

# Bioethanol Production under Simultaneous Saccharification and Fermentation (SSF) and Simultaneous Saccharification and Co-Fermentation (SSCF) As well As It's Scale up in a Stirred Tank Bioreactor

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**ABSTRACT:** An integrated approach was studied for in-house cellulase and xylanase production, from novel hyper hydrolytic enzyme producers and enzymatic hydrolysis of alkaline hydrogen peroxide pretreated *Populus deltoides* wood into bioethanol. Fermentation of lignocellulosic hydrolysate was performed under simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF) using different yeasts viz. *Saccharomyces cerevisiae* I, *Saccharomyces cerevisiae* II, *Pichia stipitis*, *Candida shehatae* and *Zymomonas mobilis* as monoculture and co-culture combination. Maximum 11.26 g/l ethanol yield achieved with co-culture of *S. cerevisiae* II + *P. stipitis* with fermentation efficiency of 44.16% under SSF. While SSCF was performed by a recombinant strain of *Clostridium thermocellum* strain DSM 1313. Co-culture combination of *S. cerevisiae* II + *P. stipitis* was applied for scale up process of bioethanol in a stirred tank bioreactor.

**KEYWORDS:** Bioethanol, *Populus deltoides*, cellulase, xylanase, pretreatment, *Clostridium thermocellum*

## I. INTRODUCTION

Ethanol produced from sugar, starch and lignocellulosic biomass is a liquid biofuel with the potential to replace some of the liquid fossil fuels used in transportation. Today fossil fuels take up 80% of the primary energy consumed in the world, of which 58% alone is consumed by the transport sector. Continuous depletion of conventional fossil fuel reserves with increasing energy demands and climate change have led to a move towards alternative, renewable, sustainable, efficient and cost-effective energy sources with smaller emissions (Nigam & Singh, 2011). Bioethanol produced from corn grain and sugarcane is currently the most common renewable fuel. However it is clear that the large scale use of bioethanol will require lignocellulosic biomass to be used as raw material. Biofuels also provide the opportunity for non oil producing countries to be self sufficient in fuel (Ohgren et al., 2007). Plant biomass consists of cellulose microfibrils embedded in lignin, hemicellulose and pectin with an altered amount of each compound among plant species and plant parts. Cellulose is crystalline glucose polymer, hemicellulose is amorphous polymer of xylose and arabinose. Whereas lignin is a large polyaromatic compound (Nigam and Singh, 2011). A variety of new technologies and methods including biochemical and thermochemical processes being developed to convert biomass to various forms of energy and chemicals. Feedstock pretreatment has become important tool to increase cellulosic ethanol production yield. Pretreatment processes break down lignin and disrupt the crystalline structure of cellulose, rendering it more accessible for enzymes to allow more ready fermentation to sugars (Wang et al., 2011). Alkaline hydrogen peroxide has been successfully developed for pretreatment of lignocellulosic materials. Alkaline solutions of

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H<sub>2</sub>O<sub>2</sub> can also solubilize a part of the hemicellulose present in the plant material which makes cellulose more accessible for the microorganisms taking part in the fermentation process. Moreover, using H<sub>2</sub>O<sub>2</sub> leads to breaking bonds between carbohydrates and lignin (Kim et al., 2001) and to reducing cellulose crystallinity (Yu et al., 2009). The sugars generated from lignocellulose are a mixture of hexoses and pentoses. The hexose sugars can readily fermented into ethanol using industrial strains of the yeast, *Saccharomyces cerevisiae*. However, yeast varieties of the genus *Saccharomyces* have not been found that can ferment pentose sugar such as xylose into ethanol, other organisms are there which can ferment xylose. In the present study, Alkaline hydrogen peroxide pretreated *P. deltooides* wood biomass was used as the substrate for bioethanol production under different Simultaneous saccharification and fermentation (SSF) and Simultaneous saccharification and co-fermentation (SSCF) as well as scale up of bioethanol in a stirred tank bioreactor.

## II. RELATED WORK

Our aims of present study were production of second generation biofuel by using lignocellulosic forest wood. This is done by using alkaline hydrogen peroxide pretreated *P. deltooides* wood under SSF and SSCF and finally scaled up in a stirred tank bioreactor. The bioethanol production steps are summarized as follows.

1. Substrate used in the study: Alkaline hydrogen peroxide pretreated *Populus deltooides* wood was used as the substrate for bioethanol production.
2. Cellulase production by mutant strain of hypercellulase producer *B. stratosphericus* N<sub>12</sub> (M) was done under submerged fermentation (SmF).
3. Xylanase production by mutant strain of hyperxylanase producer *B. altitudinis* Kd<sub>1</sub> (M) under submerged fermentation (SmF).
4. Cellulase and xylanase production by fungus *Myceliophthora thermophila* SH1 under solidstate fermentation was done.

The sub-enzymes of cellulase were measured by using standard assays. CMCase and FPase activity was measured by Reese and Mandel method (Reese and Mandel, 1963). For  $\beta$ -glucosidase Berghem and Petterson method was used (Berghem and Petterson, 1973).

Xylanase activity was measured by DNSA method (Miller, 1959).

5. The inhouse enzymes which were prepared had been mixed in the ratio of (2:2:1) i.e 2 ml of cellulase from *M. thermophila* SH1 (CMCase: 1.752 IU, FPase: 1.193 IU and  $\beta$ -glucosidase: 2.571 IU) + 2 ml of cellulase from *B. stratosphericus* N<sub>12</sub>(M) (CMCase: 2.384 IU, FPase: 2.249 IU and  $\beta$ -glucosidase: 2.451 IU) and 0.5 ml of xylanase from *B. altitudinis* Kd<sub>1</sub> (M) (71.49 IU) + 0.5 ml of xylanase from *M. thermophila* SH1 (12.59 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis. Commercial enzymes were mixed in the ratio of 1:1:1 i.e. 1ml celluclast 1.5L (CMCase: 29.75 IU, FPase: 29.75 IU), 1ml Novozyme 188 ( $\beta$ -glucosidase: 20.51 IU) + 1ml Pantopan Mono BG (xylanase: 22.64 IU) and used @ 1 ml/g of biomass.

6. SSF was done by monoculture and co-culture combinations of *Saccharomyces cerevisiae*-I, *Saccharomyces cerevisiae*-II, *Pichia stipitis*, *Candida shehatae*, *Zymomonas mobilis*, *S. cerevisiae*-I + *P. stipitis*, *S. cerevisiae*-I + *C. shehatae*, *S. cerevisiae*-II + *P. stipitis* and *S. cerevisiae*-II + *C. shehatae* at 32°C for 72 h.

7. SSCF was done by a recombinant strain of *Clostridium thermocellum* strain DSM 1313 [Accession No. |CP002416|] procured from DSM- Germany at 32°C for 72 h.

8. Ethanol prepared was estimated by Caputi and Wright method (Caputi and Wright, 1969).

9. Scale up of bioethanol production by using co-culture strains of *S. cerevisiae* II and *P. stipitis* was done in a stirred tank bioreactor.

## III. EXPERIMENTAL RESULTS

### Bioethanol production under SSF mode

In the present study, SSF was performed in which saccharification of lignocellulosic biomass was done by using inhouse enzymes i.e cellulase from *M. thermophila* SH1 + *B. stratosphericus* N<sub>12</sub> (M) and xylanase of *B. altitudinis* Kd<sub>1</sub> (M) + *M. thermophila* SH1 and results were compared with commercial enzymes used as control as the results shown in table 1. The highest ethanol yield of 11.26 g/l was observed in co-culture of *S. cerevisiae* II + *P. stipitis* with

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inhouse enzymes (fermentation efficiency of 44.16% ) followed by mono-culture of *S. cerevisiae* II and co-culture of *S. cerevisiae* I + *C. shehatae* i.e 10.31 g/l of ethanol (40.39% of fermentation efficiency). On the other hand, in case of control using commercial enzymes maximum ethanol yield of 16.65 g/l was received in co- culture of *S. cerevisiae* II +

*C. shehatae* and fermentation efficiency of 65.31%.

**Table1. Bioethanol production under SSF by using inhouse enzyme and commercial enzymes**

Strains used	Inhouse enzymes				Commercial enzymes			
	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)	Ethanol (%)	Ethanol (g/l)	Ethanol (g/g)	Fermentation Efficiency (%)
<i>Saccharomyces cerevisiae-I</i>	1.10	8.72	0.174	34.21	1.60	12.68	0.253	49.76
<i>Saccharomyces cerevisiae-II</i>	1.30	10.31	0.206	40.39	1.58	12.53	0.250	49.14
<i>Zymomonas mobilis</i>	0.79	6.32	0.125	24.57	1.50	11.89	0.237	46.65
<i>S. cerevisiae-I</i> + <i>P. stipitis</i>	1.20	9.51	0.190	37.32	1.84	14.59	0.291	57.22
<i>S. cerevisiae-I</i> + <i>C. shehatae</i>	1.30	10.31	0.200	40.43	2.0	16.02	0.320	62.82
<i>S. cerevisiae-II</i> + <i>P. stipitis</i>	1.42	11.26	0.225	44.16	1.92	15.22	0.304	59.71
<i>S. cerevisiae-II</i> + <i>C. shehatae</i>	1.26	9.99	0.19	39.18	2.10	16.65	0.333	65.31
<i>S. cerevisiae-I</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	0.79	6.32	0.125	24.57	1.29	10.23	0.204	40.12
<i>S. cerevisiae-II</i> + <i>C. shehatae</i> + <i>P. stipitis</i>	0.71	5.65	0.112	22.08	1.40	11.10	0.222	43.54
S.E.	0.04	1.38	0.021	0.27	0.273	4.383	0.696	2.629
C.D. 0.05	0.10	2.90	0.044	0.58	0.566	9.090	0.144	5.452

Inhouse enzymes: [cellulase of *M. thermophila* SH1 and *B. stratosphericus* N12 (M) + xylanase of *M. thermophila* SH1 + *B. altitudinis* Kd1 (M)]

Commercial enzymes: [Celluclast 1.5 + Novozyme188 + Pantopan Mono BG]

It was closely followed by co-culture of *S. cerevisiae* I + *C. shehatae* i.e 16.02 g/l having 62.85% of fermentation efficiency.

## Bioethanol production under SSCF mode

In the second mode, bioethanol production from NaOH + H<sub>2</sub>O<sub>2</sub> pretreated *P. deltooides* wood waste was done under Simultaneous Saccharification and Co-Fermentation (SSCF) by *Clostridium thermocellum* strain DSM 1313 [Accession No. |CP002416|].

**Table2. SSCF by a recombinant strain of *Clostridium thermocellum* DSM 1313**

Strain	<i>Clostridium thermocellum</i>
Ethanol (%)	0.30 ±0.005
Ethanol (g/g)	0.04 ± 0.001
Ethanol (g/l)	2.37 ±0.050
Fermentation efficiency (%)	7.84 ± 0.100

In table 2, 2.37 g/l of ethanol yield and fermentation efficiency of 7.84% was noticed which was meager as compared to ethanol produced in Simultaneous Saccharification and Fermentation (SSF). One of the possible reason for less yield may be due to the reason that during the fermentation of glucose along with main product other compounds such as lactate and acetate are also produced. In addition to that *C. thermocellum* also lacks the ability to convert pentose sugars efficiently to ethanol.

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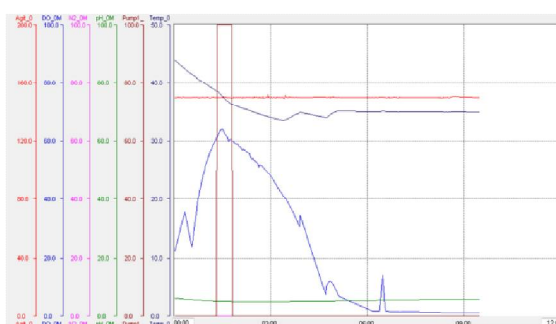
## Scale up of bioethanol production in a stirred tank bioreactor

Optimization of batch conversion of lignocellulosic hydrolysate to ethanol was carried out by co-culture of *S. cerevisiae* and *P. stipitis* which was selected from the previous study on the basis of maximum ethanol yield.

**Table3. Scale up of bioethanol production in a stirred tank bioreactor**

Time (h)	O.D.	Ethanol (%)	Ethanol (g/g)	Ethanol (g/l)	Fermentation efficiency (%)
2	0.032	0.42	0.066	3.33	12.91
4	0.041	0.51	0.080	4.04	15.65
6	0.100	1.20	0.190	9.51	37.18
8	0.110	1.31	0.206	10.31	40.31
10	0.146	1.60	0.253	12.68	49.51
12	0.146	1.60	0.253	12.68	49.51
24	0.106	1.26	0.199	9.99	38.94

Table 3 revealed the ethanol yield produced by the co-culture at different timings under anaerobic conditions. The maximum ethanol yield was observed in 10 h i.e. 12.68 g/l with the fermentation efficiency of 49.51%, which was constant upto 12 h and ethanol yield was reduced to 9.99 g/l after 24 h. The yield of ethanol is found to be proportional to fermentation time. It increases with the increase in time upto 10-12h and then declines. The time of fermentation was also reduced dramatically to 10 h for maximum ethanol yield as compared to shake flask (72 h). The environmental conditions optimized at shake flask level have initially been adapted in the bioreactor. Since a first step to improve the large scale process should be mimic of the optimal lab scale process. The results of bioreactor when compared with shake flask experiment, an improved yield with shorter fermentation time have emerged as the remarkable achievement of scale up process.



**Fig 1 Physiological behaviour of co-culture of *S. cerevisiae* and *P. stipitis* in a stirred tank bioreactor**

Fig 1 showed the physiological behaviour of co-culture of *S. cerevisiae* II+ *P. stipitis* in the stirred tank bioreactor during bioethanol production. In initial stages the level of dissolved oxygen (DO) for the co-culture combination was high and then falls down, it means the entire DO was consumed by ethanologens during ethanol production. While the other parameters viz. temperature, pH, agitation etc. were constant during the process as shown in the figure.

## IV. CONCLUSION

In the present study, development of a technology for effectively converting alkaline hydrogen peroxide pretreated *P. deltooides* wood to simple sugars by potential inhouse enzymes produced from isolated potential microorganisms i.e. *Bacillus altitudinis* Kd<sub>1</sub> (M) xylanase producer and cellulase producer *Bacillus stratosphericus* N<sub>12</sub> (M) as well as

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fungal strain *M. thermophila* SH1 and intern fermenting them to appreciable concentration of ethanol by mono culture and co-culture combinations of ethanologens in which the best combination were *S. cerevisiae-I* + *P. stipitis* The results of bioreactor when compared with shake flask experiment, an improved yield with shorter fermentation time have emerged as the remarkable achievement of scale up process that fulfills the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.

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## REFERENCES

1. Nigam, P. S., and Singh, A., "Production of liquid biofuels from renewable resources", Progress in Energy and Combustion Science, vol. 37, pp. 52-58, 2011.
2. Ohgren, K., Bura, R., Lesnicki, G., Saddler, J., and Zacchi, G., "A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam pretreated corn stover", Process Biochemistry, vol. 42, pp. 834-839, 2007.
3. Wang, H., Srinivasan, R., Yu, F., Steele, P., Li, Q., and Mitchell, B., "Effect of acid, alkali and steam explosion pretreatments on characteristics of bio-oil produced from pine wood", Energy and Fuels, vol. 25, pp. 3758-3764, 2011.
4. Kim, K. H., and Juans, H., "Supercritical CO<sub>2</sub> pretreatment of lignocellulose enhances enzymatic cellulose hydrolysis", Bioresource Technology, vol. 77, pp. 139-144, 2001.
5. Yu, J., Zhang, J. B., He, J., Li, Z. D., and Yu, Z. N., "Combination of mild physical and chemical pretreatment for enzymatic hydrolysis of rice hull", Bioresource Technology, vol. 100 No. 2, pp. 903-908, 2009.
6. Reese, E. T., Mandels, M., "Enzymatic hydrolysis of cellulose and its derivatives. In: Methods Carbohydrate Chemistry (ed. Whistler R L) 3<sup>rd</sup> edn", Academic Press, London, pp. 139-143, 1963.
7. Berghem, L. E. R., Pettersson, L. G., "Mechanism of enzymatic cellulose degradation and purification of a cellulolytic enzyme from *T. viride* active on highly ordered cellulose", Journal of Biochemistry, vol. 37 No. 1, pp. 21-30, 1973.
8. Miller, G. L., "Use of dinitrosalicylic acid reagent for determination of reducing sugars", Analytical chemistry, vol. 31, pp. 426-428, 1959.
9. Caputi, A., and Wright, D., "Collaborative study of the determination of ethanol in wine by chemical oxidation." Journal Association OFF Analytical Chemistry, vol. 52, pp. 85-88, 1969.