Enhanced ethanol production using hydrophobic resin detoxified two-step pre-treated Pine forest litter hydrolysate, mass balance equation generation and BOLT-ON process development supplementing molasses

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Abstract

High ethanol demand to make up the targets of the future gradual replacement of gasoline it is essential to look for hybrid technologies using first generation and second-generation biofuel feedstock's. The current study used a two-step sequential pre-treatment (first dilute alkali, then dilute acid) of Pine (*Pinus roxburghii*) forest litter (PFL), for bioethanol production. Further, the saccharification of pre-treated PFL was optimized through response surface method using Box–Behnken Design, wherein 0.558 g/g of reducing sugar was under optimized conditions: 12.5% (w/v) of biomass loading, 10 FPU of enzyme, 0.15% (w/v) of Tween-80 in 48 h. During fermentation using *Saccharomyces cerevisiae* NCIM 3288 strain, the ethanol titre was 22.51 g/L, which was enhanced to 27.38 g/L after detoxification of PFL hydrolysate with hydrophobic resin (XAD-4). Furthermore, the hydrolysate was supplemented with molasses (total initial sugar: 100 g/L), wherein 46.02 ± 2.08 g/L ethanol was produced with 0.482 g/g yield and 1.92 g/l/h productivity. These results, showed that BOLT-ON Technology involving integration of molasses, increases the initial sugar availability leading to enhanced ethanol production, therefore can be compatible in bio-refineries to produce high titer ethanol.

Introduction

The worldwide demand for alternative energy sources has created an opportunity for scientists to develop a cost-effective and environmentally sustainable process (Darvishi and Abolhasan Moghaddami 2019). Because of its potential to strengthen the Earth's environment by lowering greenhouse gas emissions, lignocellulosic biomass is an ideal solution for a renewable alternative to fossil fuels (Pandey et al. 2019). PFL has been identified as one of the most abundant renewable forest-based feedstock resources for lignocellulosic ethanol production (Pandey and Negi 2015a).

The lignocellulosic ethanol production process is divided into three stages: pre-treatment, enzymatic hydrolysis, and fermentation. Pre-treatment of biomass is a critical step in the breakdown of the lignin-carbohydrate complex network structures, which increases lignocellulose porosity and cellulose accessibility to enzymes in hydrolysis (Singh and Bishnoi 2012; Brar et al. 2019; Pandey et al. 2022). The alkaline pre-treatment essentially removes the lignin component from lignocellulosic biomass without affecting the other components, reduces the degree of crystallinity in biomass, and breaks the lignin-carbohydrate bonds (Agbor et al. 2011; Salehian and Karimi 2013). The lignocellulosic biomass, including the hemicelluloses portion, is transformed by diluted sulfuric acid pre-treatments into soluble sugars that can be enzymatically hydrolysed to fermentable sugars (Gupta et al. 2012). In the Lower Himalayan regions of India, chirr pine (*Pinus roxburghii*) trees are one of the most common biomass sources, and because their litter creates serious forest fires in the summer, they would make an abundant and cost-effective feedstock for ethanol production (Cotana et al. 2014; Pandey and Negi 2015a).

Various fermentation inhibitors, such as furfural, HMF, Phenolics, and acetic acid, are formed during the pre-treatment step, causing interference with microbial metabolism by causing cellular and molecular damage to them and significantly affecting ethanol production (Wallace-Salinas and Gorwa-Grauslund

2013). Most industrial yeasts, including *S. cerevisiae*, are susceptible to hydrolysis and pre-treatment inhibitors (Ask et al. 2013; Coz et al. 2016) To facilitate the yeast fermentation process, a suitable detoxifying agent such as overliming and ion exchange resin were added to the hydrolysate prior to fermentation (Tian et al. 2011). AmberliteTM XAD-4, a polystyrene-divinylbenzene resin without functional group, adsorbs phenols from aqueous solution under acidic conditions where the presence of molecular phenol species dominates, whereas adsoption decreases sharply under alkaline conditions due to the presence of dominant negatively-charged phenol species (Ku and Lee 2000).

Molasses as non-crystallizable by-products of sugar mills after sugar crystallization, contains about 40–50% of total sugars (sucrose, fructose and glucose) are preferred substrate for 1G ethanol production worldwide (Joannis-Cassan et al. 2014; Fan et al. 2018; Wu et al. 2020). India produces ~ 2.3 billion litres of ethanol using ~8.00 metric tons of molasses from about 330 distilleries (Brar et al. 2019). Currently, in India about 800 million litres of ethanol is in use as 5% blending (E5) in petrol. However, to meet the 20% blending (E20) mandate of Ministry of Petroleum and Natural Gas (MoPNG), India there is huge challenge of molasses availability as feedstock (Brar et al. 2019). Another challenge in the molasses fermentation is the need of huge amount of water for its dilution to 15–20% sugar content to make it fermentable for fermenting microorganism (Wu et al. 2020). Hence, to cope up with the future mandate, it is essential to explore some new technologies and find alternative approaches, other than existing 1G and 2G approaches. Most molasses to ethanol production studies showed the 10–12% v/v ethanol production using wild type yeast strain (Arshad et al. 2017; Wu et al. 2020).

In present study, a two-step pre-treatment, sequential alkali and acid pre-treatment (SAAP) of PFL was performed followed by optimization of enzymatic saccharification by Response surface methodology (RSM). The obtained SAAP-PFL hydrolysate was detoxified using AmberliteTM XAD-4 resin and fermented to ethanol using *S. cerevisiae* NCIM3288 strain. Mass balance equation was generated using obtained results for overall lignocellulosic to ethanol production. Furthermore, SAAP-PFL hydrolysate as supplemented with molasses and mixed fermentation was evaluated for high titre ethanol production.

Materials and Methods

Feedstock collection and compositional analysis

Pine Forest Litters (PFL), the lignocellulosic feedstock used in this study, was collected in sterile bags from Ranikhet District, Uttaranchal, India and dried at 50 ± 2^{o}C for 72 h. The dried PFL was milled and homogenized to a particle size of less than 1.0 mm. The composition of native and sequential alkali and acid pretreated (SAAP) PFL (Table 1) was analyzed using the National Renewable Energy Laboratory's (NREL) Laboratory Analysis Protocol (Meehnian et al. 2016).

Table 1
Compositional analysis of native and pretreated Rice straw

Components (% w/w)	Native PFL	Sequential alkali and acid pretreated PFL		Enzyme hydrolyzed PFL
		Composition	% removal	Composition
Cellulose	31.45 ± 1.78	41.25 ± 1.82	-31.79 ± 1.21**	11.55 ± 0.85
Hemicellulose	20.78 ± 1.02	8.72 ± 0.35	58.04 ± 2.45	7.45 ± 0.34
Total Lignin	31.48 ± 1.14	11.39 ± 0.46	63.81 ± 2.78	8.59 ± 0.41
Total Material*	83.71 ± 1.45	61.36 ± 0.81		27.59 ± 1.06
Material loss	-	22.35 ± 1.09		33.77 ± 1.03
* Total material is defined as the arithmetic sum of cellulose, hemicelluloses, and lignin content.				
** Negative sign denotes a rise in cellulose content as a result of pretreatment-induced cellulose bulging.				

Sequential dilute alkali and dilute acid Pretreatment (SAAP) of PFL (dup: abstract ?)

PFL was first pretreated with 1.0% NaOH at 121^{o}C and 15 lb pressure for 45 minutes. Following pretreatment, the solid residual PFL was neutralized with 1.0 N HCl before being washed under tap water to remove salt contamination. To remove moisture, the neutralized sample was dried at 50 ± 2 ^{o}C. The dilute alkali pretreated biomass was pretreated a second time with dilute acid.

Various concentrations of H_2SO4 (0.5, 1.0, 1.5, 2.0, and 2.5 v/v) were tested as a pretreatment for PFL that had undergone an alkali process. To maximize the yield of reducing sugar after enzymatic saccharification, the effects of three process parameters, including acid concentration (H_2SO4: 0.5, 1.0, 1.5, 2.0 and 2.5% v/v), biomass concentration (5, 10, 15, & 20% w/v), and incubation time (15, 30, 45, 60, and 75 min), were investigated and optimized by one parameter at a time approach. An experiment under similar conditions with native PFL (without alkali pretreatment) served as the control in this experiment.

Enzymatic saccharification of pretreated PFL

The crude cellulase enzyme (enzyme activity: 5.09 FPU/ml) was produced under solid state fermentation using adopting our previously reported method using locally isolated fungal strain (Pandey et al. 2016).

At 10% w/v biomass loading, SAAP-PFL was hydrolyzed in 250 ml stoppered conical flasks with crude cellulase in a 50 mM citrate buffer (pH 4.8).

The experiments were carried out for 48 h at 50^{o}C and 150 rpm with 20 FPU/g enzyme loading and 0.1% w/v Tween-80 loading. The reaction mixture was supplemented with 0.3% (w/v) sodium azide to check for microbial contamination. After 48 h, the samples were centrifuged at 10,000 rpm for 10 min to collect the hydrolysate. The reducing sugar composition in the hydrolyzate was determined using the 2, 5-dinitrosalicylic acid method (Sindhu et al. 2013). All of these experiments were performed in technical triplicates and data presented are average values.

Optimization of enzymatic saccharification of pretreated PFF by RSM

Statistical optimization is essential to see the interactive effect of parameters on the response along with individual parameters effect (Ezeilo et al. 2019; Kumar et al. 2020). The effect of four independent variables for enzymatic saccharification, including biomass loading (10-12.5% w/w), enzyme loading (5–15 FPU/g), surfactant concentration (0.1–0.2% w/w), and incubation time (36–60 h), as well as their interactions with various combinations of variables, were investigated and mathematically optimized by RSM using a Box-Behnken factorial design (BBD).

BBD with four factors and three levels of -1 (lower), 0 (middle), and + 1 (higher) (Table 2) was used for optimization, with three replicates at the centre point, and a total of 27 runs were performed (Table 3). Minitab 16 was used for the experimental design, data analysis, quadratic model construction, and generation of response surface contour plots (Minitab Inc., USA).

Table 2
Coded values and experimental range of process variables used in experimental design.

Variables	Symbol	Coded level of variables		
		Sequential alkali and acid pretreated biomass saccharification		
		-1	0	1
Biomass loading % (w/v)	X_1	10	12.5	15
Enzyme loading (FPU/g)	X_2	5	10	15
Surfactant concentration % (w/w)	X_3	0.1	0.15	0.2
Incubation Time (h)	X_4	36	48	60

Table 3
Reducing sugar yields for individual runs of the RSM design. (RO = Run order, BL = Biomass loading, EL = Enzyme loading, SC = Surfactant concentration, IT = Incubation time and TRSY = Total reducing sugars yield).

Run Order	BL (% w/w)	EL (FPU/g)	SC (% w/w)	IT (h)	TRSY (g/g)
1.	15	5	0.15	48	0.293
2.	10	15	0.15	48	0.356
3.	15	15	0.15	48	0.509
4.	12.5	10	0.1	36	0.396
5.	12.5	10	0.2	36	0.441
6.	12.5	10	0.1	60	0.444
7.	12.5	10	0.2	60	0.439
8.	10	10	0.15	36	0.281
9.	15	10	0.15	36	0.32
10.	10	10	0.15	60	0.264
11.	15	10	0.15	60	0.574
12.	12.5	5	0.1	48	0.327
13.	12.5	15	0.1	48	0.472
14.	12.5	5	0.2	48	0.264
15.	12.5	15	0.2	48	0.492
16.	10	10	0.1	48	0.286
17.	15	10	0.1	48	0.485
18.	10	10	0.2	48	0.278
19.	15	10	0.2	48	0.473
20.	12.5	5	0.15	36	0.289
21.	12.5	15	0.15	36	0.377
22.	12.5	5	0.15	60	0.317
23.	12.5	15	0.15	60	0.449
24.	12.5	10	0.15	48	0.578
25.	12.5	10	0.15	48	0.582
26.	12.5	10	0.15	48	0.582

Run Order	BL (% w/w)	EL (FPU/g)	SC (% w/w)	IT (h)	TRSY (g/g)
27.	12.5	10	0.15	48	0.582

Table 3 illustrates the trial setup for the Box-Behnken design. Response surface graphs were used to analyse the variables' optimal values for the highest degree of response in order to comprehend the impact of the individual and combined effects of the four variables. Analysis of variance (ANOVA) was used to evaluate the statistical parameters, and three additional runs using the BBD-optimal conditions were used to validate the model (Table 4).

Table 4
Analysis of variance (ANOVA) for the response surface quadratic model for SAAP-PFL saccharification. DF: Degree of freedom; Seq SS = sequential sum of squares, Adj SS = Adjusted sum of square, Adj MS = Adjusted mean square.

S.No.	Source	DF	Seq SS	Adj SS	Adj MS	F	P
1.	Regression	14	0.221245	0.320131	0.023214	19.50	0.000
2.	Linear	4	0.153215	0.039594	0.012310	9.67	0.001
3.	Square	4	0.131240	0.132431	0.029416	28.44	0.000
4.	Interaction	6	0.030216	0.024517	0.003582	3.09	0.044
5.	Residual Error	12	0.012475	0.013970	0.001291		
6.	Lack-of-Fit	10	0.012362	0.015231	0.001539	1542.44	0.092
7.	Pure Error	2	0.000001	0.000002	0.000001		
8.	Total	26	0.298451				
DF: Degree of freedom; R^2 96.25; adj R^2 91.45							

Hydrophobic resin mediated detoxification of SAAP-PFL hydrolysate

A polymeric hydrophobic resin Amberlite™ XAD-4 was procured from Sigma-Aldrich (St. Louis, MO). The SAAP hydrolysate was detoxified by treatment with polymeric hydrophobic resin XAD-4. The pH of hydrolysate was adjusted to 2.5 by using 1.0 N H_2SO_4. Initially, the resin was activated by sequential washing with milliQ water, 25% iso-propyl alcohol, 50% iso-propyl alcohol, 100% iso-propyl alcohol and milliQ water, respectively. The activated XAD-4 was mixed with hydrolyzate (5% w/v) in batch mode in the 250 ml Erlenmeyer flask containing 100 ml hydrolysate (pH = 2.5). The experiment was performed at 120 rpm and 30°C for 6 h. After treatment hydrolysate was filtered, resin was separated and regenerated by using acetone for their reuse. The detoxified hydrolysate was analyzed for reducing sugar by 2,5-dinitrosalicylic acid method (Miller et al. 1959), furfural by ethanol hydrochloric acid-aniline method (Pandey and Negi 2015b) and phenolic compounds by Folin Ciocalteu Mehod (Blainski et al. 2013).

Fermentation of SAAP-PFL enzymatic hydrolysate

Using detoxified and un-detoxified SAAP-PFL enzymatic hydrolysate, fermentation tests were carried out in 250 ml Erlenmeyer flasks that were airtight rubber corked. Cells of S. cerevisiae NCIM3288 (obtained from National collection of industrial microorganisms, National chemical laboratory, Pune, India) were first grown in yeast extract peptone dextrose (YEPD) broth at 30°C for an overnight period in order to obtain seed inoculum. For fermentation, 100 ml of hydrolysate (pH 5.5) was mixed with 0.75% w/v urea, inoculated with above grown seed inoculums (5% v/v) and incubated at 30^{o}C and 150 rpm for 24 h. 1.0 ml of sample was centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for ethanol concentration by dichromate method at 600 nm [21].

Mass balance equation generation

Mass balance of each steps involved in ethanol production such as SAAP, saccharification, detoxification and fermentation was carried out by calculating mole fraction. The mass balance equation of the overall process was generated on the basis of elements such as Carbon (C), Hydrogen (H), Nitrogen (N) and Oxygen (O) composition of the solid and liquid components of the different steps of each process.

Mixed fermentation of SAAP-PFL hydrolysate and molasses (BOLT-ON process) SAAP-PFL hydrolysate was supplemented with molasses to adjust the final sugar concentration 100 g/L in the fermentation medium (pH 5.5). For fermentation, 100 ml of fermentation medium (mixture of hydrolysate and molasses with total initial sugar of 100 g/l) was mixed with 0.5% g/L urea, inoculated with above grown seed inoculums (5% v/v) and incubated at 30^{o}C and 150 rpm for 24 h. 1.0 ml of sample was centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for ethanol concentration by dichromate method at 600 nm (Joshi et al. 2019). All the fermentation experiments were carried out in 250 ml Erlenmeyer flasks that were airtight rubber corked.

Results And Discussion

Compositional analysis of native and pretreated PFL

The compositional analysis of native and SAAP PFL were performed for structural carbohydrates and lignin content. Native PFL contained 31.45 ± 1.78% cellulose, 20.78 ± 1.02% hemicelluloses and 31.48 ± 1.14% total lignin (Table 1). The pretreated biomass contained cellulose, hemicellulose and total lignin content of 41.25 ± 1.82%, 8.72 ± 0.35% and 11.39 ± 0.46%, respectively. Mass balance analysis indicated around 22.35 ± 1.09% loss during SAAP. These findings suggested that the complicated lignocellulose network had been successfully broken down by the SAAP.

Sequential dilute alkali and dilute acid Pretreatment (SAAP) of PFL

Initially PFL was pretreated with 1.0% alkali, which had effectively broken the recalcitrant lignin outer covering. Then after, the alkali pretreated PFL was further exposed to dilute acid pretreatment for further deconstruction of lignocellulose to enhance availability of complex sugars (cellulose and hemicellulose) for enzymatic saccharification. Under optimum acid pretreatment conditions of 1.5% (v/v) H_2SO_4 concentration, 10% (w/v) biomass loading and 45 min incubation time a maximum of 0.248 g/g reducing sugar was released. The possible reason for this would be the affinity of acid towards the solubilization of hemicelluloses fraction of biomass. Similar kind of findings was also reported for the pretreatment of wheat straw (Sindhu et al. 2011).

The total material (sum of cellulose, hemicelluloses and lignin content) loss after SAAP was found to be 20.84 ± 1.44%, which implied that during pretreatment the some amount of complex sugars were hydrolyzed into simpler sugars (Table 1). The compositional analysis of SAAP-PFL implied the removal of lignin and hemicelluloses 63.81 ± 2.78% w/w & 58.04 ± 2.45% w/w, respectively. On the other hand due to breakdown of intermolecular ester cross-linkage of lignin and hemicelluloses network and increase of cavitation, the cellulose content was found to be increased.

Optimization of process parameters for enzymatic saccharification of pretreated PFL by Box-Behnken design (BBD)

The levels of variables were set to achieve maximum Reducing sugar yield in Box-Behnkken design (BBD) (Table 2). The experimental design for twenty-seven runs at different combinations of factors and their responses (reducing sugar yield) was illustrated in Table 3. The effect of each independent variable on the response (reducing sugar yield) was evaluated by fitting polynomial quadratic equations. Polynomial equations for the model used were as below:

$$\text{Predicted Reducing sugar yield (g/g)} = 0.558 + 0.260\,X_1 + 0.101\,X_2 + 6.94\,X_3 + 0.031\,X_4 - 0.017\,X_1^2 - 0.002\,X_2^2 - 25.81\,X_3^2 - 0.0005\,X_4^2 + 0.0007\,X_1X_2 - 0.006\,X_1X_3 + 0.0031\,X_1X_4 + 0.0311\,X_2X_3 + 0.0001\,X_2X_4 - 0.019\,X_3X_4$$

Where X_1, X_2, X_3 and X_4 are biomass loading, enzyme loading, surfactant concentration and incubation time respectively.

ANOVA (analysis of variance) was conducted to test statistical significance of experimental data. The model was found to be highly significant as evident from the Fisher's F-test ($p = 0.001$). The p-value denoting the significance of the coefficients was important in understanding the pattern of mutual interaction between the variables. The goodness of the fit of the model was checked by determining coefficient R^2 (96.29) which indicated that 96% of the total variation in enzymatic hydrolysis yield explained by the model (Table 4). Lack of fit analysis was carried out to measure the failure of used model to represent the data in the experimental domain. Non-significant p-value of lack of fit ($p > 0.05$) for model indicated that experimental data obtained was in good agreement with the model (Bajar et al.

2020). The surface plots were plotted by using Minitab 16 software and interaction of variables were analyzed for the determination of their optimum level to achieve maximum reducing sugar yield (Fig. 1a-1f).

The optimum conditions for the enzymatic saccharification of SAAP-PFL were 12.5% w/v biomass loading, 10 FPU/g enzyme loading, 0.15% w/v surfactant loading and 48 h incubation time, wherein a maximum of 0.548 g/g of reducing sugar was released. Untreated (native) PFL yielded 0.091 g/g of reducing sugar after being enzymatically saccharified under similar conditions. These results revealed that, in comparison to Native PFL saccharification, SAAP improved the saccharification yield by six-folds.

Detoxification of hydrolysate

The hydrolysate was detoxified by polymeric hydrophobic resin XAD-4. At 5% w/v XAD-4 loading 85.22% furfural, 68.56% HMF and 67.78% phenolics were found to be removed with 5.94% sugar loss from the hydrolysate in 2 h (Table 5). Since, the effective amount of inhibitors was removed from the hydrolysate with considerable sugar loss. These findings indicated that the hydrophobic resin XAD-4 was an effective detoxifying agent for the removal of fermentation inhibitors in order to increase fermentation efficiency.

Table 5
Composition of SAAP- PFL hydrolysate before and after detoxification with hydrophobic resin XAD-4.

S. No.	Hydrolysate components (g/L)	SAAP PFL hydrolysate	XAD treated SAAP PFL hydrolyzate (After 2 h)	% loss
1	Reducing Sugar	54.82 ± 2.11*	51.54 ± 2.39	5.94
2	Furfural concentration	0.68 ± 0.014	0.10 ± 0.003	85.22
3	5-Hydroxymethylfurfural (HMF)	0.71 ± 0.021	0.22 ± 0.002	68.56
3	Total Phenolics concentration	1.15 ± 0.041	0.37 ± 0.002	67.78
*Mean + Standard deviation (for n = 3)				

Fermentation of PFL hydrolysate for ethanol production

Ethanol fermentation of SAAP-PFL hydrolysate (with sugar concentration 54.82 g/l) and XAD-4 detoxified SAAP-PFL hydrolysate (with sugar concentration 51.54 g/l) were carried out using *S. cerevisiae* NCIM3288 and effect of detoxification on fermentation efficiency was studied. After 24 h of incubation, 20.15 ± 0.98 g/L and 21.68 ± 1.14 g/l were produced with the ethanol yield of 0.426 g/g and 0.498 g/g for un-detoxified and XAD-4 detoxified SAAP-PFL hydrolysate, respectively (Table 6). These results indicated

that the XAD-4 detoxification of hydrolysate was effective for the fermentation efficiency of S. *cerevisiae* NCIM 3288.

Table 6
Fermentation parameters obtained in fermentation of SAAP-PFL hydrolysate with *S. cerevisiae* NCIM 3288 in 24 h at 30°C. S_0; Initial sugar concentration, S_t; Residual sugar, E_t = Ethanol concentration, Yp/s; Ethanol yield (gram ethanol per gram of sugar consumed), P = Productivity (grams of ethanol produced per hour) and EE = Ethanol efficiency

S. No.	SAAP-PFL Hydrolysate type	S_0 (g/l)	S_t (g/l)	E_t (g/l)	(Yp/s) (g/g)	P (g/l/h)	EE (%)
1	Un-detoxified	54.82 ± 2.11*	7.53	20.15 ± 0.98	0.426	0.84	83.41
2	XAD-4 detoxified	51.54 ± 2.39	5.21	21.68 ± 1.14	0.498	0.90	97.45
3	Un-detoxified + Molasses	100.00 ± 3.21	6.55	42.51 ± 2.16	0.455	1.77	89.04
4	XAD-4 detoxified + Molasses	100.00 ± 3.11	4.52	46.02 ± 2.08	0.482	1.92	94.32
*Mean + Standard deviation (for n = 3)							

Mass balance analysis of overall lignocellulosic bioethanol production process

Mass balance analysis must be performed in order to determine whether the lignocellulose ethanol production process is viable (Akanksha et al. 2015; Nakanishi et al. 2017). Mass balance experiments on the sequential alkali and acid pre-treatment (SAAP), saccharification, detoxification, and fermentation processes were done in order to evaluate the lignocellulosic ethanol conversion process logically. Under optimal conditions, the cellulose content in the biomass was found to be increased by 31.79% during SAAP, and 72% of the cellulose was found to be hydrolyzed during enzymatic saccharification. The removal of inhibitors during the detoxification process was found to be approximately 73.85%, with a sugar loss of 5.94%. Fermentation efficiencies were found to be 83.41% without XAD-4 detoxification and 97.45% with detoxification, respectively.

For the developed method, the following mass balance equation was produced based on the elemental (C, H, N, and O) analysis:

Step 1: SAAP Pre-treatment of PFL

$2.66C_{1.6}H_{2.3}O$ (S) + $55.5H_2O$ (l) = $0.11C_6H_{12}O_6$ (l) + 0.049 $C_5H_{10}O_5$ (l) + $20.39C_{1.5}H_{2.1}O$ (l) + $1.19C_{3.4}H_{1.7}O$ (S) **……Equation (I)**

Step II: Hydrolysis of SAAP-PFL

$1.19C_{3.4}H_{1.7}O$ (S) + 38.88 H_2O (l) = $0.29C_6H_{12}O_6$ (l) + 0.043 $C_5H_{10}O_5$ (l) + $10.287C_{1.3}H_{4.4}O$ (l) + $0.94C_{1.4}H_{3.7}O$ (S)……**Equation (II)**

Step III: Fermentation of the SAAP-PFL hydrolysate

$0.29C_6H_{12}O_6$ (l) + 0.043 $C_5H_{10}O_5$ (l) + 10.287 $C_{1.3}H_{4.4}O$ (l) = $0.39C_2H_5OH$ (l) + 1.99 CO_2 (g) + $0.11C_6H_{12}O_6$ (l) + $0.036C_5H_{10}O_5$ (l) + $7.494C_{1.2}H_{4.3}O$ (l) + $CH_{1.75}N_{0.15}O_{0.5}$ (S)…….**Equation (III)**

Overall Mass balance equation (By summarizing all three equations):

$2.66C_{1.6}H_{2.3}O$ (S) + 94.38 H_2O (l) = $CH_{1.75}N_{0.15}O_{0.5}$ (S) (Biomass) **+** $0.39C_2H_5OH$ (l) + 1.99 CO_2 (l) + 0.22 $C_6H_{12}O_6$ (l) + 0.085 $C_5H_{10}O_5$ (l) + 20.39 $C_{1.5}H_{2.1}O$ (l) + $0.94C_{1.4}H_{3.7}O$ (S) (hydrolyzed residue) + 7.494 $C_{1.2}H_{4.3}O$ (l)

In above equation S = solid and l = liquid

The mole fraction calculations made at each step were used to create the mass balance equation above.

BOLT-ON process fermentation

Enhanced ethanol production was observed during mixed fermentation of SAAP-PPF hydrolysate supplemented with molasses, as expected due to increased initial sugar concentration (Table 6). Table 6 shows that when molasses was supplemented with XAD-4 detoxified SAAP hydrolysate during mixed fermentation, a maximum of 46.02 2.08 g/L ethanol was produced with 0.482 g/g yield and 1.92 g/l/h productivity. However, when un-detoxified hydrolysate was added to molasses, lower ethanol titer, yield, and efficiency were seen. These findings demonstrate the detoxification of XAD-4 in mixed fermentation to be successful as well. In previous studies also the enhanced ethanol production is reported during mixed fermentation of lignocellulosic hydrolysate and molasses (Brar et al. 2019; Pandey et al. 2022).

Conclusion

The sequential alkali and acid pre-treatment (SAAP) of PFL had significantly improved the enzymatic saccharification efficiency by six-folds. The polymeric resin, XAD-4 mediated detoxification drastically removed the major inhibitors such as furfural (85.22%), HMF (68.56%) and phenolics (67.78%) with very low (5.94%) sugar loss. And, therefore ethanol titre, yield and productivity was enhanced after

detoxification. During BOL-ON technology fermentation using molasses supplemented SAAP-PFL, high ethanol titre demonstrated the effectiveness of this hybrid bioethanol production technology. As a result, the developed Batch bioprocess for BOLT-ON technology would be a promising technology for industrial scale ethanol production in existing bio-refinery infrastructure. To the best our literature analysis, integration of XAD-4 detoxified and molasses has been reported first time for ethanol production in this study.

Declarations

Author contribution All authors contributed to the study conception and design. Ajay Kumar Pandey performed conceptualization, material preparation, data analysis and manuscript writing. Sangeeta Negi performed conceptualization, writing and reviewing of the manuscript. All authors read and approved the final manuscript.

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Data availability All data and materials included in the submitted manuscript will be available upon request.

Ethical approval The authors declare that there is no compliance with journal ethical standards.

Consent to participate The authors agree to participate in the submitted manuscript.

Competing interests The authors declare no competing interests.

References

1. Agbor VB, Cicek N, Sparling R et al (2011) Biomass pretreatment: Fundamentals toward application. Biotechnol Adv 29:675–685. https://doi.org/10.1016/j.biotechadv.2011.05.005
2. Akanksha K, Sukumaran RK, Pandey A et al (2015) Material balance studies for the conversion of sorghum stover to bioethanol. https://doi.org/10.1016/j.biombioe.2015.11.027
3. Arshad M, Hussain T, Iqbal M, Abbas M (2017) Enhanced ethanol production at commercial scale from molasses using high gravity technology by mutant S. cerevisiae. Brazilian J Microbiol 48:403–409. https://doi.org/10.1016/j.bjm.2017.02.003
4. Ask M, Bettiga M, Mapelli V, Olsson L (2013) The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing Saccharomyces cerevisiae. Biotechnol Biofuels 6:22. https://doi.org/10.1186/1754-6834-6-22

5. Bajar S, Singh A, Bishnoi NR (2020) Exploration of low-cost agro-industrial waste substrate for cellulase and xylanase production using Aspergillus heteromorphus. Appl Water Sci 10:153. https://doi.org/10.1007/s13201-020-01236-w
6. Blainski A, Lopes G, de Mello J (2013) Application and Analysis of the Folin Ciocalteu Method for the Determination of the Total Phenolic Content from Limonium Brasiliense L. Molecules 18:6852–6865. https://doi.org/10.3390/molecules18066852
7. Brar KK, Agrawal D, Chadha BS, Lee H (2019) Evaluating novel fungal secretomes for efficient saccharification and fermentation of composite sugars derived from hydrolysate and molasses into ethanol. Bioresour Technol 273:114–121. https://doi.org/10.1016/j.biortech.2018.11.004
8. Cotana F, Cavalaglio G, Gelosia M et al (2014) Production of Bioethanol in a Second Generation Prototype from Pine Wood Chips. Energy Procedia 45:42–51. https://doi.org/10.1016/j.egypro.2014.01.006
9. Coz A, Llano T, Cifrián E et al (2016) Physico-chemical alternatives in lignocellulosic materials in relation to the kind of component for fermenting purposes. Mater (Basel) 9. https://doi.org/10.3390/MA9070574
10. Darvishi F, Abolhasan Moghaddami N (2019) Optimization of an Industrial Medium from Molasses for Bioethanol Production Using the Taguchi Statistical Experimental-Design Method. Fermentation 5:14. https://doi.org/10.3390/fermentation5010014
11. Ezeilo UR, Wahab RA, Mahat NA (2019) Optimization studies on cellulase and xylanase production by Rhizopus oryzae UC2 using raw oil palm frond leaves as substrate under solid state fermentation. Renew Energy 1–12. https://doi.org/10.1016/j.renene.2019.11.149
12. Fan M, Zhang S, Ye G et al (2018) Integrating sugarcane molasses into sequential cellulosic biofuel production based on SSF process of high solid loading. Biotechnol Biofuels 11:1–9. https://doi.org/10.1186/s13068-018-1328-0
13. Gupta R, Kumar S, Gomes J, Kuhad RC (2012) Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. Biotechnol Biofuels 5:16. https://doi.org/10.1186/1754-6834-5-16
14. Joannis-Cassan C, Riess J, Jolibert F, Taillandier P (2014) Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup. Biomass Bioenergy 70:165–173. https://doi.org/10.1016/j.biombioe.2014.07.027
15. Joshi J, Dhungana P, Prajapati B et al (2019) Enhancement of Ethanol Production in Electrochemical Cell by Saccharomyces cerevisiae (CDBT2) and Wickerhamomyces anomalus (CDBT7). Front Energy Res 7:70. https://doi.org/10.3389/fenrg.2019.00070
16. Ku Y, Lee K-C (2000) Removal of phenols from aqueous solution by XAD-4 resin. J Hazard Mater 80:59–68. https://doi.org/10.1016/S0304-3894(00)00275-2
17. Kumar M, Pandey AK, Kumari S et al (2020) Secretome produced by a newly isolated Aspergillus flavus strain in engineered medium shows synergy for biomass saccharification with a commercial cellulase. Biomass Convers Biorefinery. https://doi.org/10.1007/s13399-020-00935-3

18. Meehnian H, Jana AK, Jana MM (2016) Effect of particle size, moisture content, and supplements on selective pretreatment of cotton stalks by Daedalea flavida and enzymatic saccharification. 3 Biotech 6:235. https://doi.org/10.1007/s13205-016-0548-x
19. Miller GL, Kuila A, Suralikerimath N et al (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal Chem 31:426–428. https://doi.org/10.1021/ac60147a030
20. Nakanishi SC, Soares LB, Biazi LE et al (2017) Fermentation Strategy for Second Generation Ethanol Production From Sugarcane Bagasse Hydrolyzate by Spathaspora passalidarum and Scheffersomyces stipitis. Biotechnol Bioeng 114:2211–2221
21. Pandey AK, Edgard G, Negi S (2016) Optimization of concomitant production of cellulase and xylanase from Rhizopus oryzae SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production. Renew Energy 98:51–56. https://doi.org/10.1016/j.renene.2016.05.071
22. Pandey AK, Kumar M, Kumari S et al (2019) Evaluation of divergent yeast genera for fermentation-associated stresses and identification of a robust sugarcane distillery waste isolate Saccharomyces cerevisiae NGY10 for lignocellulosic ethanol production in SHF and SSF. Biotechnol Biofuels 12:40. https://doi.org/10.1186/s13068-019-1379-x
23. Pandey AK, Kumar M, Kumari S, Gaur NA (2022) Integration of acid pre-treated paddy straw hydrolysate to molasses as a diluent enhances ethanol production using a robust Saccharomyces cerevisiae NGY10 strain. Renew Energy 186:790–801. https://doi.org/10.1016/j.renene.2022.01.039
24. Pandey AK, Negi S (2015a) Impact of surfactant assisted acid and alkali pretreatment on lignocellulosic structure of pine foliage and optimization of its saccharification parameters using response surface methodology. Bioresour Technol 192:115–125. https://doi.org/10.1016/j.biortech.2015.04.054
25. Pandey AK, Negi S (2015b) Impact of surfactant assisted acid and alkali pretreatment on lignocellulosic structure of pine foliage and optimization of its saccharification parameters using response surface methodology. Bioresour Technol 192:115–125. https://doi.org/10.1016/j.biortech.2015.04.054
26. Salehian P, Karimi K (2013) Alkali pretreatment for improvement of biogas and ethanol production from different waste parts of pine tree. Ind Eng Chem Res 52:972–978. https://doi.org/10.1021/ie302805c
27. Sindhu R, Kuttiraja M, Binod P et al (2011) Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresour Technol 102:10915–10921. https://doi.org/10.1016/j.biortech.2011.09.066
28. Sindhu R, Kuttiraja M, Elizabeth Preeti V et al (2013) A novel surfactant-assisted ultrasound pretreatment of sugarcane tops for improved enzymatic release of sugars. Bioresour Technol 135:67–72. https://doi.org/10.1016/j.biortech.2012.09.050
29. Singh A, Bishnoi NR (2012) Optimization of enzymatic hydrolysis of pretreated rice straw and ethanol production. Appl Microbiol Biotechnol 93:1785–1793. https://doi.org/10.1007/s00253-012-

3870-1

30. Tian S, Zhu J, Yang X (2011) Evaluation of an adapted inhibitor-tolerant yeast strain for ethanol production from combined hydrolysate of softwood. Appl Energy 88:1792–1796. https://doi.org/10.1016/j.apenergy.2010.11.037
31. Wallace-Salinas V, Gorwa-Grauslund MF (2013) Adaptive evolution of an industrial strain of Saccharomyces cerevisiae for combined tolerance to inhibitors and temperature. Biotechnol Biofuels 6:151. https://doi.org/10.1186/1754-6834-6-151
32. Wu R, Chen D, Cao S et al (2020) Enhanced ethanol production from sugarcane molasses by industrially engineered: Saccharomyces cerevisiae via replacement of the PHO4 gene. RSC Adv 10:2267–2276. https://doi.org/10.1039/c9ra08673k

Figures

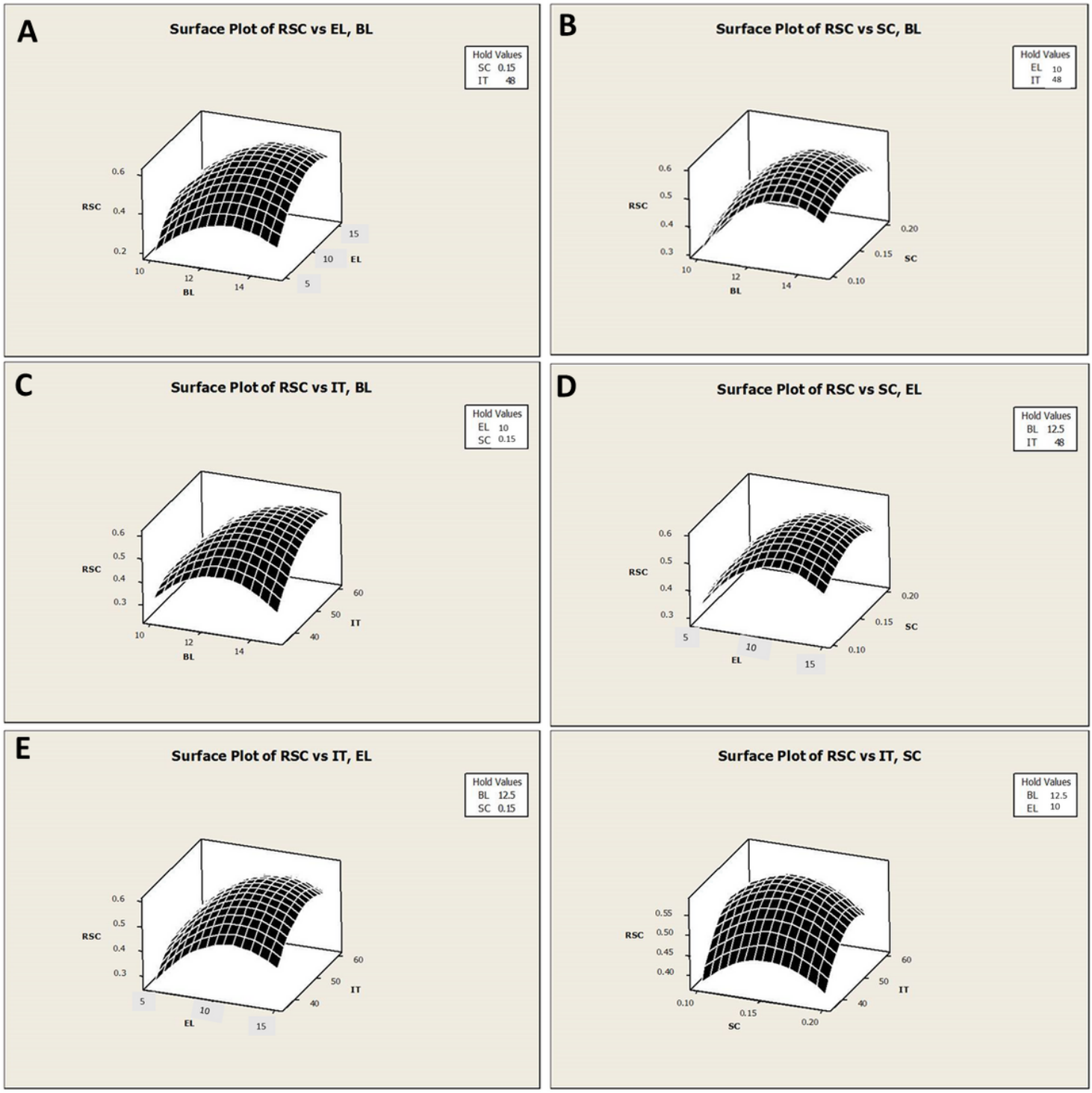

Figure 1

3D surface plots generated by Minitab Software and Box-Behnken design (BBD). Effect process parameters on saccharification of SAAP-PFF **(a)** biomass loading and enzyme loading, **(b)** surfactant concentration and biomass loading, **(c)** incubation time and biomass loading, **(d)** surfactant concentration and enzyme loading **(e)** incubation time and enzyme loading and **(f)** incubation time and surfactant concentration.