



Review

The Industrial Fermentation Process and *Clostridium* Species Used to Produce Biobutanol

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Abstract: The fermentation route for producing biobutanol from renewable plant biomass was used extensively during the last century. The key factors affecting performance in the standard batch industrial fermentation process are highlighted. Four species of *Clostridium* were utilized for the industrial production of solvents, and although they share many features in common, they also exhibit significant differences. The salient features of the existing industrial species and strains are reviewed. These include their suitability for the type of process and fermentation substrate used. The strains are also assessed with respect to their potential for future applications.

Keywords: solvent-producing *Clostridium* species; industrial acetone butanol fermentation; biobutanol

1. Introduction

The industrial Acetone-Butanol-Ethanol (ABE) fermentation process has been used for over a hundred years to produce biobutanol from renewable plant-based raw materials. During the first half of the last century, ABE fermentation was the second largest industrial fermentation process behind ethanol fermentation, and these were the only industrial processes used for producing these bulk chemicals [1]. The fermentation route using renewable raw material then came into direct competition with petrochemical processes that used non-renewable raw materials. As a result, the cost of the substrate coupled with the cost of producing, concentrating, and purifying these chemicals became the deciding factors in determining the financial viability and competitiveness of the fermentation route. In the case of ABE fermentation, the limitation of producing viable concentrations and yields of butanol became of key importance for the process to be cost-effective.

Product concentration is a measure of the amount of product produced in the fermentation, yield is a measure of the efficiency of product formation, and productivity is a measure of the rate of product formation. In ABE fermentation, product concentration can refer both to the concentration of total solvents produced and to the concentrations of butanol, acetone, and ethanol. Concentration titres are typically expressed as grams per litre (g/L). The ratio of the three solvents produced by different strains is usually expressed as a percentage. The concentration of sugar in the substrate can have a significant impact on the efficiency of the fermentation process. In general the efficiency of the fermentation tends to increase with higher set sugar concentrations up to a certain point. Yield, on the other hand, refers to the efficiency of the fermentation process in converting the fermentable sugar or starch in the raw material into solvents. It is usually expressed as a percentage of the amount of solvents that can be obtained from a set sugar concentration. Productivity in a fermentation process refers to the rate at which the desired product is produced per unit time. This is often measured as the amount of solvents produced per unit time, such as grams per litre per hour. There is often a trade-off between productivity and yield. A better yield makes the process cheaper as less sugar is needed to form the same amount of solvents, but higher productivity means faster production. As a rule of thumb, yield is more important for low value products such as bulk chemicals while productivity is more important for high value products such as pharmaceuticals.



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2. The Use of the Industrial ABE Fermentation in Different Countries/Regions

An updated history of the ABE industrial fermentation process has been published recently [2]. The fermentation route was used for solvent production in at least 17 countries/regions. The years that the process was in operation in the various countries/regions is illustrated in the timeline in Figure 1. The ABE fermentation process was first developed before WW1, as part of a quest to produce synthetic rubber. The pressing need to produce acetone for munitions during WW1 led to the rapid expansion of the industrial fermentation process in Britain. This led to the process developed by Weizmann being transferred to Canada, India, and the US, as well as the fermentation process being established in France. After WW1, the plants in the US were auctioned off, and the patents for the Weizmann strain (later named *Clostridium acetobutylicum*) and process were acquired by the Commercial Solvents Corporation (CSC) in the US. Initially the company produced butanol as a solvent for the lacquer industry and enjoyed a commercial monopoly while patent protection remained in place. A glut in sugar cane molasses that became available at this time provided an attractive cheaper alternative to maize. This required the isolation and development of new saccharolytic industrial strains capable of efficiently utilizing molasses. From the beginning of the 1930s, three other US chemical and fermentation companies are known to have established the industrial fermentation process. This was followed by the establishment of the industry in the UK, South Africa, the USSR, Japan, and Taiwan. During WW2 the industrial fermentation process again proved invaluable for the provision of strategic war materials. After the war, the production of solvents by the fermentation process reached a peak during the 1950s, with plants in 11 countries/regions in full production. During the 1960s, the use of the fermentation route went into rapid decline in the West due to the advances made in petrochemical technology resulting in their replacement by cheaper solvents produced from petroleum-based raw materials. By the end of the 1960s the ABE plants in the UK, Taiwan, Japan, and Puerto Rico had all closed. The last ABE plant in the US ended production in 1977, while the fermentation process was phased out in South Africa in 1983 and in Brazil in 1993. The fermentation process continued to be used in the USSR, China, and Egypt for the strategic production of solvents, but these plants were also eventually closed.

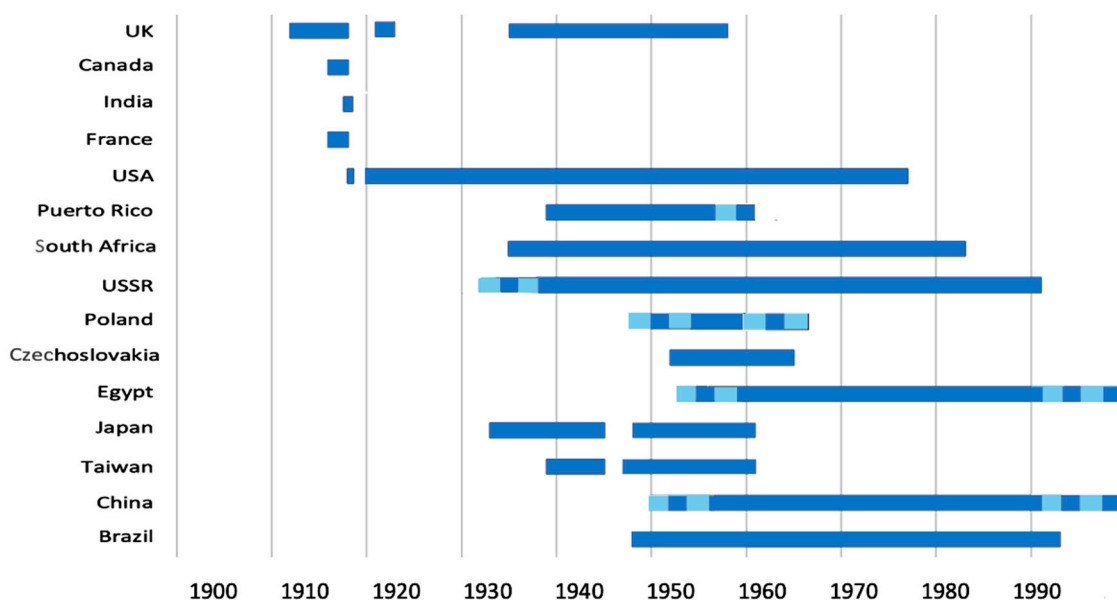


Figure 1. Timelines for the periods that the industrial fermentation process was in operation in different countries/regions. The dark blue bars indicate the year that the fermentation process began and terminated. The lighter blue bars indicate that the actual year that the fermentation process either began or was terminated is not known.

Most of the early technical information and applied research relating to the industrial fermentation process is based on the commercial processes that were operated in the US and UK. This early literature provides much of the information regarding the use of maize in the US [3,4] and later the use of molasses in the US [5–8] and the UK [9–11]. Information regarding the industrial processes used in South Africa [12,13], the USSR [14,15], Japan and Taiwan Region [16], and Mainland China [17,18] only became available later.

3. The Current Status of the Industrial Fermentation Route for Solvent Production

During the first decade of this century, there was a revival of interest in the fermentation route for solvent production, and new industrial processes were established in China, the US, and Brazil [2,19]. Most or all these newer-generation industrial processes are no longer in operation. Although there is a clear market demand for biobutanol and acetone produced by the fermentation of renewable biomass, the use of the fermentation process continues to remain at best marginally economically competitive, mainly due to the cost of agriculture-based raw materials and the low concentration of the end-products. Various initiatives aimed at overcoming the limitation of using *Clostridium* species as biocatalysts to produce n-butanol for use both as a bulk chemical and as a renewable alternative transportation fuel are being actively pursued. However, developing new-generation commercial processes remains challenging as any process will need to deliver a combination of high titre, yield, and productivity of solvents to be cost-effective.

Beginning in the 1970s, increasing numbers of scientific and technical articles and reports have been published aimed at overcoming these limitations. There are a number of excellent reviews that provide a good overview of recent developments [20–23]. These cover developments in genetic and metabolic engineering and strain improvement, as well as developments in process technology and downstream product recovery and advances in the use of alternative sources of renewable biomass for butanol production.

3.1. Developments in Genetic and Metabolic Engineering

The focus of much recent activity has been to metabolically engineer homo-butanogenic *Clostridium* strains to produce n-butanol stably and continuously. Genetic and metabolic engineering for producing second-generation biofuels such as butanol has in many cases been impeded by a lack of detailed knowledge regarding the primary metabolism and its regulation for the species. It has been recognized that developing a commercially viable process via metabolic engineering requires the detailed characterization of both the primary metabolism and the regulation of the *Clostridium* species targeted. Several metabolic strategies have already been developed to increase butanol yields, most often based on carbon pathway redirection. New biochemical data, in conjunction with quantitative transcriptomic and proteomic analyses, have been used to obtain accurate fluxomic information. This information has been used to determine the distribution of carbon and electron fluxes and to elucidate the different genes/enzymes involved in the primary metabolism, coupled with an improved understanding of the regulation of the primary metabolism under different physiological conditions. There are several recent reviews that provide a more in-depth overview of progress achieved in this area [24–26].

3.2. Developments in Process Technology and Downstream Product Recovery

Developing stable industrial strains that produce n-butanol at a high yield in a continuous culture will be a prerequisite, but it will not be sufficient for a viable commercial process as both the titres and the productivities are likely to remain too low. Using the in situ extraction of solvents and high-cell-density cultures to increase the titre, yield, and productivity of n-butanol production will also be required. To improve these parameters, several methods of butanol recovery, including gas stripping, vacuum fermentation, pervaporation, liquid–liquid extraction, perstraction, and adsorption, have been extensively investigated. However, high volumetric productivities or scaled-up industrial production will still be hard to achieve. There are a number of reviews that provide a more detailed

and comprehensive assessment of these technologies [27–29]. There have also been several strategies aimed at mitigating the limiting effect of butanol toxicity and the development of strains more tolerant to butanol [30–32]. These are discussed in later sections.

3.3. Progress in Butanol Production from Alternative Renewable Biomass

A large body of published information exists relating numerous initiatives undertaken on assessing the potential use of alternative raw materials as a substitute for maize or molasses as the primary substrate for butanol production. The search for alternative substrates has resulted in the investigation of a wide range of agricultural and food wastes as well as lignocellulosic hydrolysates. Hydrolysates produced from various sources of lignocellulosic biomass have been extensively investigated. Lignocellulosic feedstocks can be grouped into three types: agricultural residues, herbaceous and woody energy crops, and forest residues. The most extensively investigated of these potential raw materials include hydrolyzed maize cobs, maize stover, wheat and rice straw, peanut and oat hulls, and bagasse. A small proportion of hydrolysates of this type were successfully incorporated into the mash used in the commercial fermentation process operated in Russia and China [14,15,17,18].

4. Surviving Species and Strains of Industrial Solvent-Producing Clostridia

The ability to produce butanol appears to be almost exclusively confined to mesophilic, anaerobic, spore-forming species belonging to the genus *Clostridium* (*sensu stricto*). Several species of *Clostridium* are known to be able to produce butanol, but only the four known industrial species have been found to be capable of producing butanol with sufficiently high fermentation titres and yields to be commercially viable [2].

The first recognized species of solvent-producing *Clostridium* used for industrial production was *C. acetobutylicum*. This species was isolated and developed by Weizmann and patented in 1915 [33]. After WW1 the patent rights to the strain and process were acquired by CSC in the US.

During the period between WW1 and WW2, more than 20 new industrial strains of solvent-producing clostridia were patented by different companies and organizations [5,34]. Of these, more than a third were reported to produce iso-propanol in place of, or in addition to, acetone. None of these patented strains were recognized scientifically as legitimate species. As a result, it later became common practice to group most industrial strains that produced acetone together as variants of *C. acetobutylicum*, while those producing isopropanol were usually considered to be variants of *Clostridium beijerinckii* [10]. It was not until molecular genetic techniques became available for phylogenetic analysis that it was established that the existing industrial strains used for solvent production can be classified as belonging to four recognized species of *Clostridium* [35–37]. The original *C. acetobutylicum* strains used to produce solvents from maize and other starch-based substrates were found to belong to a separate phylogenetic clade. The other three saccharolytic species, *C. beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum*, used to produce solvents from molasses and other sugar-based substrates were found to be closely related and belong together in a separate distantly-related phylogenetic clade [2,38,39].

Although a significant number of industrial strains were patented and used commercially, very few of these strains have survived. It is largely due to the efforts of Elizabeth McCoy, a 20th century industrial bacteriologist in the US, that a number of these strains were maintained [2]. Through her close working relationship with CSC, McCoy isolated and patented the first successful industrial clostridial strains used to produce solvents from molasses. She also accumulated an extensive collection of more than 150 reference strains. Many of the key solvent-producing clostridial strains now held in international culture collections are originally sourced from her culture collection. A list of *C. acetobutylicum* and *C. beijerinckii* strains from her collection giving their origins is provided in Table 1. A second collection of industrial solvent-producing clostridia, referred to as the David Jones (DJ) culture collection, includes many of the industrial strains available from the international culture collections [2,38]. The collection also includes production strains

used for the commercial production of solvent by National Chemical Products (NCP) in South Africa [2,12]. These include examples of strains, some dating back to 1944, that were derived from spore stocks sent to NCP from both CSC in the US and Commercial Solvents Ltd. (CS) in the UK, as well as the later production strains propagated by NCP. More than 300 genome sequences are now available for the study of these key industrial clostridial species, sourced from both the international culture collections and the DJ culture collection which is now held by LanzaTech. Conversion tables for the DJ strains that were sequenced have been published recently [2,38].

Table 1. Strains of *C. acetobutylicum* and *C. beijerinckii* held by five international culture collections giving the original number from the Elizabeth McCoy A and B culture collection with the origin of each strain.

<i>Clostridium acetobutylicum</i> Species						
McCoy B	Strain Origin	ATCC	NRRL	NCIMB	DSM	JCM
B-3	Weizmann > CSC			6441	1733	
B-5	Weizmann > Speakman			6442		
B-10	Weizmann > Speakman			6443		
B-15	<i>C. acetonigenum</i> > Kluyver > Donker > Speakman	862 *	B-528			
B-16	Weyer Type strain	82	B-527	8052 **	792	19,013
B-27	<i>C. baconyi</i> > Castell	8529			1738	
B-28	Hall strain	3625	B-529		1737	
B-29	<i>B. butylicus</i> > Lister > Thaysen	4259	B-530	619	1731	19,012
-	Weizmann > Thaysen			2951	1732	
<i>Costridium beijerinckii</i> Species						
McCoy A	Strain Origin	ATCC	NRRL	NCIMB	DSM	JCM
A8	McCoy isolate > CSC		B-591			19,002
A13	McCoy isolate			6444		
A14	McCoy isolate	10,132	B-594	8049	1739	19,011
A14	McCoy isolate—ATCC 824T contaminant			8052		
A16	McCoy isolate > <i>C. madisonii</i>					
A21	<i>C. butylicum</i> > Beijerinck > Kluyver		B-593	9380	6423	
A38	<i>B. butylicus</i> —FB BB Fernbach > Andrewes		B-596			
A39	<i>B. fitz</i> > Pasteur Institute		B-592		6422	19,008
A39?	Donker > Reid 39-90		B-466			
A48	<i>B. bakoni</i> > Castell	8529				
A51	<i>C. multifermentans</i>	3538				
A65	<i>C. pasterianum</i> > Winogradski	861				
A67	<i>C. beijerinckii</i> —Kluyver	858		11,373	1820	1319
A67	<i>C. beijerinckii</i> > Kluyver > McCoy > McClung	25,752		9362	791	1390
A72	<i>C. amylobacter</i> > Haseehoff > Pribram		B-597			
A75	<i>B. saccharobutylicus</i> > von Kleeki > Pribram	6015				19,003
A77	<i>C. pasterianum</i> > Bezssonoff > Pribram		B-598			
A79	<i>C. butyricum</i> —Parazmonski > Pribram	6014				19,007

* The ATCC 862 strain is no longer listed. ** The NCIMB 8052 T strain was mis-labelled and is now classified as a *C. beijerinckii* strain.

Although the four species of *Clostridium* used for industrial solvent production differ phylogenetically, they share many similar characteristics. Industrial strains belonging to the *C. saccharobutylicum* and *C. beijerinckii* species in particular exhibit very similar phenotypic characteristics and are very difficult to distinguish. They are mainly differentiated by variations in the fermentation of sugars and differences in their solvent ratios, titres, and yields and in the percentage of fermentable sugar they can metabolize.

5. The Choice, Nature and Utilization of the Standard Industrial Fermentation Substrates

A key attribute of the various species and strains used for the industrial production of solvents is that they are all able to utilize a very wide range of carbohydrates as their energy

source. These include most naturally occurring mono- and di-saccharide hexose sugars and many pentose sugars, as well as starches and inulin, etc. Some species and strains also have the capability to degrade more complex carbohydrates such as hemicellulose. The different species and strains do, however, exhibit marked differences in these capabilities. The ability, performance, and effectiveness of the particular strains utilized with a particular raw material was a crucial factor in the technical and economic viability of the industrial process.

The cost of the raw material was by far the largest cost of operating commercial industrial fermentation. The choice of suitable raw materials was determined largely by cost, volume, and availability, but it was also impacted by factors like seasonality, transportation costs, tariffs, subsidies, etc. The industrial ABE fermentation process mainly used either maize or other starch-based crops or cane molasses or other sugar-based substrates. The initial fermentation substrates used for the industrial process were starch-based agricultural crops. Maize was the most used raw material, but wheat, rye, sorghum, millet, rice, potatoes, sweet potatoes, cassava, artichokes, and other starch crops were also used, along with various types of granular grain flour. Some strains only required the starch to be rendered soluble by cooking, while other strains required the hydrolysis of the starch. The increasing cost and the competition for their use as food or animal fodder made these starch-based substrates less attractive. In the 1930s, sugarcane molasses became widely used as either blackstrap or high-test molasses. Beet molasses was used mainly in the Eastern-bloc countries/regions. Citrus molasses, hydrol (maize molasses), whey, and sulphite waste liquor were also investigated as potential substrates.

5.1. The Industrial Process Using Maize and Other Starch-Based Raw Materials

Maize and most other grains were considered to contain all the nutrients needed. Biotin and p-amino benzoic acid were found to be essential growth factors for *C. acetobutylicum* but were present in the mash in sufficient amounts. The addition of either yeast stillage from ethanol fermentation or clostridial stillage from ABE fermentation to the maize mash was found to improve yields. Maize was first screened, and iron particles were removed before being ground by roller or hammer mills. In some processes, the grain was first treated to remove the germ and the maize oil extracted. When the germ was not removed, the maize oil could still be extracted after fermentation. The milled maize was usually diluted to 8–10%, which enabled the maize slurry to be pumped. Initially batch cooking processes were used, but these were superseded by continuous cooking processes. Batch cooking processes usually employed a bank of horizontal pressure vessels fitted with agitators or rakes. Maize mash was cooked at around 133 °C for 90 min under pressure and the pressure then allowed to blow down while cooling occurred in situ. In the continuous sterilization process, steam injection was used to raise the temperature to 121–126 °C and held at high temperature for 60 min before cooling through spiral pressure detention tanks or coiled piping. Both processes solubilized the starch and rendered the mash sterile. In some cases, the mash was further diluted with water after cooking.

Although the cost of maize as a raw material for the industrial fermentation process precluded its use in most countries/regions, it was the substrate of choice for the new fermentation process established by Green Biologics Ltd. (GBL) in the US that operated from 2015 to 2019 [2].

Recent economic assessments of butanol production from maize include using a hyper-butanol-producing strain of *Clostridium beijerinckii* BA101, with butanol produced in a batch reactor and recovered by distillation [40]. Another economic assessment includes a comparative analysis of the process design and economics of butanol production using corn grain and corn stover, utilizing the most recent technologies and assessing the impact of key parameters such as raw material pricing on the overall process economics. Cellulosic biomass conversion technologies are also compared [41].

5.2. The Industrial Process Using Cane Molasses and Other Sugar-Based Raw Materials

From the early 1930s, there was a shift away from the use of maize to sugar cane molasses in the USA, and from 1935 onwards, most of the fermentation plants in the West used cane molasses as the preferred substrate. Maize was, however, used again during WW2 through strategic necessity [2].

Attempts were made to ferment 50:50 maize and molasses mixtures using existing strains of *C. acetobutylicum* but resulted in poor solvent yields and abnormally long fermentation times. To overcome these problems, new strains were isolated and developed by CSC and other companies, which enabled the development of a viable fermentation process using molasses. Many of the early saccharolytic industrial clostridial strains used in the ABE fermentation process had limited ability or were unable to ferment sucrose. This required the molasses to first be inverted by heating it in the presence of acid to produce invert molasses, with the sucrose converted mainly to fructose and glucose [5,7].

The following information relating to sugar cane molasses has been accessed from a wide variety of sources including the articles referenced in the introduction relating to the industrial fermentation processes that were operated during the last century. The large amount of technical detail would make referencing challenging and unwieldy, so references for this section have been omitted.

Cane molasses generally has a higher sugar content than beet molasses. The sugar in cane molasses is about 2/3 sucrose and 1/3 inverted sugar, whereas the sugar in beet molasses is almost entirely sucrose, with the inverted sugar content never exceeding 1%. Beet molasses has a protein content of approximately 8–9%, which is much higher than cane molasses. Cane and beet molasses also vary in terms of their pH value and mineral and ash content. In addition to blackstrap molasses, high-test molasses was also often available. High-test molasses was produced when there was a glut of cane juice, and the juice was condensed directly into high-test molasses with a sugar content of around 75%. Cane juice was also used directly from the sugar mill in countries/regions where this was feasible.

Initially molasses mash was batch sterilized, but this was later replaced by continuous sterilization processes. The batch sterilization process used by various companies usually consisted of horizontal double pipe cookers fitted with a horizontal shaft fitted with rakes were used to cook the mash at 107–108 °C for one hour. There were usually four cookers needed to fill one fermenter. An example of a more continuous process: the mash was heated by steam injectors to a temperature of around 108 °C and maintained at this temperature for 60 min while being pumped under pressure through holding vessels followed by spiral cooling coils to provide a continuous mash feed for filling the fermenter at around 32–37 °C. Modifications of a continuous sterilization process developed and used by CSC were adopted by most companies. Steam injection is used to raise the temperature to 128–138 °C for 2–6 min before it is cooled to around 32–37 °C to provide a continuous mash feed for filling the fermenter.

The switch from maize to molasses provided several advantages. Molasses was usually significantly cheaper than grains, resulting in the fermentation process being more economical. Higher concentrations of sugar could be fermented with corresponding higher solvent yields, and fermentation could be completed in much shorter periods of time, resulting in increased productivity. The new strains developed for use on molasses gave more favourable solvent ratios with less ethanol. The increased ratio of butanol produced a more desirable product. The mash could be sterilized at lower temperatures and was also easier to handle. The fermentation tanks and pipework were also easier to clean, requiring only water and steam, and there was less blockage of stills. Fermentations were mostly run at a temperature of around 32 °C rather than 37 °C, which was a less favourable temperature for the development of common contaminants. When contamination did occur, the residual sugar could often be re-utilized by yeast for ethanol production.

5.3. Composition and Quality of Blackstrap Molasses

Blackstrap molasses is usually diluted with water to give a concentration between 5% and 7% fermentable sugars. Good quality blackstrap molasses consists of 75–85% of total solids. The sugar in molasses is made up primarily of sucrose and inverted sugar with 30–36% sucrose and 10–17% fructose and glucose. The term “total sugar” or “fermentable sugar” refers to the combined sucrose and inverted sugar content. There are smaller quantities of polysaccharides and gums that are mostly non-fermentable, along with 10–16% minerals and ash and a nitrogen content of 0.2–0.8%. There can be significant differences in the quality and composition of cane molasses. Quality can be affected by cane variety and yield, regional climate and soil type, cultivation and harvesting methods, and milling and sugar recovery processes. There are two main cane juice extraction methodologies used in the sugar industry: milling and diffusion technologies. Molasses sourced from the diffuser process was generally found to be more problematic as a fermentation substrate. With blackstrap molasses used for fermentation processes prior to WW2, the total sugars could be as high as 58%, and molasses with total sugar of less than 50% was deemed of poor quality. Following WW2, the improvement in extraction technology resulted in a much poorer and more variable quality of molasses. Currently molasses with a high sugar content can still sometimes be obtained, but the usual total sugar concentration is normally between 40–50%, with a much higher ash content. With a water content of 15–25%, molasses is not susceptible to microbial decomposition and can usually be stored for at least a year without deterioration. Cane molasses is usually traded in its original state with a guaranteed minimum content of water-soluble sugars, crude protein, and ash. Generally, the price of molasses is determined by its total sugar content. First-grade molasses has 50% or above fermentable sugars and a maximum of 14% ash. Second-grade and third-grade molasses contain a minimum of 44% and 40% sugar content, respectively, with a maximum of 18% ash.

Essential nutrients are not present in sufficient amounts in blackstrap molasses. It is low in nitrogen and phosphorus and requires the addition of a nitrogen source in the form of inorganic or organic nitrogen, often along with an additional source of phosphate. Inorganic nitrogen was generally provided in the form of ammonia or ammonium and nitrate salts. Organic nitrogen was provided by the addition of a variety of ingredients. The provision of a source of organic nitrogen and other organic growth factors is essential for some strains for achieving good solvent yields, concentrations, and productivity. There is general evidence that the provision of organic supplements enhances the performance of most industrial saccharolytic production strains. The practice of adding distillation slop from either ABE fermentation or ethanol fermentation to replace up to 50% of the make-up water was commonly used and provided many beneficial effects. The higher cost of including organic supplements coupled with the difficulty of accessing a suitable cheap and reliable source of organic supplement led to the acceptance of lower than optimal solvent concentrations, yields, and productivity.

6. Key Factors Affecting Performance in the Batch Industrial Fermentation Process

The standard industrial fermentation for the production of biobutanol was typically operated as a batch process with only limited modifications, and the solvents were purified by distillation. Later, semi-continuous industrial cascade fermentation processes were developed and used in both Russia and China. There are a number of key features and attributes of the industrial species and strains that determined the technical and financial viability of the particular industrial process that was used. The articles referenced in the introduction relating to the industrial fermentation processes that were operated during the last century provide background information underpinning this analysis.

6.1. Biphasic and Monophasic Batch Fermentations

Historically, industrial ABE fermentation was typically operated as a batch process and with strains belonging to both the *C. acetobutylicum* and *C. beijerinckii* and *C. saccharobutylicum* species. The fermentation occurs in two stages. During the initial acidogenesis

phase, the inoculum grows exponentially, and sugar is converted into mainly acetic and butyric acid along with CO₂ and H₂, resulting in a decrease in pH. This ensures the maximum production of ATP that supports rapid exponential growth. Towards the end of acidogenesis, the rate of H₂ production falls as the cells shift metabolic activity from acidogenesis to solventogenesis in response to the decrease in pH. The concentration of the undissociated butyric acid triggers the phase change from acidogenic to solventogenic, and the phase shift is accompanied by the cessation of growth and the initiation of sporulation. ATP production is diminished and normal binary cell division inhibited, and forespore septation occurs at one and occasionally both poles of the cell. Cell motility is usually maximal towards the end of exponential growth and is usually followed by the accumulation of granulose that results in the formation of swollen clostridial forms [42]. Solvent production, granulose production, and sporulation are interlinked under the control of the *Spo0A* gene. In the second phase, acetate and butyrate act as co-substrates for the synthesis of butanol, acetone, and ethanol. Most of the acids are consumed during the solventogenic phase, but some acids usually remain in the final fermentation product. At the end of the solventogenic phase, the concentration of butanol normally reaches a level which inhibits bacterial metabolism and the formation of mature spores. The fermentation process can take between 30 and 80 h to reach completion, depending on the temperature, the strain, and the process used. The time involved in the operation of the batch process is extended by the need for the additional time-consuming steps of transfer for the distillation, cleaning, sterilization, refilling, and re-inoculation of the bioreactor. With anaerobic fermentations, the size of the fermenter unit is not limited by oxygen or heat transfer rates, so banks of very-large-volume unstirred bioreactors can be used for production.

More recently it has been reported that the *C. saccharoperbutylacetonicum* species industrial strain N1-4 exhibits a more monophasic fermentation profile rather than the typical biphasic batch fermentation. This made this species the choice for the patented continuous industrial process developed by GBL that the company established and operated in the US [19,43,44]. Research that was carried out by GBL in the UK on the N1-4 strain shows that it exhibits a different fermentation profile [44,45]. The rate of growth of this strain is somewhat slower, and it takes longer for the batch fermentation to reach stationary phase. The cell size is also much smaller than that of the other two saccharolytic species. Instead, after a relatively short period of acetate and butyrate production during the first 10 h, the metabolism switches to using the less efficient ATP-generating butanol pathway. This allows the monophasic fermentation metabolism to continue until the sugar runs out or a butanol level is reached that results in the cessation of exponential growth through toxicity. This indicates a less efficient generation of ATP due to a reduced ability to get rid of excess reducing power as hydrogen. The limited information regarding cell morphology indicates that the cells are motile during exponential growth and that granulose is not produced. Cell degeneration was observed in a batch culture during the stationary and death phases. The N1-4 strain is known to be difficult to induce sporulation in, and spore formation normally only occurs in specialised culture medium. This alternative metabolic strategy results in higher ratios, yields, and concentrations of butanol.

6.2. Endospore Formation and the Use of Spore Stocks for Maintaining Industrial Strains

A key feature of the solvent-producing clostridial species is the ability to readily produce deeply dormant and highly resistant endospores. When propagated in the laboratory under optimal conditions, more than 80% of the vegetative cells can produce endospores. When grown under suboptimal conditions, the percentage of cells producing viable endospores can be much lower, and a large portion of the vegetative cells degenerate or lyse. With insufficient buffering, an acid crash can occur, wherein the entire population of cells are killed at low pH and no spores are produced. In full-scale industrial fermentations the high concentration of butanol produced inhibits the sporulation process. At the end of the solventogenic phase, the concentration of butanol normally reaches a level which inhibits bacterial metabolism and the formation of mature spores.

Solvent-producing clostridia are relatively easy to isolate and were most commonly derived from plants, roots, or soil rather than decaying plant material [5,7]. Promising strains were isolated and purified from single colonies and were then normally propagated and maintained as spore stocks on sterile sand, soil, silica gel, etc. Each industrial batch fermentation was started from a heat-shocked spore stock. New spore stocks continued to be propagated from fermentations that produced the best results. Dormant spores normally require a heat shock to become activated. Heat shocking also eliminates less-resistant spores but is insufficient to activate deeply dormant spores. Spore stocks can remain viable from many decades, but over time the number of viable spores shows a steady decrease. Newly propagated working spore stocks were not routinely checked for purity, and as a result many of these spore stocks became contaminated by mixtures of variants and strains. An example of this was the contamination of the NCIMB *C. acetobutylicum* Type strain with the NCIMB 8520 *C. beijerinckii* strain. Many of the spore stocks that CSC provided to NCP in South Africa during 1944–45 were not pure. Some or most of these spore stocks were not only mixtures of strain variants but were mixtures of two species, *C. saccharobutylicum* and *C. beijerinckii*. These two species were compatible, and it appears that it was somewhat random as to which species came to predominate in a particular fermentation. This was unknown at the time. This means that the historic NCP strain designations cannot be depended upon, making the tracing of the lineages and phylogenetic relationships of the surviving NCP strains complex and unreliable [2]. This issue is explored further in the section dealing with the different species.

6.3. Solvent Ratios and Productivity

Some saccharolytic strains only produce acetone, while other strains produce isopropanol or a combination of acetone and isopropanol. Most of the industrial strains that were commonly used produced acetone, although industrial strains that produced isopropanol were also utilized by some companies. The ratio of the solvents produced by the different industrial species and strains is largely determined by the genotype of the strain. The *C. acetobutylicum* industrial fermentation on maize were usually maintained at around 37 °C and gave a ratio of 60% butanol, 30% acetone, and 10% ethanol. With individual strains of saccharolytic species, the ratio of solvents produced can exhibit considerable variation. The various factors that influenced the ratios of the solvent produced included the fermenter volume and the inoculum size and ratio.

The temperature at which the full-scale fermentation is maintained has been shown to have the greatest effect on both solvent ratio and solvent productivity. The upper temperature for growth for most strains is around 40 °C, with an optimum temperature for growth usually above 35 °C. The optimum temperature for solvent production that gives the highest percentage of butanol is much lower. For the majority of saccharolytic strains, this is usually 28–33 °C. Consequently, the growth rate is diminished, and the time taken for the completion of the batch fermentation is increased, so solvent productivity is decreased. Increasing the fermentation temperature by a few degrees can shorten the fermentation time by as much as a day but results in a significant decrease in the percentage of butanol produced. The set sugar concentration, nutrient composition, pH regulation, and gas pressure can also all influence the final solvent ratio. Key factors influencing high butanol production are dependent on the optimum temperature for growth and for solvent production on the particular strain being utilized. The properties of the different industrial species and strains are discussed later.

6.4. Solvent Yields

The conversion of fermentable sugars to solvents is also to some extent determined by the genotype of the species and strain that is used. However, the variable parameters and other factors defining the features of a particular industrial process have a much larger influence. The actual true yield obtained can be difficult to determine with accuracy with full-scale industrial fermentations. With starch-based fermentations, the quantity and

quality of the starch in the wet weight or dry weight of the raw material can vary widely. This can influence the efficiency of the conversion of starch and other carbohydrates, particularly those that may be only poorly fermented. The same applies to accurately determining the precise amount of fermentable sugars in molasses. Secondly, the nature and type of cooking and sterilization of the substrate can also have a major influence due to the degradation of sugars. The amount of cell biomass produced, along with H₂ and CO₂ generated and acids and solvents produced, can vary quite widely and affect solvent yield. Acids and residual sugar remaining at the end of the fermentation can also have a significant effect in reducing solvent yields.

Theoretical maximum yields can be calculated, but these can be contentious due to the complexity of the biochemical pathways involved. Theoretical yields can be compared with the maximum yields that have been obtained in optimized laboratory-scale experiments. These values provide a good guide to the maximum, standard, and minimum acceptable solvent yields for a full-scale industrial fermentation. A solvent yield of 28% is usually considered a minimum yield for an economically viable industrial batch process. Yields of 33% or higher can be obtained with full-scale batch fermentation but can be challenging to achieve routinely. The yields for the different industrial species and strains are considered later.

6.5. Solvent Titres and Butanol Toxicity

The limited tolerance to butanol is a key factor in determining the final solvent concentration that can be obtained in a standard batch fermentation. The highest final concentration of butanol that can be tolerated by most of the industrial species and strains is around 13 g/L [11]. Under optimal conditions a few naturally occurring strains may produce butanol concentrations of around 15 g/L. The constraint imposed by butanol toxicity is discussed in more detail in the later sections dealing with the four species.

The butanol tolerance of the strain used, coupled with the ratio and yield of solvents it produces, therefore limits the initial concentration of set sugars that is used for a full-scale industrial fermentation. As an example, a strain that produces 65% butanol with a yield of 33% will produce the maximum concentration of solvents with 6% fermentable sugar. Using a higher percentage of sugar results in unused sugar remaining at the end of the fermentation. The relationship between sugar concentration and the yield, ratio, and concentration of total solvents and butanol produced by various strains is illustrated in Table 2.

Table 2. Relationship between sugar concentration and the yield, ratio, and concentration of total solvents and butanol produced by strains giving different solvent ratios.

(A)	Yield	28%	28%	30%	30%	33%	33%
Strain Ratios	Sugar	Solvents	Butanol	Solvents	Butanol	Solvents	Butanol
60:30:10	5.0%	14.0	8.4	15.0	9.0	16.0	9.6
65:32:3	5.0%	14.0	9.1	15.0	9.75	16.0	10.4
70:27:3	5.0%	14.0	9.4	15.0	10.5	16.0	11.2
75:22:3	5.0%	14.0	10.5	15.0	11.25	16.5	12.38
80:16:4	5.0%	14.0	11.3	15.0	12.0	16.5	13.2
(B)	Yield	28%	28%	30%	30%	33%	33%
Strain Ratios	Sugar	Solvents	Butanol	Solvent	Butanol	Solvents	Butanol
60:30:10	6.5%	18.2	10.92	19.5	11.7	21.45	12.87
65:32:3	6.5%	18.2	11.83	19.5	12.68	21.45	13.94
70:27:3	6.5%	18.2	12.47	19.5	13.65	21.45	15.02
75:22:3	6.5%	18.2	13.65	19.5	14.63	21.45	16.09
80:16:4	6.5%	18.2	14.56	19.5	15.6	21.45	17.16

The solvent concentration is a key aspect affecting the economics of industrial ABE fermentation. The limitation of low titres of butanol in the fermentation broth results in high product recovery costs. Any improvements in butanol tolerance can therefore potentially have far-reaching effects for the economics of the industrial fermentation process.

Butanol toxicity is largely caused by its hydrophobic nature, which causes an increase in the fluidity of the cell membrane. This affects the function of the cell membrane as the regulatory barrier between cell interior and exterior; the trans-membrane pH gradient is also destroyed, resulting in energy limitation. A common approach has been to use various strategies to isolate or generate mutants or genetically modified butanol-tolerant strains. Serial transfer and adaptation to high concentrations of butanol have been used in a number of studies. Other strategies have included random mutagenesis as well as targeted genetic modifications such as the overexpression of genes encoding heat-shock proteins, the modification of fatty acid synthesis, etc. In some studies, the strains that exhibited higher butanol tolerance and improved survival in the presence of butanol also exhibited higher butanol production. However, other studies reported that increased butanol tolerance did not result in an increase in butanol production. These findings suggest that there could be a strain-specific connection between butanol tolerance and production in some instances, while in other cases, no direct connection exists between the two mechanisms [30–32].

Various process technology approaches and techniques for the continuous removal of butanol have been widely investigated. These include cell recycling coupled with membrane filtration, liquid–liquid extraction, gas stripping, adsorption, reverse osmosis, perstraction, pervaporation, and cell immobilization. These approaches have led to significant improvements in fermentation process performance and have been extensively reviewed, for example [27–29].

Table 2 For full scale industrial ABE fermentations the total solvent yield from sugar normally ranged between about 28% and 33%. With different clostridial species and strains the ratio of butanol that is produced can range from around 60% to over 80%. Where the starting set sugar is known the expected total solvent titres and the butanol titres can be calculated. Table 2A gives the calculated solvent and butanol titres for five different strains that give butanol ratios ranging from 60–80% where the starting fixed sugar was 5%. Table 2B gives the calculated solvent and butanol titres for five same strains where the starting fixed sugar was 6.5%. For most species and strains solvent concentrations of above about 13 g/L of butanol becomes toxic and inhibits further solvent production. The calculated levels at which butanol is likely to become toxic are highlighted in red. The set sugar concentrations used for molasses varied from around 5% to up to 7%. With strains that produce a high ratio and yield butanol, the percentage of set sugar used was limited to around 5%, whereas for strains that produced around 60% butanol, set sugars of up to 7% could be used.

7. Key Features of the Four Industrial Species

7.1. The *C. acetobutylicum* Industrial Strains

The original industrial strain of *C. acetobutylicum* that was isolated and patented by Weizmann was used extensively in the UK, Canada, and the US for acetone production during WW1. After the war, CSC in the US obtained the patent and process rights and used the Weizmann strain for the commercial production of solvents until the beginning of the 1930s. After the patent lapsed, the Weizmann strain became widely available. Most of the scientific research that has been carried out on *C. acetobutylicum* has not been conducted using the Weizmann strain but has been carried out on the Type strain (ATCC 824). This strain was isolated in the US and was not used in the industrial fermentation process. Fortunately, the Type strain and the original Weizmann industrial strain are very similar regarding their performance for solvent production. There are a number of early publications that provide a general account of the industrial process using maize [3,4]. The optimised performance characteristics for the full-scale industrial process can be summarized as follows. Industrial fermentation is normally run at around 37 °C and

usually takes around 50 h for completion, but it can take up to 60 h. The starting pH is normally set around 6.0–6.5 with a final pH of 4.2–4.5. A fermentation using 9% maize mash gives an optimum solvent concentration of around 20 g/L with a butanol–acetone–ethanol ratio of 6:3:1. The fermentation gives solvent yields of around 25% on a dry grain basis and 33–34% on a total starch basis. The final butanol concentration is usually around 12 g/L. In practice industrial fermentations giving optimum performance were challenging to achieve over a prolonged period.

There are a number of different strains of *C. acetobutylicum* available from international culture collections. Strains were isolated in Europe, Britain, North America, South America, Japan, and China over a period of more than a hundred years. Currently there are 14 genome sequences published for *C. acetobutylicum*, including variants of the original Weizmann strain. Analysis revealed that all these strains are very closely related, with ANI values of near 100%. An exception is the GXAS18_1 strain isolated in China. The remarkable feature is that the genomes of all these disparate strains are so similar. This indicates that the genomes of this species are remarkably stable and highly conserved and are probably very well adapted to the environmental niche they occupy [2].

Clostridium acetobutylicum was the first solvent-producing clostridial species to have its full genome sequenced [46]. The *C. acetobutylicum* strains belong to a different phylogenetic clade to that of the saccharolytic solvent-producing clostridia. The species is characterized by a common type I *sol* operon organization (gene order *adhE*–*ctfA*–*ctfB*), with a separate *adc* operon located adjacent and being transcribed convergently. A *pdh* gene encoding pyruvate decarboxylase is present in these species, and *rnf* genes involved in the generation of an additional ion gradient from reduced ferredoxin are absent. Another major difference is that in the strains that have been investigated, the *sol* operon is located on a mega-plasmid, whereas in the other species it is located on the chromosome [46].

Promising advances have been made in the development of metabolically and genetically modified strains and improvements in process technology for the ATCC 824 strain. Examples include research carried out in Toulouse by the research group headed by Professor Soucallie [47–50]. There are also numerous other research groups that have made valuable contributions in this area. More recently there have been several publications reporting studies undertaken in China on new *C. acetobutylicum* isolates used mainly for solvent production from a range of starch-based raw material including cassava.

7.2. The Industrial Saccharolytic Solvent-Producing Species

The three species of saccharolytic clostridia were isolated and developed for use on molasses and other sugar-based raw material. They include industrial strains belonging to *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [2]. The three species are closely related phylogenetically and belong together in a separate phylogenetic clade. The members of the species in this clade encode the *sol* operon in the gene order *ald*–*ctfA*–*ctfB*–*adc* and lack a *pdh* gene [38]. As a general observation, all three species appear to perform more effectively on plant-based substrates such as molasses than they do on maize or laboratory media with glucose and/or sucrose as the sole energy source. This seems to be more marked when there is also organic nitrogen and phosphorus present.

7.3. The Diversity within *C. beijerinckii* Species

There are now over 200 genome sequences for *C. beijerinckii* species in GenBank, making this species of solvent-producing clostridia by far the largest and most diverse group. The initial molecular genetic studies based on 16S rRNA gene sequencing and DNA fingerprinting indicated that a number of subgroups of *C. beijerinckii* could be identified [36]. The comparative genomic analysis of solventogenic clostridia published in 2017 included the genome sequences for 18 *C. beijerinckii* strains [39]. The phylogenetic analysis also identified four phylogenetic clusters or subgroups within the species.

A joint LanzaTech–JGI project has added another 270 genomes from the DJ culture collection. Of these, 72% were *C. beijerinckii* genomes, consisting of 160 genomes from the

NCP industrial strain collection and 34 genomes of strains originating from the international culture collection [2,38]. The phylogenetic analysis from the expanded number of genome sequences confirmed that the *C. beijerinckii* species consists of four distinct subgroups that possibly can be considered as subspecies. These studies also confirmed that strains previously classified as *Clostridium dolis* fall within the species and that the *C. dolis* species name is invalid [39]. More recently a fifth sub-group was isolated in China from pit mud [51].

More recently there have been several reports on the diversity and evolution of the *C. beijerinckii* species, using the core genome to reconstruct the phylogeny of the species [2,38,51,52] and confirming the separation into four groups. Strains belonging to Group 1 constitute the most phylogenetically distinct and diverse cluster and contain a number of the strains that are recognized as iso-propanol producers. These include a few strains that were used for industrial solvent production, but data on these strains are limited. Strains belonging to Group 3 constitute a quite diverse assemblage that include the Type strain for *C. beijerinckii*. Most of the Group 3 strains were initially isolated from sources related to food spoilage or other public health problems and are generally quite weak solvent producers. One exception is the “*Clostridium butanolgenum*” strain that belongs in Group 3 that was isolated and used in Japan for solvent production. Most of the recognized and well-characterised industrial strains used for solvent production belong to *C. beijerinckii* Groups 2 and 4 [2].

7.4. The *C. beijerinckii* Group 2 Industrial Strains

There are currently 18 genome sequences for *C. beijerinckii* Group 2 strains. Of these, 15 were derived from three original strains isolated in the US by McCoy in 1926. Of the 22 strains that she isolated, the A-8, A-14, and A-16 strains were found to be capable of producing economically viable concentrations of solvents from molasses. It might be anticipated that the phylogenetic tree for the 15 McCoy-derived Group 2 strains would reflect these three lineages. This does not appear to be the case. The reason could be that the genomes of the McCoy strains are all very similar and different analytical tools generate somewhat different phylogenetic trees but are not capable of making fine-grained distinctions below the species level. Alternatively, some of the early McCoy strains may have become mixed up or mislabelled [2].

The A-8 strain was patented as *Clostridium saccharo-acetobutylicum* and was used by both CSC in the US and CS-GB in the UK as the first industrial strain for producing solvents from molasses. Patents for three variants, *C. saccharo-acetobutylicum* alpha, beta, and gamma, were applied for in 1933, 1934, and 1936 and issued in 1936, 1937, and 1938 [53–55]. The alpha strain was the McCoy A-8 (Number 8) strain. The beta and gamma variants were apparently separate isolates developed by CSC. This group of industrial strains are reported as being capable of producing solvent yields of around 30% from a sugar mash of 6% at an optimum temperature of 29–30 °C with fermentation times of around 72–74 h. Various examples of the McCoy A-14 strain were lodged in several international culture collections. The NCIMB 8052 strain was for a time misclassified as the *C. acetobutylicum* Type strain, resulting in a great deal of confusion. The NCIMB 8052 strain has been the most extensively studied and was the first *C. beijerinckii* strain to have its genome sequenced. In 2002, Blaschek et.al. patented a method of producing butanol using a mutant strain of *C. beijerinckii* NCIMB 8052 [56] designated BA-101. This patent reports the mutant strain as being capable of producing 18 to 21 g/L of butanol with butanol yields of 30–45% and total solvent yields from 45% to 60% from 6% glucose or starch. This mutant strain was lodged with the ATCC (ATCC 35702, strain designation SA-1). The BA-101 strain has been used extensively in the US to investigate the potential to produce solvents from a wide variety of agricultural crops and wastes using different process technologies for continuous culture and solvent extraction [22,40,57].

In 1940 McCoy applied for a patent for the A-16 strain as *Clostridium madisonii* that was granted in 1946 [58]. This strain was used under licence in Puerto Rico for the industrial

production of solvents but proved susceptible to phage infections [2,59]. The data provided in the 1946 patent indicate that this strain was capable of producing 15–16 g/L of solvents with a yield of around 29% from carbohydrate mashes of 5–6%, giving a solvent ratio of 76:20:4 butanol, acetone, ethanol. The fermentations were run at around 31 °C and took between 54–60 h to reach completion.

Another patented industrial strain belonging to Group 2 is the IFP 903 strain, along with a butanol-resistant mutant IFP 904 developed from IFP 903 [60]. At the beginning of the 1980s, France embarked on a national strategic biofuel program that included the establishment of the biobutanol fermentation process. The two agricultural crops identified as the most suitable for use in France were Jerusalem artichoke and sugar beet [2]. Research was undertaken at the Institut Français du Pétrole, and the IFP 903 strain (ATCC 39057) was isolated and used for fermenting Jerusalem artichoke liquor, giving a total solvent concentration of around 15 g/L. Further research was undertaken to improve the performance of this strain, which led to the selection of a number of mutant strains including IFP 904 (ATCC 39058), which produced 25 g/L solvents consisting of 15 g/L of butanol and 10 g/L of acetone at 34 °C after 36 h. Both the IFP 903 and 904 strains were patented as *C. acetobutylicum* but were subsequently classified as *C. beijerinckii*. The A-73 strain from the McCoy culture collection isolated in Europe in the late 1800s as *Clostridium amylobacter* has also been shown to be a member of *C. beijerinckii* Group 2.

7.5. The *C. beijerinckii* Group 4 Industrial Strains

The last industrial saccharolytic clostridial strain that was isolated and used by CSC was patented by Muller in 1940 under the name of *Clostridium granulobacter acetobutylicum* [61]. This new isolate was described as a high solvent producing strain on molasses that could give solvent concentrations of more than 23 g/L with yields above 32%. However, this strain requires a source of organic nitrogen and possibly organic phosphorus and other growth factors to achieve this high level of performance. This requirement possibly limited the use of this strain by CSC. It is not clear if the patent was for a single isolate or one of several isolates. The surviving industrial strains generated from the Muller strain were subsequently found to belong in the *C. beijerinckii* Group 4. It was later discovered that many of the *C. saccharobutylicum* BAS spore stocks that were supplied to NCP during 1944 and 1945 were mixtures of both *C. saccharobutylicum* and the *C. beijerinckii* Group 4 strains. The descendants of these CSC NCP Group 4 lineages were propagated and used by NCP as production strains. A second collection of Group 4 strains was obtained from Professor Hongo and are now part of the DJ culture collection. It is thought that these strains were isolated and characterized as part a screening program that was undertaken in Japan in a study of lysogeny and bacteriocin production in strains of *Clostridium* species [62]. What is remarkable is that the Japanese strains are all virtually indistinguishable genetically from the lineage derived from the CSC Group 4 strains. Unfortunately, due to their almost identical phenotypic characteristics, the existence of the second *C. beijerinckii* species of industrial solvent-producing clostridia was not known in the 1980s at the time the fermentation process was in operation and research was undertaken both at NCP and UCT. This makes some of the research undertaken at this time ambiguous and difficult to assess. After many of the NCP production strains were found to belong to *C. beijerinckii* Group 4, laboratory studies were undertaken at the University of Otago [63,64]. These showed very similar fermentation profiles to those described in the Muller patent and were very similar to the CSC and NCP *C. saccharobutylicum* production strains reviewed in the next section. During these investigations, one strain stood out as giving an exceptional performance on molasses medium supplemented with yeast extract. The P260 strain average solvent concentrations were around 23 g/L with solvent yields of around 32–33% on supplemented molasses medium and solvent ratios of 62% butanol, 35% acetone, and 3% ethanol and butanol titres above 14 g/L. The increased butanol tolerance of this strain appears to account for its high level of butanol when grown on molasses medium with organic supplements. With only inorganic supplements the P260 strain only produces around 17 g/L solvents.

The P260 strain was made available to Dr Nasib Quereshi at the US Department of Agriculture and has been used extensively by him and his group to investigate the possibility of producing solvents from a wide variety of agricultural crops and wastes, using different process technologies for continuous culture and solvent extraction. Similar results were obtained with P260, where high levels of solvent production were only obtained with substrates containing organic nitrogen and supplements such as food wastes and yellow top cake [65–68]. Laboratory studies undertaken on the NCP strains by GBL and the University of Cape Town [69] on the NCP *C. beijerinckii* Group 4 strains reinforced the observations that the choice of culture media, sugars, nutrient supplements, culture procedures, and fermentation temperatures gave significant variations in performance. Of the limited number of NCP strains tested in studies undertaken by GBL, the Group 4 strains that gave the best performance on molasses were obtained from the BAS/B2 spore stocks provided by CSC in 1944. Based on the various studies undertaken on the P260 strain, under optimum conditions this strain is capable of producing butanol titres in excess of 14 g/L.

As part of the joint LanzaTech–JGI genome sequencing project [2,38], the genomes of 160 of the NCP *C. beijerinckii* strains from the DJ culture collection were sequenced. Although these production strains had been sub-cultured numerous times since they were first isolated in the 1930s, the genomes were all very similar, indicating that the genomes are remarkably stable. These genomes are closely related, and the phylogenetic analysis methodologies used were not precise enough to determine the phylogenetic relationships of these genomes with certainty. In addition, the historic information and coding system used as part of the propagation of the NCP production strains does not show a good correlation with the phylogenetic analysis [2]. However, at least three slightly different genome profiles of subgroups of these strains can be identified. The majority of the genomes are around 6.14 kb with a GC content of 29.9%, but two clusters, one with a genome size of around 6.18 kb and a GC ratio around 30% and a second with a genome size of around 6.01 kb and a GC ratio of around 29.6%, can be identified. Phylogenetic analysis indicates that the bulk of the Group 4 strains were probably derived from a single ancestral isolate, but the outlying genome clusters could indicate more than one strain lineage exists.

Currently no examples of *C. beijerinckii* Group 4 industrial strains are available for use in the public domain. Arrangements are in progress to address this.

7.6. The *C. saccharobutylicum* Industrial Strains

The existing *C. saccharobutylicum* industrial strains are all derived from the original production strains isolated and developed by CSC for use on molasses [2,9]. In 1936 CSC filed the first US patent for a new industrial strain under the name of *Clostridium saccharo-butyl-aceticum-liquefaciens* [70]. This patent, granted in 1938, was followed by additional patents for two variations of this new species designated as the delta and gamma variants [71,72]. Derivatives of the original isolate and the delta and gamma variants were denoted as A, B, and C strains [9]. The A and C strains were recorded as exhibiting very similar fermentation performance profiles. They were both able to ferment molasses mashes with sugar concentrations of 7% to give total solvents of around 22 g/L with a solvent yield of 32% and above. The solvent ratio for the A strain was around 58% butanol, 38% acetone, and 4% ethanol with butanol titres of close to 13 g/L. The solvent ratio for the B strains was around 61% butanol, 35% acetone, and 4% ethanol with butanol titres also close to 13 g/L. Strains derived from the delta variants were denoted as delta B strains, delta F strains, and delta G strains. The delta variants were characterized by much higher ratios of butanol that varied from around 74–76% with acetone ratios of around 22.5–24% and ethanol ratios around 3–3.5%. The delta strains were also capable of giving total solvent yields of about 22%. Maximum solvent titres were also around 13 g/L, which meant that the set fermentable sugar in the mash was controlled to around 5.5% (Table 3). These new industrial strains were the main strains used by CSC for solvent production from molasses [7,8].

Table 3. Comparative fermentation profiles for the 3 variants of the *C. saccharobutylicum* species.

Source	Strains	Sugar %	Solvents g/L	Yield %	Butanol g/L	Butanol %	Acetone %	Ethanol %
CSC data	A strain	7	22	32	13.2	58	38	4
CSC data	Gamma C	7	21	30	12.8	61	35	4
CSC data	Delta B	5.5	17.5	32	13	74	23	3
CSC data	Delta F	5.5	17.5	32	13	75	22	3
CSC data	Delta G	5.5	18	30	13	74	23	3
CSC plant	A & Gamma	6	20	33	13	60	36	4
CSC plant	Delta B, F, G	5.5	18	33	13	74	23	3
CS-GB plant	Gamma or A	6	20	33	13.1	65	30	5
NCP plant	Delta B+F	6	18	31	12	65	32	3
NCP lab	BAS+P strains	6.5	20	31	13	56	32	3
UCT lab	P262	6	20	33	13	65.5	31	3.5
U Otago lab	BAS/B3	6	18.5	31	12	65	32.5	3
U Otago lab	BAS/SW	6	19.5	32.5	12.7	65	32	3
U Otago lab	37/3 UK	6	18	30	11.6	65	32	3
U Otago lab	162/B1 UK	6	18.9	32	12.4	66	31	3
U Otago lab	P108	6	18	30	13	72	25	3
U Otago lab	P200	6	14	23	10	61	36	3
U Otago lab	P258	6	13	22	9	70	26	4
U Otago lab	P262	6	16.6	28	10.5	71	26	3
U Otago lab	P272	6	14	23	9.4	67	27	6
GBL lab	Best strains	6	17.5	29	11	62	35	3

The original batch of CSC production spore stocks that was sent to NCP in 1944 were denoted as A strains, but, although these were used, they were never propagated by NCP. All subsequent strains provided by CSC were denoted as B strains and carried the strain codes BAS-B, BAS-B/F, BAS-2, BAS-B3, etc., indicating that they were all derivatives of the B delta strains. Many of the original spore stocks that came from the US were later found to be mixtures of *C. saccharobutylicum* and *C. beijerinckii* Group 4 strains as well as mixtures of the different delta B group strains [2,12]. This has made the tracing of the lineages and phylogenetic relationship of the NCP production strains virtually impossible, with strains bearing the same code and number often exhibiting different characteristics and genotypes [2]. In addition to the *C. saccharobutylicum* strains supplied to NCP by CSC, there are also two strains that were supplied by Commercial Solvents (CS) in the UK around 1950. These are the 37/3 immunized and 162/B1 strains. From the data in two later publications [10,11] that describe the industrial fermentation process run by CS in the UK, it is apparent that CS used derivatives of the original CSC gamma or A strain in their industrial process, giving total solvent concentration of around 20 g/L. Correspondence between CS and NCP reported the 37/3 immunized strain as a very high solvent-producing strain that had been developed by CSC in the US. The taxonomic studies performed at the University of Otago confirmed that both these strains and the NCP P249 strain derived from the 162/B1 strain were pure culture of *C. saccharobutylicum*. Phylogenetic studies undertaken on the genomes of the 162/B1 and NCP P249 strains confirmed that these strains stand out as being very different to the other NCP *C. saccharobutylicum* B strains, indicating that they are likely to be gamma strains. Unfortunately, a number of strains from the DJ culture collection that LanzaTech supplied to the JGI for genome sequencing were misclassified or mislabelled. The 37/3 strain was amongst these, and the 37/3 genome sequence that has been published is a *C. beijerinckii* Group 4 genome.

The *C. saccharobutylicum* strains were extensively used for solvent production in the US, UK, and South Africa. One difference was that the industrial fermentations in the US and UK were run at around 30 °C and took 45 h or longer to reach completion. The NCP full-scale industrial process did not have the technology required to control the fermentation temperature. The molasses mash entered the fermenters at around 34 °C, and the fermentation normally took around 30–33 h to reach completion [12,13]. Consequently, the fermentations were run at higher temperatures, and the ratio of butanol produced in the NCP process

tended to be lower. These ratios ranged quite widely but averaged around 60–67% butanol, 30–38% acetone, and 3% ethanol, with butanol titres of around 12 g/L (Table 3). Originally the solvent yields from a molasses mash with 6% set sugars was around 30–32%. With a constant deterioration in the quality of molasses in the decades following WW2, the calculated set sugars had to be increased to around 6.5%, and the average solvent yields declined to below 30% [12]. Laboratory studies were undertaken on the NCP strains by the Research and Development Division at NCP and at the University of Cape Town in the 1980s. Further laboratory-based studies were later undertaken at the University of Otago [63,64]. More recently, additional studies were carried out at the University of Cape Town [69] and by GBL in the UK (Table 3). Overall, these gave similar fermentation profiles and performance, but the choice of culture media, sugars, nutrient supplements, culture procedures, and fermentation temperatures used gave significant variations in performance. Unpublished studies undertaken by GBL in the UK on selected *C. saccharobutylicum* NCP strains on molasses medium also gave similar but slightly lower fermentation performance data to those reported in the patents [Private communication 2014].

Strains were isolated and patented by CSC and used for the industrial producing of solvents from molasses by CSC in the US, CS in the UK, and NCP in South Africa. The results are rounded off averages illustrating the optimum performance on molasses-based media. These profiles are based on the original data in the CSC patents and publications, as well as the typical performance that this species gave in the industrial plants in the US, UK, and South Africa and the results of subsequent laboratory-based trials.

As part of the joint LanzaTech–JGI genome sequencing project [2,38], the genomes of 57 of the *C. saccharobutylicum* NCP strains from the DJ culture collection were sequenced. These plus an additional four NCP genome sequences are in GenBank. The genomes are closely related, and the phylogenetic analysis methodologies used were not precise enough to determine their phylogenetic relationships with certainty. However, based on the genomic profiles and resident prophages, four subgroups of these genomes can be identified (Table 4). Subgroups 1A and 1B have slightly larger genomes, averaging around 5.12 and 5.08 KB with a GC% of 28.7, respectively, and a larger number of tRNA and rRNA genes. The largest number of genomes were Group 2 type, with an average genome size of around 4.99 kb with a GC% of 28.4. As one of the original CSC strains supplied by CSC in 1944 was a BAS/B/F strain, it seems likely that the Group 2 genomes were derived from delta F strains while the Group 1A and 1B strains were derived from delta B strains. The two Group 3 genomes stood out as being phylogenetically distinct in many features, and these are the 162/BI genome and the P249 derivative. This suggests that these strains were derived from either the original A strain or the later gamma C strain. Currently the *C. saccharobutylicum* NCP P262 Type strain is the only strain belonging to this species available from international culture collections. It is hoped that additional examples of *C. saccharobutylicum* strains will be made available.

Table 4. The averaged genome and resident prophage profiles summarised for the 61 genome sequences for *C. saccharobutylicum* species.

Subgroups	1A	1B	2	3
Genomes	11	12	35	3
Size(Mb)	5.12	5.08	4.99	4.91
GC%	28.7	28.7	28.4	28.4
CDS	4256	4167	4176	4159
Genes	4537	4473	4400	4387
Pseudogene	164	173	141	139
rRNA	39	38	12	12
tRNA	92	93	67	72
Other RNA	4	4	4	5
Prophage 1	41,439	52,015	34,321	47,627
Prophage 2	27,734	64,708	51,205	24,405
Prophage 3	48,838	48,838	104,527	48,145
Tailocin	0	0	0	38,384

Genome analysis provides support for the division of the genome profiles into four subgroups. The profile for Group 2 consists of genomes for the largest number of strains. The profiles for the Group 1 genomes are quite similar, but two variants can be distinguished. The resident prophages were typically conserved in each of the 4 groups. The genome sequences in Group 3 are for the strains supplied to NCP by CS in the UK. These strains stand out as being phylogenetically distinct from the BAS/B strains supplied to NCP by CSC in the US.

7.7. The *C. saccharoperbutylacetonicum* Industrial Strain

There is only one strain of *C. saccharoperbutylacetonicum* that has been used for the industrial production of solvents. The N1-4 strain was isolated from the soil in Japan and patented in 1959 by Hongo and Nagata based at Kyushu University [73]. Two variants of the N1-4 strain were lodged with these collections. The N1-4 strain is the Type strain, and the N1-4 HMT strain was lodged as a lysogenic derivative harbouring the inducible HM-1 prophage. Later studies have revealed the HM-1 and related phages have a very small genome size and that the phage genomes are not capable of integration into the host genome. The phage genome can become incorporated into the host endospore as a pseudo-lysogen. On germination the phage genome becomes activated, but over time the HMT strain loses the passenger HM-1 phage genome and can no longer be induced. A second strain designated N1-504 was originally reported to be a phage-resistant mutant of N1-4. Subsequent genetic studies have revealed that the genome of the N1-504 strain differs markedly from the N1-4 strain to the extent that it could be considered a separate subspecies [2]. Examples of the N1-4 strain and the N1-504 strain are held in several international culture collections.

The N1-4 strain was used by the Japanese company Sanraku Ocean for commercial solvent production after WW2 [2]. The company was founded in 1934 as Showa Shuzou and began operating an industrial process for butanol production during WW2. After the war the company resumed production at their Yatsushiro plant. Showa acquired the Mercian company in the early 1960s, merged with the Ocean Co. in 1961, and in 1962 changed its name to the Sanraku Ocean Co. [2]. The new high-butanol-producing *Clostridium* species proved to be very efficient but was found to be susceptible to phage infection. Phage contamination occurred 12 times during the period of one year, and these setbacks were reported to be partly responsible for the fermentation process being abandoned in the early 1960s [74].

The performance of the N1-4 strain reported in the 1959 US patent [73] included details for a full-scale industrial fermentation that included a 9000 L inoculum of the N1-4 culture into 100,000 L of mash consisting of 8000 kg of blackstrap molasses of 5% set sugar with the addition of 160 kg of ammonium chloride, 90 kg of calcium superphosphate, and 100 kg of calcium carbonate. The temperature of the fermentation was maintained at 30 °C for 50 h with a starting pH of 7.3. The pH was then maintained above 5.5 by adding aqueous ammonia from time to time. The performance reported in the patent for a fermentable sugar concentration of 4.71 g/L was a yield of 34.6%, with a concentration of 16.3 g/L total solvents, 12.7 g/L butanol, 6.4 g/L acetone, and 0.6 g/L ethanol, giving a ratio of butanol, acetone, ethanol of 78:18.5:3.5%. According to the performance specifications reported, an exceptionally high solvent yield of around 34.6% could be obtained in the industrial fermentation process, giving a solvent ratio of around 78% butanol with a final butanol concentration of around 12.7 g/L butanol and needed only 4.7% of fermentable sugars in the starting molasses mash. Hongo, Ogata, and their colleagues continued to study the N1-4 strain and their phages and clostocins for most of the next two decades, and they published numerous papers on these studies [74].

Due to its unique characteristics, the N1-4 strain has been used in several fundamental and applied studies. These include an assessment of performance on various alternative substrates with potential for industrial solvent production [75,76]. Improvements in process technology using the N1-4 strain include achieving high butanol production in fed-batch

fermentations using continuous butyric acid and glucose feeding and the prolonged conversion of butyrate to butanol in a two-stage continuous culture with in situ product removal [77,78].

GBL was one of a few companies that established a new industrial process for producing biobutanol this century [19,43]. In 2011 the company merged with Butylfuel in the US, which gave access to pilot-scale facilities in Columbus, Ohio. A demonstration-scale plant was subsequently built in Emmetsburg, Iowa, and in 2014 GBL purchased the Central MN Ethanol Corn distillery in Little Falls, Minnesota. The distillery was retrofitted for butanol production and began operations in 2016. In 2019, the company ran out of funds, and the plant ceased operations in July 2019 [2]. The unique features associated with a monophasic type of batch fermentation coupled with high titres of butanol resulted in the N1-4 strain being selected as the preferred solvent-producing *Clostridium* species for GBL's patented industrial fermentation platform. Their 2018 US patent [44] reported results for optimized butanol production on molasses. The monophasic industrial process used N1-4 strains that had been chemically mutated or genetically modified to produce solvents during growth. The single-stage fed-batch or continuous process used culture vessels with cell growth monitored and optimized by the addition of controlled amounts of culture media and the removal of controlled amounts of solvents such that butanol concentration in the culture vessel did not exceed 10 g/L. Culture media and nutrients were continuously added into the culture vessel, and new cells were fed in from a cell seeder to maintain cell density. In the continuous process, the cells were removed from the culture medium using a cell separator, and the solvents were removed and extracted by a solvent removal system. The residual liquid culture medium and cells were then returned to the culture vessel [44]. Both the Japanese and GBL companies ran their fermentations at around 32 °C to obtain the maximum concentration and yield of butanol. Optimum growth for N1-4 is between 33–37 °C. Batch monophasic fermentation profiles for the N1-4 strain show that all the sugars were utilized during solvent production and that when the sugars were exhausted solvent production stopped. The culture medium used consisted of 55 g/L molasses, along with yeast extract, tryptone, and inorganic salts. The initial pH was set at 6–6.5, and the pH was maintained at 5.0–5.5. These batch fermentations typically produced total solvents of around 19 g/L with 15 g/L butanol, 3 g/L acetone, and 1 g/L ethanol, with a ratio of 79%:16%:4% and a total solvent yield of around 34.5%. The butanol titre is at its maximum concentration for toxicity, and the solvent yield is close to the maximum reported. In conjunction with several other companies, GBL also investigated a wide range of alternative raw materials for their potential for biobutanol production.

The complete genome sequence for *Clostridium saccharoperbutylacetonicum* N1-4 strain DSM 14923 was first published in 2014 [79]. Currently there are four genome sequences for the N1-4 strain and two for the N1-504 strain [2]. The development and advent of mutagenesis tools for solventogenic clostridial species in recent years has allowed for increased refinements for the improvement of the N1-4 strain. Efficient CRISPR-Cas9 genome engineering systems are now available and have paved the way for elucidating the solvent production mechanism in this hyper-butanol-producing species and for engineering strains with desirable enhanced butanol-producing features, allowing for potential improvements in solvent titres and energy metabolism [80,81]. Examples of the application of these new technologies are the enhancement of sucrose metabolism through the deletion of a transcriptional repressor gene to increase solvent production [82] and the deletion of glyceraldehyde-3-phosphate dehydrogenase to increase the ATP pool and accelerate solvent production [80]. A hyper-butanol producer N1-4 strain was converted into a hyper-butyrate producer for butyrate production using glucose and lignocellulosic sugar substrates [83]. In another study targets for membrane engineering for increased butanol tolerance were investigated, and both phospholipid head group composition and membrane fluidity were identified as key targets [45].

8. Conclusions

The solvent-producing species and strains covered in this review all have proven track records as industrial production strains, making them an obvious choice for further development. The assessment of the species and strains has been limited to those surviving industrial strains that are generally available. Several Chinese solvent-producing clostridial strains have been assessed or used for biobutanol production, but these strains have not been considered as in most cases they are proprietary property and not generally accessible. It is also possible that new strains or species could be isolated with potential for industrial applications. The choice of the most appropriate industrial production strains is likely to be of key importance for the development of any new industrial process. Much will depend on whether efficient and reliable genetically modified strains can be developed for the particular species. The choice could also depend on whether standard batch fermentation or a semi continuous/continuous process is to be used and whether this is also coupled with solvent extraction and cell recycle technology. The choice is also likely to be influenced by the economics and availability of the raw material to be used and the additional nutrients and supplements required. Other factors could include the stability and reliability of the production strains and the availability of alternative back-up strains, etc. The market demands and prices for the solvents produced, coupled with the possible value of other by-products including the cell biomass and stillage and use of the gases produced, would also need to be taken into account, along with the energy and water recycling costs.

The major limitation of all the industrial strains is the constraints imposed by the toxicity of butanol. The selection of a particular strain for future industrial developments is likely to be a compromise between the balance between optimum solvent titres, yields, ratios, and productivities, with decisions being based on the case-by-case requirements for the process that is being developed.

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