

## Accepted Manuscript

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PII: S2589-014X(19)30114-8  
DOI: <https://doi.org/10.1016/j.biteb.2019.100224>  
Article Number: 100224  
Reference: BITEB 100224  
To appear in: *Bioresource Technology Reports*  
Received date: 28 March 2019  
Revised date: 30 April 2019  
Accepted date: 3 May 2019

Please cite this article as: N. Vivek, L.M. Nair, B. Mohan, et al., Bio-butanol production from rice straw – Recent trends, possibilities, and challenges, *Bioresource Technology Reports*, <https://doi.org/10.1016/j.biteb.2019.100224>

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**Bio-butanol production from rice straw – recent trends, possibilities, and challenges**

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### Abstract

Increase in concerns over greenhouse gas emissions and depletion of fossil fuels has led to the search for alternative strategies of energy. Rice straw mainly composed of cellulose, hemicelluloses, and lignin, is one of the surpluses available lignocellulosic biomass that can serve as a potential feedstock for the production of bio-butanol. One of the main challenges in the conversion of rice straw to bio-butanol is the development of an economically viable and eco-friendly pretreatment strategy for better hemicellulose and lignin removal as well as the development of hyper-productive and solvent-tolerant microbial strains for effective fermentation. This review focuses on the recent trends, challenges, and possibilities in the production of butanol utilising rice straw.

**Keywords:** Rice straw; Butanol; Pretreatment; Fermentation; Biomass

## 1. Introduction

The growing energy demands and its alarming influence on the climatic changes is a threatening global concern. Every aspect of the globe, population density, societal structure, industrial revolution, etc., has a huge impact on natural resources such as fossil fuels. Being recognized air pollution as a serious health threat, World Health Organization (WHO) spearheaded its efforts to mitigate the rate of pollution and to efficiently develop alternative energy resources to overcome the current scenario (Campbell-Lendrum and Prüss-Ustün, 2019). In par with the current global pollution index, the sustainable utilization of the waste products especially agricultural waste products are gaining much importance. Biofuels such as biodiesel, bio-butanol, bio-isobutanol, and bioethanol have emerged as a top priority renewable energy source in recent years.

Bio-butanol has globally emerged as an attractive alternative over fossil fuels and can play a major role in reducing carbon emissions (García et al., 2011). Butanol is the main component of the Acetone-Butanol-Ethanol (ABE) fermentation, which has a wide range of applications, such as solvent in industries, chemical intermediate in various reactions, extractant and as a potential biofuel (Liu et al., 2013). Butanol is a colourless, flammable four carbon straight chained alcohol with better combustion properties over alcohol based biofuels including high octane number, higher heating value, lower volatility, lesser ignition problems, inter-solubility and higher viscosity of butanol make it a suitable alternative (Ding et al., 2019).

As production of bio-butanol is currently a hot topic of research, various reports are published reviewing the metabolic regulation and physiology of microorganisms producing bio-butanol, process strategies to improve the bio-butanol production and

tolerance and various downstream processes to separate and purify bio-butanol from the fermented broth. The scope of the review is to provide the knowledge of rice straw as the potential feedstock for biobutanol production. It is explained in different sections starting with various pretreatment technologies to access the cellulosic content for efficient enzymatic hydrolysis, concept of consolidated bioprocessing, and genetic engineering tools and techniques for improved biomass valorisation of biobutanol production.

## 2. Generations of bio-butanol

The choice of raw material, the genetic makeup of the microorganism and the rate of enzyme expression determines the fermentation metabolite profile. The concept of ABE fermentation is well elucidated and its roots way back to the 18<sup>th</sup> century. Firstly in 1862, Louis Pasteur demonstrated the biological synthesis of butanol using a mixed culture. Later in 1876, Albert Fits found a pure culture of *Bacillus butylicus* that could produce butanol. In 1893, Martines Beijerinck isolated a new strain called *Granulobacter saccharobutyricum*. The first commercial butanol production plant was established in 1911 in the UK by Fernbach, Strange and Weizmann. Weizmann isolated *Clostridium acetobutylicum* a potential butanol producer from the garden soil in 1915. Although a well-established bioprocess had attained commercial interest, the development of cost effective and high yielding petrochemical processes replaced the biological process in the 1950s. Using a petrochemical derivative propylene, successive hydroformylation and hydrogenation resulted in butanol. This process is similar to aldol condensation and it was termed as Oxo process (Gheshlaghi et al., 2009; Lee et al., 2016). On the basis of different feedstocks used for the ABE fermentation, the processes can be grouped into three following types.

In the 1<sup>st</sup> generation of ABE fermentation, the starch based raw material is used as the feedstock, in which grains from starch rich crops like maize, wheat, rice, and cassava are hydrolysed and treated with glucoamylase to obtain the fermentable sugars (Ndaba et al., 2015; Gottumukkala et al., 2017). As the process is easy and can result in high yields of fermentable sugars, various processes using different microorganisms were demonstrated for bio-butanol production (Table 1). Although the volumetric yields and titers were higher, the feedstocks used here were food crops and its use could lead to the food crisis, should the bio-butanol synthesis meet the consumer demands. Due to the drawbacks of this process, research was directed to find a renewable, sustainable and non-food raw material as the source of fermentable sugars leading to 2<sup>nd</sup> generation bio-butanol process.

In this process, an abundant, low cost agro – residue is used as the feedstock that generates lesser greenhouse gases. The post-harvest agriculture residues are considered as lignocellulosic biomass due to the composition, lignin, the outer protective sheath, cellulose and hemicellulose, the inner matrix made of hexose and pentose sugars. This lignocellulosic biomass is a non-edible fraction of food crops or the plant and has no competition with the food chain. Though the lignocellulosic raw material is abundant, the initial pretreatment process is intense before generating the fermentable sugars for ABE fermentation. This intense process involves the physical as well as chemical pretreatment strategies to remove lignin and a hemicellulosic fraction of the biomass and sequential enzymatic hydrolysis of the cellulosic fraction to produce fermentable sugars. The choice of feedstock explained in this review is rice straw. Rice is widely grown in various countries such as China, India, Philippines, and Thailand as it is a major food crop. The waste associated with rice production causes a serious

environmental impact as the open field burning of rice straw is a common waste management practice in these countries. Therefore, it is crucial to consider utilizing post-harvest residue of rice as an efficient feedstock for biofuel production.

The biofuels from lignocellulosic biomass will be the potential alternative for fossil fuels, if some of the process limitations such as (i) reducing the multiple processing steps, (ii) low sugar yields after pretreatment and hydrolysis, (iii) preventing the accumulation of inhibitory molecules like acetic acid, furan derivatives and furfurals, (iv) developing of an efficient and appropriate host strain for utilizing the various sugars like hexoses and pentoses obtained from biomass concurrently but not sequentially are taken into consideration. The technical advancement and genomic characterization have led us to understand that few strains of clostridia can utilize the cellulosic fraction as the feedstock and can produce butanol. As the second generation biomass requires a large area for cultivation and also the space for storing the post-harvest residues until further processing, a new feedstock, algae, has recently gained importance. Algae are divided into two distinct types, micro, and macroalgae, however, microalgae are unicellular. As the carbohydrate composition is high in the macroalgae and is easy to cultivate in less space comparatively, they are preferable. Even seaweed can be used as one of the biosources. Research interest towards the processing of macro-algae to obtain fermentable sugars for the production of solvents is growing. However, critical parameters should be analysed to improve the process using macro-algae. It is well known in the coastal countries that large quantities of seaweed get accumulated on the beaches, resulting in environmental pollution. If the research is focused on sustainable feedstocks for the production of biofuels and value-added

chemicals via consolidated bioprocessing, the process can address the major environmental issues of an energy crisis and climate change.

### 3. Biochemistry and physiology of solventogenic clostridia during ABE fermentation

The members of genera Clostridia are the only group of microorganisms reported for butanol production along with other solvents like acetone, ethanol, and isopropanol (Ezeji 2007; Yan et al., 2016; Nanda et al., 2017). Though *C. acetobutylicum* was the potent strain isolated in 1915, later on, other strains performing ABE fermentation on different choices of substrates were isolated like *C. beijerinckii* (Maiti et al., 2016), *C. aurantibutylicum* (Zhang et al., 2018), and *C. tetanomorphum* (Gong et al., 2016). These solventogenic clostridial members have different growth pattern and metabolic physiology during carbohydrate dissimilation and later during the response to other metabolites. The physiology of ABE fermentation can be studied in two phases acidogenic and solventogenic phase (Fig. 1), this biphasic growth pattern was observed due to the changes in the surrounding habitat. Firstly, acidogenesis occurs through the exponential growth phase, where the strain produces organic acids like acetic and butyric acid, so the accumulation of these acids results in a decrease in media pH and also the cessation of microbial growth. Hence the actively dividing vegetative cell undergoes sporulation to form a stationary phase endospore where the ABE fermentation occurs (Raganati et al., 2015; Gottumukkala et al., 2017).

#### 3.1. Acidogenic phase

Two clostridial strains *C. acetobutylicum* and *C. beijerinckii* are well studied potent ABE fermentative strains, which can utilize a range of carbohydrates like arabinose, cellobiose, galactose, mannose, xylose, and glucose as carbon sources (Nanda et al., 2017). The carbohydrate dissimilation for ABE fermentation starts with



the uptake of carbon source either hexoses or pentoses through phosphoenol pyruvate (PEP) dependent phosphotransferase system (PTS), in *C. acetobutylicum* there are 13 complete PTS systems that can mediate the cellular uptake of different carbon sources (Ezeji 2007).

The hexose sugars are metabolized via the Embden – Meyerhof pathway and pentoses through pentose phosphate pathway and later through the intermediates, the carbon flux enters the glycolytic pathway to produce pyruvate molecules. 1 mole of sugar can metabolize to 2 moles pyruvate with a net production of 2 moles of adenosine triphosphate (ATP) and 2 moles of nicotinamide adenine dinucleotide (NADH). Then pyruvate is reduced to acetyl-CoA in the presence of pyruvate ferredoxin oxidoreductase and coenzyme A molecule. In the acidogenic phase, two enzymes phosphotransacetylase (EC.2.3.1.8) and acetate kinase (EC.2.7.2.1) metabolise acetate from acetyl CoA and 2 moles of acetyl CoA converts to acetoacetyl CoA mediated by acetyl CoA acetyl transferase or thiolase (EC.2.3.1.9), then in sequential reductions, acetoacetyl CoA is reduced to butyryl CoA in three different steps mediated by  $\beta$ -hydroxyl CoA dehydrogenase (EC.1.1.1.35), enoyl CoA hydratase (EC.4.2.1.17) and butyryl CoA dehydrogenase (EC.1.3.99.2). Later butyryl CoA was phosphorylated by phosphate butyryl transferase (EC.2.3.1.19) to butyryl phosphate and subsequent dephosphorylation to butyrate by butyrate kinase (EC.2.7.2.7). In acidogenic phase these are the key enzymes responsible for the production of acetate and butyrate from different carbon sources, although these enzymes are produced both in acidogenic and solventogenic phases, the concentration was observed to be minimal in the solventogenic phase (Ezeji 2007; Jaouzani et al., 2015; Ibrahim et al., 2017).

During the acidogenic phase, the microorganism is in an exponential phase, but the increased production of acetate and butyrate reduces the media pH and retards the growth of the strain. Usually, it was observed that pH maintained at 5.0 – 5.5 accelerates acidogenic phase. But the accumulation of undissociated butyric acid has a profound effect on the phase transition than the acetic acid (Nanda et al., 2017; Ibrahim et al., 2017). The lower pH and this undissociated butyric acid shifts the metabolism towards the solventogenic phase, the acids produced are re-dissimilated during the endospore formation and produce solvents acetone, butanol and ethanol in a ratio of 3:6:1 (Honicke et al., 2012). The formation of acids in the acidogenic phase results in the generation of energy packets (ATP) and redox balance and equivalents regeneration was adjusted by the formation of H<sub>2</sub>. It was reported that 10 – 70 pmol/g cell dry weight of butyryl phosphate can trigger the phase change from acidogenesis to solventogenesis, similarly 9 mM concentration of butyric acid. It was observed now, the undissociated butyric acid concentration has a positive effect and in linearity between the butanol production, and the surplus production of this acid in the medium without pH control may lead to cessation of growth and no phase change (Gottumukkala et al., 2017).

### **3.2. Solventogenic phase**

At late exponential phase and due to the accumulation of undissociated butyric acid the solventogenic clostridia performs a phase change, in which the vegetative cells undergoing duplication in acidogenesis will enter a resting phase or sporulation phase, in which the acids produced earlier are re-dissimilated to produce solvents via ABE fermentation. The physiological pH in the range of 5.0 – 5.5 favors the solventogenic phase. The key enzymes that decide the fate of butanol and ethanol are acetyl CoA

acetyltransferase or thiolase (EC. 2.3.1.9) and acetaldehyde dehydrogenase (EC.1.2.1.10), the prior enzyme converts acetyl CoA to acetoacetyl CoA, and later one reduces acetyl CoA to its respective aldehyde. If the flux is towards acetaldehyde, NADPH dependent ethanol dehydrogenase (EC. 1.1.1.1) reduces acetaldehyde to ethanol, or if the flux is towards acetoacetyl CoA, the butyryl CoA produced during acidogenesis, was reduced to butyraldehyde in the presence of butyraldehyde dehydrogenase (EC.1.2.1.57) and further reduction to butanol, which is mediated by butanol dehydrogenase (EC.1.1.1). The shift of acidogenic pathway from butyryl CoA to butyraldehyde is due to the sporulation phase, where during acidogenic pathway ATP is required to produce butyrate from butyryl CoA. In a parallel pathway, acetoacetyl CoA was converted to acetoacetate, and later to acetone in a decarboxylation reaction mediated by butyryl CoA transferase (EC.2.8.3.9) and acetoacetate decarboxylase (EC.4.1.1.4) (Ezeji 2007; Jaouzani et al., 2015; Ibrahim et al., 2017). The activation of acetoacetyl CoA transferase utilizes the butyric and acetic acid produced during acidogenesis to acetoacetate and acetone. Though to improve the butanol production during this solventogenic phase, the maximum butanol tolerance of 1.9 % was reported for *C. beijerinckii* BA 101, the accumulation of butanol during the late log phase, leads to damage of the cell membrane by disrupting the phospholipid component of the cell wall and other membrane proteins, thereby increasing the membrane fluidity (Nanda et al., 2017). The increased fluidity may have an effect on proton motive force across the membrane, confirmation of the cell, solute transport and the substrate uptake, which leads to cell collapse and eventually death of the microorganism. To counter these effects and to improve butanol tolerance, the microorganism should shift from solventogenic phase to vegetative phase, where the

ATP molecules generated could increase the saturated fatty acid synthesis and accumulation of these saturated fatty acids in the cell membrane will maintain the cell integrity. A report explaining the effect of co-factors and surfactants, where  $\text{Ca}^{2+}$  ions have a positive effect on sugar transport, butanol transport and also triggering the solventogenic phase in *C. beijerinckii* NCIMB 8052, similarly tween 80, stimulates the enzyme production efficiency and butanol tolerance ability of the microorganism (Qin et al., 2018).

#### **4. Potential of rice straw as feedstock for bio-butanol production**

The major hindrance that affects the production of bio-butanol is the cost of the substrate, which adds up to 60% of the whole process. Moving on from starch based feedstocks (food supplements) to agro residual biomass like rice straw can make the bio-butanol production economically feasible (Moradi et al., 2013). Lignocellulosic waste materials such as barley (Qureshi et al., 2010) wheat straw (Qureshi et al., 2007) corn fibre (Qureshi et al., 2008) have been extensively used in the production of bio-butanol. Rice straw is one of the lignocellulosic biomass that is abundantly available in the Asian countries with lower costs and being considered as waste residue, the biomass is rich in cellulose content and can be evaluated as a feedstock in bio-butanol production that shall overcome the problems associated with economics of substrate and pollution due to burning of residual biomass (Cao et al., 2016).

##### **4.1. Availability and composition of rice straw**

Rice remains the major agriculture crop that is being cultivated globally and is a major food crop in most of the Asian countries. This includes Africa, Asia, Europe and America where the annual production is about 731 million tons (Binod et al., 2010). India is a major rice cultivating country in Asia. China and India alone contribute

nearly half of the world output (FAO, 2014) the yield of rice varies widely among countries due to varieties used for cultivation, climatic conditions and cultivation practices. Worldwide production of paddy (rice) has risen steadily from about 200 million tons in 1960 to over 740.95 million tonnes in 2014 (FAO, 2014). Rice straw is the vegetative part of the rice plant (*Oryza sativa* L.), cut at grain harvest or after. Rice straw is a versatile by-product of rice cultivation and its yield can be estimated based on the grain production by applying a straw: grain ratio. So the estimated rice straw production amounts to approximately 740.95–1111.42 million tons per year globally. Various uses of rice straw include cattle feed, composting, thatching, Poultry litter, mushroom cultivation, packing material, Industrial uses such as manufacturing of paper, straw board, alcohol, hats and mats, ropes, baskets, etc. But, due to surplus paddy straw and problem associated with its storage, two-thirds of it being burned openly in the fields to quickly prepare it for sowing the next crop. The GHG emissions contribution through open-field burning of rice straw in India, Thailand, and the Philippines are 0.05%, 0.18%, and 0.56% (Gadde et al., 2009). Global population and its impact have led to increased cultivation of rice to meet the global food demand. Increased production had gradually seen deposition of agricultural waste products and improper waste management strategies have made the situation more critical. Owing to this current scenario efficient conversion of rice straw to a more productive end product that could be economical to farmers and eco-friendly. In recent years a great attention was given to the utilization of rice straw for production of various value-added end products like proteins (Jia et al., 2019), organic acids like Succinate (Jampatesh et al., 2019), and enzymes like laccase (Li et al., 2019) and Glucoamylase (Anto et al., 2006). Thus rice straw has become a source for product formation; this source is further used

in the production of bioethanol and bio-butanol. Composition of rice straw is depicted in Table 2.

The lignocellulosic material mainly comprises cellulose, hemicellulose, and lignin. Cellulose being the major content in rice straw its structure constitutes the monomeric D-glucose subunits that are linked together by glycosidic bonds. Based on the type of structural organization cellulose are categorized as crystalline and amorphous where a crystalline form of cellulose is more organized and amorphous is less organized. Thus the amorphous form of cellulose is more preferred as biomass for the fermentation process. Hemicellulose can be considered as a carbohydrate compound that has a complex architecture and is a combination of various small polymer units mainly pentoses, hexose and uronic acids (Scheller and Ulvskov, 2010). Hemicelluloses act as a connection network that holds the cellulose and lignin molecules providing tensile strength. Lignin is a heavily packed outer layer of the lignocellulosic biomass that protects the complex structure it is a heteropolymer complex comprising of monolignols mainly p-coumaryl, coniferyl, and sinapyl alcohols. Lignin is not a polymer that contains sugar molecules and thus it does not play a crucial role as a substrate (Grabber, 2005).

## **5. Processing of rice straw for fermentable sugars**

Agro-residual biomass like rice straw due to the complex structure should undergo few pretreatment steps and subsequent enzymatic hydrolysis to produce fermentable sugars. Conversion of rice straw into fermentable sugars includes following steps:

### **5.1. Physical Pretreatment**

#### **5.1.1. Mechanical Extrusion**

The feedstock is subjected to heating process ( $>300\text{ }^{\circ}\text{C}$ ) under shear mixing. Due to the combined effect of shearing and the heating the crystalline cellulose matrix in the biomass is being disrupted (Shafizadeh and Bradbury, 1979; Kumar and Sharma, 2017). A screw extruder is used for mechanical extrusion. Different type of extruders like a single screw and twin screw extruders have been attempted for different lignocellulosic biomass, which results in an improved saccharification rate. Extruders will provide high shear, rapid heat transfer and effective mixing in short residence time. During the passage of feedstock through extruder barrel, the physical and chemical structure will be disturbed resulting in a larger specific area, which in turn increases the accessibility of cellulose for enzyme action. An extrusion pretreatment can be combined with other pretreatment methods to increase the sugar yield. During the extrusion process lignocellulosic biomass can be treated with acid and alkali. Alkali is mostly preferred due to its delignification property and less carbohydrate damage. Among different alkali used sodium hydroxide is commonly used and can cleave ester linkages and solubilize hemicellulose and lignin (Morrison 1991; Morrison 1988). Pretreatment of rice straw using the extrusion process has been reported by Chen et al., 2011 where extrusion results in an increase in the solid loading resulting in higher monomeric xylose. The optimal condition of extrusion is 40 rpm with 3 % sulphuric acid at  $120\text{ }^{\circ}\text{C}$ , even though it is an effective method due to certain limitations like high cost and difficulties in scaling up it has not been yet commercialized (Chen et al., 2011; Zheng and Rehmann 2014).

### **5.1.2. Milling**

Milling is one of the primary step involved in the pretreatment of biomass which reduces the particle size up to 0.2 mm. Studies revealed that further reduction in size

has no effect on hydrolysis (Chang et al., 1997). Milling methods include ball milling, two roll milling, hammer milling, colloid milling, and disk milling. Among the various type of milling process most effective and popular is ball milling and wet disk milling. Ball milling was found to be effective as compared to ordinary milling (Kumar and Sharma, 2017). Wet disk milling is popularly used for lignocellulosic material due to its low energy consumption. Ball milling and wet disk milling was compared by Hiden et al., (Hiden et al., 2009) and the sugar yield without decreasing the crystallinity was found to be more for wet disk milling. Wet disk milling has more advantages like low energy consumption, more effective yield in hydrolysis and also reduction of inhibitors (Lin et al., 2010).

### **5.1.3. Ultrasound**

Ultrasound is a preferred technique for pretreatment as it reduces reaction time and chemical loading. Enhanced sugar yield was also reported by using this technique (Yachmenev et al., 2009; Yu et al., 2009). Ultrasound treatment combined with other modes of pretreatment like acid pretreatment was also cited, which shows an increase in sugar yield up to 44 % (on rice straw basis) after enzyme hydrolysis (Yoswathana et al., 2010). Ultrasound method induces physical stress via mass transfer, shear force and surface erosion as well as a chemical effect by producing oxidizing radicals.

### **5.1.4. Microwave**

Microwave is an efficient pretreatment method when applied in combination with other pretreatment methods. Huan Ma (2009) reported that microwave treatment will disrupt silicified waxy area, which partially removes silicon and lignin by breaking down lignin – hemicellulose complex (Ma et al., 2009), thus allowing the cellulose to expose more towards cellulose enzyme during hydrolysis. Enzyme hydrolysis rate was also increased



by microwave pretreatment with a higher glucose yield in the hydrolysate and lower xylose content which is more suitable for subsequent fermentation (Zhu et al., 2005). Microwave treatment of rice straw followed by lignin extraction was reported to yield 43-55 % sugar (Akhtar et al., 2016; Intanakul et al., 2003).

## **5.2. Chemical pretreatment**

### **5.2.1. Acid pretreatment**

Acid pretreatment includes the use of acids like sulphuric acid, hydrochloric acid, phosphoric acid, and nitric acid. It is one of the most effective methods reported for pretreatment of biomass. Acid can be either diluted or concentrated one; diluted acid is more preferred over concentrated acids due to various reasons like the formation of inhibitors, corrosive nature, hazardous and also requires corrosion resistant reactors (Talebnia et al., 2010). Another problem with concentrated acid is economic feasibility, to make it economically feasible some recovery steps needed to be added additionally, which make the process more complicated (Sun and Cheng, 2002). Acid pretreatment is performed either at high temperature (180 °C) for a shorter period of time or at low temperature (120 °C) for a longer period of time i.e. 30-90 minutes. Hsu et al., studied dilute acid treatment of rice straw and found that release of glucose and xylose accounted 83 % of sugar which is 44 g of sugar for 100 g of rice straw on a dry basis (Hsu et al., 2010). Performing acid pretreatment at low temperature avoids the formation of sugar degrading products like HMF and furfural, which are inhibitory to microorganisms (Saha et al., 2005). Acid pretreatment combined with other modes of treatment is also being studied in a large scale.

### **5.2.2. Alkaline pretreatment**

In this method, lignocellulosic feedstock is soaked in dilute alkaline solutions like sodium hydroxide, potassium hydroxide, ammonium hydroxide and calcium hydroxide. Among this sodium hydroxide was found to be more effective and largely studied (Kumar and Wyman, 2009). The effectiveness of alkaline treatment relies on lignin content present in the biomass feedstock. Compared to acid pretreatment, the alkaline method has more advantages like lesser sugar degradation, non-corrosive, etc. Treatment with sodium hydroxide causes swelling, which in turn leads to a decrease in polymerization and finally disrupt lignin structure (Sun and Cheng, 2002). Optimization of alkaline pretreatment for enhancing glucose yield was studied by Kim and Han, 2012. Maximum glucose yield of 254 g kg<sup>-1</sup> biomass was obtained at conditions of 2.96% NaOH concentration, 81.79 °C and 56.6 minutes. One major disadvantage of alkaline pretreatment is a neutralization step which add up to the cost of production and also loss of hemicelluloses (Bensah and Mensah, 2013). A number of studies have combined alkali treatment with other treatments like steam explosion, wet oxidation, which showed an improved result.

### 5.2.3. Organosolvent

In method aqueous organic solvents like ethanol, methanol, ethylene glycol, acetone, etc. are added to the biomass under specific conditions (Kumar and Sharma, 2017; Sun and Cheng, 2002). In addition to this catalyst like acid or bases are also added (Zhao et al., 2009). At high temperature (above 185 °C) addition of a catalyst is not necessary (Sarkanen, 1980). This process is mainly used for extraction of lignin (Kumar and Sharma, 2017). The mechanism of this pretreatment method involves three reactions. The first reaction in which ether bond cleavage of lignin occur, the second reaction is

the disruption of glycosidic bond in major hemicellulose and minor cellulose. Finally, oligosaccharides and monosaccharides dehydrate to produce HMF and furfural (Zhang et al., 2016b). By lignin removal and hemicellulose dissolution, cellulose gets exposed and are available for cellulose enzyme during enzyme hydrolysis (Koo et al., 2011; Zhang et al., 2016b) Disadvantage of this method include low boiling point, flammability, volatility, and high risk of operating (Sun and Chen, 2008). In order to prevent the inhibitory effect and production cost solvent should be recycled (Sun and Cheng, 2002).

#### **5.2.4. Ozonolysis**

Ozone gas is used as a substrate to breakdown lignin and hemicellulose in biomass and the process is termed as ozonolysis (Balat, 2011; Mood et al., 2013). Ozonolysis is performed at ambient temperature and pressure (Sun and Cheng, 2002). Ozone is soluble in water and available, but a large amount of ozone is needed which make the process expensive. Another important factor that should be noted is the moisture content of biomass. Higher moisture content leads to lower lignin oxidation. Optimum moisture content for biomass for efficient ozonolysis treatment is 30% (Taherzadeh and Karimi, 2008). One of the major advantages of this process is it does not produce toxic residues as other pretreatment methods (Kumar and Sharma, 2017).

#### **5.2.5. Ionic Liquids**

An ionic liquid treatment for biomass is a recent approach that gained importance in the last decade. Using this method biomass with different hardness can be dissolved. Ionic liquids are salts with large cation and small anions. Properties of this salt include melting point below 100 °C, nonflammable, liquid at room temperature, low volatility, and high thermal stability up to temperature 300 °C (Bensah and Mensah, 2013).

Characteristics of the ionic liquid can be changed by altering the branches of alkyl group that is integrated into cations. Steps in this treatment include solubilisation of biomass in a solvent at 90 °C to 130 °C at ambient pressure, followed by addition of water to precipitate biomass and finally washing the precipitate (Badiei et al., 2014). Nguyen et al., studied 3 different type of ionic liquids (IL) 1-butyl-3-methylimidazolium chloride ([Bmim]Cl), 1-ethyl-3-methylimidazolium chloride ([Emim]Cl), 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) and 1-ethyl-3-methylimidazolium hydrogen sulfate ([Emim]Su) for rice straw biomass. The result showed that complete dissolution for [Bmim]Cl, [Emim]Ac and [Emim]Cl but not in [Emim]Su for rice straw. The results indicate that ILs containing [Emim] cation to be more effective for rice straw than [Bmim]Cl. The smaller [Emim] cation was reported to have greater interaction with cellulose chain than bigger [Bmim] cation (Nguyen et al., 2010).

### **5.3. Physicochemical pretreatment**

#### **5.3.1. Oxidative pretreatment**

This method focuses on utilizing various oxidizing agents like ozone, hydrogen peroxide, and oxygen. Delignification occurs by the action of the aromatic ring of lignin with an oxidizing agent which led to improved digestibility as compared to alkaline treatment (Bensah and Mensah, 2013). Electrophilic substitution, side chain displacement, and oxidative cleavage of aromatic ring ether linkage occur during oxidative pretreatment (Kumar and Sharma, 2017). The various factors that contribute to lignocellulose degradation include oxidant concentration, reaction temperature, time and type of biomass used (Bensah and Mensah, 2013). The oxidative treatment leads to the conversion of lignin to acid, further this acid may act as inhibitor. so removal of acid

is needed for effective hydrolysis. Hemicellulose is badly affected by these methods which make it unavailable for fermentation. Removal of lignin increases enzyme hydrolysis by exposing cellulose. Enzyme hydrolysis yield is 95% (Kumar and Sharma, 2017).

### **5.3.2. Steam Explosion**

This method is a combination of mechanical force (pressure drop) and chemical reaction. In this hydrothermal treatment, biomass is subjected to high temperature (160-200 °C) and high pressure (0.7–4.8 MPa) for a short duration of time i.e. few seconds to a minute. After this rapid release of pressure in the system occurs, this leads to disruption of fibril and thus increases the accessibility of cellulose. In steam explosion pretreatment acetic acid produced by acetyl group of hemicellulose helps in hydrolysis of the hemicellulose into glucose and xylose monomer (Brodeur et al., 2011; Kumar & Sharma, 2017). Residence time, temperature and moisture are few factors which may affect steam explosion pretreatment.

Addition of chemicals like sulphuric acid and sulphur dioxide can improve the yield of enzymatic hydrolysis at low temperature (Jurado et al., 2009; Talebnia et al., 2010). Jin et al., experimented steam explosion of rice straw, where rice straw was steam exploded at 180, 195, 210 and 220 °C for 4-5 minutes. This mixture was then pulverized and transferred to superfine grinding using fluidized bed opposed jet mill. This is reported to have a higher hydrolytic rate and yield of reducing sugar (Jin and Chen, 2006; Taherzadeh and Karimi, 2008).

### **5.3.3. Liquid hot water**

This method is somewhat similar to the steam explosion the difference is instead if steam it uses water at high temperature and pressure. Based on the direction of flow of

biomass and water it may be divided into different types. First one is concurrent pretreatment:-both biomass and water heated to the required temperature and held at pretreatment condition. Next one is countercurrent pretreatment:-in this against the biomass hot water is pumped. Finally, the last one flows through pretreatment in which biomass remain as a stationery bed and hot water passed through biomass (Kumar and Sharma, 2017). Major plus points of this pretreatment method include non usage of chemicals and requirement of corrosion resistant material is not needed. At a temperature of 220 °C accessible surface area of cellulose and liquid hot water is able to dissolve hemicellulose complex and lignin is removed partially within 2 minutes without any application of chemicals. Liquid hot water pretreatment enlarges and makes it available for hydrolysis (Tahezadeh and Karimi, 2008).

#### **5.3.4. Wet oxidation**

Wet oxidation is carried out for dried and milled lignocellulose at 195 °C for 10-20 minutes. Further addition of sodium carbonate and water take place to reduce the by product formation. Finally, air is introduced in the system to oxidize the compound dissolved in water (Badiei et al., 2014; Pedersen and Meyer, 2009). Rapid oxidation is favoured by high temperature, pH, pressure, and catalysts (Bensah and Mensah, 2013; Schutt and Abraham, 2004). Alkaline wet oxidation reduces the formation of furfural and HMF as compared to alkaline and neutral. In rice husk, 67% of cellulose was obtained, while 89 and 70% lignin and hemicellulose were removed (Bensah and Mensah, 2013; Martín et al., 2006; Thomsen and Schmidt, 1999). Biomass such as straw, reed, have a dense coating which is removed by wet oxidation (Schmidt et al., 2002). Literature report suggests that by-product formation can be reduced by combining wet oxidation with alkaline pretreatment (Brodeur et al., 2011; Georgieva et

al., 2007; Georgieva et al., 2008; Lissens et al., 2004; Schmidt et al., 2002; Sorensen et al., 2008). Inhibitory compounds are seen to reduce by the addition of sodium carbonate and alkaline peroxide. It is also said to improve hemicellulose degradation and helps in reducing reaction temperature. Wet oxidation coupled with other pretreatments like steam explosion (wet explosion process) as also studied (Akhtar et al., 2016; Sørensen et al., 2008). Advantages of such coupling include the ability to process higher substrate loading and larger particle size (Akhtar et al., 2016; Georgieva et al., 2008).

#### 5.4. Biological pretreatment

Biological pretreatment is an eco-friendly process for effective delignification (Sindhu et al., 2016). It involves the use of microorganisms. White, brown, and soft rot fungi are largely and commonly studied organism for biological pretreatment. Among these white rot fungi is the most promising one. In 2005 Taniguchi et al. (2005) studied four types of white rot fungi. Among this *P. ostreatus* can degrade lignin fraction of rice straw in particular. The total weight losses were 25% and delignification was 41% (Binod et al., 2010b; Taniguchi et al., 2005). Lignolytic basidiomycetes, a distinct group of saprophytic fungi cause white rot in wood. It's known for mineralization of lignin years before. Most efficient white rot fungi belong to *Phanerochaete chrysosporium* due to its high growth rate and biodegradation capability (Mood et al., 2013). Enzymes such as peroxidase and laccase in white rot fungi are responsible for the degradation of lignin (Kumar et al., 2009).

Another group involves brown rot fungi, which act on cellulose and hemicellulose but do not oxidize lignin. It includes *Serpula lacrymans*, *Coniophora puteana*, *Meruliporia incrassate*, etc. soft rot fungi is another also efficient in lignocellulose degradation.

Two types of soft rot fungi type i and type ii is there. Type i is a more efficient one. Several *actinomycetes* and bacteria like *Bacillus* sp. are also reported to produce enzyme involved in lignocellulose degradation. Zhang et al. (2016a) observed *Streptomyces griseorubens* after 10 days incubation converted cellulose to reducing sugar with saccharification efficiency of 88% (Swain et al., 2019; Zhang et al., 2016a). In biological pretreatment factors which affect lignin degradation and final sugar, yield includes particle size, pretreatment time, temperature, moisture content, etc (Mood et al., 2013). Drawbacks include longer pretreatment time, continuous monitoring of microorganism is required etc. For commercializing this method more points like faster growth rate with more efficiency should be taken to account.

#### **6. Rice straw hydrolysate as a feedstock for butanol production**

As described in the previous section the fermentable sugars are obtained through any of the optimal physical, chemical pretreatment and sequential enzymatic hydrolysis of rice straw. Later the fermentable sugars are utilized by various solventogenic clostridia to perform ABE fermentation. Along with rice straw various other feedstocks like wheat straw, barley straw, corn stover, corncob, rice bran, switchgrass, etc., were utilized by clostridial strains like *C. acetobutylicum* and *C. beijerinckii*, resulting in 2.0 – 18 g/L of butanol with a volumetric yield of 0.2 – 0.4 g/g. Using the lignocellulosic biomass, different process aspects like separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were carried out for ABE fermentation. After finding the suitable feedstock for bio-butanol production, the main research interest was to develop a suitable pretreatment strategies to increase the overall yield of the fermentable sugars, similarly using various pretreatment strategies like



organosolv (Amri et al., 2014), alkaline (Moradi et al., 2013), phosphoric acid (Moradi et al., 2013), steam explosion (Ranjan and Moholkar 2011), dilute acid (Ranjan and Moholkar 2011), enzyme assisted hydrolysis (Ranjan and Moholkar 2011), acid hydrolysis (Gottumukkala et al., 2013, 2015) and dilute acid (Qureshi et al., 2008) pretreatments and subsequent enzymatic hydrolysis of the pretreated rice straw yielded butanol titers of 1.0 – 7.0 g/L. Ranjan et al., 2012 reported a maximum of 13.5 g/L butanol using rice straw hydrolysate as the feedstock consisting of 23 g/L glucose, which is obtained by acid hydrolysis of 5% rice straw. Similarly using a new pretreatment technique using deep eutectic solvent forceline, made of choline chloride and formic or acetic acid, and subsequent enzymatic hydrolysis with 50 FPU cellulase resulted in total sugar of 42.8 g/L with 87% of which is glucose, using this rice straw hydrolysate, *C. saccharobutylicum* produced 9.5 g/L butanol. From the time the second generation bio-butanol was produced using lignocellulosic biomass (i.e., biofuels from biomass are efficient), various research groups worked on either optimization of pretreatment or hydrolysis or on modes of fermentation.

The limitations of SHF or SSF in second generation bio-butanol production

- i. Multiple processing steps for lignocellulosic biomass pretreatment.
- ii. Low sugar yields due to enzyme inefficiency.
- iii. Accumulation of microbial growth inhibitors like acetic acid, ferulic acid, hydroxymethylfurfural, and other phenolics.
- iv. Low titers, yield, and productivity of butanol or other biofuels.

Though there are few limitations with SHF, the two processes hydrolysis and fermentation can be carried out at the optimal conditions, yet there is another limitation

of inhibition of cellulase activity by the sugars released during fermentation. But recently a new concept of consolidated bioprocessing (CBP) has gained much interest. With a concept of simultaneous saccharification and fermentation, CBP combines the two processes of hydrolysis and fermentation into a single unit operation.

### 6.1. Consolidated bioprocessing

Bio-butanol is considered as next generation eco-friendly biofuel with superior properties than bioethanol. Hence it is relevant to address the limitations of the current bioprocess techniques to come up with an efficient, sustainable green process. The effective way of conversion of renewable feedstocks for value added products, in this case bio-butanol production can be divided into three phases (Fig. 2), (i) initial physical or chemical pretreatment of biomass, (ii) enzymatic hydrolysis (cellulolytic) and (iii) ABE fermentation (solventogenic). Biomass constitutes 50-80% of complex carbohydrates and it is recalcitrant (Jouzani et al., 2015), hence initial pretreatment of biomass via. Physico-chemical methods are essential to disrupt the outer protective sheath and provide the access for the enzymatic hydrolysis.

Various fermentation strategies like separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF), are designed and evaluated for the efficient utilization of lignocellulosic biomass for hydrolysis and fermentation to produce biofuels and value added products. But in these strategies, each element like enzyme preparation and later fermentation is separate process, but the concept of consolidated bioprocessing (CBP) explains the single entity or a consortium of microorganisms which can perform enzyme production, hydrolysis, and fermentation in

a single pot, i.e., a single microorganism or a consortium should be able to produce cellulolytic enzymes for hydrolysis of lignocellulosic biomass and simultaneous fermentation of pentoses and hexoses derived from the biomass to value added chemicals and fuels.

If we observe the current scenario of biofuel production from lignocellulosic biomass, the major cost factor is enzyme required for hydrolysis, if we can be able to synthesize the indigenous enzyme sufficient for hydrolysis, along with reduced enzyme costs, the unit operations are reduced, for example, by introducing CBP, unit operations like enzyme hydrolysis, fermentation can be carried out in a single unit, thereby reducing the time and energy. Hence CBP in biorefineries could be efficient and economical.

Consolidated bioprocessing for bio-butanol production can be dealt through following possibilities; (i) Two stage process with cellulolytic and solventogenic microorganisms, (ii) Co-culture of cellulolytic and solventogenic microorganisms, (iii) genetic modification of either cellulolytic microorganisms with solventogenic pathway engineering or vice versa.

#### **6.1.1. Co-culture of cellulolytic and solventogenic microorganisms**

As an ideal lignocellulosic biomass utilizing microorganism, the strain should be capable of producing cellulases (cellobiohydrolase, endoglucanase,  $\beta$ -glucosidase, and phospho- $\beta$ -glucosidase), hemicellulose (endoxyranase,  $\beta$ -xylosidase, arabinofuranosidase, galactosidase and glucuronidase), pectinolytic, lignin degradation, and cell wall loosening enzymes (Jouzani et al., 2015). Till date there is no native strain available which can utilize cellulose as a sole carbon source for bio-butanol production, the strains modified using genetic engineering approaches either by heterologous or

homologous overexpression resulted in unsatisfactory butanol titres (Jiang et al., 2018). As an alternative approach, employing two mutualistic or microorganisms in synergy will be able to accomplish the task of converting the lignocellulosic biomass to fermentable sugars and later subsequent conversion to bio-butanol by ABE fermentation. Coming to the strains to be employed in the consortium to maintain the synergy, various combinations of bacteria – bacteria, yeast – yeast and yeast – bacteria were well known, for example, *Trichoderma reesei*, well known and commercialized strain for cellulase production was employed along with *E. coli* genetically modified with heterologous ABE pathway resulting in 1.8 g/L butanol from pretreated corn stover. But the genus *Clostridium*, has divergent species like cellulolytic (*C. cellulolyticum*), mesophilic (*C. cellulovorans*), and thermophilic (*C. thermocellum*) strains which can thrive on complex polysaccharides like cellulose and digest them to fermentable sugars and later has the ability to produce lactate, acetate, H<sub>2</sub>, and CO<sub>2</sub>. These strains have multienzyme complex termed as cellulosome, which mediates the degradation of insoluble substrates into simple soluble products, which are absorbed into the cell (Xiu et al., 2019). This complex cellulosome can act as a protein scaffold releasing cellulases, hemicellulases and pectinases (Salimi et al., 2010). The genes responsible for the biomass degradation are glycoside hydrolases (GH family), the genome characterization of these three clostridial strains determined, the complexity of cellulosome in *C. thermocellum* than in *C. cellulolyticum* and *C. cellulovorans*. Even the experimental results with co-culture between the *C. cellulolyticum* and *C. acetobutylicum* resulted only in acidogenesis. Though *C. Cellulolyticum* can hydrolyse biomass three times more in co-culture scenario than in mono culture, central metabolism of the strain is a limiting step for the solventogenesis. In the presence of

glucose, the pyruvate accumulation was observed and which is released into the fermentation media, if *C. acetobutylicum* is provided with the pyruvate, the flux of carbon will lead to acidogenesis resulting in butyrate and acetate (Salimi et al., 2010; Salimi et al., 2013; Gaida et al., 2016). Whereas in co-culture scenario with other two strains *C. thermocellum* ATCC 27405 and *C. cellulovorans* 743B with *C. beijerinckii* NCIMB 8052 in independent experiments resulted in 10.9 and 8.3 g/L butanol using alkali pretreated corncobs (Wen et al., 2014a; Wen et al., 2014b). These cellulolytic strains provided *C. beijerinckii* NCIMB 8052 fermentable sugars from the biomass along with butyric acid, which triggers the solventogenic phase unlike *C. cellulolyticum*. In a co-culture system with *C. saccharoperbutylacetonicum*, *C. thermocellum* produced 46.3 mU endoglucanase, 2.15 mU exoglucanase and 0.42 U  $\beta$ -glucosidase resulting in 7.9 g/L butanol using avicel (Crystalline cellulose), 2.2 g/L with untreated rice straw, 5.5 g/L with de-lignified rice straw (Kiyoshi et al., 2015). With a combination of bacteria – yeast, Wu et al., employed a solventogenic *C. beijerinckii* F-6 with *S. cerevisiae*, under the stress conditions like high temperatures and presence of organic acids, yeast secretes different amino acids, and heat shock proteins, which modulate the enhancement of metabolism in bacteria. These amino acids are hydrolysed by anaerobic bacteria for improved growth and butanol tolerance (Wu et al., 2019). It was now understood that clostridia have efficient cellulolytic, acidogenic and solventogenic strains, either can genetically engineer and construct a single strain which can perform all these functions, which is practically impossible (getting desired titres of butanol) or can employ these three strains either sequentially or together to produce butanol from biomass. Yes, the two stage fermentation (TSF) strategy was performed by co-culturing of *C. thermocellum*, *C. thermobutyricum* and *C.*

*beijerinckii* and the process was compared with separate hydrolysis and fermentation (SHF) strategy, the SHF process resulted in 96 g butanol/Kg rice straw, whereas TSF process resulted in 149 g butanol/Kg rice straw, in volumetric titres 14.9 g/L. Without any harsh process like physicochemical pretreatment and separate enzymatic hydrolysis, this process was observed to be efficient in terms of titres, economy and ease of unit operations. In a report by Nisha Singh and associates, they have reported a new isolate *Clostridium sp.*, DBT-IOC-C19 isolated from hot springs in Himalayan region, with better cellulolytic activity than *C. thermocellum* DSM 1313, though the strain was used for ethanol production (Singh et al., 2017), either the strain must be evaluated for butanol production or in co-culture strategy to improve the biomass hydrolysis. Another indigenous xylanase producing strain *Clostridium sp.*, NJP7 was also identified, which was able to produce 12.21 g/L butanol in batch and 25.58 g/L in a fed-batch mode of fermentation when supplemented with glucose as the substrate.

#### **6.1.2. Genetic modification for combined hydrolysis and ABE fermentation**

The major factor that plays a vital role in the production of butanol by any organism is the metabolic pathway and its associated reactions and the capability of the organism to utilize a wide range of substrate for production. The knowledge associated with the primary metabolic pathway of butanol production pathway can be of utmost importance to carry out the production strategies. In order to have a wider understanding of the metabolic pathway of butanol production and to further mitigate the role of genes involved and the expression of enzymes associated with this process transcriptome as well as proteomic approach was carried out to have a deeper knowledge about the molecular mechanism being associated with the *C. acetobutylicum* strain. This analysis can bring in more insight into the primary metabolism and in turn help to elucidate the

functional characterization of various enzymes that favours the production of butanol (Yoo et al., 2015). System biology approach is so crucial in deciphering the metabolic activity of the system at various set parameters and thus use of lignocellulosic biomass can also be carefully dealt with the omics approach in creating more information to improve bio-butanol production.

The genetic engineering tools can be applied either in cellulolytic strains to improve the efficiency of hydrolysis or solventogenic strains to direct the flux towards butanol production (Fig. 3) From the above explanation about cellulolytic clostridia, *C. thermocellum* has cellulosome complex (CtCel5E), a bifunctional enzyme which can produce cellulase or xylanase which digest cellulose and xylan to respective oligomers like cellobiose and xylobiose, whereas *C. cellulovorans* has CcBglA cellulosome complex which digests cellulose to glucose subunits, probable reason might be due to expression of  $\beta$ -glucosidase. A fusion construct of both these enzymes CtCel5E – CcBglA was made and expressed in yeast, which could hydrolyse and produce glucose for sustainable cell growth on pretreated rice straw (Chen et al., 2019). Similarly, glycoside hydrolases (cel A and cel D) from *Neocallimastix patriciarum*, an anaerobic cellulolytic fungus was heterologously expressed in solventogenic *C. beijerinckii* NCIMB 8052, resulting in no hydrolysis and no fermentation. Recently a metabolic and evolutionary engineering strategy was carried out in a *Clostridium cellulovorans* strain by overexpressing the *adhE1* butyraldehyde dehydrogenase and *ctfAB-adc*, CoA transferase and acetoacetate decarboxylase genes which enhanced the butanol production to 3.47 g/L (Wen et al., 2019).

*C. acetobutylicum* is a well-known butanol producer but genetic modification of this vegetative strain is tedious, as the whole solventogenic pathway genes of this

microorganism is present on pSOL1 megaplasmid (Gottumukkala et al., 2017), in the repeated transformations and electroporation, there is a chance that the vegetative strain may lose the plasmid resulting in the strain devoid of solventogenic phase. Instead of re-integrating the pSOL1 plasmid, combined gene knockout and overexpression strategies were carried out. Deletion of butyrate kinase, acetate kinase and phosphotransacetylase genes in acidogenic pathways, and overexpression of aldehyde or alcohol dehydrogenase resulted in 18.9 g/L butanol, but alcohol dehydrogenase mediates both ethanol and butanol production, but the overexpression of butanol specific butanol dehydrogenase either from *C. beijerinckii* or *C. saccharoperbutylacetonicum* could yield better titres of butanol. Lignocellulosic biomass is a mixture of hexoses and pentoses, usually any strain utilize the glucose as the primary carbon source, even the carbon catabolite repression in the presence of glucose, results in lower consumption of other reducing sugars, to improve the xylose utilization activity, heterologous expression of transaldolase, transketolase, ribose-5-phosphate isomerase and ribose-5-phosphate epimerase in *C. acetobutylicum* improved the butanol titres from 3.7 to 5.3 g/L. Similarly, along with pentoses, another aspect to which the ideal quality of the strain in CBP is resistant to inhibitors such as organic acids and phenols in the hydrolysate. It was absorbed that amino acid proline has a major role in maintenance of the cellular functions, scavenging the reactive oxygen species, the overexpression of proline biosynthetic pathway genes in *C. acetobutylicum*, strain 824 (proABC), performed exceptional by displaying tolerance to formic acid, phenols and increasing butanol titres for 3.4 fold over the wild type strain using non-detoxified rice straw hydrolysate (Liao et al., 2019). An engineered strain of *Clostridium saccharoperbutylacetonicum* was developed which could enhance acid re-



assimilation and solvent availability by utilizing acetate pretreated lignocellulosic material. Sol operon containing solventogenic genes including *ald*, NAD-dependent aldehyde dehydrogenase, *ctfA/ctfB* butyrate-acetoacetate CoA transferase subunits and *adc* acetoacetate decarboxylase for reassimilation of acid and EC cassette for carbon accumulation was overexpressed, resulting in 13.7% increases butanol titres (Wang et al., 2017).

Metabolic engineering tools and its application is gaining more impact on improved production in recent years. System biology approach can clearly highlight the complete metabolic flux and the subsequent by-product pathways being associated and more over the influence of biomass on production and the utilization of the possible sugar monomers can be easily dealt with metabolic engineering tools. Metabolic engineering of the butanol pathway is not restricted to clostridial strains there were reports on engineering for butanol production in *Pseudomonas putida* and *Bacillus subtilis* by polycistronic expression of the associated butanol pathway genes from *Clostridium acetobutylicum* and *Saccharomyces cerevisiae* (Nielsen et al., 2009). These metabolic approaches provide an overall idea about the type of metabolic engineering that is being practiced but this review focuses more on the consumption of the lignocellulosic biomass as a substrate for butanol production. Genetic engineering approach by overexpressing the genes can improve butanol production but coming to lignocellulosic materials overexpression of pathway genes alone cannot improve the butanol production the uptake of cellulose monomers by *Clostridium* is essential and some wild type strains possess the property to degrade cellulose the deficiency of cellulose degrading enzymes make it unfavourable for lignocellulosic biomass utilization and there are reports on the development of genetically modified strains that could

efficiently secrete cellulosome and thus this genetically modified can be further utilized for improving butanol production from lignocellulosic biomass.

### 6.1.3. New approaches

Along with the genetic engineering strategies, research was also focussed on the finding of new strains, which can be an ideal microorganism for CBP, i.e., a strain which can hydrolyse biomass by secreting glycoside hydrolases (GH's) and simultaneously utilize cellobiose, glucose, and xylose to produce bio-butanol. In a recent report a new strain *Clostridium* BOH3 can hydrolyse rice bran (94.5 g/L) along with sesame oil cake (36.7 g/L) to produce 13.5 g/L butanol along with 4.4 L/L hydrogen. In the fermentation media, the activity of cellulase (0.52 U/mL), xylanase (4.1 U/mL) and amylase (2.05 U/mL) was also absorbed, providing more insights of the ability of strain hydrolysing the biomass (Rajagopalan et al., 2016). In another novel approach, a two-stage process, where the cellulolytic and acidogenic microbial consortium is employed for hydrolysing the rice straw to 660 ml/L hydrogen, 6.8 g/L butyric acid and 9.52 g/L volatile fatty acids (VFA), later the fermentation broth with VFA's was used for ABE fermentation with *C. beijerinckii* NCIMB 8052, resulting in 5.8 L/L hydrogen and 13.8 g/L butanol, without acetone (Li et al., 2018). Although now discussing the second generation bio-butanol production from lignocellulosic biomass, in the first generation bio-butanol production, starch was used as the feedstock, food and fodder is the best source of starch, currently the major waste generated in the whole world after the plastic will be food, which can be utilized as the feedstock for production of value-added products. The starch from cassava as a substrate, a synthetic consortium, and co-culture technique was developed with *B. subtilis* WB161 and *C. butylicum* TISTR and *B. cereus* CGMCC 1.895 and *C. beijerinckii* NCIMB 8052

resulted in 9.71 and 6.78 g/L butanol respectively, similarly using starch from the food wastes, an amyolytic and solventogenic clostridial strain *Clostridium sp.*, HN4 was isolated from waste food materials collected from a university canteen. The strain HN4 in the presence of 60 g/L starch, additional supplementation of 3 g/L CaCO<sub>3</sub> and 5 g/L Tween 80, 17.64 g/L and 35.63 g/L butanol was produced in batch and fed-batch mode respectively (Qin et al., 2018). From the above details it is understood that *C. thermocellum* (Ct) is an efficient cellulase producer which can hydrolyse the cellulose components to cellobiose and glucose, *C. beijerinckii* (Cb) and *T. saccharolyticum* (Ts) has ability hydrolyse hemicellulose component of biomass to monosaccharides and further conversion to bio-butanol, in a genetic engineering approach, it will be able to construct a strain with all these characteristics, but what happens if all these strains are fused into a single strain?, Mohtasebi and associates has fused these three strains and also evaluated the performance of these strains in co-culture mode to understand the physiology in ABE fermentation from biomass. Two different fusants CbCt and CbCtTs were formed by protoplast fusion technique, fused CbCt and CbCtTs was able to produce 13.82 and 12.8 g/L butanol, wherein co-culture 5.79 and 6.25 g/L titres were observed respectively (Mohtasebi et al., 2019). These processes can be improved further by any of the techniques involved in CBP.

#### **6.1.4. Strain improvement for butanol tolerance**

A potent inhibitor of butanol production is the butanol accumulation in the media, as the high concentrations has adverse effects on cell growth and cell membrane fluidity. Therefore to obtain higher titres of butanol, selection of an improved butanol tolerant strain is essential.

Classical mutagenesis and adaptive evolution resulted in various butanol tolerant strains which can produce up to 21 g/L, but the genetic stability of these strains are not guaranteed, they may revert and lead to strain degeneration. Later with the advancement of molecular approaches, it was understood that a regulon *spo0A* involved in sporulation and solvent production, can be overexpressed for butanol tolerance, but the yields of butanol is very low in these strains. A histidine kinase gene *cac3319*, a part of *spo0A* regulon, was observed to have effect on butanol production, in *C. acetobutylicum* JB200, a single base deletion resulted in the gene inactivation and resulted in 21 g/L butanol titers with 67% increase in comparison with the parent strain. For further confirmation using Clostron group II intron based gene knockout, *cac3319* histidine kinase gene was knocked out in a type strain *C. acetobutylicum* ATCC 55025, the mutant strain showed 90% increased productivity (Xu et al., 2015). With this it was evident that histidine kinase has role in butanol tolerance. Similarly the role of heat shock proteins on butanol tolerance was observed in Clostridial strains, overexpression of *groESL* in *C. acetobutylicum* resulted in 85% reduction in growth inhibition and 40% improved butanol production (Abdelaal et al., 2015). From the literature point of view, if we can understand the genetic makeup of the microorganism which can improve the production of membrane proteins, lipid and cell envelope biogenesis, oxidative stress and energy supply would provide a better tolerance to the strain.

## 7. Future perspective

Although biobutanol is considered a best alternative for fossil fuels in comparison to bioethanol and biodiesel, there are few challenges and limitations to be addressed for commercialization of the process. A focussed research toward following aspects could improve the bio-butanol production efficiency resulting in an economical process:

1. Cellulolytic and solventogenic microorganisms are favourable for biobutanol production.
2. Improved reactor designs for simultaneous fermentation and product recovery would improve the titers and productivity.
3. Adaptive evolution to ensure the high product tolerance and inhibitor tolerance by the microorganism.
4. Genetic engineering of either non-solventogenic or non-cellulolytic strains, for heterologous expression of either of the physiological roles for efficient bioconversion of lignocellulosic biomass to butanol.
5. Improvement of either native or genetically engineered strains for simultaneous utilization of hexoses and pentoses without carbon catabolite repression.

## **8. Conclusion**

The bio-butanol production from rice straw is a promising area of research. Successful utilization of agro residual biomass to bio-butanol is a challenging task. Biofuel demand is increasing day by day and use of lignocellulosic biomass from rice straw can definitely help to increase its production and helps to efficiently manage the agriculture waste. Understanding the genomics and physiology of clostridial strains resulted in divergent groups which are efficient in hydrolysis and ABE fermentation. Genetic tools and systems biology approaches the CBP can be extended in delivering a tailor made monoculture or a consortium for efficient biomass hydrolysis and fermentation.

## **Acknowledgements**

The authors acknowledge the financial support by the Department of Science and Technology (DST), New Delhi under INNO-INDIGO/INDO-NORDEN project

(Sanction No. DST/IMRCD/INNO-INDIGO/INDO-NORDEN/2017(G). Narisetty Vivek acknowledges Department of Science and Technology (DST), New Delhi for providing DST-INSPIRE fellowship for doctoral studies. Narisetty Vivek and Lakshmi M Nair acknowledge Academy of Scientific and Innovative Research (AcSIR) for providing resources to carry out doctoral studies. Raveendran Sindhu acknowledges Department of Science and Technology, Government of India for sanctioning a project under DST WOS-B scheme.

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<https://patents.google.com/patent/WO2008052991A2/en?q=WO2008052991>

<https://patents.google.com/patent/US20110296747A1/en?q=US20110296747A1>

**Table 1:** List of microorganisms and different feed stocks for biobutanol production

| SI No | Process                 | Organism involved  | Butanol Yield    | Substrate                                       | Reference                        |
|-------|-------------------------|--|------------------|---|----------------------------------|
| 1     | Semi Solid              | <i>Pichia.pastoris</i>   | 9.5–<br>10.5 g/L | Corn-starch                                     | (Ding et al., 2019)              |
| 2     | Continuous fermentation | <i>Clostridium acetobutylicum</i> YM1  | 5.89 g/L         | de-oiled rice bran                              | (Al-Shorgani et al., 2019)       |
| 3     | Co-culture              | <i>Bacillus subtilis</i> and<br><i>Clostridium acetobutylicum</i> .                | 8.28 g/L         | Agave hydrolysates                              | (Oliva-Rodríguez et al., 2019)   |
| 4     | Batch                   | <i>Clostridium beijerinckii</i> .  | 7.3 g/L          | Brewer's spent grains                           | (Fernández-Delgado et al., 2019) |
| 5     | Batch                   | <i>Clostridium beijerinckii</i>  | 11.65 g/L        | Corn cob hydrolysate                            | (Zhang and Jia, 2018)            |
| 6     | Batch                   | <i>Enterococcus hirae</i>  | 6.95 g/L         | Sago effluent and oil cakes                     | (Neethu and Murugan, 2018)       |
| 7     | Batch                   | <i>Clostridium acetobutylicum</i> zzu-02<br><i>Clostridium beijerinckii</i> zzu-01 | 9.88 g/L         | Corn straws                                     | (Zhang et al., 2018)             |
| 8     | Batch                   | <i>Clostridium acetobutylicum</i>  | 83.9 g/L         | Biodegradable fraction of municipal solid waste | (Farmanbordar et al., 2018)      |
| 9     | Batch                   | <i>Clostridium beijerinckii</i> ATCC 55025   | 8.8 g/L          | Wheat bran,                                     | (Liu et al., 2010)               |
| 10    | Batch                   | <i>Clostridium beijerinckii</i>  | 7.02 ± 0.27 g/L  | Coffee silverskin                               | (Hijosa-Valsero et al., 2018)    |
| 11    | Batch                   | <i>Clostridium</i> sp. strain  | 16.62 g/L        | Glucose/galactose                               | (Shanmugam et al.,               |

| WST       |           |                                    |          | 2018)                            |
|-----------|-----------|------------------------------------|----------|----------------------------------|
| <b>12</b> | Fed Batch | <i>Clostridium beijerinckii</i>    | 54.6 g/L | (Xue et al., 2016)               |
|           |           |                                    |          | Glucose                          |
| <b>13</b> | Batch     | <i>Clostridium carboxidivorans</i> | 2.66 g/L | (Fernández-Naveira et al., 2016) |
|           |           |                                    |          | Carbon monoxide                  |
| <b>14</b> | Batch     | <i>Escherichia coli</i>            | 5.5 g/L  | (Saini et al., 2015)             |
|           |           |                                    |          | Glucose                          |
| <b>15</b> | Fed Batch | <i>Clostridium acetobutylicum</i>  | 80 g     | (Amiri et al., 2014)             |
|           |           |                                    |          | Rice straw                       |

**Table 2:** Composition of rice straw (Malik et al., 2015)

| Component (%)                    | Dry weight |
|----------------------------------|------------|
| Cellulose                        | 43         |
| Hemicellulose                    | 25         |
| Lignin                           | 12         |
| Digestible energy, mcal/kg       | 1.9        |
| Ash                              | 16         |
| Dry matter                       | 90         |
| Total digestible nutrients (TDN) | 44.0       |
| Crude protein                    | 4.5        |
| Calcium                          | 0.4        |
| Phosphorous                      | 0.08       |
| Total nitrogen                   | 0.67       |
| Potassium                        | 1.2        |
| Sulphur                          | 0.04       |
| Fat                              | 1.0        |
| Magnesium                        | 0.11       |
| Silica                           | 15.8       |
| Crude fiber                      | 29.8       |

**Table 3:** Comparison of different pretreatment methods

| SI NO                               | PRETREATMENT METHOD  | ADVANTAGE   | DISADVANTAGE   | REFERENCE   |
|-------------------------------------|----------------------|---|--|---|
| <b>I PHYSICAL METHODS</b>           |                      |   |  |   |
| 1                                   | Mechanical Extrusion | Reduces cellulose crystallinity                             | High cost and difficulties in scaling up   |   |
| 2                                   | Milling              | Reduces size and degree of crystallinity                    | High power and energy consumption  |   |
| 3                                   | microwave            | Faster fractionations and improved yield                    | Expensive and difficulties in industrial application                               |   |
| <b>II CHEMICAL METHODS</b>          |                      |   |  |   |
| 1                                   | Acid pretreatment    | Hydrolyse hemicellulose and high glucose yield              | High cost of acid and need for recovery, corrosive nature, formation of inhibitors |   |
| 2                                   | Alkali pretreatment  | Low inhibitor formation and efficient removal of lignin     | Long residence time required and high cost of alkaline catalyst                    |   |
| 3                                   | Organosolvent        | Hydrolysis lignin and hemicellulose                         | High cost due to need of cleaning of solvent from reactor.                         |   |
| 4                                   | ozonolysis           | Increase specific surface area and efficient lignin removal | Large amount of ozone requirement which make it an expensive process               | (Akhtar et al., 2016),(Brodeur et al., 2011),(Mood et al., 2013),(Cao et al., 2016; Singh et al., 2014) |
| 5                                   | Ionic liquids        | Successful lignin removal and increased surface area        | High cost  |   |
| <b>III PHYSICO CHEMICAL METHODS</b> |                      |   |  |   |



|           |                          |  |   |
|-----------|--------------------------|--|---|
| <b>1</b>  | Steam explosion          | Cost effective, lignin transformation and hemicellulose solubilisation | Toxic compound generation and partial hemicellulose degradation |
| <b>2</b>  | Liquid hot water         | Hydrolysis hemicellulose, no need of catalysts and corrosive resistant | High energy or water input                                      |
| <b>3</b>  | Wet oxidation            | Efficient lignin removal and cellulose decrystallization               | High cost of oxygen and catalyst                                |
| <b>IV</b> | <b>BIOLOGICAL METHOD</b> | Degrade lignin and hemicellulose, low energy requirement               | Hydrolysis rate is low and time consuming.                      |

**Table 4: Review of patents on bio-butanol**

| Patent number  | Title  | Butanol titre | Inventor                      |
|----------------|--|---------------|-------------------------------|
| CN102876731B   | Method for producing biological butanol by rice hull   | 8.2 g/l       | -                             |
| WO2009087680A2 | Process of production and quantification of high yield of biobutanol                               | 20 g/l        | Rangaswamy et al., 2009       |
| EP2739722B1    | Butanol fermentation using acid pretreated biomass   | -             | Rangaswamy et al., 2012       |
| CN102703523B   | Method for producing butanol by mixed fermentation of bagasse and molasses serving as raw material | 11.07 g/l     | Yirui et al., 2014            |
| US8420359B2    | Method of producing butanol  | 13.5 g/l      | Sonomoto <i>et al.</i> , 2013 |

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|                 |   |          |                           |
|-----------------|---|----------|---------------------------|
| US6358717B1     | Method of producing<br>butanol using a<br>mutant strain of<br>Clostridium<br>beijerinckii | 21 g/l   | Blaschek et al., 2002     |
| WO2008052991    | Butanol production<br>in a eukaryotic cell  | 20 mg/l  | Madeleine et al.,<br>2008 |
| US20110296747A1 | Novel method of<br>producing butanol  | 1.98 g/l | Sonomoto et al.,<br>2011  |

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**Table 5:** Details of Industrial Biobutanol producers

| SL NO | Company Name        | Feedstock   | organism   | Source  |
|-------|---------------------|---|--|---|
| 1     | Gevo                | Corn<br>Wheat<br>Sorghum<br>Barley<br>Sugarcane<br>Non-food-Cellulosic feedstock. | Yeast,<br>Genetically modified <i>E. coli</i>                            |   |
| 2     | Butamax             | Corn starch<br>corn<br>sugarcane  | Yeast  | <a href="http://www.biobutanol.com/Biobutanol-Producers-Gevo,-Butamax,-Cobalt,.html">http://www.biobutanol.com/Biobutanol-Producers-Gevo,-Butamax,-Cobalt,.html</a> |
| 3     | Cobalt technologies | Beetle-killed lodgepole pine feedstock  | <i>Clostridium</i>   |   |
| 4     | Green biologics     | corn<br>stover and<br>cobs  | Genetically modified<br><i>Clostridium</i> sp.<br><i>Geobacillus</i> sp. |   |

### Figure Captions

**Fig.1.** Illustration of acidogenic and solventogenic phases involved in vegetative and sporulation stages of clostridia utilizing both hexose and pentose sugars.

Abbreviations: EMP: Embden Meyerhof Pathway, PPP: Pentose Phosphate pathway. The numbers provided in the circular rectangle represent the enzymes mediating the respective conversion; (1) Pyruvate ferredoxin oxidoreductase, (2) Acetyl CoA acetyl transferase (3)  $\beta$ -hydroxyl CoA decarboxylase; Crotonase or enoyl CoA hydratase; Butyryl CoA dehydrogenase, (4) Butyraldehyde dehydrogenase, (5) Butanol dehydrogenase, (6) Acetaldehyde dehydrogenase, (7) NADPH dependent ethanol dehydrogenase, (8) Acetoacetyl CoA acetate or Butyrate CoA transferase, (9) Acetoacetate decarboxylase, (10) Phosphotransacetylase, (11) Acetate kinase, (12) Phosphate butyryl transferase and (13) Butyrate kinase

**Fig.2.** Concept of Consolidated Bioprocessing (CBP)

**Fig.3.** Illustration of Co-culture of cellulolytic and solventogenic Clostridia for butanol production and genetically engineered Clostridia with dual functions of being cellulolytic and solventogenic for butanol production.

**Highlights**

- Overview of bio-butanol production from rice straw.
- Recent developments in bio-butanol production.
- Challenges in bio-butanol production.

ACCEPTED MANUSCRIPT

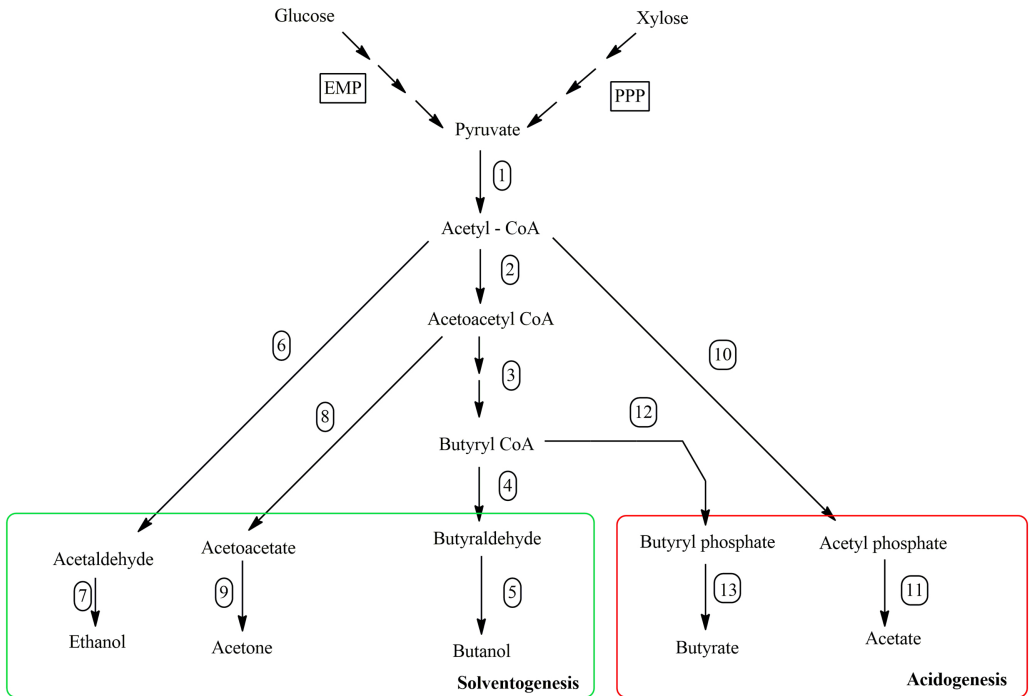


Figure 1

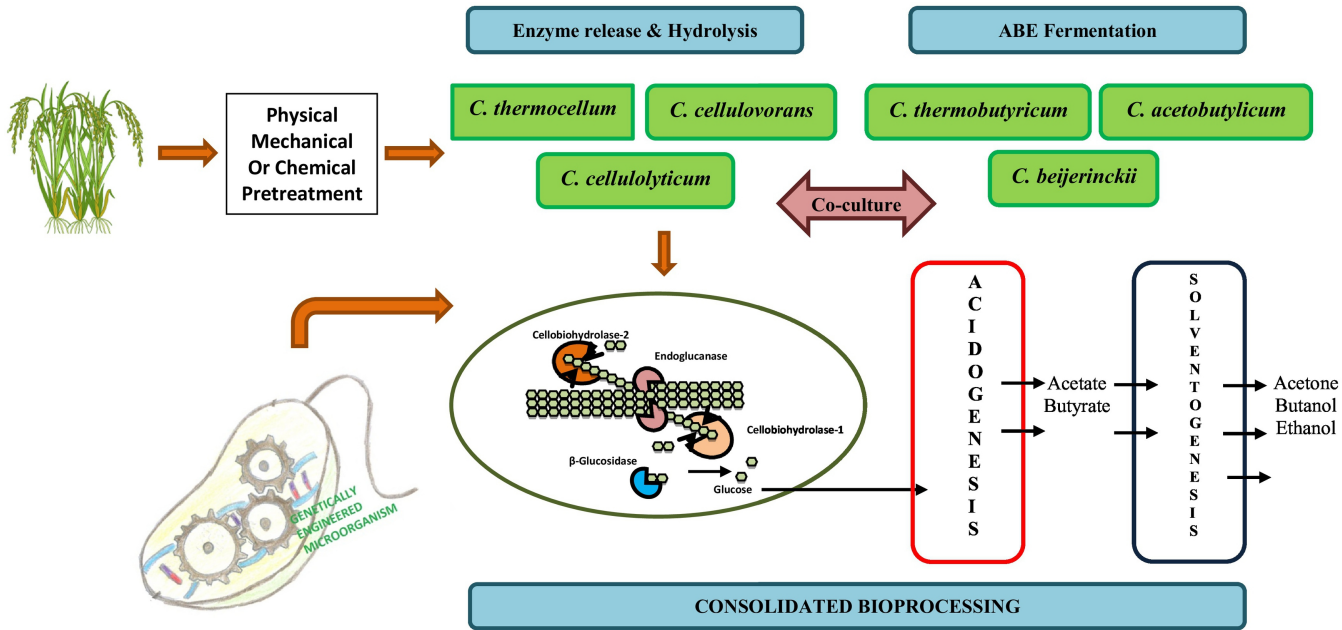


Figure 2



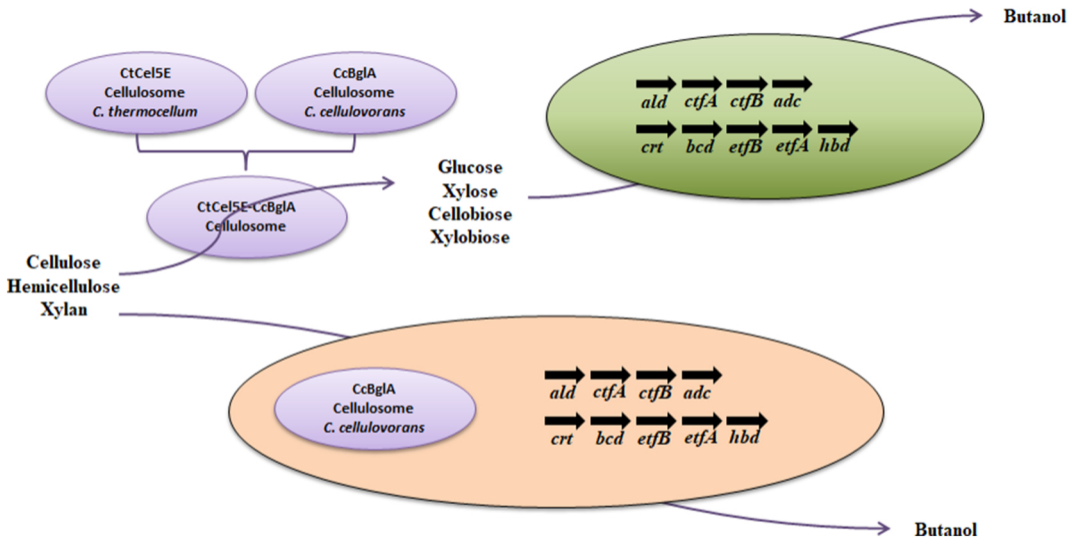


Figure 3