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Effect of phosphate and ammonium ion concentrations on solvent production in defined medium by *Clostridium acetobutylicum*

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SUMMARY

Experiments have been performed in batch fermentation, using a defined medium, to investigate the effects of phosphate and ammonium ion concentrations on solvent production using *Clostridium acetobutylicum*. Solvent production occurred under conditions of either ammonium- or phosphate-limitation, but the optimum conditions were observed to be where both of these nutrients were slightly in excess of growth requirements. A greater excess of nutrients caused the fermentation to be acidogenic rather than solventogenic.

INTRODUCTION

The acetone-butanol-ethanol (ABE) fermentation has become of increasing interest in recent years, and several review articles have been published [3,5,6]. At present, however, the process is considered to be uneconomic, the major reasons being the low reactor productivities which can be attained, and the problem of product inhibition which contributes to the high cost of product recovery (usually by distillation). Various approaches have been adopted to improve the fermentation process (e.g. continuous culture, use of immobilized cells, cell recycle), and integrated fermentation/product recovery processes have also been described (e.g. continuous culture coupled with liquid–liquid extraction, pervaporation, gas-stripping). An assessment of these approaches has been presented [3]. Despite the improvements in reactor productivity that have been achieved using these novel fermentation technologies, it is still possible that any commercial implementation of the ABE process will use traditional batch fermentation. This is because the novel technologies have not yet found any major industrial application for any fermentation product, and so little expertise is available for large-scale operation.

The batch ABE fermentation process has been described by many authors. However, although the

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initial nutrient concentrations are usually described, the nutritional status of the medium during and after solventogenesis is often not mentioned. For commercial operation in the past, it has been stated that all nutrients are generally present in excess [5], but it is not clear whether this is the optimum situation. Further, there are conflicting reports concerning the optimum nitrogen status of the medium. For example, one report has demonstrated that under conditions of nitrogen limitation, strong solventogenesis occurred after exhaustion of nitrogen from the medium [9]. In contrast, other workers have shown that insignificant solvent production occurred unless there was a minimum nitrogen concentration remaining in the medium after the growth phase was completed [7]. It has also been suggested that an excess of nitrogen is detrimental to solvent production, and that as the ratio of initial nitrogen to initial glucose decreases, the rate of solvent production increases [11]. With regard to phosphate levels, it has been reported that acids, rather than solvents, are produced under conditions of excess phosphate, while solvents are produced only after phosphate exhaustion from the medium [1].

The purpose of the present work was to investigate the effects of the nitrogen (ammonium ion) and phosphate concentrations, in a batch fermentation medium, on solventogenesis. A defined medium was used, in which lactose was used as the sugar source. This sugar was chosen because of the potential use of whey permeate as a commercial substrate for the ABE fermentation process [8].

MATERIAL AND METHODS

Culture

Clostridium acetobutylicum P262 was obtained from Professor D.R. Woods (University of Cape Town, 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, 60 g; L-cysteine hydrochloride, 1 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, 1 mg; thiamine hydrochloride, 0.02 g; *p*-aminobenzoic acid, 0.02 g. For experiments where the initial phosphate (K_2HPO_4) concentration was varied, ammonium acetate was present at 2 g/l. For experiments where the initial nitrogen (ammonium sulfate) concentration was varied, K_2HPO_4 was present at 0.4 g/l. For all experiments, the vitamin solution was sterilised separately, by membrane filtration, and then added to the medium prior to inoculation.

Inoculum preparation

Spore suspension (0.15 ml) was inoculated into Cooked Meat Medium (20 ml, Difco Laboratories, Detroit, MI) supplemented with lactose (10 g/l), and was heat-shocked at 75°C for two min. The culture was incubated anaerobically at 34°C until highly motile cells were observed (usually 17–22 h). Two ml of culture were then transferred to 100 ml of synthetic medium (identical to that to be used in the main experimental fermentation), and incubated at 34°C for approximately 18 h. A portion of this culture was then used to inoculate the main fermentation (3–4% (v/v) inoculum).

Fermentation

Fermentations were performed in a Microferm fermenter (New Brunswick Scientific Co., New Brunswick, NJ), using a 2-litre vessel with a working volume of 1.5 l. After autoclaving, the vessel, containing medium, was assembled on to 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. Fermentation proceeded at 34°C, and the culture pH was controlled at pH 5.5 by automatic addition of 3 M KOH. The culture was not agitated, except immediately prior to withdrawal of a sample.

Analyses

Analyses were performed on the supernatant liquids of samples previously centrifuged at 10 000 rpm for 10 min. Solvents and acids were determined

Table 1

Effect of ammonium ion concentration on fermentation parameters, after five days of fermentation

	Run no.					
	I	II	III	IV	V	VI
Initial ammonium (mg/l)[b]	500	650	1000	1200	1500	2000
Ammonium utilized (mg/l)[b]	500	650	1000	1200	1450	1450
Phosphate utilized (mg/l)[a]	140	185	222	250	280	260
Maximum OD_{625}	1.20	1.83	2.16	2.16	2.30	2.50
Total solvents (g/l)	2.33	2.55	5.88	5.33	7.26	4.95
Total acids (g/l)	4.26	4.46	5.23	5.35	5.12	8.33
Lactose utilized (g/l)	22.92	26.55	26.97	24.35	32.85	26.70
Total solvent yield (g solvent/g lactose utilized)	0.10	0.10	0.22	0.22	0.22	0.18
Max. observed butanol production rate (g/l · h)	0.033	0.056	0.092	0.283	0.312	0.169
Max. observed specific butanol production rate (g/g biomass ammonium · h)	0.07	0.09	0.09	0.24	0.21	0.12

[a] Initial concentration 400 mg/l (expressed as K_2HPO_4).

[b] Expressed as ammonium sulfate.

Table 2

Effect of phosphate ion concentration on fermentation parameters, after five days of fermentation

	Run no.							
	VII	VIII	IX	X	XI	XII	XIII	XIV
Initial phosphate (mg/l)[b]	20	100	150	200	250	400	600	1000
Phosphate utilized (mg/l)[b]	20	100	150	200	200	280	300	340
Ammonium utilized (mg/l)[a]	390	1430	1450	1790	1560	1700	1610	1540
Maximum OD_{625}	1.05	1.46	2.22	2.20	2.35	2.0	2.36	2.12
Total solvents (g/l)	4.82	5.76	8.93	8.95	12.00	8.73	4.25	5.30
Total acids (g/l)	1.04	4.15	2.59	3.3	1.30	2.78	8.0	8.63
Lactose utilized (g/l)	23.18	29.80	32.50	34.50	38.81	29.55	25.15	25.59
Total solvent yield (g solvent/g lactose utilized)	0.21	0.20	0.27	0.26	0.31	0.29	0.17	0.20
Max. observed butanol production rate (g/l · h)	0.015	0.112	0.137	0.262	0.233	0.143	0.100	0.100
Max. observed specific butanol production rate (g/g biomass phosphate · h)	0.75	1.12	0.91	1.31	1.16	0.51	0.33	0.29

[a] Initial concentration 2000 mg/l (expressed as ammonium acetate).

[b] Expressed as K_2HPO_4.

by gas chromatography as described previously [10], and lactose by high-performance liquid chromatography [2]. Phosphate was determined using the Molybdenum Blue method [12], and ammonium ion by a semi-micro Kjeldahl method, without prior digestion.

RESULTS

A series of batch fermentations was performed whereby the initial ammonium ion concentration of the medium was varied, while all other nutrients were maintained constant. The results of these experiments are summarised in Table 1. In Runs I to IV, the nitrogen nutrient was exhausted from the medium, while in Runs V and VI all nutrients remained in excess. The results show that as the initial concentration of the growth-limiting nutrient increased, elevated concentrations of total solvents (acetone + ethanol + butanol) were observed, until an excess of all nutrients led to a decrease in solvent production. Thus, maximum solvent production occurred when there was a slight excess of nitrogen beyond that required for growth. As expected, increases were observed in the extent of lactose utilisation and the volumetric butanol production rate (g/l · h) as the amount of biomass present increased. Surprisingly, perhaps, the specific butanol production rate, based on ammonium ion uptake, also showed an increase with increased biomass, and then decreased as the nitrogen nutrient became in excess. The total solvent yield, based on lactose utilised, followed the same trend. Under conditions of nitrogen excess, the fermentation became acidogenic rather than solventogenic.

Similar experiments were performed in which the initial phosphate concentration of the medium was varied while all other nutrients were maintained constant (Table 2). In Runs VII to X, phosphate was the growth-limiting nutrient, while in the other Runs all nutrients were present in excess. Similar trends to those reported in Table 1 were observed. Thus, increased solvent production was observed as the concentration of the growth-limiting nutrient was increased, and maximum production occurred

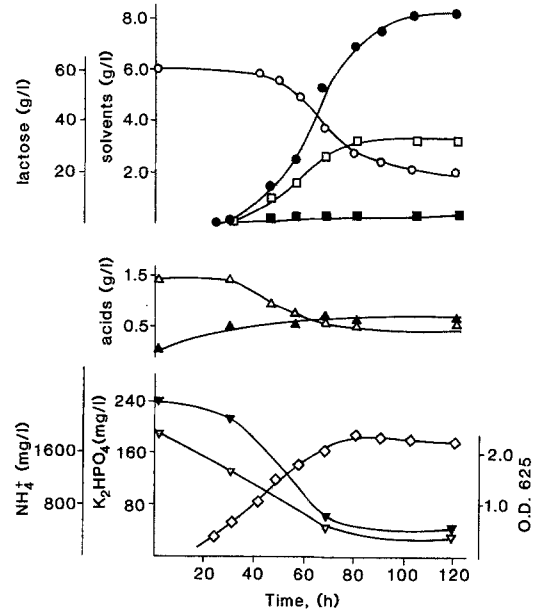

Fig. 1. Progress of fermentation Run XI. Experimental conditions are as described in the text. ○, lactose; ●, butanol; □, acetone; ■, ethanol; △, acetic acid; ▲, butyric acid; ▽, ammonium (expressed as ammonium sulfate); ▼, phosphate (expressed as K_2HPO_4); ◇, OD_{625} of culture.

when the initial phosphate concentration was in slight excess of that required for growth. As the phosphate concentration was increased further (Runs XII to XIV) the fermentation became acidogenic rather than solventogenic. Similar maxima are apparent for the data describing the solvent yield, lactose utilisation and the butanol production rate.

The experimental Run where the highest concentration of total solvents was observed was Run XI. The progress of this fermentation is shown in Fig. 1.

DISCUSSION

The purpose of this work was to determine the optimum concentrations of nitrogen and phosphate in a defined medium for solvent production by batch fermentation. The study was stimulated by the need to know whether any potential commercial substrate could be improved by the addition or removal of these nutrients, and by the apparent con-

tradictions which are in the literature. The results of the study show that solvent production can occur under conditions of either nitrogen- or phosphate-limitation, but that the optimum conditions are where the nitrogen and phosphate concentrations are just sufficient for growth, but are not growth-limiting. An excess of nutrients leads to conditions becoming acidogenic rather than solventogenic. Similar results with regard to the nitrogen nutrient have been observed previously [13], i.e. the optimum condition for solvent production occurs when the supply is just in excess of that required for growth. We believe that the present results will help to explain the apparent contradictions present in the literature.

An interesting observation from this work is the result showing that under conditions of nutrient-limitation, an increased biomass concentration leads to increased solventogenesis (refer to data for specific butanol production rate in Runs I to V and VII to X). Similar results have been reported previously [4], but the phenomenon has received little further study, despite its potential application to novel fermentation technologies.

The biochemical basis for the present results is not clear, but may involve mechanisms of catabolite regulation. In practical terms, however, it is clear that the balance of nutrients is important, and may require investigation for each particular commercial substrate.

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