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Physiological effects of the addition of *n*-dodecane as an oxygen vector during steady-state *Bacillus licheniformis* thermophillic fermentations perturbed by a starvation period or a glucose pulse

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ABSTRACT

The effect of the presence of *n*-dodecane as a potential oxygen vector during oxygen-limited continuous cultures of a *Bacillus* strain was studied, under extreme nutrient supply conditions: glucose excess, limitation and starvation. The addition of *n*-dodecane to the aqueous phase of a mechanically agitated and aerated fermentation increased the k_La by up to 35%. The *n*-dodecane additions to *Bacillus licheniformis* cells during starvation (oxygen limitation with concomitant glucose starvation) caused a severe detrimental progressive change in cell physiological state with respect to cytoplasmic membrane polarisation and permeability which was mitigated against by alleviating either the oxygen limitation (by increasing the mean energy dissipation rate or by the addition of *n*-dodecane as an oxygen vector) or by alleviating the carbon limitation (by resuming the carbon feed or by the addition of a glucose pulse). Further that during periods of excess glucose (glucose pulse) a much higher k_La was required to prevent the onset of anaerobic mixed acid fermentation than could be provided by the addition of *n*-dodecane alone. *n*-Dodecane can be used to increase the k_La when added in sufficient quantities to the aqueous phase of a mechanically agitated and aerated bioreactor but the magnitude of this increase is process and vessel geometry specific.

1. Introduction

Thermophilic aerobic wastewater treatment has many of the same benefits as thermophilic composting and sludge digestion, such as faster waste degradation rates, rapid inactivation of pathogenic micro-organisms, and low sludge yields. However, the oxygen requirements of such systems are high and can be considered to be a disadvantage when compared to anaerobic technologies [1]. Indeed, the most important consideration regarding the use of thermophilic reactors is the requirement to match the high oxygen uptake rate imposed by rapid COD consumption at high temperatures given that the saturation concentration of dissolved oxygen in the water-like growth medium is relatively low. Oxygen requirements in these systems have been estimated to be ~14% higher than for conventional aerobic processes [2] such that Rosich and Colvin [3] recommended using extremely high power inputs, gas blending and a greater tank depth to satisfy the large oxygen requirement of thermophilic treatment processes. However, these can be costly options and difficult to achieve in practice especially where available space is at a premium so the selection of alternative regimes that maximise oxygen transfer becomes one of the most critical process design choices. It is known that oxygen transfer in microbial fermentations can be enhanced by adding an organic phase with a higher affinity for oxygen i.e. an oxygen vector [4]. Oxygen vectors are defined as compounds that, when added to the growth medium, can enhance the oxygen transfer rate to the cell, resulting from the higher oxygen solubility in the organic phase when compared with a water-like growth medium [5]. In this case, oxygen transfer can occur directly to cells, or via oxygen-vectors either adsorbed or not to the air bubble surface [6,7]. Whereas no more than the saturation concentration of oxygen can be dissolved in the aqueous phase, the supply of oxygen to the aqueous phase from the gas stream may be supplemented by equilibrium partitioning of dissolved oxygen from the organic phase to the aqueous phase. Indeed a number of workers have used oxygen vectors to successfully enhance oxygen transfer and, as a consequence, have increased the biomass concentration and hence process performance in a number of different culture systems [8,9]. Examples

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of common hydrocarbon oxygen vectors include *n*-dodecane, *n*-hexadecane [10–16] perfluorocarbons [9,17,18] and a number of vegetable oils [19]. The isolation of cultures from thermophilic aerobic wastewater treatment reactors has revealed that thermophilic *Bacillus* spp. are the dominant organisms present [1,2,20]. During such highly aerobic processes oxygen requirements are often over estimated because DOT measurements are impossible to make because the DOT probe rapidly becomes coated with a biofilm. This often leads to excessive power inputs of >30 kW m^{-3} and aeration rates of ~4 vvm being used to 'ensure' oxygen-sufficient conditions [21]. Therefore in this work the effect of the presence of *n*-dodecane as a potential oxygen vector during oxygen-limited continuous cultures of a *Bacillus* strain (previously isolated from an aerobic bioremediation reactor) was studied, under extreme nutrient supply conditions: glucose excess, limitation and starvation. Such conditions are often present in large-scale fermentations (>5000 l dependent on vessel geometry) due to poor mixing [22] where cells experience rapidly changing microenvironments with respect to substrate concentrations when circulating around a bioreactor. In order to evaluate the physiological response of *Bacillus licheniformis* to the range of conditions described individual cell physiological response was measured by multi-parameter flow cytometry as well as more conventional microbiological analytical techniques and a comparison made.

2. Materials and methods

2.1. Organism

B. licheniformis was previously isolated from an aerobic wastewater treatment reactor (Sustainable Biowaste Solutions Ltd., Peterborough, UK) and deposited in the Industrial Micro-organisms Culture Collection (National Institute of Engineering, Technology and Innovation, Lisbon, Portugal), with the reference code CCMI 1034. Lyophilized cell cultures of *Bacillus cereus* NCTC1143 were also used to provide control data.

2.2. Bioreactor experiments

Cells from six nutrient agar plates grown for 24 h at 45 °C were transferred to the growth medium (GM) which was made up as follows: glucose 5 g l^{-1}; KH_2PO_4 1 g l^{-1}; $(NH_4)_2SO_4$ 1.5 g l^{-1}; yeast extract 0.25 g l^{-1}; $CaCl_2{\cdot}2H_2O$ 0.1 g l^{-1}; $MgCl_2{\cdot}7H_2O$ 0.25 g l^{-1} and supplemented with 2 ml Vishniac trace elements solution [23] to inoculate the bioreactor. After a period of batch culture, a pump was used to feed sterile GM into the fermenter at a dilution rate of 0.20 h^{-1}. Steady-state parameters were determined by periodic analysis of biomass concentration (g l^{-1}) and residual glucose concentration (g l^{-1}). All experiments were carried out in an Infors HT benchtop fermenter (Infors, Reigate, Surrey, UK), with a 1.2 l working volume, equipped with two six-bladed Rushton impellers (d = 0.048 m). The two impellers were positioned 0.042 m apart with the lower impeller situated 0.032 m above the bottom of the vessel. The vessel was fitted with three equally spaced baffles, width 0.015 m. The working volume was kept constant by using a surface dipped levelling tube linked to a variable speed peristaltic pump. Culture agitation was set at 500 rpm unless otherwise stated, and the aeration rate at 1 vvm. Continuous, DOT measurements were impossible because the polarographic dissolved oxygen probe used in this work became rapidly coated with a biofilm early on during fermentations. However, the biofilm was aseptically removed from the oxygen probe in all steady-states prior to the batch periods (starvation and glucose pulse), so that steady-state DOT readings could be taken. The temperature was controlled at 45 °C and pH was controlled automatically by the addition of 2 M NaOH or 2 M HCl on demand to 6.8 ± 0.1.

2.3. Oxygen vector

n-Dodecane (Merck, Darmstadt, Germany; oxygen solubility 54.9×10^{-3} g l^{-1} at 35 °C and atmospheric air pressure) was added to the sterile fermentation broth at different volumetric fractions 1, 2.5 and 5% (v/v) as appropriate.

2.4. k_La measurements

k_La measurements were made using a polarographic dissolved oxygen electrode (Ingold, USA) in sterile growth medium because of the problems associated with DOT probe membrane fouling. The gas–liquid mass transfer coefficient (k_La) was calculated by measuring the rate of oxygen transfer in nitrogen purged fermentation broth, at 45 °C following the method described in Ref. [24].

2.5. Glucose pulse

Once the steady-state was reached (D = 0.20 h^{-1}), the medium feed pump was switched-off, and a glucose pulse (6 g l^{-1} final concentration) containing the other GM nutrients (at the same proportion to the feed) were added to allow a period of batch growth and carbon source exhaustion to occur. *n*-Dodecane was added at either 1, 2.5 and 5% (v/v) final concentration, simultaneously with the glucose and the GM nutrients. In some cases the impeller speed was also increased from 500 to 1000 rpm at the same time as the glucose pulse was added. Samples were withdrawn from the bioreactor at regular time intervals for determination of biomass, glucose and organic acids concentration as well as for analysis by multi-parameter flow cytometry.

2.6. Starvation period

Starvation periods were carried out under the same conditions as the glucose pulse but without any nutrients added at all. *n*-Dodecane was added in the same concentrations as previously described. The dilution rate before the starvation period (0.20 h^{-1}) was kept the same for each experiment in order to try and ensure comparable physiological starting conditions in all cases (glucose pulse and starvation period). Once the steady-state was reached, the nutrient feed-line and outlet culture pumps were switched-off to allow a period of nutrient starvation to occur. Samples were withdrawn from the bioreactor at regular time intervals for determination of biomass, glucose and organic acids concentration as well as for analysis by multi-parameter flow cytometry.

2.7. Flow cytometry

The analytical methods used were essentially the same as in previous articles [21,25], so only an outline will be given here. Multi-parameter flow cytometry was used to establish the physiological state of the cells, using a Coulter Epics Elite analyser (BeckmanCoulter, UK). The advantages of multiparameter flow cytometry over the more conventional microbiological techniques such as dilution plating (c.f.u. per ml) are well documented [26,27] but, briefly, using various mixtures of fluorescent dyes it is possible to resolve an individual microbial cells physiological state beyond culturability based on the presence or absence of an intact polarised cytoplasmic membrane and the transport mechanisms across it, enabling assessment of population heterogeneity. In all cases the properties of at least 50,000 cells were measured. In addition, cell biomass ($OD_{600\,nm}$, dry