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Short communication

Evaluation of anthrax vaccine production by *Bacillus anthracis* Sterne 34F$_2$ in stirred suspension culture using a miniature bioreactor: A useful scale-down tool for studies on fermentations at high containment

Tarit K. Mukhopadhyay[a,*], Nigel Allison[b], Susan Charlton[b], Michael J. Hudson[b], Bassam Hallis[b], Annemarie King[b], Rebecca Baker[b], Sara Noonan[b], Joanne McGlashan[b], Katie West[b], M. Susana Levy[a], John M. Ward[c], Gary J. Lye[a]

[a] *The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, WC1E 7JE, UK*
[b] *Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK*
[c] *Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, UK*

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ABSTRACT

The licensed UK anthrax vaccine is produced by static cultures of *Bacillus anthracis* Sterne 34F2 in glass Thompson bottles, each batch consisting of multiple bottles grown for 24–28 h. In this work, a novel miniature bioreactor was used as a scale-down tool to investigate the possible transfer of anthrax vaccine production from static culture to stirred tank operation and to explore the effects of this change in culture conditions on process performance. It is shown that the change to stirred culture conditions is possible and that the concentration of the two main vaccine components, Protective Antigen (PA) and Lethal Factor (LF), are reached in less than half the time compared to standard Thompson bottle methods. Furthermore, because higher cell densities were attained in the miniature bioreactor, a 74% increase in antigen concentration was achieved. More detailed analysis of the stirred bioreactor results operated with and without aeration showed antigen degradation in the presence of aeration. Overall this work demonstrates the usefulness of miniaturisation techniques to perform process characterisation studies safely and without significant capital investment for large-scale containment.

1. Introduction

The use of miniaturised bioreactors is gaining increased interest in order to reduce the labour intensity and material costs of bioprocess development [1–5]. Miniature bioreactors have been created as early stage process evaluation tools and as an alternative to microtitre plates and shake flasks to overcome possible limitations in mixing and oxygen transfer capacity [6,7]. The quality of data attained combined with the small amount of required material makes miniature bioreactors particularly suited for experimentation on Advisory Committee on Dangerous Pathogens (ACDP) Class 3 pathogens. Their small footprint means that they can easily fit within an isolator or cabinet and reducing the experimental volume to millilitres allows for safer experimentation.

Anthrax is a widespread zoonotic disease that has been a prominent feature in bio-defence studies since the notorious US mail attacks of 2001 [8]. The causative agent of anthrax is the rod shaped, non-motile, spore forming bacterium, *Bacillus anthracis*. It is a Gram-positive bacterium [9] and usually grows aerobically, but is a facultative anaerobe [10]. The anthrax bacillus produces two main virulence factors; the polyglutamic acid capsule and anthrax toxin, which are encoded on two plasmids separate from the bacterial chromosome. Plasmid pX01 is a 182 kb plasmid that encodes for the three toxin component proteins: Protective Antigen (PA), Lethal Factor (LF) and Oedema Factor (EF) [11]. PA and LF combine to form lethal toxin [12] and PA and EF combine to form oedema toxin [13] in bi-partite arrangements. Thereafter the lethal and oedema toxins are internalised and exert their toxicity intracellularly. Plasmid pX02 is 93 kb in size and encodes several genes that combine to form a polyglutamic acid capsule that inhibits phagocytosis [14].

The vaccine strain used in current UK production is *B. anthracis* Sterne 34F$_2$ which lacks the pX02 plasmid but possesses the pX01 plasmid and expresses all three toxin components, including PA. PA is a major protective component of the US acellular anthrax vaccine [15,16]. It is an 83 kDa protein that is cleaved by furin at the cell surface to produce two subunits, PA$_{63}$ and PA$_{20}$; the PA$_{63}$ moiety is the activated form of the protein which heptamerises and can then bind EF or LF. Compared to PA, LF and EF are produced in smaller amounts, but are both considered important contributors to the potency of the vaccine. LF is a 90 kDa zinc metalloprotease, which

* Corresponding author. Tel.: +44 2076790438; fax: +44 2079163943.
E-mail address: ucbetkm@ucl.ac.uk (T.K. Mukhopadhyay).

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cleaves and inactivates mitogen activated protein kinase kinases [17]. EF is a calmodulin-dependent adenylate-cyclase (89 kDa) that causes a dramatic increase of intracellular cAMP levels [18].

Also encoded on pX01 is the anthrax toxin attenuator protein (AtxA). It is a central regulator which interferes with the expression of over 70 genes *in vivo* and is thought to control PA, LF and EF expression as well as capsule expression [19]. Key to this regulon is the necessity for an elevated carbon dioxide environment. Optimal production of the toxin components requires the bacteria to be grown under relatively high CO_2-tension at 37 °C [20]. It is thought that the high carbon dioxide tension has a positive effect on AtxA expression, which in turn triggers the expression of anthrax virulence factors [21,22].

The UK anthrax vaccine (anthrax vaccine precipitated, AVP) has been manufactured under license at the Health Protection Agency for more than 40 years. AVP is a cell-free alum precipitate of Sterne strain culture supernatant containing multiple vaccine antigens. Briefly, *B. anthracis* Sterne $34F_2$ is grown in Thompson bottles with a 500 ml working volume under static conditions for 24–28 h. The culture is sterilised by filtration (0.2 μm) and the supernatant combined with potassium aluminium sulphate and acidified to create a precipitate which is left to settle for approximately 1 week. This precipitate forms the basis of the anthrax vaccine [23]. A typical vaccine run consists of over 200 bottles, which take approximately 2 h to inoculate manually under containment level 3 conditions. The bottles are harvested after pH and glucose concentration meet the set harvest criteria.

In this study a novel miniature stirred bioreactor, designed in our laboratory [24], was employed to evaluate the possibility of moving anthrax vaccine production from static culture to stirred tank conditions. The miniature bioreactor used is geometrically similar to conventional fermenter designs and the results are readily scaleable to laboratory and pilot scales, making this a useful scale-down tool [25].

2. Materials and methods

2.1. Strain

Spores of the UK vaccine strain of *Bacillus anthracis* Sterne $34F_2$ (Health Protection Agency) were used in all experiments.

2.2. Standard Thompson bottle fermentation

B. anthracis spores were cultured in Thompson bottles by staff at the HPA as described in the UK Anthrax Vaccine Precipitate Product Licence (PL 1511/0058). Spores were diluted to 2×10^4 cfu ml^{-1} in 50 ml of 'Addition' medium, (60 g l^{-1} $NaHCO_3$; 20 g l^{-1} glucose; 10 mg l^{-1} $MnSO_4{\cdot}4H_2O$). This was used to inoculate Thompson bottles containing 450 ml of 'Basal' medium (5.956 g l^{-1} casamino acids; 0.518 g l^{-1} KOH; 69.5 mg l^{-1} activated carbon; 52 mg l^{-1} DL-serine; 25 mg l^{-1} $MgSO_4{\cdot}7H_2O$; 24.5 mg l^{-1} $CaCl_2{\cdot}6H_2O$; 20 mg l^{-1} L-cystine; 0.167 mg l^{-1} thiamine hydrochloride). This gave each Thompson bottle a working volume of 500 ml. Each bottle was then sealed with a permeable nitrocellulose stopper, laid flat in a 37 °C incubator and grown for 24–28 h without aeration or agitation [26]. Bottles were sacrificed sequentially at set time points in order to gain bulk values for pH and glucose concentration. The bottles were considered ready for harvest once the pH and glucose concentration had met the set harvest criteria.

2.3. Miniature stirred bioreactor fermentation

The miniature stirred bioreactor (HEL Ltd., UK) comprised of a baffled cylindrical glass bioreactor approximately 70 mm in height and 65 mm in diameter [24] with a stainless steel head plate accommodating probes, gas in and gas out lines and a sampling port. The impeller was magnetically controlled, driven from below by a solid magnetic disc at the base of the shaft and a six bladed turbine, 20 mm diameter, was located three quarters the way up. The entire bioreactor was maintained at 37 °C by placing it inside an incubator. Agitation was set at 700 rpm in order to efficiently disperse the carbon in the medium and in cases where aeration was used air was passed through a 0.22 μm filter before entering the reactor. The off gas also passed through a 0.22 μm filter in order to maintain sterility. A pH probe (Hamilton, Easyferm 9-6-52) connected to an Applikon ADI1030 controller was used in all bioreactor runs. A working volume of 80 ml was used in all runs using the same medium as used in Thompson bottle cultures.

Briefly, 72 ml of Basal medium was measured into the bioreactor and the head plate secured. The pH probe was screwed into the head plate and other ports were closed. The sampling port remained slightly open during sterilisation to prevent excess pressure build-up but was immediately sealed post sterilisation. The entire bioreactor was autoclaved using steam at 121 °C for 15 min. Once sterilised, the bioreactor was allowed to cool and moved into the 37 °C incubator. The pH probe was connected to the data logger and the bioreactor was left in the incubator for approximately 1 h to equilibrate. The bioreactor was inoculated aseptically with a pipette via a secondary port on the head plate using *B. anthracis* spores diluted in 8 ml Addition medium to give a starting concentration of 2×10^3 cfu ml^{-1}. The sampling port utilised a septum, as in many commercial bioreactors, that allowed sampling without stopping operation or removal of the bioreactor from the incubator. Instead a fixed syringe was used to create a vacuum and draw fermentation broth in collection bijou bottles. Samples were collected and cell growth monitored using optical density. Samples were sterilised by filtration (0.22 μm) and supernatants assayed immediately or after storage at −20 °C. At the end of each experimental run, the sampling port was opened and the entire reactor decontaminated by autoclaving.

2.4. ELISA (PA and LF)

Concentrations of PA and LF in supernatants were determined by antigen-capture enzyme linked immunosorbent assay (ELISA). 96-Well Immulon-2HB plates (Dynatech) were coated overnight with 2 μg ml^{-1} of Immunoglobulin G purified from hyperimmune rabbit-anti-PA serum or anti-LF serum, 100 μl well^{-1}. Plates were washed and blocked for 1 h using Phosphate Buffered Saline Solution with Tween 20 (PBST, 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4, 0.24 g l^{-1} KH_2PO_4, 2 ml l^{-1} Tween 20, pH 7.2) plus 5% foetal calf serum at 37 °C. Plates were subsequently washed with PBST and samples and standards transferred onto the plate and serially diluted. Dilutions of recombinant PA (rPA) or recombinant LF (rLF) with a starting concentration of 2 μg ml^{-1} were used to create a standard curve. All samples were run in duplicate. Plates were incubated for 1 h at 37 °C before being washed three times in PBST. 100 μl anti-PA horseradish peroxidase (HRP) conjugate diluted 1:5000 was added to each well for the PA ELISA. For the LF ELISA anti-LF-HRP was diluted 1:3000 and was added to each well. Plates were incubated for an hour at 37 °C before being washed three times in PBST and 100 μl well^{-1} of 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate (Sigma) added. The plates were incubated for 30 min or 20 min for the PA and LF ELISAs, respectively, before the enzymic reaction was stopped with 50 μl well^{-1} of 2 mol l^{-1} sulphuric acid. The plates were then read at 450 nm using a Multiskan MS plate reader (Labsystems); PA and LF were quantified by comparison with standard curves.