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The enzymatic process of lignocellulosic biomass for second generation bioethanol production, the benefits and challenges: A review

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Abstract Environmental problems as well as energy security factors are the main reason for studying economic production pathway of renewable energy. Today, production of the second generation bioethanol is widely studied around the world. The production process consists of pretreatment, hydrolysis, fermentation and purification. After pretreatment, the hydrolysis process is the key for cost effective bioethanol production. While biological hydrolysis is better than chemical hydrolysis in many respects, current biological methods are not economical and are time-consuming. Genetic engineering techniques appear to offer potential for cutting production steps and improving efficiency and speed. To this end, a particular set of enzymes, cellulases, has received an intension scrutiny by researchers. Three types of cellulases that have been used to produce glucose monomers synergically are endoglucanase, exoglucanase and β-glucosidase. This review also discuss on how recombinant enzymes can be utilized in enzymatic hydrolysis. Furthermore, the benefits and challenges of the second generation bioethanol production are also explained.

Keywords: lignocellulose, pretreatment, hydrolysis, fermentation, cellulases, bioethanol

Introduction

With increasing energy consumption and the pressing environmental need to decrease the use of fossil fuel, renewable alternative energy is sought to meet energy demand sustainably. One approach is to produce liquid fuel enzymatically through a hydrolysis process in biomass resulting in monomeric sugar, which is then fermented, becoming ethanol (Wilson, 2009).

Conversion of lignocellulosic biomass (second generation) to energy is important because the materials are abundant, inexpensive, and there is no competition for food crops (Sassner *et al.*, 2008; Singhania, 2009; Viikari *et al*., 2012; Raghavendra *et al*., 2016). Fossil based fuels and products can be

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replaced by a sustainable and renewable biomass. Biomass or lignocellulosic material is a renewable organic compounds of the entire plants which consist of cellulose, hemicellulose and lignin. The percentage composition of each component varies with different plant sources (Horn *et al*., 2012). Sun and Cheng (2002), reported that lignocellulosic plant biomass represent the largest plant sources of renewable carbon and consist of 40-55% cellulose, 25-50% hemicellulose and 10-40% lignin. The composition of these chemicals depend on wood type, softwood, hardwood or non-wood. D-glucose is a monomer of cellulose that linked by β-1,4 glycosidic bonds (Saha, 2004). Meanwhile, hemicellulose, contain several monomers that linked in highly branched of sugars. The monomers of hemicellulose are D-xylose, D-glucose, D-galactose, D-mannose and L-arabinose (Saha, 2003).

Ethanol is a promising alternative for energy source that can be produced from lignocellulosic biomass. The major steps of bioconversion of lignocellulose for ethanol production consist of pretreatment, enzymatic hydrolysis, microbial fermentation, and ethanol purification (Jing *et al.*, 2009). The pretreatment step is required to break the biomass size, its molecular structure, as well as a chemical composition to increase the hydrolysis process of carbohydrates to sugars. Moreover, pretreatment is needed to improve the efficiency of enzymes. One of the most critical factors affecting the cellulose degradation through enzymatic process is the amount of accessible surface area (Thompson *et al.*, 1992; Eibinger, 2014).

After the pretreatment process, enzymatic hydrolysis of biomass can convert polysaccharides into monosaccharides such as glucose and xylose easily. Subsequently, sugars are fermented to ethanol by use of microorganisms. Several processes for saccharification and fermentation of bioethanol production from lignocellulosic biomass have developed, these are known as Separated Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Simultaneous Saccharification and Co-Fermentation (SSCF) and Consolidated Bioprocess (CBP) (Taherzadeh and Karimi, 2007).

Fungi and bacteria are able to degrade lignocellulosic material and this process was known as bioconversion. Extracellular cellulolytic enzymes are usually produced by *Aspergillus niger* and *Trichoderma reesei* in large quantity. *T. reesei* has been known as efficient cellulase enzyme producer (Liu *et al.*, 2008). Cellulolytic enzymes including β-glucosidases, glucanases and cellobiohydrolase (exoglucanases) work together on cellulolytic residue (Dastban, 2009). Microorganisms such as *Clostridium* sp., *Trichoderma* sp., *Penicillium* sp. and *Aspergillus* sp. show highly activity of its cellulolytic and hemicellulolytic behaviour and able to ferment monosaccharides (Chandel *et al*., 2007).

Cellulases are a group of enzymes consisting of three types: cellobiohydrolase or exoglucanase (exo-1,4-β-D-glucanase, CBH, EC 3.2.1.91); endoglucanase (endo-1,4-β-D-glucanase, EG, EC 3.2.1.4); and β-glucosidase (1,4-*β*-D-glucosidase, BG, EC 3.2.1.21)(Imran *et al.*, 2016). The biological aspects of cellulosic biomass processing are becoming increasingly important, involving cellulases and cellulolytic microorganisms. In general, the three enzymes work synergistically in cellulose degradation. The cellulases from *Trichoderma* have low β-glucosidase levels but higher levels of endo and exoglucanase components. This results is limited efficiency in cellulose degradation. Meanwhile, cellulase from *Aspergillus* usually have low endoglucanase activity but higher β-glucosidase level (Kumar *et al.*, 2008).

Genetic engineering can be used to produce recombinant enzymes capable of producing cellulases to support bioethanol production using lignocellulose waste. Several cellulase genes have been identified in several microbes, especially fungi such as *T. reesei* (Dien *et al*., 2003). Characterization of gene encoding cellulases was carried out before the transformation process so that the resulting recombinant cellulase protein/enzymes can be traced.

The objective of this review is to discuss the pretreatment and hydrolysis process as well as enzymatic hydrolysis of cellulases by genetic engineering, enzyme production, and also fermentation process. Furthermore, the benefits and challenges of the second generation bioethanol production are also discussed.

Pretreatment process of lignocellulosic biomass

Various raw sources of lignocellulose need to undergo pretreatment in order to facilitate the hydrolysis process. The pretreatment process will increase cellulolytic enzyme efficiency by allowing cellulose to become more easily accessible by the cellulolytic enzymes, thereby reducing costs. This process is achieved by breaking down the cellulosic biomass into smaller components, thereby increasing product surface area, and enzymes can work more efficiently. (Mosier *et al*., 2005). The pretreatment process is carried out due to several factors such as high lignin content, large particle size and hydrolysis capability of cellulose and hemicellulose (Hendriks and Zeeman, 2009).

Pretreatment is known as one of the most expensive processing steps in cellulosic biomass to fermentable sugars conversion, and several recent review articles provide a general overview of the field (Alvira *et al*., 2010; Carvalheiro *et al.*, 2008; Hendriks and Zeeman, 2009; Taherzadeh and Karimi, 2008). The goals of pretreatment are to remove lignin and to disrupt cellulose crystalline

structure. The following criteria lead to an improvement in (enzymatic) hydrolysis of lignocellulosic material. There are increasing the surface area and porosity, removal of lignin, depolymerization and removal of hemicellulose and reduction in the crystallinity of cellulose.

The development of pretreatment techniques for lignocellulosic biomass includes biological, mechanical, chemical as well as a combination of the three methods. Combination pretreatment leads the modification of biomass, therefore (enzymatic) hydrolysis of lignocellulose can be accomplished more rapidly with greater yields.

Biological pretreatment used wood-degrading microorganisms including white-, brown- and soft-rot fungi as well as bacteria to change the chemical composition and/or structure of lignocellulose, thus the modified biomass is easier to digest by enzyme (Kurakake, 2007; Lee *et al*., 2007; Singh *et al*., 2008).

Chemical pretreatments that have studied to date have had the primary goal of improving the biodegradability of cellulose by removing lignin and hemicellulose, and to a lesser degree decreasing the degree of polymerization (DP) and crystallinity of the cellulose component (Maryana *et al*., 2016).

The initial treatment of cellulose waste is distinguished mechanically (cut, crushed, milled), physically (irradiation by microwaves, pyrolysis, gamma irradiation), physico-chemical (steam explosion, ammonia fiber explosion /AFEX, hot liquid), and chemically (O_3, H_2O_2) oxidizing agent, alkaline (NaOH, $Ca(OH)_2$), addition of acids (HCl, H_2SO_4, H_3NO_3), organic acids (malic acid, glutaric acid, etc.) and organosolv processes) (Mtui, 2009; Mood, 2013).

Hydrolysis process of lignocellulosic biomass

Breaking down the pretreated cellulosic biomass into di-monomer has been known as hydrolysis. Di-monomer or cellobiose is then further converted to simple sugar such as glucose, xylose, etc. Biological pathway for hydrolyzing biomass is usually using enzyme or in chemical pathway by using acid. The schematic flowsheet of hydrolysis process for lignocellulosic biomass is shown in Figure 1.

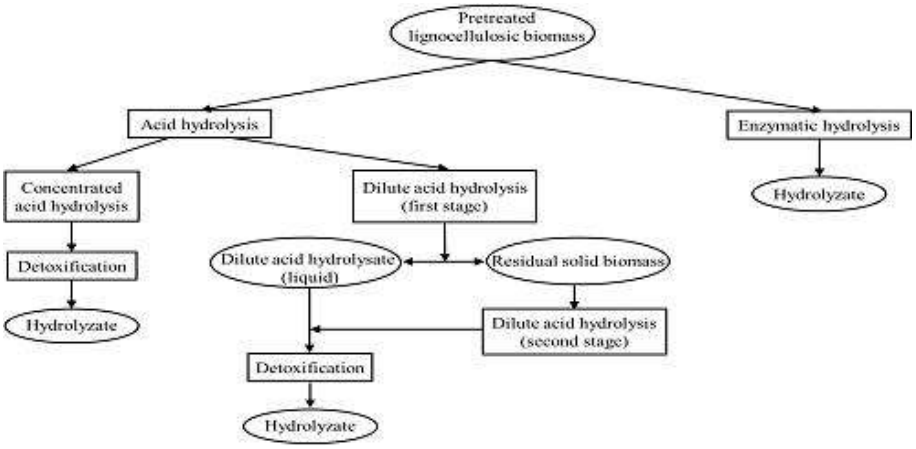

Figure 1. Schematic flowsheet of hydrolysis process for lignocellulosic biomass

Acid hydrolysis

Acid hydrolysis is one method for converting biomass to ethanol (Bransby, 2007). The use of acid for hydrolysis can penetrate lignin without prior pretreatment and also can easily convert cellulose and hemicellulose into monosaccharides (Verardi *et al*., 2012). Kinds of acid such as sulfurous, sulfuric, hydrofluoric, hydrochloric, phosphoric, formic and nitric acid can be applied for hydrolysis (Galbe and Zacchi, 2002). Furthermore, the most commonly used for hydrolysis of lignocellulose are sulfuric and hydrochloric acids (Lenihan *et al*., 2010). Concentrated acid and dilute acid hydrolysis are two types of acid hydrolysis which commonly used (Taherzadeh and Karimi, 2007). The concentrated acid hydrolysis is carried out under mild or low temperature conditions. The acid concentration used is in the range of 30-70% (Taherzadeh and Karimi, 2007). This method results in the hydrolysis of cellulose to glucose and hemicellulose, which is complete and rapidly transforms into 5-carbon sugar with partial degradation. (Devi *et al*., 2016). The crystalline region of cellulose can be reduced by strong acid and produce high yieds (i.e. 90% of theoretical glucose yield) at low temperatures (Huntley *et al.*, 2015; Iranmahboob *et al.*, 2015). However, this method of hydrolysis requires large amounts of acids and carries risks of corrosion problems to the equipment, adverse effects on the environment, and high investment and maintenance cost (Taherzadeh and Karimi, 2007; Verardi *et al*., 2012).

Dilute acid hydrolysis uses a low amount of acid and processes at high temperature to achieve acceptable rates of cellulose conversion (Sun and Cheng, 2002). However, the high temperature increases the decomposition rates of hemicellulose sugars, which subsequently results in higher amounts of toxic

compounds such as furfural and 5-hydroxymethylfurfural (HMF). These compounds inhibit yeast cells and the subsequent fermentation step, resulting in a lower ethanol production rate (Kootstra *et al.*, 2009; Larsson *et al.*, 1999). Moreover, these compounds lead to the reduction of fermentable sugars (Kootstra *et al.*, 2009). In order to avoid these drawbacks from single-stage hydrolysis, dilute-acid hydrolysis is carried out in two stages. The first hydrolysis step is conducted at mild conditions (170-190^{o}C) to hydrolyze hemicellulose and the residual solid is placed under higher conditions (200-230^{o}C) to hydrolyze cellulose in the further stage (Wyman, 1999).

Enzymatic hydrolysis

The aim of enzymatic hydrolysis is to convert polysaccharides into monosaccharides using enzymes. The comparation of the advantages between enzymatic hydrolysis and acid hydrolysis are low corrosion and toxicity problems of the hydrolyzates. The key factors affecting yields of enzyme production are strain type, culture conditions, nature of the substrate and availability of nutrients (Mojsov, 2010; Sarsan and Merugu, 2019). For example, cellulolytic fungi use cellulose as a primary carbon source. A good cellulose inducers are pure cellulose and crystalline cellulose including solka floc, avicel and cotton. However, they are not cheap and therefore cannot be used at an industrial scale (Persson *et al.*, 1991). Therefore, it is crucial to find and use a cheap substrate for cost efficiency (Khan *et al*., 2011; Moosavi-Nasab and Majdi-Nasab, 2008). Soybean hulls, wheat bran, rice straw as well as sugarcane molasses are catagorized as low-cost substrates and reported as effective for growth and enzyme production (Ellilä *et al*., 2017; Kumar *at al*., 2018; Murad and Azzaz, 2013).

Production of enzymes

Cellulose and hemicellulose can be used as carbon source or energy source by many microorganisms, as the result these compounds degrade into simpler structure. Fungi is well known for has highly efficient enzymatic system and can degrade biomass cellulose. (Sanchez, 2009). Previous study reported three types of *Trichoderma* sp. was evaluated for cellulase production (Triwahyuni *et al*., 2018). Moreover, most industrial applications derived from fungi, especially *Trichoderma* sp. and *Aspergillus* sp. (Banerjee *et al*., 2010).

Solid-state fermentation (SSF) is a method involving solids in absence (or near absence) of free water however, substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey *et al*., 2000; Pandey,

2003). This technology is cost-effective, especially for fungal cultures. Another process for enzyme production is submerged fermentation (SmF). In this fermentation, the process only using the presence of excess water. Under SmF process, fungi such as *T. reesei* and *A. niger* produce most commercial cellulases (Singhania, 2011). Table 1 shows an overview of the groups of fungal cellulolytic enzymes, location of action, mode of action and nomenclature number.

Table 1. Enzymes involved in lignocellulose degradation and their mode of action

Lignocellulosic Fraction	Enzymes	Location of action	Mode of action	E.C number
Cellulose	Endo-1,4-β-glucanases (EG)	Cellulose (amorphous regions)	Attack the amorphous regions of the cellulose and produce glucose	3.2.1.4
	Cellobiohydrolases (CBH) (Exo-1,4-β-glucanase)	Cellulose (crystallin regions)	Hydrolyze β-1,4-glycosidic bonds from chain ends, producing cellobiose as the main product	3.2.1.91
	β-glucosidase (BGL)	Cellobiose, cellodextrins	Hydrolyze soluble cellobiose and cellodextrins to glucose	3.2.1.21
Hemicellulose	Endo-xylanase	Xylan main chain	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone	3.2.1.8
	Exo-xylanase	Xylan main chain	Hydrolyzes the terminal β-1,4 xylose linkages releasing xylobiose	3.2.1.37
	β-Xylosidase	Xylooligosaccharides	Releases xylose from xylobiose and short chain xylooligosaccharides	3.2.1.32
	α-Arabinofuranosidase*	α-L-arabinofuranosyl compounds attached to the xylan main chain	Hydrolyzes terminal nonreducing α-arabino-furanose from arabinoxylan	3.2.1.55
	α-Glucuronidase*	α-1,2-linked glucuronic or 4-O-methylglucuronic acid substituents attached to xylan main chain	Release glucuronic acid from glucuroxylans	3.2.1.31
	Acetylxylan esterase*	O-Acetil groups attached to the side ends of xylan main chain	Hydrolyzes acetylester bonds in acetyl xylans, liberating acetic acid	3.2.1.6

Table 1. (con.)

Lignocellulosic Fraction	Enzymes	Location of action	Mode of action	E.C number
	Ferulic acid esterase*	feruloyl group on the arabinofuranosyl side chain attached to the terminal non-reducing xylose	Hydrolyze the ester linkages between arabinose side chain residues and phenolic acids (ferulic acid)	3.1.1.1
Lignin	Laccase (phenol oxidase)	Phenolic compounds found in the lignin structure	Oxidizes phenolic subunits of lignin	1.10.3.2
	Lignin peroxidase	Aromatic compouns found in the lignin structure	Oxidation of benzilic alcohols, cleavage of C-C bonds, cleavage of C-O bonds	1.11.1.7
	Manganase peroxidase	Phenolic compounds found in the lignin structure	Oxidation of Mn2+ to Mn3+, which then binds to an appropriate ligand, diffuses from the enzyme, and, in turn oxidizes phenolic substrates	1.11.1.13

*Known as accessory enzymes
Source: Sarrouh *et al*. (2012)

The effort to overcome the disadvantages of direct fermentation, genetic engineering can be developed to produce recombinant isolates capable of producing cellulase enzymes in an effort to support bioethanol production from lignocellulose waste. Nowadays, genetic engineering technology is preferred for the development of recombinant strains. Genetic engineering introduces new avenues for improving stability, activity, or specificity and productivity of enzymes. This allows naturally-occurring enzymes to now be produced via large-scale fermentation processes. Production of recombinant enzymes is one method that has economic value and is also environmentally friendly and sustainable.

Problems with conventional biological methods, namely time and high cost, can be solved by the introduction of enzymatic engineering. Over the past decade, genomic sequencing of cellulolytic organisms has been carried out and has provided important new information about how microorganisms degrade celluloses (Wilson, 2009). With DNA recombination, cellulase enzymes will be expressed in yeast cells, which are one of the model organisms that are often used in ethanol production. In order to increase cellulase gene expression, a vector is used to construct plasmids.

The *egl1* gene that encodes endoglucanase (EGL1) from *T. longibrachiatum* has been successful in clones, and the results are similar to

results with the *T. reesei egl1* gene (Gonzales *et al*., 1992). In addition, Adney *et al.* (2003) reported that the complete sequence of the cellobiohydrolase (Cel7A) gene originating from *T. reesei* can be expressed in *Eschericia coli* or *Pichia pastoris*, which can be produced respectively in non-dissolved and hyperglycosylated inclusion bodies.

In addition, recombinant cellobiohydrolase II (CBH II) was used to improve the enzymatic hydrolysis process of corn meal and rice straw which was treated with sodium hydroxide to increase the synergy of CBH I and CBH II in cellulase originating from *T. reesei* of 94.7% and 83.3%, respectively (Fang and Xia, 2015).

EG V (Cel5A) and EG VI (Cel6A) deficiencies have become the main factors in the enzymatic hydrolysis process. To increase Cel5A and Cel6A originating from *T. reesei*, the *Vitreoscilla* hemoglobin (VHb) gene is expressed extracellularly together in *P. pastoris* GS115. When compared with their single expressions, CMCase activity from the Cel5A and Cel6A enzymes that are expressed together is higher (Sun *et al*., 2018).

Fermentation

Several studies have been carried out to use microorganism, yeast, bacteria and fungi in the bioethanol production from biomass. Among those microbes, *Saccharomyces cerevisiae* is the most frequent used microorganism (Galbe and Zacchi, 2002). *S. cereviceae* is prefared in the bioethanol fermentation because of high yield (18%) and high ethanol tolerance (Lin *et al*., 2006). In addition, the organism has proven to be resistant to other inhibitors, and is therefore suitable for fermentation of lignocellulosic materials (Olsson *et al*., 1993, Hahn-Hägerdal *et al.*, 1994).

Raw material, pretreatment method, hydrolysis method and environmental factors such as pH, temperature, time, substrate loading, and enzyme concentration are the factors in the efficiency of fermentation process. Normal conditions for *S. cerevisiae* are pH 5.0 and a maximum temperature of 37^{o}C (Alfani *et al*., 2000). However, the fermentation performance can also be affected by many inhibitors. The mixture of inhibitors prevents the growth of fermenting organisms and decreases ethanol production. Different microorganisms have varying tolerance levels against these inhibitors. *S. cerevisiae* has evident to be the most tolerance microbe (Olofsson *et al*., 2008, Almeida *et al*., 2007).

In separate hydrolysis and fermentation, the hydrolysis products have been accumulated in the medium which will inhibit the hydrolysis process. Therefore, simultaneous of hydrolysis and fermentation as known as SSF could be one way to tackle that problem. SSF process allows the glucose produced

from hydrolysis to be fermented immediately. The concentration of glucose in SSF medium can remain low, thus the hydrolysis process continues without significant inhibition (Feng *at al*., 2012).

The benefits and challenges

Environmental problems, as well as energy security factors, are the main reasons for studying the economic production pathway of renewable energy. The use of fossil fuel releases green-house gases (GHG) into the atmosphere and contributes to the increasing atmospheric CO_2 concentration. On the other hand, fossil fuel production, especially in several countries including Indonesia, is decreasing significantly. Therefore, study in the field of biofuel as one source of renewable energy is not only important but also challenging. Bioethanol production from starch, amylose, and sugarcane molasses is known as first generation bioethanol. Meanwhile, the utilization of lignocellulosic biomass, such as waste from agricultural crops, forest and wood residue, etc. is known as second-generation bioethanol, or cellulosic ethanol. It was reported that the use of ethanol in gasoline successfully reduced GHG emissions from transportation sector by 43.5 million metric tonnes CO_2 equivalent in 2016 (Renewable Fuels Association, 2017). The main benefits for producing second generation bioethanol are listed below:

1. Second generation bioethanol does not compete against food supplies (Thompson and Meyer, 2013). Food security is a major concern in many developing nations, and a new report from The State of Food Security and Nutrition in the World states that the number of hungry people in the world reached 821 million in 2017 (WHO, 2018). Therefore, the use of lignocellulosic materials rather than starchy materials from corn or cassava will not influence food sources materials.
2. Lignocellulose biomass is a renewable and sustainable carbon source (Kim, 2010). Renewability and sustainability are very important advantages of bioethanol as fuel compared with fossil fuels. Unlike fossil fuels that take millions of years to be re-produced, plants as the main source of biomass only take several months to several years to be reproduced. Moreover, supply can be maintained or even increased while maintaining biomass as a sustainable material for producing fuel.
3. Lignocellulosic biomass is abundantly available. It is believed that biomass will play a major role in the energy sector in the near future. The estimation for carbon biomass quantity in the world is about 550 gigatons distributed across the kingdoms of life, of which carbon biomass from plants is about 450 gigatons of carbon (Bar-On, 2018). In Indonesia, oil palm biomass is abundantly available since Indonesia is the largest oil palm producer in the

world. In 2009, when plantation area was 7.3 million ha, with approximately 10 million tons of dry oil palm empty fruit bunches produced (Sudiyani, 2010). Indonesia's Statistics Agency (BPS) reported that the total area of oil palm plantations in Indonesia in 2017 was about 11.9 million hectares.

4. Bioethanol is easy to transport. Bioethanol as a fuel allows convenient handling because it is a liquid at room temperature. Moreover, the infrastructure such as fueling stations, etc. can be the same as the current gasoline distribution system, adding to convenience.

However, beside the benefits, the production of bioethanol also still faces several challenges. Production cost needs to be reduced and technology efficiency needs to be introduced. Inaccessible cellulose, the polymer of glucose, to the chemicals and enzyme because of "wrap up" with lignin and mixture with hemicellulose induce recalcitrant bioethanol second generation production. Here are some challenges to the cellulosic ethanol production:

1. Effective pretreatment process. Currently, many studies have been conducted to separate cellulose, hemicellulose and lignin. Usually physical treatment such as milling and grinding is not effective in separating lignin compared to chemical treatments such as acid, alkali, organosolv, ionic liquid and ozonolysis. Some studies reported biological pretreatment and also pretreatment by physicochemical methods such as steam explosion, liquid hot water, wet oxidation, ammonia fiber explosion, and CO_2 explosion (Mood, 2013). Effective saccharification and fermentation process. Both saccharification and fermentation are *the keys* for cost effective bioethanol production. There are two common processes for this step, simultaneous saccharification and fermentation (SSF) and separated hydrolysis and fermentation (SHF). The chemical bonds in complex network require cleavage during the hydrolysis of cellulose and hemicellulose to produce mono- or di- sugar. Some enzymes are known as molecular scissors that can convert complex carbohydrate into monomeric sugar (Gao, 2010). Some yeasts are the host of choice for the expression of fungal cellulases. One of them is *S. cereviceae* that has often been used for recombinant cellulases expression work (van Zyl *et al.,* 2007). Another alternative yeast that is also very attractive as a host is *P. pastoris* (Boer *et al*., 2000). Previous work has reported a 10-fold increase of optimized codon *T. reesei* Cel6A gene expression in *P. pastoris* compared with the native *T. reesei* Cel6A gene (*cbh*2) (Sun *et al*., 2018).
2. Biorefinery concept to reduce the cost of production. Biorefineries in bioethanol production combine production of ethanol as the main product with other value-added chemicals during the process. This concept

maximizes the value of derived chemicals from lignocellulosic biomass. In the biorefinery concept, it is possible to produce low volume but high value chemicals. Thereby, high production costs in bioethanol production can be compensated by the high value of other chemicals. Several chemicals such as furfural can be produced from hemicellulose during biomass pretreatment; glutathione during fermentation (Sudiyani *et al*., 2019) and lignin derivates such as vanillin from black liquor after delignification process. Currently, the biorefinery concept is still in its early stages; that means that in the future, new technological discoveries and inventions are possible. Hopefully, by the application of the biorefinery approach, the bottleneck in the cost of production of second generation bioethanol will be resolved in the near future.

Energy from biomass through second generation bioethanol production could play important roles in the future. Pretreatments and effective biological hydrolysis by genetically engineered microorganisms will be key for cost effective and environmental friendly second generation bioethanol production. In addition, understanding microbial genetics and behaviour of cellulase producers will be required and have to be continuously studied in order to solve the production challenges of bioethanol second generation production.

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