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Anaerobic, solvent-producing bacteria: Molecular characterisation, polysaccharolytic activity and agroindustrial waste degradation

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Anaerobic, solvent-producing bacteria: molecular characterisation, polysaccharolytic activity and agro-industrial waste degradation

INTRODUCTION

This work's main goal was to study strategies for the molecular and enzymatic characterisation of new solvent-producing mesophilic *Clostridium* isolates from Colombia and ascertain their solvent producing biotechnological potential by using a cheap agro-industrial waste as carbon source. *Bacterium* solvent production (using *Clostridium acetobutylicum*) has been studied for many decades because of the economic importance of its fermentation end-products: butanol, acetone and hydrogen.

The Weizmann strain has always given poor results, compared to a second group of industrial strains, which used inverted molasses to produce acetone and butanol, successfully isolated by North-American companies. The industrial process was stopped after the Second World War in western countries (early 1960s), but operated in South Africa until the early 1980s and is still operating in China today (Keis and Jones 1995).

The resurgence of interest in this organism has been due to projected future increases in oil prices and the growing unreliability of supply. Any process using clostridia would be of benefit to the economy (and also to the environment) in those countries where excess biomass is available as a renewable energy source; however, the bacterial solvent production process from biomass is not economically feasible with the currently available strains, needing considerable improvement in strains and a cheap carbon source. The application of molecular biology's methods to genetically improving the *Clostridium* genus and other anaerobes is generally still in a fledgling state and

practically non-existent in Colombia, despite the fact that these organisms have been used for several years as black-boxes in anaerobic digestion processes for the treatment of waste.

The Clostridium genus encompasses a collection of Gram-positive, obligatory anaerobic, non-sulphate-reducing, spore-forming, rod-shaped organisms (Cato and Stackebrandt, 1989). Over 100 species are currently recognised (Hippe *et al*., 1992), displaying a wide range of phenotypes, including psychrophiles, thermophiles and acidophiles. A group of bacteria is recognised as being a member of a species when its DNA sequence similarity (as measured by DNA-DNA hybridisation) is above a 60%-70% cut-off value, a value based on comparison of natural bacterial groups sharing many phenotypical characteristics (biotypes) (Stackenbrandt and Goebel,1994).

All solventogenic bacteria used in the ABE (acetone, butanol and ethanol) process belong to clostridial Group 1. Several independent studies have shown that the solvent-producing clostridia in themselves are a heterogeneous bacterial group, encompassing the following species: *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum, C. madisonii, C. butyricum, C. saccharo-butyl-acetonicum-liquefaciens* and others (Keis *et al*., 1995). Solvent production alone is not a useful tool for classification (George *et al*.,1983); this finding can account for significant differences in contrasting different strains originally assigned *to C. acetobutylicum.*

Molecular biology methods have recently become widely accepted as being a basic tool for taxonomy, i.e. for assigning strains to a biologically meaningful group (species). Phylogenetic trees, based on equivalent data sets of 23S and 16S bacterial rRNAs are in good agreement (Rainey *et al.,* 1993) and their overall topology is supported by alternative phylogenic markers such as elongation factors and ATPase sub-units (Ludwig and Schleifer, 1994). Lawson *et al.,* (1993) determined the 16S rRNA sequences of 17 species from the *Clostridium* genus by directly sequencing their PCR amplified genes. A recent systematic study of 55 solvent-producing strains used

biotyping and DNA fingerprinting analysis (Pulse Field Gel Electrophoresis [PFGE] of chromosomal DNA digested with restriction endonucleases) (Keis *et al.*, 1995). This study resulted in 4 phylogenetic groups being suggested, based on 16S rRNA sequencing, which were named:

I *Clostridium acetobutylicum*, containing the original Weizmann strain, the ATCC 824 type strain and other early *C. acetobutylicum* strains isolated on starch as substrate (including DSM 1731, 1732, 1733, 1737);

II *Clostridium saccharo-butyl-acetonicum-liquefaciens*, containing *C. acetobutylicum* NCP262, NRRL B643 and other NCP strains from biotype group 2;

III *Clostridium saccharoperbutylacetonicum*, containing the N1-4 (=ATCC 12564) and N1-504 (=ATCC 27022) strains from biotype groups 3 and 4; and

IV *Clostridium beijerinckii*, containing biotype groups 5, 6, 7, 8 and 9, including many former *C. acetobutylicum* (NCIMB 8052, 8049, 6444, 6445, 8653 and others), *C. beijerinckii*, *C. madisonii* and *C. saccharo-acetobutylicum* strains.

This study presents a practical basis for the future taxonomic classification of new isolates, by using simple methods such as rRNA-gene sequencing and DNA fingerprinting.

Biochemistry and physiology

Clostridia employ the Embden-Meyerhof route to break down hexose sugars (including mono-, di-, tri-, and poly-saccharides) with 1 mol hexose being converted into 2 moles pyruvate, resulting in 2 mol ATP and 2 mol reduced NADH net production (Thauer *et al.*, 1977; Gottschalk, 1986). Pentose sugars are metabolised by pentose phosphate route. Fermented pentose is converted into pentose 5-phosphate by transketolases-transaldolase enzyme, resulting in the reduction of glucose 6-phosphate and glyceraldehyde-3 phosphate which enter the glycolytic route. 3 mol pentose yield 5 mol NADH. Acid sugars, such as gluconate, are degraded via modified Entner-Doudoroff route (Andreesen and Gottschalk, 1989).

Pyruvate is a central element in the formation of fermentation products. It is usually converted into acetyl-CoA by pyruvate ferredoxin-oxide reductase in nearly all clostridial fermentation. In addition to acetyl-CoA, CO_2 and reduced-ferredoxin products are also formed during the reaction. In many *Clostridium* species the primary role of NADH ferredoxin-oxidoreductase, which requires acetyl CoA as an activator (Jungermann *et al.*, 1973), is NAD^+ regeneration by reduced ferredoxin-oxidoreductase production to produce NADPH by biosynthesis. Under appropriate NADPH conditions, the reduced ferredoxin is able to transfer electrons to iron contained in hydrogenase, permitting the use of protons as a final electron acceptor, resulting in the production of molecular hydrogen. Ferrodoxin is oxidised during this step and hydrogen gas is released from the cell.

Acetyl-CoA is a branch point between acid- versus solvent-production in *C.acetobutylicum* and it can be different, depending on the clostridial species. It can be converted into a mixture of ethanol, acetate and or butyrate. The ratio in which these products are formed depends on the amount of H_2 involved. Extra H_2 evolution relieves the fermentation redox and allows the direct conversion of part of the acetyl-CoA via acetyl phosphate into acetate. The fact that a number of clostridia produce more ethanol than acetate indicates that fermentation is not always optimised with respect to ATP yield (Andreesen *et al.*, 1989).

Several research groups have studied acetone, butanol and ethanol production (ABE fermentation) by means of *Clostridium acetobutylicum* solvent producing-strains. This fermentation occurs in two phases; acids are produced during the fist phase, the pH decreases to around 4.8. Then the solventogenic phase is switched on, and the acids are reduced to acetone, ethanol and butanol; the pH is around 5.5 during solvetogenesis. Many of the metabolic route's enzymes have now been characterised (Shen, 1993). Several genes related to the metabolic route have been isolated and sequenced. The solventogenetic shift's molecular mechanisms are still being studied.

Carbon and electron flow regulation

Final fermentation products depend on micro-organism growth conditions via the metabolic route. Various control elements, which are mainly identified in *C. acetobutylicum,* are shown in the metabolic route (

Figure 1), as well as those enzymes involved (Table 1).

CoA and its acylate derivatives (acetyl-CoA and butiryl-CoA) play a central role in regulating carbon and electron flow, this in turn being a key part of butanol and ethanol production (Jones *et al.,* 1986). An increase in acetyl-CoA or acetyl-CoA/CoA concentration stimulates conditions for electron transfer between ferredoxin cofactors and the pyrimidin nucleotide, orientating the direction of the electron flow towards reduced cofactor concentrations. Hydrogen electron flow activates NADH ferredoxin-oxidoreductase and is orientated towards solvent production.

A >0.5 increase in NADH/NAD quotient causes an almost complete inhibition of *C. acetobutylicum* glyceraldehyde 3-phosphate dehydrogenase (Girbal and Soucaille, 1995). The concentrations of NAD/NADH and acetyl-CoA/CoA have been postulated as playing key roles in regulating electron flow in the cell; they function as sensors for both ATP regeneration and H_2 production (Datta R. and J.G. Zeikus, 1985). It has not been possible to demonstrate the ATP/ADP quotient's function as regulating final product selectivity. In effect, the limiting carbon also lowers the proportion of ATP/ADP in a culture and butanol production is high (Girbal and Socaille, 1994).

Comparing concentrations in different metabolic rates during acidogenesis or solventogenesis produces regulation signals (Girbal and Soucaille, 1995). The step from acid to solvent production can thus be correlated with a drastic increase in the reduced pyrimidin nucleotide to oxidised cofactor ratio (Grupe and Gottschalk, 1992; Girbal and Soucaille, 1995). Solventogenesis also almost immediately starts to descend to ATP

levels. The solvent production shift is characterised by a rapid increase in butyryl-CoA concentration, acetyl-CoA and free CoA concentration simultaneously decreasing (Boyton et al., 1996). The *C. acetobutylicum* enzyme *adhE (aad*) has been described as playing a key role in the initiation of solventogenesis (Nair and Papoutsakis, 1994).

Gene and enzyme organisation

The *Clostridium acetobutylicum* ATCC 824 genome has been sequenced and was found to consist of 3,940,840 bp, 3,740 polypeptide-encoding ORFs and 107 stable RNA genes. The megaplasmid pSOL1, consisting of 192,000 bp, appears to encode 178 polypeptides. It has 2 prophages, apparently cryptic for 90 Kbp but 60 Kbp contain 85 and 79 genes, respectively.

It is known that the genes encoding enzymes present in the metabolic route for ABE fermentation in *C. acetobutylicum* are found predominantly in operon polycystrons or clusters. The largest transcription unit is formed by the butyryl-CoA synthase region; this embraces those genes encoded by *crt* crotonase, *bcd* butyryl-CoA dehydrogenase and *hbd* 3-hydroxyl butyryl-CoA dehydrogenase.

The cluster of genes encoding those enzymes taking part in acetone production embraces the gene for *adc* acetate-decarboxylase and the familiarly called sol operon (Dürre *et al.*, 1995; Petersen *et al.*, 1993; Nair *et al.*, 1994). The latter is composed of *aad (adhE)* aldehyde/alcohol dehydrogenase, as well as *ctfA, ctfB*, genes also encoding two CoA-acetate/butyrate CoA transferase units. This gene cluster represents an excellent tool for butanol-acetone production. The ORF 4 region shows affinity with genes encoding α-amylase (Cornillot *et al.*, 1997). ORF 5, which is upstream of the sol operon *in Clostridium acetobutylicum*, does not act as transcription repressor in *Clostridium acetobutylicum DSM* 792 but instead on enzyme involved in glycosylation-deglycosylation (Dürre *et al.*, 2001; Nölling *et al.*, 2001).

The synthetic "ace operon", which has three genes for acetone formation (*adc, ctfA* (acetate CoA-transferase) *and ctfB butyrate*-CoA transferase), is transcribed from the

adc promotor. The corresponding enzymes (acetate-decarboxylase and CoA-transferase) are best expressed at pH 4.5. However, highest solvent levels are obtained at pH 5.5. Reusing carboxylic acids is primarily done by means of acetyl-CoA transferase or butyryl-CoA transferase. Acetoacetate is decarboxylated to form acetone CO_2 by acetoacetate-decarboxylase (AADC). The resulting Acetyl-CoA and butyryl-CoA can be converted into alcohols. Forming acetone by the CoAT and AADC route is important for reassimilating carboxylic acids; the total quantity of carbohydrates can be catabolised. Strains containing the synthetic "ace operon" are expressed at elevated solvent levels (Mermelstein *et al.*, 1993; Nolling *et al.*, 2001).

The *bdhA* and *bdhB* genes are in neighbouring regions on the chromosome; they are monocystronically transcribed and differentially induced (Walter *et al.*, 1992). The expression of *bdhA* is the result of a successful shift to pH 5.5, whilst *bdhB* and sol operon genes have their maximum expression in the later stage of solventogenesis. The genes for the ptb phosphotransbutyrylase and buk butyrate-kinase enzymes are also arranged in operons . These operons' expression shows that ATP intracellular levels have a negative control (Girbal and Soucaille, 1995).

The thiolase gene and the *hydA* hydrogenase gene are not in the *C. acetobutylicum* chromosome (Nölling, *et al.*, 2001), like other gene clusters from other fermentations, and are expressed as monocystrons by transcription. These genes continue to be expressed during acidogenesis and solventogenesis in comparable levels. It is probable that thiolase and hydrogenase activity are primarily regulated at enzyme level.

Those genes involved in solventogenesis have already been identified on the megaplasmid and sequenced genome. However, those genes responsible for alcohologenesis (butanol and ethanol were being produced, but not acetone) were identified following the *C acetobutylicum* ATCC 824 genome sequence being identified,as well as a second alcohol aldehyde-dehydrogenase (CAP 0035), a piruvate-decarboxylase (CAP 0025) and an ethanol-dehydrogenase (CAP 0052), which are probably involved in this alcohologenic methabolism. As can be seen, some genes related to solventogenesis are found in the chromosome whilst others are in the

megaplasmid-pSol, suggesting that this is a very complex system, and that it would be interesting to discover whether the chromosome and megaplasmid complement each other metabolically. It has also been established that the megaplasmid has a second copy of the genes involved in PTS-type sugar transport (CAP0066-68) glicolysis (aldolase, CAP0064) and central metabolism (thiolase CAP 0078) (Nolling *et al.*, 2001).

The low-solvent-production phenomena in ABE fermentation

"Culture degeneration," which is a feature of the strain rather than of a particular batch fermentation, is associated with genetic change. This phenomenon is related to the segregation of the megaplasmid pSol in *C. acetobutylicum* ATCC *824* carrying the sol operon. This operon is made up by 4 genes for producing acetone and butanol: the *ctfA, ctfB,* and *adc* genes for producing acetone and *aad* for producing butanol (the latter gene encodes acetate-decarboxylase). The culture can degenerate if some cells lose the sol operon. These cells overgrow the plasmid-carryng cells but are then unable to produce solvents. Plasmid size has been shown to be 190 Kb and operon gene locus size 9.5 Kb (Cornillot *et al.*, 1997).

"**Acid crash**" is a phenomenon occasionally occurring in batch fermentation where the pH is not controlled in the non-dissociated acid concentration. This phenomenon occurs when non-dissociated acid concentration in the broth exceeds 50-60 mmol/l, resulting in fermentation activity becoming completely stopped and thus premature cessation of ABE production.The temperature can be reduced to avoid this phenomenon.

"Acidogenic fermentation," which occurs when batch fermentation is done at pH values close to neutrality, is due to rapid acid production followed by solventogenesis becoming inhibited when the total acid concentration reaches 240-250 mmol/l. Glucose concentration can be increased or organic nitrogen source can be reduced to avoid this phenomenon.

Polysaccharolytic enzymes

Polysaccharolytic enzymes' biotechnological potential has enforced the isolation and characterisation of a large number of anaerobic, Gram-positive, spore-forming rod-shaped bacteria, the majority of which have been allocated to the genus *Clostridium*. Polysaccharides are degraded either by secreted or cell-associated extracellular enzymes, before intracellular catabolism occurs. Saccharolytic clostridia are able to use a wide spectrum of organic carbon sources for growth (Mitchell *et al*., 1995).

Land-plant biomass is represented by starch, cellulose, hemicelluloses and lignin. Hemicelluloses identified to date are composed of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid residues. They are alkali-soluble, linear or branched, homo- or hetero-polymers. Xylan is a major component of hemicellulose; it is hydrolysed by endoxylanases and ß-xylosidases, supported by additional enzymatic activities (Schwarz *et al.,* 1995a). Several clostridia are known to synthesise xylanase enzymes and can grow on xylan as sole carbon source (Hazlewood and Gilbert, 1993). Cellulose can be degraded either by a cellulase complex (the cellulosome) or by a synergistic combination of soluble enzymes (e.g. by the low complexity, two-component *C. stercorarium* enzyme system) (Bronnenmeier *et al.,* 1990). Glucose (hexose) and xylose uptake (pentose) in *C. acetobutylicum* has been shown to be optimal during the early stages of growth and to be inhibited following butanol accumulation in the culture (Ounine *et al.,* 1985). The Emden-Meyerhof glycolytic route is the main route for hexose catabolism in the saccharolytic clostridia. Pentose use is facilitated by the pentose-phosphate route and subsequent glycolysis.

The sugars derived from **cellulose** and **hemicellulose** plant biomass components are more attractive as fuel sources than starch, since they are also non-food products and may be produced on low quality land and are abundant. Compere and Griffith (1983) demonstrated that solvents are produced by several *Clostridium* spp. strains newly isolated from xylan in a meat-broth medium or in media containing 1% (w/v) peptone (Lin and Blaschek, 1983). Lee *et al.,* (1985) demonstrated the growth of the ATCC 824 strain (and one of its mutants) on xylan as the sole carbon source in continuous culture; under their conditions, the cells used about 50% of the xylan. The use of xylan increases the range of useful biomass for solvent production.

All solventogenic bacteria used in the ABE process belong to clostridial Group I. Even though *C. cellulovorans* has been included in Group I (Lawson *et al*., 1993), it is not solventogenic but has been identified as being a very active cellulose, producing a cellulosome. This extracellular multi-enzyme complex allows the bacterium to hydrolyse crystalline cellulose efficiently. When the genomic sequence of *C. acetobutylicum* became public in 1999 it soon became clear that this micro-organism possessed a complete cellulosomal gene operon similar to that in *C. cellulovorans*, although it is not expressed (Schwarz 2001; Nolling 2001). This indicates that a solventogenic *Clostridium* able to express the cellulosomal genes should exist. One of this work's goals was to isolate solventogenic bacteria having higher polysaccharolytic enzyme activity by selecting those strains having higher degradation potential.

The newly isolated strains were used for Palm Oil Mill Effluent (POME) degradation; this waste was used as sole carbon source for producing solvents from Palm Oil Mill Effluent. Colombia has 175.000 Ha planted with African Palm (Revista El Palmicultor 2002). The quantity of effluent waste produced by palm oil extraction plants in Colombia ranges from 0.55 to 1.22 m^3 effluent/ton FFC (palm fruit processed). 30 extraction plants, having a capacity of 3 to 45 FFC tons/hour (average: 13 FFC tons/hour), are currently working in Colombia, resulting in a waste effluent release ca. 12.000 m^3 per day (Revista el Palmicultor, Fedepalma 2002).

This residue, Palm Oil Mill Effluent (POME), constitutes the main environmental hazardous contamination problem produced by the extracting plants. The residue is usually disposed of through a conventional waste-water treatment plant, thus adding considerable cost to the process, but still burdening the environment. Only a few of the residue's characteristics are known, amongst them being the DBO5/DQO ratio (= 0.61), indicating that a great part of the residue is biodegradable (*Boletín Técnico* No.11, Cenipalma, 1997). It contains several liquids, residual oil, solids in suspension and about 95% water (Devendra *et al.*, 1981).

Even though POME consists of waste material, it could be a substitute for carbon source, water, mineral salts and buffer in a bacterial medium, meaning that it could thus replace up to 60% of the process production costs (as waste material) in a local solvent production plant. It should be emphasised that POME contains a high concentration of cellulose 164.61 g/L hemicellulose 11.25 g/L and soluble carbohydrates 42.17 g/L (these values were obtained by experimental assays, see: Materials and Methods 9.1), its richness as carbon source makes it an apt candidate for use as a culture medium in fermentation.

Project objectives:

- To identify and characterise (at molecular level) a pool of newly isolated solvent-producing bacteria from Colombian sources, to carry out taxonomical classification of these strains and to determine their taxonomic relationship;
- To evaluate the strain's properties with respect to extra cellular hydrolytic enzymes and metabolic end-products; and
- To study agro-industrial residue's direct use as substrate for solvent production.

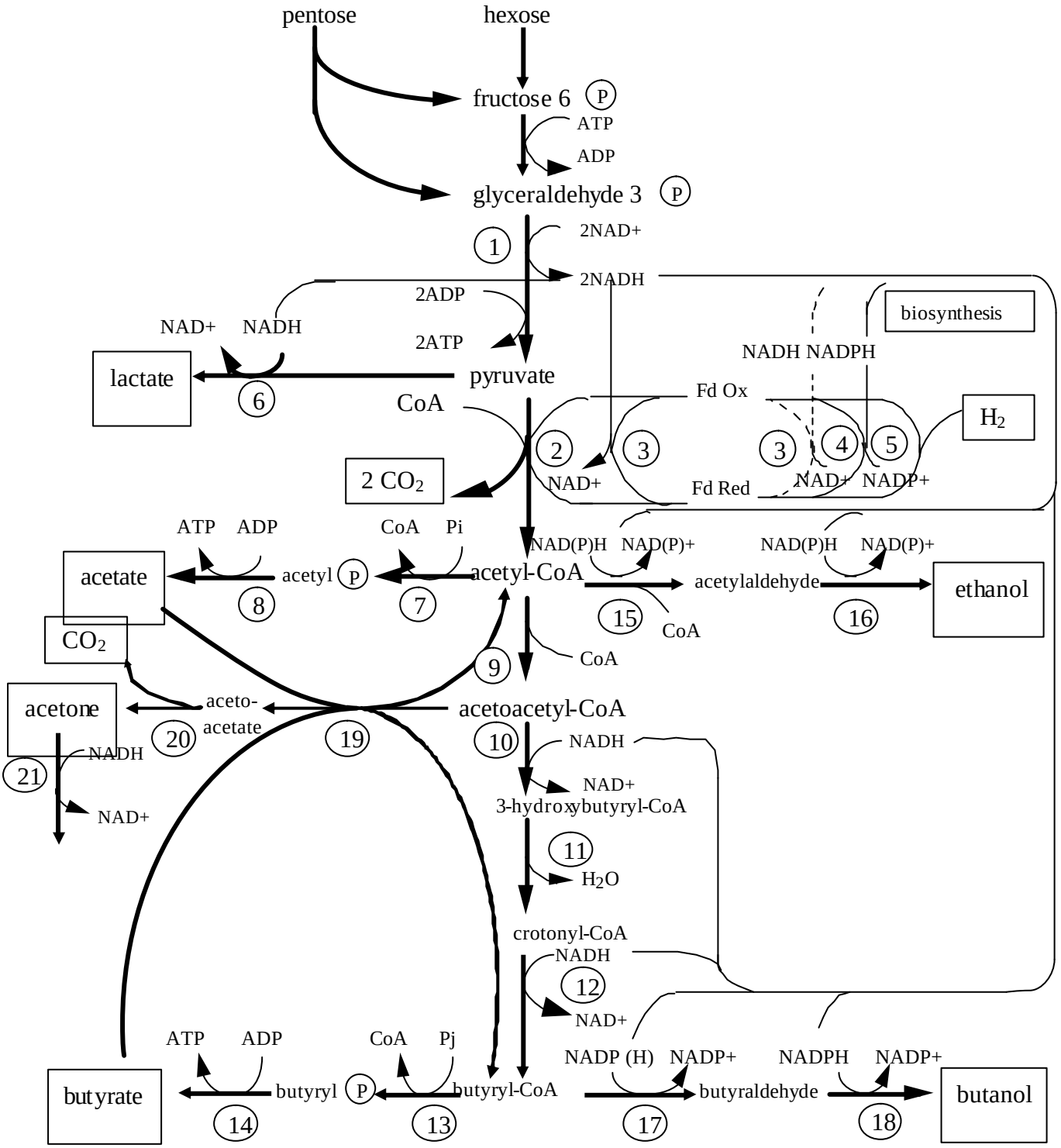


Figure 1. Biochemical pathways involved in *C. acetobutylicum* solventogenesis.

Numbers in the figure correspond to enzymes included in Table 1.

Number	Enzyme
1	glyceraldehyde–3–phosphate dehydrogenase
2	pyruvate-ferredoxin oxidoreductase
3	NADH-ferredoxin oxidoreductase
4	NADPH-ferredoxin oxidoreductase
5	hydrogenase
6	lactate dehydrogenase
7	phosphate acetyltransferase (phosphotransacetylase)
8	acetate kinase
9	thiolase (acetyl-CoA acetyltransferase)
10	3-hydroxybutyryl-CoA dehydrogenase
11	crotonase
12	butyryl-CoA dehydrogenase
13	phosphate butyltransferase (phosphotransbutyrylase)
14	butyrate kinase
15	acetaldehyde dehydrogenase
16	ethanol dehydrogenase
17	butyraldehyde dehydrogenase
18	butanol dehydrogenase
19	acetoacetyl-CoA : acetate/butyrate: CoA transferase
20	acetoacetate decarboxylase
21	isopropanol dehydrogenase

Table 1. Enzymes involved in *C. acetobutylicum* solventogenesis

I. Materials and Methods

1. Micro-organisms

Designation	Species	Origin
ATCC 824	*Clostridium acetobutylicum*	ATCC
DSM 1732	*Clostridium acetobutylicum*	DSM
DSM 792	*Clostridium acetobutylicum*	DSM
DSM 791	*Clostridium beijerinckii*	DSM
NCIMB 8052	*Clostridium beijerinckii*	NCIMB
NRRL B643	*Clostridium saccharobutylicum*	NRRL
N 1-4	*Clostridium saccharoperbutylacetonicum*	D.T. Jones
DSM 2478	*Clostridium butyricum*	DSM
DSM 523	*Clostridium kainantoi*	DSM
IBUN V517	*E. coli*	IBUN plasmid molecular marker
	Vibrio cholerae	
IBUN IV	Butanol hyper-producer mutant from *Clostridium.acetobutylicum* DSM 1732	IBUN
NRRL B643	*saccharobutylicum*	NRRL
850 M95	*Staphylococcus epidermidis*	IBUN. Molecular Epidemiology Lab

Designation	Native strains	Colombian Department	Type of crop
IBUN 13A	this study	Cundinamarca	Potato
IBUN 18A	this study	Antioquia	Chrysanthemum
IBUN 18Q	this study	Antioquia	Chrysanthemum
IBUN 18S	this study	Antioquia	Chrysanthemum
IBUN 22A	this study	Cundinamarca	Potato
IBUN 62B	this study	Cundinamarca	Grass
IBUN 62F	this study	Cundinamarca	Grass
IBUN 64A	this study	Cundinamarca	Grass
IBUN 95B	this study	Tolima	Soya
IBUN 125C	this study	Tolima	Soya
IBUN 137K	this study	Boyacá	Sugarcane
IBUN 140B	this study	Boyacá	Sugarcane
IBUN 158B	this study	Cundinamarca	tree-tomato

2. Culture Medium

2.1 RCM medium

(Reinforced Clostridia Medium, MERCK) was used to isolate native bacteria.

Yeast extract	3.0 g/L
Casein peptone	10.0 g/L
Meat extract	10.0 g/L
Glucose	5.0 g/L
Starch	10.0 g/L
Sodium chloride	5.0 g/L
Sodium acetate	3.0 g/L
L - Cysteine chlorohydrate	0.5 g/L
Agar	12.5 g/L

Completed with distilled water. pH 6.8 +/- 0.1. Sterilised at 121°C x 15 minutes

2.2 T6 broth medium

Glucose (6% w/v) used as carbon source, for solvent production experiments (Kashket and Cao, 1993)

KH_2PO_4	0.5 g/L
$MgSO_4$. $7H_20$	0.3 g/L
$FeSO_4$. $7H_20$	0.01 g/L
Ammonium acetate	3.0 g/L
Yeast extract	2.0 g/L
Tryptone	6.0 g/L
Cysteine HCl	0.5 g/L
Agar	18.0 g/L
Glucose	60.0 g/L

This was completed with distilled water. pH was adjusted to 6.5 with NaOH. The glucose had to be sterilised separately for 15 minutes at 121°C.

2.3 TYG

Hydrolysed casein	16 g
Yeast extract	4 5 g
Sodium chloride	5 g
Glucose	5 g
Cysteine HCl	0.5 g
Distilled water	Up to 1 litre

This was sterilised for 15 minutes at 121°C.

2.4 TYS

Hydrolysed casein	16 g
Yeast extract	5 g
Sodium chloride	5 g
Saccharose	5 g
Cysteine	0.5 g
Distilled water	Up to 1 litre

This was completed with distilled water and sterilised for 15 minutes at 121°C.

2.5 Milk medium for riboflavin production

Fresh milk	50 mL
Resazurin	0.05 mg

pH before sterilisation was 7.1. This medium was autoclaved in 100% N_2 for 12 minutes at 121°C.

2.6 TES Broth

Saccharose	5.0 g
Yeast extract	4.0 g
Hydrolysed casein	10.0 g
Sodium chloride	5.0 g
Cysteine	0.5 g
Calcium chloride	0.2 g
Distilled water	Up to 1 litre

The final pH was 6.8 before sterilisation. The broth was completed with distilled water and sterilised for 15 minutes at 121°C.

2.7 Basal *Clostridium* medium (CBM)

Starch	10.0 g
Yeast extract	4.0 g
Hydrolysed casein	10.0 g
Carbonate of calcium (optional)	5.0 g
Cysteine HCl	0.5 g
PABA (100 mg/mL)	1 mL
Sodium chloride	0.2 g
Distilled water	Up to 1 litre

Medium was pH 6.8 before sterilisation.

2.8 BHI Brain Heart Infusion medium for *Staphylococcus epidermidis* 850 H95 growth

BHI (Oxide)	37 g
Saccharose	150 g
Glycin	20 g
Distilled water	Up to 1 litre

2.9 LB Luria Bertani medium

Tryptone	10 g
Sodium chloride	10 g
Yeast extract	5 g
Distilled water	Up to 1 litre

2.10 Sporulation medium

Casein	8.5 g
Tryptone	8.5 g
Soy flour	3.0 g
Glucose	2.5 g
Sodium chloride	5.0 g
KH_2PO_4	2.5 g
$MnSO_4.\ H_2O$	0.03 g
Distilled water	up to 1 litre

Final pH was 6.8

2.11. Industrial medium

Industrial medium was developed using a statistical design specifically developed at IBUN for this work (Montoya, D., *et al.*, 2000 Seville Congress).

Molasses	130.0 g
Yeast extract	3.0 g
Biotin	4.0 mg
PABA	3.0 mg
KH_2PO_4	1.8 g
Mineral Stock	4.0 mL
Distilled water	Up to 1 litre

Mineral Stock

$MgSO_4.7H_2O$	1.3 g/L
$FeCl_3$	3.0 g/L
$CaCl_2.6H_20$	0.9 g/L
H_2SO_4	12.0 mL
Distilled water	Up to 1 litre

This stock was mixed and completed with distilled water. It was then sterilised at 121°C for 15 minutes.

Preparation

The molasses were dissolved in 250 mL distilled hot water, centrifuged for 30 minutes at 4,000 rpm. The supernatant was skimmed off and the pH adjusted to 3.5 with HCl 1N. This was then sterilised at 121°C for 15 minutes.

The yeast extract and K_2HPO_4 were dissolved separately in 500 mL water. They were then sterilised at 121°C for 15 minutes. Mineral stock was added and mixed in aseptic conditions. The stock was then completed to 1,000 mL with distilled water.

2.12. POME (Palm Oil Mill Effluent) Medium.

This medium was prepared by replacing the molasses in Industrial Medium (2.11) as sole carbon source by 100% v/v Palm Oil Milk Effluent. The POME Medium was thus prepared by adding the remaining industrial medium components in previously sterilised POME. The POME Medium was sterilised at 121 ºC for 15 minutes.

3. Micro-organism growth conditions

A sample of preserved spores was suspended in tubes containing 3 mL RCM medium. These were then incubated in a 70°C water-bath for 10 minutes to inactivate vegetative cells for selecting spore-formers. The tubes were subsequently incubated for 24 h at 37°C. Liquid media were poured into glass vials, flushed with nitrogen, sealed with rubber septa and aluminium crimps and autoclaved for 15 min at 121°C. Culture medium was autoclaved in a 100% N_2 atmosphere for 12 min at 121°C.

The anaerobic conditions for agar plates were obtained in an anaerobic chamber (Forma Scientific 1025) with a high purity 85% N_2, 10% H_2 and 5% CO_2 gas mixture. Palladium wafer was used as catalyst. This chamber ensured controlled anaerobic conditions by using 95% N_2 and 5% H_2 (99.9% purity). Methylene blue was used as anaerobic indicator. This indicator forms a complex with glucose presenting a blue colour on contact with oxygen and is colourless when oxygen is absent.

3.1 Anaerobic indicator composition

Glucose	4.0 g/L
Buffer (Tris hydroxymethyl aminomethane 30% water solution)	1 mL
Distilled water	100 mL
Methylene blue	2 drops
pH 8.0.	

Preparation

The components were mixed and poured into an open flask and placed in the anaerobic chamber. When the solution became transparent, the chamber was assumed to be anaerobic (1025 aerobic chamber, Forma Scientific).

4. Micro-organism isolation from soil

4.1 Sample collection from soil

- Target farms were divided into lots having similar soil, slope or crop conditions. For example, if a farm had a flat part and a sloping part, then a sample (10.0 g) was taken from the flat part and then from the sloping part.
- Between 2 to 5 samples were taken from each lot per hectare, depending on the varieties of land. Each one of these sites represented a sub-sample.
- The vegetation covering the soil at each site was completely removed.
- A shovel (or clean tool) was used to take the sample from the desired depth. If variation in colour or texture was observed, then this indicated different soil layers, which had to be shown independently and could not be mixed.
- The sub-samples were taken from the different sites (selected from each lot) and each one was independently mixed in very clean, sterile receptacles. This mixture constituted the lot's representative sample, which had to be clearly identified with the name of the farm and the lot.
- Each sample was packed in a perfectly clean plastic bag or cardboard container.
- A label was then filled in, providing all technical information pertinent to the soil sample.
- The soil samples were taken from different depths, bearing in mind that the external zones of a small plot of land could be totally aerobic, whilst the centre of the plot could still be anaerobic. This would suggest that there could be various physiological types of micro-organism within such small plots of land, indicating the existence of anoxic micro-environments at any depth whatsoever.

Samples were taken from the plant furrows; none were taken when the soil was wet. Samples were not taken from places where commercial fertiliser had been applied, or the ground fertilised with coffee pulp or compost within the last six months. Samples were not taken from places where there had been recent burning-off. Samples were collected from several depths of agriculturally cultivated soil from different locations

throughout Colombia. The description of the place, soil treatment and kind of crop were registered on a sheet giving technical information; this was later transferred to files.

The samples were air-dried for one week and sieved (2 mm mesh Fisher Scientific sieve). Humidity was determined from 10 grams of sample by drying at 80°C until constant weight was achieved. The pH was potentiometrically determined after suspending and homogenising 1 g dry sample for 20 minutes in 10 mL distilled water.

4.2 Strain isolation from soil samples

1 g soil was suspended in a sterile test-tube containing 10 mL RCM anaerobic medium in a N_2 atmosphere incubated in a 70°C water-bath for 10 minutes to inactivate vegetative cells for counter-selecting against non-spore formers. The tubes were subsequently incubated for 24 h at 37°C. Samples having gas production and increased turbidity were re-incubated for 96 h at 37°C and checked daily for growth and gas production.

500 µl of the culture were taken for evaluating acetone production in RCM and on T6 agar plates. Each colony was overlayed with a small drop of 5% sodium nitroprusiate w/v and 2% ammonium hydroxide w/v was added. A dark violet ring was formed in the presence of acetone (Calam, 1980). *C. acetobutylicum* strains producing acetone were isolated. Nitroprusiate positive cultures were purified by single colony isolation and incubated on agar plates under strict anaerobiosis at 37°C for 12-15 days.

Colonies were checked for the appearance of spores by phase-contrast microscopy (Mitchell *et al.,* 1995). Clostridial forms were restreaked for purification and re-tested for acetone production and spore formation.

4.3 Isolate conservation

Cells from colonies were inoculated into 3 mL T6 medium in tubes and then incubated in a water-bath at 70°C for 10 minutes to inactivate vegetative cells. They were

incubated for 5-7 days. Cultures were examined under the microscope for cell and spore shape and size uniformity. The cells were collected by centrifuging (4,000 rpm, 5 min) and washed with sterile isotonic sodium chloride (0.09% w/v). The pellet was suspended in 1.3 mL sterile 5% w/v UHT milk and 10% v/v glycerol mixture. 550 µl of this suspension was poured onto 1.2 g previously sterilised crystal silica gel. The vials were subsequently placed inside a vacuum dryer at room temperature for 7 days.

5. Physiological and biochemical characterisation

5.1 Isolate solvent-production profiles

A sample of preserved spores was suspended in 3 mL RCM medium, incubated in a water-bath at 70°C for 10 min (to induce spore germination) and incubated for 24 h at 37°C.
0.5 mL from vigorously growing pre-cultures was inoculated into 25 mL T6 medium in 50 mL bottles under anaerobic conditions. The concentration of butanol, ethanol and acetone in the fermentation broth was evaluated after 96 h at 37°C, as follows.

2 mL of sample, plus 1 mL of internal standard, were poured into 5 mL gas-tight vials and incubated at 50°C for 10 minutes. 1 mL of the gas phase was injected into a gas chromatography apparatus (Varian 3400®) equipped with a flame ionisation detector and connected to a recorder-integrator. The solvents were separated in a steel column (4 m length, 2 mm inside diameter) packed with Chromosorb 102; nitrogen was used as carrier gas (30 $mL.min^{-1}$) . The injector and detector temperature were 200 at a rate of 1°C/min. *n*-Propanol was used as internal standard. A mixture of standards having the following concentrations was used for quantification: 1,200 ppm ethanol, 3,600 ppm acetone, 7,200 ppm butanol and 5,000 ppm *n*-propanol. A calibration curve had to be constructed prior to sample analysis for determining the response factor.

5.1.1 Internal standard preparation: (n-propanol)

Internal standard concentration was 5,000-ppm n-propanol in distilled water. The stock solution was distributed in the following way to construct the calibration curve: 1 mL, 2 mL, 3 mL, 4 mL and 5 mL were taken and completed to 25 mL with culture medium.

Volume	**Ethanol concentration (ppm)**	**Butanol concentration (ppm)**	**Acetone concentration (ppm)**	**n-propanol concentration (ppm)**
1 mL	400	1,200	2,400	5,000
2 mL	800	2,400	4,800	5,000
3 mL	1,200	3,600	7,200	5,000
4 mL	1,600	4,800	9,600	5,000
5 mL	2,000	6,000	12,000	5,000

The series taken for constructing the calibration curve was 3 mL because its concentrations corresponded to the expected values produced by the micro-organism being studied. Retention times were calculated from the obtained data so that the solvents could be analysed and the response factor determined.

$$FR = \text{Probe } \frac{\text{Quantity}}{\text{Probe Area}} \times \frac{\text{Internal Standard Area}}{\text{Internal Standard Quantity}}$$

$$\text{Concentration} = \text{Response Factor} \times \text{Problem Area} \times \frac{\text{Probe Concentration}}{\text{Internal Standard Area}} \times \text{Internal Standard Quantity}$$

5.2 Physiological test

Tests for differentiating solventogenic *Clostridium* as described by Johnson *et al.* (1997)

5.2.1 Rifampicin sensitivity

The strains were activated from preserved spores (as described in 3.0) in 3 mL TYG medium and incubated at 37°C for 12 h. TYG agar plates were flooded with the over-night cultures and excess liquid was removed. Filter paper discs containing 10 and 100 ng rifampicin were air-dried; they were then placed onto the agar surface. The plates were incubated at 37°C for 24 h. Rifampicin sensitivity was determined by inhibiting growth around the disks.

5.2.2 Riboflavin production

Strains were activated (as described in 3.0) and inoculated into milk medium. Vials were incubated for 5 days at 37°C and then stored at 4°C for two weeks. Riboflavin (produced in the cold) was assayed by HPLC. Riboflavin dilutions in whey were used as quantitative control. The whey was filtered through a 0.22 µm Millipore filter. 5 µl of sample were injected into a HPLC apparatus (Waters) equipped with a µbond pack C-18 column (15 cm length), 0.8 mL/min flow rate at room temperature, and detected with an UV detector at 280 nm and a recorder integrator. The mobile phase consisted of 5 mL octane sulphonic acid in 95 mL 25% methanol, 0.1% TEA in water (v/v).

5.2.3 Curd formation before 24 h

1 mL of activated micro-organism was inoculated into 50 mL milk medium and incubated at 37°C for 24 h. Results were considered positive when the milk coagulated and curd was formed within 24h.

5.3 Selected strain

A total of 278 soil samples were analysed from different Colombian States (Cundinamarca, Antioquia, Santander, Boyaca, Tolima, Valle del Cauca and Huila). The total 100 isolates produced more than 2 g/L total solvents. 13 strains were selected from these strains by determining the solvents produced; all of them produced more total solvent concentration than the *Clostridium acetobutylicum* ATCC 824 reference strain (Materials and Methods Micro-organism 1).

5.4. Physiological differentiation of Clostridium species.

Biochemical tests from Bergey's manual differentiating *C. butyricum* from typical solventogenic clostridia are described as follows:

Test	***C. butyricum***	***C. acetobutylicum***	***C. beijerinckii***
Glycerol	+	-	-
Ribose	+	-	-/+
Indol	-	+	-
Gelatin	-	-	-
Catalase	-	-	-

The tests described in the Table were carried out on the three strains selected as being the greatest solvent producers.

5.4.1 Tests for glycerol, ribose and gelatin

A pre-inoculum culture was prepared in TGY medium and incubated overnight at 37ºC. 1 mL of pre-inoculum was transferred to a vial containing 10 mL TY culture medium, using the substrates required for the test (glycerol, gelatin, and ribose) as carbon source (each one at the same percentage as the glucose in the TGY medium). Culture media had been previously gassed and sterilised. The culture was incubated for 16 h at 37ºC.

A positive result was indicating by gelatine liquefying once the micro-organism had grown.

The vial was cooled to 4º for 8 h to observe liquefaction. *E. coli* V517 was used as negative control and *Vibrio cholerae* as positive control

5.4.2. Indol

The culture was serialised in 5 mL TGY medium and incubated overnight at 37ºC. 1 mL Kovac's reagent was added once the micro-organism had begun to grow. The parametylaminobenzaldehyde from this reagent reacted with the indol produced by tryptofan hydrolysis if the bacteria possessed tryptophanase activity. A reddish ring in the interface between the culture medium and the reagent's alcohol could be observed in the presence of indol.

5.4.3. Catalase

The culture was grown in 5 mL TGY medium and incubated overnight at 37ºC. 10 drops of 3% hydrogen peroxide were added to a tube containing 1 mL culture once the micro-organism had begun growing.

Micro-organisms having catalase degraded H_2O_2 and formed O_2 and H_2O with bubbling. Absence of bubbling indicated lack of catalase.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Toxicity assay

Five (5) mice were injected with 0.5 mL bacterial culture from each native strain to perform the toxicity assay. Tetanus neurotoxin was used as positive control. Assays were done prior to adding trypsin (trypsinisation at 37°C for 5 minutes) to activate the toxin, producing an identical result.

Procedure

- A pre-inoculum was made from each of the 13 native strains (as described in 3.0); the vials were incubated at 37°C o/n.
- 0.5mL of the previous culture were inoculated into 20mL TGY broth and incubated at 33°C for 7 days or until sporulation.
- Evidence of sporulation by Malachite green dying was observed by microscope.
- The pre-inoculum was then centrifuged at 12,000 rpm for 15min at 4°C.
- The supernatant was skimmed off and stored at 4°C until needed for the inoculation.
- 0.5mL of the supernatant was intra-peritoneally inoculated into two-week-old female NIH mice.
- The mice were kept under observation for 15 days.

6. Molecular characterisation

6.1 Genomic DNA isolation protocol for 16SrRNA and DNA-DNA hybridisation

Sol A: 50 mM Tris HCl (pH 8.5), 15% sucrose, 25 mM EDTA, 10 mg/mL lysozyme.
Sol B: 50 mM Tris HCl (pH 8.5), 25 mM EDTA.
Sol C: 25 mM Tris HCl (pH 8.5), 25 mM EDTA.
Sol D : 10mM Tris HCl (pH 7.6), 1mM EDTA (TE)

- Procedure
  - Each strain was activated (as described in 3.0). 1 mL of inoculum was added into 40 mL RCM (Reinforced Clostridia Medium, Merck); the vials were incubated for 24 hours at 37°C.
  - The samples were cooled on ice for 30 minutes.

- The cells were centrifuged for 25 minutes at 4,000 g.
- The pellet was dissolved in 1.2 mL lysis buffer (Sol A).
- This was incubated for 20 minutes at 37°C.
- 6.0 mL buffer B and 0.96 mL 10% SDS were added. This was incubated at 37°C for 30 minutes.
- 4.0 mL of a phenol-chloroform solution (1:1) were added and mixed to form an emulsion.
- This was centrifuged at 4,000 g for 15 minutes.
- The supernatant was skimmed off and poured into a new tube kept on ice.
- 2 volumes of cold ethanol were added and gently agitated. This was incubated at low temperature for 30 minutes.
- The nucleic acids were isolated with a small glass rod or precipitated at 8,000 g for 10 minutes. The pellet was dried by inverting the tube over blotting-paper.
- 0.8 mL buffer C and 10 µl RNAase A solution (1 mg/mL) were added and incubated at 37°C for one hour.
- Phenol-chloroform extraction was repeated.
- 2 volumes of cold ethanol and 1/10 vol NaCl (3 M) were added to the upper phase in a fresh tube.
- This was incubated over-night at room temperature then precipitated at 10,000 g for 10-15 minutes.
- The pellet was dried and washed with 70% ethanol.
- It was dried once again and suspended in TE or pure water. This was kept at low temperature.
- The samples were quantified at 260 nm. Absorbance measurements were taken at 260 nm for each sample. The samples were diluted 1:40 in ultra-pure water. The calculation was as follows:

$$\lambda\ 260 \text{ X dilution factor X } 50 = \text{concentration in ng/µl}$$

6.2. Plasmids

6.2.1 DNA isolation

Reagents

Name	**Brand**
Tris base	Gibco BRL
Disodic EDTA	Gibco BRL
Sodium dodecylsulphate (SDS)	Gibco BRL
Saccharose	Gibco BRL
Lysozyme	Merck
Glacial acetic acid	Merck
Sodium hydroxide	Merck
Potassium acetate	Merck
Chloroform for HPLC	Merck
Phenol saturated with Tris pH 8	Sigma

Restriction Enzymes

Enzyme	**10x Buffer**	**Brand**
Hind III	E	PROMEGA
Sau 3AI	B	PROMEGA
Eco 52 I	L	PROMEGA
Eco RV	D	PROMEGA
Nar I	G	PROMEGA

Stock solutions

Solution	**Concentration**
Tris HCl pH 8	1M
EDTA pH 8	0.5 M
Potassium acetate	5M
SDS	10%
Sodium hydroxide	3N

Working solutions

Solution	**Reagent**	**Composition**	**Observations**
TES buffer	Tris	25 mM	Filtered and
	EDTA	10 mM	sterile
	saccharose	15%	
10 X TBE	Tris base	108 g	Filtered and sterile
	Boric acid	55g	
	EDTA 0.5M pH 8.0	40 mL	
0.5 X TBE	10 X TBE	50 mL	Filtered and
	Water	up to 1 L	sterile
TE pH 8 buffer	Tris	10 mM	Filtered and
	EDTA	1 mM	sterile
Potassium	Potassium acetate 5M	60 mL	pH 5.5
acetate in acetic	acetic acid	11.5 mL	
acid	Water	up to 100 mL	
Alkaline-SDS	SDS (Sodium	1%	Recently
	dodecylsulphate)	0.2 N	prepared
	NaOH		

6.2.2. Micro-organism cultures and procedure for growing *Clostridium*:

E. coli V517 was grown on a plate at 37ºC overnight. Culture was kept at 4ºC. A culture colony (previously conditioned at 37ºC for one (1) hour) was taken and inoculated into 100 mL LB broth. The inoculate was grown with constant agitation at 160 rpm, 37ºC for 5 hours.

S. epidermidis was obtained from cultures in BHI medium, supplemented with 2% glycin and 15% saccharose, following the same procedure proposed for *E. coli V517*, with the exception of the time taken for incubation. *S. epidermidis* incubation time was 12 hours. *Clostridium* native strain growth conditions: 1.5% mL of the conserved *Clostridium* strain was inoculated into 15 mL RCM broth and grown as described in

3.0). 1.0 mL of these pre-inoculates was inoculated into 40 mL RCM broth. The micro-organism was grown at 37ºC until stationary phase for establishing growth kinetics. Pre-inoculate optical density was determined by spectrophotometer (Spectronic 20 Genesys TM). Readings were taken at 680 nm wavelength to enable the pre-inoculate optical density process to continue until OD = 4.7. Samples were taken until the culture reached the stationary phase (i.e. when changes in optical density became practically imperceptible).

Cell count optical densities and dilutions counted in Neubauer Chamber

Optical density	**Dilution**
0.20 – 0.35	100, 150, 400
0.40 – 0.7	50, 100, 150
0.8 – 1.2	10, 50, 100

The resulting data (obtained from counting cells in a Neubauer Chamber) was processed to obtain an average graph for the natural logarithm of the number of cells versus optical density. The number of cells necessary to see the plasmids in agarose gel is extrapolated from the graph.

6.2.3. Plasmid isolation procedure

Extraction

The methodology used in this work was standardised by basing it on the numerous protocols which have been proposed for plasmid DNA extraction in clostridia from the solventogenic group. Mahony et al. (1986) proposed a methodology which has been used in other studies with *C. acetobutylicum* (Yoshino et al., 1990; Kim et al., 1990; Kado and Liu,1981) and which became the starting point for the final developed protocol in this work. The procedure used by Minton and Morris (1981) for obtaining plasmids in *C. butyricum,* was also tested in search of better conditions for the Colombian native strains. The protocol standardised in this work and used for obtaining native *Clostridium* strains' plasmid profiles was as follows:

- Micro-organisms were grown as previously described in 3.0 until reaching the corresponding optical density.
- They were incubated at 0ºC for 20 minutes.
- Cells were collected in 50 mL Falcon tubes by centrifuging (4,500 rpm at 4ºC for 15 minutes).
- The supernatant was skimmed off and 1 mL TES buffer was added to the pellet. Each Falcon tube was shaken thoroughly on ice to ensure that the cells were well suspended. The suspended cells were transferred to 2 mL Eppendorf tubes.
- The cells were centrifuged again at 4,500 rpm for 10 minutes at 4ºC.
- The supernatant was discarded and 1 mL TES buffer was added to each Eppendorf tube (having been previously cooled), thus assuring that the cells were well suspended.
- Further centrifuging followed in the same conditions and the supernatant was discarded.
- 1 mL TES buffer (previously cooled to 0ºC) was used to suspend the cells, with constant shaking. Once the cells were well-suspended, lisozyme was added to a final 3 mg/mL concentration. The sample was incubated for 1 hour at 37ºC.
- After incubation the sample was left at 0ºC for 5 minutes, during which time the SDS (sodium dodecylsulphate)-alkaline solution was prepared.
- 400 µl SDS-alkaline solution were added to the sample. It was agitated by inversion ten times and incubated at 0ºC for 15 minutes.
- 300 µl potassium acetate solution was added and the sample was vortexed for 15 seconds at 1,600 rpm. It was incubated at 0ºC for 10 minutes.
- The tubes containing the samples were centrifuged at 11,000 rpm for 10 minutes at 4ºC.
- The supernatant was skimmed off and samples put into 2 mL Eppendorf tubes.
- A 1:1 volume of phenol:chloroform was added and agitated (by inversion) 50 times to create a good emulsion. It was then centrifuged at 11,000 rpm for 5 minutes at 4ºC. The organic phase was discarded and the aqueous part was transferred to 2 mL Eppendorf tubes. This procedure was repeated 3 times.

- A volume of chloroform was added, followed by inversion agitation in the same way as phenol:chloroform. The samples were centrifuged at 11,000 rpm for 5 minutes at 4ºC. The aqueous phase was collected and transferred to 2 mL Eppendorf tubes. This was done twice.
- Once the second washing with chloroform ended, the sample was incubated at 37ºC for 20 minutes.
- The equivalent of 2 volumes of absolute ethanol was added to the sample and it was incubated overnight at -20 ºC.
- The sample was centrifuged at 11,000 rpm for 15 minutes. The supernatant was discarded.
- The precipitated DNA was washed three times with 70% ethanol. The pellet was suspended so that it did not lose its integrity. Then the sample was centrifuged at 11,000 rpm for 5 minutes.
- The DNA was dried at 37ºC for 3 hours and finally suspended in 15 µl distilled, deionised, filtered, sterile water.

6.2.4. Electrophoresis

- The plasmid DNA was separated in 0.8% concentration agarose gels, run at 1.7 volts/cm in TBE 0.5x for 11 hours. Plasmid resolution in these conditions was good enough for subsequent analysis.
- Gel dimensions were: 25 cm long by 18 cm wide and approximate 6 mm thickness. 1,300 mL buffer was used for the run and a Bio-Rad chamber for electrophoresis.
- 6 µl samples of extracted and preserved DNA were mixed with 2 µl loading buffer (bromophenol blue and glycerol), before being loaded onto the gels.
- The samples were heated for at 65ºC for 5 minutes and then placed on ice to avoid the appearance of plasmid isoforms.

- Reproducibility: Once extraction parameters had been established, an initial probe was made with all the strains and IBUN 125C was chosen to evaluate the methodology's reproducibility, because the presence of two plasmids was

always observed in plasmid extraction. Three extractions were done on different days and those profiles obtained from the agarose gels were evaluated. The IBUN 125C strain possessed two plasmids repeatedly observed in each one of the tests. This strain was used as extraction control for the rest of the strains.

6.2.5. Plasmid DNA digestion with restriction enzymes

- DNA extracted from 40 mL culture was suspended in 15 µl water and its approximate concentration per µl was calculated from the results obtained with the agarose gels and by comparison with a quantity of known DNA from Lambda phage.
- The final reaction volume for each digestion was 20 µL. Digestion was done by incubating the reaction mixture in a water-bath at 37ºC over-night. The quantity of each component was as follows.

Digestion components with restriction enzymes

Component	**Quantity**
DNA	6 µg
Enzymes (Hind III, Sau 3Al, Eco 521, Eco RV, Nar I)	10 units of enzyme
10X Buffer (It was as supplied by the manufacturer)	2 µL
10 µg/µL Bovine serum albumin	0.5 µL
Distilled water	Up to 20 µl

DNA fragments were separated by electrophoresis on agarose gels (15 cm long by 10 cm wide) at 0.8% concentration, run for 5 hours at 2.5 volts per cm in an electrophoresis chamber (Bio-Rad) and viewed after staining with ethiduim bromide.

6.3 Pulse-field gel electrophoresis (PFGE)

→ Materials

Material	**Manufacturer**
NCERT* agarose	FMC
Triton 100X	SIGMA
EDTA	GIBCO
Boric acid	GIBCO
NaCl	GIBCO
AGAROSE	GIBCO
RNAse A	SIGMA # R 5503
N-Lauroylsarcosine	SIGMA # L 5125
Proteinase K	GIBCO-BRL 25530-015
Lysozyme	Boehringer-Mannheim # 711 454
Thiamphenicol 10 mg/mL in 70% ethanol	Life Technologies
Thermophilic Pretaq (rT41 A proteinase)	Life Sciences /GIBCO-BRL. 18061-010
PMSF (Phenylmethylsulfonyl fluoride)	Promega
EGTA	SIGMA
BssH II Life technologies	GIBCO-BRL 15468-010
Apa I Life technologies	GIBCO-BRL 15440-019
Sma I Life technologies	GIBCO-BRL 15228-018
Eco52 I	Promega R6751

→ PETT IV

Tris- HCl	10 mM
NaCl	1M

→ EC lysis buffer

Tris-HCl, pH 7.6	6 mM
NaCl	1 M
EDTA pH 8.0	100 mM
Triton 100X	0.005%
Lauroylsarcosine	0.5%(w/v)
Lisozyme	1.0 mg/mL
RNAse A	20.0 µg/mL

* RNAase was incubated for 20 minutes at 80ºC. before being used.

→ Protease reaction buffer for deproteinisation with Pretaq

Tris-HCl pH 8.0	10 mM
EDTA	0.01 mM
N-Lauroylsarcosine	1.00% w/v

→ ESP Buffer

Protease Reaction Buffer for deproteinisation with proteinase K

EDTA pH 8.0	500 mM
N-Lauroylsarcosine	1%(w/v)
Proteinase K	2 mg/mL

→ ES Buffer

Protease Reaction Buffer for deproteinisation with proteinase K

EDTA	500 mM
N-Lauroylsarcosine	1%(w/v)

→ TE buffer

Tris HCl pH 7.6	10 mM
EDTA	1mM

→ TE buffer + PMSF (Phenylmethylsulfonyl fluoride)

Tris HCl pH 7.6	10mM
EDTA 0.	1 mM
PMSF	1.0 mM
PMSF stock	100 mM in isopropylic alcohol

→ ET Buffer

Tris HCl pH 7.6	10 mM
EDTA pH 8.0	100 mM

→ EET Buffer, pH 8.0

Tris HCl	10 mM
EDTA	100 mM
EGTA	10 mM

10X TBE buffer

Tris base	108 grams
Boric acid	55g
0.5M EDTA	40 mL

pH 8.0; H_2O up to 1.0 litre

6.3.1 DNA preparation procedure

- Bacteria were grown to log-phase (ca OD 0.30).
- Thiamphenicol was added to 180 µg/mL and incubated for one more hour. The thiamphenicol stock solution was prepared in 10 mg/mL 70% ethanol.
- Culture was cooled to 0°C on ice and centrifuged (10 min at 6,000 rpm); the pellet was washed in PETT IV. OD was adjusted to 0.3, after centrifuging.
- It was centrifuged again and the pellet suspended in PETT IV: 1-2 mL PETT IV was added per 40 mL culture. OD $_{(550nm)}$ = 0.35 1x108 cell/mL.
- 400µl warm cell suspensions (at 40°C) were added to an equal volume of liquid INCERT agarose (1.5% in TE buffer) at 40°C. This was mixed and 50 µl aliquots were pipetted into each plug-forming mould. The mixture was cooled on ice for 20-30 min. It was essential to ensure that the mould was sitting on a bed of ice to ensure adequate cooling. Flame-sealed capillary tubes were used and gloves worn for handling gel plugs during this procedure.
- The plugs were transferred to sterile Eppendorf-caps (4 plugs in each cap).
- 200µl lysis buffer were added; plugs were then shaken slowly at 37°C for 1.5 to two days or until plugs appeared transparent. If the DNA plugs were not transparent, then Pretaq digestion was employed.
- The buffer was changed for ES buffer and plugs were washed twice with 200µl of this buffer and incubated at room temperature for 5 minutes each.
- Buffer was changed for 200 fresh µl ESP buffer (with 2 mg/mL Proteinase K).
- Plugs were incubated overnight at 50°C in a water bath. They were then cooled to 4°C for 10 minutes.
- Buffer was again changed for fresh ESP buffer and incubation overnight at 50°C was repeated. This was cooled to 4°C for 10 minutes.
- Buffer was changed for 200 µl TE + 2.5µl PMSF (Phenylmethylsulfonyl fluoride) (100 mM) incubated for 2 h at 37°C and slowly shaken.
- Buffer was changed for fresh TE + 2.5 µl PMSF, and slowly shaken overnight at 37°C.
- This was then changed for 200 µl TE and shaken for two hours at 37°C.

- There were 2 further changes for fresh TE and incubation at 37°C overnight.
- Plugs were was stored in 200 µl ET at 4°C.

The plugs were then ready for restriction endonuclease digestion

- Each 50µl plug (cells plus agarose) contained 150-200ng DNA; they were placed in 200µl TE buffer for 1h at 37°C.
- Buffer was changed twice more for fresh TE and incubated at 37°C for 15 minutes each time.
- This was then changed for 200 µl TE and incubated at room temperature.
- Buffer was changed for 100 µl fresh digestion buffer, 15 min at room temperature.
- This was changed for 100 µl fresh digestion buffer containing a restriction enzyme which was added up to 10 U per plug. Pre-incubation followed at 4°C for 6h and then at optimal temperature for 12 to 16 h.
- Digested DNA plugs were then loaded onto the running gel.
- A piece of non-digested DNA was used as control.

6.3.2. Pretaq digestion

- Thermus rT41A proteinase K (PRETAQ Life Sciences/Gibco-BRL).
- Plugs were incubated with 200 µl Protease Reaction Buffer and shaken 4 times for 5 minutes at 37°C.
- 200 µl Protease Reaction Buffer plus10 µl Pretaq Enzyme 3.3 U per Eppendorf-cap or 0.825U per plug was changed for deproteinisation and incubated for one and a half hours at 56°C.
- This was cooled at 4°C for 10 minutes.
- 200 µl TE buffer was changed twice and shaken three times for 5 minutes at room each time.
- DNA extraction procedure was continued from the digestion stage with proteinase K.

6.3.2 Running gel

- 1% agarose in PFG-TBE buffer is usually used for running gels. 0.5 X PFG-TBE was used here to speed up PFGE separation.
- The gel was loaded onto the electrophoresis unit filled with PFG-TBE buffer.
- Electrophoresis conditions were as follows: constant 12 V/cm voltage for 24 h, 3 sec initial time, 110 sec final time. Gene Navigator System TM from Pharmacia LKB S-751 83 Uppsala, Sweden. CHEF System. HEX electrode.

6.4 16S rRNA sequencing

6.4.1 Fragment amplification

Reactions were carried out in a thermolcycler (Hybaid Omn E) and each sample was independently amplified three times. After optimisation, the final conditions employed were: 2 min at 94°C; 10 cycles followed as described:15 s at 94°C, 30 s at 60°C and 50 s at 72°C. Another 15 cycles followed, each one consisting of 15 s at 94°C, 30 s at 60°C and 50 at 72°C (ten times). After that there were 7 mins at 72°C and then at 4°C for conserving amplification products. Each 25 µl reaction contained:

Component	**Concentration and other features**
Buffer	1X (20 mM Tris-HCl pH 8.4 and 50 mM KCl)
dNTP	0.18 Mm each
Primers	0.3 µM (each A and J)
$MgCl_2$	1.5 mM
Polymerase	1.25 U *Taq* (Promega)
Template	100 ng

It should be noted that the primers used in the amplification correspond to the A and J primers reported by Keis *et al.,* 1995, with a small modification at the 3' termini in J that allowed us to amplify a bigger gene segment. The sequencing primers (C and D)

corresponded to SEQ C and SEQ D by Keis *et al.,* (1995). The oligonucleotide primers (Keis *et al.,* 1995) were used and modified to optimise hybridisation temperature:

- pE (GAGAGTTTGAGCCTGGCTC) and pJ TTCTCCTACGGCTACCTTGTTAC) were used for amplifying the complete 16S rRNA gene (5´-3´).
- pA (GGAGCAAACAGGATTAGATACCC) and pJ were used for amplifying the partial rRNA sequence.
- The internal primers pC (GTGTCGTGAGATGTTGG), pD (TAACCCAACATCTCACG), pF (GTTTTAATCTTGCGACCGTAC), pG (GTCTTCAGGGACGATAATG) and pH (CTGCTGGCACGTAGTTAG) were used for sequencing.

6.4.2 Amplification verification

Electrophoresis was used to verify amplification efficiency and specificity. Each sample was amplified three times. One of them was loaded onto a 1% agarose gel (0.5 X TBE) and run at 3 V/cm for two hours in 0.5 X TBE buffer. The gel was stained with an ethidium bromide solution (0.1 mg/mL) for 20 minutes, destained if necessary, and photographed with a Polaroid camera.

6.4.3 PCR product recovery

PCR products were cleaned to ensure clean samples suitable for sequencing; a commercial kit (Concert Rapid PCR, Gibco BRL) was used. After cleaning, the samples were quantified by spectrophotometry at 260 nm, as described before for DNA isolation.

6.4.4. PCR fragment sequencing

An automatic sequencer (Perkin-Elmer 373, lent by the Instituto de Inmunología, Hospital San Juan de Dios) was used for direct PCR fragment sequencing. The four primers were used to sequence each fragment (the two amplification primers and those sequencing primers used by Keis *et al.,* (1995). Each sequence was carried out three times with each primer on DNA fragments amplified in both directions.

6.4.5. Sequence analysis

Sequence data was assembled with a CAP Sequence Assembly Machine (Huang and Madan,1999). This was then compared with the CLUSTAL X and the CLUSTAL W graphical interface. CLUSTAL W improved progressive multiple sequence alignment sensitivity through sequence weighting, position-specific gap penalties and weight matrix choice (Thompson *et al.,* 1994). The 16S rRNA sequence type strains were obtained from a BLAST search at EMBL (http://ncbi.nlm.nih.gov/). The MEGA programme (Kumar *et al.,* 2000) was used to establish species' relationships.

The distance matrix was generated by calculating the proportion of different nucleotides (***p***) between sequence pairs. The dendogram was constructed by Neighbour Joining, according to Jukes and Cantor's method (Nei, M. 1987).
The dendogram (Figure 17) was drawn by using Neighbor Joining from this index and the same basic topology was observed (distribution and relationship between branches) as was obtained with the ***p*** index. *C. butyricum* type strains and all the native strains are found in the first group in the Figure. The N 1-4 strain and the NCP 262 and DMS 791 strains are found in the second group, split into two. Two *Clostridium acetobutilycum* strains are found in the third group.

6.5 PCR identification of strains using a *Clostridium butyricum* specific 16S rRNA targeted oligonucleotide.

C but primer (Knabel *et al.,* 1997) and Keis's E primer (Keis *et al.,* 1995) were used for amplification. The amplification programme was carried out at 95^{o}C for 5 minutes for the first cycle and then at 95^{o}C for 30 seconds, at 52^{o}C for 30 seconds and finally at 72^{o}C for 30 seconds for the second cycle; this was repeated twenty-five times. PCR products were run in 0.8% agarose gel and, after staining the gel with ethidium bromide, viewed in a transilluminator with UV light.

6.6 DNA-DNA hybridisation

6.6.1 Chromosomal DNA preparation

Buffer	Composition
Solution A	50mM Tris:HCl
Lysis buffer	25mM EDTA
	15% sacarose
	Filtered deionised
Sol B	50mM Tris:HCl
Washing buffer	25mM EDTA
1M TE pH 8	Filtered deionised water
Sol C	10% dodecyl sodium sulphate
SDS	

V Zverlov's method was used (personal communication, June 2000) but modified in terms of agitation time with chloroform to reduce the number of extractions with this solvent and Proteinase K to the minimum.

- 120mL of an overnight cell culture (3 40mL vials per strain), grown in RCM medium at 37°C and pre-cooled for 30 minutes, were harvested by spinning at 5,000 rpm. The pellet was washed twice with 5mL of solution B.
- 5 mL of solution A and 10 mg lisozyme (2 mg/mL) were added and incubated at 37°C for 30 min.
- 0.1 volumes of a 10% SDS and Proteinase K solution were added for a final 1 mg/mL concentration. This mixture was incubated at 37°C for 1 hour, being gently agitated by inversion each 15 minutes.
- 0.33 volumes of 3M NaCl and 1 volume of chloroform were then added.
- The mixture was transferred to a 100 mL Erlenmeyer tube and agitated at 150 rpm until an emulsion was formed (about 40 min).
- It was then spun at 5,000 rpm for 10 min.

- The upper aqueous phase was skimmed off with 1,000 µl and put into an Erlenmeyer tube again to add 1 volume of chloroform; this stage was repeated twice more.
- The aqueous phase was transferred to a 50mL centrifuge tube. An inverted *Pasteur* pipette was introduced and cold isopropanol slowly added. The precipitated DNA was wound up onto the glass rod. Isopropanol continued to be added until the two phases in the tube disappeared.
- The glass rod with the DNA pellet was put into 70% ethanol for one second and left to dry until the DNA became transparent.
- The glass rod was finally put into 7 mL of water in a 15mL Falcon tube and left overnight at 4°C to suspend the DNA.
- The suspended DNA was observed and visualised by electrophoresis in 0.8%. Agarose gels. It was quantified by comparison in gel with con λ phage DNA standards of known concentration.
- The suspension was distributed in aliquots in 2mL tubes and stored at -20ºC until later use.
- 5µl of 10mg/mL RNAse were added before using the DNA to eliminate the RNA.
- The quantification of the probe for each reference strain's DNA was 50 ng/probe x 100 cm^2 membranes.

Probes preparation : 5 µg chromosomal DNA from each chosen species was digested with *Sau*3AI restriction endonuclease (5U). This enzyme completely digested the *Clostridium* DNA, generating fragments between 7 kbp and 5 bp. 86% of the generated fragments were between 7 kbp and 100 bp (less than 2% were fragments of less than 20bp), this being the optimum range for PCR labelling and using them in hybridisation experiments.

100 pg of digested DNA were used for the amplification and bound to the P2 adapter, specific for the extremes generated by the *Sau*3AI enzyme (5 unities) (Wassill *et al.*, 1998). The P2 adapter (Wassill *et al.*, 1998) was used to label the DNA fragments with digoxygenin, using the PCR DIG Probe Synthesis labelling kit (ROCHE). The amplification product was purified by precipitation with ethanol and

1/25 volumes of NaCl 3M. It was then centrifuged, washed in 70% ethanol and left to dry. The fragments were checked in a 1% agarose gel and the probe was quantified by spectrophotometry, according to Sambrook *et al.*, (1989).

6.6.2 Dot Blot and hybridisation

- These assays were done with 2 µg DNA from each strain, using a Dot Blot Manifold Filtration System (Gibco BRL) with vacuum pump, at room temperature in 200 mL NaOH at a final concentration of 0.4M. The DNA was fixed to a BioDyne nylon membrane (Pall) with ultraviolet radiation for 2 min (Cross Linker by GATC, Konstanz, Germany).

Buffer	Composition
Solution D	5x SSC
Hybridisation buffer	0.1% Lauryl sodium sarcocinate
	0.02% SDS
	1% ROCHE blocking agent
Solution E	2x SSC
Washing buffer 1	0.1% SDS
Solution F	0.5x SSC
Washing buffer 2	0.1% SDS
Solution G	100mM maleic acid
Buffer	150m M NaCl maleic acid, 7.5 pH
Solution H	100mM maleic acid
Blocking buffer	150 mM NaCl
	1% ROCHE blocking agent
Solution I	100 mM Tris HCl
Detection buffer, 9.5 pH	100 mM NaCl
	50 mM MgCl
Solution J	0.2M NaOH
Removal buffer	0.1% SDS w/v

50ng of probe were used for each 100cm² of membrane. The procedure followed for the hybridisation was that reported in the Roche Molecular Biochemicals manual (Boehringer Mannheim GmbH, 1995), with the modifications made below:

- Pre-hybridisation: The membrane was incubated with 30 mL solution D at 68°C for 2 h.
- Hybridisation: The volume needed for the probe to have 50ng per 100cm² membrane for 10 min at 95°C was denatured. This was then put immediately onto ice.
- The membrane was transferred to 10 mL of fresh solution D, pre-heated to 68°C (this being the volume necessary for the buffer to become distributed and cover the membrane.). A 10 cm by 15 cm receptacle was used.
- The probe was added and the membrane incubated with constant agitation at 68°C for 16 hours.
- Washing: Washing was done twice with solution E at room temperature with agitation for 5 minutes each time.
- Washing twice with solution F at 68°C for 15 minutes.

6.6.3. Detection

The whole procedure was done at room temperature, with constant agitation.

- The membrane was washed with solution H for 5 mins.
- 10 mL of solution H was added and the mixture incubated for 30 mins.
- This was then changed for 10 mL fresh solution H and anti-digoxygenin antibody was added in a 1:10,000 ratio. 10 µl Anti Dig AP Fab 150U fragments (ROCHE) were used.
- The membrane was incubated with the antibody for 30 mins.
- It was then washed with solution G twice for 10 minutes each time.
- The membrane was equilibrated with solution I for 5 mins.

- Once equilibrated, 10 mL fresh solution I and 25mM 100µl chemiluminescent alkaline phosphatase substrate (CDP–Star ROCHE) were added. The mixture was incubated for 10 minutes with agitation, the receptacle being protected from the light.
- After 10 minutes, the membrane was transferred to a glass slide, adding 1 mL buffer with the CDP-Star and covered with a piece of plastic film which did not interfere with the chemiluminiscence.
- A photographic film (X-Ray 10 AGFA, Gevaert) was placed directly over the Vinilpel. The film was removed after 5 seconds had passed; depending on the time, different revealing intensities were obtained.

6.6.4. Washing the membrane

The membrane was washed twice in Solution J at 68°C for 20 minutes and once at room temperature for 20 minutes, with shaking at 200 rpm to remove the probe and do a second hydridisation with a different probe. The membrane was finally washed with deionised water.

6.6.5. Determination of hybridisation percentages

The film image was acquired by using the GEL DOC system (Bio RAD, 2000a). The film was analysed with the QUANTITY ONE volume contour programme from Bio RAD kept on a fixed area so that the volumes (intensity by area) taken by the instrument varied only in terms of intensity and not area (BioRAD, 2000b). (Uvol= Unit of intensity x mm^2). The equipment used all the membrane controls to normalise the results calculated hybridisation percentages.

7. Enzyme activity determination

7.1 Cell extract and supernatant with enzyme activity

7.1.1. Micro-organism activation.

Each vial was prepared with 10 mL TYG broth in each one, under anaerobic conditions. 0.5 mL of the *Clostridium* stock spores were inoculated into them. The vials were placed in a water-bath at 70°C for 10 minutes; they were then immediately taken from the water-bath and placed into another bath containing ice. They were then incubated at 35°C for 24 hours.

7.1.2. Inocule preparation.

Vials were prepared with 25 mL TYS broth in each one, under anaerobic conditions. 1 mL of the recently cultured broth was inoculated into the TYG. This was then incubated at 35°C for 12 hours.

7.1.3. Fermentation

250 mL CMB broth with 1% starch were prepared in Erlenmeyer screw-top flasks, under anaerobic conditions. 25 mL of the recently cultured broth (TYS) was inoculated. The flasks were incubated at 35°C for 24 hours.

7.1.4. Cell harvesting.

The culture obtained in CMB with 1% starch was taken and centrifuged at 5,000 rpm for 30 minutes. The supernatant was kept in a screw-top flask and then stored at 4°C. The obtained pellet was washed twice using 0.1 M citrate buffer, pH 6.0. The culture was again centrifuged at 5,000 rpm for 30 min. 10 µl 0.1 M PMSF (Phenylmethylsulfonyl fluoride) (protease inhibitor) was added. The cell suspension was then stored in a screw-top flask at 4°C.

7.1.5. Cell extract.

The cells were broken up by sonication with 15-second pulses over a half hour period, keeping them constantly on ice. Cell rupture had to be checked microscopically. 10 µl 0.1 M PMSF (Phenylmethylsulfonyl fluoride) were then added. The sonicator used was an Autotune series high intensity ultrasonic processor, 750 Watt model.

7.2 Determination of enzyme activity

7.2.1 Dinitrosalicylic acid method (DNSA)

The dinitrosalicylic acid method was used to determine reducing sugars in supernatant and cell extract. The method was used for polymeric substrates such as: CMC, melezitose, rafinosse, chitosan, pectin, β-glucan, arabinan, avicel, xylan larch wood, xylan oat spelt, tapioca, starch and pullulan. By contrast, trials on maltose and cellobiose were determined by using a glucose oxidase kit (MERCK). (Miller, 1959)

Solution DNSA reagent

Dinitrosalicylic acid	10 g
Phenol	2 g
Na_2SO_3	0.5 g
Sodium potassium tartrate	200 g
NaOH	10 g
Distilled water	up to 1000 mL

- **DNSA calibration curve**

0-500 µg/500 µl glucose solutions were prepared. 750 µl **DNSA** reactive solution was added, mixed and heated to boiling point for 10 min. It was cooled and read at 575 nm. The mmol graph for glucose versus absorbency (575 nm) was drawn.

The enzymes were incubated with the substrate and DNSA was added to stop the reaction; the colour developed on boiling. 1M phosphate buffer (pH 5.6) was used for the supernatant. 750 µl DNSA reactive solution was added.

DNSA assay

Component	**Volume (ml)**		
	Assay	Control	Target
Enzyme	X	X*	-
Buffer 10X (1 M)	50	50	50
Substrate in 1% solution	250	250	250
Distilled water up to	500	500	500
DNSA	750	750	750

Table 2. Experimental design in DNSA assay

7.2.2 PGO assay

$$D-Glucose + H_2O + O_2 \xrightarrow{Glucose\ oxidase} Gluconate + H_2O_2$$

$$H_2O_2 + o\text{-}Dianisidin_{red} \xrightarrow{Peroxidase} o\text{-}Dianisidin_{ox} + H_2O$$

The PGO method was used to determine glucose liberated from the substrate. o-Dianisidin was used as a chromogen to read enzyme activity; o-Dianisidin red is colourless and o-Dianisidin ox. thus produces a brown colour.

Calibration curve. 0-100 µg/500 µl glucose solutions were prepared. 750 µL PGO reactive solution were added, mixed and incubated for 10 minutes at 37°C. The µmol of glucose versus absorbency (500 nm) graph was plotted. The mixture was placed in a 2 mL screw-topped Eppendorf flask for the assay.

Component	Volume (mL)		
	Assay	**Control**	**Target**
Enzyme	X	X*	-
Buffer 10X (1 M)	50	50	50
Substrate in 1% solution	250	250	250
Distilled water (500 µL)	Up to 500	up to 500	up to 500
PGO reagent	750	750	750

Table 3. Experimental design in PGO assay

PGO was added to the reaction mixture after the incubation with enzyme. 1M phosphate buffer (5.6 pH) was used for the supernatant and 1 M citrate buffer (6.0 pH) was used for the cell extract. The mixture was placed in a 2 mL Eppendorf screw-top flask for the assay. The mixture was incubated in a water-bath at 37°C for the required time, this being not greater than 14 hours. The samples were brought to the boil for 5 minutes to stop the enzymatic reaction. 50 µl PGO reactive solution were added, mixed and incubated at 37°C for 10 minutes. Absorbance was read at 500 nm.

7.2.3 Determining protein content

- Calibration curve

0-20 µg/500 µl protein solutions were prepared in 2 mL screw-topped Eppendorf tubes. 1 mL Bradford solution (Bradford , 1976) was added, mixed and allowed to stand for 2 minutes. The mixture was read at 595 nm, after two minutes. The graph of protein µg versus absorbency was drawn.

The assay consisted of a maximum of 0.1 mL protein in solution being placed in a tube for sample determination. It was completed to 0.1 mL with water.

8. Industrial medium design

8.1 Criteria used for designing Plackett-Burman matrix

IBUN IV mutant strain was grown in RCM medium to obtain maximum biomass production. Molasses were used as carbon source in constant concentration for all experiments. The concentration of molasses was theoretically calculated in relation to the glucose concentration necessary to obtain 3.8 g/L biomass and 15 g/L butanol. A total 25 g/L solvent production was considered and a 6 butanol, 3 acetone, 1 ethanol ratio was supposed, according to ABE fermentation.

Theoretic glucose concentration was calculated in 77 g/L. Molasses glucose concentration was 59.1 g/L as free glucose from molasses after hydrolysis was determined by HPLC chromatography, 130 g/L molasses concentration was thus required. The carbon and nitrogen source, amino acid and growth factor concentration interval were calculated in relation to biomass concentration obtained in vegetative medium. The range of culture medium concentrations from different components (used to design the matrix) was as follows:

Culture medium components evaluated	Concentration range tested
Molasses as carbon source	130 g/L
Yeast extract used as organic nitrogen source (Sno)	3 -10 g/L
$(NH_4)_2SO_4$ used as inorganic nitrogen source (SNi)	2.0 - 8.0 g/L
Control amino-acids: cysteine hyderochloride (Cy)	0.5 - 1.0 mg/L
thyroxin (Ty)	0.1 - 1.0 mg/L
Essential amino-acid: valin (Val)	0.1 - 1.0 mg/L
Growth factor concentration levels:	
Biotin (B)	0.05 - 1.0 mg/L
PABA (p-aminobenzoic acid) (PA)	0.1 - 2.0 mg/L
Mineral stock was used as mineral source (MS)	1 - 10 mL/L
KH_2PO_4	0.5 -2.0 g /L
Distilled water up to 1 litre	
pH 6.5, before sterilisation	

Table 4. The range of culture medium concentrations from different components used to design the matrix

Mineral Stock

$MgSO_4$. $7H_2O$	1.3 g/L
$FeCl_3$	3.0 g/L
$CaCl_2$. $6H_20$	0.9 g/L
H_2SO_4	12.0 mL/L

The Plackett-Burman matrix (Plackett and Burman, 1946) was formed by combining four high-level and four low-level culture medium concentrations **(**Table 5**)**

Assay	PABA and B	Tyr-Cys	Val	Sni	Mineral Stock	SNo	pH
1	**H**BPA	**h** TyCy	**h** Val 1	**l** Sni1	**h** MS1	**l** SNo 1	4,5
2	**H**B	**h** Ty	**l** Val 1	**h** SNi1	**l** MS 1	**l** SNo 2	5,8
3	**H**PA	**l** TyCy	**h** Val 2	**l** Sni2	**l** MS 2	**h** SNo 2	6.0
4	**L**PA	**h** Cy	**l** Val 2	**l** Sni3	**h** MS 2	**h** SNo 2	6.4
5	**H**BPA	**l** T	**l** Val 3	**h** SNi2	**h** MS 3	**h** SNo 3	4.8
6	**L**B	**l** Cy	**h** Val 3	**h** SNi3	**h** MS 3	**l** SNo 3	6.8
7	**l**PA	**h** TyCy	**h** Val 4	**h** SNi4	**l** MS 3	**h** SNo 4	5.1
8	**l**BPA	**l** TyCy	**l** Val 4	**l** Sni4	**l** MS 4	**l** SNo 4	5.5

Table 5. Placket-Burman Matrix

h high-level and **l** low-level variables were defined as:(B) biotin as growth factor; (PA) p-aminobenzoic acid and biotin; tyroxin (Tyr) and cysteine (Cys) as control variables; valin (Val) as essential amino-acid; (SNi) $(NH_4)_2SO_4$ as inorganic nitrogen source; yeast extract (SNo) organic nitrogen source; (MS) mineral stock: KH_2PO_4, $MgSO_4.7H_20$, $FeCI_3$, $CoCI_2.6\ H_2O$. Thyroxin and cysteine were used as control variables. These variable concentrations had no effect on final total solvent concentration. All experiments were done four times.

8.2. Fermentation conditions**.**

10 mL (OD=1.3) activated micro-organisms were inoculated into vials containing 40 mL culture medium. The samples were incubated at 37°C for 200 h. The effect of each variable (E_V) on solvent production was determined to define which variables

significantly influenced the process. A significant effect was obtained by comparing the absolute value of t-student t_o with t_t. Then, S $|t_o| \geq t_t$ indicated acceptance of the null hypothesis (i.e. that the independent variable (factor) had no effect on solvent response). Results are presented which have been derived from the following calculations.

$$E_v = \left(\sum \frac{hR}{n} \right) - \left(\sum \frac{lR}{n} \right)$$

$\sum \frac{hR}{n}$: Average response at high levels for each varia ble

$\sum \frac{lR}{n}$: Average of low response for each varia ble

n : Number of runs

- **T-student (t_t) was estimated as trial criteria**

$$t_o = \frac{E_v}{(S.E.eff_v)} \qquad V\,eff_v = \sum \frac{(Ed)^2}{n}$$

$$S.E.eff_v = (V\,eff_v)^{\frac{1}{2}} = 0.13$$

t_o: value calculated from trial statistics.

S.E. eff_v: standard error for the effect of each one of the variables,

V eff_v: variance of an effect,

Ed: effect of the assigned variables (thyroxin and cysteine).

8.3. Criteria for designing EVOP-Simplex (1) method

The Plackett-Burman results were used to design EVOP-Simplex experiments. Seven variables at three levels (high, medium and low) were evaluated to obtain the very best concentrations for each culture medium component. Seven matrices (or cycles) were designed, each matrix consisting of seven experiments. High and low levels were thus pre-established by Plackett-Burman application.

The first matrix of the first simplex or first cycle (Table 6). The first cycle's first assay had high concentration levels; the second assay had the lowest concentration level in the first factor and high level concentrations were repeated in the other levels. The third assay had medium level in the first factor; in the second factor it had low concentration and in the other factors, high levels were repeated. The fourth assay was designed using medium levels in the first and second factor; in the third factor, the lowest level was used and the rest of the factors were repeated. The design up to the seventh matrix assays was obtained by using this methodology.

Fermentation was repeated three times. Solvent production was determined and the assay presenting the lowest solvent production was discarded. The lowest production was obtained in Simplex assay 4 (17.43 g/L total solvents). The assay used instead of the rejected assay to design the Simplex second cycle was calculated as follows:

New assay = $2X - DIS_n$ X = S RET/n

RET: retained level;

n: number of retained levels;

X: average of retained levels;

DIS_n: level of discarded assay. Industrial culture medium final composition was obtained from the last cycle.

Assay	Biotin	PABA	KH_2PO_4	Mineral Stock	Yeast extract	pH	Total solvent g/L
1	0.5	0.4	200	0.5	500	6.4	24.59
2	0.3	0.4	200	0.5	500	6.4	20.44
3	0.4	0.2	200	0.5	500	6.4	19.09
4	0.4	0.3	160	0.5	500	6.4	17.43
5	0.4	0.3	180	0.1	500	6.4	22.74
6	0.4	0.3	180	0.3	300	6.4	21.27
7	0.4	0.3	180	0.3	400	5.8	23.90
ΣRET	2.4	1.9	1140	2.2	2700	37.8	
2 X	0.8	0.633	380	0.73	900	12.6	
DES	0.4	0.3	160	0.5	500	6.4	
2X - DES	0.4	0.33	220	0.23	400	6.2	

Table 6. First matrix from the seventh EVOP-simplex (simplex 1) cycle
Concentration in mg/L and mineral stock in mL

9. POME biodegradation methods.

9.1 Experimental determination of POME composition

Parameter	g/L
pH	4.17
Dry Material	65.38
Hemicellullose	11.25
Cellulose	164.61
Soluble Carbohydrates	42.17
Starch	25.64
Pectine	7.50
Glucose	1.80
Xylose	2.12
Arabinose	2.06
Fats and Oils	1.93

Acetic Acid	0.024
Lactic Acid	1.0
Butanol	0.04
Ethanol	0.057
Acetone	0.07
Density	1.014 g/cc

9.2. Determination of kinetic parameters

9.2.1. Biomass determination.

Cells were determined microscopically. 1 mL of sample was centrifuged at 11,000 rpm for 20 minutes. Pellet was suspended in 1mL sterilised NaCl (0.9% p/v). The pellet was washed three times to remove solids. Dilutions were done to count between 40-400 cells in Neubauer Chamber.

$$\textbf{\# cells/mL= [(S\# cells X 5)/10}^{-4}\textbf{] X dilution}$$

9.2.2. Determination of pH

5 mL fermentation supernatant were used for pH determination after calibration using the Schott pH-Meter CG 840 B potentiometer. The assay was done at room temperature.

9.2.3. Acid and solvent determination

Acids and solvents were determined in a Waters HPLC, using an Aminex HPX-87H column and two detectors (Waters 410 Differential Refractometer and Waters 996 Photodiode Array Detector). Internal and external temperatures were 30°C and 45°C respectively. 0.65 mL/min H_2SO_4 5 mM and 40 min running time were used for mobile phase. The Millennium Chromatography Manager programme was used for data processing.

Standards	Purity of standard	Retention time	Detector
Acetic acid	100%; 60.05 MW; 1.05 density	14.219	Differential refractometer (channel 410 nm)
Butyric acid	99%; 88.10 MW; 0.96 density	20.437	Photodiode array (channel 996-210 nm)
Ethanol	99.8%; 88.10 MW; 0.78 density	19.421	Differential refractometer (channel 410 nm)
Butanol	99.5%; 74.12 MW; 0.81 density	33.714	Differential refractometer (channel 410 nm)
Acetone	99.5%; 58.08 MW; 0.79 density	20.315	Photodiode array (channel 996-260 nm)

Table 7. Standards, conditions and detectors used to determine solvents and acid profiles

Sample analysis

1 mL supernatant sample was filtered first through a pre-filter and then twice through 0.45 µ (diameter pore) nitrocellulose membrane. 20 µL H_2SO_4 5N were then added to the filtered product up to 2.0 pH. 20 µL sample were injected into the chromatograph.

9.2.4 Consumed substrate

Consumed substrate was determined by DNSA assay. DNSA reagent and the calibration curve have already been described in Matherials and Methods 7.2.1. The consumed substrate has been calculated from the difference between the initial substrate concentration and the residual substrate concentration in the culture medium.

9.2.5. Determination of fermentable sugar by POME hydrolysis:

50 µl 72% H_2SO_4 were added to 1 mL POME Medium .This was then mixed at 121ºC for 15 min. The supernatant was skimmed off and DNSA was carried out in a 50 µl aliquot. The pellet was suspended in 1 mL 0.9% NaCl; 50 µl 72% H_2SO_4 were added. The cycle was repeated again and reducing sugars determined.

9.2.6. Determination of non-dissociated acids ([HA]) and acid production speed mol/L*h).

The acid production during the fermentation was determinated by HPLC and acid production speed was calculated by using times in exponential phase in producing each product, this constant being the slope value, according to the following formula:

$$\textbf{Acid production speed (mmol/L*h)} = (Y_2 - Y_1)/(X_2 - X_1)$$

Non-dissociated acid concentration in the medium was calculated according to the Henderson-Hasselbach equation:

$$pH = pK_a + \log([A^-]/[HA])$$

$$[HA] = (10^{-pH} \times C_{\text{total acids}})/(10^{-pKa} + 10^{-pH})$$

9.3 Fermentation

9.3.1 Preliminary assays

- Inoculum preparation**.** *C. saccharobutylicum* NRRL B 643 reference strain and IBUN 22 A and IBUN 140B native strains were selected for degrading the POME waste. Cells were activated as described in Materials and Methodes 3.0.

- **The degrading preliminary assays.** 25 mL POME Medium (using 1.8 mL inoculum (7.5% v/v)) 100%, 75% and 50% POME effluent as carbon source) were inoculated and incubated at 37°C for 7 and 14 days without shaking. Fermentation times, physical changes, pH and kinetic parameters were then evaluated.

9.3.2. Degradation kinetics of the IBUN 22A and IBUN IV strains in 1 litre of POME Medium.

In agreement with the preliminary assay results, 25 mL POME Medium (2.12) were inoculated with 1.85 mL pre-inoculum (7.5% v/v concentration) of previously activated (as described in 3.0) strains (IBUN 22 A, IBUN IV, separately). The vials were incubated at 33°C, 150 rpm for 8 days. Samples were taken at different hours: $t_{0,}$, t_{48}, $t_{72,}$ $t_{96,}$ $t_{120,}$ $t_{146,}$ $t_{168,}$ $t_{192,}$ $t_{216.}$ A vial (with its triplicate) was withdrawn each time. Samples were frozen and their kinetic parameters analysed when fermentation had finished.

9.3.3. Determination of POME Medium buffer capacity

Buffer capacity in POME was determined by titre curve with 1N HCl and 1N NaOH, in a 20 mL 100% POME sample. POME initial pH was 3.9; this was used as the starting point for acid and basic titres. The changes in pH were determined regarding different volumes of acid or alkali.

9.3.4. Determination of initial fermentation pH.

Buffer capacity of the POME Medium was determinated. It was seen that the region understood as being between pH 4.0 and 6.0 presented the greatest buffer capacity. To determine initial fermentation pH, assays were done at 5.04, 5.24 and 5.7 pHs. Fermentation was carried out in 25 mL vials in POME Medium. Fermentation was done at 150 rpm for seven days at 33°C.

9.3.5 Kinetics of biodegradation in 1 litre POME Medium

Fermentation kinetics were determined in one litre of POME Medium. Fermentation was done in 2-litre Erlenmeyer flasks, with one litre of culture medium, at 150 rpm for eight days at 33°C. Assays were done at 5.24 pH. Inoculum percentage was 7.5% v/v. 11 mL samples were taken each 6 hours. All assays were done in triplicate. The medium was gassed with nitrogen up to total anaerobiosis, taking around 30 minutes, and sterilised at 121 ºC for 20 minutes . The micro-organisms used were IBUN IV and IBUN 22A.

II. RESULTS

1. Micro-organism isolation from soil and solvent-production profiles

Soil samples were collected from agricultural areas in different Colombian departments (as described in Materials and Methods). 178 spore-forming, anaerobic bacteria-producing acetone isolates were purified from 155 soil samples by serial dilution. Single colonies were isolated by picking and streaking onto T6 agar plates, under strictly anaerobic conditions. The colonies of the new isolated and purifies strains were mostly circular to slightly irregular, whole, raised, dull beige, rods. Cells were rod-shaped; these were stained Gram-positive and grown anaerobically at 37°C . The solvents produced by the purified cultures from glucose in batch culture were evaluated by gas chromatography in duplicate and compared with *C. acetobutylicum* ATCC 824. The variation coefficient of all solvent production analyses ranged from 7% to 9%. The samples which grew and produced gas within 24 h were better sources for isolation of solvent-producing bacteria than those samples which grew in more than 48 h and their behaviour as a member or the *Clostridium* genus. They presented granulose accumulation, transformation from vegetative cells into clostridial cells. According to these criteria, they behaved like members of the *Clostridium* family.

The 178 isolates from 155 samples were divided into 4 groups (Figure 2) according to the solvent production profiles. Although 71% of the strains produced mainly ethanol, a number of strains presented high butanol production potential (Table 8), demonstrating the relationship between the kind of crop and the number of *Clostridium* isolates. The greatest number of solvent-producing clostridia was found in soil from industrial cotton and sugar cane cultures, although the highest average concentration of total solvent produced from this source was 5.8 g/L. On the other hand, the highest average concentration of total solvents was produced by micro-organisms isolated from grass (7.5 g/L), chrysanthemum (7.7 g/L) and tuber culture (9.5 g/L) soil.

The best solvent producers' maximum values are presented in (Table 8) . Isolation areas were classified into three types of soil pH [acidic (pH < 6.5), neutral (pH 6.5-7.5) and

alkaline (pH> 7.5)] to determine the influence of soil pH on isolation of favourable solvent-producing clostridia. The strains having the highest solvent production were isolated from soil with a pH of fewer than 6.9. Acidic pH soil generally offers a better chance of isolating a high number of solvent producers and also a potent solvent-producing bacterium. Only one strain was isolated from alkaline soil (worm breeding) with 3.8 g /L total solvent production.

Solvent production profiles of the 178 strains were studied; the results presented a wide range of variability in relation to the *Clostridium acetobutylicum* ATCC 824 type strain. One hundred (100) isolates produced more than 2 g/L total solvents and 13 of these strains produced more total solvents than ATCC 824. These were the only ones selected for further study (Table 9). The highest concentration of ethanol (19.1 g/L) was produced by the IBUN 18A isolate, which was equivalent to 1.24 moles of ethanol per mol of fermented glucose. At least 30 species of clostridia have been reported as producing ethanol in amounts ranging from traces to close to the theoretical maximum of 2 moles of ethanol per mole of fermented glucose (Jones, 1989**)**.

Differences in the distribution of the solvents produced have not been associated with species' attribution. High ethanol, acetone and butanol production was found in different strains from all four groups. The IBUN 18A strain (the most active solvent-producing strain) however did not produce butanol. As high butanol production is usually coupled with low ethanol production, then this points to the three Clostridium species having a very similar fermentation pathway.

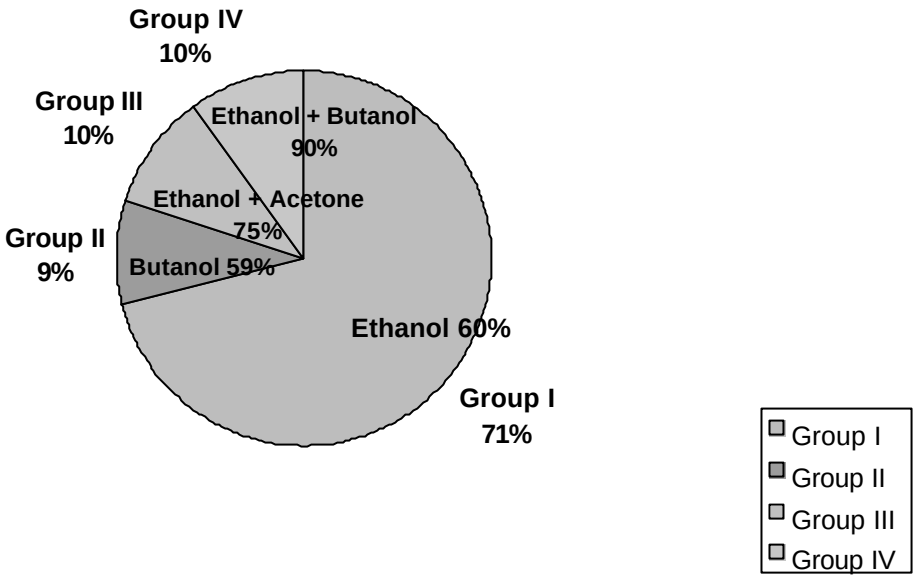


Figure 2 Solvent production profiles for all new isolates (total: 178 strains)

Four groups were classified according to final solvent concentration: Group I, 60% ethanol production; Group II, butanol production represented 59%; Group III, ethanol plus acetone production was 59%; Group IV, ethanol plus butanol production was 90% of total solvents produced from glucose in batch culture

Crop	Kind of Crop	Number of strains isolated per crop	Isolates per crop group	Percentage %
Pumpkin	Pumpkin	2	2	1.12
Coffee	Coffee	5	5	2.80
Cereal	Corn Rice	2 10	12	6.75
Fruit	Avocado Banana Lemon Mango Tree-tomato	2 2 7 1 5	17	9.55
Vegetable	Onion Lettuce Cabbage Carrot	1 4 2 5	12	6.74
Industrial Crops	Cotton Sugar-cane	19 24	43	24.15
Legume	Pea Kidney bean Soya bean	14 4 7	25	14.04
Ornamental flower	Chrysanthemum	18	18	10.11
Grass	Grass	28	28	11.79
Stubble	Stubble	10	10	5.62
Tuber	Potato	5	5	2.80
Worms*	Worm	1	1	0.56
Total			178	100

Table 8. *Clostridium spp* isolation from different crops grown in Colombia

***Clostridium* spp strains**	**Colombian department**	**Type of crop**	**Soil pH**	**Butanol g l -^{1}**	**Ethanol g l -^{1}**	**Acetone g l -^{1}**	**Total - Solvents g l -^{1}**
C acetobutylicum ATCC 824				8.4	1.2	4.4	14.0
IBUN 13[a]	Cundinamarca	Potato	4.4	3.6	0.3	12.0	15.9
IBUN 18A	Antioquia	Chrysanthemum	6.5	ND	10.1	19.1	29.1
IBUN 18Q	Antioquia	Chrysanthemum	6.5	2.8	0.1	13.5	16.4
IBUN 18 S	Antioquia	Chrysanthemum	6.5	6.0	0.9	10.0	16.9
IBUN 22 A	Cundinamarca	Potato	4.7	3.0	0.1	17.2	20.3
IBUN 62 B	Cundinamarca	Grass	4.7	1.4	N.D	16.2	17.6
IBUN 62 f	Cundinamarca	Grass	4.6	6.8	0.9	8.5	16.2
IBUN 63 D	Cundinamarca	Grass	4.7	4.5	5.9	ND	10.4
IBUN 64 A	Cundinamarca	Grass	4.7	1.4	ND	15.1	16.5
BUN 95 B	Tolima	Soya	6.6	1.1	ND	14.5	15.6
IBUN 125 C	Tolima	Rice	6.9	11.4	2.3	11.5	25.2
IBUN 137 K	Boyacá	Sugar-cane	6.2	4.4	0.3	10.6	15.3
IBUN 140 B	Boyacá	Sugar-cane	6.3	5.4	0.7	10.3	16.4
IBUN 158 B	Cundinamarca	Tree-tomato	5.9	0.8	0.1	15.6	16.5

Table 9. Solvent production from the new strains, compared to the *C. acetobutylicum* ATCC 824 strain.

The solvents produced in T6 medium, at 37^{0}C. Samples were taken 96 h after fermentation had started. ND = not detected. The pH of the soil from which the strains were isolated is shown. Numbers refer to the concentration of solvents in the fermentation broth, as determined by headspace analysis in gas chromatography (g/L)

2. Tests for differentiating between solventogenic clostridia

According to the microbiological criteria (Materials and Methods 5.2), the new isolates were mesophilic, saccharolytic isolates from the genus *Clostridium*. Consequently, physiological tests have been used to distinguish between the four different groups of solvent-producing clostridia described in Johnson *et al.*(1997): riboflavin production, curd formation from milk and rifampicin susceptibility: Group I, *Clostridium acetobutylicum* (+, +, s); Group II, *C. saccharobutylicum* NCP 262 (-, +, s); Group III, C. s*accharoperbutylacetonicum* (-, -, r), and Group IV, *C. beijerinckii* (-, +, r). Classification (according to these criteria) identified 9 strains as being *C. acetobutylicum*, 3 as C. NCP 262 and 3 as *C. beijerinckii* (Table 10)**.** This classification is, however, tentative and will have to be verified using molecular biology methodology.

Table 11 presents the results of the biochemical testing of native strains; thus gave glycerol (+), ribose (+), indol, gelatine and catalase (-) According to these results, the native strains belonged to the solventogenic *Clostridium butyricum* group.

2.1. Toxicity Test

This test was done to determine the toxicity of the thirteen native strains used in this study. Five (5) mice were inoculated with TGY medium to perform the toxicity assay for each native strain. Tetanus neurotoxin was used as positive control. A positive result was given when symptoms such as painful spasms and voluntary muscle rigidity and violent limb muscle spasms were manifested. In such cases, an autopsy must be done to observe the internal signs. Assays were done prior to adding tripsin (tripsinisation at 37°C for 5 minutes) to activate the toxin, producing an identical result. None of the native strains presented toxicity.

Strain	Milk curd (production)	Riboflavin µg/mL	Rifampicin sensitivity	Tentative classification
C. *beijerinckii* 8052	24 h	-	R	*C. beijerinckii 8052*
DSM 792	24 h	289.1	S	*C. acetobutylicum*
IBUN 13[a]	48 h	-	R	*N1-4*
IBUN 18[a]	24 h	--	S	*NCP 262*
IBUN 18Q	24 h	-	R	*N1-4*
IBUN 18 S	24 h	-	S	*NCP 262*
IBUN 22 A	24 h	-	S	*NCP 262*
IBUN 62 B	24 h	-	R	*C. beijerinckii*
IBUN 62 F	24 h	-	S	*NCP 262*
IBUN 63 D	24 h	14.7	S	*C. acetobutylicum*
IBUN 64 A	24 h	9.30	S	*C. acetobutylicum*
IBUN 95B	24 h	100.45	S	*C. acetobutylicum*
IBUN 125C	24 h	-	S	*NCP 262*
IBUN 137 K	24 h	14.9	S	*C. acetobutylicum*
IBUN 140B	24 h	13.2	S	*C. acetobutylicum*
IBUN 158B	24 h	25.8	S	*C. acetobutylicum*

Table 10. Physiological characterisation of strains.

Tentative species determination of the strains by physiological characterisation. R = resistant, S = sensitive for rifampicin.

Test	Glycerol	Ribose	Indol	Catalase	Gelatin	Avicel degradation	Vogues Proskauer
C.acetobutylicum ATCC 824	-	-	+	-	-	+	+
C. butyricum DSM 2478	+	+	-	-	-	-	-
IBUN 13[a]	+	+	-	-	-	+	-
IBUN 18[a]	+	+	-	-	-	+	-
IBUN 18Q	+	+	-	-	-	-	-
IBUN 18S	+	+	-	-	-	-	-
IBUN 22[a]	+	+	-	-	-	+	-
IBUN 62B	+	+	-	-	-	+	-
IBUN 62F	+	+	-	-	-	+	-
IBUN 64[a]	+	+	-	-	-	+	-
IBUN 95B	+	+	-	-	-	-	-
IBUN 125C	+	+	-	-	-	+	-
IBUN 137K	+	+	-	-	-	+	-
IBUN 140B	+	+	-	-	-	+	-
IBUN 158B	+	+	-	-	-	+	-

Table 11. Native strain biochemical tests

3. Molecular characterisation

3.1 Results of plasmid screening

The methodology developed demanded that the cultures had to be controlled halfway through their exponential growth phase to the higher DNAse activity following the exponential grow phase. Sample-taking was done every hour.

A theoretical approximation of the necessary conditions for plasmid detection was made before beginning the standardisation process for the extraction method. The smallest of the plasmids present in *Clostridium butyricum* NCIB 7423 was taken as a model to calculate the number of cells necessary to obtain a sufficient quantity of plasmid DNA that could be observed and thereafter digested. This was calculated from its molecular weight, assuming that each cell possessed a single copy of the plasmid. It was found necessary to use 8×10^7 cells to obtain 5.0 ng DNA plasmid so that the plasmid could be detected in agarose gels. The number of cells was determined microscopically (Materials and Methods 9.2.1) and cultures were grown until OD_{680}=0.47.

Natives strains *IBUN* 125C, *IBUN* 158B, *IBUN* 137K, *IBUN* 22A, *IBUN* 13A, *IBUN* 64A. *IBUN* 62F, *IBUN* 140B, *IBUN* 62B, *IBUN* 18A, *IBUN* 18Q, *IBUN* 18S and *IBUN* 95B, the reference strains of *Clostridium acetobutylicum* ATCC 824 and *DSM* 1732 and *Clostridium butyricum* DSM 2478 used the *S. epidermidis* M95 850 strain as an internal control and *E. coli* V 517 plasmid as molecular marker.

The strains chosen were IBUN 22 A, IBUN 140 B and *C. butyricum* 2478 as a control. 40 mL cell culture volumes were needed for the plasmids to be observed (6.2). The thirteen native isolates selected by their highest solvent production (Table 9) together with three standard strains, were handled as previously standardised. 8 plasmids from the *E. coli* V517 strain (whose Kb size was known [

Figure 3] and which were extracted under the same conditions) were used as size standard. *S. epidermidis* 850 M 95A (whose plasmid number was already known and has been reproducible in previous projects in the Molecular Epidemiology Laboratory at the Instituto de Biotecnologia, University Nacional de Colombia) was used as positive control for this methodology.

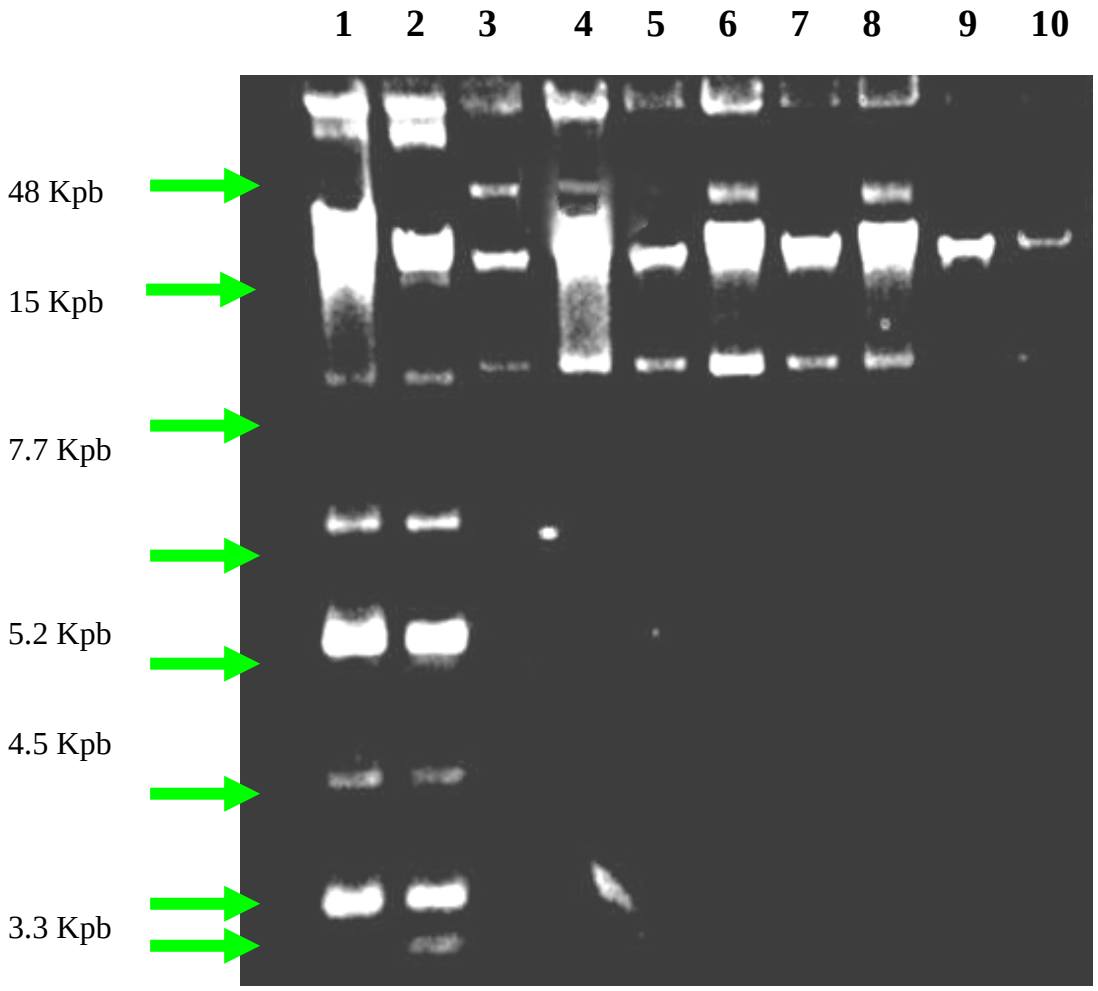


Figure 3. Plasmid gel.
Lanes 1, 2 *E. coli* V517 plamid profile as molecular marker. Lanes 3, 4: IBUN 137K. Lanes 5, 7: IBUN 18A. Lanes 6, 8: IBUN 18Q. Lane 9: *C. acetobutylicum* ATCC824. Lane 10: *C. acetobutylicum* DSM1732.

3.1.1 Plasmid profiles

The sizes of the plasmids present in each strain can be found in (Table 12). The number of large plasmid DNA fragement average base pairs consisted of one of 32.5 Kb present

in 11 strains (10 native and *C. butyricum* DSM 2478) and a 29 Kb plasmid present in IBUN 18A, IBUN 18Q and IBUN 137K native strains. The *C. acetobutylicum* ATCC 824 standard strain possessed a 20.4 Kb plasmid. The presence of smaller sized plasmid DNA fragments was observed in 8 strains (7 native strains and *C. butyricum*). Four native strains and the *C. butyricum* DSM 2478 standard strain possessed a plasmid DNA fragment whose average size was 13.7 Kb, whilst a 11.4 Kb plasmid was found in the IBUN 18A, IBUN 18Q and IBUN 137K strains.

3.1.2 Restriction and plasmid DNA assays

Chromosomal and plasmid DNA samples were digested in parallel to enable identification of the bands corresponding to plasmid digestion, as the final plasmid extract contained significant chromosomal DNA contamination. We have previously extracted the chromosomal DNA. Lambda phage cut with Hind III and Eco RI restriction enzymes was used simultaneously for restriction profiles and as molecular weight marker. Lambda phage digested with the Hind III enzyme was also used.

Chromosomal DNA present in the gels was used as a control for digested plasmid profiles. The EcoR V enzyme was chosen for evaluating all the strains, as two strong bands were generated with it in the plasmid DNA samples. The bands generated in the plasmid DNA samples (by comparison with the chromosomal DNA restriction standard) can be observed in restriction standards The remaining strains were digested in the same conditions established for the IBUN 125C strain and run in 0.8% concentration agarose gels for 5 hours at 2.5 V/cm. Profiles of plasmid fragments digested with ***Eco RV*** can be seen in Table 13

Strain	Number of plasmid DNA fragments	Size(Kb)
C. acetobutylicum ATCC 824	1	20
IBUN 13 A	1	33
IBUN 95B	1	30
IBUN 18S	1	33
IBUN 22 A	1	32
IBUN 140 B	1	32
IBUN 62B	1	31
C. butyricum DSM 2478	2	33 13
IBUN 64 A	2	33 13
IBUN 62F	2	32 13
IBUN 125C	2	33 13
IBUN 158 B	2	33 13
IBUN 137K	2	28 11
IBUN 18Q	2	28 11
IBUN 18A	2	29 11

Table 12**.** Plasmid DNA fragment size in *Clostridium spp* native strains

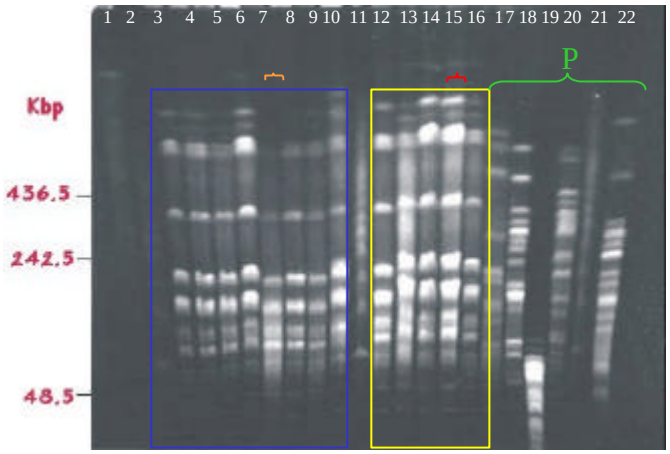
3.2 PFGE

Pulsed Field Gel Electrophoresis was used to differentiate micro-organism species. This method allowed separating long fragments (after digestion) for specifying different bands' DNA fragment standards. The enzymes were selected with 6-base-pair G+C-rich recognition sites (ApaI, SmaI, Eco52 I and BssHII). The results show fragment size ranging between 20 and 600 Kbp (Apa I) and between 40 and 1,000 Kbp (Sma I), these being fragment size and number ranges concurring with those reported by Keis *et al.*, (1995) and Wilkinson and Young (1993). Electrophoretic profiles were very similar in the majority of native strains assimilated to restriction endonuclease from both Sma I and Apa I.

Plasmid from native strain digested with *Eco RV*	**Band size bp**
IBUN 18 S	8,578 3,251
IBUN 140 B	8,601
	3,295
IBUN 13 A	9,716
	3,243
IBUN 18 A	9,808
IBUN 125 C	10,070
	3,507
IBUN 95 B	8,483
	3,309
IBUN 62 F	8,356
	3,288
IBUN 62 B	8,373
	4,015
IBUN 158 B	8,226
IBUN 137 K	8,657
	3,720

Table 13. Restriction fragments with the *Eco RV* enzyme from solventogenic clostridia strains.

Pulse Field Gel Electrophoresis.



Lane 2, 11 and 21: DNA ladder; 1: undigested control; 3: IBUN 13A; 4: IBUN 18A; 5: IBUN 18S; 6: IBUN 18Q; 7: IBUN 22A; 8: IBUN 62B; 9: IBUN 62F; 10: IBUN 64A; 12: IBUN 95B; 13: IBUN 125C; 14: IBUN 137K; 15: IBUN 140B; 16: IBUN 158B; 17: DMS 2478 (*C. butyricum*); 18: DSM, 1732 (*C. acetobutylicum*); 19: NRRL B-643 (*C. saccharobutylicum*); 20: NCIMB 8052 (*C. beijerinckii*); 22: N1-4 (*C. saccharoperbutylacetonicum*)

The number of fragments digested by SmaI was 15 and 19 by ApaI; for all new isolates, the range of fragments digested was from about 20 kb to over 600 kb. These standards were indistinguishable amongst the strains, except for 1 additional SmaI (ApaI) large-sized fragment for the IBUN 64A strain . Very few equal-sized bands could be identified in the chromosomal DNA fragment standard for DSM 1732, NRRL B643, NCIMB 8052 solventogenic clostridia, the N1-4 group and also for NCP 262.

Results and physiological standards suggest that all native strains were almost identical to each other. The PFGE results showed that the type strains conserved the same patterns as the four solventogenic groups reported by Keis *et al.,* (1995) and Johnson *et al.,* (1997) and different patterns from the native strain, except the type strain *C. butyricum* DSM 2478 which presented the same DNA macro-fragments as all native strains

3.3 16 S rRNA sequencing

3.3.1 Primers for gene amplification

Conserved regions in clostridia were identified and the positions of some primers (as reported in the literature by Kunhert *et al.*, 1996; Keis *et al.*, 1995; Lawson *et al.*, 1993) were located. The position of these primers is represented in Figure 4 (Primers 16S), according to their corresponding position in the homologous gene in *E. coli* (primer A: 774-795; primer J: 1521-1495; primer C: 1071-1087; primer D: 1091-1075) (Table 14). The possible primers' ability to be used in the same PCR with the appropriate temperature was tested. Two inner primers were also located, according to Keis *et al.*, (1995).

It should be noticed that the primers used for amplifying the second part of the 16S gene's rRNA for all native strains corresponded to the A and J primers (the J primer being modified at the 3'OH termini), allowing a decrease in annealing temperature so as to use this primer instead of the B primer reported by Keis *et al.* (1995) for amplifying a larger gene segment. The sequencing primers (C and D) corresponded to SEQ C and SEQ D, as reported by Keis *et al.* (1995). The E and F primers were used to amplify the first part of the gene to obtain the complete sequence of the 16S rRNA gene for the IBUN 22A, IBUN 125C and IBUN 64A native strains. The G and H primers (corresponding to SEQ G and SEQ H reported by Keis *et al.*, 1995) were used to sequence this part and thus obtain the 16 S ribosomal gene's complete sequence of three native strains (Figure 4).

3.3.2 Sample amplification

Chromosomal DNA was isolated (as described in 6.1) and quantified. The standard conditions for amplification were employed. A, J and F, E primers were used after testing their usefulness in generating the band of interest (i.e. a 700-bp fragment for the second part of the gene and 800 bp for the first part). This procedure was performed several times for some strains. Genomic DNA isolation from the IBUN 64A strain was substantially different and amplification was initially ineffective. In this case, a lower annealing temperature (60ºC) was necessary.

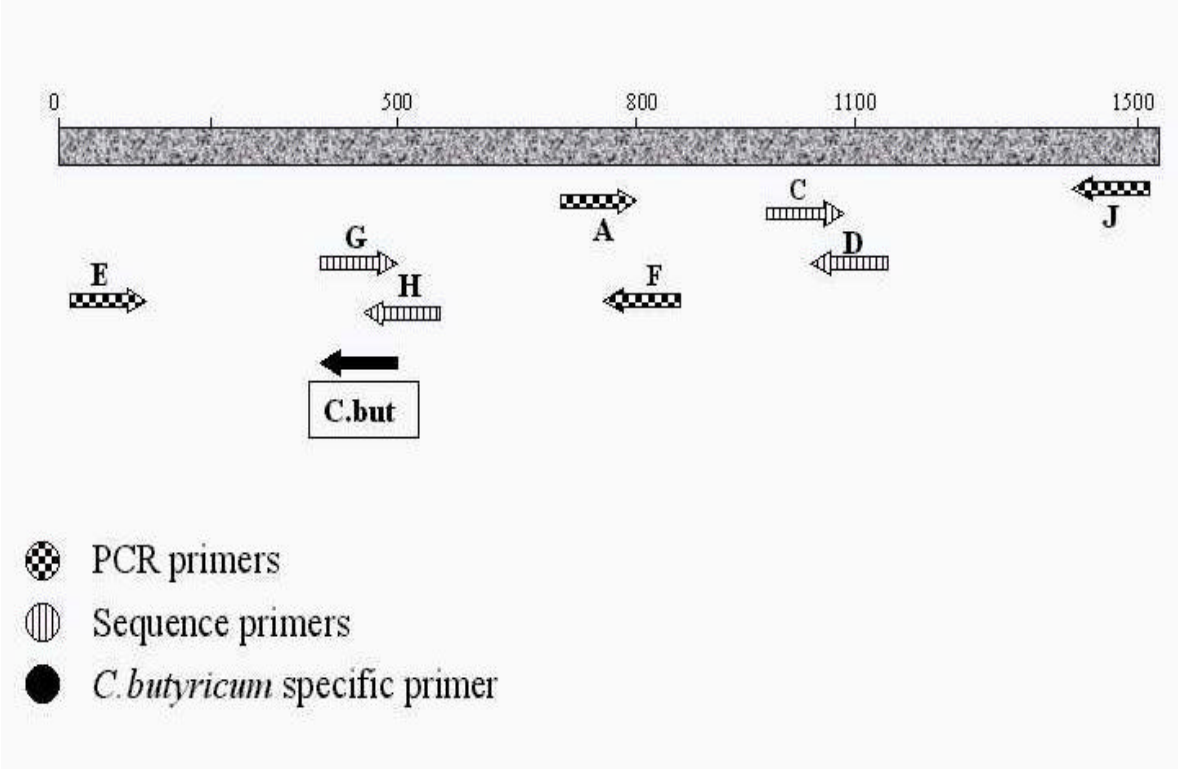


Figure 4 Primer design and sequences reported by Keis *et al.*, for 16S rRNA(1995)

pA(ggagcaaacaggattagataccc), pC (gtgtcgtgagatgttgg), pD (taacccaacatctcacg), pE (gagagtttgagcctggctc), pF (gttttaatcttgcgaccgtac), pG (gtcttcagggacgataatg) pH (ctgctggcacgtagttag), pJ modified (ttctcctacggctaccttgttac). *C. butyricum* primer reported by Knabel *et al.,* (1997)

3.3.3. DNA sequencing

A Perkin-Elmer 373 was used to sequence these strains. 4 pairs of primers were used for sequencing each part of the gene (A & J to amplify 16 S rRNA gene's second fragment; F & E to amplify the gene's first fragment; and C & D and G & H to complete 16S rRNA gene amplification) (Figure 4). The sequence obtained with the opposite primer was also checked to corroborate the sequences. Sequencing was repeated 3 times with each primer. Two type strains were sequenced to corroborate the reported sequences obtained here (i.e. DSM 791 and DSM 792). These type strains were sequenced with the 4 pairs of primers and compared with the reported sequences in the GeneBank

Strain	Sequenced nucleotide bp	Observations
DSM 791	717	Type strain
DSM 792	720	Type strain
IBUN 13 A	704	Native strain
IBUN 18 A	708	Native strain
IBUN 18 Q	709	Native strain
IBUN 18 S	686	Native strain
IBUN 22 A	711	Native strain
IBUN 62 B	699	Native strain
IBUN 62 F	713	Native strain
IBUN 64 A	672	Native strain
IBUN 95 B	717	Native strain
IBUN 125 C	713	Native strain
IBUN 137 K	705	Native strain
IBUN 140 B	708	Native strain
IBUN 158 B	715	Native strain

Table 14. 16S rRNA length fragments sequenced in each native strain

Complete gene sequences for the small ribosomal RNA subunit were obtained for the IBUN 22A (AJ289704), IBUN 64A (AJ289706) and IBUN 125C (AJ289705) strains to provide a broader data basis; these strains had the highest fermentation potential or produced the highest hydrolytic enzyme activity (Table 15). These sequences included 1,451 bp and corresponded to *E. coli* 16S rRNA, numbering 7 to 1,496. Homologous sequences were screened with a BLAST search of the EMBL data-base (Pearson, W.R. and Lipman, D.I. 1988). The most similar sequences (99.5% and 99.9%) were the 16S rRNA sequences from *C. butyricum* DSM 2478^{T} type strain and the closely related *C. kainantoi* bacterium (DSM 523).

Accession number	Bacterial strain	Base pairs	Gene
AJ289704	*Clostridium* IBUN 22A	1451	16S rRNA
AJ289705	*Clostridium* IBUN 125C	1450	16S rRNA
AJ289706	*Clostridium* IBUN 64A	1451	16S rRNA

Table 15. List of publically available DNA-sequences (GeneBank).

3.4 PCR strain identification using a 16S rRNA *Clostridium butyricum* specific targeted oligonucleotide.

The *C but* primer specific for *Clostridium butyricum* as a food contaminant has been designed by Knabel *et al.*, (1997) and could be used together with the E primer for the complementary strand to amplify the *Clostridium butyricum* 16S rRNA specific gene. The fragment size was estimated by using λ phage digested with Hind III enzyme as molecular marker. The fragment migrated below the 500 bp band. This DNA fragment size was amplified in all native strains and *Clostridium butyricum* DSM2478. However, *Clostridium acetobutylicum* ATCC 824 (amplified with a different standard) and *Clostridium acetobutylicum* DSM 1732 could not be amplified (Figure 5).

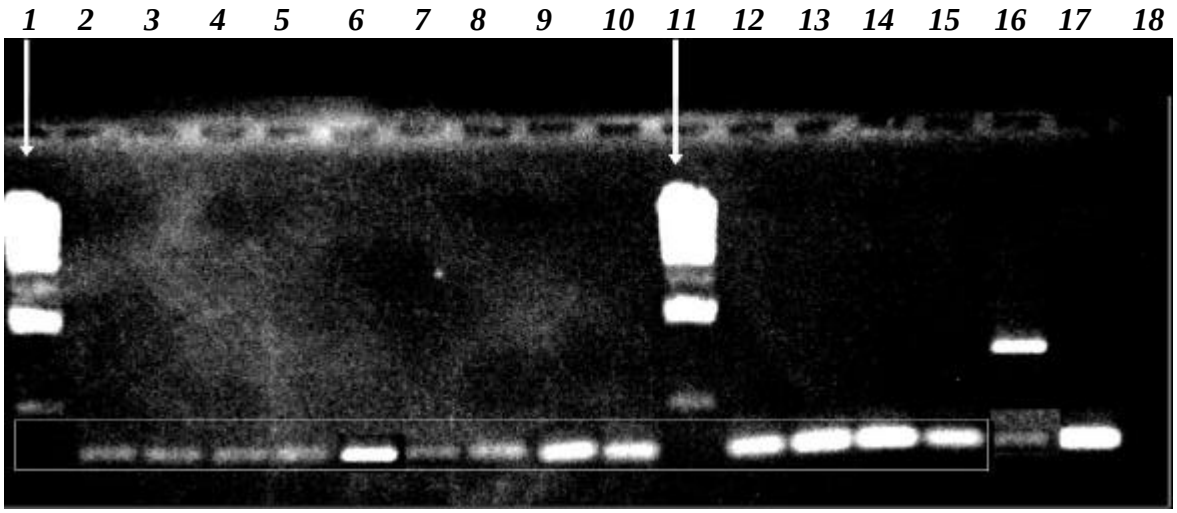


Figure 5. PCR strain identification using a 16S rRNA *Clostridium butyricum* specific targeted oligonucleotide

Lanes 1, 11 lambda HindIII marker; 2: IBUN 13A; 3: IBUN 18; 4: IBUN 18Q, 5: IBUN 18S; 6: IBUN 22A; 7: IBUN 62B; 8: IBUN 62F; 9: IBUN 64; 10: IBUN 95B; 12: IBUN 125C; 13: IBUN 137K; 14: IBUN 140B; 15: IBUN 158B; 16: *C. acetobutylicum* ATCC824; 17: *C. butyricum* DSM 2478; 18 *C. acetobutylicum* DSM 1732.

3.5. DNA-DNA hybridisation results

3.5.1. Selection of standard species-constructing probes

After analysing partial sequences from the 16S rRNA ribosomal sequences, it was observed that the 13 strains were found in the same group, closely related to *Clostridium butyricum* and relatively far away from *Clostridium acetobutylicum*, as reported by Montoya D. *et al., (*2001). *C. butyricum* DSM2478, ATCC43755, NCIMB8082 and *C. kainantoi* DSM523 strains were also found in this group. The data were insufficient to associate the native strain to the *C. butyricum* and *C. kainantoi* species, respectively. For a further differentiation, DNA probes were prepared from *C. butyricum, C. kainantoi* and native strain genomic DNA to be used in DNA-DNA hybridisation.DNA was dotted in the following pattern. A dot-blot was done with each one of the probes to test whether the probe was correctly emitting and revealed without hybridisation (Figure 6)

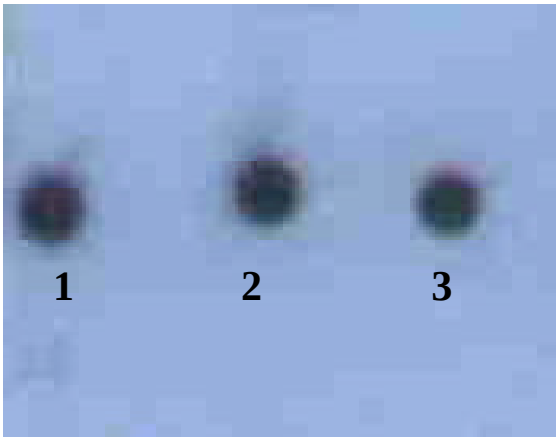


Figure 6. Chemical-luminescence test for probe emission.
Dot 1: *C. butyricum* DSM2478, Dot 2:*C, kainantoi* DSM523. Dot 3: *C. acetobutylicum DSM792*

The 62F strain was not sensitive to the methodology used for chromosomal DNA extraction; it was impossible to obtain sufficient DNA in the appropriate quantity to do the assay.(Figure 7).

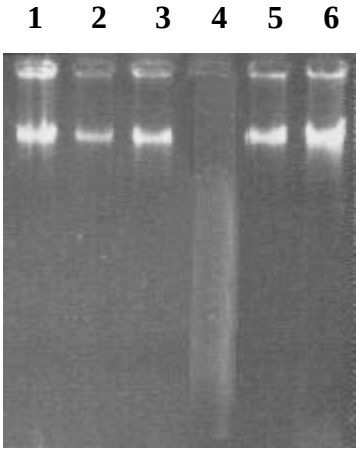


Figure 7. Chromosomal DNA extraction

Lane 1: IBUN 13A, Lane 2: IBUN 64A, Lane 3: IBUN 18S, Lane 4: IBUN 62F, Lane 5: IBUN 140B, Lane 6: DSM2478. IBUN 62F strain chromosomal DNA degradation can be seen in Lane 4**.**

Each blot-dot was done in triplicate to take the average of the three dots for each strain. Strains were included which were representative of each one of the groups of solventogenic clostridia (*C. acetobutylicum, C. sacharobutylicum* and *C. beijerinkii*) and an external group (*Anaerocellum thermophilum*). The negative standard was *Anaerocellum thermophilum.* The positive standards were DNA from each one of *C.*

butyricum DSM2478, *C. kainantoi* DSM523 and *C. acetobutylicum* DSM792, which were used as labelled probes for use in hybridisation (see Dot Blot section below).

2µg DNA from all the strains were loaded onto a gel to determine whether the intensity was the same for all. The three probes were hybridised on the same membrane to avoid differences in DNA concentration. The whole procedure was repeated on a second membrane organised in the same way by using DNA form the strains extracted on different days.

The membrane could be kept for several months. After the probe was removed, the membranes were left to dry and kept in a dry place (Boehringer Mannheim GmbH, 1995).

3.5.2. Dot Blot

Assays were done with 1µg, 1.5µg, 2µg and 2.5µg DNA using two 3 cm X 4 cm membranes for each concentration. The *C. sacharobutylicum* NI-4, *C. butyricum* DSM2478, *C. acetobutylicum* DSM792 and *C. kainantoi* DSM523 strains, the IBUN 18A strain, the external *A. thermophilum* group and water + NaOH were used for the Dots. Each membrane was hybridised with each one of the probes and the *C. sacharobutylicum* strain was taken as internal standard as it did not hybridise intensely with any one of the three probes: *C. acetobutylicum*, *C. sacharobutylicum* and *C. beijerinkii*. The *A. thermophilum* strain was taken as external standard (negative control) and the water+NaOH was taken as target (Figure 9).

These assays established that the results using 2µg DNA per dot were reproducible amongst the membranes. When less DNA was used, difficulties were found in quantifying the intensity, as well as revealing time. Differences could not be seen between the *C. acetobutylicum* and *C. butyricum* strains, possibly being due to the revealing agent's sensitivity. When more than 2µg was used, the *C. sacharobutylicum* strain had intensities similar to those of the probes which were used.

3.5.3. Quantifying hybridisation percentages

Quantification was done by using the QUANTITY ONE programme, reporting the hybridisation percentages as a ratio of intensity in the area of the dot (unit of intensity x mm^2). The positive standard in each case is the same DNA with which the probe was constructed; this representing 100% hybridisation. The negative control corresponded to the negative standard (*Anaerocellum thermophilum*) and the target (H2O + NaOH) which had 0.0% hybridisation (Table 17). The equipment did the normalisation and calculated the percentages for each strain with respect to the standards.When the equipment reported N/A, this meant that the intensity was very low, less than that of the negative control (the target). The symbol N/A means Not Acquired.

It should be born in mind that hybridisation percentages below 15% were outside the equipment's interpolation curve and that, for analysis, results close to the hybridisation percentage reported for the negative standard (external group - *A. thermophilum*) were taken as the greatest difference which could be found between the group of strains which were being analysed. The same happened with percentages greater than 95% with which the equipment became saturated. The interpolation was taken from the internal calibration curve (Bio RAD, 2000b).

Chromosomal DNA was prepared from all strains and distributed on the membrane. Two membranes and three dots were done for each strain. (Figure 8 and Table 16) contains the names of each one of the strains which were included on the membrane, with the number corresponding to their location.

Differences of intensity between the strains with each probe can be seen on the following three films (Figure 9, Figure 10 and Figure 11) show hybridisation only in those dots corresponding to *C. acetobutylicum* DSM792 and DSM1732 strains, *C. butyricum* DNA and *C. kainantoi* DNA probes, respectively.

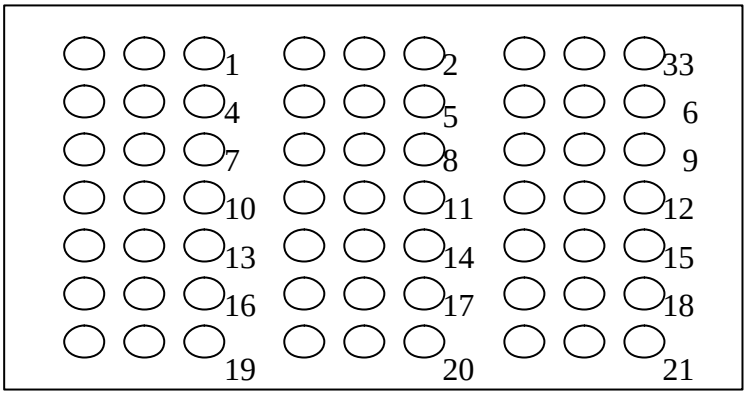


Figure 8 Organisation of the dots on the membrane

Number	Strain	Number	Strain
1	IBUN 13A	12	IBUN 158B
2	IBUN 18A	13	*C. kainantoi DSM523 Positive Control*
3	IBUN 18Q	14	*C. acetobutylicum DSM792 Positive control*
4	IBUN 18S	15	*C. beijerinkii* NCMB8052
5	IBUN 22A	16	*C. butyricum DSM2478 Positive Control*
6	IBUN 62B	17	*C. beijerinkii* DSM791
7	IBUN 64A	18	*C. saccharobutylicum* NI–4
8	IBUN 95B	19	*Anaerocellum thermophilumNegative Control*
9	IBUN 125C	20	*H2O + Na OH Target*
10	IBUN 137K	21	*C. acetobutylicum* DSM1732
11	IBUN 140B		

Table 16. DNA distribution on the membranes of those strains used.

(*) DNA provided by Vladimir Zverlov, Microbiology Department, TUM

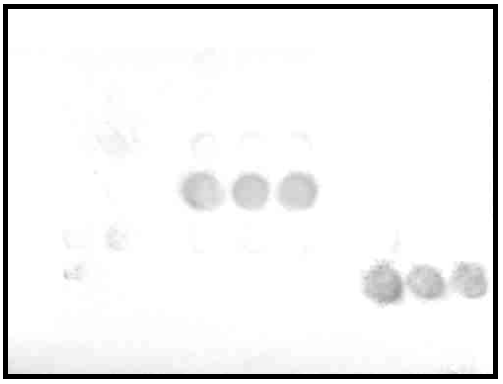


Figure 9. Film obtained from hybridisation done with *C. acetobutylicum* DNA

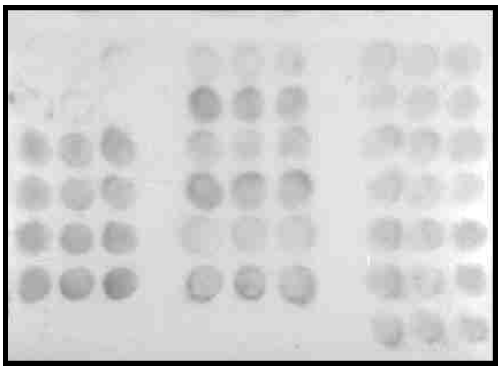


Figure 10. Film obtained from hybridisation done with *C. butyricum* DNA

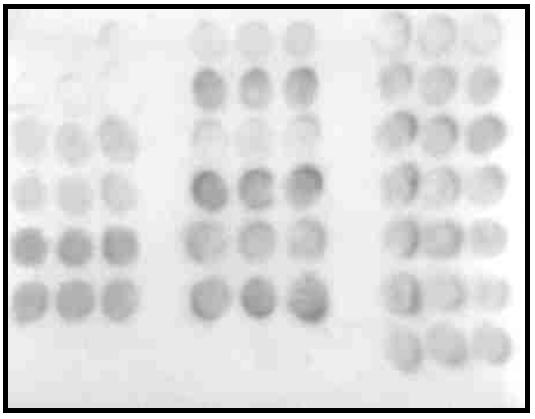


Figure 11. Film obtained from hybridisation done with *C. kainantoi* DNA

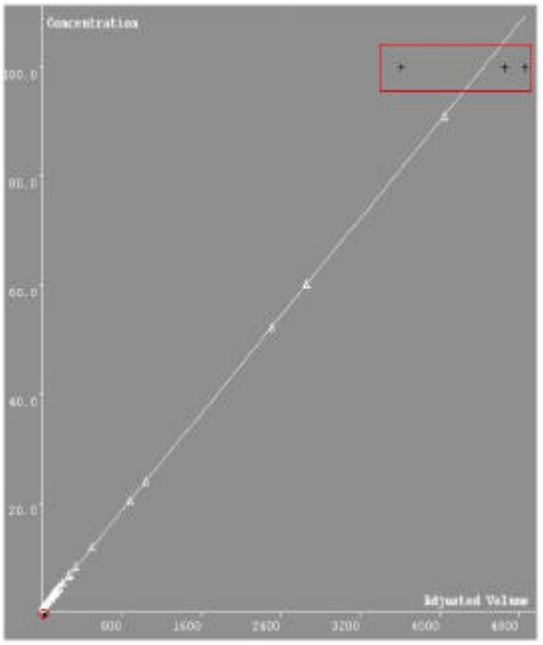


Figure 12. Hybridisation with the *C. acetobutylicum* DSM792 probe.
Each one of the points on the graph is a membrane dot. The Y axis shows the percentages of hybridisation; the X axis shows the units of area which the equipment counted. The red box shows the dots from the *C. acetobutylicum* DSM792 strain which had 100% hybridisation by corresponding to the positive standard in this case.The red circle shows the dots from the negative standard and the target.

Hybridisation with the same probe clearly separated the species: *C. beijerinckii* NMBCI8052 (<15%) and DSM791 (<15%), *C. saccarobutylicum* NI-4 (<15%), *C. butyricum* DSM2478 (<15%) and *C. kainantoi* DSM523 (<15%). Data for *A. thermophilum* (external group) were similarly less than 15%, as show in (Table 17.)

When membranes obtained with *C. kainantoi* DNA were observed, strains having different similarity indices were found. However, two of them showed hybridisation percentages equal to 70% (IBUN22A) or less than 70% (IBUN140B), indicating that they were similar to *Clostridium kainantoi*. With this same probe, 25% of the native strains had similarity percentages greater than 50%. Only 16% of the native strains had percentages greater than 50% when using *C. butyricum* DNA as probe and only IBUN 140B strain had a percentage close to 70%.

Native strain	***C. butyricum***		***C. acetobutylicum***		***C. kainantoi***	
	I	II	I	II	I	II
IBUN13A	8.75	5.30	22.5	17.5	N/A	3.30
IBUN18A	16.17	11.23	28.28	23.02	13.81	7.13
IBUN18Q	N/A	3.48	23.30	26.45	13.58	11.43
IBUN18S	1.29	N/A	29.41	26.35	N/A	3.08
IBUN22A	43.06	45.13	37.18	35.67	68.52	70.05
IBUN62B	N/A	N/A	15.86	11.43	15.65	13.04
IBUN64A	54.94	60.43	30.04	32.18	16.54	20.65
IBUN95B	N/A	4.58	18.21	20.22	4.06	N/A
IBUN125C	N/A	N/A	0.92	N/A	58.85	60.35
IBUN137K	43.59	47.40	27.62	32.56	33.87	30.56
IBUN140B	63.69	67.45	56.18	53.67	108	102
IBUN158B	N/A	N/A	0.91	N/A	26.09	19.26
C. kainantoi DSM523 **Positive control**	61.14	63.65	3.32	N/A	100	100
C. acetobutylicum DSM792 **Positive control**	36.34	42.65	100	100	69.5	62.03
C. beijerimkii NCMB8052	7.97	8.34	0.90	N/A	66.81	57.46
C. butyricum DSM2478 **Positive control**	100	100	8.43	2.34	112	97.03
C. beijerinckii DSM791	62.4	58.75	4.17	N/A	60.34	61.32
C. sacharobutylicum NI–4	30.33	33.25	3.94	N/A	50.15	57.80
Anaerocellum thermophilum **Negative control**	9.06	6.34	4.27	3.78	5.02	6.04
H_2O + NaOH **Target**	0	0	0	0	0	0
C. acetobutylicum DSM1732	25.59	20.54	91.19	96.72	63.94	62.13

Table 17. Hybridisation percentages for each strain in membrane I and II.

Each column shows the percentages for each strain on doing the hybridisation with each probe on the membrane (*C. butyricum, C. acetobutyricum, C. kainantoi*). N/A = Not acquired.

Those species used as control (*C. beijerinckii* NMBCI8052 and DSM791, *C. saccarobutylicum* NI-4 and *C. acetobutylicum* DSM792) were differentiated in those hybridisations done using *C. butyricum* DNA and *C. kainantoi* DNA as probe because they presented less than 70% hybridisation. With these it was observed that they maintained species' differentiation with the aforementioned methodology. It should be pointed out that although solventogenic clostridia hybridisation percentages with *C. kainantoi* DNA were less than 70%, they were higher than those obtained amongst strains from the solventogenic groups of Keis *et al.* (1995) and *C. butyricum* DNA.

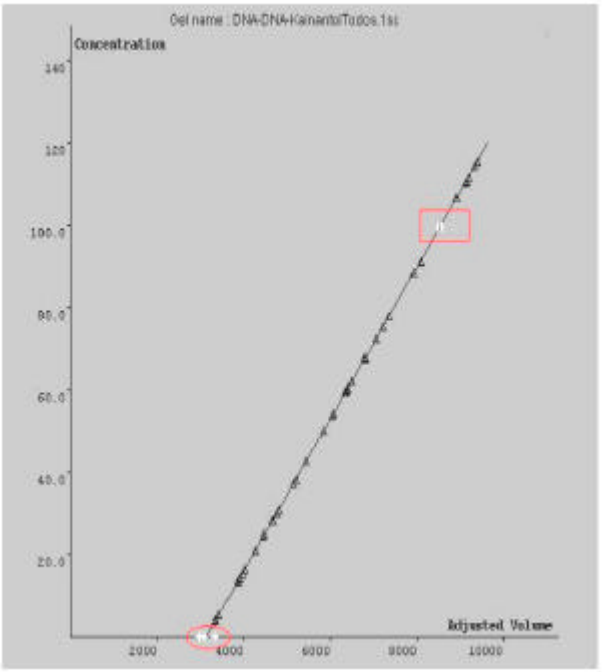


Figure 13. Hybridisation with *C. kainantoi* DSM523 probe.
Each one of the points on the graph is a membrane dot. The Y axis shows the hybridisation percentages; the X axis shows the units of area which the equipment counted The red box shows that dots from the *C. kainantoi* DSM523 strain had 100% hybridisation, being the positive standard in this case. The red circle shows dots from the negative standard and the target.

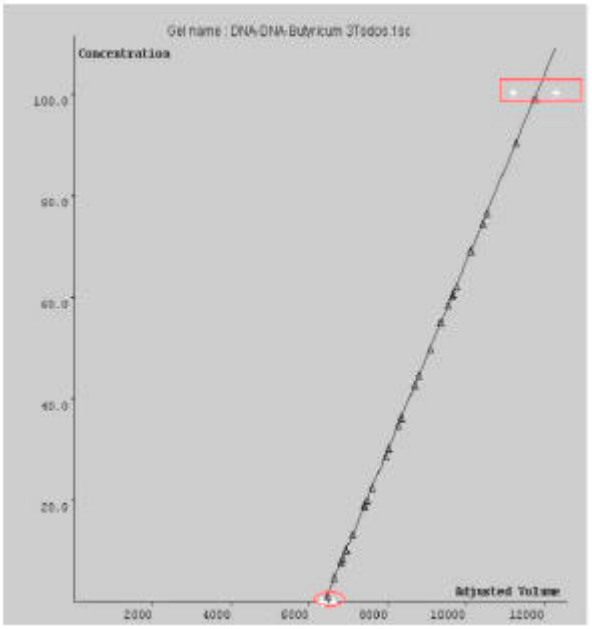


Figure 14. Hybridisation with the *C. butyricum* DSM2478 probe.

Each one of the points on the graph is a membrane dot. The Y axis shows hybridisation percentage; the X axis shows the units of area which the equipment counted . The red box shows that dots from the *C. butyricum* DSM2478 strain had 100% hybridisation, being the positive standard in this case. The red circle shows dots from the negative standard and the target.

4. Enzyme activity characterisation

Saccharolytic clostridia are capable of using a wide spectrum of organic carbon sources for growth (Hazlewood and Gilbert 1993; Mitchell *et al.*, 1995) and have the potential for simultaneous fermentation of valuable solvents, thus preventing the cost-intensive addition of enzymes in a substrate pre-treatment step. Polysaccharides are degraded (either by secreted or cell-associated extracellular enzymes) to mono- and oligo-saccharides prior to transmembrane transportation and intracellular catabolism.

Substrate use was assayed by two methods: growth on a substrate and hydrolytic activity in the culture supernatant. All strains formed colonies on TY-agar containing glucose, cellobiose or soluble starch. But, although none of the new strains was able to form colonies on xylan, barley ß-glucan, carboxymethyl-cellulose or crystalline cellulose (Avicel), high hydrolytic activity for these (and a number of other polysaccharides) was present in the culture supernatant (Table 18 and Table 19) . Soluble and raw starch, xylan and carboxymethyl-cellulose were hydrolysed with the

release of reducing sugars by all strains, pullulan, inulin, pectin and arabinan by all but one or two and barley ß-glucan and chitosan by the majority of the strains. Surprisingly, hydrolytic enzymes for maltose and cellobiose were even lower or not measurable in culture supernatants for most of the strains, although they formed colonies on starch or cellobiose. All strains grew well in liquid cultures with glycerol, raffinose, sucrose and cellobiose as sole carbohydrate (Figure 14) .

The greater part of the 13 native strains (nearly 84%) showed at least some hydrolytic activity on crystalline cellulose (Avicel). It can be seen that there was weak invertase activity on raffinose, as 37.5% of the reference strains did not show any activity and 50% had activity of less than 1 mmol glucose / [min*mg protein]. 76.9% of reference strains also presented activity less than 1 mmol glucose / [min*mg protein]. Only 7.7% reached a 2 mmol glucose / [min*mg protein] activity level, differing from the action which this enzyme presented in reference strains with saccharose when this was used as substrate. This was due to activity greater than 2 mmol glucose / [min*mg protein], corresponding to 25%. 50% presenting activity less than 2 mmol glucose / [min*mg protein]; 12.5% did not present any activity whatsoever. Activity was less for native strains, as 76.92% presented activity less than 1 mmol glucose / [min*mg protein].
The strains presenting the greatest activity in these substrates were:

- 22A 4.57mmol glucose / [min*mg protein] for avice and 140B 7.24 mmol glucose / [min*mg protein] for avicel
- B-643: 2.76 inuline and 2.77 mmol glucose / [min*mg protein] for saccharose;
- 22 A: 2.44 mmol glucose / [min*mg protein] for inuline, 2.36 mmol glucose / [min*mg protein] for saccharose and 4.55 mmol glucose / [min*mg protein] for raffinose; and
- 791: 2.07 mmol glucose / [min*mg protein] for raffinose.

Substrate	13-A	18-A	18-Q	18-S	22-A	62-B	62-F	64-A	95-B	125-C	137-K	140-B	158-B
Starch	20.53	10.89	12.26	5.58	46.07	14.54	16.55	18.26	6.25	24.13	16.51	65.71	11.63
CMC	0.41	1.02	1.41	0.39	2.71	0.95	2.10	0.38	0.55	0.76	0.76	1.28	3.55
Polyosen	0.35	0.34	0.81	0.70	0.00	0.73	1.47	0.73	0.21	0.90	0.21	1.04	0.00
Larch wood xylan	1.82	1.63	2.72	1.04	5.20	3.71	3.72	1.33	0.96	2.21	2.51	1.28	2.75
Inulin	0.34	0.28	2.21	0.35	2.44	1.33	0.86	0.00	0.36	0.49	0.49	1.31	1.62
Raffinose	0.37	0.35	0.74	0.12	4.55	0.67	0.91	0.48	0.27	0.78	2.01	1.01	0.76
Melezitose	0.00	0.31	0.93	0.00	0.62	0.67	1.30	0.65	0.29	1.03	0.62	0.88	1.13
Arabinan	0.48	0.45	0.54	0.00	7.46	0.51	0.77	0.68	0.00	0.96	0.31	1.72	2.86
Saccharose	0.18	0.31	0.93	0.23	2.36	1.01	0.21	0.56	0.00	0.35	0.62	0.75	0.79
Pullulan	0.59	0.83	1.46	0.00	1.76	1.04	1.13	0.53	0.421	0.90	0.66	0.71	0.70
Oat spelt xylan	0.95	0.55	2.78	0.514	0.33	1.48	1.93	0.78	0.81	0.34	0.89	1.65	1.62
Maltose	0.00	0.00	0.00	0.00	0.26	0.30	0.31	0.00	0.00	0.00	0.29	0.00	0.00
Cellobiose	0.00	0.00	0.00	0.34	0.20	0.30	0.32	0.00	0.35	0.00	0.36	0.51	0.35
Chitosan	0.52	0.49	0.00	0.00	3.26	2.88	0.00	0.86	0.33	1.02	0,00	1,14	1,23
Avicel	0.49	0.60	0.76	0.48	4.57	0.74	1.19	1.36	0.00	0.00	1,87	1,42	0,00
Tapioca	0.47	3.41	1.35	3.38	2.00	0.37	0.49	2.03	3.19	5.15	0,43	7,24	0,53
β-glucan	0.00	1.10	1.41	0.00	0.00	0.93	0.94	0.63	0.68	0.77	0,80	1,33	0,72
Pectin	0.45	0.35	2.26	0.61	6.18	0.95	0.86	2.12	0.00	2.00	0.79	3.63	0.82

Table 18. Native strain enzyme activity in supernatant

Substrates are: Avicel = crystalline cellulose; CMC = carboxymethylcellulose; Xylem larch wood; Xylan oat spelt;Poliosen = spruce wood hemicellulose fraction from the Organosolv process (paper pulp preparation); β-glucan = barley ß-glucan.Enzymatic activity was determined from culture supernatants from fully-grown cultures on starch as carbon substrate.

Substrate	N 1 4	B-643	791	792	824	1732	2152	8052
Starch	0.00	161.70	33.27	10.84	8.08	10.59	10.92	4.30
CMC	0.00	2.24	2.99	1.15	0.00	0.00	0.00	1.35
Polyosen	0.00	2.72	1.52	0.77	0.00	0.00	0.69	1.28
Larch wood xylan	0.00	8.68	1.72	1.70	2.53	1.59	3.49	1.04
Inulin	0.60	2.76	1.58	0.65	0.00	0.00	0.00	0.00
Raffinose	0.00	0.00	2.07	0.43	0.73	0.00	0.79	0.39
Melezitose	0.64	5.30	1.26	0.00	0.66	0.37	2.71	0.79
Arabinan	0.00	5.16	2.51	0.43	0.89	0.81	1.23	0.67
Saccharose	0.85	2.77	1.28	2.21	1.17	0.00	1.86	0.90
Pullulan	0.59	9.89	1.28	0.00	0.56	1.01	0.83	0.34
Oat spelt xylan	0.46	14.34	2.11	2.66	0.80	0.66	1.34	0.78
Maltose	0.25	0.00	0.29	0.00	0.00	0.00	0.00	0.00
Cellobiose	0.32	0.31	0.00	0.00	0.00	0.00	0.00	0.27
Chitosan	0.00	0.00	0.72	0.37	0.48	0.71	0.93	0.00
Avicel	0.00	11.70	2.74	0.49	1.58	0.81	2.31	0.53
Tapioca	0.64	5.99	0.71	3.95	1.12	1.51	6.78	2.19
β-glucan	0.00	9.31	0.51	0.85	1.96	0.37	1.23	1.96
Pectin	0.00	7.95	1.66	0.69	1.01	0.00	1.60	0.68

Table 19. Reference strain activity in supernatants.

Substrates are: Avicel = crystalline cellulose; CMC = carboxymethylcellulose; Xylan larch wood; Xylan oat spelt; Poliosen = spruce wood hemicellulose fraction from the Organosolv process (paper pulp preparation); ß-glucan = barley ß-glucan. Enzyme activity was determined from culture supernatants from fully-grown cultures on starch as carbon substrate.

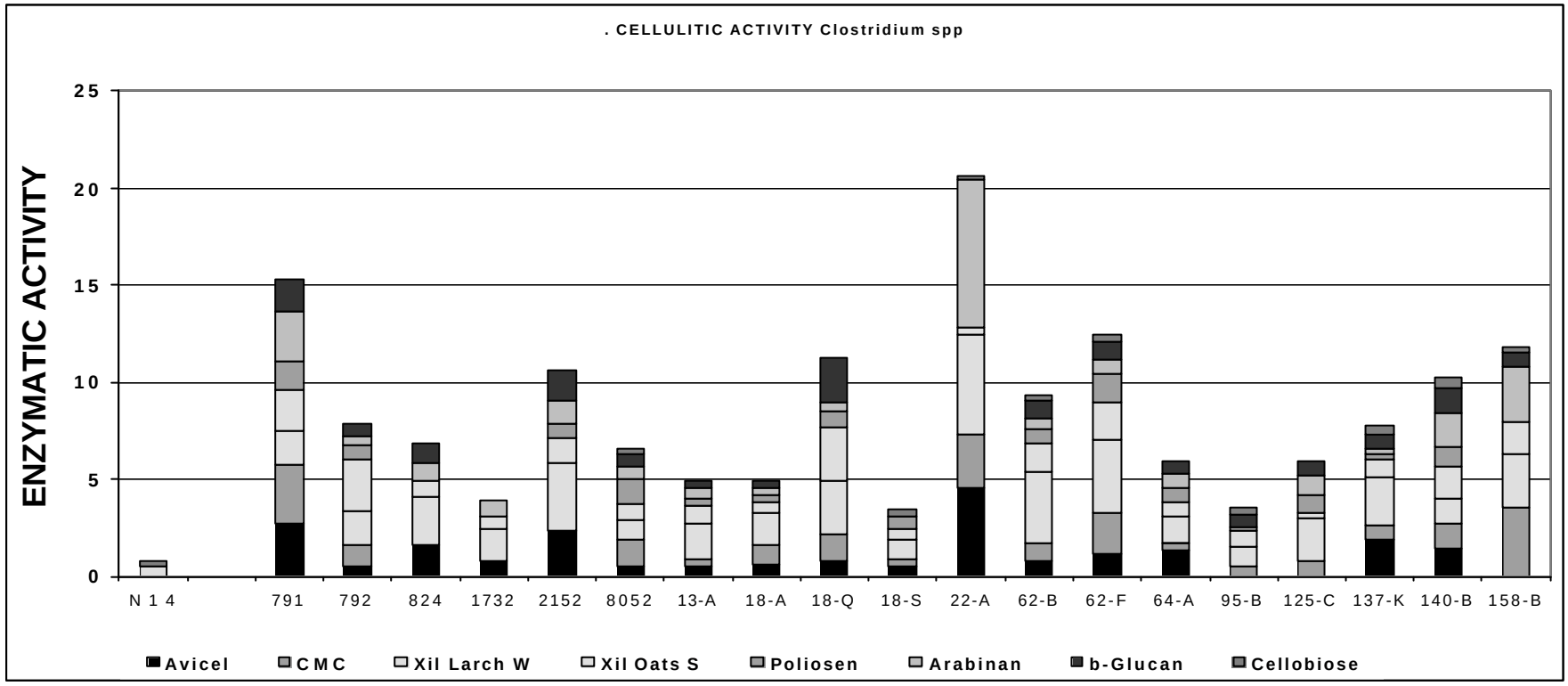


Figure 15. Hydrolytic activity in the *Clostridium spp* supernatant and some *Clostridium* type strains and native strains

Hydrolytic activity in the *Clostridium sp* supernatant and some *Clostridium* type strains: Avicel = crystalline cellulose; CMC = carboxymethylcellulose; Xylan Larch W = larchwood xylan; Xyl Oat S = xylan oat spelt; Polyosen = spruce wood hemicellulose fraction from the Organosolv process (paper pulp preparation); ß-Glucan = barley ß-glucan. Enzymatic activities were determined from culture supernatants from fully grown cultures on starch as carbon substrate. Enzyme activity is measured as being the release of reducing sugars (glucose equivalents) in µg glucose/min/mg protein.

5. Optimisation of an industrial medium

IBUN IV spontaneous mutant strain butanol hyper-producer, isolated from *Clostridium acetobutylicum* DSM 1732 (Sierra *et. al*., 1996), was used to optimise culture medium. Culture medium optimisation and strain improvement are two fundamental factors in increasing yield, as well as productivity. Co-culturing the IBUN IV with the IBUN 22A strain was thus proposed, as it would take advantage of both strains' characteristics. IBUN IV is a butanol hyper-producer and IBUN 22A has shown cellulase activity and produces acetic and butyric acids. The purpose of industrial culture medium optimisation using IBUN IV strain in optimal conditions to obtain a solvent producing strain pattern.

Medium components	**Ev Effect**	**t-Test t_0**	**5% significance**
Biotin	**- 0.733**	**- 5.64**	*****
PABA	**- 0.416**	**- 3.2**	*****
Tyrosin	**- 0.187**	**- 1.44**	**NS**
Cysteine	**- 0.023**	**- 0.179**	**NS**
Valin	**0.087**	**0.672**	**NS**
$(NH_4)_2SO_4$	**0.282**	**2.16**	**NS**
KH_2PO_4	**0.489**	**3.76**	*****
Other minerals	**0.496**	**3.819-**	*****
Yeast extract	**- 0.889**	**- 6.838**	*****
PH	**- 0. 429**	**- 3.298**	*****

Table 20. Medium components affecting ABE fermentation.

Composition ranges were obtained by the Plackett-Burman method. The effect of the medium component was significant. The NS effect of the medium component was not significant.

The Plackett-Burman method was used to evaluate seven variables in eight experiments. A fractioned factorial design structure allowed those independent variables to be identified which significantly affected the production of total solvents and also the range in which those variables could be used. Table 20 presents the results obtained by applying the matrix from the Plackett-Burman method, identifying those variables which significantly affected ABE fermentation total solvent production and concentration levels for each one of the fermentation components involved in total solvent production.(Table 21).

The EVOP-Simplex method allowed sequential exploration of a response surface so that a peak production point could be located on the same This is an application of response surface methodology concepts for improving industrial process yields. Final results in values per litre for the seventh and last Simplex (Table 22) application, indicating the industrial culture medium's final composition, were: 130 g molasses, 0.4 mg biotin, 0.3 mg PABA, 1.8 g KH_2PO_4, 3.0 g yeast extract, 4 mL mineral stock and distilled water to complete 1 litre; pH 6.1 before the medium was sterilised. Total solvent production was 24.6 g/L in the optimised culture medium, presenting an increase equivalent to 58.7% with respect to total solvent production of the same mutant strain in non-optimised culture medium (Table 23).

Selected medium component	Concentration levels in 1,000 mL
Biotin	0.3-0.5 mg
PABA	0.2- 0.4 mg
KH_2PO_4	1.6-2.0 g
Yeast extract	3.0-5.0 g
Mineral stock	1-5 mL
PH	5.8-6.4

Table 21.Industrial medium
was prepared by using selected components and their concentration levels determined by the Plackett-Burman method (Placket and Burman 1946)

Assay	Biotin	PABA	KH_2PO_4	Mineral stock	Yeast extract	pH	Total solvent production g/L
1	0.5	0.4	2.0	1.0	5.00	6.4	24.59
2	0.4	0.3	1.8	1.0	5.00	6.4	22.74
3	0.4	0.3	1.8	0.3	4.00	5.8	23.87
4	0.4	0.33	2.2	0.3	4.00	6.2	23.54
5	0.4	0.20	2.00	0.50	5.00	6.4	23.14
6	0.4	0.30	1.80	0.28	3.00	6.4	21.74
7	0.4	0.31	2.07	0.25	5.66	6.4	21.61
ΣRET	2.4	1.83	1160	1.92	2600	37.6	
2 X	0.8	0.61	387	0.64	867	12.5	
DIS	0.4	0.31	207	0.25	566	6.4	
2X - DIS	**0.4**	**0.30**	**1.80**	**0.39**	**3.01**	**6.1**	**24.62**

Table 22. Seven assays from the seventh and last Evop-Simplex (Simplex 7).
Component concentrations in g/L and mineral stocks in mL. Fermentation was done as described in Material and Methods.

Final composition of industrial medium	Concentration levels in 1,000 mL
Molasses	130g
Biotin	0.4 mg
PABA	0.3 mg
KH_2PO_4	1.8 g
Yeast extract	3.0 g
Mineral stock	4 mL
PH	6.1

Table 23. Final industrial medium concentration.
(These results were obtained from the 7^{th} Evop-Simplex.)

Glucose was used as a source of carbon in the already cited experiments. Fermentation carried out in our laboratory indicated that medium acidification occured with greater speed when glucose was used as carbon source, compared to the use of molasses (results not shown). It is probable that the results were influenced by the carbon source; thus, ammonium sulphate was not used as nutrient, unless it contributed towards neutralising a suitable pH. As can be seen in the optimisation process, pH is a factor which significantly influences total solvent production. Optimum pH was set at 6.1 before sterilisation for the conditions in this work.

It was shown that suitable use of the experimental design employed to carry out this work allowed total solvent production to be improved by 58.7%. The criteria defined to calculate carbon source and other medium nutrient concentrations were vitally important. These were calculated on knowledge concerning the metabolic route and experimental determination of maximum biomass production in vegetative medium. Once carbon source concentration had been defined, then macro- and micro-element concentrations were established. It should be pointed out that this methodology is suitable for defining the variables and their nutritional as well as environmental values, enabling other batch fermentation models to be studied.

6. POME biodegradation

Industrial Medium was optimised using IBUN IV strain and molasses as carbon source for obtaining solvent production pattern to compare with the biodegradation kinetic in POME Medium. This medium has the same composition of Industrial Medium except that the carbon source was substituted by POME, due to the high composition of cellulose and hemicellulose (Materials and Methods 9.1). The main goal was to transform POME waste into carbon source for solvent production to make the process economically feasible and to protect the environment.

6.1. Preliminary assays

The composition of POME Medium showed high carbohydrate concentration such as cellulose, hemi cellulose and total soluble carbohydrates(Materials and Methods 9.1) . Micro-organisms with cellulolytic activity would degrade cellulose polymers and use them as carbon source. Primary and secondary metabolite production depends on the micro-organisms' metabolic route.

6.2 Preliminary characterisation assay for selecting the native strains.

Optimised industrial medium (Montoya *et al.,* 2000a) was modified for preliminary POME degrading assays and for degradation kinetics. These modifications consisted of changing the 100% molasses' concentration and using POME as sole carbon source. Three POME concentrations (100%, 75% and 50% v/v in industrial medium except mollases) were previously evaluated and the best active metabolism was obtained by using 100% POME waste concentration. This means that it is possible to use palm oil mill effluent just as it flows from the industrial plant. The time for two fermentations (7 and 14 days), without stirring, at 37ºC were evaluated, as described in Materials and Methods 9.2.2. Physical changes, such as colour, particle size reduction and gas production, were assumed to give evidence of micro-organism growth. Acid and solvent production, pH changes and fermentable sugar concentration were also analysed .

Strain	% POME waste as sole carbon source	Time (days)	pH	Acetone (mmol/L)	Butyric acid (mmol/L)	Acetic acid (mmol/L)
***Clostridium saccharobutylicum* NRRL B643**	100	14	5.57	44.77	13.00	3.76
	75		5.58	61.58	19.44	6.02
	50		5.54	60.33	19.08	7.29
	100	7	5.54	ND	ND	ND
	75		5.59	ND	ND	ND
	50		5.59	ND	ND	ND
IBUN 22-A	100	14	6.26	176.24	97.97	ND
	75		6.09	143.07	71.32	ND
	50		6.28	144.45	68.26	ND
	100	7	6.04	142.16	71.13	ND
	75		6.09	167.82	81.47	ND
	50		6.32	147.10	69.78	ND
IBUN 140-B	100	14	6.06	167.59	88.12	ND
	75		6.11	59.75	31.32	ND
	50		6.09	142.52	70.41	1.19
	100	7	6.11	145.39	72.98	ND
	75		6.22	107.51	52.24	ND
	50		6.12	117.55	55.26	ND

Table 24. Preliminary assays

Acid and solvent production, pH determination after 7 and 14 days fermentation at 37ºC, without stirring, and replacing carbon source molasses by 100%, 75% and 50% POME waste. ND: not detected

The IBUN 22A strain needed biotin and mineral stock to grow in the preliminary strain characterisation. *Clostridium saccharobutylicum* NRRL B643 has shown larch wood xylan, oat spelt xylan, Avicel, CMC, starch, pectin degrading activity; it has also shown low pH change, acid and solvent production in relation to those native strains evaluated (Montoya *et al*., 2001). *Clostridium saccharobutylicum* NRRL B643 only presented physical changes after 7 days. By contrast, the IBUN 22 A and IBUN 140B strains exhibited lower growth rate, presented colour change within the first three days and important acid production, as shown below (Table 24).

The *Clostridium saccharobutylicum* NRRL B643 reference strain produced 2.60 g/L acetone 1.15 g/L butyric acid and 0.23 g/L acetic acid, whilst native strains produced butyric acid and acetone. The IBUN 22 A strain produced 10.24 g/L acetone, 8.63 g/L butyric acid and

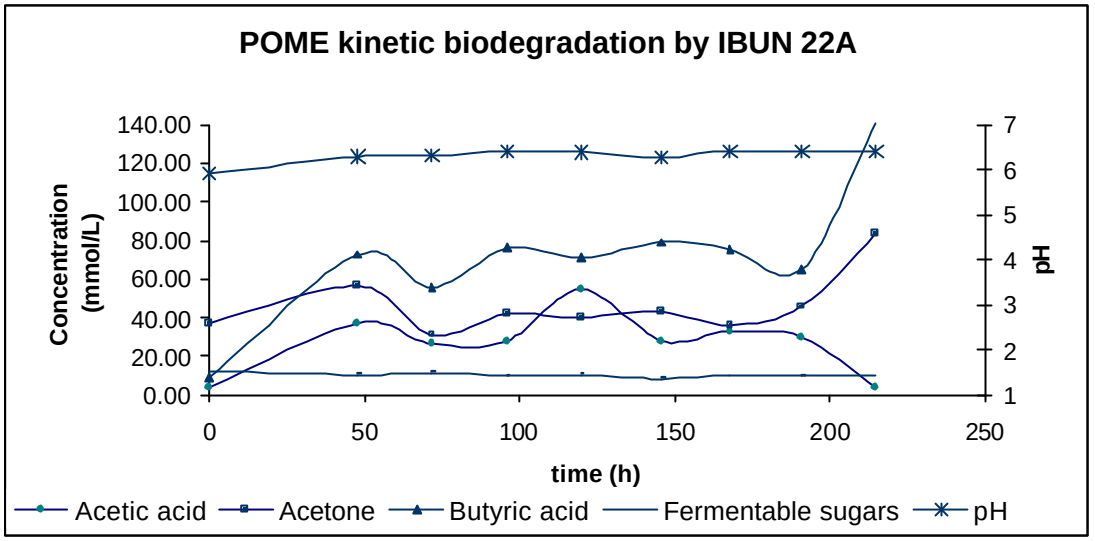
IBUN 140B produced 9.73 g/L acetone and 7.43g/L butyric acid, but no butanol or ethanol were detected. Native strain 22A was selected for degrading POME waste, due to greater metabolic activity, as shown in the above results.

6.3. IBUN 22A and IBUN IV strain kinetics grown in POME Medium. Initial pH 6.1.

The goal was not only POME waste degradation, but also to try to obtain products having the greatest value, such as butanol. It was thus proposed that the appropriate conditions for co-culture development should be found, taking both strains' characteristics (IBUN 22 A, degrading cellulose polymers and IBUN IV as hyper total-solvent producer) to produce solvents as final metabolism products. It was thus proposed to study the degrading kinetics of both strains (IBUN 22A and IBUN IV) in the same medium and equal conditions to study the fermentation parameters and design the co-culture.

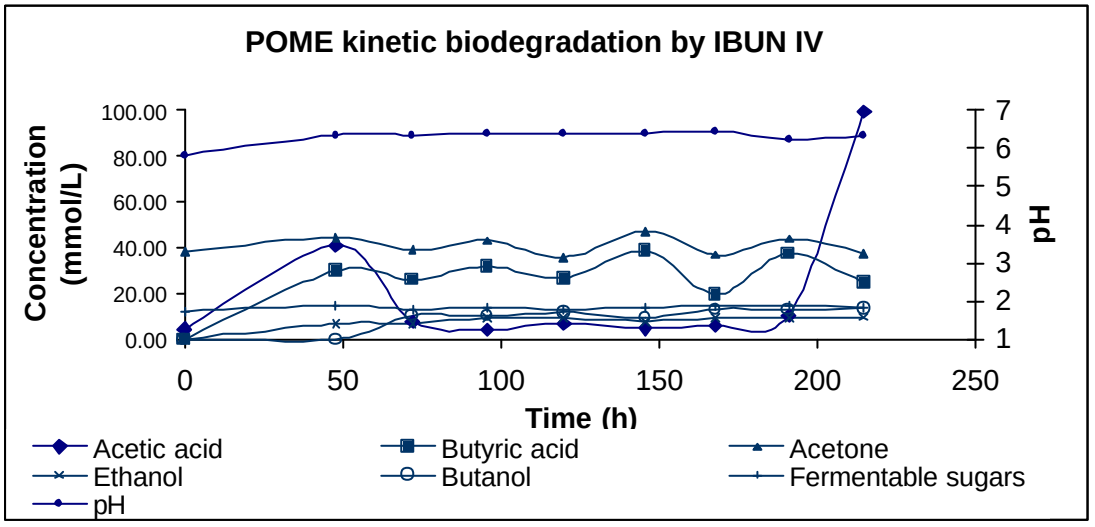
Table 25 and Table 26, show non-dissociated acids having values less than 20 mmol/L, this being necessary to start solventogenesis (Maddox *et al.,* 2000), the 22A strain presented values between 0.92-3.47 mmol/L and the IBUN IV strain between 0.34-1.86 mmol/L The total acid concentration (dissociated and non-dissociated) for the IBUN 22A strain, throughout fermentation, reached values between 103-144 mmol/L whilst IBUN IV mutant strain presented values between 25-70 mmol/L, but this reached 124 mmol/L at 215 hours.

The pH was kept within a 6.1-6.4 range during fermentation for both strains. It can thus be considered that the effluent possesses a high buffer capacity. The characteristic pH range during acidogenic phase is 4.8-5.2 and during solventogenic phase the approximate pH value is 5.5, since acids generated during primary metabolism are re-assimilated. At this fermentation pH, the acids were dissociated; it is known that in this form they do not pass through the cell membrane (Graph 1). Both fermentation performances have shown that the micro-organism only degrades the polymer when the carbon source is consumed; this behaviour can be detected by fermentable sugar analysis.



Graph. 1 POME kinetic biodegradation by IBUN 22A

Final concentration of acids and solvents (mmols/L) produced by the IBUN 22 A strain in POME Medium. Fermentation was done in 2 L Erlenmeyer flasks with 1 L of culture medium for 8 days at 33ºC with agitation (150 rpm) and at initial pH 6.1. Fermentable Sugars were determined by DNSA.



Graph. 2. POME Medium kinetic biodegradation by IBUN IV

Final concentration of acids and solvents (mmoles/L) produced by the IBUN IV strain, in POME Medium. Fermentation was done in 2 L Erlenmeyer flasks with 1 L of culture medium during 8 days at 33ºC with agitation (150 rpm) and at initial 6.1 pH.

Time (hours)	Fermentable sugar* , (mmol/L)	pH	Total solvents (mmol/L)	Total acids (mmol/L)	Non dissociated acid concentration (mmol/L)	Biomass #cell/mL
0	11.81	6.1	37.10	12.63	0.92	2.6×10^6
48	10.39	6.3	56.33	109.27	3.38	
72	11.34	6.3	31.18	81.64	2.36	3.8×10^6
96	9.77	6.4	41.67	103.87	2.53	
120	10.36	6.4	40.07	125.19	3.06	3.3×10^6
146	8.21	6.3	42.84	106.74	3.48	
168	9.89	6.4	35.73	108.01	2.50	1.3×10^6
191	9.65	6.4	45.74	94.92	2.30	
215	9.80	6.4	83.14	144.33	3.45	1.1×10^6

Table 25. Final concentration of acids and solvents (mmol/L) produced by the IBUN 22 A strain

Final concentration of acids and solvents (mmol/L) produced by the IBUN 22 A strain in POME Medium during 8 days' fermentation at 33ºC with agitation (150 rpm) and initial pH 6.1. Fermentable Sugars were determined by DNSA.

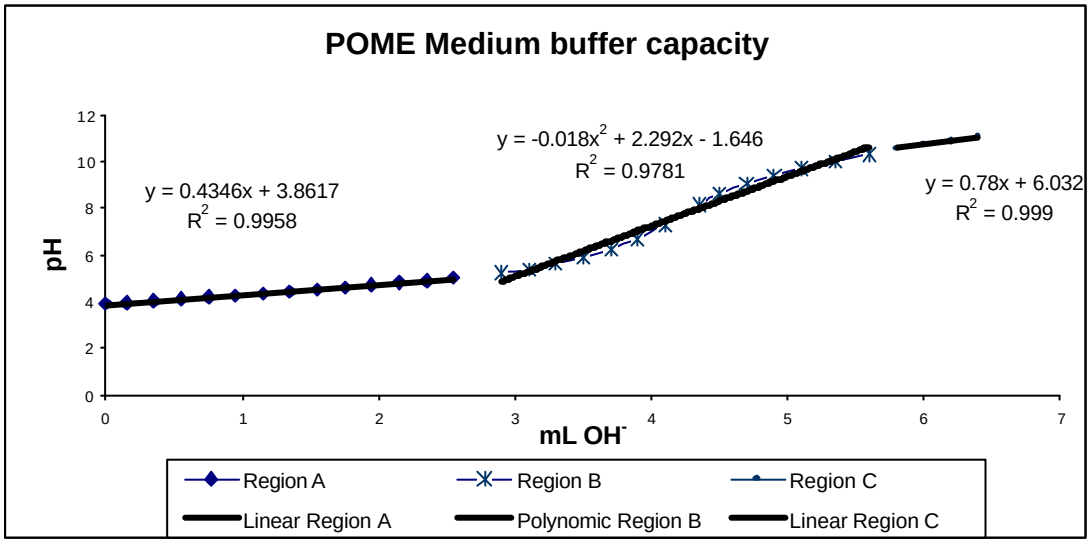
Time (hours)	Fermentable Sugars (mmol/L)	pH	Total solvents (mmol/L)	Total acids (mmol/L)	Non dissociated acid concentration (mmol/L)	Biomass #cell/mL
0	12.20	6.1	38.20	4.34	0.34	3.51×10^6
48	14.48	6.35	51.03	70.98	1.87	
72	13.19	6.35	56.42	33.84	0.93	3.19×10^6
96	13.91	6.39	62.13	36.03	0.91	
120	12.91	6.39	56.60	33.61	0.84	2.62×10^6
146	13.89	6.4	64.63	43.22	1.07	
168	14.17	6.44	59.37	25.73	0.57	9.77×10^6
191	14.48	6.25	66.43	47.68	1.63	
215	13.87	6.31	60.84	124.22	3.49	9.16×10^6

Table 26. Final concentration of acids and solvents (mmoles/L) produced by the IBUN IV strain

Final concentration of acids and solvents (mmol/L) produced by the IBUN IV strain in POME Medium during 8 days' fermentation at 33ºC with agitation (150 rpm) and initial pH 6.1. Fermentable Sugars were determined by DNSA.

6.4. Determining POME Medium **buffer capacity.**

POME Medium buffer capacity was determined as described in Materials and Methods (9.2.3). This curve revealed three pH phases throughout the titration curve. Region A with pH between 4 and 6 units showed high buffer capacity. The range of Region B pH was between 6 and 10 units, whilst Region C presented lineal behaviour the same as Region A, with pH above 10 units (Graph. 3). It should be noted that the range of pH at which these fermentations was done was between 4.8 and 6.0. It is very probable that acid re-assimilation for producing solvents could not happen in these conditions, which is in accord with results of previous fermentation at pH 6.1.

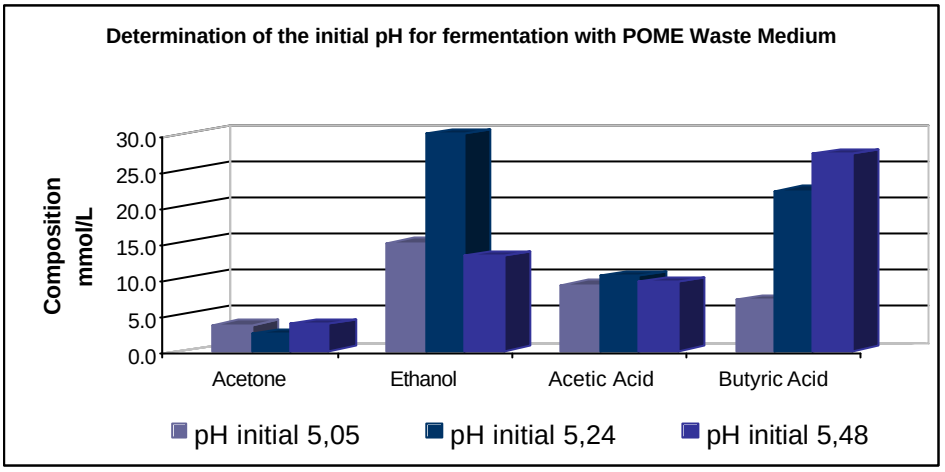


Graph. 3. POME Medium titration curve with 1N NaOH.

Three regions are observed: Regions A and C show present buffer capacity buffer

6.5. Determining initial fermentation pH in POME Medium.

Once high buffer capacity of POME Medium in pH from 4 to 6 units had been demonstrated, the experiments with IBUN IV strain were designed at three different pH (5.05, 5.24 and 5.48 units) during 160 hours at 33 °C with agitation (150 rpm).Results showed that at 5.24 pH units the total solvent production was higher and the butyric and acetic acid productions was similar to other pH values (Graph. 4)



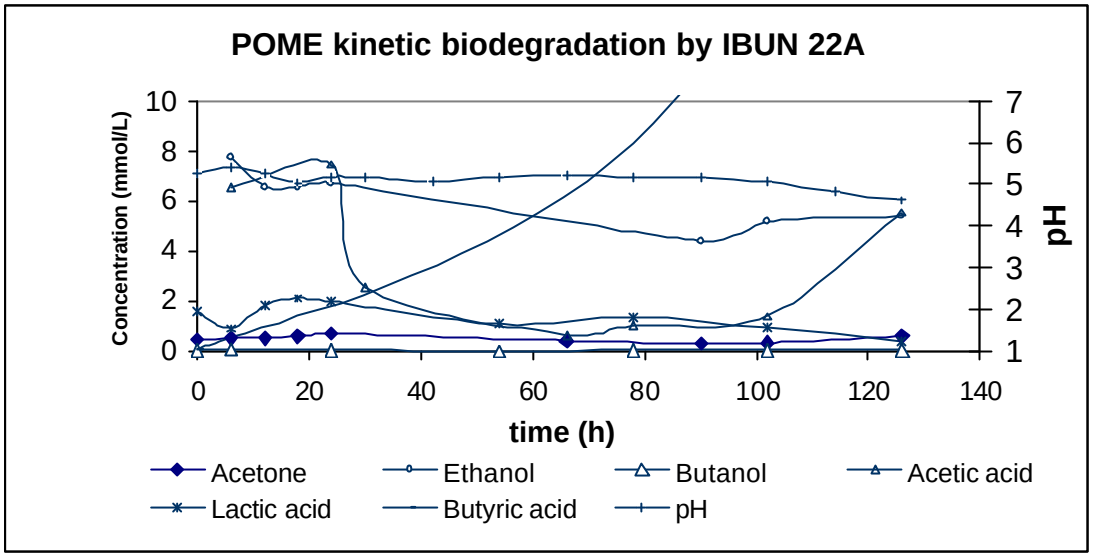
Graph. 4.Determining initial fermentation pH

Final concentration of acids and solvents (mmoles/L) produced by the IBUN IV strain, in POME Medium during 160 hours' fermentation at 33ºC with agitation (150 rpm). Initial pH 5.05, 5.24 and 5.48.

6.5. Pome Kinetic Biodegradation by IBUN 22 A and IBUN IV at 5.23 pH

After the selected initial 5.23 pH, fermentations were done in 1 L POME Medium in 2 L Erlenmeyer flasks, as described in Materials and Methods 9.2.5. The results of fermentation using IBUN 22 A and IBUN IV are presented in Graph. 5 and Graph. 6, respectively.

During the fermentations with IBUN IV and 22A strains, the concentration of cells did not change, in neither case was cells lysis observed. IBUN IV cell numbers were 10^5 and 10^6 cells/mL and cell numbers did not rise above 10^6 cells/mL with the 22A strain; the number of cells was determined microscopically. Differently to preliminary assays, preinoculum was sub-cultured in POME Medium; however, whilst cell numbers decreased, acid and solvent production did not increase, in relation to the preliminary assays.



Graph. 5 POME kinetic biodegradation by IBUN 22A.

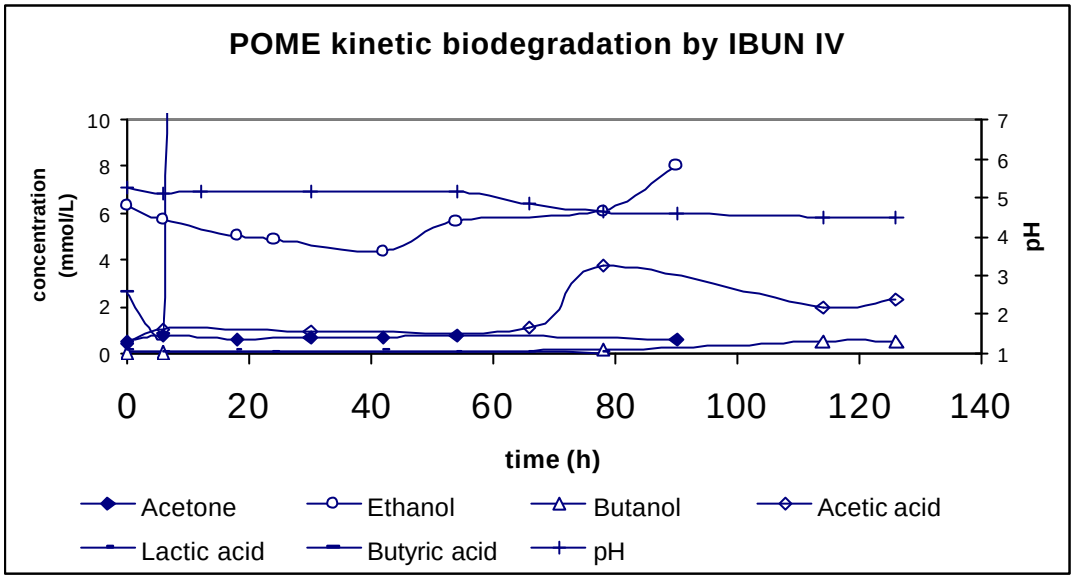
Acid and solvent profiles at 33ºC, agitation at 150 rpm for 124 hours,initial pH 5.23. Butyric acid concentration continues increasing until 500 mmol/L at 120 hours.

Time (h)	Total sugar (mmol/l)	pH	Total solvents (mmol/L)	Total acids (mmol/L)	Non-dissociated acids (mmol/l)	Biomass *108 cel/mL
0	0	5.24		1.57	0.37	0.70
6	50.58	5.4	8.30	7.41	1.37	0.95
12	43.62	5.24	7.09	7.24	0.44	1.45
18	43.45	5.04	7.14	8.03	0.71	1.43
24	40.06	5.18	7.45	9.42	2.58	1.89
90	36.69	5.16	4.86	20.03	7.50	2.20
102	36.69	5.06	5.52	21.35	7.62	6.18
126	33.8	4.61	5.70	496.39	304.19	8.00

Table 27**. Final acid and solvent concentration (mmol/L) in** POME Medium **produced by IBUN 22 A strain.**
Fermentation time 6 days, at 33ºC, in 1L Erlenmeyer flask at 150 rpm, initial pH 5.24.

Both fermentation results have shown that the range of pH during the fermentation was between 4.61-5.24. With these results it would be assumed that the POME waste possesses high buffer capacity. Thus, the results show that enzyme activities could be limited. It is known that many solventogenic enzymes have their maximum activities at acid pH. However in solventogenic phase the pH is approximately 5.5, because solventogenic enzymes present their maximum activities at this pH, in *Clostridium acetobutylicum*. As shown in molecular

characterisation, 22A native strain is not *C. acetobutylicum*.As seen in Table 27, during fermentation using IBUN22 A, the range of pH was between 5.06 and 5.24, and total acids in the same range increased from 1.57 to 21.35 mmol/L. At 126 hours of fermentation, pH was 4.61 and total acid production was 496.39 mmol/L. Besides, non-dissociated acids concentration also increased from 7.62 until 304.19 mmol/L.



Graph. 6 POME kinetic biodegradation by IBUN IV

Acid and solvent profiles at 33ºC, agitation at 150 rpm for 124 hours, initial pH 5.23. Butyric acid concentration continues increasing until 500 mmol/L at 126 hours.

Time (h)	Total sugars	pH	Total solvents	Totals acids	Non-dissociate acids	Biomass
	(mmol/l)		(mmol/L)	(mmol/L)	(mmol/l)	*10^8 cel/mL
0	0	5.25	6.85	3.06	0.73	1.00
6	46.36	5.1	6.47	1.90	0.59	1.25
12	25.66	5.18	5.66	201.97	0.70	1.28
18	40.76	5.04	4.86	423.96	0.99	1.45
24	44.07	5.02	5.06	402.28	0.20	1.52
90	15.8	4.59	6.44	342.31	230.00	1.59
102	17.84	4.82	6.29	503.98	251.29	1.81
126	0.9	4.52	8.67	502.16	331.88	1.83

Table 28**. Final acid and solvent concentration (mmol/L) in** POME Medium **produced by IBUN IV strain.**

Fermentation time was 126 hours, at 33°C, in 1L Erlenmeyer flask at 100rpm.

The pH was kept within the 5.24-4.56 range during fermentation for both strains. The concentration of non-dissociated acids increased at the end of fermentation. (Table 27 and **Table 28**) show non-dissociated acids. The 22A strain presented values 304.2 mmol/L and the IBUN IV strain 331.9 mmol/L at 126 hours. The range obtaining solventogenic phase was between 20 mmol/L and 57 mmol/L, as reported by Maddox *et al.*,(2000).

III DISCUSSION

The aim of this work was to isolate new solvent-producing strains and explore their agro-industrial waste as sole potential carbon source. The first part of the work has been orientated towards isolating and characterising new isolates from Colombian sources. Native strain cellulase activity has been evaluated in the second part, establishing criteria for selecting potent strains which are able to degrade agro-industrial waste as sole carbon source. The third part dealt with applying the most promising strain in fermenting palm oil mill effluent in a modified industrial medium.

1. Characterising new isolates

1.1. Isolating new solventogenic bacterial strains from Colombian soils.

Isolating 178 samples from soil has shown that 100 of them produce more than 2 g/L total solvents. Only 13 strains however produced more total solvent than the *Clostridium acetobutylicum* ATCC 824 type strain. Some physiological characteristics were observed during the selection process, i.e. the sporulation process is apparently involved in solvent production: According to the observations, micro-organisms having scarce spore presence resulted in low solvent-production; however, among isolates having abundant spores, solvent production did not always increase. Sporulation in *Clostridium* is reported to be associated with the triggering factors involved in the switch to solventogenesis (Woods and Jones, 1986). These authors maintain that the initiation of solventogenesis and sporulation are coupled, due to the common molecular nature and separate control mechanisms (Sauer *et al.,* 1995).

Nevertheless, it has been demonstrated that solventogenesis is not a prerequisite for sporulation in *C. acetobutylicum* ATCC 824 (Jones *et al.,* 1982). The samples which grew and produced gas within 24 h were better sources for isolating solvent producing bacteria than those samples which grew within 48 h. It is likely that log phase growth-rate in T6 medium is proportional to the rate of acidification (Kashket and Cao, 1993).

The physiological parameters of *Clostridium spp* isolated from Colombian sources were determined, as was the relationship between the type of soil and number of isolates. The highest average total solvent concentration was produced by micro-organisms isolated from grass (7.5 g/L), chrysanthemum (7.7 g/L) and tuber culture (9.5 g/L) soil. The highest average ethanol and butanol production concentration (7.9 g/L and 4.0 g/L, respectively) was found in micro-organisms isolated from coffee tuber samples' soil. The lowest average total solvent concentrations produced by *Clostridium spp* were isolated from stubble (3.6 g/L) and cereal (4.0 g/L). These results coincide with those obtained by Calam (1980). It therefore seems likely that there is no direct relationship between the number of isolates and the highest solvent concentration produced. This therefore represents the best chance for finding good solvent-producing clostridia

The physiological parameters of *Clostridium spp* isolated from Colombian sources were determined in soil from tuber, chrysanthemum and grass cultures. Soil having acidic pH offers a better chance of isolating a high number of solvent producers and also a potent solvent-producing bacterium. Only one strain was isolated from alkaline soil (worm-breeding, with 3.8 g/L total solvent production). The ethanol yield was 0.57 moles/mol fermented glucose in *C. acetobutylicum* ATCC 824 (Andreesen *et al.*, 1989). Although 12 isolates produced mainly ethanol and/or acetone, one (IBUN 125C) did achieve a high butanol production of almost 50% total solvents, corresponding to 11.4 g/L. This represents higher butanol concentration than *C. acetobutylicum* ATCC 824 (equivalent to 0.12) and is equivalent to 0.15 moles of butanol per mol glucose fermented.

The new strains were Gram-positive, spore-forming, mesophilic, anaerobic rod shaped and did not form catalase, gelatine hydrolysing protease or indol; they produced carbonic acids and alcohol from glucose, starch, sucrose and raffinose. They thus clearly belong to the saccharolytic clostridia group. Native strains were also able to grow in TYG medium without any vitamin being added, except biotin. This behaviour would be characteristic for *C. butyricum*, which has not yet been known to produce high amounts of solvents, this was thus a surprising result The IBUN 22A strain was the only one which did not grow in pure TYG medium, even if the routinely added vitamins

(biotin, thiamine and p-amino benzoic acid) were present, but it did grow in RCM medium, therefore seeming to need some additional co-factors.

The selected strains (13 native strains) presented the greatest solvent production amongst the new isolates. All these do not belong to the classical solvent-producing clostridia group. Thus, the biochemical and taxonomic classification into solventogenic clostridia groups was one of this work's goals. Classifying the new isolates by biochemical and physiological test was undertaken before their taxonomic data were available. Toxicity in some clostridia has been demonstrated; it was thus decided to carry out toxicity tests. The toxicity of the 13 native strains was evaluated by the National Health Institute, using SPF mice. The results demonstrated that these strains were non-toxigenic; such results are important because these strains are potentially useful in industry, in terms of this work for degrading agroindustrial residue produced at 12,000 m^3 per day. This indicates that biological degradation must be done with non-pathogenic micro-organisms.

The groups constructed by Johnson (1997) were used for defining three tests for classifying the solventogenic clostridia into four groups regarding: rifampicin sensitivity; riboflavin production; curd formation in milk medium before 24 h, as shown in the Results section. The native strains can be classified as being solventogenic through biochemical and physiological tests, but it was not possible to include them in any of the following four groups of strains: *C. beijerinkii* DSM 791; *C. acetobutilycum* DSM 792 (*C. acetobutilycum* ATCC 824; *C. acetobutilycum* DSM1732); *C. saccharoperbutylacetonicum* (N1-4) and *Clostridium saccharobutylicum* (NCP 262). The results are shown in Table 11 with the tentative classification. However, the results with the new isolates were not convincing and the tentative classification shown in Table 12 did not stand up to further testing.

1.2. Molecular characterisation, and taxonomic relationships of isolates from Colombian sources

Only 50% of the 157 species included in the Clostridium genus in the list published by DSM (Euzéby J. 1999) have been approved, figuring as species; the other 50% are indicated as being possible new species. 17% of the species on the list still have not been clearly denominated, not having been identified with any of the existing species (Euzéby J. 1999).

The *Clostridium* Group I is divided into 10 IA–IK subgroups (Cummins C. and Johnson J. 1971; Johnson and Francis, 1975). Group II consists of 11 relatively homogeneous species; Group III includes strains having rRNA nucleotide sequence similarity with Group I and II. (Hippe *et al.*, 1992). Johnson and Francis' 1975 classification of Groups I, II and III has been maintained, but those species included in Group IV have been divided into 16 subgroups. Group IV includes a wide range of *Clostridium* species, amongst them being some cellulolytic and thermophilic clostridia. However, the phylogeny of clostridia is still being reinvestigated, the genus currently having 19 groups (Collins M. *et al.* 1994).

Many industrially useful species are found in Group I. The **solventogenic** group has been dividided into four subgroups by molecular techniques (PFGE and ARNr 16S gene sequencing) (Keis *et al.* 1995; Johnson *et al.* 1997).

Prokaryote taxonomists currently agree that reliable classification can only be achieved by exploring taxa's internal diversity by a wide range of techniques (Roselló–Mora, 2001). Molecular characterisation was thus begun, searching for a better classification of the native strains. The question posed was, "Do these native strains belong to a new solventogenic group?" DNA–DNA techniques, 16 S rRNA sequencing and PFGE were used to answer this question.

1.2.1. New strain plasmid profile characteristics

The value of plasmids as molecular markers is limited, but could provide more information about native strain characteristics. A number of plasmids have been isolated from *Clostridium* saccharolytic species, including *C. acetobutylicum* and *C. beijerinckii* (Truffaut and Sebald 1983) and *C. butyricum* (Minton and Morris, 1981). However, all these plasmids have been reported as being cryptic.

The presence of two plasmids (*p*CB101 and *p*CB102) in *Clostridium butyricum* NCIMB 7423 and a single plasmid (*p*CB103) in *C. butyricum* SA1 has been confirmed by electron microscopy (Minton and Morris 1981). However, one study has demonstrated that two *Clostridium butyricum* strains (ATCC 43181 and ATCC 43 755) contain botulynal toxin type E. The BoNT/E gene is located in a large plasmid in the two toxigenic *C. butyricum* strains but is absent from 18 non-toxigenic strains and *C. beijerinkii* strains; this suggests a toxigenic gene transfer from *C. botulinum* E to the originally non-toxigenic *C. butyricum* strain (Hauser *et al.,* 1992).

The *p*SOL1 (210Kb) plasmid in *Clostridium acetobutylicum* ATCC 824 carries primarily metabolic genes (namely all genes necessary for acetone and butanol production), phenotypically distinguishing this strain from the non-solvent-producing butyric acid clostridia (producing butyrate and acetate as their main products). *C. acetobutylicum* ATCC 4259 (the Weizmann strain) has presented an extra chromosomal element carrying the genes required for the final acetone and butanol forming steps. It has been demonstrated that three ATCC 824 degenerate mutants, normally containing a plasmid (*p*SOL1) carrying the sol locus, do not produce solvents when this plasmid is lost (Cornillot *et al.*, 1997).

The native IBUN 13 A, IBUN 95 B, IBUN 18 S, IBUN 22 A, IBUN 140 B and IBUN 62 B strains have a plasmid DNA fragment whose average size is 32.4 Kb. The other native IBUN 64 A, IBUN 62 F, IBUN 125 C IBUN 18 A, IBUN 18 Q, IBUN 137 K and IBUN 158 B strains possess two plasmid DNA fragments, one being 33.1 Kb on average and another 13.7 Kb. Amongst the native strains, *Clostridium acetobutylicum* ATTC 824 and DSM 1732 did not show either of these two fragments (Figure 3).

The approximate values for these bands were calculated by using the Quantity One programme (BioRad Laboratories. 2000, Alfred Nobel Drive. Hercules. CA 94547, the Discovery Series TM, copyright 1998.). Two different types of profile were observed in native strains by those methods described in 6.2.2., one of them having a plasmid DNA band migrating above that for chromosomal DNA having a 33.1 Kbp size and the other (in addition to this band) presenting a second band below it, of approximately 13.1 Kbp. *E. coli* V 517 plasmids were used as molecular markers, extrapolation being done from these markers. It should be emphasised that the difference in plasmid base pairs taken from the strains, whilst being significant, was not strong enough to establish that they are different amongst themselves.

Digestions to study plasmid restriction profiles were tested with restriction enzymes *Hind* III, *Sau3*AI, *Eco52* and *Eco* RV. The results with the first enzyme showed two equal band patterns; they are probably overlapping. Digestion with *Sau*3AI cleaves plasmid DNA into fragments smaller than 400 bp; a fragment this size could not be separated by normal electrophoresis. Chromosomal and plasmid DNA were not digested by *Eco52I.* The *Eco* RV enzyme was selected for evaluating plasmid DNA; it cleaved plasmid DNA into two fragments which presented two plasmid DNA fragments (Table 13). A single fragment pattern was visualised for the majority of strains.

The two fragments observed in Figure 3 could correspond to two plasmids, one of around 13.1 Kbp and another of approximately 33.1 Kpb. However, after digetion only two fragments (8.9 and 3.4 Kpb) were visualised in the gels, due the size of these fragments could be interpreted as being a single plasmid. Thus, the lower band could contain the ccc (covalently closed-circular) forms, which are more compact and run faster; the other band could be either linear or open and circular. However samples were incubated at 65°C for 5 minutes before loading into the electrophoresis and then placed on ice to avoid the appearance of plasmid isoforms, but this treatment cannot ensure that isoform presence is avoided.

Based on the presence of two bands and the similarity of their size, these could be thought to be dimers. Another possibility is that the segment sizes are very close, being flanked by the Eco R1 restriction enzyme recognition sequence, producing similar sized fragments for both 33.1 as well as 13.7 Kbp. Even though they might be similar in size, their sequences could be different, meaning that they will have to be further sequenced and digested with more restriction enzymes in future experiments. It can also be thought that the some of the fragments could be digested in very small fragments, not being detected in the gel in those conditions in which this experiment was done, as they might only be resolved in Acrylamide gels.

1.2.2. Pulse field gel electrophoresis analysis

Chromosomal DNA analysis, using endonuclease digestion restriction patterns, has been used as a sensitive tool for defining strain relatedness (Patterson *et al.,* 1989), the chromosomal DNA macro-restriction pattern, separated by pulse field gel electrophoresis (PFGE), is usually applied to distinguish strains within a species or between closely-related species (Keis *et al.,* 1995). The restriction pattern within a species may vary considerably, as has been shown for four *C. stercorarium* strains (Schwarz *et al.,* 1995).

Pulse field gel electrophoresis was intended to define assigning the native strains to one of the group of solventogenic clostridia . The chromosomal DNA fragment macro-restriction patterns obtained by digestion with the restriction endonucleases *Sma*I and *Apa*I showed that the new strains form an extremely homogeneous group, clearly different from the solventogenic species described by Keis *et al.,* 1995. None of the 13 solvent producers had a restriction pattern similar to the well-known four solventogenic *Clostridium* groups (Keis *et al.,* 1995). This might have been due to the tight screening regime which could have excluded these strains.

However, despite isolation from different places and their great variability in fermentation and hydrolysis activity, the fragment pattern of two restriction endonuclease digests showed only little variation within the newly isolated strains; but

this was clearly different from any of the type solventogenic strains applied, including *C. butyricum* Figure 5.

The physiological and PFGE characterisation of these native strains suggests that the native strains are closely related to each other and do not belong to one of the four groups. Thus, a partial 16S rRNA gene sequence could clarify if the native strains do belong to four solventogenic groups or they belong to the *Clostridium butyricum* group, as suggested by their physiological characterisation (Keis *et al.*, 1995).

1.2.3. Partial 16S rRNA gene sequence DNA analysis

Partial sequences were obtained from all 13 new isolates;they were compared with the *Clostridium* 16sRNA gene sequences from the GeneBank. The CLUSTAL X programme (Materials and Methods 6.4.5) was used for alignment (Table 14). Sequences were aligned by their from-conserved residues; this was done with the 3'OH end (CCCTGG sequence) belonging to primer A in all cases. The alignments were done by groups (Figure 16). Native strain sequences were done first, strict alignment parameters being used, taking account that they form a very homogeneous group according to previous characterisation tests. Gaps were penalised with the relatively high value of 70. The value for the extension of each gap was 6.66 (when a gap was increased from the initial opening). There was 60% similarity for sequence alignment order [Delay Divergent Sequences] and 0.7 DNA Transition Weight.

The second group aligned consisted of the type strains from the four groups of solventogenic clostridia previously described by Keis *et al.* (1995) and Johnson *et al.* (1997), plus *Clostridium butyricum*. All were *Clostridium*, but from different species, so gaps were thus penalised with 40, 6.66 gap extension, 50% Delay Divergent Sequence and 0.5 DNA Transition Weight.

An external group was used (the third group), related to *Clostridium*, to establish parameters once the dendogram had been made. This led to ascertaining if the method used was actually differentiating the species and to knowing at which point the group of *Clostridium* species became detached from those which were being worked on. Four

Bacillus species sequences were included in the external group related to *Clostridium*: *B. macerans* (hydrolysing starch and producing gas), *B. coagulans* (an anaerobic gas producer, spore-former and lactic acid producer), *B. stearothermophyllus* (which has been found with *Clostridium* in soil samples) and finally *B. polymyxa* (anaerobic gas and acid producer in media containing glucose) (Hippe *et al.,* 1992). This group was aligned with gap penalisation equal to that of the reference strain group (second group).

Both groups above (types strains and external groups) were aligned to the group of native strains one at a time. Taking into account that they were different species, gaps were penalised with 15, a 6.66 extension gap, 50% Delay Divergent Sequences and 0.5 DNA Transition Weight.

The type strains used were: *C. beijerinckii* DSM 791, *C. acetobutilycum* DSM 792, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* DSM1732, *Saccharoperbutyl-acetonicum* N 1-4 C. and *C. saccharobutylicum* NCP 262. These strains belong to each one of those groups reported by Keis *et al.* (1995) into which the solventogenic clostridia are divided. It was found in this work that native strain 16sRNA sequences coincided 99% with the *Clostridium butyricum* sequences reported in the GeneBank Data Base; it was thus decided to include three strains from this species: *Clostridium beijerinki*i NCIMB 8082 and *C. butyricum* DSM 2478 and *C. butyricum* ATCC 43755. On the other hand, other type strains were included in the group (three more sequences), using the same morphology, physiological characteristics and growth medium conditions (such as soil), as well as the native strains as criteria.

The selected species were: *C. tyrobutyricum*, which is found to be associated with food and soil contamination; *C. kluyveri* which, when fermenting ethanol, produces hydrogen and butyrate from acetate the same as some of the native strains; and *C kainantoi* which was also included in the analysis because this species has been classified in the *C. butyricum* group by some molecular markers.

The native strains are close to *C. butyricum* (NCIMB 8082, DSM 2478 and ATCC 43755) and *C. kainantoi* DSM 523 is found in the same group as the *C butyricum* strain

in the dendogram. This shows that 16S rRNA partial sequencing is not capable of differentiating between these two species and that it is necessary to find another molecular marker (Knabel *et al.,* 1997). *C. acetobutyuricum* DSM 792 and ATCC 824 belong to another group. It was also observed that the *C. saccharoperbutylacetonicum* NI-4, *C. saccharobutylicum* NCP 262 and *C. beijerinckii* DSM 791 strains form another group. The position of the reference strains in the dendogram agrees with Keis *et al.*, (1995). *C. tyrobutyricum* and *C. kluvery* form a distant group. It is important to point out that the 64 A strain is the first to become separated from the native strains' main group Figure 17, a behaviour which has been shown using other physiological markers such as solvent production (Montoya *et al.,* 2001).

DNA sequencing of the partial 16 S rRNA gene from all strains and the complete gene from three of the new strains (IBUN 22A (AJ289704), IBUN 64A (AJ289706) and IBUN 125C (AJ289705)) did not resolve strain assignation between *C. butyricum* and C. *kainantoi*, but clearly separated them from the other solventogenic clostridia as a genetically highly homogenous group. New strains were very similar to *C butyricum* but clearly distant to the four solventogenic clostridial species. However, their fermentation pattern was distinct from the *C butyricum* species. It will be necessary to employ another marker to idetify these species more precisely.

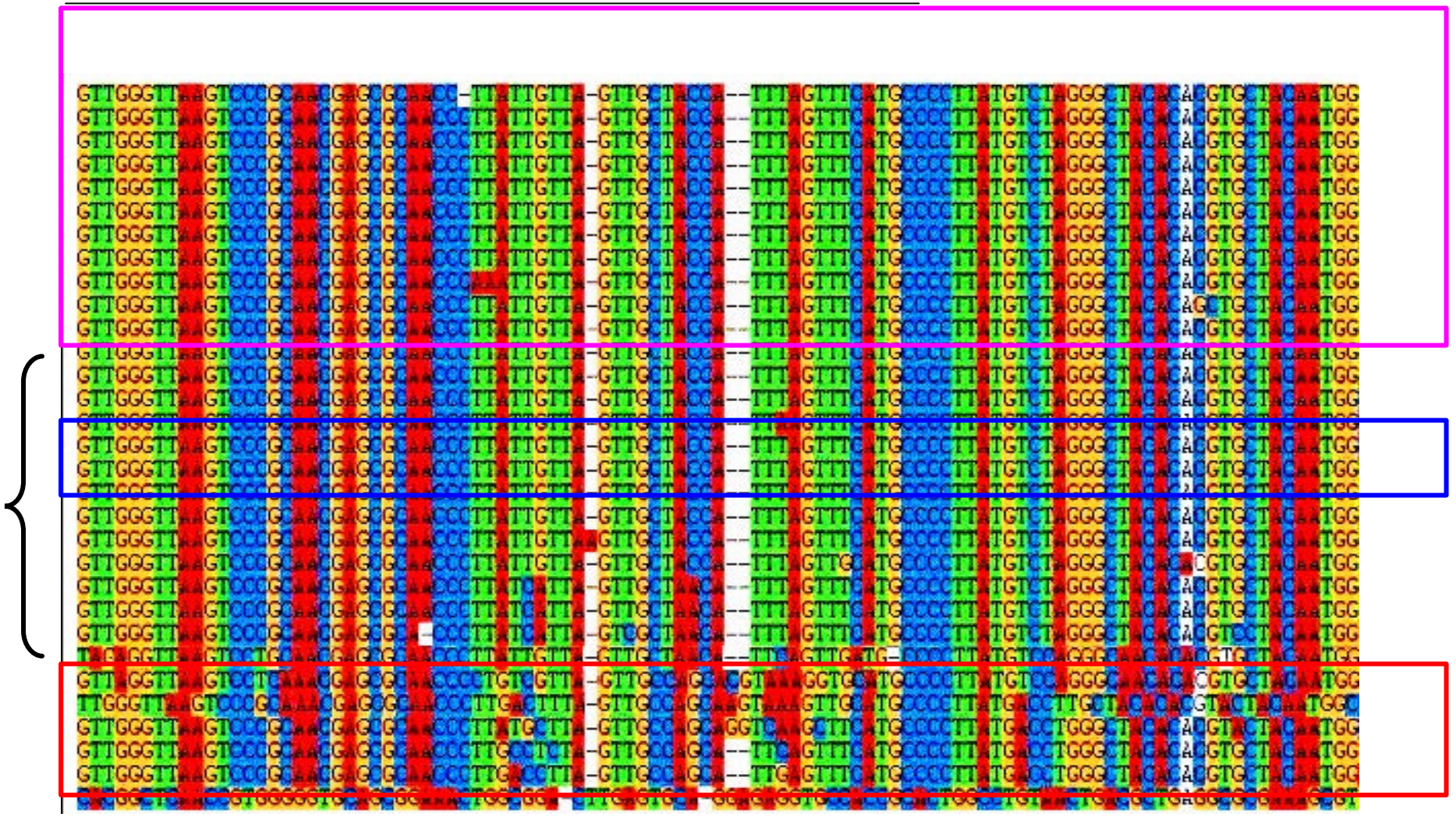


Figure 16. Multiple Alignment used to contruct the dendogram .

This figure shows the alignment including the type strains (parenthesis) ,the native strains (pink square), *C. butyricum* type strains (blue square) and the external group (red square)..

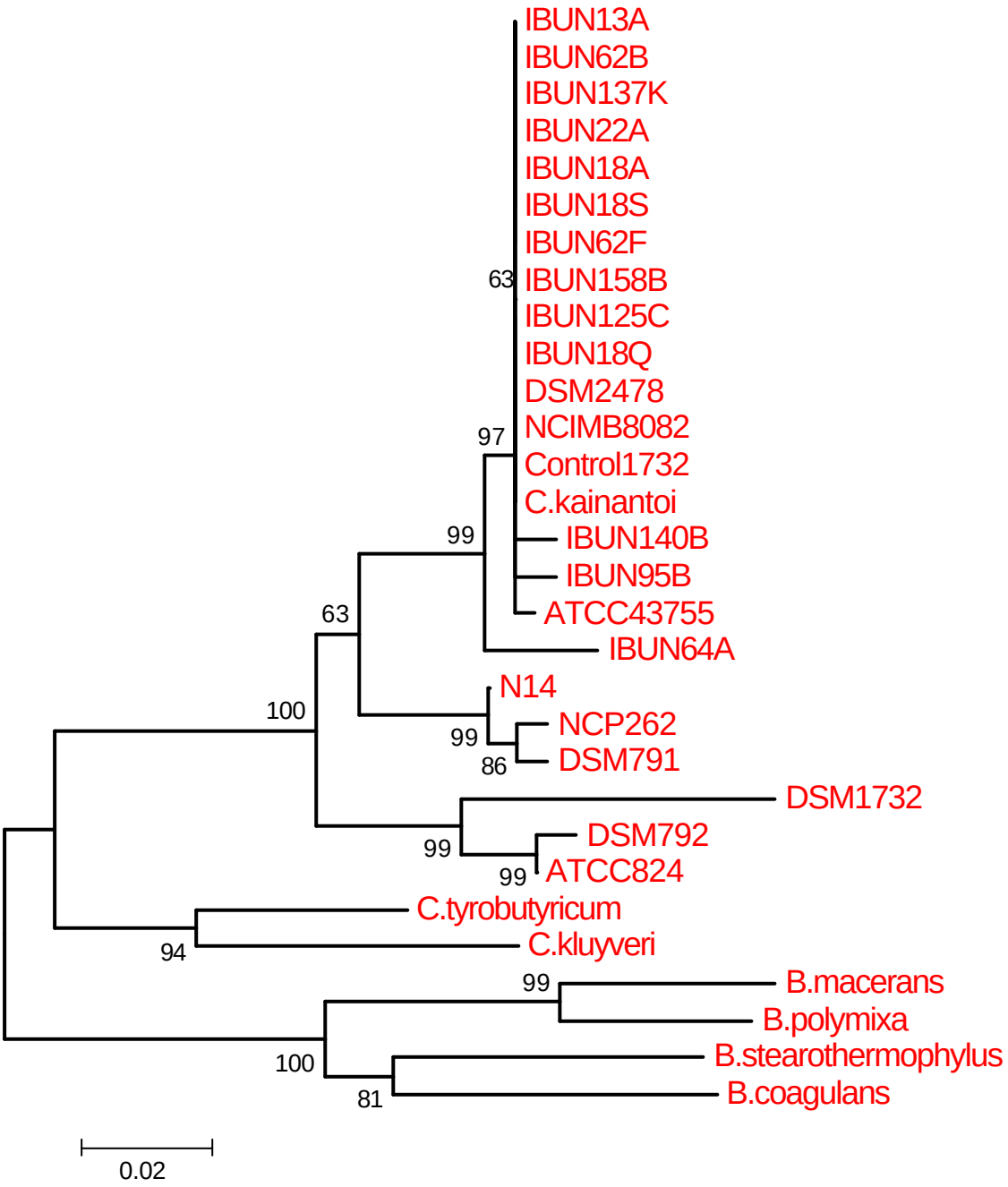


Figure 17. Dendogram for native *Clostridium* strains

The dendogram was constructed by Neighbour Joining with the p distance matrix from 16S rRNA partial sequences between 774-795 and 1521-1495, according to their corresponding position in the homologous gene in *E coli.*

1.2.4. PCR strain identification, using a 16 rRNA target oligonucleotide specific for *C. butyricum*

New strains are grouped with *C. butyricum* (NCIMB 8082, DSM 2478 and ATCC 43755) in a clustal tree of their 16SrRNA sequences. As 16S rRNA has been used for constructing species-specific oligonucleotide primers for PCR analysis of food contamination by *C. butyricum*, this test was applied to the new isolates. (Knabel *et al.*, 1997**)**. All native strains had the same amplification pattern as *C. butyricum*. By contrast, the *Clostridium acetobutylicum* DSM 1732 type strain did not present amplification and *Clostridium acetobutylicum* ATTC 824 presented a different amplification pattern. This test provided more experimental evidence, suggesting that the native strains are very closely related to *Clostridium butyricum*.

According to results shown earlier, the new strains form an extremely homogeneous group, clearly different from the solventogenic species. No member of the well-known four solventogenic clostridial species was among the best 13 solvent producers. This might be due to the tight screening regime which could have excluded those strains, as already stated.

1.2.5. DNA-DNA hybridisation

Genomic DNA-DNA hybridisation and 16S rRNA gene sequencing techniques are currently being used, amongst other techniques, for molecular characterisation (Keis *et al.*, 1995, Johnson *et al.*, 1997). As there is a non-linear correlation between DNA similarity (by DNA-DNA hybridisation) and 16S rRNA sequence homology, each method is reliable in determining a different degree of relationship. Whereas 16S rRNA sequence homology can correlate from the level of domain (above 55% homology) to the level of moderately related species (up to 97.5%), DNA hybridisation clearly differentiates between closely related species above 97.5% 16S rRNA homology (Stackebrandt and Goebel, 1994). So, despite the ease and speed of molecular biological methods (especially DNA sequencing), DNA-DNA hybridisation of total chromosomal DNA is still an indispensable method for strain differentiation. Despite the close

relationship of the 16S rRNA sequences, which would place the strains within the *C. butyricum* species, preliminary DNA-DNA hybridisation with DSM 2478 DNA data shows a degree of hybridisation which is too low to justify the assignment of the strains to the *C. butyricum* species. Further experiments in a specialised laboratory will have to be performed to show whether the strains belong to a new species (**Figure** 18).

Figure 18 shows the hybridisation percentage of the all native strains with the three probes. Type strain *C. acetobutylicum* (green circle); *C. butyricum* and *C. kainantoi* (purple circle) and native strains are in two groupes. In the first groupe (yellow) are the strains more similar to *C. kainantoi* and *C. butryricum*, the second one (blue) is composed by native strains with hybridisation percentages near to 0% with the three probes of the type strains. Besides, in this groupe is the one used as external groupe, the *Anaerobacter thermophilum.*

It is important to emphisize that native strains 140B and 22 A are not grouped with other native strains by DNA-DNA hybridisation as previously described, but they have higher hybridisation percentages with *C. kainantoi* strain probe. This can be related to the capacity of these two strains for degradating cellulose and hemicellulose polymers. Base on these characteristics, they were selected for doing preliminar assays to POME degradation.

1.2.6. Native strain toxicity.

As these microorganisms have potential application in degrading industrial waste (POME) being used as carbon source, and producing value-added products they should be used in large-scale applications. The new bacteria, having been shown to be non-toxic, can thus be considered to be safe for use in a large-scale industrial process related to production processes.

DNA-DNA Hybridisation percentages

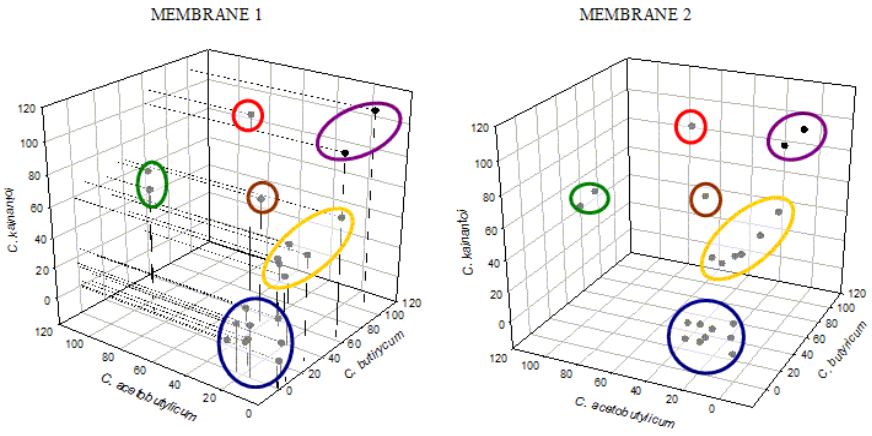


Figure 18**.** Comparison between the strains' hybridisation percentages in those membranes used. Yellow**: IBUN137K, IBUN64A, IBUN125C.** Purple: ***C. butyricum* DSM2478 y *C. kainantoi* DSM523 with closed percentages.** Green**: *C. acetobutylicum* (DSM1732 y DSM792).**
Red**: IBUN 140B. Brown: IBUN 22A.** Blue**: l IBUN13A, IBUN18A, IBUN 18Q, IBUN18S, IBUN62B, IBUN 95B, IBUN158C, and *Anaerobacter thermophilum* as the negative control.**

2 **Determining polymer degradation potential**

Eight *Clostridium* type strains and the thirteen Colombian isolates were studied for their potential to enzymatically hydrolyse polysaccharides prevalent in biomass.

Soluble starch was first used to induce exoenzyme production in the culture broth. The enzymatic activities of the clarified (or free cell) culture supernatans were assayed on different substrates such as soluble starch, pullulan, raw starch (tapioca), CMC, Avicel (microcrystalline cellulose), xylan larch wood, xylan from oat spelt, polyosen (hemicellulose mix from spruce wood), arabinan, barley β-glucan and cellobiose (units of enzyme activity are expressed as: 1 mmol glucose/[min*mg protein]).

Activity was highest on soluble starch with most of the strains (48%) between 10 and 20 units Figure 15. It is important to note that many strains exhibited relatively high activity on raw starch (the natural state of starch in biomass) and pullulan. This means that these strains could have a great potential for degrading agro-industrial waste, because the polymers in biomass are generally insoluble and the possibility of attack on this material is greater with this type of enzyme Concerning their activity on crystalline cellulose, most of the strains (nearly 50%) have between 0.01 and 1 Unit of enzyme activity; an average of 30% of the strains presented activity ranging from 1 to 2 Units, an activity which is to be considered to be high in cellulolytic organisms. If the fact that the strains were grown on starch medium is considered, then these results show that the strains may be further induced if an appropriate inducer were to be used, e.g. cellulosic substrates.

Activity on crystalline cellulose ("true cellulase") is a rare trait among bacteria. The cellulolytic strains of the anaerobic bacteria produce a cellulosome (an extracellular multi-enzyme complex). It could be possible to obtain novel cellulase activity from strains such as 22-A, 137-K and 140-B (Figure 6), which is especially intriguing in combination with their solvent production. With such strains an industrial process, producing butanol from cellulose, could be envisaged., the strains tested were able to grow on crystalline cellulose as a sole carbon source.

Another aspect for future work would concern the presence in the new strains of genes for producing a cellulosome, enabling *C. cellulovorans* from the same clostridia Group I to effectively degrade crystalline cellulose. The genes for a complete cellulosome, a 6 MDa extracellular enzyme complex, have also been detected in the *C. acetobutylicum* chromosome, but are not expressed there.

There are two types of enzymes called chitinases and glycosylhydrolases which are able to degrade chitin without previous treatment. The chitinases comprise the endochitinases which break internal sites aleatorially, generating low molecular weight GlcNAc multimers, such as chitotetraoses, chitotrioses and di-acetylquitobiose dimers, and exochitinases. The latter contains chitobiosidases which catalyse the progressive

liberation of diacetyl-chitobiose and start with chitin microfibres, and 1,4-β-acetyl-glucosaminidases which break down chitooligoscaccharides and chitobiose, generating the end product - N-acetyl-glucosamine .

Such strains with especially high hydrolytic activity on a specific substrate have a high application potential for the direct conversion of polysaccharides to solvents by improving the process and/or the strain. For example, *C. acetobutylicum* SA-1, a butanol tolerant mutant strain, was reported by Chojecki and Blaschek (1986) as showing higher starch use correlated with a higher β-amylase activity and butanol production.

Degradation of specific polymers by *Clostridium spp* could be amplified by genetic techniques for improving hydrolytic activity. This would represent a promising approach resulting in strains having a higher solvent production yield on cheaper substrates, leading to higher competitiveness for the solvent production process. On the other hand, some strains degrade a wide range of polysaccharides (e.g. the IBUN 22A strain degraded all polymers tested) and could be used for the fermentation of complex substrates such as waste material from the food industry.

3. Fermentation

3.1. Optimisation of industrial culture medium

IBUN IV strain was used in the optimisation of an industrial culture medium in which cane molasses were used as carbon source. Optimisation of the industrial culture medium and strain improvement are two fundamental factors in increasing yield, as well as productivity, in any fermentation process. The Plackett-Burman method was used to evaluate seven variables in eight experiments (Table 5). The structure of a fractioned factorial design allowed those independent variables which significantly affected the production of total solvents to be identified and also the range in which these variables could be used.

The EVOP Simplex design allowed sequential exploration of a response surface so that a peak production point could be located on the same. This is an application of response surface methodology concepts for improving industrial process yields. It was possible to enhance total solvent production by 58.7% by the appropriate use of experimental design (Table 6). The criteria for calculating carbon source concentrations and the rest of the medium components were calculated via the *Clostridium acetobutylicum* metabolic route and the total biomass production in vegetative medium. Thus the IBUN IV is a mutant isolated from *Clostridium acetobutylicum*. The influence of some medium components such as molasses being supplemented with dibasic potassium phosphate (KH_2PO_4) between 0.05 and 0.2% (Walton and Martin, 1979) is relevant. In this work, phosphate concentration was 0.18% (1.8 g/L). Girbal and Soucaille (1995) have suggested that *in vivo* low ATP levels and high NADH/NAD levels result in high butanol production in continuous culture medium. The organic nitrogen sources (such as peptone and yeast extract) increased solvent production when they were used between 3 and 4 g/L (Abou-Zeid *et al.*, 1976; Monot *et al.*, 1982). Such results coincided with this work (3g/L yeast extract).

Other authors have recommended that the mixture should be supplemented with between 0.05% and 0.2% phosphate (KH_2PO_4) (Walton and Martin, 1979). In our case, the phosphate percentage was 0.18% (1.8 g/L). It was found that ammonium sulphate did not affect solvent production significantly during culture optimisation, a result which did not coincide with other studies in which it has been stated that, to obtain high solvent production, a supply of 4:6 g/L concentration of ammonium sulphate is also necessary (Monot *et al.*, 1982).

It was found that ammonium sulphate did not affect solvent production significantly during culture optimisation, a result which did not coincide with other studies stating that a 4% to 6% concentration ammonium sulphate supply was also necessary to obtain high solvent production (Walton and Martín, 1979; Monot *et al.*, 1982).

In the experiments already cited, glucose was used as a source of carbon. Fermentation carried out in our laboratory indicates that medium acidification occurs with greater

speed when glucose is used as carbon source, compared to the use of molasses (results not shown). It is probable that the results are influenced by the carbon source; thus, ammonium sulphate will not be used as nutrient in the future, unless it contributes to neutralising acid excess to maintain a suitable pH.

As can be seen in the optimisation process, the pH is a factor significantly influencing total solvents. The optimum pH was 6.1 before sterilisation. 24.6 g/L of solvents were produced in the optimised medium, representing a 58.7% increase, with respect to the non-optimised medium (Montoya *et al.*, 2000).

3.2 POME waste degradation

The goal of POME degradation is to use it as carbon source for producing solvents and decreasing environmental contamination. It is known that carbon source represents a high percentage of solvent production's final cost; it is reasonable to expect to increase solvent production with this process. POME Medium composition was estimated. Carbohydrates were found to be present in cellulose and hemicellulose biopolymers. Native 22-A and 140-B strains **(**Figure 15**)** were selected for POME use due to their high cellulase activity; both strains produced acetone and butanol, but not ethanol. One of the advantages of using POME is that it can be taken and used straight from the production plant, to be used as 100% carbon source in POME Medium; 50% and 75% concentrations were also tested, but acid and solvent concentrations were lower.

Preliminary assays were done at initial pH 6.1, which was then selected from the industrial medium optimisation. Results of preliminary assays obtained at pH 6.1 show that type strain C. *saccharobutylicum* NRRL B643 produces less concentrations of acetone, butyric acid and acetic acid than the native stains (Table 24). The IBUN 22A strain produced 5% more acetone and 10% more butyric acid concentration than IBUN 140 B strain when using the native strains. The 22 A native strain was thus selected for POME kinetic biodegradation, using POME Medium and initial 6.1 pH. POME kinetic biodegradation using IBUN IV strain was done in the same conditions (Graph. 1 and Graph. 2).

Acidogenic fermentation could be observed (according to Maddox *et al.*, 2000) during fermentation at 6.1 pH, this being a phenomenon accomplished with pH values close to neutrality, where the main products were acids, presented in fermentation with no pH control. Here an excess in total acid production and low non-associated acid production impeded fermentation passing from the acidogenic stage to the solventogenic stage as (according to the aforementioned researchers), for solventogenesis to occur, non-dissociated acid concentration must lie between 20 and 60 mmol/L. The previous results show that it is probable that the POME Medium used had a high buffer capacity. Therefore, it required researching initial pH ranges to avoid the acidogenic phenomenon produced during this experiment.

On the other hand, Terracciano and Kashket (1993) showed that the medium's external pH reduced from 6 to 4.6 in *Clostridium acetobutylicum* (on account of the acids being obtained), but that a constant ΔpH (0.9-1.1) was maintained, the cell internal pH being more alkaline than that of the culture medium. The weak organic acids' effect on metabolism and growth generated acidification within the cell. It has been observed that the internal pH decreased to values less than 5.5 during acidogenic phase in *Clostridium acetobutylicum*, resulting in inability to produce solvents in the cell. But an increase in external pH led to a reduction in cell viability and glycolysis. As there was no decrease in external pH value in the two kinetics evaluated, then it can be presumed that the ΔpH was minimal; therefore, the H^+ flow from the exterior towards the interior of the cell and *vice versa* was also minimal, due to the fact that there was very low concentration of non-dissociated acid concentration which could have entered the cell.

The POME Medium buffer capacity was determined at pHs ranging between 3 and 12 to improve solvent production. The results show that the POME Medium presented high buffer capacity in the pH range 4.0 to 6.0 in which acidogenic and solventogenic phases occur (Graph. 3). Due to this, it was necessary to develop new experiments to determine initial fermentation pH such as different initial pH tests, all of them close to the pH reported for acidogenic-solventogenic switch. The initial pH finally selected was 5.24, because total solvents and acetic and butyric acids were also produced.

The results show differences in behaviour between IBUN 22A and IBUN IV strains related to total solvent production throughout the determined fermentation time (Table 27and Table 28). A notable change of total acid production was observed after 102 hours in IBUN 22 A fermentation, at 5.3 pH; by contrast, it occurred after 12 hours in IBUN IV fermentation. A notable increase in non-dissociated acids was produced in this last strain after 90 hours.

POME Medium degradation kinetics were done using IBUN IV and IBUN 22 A strains, once an initial 5.3 pH had been selected. After 24 hours of fermentation, pH had decreased to 4.8, but remained constant throughout the whole process, indicating that there was no re-assimilation of acids for producing solvents. According to these results, fermentation using the IBUN 22 A strain thus produced an increase of 23 times total acids and 40 times non-dissociated acids, when pH decreased from 5.06 to 4.61 after 102 hours. By contrast, when pH passed from 5.1 to 5.18, total acid production was increased 100 times with the IBUN IV strain at 12 hours and non-dissociated acids increased after 24 hours when the pH passed from 5.02 to 4.59.

The fundamental purpose of using POME as sole carbon source was to obtain greater added-value products as solvents. As can be seen in (Graph. 5 and Graph. 6) that the IBUN 22A and IBUN IV strains produced greater butyric acid concentrations than other metabolites, whilst acetic acid production was low. In both cases, ethanol production was higher than butanol and acetone production. According to these results, it can be proposed that pH must be controlled during fermentation t to achieve those conditions needed for solvent production,.

It should be remembered that during this work the 22A strain was classified as being close to *C butyricum* and IBUN IV as being a *C butyricum* DSM 1732 mutant. They did behave differently during fermentation when the carbon source was glucose and when it was POME. However, there are differences between fermentation with the two strains (see culture design in Results).

IV. CONCLUSIONS

- Native strain 16s rRNA sequences coincided 99% with the *Clostridium butyricum* sequences reported in the GeneBank data base, being different to the reference strains *C. beijerinkii* DSM 791, *C. acetobutylicum* DSM 792, *C. acetobutylicum* ATCC 824, *C. acetobutylicum* DSM1732, *C. Sacharoperbutylicum* N 1-4 and *C. saccharobutylicum* NCP 262 reported by Keis *et al.* (1995) and, using the same technique, a 16S rRNA targeted oligonucleotide specific for *Clostridium butyricum* (Knabel *et al.*, 1997) was found.

- The PFGE data corroborated the sequence data in that all strains were almost identical to each other, but different from the four solventogenic clostridia type strains (Keis *et al.*, 1995 and Johnson *et al.*, 1997).

- Despite the close relationship of the 16S rRNA sequence (which would place the strains within the *C. butyricum* species), preliminary data regarding DNA-DNA hybridisation with DSM 2478 DNA showed a degree of hybridisation which was too low to justify assigning the strains to the *C. butyricum* species. Further experiments will have to be performed to show that most of the strains could belong to a new species.

- Despite being isolated from different places and their great variability in fermentation and hydrolysis activity, all strains were able to degrade xylan (arabinoxylan and glucuronoxylan) and most strains degraded the other polymers tested. Such strains with especially high hydrolytic activity on a specific substrate have a high application potential for the direct conversion of polysaccharides to solvents by improving the process and/or the strain. Most strains degraded a wide range of polysaccharides (i.e. strain IBUN 22A

degraded all polymers tested) and could be used for the fermentation of complex substrates such as waste material from the food industry.

- Industrial Medium's experimental design and optimisation led to improving total solvent production by 58.7%.

- The IBUN 22A strain presented greater polysaccharide hydrolysing or degrading capacity when native strains were evaluated in POME Medium, according to the quantity of acid produced. Fermentation assays were done with both strains (IBUN 22 A and IBUN IV); initial fermentation pH had a greater effect on the quantity of dissociated acids. Results obtained to date showed that acidogenic fermentation occurs when initial pH is 6.1.

- Fermentation assays were also done with the above strains (IBUN 22 A and IBUN IV) at pH 5.3, during the fermentation; the pH decreased to 4.8, producing greater acid than solvent concentration, not triggering the expected solventogenesis. A notable change of total acid production was observed after 102 hours in IBUN 22 A fermentation, at 5.3 pH; by contrast, it occurred after 12 hours in IBUN IV fermentation. A notable increase in non-dissociated acids was produced in this last strain after 90 hours, but the high non-dissociated acid concentration could have been acting as an un-coupler causing increased membrane permeability leading to proton entry, in turn producing acidification inside clostridial cells.

- Solvent producing ability is influenced by substrate type and concentration, the pH, culture medium buffering capacity and culture conditions. IBUN 22 A produced ethanol as an end-product in glucose and POME medium; IBUN IV also produced ethanol and high butyric acid concentration. Fermentation pH must be controlled for co-cultures, taking advantage of the fact that IBUN 22 A produced acids by POME degradation and IBUN IV can produce solvents at a suitable pH.

- It is important to emphisize that native strains 140B and 22 A are not grouped with other native strains by DNA-DNA hybridisation as previously described, but they have higher hybridisation percentages with *C. kainantoi* strain probe. This can be related to the capacity of these two strains for degradating cellulose and hemicellulose polymers.

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