APPROACHES TO ENHANCING BIOPRODUCTION USING LIGNOCELLULOSE BIOMASS: A MAJOR FOCUS ON BIOFUEL PRODUCTION

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Abstract

The demand for an efficient utilization of abundant biomasses is growing for the production of biogas and valuable bioproducts. Lignocellulose biomass is a cheap and most abundant carbon source for the production of biofuels such as bioethanol, biobutanediol, and other bio-based chemicals. Due to its complex heterogeneity, its hydrolysis gives rise to a mixture of sugars, mainly glucose; a hexose and xylose; a pentose. Glucose is the most abundant carbohydrate monomer. Most microorganisms have evolved the ability to utilize it preferably due to carbon catabolite repression regulatory mechanism at the detriment of the pentoses. Some microbes even lack the ability to utilize them. This has led to the sequential use of these sugars and accompanying reduced productivity due to inadequate utilization of the pentoses. Also, this sequential utilization of the sugars takes time and makes the overall processes economically costly. Since lignocellulose hydrolysates comprise both hexoses and pentoses, the catabolism of these sugar mixtures to biofuels will require an efficient microbial strain capable of simultaneous utilization. The use of CCR negative mutants can achieve this. CCR negative mutants simultaneously utilize pentoses and hexoses, ensuring an improved fermentation efficacy and greater productivity, thus, making the overall bioprocess economically feasible. This article reviewed several approaches employed in creating these mutant microorganisms. A brief insight on carbon catabolite repression and phosphostransferase system were made. It also highlighted the biogas production processes, factors affecting anaerobic digestion, lignocellulosic biomass structure, challenges with their use and solutions to overcoming the challenges.

Keywords: Biogas, anaerobic digestion, gene mutation, bioengineering, lignocellulose biomass

Abbreviations:
PTS: Phosphostransferase system
CCR: Carbon catabolite repression
LB: Lignocellulosic biomass
AD: Anaerobic Digestion
OLR: Organic loading rate
HRT: Hydraulic retention time

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C/N: Carbon-to-Nitrogen
VFAs: Volatile fatty acids
AC: Adenylate cyclase
ccpA: Catabolite control protein A
cAMP: Cyclic adenosine monophosphate
CRP: cAMP receptor protein
UV: Ultraviolet

1.0 Introduction

Biofuels are replacing the use of fossil fuels (Atabani *et al.*, 2014). Fossil fuels have been linked with deleterious climatic changes (Ramaraju and Kumar, 2011). They contain long-chain hydrocarbon and have been noted for increased generation of greenhouse gases such as carbon dioxide and carbon monoxide upon combustion (Kaisan *et al.*, 2014). These are environmental pollutants that have been related to several environmental catastrophes, including global warming and public health challenges (Kaisan *et al.*, 2014). In contrast, biofuels are more environmentally friendly and a cheap renewable source of energy (Sjostron, 2003). They contain shorter chain hydrocarbons that generate relatively few greenhouse gases upon combustion.

Agricultural biomass, other than cornstarch, can be used to produce biofuel (Sawyerr *et al.*, 2019). Biofuel production requires microorganisms that can ferment the mixture of sugars derived from hemicelluloses. Lignocellulose biomass is one of the most cost-effective and abundant renewable resources (Li *et al.*, 2010). Lignocellulose from biomass can be hydrolyzed to a mixture of sugars, mainly glucose and xylose, and arabinose and galactose (Sheehan and Himmel, 1999). Consequently, a biomass-to-ethanol process requires an organism that ferments the multiple sugars derived from hemicellulose (Wang *et al.*, 2010).

It has been reported that many bacteria such as *E. coli*, other enteric bacteria, and *Bacillus subtilis* have been used to ferment lignocellulosic hydrolysates to ethanol (Nichol *et al.*, 2001). However, xylose utilization in hydrolysate fermentation is delayed, slower than fermentation of pure xylose, and is often incomplete (Dien *et al.*, 2000). Xylose, ubiquitous in lignocellulosic biomass, is particularly problematic. Metabolism of other sugars is typically delayed when glucose is present in high concentrations because glucose is a preferred carbon and energy source for *E. coli* and many other microorganisms (Bren *et al.*, 2016). Repressed use of alternate substrates is termed catabolite repression or the glucose effect (Brückner and Titgemeyer, 2002; Stlilke and Hillen, 1999).

The glucose phosphotransferase system (PTS) transports and phosphorylates glucose and represses utilization of alternate substrates so that other sugars are not efficiently taken up and metabolized by the cell when glucose, the preferred substrate, is present. Lignocellulose comprises a mixture of sugars. It is not surprising that delayed metabolism of some substrates has been observed in the fermentation of biomass hydrolysates, thus making the design of the overall bioprocess cumbersome and inefficient (Ganesh *et al.*, 2012). Solving this problem demands that carbon catabolite repression negative mutants be created as they will facilitate the simultaneous utilization of both sugars, thus, saving time and overall economic cost.

Thus, this review elucidates the different genetic approaches employed in the creation of mutants highly efficient in simultaneous fermentation of pentoses and hexoses. It briefly highlights the compositions of lignocellulose, challenges to its use, anaerobic digestion technology, and the involved microorganisms for biogas production. The principle underlying the creation of CCR mutants were also explained.

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2.0 Structure of lignocellulosic biomass

Lignocellulosic biomass (LB) comprises three types of polymers: cellulose, hemicellulose, and lignin (Olatunji *et al.*, 2021; Sjöström, 2003). Cellulose is the most abundant constituent of the lignocellulosic biomass, composed of D-glucose linked by β-(1,4) glycosidic bond (Bajpai, 2016). It is recalcitrant to hydrolysis when existing at the state of high-ordered structure and crystallinity but susceptible to degradation in its amorphous form (Chundawat *et al.*, 2011). Hemi-cellulose is the second abundant polymer in LB, which is bound to lignin and cellulose, thus increasing the compactness of the entire cellulose-hemicellulose-lignin network (Hansen & Plackett, 2008). Hemicellulose differs from cellulose by having a low molecular weight and varying in abundance in different plant species (Kumar and Dixit, 2021; Lu *et al.*, 2021). In agricultural biomass, such as grass and straw, hemicellulose is mainly xylan, while softwood hemicellulose contains mainly glucomannan (Bajpai, 2016). Lignin is another abundant polymer in LB after cellulose and hemicellulose, which has a three-dimensional amorphous polymer consisting of methoxylated phenylpropane structures (Chakar and Ragauskas, 2004). It adds strength and rigidity to cell walls and protects cellulose and hemicellulose from enzymatic hydrolysis (Ghaffar and Fan, 2013). Lignin is sparingly soluble in water and, therefore, has low degradability (Iqbal *et al.*, 2011).

3.0 Biogas Production Process

Anaerobic Digestion (AD) is the technique used to produce biogas from lignocellulosic biomass. AD involves a series of biological processes in which microorganisms convert biodegradable fractions to biogas (mixture of CH_4, CO_2, H_2S, etc.) without molecular oxygen (Sawyerr *et al.*, 2019). The AD process can be divided into four stages viz: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Angelidaki *et al.*, 2011).

3.1 Hydrolysis

This involves breaking down complex organic substances (cellulose, hemicellulose, and lignin) in lignocellulosic biomass into soluble monomers and dissolving the resulting small molecules into solution (Gerardi, 2003). Hydrolysis is the necessary first step of anaerobic digestion (Sleat *et al.*, 2006).

3.2 Acidogenesis

This involves the breaking down of the remaining component by acidogenic bacteria. In this stage, the hydrolyzed products are catabolized through different metabolic pathways and produce intermediates, including volatile fatty acids (VFAs), Hydrogen, Carbon dioxide, and alcohols (Tsapekos *et al.*, 2017).

3.3 Acetogenesis

In the third stage of AD, volatile fatty acids (VFAs) and alcohol generated from the acidogenisis stage are further used by acetogens to produce acetic acids, CO_2, and H_2 (Parawira *et al.*, 2005; Gerardi, 2003). The process is obligately syntrophic with methanogens that utilize the produced hydrogen or formate to produce methane (Batstone *et al.*, 2006).

3.4 Methanogenesis

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Methanogenesis is the last step in AD, where two groups generate methane. The first group (Acetotrophic archaea) convert acetate (CH_3COOH) to methane (CH_4) and carbon dioxide (CO_2) directly, while the second group (Hydrogenotrophic archaea) use hydrogen (H_2) (as electron donor)and carbon dioxide (CO_2) (as an electron acceptor) to produce methane (CH_4) (Appels *et al*., 2008).

4.0 Microorganisms involved in Anaerobic Digestion of Lignocellulosics

Many types of anaerobic bacteria have been found to have the ability to utilize lignocellulose as a carbon source. These can be found in genera such *as Clostridium, Ruminococcus, Fibrobacter, Acetivibrio, Butyrivibrio, Halocella, Bacteroides, Spirochaeta, Thermotoga, Echinicola, Mahella, Marinilabilia, Prevotella, Flavobacterium, and Streptomyces* (Bhujbal *et al*., 2022; Chukwuma *et al*., 2021;Tsavkelova and Netrusov, 2012). In an anaerobic digestion process operating with lignocellulosic materials as the primary substrate, the relative abundance of these different genera can vary depending on factors such as the composition of the substrate, process configuration, and operating parameters. However, the phyla Bacteroidetes and Firmicutes often dominate, followed by phyla such as Proteobacteria and Actinobacteria (Güllert *et al*., 2016). Among these organisms, studies have shown that the genus *Clostridium* plays a crucial role in Lignocellulose digestion (Güllert *et al*., 2016; Lü *et al*., 2014).

Table 1: Reported microorganisms involved in the different stages of anaerobic digestion of lignocellulosics.

AD Stage	Microorganisms	References
Hydrolysis	*Clostridium*	
	Proteus vulgaris	Tahseen *et al*., 2019
	Bacillus	Azman *et al*., 2015
	Bacteroides	
	Micrococcus	
	Staphylococcus	Smith, 1966
	Enreobacterium	Bryant, 1979
Acidogenesis	*Pseudomonas*	
	Bacillus	
	Clostridium	Fayyaz *et al*., 2014
	Micrococcus	
	Flavobacterium	Tahseen *et al*, 2019
	Escherichia	Tsapekos, 2017
	Lactobacillus	
	Desulfobacter	
	Eubacterium limosum	
	Sarcina	
	Veillonella	

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Acetogenesis	*Syntrophomonas* *Syntrophobacter* *Methanobacterium*	Fayyaz *et al*., 2014 Schink, 1997
Methanogenesis	*Methanobacterium* *Methanoplanus* *Methanospirilium* *Methanosaeta* *Methanosarcina*	Angelidaki *et al*., 2011

AD= Anaerobic Digestion

5.0 Challenges with the Use of Lignocellulosic Materials as a Substrate for Biogas Production and Solutions

During anaerobic digestion using lignocellulosic materials as feedstock, hydrolysis, the first step of the process becomes rate-limiting due to the recalcitrant nature of lignocellulose (Mulat and Horn, 2018). Furthermore, lignocellulosic materials are low in nutrients compared to other substrates, resulting in low methane yield (Li *et al*., 2013). Substrate optimization (which involves pretreatment and co-digestion) and strain improvement have been suggested to overcome these challenges (Olatunji *et al*., 2021; Mulat *et al*., 2018; Harner *et al*., 2015; Čater *et al*., 2014; Adrio and Demain, 2006).

5.1 Pretreatment of lignocellulosic biomass

Lignocellulosic biomasses are usually pretreated prior to AD to reduce the biomass recalcitrance to microbial degradation and increase the efficiency of AD conversion (Zheng *et al*., 2014). Pretreatment increases the biodegradability of lignocellulosic materials by removing lignin, hydrolyzing hemicellulose, decreasing cellulose crystallinity, increasing the porosity of materials, and making the material more accessible to microbial and enzymatic attack (Monlau *et al*., 2013). There are various pretreatment options which could be thermal, chemical, biological, or physical (Zheng *et al*., 2014). Combinations of two or more pretreatments methods can also be used. Some pretreatment methods show apparent advantages over others. For instance, hydrothermal with alkali pretreatment appears to be one of the most favorable pretreatment methods for converting lignocellulosic biomass to biogas through AD (Paul and Dutta, 2018).

5.2 Co-digestion

Lignocellulosic materials are characterized by a high Carbon-to-Nitrogen (C/N) ratio and low micronutrients and energy content (Li *et al*., 2013). Through co-digestion (digesting with other substrates), the substrate mixture can be designed to optimize the composition of nutrients, balance the C/N ratio and achieve higher methane yields (Ebner *et al*., 2016). For example, lignocellulose-rich cattle manure has been co-digested with food waste (Awasthi *et al*., 2018), and co-digestion has been shown to give enhanced methane yield compared with mono-digestion of the manure. The co-digestion of lignocellulosic materials with low C/N ratio with nitrogen-rich animal manure has been shown to improve these materials' process stability and volumetric biogas yield (Neshat *et al*., 2017; Zhang *et al*., 2013).

6.0 Factors Affecting the Anaerobic Digestion Process

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The most important factors affecting the rate of the anaerobic digestion process are temperature, pH, organic loading rate (OLR), Hydraulic retention time (HRT), and Carbon-to-Nitrogen (C/N) ratio.

6.1 Temperature

Anaerobic digestion occurs in a wide range of temperatures, from psychrophilic (<20^{o}C) mesophilic (25-40^{o}C) and thermophilic (45-60^{o}C) temperatures (Khalid *et al.*, 2011; Mathew *et al.*, 2014). Increased temperature in the bioreactor increases the product's hydrolysis, making them accessible to microorganisms, increasing the reaction kinetics in both the chemical and biological processes, shortening the reaction time, and reducing the hydraulic retention time (HRT) (Abdelgadir *et al.*, 2014). Furthermore, thermophilic temperature also increases the death rate of pathogenic bacteria, reducing the time required for pathogens' destruction in the AD process (Smith *et al.*, 2005). However, High temperature has some adverse effects on the AD process: increased temperature increases the fraction of free ammonia, which is inhibitory to microorganisms, Mesophilic temperatures are more stable than thermophilic temperatures and are more employed in the current AD facilities, but the process is achieved at a longer retention time (Ostrem *et al.*, 2004).

6.2 pH

There are three major types of bacteria involved in biogas production through AD: hydrolytic bacteria, fermentative bacteria, and methanogenic archaea. The fermentative bacteria can function in pH range from 8.5 down to pH 4, with their optimal pH range being 5.0–6.0 (Hwang *et al.*, 2004); on the other hand, methanogenic archaea can function in a pH range of 5.5 to 8.5 with an optimal range of 6.5–8.0 (Boe, 2006). pH inhibition occurs due to disruption of pH balance and increased levels of non-dissociated VFA (Batstone *et al.*, 2002). The bicarbonate produced by the methane-producing bacteria normally neutralizes the pH reduction caused by acid-producing bacteria (Liu and Tay, 2004).

6.3 C/N Ratio

All microorganisms involved in anaerobic digestion require essential elements for their growth. One of the most important elements is nitrogen, which is required to synthesize amino acids and proteins, which is converted into ammonia, a compound that is instrumental for the neutralization of the acidification process. Rajeshwari *et al.* (2000), reported that a C: N:P ratio of 100:3:1 is essential for suitable methane yield. A noticeable deviation from this ratio could lead to a deficiency of buffering capacity and a lack of nutrients for the growth of the microorganisms. Lignocellulosic biomass, such as straws, usually is carbon-rich (higher C/N ratio), while substrates such as food waste and chicken/pig manure usually have very low C/N ratios. The typical approach for balancing the C/N ratio is the co-digestion of carbon-rich and nitrogen-rich substrates under optimized mixing ratios (Abouelenien *et al.*, 2014).

6.4 Organic Loading Rate

OLR refers to the amount of organic material per unit reactor volume, which is subjected to the AD process in the reactor in a given unit period (Orhorhoro *et al.*, 2018). It can also be defined in terms of the kilograms or grams of volatile solids (VS) per day in cubic meter or litre of reactor volume. A fast microbial growth occurs at a high OLR, while at low OLR, microbial starvation and slow microbial growth occur. However, if the applied OLR is too high, the

microorganism cannot use up all produced organic acids and causes an acidic state of the digester (Liu and Tay 2004). Overloading with organic materials may also cause accumulation of volatile fatty acids (VFAs), as the methanogenic step cannot keep up with the acidogenic and acetogenic steps (Franke-Whittle *et al.*, 2014).

6.5 Hydraulic Retention Time

The Hydraulic retention time (HRT) measures the average length of time that a soluble compound remains in a constructed bioreactor (Nelabhotla *et al.*, 2020). The HRT varies in different biogas digesters and normally ranges from 10 to 30 days but is sometimes longer (Mao *et al.*, 2015). The actual size of the HRT of a bioreactor depends on many different factors, such as the characteristics of the feedstock used and the operating temperature. Due to the recalcitrant nature of lignocellulosic materials which limits hydrolysis efficiency in an anaerobic digestion process, a comparatively long HRT (>30 days) is typically needed in lignocellulosic digestion (Shi *et al.*, 2017).

6.6 Microbial Improvement

The improvement of microbial species is essential for efficient desired product formation.

6.6.1 The concept of the Carbon Catabolite Repression (CCR) and the Phosphotransferase System (PTS)

The process of sugar utilization in Gram positives and Gram-negatives, although related, follows different mechanisms. The uptake of these sugars employs the phosphotransferase system, and sugar utilized through this way is known as a PTS sugar (Saier Jr., 2015; Deutscher *et al.*, 2006; Stulke *et al.*, 1998). This sugar uptake is facilitated by a number or cascade of PTS enzymes. These enzymes include the EI and HPr. HPr has dual properties or activities in gram positives as kinase (for secondary phosphorylation at serine 46) or phosphatase (Hueck and Hillen, 1995; Stulke *et al.*, 1998). Also involved is the EII complex.

In both Gram negatives and positives and the presence of glucose, there is a transfer of ATP in the form of a phosphoryl group from PEP through enzyme 1, E1 (pyruvate kinase) to HPr, then EIIA, EIIB, and finally to the glucose (charging) through the glucose specific permease; EIIC. This is also known as primary or basal level phosphorylation (Deutscher *et al.*, 2006). As this continues, in Gram-negatives, the bulk of the EIIA is in their unphosphorylated state and triggers inducer exclusion (Brückner and Titgemeyer, 2002). This prevents the synthesis of catabolic enzymes for utilizing secondary or not preferred carbon sources, pentoses in this case.

In Gram positives, phosphorylation of glucose further leads to continued accumulation of fructose-1,6-bisphosphate, a catabolic intermediate. As a result, serine kinase is triggered, leading to autophosphorylation of Hpr at serine 46. Serine 46 dimerizes and binds to the catabolite control protein A (ccpA). The complex formed binds to the *promoter's cre* site, causing the repression of the gene for secondary or alternate carbon sources (Galinier *et al.*, 1998). These two scenarios depict the roles EIIA and Hpr serine 46 phosphocarrier protein play in the repression of catabolic genes or enzymes for alternate carbon sources making the genes that encode them possible sites for creating CCR negative mutants.

In the absence of glucose, the bulk of the $EIIA^{Glu}$ will remain in their phosphorylated state in the gram negatives. This consequently activates the membrane-bound adenylate cyclase (AC), which converts the phosphoryl group in ATP form to cyclic adenosine monophosphate (cAMP) (Stulke *et al.*, 1998). As cAMP accumulates, it binds to the catabolite or cAMP repressor or activator

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protein (CRP or CAP). cAMP is required for the functional dimerization of CRP (Tagami and Aiba,1995). The cAMP-CRP complex formed subsequently binds to the promoter, enhancing the transcription of genes for the synthesis of catabolic enzymes to utilize secondary or alternate carbon sources (Stulke *et al*., 1998). In Gram positives, once glucose concentration reduces, serine kinase activity will be lost while the activity of Hpr phosphatase will be regained (Vadeboncoeur and Pelletier, 1997). The phosphatase, in a dissociative but none hydrolytic manner, act on the serine-phosphate complex, with ccpA liberating it from the *cre* site and preventing repression for secondary carbon sources. This is known as de-repression (Deutscher *et al*., 2006).

As noted earlier, the enzymes cascade involved in PTS includes the basic PTS enzymes, which consist of 2 cytoplasmic proteins called EI and HPr. The PTS enzyme genes encode these proteins (Stulke *et al*., 1998). These genes include (i) gene *pts*I that encode EI (enzyme I) and (ii) *pts*H (*pts*HI and *pts*HII) that encode HPr (Heat-stable proteins), and the *Crr* gene that encodes EIIA, all of which constitute an operon (De Reuse and Danchin, 1988). Enzyme II (EII) is a sugar-specific permease that consists of at least three structurally distinct domains—EIIA, EIIB, EIIC, and sometimes EIID (Saier Jr and Reizer, 1992). The genes that encode them are dependent on the sugar present. For instance, *man*l gene encodes $EIIAB^{mannose}$ while *man*MN encodes for C and D domains (Abranches *et al*., 2006), and these genes are different when glucose is involved e.g., *pts*G codes for the PTS subunit $EIIBC^{Glc}$ (Zeppenfel *et al*., 2000; Nuoffer *et al*., 1988).

A good insight into the physiology of the genes involved in PTS may give ideas on ways of creating these mutant microorganisms. This is because if a gene's (parent or wild) function is known, it can be possible to deduce its phenotype when mutated (Adrio *et al*., 2006, Alberts *et al*., 2002). Therefore, creating mutants of the respective genes involved in the phosphotransferase system would likely give microbial strains that could facilitate the fermentation of mixed sugar streams, thus, eliminating diauxic growth (Nichols *et al*., 2001). With this in mind, researchers and biotechnologists seeking a solution will be able to construct or engineer microorganisms that possess pentose utilization and carbon catabolite repression negative phenotypes. This has been reportedly achieved through direct gene mutation, implantation of metabolic pathways using plasmids, physical adaption, etc., as elaborated below.

6.6.2 Approaches to Mutants Creation for Enhanced and Simultaneous Lignocellulosics Fermentation

Engineering of microorganisms for biofuel and bioproducts products has been by the modification of the existing pathway, insertion, substitution, or deletion of necessary genetic elements. Through mutagenesis, mutants capable of simultaneous utilization of pentoses and hexoses have been created as follows;

a. Inactivation of the glucose PTS (*pts*G) gene

Due to deletion mutation or inactivation of *pts*G (figure 1 and 2) gene encoding the major glucose transporter $EIIBC^{Glu}$ involved in PTS, the mutants obtained lacked the molecular machinery for carbon catabolite repression (Gosset, 2005). They also reported that many of the mutants obtained co-utilized glucose and xylose simultaneously, but at the expense of glucose utilization rates. However, this would require an increased expression of secondary glucose transporter and glucokinase to recover deficits in the PTS mutant strain.

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Inactivation of *pts*G that encodes EIIBC^{Glu} implies that the bulk of EIIA^{Glu} would remain in its phosphorylated state. In Gram-negatives, for instance, *Escherichia coli*, adenylate cyclase will be activated to convert phosphate in ATP form to cAMP, increasing its level (Reddy and Kamireddi, 1998). This prevents inducer exclusion by alerting the cell, although falsely, to an alternate carbon source. cAMP then binds catabolite or cAMP receptor protein (CRP), enhancing its functional dimerization and expression of genes for alternate carbon sources. While at the same time, glucose is used.

b. Mutation of a non-PTS *crp* gene

This simple metabolic engineering strategy involves *crp* genes (see figure 1) of efficient fermenters of lignocellulose hydrolysates. The *crp* gene, encoding the mutant cyclic adenosine monophosphate (cAMP) receptor protein CRP, which does not require cAMP for functioning, has been characterized and overexpressed in *Klebsiella oxytoca* (Ji *et al.*, 2011). The engineered recombinant could simultaneously utilize a mixture of glucose and xylose without CCR. This is because a mutant CRP does not require cAMP for functional dimerization, and in other words, a mutant CRP contributes to a non-cAMP-dependent phenotype. Upon examination, the profiles of sugar consumption and 2, 3-Butanediol (2, 3-BD) production by the engineered recombinant, in glucose and xylose mixtures showed that glucose and xylose could be consumed simultaneously to produce 2, 3-BD (Ji *et al.*, 2011). This can be attributed to the fact that in enteric bacteria, the use of sugar is transcriptionally regulated by CRP (Balat and Balat, 2009; Jojima *et al.*, 2010). The active form of CRP is a homodimer requiring cAMP for the functioning (Tagami and Aiba, 1995). It has been shown that the mutations of CRP were located within the region of the protein known to be involved in functional dimerization with cAMP (Ji *et al.*, 2011), and thus, conferred a cAMP-independent phenotype. Therefore, in the recombinant, the overexpressed CRP did not require cAMP for functional dimerization and played the regulatory role in the absence of cAMP. Thus, it could replace the native CRP and facilitate xylose uptake from mixtures of glucose and xylose. The CRP phenotype could promote xylose uptake in the presence of glucose by activating the native xylose transporters and by activating other CRP-controlled promiscuous transporters capable of xylose uptake.

c. Mutation of non-PTS Catabolite Control Protein A (ccpA)

In low-GC gram-positive, a ccpA mutant (see figure 2) has been insensitive to enzymes that drive carbon catabolite repression (Deutscher *et al.*, 1998). ccpA, like CRP or CAP in gram negatives, is a global control protein affecting a large number of catabolic genes/operons (Schumacher *et al.*, 2011). In the presence of glucose, secondary phosphorylation of the serine residue 46 of HPr occurs. The phosphorylated serine 46 has been reported to dimerize and bind to the cre sites on the promoter of the catabolic operon causing the repression of genes for alternate carbon sources (Deutscher *et al.*, 1998). This implies that ccpA plays a role in carbon catabolite repression. A ccpA mutant may, due to inactive *ccpA* gene, prevent the binding of the serine dimers and subsequent binding to the cre site, thus, derepression of other carbon catabolic genes (Zeng *et al.*, 2013; Muscariello *et al.*, 2001). This ensures that the hexoses and pentoses are utilized simultaneously.

d. Mutation of *pts*H gene

The phosphorylation of Hpr at serine 46 (secondary phosphorylation) has been directly or indirectly related to catabolite repression (Monedero *et al.*, 2001). Mutation of *pts*H gene (see

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figure 2) has been reported to confer catabolite repression resistance to some catabolic genes of gram-positive bacteria such as *Bacillus subtilis* (Lorca *et al.*, 2005; Galinier *et al.*, 1997). This mutation leads to the loss of protein kinase-catalyzed phosphorylation of Hpr, a phosphocarrier protein of the PTS.

In gram-positive bacteria, HPr, is phosphorylated by an ATP-dependent, metabolite-activated protein kinase on seryl residue 46 (Deutscher *et al.*, 1995). In a *Bacillus subtilis* mutant strain in which Ser-46 of HPr was replaced with a non-phosphorylatable alanyl residue (*ptsHl* mutation), synthesis of gluconate kinase, glucitol dehydrogenase, mannitol-l-P dehydrogenase, and the mannitol-specific PTS permease was wholly relieved from repression by glucose (Deutscher *et al.*, 1994). The observed phenotype is similar to that seen in *pts*HI mutation. There is also a possibility of mutation of *ptsH11*, which codes the primary phosphocarrier protein of HPr phosphorylated at histidine-15 (Jones *et al.*, 2008).

e. Mutation of *mlc* gene

mlc is a transcriptional regulator that controls the expression of some genes encoding enzymes of the *Escherichia coli* phosphotransferase (PTS) and phosphoenolpyruvate (PEP) system (Plumbridge, 2002). Mlc represses several glucose-related genes, including the phosphotransferase system (PTS) genes ptsHI and ptsG (Plumbridge, 2002). It also regulates genes involved in the uptake of glucose.

Mutation of the *mcl* gene by nucleotide substitution at the promoter region causes overexpression of mcl and shortage of ptsG (Nakashima *et al.*, 2012). This impedance of ptsG is known to induce a CCR-negative phenotype to the organism. They also reported that when the mcl mutant strain was fed with mixed sugars (glucose-xylose mixed sugar), the mutant strain produced 1.4-fold more isobutanol than the parent wild-type. Also, the mcl mutant strain produced similar or greater isobutanol than other CCR-negative strains (Nakashima *et al.*, 2012).

f. Deletion of the Methylglyoxal synthase gene (msgA)

Methylglyoxal synthase is an enzyme that catalyzes the formation of methylglyoxal from Dihydroxyacetone phosphate through the methylglyoxal pathway (an offshoot of glycolysis found in some prokaryotes in excess glucose condition, which converts glucose into methylglyoxal and then into pyruvate, without the production of ATP). Methylglyoxal synthase also increases the severity of catabolite repression by glucose (Yomano *et al.*, 2009). Methylglyoxal (the product of the reaction catalyzed by methylglyoxal synthase) is also believed to function as a general inhibitor of sugar metabolism during metabolic imbalance (Zhu *et al.*, 2001). Deletion of *mgsA* has been reported to increase the fermentation rate of ethanologenic *E. coli* by accelerating the co-metabolism of hexose and pentose sugars (Nieves *et al.*, 2015; Yomano *et al.*, 2009). The *msgA* deleted strain showed accelerated sugar metabolism in a mixture of glucose, xylose, arabinose, mannose, and galactose (Yomano *et al.*, 2009).

g. Implantation of the metabolic pathway

Alterations in sugar utilization patterns have also been observed when the mutant was transformed with a plasmid carrying the genes for ethanol production and tolerance, and used to ferment a sugar mixture (Chaves *et al.*, 2019; Kern *et al.*, 2007). The implantation of xylose metabolic pathway in *Corynebacterium glutamicum* using constructed plasmids (Kawaguchi *et al.*, 2006) is an example of this. This is possible mainly because the expression and regulation of

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exogenously sourced genes are not under the government of the host cell (Sanchez and Demain, 2008).

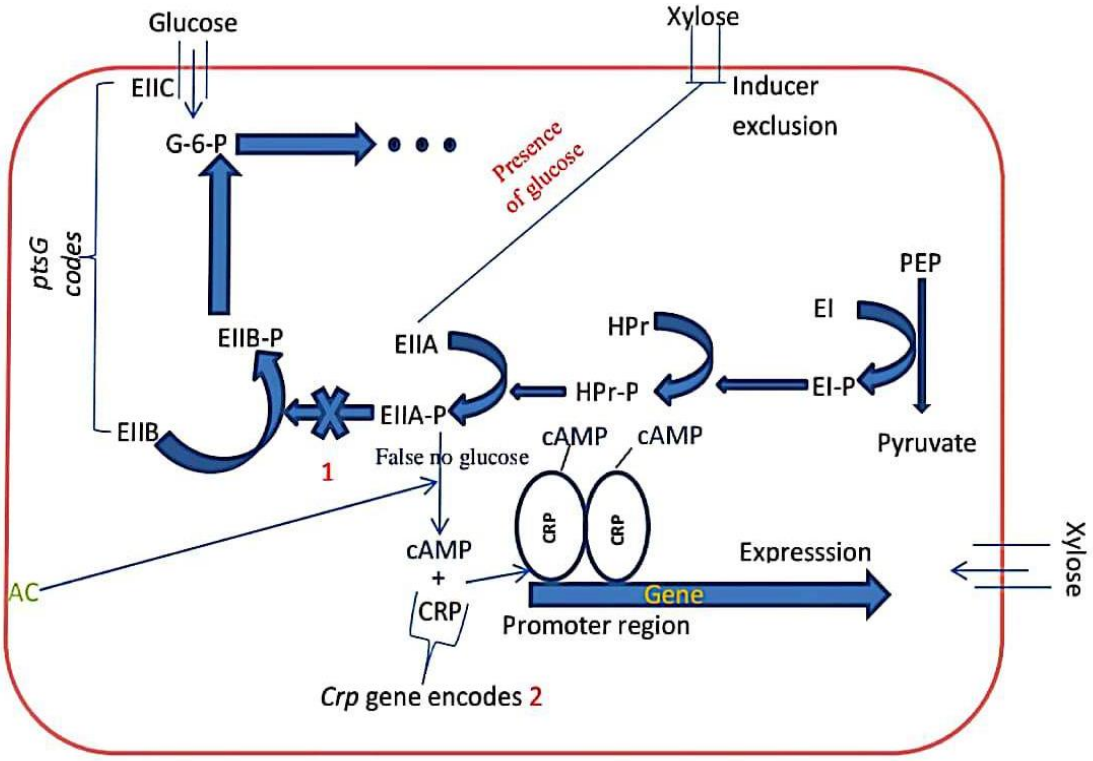

Figure 1. Catabolite repression negative mutant creation in amenable Gram-negatives. 1 and 2 are sites of mutative effects for *ptsG* and *crp* mutants, respectively. They follow similar mixed sugar fermentation pathways as indicated in gram positives below.

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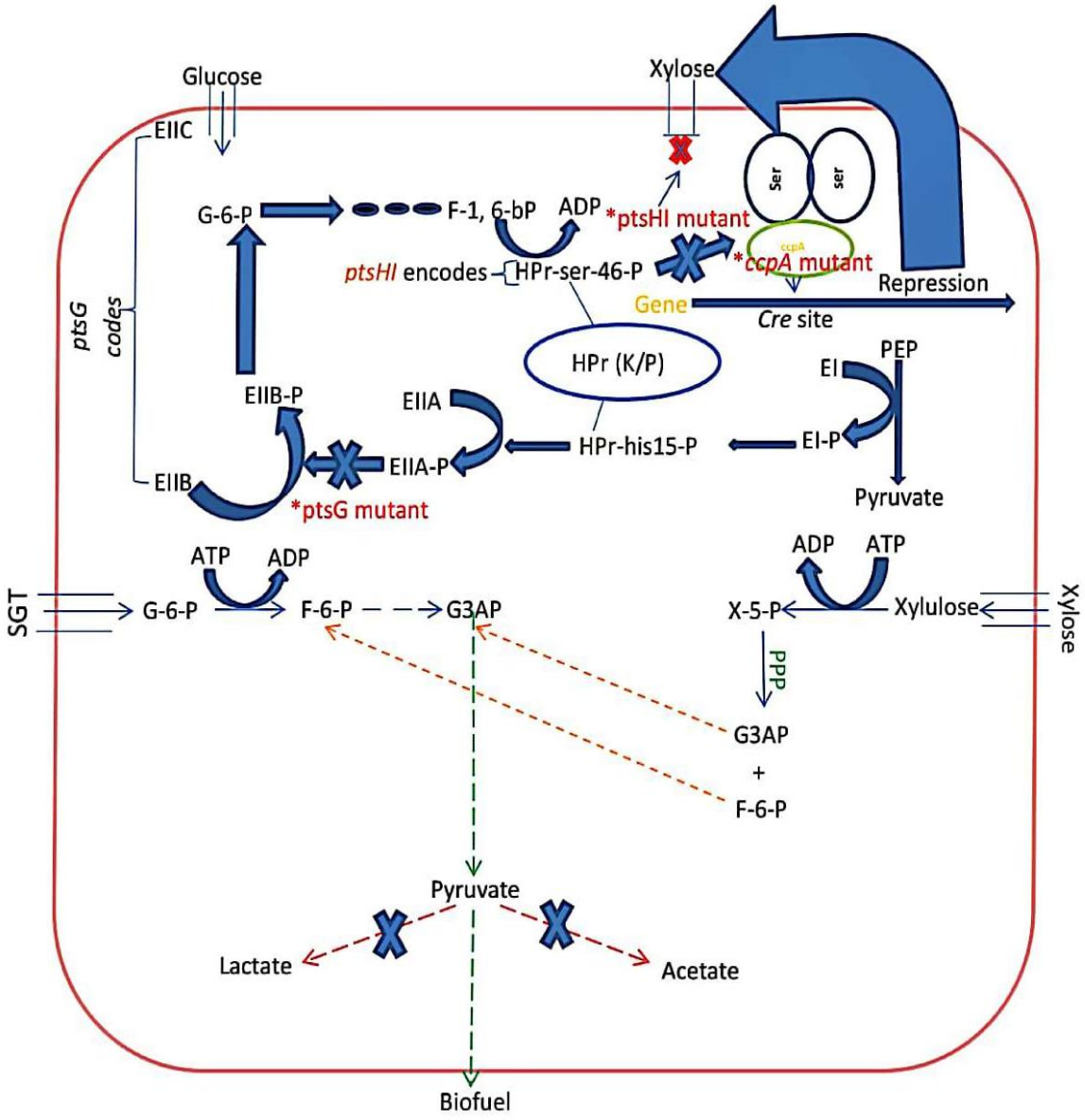

Figure 2. Sites of possible gene mutations leading to the generation of different (ptsG, ptsHI, and ccpA mutants) in amenable Gram positives and the catabolic pathways for the hemicellulose hydrolysates showing knocked out genes for undesired products formation. [SGT(secondary glucose transporters), G-6-P(glucose-6-phosphate), F-6-P(fructose-6-phosphate), G3AP(glycerol-3-acetone phosphate), X-5-P(xylulose-5-phosphate), F-16-bp(fructose-1,6-bisphosphate), ATP(adenosine triphosphate), ADP(adenosine diphosphate)]

Generally, mutagenesis has been achieved through many approaches. Ultraviolet (UV) mutation has been used to enhance *Clostridium thermocellum* (Tailliez *et al.*, 1989). Several chemical

methods have also been used to create CCR negative mutants. For instance, a CCR negative mutant has been reported after subjecting *E. coli* to a medium containing nitrosoguanidine (Ruiz-Vázquez and Cerdá-Olmedo, 1980).

Aside specific gene mutation and pathway implantation approaches discussed earlier, physiological adaptation to xylose- or lignocellulose- rich environment is a non-direct gene-based process of achieving simultaneous catabolism of lignocellulose hydrolysates and overcoming the effect of lignocellulosic inhibitors (Narayanan *et al.*, 2016). This can be achieved by prolonged subjection or exposure of a microbe to a medium containing the non-preferred sugar (in this case, a pentose such as xylose) as the only carbon source. This has generated CCR negative strains that are efficient in the utilization of hydrolysates containing glucose and xylose rather than using glucose preferentially due to changes in physiology (Nichols *et al.*, 2001).

It is also worthy of note that even after a mutant has been engineered to utilize hexoses and pentoses simultaneously, it can still produce bioproducts other than the desired one, thus, reducing the final overall quantity of the desired product (Scully and Orlygsson, 2014). This, then, means that there is the need to engineer the metabolic pathway to the final desired product. This can be facilitated by the knockout of genes responsible for the synthesis of enzymes to produce undesired products. Gene knockout is a way of creating cells with minimal function ability, an attribute efficient in the conversion of sugars to the desired final product (Jarboe *et al.*, 2010).

7.0 Prospects

It is evident that every cell's metabolic activities are driven by both endogenous and exogenous genes products if present. Understanding the roles concerned genes play in every metabolic pathway is particularly necessary. This will further guide the idea of metabolic engineering in shaping the genes to generate a particular desired product.

Several genes have been implicated in the processes that lead to biofuel generation. Manipulating these genes by mutation leads to creating mutants that are more specific to a product generation. This is supported by the fact that the knockout of genes of a parent strain reduces functionality and makes its activities more direct and specific. Consequently, mutation of one or more PTS genes and none PTS genes like *ccpA, crp,* etc., could lead to mutants with better-mixed sugar fermentation ability and increased generation of the desired product.

8.0 Conclusions

The bioconversion process of lignocellulosic to biofuels and other value-added bio-products could be successfully developed and optimized by aggressively applying these novel sciences and technologies to solve the known fundamental problems of the conversion process. Also, establishing a balance between substrate concentration and microbial substrate saccharification and fermentation capacity is important for efficient bioconversion of lignocellulose biomass. There is need to explore more microbial genetics to develop strategies towards unlocking efficient lignocellulosic biomass biorefinery as it promises huge economic benefits.

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