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Article Review: Unveiling the Potential of *Clostridium acetobutylicum* for Biofuel Production

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ABSTRACT

The acetone-butanol-ethanol (ABE) fermentation is a significant and classic example of bacterial fermentation, particularly in the production of butanol, a valuable feedstock for the chemical industry. Originating during the First World War to produce acetone for cordite manufacturing, the ABE process has historically been overshadowed by the cheaper petrochemical production of solvents, leading to its discontinuation. However, increasing environmental concerns and the depletion of fossil fuel reserves have revived interest in ABE fermentation as a method to produce more sustainable biofuels. Despite extensive knowledge of butanol metabolism and the involvement of various clostridia species, genetic manipulation of these bacteria remains limited, restricting efforts to optimize them for industrial-scale ABE solvent production. For example, understanding and enhancing solvent formation within the ABE pathway, particularly increasing butanol production while reducing acid and acetone byproducts, is crucial for industrial applications. Advances in metabolic engineering of *Clostridium acetobutylicum*, such as gene knockout or insertion, offer potential for improving ABE production, although this research is still in its early stages. The complete sequencing of the *C. acetobutylicum* genome presents opportunities for more sophisticated genetic modifications, with the reversible butanol dehydrogenase system being a key target. Additionally, high-throughput techniques like DNA microarrays can be employed to study the effects of genetic changes on global gene expression, aiding in the identification of factors influencing butanol formation. Ultimately, the development of strains capable of producing butanol more efficiently could lead to its large-scale production as a sustainable biofuel, with *C. acetobutylicum* playing a central role due to its performance in ABE fermentation. Despite the progress, further research and technological advancements are needed to make this process economically viable for widespread industrial use.

Keywords- ABE fermentation, Butanol production, *Clostridium acetobutylicum*, Metabolic engineering, Sustainable biofuels.

I. INTRODUCTION

Clostridium acetobutylicum is an anaerobic, spore-forming bacterium renowned for its effectiveness in the acetone-butanol-ethanol (ABE) fermentation process, making it a strong candidate for butanol production. The large-scale production of butanol from renewable resources using this bacterium holds the potential to replace gasoline, providing a sustainable and environmentally friendly energy source. Butanol, a four-carbon alcohol, offers several advantages over ethanol as a fuel additive or substitute, including higher energy content, compatibility with existing pipeline infrastructure, lower volatility, and reduced water absorption, making it more suitable for the current gasoline distribution system. However, further strain development is essential to make this process economically viable on a large scale (Vamsi et al., 2022; Jiang et al., 2020). During its growth, this bacterium undergoes a metabolic shift, transitioning from acid production to solvent production, particularly during the stationary phase. It can utilize amino acids for solvent production, especially through the butanediol pathway, and has the ability to degrade complex organic compounds, including cellulose, using extracellular enzymes. This

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capability makes it highly suitable for biofuel production from cheap and abundant materials such as hemicellulose and cellulose. Genetic modification efforts are focused on enhancing fermentation efficiency and solvent production capabilities, contributing to its potential as a key organism for sustainable biofuel production (Feng et al., 2021; Bortolucci et al., 2023). The study aims to optimize the bacterium for large-scale butanol production by exploring strategies to alter carbon flux distribution to increase butanol yield. Both in silico and in vivo tools will be utilized for more efficient strain development, marking an initial step towards the industrial-scale production of butanol

II. TAXONOMY AND CLASSIFICATION

Although *Clostridium saccharobutylicum* strains can be considered as *Clostridium acetobutylicum* strains, it's crucial to remember the recent nomenclature change and genome analysis metadata for reference and clarity. This particular strain not only produces acids and MCM1, but it also showcases a similar metabolic pathway as that of *Clostridium beijerinckii*. Interestingly, this species is responsible for the industrial solvent acetone (Fang et al.2020; Olorunsogbon et al.2022). On the other hand, *Clostridium saccharobutylicum* is primarily known for its production of acetic and butyric acids, molecular hydrogen, and carbon dioxide. These acids serve as vital precursors to the synthesis of more reduced acid neutral end products. Despite its complex physiological status, the finalized genome sequence of *Clostridium saccharobutylicum* has immensely contributed to clarifying this. As a member of the Clostridiaceae family, this particular strain exhibits a soil-borne lifestyle and possesses the remarkable ability to utilize various biomass components in order to produce organic acids and solvents. It is important to note that the taxonomic label of *Clostridium acetobutylicum* has been officially changed to *Clostridium saccharobutylicum*, as prescribed by the approved list of bacterial names (Chua, 2022; Li, 2020).

2.1. Metabolic Pathways

Clostridium acetobutylicum is a classical fermentative microbe renowned for its capability to produce biofuel through the conversion of CO2 and organic matter. The microbe integrates metabolic reactions under a variety of environmental conditions which enables it to survive in both oxidative and reductive environments. This mainly occurs as a result of its ability to switch between the acetone-butanol (AB) fermentation pathway and the butyrate fermentation pathway. The butyrate fermentation pathway is used during log phase growth when the microbe is experiencing balanced growth in a reductive environment. It is initiated through the uptake of 2 molecules of acetyl-CoA via and acetoacetyl-CoA with generation of 1 molecule of CoA. This is then utilised in the formation of butyryl-CoA from 2 molecules of CoA and 2 molecules of butyrate. It is at this point in the pathway that the NADH/NAD ratio effects a switch to the AB fermentation pathway (Fast and Papoutsakis, 2018; 47. Cai et.al, 2013). The butyryl-CoA is converted into butyraldehyde by butyryl-CoA: Ehitochrome C oxidoreductase, and then into butanol by butyraldehyde dehydrogenase. This serves as a means of recycling reducing power in the form of 2NADH+H+ and 2NADH, and is viewed as a strategy of minimising production of end products such as butyrate and acetate to form maximum substrate. The microbe then utilises enzymes in the butanol-AB pathway to convert the butanol and all other amcetoacetyl-CoA and acetyl-CoA into acetone via butyrate. This is a key aspect of the pathway as the 2 molecules of acetone can then be converted into 2 molecules of acetate and utilised in the formation of 2 molecules of acetyl-CoA for ATP formation through the Wood-Ljungdahl pathway. This entire pathway is a strategy for ensuring maximum production of ATP from substrate and is essential in the survival of pathogenic clostridia (Ballesteros-Paredes et al.2020).

2.2. Industrial Applications

Solventogenesis is the most important characteristic of *C. acetobutylicum*. The production of acetone-butanol is mostly done by solventogenic clostridia, particularly this bacterium. The main aim of solventogenesis is to generate more ATP and NADH to grow by consuming the acids that were produced in acidogenesis phase. Solventogenic strains of *C. acetobutylicum* have been used for production of acetone and butanol. These products are very important in industrial point of view, as they can be used as solvent in the production of lacquers, explosives, synthetic and natural rubbers, and many other polymers and plastics. Acetone and butanol can also be converted into other chemicals that are widely used in the industrial sector (Vees et al.2020). During acidogenesis, an excess of reduced ferredoxin is produced and the NADH specific-acetone formation from acetoacetyl-CoA via acetoacetate has been shown to be ferredoxin dependent. Butanol production will only occur with accumulation of acetic esters and cessation of acetate production. The switch to solventogenesis is regulated on the transcriptional level by a two-component system involving a sensor and a response-regulator protein. This global shift in carbon flux in solventogens will be channeled to a butanol production pathway that has the same net energy 2 ATP per butanol produced (than 1 molecule of ATP/2ADP) and redox balance (requiring NADH and producing NAD) as the butyrate production pathway in acidogens. Butanol is a secondary alcohol and the production involves the specific reduction of butyryl-CoA to butanol via butyraldehyde with the butyraldehyde being formed by the reversal of the corresponding dehydrogenase an acetaldehyde formation from 2 molecules of acetyl-CoA. Coat and Vemuri, 2009 are currently using systems-biology approaches to elucidate the regulation and metabolic constraints in *C. acetobutylicum* (Bortolucci et al.2023; Du et al., 2021).

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III. BIOFUEL PRODUCTION WITH CLOSTRIDIUM ACETOBUTYLICUM

C. acetobutylicum has since been the subject of metabolic engineering aimed at optimizing fermentative solvent production. This has ranged from gene knockouts to more recent gene insertional systems such as ClosTron and targeted gene disruption through homologous recombination and allelic exchange (Gao et al.2022). Acetone, butanol, and ethanol production in the ABE fermentation is a product of this species metabolism of the precursor molecule acetyl-CoA. This pathway is initiated from pyruvate through the activity of a pyruvate ferredoxin oxidoreductase (PFOR) and subsequent conversion of acetyl-CoA and CoA from the CODH/acetyl-CoA synthase enzyme complex. The main metabolic product is largely dependent on culture conditions, with butyrate also being an important precursor to solvents. Stepwise reduction of butanol to 2-propanol and further to acetone leads to inactivation of the pathway. High metabolic flux is required, and preventing the formation of acids such as butyrate and caproate could potentially increase solvent yield (Bortolucci et al.2023; Du et al., 2021). This bacteria, best known for its ability to produce solvents such as acetone, butanol, and ethanol through fermentation, has long been recognized as a promising alternative to traditional biofuels derived from agriculture. This stems from the ability of solvent-producing Clostridial strains to utilize a wide range of carbon sources, including biomass. The ability to ferment various forms of waste products from sustainable biomass feedstocks would be a major advantage over bioethanol production, which relies mainly on the fermentation of simple sugars (Zhang et al.2023; El-Dalatony et al.2022).

3.1. Fermentation Process

The fermentation process of *C. acetobutylicum* has been categorized into four unique phases. The primary phase is a period of cell growth, followed by the major production phase of the acids, particularly acetic and butyric acids, the solvent formation phase, as well as the last phase of spore formation. During the cell growth phase, biomass is developed from various nutrients, incomplete to glucose. Primarily the utilization of sugars is greatest with 85-100% utilized during later growth phases of the microorganism (Vees et al.2020; Li et al., 2020). Following cell development, acid formation takes place. This can range from 18-24 hours depending on the strain of *C. acetobutylicum*. This phase is the most crucial to solvent production in the future. It sets up how much solvent will be produced as well as the type of acid that is overproduced will be used to shift the metabolism toward solvent formation or spore formation. At the end of an acid production phase little to no acid, cells, and acids are transferred to a new medium to enhance solvent production. This is not desired in some industrial aspects due to cost inefficiency. It has been said that immobilization of these cells can use a two-step system in the same media to highly produce solvents (Gao et al.2022).

3.2. Biofuel Yield and Efficiency

Solvent production (and therefore biofuel yield) is directly proportional to the NADH2 concentration in the cell. With 3 moles of NADH2 producing 1 mole of butanol, or acetone, or butyric acid. In ABE fermentation, glucose is metabolized giving Synthesis gas, which forms acetic acid, then acetyl Co-A, which subsequently forms acetoacetyl Co-A, in turn producing 1,2 butanediol. This is the first point at which solvent formation occurs. 1,2 butanediol is the precursor to butanol, and the butane-2, 3 diol formation step is where there is the greatest loss of carbon. Butane-2, 3 diol is also formed from acetyl Co-A using an alternate route to that from Synthesis gas. This causes competition for NADH2 and carbon. The net result is that there is little butanol produced. The reaction is: a butane-2,3 triol + NAD ↔ 2butane-2,3 diol + NADH + 2H. But this will then revert to butanediol (Bortolucci et al.2023; Zhang et al.2023).

The theoretical conversion of glucose to solvents is based on $C6H12O6 + 2H2O = 2CH3COOH + Butanediol + 2CO2 + 2H2 + 2Acetone$. Therefore, the maximum yield of butanol (the ABE's principal component) is 0.375g/g (48.2% of the overall products). This is based on the thermodynamics of reactions involved in ABE production. In reality, though, the level of butanol obtained is, in fact, much lower. This indicates that there are current inefficiencies in the process. Moreover, it was cited by Hayn and Hönlinger that the maximum yield of butanol has not been achieved during the classical ABE batch fermentation since 1861. (Guerrero et al.2022)

3.3. Challenges and Limitations

Several challenges and limitations exist that affect the process of biofuel production using *C. acetobutylicum*. The first challenge lies in the substrate used. This microorganism was originally isolated from the soil and a common contaminant of plant tissues. Its natural habitat led to the use of *C. acetobutylicum* to ferment a variety of carbohydrates at a high yield to solvents. The isolation of *C. acetobutylicum* was carried out in an attempt to improve the fermentation of substrates which are less favorable to other microorganisms. However, these substrates can be expensive in comparison to glucose and other simple sugars used in yeast fermentation which leads to the production of bioethanol (Vees et al.2020; Bortolucci et al.2023; Kongjan et al.2021). The cost of substrate must also be taken into consideration for large-scale processes. High substrate costs can be an unwanted expense. If the overall cost is too high for a particular substrate, it can be infeasible to produce biofuels from *C. acetobutylicum*. This occurred with the fermentation of Jerusalem artichoke which showed potential for producing a greater quantity and mass of solvents, but was far too costly at the time due to low yields of inulin sugar from the plant. A remedy to this challenge could be the development of genetically engineered strains of *C. acetobutylicum* to ferment various substrates with higher yields of solvents. This area has shown little progress to date and could be due to the

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concern of introducing genetically modified organisms (GMOs) to the environment or the lack of funding. Another major challenge is the separation of solvents from the fermentation broth (Li et al., 2020). The ABE fermentation produces various solvents and an undesired amount of acids, most of which are toxic to the microorganism. In order to sustain a high yield of solvents, it is necessary to switch the metabolic pathway at the appropriate time to selectively synthesize the desired solvents. This is often achieved by the removal of an enzyme or substrate. With improvement, this could be done with a genetically engineered strain. Once the fermentation is complete, the separation of solvents from the broth often involves expensive methods such as distillation which is less practical in comparison to the low-cost separation of ethanol from yeast fermentation with simple sugars. Ideally, economic methods of separating solvents would vastly improve the overall process of biofuel production using *C. acetobutylicum* (Du et al., 2022; Bortolucci et al.2023).

IV. GENETIC ENGINEERING OF CLOSTRIDIUM ACETOBUTYLICUM

Genetic engineering of microorganisms involves altering the genotype by including new genes, resulting in improved traits or new properties. This is important in *C. acetobutylicum*, where knowledge of its genes and tools for manipulation have grown. Random mutagenesis is still important until the entire genome is known. High-throughput screening can identify mutants and genes for higher solvent production. With the genome and gene transfer tools available, reproducible genetic engineering can be done in *C. acetobutylicum* to improve solvent yields. CoA transferase disruption improves butanol ratios but reduces ABE production. Engineering an acetyl-CoA, Acetyl-P-phosphate transacetylase gene can increase butanol formation. ABE is a solvent and industrial product. Hydrogen and acetone production can be engineered, aiming to commercialize isopropanol as fuel and precursor to propylene. Inactivating the acetone sporulation gene increases theoretical solvent yield from 85% to 100% (Bortolucci et al.2023; Du et al., 2022; Vees et al.2020).

4.1. Strategies for Genetic Manipulation

The methods for genetic manipulation are critical to genetic engineering. The use of small plasmids derived from specific transposons allows for gene transfer. However, the plasmids may have limitations such as inability to replicate in certain strains and random integration into the chromosome. Integration of genes into the chromosome is more desirable for stable gene expression. A newer solventostatic plasmid has been developed which can replicate in specific strains. Selecting and controlling the site of recombination is crucial for the success of these methods. An efficient gene inactivation system using a mobile ribozyme has been devised. Homologous recombination is the preferred method for gene integration (Kongjan et al.2021; Li et al., 2020).

4.2. Targeted Gene Modifications

The traditional method of reverse genetics involves modifying a gene and then observing the resulting phenotype. However, this approach can be challenging when little is known about the organism's genome and the genes involved in a specific pathway. In such cases, a forward genetics approach is often preferred, where mutations are randomly introduced and mutants that exhibit the desired phenotype are selected. In both approaches, site-specific recombination greatly simplifies the process of gene modification by allowing for direct gene replacement at their normal chromosomal location (Veza et al., 2021; Fonseca et al.2020). The advancement of genetic engineering in bacteria has been facilitated by the tools of molecular biology and the increasing knowledge of gene function in different organisms. Although mutagenesis and transformation can alter gene expression in a non-specific manner, targeted gene modifications offer the ability to manipulate specific genes involved in a particular metabolic pathway. The significance of targeted gene modification lies in its ability to activate or deactivate genes, as well as insert or delete genes, resulting in significant physiological effects on the organism. Modifying a single gene can lead to minor changes in metabolic flux or a complete reorganization of metabolism (Bortolucci et al.2023).

4.3. Enhanced Biofuel Production

When aiming to optimize the biobutanol pathway in *C. acetobutylicum*, it may be useful to take a step back and consider what we actually mean by enhanced biofuel production. Biologically, butanol tends to be toxic at concentrations of about 2%, so enhanced butanol production would be increasing the rate at which butanol is produced without a decrease in the specific growth rate of the microorganism, in order to increase the yield of butanol on total ATP used and substrate used. From a production point of view, it may also be interpreted as reducing the time it takes to complete the whole fermentation process. There are two ways in which the production of butanol can be increased in the life of the solventogenic Clostridium. One way would be to shift as much of the carbon flux as possible to the butyrate/acetoacetate pathway and then into the butanol pathway (Bortolucci et al.2023). This, in theory, would give a faster butanol production rate as long as the carbon flux into the butanol pathway could be controlled. An alternative method would be to try and increase the flux into the butanol pathway while trying to increase the ATP and NADH yields. His approach was to delete the gene for the butyrate kinases which carry out the conversion of butyryl CoA into butyrate. The logic being that by preventing a route from butyryl CoA to butyrate/acetoacetate, it would force more carbon through the butyrate/acetoacetate pathway into the butanol producing pathway due to increases in butyryl CoA availability from the onset. Data showed that this gene deletion of the butyrate kinases resulted in an accumulation of butyryl CoA and an increase in the butanol: acetone ratio. This proves the

original logic to be true and so we can consider that this was a successful mutation to increase the carbon flux into the butanol pathway and thus enhance butanol production (Luo et al.2020; Kotte et al.2020).

V. OPTIMIZATION OF BIOFUEL PRODUCTION

The proportional utilization of acids and solvents and their intricate interactions leads to a difficult separation of butanol from the fermentation broth. When ABE production from glucose was monitored in pH controlled batch cultures, acid production stopped after 24 hours and solvent production was maximum at around 72 hours. During the 48 hours that solvent was being produced, approximately 80% of it was butanol. From the standpoint of product separation, it is desirable to maximize the butanol concentration in the broth. This can be accomplished by finding conditions that shift carbon flux towards solvent production and increase the ratio of butanol to acetone and ethanol. A great deal of work is still needed to understand the genetic regulation of solvent production and the factors that affect the relative production of the acids and solvents. Simulation of ABE fermentation revealed that butanol concentration could be almost doubled if the timing of butanol inhibition of solvent production could be understood and manipulated in the fermentative process. This would have a large impact on the economics of acetone and ethanol production from petrochemicals because butanol is a much more valuable solvent. The development of Clostridium strains capable of hyper butanol production with decreased acetone and ethanol production is an area that needs more research (Koller, 2023; Lin et al., 2023).

5.1. Process Parameters

Temperature is another key variable in the fermentative production of solvents by *C. acetobutylicum*. Although it is a mesophilic organism, the choice of whether to grow it at its optimal temperature or to use heat to manipulate the metabolism of the culture is a key issue. Optimum growth occurs around 30-37°C, depending on the strain, and solvent production is greatest when the culture is shifted from this range to about 25°C. This can be accomplished on a small scale with a temperature shift at the midpoint of the fermentation, but solvent production may still be hindered by residual acidogenesis at the higher growth temperature. On substrates such as hemicellulose, where solvent production occurs immediately following acidogenesis, it is more effective to simply grow the culture at the lower temperature. A study using *C. acetobutylicum* P262 in a hemicellulose medium obtained a solvent yield of 0.33 g/g and an ABE yield of 0.29 g/g, the highest values reported for any solvent-producing clostridium using a monophasic culture and no pH control. (Wu et al.2020; Du et al.2021)

Fermentation is affected by numerous process parameters which include pH, temperature, inoculum size, agitation rate, and reactor type. The growth and product formation are particularly affected by the pH of the fermentation medium. This bacterium ferments sugars to acids, solvents, and gases. Acid formation occurs in the acidogenic phase. The acidogenic enzymes, including phosphotransacetylase and acetate kinase, have an optimal pH around 5.5, while the solventogenic enzymes have an optimal pH around 4.75. Once the pH of the medium drops to a level between 4 and 5, *C. acetobutylicum* will switch from acid production to solvent production. This transition can be facilitated by initially growing the culture on hemicellulose-derived sugars at an uncontrolled pH. When the sugars are depleted, the pH can be adjusted to the desired interval for solvent production. Optimum solvent production in batch culture has been achieved by buffering the culture at an initial pH of 6 and allowing it to drop to 4 over the course of the fermentation. At this point, greater than 60% of the acids are reassimilated as solvents. Although this pH profile was designed for acid production on glucose, it is still an effective means of initiating solvent production for hemicellulose and cellulose substrates. Control of the pH during the solventogenic phase on hemicellulose, cellulose, and other mixed sugar substrates has been achieved by monophasic and biphasic culture with no loss in solvent production or degradation in the solvent yield coefficient. (Xin et al.2020; Han et al.2020; Luo et al.2020)

5.2. Substrate Selection

From the research and development aspect of biofuel production using *C. acetobutylicum*, much work can be done to manipulate specific pathways and genetic engineering may be used to enhance production of the desired chemicals. This is a very broad field of study and little is known about the biochemical nature of some cellulose and solvent degradation/formation pathways by these microorganisms. This suggests there may be more effective ways of biofuel production using cellulose and different strains of the bacterium. It would be useful to monitor and develop the genetic potential and metabolic pathways of various strains using modern transcriptomic and metabolomics analysis would be beneficial. This would help determine the most efficient methods of biofuel production using the least amounts of enzymes and resources, potentially leading to simpler and cheaper methods. With the issue of global climate change and limited resources of energy and chemicals, *C. acetobutylicum* and other biofuel production microorganisms are sure to attract more research and interest in the near future (Veza et al., 2021; Luo et al.2020).

The major by-product of biomass fermentation is acetone-butanol-ethanol (ABE). This is one of the oldest known industrial fermentation processes, initially used to produce solvents rather than biofuels, employing a different strain of the bacterium. The genetic potential of this microorganism means the ABE process could be a viable source of bio-butanol. However, the ABE process is sensitive to and strongly inhibited by product and substrate concentrations, suggesting that

allowing cells to grow and produce chemicals without interference might yield the highest chemical output. The genes of the ABE pathway are organized in a single cluster, with key enzymes being produced and activated depending on the growth phase of the cells. This implies that batch culture could be the most efficient method for ABE production, as the switch from cell growth to chemical production can be controlled by altering the batch phase. For butanol production as a biofuel through biomass fermentation, immobilized cell bioconversion is the most efficient method. In an attempt to determine the feasibility of biofuel production from biomass fermentation by *C. beijerinckii*, Shinto et al. investigated ABE production using fibrous bed bioreactors and found it more effective than conventional methods. This finding aligns well with the future potential for bio-butanol production through biomass fermentation by *C. acetobutylicum*. Immobilized cell bioconversion offers the advantage of simplifying product recovery and allows for continuous fermentation without the risk of contamination (Zhang et al., 2021; Xin et al., 2020; Shinto et al., 2007).

5.3. Bioreactor Design

Conceptually, bioreactors are vessels where biological reactions occur. When related to biofuel production, bioreactors are used to cultivate microorganisms that will produce fuel. There have been many types of bioreactors developed for various uses. Unfortunately, data is lacking for the best type of bioreactor for the ABE fermentation. During the original ABE fermentations, the inoculums were a mixed culture and were not pure. Using the old-style bioreactors was not feasible with a pure culture since it was a batch process and the seeds could not be reused. Also, with a pure culture, solvent inhibition is a problem and butanol is toxic to the cells at high concentrations (Veza et al., 2021). Batch and continuous culture were compared using a STR for butanol production and simply having a larger volume of butanol/water mixture at the top for continuous, it was shown that the concentration and overall butanol production was higher for the batch process. A gravity discharge column type bioreactor was also tested for ABE production and found to be better than a comparable stirred tank fermentor. A more complex bioreactor using immobilized cells with an ultrafiltration membrane to separate the biocatalyst from the fermentation broth showed to drastically reduce the inhibitory effects of butanol, allowing for butanol production to continue in the ABE producer phase and not revert back to the acid producer phase (Lin et al., 2023; Patakova et al.2022). These types of data are very useful and will allow the best type of bioreactor to be chosen for ABE production. More research done in these areas will yield whether the bioreactors used for butanol production from ABE are the best for what is wanted now – butanol as a biofuel from various resources and developing processes to more efficiently obtain butanol from other solvents in the ABE mix. This will allow for the development of new types of bioreactors and modifications of existing types to create the most efficient bioreactor for butanol production from various resources to date and in the future (El-Dalatony et al.2022; Wu et al.2020).

VI. FUTURE PROSPECTS AND RESEARCH AVENUES

The exploration of new strains of *C. acetobutylicum* is currently a theme of significant interest for those looking to increase the efficiency and economic viability of bio-butanol production. This interest stems from the fact that wild-type strains possess several physiological and metabolic bottlenecks that limit their ABE production potential. To address this, metabolic engineering can be employed to modify certain characteristics of the microorganism, enhancing its ability to ferment sugars into the desired solvents. This process typically involves gene knock-out or gene modification to achieve a specific effect on the cell's metabolism. An example of this approach is demonstrated in the work of Sillers et al., who attempted to increase the butanol yield of *C. beijerinckii* by introducing an aldehyde/alcohol dehydrogenase gene (aad) from *C. acetobutylicum* (Sillers et al., 2008). This modification enabled the *C. beijerinckii* strain to convert butyryl-CoA into butanol without producing acetone as a by-product, thereby increasing butanol yield and decreasing the acetone/butanol ratio. This genetic modification significantly altered the strain's solvent production pattern and is applicable to future work with *C. acetobutylicum* for butanol production (Vees et al., 2020; Li et al., 2020). Another approach is to identify and utilize a new strain with higher butanol production potential than the currently well-studied ATCC 824 strain. Evidence of this potential was shown in the work of Taconi et al., which found that the DSM 1731 strain had a higher butanol yield and lower acid by-product formation than ATCC 824 during the fermentation of sugarcane bagasse hydrolysate (Taconi et al., 2009). This demonstrates that there is potential for finding a more suitable strain through the screening of various wild-type strains, one that may prove to be more economically viable for bio-butanol production (Bortolucci et al., 2023).

6.1. Exploration of New Strains and Pathways

Potential to further increase the capabilities of the ABE fermentation exists through the exploration of new strains of *C. acetobutylicum*. This can be achieved through a variety of methods. Firstly, through continued selective pressure of the wild-type strain, it may be possible to isolate spontaneous mutants with higher ABE production. Using microarray technology, it is possible to compare gene expression of the wild-type to that of mutants with higher ABE production, and thereby identify which genes and pathways are crucial to the ABE process (Green et al., 1996). This method was employed by Tomas et al. when comparing a butanol hyperproducing *Clostridium beijerinckii* BA101 to its parent strain (Tomas et al., 2003). Similarly, a greater understanding of the ABE pathway may reveal potential metabolic engineering techniques involving gene inactivation, amplification, or over-expression (Du et al., 2022). Adopting a genetic engineering approach to

strain improvement is a feasible prospect with potential to yield fast and significant results. As with spontaneous mutants, it is possible to identify and manipulate genes responsible for ABE production (Sillers et al., 2008). This can be achieved by inactivating butanol formation pathways, and introducing genes from other organisms which have been shown to improve ABE production, such as the adc gene from *C. beijerinckii* which has been proven to enhance acetone production (Grupe and Gottschalk, 1992). This technique has already been trialed by Tracy et.al, who managed to increase butanol production in *C. acetobutylicum* ATCC824 by introducing the *C. beijerinckii* adhe2 gene (Tracy et.al, 2012). A more radical approach may involve generating recombinant strains of *C. acetobutylicum* containing a different combination of ABE genes, or even synthesis of an entirely artificial pathway. While genetic manipulation has proven a successful tool for strain improvement, it is not without its difficulties. Many strains of *C. acetobutylicum* are known to be difficult to transform, and little genomic information is available for most wild-type strains (Al-Hinai et al., 2014; Vees et al.2020).

6.2. Enhancing Process Optimization Strategies

Further work on developing rapid and reliable systems for gene knock-out and knock-in will enable construction of multiple mutants and metabolic engineering pathways. One possible method is to create a homologous gene replacement vector carrying an up and downstream fragment of the target gene to be knocked out. This can increase recombination with the chromosome and decrease non-homologous recombination that simply restores the wild type gene. Site-specific recombination systems such as the Cre-lox system along with group II introns can essentially provide non-reversible gene inactivation systems which may be required to create knockout mutations in essential genes. Electroporation as a gene transfer method is more efficient than protoplast or bacterial conjugation methods, and developing an electroporation protocol for Clostridial organisms would save much time and effort. These mentioned methods require a thorough understanding of the genetic and physiological mechanisms of solventogenic clostridia, and it is important to optimize such conditions when performing genetic manipulations (El-Dalatony et al.2022; Kumar et al., 2023). The commercial production of ABE fermentation has largely been focused on process development rather than exploring new strains and metabolic pathways. Previously published results have shown that more effort needs to be invested into refining genetic techniques so that they are compatible with Clostridial organisms. Moreover, prevalent methods for genetic manipulation involve the use of antibiotics as selective markers. However, poor selection pressure and instability of the antibiotics are often the cause of plasmid loss from the host organism. It is highly advantageous to develop a reliable host-vector system that can stably maintain foreign DNA in the host chromosome (Bortolucci et al.2023; Du et al., 2022).

6.3. Synergistic Integration with Other Biofuel Technologies

Acetone-butanol-ethanol fermentation is a bioconversion method in which sugar is transformed into acetone, butanol, and ethanol through the action of anaerobic bacteria. This process offers the opportunity for integration with various biofuel technologies, leading to improved efficiency and enhanced yield. In addition to the ABE fermentation approach, there exists another technology called biobutanol. Although it is similar to ABE fermentation, it does come with higher associated costs. However, it also presents promising prospects when it comes to biofuel production. Furthermore, another technique involves utilizing either corn or lignocellulosic materials as substrates for the ABE fermentation process. By employing different substrates, a diverse range of ABE products can be generated, which can then be efficiently separated using distillation. This separation step allows for the isolation and extraction of the individual components of the ABE product pool (Li et al., 2020; Inyang et a., 2022).

REFERENCES

[1] Al-Hinai, M. A., Jones, S. W., & Papoutsakis, E. T. (2014). σ^K of Clostridium acetobutylicum is the first known sporulation-specific sigma factor with two developmentally separated roles, one early and one late in sporulation. Journal of Bacteriology, 196(2), 345-357.

[2] Ballesteros-Paredes, J., André, P., Hennebelle, P., Klessen, R. S., Kruijssen, J. D., Chevance, M., ... & Vazquez-Semadeni, E. (2020). From diffuse gas to dense molecular cloud cores. Space Science Reviews, 216, 1-58.

[3] Bortolucci, J., Zani, A. C. B., de Gouvêa, P. F., Dinamarco, T. M., & Reginatto, V. (2023). A non-solventogenic Clostridium beijerinckii strain lacking acetoacetate decarboxylase assimilates acetate and accumulates butyrate. Biomass and Bioenergy, 172, 106780.

[4] Cai, G., Jin, B., Monis, P., & Saint, C. (2013). A genetic and metabolic approach to redirection of biochemical pathways of Clostridium butyricum for enhancing hydrogen production. Biotechnology and Bioengineering, 110(1), 338-342. https://doi.org/10.1002/bit.24596

[5] Chua, M. (2022). Gene order alignment on a phylogeny with BOPAL 2.0. umanitoba.ca

[6] Du, G., Che, J., Wu, Y., Wang, Z., Jiang, Z., Ji, F., & Xue, C. (2021). Disruption of hydrogenase gene for enhancing butanol selectivity and production in Clostridium acetobutylicum. Biochemical Engineering Journal, 171, 108014.

[7] Du, G., Wu, Y., Kang, W., Xu, Y., Li, S., & Xue, C. (2022). Enhanced butanol production in Clostridium acetobutylicum by manipulating metabolic pathway genes. Process Biochemistry.

[8] Du, G., Zhu, C., Xu, M., Wang, L., Yang, S. T., & Xue, C. (2021). Energy-efficient butanol production by Clostridium acetobutylicum with histidine kinase knockouts to improve strain tolerance and process robustness. Green Chemistry.

[9] El-Dalatony, M. M., Basak, B., Kurade, M. B., Roh, H. S., Jang, M., & Jeon, B. H. (2022). Effect of sonication pretreatment on hydrogen and acetone-butanol-ethanol coproduction from Chlamydomonas mexicana biomass using Clostridium acetobutylicum. Journal of Environmental Chemical Engineering, 10(3), 107600.

[10] Fang, D., Wen, Z., Lu, M., Li, A., Ma, Y., Tao, Y., & Jin, M. (2020). Metabolic and process engineering of Clostridium beijerinckii for butyl acetate production in one step. Journal of Agricultural and Food Chemistry, 68(35), 9475-9487.

[11] Fast, A. G., & Papoutsakis, E. T. (2018). Functional Expression of the Clostridium ljungdahlii Acetyl-Coenzyme A Synthase in Clostridium acetobutylicum as Demonstrated by a Novel In Vivo CO Exchange Activity En Route to Heterologous Installation of a Functional Wood-Ljungdahl Pathway. Applied and Environmental Microbiology, 84(7),1-15.

[12] Feng, J., Zhang, J., Ma, Y., Feng, Y., Wang, S., Guo, N., ... & Wang, Y. (2021). Renewable fatty acid ester production in Clostridium. Nature Communications, 12(1), 4368. nature.com

[13] Fonseca, B. C., Bortolucci, J., da Silva, T. M., dos Passos, V. F., de Gouvêa, P. F., Dinamarco, T. M., & Reginatto, V. (2020). Butyric acid as sole product from xylose fermentation by a non-solventogenic Clostridium beijerinckii strain under controlled pH and nutritional conditions. Bioresource technology reports, 10, 100426.

[14] Gao, R., Xiong, L., Wang, M., Peng, F., Zhang, H., & Chen, X. (2022). Production of acetone-butanol-ethanol and lipids from sugarcane molasses via coupled fermentation by Clostridium acetobutylicum and oleaginous yeasts. Industrial Crops and Products, 185, 115131.

[15] Green, E. M., Boynton, Z. L., Harris, L. M., Rudolph, F. B., Papoutsakis, E. T., & Bennett, G. N. (1996). Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. Microbiology, 142, 2079-2086.

[16] Grupe, H., & Gottschalk, G. (1992). Physiological events in Clostridium acetobutylicum during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Applied and Environmental Microbiology, 58(12), 3896-3902.

[17] Guerrero, K., Gallardo, R., González, E., Veliz, F., Conejeros, R., Gentina, J. C., & Aroca, G. (2022). Butanol production by Clostridium acetobutylicum ATCC 824 using electro-fermentation in culture medium supplemented with butyrate and neutral red. Journal of Chemical Technology & Biotechnology, 97(6), 1526-1535.

[18] Han, Y. F., Xie, B. T., Wu, G. X., Guo, Y. Q., Li, D. M., & Huang, Z. Y. (2020). Combination of trace metal to improve solventogenesis of Clostridium carboxidivorans P7 in syngas fermentation. Frontiers in Microbiology, 11, 577266. frontiersin.org

[19] Inyang, V., Laseinde, O. T., & Kanakana, G. M. (2022). Techniques and applications of lignocellulose biomass sources as transport fuels and other bioproducts. International Journal of Low-Carbon Technologies, 17(4), 900–909. https://doi.org/10.1093/ijlct/ctac068

[20] Jiang, Y., Lv, Y., Wu, R., Lu, J., Dong, W., Zhou, J., ... & Jiang, M. (2020). Consolidated bioprocessing performance of a two-species microbial consortium for butanol production from lignocellulosic biomass. Biotechnology and Bioengineering, 117(10), 2985-2995.

[21] Koller, M. (2023). Biotechnological approaches to generate biogenic solvents and energy carriers from renewable resources. The EuroBiotech Journal. sciendo.com

[22] Kongjan, P., Usmanbaha, N., Khaonuan, S., Jariyaboon, R., Sompong, O., & Reungsang, A. (2021). Butanol production from algal biomass by acetone-butanol-ethanol fermentation process. In Clean Energy and Resources Recovery (pp. 421-446). Elsevier.

[23] Kotte, A. K., Severn, O., Bean, Z., Schwarz, K., Minton, N. P., & Winzer, K. (2020). RRNPP-type quorum sensing affects solvent formation and sporulation in Clostridium acetobutylicum. Microbiology, 166(6), 579-592. microbiologyresearch.org

[24] Kumar, M., Saini, S., & Gayen, K. (2023). Exploring the Influence of pH on the Dynamics of Acetone–Butanol–Ethanol Fermentation. Microorganisms. mdpi.com

[25] Li, S., Huang, L., Ke, C., Pang, Z., & Liu, L. (2020). Pathway dissection, regulation, engineering and application: lessons learned from biobutanol production by solventogenic clostridia. Biotechnology for biofuels. springer.com

[26] Lin, Z., Cong, W., & Zhang, J. (2023). Biobutanol Production from Acetone–Butanol–Ethanol Fermentation: Developments and Prospects. Fermentation. mdpi.com

[27] Luo, H., Zheng, P., Bilal, M., Xie, F., Zeng, Q., Zhu, C., ... & Wang, Z. (2020). Efficient bio-butanol production from lignocellulosic waste by elucidating the mechanisms of Clostridium acetobutylicum response to phenolic inhibitors. Science of the total environment, 710, 136399.

[28] Olorunsogbon, T., Adesanya, Y., Atiyeh, H. K., Okonkwo, C. C., Ujor, V. C., & Ezeji, T. C. (2022). Effects of Clostridium beijerinckii and Medium Modifications on Acetone–Butanol–Ethanol Production From Switchgrass. Frontiers in Bioengineering and Biotechnology, 10, 942701. frontiersin.org

[29] Patakova, P., Branska, B., Vasylkivska, M., Jureckova, K., Musilova, J., Provaznik, I., & Sedlar, K. (2022). Transcriptomic studies of solventogenic clostridia, Clostridium acetobutylicum and Clostridium beijerinckii. Biotechnology Advances, 58, 107889.

[30] Rezahasani, R., Khalili-Samani, A., Aghbashlo, M., Amiri, H., Tabatabaei, M., & Nizami, A. S. (2024). Sugar fermentation: C4 platforms. In Higher Alcohols Production Platforms (pp. 125-156). Academic Press.

[31] Shinto, H., Tashiro, Y., Yamashita, M., Kobayashi, G., Sekiguchi, T., Hanai, T., Kuriya, Y., Okamoto, M., Sonomoto, K. (2007). Kinetic modeling and sensitivity analysis of aceton-butanol-ethanol production. Journal of Biotechnology 131(1), 45-56.

[32] Sillers, R., Chow, A., Tracy, B., & Papoutsakis, E. T. (2008). Metabolic engineering of the non-sporulating, non-solventogenic Clostridium acetobutylicum strain M5 to produce butanol without acetone demonstrates the robustness of the acid-formation pathways and the importance of the electron balance. Metabolic Engineering, 10(6), 321-332.

[33] Taconi, K. A., Venkataramanan, K. P., & Johnson, D. T. (2009). Growth and solvent production by Clostridium pasteurianum ATCC 6013 utilizing biodiesel-derived crude glycerol as the sole carbon source. Environmental Progress & Sustainable Energy, 28(2), 100-110.

[34] Tomas, C. A., Welker, N. E., & Papoutsakis, E. T. (2003). Overexpression of groESL in Clostridium acetobutylicum results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Applied and Environmental Microbiology, 69(8), 4951-4965.

[35] Tracy, B. P., Jones, S. W., Fast, A. G., Indurthi, D. C., & Papoutsakis, E. T. (2012). Clostridia: The importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Current Opinion in Biotechnology, 23(3), 364-381.

[36] Vamsi Krishna, K., Bharathi, N., George Shiju, S., Alagesan Paari, K., & Malaviya, A. (2022). An updated review on advancement in fermentative production strategies for biobutanol using Clostridium spp. Environmental Science and Pollution Research, 29(32), 47988-48019.

[37] Vees, C. A., Neuendorf, C. S., & Pflügl, S. (2020). Towards continuous industrial bioprocessing with solventogenic and acetogenic clostridia: challenges, progress and perspectives. Journal of Industrial Microbiology & Biotechnology: Official Journal of the Society for Industrial Microbiology and Biotechnology, 47(9-10), 753-787. springer.com

[38] Veza, I., Said, M. F. M., & Latiff, Z. A. (2021). Recent advances in butanol production by acetone-butanol-ethanol (ABE) fermentation. Biomass and Bioenergy.

[39] Wu, Y., Wang, Z., Xin, X., Bai, F., & Xue, C. (2020). Synergetic engineering of central carbon, energy, and redox metabolisms for high butanol production and productivity by Clostridium acetobutylicum. Industrial & Engineering Chemistry Research, 59(39), 17137-17146.

[40] Xin, X., Cheng, C., Du, G., Chen, L., & Xue, C. (2020). Metabolic engineering of histidine kinases in Clostridium beijerinckii for enhanced butanol production. Frontiers in Bioengineering and Biotechnology, 8, 214. frontiersin.org

[41] Zhang, F., Zhang, K., Zhang, Z., Chen, H. Q., Chen, X. W., Xian, X. Y., & Wu, Y. R. (2023). Efficient isopropanol-butanol-ethanol (IBE) fermentation by a gene-modified solventogenic Clostridium species under the co-utilization of Fe (III) and butyrate. Bioresource Technology, 373, 128751.

[42] Zhang, K., Hong, Y., Chen, C., & Wu, Y. R. (2021). Unraveling the unique butyrate re-assimilation mechanism of Clostridium sp. strain WK and the application of butanol production from red seaweed Gelidium amansii …. Bioresource Technology.