ISSN Online: 2349-7785

Production of Bioethanol from Enzymatic and Dilute Acid Hydrolysate of *Lantana camara* in Batch Fermentation

Vikash Kandari, Irshita Bajpayee, Barkha Kamal, Vikas S. Jadon and Sanjay Gupta*
Department of Biotechnology and Biochemistry, SBSPGI, Balawala Dehradun, Uttarakhand, India

Received 12 October 2014; accepted in revised form 19 November 2014

Abstract: The presence of 62.2 % of total carbohydrates in *Lantana camara* makes it a potential and low-cost renewable material for bioethanol production. The wood dust slurry at 10 % (w/v) solid content when treated with 3 % (w/v) NaOH at room temperature for 4 hours resulted in 90.13 % delignification with phenolics yield of 268±15.79 mg/g of dry weight of substrate. Acid hydrolysis (2 % w/v sulfuric acid, 24 h) of delignified *L.camara* wood dust produced 181.86 mg/g total sugars along with fermentation inhibitors furfural and HMF (134±3.4 mg/l). The hydrolysate obtained after dilute acid hydrolysis was subsequently detoxified sequentially by the different detoxification methods such as ethyl acetate differential extraction, overliming, vaccum evaporation and activated charcoal to diminish the inhibition effect of different inhibitors. The cellulase enzyme prepared from *Trichoderma viride* was used to study the saccharification of the cellulose fiber from *Lantana camara* wood dust. The optimal saccharification yield (0.899 g of sugar/g of cellulose) was achieved after 12 h of incubation at optimal performance parameters when almost 82 % of cellulose depolymerization was obtained, resulting in sugar concentration of 45.75 g/l in hydrolysis medium. Fermentation of enzymatic and acid hydrolysates with *Saccharomyces cerevisiae* VS1 and *Saccharomyces cerevisiae* VS3 respectively gave rise to 43.56 g/L and 26.38 g/L of ethanol with corresponding yields of 0.44g/g and 0.26g/g after 24 and 48 h, respectively.

Key words: *Saccharomyces cerevisiae,* Depolymerization, Hydrolysis.

Introduction

The limited availability of oil reserves and growing world-wide energy demands has resulted in increasing energy prices. Furthermore, the utilization of fossil fuels has negative impacts such as air pollution and the generation of the green house gas carbon dioxide, which is presumed to be one of the main anthropogenic contributors to the global warming effect [1]. Ethanol is a renewable oxygenated fuel. Developing ethanol as fuel, beyond its current role as fuel oxygenate, will require developing lignocellulosic biomass as a feedstock because of its abundance and low cost [2]. *Lantana camara* is one of the world's 100 worst weeds and is invasive in over 60 countries [3]. The approximate total biomass produced by *L. camara* per year ranges from 15 to 17 tonnes/ha [4]. In the present study, *L.camara* was chosen as the raw lignocellulosic material due to its vast abundance, low cost and being rich in fermentable carbohydrates, it is likely to offer a potential feedstock for ethanol production.

Current technology for conversion of lignocellulose to ethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by a microorganism [5]. A pretreatment step is essential to disrupt the cell wall macromolecular complexes, to remove hemicellulose and/or lignin and to increase the surface area accessible to hydrolytic enzymes [6,7].

*Corresponding author (Sanjay Gupta)
E-mail: < sanjay_gupta9999@rediffmail.com >

Delignification is essential to promote enzymatic digestibility of lignocellulosic materials by alkali which causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and crystallinity which provokes lignin structure disruption [8,9]. Among different pretreatment methods, chemical pretreatment using dilute acid is reported as one of the leading pretreatment technologies [10,11]. The advantage of acid pretreatment is the solubilization of hemicellulose, thus making the cellulose more accessible for the enzymes [12]. Depending on the process temperature, some sugar degradation compounds such as furfural, HMF and aromatic lignin degradation compounds are detected and affect the microorganism in the fermentation step [13,14]. Therefore, an additional operation of detoxification is required prior to fermentation [15,16]. These methods include overliming [17], ion exchange [18], detoxification with laccase[19] and biological detoxification [20]. Enzymatic hydrolysis by cellulolytic enzymes [21,22] is another most important step in bioconversion of lignocelluloses into ethanol. The sugar syrup obtained after cellulosic hydrolysis is used for ethanol fermentation [23]. In the fermentation process, the hydrolytic products including monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) will be fermented to valuable products such as ethanol by *Saccharomyces cereviseae,* shows high ethanol productivity, high tolerance to ethanol and tolerance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass [24].

The aim of the present work was to explore the possibility of using saw dust of *Lantana camara*, for the production of fermentable sugar which in turn would provide cheap substrate for the production of ethanol. Further, the attempt was made to ferment hexose sugar hydrolysate alongwith pentose sugar hydrolyaste by *Sacccharomyces cerevisiae.*

Materials and methods

Biomass collection and preparation

Lantana camara was collected from the local area of the village Balawala, Dehradun, India. Dried stem wood and branches were cut into small pieces and the material was further finely grounded by chipping and milling using a laboratory knife mill to attain a particle size of 2-4 mm. The obtained wood dust of *Lantana camara* was screened through sieves of different pore sizes to obtain particles of uniform mesh size viz. 40 and 60. The wood dust was washed repeatedly with water and dried overnight at 60-70ºC.

Biomass composition analysis

The chemical composition of *Lantana camara* was analysed for cellulose, hemicellulose, lignin, ash and moisture content. The chemical analysis of *Lantana.camara* wood dust was performed by following the TAPPI (Technical Association of Pulp and Paper Institute) protocols [25].

Optimization of pretreatment of lignocellulosic material

Delignification and neutralization

The *L.camara* wood dust suspension (10 %, w/v) was treated with varying concentration of sodium hydroxide (1.0-4.0 %, w/v) at room temperature. The time of incubation was also varied from 2-10 hrs. After respective periods of incubation for delignification, the wood dust suspension was neutralized to pH 7.0 using concentrated sulfuric acid. The delignified biomass was then filtered through double layered muslin cloth and the biomass residue was washed with tap water and dried overnight at 60ºC.

Optimization of acid pretreatment

The optimization of acid hydrolysis was carried out at room temperature with different acids (sulfuric acid, hydrochloric acid, phosphoric acid and nitric acid) at different concentrations (1-5 % for dilute acid hydrolysis and 80-100 % for concentrated acid hydrolysis) for 24 hours to the suspension obtained after delignification and neutralization at 10 % (w/v) solid content (*Lantana camara w*ood dust) [21,23].

Detoxification of hydrolysate

In the present study, the hydrolysate obtained after dilute acid hydrolysis was subsequently detoxified sequentially by the different detoxification methods such as ethyl acetate differential extraction, overliming, vaccum evaporation and activated charcoal to diminish the inhibition effect

of different inhibitors by the method of Canteralla *et al* [26].

Enzymatic hydrolysis of delignified *Lantana camara*

Cellulase and β-glucosidase enzyme was prepared by *Trichoderma viride* and *Aspergillus wentii* respectively by solid state fermentation utilizing pretreated/delignified *Lantana camara* wood dust as substrate.

The enzymatic hydrolysis of cellulose was performed in 250 ml Erlenmeyer flask. 2.5 gm, 5.0 gm and 10.0 gm cellulose material was suspended separately in 100 ml of 50 mM citrate buffer, pH 4.8 containing 100 units of partially purified cellulase enzyme. The suspension was incubated for varying intervals of time from 0 to 48 h at 50°C with constant shaking at the rate of 150 rpm. In order to prevent bacterial contamination, 0.01 % (v/v) toluene was added. At indicated time intervals, samples were drawn and immersed in a boiling water bath for few min. to stop the enzyme action. The residual material was removed by centrifugation for 10 min. at 5000 rpm. The supernatant was decanted off and reducing sugar was expressed as glucose. Whenever large amount was to be hydrolysed, multiple incubations were carried out or procedure was scaled up to 5 litre.

Ethanol Fermentation

Microorganisms

Saccharomyces cerevisiae VS1, VS2, VS3 were isolated from the soil samples collected from the hot regions, where boiling effluent was drained out from the distillation column of the nearby local distillery of Dehradun Uttarakhand India. The organisms were identified as *Saccharomyces cerevisiae* in our lab [27]. It was maintained on Yeast extract, Peptone Dextrose Agar (YEPDA). The strains were further acclimatized to ferment hemicellulosic acid hydrolysate fraction of *Lantana camara* biomass [27]. For inoculum preparation, *Saccharomyces cerevisiae* VS1 and VS3, from slants were subcultured on petriplates containing nearly 15 ml of YEPDA (Yeast Extract, Peptone, Dextrose, Agar) medium and incubated at 30°C for 24 hours. The cell were aseptically harvested in YEPD broth and incubated at 30°C for 24 hours at 150 rpm. 10% inoculum containing nearly 10^6-10^7 cell/ml was used to inoculate the fermentation in culture in the flasks for production of alcohol.

Fermentation

The sugar solution produced from acid and enzymatic hydrolysis was concentrated in a rotary vaccum to give 10 % (w/v) concentration. The fermentation of acid, enzymatic and mixed hydrolysates was carried out separately in Erlenmeyer flasks. The enzymatic hydrolysate (45.75 g/L sugars, pH 5.5) was inoculated with *Saccharomyces cerevisiae* VS1 (5 %, v/v) and incubated at 40°C for 24 hours under static conditions. The detoxified xylose rich hydolysate obtained after dilute acid hydrolysis containing 15.38 g/l of sugar was used for fermentation with *Saccharomyces cerevisiae* VS3 (5 %, v/v), a thermotolerant yeast isolated in our laboratory adapted and modified for fermenting pentose sugars [27]. The acid hydrolysate containing 15.38 g/l hemicellulosic sugars and enzymatic hydrolysate containing 45.75 g/l of cellulosic sugars were mixed in equal volumes to give mixed fraction. The mixed hydrolsate has 30.57 g/l of sugar concentration. The fermentation of mixed hydrolysate was carried out by co-culturing *Saccharomyces cerevisiae* VS1 and *Saccharomyces cerevisiae* VS3. Samples were withdrawn at regular intervals of 4h and centrifuged at 10,000 g for 15 min. at 4°C. The cell free supernatant was used to determine ethanol concentration.

Analytical methods

Total reducing sugars were measured by dinitrosalicyclic acid (DNS) method of Miller [28]. Total phenolics released during delignification were determined by the Folin-Ciocalteau reagent method using Vanillin as a standard [29]. Total furans were estimated by the UV absorbance method [17]. The percentage enzymatic saccharification and hydrolysis yield was calculated by Caputi [30].

$$\text{Saccharification (\%)} = \frac{\text{Amount of Glucose (mg/ml) x 100}}{\text{Total concentration (mg/ml) in the substrate}}$$

$$\text{Hydrolysis yield } Y_{cellulose}\,(\%) = \frac{\text{Cellulose}_S\,(g) - \text{Cellulose}_R\,(g)\ X\ 100}{\text{Cellulose}_S\,(g)}$$

where, cellulose$_S$ (g) is the amount of cellulose in the substrate, Cellulose $_R$(g) is the amount of the cellulose in the residual solid after the process.

The ethanol content of the fermented extract was estimated colorimetrically after distillation by the method described by Caputi [30]. The ethanol yield ($Y_{ethanol}$) was calculated assuming that 1 g of glucose present in the liquid would theoretically give 0.511 g of ethanol and 1 g of cellulose gives 1.11 g of glucose [31]. The ethanol yield ($Y_{ethanol}$) was calculated according to

$$(Y_{ethanol}) = \frac{\text{Ethanol produced (g) X 100}}{\text{Cellulose}_S\,(g) - \text{Cellulose}_R\,(g)\ X\ 0.568 - \text{Glucose (g) X } 0.511}$$

Glucose is the unfermented glucose in the liquor. The factor of 0.568 is the theoretical conversion factor for ethanol from cellulose by *S. cerevisiae* [32].

Statistical analysis

All the experiments were performed in triplicate and the results are presented as mean± standard deviation [33].

Results and discussion

Compositional analysis of Lantana camara

The comminuted and oven dried wood dust of *Lantana camara* was found to contain cellulose (44.50±3.0 %), hemicellulose (17.81±1.1 %), lignin (29.80±2.2 %), ash (6.84±0.43 %) and moisture (2.67±0.35 %).The presence of 62.2 % of holocellulose makes it a potential material for bioethanol production.

Pretreatment of lignocellulosic material

Delignification of L. camara

The lignin removal is essential to improve the enzymatic hydrolysis of *L.camara* as it increases the accessibility of enzyme to cellulose. The chemical delignification of *Lantana camara* wood dust displayed a continuous increase in phenolics with an increase in sodium hydroxide from 1.0-4.0 % (w/v). However, above the 3 % (w/v) concentration of alkali, there was no significant increase in phenolics content. The wood dust slurry at 10 % (w/v) solid content when treated with 3 % (w/v) NaOH at room temperature for 4 hours resulted in 90.13 % delignification with phenolics yield of 268 ± 15.79 mg/g of dry weight of substrate. Similar, results were reported by alkaline pretreatment of chopped rice straw by 2 % NaOH at 20 % solid loading rate [34]. The main effect of sodium hydroxide pretreatment on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity of biomass [35,36]. Further, a high degree of delignification by sodium sulphite and sodium chlorite was reported by the previous workers[4,37,38].

Since, in the present study the delignification has been performed at room temperature but with a relatively longer time of four hours, it causes very less sugar degradation or loss as shown in Table 1.

Acid pretreatment

In the present set of experiments, the dilute mineral acid i.e. 2.0 (%, w/v) sulfuric acid pretreatment, hemicelluloses content of delignified *Lantana camara* wood dust decreased by almost 80.35% followed by hydrochloric, phosphoric and nitric acid as shown in Table. 2.

In the present study for pretreatment of *Lantana camara* wood dust, almost 92.0 % hemicelluloses depolymerization was obtained at room temperature after 24 h of residence time using 2 (%, w/v) sulfuric acid as hydrolyzing agent (Table 3). The result shows that by employing 3 (%, w/v) of sulfuric acid for acid hydrolysis of delignified substrate, there is an increase in the total concentration of furfural and hydroxymethyl furfural in the hydrolysate.

Table 1. Delignification of *Lantana camara* wood dust with different concentration of sodium hydroxide at room temperature

NaOH (%, w/v)	Time(h)	Phenolics Yield (mg/g dry biomass)	Delignification (%, w/w)	Sugar Yield (mg/g)
1	2	23.63±0.89	7.70	-nd-
	4	66.52±4.40	22.32	-nd-
	6	81.62±9.25	27.38	-nd-
	8	103.56±8.15	34.75	-nd-
	10	132.57±14.26	44.29	8.64
2	2	53.35±6.24	17.90	-nd-
	4	96.66±9.37	32.43	-nd-
	6	131.22±13.18	44.03	8.27
	8	197.18±11.52	66.16	11.63
	10	223.64±10.41	75.03	13.44
3	2	182.19±12.67	61.13	10.64
	4	268.61±15.79	90.13	12.23
	6	268.31±17.11	90.03	13.10
	8	266.40±11.19	89.39	13.63
	10	265.33±12.23	89.03	13.95
4	2	226.09±17.44	75.86	11.50
	4	268.17±15.10	89.31	13.65
	6	268.93±18.22	90.24	13.41
	8	269.11±14.91	90.30	14.14
	10	268.45±19.14	90.08	14.00

Results are mean ± SD of 3 replicates

Table 2. Different concentration acid pretreatment of *Lantana c amara* wood dust at room temperature for 24 hours

Acid	Concentration (%, w/v)	Sugar Yield (mg/g)	Furfural+HMF Concentration (mg/L)
Sulfuric	1	125.67±3.29	103.43
	2	180.25±0.54	134.15
	3	182.36±0.73	178.44
	5	171.54±0.4.32	192.65
	80	155.42±2.44	418.54
	90	143.65±1.91	602.18
	100	131.79±2.02	815.25
Hydrochloric	1	141.24±3.45	146.67
	2	148.63±4.05	192.31
	3	152.84±2.25	289.67
	5	167.91±5.20	295.04
	80	141.59±3.25	642.44

table 2. (continued).

Acid	Concentration (%, w/v)	Sugar Yield (mg/g)	Furfural+HMF Concentration (mg/L)
	90	161.15±4.23	715.53
	100	150.59±1.78	754.41
Phosphoric	1	99.19±0.05	95.19
	2	95.42±0.12	143.24
	3	104.25±1.23	155.81
	5	130.12±3.52	185.13
	80	97.63±1.23	350.29
	90	91.64±1.02	455.25
	100	87.48±0.53	495.91
Nitric	1	85.19±1.57	107.81
	2	90.66±0.08	155.45
	3	95.15±1.46	171.09
	5	105.12±2.43	175.22
	80	115.10±1.46	502.13
	90	92.67±0.23	767.11
	100	90.10±1.08	835.56

Results are mean ± SD of 3 replicates

Table 3. Acid pretreatment of *Lantana camara* wood dust with different concentration of sulfuric acid at room temperature

Sulfuric acid (%, w/v)	Time (h)	Sugar Yield (mg/g)	Hemicellulose Depolymerisation (%)	Total (Furfural+HMF) Concentration (mg/L)
1	6	77.37	39.13	-nd-
	12	101.61	51.39	-nd-
	18	119.40	60.39	94±4.0
	24	127.52	64.50	103±3.5
	30	132.21	66.87	116±4.2
2	6	101.72	51.45	-nd-
	12	154.92	78.36	126±2.6
	18	168.51	85.23	131±4.7
	24	181.86	91.99	134±3.4
	30	182.43	92.22	152±2.5
3	6	142.34	72.00	115±3.3
	12	166.85	84.39	144±4.2
	18	181.22	91.66	165±1.9
	24	179.48	90.78	178±3.4
	30	178.15	90.11	188±4.1

Results are mean ± SD of 3 replicates

The trials were conducted for hemicellulose depolymerization at higher temperature ranges (100- 140°C) with shorter interval of residence time (30-90 min). The results showed an enhanced sugar yield corresponding to an increase in depolymerization and decreased solid recovery. The maximum sugar yield was obtained when *Lantana camara* was hydrolysed with 2 % (w/v) sulphuric acid at 120°C for 60 min giving almost 88 % depolymerization of hemicellulosic fraction. No significant increase in sugar was obtained when pretreatment was carried out at higher temperature using 2 (%, w/v) of sulfuric acid (Table 4). This may be due to degradation of sugar in severe conditions. Increasing, the pretreatment period beyond 1 h, resulted in a decrease in the hydrolysed sugar concentration. This may be correlated to the degradation of sugars i.e. formation of furfural and hydroxyl-methyl furfural (HMF) at higher acid concentration with increase in time leads to an increase in total concentration of furfural and hydroxymethyl furfural in the acid hydrolysate.

Detoxification of hydrolysate

From the results obtained (Table 5.), it was observed that differential ethyl acetate extraction reduced 65 % of the total phenolics and resulted in 6.9 % decline in sugar yield. Overliming reduced the total furfural and hydroxymethyl furfural by 76 % and there was further 4.7 % decline in sugar yield. Subsequent detoxification with activated charcoal resulted in further reduction of total phenolics concentration by 54.02 % while the

Table 4. Acid pretreatment of *Lantana camara* wood dust with (2 %, w/v) of sulfuric acid at different temperatures

Temperature(°C)	Time(h)	Sugar Yield (mg/g)	Hemicellulose Depolymerisation(%)	Total (Furfural+HMF) concentration
100	30	101.54	51.36	112±2.6
	60	129.71	65.61	135±3.5
	90	148.51	75.12	174±2.8
120	30	115.32	58.33	146±2.6
	60	174.14	88.08	167±2.2
	90	171.25	86.62	178±3.1
140	30	124.15	62.80	164±3.5
	60	175.29	88.66	193±4.2
	90	165.55	83.74	216±3.6

Results are mean ± SD of 3 replicates

Table 5. Detoxification of delignified/pretreated acid hydrolysate of *Lantana camara* wood dust

Detoxification method	Sugar Yield (g/g)	Furfural+HMF concentration (mg/L)	Phenolics concentration (mg/g)
Control	181.86±7.25	134.15±2.65	268.61±11.25
Ethyl acetate extraction	169.23±4.23	127.89±1.68	94.12±2.20
Overliming by Ca(OH)2	161.20±3.65	51.17±0.12	83.76±3.40
Activated charcoal treatment	153.85±2.35	-nd-	38.51±5.25

Results are mean ± SD of 3 replicates

furfural and hydroxymethyl furfural concentration was not detected by the UV absorbance method [17]. The complete sequential detoxification by different methods lead to the removal of almost 86 % phenolics and 70 % of total furfural and hydro-xymethyl furfural from the dilute alkali delignified/dilute acid treated hydrolysate of *Lantana camara* wood dust. However, a 15 % decline in sugar yield was observed which was quite high. The results of the present study are in agreement with the work of the previous workers [4,23,39,40,41], where overliming and activated charcoal were employed to remove the inhibitors from the acid hydrolysate to strongly improve the fermentability of dilute acid hydrolysate from lignocellulosic substrates in the production of bioethanol.

Enzymatic hydrolysis of cellulosic material

During the course of enzymatic saccharification, a perpetual increase in sugar concentration was observed till 12-18 h which on prolonged incubation remained almost constant (Fig.1). The optimal saccharification yield (0.899 g of sugar/g of cellulose) was achieved after 12 h of incubation at optimal performance parameters. At this point of time almost 82 % of cellulose depolymerization was obtained, resulting in sugar concentration of 45.75 g/l in hydrolysis medium. The maximal rate of saccharification of 0.116 g/g/h was achieved after 6 h of incubation, which thereafter started declining. These results were in agreement to the results reported by the previous workers where they observed the maximum rate of hydrolysis in 4-8 h of enzyme substrate incubation [4,40,42]. The decline in hydrolysis rate could be due to the increasing resistance of the substrate during the course of hydrolysis or other factors [15,43,44,45].

Fermentation

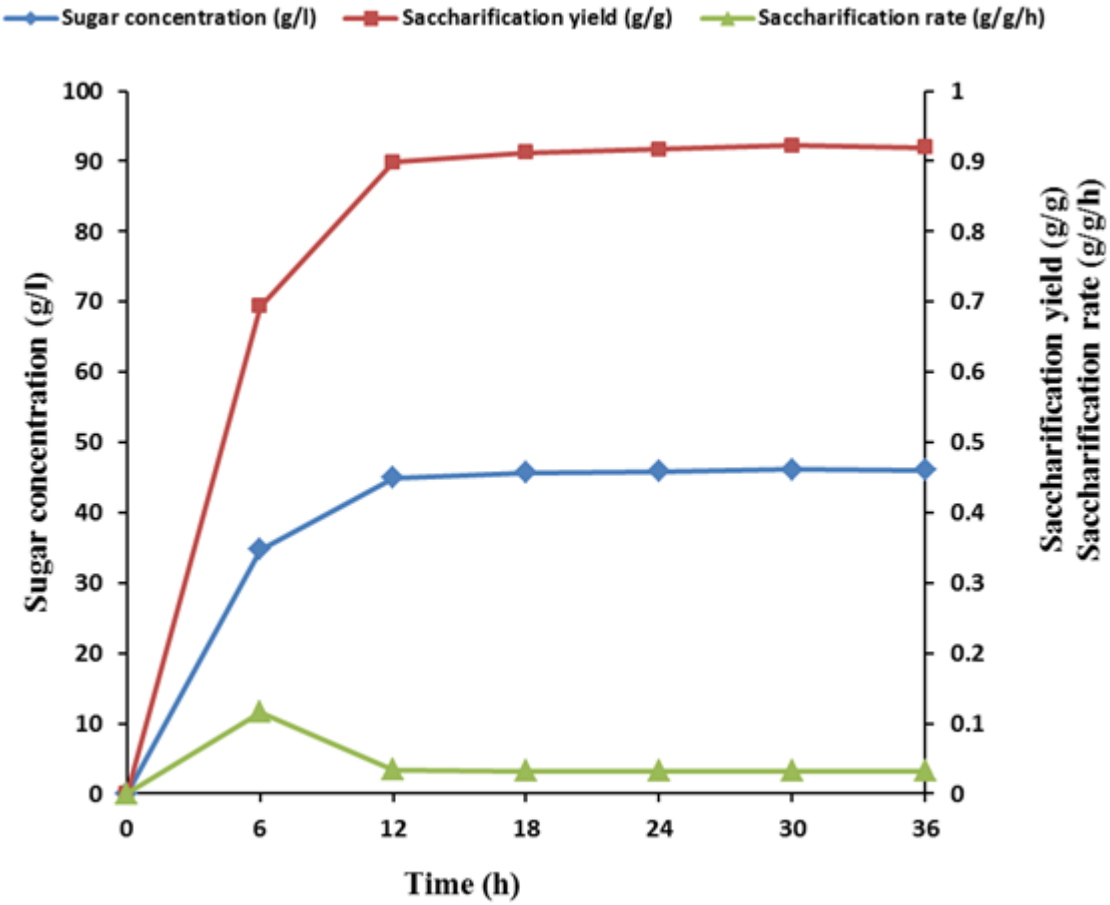

Fig. 1. Time course profile of enzymatic saccharification of pretreated/delignified *Lantana camara* wood dust

The acid and enzymatic hydrolysates were fermented with *Saccharomyces cerevisiae* VS1 and *Saccharomyces cerevisiae* VS3 respectively. Fig. 2 shows the time course study of alcohol production by *Saccharomyces cerevisiae* VS1 using 15 % (w/v) medium of pretreated/delignified *Lantana camara* wood dust enzymatic hydrolysate. The enzymatic hydrolysate, when fermented with *S.cerevisiae*, resulted in ethanol production of 43.56 g/L with yield 0.44 g/g and fermentation efficiency of 85.22 % after 24 h of incubation. The maximum productivity of 2.72 g/l/h was obtained after 12 h of fermentation. The maximum biomass accumulation was also observed within 12 h of fermentation with a biomass yield of 0.18 g/g of sugar. Gupta *et al.*,[38] reported an ethanol yield of 0.45 g/g of sugar from the fermentation of enzymatic hydrolysate obtained from the wood of *Prosopis juliflora.* Singh and Bishnoi [46] reported an yield of 0.50, 0.47 and 0.48 by the strains of *Saccharomyces cerevisiae, Schefferesomyces stipitis* and co-culture of both the strains, respectively.

From the data it was very clear that 4-6 h after the incubation at optimal performance parameters, the extracellular excretion of ethanol was observed into the medium. Following lag period of 2-4 h the concentration of ethanol in the fermentation medium increases steadily. After 4 h of fermentation, the rate of ethanol production increased rapidly and attains the maximal value between 16-24 h of incubation. These results are in agreement with the results obtained by Pasha[47] and Li [48], who found that the maximum ethanol productivity was obtained after 24-48 hours of fermentation. However, another obtained the maximal ethanol productivity employing *Saccharomyces cerevisiae* grown on the wood enzymatic hydrolysate of *Prosopis juliflora* after 12 h of culture incubation[38]. In the present study, the overall production of the ethanol seems to follow sigmoid type of kinetics. Further, it was observed that sugar uptake by the yeast cells was completed within 2-12 h of incubation which was correlated as rapid increase of biomass of yeast cells in the first 12 h of incubation. The time course data on the microbial activity of ethanol production conforms to the classical scheme of Gaden [49] i.e. process is considered as the type I fermentation. The process was characterized by the single rate maxima, representing the growth of yeast cells accompanied by the high substrate consumption

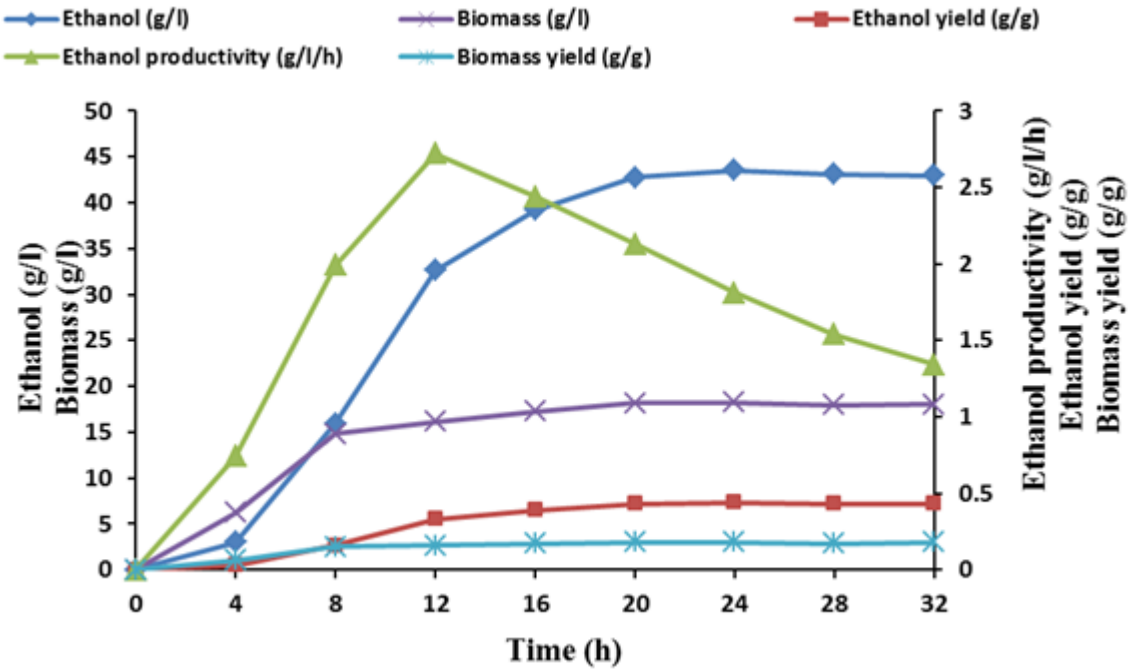

Fig. 2. Time course profile of ethanol production under optimal fermentation condition utilizing enzymatic hydrolysate by *Saccharomyces cerevisiae*

and simultaneously increased rate of ethanol production. The reduced growth rate of yeast culture corresponds to the reduced rate of production of ethanol. Further, decline in ethanol production after 30 h incubation can be due to consumption of accumulated ethanol by the organism [50].

The 1 litre acid hydrolysate containing 100 g of sugars was used to produce 26.38 gl^{-1} of ethanol in 48 h of fermentation at 40°C. This gives an ethanol yield of 0.263 g of ethanol per g of sugar with a fermentation efficiency of 51.62 %. The productivity of 0.549 g/l/h was obtained after 48h of fermentation (Fig 3). These results are in agreement with the results obtained by the previous workers utilizing the different strains of yeast for conversion of different types of feedstock employing different hydrolysis strategies for release of fermentable sugars and subsequently utilizing it for the production of bioethanol[13,51-56].

However, the fermentation efficiency was quite low in the present study as a lot of amount of sugar was not utilized for the conversion to ethanol. However, in case of mixed hydrolysate (pentose and hexose sugar) containing 100g of sugars was used to produce 39.15 gl^{-1} of ethanol in 40 h of fermentation at 40°C. This gives an ethanol yield of 0.392 g of ethanol per g of sugar with a fermentation efficiency of 76.59 % (Fig 4). The productivity of 0.932 g/l/h was obtained after 42h of fermentation.

The co-culturing of two strains of *Saccharomyces cerevisiae* VS1 and VS3 did not enhance ethanol yield than the cellulosic enzymatic hydolysate fermentation as shown in Fig. 5. However, we were able to ferment the mixed hydrolysate to give a better and more economical way of bio conversion of lignocellulosic biomass.

Conclusion

Ethanol made from *Lantana camara* provides unique environmental, economic strategic benefits and can be considered as a safe and cleanest liquid fuel alternative to fossil fuels. Also ethanol production from *L.camara* will serve as an effective method of weed management. The present study demonstrates that pretreatment (delignification and acid pretreatment) was effective in removing most of the non-cellulosic materials, making cellulose more accessible to enzymes that convert it into fermentable sugars. The hexose sugars can be fermented to ethanol

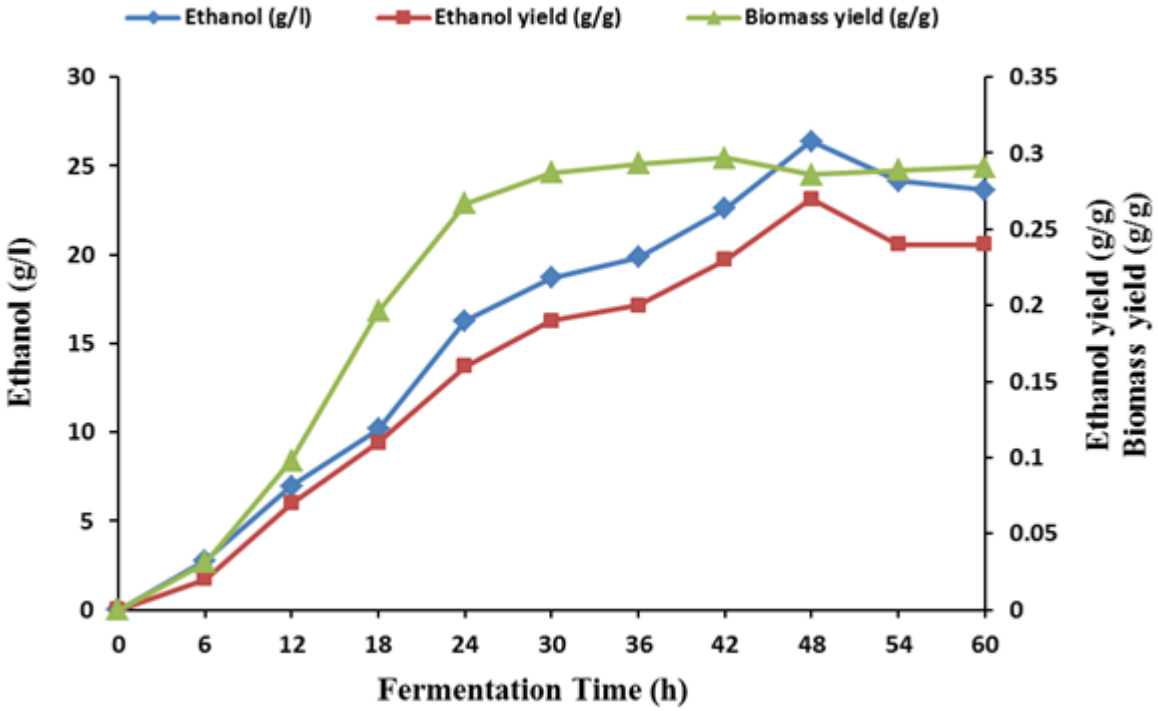

Fig. 3. Fermentation of acid hydrolysate by *Saccharomyces cerevisiae* VS3

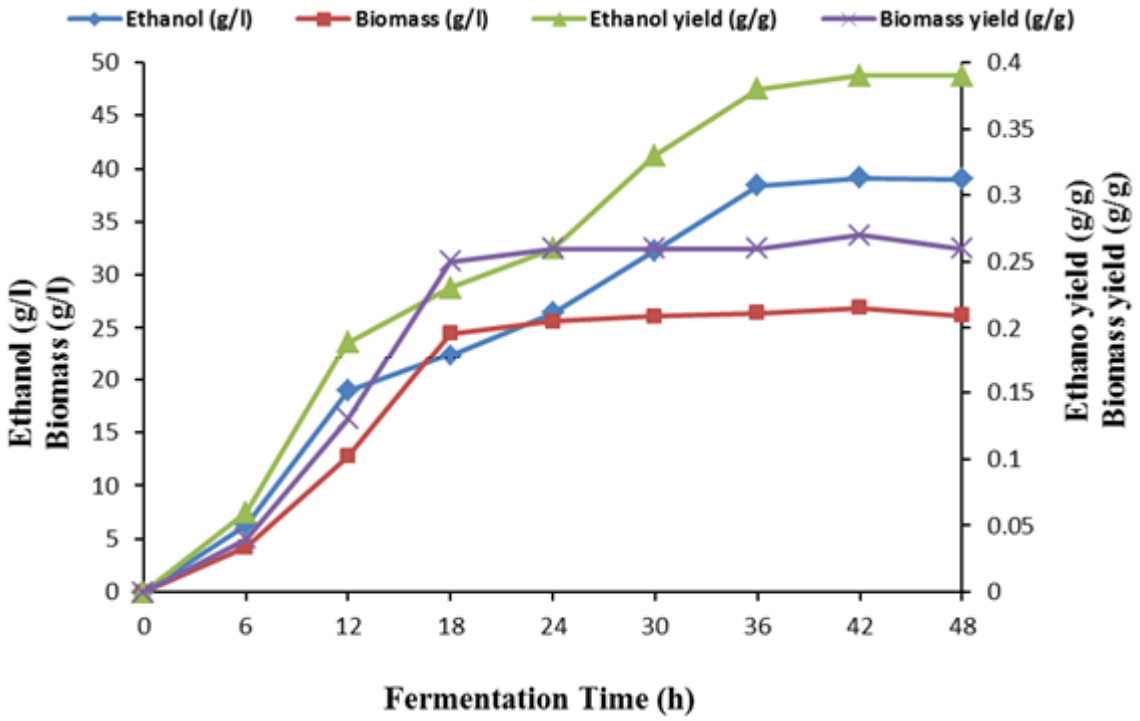

Fig. 4. Fermentation of mixed hydrolysate by co-culturing *Saccharomyces cerevisiae* VS1 and *Saccharomyces cerevisiae* VS 3

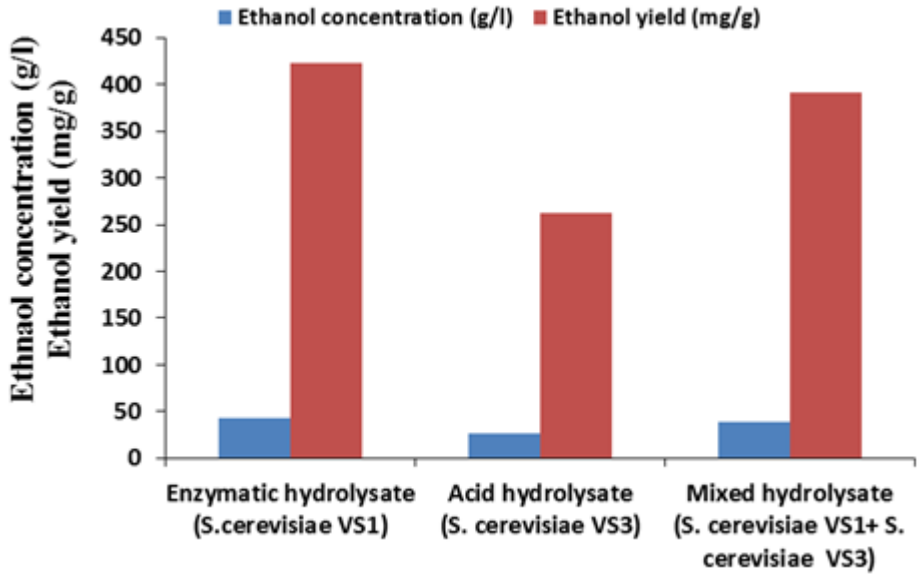

Fig. 5. Comparative ethanol production and ethanol yield by *Saccharomyces cerevisiae* VS1, VS3 and in co-culture condition in fermentation of enzymatic, acid and mixed hydrolysate

while, pentose sugar had little difficulty in getting femented. Further, the mixed hydrolysate comprising of enzymatic and acid hydrolysate showed a comparable fermentable efficiency using co-culturing different strains of *Saccharomyces cerevisiae* VS1 and VS3. However, the overall economic feasibility is certainly dependent on the efficient utilization of all main components of the lignocellulosic wastes i.e. cellulose, hemicelluloses and lignin.

References

1. **Maas, R.H.W., Bakker, R.R., Boersma, A.R., Bisschops, I., Pels, J.R, Jonge, E.D, Weusthuis, R.A., Reith, H. (2008).** Pilot–scale conversion of lime–treated wheat straw into bioethanol: quality assessment of bioethanol and valorization of side streams by anaerobic digestion and combustion. Biotechnol. Biofuels., 1: 14
2. **Saha, B.C., Cotta, M.A. (2006).** Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnol. Prog., 22: 449-453
3. **Babu, S., Love A., Babu, C.R. (2009).** Ecological restoration of lantana-invaded landscapes in corbett tiger reserve. India. Ecological Rest., 27: 467-477
4. **Kuhad, R.C., Gupta, R., Khasa, Y.P., Singh A. (2010).** Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation. Bioresour. Technol., 101: 8348-8354
5. **Fatma, H., Abd E. Z., Fadel, M. (2010).** Production of bioethanol via enzymatic saccharification of rice straw by cellulase produced by *Trichoderma reesei* under solid state fermentation. New York Science Journal, 3(4): 72-78
6. **Zhang, M., Wang, F., Su, R., Qi, W., He, Z. (2010).** Ethanol production from high dry matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment. Bioresour. Technol. 101(13): 4959-4964
7. **Kumar, R., Wyman, C.E. (2009).** Cellulase adsorption and relationship to features of corn stover solids produced by leading pretreatments. Biotechnol. Bioeng. 103: 252-267
8. **Wei, Z., Zili, Y., Jiangfeng, H., Fengcheng, L. , Bo, H., Ming, L, Shufen, H., Yezi, L.V., Wei, S., Arthur, R., Fan, H., Junhua, P., Liangcai, P. (2013).** Three lignocellulose features that distinctively affect biomass enzymatic digestibility under NaOH and H_2SO_4 pretreatments in *Miscanthus*. Bioresour. Technol. 130(13): 30-37
9. **Taherzadeh, M.J., Karimi, K. (2008).** Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int. J. Mol. Sci., 9: 1621-1651
10. **Moiser, N., Wyman,C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. (2005).** Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol., 96(6): 673-686
11. **Qi B., Chen, X., Wan, Y. (2010).** Pretreatment of wheat straw by nonionic surfactant– assisted dilute acid for enhancing enzymatic hydrolysis and ethanol production. Bioresour. Technol., 101: 4875-4883
12. **Hendriks, A.T.W.M., Zeeman, G. (2009).** Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol., 100: 10-18
13. **Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V. (2005).** Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Proc. Biochem., 40: 3693-3700
14. **Alvira, P., Tomas–pejo, E., Ballesteros, M., Negro, M.J. (2010).** Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis, a review. Bioresour. Technol., 101: 4851-4861
15. **Hsu, T.C., Guo, G.L, Chen, W.H., Hwang, W.S. (2010).** Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresour. Technol., 101: 4907-13.
16. **Kasthuri, D., Gowdhaman, D., Ponnusami, V. (2012).** Production of ethanol from Water Hyacinth (*Eichhornia crassipes*) by *Zymomonas mobilis* CP4:Optimization Studies. Asian. J. Scientific Res., 5: 285-289
17. **Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O, (2000).** Effects of $Ca(OH)_2$ treatments (“overliming’’) on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnol. Bioeng., 69: 526-536
18. **Nilvebrant, N., Reimann, A., Larsson, S., Jonsson, L.J. (2001).** Detoxification of lignocellulose hydrolysates with ion exchange resins. Appl. Biochem. Biotechnol. 91-93: 35-49

19. **Martin, N. C., Galbe, M., Wahlbom. C.F., Hagerdal, B.H., Jonsson, L.J. (2002).** Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*. Enz. Microb. Technol. 31: 274-282
20. **Lopez, M.J., Nichols, N.N., Dien, B.S., Moreno, J., Bothast, R.J. (2004).** Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl. Microb. Biotechnol. 64: 125-131
21. **Sun, Y., Cheng, I. (2002).** Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol., 83: 1-11
22. **Singhania, R.R., Sukumaran, R.K., Pillai, A, Prema, P., Pandey, A. (2006).** Solid - state fermentation of lignocellulosic substrates for cellulase production by *Trichoderma reesei* NRRL 11460. Indian J. Biotechnol., 5(4): 332-336
23. **Chandel, A.K., Chan, E.S., Rudravaram, R., Narasu, M.L., Rao, L.V, Ravindra, P. (2007).** Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol. Mol. Biol. Rev., 2(1): 14-32
24. **Tomas-pejo, E., Oliva, J.M., Ballesteros, M., Olsson, L. (2008).** Comparison of SHF and SSF processes from steam–exploded wheat straw for ethanol production by xylose-fermenting and robust glucose–fermenting *Saccharomyces cerevisiae* strains. Biotechnol. Bioeng., 100(6): 1122-1131
25. **TAPPI, Test Methods. Atlanta (USA). (1992).** Technical Association for Paper and Pulp Industries (TAPPI) Publication.
26. **Cantarella, M, Cantarella, L, Gallifuco, A, Spera, A, Alfani, F (2004).** Effect of inhibitor releasedduring steam explosion treatment of popolar wood on subsequent enzymatic hydrolysis and SSF. Biotechnol. Prog., 20: 200-206
27. **Joshi, V. (2011).** Studies on uilization of hemicelluloses component of residue for biofuel production. Master Thesis Biotechnology: H.N.B Garhwal University, India.
28. **Miller, G.L. (1959).** Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.
29. **Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999).** Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. Meth. Enzymol., 299: 152-178.
30. **Caputi, A.J.R., Ueda, M., Brown, T. (1968).** Spectrophotometric determination of ethanol in wine. Am. J. Enol. Vitic., 19: 160-165.
31. **Dadi, A.P., Varanasi, S., Schall, A.A. (2006)**. Enhancement of cellulose sacchariûcation kinetics using an ionic liquid pretreatment step. Biotechnol. Bioeng., 95(5): 904-910.
32. **Varga, E., Klinke, H.B., Reczey, K, Thomsen, A.B. (2004).** High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnol. Bioeng., 88(5): 567-574.
33. **Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang,, J., Li, Q., Tu, Y., Yu, B., Wang, Y., Jiang, J., Qin, J., Peng, L. (2012).** Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H_2SO_4 pretreatments in Miscanthus. Biotechnol. Biofuel 5: 58.
34. **Zhang, Q.Z., Cai, W.M. (2008).** Enzymatic hydrolysis of alkai–pretreated rice straw by *Trichoderma reesei* ZM4–F3. Biomass Bioenerg., 32(12): 1130-1135.
35. **Zhu, J.Y., Pan, X., Zalesny, R.S J. (2010).** Pretreatment of woody biomass for biofuel production: energy efficiency, technologies, and recalcitrance. Appl. Microbiol. Biotechnol., 87(3): 847-857.
36. **Brodeur, G., Yau, E, Badal, K., Collier, J., Ramachandran, K.B, Ramakrishnan, S. (2011).**Chemical and physicochemical pretreatment of lignocellulosic biomass: A Review, Enzyme Res., Vol. 2011: Article ID 787532, Epub 2011 May 24.
37. **Gupta, R., Khasa, Y.P., Kuhad, R.C. (2011).** Evaluation of pretreatment methods in improving

the enzymatic saccharification of cellulosic materials. Carbohydr. Polym., 84: 1103-1109.
38. **Gupta, R., Kumar, S., Gomes, J., Kuhad, R.C. (2012).** Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. Biotechnol. Biofuels, 5: 16.
39. **Martinez, A., Rodriguez, M.E., Wells, M.L., York, S.W., Preston, J.F., Ingram, L.O. (2001).** Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol. Prog., 17: 287-293.
40. **Gupta, R., Sharma, K.K., Kuhad, R.C. (2009).** Separation hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis* NCIM 3498. Bioresour. Technol., 100: 1214-1220.
41. **Meinita, M.D., Hong, Y.K., Jeong, G.T, (2012).** Detoxification of acidic catalyzed hydrolysate of *Kappaphycus alvarezii* (cottonii). Bioprocess. Biosyst. Eng., 35(1-2): 93-98.
42. **Obama, P., Ricochong, G., Muniglia, L., Brosse, N. (2012).** Combination of enzymatic hydrolysis and ethanol organosolv pretreatment: effect on lignin structures, delignification yields and cellulose–to–glucose conversion. Bioresour. Technol., 112: 156-163.
43. **Zhu, S., Wu Y., Yu Z., Wang, C., Yu F., Jin, S., Ding, Y., Chi, R., Liao, J., Zhang, Y. (2006).** Comparison of three microwave/chemical pretreatment processes for enzymatic hydrolysis of rice straw. Biosystems. Eng., 93(3): 279-283.
44. **Yang, J., Zhang, X., Yong, Q., Yu, S. (2010).** Three-stage hydrolysis to enhance enzymatic saccharification of steam–exploded corn stover. Biores. Technol., 101(13): 4930-4935.
45. **Shuai, L., Xuejun, P. (2012).** Hydrolysis of cellulose by cellulase-mimetic solid catalyst. Energy Environ. Sci., 5: 6889-6894.
46. **Singh, A., Bishnoi, N.R. (2012).** Optimization of enzymatic hydrolysis of pretreated rice straw and ethanol production. Appl. Microbiol. Biotechnol., 93(4): 1785-93.
47. **Pasha, C., Nagavalli, M., Rao, L.V. (2007).** *Lantana camara* for fuel ethanol production using thermotolerant yeast. Lett. Appl. Microbiol., 44: 666-672.
48. **Li, Y., Gao, K., Tian, S., Zhang, S, Yang, X. (2011).** Evaluation of *Saccharomyces cerevisiae* Y5 for ethanol production from enzymatic hydrolysate of non–detoxified steam–exploded corn stover. Bioresour. Technol., 102: 10548-10552.
49. **Gaden, E.J. (1959).** Fermentation process kinetics. J. Biochem. Microbiol. Technol. Eng.,1(4): 413-429.
50. **Ramon-Portugal, F., Pingaud, H., Strehaiano, P. (2004).** Metabolic transition step from ethanol consumption to sugar/ethanol by *Saccharomyces cereviseae*. Biotechnol. Lett., 26: 1671-1674.
51. **Ohgren, K., Bengsston, O., Gorwa-Grauslund, M.F., Galbe, M., Hahn-Hagerdal, B., Zacchi, G. (2006).** Simultaneous saccharification and co-fermentation of glucose and xylose in steam–pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. J. Biotechnol., 126: 488-498.
52. **Saha, B.C., Catta, M.A. (2007).** Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol. Enzyme Microb. Technol., 41(4): 528-532.
53. **Cara, C., Ruiz, E., Oliva, J.M., Saez, F., Castro, E. (2008).** Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresour. Technol., 99: 1869-1876.
54. **Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. (2009).** Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res., 48: 3713-3729.
55. **Chen, K., Iverson, A.G., Garza, E.A., Grayburn, W.S., Zhou, S. (2010).** Metabolic evolution of non-transgenic *Escherichia coli* SZ420 for enhanced homoethanol fermentation from xylose. Biotechnol. Lett., 32(1): 87-96.
56. **Girio, F.M., Fonesca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Lukasik, R. (2010).** Hemicelluloses for fuel ethanol: A review. Bioresour. Technol., 101(13): 4775-4800.