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Challenges in cellulase bioprocess for biofuel applications

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ABSTRACT

Increasing population and industrialization caused increased demand for liquid fossil fuels which in turn increases the greenhouse gas emission. Bioethanol produced from lignocellulosic biomass via enzymatic route is a potential alternative to fossil fuels and is environmentally sustainable. Cellulases have been regarded as the limiting factor for bioethanol production from lignocellulosic biomass via enzymes. In the last few decades advances in bioprocesses led to reduction in the cost of cellulases by several folds, enabling bioethanol production to become cost-effective. This is the reason for existence of commercial plants for bioethanol production, however; still there are scope for further improvement in bioprocess for cellulase production and research is ongoing worldwide. Researchers face huge challenge while moving from flask and bioreactor research outcomes from a laboratory scale to the pilot scale production, which has been rarely discussed. This review will present those challenges and its probable solutions. Though commercial cellulases are available, it is highly required to have in-house cellulase production technology to be self-reliant. On-site and integrated cellulase production configuration is popular as it seems to be cost-effective. This review will address advances in bioprocesses and challenges for cellulase production which have surfaced in the last decade.

1. Introduction

Cellulases have been regarded as the crucial factor since decades for the sustainable production of bioethanol via lignocellulosic (LC) biomass. It has been one of the most demanded industrial enzymes due to several versatile applications such as in detergent industry, paper and pulp industry, textile industry, food and beverages industry, biomass to commodities and chemicals industry, etc., however, has gained utmost fame and came in the forefront due to its importance in bioethanol production from LC biomass via enzymatic route. Cellulases are glycosyl hydrolases and are complex enzyme containing majorly three important components as exocellulase/cellobiohydrolase, endoglucanase and beta-glucosidase which acts in a synergistic manner to hydrolyse cellulose completely into its monomer, glucose. Cellulose has been regarded as the most abundant, inexhaustible raw material available on the planet to be utilized by mankind for their benefit. It is a biopolymer where its monomer glucose is linked with β, 1–4 linkage. Its complete degradation gives glucose which can be converted into ethanol via fermentation. Bioethanol production from LC biomass seemed to be a subject of research only, far from reality due to high cost of cellulase and its low titre of production. It has been a limiting factor in lignocellulosic biorefinery; also because of its vast amount which is required for the degradation of lignocellulose accounting for 40–100 folds higher as that required for hydrolysis of starch [1,2]. Still enzyme biorefinery platforms have been the preferred way for biofuel production being a sustainable solution for the environment [3]. In the last few decades due to advances in bioprocesses for cellulase production; cost of cellulase has been reduced with improved titres and properties resulting in commercialization of bioethanol production in various parts of the globe.

Both solid-state fermentation (SSF) and submerged fermentation (SmF) have been employed exclusively for cellulase production at lab scale [4–9]. Though fungi, yeast, bacteria, and actinomycetes are known to produce cellulase; filamentous fungi are the most exploited source for cellulases. SSF depicts closely the natural habitat of these filamentous fungi, due to which these are better adapted, producing higher enzyme titres which may be extracted or may be employed for biomass

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List of abbreviations:

SSF	Solid state fermentation
LC	Lignocellulose
CCR	Carbon Catabolite Repression
BGL	Beta glucosidase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
PCV	Packed cell volume
FTFs	Fusion transcription factors
MESP	Minimum ethanol selling price
FPU mL-1	FPU (activity) per millilitre
FPU L-1	FPU (activity) per litre
SmF	Submerged fermentation
CBP	Consolidated bioprocessing
LPMOs	Lytic polysaccharide monooxygenases
SWO	Swollenin
DO	Dissolved oxygen
DES	Di-ethyl sulphonate
EMS	Ethyl methyl sulphonate
VVM	Volume (L-1 gas) Volume (L-1 Liquid) min-1
FPU	Filter paper unit
FPU L-1 h-1	FPU productivity per litre per hour
U g-1	unit (activity) per gram (substrate)

hydrolysis directly without extraction [7,10]. Research has been done on both types of bioprocess and continuing, however the handling and monitoring at large scale is always a challenge for SSF due to which submerged fermentation enjoys importance for commercial cellulase production [11].

Adsul et al. [12–15] presented improvement in cellulase production from ~25 FPUs $h^{-1}L^{-1}$ to ~100 FPUs $h^{-1}L^{-1}$ by adopting multifaceted approaches such as media engineering (utilizing cheaper carbon sources and removing all the salts from the media strategically), bioprocess optimization and strain improvement via mutation and screening the best one with all cellulase components including BGL [12–15]. Fig. 1 shows various strategies adopted for improving bioprocess of cellulase production. *Penicillium funiculosum* was mutated via UV and Ethyl methyl sulphonate (EMS) and with stringent screening with specified medium, hyper cellulase producing mutants were obtained. *P. funiculosum* MRJ-16 has been obtained after several series of mutations, which produced 3 times higher cellulases than the original parents [12–15]. After media engineering, bioprocess optimization and strain improvement ~100 FPUs h^{-1} productivity have been achieved. Even the wastewater produced after pretreatment could be utilized for media preparation for cellulase production as the inhibitors generated were found to have negligible effect on cellulase production via MRJ-16 or it can be said that the strain was resistant to inhibitors. Cellulase production from shake-flask level to large scale industrial level journey was tough as very limited information is available in public domain about the real large-scale challenges. Great challenges are being faced while transitioning from laboratory to industrial scale during commercial process development as when the system size increases, many properties change nonlinearly related to size [16]. Authors have tried to put their own experience, the challenges they faced during their journey in submerged fermentation from laboratory to pilot and/or industrial scale along with critical analysis of available literature.

2. Bioprocess aspects for cellulase production

Economic feasibility of bioethanol production from LC biomass via enzymatic route depends on efficient bioprocess technologies for large scale cellulase production. The major cost component in the bioethanol production process is contributed by cellulase, which is the main reason for rejuvenated interest among investigators worldwide to further improve the cost-effectiveness of cellulase. Advances in bioprocess have enabled the bioethanol process to become one among sustainable bioeconomies. Multifaceted approach has been adopted for improving titres of cellulase such as employing advanced bioprocess technologies, adopting integrated enzyme production technology, using cheaper raw materials as substrate for enzyme production, strain improvement by mutation/bioengineering, etc., [5,8,17–20]. A significant part of research has addressed the strategies for bioprocess improvement to enhance the yield and specific activities of cellulases as well [6]. Table 1 shows bioprocess strategies adopted for cellulase production by

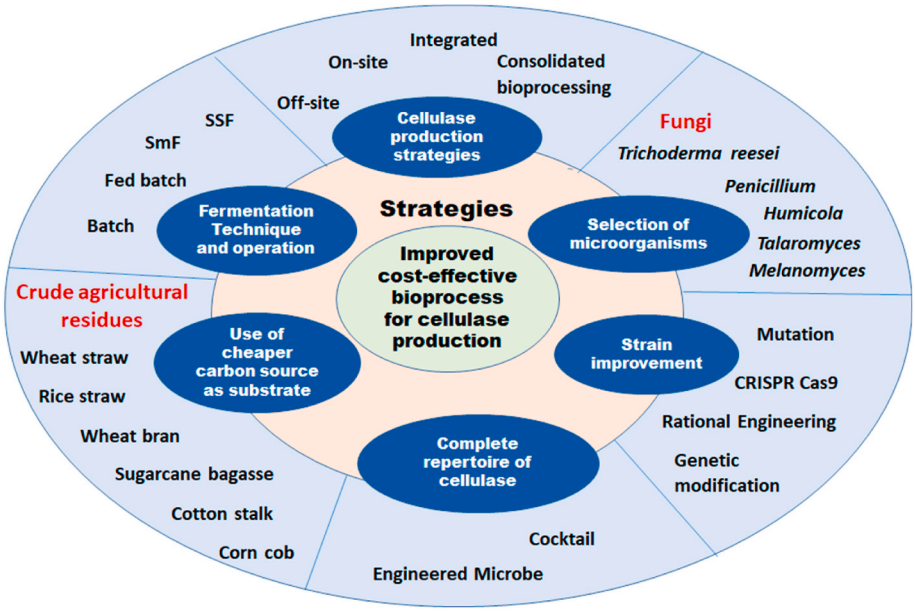

Fig. 1. Overview of the scheme of cost-effective cellulase production for application in 2G bioethanol.

Table 1
Details of bioprocess (SmF) employed for cellulase production in various recent studies.

Bioprocess	Microorganisms	Substrate/medium	Bioprocess parameters	Bioreactor/volume	Enzyme titers (FPase/CMCase/ BGL and/or protein)	Reference
SmF	*Penicillium funiculosum*	Acid pretreated sugarcane baggase 20 g L^{-1}/Mandel & Weber	inoculation: 5–10% (v/v), temperature 30 °C, agitation 200–350 rpm & pH 5.0, Aeration 0.5–1 vvm to attain 20–40% O_2 level	Biostat Braun Biotech International, Germany; working vol. 7–10 L	1.35 FPU, 10.252 CMCase, 2.260 BGL mL^{-1}	[21]
SmF	*Trichoderma harzianum*	Delignified sugarcane bagasse	Working vol. 1.5 L, temperature 29 °C, Aeration to get O_2 level below 30%, pH 5	3.0 L BioFlo 115 Fermenter (New Brunswick Scientific Co., USA)	0.69 FPU, 9.71 CMCase mL^{-1}	[22]
SmF, batch	*Trichoderma harzanium* ATCC® 20846™	Finely powdered surgical wate and cardboard waste (1:1) 10 g L^{-1} in Vogel's medium	28 ° C, aeration 1 vvm-0.5vvm based on foaming, Anchor type impeller used, agitation and DO% set in cascade mode, DO% set to max 100 and min 40, pH 5.5, end time 120h	Bioeng KLF Advanced Bioreactors M/s (3.2 L) Bioeng Switzerland, working vol 1L (800 ml of media & 20% inoculum	1.85 FPU at 120 h 11.75 CMCase 3170 BGL mL^{-1}	[23]
Exponential fed batch	*Trichoderma harzanium* ATCC® 20846™	Finely powdered surgical wate and cardboard waste (1:1) 10 g L^{-1} in Vogel's medium	28 ° C, aeration 1 vvm-0.5vvm based on foaming, Anchor type impeller used, agitation and DO% set in cascade mode, DO% set to max 100 and min 40, pH 5.5, Four feedings were performed after 120 h till the end time 220 h, final broth volume 1.5L	Bioeng KLF Advanced Bioreactors M/s (3.2 L) Bioeng Switzerland, working vol 1L (800 ml of media & 20% inoculum	1.94 FPU at 220 h 12.12 CMCase 2994 BGL mL^{-1}	[23]
Pulse fed batch	*Trichoderma harzanium* ATCC® 20846™	Finely powdered surgical wate and cardboard waste (1:1) 10 L^{-1} in Vogel's medium	28 ° C, aeration 1 vvm-0.5vvm based on foaming, Anchor type impeller used, agitation and DO% set in cascade mode, DO% set to max 100 and min 40, pH 5.5 m, pulse feeding was done	Bioeng KLF Advanced Bioreactors M/s (3.2 L) Bioeng Switzerland, working vol 1L (800 ml of media & 20% inoculum	2.12 FPU at 198 h 13 CMCase 3020 BGL mL^{-1}	[23]
pH stat fed batch	*Trichoderma harzanium* ATCC® 20846™	Finely powdered surgical wate and cardboard waste (1:1) 10 g L^{-1} in Vogel's medium	28 ° C, aeration 1 vvm-0.5vvm, impeller used anchor type, agitation and DO% set in cascade mode, DO% set to max 100 and min 40, pH 5.5, two different acidic and basic feed was provided after negligible C source was left behind till the final volume of broth reached to 1.5 L, end time 196 h	Bioeng KLF Advanced Bioreactors M/s (3.2 L) Bioeng Switzerland, working vol 1L (800 mL of media & 20% inoculum	1.92 FPU at 196 h, 12.38 CMCase, 2993 BGL mL^{-1}	[23]
SmF, Batch fermentation	*T reesei* RUT C-30 DES-15 a mutant by DES mutagen	(Vol. %: Avicel 3.3, CSL 1.7, GL-0.25 in salt solu. with initial pH 5.5)	Batch fermentation, pressure 0.05 Mpa, pH 5.0, temp 26 °C, 30% DO maintained by varying agitation speed and aeration rate	BIOTECH-5BG, Shanghai Baoxing Bio-Engineering Equipment Co. Ltd., China 5L fermenter	11.86 FPU mL^{-1}	[24]
SmF, Fed-batch fermentation with lactose as feeding	*T reesei* RUTC-30	20 L production medium (Vol. g L^{-1}: CL 50, YE 10, LT 225, LBA 1.5); 1L growth medium (Vol. g L^{-1}: Glc 10, CL 50, YE & Salt sol. 10)	20% DO was maintained by 8L min^{-1} flow of air by diffusing through stones. The bioreactor (30L) equipped with control system for pH, aeration, temperature, antifoam, & agitation along with an automated monitoring	35 L Air lift fermenter filled with 20L of production media and 1L of growth media	It is a model-based study	[25]
Batch fermentation	*T reesei* RUTC-30	(Vol. g L^{-1}: Avicel 20.0; LT 10.0; YE & salt solu. 20.0)	2 M H_3PO_4 and 10% ammonia solutions to control pH. 1.0 mL L^{-1} of J647 antifoam was used, aeration 0.7vvm and DO above 20% in an agitation cascade model (400–1000 rpm)	BioFlo/CelliGen 115 systems (Eppendorf, Hamburg, Germany) 3.0 L vessels, water-jacketed, working volume 1.0–1.5 L.	80.6 g L^{-1} (0.24 g L^{-1} h−1)	[26]
Fed-Batch fermentation	*T reesei,* (engineered)	Soybean hulls, sugarcane bagasse and sugarcane molasses	0.7 VVM aeration with compressed air was maintained, pH 4.0–5.0 using 2 M H_3PO_4 and 15% ammonia and DO above 20% in a cascade mode (400–1000 rpm). 1–1.2 L was the initial volume used, inoculated with 1:10 volume of 3–7 days old inoculum	BioFlo/CelliGen 115 system 3.0-L vessels (Eppendorf) and water-jacketed	27 g L^{-1} extracellular protein was produced in 10 days	[27]
Batch fermentation	*Penicillium funiculosum* MRJ-16	Avicel, CSL	36–48 h old 30% PCV mycelial inoculum inoculated	Stirred tank bioreactor 7 L glass jacketed vessel with 5 L working volume	100 FPU $L^{-1}h^{-1}$ cellulase productivity, 9 ± 0.5 FPU mL^{-1}	[14]

Note: CL-cellulose; Glc-glucose, GL-glycerol; LBA-lactobionic acid; YE-yeast extract; LT-lactose; CSL-corn steep liquor.

submerged fermentation.

In nature, the growth and cellulose utilization of filamentous fungi elaborating cellulases resembles solid-state fermentation [28,29]. Prevot et al. [30] compared cellulase production by SmF and SSF, employing *T. reesei* RUT-C30 as producer micro-organisms and wheat bran as the substrate under similar operational conditions. Author declared SSF technique better than SmF owing to superior performance. Singhania et al. [7] presented a strategy of single pot fermentation where cellulase is being produced by fungal strain via SSF and the fermented matter was used for further fermentation to ethanol serving as source of enzyme and the leftover substrate to be utilized for hydrolysis. Though it looks attractive, it was demonstrated successfully only in shake flask level and must be validated in large scale studies. Albeit the challenges of mass transfer in large scale as well as difficulty in automatic monitoring are always associated with SSF [4]. SmF offers several advantages such as online monitoring and sophisticated bioreactors are available permitting easy mass transfer making the handling easy and is the main reason that large commercial facilities employ SmF for cellulase production. SmF also permits the easy acquisition of enzymes secreted extracellularly for downstream bioprocessing [31]. Mostly SmF is employed for large scale cellulase production by filamentous fungi. *T. reesei,* the most exploited microorganism for cellulase has been also verified maximum in SmF. *T. reesei's* ability to grow homogeneously in liquid medium makes it suitable for SmF [9,20]. Sukumaran et al. [17] and Mathew et al. [32] have presented bioprocess development for cellulase production by fungi. Many external factors influence operation in submerged cultivation when fungal culture is involved.

2.1. Fungal morphology

Hyphal morphology of fungi is an important characteristic which is influenced by changes in external factors [33] and plays an important role in protein secretion. An optimal morphology is critical for optimum cellulase production by fungi [34]. Smaller and more branched hyphae are regarded as better for protein secretion. Process of mycelial branching causes more growing tips to emerge which are involved in protein secretion [35]. Increasing the number of active mycelial tips have the potential to improve overall protein yield which may be possible by any external or internal factors [36]. Ahamed and Vermette [37,38] reported a method that could be employed to help improve cellulase activity by manipulating agitation rates along with culture medium composition, the number of hyphae tips and hyphal branching could be controlled. Bach C [39] also studied the effect of agitation on *T. reesei* morphology and tried to establish a relationship between agitation rate, morphology and cellulase production. However, the breakage of mature (secondary) mycelia due to increased agitation also leads to reduced cellulase production [24], thus stir rate and impeller used becomes critical with fungal fermentation. Nevertheless, protein secretion was just double due to increased branching and number of hyphal tips without increase in fungal biomass which is definitely positive as dense biomass causes increased viscosity leading to the poor oxygen transfer. It is generally believed that filamentous fungi primarily secrete protein through young hyphal tips [40], as at this site the cell wall facilitates rapid protein secretion being more porous [41].

Starvation and anaerobic stress results in autophagy causing the growth retardation of the mycelia [42]. Similarly, an increase may be observed in lag phase in case of biomass during fed-batch feeding when the concentration of glucose decreases as it takes time to adapt for the uptake of cellulose as a substrate [42]. The reason behind this is the occurrence of vacuolation and the utilization of the endogenous carbon by the mycelia [43]. Thus, it becomes critical to choose the right time for feeding in a fed batch process.

2.2. Aeration

Aeration is one of the most critical parameters for large scale aerobic industrial bioprocess. Since oxygen is sparingly soluble in water, dissolved oxygen (DO) plays an important role in cultivation of aerobic microorganisms in bioreactor and a continuous supply is needed. The DO concentration in an aerobic bioprocess depends on the oxygen transfer rate from the gas phase to the liquid, on the rate at which oxygen is transported where it is consumed (into the cells), and on the rate of oxygen uptake for growth of the microorganism, production and maintenance as well [44]. The oxygen transfer rate must be known to achieve scale-up and an optimum design operation of bioreactors.

In this aerobic bioprocess with filamentous fungi, oxygen transfer rate is critical and could be the growth limiting factor.

The classical method of aeration for bioreactors is performed by sparging (bubbling) gas through the culture medium. Air or mixed gases are forced through small pores of sparger or its tip with slight over pressure. The smaller the bubbles the larger the surface area which improves oxygen diffusion through the bubble to liquid contact. Usually, 0.5–2 vvm air flow rate is used in stirred tank bioreactors in these bioprocesses [9,11,45]. Bubble column and stirred tank reactors of various types are usually employed for aerobic fermentations. Stirred tank bioreactors usually provide excellent mixing with high values of mass and heat transfer rates, which may be the reason for the first choice for commercial production of cellulases, however the pressure at the bottom of the bioreactor will depend on the liquid filled which may vary from 1 to 3 bar when compared with the top layer of liquid in the fermenter [39]. In addition, impeller design, air flow rate and stir rate together influence aeration. It is critical to maintain DO level during growth of fungi but due to increased viscosity it becomes a challenge and also the increased stir rate may prove detrimental for fungi as it causes breakage of mycelia/hyphae.

Tip velocity of impeller depends on its diameter and its speed, and can be given as,

$$V_{tip} = \pi \times D \times N$$

where D is the diameter of impeller and N is the rotation speed S^{-1}. Hence impeller speed must be decided carefully based on its diameter and velocity at the tip so as not to cause mycelial breakage.

The volumetric oxygen transfer coefficient (kLa) indicates the efficiency of oxygen supply to microorganisms in a bioreactor and is one of the most important parameters in aerobic biotechnological process. The oxygen consumption by the fungi depends on its local environment. Oxygen uptake rate (OUR) is growth associated and can be represented best as;

$$OUR = Y_{xo}.\mu.X, \quad \mu = \frac{\mu_{max}.C_s}{C_s + K_s}$$

Where, Y_{xo} is the yield of oxygen on biomass, μ is the microbial growth rate, X is the biomass concentration, μ_{max} is the maximum growth rate, C_s is the substrate concentration and K_s is the half saturation concentration of the given substrate.

Frothing is another challenge which could be controlled to an extent by the addition of anti-frothing agent, however, aeration and agitation needs to be controlled as well. Increased pressure in the head space could help but then the bioreactor and the microorganism must be capable to bear that. Few researchers have even suggested addition of H_2O_2 in the medium to keep oxygen in high levels [46]. Hydrogen peroxide is a relatively mild oxidizing reagent and by weight, 47% of it is an oxidant which produces oxygen and water as its only by-products [47,48], however the effect of H_2O_2 over the culture must be analysed beforehand. Recently, nanobubble technology appears interesting as nanosized air bubble will remain in the liquid medium until it is utilized by the microorganisms. Due to its small size and volume it will not travel to the surface and get disrupted, rather stay back for utilization [49].

2.3. Biomass estimation

While working on cellulase production via filamentous fungi in bioreactor, biomass estimation becomes a challenge; majorly because the agricultural residues are employed as a substrate due to which the media contains suspended particles from the beginning of the bioprocess itself. It was stated "without doubt, the single most vital yet most problematical value sought during fermentation is biomass estimation" [50]. Deriving an accurate result in biomass determination remains a major challenge in SmF when agricultural residues or insoluble carbon sources are used. Fungi being well-characterised microorganisms are employed widely for cellulase production via SSF or SmF due to their ability to produce high titers of cellulase. Complete recovery of the biomass is not possible due to the compressed structure of agricultural residues and the mycelia which may not allow its separation. Since the use of a direct technique such as the dry weight determination method is impractical the only alternative is to use the indirect techniques of biomass estimation. Many promising indirect estimation techniques are available, such as (i) measuring cell components which are not present in the substrate such as glucosamine content determination; (ii) measuring biomass component commonly available in both substrate and biomass such as total protein and nucleic acid estimation; (iii) measuring other secondary metabolites such as ergosterol determination; (iv) determining metabolic activity; (v) determining images from direct microscopic observation and (vi) estimating biomass from the substrate matrix [51,52]. These are all time-consuming methods. In order to have a quick estimation of fungal biomass during bioprocess, mycelia volume could be estimated by centrifugation at 4000 rpm for 5 min to give packed cell volume (PCV). It could be very well used as a quick indicator of the measurement of fungal biomass available in the bioreactor, though it does not give accurate measurement. Although significant advances have been achieved and various techniques are available; still, the progress remains quite unsatisfactory. The evaluation of microbial growth becomes a challenge being impractical and inaccurate and more laborious as well. Essentially, this remains another critical issue for growth monitoring. The information of the growth profile of fungal culture throughout any bioprocesses constitutes an extremely essential parameter in determination of kinetic variables and thereby subsequently, scale-up of the bioprocess.

Inoculum is another challenge while moving to a large-scale production process. Spore as well as mycelial inoculum could be used. Spores could be counted and hence reproducibility of cellulase activity is higher in this case. It is homogenous and usually 10^6 -10^8 spores per 100 ml is inoculated; however, generating such a huge number of spores is impossible for several thousand litres of production media. Hence mycelial inoculum is preferred for scale-up. Inoculum preparation itself is an independent unit. For inoculating large scale media in a fermenter, 20–30% PCV of mycelia will be sufficient [9]. PCV determination could be used as indication of phases of fungal fermentation too. In course of fermentation usually PCV increases initially, and reaches up to 70 or even 80% PCV, challenging aeration; and DO drops at that point indicating starvation of the culture. During the end of fermentation PCV again decreases indicating death of the culture and dissolution of the mycelia. Hence, PCV could be used as a quick indicator of mycelial biomass estimation.

3. Various approaches for cellulase production

The cost of cellulase production is the major hindrance and reducing the cost is the main key to economic viability of ethanol bioprocess. There are three popular approaches that have been adopted for cellulase production; as offsite, on-site and integrated cellulase production. Usually, the traditional cellulase production technology is offsite which means that cellulase is purchased or produced in far off facilities and needs transportation and formulation with stabilizers for stability due to the need of storage. Glucose and nutrient solutions are used for cellulase production in off-site and on-site configuration. In on-site configuration the production facility of cellulase remains in a nearby ethanol facility which may bypass the transportation as well as formulation need, hence proving to be cost effective as compared to off-site configuration. When the cellulase production facility in on-site configuration uses cheaper pre-treated cellulosic biomass as substrate instead of glucose, which might be similar pre-treated biomass as used for ethanol production, then it becomes an integrated approach. Integrated configuration has been advocated as the most cost-effective configuration for cellulase production among three Johnson et al. [53]. Fig. 2 depicts the various configurations of cellulase production. Along with these three consolidated bioprocessing is also an interesting configuration where all the process is operated in the similar vessel. In a single bioreactor enzyme production, saccharification and ethanol conversion is accomplished by either a single microorganism or by a consortium. Though it looks fascinating, it has its own challenges to be addressed.

3.1. Offsite cellulase production

Off-site configuration is a traditional cellulase production process which is usually situated far off the central ethanol facility or is purchased. Cost of cellulase is higher as it includes the cost of transportation, clarification and stabilizers also. Usually, the purchased enzyme is considered in this configuration. It could have been produced by batch or fed-batch operation strategy.

In SmF, fed batch and batch strategy, both have been employed for cellulase production where fed batch process is considered tricky and limitations that arise during a batch SmF could be avoided while performing fed-batch operations, to a considerable extent. That too pulse feed fed-batch strategy in comparison to the pH stat fed-batch and exponential fed-batch processes, was high yielding and economical. For cellulase production a fed-batch strategy from a mixture of waste cotton-cardboard was claimed to be demonstrated for the first time [23]. Pulse feeding fed batch was found better than batch fermentation and other fed batch strategy in which intermittent pulse feeding was done after 120 h at every 24 h interval. It presents an interesting study however the enzyme titres do not look impressive as *T harzianum* strain is wild type. The operational costs of the process provided in the work and noteworthy features of fed-batch fermentations could serve as incredible data for scale-up of the process [23]. While doing fed batch fermentation, fresh nutrients must be supplied just before it gets depleted in the medium to have continuity of healthy growth of microorganisms. Once ammoniacal nitrogen level reaches below 400ppm–200 ppm, nitrogen source must be supplemented as is required for growth and amino acid synthesis.

3.2. On-site cellulase production

On-site cellulase production presents advantages over the offsite production process as it enables the technology to move towards economic feasibility. Also, on-site configuration is considered environmentally sustainable due to its substantially reduced green-house gases emissions than those employing purchased enzymes [54]. When the cellulase production facility is integrated with the bioethanol process at the site, it is referred to as on-site cellulase production. Enzyme transport also adds to the cost of the technology which could be circumvented by adopting on-site configuration [22,55,56]. This can also reduce the cost by eliminating the need of stabilizers and clarifiers as long-term storage needs can be bypassed, and whole fermentation broth without any extraction could be employed in hydrolysis bypassing downstream processing step [7,57]. However, in such cases enzymes must be stable.

Though there is no major difference between on-site production process and off-site production process of purchased cellulase, but still the on-site production process was found to be more energy efficient. Market prices of commercial enzymes available determines the economic advantage of produced enzymes [53]. Information available is

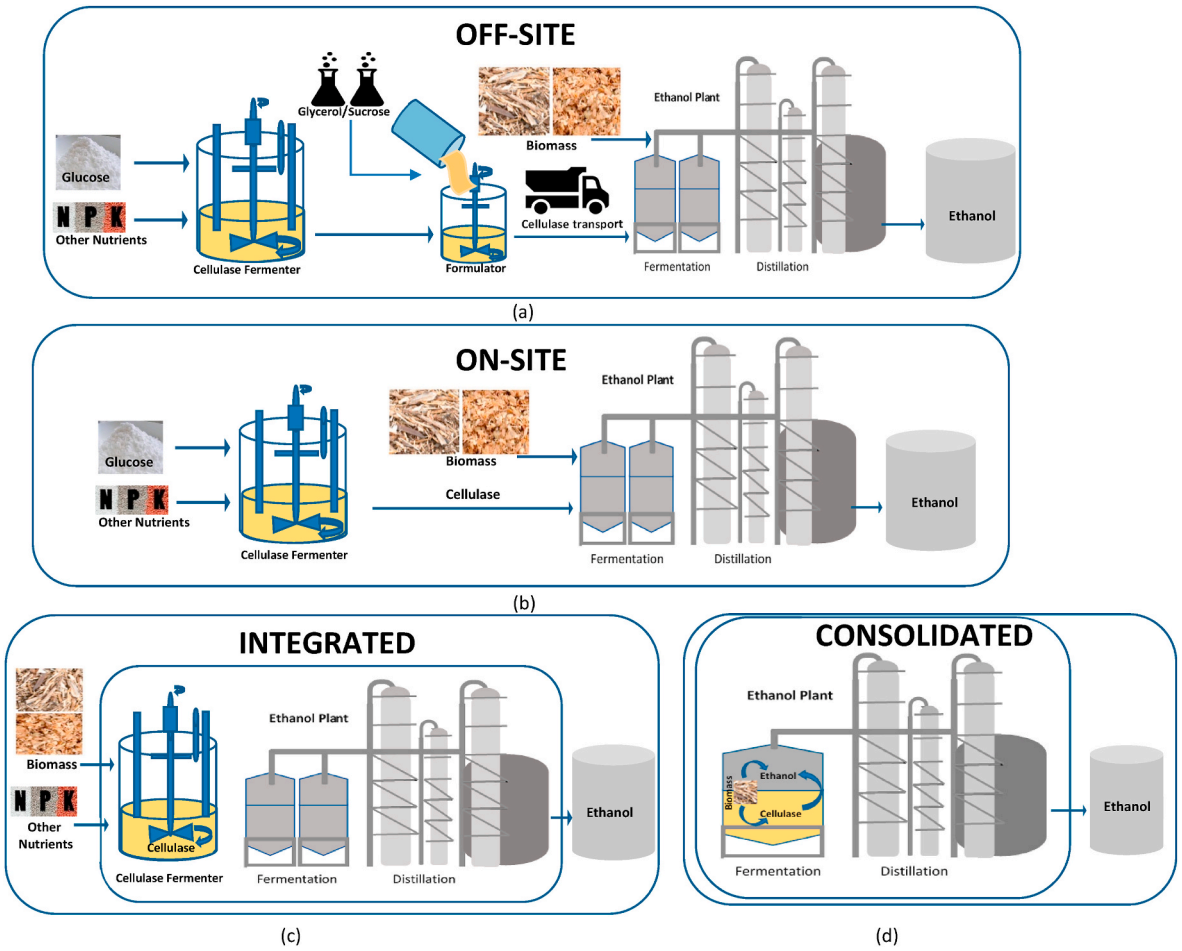

Fig. 2. Different production strategies of commercial of cellulases for application of 2G bioethanol.

scarce on the cost statistics to produce commercial cellulases [1]. Strain improvement may lead to more economic feasibility by developing potent and robust cellulase producers. Hence, on-site cellulase production process must further improve by focusing on strain improvement, specific activity, protein yield, whole process optimization and process parameters such as requirement of oxygen and residence time [58]. A detailed techno-economic model analysis on on-site production process of cellulase located at a corn ethanol mill assumed cost of about \$580 t^{-1} glucose as a carbon source. According to it, the carbon source itself accounted for half of the total cost of enzyme (\$4.24 kg^{-1}) whereas 21% and 13% of the cost was contributed by equipment and the electricity used, respectively [55]. Siqueira et al. [59] have reviewed advances in on-site cellulase production using plant biomass and have presented its current advances for cellulase applications for lignocellulosic biomass to fermentable sugars conversion and finally to bio-ethanol production.

3.3. Integrated cellulase production

Most of the time 'integrated cellulase production' configuration is replaced as 'on-site configuration' employing cheaper pre-treated biomass as substrate which is used for ethanol production. It is often envisioned that lignocellulosic biomass itself, which is an inexpensive raw material, could be used as a carbon source for enzyme production [1,19,22,60–64]. It could be cost-effective and the hydrolytic efficiency of produced enzymes for biomass hydrolyse could also be superior to those enzymes which are produced on different carbon sources [59]. It is an integrated configuration of cellulase production that could significantly reduce the cost of the whole process by using low-cost substrates, avoiding downstream processing, enzyme storage and transportation [65,66]. Integrated configuration increases the value of the product because of the amount of biomass which is diverted from the production stream of bioethanol. Thus, for the cellulosic ethanol process, the minimum ethanol selling price (MESP) would highly depend on the cost of enzyme (\$ kg^{-1}) [67].

3.4. Consolidated bioprocessing

Consolidated bioprocessing seems to be quite promising for efficient ethanol production from lignocellulosic biomass in one bioreactor, where single microorganism or consortium is capable of producing all the cellulolytic enzymes required for complete breakdown of biomass producing sugars and thereby fermenting it into ethanol. This strategy can reduce the cost of bioreactor and the enzyme, which are the major obstructions to low-cost bioprocess [68]. Compared to the traditional ethanol production process from lignocellulosic biomass, 25% of the cost of ethanol produced via CBP can be reduced. A comparative cost analysis was conducted on ethanol production considering capital, utilities, raw materials, and yield loss expenditures which resulted in a projection of \$0.04 gal^{-1} for CBP. Simultaneously, \$0.19 gal^{-1} was projected for saccharification and co-fermentation [69]. It could be a gamechanger bioprocess for ethanol production from biomass in one go.

The concept is well proven but the titres are quite low at the present moment when compared to other cellulase production strategies which has limited it just in the laboratory. Research is going on and bioengineering could be an excellent tool to enable ethanol producing yeast strain to produce cellulolytic enzymes or cellulolytic microorganisms to produce ethanol. However, the main challenges in bioengineering include the adversative impacts of the co-expression of multiple

unwanted genes, the improper folding of proteins which can prevent their secretion, the modulation of the different genes' expression at the inappropriate levels along with inadequate fermentation pathway [68,70].

Hence, for each configuration, focus must be on cellulase producing strain improvement for further reduction of cost [71]. The development of genetically modified systems, redesigned promoters, and the use of native transcription factors or artificial ones in metabolic engineering are recent advances in genetics that are leading to the development of superior enzyme producers.

4. Genetic and metabolic engineering for improving cellulase production capacity of filamentous fungi for biofuel applications

Aerobic microorganisms are known to produce cellulases. Fungi as well as bacteria produce cellulase when cellulose is available as a sole carbon source. Most of the potent cellulase producers are filamentous fungi and industrially exploited strains for cellulase production are *Trichoderma reesei, Penicillium* sp., *Aspergillus niger, Humicola insolens, Talaromyces emersonii, etc. T. reesei RUT C*-30 has been enjoying the top position among best cellulase producers which has continuously undergone improvement since the last 70 years. This strain is available in the public domain for research and has been extensively studied and modified for hyperproduction. Recently, *Penicillium* sp. is also giving tough competition to *Trichoderma* in race [72]. Though *T. reesei* RUT C30 secretes cellobiohydrolase II about 80% of the total protein, its cellulase contains lesser beta-glucosidase and it also exhibits glucose inhibition. Usually, *Aspergillus niger* is used for beta-glucosidase production, however *Penicillium* sp. such as *P. oxalicum* [45], *P. janthinellum* [11], *P. funiculosum* [14] has been found competent to *T. reesei* as produces high titres of cellulases with higher glucose tolerant beta-glucosidase which is a limiting component in *T. reesei*. Bacteria are also known to produce cellulosomes having cellulolytic activity and have gained importance due to several properties such as more penetrating power into the substrate, however its low titre have limited its employment for commercial production.

It has been a challenge to develop a bioprocess employing wild microorganisms, which does not generally produce the complete repertoire of cellulase. Hence, researchers have been engaged in investigating the cellulolytic enzyme production, employing genetically engineered microorganisms. Genetic modifications in microorganisms to improve strain for improved cellulase and β-glucosidase titres as well as properties has been studied exclusively [9,20,73]. Improved strains by genetic modification or mutation are screened which produces higher titres of enzyme with improved properties as compared to its parent. Mutation is a classical approach but has been successfully employed for filamentous fungi for improved production. Raghuwanshi et al. [74] generated mutants of *Trichoderma asperellum* by UV irradiation and the compared for cellulase production. It was found that enzymatic activity was increased for mutants generated in comparison to wild strain from 1.60 to 2.20 U g^{-1} for FPase, from 10.25 to 13.13 U g^{-1} for CMCase, and from 6.32 to 9.20 U g^{-1} for β-glucosidase. Similarly, *Penicillium janthinellum* was mutated and a mutant strain was developed employing ultra violet (UV) light and ethyl methyl sulphonate (EMS) by Adsul et al. [75]. A mutant of *P. janthinellum* named EMS-UV-8, produced 3.2 U mL^{-1} of FPase activity in comparison to 1.5 U mL^{-1} produced by the parent strain. *Penicillium* sp was mutated by series mutation to obtain improved strain which could produce 3 times higher cellulase than its parental counterparts which allows us to believe that mutations could be an exciting approach for enhancement in production of cellulase [13,15].

Ronglin et al. [24] further improved *T. reesei* RUT-C30 via mutation with DES (di-ethyl sulfonate) and obtained several mutant and on screening DES-15 mutant was found superior having 11.86 FPU ml^{-1} which was 66% higher than its parent's 7.11 FPU ml^{-1} cellulase activity which was attributed to the smaller and branched hyphae. Several studies have addressed the absence of enough β-glucosidase activity to be the major drawback of *T. reesei* secretomes [9]. Cellobiose gets accumulated during hydrolysis due to the lack of β-glucosidase which in turn hampers the other key cellulase components activities such as endoglucanases and cellobiohydrolases. Directed evolution was applied to *A. niger* BGL1 by expressing a library mutant BGLs in *S. cerevisiae* and identified improved enzymes by using a two-step functional screening. In β-glucosidases transglycosylation reaction usually occurs at high susbtrate concentration which causes inhibition in case of BGL1, however, it was dramatically reduced by a key substitution at Tyr^{305} [76]. *T. reesei* strains have been genetically modified to overexpress native [77,78] and heterologous [26,27,79–84] β-glucosidases in several previous studies.

Strain improvement is an ever-going process and even after 70 years of research and evolution of *T. reesei* RUTC-30 which is available in public domain researchers keep modifying the strain for further improvement [26]. *T. reesei* was genetically engineered by introducing a heterologous β-glucosidase gene from *Talaromyces emersonii* to produce enzymes even in the existence of repressing sugars. It enabled the production strain to produce enzymes with improved hydrolytic efficiency. From *A. niger* an invertase gene was also added further in *T. reesei* so that from sugarcane molasses it may consume sucrose directly, bypassing the requirement to invert sucrose by utilizing acid or other means [27]. It was claimed that with minimum genetic modification and media engineering, significant improvement in cellulase production was achieved via modified *T. reesei* strain [27].

The CRISPR-Cas9 system is a powerful genome-editing tool to facilitate genetic modification of genomes in different organisms which has been explored recently in filamentous fungi too [26,85–87]. Liu et al. [86] demonstrated the establishment of a CRISPR -Cas9 system in *T. reesei* by *in vitro* RNA transcription and specific codon optimization. In target genes, site-specific mutations were generated through efficient homologous recombination, even using short homology arms. This system provides a promising and applicable approach to target multiple genes simultaneously. *T. reesei* RUT-C30 was engineered by introducing six genetic modifications and CRISPR-Cas9 system was employed which resulted in a significant enhancement in protein secretion rates by *T. reesei* RUT-C30 and overcame deficiency of β-glucosidase while permitting the sucrose consumption and the requirement of inducers for enzyme production was also eliminated. These six modifications include the constitutive expression of a mutated allele of the transcription factor also the master regulator XYR1 of cellulase, the expression of two heterologous enzymes as the invertase SUC1 from *Aspergillus niger* and the β-glucosidase CEL3A from *T. emersonii*, and the deletion of three genes such as the one encoding the repressor ACE1 of cellulase and the other two are for the extracellular proteases PEP1 and SLP1 [26].

As the CRISPR-Cas9 system has been proved to be a powerful tool enabling precise and desirable genome-manipulation for filamentous fungi. It may accelerate strain improvement as well as studies on functional genomics in these filamentous fungi.

Strain improvement has a huge scope for further improvement in cellulase production thereby decreasing the cost of cellulases causing economic feasibility of the whole process. Singhania et al. [20] and Singh et al. [9] have presented genetic modification as an important tool for strain improvement for higher cellulase as well as β-glucosidase production titres along with its improved properties. An emphasis has been witnessed on the use of genome editing and synthetic biology as emerging tools for understanding mechanisms and developing strains for lignocellulosic bioconversion. The methods of genome editing, such as CRISPR-Cas9, as well as synthetic biology, which accelerate mechanisms elucidation and strain development will further facilitate cost-effective cellulase production and thereby biofuel production. Table 2 gives an account of genetic modifications for improving cellulase titres or properties.

Table 2
Various genetic modification to improve titres of whole cellulase/components or its properties.

Source organism/gene	Host	Strategy used	Recombinant properties	References
bgl from *Aspergillus niger*/Tr*LPMO* from *Trichoderma Reesei*	*Penicillium verruculosm*	Heterologous expression of *bgl* and *lpmo* under the control of gla 1 promoter	Production of heterologous enzyme simultaneously.	[88]
Penicillium oxalicum	*Penicillium oxalicum*	Overexpressed BGL under constitutive and inducible promoter	65 folds higher yield compared with wild type	[89]
Penicillium funiculosum NCL1, bgl gene	*Pichia Pastoris*	Cloned and expressed *bgl*	rBGL shows high substrate conversion rate of 2083 lmol min^{-1} mg^{-1} with cellobiose, glucose tolerance up to 400 mM conc. and optimum pH 5.0 and temp 60 ^{o} C	[90]
Aspergillus nidulans, Promoter gpdA	*Penicillium oxalicum*	Amplifying induction along with depression (knock out strategy)	Enhanced cellulase production	[91]
Neotermes koshunensis	*Aspergillus oryzae*	Overexpressed BGL	Presence of 200 mM glucose stimulated rBGL by 1.3-fold, km and Vmax were 0.77 mM and 16 U/mg respectively. Active at pH 5.0–9.0.	[92]
Bos frontalis metagenome CMC-1	*E. coli*	Directed evolution strategy of error prone PCR	CMC-1 activity enhanced 2 folds	[93]
CBH gene	*S. cerevisiae*	By disrupting cell wall protein (CWP2)	85.9% increase in cellobiohydrolase activity	[94]
XYR1, CEL3A from *T emersonii* and SUC1 from *A niger,* deletion, ACE1, SLP1 and PEP1	*Trichoderma RUT C30*	CRISPR-Cas9 (6 gene modification)	Remarkable increase in protein secretion, 72- fold BGL and 42-fold xylanase	[26]
Penicillium verruculosum EGLII	*Penicillium verruculosum*	Structure based disculphide bond (DSB) engineering	Increased ~20% thermostability and 15–21% sp activity	[95]
Gloeophyllum trabeum CBS 900.73, Cel5	*Pichia Pastoris*	Site directed mutagenesis	Increase in K_{cat} and K_M by 45 and 52% respectively	[96]
Trichoderma reesei Cel5a	*Pichia Pastoris*	Site directed mutagenesis for elimination of disulphide bond	Increased Catalytic efficiency to 1.3-fold and thermal stability to 2.4-fold at 80^{o} C	[97]
Aspergillus niger BGL1	*S. cerevisae*	Directed evolution	Two-fold increase in cellulase activity	[76]
Trichoderma reesei Cel7B	*Neurospora crassa*	Structure guided evolution	Increased 4-fold CMCase, 2-fold FPase, 3-fold BGL and 2-fold activity at 60^{o} C	[98]
Trichoderma reesei XYR1 & BGL1	*T. reesei* QM9414	Rational Engineering	Increased 102% FPase in glucose medium, without inducers	[99]
Myceliophthora thermophila eg7	*Pichia pastoris*	Cloned and expressed eg7	Increased thermostability	[100]
Penicillium decumbens	*Trichoderma reesei* (RUT-C-30)	Agrobacterium mediated transformation	Enhanced β-glucosidase activity	[79]
Aspergillus nidulans AN3046LPMO	protein secreting vector pEXPYR	Cloned and expressed in pEXPYR a protein secreting vector using ligation free cloning	Enhanced LPMO level in cellulase produced	[101]

5. Carbon source and induction

Carbon source is a critical parameter for cellulase production. Cellulases are inducible enzymes and the cellulosic materials are considered as the most effective natural inducers for cellulase production by microorganisms. Usually, cellulosic carbon source is required for cellulase production as in occurrence of easily available carbon source, for example, glucose, the microorganisms does not need to produce cellulase. Cellulose as pure as avicel to crude biomass such as rice straw, wheat straw, sugarcane bagasse, cotton stalk, rice husk, corn cob, cotton, etc, has been employed as carbon source for cellulase production. Most of these carbon sources are able to induce cellulase production in fungi. Fruit waste has also been utilized as carbon sources for cellulase production as they could also serve as cheaper carbon source, however due to the low cellulose content it is usually supplemented with cellulose. Recent publication shows technological advances for improving fungal cellulase production utilizing fruit waste for biofuel application [102]. However, there is a limit to which these carbon source can be added to the production medium, for example soybean hull could be added to 100–140 g L^{-1} and it is the limit, as excessive soybean hull can lead to compromising aeration in the medium and retarded microbial growth [27].

Insolubility of cellulosic material causes several other complications too during fermentation. Absorption of cellulase on to biomass causes enzyme loss and cell biomass measurement, continuous feeding/sampling, agitation and aeration of the fermentation broth as well as downstream processing becomes a challenge [103]. Cellulase production highly depends on the hydrolysis of cellulosic substrate due to which the whole process becomes time-consuming. Cellulase production being induced by insoluble cellulosic substrate is very energy-intensive as it requires vigorous mixing along with intensive aeration of the viscous non-Newtonian fermentation broth [103]. These emphasizes the significance of use of soluble inducers for cellulase production. Hence it necessitates utilization of additional soluble carbon source for achieving even high titers.

Remarkable studies have been done in 1980s and 1990s on some soluble carbon sources like sophorose [104], cellobiose [105], galactose, l-sorbose [106], and lactose [107] and showed that, these are able to induce cellulase production in fungi. Even though sophorose proved to be a strong inducer of cellulase but its high cost prevented its application for cellulase production [104]. Among all the above inducers lactose is inexpensive and has been exploited commercially for cellulase production, although the mechanism of induction is still a subject of research. It was assumed that lactose was hydrolysed extracellularly to give galactose and glucose and thus boosted *T. reesei's* growth [108] however, repressed cellulase production indicates that cellulase production is not growth related. Thus, for cellulase induction either the intracellular presence or the uptake and/or metabolism of lactose is essential. The previous assumptions that in T. reesei the lactose metabolism proceeds only via extracellular hydrolysis and subsequent metabolism on uptake of the monomer glucose and galactose [109,110] is not convincing. It is reported that lactose induces xyr1 in *T. reesei* which could increase cellulase production in its presence [111]. Cheaper inducers may also be generated by incubating β-glucosidase (BGL) having transglycosylation activity with sugar/glucose syrup. Transglycosylation reactions may be dependent on glucose concentration and other factors like temperature. Incubating glucose syrup with BGL may give dimers (cellobiose), sophorose, etc. which may induce cellulase production [19].

Another way of using soluble carbon sources could be rational engineering of cellulase producers. T. reesei is widely employed in the industry for cellulase production and Zheng et al. [99] have described rational engineering of *T. reesei* QM9414 to achieve an extraordinary

higher cellulase production in glucose. Overexpression of master regulator XYR1 by the copper repressible promoter Ptcu1 was implemented first to attain production of cellulase with full capacity eliminating the need for inducing sugars in the context of CCR (carbon catabolite repression) for enzyme production. The engineered strain could efficiently produce cellulase on glucose and to compensate for low BGL titres, the bgl1 gene was overexpressed. Authors reported 102% enhancement in FPase activity when compared with the *T. reesei* RUT-C30 strain [99]. Efforts have been made to find inducers for *Penicillium* strains as well [112]. Though few inducers have been reported but none of them induces cellulase production significantly.

6. Regulation of cellulase production

It is evident that microorganisms produce cellulases to satisfy their own needs and when cellulose are converted into glucose, the cellulase production machinery gets shut down hence, they produce cellulases economically. This is the reason that crude cellulosic biomass, either native or pretreated, serves as a cheaper carbon source and can induce cellulase producing machinery in microorganisms.

Fungi produce cellulases under inducible condition (in presence of cellulosic substrate) for their own need and it switches off the cellulase producing machinery once it gets available sugars. The fungus exhibits a phenomenon in the occurrence of an easily available carbon source such as glucose which is called as Carbon Catabolite Repression (CCR). In the bioreactor, during the course of the bioprocess when cellulase production starts it hydrolyses the substrate mixture, releasing sugars mainly glucose which result in catabolite repression [113]. In fungus a mechanism is mediated by a CRE-1 gene due to which it prefers to avoid spending excessive energy needed to synthesis cellulase complex; and prefers to utilize the available sugars such as glucose in the media. This phenomenon is present usually in all the wild type fungal strain, hence bioengineered strains with truncated CRE-1 gene are preferred. *T. reesei* RUT C-30 has truncated CRE-1 gene and is the reason for hyper cellulase production. However, it has been debated that CRE-1 is also essential for normal hyphal growth and early efforts were done for Cre1 modification to release the CCR. CRE-1 modification may lead to arrested hyphal growth of fungi and reduced accumulation of biomass affecting cellulase production negatively [114]. Novel fusion transcription factors (FTFs) were designed to overcome the above issue so as to attenuate or release CCR inhibition in cellulase transcription, while to maintain normal hyphal growth the Cre1 was left intact. In all the transformants transcription levels of a major cellulase gene *cel7a*, were significantly elevated when grown on a media using lactose as a sole carbon source [114].

7. Challenges in hydrolysis of biomass by cellulase

Concentrated sugar syrup is required for bioethanol production as 4% ethanol is must for economic distillation. For these high solid loadings up to 15% w/w biomass is required in the reaction mixture; resulting in increased concentration of sugar and improvement in both capital and operational cost enabling the process to become more economically feasible [115]. Biomass consists of cellulose, hemicellulose and lignin majorly which is hydrolysed by biomass degrading complex enzymes as cellulase and hemicellulases; each consisting of several components [116]. Cellulases composed of cellobiohydrolase, endoglucanase, beta-glucosidase and LPMOs as well, likewise hemicellulases also contain several components acting together to hydrolyse hemicellulose completely. Even components of cellulase and hemicellulose influence each other's action. The major end products are glucose and xylose, however they may present in dimer forms also.

7.1. Product inhibition (regulation of cellulase action)

High solid loading is required for concentrated sugar syrup but leads to several other challenges such as product inhibition. End product inhibition in cellulases, β-glucosidase, and hemicellulases as well have been the focus of rigorous study [43,117–121]. It is well known that both cellobiohydrolases and endoglucanases gets inhibited by cellobiose directly [43,117,119] which binds to the enzymes's carbohydrate-binding module [122] and/or to its catalytic module [121]. Glucose the end product of cellulase inhibits majorly β-glucosidase and also inhibits cellobiohydrolases and endoglucanases to a lesser extent, similarly by binding to either the catalytic module or to the carbohydrate binding module and/or both, of these enzymes [117,120,122]. It is also important to mention here that even xylans, short xylo-oligosaccharides, xylobiose and hemicellulose-derived monosaccharides, have also been shown to inhibit cellulases actions [123–127]. The access of cellulases could probably be prevented to the cellulose chain by following inhibitory mechanisms of adsorption [123]. Furan derivatives and phenolic compounds resulting due to pre-treatment of biomass may also hamper hydrolytic efficiencies of these enzymes [128].

Thus high-solids hydrolysis is a major challenge which needs to be resolved by the intervention of scientific and technological advances including the development of highly efficient enzyme formulations, enzyme and biomass feeding strategies, and process strategies to overcome the end-product inhibition [115].

7.2. Lignin inhibition

Pretreatment of biomass is aimed at reducing the recalcitrance of biomass towards enzyme action. Lignin has been regarded as the roadblock for economically efficient hydrolysis of biomass [129]. Lignin is a relatively hydrophobic macromolecule which is a cross-linked phenolic, considered detrimental to the cellulases action. Pseudo lignin or humins are also known to negatively affect the rate of cellulase action on cellulose due to non-productive binding of cellulase to cellulose structure and/or hindering the access to cellulose by forming the physical barrier [130–132].

In presence of lignin hydrolysis yield could be decreased or even increased also, depending on lignin with varied characteristics which is produced due to various pretreatment of biomass under different conditions [133]. Huang et al. [133] suggested that the lignin hydrophobicity and the negative zeta potential controls the lignin inhibition and simulation effect, respectively. Lignin's presence was also reported to increase the rate of hydrolysis of avicel [134] probably due to presence of LPMOs. Cellulases used in these studies comprises LPMOs, which could be held responsible for the increased hydrolysis efficiency, as suggested by Cannella et al. [135]. Lignin, acting as a reducing agent, was able to activate LPMOs which also indicates a relationship between the redox cycles in lignin and oxidative breakdown of cellulose. A recent article on understanding the mechanism of inhibition factors for lignocellulose biomass hydrolysis gives an insight for improving the overall process efficiency [136].

7.3. Cocktail

As it is difficult to acquire all the cellulase component from single microbial strain in an optimum proportion so as to have maximum efficiency; it has been also produced separately and mixed afterwards to obtain a cocktail containing all the enzymatic component in optimum ratio to act synergistically to hydrolyse biomass [137,138]. Adsul et al. [139] mixed three different extracts of enzymes produced by *Trichoderma reesei* RUT-C30, *P. janthinellum* EMS-UV-8 and *Aspergillus tubingensis* under similar culture conditions employing avicel-wheat bran as carbon source by SmF process. All these three fungi were rich in different cellulase components and the idea of mixing was to bring all the components together in such a ratio that the hydrolysis efficiency of the cocktail must be superior to the individual enzymes. Heterologous BGL (β-glucosidase) is usually added to *T. reesei's* cellulase [137,138,

140] to improve its hydrolytic efficiency as it is deficient in BGL components. In most cases, the blends were found superior with higher activities as compared to each crude extract. All the three extracts when mixed together exhibited 16.9 U g^{-1} FPase activity, 162 U g^{-1} CMCase activity and 33 U g^{-1} β-glucosidase activity.

To cellulase, addition of hydrolytic enzymes such as pectinase [141], tannase [96] and non-hydrolytic enzymes such as laccase, manganese peroxidase, lignin peroxidase also has great potential to improve enzyme cocktail. However, these non-hydrolytic/oxidative enzymes showed positive and negative impact as well, when added to cellulase for hydrolysis of biomass [142,143].

Beyond major cellulolytic components, there are non-hydrolytic proteins like swollenin (Expansin like protein of fungi) which have been reported to increase hydrolytic efficiency of cellulase when added into it [52]. For example, in the hydrolysis of hot water pretreated miscanthus biomass, a 100% increase was resulted by employing a cocktail of Celluclast®, Novozyme® 188 and a purified swollenin from *T. harzianum* [52]. However, on pure celluloses swollenin from *T. reesei* was principally inactive [144]. The reason could be explained as, that after drying the material, cellulose nanocrystals was surrounded by a remnant hydration shell. Incubation of these material with *T. reesei* swollenin suggested the maintenance of a hydration layer by these proteins which could conceal hydrophobic spots that are bare in an environment where there was no free water, mitigating the unproductive binding of cellulases [144]. Even though, enzyme formulations comprising swollenin and expansin-like proteins seems lucrative; studies reporting the high levels of swollenin employment with the enzyme cocktails for the hydrolysis of lignocellulose materials are scarce.

At high solid loadings, LPMOs were found to be highly important as these oxidative enzymes were able to enhance the conversion of lignocellulosic biomass significantly. These shows positive impact on biomass hydrolysis, however on pure cellulase the impact is negligible as lignin's presence in pretreated or native biomass might act as reducing agents facilitating LPMOs action. At present, industrial cellulase preparations for biomass degradation are generally produced from filamentous fungi [145] and comprise a mixture of glycoside hydrolases with other accessory proteins that are required for synergistic action with cellulases [146].

Enzyme giant such as 'Novozyme' have come up with series of cellulase cocktail specifically for biomass hydrolysis, the first of being Celluclast, which was improved further by supplementing it with heterologous β-glucosidase and is available as CellicCtec2. Novozyme kept on refining its cellulolytic cocktail and came up with advanced CellicCtec3 which was found superior to even CellicCtec2 as has truly been reported to be supplemented with additional auxiliary enzymes specially LPMOs [20,147].

7.4. Thermostability

Thermostability has been the most desirable trait of cellulases for industrial application [148], especially for bioethanol application as it will drastically reduce the dosage of enzyme resulting in its economic utilization. Higher operating temperature for enzymatic hydrolysis of biomass allows faster reaction rate with reduced dosage making it more feasible. Several researchers have tried to modify microorganisms to obtain thermostable cellulases which could retain its catalytic efficiency even at 70° centigrade [73). Isolation of microorganisms from hot habitat has been thought as the way of getting thermostable enzymes, however, via genetic modification potent thermostable producing microorganisms have been developed. Along with few amino acids which are responsible for thermostability, the disulfide bonds are also known to impact the enzymes thermostability [149]. A novel thermostable cellulase GH45 (TaCel45) was studied from *Thielavia arenaria* XZ7 which is a thermophilic fungus and observed that at C12–C48 the disulfide bond was critical for refolding and thermal adaptation [150]. In the catalytic module of *Talaromyces emersonii* Cel7A, additional disulfide bridges were introduced which resulted in mutants (G4C/A70C, N54C/P191C and T243C/A375C) with improved thermostability [151]. Hence enhanced thermostability of cellulases drive the saccharification process towards economic feasibility as working at enhanced temperature would require lesser enzymes with faster reaction.

8. Integration process for second generation biorefineries

The process integration in the development of second generation biorefineries is a fundamental part in reducing costs and energy for biofuels and high added value compounds in terms of circular bioeconomy [152]. Today, there is little information available on the economy and energy used on an industrial scale, especially in the integration of biorefineries for biofuels such as bioethanol and enzyme production.

One of the most promising pretreatments in the integration and development of biorefineries is hydrothermal processing for biofuels, high added value compounds and enzymes production [153,154]. Fig. 3 shows a possible scenario in the development of a second generation biorefinery with the integration of energy and enzyme production. Larnaudie et al. [155] studied the techno-economic analysis of hydrothermal pretreatment for switchgrass biorefineries. They reported that the enzyme loading, high solid loading processing (pretreatment, hydrolysis and fermentation), enzymatic hydrolysis and fermentation strategy had a high impact on the minimum ethanol selling price (MESP). In addition, they showed the integration of energy (co-generation of electricity from lignin) in 2 scenarios: 1) producing only ethanol (cellulose fraction) and electricity and 2) producing ethanol, electricity, and high added value compounds (oligomers, furfural, acetic acid, and formic acid from hemicellulose fraction). Therefore, they concluded that scenario 2 was less than scenario 1 respect to the MESP. An important proposal in the development of biorefineries should be the co-production of enzymes, to reduce the production cost and the integration of energy in the process [154] (Fig. 3).

In a biorefinery biochemical platform, the chemical composition of biomass, enzyme loading, enzymatic hydrolysis and fermentation strategy using high solid loading have a significant effect on environmental performance (use of natural resources and emissions generated during the process). In a recent work, Larnaudie et al. [156] studied the life cycle assessment for bioethanol production from switchgrass using hydrothermal pretreatment in the context of biorefinery concept. They concluded that the biorefinery strategy in the production of ethanol, electricity, furfural, acetic acid, and formic acid had a good performance in terms of environmental impacts. However, the enzyme loading had an important effect on environmental performance compared to other process factors (chemical composition of feedstock, fermentation and enzymatic efficiency and solid loading processing). Therefore, it is important to reduce the enzyme loading in the biochemical processes, but also the co-production of enzymes from the substrates obtained after hydrothermal processing can be the key to reducing costs and environmental aspects and energy integration. Silva and Filho [157] reviewed the pretreatments technologies for improve the enzyme production on-site using the cellulosic and hemicellulosic fraction as carbon source and filamentous fungi, and they concluded that this strategy contributes to reducing enzyme production costs. The integration of bioprocess in a biorefinery concept can lead to an economically viable approach in the biofuels production due to the high cost of commercial enzymes [59].

Different authors have reported the production of enzymes using hydrothermally pretreated substrates as inducers in the production of cellulases and hemicellulases. Zhao et al. [158] produced on-site cellulase using a mixed culture of *T. reesei* and *A. niger* using hydrothermal pretreated corn stover, producing 3.42 FPIU mL^{-1} of cellulase. Michelin et al. [159] produced cellulases (3.5 FPU mL^{-1}) from hydrothermal pretreated solids rich in cellulose and lignin using *Trichoderma reesei*. The corncob was pretreated at 180-200 °C for 10–50 min, obtaining high cellulose content between (60–65 g/100 g of raw material). Also, the

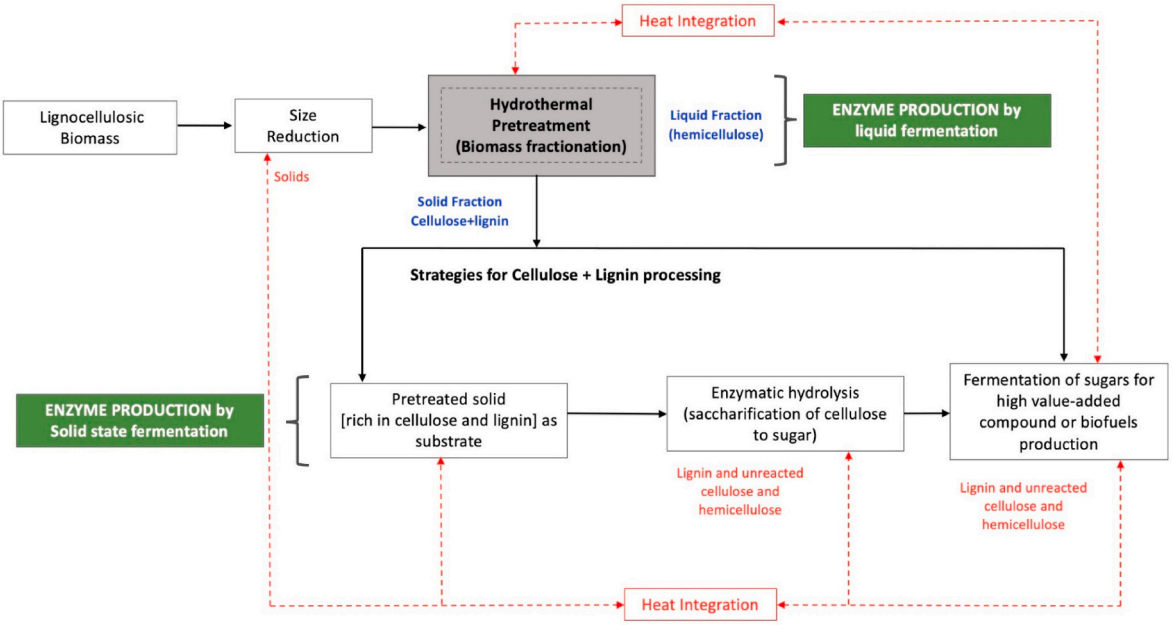

Fig. 3. Process diagram for biorefineries using hydrothermal pretreatment: Integrated enzyme production and heat integration.

hemicellulosic fraction after hydrothermal pretreatment rich in xylooligosaccharides, xylose and arabinose can be used as a carbon source for the production of hemicellulases. Michelin et al. [160] reported the production of xylanases (750 IU) and β-xylosidase (30 IU) by *Aspergillus terricola* using corncob hydrothermally pretreated (200 °C for 30 min) as substrate.

Regarding the integration of energy in hydrothermal processes, in a recent work Ruiz et al. [161] proposed two sections (Fig. 1) for heat recovery and integration (steam production, hot water and power: co-generation) from burning lignin and unreacted cellulose and hemicellulose. However, more studies are necessary to determine the global energy balances at the pilot and industrial level. Strategies such as pinch analysis can be important to minimize the energy to be used in the processes of second generation biorefineries.

9. Future perspective and conclusions

Cellulase production is the most important and integral part of bioethanol production bioprocess from cellulosic biomass. Filamentous fungi are the potent source for cellulase production as they produce a complete repertoire of cellulase with higher titres. *T. reesei* is the most exploited one and is employed for commercial cellulase production. Fungal fermentation causes several challenges being highly aerobic like increased viscosity causing difficulty in agitation and aeration and thereby reducing mass transfer. Few additives in medium like H_2O_2 may improve DO which needs to be explored for each bioprocess. Impeller design plays a significant role in resolving these issues. Nanobubble technology looks promising in solving aeration issue and mass transfer as well because the air bubble having nano size would not get disrupted by reaching to the surface of the medium and remains suspended there until utilized. It could be revolutionary for fungal fermentation. However, few fungal cultures grow homogeneously like bacteria and with the employment of soluble carbon sources, this issue could be controlled to a small extent. Then proper mixing by suitable impeller designing and proper aeration is important. This needs interference from biological engineers. Stirred tank reactors are commonly used for large scale cellulase production. Fungi with short and highly branched hyphae would be advantageous as increased number of mycelial tips lead to secrete more protein from it. A recent rapid advance has been made in improving the enzyme production of filamentous fungi through metabolic engineering. The methods and tools used for developing superior enzyme producers includes genetic modification systems, promoter selection and design as well as metabolic engineering using native or artificial transcription factors. Genetic modifications have brought revolutionary development in producing cost-effective cellulases, however cocktail formulation of different counterparts of cellulase is also an excellent way of getting highly efficient cellulase. Still harbouring different genes of interest in a single host would pave the way to cost-effective cellulase production. CRISPR-Cas9 and more advanced techniques of genetic modifications are coming up and their intervention will definitely open up the way for cheaper bioprocesses developed with those microorganisms.

Off-site configuration is a traditional way where purchased enzymes are used for the bioethanol process. There are enzyme giants like Novozymes, Danisco, Genencore, etc, but they usually tie up with bioethanol producing companies. For example, 'Genencore' is now a part of DUPONT. It necessitates in-house cellulase production technology. On-site cellulase production process will be cost-effective as stabilization and transport steps could be bypassed saving cost of stabilizers and transport. Integrated configuration is even more cost-effective utilizing the cheaper crude biomass for cellulase production, the same being used for hydrolysis thereby producing ethanol. CBP is an emerging technology having the ability to surpass all the emerging technologies for bioethanol production. Though, CBP is a proven concept it is limited by its low titres. Engineering a single microbe or finding a suitable consortium to do all the job starting from enzyme production till the ethanol fermentation with decent rate would lead to the success of the bioethanol process. It's a fascinating technology though far from commercialization. Overall cellulase production bioprocess has achieved tremendous growth and success, still there is a long way to go to produce ethanol from biomass at a competitive price to liquid petroleum fuels.

Cellulase production is an integral and most important part of bioethanol production from biomass-based bioprocess. Scale-up challenges need to be addressed in the initial phase of the technology. Integrated cellulase production is the most popular and cost-effective configuration. Genetic modification via intervention of most advanced technologies pave the way for cost-effective bioprocess of cellulase production along with advancements like using the whole broth by avoiding the downstream processing.

Credit author statement

Conceptualization- RRS and, AKP; Visualization- RRS and AKP; Methodology- HAR, AKP, and RRS; Validation- AKP, CWC and CDD; Formal draft preparation- RRS, MKA and HAR; Data curation- AKP, CWC and CDD; Writing—original draft preparation- RRS, MKA, HAR and AKP; Writing—review and editing, RRS and AKP; Supervision- RRS and AKP. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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