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REVIEW PAPER

Prospects of reusable endogenous hydrolyzing enzymes in bioethanol production by simultaneous saccharification and fermentation

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Abstract−This study was conducted to evaluate the presence, origination and classification of various hydrolyzing enzymes from malt and their specified hydrolyzing effects on various substrates for bioethanol production and to link these characteristics with the future prospects of bioethanol production. These enzymes are categorized as cell wall, starch, protein, lipid, polyphenol and thiol hydrolyzing enzymes based on their substrate specificity. Waste from beer fermentation broth (WBFB) has been evaluated as a rich source of malt derived hydrolyzing enzymes with significant self potential for bioethanol production. However, yeast cells cannot survive at the high temperature required for the saccharification activities of hydrolyzing enzymes during simultaneous saccharification and fermentation (SSF). This dilemma might be resolved by bioethanol production at elevated temperatures via cell-free fermentation systems in the presence of malt hydrolyzing enzymes. Moreover, emerging technologies such as genetic engineering in biomass and biotransformation in cell-free enzymatic systems will likely hasten bioethanol production in the near future. The present study adds new dimensions to eco-friendly bioethanol production from renewable and waste energy resources based on the specific hydrolyzing activities of malt enzymes.

Key words: Hydrolyzing Enzymes, Waste from Beer Fermentation Broth, Bioethanol, Cell-free Fermentation, Genetic Engineering, Biotransformation

INTRODUCTION

Malting processes stimulate certain changes inside seed embryos starting from the activation of endogenous phytohormone production [1]. These phytohormones play a significant role in the biosynthesis and activation of hydrolytic enzymes involved in the degradation of storage compounds into their constituent monomers [2]. The hydrolyzing enzymes expressed in malted cereal are classified into cell wall, starch, protein, lipid, polyphenol, and thiol hydrolyzing enzymes based on their substrate specificity. Although barley malt is most commonly used, other food commodities including rice [3], oats [4], corn [5] and sorghum [6] have also been utilized for the production of malt. Through the activity of hydrolyzing enzymes, higher yields of carbohydrates and monomers of all other storage macromolecules can be obtained during mashing [7]. The hydrolyzed products can then be converted into different products (most importantly biofuels) through fermentation by microorganisms.

Bioethanol is considered an important renewable bioenergy source that contributes to global energy requirements while reducing overall green house gases production [8-10]. When bioethanol was first produced as an energy source, starch based ethanol was produced using hydrolyzing enzymes throughout the process. After pretreatment of lignocellulosic biomass and its (lignocellulosic biomass) higher availability, ethanol production from cellulose was started in parallel. With the continuous development in bio-processing and engineering during the last decade, the annual global bioethanol production increased from 18.45 billion liters to 83.00 billion liters, and it is expected to be more than 100 billion liters by 2015 [11].

The global food crisis and expensive pretreatment processes have led to the continual search for inexpensive sources for biofuels production. Different waste sources, including municipal sludge, food wastes, and wastes from the paper and brewing industry have been utilized for bioethanol production [11-14]. WBFB is a semi-solid waste material enriched with malt hydrolyzing enzymes that has shown tremendous potential for bioethanol production [14,15]. WBFB has the potential to carry out the entire process without additional exogenous enzymes, substrates, or microbial species [14].

Because yeast cells cannot ferment starch or cellulose directly into bioethanol [16], saccharification processes are necessary for the hydrolysis of biomass containing polysaccharides into monomers. Exogenous hydrolyzing enzymes are primarily used in bioreactors for saccharification and are considered to be more expensive than endogenous enzymes [17]. The optimum temperatures of hydrolyzing enzymes are generally >50 °C [18]. Once hydrolyzed, simple sugars can be fermented by microorganisms for bioethanol production. Malt hydrolyzing enzymes are also composed of proteins, lipids and other hydrolyzing enzymes that provide nutrients and certain precursors of macromolecules required for yeast cell proliferation. The products of hydrolyzing enzymes (hexose sugars, amino acids, fatty acids, etc.) are essential to the vegetative growth of yeast cells and enhance its fermentation potential [19,20]. However, the acceding quantities of sugars adversely affect the enzyme kinetic owing to their inhibition effect. There exists a tremendous need of parallel fermentation of the produced sugars to ethanol in order to minimize the enzymatic inhibition effects. Therefore, carrying out the entire operation simultaneously (SSF) can overcome the dilemma. Since cellulase is inhibited by glucose as it is formed,

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quick conversion of the glucose into ethanol by microbial species can accelerate the production rate, and produce greater ethanol concentrations than possible for separate hydrolysis and fermentation (SHF) [21]. Additionally, combining hydrolysis and fermentation eliminates the need for separate reactors, which results in cost reductions. Thus, SSF is thought to be the best process for enzymatic conversion of cellulose to ethanol [22,23].

The difference in temperature optima of both saccharification enzymes (>50 °C) and microbial cell growth (<35 °C) is the bottleneck for bioethanol production through SSF [18]. As temperature increases, the membrane permeability of fermenting microorganisms continuously increases, eventually resulting in cell lysis and secretion of the internal matrix (various enzymes, nutrients, working machinery etc.) into the surrounding medium [24]. Accordingly, a specific system should be investigated to enable SSF at elevated temperature without the need to consider the restrictions of cell viability. Cell-free fermentation, which has been described in detail elsewhere [25], has been suggested as a reasonable and reliable approach for conducting SSF using WBFB as a source of hydrolyzing enzymes, microbial cells, and nutrients. Although the final bioethanol production of cell-free fermentation has been found to be less than that of conventional microbial fermentation, cell-free bio-systems will be the most applicable and reliable strategy for bioethanol production in the near future owing to current developments such as biotransformation in the metabolic pathway [26].

There is a great deal of literature regarding the identification and isolation of malt derived hydrolyzing enzymes with respect to use in the brewing industry [27-34]. However, the effective role of such hydrolyzing enzymes in bioethanol production has not yet been reported. Here, we evaluate various hydrolyzing enzymes from malt, their presence in waste materials (WBFB) and their effective role in bioethanol production. Overall, this study signifies the importance of the effects of hydrolyzing enzymes on the viability of live cells and identifies new avenues for future research regarding bioethanol and other biofuels production.

HYDROLYZING ENZYMES FROM MALT

Metabolic pathways and bioprocesses are exclusively controlled by enzymes present either endogenously in the processing media or added exogenously under definite circumstances. Accordingly, it is essential to understand the characteristics and specified activity of enzymes before subjecting them to biological processes. Studies have been conducted to describe various malt enzymes that are directly or indirectly involved in bioethanol production from various feedstocks. Malt is any germinated cereal grain that has been processed to dry in the presence of hot air. The process used for converting cereal grains (most often barley and wheat) into malt and triggering the expression of different endogenous hydrolyzing enzymes, which are primarily used in the brewing industry, is called malting [35]. Malting process consists of two basic steps, soaking grains in water to increase water contents (42-46%) and then drying them with hot dry air after initial germination. Malting causes the initial stimuli to be generated for expression of starch hydrolyzing enzymes involved in saccharification of grain starch into monosaccharides such as glucose or fructose, as well as disaccharides that are supplied for initial growth and development. Some proteases developed in cereal grains during malting process have been found to break the peptide bonds between amino acids and thus convert the long peptides into short chains or even amino acids [36].

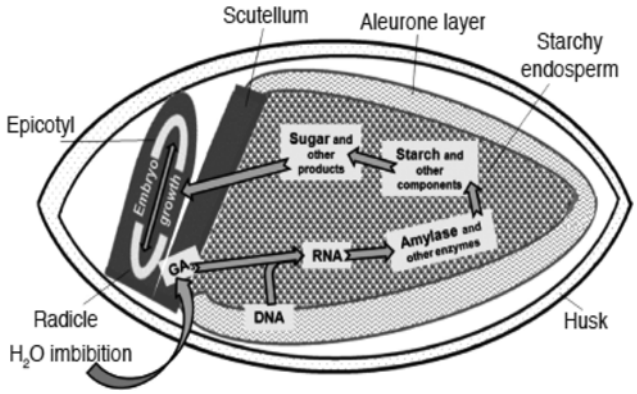

Fig. 1. Overview of all physiological changes in barley grain during germination. This figure is reproduced from [37] with permission. Copyright 2011 Biotechnology, Agronomy, Society and Environment (BASE).

The quality of malting processes primarily depends on the physiological changes that occur inside the grains during germination (Fig. 1). Soaking causes gibberellins synthesis to be induced by the scutellum, which triggers the activation of genes for amylase and other enzymes to hydrolyze starch, proteins, etc., into subunits readily

Table 1. Major key enzymes of malt secreted in fermentation broth during brewing

Substrate	Class	Enzyme	Reference
Cell wall	1	Endoglucanase	[39-41]
		Xylanase	[40,42,43]
		Arabinofuranosidase	[40]
		Feruloyl esterase	[40]
		Acetoxylan esterase	[40]
		Carboxypeptidase	[44,45]
		Exo-β-glucanase	[40]
		β-glucosidases	[40,31]
		Glucanase	[37-46]
Starch	2	α-amylase	[31,40,42,47]
		β-amylase	[40,42,48]
		Limit dextrinase	[31,40,49]
		α-glucosidase	[40]
Protein	3	Endo-peptidase	[40,50]
		Carboxypeptidase	[40,42,45,51]
		Proteases	[42]
		Lipid transfer proteins	[27,52]
Lipids	4	Lipase	[53,54]
		Lipoxygenase	[42,55]
		Hydroperoxide lyase	[40]
		Hydroperoxide isomerase	[40]
		Hydrase	[40]
Phytin	5	Phytase	[56]
Polyphenol	6	Peroxidase	[57]
Thiols	7	Thiol oxidase	[40]

available for embryo growth. The expression of genes encoding different hydrolyzing enzymes is one of the most important internal changes that occur during this process. These enzymes are primarily involved in the hydrolysis of three main components of grain, starch, protein and cell wall polysaccharides [38]. Table 1 provides the classification of these hydrolyzing enzymes expressed upon germination during the malting process based on their nature and specific activity.

1. Cell Wall Hydrolyzing Enzymes

The cell wall, which plays a role in maintenance of the integrity of the cell, is the first barrier that protects the cell from external environmental changes (stress, pathogens, insects, etc.) [17]. Cell walls of the starchy endosperm of barley are composed of (1→3, 1→4)-β-D-glucans (75%), arabinoxylane (20%), cellulose (2%), glucomann (2%) and traces of acetic and ferulic acids. Aleurine cells are composed of arabinoxylane (71%), (1→3, 1→4)-β-D-glucans (26%) with 3% cellulose and glucomannam [58,59]. (1→3, 1→4)-β-D-glucans are also referred to as β-glucane, which is a linear polysaccharide composed of an un-branched chain of D-glucose residues linked together by β-(1→4) and β-(1→3) bonding with a ratio of 3.2 : 1 to 6.6 : 1 [37]. Based on their solubility in water, these compounds are classified as either soluble or insoluble [60]. The molecular weight of β-glucane varies from 800-1,220 kDa [61]. Arabinoxylane is a hemicellulose primarily found in primary and secondary cell walls of cereal grains or woody plants [62]. The backbone of arabinoxylans consists of D-xylanopyranosyl units linked together by β-(1→4) bonds and a branch of single L-arabinofuranose linked by α-(1→2) or α-(1→3) (Fig. 2).

A number of cell wall hydrolyzing enzymes from malt have been reported in previous studies, including endo-gluconase, exo-gluconase, arabinofuranosidase, esterase [40], carboxypeptidase [44,45], xylanase [40,43] and β-glucosidase [31]. The cell wall hydrolyzing enzymes are glycosyl hydrolases, which comprise one of the two main classes of carbohydrate active enzymes [37]. The presence of malt hydrolyzing enzymes in WBFB has been reported in previous studies [14,15].

The solubilization of β-glucans is an initiation step in malt cell wall hydrolysis. The entire group of enzymes involved in this solubilization process can be described by the term "solubilase." As shown in Fig. 3, there are two pathways that lead to the solubilization of β-glucans [37,63]. Specifically, β-glucans are either directly hydrolyzed by β-glucan exohydrolases into glucose or hydrolyzed by a number of enzymes involved in removal of the outer layer of the cell wall and then into glucose. Enzymes participating in the breakdown of β-glucans include (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidase [61,64,65]. Four enzymes, endo-β-(1→4)-xylanase, exoxylanase, β-xylosidase and α-arabinofurnosidase, are expected to be involved in the hydrolysis of arabinoxylan [66]. The complete hydrolysis of arabinoxylan is shown in Fig. 4. Arabinoxylane hydrolyzing enzymes are relatively more active during brewing than in the malting process because they are not fully hydrolyzed during malting [7]. Although exoxylanase cleaves the outer β-(1→4) xylosidic linkages, endo-β-(1→4)-xylanase attacks inner β-(1→4) xylosidic linkages in arabinoxylan polymer, separating the arabinofuranosyl residues [67]. β-xylosidase catalyzes the hydrolysis of β-(1→4) xylosidic bonding within xylo-oligosacchrides, while arabinofuranosidase cleaves the α-(1→2) and α-(1→3) linkage formed between arabinofuranose units. It has also been reported that acetyl esterase and ferulic acid esterase have a great influence on arabinoxylane hydrolysis [68,69].

2. Starch Hydrolyzing Enzymes

Starch, which is the most abundant polysaccharide, is made up of D-glucopyranose chains linked together via α-(1→4) and α-(1→6) glycosidic bonds. The backbone of the starch is linear and called amylose, while branches that arise from the linear backbone are called amylopectin. Starch is the most common polysaccharide produced by plants and is common in staple foods such as wheat,

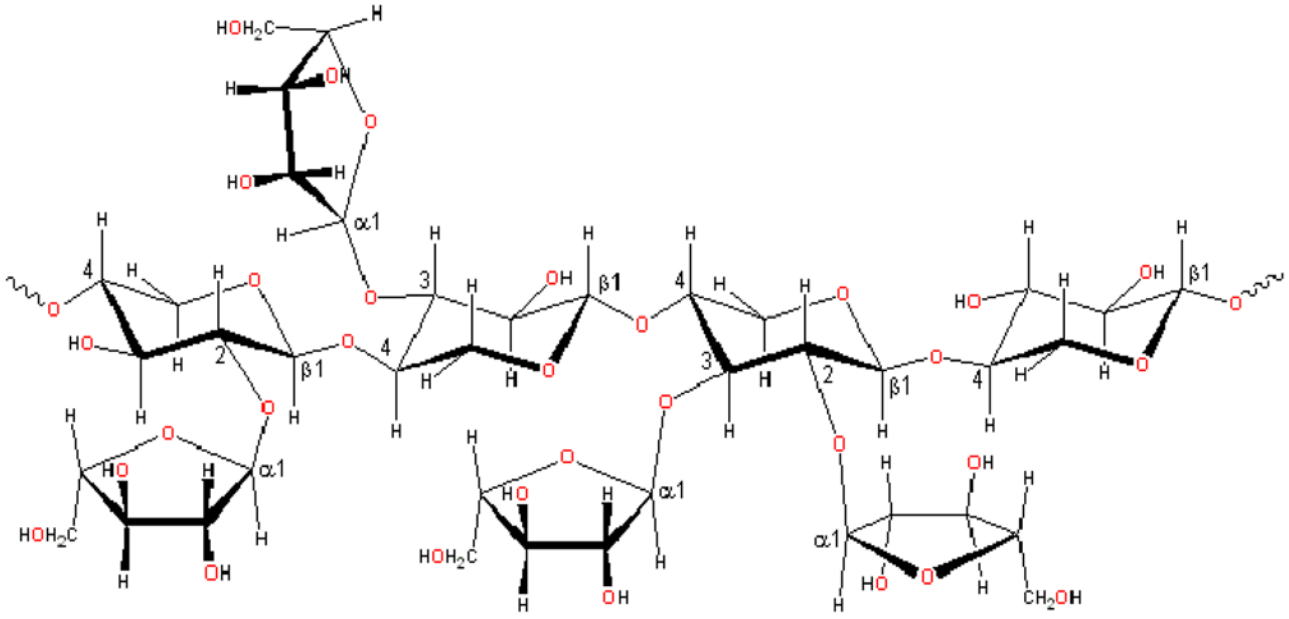

Fig. 2. Arabinoxylans consist of α-L-arabinofuranose residues attached as branch-points to α-(1→4)-linked D-xylopyranose polymeric backbone chains.

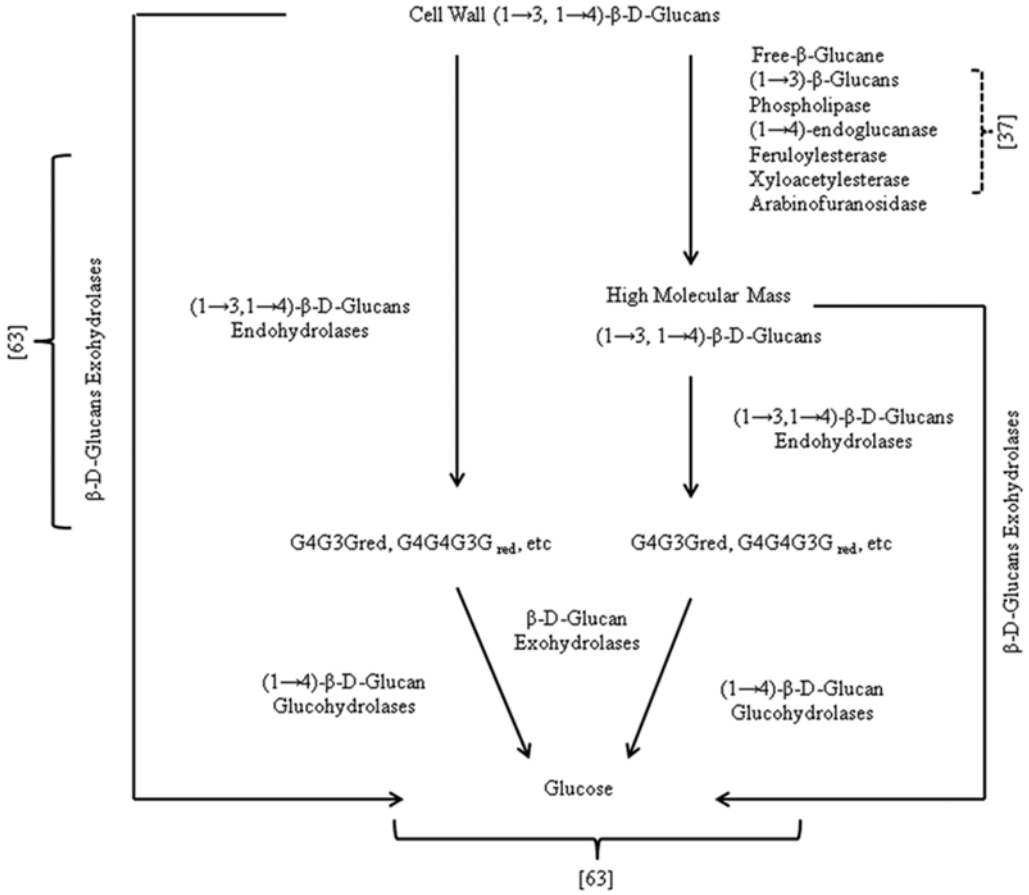

Fig. 3. Diagrammatic illustration of enzymatic hydrolysis of cell wall (1→3), (1→4)-*β*-D-glucans, based on previous reports. In this diagram, enzymes believed to be involved in the release of (1→3), (1→4)-*β*-D-glucans from cell walls and the complete hydrolysis of the polysaccharide to glucose are shown. In intermediate oligosaccharides, G indicates a *β*-D-glucosyl residue, 3 denotes (1→3) linkages, 4 indicates (1→4) linkages, and red denotes the reducing end.

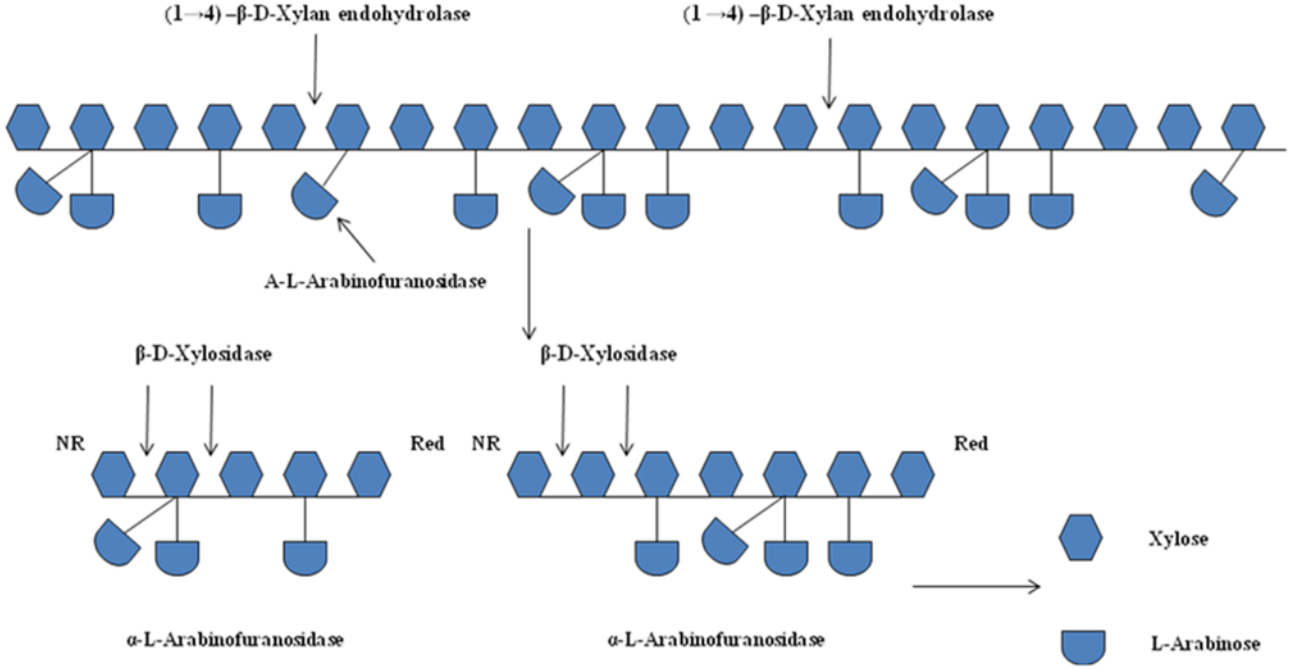

Fig. 4. Arabinoxylan structural model with proposed sites of action of major arabinoxylan hydrolyzing enzymes and sequential stages of complete de-polymerization.

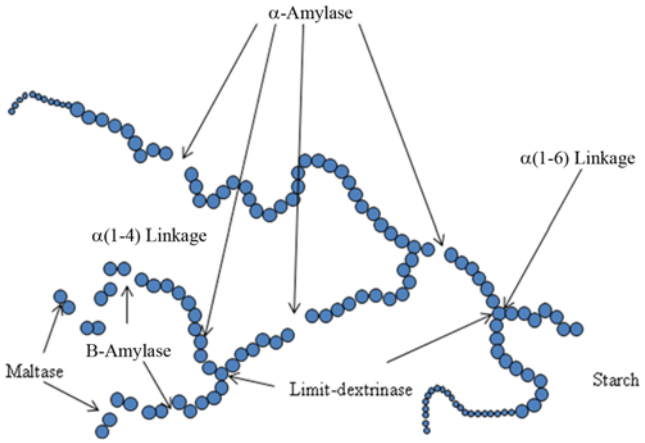

Fig. 5. Medium sized dextrins are produced from starch as result of α-amylase activity and later as result of β-amylase, α-glucosidase and limit dextrinase activity, which hydrolyzes the starch into glucose monomers.

corn and barley. Payen and Perzos (1833) observed that alcohol precipitate of malt extract (termed diastase) had the potential to liquefy the starch into simple sugars [70]. It was later found that malt diastase has two different catalytic activities [71]; thus, it was classified as α-amylase (or dextrinogen) and β-amylase (or saccharogen) [72]. Various studies have illustrated the presence and involvement of hydrolyzing enzymes from malt in starch degradation, the most important being α-amylase, β-amylase, limit dextrinase and β-glucosidase [31,40].

α-amylase is a key enzyme in the metabolism of organisms that use starch as energy sources that also has the potential for use in production of starch derivatives on an industrial scale. In the malting process, α-amylase is produced at the beginning of germination, after which it migrates to, and is stored in, the mature endosperm of seeds [73]. α-amylase acts as an endo-acting enzyme that randomly cleaves α-(1→4) glycosidic linkages in starch producing medium sized dextrins as hydrolysis products (Fig. 5). This hydrolyzing activity of α-amylase peaks in the first 3 to 4 days of seed germination during the malting process [74]. α-amylase of barley malt possesses two isozymes, AMY1 and AMY2, which can be distinguished by their PI values (AMY1=4.7-4.9 and AMY2=5.9-6.1) [75].

Malt β-amylase is a typical exo-acting enzyme involved in the cleavage of α-(1→4) glucosidic bonds at the non-reducing end of linear chains in starch and other polysaccharides. As a result of its catalytic activity, β-maltose and β-glucose are produced successively [76,77]. β-amylase continues its catalytic activity until α-(1→6) linkages in starch molecules are reached. Glucosidases also behave as exo-acting enzymes and cleave the α-(1→4) glycosidic linkage in starch molecules. The main advantage of glucosidase over β-amylase is its ability to bypass the side chain at α-(1→6) bonds [78].

Limit dextrinase (EC 3.2.1.41) is expressed in the aleurone layer of barley grain and secreted into the endosperm during the malting process [29]. Malt limit dextrinase only has the ability to break the amylopectin α-(1→6) linkages of branched dextrins and completes hydrolysis of starch into its monomeric sugar at the end of the starch hydrolyzing reaction [79]. In short, the initial solubilization of starch in malt is mainly catalyzed by α-amylase and the hydrolysis of the resulting dextrins to oligosaccharides and glucose is subsequently carried out by the synergistic action of α-amylase, β-amylase, limit dextrinase and glucosidase [1,32].

3. Protein Hydrolyzing Enzymes

Cereal grain proteins are either water-insoluble hordeins or water-soluble albumins. Hordeins are considered storage proteins, while albumins are required for storage protein hydrolysis. Similar to polysaccharides hydrolysis during malting, the hydrolysis of polypeptides is also an essential catalyzed reaction for production of amino acids and di-tri peptides required for initial seed growth (germinating seedling). Typical malted barley constitutes about 9-11% of proteins by dry weight [80]. In the brewery industry, malted cereal is further processed for production of alcoholic beverages (e.g., malt barley for beer production). Protein is the constituent of all beers, representing 0.5% of the final product. During fermentation, malt proteins are involved in foam formation and stabilization and are required for the nutrition of yeast cells [78,81].

Certain proteolytic enzymes synthesized during germination are involved in the production of free amino acids (FAN), di-tri amino acids. These enzymes can provide a greater exposed surface area for starch hydrolyzing enzymes [82]. The production of free amino nitrogen (FAN), di-tri amino acids as result of proteolytic activity is a protein modification that ultimately results in seed softening and increased friability being required for effective growth of seedlings [83]. The enzymes involved in this protein modification are shown in Table 1. All enzymes share the same substrate (polypeptide), but have different action sites. The final product, FAN, di-tri amino acids, is produced as result of their synergistic action.

A portion of the enzymes involved in protein modification already exists in mature barley grain, while some are synthesized *de novo* in aleurone cells during germination [84,85]. Endopeptidases and exopeptidases are two principal groups of malt proteolytic enzymes. Endo-peptidases cleave the bonds between two amino acids in a protein molecule at random, producing relatively smaller peptide chains, while exopeptidases attack these smaller peptide chains and cleave the links between terminal amino acids. Therefore, exopeptidase activity ultimately leads to protein molecules being hydrolyzed into FAN or di-tri amino acids. Endopeptidase has a lower optimum temperature; thus, most of these enzymes are degraded during the malting process. However, exopeptidase can withstand high temperatures and complete the protein hydrolysis process. Exopeptidase consists of two major enzymes, carboxypeptidase and aminopeptidase, which both have different active sites. Specifically, carboxypeptidase has the ability to hydrolyze protein molecules from the carboxyl end, while aminopeptidase works from the amino end [78,86].

4. Lipid Hydrolyzing Enzymes

Lipids comprise a complex constituent of cereal crops with varying properties. Their applications include use as a natural food reservoir (natural glycosides), source of hydrocarbons (waxes), cofactor in intermediate metabolism (quinines) and structural component of cells (polar glycerides). Grain lipids consist of 65-78% natural lipids, 7-13% glycolipids and 15-26% phospholipids [87]. Lipids in cereal grains are associated with starch granules on their surface or within the starch structure [88]. The lipids inside the starch granules and

inclusion bodies containing polysaccharides play important roles in altering the gelatinization properties of starch granules [89]. The number of biopolymers hydrolyzed is governed by hydrolyzing enzymes in the initial stage of barley grain germination during malting. The hydrolysis of numerous biopolymers is governed by respective hydrolyzing enzymes in the initial stage of barley grain germination during malting. However, the metabolism of lipids is somehow different from that of all other macromolecules. Although the grain already possesses lipid hydrolyzing activity, this activity increases by up to four times during malting [88,90].

Lipid hydrolyzing enzymes in malted barley include lipase [53], lipoxygenase [55], hydroperoxidase lyase, and hydroperoxide isomerase [40,91]. Lipases are hydrolyzing enzymes that catalyze esters of long chain aliphatic fatty acids to produce free fatty acids and glycerol [92]. Their ability to hydrolyze insoluble fatty acyl esters makes lipases different from all other esterases [93]. Barley grain already possesses lipases, but its efficacy increases many fold during malting [87]. Cereals contain storage fats or oils called lipid bodies that are hydrolyzed into free fatty acids by lipase. The hydrolysis of lipid bodies is directly related to the concentration of lipase in the aleurone and scutellum of grain, including both pre-existing and newly synthesized lipase in response to physical and chemical changes [94-96]. Free fatty acids produced by triglycerides hydrolysis are readily utilized as metabolic energy in the synthesis of glucose when grains containing soluble sugars are depleted or as precursors for phospholipids synthesis involved in cell membrane proliferation [97,98].

The enzyme lipoxygenase in malt can hydrolyze the dioxygenation of polyunsaturated fatty acids (linoleic and linolenic acid) into hydroperoxy acids. This hydrolyzed product is used as a precursor for the production of stale testing aldehydes used in the wine brewing industry [99]. Barley genetic material encodes two types of lipoxygenases secreted in embryonic tissues, LOX-1 and LOX-2. LOX-1 is already present in raw barley and its expression increases with germination, while LOX-2 is only expressed during germination of barley grain [99]. Lipoxygenase is heat sensitive and loses most of its activity (96-98%) during the Kilning process [53-54,100]. Even with such a low hydrolyzing activity after the kilning process, almost 30% of malt lipids are hydrolyzed by lipoxegenase during mashing [101]. This enzyme also plays a crucial role in senescence, wounds and infection, and resistance against pests [102-104].

Hydroperoxide lyase (HPL), which is very common in plants, is known for catalysis of the hydrolysis reaction of fatty acid hydroperoxides into oxo-acids and aldehydes. HPL is a member of the cytochrome P450 family that plays a major role in phytooxylipis synthesis as a result of interactions among plant herbivores [105-107]. HPL is divided into two groups based on its substrate activity, 13-HPL (CYP74B) and 9-/13-HPL (CYP74C) [104-105]. Owing to its alteration of the constituents of volatile aldehydes, HPL plays an important role in determination of the characteristics of food products [109,110]. Intensive studies have been undertaken to investigate the effects of dicot HPL on food quality, but less data pertaining to HPL activity in monocots is available, especially malt [111]. The hydroxyperoxide isomerase found in malt [89] catalyzes the conversion of hydroperoxylinoleic acid into α- or β-ketols. The locus of its expression changes with germination, with the enzyme only being expressed in the embryo as the dormant form during early development, but found in the embryo, acrospires and the rootlets of germinated barley during later stages [90]. Hydrase enzymes have also been reported in malt [40].

5. Other Hydrolyzing Enzymes

In addition to cell wall, starch, protein and lipid hydrolyzing enzymes, many other hydrolyzing enzymes are already present in malted barley grains or expressed during the malting process. These hydrolyzing enzymes include phytase, peroxidase, thiol oxidase [40] and nuclease [30]. Pytate (*myo*-inositol hexakisphosphate, $InsP_6$) is a major constituent of barley that serves as a source of phosphorus and is mainly concentrated in the external layer of grains. Phytase is involved in the hydrolysis of phytate into phytic acid [112], inorganic phosphate and myo-inosito during the malting process [113] and thus decreases its total contents in the final product. Two types of phytase, 3- and 6-phytase, are renowned for their dephosphorylation of phytate. 3-phytase is found in microbial species, while 6-phtase is primarily found in seeds of higher plants including barley [114,115]. Inorganic phosphate as a constituent of nucleotides may also be utilized by metabolic machinery during *in vivo* nucleotides (purine and pyramidine) synthesis.

BIOETHANOL PRODUCTION

Owing to the high reliance on fossil fuels, expansion of the human population and rapid evolution in industrial sectors, the global fossil fuel reservoir is rapidly being depleted. The average annual global oil production has been decreasing annually, and this will become more serious in the near future [116,117]. The surge in fossil fuels combustion gave rise to certain environmental concerns in recent decades, the most prominent being the drastic increase in greenhouse gasses in the earth's atmosphere [118]. The rapid depletion of fossil fuels coupled with environmental concerns associated with their use has resulted in investigation of alternative renewable sources receiving a great deal of attention [119,120]. The major issue associated with sustainable industrial evolution is the transition from fossil fuels to renewable fuels in different sectors, such as energy and fuel, chemical and all other related industries. Renewable sources have the potential to replace petroleum based fuels in the near future [121,122].

Bioethanol is one of the most common and well known renewable fuels contributing to diminished hazardous environmental impacts generated by the worldwide consumption of fossil fuels. Process engineering has been applied to the design of environmentally friendly and cost effective technologies for bioethanol production [123], and the bioethanol production capacity has been increasing continuously. In 2001, the global annual production of bioethanol was 18.45 billion liters, and this grew to 39.24 billion liters in 2006 and is expected to reach 100 billion liters in 2015 [124-127]. Biofuel obtained from biomass is categorized into generations based on the technologies adopted for its production and the type of biomass used as the source. Fig. 6 provides a schematic diagram of first and second generation biofuel production.

Bioethanol production is directly dependent on the enzyme activity. Familiarization with the enzymes' nature and understanding their particular role in the metabolic pathway should advance the production of bioethanol. The enzymes involved in the bioethanol production process are related to the precursor feedstock. Based on the

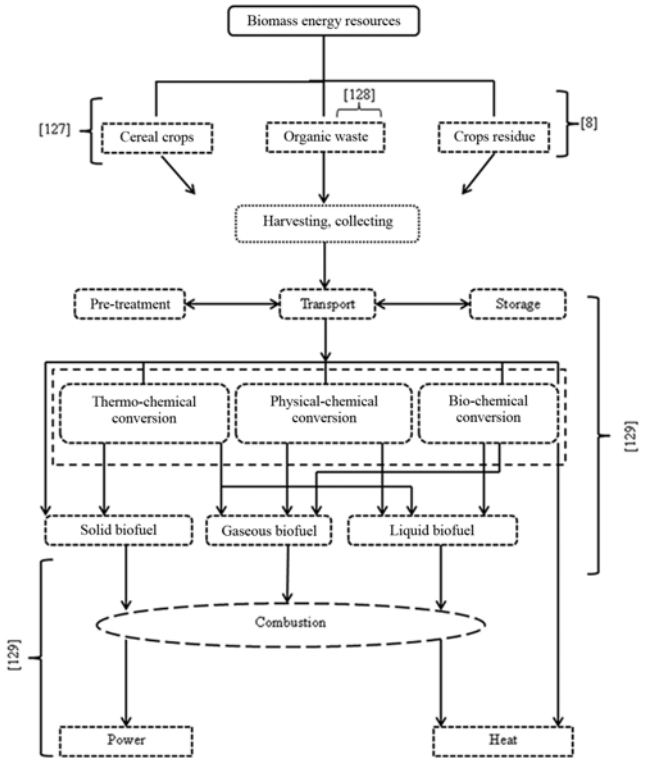

Fig. 6. Basic pathway for first and second generation biofuel production with concern technological approach and biomass feedstock, prepared based on previous reports.

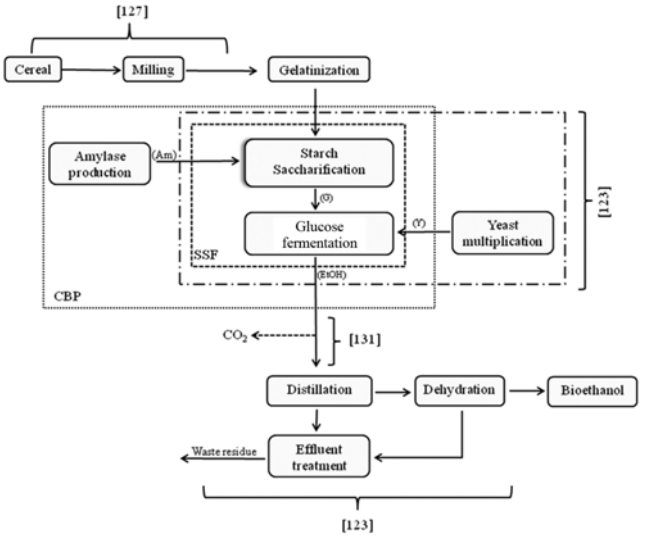

Fig. 7. Generic block diagram of fuel ethanol production from cereal grain (starchy materials) based on previous reports. Possibilities for reaction-reaction integration are shown inside the boxes: SSF, simultaneous saccharification and fermentation; SSYPF, simultaneous saccharification, yeast propagation and fermentation; CBP, consolidated bioprocessing. Main stream components: Am, amylases; G, glucose; Y, yeast; EtOH, ethanol.

feedstock, ethanol production has been classified into first and second generation bioethanol production. However, the shifting of feedstock is a dynamic process that occurs with the requirements of bioethanol production with environmental and economic perceptions. In fact, the need for bioethanol production has increased continually, leading to the origination of different procedures, pathways, feedstocks and enzymes during the process. The requirements and the developmental procedure for eco-friendly bioethanol are discussed herein.

1. First Generation Bioethanol

Ethanol produced using starchy food crops is usually referred as to first generation bioethanol. Sugar-containing crops (sugar cane, wheat, beet roots, fruit, palm juice) and starch-containing crops (grains such as wheat, barley, rice, sweet sorghum, and corn, and root plants such as potato and cassava) are two major classes of crop usually preferred for the production of first generation bioethanol [130]. Bioethanol produced using grain crops (wheat, barley, rice, sweet sorghum, corn, etc.) is also known as grain ethanol and its production is illustrated in Fig. 7.

The production of first generation bioethanol consists of two basic steps, saccharification of crops containing polysaccharides and the subsequent fermentation of reducing sugars obtained as a result of saccharification (Fig. 7). Bioethanol production from cereal crops is primarily dependent upon the polysaccharides content in grain. In cereal crops, the saccharification process involves the hydrolysis of starch into its reducing sugars. Starch consists of amylose and amylopectin. Amylose is a linear chain of glucose linked with α-(1→4) glycosidic bonds, while amylopectin is composed of glucose chains and arises as branches from the backbone of starch molecules bound by β-(1→6) glycosidic linkages. Amylase makes the major contribution to starch hydrolysis and plays an important role in the biotechnological approach to the starch saccharification process [33]. Certain enzymes from microorganisms have been adopted for effective hydrolysis of starch into reducing sugars on an industrial scale [33]. During brewing, malt endogenous starch hydrolyzing enzymes play a major role in the saccharification of grain starch into its reducing sugars [132]. These starch hydrolyzing enzymes from malt found in WBFB have also been evaluated for use in saccharification processes during bioethanol production [15]. Starch hydrolyzing enzymes from malt reported in WBFB are given in Table 1. Processing adjuncts (unmalted cereal grains) for bioethanol production requires the continuous supply of exogenous enzymes for effective saccharification as the adjuncts are deficient in endogenous hydrolyzing enzymes [33].

Valuable products such as amino acids, fatty acids and reducing sugars, which are essential to fermentative microorganism proliferation, have been obtained during malting by endogenous hydrolyzing enzymes. The selection of fermentative microorganisms primarily depends on the composition of sugar molecules in a raw material. *Saccharomyces cerevisiae* is the most preferable microorganism for fermentation of hexoses, while *Kluyveromyces fragilis* or *Candida* sp. has been adopted for fermentation of lactose or pentose [133]. *S. cerevisiae* converts hexose sugar into ethanol and CO_2 through glycolysis via the following reaction:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

A variety of enzymes are involved in hydrolysis of glucose into ethanol during the glycolysis process.

2. Second Generation Bioethanol

The production of second generation bioethanol, also referred to

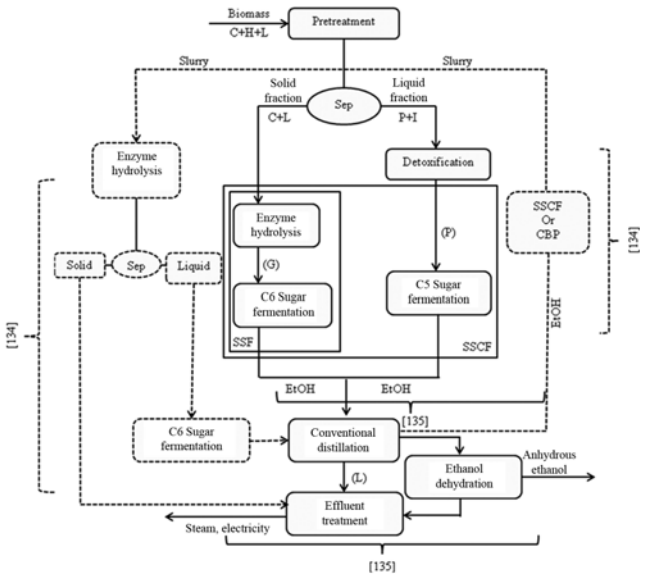

Fig. 8. Generic block diagram of second generation bioethanol production from lignocellulosic biomass with representation of SSCF (simultaneous saccharification and co-fermentation), CBP (Consolidated bioprocess), and SSF (simultaneous saccharification and fermentation) based on previous reports. Main stream components: C, cellulose; H, hemicellulose; L, lignin; G, glucose; P, pentoses; I, inhibitors; EtOH, ethanol.

as the production of bioethanol from biomass, uses an organic carbon source that is rapidly renewed as part of the carbon cycle (Fig. 8). Ethanol derived from biomass is considered one of the most advanced and reliable approaches for solution of the expected fuel crisis. The utilization of sugar and cereal crops was common in early methods of bioethanol production. However, the rapid increase in use of such feedstocks for bioethanol has had an adverse impact on global food supplies, leading to a food versus fuel debate. The use of lignocellulosic biomass for bioethanol production has reduced this conflict to some extent [136]. Indeed, the advent of new technology and pretreatment procedures will make possible widespread utilization of lignocellulosic biomass for bioethanol production.

Lignocellulosic biomass, which is processed for bioethanol production, primarily includes the residues of cereal crops such as corn stover, barley straw, wheat straw and rice straw. These crop residues are mainly composed of cellulose, hemicellulose, lignin and protein. The cellulose and hemicellulosic composition plays a vital role in the production of bioethanol, as they are hydrolyzed into sugar via hydrolysis processes and thus readily available for fermentative organisms [121,137]. The percentage of lignocellulosic biomass and their potential for bioethanol production is summarized in Table 2. Corn stover, barley and wheat straw are the most preferable lignocellulosic biomasses due to their reasonably high potential for bioethanol production. Asia is one of the richest regions for lignocellulosic biomass production based on availability. Indeed, almost 932.81 million tons of lignocellulosic biomass is produced annually in Asia, which is about 60% of the worldwide lignocellulosic biomass production. Moreover, lignocellulosic biomass is rich in lignin contents, and the residue obtained from fermenters after bioethanol production has the potential for use in the generation of electricity and steam power [8].

Owing to the importance of crop residue for long term sustainable crop yields, the removal of crop residue from fields is a sensitive issue. In most cases, the availability of crop residues is directly related to soil erosion and soil organic composition [146]. Due to its importance, the United States Department of Agriculture has published a complete guideline that considers all other parameters directly or indirectly affecting sustainable crop yield [147]. Since excess crop residue on the soil surface also affects crop yield, 70% collection is currently the preferable limit for industrial processing [148].

To overcome the risk to food security, lignocellulosic material is the most preferable feedstock for bioethanol production. However, many hurdles must be overcome before this material is utilized for microbial ethanol fermentation, among which the process of pretreatment is the most challenging. Indeed, considerable effort is still required to improve pretreatment methods to maximize glucose conversion to ethanol. The recalcitrant and heterogeneous structure of lignocellulosic biomass resists enzymatic saccharification, resulting in reduction of sugars as the final product [149,150]. To provide maximum access to cellulase enzymes and their catalytic activity, pretreatment of lignocellulosic biomass is essential to improve glucose yield and its fermentation into ethanol [151,152]. The rate of hydrolysis of this lignocellulosic biomass is directly affected by the porosity of the biomass, crystallinity of cellulose fiber and composition of lignin and hemicellulose [153]. Delignification, solubilization of hemicellulose, reduced cellulose crystallinity and an increased fraction of amorphous cellulose occurs during pretreatment of lignocellulosic biomass, resulting in escalating enzymatic hydrolysis of cellulose into glucose [154]. However, pretreatment conditions and the requirement for special equipment with high energy input makes

Table 2. Percentage composition of lignocellulosic biomass in various sources and their potential for bioethanol production

Feed stock	Composition (%)						References
	Cellulose	Hemi-cellulose	Lignin	Protein	Ash	Ethanol yield (L/kg of dry mass)	
Corn stover	42.60	21.30	15.10	4.00	4.30	0.29	[8,121,138]
Barley straw	33.25	20.36	17.13	3.62	2.18	0.31	[8,139]
Wheat straw	34.20	23.68	13.88	2.33	2.36	0.29	[8,139]
Rice straw	23.47	19.27	9.90	2.20	12.40	0.28	[8,138,140,141]
Bagasse	52.42	25.80	18.40	3.00	2.73	0.28	[8,138,142]
Oat straw	14.15	17.13	12.85	5.34	2.19	0.26	[8,139]
Brewery waste	13.14±0.35	29.96±1.03	7.12±0.26	0.07	5.76±0.14	0.11	[143-145]

Table 3. Recent proposed methods for pretreatment and hydrolysis of different feedstocks and final sugar concentration

Feed stock	Pretreatment	Hydrolysis	Yield of sugars	Reference
Corn stover	Soaking pretreatment 1% NaOH+8% NH_4OH, 50 °C, 48 h	Enzyme (Celluclast 1.5 L at 30 FPU/g of substrate and Novozyme 188 at 45 CBU/g of substrate) were used at 50 °C, pH 4.8 and at 150 rpm	Glucose 78.5% Xylose 69.3%	[156]
Barley straw	Alkaline peroxide (2.5% H_2O_2, pH 11, 35 °C, 24 h)	Cocktail of 3 commercial enzyme (cellulase, beta-glucosidase, and hemicellulase) at 0.15 ml/g of straw at 45 °C, pH 5.0, 120 h	Glucose, 395±0 mg Xylose, 176±0 mg Arabinose, 32±0 mg total sugars, 604±0 mg 94% yield	[157]
Wheat straw	Air dried, milled, and grinded to a size lower than 0.5 mm, diluted acid treatment (1.2% H_2SO_4 at 130 °C, pH 5, 150 min)	Celluclast 1.5 L at 10 FPU/g polysaccharides supplemented with Novozym 188 at 0.2 mL/g polysaccharides 55 °C, pH 4.8, 130 rpm	Glucose ~53%	[158]
Rice straw	Microwave alkali pretreatment (washing, air drying at 50 °C and chopping to 1 mm size rage, 2.7% NaOH in Microwave oven for 20 min)	Cellulase loading around 10 FPU/g biomass and BGL loading of 100 IU/g, with tween 80 at 50 °C, pH 4.5 and 100 rpm	Total sugar 84%	[159]
Bagasse	Ball milling (4 hr)	Acremonium cellulose at 5 FPU/g substrate of celluloase and 20 U/g substrate of xylanase from optimase BG at 45 °C, pH 5.0 for 72 h	Glucose 89.2±0.7% Xylose 77.2±0.9%	[160]
Oat straw	Soaking in 15% aqueous Ammonia	Cellulase at 25 FPU/g of substrate and glucosidase at 50 CBU/g of substrate	Glucose 93.5%	[161]

this a costly approach with the potential for the formation of products that inhibit subsequent microbial fermentation [155]. Table 3 shows the different feedstocks and their suitable pretreatment processes, as well as the types of enzymes involved in biomass hydrolysis and the final yield of reducing sugars available for fermentation into ethanol.

BIOETHANOL PRODUCTION FROM WASTE MATERIALS

Although starch crops and their residues are considered promising sources for bioethanol production, their continuous availability is questionable due to their use in the global food and animal feed supply. Bioethanol production from lignocellulosic biomass is always preferable, but the uncertainty associated with techno-economic feasibility due to its pretreatment costs has prevented its commercialization on a large scale. The cost of biomass is almost 31.3% of the overall operation costs of biofuel production [162]. Owing to recent developments in pretreatment technologies, massive research is underway to identify the least expensive feedstock for bioethanol production. Thus, lower feedstock costs and cost-effective pretreatment technologies will result in a significant decrease in the costs associated with production of cellulosic ethanol. Accordingly, a wide variety of waste material has been investigated to identify the least expensive alternative feedstocks for bioethanol production.

Owing to its abundance and cellulosic composition, municipal solid waste is considered an attractive feedstock for sustainable production of bioethanol [12,163,164]. Specifically, municipal solid waste has a very low cost, is a rich source of carbohydrates (51.3%) and is available throughout the year [165]. Moreover, use of municipal solid waste for fuel production is eco-friendly. Accordingly, governments throughout the world have turned their attention to the recycling of municipal solid waste to conserve natural resources

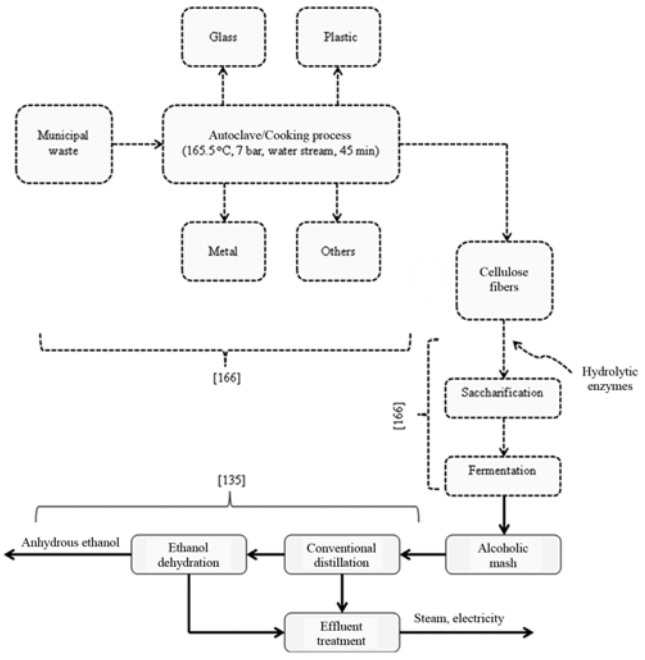

Fig. 9. Flow chart of bioethanol production employing municipal waste material as feedstock based on previous reports.

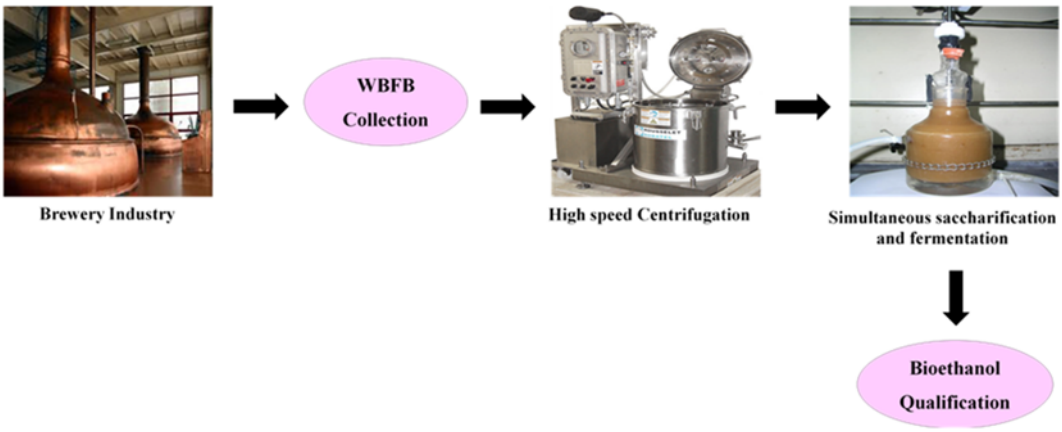

Fig. 10. Waste from beer fermentation broth is evaluated for bioethanol production via SSF at gradually increasing temperatures from 25 °C to 67 °C during culture at 150 rpm.

and investigate how to turn these solid wastes into biofuels, including ethanol. A flow chart for assessment of the potential of municipal waste for bioethanol production is shown in Fig. 9. Unnecessary contents including heavy metals from municipal waste must be removed by employing different pretreatment strategies. Exogenous cellulases are then employed for effective hydrolysis of cellulose into glucose, which is subsequently used for microbial fermentation into ethanol. The non-cellulosic portion often results in low digestibility of pretreated solid or requires large amounts of expensive exogenous hydrolyzing enzymes to make the sugar available for fermentation [164]. Overall, the biggest challenge in using municipal solid waste for biofuel production is designing a strategy to ensure bioconversion of the remaining non-cellulosic portion of feedstock into bioethanol.

Since waste material obtained from food industries such as kitchen garbage is considered a cost-competitive rich source of carbon, intensive research is underway to develop methods of using this material in bioethanol production [13,167]. When employing kitchen waste, the liquid portion is utilized for biodiesel production, while the solid portion is used for bioethanol production after pretreatment (boiling, crushing, etc.). Glucoamylase and protease hydrolyzing enzymes are used for depolymerization of polymers into its monomers, which are then utilized by yeast cells for growth, resulting in fermentation and the production of bioethanol [167]. A number of technologies for efficient and cost effective bioethanol production have been introduced, including continuous membrane bioreactors with cell recycling or membrane retention and employment of genetically modified yeast strains [163,164]. Although waste from the food industry is a cost-effective feedstock for bioethanol production, the pretreatment of waste material and hydrolyzing enzymes employed for the saccharification process make it an expensive approach for bioethanol production [167].

A semi-solid residue, WBFB, has also been evaluated as feedstock for the production of bioethanol through SSF without any extra addition of saccharification enzymes, microbial cells or carbohydrates [14,15]. During malting at the beginning of beer production, many hydrolyzing enzymes are produced. The malting process activates different enzymatic pathways, including starch hydrolysis. The product of starch hydrolysis is then supplied to endosperms, which are essential to the primary growth of plants. Malted barley is preferred by the brewing industry because it is a rich source of starch and proteins that also contributes to the supply of cytolytic, proteolytic and amylolytic enzymes required during brewing [34]. Thus, it is expected that WBFB would be a rich source of malt hydrolyzing enzymes. Most malt-derived hydrolyzing enzymes, including cell wall hydrolyzing enzymes, starch hydrolyzing enzymes, lipid hydrolyzing enzymes, protein hydrolyzing enzymes and a few others have been identified in WBFB.

High saccharide content, malt hydrolyzing enzymes and yeast cells make WBFB one of the most suitable and cost effective feedstocks for bioethanol production. In addition to its availability throughout the year and the fact that there is almost no cost associated with its pretreatment, a high concentration (103.8 g/L) of ethanol is usually produced from WBFB [14]. Additionally, the hydrolyzing enzymes existing in WBFB have the potential for use as exogenous enzymes for nullification of the processing costs of other carbohydrate containing feedstocks for bioethanol production [14,15]. Overall, the high availability, cost-effective pretreatment, pre-existing yeast cells, and high levels of carbohydrates and malt hydrolyzing enzymes make WBFB the most attractive feedstock among all waste materials currently employed for bioethanol production. Fig. 10 summarizes WBFB processing for bioethanol production.

POSSIBILITY OF APPLICATION OF MALT HYDROLYZING ENZYMES TO BIOETHANOL PRODUCTION

As mentioned earlier, the biological processes in bioethanol production are controlled by the enzymes. The multiplicity of feedstocks and availability of various enzymes have made it possible to develop numerous methods for bioethanol production. Malt contains almost all enzymes produced from different feedstock that can be utilized for bioethanol production. The need, quantity, specificity and activity of the enzymes involved in bioethanol production differ with feedstocks. Understanding the involvement and working mechanism of these enzymes is very important to the commercial scale up of bioethanol production. Accordingly, a detailed discussion based on the applications of particular enzymes in the meta-

bolic pathways involved in the production of ethanol from different feedstocks of first and second generation bioethanol is provided below.

Cell wall hydrolyzing enzymes including endo-gluconase, exo-gluconase, arabinofuranosidase, esterase, carboxypeptidase, xylanase and β-glucosidase play crucial roles in bioethanol production. The expression of cell wall hydrolyzing enzymes reflects the beginning of all modifications that occurred in cereal grains during the malting process. By using starchy endosperm for bioethanol production, the arabinoxylans and (1→3, 1→4)-β-D-glucans and cellulose are targeted to for complete hydrolysis into their monomers. The hydrolysis of β-glucans is directly related to the expression and catalytic activity of the cell wall hydrolyzing enzymes, (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidase [61,64,65]. Due to the synergistic action of these enzymes, most cell wall components (cellulose, (1→3, 1→4)-β-D-glucans, arabinoxylans) are hydrolyzed into hexose sugars that are readily available for microbial fermentation. Therefore, a greater hydrolyzing enzyme activity is associated with a higher final bioethanol production through microbial fermentation under standard conditions. During the production of second generation bioethanol, cell wall hydrolyzing enzymes make the same contribution to fermentable sugar production, but lignin, the main component of lignocellulosic biomass, reduces the activity of saccharification enzymes [149].

The production of first generation bioethanol is well known because of the use of starchy cereal crops as feedstock. During fermentation of starch molecules, the α-(1→4) and α-(1→6) bonds must be cleaved to generate reducing sugar (glucose) to be fermented into ethanol. There are numerous hydrolyzing enzymes capable of cleaving these bonds to obtain glucose as a final product. Amylases are important industrial enzymes that are well known for their synergistic action in the hydrolysis of starchy biomass into reducing sugars [170]. This complex of amylolytic enzymes is composed of exoamylases (β-amylase and glucosidase), endoamylases (α-amylase) and the debranching enzymes (limit dextrinase). One of the two main processes that significantly affect the final cost of bioethanol production from starch is the requirement for a large amount of exogenous amylase enzymes. α-amylase, β-amylase, limit dextrinase and β-glucosidase are well characterized enzymes involved in the hydrolysis of starchy biomass. Accordingly, optimizing the malting conditions for over expression of these hydrolyzing enzymes might be a reliable approach to cost-effective ethanol production, leading to maximization of the hydrolysis of starch into glucose.

Protein hydrolyzing enzymes from malt play an important role in bioethanol production when yeast cells are used as the fermenting microorganism. Yeast requires nutrients such as peptides and amino acids for its growth and thus for continuous fermentation of the reducing sugars of the growth media into ethanol. Malt hydrolyzing enzymes characterized for polypeptide hydrolysis are preexisting in cereal grains, and their expression is enhanced in aleurone layer cells as a result of the malting process. Through the synergistic action of exo- and endo-peptidases, protein is finally hydrolyzed into FAN (short peptides, amino acids). Therefore, proteolytic enzymes are responsible for the continuous supply of nutrients for rapid yeast cell proliferation during fermentation. In short, proteolytic enzymes are directly related to the efficiency with which yeast cells ferment reducing sugars during bioethanol production. Conversely, proteolytic enzymes also affect the starch hydrolytic enzyme activity. As starch is in a complex with proteins in cereal grain, more surface area is provided to starch hydrolyzing enzymes. Accordingly, abundant reducing sugars are obtained for bioethanol production via yeast cell fermenting machinery.

Lipid hydrolyzing enzymes play a more significant role in biodiesel production than in bioethanol production. In biodiesel production, these lipid hydrolyzing enzymes are involved in transesterification of vegetable oils or fats, which has several advantages over conventional chemically catalyzed esterification [171]. Lipases are similar to other enzymes with a proteinous nature, and are involved in the hydrolysis of triglycerides into di-, mono-glycerides, fatty acids and glycerol. Little literature describing the direct importance of lipid hydrolyzing enzymes in bioethanol production is available. However, it is predicted that free fatty acids are readily utilized for the generation of energy required for the synthesis of glucose in case of soluble glucose depletion [97].

CELL-FREE ETHANOL PRODUCTION

The activity of enzymes is highly sensitive to the pH and temperature of the media. Bioethanol production involves enzymes for saccharification and fermentation (endogenous/body enzymes). The temperature specificity of enzymes varies widely from 25 °C to 90 °C [172], and hydrolyzing activities usually favor much higher temperatures than fermenting activities. Taking into account the suitability for production, the process is controlled at certain specified temperatures. If bioethanol production is carried out in one pot at a higher temperature, it could cause the death of live cells [24]. The role of endogenous enzymes from the dead microorganisms was unclear for a long time. However, it is proposed that the production of ethanol and CO_2 occurred by utilization of glucose via the metabolic machinery of yeast cells, which led to the concept of cell-free enzyme system for bioethanol production [25].

1. Cell-free Enzyme System

Cell-free enzymes systems have been adopted for the production of various bio-products [173], Polymyxin E [174], and Tyrocidine [175]. Despite in vivo cytotoxic and regulatory protein synthesis, the interest in cell-free systems is primarily due to their ability to produce certain unstable proteins that are usually not expressed under normal circumstances inside the cell [176,177]. Cell-free enzyme systems for bioethanol production involve the expression of enzymes and all other related polypeptides in combinatorial manners. It is known that a single enzyme is responsible for catalysis of a simple biochemical reaction. Approximately 4,800 enzymes catalyzing specific reactions have been found and classified in the Brenda database [178]. The most important consideration when employing this approach is to evaluate the optimal conditions for the activities of all concerned enzymes. Because bioethanol production entails a number of reactions, several enzymes are involved in individual specific reactions that ultimately convert the biomass containing polysaccharides into bioethanol.

The entire process of biomass conversion into bioethanol primarily consists of two processes, saccharification and fermentation. The saccharification process is simply depolymerization of polysaccharides into their monomer, glucose molecules, which are readily

available for the fermentation into ethanol by glucoside hydrolases (GHs) [179].

High concentration of bioethanol in a culture medium has a toxic effect on yeast cell viability [180], which is a major limitation of bioethanol production through yeast cell culture. To overcome this problem, a great deal of research work has been conducted to improve yeast strains by enabling them to tolerate high ethanol concentrations and cell-free enzyme systems [181,182]. The production of functional proteins involved in bioethanol production has been achieved via cell-free protein expression, which is considered to be a simple and high throughput methodology [179]. Cell-free protein expression systems involve the translational machinery for encoding genetic information as well as endogenous protein synthesis, which is essential to metabolic pathways that include glycolysis [183], oxidative phosphorylation [184] and amino acid synthesis [185]. To generate ATP through glycolysis, glucose was evaluated as an economical approach for comparison to phosphate based compounds such as creatine phosphate and phosphoenolpyruvate [184].

Cell-free enzyme systems are initiated via utilization of a small fraction of sugars such as glucose or xylose as an energy source. Once a cell-free synthesized GH enzyme is activated, it hydrolyzes all supplied glycan substrates into glucose, thus acting as a source of energy for more ATP generation and subsequent synthesis of additional enzymes via the cell-free system [179]. The sugar produced as result of catalytic activity of cell-free synthesized GH is used as an energy source for further protein translation via the cell-free system, as well as converted into acidic byproducts, resulting in a decrease in the cell-free mixture pH. The decrease in pH has a positive effect on the activity of many GHs with optimum activities that range from pH 4.0 to 6.5 [186].

2. SSF and Cell-free Enzyme System using WBFB

Simultaneous saccharification and fermentation (SSF) of reducing sugar (glucose) is an attractive and reliable approach to a high yield of bioethanol. The reducing sugar concentration and culture conditions (<35 °C) play a critical role in bioethanol production. The temperature of the culture broth is one of the most important parameters impacting bioethanol production via SSF [187].

WBFB is as potent source of malt hydrolyzing enzyme and yeast cells that has been utilized for bioethanol production, even at high temperatures (>60 °C). Despite the yeast cell lysis that occurs at high temperature, bioethanol production was observed using WBFB as the culture media [14,15]. This is likely because, at high temperature in the presence of malt hydrolyzing enzymes extract (containing cell wall hydrolyzing enzymes), the cell wall of the microbes breaks down and the cell matrix containing all of translation machinery and related polypeptides involved in cell-free protein expression system is secreted into the culture broth [14,15]. Indeed, high temperature SSF for bioethanol production may only be possible via cell-free biosystems composed of dead yeast cells in the presence of malt derived enzymes.

The final product of malt derived starch and cell wall hydrolyzing enzymes is simple sugar (glucose or xylose), which is required for initiation of the expression of proteins (including enzymes) involved in the fermentation process. Simple sugars are not only utilized as a source of energy for initiation of the fermentation process, but also in the expression of fermentation related enzymes (GHs) [179]. WBFB enriched in malt hydrolyzing enzymes does not require an external supply of simple sugar or saccharide hydrolyzing enzymes for either initiation or proliferation of fermentation via the cell-free enzyme system. The conversion of β-(1→4) glycosidic bond linked cellulose into α-(1→4)-glycosidic bond linked starch can be accomplished via a cell-free biosystem in the presence of malt hydrolyzing enzymes without the addition of external enzymes [188].

The translation machinery of yeast cells requires free amino acids for the translation of polypeptide sequences involved in bioethanol production. WBFB enable the continuous supply of reducing sugars for bioethanol production and offer malt derived protein hydrolyzing enzymes that provide free amino acids for yeast translation machinery for the synthesis of enzymes involved in metabolic pathways. It can be concluded that, in the case of utilization of WBFB as feedstock for bioethanol production, the presence of malt derived hydrolyzing enzymes directly influences the efficiency of yeast cell-free biosystems for bioethanol production. Moreover, due to the high and regular supply of hexose sugars, bioethanol may be obtained in higher amounts than the 9 mmol L^{-1} min^{-1} already reported in the literature for cell-free enzyme systems [189]. It can also be assumed that malt-derived lipid hydrolyzing enzymes contribute to bioethanol production via cell-free biosystems. These hydrolyzing enzymes produce free fatty acids via the hydrolysis of malt containing triglycerides, which are readily utilized as energy resources for regulation of the metabolic pathway for hexose sugar biosynthesis in cases of its depletion in reaction mixtures [97].

CONCLUDING REMARKS; FUTURE CHALLENGES AND PROSPECTS

Being a complex and versatile process, bioethanol production has passed through a series of steps before attaining its current status. With emerging energy requirements, environmental consequences, food disasters and rapid economy changes, there is a need for development of new approaches for efficient bioethanol production. The aforementioned discussion of the enzymes, feedstocks and processing strategies involved in bioethanol production indicates that a great deal of effort is still required to attain economically and environmentally friendly bioethanol. Certain issues must be resolved before achieving the goal of having bioethanol as a sole alternative fuel. Herein, we summarize the present challenges to bioethanol production.

Bioethanol production is leading to a significant reduction in our dependence on fossil fuels by enabling replacement of conventional gasoline [119]. In 2011, the global production of bioethanol forecasted by the Global Renewable Fuels Alliance (GRFA) was about 88.7 billion liters, which is sufficient to replace one million barrels of crude oil per day [190]. Regardless of such improvements in global bioethanol production, a number of challenges and barriers to commercialization of bioethanol production remain. For example, utilization of feedstocks for biofuels production has escalated global food prices over the last few years [191]. Accordingly, immense research is already underway to address these challenges and enable bioethanol production on an industrial scale.

The cost of cellulosic bioethanol production is highly dependent on the expenses of cellulase enzymes and the biomass pretreatment process. Cellulase enzymes and the pretreatment process are essential for the breakdown of biomass into its intermediates and in

removal of lignin for microbial fermentation. Both of these factors are responsible for an escalation in cellulosic bioethanol prices to almost 2 to 3 times that of bioethanol produced using cereal grains only [17]. Accordingly, genetic engineering of plants at a commercially accepted level is considered to be the most promising strategy for improvement of bioethanol production while reducing its cost as well. Through genetic engineering, it may be possible to enhance the expression of all hydrolyzing enzymes, including cellulase, thereby reducing the need for exogenous hydrolyzing enzymes in bioreactors during production [192,193].

The dominant issue preventing large scale bioethanol production was initially the high energy input required relative to its output. However, developed technologies have led to a significant increase in bioethanol production using lower amounts of energy. According to the United States Department of Agriculture, the ratio of input-output for bioethanol is about 1 : 1.34 [194]. It is expected that further improvements in bioprocess engineering will lead to a higher input-output ratio in the near future.

The SSF strategy for bioethanol production has a strong influence on the final production, but there are still many obstacles to be overcome to obtain the maximum yield [188]. Continuous thermal stress and high concentration of ethanol in the medium greatly influence the cell viability [195,196]. Development of ethanol and thermo-resistant yeast strains is the first priority to enhanced bioethanol production. Through genome modification, the development of strains that are thermally stable and adaptable to higher ethanol concentrations has been accomplished to a certain extent, but the problem has yet to be completely resolved. Therefore, a great deal of research is still needed.

Cell-free biosystems are considered an attractive approach to bioethanol production because they address the loss of cell viability that occurs at high temperature or in the presence of high ethanol concentrations. However, such systems are still not reliable or productive. Current development of cell-free biosystem strategies such a biotransformation via a cell-free synthetic (enzymatic) pathway has the potential for future advances in this technology [26]. Thus, it is suggested that the combination of biotransformation of the biomass (plant) genome and cell-free enzymatic pathways will greatly influence global bioethanol production in the near future.

Bioethanol production from various substrates is most commonly controlled by hydrolyzing enzymes. Herein, the presence of various hydrolyzing enzymes in malt and their specific saccharifying activities in various substrates for bioethanol production were evaluated. WBFB, which is a waste product enriched with substrates and hydrolyzing enzymes, was found to have potential for use in bioethanol production. However, the complex synthetic pathway to production using this material faces several hurdles regarding optimization owing to the number of enzymes involved. Among the different approaches that have been taken to resolve this dilemma, cell-free enzyme systems might prove to be the most successful. Genetic engineering and biotransformation in the cell-free enzymatic pathway will likely lead to successful biofuel technologies.

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TERMINOLOGY

SSF : simultaneous saccharification and fermentation
WBFB : waste from beer fermentation
AMY: amylase
FAN : free amino nitrogen
LOX : lipoxygenase
HPL : hydroperoxide lyase
GHs : glucoside hydrolases
ATP : adenosine triphosphate
SSYPF : simultaneous saccharification, yeast propagation and fermentation
CBP : consolidated bioprocessing
SSCF : simultaneous saccharification and co-fermentation

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