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Effect of controlled substrate feeding on butyric acid production by *Clostridium tyrobutyricum*

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SUMMARY

Production of butyric acid from wheat flour hydrolysate was studied with *Clostridium tyrobutyricum*. The mode of substrate supply was found a key parameter for fermentation performance as large improvements were obtained by feeding with a non-limiting supply of substrate. With this procedure, increases in product concentration and productivity but also in selectivity and yield for butyrate were obtained. Substrate feeding controlled by the rate of gas production was found preferable to constant rate feeding for reason of convenience and flexibility. In these conditions, a butyrate concentration of 62.8 g l^{-1} was obtained with a productivity of 1.25 g l^{-1} h^{-1}, a selectivity of 91.5% and a yield of 0.45 g per g of glucose.

INTRODUCTION

Butyric acid and some of its esters are used in the food and perfumes industries, because of their aromatic properties. Many processes have been proposed for butyrate production by fermentation during the first half of this century [14,15]. In general, the most studied butyrate-producing microorganisms belonged to the genus *Clostridium*. The utilization of a large range of fermentation substrates including hydrolysates of waste cellulosic material [8,9], lactose from whey [7], marine algae [5], molasses [2,10], cellulosic materials [6] has been described. Nevertheless, fermentation processes have not been used commercially because of the low concentration of butyric acid in the fermentation mash (20–30 g l^{-1}) and because of the acetate production which was produced simultaneously with butyrate.

Presently, butyric acid is produced entirely by the petrochemical way. However, the use of butyric acid or its esters as additives, in particular in food industry, makes its origin not indifferent as consumers preferences are for natural products.

The aim of our work was to define a fermentation process on wheat flour hydrolysate leading to a mash with a high concentration of butyrate while minimizing the concentration of acetate.

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MATERIALS AND METHODS

Microorganisms. Clostridium tyrobutyricum used in the present work was isolated in the laboratory from sheep rumen. It was deposited at the culture collection of Institut Pasteur, Paris under the number CIP I-776. It was maintained on the potato medium described by Calam [4].

Culture medium. The carbon source was wheat flour hydrolysate (380 g l^{-1} of glucose) obtained by enzymatic liquefaction and saccharification of wheat flour using commercial enzymes (Novo, Denmark) according to the prescriptions of the manufacturer. The hydrolysate was then centrifuged to eliminate gluten.

The fermentation broth contained the following supplementations: yeast extract (Biomérieux, France), 5 g l^{-1} (except mentioned otherwise); $FeSO_4 \cdot 7\ H_2O$, 30 mg l^{-1}; KH_2PO_4, 1.5 g l^{-1}; $MgSO_4 \cdot 7\ H_2O$, 0.6 g l^{-1}; $(NH_4)_2SO_4$, 1 g l^{-1}. Media were sterilized at 110 °C for 40 min.

Fermentation set-up. A 6-liter fermentor (Biolafitte, France) equipped with pH regulation was used, it was connected to a volumetric gas meter (1 dm^3), FLONIC, Montrouge, France). This gas meter was equipped with devices triggering data acquisition (pH, gas volume, gas production rate) on an Apple II microcomputer (Apple, Cuppertino, U.S.A.) for each liter of gas produced in the reactor [1]. The microcomputer could also control a substrate feeding pump (Minipuls 3, Gilson, France) in response to the gas production rate via a RS-422A interface card (Gilson, Middleton, U.S.A.).

Fermentation modes. Preculture was carried out in an anaerobic chamber at 34 °C for 18 h on 200 ml of culture medium containing 30 g l^{-1} of glucose, in an erlenmeyer flask fitted with a side tube which was connected to the fermentor during preculture transfer. Anaerobiosis was obtained by flushing the fermentor headspace with N_2 until culture was inoculated. The culture was operated at 34 °C under low stirring (300 rpm) and pH was regulated at 6.5 by addition of 5 N NH_4OH. During the fermentation, performed at atmospheric pressure, the gas produced was circulated through a gas-washing bubbler.

Three modes of substrate supply were tested:

(1) Batch culture: the wheat flour hydrolysate was diluted to obtain a glucose concentration of 130 g l^{-1} in a 4-l working volume.

(2) Constant rate fed-batch culture: the wheat flour hydrolysate was diluted to obtain an initial glucose concentration lower than 10 g l^{-1} in a working volume of 2.3 l. Additional substrate was provided directly from the wheat flour hydrolysate at a constant addition rate of 20 g of glucose per hour.

(3) Controlled fed-batch culture: continuous feeding of substrate was controlled by the rate of gas production according to a defined ratio of 3 g of glucose injected by liter of gas produced. Feeding was automatically started and stopped after production of a defined gas volume.

Analytical assays. Acetate and butyrate were determined on centrifuged samples using a gas chromatograph (Girdel, France), on a 2 m column (Porapak Q) as previously described [11]. Glucose was determined with the glucose dehydrogenase method [3] using a commercial kit, (Boehringer, Mannheim, F.R.G.).

Fermentation parameters. Yield for butyrate is defined as the ratio (w/w) of butyrate produced to glucose utilized. The overall volumetric productivity (g l^{-1} h^{-1}) for butyrate is expressed as the ratio of final butyrate concentration to the fermentation time. Selectivity for butyrate is defined as the weight percentage of butyrate to the sum acetate plus butyrate.

RESULTS

Batch fermentation

Clostridium tyrobutyricum CIP I-776, used in the present study had been selected in preliminary work involving 62 *Clostridium* strains on the criteria of selectivity and productivity for butyrate and of final product concentration in batch cultures with glucose as the carbon source. The time course of a batch culture on wheat flour hydrolyzate at an initial glucose concentration of 130 g l^{-1} is presented in Fig. 1. Due to an initial lag time of 30 hours the productivity for butyrate was low as shown in Table 1 which presents the fermentation characteristics in particular the main criteria of overall performance, productivity, product concentration and selectivity.

Fed-batch fermentation with constant rate substrate supply

Batch cultures carried out at lower initial glucose concentrations did not exhibit important lag periods. For this reason, fed-batch supply of the substrate was varied. The kinetics of a fed-batch fermentation with a non limiting constant rate supply of substrate is presented in Fig. 2. Substrate feeding was started when initial glucose was partially utilized. No initial lag time was observed. Glucose concentration in the fermentor was not limiting but did not exceed 30 g l^{-1}. A remarkable difference with the batch operation was that acetate which reached 4.1 g l^{-1}, (total amount 17.9 g), decreased to 2.1 g l^{-1} at the end of the fermentation (total amount 9.6 g). The lower final concentration of acetate implied acetate reutilization and did not only result from a simple dilution effect due to the increase of the fermentation volume as shown by the decrease in the total amount of acetate. The results shown in Table 1 demonstrate also an important improvement in all criteria of fermentation performance productivity, butyrate concentration, selectivity (and yield) with respect to the batch fermentation.

Fed-batch fermentation with controlled substrate supply

As substrate supply by constant rate feeding required prior knowledge of the proper rate to use and determination of the time to start and to stop substrate supply, a more flexible mode of feeding in which the rate of substrate injection could be controlled by a parameter reflecting the fermentation velocity was devised. The gas pro-

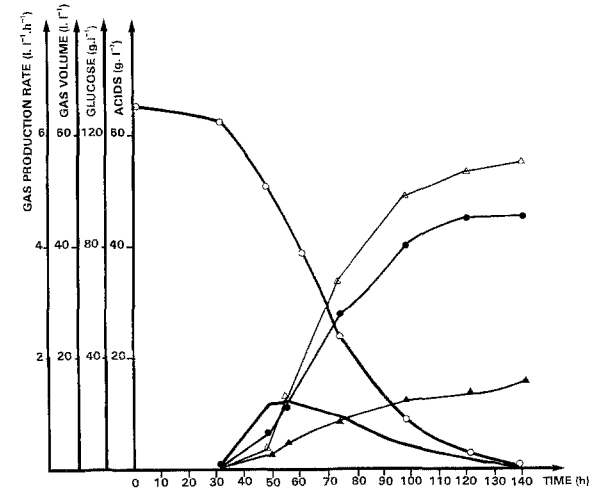

Fig. 1. Batch culture of *C. tyrobutyricum*. (○———○) Residual glucose concentration; (△———△) gas volume; (———) gas production rate; (●———●) butyrate; (▲———▲) acetate.

TABLE 1

Butyric fermentation performance[a] with *C. tyrobutyricum* using different modes of substrate supply

Modes of substrate supply	Butyrate ($g\ l^{-1}$)	Acetate ($g\ l^{-1}$)	Butyrate yield ($g\ g^{-1}$)	Butyrate productivity ($g\ l^{-1}\ h^{-1}$)	Selectivity for butyrate (%)
Batch[c]	45	15	0.34	0.32	75
Fed-batch at constant feeding rate[d]	55.3	2.1 (4.1)[b]	0.44	1.17	96.3
Fed-batch at feeding rate controlled by gas production rate[e]	62.8	5.8 (7.9)[b]	0.45	1.25	91.5
Fed-batch at feeding rate controlled by gas production rate[f]	72	2.4 (3.7)[b]	0.48	0.6	96.7

[a] Figures refer to final fermentation values except where indicated. [b] Maximal values observed during fermentation. [c] See Fig. 1. [d] See Fig. 2. [e] See Fig. 3. [f] See Fig. 4.

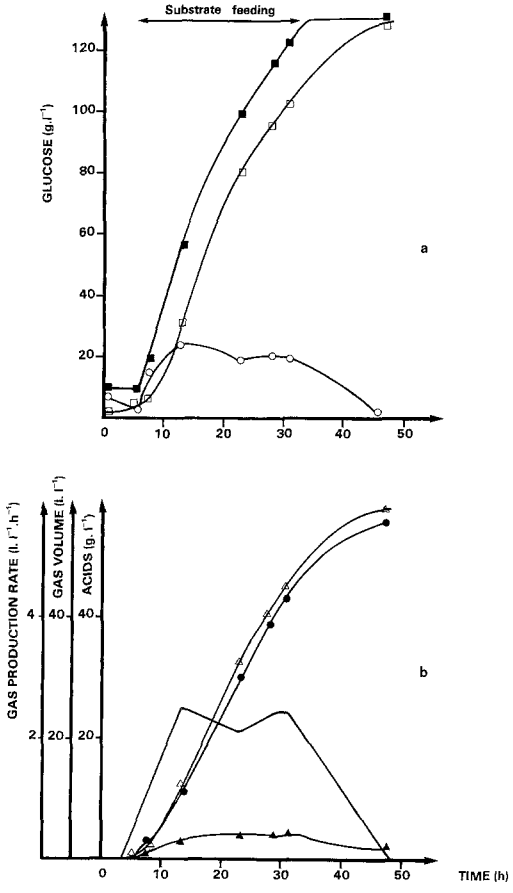

Fig. 2. Fed-batch culture of *C. tyrobutyricum* at constant feeding rate. Initial glucose was $6.8\ g\ l^{-1}$. Glucose feeding was realized at a constante rate of 20 g per h. (a) Glucose supplied (■———■), glucose utilized (□———□), residual glucose (○———○). (b) Gas volume (△———△), gas production rate (———), butyrate (●———●), acetate (▲———▲).

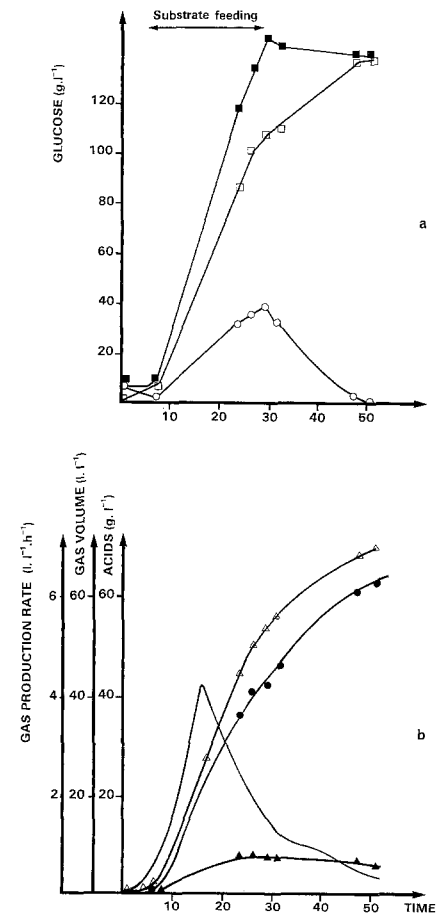

Fig. 3. Fed-batch culture of *C. tyrobutyricum* with glucose feeding rate controlled by gas production. Initial glucose was $6\ g\ l^{-1}$. (a) Glucose supplied (■———■), glucose utilized (□———□), residual glucose (○———○). (b) Gas volume (△———△), gas production rate (———), butyrate (●———●), acetate (▲———▲).

TABLE 2

Influence of feeding end point on fermentation performance[a]

Total gas production commanding feeding end (l)	Residual glucose ($g\ l^{-1}$)	Fermentation time (h)	Butyric acid ($g\ l^{-1}$)	Acetic acid ($g\ l^{-1}$)	Glucose utilized ($g\ l^{-1}$)	Gas volume ($l\ l^{-1}$)
200	0	47.5	53.4	4.9 (6.0)[b]	134.7	64.3
236	0	50	62.8	5.8 (7.9)[b]	139.8	69.2
270	25	63	53.5	4.3 (7.2)[b]	123.4	60.4

[a,b] As in Table 1.

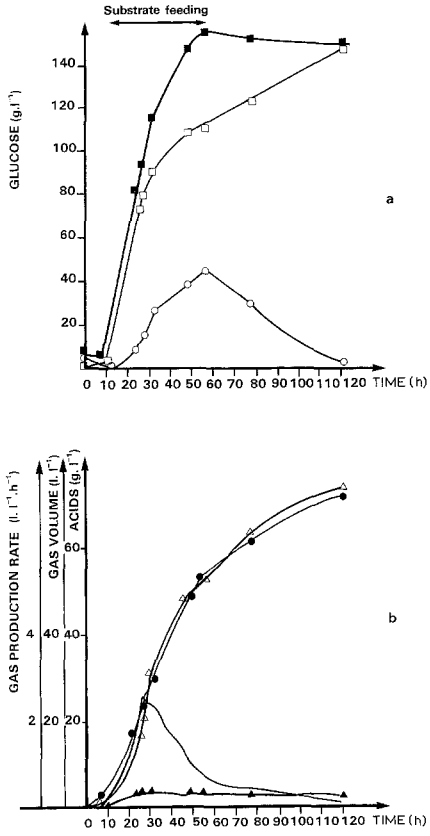

Fig. 4. Fed-batch culture of *C. tyrobutyricum* with glucose feeding rate controlled by gas production in a corn-steep supplemented medium. Medium was supplemented with 5 $g\ l^{-1}$ powdered corn-steep liquor (Roquette, France) instead of yeast extract. Initial glucose was 4.2 $g\ l^{-1}$. (a) Glucose supplied (■———■), glucose utilized (□———□), residual glucose (○———○). (b) Gas volume (△———△), gas production rate (————), butyrate (●———●), acetate (▲———▲).

duction rate which was recorded for every liter of gas produced was chosen for this purpose. The substrate feeding pump was connected to the volumetric gas meter via a microcomputer so that the rate of continuous glucose feeding was readjusted at every liter of gas produced. The ratio of 3.0 g of glucose per liter of gas produced allowed a non-limiting substrate supply. Substrate injection was automatically started after production of 4 l of gas and stopped after production of 236 l of gas for the whole reactor. The time course of a fed-batch culture in these conditions is presented in Fig. 3. As in the previous experiment, glucose concentration was non-limiting but did not exceed 40 $g\ l^{-1}$ and no lag time was observed. As shown in Table 2 feeding interruption after production of 236 l of gas allowed optimal production of butyrate while insuring satisfactory exhaustion of glucose. Feeding interruption at other values of gas volumes (200 l or 270 l) lowered the final performance either because of glucose limitation or because of inhibition by excess glucose. Accumulation of acetate and subsequent reutilization was also observed in these experiments. It is apparent that butyrate production in optimal conditions was better in the case of controlled substrate supply than with a constant rate of feeding (see Table 1).

The flexibility of substrate feeding controlled by gas production was tested in fermentation optimization work involving changes in culture conditions and in fermentation kinetics. Fig. 4 illustrates a fermentation where corn steep liquor was tested as supplementation instead of yeast extract. The fermentation pattern observed in previous fed-batch experiments (absence of a lag period and of excessive accumulation of glucose, acetate reutilization) was again apparent but the fermentation time was increased. However, controlling substrate supply by gas production allowed to adjust it to the require-

ments of a different fermentation kinetics. The results of Table 1 also show that in spite of a lower productivity, supplementation with corn steep liquor, which lowers the cost of the fermentation medium, led to satisfactory results regarding product concentration and selectivity.

DISCUSSION

As shown in the results presented above, fed-batch supply of substrate greatly increased the performance of butyrate production. This procedure relieved the inhibition by excess substrate which took place in batch fermentation as shown by the suppression of the initial lag phase of the culture. In these conditions, both productivity for butyrate and final product concentration increased.

A further positive effect of fed-batch supply was the higher selectivity of butyrate production which was observed in these conditions. This higher selectivity resulted in particular of acetate reutilization in confirmation of results previously obtained with another strain using glucose as the carbon source [12]. However, in the present study the mode of fed-batch supply was important for optimal operation. When constant rate feeding was used, good performance could be achieved as shown above with a non-limiting supply of substrate but the technique was somewhat difficult to control because it is not self-adjusting. In some experiments excessive substrate accumulation was observed when, due to inoculum variability, the fermentation started more slowly than expected. Other experiments, not detailed above, showed that glucose-limited fed-batch supply, which is self-adjusting, while promoting selectivity of butyrate production, was less satisfactory in terms of final product concentration.

The best compromise was obtained with substrate feeding controlled by the gas production rate and by total gas production. This mode of substrate feeding yielded the highest butyrate concentrations. It also provided better reliability and convenience than constant rate feeding as glucose supply automatically adjusted to different fermentation velocities whether due to the physiological state of the inoculum or to a different composition of the fermentation medium as illustrated in the case of the fermentation with corn steep liquor. In fact, the overall performance obtained clearly exceeds the values reported in the literature [13].

The results presented above constitute a good example of the improvements that can be obtained through piloting a key parameter of the fermentation process by a suitable sensor of metabolic activity. The results also show that recording of total gas production can readily be used for this purpose. Being simple and convenient, it deserves more widespread use in gas-evolving anaerobic fermentations.

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