# Lignocellulosic ethanol production from wild grass and water hyacinth involving recombinant *Clostridium thermocellum* cellulase and hemicellulase

***A Thesis***

***Submitted in Partial Fulfillment of the requirements for the Degree of***

**DOCTOR OF PHILOSOPHY**

***by***

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***Under supervision of***

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## STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of Professor Arun Goyal and Dr. Debasish Das.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

*December, 2013* *Saprativ P. Das*

**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**

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# CERTIFICATE

It is certified that the work described in this thesis entitled **"Lignocellulosic ethanol production from wild grass and water hyacinth involving recombinant *Clostridium thermocellum* cellulase and hemicellulase"** by Mr. Saprativ P. Das for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision mainly in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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# SYNOPSIS

## Introduction

Rapid diminution in the accessibility of fossil fuels poses a serious need for sustainable development of alternative energy source. Depletion of oil supply reserves as well as rise in the greenhouse gas emission has glimmered renewed interest in fuel production from renewable resources (Balat *et al.,* 2011). Lignocellulosic ethanol has gained tremendous significance in recent years due to its projection as a feasible alternative to petroleum based fuels. The choice of feedstock for bio-ethanol production is a major concern since the biomass either directly or indirectly competes with the food crops. Cost-effective fermentation of lignocellulosic hydrolysate to bioethanol not only necessitates the hydrolytic activities of various cellulolytic enzymes but also efficient mixed sugar utilization by various fermentative microbes (Alper *et al.,* 2009). Conspicuously, the rate and yield of glucose, xylose and arabinose fermentation to ethanol must be enhanced. Ethanol as a fuel has several advantages over fossil fuels such as greater air-fuel ratio, higher energy density and added specific energy with heat of vaporization. As ethanol has a high octane number than petrol, no pre ignition occurs on use of fuel ethanol (Balat *et al.,* 2011). Hence, broad use of ethanol has being done as an economical fuel additive with gasoline. Northern India has abundance of various weeds such as wild grass (*Achnatherum hymenoides*) and water hyacinth (*Eichhornia crassipes*). Production of bioethanol from land and water weeds not only provides the long-term sustainable solution in the form of low value feedstock but also solve the problem of weed management.

Although lignocellulose is the most abundant plant material resource, the major drawback in its susceptibility has been curtailed by its rigid structure. As a result, an effective pretreatment is needed to liberate the cellulose from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step. The available pre-treatment techniques include steam explosion, alkali pretreatment, acid hydrolysis, ammonia fiber expansion, organosolv pretreatment, alkaline wet oxidation, microwave assisted alkali pretreatment (MAA) (Kumar *et al.,* 2009). For more than three decades, lignocellulose degrading fungal enzymes are in use. But, the main drawback is the high cost of the commercially available *Trichoderma reesei* cellulolytic enzymes (Chowdary *et al.,* 2001). The progression of recombinant DNA technology along with molecular biology has introduced hydrolytic enzyme production and its overexpression in transformed cells. These recombinant enzymes can be further used in the degradation of cellulose and hemicellulose into simple sugars which can be subsequently converted to bioethanol (Adlakha *et al.,* 2011). *Clostridium thermocellum* exhibits one of the highest rates of cellulose utilization known, and the cellulosome of the bacterium is reported to display a 50-fold higher specific activity against crystalline cellulose than the corresponding *Trichoderma* system (Demain *et al.,* 2005). According to the CAZy database, glycoside hydrolase family 5 (GH5) and family 43 (GH43) are enzymes with varying substrate specificity including cellulase (Taylor *et al.,* 2005) and hemicellulase activity (Ahmed *et al.,* 2013). *Saccharomyces cerevisiae* possess the intrinsic ability of utilizing various substrates for ethanol production apart from high ethanol tolerance and endurance to metabolic inhibitions (Cheng *et al.,* 2007). The

hexose fermenting, gram negative bacterium *Zymomonas mobilis* utilizes the Entner-Doudoroff (ED) pathway for ethanol production (Furher *et al.,* 2005). *Candida shehatae* has key enzymes, xylitol dehydrogenase and xylose reductase enabling it to metabolize pentose sugars for ethanol production through the pentose phosphate pathway (Chandel *et al.,* 2007). The single-stage simultaneous saccharification and fermentation (SSF) decreases the processing time and reduces the end-product inhibition of enzyme, which in turn leads to rise in ethanol production (Soderstrom *et al.,* 2005). Taguchi method is based on "Orthogonal Array" experiments, which gives much reduced "variance" in the results Oliveira and Alves, 2000). Orthogonal Arrays (OA) provide a set of well balanced (minimum) experiments and has a set of combination of parameters' levels. For each combination, the signal-to-noise ratio (S/N), which is the logarithmic function of desired output serves as objective function for optimization that is used finally in data analysis and prediction of optimum results. Increment in substrate concentration along with hydrolytic enzyme loadings and fermentative microbe inoculum volume enhances ethanol titre and yield (Zhang *et al.,* 2010). The scale-up approach from shake flask to an automated bioreactor makes the stringent monitoring of important process parameters possible (Li *et al.,* 2009). The SSF process parameters such as pH and aeration significantly affect the bioconversion process and in turn, the fermentation dynamics and final ethanol titre (Sunitha *et al.,* 1999). Distillation, rotary vacuum evaporation, pervaporation are some of the commonly used fermentation product recovery processes (Palmqvist *et al.,* 1996).

**Present work**

The present investigations are carried out on "**Lignocellulosic ethanol production from wild grass and water hyacinth involving recombinant *Clostridium thermocellum* cellulase and hemicellulase**". In the present study, production and enhancement of activity of recombinant cellulase and hemicellulase from *Clostridium thermocellum* expressed in *Escherichia coli* was carried out. Selection of cellulose and hemicellulose rich substrates and efficient pretreatment process for bioethanol production was done. The best simultaneous saccharification and fermentation (SSF) trial of pretreated wild grass and water hyacinth from different combinations of hydrolytic enzymes and fermentative microbes was identified. The optimization of simultaneous saccharification and fermentation process by Taguchi orthogonal array design was accomplished. The simultaneous saccharification and fermentation in shake flask at higher concentration of pretreated wild grass and water hyacinth and scale up in bioreactor under Taguchi optimized conditions was performed and also effective ethanol recovery was achieved. The thesis comprises 6 Chapters.

**Chapter 1** is the "General Introduction" which embodies the brief review of literature dedicated to the search for alternative and sustainable energy sources to balance the continuous shortage of fuel and the environmental threats caused by the extreme exploitation of non-renewable sources. It mainly focuses on bioethanol production from lignocellulosic biomass involving recombinant *C. thermocellum* cellulase and hemicellulase belonging to different glycoside hydrolase (GH) families. It illustrates

the importance of structural carbohydrates in plants with its effective breakdown by efficient pretreatment strategy. It also describes the involvement of fungal and recombinant bacterial hydrolytic enzymes for saccharification. This chapter elaborately reviewed carbohydrate-active enzymes, especially GHs that are important plant cell wall degrading enzymes and are pivotal to many biological and industrial processes. The chapter reviews extensively, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase). Various types of fermentation *viz*., solid state fermentation, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are also described. The potential applications of *S. cerevisiae, Z. mobilis* and *C. shehatae* are also reviewed.

**Chapter 2** describes the augmentation of the cell biomass of different *Escherichia coli* cells harboring *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) gene and, in turn, their enzymatic activities by using repetitive batch strategy in various growth media like Luria Bertani (LB), Terrific Broth (TB) and LB with glucose at shake flask level. For GH5 cellulase production, in LB medium with batch mode, the enzyme activity, protein concentration and the cell OD were 2.2 U $mg^{-1}$, 0.18 mg $mL^{-1}$ and 1.4. A cell OD (2.8), protein concentration (0.37 mg $mL^{-1}$) and enzyme activity (4.5 U $mg^{-1}$) with an increment of 2-fold was observed in LB medium with repetitive batch mode as compared to batch mode (Table 1). The cell free extract of recombinant GH5 cellulase displayed a 35 kDa band on SDS-PAGE. Zymogram study confirmed the cellulase activity of GH5. In case of terrific broth (TB) with batch mode, the enzyme activity of 2.4 U $mg^{-1}$, protein concentration

of 0.20 mg $mL^{-1}$ and the cell OD of 4.8 was obtained (Table 1). On the other hand, in repetitive batch mode, the enzyme activity, protein concentration and cell OD achieved was 5.0 U $mg^{-1}$, 0.40 mg $mL^{-1}$ and 7.5 respectively (Table 1). Thus, a 2-fold increment both in activity of the enzyme and protein concentration along with 1.6-fold rise in cell biomass was gained in repetitive batch mode as compared with batch mode. Thus, as compared to LB medium in batch and repetitive batch modes, a 3.4- and 2.7-fold augmentation in cell biomass were obtained for TB in respective modes. In batch mode LB medium with glucose, the recombinant GH5 cellulase activity of 2.8 U $mg^{-1}$ with protein concentration of 0.37 mg $mL^{-1}$ and highest cell OD of 6.0 was obtained. The repetitive batch mode yielded the specific activity (5.7 U $mg^{-1}$), protein concentration (0.45 mg $mL^{-1}$) and cell OD of 9.6 (Table 1). Thus, a 4.2-fold and 3.4-fold escalation in cell biomass was obtained in LB medium with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes.

**Table 1** Batch and repetitive batch culture for cell biomass and GH5 cellulase activity in LB, TB and LB with glucose media.

| **Mode** | **Maximum Cell Biomass (OD at 600nm)*** | **Protein concentration (mg $mL^{-1}$)*** | **Specific Activity (U $mg^{-1}$)*** |
|---|---|---|---|
| Batch (LB medium) | 1.4 ± 0.04 | 0.18 ± 0.04 | 2.2 ± 0.05 |
| Repetitive Batch (LB medium) | 2.8 ± 0.05 | 0.37 ± 0.05 | 4.5 ± 0.04 |
| Batch (TB medium) | 4.8 ± 0.07 | 0.20 ± 0.09 | 2.4 ± 0.01 |
| Repetitive Batch (TB medium) | 7.5 ± 0.09 | 0.40 ± 0.07 | 5.0 ± 0.06 |
| Batch (LB with glucose) | 6.0 ± 0.02 | 0.23 ± 0.02 | 2.8 ± 0.07 |
| Repetitive Batch (LB with glucose) | 9.6 ± 0.05 | 0.45 ± 0.06 | 5.7 ± 0.08 |

***values are mean ± SE (n=3)*

For GH43 hemicellulase (α-L-arabinofuranosidase) production, the batch mode LB medium yielded the enzyme activity, protein concentration and the cell OD

of 1.9 U $mg^{-1}$, 0.20 mg $mL^{-1}$ and 1.6, respectively (Table 2). Repetitive batch LB medium gave an enzyme activity of 3.0 U $mg^{-1}$, protein concentration of 0.26 mg $mL^{-1}$ and cell OD of 2.5 (Table 2). A 1.6-fold augmentation both in enzyme activity and cell biomass was obtained in repetitive batch mode as compared with batch mode. The cell free extract of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) displayed a 34 kDa band on SDS-PAGE. Zymogram study confirmed the hemicellulase activity of GH43. In terrific broth (TB) with batch mode, the enzyme activity, protein concentration and cell OD obtained were 2.0 U $mg^{-1}$, 0.21 mg $mL^{-1}$ and 3.7, respectively (Table 2). In repetitive batch mode an enzyme activity (3.4 U $mg^{-1}$), protein concentration (0.28 mg $mL^{-1}$) and cell OD (5.9) displaying around 2-fold increase in enzyme activity was obtained as compared with batch mode. In batch mode LB medium with glucose, GH43 hemicellulase (α-L-arabinofuranosidase) activity of 2.2 U $mg^{-1}$ with protein concentration of 0.24 mg $mL^{-1}$ and cell OD of 5.2 was obtained (Table 2). The repetitive batch mode yielded specific activity (3.7 U $mg^{-1}$), protein concentration (0.32 mg $mL^{-1}$) and cell OD of 8.1 (Table 2).

**Table 2** Batch and repetitive batch culture for cell biomass and GH43 hemicellulase (α-L-arabinofuranosidase) activity in LB, TB and LB with glucose media

| **Mode** | **Maximum Cell Biomass (OD at 600nm )*** | **Protein concentration (mg $mL^{-1}$)*** | **Specific Activity (U $mg^{-1}$)*** |
|---|---|---|---|
| Batch (LB medium) | 1.6 ± 0.03 | 0.20 ± 0.07 | 1.9 ± 0.02 |
| Repetitive Batch (LB medium) | 2.5 ± 0.06 | 0.26 ± 0.05 | 3.0 ± 0.05 |
| Batch (TB medium) | 3.7 ± 0.01 | 0.21 ± 0.02 | 2.0 ± 0.05 |
| Repetitive Batch (TB medium) | 5.9 ± 0.03 | 0.28 ± 0.01 | 3.4 ± 0.08 |
| Batch (LB with glucose) | 5.2 ± 0.02 | 0.24 ± 0.08 | 2.2 ± 0.04 |
| Repetitive Batch (LB with glucose) | 8.1 ± 0.04 | 0.32 ± 0.09 | 3.7 ± 0.06 |

***values are mean ± SE (n=3)*

Thus, a 3.2 fold escalation in cell biomass was obtained in LB medium with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes.

**Chapter 3** elucidates the selection of cellulose and hemicellulose rich substrate among leafy substrates of various agricultural and forest residues on the basis of structural carbohydrate composition. The efficiency of different pretreatments was evaluated in terms of complex carbohydrate breakdown of lignocellulosic wild grass and water hyacinth. The efficiency of mixed microwave assisted alkali (MAA) with organosolv pretreatment in degradation of wild grass and water hyacinth was confirmed by field emission scanning electron microscopy (FESEM) and FT-IR analyses. The efficacy of recombinant *C. thermocellum* hydrolytic GH5 cellulase along with *S. cerevisiae* in terms of bioethanol production was determined by two modes of fermentation, SHF and SSF. Also, the efficiency of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated by SHF and SSF modes of fermentation.

Among nine different substrates (leafy biomass), the maximum cellulose content was observed in wild grass (51.70%, w $w^{-1}$) followed by jamun (40.36%, w $w^{-1}$), eucalyptus (39.68%, w $w^{-1}$), bamboo (37.30%, w $w^{-1}$), mango (33.16%, w $w^{-1}$), poplar (31.40%, w $w^{-1}$), water hyacinth (30.07%, w $w^{-1}$), asoka (26.62%, w $w^{-1}$) and lowest in neem (24.64%, w $w^{-1}$) (Table 3). On the other hand, the maximum hemicellulose content was found in water hyacinth (44.52%, w $w^{-1}$) followed by poplar (43.04%, w $w^{-1}$), mango (42.98%, w $w^{-1}$), neem (41.84%, w $w^{-1}$), eucalyptus

(40.44%, w $w^{-1}$), bamboo (35.04%, w $w^{-1}$), jamun (32.22%, w $w^{-1}$), wild grass (30.90%, w $w^{-1}$), and lowest in asoka (30.06%, w $w^{-1}$) (Table 3). Lignin was found to be maximum in bamboo (27.65%, w $w^{-1}$) followed by jamun (27.40%, w $w^{-1}$), poplar (25.23% w $w^{-1}$), neem (23.52%, w $w^{-1}$), mango (22.76%, w $w^{-1}$), asoka(21.81%, w $w^{-1}$), eucalyptus (19.88%, w $w^{-1}$), wild grass (18.07%, w $w^{-1}$) and water hyacinth (29.40%, w $w^{-1}$) (Table 3).

**Table 3** Cellulose, hemicellulose and lignin content (%) of various lignocellulosic leafy biomass

| **Substrates (leafy biomass)** | **Cellulose* (%, w $w^{-1}$)** | **Hemicellulose* (%, w $w^{-1}$)** | **Lignin* (%, w $w^{-1}$)** |
|---|---|---|---|
| Jamun (*Syzygium cumini*) | 40.36 ± 0.45 | 32.22 ± 0.52 | 27.40 ± 0.49 |
| Neem(*Azadirachta indica*) | 24.64 ± 0.44 | 41.84 ± 0.48 | 23.52 ± 0.40 |
| Asoka (*Saraca indica*) | 26.62 ± 0.32 | 30.06 ± 0.50 | 21.81 ± 0.50 |
| Bamboo (*Bambusa dendrocalmus*) | 37.30 ± 0.50 | 35.04 ± 0.47 | 27.65 ± 0.42 |
| Poplar (*Populus nigra*) | 31.40 ± 0.40 | 43.04 ± 0.38 | 25.23 ± 0.46 |
| Wild grass (*Achnatherum hymenoides*) | 51.70 ± 0.43 | 30.90 ± 0.55 | 18.07 ± 0.56 |
| Eucalyptus (*Eucalyptus marginata*) | 39.68 ± 0.49 | 40.44 ± 0.50 | 19.88 ± 0.45 |
| Mango (*Mangifera indica*) | 33.16 ± 0.38 | 42.98 ± 0.43 | 22.76 ± 0.50 |
| Water hyacinth (*Eichhornia crassipes*) | 30.07 ± 0.24 | 44.52 ± 0.45 | 29.40 ± 0.32 |

******values are mean ± SE (n=3)*

Wild grass containing highest cellulose content, 51.7% (w $w^{-1}$) with 30.9% (w $w^{-1}$) hemicellulose and water hyacinth encompassing highest hemicellulose content, 44.5% (w $w^{-1}$) with 30% (w $w^{-1}$) cellulose were selected as the most sustainable substrates for bioethanol production. Nine pretreatments were employed for the efficient breakdown of structural carbohydrates in wild grass. Microwave assisted alkali (MAA) pretreatment resulted in maximum loosening of cellulose (47.50%) as compared to steam explosion (48.91%), alkali (50.01%), wet oxidation (49.17%), phosphoric acid – acetone pretreatment (48.80%), AFEX (49.60%), organosolv (48.80%), pH controlled hot water (50.03%) and dual step dual temperature (DSDT)

mild acid hydrolysis (49.50%). Organosolv pretreatment degraded hemicellulose to the maximum extent of 27.02%. Hemicellulose was broken down to least extent in phosphoric acid – acetone pretreatment (28.12%) as compared to alkali (28.10%), wet oxidation (28.04%), pH controlled hot water (27.78%), steam explosion (48.91%), MAA pretreatment (27.50%), AFEX (27.42%) and DSDT mild acid hydrolysis (27.40%), etc. Lignin was broken down to maximum extent by MAA (15.60%) followed by organosolv (16.04%) pretreatments. The mixed MAA and organosolv pretreatment strategy loosened maximum cellulose content of 46.30%, hemicellulose of 26.50 % and lignin of 15.20%. Water hyacinth was also subjected to nine different pretreatments. MAA pretreatment resulted in maximum loosening of cellulose (27.30%) as compared to steam explosion (28.60%), alkali (29.80%), wet oxidation (28.70%), phosphoric acid-acetone pretreatment (28.10%), AFEX (29.20%), organosolv (28.40%), pH controlled hot water (29.60%) and dual step dual temperature (DSDT) mild acid hydrolysis (29.10%). Organosolv pretreatment degraded hemicellulose to the maximum extent of 34.01%. Lesser hemicellulose breakdown was obtained by wet oxidation (39.60%) followed by phosphoric acid-acetone pretreatment (39.50 %), alkali (39.14%), AFEX (37.28%), steam explosion (37.24%), DSDT mild acid hydrolysis (36.20%), pH controlled hot water (36.02%), MAA pretreatment (35.80%). Lignin was broken down to maximum extent by MAA (16.24%) followed by organosolv (17.80%) pretreatments. The mixed MAA and organosolv pretreatments loosened cellulose (26.10%), hemicellulose, 33.20 % and lignin, 15.64%. The surface morphology analysis of wild grass and water hyacinth by FESEM revealed increase in porosity and structural destabilization with mixed

microwave assisted alkali (MAA) and organosolv pretreatment (Fig. 1, Fig. 2). The porosity increment over the surface of the pretreated substrate enhanced the enzyme accessibility towards the substrate with a substantial release of utilizable reducing sugar for improved ethanol yield.

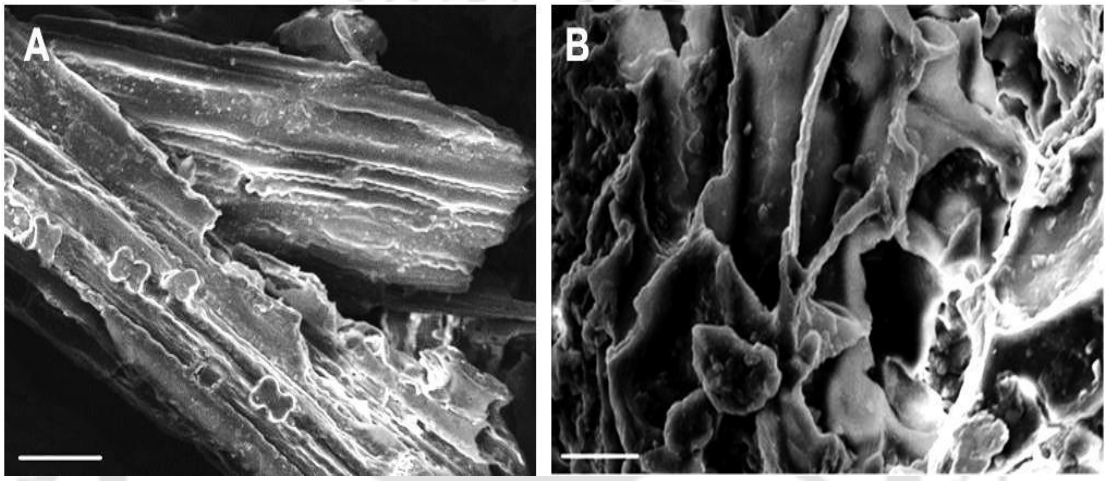


**Fig. 1** FESEM images of (A) Untreated (B) Mixed microwave assisted alkali (MAA) with organosolv pretreated wild grass (*Achnatherum hymenoides*). All images are shown at magnification-scale bar: 30 µm.

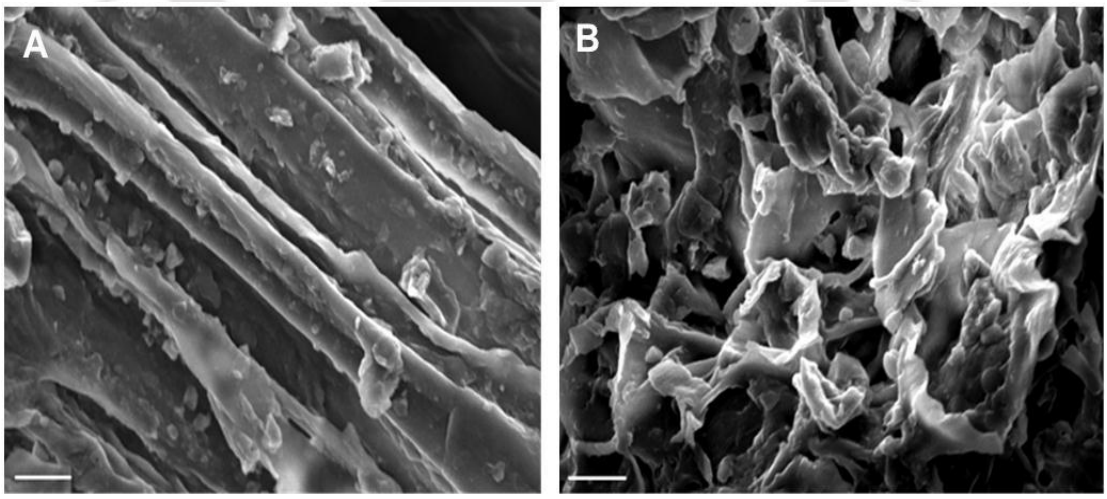


**Fig. 2** FESEM images of (A) Untreated (B) Mixed microwave assisted alkali (MAA) with organosolv pretreated water hyacinth (*Eichhornia crassipes*). All images are shown at magnification-scale bar: 20 µm.

The FT-IR analyses of the two substrates, wild grass and water hyacinth displayed a peak of xylose, glucose and arabinose at 995, 1033, 1152 $cm^{-1}$, respectively, confirming the significant breakdown of complex cellulose and hemicellulose to monomeric sugars by mixed pretreatment strategy.

The SSF experiments involving mixed pretreated 1% (w $v^{-1}$) wild grass along with recombinant GH5 cellulase and *S. cerevisiae* yielded a 1.2-fold higher ethanol titre of 0.67 g $L^{-1}$ as compared to ethanol concentration of 0.56 g $L^{-1}$ shake flask SHF (Table 4).

**Table 4** Shake flask SHF and SSF employing GH5 cellulase and *S. cerevisiae* on mixed MAA and Organosolv pretreated 1% (w $v^{-1}$) wild grass.

| Mode of fermentation | Reducing sugar* (g $L^{-1}$) | Ethanol yield (g of ethanol g of substrate$^{-1}$) | Ethanol titre* (g $L^{-1}$) |
|---|---|---|---|
| SHF | 0.89 ± 0.09 | 0.076 | 0.56 ± 0.02 |
| SSF | 1.26 ± 0.06 | 0.092 | 0.67 ± 0.04 |

***the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

Similarly, the SSF experiments involving mixed pretreated 1% (w $v^{-1}$) water hyacinth along with recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* yielded a 1.2-fold higher ethanol titre of 0.55 g $L^{-1}$ as compared to ethanol concentration of 0.45 g $L^{-1}$ shake flask SHF (Table 5).

**Table 5** Shake flask SHF and SSF employing GH5 cellulase and *S. cerevisiae* on mixed MAA and Organosolv pretreated 1% (w $v^{-1}$) water hyacinth.

| Mode of fermentation | Reducing sugar* (g $L^{-1}$) | Ethanol yield (g of ethanol g of substrate$^{-1}$) | Ethanol titre* (g $L^{-1}$) |
|---|---|---|---|
| SHF | 0.62 ± 0.07 | 0.075 | 0.45 ± 0.03 |
| SSF | 0.74 ± 0.06 | 0.092 | 0.55 ± 0.08 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3).*

**Chapter 4** illustrates the independent shake flask SSF trials involving different combinations of hydrolytic enzymes and fermentative microbes were performed on 1% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth. The hydrolytic performance of recombinant *C. thermocellum* GH5 cellulase was compared with the *T. reesei* cellulase in terms of reducing sugar formed in SSF experiments. The bioethanol producing capability of *S. cerevisiae* was compared with *Z. mobilis* in terms of ethanol titre. Also, the competence of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated in fermentation trials of wild grass and water hyacinth. Finally, a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae, C. shehatae*) system was employed to yield better ethanol titre. The mixed MAA and organosolv pretreatments of wild grass gave cellulose (46.30%), hemicellulose (26.50%) and lignin (15.20%). The mixed MAA and organosolv pretreatments of water hyacinth contributed cellulose (26.10%), hemicellulose (33.20%) and lignin (15.64%).

The saccharification efficiency of recombinant *C. thermocellum* GH5 cellulase was compared with the fungal *T. reesei* cellulase in terms of reducing sugar formed in SSF trials of wild grass and water hyacinth. In case of wild grass, a 5% increase in reducing sugar concentration was obtained by GH5 cellulase as compared to *T. reesei* cellulase. In water hyacinth, a 15% rise in reducing sugar concentration was achieved by GH5 cellulase as compared to *T. reesei* cellulase. The fermentation capability of *S. cerevisiae* was compared with *Z. mobilis* in terms of ethanol titre obtained in SSF experiments of wild grass and water hyacinth. In case of wild grass, a 40% upsurge in

ethanol titre was obtained by *Z. mobilis* as compared to *S. cerevisiae*. In water hyacinth, a 20% augmentation in ethanol concentration was attained by *Z. mobilis* as compared to *S. cerevisiae*. The hydrolytic capability of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated in fermentation trials of wild grass and water hyacinth. Due to low hemicellulose content of wild grass, GH43 hemicellulase yielded less reducing sugar (0.49 g $L^{-1}$), whereas, GH43 hemicellulase gave a higher amount of reducing sugar (0.55 g $L^{-1}$) owing to greater hemicellulose fraction of water hyacinth.

**Table 6** SSF combination of mixed pretreated 1% (w $v^{-1}$) wild grass involving different hydrolytic enzymes and fermentative microbes.

| SSF combination | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$)* |
|---|---|---|---|
| *T. reesei* + *S. cerevisiae* | 1.22 ± 0.04 | 0.61 ± 0.01 | 0.083 |
| GH5 + *S. cerevisiae* | 1.26 ± 0.06 | 0.67 ± 0.04 | 0.092 |
| *T. reesei* + *Z. mobilis* | 1.30 ± 0.02 | 0.82 ± 0.03 | 0.113 |
| GH5 + *Z. mobilis* | 1.36 ± 0.05 | 0.94 ± 0.07 | 0.129 |
| GH43 + *C. shehatae* | 0.49 ± 0.06 | 0.40 ± 0.04 | 0.054 |
| GH5 + GH43 + *Z. mobilis* | 1.60 ± 0.01 | 1.29 ± 0.01 | 0.177 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* | 1.70 ± 0.09 | 1.50 ± 0.06 | 0.206 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

The SSF trial of mixed pretreated 1% (w $v^{-1}$) wild grass involving *T. ressei* cellulase and *S. cerevisiae* gave reducing sugar concentration of 1.22 g $L^{-1}$, ethanol titre of 0.61 g $L^{-1}$ and an ethanol yield of 0.083 (g of ethanol g of substrate$^{-1}$) (Table 6). Whereas, the SSF experiment of wild grass involving GH5 cellulase and *S. cerevisiae* provided a reducing sugar concentration of 1.26 g $L^{-1}$, ethanol titre of 0.67

g $L^{-1}$ and an ethanol yield of 0.092 (g of ethanol g of substrate$^{-1}$) (Table 6) displaying a 11% percent increase in the ethanol yield and proved the effectiveness of GH5 cellulase over *T. ressei* cellulase with *S. cerevisiae*.

The SSF trial of wild grass involving *T. reesei* cellulase and *Z. mobilis* contributed a reducing sugar concentration of 1.30 g $L^{-1}$, ethanol titre of 0.82 g $L^{-1}$ and an ethanol yield of 0.113 (g of ethanol g of substrate$^{-1}$) (Table 6). Contrastingly, the SSF experiment of wild grass involving GH5 cellulase and *Z. mobilis* offered a reducing sugar concentration of 1.36 g $L^{-1}$, ethanol titre of 0.94 g $L^{-1}$ and an ethanol yield of 0.129 (g of ethanol g of substrate$^{-1}$) (Table 6) presenting a 14% percent escalation in the ethanol yield and proved the efficiency of GH5 cellulase over *T. ressei* cellulase with *Z. mobilis.*

The SSF trial of wild grass involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* gave a reducing sugar concentration of 0.49 g $L^{-1}$, ethanol titre of 0.40 g $L^{-1}$ and an ethanol yield of 0.054 (g of ethanol g of substrate$^{-1}$) (Table 6). The SSF experiment of wild grass involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* gave a reducing sugar concentration of 1.59 g $L^{-1}$, ethanol titre of 1.29 g $L^{-1}$ and an ethanol yield of 0.177 (g of ethanol g of substrate$^{-1}$) (Table 6) exhibiting a 37% percent upturn in the ethanol yield as compared with SSF trial of GH5 cellulase and *Z. mobilis.*

The SSF trial of wild grass involving the combination of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae* and *C. shehatae* furnished the maximum reducing sugar concentration of 1.70 g $L^{-1}$, maximum ethanol titre of 1.50 g $L^{-1}$ and an ethanol yield of 0.206 (g of ethanol g of

substrate$^{-1}$) (Table 6). A 2.3-3.2 fold increment was observed in ethanol titre using a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae*, *C. shehatae*) system as compared to single enzyme-single culture systems for wild grass.

The SSF trial of mixed pretreated 1% (w v$^{-1}$) water hyacinth involving *T. ressei* cellulase and *S. cerevisiae* gave a reducing sugar concentration of 0.47 g L$^{-1}$, ethanol titre of 0.37 g L$^{-1}$ and an ethanol yield of 0.062 (g of ethanol g of substrate$^{-1}$) (Table 7). Interestingly, the SSF experiment of water hyacinth involving GH5 cellulase and *S. cerevisiae* provided a reducing sugar concentration of 0.51 g L$^{-1}$, ethanol titre of 0.39 g L$^{-1}$ and an ethanol yield of 0.066 (g of ethanol g of substrate$^{-1}$) (Table 7) revealing a 6% percent rise in ethanol yield and evidenced the efficacy of GH5 cellulase over *T. ressei* cellulase with *S. cerevisiae*.

**Table 7** SSF combination of mixed pretreated 1% (w v$^{-1}$) water hyacinth involving different hydrolytic enzymes and fermentative microbes.

| SSF combination | Reducing sugar (g L$^{-1}$)* | Ethanol titre (g L$^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$)* |
|---|---|---|---|
| *T. reesei* + *S. cerevisiae* | 0.47 ± 0.04 | 0.37 ± 0.03 | 0.062 |
| GH5 + *S. cerevisiae* | 0.51 ± 0.07 | 0.39 ± 0.01 | 0.066 |
| *T. reesei* + *Z. mobilis* | 0.58 ± 0.01 | 0.41 ± 0.05 | 0.069 |
| GH5 + *Z. mobilis* | 0.64 ± 0.02 | 0.47 ± 0.03 | 0.079 |
| GH43 + *C. shehatae* | 0.74 ± 0.06 | 0.55 ± 0.08 | 0.092 |
| GH5 + GH43 + *Z. mobilis* | 1.13 ± 0.05 | 0.82 ± 0.06 | 0.138 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* | 1.20 ± 0.08 | 1.00 ± 0.02 | 0.168 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

The SSF trial of water hyacinth involving *T. reesei* cellulase and *Z. mobilis*

contributed a reducing sugar concentration of 0.58 g $L^{-1}$, ethanol titre of 0.41 g $L^{-1}$ and an ethanol yield of 0.069 (g of ethanol g of substrate$^{-1}$) (Table 7). On the other hand, the SSF experiment of water hyacinth involving GH5 cellulase and *Z. mobilis* gave a reducing sugar concentration of 0.64 g $L^{-1}$, ethanol titre of 0.47 g $L^{-1}$ and an ethanol yield of 0.079 (g of ethanol g of substrate$^{-1}$) (Table 7) exhibiting an increase of 14% percent in ethanol yield and proved the competence of GH5 cellulase over *T. ressei* cellulase with *Z. mobilis.*

The SSF trial of water hyacinth involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* gave a reducing sugar concentration of 0.74 g $L^{-1}$, ethanol titre of 0.55 g $L^{-1}$ and an ethanol yield of 0.092 (g of ethanol g of substrate$^{-1}$) (Table 7). The SSF experiment of water hyacinth involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* gave a reducing sugar concentration of 1.13 g $L^{-1}$, ethanol titre of 0.82 g $L^{-1}$ and an ethanol yield of 0.138 (g of ethanol g of substrate$^{-1}$) (Table 7) exhibiting a 22% percent improvement in the ethanol yield as compared with the SSF trial of GH5 cellulase and *Z. mobilis.* The SSF trial of water hyacinth involving the combination of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae* and *C. shehatae* furnished a maximum reducing sugar concentration of 1.21 g $L^{-1}$, maximum ethanol titre of 1.0 g $L^{-1}$ and an ethanol yield of 0.168 (g of ethanol g of substrate$^{-1}$) (Table 7). A 1.8-2.7 fold increment was observed in ethanol titre using a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae*, *C. shehatae*) system as compared to single enzyme-single culture system for water hyacinth.

**Chapter 5** emphasizes on the Taguchi optimization of different fermentation process parameters such as mixed recombinant *C. thermocellum* enzymes' volume, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase), mixed cultures' inoculum volume (*S. cerevisiae, C. shehatae*), pH and temperature on bioethanol production from mixed pretreated wild grass and water hyacinth independently with subsequent validation of the models at shake flask level. The mixed MAA and organosolv pretreatment strategy was employed for the improvement in breakdown of the structural carbohydrates *viz.,* cellulose and hemicellulose of the two substrates, wild grass and water hyacinth. The mixed consortium of recombinant *C. thermocellum* hydrolytic enzymes aided the significant breakdown of complex carbohydrates such as cellulose and hemicellulose. The mixed culture of bioethanol producers, *S. cerevisiae* and *C. shehatae* were engaged for efficient ethanol production owing to their capability of utilizing both hexose and pentose sugars.

In case of wild grass, the optimized process parameters in 100 mL of fermentation medium were (%, v $v^{-1}$): 1.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 0.25, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 4.3 and temperature, 35°C (Fig. 3). pH with *p*-value 0.001 was found to be the most significant factor affecting wild grass shake flask SSF.

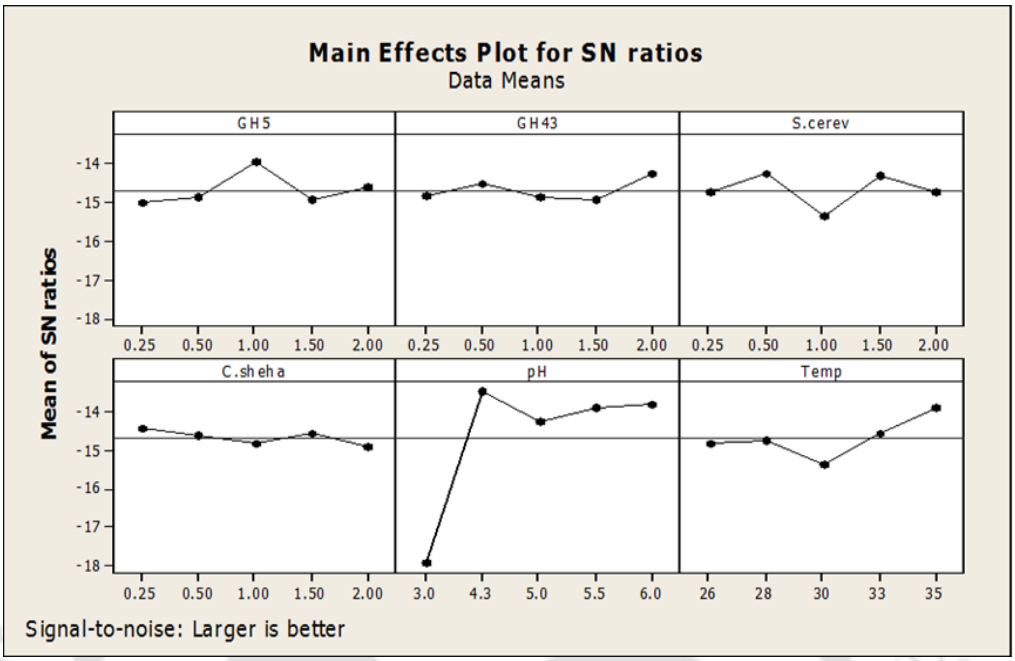


**Fig. 3** Main effect plots for S/N ratios with larger the better objective function of Taguchi optimized fermentation process parameters for wild grass.

The ethanol titre obtained in Taguchi optimized shake flask SSF was 2.0 g $L^{-1}$ implying a 1.3-fold increase as compared to ethanol titre of 1.5 g $L^{-1}$ in unoptimized shake flask SSF (Table 8).

**Table 8** Comparison of unoptimized and Taguchi optimized SSF combinations with wild grass.

| SSF combination | Substrate concentration (%, w $v^{-1}$) and mode of SSF | Reducing sugar* (g $L^{-1}$) | Ethanol yield (g of ethanol g of substrate$^{-1}$) | Ethanol titre* (g $L^{-1}$) |
|---|---|---|---|---|
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (unoptimized) | 1% shake flask | 1.70 ± 0.09 | 0.206 | 1.50 ± 0.06 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized) | 1% shake flask | 2.31 ± 0.05 | 0.274 | 2.0 ± 0.04 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

In case of water hyacinth, the optimized process parameters in 100 mL of fermentation medium were (%, v $v^{-1}$): 2.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 2.0, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 5.4 and temperature, 35°C (Fig. 4). pH with *p*-value 0.010 was found to be the most significant factor affecting water hyacinth shake flask SSF.

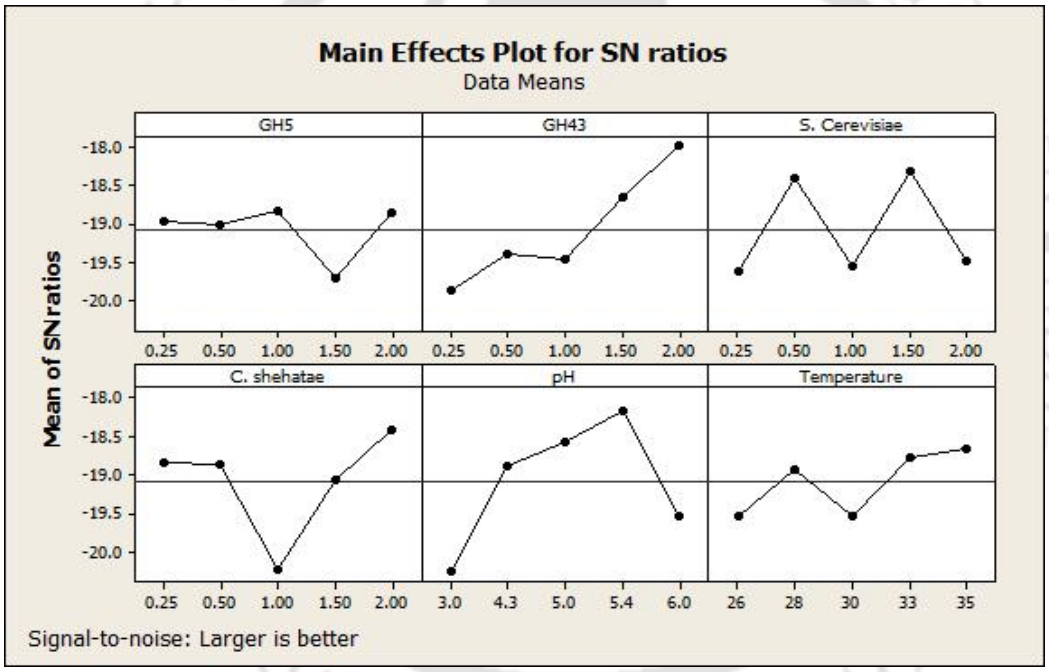


**Fig. 4** Main effect plots for S/N ratios with larger the better objective function of Taguchi optimized fermentation process parameters for water hyacinth.

The ethanol titre obtained in Taguchi optimized shake flask SSF implied a 1.2-fold increase as compared to unoptimized shake flask SSF (Table 9).

**Table 9** Comparison of unoptimized and Taguchi optimized SSF combinations using water hyacinth.

| SSF combination | Substrate concentration (%, w $v^{-1}$) and mode of SSF | Reducing sugar* (g $L^{-1}$) | Ethanol yield (g of ethanol g of substrate$^{-1}$) | Ethanol titre* (g $L^{-1}$) |
|---|---|---|---|---|
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (unoptimized) | 1% shake flask | 1.20 ± 0.08 | 0.168 | 1.00 ± 0.04 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized) | 1% shake flask | 1.41 ± 0.05 | 0.205 | 1.22 ± 0.03 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3).*

**Chapter 6** describes shake flask SSF trials involving mixed hydrolytic enzymes and mixed fermentative microbes on 1% and 5% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth. The mixed consortium of recombinant *C. thermocellum* hydrolytic GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were employed for effective saccharification. The mixed culture system comprising *S. cerevisiae* and *C. shehatae* were engaged for utilization of hexose and pentose sugars for bioethanol production. The shake flask SSF with 5% (w $v^{-1}$) wild grass and water hyacinth were separately, scaled-up in a bioreactor under controlled process parameters. The controlled conditions of pH and aeration were the additional parameters employed in the bioreactor as compared with shake flask that could give improved ethanol titre and yield. Subsequently, the bioethanol obtained from separate bioreactor SSF of wild grass and water hyacinth was recovered by rotary vacuum evaporator and purified.

The shake flask SSF of mixed pretreated 1% (w $v^{-1}$) wild grass involving mixed enzyme-mixed culture system gave reducing sugar concentration of 2.31 g $L^{-1}$, ethanol titre of 2.0 g $L^{-1}$ and an ethanol yield of 0.274 (g of ethanol g of substrate$^{-1}$) (Table 10).

**Table 10** Comparison of SSF combinations involving recombinant hydrolytic enzymes and fermentative microbes with wild grass.

| **Substrate concentration and Mode of fermentation** | **Reducing sugar (g $L^{-1}$)*** | **Ethanol titre (g $L^{-1}$)*** | **Ethanol yield (g of ethanol g of substrate$^{-1}$)*** |
|---|---|---|---|
| 1%, Shake flask | 2.31 ± 0.05 | 2.0 ± 0.04 | 0.274 |
| 5%, Shake flask | 13.85 ± 0.03 | 10.9 ± 0.06 | 0.299 |
| 5%, Bioreactor | 23.02 ± 0.05 | 18.0 ± 0.07 | 0.494 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3).*

Whereas, the SSF experiment of mixed pretreated 5% (w $v^{-1}$) wild grass in shake flask involving the same mixed enzyme-mixed culture system provided a reducing sugar concentration of 13.85 g $L^{-1}$, ethanol titre of 10.9 g $L^{-1}$ and an ethanol yield of 0.299 (g of ethanol g of substrate$^{-1}$) (Table 10) displaying a 5.4 fold improvement in ethanol concentration and 9% escalation in ethanol yield. This confirmed that increasing the substrate concentration along with enzyme loadings and inoculum volume improves the ethanol titre.

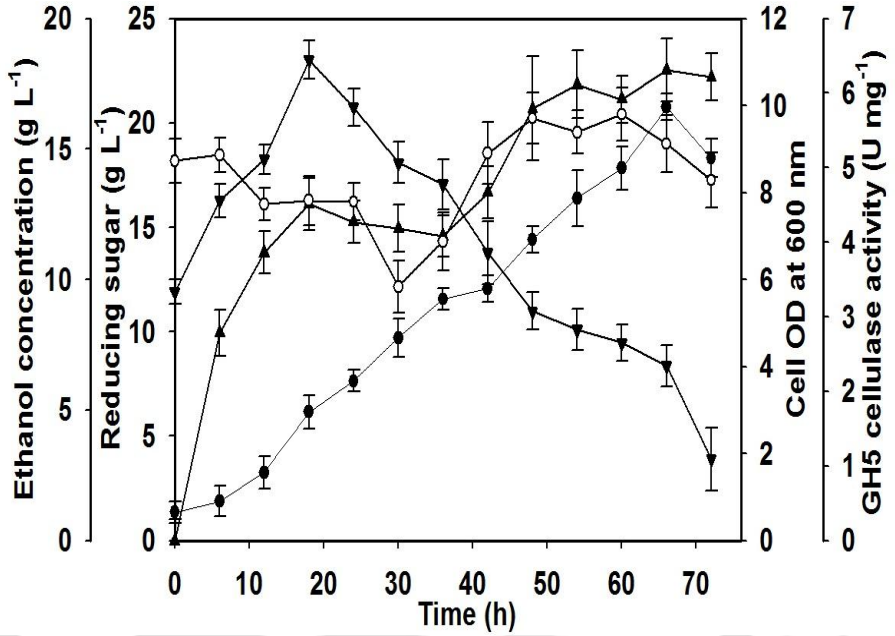


**Fig. 5** SSF profile of 5% (w $v^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in bioreactor. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 1 L medium contained in 3 L bioreactor; initial pH 4.3; temperature 35°C, agitation 120 rpm and aeration 1 vvm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown).

The SSF trial of mixed pretreated 5% (w $v^{-1}$) wild grass in a bioreactor with controlled pH and aeration and same mixed enzyme-mixed culture system contributed a maximum reducing sugar concentration of 23.02 g $L^{-1}$, ethanol titre of 18.0 g $L^{-1}$ and an ethanol yield of 0.494 (g of ethanol g of substrate$^{-1}$) (Table 10, Fig. 5). A 1.6-fold increase both in ethanol titre and yield was witnessed on scaling up the shake flask SSF with 5% (w $v^{-1}$) substrate concentration to bioreactor level (Table 10). The HPAEC pattern of monosaccharides in sugar hydrolysate *viz.*, arabinose, glucose and xylose obtained from bioreactor SSF of wild grass at different time intervals clearly detected the presence of reducing sugar in the fermentation broth.

92.1 (%, v $v^{-1}$) of partially purified ethanol from bioreactor SSF of wild grass was recovered by rotary evaporator with 23% purification efficiency.

The shake flask SSF trial of mixed pretreated 1% (w $v^{-1}$) water hyacinth involving mixed enzyme-mixed culture system offered reducing sugar concentration of 1.41 g $L^{-1}$, ethanol titre of 1.22 g $L^{-1}$ and an ethanol yield of 0.205 (g of ethanol g of substrate$^{-1}$) (Table 11).

**Table 11** Comparison of SSF combinations involving recombinant hydrolytic enzymes and fermentative microbes with water hyacinth.

| **Substrate concentration and Mode of fermentation** | **Reducing sugar (g $L^{-1}$)*** | **Ethanol titre (g $L^{-1}$)*** | **Ethanol yield (g of ethanol g of substrate$^{-1}$)*** |
|---|---|---|---|
| 1% Shake flask | 1.41 ± 0.05 | 1.22 ± 0.03 | 0.205 |
| 5% Shake flask | 9.74 ± 0.04 | 6.20 ± 0.03 | 0.209 |
| 5% Bioreactor | 19.68 ± 0.07 | 13.70 ± 0.05 | 0.461 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3).*

Contrastingly, the shake flask SSF trial of mixed pretreated 5% (w $v^{-1}$) water hyacinth with the same mixed enzyme-mixed culture system contributed a reducing sugar concentration of 9.74 g $L^{-1}$, ethanol titre of 6.20 g $L^{-1}$ and an ethanol yield of 0.209 (g of ethanol g of substrate$^{-1}$) exhibiting a 5 fold enhancement in ethanol concentration and 2% rise in ethanol yield (Table 11). This confirmed that increasing the substrate concentration along with enzyme loadings and inoculum volume enhances the ethanol concentration and yield.

Finally, the SSF trial of mixed pretreated 5% (w $v^{-1}$) water hyacinth in a bioreactor with same mixed enzyme-mixed culture system supplemented with controlled parameters of pH and aeration yielded a maximum reducing sugar

concentration of 19.68 g $L^{-1}$, ethanol titre of 13.7 g $L^{-1}$ and an ethanol yield of 0.461 (g of ethanol g of substrate$^{-1}$) (Table 11, Fig. 6).

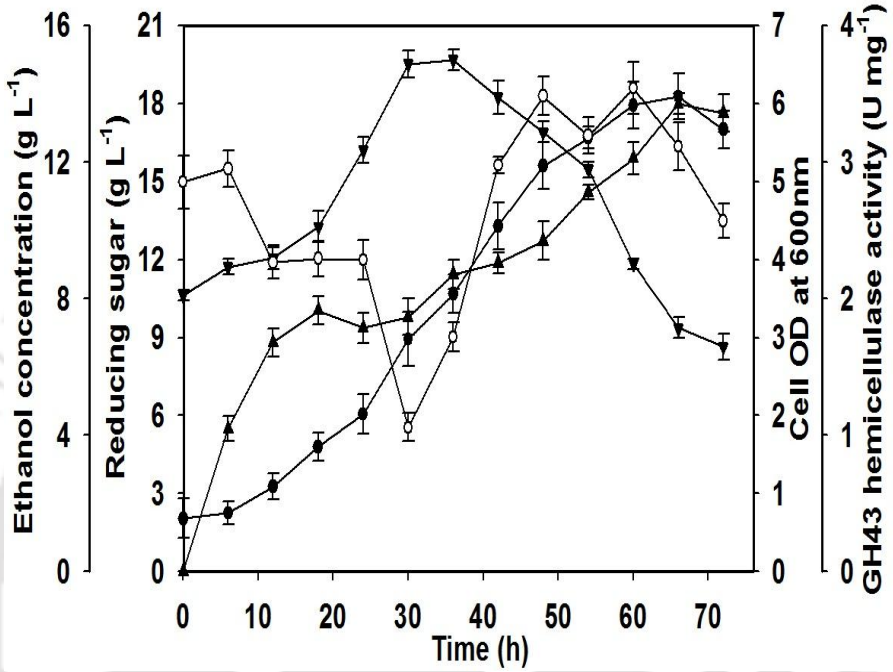


**Fig. 6** SSF profile of 5% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in bioreactor. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 1 L medium contained in 3 L bioreactor; initial pH 5.4; temperature 35°C, agitation 120 rpm and aeration 1 vvm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown).

A 2.2 fold upsurge both in ethanol titre and yield (Table 11) was observed on scaling up the shake flask SSF with 5% (w $v^{-1}$) substrate concentration in an automated bioreactor. The HPAEC pattern of monosaccharides in sugar hydrolysate *viz.*, arabinose, glucose and xylose obtained from bioreactor SSF of water hyacinth at different time intervals clearly proved the presence of reducing sugar in the fermentation broth. 94 (%, v $v^{-1}$) of partially purified ethanol from bioreactor SSF of

water hyacinth was recovered by rotary evaporator with 22% purification efficiency. The effective breakdown of the complex lignocellulosic weeds, wild grass and water hyacinth by mixed pretreatment strategy along with recombinant hydrolytic enzymes and fermentative microbes in SSF at bioreactor level will yield an improved titre of the fuel for tomorrow.

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## CONTENTS

# Chapter 1

## General Introduction

### 1. Quest for alternative energy

Energy security, galloping oil price, resource depletion and climate change are some of the greatest challenges looming large on the future of humanity (Balat *et al.,* 2011). The indiscriminate use of fossil fuels coupled with rapid pace of industrialization has played havoc with the nature (Macnae *et al.,* 1969). The 'greenhouse effect' caused by the ever increasing carbon dioxide levels in the air and depleting ozone layer has resulted in extensive climate changes. The combustion of fossil fuels is responsible for 73% of the $CO_2$ production (Hameed *et al.,* 1988). The phenomenal increase in human population and industrialization has significantly pushed up the global demand for energy (Balat *et al.,* 2011). This, coupled with ever depleting oil reserves have necessitated the search for alternative energy sources especially the renewable ones (Stockdale *et al.,* 2001). Scientists have now started looking at recycling the waste biomass for products like ethanol and biodiesel. The global demand for energy continues to grow due to rapidly expanding human

population and increase of the industrial prosperity in developing countries (Balat *et al.,* 2011).

The search for alternative and sustainable energy sources has become the most important need of mankind to balance the continuous shortage of fuel and the environmental threats caused by the extreme exploitation of non-renewable sources (Stockdale *et al.,* 2001).

Environmental concerns and the depletion of oil reserves have resulted in governmental actions and incentives to establish greater energy independence by promoting research on environmentally benign and sustainable biofuels. Utilization of biomass as the starting material for various chemicals and for the production of fuels has received considerable interest in recent years.

With the price of crude petroleum oil in the world market escalating to more than US$96 per barrel due to political instabilities in many oil-exporting countries, the quest for renewable energy supply that is affordable and environment friendly is inevitable (Tan *et al.,* 2008). Use of fossil fuels such as diesel and gasoline substantially increases the greenhouse gases (GHGs), accelerating the environmental degradation, hastening global warming, causing acid rain, shift in climate zones and seasons, melting of polar ice caps and glaciers, rise in sea levels and endangering human health and subsequently contributes to environmental issues. The search for sustainable methods to produce transportation fuels is driven by concerns associated with its supply and demand and its impact on climate change and greenhouse gas (GHG) emissions. With the crude becoming scarce and expensive, the quest for renewable energy sources at affordable price and in an environment friendly and sustainable way, is gathering momentum. Over the last ten years biofuels production

has increased dramatically. The increment in production has been driven by governmental interventions (Omer, 2008). The blending targets for ethanol and biodiesel in gasoline and petroleum diesel were proposed at 10% and 20% by 2011-2012 respectively (Sukumaran *et al.*, 2010). However, large scale implementation of the blending idea would need tremendous scaling up of the production of biofuels particularly from biowaste.

## 1.1 Lignocellulosic biomass

Lignocellulosic biomass is renewable, inexpensive and is abundantly available with its annual production estimated in $1x10^{10}$ metric tonne (MT) worldwide. Biowaste like crop residue, grasses, saw dust and wood chips, are the most common lignocellulose containing material (Sánchez and Cardona, 2008). Extensive research with reproducible outcomes has been carried out using variety of these farm and industrial wastes to produce ethanol (Ghatak *et al.*, 2008). Although, bioconversion of lignocellulose to ethanol offers numerous benefits, its development on commercial scale is largely limited by operational cost and the need for process optimization. Operation cost can be appreciably reduced by availability and efficient utilization of the raw material, maximum productivity, high ethanol concentration in the distillation feed and integration of the process with the already existing infrastructure. Ethanol production from lignocellulosic biomass involves following steps:

a. an effective pretreatment for the separation of lignin residue,
b. hydrolysis of cellulose and hemicellulose,
c. sugar fermentation, and
d. efficient recovery with purification of ethanol to meet fuel specifications.

The task of hydrolyzing lignocellulose to fermentable monosaccharides is still a technical challenge because the digestibility of cellulose is hindered by many physico-chemical, structural and compositional factors (Tan *et al.,* 2008), which make pretreatment an essential step for obtaining easily fermentable sugars in the hydrolysis step.

Very few studies have so far been reported on the effect of bioethanol production on agricultural economy. At present conventional crops like corn, sugar beet, tapioca, sweet potato, cassava and sweet sorghum are being used as the feed stock for ethanol production. Production of ethanol from agricultural crops would imply more land being allotted for their cultivation entailing on the arable land available for other crops (Martin *et al.,* 2008). A detailed survey of sugar sources for ethanol production revealed that only sugarcane molasses offered competitive feedstock and processing costs to established corn-based technologies (Fig. 1.1). But the annual capital cost investments are comparable for corn, sugarcane and sugarbeet molasses as challenging feedstocks (Shapouri *et al.,* 2006). The International Food Policy Research Institute (IFPRI), USA reported that if the world's major biofuel producing countries expanded their biofuel production based on their current ''first-generation bioethanol technologies" and future targets, it will significantly increase world prices of feedstock crops as well as other agricultural commodities.

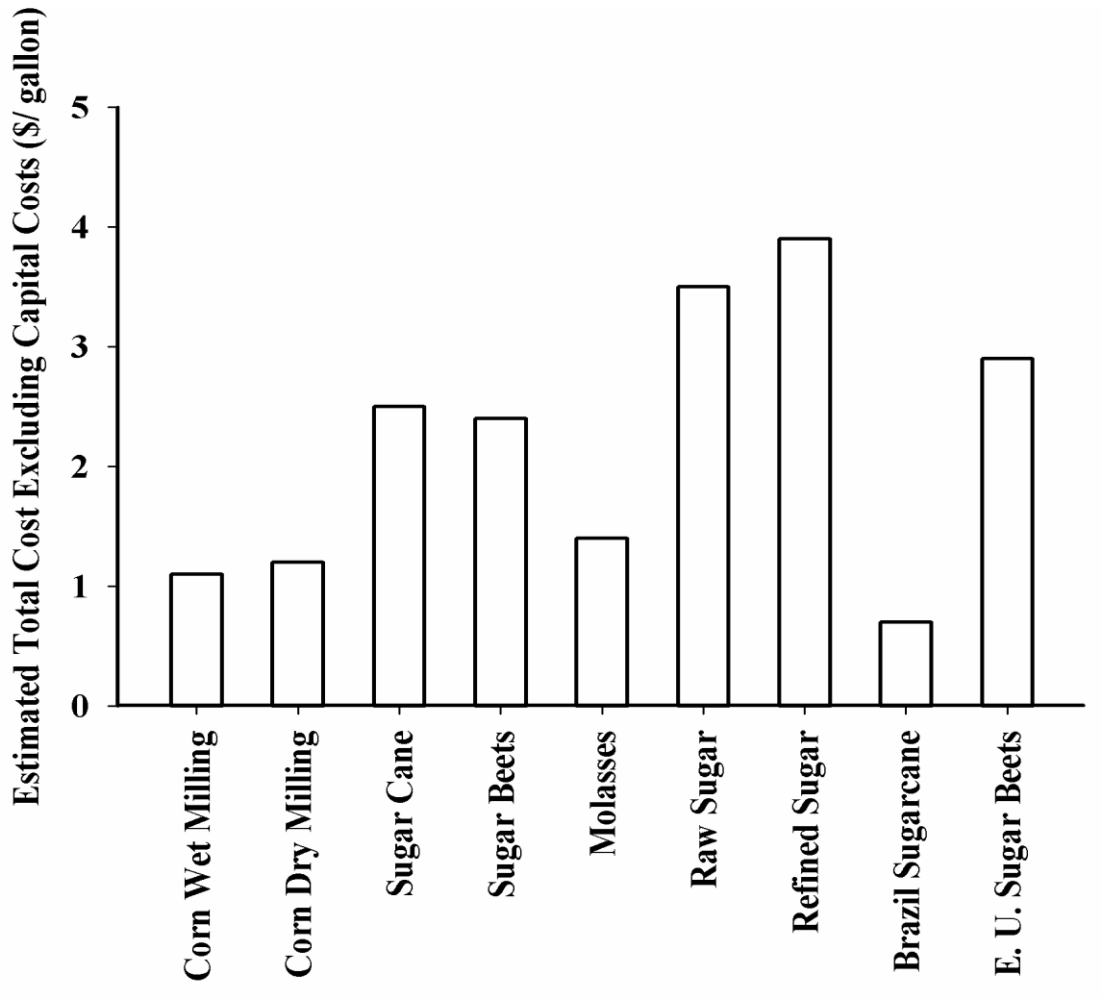


**Fig. 1.1** Estimated ethanol production costs (Shapouri *et al.,* 2006).

Biofuel development can also have significant impact on the structure and distribution of agricultural production, trade, poverty and the welfare of different households (Shapouri *et al.,* 2006). Agricultural residues and wastes such as leafy biomass of plants have not yet been examined as feed-stock for bioethanol despite of their high cellulose and hemicellulose content. Northern India has rich plantation of trees like Jamun (*Syzygium cumini*), Asoka (*Saraca indica*), Bamboo (*Bambusa dendrocalmus*), Poplar (*Populus nigra*), Eucalyptus (*Eucalyptus marginata*) and also shrubs like wild grass (*Achnatherum hymenoides*), thatch grass (*Hyparrhenia rufa*)

and weeds like water hyacinth (*Eichhornia crassipes*). The voluminous leaf fall from these plants can form an easily available raw material for the production of bioethanol (Pandey *et al.*, 2000; Das and Singh, 2004). Under the climatic conditions of north-east India, a daily average water-hyacinth biomass productivity of 0.26 ton of dry biomass per hectare in all seasons has been reported (Singh *et al.*, 1984; Ganguly *et al.*, 2012). Owing to its high hemicellulose content and being a non-competitor to food crops, water hyacinth can form a potential source for bioethanol production.

## 1.2 Structural carbohydrates in plants

Lignocellulosic biomass consists of a complicated matrix made up of cellulose and lignin bound by hemicellulose chains.

### 1.2.1 Cellulose

Cellulose is the most abundant biological organic compound on earth (Wolfe *et al.*, 2002). The cellulose are linear homopolymer consisting of regio- and enantio-selective D-glucopyranose (also known as anhydroglucose units) chains linked together by β-(1→4)-glycosidic linkages (Demain *et al*., 2005). The chemical structure of cellulose is shown in Fig. 1.2 (Somerville, 2006). Cellulose molecules are joined together by hydrogen bonds to give larger units which are ordered in crystalline manner. However, the percentage of cellulose in plant varies depending on the origin. The agriculture residues, water plants and grasses contain cellulose.

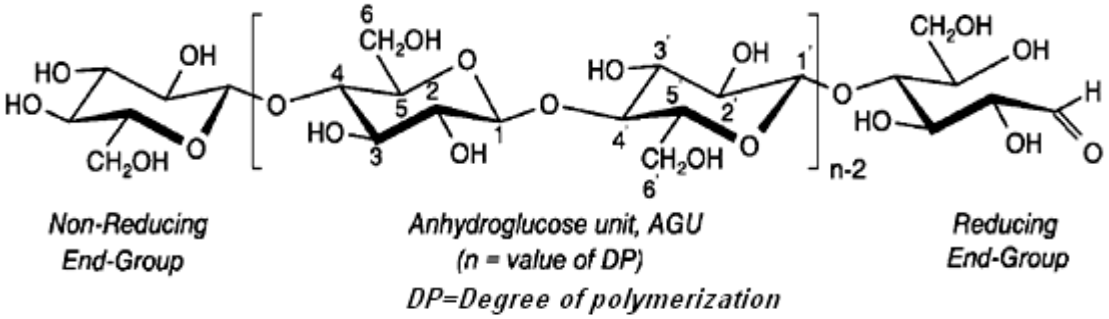


**Fig. 1.2** Chemical structure of cellulose displaying β-(1→4) glycosidic linkages, reducing end and non-reducing end (Somerville, 2006).

The Table 1.1 illustrates the percentage of cellulose, hemicellulose and lignin present in different sources. Cotton, henequen plant and sunn hemp showed very high amount of cellulose content (Table 1.1).

**Table 1.1** The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes[a].

| Lignocellulosic materials | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| Hardwoods stems | 40–55 | 24–40 | 18–25 |
| Softwood stems | 45–50 | 25–35 | 25–35 |
| Nut shells | 25–30 | 25–30 | 30–40 |
| Corn cobs | 45 | 35 | 15 |
| Grasses | 25–40 | 35–50 | 10–30 |
| Paper | 85–99 | 0 | 0–15 |
| Wheat straw | 30 | 50 | 15 |
| Sorted refuse | 60 | 20 | 20 |
| Leaves | 15–20 | 80–85 | 0 |
| Cotton seed hairs | 80–95 | 5–20 | 0 |
| Newspaper | 40–55 | 25–40 | 18–30 |
| Waste papers from chemical pulps | 60–70 | 10–20 | 5–10 |
| Primary wastewater solids | 8–15 | NA[b] | 24–29 |
| Swine waste | 6.0 | 28 | NA[b] |
| Coastal Bermuda grass | 25 | 35.7 | 6.4 |
| Switch grass | 45 | 31.4 | 12.0 |

[a] Source: Reshamwala *et al. (1995)*, Cheung and Anderson *(1997)*, Boopathy *(1998) and* Dewes and Hunsche *(1998)*, [b] *NA – not available.*

### 1.2.2 Hemicellulose

Hemicelluloses are usually hetero-polysaccharides present in plant cell walls containing β-(1→4)-linked backbones of glucose, mannose or xylose (Scheller and Ulvskov, 2010) (Fig. 1.3). They commonly occur in the nature as xylans, xyloglucans, arabinogalactans, mannans, arabinoxylans, glucomannans, β-(1→3, 1→4)-glucans and pectins (Schadel *et al.*, 2009). Hemicelluloses are known to be synthesized in the Golgi apparatus (membranes) by various glycosyltransferases. Many glycosyl-transferases involved in the process of biosynthesis of xyloglucans and mannans are reported (Schadel *et al.*, 2009). The predominant hemicellulose in many primary walls is xylan. Other hemicelluloses found in primary and secondary walls include glucuronoxylan, arabinoxylan, glucomannan and galactomannan (Schadel *et al.*, 2009).

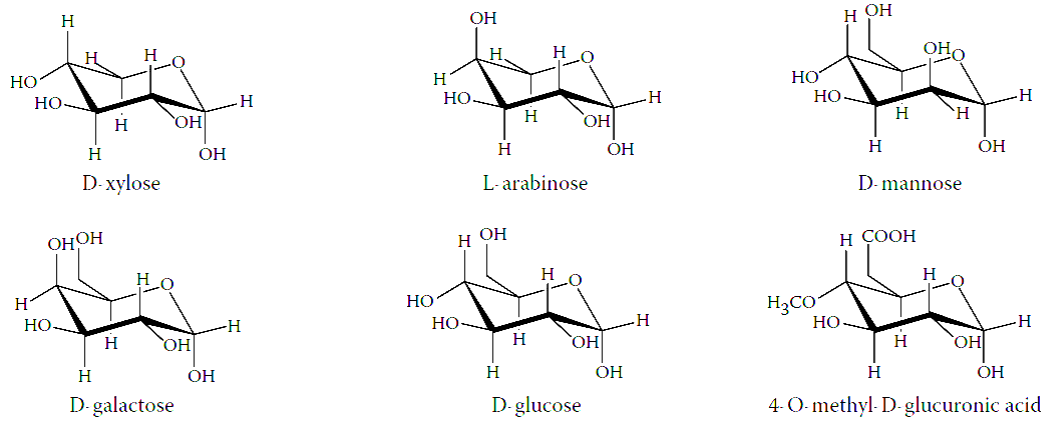


**Fig. 1.3** Chemical structures of sugar components of hemicelluloses (Scheller and Ulvskov, 2010).

### 1.2.3 Lignin

The main building blocks of lignin are the hydroxycinnamyl alcohols (or monolignols), coniferyl alcohol and sinapyl alcohol, with typically minor quantities of p-coumaryl alcohol (Fig. 1.4). Lignins are large group of aromatic polymers that result from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids as shown in Fig. 1.4 (Boerjan *et al*., 2003; Ralph *et al*., 2004). Lignin is found in all vascular plants, mostly between the cells and also within the cells and in the cell walls. It usually occurs as complex structure bound to the hemicelluloses in wood.

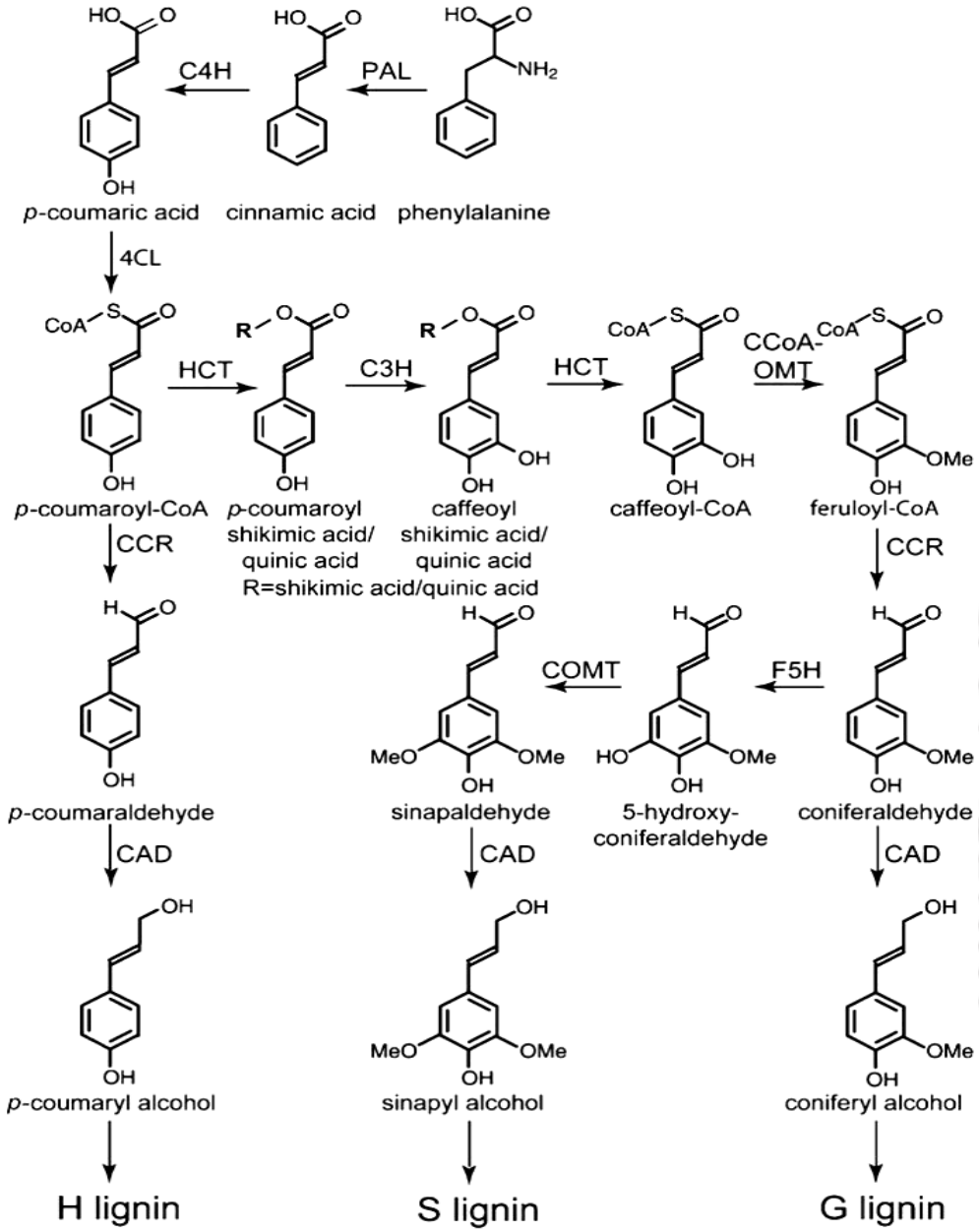


**Fig. 1.4** The main biosynthetic pathway for synthesis of the monolignols p-coumaryl, coniferyl, and sinapyl alcohol (Boerjan *et al*., 2003). Phenylalanine ammonia-lyase (PAL); 4-coumarate:CoA ligase (4CL); Cinnamate-4-hydrolyase (C4H); p-coumarate-3-hydrolyase (HCT), p-hydroxycinnamoyl-CoA:Quinate/Shikmate-p-hydroxycinnamoyltranferase (CCoAOMT), Cinnamoyl CoA reductase ferrulate-5-hydroxylyase (F5H), Caffeoyl-CoA-O-methyltransfersae (CCR), Cinnamoyl alcohol dehydrogenase (CAD) Caffeic acid-O-methyltransferase (COMT), (Borjan *et al*., 2003 and Ralph *et al*., 2004).

### 1.3 Pretreatment

Pretreatment is one of the most important step involved in the bioethanol production from lignocellulosic biomass. Pretreatment methods refer to the solubilisation and separation of the complex components in biomass. It makes the remaining solid biomass more accessible to further enzymatic hydrolysis or biological treatment (Demirbas, 2005). It plays a critical role in determination of the final yield of ethanol. The first attempt at commercializing a process for ethanol from wood was done in Germany in 1898. It involved the use of dilute acid pretreatment to hydrolyze the cellulose to glucose, and was able to produce 7.6 liters of ethanol per 100 kg of wood waste (Katzen and Schell, 2006). Lignocellulosic biomass consists of a complicated matrix made up of cellulose and lignin bound by hemicellulose chains. Pretreatment is done to break this matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose and hemicellulose for enzymatic attack (Sanchez and Cardona, 2008). Pretreatment is primarily done to assist the formation of sugars directly or subsequently by hydrolysis; to avoid the loss and/or degradation of sugars formed, to limit formation of products that are inhibitory to the production of bioethanol and to reduce energy demands and costs. Pretreatment techniques can be generally categorized into physical, chemical, physicochemical and biological treatments. In general a combination of these processes is used in the pretreatment step (Sarkar *et al*., 2012).

### 1.3.1 Physical pretreatment

#### *1.3.1.1 Mechanical size reduction*

The first step for ethanol production from agricultural solid wastes is comminution of the selected substrates by means of milling, grinding or chipping. This improves the surface to volume ratio making the biomass more accessible to chemical or biological treatment and also reduces cellulose crystallinity and improves the efficiency of downstream processing (Cheng and Sun, 2002). Wet milling, dry milling, vibratory ball milling and compression milling are usually done for this purpose. The initial and final particle sizes, moisture content and the nature of waste (hardwood, softwood, fibrous, etc.) determines the power requirements for mechanical comminution of agricultural materials (Talebnia *et al*., 2010). Albeit size reduction provide better results, very fine particle size may impose negative effects on the subsequent processing such as pretreatment and enzymatic hydrolysis (Bjerre *et al*., 1996).

#### *1.3.1.2 Pyrolysis*

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300˚C, cellulose rapidly decomposes to gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Brad-bury, 1979). The decomposition is much slower with less volatile product formation at lower temperatures. Mild acid hydrolysis (1 N sulphuric acid, 97˚C, 2.5 h) of the residues from pyrolysis pretreatment had resulted in 80–85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan *et al.*,

1987). The process can be enhanced in the presence of oxygen (Shafizadeh and Bradbury, 1979). When sodium carbonate is added as a catalyst, the decomposition of pure cellulose occurs at a lower temperature (Shafizadeh and Lai, 1975).

### 1.3.2 Physicochemical pretreatment

#### *1.3.2.1 Steam explosion*

Steam explosion is a promising and simple method of pretreatment which makes biomass more accessible to cellulase attack (Sharma *et al*., 2007). This method of pretreatment does not employ any catalyst and fractionates the biomass to yield levulinic acid, xylitol and alcohols (Balat *et al*., 2008). In this method, 60% moisture is maintained in 1 g of biomass (600 µL water for 1 g biomass) which is then heated using high pressure steam (20-50 bar, 160-290ºC) for a few minutes; the reaction is then stopped by sudden decompression to atmospheric pressure (Sharma *et al*., 2007). When steam is allowed to expand within the lignocellulosic matrix it separates the individual fibres (Balat *et al*., 2008). The high recovery of xylose (45-65%) makes the steam-explosion pretreatment economically attractive.

#### *1.3.2.2 Ammonia fibre expansion*

Ammonia fibre expansion (AFEX) is an alkaline thermal pretreatment which exposes the lignocellulosic materials to high temperature and pressure followed by rapid pressure release (Mosier *et al*., 2005). It involves liquid ammonia and steam explosion (Balat *et al*., 2008). This system exposes the polymers (hemicellulose and cellulose) to enzymatic attack without liberating any sugars. This pretreatment has the drawbacks of being less efficient for biomass containing higher lignin contents (e.g.

softwood, newspaper) as well as of causing solubilisation of only a very small fraction of solid material particularly hemicellulose.

### 1.3.3 Chemical pretreatment

#### *1.3.3.1 Acid hydrolysis*

Concentrated acids such as sulphuric acid ($H_2SO_4$) and hydrochloric acid (HCl) have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acids must be removed after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995). Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulphuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian *et al.*, 1997). At moderate temperature, direct saccharification suffers from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994). Recently developed dilute acid hydrolysis processes use less severe conditions and achieve high xylan to xylose conversion yields. There are primarily two types of dilute acid pretreatment processes: high temperature (T greater than 160˚C), continuous-flow process for low solids loading (5-10% [weight of substrate/weight of reaction mixture]) (Converse *et al.,* 1989), and low temperature (T less than 160˚C), batch process for high solids loading (10–40%) (Cahela *et al.*, 1983; Esteghlalian *et al.*, 1997). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or

AFEX. A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes.

### *1.3.3.2 Alkaline hydrolysis*

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan *et al.,* 1987). The alkaline hydrolysis is based on the mechanism of saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, as in lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute sodium hydroxide (NaOH) treatment of lignocellulosic materials cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Fan *et al.,* 1987). The digestibility of sodium hydroxide treated hardwood increased from 14% to 55% with the decrease in lignin content from 24-55% to 20% (Millet *et al.,* 1976). However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet *et al.,* 1976). Dilute NaOH pretreatment was also effective for the hydrolysis of straws that have relatively low lignin content of 10-18% (Bjerre *et al.,* 1996).

### *1.3.3.3 Wet oxidation*

Wet oxidation is well suited for loosening the surface structures of the lignocellulosic biomass aiding in hemicellulose degradation (Pedarson *et al.,* 2002).

In wet oxidation, the feedstock material is mixed with water (1 L of water per 6 g of biomass) and heated in a high pressure reactor either in the presence of oxygen or nitrogen at temperatures above 120˚C (Martín *et al*., 2007). This technique facilitates the transfer of partially hydrolysed hemicellulose molecules from solid phase to the liquid phase. Hemicellulose hydrolysis causes the release of sugar oligomers into the broth (Ahring *et al*., 1999).

#### *1.3.3.4 Organosolv pretreatment*

Organic solvent or organosolv pulping processes are alternative methods for the delignification of lignocellulosic materials. This technique uses organic solvent/water mixtures and thus eliminates the need to burn the liquor and allows the isolation of the lignin (by distillation of the organic solvent). Examples of such pretreatment include using 90% formic acid and pressurized carbon dioxide in combination (50% alcohol/water mixture and 50% carbon dioxide) (Cardona *et al*., 2010). Other various organic solvents which can be used for delignification are methanol, ethanol, acetic acid, performic acid, per-acetic acid and acetone (Zhao *et al*., 2009).

### 1.3.4 Biological pretreatment

Biological pretreatment process was performed to prevent the loss of cellulose, by a cellulase-less mutant of *Sporotrichum pulverulentum* developed for the degradation of lignin in wood chips (Ander and Eriksson, 1977). Hatakka (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks.

Similar conversion was obtained in the pretreatment by *Phanerochaete sordida* 37 and *Pycnoporus cinnabarinus* 115 in four weeks (Hatakka, 1983). However, further studies illustrated the use of brown-, white-, and soft-rot fungi in the degradation of lignin and hemicellulose in waste materials (Fan *et al.,* 1987). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are most effective Basidiomycetes for biological pretreatment of lignocellulosic materials (Fan *et al.,* 1987). The white-rot fungus *P. chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases for the degradation of wood cell walls (Waldner *et al.,* 1988). These enzymes were produced during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Akin *et al.,* (1995) also reported the improvement in biodegradation of Bermuda grass stems by 29-32% using white-rot fungi *Ceriporiopsis subvermispora* and 63-77% using *Cyathus stercoreus* after 6 weeks. The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

### 1.4 Saccharification

Saccharification involves the conversion of complex carbohydrates to simple monomers and can be considered as a critical step in bioethanol production. It encompasses the use of hydrolysing enzymes and requires less energy and mild environment conditions (Ferreira *et al.,* 2009). Saccharification is carried out by enzymes that are highly substrate specific. Cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively to release hexose or

pentose sugars that can be fermented to bioethanol. Cellulose contains several glucose moieties in long chains and hemicellulose contains different sugar units such as mannan, xylan, glucan, galactan and arabinan. Cellulase enzymes involve endo and exoglucanase and β-glucosidase. While endoglucanases (endo-β-(1→4)-D-glucanhydrolase or E.C. 3.2.1.4) attack the low crystallinity regions of the cellulose fibre, exoglucanases (exo-β-(1→4)-D-glucan cellobiohydrolase or E.C. 3.2.1.91) remove the cellobiose units from the free chain ends. These cellobiose units are finally hydrolysed to glucose by β-glucosidase (E.C. 3.2.1.21) (Sanchez *et al*., 2008). Hemicellulolytic enzymes are more complex and are a mixture of at least eight enzymes such as endo-β-(1→4)-D-xylanases, exo-β-(1→4)-D-xylocuronidases, α-L-arabinofuranosidases, endo-β-(1→4)-D-mannonases, β-mannosidases, acetyl xylan esterases, α-glucoronidases and α-galactosidases. Hemicellulose gives rise to several pentoses and hexoses. Several species of *Clostridium, Cellulomonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces* are able to produce cellulase enzyme. Mesophilic strains producing cellulases like *Fusarium oxysporium, Piptoporus betulinus, Penicillium echinulatum, P. purpurogenum, Aspergillus niger* and *A. fumigatus* have also been reported (Sharma *et al.,* 2001; Valaskova and Baldrian, 2006; Martins *et al.,* 2008). The cellulases from *Aspergillus* usually have high β-glucosidase activity but lower endoglucanase levels, whereas, *Trichoderma* has high endo and exoglucanase components with lower β-glucosidase levels, and hence has limited efficiency in cellulose hydrolysis. Many fungi such as *Trichoderma, Penicillium, Fusarium, Phanerochaete, Humicola, Schizophillum* sp. also have been reported for cellulase production.

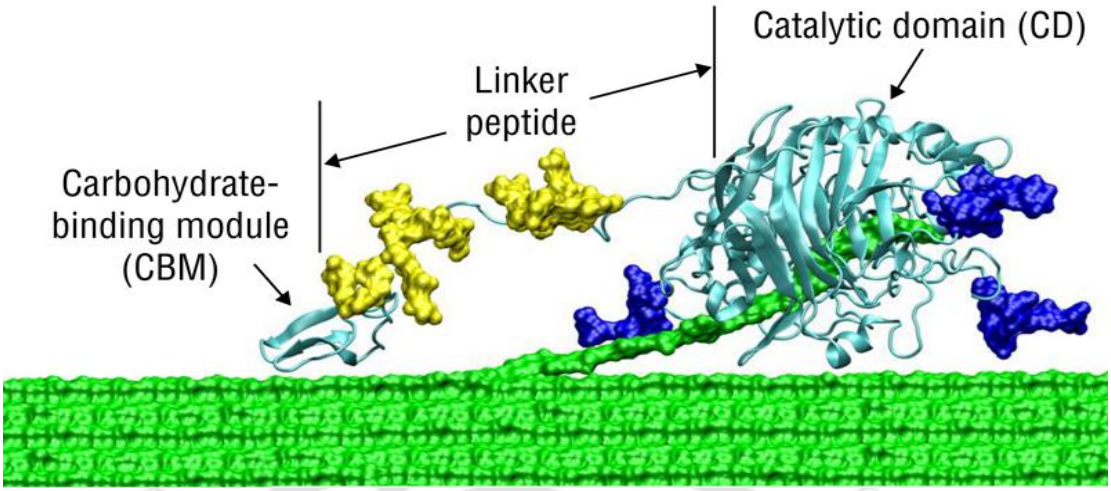


**Fig. 1.5** Family 7 cellobiohydrolase -3 sub-domains of *Trichoderma reesei* (Sandgren *et al*., 2001).

Among the various cellulolytic microbial strains, *Trichoderma* is one of the most well studied cellulase producing fungal strains (Xu *et al*., 1998). *Trichoderma* produces at least two cellobiohydrolases and five endoglucanases and three endoxylanases (Sandgren *et al*., 2001) (Fig. 1.5). However, *Trichoderma* lacks β-glucosidase activity that plays an efficient role in polymer conversion (Kovács *et al*., 2009).

### 1.4.1 *Clostridium thermocellum*

*Clostridium thermocellum* is an anaerobic, thermophilic, cellulolytic and ethanologenic, gram-positive bacterium capable of directly converting cellulosic biomass into ethanol (Bayer *et al*., 2000). The general cellular structure of *C. thermocellum* is similar to most rod-shaped bacteria as shown in scanning electron microscopic (SEM) images (Fig. 1.6 from Lamed *et al*., 1987). The unstained *C. thermocellum* YS cells show typical rod-shaped cells (Fig. 1.6A). However, the fibrous and protuberant structures on the cell surface are visible only when the cell is

stained with cationized ferritin as shown for *C. thermocellum* ATCC 27405 (Fig. 1.6B). The cationized ferritin (CF) stained *C. thermocellum* YS structure as seen in Transmission Electron Microscope (TEM) is displayed in Fig. 1.7. The TEM image of *C. thermocellum* clearly showed fibrous and protuberant structures on the cell surface (Fig. 1.7). The three major types of labelling: the monolayer (m) of CF particles which envelop the entire cell surface, the fibrous structures (f) which sometimes connect two adjacent cells, and the nodulous protuberances (p) which appear in large numbers over the entire cell surface (Fig. 1.7). In tangentially sectioned areas of the cell surface (t), there are indications that the protuberances may be interconnected secondarily by low-lying structures (Fig. 1.7).

The usefulness of *C. thermocellum* cellulosomal enzymes lies in its capability to directly convert the cellulose biomass into a usable energy source *viz*., bio-fuel or ethanol. However, there are some disadvantages of *C. thermocellum* to practical applications owing to low ethanol yield, at least partially due to branched fermentation pathways that produce acetate, formate and lactate along with ethanol. The research has been directed to optimizing the ethanol-producing metabolic pathway for creating more efficient biomass conversion (Zhang and Lynd, 2005). Biotechnological research has shown that the cellulose degrading bacteria produce a large, complex cellulase system known as the cellulosome which consists about 20 catalytic proteins that are involved in the adherence of bacterium to cellulose, breakdown and regulation of cellulose degradation and the transport of sugar monomers (Bayer *et al*., 2000).

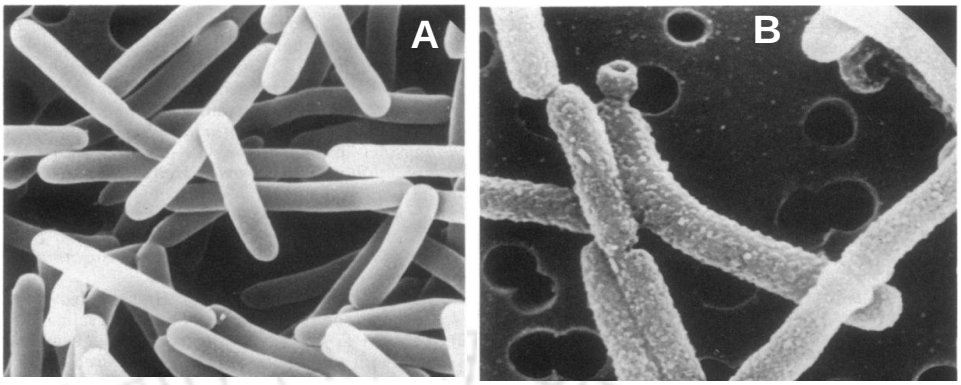


**Fig. 1.6** Scanning electron microscope (SEM) images of *Clostridium thermocellum,* **A)** showing normal rod shaped cells of *Clostridium thermocellum* YS and **B**) shows cationized ferritin (CF) stained *Clostridium thermocellum* ATCC 27405 rod shaped cells with protuberant structures on cell surface (Lamed *et al*., 1987).

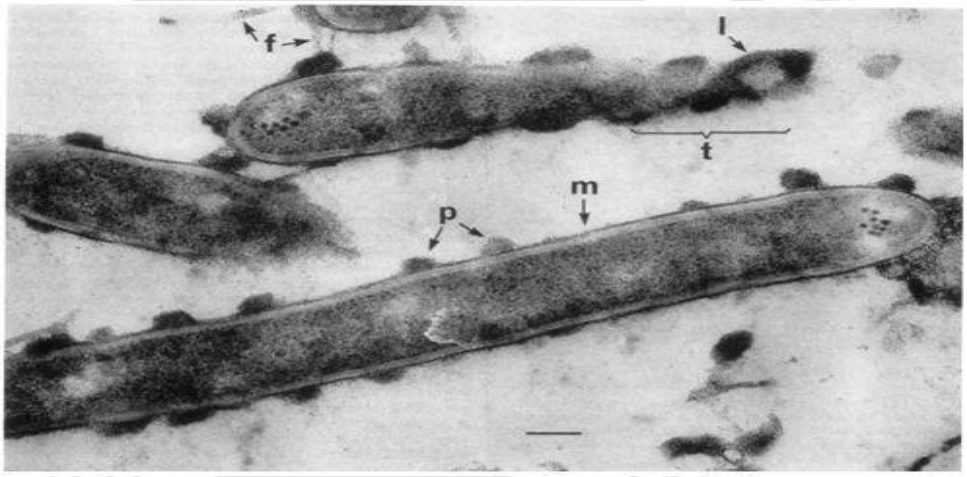


**Fig. 1.7** Transmission electron microscope (TEM) image of cationized ferritin (CF) stained *C. thermocellum* YS grown on cellobiose. The three major types of labelling: the monolayer (m) of CF particles encompassing the entire cell surface, the fibrous structures (f) connecting two adjacent cells and the nodulous protuberances (p) over the entire cell surface. In tangentially sectioned areas of the cell surface (t), the protuberances may be interconnected secondarily by low-lying structures (Bayer and Lamed, 1986).

Cellulosomes are exocellular, multienzyme complex produced by *C. thermocellum,* which comprises numerous cellulases and hemicellulases organized around a scaffolding protein.

### *1.4.1.1 Cellulosome structure*

The cellulosome is a macromolecular complex, whose components interact in a synergistic manner to catalyze the efficient degradation of cellulose (Bayer *et al.,* 2007). The cellulosome complex comprises numerous kinds of cellulases and related enzyme subunits, which are assembled into the complex by virtue of a unique type of scaffolding subunit known as scaffoldin (Bayer *et al*., 2004). The cellulosomal enzymes from *C. thermocellum* range in molecular size from about 40 to 180 kDa (Fontes and Gilbert, 2010). The realization about the multienzyme complex (cellulosome) of *C. thermocellum* occurred gradually with discoveries of different catalytic and binding domains and dockerins, first in clostridial species and, subsequently in other bacteria and fungi (Doi and Kosugi, 2004). Each of the cellulosomal subunits consist of a multiple set of modules, two classes (dockerin domains on the enzymes and cohesin domains on scaffoldin) of which govern the incorporation of the enzymatic subunits into the cellulosome complex (Bayer *et al*., 1998). The cellulosomal enzymes are usually members of the glycosyl hydrolase families of enzymes, which hydrolyze oligosaccharides and polysaccharides (Fontes and Gilbert, 2010).

### 1.4.2 Carbohydrate-active enzymes

Carbohydrates are dynamic molecules that are constantly synthesized and broken down. There are varieties of enzymes involved in the synthesis as well as breakdown of carbohydrates. The glycosyltransferases (GTs) are mainly involved in the formation of the glycosidic bond or biosynthesis of carbohydrates. The polysaccharide lyases (PLs), carbohydrate esterase (CEs) and glycoside hydrolases (GHs) are concerned with the breakdown of polysaccharides. In summary, the carbohydrate-active enzymes are grouped into 262 families based on amino acid sequence similarity and are listed in the continually updated carbohydrate-active enzyme (CAZy) database (www.cazy.org). Out of 262 carbohydrate-active enzymes, about 132 families belong to GHs (Cantarel *et al*., 2009). These 132 GH families contain nearly 2243 species of bacteria, 155 species of archaea and 71 species of eukaryote (www.cazy.org). A close inspection of the genomes listed within the database reveals the fact that 1-3% of the genome of most organisms is devoted to encoding glycosyltransferases (GTs) and glycoside hydrolases (GHs) (www.cazy.org). The information available at CAZy database provide a wealth of gene sequences (many yet to be characterized) to study the structure and function of carbohydrate-active enzymes (Cantarel *et al*., 2009).

#### *1.4.2.1 Glycosyltransferases*

Glycosyltransferases (GTs) catalyse the transfer of any sugar moieties from activated donor molecules to specific acceptor molecules with the formation of glycosidic bonds (Sinnot, 1990). These enzymes utilize 'activated' sugar phosphates as glycosyl donors and catalyze glycosyl group transfer to a nucleophilic group, usually

an alcohol (Campbell *et al*., 1997). The product of glycosyl transfer may be an O-, N-, S-, or C-glycoside; the glycoside may be part of a monosaccharide, oligosaccharide or polysaccharide (Lairson *et al*., 2008). As of now, nearly 94 families of GTs have been recognized and are listed in the CAZy database (http://www.cazy.org/GlycosylTransferases.html). There are almost over 8000 gene sequences in GenBank and the crystal structure of 36 GTs has been solved till date (http://www.cazy.org/GlycosylTransferases.html). Glycosyltransferases family 1 (GT1) shows known activities of UDP-glucuronosyltransferase (EC 2.4.1.17), UDP-glucose: 4-hydroxybenzoate 4-O-b-glucosyltransferase (EC 2.4.1.194) involving inverting mechanism, glycosyltransferases family 5 (GT5) shows activities of starch glucosyltransferase (EC 2.4.1.21), NDP-Glc: starch glucosyltransferase (EC 2.4.1.242) involving retaining mechanism, glycosyltransferases family 74 (GT74) shows activities of α-(1→2)-L-fucosyltransferase (EC 2.4.1.69) involving inverting (inferred) mechanism Many GTs have been reported from wide range of bacterial population *viz., Acidophilium, Actinoplanes, Bacillus, Clostridium, Gloeobacter, Lactobacillus,* (Coutinho *et al*., 2003).

### *1.4.2.2 Polysaccharide lyases*

Polysaccharide lyases (PLs) are group of enzymes that cleave the uronic acid-containing polysaccharide chains following a β-elimination mechanism liberating an unsaturated hexenuronic acid residue and a new reducing end (http://www.cazy.org/Polysaccharide-Lyases.html). Based on amino acid sequence similarities, the CAZy database contains 22 families of these classified enzymes, reflecting their structural features (Lombard *et al*., 2010). As of now, more than one

thousand gene sequences of PLs have been deposited in the GenBank and out of them 546 sequences are from bacteria. Nearly 98 PLs have been characterized till date and the crystal structures of only 12 PLs have been solved (http://www.cazy.org/Polysaccharide-Lyases.html). These enzymes operate according to the general *syn*- and *anti*-elimination mechanisms. Polysaccharide lyase family 1 (PL1) are known to show activities of pectate lyase (EC 4.2.2.2), exo-pectate lyase (EC 4.2.2.9), pectin lyase (EC 4.2.2.10). Polysaccharide lyase family 10 (PL10) displays activities of pectate lyase (EC 4.2.2.2) and polysaccharide lyase family 16 (PL16) shows activities of hyaluronan lyase (EC 4.2.2.1).

### *1.4.2.3 Carbohydrate esterase*

The carbohydrate esterases (CEs) catalyze the de-O or de-N-acylation of substituted saccharides. Two types of substrates have been considered for carbohydrate esterases: i) those in which the sugar plays the role of the "acid", such as pectin methyl esters for 4-O-methyl-glucuronoyl methylesterase from *Schizophyllum commune* (Li *et al*., 2007) and ii) those in which the sugar behaves as the alcohol, such as acetylated xylan for acetyl xylan esterase (family 1 and 2 CEs) from *Clostridium thermocellum* ATCC 27405 (Montanier *et al*., 2009). A number of possible reaction mechanisms are involved: the most common is a Ser-His-Asp catalytic triad catalyzed deacetylation analogous to the action of classical lipase and serine proteases but other mechanisms such as a $Zn^{2+}$ catalyzed deacetylation prevails in some families**.** Carbohydrate esterase family 1 (CE1) shows activities of cinnamoyl esterase (EC 3.1.1.-); feruloyl esterase (EC 3.1.1.73); carboxylesterase (EC 3.1.1.1) possessing (α/β/α)-sandwich structure, carbohydrate esterase family 2 (CE2) shows

activities of acetyl xylan esterase (EC 3.1.1.72), carbohydrate esterase family 5 (CE5) shows activities of cutinase (EC 3.1.1.74) possessing (α/β/α)-sandwich structure, carbohydrate esterase family 14 (CE14) shows activities of N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-a-D-glucopyranoside deacetylase (EC 3.5.1.89) and diacetylchitobiose deacetylase (EC 3.5.1.-), etc.

### *1.4.2.4 Glycoside hydrolase*

Glycoside hydrolases (GHs) catalyse the hydrolysis of the glycosidic linkage of glycosides, and lead to the formation of hemiacetal or hemiketal and the corresponding free aglycone. GHs can catalyze the hydrolysis of O-, N- and S-linked glycosides. GHs are also referred to as glycosidases, and glycosyl hydrolases. The GHs are group of enzymes that exists in most of the living organism (Cantarel *et al*., 2009). The carbohydrate-active enzymes database (CAZy) provides an updated list of the glycoside hydrolase families. They are classified into different families based on homology of their primary sequence. As of now, the GHs are grouped into 130 families based on amino acid sequence with more than 30,000 entries in the CAZy database (www.cazy.org). GHs are also grouped into 14 clans based on the fold of proteins as it was found to be better conserved than their amino acid sequence (Cantarel *et al.*, 2009). Even the closely related catalytic GHs are very often found to differ in substrate specificity (http://www.cazy.org/), while enzymes with very different enzyme activities are found among glycoside hydrolase homologs (Coutinho *et al.*, 2003). Small changes in the primary structure of glycoside hydrolases are able to change their substrate specificity (Andrews *et al.*, 2000). Unlike other CAZymes (GTs, PLs, and CEs), the structural data on GHs has clearly displayed several

different folds, such as $(\alpha/\alpha)_6$, β-helix, β-propeller, β-jelly roll and the $(\alpha/\beta)_8$ TIM barrel motif. The $(\alpha/\beta)_8$ TIM barrel is found in the majority of GHs listed in CAZy database (Cantarel *et al.*, 2009). Glycoside hydrolase family 1 (GH1) are known to show activities of β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.23), β-mannosidase (EC 3.2.1.25). Glycoside hydrolase family 26 (GH26) shows activities of β-mannanase (EC 3.2.1.78). Glycoside hydrolase family 30 (GH30) displays activities of glucosylceramidase (EC 3.2.1.45), β-xylosidase (EC 3.2.1.37), β-fucosidase (EC 3.2.1.38), etc. Glycoside hydrolase family 39 (GH39) exhibits activities of α-L-iduronidase (EC 3.2.1.76) and β-xylosidase (EC 3.2.1.37).

GHs are important plant cell wall degrading enzymes and thus cell wall degradation by microbial enzymes is pivotal to many biological and industrial processes (Gilbert *et al.*, 2010). The polysaccharides of plant cell walls are relatively recalcitrant to enzymatic degradation and due to this the microbes, with time, have evolved and developed a complex enzymatic systems in order to encounter this problem. For example, *Clostridium thermocellum* and *Clostridium cellulolyticum* secrete a mega-dalton multi-modular enzyme complex called the "cellulosome" (Fontes and Gilbert, 2010). The cellulosome is a macromolecular complex, whose components interact in a synergistic manner to catalyze the efficient degradation of cellulose and hemicellulose. The cellulosome complex is composed of numerous kinds of cellulases and related enzyme subunits, which are assembled into the complex by virtue of a unique type of scaffolding subunit (Bayer *et al.*, 1998; Bayer *et al.*, 2004).

***1.4.2.4.1 Glycoside hydrolases and their modular nature***

A module is defined as a contiguous amino acid sequence within a larger sequence folding independently having an individual function but together increases the overall efficiency of the enzyme (Doi and Kosugi, 2004). The GHs are often found to exhibit a modular architecture comprising a catalytic module fused or attached with one or more ancillary modules via linker peptides. The ancillary modules are usually the carbohydrate binding modules (CBMs). Fig. 1.8 displays typical example of a modular family 33 glycoside hydrolase (GH33) from *Micromonospora viridifaciens* exhibiting a catalytic GH33 module which is attached to a non-catalytic family 22 carbohydrate binding module (CBM22) via a linker. The catalytic GH33 from *Micromonospora viridifaciens* was found revealing sialidase activity (Gaskell *et al*., 1995).

***1.4.2.4.2 Glycoside hydrolases and their activity***

The glycosidic bond linking two carbohydrates together or a carbohydrate to another compound is generally a very stable bond with an estimated half-life of 5 million years (Wolfenden *et al*. 1998). The GHs act to hydrolyze these glycosidic bonds and thereby increase bond cleavage efficiency up to ~ $10^{17}$ fold (Wolfenden *et al*. 1998). The substrates degraded by GHs include plant cell wall polysaccharides such as cellulose, hemicellulose and lignin.

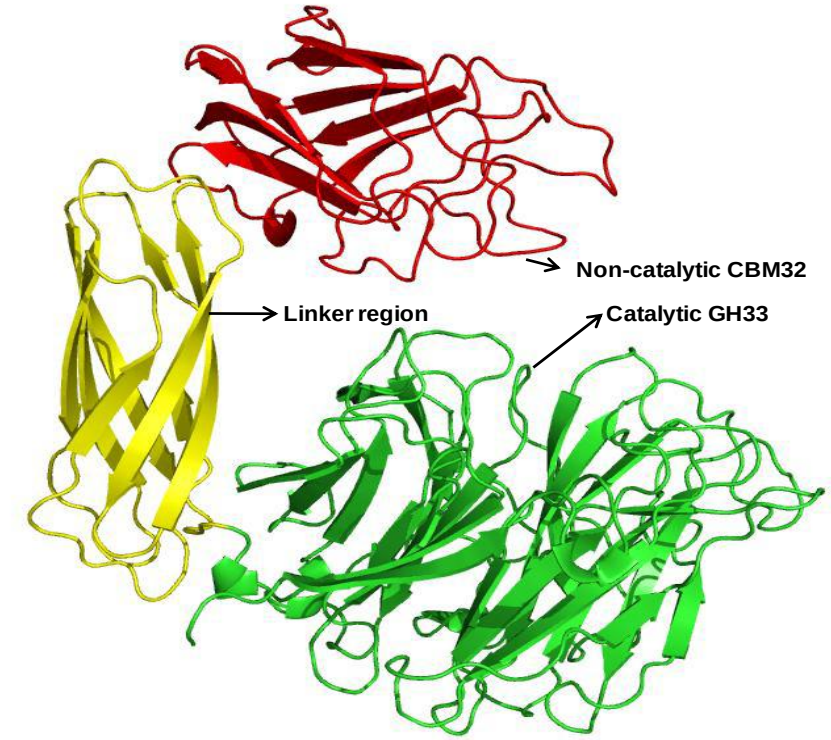


**Fig. 1.8** The 3-dimensional structure (PDB Code 1EUT) of full length family 33 glycoside hydrolase (*Mv*GH33, sialidase) from *Micromonospora viridifaciens* (Gaskell *et al.*, 1995) is shown. The catalytic GH33 is shown in green; linker in yellow and carbohydrate-binding module (CBM32) is in red.

### *1.4.2.4.3 Family 5 glycoside hydrolases*

As per CAZy database, glycoside hydrolase family 5 (GH5) are known to show activities of chitosanase (EC 3.2.1.132); β-mannosidase (EC 3.2.1.25); endo-β-(1→4)-glucanase / cellulase (EC 3.2.1.4); glucan β-(1→3)-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); glucan endo-β-(1→6)-glucosidase (EC 3.2.1.75); mannan endo-β-(1→4)-mannosidase (EC 3.2.1.78); endo-β-(1→4)-xylanase (EC 3.2.1.8); cellulose β-(1→4)-cellobiosidase (EC 3.2.1.91); β-(1→3)-mannanase (EC 3.2.1.-); xyloglucan-specific endo-β-(1→4)-glucanase (EC 3.2.1.151); mannan transglycosylase (EC 2.4.1.-); endo-β-(1→6)-galactanase (EC 3.2.1.164); endoglycoceramidase (EC 3.2.1.123); β-primeverosidase (EC 3.2.1.149); β-

glucosylceramidase (EC 3.2.1.45); hesperidin 6-O-α-L-rhamnosyl-β-glucosidase (EC 3.2.1.168); exo-β-(1→4)-glucanase / cellodextrinase (EC 3.2.1.74).

Cellulases are divided into three major groups of enzymes, which belong to the EC 3.2.1.X class; Endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidase. According to modern concepts most cellulolytic enzymes comprise multidomain proteins containing at least three separate structural elements of different functions, i.e. catalytic domain, cellulose biding domain and inter domain linker. Cellulases can be obtained from several different origins both from aerobic and anaerobic organisms and both from bacteria and fungi. Many cellulases have affinity for several substrates such as chitin, mannan, xylan and cellulose (Rabinovich *et al.*, 2002).

***1.4.2.4.4 Endoglucanases***

Endoglucanases (endo-cellulases), are enzymes of the EC 3.2.1.4 class that have a cleft- shaped active site (Warren, 1996) and hydrolyze the internal β-(1→4)-D glucosidic bonds in cellulose, lichenin and cereal β-glucans. They are produced as many multiforms and other variations in fungal and bacterial systems. The former generally produce different kinds of cellulases (endo or exo, β-glucosidase) whereas the latter mainly produce endoglucanases.

These enzymes act fundamentally by two different mechanisms, which are characterized by the stereo chemical outcome of the degradation reaction. If the stereochemistry of the linkage at the anomeric centre is inverted in the product, forming terminal α-glucose, then the enzyme is an inverting enzyme (Fig. 1.9). On the other hand, if the stereochemistry of the linkage at the anomeric centre is retained

in the product, forming terminal β-glucose, then the enzyme is a retaining enzyme (Withers *et al*., 1986).

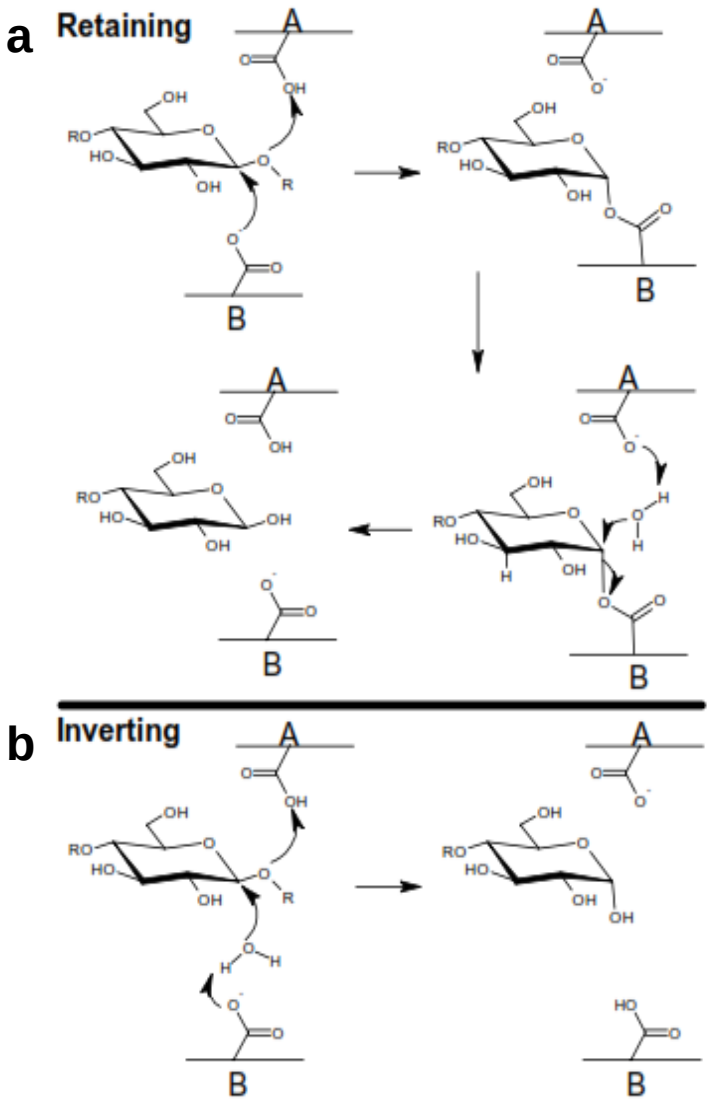


**Fig. 1.9** Reaction mechanisms for cellulases. In the retaining mechanism (a) an enzyme- substrate complex is formed where the substrate is covalently bound to the enzyme. In this mechanism the anomeric carbon will retain its β-configuration. The inverting mechanism (b) changes the anomeric configuration to α-configuration.

The inverting enzymes use a single-displacement mechanism, in which water attacks directly at the anomeric centre, displacing the leaving group in a general acid/base catalyzed process via a transition state with considerable oxo-carbanion

character (Withers *et al*., 1986). Retaining enzymes employ a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate. The first step involves attack of an enzyme nucleophile at the anomeric centre with general acid-catalyzed displacement of the leaving group to yield a covalent glucosyl-enzyme acyl intermediate.

The second step involves a water attack on the anomeric centre of this intermediate in a general base-catalyzed process (Sinnott, 1990). The water in the second step can be substituted for a glucosyl group which then, instead of an attack, will result in the formation of another oligosaccharide. This capability of retaining enzymes is called transglucosylation and has been investigated and reported on several occasions in the literature (Harjunpaa *et al*., 1999).

When unmodified cellulose is the substrate, the endoglucanases have lower activity towards the crystalline regions of the cellulose. This is caused by pervasive hydrogen bonding between the cellulose chains in these regions, which greatly limit their activity.

The degree of polymerization (DP) is usually defined as the number of monomeric units in a macromolecule or polymer or oligomer molecule. Amorphous regions of cellulose allow great penetration by the enzyme facilitating the endoglucanases to effectively hydrolyse the internal bonds in these regions randomly, creating short cello-oligomer chains with low DP. The oligomers formed might also tend to be longer in presence of crystalline domains. Endoglucanases have been shown not to have any activity towards smaller oligomers, i.e. for oligomers with DP $\leq$ 3. However, the cellulose chain might have to be subjected for prolonged hydrolysis depending on the type of endoglucanase.

The extent of this "pre requisite" in chain length for effective hydrolysis reflects the degree of specificity of the enzyme.

#### *1.4.2.4.5 Exoglucanases*

*Exoglucanases (exo-cellulases),* also called cellobiohydrolases abbreviated CBH, belong generally to either EC 3.2.1.91 or 3.2.1.74 classes, and usually contain a tunnel-like active site (Warren, 1996). They attack cellulose from the reducing or non-reducing end, generally producing cellobiose but some glucose and longer oligomers can also be formed (Ryu and Mandels, 1980). Since the active site is shaped as a tunnel, it is believed that the substituents around the linkage to be hydrolyzed in effect pose steric obstacles, which limit the extent to which these enzymes can hydrolyze a given polymer. Thus, since this type of enzyme attacks the ends of the cellulose chains, hydrolysis may progress until a substituent appears. By contrast, endo-glucanases with a cleft-shaped active site are believed to be more tolerant towards the presence of substituents since they attack the cellulose "from the side" of the chain instead of from the ends. Three-dimensional structures of several enzymes have confirmed this hypothesis (Spezio *et al.*, 1993). Exoglucanases are distinguished from β-glucosidase by the inversion of the products (Coughlan, 1985).

#### *1.4.2.4.6 β-glucosidase*

The third type of enzyme that can hydrolyze a cellulose chain is called β-*glucosidase* and is an exo-enzyme that hydrolyzes unmodified cellobiose to glucose starting from the non-reducing end. The activity of cellobiose is crucial for the

hydrolysis of cellulose to individual glucose units (Wood, 1985). This is mainly done because β-glucosidase further hydrolyses the final products of the endo-and exo-glucanases that may have hindered their hydrolysis through product inhibition (Wirick, 1968). Since endoglucanases only hydrolyze cellodextrins with chain length of DP 3 and higher, the presence of β-glucosidase in a cellulase batch can be verified by hydrolysing cellobiose. If the cellobiose remains intact and no traces of glucose are found, the cellulose batch is considered to be free from β-glucosidase. Distinguishing β-glucosidase from exoglucanase is more difficult since inversion and activity towards longer substrates is the only difference.

***1.4.2.4.7 Synergism in a Multi-Enzyme Approach***

The complete hydrolysis of unmodified cellulose to glucose requires a combination of enzymes (endo-, exo-glucanase and β-glucosidase). During the hydrolysis of native unmodified cellulose, the endoglucanases attack the cellulose in a random manner creating low amount of new reducing ends. This reaction is followed by the hydrolysis with exo-glucanases, which attack the cellulose from either end, depending on which type of enzymes, releasing a higher number of new reducing ends. Finally, the β-glucosidase completes the hydrolytic process through the formation of glucose from cellobiose. It is considered that all three enzymes work in a synergistic manner for hydrolysis of both native and modified cellulose (Mansfield *et al*., 1999).

The endoglucanases can hydrolyze the interior parts, liberating free modified and/or unmodified glucose chains which in turn will be attacked by the exo-glucanases and the β-glucosidase. Thus, the use of a combination of all three

enzymes will hydrolyze modified cellulose to a higher extent than the use of only one of the enzyme types by itself. An important fact that has to be addressed is that the substituents of modified cellulose in most cases hinder the enzyme from gaining access to the cellulose chain and forming an adequate transition state for hydrolysis to occur. Hence, complete degradation of modified cellulose to monomers using a combination of these three enzyme types is not possible.

Sequential separate hydrolysis with an endoglucanase followed by an exoglucanase has been shown to give much less extensive cellulose hydrolysis than that achieved by incubation with both enzymes simultaneously (Shepherd *et al.*, 1981). This phenomenon has been suggested to be due to a special synergistic effect, where each enzyme speeds up the action of the other, with a resulting increase of hydrolysis yield.

#### *1.4.2.4.8 Family 43 glycoside hydrolases*

According to CAZy database, the family 43 glycoside hydrolase (GH43) includes enzymes with the different activities like β-xylosidase (EC 3.2.1.37), β-(1→3)-xylosidase (EC 3.2.1.-), xylanase (EC 3.2.1.8), galactan β-(1→3)-galactosidase (EC 3.2.1.145), α-L-arabinofuranosidase (EC 3.2.1.55) and arabinanase (EC 3.2.1.99).

#### *1.4.2.4.9 α-L-arabinofuranosidases*

The most recent classification scheme based on amino acid sequences, primary structure similarities and hydrophobic cluster analysis has classified α-L-arabinofuranosidases (α-L-Ara*f*) into five glycosyl hydrolase (GHs) families i.e. GH30

(Valenzuela *et al.*, 2012; Zhou *et al*., 2012), GH43 (Cartmell *et al*., 2011; Jiang *et al.*, 2012), GH51 (Souza *et al.,* 2011), GH54 (Miyanaga *et al.*, 2004) and GH62 (Pons *et al.*, 2004). The α-L-arabinofuranoside arabinofuranohydrolases (α-L-Ara*f*, EC 3.2.1.55) are the enzymes involved in the hydrolysis of L-arabinosyl linkages. They form an array of GHs required for the complete degradation of arabinose containing polysaccharides (Saha, 2003). Such enzymatic hydrolysis release soluble substrates which are utilized by both prokaryotic and eukaryotic microorganisms (Bayer *et al*., 2000). The α-L-Ara*fs* do not distinguish between the saccharides linked to the arabinofuranosyl moiety, which enables them to exhibit wide substrate specificity (Saha, 2003). The α-L-Ara*f* specifically catalyze the hydrolysis of terminal non-reducing- α-L-(1→2)-, α-L-(1→3)-, and α-L-(1→5)-arabinofuranosyl residues from different oligosaccharides and polysaccharides (Numan and Bhosle, 2005). Effective hydrolysis of α-L-arabinofuranosyl residues from various homo-hemicellulosic polysaccharides like branched arabinans, debranched arabinans, heteropolysaccharides, arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, pectin and different glycoconjugates is carried out by α-L-Ara*f* (Cartmell *et al*., 2011; Mckee *et al*. 2012).

### 1.5 Fermentation

Fermenting microorganisms are used for the conversion of monomeric sugars to ethanol. Different organisms such as bacteria, yeast and fungi can be used for the conversion, however the most frequently used organism in industrial processes are the robust yeast *Saccharomyces cerevisiae* (baker's yeast).

### 1.5.1 Solid-state fermentation

Solid-state fermentation (SSF) is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of micro-organism (Pandey, 1992; Pandey *et al.*, 2000). SSF stimulated the growth of micro-organisms in nature on moist solids which formed the basis of various fermentation techniques in ancient times. SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid-state processes have lower energy requirements, produce lesser wastewater and are environmental-friendly as they resolve the problem of solid wastes disposal. There are several important aspects, which should be considered in general for the development of any bioprocess in SSF. These include selection of suitable micro-organism and substrate, optimization of process parameters and isolation and purification of the product. Going by theoretical classification based on water activity, only fungi and yeast were termed as suitable micro-organisms for SSF. It was thought that due to high water activity requirement, bacterial cultures might not be suitable for SSF. However, experience has shown that bacterial cultures can be well managed and manipulated for SSF processes (Selvakumar and Pandey, 1999). During SSF, a large amount of heat is generated, which is directly proportional to the metabolic activities of the micro-organism. The solid materials/ matrices used for SSF have low thermal conductivities; hence heat removal from the process could be very slow. Sometimes accumulation of heat is high, which denatures the product formed and gets accumulated in the bed. Temperature in some locations of the bed could be 20ºC higher than the incubation temperature. In the early phases of SSF, temperature

and concentration of oxygen remain uniform throughout the substrate but as the fermentation progresses, oxygen is slowly depleted, anaerobiosis sets in and air transfer gets restricted, resulting in the generation and accumulation of heat. The temperature of the substrate is an important factor in SSF as it directly affects the growth of the micro-organism with formation and germination of spore, ultimately regulating the quantity and quality of the intended product. High moistures results in decreased substrate porosity, which in turn prevents oxygen penetration. This may help bacterial contamination. On the other hand, low moisture content may lead to poor accessibility of nutrients resulting in poor microbial growth. Therefore, the moisture content of the substrate has to be carefully regulated for efficient SSF. This is more important because water activity ($a_w$) of the substrate has determining influence on microbial activity. Water activity or '$a_w$' is defined as the vapour pressure of a liquid divided by that of pure water at the same temperature. In short, the type of micro-organisms that can be grown in SSF systems is determined by $a_w$. Water relations in SSF must be critically evaluated.

### 1.5.2 Separate hydrolysis and fermentation

The enzymatic hydrolysis step is often in close collaboration with the following fermentation step in the ethanol production. The concept of separate hydrolysis and fermentation (SHF) involves a separation of the hydrolysis and fermentation by running the reactions in separate units. Pretreated lignocellulosic material is first degraded, in a separate unit, to monomeric sugars by differnet hydrolytic enzymes and then fermented to ethanol in a separate unit. The main advantage of this method is that the two processes (hydrolysis and fermentation) can

be performed at their own individually optimal conditions. The fact that cellulases have their functional optima between 45-50°C and the commonly used fermenting organism has an optimum temperature of 30-37$^0$C (Taherzadeh and Karimi, 2007), makes the SHF concept more relevant. Another advantage with SHF is the possibility to run the fermentation process in a continuous mode with cell recycling. This is possible because lignin residue removal can occur before fermentation. The removal of lignin residue becomes more difficult if it is mixed with the yeast (Taherzadeh and Karimi, 2007). The major drawback of SHF is that the end products i.e. glucose and cellobiose released in cellulose hydrolysis strongly inhibit the cellulase efficiency. Glucose inhibits β-glucosidase which accelerates cellobiose formation since β-glucosidase catalyzes the hydrolysis of cellobiose to glucose. Due to the relatively long residence time of hydrolysis process from one to four days, there is a greater risk of microbial contamination of the sugar solution (Taherzadeh and Karimi, 2007). SHF as an industrial application in a large scale plant gives rise to several alternatives since the process can be designed in various ways. Fig. 1.10 represents a schematic picture of the SHF process (Taherzadeh and Karimi, 2007). After pretreatment, the slurry can be filtered to obtain a separation of the prehydrolysate and the solids. The sugars, mainly pentoses that have been released from the hemicellulose during pretreatment will be removed and only the solid part i.e. cellulose and lignin, are supplied to the enzymatic hydrolysis. It is also possible to wash the slurry prior to enzymatic hydrolysis to remove toxic degradation products derived from the pretreatment process (Lu *et al.*, 2010). However, these methods require extra processing steps and increase the water consumption. This can be avoided by utilizing the whole slurry for the enzymatic hydrolysis (Horn and Eijsink, 2010).

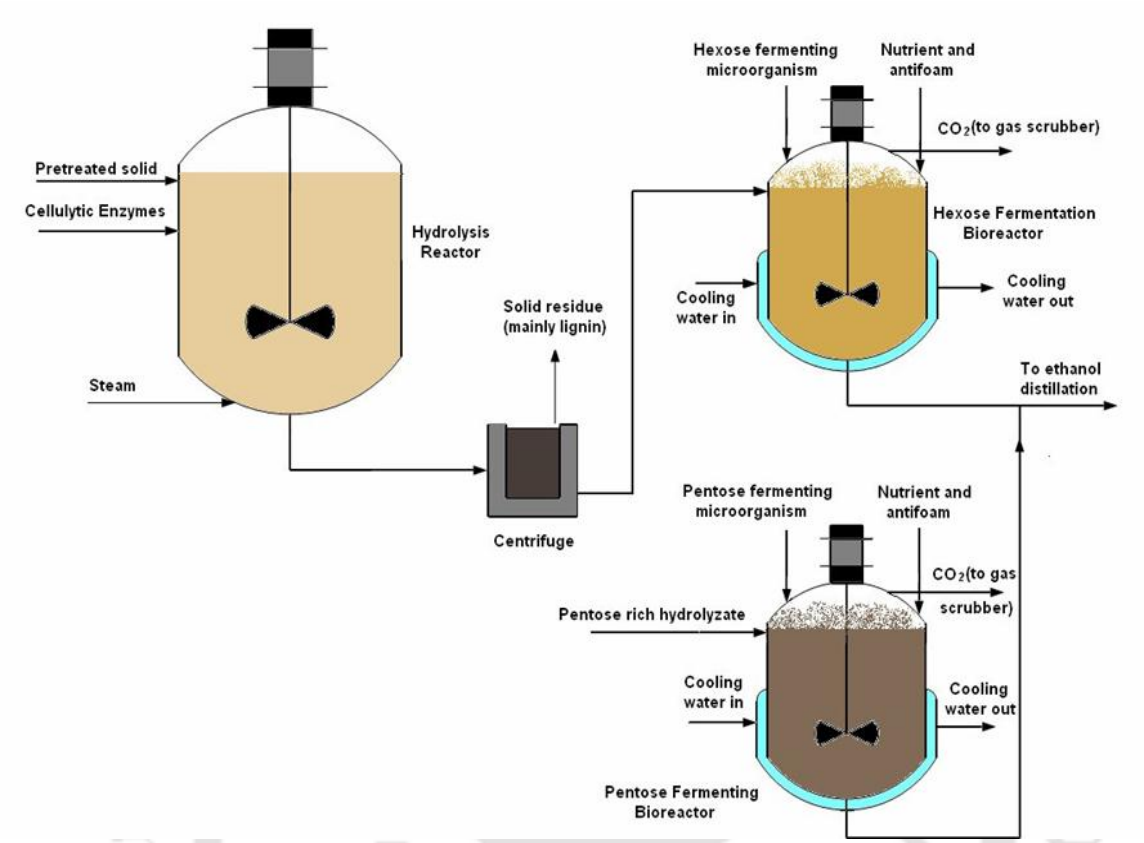


**Fig. 1.10** Schematic representation of bioethanol process employing separate hydrolysis and fermentation (SHF) (Taherzadeh and Karimi, 2007).

A part of the released glucose could be utilized to produce enzymes in a separate reactor. The other alternative is to buy already made enzyme mixes from industrial suppliers (Hamelinck *et al.*, 2005). After enzymatic hydrolysis, lignin is removed before fermentation of the hydrolysate. The hemicellulosic sugars released from the pretreatment and glucose released in the enzymatic hydrolysis can either be fermented together or separately. The ethanol broth is then further transported to distillation and purification (Palmqvist *et al.*, 1996).

### 1.5.3 Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) processes firstly described by Takagi *et al.,* (1977), combine enzymatic hydrolysis of cellulose with simultaneous fermentation of the sugars obtained to ethanol (Fig. 1.11).

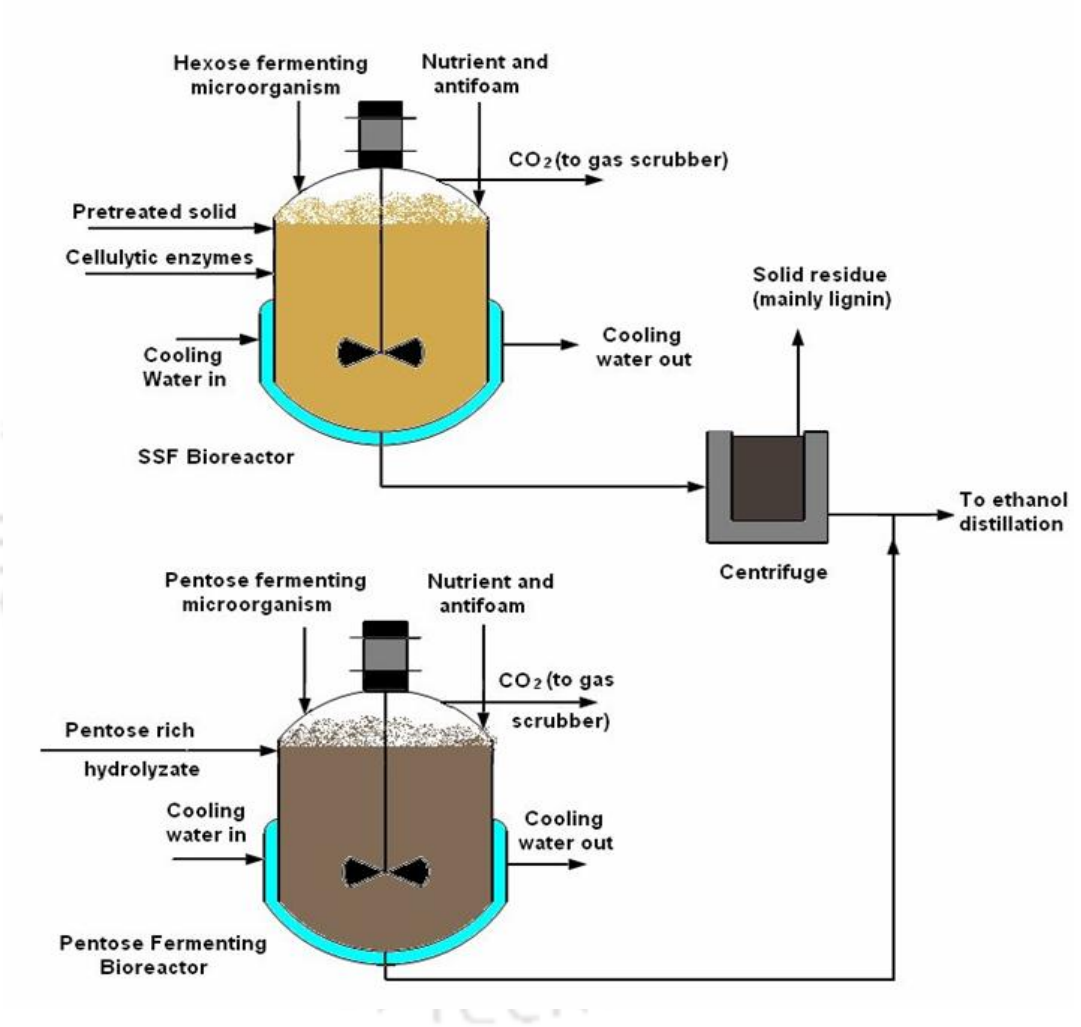


**Fig. 1.11** Schematic representation of a bioethanol process employing simultaneous saccharification and fermentation (SSF) (Taherzadeh and Karimi, 2007).

In the SSF process, the stages are virtually the same as in separate hydrolysis and fermentation systems, except that both are performed in the same reactor. Thus, the presence of yeast together with the cellulolytic enzyme complex reduces the

accumulation of sugars within the reactor, thereby increasing yield and saccharification rate with respect to separate saccharification and fermentation (Wyman *et al.,* 1999). Another advantage of this approach is that a single fermenter is used for the entire process, thereby curbing the investment costs. In addition, the presence of ethanol in the culture medium causes the mixture to be less vulnerable to invasion by undesired microorganisms.

SSF requires compatible saccharification and fermentation conditions, with a similar pH, temperature and optimum substrate concentration. One problem associated to SSF is the different optimum temperatures for saccharification and fermentation. The overall ethanol yield in SSF has been reported to be higher than if the enzymatic hydrolysis and fermentation are carried out separately (SHF) (Wingren *et al.,* 2003). However, not only the yield but also the ethanol concentration is important, because the distillation costs decrease as a function of the final ethanol concentration (Sassner *et al.,* 2008). To increase the ethanol concentration, a high content of water-insoluble solids (WIS) is needed, but very high WIS content leads to a high viscosity of the medium which makes mixing cumbersome. Maximum WIS content for each feedstock, which can be processed in an SSF set up, depends upon its type and quality. In addition to WIS content, the liquid obtained after pretreatment contains a number of compounds with inhibitory effect on the yeast, and potentially also the enzymes (Almeida *et al.,* 2007). Increasing the substrate concentration along with enzyme loadings and inoculum are reported to enhance ethanol titre and yield (Zhang *et al.,* 2010).

Instead of a batch SSF process, a fed-batch SSF process can instead be used.

In this way the following advantages are gained: a) The viscosity of the medium can be maintained low due to a gradual feeding of new material to the reactor, in which the viscosity decreases due to enzymatic degradation (Hodge *et al.,* 2009); b) the effect of toxicity of the hydrolysate can be decreased as a result of both adaptation of the yeast and gradual biological detoxification; c) there may be a beneficial effect on the xylose uptake from a changed concentration ratio of xylose to glucose in the medium (Zhang *et al.,* 2009).

The choice of substrate is an important element in the SSF process. Several cellulosic substrates have been evaluated in the SSF process, including sugar cane bagasse, rice straw, wheat straw, wood fractions, and paper mill byproducts. Recently, several studies on different raw materials have been carried out using simultaneous saccharification and co-fermentation (SSCF) with a genetically engineered strain of *Saccharomyces cerevisiae,* TMB3400, capable of fermenting glucose and xylose (Olofsson *et al.,* 2010). Yeast selection for SSF is an important parameter. The use of thermotolerant yeasts capable of fermenting glucose to ethanol at temperatures above 40°C, which are closer to the optima for the activity of the cellulolytic complex in the range of 40-45°C, is therefore advisable when employing coupled SSF processes. Although an increase in temperature can speed up the hydrolysis, loss of cell viability counters these gains. Temperature range of 35 to 40°C still appears to be optimum for the SSF process (Spindler, 1989). Cellobiose-fermenting yeast has also been studied because additional β-glucosidase activity can speed up the SSF reaction. In general, *Saccharomyces cerevisiae,* a competitive glucose fermenter with faster rate of fermentation performs well if the enzyme preparation is enhanced with β-glucosidase.

Better yield, rate and concentrations are provided by the mixed culture of *Bacillus clausenii* and *S. cerevisiae,* provided the enzyme is lower in β-glucosidase (Taherzadeh and Karimi, 2007).

### 1.5.4 Hexose fermenting *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae,* a glucose utilizing yeast is used extensively in batch fermentations to convert sugars to ethanol for the production of beverages and biofuels. The metabolic pathway of ethanol biosynthesis in *S. cerevisiae* is represented in Fig. 1.12. *S. cerevisiae* is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, producing over 50 mM of ethanol per h per g of cell protein (Dombek and Ingram, 1986). However, this high rate is maintained for only a brief period during batch fermentation and declines progressively as ethanol accumulates in the surrounding broth (Casey and Ingledew, 1986).

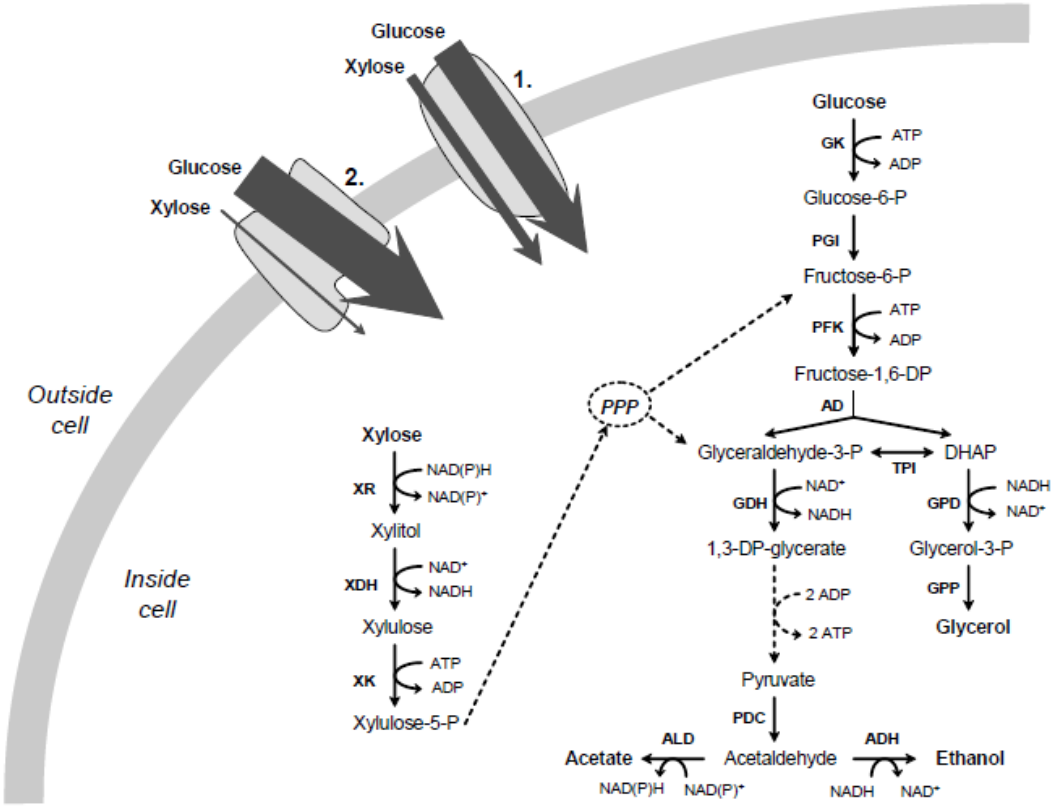


**Fig. 1.12** Metabolic Pathway of Ethanol Biosynthesis in *Saccharomyces cerevisiae* (Casey and Ingledew, 1986).

An ethanol concentration of 3.2 g $L^{-1}$ was achieved in a SSF process using corn stover and natural cellulase along with *S. cerevisiae* (Varga *et al.*, 2002). An ethanol yield of 0.29 (g $g^{-1}$) has been reported from SSF process of pretreated olive tree wood and sunflower stalks by *S. cerevisiae* (Castro *et al.*, 2006). A requirement of lipids for biosynthesis (Casey *et al.*, 1984) and molecular oxygen have been reported essential in many fermentation broths for the maintenance of high fermentative activity. Magnesium is an essential co-factor for many of the glycolytic enzymes and has also been identified as a limiting nutrient in fermentation broth containing peptone and yeast extract (Dombek and Ingram, 1986). Supplying these

nutritional needs reduces but does not eliminate the decline in fermentative activity during batch fermentation. Using recombinant cellulase from *Clostridium thermocellum*, an ethanol yield of 0.14 (g $g^{-1}$) was achieved employing *S. cerevisiae* on Jamun (*Syzygium cumini*) leafy biomass as the substrate (Mutreja *et al.*, 2011). A maximum ethanol yield of 0.480 (g $g^{-1}$) was obtained using *S. cerevisiae* with water hyacinth as substrate for bioethanol fermentation (Guragain *et al.*, 2011). 4.4 g $L^{-1}$ of ethanol has been reported from *T. reesei* cellulase and baker's yeast *S. cerevisiae* with water hyacinth as substrate (Aswathy *et al.*, 2010).

### 1.5.5 Hexose fermenting *Zymomonas mobilis*

*Zymomonas mobilis* is a rod-shaped, gram negative, non-spore forming, motile bacteria (Fig. 1.13). It uses Entner-Doudoroff pathway to rapidly produce ethanol. The key enzymes for ethanol fermentation: alcohol dehydrogenase and pyruvate decarboxylase has been reported to be best expressed in *Zymomonas mobilis* (Oyeleke and Zibrin, 2009; Santos *et al.*, 2010). As a consequence, in recent years, research is focused on *Z. mobilis*, as promising alternative ethanol producer because of its high glucose uptake and high ethanol tolerance. A SSF experiment involving 30% (w $w^{-1}$) solid content with commercial cellulase enzyme and *Zymomonas mobilis* as fermentative organism yielded an ethanol concentration of 60 g $L^{-1}$ (Santos *et al.*, 2010). Nakamura *et al*, (2008) obtained an ethanol yield of 0.09 (g $g^{-1}$) in a SSF process from paper sludge waste using *Z. mobilis*.

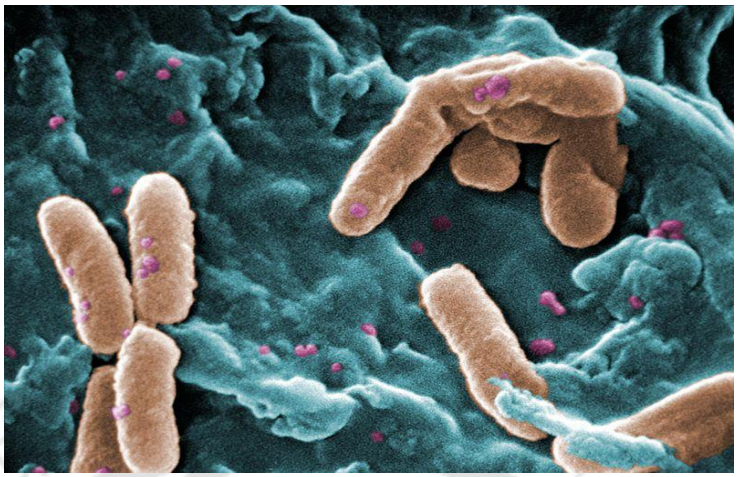

**Fig. 1.13** Scanning electron micrograph of *Zymomonas mobilis* (Oyeleke and Zibrin, 2009).

### 1.5.6 Pentose fermenting *Candida shehatae*

Among the 200 species of yeast examined for the production of ethanol from xylose, *Candida shehatae* presents the best performances in terms of yield and productivity (Kadam and Schimdt, 1997). *Candida shehatae* is a xylose fermenting fungal organism that shows greater ethanol tolerance. They ferment xylose by first converting into pentitol xylitol by the action of xylose reductase. This is then converted in to xylulose by xylulose dehydrogenase which then enters the pentose phosphate pathway.


**Fig. 1.14** Scanning electron micrograph of *Candida shehatae* (Du Preez, 1994).

An ethanol concentration of 44 g $L^{-1}$ with a yield of 0.34 g $g^{-1}$ was obtained employing *C. shehatae* in fed-batch culture with xylose (Du Preez *et al.*, 1989). Such performance proved *C. shehatae* as an interesting candidate for industrial fuel-ethanol production from lignocellulosic raw materials. Crabtree effect is the phenomenon of inhibition of oxygen consumption by addition of glucose with high aerobic glycolysis. It is due to increased competition of glycolysis for inorganic phosphate and NAD limiting their availability for phosphorylation and oxidation. *C. shehatae* is Crabtree-negative yeast (Hahn-Hagerdal *et al.*, 2006) that mainly produces biomass and carbon dioxide when there is no nutritional limitation, but can produce ethanol in oxygen-limited conditions. Du Preez, (1994) suggested that the oxygen availability and particularly the specific oxygen uptake rate (OUR) would be the parameter that determines the partitioning of the carbon flux between ethanol, growth and the production of polyols (xylitol, arabitol, ribitol and glycerol). Alexander *et al.*, (1988) attempted the characterization of *C. shehatae* in a chemostat in order to control the specific OUR by controlling the stirring rate, the airflow rate and the biomass

concentration. Chandel *et al.,* (2007) obtained ethanol yield of 0.48 g $g^{-1}$ from sugarcane bagasse using commercial enzymes and *C. shehatae.*

Substrate fermentation in automated bioreactors enables close monitoring of the process parameters *viz.,* pH and aeration that significantly affect the fermentation dynamics reflected in terms of final ethanol titre (Sanchez *et al.*, 1997). Distillation, rotary vaccum evaporation and pervaporation are some of the commonly used fermentation product recovery processes (Palmqvist *et al.*, 1996). Reddy *et al.,* (2010) obtained an ethanol concentration of 2.2 g $L^{-1}$ from 1% (w $w^{-1}$) of banana waste using a coculture of *C. thermosaccharolyticum* HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937.

## 1.6 Objectives of the present study

### 1.6.1 Why study lignocellulosic ethanol production using naturally and recombinant *Clostridium thermocellum* hydrolytic enzymes

The reason for studying lignocellulosic ethanol production involving naturally isolated and recombinant *Clostridium thermocellum* hydrolytic enzymes are summarized as given below:

1. Agricultural residues (leafy biomass) primarily contain cellulose followed by hemicellulose and lignin. Therefore, development of an effective pretreatment strategy becomes essential for liberation of hexoses and pentoses from lignin seal.
2. Lignocellulose degrading fungal enzymes have been in use at industrial level for more than three decades. However, the main drawback is the high cost of the commercially available *Trichoderma reesei* cellulolytic enzymes (Schulein, 1988).
3. The genome of *Clostridium thermocellum* encompasses a complex cellulosome having a large number of cellulose and hemicellulose degrading enzymes.
4. The cellulosome of *Clostridium thermocellum* is known to have one of the highest rates of cellulose utilization till date reported, that displays 50-fold higher specific activity than the corresponding *Trichoderma reesei* system against crystalline cellulose (Fontes and Gilbert, 2010).
5. Family 5 glycoside hydrolase (GH5) are generally cellulases that aid in successful degradation of cellulose into hexose sugar glucose that can be utilized for bioethanol production.
6. Family 43 glycoside hydrolase (GH43) generally classified as hemicellulases are essential for complete degradation of xylans or arabinoxylans into respective pentose sugars that can be utilized for bioethanol production.

The present study investigated the comparative performance of fungal and recombinant *C. thermocellum* hydrolytic enzymes along with fermentative microbes like *S. cerevisiae* and *C. shehatae* for efficient bioethanol production. Different pretreatment methods were employed on cellulose and hemicellulose rich substrates for effective separate hydrolysis and fermentation (SSF) and simultaneous saccharification and fermentation (SSF) trials carried out at shake flask level. Shake flask SSF experiments with increased substrate concentration was scaled up to a laboratory scale bioreactor under controlled process parameters of pH, temperature, aeration and agitation. Rotary vaccum evaporator was employed for the effective recovery of bioethanol.

The following objectives were specified for the research work:

### 1.6.2 Specific Objectives

1. Production and enhancement of activity of recombinant cellulase and hemicellulase from *Clostridium thermocellum* expressed in *Escherichia coli.*
2. Selection of cellulose and hemicellulose rich substrates and efficient pretreatment process for bioethanol production.
3. Identification of best Simultaneous Saccharification and Fermentation (SSF) of pretreated wild grass and water hyacinth from different combinations of hydrolytic enzymes and fermentative microbes.
4. Optimization of Simultaneous Saccharification and Fermentation (SSF) process by Taguchi Orthogonal array design.
5. Simultaneous Saccharification and Fermentation in shake flask at higher concentration of pretreated wild grass and water hyacinth and scale up in bioreactor under Taguchi optimized conditions with effective ethanol recovery.

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# Chapter 2

## Production and enhancement of activity of recombinant cellulase and hemicellulase from *Clostridium thermocellum* expressed in *Escherichia coli*

### 2.1 Introduction

Lignocellulose degrading fungal enzymes are in use for more than three decades. But the elevated price of the commercially available *Trichoderma reesei* cellulolytic enzymes is the major drawback in its usage (Chowdary *et al.*, 2001). In the recent times, there has been renewed interest in the liberation of fermentable sugars from lignocellulosic biomass by utilizing mixtures of enzymes that hydrolyze biomass for ethanol and other biofuels (Banerjee *et al.*, 2010; Gao *et al.*, 2010). The progression of recombinant DNA technology along with molecular biology has introduced hydrolytic enzyme production and its overexpression in transformed cells. These enzymes can be subsequently used for converting structural carbohydrates to simple sugars (Adlakha *et al.*, 2011).

The cellulosome of the bacterium *Clostridium thermocellum* exhibits one of the highest rates of cellulose utilization known and displays a specific activity against crystalline cellulose that is 50-fold higher than the corresponding *T. reesei* system

(Demain *et al.,* 2005). *C. thermocellum* contains genes coding for exocellular multienzyme complexes called cellulosomes exhibiting both endoglucanase and exoglucanase activities that can be cloned into expression vectors (Guglielmi and Beguin, 1998).

Glycoside hydrolases are a group of enzymes with varying substrate specificity, which also includes cellulases and hemicellulases. According to CAZy database, glycoside hydrolase family 5 (GH5) exhibit activities of chitosanase (EC 3.2.1.132); β-mannosidase (EC 3.2.1.25); cellulase (EC 3.2.1.4); glucan-β-(1→3)-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); glucan endo-β-(1→6)-glucosidase (EC 3.2.1.75); mannan endo-β-(1→4)-mannosidase (EC 3.2.1.78); cellulose β-(1→4)-cellobiosidase (EC 3.2.1.91); β-(1→3)-mannanase (EC 3.2.1.-); mannan transglycosylase (EC 2.4.1.-); endo-β-(1→6)-galactanase (EC 3.2.1.164); exo- β-(1→4)-glucanase / cellodextrinase (EC 3.2.1.74); chitosanase (EC 3.2.1.132); cellulase (EC 3.2.1.4), whereas glycoside hydrolase family 43 displays activities of β-xylosidase (EC 3.2.1.37); β-(1→3)-xylosidase (EC 3.2.1.-); α-L-arabinofuranosidase (EC 3.2.1.55); arabinanase (EC 3.2.1.99) and xylanase (EC 3.2.1.8).

Employing active biomass hydrolyzing enzymes at extremes of temperature and pH may be advantageous for industrial scale production of fermentable sugars from lignocellulosic biomass, because these conditions facilitate overcoming biomass recalcitrance and prevent the growth of contaminating microorganisms (Blumer-Schuette *et al.,* 2008). *Escherichia coli* have been the work-horse of gene expression for many years and is the first-line system for producing recombinant proteins. The reason for this is that many different host-vector systems are readily available, the organism is simple to culture, growth is rapid and recovery of the recombinant protein

is relatively straightforward, particularly with the use of affinity tags. Commercially, *E. coli* is generally recognized as a safe organism and has been proved to be an economically viable means for producing protein products. The optimal growth temperature for *E. coli* is 37°C and pH between 6.4 and 7.2. The bacterium is generally grown under aerobic conditions since anaerobic growth provides less energy for metabolic processes such as protein synthesis (Xu *et al.*, 1999).

As compared to animal cell expression systems, *E. coli* is easily transformed and possesses a relatively low sensitivity to mixing shear rates and process disturbances in pH and dissolved oxygen (DO) (Lee, 1996). These advantages are decisive unless it is desired to produce large, complex proteins or those requiring post-translational modification to become active, both of which are not directly possible using *E. coli* expression (Lee, 1996). However, not all proteins are accumulated to maximal levels in *E. coli* and production typically requires optimization. The goal for optimizing production of recombinant proteins is to produce the highest amount of functional product per unit volume per unit time. For *E. coli*, or any other fermentation system, the level of intracellular accumulation of a recombinant protein is dependent on the final cell density and the specific activity of the protein, or, in other words, the level of accumulation relative to total protein. Four strategies are typically taken for optimizing the production of a recombinant protein. These are: choice of culture medium, mode of cultivation, strain improvement, and expression system control for reviews on optimizing strategies (Kleman and Strohl, 1994). Much of the effort aimed at increasing recombinant protein production in bacterial strains has been directed at maximizing the biomass production and little is known about the effects of media composition on the expression of recombinant

proteins. However, it is well known that the production of secondary metabolites in microbial strains can depend on the composition of the medium in which the organism is grown. Despite this, little attention has been paid to the effects of medium formulation on the accumulation of recombinant proteins.

The primary processing problem in recombinant *E. coli* fermentation is the production of organic acids such as acetic acid, which can inhibit both growth and recombinant protein production (Doelle *et al.,* 1982). Excretion of unwanted organic acids by *E. coli* is a result of fermentative metabolism that occurs in response to insufficient oxygen or the presence of excess carbon substrate (generally glucose). The latter limitation is known as the Crabtree effect (Han *et al.,* 1992). It has been attributed to both saturation of the TCA cycle and saturation of the electron transport phosphorylation process (Konstantinov *et al.,* 1990). Out of a variety of acid by-products, acetate has been shown to possess the most detrimental effect on process performance (Luli and Strohl, 1990). The biomass production of cells is inhibited by acetate toxicity which retards the recombinant proteins' expression and consequently, the cell density owing to the variations in pH, oxygen availability and limiting substrate availability (Shiloach and Bauer, 1975). There are several modes of action by which acetic acid production has been suggested to undermine process performance. In the absence of pH control, acetic acid production will lower pH and inhibit culture growth. However, the control of pH is not sufficient to avoid the detrimental effects of acetate because in sufficient quantity, acetate can reduce growth rate and place an absolute limit on cell growth (Shiloach and Bauer, 1975).

Oscillations in reactor glucose concentrations, even at low levels, have been shown to increase acetic acid production (Neubauer *et al.,* 1995). Hence, avoidance of

the Crabtree effect requires control of either growth rate or reactor glucose concentration at values below the threshold for acetate production (Kleman *et al.*, 1991). Significant acetate production will also decrease the biomass yield through diversion of carbon away from biomass production, and hence decrease process efficiency. Although the exact mechanism remains unclear, acetate has also been shown to decrease recombinant protein production, thus reducing the efficiency of recombinant expression (Lee, 1996). To limit acetic acid production, both oxygen limitation and the Crabtree effect must be avoided. Oxygen limitation is straightforward to avoid, provided means to measure in-situ oxygen concentration are present and the aeration capacity of the reactor can be increased sufficiently to keep pace with culture requirement. Repetitive-batch and fed-batch strategies have proven to be the most robust techniques to maintain growth rates just below the acetate threshold, particularly through exponential growth. The challenge is to manage this control through careful manipulation of the nutrient feed. Scheduled feed addition (without feedback control) may be employed, although such addition will result in non-optimal biomass productivity because optimal performance requires the glucose addition rate be maintained at levels just below the threshold for acetate production (O' Connor *et al.*, 1992).

The regulation of glucose in transcription of functional genes under T7 promoter control has been quite common in *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) plysS cells. As compared to wild type lac promoter, the lambda DE3 prophage encoding T7 RNA polymerase in pET expression hosts have three point mutations (Fig. 1.1). Two point mutations in the –10 region increase promoter strength and decrease its dependence on CAP/cAMP stimulation for full activation. The third-point

mutation is located in the CAP/cAMP binding site and decreases the affinity for CAP/cAMP. This mutation reduces, but does not eliminate, sensitivity to catabolite repression.

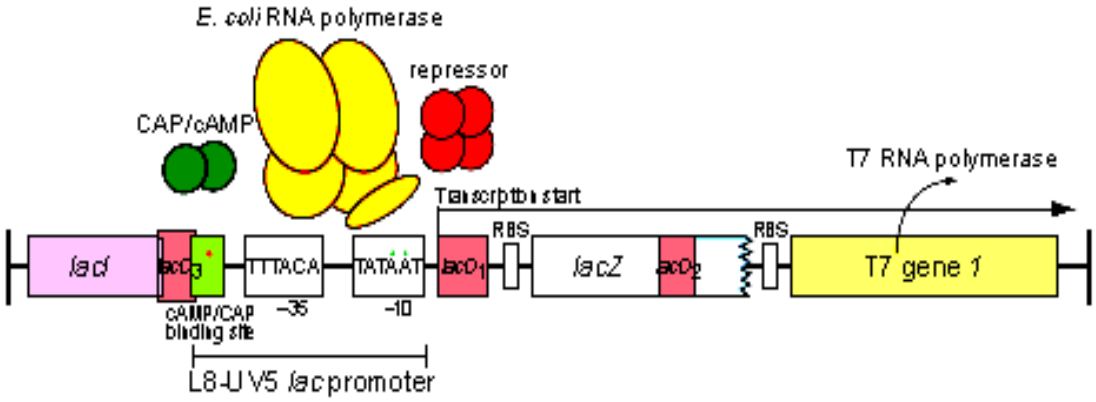


**Fig. 1.1** Transcriptional control of T7 gene 1 in λDE3 lysogens (Grossman *et al.*, 1998).

The recombinant *E. coli* cells can be grown to high densities in common media such as Luria Bertani (LB) (Curless *et al.*, 1990; Lin and Swartz, 1992), synthetic M9 minimal salt medium (Tripathi *et al.*, 2009), Terrific broth (TB) (Lim *et al.*, 2000; Tripathi *et al.*, 2008), Super broth (SB) (Madurawe *et al.*, 2000) and LB medium supplemented with glucose (Grossman *et al.*, 1998).

In the present study, an effort was made to augment the cell biomass of different *E. coli* cells harboring *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) gene and, in turn, the improvements of enzymatic activities by using repetitive batch strategy in various growth media like LB, TB and LB with glucose at shake flask level.

## 2.2 Materials and Methods

### 2.2.1 Reagents, chemicals and substrates

Carboxy methyl cellulose (CMC), kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma Aldrich (St. Louis, USA). Rye arabinoxylan was procured from Megazyme International Ltd. (Ireland). The analytical grade reagents and chemicals *viz.,* sodium chloride, ampicillin, sodium acetate, glucose, yeast extract, peptone, tryptone, di-potassium phosphate, mono-potassium phosphate, glycerol, sodium carbonate, sodium bicarbonate, sodium potassium tartarate, sodium sulphate, copper sulphate, ammonium molybdate and sodium arsenate were purchased from Himedia Pvt. Ltd. India. Phosphoric acid was procured Qualigens India Pvt. Ltd. Coomassie Brilliant Blue G-250 and Coomassie Brilliant Blue R-250 were purchased from Amresco LLC, USA.

### 2.2.2 Microorganisms and culturing conditions

The family 5 glycoside hydrolase (GH5) gene from *Clostridium thermocellum* was cloned in expression vector pET-21a(+) and resulting recombinant plasmid was used for transformation of *E. coli* BL21 (DE3) cells for expression of the enzyme as reported earlier (Taylor *et al.,* 2005). The recombinant GH5 cellulase is now available commercially at NZY Tech, Lda, Lisbon, Portugal. The *E. coli* BL21 (DE3) pLysS cells transformed by family 43 glycoside hydrolase (GH43) gene from *Clostridium thermocellum* was cloned in pET-28a(+) expression vector and expressed as reported earlier (Das *et al.,* 2012). These cells were cast-off as a source of recombinant GH43 hemicellulase (α-L-arabinofuranosidase). These *E. coli* BL21 (DE3) cells containing

GH5 (Taylor *et al.*, 2005) and *E. coli* BL21 (DE3) pLysS cells containing GH43 (Das *et al.*, 2012) were preserved in LB medium as glycerol stock at -80°C in the laboratory of Prof. Arun Goyal at Department of Biotechnology, IIT Guwahati.

### 2.2.3 Batch Production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani medium

The recombinant *C. thermocellum* GH5 cellulase production was initiated by inoculating 50 µL of the *E. coli* BL21 (DE3) culture from glycerol stock into 5 mL of Luria-Bertani (LB) medium (pH 7.2) supplemented with 100 µg $mL^{-1}$ ampicillin and incubated at 37°C and 180 rpm for 16 h. The chemical composition of LB medium is given in Table 2.2.1. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min.

**Table 2.2.1** Chemical composition of LB medium.

| Constituents | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Tryptone | 1 |
| Yeast extract | 0.5 |
| NaCl | 1 |

One percent (v $v^{-1}$) of this culture inoculum was transferred aseptically to 200 mL of LB medium in 500 mL flask containing 100 µg $mL^{-1}$ ampicillin and was incubated at 37°C, 180 rpm till the culture reached mid-exponential phase (OD at 600 nm ~ 0.6). This culture was then induced with 1 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) followed by further 12 h incubation at 37°C for over-expression of recombinant protein (Taylor *et al.*, 2005). 50 µg $mL^{-1}$ kanamycin was used as a selective marker for *E. coli* BL21 (DE3) pLysS cells containing GH43 hemicellulase (α-L-arabinofuranosidase) (Das *et al.*, 2012). Similar production

process was followed for GH5 cellulase except, the incubation period was 24°C, 180 rpm and 24 h for over-expression of GH43 after induction with 1 mM IPTG.

### 2.2.4 Repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani medium

During repetitive batch mode, the first step remained similar as followed in batch mode (Section 2.2.3) and at early stationary phase, 198 mL of culture medium containing the *E. coli* cells (GH5 or GH43) was removed. This was centrifuged and cell mass was further processed for the isolation of enzymes. To remaining 2 mL broth, 198 mL of fresh medium was added. Similar protocol was followed in consecutive three batches where remaining 2 mL of medium comprising induced cells were castoff as inoculum. The schematic diagram of repetitive batch strategy using different media is represented in Fig. 2.2.1.

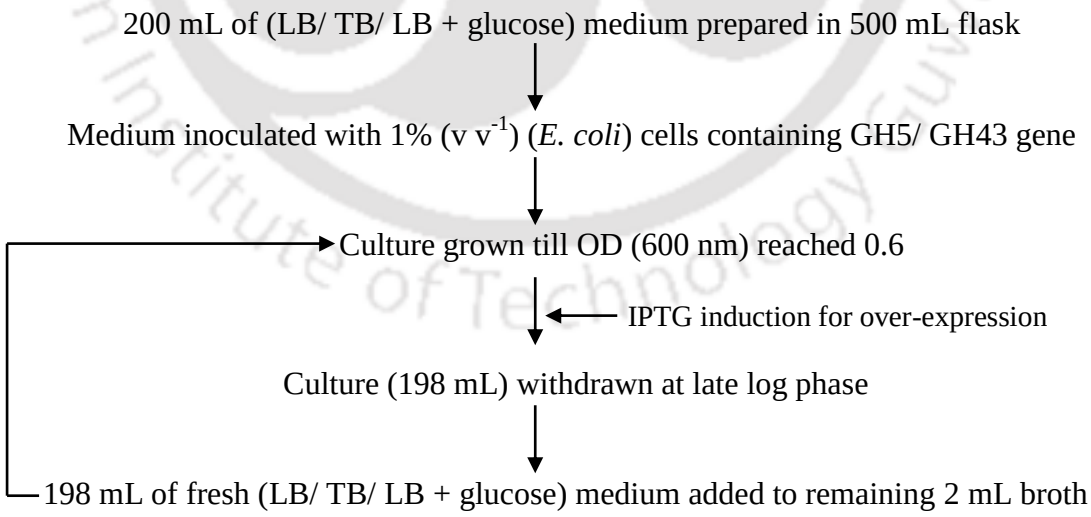


**Fig 2.2.1** Schematic presentation of repetitive batch strategy of *E. coli* cells in LB/ TB/ LB + glucose medium.

Two hundred millilitre of LB medium containing *E. coli* cells (GH5 or GH43) were harvested by centrifugation (8,510***g***, 4°C for 30 min) and the resulting pellets were resuspended in 10 mL of 50 mM sodium phosphate buffer (pH 7.0). Each of the resuspended cell pellets was subjected to sonication (Vibra Cell, SONICS, Newtown, CT, USA) in an ice-bath separately for 15 min. The sonicated cells were centrifuged (19,650***g***, 4°C for 30 min). The cell pellet was discarded and the cell free extract containing the crude recombinant enzymes, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were analysed by 12% SDS-PAGE. The enzyme activities of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were determined as described in Sections 2.2.12.1 and 2.2.12.2, respectively.

### 2.2.5 SDS-PAGE analysis of recombinant proteins

The recombinant proteins were separated on Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis SDS-PAGE gel on the basis of their respective molecular size. PAGE is an analytical method used to separate components of a protein mixture based on their size (Laemmli, 1970; Sambrook *et al*., 1989). The PAGE makes use of the fact that a charged molecule migrates in an electric field in the direction of an electrode with opposite charge. But this method cannot be used to determine the molecular weight of proteins because the mobility of a substance in the gel depends on both charge and size. Therefore, the proteins were treated with SDS so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, they migrate towards the anode and are separated based on size. The migrated proteins were then visualized using a staining

solution containing Coomassie brilliant blue R-250 dye which binds with proteins (Sambrook *et al*., 1989).

### *2.2.5.1 Preparation of SDS-PAGE gel*

The polyacrylamide gels are prepared by copolymerization of acrylamide and bis-acrylamide ("bis," N,N'-methylene-bisacrylamide). The copolymerization reaction is basically a vinyl addition reaction initiated by a free radical-generator *viz.* Ammonium per sulphate (APS) in presence of N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) which acts as a catalyst (Chrambach, 1985). The components of SDS-PAGE are acrylamide solution 30% (w $v^{-1}$), resolving gel buffer (Tris-HCl, pH 8.8), a stacking gel buffer (Tris-HCl, pH 6.8), SDS 10 % (w $v^{-1}$), APS 10% (w $v^{-1}$), TEMED, sample loading buffer (pH 6.8) and electrophoretic running or tank buffer comprising Tris glycine and SDS, pH 8.3-8.5. The composition of each component of SDS-PAGE gels and buffers are described below in Sections 2.2.5.2-2.2.5.6.

### *2.2.5.2 Preparation of acrylamide solution*

0.8 g of bis-acrylamide was weighed and transferred into an amber colour bottle and dissolved in 50 mL of ultra-pure deionized water (18 MΩcm, Millipore, Milli-Q water purification system) on a magnetic stirrer (IKA, C-MAG HS7). After completely dissolving bis-acrylamide, 29.2 g of acrylamide was added to it to make 30% (w $v^{-1}$) acrylamide solution and stirred on a magnetic stirrer. The final volume was adjusted to 100 mL with ultra-pure water by keeping the measuring cylinder (100 mL) wrapped with aluminium foil as acrylamide is light sensitive. The acrylamide solution was then filtered (Whatman No. 1) under dark condition and stored at 4°C.

### *2.2.5.3 Polymerization of SDS-PAGE gel*

The resolving gel and stacking gels were prepared following protocols from Sambrook *et al*. (1989) using the composition as described in Tables 2.2.2 and 2.2.3 given below. The resolving gels were prepared by adding all the components in the order as mentioned in Table 2.2.2, in a 25 mL beaker, by keeping acrylamide concentration to 12% (w $v^{-1}$) for GH5 cellulase and GH43 hemicellulase hemicellulase (α-L-arabinofuranosidase). Similarly, the stacking gel was prepared by dissolving all the components mentioned in Table 2.2.3. The acrylamide concentration in the stacking gel was kept at 4% (w $v^{-1}$).

**Table 2.2.2** Composition of components for preparation of resolving gel (12%).

| Components | Volume (mL) |
|---|---|
| Acrylamide solution (30%, w $v^{-1}$) | 4.0 |
| Deionized water | 0.7 |
| SDS (10%, w $v^{-1}$) | 1.0 |
| Glycerol (50%, v $v^{-1}$) | 1.0 |
| 1.5 M Tris-HCl (pH 8.8) | 3.3 |
| APS (10%, w $v^{-1}$) | 0.1 |
| TEMED | 0.01 |

**Table 2.2.3** Composition of components for preparation of stacking gel (4%).

.

| Components | Volume (mL) |
|---|---|
| Acrylamide solution (30%, w $v^{-1}$) | 0.7 |
| Deionized water | 2.8 |
| SDS (10%, w $v^{-1}$) | 0.5 |
| 0.5 M Tris-HCl (pH 6.8) | 1.0 |
| APS (10%, w $v^{-1}$) | 0.05 |
| TEMED | 0.005 |

### *2.2.5.4 Preparation of SDS-PAGE running buffer*

The SDS-PAGE gels were run using a 1x running or tank buffer prepared from the 5x stock solution as described below in Table 2.2.4. 15.14 g of Tris free base and 94 g of glycine were dissolved in 800 mL of deionized water. To this 50 mL of 10% (w $v^{-1}$) SDS was added and the final volume was adjusted to 1 litre. The final pH of the buffer was adjusted to 8.3. The 5x buffer was filtered (Whatman, Filter No. 1) and stored at 4°C.

**Table 2.2.4** Composition of 5x Tris-Glycine, running or tank buffer.

| Components | Final concentration (5x buffer) |
|---|---|
| Tris base | 0.125 M |
| Glycine | 1.25 M |
| SDS | 0.5 % (w $v^{-1}$) |

### *2.2.5.5 Preparation of sample buffer*

5x sample loading buffer was prepared by dissolving the components while keeping the concentration of components as described in Table 2.2.5 and the pH of the buffer was adjusted to 6.8 (Laemmli, 1970). The components were dissolved in the order as mentioned in Table 2.2.5 to make 5x sample buffer. However, the final concentration while loading to a SDS-PAGE gel was always kept to 1x by mixing 4 volumes of sample (protein) with 1 volume of 5x sample buffer. In case of 5x sample loading buffer for non-denaturing SDS-PAGE β-mercaptoethanol was not added.

**Table 2.2.5** Composition of 5x sample loading buffer.

| Components | Final concentration (5x buffer) |
|---|---|
| Tris-HCl (pH 6.8) | 62.5 mM |
| Glycerol | 20.0 (%, v $v^{-1}$) |
| SDS | 2.0 (%, w $v^{-1}$) |
| Bromophenol Blue | 0.025 (%, w $v^{-1}$) |
| β-mercaptoethanol | 5.0 (%, w $v^{-1}$) |

### *2.2.5.6 Preparation of staining and destaining solutions*

The proteins on the SDS-PAGE gel were visualized using a staining solution that contained Coomassie Brilliant Blue (CBB) R-250 dye, which is a disulfonated triphenylmethane (Fig. 2.2.2). The CBB R-250 dye (detection range of 100-1000 ng of protein) formed a non-covalent complex with proteins, based on a combination of Van der Waals forces and electrostatic interactions (Neuhoff *et al*., 1985). The negatively charged anionic form of the dye is stabilized by formation of a blue colour protein-dye complex which may then be seen on gel (Meyer and Lambert, 1965). The staining solution (100 mL) was prepared by dissolving 250 mg or 0.25% (w $v^{-1}$), of CBB R-250 dye in 50 mL of deionized water in an amber colour bottle by keeping on a magnetic stirrer for overnight. The solution was filtered (Whatman, Filter No. 1), then 40 mL of methanol and 10 mL of glacial acetic acid were added to finally make the ratio 5:4:1 (deionized water : methanol : glacial acetic acid). The destaining solution was prepared by mixing deionized water: methanol: glacial acetic in 5:4:1 ratio. The gels were destained by immersing in destaining solution with gentle shaking and change of buffer every 30 min, until the protein bands were clear.

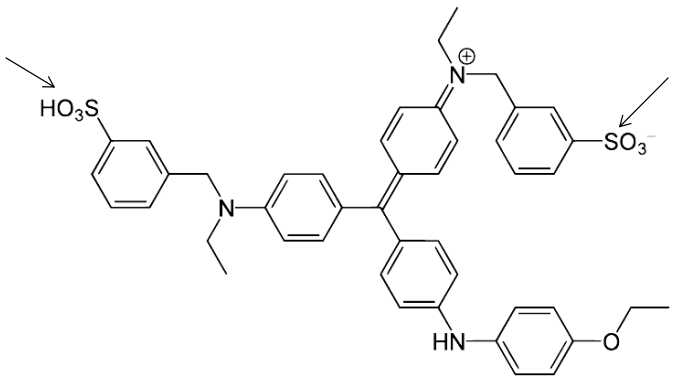


**Fig. 2.2.2** Chemical structure of Coomassie Brilliant Blue dye R-250 showing disulfonated triphenylmethane ring.

### 2.2.6 Denaturing SDS-PAGE of GH5 and GH43

The recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were analyzed in two separate 12% (w $v^{-1}$) denaturing SDS-PAGE gels. 200 µL each of uninduced and induced *E. coli* cells of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were centrifuged at 19,650***g*** and the resulting pellets were washed thrice with distilled water to remove LB medium. Each sample was diluted with 25 µL of water and kept at 4ºC. Cell free extracts of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were obtained by cell harvesting followed by sonication and centrifugation as described earlier in Section 2.2.4. 25 µL of each of these uninduced, induced, cell free supernatant and cell pellet of *E. coli* cells after sonication were mixed with 5 µL of sample buffer (1.5 M Tris-Cl, pH 6.8, 10 mL glycerol, 2 g SDS, 0.01% (w $v^{-1}$), Bromophenol Blue and 5 mL β-mercaptoethanol) as described in Section 2.2.5.5 (Laemmli, 1970), boiled in a boiling water bath for 5 min and loaded on the gel. 5 µL of prestained Page Ruler protein marker (10-200 kDa, Fermentas, USA) was loaded on the gel. The electrophoresis was carried out using Mini-PROTEAN 3 Cell electrophoresis system (Bio-Rad, California, USA) with a constant current of 2.5 mA per lane using 1x running buffer as described in Section 2.2.5.4. The post electrophoresis process was followed by staining the gels with Coomassie Brilliant Blue R-250 (CBB) for 20 min. After the completion of staining, each gel was destained with destaining solution (methanol: water: acetic acid:: 5: 4: 1) till the protein bands were clearly visible. Both the gels were visualized under white light epi-illuminator using a gel documentation system (Gel Logic 1500 imaging system, Carestream health, Inc., USA).

### 2.2.7 Non-denaturing SDS-PAGE of GH5 and GH43 for activity staining

Zymogram study of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) was separately performed, by using 0.5% (w $v^{-1}$) caboxymethyl cellulose (CMC) and rye arabinoxylan, respectively, using non-denaturing SDS-PAGE (12%, w $v^{-1}$). The cell free extracts of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were obtained by cell harvesting followed by sonication and centrifugation as described in Section 2.2.4. 25 µL of each of these cell free extracts mixed with 5 µL, 5x sample buffer (1.5 M Tris-Cl, pH 6.8, 10 mL glycerol, 2 g SDS, 0.01% (w $v^{-1}$) Bromophenol blue as described in Section 2.2.5.5, with no reducing agent β-mercaptoethanol and no boiling were loaded on the gel. The polyacrylamide gel electrophoresis was carried out using Mini-PROTEAN 3 Cell electrophoresis system (Bio-Rad, California, USA) with a current of 2.5 mA per lane using 1x running buffer as described in Section 2.2.5.4. After the completion of electrophoresis both the gels were incubated in 2.5% (v $v^{-1}$) TritonX100 at 25ºC for 1 h followed by incubation in 50 mM sodium acetate buffer of pH 4.3 and 5.4, respectively, for GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) for another 1 h. As in both the cases SDS-PAGE was used which might affect the activity of these enzymes, thus Triton X-100 treatment was done to partially renature these proteins to maintain the activity against CMC and rye arabinoxylan, respectively. Then, the gels were incubated in preheated 50 mM sodium acetate buffer (pH 5.0) at 50°C for 30 min and then stained with 0.1% (w $v^{-1}$) Congo red for 45 min following the method of Aboul-Enein *et al*., (2010). After Congo red staining, the gels were counter stained with 1N HCl (Ruijssennars and Hartmans, 2001).

### 2.2.8 Batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Terrific broth

The recombinant *C. thermocellum* GH5 cellulase production was initiated by inoculating 50 µL of the *E. coli* BL21 (DE3) culture from glycerol stock into 5 mL of Terrific broth (TB) (pH 7.2) supplemented with 100 µg $mL^{-1}$ ampicillin and incubated at 37°C and 180 rpm for 16 h. The composition of Terrific broth (TB) is given in Table 2.2.6. The medium was sterilized by autoclaving at 120ºC and 15 psi for 20 min.

**Table 2.2.6** Composition of Terrific broth (TB).

| Constituents | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Tryptone | 1.2 |
| Yeast extract | 2.4 |
| Dipotassium phosphate | 0.94 |
| Monopotassium phosphate | 0.22 |
| Glycerol | 0.4 |

1% (v $v^{-1}$) of this culture inoculum was transferred aseptically to 200 mL of TB in 500 mL flask containing 100 µg $mL^{-1}$ ampicillin and was incubated at 37°C, 180 rpm till the culture reached mid-exponential phase (OD at 600 nm ~ 0.6). This culture was then induced with 1 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) followed by further 12 h incubation at 37°C for over-expression of recombinant protein (Taylor *et al.*, 2005). 50 µg $mL^{-1}$ kanamycin was used as a selective marker for *E. coli* BL21 (DE3) pLysS cells containing GH43 hemicellulase (α-L-arabinofuranosidase) (Das *et al.*, 2012). Similar production process was followed for GH5 cellulase except, the incubation period was 24°C, 180 rpm and 24 h for over-expression of GH43 after induction with 1 mM IPTG.

### 2.2.9 Repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Terrific broth (TB)

During repetitive batch mode, the first step remained similar as followed in batch mode (section 2.2.8) and then approximately, there was removal of 198 mL of culture medium when the *E. coli* cells (GH5 or GH43) reached late log phase or early stationary phase. This was centrifuged and cell mass was further processed for the isolation of enzymes separately. To remaining 2 mL broth, 198 mL of fresh medium was added. Similar practice was followed in consecutive three batches where remaining 2 mL of medium comprising induced cells were castoff as inoculum. The schematic diagram of repetitive batch strategy using different media is represented in Fig. 2.2.1.

Two hundred millilitre of TB containing *E. coli* cells (GH5 or GH43) were harvested by centrifugation (8,510***g***, 4°C for 30 min) and the resulting pellets were subsequently resuspended in 10 mL of 50 mM sodium phosphate buffer (pH 7.0). Each of the resuspended cell pellets were subjected to sonication (Vibra Cell, SONICS, Newtown, CT, USA) in an ice-bath separately for 15 min. The sonicated cells were centrifuged (19,650***g***, 4°C for 30 min). The cell pellet was discarded and the cell free extract containing the crude recombinant enzymes, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were analysed for enzyme activity. The enzyme activities of both the crude enzymes were measured using Nelson Somogyi methodology (Nelson, 1944; Somogyi, 1945) as described in Sections 2.2.12.1 and 2.2.12.2.

### 2.2.10 Batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in LB with glucose medium

The recombinant *C. thermocellum* GH5 cellulase production was initiated by inoculating 50 µL of the *E. coli* BL21 (DE3) culture from glycerol stock into 5 mL of LB with glucose medium (pH 7.2) supplemented with 100 µg $mL^{-1}$ ampicillin and incubated at 37°C and 180 rpm for 16 h. The chemical composition of LB with glucose medium is given in Table 2.2.7. The medium was sterilized by autoclaving at 120ºC and 15 psi for 20 min.

**Table 2.2.7** Chemical composition of LB with glucose medium.

| Constituents | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Tryptone | 1 |
| Yeast extract | 0.5 |
| NaCl | 1 |
| Glucose | 1 |

1% (v $v^{-1}$) of this culture inoculum was transferred aseptically to 200 mL of LB medium supplemented with glucose in 500 mL flask containing 100 µg $mL^{-1}$ ampicillin and was incubated at 37°C, 180 rpm till the culture reached mid-exponential phase (OD at 600 nm ~ 0.6). This culture was then induced with 1 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) followed by further 12 h incubation at 37°C for over-expression of recombinant protein (Taylor *et al.*, 2005). 50 µg $mL^{-1}$ kanamycin was used as a selective marker for *E. coli* BL21 (DE3) pLysS cells containing GH43 hemicellulase (α-L-arabinofuranosidase) (Das *et al.*, 2012). Similar production process was followed for GH5 cellulase except, the

incubation period was 24°C, 180 rpm and 24 h for over-expression of GH43 after induction with 1 mM IPTG.

### 2.2.11 Repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in LB medium with glucose

During repetitive batch mode, the first step remained similar as followed in batch mode (section 2.2.10) and then approximately, there was removal of 198 mL of culture medium when the *E. coli* cells (GH5 or GH43) reached late log phase or early stationary phase. This was centrifuged and cell mass was further processed for the isolation of enzymes separately. To remaining 2 mL broth, 198 mL of fresh medium was added. Similar practice was followed in consecutive three batches where remaining 2 mL of medium comprising induced cells were castoff as inoculum. The schematic diagram of repetitive batch strategy using different media is represented in Fig. 2.2.1.

Two hundred millilitre of LB with glucose medium containing *E. coli* cells (GH5 or GH43) were harvested by centrifugation (8,510***g***, 4°C for 30 min) and the resulting pellets were subsequently resuspended in 10 mL of 50 mM sodium phosphate buffer (pH 7.0). Each of the resuspended cell pellets were subjected to sonication (Vibra Cell, SONICS, Newtown, CT, USA) in an ice-bath separately for 15 min. The sonicated cells were centrifuged (19,650***g***, 4°C for 30 min). The cell pellet was discarded and the cell free extract containing the crude recombinant enzymes, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were analysed for enzyme activity. The enzyme activities of both the crude enzymes were measured

using Nelson Somogyi methodology (Nelson, 1944; Somogyi, 1945) as described in Sections 2.2.12.1 and 2.2.12.2.

### 2.2.12 Analytical methods

#### *2.2.12.1 Recombinant GH5 cellulase activity assay*

The recombinant GH5 cellulase assay was performed by incubating the enzyme (10 µL) in a 100 µL reaction mixture containing 1% (w $v^{-1}$) CMC in 20 mM sodium acetate buffer (pH 4.3) at 50°C for 10 min. The mixture was assessed for the released reducing sugars as reported earlier (Nelson, 1944; Somogyi, 1945). The amount of released reducing sugar was used to determine the enzyme activity. To 100 µL of reaction mixture containing the released reducing sugar, 100 µL of reagent D (as described in Section 2.2.12.3) was added. The solutions were mixed and heated for 20 min in the boiling water bath. After 20 min of boiling, the solution was ice cooled and 100 µL of reagent C (Section 2.2.12.3) was added. The colour developed rapidly with the evolution of carbon dioxide. The mixture was diluted by adding 700 µL water to make up the volume to 1 mL and the optical density (OD) was measured at 500 nm on a UV-Visible spectrophotometer (Varian, Cary 100 Bio). D-glucose in the range of 50-500 µg $mL^{-1}$ was used for generating the standard plot as described in section 2.2.12.4.

#### *2.2.12.2 Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity assay*

GH43 hemicellulase (α-L-arabinofuranosidase) activity was determined by incubating 10 µL of enzyme in a 100 µL reaction mixture containing 1% (w $v^{-1}$)

rye arabinoxylan in 100 mM sodium acetate buffer (pH 5.4) at 50°C for 10 min. The mixture was assessed for the released reducing sugars as reported earlier (Nelson, 1944; Somogyi, 1945) and as described in Section 2.2.12.3. The amount of released reducing sugar was used to calculate the enzyme activity. To 100 µL of reaction mixture, 100 µL of reagent D was added, mixed and heated for 20 min in boiling water bath. After cooling it to room temperature 100 µL of reagent C (Section 2.2.12.3) was added. The colour developed rapidly with the evolution of carbon dioxide. The mixture was diluted by adding 700 µL water to make up the volume to 1 mL and the OD was measured at 500 nm on a UV-Visible spectrophotometer (Varian, Cary 100 Bio). L-arabinose in the range of 10-250 µg $mL^{-1}$ was used for generating the standard plot as described in Section 2.2.12.4.

### *2.2.12.3 Preparation of reagents for reducing sugar estimation*

***Reagent A***

**Table 2.2.8** Chemical composition of Reagent A.

| Constituents | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Sodium carbonate anhydrous | 6.25 |
| Sodium potassium tartarate | 6.25 |
| Sodium bicarbonate | 5.0 |
| Sodium sulphate anhydrous | 50.0 |

The above mentioned components were dissolved in 100 mL of deionized water and the final volume was adjusted to 250 mL. The solution was filtered (Whatman No. 1) and stored at a temperature between 30-37°C.

***Reagent B***

Reagent B was prepared by dissolving 15 g of copper sulphate ($CuSO_4$) in 50 mL deionized water and one or two drops of concentrated sulphuric acid was

added to it. The final volume was made up to 100 mL with deionized water and the solution was filtered (Whatman No. 1) and stored at room temperature.

***Reagent C***

Reagent C was prepared in two steps by under dark condition as it is light sensitive. First, 2.5 g of ammonium molybdate was dissolved in 45 mL of deionized water in 100 mL beaker and 2.1 mL of concentrated sulphuric acid was added to it. In another beaker 0.3 g of sodium arsenate was dissolved in 2.5 mL of deionized water. Now, this solution was added to ammonium molybdate solution and the contents were mixed (total volume was around 50 mL). The solution was filtered (Whatman No. 1) under dark conditions and stored at 37°C. The solution was used after 24 h incubation.

***Reagent D***

Reagent D was prepared by mixing reagent A and reagent B in the ratio 25:1. Reagent D was always prepared freshly for use in the assay.

### *2.2.12.4 Generation of standard plot of D-glucose and L-arabinose*

The standard plot of D-glucose was prepared by varying the concentration of D-glucose from 50-500 µg $mL^{-1}$. The reaction volume was kept to 100 µL with 50 mM sodium acetate buffer pH 4.3. The reaction mixture containing buffer and D-glucose was incubated (in a 1.5 mL microcentrifuge tube) at 50°C for 15 min and then 100 µL of reagent D (Section 2.2.12.3) was added to it. The reaction mixture was then boiled for 20 min and cooled. 100 µL of reagent C (Section 2.2.12.3) was added and the contents were mixed. Then 700 µL of deionized water was added to make the final volume to 1 mL. The OD at 500 nm wavelength was measured using UV-Visible

spectrophotometer (Varian, Cary 100 Bio) against a buffer blank. A standard plot of OD versus D-glucose concentration (µg $mL^{-1}$) was generated and 1 OD equivalent of D-glucose (µg $mL^{-1}$) was calculated. The 1 OD equivalent of D-glucose (µg $mL^{-1}$) was converted into mg $mL^{-1}$ terms for calculation of enzyme activity.

The standard plot of L-arabinose was prepared by varying the concentration of L-arabinose from 10-250 µg $mL^{-1}$. The reaction volume was kept to 100 µL with 50 mM sodium acetate buffer pH 5.4. The reaction mixture containing buffer and L-arabinose was incubated (in a 1.5 mL microcentrifuge tube) at 50°C for 15 min and then 100 µL of reagent D (Section 2.2.12.3) was added to it. The reaction mixture was then boiled for 20 min and cooled. 100 µL of reagent C (Section 2.2.12.3) was added and the contents were mixed. Then 700 µL of deionized water was added to make the final volume to 1 mL. The OD was measured at 500 nm wavelength using UV-Visible spectrophotometer (Varian, Cary 100 Bio) against a buffer blank. A standard plot of OD versus L-arabinose concentration (µg $mL^{-1}$) was generated and 1 OD equivalent of L-arabinose (µg $mL^{-1}$) was calculated. The 1 equivalent of L-arabinose (µg $mL^{-1}$) was converted in mg $mL^{-1}$ terms for calculation of enzyme activity.

#### *2.2.12.5 Calculation of enzyme activity of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase)*

The activity of the enzyme was expressed as U $mL^{-1}$ and the specific activity as U $mg^{-1}$ of protein. One unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (glucose or arabinose) per min. The enzyme activity of recombinant GH5 cellulase was calculated as described below,

$$\text{Enzyme activity (U mL}^{-1}) = \frac{\Delta A_{500} \times C \times V}{180 \times t \times v} = (\mu \text{ mole min}^{-1} \text{ mL}^{-1})$$

where,

$\Delta A_{500}$ = change in OD of the sample at 500 nm
C = 1 OD equivalent is D-glucose concentration from standard plot (µg $mL^{-1}$)
V = volume of the reaction mixture (mL)
t = time of reaction (min)
180 = molecular weight of D-glucose
v = volume of the enzyme taken in assay (mL) for reducing sugar estimation.

The enzyme activity of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) was calculated as described below,

$$\text{Enzyme activity (U mL}^{-1}) = \frac{\Delta A_{500} \times C \times V}{150 \times t \times v} = (\mu \text{ mole min}^{-1} \text{ mL}^{-1})$$

where,

$\Delta A_{500}$ = change in OD of the sample at 500 nm
C = 1 OD equivalent is L-arabinose concentration from standard plot (µg $mL^{-1}$)
V = volume of the reaction mixture (mL)
t = time of reaction (min)
180 = molecular weight of L-arabinose
v = volume of the enzyme taken in assay (mL) for reducing sugar estimation.

***2.2.12.6 Protein concentration estimation***

The concentration of protein was detected by mixing the enzyme (10 µL) with distilled water (90 µL) in a total reaction volume (100 µL) with final addition of 1 mL Bradford reagent (Bradford, 1976). The reaction mixture was kept at 25°C for 20 min and OD was determined using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) at 595 nm. A Bovine serum albumin (BSA) standard curve was prepared by varying the concentration of BSA from 0.01-0.2 mg $mL^{-1}$ to determine the protein concentration. The specific activity (U $mg^{-1}$) of the cell free supernatant was calculated from enzyme activity and protein concentration.

### *2.2.12.7 Preparation of Bradford's reagent*

Bradford assay involves the spectral properties of Coomassie Brilliant Blue G-250 to estimate the amount of protein in a solution (Bradford, 1976). 100 mg 0.01% (w $v^{-1}$) Coomassie Brilliant Blue G-250 was weighed and dissolved in 50 mL 95% ethanol (in an amber colour bottle). 100 mL 85% (w $v^{-1}$) phosphoric acid was added to it. A magnetic bead was placed inside the bottle and the contents were mixed properly by keeping on magnetic stirrer until the dye completely dissolved. The dye was finally diluted to 1 L with deionized water, filtered (Whatman, No. 1 paper) under dark conditions and stored at 4°C.

The concentration of protein was calculated as follows:

$$\text{Protein concentration (mg mL}^{-1}) = \frac{\Delta A_{595} \times C \times V}{v} = (\text{mg mL}^{-1})$$

where,

$\Delta A_{595}$ = change in OD of the sample
C = 1 OD equivalent of BSA from standard plot
V = volume of the reaction mixture (mL)
v = volume of the enzyme used for assay (mL)

## 2.3 Results and Discussion

Media composition plays a significant role in higher production of recombinant proteins (Tripathi *et al.*, 2009). Use of chemically defined medium is a common practice in producing recombinant proteins as these media attain more consistent titres, allow easier process control and monitoring, and simplify downstream recovery of the target protein (Lim and Jung, 1998; Cserjan-Puschmann *et al.*, 1999; Zhang and Greasham, 1999). Overall enzyme synthesis and their activities depend upon the IPTG induction of *lac* constitutive system and the cell biomass productivity. Repetitive batch cultivation mode was used for maintaining the ideal conditions supporting the growth of recombinant *E. coli* cells and thus, the maximum production of cellulase and hemicellulase. The cell biomass increased to a higher extent owing to the consecutive use of induced cells in the following repetitive batches increasing the enzyme production during repetitive batch fermentation.

### 2.3.1 Production of GH5 cellulase by batch and repetitive batch modes in LB, TB and LB with glucose media

The consequence of batch and repetitive batch mode operations on the synthesis of recombinant *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were studied at shake flask level. The dynamic profiles of cell biomass (OD at 600 nm ~ 0.6) and specific activity (U $mg^{-1}$) of recombinant GH5 cellulase were studied in the batch and repetitive batch mode in three media, respectively. A constant volume repetitive batch operation was designed which used the IPTG induced *E. coli* cells as the inoculum for the subsequent batch operation.

In LB medium with batch mode, the maximum cell OD at 600 nm, activity of GH5 cellulase and protein concentration obtained were 1.4, 2.2 U $mg^{-1}$ and 0.18 mg $mL^{-1}$, respectively (Table 2.3.1, Fig. 2.3.1).

**Table 2.3.1** Batch and repetitive batch culture for cell biomass and GH5 cellulase activity in LB, TB and LB with glucose media.

| Mode | Maximum Cell Biomass (OD at 600nm)* | Protein concentration (mg $mL^{-1}$) | Specific Activity (U $mg^{-1}$)* |
|---|---|---|---|
| Batch (LB medium) | 1.4 ± 0.04 | 0.18 ± 0.04 | 2.2 ± 0.05 |
| Repetitive Batch (LB medium) | 2.8 ± 0.05 | 0.37 ± 0.05 | 4.5 ± 0.04 |
| Batch (TB medium) | 4.8 ± 0.07 | 0.20 ± 0.09 | 2.4 ± 0.01 |
| Repetitive Batch (TB medium) | 7.5 ± 0.09 | 0.40 ± 0.07 | 5.0 ± 0.06 |
| Batch (LB with glucose) | 6.0 ± 0.02 | 0.23 ± 0.02 | 2.8 ± 0.07 |
| Repetitive Batch (LB with glucose) | 9.6 ± 0.05 | 0.45 ± 0.06 | 5.7 ± 0.08 |

*****values are mean ± SE (n=3)*

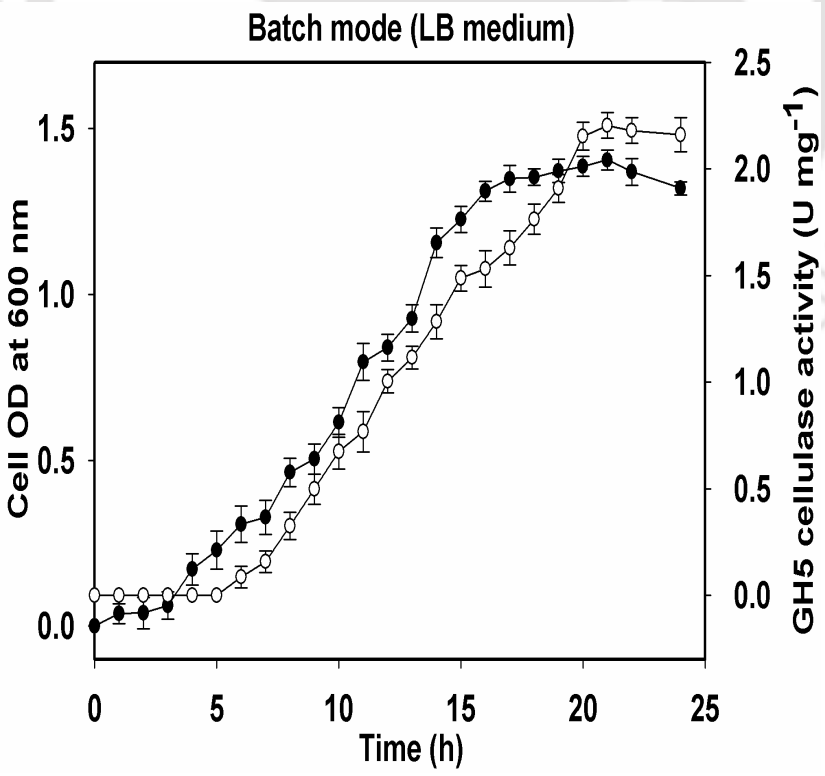


**Fig. 2.3.1** Batch mode dynamic profile of cell growth and recombinant GH5 cellulase activity in LB medium. (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

In the second cycle of repetitive batch fermentation, a 1.6-fold increase in both cell OD 600 nm (2.4) and specific enzyme activity (3.5 U $mg^{-1}$) was observed when compared with the batch mode fermentation (Fig. 2.3.2).

Finally, the maximum cell OD at 600 nm (2.8), protein concentration (0.37 mg $mL^{-1}$) and enzyme activity (4.5 U $mg^{-1}$) with a 2-fold increment in all was observed in the third cycle of repetitive batch mode when compared with batch mode production of recombinant GH5 cellulase (Table 2.3.1, Fig. 2.3.2).

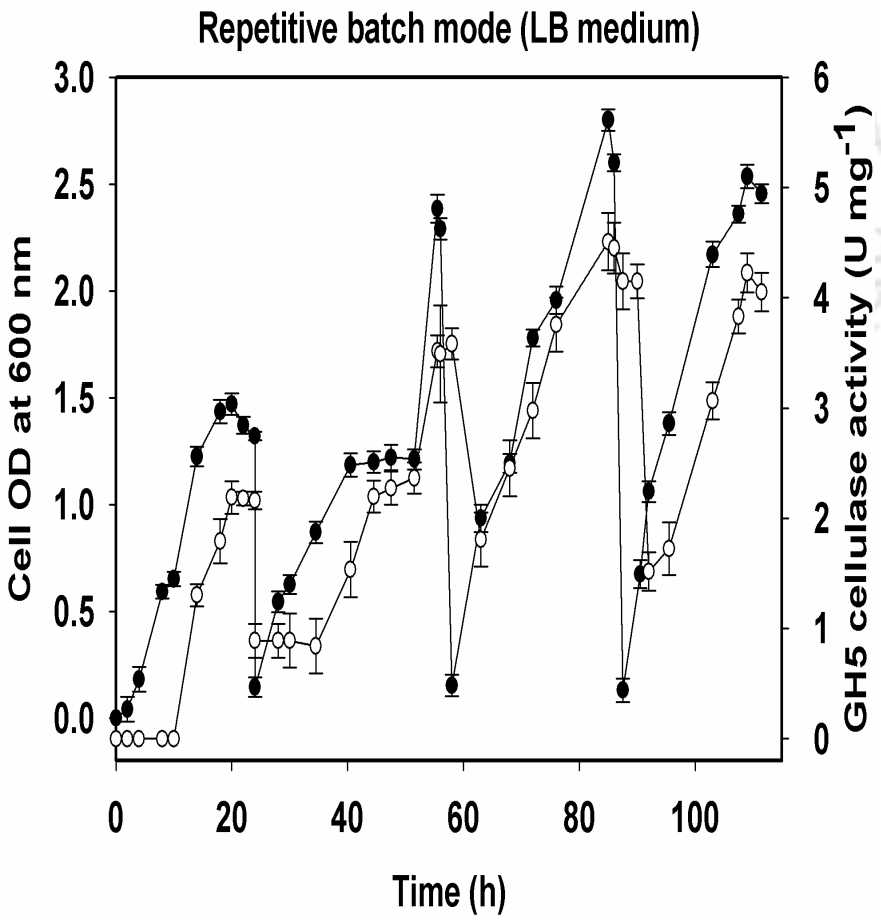


**Fig. 2.3.2** Repetitive batch mode dynamic profile of cell growth and recombinant GH5 cellulase activity in LB medium. (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

The cell free extract obtained after cell harvesting and sonication of recombinant GH5 cellulase displayed a 35 kDa band on 12% SDS-PAGE (Fig. 2.3.3, Lane 3).

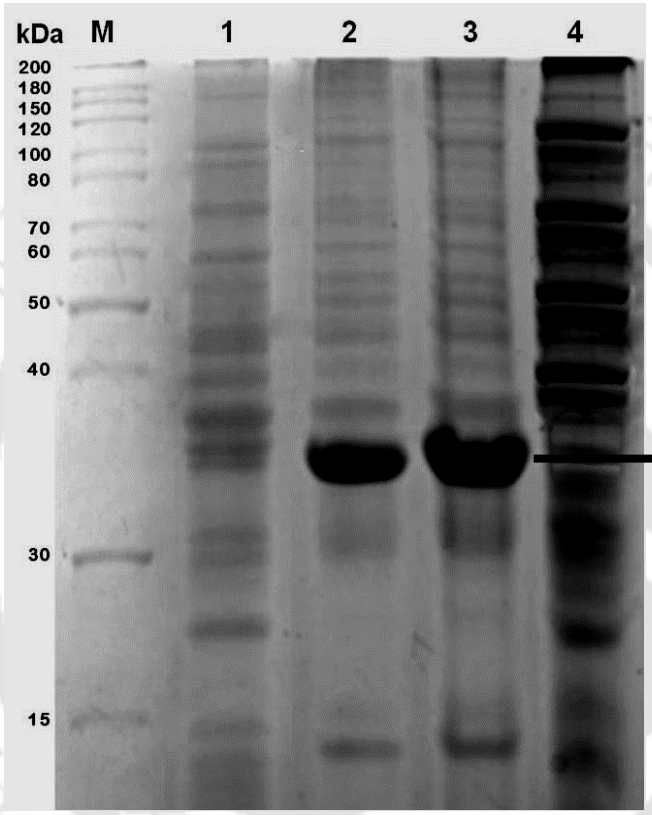


**Fig. 2.3.3** SDS-PAGE (12%) showing over-expression of GH5 cellulase. Lane M: Page Ruler protein marker (10-200 kDa, Fermentas, USA), lane 1: uninduced *E. coli* cells, lane 2: induced *E. coli* cells, lane 3: cell free supernatant from *E. coli* cells, lane 4: cell pellet of *E. coli* cells after sonication.

Separate SDS-PAGE gel was used in the zymogram study to show the active band of GH5 cellulase against CMC (Fig. 2.3.4). GH5 showed an active band around 35 kDa with congo red staining and counter staining with 1 N HCl (Fig. 2.3.4). GH5 cellulase action displayed a clear zone in the 12% SDS-PAGE containing CMC

(0.5%, w $v^{-1}$) as substrate. Enzyme activity was detected as clear zones against red (after staining with Congo red) and blue background (after counter stained with 1 N HCl). The results clearly confirmed that GH5 has cellulase activity.

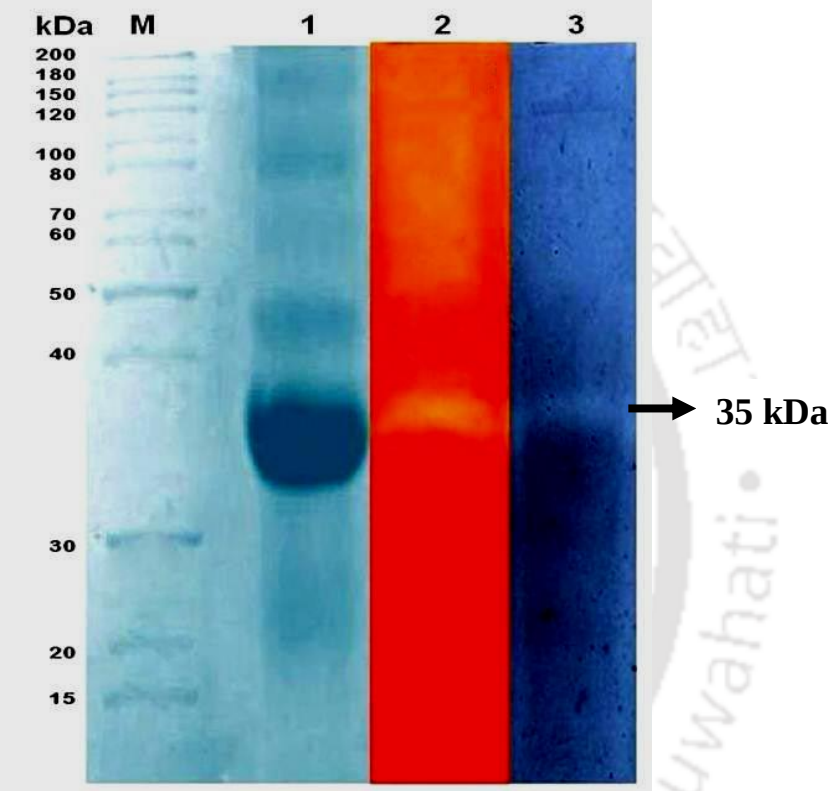


**Fig. 2.3.4** Zymogram of GH5 cellulase using 12% SDS-PAGE. Lane M: Page Ruler protein marker (10-200 kDa, Fermentas, USA), Lane 1: cell free supernatant containing GH5 cellulase, Lane 2: congo red staining, Lane 3: 1 N HCl counter staining.

In case of terrific broth (TB) with batch mode, the maximum enzyme activity of 2.4 U $mg^{-1}$, protein concentration of 0.20 mg $mL^{-1}$ and the cell OD of 4.8 was obtained (Table 2.3.1). On the other hand, in repetitive batch mode, the maximum enzyme activity, protein concentration and cell OD achieved were 5.0 U $mg^{-1}$, 0.40 mg $mL^{-1}$ and 7.5 respectively (Table 2.3.1). Thus, a 2-fold increment both in activity

of the enzyme and protein concentration along with 1.6-fold rise in cell biomass was gained in repetitive batch mode as compared to batch mode (Table 2.3.1). Thus, the TB medium in batch and repetitive batch modes resulted in a 3.4- and 2.7-fold augmentation in cell biomass as compared to LB medium in the respective modes (Table 2.3.1).

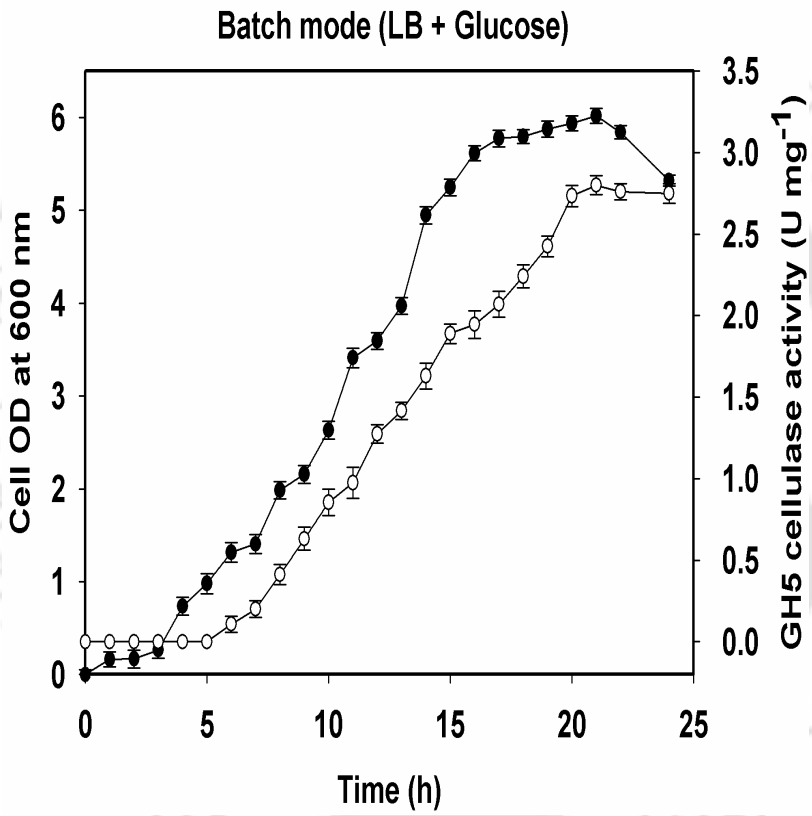


**Fig. 2.3.5** Batch mode dynamic profile of cell growth and recombinant GH5 cellulase activity in LB medium supplemented with glucose (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

In batch mode LB medium supplemented with glucose, the maximum GH5 cellulase activity of 2.8 U $mg^{-1}$ with protein concentration of 0.37 mg $mL^{-1}$ and highest cell OD of 6.0 (Table 2.3.1, Fig. 2.3.5) was obtained. In 2$^{nd}$ cycle of repetitive

batch mode, a greater enzyme activity of 4.5 U $mg^{-1}$ and cell OD of 8.15 was achieved (Fig. 2.3.6). Finally, the 3rd batch yielded the maximum specific activity (5.7 U $mg^{-1}$), protein concentration (0.45 mg $mL^{-1}$) and cell density of 9.6, respectively (Table 2.3.1, Fig. 2.3.6).

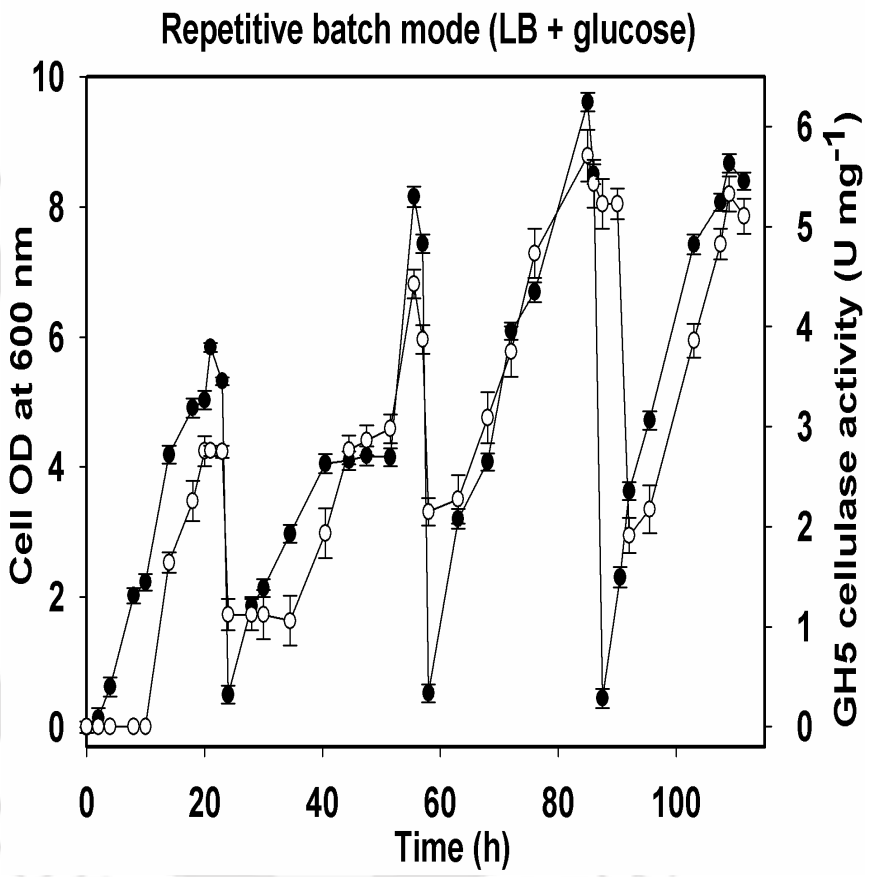


**Fig. 2.3.6** Repetitive batch mode dynamic profile of cell growth and recombinant GH5 cellulase activity in LB medium supplemented with glucose. (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

Thus, a 4.2-fold and 3.4-fold escalation in cell biomass was obtained in LB medium with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes (Table 2.3.1).

### 2.3.2 Production of GH43 hemicellulase (α-L-arabinofuranosidase) by batch and repetitive batch modes in LB, TB and LB with glucose medium

The batch mode LB medium for recombinant GH43 hemicellulase (α-L-arabinofuranosidase) production yielded a maximum enzyme activity, protein concentration and the cell OD of 1.9 U $mg^{-1}$, 0.20 mg $mL^{-1}$ and 1.6, respectively (Table 2.3.2).

**Table 2.3.2** Batch and repetitive batch culture for cell biomass and GH43 hemicellulase (α-L-arabinofuranosidase) activity in LB, TB and LB with glucose media.

| Mode | Maximum Cell Biomass (OD at 600nm )* | Protein concentration (mg $mL^{-1}$) | Specific Activity (U $mg^{-1}$)* |
|---|---|---|---|
| Batch (LB medium) | 1.6 ± 0.03 | 0.20 ± 0.07 | 1.9 ± 0.02 |
| Repetitive Batch (LB medium) | 2.5 ± 0.06 | 0.26 ± 0.05 | 3.0 ± 0.05 |
| Batch (TB medium) | 3.7 ± 0.01 | 0.21 ± 0.02 | 2.0 ± 0.05 |
| Repetitive Batch (TB medium) | 5.9 ± 0.03 | 0.28 ± 0.01 | 3.4 ± 0.08 |
| Batch (LB with glucose) | 5.2 ± 0.02 | 0.24 ± 0.08 | 2.2 ± 0.04 |
| Repetitive Batch (LB with glucose) | 8.1 ± 0.04 | 0.32 ± 0.09 | 3.7 ± 0.06 |

*****values are mean ± SE (n=3)*

The repetitive batch mode of LB medium gave a highest enzyme activity of 3.0 U $mg^{-1}$, protein concentration of 0.26 mg $mL^{-1}$ and cell OD at 600nm ~ 2.5, respectively (Table 2.3.2). A 1.6-fold augmentation both in enzyme activity and cell biomass was obtained in repetitive batch mode as compared to batch mode (Table 2.3.2).

The cell free supernatant obtained after cell harvesting and sonication of the recombinant GH43 hemicellulase (α-L-arabinofuranosidase) displayed a 34 kDa band in 12% SDS-PAGE (Fig. 2.3.7, Lane 3).

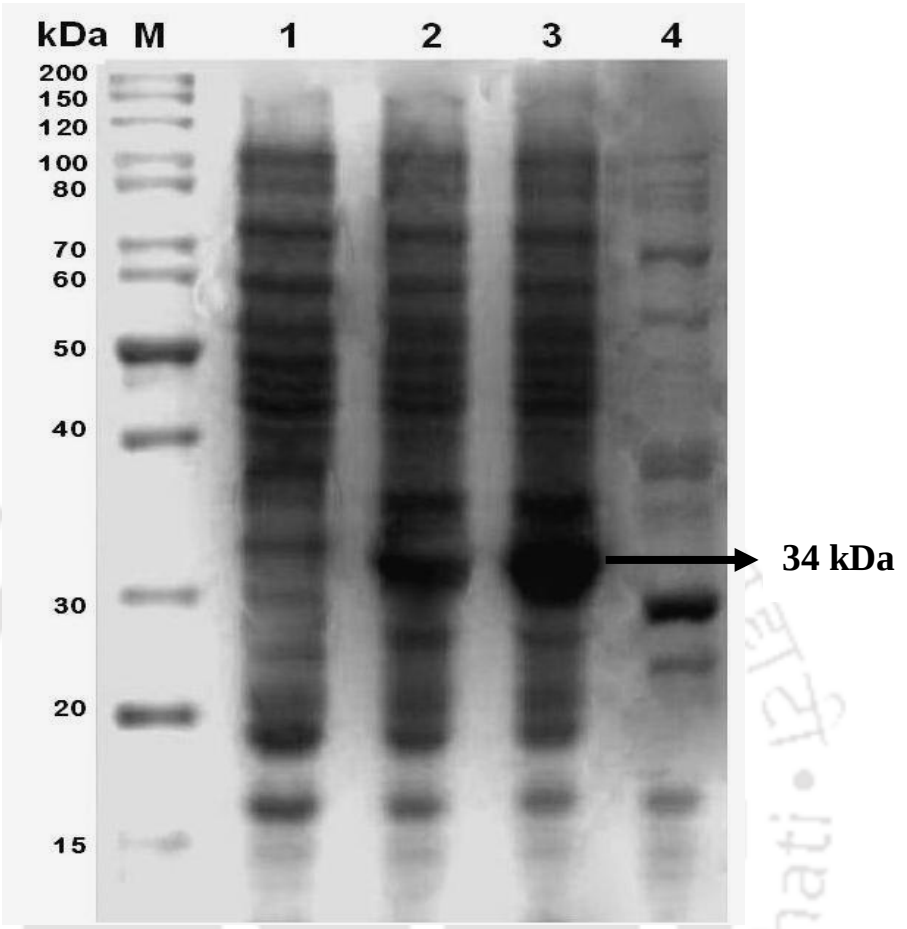


**Fig. 2.3.7** SDS-PAGE (12%) showing over-expression of GH43 hemicellulase (α-L-arabinofuranosidase). Lane M: Page Ruler protein marker (10-200 kDa, Fermentas, USA), lane 1: uninduced *E. coli* cells, lane 2: induced *E. coli* cells, lane 3: cell free supernatant from *E. coli* cells, lane 4: cell pellet of *E. coli* cells after sonication.

Separate SDS-PAGE gel was used in the zymogram study to display the active band of GH43 hemicallulase (α-L-arabinofuranosidase) against rye arabinoxylan (Fig. 2.3.8). GH43 displayed an active band around 34 kDa with congo red staining and counter staining with 1 N HCl (Fig. 2.3.8). GH43 hemicellulase (α-L-arabinofuranosidase) action exhibited a clear zone in the 12% SDS-PAGE containing rye arabinoxylan (0.5%, w $v^{-1}$) as substrate. Enzyme activity was detected as clear

zones against red (after staining with Congo red) and blue background (after counter stained with 1 N HCl). The results confirmed that GH43 has hemicellulase (α-L-arabinofuranosidase) activity.

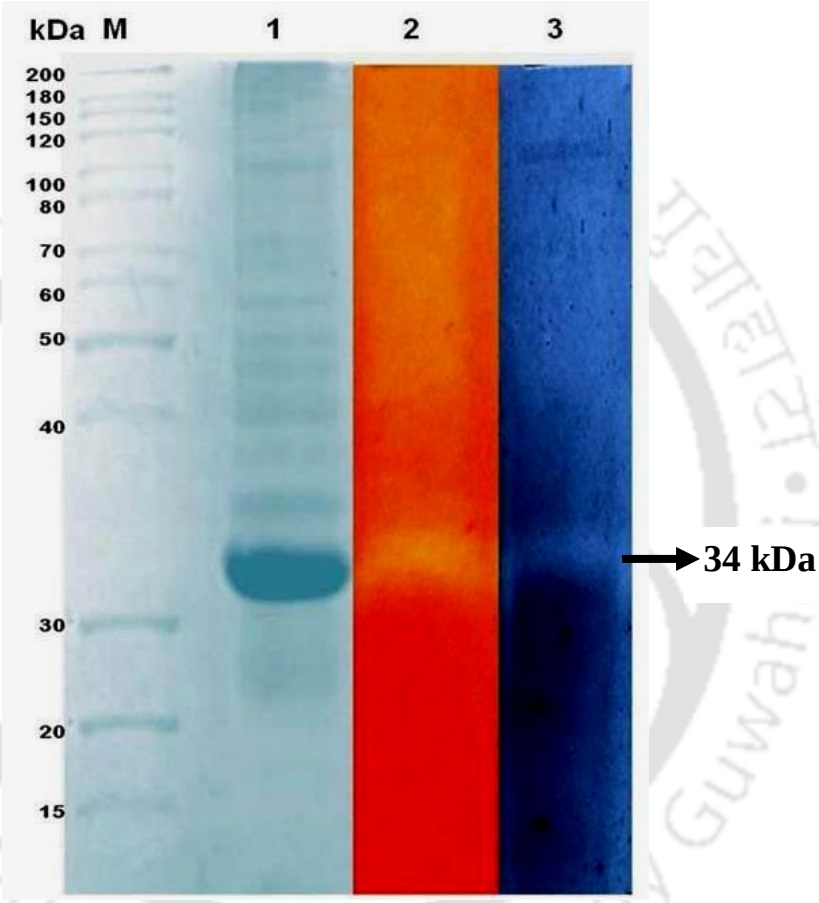


**Fig. 2.3.8** Zymogram of GH43 hemicellulase (α-L-arabinofuranosidase) using 12% SDS-PAGE. Lane M: Page Ruler protein marker (10-200 kDa, Fermentas, USA), Lane 1: cell free supernatant of GH43 hemicellulase (α-L-arabinofuranosidase), Lane 2: congo red staining, Lane 3: 1 N HCl counter staining.

In terrific broth (TB) with batch mode, the maximum enzyme activity, protein concentration and the cell OD were 2.0 U $mg^{-1}$, 0.21 mg $mL^{-1}$ and 3.7, respectively (Table 2.3.2, Fig. 2.3.9).

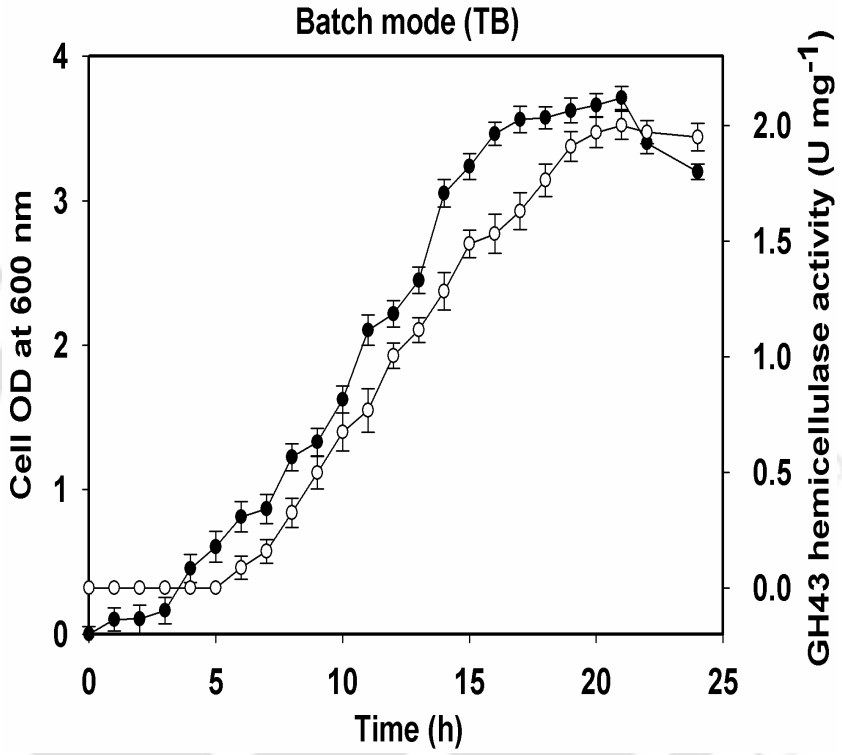


**Fig. 2.3.9** Batch mode dynamic profile of cell growth and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity in Terrific broth (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

In the second cycle of repetitive batch fermentation using TB, a 1.3-fold increase in both specific enzyme activity (2.7 U $mg^{-1}$) and cell OD of 5.0 was observed while compared with the batch mode fermentation (Fig. 2.3.10). Finally, a maximum cell OD (5.9), protein concentration (0.28 mg $mL^{-1}$) and enzyme activity (3.4 U $mg^{-1}$) with an increment of 2-fold was observed in third cycle of repetitive

batch mode when compared with batch mode production of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (Table 2.3.2, Fig. 2.3.10).

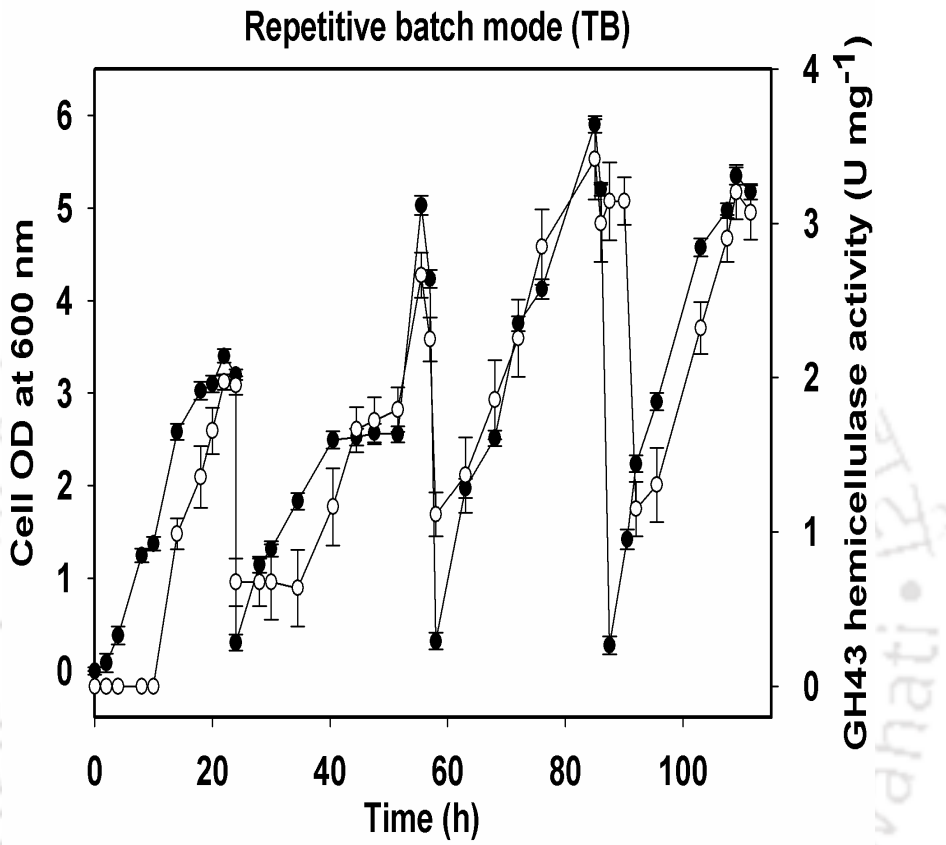


**Fig. 2.3.10** Repetitive batch mode dynamic profile of cell growth and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity in Terrific broth (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

In batch mode LB medium supplemented with glucose, the maximum recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity of 2.2 U $mg^{-1}$ with protein concentration of 0.24 mg $mL^{-1}$ and highest cell OD at 600 nm ~5.2 (Table 2.3.2, Fig. 2.3.11) was obtained.

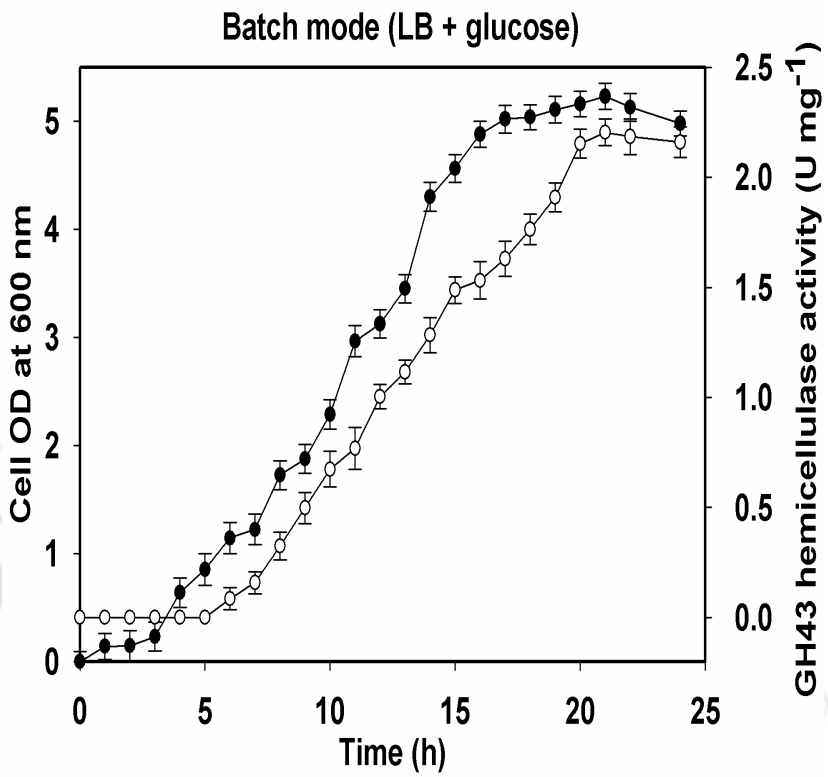


**Fig. 2.3.11** Batch mode dynamic profile of cell growth and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity in LB medium supplemented with glucose (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

In 2nd cycle of repetitive batch mode, a greater enzyme activity of 2.9 U $mg^{-1}$ and cell OD of 6.9 was achieved (Fig. 2.3.12). Finally, the 3rd batch in repetitive mode yielded the maximum specific activity (3.7 U $mg^{-1}$), protein concentration (0.32 mg $mL^{-1}$) and cell density of 8.1 (Table 2.3.2, Fig. 2.3.12). Thus, a 3.2-fold escalation in cell biomass was obtained in LB medium added with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes.

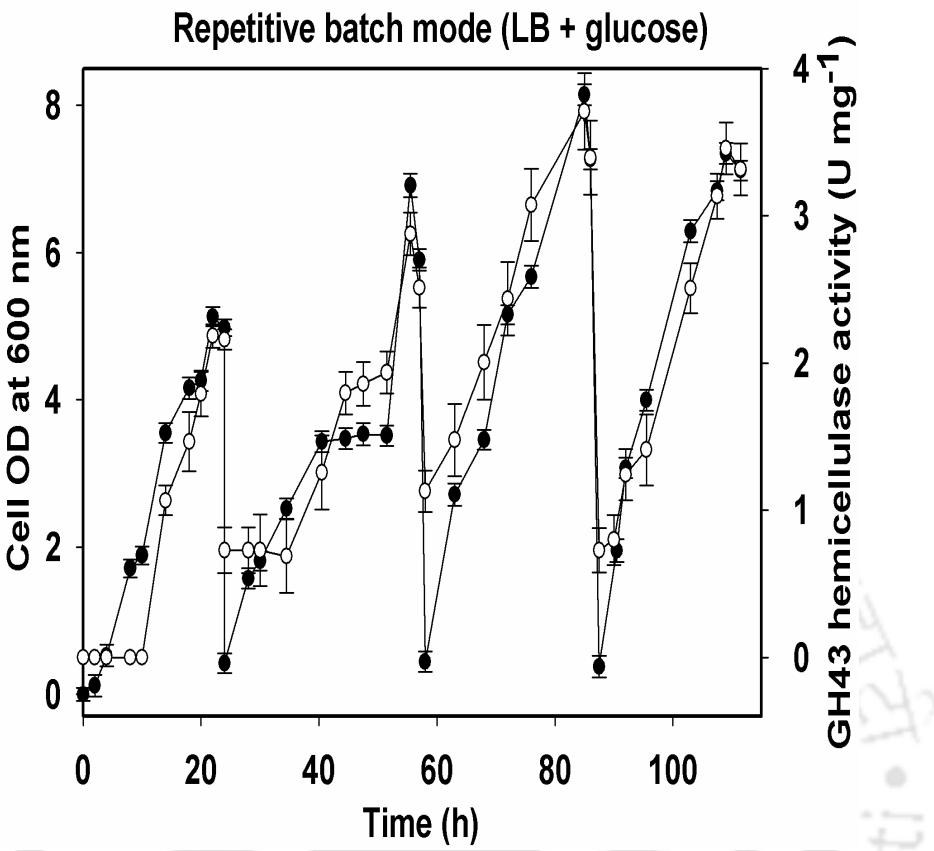


**Fig. 2.3.12** Repetitive batch mode dynamic profile of cell growth and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) activity in LB medium supplemented with glucose (●) cell OD measured at 600 nm and (○) specific activity (U $mg^{-1}$) with time.

The recombinant *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) presented highest cell densities, concentration of proteins and enzyme activities in LB medium supplemented with glucose (Table 2.3.1, Table 2.3.2). In Terrific broth, moderate cell densities and protein production were observed (Table 2.3.1, Table 2.3.2). In LB medium, lowest cell density with lowest protein concentrations were achieved (Table 2.3.1, Table 2.3.2).

The regulation of recombinant *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) plysS cells harboring *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-

arabinofuranosidase) genes were controlled by a strong bacteriophage promoter T7. The highest achievement of cell biomass and recombinant proteins were due to the underlying molecular mechanisms as described earlier by Grossman *et al.,* (1998). The production of the T7 RNA polymerase in expression hosts (λDE3 lysogens) is regulated by a lac promoter derivative, the *E. coli* L8-UV5 lac promoter. The lambda DE3 prophage encoding T7 RNA polymerase in pET expression hosts carries the L8-UV5 promoter having three point mutations that distinguishes it from the wild type lac promoter. This mutation reduces the sensitivity to catabolite repression (Grossman *et al.,* 1998). The net effect of the three-point mutations is the creation of a stronger promoter that is less sensitive to the glucose effect. This allows strong IPTG induction of T7 RNA polymerase expression even in the presence of glucose. As first described by Grossman *et al.,* (1998), yet another level of regulation can be employed with the pET system by exploiting the glucose effect described above, i.e., supplementing standard medium such as LB with glucose to keep cAMP levels low. Apparently, although the L8-UV5 promoter is less dependent on CAP/cAMP stimulation than the wild type lac promoter, in practice there is still a significant reduction in basal transcription in the presence of glucose. Thus, glucose supplementation with LB media was a necessity for overproduction of recombinant *C. thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase). Terrific broth (TB) being rich in tryptone, yeast extract, and phosphate salts also well supported the growth and overproduction of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase). Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during

synthesis of recombinant protein (Lim *et al.*, 2000). Similarly, a high concentration of phosphate is known to be important for attaining high cell densities of *E. coli*, as phosphate can easily become a limiting nutrient when provided in low doses (Korz *et al.*, 1995). In addition to providing a source of phosphate, the phosphate salts in the media provided a buffering capacity against pH fluctuations that could adversely affect normal metabolic activity. The relatively poor performance of LB versus those of complex media such as LB with glucose and TB has been discussed for other recombinant proteins (Lim *et al.*, 2000; Tripathi *et al.*, 2008) and is attributed to lower amounts of readily accessible carbon and nitrogen in LB (Lim *et al.*, 2000). Moreover, the lack in pH control in absence of phosphate might deregulate the biomass growth and recombinant protein production. As in the case of LB with glucose similar effect might hamper the overproduction of proteins but glucose regulation prevailed over the minimization effect of biomass and protein production by pH fluctuations.

## 2.4 Conclusions

This study reported for the first time the enhanced production of recombinant *C. thermocellum* hydrolytic GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in repetitive batch mode in different media. For GH5 cellulase production, in LB medium with batch mode, the enzyme activity, protein concentration and the cell OD were 2.2 U $mg^{-1}$, 0.18 mg $mL^{-1}$ and 1.4. A cell OD (2.8), protein concentration (0.37 mg $mL^{-1}$) and enzyme activity (4.5 U $mg^{-1}$) with an increment of 2-fold was observed in LB medium with repetitive batch mode as compared to batch mode. The cell free extract of recombinant GH5 cellulase displayed a 35 kDa band on SDS-PAGE. Zymogram study confirmed the cellulase activity of GH5. In case of terrific broth (TB) with batch mode, the enzyme activity of 2.4 U $mg^{-1}$, protein concentration of 0.20 mg $mL^{-1}$ and the cell OD of 4.8 was obtained. On the other hand, in repetitive batch mode, the enzyme activity, protein concentration and cell OD achieved was 5.0 U $mg^{-1}$, 0.40 mg $mL^{-1}$ and 7.5 respectively. Thus, a 2-fold increment both in activity of the enzyme and protein concentration along with 1.6-fold rise in cell biomass was gained in repetitive batch mode as compared with batch mode. Thus, as compared to LB medium in batch and repetitive batch modes, a 3.4- and 2.7-fold augmentation in cell biomass were obtained for TB in respective modes. In batch mode LB medium with glucose, the recombinant GH5 cellulase activity of 2.8 U $mg^{-1}$ with protein concentration of 0.37 mg $mL^{-1}$ and highest cell OD of 6.0 was obtained. The repetitive batch mode yielded the specific activity (5.7 U $mg^{-1}$), protein concentration (0.45 mg $mL^{-1}$) and cell OD of 9.6. Thus, a 4.2-fold and 3.4-fold escalation in cell biomass was obtained in LB

medium with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes.

For GH43 hemicellulase (α-L-arabinofuranosidase) production, the batch mode LB medium yielded the enzyme activity, protein concentration and the cell OD of 1.9 U $mg^{-1}$, 0.20 mg $mL^{-1}$ and 1.6, respectively. The repetitive batch mode of LB medium gave an enzyme activity of 3.0 U $mg^{-1}$, protein concentration of 0.26 mg $mL^{-1}$ and cell OD of 2.5. A 1.6-fold augmentation both in enzyme activity and cell biomass was obtained in repetitive batch mode as compared with batch mode. The cell free extract of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) displayed a 34 kDa band on SDS-PAGE. Zymogram study confirmed the hemicellulase activity of GH43. In terrific broth (TB) with batch mode, the enzyme activity, protein concentration and cell OD obtained were 2.0 U $mg^{-1}$, 0.21 mg $mL^{-1}$ and 3.7, respectively. Whereas in repetitive batch mode an enzyme activity (3.4 U $mg^{-1}$), protein concentration (0.28 mg $mL^{-1}$) and cell OD (5.9) displaying around 2-fold increase in enzyme activity was obtained as compared with batch mode. In batch mode LB medium with glucose, GH43 hemicellulase (α-L-arabinofuranosidase) activity of 2.2 U $mg^{-1}$ with protein concentration of 0.24 mg $mL^{-1}$ and cell OD of 5.2 was obtained. Finally, the repetitive batch mode yielded specific activity (3.7 U $mg^{-1}$), protein concentration (0.32 mg $mL^{-1}$) and cell OD of 8.1. Thus, a 3.2-fold escalation in cell biomass was obtained in LB medium with glucose as a co-substrate in batch and repetitive batch modes as compared to LB medium without glucose in both modes. The enhanced production of recombinant enzymes in LB medium supplemented with glucose will aid in effective saccharification of various lignocellulosic substrates to transform into a value-added product, bioethanol.

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# Chapter 3

## Selection of cellulose and hemicellulose rich substrates and efficient pretreatment process for bioethanol production

### 3.1 Introduction

Rapid diminution in the accessibility of fossil fuels poses a serious need for sustainable development of alternative energy source. Depletion of oil supply reserves as well as rise in the greenhouse gas emision has glimmered renewed interest in biofuel production from renewable resources. To that end, ethanol fermentation from lignocellulosic substrates has been gaining significant concern in the scientific community. Ethanol as a fuel has several advantages over fossil fuels such as greater air-fuel ratio, higher energy density and added specific energy with heat of vapourization (Oliviera *et al.,* 2005). Since ethanol has a high octane number than petrol, no pre ignition occurs on use of fuel ethanol hence, it is used as an economical fuel additive with petrol (Dien *et al.,* 2002; Oliviera *et al.,* 2005).

Lignocellulosics from plant sources are composed of heterogeneous complex of carbohydrate polymers *viz.,* cellulose, hemicellulose along with lignin (Sun and Cheng, 2002). The common agricultural residues and wastes, contain 40-60% (w $w^{-1}$)

cellulose that is high molecular weight polymer consisting of glucose chains rigidly held together as bundles of fibres (Sun and Cheng, 2002). Hemicelluloses (20-40%, w $w^{-1}$) found in agricultural residues are shorter polymers of various sugars that bind cellulose bundles together (Sun and Cheng, 2002). Lignin (10- 30%, w $w^{-1}$) present in plant cell wall consists of a three-dimensional polymer of propyl-phenol that is bound to hemicellulose to provide rigidity (Ralph *et al*., 2001). The structural carbohydrate analysis of various agricultural residues (leafy biomass) showed that the percent fraction of cellulose is maximum followed by hemicellulose and lignin (Sun and Cheng, 2002). Easy and ample availability of leaves from trees of jamun (*Syzygium cumini*), asoka (*Saraca indica*), bamboo (*Bambusa dendrocalamus*), poplar (*Populus nigra*) and eucalyptus (*Eucalyptus marginata*) and weeds like wild grass (*Achnatherum hymenoides*) and water hyacinth (*Eichhornia crassipes*) in northern India envisaged interest in exploiting these as substrates for making and retrieval of many valuable products such as bioethanol (Das *et al.*, 2012).

Albeit lignocellulose being the most abundant renewable resource available, its rigid structure and crystalline nature prevents it from its efficient utilization for hydrolysis (Sun and Cheng, 2002). An effective pretreatment strategy is necessary for the liberation of the cellulose and hemicellulose from the lignin seal so as to render it accessible for a subsequent hydrolysis. To date, a fair number of readily available pre-treatment techniques are reported in literature (Barrett *et al.*, 2009). Physical pretreatment, often called size reduction breaks down the substrate physically. Chemical pretreatment disrupts chemical bonds aiding in enhanced enzymatic attack to the carbohydrate polymers (Barrett *et al.*, 2009). Steam explosion is a prompt expansion operation where biomass treated with high-pressure steam is rapidly

discharged to a vessel operated at lower pressure (Sharma *et al.,* 2007). Alkali pretreatment refers to the application of alkaline solutions such as sodium hydroxide and calcium hydroxide to remove lignin and a part of the hemicellulose and efficiently increase the accessibility of enzyme to the cellulose (Okeke and Obi, 1995). Wet oxidation separates the cellulosic fraction from lignin and hemicellulose (Palonen *et al.,* 2003). Phosphoric acid ($H_3PO_4$) – acetone pretreatment of lignocellulosic biomass can effectively remove hemicellulose, producing C5 sugar monomers in the liquid (Li *et al.,* 2009). Pretreatment by ammonia fibre expansion (AFEX) method has the advantage over others that it does not produce inhibitors for the downstream processes at high temperature (> 90°C) and pH (< 12.0), which, minimizes the formation of degraded products from sugar resulting in higher yields (Holtzapple *et al.,* 1991). Organosolv pretreatment using an organic or aqueous organic solvent decomposes the network of lignin and possibly a part of the hemicellulose (Geng *et al.,* 2012). The pH controlled hot water and dual step dual temperature (DSDT) mild acid hydrolysis pretreatment under high pressure can penetrate into the biomass, hydrate cellulose, and remove hemicellulose and part of lignin (Mosier *et al.,* 2005; Bosch *et al.,* 2010). Microwave assisted alkali (MAA) pretreatment loosens cellulose more effectively than hemicellulose and lignin (Zhu *et al.,* 2006).

Electron microscopy and Fourier transform infrared (FT-IR) spectroscopy have been used for the analysis of morphological and structural modifications in the biomass after the pretreatment (Nada *et al.,* 1998; Kelly *et al.,* 2004; Rodrigues *et al.,* 2007; Rezende *et al.,* 2011). The hydrolytic activity of cellulases with the maximum release of utilizable sugars is a crucial factor in bioethanol production. The prime

hindrance in the usage of commercial fungal enzymes is, due to its high cost. Also, there is absence of prominent β-glucosidase activity in most of the readily available enzymatic pools, directed towards an efficient saccharification process (Schulein, 1988). The thermophilic *Clostridium thermocellum* cellulosome displays 50-fold higher specific activity than the corresponding *Trichoderma reesei* system against crystalline cellulose (Demain *et al.,* 2005). Glycoside hydrolases are a group of enzymes which includes cellulases and hemicellulases. According to CAZy database, glycoside hydrolase family 5 (GH5) exhibits activities of cellulase (EC 3.2.1.4); licheninase (EC 3.2.1.73), glucan endo-β-(1→6)-glucosidase (EC 3.2.1.75) and cellulose β-(1→4)-cellobiosidase (EC 3.2.1.91), whereas glycoside hydrolase family 43 displays activities of β-(1→3)-xylosidase (EC 3.2.1.-), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), xylanase (EC 3.2.1.8) and arabinanase (EC 3.2.1.99). *Saccharomyces cerevisiae* possesses the intrinsic ability of utilizing various substrates for ethanol production apart from high ethanol tolerance and endurance to metabolic inhibitions (Casey and Ingledew, 1986). *Candida shehatae* possesses key enzymes, xylitol dehydrogenase and xylose reductase to metabolize pentose sugars for ethanol production by pentose phosphate pathway (Kadam and Schimdt, 1997). In simultaneous saccharification and fermentation (SSF) process, the enzymatic hydrolysis of complex polysaccharides and the fermentation of monomeric sugars are performed in a single step whereas in separate hydrolysis and fermentation (SHF) the hydrolysis and fermentation are performed in separate reactors (Hamelinck *et al.,* 2005; Sangkharak *et al.,* 2011).

In the present study, cellulose and hemicellulose rich substrate was selected among leafy substrates of various agricultural and forest residues on the basis of

structural carbohydrate composition. The efficiency of different pretreatments was evaluated in terms of complex carbohydrate breakdown of lignocellulosic wild grass and water hyacinth. The efficiency of mixed microwave assisted alkali (MAA) with organosolv pretreatment in degradation of wild grass and water hyacinth was confirmed by field emission scanning electron microscopy (FESEM) and FT-IR analyses. The efficacy of recombinant *C. thermocellum* hydrolytic GH5 cellulase along with *S. cerevisiae* in terms of bioethanol production was determined by two modes of fermentation, SHF and SSF. Also, the efficiency of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated by SHF and SSF modes of fermentation.

## 3.2 Materials and Methods

### 3.2.1 Reagents and chemicals

Carboxy methyl cellulose (CMC), kanamycin and isopropyl-β-D-thiogalactopyranoside (IPTG) was procured from Sigma Aldrich (St. Louis, USA). Rye arabinoxylan was purchased from Megazyme International Ltd. (Ireland). The analytical grade reagents and chemicals *viz.*, sodium chloride, sodium acetate, ampicillin, glucose, peptone, tryptone, yeast extract, malt extract, sodium carbonate, sodium sulphate, sodium bicarbonate, sodium potassium tartarate, ammonium molybdate, copper sulphate, potassium dichromate, sodium arsenate, sodium hydroxide, acetic acid, hydrogen peroxide, potassium dihydrogen phosphate, ammonium sulphate, magnesium sulphate heptahydrate and agar were purchased from Himedia Pvt. Ltd. India. Phosphoric acid and hydrochloric acid were procured from Qualigens India Pvt. Ltd. Coomassie Brilliant Blue G-250 was purchased from Amresco LLC, USA. Sulphuric acid and liquid ammonia were obtained from Merck India Pvt. Ltd.

### 3.2.2 Microorganisms and culturing conditions

The culturing and maintenance of *E. coli* BL21 (DE3) cells containing family 5 glycoside hydrolase (GH5) gene and *E. coli* BL21 (DE3) pLysS cells harbouring family 43 glycoside hydrolase (GH43) gene from *Clostridium thermocellum* was done autonomously as described in Chapter 2, Section 2.2.2 earlier. The predominantly aerobic fermentative microbes, *Saccharomyces cerevisiae* (NCIM no: 3215) and *Candida shehatae* (NCIM no: 3500) were procured from National Chemical Laboratory, Pune, India. They were maintained separately at 4°C in 5 mL of MGYP (Malt extract Glucose Yeast extract Peptone, pH 5.0) slants (Wickerman, 1951). The

chemical composition of MGYP medium is described in Table 3.2.1. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min.

**Table 3.2.1** Chemical composition of MGYP medium.

| Constituent | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Malt extract | 0.3 |
| Glucose | 1.0 |
| Yeast extract | 0.3 |
| Peptone | 0.5 |
| Agar | 2.0 |

One loopful of these slant cultures were inoculated independently into 50 mL of GYE (Glucose Yeast extract) medium (pH 5.0) contained in 250 mL Erlenmeyer flask. The chemical composition of GYE medium is described in Table 3.2.2. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min.

**Table 3.2.2** Chemical composition of GYE medium.

| Constituents | Concentration (g 100 $mL^{-1}$) |
|---|---|
| Glucose | 1.0 |
| Yeast extract | 0.1 |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 0.1 |
| Ammonium sulphate [$(NH_4)_2SO_4$] | 0.5 |
| Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) | 0.05 |

Each of the culture was incubated in shaking incubator at 30°C, 120 rpm for 48 h prior inoculation into SHF and SSF medium. The SHF and SSF medium comprised of 100 mL working volume of sodium acetate buffer (pH 4.3 or pH 5.4, 20 mM) in 250 mL Erlenmeyer flask supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$). The aliquots measuring 1 mL from each of actively growing culture of

*S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) were aseptically inoculated into two separate SHF and SSF medium. The cell count of the actively growing *S. cerevisiae* and *C. shehatae* was measured using a haemocytometer.

### 3.2.3 Repetitive batch production of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani (LB) medium with glucose

The repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) into separate Luria-Bertani (LB) medium supplemented with glucose was done to yield higher enzyme activity and protein concentration as described earlier in Chapter 2, Section 2.2.11. The recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was used for saccharification in SHF and SSF experiments of wild grass as described later in Sections 3.2.7 and 3.2.9. The recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was used for saccharification in SHF and SSF experiments of water hyacinth as described later in Sections 3.2.8 and 3.2.10.

### 3.2.4 Substrates

The leafy substrates of various agricultural and forest residues such as jamun (*Syzygium cumini*), neem (*Azadirachta indica*), asoka (*Saraca indica*), bamboo (*Bambusa dendrocalamus*), poplar (*Populus nigra*), wild grass (*Achnatherum hymenoides*), eucalyptus (*Eucalyptus marginata*) and mango (*Mangifera indica*) were kindly gifted by Prof. Dinesh Goyal, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab, India (Fig. 3.2.1). The

leaves and petioles of water hyacinth (*Eichhornia crassipes*) were collected from the lakes within the campus of IIT Guwahati, Assam, India (Fig. 3.2.1).


**Fig. 3.2.1** Source of substrate (leaves) selected for bioethanol production. The outer panel displays normal leaves and the inset depicts the dried and ground form of the leaves (A) Jamun (*Syzygium cumini*) (B) Neem (*Azadirachta indica*) (C) Asoka (*Saraca indica*) (D) Bamboo (*Bambusa dendrocalamus*) (E) Poplar (*Populus nigra*) (F) Wild grass (*Achnatherum hymenoides*) (G) Eucalyptus (*Eucalyptus marginata*) (H) Mango (*Mangifera indica*) (I) Water hyacinth (*Eichhornia crassipes*).

The leafy substrates and leaves with petioles were cut into small pieces of length 4-5 cm with cutter and scissors and ground in a mixer grinder (Philips Classic,

HL 1606/03). They were washed with water to remove unwanted dust particles and finally, oven dried at 60°C (Fig. 3.2.1). The substrates were further ground in a mixer grinder for fine powder and passed through a 1 mm mesh size sieve prior to the pretreatment.

### 3.2.5 Pretreatment of substrates

Lignocellulosic biomass comprise of a complex matrix made up of cellulose and lignin bound by chains of hemicellulose. Pretreatment degrades this matrix reducing the degree of crystallinity of the cellulose with subsequent rise in the amorphous cellulose and hemicellulose fraction for effective enzymatic action (Sanchez and Cardona, 2008). The finely powdered wild grass and water hyacinth were subjected independently to nine pretreatment strategies and one mixed pretreatment strategy.

#### *3.2.5.1 Steam explosion*

One gram each of the dry ground wild grass and water hyacinth was taken separately in 100 mL Erlenmeyer flask. In an autoclave, the flasks were kept at 121°C and 15 psi for 1 h. The autoclave was exposed to sudden steam depressurization by completely opening the steam exhaust valve, intending to gain maximum quantity of fermentable sugars in least treatment time (Sharma *et al.*, 2007).

#### *3.2.5.2 Alkali treatment*

One gram each of the powdered wild grass and water hyacinth was retained in a 100 mL Erlenmeyer flask, adding 20 mL of 0.5 M sodium hydroxide. Then,

autoclaving of the mixture was done at 115°C and 15 psi for 10 min (Okeke and Obi, 1995). Subsequently, the mixture was cooled to room temperature and washed with distilled water. Each washing was followed by centrifugation (5,876***g***, 10 min, 25ºC). The residues were washed with distilled water until a pH of 7 was reached. Subsequently, the residues were dried at 50°C in an oven for 12 h.

### *3.2.5.3 Wet oxidation*

One gram each of the powdered wild grass and water hyacinth was mixed with 17 mL of water. The pH of the mixture was adjusted to 3.5 with the addition of 0.03 mL of concentrated sulphuric acid (36.5%, w $w^{-1}$). The wet oxidation of each mixture was carried out independently in a 0.5 L high pressure reactor (Amar Equipments, Mumbai, India) by continuous circulation of oxygen gas (at 12 bar) following Palonen method (Palonen *et al.*, 2003) with modification in residence time (1 h) and temperature (120ºC). Subsequently, cooling of each substrate was done to room temperature (25ºC). Finally, vacuum filtration of each substrate was done using a vacuum filtration unit (Millipore, Massachusetts, USA) with nylon membrane having pore size of 0.45 µm. After filtration, the left over residue of each substrate was collected and dried at 50°C in an oven for 12 h.

### *3.2.5.4 Phosphoric acid ($H_3PO_4$) - acetone*

One gram each of the powdered wild grass and water hyacinth was mixed with 8 mL of concentrated phosphoric acid in two separate 250 mL Erlenmeyer flask (Li *et al.*, 2009). Each of the mixture was then incubated at 50°C and 120 rpm for 1 h. The slurry was then poured into 24 mL chilled acetone and the mixture was centrifuged at

5,876***g*** for 10 min and 25ºC. The supernatant was discarded and the pellet collected was washed three times with distilled water. Before third wash, the pH of the resuspended pellet in water was adjusted to 5-6 using 1 N NaOH. Then, each pellet was dried at 50°C in an oven for 12 h.

### *3.2.5.5 Ammonia fibre expansion (AFEX)*

The ammonia fibre explosion (AFEX) treatment of each of the powdered wild grass and water hyacinth was carried out by following the method of Holtzapple (Holtzapple *et al.,* 1991) with modification in reaction vessel, residence time and temperature. 1 g each of the powdered substrate was treated with 2.5 mL of ammonia solution in crucible. The crucibles were covered with aluminium foil restricting the entry of air and kept at 100°C for 1 h in a hot air oven. Then, the aluminium foil was removed and the samples were kept in a fumigating hood overnight to remove the residual excess ammonia.

### *3.2.5.6 Organosolv pretreatment*

One gram each of the powdered wild grass and water hyacinth was treated with ethanol: water mixture (70:30 v $v^{-1}$) containing 1% of concentrated sulphuric acid, hydrochloric acid, acetic acid and phosphoric acid at $70^0$C for 1 h. The pretreated substrates were then washed initially with 95% (v $v^{-1}$) ethanol at $60^0$C for 4 h and then 70% (v $v^{-1}$) ethanol at $30^0$C for 1 h. The residues were then treated with hydrogen peroxide for 16 h and again washed with 70% ethanol at $30^0$C for 1 h (Geng *et al.,* 2012). Then, each residue was dried at 50°C in an oven for 12 h.

### *3.2.5.7 pH controlled hot water pretreatment*

One gram each of the powdered wild grass and water hyacinth was mixed with 20 mL of water at pH 4.0 (adjusted by 1 mL of concentrated sulphuric acid, 36.5%, w $w^{-1}$). The pH controlled hot water pretreatment of each mixture was carried out in a 0.5 L high pressure reactor (Amar Equipments, Mumbai, India) by continuous circulation of steam following the method described by Mosier *et al.,* 2005 with modification in pressure (8 bar), residence time (40 min) and temperature (90°C). Subsequently, each mixture was cooled down to room temperature (25ºC). Thereafter, vacuum filtration of each substrate was done using a vacuum filtration unit (Millipore, Massachusetts, USA). A nylon membrane of pore size 0.45 µm was used for filtration. Then, the left over residue of each substrate was collected and dried in an oven (50°C) for 12 h.

### *3.2.5.8 Dual step dual temperature (DSDT) mild acid hydrolysis*

One gram each of the powdered wild grass and water hyacinth was mixed separately with 1% (v $v^{-1}$) dilute sulphuric acid adjusted at pH 3.5. The dual step dual temperature (DSDT) mild acid hydrolysis of each mixture was carried out independently in a 0.5 L high pressure reactor (Amar Equipments, Mumbai, India) by continuous circulation of steam (at 10 bar) following the method of Bosch *et al.,* (2010) with modification in residence time and temperature (8 min at 90°C and 6 min at 99°C). Finally, each substrate was filtered using a vacuum filtration unit (Millipore, Massachusetts, USA) with a nylon membrane of pore size (0.45 µm). The residue of each substrate was collected and dried in an oven at 50°C for 12 h.

### *3.2.5.9 Microwave assisted alkali (MAA) pretreatment*

Twenty gram each of the powdered wild grass and water hyacinth was suspended separately in 160 mL of 1% (v $v^{-1}$) sodium hydroxide solution in two 500 mL beakers. Each beaker was positioned at the centre of a rotating circular glass plate in a domestic microwave oven for microwave treatment. The applied microwave power was 900 W for 25 min (Zhu *et al.*, 2006). Then, each substrate was filtered by a vacuum filtration unit (Millipore, Massachusetts, USA) with a nylon membrane of pore size (0.45 µm). Then, the residue of each substrate was collected and dried in an oven at 50°C for 12 h. Finally, 1 g of each substrate was subjected for further studies.

## 3.2.6 Mixed microwave-assisted alkali (MAA) and organosolv pretreatment strategy

Mixed microwave-assisted alkali (MAA) pretreatment loosens the compact structure of cellulose and aids in its hydrolysis to glucose (Zhu *et al.*, 2006). Organosolv pretreatment with the assistance of different organic acids benefits in relaxing the complex hemicellulose for its efficient hydrolysis to xylose (Geng *et al.*, 2012). Owing to the substantial quantities of cellulose and hemicellulose in wild grass and water hyacinth, the lignocellulosic substrates were subjected to mixed MAA and organosolv pretreatment strategy.

One gram (dry powder) each of wild grass (*A. hymenoides*) and water hyacinth (*E. crassipes*) was independently subjected to microwave assisted alkali (MAA) pretreatment as described earlier in Section 3.2.5.9. The microwave assisted alkali (MAA) pretreated wild grass and water hyacinth was further subjected separately to

organosolv pretreatment as described earlier in Section 3.2.5.6. Each of the mixed MAA and organosolv pretreated substrate was subsequently subjected for fermentation experiments.

### 3.2.7 Separate hydrolysis and fermentation (SHF) of wild grass in shake flask

Separate hydrolysis and fermentation (SHF) of wild grass in shake flask was carried out using 1 g of mixed MAA and organosolv pretreated substrate in 100 mL of sodium acetate buffer (pH 4.3, 20 mM) in 250 mL flask. 1 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was added to the flask. The saccharification was carried out at 50°C, 120 rpm for 36 h. Then, the medium was centrifuged at 5,476***g*** at 25°C for 15 min and the supernatant was collected. The supernatant supplemented with (0.1%, w $v^{-1}$) each of yeast extract and peptone was used as fermentation medium. 1 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) was added to the flask containing fermentation medium. The fermentation was carried out at 30°C, 120 rpm for three days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$) and ethanol concentration (g $L^{-1}$).

### 3.2.8 Separate hydrolysis and fermentation (SHF) of water hyacinth in shake flask

Separate hydrolysis and fermentation (SHF) of water hyacinth at shake flask level was carried out using 1 g of mixed MAA and organosolv pretreated substrate in 100 mL of 20 mM sodium acetate buffer (pH 5.4) in 250 mL conical flask. 1 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added to the flask. The saccharification was carried out at 50°C, 120 rpm

for 36 h. Then, the medium was centrifuged at 5,476***g*** at 25°C for 15 min and the supernatant was collected. The supernatant was supplemented with (0.1%, w $v^{-1}$) each of yeast extract and peptone and was used as fermentation medium. 1 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added to the flask containing above fermentation medium. The fermentation was carried out at 30°C, 120 rpm for three days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$) and ethanol concentration (g $L^{-1}$).

### 3.2.9 Simultaneous saccharification and fermentation (SSF) of wild grass at shake flask level

Simultaneous saccharification and fermentation (SSF) of wild grass in shake flask was carried out using 1 g each of mixed MAA and organosolv pretreated substrate in 100 mL of sodium acetate buffer (pH 4.3, 20 mM) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL flask. 1 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was added to the flask for hydrolysis. 1 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) was added to the flask containing fermentation medium. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for three days. The sample (2 mL) were collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$)

### 3.2.10 Simultaneous saccharification and fermentation (SSF) of water hyacinth at shake flask level

Simultaneous saccharification and fermentation (SSF) of water hyacinth in

shake flask was carried out using 1 g each of mixed MAA and organosolv pretreated substrate in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL conical flask. 1 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added to the flask for saccharification. 1 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added to the flask containing fermentation medium. Both the enzyme and the fermentative microbe were added simultaneously. The fermentation was carried out at 30°C, 120 rpm for three days. The sample (2 mL) were collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

### 3.2.11 Analytical methods

#### *3.2.11.1 Structural carbohydrates estimation*

The structural carbohydrates like cellulose, hemicellulose and lignin of jamun, neem, asoka, bamboo, poplar, wild grass, eucalyptus, mango and water hyacinth were estimated by standardized methods of NREL, USA (Sluiter *et al.*, 2008). 0.3 g of dry substrate (lignocellulosic leafy biomass) was mixed with 3 mL of 27 N sulphuric acid and incubated at 30°C for 1 h. Then 84 mL of distilled water was added to lower down sulphuric acid concentration to 1.5 N. The sample was autoclaved at 121°C for 1 h. The substrate was cooled to room temperature and the treated biomass was filtered using a vacuum filtration unit. The residue was weighed which was lignin (Acid Insoluble Lignin). The filtrate was collected and pH was neutralized by addition of 1 M $CaCO_3$. Finally, the filtrate was assayed for reducing sugar which was glucose

from where cellulose was calculated. (1 g cellulose = 1.1 g of glucose). The remaining content was the hemicellulose.

### *3.2.11.2 Measurement of cell growth*

The cell growth of *S. cerevisiae* or *C. shehatae* during fermentation was estimated with the withdrawl of initial medium along with the pretreated substrate prior to inoculation as blank. Then, the fermentative microbe *S. cerevisiae* or *C. shehatae* was inoculated into the fermentation medium. With the advancement of fermentation (SHF and SSF), the absorbance of the broth samples containing the substrate along with the microbial cells was measured against the above blank. The change in cell OD at 600 nm was measured as cell growth.

### *3.2.11.3 FESEM Analysis*

The field emission scanning electron microscopy (FESEM) analysis was performed for each of the untreated and mixed microwave assisted alkali (MAA) with organosolv pretreated wild grass and water hyacinth. 25 µL of each substrate (0.05 g $L^{-1}$) was placed over the glass slide, dried and coated with gold film using a SC7620"Mini", Polaron Sputter Coater, Quorum Technologies, Newhaven, England and analyzed under the field emission scanning electron microscopy (FESEM-Carl Zeiss, SIGMA VP instrument). The images were obtained for each of the untreated and pretreated wild grass and water hyacinth.

### *3.2.11.4 FT-IR spectroscopy analysis*

Fourier transform infrared (FT-IR) spectroscopy analysis of untreated and mixed microwave assisted alkali (MAA) with organosolv pretreated wild grass and water hyacinth was carried out in FT-IR spectrometer (Spectrum Two, Perkin Elmer, USA). The samples were pelleted by dispersing 1 mg each of dried (45°C for 18 h) untreated and pretreated substrate with 3 mg of potassium bromide (Sigma, USA) in 1:3 ratio. Three replicate of the samples were prepared to increase the reproducibility of the analysis of samples. The samples were verified by 30 scans per sample in iteration with resolution 4 $cm^{-1}$ and data interval 0.1 $cm^{-1}$.

### *3.2.11.5 Recombinant GH5 cellulase assay*

The reducing sugar estimation and recombinant GH5 cellulase assay was performed as described earlier in Chapter 2, Section 2.2.12.1.

### *3.2.11.6 Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay*

The reducing sugar estimation and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay was performed as described earlier in Chapter 2, Section 2.2.12.2.

### *3.2.11.7 Protein content determination*

The protein content was determined as described earlier in Chapter 2, Section 2.2.12.6.

### *3.2.11.8 Ethanol estimation by Gas chromatography and Dichromate assay*

The ethanol obtained from SHF and SSF experiments were estimated by Gas chromatography (GC) and Dichromate assay. The ethanol fraction in fermentation broth was determined by gas chromatography furnished with flame ionization detector (GC-FID, Varian 450) and Porapaq (Hayesep) Q packed column (3.0 m x 2.0 mm i.d., 80-100 mesh, Varian). A constant flow rate (55 $cm^3$ $min^{-1}$) of nitrogen was used as the carrier gas and the oven temperature was kept constant at 150°C for 20 min as per the method of Bandaru *et al.*, (2006). Both the injector and detector temperature were maintained at 170°C. The injection volume used for ethanol analysis was 1 µL.

The dichromate method was also employed to detect the ethanol content by its conversion to acid following dichromatic reaction (Fletcher and Van Staden, 2003). The cell free supernatant of fermentation broth (1 mL) was mixed with 0.115 M potassium dichromate (2 mL) with final addition of 9 mL distilled water. The 12 mL reaction mixture was kept for 10 min in a boiling water bath. Finally, the absorbance of the cooled sample was measured against a blank of potassium dichromate as standard using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) at 600 nm.

#### *3.2.11.8.1 Generation of standard plot of GC analysis for ethanol estimation*

The standard plot of ethanol by GC analysis was prepared by varying the concentration of ethanol from 0 - 40 g $L^{-1}$. Nitrogen was used as carrier gas at a constant flow rate of 55 $cm^3$/min. The oven temperature was maintained isothermally at 150°C for 20 min. An injection volume of 1 µL was used for analysis with the injector and detector temperature kept at 170°C. A standard plot of area (mV. min)

versus ethanol concentration (g $L^{-1}$) was generated where, y = 1 mV. min corresponds to x = 0.0187 (g $L^{-1}$) of ethanol concentration with a $R^2$ value of 0.9966 (Fig. 3.2.2).

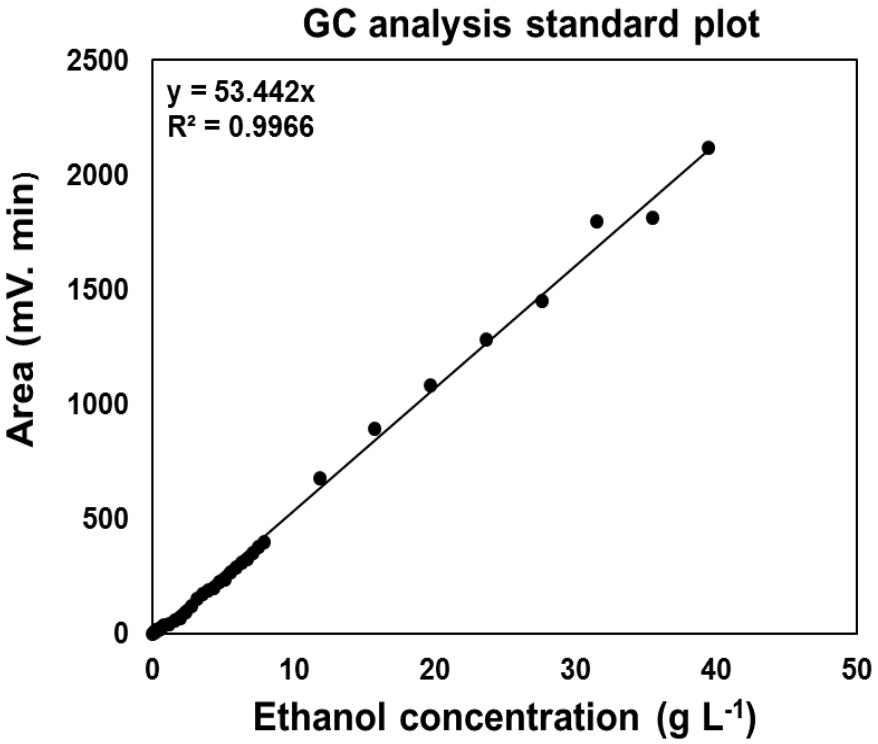


**Fig. 3.2.2** Standard plot of GC analysis for ethanol estimation.

***3.2.11.8.2 Generation of standard plot of Dichromate assay for ethanol estimation***

The standard plot of ethanol by Dichromate assay was prepared by varying the concentration of ethanol from 0-40 g $L^{-1}$. For dichromate assay, 1 mL of the ethanol at particular concentration (0-40 g $L^{-1}$) was mixed with 2 mL of $K_2Cr_2O_7$ (0.115 M) and 9 mL of distilled water. The reaction mixture (12 mL) was heated in a boiling water bath for 10 min. The sample was cooled and the absorbance was measured at 600 nm against a blank with dichromate as standard using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45). A standard plot of optical

density (OD) versus ethanol concentration (g $L^{-1}$) was generated where, y = 1 OD at 600 nm corresponds to x = 2.885 (g $L^{-1}$) of ethanol concentration with a $R^2$ value of 0.9944 (Fig. 3.2.3).

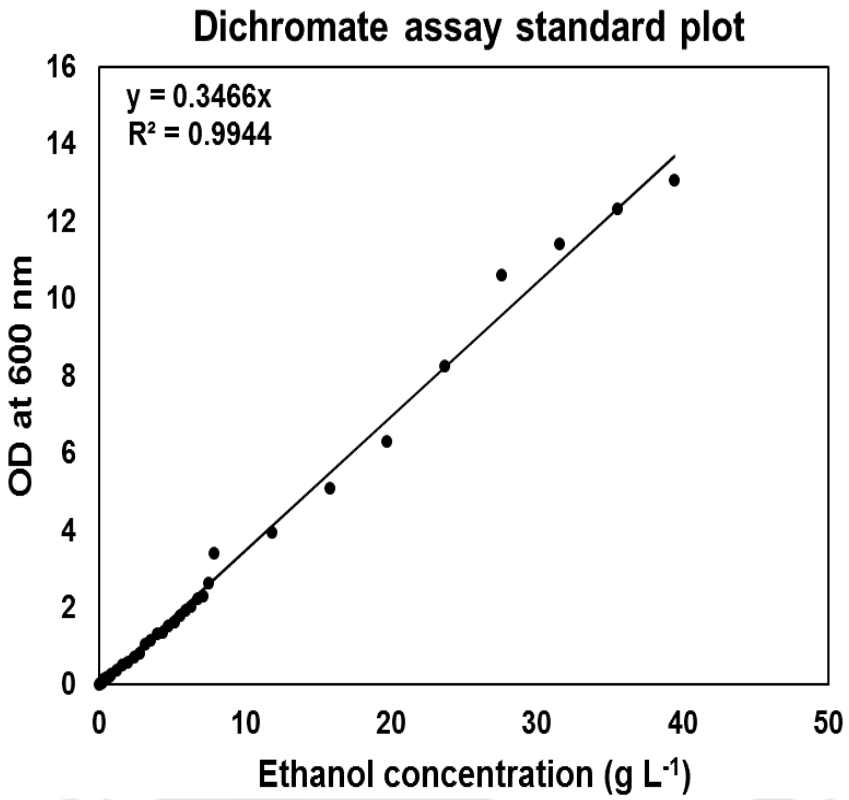


**Fig. 3.2.3** Standard plot of Dichromate assay for ethanol estimation.

***3.2.11.9 Determination of ethanol yield***

*Saccharomyces cerevisiae* is capable of very rapid rates of glycolysis and ethanol production under optimal conditions, producing over 50 mM of ethanol per h per g of cell protein (Dombek and Ingram, 1986). The hexose sugar to ethanol conversion with molar output is given in Eq. 1

$$\mathbf{C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2} \quad \textbf{… Eq. 1}$$

Therefore, $12 \times 6 + 1 \times 12 + 16 \times 6 \longrightarrow 2 \times (12 \times 2 + 1 \times 5 + 16 + 1) + 2CO_2$

$72 + 12 + 96 \longrightarrow 2 \times (24 + 5 + 16 + 1)$

$180 \longrightarrow 2 \times (46)$

180 g glucose $\longrightarrow$ 92 g of ethanol

**Therefore, ethanol yield $\longrightarrow$ 92 / 180 = 0.511**

*Candida shehatae* is a xylose fermenting fungal organism that shows greater ethanol tolerance (Kadam and Schimdt, 1997). The pentose sugar to ethanol conversion with molar output is given in Eq. 2

$$\mathbf{3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2} \quad \textbf{… Eq. 2}$$

Therefore, $3 \times (12 \times 5 + 1 \times 10 + 16 \times 5) \longrightarrow 5 \times (12 \times 2 + 1 \times 5 + 16 + 1) + 5CO_2$

$3 \times (60 + 10 + 80) \longrightarrow 5 \times (24 + 5 + 16 + 1)$

$3 \times (150) \longrightarrow 5 \times (46)$

$450 \longrightarrow 230$

**Therefore, ethanol yield $\longrightarrow$ 230 / 450 = 0.511**

In our SHF and SSF trials, the ethanol yield (g of ethanol g of substrate$^{-1}$) was calculated by dividing the maximum ethanol concentration (g $L^{-1}$) with initial cellulose and hemicellulose concentration (g $L^{-1}$) of the pretreated substrate (lignin was not taken into account). When these ethanol yields are compared with the theoretical 0.51 g ethanol per g of sugar (glucose) yield, since, the residual cellulose and hemicellulose contents after fermentation were not determined in our fermentation studies, thus the amount of cellulose and hemicellulose consumed could not be calculated. Similar method for calculation of ethanol yield is reported earlier (Kadar *et al.*, 2004).

### 3.3 Results and Discussion

The techno-economic feasibility of lignocellulosic ethanol production by SHF and SSF process depends on efficient consumption of both monomeric sugars derived from complex cellulose and hemicellulose moieties of various agricultural residues. Release of simple sugars from varied range of leafy substrates with structural polysaccharide content requires the effective action of saccharifying enzyme. Further, an efficient pretreatment method for loosening the complex carbohydrate structure and the lignin content removal with accessibility to hydrolytic enzymes also becomes essential to enrich the availability of utilizable forms of reducing sugars from substrates.

#### 3.3.1 Composition analysis of substrates for bioethanol production

The structural carbohydrate composition of raw substrates (leafy biomass) involved in the current study determined by standard methods of NREL, USA (Sluiter *et al.*, 2008) is shown in Table 3.3.1. The maximum cellulose content was observed in wild grass (51.70%, w $w^{-1}$) followed by jamun (40.36%, w $w^{-1}$), eucalyptus (39.68%, w $w^{-1}$), bamboo (37.30%, w $w^{-1}$), mango (33.16%, w $w^{-1}$), poplar (31.40%, w $w^{-1}$), water hyacinth (30.07%, w $w^{-1}$), asoka (26.62%, w $w^{-1}$) and lowest in neem (24.64%, w $w^{-1}$) (Table 3.3.1). The maximum hemicellulose content was found in water hyacinth (44.52%, w $w^{-1}$) followed by poplar (43.04%, w $w^{-1}$), mango (42.98%, w $w^{-1}$), neem (41.84%, w $w^{-1}$), eucalyptus (40.44%, w $w^{-1}$), bamboo (35.04%, w $w^{-1}$), jamun (32.22%, w $w^{-1}$), wild grass (30.90%, w $w^{-1}$) and lowest in asoka (30.06%, w $w^{-1}$) (Table 3.3.1). On the other hand, the maximum lignin content was found in

bamboo (27.65%, w $w^{-1}$), jamun (27.40%, w $w^{-1}$), poplar (25.23% w $w^{-1}$ ), neem (23.52%, w $w^{-1}$), mango (22.76%, w $w^{-1}$ ), asoka (21.81%, w $w^{-1}$), eucalyptus (19.88%, w $w^{-1}$), wild grass (18.07%, w $w^{-1}$) and lowest in water hyacinth (29.40%, w $w^{-1}$) (Table 3.3.1).

**Table 3.3.1** Cellulose, hemicellulose and lignin content (%) of various lignocellulosic leafy biomass.

| **Substrates (leafy biomass)** | **Cellulose (%, w $w^{-1}$)*** | **Hemicellulose (%, w $w^{-1}$)*** | **Lignin (%, w $w^{-1}$)*** |
|---|---|---|---|
| Jamun (*Syzygium cumini*) | 40.36 ± 0.45 | 32.22 ± 0.52 | 27.40 ± 0.49 |
| Neem(*Azadirachta indica*) | 24.64 ± 0.44 | 41.84 ± 0.48 | 23.52 ± 0.40 |
| Asoka (*Saraca indica*) | 26.62 ± 0.32 | 30.06 ± 0.50 | 21.81 ± 0.50 |
| Bamboo (*Bambusa dendrocalmus*) | 37.30 ± 0.50 | 35.04 ± 0.47 | 27.65 ± 0.42 |
| Poplar (*Populus nigra*) | 31.40 ± 0.40 | 43.04 ± 0.38 | 25.23 ± 0.46 |
| Wild grass (*Achnatherum hymenoides*) | 51.70 ± 0.43 | 30.90 ± 0.55 | 18.07 ± 0.56 |
| Eucalyptus (*Eucalyptus marginata*) | 39.68 ± 0.49 | 40.44 ± 0.50 | 19.88 ± 0.45 |
| Mango (*Mangifera indica*) | 33.16 ± 0.38 | 42.98 ± 0.43 | 22.76 ± 0.50 |
| Water hyacinth (*Eichhornia crassipes*) | 30.07 ± 0.24 | 44.52 ± 0.45 | 29.40 ± 0.32 |

***values are mean ± SE (n=3)***

The stovers of Bermudagrass, reed and rapeseed were reported to contain cellulose content of 47.8%, 39.5%, 27.6% and lignin content of 19.4%, 24.0%, 18.3%, respectively (all values are in w/w) (Li *et al.,* 2009). Cellulose content of rice husk reported was 30 (%, w $w^{-1}$) which is lower than the cellulose contents of various substrates used in the present study (Mansilla *et al.,* 1998).

Among nine substrates, wild grass containing highest cellulose content, 51.7% (w $w^{-1}$) with 30.9% (w $w^{-1}$) hemicellulose and water hyacinth encompassing highest hemicellulose content, 44.5% (w $w^{-1}$) with 30% (w $w^{-1}$) cellulose were selected as the most sustainable substrates for bioethanol production.

### 3.3.2 Pretreatment of substrates

#### *3.3.2.1 Structural carbohydrate determination of untreated and pretreated wild grass*

The structural carbohydrates *viz.*, cellulose, hemicellulose and lignin of wild grass estimated by standardized methods of NREL, USA (Sluiter *et al.*, 2008) after each individual pretreatment and mixed pretreatment is shown in Table 3.3.2. The cellulose, hemicellulose and lignin content (%, w $w^{-1}$) in untreated wild grass (*Achnatherum hymenoides*) accounted to be 51.70, 30.92 and 18.71 respectively.

**Table 3.3.2** Cellulose, hemicellulose and lignin content (%, w $w^{-1}$) of wild grass (*Achnatherum hymenoides*) after different pretreatments.

| Type of pretreatment | Cellulose (%, w $w^{-1}$)* | Hemicellulose (%, w $w^{-1}$)* | Lignin (%, w $w^{-1}$)* |
|---|---|---|---|
| Control (Untreated) | 51.70 ± 0.36 | 30.92 ± 0.26 | 18.71 ± 0.27 |
| Steam explosion | 48.91 ± 0.27 | 27.80 ± 0.37 | 17.65 ± 0.48 |
| Alkali (NaOH) treatment | 50.01 ± 0.43 | 28.10 ± 0.54 | 16.24 ± 0.39 |
| Wet oxidation | 49.17 ± 0.38 | 28.04 ± 0.38 | 16.50 ± 0.39 |
| Phosphoric acid ($H_3PO_4$) – acetone | 48.80 ± 0.28 | 28.12 ± 0.32 | 17.60 ± 0.43 |
| Ammonia fibre expansion (AFEX) | 49.60 ± 0.32 | 27.42 ± 0.28 | 16.31 ± 0.54 |
| Organosolv | 48.80 ± 0.39 | 27.02 ± 0.32 | 16.04 ± 0.36 |
| pH controlled hot water | 50.03 ± 0.52 | 27.78 ± 0.45 | 17.50 ± 0.23 |
| †DSDT mild acid hydrolysis | 49.50 ± 0.43 | 27.40 ± 0.37 | 17.45 ± 0.34 |
| Microwave assisted alkali (MAA) | 47.50 ± 0.37 | 27.50 ± 0.33 | 15.60 ± 0.37 |
| MAA + Organosolv | 46.30 ± 0.25 | 26.50 ± 0.28 | 15.20 ± 0.51 |

†DSDT- Dual step dual temperature **values are mean ± SE (n=3)*

There was a sudden explosive decompression in structural carbohydrate composition of wild grass on undergoing steam explosion pretreatment resulting in cellulose, 48.91%, hemicellulose, 27.80% and lignin degradation to 17.65% (Table 3.3.2). However, with sodium hydroxide pretreatment, the final cellulose and

hemicellulose dissolution was low having 50.01% and 28.10%, but the removal of lignin was much effective with 16.24% (Table 3.3.2). The effective removal of lignin accomplished by wet oxidation, phosphoric acid ($H_3PO_4$) – acetone, AFEX, organosolv, pH controlled hot water and dual step dual temperature (DSDT) mild acid hydrolysis were 16.50, 17.60, 16.31, 16.04, 17.50 and 17.45%, respectively. Lignin was degraded to the maximum extent (15.60 %) by microwave assisted alkali (MAA) and organosolv (16.04%) pretreatments (Table 3.3.2.). MAA pretreatment resulted in maximum loosening of cellulose (47.50%) as compared to wet oxidation (49.17%), phosphoric acid – acetone pretreatment (48.80%), AFEX (49.60%), organosolv (48.80%), pH controlled hot water (50.03%) and dual step dual temperature (DSDT) mild acid hydrolysis (49.50%) (Table 3.3.2). Organosolv pretreatment loosened hemicellulose to a maximum extent of 27.02% (Table 3.3.2). Hemicellulose was broken down to least extent in phosphoric acid – acetone pretreatment (28.12%) as compared to wet oxidation (28.04%), pH controlled hot water (27.78%), MAA pretreatment (27.50%), AFEX (27.42%) and DSDT mild acid hydrolysis (27.40%) (Table 3.3.2). The MAA and organosolv pretreatments independently degraded maximum lignin with subsequent loosening of cellulose and hemicellulose (Table 3.3.2). The mixed MAA and organosolv pretreatments employed for the concomitant decrease in cellulose crystallinity and maximum hemicellulose breakdown with the simultaneous lignin removal resulted in cellulose content of 46.30%, hemicellulose of 26.50 % and lignin of 15.20%, respectively (Table 3.3.2.). The mixed pretreated wild grass was further subjected to FESEM and FT-IR analyses and different fermentation experiments.

### *3.3.2.1 Structural carbohydrate determination of untreated and pretreated water hyacinth*

The structural carbohydrates *viz.*, cellulose, hemicellulose and lignin of water hyacinth estimated by standardized methods of NREL, USA (Sluiter *et al.*, 2008) after each individual pretreatment and mixed pretreatment is shown in Table 3.3.3. The cellulose, hemicellulose and lignin content (%, w $w^{-1}$) in untreated water hyacinth (*Eichhornia crassipes*) was 30.07, 44.52 and 29.40.

**Table 3.3.3** Cellulose, hemicellulose and lignin content (%) of water hyacinth (*Eichhornia crassipes*) after different pretreatments.

| Type of pretreatment | Cellulose (%, w $w^{-1}$)* | Hemicellulose (%, w $w^{-1}$)* | Lignin (%, w $w^{-1}$)* |
|---|---|---|---|
| Control (Untreated) | 30.07 ± 0.24 | 44.52 ± 0.45 | 29.40 ± 0.32 |
| Steam explosion | 28.60 ± 0.34 | 37.24 ± 0.39 | 19.51 ± 0.36 |
| Alkali (NaOH) treatment | 29.80 ± 0.43 | 39.14 ± 0.37 | 18.50 ± 0.41 |
| Wet oxidation | 28.70 ± 0.32 | 39.60 ± 0.43 | 18.90 ± 0.51 |
| Phosphoric acid ($H_3PO_4$) – acetone | 28.10 ± 0.40 | 39.50 ± 0.23 | 19.45 ± 0.42 |
| Ammonia fibre expansion (AFEX) | 29.20 ± 0.49 | 37.28 ± 0.46 | 18.60 ± 0.32 |
| Organosolv | 28.40 ± 0.43 | 34.01 ± 0.37 | 17.80 ± 0.39 |
| pH controlled hot water | 29.60 ± 0.47 | 36.02 ± 0.41 | 19.35 ± 0.34 |
| †DSDT mild acid hydrolysis | 29.10 ± 0.23 | 36.20 ± 0.50 | 19.20 ± 0.42 |
| Microwave assisted alkali (MAA) | 27.30 ± 0.34 | 35.80 ± 0.27 | 16.24 ± 0.23 |
| MAA + Organosolv | 26.10 ± 0.23 | 33.20 ± 0.29 | 15.64 ± 0.21 |

†DSDT- Dual step dual temperature **values are mean ± SE (n=3)*

Steam explosion pretreatment resulted in lesser breakdown of cellulose (28.60%), considerable degradation of hemicellulose (37.24%) and lignin (19.51%) (Table 3.3.3). However with sodium hydroxide pretreatment, the final cellulose and hemicellulose dissolution was low having 29.80 % and 39.14 %, but the removal of lignin was much effective with 18.50% (Table 3.3.3). The effective removal of lignin obtained by wet oxidation, phosphoric acid ($H_3PO_4$) – acetone, AFEX, organosolv, pH controlled hot water and dual step dual temperature (DSDT) mild acid hydrolysis

were 18.90, 19.45, 18.60, 17.80, 19.35, and 19.20% respectively. Lignin was degraded to the maximum extent (16.24%) by microwave assisted alkali (MAA) and organosolv (17.80%) pretreatments (Table 3.3.3.). MAA pretreatment resulted in maximum loosening of cellulose (27.30%) as compared to wet oxidation (28.70%), phosphoric acid – acetone pretreatment (28.10%), AFEX (29.20%), organosolv (28.40%), pH controlled hot water (29.60%) and dual step dual temperature (DSDT) mild acid hydrolysis (29.10%) (Table 3.3.2). Organosolv pretreatment relaxed the complex hemicellulose to the maximum extent of 34.01% (Table 3.3.3). Lesser hemicellulose breakdown was obtained by wet oxidation (39.60%) as compared to phosphoric acid – acetone pretreatment (39.50 %), AFEX (37.28%), and DSDT mild acid hydrolysis (36.20%), pH controlled hot water (36.02%), MAA pretreatment (35.80%) (Table 3.3.3). Lignin was degraded to maximum extent by MAA and by organosolv pretreatments with consequent loosening of cellulose and hemicellulose independently. The mixed MAA and organosolv pretreatments employed for the simultaneous loosening of cellulose and hemicellulose with efficient lignin removal resulted in cellulose (26.10%), hemicellulose, 33.20 % and lignin, 15.64%, respectively (Table 3.3.3). The mixed pretreated water hyacinth was further subjected to FESEM and FT-IR analyses and different fermentation experiments.

### 3.3.3 FESEM and FT-IR analysis of wild grass (*Achnatherum hymenoides*)

The FESEM image of the untreated substrate revealed structural eveness and displayed negligible surface porosity (Fig. 3.3.1A). The surface morphology analysis of wild grass revealed increase in porosity and structural destabilization with mixed microwave assisted alkali (MAA) and organosolv pretreatment (Fig. 3.3.1B). The porosity

increment over the surface of the pretreated substrate enhanced the enzyme accessibility towards the substrate with a substantial release of utilizable reducing sugar for improved ethanol yield.

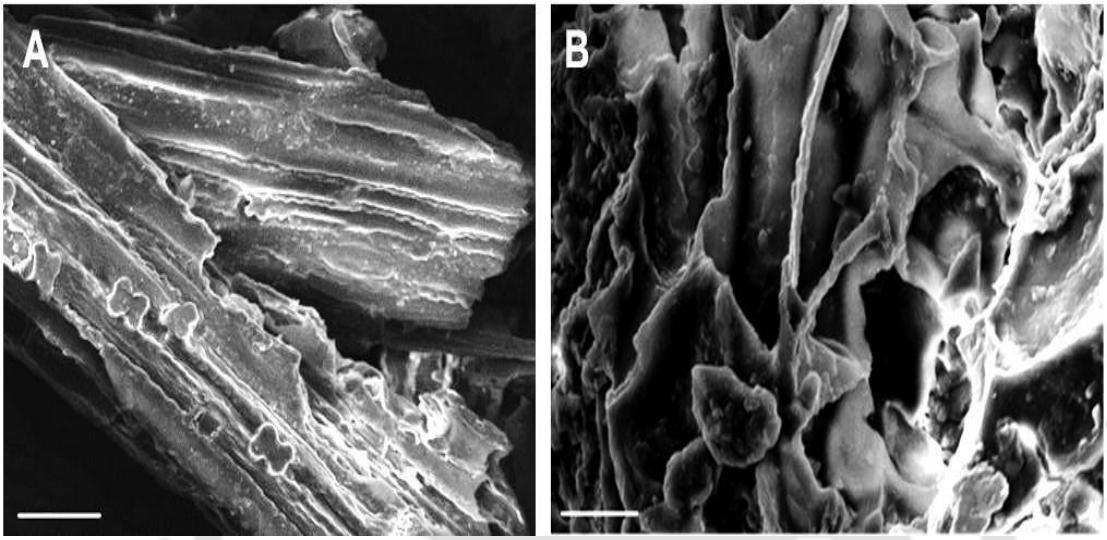


**Fig. 3.3.1** FESEM images of (A) Untreated (B) Mixed microwave assisted alkali (MAA) with organosolv pretreated wild grass (*Achnatherum hymenoides*). All images are shown at magnification-scale bar: 30 µm.

The FT-IR spectral analysis of both untreated and pretreated wild grass samples were performed (Fig. 3.3.2). The H-bond transmittance of OH stretching supposed to arise from the glycosidic bonds of cellulose or hydroxyphenyl, guaiacyl and syringyl groups of lignin released from pretreated wild grass was shifted to higher wave number from 3416 $cm^{-1}$ to 3426 $cm^{-1}$ in the treated sample (Fig 3.3.2). The analysis of carbohydrate specific fingerprint of FT-IR spectra obtained for mixed pretreated wild grass is depicted in Table 3.3.4. The shift was due to change of hydrogen bonding energy in the system of internal and intermolecular interactions (Mothe and Miranda, 2009). Two independent peaks at 2967 and 2900 $cm^{-1}$ appeared

in pretreated sample due to C-H stretching of aliphatic structures as also reported by Cao and Tang (2002).

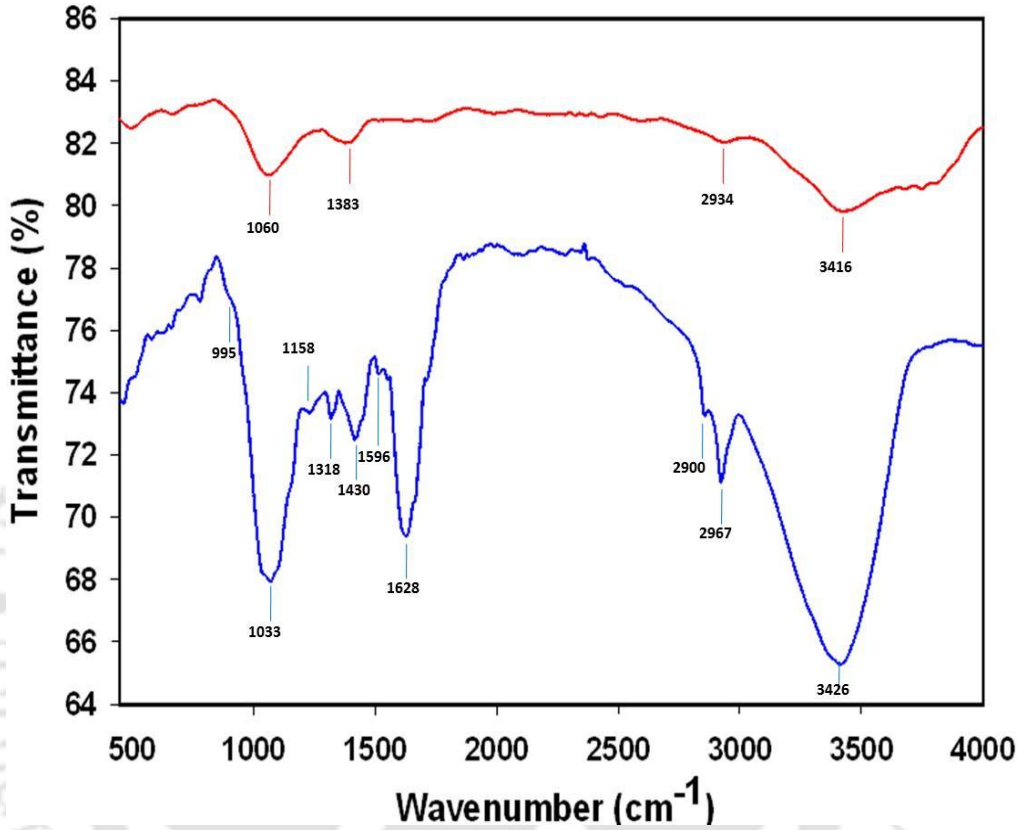


**Fig. 3.3.2** Fourier transform infrared (FT-IR) spectroscopic analysis of untreated wild grass (*Achnatherum hymenoides*) **(–)** and Mixed microwave assisted alkali (MAA) with organosolv pretreated wild grass **(–)**.

**Table 3.3.4** FT-IR analysis of mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass (*Achnatherum hymenoides*).

| **Name of characteristic group** | **Wave number ($cm^{-1}$)** |
|---|---|
| OH | 3426 |
| Aliphatic C-H stretch | 2967, 2900 |
| Aromatic ring stretch of lignin | 1628 |
| Aromatic skeletal vibration plus C=O stretch | 1596 |
| C-H (Crystalline cellulose) | 1430 |
| $CH_2$-wagging vibrations in cellulose and hemicellulose | 1318 |
| Antisymmetric stretching of C-O-C in cellulose and hemicellulose | 1160 |
| C - O stretch vibration of Glucose | 1033 |

The typical peaks between 1152 and 995 $cm^{-1}$ were of arabinose and xylose counterparts of arabinoxylan (Kacurakova *et al.,* 1998) attributed to the stretching and bending vibrations of C-O, C-C and C-OH. A more contrasting peak was observed at lower wavenumber 1033 $cm^{-1}$ as compared to untreated sample at 1060 $cm^{-1}$ which arose due to C - O stretch vibration of glucose from cellulose hydrolysis in the pretreated sample. Glucose is reported to give maximum peak intensity at 1033 $cm^{-1}$ due to C-O stretch vibration (Adina *et al.,* 2010). Therefore, the mixed microwave assisted alkali (MAA) and organosolv pretreatment process was significant in the release of free sugars from wild grass for fermentation to ethanol.

### 3.3.4 FESEM and FT-IR analysis of water hyacinth (*Eichhornia crassipes*)

The FESEM image of the untreated water hyacinth displayed structural eveness and minor surface porosity (Fig. 3.3.3A). The surface morphology study of water hyacinth revealed porosity increment and structural deterioration with mixed microwave assisted alkali (MAA) and organosolv pretreatment (Fig. 3.3.3B). The increase in porosity over the surface of the pretreated substrate improved the enzyme accessibility for enhanced hydrolysis with a considerable release of utilizable reducing sugar for efficient yield of ethanol.

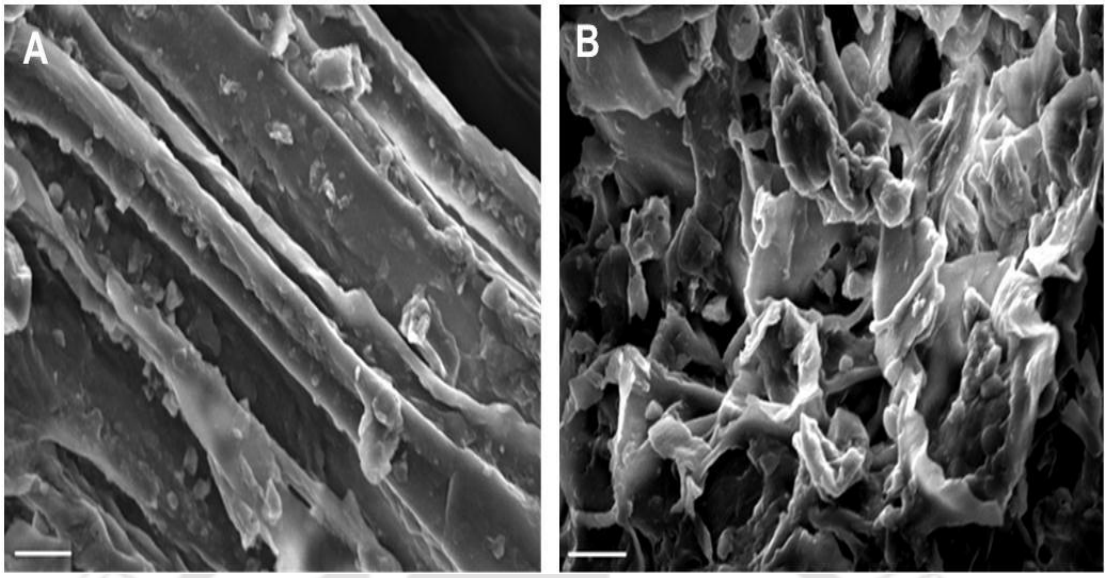


**Fig. 3.3.3** FESEM images of (A) Untreated (B) Mixed microwave assisted alkali (MAA) with organosolv pretreated water hyacinth (*Eichhornia crassipes*). All images are shown at magnification-scale bar: 20 µm.

The FT-IR spectra of both untreated and mixed microwave assisted alkali (MAA) with organosolv pretreated water hyacinth are illustrated in Fig. 3.3.4. The analysis of carbohydrate specific fingerprint of FT-IR spectra obtained for mixed pretreated water hyacinth is depicted in Table 3.3.5. The absorption at 3384 $cm^{-1}$ is attributed to the hydroxyl stretching vibrations, and the band at 2930 $cm^{-1}$ is due to the C-H stretching of methyl groups. Similar identifications were reported previously by Sun *et al.*, (2012) with little peak shifts and more pronounced peaks as in case of pretreated sample while comparing with the spectra of untreated water hyacinth. The band around 1642 $cm^{-1}$ was probably due to the bending mode of water, since the hemicelluloses have a strong affinity for water, and these macromolecules in the solid state may have disordered structures that can be easily hydrated (Kacurakova *et al.*, 1994; Chaikumpollert *et al.*, 2004).

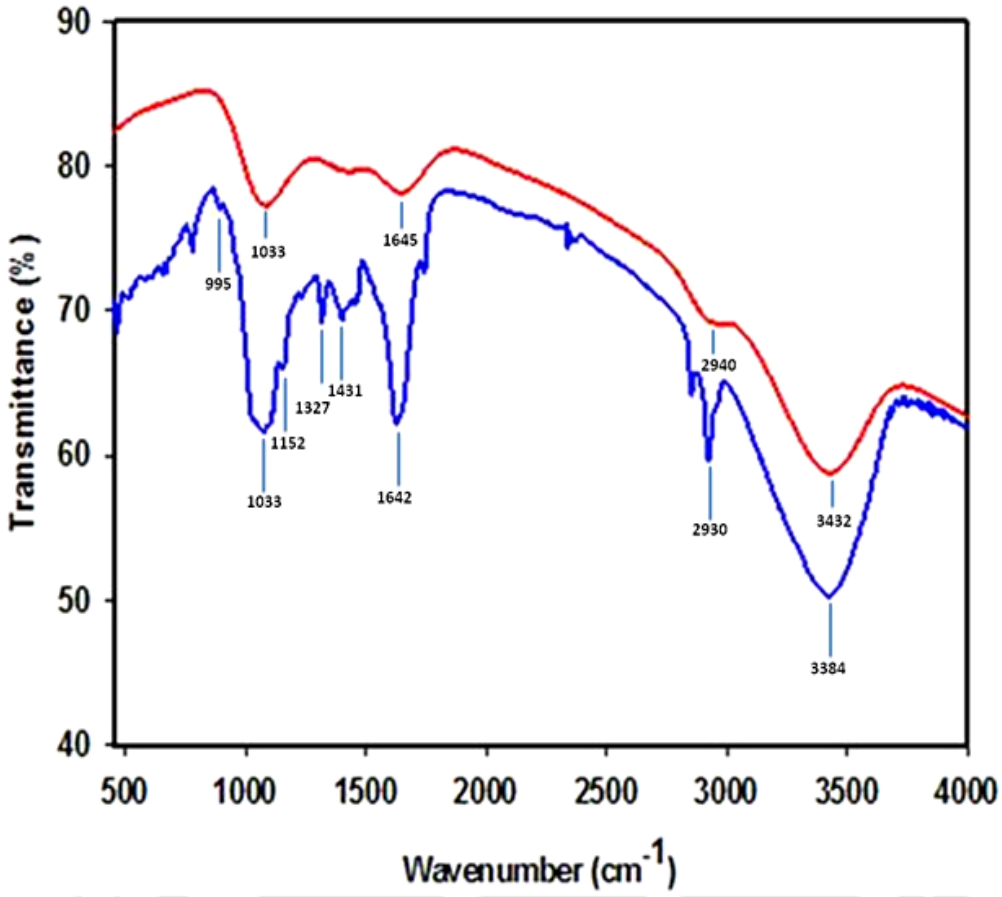


**Fig. 3.3.4** Fourier transform infrared (FT-IR) spectroscopic analysis of untreated water hyacinth **(–)** and Mixed microwave assisted alkali (MAA) with organosolv pretreated water hyacinth (*Eichhornia crassipes*) **(–)**.

**Table 3.3.5.** FT-IR analysis of mixed microwave assisted alkali (MAA) and organosolv pretreated water hyacinth (*Eichhornia crassipes*).

| Name of characteristic group | Wave number ($cm^{-1}$) |
|---|---|
| OH | 3426 |
| Aliphatic C-H stretch | 2967, 2900 |
| Aromatic ring stretch of lignin | 1628 |
| Aromatic skeletal vibration plus C=O stretch | 1596 |
| C-H (Crystalline cellulose) | 1430 |
| $CH_2$-wagging vibrations in cellulose and hemicellulose | 1318 |
| Antisymmetric stretching of C-O-C in cellulose and hemicellulose | 1160 |
| C - O stretch vibration of Glucose | 1033 |

The bands at 1327 and 1431 $cm^{-1}$ represent C-H wagging and OH bending and C-H, OH bending, respectively (Kacurakova and Mathlouthi, 1996). None of these peaks were observed in untreated water hyacinth. The typical peaks between 1152 and 995 $cm^{-1}$ attributed to the stretching and bending vibrations of C-O, C-C and C-OH were of arabinose and xylose counterparts of arabinoxylan (Kacurakova *et al.*, 1998). None of the peaks were observed as in case of untreated sample. A more pronounced peak at 1033 $cm^{-1}$ was attributed to glucose as in case of pretreated sample as identified earlier by Adina *et al.*, (2010). Therefore, mixed pretreatment process was efficient to release the monomeric sugar moieties from complex water hyacinth for ethanol production.

### 3.3.5 Separate hydrolysis and fermentation (SHF) of wild grass at shake flask level

With the objective to evaluate the offline measurements at shake flask level, SHF experiment was carried out involving recombinant cellulase (GH5) along with *S. cerevisiae* on mixed pretreated wild grass (*Achnatherum hymenoides)*.

**Table 3.3.6** Shake flask SHF and SSF employing GH5 cellulase and *S. cerevisiae* on mixed MAA and Organosolv pretreated 1% (w $v^{-1}$) wild grass.

| **Mode of fermentation** | **Reducing sugar (g $L^{-1}$)*** | **Ethanol titre (g $L^{-1}$)*** | **Ethanol yield (g of ethanol g of substrate$^{-1}$)** |
|---|---|---|---|
| SHF | 0.89 ± 0.09 | 0.56 ± 0.02 | 0.076 |
| SSF | 1.26 ± 0.06 | 0.67 ± 0.04 | 0.092 |

***the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

The dynamic profile of SHF displayed the growth of fermentative microbe *S. cerevisiae*, sugar utilization and rate of ethanol formation (Fig. 3.3.5).

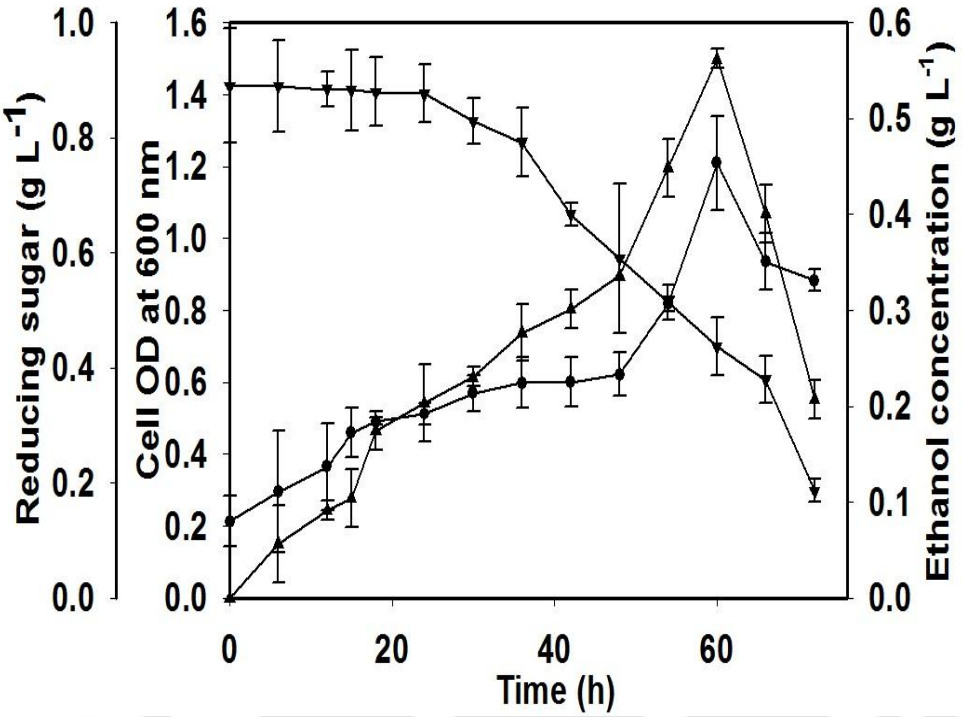


**Fig. 3.3.5** SHF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase and *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$) and (▼) reducing sugar (g $L^{-1}$) with time (h). SHF was carried out in 100 mL in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

After the initial adaptation phase with cell OD of 0.37 at 16 h. there was an increment in cell OD from 48 - 60 h (Fig. 3.3.5). The cell growth exhibited the exponential growth pattern with OD of 0.9 at 54 h and a maximum OD of 1.3 at 60 h (Fig. 3.3.5). There was a subsequent decrease in reducing sugar concentration from 0.89 g $L^{-1}$ (Table 3.3.6) during initial stage of SHF to 0.7 g $L^{-1}$ at 42 h with a later exponential decrease to 0.48 g $L^{-1}$ at 60 h (Fig. 3.3.5). The ethanol production was growth associated reaching a maximum ethanol concentration of 0.56 g $L^{-1}$ with ethanol yield of 0.076 g of ethanol g of substrate$^{-1}$ at 60 h (Table 3.3.6, Fig. 3.3.5).

### 3.3.6 Simultaneous saccharification and fermentation (SSF) of wild grass at shake flask level

SSF experiment was performed involving 1% (w $v^{-1}$) mixed pretreated wild grass as substrate and recombinant cellulase (GH5) as saccharifying enzyme along with *S. cerevisiae* for bioethanol production.

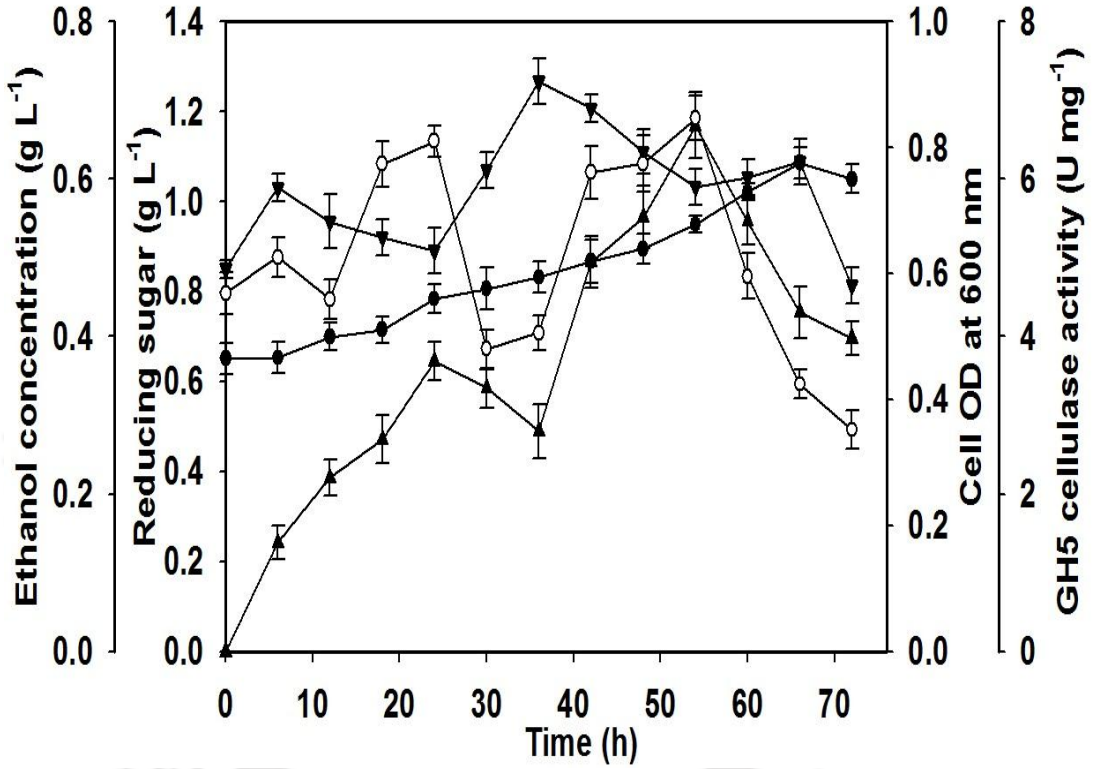


**Fig. 3.3.6** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

The dynamic profile of SSF exhibited three distinct phases in terms of growth of fermentative microbes, specific activity of enzyme, release of utilizable sugar and rate of ethanol formation (Fig. 3.3.6). In the first phase, there was a drop in reducing sugar after a short initial accumulation phase. A decrease in sugar concentration was

found to be concomitant with simultaneous increase in growth and ethanol concentration. In the second phase of fermentation, the organisms were still in their exponential phase reaching a maximum OD of 0.8 at 66 h (Fig. 3.3.6). Interestingly, a decrease in ethanol concentration was observed with the substantial accumulation of reducing sugar (1.26 g $L^{-1}$) in the broth (Table 3.3.6, Fig. 3.3.6). The third and final phase of fermentation was marked with a steep rise in ethanol concentration attaining a maximum titre of 0.67 g $L^{-1}$ and yield of 0.092 g of ethanol g of substrate$^{-1}$ (Table 3.3.6, Figure 3.3.6). This phase displayed a continuous sugar withdrawl from the broth. There was a drop in cell biomass concentration after 66 h indicating achievement of senescence. The dynamic profile of reducing sugar showed a sinusoidal behaviour attributing to a delicate balance between the rate of saccharification for reducing sugar release and the extent of its utilization for growth and ethanol formation. It was found that the reducing sugar concentration increment was associated with decline in specific enzyme activity and vice-versa. The ethanol titre (g $L^{-1}$) obtained from SSF experiment involving mixed pretreated wild grass clearly proved SSF process better over SHF with enhanced ethanol yield.

### 3.3.7 Separate hydrolysis and fermentation (SHF) of water hyacinth at shake flask level

The shake flask SHF experiment on mixed pretreated water hyacinth (*E. crassipes*) involving recombinant hemicellulase (GH43) (α-L-arabinofuranosidase) along with *C. shehatae* exhibited a complex interplay between cell growth, rate of sugar utilization and ethanol formation (Fig. 3.3.7).

**Table 3.3.7** Shake flask SHF and SSF employing GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* on mixed MAA and Organosolv pretreated 1% (w $v^{-1}$) water hyacinth**.**

| Mode of fermentation | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|
| SHF | 0.62 ± 0.07 | 0.45 ± 0.03 | 0.075 |
| SSF | 0.74 ± 0.06 | 0.55 ± 0.08 | 0.092 |

***the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)***

The cell growth of *C. shehatae* exhibited the exponential growth pattern with a maximum OD of 1.1 at 66 h (Fig. 3.3.7). There was a subsequent decrease in reducing sugar concentration from 0.62 g $L^{-1}$ (Table 3.3.7) during initial stage of SHF to 0.46 g $L^{-1}$ at 42 h with a later exponential decrease to 0.26 g $L^{-1}$ at 66 h (Fig. 3.3.7). A maximum ethanol concentration of 0.45 g $L^{-1}$ was achieved at 60 h of fermentation with an ethanol yield of 0.075 g of ethanol g of substrate$^{-1}$ (Table 3.3.7, Fig. 3.3.7). There was no ethanol formation thereafter revealing the consumption of sugar for the maintenance of *C. shehatae*. Thus, the growth concomitant ethanol production exhibited an inverse relationship with reducing sugar utilization. Similar fermentation kinetics has been reported earlier (Sharma, 1971).

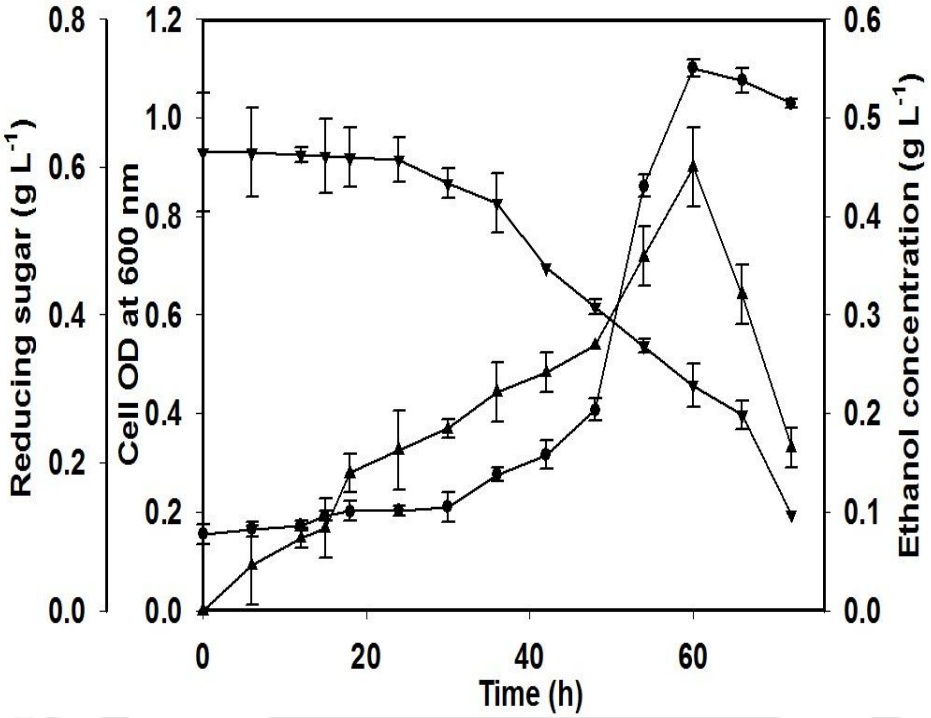


**Fig. 3.3.7** SHF profile of 1% (w $v^{-1}$) water hyacinth using GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$) and (▼) reducing sugar (g $L^{-1}$) with time (h). SHF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

### 3.3.8 Simultaneous saccharification and fermentation (SSF) of water hyacinth at shake flask level

In case of SSF involving mixed pretreated water hyacinth along with recombinant hemicellulase (GH43) (α-L-arabinofuranosidase) and *C. shehatae* (Fig. 3.3.8), the first growth phase corresponded to an initial 18 h of fermentation with a cell OD of 0.74. During this phase, the utilization of sugar that is produced from the enzyme hydrolysis of substrate was used by the micro-organism for its growth. The second distinct growth phase was observed during 54 h of fermentation with a cell absorbance of 1.26.

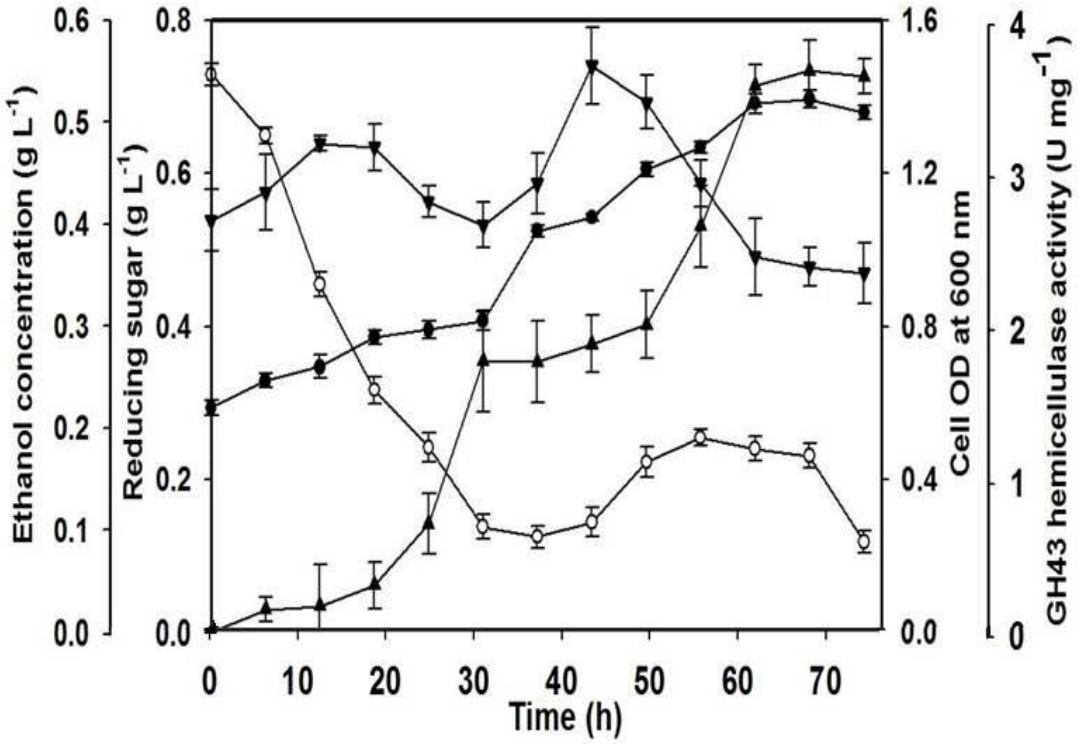


**Fig. 3.3.8** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

The biomass production (cell OD at 600 nm) reached its peak value of 1.39 at 60 h of fermentation, and thereby a decrease in growth was observed, indicating achievement of senescence. The ethanol formation was proportional to the increase in biomass. A maximum ethanol titre of 0.55 g $L^{-1}$ was achieved (Table 3.3.7, Fig. 3.3.8) at 66 h of fermentation and after that it remained constant. The ethanol yield (g of ethanol g of substrate$^{-1}$) obtained was 0.092 (Table 3.3.7). Interestingly, the dynamic profile of reducing sugar showed a sinusoidal behaviour attributing to a delicate balance between the saccharification rate for release of reducing sugar and the rate of

its subsequent utilization by *C. shehatae* growth and ethanol formation. It is important to note that the increase in reducing sugar concentration (0.74 g $L^{-1}$) was found to be concomitant with decrease in specific activity of enzyme and vice-versa. The ethanol titre (g $L^{-1}$) obtained from SSF experiments involving pretreated water hyacinth clearly proved SSF process better over SHF with enhanced ethanol yields by eliminating end product inhibition and excluding separate reactors' obligation. Similar findings are reported in literature (Hamelinck *et al.,* 2005).

The pretreatment of lignocellulosic biomass is phenomenal in the release of cellulosic, hemicellulosic and lignin content from the complex carbohydrate reservoir. Comparing the various pretreatment outcomes with the untreated wild grass and water hyacinth (Table 3.3.2, Table 3.3.3), the highest degradation of lignin content along with maximum loosening of cellulose and hemicellulose was achieved by MAA and organosolv pretreatments. Little disintegration of cellulose, hemicellulose and lignin was achieved after steam explosion, alkali (NaOH) treatment, wet oxidation, phosphoric acid ($H_3PO_4$) – acetone, ammonia fibre expansion (AFEX), pH controlled hot water, dual step dual temperature (DSDT) mild acid hydrolysis. The higher denaturation of lignin associated with maximum cellulose loosening in MAA pretreatment in contrast to other pretreatments may be due to reduced crystallinity of secondary fibre (SF) of plant cell wall material with consequent rapid increment in the inter-planar crystal spacing. These changes were attributed to the synergistic effect mechanism of microwave irradiation, $Na^+$, $OH^-$ and water molecules in alkali liquor on SF supra molecular structure. These changes resulted in the improvement of water retention value and reactivity of SF. These mechanisms of deconstruction of lignocellulosic material into high individual components were described earlier (Zhu *et al.,* 2006).

They reported that in enzymatic hydrolysis of pretreated rice straw, the pretreatment by MAA had the highest hydrolysis rate and glucose content in the hydrolysate. It was reported earlier that the highest achieved lignin breakdown along with hemicellulose deconstruction in organosolv pretreatment process was due to solubilization of solid biomass at elevated temperature with further decomposition of a significant percentage of the soluble carbohydrates to by-products such as furfural or 5-hydroxymethylfurfural (HMF) (Rolz *et al.,* 1986). So, use of these pretreatments in combination (MAA with Organosolv) phenomenally reduced the efforts of using two different pretreatments separately to achieve highest lignin removal and complex carbohydrates deconstruction into simple sugars. Fourier transform infrared (FT-IR) spectroscopic analysis confirmed the efficiency of mixed pretreatment in significant release of free glucose, xylose and arabinose moieties from complex lignocellulosic wild grass and water hyacinth.

The ethanol titre (g $L^{-1}$) obtained from SSF trial involving mixed pretreated wild grass and water hyacinth evidenced SSF process efficient over SHF with enhanced ethanol yield by eliminating end product inhibition excluding separate reactors' obligation. Similar findings are reported in literature (Hamelinck *et al.,* 2005). The values of ethanol concentration and yield obtained in our study are comparable with other reported literature. Some researchers have reported an ethanol titre of 1 g $L^{-1}$ using crude unprocessed cellulase from *T. reesei* from 1% (w $v^{-1}$) substrate (Lever *et al.,* 2010). Castro *et al.,* (2006) obtained an ethanol yield of 0.29 (g $g^{-1}$) from pretreated olive tree wood and sunflower stalks as substrates by an SSF process. An ethanol titre of 0.9 g $L^{-1}$ was obtained from kinnow waste and banana peels as substrates by simultaneous saccharifi cation and fermentation (Sharma *et al.,*

2007). Nakamura *et al.*, (2008) obtained an ethanol yield of 0.09 (g $g^{-1}$) in a SSF process from paper sludge waste using *Zymomonas mobilis*. Using recombinant cellulase from *Clostridium thermocellum*, researchers obtained an ethanol yield of 0.14 (g $g^{-1}$) employing *S. cerevisiae* on Jamun (*Syzygium cumini*) leafy biomass as the substrate (Mutreja *et al.*, 2011).

### 3.4 Conclusions

This study reported for the first time the efficiency of mixed pretreatment strategy of microwave assisted alkali (MAA) along with organosolv in the breakdown of complex lignocellulosic wild grass and water hyacinth. The efficacy of recombinant *C. thermocellum* hydrolytic GH5 cellulase along with *S. cerevisiae* on cellulose rich wild grass in terms of ethanol titre was determined by two modes of fermentation, SHF and SSF. The efficiency of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* on hemicellulose rich water hyacinth for ethanol production was evaluated by SHF and SSF modes of fermentation.

Among nine different substrates (leafy biomass), the maximum cellulose content was observed in wild grass (51.70%, w $w^{-1}$) followed by jamun (40.36%, w $w^{-1}$), eucalyptus (39.68%, w $w^{-1}$), bamboo (37.30%, w $w^{-1}$), mango (33.16%, w $w^{-1}$), poplar (31.40%, w $w^{-1}$), water hyacinth (30.07%, w $w^{-1}$), asoka (26.62%, w $w^{-1}$) and lowest in neem (24.64%, w $w^{-1}$). On the other hand, the maximum hemicellulose content was found in water hyacinth (44.52%, w $w^{-1}$) followed by poplar (43.04%, w $w^{-1}$), mango (42.98%, w $w^{-1}$), neem (41.84%, w $w^{-1}$), eucalyptus (40.44%, w $w^{-1}$), bamboo (35.04%, w $w^{-1}$), jamun (32.22%, w $w^{-1}$), wild grass (30.90%, w $w^{-1}$), and lowest in asoka (30.06%, w $w^{-1}$). Lignin was found to be maximum in bamboo (27.65%, w $w^{-1}$) followed by jamun (27.40%, w $w^{-1}$), poplar (25.23% w $w^{-1}$), neem (23.52%, w $w^{-1}$), mango (22.76%, w $w^{-1}$), asoka(21.81%, w $w^{-1}$), eucalyptus (19.88%, w $w^{-1}$), wild grass (18.07%, w $w^{-1}$) and water hyacinth (29.40%, w $w^{-1}$). Wild grass containing highest cellulose content, 51.7% (w $w^{-1}$) with 30.9% (w $w^{-1}$) hemicellulose

and water hyacinth encompassing highest hemicellulose content, 44.5% (w $w^{-1}$) with 30% (w $w^{-1}$) cellulose were selected as the most sustainable substrates for bioethanol production.

Nine pretreatments were employed for the efficient breakdown of structural carbohydrates in wild grass. Microwave assisted alkali (MAA) pretreatment resulted in maximum loosening of cellulose (47.50%) as compared to steam explosion (48.91%), alkali (50.01%), wet oxidation (49.17%), phosphoric acid – acetone pretreatment (48.80%), AFEX (49.60%), organosolv (48.80%), pH controlled hot water (50.03%) and dual step dual temperature (DSDT) mild acid hydrolysis (49.50%). Organosolv pretreatment degraded hemicellulose to the maximum extent of 27.02%. Hemicellulose was broken down to least extent in phosphoric acid-acetone pretreatment (28.12%) as compared to alkali (28.10%), wet oxidation (28.04%), pH controlled hot water (27.78%), steam explosion (48.91%), MAA pretreatment (27.50%), etc. Lignin was broken down to maximum extent by MAA (15.60%) followed by organosolv (16.04%) pretreatments. The mixed MAA and organosolv pretreatment strategy loosened maximum cellulose (46.30%), hemicellulose (26.50%) and lignin removal of 15.20%. Water hyacinth was also subjected to nine different pretreatments. MAA pretreatment resulted in maximum loosening of cellulose (27.30%) as compared to steam explosion (28.60%), alkali (29.80%), wet oxidation (28.70%), phosphoric acid-acetone pretreatment (28.10%), AFEX (29.20%), organosolv (28.40%), pH controlled hot water (29.60%) and dual step dual temperature (DSDT) mild acid hydrolysis (29.10%). Organosolv pretreatment degraded hemicellulose to the maximum extent of 34.01%. Lesser hemicellulose breakdown was obtained by wet oxidation (39.60%) as compared to phosphoric acid-

acetone pretreatment (39.50 %), alkali (39.14%), AFEX (37.28%), steam explosion (37.24%), etc. Lignin was broken down to maximum extent by MAA (16.24%) followed by organosolv (17.80%) pretreatments. The mixed MAA and organosolv pretreatments loosened cellulose (26.10%) and hemicellulose, 33.20% with maximum lignin removal of 15.64%.

The surface morphology analysis of wild grass and water hyacinth by FESEM revealed increase in porosity and structural destabilization with mixed microwave assisted alkali (MAA) and organosolv pretreatment. The porosity increment over the surface of the pretreated substrate enhanced the enzyme accessibility towards the substrate with a substantial release of utilizable reducing sugar for improved ethanol yield. The FT-IR analyses of the two substrates, wild grass and water hyacinth displayed a peak of xylose, glucose and arabinose at 995, 1033, 1152 $cm^{-1}$, respectively, confirming the significant breakdown of complex cellulose and hemicellulose to monomeric sugars by mixed pretreatment strategy.

The SSF experiments involving mixed pretreated 1% (w $v^{-1}$) wild grass along with recombinant GH5 cellulase and *S. cerevisiae* yielded a 1.2-fold higher ethanol titre of 0.67 g $L^{-1}$ as compared to ethanol concentration of 0.56 g $L^{-1}$ shake flask SHF. Similarly, the SSF experiments involving mixed pretreated 1% (w $v^{-1}$) water hyacinth along with recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* yielded a 1.2-fold higher ethanol titre of 0.55 g $L^{-1}$ as compared to ethanol concentration of 0.45 g $L^{-1}$ shake flask SHF. The effective breakdown of complex carbohydrates by mixed pretreatment strategy along with recombinant hydrolytic cellulase and hemicellulase in SSF will convert these lignocellulosic weeds into the fuel for tomorrow.

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# Chapter 4

## Identification of best Simultaneous Saccharification and Fermentation of pretreated wild grass and water hyacinth from different combinations of hydrolytic enzymes and fermentative microbes

### 4.1 Introduction

As the prices of fossil fuels continue to rise and the concern over global warming heightens, finding alternative renewable sources of energy becomes more and more imperative. An upsurge in ethanol production using the corn starch-based technology is not practical because corn production for ethanol competes for the limited agricultural land needed for food and feed production (Wright, 1998). Considering the abundance of lignocellulosic biomass, the potential significance of its conversion to fuel such as ethanol has been recognized since long. Utilization of cheap lignocellulosic biomass and other renewable substrates make bioethanol much competent than fossil fuels (Girio *et al.,* 2010). In comparison to fossil fuels, ethanol as a fuel has an added specific energy with heat of vapourization, higher energy density and greater air-fuel ratio (Oliveira *et al.,* 2005).

The plant lignocellulosic biomass is composed of complex heterogeneous carbohydrate polymers *viz.,* cellulose, hemicelluloses along with lignin (Sun and

Cheng, 2002). Ethanol production from lignocellulosic biomass requires the breakdown of the solid material to simple sugars that can be consumed by microorganisms. The conversion comprises three processes: effective pretreatment for delignification, hydrolysis of intricate cellulose and hemicellulose in the lignocellulosic materials to fermentable reducing sugars, and fermentation of the sugars to ethanol. With respect to hemicellulose, cellulose loosening and lignin degradation is more efficiently achieved by microwave assisted alkali (MAA) pretreatment (Zhu *et al.*, 2006). The lignin network and possibly a part of the hemicellulose are degraded effectively by organic solvent-mediated organosolv pretreatment (Geng *et al.*, 2012). The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria (Wright, 1998). With respect to various pretreatments, the utility of enzymatic hydrolysis with the aid of microbes has many advantages such as reduced energy use, no release of toxic chemicals and diminished generation of products lethal to the environment as well as mankind (Wright, 1998).

The land weed *viz.*, wild grass (*Achnatherum hymenoides*) and water weed, water hyacinth (*Eichhornia crassipes*) are abundantly available in northern India. Easy accessibility of this lignocellulosic biomass has aroused interest to exploit them as substrates for the retrieval of many value-added products such as bioethanol (Das *et al.*, 2012; Das *et al.*, 2013). The cost of cellulase production profoundly influences the economics of the entire ethanol production process. *Trichoderma reesei* is a bio technically important filamentous fungus used commercially in enzyme production. *T. reesei* Rut C-30 is the most commonly used fungal strain for industrial cellulase production (Szijarto *et al.*, 2004). *T. reesei* has been widely accepted as the organism

capable of producing the hydrolytic enzymes in considerable quantities, appropriate for lignocellulosic biomass digestion (Balat *et al.*, 2008). *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles have been constructed for specific industrial applications, *viz.*, biostoning and biofinishing of cotton (Gusakov, 2011). The prime hindrance in the usage of commercial fungal enzymes is, lies in its high cost. Also, there is absence of prominent β-glucosidase activity in most of the readily available enzymatic pools, directed towards an efficient saccharification process (Schulein, 1988).

The cellulosome of *Clostridium thermocellum*, a gram-positive thermophilic bacterium displays a specific activity that is 50-fold higher than the corresponding *Trichoderma reesei* system against crystalline cellulose (Demain *et al.*, 2005). A widespread group of enzymes hydrolysing the glycosidic bond between two or more carbohydrates are glycoside hydrolases (GHs). The structurally related enzymes from same GH family may have different substrate specificities. These substrate diversities reflect their significance in specific biological processes at different stages of a plant owing to the fact that the GHs in plant genomes often belong to multi-gene family (Barbosa *et al.*, 1994; Fuhrer *et al.*, 2005; Seo *et al.*, 2005). GHs are a group of enzymes that include cellulases and hemicellulases. According to CAZy database (http://www.cazy.org/fam/acc_GH.html), glycoside hydrolase family 5 (GH5) displays activities of cellulase (EC 3.2.1.4); glucan β-(1→3)-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73), exo-β-(1→4)-glucanase (EC 3.2.1.74); glucan endo-β-(1→6)-glucosidase (EC 3.2.1.75) and cellulose β-(1→4)-cellobiosidase (EC 3.2.1.91), whereas glycoside hydrolase family 43 displays activities of β-(1→3)-xylosidase (EC 3.2.1.-), xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-

arabinofuranosidase (EC 3.2.1.55), arabinanase (EC 3.2.1.99) and galactan β-(1→3)-galactosidase (EC 3.2.1.145).

The hexose fermenting, gram negative bacterium *Zymomonas mobilis* utilizes the Entner-Doudoroff (ED) pathway for ethanol production. Glucose, which gets converted to glyceraldehyde-3-phosphate-to-pyruvate and pyruvate-to-ethanol, provides most of the ATP required for cell activities, about one mole of ATP per mole of glucose (Swings and De ley, 1977; Rogers *et al.,* 1980), a much lower level than the traditional ethanol producing yeast, *Saccharomyces cerevisiae*. The organism thus appears to maintain a high level of glucose flux through the pathways to compensate its low ATP yield (Barnell *et al.,* 1990), for which large amounts of enzymes related to the pathway are expressed, constituting 30-50% of total soluble proteins of cells (Swings and De ley, 1977). As a consequence, it performs less biomass formation and efficient production of ethanol compared to *S. cerevisiae* (Cromie and Doelle, 1980). *Z. mobilis*, which has a relatively compact genome with 2000 genes possesses incomplete Embden-Meyerhof-Parnas (EMP) pathway and incomplete TCA cycle due to a lack of genes for 6-phosphofructokinase, 2-oxoglutarate dehydrogenase complex and malate dehydrogenase (Swings and De ley, 1977; Barnell *et al.,* 1990; Seo *et al.,* 2005) but possesses strong activities of ED pathway (Furher *et al.,* 2005). As a consequence, in recent years, research is focused on *Z. mobilis*, as promising alternative ethanol producer because of its high glucose uptake and greater ethanol tolerance.

Apart from high ethanol tolerance and survival to metabolic inhibitions, the hexose fermenting yeast, *S. cerevisiae* possesses the inherent ability of utilizing various cellulosic biomasses for ethanol production (Casey and Ingledew, 1986).

*Candida shehatae*, a pentose utilizing yeast and having xylitol dehydrogenase and xylose reductase utilizes the pentose phosphate pathway to metabolize pentose sugars for ethanol production (Kadam and Schimdt, 1997; Chandel *et al.*, 2007). Simultaneous saccharification and fermentation (SSF) is a single step technique that consolidates hydrolysis of the complex cellulose and hemicellulose along with the fermentation of the reducing sugar produced (Sangkharak *et al.*, 2011).

In the present study, independent shake flask SSF trials involving different combinations of hydrolytic enzymes and fermentative microbes were performed on 1% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth. The hydrolytic performance of recombinant *C. thermocellum* GH5 cellulase was compared with the *T. reesei* cellulase in terms of reducing sugar formed in SSF experiments. The bioethanol producing capability of *S. cerevisiae* was compared with *Z. mobilis* in terms of ethanol titre. Also, the competence of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated in fermentation trials of wild grass and water hyacinth. Finally, a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae, C. shehatae*) system was employed to yield better ethanol titre.

## 4.2 Materials and Methods

### 4.2.1 Reagents, chemicals and substrates

Carboxy methyl cellulose (CMC), isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were obtained from Sigma Aldrich (St. Louis, USA). Rye arabinoxylan was procured from Megazyme International Ltd. (Ireland). The analytical grade reagents and chemicals *viz.*, ampicillin, glucose, peptone, tryptone, sodium chloride, sodium acetate, yeast extract, malt extract, potato dextrose broth (PDB), sodium carbonate, sodium sulphate, sodium bicarbonate, sodium potassium tartarate, ammonium molybdate, copper sulphate, potassium dichromate, ammonium sulphate, sodium arsenate, sodium hydroxide, magnesium sulphate heptahydrate, potassium dihydrogen phosphate and agar were purchased from Himedia Pvt. Ltd. India. Phosphoric acid was purchased from Qualigens India Pvt. Ltd. Coomassie Brilliant Blue G-250 was purchased from Amresco LLC, USA. Sulphuric acid was acquired from Merck India Pvt. Ltd. Lignocellulosic biomass wild grass (*A. hymenoides*) and the leaves along with petioles of water hyacinth (*E. crassipes)* were collected from the campus of Indian Institute of Technology Guwahati, India. The preliminary treatment of substrates was carried out as described earlier in Chapter 3, Section 3.2.4.

### 4.2.2 Microorganisms and culturing conditions

The culturing and maintenance of *E. coli* BL21 (DE3) cells harbouring the pET-21a(+) plasmid containing a gene encoding family 5 glycoside hydrolase (GH5), a cellulase and *E. coli* BL21 (DE3) pLysS cells harbouring pET-28a(+) plasmid

containing a gene encoding family 43 glycoside hydrolase (GH43), a hemicellulase from *Clostridium thermocellum* was done as described in Chapter 2, Section 2.2.2.

*Trichoderma reesei* (MTCC 164) and *Zymomonas mobilis* (MTCC 2427) were procured from Institute of Microbial Technology (IMTECH), Chandigarh, Punjab, India. *T. reesei* was maintained at 4°C in 15 mL of PD (Potato Dextrose, pH 5.4) agar slants. The chemical composition of PD medium is described in Table 4.2.1. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min.

**Table 4.2.1** Chemical composition of PD agar medium.

| **Constituent** | **Concentration (g 100 mL$^{-1}$)** |
|---|---|
| Potato dextrose powder | 2.4 |
| Agar | 2.0 |

One hundred millilitre of PD (Potato Dextrose) broth (pH 5.4) contained in 250 mL Erlenmeyer flask was sterilized by autoclaving at 121°C and 15 psi for 20 min. One millilitre of *T. reesei* spore suspension (5 x $10^7$ spores mL$^{-1}$) was inoculated into the PD medium. The spore count of *T. reesei* was measured using a haemocytometer. The culture was incubated at 28°C, 120 rpm for 48 h. The broth was centrifuged at 10,000***g*** for 15 min and the cell free supernatant obtained was filtered twice using a vacuum filtration unit (Millipore, Massachusetts, USA) with nylon membrane having pore size of 0.22 µm. Finally, the filtered supernatant containing the cellulase enzyme (9.8 U mg$^{-1}$, 0.82 mg mL$^{-1}$) was used in SSF experiments of wild grass and water hyacinth.

The predominantly hexose utilizing aerobic fermentative microbe, *Z. mobilis* was maintained at 4°C in 5 mL of GYE (Glucose Yeast extract, pH 5.0) slants. The

chemical composition of GYE medium is described in Table 4.2.2. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min.

**Table 4.2.2** Chemical composition of GYE medium.

| **Constituents** | **Concentration (g 100 $mL^{-1}$)** |
|---|---|
| Glucose | 2.0 |
| Yeast extract | 1.0 |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 0.2 |
| Ammonium sulphate [$(NH_4)_2SO_4$] | 0.8 |
| Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) | 0.05 |
| Agar | 2.0 |

One loopful of the slant culture was inoculated into 50 mL of GYE (Glucose Yeast extract) medium (pH 5.0) contained in 250 mL Erlenmeyer flask. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. The culture was incubated in shaking incubator at 30°C, 120 rpm for 48 h prior inoculation into SSF medium. The SSF medium comprised of 100 mL working volume of sodium acetate buffer (pH 4.3 or pH 5.4, 20 mM) in 250 mL Erlenmeyer flask supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$). The aliquots measuring 1 mL from actively growing culture of *Z. mobilis* (2.1 x $10^6$ cells $mL^{-1}$) was aseptically inoculated into SSF medium. The cell count of the actively growing *Z. mobilis* was measured using a haemocytometer.

The culturing and maintenance of aerobic fermentative microbes, *Saccharomyces cerevisiae* (NCIM No. 3215) and *Candida shehatae* (NCIM No. 3500) procured from National Chemical Laboratory (NCL), Pune, India was carried out as described in Chapter 3, Section 3.2.2 earlier.

### 4.2.3 Repetitive batch production of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani (LB) medium with glucose

The repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in separate Luria-Bertani (LB) medium supplemented with glucose was done to yield higher enzyme activity and protein concentration as described earlier in Chapter 2, Section 2.2.11. The recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was used for saccharification in SSF experiments of wild grass and water hyacinth. The recombinant GH43 hemicellulase (α-L-arabinofuranosidase) having specific activity 3.7 U $mg^{-1}$ (0.32 mg $mL^{-1}$) was used for saccharification in SSF experiments of wild grass and water hyacinth.

### 4.2.4 Mixed microwave-assisted alkali (MAA) and organosolv pretreatment strategy

The mixed microwave-assisted alkali (MAA) and organosolv pretreatment of two substrates *viz*., wild grass and water hyacinth were performed as described earlier in Chapter 3, Section 3.2.6.

### 4.2.5 Simultaneous saccharification and fermentation (SSF) trials of wild grass and water hyacinth at shake flask level

Simultaneous saccharification and fermentation (SSF) experiments in 250 mL shake flask were performed independently on 1% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass or water hyacinth employing various combinations of hydrolytic enzymes and fermentative microbes as described in following Sections 4.2.5.1, 4.2.5.2, 4.2.5.3, 4.2.5.4, 4.2.5.5, 4.2.5.6 and 4.2.5.7.

The saccharifying enzymes used were *T. reesei* cellulase, recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase), whereas, the fermenting microbes were *Z. mobilis, S. cerevisiae* and *C. shehatae* as described in the Sections 4.2.5.1, 4.2.5.2, 4.2.5.3, 4.2.5.4, 4.2.5.5, 4.2.5.6 and 4.2.5.7.

### *4.2.5.1 SSF of wild grass and water hyacinth involving T. ressei cellulase and S. cerevisiae*

Owing to the presence of considerable amount of cellulose in wild grass and water hyacinth, SSF trials were employed on these substrates involving the cellulase from fungus *T. reesei* for saccharification and *S. cerevisiae* for bioethanol production. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 0.6 mL of *T. reesei* cellulase (9.8 U $mg^{-1}$, 0.82 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) was added to each flask for fermentation. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.2 SSF of wild grass and water hyacinth involving recombinant GH5 cellulase and S. cerevisiae*

To compare the efficiency of the recombinant bacterial cellulase with that of fungal cellulase in terms of production of reducing sugar and in turn, the ethanol titre, SSF experiments of wild grass and water hyacinth involving recombinant GH5 cellulase and the hexose utilizing yeast, *S. cerevisiae* were set. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of sodium acetate buffer (pH 4.3, 20 mM) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 1 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) was added to each flask for fermentation. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.3 SSF of wild grass and water hyacinth involving T. ressei cellulase and Z. mobilis*

SSF trials of wild grass and water hyacinth were carried out to determine the efficacy of the hexose utilizing bacteria, *Z. mobilis* with that of *S. cerevisiae* in terms of ethanol concentration achieved. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone

(0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 0.6 mL of *T. ressei* cellulase (9.8 U $mg^{-1}$, 0.82 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *Z. mobilis* (2.1 x $10^6$ cells $mL^{-1}$) was added to each flask for fermentation. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.4 SSF of wild grass and water hyacinth involving recombinant GH5 cellulase and Z. mobilis*

SSF trials of wild grass and water hyacinth were accomplished employing recombinant GH5 cellulase and *Z. mobilis* to compare the efficiency of the bacterial cellulase to that of fungal cellulase by the detection of reducing sugar formed with its utilization by the bacterial bioethanol producer. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of sodium acetate buffer (pH 4.3, 20 mM) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 1 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *Z. mobilis* (2.1 x $10^6$ cells $mL^{-1}$) was added to each flask for fermentation. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at

every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.5 SSF of wild grass and water hyacinth involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and C. shehatae*

As wild grass and water hyacinth contains substantial quantity of hemicellulose, SSF trials were employed on these substrates involving the recombinant *C. thermocellum* GH43 hemicellulase (α-L-arabinofuranosidase) for saccharification and the pentose utilizing yeast, *C. shehatae* for bioethanol production. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in two independent 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 1 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added to each flask for fermentation. Both the enzyme and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.6 SSF of wild grass and water hyacinth involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and Z. mobilis*

Further, SSF experiments of wild grass and water hyacinth were done

involving a mixed enzyme system comprising of recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) for the improved saccharification of complex cellulose and hemicellulose along with *Z. mobilis* for bioethanol production. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of sodium acetate buffer (pH 5.0, 20 mM) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 0.5 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) along with 0.5 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added to each flask for hydrolysis. 1 mL of *Z. mobilis* (2.1 x $10^6$ cells $mL^{-1}$) was added to each flask for fermentation. The enzymes and the fermentative microbe were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *4.2.5.7 SSF of wild grass and water hyacinth involving GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), S. cerevisiae and C. shehatae*

Finally, SSF trials of wild grass and water hyacinth were carried out involving mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)] and mixed culture (*S. cerevisiae, C. shehatae*) system for improved saccharification and efficient bioethanol production. One gram each of mixed MAA and organosolv pretreated wild grass and water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.0) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone

(0.1%, w $v^{-1}$) in two separate 250 mL Erlenmeyer flasks. Each medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 0.5 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) along with 0.5 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added to each flask for hydrolysis. 0.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) along with 0.5 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added to each flask for fermentation. The enzymes and the fermentative microbes were added at the same time. The fermentation was carried out at 30°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

### 4.2.6 Analytical methods

#### *4.2.6.1 Structural carbohydrates estimation*

The structural carbohydrates like cellulose, hemicellulose and lignin of untreated and mixed MAA with organosolv pretreated of two substrates, wild grass and water hyacinth were estimated as described in Chapter 3, Section 3.2.11.1.

#### *4.2.6.2 Measurement of cell growth during SSF*

The cell growth of the fermentative microbes, *S. cerevisiae* and *C. shehatae* were measured as described in Chapter 3, Section 3.2.11.2.

#### *4.2.6.3 Trichoderma reesei cellulase assay*

The *Trichoderma reesei* cellulase assay was performed by incubating the enzyme (10 µL) in a 100 µL reaction mixture containing 1% (w $v^{-1}$) CMC in 20 mM

sodium acetate buffer (pH 5.4) at 50°C for 10 min. The mixture was assessed for the released reducing sugars as reported earlier (Nelson, 1944; Somogyi, 1945). The amount of released reducing sugar was used to determine the enzyme activity. To 100 µL of reaction mixture containing the released reducing sugar, 100 µL of reagent D (as described in Chapter 2, Section 2.2.12.3) was added. The solutions were mixed and heated for 20 min in the boiling water bath. After 20 min of boiling, the solution was ice cooled and 100 µL of reagent C (as described in Chapter 2, Section 2.2.12.3) was added. The colour developed rapidly with the evolution of carbon dioxide. The mixture was diluted by adding 700 µL water to make up the volume to 1 mL and the optical density (OD) was measured at 500 nm on a UV-Visible spectrophotometer (Varian, Cary 100 Bio). D-glucose in the range of 50-500 µg $mL^{-1}$ was used for generating the standard plot as described earlier in Chapter 2, Section 2.2.12.4.

### *4.2.6.4 Calculation of enzyme activity of T. reesei cellulase*

The activity of the enzyme was expressed as U $mL^{-1}$ and the specific activity as U $mg^{-1}$ of protein. One unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (glucose) per min. The enzyme activity of *T. reesei* cellulase was calculated as described below,

$$\text{Enzyme activity (U mL}^{-1}) = \frac{\Delta A_{500} \times C \times V}{180 \times t \times v} = (\mu \text{ mole min}^{-1} \text{ mL}^{-1})$$

where,

$\Delta A_{500}$ = change in OD of the sample at 500 nm
C = 1 OD equivalent is D-glucose concentration from standard plot (µg $mL^{-1}$)
V = volume of the reaction mixture (mL)
t = time of reaction (min)
180 = molecular weight of D-glucose
v = volume of the enzyme taken in assay (mL) for reducing sugar estimation.

#### *4.2.6.5 Recombinant GH5 cellulase assay*

The reducing sugar estimation and recombinant GH5 cellulase assay was performed as described earlier in Chapter 2, Section 2.2.12.1.

#### *4.2.6.6 Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay*

The reducing sugar estimation and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay was performed as described earlier in Chapter 2, Section 2.2.12.2.

#### *4.2.6.7 Protein content determination*

The protein content was determined as described earlier in Chapter 2, Section 2.2.12.6.

#### *4.2.6.8 Ethanol content determination by Gas chromatography and Dichromate method*

The ethanol obtained from SSF experiments were estimated by Gas chromatography (GC) and Dichromate assay as described earlier in Chapter 3, Section 3.2.11.8.

#### *4.2.6.9 Determination of ethanol yield*

The ethanol yield was calculated as described earlier in Chapter 3, Section 3.2.11.9.

### 4.3 Results and Discussion

The techno-economic feasibility of an efficient SSF process relies on the enhanced saccharification of complex lignocellulosic substrates by efficient hydrolytic enzymes that can lead to improved bioethanol production when subjected to fermentation by microbes. The structural carbohydrate composition of wild grass (*A. hymenoides*) showed more amount of cellulose (51.70%, w $w^{-1}$) followed by hemicellulose (30.92% w $w^{-1}$) and lignin (18.71% w $w^{-1}$) (Table 4.3.1) whereas water hyacinth revealed greater amount of hemicellulose (44.52%, w $w^{-1}$) followed by cellulose (30.07%, w $w^{-1}$) and lignin (29.40%, w $w^{-1}$) (Table 4.3.1) suggesting wild grass and water hyacinth as the potential substrates for SSF mediated bioethanol production.

**Table 4.3.1** Cellulose, hemicellulose and lignin content (%, w $w^{-1}$) of untreated and mixed pretreated wild grass and water hyacinth.

| Substrate | Type of pretreatment | Cellulose (%, w $w^{-1}$)* | Hemicellulose (%, w $w^{-1}$)* | Lignin (%, w $w^{-1}$)* |
|---|---|---|---|---|
| Wild grass (*A. hymenoides*) | Untreated | 51.70 ± 0.36 | 30.92 ± 0.26 | 18.71 ± 0.27 |
| | MAA† + Organosolv | 46.30 ± 0.25 | 26.50 ± 0.28 | 15.20 ± 0.51 |
| Water hyacinth (*E. crassipes*) | Untreated | 30.07 ± 0.24 | 44.52 ± 0.45 | 29.40 ± 0.32 |
| | MAA† + Organosolv | 26.10 ± 0.23 | 33.20 ± 0.29 | 15.64 ± 0.21 |

† Microwave assisted alkali **values are mean ± SE (n=3)*

The mixed MAA and organosolv pretreatments of wild grass gave cellulose (46.30%), hemicellulose (26.50%) and lignin (15.20%) (Table 4.3.1). The mixed MAA and organosolv pretreatments of water hyacinth contributed cellulose (26.10%), hemicellulose (33.20%) and lignin (15.64%) (Table 4.3.1). The SSF trials performed on mixed pretreated wild grass and water hyacinth involving different combinations

of hydrolytic enzymes and fermentative microbes provided improved reducing sugar concentration and enhanced bioethanol production.

### 4.3.1 Simultaneous saccharification and fermentation of wild grass

The results of various SSF experiments performed on mixed microwave assisted alkali (MAA) and organosolv pretreated 1% (w $v^{-1}$) wild grass involving different hydrolytic enzymes and fermentative microbes are presented in Table 4.3.2.

**Table 4.3.2** SSF combination of mixed pretreated 1% (w $v^{-1}$) wild grass involving different hydrolytic enzymes and fermentative microbes.

| SSF combination | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|
| *T. reesei* + *S. cerevisiae* | 1.22 ± 0.04 | 0.61 ± 0.01 | 0.083 |
| GH5 + *S. cerevisiae* | 1.26 ± 0.06 | 0.67 ± 0.04 | 0.092 |
| *T. reesei* + *Z. mobilis* | 1.30 ± 0.02 | 0.82 ± 0.03 | 0.113 |
| GH5 + *Z. mobilis* | 1.36 ± 0.05 | 0.94 ± 0.07 | 0.129 |
| GH43 + *C. shehatae* | 0.49 ± 0.06 | 0.40 ± 0.04 | 0.054 |
| GH5 + GH43 + *Z. mobilis* | 1.60 ± 0.01 | 1.29 ± 0.01 | 0.177 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* | 1.70 ± 0.09 | 1.50 ± 0.06 | 0.206 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

#### *4.3.1.1 SSF involving T. ressei cellulase and S. cerevisiae*

The simultaneous saccharification and fermentation trial of 1% (w $v^{-1}$) mixed pretreated wild grass involving *T. ressei* cellulase and *S. cerevisiae* exhibited divergent phases in terms of growth of fermentative microbes, specific activity of enzyme, release of utilizable sugar and rate of ethanol formation. In the initial time

interval from 0 - 24 h, there was a continuous increase in ethanol titre and cell OD from the initial 0.39 to 0.56 (Fig. 4.3.1).

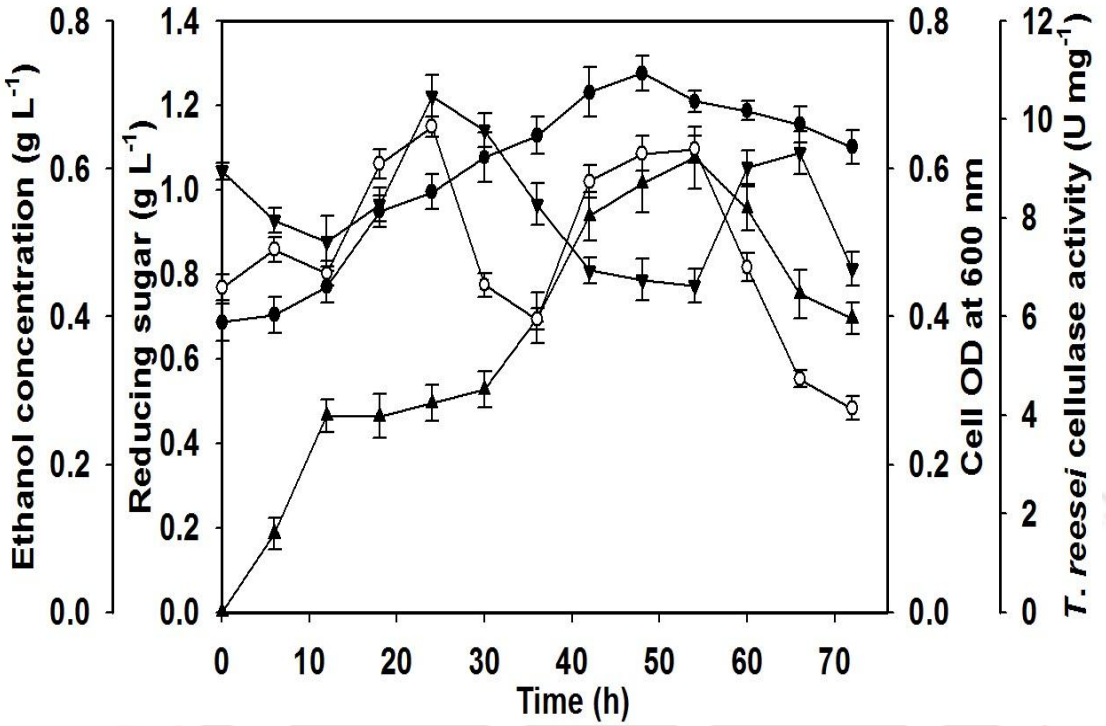


**Fig. 4.3.1** SSF profile of 1% (w $v^{-1}$) wild grass using *T. reesei* cellulase, *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of *T. reesei* cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

Simultaneously, a decrease in reducing sugar level was also observed in the initial 12 h (0.87 g $L^{-1}$) but thereafter, an increment was observed with a maximum reducing sugar concentration of 1.22 g $L^{-1}$ at 24 h (Table 4.3.2, Fig. 4.3.1). In the later hours, the cell OD continued to increase exponentially reaching a maximum of 0.72 at 48 h and a maximum ethanol titre of 0.61 g $L^{-1}$ was achieved at 54 h of SSF (Table 4.3.2, Fig. 4.3.1). The ethanol yield (g of ethanol g of substrate$^{-1}$) obtained was 0.083 (Table 4.3.2). However, the reducing sugar showed a continuous decline in the

fermentative medium till 54 h but then, a slight increment (1.08 g $L^{-1}$) was observed at 66 h. A decrease in ethanol concentration reaching a minimum level of 0.39 g $L^{-1}$ at 72 h with a cell OD of 0.62 was observed at 66 h which connoted the attainment of senescence (Fig. 4.3.1). In the initial 18 h, the specific activity of fungal cellulase was 9.8 U $mg^{-1}$ and later it declined with the progress of SSF (Fig. 4.3.1).

### *4.3.1.2 SSF of wild grass involving GH5 cellulase and S. cerevisiae*

The SSF profile of wild grass involving GH5 cellulase and *S. cerevisiae* displayed three separate phases in terms of growth of *S. cerevisiae*, specific activity of enzyme, release of utilizable sugar and rate of ethanol formation (Fig. 4.3.2). In the first phase, there was a descent in reducing sugar after an initial accumulation phase. A diminution in sugar content was found to be concomitant with simultaneous rise in growth and ethanol concentration.

In the second phase of fermentation, the bioethanol producer was in its exponential phase reaching a maximum OD of 0.8 at 66 h (Fig. 4.3.2). A reduction in ethanol concentration was observed with the significant accumulation of reducing sugar (1.26 g $L^{-1}$) (Table 4.3.2, Fig. 4.3.2). The decline in specific enzyme of GH5 cellulase activity between 24 h and 30 h might be due to the increase in reducing sugar concentration that lowered the enzyme activity through involvement of feedback inhibition (Fig. 4.3.2). The final phase of SSF was marked with a sharp rise in ethanol titre attaining a maximum of 0.67 g $L^{-1}$ and yield of 0.092 g of ethanol g of $substrate^{-1}$ (Table 4.3.2, Fig. 4.3.2). This phase presented a continuous sugar withdrawl from the broth. There was a fall in cell OD after 66 h indicating attainment of senescence.

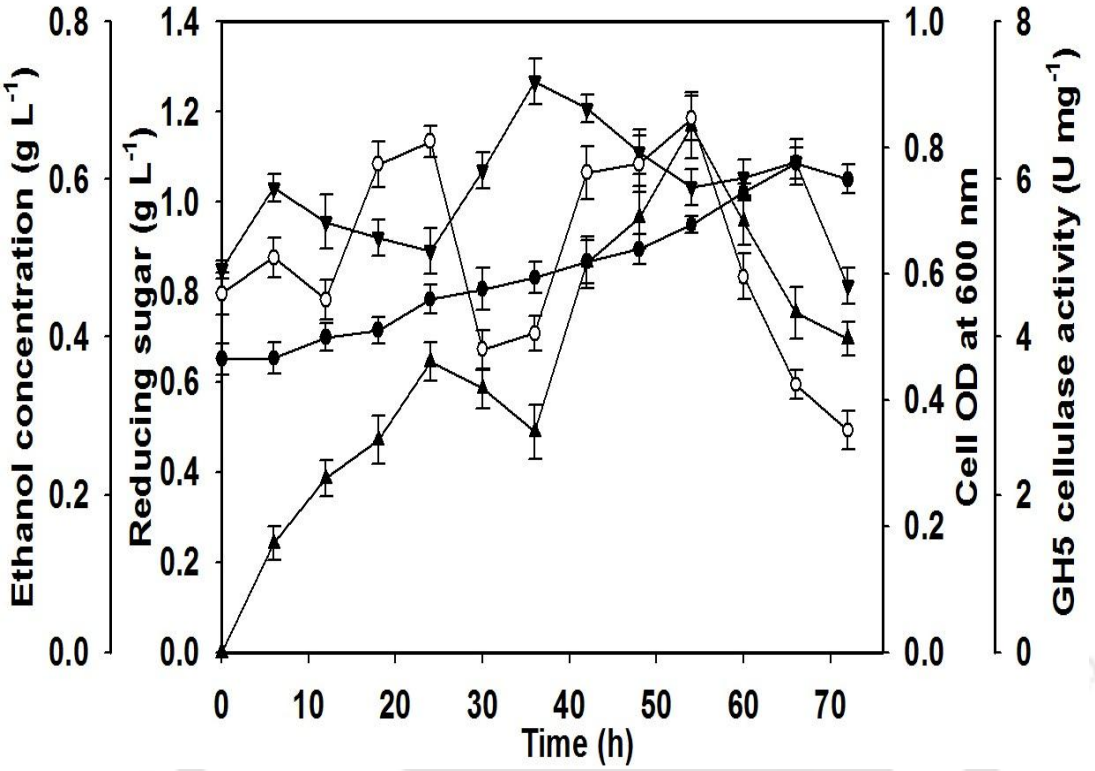


**Fig. 4.3.2** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

The dynamic profile of reducing sugar exhibited a sinusoidal behaviour ascribing to a balance between the rate of saccharification with sugar utilization for growth and ethanol formation. It was found that the reducing sugar concentration rise was related with drop in enzyme activity and vice-versa. A 1.1-fold increment in ethanol titre was observed employing GH5 cellulase on wild grass as compared to *T. reesei* cellulase with *S. cerevisiae.*

### *4.3.1.3 SSF of wild grass involving T. reesei cellulase and Z. mobilis*

The hexose utilizing bacterium, *Z. mobilis* exhibited a log growth phase from initial stages of fermentation reaching cell OD of 0.64 at 30 h and then, a maximum cell biomass (OD) of 0.83 at 60 h (Fig. 4.3.3). During initial stage of SSF, the sugar concentration in the fermentation medium increased to 0.96 g $L^{-1}$ (12 h) from initial 0.78 g $L^{-1}$ (0 h) (Fig. 4.3.3).

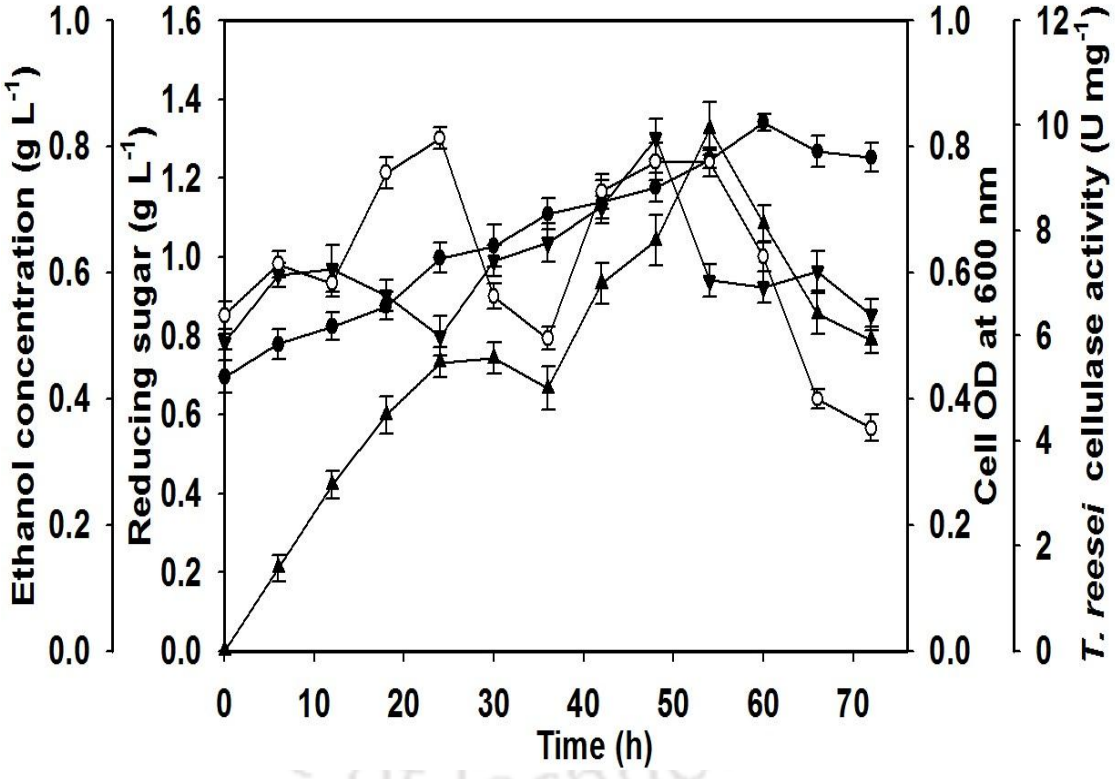


**Fig. 4.3.3** SSF profile of 1% (w $v^{-1}$) wild grass using *T. reesei* cellulase, *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of *T. reesei* cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

Later a steep decline in reducing sugar concentration (0.79 g $L^{-1}$) at 24 h depicted a descent in the rate of hydrolysis (Fig. 4.3.3). A maximum specific activity

of *T. reesei* cellulase (9.7 U $mg^{-1}$) was obtained at 24 h of SSF (Fig. 4.3.3). Thereafter, with the steady increase in reducing sugar concentration, there occurred a feedback inhibition that might have caused a sharp decline of specific activity between 24 -36 h. After 36 h, a regain of specific activity of *T. reesei* cellulase was noticed with an exponential increase in cell OD implying the utilization of the reducing sugar by *Z. mobilis.*

The later stages of fermentation showed an inverse association between rates of hydrolysis, sugar formation with subsequent utilization by *Z. mobilis* for ethanol formation. The maximum ethanol titre was attained at 54 h (0.82 g $L^{-1}$) and an yield of 0.113 (g of ethanol g of $substrate^{-1}$) (Table 4.3.2, Fig. 4.3.3). A maximum reducing sugar concentration (1.30 g $L^{-1}$) was obtained at 48 h (Table 4.3.2, Fig. 4.3.3). Thereafter, a gradual decrease in ethanol concentration along with the sugar level after 60 h depicted the decline phase of the fermentative microbe.

#### *4.3.1.4 SSF of wild grass involving GH5 cellulase and Z. mobilis*

In the SSF trial involving recombinant GH5 cellulase along with *Z. mobilis*, the cell OD of *Z. mobilis* continued to grow from the initial value of 0.41 at 0 h to the maximum OD of 0.86 at 66 h (Fig. 4.3.4). A biphasic fermentation kinetics was observed in terms of ethanol titre. An inverse relationship was observed for decrease in sugar concentration with simultaneous increase in growth and ethanol concentration for the first phase. In the second phase of fermentation, a substantial accumulation of reducing sugar (1.36 g $L^{-1}$) at 42 h in the broth was observed (Table 4.3.2, Fig. 4.3.4). A decrease in ethanol concentration was observed at 36 h (0.32 g $L^{-1}$) form 0.36 g $L^{-1}$ at 30 h, which later increased to 0.57 g $L^{-1}$ at 48 h. (Fig. 4.3.4).

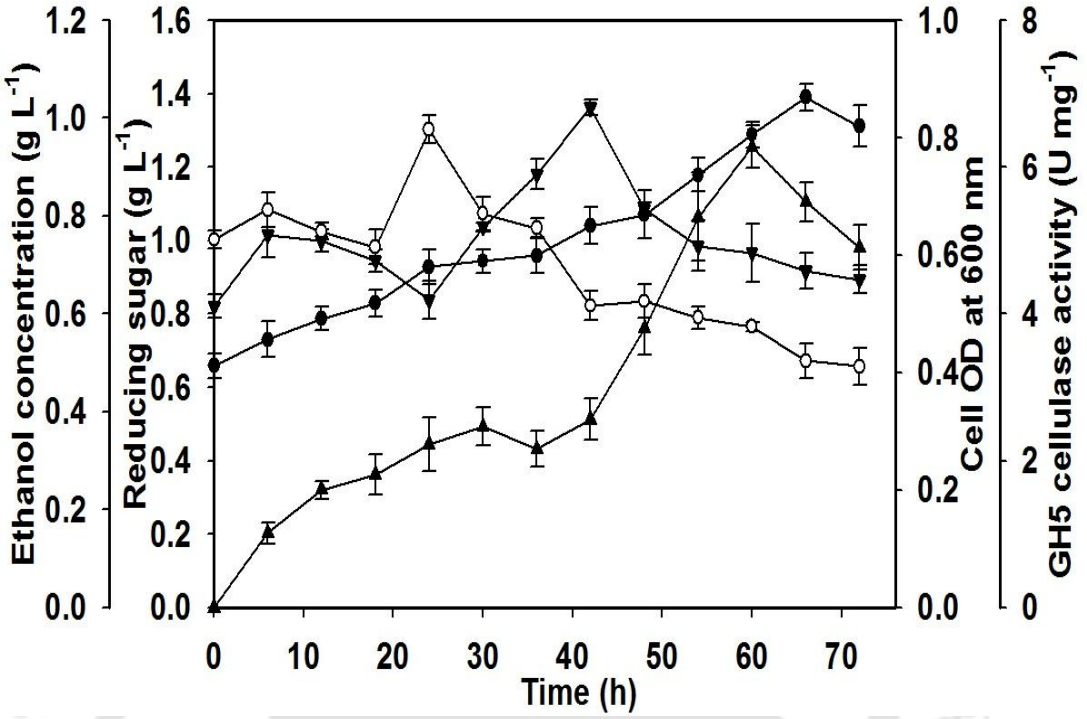


**Fig. 4.3.4** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

The third and last phase of fermentation was marked with a rise in ethanol concentration attaining a maximum titre of 0.94 g $L^{-1}$ at 66 h and a yield of 0.129 g of ethanol g of $substrate^{-1}$ (Table 4.3.1, Fig. 4.3.4). This phase showed a continuous decline in the sugar level reaching a minimum level of 0.89 g $L^{-1}$. There was a decrease in cell OD after 66 h indicating attainment of senescence. It was found that the specific enzyme activity attained its maximum value of 6.5 U $mg^{-1}$ at 24 h and subsequently decreased to a minimum level of 3.3 U $mg^{-1}$ at 72 h in due course of SSF. A 1.14-fold increment in ethanol titre was observed employing GH5 cellulase as compared to *T. reesei* cellulase with *Z. mobilis*.

### *4.3.1.5 SSF of wild grass involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and C. shehatae*

The SSF profile of wild grass involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* exhibited a complex interplay among growth of fermentative microbe and rate of ethanol formation. For the initial phase, the organism continued to grow in log phase with the initial OD of 0.36 at 0 h to maximum of 0.61 by the end of 60 h (Fig. 4.3.5).

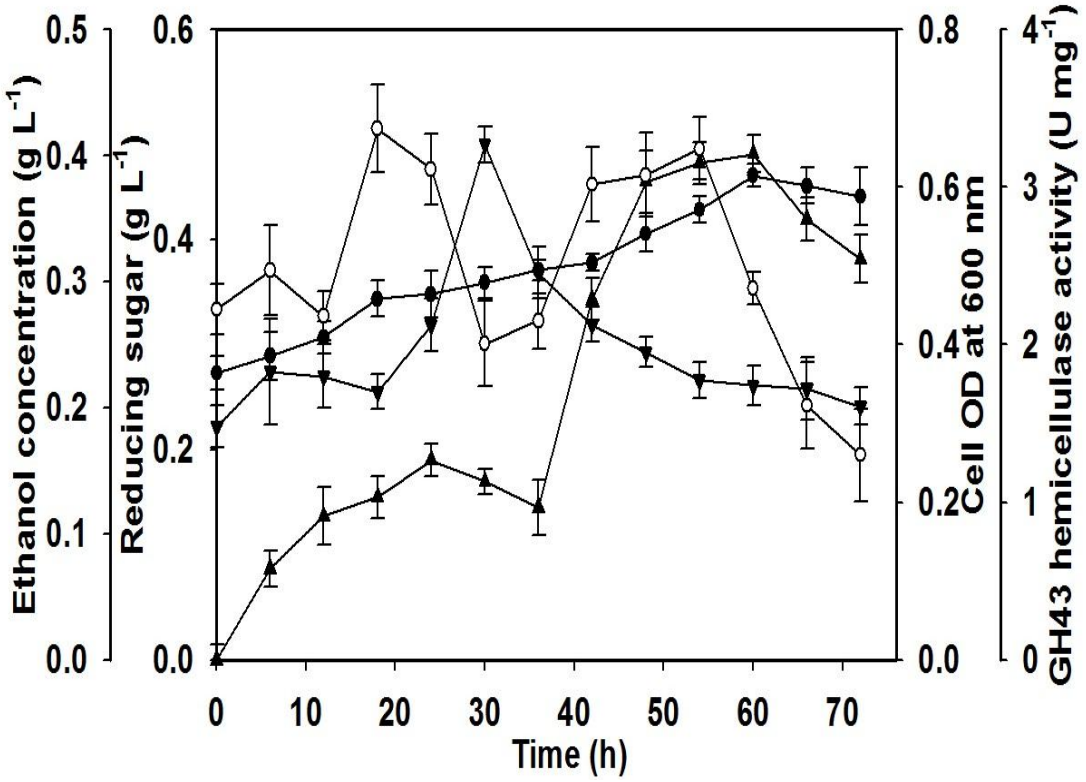


**Fig. 4.3.5** SSF profile of 1% (w $v^{-1}$) wild grass using GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

A slight increase of reducing sugar from initial 0.22 to 0.27 g $L^{-1}$ with a steep decline to 0.25 g $L^{-1}$ by the end of 18 h was observed (Fig. 4.3.5). The ethanol concentration increased steadily reaching 0.16 g $L^{-1}$ at 24 h. However, post saccharification period between 24-36 h showed a decline in ethanol concentration (0.12 g $L^{-1}$) (Fig. 4.3.5). But later stages of fermentation exhibited an inverse relationship between rates of hydrolysis, sugar formation with subsequent utilization by C. *shehatae*. A maximum reducing sugar concentration of 0.49 g $L^{-1}$ was obtained during 30 h of SSF (Table 4.3.2, Fig. 4.3.5). The increase in ethanol titre and cell biomass was observed with the maximum of 0.40 g $L^{-1}$ and 0.61 respectively, at 60 h with subsequent decline in reducing sugar (0.26 g $L^{-1}$) at 60 h (Table 4.3.2, Fig. 4.3.5). The ethanol yield (g of ethanol g of substrate$^{-1}$) obtained was 0.054 (Table 4.3.2). The specific activity of GH43 hemicellulase showed a maximum value of 3.4 U $mg^{-1}$ at 36 h and thereby, a decrease (Fig. 4.3.5). After 60 h of SSF, a decrease in both sugar and ethanol concentration was observed.

#### *4.3.1.6 SSF of wild grass involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and Z. mobilis*

The delicate balance in terms of growth of *Z. mobilis*, specific activities of enzymes, release of utilizable sugar and in turn, ethanol formation was depicted in the SSF profile of wild grass utilizing both recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) for saccharification (Fig. 4.3.6). In the first phase, there was a drop in reducing sugar concentration. Simultaneously a substantial increase in cell OD and ethanol concentration of 0.49 and 0.37 g $L^{-1}$, respectively was achieved at the end of 18 h (Fig. 4.3.6). In the next phase of fermentation, *Z. mobilis*

continued to be in its exponential phase reaching a maximum OD of 0.84 at 66 h (Fig. 4.3.6). The incessant increase in ethanol concentration was observed till 54 h with a maximum titre of 1.29 g $L^{-1}$ and yield of 0.177 (g of ethanol g of substrate$^{-1}$) (Table 4.3.2, Fig. 4.3.6).

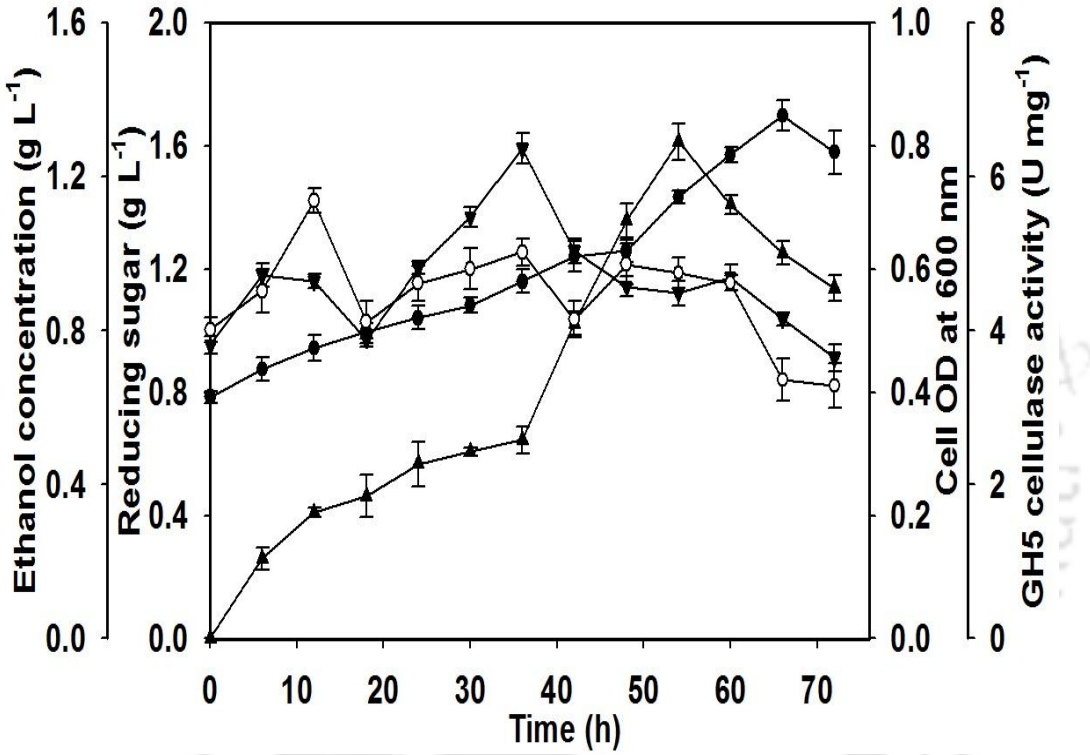


**Fig. 4.3.6** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.0; temperature 30°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

Interestingly, an increase in sugar level was depicted with the maximum value of 1.60 g $L^{-1}$ at 36 h and later steady decrease with a minimum of 0.9 g $L^{-1}$ during the 72 h (Table 4.3.2, Fig. 4.3.6). It was found that the reducing sugar concentration increment was associated with increase in specific activity of GH5 cellulase till 60 h.

Thereafter, a drop in GH5 cellulase activity with the simultaneous decrease in cell OD after 66 h showed the completion of SSF.

#### *4.3.1.7 SSF of wild grass involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), S. cerevisiae and C. shehatae*

The dynamic profile of SSF involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* for ethanol production from mixed pretreated 1% (w $v^{-1}$) wild grass in shake flask level is exhibited in Fig. 4.3.7.

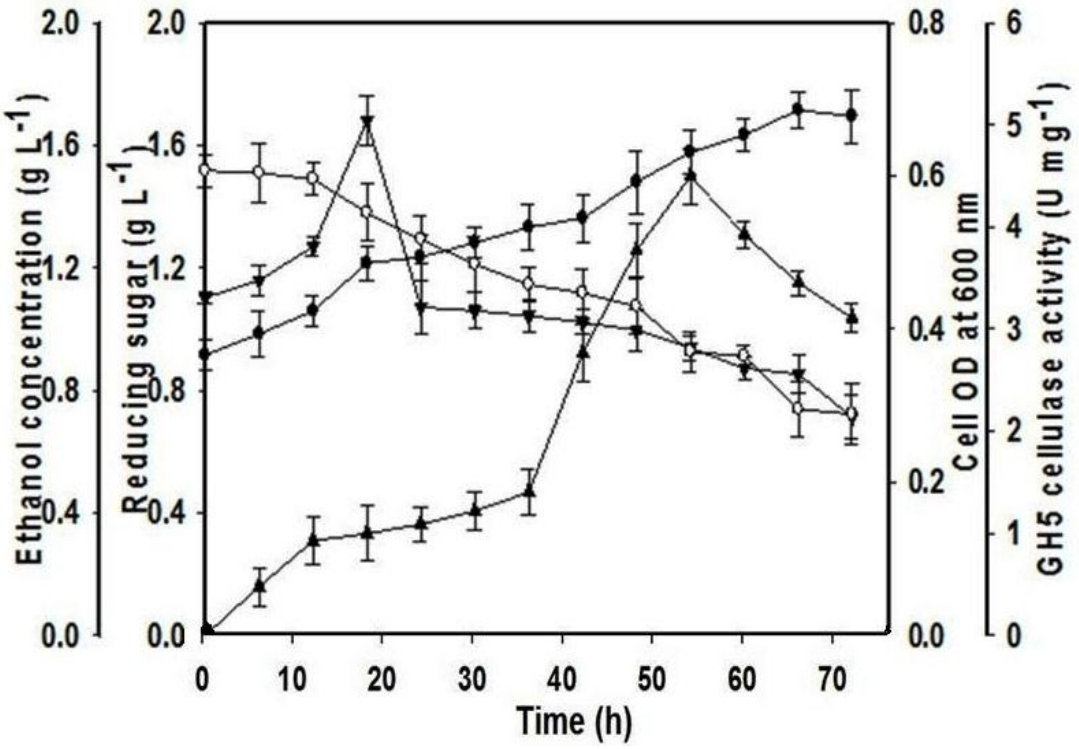


**Fig. 4.3.7** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.0; temperature 30°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited no lag phase in their growth with steady rise till 66 h and small decline thereafter (Fig. 4.3.7). The growth-associated ethanol formation was initiated from 12 h of SSF with a gradual escalation till 36 h after which a sharp augmentation was observed till 54 h (Fig. 4.3.7). The maximum ethanol titre accomplished was 1.50 g $L^{-1}$ (Table 4.3.2, Fig. 4.3.7) with a yield of 0.206 (g of ethanol g of substrate$^{-1}$). Thereafter, a decrease in ethanol production was witnessed. The preliminary phase of SSF signified an accumulation of available sugars till 18 h. The maximum reducing sugar concentration was 1.70 g $L^{-1}$ (Table 4.3.2, Fig. 4.3.7). The activities of both the recombinant enzymes decreased with advancement in fermentation. As wild grass contains more cellulose, the dynamic profile of only recombinant GH5 cellulase is shown in Fig. 4.3.7. Interestingly, the growth of fermentative microbes and ethanol production depicted an inverse relationship with hydrolytic enzyme activity and, in turn, the released reducing sugars representing the fact of sugar utilization by the bioethanol producers for growth and ethanol formation (Fig. 4.3.7).

*S. cerevisiae* provides a better choice for the production of ethanol in the large scale cultivation, due to their inherent ability to utilize various substrates, high ethanol tolerance and its robustness to withstand range of metabolic inhibitions (Casey and Ingledew, 1986). *Z. mobilis* is also an equal competitor in the ethanol production with reduced overall cell biomass and enhanced ethanol productivity than *S. cerevisiae.* While, both *S. cerevisiae* and *Z. mobilis* are known to utilize hexoses predominantly, the key enzymes for ethanol fermentation: alcohol dehydrogenase and pyruvate decarboxylase was reported to be best expressed in *Z. mobilis* (Sprenger, 1996). Therefore, *Z. mobilis* was also used with the aim to achieve higher ethanol yield.

A 1.1-fold rise in ethanol concentration was obtained from GH5 cellulase on wild grass as compared to *T. reesei* cellulase with *S. cerevisiae*. Owing to greater ethanol tolerance and sugar utilization by *Z. mobilis*, a 1.15-fold rise in ethanol titre was obtained by GH5 cellulase as compared to *T. reesei* cellulase with *Z. mobilis*. The structural carbohydrate composition of wild grass revealed significant cellulose along with hemicellulose. To further improve the ethanol yield obtained by single enzyme-single culture system, utilization of large fraction of unused substrate (hemicellulose) for fermentation became necessary. Therefore, SSF experiments were set with a mixed enzyme system comprising of recombinant *C. thermocellum* GH5 cellulase and GH43 hemicellulase along with *Z. mobilis*. Although, SSF was carried out with mixed hydrolytic enzymes releasing both hexose and pentose sugars, but the absence of pentose utilizing microbe caused not much increase in ethanol titre. Therefore, a mixed culture system was employed along with mixed enzyme for better ethanol yield. The effective fermentation by *C. shehatae* along with *S. cerevisiae* increased the overall ethanol titre and yield, as the pentose sugars were utilized by *C. shehatae*. The maximum ethanol titre obtained was 1.50 g $L^{-1}$ with a yield of 0.206 (g of ethanol g of pretreated substrate$^{-1}$). A 2.3-3.2 fold increment in ethanol titre and 2.2-3.8 fold rise in ethanol yield was observed using a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]- mixed culture (*S. cerevisiae*, *C. shehatae*) system as compared with single enzyme-single culture systems.

The ethanol titre values attained in our study are analogous with that reported in literature. An ethanol yield of 0.09 (g $g^{-1}$) in a SSF process from paper sludge waste using *Zymomonas mobilis* was reported by Nakamura *et al.*, (2008). The recombinant cellulase from *C. thermocellum* presented an ethanol concentration of 1.4 g $L^{-1}$ from

1% (w $v^{-1}$) Jamun (*Syzygium cumini*) leafy biomass (Mutreja *et al.*, 2011). An ethanol titre of 0.47 g $L^{-1}$ was obtained from 1% (w $v^{-1}$) bermuda grass employing commercial cellulase and *S. cerevisiae* (Li *et al.*, 2009).

### 4.3.2 Simultaneous saccharification and fermentation of water hyacinth

The results of various SSF trials performed on mixed microwave assisted alkali (MAA) and organosolv pretreated 1% (w $v^{-1}$) water hyacinth involving different hydrolytic enzymes and fermentative microbes are presented in Table 4.3.3.

#### *4.3.2.1 SSF of water hyacinth involving T. ressei cellulase and S. cerevisiae*

As water hyacinth contained a considerable amount of cellulose, SSF trials were employed on 1% (w $v^{-1}$) substrate involving *T. ressei* cellulase and *S. cerevisiae.* Different stages in the growth of fermentative microbes, specific enzyme activity, release of utilizable sugar and ethanol formation was exhibited by the SSF profile.

**Table 4.3.3** SSF combination of mixed pretreated 1% (w $v^{-1}$) water hyacinth involving different hydrolytic enzymes and fermentative microbes.

| SSF combination | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|
| *T. reesei* + *S. cerevisiae* | 0.47 ± 0.04 | 0.37 ± 0.03 | 0.062 |
| GH5 + *S. cerevisiae* | 0.51 ± 0.07 | 0.39 ± 0.01 | 0.066 |
| *T. reesei* + *Z. mobilis* | 0.58 ± 0.01 | 0.41 ± 0.05 | 0.069 |
| GH5 + *Z. mobilis* | 0.64 ± 0.02 | 0.47 ± 0.03 | 0.079 |
| GH43 + *C. shehatae* | 0.74 ± 0.06 | 0.55 ± 0.08 | 0.092 |
| GH5 + GH43 + *Z. mobilis* | 1.13 ± 0.05 | 0.82 ± 0.06 | 0.138 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* | 1.20 ± 0.08 | 1.00 ± 0.02 | 0.168 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

In the initial phase of fermentation, till 24 h, there was a continuous increase in ethanol concentration and cell OD to 0.21 g $L^{-1}$ and 0.42, respectively (Fig. 4.3.8). An accumulation of reducing sugar with maximum of 0.47 g $L^{-1}$ (Table 4.3.3) at 24 h was observed from the initial decrease between 6 to 12 h (Fig 4.3.8). The later phase of SSF depicted a biphasic increment of ethanol titre with 0.21 g $L^{-1}$ at 30 h and a maximum of 0.37 g $L^{-1}$ at 54 h with a yield of 0.062 (g of ethanol g of substrate$^{-1}$) (Table 4.3.3, Fig. 4.3.8).

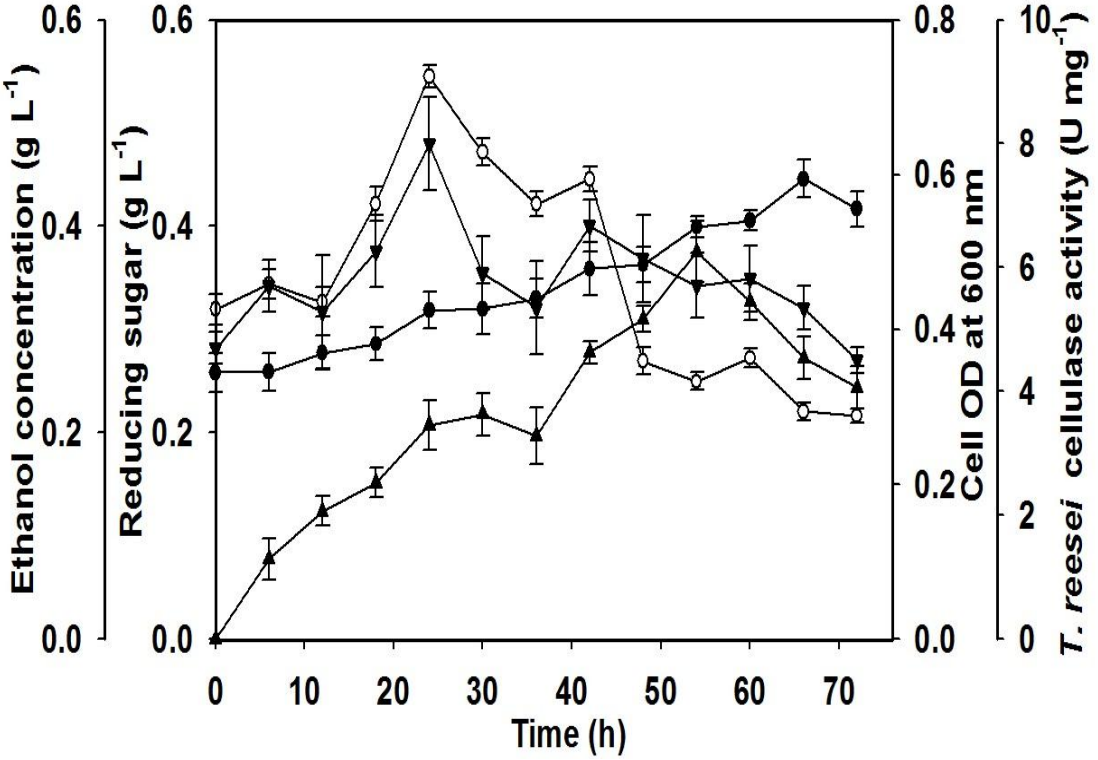


**Fig. 4.3.8** SSF profile of 1% (w $v^{-1}$) water hyacinth using *T. reesei* cellulase, *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of *T. reesei* cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

Interestingly an increase in reducing sugar of 0.40 g $L^{-1}$ was observed at 42 h which later showed an exponential decline to a minimum 0.27 g $L^{-1}$ at 72 h due to its consumption by *S. cerevisiae*, as a sole carbon source for effective fermentation (Fig. 4.3.8). The specific activity of *T. reesei* cellulase showed an exponential increase of 9.7 U $mg^{-1}$ at 24 h (Fig. 4.3.8). However, a decline of specific activity was observed in the later stage of SSF. The maximum cell OD of 0.59 was depicted at 66 h declining to 0.55 at 72 h (Fig 4.3.8).

### *4.3.2.2 SSF of water hyacinth involving GH5 cellulase and S. cerevisiae*

SSF of water hyacinth involving GH5 cellulase and *S. cerevisiae* exhibited an exponential growth pattern with a maximum cell OD of 0.68 at 66 h (Fig. 4.3.9). The enzyme activity profile displayed a sinusoidal behaviour. There was a decrease in reducing sugar concentration from 0.41 g $L^{-1}$ at 6 h to 0.35 g $L^{-1}$ at 24 h (Fig. 4.3.9). The sugar content substantially increased to 0.51 g $L^{-1}$ (Table 4.3.3, Fig. 4.3.9) and finally, experienced an exponential decrease to 0.41 g $L^{-1}$ at 60 h (Fig. 4.3.9). A maximum ethanol concentration of 0.39 g $L^{-1}$ was achieved at 42 h of fermentation with an ethanol yield of 0.066 g of ethanol g of $substrate^{-1}$ (Table 4.3.3, Fig. 4.3.9). The growth associated with ethanol production displayed an inverse relationship with reducing sugar consumption. The specific activity of GH5 cellulase showed a sinusoidal behaviour attributing to a gentle balance between the rates of saccharification for the release of reducing sugar.

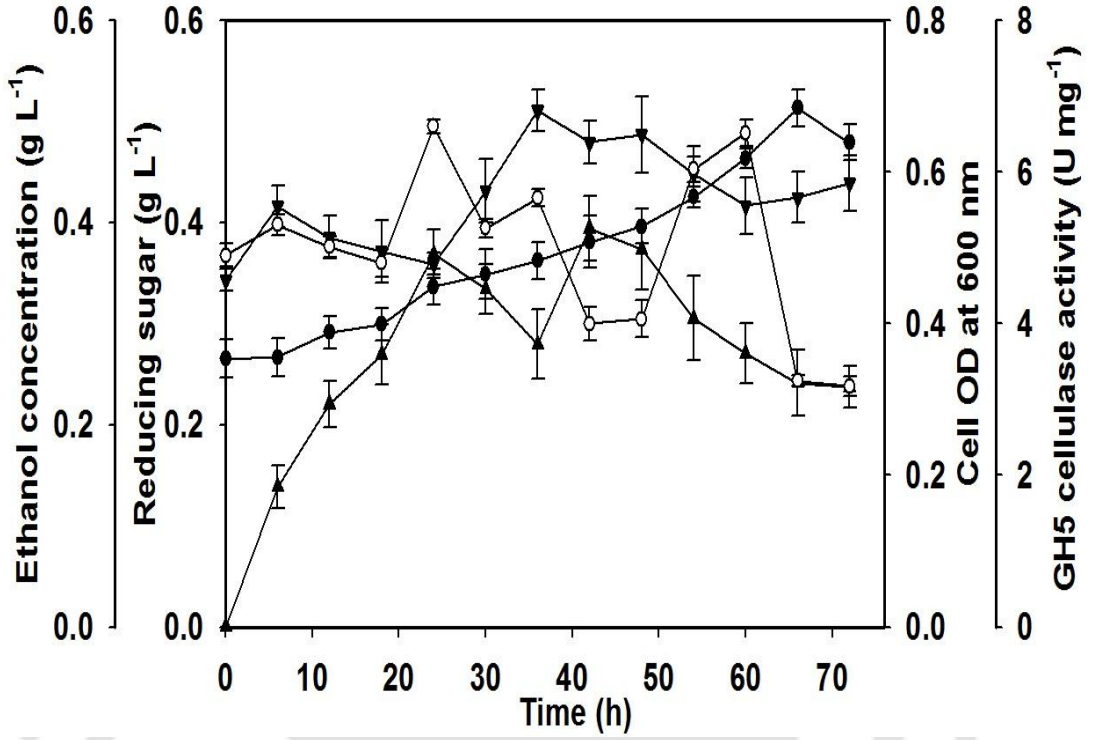


**Fig. 4.3.9** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH5 cellulase, *S. cerevisiae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

A 6.5% increase in ethanol yield was observed with the combination of recombinant bacterial GH5 cellulase (0.066 g $g^{-1}$ substrate) as compared with the combination of fungal *T. reesei* cellulase and *S. cerevisiae* (0.062 g $g^{-1}$ substrate) on mixed pretreated water hyacinth as described in Section 4.3.2.1

### *4.3.2.3 SSF of water hyacinth involving T. reesei cellulase and Z. mobilis*

SSF trials performed with *T. reesei* cellulase and *Z. mobilis* on water hyacinth (*E. crassipes*) showed a complex relationship within growth, sugar utilization and rate of ethanol formation with specific activity of *T. reesei* cellulase. The dynamic SSF profile showed an exponential log phase reaching the maximum OD of 0.75 at 54 h (Fig. 4.3.10).

A slight decline in ethanol concentration (0.29 g $L^{-1}$) was observed at 30 h. A considerable increase of ethanol titre with a maximum value of 0.41 g $L^{-1}$ and yield of 0.069 g of ethanol g of substrate$^{-1}$ was achieved at 54 h (Table 4.3.3, Fig. 4.3.10).

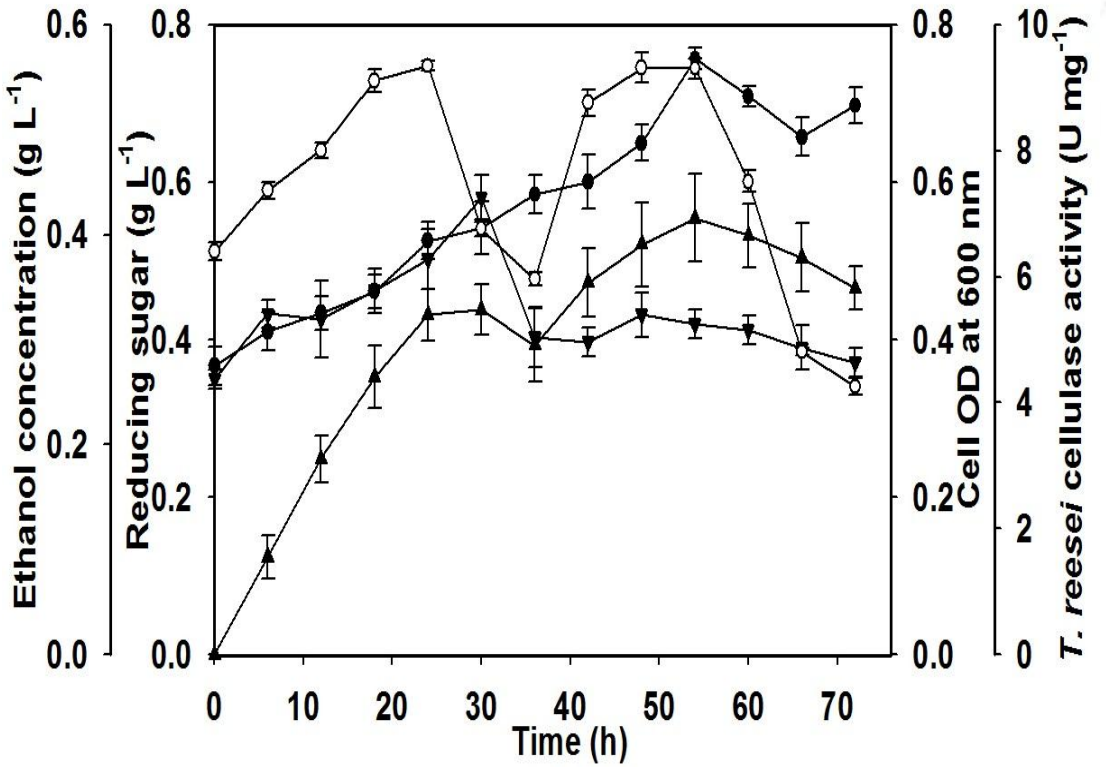


**Fig. 4.3.10** SSF profile of 1% (w $v^{-1}$) water hyacinth using *T. reesei* cellulase and *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of *T. reesei* cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

The reducing sugar continued to increase at regular interval with a slight decline from 0.43 g $L^{-1}$ (6 h) to 0.42 g $L^{-1}$ (12 h) and reaching a maximum (0.58 g $L^{-1}$) at 30 h (Table 4.3.3, Fig. 4.3.10). The specific activity showed a sinusoidal behaviour reaching a maximum of 9.34 U $mg^{-1}$ at 54 h and later declined to 4.2 U $mg^{-1}$ at 72 h (Fig. 4.3.10). No subsequent increase in cell biomass with a decline in ethanol concentration along with reducing sugar was observed after 54 h displaying the achievement of stationary phase.

### *4.3.2.4 SSF of water hyacinth involving GH5 cellulase and Z. mobilis*

The dynamic profile of SSF showed an increment in cell OD in sigmoidal manner achieving a maximum of 0.85 at 60 h of fermentation (Fig. 4.3.11). There was an initial increase in reducing sugar level from 0.38 g $L^{-1}$ (0 h) to 0.47 g $L^{-1}$ (6 h) with a marginal decrease at 24 h implying the utilization of sugar for growth and metabolism by fermentative microbe (Fig. 4.3.11). Simultaneously, a little increase in ethanol titer was observed in earlier phase which later increased exponentially till 30 h (0.22 g $L^{-1}$) (Fig. 4.3.11). After 30 h, a slight decrease in ethanol concentration was observed till 36 h which again retraced back to its exponential path with a maximum value of 0.47 g $L^{-1}$ at 60 h (Table 4.3.3, Figure 4.3.11). A maximum ethanol yield (g of ethanol g of substrate$^{-1}$) of 0.079 was obtained (Table 4.3.3). A maximum reducing sugar content of 0.64 g $L^{-1}$ was obtained at 48 h of SSF (Table 4.3.3, Fig. 4.3.11). After 60 h, a decline in cell OD and ethanol concentration depicted the achievement of decline phase.

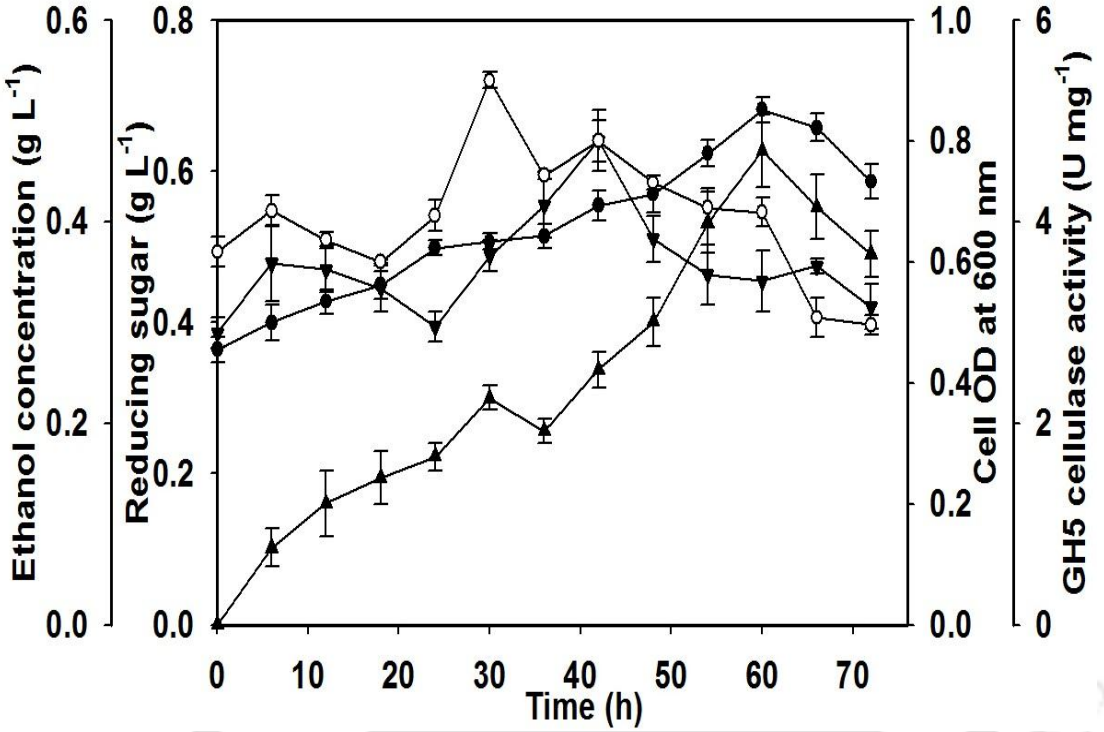


**Fig. 4.3.11** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH5 cellulase and *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

A 1.14-fold increment in ethanol titre was observed employing GH5 cellulase as compared with that from *T. reesei* cellulase along with *Z. mobilis.*

#### *4.3.2.5 SSF of water hyacinth involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and C. shehatae*

The first growth phase corresponded to an initial 18 h of fermentation with a cell OD of 0.74 in SSF trial involving mixed pretreated water hyacinth along with recombinant hemicellulase (GH43) (α-L-arabinofuranosidase) and *C. shehatae* (Fig. 4.3.12).

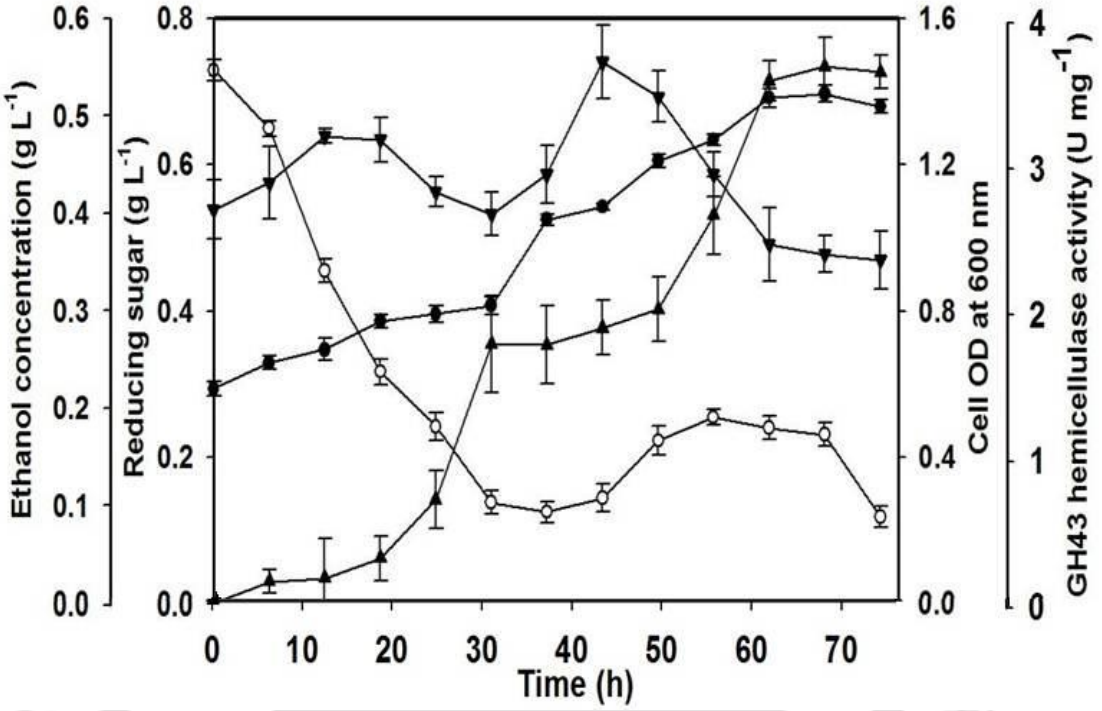


**Fig. 4.3.12** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 30°C and shaking 120 rpm. Values are mean ± SE (n=3).

During this phase, the sugar was utilized by the micro-organism for its growth. The second distinct growth phase was witnessed at 54 h of fermentation with a cell OD of 1.26. The cell biomass touched its peak value of 1.39 at 60 h, and thereby growth decreased, indicating senescence. A maximum ethanol concentration of 0.55 g $L^{-1}$ was achieved (Table 4.3.3, Fig. 4.3.12) at 66 h of fermentation. The ethanol yield (g of ethanol g of $substrate^{-1}$) obtained was 0.092 (Table 4.3.3). The reducing sugar profile showed a sinusoidal behaviour ascribing to balance between the rate of saccharification and ethanol formation. It is important to note that the increase in

reducing sugar concentration (0.74 g $L^{-1}$) was found to be concomitant with decrease in specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) and vice-versa.

### *4.3.2.6 SSF of water hyacinth involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and Z. mobilis*

The SSF involving recombinant cellulase (GH5) along with hemicellulase (GH43) and *Zymomonas mobilis* for fermentation exhibited a maximum biomass of 0.82 at 60 h (Fig. 4.3.13).

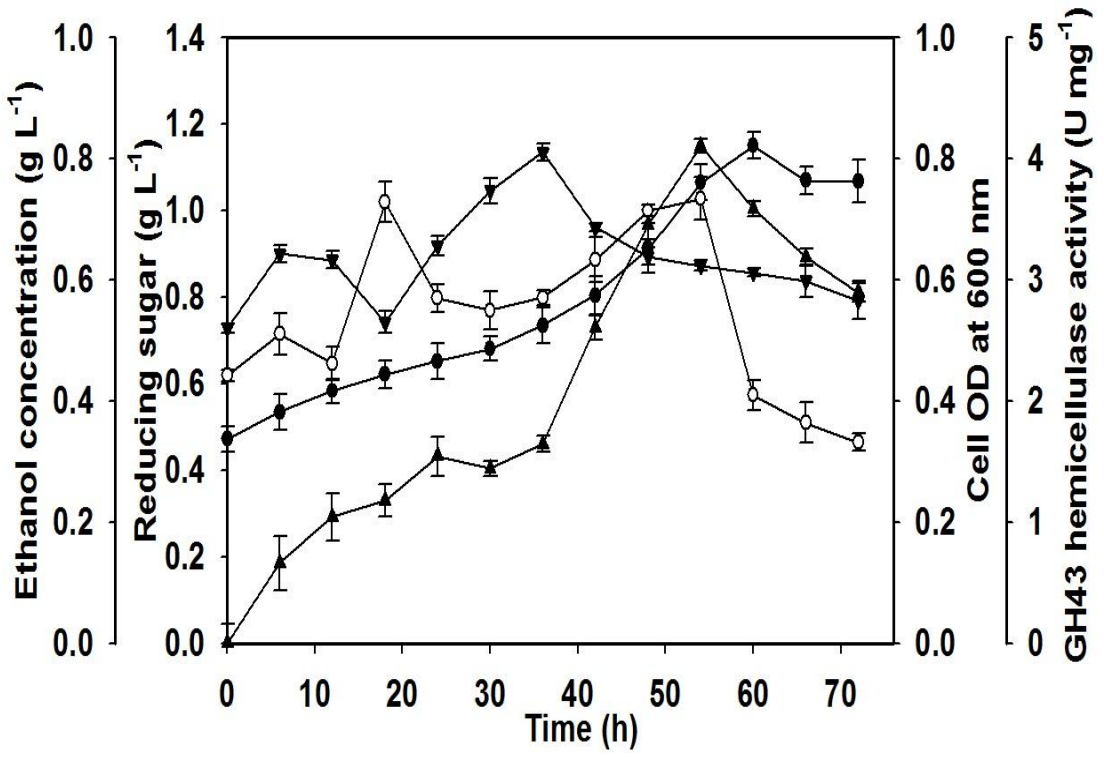


**Fig. 4.3.13** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.0; temperature 30°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

During this phase, the utilization of sugar that was produced from the hydrolysis of substrate by the enzymes was used by *Z. mobilis* for its growth and later in the production of bioethanol. The ethanol formation was proportional to the increase in cell biomass. A maximum ethanol titre of 0.82 g $L^{-1}$ was achieved (Table 4.3.3, Fig. 4.3.13) at 54 h of fermentation followed by a gradual decrease thereafter. The ethanol yield (g of ethanol g of substrate$^{-1}$) obtained was 0.138 (Table 4.3.3). Interestingly, the dynamic profile of reducing sugar showed an initial increase for first 6 h and then, a slight decrease at 18 h (0.74 g $L^{-1}$) (Fig. 4.3.13). With further progress of SSF the reducing sugar showed a remarkable increase reaching a maximum value of 1.13 g $L^{-1}$ at 36 h (Table 4.3.3, Fig. 4.3.13).

There was a concomitant decrease in total reducing sugar, with the increase of specific activity reaching maximum of 3.66 U $mg^{-1}$ at 54 h and then declined to 1.65 U $mg^{-1}$ at 72 h (Fig. 4.3.13). The growth remained constant after 66 h of SSF process, indicating achievement of stationary phase.

#### *4.3.2.7 SSF of water hyacinth involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), S. cerevisiae and C. shehatae*

The dynamic SSF profile involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* for ethanol production from mixed pretreated 1% (w $v^{-1}$) water hyacinth at shake flask level is depicted in Fig. 4.3.14. The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited negligible lag phase of growth with steady rise of cell OD till 60 h and then a slight decline (Fig. 4.3.14).

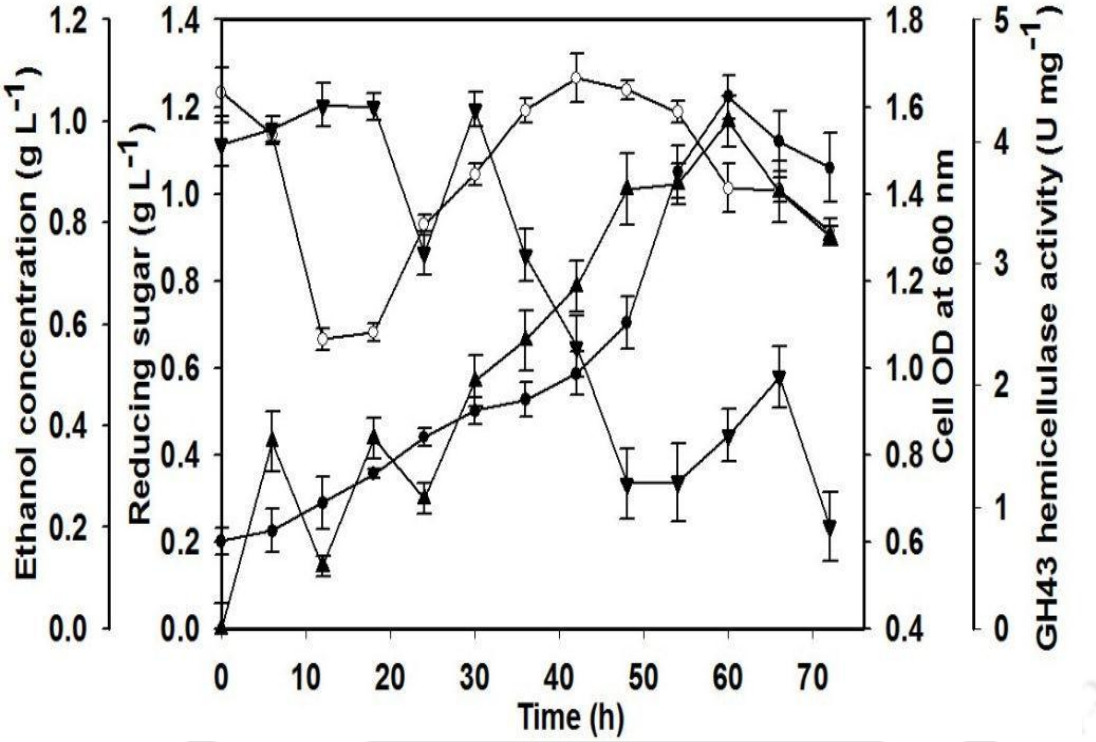


**Fig. 4.3.14** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.0; temperature 30°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

The ethanol formation began from 18 h of SSF with a sharp rise was till 60 h (Fig. 5.3.2). The maximum ethanol concentration accomplished was 1.0 g $L^{-1}$ (Table 4.3.3, Fig. 4.3.14) with a yield of 0.168 (g of ethanol g of substrate$^{-1}$). The early phase of SSF signified sugar accumulation during 18 h and 36 h and with a decline thereafter. The maximum reducing sugar concentration was 1.20 g $L^{-1}$ (Table 4.3.3, Fig. 4.3.14). The activities of both the recombinant enzymes reduced with advancement in SSF. The dynamic profile of only recombinant GH43 hemicellulase (α-L-arabinofuranosidase) has been shown in Fig. 4.3.14 due to more hemicellulose

content of water hyacinth. There was a substantial increase in GH43 hemicellulase (α-L-arabinofuranosidase) activity reaching a maximum of 3.8 U $mg^{-1}$ at 42 h and there after showed a decline to 2.8 U $mg^{-1}$ at 72 h.

*S. cerevisiae* has been the routine hexose utilizing microbe employed for the large scale production of ethanol due to its robustness to endure range of metabolic inhibitions (Casey and Ingledew, 1986). A slightly higher ethanol concentration was observed employing GH5 cellulase on water hyacinth as compared with *T. reesei* cellulase along with *S. cerevisiae* (Table 4.3.3). A 1.14-fold rise in ethanol titre was obtained involving GH5 cellulase on water hyacinth as compared with *T. reesei* cellulase with *Z. mobilis.* As compared with *S. cerevisiae* in SSF experiments of water hyacinth, *Z. mobilis* showed better performance due to its potential of having high ethanol tolerance. A 1.2-fold rise in ethanol titre was achieved by *Z. mobilis* with GH5 cellulase as compared to *S. cerevisiae* with the same hydrolytic enzyme (Table 4.3.3). Also, *C. shehatae* along with GH43 hemicellulase (α-L-arabinofuranosidase) yielded a much better ethanol titre due to the high hemicellulose content of water hyacinth. Employing a mixed enzyme system comprising of GH5 cellulase along with GH43 hemicellulase and *Z. mobilis* improved the ethanol titre by 1.74-fold as compared to that of GH5 cellulase and *Z. mobilis* (Table 4.3.3). The involvement of *C. shehatae* for utilization of pentose sugars released from hemicellulose hydrolysis along with *S. cerevisiae* and mixed enzyme system augmented the ethanol titre. A 1.8-2.7 fold increment was observed in ethanol titre using a mixed enzyme [GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase)] mixed culture (*S. cerevisiae* and *C. shehatae*) system as compared with single enzyme-single culture systems (Table 4.3.3).

The ethanol titre values achieved in our study are comparable with the outcomes reported in literature. Using a coculture of *Clostridium thermosaccharolyticum* HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937, scientists obtained an ethanol concentration of (2.2 g $L^{-1}$) from 1% (w $v^{-1}$) of banana waste (Reddy *et al.,* 2010). Das *et al.,* (2012) reported an ethanol titre of 2.1 g $L^{-1}$ from 1% (w $v^{-1}$) mango leaves with recombinant GH43 hemicellulase from *C. thermocellum* and *C. shehatae.* Some researchers reported an ethanol concentration of 2.1 g $L^{-1}$ using commercial cellulolytic enzyme and 1% (w $v^{-1}$) sunflower stalks (Ruiz *et al.,* 2006). The leafy biomass of mango contributed an ethanol titre of 1.33 g $L^{-1}$ using naturally isolated cellulase and recombinant enzymes from *C. thermocellum* (Das *et al.,* 2013). *Z. mobilis* upon fermentation from 1% (w $v^{-1}$) sugarcane bagasse contributed an ethanol titre of 0.31 g $L^{-1}$ (Wirawan *et al.,* 2012). An ethanol yield of 0.19 (g g of substrate$^{-1}$) was achieved from acid hydrolysed water hyacinth involving *Pichia stipits* (Nigam, 2002).

## 4.4 Conclusions

This study reported shake flask SSF involving different combinations of hydrolytic enzymes and fermentative microbes on mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth. The mixed MAA and organosolv pretreatments of wild grass gave cellulose (46.30%), hemicellulose (26.50%) and lignin (15.20%). The mixed MAA and organosolv pretreatments of water hyacinth contributed cellulose (26.10%), hemicellulose (33.20%) and lignin (15.64%).

The saccharification efficiency of recombinant *C. thermocellum* GH5 cellulase was compared with the fungal *T. reesei* cellulase in terms of reducing sugar formed in SSF trials of wild grass and water hyacinth. In case of wild grass, a 5% increase in reducing sugar concentration was obtained by GH5 cellulase as compared to *T. reesei* cellulase. In water hyacinth, a 15% rise in reducing sugar concentration was achieved by GH5 cellulase as compared to *T. reesei* cellulase. The fermentation capability of *S. cerevisiae* was compared with *Z. mobilis* in terms of ethanol titre obtained in SSF experiments of wild grass and water hyacinth. In case of wild grass, a 40% upsurge in ethanol titre was obtained by *Z. mobilis* as compared to *S. cerevisiae*. In water hyacinth, a 20% augmentation in ethanol concentration was attained by *Z. mobilis* as compared to *S. cerevisiae*. The hydrolytic capability of *C. thermocellum* hydrolytic GH43 hemicellulase (α-L-arabinofuranosidase) along with *C. shehatae* was evaluated in fermentation trials of wild grass and water hyacinth. Due to low hemicellulose content of wild grass, GH43 hemicellulase yielded less reducing sugar (0.49 g $L^{-1}$), whereas, GH43 hemicellulase gave a higher amount of reducing sugar (0.55 g $L^{-1}$) owing to greater hemicellulose fraction of water hyacinth.

In conclusion, a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae, C. shehatae*) system was engaged to achieve improved ethanol titre.

The SSF trial of mixed pretreated 1% (w $v^{-1}$) wild grass involving *T. ressei* cellulase and *S. cerevisiae* gave reducing sugar concentration of 1.22 g $L^{-1}$, ethanol titre of 0.61 g $L^{-1}$ and an ethanol yield of 0.083 (g of ethanol g of substrate$^{-1}$). Whereas, the SSF experiment of wild grass involving GH5 cellulase and *S. cerevisiae* provided a reducing sugar concentration of 1.26 g $L^{-1}$, ethanol titre of 0.67 g $L^{-1}$ and an ethanol yield of 0.092 (g of ethanol g of substrate$^{-1}$) displaying a 11% increase in the ethanol yield and proved the effectiveness of GH5 cellulase over *T. ressei* cellulase with *S. cerevisiae*.

The SSF trial of wild grass involving *T. reesei* cellulase and *Z. mobilis* contributed a reducing sugar concentration of 1.30 g $L^{-1}$, ethanol titre of 0.82 g $L^{-1}$ and an ethanol yield of 0.113 (g of ethanol g of substrate$^{-1}$). Contrastingly, the SSF experiment of wild grass involving GH5 cellulase and *Z. mobilis* offered a reducing sugar concentration of 1.36 g $L^{-1}$, ethanol titre of 0.94 g $L^{-1}$ and an ethanol yield of 0.129 (g of ethanol g of substrate$^{-1}$) presenting a 14% escalation in the ethanol yield and proved the efficiency of GH5 cellulase over *T. ressei* cellulase with *Z. mobilis.*

The SSF trial of wild grass involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* gave a reducing sugar concentration of 0.49 g $L^{-1}$, ethanol titre of 0.40 g $L^{-1}$ and an ethanol yield of 0.054 (g of ethanol g of substrate$^{-1}$). The SSF experiment of wild grass involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* gave a reducing sugar concentration of 1.59 g $L^{-1}$, ethanol titre of 1.29 g $L^{-1}$ and an ethanol yield of 0.177 (g

of ethanol g of substrate$^{-1}$) exhibiting a 37% upturn in the ethanol yield as compared with SSF trial of GH5 cellulase and *Z. mobilis.*

The SSF trial of wild grass involving the combination of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae* and *C. shehatae* furnished the maximum reducing sugar concentration of 1.70 g L$^{-1}$, maximum ethanol titre of 1.50 g L$^{-1}$ and an ethanol yield of 0.206 (g of ethanol g of substrate$^{-1}$). A 2.3-3.2 fold increment was observed in ethanol titre using a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]- mixed culture (*S. cerevisiae, C. shehatae*) system as compared to single enzyme-single culture systems for wild grass.

The SSF trial of mixed pretreated 1% (w v$^{-1}$) water hyacinth involving *T. ressei* cellulase and *S. cerevisiae* gave a reducing sugar concentration of 0.47 g L$^{-1}$, ethanol titre of 0.37 g L$^{-1}$ and an ethanol yield of 0.062 (g of ethanol g of substrate$^{-1}$). Interestingly, the SSF experiment of water hyacinth involving GH5 cellulase and *S. cerevisiae* provided a reducing sugar concentration of 0.51 g L$^{-1}$, ethanol titre of 0.39 g L$^{-1}$ and an ethanol yield of 0.066 (g of ethanol g of substrate$^{-1}$) revealing a 6% rise in ethanol yield and evidenced the efficacy of GH5 cellulase over *T. ressei* cellulase with *S. cerevisiae*.

The SSF trial of water hyacinth involving *T. reesei* cellulase and *Z. mobilis* contributed a reducing sugar concentration of 0.58 g L$^{-1}$, ethanol titre of 0.41 g L$^{-1}$ and an ethanol yield of 0.069 (g of ethanol g of substrate$^{-1}$). On the other hand, the SSF experiment of water hyacinth involving GH5 cellulase and *Z. mobilis* gave a reducing sugar concentration of 0.64 g L$^{-1}$, ethanol titre of 0.47 g L$^{-1}$ and an ethanol yield of 0.079 (g of ethanol g of substrate$^{-1}$) exhibiting an increase of 14% percent in ethanol

yield and proved the competence of GH5 cellulase over *T. ressei* cellulase with *Z. mobilis.*

The SSF trial of water hyacinth involving recombinant GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* gave a reducing sugar concentration of 0.74 g $L^{-1}$, ethanol titre of 0.55 g $L^{-1}$ and an ethanol yield of 0.092 (g of ethanol g of substrate$^{-1}$). The SSF experiment of water hyacinth involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase) and *Z. mobilis* gave a reducing sugar concentration of 1.13 g $L^{-1}$, ethanol titre of 0.82 g $L^{-1}$ and an ethanol yield of 0.138 (g of ethanol g of substrate$^{-1}$) exhibiting a 22% percent improvement in the ethanol yield as compared with the SSF trial of GH5 cellulase and *Z. mobilis.*

The SSF trial of water hyacinth involving the combination of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae* and *C. shehatae* furnished a maximum reducing sugar concentration of 1.21 g $L^{-1}$, maximum ethanol titre of 1.0 g $L^{-1}$ and an ethanol yield of 0.168 (g of ethanol g of substrate$^{-1}$). A 1.8-2.7 fold increment was observed in ethanol titre using a mixed enzyme [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)]-mixed culture (*S. cerevisiae, C. shehatae*) system as compared to single enzyme-single culture system for water hyacinth. The effective breakdown of the complex lignocellulosic weeds by mixed pretreatment strategy along with recombinant hydrolytic enzymes and fermentative microbes in SSF will yield a value-added product.

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# Chapter 5

## Optimization of Simultaneous Saccharification and Fermentation (SSF) process by Taguchi Orthogonal array design

### 5.1 Introduction

The production of lignocellulosic ethanol is dependent on vital process parameters such as temperature, pH, hydrolytic enzyme volume and fermentative microbe's inoculum volume (Latifian *et al.,* 2007). The performance of multiple experiments by analyzing one variable at a time (OVAT) approach is time consuming and laborious for identifying various independent variable with their effect (Vishwanatha *et al.,* 2010). Statistically based experimental design *viz.,* Placket-Burman design, Box-Behnken design and Taguchi orthogonal array design summarizes the collection and sorting of variables to be taken for consideration, determining the variable amount, analyzing the variable at different parameters and finally, the effect of variable error. Better quality at low cost is the main aim for generation of Taguchi design of experiments (DOE) approach to maximize robustness of products and process (Antony *et al.,* 1998). Taguchi experimental design is fast and

considerable way of optimization conferring remarkable outcome in simultaneous study of many factors, generating quantitative information with less experimental trial, making its mark in quality products supplemented with better process performance rendering high yield and better stability (Stamitis, 1977; Taguchi, 1986). The basic principle involved is the encompassment of large experimental data as orthogonal (unbiased) array in determining the effect of various factors which govern the reaction happening, ensuing in experimental error reduction with improved producibility (efficiency) of experimental outcome. Taguchi design established the importance of statistically aligned experiments in speculating the settings of product (and/or processes) on various parameters (Byrne and Taguchi, 1987; Oliveira and Alves, 2000).

Cost-effective fermentation of lignocellulosic hydrolysate to a value-added product, bioethanol necessitates the conspicuous enhancement in the activities of various hydrolytic enzymes along with efficient mixed sugar utilization by various fermentative microbes (Sun and Cheng, 2002). Simultaneous saccharification and fermentation (SSF) is a single step combination of enzymatic hydrolysis of complex polysaccharides with concurrent fermentation of derived monosaccharides (glucose, arabinose and xylose) to ethanol (Ballesteros *et al.*, 2004). The north-east part of India has a wide abundance of lignocellulosic substrate *viz.*, wild grass (*Achnatherum hymenoides*), rich in cellulose and hemicellulose (Das *et al.*, 2012). Singh *et al.*, (1984) reported a daily average water-hyacinth biomass productivity of 0.26 ton of dry biomass per hectare in all seasons under the climatic conditions of Assam. Water hyacinth (*Eichhornia crassipes)* is a typical menace infesting large areas of water bodies causing ecological and socio-economic problems which include diminution of

biodiversity, blockage of rivers and drainage system, and depletion of dissolved oxygen. The possibility of converting water hyacinth to biogas or fuel ethanol is currently an area of great research interest (Ganguly *et al.,* 2012). Water hyacinth having high hemicellulose content can be a potential source for bioethanol production. As compared to the commercially employed hydrolytic enzyme of the corresponding *Trichoderma* system, the cellulosome of the anaerobic thermophilic bacterium, *Clostridium thermocellum* exhibits a 50-fold higher specific activity against crystalline cellulose (Demain *et al.,* 2005) The advancement in molecular biology has familiarized new area of enzyme production in transformed cells with over expression and their subsequent use for the breakdown of structural carbohydrates *viz.,* cellulose and hemicellulose into simple sugars (Adlakha *et al.,* 2011).

According to CAZy database, glycoside hydrolase family 5 (GH5) exhibits activities of chitosanase (EC 3.2.1.132); β-mannosidase (EC 3.2.1.25); cellulase (EC 3.2.1.4); glucan-β-(1→4)-glucosidase (EC 3.2.1.58); licheninase (EC 3.2.1.73); glucan endo-β-(1→6)-glucosidase (EC 3.2.1.75); mannan endo-β-(1→4)-mannosidase (EC 3.2.1.78); cellulose β-(1→4)-cellobiosidase (EC 3.2.1.91); β-(1→3)-mannanase (EC 3.2.1.-); mannan transglycosylase (EC 2.4.1.-); endo-β-(1→6)-galactanase (EC 3.2.1.164); exo-β-(1→4)-glucanase / cellodextrinase (EC 3.2.1.74); chitosanase (EC 3.2.1.132), cellulase (EC 3.2.1.4), whereas glycoside hydrolase family 43 displays activities of β-xylosidase (EC 3.2.1.37), β-(1→3)-xylosidase (EC 3.2.1.-); α-L-arabinofuranosidase (EC 3.2.1.55), arabinanase (EC 3.2.1.99) and xylanase (EC 3.2.1.8). A number of available pre-treatment techniques are used for liberating the cellulosic and hemicellulosic components from the lignin moieties and in turn, rendering the accessibility to a better hydrolysis step (Barrett *et*

*al.,* 2009). *Saccharomyces cerevisiae* has the inherent ability of utilizing hexose sugars, considerable product tolerance and resistance to metabolic inhibitions in ethanol production. Xylitol dehydrogenase and xylose reductase are the prime enzymes of *Candida shehatae* that enables it to utilize pentose sugars for ethanol production (Chandel *et al.*, 2007).

The present study emphasizes on the Taguchi optimization of different fermentation process parameters such as mixed recombinant *C. thermocellum* enzymes' volume, GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase), mixed cultures' inoculum volume (*S. cerevisiae, C. shehatae*), pH and temperature on bioethanol production from mixed pretreated wild grass and water hyacinth independently with subsequent validation of the models at shake flask level.

## 5.2 Materials and Methods

### 5.2.1 Reagents, chemicals and substrates

Carboxy methyl cellulose (CMC) and kanamycin were procured from Sigma Aldrich (St. Louis, USA). Rye arabinoxylan was purchased from Megazyme International Ltd. (Ireland). The analytical grade reagents and chemicals *viz.*, sodium chloride, sodium acetate, glucose, peptone, yeast extract, ampicillin, potassium dichromate, sodium carbonate, sodium bicarbonate, sodium potassium tartarate, sodium sulphate, copper sulphate, ammonium molybdate and sodium arsenate were purchased from Himedia Pvt. Ltd. India. Ethanol was obtained from Merck Pvt. Ltd. India. Phosphoric acid was purchased from Qualigens India Pvt. Ltd. Coomassie Brilliant Blue G-250 was procured from Amresco LLC, USA. Lignocellulosic substrate wild grass (*A. hymenoides*) and the leaves and petioles of water hyacinth (*E. crassipes)* were collected from the campus of Indian Institute of Technology Guwahati, India. The preliminary treatment of the substrates was carried out as described earlier in Chapter 3, Section 3.2.4.

### 5.2.2 Microorganisms and culturing conditions

The culturing and maintenance of *E. coli* BL21 (DE3) cells containing family 5 glycoside hydrolase (GH5) gene and *E. coli* BL21 (DE3) pLysS cells harbouring family 43 glycoside hydrolase (GH43) gene from *Clostridium thermocellum* was done as described in Chapter 3, Section 2.2.2 earlier. The culturing and maintenance of predominantly aerobic fermentative microbes, *Saccharomyces cerevisiae* (NCIM no: 3215) and *Candida shehatae* (NCIM no: 3500) procured from National Chemical Laboratory, Pune, India was done as described in Chapter 3, Section 3.2.2 earlier.

### 5.2.3 Repetitive batch production of recombinant GH5 cellulase in Luria-Bertani (LB) medium supplemented with glucose

The repetitive batch production of recombinant GH5 cellulase in Luria-Bertani (LB) medium supplemented with glucose was done to yield higher enzyme activity and protein concentration as described earlier in Chapter 2, Section 2.2.11. The recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was used for cellulose hydrolysis in SSF experiments of wild grass and water hyacinth as described later in Section 5.2.6.

### 5.2.4 Repetitive batch production of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) in LB medium supplemented with glucose

The repetitive batch production of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani (LB) medium supplemented with glucose was done to yield higher enzyme activity and protein concentration as described in Chapter 2, Section 2.2.11 earlier**.** The recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was used for hemicellulose hydrolysis in SSF experiments of wild grass and water hyacinth as described later in Section 5.2.6.

### 5.2.5 Mixed microwave-assisted alkali (MAA) and organosolv pretreatment strategy

The mixed microwave-assisted alkali (MAA) and organosolv pretreatment of two substrates, wild grass and water hyacinth were performed as described earlier in Chapter 3, Section 3.2.6.

### 5.2.6 Simultaneous saccharification and fermentation (SSF) of pretreated wild grass and water hyacinth

One percent (w $v^{-1}$) each of the mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass (*A. hymenoides*) and water hyacinth (*E. crassipes*) were autoclaved separately in 250 mL Erlenmeyer flasks containing 100 mL working volume of 20 mM sodium acetate buffer (pH 5.0) supplemented with 0.1%, w $v^{-1}$ each of yeast extract and peptone. Then, 0.5 mL each of crude recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) were added as the mixed enzymatic consortium for hydrolysis to the two flasks. At the same time, 0.5 mL each of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) inoculum were added for fermentation to the two flasks. The flasks were kept at 30°C and 120 rpm for 72 h and the sample was collected at every 6 h interval. The monitoring of SSF dynamic profiles were done with the measurement of the cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

### 5.2.7 Optimization of process parameters of simultaneous saccharification and fermentation (SSF) involving pretreated wild grass by Taguchi method

#### *5.2.7.1 Statistical optimization using Taguchi Orthogonal Array design*

Taguchi experimental design matrix, a standard orthogonal array $L_{25}$ ($6^5$) was used to examine six factors *viz.*, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) volume (mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$,0.32 mg $mL^{-1}$) volume (mL), *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) inoculum volume (mL), *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) inoculum volume (mL), pH and temperature (°C) in five levels, *viz.*, Level 1 to Level 5 (Table 5.2.1) in SSF

experiments involving mixed pretreated 1% (w $v^{-1}$) wild grass at shake flask level. The L and the subscript (25) represent the Latin square and the number of experimental runs, respectively. The levels of the factors studied and the layout of the $L_{25}$ Taguchi's orthogonal array are represented in Tables 5.2.1 and 5.2.2. Each of the twenty-five simultaneous saccharification and fermentation (SSF) experiments denoted as 'runs' were carried out as per the defined values of six different parameters in five levels (Table 5.2.2). All the SSF experiments were carried out in 100 mL of fermentation media at 120 rpm for 72 h at varying tempertatures (Table 5.2.1) with sample collection at every 6 h interval.

**Table 5.2.1** Factor (Parameter) and levels in Taguchi Experimental Design for shake flask SSF process employing mixed pretreated 1% (w $v^{-1}$) wild grass at 120 rpm.

| **Factor/Parameter** | **Levels** | | | | |
|---|---|---|---|---|---|
| Recombinant GH5 cellulase* (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase)* (3.7 U$mg^{-1}$, 0.32 mg $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| *S. cerevisiae** (3.6 x $10^8$ cells $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| *C. shehatae** (2.1 x $10^8$ cells $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| pH | 3.0 | 4.3 | 5.0 | 5.5 | 6.0 |
| Temperature (°C) | 26.0 | 28.0 | 30.0 | 33.0 | 35.0 |

** indicates the values of levels in (%, v $v^{-1}$)*

**Table 5.2.2** Matrix layout of the $L_{25}$ Taguchi orthogonal array design for 1% (w $v^{-1}$) mixed pretreated wild grass at 120 rpm.

| Run/ Expt. No. | Recombinant GH5 cellulase* | Recombinant GH43 hemicellulase* | *S. cerevisiae** | *C. shehatae** | pH | Tempe -rature |
|---|---|---|---|---|---|---|
| 1 | 0.25 | 1.0 | 1.0 | 1.0 | 5.0 | 30 |
| 2 | 0.5 | 1.5 | 2.0 | 0.25 | 4.3 | 30 |
| 3 | 1.0 | 2.0 | 0.5 | 1.5 | 3.0 | 30 |
| 4 | 1.5 | 0.25 | 1.5 | 0.5 | 6.0 | 30 |
| 5 | 2.0 | 0.5 | 0.25 | 2.0 | 5.5 | 30 |
| 6 | 0.25 | 0.25 | 0.25 | 0.25 | 3.0 | 26 |
| 7 | 0.5 | 0.5 | 1.0 | 1.5 | 6.0 | 26 |
| 8 | 1.0 | 1.0 | 2.0 | 0.5 | 5.5 | 26 |
| 9 | 1.5 | 1.5 | 0.5 | 2.0 | 5.0 | 26 |
| 10 | 2.0 | 2.0 | 1.5 | 1.0 | 4.3 | 26 |
| 11 | 2.0 | 0.25 | 2.0 | 1.5 | 5.0 | 28 |
| 12 | 1.5 | 2.0 | 1.0 | 0.25 | 5.5 | 28 |
| 13 | 1.0 | 1.5 | 0.25 | 1.0 | 6.0 | 28 |
| 14 | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 28 |
| 15 | 0.25 | 0.5 | 0.5 | 0.5 | 4.3 | 28 |
| 16 | 0.25 | 1.5 | 1.5 | 1.5 | 5.5 | 33 |
| 17 | 0.5 | 2.0 | 0.25 | 0.5 | 5.0 | 33 |
| 18 | 1.0 | 0.25 | 1.0 | 2.0 | 4.3 | 33 |
| 19 | 1.5 | 0.5 | 2.0 | 1.0 | 3.0 | 33 |
| 20 | 2.0 | 1.0 | 0.5 | 0.25 | 6.0 | 33 |
| 21 | 0.25 | 2.0 | 2.0 | 2.0 | 6.0 | 35 |
| 22 | 0.5 | 0.25 | 0.5 | 1.0 | 5.5 | 35 |
| 23 | 1.0 | 0.5 | 1.5 | 0.25 | 5.0 | 35 |
| 24 | 1.5 | 1.0 | 0.25 | 1.5 | 4.3 | 35 |
| 25 | 2.0 | 1.5 | 1.0 | 0.5 | 3.0 | 35 |

** indicates the values of levels in (%, v $v^{-1}$)*

### *5.2.7.2 Analysis of the Taguchi orthogonal array experiments (runs)*

The MINITAB® statistical software package, (Design Expert, version 8.0) was used to determine the outcomes of the fermentation runs. The signal-to-noise ratio (S/N), which is the logarithmic function of desired output served as objective function for optimization.

For each run, S/N ratio corresponding to larger-the-better objective function was computed using relation in Eq. 1

$$\frac{S}{N} = -10\log_{10}\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2} \quad \ldots\ldots\ldots\ldots\ldots (\mathbf{Eq.1})$$

where, '$\mathbf{y_i}$' is the signal and '**n**' is the number of repetitions in each experiment.

The response values in term of ethanol titre (%, v $v^{-1}$) and S/N ratios of Taguchi experimental design in 25 runs were analysed to extract independently the main effects of the factors; the analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The analysis of variance (ANOVA) for the responses of ethanol production was carried out according to each factor's contribution by the Taguchi method. The factors in the experimental design considered to be statistically significant at 95% confidence limit were used to determine the ratio (F) and the p value ($p < 0.05$).

### *5.2.7.3 Validation of the experimental model*

The model was validated by performing the SSF trial employing Taguchi optimized fermentation process parameters on mixed pretreated 1% (w $v^{-1}$) wild grass in 100 mL of fermentation medium. The best fermentation process parameters comprised of 1.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 0.25 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 4.3 and temperature of 35°C. The fermentation was carried out at 120 rpm for 72 h with 6 h sample collection interval. The validation of the experimental model was executed by determining the ethanol titre (%, v $v^{-1}$).

## 5.2.8 Optimization of process parameters of simultaneous saccharification and fermentation involving pretreated water hyacinth by Taguchi method

### *5.2.8.1 Statistical optimization using Taguchi Orthogonal Array design*

A standard Taguchi orthogonal array $L_{25}$ ($6^5$) experimental design matrix was used to examine six factors *viz.*, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) volume (mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) volume (mL), *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) inoculum volume (mL), *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) inoculum volume (mL), pH and temperature (°C) in five levels, *viz.*, Level 1 to Level 5 (Table 5.2.3) in SSF experiments involving mixed pretreated 1% (w $v^{-1}$) water hyacinth at shake flask level.

**Table 5.2.3** Factor (Parameter) and levels in Taguchi Experimental Design for shake flask SSF process employing mixed pretreated 1% (w $v^{-1}$) water hyacinth at 120 rpm.

| **Factor/Parameter** | **Levels** | | | | |
|---|---|---|---|---|---|
| Recombinant GH5 cellulase* (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase)* (3.7 U$mg^{-1}$, 0.32 mg $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| *S. cerevisiae** (3.6 x $10^8$ cells $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| *C. shehatae** (2.1 x $10^8$ cells $mL^{-1}$) | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 |
| pH | 3.0 | 4.3 | 5.0 | 5.4 | 6.0 |
| Temperature (°C) | 26.0 | 28.0 | 30.0 | 33.0 | 35.0 |

** indicates the values of levels in (%, v $v^{-1}$)*

The levels of the factors studied and the layout of the $L_{25}$ Taguchi's orthogonal array are presented in Tables 5.2.3 and 5.2.4. Each of the twenty-five simultaneous saccharification and fermentation (SSF) experiments denoted as 'runs' were carried out as per the defined values of six different parameters in five levels (Table 5.2.4).

**Table 5.2.4** Matrix layout of the $L_{25}$ Taguchi orthogonal array design for 1% (w $v^{-1}$) water hyacinth at 120 rpm.

| Run/ Expt. No. | Recombinant GH5 cellulase* | Recombinant GH43 hemicellulase* | *S. cerevisiae** | *C. shehatae** | pH | Tempe -rature |
|---|---|---|---|---|---|---|
| 1 | 0.25 | 0.25 | 0.25 | 0.25 | 3.0 | 26 |
| 2 | 0.25 | 0.5 | 0.5 | 0.5 | 4.3 | 28 |
| 3 | 0.25 | 1.0 | 1.0 | 1.0 | 5.0 | 30 |
| 4 | 0.25 | 1.5 | 1.5 | 1.5 | 5.4 | 33 |
| 5 | 0.25 | 2.0 | 2.0 | 2.0 | 6.0 | 35 |
| 6 | 0.5 | 0.25 | 0.5 | 1.0 | 5.4 | 35 |
| 7 | 0.5 | 0.5 | 1.0 | 1.5 | 6.0 | 26 |
| 8 | 0.5 | 1.0 | 1.5 | 2.0 | 3.0 | 28 |
| 9 | 0.5 | 1.5 | 2.0 | 0.25 | 4.3 | 30 |
| 10 | 0.5 | 2.0 | 0.25 | 0.5 | 5.0 | 33 |
| 11 | 1.0 | 0.25 | 1.0 | 2.0 | 4.3 | 33 |
| 12 | 1.0 | 0.5 | 1.5 | 0.25 | 5.0 | 35 |
| 13 | 1.0 | 1.0 | 2.0 | 0.5 | 5.4 | 26 |
| 14 | 1.0 | 1.5 | 0.25 | 1.0 | 6.0 | 28 |
| 15 | 1.0 | 2.0 | 0.5 | 1.5 | 3.0 | 30 |
| 16 | 1.5 | 0.25 | 1.5 | 0.5 | 6.0 | 30 |
| 17 | 1.5 | 0.5 | 2.0 | 1.0 | 3.0 | 33 |
| 18 | 1.5 | 1.0 | 0.25 | 1.5 | 4.3 | 35 |
| 19 | 1.5 | 1.5 | 0.5 | 2.0 | 5.0 | 26 |
| 20 | 1.5 | 2.0 | 1.0 | 0.25 | 5.4 | 28 |
| 21 | 2.0 | 0.25 | 2.0 | 1.5 | 5.0 | 28 |
| 22 | 2.0 | 0.5 | 0.25 | 2.0 | 5.4 | 30 |
| 23 | 2.0 | 1.0 | 0.5 | 0.25 | 6.0 | 33 |
| 24 | 2.0 | 1.5 | 1.0 | 0.5 | 3.0 | 35 |
| 25 | 2.0 | 2.0 | 1.5 | 1.0 | 4.3 | 26 |

** indicates the values of levels in (%, v $v^{-1}$)*

All the SSF experiments were carried out in 100 mL of fermentation media at 120 rpm for 72 h with sample collection at every 6 h interval.

### *5.2.8.2 Analysis of the Taguchi orthogonal array experiments (runs)*

The MINITAB® statistical software package, (Design Expert, version 8.0) was used to determine the outcomes of the fermentation runs. Similar analytical procedure was followed as described in section 5.2.6.2.

### *5.2.8.3 Validation of the experimental model*

The model was validated by performing the SSF trial employing Taguchi optimized fermentation process parameters on mixed pretreated 1% (w $v^{-1}$) water hyacinth in 100 mL of fermentation medium. The best fermentation process parameters comprised of 2.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 2.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 5.4 and temperature of 35°C. The fermentation was carried out at 120 rpm for 72 h with 6 h sample collection interval. The validation of the experimental model was executed by determining the ethanol titre (%, v $v^{-1}$).

## 5.2.9 Analytical methods

### *5.2.9.1 Structural carbohydrates estimation*

The structural carbohydrates like cellulose, hemicellulose and lignin of untreated and mixed MAA with organosolv pretreated of two substrates, wild grass and water hyacinth were estimated as described in Chapter 3, Section 3.2.11.1.

#### *5.2.9.2 Measurement of cell growth during SSF*

The cell growth of the fermentative microbes, *S. cerevisiae* and *C. shehatae* were measured as described in Chapter 3, Section 3.2.11.2.

#### *5.2.9.3 Recombinant GH5 cellulase assay*

The reducing sugar estimation and recombinant GH5 cellulase assay was performed as described earlier in Chapter 2, Section 2.2.12.1.

#### *5.2.9.4 Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay*

The reducing sugar estimation and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay was performed as described earlier in Chapter 2, Section 2.2.12.2.

#### *5.2.9.5 Protein content determination*

The protein content was determined as described earlier in Chapter 2, Section 2.2.12.6.

#### *5.2.9.6 Ethanol content determination by Gas chromatography and Dichromate method*

The ethanol obtained from SSF experiments were estimated by Gas chromatography (GC) and Dichromate assay as described earlier in Chapter 3, Section 3.2.11.8.

#### *5.2.9.7 Determination of ethanol yield*

The ethanol yield was calculated as described earlier in Chapter 3, Section 3.2.11.9.

## 5.3 Results and Discussion

The improved saccharification of cellulosic and hemicellulosic components of lignocellulosic biomass by different hydrolytic enzymes with simultaneous consumption of monomeric sugars by fermentative microbes is the techno-economic viability of an efficient SSF process. The structural carbohydrates determination of wild grass (*A. hymenoides*) revealed greater amount of cellulose (51.70 %, w $w^{-1}$) followed by hemicellulose (30.9 % w $w^{-1}$) whereas water hyacinth revealed greater amount of hemicellulose (44.52 %, w $w^{-1}$) followed by cellulose (30.07 % w $w^{-1}$) suggesting wild grass and water hyacinth as the suitable candidates for SSF based bioethanol production. The mixed MAA and organosolv pretreatments of wild grass loosened cellulose content of 46.30%, hemicellulose of 26.50 % and degraded maximum lignin of 15.20%. The mixed MAA and organosolv pretreatments of water hyacinth loosened cellulose (26.10%) and hemicellulose (33.20%) with a maximum lignin removal of 15.64%.

### 5.3.1 Unoptimized simultaneous saccharification and fermentation (SSF) experiments of mixed pretreated 1% (w $v^{-1}$) wild grass at shake flask level

The dynamic profile of SSF involving unoptimized process parameters for ethanol production from mixed pretreated 1% (w $v^{-1}$) wild grass at shake flask level is presented in Fig. 5.3.1. The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited negligible lag phase in their growth with steady increase till 66 h with slight decrease thereafter (Fig. 5.3.1). The growth-associated ethanol formation began from 12 h of SSF with a gradual increase till 36 h after which a sharp rise was observed till 54 h (Fig. 5.3.1). The maximum ethanol titre achieved was 1.50 g $L^{-1}$ (Table 5.3.1, Fig.

5.3.1) with a yield of 0.206 (g of ethanol g of substrate$^{-1}$). Thereafter, a decrease in ethanol production was witnessed.

**Table 5.3.1.** Comparison of unoptimized and Taguchi optimized SSF combinations with wild grass.

| SSF combination | Substrate concentration (%, w v$^{-1}$) and mode of SSF | Reducing sugar (g L$^{-1}$)* | Ethanol titre (g L$^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|---|
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (unoptimized) | 1%, shake flask | 1.70 ± 0.09 | 1.50 ± 0.06 | 0.206 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized) | 1%, shake flask | 2.31 ± 0.05 | 2.0 ± 0.04 | 0.274 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

The initial phase of the SSF represented an accumulation of available sugars till 18 h with a gradual decline. The maximum reducing sugar concentration was 1.70 g L$^{-1}$ (Fig. 5.3.1). The activities of both the recombinant enzymes decreased with progress in fermentation. The dynamic profile of only recombinant GH5 cellulase has been shown in Fig. 5.3.1 as wild grass contains more cellulose. Interestingly, the microbial growth and ethanol production shared an inverse relationship with enzyme activities and, in turn, the reducing sugars released clearly demonstrating the fact of sugar utilization by the organisms for growth and ethanol formation (Fig. 5.3.1).

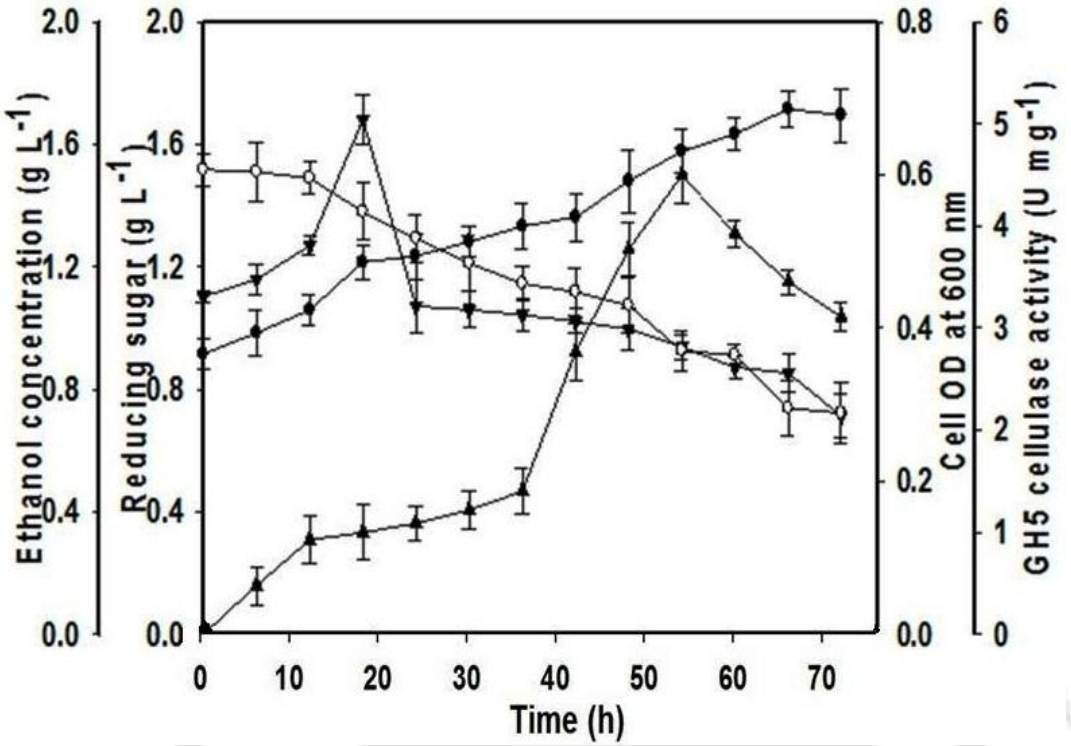


**Fig. 5.3.1** SSF profile of 1% (w $v^{-1}$) of mixed MAA and organosolv pretreated wild grass (*Achnatherum hymenoides*) using unoptimized fermentation process parameters *viz.*, recombinant GH5 cellulase, recombinant GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae*, *C. shehatae*, pH and temperature at shake flask level showing variation of (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (o) specific activity (U $mg^{-1}$) of GH5 cellulase with time (h). Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

### 5.3.2 Unoptimized SSF experiments of mixed pretreated 1% (w $v^{-1}$) water hyacinth at shake flask level

The dynamic profile of SSF involving unoptimized process parameters for ethanol production from mixed pretreated 1% (w $v^{-1}$) water hyacinth at shake flask level is represented in Fig. 5.3.2. The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited no growth lag phase with steady increase till 60 h and a slight decrease thereafter (Fig. 5.3.2). The ethanol formation began from 18 h of SSF with a sharp

rise was till 60 h (Fig. 5.3.2). The maximum ethanol titre achieved was 1.0 g $L^{-1}$ (Table 5.3.2, Fig. 5.3.2) with a yield of 0.168 (g of ethanol g of substrate$^{-1}$). The initial phase of the SSF represented an accumulation of sugars at 18 and 36 h with a decline thereafter. The maximum reducing sugar concentration was 1.20 g $L^{-1}$ (Fig. 5.3.2).

**Table 5.3.2** Comparison of unoptimized and Taguchi optimized SSF combinations using water hyacinth.

| SSF combination | Substrate concentration (%, w $v^{-1}$) and mode of SSF | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|---|
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (unoptimized) | 1%, shake flask | 1.20 ± 0.08 | 1.00 ± 0.04 | 0.168 |
| GH5 + GH43 + *S. cerevisiae* + *C. shehatae* (Taguchi optimized) | 1%, shake flask | 1.41 ± 0.05 | 1.22 ± 0.03 | 0.205 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

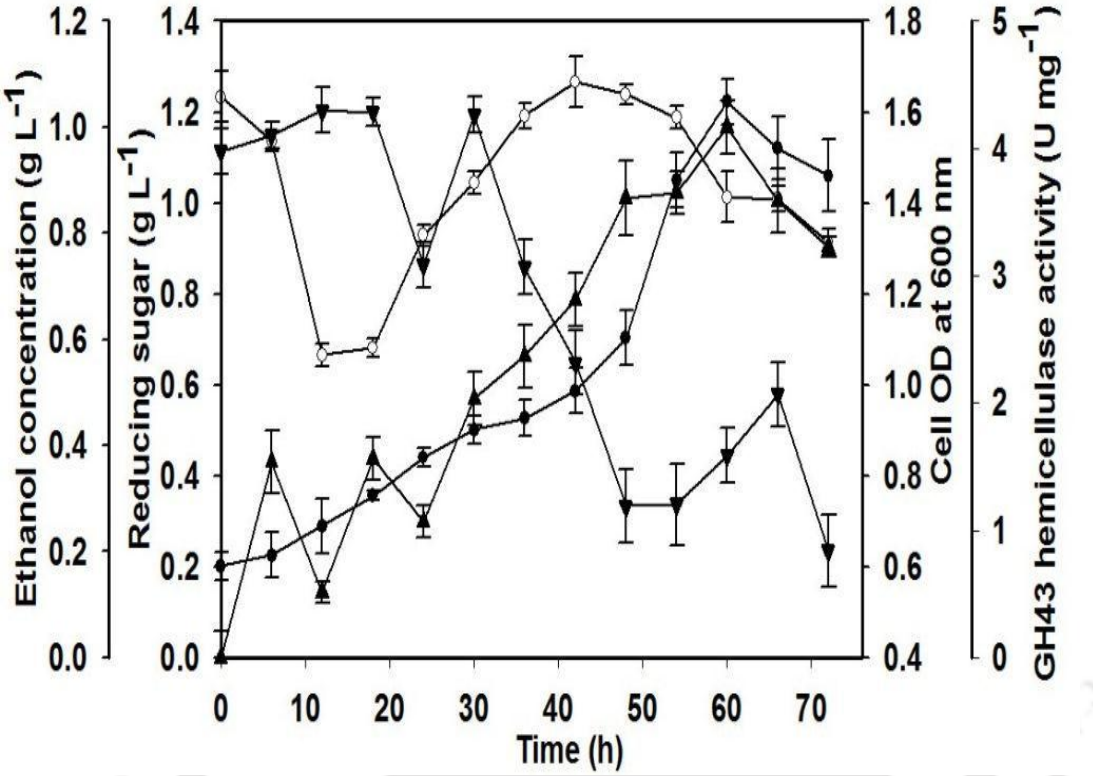


**Fig. 5.3.2** SSF profile of 1% (w $v^{-1}$) of mixed MAA and organosolv pretreated water hyacinth (*Eichhornia crassipes*) using unoptimized fermentation process parameters *viz.*, recombinant GH5 cellulase, recombinant GH43 hemicellulase (α-L-arabinofuranosidase) along with *S. cerevisiae*, *C. shehatae*, pH and temperature at shake flask level showing variation of (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (o) specific activity (U $mg^{-1}$) of GH43 cellulase with time (h). Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

The activities of both the recombinant enzymes decreased with progress in SSF. As water hyacinth contains more hemicellulose, the dynamic profile of only recombinant GH43 hemicellulase (α-L-arabinofuranosidase) has been shown in Fig. 5.3.2.

### 5.3.3 Optimization of process parameters of simultaneous saccharification and fermentation (SSF) involving pretreated wild grass by Taguchi method

Taguchi experimental design is a good positive option for the optimization of biotechnological processes. The fermentation process parameters *viz.*, temperature, pH, hydrolytic enzyme volume and fermentative microbe's inoculum volume play an important role in lignocellulosic ethanol production (Latifian *et al.*, 2007). In case of wild grass SSF, the influence of 6 factors on the SSF process was tested by Taguchi experimental design in 25 runs (Tables 5.2.1 and 5.2.2). The response values in terms of ethanol titre (%, v $v^{-1}$) and S/N ratios of Taguchi experimental design in 25 runs, for the six factors, *i.e.*, recombinant GH5 cellulase volume, recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume, *S. cerevisiae* inoculum volume, *C. shehatae* inoculum volume, pH and temperature (°C) chosen for optimization of ethanol production by SSF process (Table 5.3.3) showed the efficiency of ethanol concentration ranging from 0.120 – 0.251 v $v^{-1}$ corresponding to the combined effect of the six factors in their specific ranges. The experimental results suggest that these factors at optimum level strongly support the production of ethanol. In run (expt. 6), with a combination of recombinant GH5 cellulase volume (0.25 mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume (0.25 mL), *S. cerevisiae* inoculum volume (0.25 mL), *C. shehatae* inoculum volume (0.25 mL), pH (3) and temperature (26°C), an ethanol concentration of 0.120 (%, v $v^{-1}$) was observed (Table 5.3.3, Fig. 5.3.3) with lowest S/N ratio of -18.39.

**Table 5.3.3** Response values and S/N ratio of $L_{25}$ Taguchi orthogonal array design for SSF of wild grass.

| Run/ Expt. No | Response (ethanol titre, %, v $v^{-1}$)* | S/N ratio |
|---|---|---|
| 1 | 0.153 ± 0.05 | -16.32 |
| 2 | 0.189 ± 0.04 | -14.46 |
| 3 | 0.143 ± 0.09 | -16.92 |
| 4 | 0.188 ± 0.02 | -14.48 |
| 5 | 0.183 ± 0.07 | -14.76 |
| 6 | 0.120 ± 0.08 | -18.39 |
| 7 | 0.187 ± 0.06 | -14.55 |
| 8 | 0.210 ± 0.01 | -13.55 |
| 9 | 0.184 ± 0.03 | -14.72 |
| 10 | 0.225 ± 0.06 | -12.95 |
| 11 | 0.191 ± 0.05 | -14.38 |
| 12 | 0.194 ± 0.07 | -14.26 |
| 13 | 0.209 ± 0.08 | -13.59 |
| 14 | 0.123 ± 0.08 | -18.23 |
| 15 | 0.217 ± 0.04 | -13.27 |
| 16 | 0.202 ± 0.05 | -13.87 |
| 17 | 0.201 ± 0.03 | -13.95 |
| 18 | 0.206 ± 0.02 | -13.74 |
| 19 | 0.124 ± 0.01 | -18.09 |
| 20 | 0.219 ± 0.08 | -13.16 |
| 21 | 0.217 ± 0.09 | -13.27 |
| 22 | 0.218 ± 0.07 | -13.24 |
| 23 | 0.251 ± 0.03 | -11.99 |
| 24 | 0.222 ± 0.02 | -13.05 |
| 25 | 0.127 ± 0.05 | -17.94 |

**the values correspond to the maximum ethanol at a particular time, values are mean ± SE (n=3)*

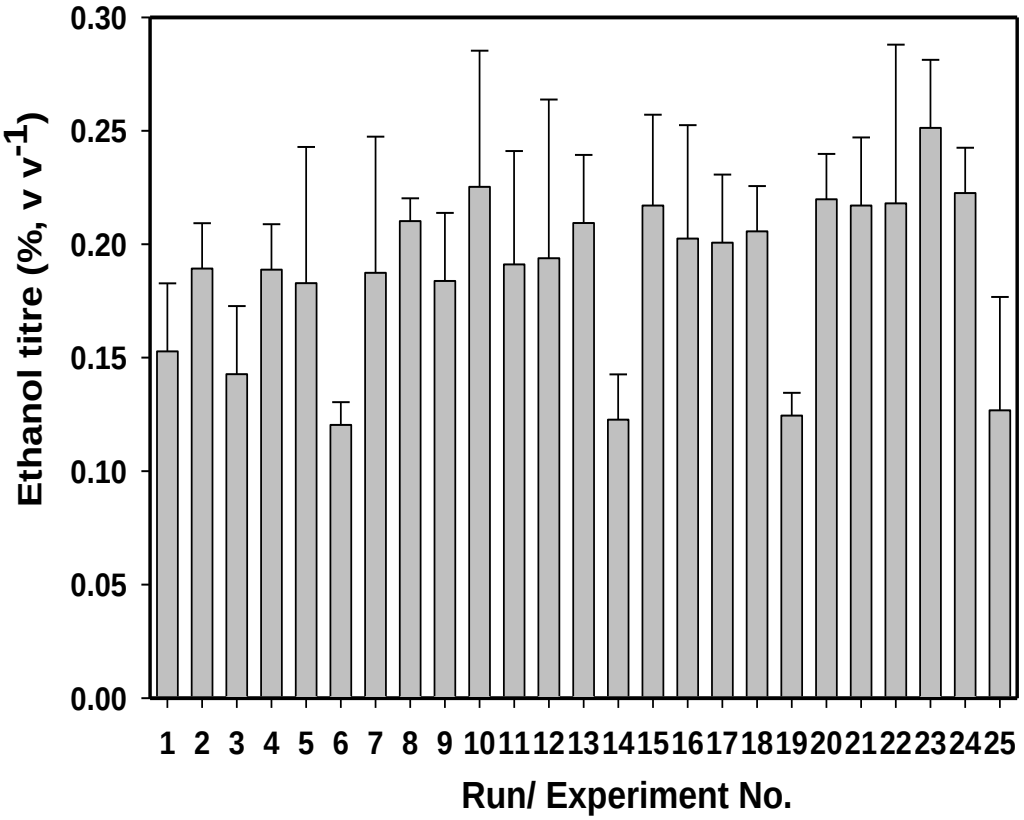


**Fig. 5.3.3** Comparative results of response in terms of ethanol titre (%, v $v^{-1}$) of wild grass SSF Taguchi $L_{25}$ orthogonal array of experiments.

A maximum ethanol titre of 0.251 (%, v $v^{-1}$) ethanol was observed in run (expt. 23) with a combination of recombinant GH5 cellulase volume (1.0 mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume (0.5 mL), *S. cerevisiae* inoculum volume (1.50 mL), *C. shehatae* inoculum volume (0.25 mL), pH (5) and temperature (30°C) with the best response and maximum S/N ratio (-11.99) (Table 5.3.3, Fig. 5.3.3).

The Taguchi optimized fermentation process parameters for wild grass are shown in Fig. 5.3.4. The best process parameters in 100 mL of fermentation medium comprised 1.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 0.25 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 4.3 and temperature of 35°C.

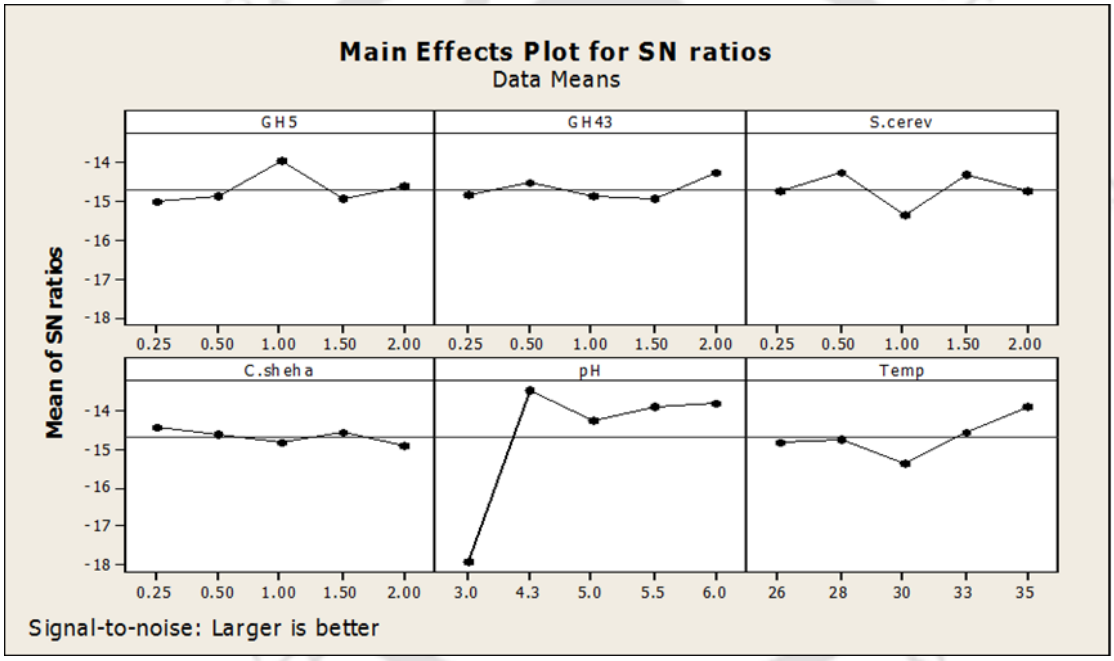


**Fig. 5.3.4** Main effect plots for S/N ratios with larger the better objective function of Taguchi optimized fermentation process parameters for wild grass.

The analysis of variance (ANOVA) for the responses of ethanol production was carried out according to the factors' contribution by the Taguchi method (Table 5.3.4).

**Table 5.3.4** Analysis of Variance for the responses of ethanol production from SSF of wild grass.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Recombinant GH5 cellulase | 1 | 0.0000660 | 0.0000660 | 0.0000660 | 0.08 | 0.783 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) | 1 | 0.0000637 | 0.0000637 | 0.0000637 | 0.08 | 0.786 |
| *S. cerevisiae* | 1 | 0.0000000 | 0.0000000 | 0.0000000 | 0.00 | 0.996 |
| *C. shehatae* | 1 | 0.0002732 | 0.0002732 | 0.0002732 | 0.32 | 0.576 |
| pH | 1 | 0.0150152 | 0.0150152 | 0.0150152 | 17.85 | 0.001 |
| Temp | 1 | 0.0012587 | 0.0012587 | 0.0012587 | 1.50 | 0.237 |
| Error | 18 | 0.0151394 | 0.0151394 | 0.0008411 | | |
| Total | 24 | 0.0318162 | | | | |

*DF- Degrees of freedom, SS-Sum of squares, MS-Mean of Squares*

From the calculated ratios (F), it can be inferred that the factors considered in the experimental design are statistically significant at 95% confidence limit. Table 5.3.5 represented the contribution of selected factors on bioethanol production.

**Table 5.3.5** Rank and significance of various factors for SSF from wild grass.

| Factor/Parameter | Rank | *p*-value |
|---|---|---|
| Recombinant GH5 cellulase* (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) | 4 | 0.783 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase)* (3.7 U$mg^{-1}$, 0.32 mg $mL^{-1}$) | 5 | 0.786 |
| *S. cerevisiae** (3.6 x $10^8$ cells $mL^{-1}$) | 3 | 0.996 |
| *C. shehatae** (2.1 x $10^8$ cells $mL^{-1}$) | 6 | 0.576 |
| pH | 1 | 0.001 |
| Temperature | 2 | 0.237 |

*$p < 0.05$*

It can be observed that on the basis of *p*-value ($p < 0.05$), pH with rank 1 is the most significant of all other factors and shows highest positive impact on the ethanol production. *C. shehatae* inoculum volume showed least impact on ethanol production among the factors studied with the assigned variance of values.

### *5.3.3.2 Validation of Taguchi experimental model for SSF of wild grass*

The validation of Taguchi experimental model is represented in Table 5.3.6. It was observed that the response (ethanol %, v $v^{-1}$) (0.254) as well as S/N ratio (-10.95) for Taguchi optimum values was more than experimental optimum values for ethanol concentration (0.251%, v $v^{-1}$) and S/N ratio (-11.99). This validated the Taguchi optimized SSF process parameters for wild grass bioethanol production. Thus, there was a 1.3-fold increase in ethanol titre with Taguchi optimized SSF process parameters as compared to unoptimized parameters (Table 5.3.1, Fig. 5.3.5).

**Table 5.3.6** Validation of Taguchi experimental data values for SSF from wild grass.

| Factor/ Parameter | Taguchi optimum | Experiment optimum |
|---|---|---|
| Recombinant GH5 cellulase ($5.7\ U\ mg^{-1}$, $0.45\ mg\ mL^{-1}$) (%, v $v^{-1}$) | 1.0 | 1.0 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) ($3.7\ Umg^{-1}$, $0.32\ mg\ mL^{-1}$) (%, v $v^{-1}$) | 2.0 | 0.5 |
| *S. cerevisiae* ($3.6 \times 10^8$ cells $mL^{-1}$) (%, v $v^{-1}$) | 1.5 | 1.5 |
| *C. shehatae* ($2.1 \times 10^8$ cells $mL^{-1}$) (%, v $v^{-1}$) | 0.25 | 0.25 |
| pH | 4.3 | 5.0 |
| Temperature (°C) | 35 | 35 |
| S/N ratio | -10.95 | -11.99 |
| Response experimental Ethanol titre (%, v $v^{-1}$) | 0.254 | 0.251 |
| Response predicted (%, v $v^{-1}$) Ethanol titre (%, v $v^{-1}$) | 0.270 | 0.267 |
| Ethanol titre (g $L^{-1}$) | 2.00 | 1.98 |
| Ethanol yield (g of ethanol g of $substrate^{-1}$) | 0.274 | 0.271 |

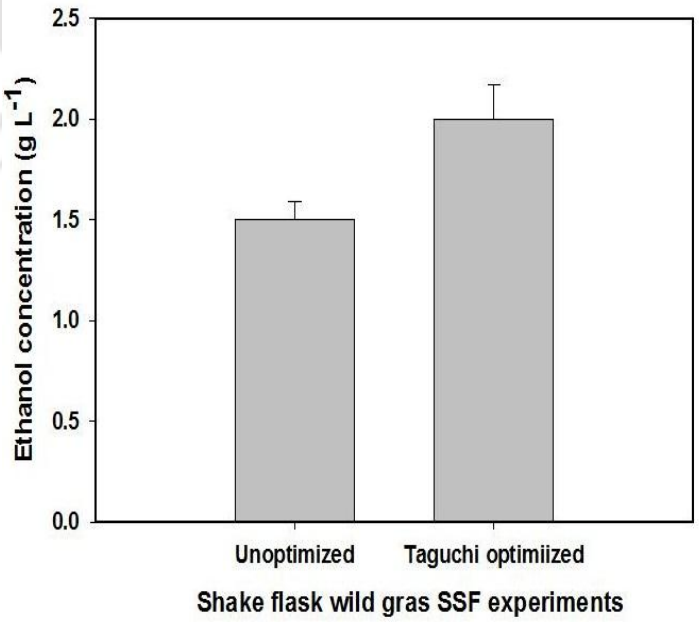


**Fig. 5.3.5** Ethanol titre (g $L^{-1}$) of wild grass for unoptimized and Taguchi optimized shake flask SSF experiments.

### 5.3.4 Optimization of process parameters of simultaneous saccharification and fermentation involving pretreated water hyacinth by Taguchi method

Temperature, pH, hydrolytic enzyme volume and fermentative microbe's inoculum volume are the process parameters that play a vital role in lignocellulosic ethanol production (Latifian *et al.*, 2007). In case of bioethanol production from water hyacinth by SSF, the influence of 6 factors on the SSF process was tested by Taguchi experimental design in 25 runs (Tables 5.2.3 and 5.2.4). The response values in terms of ethanol titre (%, v $v^{-1}$) and S/N ratios of Taguchi experimental design in 25 runs, for the six factors, *i.e.*, recombinant GH5 cellulase volume, recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume, *S. cerevisiae* inoculum volume, *C. shehatae* inoculum volume, pH and temperature (°C) selected for optimization of bioethanol production from water hyacinth by SSF process displayed the efficiency of ethanol production ranging from 0.075 (%, v $v^{-1}$) – 0.149 (%, v $v^{-1}$) conforming to the combined effect of the six factors in their specific ranges (Table 5.3.7). The experimental outcomes suggest that these factors at optimum level strongly support the production of ethanol. In run (expt. 17), with a combination of recombinant GH5 cellulase volume (1.5 mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume (0.50 mL), *S. cerevisiae* inoculum volume (2.0 mL), *C. shehatae* inoculum volume (1.0 mL), pH (3) and temperature (33°C), an ethanol concentration of 0.075 (%, v $v^{-1}$) was observed (Table 5.3.7, Fig. 5.3.6) with lowest S/N ratio of -22.45.

**Table 5.3.7** Response values and S/N ratio of $L_{25}$ Taguchi orthogonal array design for SSF of water hyacinth.

| Run/ Expt. No. | Response (Ethanol titre, %, v $v^{-1}$)* | S/N ratio |
|---|---|---|
| 1 | 0.082 ± 0.02 | -21.68 |
| 2 | 0.125 ± 0.06 | -18.07 |
| 3 | 0.089 ± 0.05 | -20.92 |
| 4 | 0.149 ± 0.03 | -16.56 |
| 5 | 0.131 ± 0.06 | -17.65 |
| 6 | 0.113 ± 0.01 | -18.96 |
| 7 | 0.092 ± 0.07 | -20.71 |
| 8 | 0.112 ± 0.08 | -18.99 |
| 9 | 0.111 ± 0.09 | -19.01 |
| 10 | 0.134 ± 0.05 | -17.45 |
| 11 | 0.114 ± 0.06 | -18.92 |
| 12 | 0.137 ± 0.07 | -17.22 |
| 13 | 0.113 ± 0.01 | -18.96 |
| 14 | 0.096 ± 0.04 | -20.39 |
| 15 | 0.116 ± 0.02 | -18.70 |
| 16 | 0.095 ± 0.08 | -20.43 |
| 17 | 0.075 ± 0.07 | -22.45 |
| 18 | 0.100 ± 0.06 | -19.99 |
| 19 | 0.127 ± 0.05 | -17.88 |
| 20 | 0.129 ± 0.09 | -17.77 |
| 21 | 0.107 ± 0.07 | -19.38 |
| 22 | 0.118 ± 0.03 | -18.58 |
| 23 | 0.119 ± 0.02 | -18.48 |
| 24 | 0.107 ± 0.04 | -19.43 |
| 25 | 0.120 ± 0.08 | -18.41 |

**the values correspond to the maximum ethanol at a particular time, values are mean ± SE (n=3)*

A maximum ethanol titre of 0.149 (%, v $v^{-1}$) ethanol was observed in run (expt. 4) with a combination of recombinant GH5 cellulase volume (0.25 mL), recombinant GH43 hemicellulase (α-L-arabinofuranosidase) volume (1.50 mL), *S.*

*cerevisiae* inoculum volume (1.50 mL), *C. shehatae* inoculum volume (1.50 mL), pH (5.4) and temperature (33°C) with the best response and maximum S/N ratio (-16.56) (Table 5.3.7, Fig. 5.3.6).

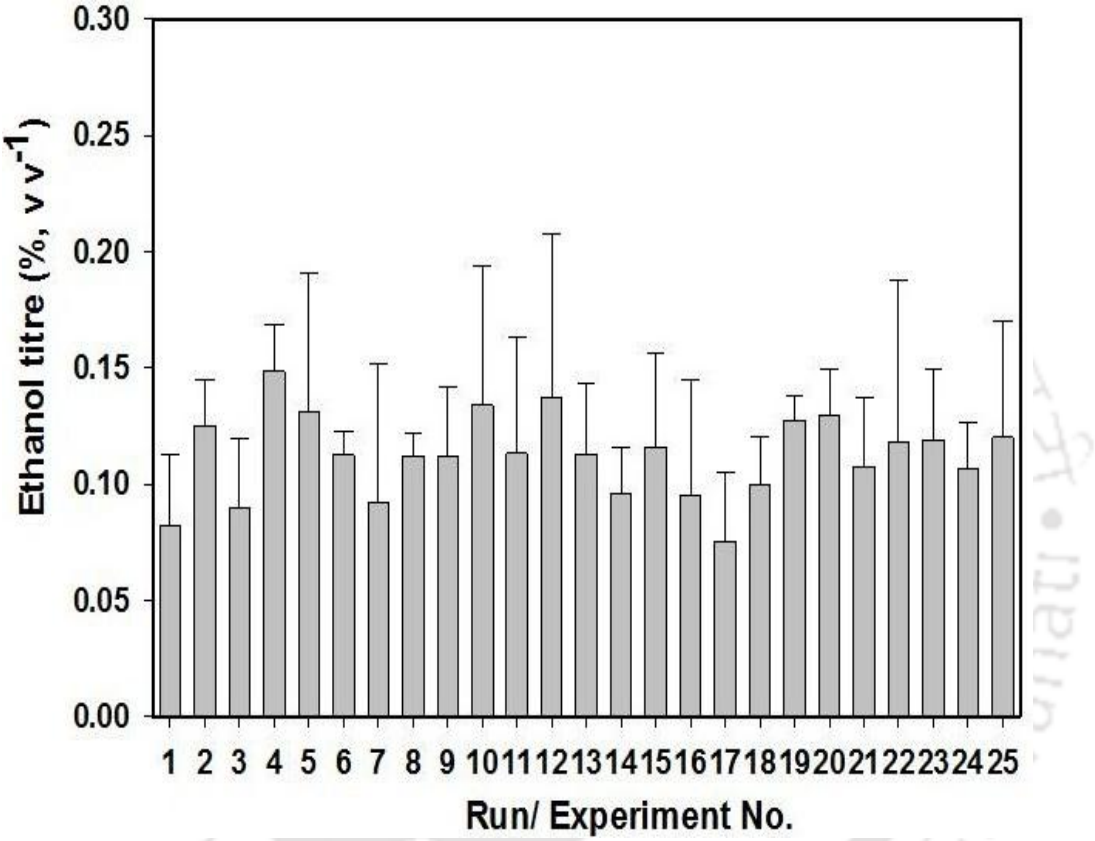


**Fig. 5.3.6** Comparative results of response in terms of ethanol titre (%, v $v^{-1}$) of water hyacinth SSF Taguchi $L_{25}$ orthogonal array of experiments.

The main effect plots for S/N ratios with 'larger is better' the objective function of Taguchi optimized fermentation process parameters for water hyacinth is presented in Fig. 5.3.7. The best fermentation process parameters in 100 mL of SSF medium encompassed 2.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 2.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 5.4 and temperature of 35°C.

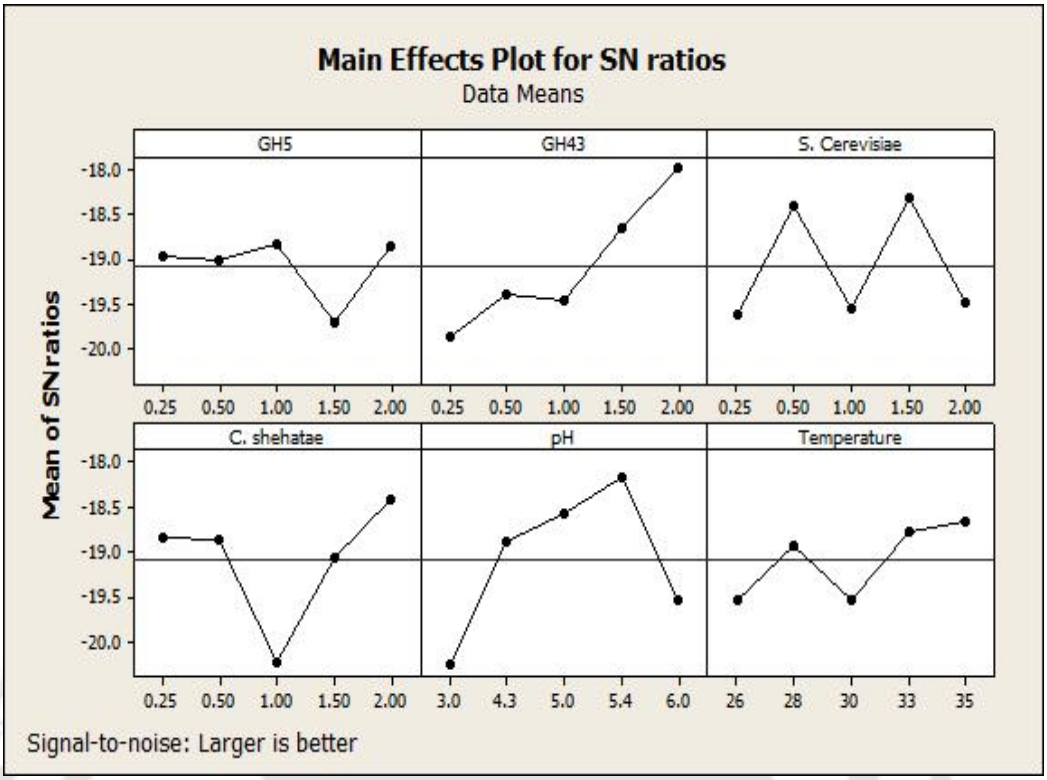


**Fig. 5.3.7** Main effect plots for S/N ratios with larger the better objective function of Taguchi optimized fermentation process parameters for water hyacinth.

The analysis of variance (ANOVA) for the responses of ethanol production from SSF of water hyacinth was carried out according to the factors' contribution by the Taguchi method (Table 5.3.8).

From the calculated ratios (F), it can be inferred that the factors considered in the experimental design are statistically significant at 95% confidence limit. Table 5.3.9 illustrated the influence of selected factors on bioethanol production.

**Table 5.3.8** Analysis of Variance for the responses of ethanol production from SSF of water hyacinth.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Recombinant GH5 cellulase | 1 | 0.0000837 | 0.0000837 | 0.0000837 | 0.96 | 0.340 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) | 1 | 0.0004547 | 0.0004547 | 0.0004547 | 0.92 | 0.351 |
| *S. cerevisiae* | 1 | 0.0003337 | 0.0003337 | 0.0003337 | 0.512 | 0.485 |
| *C. shehatae* | 1 | 0.0003377 | 0.0003377 | 0.0003377 | 0.995 | 0.332 |
| pH | 1 | 0.0005192 | 0.0005192 | 0.0005192 | 8.050 | 0.010 |
| Temp | 1 | 0.0001612 | 0.0001612 | 0.0001612 | 2.002 | 0.174 |
| Error | 18 | 0.0056715 | 0.0056715 | 0.0003150 | | |
| Total | 24 | 0.007562 | | | | |

*DF- Degrees of freedom, SS-Sum of squares, MS-Mean of Squares*

It can be witnessed on the basis of *p*-value ($p < 0.05$), pH with rank 1 is the most significant of all other factors and showed highest positive impact on ethanol production. The inoculum volume of *S. cerevisiae* exhibited least impact on bioethanol production.

**Table 5.3.9** Rank and significance of various factors for SSF from water hyacinth.

| Factor/Parameter | Rank | *p*-value |
|---|---|---|
| Recombinant GH5 cellulase* (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) | 4 | 0.340 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase)* (3.7 U$mg^{-1}$, 0.32 mg $mL^{-1}$) | 5 | 0.351 |
| *S. cerevisiae** (3.6 x $10^8$ cells $mL^{-1}$) | 6 | 0.485 |
| *C. shehatae** (2.1 x $10^8$ cells $mL^{-1}$) | 3 | 0.332 |
| pH | 1 | 0.010 |
| Temperature | 2 | 0.174 |

*$p < 0.05$*

### 5.3.4.2 Validation of Taguchi experimental model for SSF of water hyacinth

The validation of Taguchi experimental model is represented in Table 5.3.10. It was observed that the response (ethanol %, v $v^{-1}$) (0.151) as well as S/N ratio (-16.40) for Taguchi optimum values was more than experimental optimum values for ethanol production (0.149%, v $v^{-1}$) and S/N ratio (-16.56). This validated the Taguchi optimized SSF process parameters for bioethanol production from water hyacinth. Thus, there was a 1.2-fold rise in ethanol concentration with Taguchi optimized SSF process parameters as compared to unoptimized parameters (Table 5.3.2, Fig. 5.3.8).

**Table 5.3.10** Validation of Taguchi experimental data values for SSF from water hyacinth.

| Factor/ Parameter | Taguchi optimum | Experiment optimum |
|---|---|---|
| Recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) (%, v $v^{-1}$) | 2.0 | 0.25 |
| Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U$mg^{-1}$, 0.32 mg $mL^{-1}$) (%, v $v^{-1}$) | 2.0 | 1.5 |
| *S. cerevisiae* (3.6 x $10^8$ cells $mL^{-1}$) (%, v $v^{-1}$) | 1.5 | 1.5 |
| *C. shehatae* (2.1 x $10^8$ cells $mL^{-1}$) (%, v $v^{-1}$) | 2.0 | 1.5 |
| pH | 5.4 | 5.4 |
| Temperature (°C) | 35 | 33 |
| S/N ratio | -16.40 | -16.56 |
| Response experimental Ethanol titre (%, v $v^{-1}$) | 0.151 | 0.149 |
| Response predicted (%, v $v^{-1}$) Ethanol titre (%, v $v^{-1}$) | 0.168 | 0.165 |
| Ethanol titre (g $L^{-1}$) | 1.22 | 1.16 |
| Ethanol yield (g of ethanol g of substrate$^{-1}$) | 0.205 | 0.195 |

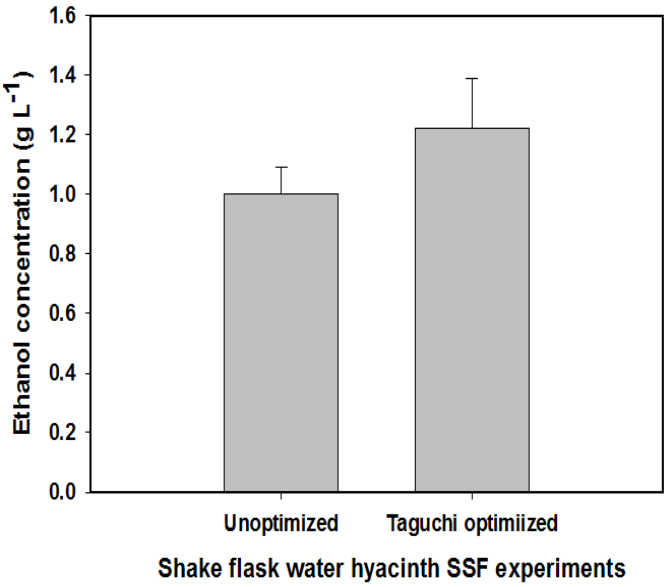


**Fig. 5.3.8** Ethanol titre (g $L^{-1}$) of water hyacinth for unoptimized and Taguchi optimized shake flask SSF experiments.

These experiments supported the analysis of the main effect of each constituent of the medium. The Taguchi SSF experiments provided basic information for the improvement of the ethanol production efficiency.

Several scientists have reported that the transport of chemical products and enzymes across the cell membrane is affected by the pH of the fermentation medium, influencing many enzymatic reactions (Latifian *et al.,* 2007; Liang *et al.,* 2010). The statistical outcomes in our research also confirmed fermentation medium pH to be an important factor affecting SSF. Similar findings have been reported in literature (Latifian *et al.*, 2007).

The dynamic profiles of various offline measurements from various SSF batch runs established a complex interplay between the rates of saccharification by the mixed recombinant enzymes, utilization of sugar by bioethanol producers and finally,

the formation of ethanol (Fig. 5.3.1 and 5.3.2). The reducing sugar profile was inversely proportional to the rate of ethanol formation. The repressed enzyme activities in the later stages of fermentation might be attributed to sugar accumulation in the broth. A depleted reducing sugar concentration was observed without any further upturn in ethanol titre during the late log phase indicating the sugars utilization only for maintenance and endurance of the fermentative microbes (Fig. 5.3.1 and 5.3.2).

The ethanol titre values obtained in our study are comparable with the findings reported in literature. An ethanol titre of 2.1 g $L^{-1}$ has been reported from 1% (w $v^{-1}$) mango leaves with recombinant GH43 hemicellulase from *C. thermocellum* and *C. shehatae* (Das *et al.,* 2012). The coculture of *C. thermosaccharolyticum* HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937 provided an ethanol concentration (2.2 g $L^{-1}$) from 1% (w $v^{-1}$) of banana waste (Reddy *et al.,* 2010). The recombinant cellulase from *Clostridium thermocellum* offered an ethanol titre of 1.4 g $L^{-1}$ from 1% (w $v^{-1}$) Jamun (*Syzygium cumini*) leafy biomass (Mutreja *et al.,* 2011). A SSF process from 6% (w $w^{-1}$) solka floc employing commercial cellulase and *Kluyveromyces marxianus* contributed an ethanol yield of 0.337 (g $g^{-1}$) (Kadar *et al.,* 2004). An ethanol titre of 1 g $L^{-1}$ from 1 (%, w $v^{-1}$) wheat straw using crude unprocessed *Trichoderma reesei* cellulase has been reported (Lever *et al.,* 2010). Das *et al.,* (2013) has reported an ethanol concentration of 1.52 g $L^{-1}$ from SSF experiments employing organosolv pretreated mango leaves along with naturally isolated *B. subtilis* cellulase, recombinant *C. thermocellum* hemicellulase (GH43) and *Candida shehatae.*

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## 5.4 Conclusions

This study reported for the first time the independent statistical optimization and validation of different fermentation process parameters for bioethanol production from mixed MAA and organosolv pretreated 1% (w $v^{-1}$) wild grass (*A. hymenoides*) and water hyacinth (*E. crassipes*) using Taguchi orthogonal array design at shake flask level *viz.*, mixed recombinant *C. thermocellum* hydrolytic enzymes' volume along with mixed fermentative microbes' inoculum volume, pH and temperature.

The mixed MAA and organosolv pretreatment strategy was employed for the improvement in breakdown of the structural carbohydrates *viz.*, cellulose and hemicellulose of the two substrates, wild grass and water hyacinth. The mixed consortium of recombinant *C. thermocellum* hydrolytic enzymes aided the significant breakdown of complex carbohydrates such as cellulose and hemicellulose. The mixed culture of bioethanol producers, *S. cerevisiae* and *C. shehatae* were engaged for efficient ethanol production owing to their capability of utilizing both hexose and pentose sugars.

In case of wild grass, the optimized process parameters in 100 mL of fermentation medium were (%, v $v^{-1}$): 1.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 0.25, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 4.3 and temperature, 35°C. pH with *p*-value 0.001 was found to be the most significant factor affecting wild grass shake flask SSF. The ethanol titre obtained in Taguchi optimized shake flask SSF was 2.0 g $L^{-1}$ implying a 1.3-fold increase as compared to ethanol titre of 1.5 g $L^{-1}$ in unoptimized shake flask SSF.

In case of water hyacinth, the optimized process parameters in 100 mL of fermentation medium were (%, v $v^{-1}$): 2.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 2.0, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 5.4 and temperature, 35°C. pH with *p*-value 0.010 was found to be the most significant factor affecting water hyacinth shake flask SSF. The ethanol titre obtained in Taguchi optimized shake flask SSF was 1.22 g $L^{-1}$ implying a 1.2-fold increase as compared to ethanol titre of 1.0 g $L^{-1}$ in unoptimized shake flask SSF.

In essence, the statistical optimization of fermentation process parameters involving recombinant enzymes can transform the grass weed, *A. hymenoides* and water weed, *E. crassipes* into the fuel of tomorrow, bioethanol.

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# Chapter 6

## Simultaneous Saccharification and Fermentation in shake flask at higher concentration of pretreated wild grass and water hyacinth and scale up in bioreactor under Taguchi optimized conditions with effective ethanol recovery

### 6.1 Introduction

The present scenario with tremendous anthropogenic climate fluctuation has revealed large utilization of fossil fuels with high $CO_2$ emission (Olsson, 1996). The existing fossil fuel depletion has rendered switch over to alternative renewable energy source to meet the energy demand at economic level. Alternative approaches towards effective and cleaner technologies are undertaken in the large scale production of second generation bioethanol from lignocellulosic biomass (Alper *et al.*, 2009; Gupta *et al.*, 2011; Zaldivar *et al.*, 2001). Agricultural and waste leafy lignocellulosic biomass has emerged to be promising raw materials for bioconversion process due to easy availability and non-competitor with food crops (Duff and Murray, 1996; Lin and Tanaka, 2006). The biological processes undergoing the breakdown of complex lignocellulosic biomass for enhanced bioethanol production requires effective pretreatment for delignification to release cellulose and hemicellulose, efficient

saccharification of the carbohydrate polymers *viz.*, cellulose and hemicellulose to produce free sugars and fermentation of mixed hexose and pentose sugars (Lynd, 1996).

Northern India has abundance of various weeds such as wild grass (*Achnatherum hymenoides*) and water hyacinth (*Eichhornia crassipes*). Production of bioethanol from land and water weeds not only provides the long-term sustainable solution in the form of low value feedstock but also solve the problem of weed management (Das *et al.,* 2012; Das *et al.,* 2013). These lignocellulosic biomasses contain 40-60% (w $w^{-1}$) cellulose and 20-40% (w $w^{-1}$) hemicellulose and 10- 30% (w $w^{-1}$) lignin (Dewes and Hunsche, 1998; Sun and Cheng, 2002). The microwave assisted alkali (MAA) pretreatment effectively loosens the cellulose and degrades lignin from the lignocellulosic biomass (Zhu *et al.*, 2006). Organosolv pretreatment break down lignin to a significant amount along with hemicellulose release (Geng *et al.*, 2012).

The anaerobic thermophilic bacterium, *Clostridium thermocellum* produces an extensive repertoire of glycoside hydrolases that are assembled into high molecular weight cellulase and hemicellulase complex which is termed the cellulosome (Fontes and Gilbert, 2010). As compared to the analogous *Trichoderma reesei* system, a 50-fold higher specific activity against crystalline cellulose is reported by *C. thermocellum* cellulosome (Demain *et al.,* 2005). Diverse substrate specificities with importance in definite biological routes may be exhibited by structurally related enzymes from same glycoside hydrolase (GH) family (Barbosa *et al.,* 1994; Fuhrer *et al.,* 2005; Seo *et al.,* 2005). Glycoside hydrolase family 5 (GH5) and family 43 (GH43) are enzymes with varying substrate specificity including cellulase (Bharali *et*

*al.,* 2005; Taylor *et al.,* 2005) and hemicellulase activity (Ahmed *et al.,* 2013), respectively that can be utilized for deriving monomeric sugars out of lignocellulosic biomass. *Saccharomyces cerevisiae*, the most frequently used fermentative microorganism for ethanol production is capable of fermenting only hexose sugars and cannot ferment pentoses (Cheng *et al.,* 2007; Kumar *et al.,* 2009). Apart from cellulose, lignocellulosic biomass contains complex hemicellulosic polymer of pentose sugars. *Candida shehatae* having xylose reductase and xylitol dehydrogenase is well-known for pentose sugar conversion to ethanol from lignocellulosic hydrolysate (Alexander *et al.,* 1988; Chandel *et al.,* 2007).

The single-stage simultaneous saccharification and fermentation (SSF) decreases the processing time and reduces the end-product inhibition of enzyme, which in turn leads to rise in ethanol production (Ballesteros *et al.,* 1991; Alfani *et al.,* 2000; Wyman, 2003; Soderstrom *et al.,* 2005). Increment in substrate concentration along with hydrolytic enzyme loadings and fermentative microbe inoculum volume enhances ethanol titre and yield (Zhang *et al.,* 2010). The scale-up approach from shake flask to bioreactor provides the ability to continuously improve and maintain fermentative microbe's productivity along with maximum ethanol production (Li *et al.,* 2009). The performance of trials in an automated bioreactor makes the stringent monitoring of important process parameters possible. The SSF process parameters such as pH and aeration significantly affect the bioconversion process and in turn, the fermentation dynamics and final ethanol titre (Du Preez, 1994; Sanchez *et al.,* 1997; Sunitha *et al.,* 1999).

The high performance anion exchange chromatography (HPAEC) is a useful analytical tool for carbohydrate determination (Rocklin *et al.,* 1998). This technique

helps in the analysis of all classes of mono-, oligo- and poly- saccharides, amino sugars, alditols based on their structural features such as size, composition, anomericity and linkage isomerism (Borromei *et al.,* 2009). Monosaccharide separations are normally performed on CarboPac PA1 and CarboPac PA10 columns, which have high selectivity for mono- and di-saccharide separations (Martinez *et al.,* 2009). Distillation, rotary vacuum evaporation, pervaporation are some of the commonly used processes for bioethanol recovery (Palmqvist *et al.,* 1996; Tao *et al.,* 2003; Cho and Jeon, 2006; Bastidas *et al.,* 2012).

In the present study, shake flask SSF trials involving mixed hydrolytic enzymes and mixed fermentative microbes were performed on 1% and 5% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth. The mixed consortium of recombinant *C. thermocellum* hydrolytic GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) were employed for effective saccharification. The mixed culture system comprising *S. cerevisiae* and *C. shehatae* were engaged for utilization of hexose and pentose sugars for bioethanol production. The shake flask SSF with 5% (w $v^{-1}$) wild grass and water hyacinth were separately, scaled-up in a bioreactor under controlled process parameters. The controlled conditions of pH and aeration were the additional parameters employed in the bioreactor as compared with shake flask that could give improved ethanol titre and yield. Subsequently, the bioethanol obtained from separate bioreactor SSF of wild grass and water hyacinth was recovered by rotary vacuum evaporator.

## 6.2 Materials and Methods

### 6.2.1 Reagents, chemicals and substrates

Isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin and carboxy methyl cellulose (CMC) were purchased from Sigma Aldrich (St. Louis, USA). Rye arabinoxylan was obtained from Megazyme International Ltd. (Ireland). The analytical grade reagents and chemicals *viz.*, agar, glucose, tryptone, peptone, yeast extract, malt extract, sodium carbonate, sodium chloride, sodium acetate, sodium sulphate, sodium potassium tartarate, sodium bicarbonate, copper sulphate, ammonium molybdate, potassium dichromate, ammonium sulphate, sodium arsenate, potassium dihydrogen phosphate, magnesium sulphate heptahydrate, sodium hydroxide, acetic acid and ampicillin were purchased from Himedia Pvt. Ltd. India. Phosphoric acid and hydrochloric acid was obtained from Qualigens India Pvt. Ltd. Coomassie Brilliant Blue G-250 was procured from Amresco LLC, USA. Sulphuric acid was purchased from Merck India Pvt. Ltd. Lignocellulosic biomass wild grass (*A. hymenoides*) and the leaves along with petioles of water hyacinth (*E. crassipes)* were collected from the campus of Indian Institute of Technology Guwahati, India. The preliminary treatment of substrates was carried out as described earlier in Chapter 3, Section 3.2.4.

### 6.2.2 Microorganisms and culturing conditions

The culturing and maintenance of *E. coli* BL21 (DE3) cells harbouring the pET-21a(+) plasmid containing a gene encoding family 5 glycoside hydrolase (GH5), a cellulase and *E. coli* BL21 (DE3) pLysS cells harbouring pET-28a(+) plasmid containing a gene encoding family 43 glycoside hydrolase (GH43), a hemicellulase

from *Clostridium thermocellum* was done as described earlier in Chapter 2, Section 2.2.2. The culturing and maintenance of predominantly aerobic fermentative microbes, *Saccharomyces cerevisiae* (NCIM no: 3215) and *Candida shehatae* (NCIM no: 3500) obtained from National Chemical Laboratory, Pune, India was accomplished as described in Chapter 3, Section 3.2.2 earlier.

### 6.2.3 Repetitive batch production of GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in Luria-Bertani (LB) medium with glucose

The repetitive batch production of recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) in separate Luria-Bertani (LB) medium supplemented with glucose was done to yield higher enzyme activity and protein concentration as described earlier in Chapter 2, Section 2.2.11. The recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) was used for saccharification of cellulose in SSF experiments of wild grass and water hyacinth as described later in Sections 6.2.5.1, 6.2.5.2, 6.2.6.1 and 6.2.6.2. The recombinant GH43 hemicellulase (α-L-arabinofuranosidase) having specific activity (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was used for hemicellulose hydrolysis in SSF experiments of wild grass and water hyacinth as described later in Sections 6.2.5.1, 6.2.5.2, 6.2.6.1 and 6.2.6.2.

### 6.2.4 Mixed microwave-assisted alkali (MAA) and organosolv pretreatment strategy

The mixed microwave-assisted alkali (MAA) and organosolv pretreatment of wild grass and water hyacinth were performed as described earlier in Chapter 3, Section 3.2.6.

### 6.2.5 Simultaneous saccharification and fermentation (SSF) trials of wild grass

Simultaneous saccharification and fermentation (SSF) experiments of mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass was performed separately in 250 mL shake flasks and 3 L bioreactor. The SSF experiments were performed employing Taguchi optimized fermentation process parameters for 1% (w $v^{-1}$) wild grass as described in Chapter 5, Section 5.2.7.3. For 5% (w $v^{-1}$) wild grass, the Taguchi optimized fermentation process parameters *viz.*, hydrolytic enzymes' volume and fermentative microbes' inocula volume were scaled-up accordingly. The hydrolytic enzymes used were recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase), whereas, the fermenting microbes were *S. cerevisiae* and *C. shehatae* as described in the Sections 6.2.5.1, 6.2.5.2 and 6.2.5.3.

#### *6.2.5.1 SSF of 1% (w $v^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The SSF trial of 1% (w $v^{-1}$) wild grass was performed using Taguchi optimized fermentation process parameters as described in Chapter 5, Section 5.2.7.3. The Taguchi optimized fermentation process parameters were 1.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 0.25 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 4.3 and temperature of 35°C. 1 g of mixed MAA and organosolv pretreated wild grass was taken in 100 mL of 20 mM sodium acetate buffer (pH 4.3) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL Erlenmeyer flask.

The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 1.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for cellulose and hemicellulose hydrolysis. Then, 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 0.25 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added at the same time. The fermentation was carried out at 35°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *6.2.5.2 SSF of 5% (w $v^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

Five grams of mixed MAA and organosolv pretreated wild grass was taken in 100 mL of 20 mM sodium acetate buffer (pH 4.3) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL Erlenmeyer flask. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 5.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and 10.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for hydrolysis. Then, 7.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 1.25 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added at the same time. The fermentation was carried out at 35°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$) of enzymes.

#### *6.2.5.3 SSF of 5% (w $v^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in bioreactor*

Fifty grams of mixed MAA and organosolv pretreated wild grass was taken in 1 L of 20 mM sodium acetate buffer (pH 4.3) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 3 L bioreactor (Applikon, Bio Console ADI 1025, Schiedam, Netherlands). The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 50.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and 100.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for saccharification. Then, 75.0 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 12.5 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added at the same time. The batch fermentation was carried out at 35°C, 120 rpm for 3 days. The aeration rate was controlled at 1 vvm by a mass flow controller to maintain dissolved oxygen (DO) level of minimum 40% for the efficient growth of fermentative microbes. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$). The pH was upheld at a set point of 4.3 by addition of 1N HCl and 1N NaOH. Thus, pH excursions of the organism below the set point were not permitted owing to its sensitivity for such changes. After the completion of SSF process, vaccum filtration of the fermentation broth was done using a vacuum filtration unit (Millipore, Massachusetts, USA) with nylon membrane having pore size of 0.45 µm. Subsequently, the filtrate collected was subjected to further recovery of ethanol by vacuum evaporation as described later in Section 6.2.7.

### 6.2.6 Simultaneous saccharification and fermentation (SSF) trials of water hyacinth in shake flask and bioreactor

Simultaneous saccharification and fermentation (SSF) experiments of mixed microwave assisted alkali (MAA) and organosolv pretreated water hyacinth was performed independently in 250 mL shake flasks and 3 L bioreactor. The SSF experiments were attained using Taguchi optimized fermentation process parameters for 1% (w $v^{-1}$) water hyacinth as described in Chapter 5, Section 5.2.8.3. For 5% (w $v^{-1}$) water hyacinth, the Taguchi optimized fermentation process parameters *viz.*, hydrolytic enzymes' volume and fermentative microbes' inocula volume were scaled-up accordingly. The hydrolytic enzymes used were recombinant GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase), whereas, the fermenting microbes were *S. cerevisiae* and *C. shehatae* as described in the Sections 6.2.6.1, 6.2.6.2 and 6.2.6.3.

#### *6.2.6.1 SSF of 1% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The SSF experiment of 1% (w $v^{-1}$) water hyacinth was performed using Taguchi optimized fermentation process parameters as described in Chapter 5, Section 5.2.8.3. The Taguchi optimized fermentation process parameters were 2.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$), 2.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$), 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$), 2.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$), pH of 5.4 and temperature of 35°C. 1 g of mixed MAA and organosolv pretreated water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL

Erlenmeyer flask. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 2.0 mL each of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for saccharification. Then, 1.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 2.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added simultaneously. The fermentation was carried out at 35°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$).

#### *6.2.6.2 SSF of 5% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

Five grams of mixed MAA and organosolv pretreated water hyacinth was taken in 100 mL of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 250 mL Erlenmeyer flask. The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 10.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and 10.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for saccharification. Then, 7.5 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 10.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added at the same time. The fermentation was carried out at 35°C, 120 rpm for 3 days. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$) of enzymes.

### *6.2.6.3 SSF of 5% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in bioreactor*

Fifty grams of mixed MAA and organosolv pretreated water hyacinth was taken in 1 L of 20 mM sodium acetate buffer (pH 5.4) supplemented with yeast extract (0.1%, w $v^{-1}$) and peptone (0.1%, w $v^{-1}$) in 3 L bioreactor (Applikon, Bio Console ADI 1025, Schiedam, Netherlands). The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min. 100.0 mL of recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$) and 100.0 mL of recombinant GH43 hemicellulase (α-L-arabinofuranosidase) (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$) was added for hydrolysis. Then, 75 mL of *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$) and 100.0 mL of *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$) was added for fermentation. Both the enzymes and the fermentative microbes were added at the same time. The batch fermentation was carried out at 35°C, 120 rpm for 3 days. The aeration rate was controlled at 1 vvm by a mass flow controller to maintain dissolved oxygen (DO) level of minimum 40% for the efficient growth of fermentative microbes. The sample (2 mL) was collected at every 6 h for estimation of cell OD at 600 nm, reducing sugar (g $L^{-1}$), ethanol concentration (g $L^{-1}$) and specific activity (U $mg^{-1}$). The pH was maintained at a set point of 5.4 by addition of 1N HCl and 1N NaOH. Consequently, pH excursions below the set point were not allowed owing to its sensitivity for such changes. After the accomplishment of SSF, vaccum filtration of the fermentation broth was carried out using a vacuum filtration unit (Millipore, Massachusetts, USA) with nylon membrane of pore size (0.45 µm). The filtrate collected was subjected to further recovery of ethanol by vacuum evaporation as described later in Section 6.2.7.

### 6.2.7 Recovery of partially purified ethanol from bioreactor SSF of wild grass and water hyacinth

The filtered fermentation broth containing bioethanol from separate bioreactor SSF experiments of wild grass and water hyacinth were independently concentrated under vacuum in a rotary evaporator (Buchi Rotavapor R-200, Switzerland). The process was carried out in a 2 L round bottom evaporation flask encompassing 1 L working volume of fermentation broth. The heating was done in a water bath (Buchi Heating Bath B-490) for 3 h at 78.5°C. Finally, the distillate containing the partially purified ethanol was collected and estimated by Gas chromatography (GC) and Dichromate assay as described in Chapter 3, Section 3.2.9.8. The purification process efficiency of ethanol obtained by rotary evaporator was calculated by using the following equation,

$$\text{Purification process efficiency (\%)} = \frac{\text{volume of partially purified ethanol in distillate (mL L}^{-1})}{\text{crude ethanol in fermentation broth (mL L}^{-1})} \times 100$$

### 6.2.8 Analytical methods

#### *6.2.8.1 Measurement of cell growth during SSF*

The cell growth of *S. cerevisiae* or *C. shehatae* during fermentation was estimated with the withdrawl of initial medium along with the pretreated substrate prior to inoculation as blank. Then, the fermentative microbe *S. cerevisiae* or *C. shehatae* was inoculated into the fermentation medium. The remaining procedure of cell growth estimation was accomplished as described earlier in Chapter 3, Section 3.2.11.2.

### *6.2.8.2 High pressure anion exchange chromatography (HPAEC) analysis of polysaccharides hydrolyzed by GH5 cellulase and GH43 hemicellulase*

High pressure anion exchange chromatography (HPAEC) was executed independently to detect monosaccharides released by enzymatic degradation of complex polysaccharides from wild grass (*A. hymenoides*) and water hyacinth (*E. crassipes*) during separate bioreactor SSF trials. A CARBOPACK$^{TM}$ PA-20 column (Dionex) (3 x 150 mm) was used as described by Van Gool *et al.*, (2011) with modification in flow rate. The ion chromatography system (ICS-3000, Dionex, California, USA) was kept at 30°C with a loop size of 25.0 µL and flow rate of 0.5 mL min$^{-1}$ throughout the analyses. The elution of reducing sugars were performed with 100.0 mM NaOH and analyzed by pulsed amperometric detector (PAD) in tandem with Dionex (ICS-3000). The HPAEC profiles of the hydrolyzed product, glucose by GH5 cellulase and arabinose by GH43 hemicellulase (α-L-arabinofuranosidase) were studied at 0, 18, 36, 54 and 72 h, respectively. Arabinose, glucose and xylose were used as standard (1.2 mg mL$^{-1}$ final concentration of each sugar in the standard mixture). The crude sample (200 µL) of fermentation broth obtained from bioreactor SSF was diluted with 400 µL of ultrapure water and centrifuged at 15,493***g*** for 15 min. The supernatant (500 µL) was filtered through 0.2 µm membrane and subsequently 25.0 µL sample was injected manually into HPAEC-PAD.

### *6.2.8.3 Recombinant GH5 cellulase assay*

The reducing sugar estimation and recombinant GH5 cellulase assay was performed as described earlier in Chapter 2, Section 2.2.12.1.

### *6.2.8.4 Recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay*

The reducing sugar estimation and recombinant GH43 hemicellulase (α-L-arabinofuranosidase) assay was performed as described earlier in Chapter 2, Section 2.2.12.2.

### *6.2.8.5 Protein content determination*

The protein content was determined as described earlier in Chapter 2, Section 2.2.12.6.

### *4.2.6.6 Ethanol content determination by Gas chromatography and Dichromate method*

The ethanol obtained from SSF experiments of wild grass and water hyacinth were estimated by Gas chromatography (GC) and Dichromate assay as described earlier in Chapter 3, Section 3.2.11.8.

### *4.2.6.7 Determination of ethanol yield*

The ethanol yield was calculated as described earlier in Chapter 3, Section 3.2.11.9.

## 6.3 Results and Discussion

Rate limiting step in simultaneous saccharification and fermentation (SSF) process is the production of utilizable reducing sugars from the complex lignocellulosic residues and removing the non-utilizable metabolites (Zhang *et al.*, 2010). The structural carbohydrate composition of untreated and pretreated wild grass and water hyacinth was done as described earlier in Chapter 3, Section 3.2.9.1. Wild grass (*A. hymenoides*) displayed cellulose (51.70%, w $w^{-1}$), hemicellulose (30.92% w $w^{-1}$) and lignin (18.71% w $w^{-1}$) whereas water hyacinth (*E. crassipes*) showed cellulose 30.07%, w $w^{-1}$, hemicellulose (44.52%, w $w^{-1}$) lignin (29.40%, w $w^{-1}$) signifying both wild grass and water hyacinth as the potential lignocellulosic biomass for bioethanol production. The mixed MAA and organosolv pretreatments of wild grass provided cellulose (46.30%), hemicellulose (26.50%) and lignin (15.20%). The mixed MAA and organosolv pretreatments of water hyacinth contributed cellulose (26.10%), hemicellulose (33.20%) and lignin (15.64%).

### 6.3.1 Simultaneous saccharification and fermentation of wild grass

The outcome of SSF experiments performed on mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass at different substrate concentration in shake flask and bioreactor are displayed in Table 6.3.1.

#### *6.3.1.1 SSF of 1% (w $v^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The dynamic profile of SSF involving recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* for ethanol production from mixed pretreated 1% (w $v^{-1}$) wild grass in shake flask level is

exhibited in (Fig. 6.3.1). The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited no lag phase in their growth with an exponential increase till 66 h and small decline thereafter (Fig. 6.3.1). The growth-associated ethanol formation was initiated from 0-12 h of SSF with a gradual escalation till 36 h after which a sharp augmentation was observed till 60 h (Fig. 6.3.1). The maximum ethanol titre accomplished was 2.0 g $L^{-1}$ with a yield of 0.274 (g of ethanol g of substrate$^{-1}$) at 60 h (Table 6.3.1, Fig. 6.3.1).

**Table 6.3.1** Comparison of SSF combinations involving recombinant hydrolytic enzymes and fermentative microbes with wild grass.

| Substrate concentration and Mode of fermentation | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|
| 1%, Shake flask | 2.31 ± 0.05 | 2.0 ± 0.04 | 0.274 |
| 5%, Shake flask | 13.85 ± 0.03 | 10.9 ± 0.06 | 0.299 |
| 5%, Bioreactor | 23.02 ± 0.05 | 18.0 ± 0.07 | 0.494 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

Thereafter, a decrease in ethanol production was witnessed. The preliminary phase of SSF signified an accumulation of reducing sugars till 12 h. The maximum reducing sugar concentration was 2.31 g $L^{-1}$ (Table 6.3.1, Fig. 6.3.1) at 6 h. The level of reducing sugar showed a gradual decline till 42 h and later showed a much steeper decrease reaching the minimum level of 1.18 g $L^{-1}$ at 72 h (Fig. 6.3.1). As cellulose is in major amount in wild grass, the profile of only GH5 cellulase activity is shown. The GH5 cellulase activity showed a sinusoidal behavior with advancement in fermentation. The lesser enzyme activity of 3.97 U $mg^{-1}$ at 12 h and 2.84 U $mg^{-1}$ at 18 h might be due to inhibition by accumulated reducing sugar in initial hours of SSF (Fig. 6.3.1).

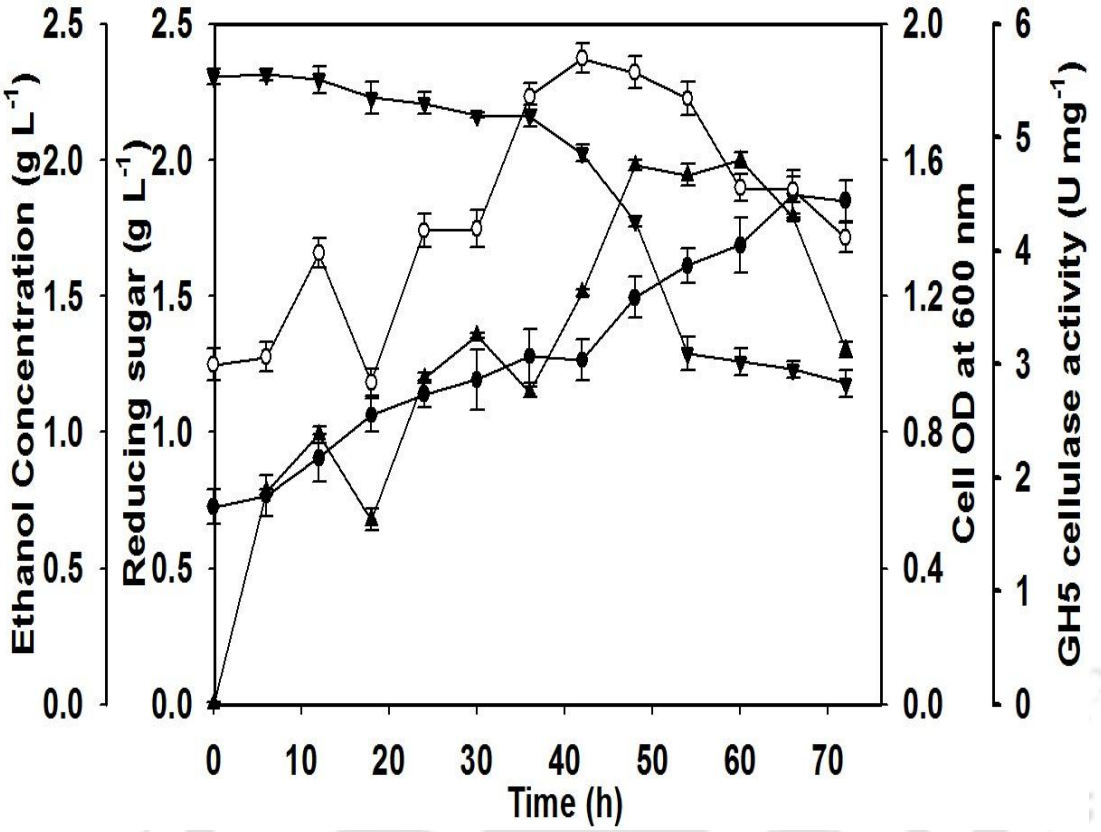


**Fig. 6.3.1** SSF profile of 1% (w $v^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 35°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

After 18 h there was a gradual increment reaching up to the maximum value of 5.69 U $mg^{-1}$ at 42 h (Fig. 6.3.1). Thereafter, a decrease in GH5 cellulase activity was observed with a minimum value of 4.11 U $mg^{-1}$ at the end of SSF (Fig. 6.3.1). Interestingly, the growth associated ethanol production depicted an inverse relationship with the released reducing sugars representing the fact that sugar was

utilized by the *S. cerevisiae* and *C. shehatae* for growth and ethanol formation (Fig. 6.3.1).

### *6.3.1.2 SSF of 5% (w v$^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The SSF profile exhibited a single exponential growth phase of the fermentative microbes with 5% (w v$^{-1}$) wild grass in shake flask. The cell OD initially remained low with an initial value 0.67 at 0 h followed by an exponential increase to a maximum value of 6.1 at 60 h of fermentation (Fig. 6.3.2).

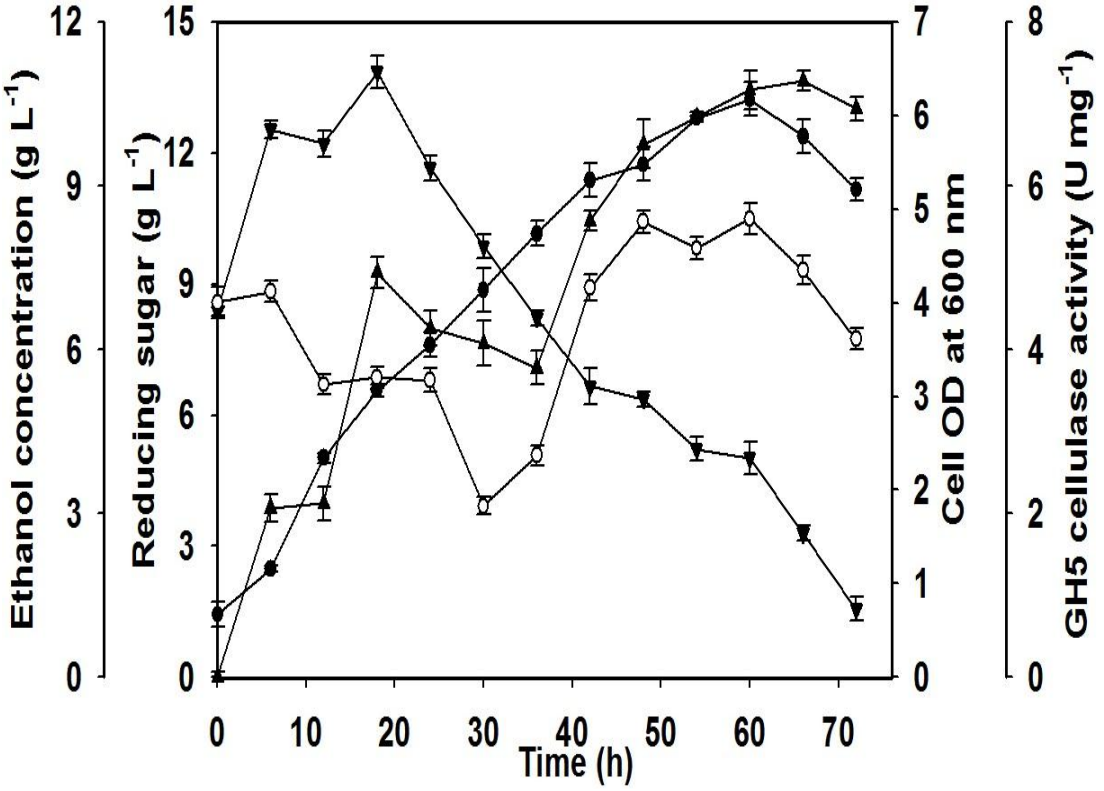


**Fig. 6.3.2** SSF profile of 5% (w v$^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g L$^{-1}$), (▼) reducing sugar (g L$^{-1}$) and (○) specific activity of GH5 cellulase (U mg$^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 4.3; temperature 35°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

The dynamic profile of reducing sugar showed a substantial accumulation in the initial phase of fermentation (13.85 g $L^{-1}$ at 18 h), followed by a sharp decrease in concentration concomitant with exponential phase of growth (Table 6.3.1, Fig. 6.3.2). During initial phase of fermentation there was an increase in ethanol titre of 7.40 g $L^{-1}$ at 18 h, but a substantial decrease was observed reaching 5.6 g $L^{-1}$ at 36 h. Thereafter, a steady increase in ethanol titre was observed with maximum concentration of 10.91 g $L^{-1}$ at 66 h (Table 6.3.1, Fig. 6.3.2). The rate of ethanol formation was found to be proportional with the rate of substrate utilization and cell growth (Fig. 6.3.2). Only the specific activity profile of GH5 cellulase is shown, because wild grass contains more cellulose. The specific activity of GH5 cellulase was 4.7 U $mg^{-1}$ in the initial 6 h and after that a decline occurred due to inhibition by accumulated reducing sugar, then after 42 h it showed a sinusoidal increase reaching to a maximum of 5.7 U $mg^{-1}$ at 60 h. The ethanol yield obtained was 0.299 (g of ethanol g of substrate$^{-1}$) (Table 6.3.1). At 72 h, the concentration of reducing sugar reached to the minimum level of 1.55 g $L^{-1}$ along with the decrease in cell OD of 5.2. Interestingly, the inverse relationship between reducing sugar concentration and specific activity of the enzyme was found to be more prominent with the increase in substrate concentration. A 5.4-fold upsurge in ethanol concentration and 9% rise in ethanol yield were observed on increasing the substrate concentration from 1% to 5% in the shake flask (Table 6.3.1).

#### *6.3.1.3 SSF of 5% (w $v^{-1}$) wild grass involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in bioreactor*

The control and monitoring of SSF process parameters is possible in a bioreactor. The dynamic profile of bioreactor SSF employing 5% (w $v^{-1}$) wild grass along with mixed enzyme-mixed culture system is depicted in Fig. 6.3.3. The mixed

culture of fermentative organisms followed an exponential growth, remaining in the lag phase for initial 6 h with an initial cell OD of 0.65 at 0 h (Fig 6.3.3). There was an increase in biomass concentration as the organisms entered the log phase until the 66 h, then reached a maximum cell OD of 10, and finally, followed a decline in the growth. Ethanol formation was observed in two distinct phases. The first phase of ethanol production recorded a titre of 12.89 g $L^{-1}$ at 18 h of fermentation followed by a slight decrease in the rate of ethanol synthesis till 36 h (Fig 6.3.3).

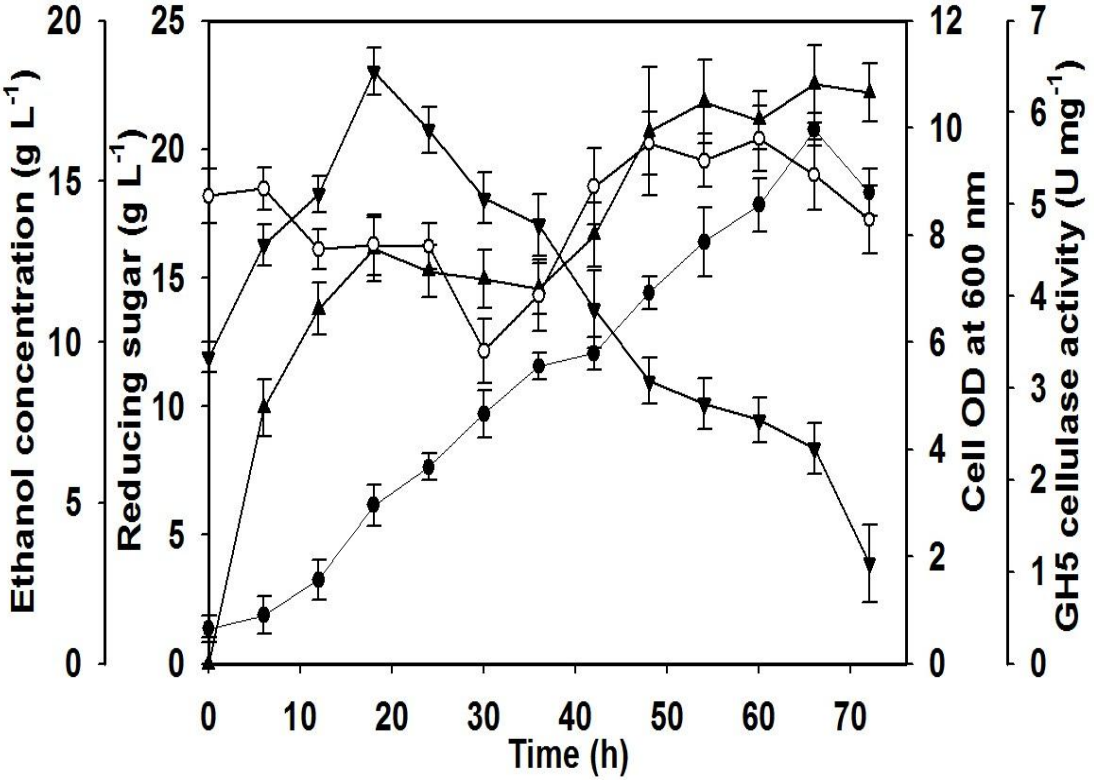


**Fig. 6.3.3** SSF profile of 5% (w $v^{-1}$) wild grass using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in bioreactor. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH5 cellulase (U $mg^{-1}$) with time (h). SSF was carried out in 1 L medium contained in 3 L bioreactor; initial pH 4.3; temperature 35°C, agitation 120 rpm and aeration 1 vvm. Similar specific activity profiles were obtained for recombinant hemicellulase (GH43) (data not shown). Values are mean ± SE (n=3).

The second and final phase of fermentation witnessed an increase in ethanol titre of 16 g $L^{-1}$ at 48 h and finally a maximum value of 18 g $L^{-1}$ with an ethanol yield of 0.494 (g of ethanol g of substrate$^{-1}$) at 66 h and then showed decline till the end of the SSF process (Table 6.3.1, Fig. 6.3.3). The dynamic profile of only recombinant GH5 cellulase has been shown in Fig. 6.3.3 as wild grass contains more cellulose. The initial specific activity was maximum (5.2 U $mg^{-1}$) at 6 h and later declined to 3.9 U $mg^{-1}$ till 30 h as the increased sugar concentration inhibited the enzyme activity of GH5 cellulase. Afterwards the specific activity of GH5 cellulase increased and reached a maximum 5.7 U $mg^{-1}$ at 60 h, which, declined thereafter. The reducing sugar concentration escalated during the initial 18 h reaching a maximum value of 23.0 g $L^{-1}$ (Table 6.3.1, Fig. 6.3.3). A 75% repressive effect on the activity of cellulase was reported by a glucose concentration of 20 g $L^{-1}$ (Oh *et al.*, 2000). The uptake of sugar by the fermentative microbes for their growth, maintenance and production of ethanol after 36 h accounted for the drop in sugar concentration for the rest of the period of fermentation process. The dynamic profile of SSF exhibited an inverse relationship between rates of sugar utilization and ethanol formation.

The degradation products released from cellulosic and hemicellulosic content of wild grass by saccharification in a bioreactor were detected by HPAEC-PAD (Fig. 6.3.4A, B, C, D, E and F). The retention time for different monosaccharide sugars *viz.* arabinose, glucose and xylose used as standard were 3.71, 4.23 and 4.98 min, respectively (Fig. 6.3.4A). The HPAEC profile of the monosaccharides at 0 h witnessed little amount of xylose that might have released along with arabinose and glucose during pretreatment (Fig. 6.3.4B).

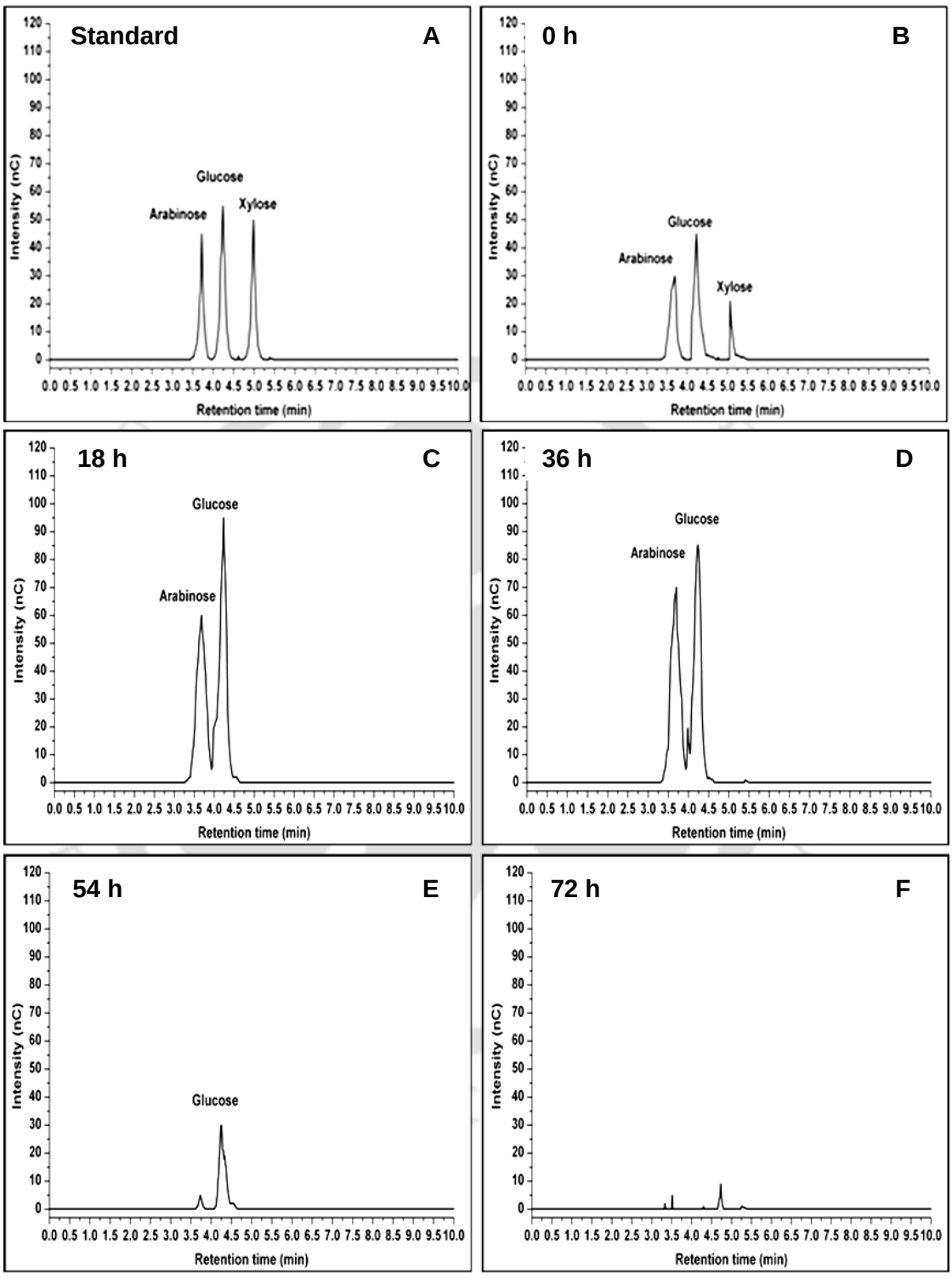


**Fig. 6.3.4** HPAEC Profiles of monosaccharides obtained from bioreactor SSF of 5% (w $v^{-1}$) wild grass. The chromatogram of sugar hydrolysate *viz.*, arabinose, glucose and xylose were obtained at different time intervals by HPAEC-PAD (A) Standards (B) 0 h (C) 18 h (D) 36 h (E) 54 h (F) 72 h.

Surprisingly, no xylose was detected in the subsequent stages of SSF due to its uptake by *C. shehatae* (Fig. 6.3.4C, D, E and F). The HPAEC profile at 18 h detected substantial amount of glucose and arabinose released by mixed enzyme consortium (GH5 cellulase and GH43 hemicellulase) (Fig. 6.3.4C). The HPAEC profile at 36 h also exhibited considerable amount of monosaccharide sugars glucose and arabinose (Fig. 6.3.4D). At 54 h, the HPAEC profile exhibited much reduced amount of glucose and trace quantity of arabinose due to their utilization by *S. cerevisiae* and *C. shehatae* for ethanol formation (Fig. 6.3.4E). The HPAEC pattern at 72 h showed negligible amounts of monosaccharide sugars due to their consumption by fermentative yeasts for growth, maintenance and ethanol formation (Fig. 6.3.4F). A 1.6-fold augmentation both in ethanol titre and yield was observed on scaling up the shake flask SSF with 5% (w $v^{-1}$) substrate concentration to bioreactor level.

The controlled pH 4.3 and aeration rate 1 vvm significantly affected the growth and ethanol titre. A threshold dissolved oxygen (DO) level of minimum 40% was upheld by 1 vvm aeration rate for the effective growth of fermentative organisms and in turn a good product yield. Addition of *C. shehatae* along with *S. cerevisiae* increased the overall ethanol yield as the pentose sugars released from lignocellulosic hydrolysis were metabolized by *C. shehatae*.

The ethanol titre outcomes obtained in our study are analogous with that in literature. 1% (w $v^{-1}$) *Mangifera indica* leaves with recombinant *C. thermocellum* GH43 hemicellulase and *Candida shehatae* yielded an ethanol titre of 2.1 g $L^{-1}$ (Das *et al.,* 2013). A SSF experiment involving 5% (w $w^{-1}$) solid content with commercial cellulase enzyme and *Zymomonas mobilis* as fermentative organism gave an ethanol concentration of 1 g $L^{-1}$ in shake flask (Santos *et al.,* 2010). An ethanol yield of 0.474

g of ethanol g of substrate$^{-1}$ was obtained from 50 g bermuda grass employing commercial cellulase and *S. cerevisiae* (Li *et al.,* 2009). Kadar *et al.,* (2004) reported an ethanol yield of 0.337 (g g$^{-1}$) in a shake flask SSF process from 6% (w w$^{-1}$) solka floc employing commercial cellulase and *Kluyveromyces marxianus.* Zhang *et al.,* (2010) reported an ethanol yield of 0.463 g of ethanol g of substrate$^{-1}$ with 19% (w w$^{-1}$) dry corncorb and commercial cellulolytic enzymes in a bioreactor.

#### *6.3.1.4 Recovery of partially purified ethanol and purification process efficiency determination from bioreactor SSF of wild grass*

The ethanol from fermentation broth was recovered using a rotary vacuum evaporator. The crude ethanol obtained in SSF studies employing mixed recombinant enzymes (GH5 cellulase, GH43 hemicellulase) and mixed cultures (*S. cerevisiae, C. shehatae*) using 5% (w v$^{-1}$) mixed MAA with organosolv pretreated wild grass at bioreactor level was 22.81 mL L$^{-1}$ i.e, 18 g L$^{-1}$ (Table 6.3.1). 1 L fermentation broth with crude ethanol on vacuum evaporation yielded 5.70 mL of distillate containing 5.25 mL i.e., 92.1% (v v$^{-1}$) of partially purified ethanol. Finally, the efficiency of purification was estimated to be 23%. The remaining ethanol in the broth obtained with the water condensates could be recovered through repeated distillation. In the large scale operations, multiple distillation steps are included to obtain 100% recovery from the water condensates (Palmqvist *et al.,* 1996). The minimum evaporation loss in the condensate collector with maximum recovery can be achieved by a rotary vacuum evaporator equipped with multiple condenser units.

### 6.3.2 Simultaneous saccharification and fermentation of water hyacinth

The consequences of SSF experiments accomplished on mixed microwave assisted alkali (MAA) and organosolv pretreated water hyacinth at different substrate concentration in shake flask and bioreactor are displayed in Table 6.3.2.

**Table 6.3.2** Comparison of SSF combinations involving recombinant hydrolytic enzymes and fermentative microbes with water hyacinth.

| Substrate concentration and Mode of fermentation | Reducing sugar (g $L^{-1}$)* | Ethanol titre (g $L^{-1}$)* | Ethanol yield (g of ethanol g of substrate$^{-1}$) |
|---|---|---|---|
| 1%, Shake flask | 1.41 ± 0.05 | 1.22 ± 0.03 | 0.205 |
| 5%, Shake flask | 9.74 ± 0.04 | 6.20 ± 0.03 | 0.209 |
| 5%, Bioreactor | 19.68 ± 0.07 | 13.70 ± 0.05 | 0.461 |

**the values correspond to the maximum reducing sugar and maximum ethanol at a particular time, values are mean ± SE (n=3)*

#### *6.3.2.1 SSF of 1% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The dynamic profile of SSF comprising recombinant GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* for ethanol production from mixed pretreated 1% (w $v^{-1}$) water hyacinth at shake flask level is depicted in Fig. 6.3.5. The mixed cultures of *S. cerevisiae* and *C. shehatae* exhibited negligible lag phase of growth with steady rise of cell OD till 60 h and then a slight decline (Fig. 6.3.5). A maximum OD of 1.5 was reached at 60 h of SSF (Fig. 6.3.5). The ethanol formation began initially at 0 h and at 30 h reached a titre of 1.02 g $L^{-1}$ with a slight decline to 0.99 g $L^{-1}$ at the 36 h, and after that a sharp rise was observed till 54 h was (Fig. 6.3.5). The maximum ethanol concentration accomplished was 1.22 g $L^{-1}$ with a yield of 0.205 (g of ethanol g of substrate$^{-1}$) (Table 6.3.2, Fig. 6.3.5).

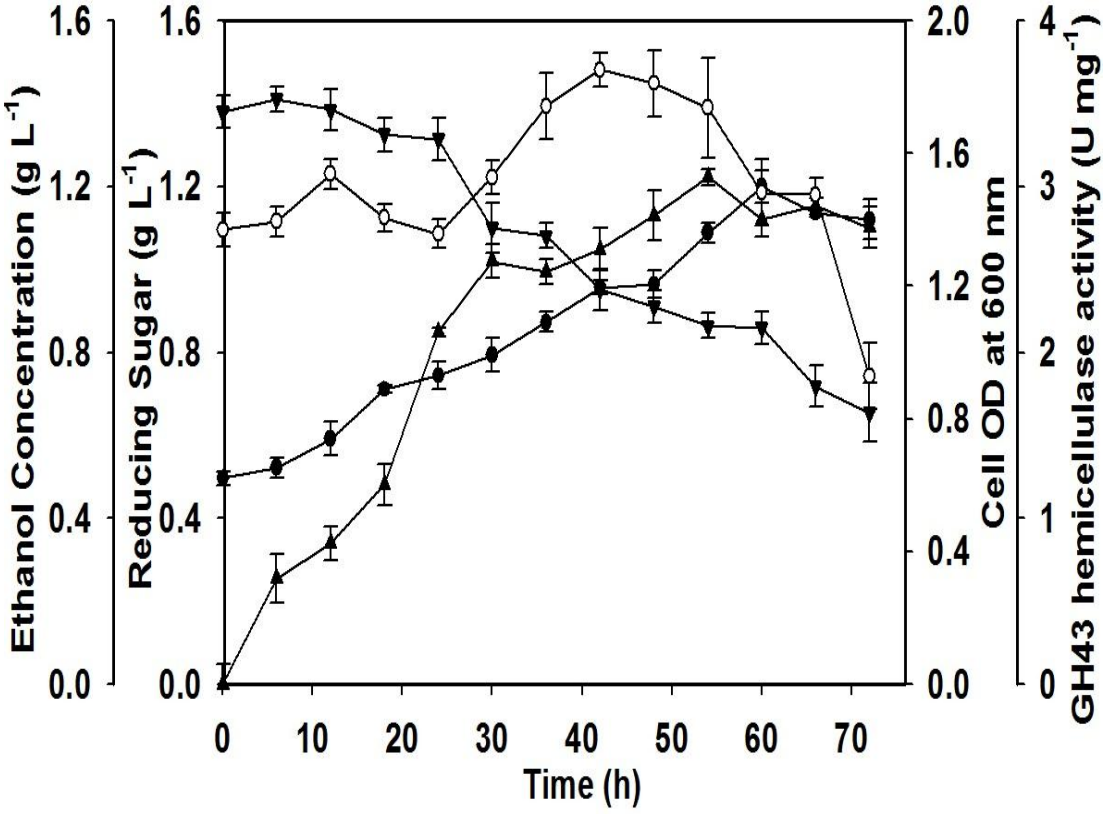


**Fig. 6.3.5** SSF profile of 1% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 35°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

The early phase of SSF showed sugar accumulation during first 12 h and then a decline. The maximum reducing sugar concentration was 1.41 g $L^{-1}$ (Table 6.3.2, Fig. 6.3.5). A minimum level of 0.65 g $L^{-1}$ reducing sugar concentration was attained at 72 h marked by the end of SSF experiment. The dynamic profile of only recombinant GH43 hemicellulase (α-L-arabinofuranosidase) has been shown in Fig.

6.3.5 due to more hemicellulose content of water hyacinth. The GH43 hemicellulase activity was less during initial hours of SSF when the reducing sugar concentration was more thereby, imposing an inhibitory effect on the enzyme activity. There was a substantial increase in GH43 hemicellulase (α-L-arabinofuranosidase) activity reaching a maximum of 3.7 U $mg^{-1}$ at 42 h and thereafter showed a decline to 1.8 U $mg^{-1}$ at 72 h. The growth associated ethanol formation and GH43 hemicellulase activity shared an inverse relationship with the reducing sugar concentration.

#### *6.3.2.2 SSF of 5% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in shake flask*

The SSF process in shake flask using mixed pretreated 5% ($wv^{-1}$) water hyacinth, mixed recombinant enzymes and mixed bioethanol producers showed that the growth of the microbes remained in the lag phase for initial 6 h with OD of 0.52 (Fig. 6.3.6). However, there was a steady increase in cell biomass from 12 to 72 h due to the presence of pentose sugars along with hexoses reaching a maximum cell OD of 3.0 at 60 h (Fig. 6.3.6). The initial ethanol titre was low and gradually started increasing at 12 h with 1.04 g $L^{-1}$ and increased to 1.59 g $L^{-1}$ at 18 h (Fig. 6.3.6). The maximum ethanol concentration attained was 6.2 g $L^{-1}$ with a yield coefficient of 0.209 (g of ethanol g of substrate$^{-1}$) at 60 h (Table 6.3.2, Fig. 6.3.6). The amount of sugars (hexoses and pentoses) released from the substrate accounted to the effectiveness of the pretreatment method and the enzyme activity. The initial amount of reducing sugars (hexoses and pentoses) in the fermentation broth was high at 12 h (Fig. 6.3.6). The reducing sugar showed a sinusoidal behaviour with an initial increase depicting a maximum concentration of 9.74 g $L^{-1}$ at 12 h (Table 6.3.2, Fig. 6.3.6). The

sugars released after hydrolysis of the pretreated substrate by the enzymes were initially used by the microbes for acclimatization and growth and this is evident from the low ethanol titre obtained during that period. Afterwards a sinusoidal pattern of increment and decline of reducing sugar was observed till 42 h. Thereafter, a regular decline was observed as time progressed reaching a minimum level of 0.99 g $L^{-1}$ at 72 h (Fig. 6.3.6).

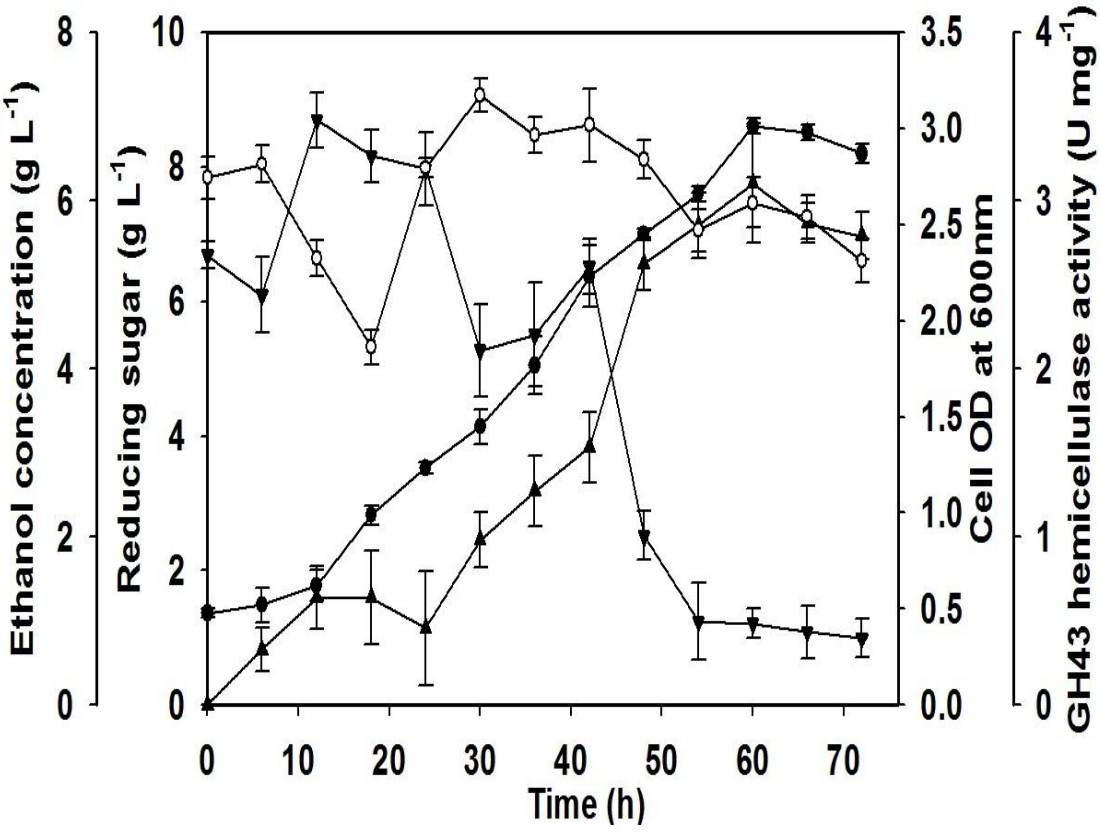


**Fig. 6.3.6** SSF profile of 5% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in shake flask. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 100 mL medium contained in 250 mL Erlenmeyer flask; initial pH 5.4; temperature 35°C and shaking 120 rpm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

An intriguing relationship was detected between sugar concentration and enzyme activities of recombinant GH5 cellulase and GH43 hemicellulase (Fig 6.3.6). As water hyacinth contains more hemicellulose, the dynamic profile of only recombinant GH43 hemicellulase was shown (Fig. 6.3.6). The organisms consumed more amounts of sugars for their growth in a new environment and likewise, the enzyme activity remained high (2.9 U $mg^{-1}$) as the reducing sugar level and in turn, the feedback inhibition was low during the early stages (6 h) of the experiment. Thereafter, enzyme activity of recombinant GH43 hemicellulase decreased to 2.2 U $mg^{-1}$ at 18 h, as it was inhibited by the presence of reducing sugar released from hemicellulose (Fig. 6.3.6). Similar findings on feedback inhibition of enzyme by increased reducing sugar concentration have been reported in the literature (Govindaswamy and Vane, 2007; Andric *et al.*, 2010. After 18 h, there was an increase in specific activity of GH43 hemicellulase reaching a maximum 3.54 U $mg^{-1}$ at 30 h (Fig. 6.3.6). Towards the end of SSF experiment (60 h), an elevated rate of sugar depletion was observed with the simultaneous decline in specific activity. This was due to the increase in uptake of nutrients by the organisms for maintenance and survival during 60-72 h of SSF (Fig. 6.3.6). A 5-fold increase in ethanol concentration and 2% rise in ethanol yield were detected on increasing the substrate concentration from 1% to 5% at shake flask level (Table 6.3.1).

#### *6.3.2.3 SSF of 5% (w $v^{-1}$) water hyacinth involving GH5 cellulase, GH43 hemicellulase, S. cerevisiae and C. shehatae in bioreactor*

The SSF experiment was scaled up with 5% (w $v^{-1}$) pretreated substrate water hyacinth from shake flask to bioreactor level. The growth profile of the organism in

the bioreactor followed a sinusoidal behaviour (Fig. 6.3.7). The organisms remained in the lag phase with cell OD of 0.75 at 6 h (Fig. 6.3.7). The exponential growth of the fermentative microbe was observed and it continued to grow till the 66 h, after which it entered the stationary phase.

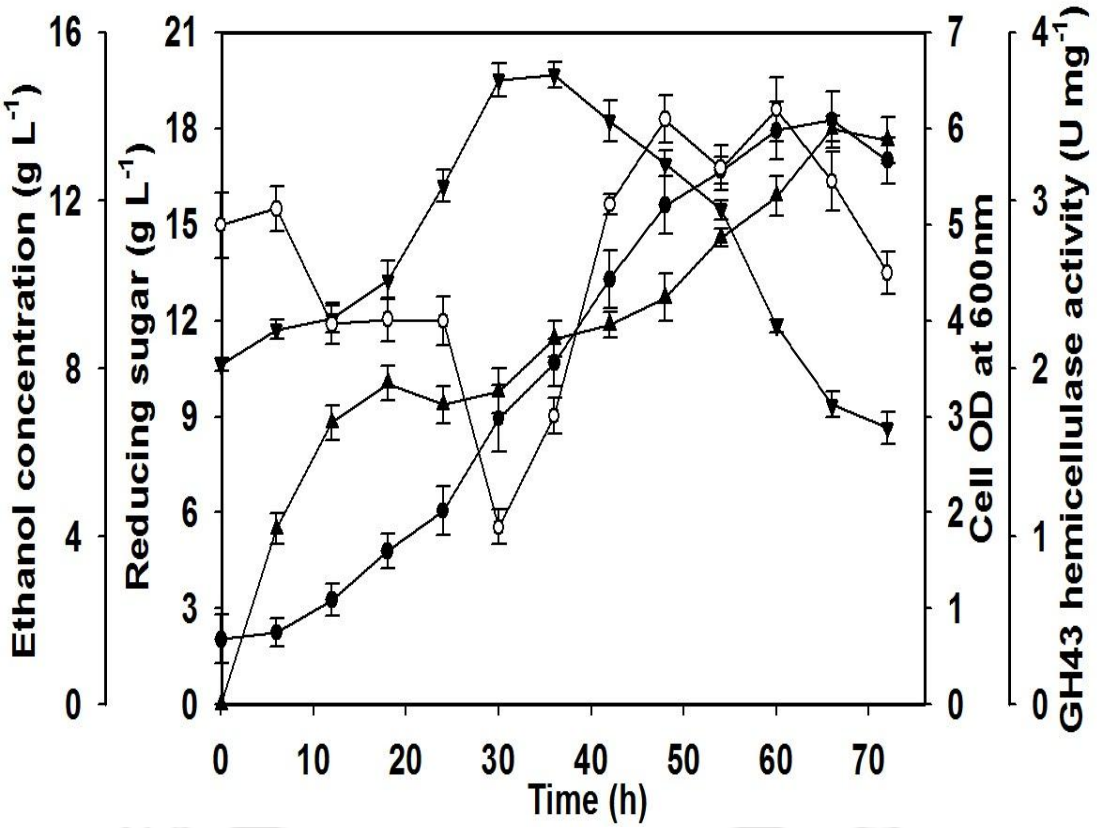


**Fig. 6.3.7** SSF profile of 5% (w $v^{-1}$) water hyacinth using GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase), *S. cerevisiae* and *C. shehatae* in bioreactor. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g $L^{-1}$), (▼) reducing sugar (g $L^{-1}$) and (○) specific activity of GH43 hemicellulase (α-L-arabinofuranosidase) (U $mg^{-1}$) with time (h). SSF was carried out in 1 L medium contained in 3 L bioreactor; initial pH 5.4; temperature 35°C, agitation 120 rpm and aeration 1 vvm. Similar specific activity profiles were obtained for recombinant cellulase (GH5) (data not shown). Values are mean ± SE (n=3).

The ethanol concentration displayed a rapid increase, reaching a titre 7.65 g $L^{-1}$ at 18 h and then a slow rise reaching 9.68 g $L^{-1}$ at 48 h (Fig. 6.3.7). The ethanol titre continued to increase and reached a maximum 13.70 g $L^{-1}$ with a yield coefficient of

0.461 (g of ethanol g of substrate$^{-1}$) at 66 h (Table 6.3.2, Fig. 6.3.7). The specific activity of only recombinant GH43 hemicellulase was depicted in the dynamic profile of SSF due to more hemicellulose content of water hyacinth. The recombinant GH43 hemicellulase activity showed a sigmoidal behaviour with an initial value 2.95 U mg$^{-1}$ at 6 h, which decreased to 1.05 U mg$^{-1}$ at 30 h, due to inhibitory effect of higher reducing sugar concentration (Fig. 6.3.7). A reducing sugar concentration of 20 g L$^{-1}$ having 75% repressive effect on the enzyme activity has been reported (Oh *et al.*, 2000). A gradual increase was observed with 3.56 U mg$^{-1}$ at 60 h which, finally decreased to 2.16 U mg$^{-1}$ at 72 h. The reducing sugar concentration gradually increased from 12.05 g L$^{-1}$ at 12 h while the biomass and ethanol concentration augmented concomitantly. The reducing sugar reached a maximum 19.68 g L$^{-1}$ at 36 h (Table 6.3.2, Fig. 6.3.7). After 36 h there was a continuous decline in reducing sugar concentration reaching a minimum level of 9.0 g L$^{-1}$ at 72 h (Fig. 6.3.7).

The degradation products released from hydrolysis of *E. crassipes* in bioreactor SSF were analysed by HPAEC-PAD (Fig. 6.3.8A, B, C, D, E and F). The retention time for different standard monosaccharide sugars arabinose, glucose and xylose were 3.71, 4.23 and 4.98 min (Fig. 6.3.8A). Some amount of xylose probably released during mixed MAA and organosolv pretreatment along with arabinose and glucose was witnessed by the HPAEC profile of the monosaccharides at 0 h (Fig. 6.3.8B). Surprisingly, the later stages of SSF showed no xylose due to its uptake by *C. shehatae* (Fig. 6.3.8C, D, E and F). Substantial amounts of arabinose and glucose released by mixed enzyme consortium (GH5 cellulase, GH43 hemicellulase) was sensed at 18 h profile (Fig. 6.3.8C). The maximum amounts of reducing sugars were observed at 36 h of the profile (Fig. 6.3.8D).

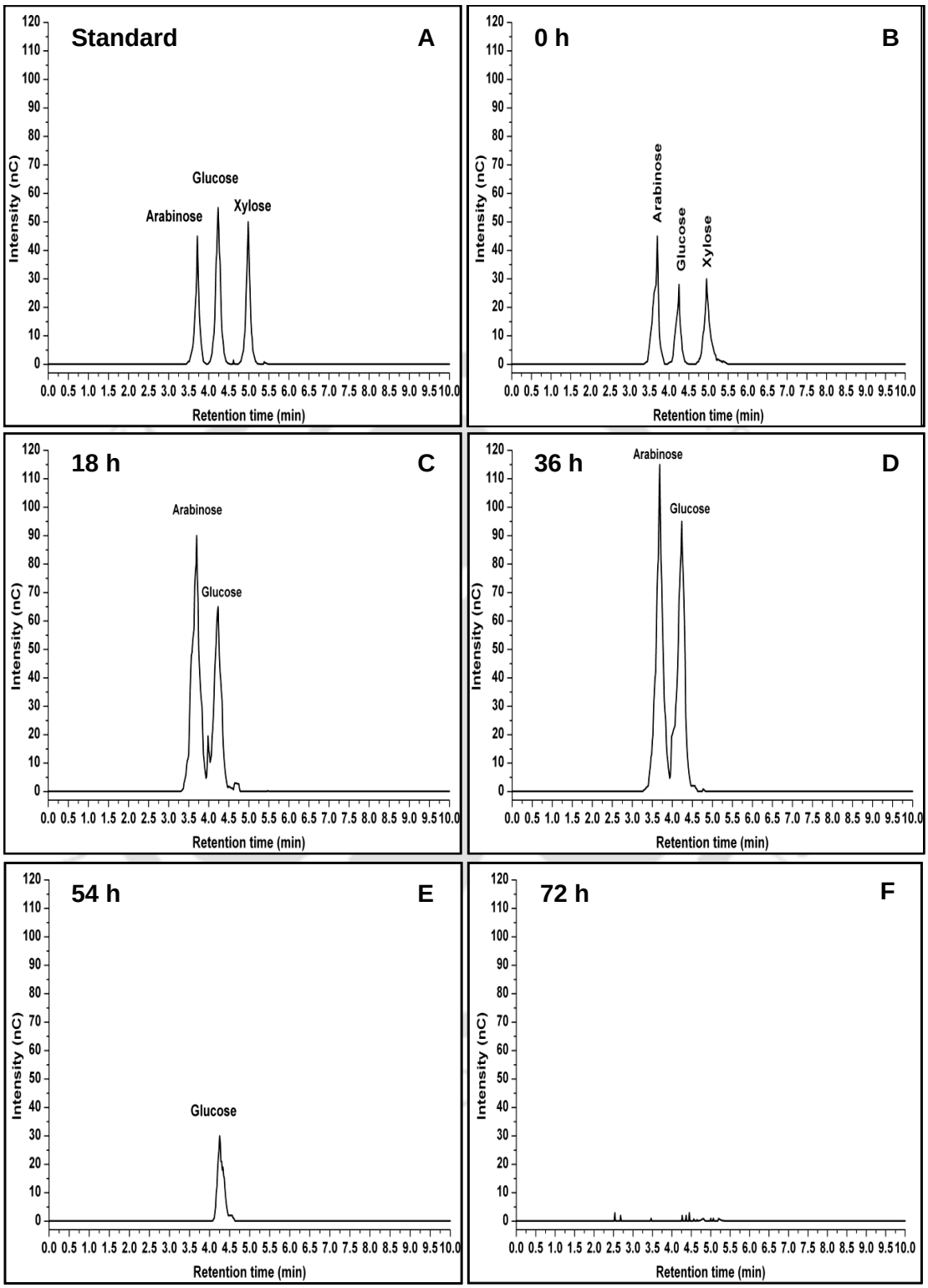


**Fig. 6.3.8** HPAEC Profiles of monosaccharides obtained from bioreactor SSF of 5% (w $v^{-1}$) water hyacinth. The chromatogram of sugar hydrolysate *viz.*, arabinose, glucose and xylose were obtained at different time intervals by HPAEC-PAD (A) Standards (B) 0 h (C) 18 h (D) 36 h (E) 54 h (F) 72 h.

The HPAEC profile exhibited small amount of glucose and no arabinose due to their consumption by fermentative microbes for ethanol formation at 54 h (Fig. 6.3.8E). The 72 h HPAEC pattern displayed negligible amount of monosaccharides due to their complete utilization by bioethanol producers for growth and maintenance (Fig. 6.3.8F). The aeration rate of 1.0 vvm in bioreactor maintained a threshold dissolved oxygen (DO) level of 40% for the efficient growth of *S. cerevisiae* and *C. shehatae*. Using 5% (w $v^{-1}$) substrate, an improved ethanol titre (14.39 g $L^{-1}$) with yield of 0.461 (g of ethanol g of $substrate^{-1}$) in bioreactor batch fermentation displayed a 2.2-fold rise in ethanol titre and yield as compared with shake flask SSF experiments (6.2 g $L^{-1}$, 0.209 g of ethanol g of $substrate^{-1}$) (Table 6.3.2).

The ethanol concentration results achieved in our research are comparable with reported literature. An ethanol titre of 0.2 g $L^{-1}$ and yield of 0.066 (g $g^{-1}$) has been reported from 1% (w $v^{-1}$) acid hydrolysed water hyacinth employing *S. cerevisiae* in shake flask (Masami *et al.*, 2008). The acid hydrolysed 1% water hyacinth in shake flask involving *Pichia stipits* contributed an ethanol yield of 0.19 (g g of $substrate^{-1}$) (Nigam, 2001). Ma *et al.*, (2010) obtained an ethanol yield of 0.192 (g $g^{-1}$) from 5 g water hyacinth in shake flask employing commercial cellulase and *S. cerevisiae.* Aswathy *et al.*, (2010) reported an ethanol titre of 4.4 g $L^{-1}$ using *T. reesei* cellulase and baker's yeast *S. cerevisiae* from 5% water hyacinth as substrate in shake flask. The ethanol concentration (3.36 g $L^{-1}$) was reported from 50 g $L^{-1}$ pretreated sugarcane bagasse under optimized process conditions in aerobic batch fermentation in a lab scale reactor (Sasikumar and Viruthagiri, 2010). An ethanol yield of maximum 0.480 (g $g^{-1}$) was produced from water hyacinth as substrate (Guragain *et al.*, 2011). The ethanol titre of 16.9 g $L^{-1}$ was obtained from 6% water hyacinth by

commercial cellulase along with *S. cerevisiae* and *Pichia stipitis* at shake flask level (Takagi *et al.*, 2012).

#### *6.3.2.4 Recovery of partially purified ethanol and purification process efficiency determination from bioreactor SSF of water hyacinth*

The crude ethanol obtained in bioreactor SSF studies with 5% (w $v^{-1}$) mixed MAA with organosolv pretreated water hyacinth engaging mixed recombinant enzymes (GH5 cellulase, GH43 hemicellulase) and mixed cultures (*S. cerevisiae, C. shehatae*) was 17.36 mL $L^{-1}$ i.e, 13.7 g $L^{-1}$ (Table 6.3.2). One litre of fermentation broth with crude ethanol on vacuum evaporation yielded 4.05 mL of distillate containing 3.81 mL i.e., 94.0% (v $v^{-1}$) of partially purified ethanol. Finally, the purification efficiency was estimated to be 22%. The multiple distillation steps can be engaged to obtain 100% ethanol recovery from the water condensates in the large scale operations as reported earlier (Palmqvist, 1996).

## 6.4 Conclusions

Simultaneous saccharification and fermentation **(**SSF) trials in shake flask involving mixed hydrolytic enzymes [GH5 cellulase, GH43 hemicellulase (α-L-arabinofuranosidase)] and mixed fermentative microbes (*S. cerevisiae, C. shehatae*) on 1% and 5% (w $v^{-1}$) mixed microwave assisted alkali (MAA) and organosolv pretreated wild grass and water hyacinth were performed. Subsequently, the shake flask SSF with 5% (w $v^{-1}$) wild grass and water hyacinth were independently scaled-up in a bioreactor under controlled process parameters. Bioethanol obtained from separate bioreactor SSF runs using wild grass and water hyacinth was recovered by rotary vacuum evaporator.

The cellulose, hemicellulose and lignin of mixed pretreated wild grass was 46.30%, 26.50% and 15.20%, respectively whereas the cellulose, hemicellulose and lignin of mixed pretreated water hyacinth was 26.10% cellulose, 33.20% hemicellulose and 15.64 lignin, respectively.

The shake flask SSF run of mixed pretreated 1% (w $v^{-1}$) wild grass involving mixed enzyme-mixed culture system gave reducing sugar concentration of 2.31 g $L^{-1}$, ethanol titre of 2.0 g $L^{-1}$ and an ethanol yield of 0.274 (g of ethanol g of $substrate^{-1}$). Whereas, the SSF experiment of mixed pretreated 5% (w $v^{-1}$) wild grass in shake flask involving the same mixed enzyme-mixed culture system provided a reducing sugar concentration of 13.85 g $L^{-1}$, ethanol titre of 10.9 g $L^{-1}$ and an ethanol yield of 0.299 (g of ethanol g of $substrate^{-1}$) displaying a 5.4-fold improvement in ethanol concentration and 9% escalation in ethanol yield. This confirmed that increasing the substrate concentration along with enzyme loadings and inoculum volume improves the ethanol titre.

The SSF trial of mixed pretreated 5% (w $v^{-1}$) wild grass in a bioreactor with controlled pH and aeration and same mixed enzyme-mixed culture system contributed a maximum reducing sugar concentration of 23.02 g $L^{-1}$, ethanol titre of 18.0 g $L^{-1}$ and an ethanol yield of 0.494 (g of ethanol g of substrate$^{-1}$). A 1.6-fold increase both in ethanol titre and yield was witnessed on scaling up the shake flask SSF with 5% (w $v^{-1}$) substrate concentration to bioreactor level. The HPAEC pattern of monosaccharides in sugar hydrolysate *viz.*, arabinose, glucose and xylose obtained from bioreactor SSF of wild grass at different time intervals clearly detected the presence of reducing sugar in the fermentation broth. 92.1 (%, v $v^{-1}$) of partially purified ethanol from bioreactor SSF of wild grass was recovered by rotary evaporator with 23% purification efficiency.

The shake flask SSF trial of mixed pretreated 1% (w $v^{-1}$) water hyacinth involving mixed enzyme-mixed culture system offered reducing sugar concentration of 1.41 g $L^{-1}$, ethanol titre of 1.22 g $L^{-1}$ and an ethanol yield of 0.205 (g of ethanol g of substrate$^{-1}$). Contrastingly, the shake flask SSF trial of mixed pretreated 5% (w $v^{-1}$) water hyacinth with the same mixed enzyme-mixed culture system contributed a reducing sugar concentration of 9.74 g $L^{-1}$, ethanol titre of 6.20 g $L^{-1}$ and an ethanol yield of 0.209 (g of ethanol g of substrate$^{-1}$) exhibiting a 5-fold enhancement in ethanol concentration and 2% rise in ethanol yield. This confirmed that increasing the substrate concentration along with enzyme loadings and inoculum volume enhances the ethanol concentration and yield.

Finally, the SSF trial of mixed pretreated 5% (w $v^{-1}$) water hyacinth in a bioreactor with same mixed enzyme-mixed culture system supplemented with controlled parameters of pH and aeration and funded a maximum reducing sugar

concentration of 19.68 g $L^{-1}$, ethanol titre of 13.7 g $L^{-1}$ and an ethanol yield of 0.461 (g of ethanol g of substrate$^{-1}$). A 2.2-fold upsurge both in ethanol titre and yield was observed on scaling up the shake flask SSF with 5% (w $v^{-1}$) substrate concentration in an automated bioreactor. The HPAEC pattern of monosaccharides in sugar hydrolysate *viz.*, arabinose, glucose and xylose obtained from bioreactor SSF of water hyacinth at different time intervals clearly proved the presence of reducing sugar in the fermentation broth. 94 (%, v $v^{-1}$) of partially purified ethanol from bioreactor SSF of water hyacinth was recovered by rotary evaporator with 22% purification efficiency.

The effective breakdown of the complex lignocellulosic weeds, wild grass and water hyacinth by mixed pretreatment strategy along with recombinant hydrolytic enzymes and fermentative microbes in SSF at bioreactor level will yield an improved titre of the fuel for tomorrow.

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## Conclusions

A long-term sustainable solution of weed management from low-value biomass feedstock was developed. Bioethanol was produced from wide-spread lignocellulosic weeds in northern India *viz.*, wild grass and water hyacinth. A very important facet in the field of the integrated production of bioethanol under the biorefinery concept was established. The aspects concerning bioethanol production from chemical and physical pretreatment of biomass to laboratory optimization of fermentation process, bioreactor scale-up with bioethanol recovery and purification were addressed.

Repetitive batch strategy in different growth media augmented the cell biomass and enzymatic activities of respective *E. coli* cells harbouring *Clostridium thermocellum* GH5 cellulase and GH43 hemicellulase (α-L-arabinofuranosidase) genes. For GH5 cellulase production in TB medium, a 3.4- and 2.7-fold augmentation in cell biomass was obtained in batch and repetitive batch modes as compared to LB medium in both modes. A 4.2-fold and 3.4-fold escalation in cell biomass was obtained in LB medium with glucose in batch and repetitive batch modes as compared to LB medium without glucose in both modes. For GH43 hemicellulase production, a 3.2-fold escalation in cell biomass was obtained in LB medium with glucose in batch and repetitive batch modes as compared to LB medium without glucose in both modes. In case of fed batch with glucose as a co-substrate, the biomass increased to a greater extent probably avoiding the acetate dependent growth repression and the consecutive use of induced cells in the following repetitive batches increased the productivity of enzymes.

Wild grass (*Achnatherum hymenoides*) containing highest cellulose content, 51.7% (w $w^{-1}$) and water hyacinth (*Eichhornia crassipes*) encompassing highest hemicellulose content, 44.5% (w $w^{-1}$) were selected as most sustainable among nine different substrates for bioethanol production. A mixed pretreatment strategy comprising of microwave assisted alkali (MAA) and organosolv pretreatments were employed prior to saccharification among nine pretreatments as microwave assisted alkali (MAA) pretreatment and organosolv pretreatment degraded lignin independently to the maximum extent. The FT-IR analyses of both substrates displayed a peak of xylose, glucose and arabinose at 995, 1033, 1152 $cm^{-1}$, respectively, confirming the significant breakdown of complex cellulose and hemicellulose to monomeric sugars by mixed pretreatment strategy. The shake flask SSF experiments involving mixed pretreated 1% (w $v^{-1}$) wild grass with GH5 cellulase and *S. cerevisiae* yielded a 1.2-fold higher ethanol titre over shake flask SHF. The SSF experiments involving mixed pretreated 1% (w $v^{-1}$) water hyacinth along with GH43 hemicellulase (α-L-arabinofuranosidase) and *C. shehatae* in shake flask gave a 1.2-fold higher ethanol titre as compared to that in shake flask SHF.

SSF trial of wild grass involving GH5 cellulase and *S. cerevisiae* displayed an 11% increase in the ethanol yield over *T. reesei* cellulase and *S. cerevisiae*. SSF experiment involving GH5 cellulase and *Z. mobilis* presented a 14% escalation in the ethanol yield confirming the efficiency of GH5 cellulase over *T. reesei* cellulase with *Z. mobilis*. The SSF experiment of wild grass involving mixed enzymes, recombinant GH5 cellulase, GH43 hemicellulase and *Z. mobilis* exhibited a 37% upturn in the ethanol yield as compared with that of single enzyme, GH5 cellulase and *Z. mobilis*. A 2.3-3.2 fold increment was observed in ethanol titre using a mixed enzyme [GH5

cellulase, GH43 hemicellulase]- mixed culture (*S. cerevisiae*, *C. shehatae*) system as compared to single enzyme-single culture systems for wild grass. SSF experiment of water hyacinth involving GH5 cellulase and *Z. mobilis* offered a 14% increase in the ethanol yield over *T. reesei* cellulase with *Z. mobilis*. The SSF trial involving mixed enzymes and *Z. mobilis* exhibited a 22% upturn in the ethanol yield as compared with that of GH5 cellulase and Z. mobilis. A 1.8-2.7 fold increment was observed in ethanol titre using a mixed enzymes-mixed culture system as compared to single enzyme-single culture system for water hyacinth.

The Taguchi optimized process parameters for wild grass in shake flask SSF with 100 mL of fermentation medium were (%, v $v^{-1}$): 1.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 0.25, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 4.3 and temperature, 35°C. In case of water hyacinth, the optimized process parameters for 100 mL of fermentation medium in shake flask SSF were (%, v $v^{-1}$): 2.0, recombinant GH5 cellulase (5.7 U $mg^{-1}$, 0.45 mg $mL^{-1}$); 2.0, recombinant GH43 hemicellulase (3.7 U $mg^{-1}$, 0.32 mg $mL^{-1}$); 1.5, *S. cerevisiae* (3.9 x $10^8$ cells $mL^{-1}$); 2.0, *C. shehatae* (2.7 x $10^7$ cells $mL^{-1}$); pH, 5.4 and temperature, 35°C. pH with *p*-value 0.001 and 0.010 was found to be the most significant factor affecting wild grass and water hyacinth SSF, respectively. The ethanol titres obtained in optimized shake flask SSF of wild grass and water hyacinth exhibited a 1.3-fold and 1.2-fold rise, respectively as compared with unoptimized conditions.

Shake flask SSF trial of mixed pretreated 5% (w $v^{-1}$) wild grass involving mixed enzyme-mixed culture system displayed a 5.4-fold improvement in ethanol concentration and 9% escalation in ethanol yield over 1% (w $v^{-1}$) substrate confirming

the increase in the substrate concentration along with enzyme loadings and inoculum volume improves the ethanol titre. A 1.6-fold increase both in ethanol titre and yield was witnessed on scaling up the shake flask SSF with 5% (w $v^{-1}$) wild grass to bioreactor level. The shake flask SSF trial of mixed pretreated 5% (w $v^{-1}$) water hyacinth with mixed enzyme-mixed culture system exhibited a 5-fold enhancement in ethanol concentration and 2% rise in ethanol yield as compared with 1% (w $v^{-1}$) substrate. A 2.2-fold upsurge both in ethanol titre and yield was observed on scaling up the shake flask SSF with 5% (w $v^{-1}$) water hyacinth in an automated bioreactor. The HPAEC pattern of monosaccharides *viz.*, arabinose, glucose and xylose in sugar hydrolysate obtained from bioreactor SSF of both wild grass and water hyacinth at different time intervals clearly detected the presence of reducing sugar in the fermentation broth. 92.1 and 94 (%, v $v^{-1}$) of partially purified ethanol from bioreactor SSF of wild grass and water hyacinth was recovered by rotary evaporator with 23% and 22% purification efficiency, respectively.

In conclusion, biotechnological approaches were exploited to produce the fuel for tomorrow, bioethanol from land and water weeds.

## Future prospects

- Optimized pretreatment can be employed on the lignocellulosic substrate aiding in effective hydrolysis.
- The storage time (half-life) of enzyme can be enhanced by covalent immobilization for competent saccharification.
- Effective hydrolysis of substrate can be obtained in multistage process using cocktail of hydrolytic cellulases and xylanases.
- Metabolic pathway study can be targeted for enhanced second-generation bioethanol production along with some useful by-products.
- Life cycle assessment /economics of the entire process can be performed.

## List of publications

### Published/In press/Accepted

1. **Saprativ P. Das,** Rajeev Ravindran, Shadab Ahmed, Debasish Das, Dinesh Goyal, Carlos M.G.A.Fontes and Arun Goyal **(2012)** Bioethanol production involving recombinant *C. thermocellum* hydrolytic hemicellulase and fermentative microbes. *Applied Biochemistry and Biotechnology*, 167, 1475-1488.
2. **Saprativ P. Das,** Rajeev Ravindran, Deepmoni Deka, Mohammad Jawed, Debasish Das and Arun Goyal **(2013)** Bioethanol production from leafy biomass of mango (*Mangifera indica*) involving naturally isolated and recombinant enzymes. *Preparative Biochemistry & Biotechnology*, 43, 717-734.
3. **Saprativ P. Das,** Arabinda Ghosh, Ashutosh Gupta, Debasish Das and Arun Goyal **(2013)** Lignocellulosic fermentation of wild grass employing recombinant hydrolytic enzymes and fermentative microbes with effective bioethanol recovery. *Biomed Research International*, 2013, ID 386063, 14 pages, doi: 10.1155/2013/386063.
4. Deepmoni Deka, **Saprativ P. Das**, Debasish Das, Mohammad Jawed and Arun Goyal **(2013)** Enhanced cellulase production from *Bacillus subtilis* by optimizing physical parameters for bioethanol production. *ISRN Biotechnology*, 2013, ID 965310, 11 pages, doi:10.5402/2013/965310.
5. **Saprativ P. Das,** Deepmoni Deka, Arabinda Ghosh, Debasish Das, Mohammad Jawed and Arun Goyal **(2013)** Scale up and efficient bioethanol production involving recombinant cellulase (Glycoside hydrolase family 5) from *Clostridium thermocellum. Sustainable Chemical Processes*, 2013, 1:19 doi: 10.1186/2043-7129-1-19.
6. **Saprativ P. Das**, Rajeev Ravindran, Arabinda Ghosh, Deepmoni Deka, Debasish Das, Mohammad Jawed, Carlos M.G.A. Fontes and Arun Goyal **(2013)** Efficient pretreatment for bioethanol production from water hyacinth (*Eichhornia crassipes*) involving naturally isolated and recombinant enzymes and its recovery. *Environmental Progress & Sustainable Energy* (doi:10.1002/ep.11885).
7. **Saprativ P. Das,** Debasish Das and Arun Goyal **(2013)** Statistical optimization of fermentation process parameters by Taguchi orthogonal array design for improved bioethanol production. *Journal of Fuels* (in press).

## List of conferences

**International**

1. **Saprativ P. Das**, Debasish Das, Dinesh Goyal and Arun Goyal (**2010**) Simultaneous Saccharification and Fermentation (SSF) process involving recombinant *Clostridium thermocellum* cellulase expressed and isolated from *E. coli*. (International Conference on Genomic Sciences, VII Convention of Biotech Research Society of India), Nov 12-14, 2010, Madurai Kamaraj University, Tamil Nadu, India.
2. **Saprativ P. Das**, Rajeev Ravindran, Debasish Das and Arun Goyal **(2011)** Bioethanol production by Simultaneous Saccharification and Fermentation (SSF) using statistically optimized parameters and mixed enzymes and cultures. (International Conference on New Horizons in Biotechnology, NHBT-BRSI 2011), Nov 21-24, 2011. National Institute for Interdisciplinary Science and Technology- CSIR, Trivandrum, Kerala, India.
3. Rajeev Ravindran, **Saprativ P. Das**, Deepmoni Deka and Arun Goyal **(2011)** Bench scale bioethanol production involving recombinant *C. thermocellum* hydrolytic enzymes and fermentative microbes. (52$^{nd}$ Annual Conference of AMI, Nov 3-6, 2011. International Conference on Microbial Biotechnology for Sustainable Development), Panjab University, Chandigarh, Punjab, India.
4. **Saprativ P. Das**, Rajeev Ravindran, Debasish Das, Dinesh Goyal and Arun Goyal **(2011)** Reactor scale-up for lignocellulosic fermentation employing different hydrolytic enzymes and bioethanol producers. (International Conference on Yeast Biology), Dec 10-13, 2011. Indian Institute of Technology Bombay, Mumbai, India.
5. Rajeev Ravindran, **Saprativ P. Das**, Deepmoni Deka and Arun Goyal **(2012)** Lignocellulosic biomass as a sustainable source for bioethanol production. (International Conference on Environmentally Sustainable Urban Ecosystems), Feb 24-26, 2012, Indian Institute of Technology Guwahati, Guwahati, India.
6. Deepmoni Deka, **Saprativ P. Das**, Rajeev Ravindran and Arun Goyal **(2012)** Water hyacinth as a potential source of biofuel for sustainable development. (International Conference on Environmentally Sustainable Urban Ecosystems), Feb 24-26, 2012, Indian Institute of Technology Guwahati, Guwahati, India.
7. **Saprativ P. Das**, Rajeev Ravindran, Debasish Das and Arun Goyal **(2012)** Enhanced bioethanol production using mixed pretreatment along with mixed recombinant hydrolytic cellulase (GH5) and hemicellulase (GH43) from *Clostridium thermocellum*. (International Workshop and Conference on Renewable Energy and Climate Change), Apr 5-7, 2012, Madurai Kamaraj University, Madurai, Tamilnadu, India**.**
8. **Saprativ P. Das**, Deepmoni Deka, Rajeev Ravindran, Debasish Das, Mohammad Jawed and Arun Goyal **(2012)** Lignocellulosic fermentation of water hyacinth involving mixed pretreatment and different hydrolytic enzymes. (International Workshop and Conference on Renewable Energy and Climate Change), Apr 5-7, 2012, Madurai Kamaraj University, Madurai, Tamilnadu, India**.**

9. **Saprativ P. Das**, Rajeev Ravindran, Debasish Das, Carlos M. G. A. Fontes and Arun Goyal **(2012)** Efficient pretreatment for lignocellulosic ethanol fermentation involving recombinant hydrolytic GH5 cellulase and GH43 hemicellulase from *Clostridium thermocellum.*(8th International Conference on Renewable Resources & Biorefineries), Jun 4 – 6, 2012, Toulouse, France.

10. Rajan Choudhary, **Saprativ P. Das**, Anil Kumar Verma, Debasish Das and Arun Goyal **(2013)** Efficient bioethanol production from lignocellulosic leafy biomass of poplar (*Populus nigra*). (International Conference on Conserving Biodiversity for Sustainable Development), Aug 16-18, 2013, National Institute of Technology Rourkela, Orissa, India.

11. **Saprativ P. Das**, Arabinda Ghosh, Ashutosh Gupta, Debasish Das and Arun Goyal **(2013)** Approaches for identification of a combination of hydrolytic enzymes and fermentative microbes for bioethanol production from wild grass. (Indraprastha International Conference on Biotechnology), Oct 22-25, 2013, Guru Gobind Singh Indraprastha University, New Delhi, India.

12. Ashutosh Gupta, **Saprativ P. Das**, Rajan Choudhary, Debasish Das and Arun Goyal **(2013)** Application of recombinant hydrolytic enzymes for bioethanol production from leafy biomass of bamboo (*Bambusa dendrocalamus*) (52$^{nd}$ Annual Conference of AMI, Nov 17-20, 2013. International Symposium on Frontier Discoveries and Innovations in Microbiology and its Interdisciplinary Relevance), Maharshi Dayanand University, Rohtak, Haryana, India.

13. **Saprativ P. Das**, Ashutosh Gupta, Debasish Das and Arun Goyal **(2013)** Bench scale bioethanol production from *Eichhornia crassipes* involving statistical optimization of fermentation process parameters by Taguchi orthogonal array design. (International conference on advances in biotechnology & bioinformatics, ICABB-BRSI 2013), Nov 25-27, 2013, Dr. D. Y. Patil Vidyapeeth, Pune, India.

14. Ashutosh Gupta, **Saprativ P. Das**, Rajan Choudhary, Debasish Das and Arun Goyal **(2013)** Bioethanol production from *Populus nigra* involving recombinant acetylxylanesterase (Axe) from *Clostridium thermocellum.* (International conference on advances in biotechnology & bioinformatics, ICABB-BRSI 2013), Nov 25-27, 2013, Dr. D. Y. Patil Vidyapeeth, Pune, India.

15. Ashutosh Gupta, **Saprativ P. Das**, Debasish Das and Arun Goyal **(2013)** Dual-stage saccharification of sugarcane bagasse for bioethanol production by mixed recombinant *Clostridium thermocellum* enzymes. (82$^{nd}$ Annual Meeting of Society of Biological Chemists, India and International conference on genomes: Mechanism and function), Dec 2-5, 2013, University of Hyderabad, Andhra Pradesh, India.

16. **Saprativ P. Das**, Ashutosh Gupta, Debasish Das and Arun Goyal **(2014)** Utilization of recombinant *Clostridium thermocellum* enzymes cocktail in two-stage hydrolysis of corn cob for bioethanol production. (27$^{th}$ International Carbohydrate Symposium), Jan 12-17, 2014, Indian Institute of Science, Bangalore, India *(abstract accepted).*

17. Ashutosh Gupta, **Saprativ P. Das**, Debasish Das and Arun Goyal **(2014)** Enhanced bioethanol production by two-stage saccharification from poplar

(*Populus nigra*) involving recombinant saccharifying enzymes from *Clostridium thermocellum*. (CARBO-XXVIII, International Conference on Challenges in Chemistry and Biology of Carbohydrates), Jan 20-22, 2014, Forest Research Institute, Dehradun, India *(abstract accepted)*.

**18.** Ashutosh Gupta, **Saprativ P. Das**, Debasish Das and Arun Goyal **(2014)** Simultaneous saccharification and fermentation (SSF) of corn cob involving recombinant *Clostridium thermocellum* acetyl-xylan esterase (CARBO-XXVIII, International Conference on Challenges in Chemistry and Biology of Carbohydrates), Jan 20-22, 2014, Forest Research Institute, Dehradun, India *(abstract accepted)*.

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# VITAE

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