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Polysaccharides of Starchy and Lignocellulose Materials and their Use in Ethanol Production: Enzymes and other Factors Affecting the Production Process

Szymon Lugowoj*, Maria Balcerek

Institute of Fermentation Technology and Microbiology, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Wolczanska 171/173, 90-530 Lodz, Poland

Abstract

Background and Objective: Nowadays, production of ethanol involves many kinds of plant based materials, from conventionally used starchy materials such as rye, wheat, corn and barley to lignocellulose materials serving in second-generation bioethanol production. While raw materials containing simple sugars do not require such complex mashing processes, starchy and lignocellulose materials need significant processing. This review provides an in-depth description of the structures of starchy raw materials commonly used in production of first-generation ethanol. Furthermore, the review describes the structure of lignocellulose biomasses used for second-generation bioethanol production.

Results and Conclusion: Methods commonly used in distilleries to release starch from plant raw materials belong to pressure-thermal pretreatments known as steaming or pressureless liberation of starch methods. Literature shows that amylolysis is strongly determined by the morphology of starch granules. The larger the specific surface area of granules, the greater their susceptibility to amylolysis. The key stage in preparation of starch raw materials for fermentation is starch hydrolysis, which consists of two steps of liquefaction and saccharification. Several species of bacteria (e.g. *Bacillus licheniformis*) and fungi (e.g. *Aspergillus niger*) are available that are capable of producing enzymes necessary for starch hydrolysis. Enzymes needed for starch hydrolysis are divided into 1) liquefying enzymes such as α-amylase produced by *Aspergillus niger* and *Bacillus licheniformis* or can be found in malt and 2) saccharifying enzymes such as glucoamylase, β-amylase and maltogenic α-amylase of fungal, bacterial and malt origins. Proteases and phytases are used to support mashing process hydrolases of non-starch polysaccharides (xylanase and pullulanase).

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***Corresponding author:**

Szymon Lugowoj*
Institute of Fermentation Technology and Microbiology, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Wolczanska 171/173, 90-530 Lodz, Poland

Tel: 795171137
E-mail: szymon.lugowoj@dokt.p.lodz.pl

1. Introduction

The ethanol industry is an important contributor to national economies and their energy security strategies, as well as playing significant roles in responses to climate changes [1]. First-generation biofuels [ethanol and biodiesel] are based on grains, beets and sugarcanes. In recent years, significant investments have been carried out in second-generation biofuels [e.g. cellulosic ethanol] from lignocellulose materials. While raw materials containing simple sugars or disaccharides can be fermented directly by yeasts, starchy and lignocellulose materials need significant processing. Until the 1980s, the major amylolytic enzymes used in starch mashing processes included various types of malts, which are still used in industries such as whisky production due to the characteristic tastes they provide. With

technological advancements, manufacturers switched to use bacterial preparations, especially for the second-generation biofuels and biochemicals. Currently, enzymes used in mashing processes mostly include enzymes of fungal and bacterial origins [1]. There are two major types of enzymes used in the production of ethanol. Liquefying enzymes are used for the dextrinization of starch. Saccharification enzymes are used for further degradation of dextrin to fermentable sugars. Another group includes supporting enzymes used by distillers to improve mashing, fermentation dynamics, fermentation efficiency and ethanol yield [2]

2. Starchy Raw Materials

2.1. Origin and structure of starch

Cereals such as wheat, corn, rye and barley are the major raw materials used in the production of first-generation ethanol in Europe. Cereals contain starch in their endosperm polysaccharides, which forms granules with various sizes due to its organization. This organization can form concentric alternating semi-crystalline and amorphous layers [3,4]. Efficiency of starch hydrolysis mostly depends on the origin and cultivator of the substrate as well as the enzyme origin. Various starch structures result in its susceptibility to enzymes [5]. Amylolysis is strongly determined by the morphology of the starch granule, including its shapes, sizes, pores, channels and amylose contents, as well as protein and lipid-bound complexes. These characteristics depend on the origin of the raw materials and weather conditions when they are cultivated [6] Scanning electron microscope (SEM) analysis of randomly selected large (> 20 μm) starch granules from raw materials such as rye, wheat and triticale grain reveals relatively smooth surfaces with light furrows and shallow depressions (Fig. 1[A]-[C]) and characteristic grooves for cereals (Fig. 1[D]-[F]) [6].

Large granules of wheat and triticale starch are flatter than the large granules of rye. In all cases, the small granules are spherical in shape. Such surface irregularities can increase areas potentially available to enzymes. The larger the specific surface area of granules, the greater their susceptibility to amylolysis [7]. Sizes of starch granules may affect activity of α-amylase and susceptibility of the granules to hydrolysis [8]. Langenaeken et al. [9] showed that barley starch large granules were gelatinized faster than smaller starch granules at lower temperatures. Larger granules started to gelatinize at 62 °C, whereas the smaller granules showed no signs of gelatinization even after 30 min. The authors concluded that the faster gelatinization of larger granules might allow the enzymes to carry out hydrolysis faster. However, smaller granules were more prone to amylolysis, due to their relatively higher surface areas per unit of mass available for the enzyme adsorption [10].

2.2. Technologies used in starch processing

Efficiency of ethanol production from starchy raw materials is affected by type and chemical composition of the raw materials as well as processing methods. Physico-chemical parameters of the raw materials that determine the course and efficiency of starch hydrolysis and fermentation include starch content, size of starch granules, ratio of amylose to amylopectin, protein content and content of non-starch polysaccharides e.g. xylan, which may cause excessive viscosity of distillery mashes. Processing factors include type of pretreatment (pressure cooking or milling and pressureless starch liberation), gravity of the mashes, starch gelatinization temperature and time, viscosity, enzymes used in mashing, initial pH of the mashes and fermentation temperature.

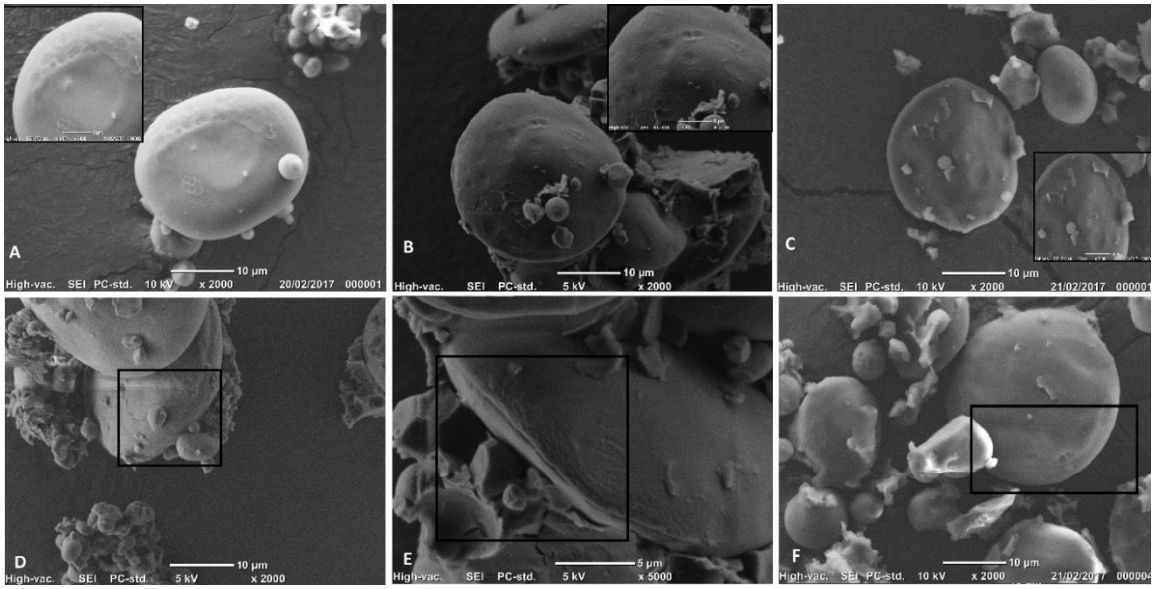

Figure 1. Scanning electron micrographs of cereal starch granules (A, D, rye; B, E, wheat; C, F, triticale) isolated from raw materials [6]

These factors usually interact and include combined effects on the efficiency of starch hydrolysis and fermentation as well as production costs [2,5]. Two major methods are used in distillery industries to release starch from plant raw materials. The first method includes pressure-thermal pretreatment using Henze St eamer at 0.4 mPa for 45 min at 151 °C. The major goal of this method is to gelatinize and liquefy starch granules to ensure starch saccharification (hydrolysis) into fermentable sugars. After this pretreatment, a drain valve in the steamer is opened and the liquid starch mass is rapidly transferred to the mash tun. In this stage of the process, the structure of the rye grain cell is destroyed, enabling starch release.

Significant energy requirements associated with steaming can effectively decrease using pressureless liberation of starch (PLS) method, which needs mechanical comminution of the raw materials [11]. One of the important stages in preparation of raw materials for hydrolysis and fermentation is milling because it defines accessibility of the starch granules for amylolysis. The major factors that affect characteristics of the kernels during milling include hardness and vitreousness of the kernels. Middling and flour size and distribution is defined by the cereal kernel hardness [12]. Studies on three cereal grain species showed that rye grain is the hardest grain within rye, wheat and triticale grains [6]. Grinding efficiency may be affected by protein contents in the grain. Grinding harder grain, especially grains with higher protein contents, results in greater damages to starch granules than milling grain significantly softer, because further mechanical energies are needed to break the structure of hard grains [13]. Regardless of whether raw materials were pretreated by steaming or mechanical milling and mixing with water, the next stage of the technological procedure is mashing such as enzymatic hydrolysis of starch to fermentable sugars. Scheme of starch hydrolysis is presented in Figure 2.

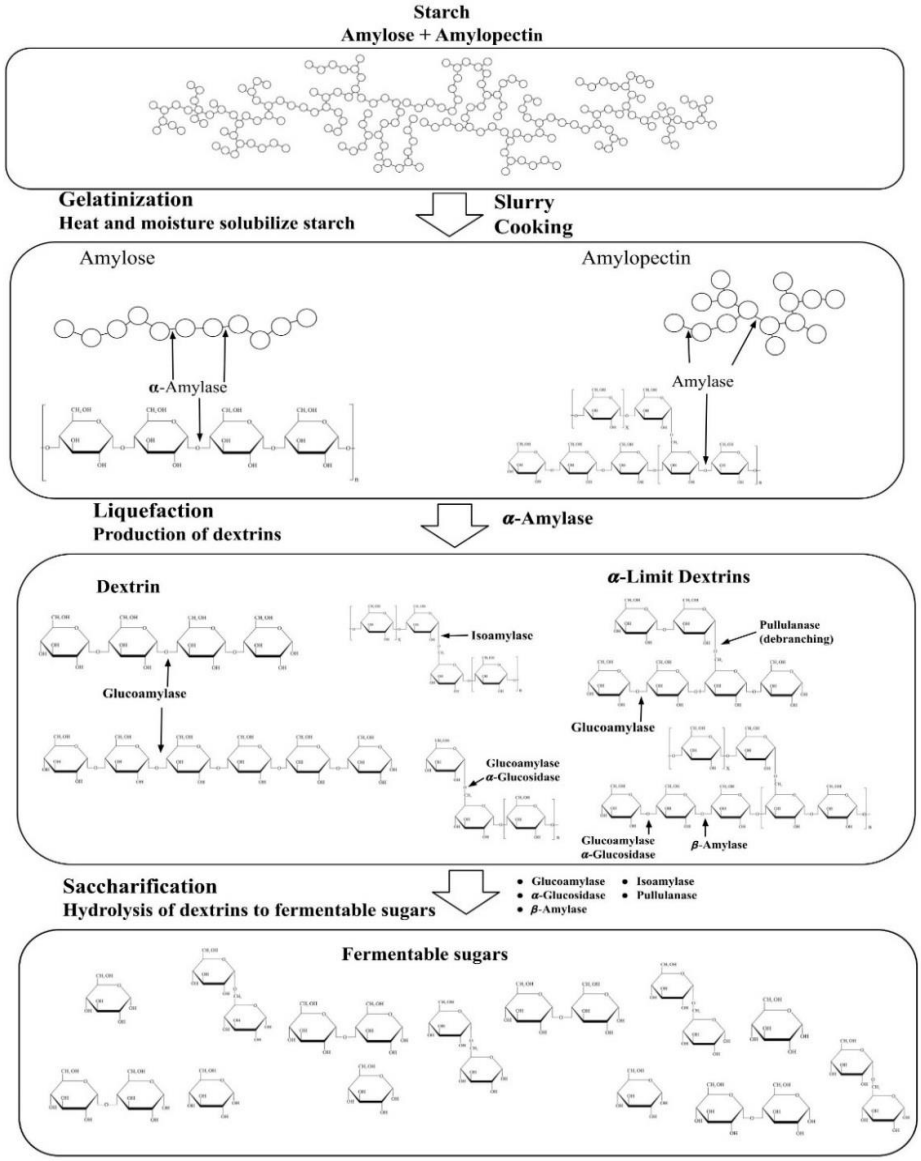

Figure 2. Scheme of hydrolysis of starch with use of enzymes and thermal processing

In this stage, steamed mass transferred to the mash tun must be cooled down to a temperature of approximately 85-90 °C. Mixture of ground cereal grains and water must be heated to a similar temperature. Once the steamed mass or the mixture of ground cereal grains and water reaches the temperature, an amylolytic enzyme preparation containing the endoenzyme α-amylase is added. This stage of the process is known as liquefaction due to the changes in rheological properties of the media caused by starch hydrolysis to intermediate products such as dextrins. This stage is especially important for the efficient starch hydrolysis and is carried out for approximately 60-90 min. In the PLS method, α-amylase may be introduced at the beginning even before the heating process for pre-liquefaction of starch [14]. During conventional enzymatic liquefaction, granular starch is gelled. Heating to approxi-mately 85 °C disturbs physical nature of the granules and opens the crystal structure, making it amenable to enzymatic digestion [15].

The second stage of mashing is the saccharification of dextrins to fermentable sugars. Therefore, an amylo-glucosidase is added at 0.06-0.08% by weight of the cereal. Time needed for dextrin saccharification is usually 45-90 min. keeping the temperature of the mashed mass at 60-65 °C for a long time is undesirable, especially when the mass has not previously been subjected to pressure-thermal treatments. Sterilization is achieved above 120 °C; however, temperature of 90 °C used in the PLS method inactivates most viable vegetative forms of the microorganisms, except for spores which are heat-resistant. Burgess et al. [16] showed that the germination of spores from the *Bacillus* genus was initiated after heat activation at 65-70 °C. To prevent risks linked to microbial hazards during starch saccharification, special attention should be paid to the method of starchy raw material processing known as simultaneous saccharification and fermentation (SSF). With this method, an amyloglucosidase enzyme preparation is added when the previously liquefied mass reaches a temperature of approximately 60-65 °C. The medium is immediately cooled down to a fermentation temperature of nearly 30 °C. Saccharification enzymes begin to act and generate simple sugars, which are simultaneously fermented by the yeast. This prevents accumulation of glucose in the mash, causing osmotic stress that inhibits activity of the yeast. Compared to separate hydrolysis and fermentation, the SSF method needs lower investment costs, simplifies technological operations, decreases the time needed for saccharification during the mashing process and cooling the mash to an optimal temperature for yeast inoculation, lowers the osmotic stress on yeast cells caused by a high concentration of sugar in the medium and enables use of mash with a higher dry matter content (> 18% w w^{-1}); thereby, saving water [17,18].

In the two methods of conventional ethanol production, the initial stage (e.g. amylolysis) is expensive because of the high temperatures necessary to gelatinize starch. Recently, granular starch hydrolyzing enzymes such as α-amylase and glucoamylase mixtures have been developed to hydrolyze native starch into fermenting sugars at temperatures below gelatinization. These enzymes can be used in SSF, enabling use of uncooked native starch and further decreasing costs of preparing mashes for fermentation [19]. Hydrolysis is carried out using enzymes with the ability to hydrolyze native starch below the temperature of starch gelatinization. Hydrolysis of native starch occurs through adsorption of the enzyme on the surface of the starch grain. Enzyme penetrates its channels and then penetrates the grain [20]. Strak-Graczyk and Balcerek [21] demonstrated that amylases attached to the granule surface and created holes in various places, which narrowed toward the center of the granule. While the fermentation process continued, new holes were formed and old holes grew and deepened, revealing the structure of the starch (Fig. 3).

Eventually, the residue forms a honeycomb structure consisting of pentagons and hexagons (72 h). Patches remain in the samples and starch molecules that are not completely digested retain their granular shape. Amylases may digest entire surface of the granule or only parts of its surface. They can hollow out channels from particular points on the surface toward the center point of the starch granule. Regions with amorphous rings, which are much less organized, are more susceptible to enzyme attack, whereas crystal forms seem much more resistant to enzymatic erosion [22]. Xu et al. [23] used raw corn and cassava flours as raw materials for fermentation to investigate the SSF of raw starch into ethanol using novel raw starch-digesting glucoamylase purified from *Penicillium oxalicum* GXU20.

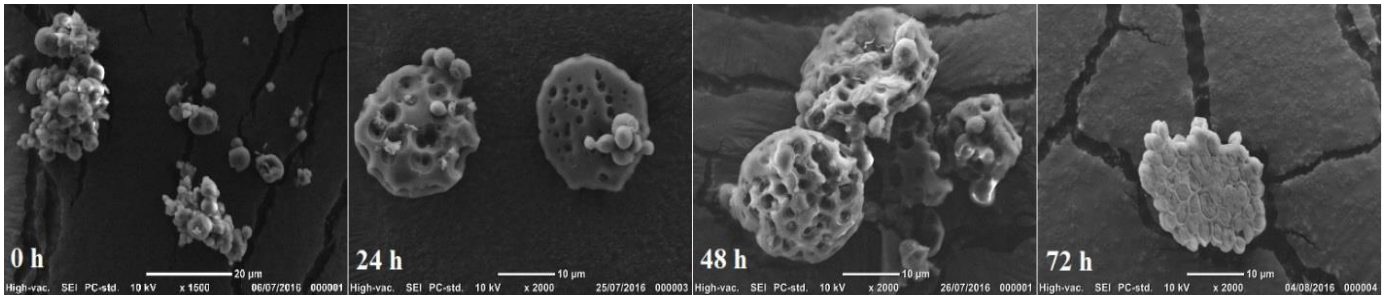

Figure 3. SEM images (×1500 and ×2000) of native rye starch hydrolysis during SSF [21]

Their study showed efficiency of the fermentation as 95.1%. The key factors, which determine enzymatic hydrolysis of native starch granules, include supramolecular structure, crystallinity and presence of complexing agents [24]. Uthumporn et al. [25] investigated effects of heat treatment below the gelatinization temperature on the susceptibility of various types of starches such as corn, potato and mung bean starch to granular starch hydrolysis (35 °C). The starches were hydrolyzed in the granular state in the presence of the granular starch hydrolyzing enzyme for 24 h. As a control sample, starch was hydrolyzed after treatment at 50 °C for 30 min under a similar condition. The heat-treated starch showed increases in the level of dextrose equivalent compared to the starch in native form. The SEM micrographs showed further porous granules and erosions on the surface of the heat-treated starch, compared with native starch. No changes were reported in X-ray analysis, but sharper peaks for all the starches suggested that hydrolysis occurred in the amorphous region. Amylose content and swelling power of the heat-treated starches were significantly altered by hydrolysis.

A producer of native starch hydrolyzing enzyme preparations used in ethanol production recommends activetion of starch. A mixture of milled cereal grains and water or thin stillage is heated to a temperature not exceeding the temperature of starch gelatinization. This is then treated with fungal/acid α-amylase to activate (e.g. partially hydrolyse) the starch granules [26]. A previous study [27,28] showed that activation of cereal starches with acid α-amylase at increased temperatures (not exceeding the gelatinization temperature) was not necessary for efficient saccharification and fermentation. Moreover, starch can physically be modified by ultrasonication, which leads to increased effectiveness of water diffusivity, the sponge effect, formation of microscopic channels and forced heat and mass transfers [29]. Starch modification can improve the efficiency of hydrolysis, increasing the ethanol yield from the raw materials. Pietrzak and Kawa-Rygielska [30] reported that sonication improved efficiencies of waste bread starch hydrolysis and fermentation. Abedi et al. [31] investigated appropriateness of ultrasonication for pre-gelatinization of wheat and tapioca starches as well as their physicochemical characteristics. Results showed that swelling and solubility of starch treated with ultrasound increased as a function of the process parameters. As Tse et al. have suggested for the production of first-generation ethanol, yields differ depending on the raw materials. For sugar beet and sugarcane, yields include 11 and 7-7.5 L 100 kg^{-1}, respectively. For cereals such as maize, wheat, and rice, yields include 40, 34 and 43 L/100 kg, respectively [32].

3. Enzymes Needed for Starch Hydrolysis

3.1. Liquefying enzymes

An important step in ethanol production includes providing starch for microorganisms to carry out ethanol fermentation. Structure of the starch grains makes it almost impossible to carry out efficient processes without preliquefaction since hydrogen bonds occur between the starch molecules or within a single starch molecule [33]. To provide starch to the enzymes, gelatinization must be carried out. At high temperatures (65 °C), hydrogen bonds are degraded. Thus, starch is soluble and available for the enzyme hydrolysis. Liquefaction of starch by amylolytic enzymes not only results in further processing but also lowers viscosity of the slurry, which prevents mash from burning and increases efficiency of the process. For centuries, malt has served as one of the major sources of amylolytic enzymes in production of alcoholic beverages. Malt contains a wide spectrum of amylolytic enzymes such as α-amylase, β-amylase (EC 3.2.1.2), limit dextrinase (dextrin α-1,6-glucanohydrolase, EC 3.2.1.41), α-glucosidase (maltase, EC 3.2.1.20), proteases, glucanases, phosphatases and lipases [34]. To make activity of the native enzymes of plant origins (malt enzymes) as high as possible, it is necessary to strictly ensure optimal conditions such as appropriate temperature and pH during starch hydrolysis, which include 70-75 °C for α-amylase and 55-65 °C for β-amylase. To preserve high activity of these amylolytic malt enzymes in mashing processes, lower temperatures (not exceeding 50-56 °C) are often used. Advantage of the malt-based mashing process includes multitude of enzymes that hydrolyze cereal starch and non-starch structural cell components, providing nutrients to the yeast [35]. Nowadays, malt enzymes are often replaced with commercial enzyme preparations. The most commonly used enzyme in ethanol industries is α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) [36]. The molecular weight of α-amylase is 57.6 kDa and is structured with three consecutive domains of A, B and C. The largest barrel-shaped Domain A is connected to Domain C by a simple polypeptide chain. Domain C is composed of beta-sheets. Between these domains is Domain B, which is connected to Domain A via a disulfide bond [37]. The major function of α-amylase is to cut α-1,4 glycosidic bonds, resulting in dextrinization of starch molecules and it also gives some quantities of maltose and glucose, which are fermentable sugars [38,39]. The α-amylase produced in large quantities by microorganisms includes additional characteristics, including pH stability, high enzyme yield, secretion of the product out of the cell, thermostability and long shelf-life [37,40].

Usually, α-amylase is produced by microorganisms such as bacteria and fungi. The major advantage of the enzyme production by fungi is that α-amylase is secreted out of the cell walls of the microorganisms [41]. Fungi that produce α-

amylase include *Aspergillus niger*, *A. oryzae* and *A. awamori* [42]. Studies on novel extremophilic bacteria have led to the isolation of α-amylase from bacteria in Manikaran Hot Springs, called *Geobacillus bacterium* [43]. This thermophilic α-amylase, which includes the highest activity at 80 °C, has been verified to include more structural flexibility than that the mesophilic enzyme does. Other α-amylases of bacterial origin widely used in industries include those produced by *B. licheniformis*, which are stable at 85 °C for 1 h, and those produced by *B. amyloliquefaciens* and *B. stearothermophilus*, which have the optimum activities at 70–80 °C and pH 5.0-6.0 [40]. The α-amylase most commonly used in ethanol production industries originated from *Bacillus* species due to its thermostability character. Species such as *B. amyloliquefaciens*, *B. stearothermophilus* and *B. licheniformis* are used as well [44]. Naturally, α-amylases with various characteristics can be found, including the socalled cold-stable α-amylase isolated from *Pseudoalteromonas* M175 in Antarctic Sea ice [45]. This type of α-amylase is used mostly for saving energy in industries such as detergent production or baking industries [46]. Another desirable characteristic for amylase enzymes includes its stability at low pH. Resistance to low pH eliminates the need to adjust pH during the mashing process and a low-acidic environment can improve degradation of starch. Acidic α-amylases have been detected in *B. acidocaldarius* [47] and *G. bacterium* [48], which can carry out hydrolysis under such conditions. Other microorganisms, including plants and animals, are capable of producing α-amylases (Table 1) [42,48-50].

3.2. Saccharifying enzymes

After starch liquefaction/dextrinization, the achieved medium consists of a high volume of dextrin, glucose polymers of various lengths and small quantities of easily fermentable sugars. The goal of the saccharification stage is to enzymatically hydrolyze dextrins to fermentable sugars such as maltose and glucose to ensure efficient fermentation with a high ethanol yield. Enzymes used for the saccharification of starch or dextrin chains include maltogenic α-amylase [1,4-α-glucan maltohydrolase (EC 3.2.1.133) and β-amylase (EC 3.2.1.2)]. The former enzyme catalyzes hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides, removing successive α-maltose residues from the non-reducing ends of the chains. It acts on starch and associated polysaccharides and oligosaccharides. The product of its action is α-maltose. The latter enzyme catalyzes hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides, removing successive maltose units from the non-reducing ends of the chains. It acts on starch, glycogen and associated polysaccharides and oligosaccharides, producing β-maltose by inversion. The term β is linked to the initial anomeric configuration of the free sugar group released, not to the configuration of the linkage hydrolyzed. In contrast to α-amylase, maltogenic α-amylase and β-amylase do not act randomly but act in a specific order, liberating maltose by consistently disconnecting two molecules of glucose [51].

For a long time, barley (*Hordeum vulgare* L.) malt was used as a source of β-amylase. Its grain enzyme includes one polypeptide chain in two forms, one in mature grains (59.7 kDa) and the other as an isoform (56.0 kDa) converted during the process of germination [52]. One of the characteristics of β-amylase is its thermal instability, which severely limits its use in ethanol industries, especially where malt is not used and temperature of the process exceeds the thermal optimum of the active enzyme, requiring time and energy to cool down the mash. Enzyme preparations of thermostable β-amylases from bacterial origins are available isolated from various bacteria such as *Clostridium thermosulfurogenes* SV2 with an optimum β-amylase activity at 70 °C and pH 6.0 [53]. Glucoamylase (glucan 1,4-glucosidase, EC 3.2.1.3), also called γ-amylase, is one of the most widely used biocatalysts worldwide. It has been classified with $\boldsymbol{\alpha}$-amylase and β-amylase into glycoside hydrolase families, including glucoamylase to 15, α-amylase to 13 and β-amylase to 14. The most important characteristic of glucoamylase is its ability to release glucose as the major product of starch and dextrin hydrolysis. Moreover, enzyme is capable of hydrolysis of α-1,4 and α-1,6 glucosidic bonds [54,55]. The major sources of glucoamylase for industrial use are the fungal species of *A. awamori* and *A. niger*. Other microorganisms, which show the ability to produce glucoamylase, include the bacterial species of *B. stearothermophilus* and *Flavobacterium* and yeast species of *Saccharomyces cerevisiae* var. *diastaticus* and *Lipomyces starkeyi* [54].

Forms of glucoamylase produced by the fungi differ in terms of stability, molecular weight and their ability to process raw starch [56]. Monma et al. [57] and Nagasaka et al. [58] identified six various forms of fungal glucoamylase. However in commercially used fungal species such as *A. awamori* and *A. niger*, enzymes occur in two forms of GAI and GAII. These two forms differ in size and only the larger one (GAI) can process raw starch [53]. The most studied form of glucoamylase originates from *A. awamori* var. X100. In this fungus, the major form of the enzyme is GAI, which consists of three various domains of one linker domain (13kDa) and two consecutive globular functional domains. The catalytic domain on the N-terminal end (55kDa) includes an optimum pH of 5.0 [59]. It becomes inactive at 70 °C and mild acidic pH due to changes in its structure and as the catalytic center loses integrity [60]. However, studies have been carried out to increase the thermostability of glucoamylase via bioengineering. One of these studies included replacement of glycine with another amino acid to decrease the number of possible conformations while the protein is unfolded [59]. To maximize the efficiency of glucoamylase, the highlighted method of SSF was developed [61]. Sources and action parameters of amylolytic enzymes are presented in Table 1.

Table 1. Sources and working parameters of amylolytic enzymes

Enzyme	E.C. number	Source of enzyme	Working temperature °C	Working pH	References
α-amylase	EC 3.2.1.1	Malt	70–75	5.0-6.1	34
		Aspergillus niger	45-95	5.0-7.0	41
		Aspergillus oryzae	45-95	5.5- 10.0	41
		Aspergillus awamori	45-95	4.0-9.0	41
		Bacillus licheniformis	75-85	6.5-8.0	39,43
		Bacillus amyloliquefaciens	70–80	5.0-6.0	39,43
		Bacillus stearothermophilus	70–80	5.0-6.0	39,43
		Alicyclobacillus acidocaldarius	70-90	5.0-8.0	46
		Geobacillus bacterium	40-90	3.0-10.0	42
		seudoalteromonas sp. M175	10-40	7.0-9.0	44
β-amylase	EC 3.2.1.2	Malt	55-65	4.0-5.5	51
		Clostridium thermosulfurogenes SV2	70	5.0-7.0	52
		Aspergillus niger	55	3.0-6.0	53
		Aspergillus awamori	55-60	3.0-11.0	53
		Bacillus stearothermophilus	70-80		53,101
			-		
		Flavobacterium sp.	-	5.5-6.5	53
		Saccharomyces cerevisiae var. diastaticus	-	5.0	53
		Lipomyces starkeyi	-		53

4. Hydrolases of Non-Starch Polysaccharides [NSP] as Enzymes Supporting Starchy Raw Material Mashing

4.1. Xylanases

Xylanase (endo-1,4-*β*-xylanase, EC 3.2.1.8) is responsible for the degradation of 1,4-*β*-D-xylosidic bonds in xylan. It is produced by a great variety of organisms from bacteria to arthropods [62]. Many organisms such as *A. niger* produce up to 15 different xylanases with different specific activities, yields and structures [63,64]. The substrate hydrolyzed by xylanase is xylan, which creates a complex of hemicellulose with other polycarbohydrates such as glucomannan, galactoglucomannan, arabinogalactan and xyloglucan [65]. Hemicellulose is one of three major components (with cellulose and lignin) of the cell walls of plants [66]. Xylanase belongs to Glycoside Hydrolase Families 5, 7, 8, 10, 11 and 43. These families include different characteristics. They differ in their number of members, folds in the protein structure and their catalytic mechanisms. Due to the great variety of forms and isoforms of xylanase, this enzyme can be detected in several microorganisms, including *A. niger* (Family 11), *Cellulomonas fimi* (Family 10), *Bacillus* KK-1 (Family 8) and *C. cellulovorans* (Family 5) [61]. Xylan, a polymer of xylose and to a minor extent mannose, is a component of plant cell walls and cereal grains used as raw materials in ethanol production. Xylans are partially dissolved in water, forming a highly viscous suspension. In ethanol production technologies, endo-1,4-β-xylanase is used to decrease the viscosity of sweet mashes, especially those achieved by pressureless methods. It also facilitates technological processes by preventing burning, which decreases the content of fermentable sugars and hence fermentation efficiency.

Hydrolysis of non-starch polysaccharides results in a higher content of fermentable sugars, which can boost efficiency of the process. Combined action of xylanase and α-amylase results in attacks on pentosans and β-glucans. Therefore, starch grains become more accessible to amylases, improving course and efficiency of fermentation [67,68]. Use of xylanase in ethanol production includes numerous advantages and is a promising method for further optimization of the process, maximizing the ethanol yield. Balcerek and Pielech-Przybylska [69] showed that addition of xylanolytic enzymes could assist the process of ethanol production. In their study, endo-1,4-β-xylanase preparation Shearzyme 500 l from *A. oryzae* was used at various doses. The first significant improvement included decreases in viscosity of the sweet mash by 97-99%, compared to the reference mashes. Moreover, use of endo-1,4-β-xylanase preparation included effects on composition of the fermented mashes and ethanol yield. Sugar intake increased significantly from 88.8% in the reference mashes to approximately 92%. As a result, the ethanol content was significantly higher, increasing from 78.7% of the theoretical value in the reference mashes to 85.2-88.5%.

4.2. Proteases

During ethanol production, yeasts used in the fermentation process need to be supplemented with nitrogen and phosphorus to produce sufficient quantities of biomass, enabling efficient fermentation. These compounds can be provided with no supplementation with mineral compounds using proteases during the mashing processes. Proteases are a subclass of enzymes (E.C. 3.4.) that are responsible for the

hydrolysis of peptide bonds in proteins [70]. Proteases are generally divided into two groups. Exoproteases act from the C- or N-terminal ends of the protein, disconnecting single amino acids or peptides. Another group, polypeptides, hydrolyze molecules in the middle of the polypeptide chain [1]. Numerous studies have shown that addition of proteases can increase free amino acid nitrogen [FAN] content and hence ethanol yield. Lantero and Fish [71] showed that addition of proteases increased the rate of corn fermentation. Digestion with a neutral protease was reported to increase the content of FAN in corn mashes by up to 60% and in sorghum mashes by up to 30% [92]. Gohel et al. [72] reported that addition of proteases in SSF of Indian sorghum mashes with 30% d.m. increased efficiency of ethanol production from 88.1% (mashes supplemented with urea) to 93.12% (mashes digested with protease). In mashes with 35% d.m., ethanol yield increased from 86.79% (mashes supplemented with urea) to 93.25% [72].

4.3. Pullulanases

During the mashing of starch, molecules of pullulan are released. Pullulan is a homopolymer of glucose. The monomer of pullulan is composed of α-1,4 linked molecules of glucose, connected by α-1,6 glucosidic bonds [73]. Pullulanase [EC 3.2.1.41] can be classified into two groups, depending on the preferred substrate and end product of hydrolysis. Pullulanase type I is responsible for degrading α-1,6 bonds. Its preferred substrates include oligo and polysaccharides and molecules of maltotriose that produce pullulan via hydrolysis [74,75]. Pullulanase type II is able to hydrolyze α-1,4 and α-1,6-glucosidic bonds, meaning that not only pullulan but also starch can be used as substrates. Products of pullulanase type II include a mixture of other saccharides such as glucose and maltose in addition to maltotriose [76-78]. Furthermore, pullulanase can be classed within debranching enzymes, which are able to hydrolyze α-1,6-glucosidic bonds in amylopectin and glycogen to make the molecules of solutions further linear. Debranching enzymes are divided into two major groups, depending on their direct or indirect activity [75]. Used with glucoamylase, pullulanase can increase the yield of D-glucose by up to 98% of the theoretical value. Together with β-amylase, pullulanase can increase the content of maltose by up to 25% of the theoretical value [79,80]. Type II pullulanase can improve the mashing process that owes to its ability to hydrolyze α-1,6-glucosidic bonds, debranching amylopectin molecules and making them further accessible for saccharification enzymes [75]. Type II pullulanase is more important for the ethanol production industries due to its preferred substrate, which significantly increases concentration of easily fermentable sugars, resulting in a further efficient mashing process. Further attention has been paid by the researchers to pullulanase of microbiological origin due to their specific action on α-1,6-linkages in pullulan. Pullulanase is synthesized by a great variety of microorganisms such as *B. acidopullulyticus*, *G. stearother-mophilus*, *B. cereus* FDTA-13 and *Klebsiella planticol*a [76] as well as *Bacillus* AN-7, which produces thermostable pullulanase with a thermal optimum of 90 °C due to its thermophility. Enzymes with similar characteristics can be detected in *B. thermoleovorans* 95-1 and *Micrococus* Y-1 [75].

4.4. Phytases

In the process of ripening, cereal accumulates a compound called phytic acid [myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate], which is composed of nearly 1-2% of the grain and contains up to 85% of the total phosphorus content [81]. The major portion of phytic acid is in the form of phytate, which is a great source of energy and phosphorus [82]. Microorganisms and animals are able to use this source only via a group of enzymes called phytases [myo-inositol-1,2,3,4,5,6-hexakisphosphate phosphohydrolases]. Phytases can be classified based on their E. C. number. This is based on which carbon at the phytate myo-inositol ring dephosphorylation is initiated. There are three major groups of 3-phytases [E.C. 3.1.3.8], 6-phytases [E.C. 3.1.3.26] and 5-phytases [E.C. 3.1.3.72] [83]. Many microbiological sources of phytases are available. The most common source includes an enzyme preparation of fungal origin such as Natuphos 5000G by BASF, which is isolated from *A. niger* and is active at up to 85 °C [64]. Another example of a phytase preparation of fungal origin is Phyzyme XP 10000TPT by DuPont Danisco with a thermal stability up to 95 °C [65]. Due to the high content of phytic acids in cereal grains [84], phytases can be used as substitutes for additional supplementation with phosphorus and other compounds that are important for the yeast biomass growth. In the first stage of the fermentation process, yeasts can accumulate up to 2.15% (of dry matter) phosphorus. At the end of the fermentation process, phosphorus content decreases to only 1% [85], decreasing efficiency of the process and ethanol yield. Sources of supportive enzymes described in this study are presented in Table 2.

5. Lignocellulose Biomass as a Raw Material for Second-generation Bioethanol Production

First-generation bioethanol is produced from food-based crops containing starch (e.g., cereal grain, maize and cassava) or saccharose (sugar beet and sugarcane) worldwide. Therefore, the production conflicts with the use of crops and arable lands for food and feed supplies. Moreover, production of ethanol from sugarcane competes with the sugar market, leading to decreases in biofuel production in countries such as Brazil, which is one of the most important ethanol/sugar producers worldwide. A similar paradox limits the production of corn ethanol as the

value of food increases in the world market. Although free sugar-based and starch-based ethanol are the most common biofuels, their raw materials become further expensive, leading to a greater interest in second-generation biofuels produced from waste lignocellulosic biomasses [62,82]. Lignocelluloses are composed of three consecutive components of cellulose, hemicellulose and lignin. Cellulose generally comprises up to 45% of dry matter in wood. It is a homopolymer consisted of anhydro glucopyranose units linked by β-1,4 bonds [86]. Cellulose can be found in three forms of long fibril form, crystal form as crystalline cellulose and non-organized chain form of amorphous cellulose. The latter form is the most susceptible form to enzymatic degradation [87]. Two other components of lignocellulosic biomass [hemicellulose and lignin] act as covers for microfibrils created from cellulose chains. Hemicellulose is a complex carbohydrate from various sugars such as D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, and 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. All these components are linked together with β-1,4 and occasionally β-1,3-glycosidic bonds. Depending on the type of wood, predominant components vary. The predominant component is glucuronoxylan in hardwood and glucomannan in softwood. Hemicellulose consists of 25-30% of the dry matter in wood [88]. The third component of this complex is lignin, which is responsible for the cell wall impermeability, structural support and resistance to oxidative stress. Lignins are synthesized via the oxidative coupling of p-hydroxycinnamyl alcohol monomers and their associated compounds [89].

Lignocellulosic biomass is not available for fermentation via the traditional mashing processes. Structure of the plant-based biopolymers makes it almost impossible for the enzymes to carry out rapid and efficient processes with no pretreatments. The most basic kind of physical pretreatment is mechanical size reduction. This method is based on milling, chopping and grinding of the biomass to decrease its size and affect the crystalline structure of cellulose [90].

Size of the particles is connected to the type of raw materials. If the particles are too fine, this may result in a lower efficiency due to the formation of clumps that prevent enzymes from accessing the substrates [91]. Another type of physicochemical pretreatments is steam explosion, which is similar to the pressure-thermal method for processing starch. Biomass is heated to 160–290 °C for a few minutes at 20–50 bar using steam. Reaction is then stopped by sudden decompression to atmospheric pressure [92].

Other popular kinds of pretreatments include chemical methods (acids, bases and organic solvents). Acid pretreatment can be carried out using diluted or concentrated acids at temperatures from 130 to 210 °C with various types of acids such as sulfuric acid, hydrochloric acid, nitric acid and phosphoric acid [93]. Unfortunately, acidic pretreatments may result in creation of compounds such as acetic acid, furfural and 5-hydroxymethylfurfural, which inhibit the growth of microorganisms [94]. A similar method to the acidic method is the alkaline method, which disrupts the cell wall by dissolving hemicelluloses, lignin and silica. Alkaline pretreatments act via hydrolyzing uronic and acetic esters and swelling cellulose, which decreases its crystallinity [91]. Alkaline pretreatments with 1.5% w v^{-1} NaOH at 20 °C for 144 h can result in the release of up to 60% of the lignin and 80% of the hemicellulose from lignocellulose biomasses [95]. Tse et al. showed that yields of second-generation ethanol could be high and sometimes higher than the yields of first-generation ethanol. For example, 36.2-45.6 L 100 kg^{-1} of ethanol could be produced from corn stover. Waste materials such as wheat straw and sugarcane bagasse could be used to produce ethanol with yields of 40.6 L 100 kg^{-1} and 31.8-50 L 100 kg^{-1}, respectively [32].

Table 2. Sources and working parameters of supportive enzymes used in ethanol production

Enzyme	E.C. number	Source of enzyme	Working temperature [°C]	pH range for activity	Reference
Xylanase	EC 3.2.1.8	*Aspergillus niger*	*20-100*	*4,5-9.0*	60
		Cellulomonas fimi	15-80	4.0-9.0	60
		Bacillus sp. KK-1	*10-55*	*5.0-8.0*	60
		Clostridium cellulovorans	30-70	4.0-.8.0	60
Pullulanase	EC 3.2.1.41	*Bacillus acidopullulyticus*	*40-80*	*3.0-7.0*	108
		Bacillus thermoleovorans 95-1	-	-	82
		Bacillus cereus FDTA-13	*55-80*	*5.5-6.5*	75
		Bacillus sp. AN-7	*40-110*	*4.5-7.0*	82
		Geobacillus stearothermophilus	*20-80*	*4.5-9.5*	76
		Klebsiella planticola	-	-	75
		Micrococcus sp. Y-1	*50-60*	*8.0-10.0*	82
Phytases	E.C. 3.1.3.8 E.C. 3.1.3.26 E.C. 3.1.3.72	*Aspergillus niger*	*55-80*	*2.0-6.0*	64
		Saccharomyces pombe	*>95*	*3.0-6.0*	66

5.1. Cellulases and cellobiases

Cellulases, the enzymes responsible for the degradation of cellulose, are traditionally divided into two groups. Exoglucanases (E.C.3.2.1.91) cut the chain from the ends, liberating cellobiose. Endoglucanases (E.C.3.2.1.4) hydrolyze the cellulose chain from within. Most cellulases are composed of three domains of a large and spherical domain which is catalytically active, an elongated linker domain, and a spherical domain with cellulose-binding characteristics [1]. Cellulases are produced by fungi and bacteria. However, fungi are more widely used due to their ability to secrete enzyme molecules out of their cells, which ensures easy extraction and purification of the enzymes. Recent studies have shown that use of bacteria as enzyme sources may include advantages. Bacteria can produce further complex enzymes and complex systems of enzymes. Production of further supportive enzymes can potentially maximize the efficiency of cellulose hydrolysis, resulting in better use of raw materials and higher yields, compared to fungi. In addition, bacteria inhabit further diverse environments, creating the possibility of extremophile bacteria to produce cellulase with additional characteristics such as thermos-tability [96]. Hydrolysis of cellulose is carried out by an often complex system of enzymes; in which, activity of one enzyme is interconnected with the activity of another enzyme.

Exoglucanases hydrolyze cellulose to cellobiose; however, high concentrations of cellobiose can inhibit activity of the whole enzymatic system responsible for the hydrolysis of cellulose [97]. Therefore, its cleavage by cellobiase (β-glucosidase, EC 3.2.1.21) to glucose is important to ensure efficient second-generation bioethanol production. Cellobiase is responsible for the degradation of variously β-linked diglucosides and aryl β-glucosides, resulting in degradation of cellobiose to glucose [97]. Scheme of enzymatic hydrolysis of cellulose is presented in Figure 4.

Cellobiohydrolase and endoglucanase are inhibited by cellobiose, verifying that cellobiase controls the whole process of cellulose degradation. Cellobiase not only produces fermentable sugars (glucose) from indirect substrates but also increases the efficiency of other enzymes involved in hydrolysis [98]. Cellulolytic enzyme preparations may support ethanol production from starchy raw materials. They contribute to decreased mash viscosity, facilitate technological processes and release glucose, which can increase ethanol efficiency [99]. Use of enzyme complex preparations containing xylanase, cellulase and endo-β-1:3,1: 4-glucanase is particularly recommended for very high gravity (VHG) mashes [100]. Due to the nature of ethanol production process, one of the most desirable characteristics of enzyme preparations is thermostability [102]. Liang et al. [103] reported that cellulase originating from a novel *Brevibacillus* strain JXL isolated from swine slurry was able to resist 100 °C for 1 h while preserving 50% of its activity. Moreover, the strain was capable of using a broad spectrum of carbon sources, including crystalline cellulose, xylan and carboxymethyl cellulose (CMC). Studies have investigated the possibility of extracting genes responsible for the production of cellulase from a bacterial cell and incorporating them into a yeast cell. Yanase et al. [104] reported a successful and efficient process of ethanol production using amorphous cellulose as a raw material for genetically modified yeasts. The microorganism included *S. cerevisiae* MT8-1 with inserted genes responsible for the production of enzymes such as endoglucanases, cellobiohydrolases from *Trichoderma reesei* and β-glucosidases from *A. aculeatus*.

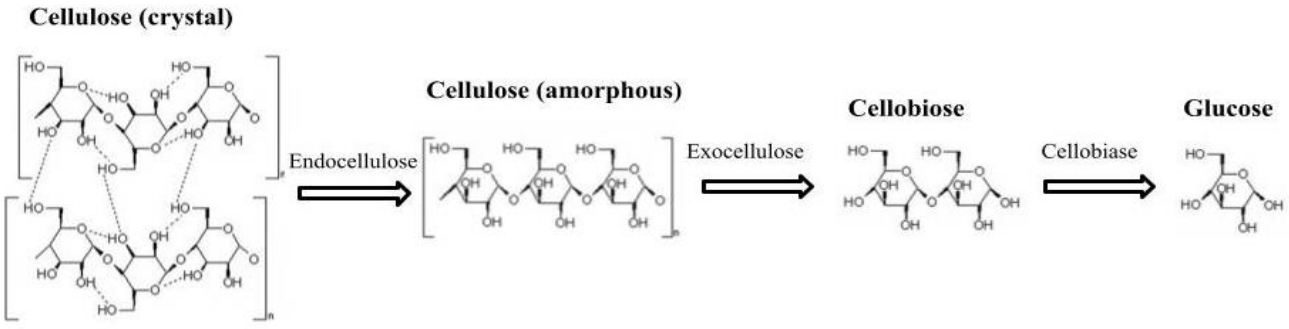

Figure 4. Scheme of enzymatic hydrolysis of cellulose

6. Future prospects

Ethanol production is still a developing branch of industries. Producers try to enhance efficiency of the technology used in ethanol production in each step of the production processes, from cultivation techniques for the growth of raw materials to the distillation and rectification processes. First-generation bioethanol from crops containing starch or saccharose is currently the preferred option for commercial ethanol production. Second-generation bioethanol production processes become competitive. Further process developments can lower production costs (e.g. cost of enzymes and energy), minimize the quantity of wastes and increase use of byproducts (e.g. for the production of electricity and biogas). Integrating first and second-generation bioethanol productions can maximize ethanol yields, while requiring less capital investments.

The greatest advances can be seen in second and third-generation bioethanol productions. Lopez-Linares et al. [105] studied microwave pretreatment of lignocellulosic biomass for second-generation biobutanol production. Up to 64% of hemicellulose sugars were recovered in solvent-free media. Glucose production via enzymatic hydrolysis increased by 70%. This verifies that microwave pretreatment not only limits heating but also increases the efficiency of biobutanol and bioethanol productions in combination with other forms of pretreatments. Using microalgae, third-generation bioethanol production is described as the most advantageous method since it needs less land and can capture CO_2. del Rio et al. [106] showed the possibility of using micro and macroalgae as potential raw materials. However, use of microalgae needs improvements to decrease costs and increase economic sustainability. Other raw materials are assessed in the beverage production of the ethanol industries, where the use of unconventional raw materials can be attractive to consumers. For example, Lugowoj et al. [107] demonstrated that use of buckwheat has potentials as alcoholic beverages due to its similar chemical composition to conventionally used materials. Researchers have reported that the efficiency of ethanol production from buckwheat can reach 85% of the theoretical efficiency. Moreover, it can include original organoleptic characteristics. With the proper optimization, buckwheat can compete with feedstock used in major industries.

7. Conclusions

Ethanol is an important substance for several industries, including pharmaceutical, transport and alcoholic beverage industries. This review has described polysaccharides of plant origins and their uses in ethanol production. Furthermore, the review has described the structure of the feedstocks used in first and second-generation ethanol productions. Pretreatment processes have also been discussed, especially use of basic and supportive enzymes as well as techniques and materials. Further studies are necessary to optimize current processes and discover novel raw materials. Enzymes with superior characteristics and energy and environment-saving technologies can enable cleaner and more-efficient ethanol productions.

8. Conflict of Interest

The authors report no conflicts of interest.

9. Authors Contributions

SŁ contributed significantly to the study for bibliographic sources as well as writing of the manuscript and proposal. MB contributed to complement information collected previously and reviewed the document providing ideas on the distribution of items within the manuscript. All authors read and approved the final manuscript.

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پلی ساکاریدهای مواد نشاسته ای و لیگنوسلولزی و استفاده از آنها در تولید اتانول: آنزیم‌ها و سایر عوامل موثر بر فرایند تولید

شیمون لوگووی*، ماریا بالسرک

انستیتو میکروبیولوژی و فناوری تخمیر، دانشکده زیست‌فناوری و علوم غذایی، دانشگاه صنعتی لودز، Wolczanska 171/173، ۹۰-۵۳۰ لودز، لهستان

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- نشاسته
- سلولوز
- آمیلازها
- آنزیم‌های کمکی

***نویسنده مسئول**

شیمون لوگووی

انستیتو میکروبیولوژی و فناوری تخمیر، دانشکده زیست‌فناوری و علوم غذایی، دانشگاه صنعتی لودز، Wolczanska 171/173، ۹۰-۵۳۰ لودز، لهستان

تلفن: ۷۹۵۱۷۱۱۳۷

پست الکترونیک:
szymon.lugowoj@dokt.p.lodz.pl

چکیده

سابقه و هدف: امروزه، انواع بسیاری از مواد گیاهی در تولید اتانول مورد استفاده قرار می‌گیرند، از مواد نشاسته‌ای معمولی مانند چاودار، گندم، ذرت و جو گرفته تا مواد لیگنوسلولزی که در تولید اتانول زیستی نسل دوم کاربرد دارند. مواد خام حاوی قندهای ساده به چنین فرآیندهای له کردن پیچیده‌ای نیاز ندارند، در حالی‌که مواد نشاسته‌ای و لیگنوسلولزی نیازمند به فرایند مهمی می‌باشند. این مقاله مروری توصیفی عمیق از ساختار مواد خام نشاسته‌ای که معمولاً در تولید اتانول نسل اول استفاده می شود، ارائه می دهد. علاوه بر این، ساختار زی‌توده‌های[۱] لیگنوسلولزی مورد استفاده برای تولید اتانول زیستی نسل دوم را توصیف می‌کند.

یافته‌ها و نتیجه‌گیری: روش‌هایی که معمولاً در کارخانه‌های تقطیر برای آزادسازی نشاسته از مواد خام گیاهی استفاده می‌شوند، شامل پیش تیمارهای حرارتی تحت فشار است که به روش‌های بخاردهی یا آزادسازی بدون فشار نشاسته معروف می‌باشند. بازنگری منابع نشان می دهد مورفولوژی دانه های نشاسته بر آمیلولیز تاثیر بسیاری دارد. هر چه سطح ویژه ریزدانه‌ها[۲] بزرگتر باشد، حساسیت آنها به آمیلولیز بیشتر می شود. مرحله کلیدی در آماده‌سازی مواد خام نشاسته برای تخمیر، آبکافت[۳] نشاسته می‌باشد که از دو مرحله مایع سازی و ساکاره سازی[۴] تشکیل شده است. چندین گونه از باکتری‌ها (مانند باسیلوس لیکنیفورمیس) و قارچ‌ها (مانند آسپرژیلوس نایجر) در دسترس می‌باشند که توانایی تولید آنزیم‌های لازم برای آبکافت نشاسته را دارند. آنزیم‌های مورد نیاز برای آبکافت نشاسته به ۱) آنزیم-های مایع‌کننده مانند آلفا-آمیلاز تولید شده توسط *آسپرژیلوس نایجر* و *باسیلوس لیشنیفورمیس* یا موجود در مالت و ۲) آنزیم‌های ساکاره‌کننده مانند گلوکوآمیلاز، بتا-آمیلاز و آلفا-آمیلاز مالتوژنیک با منشا باکتریایی، قارچی و مالت تقسیم می‌شوند. برای کمک به فرآیند له کردن هیدرولازهای پلی‌ساکاریدهای غیر نشاسته‌ای (زیلاناز و پولولاناز) از پروتئازها و فیتازها استفاده می‌شود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] biomasses
[۲] granules
[۳] hydrolysis
[۴] saccharification