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# Review: Advances in Magnetic Biochar-Based Cellulase Immobilization Systems for Enhancing Hydrolysis Efficiency in Second-Generation Bioethanol

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**ABSTRACT.** Second-generation bioethanol production utilizes abundant lignocellulosic biomass, such as agricultural residues and woody materials, without competing with food resources. Enzymatic hydrolysis of cellulose into glucose using cellulase is considered more environmentally friendly than conventional chemical methods. However, the practical application of free cellulase is limited by low operational stability, sensitivity to temperature and pH variations, and inhibition caused by compounds generated during biomass pretreatment. To overcome these limitations, enzyme immobilization on solid supports has been widely investigated to enhance enzyme stability and enable enzyme reuse. The incorporation of magnetic particles, commonly $Fe_3O_4$, into biochar produces magnetic biochar, which allows rapid separation from the reaction medium using an external magnetic field and facilitates enzyme recovery and reutilization. This review summarizes studies published between 2020 and 2025 on the application of magnetic biochar as a support for cellulase immobilization to improve lignocellulosic hydrolysis efficiency. Reported findings demonstrate high enzyme loading capacities of up to approximately 484 mg $g^{-1}$ biochar, glucose yields exceeding 90%, and retention of more than 80% enzymatic activity after 5–10 reuse cycles. Overall, magnetic biochar-based cellulase immobilization significantly improves the thermal and pH stability of cellulase, supporting a more efficient and economically viable saccharification process for second-generation bioethanol production.

## 1. INTRODUCTION

Second-generation bioethanol is produced from lignocellulosic biomass, such as agricultural residues and woody wastes, which are not used as food resources. Lignocellulosic biomass consists of three main components: cellulose, hemicellulose, and lignin. Among these components, only cellulose and hemicellulose can be converted into fermentable sugars for bioethanol production [1]. Struktur lignoselulosa yang kompleks menyebabkan material ini bersifat sangat resisten terhadap degradasi, sehingga diperlukan tahap pra-perlakuan (pretreatment) untuk mengganggu matriks lignin dan meningkatkan luas permukaan selulosa sebelum proses hidrolisis enzimatik. Dalam sistem biorefinery, hidrolisis enzimatik menggunakan selulase umumnya dipandang sebagai metode yang lebih ramah lingkungan karena berlangsung pada kondisi operasi yang relatif ringan, tidak melibatkan penggunaan asam atau basa kuat, serta menghasilkan produk samping berbahaya dalam jumlah minimal [2].

Cellulase is an enzymatic system composed of three main components: endoglucanase, exoglucanase (or exocellulase), and β-glucosidase. These enzymes act synergistically in the degradation of cellulose. Endoglucanase cleaves internal β-1,4-glycosidic bonds within the cellulose chain, generating new chain ends. Exoglucanase subsequently hydrolyzes cellulose by releasing cellobiose or glucose units from the chain ends. Finally, β-glucosidase converts cellobiose and short-chain oligosaccharides into free glucose that can be readily fermented. The coordinated action of these three enzymes is essential for achieving efficient cellulose conversion in second-generation bioethanol production [1-2].

Free cellulase enzymes are highly sensitive to environmental conditions. Fluctuations in pH, exposure to elevated temperatures, and the presence of inhibitory compounds generated during biomass pretreatment can rapidly reduce enzymatic activity. Such inhibitors, including furfural, hydroxymethylfurfural, and various phenolic compounds, may interact with the enzyme's active sites or disrupt its structure, leading to a significant decline in

catalytic performance. As a result, the use of free cellulase often results in low hydrolysis efficiency and increased enzyme consumption, which ultimately raises the overall cost of second-generation bioethanol production [3].

A strategy is required to enhance enzyme stability against variations in temperature and pH while enabling enzyme reuse. One of the most promising approaches is enzyme immobilization, which involves anchoring cellulase onto a solid support so that the enzyme remains bound while retaining its catalytic activity. Through immobilization, the enzyme can be protected from denaturation and enzyme loss during the reaction process can be minimized, thereby improving the efficiency and sustainability of the hydrolysis process [4]. and enables easy separation of the enzyme from the reaction system, particularly when magnetic materials are employed [7].

Numerous studies have reported that cellulase immobilization has the potential to significantly transform lignocellulosic hydrolysis processes. This approach not only enhances substrate conversion efficiency but also improves enzyme stability under harsh operating conditions, such as fluctuations in temperature and pH. In addition, immobilization enables enzyme reuse over multiple reaction cycles without a substantial loss of activity, thereby reducing enzyme consumption and improving the overall sustainability and economic feasibility of second-generation bioethanol production.

## 2. METHODOLOGY OF THE REVIEW

This article is prepared as a systematic literature review that compiles and analyzes recent research findings published between 2020 and 2025 concerning the immobilization of cellulase on magnetic biochar supports in the context of second-generation bioethanol production. Literature was collected through searches of various scientific databases and online scholarly sources, Web of Science, and Google Scholar. The literature search employed combinations of relevant keywords such as *"cellulase immobilization"*, *"magnetic biochar"*, *"second-generation bioethanol"*, and related terms. The review primarily focuses on experimental studies that investigate the preparation and modification of magnetic biochar, cellulase immobilization techniques, and the evaluation of system performance based on hydrolysis efficiency, enzyme stability, and reusability.

The selected articles were chosen based on their relevance to the research topic and the completeness of the experimental data presented. Findings from the selected studies were then comparatively analyzed to identify research trends, common approaches, existing challenges, and potential directions for further development of magnetic biochar-based cellulase immobilization systems.

## 3. RESULTS AND DISCUSSION

The discussion focuses on recent developments in the use of magnetic biochar as a support for cellulase immobilization, highlighting the relationship between the characteristics of the support material, immobilization methods, and the resulting enzyme performance. The key parameters analyzed include enzyme loading capacity, lignocellulosic hydrolysis efficiency, enzyme thermal and pH stability, and the reusability of the immobilized systems. Through a comparative analysis of various studies, this section aims to identify common trends, strengths, and limitations of the reported approaches, while also highlighting the challenges that remain in the application of magnetic biochar-based cellulase immobilization systems. Furthermore, the discussion provides insights into potential future developments to enhance the efficiency and sustainability of second-generation bioethanol production processes.

### *3.1 Second-Generation Bioethanol*

Second-generation (2G) bioethanol is produced using lignocellulosic biomass as feedstock, including straw residues, sawdust, cassava, and various agricultural residues [1]. Unlike first-generation bioethanol, which is produced from sugar- or starch-based feedstocks, second-generation bioethanol does not directly compete with food commodities and is therefore considered more sustainable. The conversion of lignocellulosic biomass into second-generation bioethanol generally involves several key stages. The initial stage is pretreatment, such as steam explosion or dilute acid treatment, which aims to disrupt the lignocellulosic matrix and increase the accessibility of cellulose and hemicellulose. This is followed by enzymatic hydrolysis using cellulase and hemicellulase to convert complex polysaccharides into monomeric sugars. The sugars produced are then fermented by microorganisms to generate ethanol as the final product [14].

One of the main challenges in second-generation bioethanol production is the low efficiency of the saccharification process, as lignin content and the high crystallinity of cellulose significantly hinder enzyme penetration and accessibility. In this context, the availability of cellulase enzymes with high stability and

reusability becomes crucial for improving overall process efficiency. Broda *et al.,* 2022 [9] reported that second-generation biorefinery systems have strong potential to meet energy demands in a sustainable manner without competing with food resources, through the efficient utilization of all lignocellulosic components. Although second-generation bioethanol production has not yet been widely commercialized, research efforts continue to focus on improving the efficiency of the hydrolysis stage.

*3.2 Cellulase Enzymes*

Cellulase is a multiprotein enzyme predominantly produced by fungi, such as *Trichoderma reesei*, and certain bacteria. Its main components include endoglucanase, which cleaves glycosidic bonds within the cellulose chain; exocellulase, which releases cellobiose from the chain ends; and β-glucosidase, which converts cellobiose into glucose [12]. The three components of cellulase function synergistically, with each playing a specific and complementary role, making their collaboration essential for achieving optimal cellulose degradation. However, free cellulase enzymes exhibit certain limitations, as they are prone to denaturation under extreme pH conditions or elevated temperatures. In addition, these enzymes tend to adsorb onto lignin surfaces present in biomass, which consequently diminishes their effectiveness in breaking down cellulose into simple sugars [3].

Free cellulase enzymes have another limitation because they cannot be recovered from the reaction mixture, which prevents their reuse and requires the addition of fresh enzymes for each new reaction. For instance, Mo and colleagues reported that free cellulase lost more than 50% of its activity under conditions of 60°C or within a pH range of 4–8 [3]. Therefore, to enhance the performance and stability of cellulase in cellulose degradation processes, it is necessary to apply effective stabilization methods. One approach involves enzyme modification through protein engineering to improve its stability and activity. However, a more common and practical strategy is to immobilize cellulase on a solid support, allowing the enzyme to be retained and anchored on the surface of the supporting material. This technique not only helps maintain enzyme activity under challenging conditions but also enables the enzyme to be reused over multiple reaction cycles, thereby improving the overall efficiency and cost-effectiveness of the process [4].

The application of immobilization techniques helps to preserve the secondary and tertiary structures of cellulase, ensuring that the enzyme's conformation remains stable even under challenging environmental conditions. Moreover, immobilization can reduce non-specific interactions between the enzyme and lignin present in biomass, which often lead to decreased enzymatic activity. As a result, both the activity and stability of immobilized enzymes are generally higher compared to free cellulase, making them more effective and reliable for industrial applications that require repeated reactions or operation under extreme conditions [13].

*3.3 Principles and Techniques of Enzyme Immobilization*

Enzyme immobilization can be achieved through various binding mechanisms, including physical adsorption and covalent attachment. Physical adsorption occurs via weak interactions such as van der Waals forces or hydrogen bonds between the enzyme and the support surface, without requiring modification of the supporting material. However, these bonds are relatively weak, making the enzyme prone to leaching during the reaction. In contrast, covalent attachment involves the formation of strong chemical bonds between amino groups or the protein of cellulase and active groups on the support surface, for example using glutaraldehyde as a crosslinker. This covalent binding enhances enzyme stability and resistance to desorption and harsh environmental conditions, generally outperforming physical adsorption in terms of enzyme stability. Other techniques include cross-linked enzyme aggregates (CLEAs) and encapsulation. In the context of magnetic biochar, the most common approaches involve anchoring cellulase onto the biochar surface through chemical reactions, such as with glutaraldehyde, or via adsorption into large pores to maintain enzyme activity [15].

Immobilization of enzymes on nanoscale materials, such as magnetic nanoparticles, offers significant advantages in the development of nanobiocatalysts. The exceptionally large surface area of these nanomaterials allows for a higher loading of enzymes, thereby increasing the catalytic capacity of the system. Furthermore, the nanoscale structure facilitates more efficient diffusion of reactants to the enzyme's active sites, accelerating reaction rates and enhancing the overall performance of the catalytic system. This approach not only improves enzyme activity and stability but also enables more effective reuse of enzymes in industrial applications or biotechnological processes [4].

The recovery of enzymes from aqueous suspensions can be achieved using a relatively small magnetic field, making enzyme separation after the reaction easier and faster. This significantly reduces enzyme loss during the

process, enables enzyme reuse, and extends the overall lifespan of the catalyst. Immobilization of cellulase on nanostructures, including magnetic particles, has proven to be a crucial strategy for enhancing catalytic efficiency, enzyme stability, and reusability over multiple reaction cycles. This approach is particularly relevant in industrial and biotechnological applications, where high enzyme performance, maintained stability, and effective reusability are key factors for successful process implementation [7]

*3.4 Characteristics of Magnetic Biochar*

Biochar is a form of solid carbon produced through the pyrolysis of dry biomass, such as wood, coconut shells, or rice husks, at high temperatures in the absence of oxygen. Its structure is highly porous, ranging from macropores to micropores, and possesses a high specific surface area, which can reach up to approximately 1600 $m^2/g$. Characteristics allow biochar to accommodate a large amount of enzyme and facilitate the diffusion of reactants to the enzyme's active sites, enhancing catalytic efficiency in various biotechnological processes. In addition to its excellent physical properties, biochar offers practical and economical as an enzyme support. The raw materials are relatively inexpensive and readily available, the preparation process is straightforward, and biochar exhibits good thermal stability, allowing enzymes to remain active under harsh operating conditions. The combination of high surface area, porous structure, and thermal stability makes biochar an attractive and efficient immobilization support for both industrial applications and biotechnological research [6].

The surface of biochar can be activated, for example using an alkaline treatment, to increase the number of functional groups such as amino and carboxyl groups, thereby enhancing its ability to form stronger bonds with enzymes. However, pure biochar has certain limitations, including a relatively low number of active sites for covalent binding and difficulties in separating it from the reaction mixture. To overcome these issues, magnetic materials, typically $Fe_3O_4$ particles, are incorporated into the biochar. Magnetic biochar is produced by mixing a solution of iron ions ($Fe^{2+}/Fe^{3+}$) with biomass or biochar, followed by pyrolysis, which results in $Fe_3O_4$ particles being impregnated within the solid structure. These magnetic particles impart strong magnetic properties to the biochar, allowing it to be easily recovered and separated from suspensions using a permanent magnet, thereby facilitating enzyme reuse in immobilization applications [7].

$Fe_3O_4$ is selected as a magnetic material due to its non-toxic nature, relatively low cost, and its ability to provide additional surface area for effective enzyme immobilization. Although other metals such as nickel (Ni) or cobalt (Co) can also be used as magnetic supports, their application is limited due to higher costs or potential toxicity. According to Zhao and colleagues (2021), composite magnetic biochar retains a high carbon content and a highly porous structure, thereby maintaining excellent adsorption capacity and enhancing catalytic performance. The added advantage of facile magnetic separation makes composite magnetic biochar a major focus in current research, particularly for enzyme immobilization applications that require high stability and reusability [7].

As an example of this preparation method that sugarcane bagasse was first activated using a NaOH solution, followed by pyrolysis to produce biochar. Subsequently, magnetic $Fe_2O_3$ particles were synthesized onto the biochar surface using glutaraldehyde and chitosan, resulting in a stable magnetic biochar suitable for enzyme immobilization. This approach demonstrates how the combination of chemical activation, pyrolysis, and the incorporation of magnetic materials can enhance the biochar's capacity to retain enzymes and support higher catalytic performance [6].

After cellulase is immobilized on magnetic biochar, the separation of the enzyme from the suspension becomes highly practical and efficient, requiring only the application of a magnetic field and eliminating the need for conventional filtration. Magnetic biochar possesses several advantageous characteristics, including high porosity, controllable pore volume, and a chemically modifiable surface. The combination of these properties makes it an ideal support for enzyme immobilization, as it not only retains the high adsorption capacity typical of biochar but also facilitates rapid enzyme recovery through magnetic separation. Consequently, magnetic biochar enables repeated enzyme usage, enhances catalytic stability and efficiency, and provides greater flexibility for industrial and biotechnological applications [6-7].

*3.5 Effectiveness of Magnetic Biochar as an Immobilization Support*

The immobilized cellulase exhibited excellent initial catalytic activity, reaching 330.9 mg of glucose per gram of substrate (CMC) within 24 hours. Moreover, the immobilized enzyme retained a significant portion of its catalytic capacity even after repeated use, maintaining approximately 86% of its initial activity following ten

successive cycles. These results demonstrate that immobilization on magnetic biochar not only preserves the enzyme's initial performance but also enhances stability and durability during repeated applications, highlighting its strong potential for industrial processes where enzyme efficiency and reusability are critical factors [10].

In a single usage cycle, there is minimal loss in performance if the magnetic biochar is promptly separated from the suspension after the reaction, ensuring that the enzyme remains effective for reuse. This highlights the importance of rapid magnetic separation in maintaining the catalytic stability of immobilized enzymes. $Fe_3O_4$/Graphene Oxide/Chitosan composite was able to immobilize cellulase from *Aspergillus niger* with an efficiency of approximately 86%, indicating that nearly all of the enzyme was successfully bound to the magnetic support. This approach underscores the significance of magnetic composite materials with high surface area and sufficient functional groups to facilitate enzyme binding, thereby enabling efficient immobilization and enhancing the reusability of enzymes across multiple reaction cycles [10].

The use of magnetic biochar as an immobilization support for cellulase has demonstrated highly promising results in bioethanol production. For instance, ethanol productivity reached 7.32 g/L from straw after four usage cycles, with approximately 99% of the enzyme's activity retained, indicating high stability and reusability. Similar findings were reported by Qiu et al. (2024), who observed that magnetic biochar with a high $Fe_3O_4$ density was capable of loading endoglucanase up to approximately 484 mg/g. This system produced a glucose yield of 932.5 mg/g in a biphasic IL/air setup, while maintaining enzyme activity constant over ten successive cycles. These results demonstrate that magnetic biochar not only enhances enzyme loading capacity but also supports high catalytic efficiency and stable reusability in biomass-based bioethanol production processes [8].

The use of cellulase immobilized on magnetic biochar resulted in a high glucose hydrolysis efficiency, reaching 90.5% from the lignocellulosic residue of *Cardamine violifolia*. Moreover, after ten usage cycles, the glucose yield remained at 46.4%, which is significantly higher than that achieved with free enzymes that cannot be separated from the substrate. These findings indicate that immobilization on magnetic biochar not only enhances the initial hydrolysis efficiency but also preserves the catalytic activity of the enzyme over multiple cycles, providing a clear advantage in terms of stability and reusability compared to free enzymes [9].

In terms of stability, immobilization on magnetic biochar consistently helps maintain the integrity of cellulase, protecting the enzyme from denaturation caused by high temperatures and extreme pH conditions [3]. This contributes to enhancing the reliability of the hydrolysis reaction. Several key studies on the immobilization of cellulase on magnetic biochar are summarized in the following table, highlighting the enzyme efficiency, stability, and reusability observed in various tested systems.

**Table 1.** Hydrolysis Efficiency and Reusability of Cellulase on Magnetic Biochar Supports

| Study (Year) | Biochar (Material & Preparation Method) | Enzyme (Type) | Hydrolysis Efficiency / Result | Stability & Reusability | Magnetic Separation |
|---|---|---|---|---|---|
| Mo et al., (2020) | Sugarcane bagasse, NaOH activation, pyrolysis at 800°C, $Fe_2O_3$ impregnation with chitosan (calcination) | Cellulase (*Trichoderma*) | 330.9 mg glucose/g CMC (24 h); ~73% of initial activity retained | ~86% of initial activity after 10 cycles | Very easy ($Fe_2O_3$) |
| Qiu et al., (2024) | Biochar from poplar, bamboo, straw; $Fe^{3+}$ ion impregnation, pyrolysis to generate $Fe_3O_4$ | Endoglucanase (Cel5A) | 932.5 mg glucose/g (biphasic IL system); enzyme loading ~484 mg/g. | Activity remained constant after 10 cycles | Easy ($Fe_3O_4$) |
| Wang et al., (2025) | *Cardamine violifolia* + Zn/Mg/Fe ($Cl_2$) followed by pyrolysis | Cellulase (*A. niger*) | 90.54% glucose yield from biomass; enzyme loading ~483 mg/g. | Yield remained 46.4% after 10 cycles | Easy ($Fe_3O_4$) |
| John et al., (2023 | GO/$Fe_3O_4$/Chitosan composite (in situ biosynthesis) | Cellulase (*Aspergillus*) | Ethanol ~7.3 g/L from bran; enzyme immobilization. | 99% of activity retained after 4 cycles | Very easy ($Fe_3O_4$) |

The table above demonstrates that all magnetic biochar-based systems are capable of enhancing the performance of cellulase compared to free enzymes. Factors influencing this effectiveness include the method of biochar preparation, such as activation or impregnation techniques that increase surface area; the type of cellulase used; catalytic efficiency, measured in terms of glucose or ethanol yield; and enzyme stability over multiple reaction cycles. A key advantage of these systems is the magnetic property of biochar, which allows for rapid separation of the immobilized enzyme after the reaction, enabling its reuse without significant loss of performance. Overall, magnetic biochar has proven to be a highly effective support for lignocellulosic hydrolysis processes, combining the high porosity typical of biochar with the convenience of magnetic separation, thereby promoting enzyme efficiency, stability, and reusability in both industrial and biotechnological applications.

## 4. CONCLUSION

Immobilization of cellulase on magnetic biochar has been shown to significantly enhance the hydrolysis efficiency of lignocellulosic biomass. Magnetic biochar combines a high surface area, providing abundant sites for enzyme binding, with magnetic properties that facilitate easy enzyme separation, thereby improving thermal and pH stability as well as the reusability of cellulase. Recent studies have reported glucose or ethanol hydrolysis yields exceeding 90%, with enzyme activity retention above 80% even after multiple cycles of use. These results highlight the great potential of magnetic biochar-based immobilization technology for more cost-effective and sustainable second-generation bioethanol biorefineries. Future research is recommended to optimize large-scale processes, explore novel biochar materials, and integrate immobilization with other enzymes such as laccase or xylanase to maximize the breakdown of complex lignocellulosic structures. With these advancements, the production of second-generation bioethanol is expected to become more efficient, supporting the global transition toward renewable energy sources.

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