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# Effect of biomass concentration on the specific solvent productivity of *Clostridium acetobutylicum* in chemostat culture

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## SUMMARY

Using a defined medium in chemostat culture, an inverse relationship between the biomass concentration and the specific butanol productivity has been observed. It is suggested that this is due to the cell population not being homogeneous, and that a change in the nutrient balance leads to a change in the relative proportions of acidogenic, solventogenic and inert cells (spores).

## INTRODUCTION

The acetone-butanol-ethanol (ABE) fermentation has potential commercial application, but the process economics are adversely affected by low reactor productivities and the problem of product inhibition. In recent years increasing attention has been paid to the application of novel fermentation technologies (e.g. cell immobilization, cell recycle techniques) to this fermentation process [1,7,12]. One of the justifications for these studies is that the attainment of a high cell density within the reactor will allow increased productivities to be achieved. It is often assumed in this approach that each individual cell within the reactor will perform at least as well under conditions of high cell density as under conditions of low cell density. Perusal of the literature, however, suggests that this may not necessarily be so. For example, during studies using a phosphate-limited chemostat, it was shown that at higher phosphate concentrations the fermentation became more solventogenic, suggesting that solventogenesis is favoured at high cell densities [2]. However, by calculation of specific production rates from the data provided, it appears that the reverse may be true, i.e. as the concentration of the limiting nutrient, and thus biomass, increases, so the specific rate of solvent production decreases. Similar results have been reported from another laboratory, using both nitrogen- and phosphate-limited chemostats [11]. Thus, there is evidence of an inverse relationship between the biomass concentration and the specific rate of solvent production, at least in chemostat culture.

In contrast, there are reports showing that in a nitrogen-limited chemostat, there is a direct relationship between the limiting nutrient concentration and the specific solvent production rate [14,15]. It has also been stated that under phosphate-limited conditions the maintenance of a suitably high biomass concentration is a prerequisite for solvent production [15]. Furthermore, from experiments in turbidostat culture, it has been reported that low cell densities favour acidogenesis, while high cell densities are required for solventogenesis [6].

The purpose of the present work was to reinvestigate the effect of cell density on specific solvent productivity. This effect is important in the development of novel fermentation technologies in that there may be little point in maximising the cell density within a reactor if each individual cell performs only poorly. Further, it is fundamental to fermentation studies that techniques be developed to maximise the performance of each individual cell within the reactor. A defined medium was used in which lactose was the sugar source. This sugar was chosen because of the potential use of whey permeate as a commercial substrate for the ABE fermentation process [10]. The technique of chemostat culture was used since this allows precise control of biomass concentration.

## MATERIALS AND METHODS

### *Culture*

*Clostridium acetobutylicum* P262 was obtained from Professor D.R. Woods (University of Cape Town, Re-

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public of South Africa), and was maintained as a spore suspension in distilled water at 4 °C.

*Medium*

The fermentation medium contained the following, per litre of distilled water: lactose, 50 g; NaCl, 1.0 g; cysteine HCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; biotin, 0.1 mg; thiamine HCl, 2.0 mg; *p*-aminobenzoic acid, 8.0 mg. For nitrogen-limited cultures, $K_2HPO_4$ was present at 0.15 g/l, and the concentration of ammonium acetate was varied as appropriate. For phosphate-limited cultures, ammonium acetate was present at 0.3 g/l, and the concentration of $K_2HPO_4$ was varied as appropriate. For all experiments, the vitamin solution was sterilised separately, by membrane filtration, and then added to the bulk medium.

*Fermentation*

A new fermentation was prepared for each steady state, using an inoculum prepared as described previously [9]. Experiments were performed in a Microferm fermenter (New Brunswick Scientific Co., New Brunswick, NJ), using a 2-l vessel with a working volume of 1.0 l. After autoclaving, the vessel, containing medium, was assembled onto the fermenter unit while still hot, and sterile oxygen-free nitrogen gas was flushed across the medium surface during cooling. This gas stream was maintained after inoculation until visible gassing due to bacterial growth was observed. After inoculation, fermentation proceeded under batch conditions at 34 °C, and the culture pH was controlled at pH 5.0 by automatic addition of 4M KOH. The medium used for this batch phase contained the limiting nutrient at a concentration twice that of its concentration in the feed medium during chemostat operation. After 22 h of batch culture, continuous feeding was commenced at a dilution rate of 0.056 $h^{-1}$, and the culture was controlled at pH 4.5. The culture was considered to be in a steady state when, after at least three residence times, successive samples taken over two residence times showed consistency in lactose, nitrogen, phosphate, solvent and acid concentrations in the culture fluid.

*Analyses*

Analyses were performed on the supernatant liquids of samples previously centrifuged at 10000 rpm for 10 min. Solvents and acids were determined by gas chromatography as described previously [12], and lactose by high performance liquid chromatography [4]. Phosphate was determined using the Molybdenum Blue method [16], and ammonium ion by a semi-micro Kjeldahl method without prior digestion.

Cell mass was determined by estimating the nitrogen or phosphate content of washed cells, and multiplying by the appropriate factor. The nitrogen content of the cells was determined as 6.0–6.5%, and the phosphate content as 4.5–5.0%, on a dry basis.

## RESULTS

Preliminary experiments had established that a dilution rate of 0.056 $h^{-1}$ and a culture pH value of pH 4.5 were suitable to allow both solvent and acid production to occur. Hence these parameters were kept constant in all experiments.

A series of experiments was performed to study the effect of nitrogen limitation on solvent production, and the results are recorded in Table 1 (data for acetone/ethanol and acetate are not provided as they followed similar trends to butanol and butyrate, respectively). For volumetric rates, the results were as expected. Thus, as the concentration of the limiting nutrient, and hence biomass concentration, was increased, so the rates of lactose uptake and butanol and butyrate production increased. In terms of *specific* productivities, however, there was an inverse relationship between biomass concentration and the appropriate uptake and production rates.

Similar experiments were performed in a phosphate-limited chemostat, and comparable results were observed (Table 2). Thus, although the volumetric rates increased with increasing biomass concentration, the specific rates displayed a decrease.

## DISCUSSION

This work has attempted to assess the effect of varying cell density on the specific butanol productivity of *C. acetobutylicum*. The reason for the study was to gather fundamental information which can be used in the development of novel fermentation technologies which often rely for their effectiveness on the achievement of high cell densities. The experimental technique used was chemostat culture. The reason for using this technique rather than turbidostat culture was that it allows precise control of biomass concentration.

The results demonstrate that there is an inverse relationship between biomass concentration and specific productivity. There are two possible explanations for this effect. First, each individual cell in the population may perform equally less well as the total biomass increases, perhaps due to competition for nutrients. Secondly, the population within the reactor may not be homogeneous, i.e. not all of the cells are actively solventogenic, and the proportions of the different cell types may vary with the biomass concentration. This latter hypothesis has been postulated previously [3,5,13], and has been supported by

TABLE 1

Steady state fermentation parameters during nitrogen-limited chemostat experiments

| | Run No. | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Initial ammonium (mg/l)[a] | 46 | 104 | 185 | 300 |
| Ammonium utilized (mg/l)[a] | 46 | 104 | 185 | 243 |
| Cell mass (g/l) | 0.13 | 0.30 | 0.53 | 0.71 |
| Lactose uptake rate (mg/l h) | 200 | 310 | 390 | 420 |
| Butanol production rate (mg/l h) | 4.5 | 6.7 | 7.8 | 6.7 |
| Butyrate production rate (mg/l h) | 15.1 | 22.3 | 31.9 | 29.7 |
| Lactose uptake rate (g/g cell h) | 1.5 | 1.0 | 0.7 | 0.6 |
| Butanol production rate (mg/g cell h) | 34.6 | 22.3 | 14.7 | 9.4 |
| Butyrate production rate (mg/g cell h) | 116 | 74 | 60 | 42 |

[a] Expressed as ammonium acetate.

TABLE 2

Steady state fermentation parameters during phosphate-limited chemostat experiments

| | Run No. | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Initial phosphate (mg/l)[a] | 22 | 37 | 59 | 94 |
| Phosphate utilized (mg/l)[a] | 22 | 37 | 59 | 81 |
| Cell mass (g/l) | 0.26 | 0.43 | 0.68 | 0.94 |
| Lactose uptake rate (mg/l h) | 184 | 314 | 309 | 340 |
| Butanol production rate (mg/l h) | 6.2 | 5.7 | 6.9 | 9.0 |
| Butyrate production rate (mg/l h) | 22.3 | 24.2 | 23.8 | 28.4 |
| Lactose uptake rate (g/g cell h) | 0.70 | 0.73 | 0.45 | 0.36 |
| Butanol production rate (mg/g cell h) | 23.8 | 13.3 | 10.1 | 9.6 |
| Butyrate production rate (mg/g cell h) | 86 | 56 | 35 | 30 |

[a] Expressed as $K_2HPO_4$.

microscopical examination of the cultures, which reveals the presence of three types of cells. These are, first, actively growing, vegetative, acidogenic cells; secondly, non-growing, clostridial, solventogenic forms; thirdly, inert spores. The exact determinants governing the interconversions of these cell types are not known, but it is now well-established that both sporulation and solventogenesis are strongly influenced by the *balance* of nutrients, and this can also influence the total biomass concentration [8,9]. In the present work, microscopical examination of the cultures was not performed, but it can be postulated that the observed decrease in specific butanol productivity with increasing biomass concentration is due to a shift in the proportions of different cell types caused by changes in the nutrient balance. The apparently contradictory results described by other workers [6,14,15] are probably caused by the same effect. The problem remains, however, of the exact nature of the determinants responsible for interconversion of the cell types. A target for further study will be to identify those factors which favour the production of the non-growing, clostridial, solventogenic cell types. In the longer term, it is hoped that

such information can be applied to novel fermentation technologies, and so allow the development of stable and productive reactor systems.

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