Parameters Affecting Solvent Production by Clostridium pasteurianum

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The effect of pH, growth rate, phosphate and iron limitation, carbon monoxide, and carbon source on product formation by Clostridium pasteurianum was determined. Under phosphate limitation, glucose was fermented almost exclusively to acetate and butyrate independently of the pH and growth rate. Iron limitation caused lactate production (38 mol/100 mol) from glucose in batch and continuous culture. At 15% (vol/vol) carbon monoxide in the atmosphere, glucose was fermented to ethanol (24 mol/100 mol), lactate (32 mol/100 mol), and butanol (36 mol/100 mol) in addition to the usual products, acetate (38 mol/100 mol) and butyrate (17 mol/100 mol). During glycerol fermentation, a completely different product pattern was found. In continuous culture under phosphate limitation, acetate and butyrate were produced only in trace amounts, whereas ethanol (30 mol/100 mol), butanol (18 mol/100 mol), and 1,3-propanediol (18 mol/100 mol) were the major products. Under iron limitation, the ratio of these products could be changed in favor of 1,3-propanediol (34 mol/100 mol). In addition, lactate was produced in significant amounts (25 mol/100 mol). The tolerance of C. pasteurianum to glycerol was remarkably high; growth was not inhibited by glycerol concentrations up to 17% (wt/vol). Increasing glycerol concentrations favored the production of 1,3-propanediol.

The conversion of renewable biomass to neutral fuels and solvents may be an important supplement to chemically synthesized products in the future. Some species of the genus Clostridium are known for their ability to produce solvents such as acetone, butanol, and ethanol. Clostridium acetobutylicum is one of the best-known members of this group, and the acetone-butanol fermentation carried out by this microorganism was the basis for production of these solvents on an industrial scale for a long period of time (reviewed in references 7 and 26).

Clostridium pasteurianum, on the other hand, is known as a classical acid producer and usually ferments carbohydrates to butyrate, acetate, carbon dioxide, and molecular hydrogen (19). However, there are some recent reports on solvent production by C. pasteurianum. When grown in media of high glucose concentrations (e.g., 12.5% [wt/vol]), significant quantities of acetone, butanol, and ethanol are produced (21). Furthermore, an algal biomass mixture supplemented with 4% (wt/vol) glycerol is converted by C. pasteurianum to butanol, 1,3-propanediol, and ethanol (36).

In a minimal medium with glycerol as the sole carbon and energy source, C. pasteurianum ferments more than half of the substrate supplied to butanol (22). These results clearly indicate that C. pasteurianum has the ability to switch from acid to solvent production under certain growth conditions, similar to C. acetobutylicum. However, these conditions have not been determined in detail, and it is not clear whether the growth parameters which have been shown to favor solvent production in C. acetobutylicum (6, 8, 30, 34) influence the regulation of metabolism in C. pasteurianum in a comparable fashion. Therefore, several of these conditions (low pH, phosphate and iron limitation, and carbon monoxide) were applied to C. pasteurianum and their effect on product formation was determined during glucose and glycerol fermentation in batch and continuous culture.

MATERIALS AND METHODS

Organism and growth conditions. The organism used was a spontaneous asporogenous mutant of C. pasteurianum DSM 525, repeatedly selected during continuous cultivation in a phosphate-limited chemostat, as described for C. acetobutylicum by Meinecke et al. (32). The asporogenous strain had the same product pattern as the wild type in batch culture under different conditions. Stock cultures were maintained on chopped-meat medium (23) supplemented with 20 g of glucose per liter.

The standard minimal medium for batch cultures contained the following ingredients in 1 liter of distilled water (modified from reference 29): K2HPO4, 1.74 g; NH4Cl, 0.66 g; MgSO4 - 7H2O, 0.251 g; KCl, 0.596 g; Fe-Na-EDTA, 69 mg; NaHCO3, 6 g; p-aminobenzoic acid, 4 mg; biotin, 0.24 mg; yeast extract, 0.5 g; resazurin, 1 mg; cysteine-HCl, 0.5 g; and glucose · H2O, 20 g, or glycerol, 20 to 200 g, as indicated. The pH was adjusted to 7.0 with KOH. Glucose and glycerol were sterilized separately as anaerobic stock solutions and added to the autoclaved medium. The gas phase contained 80% (vol/vol) N2 and 20% (vol/vol) CO2. For experiments with CO, the carbonate buffer was substituted by a sodium phosphate buffer (50 mM) and the gas phase contained only N2 in addition to CO (0 to 15%, vol/vol). Since the solubility of CO in the medium is low, a liquid-to-gas ratio of 1:13 (vol/vol) was chosen, and the cultures were shaken on a rotary shaker (160 rpm). For batch culture experiments under iron limitation, the composition of the standard minimal medium was changed as follows: no yeast extract or Fe-Na-EDTA was added, and the medium was supplemented with FeSO4 · 7H2O, as indicated in Results.

The standard minimal medium was also used for the phosphate-limited chemostat experiments, except that yeast extract, cysteine-HCl, and NaHCO3 were omitted, 35 mg of Na2S2O4 was added, only 0.087 g of K2HPO4 (0.5 mM) was used, and 40 g of glucose · H2O or glycerol was added. To obtain an iron-limited medium, the phosphate-limited me-
dium was changed as follows: no Fe-Na-EDTA, 0.435 g of K$_2$HPO$_4$, and 0.47 to 2.77 mg of FeSO$_4$·7H$_2$O were added per liter. The media were sterilized by filtration through a Seitz filter. EKS size 14 cm, with an N$_2$ pressures of 3 × 10$^8$ Pa. The continuous culture experiments were performed in a Biostat M fermentor (Braun, Melsungen, Federal Republic of Germany [FRG]) with a 1-liter working volume as described previously (5).

Optical density. The OD$_{578}$ was measured in a PM 4 spectrophotometer (Zeiss, Oberkochen, FRG). The light path was 1 cm. Samples containing resazurin were decolorized with Na$_2$S$_2$O$_4$.

Determination of substrates and products. Glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase system (Boehringer GmbH, Mannheim, FRG). Glycerol was determined as described by Eggstein and Kuhlmann (14). The concentration of phosphate was measured colorimetrically in the molybdate-vanadate complex (Boehringer). Iron was determined as the bathophenanthroline complex (Boehringer).

l- and d-lactate were determined enzymatically as described by Bergmeyer (9). l- and d-lactate were produced in nearly equal quantities; therefore, total lactate concentrations are given. Other fermentation products such as acetate, ethanol, butyrate, butanol, and 1,3-propanediol were determined by gas chromatography as described previously (4). H$_2$ was determined as described by Deppenmeier et al. (13).

RESULTS

Effect of pH and growth rate on glucose fermentation under phosphate limitation. C. pasteurianum was grown in continuous culture under phosphate limitation at different pH values and growth rates. These conditions were chosen at the beginning of our investigations, since they allow C. acetobutylicum to modulate product formation at will: at pHs above 5 and high growth rates (e.g., 1.5 h$^{-1}$), butyrate and acetate are the major products, whereas at pHs below 5 and at low growth rates (e.g., 0.05 h$^{-1}$), acetone and butanol dominate (5). As shown in Fig. 1, C. pasteurianum responded quite differently to changes of the pH and growth rate under phosphate limitation. First of all, C. pasteurianum was not able to grow over a pH range comparably wide to that for C. acetobutylicum. At pHs below 5.5, growth and substrate turnover already started to decrease, and at pHs below 4.8, steady-state conditions could not be obtained anymore. The pH had almost no effect on the production of solvents (ethanol and butanol) which were produced at low concentrations (2 to 5 mM) over the pH range from 4.8 to 7.0. In addition, no significant solvent production could be initiated at low growth rates, although the butyrate concentration reached high levels (90 mM) at a growth rate of 0.03 h$^{-1}$, which allows solvent formation by C. acetobutylicum even at neutral pH (24).

Effect of carbon monoxide. Carbon monoxide is known to be a strong inhibitor of hydrogenase activity (11, 20), and consequently the fermentation of C. acetobutylicum can be modulated (less H$_2$, acetate, and butyrate and more butanol and ethanol [31]), and butanol formation can be initiated by CO (34). To examine the effect of CO on product formation by C. pasteurianum, experiments in batch culture with initial headspace CO concentrations from 1 to 15% (vol/vol) were conducted. The results on growth, substrate conversion, and product concentrations after 27 h of incubation at 37°C are shown in Fig. 2. Growth started to be inhibited at initial headspace concentrations of greater than 5% (vol/vol) CO, as indicated by lower final optical density and glucose consumption. An effect of CO on product formation by C. pasteurianum could already be observed at 1% (vol/vol) CO in the atmosphere. Increasing CO concentrations resulted in enhanced solvent (ethanol and butanol) and lactate formation and less acetate and butyrate production. At 15% (vol/vol) CO, C. pasteurianum converted glucose to butyrate (17 mol/100 mol), acetate (38 mol/100 mol), ethanol (24 mol/100 mol), butanol (36 mol/100 mol), and lactate (32 mol/100 mol). Without CO, butyrate (69 mol/100 mol) and acetate (52 mol/100 mol) were the major products, whereas ethanol, butanol, and lactate were only produced in low amounts. Moreover, H$_2$ production decreased from 1.9 mol/mol of glucose without CO to 0.9 mol/mol of glucose in the presence of 15% (vol/vol) CO.

Product formation from glucose under iron limitation. Iron concentrations of up to 10 μM were found to be growth limiting for C. pasteurianum (data not shown). Similar results had been obtained by Schönheit et al. (38). To investigate the influence of iron limitation on glucose fermentation, cells were grown at 5.7 and 8.7 μM ferrous ions present in the medium, and product formation was compared with that under conditions of excess iron (25.7 μM). Iron limitation had a pronounced effect on glucose fermentation;
significant amounts of lactate were produced, which became the major product (77 mol/100 mol of glucose) at an iron concentration of 5.7 μM, mostly at the expense of butyrate (Table 1). Solvent (ethanol and butanol) production was not stimulated under these conditions. Instead, the butanol yield increased slightly with higher growth and glucose consumption at higher iron concentrations. In addition, iron limitation did not cause decreased hydrogen production in continuous culture, in which the products were similar to those found in batch culture (data not shown).

**Fermentation pattern with glycerol as substrate.** When grown on minimal medium with glycerol as the only energy and carbon source in batch culture, *C. pasteurianum* showed a drastic change in product pattern compared with that during glucose fermentation. The effect of different amounts of glycerol in the medium on growth and the products formed is shown in Fig. 3. The tolerance of *C. pasteurianum* to glycerol was remarkably high. Good growth and substrate consumption were obtained at glycerol concentrations of up to 17% (wt/vol). *C. pasteurianum* was able to ferment maximally 300 mmol of glycerol per liter under the conditions employed. It is obvious that glycerol fermentation resulted in the almost exclusive production of ethanol, butanol, and 1,3-propanediol. Acetate and butyrate were produced only in trace amounts. Interestingly, the ratio of the solvents changed with increasing glycerol concentrations. At up to 8% (wt/vol) glycerol in the medium, almost equal amounts were converted to ethanol, butanol, and 1,3-propanediol. Increasing the glycerol concentration further favored the production of 1,3-propanediol at the expense of ethanol. Thus, at 17% (wt/vol) glycerol, the concentrations of 1,3-propanediol, butanol, and ethanol after growth of *C. pasteurianum* were 132, 45, and 30 mM, respectively.

**Effect of iron limitation on glycerol fermentation.** Since iron limitation had changed the product pattern of *C. pasteurianum* during glucose fermentation, its influence on glycerol fermentation was also determined. *C. pasteurianum* was grown at two limiting iron concentrations (3 and 6 μM) with 4% (wt/vol) glycerol as the substrate, and product formation was compared with that found under conditions of excess iron (23 μM) (Fig. 4). Butyrate and acetate were produced again in trace amounts under all conditions and are not shown in Fig. 4. However, 1,3-propanediol was the major product at the lowest iron concentration (43 mol/100 mol of glycerol). Furthermore, lactate was produced in significant amounts (20 mol/100 mol) under these conditions, whereas

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**TABLE 1. Effect of iron-limited growth on product formation by *C. pasteurianum* during glucose fermentation in batch culture**

<table>
<thead>
<tr>
<th>Iron concn (μM)</th>
<th>Growth (OD$_{578}$)</th>
<th>Glucose consumed (mM)</th>
<th>Products formed (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>5.7</td>
<td>3.1</td>
<td>73</td>
<td>4 (5.5)</td>
</tr>
<tr>
<td>8.7</td>
<td>6.0</td>
<td>112</td>
<td>7 (6.3)</td>
</tr>
<tr>
<td>25.7</td>
<td>7.5</td>
<td>127</td>
<td>8 (6.3)</td>
</tr>
</tbody>
</table>

* Samples were taken 24 h after inoculation.
* The values represent the total iron content of the medium. Without the addition of FeSO$_4$, the medium contained about 5.7 μM iron.
* Values in parentheses represent moles of product per 100 mol of glucose fermented.

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**FIG. 2.** Effect of initial headspace CO concentration on product formation, glucose consumption and growth of *C. pasteurianum* in batch culture. Products were analyzed 27 h after inoculation. C, OD$_{578}$; +, glucose consumption; Δ, ethanol; □, acetate; ▲, butanol; ○, butyrate; ▽, lactate; ■, H$_2$.

**FIG. 3.** Influence of glycerol concentration on growth (A) and fermentation products (B) of *C. pasteurianum* in batch culture. OD$_{578}$ (●) and fermentation products were determined 36 h after inoculation.
ethanol production decreased from 40 mol/100 mol (23 μM iron) to 10 mol/100 mol (3 μM iron). It was obvious that ethanol production at high iron concentrations continued vigorously after growth had ceased 16 to 20 h after inoculation. Comparison of glycerol fermentation in continuous culture under phosphate and iron limitation gave some interesting results (Table 2). First, a decrease in ethanol formation from 30 mol/100 mol of glycerol (phosphate limitation) to 4 mol/100 mol (iron limitation [10.8 μM]) was observed, whereas butanol production increased from 18 to 30 mol/100 mol. Second, a further decrease in the iron concentration from 10.8 to 6.8 and 2.5 μM favored the formation of lactate (10 and 25 mol/100 mol, respectively) and of 1,3-propanediol (17 and 34 mol/100 mol, respectively) at the expense of butanol. Third, glycerol consumption and growth are not coupled under iron limitation, as indicated by increasing substrate turnover at lower cell densities, which were determined by the available iron.

**DISCUSSION**

**Glucose fermentation.** Several parameters affecting solvent production by *C. pasteurianum* were examined in this study. Neutral solvents such as butanol and ethanol are more reduced than the corresponding fatty acids. Therefore, the switch in carbon flow from acids to solvents has to be linked to a change in electron flow. Consequently, in *C. acetobutylicum* for example, the shift from acid to solvent production is accompanied by a decrease in hydrogen production (26). Our results confirm the important role of the hydrogenase in *C. pasteurianum* as well in maintaining the internal redox balance. Inhibition of hydrogen production by CO forced the cells to produce butanol, ethanol, and lactate. Since inhibition of the hydrogenase of *C. pasteurianum* by CO has been demonstrated previously (2, 3, 15, 16), the observed effect on product formation can be attributed to a less active hydrogenase under these conditions. The effect of inhibition of hydrogenase by CO on product formation by *C. pasteurianum* had not been determined in the above-mentioned earlier work. Inhibition of the hydrogenase likely results in a higher pool of reduced ferredoxin in the cells, which is reoxidized via NAD(P)H-ferredoxin oxidoreductase (27, 28, 37). The reducing equivalents of NAD(P)H are then regenerated by the use of butanol, ethanol, and lactate as electron sinks. Thus, the response of *C. pasteurianum* to CO is similar to that of *C. acetobutylicum* (12, 30, 35).

A *C. acetobutylicum*-like product pattern (8) was also observed under iron limitation at neutral pH; lactate became the major product. The activity of the hydrogenase, an iron-containing enzyme (11), was not affected under these conditions, since unchanged H₂ formation was observed. Furthermore, no significant amounts of butanol or ethanol were formed as after inactivation of the hydrogenase by CO. Thus, the point(s) of interference of iron limitation with the metabolic routes of *C. pasteurianum* can only be speculated upon. Possible targets could be the pyruvate-ferredoxin oxidoreductase, which is an iron-sulfur protein in *C. acidurici* (39) and *C. acetobutylicum* (33), or other enzymes involved in the formation of butyrate, which was produced

![FIG. 4](http://aem.asm.org/) Effect of iron limitation on growth and product formation by *C. pasteurianum* during growth on glycerol (4% [wt/vol]) in batch culture. (A) 2.8 μM; (B) 5.8 μM; (C) 22.8 μM total iron content. Without the addition of FeSO₄, the medium contained 2.8 μM iron. ▲, ethanol; ●, butanol; ○, lactate; ■, 1,3-propanediol.

**TABLE 2.** Product formation and growth with glycerol as the substrate in continuous culture under phosphate and iron limitation

<table>
<thead>
<tr>
<th>Limitation</th>
<th>Growth (OD₅₇₀)</th>
<th>Glycerol consumed (mM)</th>
<th>Products formed (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>Phosphate (0.5 mM)</td>
<td>5.7</td>
<td>335</td>
<td>100 (30)</td>
</tr>
<tr>
<td>Iron⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.8 μM</td>
<td>5.8</td>
<td>335</td>
<td>14 (4)</td>
</tr>
<tr>
<td>6.8 μM</td>
<td>5.5</td>
<td>350</td>
<td>13 (4)</td>
</tr>
<tr>
<td>2.5 μM</td>
<td>3.75</td>
<td>390</td>
<td>17 (4)</td>
</tr>
</tbody>
</table>

⁹ The medium contained 4% (wt/vol) glycerol (435 mM). The dilution rate was 0.05 h⁻¹. The pH in the phosphate-limited culture was 7.0, and in the iron-limited chemostat it was 6.7.

⁸ Values in parentheses represent moles of product per 100 mol of glycerol fermented.

⁹ The values represent the total iron content of the medium. Without the addition of FeSO₄, the medium contained about 0.8 μM iron.
in low amounts under iron limitation. In experiments with *C. acetobutylicum*, phosphate proved to be a suitable and reliable growth-limiting factor to ensure a high substrate turnover with a high yield of solvents. The fact that phosphate limitation was not a suitable condition for solvent formation by *C. pasteurianum* might be connected to the inability of this organism to grow at low pHs. In *C. acetobutylicum*, solvent formation under phosphate limitation was initiated only at pHs below 5 (5). Therefore, the pH obviously does not play an important role in the regulation of solvent formation in *C. pasteurianum* as it does in *C. acetobutylicum*.

In general, our results demonstrate the flexibility of *C. pasteurianum* in changing product formation from glucose depending on the growth conditions. As with *C. acetobutylicum*, interference with the electron flow caused by CO or iron-limited growth results in the formation of more reduced compounds compared with the well-known acetate and butyrate production from glucose under standard conditions.

**Glycerol fermentation.** The fermentation of glycerol as a sole carbon and energy source can be accomplished by several *Klebsiella* and *Citrobacter* strains (25) and by solventogenic clostridia (18). Part of the glycerol used as the carbon and energy source is oxidized to dihydroxyacetone by glycerol dehydrogenase and phosphorylated by dihydroxyacetone kinase. Further catabolism proceeds through glycolysis. The remaining part of glycerol is reduced to 1,3-propanediol after dehydration to 3-hydroxypropionaldehyde. These pathways have been established for *Klebsiella pneumoniae* (1, 17). The existence of a specific 1,3-propanediol dehydrogenase has been demonstrated for *C. pasteurianum* LMG 3285 (22). The possible pathways present in *C. pasteurianum* during glycerol and glucose fermentation are shown in Fig. 5. During glycerol oxidation, one additional NADH is generated per acetyl-coenzyme A (CoA) formed. Furthermore, the disposal of electrons via pyruvate-ferredoxin oxidoreductase or NADH-ferredoxin oxidoreductase and hydrogenase might be affected by the corresponding NADH and acetyl-CoA levels during glycerol fermentation. Both substances are important for the regulation of ferredoxin oxidoreductases (27, 28, 37). Thus, the oxidation-reduction state has to be balanced by the formation of
reduced compounds such as lactate, ethanol, butanol, and 1,3-propanediol. C. pasteurianum LMG 3285 converted only 6% of the glycerol to 1,3-propanediol, and butanol was used as the major electron sink. In this connection, our results obtained with glycerol fermentation under iron limitation are of special interest. Iron limitation somehow inhibited the formation of butanol and ethanol. It is possible that the alcohol dehydrogenases involved are iron dependent. Such an enzyme has been identified in the related organism C. acetobutylicum (41). Thus, the 1,3-propanediol pathway had to be highly active to regulate the internal redox balance. The same situation has been found in Clostridium butyricum, in which an ethanol and butanol pathway is absent or at least very weak (22).

Another advantage of iron limitation with respect to a possible biotechnological use of a Clostridium strain for the production of 1,3-propanediol is the uncoupling of growth and glycerol turnover, leading to the desired higher product formation rates. Whether Clostridium species will ever be used in an industrial fermentation process depends on the 1,3-propanediol yield. Maximally, 66 mol of 1,3-propanediol can be produced per 100 mol of glycerol, which is achieved by Clostridium species (25). C. pasteurianum and C. butyricum have the advantage that they are nonpathogenic. The 1,3-propanediol yield of C. butyricum is already close to the maximum (22). C. pasteurianum, on the other hand, tolerates high concentrations of glycerol (up to 17% [wt/vol]), whereas growth of C. butyricum is already inhibited at 8% (wt/vol) glycerol (10). Conditions have to be found under which C. pasteurianum converts glycerol in high concentrations into products such as 1,3-propanediol. With the progress made in recent years in the genetics of clostridia (40), it should be possible to isolate suitable, e.g., lactate-negative, mutants of C. pasteurianum which might have some biotechnological potential.

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