

Comparative Fermentation Studies of Industrial Strains Belonging to Four Species of Solvent-Producing Clostridia

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Abstract

Industrial and culture collection strains of solvent-producing clostridia, classified as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum* were utilised in a comparative study of fermentation performance in a laboratory fermentation medium, a molasses fermentation medium, and a maize fermentation medium under standardised culture conditions. At least one representative strain was selected from each of the sub-groups within the four species. Preliminary evaluations were first undertaken for the three different fermentation media to determine the most appropriate media formulations, carbohydrate concentrations, and culture conditions for comparison of the solvent-producing ability of these strains. Standardised fermentation media and culture conditions were then selected for each of the comparative fermentation studies. These included TYA medium containing 4% glucose, a supplemented molasses medium containing 6% fermentable sugars, and a supplemented maize mash medium containing 8% maize. Additional comparative fermentation studies on industrial strains belonging to two species of solvent-producing clostridia were carried out in molasses containing higher concentrations of fermentable sugars, and the sugar concentrations supporting maximum levels of solvent production were determined. Although all the strains tested grew in the maize fermentation medium and degraded starch, only a few strains produced consistently high solvent levels. Optimum starch utilisation and solvent production was obtained at a maize concentration of 80 g/l. Pretreatment of the maize by milling or saccharification decreased the buffering capacity of the medium and resulted in decreased solvent production. Decreasing the time used to gelatinise the starch had little effect. Solvent yields and concentrations obtained in this study were compared with various published data in the scientific and patent literature and appeared to closely simulate

the results obtained in the industrial fermentation process. The fermentation performances of individual strains could provide useful comparative data for the selection and development of strains for use on various commercial fermentation substrates.

Introduction

The acetone-butanol (AB) fermentation process was widely used in the earlier part of this century for the industrial production of solvents (Jones and Woods, 1986). The fermentation process, developed during the First World War for munition production, utilised starch as the fermentation substrate. The commercial AB fermentation process originally established by the Commercial Solvents Corporation (CSC) in the USA continued to use maize as the fermentation substrate until the early 1930's when the widespread availability of cheap molasses from the sugar industry provided a strong incentive to switch substrates (Hastings, 1971). However, although considerable effort was invested in attempting to utilise the existing starch-fermenting strains for use on molasses this was never successfully achieved (McCutchan and Hickey, 1954; Hastings, 1971). The poor performance obtained with the maize strains prompted a quest for new isolates that would provide economically viable solvent yields.

In 1935, the patent protecting the CSC process monopoly lapsed, and a number of new companies entered the market worldwide. During the 1930's and 1940's a substantial number of new saccharolytic clostridial strains were isolated and patented by different companies (Beesch, 1952; McCutchan and Hickey, 1954; Ross, 1961; Walton and Martin, 1979; Jones and Keis, 1995). Among the first strains to be patented were a number that, under optimal conditions, were able to utilise between 4-6% fermentable sugars producing solvent concentrations of 14-18 g/l with solvent yields from 25-30%. Early studies revealed that molasses was nutritionally deficient as a fermentation substrate and supplementation with an additional source of nitrogen and a buffering agent became common practice (Beesch, 1952; McCutchan and Hickey, 1954). It is apparent from both the scientific and patent literature that many of the newly isolated strains had serious limitations. Although some were used commercially, in a number of instances sucrose utilisation was poor and it was necessary to invert the sugars. With some strains, long fermentation times (up to 72 h) resulted in lower productivity, and sugar concentrations were limited to around 6% set sugars (McCutchan and Hickey, 1954). An ongoing quest for more efficient strains that could use higher concentrations of fermentable sugars to produce higher solvent yields with shorter fermentation times continued. Strains producing higher ratios of butanol and

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exhibiting greater stability and resistance to phage infection were also isolated and patented. By the late 1930's and early 1940's a number of new strains that had substantially improved characteristics were being used for commercial solvent production (McCutchan and Hickey, 1954; Hastings, 1971; Walton and Martin, 1979). The temperature optima for most of these saccharolytic strains were reported to be between 30-33°C and they functioned at wider pH ranges than the original starch-fermenting strains (McCutchan and Hickey, 1954; Ross, 1961). Strains that were able to utilise up to 7.5% fermentable sugars to give reproducible solvent concentrations of 18-23 g/l and yields of 30-33% were reported to be in use at CSC (McCutchan and Hickey, 1954; Walton and Martin, 1979). Some of these later strains allowed fermentation times to be as short as 30 h. Solvent-producing strains that gave butanol ratios as high as 70% were later isolated and patented in Japan (Hongo, 1960).

Unfortunately, many of these industrial strains do not appear to have survived the demise of the fermentation process which occurred in most countries in the Western World in the early 1960's. However, a number of these solvent-producing strains are obtainable from international culture collections (Jones and Keis, 1995). The most comprehensive collection of industrial strains to have survived are those maintained at National Chemical Products (NCP) Ltd in South Africa. This company continued to operate the AB fermentation process until the early 1980's (Spivey, 1978). This collection, which includes a range of the strains used in the NCP fermentation process as well as a number of original strains supplied by CSC and Commercial Solvents (Great Britain) is now housed in the Department of Microbiology at the University of Otago, New Zealand, and a selection of these strains were included in this study along with standard culture collection strains.

Until recently, the taxonomic and phylogenetic relationships of these various solvent-producing clostridial strains remained obscure and standard practice was to regard all existing strains as variants of *Clostridium acetobutylicum* or *Clostridium beijerinckii*. However, a number of systematic studies (Keis *et al.*, 1995; Johnson *et al.*, 1997) have revealed that the industrial solvent-producing clostridia comprise four species. All of the original starch-fermenting strains were found to belong to a single species, *C. acetobutylicum*. This species is phylogenetically distinct and only very distantly related to the other three more closely related species containing the later generation of sugar-fermenting industrial strains. The majority of these saccharolytic strains have been identified as belonging to the *C. beijerinckii* species which contains at least 17 distinct sub-groups based on genomic DNA/DNA hybridisation and DNA fingerprint patterns. Existing strains belonging to the other saccharolytic species can both be sub-divided into two genomic DNA fingerprint groups (Keis *et al.*, 1995).

The industrial production of solvents from both maize and molasses is well documented in the literature (Gabriel, 1928; Beesch, 1953; McCutchan and Hickey, 1954; Ross, 1961; Hastings, 1971; Spivey, 1978; Walton and Martin, 1979). However, there are limited data comparing the fermentation performance of individual industrial strains of solvent-producing clostridia using maize and molasses as fermentation substrates under comparative laboratory conditions.

The aim of this study was to establish supplemented maize and molasses fermentation media that would allow the various strains of solvent-producing clostridia investigated to produce high yields of solvents under laboratory conditions, equivalent to the maximum yields reported. Several parameters reported to affect fermentation performance were also investigated, and the effect that these had on the total solvent production was examined. The solvent-producing abilities of different strains of *C. acetobutylicum*, *C. beijerinckii*, *Clostridium saccharoperbutylacetonicum*, and *C. saccharobutylicum* (new nomenclature submitted for publication) were then compared using these maize and molasses media and a standard laboratory fermentation medium containing glucose.

In order to obtain results that could be compared directly it was necessary to use a common set of conditions. While the particular set of culture conditions utilised for comparative studies might be close to optimum for some species and strains, it has to be accepted that it is unlikely that the specific conditions used would be optimal for all strains tested. A second problem encountered is that in some instances laboratory-scale fermentations do not always produce levels of solvents comparable to those obtained in industrial-scale fermentations (van der Westhuizen, 1982).

Results

Development of Fermentation Media for Comparative Studies

To facilitate comparative fermentation studies, a laboratory fermentation medium that would enable most strains to produce consistent and apposite concentrations and yields was required. A number of trials were conducted on different fermentation media reported in the literature and a TYA glucose medium modified from that utilised by Hongo and Murata (1965) was selected as a versatile fermentation medium that supported good solventogenic fermentations with the various test strains used.

Preliminary studies were conducted with TYA medium containing 4%, 5%, 6%, and 7% glucose to determine the most appropriate glucose concentration. Initially five test strains known to be capable of producing high concentrations and yields of solvents comprising two strains of *C. beijerinckii* (NCIMB 8052 and NRRL B592) and one strain from each of *C. acetobutylicum*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*, (ATCC 824, NCP P262, and N1-4, respectively) were used. With all five of the test strains used, solvent yields were observed to decrease as the concentration of glucose was increased from 4% to 7% (Table 1). The ratio of butanol to acetone produced also decreased in all five strains as the glucose concentrations were increased from 4% to 7% (data not shown). However, the concentration of sugars at which solvent yields began to decrease and the actual rate of decrease differed in the five test strains used. As the TYA medium containing 4% glucose produced good growth, gave the highest yields of solvents, and produced acceptable solvent concentrations with all of the strains tested, this concentration of glucose was selected for the comparative fermentation study.

Preliminary studies were also conducted to develop a supplemented molasses fermentation medium using two

Table 1. Effect of Increasing Glucose Concentration in TYA Medium on Solvent Concentration and Yield

Strain ^a	Glucose concentration							
	4%		5%		6%		7%	
	Concentration (g/l)	Yield (%)	Concentration (g/l)	Yield (%)	Concentration (g/l)	Yield (%)	Concentration (g/l)	Yield (%)
<i>C. acetobutylicum</i> ATCC 824 ^T	12.0	30.0	15.7	31.5	18.5	30.8	13.5	19.3
<i>C. saccharobutylicum</i> NCP P262	12.8	32.0	15.2	30.4	16.8	28.0	16.5	23.6
<i>C. saccharoperbutylacetonicum</i> N1-4	13.0	32.5	14.3	28.6	15.7	26.2	9.8	14.0
<i>C. beijerinckii</i> NCIMB 8052	11.2	28.0	14.8	29.6	11.2	18.7	10.5	15.0
NRRL B592	11.3	28.3	11.5	23.0	12.7	21.2	9.9	14.1

^a T=Type strain

strains, *C. beijerinckii* ATCC 8052 and *C. saccharobutylicum* NCP P262. Commonly utilised formulations for fermentation media containing molasses usually included additional inorganic nitrogen and a buffering agent (Beesch, 1952; McCutchan and Hickey, 1954). In addition a source of phosphorus and organic nitrogen were often included for laboratory studies. Before deciding on the formulation and the fermentation conditions to be used for the comparative molasses fermentations, a number of parameters were investigated. These included different sources of Australian blackstrap molasses, the nature and concentration of the inorganic nitrogen and phosphorus, and the concentration of the yeast extract and buffering agent. Variation in the volume of the fermentation media and fermentation container, and the temperature of incubation were also investigated. Within the range of the variations tested no significant differences in performance were observed (data not shown). A variety of common ammonium compounds were investigated as a source of inorganic nitrogen. Substituting one compound for another had little effect on the outcome of the fermentation. Fermentations with strain NCP P262 were carried out at 30°C, 33°C, and 37°C. No significant difference was observed in solvent concentrations and yields in the fermentations run at 30°C and 33°C. However, fermentations run at 37°C did exhibit a significant decrease in solvent concentrations and yields. Trials were also undertaken with the four strains NCIMB 8052, NRRL B591, NCP P258, and NCP P262 in molasses fermentation medium ranging in concentration from 4% fermentable sugars to 8% fermentable sugars. All four strains exhibited a drop-off in solvent yield as the molasses concentration increased. The two *C. beijerinckii* strains also exhibited a drop-off in solvent production above 6% of fermentable sugars. As a consequence of these studies, molasses fermentation medium containing 6% fermentable sugars was adopted for the comparative fermentation studies.

Comparative Fermentations in TYA Medium and Molasses Medium

A single representative strain from each genomic DNA fingerprint sub-group from the four solvent-producing

species was included in a comparative fermentation study using both TYA medium and the molasses fermentation medium. The results obtained are presented in Table 2.

The fermentation procedures and conditions used were the same as those used in the initial trials to assess the effect of sugar concentration. The 24 representative strains from the four species grown in 4% glucose TYA medium exhibited a wide range of solvent concentrations and yields. Representative strains of *C. acetobutylicum*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* all produced solvent yields above 30% (calculated as the amount of glucose (g) converted to solvents, expressed as a percentage) with solvent concentrations above 13 g/l. None of the representative strains of the 17 sub-groups of *C. beijerinckii* that were selected produced comparable solvent yields. The highest solvent concentrations and yields were obtained with strains NRRL B592, NCIMB 8052, ATCC 39058, and ATCC 11914 that all produced maximum solvent concentrations of just over 11 g/l and solvent yields of around 28%. About half of the *C. beijerinckii* strains produced solvent yields and concentrations well below these levels and a few of the strains tested only produced trace amounts of solvents. Five of the *C. beijerinckii* strains tested produced significant amounts of isopropanol (Table 2).

The fermentation profiles of ten industrial strains belonging to the same genomic DNA fingerprint group as NCIMB 8052 were also compared in 4% glucose TYA medium. These included strains NCIMB 8052, NCIMB 8049, NCIMB 6444, NCIMB 6445, NCIMB 8653, NRRL B591, NRRL B594, NRRL B597, ATCC 10132, and DSM 1739. None of these closely related strains produced solvent concentrations or yields that were substantially higher than those produced by the NCIMB 8052 representative strain included in Table 2.

In many cases the representative test strains did not perform as well in the TYA fermentation medium as they did in the supplemented molasses medium containing 6% fermentable sugars. The 24 representative strains again produced a wide range of solvent levels in the molasses fermentation medium.

None of the three strains of *C. acetobutylicum* included

Table 2. Comparative Fermentations of Strains from the Four Species in TYA Medium and Molasses Medium

Sub-group ^a	Strain ^b	TYA medium 4% glucose		Molasses medium ^c 6% fermentable sugars	
		Concentration (g/l)	Yield (%)	Concentration (g/l)	Yield (%)
	<i>C. acetobutylicum</i>				
1	ATCC 824 ^T	12.5	31.2	7.8	13.0
2	ATCC 4259	13.2	33.0	9.5	15.8
3	DSM 1732	12.9	32.5	4.1	6.8
	<i>C. saccharobutylicum</i>				
1	NCP P262	13.4	33.5	17.9	29.8
2	NCP P258	13.7	34.4	18.3	30.5
	<i>C. saccharoperbutylacetonicum</i>				
1	N1-4	13.1	32.8	4.9	8.2
2	N1-504	9.8	24.5	15.6	26.0
	<i>C. beijerinckii</i>				
1	NCP P260	9.5	23.8	18.9	31.5
2	NRRL B592	11.3 ^d	28.3	11.1	18.5
3	NRRL B593	9.5	23.8	11.5	19.2
4	NCIMB 8052	11.2	28.0	11.0	18.3
5	ATCC 39058	11.1	27.8	10.7	17.8
6	ATCC 6014	10.5	26.4	11.7	19.7
7	ATCC 11914	11.0	27.4	6.2	10.3
8	NCIMB 11373	8.5	21.1	4.0	6.6
9	IAM 19015	7.6 ^d	19.0	12.0	20.0
10	ATCC 6015	7.7	19.3	9.6	16.6
11	ATCC 17795	6.1	15.2	6.1	10.2
12	ATCC 14823	4.4 ^d	11.0	0.5	0.8
13	NCIMB 12404	2.1	5.1	0.1	1.6
14	NCIMB 9581	3.3 ^d	8.3	ND	ND
15	NCIMB 9579	3.4	8.4	1.2	2.0
16	NCIMB 9362 ^T	3.0	7.5	0.7	1.2
17	NCIMB 9503	0.9	2.1	0.1	0.8

^a Sub-groups determined by Keis *et al.* (1995).

^b T=Type strain

^c ND= not determined

^d Isopropanol produced

in the comparative fermentation study performed well in the molasses medium with all three producing less than 10 g/l of solvents with corresponding low solvent yields. The two strains of *C. saccharobutylicum* tested showed some variation in solvent production but in both sub-groups the maximum yields obtained were around 30% with solvent concentrations of around 18 g/l. Of the two strains of *C. saccharoperbutylacetonicum* tested one produced good levels of solvents while the other strain performed poorly under the test conditions used.

Of the 16 representative strains of *C. beijerinckii* tested in the molasses medium, only the NCP P260 strain produced solvent yields above 20%. This strain consistently produced solvent yields above 30% and solvent concentrations above 18 g/l. Seven of the strains representing the different sub-groups produced solvent levels from 9-12 g/l while eight of the strains produced solvent concentrations ranging from trace levels to just over 6 g/l.

Comparison of NCP Industrial Strains on Molasses Medium

In addition, a separate comparative fermentation study was undertaken with 38 industrial strains originating from the

NCP strain collection listed in Table 3. Of these strains, 19 have been classified as *C. beijerinckii*, and 19 as *C. saccharobutylicum* on the basis of 16S rRNA sequence data, genomic DNA fingerprint patterns, and biotype characteristics (Shaheen, 1997). The 19 strains of *C. saccharobutylicum* can be further sub-divided into two DNA fingerprint groups with 14 strains belonging to Group I and five strains belonging to Group II. As the standard industrial fermentation process operated by NCP utilised molasses containing around 6.5% fermentable sugars (Spivey, 1978) the concentration of fermentable sugars was increased from 6% to 6.5% for these studies. The levels of solvent concentrations and yields obtained with the 38 industrial production strains belonging to the three groups again varied quite considerably under the conditions used. Within each group there were a number of strains which only exhibited mediocre solvent-producing ability while there were three or four strains in each group that produced high levels of solvents. The results obtained in these comparative fermentation studies are summarised in Table 4. Two of the strains belonging to the *C. beijerinckii* group exhibited solvent yields in excess of 30% while five of the strains belonging to the *C. saccharobutylicum* group exhibited yields in the range of 27-30%.

Table 3. Industrial Strains from the NCP Strain Collection

<i>C. saccharobutylicum</i>	<i>C. beijerinckii</i>
Group 1	Group 3
BAS/B3 CSC 1944	BAS/B SW 136 NCP 1946
37/3 IMMUNISED GS GB 1950	BAS/B/SW/336 NCP 1946 (B)
NCP P108	BAS/B/136 NCP 1946
NCP P172(S)	NCP P106
NCP P195	NCP P172(B)
NCP P199	NCP P193
NCP P200(S)	NCP P200(B)
NCP P202(S)	NCP P202(B)
NCP P220	NCP P254(B)
NCP P249	NCP P259
NCP P254(S)	NCP P260
NCP P262	NCP P261
NCP P265(S)	NCP P263
NCP P268	NCP P264
	NCP P265(B)
Group 2	NCP P270
NRRL B643 CSC 1946	NCP P271
BAS/B3/SW/336(S)	NCP P272(B)
162/BI NCP 1960	NCP P280
NCP P258	
NCP P272(S)	

The Effect of Increasing Sugar Concentrations in Molasses Medium

The preliminary studies undertaken on optimising the supplemented molasses fermentation medium supported the data contained in the patent and scientific literature that states that many strains of solvent-producing clostridia cannot effectively utilise molasses when the concentration of fermentable sugars exceeds 6 to 6.5%. Exceptions to this are two types of novel industrial strains patented by CSC in the late 1930's and early 1940's. Both of these types represented by *C. saccharobutylicum* P258 and P262 and *C. beijerinckii* P260 were reported to be able to produce high solvent concentrations and yields in molasses

Table 4. Comparative Fermentations of 38 Industrial Strains from the NCP Strain Collection in Molasses Medium Containing 6.5% Fermentable Sugars

NCP industrial strains	Solvent concentration (g/l)	Solvent yield (%)	Acetone ratio (%)
<i>C. saccharobutylicum</i> Group I			
Range of the 14 strains tested (listed in Table 3)	13-19	20-29	25-38
Average of the 14 strains tested	14.4	22.2	29
Average of the best performing strain (NCP P108) ^a	18.6	28.6	31
<i>C. saccharobutylicum</i> Group II			
Range of the five strains tested (listed in Table 3)	14-20	22-30	25-38
Average of the five strains tested	16.5	25.3	28
Average of the best performing strain (BAS/B3/SW/336(S)) ^a	19.6	30.0	31
<i>C. beijerinckii</i>			
Range of the nine best performing strains ^b	13-22	25-34	24-36
Average of the nine best performing strains	17.7	26.2	31
Average of the best performing strain (NCP P260) ^a	21.9	33.4	35
Average of the other ten strains	12.0	18.5	19

^aAverages determined from a minimum of three fermentations per strain

^bStrains were BAS/B/136, NCP P106, NCP P172(B), NCP P200(B), NCP P202(B), NCP P254(B), NCP P260, NCP P264, and NCP P270.

substrates containing up to 7.5% fermentable sugars. These patented strains were utilised as the main industrial production strains by CSC and the three groups of industrial solvent-producing strains utilised at NCP (Table 3) are known to be derived from strains provided by CSC in 1944 (Jones and Keis, 1995).

A series of fermentations were undertaken to determine the concentration of molasses that would limit solvent production in the two NCP species. The NCP P260 strain was selected as the representative strain for the *C. beijerinckii* species. This strain was inoculated into supplemented molasses fermentation media with fermentable sugar concentrations ranging from 6% to over 8% fermentable sugars. The results obtained from three fermentations at each fermentable sugar concentration are given in Table 5. These results showed that solvent concentration continued to increase as sugar concentration was increased up to 7.5% while solvent yields remained fairly constant at around 31.5%. At a sugar concentration of 7.8% one of the fermentations proceeded normally giving a final solvent concentration of 23.4 g/l with a solvent yield of 30% while the other two fermentations failed to produce normal solventogenic fermentations. Solvent production was inhibited in all fermentations above this sugar level. A noticeable effect on fermentation profiles was the production of decreasing ratios of butanol to acetone as the concentration of fermentable sugars was increased (data not shown).

Similar fermentations were performed with six of the top performing NCP *C. saccharobutylicum* strains (Group I strains: NCP P260, BAS/B3, and 37/3; Group II strains: NCP 258, BAS/B3/SW/336, and NRRL B643) (data not shown). Although all of these strains continued to produce solvents in molasses medium containing up to 8% fermentable sugars the solvent concentrations and yields produced were significantly less than those obtained with the NCP P260 strain. In molasses medium containing 7.5% fermentable sugars these six strains produced solvent concentrations ranging between 10.2 and 15.5 g/l with solvent yields ranging from 15.7% to 20.5%. These six strains also exhibited a decrease in the butanol to acetone ratio as the fermentable concentration of the molasses medium increased.

Development of a Maize Fermentation Medium

C. saccharobutylicum NCP P262 was selected as the initial test strain for the development of a maize mash fermentation medium. When this strain was grown in maize mash alone (80 g of maize/l), the final concentration of solvents obtained was only 1 g/l. In this fermentation

 Table 5. Effect of Increasing Sugar Concentration on Solvent Production by *C. beijerinckii* NCP P260 Grown in Molasses Medium: Results of Three Fermentations

Fermentable sugars (g/l)	Solvent concentration (g/l)				Solvent yield (%)		
	1	2	3	4	1	2	3
59.8	19.3	18.9	18.6	32.3	31.6	31.1	
64.4	20.9	20.5	19.6	32.5	31.8	30.4	
69.0	22.5	21.6	21.3	32.6	31.3	30.9	
73.6	24.4	23.9	22.6	33.2	32.5	30.7	
78.2	23.4	4.4	1.5	30.0	5.6	1.9	
82.8	9.1	0.1	0.1	11.0	-	-	
87.4	0.1	0.1	0.1	-	-	-	

Table 6. Comparative Fermentations of Strains from the Four Species in Maize Mash Medium

Strain ^a	Solvent concentration (g/l)	Solvent yield (%)
<i>C. acetobutylicum</i>		
NCIMB 619 (=ATCC 4259)	19.6	24.5
NCIMB 6443	19.5	24.4
NRRL B527 ^T	19.5	24.5
ATCC 824 ^T	19.2	24.0
NCIMB 2951 (=DSM 1732)	17.9	22.4
ATCC 3625	11.6	14.5
NCIMB 6441	11.4	14.3
ATCC 8529	10.0	12.6
NCIMB 6442	6.8	8.5
<i>C. saccharobutylicum</i>		
NCP P262	11.3	14.1
NCP P258	10.8	13.5
NCP P286	10.2	12.8
<i>C. saccharoperbutylacetonicum</i>		
N1-4	14.2	17.8
N1-504	13.1	16.3
<i>C. beijerinckii</i>		
NRRL B592	16.2	20.8
NRRL B593	14.1	17.6
NCP P259	14.6	18.3
NCP P264	14.5	18.1
NCP P260	11.3	14.0
NCIMB 8653 ^b	11.2	14.0
NCIMB 6445 ^b	11.1	13.9
NCIMB 6444 ^b	3.3	4.1
ATCC 14823	0.5	0.6
ATCC 10132 ^b	0.1	0.1

^a T=Type strain

^b Same genomic DNA fingerprint group as NCIMB 8052

medium the pH of the mash decreased from 5.4 to 4.4 within 4 h of inoculation and cell degeneration occurred. These results suggested that the buffering capacity of the maize mash on its own was insufficient to prevent the pH of the mash falling below a critical threshold level for this strain. Various combinations of buffering agents were added to the maize mash and the addition of a combination of calcium carbonate, ammonium phosphate and dried brewers yeast resulted in a 10-fold enhancement of the final concentration of solvents. Maximum solvent concentrations were obtained with 10 g/l of dried brewers yeast, 2 g/l of calcium carbonate and 1 g/l of ammonium phosphate. This was adopted as the standard supplemented maize medium (SMM) for later studies.

Substitution of dried yeast with yeast extract resulted in a slight decrease in the solvent levels while substitution with a variety of other ammonium and phosphate compounds did not affect solvent production.

Comparison of Fermentation Characteristics on Maize Medium

Twenty-four representative strains from the four solvent-producing species were evaluated for their ability to produce solvents from starch using SMM. These included nine strains of *C. acetobutylicum*, three strains of *C. saccharobutylicum*, two strains of *C. saccharoperbutylacetonicum*, and ten strains of *C. beijerinckii* (Table 6). All of the strains tested grew well on SMM and utilised starch without the need for prior saccharification.

Of the nine *C. acetobutylicum* strains tested, four strains, ATCC 824, NCIMB 619, NCIMB 6443, and NRRL B527, produced over 19 g of solvents/l in SMM. The minimum pH attained during the fermentation by these strains varied between 4.4 and 4.6, indicating that these strains were able to tolerate lower pH levels than strains from the other three saccharolytic species without affecting solvent production. The strains which produced the highest solvent levels were tested for reproducibility and two strains, ATCC 824 and NCIMB 6443, were found to consistently produce solvent levels between 18 and 23 g/l in SMM with solvent yields on dry maize meal of around 24.5% (amount of dry maize meal converted to solvents, expressed as a percentage). The three strains of *C. saccharobutylicum* tested consistently produced around 11 g/l solvents and the two strains of *C. saccharoperbutylacetonicum* around 14 g/l solvents. Solvent production in the ten *C. beijerinckii* strains tested ranged from trace amounts (ATCC 10132 and ATCC 14823) up to levels of around 16 g/l produced by strain NRRL B592. Most of the other *C. beijerinckii* strains tested produced solvent concentrations in the range of 11 to 14.5 g/l.

The morphology and physiology of *C. acetobutylicum* ATCC 824 and NCIMB 6443 during fermentation in SMM were monitored. Neither strain showed clostridial stage formation, granulose accumulation, or forespore development in the maize medium. In these fermentations, the pH of the medium gradually decreased, reaching a minimum value of pH 4.4–4.5 at about 20 h. The concentrations of organic acids also reached their highest levels at this time (6–8 g/l) and then gradually decreased to between 1–4 g/l by the end of the fermentation. Final solvent concentrations of approximately 19 g/l were attained after 42–46 h.

Efficiency of Carbohydrate Utilisation in Maize Medium

The efficiency of carbohydrate utilisation was examined over a range of maize concentrations from 20–100 g/l using ATCC 824 as the test strain. A small amount of unfermented starch remained at the end of the fermentation over the entire range of maize concentrations investigated. At maize concentrations of 80 g/l or less, a basal level of maize equivalent to approximately 2 g of glucose/l remained unhydrolysed. The optimum carbohydrate utilisation (around 97%) occurred at maize concentrations of between 60–80 g/l and optimum solvent concentrations and yields were obtained at maize concentrations of 80 g/l. At 20 g of maize/l maize efficiency of utilisation dropped to around 91%. As the maize concentration was increased above 80 g/l, the amount of residual starch increased and at 100 g of maize/l, residual starch produced a glucose concentration of 15 g/l giving an efficiency of only 84%.

Effect of Pretreatment of Maize

The effect of various types of pretreatment of the maize on the efficiency of the fermentation was investigated. A reduction of the particle size of the maize by milling decreased the buffering capacity of the maize mash. When the maize was ground to a fine powder (approximately 40–60 mesh), the final pH decreased to between pH 4.4 and 4.5 compared with a final pH in the control culture of pH 4.9. In these cultures, the solvent yield was 6–63% lower than the control culture and final solvent concentrations

dropped by between 6 and 16 g/l. The effect of a reduction in the time required for sterilisation and gelatinisation of the maize was investigated by autoclaving for 5, 10, 15, 20, 25, 30, and 60 min at 121°C. The reduction of the time to 5 min at 121°C did not have any significant effect on the outcome of the fermentation and in these experiments solvent concentrations and yields did not vary by more than 4% from the mean values.

The effect of enzymatically saccharifying the maize both prior to and simultaneously with the fermentation was also evaluated. In experiments where saccharification preceded fermentation, the free glucose concentration in the mash ranged from 11–63 g of glucose/l (13–76% of the total glucose available from starch). During the subsequent fermentation, the pH of the mash decreased to between pH 4.2 and 4.3 and less than 1 g/l of solvents were obtained. The addition of enzymes at the start of the fermentation also resulted in a decrease in the pH to pH 4.3 or below and little or no solvent production was obtained, with the exception of one fermentation where the culture pH remained at pH 4.8 and a final solvent concentration of 23 g/l was obtained.

Discussion

The aim of this study was to undertake a comparison of the solvent-producing ability of representative culture collection and industrial clostridial strains under standardised conditions. Fermentation substrates and culture conditions were selected to provide conditions that might be expected to allow close to maximum solvent concentrations and solvent yields to be achieved. However, in order to allow direct comparison it was necessary to utilise a common set of culture conditions for all strains. While the particular set of culture conditions selected might have been close to optimum for some strains and species it is unlikely that these conditions would be optimal for all. Optimisation of growth and fermentation conditions for all strains was not feasible. Facilities for undertaking large numbers of bench-scale fermentations were not available. Therefore, individual fermentations were performed in an anaerobic glove cabinet at 33°C with no stirring or pH control, conditions not dissimilar to culture conditions employed in the standard industrial batch-fermentation processes. Although pH and the production of acetate, butyrate, ethanol, isopropanol, acetone, and butanol were monitored in all fermentations this data has not been included. Limitation of the data presented, to solvent concentrations and yields only, was done for brevity, and because the additional data did not add significantly to the conclusions. In many fermentations the fermentation profile and changes in cell morphology were also monitored over time but this data has been omitted for similar reasons.

The TYA medium was selected as a general laboratory culture medium for comparative studies as in the preliminary trials this culture medium produced better results than other culture media tested. However, it is likely that TYA medium was not ideal for many strains. The most likely reason for this is the limiting buffering capacity of the TYA medium. Increasing the sugar concentration in the TYA medium resulted in the production of higher acid levels and there was evidence of poor conversion to solventogenesis and acid crash symptoms in a number of fermentations. It is possible that higher solvent

concentrations and yields could have been obtained in this culture medium if adequate buffering capacity could have been provided or pH control utilised. As high solvent yields and acceptable solvent concentrations were obtained in TYA medium containing 4% glucose with the test strains, comparative studies were restricted to this sugar concentration. The results obtained in TYA medium for the most part correlated reasonably well with the results obtained when the same strains were grown in molasses and maize medium, with a few notable exceptions. Although the *C. acetobutylicum* strains performed well in both the TYA medium and maize medium, they did not perform well in molasses medium. This finding supports similar observation in the literature (McCutchan and Hickey, 1954; Ryden, 1958; Hastings, 1971; Walton and Martin, 1979). The NCP strains identified as *C. beijerinckii* did not perform well in the TYA medium but gave good results on both the molasses and maize fermentation substrates. The differences highlight the pitfalls of comparing the performance of strains under any one specific set of culture conditions.

The aim of the comparative studies on the molasses substrate was to achieve solvent concentrations and yields that were comparable to those reported in the industrial fermentation process. In developing the molasses fermentation medium, an attempt was made to duplicate culture conditions utilised in the industrial fermentation process as closely as possible while ensuring that the nutrient supplementation was of a sufficient level not to disadvantage any specific molasses-fermenting strain or group. For comparative purposes a fermentable sugar concentration of 6% was used as the performance of a number of strains decreased significantly at higher sugar concentrations. The comparative studies on molasses revealed that strains of *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* performed well on this substrate. Of the *C. beijerinckii* strains tested the industrial strains belonging to the NCP group and the NRRL B592 strain performed well while industrial strains belonging to the group represented by strain NCIMB 8052 only performed moderately well. Other strains of *C. beijerinckii* and the strains of *C. acetobutylicum* tested did not perform well in the molasses fermentation medium.

As the industrial fermentation process operated by NCP utilised molasses substrates with a fermentable sugar concentration of around 6.5%, comparative fermentation profiles for the 38 strains held in the culture collection were undertaken at this higher sugar concentration. Although the majority of the strains belonging to the two species that were tested were derived from production strains utilised in the factory at one time or another, considerable variation of performance was observed. The best performing strains produced solvent concentrations and yields equal to the best results recorded by NCP. However, some of the strains performed indifferently or poorly in the molasses fermentation medium confirming that the ability to produce significant levels of solvents can become decreased or lost over time. It is possible that enhanced solvent production could be recovered in some of these strains by subjecting them to several cycles of heat-shock and endospore selection that was common practice (Beesch, 1953; McCutchan and Hickey, 1954).

The results obtained in the studies which involved increasing the fermentable sugar concentrations in

molasses, using NCP P260, are significant in that solvent concentrations continued to increase while a constant high solvent yield was maintained until the sugar concentration reached around 7.8%. The P260 strain, along with a few closely related NCP strains, emerged as the most effective solvent-producing strain on molasses and consistently produced solvent concentrations and yields equivalent to or exceeding the very best levels achieved at NCP. The NCP *C. saccharobutylicum* strains tested in this study were not able to produce the same high level of solvent production at elevated sugar concentrations.

In the industrial AB fermentation process, maize mash was normally used without further nutritional supplementation as the maize was considered to contain all the nutrient requirements for growth and solvent production (Gabriel, 1928; Walton and Martin, 1979). In the laboratory-scale experiments with the test strain, low solvent yields and concentrations were obtained when the fermentation substrate consisted of maize mash alone. The low levels of solvents produced in this fermentation medium were associated with low pH levels during the fermentation and appeared to be due to insufficient buffering capacity. The addition of a combination of buffering agents to the maize resulted in greatly enhanced yields of solvents. With a number of *C. acetobutylicum* strains the yields and final concentrations of solvents obtained in the buffered maize mash medium were comparable with the maximum values reported for the industrial fermentation process using maize as the raw material (Beesch, 1953; Walton and Martin, 1979). In addition to enhancing the buffering capacity of the maize mash, the buffering agents provide additional nutrients, but their specific role in enhancing solvent yields and concentrations was not investigated further.

There is little published information on the ability of the four industrial solvent-producing species to ferment starched-based substrates. Of the 24 solvent-producing strains tested, all were able to degrade starch effectively and grew on raw maize starch without the requirement for saccharification. Only two of the strains tested failed to produce any significant levels of solvents and 20 of the strains produced in excess of 10 g/l solvents in SMM. However, the performance of a number of strains appeared to be adversely affected by the low pH levels that were recorded in the SMM. The fermentation profiles were similar to those obtained in a molasses medium previously reported by Jones (1982) but the longer fermentation times observed in SMM were apparently a result of a decrease in the specific growth rates of strains compared with their growth rates in molasses.

In the industrial fermentation process, maize concentrations varying between 60 and 100 g/l were used (Beesch, 1953; McCutchan and Hickey, 1954; Walton and Martin, 1979), but little information is available regarding the maximisation of starch utilisation. The results of this study showed that a maize concentration of 80 g/l gave both the maximum starch utilisation and the maximum solvent production. With maize concentrations above this level, escalating amounts of residual starch remained and the starch utilisation decreased, while at concentrations below this level, the amount of residual starch at the end of the fermentation remained low but the concentration of solvents decreased. The efficiency of starch utilisation was not enhanced by enzymatic saccharification of the starch either prior to or during the fermentation or by size reduction

of the maize particles. On the contrary, both these treatments appeared to have a detrimental effect on the fermentation by adversely affecting the buffering capacity of the SMM. Starch utilisation was not affected by a 12-fold decrease in the sterilisation and gelatinisation time. A reduction in cooking time may significantly reduce the cost of solvent production as maize was routinely cooked for between 60 and 90 min at 130 – 133°C (Beesch, 1953; Walton and Martin, 1979). Although no problem with contamination was encountered during these experiments, increased contamination could be a major limitation in reducing the cooking time.

The results of this study indicate that many strains of solvent-producing clostridia are able to utilise raw maize starch for solvent production with a high level of efficiency. The inability of some strains to produce high final concentrations of solvents appeared to be due to an inability to degrade all of the starch substrate in combination with the inability of the fermentation medium to maintain the pH of above a level necessary for cell viability.

Although a number of the *C. acetobutylicum* strains tested performed well in the TYA and SMM none of the strains performed well on molasses substrates. A number of strains currently held in culture collections have retained the ability to produce high levels of solvents comparable to those reported in the literature. Therefore, *C. acetobutylicum* appears to be the species with the greatest potential for starch-based substrates.

The majority of *C. saccharobutylicum* strains tested performed well on TYA and molasses medium, but performed only moderately well on the maize medium. The results obtained, even with the best strains, were slightly less than those reported in the literature and those recorded at NCP, suggesting that the conditions used in this study might not have been optimal. The strains belonging to this species appear to be good all-round performers with potential for use in mixed agricultural and waste-based substrates.

The *C. saccharoperbutylacetonicum* strains performed well in all three fermentation media tested. Although not the top performer on either the sugar- or starch-based substrates, they performed adequately and are therefore versatile strains that could prove to be particularly useful on mixed substrates. In addition, the high proportion of butanol produced could be advantageous.

Amongst the *C. beijerinckii* strains tested, the NRRL B592 strain also stood out as a good all-round performer with potential for use on mixed substrates. The industrial strains originating at CSC and utilised by NCP were by far the most effective strains on molasses substrates, capable of maintaining high concentrations and yields of solvents at fermentable sugar concentrations as high as 7.5%. The NCP strains also performed moderately well on maize substrates but not in the TYA medium. This suggests that these strains may have more demanding growth requirements than other strains. The other group of *C. beijerinckii* strains originating as industrial isolates, represented by the NCIMB 8052 strain, did not perform particularly well in any of the three fermentation substrates used in this study. From the information contained in the literature it would appear that these early isolates were only moderately successful as industrial solvent-producers. However, it is possible that either the particular culture conditions used were not suitable, or that these strains have lost solvent-producing ability.

A number of intrinsic limitations are encountered when attempting to compare the solvent-producing performance of strains used in this study with performances reported in the literature. These included the validation of the identity of the strains (Jones and Keis, 1995) and that in some cases more than 50 years separate the current study and publication of the original data. In addition, in many cases the published data was generated under fermentation conditions that differed from those used in this study.

Experimental Procedures

Microorganisms

The solvent-producing clostridial strains used in this study, obtained from type culture collections, are listed in Table 7. The industrial production strains originating from the NCP strain collection included in this study, are listed in Table 3.

Fermentation Media and Culture Conditions

The methods used for the maintenance and propagation of the clostridial strains, the modified *Clostridium* basal medium (CBM) and the modified tryptone yeast acetate glucose medium (TYA medium) used in this study have been described previously (Keis *et al.*, 1995). An anaerobic glovebox (Forma Scientific Industries, Marietta, Ohio) was used for anaerobic incubation and manipulation. Unless otherwise stated, batch fermentations were carried out in two hundred ml volumes inoculated with 10 ml of actively growing culture (0.4 OD₆₀₀) and incubated anaerobically at 33°C without stirring or pH control.

The supplemented molasses medium contained varying concentrations of Australian blackstrap molasses (Healtheries Ltd., New Zealand) with 10 g/l yeast extract, 3.2 g/l calcium carbonate, 2 g/l ammonium sulphate, and 1 g/l calcium phosphate. The concentration of fermentable sugars utilised in the molasses medium ranged from 6 to 9.5%.

The supplemented maize medium (SMM) contained 80 g/l of commercial degermed white maize meal of a particle size approximately equal to 20 mesh. The buffering capacity and nutrient composition of the maize mash was enhanced by the addition of 1 g/l ammonium phosphate, 2 g/l calcium carbonate, and 10 g/l dried brewers yeast.

All media were adjusted to pH 6.5 prior to autoclaving. The media were autoclaved (121°C at 15 kPa) for 15 min except SMM which was autoclaved for 60 min, unless otherwise stated. After sterilisation, the media were transferred to an anaerobic glovebox while still warm and allowed to reduce overnight.

Pretreatment of Maize

The enzyme saccharification of the maize mash was carried out by the addition of α -amylase (Termamyl 120L, Novo Industria, Denmark) and glucoamylase (AMG 150L, Novo Industria, Denmark). To examine the effect of saccharification prior to fermentation, the α -amylase (120 KNU/g) and/or glucoamylase (150 Ag/ml) were added before inoculation to give a final concentration of 2% (v/v) and the SMM was incubated under the conditions recommended by the manufacturers (α -amylase at 90°C for 30 min, glucoamylase at 60°C for 60 min). To examine the effect of simultaneous saccharification and fermentation, either or both of the enzymes were added at the time of inoculation to give a final concentration of 1% (v/v) and the SMM was incubated at 33°C.

The residual starch content of SMM was determined as the concentration of glucose released after hydrolysis with 2% (v/v) α -amylase and 2% (v/v) glucoamylase at 60°C for 2 h. The glucose concentration in the supernatant was measured using a Beckman Glucose Analyser 2 (Beckman Instruments). The starch content of the unfermented maize was determined by analysing triplicate samples of maize mash containing five different concentrations of maize ranging from 20 to 100 g/l and averaging the results.

Gas Chromatography

The acids and solvents produced during the fermentations were measured by gas chromatography using a Hewlett Packard HP5890 series II GC (Hewlett Packard) fitted with a wide-bore carbowax capillary column (Econocap 19659, Alltech Associates) using conditions and procedures described previously (Long *et al.*, 1984).

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Table 7. Bacterial Strains from Type Culture Collections

Species	Strain ^a	Reclassified designation (Keis, 1996)
American Type Culture Collection (ATCC) strains		
<i>C. acetobutylicum</i>	ATCC 824 ^T	
<i>C. acetobutylicum</i>	ATCC 4259	
<i>C. acetobutylicum</i>	ATCC 2951	
<i>C. acetobutylicum</i>	ATCC 3625	
<i>C. acetobutylicum</i>	ATCC 10132	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	ATCC 39058	<i>C. beijerinckii</i>
<i>C. beijerinckii</i>	ATCC 6014	
<i>C. beijerinckii</i>	ATCC 6015	
<i>C. beijerinckii</i>	ATCC 11914	
<i>C. beijerinckii</i>	ATCC 14823	
<i>C. beijerinckii</i>	ATCC 17795	
Deutsche Sammlung von Mikroorganismen (DSM) strains		
<i>C. acetobutylicum</i>	DSM 1732	
<i>C. acetobutylicum</i>	DSM 1739	<i>C. beijerinckii</i>
National Collection of Industrial and Marine Bacteria (NCIMB) strains		
<i>C. acetobutylicum</i>	NCIMB 619	
<i>C. acetobutylicum</i>	NCIMB 2951	
<i>C. acetobutylicum</i>	NCIMB 6441	
<i>C. acetobutylicum</i>	NCIMB 6442	
<i>C. acetobutylicum</i>	NCIMB 6444	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NCIMB 6445	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NCIMB 8049	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NCIMB 8052	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NCIMB 8653	<i>C. beijerinckii</i>
<i>C. beijerinckii</i>	NCIMB 9362 ^T	
<i>C. beijerinckii</i>	NCIMB 9503	
<i>C. beijerinckii</i>	NCIMB 9579	
<i>C. beijerinckii</i>	NCIMB 9581	
<i>C. beijerinckii</i>	NCIMB 11373	
<i>C. beijerinckii</i>	NCIMB 12404	
Northern Regional Research Laboratory (NRRL) strains		
<i>C. acetobutylicum</i>	NRRL B527	
<i>C. acetobutylicum</i>	NRRL B591	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NRRL B594	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NRRL B597	<i>C. beijerinckii</i>
<i>C. acetobutylicum</i>	NRRL B643	<i>C. saccharoperbutylacetonicum</i>
<i>C. beijerinckii</i>	NRRL B592	
<i>C. beijerinckii</i>	NRRL B593	
Other strains		
<i>C. butanologenum</i>	IAM 19015	<i>C. beijerinckii</i>
<i>C. saccharoperbutylacetonicum</i>		N1-4
<i>C. saccharoperbutylacetonicum</i>		N1-504

^aT=Type strain

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