Simultaneous Saccharification and Fermentation of Lignocellulosic Biomass

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Abstract In recent years, with the growing concerns over the depletion of natural resources and food security, researchers are focusing on abundantly available non-food crops such as lignocellulosic biomass as alternative reserves for bioenergy. Since lignocellulosic biomass are a rich source of carbohydrates they can be used to produce various biological products through different fermentation strategies such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) along with consolidated bioprocessing (CBP). Among these, SSF has increased popularity for its cost-effectiveness and high product yield. The major advantages of SSF over SHF are the reduction in end product inhibition during saccharification, use of a single reactor for its operation and utilization of various lignocellulosic substrates under different pretreatment conditions that result in high product yield in short incubation time. However, certain drawbacks exist in SSF such as negotiation with the process parameters mainly temperature and pH; inability to utilize pentoses and low ethanol tolerance of fermenting strains. To overcome these limitations the authors are trying to emphasize a consolidated bioprocessing approach for utilization of pentoses and hexoses for improved bioenergy and other value-added product generation.

Keywords Lignocellulosic biomass · Simultaneous saccharification and fermentation · Biofuels

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1 Introduction

Need for alternative energy and cleaner air is increasing with every passing day due to rapidly growing industrialization, overpopulation, alarming GHG pollution due to the use of private transportation over public and multiple vehicles running from the same household. Understanding the need of the day, various biofuels have been produced with the advent of new technologies aiming at improved yield, low investment, higher biomass conversion efficiency and valorization of the by-products. The production of biofuels with comparable energy density to that of petroleum fuels requires a copious, low cost, and sustainable raw material that could suffice the incessant global demand. Lignocellulosic biomass comprising of recalcitrant lignin (10–25% w/w), crystalline and amorphous celluloses (40–50% w/w) and hemicelluloses (20–30% w/w) as the major entities is abundantly produced annually in the tune of 20×10^{10} tonnes that can serve as the potent raw material for various biofuel generation without disturbing the food-fodder supply chain (Zahid et al. 2014). The conversion of the biomass to value added products can be achieved by understanding the biochemical composition of the biomass, type and quality of the product desired and framing the minimum number of steps to economically recover the product. The fundamental steps in lignocellulosics to biofuel conversion include pretreatment, saccharification, and fermentation. Biomass pretreatment is performed prior to saccharification with the objective to degrade the lignin, reduce the degree of polymerization of holocelluloses, decrease the crystalline cellulose, and increase its amorphous counterpart and improve the yield and productivity of the reducing sugars upon enzymatic hydrolysis (Taherzadeh and Karimi 2007). The different methods of pretreatment such as physical (milling, pyrolysis, and irradiation), chemical (acid, alkali, and ionic liquid), physicochemical (ammonia fibre explosion, acid/alkali treatment-sonication) and enzymatic (laccase, lignin peroxidase, manganese peroxidase) are practiced of which enzymatic pretreatment is of particular interest when both celluloses and hemicelluloses are to be recovered with minimum loss under mild conditions. Saccharification is the hydrolysis of cellulose and hemicellulose polymers to easily fermentable pentoses and hexoses using hemicellulases and cellulases of fungal/bacterial origin. Fermentation of these reducing sugars to value-added deliverables such as bioethanol can be achieved by using wild-type hexose/pentose-fermenting strains or genetically engineered mixed sugar fermenting strains either in free or immobilized state. It is imperative to comprehend that hindrance at any of these unit operations ultimately affects the product thus an integrated approach must be followed to improve ethanol yield. In an attempt to reduce the fermentation time and increase the amount of substrate processed per given volume of hydrolyzing enzymes and fermenting cells different fermentation strategies such as sequential/separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP), etc., have been adopted world-wide. The present chapter emphasizes on various aspects of SSF viz. lignocellulosic substrates for SSF, biological agents involved

and the factors effecting the process, different modes of operation for commercialization, constraints in SSF, their mitigation strategies and the major commercial products generated during fermentation in SSF.

1.1 Lignocellulosic Substrates

The first challenging step towards commercial ethanol production is the selection of the raw lignocellulosic biomass and its collection, transportation and storage for large-scale ethanol production. Type of lignocellulosic material chosen decides the time required for pretreatment which depends on the lignin content of the biomass and for those biorefining industries which rely on contract farming for their biomass needs, cost of biomass would be the most influential factor affecting the cost of the entire process. Therefore, a thorough knowledge on available feedstocks in the local regions is essential. The abundantly available lignocellulosic biomass in general can be classified into edible crop varieties such corn, sugarcane, sugar beet, wheat, pineapple, sorghum, etc. These food crops have high holocellulose and low lignin content and thus could be easily hydrolyzed and fermented to ethanol without any pretreatment of the biomass. But it is essential that the feedstock for commercial ethanol production should be sustainable, available and must have nil or barely any consumption as food commodity so as to avoid the food versus fuel controversy. However, the residues from these food crops such as bagasse, rice husks, wheat straw, wheat bran, sugarcane tops, coconut shells, maize cobs, jute sticks, chilly stalk, cotton stalk, etc., considered as fodder/grazable crop residues can be used to produce biofuels. But as these residues are limited to a particular season after harvesting period their availability cannot be ensured throughout the year. Moreover, the agricultural residues are produced in decentralized fashion and thus incur high transportation cost making it uneconomical to use agricultural residues as the primary substrates for biofuel production. Also, these residues vary as a function of regional production, harvesting, processing and storage methods.

Other important feedstocks for biofuel production are forest residues which includes non-harvested biomass or that obtained from commercial hardwood and softwood processing locations, from thinning of forests done as a part of management operations and from dead and dying trees (wood chips, sawdust, dried leaves, tree barks, etc.). These residues can contribute 65% of the biomass energy potential. Several reports have stated their use for bioenergy production at district level through suitable designs of decentralized smaller plants. Nevertheless, the limitations such as the extraction costs, transportation to centralized processing plants make forest fuels expensive.

Therefore, considering these limitations it is appropriate to use the whole plant of non-edible lignocellulosic varieties for fuel production such as *Parthenium* sp., *Miscanthus giganteus,* napier leaves, purple guinea, *Saccharum spontaneum*, switchgrass, silver grass, etc., which either do not have food applications due to the presence of toxic compounds or the same demand as food crops. Example includes

Table 1 Biochemical composition of various lignocellulosic substrates (% dry weight)

S.no.	Lignocellulosic biomass	Cellulose	Hemicellulose	Lignin	References
1	Newspaper	25–40	40–55	18–30	Limayem and Ricke (2012)
2	Switch grass	30–35	40–45	12	
3	Waste papers from chemical pulps	12–20	50–70	6–10	
4	Nut shells	25–30	25–30	30–40	Sun and Cheng (2002)
5	Corn cobs	45	35	15	
6	Coastal Bermuda grass	25	35.7	6.4	
7	Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7	
8	Swine waste	6.0	28	NA	
9	Primary wastewater solids	8–15	NA	24–29	
	Sugarcane leaves	36%	28%	20%	Shields and Boopathy (2011)
10	*Lantana camara*	47.25	18.23	19.25	Kuila et al. (2011)
11	*Ricinus communis*	42		19.8	Mukhopadhyay et al. (2011)
12	*Saccharum spontaneum*	38.7	29	17.46	Rajak and Banerjee (2015)

NA Not available

Lantana camara, which contains toxin of the family Lantadene whereas *Ricinus communis* and Jatropha contain ricin and forbol toxin respectively. Thus, non-edible lignocellulosics are gaining more importance due to their less/no competition as food/fodder and sustainable nature. The biochemical composition of various lignocellulosics is summarized in Table 1. Also, a mixture of these lignocellulosics can be employed to ease the laborious process of collection of huge quantity of single type biomass and to run the biorefinery in all seasons with the available lignocellulosic mixtures.

2 Biological Agents

The major biological agents that are involved in saccharification and fermentation process include cellulolytic enzymes that hydrolyze holocelluloses into simple sugars and ethanologenic microorganisms that are involved in ethanol production.

2.1 Cellulase and Xylanase and Their Types

Lignocellulosics are predominantly composed of cellulose, a homopolymer of glucose units linked by β 1-4 glycosidic bonds and hemicellulose which is a

heteropolymer constituting various proportions of monosaccharide units such as D-xylose, L-arabinose, D-glucose, D-mannose, D-galactose, D-glucuronic acid, and D-galacturonic acid. In order to obtain simple sugars for ethanol production, these polymers should be broken down by cellulase and xylanase. These enzymes are produced by numerous microbial sources viz., yeast, bacteria, protozoans, snails, crustaceans, and fungi among which *Trichoderma* sp. is the most prominent fungal source having efficiency to produce both the enzymes at a time. Cellulase and xylanase are classified (Sadhu and Maiti 2013) depending on their mode of action, structural properties, and substrate specificity which are tabulated as in Table 2.

Trichoderma reesei produces two exoglucanases- CBHI and CBHII, seven β- glucosidases-BGI-BGVII and eight endoglucanases- EGI-EGVIII. Cellulase system of *Humicola insolens* is homologous to that of *T. reesei* and contains seven cellulases (CBHI and CBHII, EG-I, II, III, V, VI). The isotypes of exo, endo and β-glucosidases differ from each other in the molecular weight, topology, pH and isoelectric point. For instance, CBHI and CBHII mainly differ in their molecular weight, i.e., 52.2 and 47.2 KDa respectively. BGI and BGII on the other hand differ in their secondary structures, i.e., α and β barrel shaped structures respectively. The optimal pH for different types of cellulases varies with the substrate mostly in the range of 4.2–5.2 and isoelectric point are in the range of 4.5–7.2. Cellulase is an inducible enzyme whose production is controlled by activation and repression mechanisms. In *T. reesei*, genes are coordinately regulated, where cellulolytic enzyme is induced in the presence of cellulose-rich substrates and repressed in the presence of excess glucose. The most probable inducers of *Trichoderma* sp. cellulase system are sophorose and lactose.

A wide range of microorganisms can produce xylanases of which bacteria and fungi are proficient producers. There is a significant difference between the bacterial and fungal xylanases. The bacterial xylanases show low activity than that of the fungal xylanases and additionally they do not undergo post translational modifications. In order to breakdown heteropolymeric xylan, synergistic effect should exist between the xylan degrading enzymes.

Fungal endoxylanases are mostly glycosylated single subunit proteins with molecular weight ranging from 8.5 to 85 kDa and isoelectric point between 4.0 and 10.3. ß-xylosidases may exist as mono, di or tetramer with 26–360 kDa. Arabinofuranosidases mostly exist as monomers, but dimeric, tetrameric, and octameric forms have also been found. The molecular weights are in the range of 53–495 kDa, pI from 3.6 to 9.3 and optimum pH ranges from 2.5 to 6.9.

2.2 Yeasts and Other Microbes for Fermentation

Saccharomyces cerevisiae is most commonly employed strain for the commercial ethanol production. However, ethanol producing bacteria (EPB) like *Zymomonas mobilis* is attracting much attention owing to its faster growth rate, high sugar uptake, high ethanol tolerance up to 16% (v/v) and ability to ferment under low/no

Table 2 Classification of cellulase and xylanase

Enzyme	EC number	Mode of action
Cellulase		
• Endocellulase (Endoglucanse (EG), Endo-1,4-β-glucanse, Carboxymethyl cellulase, β-1,4-endoglucon hydrolase)	EC 3.2.1.4	Random cleavage of cellulose at amorphous sites yielding glucose and cellooligosaccharides
• Exocellulase (cellobiohydrolase (CBH) or glucanase)	EC 3.2.1.91	Releases cellobiose either from reducing or non-reducing ends of cellulose chain by hydrolyzing 1,4-β glycosidic linkages
• Cellobiases (β-D-glucosidase (BG), gluco-hydrolases, cellobiase)	EC 3.2.1.21	Releases glucose from cellobiose and short chain cellooligosaccharides
• Oxidative cellulase	NA	Deploymerize cellulose by radical reaction
• Cellulose phosphorylase	NA	Depolymerize cellulose using phosphates instead of water
Xylanase		
• Endo-β-(1,4)-D-xylanase (β-(1,4)-D-xylan xylanohydrolase)	EC 3.2.1.8	Randomly act on xylan to produce xylooligosaccharides of various chain lengths
(a) Non-arabinose liberating endoxylanases-I		Cannot act on L-arabinosyl initiated branch points at β-(1,4) linkages and produce only xylobiose and xylose by breaking xylooligosaccharides
(b) Non-arabinose liberating endoxylanases-II		Cannot cleave branch points at α-(1,2) and α-(1,3) and produces xylooligosaccharides
(c) Arabinose liberating endoxylanase-I		Produces xylobiose, xylose and arabinose by cleaving xylan at branch point
(d) Arabinose liberating endoxylanases-II		Produces xylooligosaccharides and arabinose by cleaving at branch point
• Exo-β-(1,4)-D-xylanase (β-(1,4)-D-xylan xylohydrolase)	NA	Remove single xylose units from the non-reducing end of the xylan chain
• β-xylosidase (xylobiase)	EC 3.2.1.37	Hydrolyzes xylobiose and xylooligosaccharides
• α-Glucuronidase	NA	Hydrolyzes α-1,2 bond present between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan

NA Not available

oxygen conditions. Some natural ethanologenic yeast species such as *Pichia stipitis, Candida shehatae*, *Kluyveromyces marxianus,* and *Pachysolen tannophilius* appeared to have efficiency in utilizing pentoses which can be used to co-ferment along *S. cerevisiae* for complete lignocellulosic ethanol fermentation.

Fermenting pentose sugars present within the saccharified broth is one of the main bottlenecks which restrict the commercialization of ethanol production. In

order to circumvent this problem, Microbial Biotechnology and Downstream Processing laboratory, IIT Kharagpur has isolated a new pentose fermenting strain from the local soil of IIT Kharagpur which could utilize both C5 and C6 sugars. The process was further optimized for SSF of *L. camara* and *R. communis* and the optimized conditions yielded 28.77 and 35.48 g/L ethanol respectively. The result obtained is competitive with the reported literature. Similar attempt was made by Silva et al. (2011) in which *P. stipitis* NRRLY7124 was grown on xylose (90 g/L) as the carbon source under aeration rate of 0.25 vvm at 250 rpm. Under these experimental conditions, the ethanol production was observed to be 26.7 g/L.

Suriyachai and co-workers conducted a study using pretreated rice straw and subjected it to simultaneous saccharification and co-fermentation (SSCF) using *S. cerevisiae* and *Schefferomyces stipitis*. Under optimized SSCF conditions, i.e., 0.31:0.69 cell ratio (*S. cerevisiae*: *S. stipitis*) at 33.1 °C under agitation speed of 116 rpm, the maximum ethanol concentration was found to be 28.6 g/L at 10% biomass concentration in 72 h (Suriyachai et al. 2013). This comparative analysis indicates the efficiency of the newly isolated strain for SSF. The work conducted with this strain revealed that inoculum volume 9–10% (v/v), substrate concentration 18–19% (w/v), incubation time 48 h, 37 °C, inoculum age 48 h are optimum conditions for maximum ethanol yield from substrates like *L. camara* and *R. communis*. As this strain works at mild environmental conditions it has wide scope for industrial ethanol production. However, further improvement in both the ethanol concentration and the process parameters namely, substrate loading and incubation time is vital such that more amount of substrate can be converted to ethanol in shorter incubation time to make it commercially feasible.

In order to compete with the ethanologenic microorganisms, the wild strain of *S. cerevisiae* which can only ferment glucose should be genetically modified so that it can ferment both pentose and hexose sugars for enhanced ethanol production. Employing the microorganisms having both cellulolytic and ethanologenic activity for SSF is another viable alternative for cost effective ethanol production.

2.3 Yeast Growth Studies

2.3.1 Immunofluorescence and FACS Analyses

Immunofluorescence is performed to visualize the cellular features of yeast by conjugating the dyes like fluorescein isothiocyanate and rhodamine B with monospecific antibodies which are raised against yeast structural proteins. Confocal laser beam immunofluorescence microscopy can be used to detect the intracellular localization of proteins in the yeast cell and for its three dimensional ultrastructural information. Fluorescence-activated cell sorting (FACS) helps in studying the cell cycle of yeast and in monitoring changes in organelle biogenesis. Scanning electron

microscopy (SEM) and transmission electron microscopy (TEM) are useful in revealing the surface topology and intracellular fine structures of yeasts (Walker 1998).

2.3.2 Cytometric and Spectrophotometric Analyses

The density of cells in a yeast culture can be determined by direct counting in a haemocytometer chamber and by measuring optical density at 600 nm in spectrophotometer. Wild-type yeast strains with OD_{600} of 1 correspond to $\sim 3 \times 10^7$ cells/mL. Mutations affect the cell size or shape, thereby altering the OD. Some mutant strains exhibit clumpy phenotype resulting in inaccurate density measurements. In such situation, the clumps should be dispersed by mild sonication prior to counting and density measurement. Another method practiced for cytological and physiological studies of the yeast is flow cytometry. It has been developed to determine size, membrane potential, intracellular pH, and levels of cellular components such as DNA, surface receptors, protein, and calcium.

3 Fermentation Strategies

The reducing sugar-rich hydrolyzate of lignocellulosic biomass obtained after enzymatic saccharification is composed of C6 sugars (glucose, mannose, and galactose) and C5 sugars (xylose and arabinose) which theoretically yield 0.51 g ethanol per 1 g glucose/xylose. However, the molecular conversion of glucose to ethanol is slightly higher than xylose (i.e., 1 glucose molecule gives 2 molecules of ethanol and 1 xylose molecule gives 1.67 molecules of ethanol) (Okamoto et al. 2014). To reach the theoretical conversion of the substrate to ethanol the following fermentation strategies can be adopted. The various parameters to be considered for selecting a particular fermentation strategy are incubation time, inoculum volume, temperature, labour involved, and substrate loading into the bioreactor per batch, all of which directly influence the overall ethanol yield.

3.1 Separate Hydrolysis and Fermentation (SHF)

The process of conducting saccharification and fermentation of pretreated lignocellulosic biomass in separate tanks under different reaction conditions is defined as SHF. The holocellulolytic enzymes (cellulases and xylanases) efficiently hydrolyze at 45–50 °C while the generally used fermenting strains produce ethanol between 30 and 37 °C. SHF gives the liberty to conduct both the unit operations under their respective optimum conditions. Based on the substrate used and the microbial source of the enzymes, the reaction conditions for conducting separate hydrolysis

may slightly vary. The major disadvantage in SHF is that the hydrolysis products mainly glucose and its corresponding disaccharide, cellobiose inhibit cellulase action. But the cellulase inhibitory concentration of cellobiose is slightly higher than glucose indicating that glucose has stronger inhibitory effect on cellulase. When cellobiose concentration in the hydrolyzate was 6 g/L the residual cellulase activity was 40% of the initial activity and when glucose concentration was 3 g/L, the residual activity of β-glucosidase unit of cellulase was 25% of the initial activity (Taherzadeh and Karimi 2007). Another constraint in SHF is the hydrolysis of holocelluloses to sugars followed by their separation from saccharified biomass and then separate fermentation of sugars to ethanol which is a two step process that is laborious, additional cost incurring, and time taking. These drawbacks can be avoided by simultaneous conversion of sugars to ethanol within the same reactor.

3.2 Simultaneous Saccharification and Fermentation (SSF)

SSF is the process of conducting saccharification of the pretreated lignocellulosic biomass and the concomitant fermentation of reducing sugars to ethanol in the same fermenting vessel. This phenomenon is practically feasible when the optimum working temperatures of cellulases/xylanases meet as closely as possible to that of fermenting microbial strain because the raw material for fermentation is the end product of saccharification. Thermophilic bacterial and yeast cells such as *C. acidothermophilum* and *K. marxianus* can be used as fermenting strains for conducting SSF without compromising the optimal temperature of hydrolysis.

The major advantage with SSF is that it can be adopted to process any cellulose-rich biomass without the problem of cellulase inhibition by glucose or cellobiose. This is due to the fact that before reaching the inhibitory concentrations of cellobiose/glucose, these sugar molecules are concurrently fermented to high-energy density ethanol molecules. Therefore, SSF improves the ethanol yield in shorter incubation time, reduces the cost of investment and operation as one reactor suffices the work of two thereby cutting down the labour involved in separation of residual biomass from the sugar rich hydrolyzate. Also, it checks the microbial contamination of sugars due to the presence of ethanol in the same vessel (Ohgren et al. 2007). The main drawback in this process is that though hexoses are efficiently converted to ethanol, pentoses are either neglected (when working with only hexose fermenting strain such as *S. cerevisiae*) or separated after pretreatment (such as dilute acid) into separate tank to be fermented to ethanol using a pentose utilizing strain. Genetically engineered mixed sugar utilizing strains can solve this issue and enable fermentation of both the sugar types within the same reactor.

Moreover, the use of lytic polysaccharide monooxygenases (LPMOs), an oxidative metalloenzyme along with cellulase is found to enhance the cellulose degradation in lignocellulosic biomass. LPMO requires molecular oxygen as electron donor for carrying out oxidation of C_1 or C_4 in the scissile β-1,4-glycosidic bonds. When SSF for ethanol production is carried out using this enzyme cocktail

and yeast, the fermenting strain competes with LPMO in the cocktail for the molecular oxygen, thereby creating anoxic environment detrimental to LPMO. Hence the processing strategy has been shifted to SHF instead of SSF where the conditions are more favourable (Cannella and Jorgensen 2014). In another study, Muller et al. (2016) reported that the combination of LPMO-containing cellulase cocktail and fermenting microorganism resulted in maximum lactic acid production under SHF over SSF. These studies indicate that the oxidative and hydrolytic enzyme cocktail works well with SHF rather than SSF strategy.

3.3 Non-isothermal Simultaneous Saccharification and Fermentation (NSSF)

When thermophilic microbial strains are used for SSF as a substitute of *S. cerevisiae* to ferment at 45–50 °C in order to reach the activation energy of cellulase/xylanase, the yield of the main end product viz. ethanol was found to drastically decrease and other by-products such as acetic acid and lactic acid was increased rendering the entire process uneconomical. In an attempt to increase the ethanol yield and overcome the drawbacks of SHF (i.e., cellulase inhibition by hydrolysis products) and SSF (i.e., deviation from optimal temperature), a novel strategy of non-isothermal simultaneous saccharification and fermentation (NSSF) was proposed. This process involves a presaccharification step either in the same or in separate reactor vessel at optimum temperature (50 °C) to maximize the reducing sugar yield. It was reported that cellulase activity increased 2–3 times when the reaction temperature was gradually increased from 30 to 50 °C. The effluent after saccharification comprising of pentoses, hexoses and un-hydrolyzed biomass is pumped to another fermenter vessel or inoculated in the same vessel with microbial cells and maintained at 30–37 °C which is the optimum temperature for metabolism of mesophilic ethanologenic strains. This process was reported to reduce the volume of enzyme needed for hydrolysis by 30–40% by improving the enzyme-substrate kinetics and drastically decreased the fermentation time from 4 days through SSF to 40 h by NSSF (Wu and Lee 1988).

3.4 Simultaneous Saccharification and Co-fermentation (SSCF)

A novel co-fermentation strategy called as SSCF which is the further improvement of SSF has been adopted where the loss of pentose sugars (xylose and arabinose) obtained upon hydrolysis of hemicelluloses is well addressed. The pentose-rich fraction is integrated with hexose stream within the same reactor and fermented using genetically engineered variants of *Z. mobilis, Escherichia coli, S. cerevisiae,*

etc., having genes, enzymes cascades, and sugar transport systems for both hexose and pentose fermentation (Bothast et al. 1999). These strains first metabolize glucose to their primary metabolic product (ethanol) and then use pentose for fermentation. This integrated approach allows maximum utilization of the lignocellulosic biomass and improves the ethanol yield compared to SHF and SSF. Although, genetically engineered strains are used for hexose and pentose sugar utilization, high concentration of glucose can inhibit xylose metabolism making co-fermentation of sugars challenging. This is due to the competition between glucose and xylose for the same transport system to enter into the cell (Meinander and Hahn Hagerdal 1997). Moreover, the affinity of glucose towards the glucose transport system is 200 fold higher than xylose (Kotter and Ciriacy 1993). In order to overcome this problem, continuous mode of co-fermentation can be adopted by adjusting the dilution rate so as to keep glucose concentration in the system below 2.3 g/L for rapid fermentation of both glucose and xylose (Chen 2011).

On the other hand, when co-fermentation of sugars is done using two different strains for hexose and pentose fermentation, compatibility of these strains also needs to be investigated since it may cause end-product inhibition wherein ethanol produced from glucose may inhibit the xylose fermenting strain due to its low ethanol tolerance.

Besides, it is also important to consider that ethanol yield beyond 30 g/L is inhibitory to the cellulase activity (Wyman 1996). In this process the ethanol yield may shoot up beyond the inhibitory concentration as there is an opportunity for mixed sugar utilization depending on the holocellulosic content of the biomass and the reaction conditions. Therefore, continuous stirred tanks reactor systems are ideal in such circumstances where the ethanol produced in the fermentation broth is continuously drawn out of the system. Another mode of operation is fed-batch mode of co-fermentation, where the initial viscosity of the reactants is maintained low to allow easy mixing and avoid mass transfer limitations with in the fermenter thus leading to high ethanol yield (Liu and Chen 2016).

3.5 Direct Microbial Conversion (DMC)/Consolidated Bioprocessing (CBP)

Hydrolysis step of the ethanol fermentation process is the major constraint in ethanol commercialization due to the high cost of the enzymes used for saccharification. Scientists have come up with a probable solution to this expenditure to combine holocellulolytic enzyme production with that of ethanol production rendering the process self-sufficient from start to finish. This approach is advantageous as it reduces the cost for biomass processing and decreases the number of fermenters needed for plant operation as it is a single step system (Mbaneme-Smith and Chinn 2014). The direct microbial conversion of the biomass can be achieved by using genetically engineered strains with excellent cellulolytic and ethanologenic activities. Therefore, an efficient ethanol producing strain can be modified to

express genes for cellulases and xylanases or use the finest holocellulolytic strain and metabolically engineer it as a superbug being able to ferment both hexoses and pentoses (Linger and Darzins 2013). Several anaerobic microbes such as *Clostridium thermocellum*, *Neurospora crassa*, *Paecilomyces* sp. and *Monilia* sp. with cellulolytic activities and high temperature resistance have been reported to have the potential of DMC. Thermophile namely *Caldicellulosiruptor* sp. is an important DMC strain as it can directly hydrolyze the raw lignocellulosic biomass without any delignification step.

The challenge in CBP system is that the microorganisms within the fermenter come across various toxic compounds during biomass pretreatment such as phenolics, furan derivatives, etc. that inhibit their growth and metabolism and ultimately affect the ethanol yield (Hasunuma and Kondo 2012). In this context, detoxification of the hydrolyzate is being used nowadays, but however this additional step adds to the cost of the process on a large scale basis. To avoid this additional step, genetically engineered inhibitor tolerant variants of *S. cerevisiae* have been developed (Larsson et al. 2001).

4 Factors Affecting SSF

There is a tremendous need to select the factors which have significant effect on the ethanol yield. Solid and enzyme loading, yeast concentration, temperature, incubation time, pH and additives have profound influence on ethanol production which is discussed in detail.

4.1 Solid Loading

One of the major factors that can contribute to high ethanol production is optimum solid loading. Increase in solid loading should lead to increased ethanol yield but, in practice, that does not occur and is a challenging task for the scientific community to resolve it. Generally, increase in solid loading increases the reducing sugar concentration up to certain percentage of solid loading and after that it declines. High solid loading results in improper mixing of substrate and enzyme that may lead to limited cellulose conversion to reducing sugars. Moreover, increased viscosity of fermentation broth and mass transfer limitations reduces the efficiency of enzymatic hydrolysis and fermentation. The viscosity problem can be well addressed by adding the substrate gradually rather than adding at once.

4.2 Enzyme Loading

The enzyme loading during SSF should be minimized to reduce the cost of the ethanol production process. The enzyme concentration should be maintained in such a way that it can interact with the maximum substrate available in its vicinity. A decrease in enzyme loading results in less accessibility of enzyme towards the available substrate leading to reduced ethanol yield and subsequently increases the duration of ethanol production. According to techno-economical calculations, 50% reduction of enzyme loading is beneficial if the decrease in the product yield is nearly 6–7% and increase in the residence time is not more than 30% (Sassner et al. 2008).

4.3 Incubation Time

During SSF, the reducing sugar obtained due to hydrolysis of polysaccharides by cellulolytic enzymes is utilized by the ethanologenic microorganisms simultaneously for their growth and ethanol production. The production of ethanol gradually increases and after certain incubation period it declines. The probable reason for the decline in ethanol production is the enzyme inactivation or product inhibition of fermenting strain. Kitagaki et al. (2007) reported that yeast cell has capacity to tolerate ethanol up to certain concentration, above which its growth gets inhibited leading to the damage of the cell. Ethanol-induced yeast cell death occurs due to stress and changes in protein structure, membrane fluidity, mRNA export from the nucleus.

4.4 Temperature

Temperature has immense effect on activity of cellulolytic enzymes, growth and other metabolic activities of yeast cell. The optimum temperature required for the cellulolytic enzymes (50 °C) and yeast (30–37 °C) are different and if SSF is to be performed then there should be a compromise between the two optimal temperatures which may affect the ethanol yield. Ethanol production reduced considerably at high temperature. The plausible reasons might be denaturation of cellulase, shortened exponential phase of the yeast, change in membrane fluidity, increased accumulation of ethanol in the cell, etc. Through employment of thermotolerant yeast strains like *S. uvarum, Fabospora fragilis, Candida brassicae, Candida lusitaniae, and Kluyveromyces marxianus* the temperature of SSF can be maintained closer to that of optimal temperature of cellulolytic enzymes.

4.5 pH

Each and every microorganism possesses a pH range for its growth and activity and deviation from the optimum value results in decrease in their growth and product formation. During SSF, due to high pH, cellulase is destabilized and yeast loses its osmotic balance. The optimum pH for *S. cerevisiae* BY4742 lies in the range of 4–5. The pH lower than 4, prolongs the incubation period for ethanol production and the pH above 5 reduces the ethanol yield substantially. The pH below 4 and above 5 favours the formation of acetic acid and butyric acid respectively (Lin et al. 2012).

4.6 Inoculum Volume

The inoculum volume has impact on the duration of lag phase, specific growth rate, and ethanol production. The higher inoculum loading decreases the lag phase duration. The increase in inoculum volume leads to gradual increase in ethanol yield up to certain extent and after that it does not show a significant increase in ethanol yield. In the industrial perspective, low yeast loading with more ethanol yield is advantageous. The substrate for yeast production also plays a key role in the cost of ethanol production. When yeast is cultured using expensive substrate/medium, higher yeast loading during SSF is not economical. Though volumetric productivity of ethanol is dependent upon yeast loading, the enzymatic hydrolysis is the rate determining factor during SSF.

4.7 Effect of Additives

Supplementing of growth medium of *S. cerevisiae* with additives like yeast extract, peptone, malt extract, and ammonium sulphate enhances the ethanol yield. Supplements also improve the sugar utilization by the yeast cells which might be possible reason for higher ethanol productivity. Yeast extract provides cofactors like biotin and riboflavin to enhance the growth of yeast. Though these supplements enhance the ethanol yield, they cannot be used at industrial scale as they are very expensive. Among the low-price supplements to the yeast medium like sunflower, safflower oil seed meal cakes, wheat mash, groundnut, soy flour, safflower oilseed meal cake resulted in higher ethanol yield. The safflower oil cake is rich in polyunsaturated fats and unsaturated fatty acids which rendered higher ethanol tolerance to yeast (Ding et al. 2009). Also, reducing sugar-rich hydrolyzate obtained after saccharification of lignocellulosic biomass can be used as a cost effective and sustainable substitute for commercial media for the growth of ethanologenic microorganisms.

5 Modes of Fermentation for SSF

The ethanol production with yeast majorly depends on the substrate utilized for its production and mode of fermentation process. Fermentation can be performed majorly by batch, fed-batch, and continuous modes and its selection depends on the kinetic properties of the microorganism and cost of the process.

5.1 Batch Fermentation

Batch fermentation is a traditional and most commonly practiced process for ethanol production due to its low investment cost, easy feedstock management, and its flexibility. During this process, feed constituting substrate, yeast and other nutrients required for ethanol production are charged into the fermenter and after specific incubation time, entire ethanol is recovered. The high sugar concentration imparts substantial osmotic stress on yeast, thus slowing down the rate of fermentation leading to low productivity. The other disadvantages of the process are labour intensive and time consuming as considerable amount of time is wasted in each batch for cleaning, sterilization, inoculum growth, and harvesting. The problem associated with the high sugar loading can be addressed either by fed-batch addition of sugars or by employing a continuous fermentation process in which yeast cells are not subjected to osmotic stress.

5.2 Continuous Fermentation

In the continuous fermentation process, feed is pumped continuously into the fermenter where microorganisms are active. During this process, the addition of feed into the fermenter and removal of fermented broth containing ethanol, biomass, and residual sugars occurs at the same rate which leads to the maintenance of constant liquid volume inside the fermenter. Though the process is less labour intensive, contamination is a serious problem with this process since the system is interrupted several times. Another serious issue with this process is the loss of active yeast cells during removal of fermentation broth. The problem can be addressed by growing yeast at the same rate as that of the dilution rate to avoid washout of the cells or by employing the flocculated yeast or by immobilized yeast cells (Taherzadeh et al. 2001).

The productivity of the process can be improved by reusing the enzymes and inoculum involved in ethanol production. In order to recycle the cells, the concentration of non-yeast insoluble solids should be low and high yeast cell viability should be maintained. The cells can be separated from the medium through centrifugation or sedimentation and reused for the subsequent batches till the cells

tolerate the ethanol concentrations. To improve the fermentability, high ethanol tolerant strain should be employed. The thermotolerant *S.cerevisiae* (IR2-9a) produce significantly high ethanol of 28 g/L from bleached kraft pulp compared to native strain of *S.cerevisiae* (16 g/L) (Edgardo et al. 2008)

5.3 Fed-Batch Fermentation

The fed-batch mode of operation is considered as a combination of both batch and continuous operations and is predominantly practiced in alcohol industries. During this process, yeast inoculum is added initially to a small amount of media and then fresh medium is added continuously at regular intervals without removing the fermented broth. Intermittent pumping of substrate maintains the sugar concentration in the reactor and prevents the osmotic stress on the yeast thereby enhancing ethanol yield. Implementation of cell recycling along with fed-batch cultivation can improve the volumetric productivity of ethanol (Sanchez and Cardona 2008). Besides, it is suitable for dilute acid hydrolyzate fermentation as high concentrations of inhibitors can be avoided during the process (Taherzadeh et al. 2000).

The ethanol productivity can be enhanced by concentrating the cell biomass in the fermenter through immobilization and recirculation of cells. The process can be made economical by improving ethanol productivity using small fermenter and coculture of microorganisms for effective substrate utilization.

6 Major Commercial Products of SSF

Over the last few decades, SSF has shown an increasing trend in bioprocesses and bioproducts and has been attributed for producing primary metabolites that has numerous practical advantages.

6.1 Ethanol

Bioethanol production by SSF from lignocelluloses (energy crops, forest, and agricultural residues) shows prospective advantage over first generation bioethanol from an environmental and sustainable perspective. Though ethanol can be produced by SHF, SSF due to its reduced investment cost, low water requirement and reduction in end-product inhibition of the enzymatic hydrolysis makes it a preferred method. During ethanol production by SSF, there are other co products formed along with it, such as lactic acid, acetic acid. Vincent et al. (2011) reported the production of ethanol using corn stover using *S. cerevisiae* and *E. coli* K011 which showed highest ethanol concentration of 2.29 g/100 g corn stover and 4.79 g/100 g

corn stover respectively. Acetic acid and lactic acid were also monitored with acetic acid production in the range of 0.45 and 0.78 g/100 g corn stover while no lactic acid was detected. The major constraint in the ethanol production process is the separation of ethanol from fermented broth defined as distillation. In order to overcome this constraint various separation technologies have been investigated so as to separate ethanol in best possible way so that ethanol recovery cost is reduced. Due to high energy requirements in distillation, various other separation technologies have been investigated for energy efficiency. Fermentation broth containing low ethanol concentration can be separated by applying pervaporation which economically viable than distillation. It is the process employed for separation of ethanol from the fermented broth through partial vaporization using porous/non-porous membranes.

6.2 Butanol

Butanol, a colourless liquid is miscible with organic solvent and is less hydroscopic and less corrosive than ethanol. It can be used for many purposes such as fuel replacing gasoline and fuel additives, solvents for certain pharmaceutical products and as diluents for brake fluid. Biobutanol was first industrially synthesized during 1912–1914 by Acetone–Butanol–Ethanol (ABE) fermentation of cereal grains and molasses using *Clostridium acetobutylicum* (Jones and wood 1986). Few strains producing high biobutanol yield was also identified such as *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* (Keis et al. 2001). Su et al. (2015) compared the efficiency of SHF and SSF by using the hydrolyzate of sugarcane bagasse using *C. beijerinckii* NCIMB 8052 and demonstrated that SSF was able to produce high butanol concentration of 6.4 g/L and total ABE of 11.9 g/L which was comparatively higher than SHF.

7 Scale up, Mass Balance, and Economic Feasibility of SSF

In the present scenario, development of economical technologies for production of biofuels and other value added platform chemicals and biochemicals from lignocelluloses is an important issue among the researchers, private companies and government. Scaling up is a major task and is the basic step in making a process practically applicable on an industrial scale. The different factors that help in the scaling up of the process are the laboratory experiments, derivation of kinetic correlation, mathematical modelling, design and operation of pilot plant. Process engineering tools are very much required along with innovative process configuration that is aimed at reducing energy and developing an ecofriendly technology.

For scaling up of a process, mass balance is very important criteria in processing so as to maximize yield of product and minimize the investment cost. When designing a new process or investigating a present one, mass balance is an important factor that helps in calculating the mass flow rates passing through different physical, chemical or biological processes.

Economic feasibility is an important aspect when adopting a particular process. Ideally the cost of investment must be lower than that of the market price for ethanol which is determined by type and composition of the feedstock, cost of biomass, ethanol plant volumetric capacity, and biomass conversion efficiency. Therefore, it is crucial to evaluate the cost of the process at each unit operation specially the rate limiting step to calculate the process economics and anticipate the commercial feasibility.

The economic feasibility of 2G ethanol from lignocellulosics is primarily influenced by an effective strategy to achieve minimal ethanol selling price (MESP) and second, by the various value added platform chemicals and other biochemicals that can be derived from the residues obtained after ethanol production. The maximum profitability in form of multiple products starting from the same amount of biomass can lead to an integrated bioprocessing approach. Some of the commercially significant deliverables that can be produced from the residues of lignocellulosic ethanol refinery include lactic acid, acetic acid, xylitol, sorbitol, hydroxy methyl furfural, furfurals, alkanes, ethylene glycol, alkenes, glycolic acid, acetone, and ethylene. Apart from these low value-high yield products, high value-low yield lignin degradation products like guaiacols, resins, catechols, syringaldehyde, benzene, quinoline, vanillic acid and vanillin may also be produced (Avanthi et al. 2016). Besides, the biomass obtained after SSF may be used for the production of fuels such as biogas/biomethane and biohydrogen. Carbon dioxide separated from biogas may be used for the growth of microalgae which are the potential lipid source for biodiesel production whereas methane enriched gas has improved calorific value which is pollution free too. The solid residue obtained after these high energy density fuel production can further be fortified with nitrogen, phosphorous, and potassium (NPK) levels using cyanobacteria and used as biomanure to enrich the soil quality of marginal lands (Chintagunta et al. 2015). One such holistic attempt made by Ghosh and co-workers for the generation of fuels and chemicals from sugarcane bagasse where thermophilic yeast, *Kluyveromyces* sp. IIPE453 (MTCC 5314) was used to ferment the hexose stream obtained from the saccharification of sugarcane bagasse to ethanol. The unutilized pentose fraction was used for the thermophilic seed culture preparation and furfural generation whereas the residual solid biomass was subjected to gasification to produce electrical energy. From this integrated study it was found that 1 kg of sugarcane bagasse could yield 366 mL of ethanol, 149 g furfural and 0.30 kW of electrical energy (Ghosh et al. 2015).

8 Conclusion and Perspectives

Rapidly growing global demand for transportation fuels and enormous depletion of fossil fuels necessitates development of an effective biomass to biofuel production technology. Lignocellulosic biomass is renewable, abundantly available resource holding tremendous potential in meeting energy needs and providing environmental benefits. Technical knowledge is needed to design the process and to deal with complexity of biomass for ethanol production. The process of ethanol production includes appropriate pretreatment, saccharification, fermentation, distillation and removal of inhibitory byproducts. The ethanol production can be made economical by praticing SSF, SSCF, PSSF, or CBP and by producing efficient enzymes through biotechnological intervention. Utilizing substrate without or with slight pretreatment, fermenting reducing sugar with co-cultures or with the engineered strain having capability to utilize both pentoses and hexoses can also be implemented to improve the ethanol yield. Adoption of non-polluting, renewable energy sources in combination with strategies such as biodiversity studies, metagenomics, metabolic engineering and systems biology can improve biofuel yield.

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