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Cryopreservation of *Streptococcus mutans* for microcalorimetry based applications

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Abstract

Cryopreservation is shown to be a suitable technique for storing *Streptococcus mutans* prior to analysis of chemicals for putative antibacterial properties by microcalorimetry. Excellent recovery of cryopreserved cells was obtained and their response in the microcalorimeter was highly reproducible. Linear responses were obtained when the concentration of fluoride was varied in assays. Similar results were obtained with natural products claimed to have antimicrobial properties. It is proposed that microcalorimetry with cryopreserved cells could be used as a method for rapidly assessing the potential of individual or mixtures of components for commercial preparations designed to control oral microflora. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Antimicrobials; Cryopreservation; Microcalorimetry; Oral microbiology; *Streptococcus mutans*

1. Introduction

Microcalorimetry can be used to measure the heat associated with chemical or biochemical reactions [1–3]. It has proven to be a useful tool for examining many types of cellular activities in a wide range of organisms [4–6]. In many circumstances, microcalorimetry can reveal facets of a process that would not be detected by other methods of biochemical analysis. For example, Wentzien *et al*. [7] investigated the stages that lead to adhesion during the formation of biofilms containing a species of *Thiobacillus*. Application of this technique to the study of metabolic processes has also yielded important information and shown the advantages of non-invasive methods for studying metabolism [8–10].

The oral care market is one of the most important in the pharmaceutical sector, toothpaste sales alone were worth nearly $1 billion per annum a decade ago [11]. Most oral care products have bioactive agents, such as fluoride, to control the microbial flora in the mouth. In recent years, there has been a move to incorporate natural products into toothpastes and mouthwashes to replace constituents or enhance the action of a formulation. For example, compounds from *Camellia sinensis* (green tea) can have synergistic effects with other agents used in caries control [12]. Currently, there is a need to analyse the interaction of mixtures of existing and newly identified antimicrobial agents with caries forming micro-organisms as an aid to product formulation. Microcalorimetry is ideal for this type of application.

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Strains of *Streptococcus mutans* have long been implicated in the formation of dental plaque and cariogenicity [13]. Much information has accumulated on the metabolism of these bacteria and how antimicrobial agents, especially fluoride, inhibits their metabolism. Natural products from plants have been shown to be active against *S. mutans* and in some cases the site where they inhibit metabolism has been identified [14].

For the day to day use of microcalorimetry for the assessment of chemicals and chemical mixtures active against oral bacteria, it is essential to have a supply of micro-organisms that are readily available and with a uniform response to bioactive agents. Of the many ways by which bacteria can be preserved, cryopreservation at $-196^{\circ}C$ was selected as the focus of this study to provide *S. mutans* for use in assessment of antimicrobial agents that affect the growth and survival of this bacterium. The reasons for selecting this method of preservation included easy preparation and storage of bacterial populations, quick recovery of bacteria for use in assays, likely lack of variability within a batch of preparations [15], and likely applicability for other oral microbes.

2. Materials and methods

2.1. Organism and growth conditions

S. mutans NCTC 10832 was obtained from the National Collection of Type Cultures in freeze dried form and revived using the method provided. The nature of the bacterium was confirmed and monitored using the Gram stain procedure and the API 20 Strep system (bioMérieux UK, Basingstoke, Hampshire, UK). Cultures were routinely maintained by subculture on brain heart infusion (BHI) agar slants. Cultures for cryopreservation were grown in liquid culture on either BHI, tryptone soya broth (TSB) or on modified van der Hoeven's medium (VDH) [16]. VDH is a fully defined growth medium that was modified by increasing the concentration of K_2HPO_4, NaH_2HPO_4 and Na_2CO_3 to 0.77, 0.53 and 2.0 g l^{-1}, respectively.

All media were sterilised by autoclaving at $121^{\circ}C$ for 15 min. Batch cultures of *S. mutans* were grown in Erlenmeyer flasks (50 ml media in 250 ml flasks, 500 ml media in 2 l flasks). Starter cultures (50 ml) were inoculated with a loop of cells from BHI agar slants. Experimental flasks were inoculated with an overnight (14 h) starter culture (1% v/v). Where cultures were grown in vessels containing 15 l of growth medium, a 3.3% v/v inoculum was employed. All incubations were at $37^{\circ}C$, shake flasks were incubated at 60 rpm in an orbital incubator (Gallenkamp cooled orbital incubator). Culture turbidity was monitored by taking absorbance readings at 600 nm.

2.2. Cryopreservation and recovery of bacteria

The first part of the procedure involved the separation of cells from culture media. It was found that centrifugation at $4000 \times g$ for 10 min formed a pellet that was easy to resuspend in cryopreservation media. Cells were resuspended in BHI, or 1/4 strength Ringer solution (Oxoid) with (i) no additives, (ii) plus 10% v/v dimethyl sulfoxide (DMSO), or (iii) plus 10% v/v glycerol. Total viable counts on resuspended cells were performed using the spread plate method where the resuspended cells were serially diluted with 1/4 strength Ringer solution and 0.5 ml aseptically plated on tryptone soya agar. Typically between 8 and 9×10^9 cells ml^{-1} were taken to the next stage of the process. Suspensions of cells for cryopreservation were stirred continuously and aliquots (2.0l ml) were rapidly dispensed into polypropylene ampoules, which were then capped. To monitor the freezing process, a thermometer was placed in a specially designed ampoule containing preservation media without cells. The ampoules were held evenly spaced on cooling bars and placed 10 cm above liquid nitrogen that had been placed in a suitable container (Fig. 1). The temperature change in the ampoules was monitored every minute until the temperature was approximately $-80^{\circ}C$, at which time the ampoules were lowered into the liquid nitrogen for storage. The rates of cooling obtained during this process, from a starting temperature of $21^{\circ}C$, were between -8 and $-12^{\circ}C\ min^{-1}$ with 1/4 strength Ringer solution plus 10% v/v DMSO.

Frozen cell suspensions were thawed by placing the ampoules into a water bath, set at $40^{\circ}C$, for 2 min. The ampoules were then removed from the water bath, wiped dry and then agitated using a vortex mixer for 1 min. Nearly 100% recovery of viable cells was obtained using this procedure (a typical recovery was 97% of viable cells originally present).

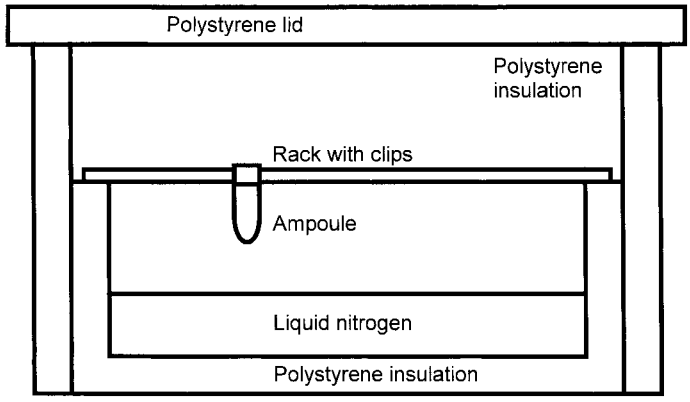

Fig. 1. An aluminium tank with polystyrene insulation was used for the large batch cryopreservation procedure. Liquid nitrogen (−196°C) was poured into the aluminium tray (internal area of 30 cm×50 cm) to a pre-determined depth (5 cm). Bars placed over the liquid nitrogen held cryogenic ampoules containing the 25 fold concentrated homogeneous cell suspension aliquots to be frozen. The height of the ampoules above the liquid nitrogen was consistent (10 cm) allowing uniform cooling in the nitrogen vapour.

2.3. Microcalorimetry

All experiments were carried out in an LKB 10700-1 flow calorimeter (Thermometric AB, Sweden). A Keithley 155 Null detector microvoltmeter (Keithley Instruments GMBH, Germany) was employed to amplify the signal. The amplified signal was recorded on a chart as percentage amplifier detection against time. The microcalorimeter contained one flow-through type reaction vessel operated at a constant flow rate and another vessel that could be used for mixing. All experiments were undertaken in a constant temperature room (21±0.1°C), the calorimeter unit being in a thermostated air bath set at 30°C. A bubble of air was allowed to enter the flow system before an experiment; this showed the boundary between the liquids, acted as a marker and also helped to clean the reaction vessel before the new liquid entered. Phosphate buffer (16 mM) pH 7.2 with 12.2 mM glucose and 20% v/v BHI broth were continuously stirred outside the microcalorimeter while being pumped through the microcalorimetric reaction vessel. When the closed loop was formed (outer stirred vessel — microcalorimetric flow-through reaction vessel — outer stirred vessel) 0.4 ml *S. mutans* was added to the outer stirred vessel. Following a pumping time lag and thermal lag totaling 3 min, the heat output of the micro-organisms was registered. 20 min after addition of the bacteria, putative antimicrobial agents were added. Any decrease in heat output from this stable upper baseline was defined as 'biological response'. This 'response' was mathematically corrected to compensate for the dilution effect of adding a defined volume of liquid. The plant extracts were of a crude aqueous nature.

3. Results and discussion

3.1. Growth and cryopreservation of S. mutans NCTC 10832

The cryopreservation of *S. mutans* was compared with media that have previously been used for batch cultures of this type of bacterium. The key parameters evaluated were biomass yield, resultant homogeneous or heterogeneous growth, and recovery of viable cells after cryopreservation. In the latter case, both cell viability and metabolic activity (heat output during glucose metabolism) were used as measures of successful recovery. Poor growth of this strain of *S. mutans* was obtained on the fully defined media described by van der Hoeven *et al.* [16]. This was unfortunate, as it would have provided a medium where nutrient carry over could be greatly reduced and where the batch to batch chemical composition would vary only slightly. The best biomass yield and homogeneous growth were obtained in BHI (Fig. 2). Increasing the size of the inoculum from 0.5% (v/v) to 4% (v/v) for the batch cultures grown on BHI

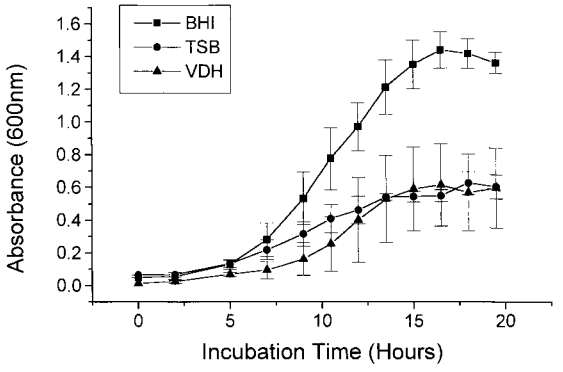

Fig. 2. The graph shows the growth curves of *S. mutans* shaker cultures (60 rpm) grown on BHI (■) broth, TSB (●) or VDH — a fully defined medium (▲) at 37°C. 1.0 ml of a late exponential culture was used to inoculate pre-warmed Erlenmeyer flasks containing 50 ml of each medium and absorbance readings were recorded at 600 nm. Standard deviations from eight data sets are shown.

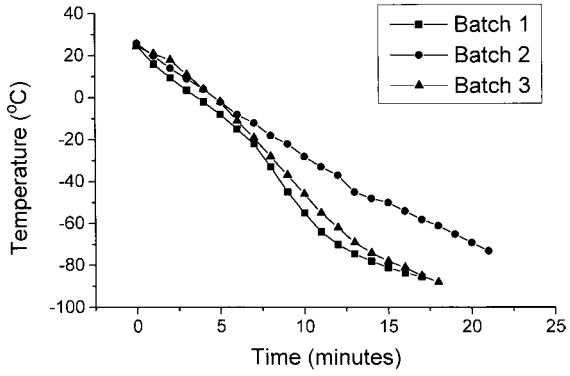

Fig. 4. The graph shows the profile of the *S. mutans* cooling curves above the liquid nitrogen. A thermometer was placed in a specially designed cryogenic ampoule containing the resuspension medium (1/4 Ringer+10% DMSO) without micro-organisms. When the temperature reached −80°C, the ampoules were removed from the cooling bars and placed in the liquid nitrogen. The viability and condition of the micro-organisms in the cryogenic ampoules within a batch were the same.

decreased the lag phase duration time from 10.2 to 4.1 h.

Cells harvested from batch cultures grown on BHI where the growth rate was just starting to decrease were cryopreserved in a variety of media (Fig. 3). The greatest viable counts, but also greatest variability in recovery, was obtained when BHI was used as the cryopreservation medium. Ringer solution (1/4 strength) with or without the addition of 10% (v/v) DMSO or glycerol gave comparable recoveries, allowing the possibility of omitting carbon sources that could increase the metabolic activity of thawed preparations. Recovery of cells, in terms of viable counts, was probably low because viable count procedures underestimate the number of individual cells present due to chains of *S. mutans* produced during growth. Attempts to break up cell aggregates before cryopreservation were unsuccessful.

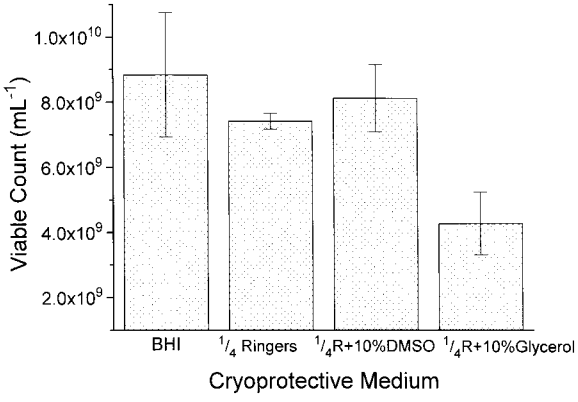

Fig. 3. The graph shows viable counts of *S. mutans* following cryopreservation and thawing in four different cryoprotective media. The micro-organisms were harvested from BHI broth culture by centrifugation before freezing and resuspended in one of the cryoprotective media to form a 25 fold concentrated, homogeneous suspension. Standard deviations from two data sets are shown.

Fig. 4 shows the cooling curves for three separate cryopreservation sequences using 1/4 Ringer+ DMSO. Using the apparatus shown in Fig. 1, this procedure can be performed with little batch to batch variation. Recovery of viable cells was similar in all cases and preparations were stored and tested for 23 months without significant loss of viability.

3.2. Microcalorimetry of cryopreserved cultures of S. mutans

Fig. 5 shows a stylised trace from a typical microcalorimetry experiment as performed using the protocols described earlier. Heat output reaches a steady state of mean 70% and standard deviation 5.4% eventually declining as the carbon/energy source (e.g. glucose) is depleted, product inhibition of metabolic activity occurs or due to loss of activity of a key

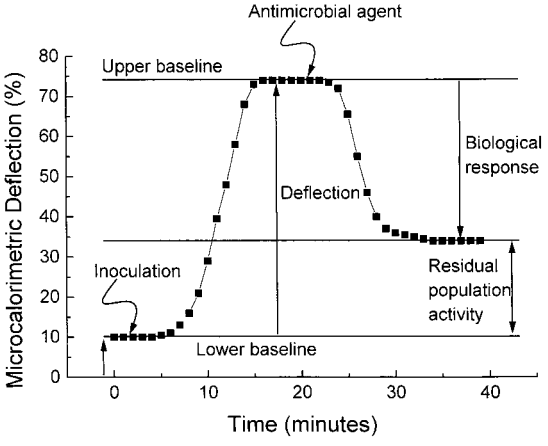

Fig. 5. The graph shows a typical microcalorimetric trace when the microcalorimetric flow medium was inoculated with 0.4 ml *S. mutans*. Should an antimicrobial agent be added to the flow medium, then the drop in heat output from the stable upper baseline would be termed 'biological response'.

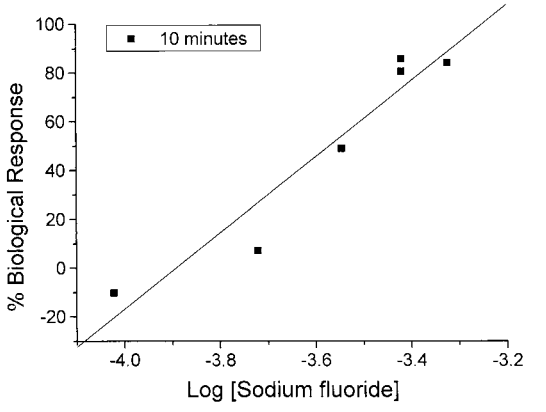

Fig. 6. The graph shows a plot of the microcalorimetrically measured biological response against the logarithm of the dose of sodium fluoride. The line of best fit through the linear portion of the LDR curve is shown.

enzyme in the metabolic processes that result in heat generation. All the experiments performed in this study resulted in a similar pattern of heat generation, although the factors that ultimately lead to a decline in heat output were not identified in this study.

Only the metabolism of glucose (12.2 mM) gave rise to a measurable signal in the microcalorimeter, reflecting the exothermic energy generation from this sugar in *S. mutans*, glycolysis. Cells of *S. mutans* cryopreserved in 1/4 Ringer and 10% DMSO retained 95.8 and 96.8% of the heat output of non-cryopreserved cells after 20 and 30 min incubation, respectively. Typically, it took about 10 min for a steady state to be achieved. Decline in heat output from this steady state value was not seen during a 4 h extended run. Addition of an antimicrobial agent causes a biological response, but may not reduce the heat output to the lower baseline. Heat output above the lower baseline is termed 'residual population activity'.

The inhibition of heat output by fluoride depended on its concentration and the time it was in contact with the cells flowing through the calorimeter. Using natural plant extracts, it was shown that many were capable of inhibiting the heat output from the metabolism of glucose by *S. mutans*. Therefore, this technique can be used as a way for evaluating the usefulness of individual and mixtures of such extracts in formulations that are used to control or stop the growth of this cariogenic bacterium. Assessing antimicrobial activity of a putative antimicrobial agent has been determined by minimum inhibitory concentrations (MICs), agar diffusion assays or the impedance/conductance technique. The advantage of microcalorimetry is that the micro-organisms can be challenged with various concentrations of the agent and any response is immediately registered.

Fig. 6 shows the responses obtained using various concentrations of sodium fluoride, recorded just after the attainment of steady-state heat output. A line of best fit is drawn through the linear portion of the sigmoidal log[dose] response (LDR) curve. Iodoacetamide, (that also inhibits glycolysis) gave a similar response, but over a much lower concentration range (1 μM inhibited heat output by 60%). At the lower end of the range of fluoride concentrations tested heat output was greater than the controls. This could reflect the complex range of metabolic activities affected by fluoride resulting in an increased diversion of metabolites through heat generating reactions. Thus, microcalorimetry can be used to indicate a wider range of metabolic events than merely percentage inhibition. Indeed, microcalorimetry has provided useful information for the synthetic designing of drugs [17] and has been used in deriving quantitative structure activity relationships (QSARs) [18].

The response of *S. mutans* to a range of natural products at varying concentrations was also tested. Typical responses were obtained with thyme aqueous

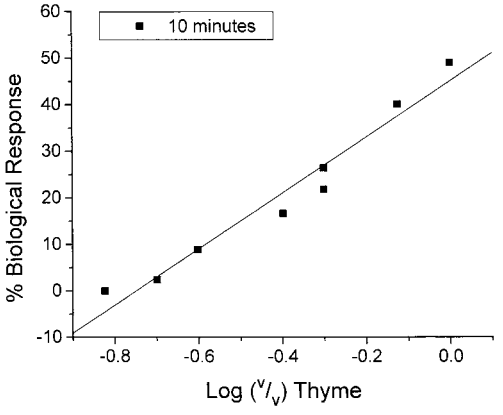

Fig. 7. The graph shows a plot of the microcalorimetrically measured biological response against the logarithm of the dose of Thyme (crude aqueous extract). The line of best fit through the linear portion of the LDR curve is shown.

extracts (from the plant *Coridothymus capitatus*) which are claimed to have antimicrobial activity [19] (Fig. 7). Such preparations show a significant but much lower inhibition of heat output than sodium fluoride. The character and extent of the response of bacteria to an antibacterial agent is reproducible and different for each antibacterial agent studied [20]. The microcalorimetric determination of antibacterial efficacy requires fewer than 60 min to quantitate, whereas classical microbiological techniques could need a period of 48 h growth to form a visible colony-forming-unit on an agar plate. By adding mixtures of such plant extracts with the formulation components used in mouthwashes, it is possible to use microcalorimetry to detect synergistic and antagonistic interactions. Therefore, this technique could be used in conjunction with other methodologies (e.g. viable cell counting, enzyme activities) and can be used to assess the inhibitory properties of individual components in complex formulation mixtures on *S. mutans*. This investigation could be readily expanded to include the effects of antimicrobials on other oral microorganisms.

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