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From Sugarcane Bagasse to Second-Generation Bioethanol: Integrating Alkaline Pretreatment, Trichoderma reesei Saccharification, and Saccharomyces cerevisiae–Scheffersomyces stipitis Co-Fermentation

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Abstract

The transition toward renewable fuels has increased interest in second-generation bioethanol obtained from lignocellulosic residues. Sugarcane bagasse is particularly attractive because it is produced in large quantities by the sugar industry and contains substantial fractions of cellulose and hemicellulose that can potentially be converted into fermentable sugars. Its utilization, however, is complicated by the highly organized association of cellulose, hemicellulose, and lignin, which restricts enzymatic accessibility and makes pretreatment necessary. Alkaline treatment offers a practical strategy for bagasse because it promotes fiber swelling, disruption of lignin–carbohydrate associations, and partial delignification while preserving much of the cellulose fraction. The carbohydrates exposed after pretreatment must then be converted into soluble sugars through enzymatic saccharification. Trichoderma reesei is an established producer of extracellular cellulolytic and hemicellulolytic enzymes and therefore represents a suitable biological source of enzymes for this stage. Subsequent fermentation presents another difficulty because conventional Saccharomyces cerevisiae efficiently ferments glucose but does not normally utilize xylose effectively, whereas Scheffersomyces stipitis possesses a native xylose-fermenting metabolism. Their combination therefore provides a possible division of metabolic labor between hexose and pentose fermentation. Nevertheless, differences in oxygen requirements, temperature optima, inhibitor tolerance, and growth kinetics make an uncontrolled three-organism culture biologically problematic. This review evaluates a staged process involving alkaline pretreatment, T. reesei-derived enzymatic saccharification, and controlled S. cerevisiae–S. stipitis co-fermentation. Particular attention is given to pretreatment selectivity, inhibitor formation, enzyme composition, mixed-sugar metabolism, oxygen transfer, population stability, process monitoring, ethanol recovery, scale-up, and techno-economic relevance. The available evidence supports the individual functional roles of the three microorganisms and the feasibility of mixed-yeast fermentation, but direct experimental proof of superiority of the complete three-organism sugarcane-bagasse process remains limited. Consequently, the system should be approached as a scientifically testable bioprocess strategy rather than as an already optimized technology.

Keywords: *sugarcane bagasse; second-generation bioethanol; lignocellulosic biomass; alkaline pretreatment; Trichoderma reesei; cellulase; Saccharomyces cerevisiae; Scheffersomyces stipitis; xylose fermentation; co-fermentation; microbial consortium..*

1. Introduction

The increasing requirement for sustainable transportation fuels and the need to reduce dependence on fossil resources have intensified interest in renewable biofuels. Bioethanol is among the most established alternatives and can be produced from carbohydrate-rich biomass. While first-generation ethanol primarily relies on readily fermentable sugars or starch, second-generation bioethanol utilizes structural carbohydrates present in lignocellulosic residues, possibly increasing fuel recovery without directly depending on food-associated feedstocks [1]. Sugarcane-processing industries are particularly suitable for this approach because existing mills generate large quantities of lignocellulosic residues and already possess infrastructure for biomass handling, fermentation, steam generation and distillation [2] [3].

Sugarcane bagasse, the fibrous residue remaining after juice extraction, consists mainly of cellulose, hemicellulose and lignin and represents an important

feedstock for second-generation ethanol production [1]. Cellulose provides glucose after hydrolysis, whereas the xylan-rich hemicellulose fraction represents an important source of xylose [4]. However, these carbohydrates are embedded within a recalcitrant plant cell wall structure in which cellulose crystallinity, hemicellulose shielding, and lignin restrict enzymatic accessibility [5] [6]. Effective conversion therefore requires pretreatment before enzymatic saccharification. Pretreatment modifies lignocellulosic structure to improve enzyme accessibility while ideally preserving fermentable carbohydrates and minimizing inhibitor formation [7]. Mechanical, acidic, hydrothermal, steam-explosion, alkaline, and organosolv approaches have been investigated, although their effectiveness varies with feedstock composition and operating severity [8] [9]. For sugarcane bagasse, alkaline pretreatment is particularly attractive because it promotes fiber swelling, disrupts lignin-associated linkages, and partially removes lignin, thereby improving enzymatic accessibility [10] [11]. Nevertheless, pretreatment conditions must be carefully controlled because excessive processing can cause carbohydrate losses and generate compounds that interfere with subsequent fermentation [12] [13].

Following pretreatment, exposed structural carbohydrates must be converted into fermentable sugars. *Trichoderma reesei* is one of the most extensively studied cellulase-producing fungi and possesses a specialized extracellular enzyme system for lignocellulose degradation [14]. Its cellulolytic system includes endoglucanases, cellobiohydrolases, and β-glucosidases that act cooperatively to convert cellulose into glucose, while xylanases and accessory enzymes contribute to hemicellulose hydrolysis [15]. The organism has therefore been extensively developed for industrial enzyme production, including strains with enhanced cellulase secretion [16]. However, enzyme composition remains important because inadequate β-glucosidase or hemicellulase activity can restrict complete carbohydrate conversion [15].

Efficient fermentation of the resulting hydrolysate requires utilization of both glucose and xylose. *Saccharomyces cerevisiae* is highly efficient in glucose fermentation and possesses desirable industrial characteristics, including rapid ethanol production and comparatively high ethanol tolerance, but conventional strains do not efficiently ferment xylose [17]. In contrast, *Scheffersomyces stipitis* is naturally capable of xylose fermentation and can therefore complement the glucose-fermenting capacity of *S. cerevisiae* [18]. This metabolic complementarity provides a rationale for co-fermentation, although its effectiveness depends strongly on inoculum ratio, oxygen availability, substrate composition, and hydrolysate toxicity [19].

Integrating these biological functions offers a logical pathway for more complete utilization of sugarcane-bagasse carbohydrates: sugarcane bagasse → alkaline pretreatment → *T. reesei*-mediated saccharification → glucose- and xylose-rich hydrolysate → *S. cerevisiae–S. stipitis* co-fermentation → ethanol. However, the organisms have different physiological requirements, making a staged process more realistic than unrestricted simultaneous cultivation.

Accordingly, this review critically examines the integration of alkaline pretreatment, *T. reesei*-derived enzymatic saccharification, and *S. cerevisiae–S. stipitis* co-fermentation, with particular emphasis on carbohydrate recovery, enzyme efficiency, mixed-sugar utilization, oxygen control, inhibitor tolerance, and overall process feasibility.

2. Review Approach and Scope

This article presents a critical narrative review integrating current evidence on sugarcane bagasse composition, pretreatment, *Trichoderma reesei*-mediated enzymatic saccharification, and glucose–xylose fermentation by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*. Evidence obtained directly from sugarcane bagasse is prioritized, while studies involving other lignocellulosic substrates or synthetic sugar mixtures are used primarily to support mechanistic understanding and process interpretation. Particular consideration is given to strain-specific differences, since enzyme production and fermentation performance can vary considerably among microbial strains. The review also emphasizes standardized evaluation of biomass composition, enzyme activity, individual sugar concentrations, inhibitor formation, and ethanol production to enable meaningful comparison among pretreatment, saccharification, and fermentation strategies. Collectively, these considerations provide the basis for critically assessing the feasibility of integrating alkaline pretreatment, fungal saccharification, and mixed-yeast fermentation for second-generation bioethanol production.

3. Sugarcane Bagasse as a Lignocellulosic Feedstock

Sugarcane bagasse is a heterogeneous lignocellulosic material composed primarily of cellulose, hemicellulose, and lignin, together with smaller amounts of extractives, ash, and residual soluble compounds. Cellulose consists of β-1,4-linked glucose units organized into fibrillar structures, while the hemicellulose fraction of bagasse is rich in xylan and therefore represents an important potential source of xylose [6] [7]. Consequently, both cellulose-derived glucose and hemicellulose-derived xylose contribute to the total fermentable-carbohydrate potential of bagasse. However, its composition varies with cultivar, harvesting, milling, storage, and analytical conditions; therefore, the actual biomass used experimentally should be characterized rather than relying solely on literature values.

Efficient utilization of these carbohydrates is restricted by biomass recalcitrance. Cellulose crystallinity, limited surface accessibility, hemicellulose coverage, lignification, and interactions among cell-wall components restrict enzyme penetration and hydrolysis [7] [30]. Enzymatic conversion is further influenced by enzyme adsorption, accessible cellulose surface, chain-end availability and product inhibition [31]. Residual lignin can additionally reduce saccharification through non-productive adsorption of cellulases, xylanases and β-glucosidases [32]. These structural limitations explain why untreated bagasse generally requires pretreatment before efficient enzymatic hydrolysis.

Importantly, changes in biomass composition after pretreatment should be interpreted using component-specific mass balances.

An increase in the percentage of cellulose in the recovered solid may result from removal of lignin and hemicellulose rather than increased cellulose recovery. Similarly, hemicellulose solubilization can transfer potentially fermentable xylose into pretreatment and washing streams. Therefore, carbohydrate recovery should ideally be followed throughout the sequence raw bagasse → pretreatment fractions → enzymatic hydrolysate → fermentation products and residual sugars, allowing improvements in saccharification and ethanol production to be evaluated against the carbohydrate originally present in the feedstock.

4. Pretreatment of Sugarcane Bagasse

Pretreatment is a critical step in lignocellulosic bioethanol production because it reduces biomass recalcitrance and improves enzymatic accessibility while ideally preserving fermentable carbohydrates [8] [9]. An effective pretreatment should enhance subsequent saccharification, minimize carbohydrate degradation and inhibitor formation, and limit excessive chemical, energy, and water requirements [10] [11] [12] [13]. Several approaches, including dilute-acid, liquid-hot-water, steam-explosion, organosolv, and biological pretreatments, have been investigated for lignocellulosic biomass [8] [10]. Although these methods can effectively disrupt biomass structure, their application may involve carbohydrate degradation, inhibitor formation, high energy requirements, or additional chemical-recovery costs [14] [16] [17].

For sugarcane bagasse, alkaline pretreatment is particularly relevant because sodium hydroxide promotes fiber swelling, disrupts lignin-associated ester linkages, and partially solubilizes lignin, thereby increasing accessibility of cellulose and hemicellulose to hydrolytic enzymes [4] [9] [15]. Its effectiveness depends on NaOH concentration, temperature, residence time, particle size, solids loading, and liquid-to-solid ratio. These variables should therefore be optimized collectively, preferably using factorial or response-surface approaches, with recoverable glucose and xylose as major performance indicators rather than lignin removal alone. Importantly, an apparent increase in cellulose percentage after treatment may result from removal of other biomass components and should not be interpreted as complete cellulose recovery without an appropriate mass balance.

Pretreatment can also influence downstream fermentation through the formation or release of inhibitory compounds. Furfural and HMF may arise from degradation of pentoses and hexoses, respectively, while acetic acid can originate from acetylated hemicellulose and phenolic compounds from lignin disruption [17] [18] [19]. Their combined presence can inhibit yeast growth and reduce ethanol productivity. Although alkaline processing generally produces a different inhibitor profile from severe acidic treatments, it should not be considered inhibitor-free. Washing or detoxification may reduce inhibitory effects but can also cause fermentable-sugar losses and increase water and processing requirements [20] [21]. Therefore, alkaline pretreatment should ultimately be optimized according to carbohydrate recovery, enzymatic digestibility, inhibitor burden, and downstream ethanol production, rather than structural delignification alone.

5. Fungal Saccharification Using *Trichoderma reesei*

Following pretreatment, enzymatic saccharification is required to convert the exposed structural carbohydrates of sugarcane bagasse into fermentable sugars. *Trichoderma reesei* is one of the most extensively studied fungi for this purpose because of its high extracellular protein-secretion capacity and ability to produce a broad range of carbohydrate-degrading enzymes [22]. Continuous strain improvement has further enhanced cellulase productivity, making *T. reesei* an important organism for both commercial enzyme production and lignocellulosic biorefineries [23].

Cellulose degradation by *T. reesei* depends on the cooperative activity of several enzymes rather than a single cellulase. Endoglucanases hydrolyze internal β-1,4-glycosidic bonds within accessible cellulose regions and generate new chain ends [24]. Cellobiohydrolases subsequently act on these chains and progressively release cellobiose, while β-glucosidases convert cellobiose and short cello-oligosaccharides into fermentable glucose [28]. The coordinated action of these enzymes is essential because accumulation of cellobiose can inhibit cellulase activity and restrict complete cellulose conversion [30]. Native *T. reesei* preparations may therefore require β-glucosidase supplementation when this activity is insufficient relative to other cellulase components [31]. In addition, xylanases and accessory enzymes facilitate degradation of the hemicellulose surrounding cellulose fibers and contribute to the release of xylose [29]. This is particularly relevant to the proposed process because hemicellulose-derived xylose can subsequently serve as a substrate for *Scheffersomyces stipitis*. Pretreated sugarcane bagasse can also be employed as a relatively inexpensive carbon source and inducer for *T. reesei* enzyme production. However, cellulase production is strongly influenced by fungal strain, substrate characteristics, nutrient composition, initial pH, cultivation temperature, aeration, agitation, and fermentation time [25]. Enzyme production may increase during active fungal growth and subsequently decline because of nutrient depletion, changes in culture conditions, or extracellular enzyme degradation [26]. Consequently, the optimum harvesting period should be determined experimentally rather than selecting a fixed cultivation time. On-site cellulase production using lignocellulosic substrates has been investigated as a strategy for reducing dependence on externally manufactured enzyme preparations, although additional requirements for aeration, cultivation, and process control must also be considered [27].

Evaluation of the crude enzyme preparation should be based on functional activity rather than fungal biomass or culture-supernatant volume. Filter-paper activity provides a standardized estimate of overall cellulase activity and remains widely used for characterization of cellulolytic preparations [33]. Endoglucanase, β-glucosidase, and xylanase activities should also be determined separately because a high total cellulase value does not necessarily indicate an optimally balanced enzyme cocktail. Reducing-sugar assays using the dinitrosalicylic acid method can provide convenient preliminary measurements of hydrolytic activity [34]. However, these assays do not distinguish individual sugars; therefore, chromatographic determination of glucose, xylose, and cellobiose is preferable for evaluating actual carbohydrate conversion.

Enzymatic saccharification with fungal cellulases is generally favored under moderately acidic conditions, commonly near pH 4.8, and at temperatures around 45–50°C, although optimum conditions depend on the particular enzyme preparation and substrate [28]. Enzyme adsorption, cellulose accessibility, lignin interactions, and product inhibition can substantially influence hydrolysis efficiency [30]. Increasing solids loading can increase the concentration of fermentable sugars and potentially improve final ethanol titer, but excessive solids may also increase viscosity, impair mixing and limit mass transfer. Enzyme dosage should therefore be expressed according to catalytic activity, preferably as FPU per gram of glucan, rather than simply as the volume of crude enzyme added.

The temperature requirements of enzymatic saccharification are also higher than those normally preferred by *S. cerevisiae* and *S. stipitis*. This physiological difference supports the use of a staged process in which enzymatic hydrolysis is completed or substantially advanced before yeast fermentation. Structural carbohydrates should be quantified before and after pretreatment using standardized analytical procedures so that glucan and xylan recovery can be calculated [35]. The resulting liquid hydrolysate should similarly be analyzed for individual sugars and relevant degradation products [36]. Ultimately, the effectiveness of the *T. reesei* stage should be judged by the recovery of fermentable glucose and xylose from the carbohydrates originally present in sugarcane bagasse, providing a direct link between fungal saccharification and subsequent mixed-sugar ethanol fermentation.

6. Fermentation of Glucose and Xylose

Efficient fermentation of lignocellulosic hydrolysates requires utilization of both cellulose-derived glucose and hemicellulose-derived xylose. *Saccharomyces cerevisiae* remains the principal organism used for industrial ethanol production because of its rapid glucose fermentation, comparatively high ethanol tolerance, and ability to perform under acidic fermentation conditions. However, conventional *S. cerevisiae* strains do not possess an efficient native pathway for xylose fermentation, which can leave a substantial fraction of the pentose sugars in lignocellulosic hydrolysates unutilized [43]. Although metabolic engineering has enabled *S. cerevisiae* strains to ferment xylose through introduced xylose-reductase/xylitol-dehydrogenase or xylose-isomerase pathways, their performance can still be affected by transport limitations, redox imbalance, and competition between glucose and xylose metabolism [41]. Considerable strain-engineering efforts have improved these characteristics [42], but such performance should not be assumed for conventional industrial or baker's yeast strains.

Scheffersomyces stipitis, formerly known as *Pichia stipitis*, provides a complementary function because it naturally metabolizes xylose and can convert it into ethanol [37]. This capability is particularly relevant for sugarcane bagasse, where hydrolysis of the xylan-rich hemicellulose fraction can release substantial quantities of xylose. Nevertheless, ethanol production by *S. stipitis* is highly dependent on environmental conditions, particularly oxygen availability [38].

Excessive aeration can favor respiratory metabolism and biomass formation rather than ethanol production, whereas severe oxygen limitation may restrict growth and efficient xylose utilization [39]. Thus, controlled oxygen-limited or microaerobic conditions are generally more appropriate than simply classifying the fermentation as aerobic or anaerobic. Oxygen transfer should therefore be carefully controlled during experimental optimization.

Mixed-sugar fermentation presents an additional challenge because glucose is generally utilized preferentially, while substantial xylose consumption may occur later in the fermentation. This sequential pattern can prolong fermentation and reduce overall productivity. The complementary metabolic capabilities of *S. cerevisiae* and *S. stipitis* therefore provide the rationale for their use as a co-culture: *S. cerevisiae* can rapidly convert glucose into ethanol, while *S. stipitis* can contribute to utilization of the remaining xylose. However, this combination should be regarded as a functional association rather than an automatically synergistic microbial consortium.

Co-culture performance depends strongly on the relative abundance of the two yeasts. An excessive proportion of *S. cerevisiae* may promote rapid glucose depletion but leave insufficient *S. stipitis* biomass for subsequent xylose utilization. Conversely, a very high proportion of *S. stipitis* may alter oxygen demand and reduce the initial rate of glucose fermentation. Dynamic modeling has demonstrated that inoculum ratio, sugar composition, and oxygen availability interact strongly in *S. cerevisiae*–*S. stipitis* fermentations [45]. Model-based studies have similarly indicated that optimization of the relative yeast populations can improve mixed-sugar conversion and ethanol productivity [46]. However, the optimum ratio is strain- and process-dependent and should therefore be determined experimentally rather than adopted directly from another study.

Experimental investigations using lignocellulosic hydrolysates support the feasibility of this co-fermentation strategy. Co-cultivation of *S. cerevisiae* and *S. stipitis* has been reported to improve utilization of glucose–xylose mixtures compared with glucose-fermenting yeast alone under appropriate conditions [47]. Strategies such as cell recycling can further maintain metabolically active yeast populations and improve fermentation productivity [48]. High-cell-density cultivation of *S. stipitis* has also been explored to overcome its comparatively slower xylose utilization [49], while immobilization represents another approach for retaining sufficient microbial biomass during repeated mixed-sugar fermentation [50]. These approaches demonstrate the potential of mixed-yeast systems but also introduce additional process complexity.

The chemical composition of the hydrolysate must also be considered because lignocellulosic fermentation occurs in the presence of compounds other than fermentable sugars. Organic acids, furans, phenolics, and salts generated or released during pretreatment can reduce yeast growth and ethanol productivity. *S. cerevisiae* generally possesses considerable industrial robustness, whereas *S. stipitis* can be more sensitive to hydrolysate composition and changes in cultivation conditions [52].

Consequently, successful co-fermentation requires simultaneous optimization of sugar concentration, inoculum ratio, nutrient availability, oxygen transfer, pH, and inhibitor tolerance. Current developments in lignocellulosic ethanol production similarly emphasize that efficient mixed-sugar metabolism must be combined with resistance to the chemical stresses encountered in real biomass hydrolysates [53].

Therefore, the proposed *S. cerevisiae–S. stipitis* co-culture should not be considered superior solely because the two organisms possess complementary metabolic pathways. Its advantage must be demonstrated experimentally against the corresponding monocultures under equivalent conditions. Improvements should be evaluated through combined glucose and xylose consumption, ethanol titer, ethanol yield, volumetric productivity, and residual sugar concentration. Such comparisons will establish whether inclusion of *S. stipitis* provides a genuine improvement in carbohydrate recovery and ethanol production from sugarcane-bagasse hydrolysate.

7. Integration of the Three Microbial Functions

The proposed combination of *Trichoderma reesei*, *Saccharomyces cerevisiae*, and *Scheffersomyces stipitis* is based on complementary biological functions: *T. reesei* provides cellulolytic enzymes for carbohydrate release, *S. cerevisiae* efficiently converts glucose to ethanol, and *S. stipitis* extends fermentation toward hemicellulose-derived xylose. However, metabolic complementarity does not necessarily imply that all three microorganisms can perform optimally when cultivated simultaneously. Their different requirements for oxygen, temperature, and substrate availability represent major barriers to direct three-species fermentation.

Active growth and cellulase production by *T. reesei* require aerobic conditions, whereas ethanol production by the yeasts generally benefits from restricted oxygen availability. This incompatibility is particularly important for *S. stipitis*, in which oxygen availability strongly influences the distribution of carbon between biomass formation, respiration, and ethanol production. Temperature requirements create an additional conflict because *T. reesei*-derived cellulases generally exhibit strong hydrolytic activity at temperatures around 45–50°C, whereas conventional *S. cerevisiae* and *S. stipitis* fermentations are commonly performed closer to 30°C. Maintaining a single temperature would therefore require a compromise between enzyme activity and yeast performance. Moreover, metabolically active *T. reesei* could consume part of the soluble sugars released during hydrolysis, reducing the amount of carbohydrate available for ethanol formation.

These physiological differences support a staged processing strategy in which fungal enzyme production, enzymatic saccharification, and yeast fermentation are separated. A practical sequence would involve aerobic *T. reesei* cultivation → recovery of cell-free cellulolytic enzymes → enzymatic hydrolysis of pretreated bagasse → adjustment of hydrolysate conditions → controlled *S. cerevisiae–S. stipitis* co-fermentation. Such separation allows each biological function to operate under more favorable conditions and enables the performance of individual stages to be evaluated independently.

Separate hydrolysis and fermentation (SHF) is particularly suitable for initial proof-of-concept studies because enzymatic hydrolysis can be conducted near the preferred temperature and pH of the cellulase preparation before the hydrolysate is cooled and adjusted for yeast fermentation. Its major limitation is the accumulation of glucose during saccharification, which can contribute to product inhibition of cellulases. Nevertheless, the ability to independently optimize and diagnose hydrolysis and fermentation makes SHF valuable for establishing the feasibility of the proposed microbial system.

Greater process integration can subsequently be explored. Simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis and glucose fermentation, allowing released glucose to be rapidly consumed and thereby reducing cellulase product inhibition [54]. Simultaneous saccharification and co-fermentation (SSCF) extends this strategy toward combined C6 and C5 sugar utilization. However, both approaches require compromises in temperature, enzyme activity, yeast physiology, and oxygen transfer. Consolidated bioprocessing (CBP) represents the highest degree of integration by combining enzyme production, biomass hydrolysis, and fermentation within a single biological system [57]. Although substantial progress has been achieved in strain engineering and CBP development [58], simultaneously obtaining high cellulase production, efficient polysaccharide hydrolysis, robust mixed-sugar fermentation, and high ethanol tolerance remains challenging.

Process integration becomes even more difficult at high substrate concentrations. High-gravity fermentation is desirable because increased sugar concentrations can produce higher ethanol titres and potentially reduce downstream distillation requirements [55]. However, increasing biomass solids also raises viscosity, restricts mixing and mass transfer, and can reduce enzymatic conversion [56]. Limited water availability and accumulation of hydrolysis products further influence conversion at high solids [59]. Improved cellulolytic enzyme preparations can partly enhance performance, but they do not completely eliminate these physical limitations [60].

Therefore, a staged SHF-based configuration is the most appropriate starting strategy for the proposed three-microorganism system. It permits independent optimization of *T. reesei* enzyme production, bagasse saccharification, and *S. cerevisiae–S. stipitis* fermentation before attempting more complex SSF, SSCF, or consolidated configurations. Process consolidation should subsequently be considered only when it provides measurable improvements in overall carbohydrate utilization, ethanol yield, productivity, or process economics.

8. Process Monitoring and Performance Criteria

Accurate process monitoring is essential for determining whether pretreatment, saccharification, and fermentation genuinely improve the conversion of sugarcane bagasse into ethanol. Visible biomass degradation, reducing-sugar release, or detection of ethanol alone cannot demonstrate efficient overall conversion.

The concentrations of major carbohydrates, particularly glucose, xylose, and cellobiose, should therefore be monitored throughout enzymatic hydrolysis and fermentation. The DNS assay provides a convenient method for preliminary measurement of reducing sugars [34], but it does not distinguish individual carbohydrates and may be affected by the complex composition of lignocellulosic hydrolysates. High-performance liquid chromatography (HPLC) is therefore preferable for the definitive quantification of glucose, xylose, arabinose, and cellobiose, together with relevant fermentation products and pretreatment-derived compounds [36].

Analysis of the solid biomass is equally important. Raw and pretreated bagasse should be characterized for glucan, xylan, lignin, ash and extractives using standardized compositional procedures [35]. These measurements allow changes produced by pretreatment to be interpreted through actual component recovery rather than percentage composition alone. Monitoring both solid and liquid fractions also permits carbohydrate losses during pretreatment, washing and hydrolysis to be incorporated into the overall process mass balance.

Fermentation performance should be assessed using multiple parameters rather than ethanol concentration alone. Initial and residual glucose and xylose concentrations provide direct evidence of C6 and C5 sugar utilization, while ethanol titre indicates the final product concentration. Ethanol yield should be expressed relative to the amount of fermentable sugar consumed, and volumetric productivity should describe the rate of ethanol formation. The theoretical ethanol yield from glucose is approximately 0.511 g ethanol per g glucose, providing a useful basis for estimating fermentation efficiency [52]. Similar stoichiometric considerations can be applied when evaluating xylose conversion [53]. Monitoring xylitol is particularly useful in *S. stipitis*-containing fermentations because diversion of xylose toward xylitol can reduce ethanol recovery, while glycerol can provide additional information on cellular stress and redox metabolism.

Population dynamics should also be considered during *S. cerevisiae–S. stipitis* co-fermentation. Total optical density cannot distinguish the contribution of each yeast, and the initial inoculum ratio may change substantially during fermentation. Species-specific monitoring through differential viable counting, molecular methods or another validated discriminatory approach is therefore preferable when available. Measuring population composition at several fermentation stages can help determine whether poor xylose conversion results from metabolic limitations or progressive loss of the *S. stipitis* population.

Overall process performance should ultimately integrate carbohydrate recovery, glucose and xylose utilization, ethanol titre, ethanol yield, volumetric productivity, fermentation efficiency and ethanol recovery per unit of initial dry bagasse. This is important because excellent performance at one stage does not necessarily translate into a high overall process yield. For example, extensive pretreatment may improve saccharification while simultaneously causing carbohydrate losses, whereas high sugar consumption during fermentation may be accompanied by increased biomass or by-product formation.

Ethanol recovery and product quality represent the final analytical considerations. Distillation can effectively concentrate ethanol from fermentation broth, but conventional ethanol–water distillation alone cannot readily produce anhydrous ethanol because of azeotropic limitations. Industrial dehydration therefore commonly requires additional separation technologies, including molecular-sieve-based processes [65]. The energy demand of ethanol recovery is strongly influenced by fermentation titre, providing an additional reason to maximize ethanol concentration while maintaining high conversion efficiency.

Finally, ethanol identity and quality should be established analytically rather than inferred from odour, flammability or boiling behaviour. ASTM D4806 specifies requirements relevant to denatured fuel ethanol intended for gasoline blending [67], while ASTM D5501 describes gas-chromatographic determination of ethanol and methanol content in fuel ethanol [68]. Accordingly, experimentally produced ethanol should be described as bioethanol or recovered ethanol unless its composition and relevant quality parameters have been demonstrated to meet an applicable fuel specification.

9. Techno-Economic and Environmental Considerations

The industrial feasibility of second-generation bioethanol depends not only on efficient microbial conversion but on the performance of the entire production system. Sugarcane bagasse offers an important advantage because it is generated within existing sugar mills, where biomass handling, steam and electricity generation, fermentation and distillation infrastructure may already be available. Integration of second-generation ethanol production with conventional sugarcane processing can therefore reduce some infrastructure and utility requirements compared with completely stand-alone facilities [61]. Techno-economic assessments further demonstrate that carbohydrate recovery, process configuration, energy integration and co-product utilization strongly influence the economic performance of bagasse-based ethanol production [62].

Pretreatment and enzymatic saccharification represent particularly important cost-sensitive stages. Chemical consumption, heating, neutralization and extensive washing can increase operating costs and generate wastewater requiring further treatment. Enzyme expenditure is another major concern because high cellulase loading can substantially affect the cost of lignocellulosic ethanol [66]. On-site production of *T. reesei* enzymes using bagasse or other low-cost substrates may reduce dependence on externally produced commercial enzymes, but additional requirements for fungal cultivation, aeration and process control must be included when assessing its economic benefit. Likewise, increasing glucose and xylose recovery is valuable only when the additional ethanol produced compensates for the associated enzyme, chemical, energy and processing requirements.

Bagasse should also not be considered a zero-value waste material. Sugar mills commonly use it as a fuel for cogeneration of process steam and electricity; consequently, diverting bagasse toward ethanol production may reduce the amount available for energy generation or electricity export [61].

Process evaluation should therefore consider the relative value of ethanol production, electricity generation and other possible bagasse-derived products rather than assigning the feedstock no opportunity cost. Comprehensive process-design studies similarly demonstrate that feedstock handling, pretreatment, enzymes, fermentation, product recovery, utilities and wastewater treatment collectively determine the economics of lignocellulosic ethanol [64].

Environmental benefits must likewise be assessed across the complete life cycle. Greenhouse-gas performance can be influenced by agricultural inputs, chemical and water consumption, process-energy requirements, electricity generation, product recovery and allocation of environmental burdens among co-products [63]. A process that produces a higher ethanol yield at laboratory scale may therefore not necessarily provide superior environmental performance if it requires substantially greater chemical, water or energy inputs. Recovery and reuse of water and alkali, energy integration and productive utilization of lignin-rich residues could improve the environmental and economic performance of an integrated biorefinery.

Ultimately, commercial viability requires simultaneous optimization of ethanol yield, pentose utilization, enzyme demand, chemical consumption, water use, energy requirements, waste generation and co-product recovery. Laboratory improvements in saccharification or fermentation should therefore be interpreted within this broader process context. Commercial experience with lignocellulosic biofuels demonstrates that achieving efficient biological conversion is essential but does not by itself overcome the economic and engineering challenges associated with large-scale production [69].

10. Proposed Experimental Framework

The proposed experimental framework should evaluate a staged microbial strategy involving *Trichoderma reesei*, *Saccharomyces cerevisiae*, and *Scheffersomyces stipitis* for conversion of alkaline-pretreated sugarcane bagasse into bioethanol. The primary objective should be to determine whether combining fungal saccharification with glucose–xylose co-fermentation improves utilization of the carbohydrates originally present in bagasse. Raw bagasse should first be dried, milled to a defined particle size, and characterized for glucan, xylan, lignin, ash, and extractives using standardized biomass-analysis procedures [35]. All calculations should be expressed on a dry-biomass basis to permit reliable comparison of carbohydrate recovery throughout the process.

Alkaline pretreatment should subsequently be optimized by varying major operating parameters such as NaOH concentration, temperature, and residence time. Conditions around 1–3% NaOH, 100–121°C, and 30–90 min can provide an initial experimental range, although these values should be treated as screening conditions rather than universal optima. Alkaline treatment has been shown to modify the lignin-rich structure of sugarcane bagasse and increase enzymatic accessibility [4]. Following pretreatment, the recovered solid and liquid fractions should be retained so that carbohydrate losses are incorporated into the material balance. The optimum treatment should therefore be selected according to recoverable glucose and xylose, enzymatic digestibility, inhibitor burden, and chemical requirements rather than lignin removal alone [15].

Pretreated bagasse can then be employed as a substrate for cellulolytic enzyme production by *T. reesei*. A characterized cellulase-producing strain such as RUT-C30 may provide a suitable reference organism [23]. Fungal cultivation can initially be performed under moderately acidic conditions at approximately 28–30°C with adequate aeration, while enzyme production is monitored over several days. Filter-paper cellulase, endoglucanase, β-glucosidase, and xylanase activities should be measured so that the enzyme preparation is harvested near its maximum functional activity [33]. Fungal biomass should subsequently be removed by filtration or centrifugation before the crude enzyme preparation is used for saccharification.

Enzymatic hydrolysis of pretreated bagasse may initially be performed at approximately pH 4.8 and 50°C, with enzyme dosage standardized as FPU per gram of glucan rather than crude-supernatant volume. Glucose, xylose, and cellobiose should be monitored during hydrolysis to determine the extent and kinetics of carbohydrate conversion. A commercial cellulase preparation at an equivalent activity should be included as a benchmark, together with a no-enzyme control. If substantial cellobiose accumulates, β-glucosidase supplementation can be investigated to determine whether insufficient β-glucosidase activity limits cellulose conversion [24]. Final hydrolysates should preferably be analysed chromatographically for glucose, xylose, cellobiose, and relevant pretreatment-derived inhibitors [36].

For fermentation, *S. cerevisiae* and *S. stipitis* should be cultivated separately and standardized according to viable cell concentration or biomass before inoculation. The hydrolysate should be adjusted to approximately pH 5 and 30°C before yeast addition. Experimental treatments should include *S. cerevisiae* monoculture, *S. stipitis* monoculture, an uninoculated control, and selected co-culture ratios such as 3:1, 1:1, 1:3, and 1:9. The total starting viable biomass should remain equivalent among treatments so that differences cannot be attributed simply to inoculum size. Because the relative abundance of the two yeasts can strongly influence mixed-sugar conversion, inoculum ratio should be treated as an experimental variable rather than assuming a universal optimum [45].

Fermentation may initially be monitored for approximately 72 h, with periodic determination of glucose, xylose, ethanol, xylitol, glycerol, and relevant inhibitory compounds. Oxygen availability should be carefully controlled because *S. stipitis* requires an oxygen-limited environment for effective xylose fermentation, while excessive aeration can increase respiratory metabolism [38]. Where bioreactors are available, dissolved oxygen and aeration should be recorded; in shake-flask experiments, working volume, agitation rate, flask size, and closure conditions should be standardized [39]. Species-specific viable counting or molecular monitoring is preferable because total optical density cannot distinguish *S. cerevisiae* from *S. stipitis*.

Appropriate controls are essential for determining the contribution of each process stage. Untreated bagasse subjected to enzymatic hydrolysis should provide a reference for evaluating pretreatment, while pretreated bagasse without enzyme should indicate background sugar release. Commercial cellulase can be used to assess the performance of the crude *T. reesei* enzyme preparation.

Individual yeast monocultures should provide the principal controls for determining whether co-culture genuinely improves mixed-sugar utilization. Fermentation of a synthetic glucose–xylose medium may additionally help distinguish intrinsic yeast limitations from inhibition caused by the bagasse hydrolysate.

Process performance should be assessed using complete mass-balance and fermentation calculations. Solid recovery can be calculated as the dry mass recovered after pretreatment divided by the initial dry bagasse mass and multiplied by 100, while glucan recovery can be determined from the recovered biomass mass and its glucan fraction relative to the glucan initially present. Enzymatic glucan conversion can be calculated as 0.90 × glucose released ÷ initial glucan × 100, and xylan conversion as 0.88 × xylose released ÷ initial xylan × 100. Ethanol yield ($Y_p/_s$) should be calculated as ethanol produced ÷ total glucose and xylose consumed, while fermentation efficiency can be expressed as $Y_p/_s$ ÷ 0.511 × 100. Volumetric ethanol productivity (Q_p) can be determined as (P_t − P_0) ÷ t, and overall ethanol yield should be expressed as ethanol produced ÷ initial dry bagasse mass. These calculations are important because they integrate losses occurring during pretreatment, saccharification, and fermentation rather than evaluating each operation independently [64].

All major experiments should include independent biological replicates and appropriate statistical analysis. Pretreatment optimization may be performed using response-surface methodology or another multifactor experimental design, while fermentation data can be evaluated using factorial or repeated-measures approaches where appropriate. Results should report measures of variability together with statistical significance and effect size. Finally, the predicted optimum should be tested independently to confirm that the observed improvement is reproducible. This framework would enable the proposed three-microorganism strategy to be evaluated on the basis of carbohydrate recovery, mixed-sugar utilization, ethanol yield, productivity, and overall conversion of initial bagasse into ethanol, rather than ethanol production alone.

11. Critical Research Gaps

Despite strong evidence supporting the individual roles of *Trichoderma reesei*, *Saccharomyces cerevisiae*, and *Scheffersomyces stipitis*, direct evidence demonstrating that their complete integration consistently improves ethanol recovery from sugarcane bagasse remains limited. *T. reesei* is well established as a source of cellulolytic enzymes [22], while *S. stipitis* provides native xylose-fermenting capacity [37]. Similarly, experimental studies demonstrate the feasibility of *S. cerevisiae–S. stipitis* co-fermentation [47]. However, these findings establish biological feasibility rather than proving synergy of the complete three-function process. Direct comparison with appropriate monoculture and process controls is therefore required before superiority can be established.

Another important limitation is the difference between synthetic sugar media and actual bagasse hydrolysates. Defined glucose–xylose mixtures are valuable for studying microbial interactions, but real hydrolysates contain variable concentrations of sugars together with organic acids, furans, phenolics, salts, and other pretreatment-derived compounds [17]. These components can interact to inhibit yeast growth and fermentation [19]. Consequently, inoculum ratios and fermentation conditions optimized in synthetic media should be re-evaluated using authentic bagasse hydrolysates.

Oxygen regulation represents a particularly important challenge for *S. stipitis*. Xylose utilization and ethanol formation are highly sensitive to oxygen availability [38], yet terms such as “microaerobic” or “oxygen-limited” are frequently difficult to compare among experimental systems. Flask geometry, working volume, agitation, biomass concentration, and reactor configuration can substantially alter oxygen transfer [39]. Future studies should therefore relate fermentation performance to measurable parameters such as dissolved oxygen, gas-flow rate, or the volumetric oxygen-transfer coefficient (kLa).

Population stability represents another unresolved aspect of mixed-yeast fermentation. The initial *S. cerevisiae–S. stipitis* inoculum ratio may change considerably because the organisms differ in growth rate, sugar preference, oxygen requirement, and inhibitor tolerance. Monitoring only total optical density can therefore conceal progressive dominance by one organism. Species-specific viable counts or molecular methods should be used to determine whether both populations remain functionally active throughout fermentation [48].

The composition of the *T. reesei* enzyme cocktail also requires further optimization. High overall cellulase activity does not necessarily indicate sufficient β-glucosidase or xylanase activity for complete bagasse deconstruction [24]. Cellobiose accumulation may indicate inadequate β-glucosidase activity, whereas poor xylose release may reflect insufficient hemicellulose degradation. Individual enzyme activities should therefore be related directly to glucose, xylose, and cellobiose release rather than relying solely on total cellulase measurements [33].

Finally, most laboratory studies are conducted at relatively low biomass concentrations, whereas industrially relevant ethanol production requires higher solids loading and higher final ethanol titres. Increasing solids concentration introduces viscosity, mixing, mass-transfer, and enzyme-distribution limitations [55]. These constraints become increasingly significant during scale-up [56] and can reduce fractional carbohydrate conversion even when more substrate is present [59]. Future validation should therefore progress from laboratory proof-of-concept experiments toward controlled high-solids and larger-scale systems. Economic and environmental advantages should only be claimed after appropriate mass, energy, chemical, water, and co-product balances have been established [64] [69].

12. Future Perspectives

Future development of bagasse-based bioethanol should focus on improving microbial robustness, enzyme efficiency, process integration, and overall carbon recovery. Adaptive laboratory evolution of *S. stipitis* in progressively more challenging bagasse hydrolysates could potentially improve tolerance to pretreatment-derived inhibitors while retaining xylose-fermenting capacity.

In parallel, metabolic engineering of *S. cerevisiae* offers a longer-term route toward efficient fermentation of both glucose and xylose by a single industrially robust organism [40]. Improvements in xylose transport, pentose-phosphate metabolism, and intracellular redox balance have already demonstrated the potential of this approach [42]. Nevertheless, the two-yeast system remains valuable as an experimentally accessible strategy for exploiting complementary glucose and xylose metabolism without requiring extensive genetic modification.

Improving the stability and density of fermenting populations represents another promising direction. Cell recycling and high-cell-density cultivation can maintain sufficient metabolically active biomass and reduce repeated growth requirements [48]. Immobilization may similarly improve cell retention during repeated fermentation cycles [50]. However, such approaches require long-term evaluation because contamination, declining viability, mass-transfer limitations, and changes in the relative abundance of the two yeasts may reduce performance over repeated cycles.

Future improvement of *T. reesei* should focus on enzyme quality as well as total enzyme titre. A balanced preparation containing adequate cellulase, β-glucosidase, xylanase, and accessory activities would promote more complete recovery of both cellulose- and hemicellulose-derived sugars [24]. Improving extracellular enzyme stability, including through reduction of unwanted proteolytic degradation, may further increase usable enzyme activity [26]. On-site enzyme production using bagasse-derived substrates also remains attractive as a potential strategy for reducing dependence on externally manufactured cellulases [27].

Process development should progress gradually from separate hydrolysis and fermentation toward greater integration. Once individual biological stages have been optimized, temperature-shift fermentation, enzyme-fed simultaneous saccharification and co-fermentation, or other partially consolidated configurations could be investigated [54]. More advanced consolidated bioprocessing may eventually reduce the number of unit operations, although combining enzyme production, saccharification, mixed-sugar fermentation, and ethanol tolerance within a single compatible biological system remains challenging [57] [58].

A broader biorefinery approach may further improve resource utilization by integrating ethanol production with energy generation and productive use of lignin- and hemicellulose-derived streams. Integration with existing sugar mills is particularly attractive because utilities, biomass handling, and distillation infrastructure can potentially be shared [61]. However, valorization of additional streams should be supported by realistic mass balances, product-recovery requirements, market value, and life-cycle assessment rather than assuming that every additional co-product automatically improves sustainability [63].

Finally, advanced process monitoring and modeling could improve control of the proposed microbial system. Online measurement of pH, dissolved oxygen, off-gas composition, sugar concentration, and microbial populations could provide a more detailed understanding of fermentation dynamics than endpoint ethanol measurements alone.

Predictive models incorporating glucose and xylose consumption, viable population changes, and oxygen transfer could eventually support dynamic aeration, feeding, and inoculum-control strategies. Such developments could transform the proposed system from a laboratory proof of concept into a more controlled and scalable platform for maximizing conversion of sugarcane-bagasse carbohydrates into second-generation bioethanol.

13. Conclusion

Sugarcane bagasse is a promising lignocellulosic feedstock for second-generation bioethanol because of its substantial cellulose and hemicellulose content [1]. However, its structural recalcitrance requires effective pretreatment and enzymatic hydrolysis before fermentation [7]. Alkaline pretreatment can improve carbohydrate accessibility through partial delignification and structural disruption [15], while *Trichoderma reesei* provides cellulolytic enzymes that release fermentable sugars from the pretreated biomass [24]. The resulting glucose- and xylose-containing hydrolysate can potentially be fermented using the complementary metabolic capabilities of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*. While *S. cerevisiae* efficiently ferments glucose, *S. stipitis* provides native xylose-fermenting capacity [37]. Co-culture studies support the feasibility of combining these functions, although performance depends strongly on inoculum ratio, oxygen availability, hydrolysate composition, and strain characteristics [45]. Because the three microorganisms have different physiological requirements, a staged process involving *T. reesei* enzyme production → enzymatic saccharification → *S. cerevisiae*–*S. stipitis* co-fermentation represents a practical strategy for further investigation. Its effectiveness should be demonstrated through glucose and xylose utilization, ethanol yield, productivity, and overall ethanol recovery from the initial dry bagasse. Thus, the proposed system provides a scientifically promising approach for improving carbohydrate utilization in bagasse-based bioethanol production, but its technical, economic, and environmental advantages require further experimental validation.

Ethical Considerations

This review article is based exclusively on previously published literature and publicly available scientific information. No experiments involving human participants or animals and no collection of personally identifiable information were undertaken as part of this work. Therefore, institutional ethical approval and informed consent were not required. The authors have endeavoured to maintain research integrity through appropriate attribution, accurate representation of published findings, and responsible synthesis of the available literature.

Conflict of Interest

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Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, the authors used generative artificial intelligence (AI)-assisted tools for language editing and refinement, including improvements in grammar, sentence structure, clarity, readability, and academic presentation. These tools were not used as a substitute for the authors' scientific judgment, critical interpretation, or evaluation of the literature. All AI-assisted text was subsequently reviewed, verified, and edited by the authors. The authors take full responsibility for the accuracy, integrity, originality, appropriate attribution of sources, and final content of the manuscript.

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