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Screening and Adaptive Evolution of Clostridium for Butanol Synthesis by ABE Fermentation from Cellulosic Biomass: A Review

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ABSTRACT

Cellulosic biomass has recently been given considerable attention as the most common renewable feedstock for biofuel manufacture. However, because of lignocelluloses' complex structure, it must be processed in several steps, which is expensive and time-consuming. By fermenting cellulosic biomass, Gram-positive Clostridium species can naturally produce butanol. Therefore, novel microbial biocatalysts with a higher butanol tolerance are required for the industrial-scale production of butanol. Due to its natural capability to break down cellulose, the Clostridium bacterium shows excellent potential as a strain isolated from lignocellulosic feedstocks, agricultural wastes and converted into butanol. Other species rather than Clostridiun can be used to produce butanol by adaptive evolution. But, compared to solvent-producing clostridia, other species such as *Escherichia coli* and *Saccharomyces cerevisiae* can adaptively change the butanol pathway that might be a

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solution for eliminating the formation of major by-products, acetone and ethanol, so that butanol yield can be improved significantly. Butanol (C_4H_9OH) is generated primarily using fermentation techniques and is a powerful industrial solvent. Fermenting cellulosic butanol requires cellulases to break down lignocellulose into fermentable sugars. Solventogenic Clostridia lack efficient cellulase secretion abilities, but cellulolytic Clostridia have the innate capability to degrade lignocellulose and generate not just ethanol and acetate, but also butyrate and potentially even n-butanol. The n-butanol production process frequently utilized is the ABE fermentation. Also, different target genes employed to manipulate Clostridium's metabolism in the development of novel microorganisms, and potent synthetic biology enzymes that exhibit high activity, high production, and butanol robustness are additionally explored. Sometimes, it is crucial to highlight all challenges encountered and utilize all of the experiences gained to develop a cost-effective and high-yielding procedure. The objective of this review is to examine the isolation and evolutionary adaptation of Clostridium species to produce butanol from cellulosic biomass.

Keywords: *Screening, Adaptive evolution, Clostridium, butanol synthesis, cellulosic biomass*

1. Introduction

Increased demand brought on by fast economic expansion, particularly in emerging nations like China and India, is the primary cause of the current energy problem. By 2030, India is anticipated to overtake China as the energy consumption that ranks third on a global scale (Zhang et al., 2020). The need to combat global warming and address energy scarcity on a global level has spurred the evolution of sustainable fuel sources like biomass, solar, wind, and Hydro alternatives to fossil fuels (Re and Mazzoli, 2023). Petroleum and coal, which are fossil fuels made from biomass, now provide most of society's energy demands (Figure 1) (Zhang et al., 2020). However, India's oil reserves are very limited, and the future of oil imports from the unstable Middle East is unclear owing to ongoing depletion and worries about national security (Srivastava et al., 2020). Hence, the discovery and use of alternative fuel sources are required owing to several challenges, including environmental, social, political, and geological ones.

By 2050, biomass is predicted to provide about half of all energy needs from alternative sources, with biodiesel becoming increasingly crucial for lowering carbon emissions (Srivastava et al., 2020; Tabassum et al., 2022). The current value of the energy markets is around $1.5 trillion.

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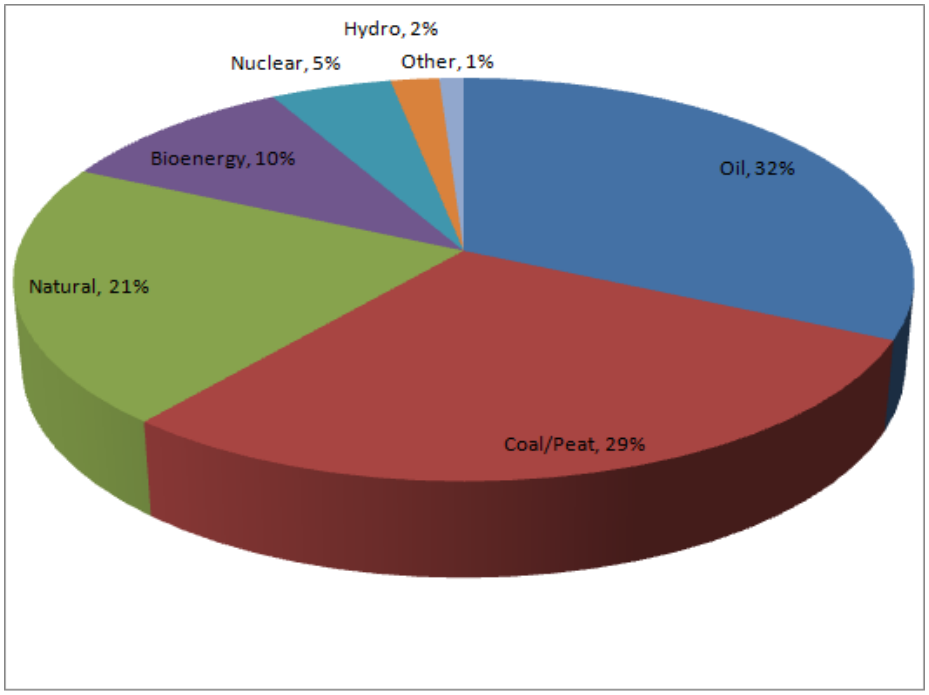

Fig 1: World's primary energy demand (Ho et al., 2014)

Biofuel industry has proliferated recently (Panahi et al., 2020). Due to its capacity as a direct substitute fuel, n-butanol has garnered significant research attention in recent decades. Solventogenic Clostridia are a common source for the production of butanol, an essential fuel and a promising substitute for renewable gasoline.

Lignocellulosic biomass, which encompasses waste from farming, cities, and industries, presents a substitute, ample, and cost-effective raw material for fermentation. The generation of microbial butanol through cellulosic biomass includes several processes, including cellulase secretion, cellulose enzymatic hydrolysis, pre-treatment, and co-fermentation of hexose and pentose. SDMA (S), coniferyl ethanol (G), and P-coumaric ethanol (H) are the three main phenolic components of lignocellulose, together with the three 5- and 6-carbon sugars xylose, hemicellulose, galactose, mannose, dextrose, and arabinose (Fig.2).

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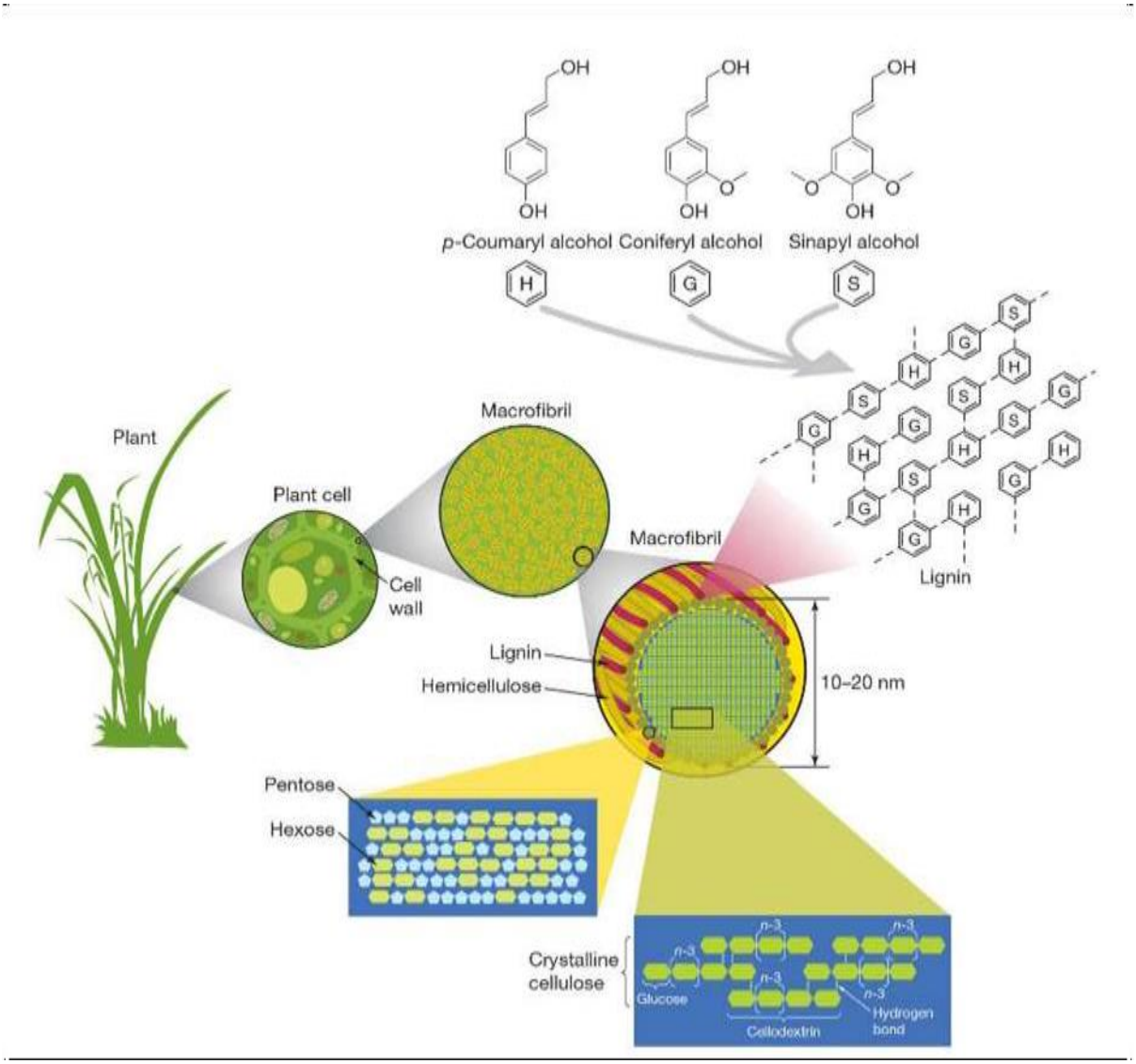

Fig. 2. The structure of lignocellulosic biomass (Salehi and Taherzadeh, 2015).

Gram-positive Clostridium species may produce butanol by fermenting a variety of substrates. Consequently, to produce butanol economically at industrial scales, innovative microbial biocatalysts with increased butanol tolerance are needed. In this study, it was described how microorganisms may be isolated and evolutionary adapted to butanol.

Cellulosic butanol fermentation incurs high costs due to the need for cellulases to saccharify lignocellulose, a process that solventogenic Clostridia cannot efficiently carry out by themselves, as noted by Wen et al. (2019). On the other hand, cellulolytic Clostridia are capable of naturally breaking down lignocellulose and generating butanol, ethanol, acetate, and even butyrate. Clostridia, specifically *Clostridium acetobutylicum* or *C. beijerinckii*, have employed ABE fermentation, to generate n-butanol as the primary product (Wen et al., 2020). The development of butanol exemplifies the end-product inhibition, whereby butanol titers exceeding 20 g/L become lethal to the microbes. This toxicity disturbs the membrane cell system, impeding development and ultimately hindering

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butanol generation. As a result of the lack of butanol engagement and efficiency in the broth, separating the butanol from the mixture presents a significant challenge (Cui et al., 2021).

The present comprehension of the cellulose-digesting enzymes and butanol metabolic pathways in cellulolytic organisms has been enhanced through the process of screening and isolation to produce butanol. In addition, genetic manipulation techniques specific to Clostridia offer an opportunity to engineer these metabolically adept microbes for n-butanol production. Therefore, this review aims to highlight the production of butanol, with a focus on adaptive evolution and ABE fermentation within native cellulolytic Clostridia.

2. Isolation and screening of solventogenic bacterium strains

Low butanol tolerance of solventogenic strains inhibits their butanol synthesis during fermentation at industrial scale. Isolation and characterisation are conducted as an effort to identify microorganisms with a high tolerance for butanol.

Butanol can be made by Gram-positive Clostridium species by fermenting different substrates. Therefore, novel microbial biocatalysts with higher butanol tolerance are required in order to manufacture butanol commercially at industrial scales. The isolation and adaptation of different microorganisms to butanol were discussed in this work (Liu et al., 2011). Eleven strains, including *Lactobacillus amylovorus* strain, *Pedioco ccusparvulus*, *Lactobacillus crispatus*, and *Weissella confusa*, were discovered to be capable of growing in 3-4% butanol after extensive adaption (Liu et al., 2011). These strains may be used to investigate how tolerance works as well as to find certain genes that respond to butanol stress in order to create butanol-tolerant microorganisms. 52 environmental samples in all were gathered and analysed for bacteria able to use plant biomass for solvent synthesis, according to a study by Berezina et al (2009). In cultures from potato, silt, and rye grain, acetone, butanol, and butyric acid were found. Following purification of single colonies on plates, 48 colonies were isolated. Novel isolate of solventogenic bacterium Clostridium saccharobutylicum displayed excellent hemicellulolytic activity, producing acetone and butanol. Among the industrial production strains, four unique species of clostridia were identified: Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharoperbutylacetonicum, and Clostridium saccharobutylicum (Al-Shorgani et al., 2016). Two strains with greater butanol tolerance than frequently used solventogenic Clostridium acetobutylicum were obtained using evolution and screening techniques (Liu et al., 2011).

3. Adaptive evolution approach for butanol production

Microorganisms may survive in hostile environments and swiftly adapt to new environmental circumstances, particularly via artificial evolution. Adaptive evolution has therefore become a significant tool for biotechnological applications in industrial processes, including as activating latent pathways, increasing resistance to harsh environments, exploiting non-native substrates, and enhancing productivity (Portnoy et al., 2011, Wang et al., 2016). For instance, Abdelaal et al. (2019) revealed that evolutionary adaptation triggered the butanol metabolism route in E.coli, resulting in a high butanol output. In a long-term experimental adaptive evolution procedure, the model cyanobacterium *Synechocystis* sp. PCC 6803 treated with steadily increased butanol concentration from 0.2% to 0.5% (v/v) achieved a 150% increase in butanol tolerance (Wang et al., 2014).Compared to solvent-producing clostridia, designing heterologous species such as Escherichia

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coli and Saccharomyces cerevisiae with butanol pathways might be a solution for reducing the generation of primary by-products, acetone and ethanol in order to greatly increase butanol output (Xue et al., 2013). Continuous operation is more productive for large-scale production of butanol as a biofuel than batch fermentation, but a single chemostat bioreactor and tanks-in-series cannot reach this aim for biphasic ABE fermentation.

In fact, a boost in butanol titer with ABE fermentation may greatly reduce energy consumption for medium sterilisation and stillage treatment due to the ability to employ concentrated medium and, as a result, a decrease in production system mass flow. Optimizing systems for different food waste and new strains is essential.

4. Butanol production through ABE fermentation

Over a hundred years ago, the solventogenic bacterium *Clostridium acetobutylicum* was used to industrially produce butanol through ABE fermentation of sugar or starch in a ratio of 3:6:1 for acetone, butanol, and ethanol, respectively. Recently, interest has increased in generating butanol from inexpensive renewable feedstocks due to the depleting supply and growing crude oil prices (Jang et al., 2012). The end-product of ABE fermentation, i.e., butanol, a biofuel that has better features than ethanol, including a greater energy density, reduced volatility, and good suitability with current energy networks and engines with self-ignition (Zhang et al., 2020; Wen et al., 2020). The ABE fermentation using *Clostridium* spp. from food waste (Fig 3; Jen et al., 2020), maize (starch) and black treacle (sucrose and glucose) was formerly the second-biggest fermentation process globally (Wen et al., 2014; Jin et al., 2020). Nevertheless, starch feedstocks account for roughly 70% of the total production costs in the traditional ABE fermentation method, which is now used in China (Bao, 2021).

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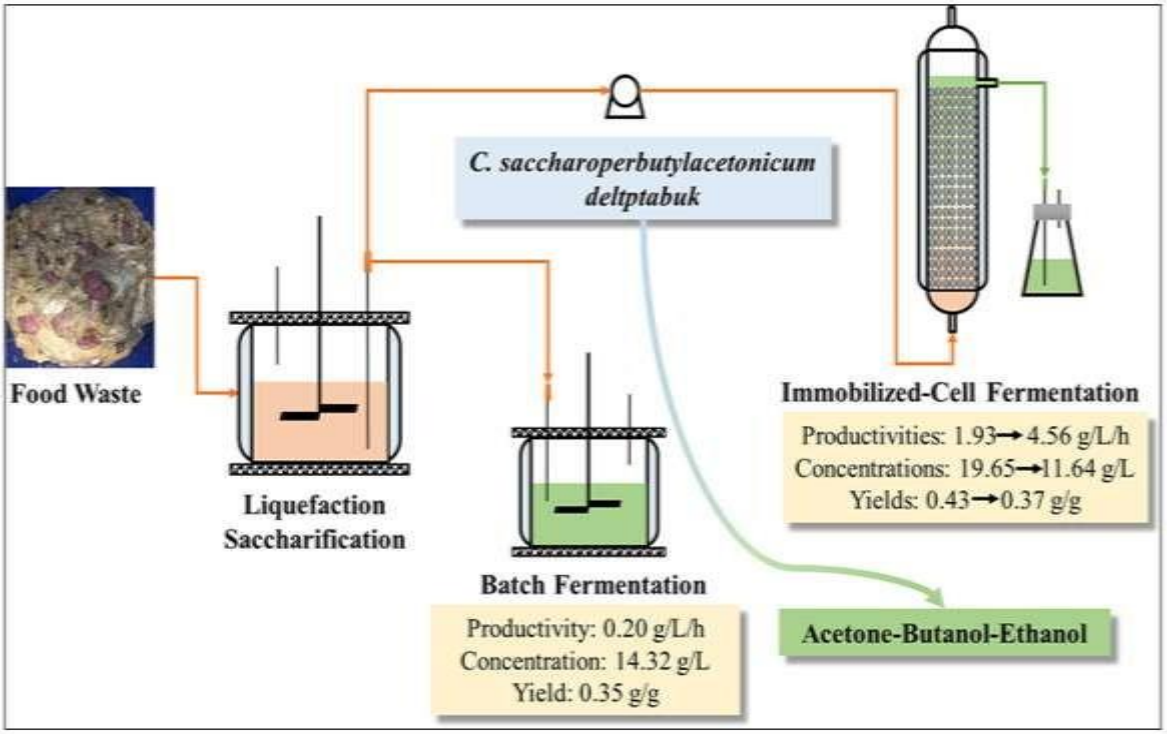

Fig. 3. Sustainable and economical production of butanol via acetone-butanol-ethanol (ABE) fermentation (Jin et al., 2020).

Moreover, the feedstocks' carbon content is converted to CO_2 at around 34%, leading to a relatively poor output yield. Potential new feedstocks for ABE fermentation, such as food waste due to their high starch content and abundance, have been identified for future use (Zhang et al., 2020). Moreover, recent studies have demonstrated the implementation of butanol production in three cellulolytic clostridia, including the mesophilic *Clostridium cellulolyticum*, which has been discussed in several reviews (Wen et al., 2019; Re and Mazzoli, 2023).

ABE production in work by Wen et al. (2014) was 5.68g/L of ABE (acetone 1.11, butanol 4.11, and ethanol 0.46) with *C. beijerinckii* and *C. cellulovorans* by using corn cob as substrate. On the other hand, *C. beijerinckii* ABE fermentation of cellulose yielded >4.5 g/L ABE, likely because of lack of rhamnose uptake. As a result, *C. acetobutylicum* struggled to ferment the hydrolysates effectively, as Van der Wal et al. (2013) observed.

However, butanol generation is not feasible economically by the ABE fermentation procedure. Severe end-product toxicity hinders butanol production in ABE fermentation, as highlighted by Bao (2021). Consequently, three interconnected challenges must be addressed to produce butanol efficiently:

(i) end-product toxicity,

(ii) the difficulty in separating butanol due to its low titer, and

(iii) ensuring a sufficient supply of feedstock.

5. Genetic manipulation of Clostridium for the purpose of butanol production.

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The challenges of ABE fermentation can be reduced using engineered cellulolytic microorganisms that can increase butanol production. It can overcome the challenges associated with energy and rural unemployment. The genetic modification required to engineer butanol production in the *C. cellulovorans* bacterium was more limited as it naturally possesses a butyryl-CoA pathway, a precursor for butanol production, as noted by Re and Mazzoli (2023). Overall, metabolic engineering techniques and the advancement of alternative strains of microbial that enable straight production of butanol through the fermentation of (hemi) cellulose generation have primarily relied on manipulation of genes. Genetic engineering is a three-step process:

- a quantitative study of how the organism works.
- genetic treatments meant to produce a particular result.
- an experiment to test the result.

Examples of these processes are shown by using thermophilic bacteria to produce butanol. Although, *Thermoanaerobacterium* and *Thermosaccharolyticum* were engineered to ferment crystalline cellulose, they only produced very low butanol concentration (<0.5 g/L) (Wen et al., 2020). So far, Clostridium has been metabolically engineered and used for butanol production in bulk (Re and Mazzoli, 2023).
The primary strain of ABE generating clostridia is *C. acetobutylicum* ATCC 824; genetic tools have been created that enable gene manipulation described in Table.1 (Wen et al., 2020). Other industrial microorganisms, including *E. coli*, *S. cerevisiae*, *P. putida*, *B. subtilis*, and *L. brevis*, have also expressed the n-butanol biosynthetic pathway for the manufacture of n-butanol (Holwerda et al., 2020). However, despite their advantageous characteristics, such as rapid growth of cell, good understood genetics, and average to elevated tolerance of butanol, these bacteria cannot directly utilize cellulose and are frequently tricky to utilize with lignocellulosic hydrolysates.Microbial consortia make up 99% of microorganisms in natural environments (Re andMazzoli, 2023). These groups of microorganisms can do more complex assignments than single variants of microorganisms via decoupling them into distinct strains (Cui et al., 2021; Bao et al., 2021).
Genetic manipulation techniques for metabolic enhancement have been used to enhance and improve strains or consortia for more efficient bioprocessing (Wen et al., 2020). To assess butanol resistance or improve the microbial community's producer composition, omics analysis, metabolic modelling, and other approaches have been applied. Cellulolytic Clostridium has used metabolic engineering in a cooperative culture of *C. cellulovorans* with *C. beijerinckii*, *C. cellulovorans*, which break down lignocellulose into fermentable sugars to promote the production and solvent fermentation of the sugars. In contrast, the sugars reassimilate and detoxicate butyrate for *C. cellulovorans*. Engineered organisms achieved higher butanol titers (i.e., 3.06 g/L) through cellulose fermentation, as reported by Bao et al. (2021). In the multicellular system, *C. cellulovorans* and *C. beijerinckii* formed a unique feeding-detoxification connection. They generated 8.30 g/l of n-butanol from an alkali-extracted concentration of 68.6 g/L deshelled corn cobs (AECC) in under 80 hours, demonstrating the potential of synthetic consortia in the biorefinery is promising (Wen et al., 2020).

TABLE 1: Production of n-butanol from different strains of Clostridium with genetic modification

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Microbial culture	Genetic engineering approach	Reactant	Concentration	Productivity	Reference
			(g/liter)a	(g/liter/h)	
C. acetobutylicum **American Type Culture Collection 824**	Optimizing the overexpression of chimeric cellulosomal operons	cellulose microfibrils	ND	ND	Wen et al., 2020
	Attaching carrier modules to the N-termini of foreign cellulases via grafting	cellulose microfibrils	ND	ND	Wen et al., 2019
	Incorporating synthetic cellulosomal operons into the genetic material through allele-coupled exchange	Cellohexose	ND	ND	Kovacs et al., 2013
	Using the natural sortase system to attach the recombinant cellulosome to the surface of the cell	raw wheat straw without any processing	ND	ND	Wilson et al., 2016
C. cellulolyticum **American Type Culture Collection 35319**	Implementing a pathway that depends on CoA	cellulose microfibrils	0.12	0.00025	Gaida et al., 2016
C. cellulovorans **DSM 743B**	Enhancing the expression of adhE2 obtained from *C. acetobutylicum* ATCC 824	cellulose microfibrils	1.42	0.0056	Yang et al., 2015
	Integrated genetic and evolutionary engineering	Corn cobs (alkali extracted)	3.47	0.0413	Wen et al., 2020

4. Conclusion

In conclusion, Clostridium species have been utilized for ABE fermentation to generate Acetone: Butanol: Ethanol derived from cellulose substrate. Adaptive evolution has therefore become a significant tool for biotechnological applications in industrial processes. Mostly, adaptive laboratory evolution is used to produce mutants with certain characteristics under predetermined pressure. Cellulose, flammable by-products of ABE fermentations are evaluated to ferment into a high yield, titer, and productivity of n-butanol. The most significant possible value that could be attained is 0.63 g/g of cellulose, boosting butanol production overall by 50%. The adaptively evaluated Clostridium strains

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may be employed for fermentation at a high-cell density approach combined with the online

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recovery of butanol to produce n-butanol from lignocellulosic biomass economically. If equivalent efficiency and butanol are achieved, a butanol fermentation method using manipulated Clostridium employing cellulosic biomass, such as maize stover, as inexpensive substrates would be far cost effective. The integrated process, with an increase in yield and productivity, has the potential to decrease the price of biobutanol by over 50% compared to the current ABE fermentation method using maize as a feedstock. Large-scale, cost-effective biobutanol not only helps to alleviate the oil crisis, but it may also have positive effects on the environment by reducing emissions and on agriculture by increasing farmer income.

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