Bioethanol from Lignocellulosic Biomass: A review

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Abstract: The perspectives of biobased fuels as options for partial fossil fuels substitution has encouraged research on the availability of biomass feedstock and development of efficient conversion processes. In the case of fuels for transport, bioconversion of lignocellulosic materials to ethanol has been recognized as one of the promising routes of producing competitive substitutes to gasoline.

Lignocellulose is the most abundant natural renewable resource and is one of the preferred choices for the production of bioethanol. As a substrate for bioethanol production it has a barrier in its complex structure, which resists hydrolysis. For lignocellulose to be amenable to fermentation, treatments are necessary that release monomeric sugars, which can be converted to ethanol by microbial fermentation. The current state of the art on acid and enzymatic hydrolysis of lignocellulose and subsequent microbial fermentation to ethanol are described in this work. Approaches for detoxification of the lignocellulose hydrolysate for effective fermentation to ethanol are also described.

Keywords: lignocellulosic material, enzymatic hydrolysis, cellulose, hemicelluloses, lignin.

1. Introduction

The use of ethanol as a fuel has a long history, starting in 1826 when Samuel Morey used it with the first American prototype of the internal combustion engine.

The renewal of interest in fuel ethanol started, however, from the 1973–1974 world oil crisis when the Brazilian government launched its pro-alcohol strategic program to substitute a large share of imported oil. Later on, another U.S. federal program guaranteed loans for investment in ethanol plant construction. Brazil and the United States are still the two main producers and users of fuel ethanol worldwide.

Ethanol has good properties for internal combustion engines. Its average octane number of 99 is high compared to 88 for regular gasoline. Fuel ethanol is used in several manners in internal combustion engines: as 5% to 25% anhydrous ethanol blends with gasoline (5% maximum in Europe and India, 10% in the United States and China, 20 to 25% mandatory blends in Brazil), as pure fuel (100% of hydrated ethanol) in dedicated vehicles, or up to 85% in FFVs (Flexible fuelled vehicles).

When anhydrous bioethanol is blended with gasoline in small proportion (up to 15%), the influence of the lower heating value has no significant effect. For higher blend levels, the fuel economy is reduced compared to that with conventional gasoline.

Bioethanol can be produced from a large variety of carbohydrates: monosaccharides, disaccharides, and polysaccharides. The large-scale biomass-to-ethanol industry mostly uses the following feedstocks: sweet juice (e.g., sugarcane, sugar beet juice, or molasses) and starch (e.g., corn, wheat, barley, cassava). Ethanol is also commercially produced in the pulp and paper industry as a by-product of an acid-based conversion process [1].

The feedstock for bioethanol production is currently based mostly on agricultural crops, which can be devoted to both food and ethanol markets or dedicated solely to ethanol, that is, crops cultivated on fallow or undeveloped lands.

In case of a high world production of bioethanol, the correlation between food and ethanol markets may generate a high volatility of agricultural crops with regard to fluctuations in energy prices. Figure 1 outlines a generic biomass-to-ethanol process.
Fig. 1. Schematic outline of the biomass-to-ethanol process, [2].

One or more steps may be omitted and several may be combined, depending on the feedstock and the conversion technology. Once the biomass is delivered to the ethanol plant, it is stored in a warehouse and conditioned to prevent early fermentation and bacterial contamination.

Through pretreatment, carbohydrates are extracted or made more accessible for further extraction. During this step, simple sugars may be made available in proportions depending on the biomass and the pretreatment process. A large portion of fibers may remain for conversion to simple sugars through hydrolysis reactions or other techniques.

In the fermentation step, batch operations may be used in which the hydrolysate, the yeasts, nutriments, and other ingredients are added from the beginning of the step. In a fed batch process, one or more inputs are added as fermentation progresses.

Continuous processes in which ingredients are constantly input and products removed from the fermentation vessels are also used [3]. In efficient processes, the cell densities may be made high by recycling or immobilizing the yeasts in order to improve their activity and increase the fermentation productivity. The fermentation reactions occur at temperatures between 25 and 30°C and last between 6 and 72 h depending on the composition of the hydrolysate, and the type, density, and activity of the yeasts. The broth typically contains 8 to 14% of ethanol on a volume basis. Above this latter concentration, inhibition of yeasts may occur that reduces their activity. The distillation step yields an azeotropic mixture of 95.5% alcohol and 4.5% water that is the “anhydrous” ethanol or “hydrated” ethanol which is then dehydrated to obtain an “anhydrous” ethanol with 99.6% alcohol and 0.4% water.

The remaining flow from the distillation column can be valorized to produce co-products, which may include process steam and electricity, products for feeding animals, more or less concentrated stillage used as fertilizer, and other valuable by-products. In 2005, around 36 billion liters of fuel bioethanol were produced in the world; Brazil and the United States provided 86% of the production [3].

Modern lignocellulosic biomass-to-ethanol processes are envisaged to provide a significant percentage of bioethanol in the long term due to the expected low cost of the feedstock (agricultural and forestry residues) and to their high availability.

Lignocellulosics are the most abundant source of unutilized biomass. Their availability does not necessarily impact land use. Agricultural or forestry residues are available though their collection is costly. However, conversion of lignocellulosic materials to ethanol is more complex. Lignocellulose is composed mainly of cellulose, hemicelluloses, and lignin (Figure 2) [4].

Fig. 2. Structure of plant cell walls, [4].
Lignocellulose does not compete with food. Typical sources of lignocellulosic biomass are bagasse of sugarcane or sweet sorghum, corn stover, grasses, woody biomass, industrial wastes, and dedicated woody crops (poplar). Table 1 gives proportions of each component in a typical lignocellulosic biomass [5].

Table 1. Typical Proportion of Cellulose, Hemicellulose, and Lignin in Lignocellulosic Biomass [5].

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>40-60</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>20-40</td>
</tr>
<tr>
<td>Lignin</td>
<td>10-25</td>
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</table>

Cellulose is typically found in the walls of plant cells, which have secondary thickening. These cell walls also contain pectin, lignin, and hemicellulose. It is now well established that lignocellulose-containing biomass is a potential renewable resource for the production of single cell protein, glucose, or ethanol. For example, one ton of dry sugarcane bagasse is theoretically reported to generate 424 liters of ethanol [6].

However, the hydrolysis of cellulose by enzymes is a complex phenomenon and is affected both by the structure and reaction conditions. Unlike a homopolymer like starch, which is easily hydrolyzed, lignocellulose contains cellulose (23–53%), hemicellulose (20–35%), polyphenolic lignin (10–25%), and other extractable components [6].

The biodegradation of heterogeneous insoluble substrates like lignocellulosic materials is a slow process. Reducing the time to achieve satisfactory sugar yields will therefore have a large impact on the process economy. For this purpose, the lignocellulosics require specific pretreatments to overcome both the physical and chemical barriers to increase their accessibility to enzymes for hydrolysis.

Pretreatment refers to the solubilization and separation of one or more of the four major components of biomass, hemicellulose, cellulose, lignin, and extractives, and make the remaining solid biomass accessible to further chemical or biological treatment.

1.1. Enzymatic Hydrolysis of Lignocellulosic Materials: the barriers

The enzymatic hydrolysis of a solid substrate is a slow process. For example, the cellulose in the lignocellulosic materials is normally not easily degradable by the extracellular hydrolytic enzymes to any appreciable extent. This is because the cellulose molecules are not found individually but are linked together to form microfibrils.

The separate molecules are linked by hydrogen bonding into a highly ordered crystalline structure. Some parts of the microfibrils have a less ordered, noncrystalline structure and are referred to as amorphous regions. The high molecular weight and ordered tertiary structure make natural cellulose insoluble in water. The crystalline regions of the cellulose are more resistant to iododegradation compared to amorphous regions. Another important factor is the degree of polymerization (DP). Cellulose of low DP will obviously be more susceptible to cellulolytic enzymes, particularly exocellulases [7].

Cellulose does not occur alone but is associated with lignin and hemicelluloses. Lignin is heterogeneous in bond type and most of the bonds are not amenable to hydrolytic cleavage. It is insoluble and difficult to wet. Thus, the presence of lignin is always deleterious to cellulose degradation. The rate of cellulolysis is inversely related to the lignin content and is also related to the type of lignin and its association with cellulose. In general, the plant cell walls are subdivided as primary (PW) and secondary walls (SW). The distribution of the cellulose, hemicelluloses, and lignin varies considerably among these layers. The secondary wall is composed of SW1, SW2, and SW3 where SW2 is usually thicker than the others and contains the major portion of cellulose. The middle lamella, which binds the adjacent cells, is almost entirely composed of lignin (Figure 3) [7].

![Fig. 3. Diagrammatic sketch of wood cell wall showing the thin primary cell wall (PW) and the three layers of secondary cell wall (SW-1, SW-2, and SW-3) and the middle lamella (ML), [7].](image-url)
significant amount of the original enzyme and restricts the reuse of these enzymes on added, fresh substrate. All these factors serve to limit the availability of the glycoside bonds to the hydrolytic enzymes [7].

Most potential substrates for cellulose bioconversion are heavily lignified. Thus, most of the cellulose in nature is unsuitable for bioconversion unless effective and economically viable procedures are developed to remove or modify lignin. The essential feature of any successful pretreatment is to decrease the protective association between the lignin and the cellulose. The susceptibility of cellulosic substrates has to be increased in order to improve the enzymatic saccharification rate in a bioreactor.

Many investigators have examined various pretreatments for improving the biodegradation of the potential substrates. The pretreatment is important from the viewpoint of utilization of natural cellulose as forage for ruminant animals or as a feedstock for the biotechnological industry [8], [9].

There is only a limited understanding of how these pretreatments enhance hydrolysis of the lignocellulosic substrates. The number of glucose residues that are accessible to the rather large cellulase enzymes governs the rate of hydrolysis of the cellulose. The rate of biodegradation of cellulose is not related to the concentration in terms of weight or volume but rather must be associated with the surface area. Any reduction in the time needed to obtain a satisfactory sugar yield, therefore, will have a significant impact on the process economics. For this purpose, several methods have been described in the literature for increasing the accessibility/availability and hydrolysis of cellulose [10], [11], [12], [13].

1.2. Types of Pretreatment

A large number of pretreatments have been tried by many investigators, which can be broadly classified into physical, chemical, physicochemical, and biological (Table 2). Sometimes a combination of two or more pretreatments is employed. These pretreatments open the structure of the potential cellulose substrate. An efficient pretreatment method is one that increases accessibility to the cellulase and enhances the complete solubilization of the polymer to monomer sugars without formation of degradation products. In addition, the process should be inexpensive, less energy intensive, and not cause any serious pollution [7].

| Table 2. Pretreatment Methods [7]. |
|------------------------------ |--------------------------------- |
| Physical                      | Ball milling, two-roll milling, hammer milling, colloid milling, vibro energy milling, pyrolysis, γ-irradiation, microwave irradiation |
| Chemical                      | Alkali- NaOH, NH₃, ammonium sulfate |
|                              | Acid-H₂SO₄, H₃PO₄, HCl          |
|                              | Gases-CO₂, NO₂, SO₂            |
|                              | Oxidizing agents-H₂O₂, ozone   |
|                              | Cellulose dissolving agents-cadonex, phosphoric acid/acetone, ionic liquids |
|                              | Solvent extraction-ethanol-water, benzene-water, ethylene-glycol |
| Physicochemical               | Steam explosion (SE), SO₂-catalyzed SE, CO₂ explosion, SC- CO₂ explosion, ammonia freeze explosion (AFEX) |
| Biological                    | Fungi                           |

1.2.1. Physical treatments

Physical treatments such as grinding, milling, high temperature, freeze/thaw cycles, and radiation are aimed at size reduction and mechanical decrystallization. Mechanical methods such as ball milling, two roll milling, colloid milling, and nonmechanical methods such as α-irradiation, high-pressure steaming, and pyrolysis have all been attempted to change one or more structural features of the cellulose and enhance the hydrolysis. Most of these methods are limited in their effectiveness and often expensive [7].

Milling reduces the particle size and crystallinity and increases the surface area and the bulk density. This method can be used for a variety of substrates but is highly energy intensive. Ball milling and two-roll milling have been found to increase the susceptibility of the cellulose to enzyme action. Fitz milling results in size reduction without changing the crystallinity and wet milling results in fibrillation and delamination of the cellulose with no change in the chain length and crystallinity due to the plasticizing action of water [7].

Effect of Temperature.; Freezing cellulosic materials in water suspension at -75°C is reported to enhance chemical reactivity (as measured by dye absorption). The effect was more pronounced with repeated freezing and thawing cycles. The cryomilled cotton cellulose obtained by hammer milling in liquid nitrogen showed 36% more hydrolysis compared to untreated sample.

Pyrolysis involves heating the biomass at 200°C and is reported to increase hydrolysis. The type of gaseous atmosphere during pyrolysis affects the reaction. Pyrolysis in the presence of oxygen results in depolymerization, oxidation, and dehydration. In inert atmosphere,
depolymerization is slow and by-product formation decreases [13].

**Effect of \(\gamma\)-Irradiation:** High energy radiation was found to enhance in vitro digestibility as well as acid/ enzymatic hydrolysis of the cellulose. The radiation treatments are effective in breaking the lignin-cellulose complex as evidenced by the increased presence of phenolics in the irradiated samples. The irradiation is reported to cause increase in the surface area, while its effect on the crystallinity of the cellulose is controversial. Irradiation in the presence of oxygen, milling, or the addition of nitrate salts, or treatment with acid or alkali prior to irradiation increased the digestibility of the treated sample [14].

### 1.2.2. Chemical Pretreatments

There are two types of swelling of cellulose, intercrystalline and intracrystalline. Intercrystalline swelling can be affected by water and is a prerequisite for any microbial reaction to occur. Intracrystalline swelling requires a chemical agent that is capable of breaking the hydrogen bonds of the cellulose. Aqueous solutions of acid and alkali belong to this group of chemical agents.

Chemical pretreatment approaches have gained significant attention to increase the accessibility to hydrolytic attack. A wide variety of chemicals as pretreatment agents have been reported in the literature, which include cellulose solvents, sodium hydroxide, aqueous ammonia, calcium hydroxide plus calcium carbonate, phosphoric acid, alkaline hydrogen peroxide, sulfur dioxide, carbon dioxide, inorganic salts with acidic properties, ammonium salts, Lewis acids and organic acid anhydrides, acetic acid, formic acid, sulfuric acid, organic solvents, \(n\)-butylamine, \(n\)-propylamine, and alcohols such as methanol, ethanol, or butanol in the presence of an acid or alkaline catalyst [9].

Chemical pretreatments are generally more effective in solubilizing a greater fraction of lignin while leaving behind much of the hemicellulose in an insoluble polymeric form and opening up the crystalline cellulosic substrate.

The pulping of wood by the paper industry is one of the earliest methods used for delignification; however, pulping is an expensive method to use as a pretreatment for lignocellulose [7].

### 1.2.3. Physicochemical Pretreatments

Several pretreatment processes combine physical and chemical methods. In this regard, high pressure steaming, with or without rapid decompression (explosion), has been claimed as one of the most successful options for fractionating wood into its three major components and enhancing the susceptibility of the cellulose to enzymatic attack. Several patents have been granted to this process and many pilot plants of different capacities have been developed for either commercial or research purposes, located in various parts of the world, such as in Canada, the United States, Spain, Sweden, France, Italy, Japan, and Brazil [9].

- Physicochemical Pretreatments:
  - Steam Treatment (Autohydrolysis)
  - Acid-Catalyzed Steam Explosion
  - Ammonia and Steam Explosion
  - \(\text{CO}_2\)-Catalyzed Steam Explosion
  - \(\text{SO}_2\)-Catalyzed Steam Explosion
  - Supercritical Carbon Dioxide (SC-CO\(_2\))

### 1.2.4. Biological Pretreatments

In these pretreatments, the natural wood attacking microorganisms that can degrade lignin are allowed to grow on the biomass, resulting in lignin degradation. The main biological pretreatments include fungi and their enzymes. There is significant loss of the xylan and mannan components of the hemicellulose during the lignin hydrolysis.

Reductions up to 65% in the lignin content of cotton straw have been reported using white-rot fungi. This is the most promising organism for biological pretreatment of lignocellulose. The various means to use these organisms are: use of naturally occurring white-rot fungi; use of cellulose-less mutants as efficient lignin degraders and/ or to repress the enzymes that degrade wood carbohydrates. A white-rot fungus was used to remove 42% lignin, 2% glucan (including cellulose), and 30% hemicellulose of birch wood [13].

### 1.3. Hydrolysis of Lignocellulosic Biomass

The most commonly considered hydrolysis processes are the concentrated hydrochloric acid process, the two-step dilute acid hydrolysis, and enzymatic hydrolysis.

During the hydrolysis of lignocellulosic materials a wide range of compounds are released which are inhibitory to microbial fermentation. The composition of the inhibitors differs depending on the type of lignocellulosic hydrolysates [15].
1.3.1. Acid Hydrolysis

Dilute acid hydrolysis of biomass is, by far, the oldest technology for converting biomass to ethanol. The first attempt at commercializing a process for producing ethanol from the wood was carried out in Germany in 1898. It involved the use of dilute acid to hydrolyze the cellulose to glucose, and was able to produce 7.6 liters of ethanol per 100 kg of wood waste. The hydrolysis occurs in two stages to accommodate the differences between the hemicellulose and the cellulose [16] and to maximize the sugar yields from the hemicellulose and cellulose fractions of the biomass. The first stage is operated under milder conditions to hydrolyze the hemicellulose, while the second stage is optimized to hydrolyze the more resistant cellulose fraction. The liquid hydrolysates are recovered from each stage, neutralized, and fermented to ethanol [16].

Concentrated Acid Hydrolysis: This process is based on concentrated acid decrystallization of the cellulose followed by dilute acid hydrolysis to sugars at near theoretical yields. The separation of acid from the sugars, acid recovery, and acid reconcentration are critical operations. The fermentation converts sugars to ethanol. A process was developed in Japan in which the concentrated sulfuric acid was used for the hydrolysis. The process was commercialized in 1948. The remarkable feature of their process that was way ahead of its time, achieved 80% recovery of acid [17].

1.3.2. Enzymatic Hydrolysis of Lignocellulosic Biomass

The enzymatic hydrolysis or saccharification of lignocellulosic biomass is preceded by a pretreatment process in which the lignin component is separated from the cellulose and hemicellulose to make it amenable to the enzymatic hydrolysis. The lignin interferes with hydrolysis by blocking the access of the cellulases to the cellulose and by irreversibly binding the hydrolytic enzymes. Therefore, the removal of the lignin can dramatically increase the hydrolysis rate [18].

Recently, the enzymatic hydrolysis of lignocellulosic biomass has been optimized using enzymes from different sources and mixing in an appropriate proportion using a statistical approach of factorial design. A twofold reduction in the total protein required to reach glucan to glucose and xylan to xylose hydrolysis targets (99% and 88% conversion, respectively), thereby validating this approach toward enzyme improvement and process cost reduction for lignocellulose hydrolysis [19], [20].

1.4. Detoxification of lignocellulosic hydrolysates

Biological, physical, and chemical methods have been employed for detoxification (the specific removal of inhibitors prior to fermentation) of lignocellulosic hydrolysates [21]. The methods of detoxification change depending on the source of the lignocellulosic hydrolysate and the microorganism being used. The lignocellulosic hydrolysates vary in their degree of inhibition and different microorganisms have different inhibitor tolerances. Several reports on adaptation of yeasts to inhibiting compounds in lignocellulosic hydrolysates are found in the literature [22], [23], [24].

1.4.1. Biological Detoxification Methods

Biological methods of treatment make use of specific enzymes or microorganisms that act on the toxic compounds present in hydrolysates and change their composition. Treatment with the enzymes peroxidase and laccase, obtained from the ligninolytic fungus Trametes versicolor, has been shown to increase maximum ethanol productivity in a hemicellulose hydrolysate of willow two to three times due to their action on acid and phenolic compounds [25].

The filamentous soft-rot fungus Trichoderma reesei has been reported to degrade inhibitors in a hemicellulose hydrolysate obtained after steam pretreatment of willow, resulting in around three times increased maximum ethanol productivity and four times increased ethanol yield. Acetic acid, furfural, and benzoic acid derivatives were removed from the hydrolysate by treatment with Trichoderma reesei [26].

1.4.2. Physical Detoxification Methods

Hydrosyate concentration by vacuum evaporation is a physical detoxification method for reducing the concentration of volatile compounds such as acetic acid, furfural and vanillin present in the hydrolysate. However, physical detoxification increases moderately the concentration of nonvolatile toxic compounds and consequently the degree of fermentation inhibition [15].
1.4.3. Chemical Detoxification Methods

Chemical detoxification includes precipitation of toxic compounds and ionization of some inhibitors under certain pH values, the latter being able to change the degree of toxicity of the compounds [27]. Toxic compounds may also be adsorbed on activated charcoal [28], [29], on diatomaceous earth [30] and on ion exchange resins [31], [32].

1.5. Fermentation of Lignocellulosic Biomass to Ethanol

The hydrolysis of lignocellulosic biomass yields reducing sugars. Once the sugars are available, its fermentation to ethanol is not a difficult task as many technologies have been developed. Essentially, there are three different types of processes by which this can be achieved, namely,

1. Separate hydrolysis and fermentation (SHF)
2. Direct microbial conversion (DMC)
3. Simultaneous saccharification and fermentation (SSF)

SSF has been shown to be the most promising approach to biochemically convert cellulose to ethanol in an effective way [33].

1.5.1. Separate Hydrolysis and Fermentation (SHF)

This is a conventional two-step process where the lignocellulose is hydrolyzed using enzymes to form reducing sugars in the first step and the sugars thus formed are fermented to ethanol in the second step using *Saccharomyces* or *Zymomonas* [34], [35]. The advantage of this process is that each step can be carried out at its optimum conditions.

1.5.2. Direct Microbial Conversion (DMC)

This process involves three major steps, namely, enzyme production, hydrolysis of the lignocellulosic biomass, and the fermentation of the sugars, all occurring in one step [36]. The relatively lower tolerance of the ethanol is the main disadvantage of this process. A lower tolerance limit of about 3.5% has been reported as compared to 10% of ethanologenic yeasts. Acetic acid and lactic acid are also formed as by-products in this process in which a significant amount of carbon is utilized [37]. *Neurospora crassa* is known to produce ethanol directly from cellulose/hemicellulose, because it produces both cellulase and xylanase and also has the capacity to ferment the sugars to ethanol anaerobically [38].

1.5.3. Simultaneous Saccharification and Fermentation (SSF)

The saccharification of lignocellulosic biomass by enzymes and the subsequent fermentation of the sugars to ethanol by yeast such as *Saccharomyces* or *Zymomonas* take place in the same vessel in this process [39]. The compatibility of both saccharification and fermentation processes with respect to various conditions, such as pH, temperature, substrate concentration, etc., is one of the most important factors governing the success of the SSF process. The main advantages of using SSF for ethanol bioconversion are:

- Enhanced rate of lignocellulosic biomass (cellulose and hemicellulose) due to removal of the sugars that inhibit cellulase activity
- Lower enzyme loading
- Higher product yield
- Reduced inhibition of the yeast fermentation in case of continuous recovery of the ethanol
- Reduced requirement for aseptic conditions, resulting in increasing economics of the process [40], [41], [33], [42].

Because several inhibitory compounds are formed during hydrolysis of the raw material, the hydrolytic process has to be optimized so that inhibitor formation can be minimized. SSF seems to offer a better option for commercial production of ethanol from lignocellulosic biomass. *Penicillium funiculosum* cellulase and *Saccharomyces uvarum* cells have been reported to be used for SSF [40].

2. Conclusions

Although bioethanol production has been greatly improved by new technologies there are still challenges that need further investigation. These challenges include maintaining a stable performance of the genetically engineered yeasts in commercial-scale fermentation operations and integrating the optimal components into economic ethanol production systems.

Metabolic engineering and other classical techniques such as random mutagenesis address the further enhancement of microorganism capabilities by adding or modifying traits such as tolerance to ethanol and inhibitors, efficient hydrolysis of cellulose/hemicellulose, thermostolerance, reduced need for nutrient
supplementation, and improvement of sugar transport. The improvement achieved in the fermentation step with the help of metabolic engineering is just one of the aspects of an integrated process. Keeping a realistic perspective one can conclude that several pieces still remain to be properly assembled and optimized before an efficient industrial configuration is acquired.

References


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