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C. O'Cleirigh · J. T. Casey · P. K. Walsh · D. G. O'Shea

Morphological engineering of *Streptomyces hygroscopicus* var. *geldanus*: regulation of pellet morphology through manipulation of broth viscosity

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Abstract Actinomycetes, especially members of the genus *Streptomyces*, are responsible for producing the majority of known antibiotics. The production of antibiotics by filamentous organisms is often dependent on the morphology and size distribution of the pellet population within the culture. Particle interaction and subsequent pellet formation are primarily dependent on the rate of collision of particles in culture, which is in turn, a function of fluid turbulence. The microbial polysaccharide xanthan gum was used to artificially regulate the apparent viscosity (μ_a) of *S. hygroscopicus* fermentation broths with the aim of controlling particle interaction, aggregation and hence pellet formation. An increase in both pellet count and biomass concentration from approximately 2,000 to 8,000 pellets ml^{-1} and 0.9–2.1 gl^{-1} dry weight of biomass, as well a decrease in the mean pellet volume from 0.014 to 0.004 mm^3 was observed in cultures supplemented with 3 gl^{-1} xanthan gum. The addition of xanthan gum significantly alters fluid rheology by increasing the μ_a. Counter-intuitively, an increase in the μ_a within the experimental range examined resulted in an increase in the rate of gas–liquid mass transfer. This was attributed to the predominantly diffusive nature of oxygen transfer in shake flask cultures.

Introduction

The genus *Actinomyces* is an important group of microbes due to their ability to produce commercially valuable secondary metabolites (Okami and Hotta 1988; Prosser and Tough 1991). The actinomycete *Streptomyces hygroscopicus* var. *geldanus* produces a range of antibiotic compounds depending on environmental and nutritional conditions. One of the compounds produced by this organism is Geldanamycin, a benzoquinone ansamycin, first isolated and studied for its broad-spectrum antibiotic activity against Protozoa, bacteria and fungi (DeBoer et al. 1970; DeBoer and Dietz 1976). Although initially isolated and studied for its antibiotic properties, the recent focus on Geldanamycin has been for its potential anti-cancer uses.

Filamentous organisms, both bacterial and fungal, possess the ability to grow in a range of morphological forms in submerged fermentations depending on process conditions such as shear rate, inoculum size, broth rheology, oxygen and nutrient availability and culture vessel design (Hotop et al. 1993; Sinha et al. 2001; Tamura et al. 1997; Whitaker 1992). Product formation in submerged fermentations of filamentous microbes is dependent not only on the level of biomass present but also the morphological profile of the culture (Braun and Vecht-Lifshitz 1991; Vecht-Lifshitz et al. 1992; Treskatis et al. 1997).

The morphology of these organisms may vary from a free filamentous suspension to pellets depending on the degree of aggregation (Nielsen 1996; Prosser and Tough 1991). The formation of pellets in submerged fermentations begins as a result of the aggregation of spores and/or hyphae. Aggregation between individual spores or hyphal elements may be influenced by the composition, structure, hydrophobicity or charge of the cell wall, as well as the presence of extracellular polymeric substances (Atkinson and Daoud 1976). Hyphal growth results in the formation of a branched structure, which facilitates the permanent entanglement of aggregated particles. This accounts for the three-dimensional structure of microbial pellets (Prosser and Tough 1991).

The kinetics of particle aggregation often depend on the existence of an energy barrier between colliding particles; if the collision energy is not sufficient then the particles will not aggregate (Lu et al. 1998). Once a collision has occurred the aggregation mechanism of fine particles in turbulent systems can be: coagulation by means of compression of the electrical double layer, polymer aggregation by means of ion bridging between cell surface molecules or hydrophobic aggregation by means of inter-

C. O'Cleirigh · J. T. Casey · P. K. Walsh · D. G. O'Shea (✉)
Bioprocess Engineering Research Group,
National Institute for Cellular Biotechnology
and School of Biotechnology,
Dublin City University,
Dublin, 9, Ireland
e-mail: donal.oshea@dcu.ie
Fax: +353-1-7005412

action between particles. Collision energy is primarily a function of fluid turbulence, which is in turn impacted on by broth rheology, the control of which enables regulation of particle interaction.

Viscosity is the resistance of a fluid to deformation under shear stress and is related to the resistance to motion in a fluid, a measure of drag force. From a bioprocessing point of view, the rheology of a fermentation broth is of interest given that it impacts on fluid mixing, which in turn affects the rates of heat and mass transfer (Doran 1995). Viscosity affects the motion of particles within a fermentation broth through its impact on drag forces within the fluid and also the Reynolds number. An increase in broth apparent viscosity (μ_a) results in an increase in drag forces and a decrease in the Reynolds number. Consequently the turbulence within the system decreases, which in turn reduces the rate of particle collision, interaction and subsequent aggregation.

The regulation of particle collision and subsequent aggregation through the control of broth rheology and its impact on culture morphology and physiology was identified as a possible control strategy for culture morphology. The work reported in this paper describes the use of varying concentrations of xanthan gum to create fermentation media of artificially high viscosity in order to potentially regulate particle collision and thereby enable the morphological engineering of *S. hygroscopicus* var. *geldanus* cultures.

Materials and methods

Cultivation of *S. hygroscopicus geldanus*

Spore production

Streptomyces hygroscopicus var. *geldanus* NRRL 3602 was grown on Bennett's agar containing the following: technical agar (Oxoid no. 3), 20 g l^{-1}; yeast extract (Oxoid), 1 g l^{-1}; Lab-lemco beef extract (Oxoid), 1 g l^{-1}; n-z-amine a (Sigma-Aldrich), 2 g l^{-1}; dextrose monohydrate, 10 g l^{-1}. Incubation of the organism on 14 g of solid media in a 250-ml Erlenmeyer flask for a period of 21 days at 28°C resulted in the production of aerial spores. A spore suspension, for subsequent use as an inoculum, was prepared by washing the surface of the solid media at 100 r.p.m. on an orbital shaker for 1 h and 4°C using 20 ml of the following solution: yeast extract (Oxoid), 3 g l^{-1}; bacteriological peptone (Oxoid), 5 g l^{-1}; magnesium sulphate heptahydrate, 1 g l^{-1}.

Liquid culturing

Bennett's medium used for growth in liquid culture contained the following: yeast extract (Oxoid), 1 g l^{-1}; Lab-lemco beef extract (Oxoid), 1 g l^{-1}; NZamine A (Sigma-Aldrich), 2 g l^{-1}; dextrose monohydrate, 10 g l^{-1}.

Table 1 Concentration range of xanthan gum-supplemented fermentation broths and the rheological characteristics of the resultant broths

Xanthan gum (gl^{-1})	Flow behaviour index(n) (–)	Fluid consistency index (N s^n m^{-2})	Apparent viscosity (10^{-3} N s m^{-2}) at a shear rate of 28 s^{-1}
Control	0.99	0.91	0.90
0.1	0.90	1.70	1.38
0.5	0.89	2.84	1.95
1.0	0.82	7.19	3.79
2.0	0.86	8.53	5.12
3.0	0.84	19.57	11.34
4.0	0.75	49.21	21.52
5.0	0.62	119.62	36.21

Xanthan gum was added to the media prior to autoclaving at concentrations shown in Table 1. The organism was cultivated on an orbital shaker at 150 r.p.m. and 28°C using 100 ml of Bennett's media in 250-ml Erlenmeyer flasks. A set of five flasks was prepared for each xanthan gum concentration. All cultures were inoculated with 1% (v/v) of 10^6 ml^{-1} spore suspension.

Sample preparation, image capture and image analysis

The sample preparation involving Safranin staining, image capture using a flatbed scanner and image analysis utilising Optimas software was as previously described (O'Cleirigh et al. 2003).

Biomass concentration

The dry weight of biomass in fermentation broth was determined using the following procedure. A 20-ml sample of the fermentation broth was placed in a plastic universal container and subjected to two centrifugation cycles at 3,500 r.p.m. for 10 min. After the first cycle the supernatant was removed and the pellet was resuspended with distilled water to a final volume of 20 ml. After the second cycle, the supernatant was again removed but the pellet was resuspended in 2 ml ethanol and transferred to a pre-washed and pre-weighed glass universal. The glass universal was placed in a 105°C oven for 24 h before weighing and determination of the biomass concentration.

Viscosity determination

The determination of the fermentation broth μ_a was achieved using a Cone and Plate Brookfield Rotational DV-I+ Viscometer (Brookfield, Middleboro, Mass.). Solutions of xanthan gum are non-Newtonian power law

fluids, the μ_a of which is dependent on shear rate. The viscometer facilitated the calculation of the μ_a over a wide range of shear rates.

The rheology of a non-Newtonian power law fluid is characterised by the flow behaviour index (n), the fluid consistency index (K) and theμ_a. Table 1 illustrates the relationship between n, K, μ_a and the concentration of xanthan gum in solution. Increasing xanthan gum concentration results in a decrease in the n and an increase in the K and μ_a. In xanthan gum solutions, n, K and μ_a are concentration dependent.

Shear rate determination

The shear rate in 100 ml of nutrient broth in a 250-ml Erlenmeyer flask agitated on an orbital shaker at 150 r.p.m. is assumed to be 28 s^{-1}, as was determined by Fujita el al. (1994).

Gas–liquid mass transfer coefficient determination

The gas–liquid mass transfer coefficient (K_La) was determined using the dynamic gassing out method (Doran 1995). At 20°C, 100 ml of the relevant solution was aliquoted into a 250-ml Erlenmeyer flask and de-oxygenated by sparging pure nitrogen through the liquid phase. The headspace in the flask was then sparged with air before the dissolved oxygen probe was positioned in the centre of the flask. The flask was placed on an orbital shaker and agitated at 150 r.p.m. while recording the % saturation at 20-s intervals, using a dissolved oxygen metre (WTW, Weilheim, Germany).

Results

Regulation of *S. hygroscopicus* morphology using xanthan gum

As mentioned previously, spores of *S. hygroscopicus* are coagulative and hence tend to aggregate to form pellets. The day-7 pellet count per millilitre of solution and mean pellet volume are given in Fig. 1. The pellet count increases to a maximum at a concentration of 3 gl^{-1} xanthan gum and then decreases in excess of that point, whereas the mean pellet volume decreases with respect to increasing xanthan gum concentration. The addition of 3 gl^{-1} xanthan gum to the fermentation media increases the pellet count by a factor of approximately 4 while correspondingly decreasing the mean pellet volume by a factor of 3.5 from control behaviour. As is the case with the pellet count per millilitre of solution the maximum concentration of biomass is produced in the 3 gl^{-1} xanthan gum cultures. The biomass concentration increases to an optimal 2.5-fold at 3 gl^{-1} xanthan gum and decreases in excess of that. An increase in biomass production is accompanied by a five-fold increase in the rate of glucose consumption over the same range, however the yield ($Y_{x/s}$) decreases with respect to increasing xanthan gum concentration.

The correlation between increased pellet count, reduced mean pellet volume and the concentration of xanthan gum may also be seen in the morphological profiles shown in Fig. 2. This figure contains both images of the stained microbial biomass and the corresponding size distribution profiles for a range of xanthan gum concentrations. Increasing xanthan gum concentration and hence μ_a results in a narrower size distribution with a single mode, i.e. more pellets with a reduced mean volume. Decreasing the μ_a returns the system to control-like behaviour with a broader

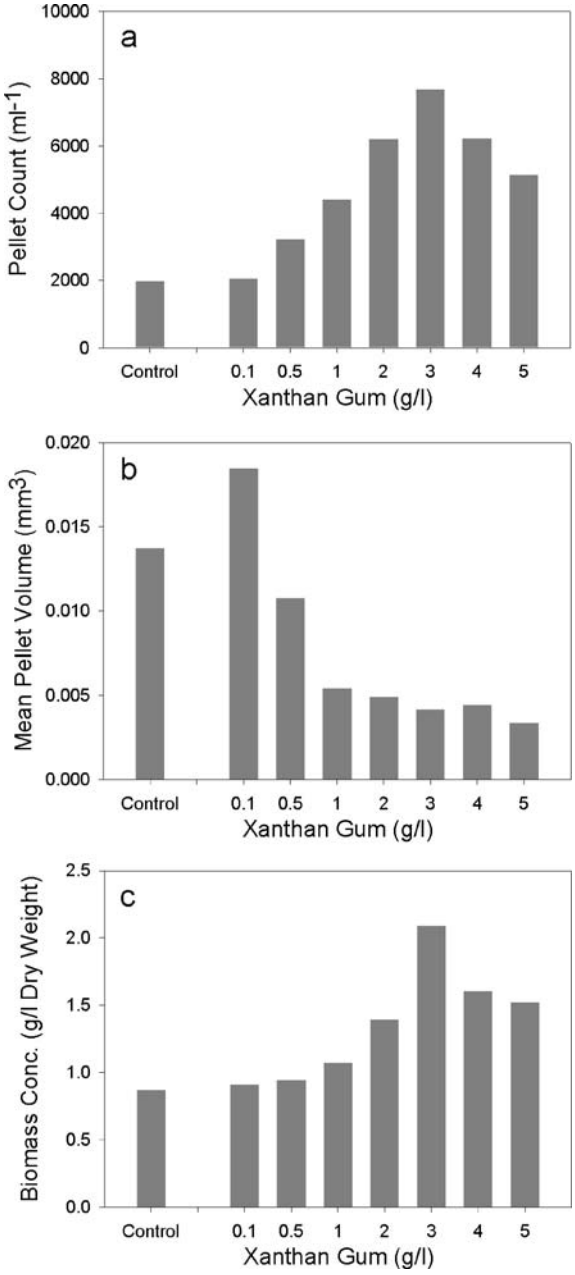

Fig. 1 Impact of adding varying concentrations of xanthan gum to *Streptomyces hygroscopicus* fermentation broths on the day-7 pellet count (**a**), mean pellet volume (**b**) and dry weight of biomass (**c**)

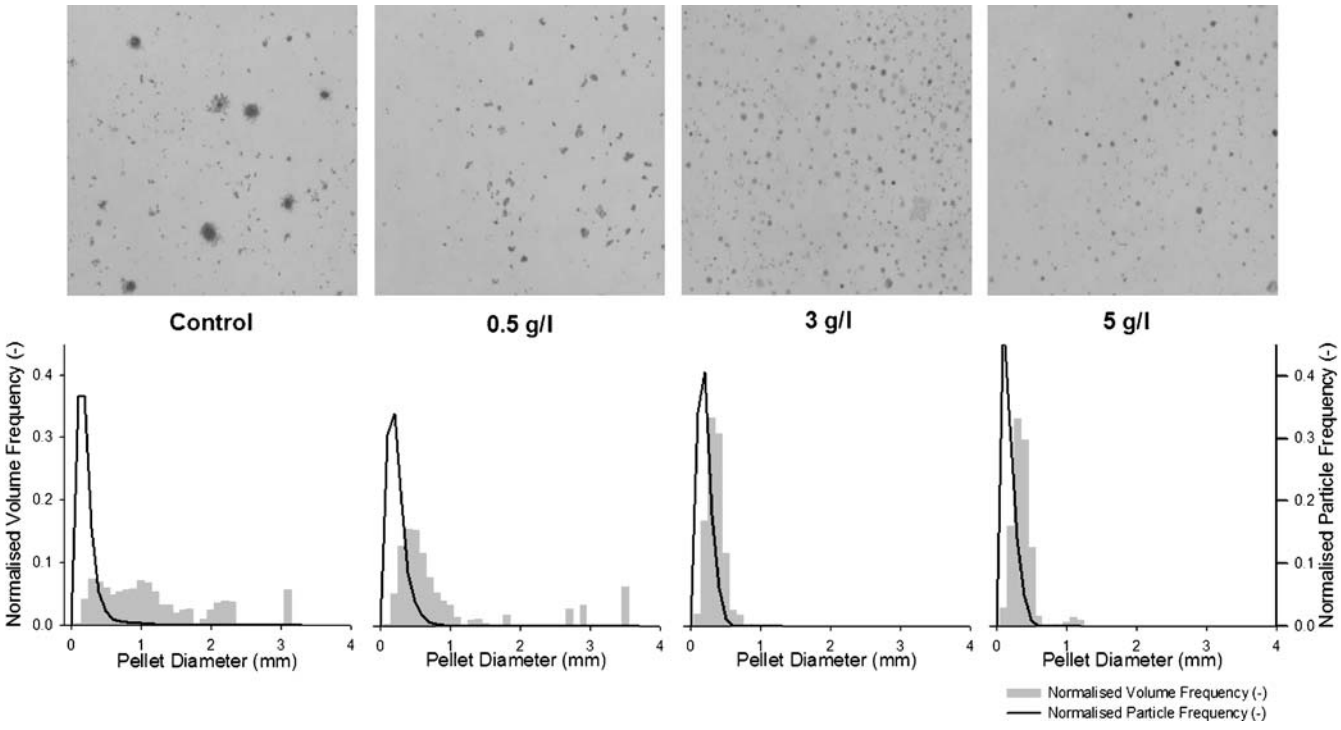

Fig. 2 Stained microbial biomass and normalised volume and particle frequency distributions for control (1 in 5 dilution), 0.5 gl^{-1} (1 in 10 dilution), 3 gl^{-1} (1 in 10 dilution) and 5 gl^{-1} xanthan gum (1 in 10 dilution) for day-7 fermentation samples

distribution and the potential for multi-modality, as can be seen in the culture supplemented with 0.5 gl^{-1} xanthan gum. Increasing μ_a also increases the homogeneity of the cultures as can be seen by the increased overlap on the normalised particle and volume frequencies in the cultures supplemented with 3 and 5 gl^{-1} xanthan gum.

Influence of xanthan gum concentration on μ_a and gas–liquid mass transfer

The morphological regulation of *S. hygroscopicus* cultures through the addition of varying concentrations of xanthan gum artificially controls broth μ_a as shown in Fig. 3. Increasing concentration results in an exponential increase in broth μ_a, the level of which does not alter over the course of the experimental time period. The addition of xanthan gum to the fermentation broth and its impact on μ_a also affects the K_La of the system. Counter-intuitively, it is observed that increasing μ_a steadily increases K_La by up to 37% at 2 gl^{-1} xanthan gum. At levels in excess of 2 gl^{-1}, a steady decrease in K_La is observed with respect to xanthan gum concentration.

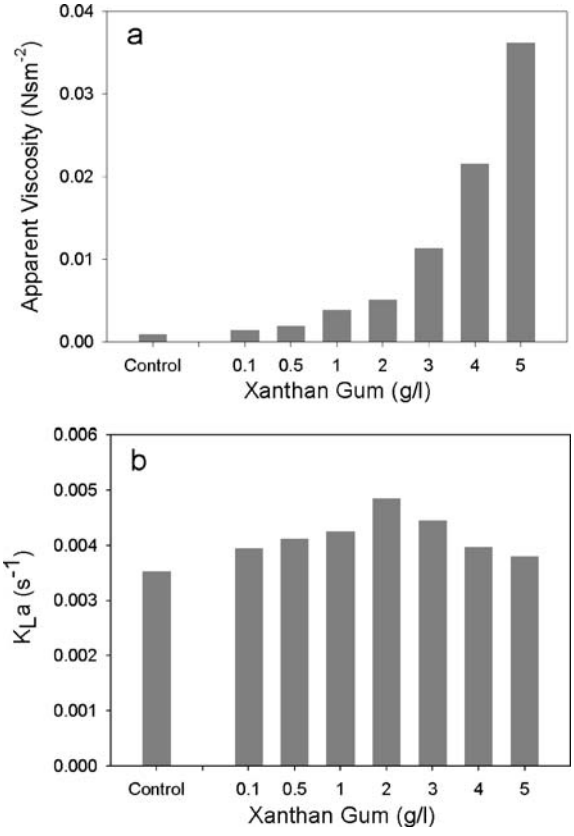

Fig. 3 Impact of adding varying concentrations of xanthan gum to *S. hygroscopicus* fermentation broths on apparent viscosity (**a**) and gas–liquid mass transfer (**b**)

Discussion

Increasing xanthan gum concentration in *Streptomyces hygroscopicus* shake flask fermentations, up to an optimal value of 3 gl^{-1}, results in a fourfold increase in pellet count with respect to the control, as indicated in Fig. 1. This is

assumed to be due to reduced particle-to-particle interaction and subsequent aggregation. Furthermore, given the increase in pellet number with respect to the control as a result of reduced particle-to-particle interaction, the mean pellet volume decreases. This results in more individual spores or smaller hyphal aggregates developing into pellets without subsequent aggregation. Figure 2 indicates that the formation of pellets and the resulting population size distribution is regulated by the μ_a of the culture. As well as ensuring a narrower more normal distribution, increasing the μ_a ensures a more homogeneous population, as evidenced by the similarity of the normalised particle and volume frequencies in the 3 and 5 gl^{-1} distributions.

In many systems compound formation is heavily dependent on the morphology of the organism, with many microbes capable of producing a variety of compounds depending on pellet size distribution (Braun and Vecht-Lifshitz 1991; Treskatis et al. 1997). The ability to engineer the morphological profile of a culture as shown in Figs. 1 and 2, by regulating the μ_a, to produce a specific compound of interest, is of great potential benefit in *Streptomyces* fermentations.

The addition of xanthan gum also increases biomass production by up to 2.5-fold, with respect to the control, at an optimum concentration of 3 gl^{-1} xanthan gum. A four-fold increase in pellet count, coupled with a 2.5-fold increase in biomass concentration indicates that optimising biomass growth is achieved in two ways. Firstly, by inhibiting particle aggregation the development of individual spores into smaller pellets is achieved, with respect to the control and secondly, by increasing the oxygen transfer to those pellets once they have formed, through an increase in K_La. By regulating particle interaction and increasing oxygen transfer through the addition of an inert, viscosity-regulating agent, it is possible to control the number and size distribution of pellets formed, while not compromising the amount of biomass generated.

Increasing viscosity results in a reduction in the Reynolds number, and hence fluid mixing; this in turn should also mean a decrease in the rate of gas–liquid mass transfer. Previous research has shown that increasing broth μ_a using xanthan gum has resulted in a decreasing rate of oxygen transfer in stirred tank reactors (García-Ochoa and Goméz 1998; García-Ochoa et al. 1998). However this does not appear to be the case with shake flask systems where the addition of xanthan gum increases the rate of oxygen transfer in the range investigated as shown in Fig. 3. It has been previously shown that increasing xanthan gum concentration can increase the rate of oxygen diffusion in non-agitated solutions (Ho and Ju 1988). In an aerated, stirred tank reactor, oxygen transfer is achieved by means of bubble dispersion, which does not occur in shake flasks. It is proposed that in agitated shake flask systems at the shear rate investigated, where fluid mixing is different to in a stirred tank reactor, the driving force behind oxygen transfer is active diffusion within a fluid in motion and thus an increase in xanthan gum concentration increases the rate of gas–liquid mass transfer up to an optimum at 2 gl^{-1}xanthan gum. At concentrations in excess of this point the viscosity of the broth increases sharply as illustrated in Fig. 3 and results in the reduction of bulk fluid mixing and consequently a decrease in the rate of gas–liquid mass transfer.

The μ_a of any solution is dependent on the rheological characteristics, n, K, and the shear rate experienced in the system. Given the concentration dependence of n and K as shown in Table 1 and the shear rate experienced in the system it is possible to determine the μ_a of a variety of xanthan gum suspensions over a range of concentrations and shear rates. The dependence of μ_a on both concentration and shear rate enabled the formulation of fermentation media with desirable rheological characteristics. Table 1 shows the range of μ_a values for each of the concentrations of xanthan gum used.

The viscosity of a solution governs the resistance to motion within the fluid and in the case of the biological system in question the motion of particles in solution. The kinetics of aggregation rely on the chaotic collision of particles within this solution. Introduction of a viscous agent into the solution increases the resistance to motion and reduces the Reynolds number, thereby altering the flow characteristics of the fluid and decreasing turbulence, which in turn decreases chaotic particle collision and subsequent aggregation. Increased viscosity ensures a narrower size distribution of smaller pellets and reduced flask-to-flask variability. An increase in broth viscosity is not only responsible for regulating particle aggregation, but also the rate of oxygen transfer, both of which are believed to be responsible for the elevated levels of biomass in cultures supplemented with xanthan gum. The regulation of broth μ_a through the addition of a non-Newtonian agent, such as xanthan gum, may be used to morphologically and physiologically engineer submerged cultures of filamentous organisms.

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