also a problem towards the end of the fermentation where the pH decreases if it's not regulated. [40]

4.9.5 Cultivation of *S. hygroscopicus* with addition of Liquid-Core Capsules

In the *S. hygroscopicus* cultures with addition of 10 ml Liquid-Core capsules no GM could be detected in the broth over 24 days of cultivation. In the control culture though a GM production of 67 mg/l GM could be measured after 21 days. Therefore this result is consistent with GM being perstracted from the culture broth by the added liquid-core capsules. As the recovery of the GM from the capsules has not yet been developed and as the measured GM concentrations in different cultivations have shown a large variation, although the same cultivation conditions were chosen, the minimum perstracted amount of GM in this case can only be assumed: With the assumption that the culture with addition of capsules produced the same amount of GM as the control culture, a perstraction of 0.67 $mg_{GM}/ml_{capsules}$ could be achieved. Although it must be mentioned, that the perstracted amount of GM is estimated at much a higher value, because the maximal capacity of the capsules was not reached in this experiment and also because of the higher stability of GM in the capsular environment. This result shows also, that through addition of Liquid-Core capsules no GM (soluble, insoluble or intracellular) remains in the broth and therefore in direct contact with the cells.

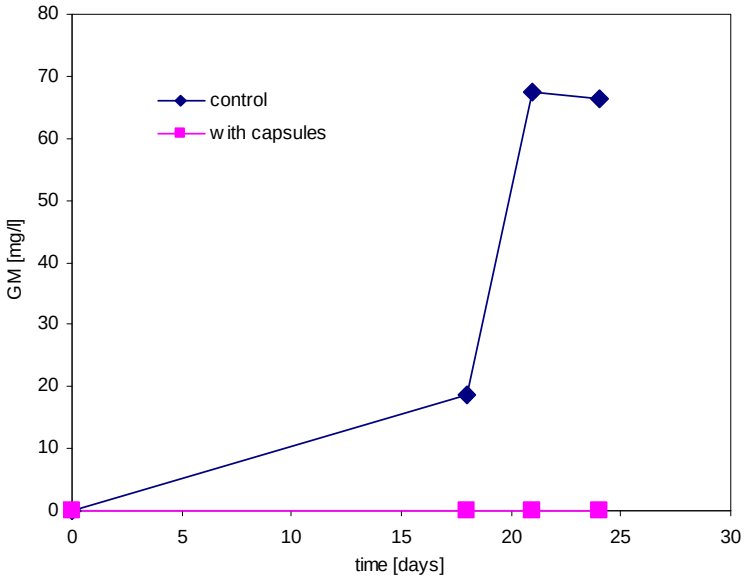


***Figure 12**: GM concentration in the broth during a S. hygroscopicus cultivation with and without addition of Liquid-Core capsules.*

5 Conclusions and preview

5.1 Feasibility of applying Liquid-Core capsules for the ISPR of GM

The feasibility of recovering GM through Liquid-Core capsules from a fermentation broth could be evaluated. It could be shown that ISPR of GM from a *S. hygroscopicus* culture is sensible because of the instability of GM in the culture broth through spontaneous degradation and through degrading proteins released by the cells at a certain growth phase.

To identify the proteins which degrade GM and to investigate at which growth phase they are released, samples throughout the cultivation of S. hygroscopicus could be analysed with SDS-PAGE and the dry weight of the cells could be measured, to follow the protein production and the growth of the cells.

Perstraction of GM with Liquid-Core capsules from the culture broth of *S. hygroscopicus*, would allows fast recovery of GM from the broth and keep GM in an environment where it's more stable than in culture broth, as it's protected from oxidation and from degrading proteins. Also the perstraction of GM through Liquid-Core capsules allows an integrated downstream process, as they can be added directly to the fermentation broth and be separated easily from the broth through filtration.

The next step of this work would consist of the evaluation, if the GM production with Liquid-Core capsules is higher than without. This would require the measurement of the GM concentration in the capsules. Therefore the capsules would need to be dissolved or the back-extracted.

Another interesting point would be to examine the stability of the perstracted GM inside the capsules while the degrading proteins are produced. It would be necessary to investigate if these proteins would also have a degrading effect on GM when GM is encapsulated, as the typical molecular weight cut-off of such liquid-core capsules would allow the penetration of proteins through the capsule wall. Although it would be expected that the proteins wouldn't penetrate into the oil core, as they require H_2O for activity, but they would be able to remain on the alginate-oil interface.

5.2 Recovery of GM from liquid-core capsules

A next step of the ISPR of GM through Liquid-Core capsules would be the recovery of GM from the Liquid-Core capsules. This could be achieved either through disruption of the capsules or through back-extraction of GM from the capsules. A disruption of the capsules could be achieved through washing the capsules in a sodium citrate solution, as citrate ions chelate with the calcium ions in the alginate membrane, what would cause the disruption of the capsule wall. The capsule oil would be liberated into the sodium citrate solution. This

would lead to a two phase system in which the oil would need to be separated from the aqueous solution. This wouldn't be ideal, as it would require many other separation steps, until the pure GM could be isolated. Another possibility of recovering GM from the Liquid-Core capsules would be the back-extraction of GM with Dichloromethane (DCM) from the capsules. In this case the capsules could be washed with DCM, which would cause the solubilisation of GM in DCM, as the solubility of GM in DCM is 10 g/l [41]. But also the oil would be solubilised with DCM, as DCM is miscible with DBS. The solubility of DCM in water is 13 g/l, which is very low and has a boiling point of 39℃. This would allow an evaporation of the DCM to recover GM. A small water solubility of DCM is necessary, so that DCM can penetrate through the Alginate wall of the capsules. As DCM is miscible with DBS, this would mean that the capsules would possibly not be reusable anymore, because DCM would remain in the core.

An optimal recovery process would be one where the capsules could be reused. This would be possible when the extracting solvent wouldn't be miscible with the liquid-core of the capsules. As OA isn't miscible with DCM, it would be a possibility to encapsulate OA.

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I'd like to thank following people from HES-SO and DCU for giving me the opportunity to carry out my diploma work in Dublin and for supporting me during this time: Professor Ian Marison, who offered me this opportunity, gave me the possibility to work in a good atmosphere and gave me a high quality scientific support, Professor Simon Crelier, who prepared me for this work and was always at disposal to answer to my questions and Micheal Whelehan for his help and support through this work, for answering to my questions and for his corrections. Also I would like to thank people from other laboratories in DCU, who permitted me to use their equipment. Special recognition goes also to Mary Rafter for organising my accommodation and who permitted me a pleasant stay.

7 Abbrevations

ACN: Acetonitrile

℃: degrees Celcius

DBS: Dibutyl sebacate

DCM: Dichloromethane

g: Gramms

GM: Geldanamycin

h: Hours

Hz: Hertz

ISPR: In-Situ Product Removal

K: Kelvin

K: partition co-efficient

kHz: kilo Hertz

kV: kilo Volt

l: Liters

M: Molar

MeOH: Methanol

MG: Miglyol

ml: milli Liters

µl: micro Liters

mM: milli Molar

µm: micro Meters

MOPS: 3-(N-morpholino)propanesulfonic acid

min: Minutes

nm: nano meters

OA: Oleic Acid

pH: per Hydron or per Hydrogen

RP-HPLC: Reversed Phase High Pressure Liquid Chromatography

S.: *Streptomyces*

UV: Ultraviolet

var. : variety

v/v: volume per volume

w/v: weight per volume

8 Definitions

Polyketide: Polyketides are secondary metabolites from bacteria, fungi, plants, and animals. They are formally derived from the polymerization of acetyl and propionyl subunits in a similar process to fatty acid synthesis. They also serve as building blocks for a broad range of natural products or are derivatized.

Secondary metabolites seem to be unnecessary for an organism's ontogeny, but appear to have applications such as defense and intercellular communication.

Polyketides are a rich source of many pharmaceuticals, including antibiotics, anticancer drugs, cholesterol-lowering drugs, immunosuppressant and other therapeutics. However, polyketides are made in very small amounts in microorganisms and are difficult to make or modify chemically.

Ansamycin: An anticancer drug that belongs to the family of drugs called antineoplastic antibiotics. Ansamycin is a class of an antibiotic with ansa rings which contains an aliphatic bridge connecting two nonadjacent positions of an aromatic nucleus.

Antineoplastic: A type of anticancer drug that blocks cell growth by interfering with DNA, the genetic material in cells, also called an anticancer antibiotic or antitumor antibiotic.

Hepatotoxity: from hepatic toxicity; a chemical-driven liver damage.

L-1210: Mouse lymphocytic leukemia established from the methylcholanthrene-induced tumor in a DBA strain mouse in 1949. Morphology: single round, lymphoblast-like cells in suspension.

KB cells: A cell line derived from a human carcinoma of the nasopharynx, used as an assay for antineoplastic agents.

Tetrahymena pyriformis: A species of ciliate protozoa used extensively in genetic research.

Crithidia fasciculate: A species of monogenetic, parasitic protozoa usually found in insects.

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10 Appendix

10.1 Microcapsule / Bead Parameter + Result Sheet:

Table 7: *Microcapsule / Bead Parameter Sheet*

Experiment No.		
Parameters		
Type of Alginate (w/v) + Buffer + pH		
Age of Alginate (Days)		
Core (Oil) Type (% purity)		
Polymerisation Solution (Type)		
Reaction Vessel (Type)		
Stirring Bar Type (mm)		
Alginate Vol. (syringe ml)		
Oil Vol. (Glass Syringe ml)		
Vol. of Poly solution (ml)		
Impact Height (mm) (No agitation of polymerisation solution)		
Alginate flow rate (ml/min)		
Oil flow rate (ml/min)		
Alginate Temp. (^{o}C)		
Frequency (Hz) ***(Exact value)***		
Amplitude (%)		
Stirring speed (on stirring plate)		
Voltage (kV)		
Length of Polymerisation (min)		
Washing Solution		
Results	**%**	**St. Dev. + St. Dev.**
Diameter of microcapsule Average (mm)		
Diameter of oil core Average (mm)		
Diameter of Alginate shell Average (mm)		
Mechanical Resistance Average		
Shape of Microcapsules Average		
Mono-dispersity (homogeneity)		

Table 8: *Result Sheet for the production of Liquid-Core Microcapsules*

	Dia. of capsule [µm]	Dia. of oil core [µm]	Dia. of Alginate shell [µm]
Picture 1	696	548	148
	767	551	216
	667	567	100
Picture 2	719	551	168
	741	557	184
	703	516	187
Picture 3	722	554	168
	706	583	123
	667	542	125
	697	599	97.6
	697	577	119.6
	674	532	142
Picture 4	697	526	170.93
	690	558	132.08
	738	542	196.2
Picture 5	703	519	183.8
	726	546	180
	690	558	132.08
	742	545	196.75
	703	548	155.05
	713	570	142.7
Picture 6	732	577	155
	858	667	190.85
	706	510	196.72
	841	651	190
Picture 7	655	529	125.77
	748	536	212.4
	748	561	187
	690	490	199.95
	739	554	184.52
Picture 8	732	558	174.15
	703	526	177.33
	700	551	149
	684	493	190.71
	684	529	154.71
	716	581	135.45
Picture 9	680	555	125.77
	729	548	180.6
	735	558	177.3
	690	551	138.68
Picture 10	693	561	132.22
	690	558	132.08
	713	539	174.15
	713	545	167.7
	768	555	212.85
	674	548	126
	713	590	122.55

	729	548	180.85
Picture 11	813	642	170.93
	642	535	106.42
	713	568	145.12
	684	526	158.03
	735	558	177.38
	648	558	90.08
Picture 12	742	558	183.83
	710	551	158.03
	680	539	141.9
Picture 13	664	574	90
	742	545	196.75
	671	545	125.78
	719	526	193.5
	697	568	129
	700	574	125.77
Average [µm^3]	712	554	158
Std. Dev. [µm^3]	39	30	32
Volume [µm^3]	1.8924E+08	8.9175E+07	1.0007E+08
Volume [m^3]	1.8899E-10	8.90285E-11	9.99619E-11
Volume [%]	100	47.12265	52.87735

10.2 Standard curves of GM in ACN

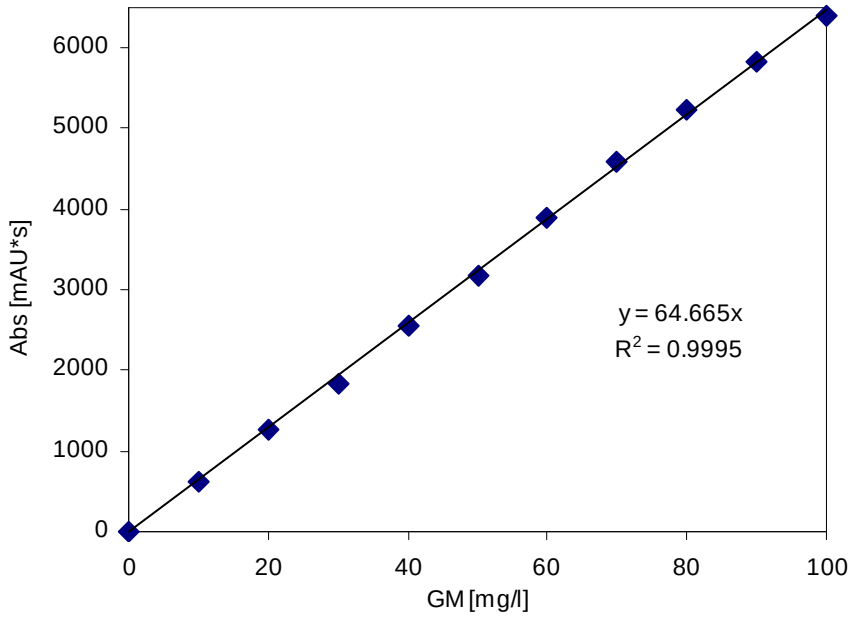


Figure 12: *Standard curve of GM in ACN from 10 to 100 mg/l.*

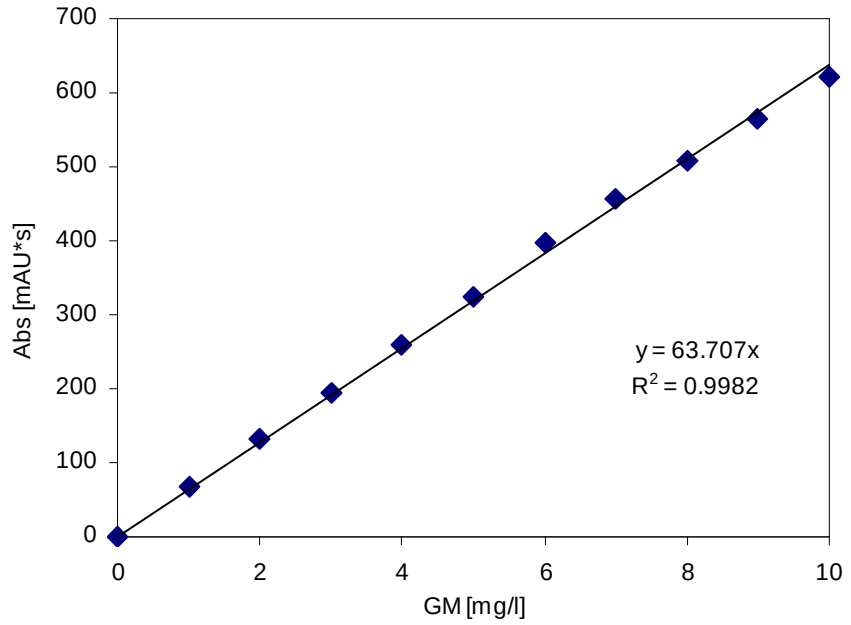


Figure 13: *Standard curve of GM in ACN from 1 to 10 mg/l.*

10.3 Standard curve of GM in OA

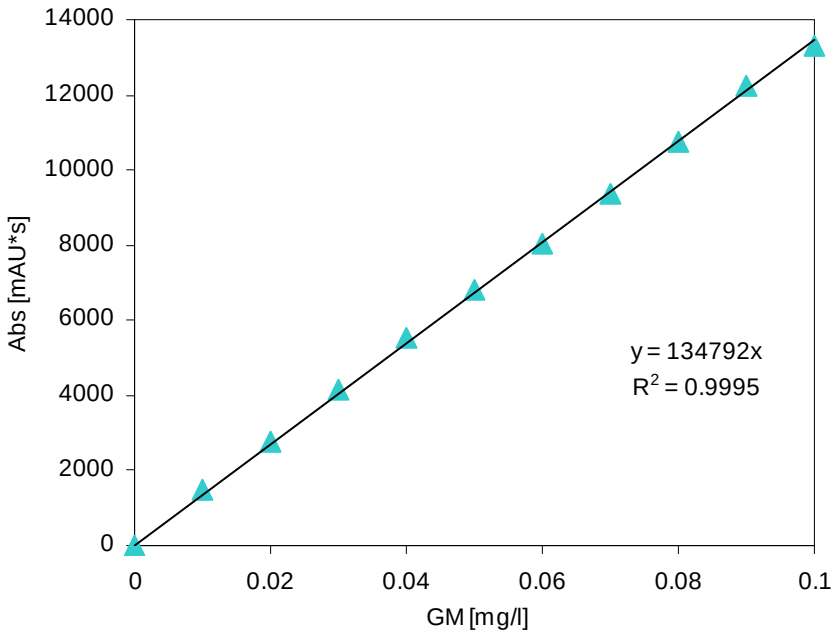


Figure 14: *Standard curve of GM in OA from 10 to 100 mg/l.*

10.4 Standard curve of GM in DBS

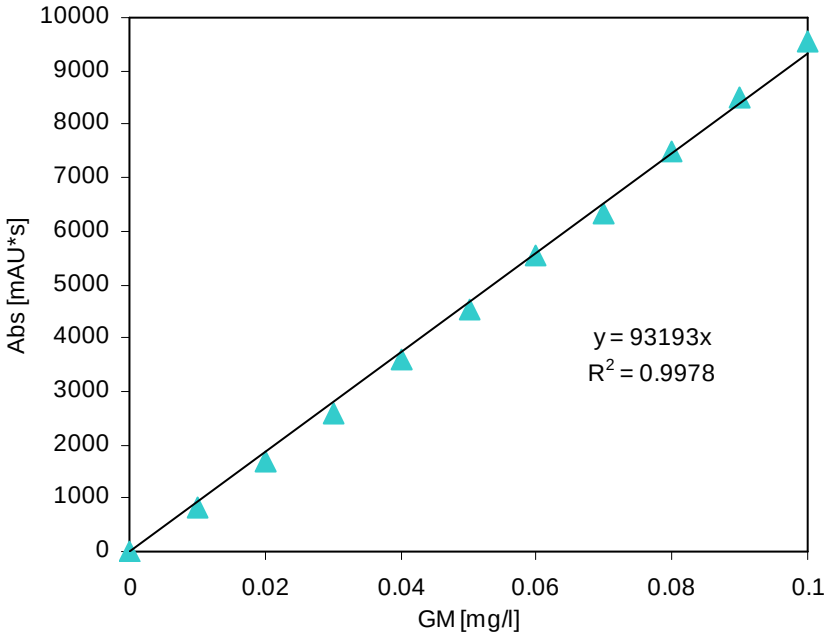


Figure 15: *Standard curve of GM in DBS from 10 to 100 mg/l.*

10.5 Kinetic adsorption models

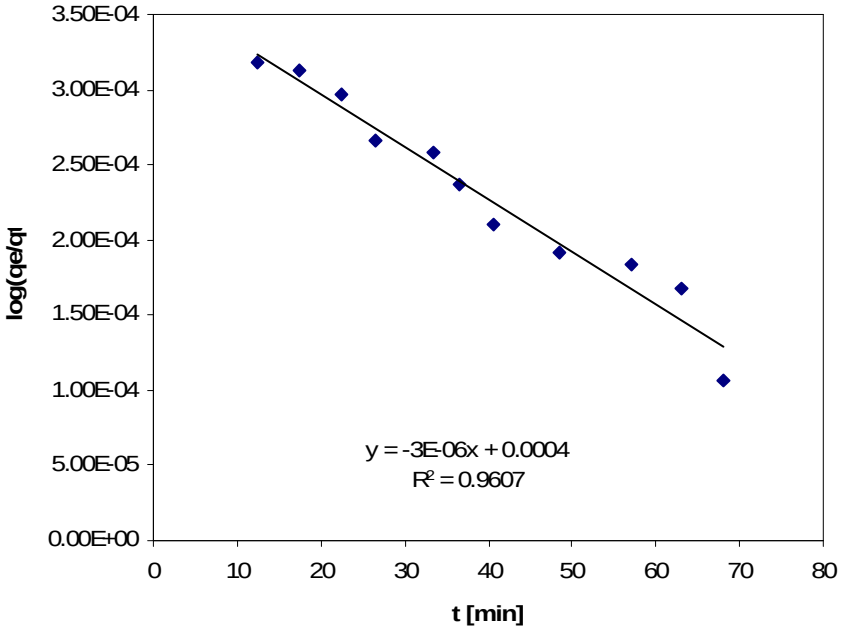


Figure 16: *Pseudo first order kinetic model*

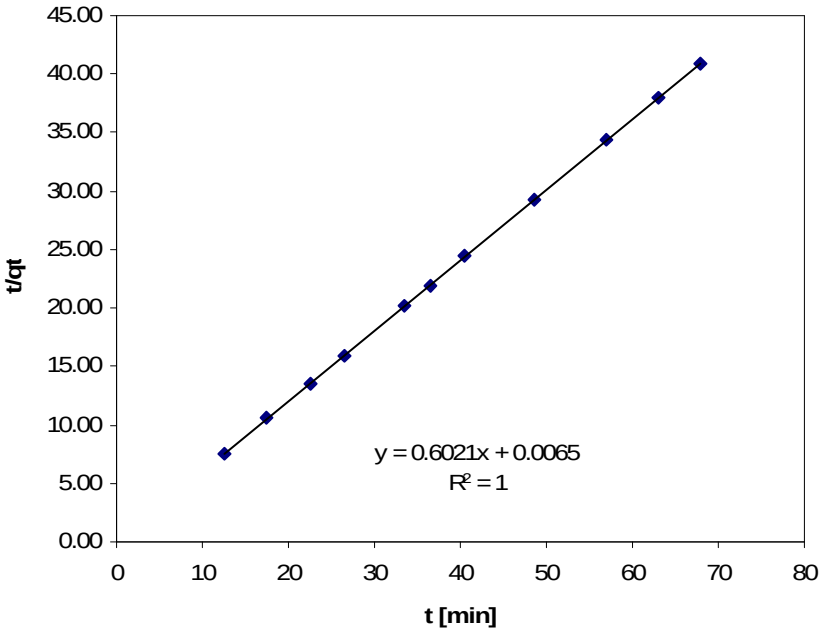


Figure 17: *Pseudo second order kinetic model*

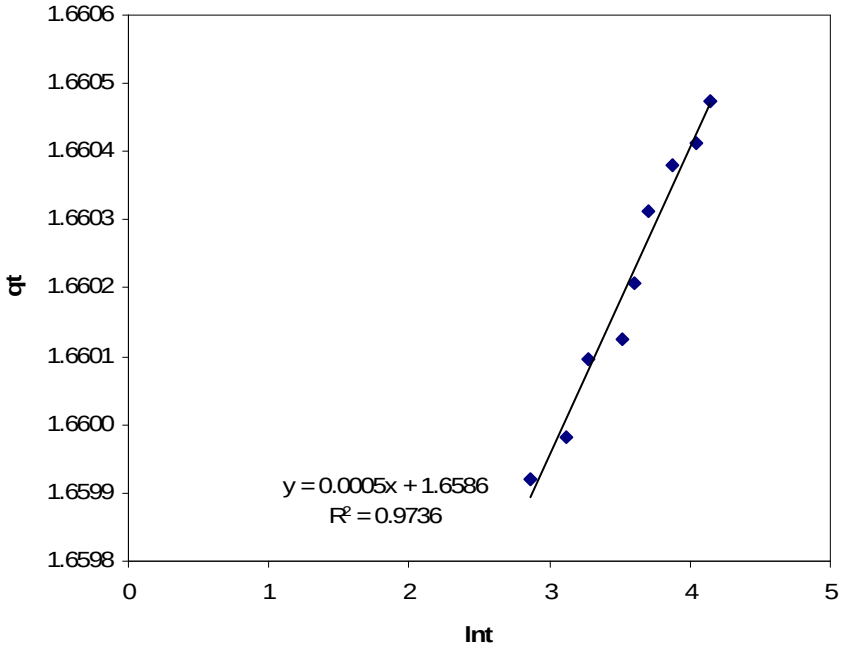


Figure 18: *Elovich kinetic model*

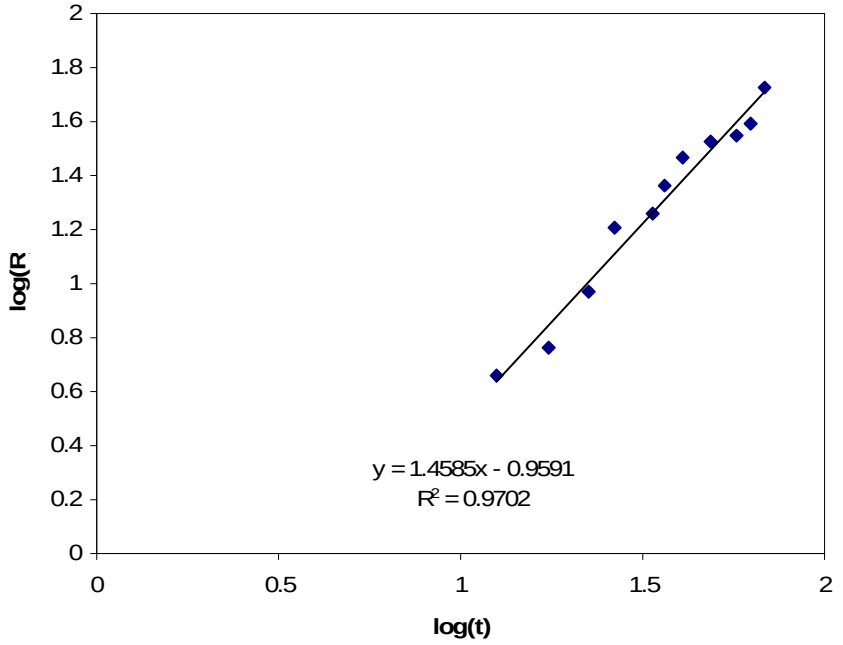
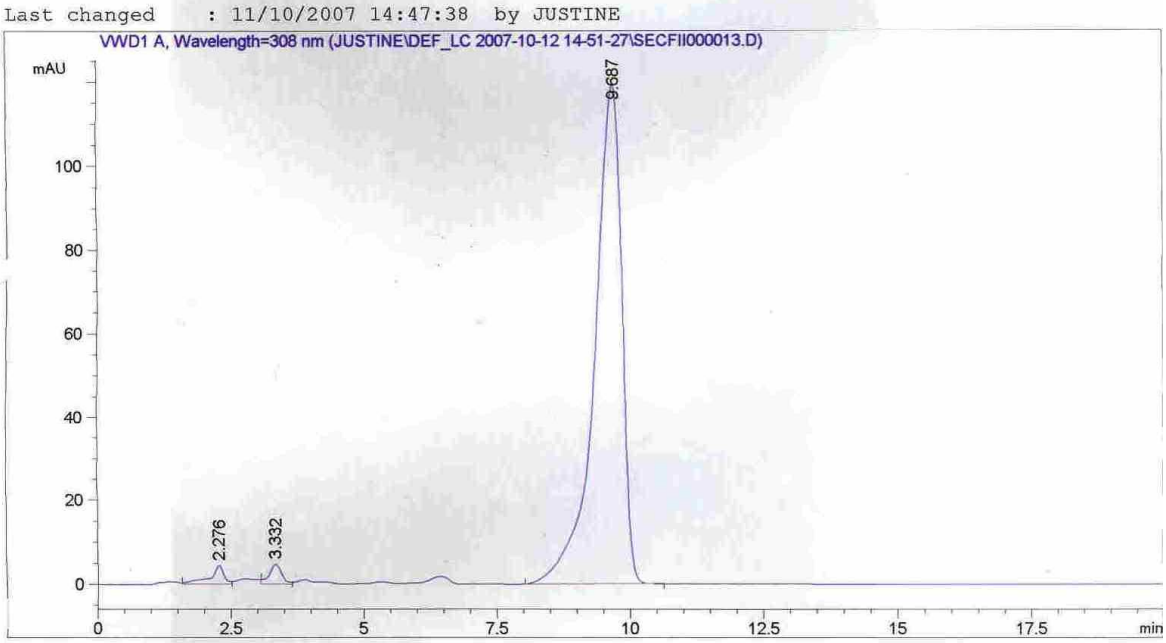


Figure 19: *Intraparticle diffusion model*

10.6 HPLC Chromatograms

Data File C:\CHEM32\1\DATA\JUSTINE\DEF_LC 2007-10-12 14-51-27\SECFII000013.D
Sample Name: 13 t72 control 20mg/l GM in ACN

```
Acq. Operator    : JUSTINE                          Seq. Line :  13
Acq. Instrument  : Instrument 1                      Location : Vial 63
Injection Date   : 12/10/2007 19:19:27                   Inj :   1
                                                   Inj Volume : 50 µl
Acq. Method      : C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-10-12 14-51-27\JUSTINE GM
                   DETECTION.M
Last changed     : 11/10/2007 14:47:38  by JUSTINE
Analysis Method  : C:\CHEM32\1\DATA\JUSTINE\DEF_LC 2007-10-12 14-51-27\SECFII000013.D\DA.M
                   (JUSTINE GM DETECTION.M)
Last changed     : 11/10/2007 14:47:38  by JUSTINE
```



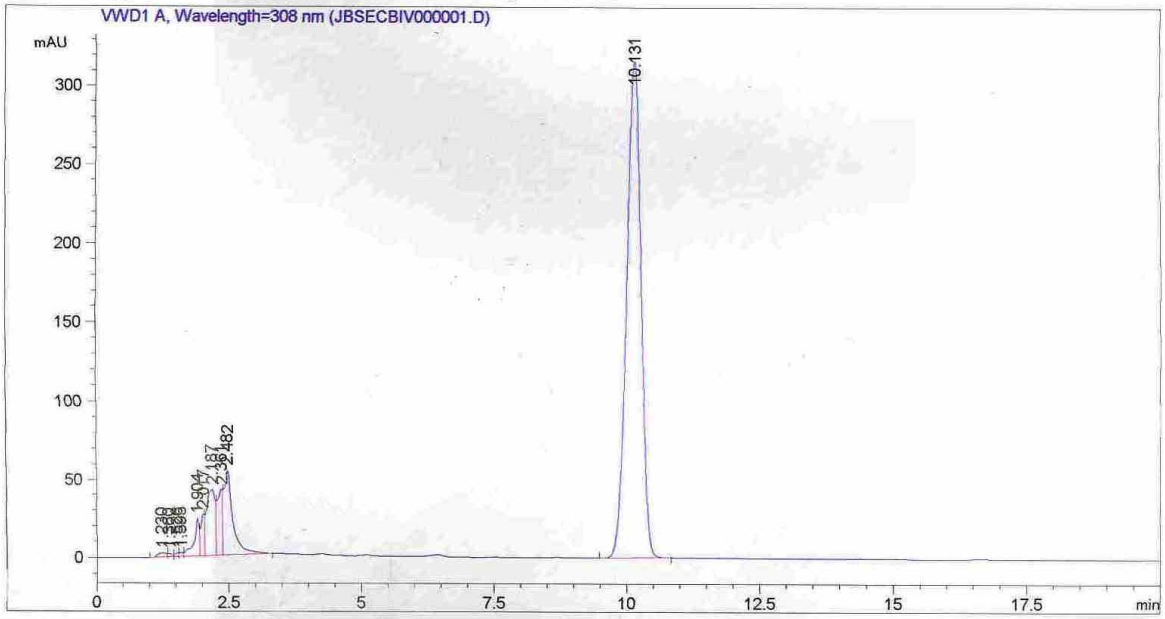
Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: VWD1 A, Wavelength=308 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   2.276 VV    0.2648   85.84308    4.47013   1.9558
   2   3.332 VV    0.2472   78.04763    4.74634   1.7782
   3   9.687 BB    0.5529 4225.16455  119.30896  96.2659

Totals :                   4389.05526  128.52543
```

Image 12: *HPLC chromatograms of GM solution (20 mg/l) in ACN after 72 hours at -20℃.*

Data File C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 17-22-58\JBSecBIV000001.D
Sample Name: 1 0.1g/l GM in DMSO

Acq. Operator	: Justine	Seq. Line :	1
Acq. Instrument	: Instrument 1	Location :	Vial 1
Injection Date	: 04/09/2007 17:24:41	Inj :	1
		Inj Volume :	50 µl
Sequence File	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 17-22-58\DEF_LC.S		
Method	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 17-22-58\JUSTINE GM DETECTION.M		
Last changed	: 03/09/2007 12:31:26 by Justine		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=308 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.230	BV	0.1547	31.08963	2.72901	0.4023
2	1.360	VV	0.0774	12.32718	2.28924	0.1595
3	1.526	VV	0.0764	10.99700	2.29014	0.1423
4	1.595	VV	0.0679	12.77134	2.64901	0.1653
5	1.904	VV	0.0941	168.73444	23.88014	2.1836
6	2.017	VV	0.0703	130.12923	26.54432	1.6840
7	2.187	VV	0.1479	480.72647	42.54054	6.2211

Instrument 1 04/09/2007 17:44:57 Justine Page 1 of 2

Image 13: *Page 1 of HPLC chromatogram of fresh GM solution (0.1 g/l) in DMSO.*

Data File C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 17-22-58\JBSecBIV000001.D
Sample Name: 1 0.1g/l GM in DMSO

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
8	2.361	VV	0.0891	280.29752	42.23222	3.6273
9	2.482	VB	0.1577	648.02570	54.46378	8.3861
10	10.131	BB	0.2923	5952.28857	316.40402	77.0285
Totals :				7727.38707	516.02243	

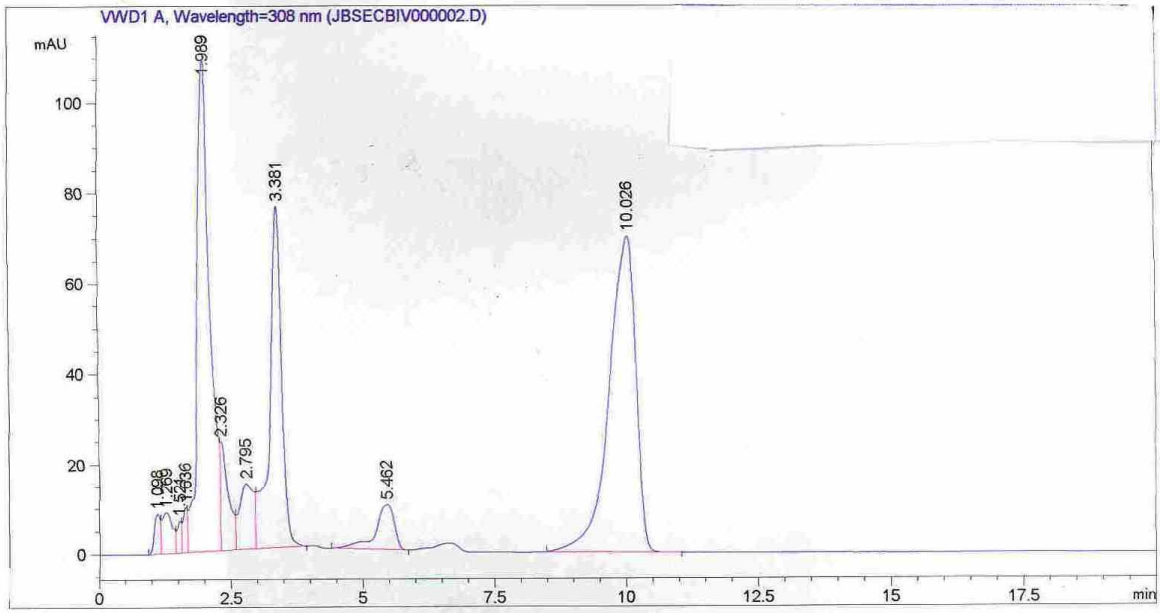
*** End of Report ***

Instrument 1 04/09/2007 17:44:57 Justine Page 2 of 2

Image 14: *Page 2 of HPLC chromatogram of fresh GM solution (0.1 g/l) in DMSO.*

Data File C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 12-43-51\JBSecBIV000002.D
Sample Name: 1 GM-SOL

Acq. Operator	: Justine	Seq. Line	: 2
Acq. Instrument	: Instrument 1	Location	: Vial 2
Injection Date	: 04/09/2007 13:08:58	Inj	: 1
		Inj Volume	: 50 µl
Sequence File	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 12-43-51\DEF_LC.S		
Method	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 12-43-51\JUSTINE GM DETECTION.M		
Last changed	: 03/09/2007 12:31:26 by Justine		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=308 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.098	BV	0.1257	71.35349	8.89001	1.0836
2	1.269	VV	0.2032	125.09534	9.06770	1.8998
3	1.521	VV	0.0809	41.42764	7.12568	0.6291
4	1.636	VV	0.0893	63.13857	10.05558	0.9589
5	1.989	VV	0.2451	1844.72339	109.19933	28.0149
6	2.326	VV	0.1407	250.39954	24.28545	3.8027
7	2.795	VV	0.2669	273.47955	14.67822	4.1532

Instrument 1 04/09/2007 13:29:14 Justine Page 1 of 2

Image 15: *Page 1 of HPLC chromatogram of 5 day old GM solution (0.1 g/l) in DMSO.*

Data File C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-09-04 12-43-51\JBSecBIV000002.D
Sample Name: 1 GM-SOL

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
8	3.381	VB	0.2357	1198.84497	75.72394	18.2063
9	5.462	BV	0.3835	247.89302	9.91361	3.7646
10	10.026	BB	0.5617	2468.43042	70.19999	37.4869
Totals :				6584.78593	339.13951	

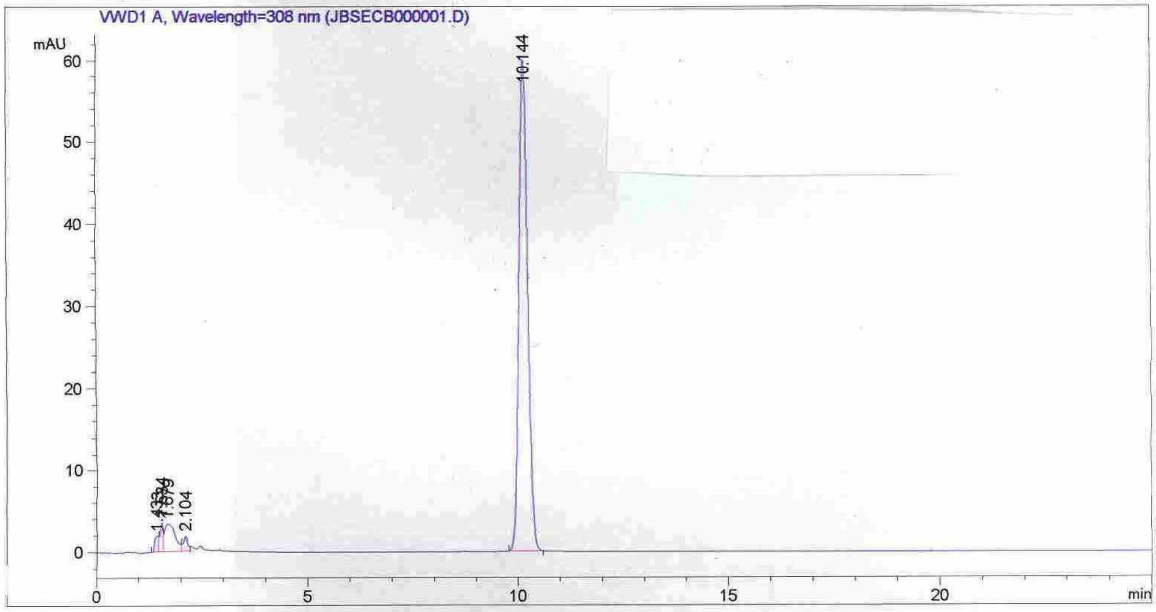
*** End of Report ***

Instrument 1 04/09/2007 13:29:14 Justine Page 2 of 2

Image 16: *Page 2 of HPLC chromatogram of 5 day old GM solution (0.1 g/l) in DMSO.*

Data File C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-08-31 16-50-11\JBSecB000001.D
Sample Name: 1

Acq. Operator	: Justine	Seq. Line	: 1
Acq. Instrument	: Instrument 1	Location	: Vial 1
Injection Date	: 31/08/2007 16:51:57	Inj	: 1
		Inj Volume	: 50 µl
Sequence File	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-08-31 16-50-11\DEF_LC.S		
Method	: C:\Chem32\1\DATA\JUSTINE\DEF_LC 2007-08-31 16-50-11\JUSTINE GM DETECTION.M		
Last changed	: 30/08/2007 12:33:56 by Justine		



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=308 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.433	BV	0.1053	12.61980	1.93298	1.3015
2	1.534	VV	0.0671	16.97983	3.57370	1.7512
3	1.679	VV	0.2758	58.37219	3.35526	6.0201
4	2.104	VV	0.1040	12.90525	1.76949	1.3310
5	10.144	BB	0.2256	868.74188	60.03262	89.5962
Totals :				969.61895	70.66404	

Image 17: *HPLC chromatogram of fresh GM solution (10 mg/l) in H_2O with 2% DMSO.*