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Comparative investigations of growth and solvent formation in '*Clostridium saccharoperbutylacetonicum*' DSM 2152 and *Clostridium acetobutylicum* DSM 792

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The butanol and acetone-producing strain DSM 2152, invalidly described as '*Clostridium saccharoperbutylacetonicum*' is compared with the type strain *C. acetobutylicum*, DSM 792, with respect to solvent and acid formation at varying pH values and growth rates. Batch cultures, product-limited chemostat and pH-auxostat cultures were used for characterization. Under all conditions strain DSM 2152 produced much lower amounts of butyric and acetic acids than the type strain. The pH optimum for solvent formation was higher, ie 5.5 instead of 4.5. Solvent formation occurred at higher dilution rates, but below 0.1 h^{-1} a lower solvent concentration was obtained, indicating that acid production was too low to provide a sufficient amount for acetone formation. The results are discussed in the light of recent publications on the taxonomy of butanol-acetone producing clostridia using 16S rRNA sequence analysis and other nucleic acid data. The presently suggested 'phylogenetic' classification of the collective species, *C. acetobutylicum*, is also reflected in the fermentation characteristics.

Keywords: butanol-acetone fermentation; '*Clostridium saccharoperbutylacetonicum*'; *Clostridium acetobutylicum*; chemostat; pH-auxostat

Introduction

In 1960 Hongo patented a butanol- and acetone-producing isolate and (invalidly) described it as a new species, '*Clostridium saccharoperbutylacetonicum*' [6]. The strain was distinguished by a higher butanol/acetone ratio than with other industrial strains, ie 4:1 *vs* 2:1. It showed unusually large clostridial forms (spore-containing cells) and differences in substrate utilization. In view of the numerous and no longer valid species descriptions in the butanol/acetone patent literature during the period of industrial solvent production, Reysset *et al* [12] treated the strain as *C. acetobutylicum*. On the other hand Soni *et al* [15–17] employed the original designation, whilst Schoutens and Kossen [14] treated the strain as an undefined *Clostridium* sp.

Recently the taxonomy of the butanol and acetone/isopropanol-producing clostridia was reassessed using 16S rDNA sequences and genome sizes as well as DNA fingerprints and biotyping [7,9,19,20]. The existing strains were classified into four groups of species rank, the Hongo strain N1–4 and its derivatives forming one of them. Two of the groups, the *C. beijerinckii* group and an unnamed one represented by the molasses-fermenting industrial strains were closely related to strain N1–4 whereas the starch-fermenting strains were phylogenetically distant from the other groups. It was proposed to retain the original name for strain N1–4 and to reserve the present species *C. acetobutylicum* for the starch-fermenting strains.

A phenotypic characterization of the groups/species has still to be worked out. In particular it would be of interest if the suggested classification is also reflected in the existing differences in the fermentation characteristics, ie solvent ratio, acid formation and pH dependence. Unfortunately the literature on butanol-acetone fermentation does not provide the required data for comparison since media and culture conditions used by different research groups differ considerably especially in supplementation with complex substances and in the pH regimes.

In this study the fermentation parameters of '*C. saccharoperbutylacetonicum*' are compared to those of the type strain of *C. acetobutylicum* using identical media and growth conditions. Both batch and continuous cultures were used to record solvent and acid production over a wide range of pH and growth rates. The continuous cultures were run as product-limited chemostat cultures, which appear to be the most suitable version for industrial fermentation and as pH-auxostat cultures which allow an evaluation of the culture performance by growth rate measurement.

Materials and methods

Microorganisms

The two butanol-acetone producing strains compared in this investigation were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ. The Hongo strain was represented by the derivative N1–504 and listed as *Clostridium* sp DSM 2152 which corresponds to ATCC 27022; the type strain of *Clostridium acetobutylicum* was listed as DSM 792 and corresponds to ATCC strain 824.

Medium and culture conditions

The medium contained per L of deionized water: KH_2PO_4, 1 g; K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g;

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$CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4$, 5 mg, trace element solution SL 7 [3], 2 ml; glucose, 28 g for batch and 60 g for continous culture; $CaCO_3$, 2 g (only for precultures).

Precultures were grown in 100-ml screw-cap bottles with rubber septa under nitrogen using 50 ml of degassed medium and were inoculated from dry spore sand. The incubation temperature was 37°C. At least two transfers were prepared before a fermentor was inoculated.

All fermentations were carried out in a 1-L fermentor manufactured by BCC, Göttingen, Germany, with pH and temperature control. For continuous culture the medium was dispensed using peristaltic pumps. Theoretical concept and practical performance of the pH auxostat culture has been previously described [2]. In this type of continuous culture the medium supply is regulated by alkali addition necessary to maintain the pH in an acid-producing culture. By variation of the alkali flow rate, the medium volume which enters the fermenter during the pH adjustment period is determined.

Analytical methods

Glucose consumption was measured using a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). The fermentation products were analyzed gas-chromatographically using Chromosorb 101 as column material and N_2 as carrier gas; the oven temperature was 180°C. The cell mass is given as dry weight: the cells were centrifuged at 17 500 × *g* in preweighed steel tubes, washed twice and dried at 80°C for 48 h.

Results

Product formation in batch cultures

Batch cultures of '*C. saccharoperbutylacetonicum*' DSM 2152 showed obvious differences from *C. acetobutylicum* DSM 792 at different pHs (Figure 1). With strain DSM 2152, the pH up to which predominantly solvents are produced was about one unit higher than in DSM 792, 5.5 *vs* 4.5. The intermediate accumulation of butyric and acetic acids was very low in DSM 2152 under conditions of solvent formation, compared to DSM 792. A low fraction of acetone in relation to butanol which, according to the original description [6], should be the determinative trait of the strain was confirmed only for pH 5.5 and higher, while at the lowest pH at which growth was observed the acetone: butanol ratio was close to that of the comparative strain. In both strains butanol formation is never completely suppressed, not even at the highest pH tested. Table 1 compares characteristic fermentation data of the two cultures with corresponding published values. It shows satisfying correspondence between the different cultures of the type strain (DSM 792 should be identical to ATCC 824) and between DSM 2152 and B18.

Product formation in chemostat culture

Determination of the optimum pH and growth rate dependency of product formation was the aim of the chemostat culture experiments. The cultures were grown with excess of nutrients so that the resulting steady states must have been brought about by inhibition of the products. Represen-

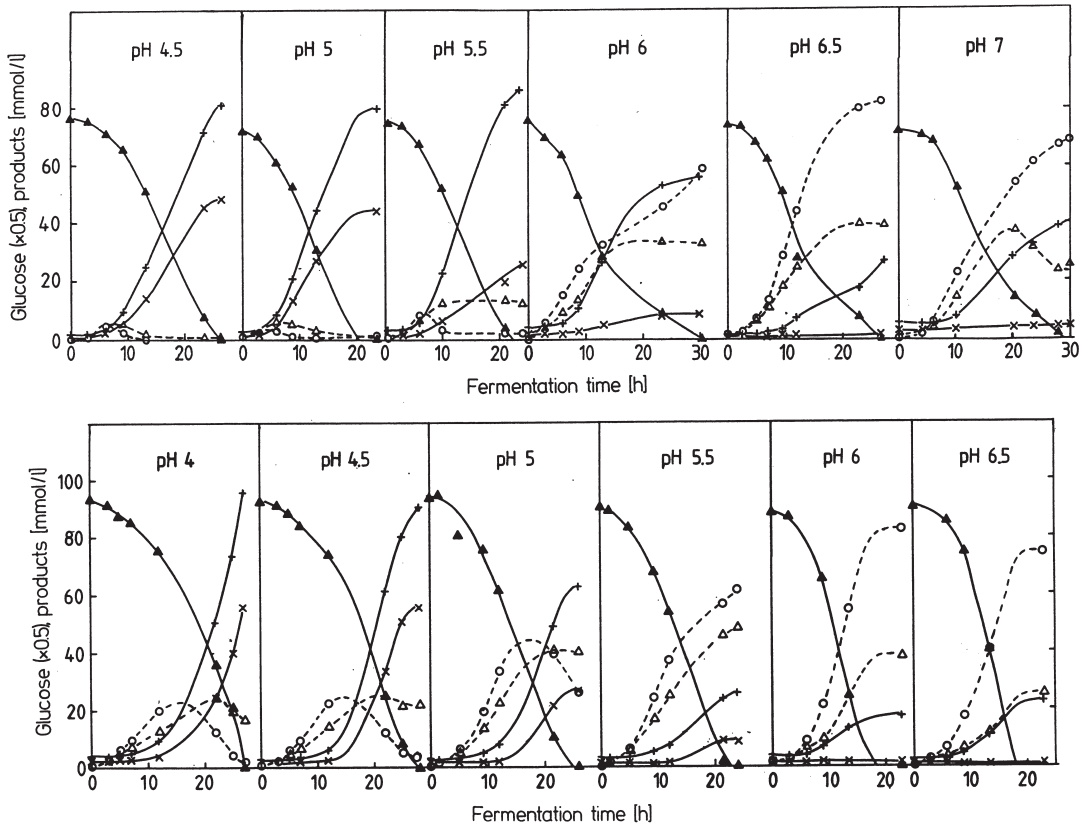

Figure 1 Substrate consumption and product formation in batch cultures of strains DSM 2152 (upper panel) and DSM 792 (lower panel) at various pH values. Glucose (▲), butanol (+), acetone (×), butyric acid (○), acetic acid (△).

Table 1 Maximum butyric acid concentration and final butyric acid and butanol yield in batch cultures of strains DSM 2152 and DSM 792 compared to published data. Glucose concentration 2.8% for the DSM strains, 5.5% for ATCC 824 and 6% for strain B18

pH	Butyric acid maximum (mmol L^{-1})				Final butyric acid yield (mol per 100 mol of glucose)				Final butanol yield (mol per 100 mol of glucose)			
	DSM 2152	DSM 792	B18[b]	ATCC 824[a]	DSM 2152	DSM 792	B18	ATCC 824	DSM 2152	DSM 792	B18	ATCC 824
4	nt	23	nt	nt[c]	nt	1	nt	nt	nt	52	nt	nt
4.5	5	24	8	34	0	3	0	5	53	49	34	51
5	3	45	11	69	0	14	0	13	55	33	49	56
5.5	10	–[d]	24	106	1	34	9	22	57	14	53	33
6	–	–	50	–	38	47	12	61	36	10	44	4
6.5	–	–	–	nt	54	41	55	nt	18	13	5	nt
7	–	nt	–	nt	44	nt	48	nt	26	nt	1	nt

[a]Data derived from [10], ATCC 824 = DSM 792, synthetic medium.
[b]Data derived from [5], B18 is a mutant from NRRL B643, medium with 8 g yeast extract, 2.2 g casein hydrolysate and 1 g asparagine L^{-1}.
[c]Not tested.
[d]Butyric acid maximum not found.

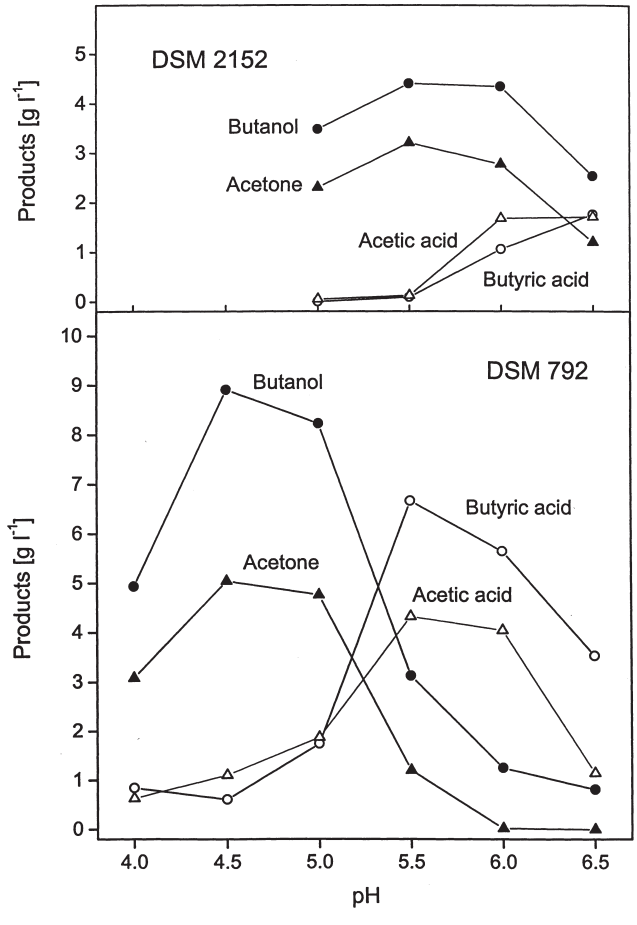

Figure 2 The effect of pH on product formation in chemostat cultures by strains DSM 2152 and DSM 792. Dilution rate 0.085 h^{-1}.

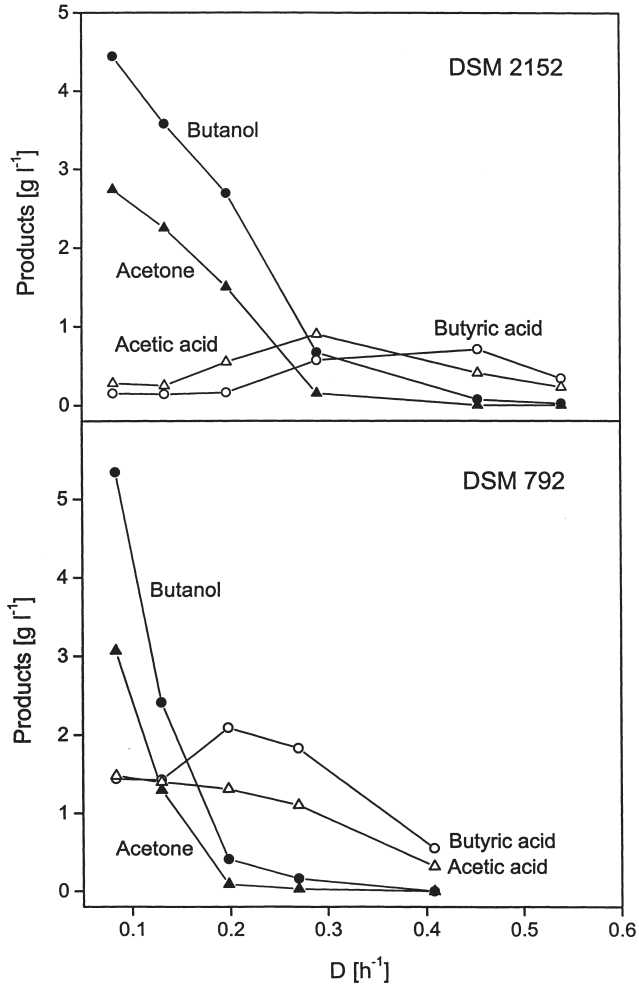

Figure 3 Product formation by strains DSM 2152 and DSM 792 at steady states of increasing dilution rate, pH 5.5 for DSM 2152 and 4.5 for DSM 792.

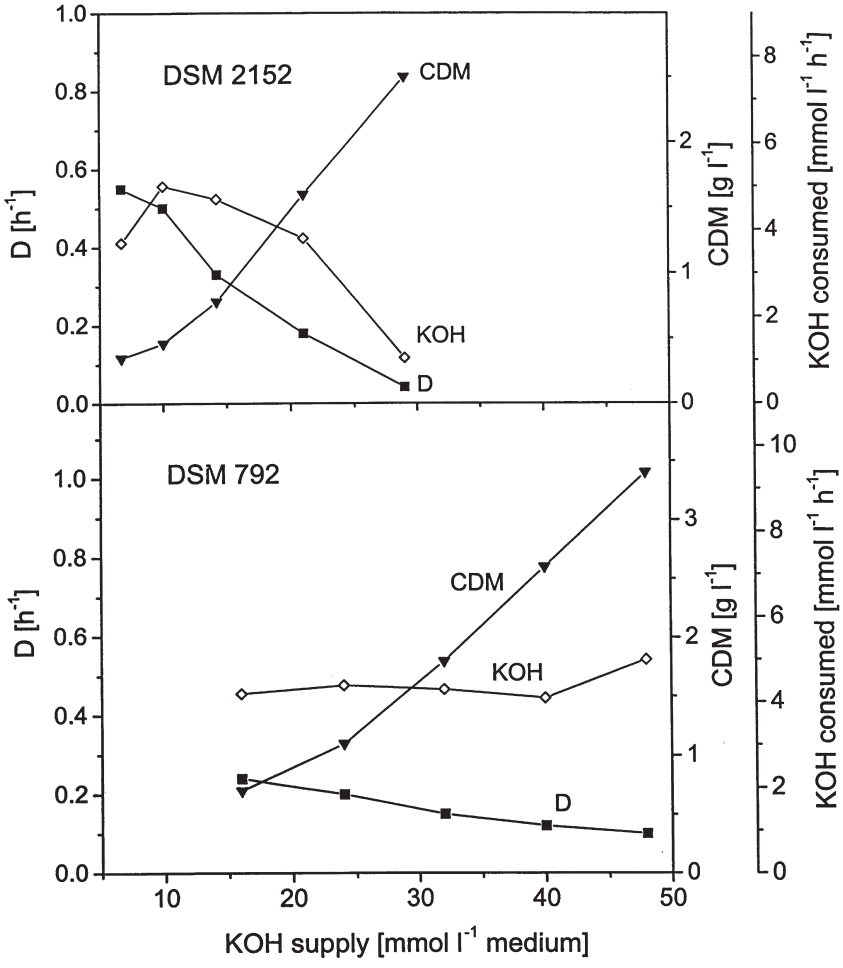

Figure 4 pH-auxostat cultures of strains DSM 2152 and DSM 792 as influenced by the amount of alkali supplied per volume of medium: resulting dilution rate, cell concentration and alkali consumption. D = dilution rate, CDM = cell dry mass.

tative steady state samples were taken after at least five volume changes had passed and two consecutive turbidity measurements gave equal values. The steady state product composition at various pH values is shown in Figure 2 for the two strains using a dilution rate of 0.085 h^{-1}. As in the batch culture, the optimum pH for solvent formation differed by about one pH unit, being 5.7 for DSM 2152 and 4.7 for DSM 792. In each culture acid production steeply increased beginning with these pHs. At pH values at and below the solvent optimum, the acid content was almost zero with strain DSM 2152 while with DSM 792 at least 1–2 g L^{-1} of each acid were always present in the culture.

Figure 3 shows that at its optimum pH, strain DSM 2152 produces solvents up to a dilution rate (growth rate) of 0.3 h^{-1}, but only of 0.2 h^{-1} in strain 792. Acid formation remains low under all dilution rates in the Hongo strain in comparison to the type strain of *C. acetobutylicum*. At the lowest dilution rate, overall product formation is comparatively lower in the Hongo strain which might explain its low productivity in the pH experiment (Figure 2).

Growth and product formation in a pH-auxostat culture

In the pH-auxostat modification used here [2,11,18], the amount of alkali supplied per volume of medium should be proportional to cell density and substrate conversion. Figure 4 shows that this is realized for both strains except for a very low alkali (KOH) to medium ratio. The strains differ however in the amount of KOH neccessary to achieve a certain cell density; DSM 2152 requires much less alkali than the type strain, reflecting lower production of acids. Therefore the KOH consumption in DSM 2152 at high cell density and in the solvent production phase is so decreased that the function of the pH-auxostat comes to its limits, whereas DSM 792 consumes KOH at a constant rate. The resulting dilution rate falls rapidly within the active KOH supply range for strain DSM 2152, while it decreases slowly in the comparative strain. Figure 5 shows the products in this fermentation and confirms the differences in acid production in the two strains. It also shows that reduced acetone production is not seen in continuous culture (see also Figure 3).

In Figure 6 the steady state product concentrations of the pH-auxostat experiment are plotted against the dilution rates. Corresponding to the chemostat results, solvent formation in strain DSM 2152 extends to higher growth rates than in strain DSM 792 but strain DSM 792 is superior in productivity at low growth rate.

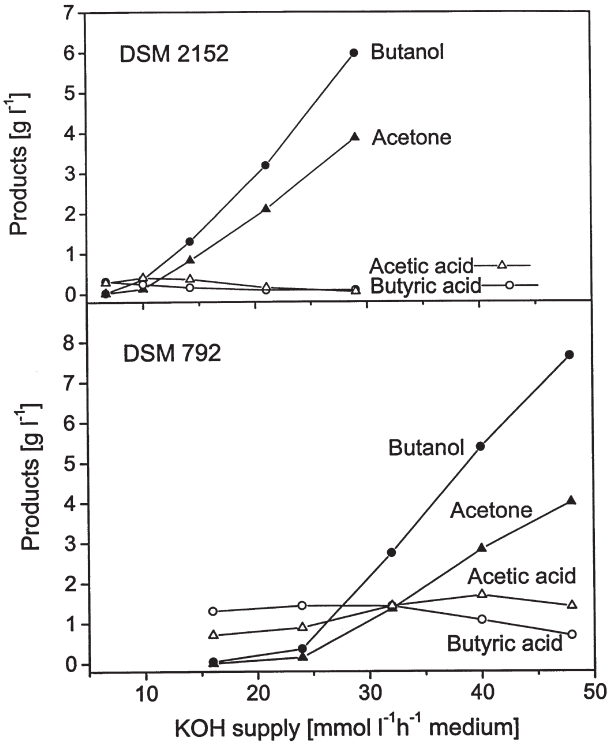

Figure 5 pH-auxostat cultures of strains DSM 2152 and DSM 792 as dependent on the amount of alkali supplied per volume of medium: products formed.

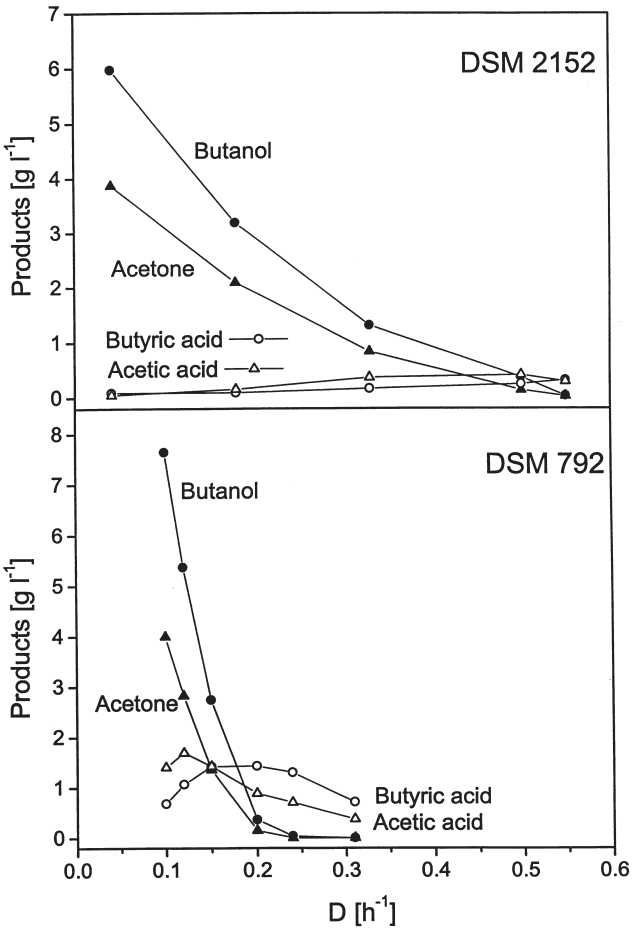

Figure 6 pH-auxostat cultures of strains DSM 2152 and DSM 792: products formed plotted against the dilution rates resulting from different alkali/medium ratios.

Discussion

Although a typical butanol-acetone-ethanol producing *Clostridium* strain DSM 2152, '*C. saccharoperbutylacetonicum*' differs remarkably from the type strain of *C. acetobutylicum*. In batch culture at low pH a very small amount of butyric and acetic acids is necessary to induce solvent formation. In continuous culture the fraction of acids present is also very low at comparable pH and dilution rate. At low dilution rate DSM 2152 was inferior to *C. acetobutylicum* DSM 792, because acid formation was too low to provide sufficient substrate for the CoA transferase to form acetone so that solvent production was diminished. The pH optimum for solvent production was about one unit higher than in the type strain.

The fermentative properties of the strains that were assigned to the collective species *C. acetobutylicum* cannot be easily compared because the available data originate from different culture conditions and experimental designs. There are sufficient indications that at least one of the investigated characteristics, the optimum pH range for solvent production, is in accord with the classification obtained from nucleic acid data [9]. A narrow pH optimum around 4.5 and high tolerance to acidic conditions which have been found for strain DSM 792 [1, see also 10] are restricted to the newly defined *C. acetobutylicum* which comprises the industrial starch-fermenting strains [9]. The same range was also reported for a continuous culture of strain DSM 1731 (ATCC 4259), the putative Weizmann patent strain. A broader pH optimum around pH 5.5 as shown here for '*C. saccharoperbutylacetonicum*' occurs in the other three groups of the original species which are also phylogenetically closely related. For instance, strain NCIMB 8052, which was recently identified as *C. beijerinckii* [19] showed much better growth and solvent production at pH 5.5 than at pH 5.0 and no growth at pH 4.0 [18]. The industrial molasses-fermenting strains, eg strain P262, are generally regarded as producing solvents at a higher pH range [8], and for mutant B18, which was generated from NRRL B643, a pH optimum of 5.5 in batch culture was demonstrated.

Reduced production of acids, however, is not a relevant character for species discrimination, as has been shown for the low-acid strain B18 which was selected after ethyl methanesulfonate treatment. Its pH-dependent acid formation [5] is similar to that of DSM 2152 (Table 1) whereas the parent strain behaves as the type strain in this respect [13]. Strain DSM 2152 is a mutant, too, which was selected for phage resistance.

Soni *et al* [15] reported that '*C. saccharoperbutylacetonicum*' produces more butanol than *C. acetobutylicum*. The present investigation does not allow a definite assessment about the maximum performance of the Hongo strain, but from the continuous culture experiments it appears that overall product formation is similar to the type strain of *C. acetobutylicum* at all growth rates and that only the solvent fraction is higher. As inhibition by butanol and butyric acid

is comparable in both strains [4,16] and butyric acid is a stronger inhibitor than butanol, there might be some advantage for strain DSM 2152 to reach a higher end concentration of butanol. The strain is a candidate for genetic improvement supplementary to the type strain of *C. acetobutylicum* which is the subject of a rapid development in DNA recombination.

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