Frontiers in Bioscience Elite

Front. Biosci. (Elite Ed) **2024**; 16(1): 5
https://doi.org/10.31083/j.fbe1601005

Review

Approaches for Producing Fungal Cellulases Through Submerged Fermentation

Madiha Nazir[1,†], Attia Iram[2,†], Deniz Cekmecelioglu[2], Ali Demirci[2,*]

[1]Microbiology, North Carolina State University, Raleigh, NC 27607, USA
[2]Department of Agricultural and Biological Engineering, Penn State University, University Park, PA 16802, USA
*Correspondence: demirci@psu.edu (Ali Demirci)
[†]These authors contributed equally.

Academic Editor: Suresh G. Joshi
Submitted: 5 October 2023 Revised: 22 November 2023 Accepted: 28 November 2023 Published: 31 January 2024

Abstract

Fungal cellulases are the most sought-after biological molecules produced from microbial sources in the last four decades. Owing to their emerging applications in the bioenergy industry for hydrolyzing cellulose, for which they are the most abundant source on this planet, research trends are shifting heavily toward adapting to submerged fermentation. However, filamentous fungal species, which are efficient cellulase producers, are well-adapted to low-moisture solid support as the substrate, such as in nature. Therefore, various fermentation strategies are currently being investigated to adapt them to submerged fermentation for large and high-quality production of cellulases. Emerging research trends, such as the use of inexpensive feedstocks, nutrient and/or culture optimization, innovative bioreactor designs, microparticle-assisted fungal growth, and innovative genetic engineering approaches, are some of the recent efforts by researchers to exploit the full potential of these biological molecules. This review discusses some of these strategies and their success rates in various research conditions. In addition, specific focus was provided to both increasing the market value of cellulases and the innovative strategies required to enhance their production on an industrial scale.

Keywords: cellulase; fungal cellulases; feedstock; Distillers' Dried Grains with Soluble (DDGS); biofilm reactors; culture optimization; *Aspergillus*; *Trichoderma*

1. Introduction

Enzymes are protein-structured biocatalysts capable of catalyzing specific reactions in living systems. However, enzymatic applications in various industrial sectors have gained interest since their discovery outside of cells. Their major advantages over chemical catalysts are their specificity, mild condition requirements, and rate of acceleration. However, other benefits that make them preferable in various industries are their environmentally friendly nature, use in various applications, and lower energy requirements [1]. Enzymes can be named according to their substrate and catalytic actions followed by the suffix—ase. For example, cellulases hydrolyze cellulose. Thus, they are called 1,4 beta-D-glucan 4-glucanohydrolase or simply cellulases.

Cellulose is the most abundant plant biomass on this planet [2]. The lignocellulosic biomass comprises 40–60% cellulose, 10–40% hemicellulose, and 15–30% lignin and can provide 14% of the world's total energy requirement, which is currently met by fossil fuels [2–4]. According to some speculations, if adapted to its full potential, bioenergy from biomass can be sufficiently generated to satisfy 27% of the world's transportation fuel by 2035. However, prominent problems in the current infrastructure remain troublesome in realizing the full potential of lignocellulosic biomass.

Cellulose also occupies the largest fraction of lignocellulosic biomass. However, it requires an extensive pretreatment step to release simple sugars (glucose) before they can be converted to transportation fuels, such as ethanol [5]. Cellulases are a group of enzymes that can be produced from microbial sources and can help reduce the energy load and cost of pretreatment [6]. As per the International Union of Biochemistry and Molecular Biology (IUBMB), cellulases are grouped into three categories: endocellulases, exocellulases (cellobiohydrolases), and cellobiases (β-glucosidases) [7]. Endocellulases attack glycosidic bonds within the cellulose chain, while exocellulases hydrolyze terminal ends. Cellobiases act on cellobioses to convert them into glucose. Interestingly, cellobiose inhibits cellulases, while the readily ongoing conversion process to glucose reduces the inhibition of this product [5,8]. Therefore, a cocktail of the three main enzymes yields efficient hydrolysis of cellulose compared to a single enzyme. Hence, microbial strains that can secrete all three types are preferable to single cellulase-producing microbial strains for the industry.

Cellulases are produced by a wide range of microbial species across the global niche [6]. While some bacterial species, such as *Bacillus subtilis*, are being explored for cellulase production, the main industrial producers are filamentous fungal species belonging to *Penicillium*, *Aspergillus*, and *Trichoderma* [6,7,9,10]. However, research

Copyright: © 2024 The Author(s). Published by IMR Press.
This is an open access article under the CC BY 4.0 license.

Publisher's Note: IMR Press stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

is ongoing to identify the best strains that can produce various types of cellulases without the need for expensive feedstocks and culture conditions [5]. Microbial production of cellulases and other products that add value is carried out by solid-state and submerged fermentations, as explained elsewhere [5,11,12]. The filamentous fungi, which are also the top industrial producers of cellulases, prefer solid-state fermentation (SSF) as they have adapted to low-moisture environments, such as decaying wood [5,12]. However, SSF is not the preferred mode of the industrial production of microbial bioproducts due to several underlying problems associated with expanding the process [5]. Therefore, submerged fermentation (SmF) is more appropriate for industrial implementation. However, a major barrier with the fungal SmF mode is the low enzyme activity, which is still under extensive research [13]. Therefore, this review focuses on novel approaches to produce fungal cellulases under submerged fermentation. These are namely the use of innovative or renewable feedstocks [14], media/culture optimization via statistical optimization techniques, such as response surface methodology (RSM) to enhance enzyme activities [10,13], the use of innovative additives such as microparticles [15], and genetic engineering [16]. All these topics are discussed in detail, along with the market value of cellulases, which is expected to increase in the near future following the adaptation of greener energy options [7]. This review also highlights some of the ideal approaches to increase the production of fungal cellulases on an industrial scale.

1.1 Fungal Cellulases as a Key to Second-Generation Biofuels

The constant depletion of fossil fuels and the air pollution resulting from burning fossil fuels exacerbate the current world. It is envisioned that the consumption of the globally most abundant source of biomass is lignocellulose, while the eco-friendly hydrolysis by cellulases makes these enzymes highly desirable in bioenergy (biofuels, viz. methane and ethanol) research and industry. Biofuels generated using lignocellulosic biomass are second-generation biofuels, which are superior to the food crop-dependent first-generation biofuels [17]. Conversely, the complexity of the lignocellulosic biomass due to cellulose, lignin, and hemicellulose is a challenge that manufacturers of second-generation biofuels face. The multistep transformation of the lignocellulosic biomass into usable and value-added commodities is not as easy as it sounds. The biggest challenge is to break down the raw biomass into simple sugars, followed by the bioconversion of these components into biofuels and value-added commodities [18]. Environmentally hazardous chemical hydrolysis is fully replaceable by eco-friendly enzymatic hydrolysis [19,20]. One eighty billion tons of raw lignocellulosic biomass on our planet makes it the most desirable inexpensive raw material, and the cellulases, in turn, have a plethora of applications in industry [21]. However, the high production prices of the cellulases are a bottleneck to making this commercially viable. It is envisaged that using lignocellulosic biomass for ethanol production in the transportation sector would make cellulases the most in-demand industrial enzyme. The greatest potential of fungal cellulolytic enzymes lies in ethanol production from biomass through the enzymatic hydrolysis of cellulose; however, low thermostability and low titer cellulase production results in high enzymatic costs [21,22]. Nonetheless, merchantable production of ethanol from lignocellulosic biomass depends on the production of cellulases, whose finances require upgrading.

1.2 Other Industrial Applications of Cellulases

The importance of microbial cellulases in prospective applications to the lignocellulosic biomass started gaining attention in the early 1950s. The widespread need for cellulases is because the cellulose polysaccharide constitutes 50% of the globally most abundant source of biomass, i.e., lignocellulose, thereby making it an industrially desired raw material for manufacturing products that add value to the bioenergy sector. Annually, 100 billion tons of lignocellulosic biomass is accumulated from various agricultural and waste resources. Efficient and sustainable utilization of lignocellulosic biomass can be achieved by cellulolytic hydrolysis by cellulases. Indeed, Anselme Payen first achieved the discovery and isolation of cellulolytic enzymes from plants [23]. Marine algae viz. *Ulva* and numerous prokaryotes, i.e., bacteria from the genus *Glucanobacter* and *Agrobacterium*, also contribute to the hydrolysis of lignocellulosic biomass [24,25]. However, fungi were concluded to be the dominant and efficient cellulase producers globally.

Cellulases have played a significant role as biocatalysts for many decades, which has enlightened their possible manufacturing applications. Value-added products from lignocellulosic biomass by employing cellulases are a recent concept. Industrial demand for fungal cellulases has risen significantly over the last few decades, especially for strains capable of producing stable enzymes under harsh industrial conditions. The possibility of actively converting cellulose into simpler components for conversion to transportation fuel can make cellulases the most desirable industrial class of enzymes [26].

Widespread applications of cellulases in food are diverse, viz. cereal grains, polishing, feed supplements, and flavor enhancements. Moreover, cellulases are involved in improving the digestibility of animal feeds [27]. Flavonoid extraction from the seeds and flowers is also carried out by cellulases. The food sector utilizes cellulases for bakery, juice, and wine processing, as cellulases provide enhanced wine filtration, improved pulp hydrolysis in the juice industry, and enriched texture and quality of bakery products [28]. Cellulases are efficient and preferred in extraction because they ensure less heat damage and higher yields.

The major cellulase contributors to the food industry originate from *Aspergillus* and *Trichoderma* [29]. Additionally, cellulases are commercially produced by companies such as Novozymes, Badische Anilin- und Sodafabrik (BASF), DuPont Danisco, and Dutch State Mines (DSM) [30].

Biofuel production using lignocellulosic biomass hydrolyzed by cellulases has the potential to be the world's largest industry, yet requires these enzymes. Further, the pulp and paper industry has great market demand for stable cellulases. Cellulase cocktails are very effective in sustainable, environment-friendly detergents, and the utilization of cellulases in the textile, paper, and detergent industries is massive. Hence, demand has increased [31,32]. In the textile industry, pumice stone washing of the jeans reduced the capacity of the jean load, while a fifty percent higher jean load can be achieved by replacing stone washing with cellulases [33]. Additionally, acidic *T. reesei* endoglucanase II is employed in nontoxic fabric biopolishing [34].

1.3 Current Market Value of Cellulases

Cellulases comprise approximately 3/4 of the total demand for industrial enzymes in many industries mentioned above, making them a frontrunner in global enzyme production and consumption [35]. The most recent global market value of cellulases was estimated at 1.621 billion dollars in 2022, which is expected to increase at a compounded annual growth rate (CAGR) of 6.94% from 2022 to 2032 [36]. In comparison, global biofuel enzymes are projected to increase from 905.2 million dollars (2020) to 1.3 billion dollars by 2026 with a CAGR of 7.3% [36], while the total enzyme market will grow from 10 billion dollars in 2019 to 14.7 billion in 2024 [37]; the CAGR of industrial enzymes is placed at 6.5% in terms of value [38].

The extensive research on cellulases by different manufacturers in the past two decades has led to the production of diverse blends of cellulases. The current market is dominated by Novozymes (Denmark) and Genencor, which have merged with DuPont (Netherlands), DSM (Netherlands), Solvay enzymes (Germany), and Dyadic (USA) [38]. In fact, 75% of the entire industrial enzyme market, not just cellulases, is concentrated within the first three hosts listed [39]. The indices used to indicate enzymatic activity are diverse; thus, the market value of cellulases is not standardized based on the quality of the product [40]. The inconsistencies between commercial indices used in the market present another major hurdle: inappropriate production improvement and economic analyses of biofuel production from cellulases.

Owing to the diverse composition of the lignocellulosic biomass obtained from various sources, a specific cocktail of enzymes cannot provide a widespread solution. This allows us to identify crucial limitations in producing the ideal enzyme or ideal blend of enzymes that combine for a specific application. The Carbohydrate-Active EnZymes (CAZy) database provides substantial information and plays a fundamental role in the improvisations of industrially needed enzyme preparations. The three approved approaches for preparing the desired mix of enzymes are (i) crude enzyme preparations from microbial consortia, (ii) defined cellulolytic enzyme preparation obtained from synergistic microbial interactions by fungal decomposers, and (iii) single component enzymes obtained from simple synergistic hydrolyzing systems for specific applications [41]. Next-generation biological modification tools (-omics centered and high-throughput selection) are the driving force in characterizing these enzymes and promoting the progress in improved production of cellulases from diverse fungal strains along with studies on their synergism, the ideal cellulase cocktail, and relevant applications in the desired industry.

Due to the inducible expression of the cellulases, the chief expensive and demanding feature of industrial fungal cellulase production is delivering a suitable inducer. Additionally, bacterial cellulases are less desirable compared to fungal cellulases since bacterial cellulases mostly lack either of the three cellulolytic activities, particularly exoglycanase activity. From an economical and ease of process point of view, fungal cellulases are preferred due to simpler downstream processing. Fungal cellulases also have higher activities than their bacterial counterparts.

1.4 Cost Implications of Producing Cellulases From Renewable Resources

There are many economic challenges associated with producing cellulases, thereby making their applications expensive. Therefore, enzyme industries aim to reduce production costs. Hence, sustainable, economically reasonable, and readily procurable sources of nutrients for cellulase-producing microbes can significantly lower enzyme costs [42]. However, the economic repercussions of producing cellulases from renewable biomass are manyfold and encompass multistep evaluations of the production process, from raw material selection to the market dynamics affecting the final product price.

Owing to its abundance and diversity, lignocellulosic biomass is an inexpensive and attractive substrate for cellulase production [43]. However, sole dependence on conventional lignocellulosic biomass, such as forest residues, municipal waste, and agro-industrial resources, may eventually raise questions about land management and affordable availability due to their abundant applications [44]. Additionally, lignocellulosic pretreatment is an energy-demanding and expensive affair. Therefore, it is also advisable to explore unconventional resources, such as naturally growing unwanted weeds. For instance, *Parthenium hysterophorus* is among seven globally abundant and unwanted weeds growing in Australia, Asia, America, and Africa [45]. Microbial fermentation of such renewable biomass would also allow environmental protection from these weeds and economic cellulase production.

Table 1. Some of the cellulase studies conducted over the last five years.

Study specifications	Results (SSF vs. SmF, respectively)	Ref
Evaluation of two fungal strains with rice straw	6.4 IU/g and 3.8 IU/g FPase*	[52]
Implementation of sequential fermentation (SF) technique by *Aspergillus niger* with tree leaves	SF (combined SSF and SmF) increased enzyme production by 140%	[53]
Study of porous materials as inert carriers in SSF	0.368 FPU/mL and 0.262 FPU/mL	[54]
Production with surgical cotton–cardboard mixture	3.230 IU/mL and 1.94 IU/mL Fpase	[55]
Life Cycle Assessment (LCA) for recovering cellulase from coffee husks	The environmental impact of SmF was higher than SSF	[56]
Evaluation of various fungal isolates	903.7 IU/mL and 800 IU/mL	[57]

*Filter Paper Cellulase Assay; SSF, solid-state fermentation; SmF, submerged fermentation.

Finally, Life Cycle Analysis (LCA) should be included to evaluate the environmental cost of utilizing various renewable resources. Life cycle assessment of cellulase production from solid-state fermentation of coffee husks has been reported [46]. Similarly, a techno-economic evaluation of cellulase production by submerged fermentation was reported to compare the batch versus fed-batch process feasibility, whereby the fed-batch was concluded to be a more economically viable process [47]. These studies imply that the feasibility of using renewable resources depends on overall process energy costs, fermentation time, and additional nutrient components. Cellulases are inducible enzymes, and the addition of lactose has been widely explored to induce their production, which also increases the final costs. There is no doubt that renewable resources and biomass management align with sustainability goals, yet the long-term availability and stability should also be considered.

2. Types of Fungal Fermentations for Cellulase Productions

Fungi are a diverse group of microorganisms that contain several types of cells, ranging from yeast and molds to mushrooms. These saprophytic microorganisms are best known for their wide adaptability strategies across different niches on the planet, making them also one of the oldest domesticated microorganisms by humans [48]. Roughly, fungi can be divided into filamentous (molds) or non-filamentous microorganisms (yeasts). Cellulases are hydrolytic enzymes best known to be produced from wood-rot fungi. Two of the best-known genera for this purpose are *Aspergillus* and *Trichoderma,* containing several species that can depolymerize the cellulosic materials effectively because of their ability to secrete the complete cellulase systems [6,49]. Two types of fermentations are currently employed for producing cellulases by fungal strains: solid-state fermentation (SSF) and submerged fermentation (SmF). SSF is the application of a solid substrate that can be lignocellulosic in nature for the growth of mycelia. The SSF mode does not have free water in the fungal media but contains enough moisture to sustain growth across the solid substrate; the solid substrate can also provide nutrients for growth. Conversely, SmF contains free water, and the mycelial structures are suspended in the fungal media. Interestingly, both types of fermentation have their merits and demerits in terms of production efficiency, cost, and environmental impact. From an industrial point of view, SmF is the most widely used method due to its relative feasibility for scale-up [50].

The evaluation of fermentation type (SSF vs. SmF) for fungal cellulase production has been one of the most prominent research topics since realizing the importance of these enzymes in the biofuel industry [51]. It has been widely accepted that while SmF is the most feasible method for controlling fermentation at industrial scales, SSF has produced higher enzyme activities in various comparative studies. Table 1 (Ref. [52–57]) shows some of these comparative studies conducted in the last five years. Each method has its advantages and challenges over the other, as explained below. However, the main production method used on an industrial scale must have some inherent properties, which should be improved via intensive research. Therefore, in this review, strategies to overcome the challenges of SmF are discussed in detail in later sections.

3. Challenges in the Production of Fungal Cellulases by SSF

The inefficient distribution of resources, such as nutrients, oxygen, and moisture, is the most notable challenge in the production and adaptation of fungal cellulases under SSF [49]. Consequently, the fungal cells do not grow to their fullest extent, thereby limiting the production scale. In addition, successful industrial adaptation requires a large space and workforce to control fermentation parameters. To solve the problem of distribution, strategies such as rotary drums are employed [58]. However, fungal cells are prone to shear, thereby rendering such strategies inefficient [49]. Heat dissipation is another huge issue while operating SSF. The metabolic heat generated by the fungal cells during growth and enzyme production must be dissipated from the fermentation reactor. If the dissipation does not occur efficiently, the cells and the enzymes can become denatured. Therefore, small-scale bioreactors, which can be controlled efficiently, lead to larger yields from SSF than SmF (Ta-

Table 2. A list of innovative strategies for the production of fungal cellulases under submerged fermentation.

Strategy	Parameters	Ref
Non-conventional, economical, and renewable feedstocks	Distillers Dried Grains with Soluble (DDGS)	[6,10,14,65]
	Rice straw	[66]
	Municipal solid waste	[67]
	Potato waste	[68]
	Wheat straw	[69]
	Green seaweed	[70]
	Sugarcane bagasse	[71–73]
	Pea hulls	[74]
	Coir (coconut husks)	[75]
Nutrient-based optimization	Pretreatment of biomass	[76]
	Nitrogen source optimization	[10]
	Carbon source optimization	[9]
	Mineral evaluation and optimization	[10]
Culture parameter optimization	Temperature	[77]
	pH	[78]
	Agitation	[13]
	Aeration	[13]
Biofilm reactors	Multispecies	[79]
Mixed cultures	Co-cultures of different fungal species	[80]
Microparticle-assisted enhancement	Aluminum oxide, magnesium silicate, *etc.*	[15]
Thermophilic fungi	*Rasamsonia emersonii*	[16]

ble 1) yet are still inadequate for industrial adaptations [59]. Hence, SmF has been widely investigated to overcome the ultimate challenge of extensive industrial adaptation.

4. Pros and Cons of the Production of Fungal Cellulases by SmF

A prominent challenge of SmF is the decreased enzyme activities obtained in fungal enzyme productions (Table 1). The main reason behind this is the shear stress of agitation on mycelial cells [60], the formation of large mycelial clumps [13], and carbon catabolic repression [61]. Cellulase production by filamentous fungal species is a natural phenomenon that has been adapted due to the absence of simple sugars in the environment. Cellulases are then secreted to break down the complex cellulosic material and convert them into simple sugar monomers. However, in the presence of simple sugars, the carbon catabolic repression causes a reduction in the levels of cellulase production by the fungal species. Therefore, complex yet biologically degradable material can induce enzyme production in fungal fermentation systems. Consequently, choosing a suitable feedstock that can induce enzyme production is imperative. Another challenge in the submerged fermentation systems is the variability in the reactions of different microbial strains toward different feedstock, media composition, and fermentation conditions. Therefore, it is essential to optimize fermentation conditions according to the specific strain and/or feedstock. Excessive foaming during the aeration and agitation of the aerobic fermentations is another major issue in SmF [62]. While foaming can be controlled with the help of antifoaming agents, adding such chemicals can affect the viscosity of the culture media and decrease the oxygen hold-up due to the increased viscosity. Similar problems arise due to the excessive growth of the fungal mycelium in the media [62], while product inhibition is another problem in the liquid media. Cellulases can break down the cellulosic components in the feedstocks, releasing simple sugars, which can have inhibitory effects on the enzymes. Finally, a lower dissolved oxygen uptake due to the production of denser mycelial clumps can also decrease the overall productivity of fungal fermentation systems for cellulase production. Notably, all these challenges are currently being addressed in research studies, and some of the recently proposed solutions are provided in the subsequent sections.

5. Novel Approaches to Solving the Challenges in SmF for Fungal Cellulase Production

Fungal cellulase production by SmF is growth-associated [63], and filamentous fungal strains tend to adhere to surfaces for optimal growth. The enzymes are produced due to the scarcity of simple fermentable sugars in the environment, and fungal strains release the enzymes to break the complex lignocellulosic biomass around them and release simple sugars for growth. Therefore, the growth of fungal biomass in the submerged state can be considered unconventional or unnatural, making this process dif-

ficult for microbial adaptation. However, denser mycelial growth also hinders further growth and enzyme production in the submerged bioreactors, presenting several problems related to process design and efficient adaptation on industrial scales [64]. The denser mycelial growth is also associated with poor microbial performance due to the inefficient distribution of resources, such as nutrients and oxygen. However, despite all these challenges, submerged fermentation remains the preferred mode for large scales due to the relative ease in scaling up and the better control of process conditions. Therefore, researchers in the last few decades have dedicated many studies to solving the underlying problems associated with fungal cellulase production in submerged fermentation conditions. The most common strategies successfully implemented in fungal cellulase production under submerged fermentation are listed in Table 2 (Ref. [6,9,10,13–16,65–80]).

5.1 Non-Conventional, Economical, and Renewable Feedstocks

To manufacture value-added products via fermentation, the most common and simplest feedstocks are readily fermentable sugars, such as glucose, fructose, sucrose, galactose, alongside many other mono, di, or oligosaccharides [81–84]. However, decades of research have shown that each product can be produced in higher amounts using certain types of feedstocks compared to others [81,83,85]. For example, in the case of citric acid, simple sugars such as glucose and fructose have been shown to be ideal feedstocks compared to others [85]. The definition of a microbial feedstock is also ambiguous as it sometimes contains all the nutrient requirements of the microbial species, whereas sometimes, additional macro or micronutrients are required in a prepared feedstock. As mentioned earlier, fungal cellulase production depends on the presence of cellulose in the media [5]. However, cellulose is not soluble in the medium, creating an accessibility problem for the fungal cells under submerged fermentation. Conversely, simple sugars such as glucose are also not feasible for fungal cellulase production because they do not induce cellulase production in fungal cells. Therefore, media containing soluble cellulose fibrils can induce enzyme production. Indeed, Iram *et al*. [9] recently proved the theoretical basis of this concept.

Several low-cost feedstocks containing cellulose in the media are ideal to meet the above-mentioned conditions. Some famous examples include sugarcane bagasse [71–73], wheat or rice straw [66,69], corn cobs or husks [86], and many other agricultural waste products, as mentioned in Table 2. The main problem with all high cellulose feedstocks is that they need harsh pretreatments to release simple cellulose fibrils for enzyme induction and simple fermentable sugars for the initial growth of the fungal species. In addition, all such feedstocks also lack the essential nitrogen sources required to produce microbial proteins efficiently. Distillers' Dried Grains with Soluble (DDGS) are a byproduct of bioethanol production, which shows high potential to produce fungal cellulases with the help of optimization strategies mentioned below [14]. DDGS contains 26–33% proteins that help produce fungal enzymes, although it also has fibers (33–40%) that induce cellulase production [14]. The enzyme production (0.66 IU/mL) was highest in mildly acid-treated DDGS, compared to crystalline cellulose (0.4 IU/mL) or glucose (0.3 IU/mL) [9]. Conclusively, DDGS is an ideal feedstock for fungal enzyme production under SmF.

5.2 Nutrient Optimization

Nutrient optimization means making a microbial medium recipe that is best for a particular microbial outcome or product. Thus, for fungal cellulase production, cellulase activity, yield, and stability are some of the crucial factors. Alternatively, growth is not a desired factor, as dense mycelial clumps in the media will hinder the effective distribution of nutrients and dissolved oxygen in the media [5,12]. Any nutrient element such as nitrogen source, minerals, or vitamins can positively or negatively affect the desired outcome. Therefore, it is crucial to know the effect (positive or negative) of each element and the ideal concentration in the media.

Many statistical techniques, such as the Plackett–Burman design (PBD), can be used to screen the effectiveness of each nutrient element. In addition, a range of concentrations can be optimized for various media elements using statistical optimization techniques, such as the response surface methodology (RSM). RSM is a method used to set pre-determined variables in such combinations that the desired level (maximum, minimum, or a specific value) can be obtained throughout a low number of experiments. The method is a fractional factorial design, which also provides response values at corresponding values of the dependent variables [87]. After an initial screening via PBD or other methods, RSM can be applied to optimize three or more variables for one or more response variables. This technique has been extensively used in the past few decades to optimize nutrient formulations to obtain maximum levels of specific fermentation products, as described in an example below.

In a study by the authors, statistical optimization of various minerals and nitrogen sources was evaluated using DDGS hydrolysate as the main feedstock. It was determined that additional minerals do not significantly positively affect fungal cellulase production under submerged fermentation [10]. This could be because DDGS, a byproduct of a microbial fermentation process (bioethanol production), already has some minerals. In addition, the pretreatment and subsequent neutralization step can further enhance the salt concentrations in DDGS hydrolysates. On the other hand, RSM helped produce a 262% increase in cellulase production [10].

Many other studies show the positive effect of nitrogen source amendment and optimization on fungal enzyme production. For example, in a study by Acharya *et al*. [88], peptone and ammonium sulfate were optimized to maximize cellulase production. The maximum cellulase activity was obtained at 0.125% peptone and 0.14% ammonium sulfate. On the other hand, using only 0.03% urea produced 0.15 IU/mL of cellulase activity. The main feedstock in this study was sawdust, and the concentration of sawdust was also optimized. Similar results were also obtained by Ghori *et al*. [89], where urea showed significant enzyme production. All such studies show the efficacy of using inexpensive feedstocks to analyze and optimize nitrogen sources for maximum enzyme production.

5.3 Culture Parameters Optimization

Culture parameters are pH, temperature, aeration, agitation, and inoculum size. All such parameters directly influence growth rate, enzyme production, and the production of undesirable products. There are many studies where such parameters have been evaluated and optimized using RSM for maximum fungal cellulase production under submerged fermentation. The main difference between these studies is the choice of feedstock and fermentation scale (shake-flask or benchtop bioreactors or pretreatment method before the fermentation stage). All such parameters, however, show a direct influence on cellulase production. Using DDGS as the main feedstock, Iram *et al*. [13] optimized the effect of inoculum size (1–10%), aeration (0.5–2 vvm), and agitation (100–500 rpm) in benchtop bioreactors (1.5 L) for fungal cellulase production under the submerged condition [13]. Cellulase production increased by 37% following fermentation parameter optimization. The optimum conditions were 6.5% inoculum size with 1.4 vvm aeration and 310 rpm agitation.

Several other studies have shown the effect of fermentation parameter optimization on cellulase production [5,88]. In the study by Acharya *et al*. [88], pH, inoculum size, temperature, and agitation were evaluated for maximum enzyme production. An optimal pH of 4, a temperature of 28 °C, and an inoculum size of 10 discs were reported.

The effect of agitation is crucial for fungal fermentation under submerged conditions for several reasons. Agitation directly affects the distribution of nutrients and dissolved oxygen in the media [90]. However, it also affects negatively by imposing shear stress on the fungal mycelial structures. Therefore, optimizing this culture parameter is crucial [12]. In addition to the agitation rate, the selection and design of the impeller are also important as it can influence the shear stress on microbial cells [91]. The impeller helps in the dispersal of oxygen bubbles in the media. There are several types of impellers, yet the two main types are Rushton (radial flow) impellers and axial flow impellers. Flat-blade or Rushton impellers have shown high shear stress to mycelial mass in submerged fermentation [92]. Therefore, innovative impeller designs specifically for fungal fermentation have been reported to reduce the shear stress on fungal morphology [92]. Fig. 1 (Ref. [92]) shows one of the innovative impellers designed specifically for submerged fungal fermentation.

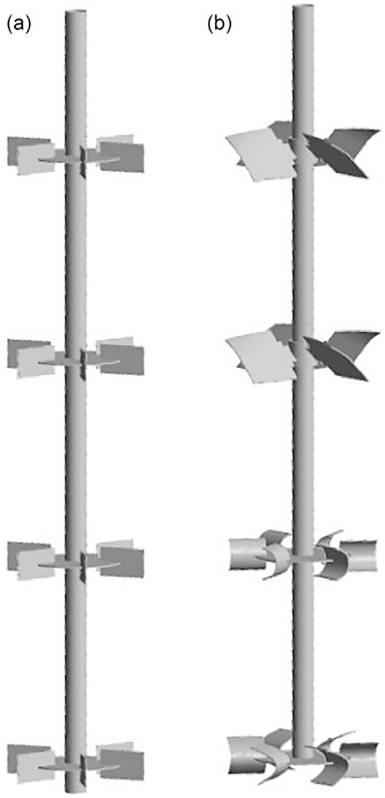

Fig. 1. Impeller design for fungal submerged fermentation. (a) Conventional impeller. (b) Novel impeller. Reproduced with permission from [92].

5.4 Study and Modeling of Enzyme and Growth Kinetics

Among various techniques to increase the productivity of the enzymes for a particular industrial product, enzyme kinetics can help better interpret results and optimize the production process. Enzyme kinetics involves modeling the rate of enzymes with respect to substrate conversion. Furthermore, quantifying enzyme–substrate interactions can contribute to a better understanding of hydrolysis, yield, and any reasons that may reduce the reaction rate. Due to the heterogeneous nature of the lignocellulosic biomass, the study of cellulase kinetics has always been simplified with different assumptions [93]. Different sets of assumptions result in varying degrees of model accuracy, yet all ensure different strategies to improve the degree of hydrolysis. For example, in a study by Paulraj *et al*. [94] enzyme kinetics were used to assess the effect of inorganic salts on cellulase activity.

In fermentation process improvement, growth kinetics also play an important role in defining the quality of the desired product. Predominantly, enzyme kinetics (e.g., substrate consumption) are closely correlated with the growth of the microorganism. In a study by Sasikumar and Viruthagiri [95] growth kinetics were used to predict the constants in the polysaccharide fermentation process. Similarly, in a study by these authors, substrate conversion was correlated to cellulase production trends, and it was concluded that higher enzyme activity results from low substrate concentration in the fungal media [12]. Therefore, it can be supposed that modeling enzyme and growth kinetics can provide useful insights into the control of the cell mass and enzyme activity in fungal fermentation systems.

5.5 Use of Suitable Fungal Strains or Mixed Cultures

Cellulase is a set of several different enzymes that act in synergy for the effective hydrolysis of cellulose. However, not all fungal species or their specific strains can produce all these enzymes, with most strains only producing a subset [6]. Therefore, for effective adaptation at industrial scales, microbial strains that can produce many different types of cellulases are preferred due to the production of an efficient enzyme cocktail. For example, *Trichoderma reesei* RUT-C30, which is a top producer of cellulase enzymes, produces higher amounts of exoglucanases [6]. Therefore, screening the best fungal strains for the desired cellulase is extremely important. With DDGS as the main fermentation feedstock, several *A. niger* and *T. reesei* strains showed promise for the production of many different types of fungal cellulases [6].

In addition to the ideal strains, a mixture or a co-culture of several fungal strains has also been explored for maximum cellulase production under submerged fermentation [80,96]. While the efficacy of using multiple microbial strains for a specific outcome has been proven many times for maximum product formation, undesirable microbial metabolic interactions can also hinder optimal production. Therefore, co-cultures should be designed by their synergetic effect on each other to produce the maximum output.

5.6 Use of Biofilm Reactors

Biofilm reactors are a special type of bioreactors, whereby the microbial species are facilitated with solid or semi-solid surfaces where they can form biofilms for the growth and production of desired products [97]. Several microbial species can form biofilms following mutual interactions to create the desired product formation. However, in the case of filamentous fungal species, the main incentive is to give fungus support for mycelial growth while tuning its environment via fermentation parameter optimization for maximum productivity. A recent example of this is shown in the study by Xiros and Studer [79], where they used multiple fungal species for cellulase activity. However, the concept is still in its infancy and should be explored further to assess the full potential of this technique for fungal submerged cellulase production.

5.7 Microparticle-Enhanced Fermentation

Recently, a newer technique where the microbial medium is supplemented with inert microparticles, such as aluminum oxide, magnesium silicate, or titanium oxide, to control the morphology of filamentous fungi under submerged fermentation has been explored [15]. Several studies have been shown to increase microbial product formation, while decreasing the mycelial clumps in liquid media. Some examples include the studies conducted by Coban *et al*. [98] on phytase production and Coban and Demirci [99] on lactic acid production. Iram *et al*. [100] assessed the effect of microparticles (e.g., aluminum oxide and magnesium silicate) on fungal cellulase production using a shake-flask and 1.5 L benchtop bioreactor scales. The addition of microparticles increased cellulase production by several folds while decreasing the size of mycelial clumps in the media, resulting in a better distribution of nutrients and dissolved oxygen. These results show the positive effect of microparticles on fungal cellulase production under submerged fermentation.

5.8 Genetic Engineering

Wild-type strains of fungi have been studied tremendously for cellulase production. The research studies underline the major drawbacks of wild-type strains, such as the unavailability of hyper-producing efficient strains, incapacity of industrial-scale production, and limited tolerance to extreme conditions in industrial settings. Improvement in the catalytic properties of cellulases can withstand industrial-scale requirements. The high demand for improved production and desired properties of cellulases underlines the importance of tailored genetic manipulations to construct novel fungal strains that can be exploited industrially. Time-consuming and tedious conventional genetic manipulations are also not viable solutions.

Innovative sequencing methods and molecular-level tools in the present times reinforce the efficacy of genetic manipulations of filamentous fungi for improved properties and industrial-scale production. Moreover, next-generation sequencing has enabled access to the genome sequences of several industrially appropriate filamentous fungal strains to design relevant studies. The most rigorously studied and the major cellulase-producer filamentous fungi include *Trichoderma reesei* [101], *Aspergillus niger* [22,102], *Neurospora crassa* [103], and *Penicillium oxalicum* [104]. Natural processes are slow owing to the scarcity of bioavailable nutrient components; therefore, industrial scale-up is required to speed up the processes and cell factory manipulations to enhance their enzyme production speeds and capacities.

Table 3. List of genetic engineering tools implemented on fungal strains for improving cellulase production by fermentation.

Strain	Genetic improvement strategy	Target genes/property	Reference
T. reesei, *A. niger*	Promoter engineering	Strong inducible promoters from genes *cbh1* and *cbh2*	[105–107]
A. nidulans, *N. crassa*, *Aspergillus*, *Myceliophthora*, *Penicillium oxalicum*	Genetic engineering of selective markers	Nutritional marker genes (*pyrG, pyr4*, *trpC*, *acuD*)	[108–110]
T. koningii, *T. reesei*	Genetic engineering of transcription regulators	Enzymatic regulatory induction, RNA interference	[111–113]
T. reesei, *A niger*	Genetic engineering fermentation friendly morphology	Enhanced agitation speed results in higher pellet diameter and higher cellulase productivity	[102,114,115]
A. fumigatus, *P. chrysogenum*	CRISPR–Cas9-based genome editing system	Targeted gene editing (deletion, addition, or inhibitions)	[116–119]

CRISPR–Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9.

Implementation of metabolic engineering for efficient production and improved catalytic properties of cellulolytic enzymes by fungal strains is linked to understanding the mode of action of cellulases and their transcription level expression. As opposed to traditional genome editing tools, CRISPR/CAS has been exploited to test multiple target sites (Table 3, Ref. [102,105–119]). Clustered, regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR-CAS) technology has emerged for developing competent genetically modified cellulolytic fungi strains. The preferred level of cellulase production and the futuristic approaches require a better understanding of the mode of action of cellulases and transcription level regulation of enzyme expression, which has progressed by utilizing technology such as CRISPR-CAS. Genetic manipulations are also employed to improve the enzymes' properties, namely stereospecificity, enantioselectivity, substrate specificity, enzyme activity, enzyme stability, and tolerance to environmental factors.

6. Conclusions

Cellulases are a group of enzymes that are crucial in biomass hydrolysis prior to their subsequent use in the generation of biofuels. Compared to other microbial species, fungal species produce high-quality cellulases. However, their effective adaptations at industrial scales require improvements in submerged fermentation. Fungal species produce low-quality enzymes with lower activities in submerged fermentations. New and innovative approaches are being developed to improve the activity of such enzymes under submerged fermentation methods. Some highly cited examples are innovative feedstocks, nutrient or culture optimization, innovative bioreactor designs, such as the use of biofilms, microparticle-enhanced fermentation modes, the use of thermophilic fungi, and genetic engineering.

7. Future Perspectives

Future studies should also focus on analyzing the potential of such approaches individually and assessing the combined effect of different approaches. For example, the choice and concentrations of microparticles should be assessed with a specific feedstock (e.g., DDGS) alongside media and culture optimization strategies. Similarly, genetically modified producers can be used to assess the effect of a particular feedstock, or a microbial strain can be genetically tuned to use a particular feedstock. All such research trends will surely provide new ways to reduce the excessive utilization of cellulases on industrial scales.

Author Contributions

AD and DC designed the research study. MN and AI performed the research. AD and DC provided help and advice on review. MN and AI analyzed the data. MN and AI wrote the manuscript. AD and DC finalized the results and conclusions. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This work was supported in-part by the USDA National Institute of Food and Agriculture and Hatch Appropriations under Project #PEN04850 with Accession #7005668.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Chapman, J, Ismail, AE, Dinu, CZ. Industrial applications of enzymes: Recent advances, techniques, and outlooks. Catalysts. 2018; 8: 238.

[2] Wu X, Luo N, Xie S, Zhang H, Zhang Q, Wang F, *et al*. Photocatalytic transformations of lignocellulosic biomass into chemicals. Chemical Society Reviews. 2020; 49: 6198–6223.

[3] Cai J, He Y, Yu X, Banks SW, Yang Y, Zhang X, *et al*. Review of physicochemical properties and analytical characterization of lignocellulosic biomass. Renewable and Sustainable Energy Reviews. 2017; 76: 309–322.

[4] Iram A, Berenjian A, Demirci A. A Review on the Utilization of Lignin as a Fermentation Substrate to Produce Lignin-Modifying Enzymes and Other Value-Added Products. Molecules. 2021; 26: 2960

[5] Iram A, Cekmecelioglu D, Demirci A. Ideal Feedstock and Fermentation Process Improvements for the Production of Lignocellulolytic Enzymes. Processes. 2021; 9: 38.

[6] Iram A, Cekmecelioglu D, Demirci A. Screening of bacterial and fungal strains for cellulase and xylanase production using distillers' dried grains with solubles (DDGS) as the main feedstock. Biomass Conversion and Biorefinery. 2021; 11: 1955–1964.

[7] Singh A, Bajar S, Devi A, Pant D. An overview on the recent developments in fungal cellulase production and their industrial applications. Bioresource Technology Reports. 2021; 14: 100652.

[8] Dashtban M, Schraft H, Syed TA, Qin W. Fungal biodegradation and enzymatic modification of lignin. International Journal of Biochemistry and Molecular Biology. 2010; 1: 36–50.

[9] Iram A, Cekmecelioglu D, Demirci A. Comparison of common carbon sources with unhydrolyzed, dilute acid and steam hydrolyzed distillers' dried grains with solubles for lignocellulolytic enzyme productions by fungal strains. Fuel. 2023; 340: 127572.

[10] Iram A, Cekmecelioglu D, Demirci A. Salt and nitrogen amendment and optimization for cellulase and xylanase production using dilute acid hydrolysate of distillers' dried grains with solubles (DDGS) as the feedstock. Bioprocess and Biosystems Engineering. 2022; 45: 527–540.

[11] Iram A, Ozcan A, Turhan I, Demirci A. Production of Value-Added Products as Food Ingredients via Microbial Fermentation. Processes. 2023, 11: 1715.

[12] Iram A, Cekmecelioglu D, Demirci A. The Effects of Dilution, Aeration, and Agitation on Fungal Cellulase and Xylanase Production in the Fermentation Media Based on Distillers Dried Grains with Solubles Using Stirred Tank Bioreactors. Industrial Biotechnology. 2023; 19: 229–236.

[13] Iram A, Cekmecelioglu D, Demirci A. Optimization of the fermentation parameters to maximize the production of cellulases and xylanases using DDGS as the main feedstock in stirred tank bioreactors. Biocatalysis and Agricultural Biotechnology. 2022; 45: 102514.

[14] Iram A, Cekmecelioglu D, Demirci A. Distillers' dried grains with solubles (DDGS) and its potential as fermentation feedstock. Applied Microbiology and Biotechnology. 2020; 104: 6115–6128.

[15] Iram A, Özcan A, Yatmaz E, Turhan I, Demirci A. Effect of Microparticles on Fungal Fermentation for Fermentation-Based Product Productions. Processes. 2022; 10: 2681.

[16] Singh V, Raheja Y, Basotra N, Sharma G, Tsang A, Chadha BS. CRISPR/Cas9 Mediated Gene Editing of Transcription Factor ACE1 for Enhanced Cellulase Production in Thermophilic Fungus Rasamsonia Emersonii. Fungal Biology and Biotechnology. 2023; 10: 1–14.

[17] Aro E. From first generation biofuels to advanced solar biofuels. Ambio. 2016; 45: 24–31.

[18] Wi SG, Cho EJ, Lee D, Lee SJ, Lee YJ, Bae H. Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. Biotechnology for Biofuels. 2015; 8: 228.

[19] Feng X, Yao Y, Xu N, Jia H, Li X, Zhao J, *et al*. Pretreatment Affects Profits From Xylanase During Enzymatic Saccharification of Corn Stover Through Changing the Interaction Between Lignin and Xylanase Protein. Frontiers in Microbiology. 2021; 12: 754593.

[20] Song H, Gao Y, Yang Y, Xiao W, Liu S, Xia W, *et al*. Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates. Bioresource Technology. 2016; 219: 710–715.

[21] Deng W, Feng Y, Fu J, Guo H, Guo Y, Han B, *et al*. Catalytic conversion of lignocellulosic biomass into chemicals and fuels. Green Energy & Environment. 2023; 8: 10–114.

[22] Alabdalall AH, Almutari AA, Aldakeel SA, Albarrag AM, Aldakheel LA, Alsoufi MH, *et al*. Bioethanol Production from Lignocellulosic Biomass Using Aspergillus Niger and Aspergillus Flavus Hydrolysis Enzymes through Immobilized S. Cerevisiae. Energies. 2023; 16: 823.

[23] Wisniak, J. Anselme Payen. Educación Química. 2018 16: 568.

[24] Costa AFS, Almeida FCG, Vinhas GM, Sarubbo LA. Production of Bacterial Cellulose by Gluconacetobacter Hansenii Using Corn Steep Liquor as Nutrient Sources. Frontiers in Microbiology. 2017; 8: 282716.

[25] Kraan S. Algal polysaccharides, novel applications and outlook. Carbohydrates-comprehensive studies on glycobiology and glycotechnology. IntechOpen. 2012.

[26] Patel AK, Pandey A, Singhania RR. Production of Celluloytic Enzymes for Lignocellulosic Biomass Hydrolysis. Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels. 2019; 66: 401–426.

[27] Dhiman TR, Zaman MS, Gimenez RR, Walters JL, Treacher R. Performance of dairy cows fed forage treated with fibrolytic enzymes prior to feeding. Animal Feed Science and Technology. 2002; 101: 115–125.

[28] de Carvalho LMJ, de Castro IM, da Silva CAB. A study of retention of sugars in the process of clarification of pineapple juice (Ananas comosus, L. Merril) by micro- and ultra-filtration. Journal of Food Engineering. 2008; 87: 447–454.

[29] Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, Madhavan A, *et al*. Applications of Microbial Enzymes in Food Industry. Food Technology and Biotechnology. 2018; 56: 16–30.

[30] Businesswire.com. (n.d.). Top 5 Vendors in the Global Food Enzymes Market from 2016 to 2020: Technavio | Business Wire. Available at: https://www.businesswire.com/news/home/20160929005423/en/Top-5-Vendors-Global-Food-Enzymes-Market (Accessed: 3 September 2023).

[31] Singh S, Singh VK, Aamir M, Dubey MK, Patel JS, Upadhyay RS, *et al*. Cellulase in Pulp and Paper Industry. New and Future Developments in Microbial Biotechnology and Bioengineering. 2016; 99: 152–162.

[32] Chakraborty D, Shelvapulle S, Reddy KR, Kulkarni RV, Puttaiahgowda YM, Naveen S, *et al*. Integration of biological pretreatment methods for increased energy recovery from paper and pulp biosludge. Journal of Microbiological Methods. 2019; 160: 93–100.

[33] Sreenath HK, Shah AB, Yang VW, Gharia MM, Jeffries TW. Enzymatic polishing of jute/cotton blended fabrics. Journal of Fermentation and Bioengineering. 1996; 81: 18–20.

[34] Heikinheimo L, Buchert J, Miettinen-Oinonen A, Suominen P. Treating Denim Fabrics with Trichoderma Reesei Cellulases. Textile Research Journal. 2000; 70: 969–973.

[35] Jayasekara S, Ratnayake R. Microbial Cellulases: An Overview

and Applications. Cellulose. 2019; 22: 92.
[36] Ranjan R, Rai R, Bhatt SB, Dhar P. Technological road map of Cellulase: a comprehensive outlook to structural, computational, and industrial applications. Biochemical Engineering Journal. 2023; 198: 109020.
[37] MarketsandMarkets. Technical Enzymes Market. 2022. Available at: https://www.marketsandmarkets.com/Market-Reports/enzyme-market-46202020.html?gad_source=1&gclid=CjwKCAiAg9urBhB_EiwAgw88mRLRFefnzZVbjleE7GJNS5ta5LX56MMOTgXIv4bh7mVSIBH4dCz7GBoCSEYQAvD_BwE (Accessed: 1 September 2023).
[38] Rodrigues VJ, Odaneth AA. Industrial application of cellulases. Current Status and Future Scope of Microbial Cellulases. 2021; 8: 189–209.
[39] Novozymes. (n.d.). The Novozymes Report 2022. 2022. Available at: https://investors.novozymes.com/investors/financial-reports/annual-reports/default.aspx (Accessed: 3 September 2023).
[40] Mendes FB, Ibraim Pires Atala D, Thoméo JC. Is cellulase production by solid-state fermentation economically attractive for the second generation ethanol production? Renewable Energy. 2017; 114: 525–533.
[41] Champreda V, Mhuantong W, Lekakarn H, Bunterngsook B, Kanokratana P, Zhao X, *et al*. Designing cellulolytic enzyme systems for biorefinery: from nature to application. Journal of Bioscience and Bioengineering. 2019; 128: 637–654.
[42] Singhania RR, Sukumaran RK, Pandey A. Improved Cellulase Production by Trichoderma reesei RUT C30 under SSF through Process Optimization. Applied Biochemistry and Biotechnology. 2007; 142: 60–70.
[43] Verma N, Bansal MC, Kumar V. Pea Peel Waste: A Lignocellulosic Waste and Its Utility in Cellulase Production by Trichoderma Reesei under Solid-State Cultivation. Bioresources. 2011; 6: 1505–1519.
[44] Emanuela Barbiroglio. (2019, September 10). Land use puts huge pressure on Earth's resources. Here's what needs to change. 2019. Available at: https://ec.europa.eu/research-and-innovation/en/horizon-magazine/land-use-puts-huge-pressure-earths-resources-heres-what-needs-change (Accessed: 10 September 2019).
[45] Patel S. Harmful and beneficial aspects of Parthenium hysterophorus: an update. 3 Biotech. 2011; 1: 1–9.
[46] Catalán E, Komilis D, Sánchez A. Environmental impact of cellulase production from coffee husks by solid-state fermentation: a life-cycle assessment. Journal of Cleaner Production. 2019; 233: 954–962.
[47] Taiwo AE, Tom-James A, Falowo OA, Okoji A, Adeyi O, Olalere AO, *et al*. Techno-economic analysis of Cellulase Production by Trichoderma reesei in Submerged Fermentation Processes using a Process Simulator. South African Journal of Chemical Engineering. 2022; 42: 98–105.
[48] Kavanagh K. Fungal Fermentation Systems and Products. Biology and Applications. 2011; 125–146.
[49] Yoon LW, Ang TN, Ngoh GC, Chua ASM. Fungal solid-state fermentation and various methods of enhancement in cellulase production. Biomass and Bioenergy. 2014; 67: 319–338.
[50] Singhania RR, Ruiz HA, Awasthi MK, Dong C, Chen C, Patel AK. Challenges in cellulase bioprocess for biofuel applications. Renewable and Sustainable Energy Reviews. 2021; 151: 111622.
[51] Hemansi, Chakraborty S, Yadav G, Saini JK, Kuhad RC. Comparative Study of Cellulase Production Using Submerged and Solid-State Fermentation. New and Future Developments in Microbial Biotechnology and Bioengineering. 2019; 60: 99–113.
[52] Singh A, Bajar S, Devi A, Bishnoi NR. Evaluation of cellulase production from Aspergillus niger and Aspergillus heteromorphus under submerged and solid-state fermentation. Environmental Sustainability. 2021; 4: 437–442.
[53] Santos GB, de Sousa Francisco Filho Á, Rêgo da Silva Rodrigues J, Rodrigues de Souza R. Cellulase production by Aspergillus niger using urban lignocellulosic waste as substrate: Evaluation of different cultivation strategies. Journal of Environmental Management. 2022; 305: 114431.
[54] Wang L, Lin X, Zhou Y, Chen H. Porous inert material as promising carrier enhanced cellulase production from Trichoderma reesei in solid-state fermentation. Process Biochemistry. 2022; 122: 316–322.
[55] Kumar Ramamoorthy N, Sambavi TR, Renganathan S. A study on cellulase production from a mixture of lignocellulosic wastes. Process Biochemistry. 2019; 83: 148–158.
[56] Catalán E, Sánchez A. Solid-State Fermentation (SSF) versus Submerged Fermentation (SmF) for the Recovery of Cellulases from Coffee Husks: A Life Cycle Assessment (LCA) Based Comparison. Energies. 2020; 13; 2685.
[57] Patel PS, Desai RG. Study of Cellulase by Isolated Fungal Culture from Natural Resources and Application in Bio-Ethanol Production. International Journal of Applied Science and Technology. 2019; 7: 2277–2285.
[58] Kim S, Kim CH. Production of cellulase enzymes during the solid-state fermentation of empty palm fruit bunch fiber. Bioprocess and Biosystems Engineering. 2012; 35: 61–67.
[59] Durand A. Bioreactor designs for solid state fermentation. Biochemical Engineering Journal. 2003; 13: 113–125.
[60] Gamarra NN, Villena GK, Gutiérrez-Correa M. Cellulase production by Aspergillus niger in biofilm, solid-state, and submerged fermentations. Applied Microbiology and Biotechnology. 2010; 87: 545–551.
[61] Suto M, Tomita F. Induction and catabolite repression mechanisms of cellulase in fungi. Journal of Bioscience and Bioengineering. 2001; 92: 305–311.
[62] Korsa G, Konwarh R, Masi C, Ayele A, Haile S. Microbial Cellulase Production and Its Potential Application for Textile Industries. Annals of Microbiology. 2023; 73: 13.
[63] Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme and Microbial Technology. 2010; 46: 541–549.
[64] Gibbs PA, Seviour RJ, Schmid F. Growth of Filamentous Fungi in Submerged Culture: Problems and Possible Solutions. Critical Reviews in Biotechnology. 2000; 20: 17–48.
[65] Iram A, Cekmecelioglu D, Demirci A. Integrating 1G with 2G Bioethanol Production by Using Distillers' Dried Grains with Solubles (DDGS) as the Feedstock for Lignocellulolytic Enzyme Production. Fermentation. 2022; 8: 705.
[66] Kang S. Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass. Bioresource Technology. 2004; 91: 153–156.
[67] Martău GA, Unger P, Schneider R, Venus J, Vodnar DC, López-Gómez JP. Integration of Solid State and Submerged Fermentations for the Valorization of Organic Municipal Solid Waste. Journal of Fungi. 2021; 7: 766.
[68] Ben Taher I, Bennour H, Fickers P, Hassouna M. Valorization of Potato Peels Residues on Cellulase Production Using a Mixed Culture of Aspergillus niger ATCC 16404 and Trichoderma reesei DSMZ 970. Waste and Biomass Valorization. 2017; 8: 183–192.
[69] Anita S, Namita S, Bishnoi NR. Production of Cellulases by Aspergillus Heteromorphus from Wheat Straw under Submerged Fermentation. International Journal of Environmental Science and Engineering. 2009; 1: 23–26.
[70] Bentil JA, Thygesen A, Lange L, Mensah M, Meyer AS. Green

seaweeds (Ulva fasciata sp.) as nitrogen source for fungal cellulase production. World Journal of Microbiology and Biotechnology. 2019; 35: 82.
[71] Camassola M, Dillon AJP. Biological pretreatment of sugar cane bagasse for the production of cellulases and xylanases by Penicillium echinulatum. Industrial Crops and Products. 2009; 29: 642–647.
[72] Namnuch N, Thammasittirong A, Thammasittirong SNR. Lignocellulose Hydrolytic Enzymes Production by Aspergillus Flavus KUB2 Using Submerged Fermentation of Sugarcane Bagasse Waste. Mycology. 2021; 12: 119–127.
[73] Cunha FM, Kreke T, Badino AC, Farinas CS, Ximenes E, Ladisch MR. Liquefaction of sugarcane bagasse for enzyme production. Bioresource Technology. 2014; 172: 249–252.
[74] Sirohi R, Singh A, Tarafdar A, Shahi NC, Verma AK, Kushwaha A. Cellulase Production from Pre-treated Pea Hulls Using Trichoderma reesei under Submerged Fermentation. Waste and Biomass Valorization. 2019; 10: 2651–2659.
[75] Mrudula S, Murugammal R. Production of Cellulase by Aspergillus Niger under Submerged and Solid State Fermentation Using Coir Waste as a Substrate. Brazilian Journal of Microbiology. 2011; 42: 1119–1127.
[76] Iram A, Cekmecelioglu D, Demirci A. Optimization of dilute sulfuric acid, aqueous ammonia, and steam explosion as the pretreatments steps for distillers' dried grains with solubles as a potential fermentation feedstock. Bioresource Technology. 2019; 282: 475–481.
[77] Abd Elrsoul R, Bakhiet SEA. Optimization of Factors Influencing Cellulase Production by Some Indigenous Isolated Fungal Species. Jordan Journal of Biological Sciences. 2018; 11: 31–36.
[78] Xie H, Wu B, Liu G, Li X. Optimization of in situ cellulase production from Penicillium oxalicum P-07 under submerged fermentation conditions with different cellulose types. Journal of Environmental Chemical Engineering. 2023; 11: 110290.
[79] Xiros C, Studer MH. A Multispecies Fungal Biofilm Approach to Enhance the Celluloyltic Efficiency of Membrane Reactors for Consolidated Bioprocessing of Plant Biomass. Frontiers in Microbiology. 2017; 8: 1930.
[80] Ikram-ul-Haq MMJ, Khan S, Siddiq Z. Cotton Saccharifying Activity of Cellulases Produced by Co-Culture of Aspergillus Niger and Trichoderma Viride. Research Journal of Agriculture and Biological Sciences. 2005; 1: 241–245.
[81] Pérez-García F, Max Risse J, Friehs K, Wendisch VF. Fermentative production of L-pipecolic acid from glucose and alternative carbon sources. Biotechnology Journal. 2017; 12: 1600646.
[82] Paulino TP, Cardoso M, Bruschi-Thedei GCM, Ciancaglini P, Thedei G. Fermentable and non-fermentable sugars: a simple experiment of anaerobic metabolism. Biochemistry and Molecular Biology Education. 2003; 31: 180–184.
[83] Ferreira Rosa PR, Santos SC, Silva EL. Different ratios of carbon sources in the fermentation of cheese whey and glucose as substrates for hydrogen and ethanol production in continuous reactors. International Journal of Hydrogen Energy. 2014; 39: 1288–1296.
[84] Jorge JMP, Pérez-García F, Wendisch VF. A new metabolic route for the fermentative production of 5-aminovalerate from glucose and alternative carbon sources. Bioresource Technology. 2017; 245: 1701–1709.
[85] Mores S, Vandenberghe LPDS, Magalhães Júnior AI, de Carvalho JC, de Mello AFM, Pandey A, *et al*. Citric acid bioproduction and downstream processing: Status, opportunities, and challenges. Bioresource Technology. 2021; 320: 124426.
[86] Liming X, Xueliang S. High-yield cellulase production by Trichoderma reesei ZU-02 on corn cob residue. Bioresource Technology. 2004; 91: 259–262.
[87] Thomareis AS, Dimitreli G. Techniques used for processed cheese characterization. Processed Cheese Science and Technology. 2022; 11: 295–349.
[88] Acharya PB, Acharya DK, Modi HA. Optimization for Cellulase Production by Aspergillus Niger Using Saw Dust as Substrate. African Journal of Biotechnology. 2008; 7: 4147–4152.
[89] Ghori MI, Ahmed S, Malana MA, Jamil A. Corn Stover-Enhanced Cellulase Production by Aspergillus Niger NRRL 567. African Journal of Biotechnology. 2011; 10: 5878–5886.
[90] Fadzilah K, Mashitah MD. Cellulases Production in Palm Oil Mill Effluent: Effect of Aeration and Agitation. Journal of Applied Sciences. 2010; 10: 3307–3312.
[91] Buffo MM, Corrêa LJ, Esperança MN, Cruz AJG, Farinas CS, Badino AC. Influence of dual-impeller type and configuration on oxygen transfer, power consumption, and shear rate in a stirred tank bioreactor. Biochemical Engineering Journal. 2016; 114: 130–139.
[92] Yang Y, Xia J, Li J, Chu J, Li L, Wang Y, *et al*. A novel impeller configuration to improve fungal physiology performance and energy conservation for cephalosporin C production. Journal of Biotechnology. 2012; 161: 250–256.
[93] Bansal P, Hall M, Realff MJ, Lee JH, Bommarius AS. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnology Advances. 2009; 27: 833–848.
[94] Paulraj Gundupalli M, Sahithi S T A, Cheng Y, Tantayotai P, Sriariyanun M. Differential effects of inorganic salts on cellulase kinetics in enzymatic saccharification of cellulose and lignocellulosic biomass. Bioprocess and Biosystems Engineering. 2021; 44: 2331–2344.
[95] Sasikumar E, Viruthagiri T. Optimization of Process Conditions Using Response Surface Methodology (RSM) for Ethanol Production from Pretreated Sugarcane Bagasse: Kinetics and Modeling. BioEnergy Research. 2008; 1: 239–247.
[96] Peláez RDR, Wischral D, Cunha JRB, Mendes TD, Pacheco TF, Siqueira FGd, *et al*. Production of Enzymatic Extract with High Cellulolytic and Oxidative Activities by Co-Culture of Trichoderma Reesei and Panus Lecomtei. Fermentation. 2022; 8: 522.
[97] Arvin E, Harremoës P. Concepts and Models for Biofilm Reactor Performance. Water Science and Technology. 1990; 22: 171–192.
[98] Coban HB, Demirci A, Turhan I. Microparticle-enhanced Aspergillus ficuum phytase production and evaluation of fungal morphology in submerged fermentation. Bioprocess and Biosystems Engineering. 2015; 38: 1075–1080.
[99] Coban HB, Demirci A. Enhancement and modeling of microparticle-added Rhizopus oryzae lactic acid production. Bioprocess and Biosystems Engineering. 2016; 39: 323–330.
[100] Iram A, Cekmecelioglu D, Demirci A. Microparticle-enhanced lignocellulolytic enzyme production using DDGS as the main fermentation feedstock. In ASABE Annual International Meeting. American Society of Agricultural and Biological Engineers. 2023.
[101] Peterson R, Nevalainen H. Trichoderma reesei RUT-C30 – thirty years of strain improvement. Microbiology. 2012; 158: 58–68.
[102] Driouch H, Hänsch R, Wucherpfennig T, Krull R, Wittmann C. Improved enzyme production by bio-pellets of Aspergillus niger: targeted morphology engineering using titanate microparticles. Biotechnology and Bioengineering. 2012; 109: 462–471.
[103] Matsu-ura T, Baek M, Kwon J, Hong C. Efficient gene editing in Neurospora crassa with CRISPR technology. Fungal Biology and Biotechnology. 2015; 2: 4.
[104] Jiang B, Zhang R, Feng D, Wang F, Liu K, Jiang Y, *et al*. A Teton and Cre-loxP Based Genetic Engineering System for Convenient Recycling of Selection Markers in Penicillium oxalicum. Frontiers in Microbiology. 2016; 7: 485.

[105] Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in Trichoderma reesei by heterologous expression of a beta-glucosidase gene from Penicillium decumbens. Enzyme and Microbial Technology. 2011; 49: 366–371.
[106] Li Y, Zhang X, Xiong L, Mehmood MA, Zhao X, Bai F. On-site cellulase production and efficient saccharification of corn stover employing cbh2 overexpressing Trichoderma reesei with novel induction system. Bioresource Technology. 2017; 238: 643–649.
[107] Zou G, Shi S, Jiang Y, van den Brink J, de Vries RP, Chen L, *et al*. Construction of a cellulase hyper-expression system in Trichoderma reesei by promoter and enzyme engineering. Microbial Cell Factories. 2012; 11: 21.
[108] Gruber F, Visser J, Kubicek CP, de Graaff LH. The development of a heterologous transformation system for the cellulolytic fungus Trichoderma reesei based on a pyrG-negative mutant strain. Current Genetics. 1990; 18: 71–76.
[109] Gao L, Gao F, Wang L, Geng C, Chi L, Zhao J, *et al*. N-Glycoform Diversity of Cellobiohydrolase i from Penicillium decumbens and Synergism of Nonhydrolytic Glycoform in Cellulose Degradation. Journal of Biological Chemistry. 2012; 287: 15906–15915.
[110] Beri RK, Turner G. Transformation of Penicillium chrysogenum using the Aspergillus nidulans amdS gene as a dominant selective marker. Current Genetics. 1987; 11: 639–641.
[111] Benocci T, Aguilar-Pontes MV, Zhou M, Seiboth B, de Vries RP. Regulators of plant biomass degradation in ascomycetous fungi. Biotechnology for Biofuels. 2017; 10: 152.
[112] Gupta VK, Steindorff AS, de Paula RG, Silva-Rocha R, Mach-Aigner AR, Mach RL, *et al*. The Post-genomic Era of Trichoderma reesei: what's next? Trends in Biotechnology. 2016; 34: 970–982.
[113] Wang S, Liu G, Wang J, Yu J, Huang B, Xing M. Enhancing cellulase production in Trichoderma reesei RUT C30 through combined manipulation of activating and repressing genes. Journal of Industrial Microbiology & Biotechnology. 2013; 40: 633–641.
[114] Yu L, Chao Y, Wensel P, Chen S. Hydrodynamic and kinetic study of cellulase production by Trichoderma reesei with pellet morphology. Biotechnology and Bioengineering. 2012; 109: 1755–1768.
[115] Novy V, Schmid M, Eibinger M, Petrasek Z, Nidetzky B. The micromorphology of Trichoderma reesei analyzed in cultivations on lactose and solid lignocellulosic substrate, and its relationship with cellulase production. Biotechnology for Biofuels. 2016; 9: 169.
[116] Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, *et al*. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nature Biotechnology. 2016; 34: 184–191.
[117] Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science. 2014; 346: 1258096.
[118] Fuller KK, Chen S, Loros JJ, Dunlap JC. Development of the CRISPR/Cas9 System for Targeted Gene Disruption in Aspergillus fumigatus. Eukaryotic Cell. 2015; 14: 1073–1080.
[119] Pohl C, Kiel JA, Driessen AJ, Bovenberg RA, Nygård Y. CRISPR/Cas9 Based Genome Editing of Penicillium chrysogenum. ACS Synthetic Biology. 2016; 5: 754–764.