REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1. Bioethanol as a renewable fuel

Global concerns over energy security and climate change have necessitated the application of non-conventional resources such as lignocellulosic biomass for generating energy and fuel. The fuel generated from plant or algae biomass is called biofuel, such as bioethanol and biodiesel. Lower cost, surplus availability and renewable nature has made lignocellulosic biomass (LCB) the most appropriate and sustainable feedstock for generating biofuel and other value-added material (Aditiya et al., 2016). Some of the common examples of the globally abundant LCBs are rice straw, wheat straw and sugarcane bagasse (SCB). The total LCB production in India alone exceeds 680 metric ton per annum which accounts for production of approximately 52 billion liters of bioethanol (Jain & Agrawal, 2018). As 40% of the world's energy consumption is in the form of the liquid fuels (Tan et al., 2008), bioethanol has been considered as an alternative to supplement conventional fuels. Bioethanol is a promising renewable and an alternate source of energy produced from lignocellulosic biomass residue and is a sustainable solution to the problems of diminishing petroleum reserves, issues over national security and environmental deterioration due to GHG emissions. Unlike gasoline, bioethanol is an eco-friendly fuel and causes 90% reduction in greenhouse gas emissions compared to petroleum (Sánchez & Cardona, 2008).

Bioethanol is used as a transport fuel, mainly as biofuel additive for gasoline. One overwhelming advantage of bioethanol for the environment is its potential to be carbon neutral on a lifecycle basis – means carbon dioxide (CO_2) emitted during its use is offset by the absorption from the atmosphere during its growth (Sánchez & Cardona, 2008). Ethanol has a higher octane number (113) than petrol (87-93) (https://ethanolrfa.org/) as result pre-ignition does not occur when ethanol is used (Agarwal, 2007; Balat et al., 2008). It improves

the efficiency of engines when compared to petroleum and this enhances its appropriateness for use in advanced vehicles' engines (Balat et al., 2008; Masum et al., 2013).

Ethanol is burnt completely so that hydrocarbon and carbon monoxide emission is drastically lower as compared to petrol. Furthermore, extraction of fossil fuels is hazardous to environment and public health. Ethanol can be mixed with petrol for up to 10-15% (E10 or E15) without alterations to conventional motor engines (Moriarty & Yanowitz, 2015). Countries like United States and Brazil contribute major fraction in world's bioethanol production. The practice of blending ethanol (E5) in India started in 2001. Currently, there is blending of 10% (E10) in petrol. Ministry of petroleum and natural gas, government of India has mandate for increase the blending of ethanol to 20 % (E20) by 2030. Apart from its environmental benefits, the use of bioethanol as a fuel also has economic benefits like creating new jobs, supporting agrarian economy and helps meet the energy needs of developing countries (Quintero et al., 2013).

2.2. Lignocellulosic biomass as feedstock for second generation bioethanol

Lignocellulose is a renewable organic material and is the most promising feedstock considering its great availability, low cost and non-competence with the food demands. It is a natural complex of three biopolymers: cellulose (polymer of α-d-glucose), hemicellulose (heteropolymer of C5 and C6 sugars) and lignin (heteropolymer of phenylpropanoid units) (Juturu & Wu, 2014; Kuhad et al., 1997; Singhania et al., 2009). Numerous lignocellulosic biomasses can be successfully utilized for producing bioethanol. Some of them with their compositions are listed in Table 2.1.In general, the lignocellulosic biomass contains 40-45 % cellulose, 20 to 35% hemicelluloses and 5 to 30% lignin. In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues, in varied proportion based on the source (Menon & Rao, 2012). 2.9 $\times 10^3$ million tons of lignocellulosic residues are available that are waste products of cereal crops while waste from

pulses and oil crops amounts to be around 3×10^3 million tons. Similarly, plantation also generates waste that generates almost 5.4×10^2 million tons of lignocellulosic waste annually (Saini et al., 2015b; Singhania et al., 2009). The different types of lignocellulosic feedstock commonly used for ethanol production are sugarcane bagasse, wheat straw, rice straw, cotton stalk, rice husks, wheat straw, cotton stalks, corncob, coconut shells and municipal solid waste (MSW), forestry waste counting bark and wood chips. Few prominently used lignocellulosic feedstock are shown in Figure 2.1. The availability of lignocellulosic biomass in Indian context is shown in Figure 2.2.

Sugarcane bagasse (SCB) is one of the largest agricultural remnants in the world and according to the Indian state, it produces 100 million tons per year annually from 600 sugar mills in India (Konde et al., 2021). Since SCB mainly consists of cellulose, hemicellulose, and lignin, it can easily be utilized for generating bioethanol after pretreatment.

Therefore, the efficient use of residual bagasse requires immediate attention from the sugar industry and the scientific community around the world. While newly developed technologies have demonstrated promising prospects for the sustainable transformation of SCB into ethanol and additive chemicals, there is a lack of consensus among the scientific community on technological understanding and commercial use of current SCB transformation technologies (Gao et al., 2018).

Industries and research laboratories in India are working on the development of technology to produce bioethanol from lignocellulosic biomass. Significantly, India is the fourth largest producer of ethanol; therefore, the use of SCB for ethanol production will improve this industry. Therefore, recent efforts led by the Indian government led to the possible growth of SCB technology into a commercial level unit. However, to achieve this goal, Indian industries will need to regard the SCB as a resource for residual waste (Jain and Aggarwal 2018).

Figure 2.1: Different lignocellulosic feedstock used worldwide for bioethanol production. (a-Sugarcane bagasse, b-Wheat Straw, c-Rice Straw, d-Cotton stalk, e-*Prosopis juliflora*, f-*Lantana camara*, g-Willow, h-*Gracillaria verrucosa*)

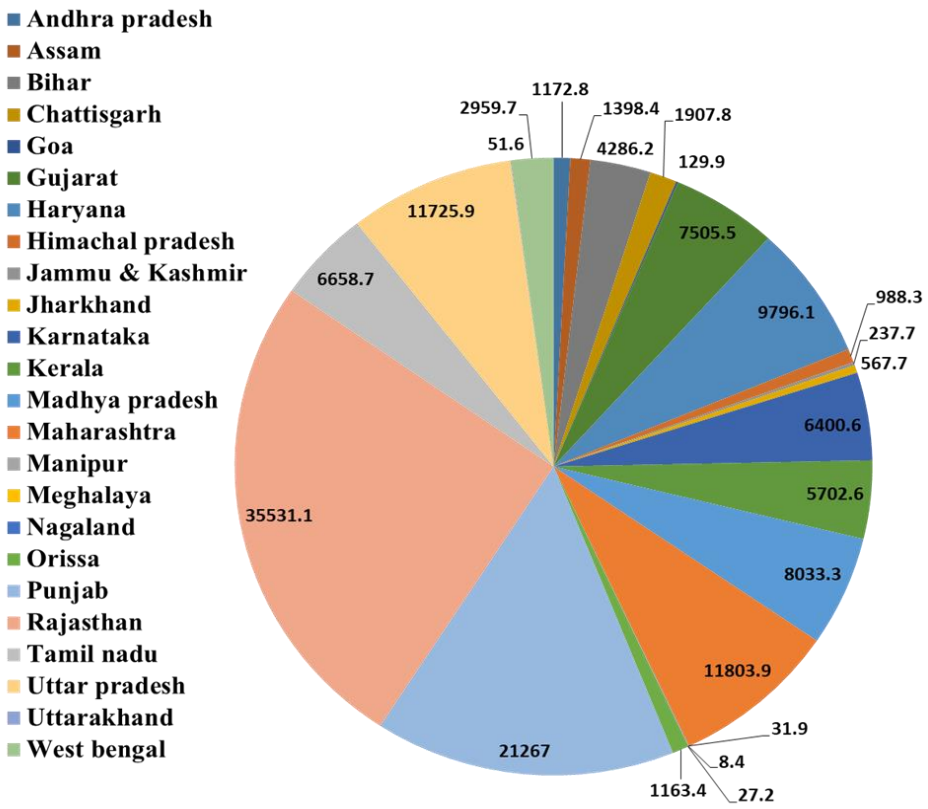

Figure 2.2: Availability of lignocellulosic biomass (kilo ton per year) in India (Hemansi et al., 2019)

2.3. Second generation bioethanol process

Second generation bioethanol is derived from lignocellulosic resources in contrast to first generation bioethanol derived from sugar or starch based resources. The conversion of lignocellulosic biomass to bioethanol is a multi-step process. The structural carbohydrate polymers in lignocellulose, i.e. cellulose and hemicellulose are first depolymerized through pretreatment and saccharification and the obtained monomeric sugars are subsequently fermented to ethanol. Lignocellulose conversion to bioethanol can be carried out in various manners, such as by employing biochemical/microbial/enzymatic route (Kang et al., 2014).

The biochemical conversion route of lignocellulosic biomass to ethanol is milder and environmental friendly and broadly comprises four sequential steps: deconstruction of biomass (pretreatment), saccharification, conversion of sugar to ethanol (fermentation) and purification of the product (Figure 2.3) (Kuhad et al., 1997; Lynd et al., 1999).

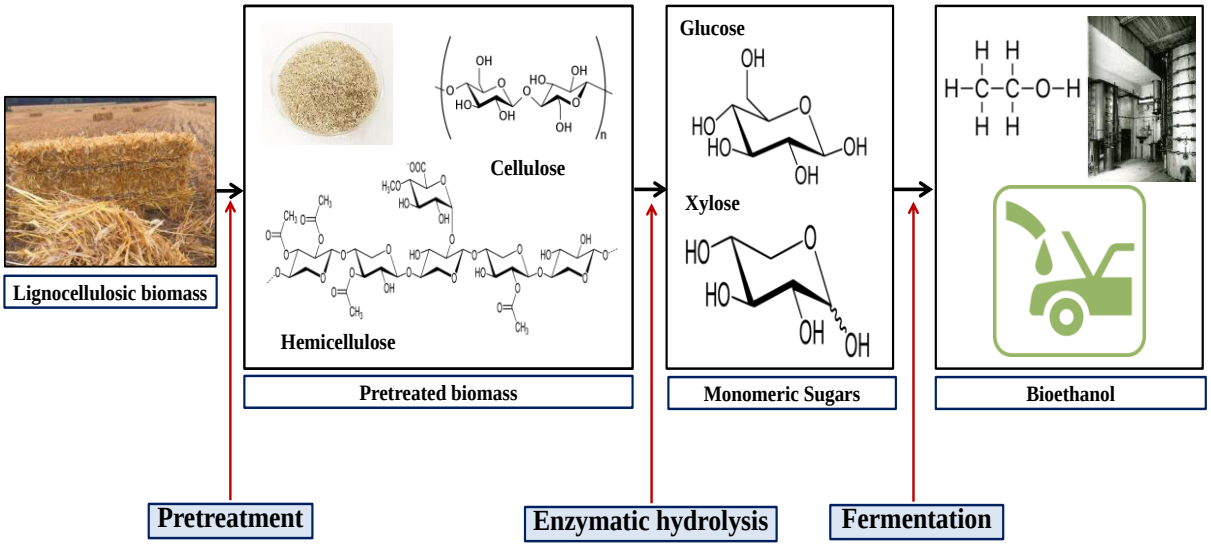

Figure 2.3: Schematic illustration of process of conversion of lignocellulosic biomass to bioethanol

Table 2.1: Composition of various lignocellulosic biomass used for bioethanol production (Hemansi et al., 2018)

Substrate	% composition (dry wt.)			Substrate	% composition (dry wt.)		
	Hexosans	**Pentosans**	**Lignin**		**Hexosans**	**Pentosans**	**Lignin**
Bamboo	49-50	18-20	23	Oat straw	41	16	11
Banana waste	13.2	14.8	14	Olive tree waste	25.2	15.8	19.1
Barley hull	34	36	19.3	Paper	85-99	0-5	0-15
Barley pulp	69.9	18.3	10.9	Pepper stalks	35.7	26.2	18.3
Bean stalks	31.1	26.0	16.7	Pine	41	10	27
Bermuda grass	25	35.7	6.4	Poplar	40	14	20
Birch wood	40	33	21	Reed	49.40	31.50	8.74
Chilli stalks	37.5	28.3	17.3	Rice husk	36	15	19
Coffee pulp	33.7-36.9	44.2-47.5	15.6-19.1	Rice straw	32	24	13
Corn cobs	42	39	14	Rye straw	31	25	7
Corn Stover	38	26	19	Salix	41.5	22-25	25
Cotton seed hair	80-95	5-20	0-5	Saw dust	55	14	21
Cotton stalks	41.7	27.3	18.7	Soft wood stem	45-50	25-35	25-35
Douglas fir	35-48	20-22	15-21	Sorghum straw	33	18	15
Eucalyptus	45-51	11-18	29	Soybean stalks	34	25	20
Flax sheaves	35	24	22	Spruce	45	26	28
Grapevine stems	43.1	19.4	26.6	Sugarcane bagasse	33	30	29
Grasses	25-40	35-50	10-30	Sweet sorghum	23	14	11
Groundnut shells	38	36	16	Switch grass	37	29	19
Hemp	53.86	10.60	8.76	Waste paper	60-70	10-20	5-10
Jute fibers	45-53	18-21	21-26	Water hyacinth	18.4	49.2	-
Miscanthus	43	24	19	Wheat straw	30	24	18
Municipal solids	8-15	NA	24-29	Willow	55.9	14	19

2.3.1. Pretreatment of lignocellulosic biomass

Production of bioethanol from second generation biomass requires efficient depolymerization of structural carbohydrate polymers to be fermented to ethanol. However, lignocellulosic biomass has evolved complex structural and chemical mechanisms, which provide recalcitrance to its structural sugars from the microbial and enzymatic attack. Therefore, a deconstruction of biomass is required to change the biomass size and structure as well as chemical composition so that hydrolysis of the carbohydrate portion to monomeric sugars can be attained rapidly with higher yields. The main aims of pretreatment are as follows:

(1) To improve sugar yields during enzymatic hydrolysis by reduction of crystallinity of cellulose and enhanced porosity of the biomass;

(2) To minimize the emergence of fermentation inhibitors during deconstruction;

(3) To retrieve lignin from hydrolysate for converting it into valuable by-products and

(4) To make the process economic by making the operation easier (Aditiya et al., 2016).

Broadly, pretreatment strategies are categorized into physical, physico-chemical, chemical and biological. With every different feedstock used for bioethanol production, the selection of pretreatment method varies due to distinct chemical composition and physical structure of feedstock. Factors like cellulose crystallinity, lignin content, cell wall porosity, hemicellulose side chain branching and crosslinking are critical in choosing the pretreatment method. Most chemical pretreatment modifies cellulose ultrastructure through certain physico-chemical modification, though it is possible to fractionate cellulose, hemicellulose and lignin by using pretreatment with some catalysts. A list of common pretreatment strategies used and their advantages and disadvantages are shown in Table 2.2.

Table 2.2: Various pretreatment strategies with their specifications (Hemansi et al., 2018)

Pretreatment	**Action**	**Advantages**	**Disadvantages**
Dilute Acid	Hydrolyses hemicelluloses, Alters lignin structure	Hemicellulosic removal	Low removal of lignin, Low enzymatic hydrolysis (30-40%), Inhibitor generation
Dilute Alkali	Eliminates lignin and hemicelluloses, Enhances surface area exposure for enzyme access	High digestibility, high lignin removal	Hemicellulosic sugar loss, low enzymatic hydrolysis (50-60%), Inhibitor generation
Ammonia fibre expansion (AFEX)	surface area for access to enzyme upsurges after treatment, removes out hemicellulose and lignin	Small amount of inhibitors formation	Not proficient for biomass with high level of lignin, high price of ammonia
Ionic liquid	Decreases cellulose crystallinity, removes lignin	High dissolution, green solvents	applications at large-scale are under exploration
Alkaline peroxide	Removes lignin and solubilizse most of the hemicellulose	Cellulose isolation	Loss of hemicellulosic sugars, loss of lignin
Acid-chlorite	Reduces lignin content	Isolation of hemicellulose and cellulose	Loss of lignin, Costly method of pretreatment
Ammonia	Opens up cell wall and exposes celluloses and	Lignin removal (partial)	Hemicellulosic sugar loss, Low enzymatic

	hemicelluloses		hydrolysis (50-60%), Inhibitor generation
Steam explosion	Causes lignin transformation, and causes hemicelluloses solubilisation	Deconstruction of structural polymer, recovery of lignin, lower loss of hemicellulose, less amount of inhibitors generated, higher yield of hemicellulose and, economic process	Generation of inhibitors, generation of inhibitory compounds, partial hemicellulose degradation, disrupted lignin-carbohydrate matrix is lacking
Biological	Degrades lignin and hemicellulose	Partial deconstruction of lignocellulosics, low energy consumption	Longer fermentation time

The biochemical conversion of the cellulose (or hemicellulose) requires action of lignocellulolytic enzymes, thereby releasing mono-, di- and oligo-saccharides which are further fermented by microbes to the desired product. Complete as well as economic conversion of LCBs to its constituent saccharides is still considered a major challenge due to inherent biomass recalcitrance (Agbor et al., 2011). For counteracting the recalcitrance, LCB must be deconstructed using physical-chemical processes prior to its enzymatic hydrolysis by lignocellulolytic enzymes. Pretreatment enhances accessibility of biomass components to lignocellulolytic enzymes, thereby, resulting in maximum product recovery from LCB and improved economics of lignocellulosic biorefineries (Kumar et al., 2009).

More commonly employed pretreatment methods are dilute-acid (DA), alkali (DB) and steam-explosion. Dilute sulphuric acid pretreatment results in breakdown of the rigid structure of LCB by solubilization of its hemicelluloses (Martiniano et al., 2014). DB pretreatment involves hydroxides of sodium, potassium, calcium, and ammonium and

facilitates the delignification, swelling of cellulose and partial decrystallization and solubilisation of cellulose and hemicellulose, respectively (Brodeur et al., 2011; Silverstein et al., 2007). DA pretreatment employing sodium hydroxide is more commonly applied for effective disruption and removal of lignin from LCB (Brodeur et al., 2011; Kumar et al., 2009). The pretreatment processes should enhance the relative proportion of cellulose and reduce both the hemicellulose and lignin contents of LCB for better conversion of cellulose during enzymatic hydrolysis, which is generally not possible when a single step pretreatment is used alone. Therefore, sequential acid-alkali (DAB) pretreatment has also been attempted as a better method for pretreating various lignocellulosic agro-wastes, such as rice straw (Kim et al., 2013), corn stover (Lee et al., 2015), oil palm fruit waste (Kim et al., 2012) and SCB for enhancing the sugar yield during hydrolysis (Giese et al., 2013; Isaac et al., 2018; Philippini et al., 2019). In this method, cellulignin obtained after acid pretreatment is further treated with alkali for lignin removal which in turn maximises the surface area of cellulose available for cellulase during hydrolysis for biorefining operations (Chandel et al., 2014; Keshav et al., 2016). Previous study on sequential DAB pretreatment of SCB reported a final cellulose content of 76.5% along with 20.0% lignin (Chandel et al., 2014). Even this much lignin content could still cause hindrance in economic hydrolysis of the biomass. Therefore, sequential DAB pretreatment of SCB needs further investigations. Furthermore, dilute alkali-acid pretreatment of LCBs such as cotton stalk has also been reported (Rocha et al., 2012), but the studies are only a few in the literature. Pretreatment induces macro to nano-scale structural changes in lignocellulosic plant biomass, including removal and reorganization of constituents, increased pore size and density, altered crystallinity, etc. Such structural variations in LCB are monitored by analytical tools spanning multiple length scales, such as scanning electron microscopy (SEM), Fourier transforming infrared spectroscopic (FT-IR) method, wide or small angle X-ray diffraction (XRD) method, thermo-gravimetry (TGA),

etc. Recently, small angle neutron scattering (SANS) has emerged as a powerful technique for deeper understanding of biomass recalcitrance (Pingali et al., 2017; Yuan et al., 2017), but the reports on application of SANS for lignocellulose monitoring during pretreatment are still very scarce. Further studies on pore size and distribution using SANS are crucial for better understating of the lignocellulose deconstruction, especially for the surplus crop residues like sugarcane bagasse (SCB).

2.3.2. Enzymatic hydrolysis of lignocellulosic biomass

The hydrolysis of pretreated biomass is the most crucial step in the bioethanol production process. Although hydrolysis of biomass can be accomplished by using acid or enzymes, saccharification using enzymes is preferred due to milder processing conditions and environment-friendly nature. Depolymerization of biomass via enzymatic hydrolysis is a multi-enzymatic process with high complexities. In nature, lignocellulosic biomass can be depolymerized by a number of hydrolytic enzymes that are produced by diverse fungi and bacteria. Cellulases are the representative class of enzymes involved in depolymerizing lignocellulosic substrate by synergistic action of all three enzymes present in the complex.

Cellulase complex consists of exoglucanases (cellobiohydrolases, CBH), endoglucanases (EG) and β-glucosidases (cellobiase, BG) (Behera & Ray, 2016). EG acts upon cellulose chains and hence creates two types of reactive ends for CBHs. CBH I acts on reducing ends and CBH II on non-reducing ends of cellulose fragments thereby, catalysing step wise degradation of cellulose to cellobiose. BG utilizes cellobiose and converts it into glucose (Kuhad et al., 2011). CBH gets inhibited by cellobiose, therefore; BG plays a key role in reducing end-product inhibition and depolymerizing the cellulose completely. Modular structure with concluding catalytic and carbohydrate binding molecules (CBM) is a common feature of most of cellulases. The carbohydrate binding molecules facilitate hydrolysis of biomass by fetching the catalytic domain in contiguity to the insoluble cellulose. Thus, the

rate of enzymatic hydrolysis of the biomass is subjective to the substrate properties and catalytic performance both. The scheme of mechanistic action of cellulases over cellulose is shown in Figure 2.4.

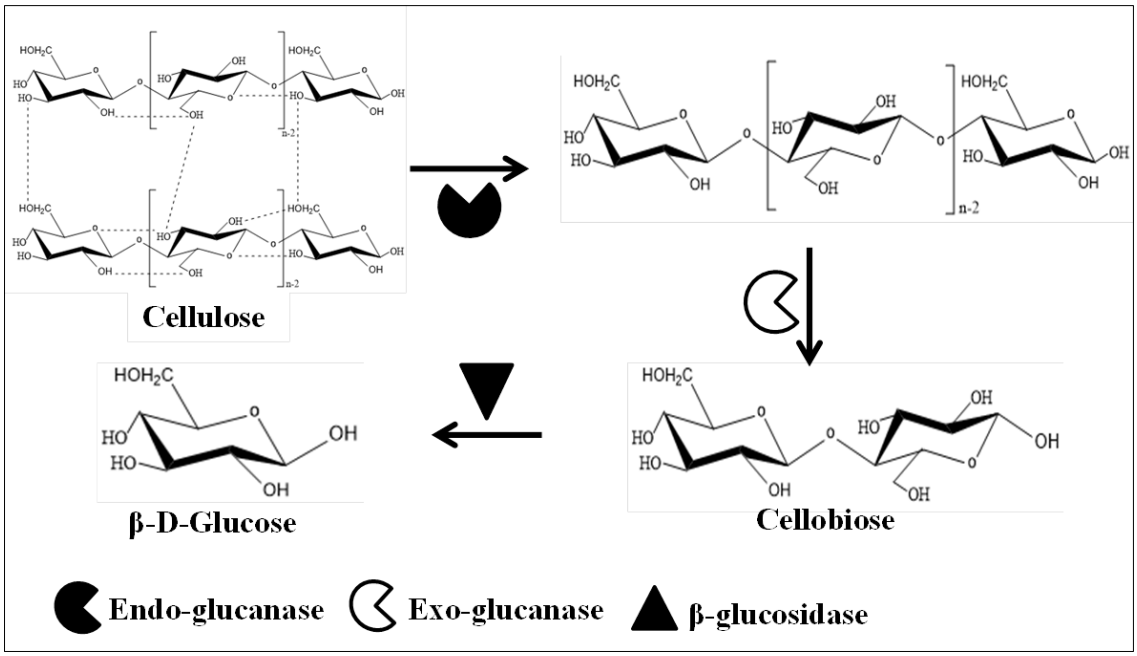

Figure 2.4: Schematic diagram showing mechanism of enzymatic hydrolysis (Hemansi et al., 2019)

Although saccharification using enzymes has more scope for improvement than those using chemicals, the high cost of cellulases is still a technical barrier (Culbertson et al., 2013; Hong et al., 2013). Fall in the cost of cellulase could be obtained by (a) intensive efforts which enquire more than a few aspects of enzymes with improved hydrolytic properties such as binding affinity, thermostability, etc. (b) by improvement of technologies for which are proficient for hydrolysis including of superior cocktails of enzyme and conditions for hydrolysis. In addition to enzyme characteristics, substrate features such as the degree of polymerization, cellulose crystallinity and the existence of lignin and hemicellulose also affect the enzymatic hydrolysis.

Therefore, to improve the overall process, upgrading in cellulase performance and enhancing the substrate-enzyme interaction are prerequisite. Industrially, among all probable strategies, the optimization of the characteristics of cellulases like thermostability and end-product

inhibition is crucial for large scale application. Also, optimizing production medium by altering its components is an approach to enhance the enzymatic hydrolysis. Development of multi-enzyme cocktail secreted by various strains of fungi is also a good choice for improving the performance of cellulase as a complete system. Several studies have reported that synergistic action of cellulase is linked with the ratio of every enzyme in the system (Berlin et al., 2007; Hemansi et al., 2018).

The constraint of cellulases is constantly increasing due to its miscellaneous applications; high demand also produces some challenges. The recalcitrant nature of lignocellulosic biomass is a major complication.

2.3.3. Fermentation

As compared to simpler fermentation process of sugars derived from food-based feedstock, crop-waste based feedstock to ethanol conversion process is very tedious and involves many critical steps. Pentose-rich sugar syrup and hexose rich sugars coming from hydrolysis of hemicellulose and cellulose, respectively, are the major substrates after initial hydrolysis that can be further fermented to produce ethanol. There are many desirable characteristics of an ideal fermenting microorganism, such as high conversion efficiency both with respect to substrate utilized and time, robustness against inhibitory compounds and ability to withstand high ethanol concentrations.

Several laboratories have established the process of utilizing pentose sugars as well as hexose sugars by various yeasts, fungi and bacteria for the production of fermentation products including alcohols. Among these, the most common and efficient glucose fermenting microbes are brewer's yeast *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Hahn-Hägerdal et al., 2006), while for pentose fermentation are *Pichia stipitis* and *Candida shehatae.*

The process of ethanol production not always requires aerobic conditions. It is required only for the production of biomass (Agbogbo & Wenger, 2007). Further to enhance the ethanol production from pentose sugars, different detoxification strategies have been used by various researchers (Chandel et al., 2007). The elimination of inhibitors from fermentation broth considerably improved the yield and productivity of ethanol as compared to un-detoxified hydrolysate. Moreover, utilization of all the sugars including hexoses (C6; glucose, galactose, and mannose) and pentoses (C5 sugars; xylose and arabinose) in a single reactor can be another option to reduce the cost of producing cellulosic bioethanol.

Scientists around the world have employed different fermentation strategies for cost-effective processes for ethanol production from lignocellulosic biomass in a single reactor. These processes include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), consolidated bioprocessing (CBP), and simultaneous saccharification, filtration and fermentation (SSFF). All the processes have been shown in Figure 2.5.

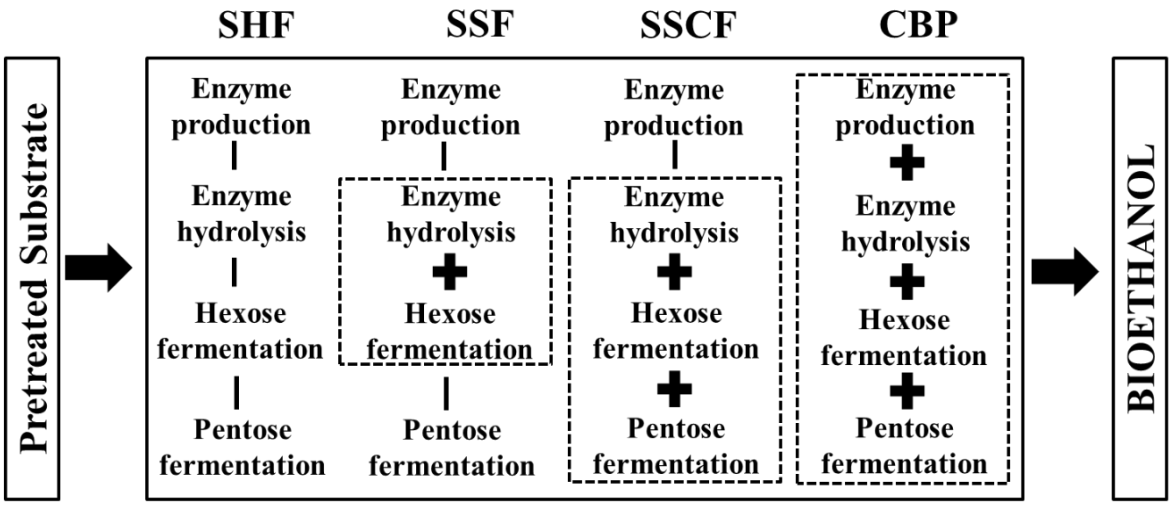

Figure 2.5: Overview of various fermentation strategies (Hemansi et al., 2019)

Above mentioned methods (SSF, SSCF, and CBP) are preferred over separate enzymatic deconstruction and fermentation (SHF) strategy. Despite it, in the current scenario, SHF is the mostly used method for bioethanol production. During the first step of SHF, cocktail of lignocellulolytic enzymes is produced so that lignocellulosic biomass can be converted into syrup of monomeric sugars (hexoses/pentoses). This solution is further used to produce bioethanol with the help of pentose/hexose fermenting microbes in a separate step. For the first step, i.e. hydrolysis, optimum temperature ranges from 45 to 50 °C, whereas for fermentation, the optimal range is near 30 °C, so both steps are performed sequentially. In SSF, the enzymatic hydrolysis of pretreated lignocellulosic biomass to release monomeric sugars for subsequent microbial conversion to ethanol is performed in the same vessel. Hallmark of this type of process is the compromise between optimum temperatures of both hydrolysis and fermentation (Choudhary et al., 2016). SSF is important over SHF as it delimits repression of cellulases (by glucose) via feedback inhibition, so improves the efficiency of saccharification as well as ethanol yield.

This is interesting to note that while performing chemical-based pretreatments, generation of various fermentation inhibitors (furfural, hydroxymethyl furfural, phenolics, acetic acid, etc.) takes place. Therefore, prior to fermentation, removal of these inhibitors seems necessary. Several detoxification strategies such as liming, activated charcoal adsorption, ion-exchange resin treatment and enzymatic detoxification have been used to remove these fermentation inhibitors. An alternative and more sustainable way to tackle the problem of inhibitors is to use inhibitor resistant or tolerant enzymes and microbial strains.

2.4. Challenges associated with bioethanol generation process

Bioethanol is generated by harvesting the plant materials that are otherwise thrown away, then using it to create fuel without adding chemicals or significant amounts of greenhouse gases into the atmosphere. Lignocellulosic biomass is a promising feedstock for bioethanol

production. But there are challenges associated with making biodegradable lignocellulosic biomass, which result in lower ethanol yields than the ideal process. Therefore, technological bottlenecks in commercial production of lignocellulosic ethanol need to be addressed to make ethanol production cost-effective.

2.4.1. Challenges related to feedstock

Major challenges related to feedstock are its collection, storage and transportation. It is also essential to make the feedstock available throughout the year. As the agricultural fields are distributed unevenly within a country, it is very difficult to collect and store the biomass at a single location for round the year use. Since, location of the industries may not be closer to the biomass generation site; the transportation of biomass to distant places can significantly increase the overall bioethanol production process cost (Saini et al., 2015).

2.4.2. Challenges involve with hydrolytic enzyme

Enzyme related challenges during bioethanol production include restricted enzyme mobility, reduced adsorption of cellulase onto cellulose, unproductive binding that hinders the enzymatic action on biomass. High levels of end products like cellobiose and glucose inhibit cellulases by feedback mechanism and lower the rate and yield of saccharification. To achieve desired saccharification efficiency, more enzyme dosages are required, which increases the overall cost. Therefore, the crucial objective for decreasing the enzyme cost is the high level production of in-house enzymes by employing hyper enzyme producing strains, inexpensive raw material and cost-efficient production technologies (Hemansi et al., 2018).

2.4.3. Challenges during biomass pretreatment

One of the significant disadvantages of the pretreatment process is the generation of a variety of chemical compounds due to undesired degradation of its LCB components, formation of by products, use of chemicals/solvents and heat (Figure 2.6). Major degradation products

formed are furans, phenolics, weak acids, etc. (Mankar et al., 2021; Wang et al., 2018). These inhibitory compounds affect the normal functioning of both enzymes and fermenting microorganisms, thereby, inhibiting hydrolysis and fermentation processes, respectively (Bhatia et al., 2021; Koppram et al., 2014). Furans such as, furfural and 5-hydroxymethyl-2-furaldehyde (HMF), inhibit yeast fermentation by blocking their oxidative metabolism and cell-biomass formation (Wang et al., 2018). Acetic acid, the major weak acid generated during pretreatment, causes yeast cell membrane disintegration and increased osmotic pressure, thereby reducing carbohydrate metabolism and fermentation. Similarly, phenolic compounds also reduce fermentation efficiency by affecting yeast growth. The most notable phenolic compounds include vanillin, catechol, coniferyl alcohol and aldehyde, syringaldehyde, and p-coumaric acid. These inhibitors are present in the pretreatment slurry/hydrolysate and also remain adsorbed to the biomass after filtration or centrifugation for solid recovery, thereby, inhibiting the fermenting microbes and decreasing the ethanol yield and productivity (Wang et al., 2018). Therefore, washing of the biomass after pretreatment has become more or less a necessary operational step for removal of these inhibitors prior to hydrolysis and fermentation. But it costs enormous amount of clean water and generates chemical rich waste water needing further treatment (Lyu et al., 2020). Detoxification is also not an economic option, as it causes loss of fermentable sugars, and increases operational time and complexity (Shibuya et al., 2017; Yu et al., 2020). Therefore, there is a need of robust yeast strains which can ferment the glucose in presence of these inhibitors.

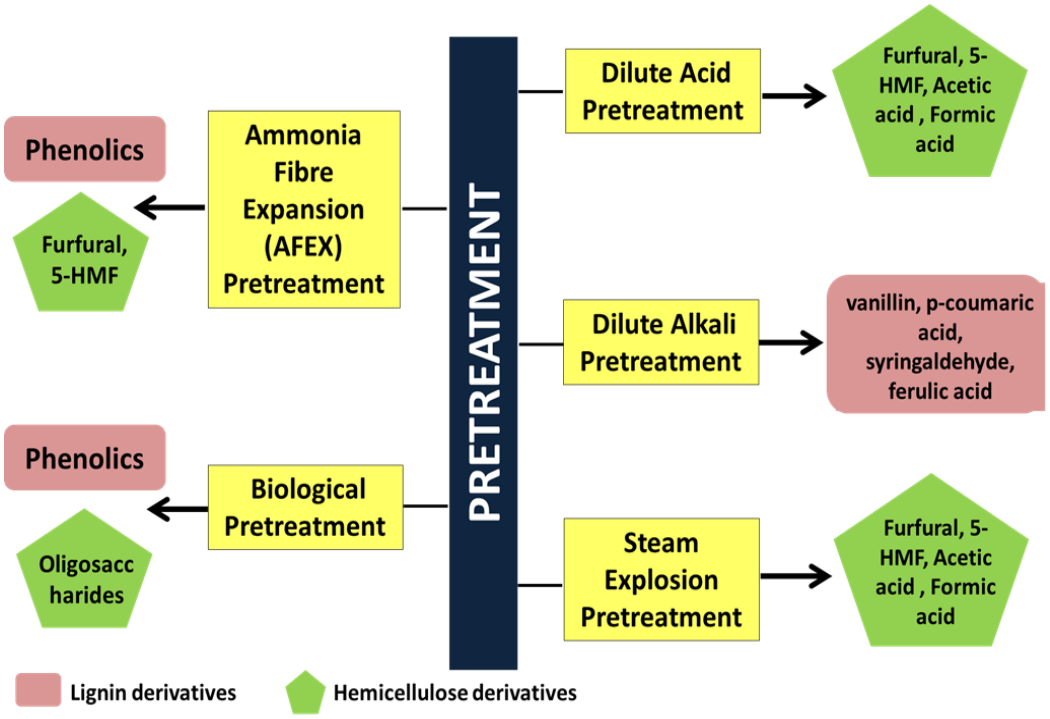

Figure 2.6: Various inhibitors generated during pretreatment of lignocellulosic biomass

2.4.4. Challenges during fermentation

Slower glucose consumption by yeasts creates an osmotic stress for the cells. Few compounds which releases during pretreatment of lignocellulosic biomass, act as inhibitors to the yeast cells while fermentation. These compounds decrease the fermentation efficiency of the cells by increasing their lag phase. Moreover, the presence of a high amount of toxic inhibitors has an impact on the performance of both enzymes and yeast. Due to the high viscosity of substrate solutions, poor mixing and heat transfer difficulties occur. In the SSF process, a compromise between ideal saccharification and fermentation temperatures leads to poor overall conversion of biomass to ethanol.

During cellulosic ethanol fermentation, SSF is the configuration of choice, as it provides many advantages including faster metabolism, high productivity, alleviation of enzyme inhibition by feedback mechanisms, less contamination, lower costs of cooling, and less energy constraints in mixing and recovery of product (Arora et al., 2019). However, use of conventional mesophilic yeasts like *Saccharomyces cerevisiae*, decreases the overall bioconversion efficiency of SSF due to larger mismatch between optimal temperatures of hydrolysis and fermentation processes. Thermotolerant yeasts like *Klyuveromyces marxianus*

provide an upper edge during SSF, as the fermentation can be carried out at a temperature (≥ 42 °C) which is near the optimal range of hydrolysing enzymes (around ≥ 50 °C) (Saini et al., 2015a). Though, thermotolerant yeasts exhibit better ethanol production under temperature stress, low tolerance to multiple fermentation inhibitors at high temperature remains bottleneck to realising their full potential in lignocellulosic biorefineries (Arora et al., 2019; Hemansi et al., 2021).

Microbial strain improvement can be carried out using genetic engineering, metabolic engineering, and synthetic biology based rational approaches, which requires the accurate knowledge about underlying principles of tolerance and the target genes or their metabolic functions (Wang et al., 2018). An alternate strain improvement strategy based upon 'evolutionary engineering' principle is known as 'adaptive laboratory evolution (ALE)' (Mo et al., 2019; Qureshi et al., 2015; Wu et al., 2021). ALE relies upon forced selection of the specific phenotypes after accumulation of spontaneous mutations, generation after generation under constant selection pressure, for developing an improved microbial strain (Yamakawa et al., 2018). It is a powerful strategy to acquire desired phenotype of inhibitor tolerance in the fermenting microorganism by cultivating it under progressively increasing levels of inhibitors followed by screening of the tolerant strains (Sandberg et al., 2019). Ideally, the developed strain would show better growth, and improved ethanol yield, titer and productivity in the presence of inhibitors. Tolerance engineering in fermenting microbes by ALE doesn't require detailed understanding of the inhibitory mechanism and complex interaction of inhibitor with biochemical or genetic networks. Moreover, the metabolic burden exerted due to heterologous expression of genes/proteins and the need for stringent control of genetic expression required in the rational approaches can be avoided by evolving tolerant strains through ALE (Mohamed et al., 2017). Most of the previous studies on ALE applied to improve biofuel fermentation have focused on improving microbial tolerance to a single

stress, such as phenolic compounds, furfural, ionic liquids, ethanol, and acetic acid (Matsusako et al., 2017; Shui et al., 2015; Wang et al., 2018). However, only a few studies on ALE have concentrated on simultaneous improvement of tolerance to multiple stresses and inhibitors, which is practically more advantageous (Wallace-Salinas & Gorwa-Grauslund, 2013).

2.5. Strategies to address current challenges during bioethanol production process

2.5.1 Strain engineering of microbes for improved bioethanol production

One of the major challenges for economic conversion of lignocellulose to bioethanol is to generate robust yeast strains. These strains should be able to cope with inhibitory compounds as well as fermentation conditions while keeping proper catalytic functions for biomass conversion to ethanol. Here, we have tabulated some of the methods such as adaptive evolution, random mutagenesis and metabolic engineering as strategies for acquiring stress tolerant strains suitable for industrial use (Table 2.3).

Control of a strong constitutive promoter showed particularly high ethanol production from xylose and low xylitol yield by fermentation of not only xylose as the sole carbon source, but also a mixture of glucose and xylose (Watanabe et al., 2007). Additionally, an ethanologenic *E. coli* mutant that is, devoid of foreign genes, has also been developed by combining the activities of pyruvate dehydrogenase and the fermentative alcohol dehydrogenase and the mutant was found able to ferments glucose or xylose to ethanol with 82% ethanol yield under anaerobic conditions (Kim et al., 2007).

Table 2.3: Various strategies employed for improving tolerance of fermenting microorganisms

Strategy	Potential drawbacks/Considerations	Approaches	References
Evolutionary engineering	The inhibition problems vary depending on the feedstock and pretreatment	The Adaptive evolution with specific hydrolysate and inhibitors of lignocellulosic	Almario et al. (2013); Koppram et al. (2012); Smith et al. (2014)
Metabolic engineering/genetic engineering	Genetically modified microorganisms-based process	Phenolics, furfural and carboxylic acid resistance engineering	Larsson et al. (2001); Sanda et al. (2011); Wang et al. (2013)
Microorganism selection	Specific productivity and product yields should be selected primarily	Screening of natural or industrial microbial collections	Favaro et al. (2013); Wimalasena et al. (2014)
Conditioning/Det-oxification	More chemicals are needed; some methods require a further step in the process	Chemical additives, such as alkaline therapy, reduction agents, polymers	Alriksson et al. (2011); Alriksson et al. (2006); Cannella et al. (2014)
Feedstock selection and engineering	Wanted to use a wide variety of feedstocks; option for biorefinery, & ,short rotation crops through sugar platform process	Use of less recalcitrant feedstocks and feedstocks that generate less pretreatment inhibitors	Larsen et al. (2012); Studer et al. (2011).
Culturing schemes	Effects on productivity and product output; inoculums adds to industrial process costs	SSF/CBP reduces sugar inhibition of feedstock; uses large sizes of inoculum	den Haan et al. (2013); Hoyer et al. (2010); Olofsson et al. (2010); Olson et al. (2012); Pienkos and Zhang (2009)
Bioabatement	Could take time and affect the sugar content	Microbial treatment	Cao et al. (2013).

Adaptive laboratory evolution (ALE), also known as evolutionary engineering, is a continuous process which is based on the selection of desired strains (Sauer, 2001). ALE requires three key components: (1) select for desired phenotypes; (2) evolve organisms; and (3) generate progeny within a lab that can be screened, according to the selection criteria. It is the approach through which diversity in performance of the microbial strains can be developed which is actually the initial point for metabolic engineering. It is an excellent tool for the production of yeast strains with various biotechnological applications such as production of fuels and chemicals (Querol et al., 2003). Lack of complete understanding of the physiology of microbes and exploiting evolution phenomena naturally to develop strains of interest are driving researchers to prefer this approach. This method can be employed easily on classical or random mutants that could be selected by providing selective pressure for growing on higher inhibitory conditions.

Environmental stress induces several alterations in bacterial or yeast genome and fitter strains that are adapted evolutionarily survived and selected (Galhardo et al., 2007). Connecting growth with production could be achieved with environmental manipulations, and has been seen quick improvements in microbial strains in several cases. The method is being successful in improving industrial yeasts strains which are not considered as GMO and can be commercialised easily. Adaptive evolution can be accomplished by delivering continuous culture conditions via chemostat and controlling the duplication rate of yeast strains, by keeping the environment fixed with the desired metabolites or inhibitors concentrations (Steensels & Verstrepen, 2014).

The technique adaptive evolution is suitable for microbes due to numerous reasons such as less generation time, less generation time, reproducibility, easy maintenance, large population size etc., (Elena & Lenski, 2003). Moreover, it has advantages in process which is simple passaging and do not require specialized equipment. However, the simple manipulations can be easily automated, and thus scaled up to hundreds of simultaneous experiments. It has been

successfully proven as a foundation of molecular & mechanistic cores of evolution. Albeit, variability in selection, making the organism cripple and improved in single feature are few drawbacks of this technique.

Several studies have reported improvement of bioethanol producing microorganisms by adopting ALE as shown in Table 2.4. The major applications of adaptive evolution in yeasts can be categorized into two; one for substrate utilization (glucose/xylose/co-fermentation) and another stress resistance against various inhibitors of the process. In order to enhance the yield of ethanol, it is required that yeast strain should utilize both pentoses and hexoses. Generally, single strain could not be able to utilize both of the sugars simultaneously. Recent studies have been concentrating on producing ethanol using *S. stipitis* (Melo-Ferreira et al., 2014) by xylose at industrial scale. The genes involved in this pathway XYL2 and XYL1 were transferred in *S. cerevisiae* using metabolic engineering and a recombinant strain developed which could utilize both glucose and xylose. Developing xylose utilizing strain through evolutionary engineering was also attempted by many researchers. In a study by Sonderegger and Sauer (2003), mutant with xylose utilization and 19% enhanced ethanol yield was developed using selection of strain after 460 generations under chemostat. Moreover, in spite of general fact that *S. cerevisiae* does not utilize xylose as sole carbon source, applying this evolutionary engineering approach, in a study by Attfield and Kletsas (2000), *S. cerevisiae* strains were growing on xylose

S. cerevisiae has been successfully evolved via adaptive evolution for more efficient lactose and arabinose fermentations (Guimarães et al., 2008). The genetic foundation for the lactose-fermentative adaptive strain's enhanced phenotype was investigated, and mutational events such as deletions in the promoter sequence of LAC genes, plasmid copy number reductions, and transcriptional differentiations were discovered (Guimarães et al., 2008). For multiple-stress resistant yeast mutants, batch selection for freezing–thawing stress resistance found to be the optimum technique. The best evolved strain had a 62-fold increase in ethanol stress

resistance, an 89-fold increase in temperature tolerance, and a considerable increase in freezing–thawing and oxidative stress tolerance (102-fold and 1429-fold, respectively).

In a study by Shui et al. (2015), furfural- and acetic acid-tolerant bacterial strains were developed by ALE. Under a 3 g/L furfural stress environment, the best mutant ZMF3-3 obtained a theoretical ethanol production of 94.84%, significantly higher than the 9.89% yield from ZM4. Given that furfural creates DNA-damaging free radicals in hydrolysates, the underlying mechanism could be that the hydrolysate acts as a mutagen capable of generating genetic variety in the chosen strain.

Furthermore, evolutionary adaptation procedures have been used to improve the fermentation capabilities of recombinant strains. By sub-culturing xylose-fermenting recombinant strains of *Z. mobilis* 39767 in a medium containing 10-50 percent hydrolysate, Lawford and his team improved the strains' tolerance to higher concentrations of acetic acid, and the adapted isolates showed a significant increase in ethanol productivity when compared to un-adapted strains (Lawford & Rousseau, 1999). Similarly, employing a long-term adaptation method of different serial selections for liquid and solid medium, a modified *E. coli* KO11 was created to withstand high ethanol concentrations. The mutants (LY01, LY02, and LY03) showed a survival rate of more than 50% in 10% ethanol (0.5 min exposure) and a reduction in fermentation time (Yomano et al., 1998) . Almost every prior attempt at evolutionary adaptation began with genetic engineering, which was then followed by adaptive selection (Kuyper et al., 2005; Sonderegger & Sauer, 2003; Wisselink et al., 2009).

However, a new technique involving genetic engineering, EMS mutation, and two-step evolutionary adaptation (under consecutive aerobic and oxygen-limited settings) has recently been tried (Liu & Hu, 2010). In comparison to the parental strain, the strain generated thus demonstrated a four-fold increase in its specific growth rate. Surprisingly, the activity of key xylose metabolism enzymes (XR, XDH, and XK) remains intact, implying that chemical mutagenesis and evolutionary adaption may have resulted in a new genetic characteristic that makes mutants capable of xylose metabolism (Liu & Hu, 2010).

Table 2.4: Strain improvement of ethanol producing yeasts for inhibitor tolerance via adaptive laboratory evolution

Inhibitory Conditions	**Fermentation Process**	**Microorganism**	**Improvements achieved**	**References**
High sugar (Osmotic stress)	Batch fermentation	*Saccharomyces cerevisiae*	Utilising 50% Glucose for ethanol production	Zhang et al. (2019)
Alternate substrate (Xylose) (Glucose+Xylose)	Batch and Continuous fermentation	*Saccharomyces cerevisiae*	Increased specific consumption of xylose	Koppram et al. (2012)
Inhibitory compounds (Acetic acid, Furfural, HMF Vanillin, Formic acid)	Batch and Continuous fermentation	*Saccharomyces cerevisiae*	Increased conversion of inhibitors, 50% increased ethanol productivity during fermentation	Koppram et al. (2012)
Ethanol	Batch fermentation	*Klyuveromyces marxianus*	Increased ethanol tolerance from 6% to 10%	Mo et al. (2019)
Ethanol + Temperature	Batch fermentation	*Saccharomyces cerevisiae*	Utilising 50% Glucose for ethanol production	Zhang et al. (2019)
Xylose + Acetic acid	Batch	*Saccharomyces cerevisiae*	Tolerating 20% ethanol, growing at 40°C temperature**, e**nhanced production of ethanol from xylose while tolerating 5 g/L of acetic acid	Ko et al. (2020)

2.5.2. Improvisation of fermentation process

A meaningful way to counteract the challenge of a higher cost of bioethanol production is the application of the SSF process by reducing the number of vessels, minimizing the intermittent operations, lowering the processing steps, reducing the enzyme inhibition, and minimizing the overall production time. An SSF process with high solid loading (above 100 g/L) is expected to significantly decrease bioethanol yield by increasing the viscosity, elevates stress for the microbes and enzymes via increased inhibitor concentrations and low mass and heat

exchange (Wingren et al., 2003). However, it is possible to avoid such inhibitions by combining SSF with intermittent feeding, i.e., carrying out the SSF process in a fed-batch mode.

During the fed-batch SSF process, the biomass feedstock is added intermittently in the fermenter, which does not allow the viscosity of the slurry to increase much, as the biomass is continuously liquified to sugars, which in turn are fermented to ethanol by the yeasts (Rudolf et al., 2005). Thus, there is no build-up of glucose or other sugars, and mixing and heat transfer are improved, thereby increasing the efficiency of enzymes and yeasts. The solid loading can be increased up to 200 g/L or even more by using the fed-batch mode, which is more than 2-fold of the solid loading employed in the batch mode. This intermittent substrate loading maintains the slurry viscosity at manageable levels, besides obtaining much better conversion yields and ethanol titers than the batch-SSF process. Moreover, low levels of free glucose in the slurry during FBSSF allow better conditions for co-fermentation of pentose and hexose sugars into ethanol using the recombinant microorganisms or the mixed microbial cultures. Further, the enzymes and the fermenting microorganisms encounter a significantly lesser concentration of the inhibitors during the FBSSF process, especially during the initial phase (Hoyer et al., 2010; Tomás-Pejó et al., 2009). The yeasts cannot only metabolize low concentrations of some inhibitors (furfural or HMF) but also get better adapted when the inhibitor concentrations are at lower levels (Hodge et al., 2008; Taherzadeh et al., 2000). FBSSF is also more effective in making the bioethanol production economics by allowing optimal dosage of enzyme and yeast inoculum (Gao et al., 2014; Wanderley et al., 2013; Zhang & Zhu, 2017).

Several studies demonstrated advantages of fed-batch over batch SSF **(Table 2.5)**. Lesser enzyme feedback inhibition and increased substrate loading are the main factors that improve the ethanol yield in fed-batch SSF (Gao et al., 2018). In most of the studies, commercial

enzyme formulations, mainly Cellic, CTec2 alone or in combination with glucosidase and other accessory enzymes, have been used. The use of thermotolerant yeast and cold-active cellulases can further improve the ethanol yield under SSF (Choudhary et al., 2017; Hemansi et al., 2021; Saini et al., 2015a).

Another research study compared ethanol titers and yields of batch and fed-batch SSF using sweet sorghum bagasse at high solid loadings in which biomass was fed either with or without proportional quantities of the enzyme and yeast inoculum, and fed-batch SSF proved much better than batch SSF (Darkwah et al., 2016). Fed-batch SSF has also been carried out using paddy straw as feedstock for bioethanol production (Shengdong et al., 2006). The fed-batch SSF was performed using the parameters statistically optimized in a batch SSF, and significantly higher ethanol titers were obtained than the batch processes by mitigating the mixing and mass transfer related problems of the batch process when using high biomass (Shengdong et al., 2006). In a recent study, Gao et al. (2018) produced a higher concentration of bioethanol by using high solid loading of the feedstock, i.e., sugarcane bagasse, by carrying out the SSF process fed-batch mode. The authors also reported better titer (75.57 g/L) and productivity of ethanol (Gao et al., 2018)

Table 2.5: Advantages of using fed-batch simultaneous saccharification and fermentation process for bioethanol production.

S. No.	Advantages	References
1.	As the sugars, generated during hydrolysis, are utilised and converted to ethanol instantaneously, the enzyme inhibition is mitigated.	Gao et al. (2014); Wanderley et al. (2013); Zhang and Zhu (2017)
2.	The process gets economic as enzyme loading can be controlled.	
3.	Mixing of the substrate is increased.	Stanbury and Whitaker (1984); Zhang and Zhu (2017)
4.	reduced viscosity due to pulsed addition of substrate, resulted in minimized content of insoluble solids in the medium	
5.	Reduction of substrate inhibition.	
6.	Ethanol yield can be enhanced by increasing the loading	
7.	overcome mass transfer problems	
8.	Reduction in power due to use of single vessel for hydrolysis and fermentation.	Hodge et al. (2008); Taherzadeh and Karimi (2008)
9.	Also, by using thermotolerant yeast for fermentation, energy can be saved which would have used for cooling down the vessel.	
10.	decreased fermentation time	
11.	higher productivity	Sotaniemi et al. (2016)
12.	higher dissolved oxygen in the medium	
13.	reduced toxic effects of the medium components	
14.	Increased yeast viability	