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New insight from cane sugar industry waste utilization (press mud) for cleaner biobutanol production by using *C. acetobutylicum* NRRL B-527

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Abstract

In the present study, press mud a sugar industry waste was explored for biobutanol production to strengthen agricultural economy. The fermentative production of biobutanol was investigated via series of steps *viz.* characterization, drying, acid hydrolysis, detoxification, and fermentation. Press mud contains adequate amount of cellulose (22.3%) and hemicellulose (21.67%) on dry basis and hence it can be utilized for further acetone-butanol-ethanol (ABE) production. Drying experiments were conducted in the temperature range of 60-120 °C to circumvent microbial spoilage and enhance storability of press mud. Furthermore, acidic pretreatment variables *viz.* sulfuric acid concentration, solid to liquid ratio, and time were optimized using response surface methodology. The corresponding values were found to be 1.5% (v/v), 1:5 g/mL, and 15 min, respectively. In addition, the detoxification studies were also conducted using activated charcoal that removed almost 93-97% phenolics and around 98% furans, which were toxic to microorganism during fermentation. Finally, the batch fermentation of detoxified press mud slurry (sample dried at 100 °C and pretreated) using *Clostridium acetobutylicum* NRRL B-527 resulted in higher butanol production of 4.43 g/L with total ABE of 6.69 g/L.

Keywords
Biobutanol, Detoxification, Drying, Fermentation, Press mud, Pretreatment

1. Introduction
The rapid escalating energy demand, flourishing environmental issues, and highly fluctuating market prices of crude oils lead to the development of advanced biofuels as a favorable choice over fossil fuels [1]. Moreover, the renewability and biodegradability of biofuels make them a promising substitute for traditional fuels [2]. Among different biofuels, biobutanol exhibits superior fuel properties such as higher energy content, lower

hygroscopy, less corrosive to contact parts over bioethanol and biomethanol [3]. Butanol is commonly used as a solvent and reactant to synthesize esters in chemical and allied industries, apart from its use as fuel additive [4,5]. Butanol business has grown considerably during last few years and its global market is forecasted to grow exponentially in next decade, especially in Asia-Pacific region. The global market for butanol was estimated at $3.0 billion in 2013 with 7.7% annual growth, which is projected to reach $4.3 billion in 2018[6].

The most prevalent route for biological production of butanol is by historic acetone-butanol-ethanol (ABE) fermentation using *Clostridium spp*. [7]. The most common steps involved in biobutanol production are shown in Fig. 1. Conventional feedstocks such as corn and molasses were widely used for ABE fermentation [1, 8]. However, due to food versus fuel crisis, second generation feedstocks gained more importance. Therefore, different lignocellulosic materials have been utilized for ABE production such as barley straw [9, 10], corn stover [11], wheat straw, switchgrass [12] and spruce chips [13]. Nowadays lignocellulosic biomass is catching the eye of many researchers worldwide as an attractive feedstock [14-16]. Therefore press mud as a low cost feedstock, which is abundantly available with large hollocellulose content could also be utilized in production of value added biochemicals. Hence, in the context of Indian sugar mill growth and investment, press mud is also considered as a promising feedstock for biofuel production [17].

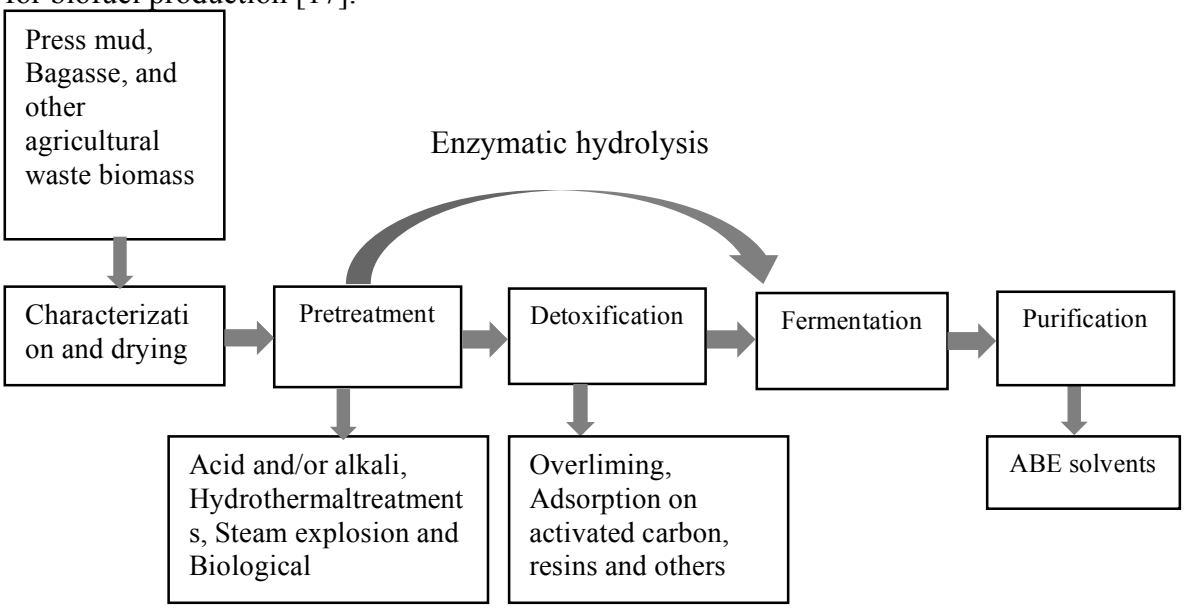

Fig. 1. Important steps involved in biobutanol production

India has major share in sugarcane industry and become the second largest producer of sugarcane in the world. It produces around 300-350 million metric tons sugarcane annually to meet the national and worldwide demand of sweeteners. As on year 2015, India had 703 sugar mills widely spread over different states such as Maharashtra, Uttar Pradesh, Karnataka, Tamil Nadu, Gujarat and Andhra Pradesh [18].

During sugar production in industry, the sugarcane juice is clarified to remove dissolved and suspended solid substances. The left over cake after clarification/filtration step is termed as 'press mud' [17]. With harvesting of 1000 kg sugarcane in a mill produces approximately 176 kg trash (sugarcane agricultural residue) and 824 kg stalks.

During its large scale processing, cane's stalks produces 104 kg sugar, 231 kg bagasse, and 26 kg molasses. In addition, 430 kg liquid effluents and 33 kg press mud is also generated as by-products [19]. India produces around 8-10 million tons press mud every year as a waste, which is being used as manure during farming [20]. However, most of the sugar mills spray large amount of spent solvents, which are generated during industrial operations, on piles of press mud [21]. Hence, press mud intermingled with spent solvents may harm the soil fertility when it is used as manure. Therefore, the use of press mud in production of value added biomolecules is thought desirable. Consequently, it will enhance the economics of sugarcane industries and ultimately contribute to the welfare of farmers.

Press mud contains approximately 5-15% sugars along with 70-80% moisture on wet basis that promotes it to be a competitive feedstock for biofuel production [22]. Kuruti et al. [17] produced bioethanol and volatile fatty acids by using press mud as a feedstock. However, due to high moisture content and considerable presence of sugars, press mud is highly susceptible for microbial spoilage and also leads to heavy transportation cost. Therefore, it is advantageous to dry, press mud for enhancing its storability, which further reduce packaging and transportation cost [23]. Sathish and Vivekanandan, [24] also showed that drying of press mud improved biogas yield and performance.

Due to complexity of lignocellulosic biomass, the pretreatment method is used to breakdown complex structure into fermentable sugars. During this process, numerous inhibitors are known to be formed which are lethal to microorganisms affecting fermentation process and can be removed by detoxification. Hence, the pretreatment and detoxification are important steps in biosynthesis of butanol [25]. Besides, the thrust areas in butanol production *viz.* selectivity, tolerance and process development to further improve solvent yield and productivity by economic way are also needs to be addressed. Therefore, significant efforts have been made in commercial production of biobutanol or retrofitting same existing bioethanol plants for butanol production to reduce the capital investment [26].

The objective of current work was to produce butanol from press mud as a substrate by using *C. acetobutylicum* NRRL B-527. This work is categorized into three parts as: (i) characterization and drying studies of press mud (ii) acid hydrolysis and detoxification studies to release maximum fermentable sugars, and (iii) fermentative production of biobutanol for higher yield and productivity. In the present manuscript, major emphasis is given on the drying study and behavioral moisture change in press mud samples. This study puts emphasis on understanding the underlying mechanisms involved in drying process of press mud under the given experimental conditions with the help of mathematical modeling. Furthermore, the use of press mud as potential feedstock for ABE fermentation to assess biobutanol production with appropriate pretreatment method was also carried out. To the best of our knowledge, no attempts have been made on biobutanol production using press mud as a feedstock, till date. The detailed fermentation and process development studies for ABE will be carried out in subsequent parts of this project and will be presented in future manuscripts.

2. Material and methods

2.1 Materials

D-glucose, peptone, sodium chloride, soluble starch, L-cysteine hydrochloride, magnesium sulfate, manganese sulfate, iron sulfate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium acetate, biotin, thiamin, p-aminobenzoic acid, glycerol, sodium hydroxide, 3,5- dinitrosalicyclic acid, sodium potassium tartarate, acetic acid, butyric acid, acetone, butanol, isopropanol were purchased from SRL Ltd, India. Sodium acetate and phenol were obtained from Sigma Aldrich, India. Hydrochloric acid and sulfuric acid were procured from Avra Synthesis Ltd, India. Yeast extract and meat extract was purchased from Himedia, India. Gallic acid, anthrone powder, Folin-Ciocalteu reagent, sodium carbonate, coomassie brilliant blue dye, phosphoric acid and activated charcoal were obtained from HPLC, India. All the chemicals and solvents were of analytical grade (AR).

2.2 Microorganism and maintenance

C. acetobutylicum NRRL B-527 was generously gifted by ARS (Agriculture Research Services) culture collection, USA. The lyophilized cells were revived by using sterile revival medium (RM) containing (g/L): glucose (5.0), peptone (10), beef extract (10), yeast extract (3.0), sodium chloride (5.0), soluble starch (1.0), L-cysteine hydrochloride (0.5), sodium acetate (3.0), and resazurin (0.001) at pH 6.8. The medium was purged with nitrogen to remove dissolved oxygen, sealed, and autoclaved at 121 °C for 20 min and cooled at room temperature. L-cysteine hydrochloride was added separately by filter sterilization. The sterile RM bottles (air tight glass bottles with rubber cork) with working volume of 80 mL were used to inoculate lyophilized cells for 48 h at 37±2 °C. Furthermore, the spore suspension of *C. acetobutylicum* NRRL B-527 was prepared by inoculating actively growing cells into sterile 6% (w/v) starch solution and incubated anaerobically for 8-10 days at 37°C. The spore suspension was stored in cool and dry place at room temperature (25 °C) for further use. Alternatively, the actively growing cells were also stored in glycerol solution (70% v/v) at -20°C.

2.3 Characterization of feedstock

Fresh press mud sample was obtained from 'Sahyadri Sugar Mill', Karad, Maharashtra, India, and stored at 4 °C until further processing. Proximate analysis of press mud was carried out after oven drying at 60 °C for 24 h. The press mud samples were further subjected to determination of moisture, ash, and protein content according to standard methods [27]. Simultaneously, the lignin content was determined by Klason method [28]. For cellulose estimation, 10 mL of 67% (v/v) sulfuric acid (H_2SO_4) was added into known amount (1 g) of press mud sample. After 1 h incubation, the equivalent amount of glucose produced was quantified by 'Anthrone'reagent [29]. Besides, hemicellulose was also determined according to method reported by Gao et al. [30]. All the experiments were performed in triplicates and results reported are average ± standard deviation of all values.

2.4 Drying of press mud

2.4.1 Experiment and procedures

A hot air oven (Bio-Technic BIT-30, India) (400 mm × 200 mm × 380 mm) with maximum output temperature of 200 °C was used for drying experiments. A predetermined quantity (101±0.5g) of press mud sample was spread over a glass plate (diameter, 150mm) with sample thickness of 15mm. Drying experiments were carried out

in the temperature range of 60-120 °C. Samples were weighed and the thickness of samples was recorded after every 10 min for each temperature. The experiment was continued until all the moisture was removed (till constant sample weight) from the press mud sample. All the experiments were performed in triplicates and results reported are average ± standard deviation of all values. Moisture content, moisture ratio, and drying rate of press mud were calculated with the help of data received.

2.4.2 Data analysis

The experimental moisture content of press mud was estimated for a given time by following expression:

$$M = \frac{W_t - W_d}{W_d} \quad (1)$$

where M is the moisture content for a given time (g /g dry solid), W_t is a mass for a given time t (g), W_d is a mass bone dry solid (g). The experimental drying data are analyzed using following moisture ratio equation [31]:

$$MR = \frac{M - M_e}{M_o - M_e} \quad (2)$$

where MR is the dimensionless moisture ratio, M is the moisture content for a given time (g/g dry solid), M_o is the initial moisture content (g/g dry solid), M_e is the equilibrium moisture content (g/g dry solid). The experimental drying rate was determined by using following expression [32]:

$$\text{Drying rate} = \frac{M_{t2} - M_{t1}}{t_2 - t_1} \quad (3)$$

where t_1 and t_2 are the drying times (min), M_{t1} and M_{t2} are moisture contents of press mud at time t_1 and t_2, respectively.

Drying experiments of press mud were carried out in the temperature range of 60-120 °C to discern its kinetics. The following drying rate expression can be written:

$$-r_M = -\frac{dM}{dt} = kM^n \quad (4)$$

Where r_M is drying rate, M is moisture content expressed as (g moisture/g dry solid), k is specific reaction constant and n is the order of drying with respect to moisture content of press mud. An integral method of analysis was followed to determine the value of n. It was found that the rate of drying was independent of moisture content and therefore equation 1 reduces to following:

$$-r_M = -\frac{dM}{dt} = kM^0 = k \quad (5)$$

An integration of equation 5 between the limit M_0 (at time 0) and M (at time t) yields following equation:

$$M_0 - M = kt \quad (6)$$

The temperature dependence of a rate constant can be expressed using Arrhenius equation:

$$k = k_0 \exp\left(-\frac{E}{RT}\right) \quad (7)$$

Where R is the universal gas constant (8.314 kJ/ (mol·K)), E is an activation energy (kJ/mol), k_0 is Arrhenius factor (g moisture/ (g dry solid·h)) and T is the absolute temperature (K). Both the kinetic parameters (E and k_0) can be estimated from a slope and intercept of plot ln k versus 1/T.

2.5 Pretreatment process optimization

2.5.1 Experimental design

The response surface methodology (RSM) using Box Behnken design (BBD) was used for the optimization of pretreatment conditions *viz.* concentration of H_2SO_4, solid: liquid ratio and time of acid hydrolysis. Experimental designs were generated using statistical software Design-Expert version 9 (Stat-Ease' Inc., Minneapolis, MN, USA). The BBD is a second order multivariate technique based on three-level incomplete factorial designs. Each variable in the design was studied at three different levels when all other variables were set at a central coded value of zero [33]. The search ranges for pretreatment conditions were, concentration of H_2SO_4 (0.5, 1.0, 1.5% v/v), solid: liquid ratio (1:5, 1:10, 1:15 g/mL), and time (15, 30, 45 min). Based on these, BBD was used to generate 17 experiments by varying the operational set point parameters (data not shown).

2.5.2 Acid hydrolysis

The dried press mud samples were subjected to pre-optimized pretreatment conditions for breakdown of complex structures (cellulose, hemicellulose) of the material. Acid hydrolysis was carried out in an autoclave at 121 °C under specified conditions. The pretreated press mud was filtered and analyzed for its total sugar content. Pretreatment procedure was carried out for all press mud samples, which were dried earlier at temperature range of 60-120 °C. A control experiment with non-dried press mud sample of 4.2 g (equivalent to 1 g of dried sample) was also carried out for acid hydrolysis study.

2.6 Detoxification of press mud hydrolysates

Pretreatment method at higher temperatures is known to produce some fermentation inhibitors. Depending on type of pretreatment and other process parameters (time and temperature), lignin is degraded into phenolics and the fermentable sugars mainly into 5-hydroxyl methyl furfural (HMF), furfural, acetic, levulinic and ferulic acids which either hinder fermentation process or slows down reaction rates [34]. Thus, detoxification

methods (over-liming and activated charcoal (AC)) were studied individually and in combinations. The detoxification by over-liming was carried out as reported by Harde et al. [35]. The calcium hydroxide was gradually added in hydrolysate at 50 °C and mixed by using magnetic stirrer till pH reached to 9-10. Further, the mixture was filtered after 30 min and filtrate was used for inhibitor analysis. Alternatively, the AC treatment reported by Yamamoto et al. [36] was also used in which, the pH of hydrolysate was adjusted in between 9-10 using NaOH solution followed by treatment with 5% (w/v) AC for 2 h. The detoxification was carried out at 60 °C with continuous shaking at 200 rpm in orbiter shaker. Further, the detoxified hydrolysate was filtered and used for ABE fermentation. Finally, the combination of over liming and AC treatment wherein the hydrolysate was first treated with lime followed by treatment with AC was also carried out. The total phenolics from hydrolysates were determined before and after detoxification treatments according to Harde et al. [35]. Folin-Ciocalteu was used as an analyzing reagent while gallic acid was used as a standard sample (0.02-0.1 mg/mL). Absorbance of sample was measured in UV-visible spectrophotometer (Lab India-3000^{+}) at 765nm. In addition, the total furans were determined using spectrophotometric method based on the difference in absorbance values measured at 284 and 320 nm [37].

2.7 ABE fermentation

2.7.1 Inoculum preparation and production medium

A seed inoculum was prepared by freeze-thaw techniques by using sterile 80 mL reinforced clostridial medium (RCM) with 2% (v/v) spore culture inoculation [35]. The RCM contained (g/L): meat extract (10), peptone (5.0), yeast extract (3.0), glucose (30), starch (1.0), sodium chloride (5.0), sodium acetate (3.0) and L-cysteine (0.5) and the pH was adjusted to 6.8. RCM culture bottles were incubated for 18-20 h at 37 °C and further used as inoculum in production medium. The production medium (P2) reported by Bankar et al. [38] was used, which contained (g/L): glucose (60), magnesium sulfate (0.2), sodium chloride (0.01), manganese sulfate (0.01), iron sulfate (0.01), dipotassium hydrogen phosphate (0.5), potassium dihydrogen phosphate (0.5), ammonium acetate (2.2), biotin (0.01), thiamin (0.1) and p-aminobenzoic acid (0.1). The pH was adjusted to 6.5 with hydrochloric acid, if necessary. The medium was then autoclaved at 121 °C for 20 min and cooled.

2.7.2 Fermentation

Fermentation experiments were carried out by using hydrolysates obtained from dried press mud samples with the help of *C. acetobutylicum* NRRL B-527. The batch experiments were performed in 100 mL airtight glass bottles with 80 mL detoxified press mud slurry supplemented with other nutritional components of P2 medium. The total sugar concentration was adjusted to remain constant at 60 g/L for better comparison in diversified experiments. Cane molasses (another sugar rich byproduct of sugar processing industries) was used to supplement the medium to adjust final sugar concentration. Fermentation was initiated by inoculating 5% (v/v) 20 h old inoculum (OD_{560}1.36) into anaerobic glass bottles and incubated at 37 °C for 96 h [35]. The P2 medium was used as a control. After 96 h, the samples were taken out for subsequent solvent and sugar utilization analysis.

2.8 Analytical methods

The total sugars were quantified by using phenol-sulfuric acid method as explained by DuBois et al. [39]. Individual sugars at beginning and after fermentation were determined

by using high-performance liquid chromatography (HPLC) system (Dionex India Ltd.) equipped with a refractive index detector. An ion exclusion column (Biorad Aminex, HPX 87-H) was used at a temperature of 30 °C with 0.008 M H_2SO_4 as a mobile phase, at flow rate of 0.6 mL/min and sample volume of 20 µL. Scanning electron microscopy (SEM) (JSM-6380LA, JEOL, Japan) was used to observe the morphological changes during press mud drying and hydrolysis processing. The sample was mounted on brass stub using carbon tape and charged by sputter-coating with platinum. Imaging was carried out at 5 kV of beam voltage. Solvents (acetone, butanol and ethanol) and acids (acetic acid and butyric acid) were quantified as described by Bankar et al. [40]. Gas chromatography (Agilent Technologies7890B) equipped with a flame ionization detector and AB-INNOWAX capillary column (30 m × 0.32 mm × 1 µm) was used. Injector and detector temperatures were maintained at 200 °C and 250 °C, respectively. The sample of 0.5 µL was used for solvent analysis.

3. Results and discussion

3.1 Characterization of feedstock

Press mud samples were characterized to determine its composition. It was observed that press mud contains 22.3% cellulose, 21.67% hemicellulose and 12.90% lignin on dry basis. These values are in close agreement with the report on press mud characterization by Kumar et al. [41]. Further, the proximate analysis of press mud showed to contain higher moisture content of 76.19% (w/w) with total volatile solids of 80.60% (w/w), ash 19.4% (w/w) and protein content to be 0.16% on dry basis. The proximate analysis values match very well with other reports on press mud studies [17, 22]. The significant amount of cellulose and hemicellulose content indicates that press mud has a huge potential to be used as feedstock for ABE production.

3.2 Drying kinetics

Based on compositional analysis of press mud, it was noted that it contained almost 75-80% (w/w) moisture which can cause microbial spoilage and also incur dilution effect during acid hydrolysis. Hence, drying study of press mud is of utmost importance to reduce moisture level in order to enhance storability and to minimize transportation cost. Additionally, different drying temperatures (60-120°C) were evaluated to check the effect of drying temperatures on fermentable sugar degradation.

From a plot of (M_0 – M) against time (t) (figure not shown), it is clear that the drying rate follows zero order with respect to moisture content, indicating the validity of equation 5. The value of specific rate constant (k) can be estimated from a slop of straight line. The drying of press mud occurs entirely within the constant drying period. In this period, the rate of transfer of moisture from interior to surface is equal to the rate of evaporation of moisture, so as to maintain the drying rate constant. The profiles of experimental moisture content as a function of time, during drying of press mud are shown in Fig. 2. All the profiles show linear relationship between moisture content and drying time to reveal the constant rate of press mud drying. Similar findings were reported by Gornicki and Kaleta, [42] during drying of carrot cubes. Hassini et al. [43] studied the drying kinetics of potato, and demonstrated that drying curves to exhibit a heating up, constant rate and falling rate periods. Further, it was observed that, with increase in drying temperature, the drying time for constant moisture content was decreased. The time required to achieve moisture content lower than 0.10 g moisture/g

dry solid at 60 °C (21 h), which is nearly doubled the time needed to reach same moisture content at 80 °C (11 h), and fairly four times the time required at 120 °C (5 h).

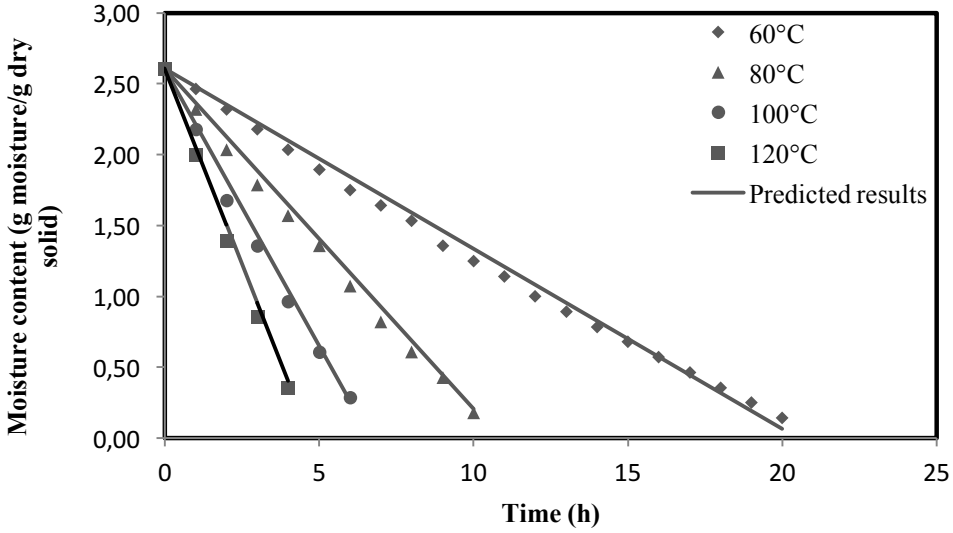

Fig. 2. Moisture content versus drying time at four different temperatures: declination of moisture content of press mud with respect to time for a given temperature. As drying temperature increases; time required to reduce moisture level decreases

Similar relation between drying temperature and time was reported by Galvez et al. [44] during drying of olive cake. Fig. 2 also shows that predicted values from equation 6 matches very well with experimental values of moisture content with standard deviation less than 3.0 %. The temperature dependence of a rate constant can be illustrated using equation 7. A straight-line graph of ln k against the reciprocal of absolute temperature (figure not shown) resulted into quantitative estimation of activation energy and Arrhenius factor, with slope and intercept, respectively. The values of activation energy and Arrhenius factor were found to be 26.50 kJ/mol and 2.07×10^3 g moisture/(g dry solid·h), respectively. Likewise, Akdas and Baslar, [45] also studied dehydration and degradation kinetics for mandarin slices and calculated activation energies under vacuum and oven dried conditions. The rate of drying for a given temperature, thus, can be written as follows:

$$-r_M = -\frac{dM}{dt} = 2.07 \times 10^3 \times \exp\left(-\frac{3215}{T}\right) \quad (8)$$

The experimental values of drying rate for a given temperature fits well with the values obtained from equation 8 (data not shown). The experimental and predicted values obtained after model simulation shows a standard deviation of 0.5%.

3.3 Pretreatment process optimization

3.3.1 Box Behnken design

Based on literature studied, the acid concentration, solid to liquid (S:L) ratio and time were thought to be an important factors that affect total sugar yield in a pretreatment process of biomass [46, 47]. Therefore, these factors were further optimized with the help of a statistical tool such as three-level factorial design using response surface

methodology (RSM) (data not shown). The acid concentration (0.5, 1.0, 1.5%), S:L ratio (1:5, 1:10, 1:15 g/mL) and time (15, 30, 45 min) were used as input factors and statistical analysis was performed to test the interactions between variables. According to analysis of variance (ANOVA), the quadratic model was significant and having F-value of 31.51. Among the test variables, H_2SO_4 concentration, solid to liquid ratio showed significant effect on maximum sugar release from press mud samples along with other significant interactions of model parameters. The validation experiment resulted in optimum acid concentration of 1.5% (v/v), S:L ratio of 1:5 (g/mL) and pretreatment time of 15 min for higher sugar release. The aforesaid optimized conditions resulted in total sugar (TS) release to be 23.58 g/L, which is very close to the predicted values offered by simulated model (23.04 g/L).

3.3.2 Acid hydrolysis

Pretreatment is an essential and fundamental step to disrupt cellulose, hemicellulose and lignin matrix for successful hydrolysis and effective fermentation operations [48]. To date, many pretreatment technologies have been developed such as acid, alkali, steam explosion, organosolv, biological pretreatment, ionic liquids, and others [49, 50]. The appropriate pretreatment method is expected to maximize sugar release and minimize the generation of fermentation inhibitor compounds.

Previously dried press mud samples were used for acid hydrolysis treatment to study the effect of drying temperature on fermentable sugar release. For efficient release of fermentable sugars; previously optimized conditions (acid concentration, 1.5% (v/v); solid to liquid ratio, 1:5 (g/mL) and time,15 min) were used during hydrolysis process. The total sugar release data (Fig. 3) obtained after acid hydrolysis, showed that the press mud sample drying at higher temperature has no deleterious effect on fementable sugar release.

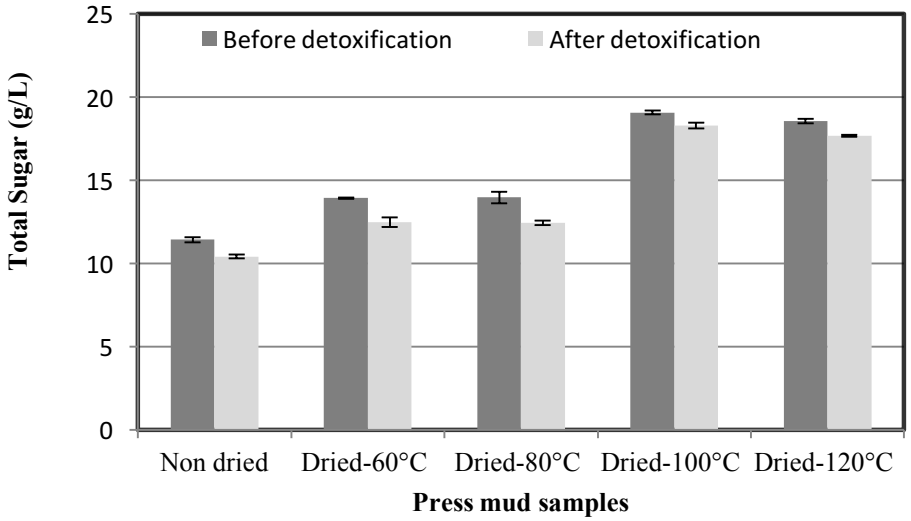

Fig. 3. Total sugar content, before and after detoxification of press mud hydrolysates: samples dried at different temperatures range (60-120 °C) show efficient sugar release during acid hydrolysis than non-dried sample

The highest total sugar release of 19.08 g/L was observed in the sample which was dried at 100°C. This may be due to the changes in lignin distribution, resulting

hemicellulose availability to acidic treatment. Further, press mud samples (sun dried and oven dried at 100 °C followed by acid treatment) were characterized by using SEM, to study the morphological changes during drying and hydrolysis operations. As can be seen from Fig. 4A (sun dried sample), the material has intact structure without any disruption on its surface. Comparing Fig. 4A and 4B (dried at 100 °C), a partial disorganization with curly structure in the material is verified.

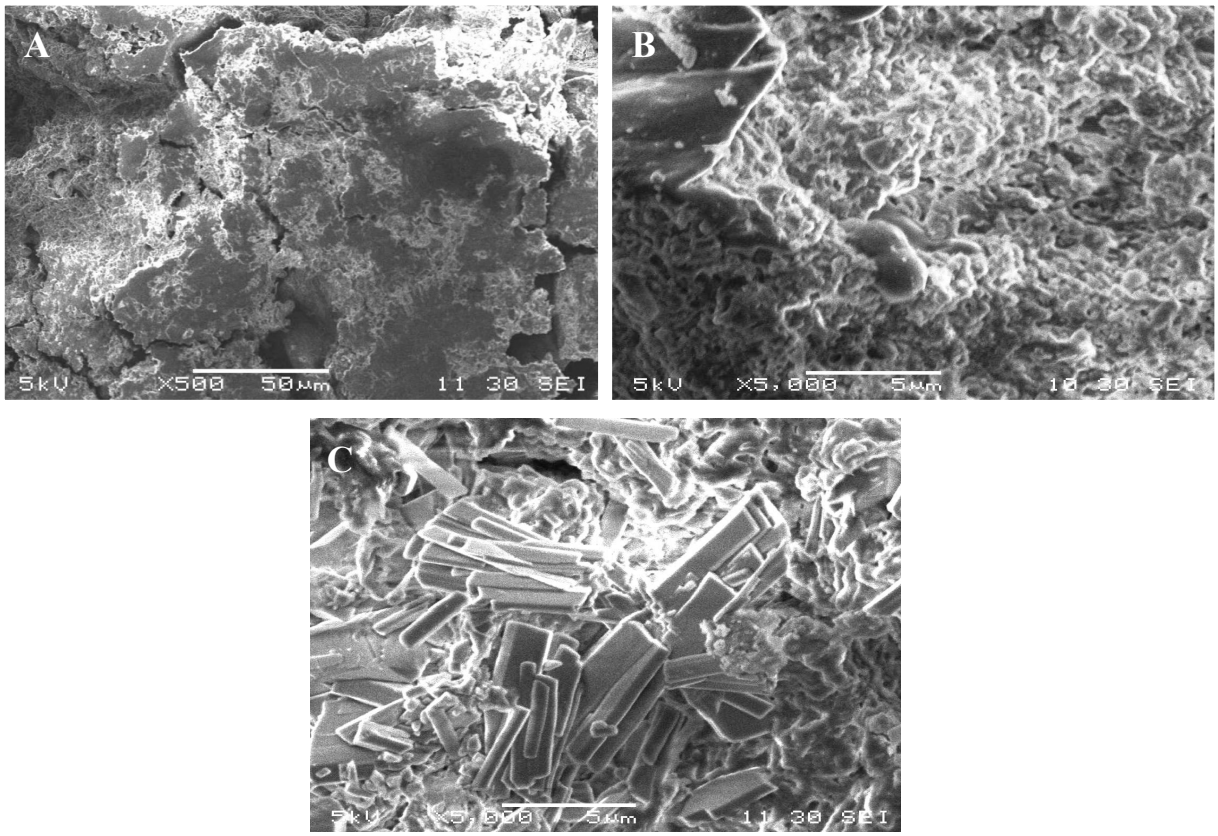

Fig. 4. SEM images of press mud after drying and acidic treatment: A- sun dried (control) sample: smooth and intact surface; B- sample dried at 100 °C: partially disordered with curly appearance on surface; C- acid treated sample: completely ruptured structure

On the other hand, acid treated material (Fig. 4C) showed occasioning ruptures and fissures along with great disorder in biomass. Koo et al. [51] also confirmed that morphological changes during biomass pretreatment have substantial effect in sugar release. Besides, drying at lower temperature takes prolong time that is presumed to have hardening effect on material that ultimately affect acid hydrolysis and therefore results in lower sugar release. The non-dried sample (wet), even after hydrolysis process was able to release only 11.43 g/L total sugars. As mentioned earlier, presence of moisture either leads to microbial spoilage (on storage) or show dilution effect, which might be the reason for lowered sugar release in non-dried samples. Furthermore, the inhibitors namely phenolics and acetic acid were generated during acid hydrolysis (Table 1).

In addition, the furans were also formed in negligible amount (0.2-0.5 mg/L) during the process. Kuruti et al. [17] carried out acid thermal hydrolysis of press mud and

showed its beneficiary effect in terms of volatile fatty acid intensification. Gonzalez et al. [52] also carried out hot water pretreatment of press mud and reported biomass solubilization and higher methane yield.

3.4 Detoxification of hydrolysates

The pretreatment and hydrolysis of lignocellulosic biomass and its neutralization process generates various types of inhibitors such as furfural, 5-hydroxy methyl furfural (HMF) and phenolics [53]. Acetic acid is another well-known inhibitory compound being produced during acid hydrolysis treatment. The un-dissociated form of acetic acid largely affects microorganisms than dissociated form [54]. The neutralization process which was used to maintain the initial pH (6.5) of pretreated hydrolysate prior to fermentation also leads to salt formation that subsequently affects ABE production [1, 53]. Hence, three detoxification methods (over-liming, activated charcoal (AC), and combination of both) were performed to understand its effect on inhibitor removal. Over-liming showed removal of total furans and total phenolics in the range of 55-65% and 60-65% from each hydrolysates, respectively. On the other hand, AC treatment efficiently removed more than 93% of phenolics in all hydrolysate with acceptable (4-8%) sugar loss (Fig. 3). Similarly, the total furan concentrations were greatly reduced (around 98%) by using AC detoxification method, while acetic acid concentration remained unaffected even by either treatment. Hodge et al. [55] also reported that more than 85% furan and 86-98% phenolics have been removed while acetic acid concentration remained unchanged after AC treatment during softwood processing. These results are in very close agreement with present study. The inhibitory compounds before and after AC detoxification treatment (for five pretreated hydrolysates) are summarized in Table 1. Almost similar percent inhibitor removal was observed when combinations of two treatments (over-liming plus AC) were used.

Table 1

Effect of activated charcoal (AC) detoxification on inhibitor removal from press mud hydrolysates: total phenolics greatly reduced by AC treatment while acetic acid remained unaffected. H: Hydrolysate; H+AC: Detoxified hydrolysate

Inhibitors	AC treatment	Non-dried	Dried 60 °C	Dried 80 °C	Dried 100 °C	Dried 120 °C
Phenolics (g/L)	H	1.45±0.25	0.85±0.05	0.83±0.030	1.69±0.12	2.00±0.43
	H+AC	0.08±0.001	0.05±0.006	0.05±0.002	0.04±0.001	0.05±0.003
Acetic acid (g/L)	H	0.82±0.001	0.53±0.005	0.94±0.006	0.99±0.004	1.07±0.006
	H+AC	0.82±0.004	0.53±0.002	0.94±0.001	0.99±0.002	1.07±0.005

Many researchers listed wide variety of detoxification processes such as over-liming with calcium hydroxide, adsorption by AC and/or ion exchange resins, and biological treatment [56] for efficient inhibitor removal. However, each method has its own characteristics and impacts on fermentable sugars. Ezeji et al. [57] reported that, although anion exchange treatment removed more inhibitors, it leads to larger sugar loss of about 11% in comparison to over-liming (about 3%). Though, over-liming using calcium hydroxide is cheap and reliable process it has been observed that excessive salt formation take place during adjustment of pH from 10 to 6.5 (for fermentation) which may be toxic to microorganisms and also adds additional step of centrifugation. It also has several disadvantages such as difficulty in slurring the lime at high concentrations, controlling

lime addition, disposal of filter cake, and equipment scaling due to 30-40% of remaining soluble calcium after over-liming. In the present study, AC detoxification was found to be beneficial to remove inhibitors compared to over-liming. This may be due to initial pH adjustment during AC treatment and high adsorption capacity of AC used. Yamamoto et al. [36] also showed that AC treated hydrolysate from forest biomass was able to produce solvents, which were comparable with glucose control sample, signifying effectiveness of AC detoxification. In addition, the use of AC treatment for detoxification may be cost effective, if either cheap sources of char or efficient regeneration methods are employed. Interestingly, certain inhibitors such as furfural and HMF also act as a stimulator during ABE fermentation, resulting in increased productivity and yield [1]. Ezeji et al. [57] demonstrated that addition of furfural and HMF stimulated the growth of *C. beijerinckii* BA101, whereas phenolic acids inhibited the growth by damaging the hydrophobic sites of bacterial cells.

3.5 ABE fermentation

ABE production results from biphasic fermentation which involves sequential acidogenic and solventogenic phases. The former takes place in exponential growth phase where the substrate is converted to acetic acid, butyric acid, H_2 and CO_2 while latter takes place in stationary phase in which the formed acids are reassimilated into acetone, butanol and ethanol [38].

Dried press mud samples which were hydrolysed and detoxified were used in batch fermentation of *C. acetobutylicum* NRRL B-527 to produce biobutanol. Indeed, an adequate balance of organic and inorganic nutrients is also required for growth and solvent production [58]. Hence, all hydrolysates were supplemented with other nutritional components of P2 medium. Besides, literature report explains the necessity of dilute detoxified hydrolysates for further improvements in solvent production (by diluting the inhibitors) [59]. At the same time, sufficient amount of fermentable sugars are also essential to avoid substrate deficiency, which may results into accumulation of acids instead of solvents. Therefore, cane molasses was incorporated in order to dilute the slurries along with sugar adjustment to be around 60 g/L for better comparison. Additionally, the use of cane molasses as a supplement may fulfill the criteria of bio-refinery process. Furthermore, all slurries (two fold diluted with cane molasses) were mainly consisted the mixture of hexose (glucose and fructose) and pentose (xylose and arabinose). The detailed sugar composition at beginning and after fermentation is shown in Fig. 5.

According to previous experience, the ABE fermentation experiments were performed till 96 h [35]. In this time period; initial 10 h has been considered as lag phase followed by acidogenesis in mid exponential growth phase. The fermentation shifted from acidogenesis to solventogenesis at approximately 17-20 h which corresponds to late exponential growth phase and continues throughout stationary phase beyond 80 h during fermentation [35, 60]. Fig. 5 shows the sugar consumption pattern during aforesaid course of fermentation for differently treated press mud slurries. As expected, glucose was found to be the most preferred substrate followed by fructose, xylose and arabinose. Although, pentose and hexose sugar consumption was almost equal during growth of microorganisms; hexose sugars are preferred over pentose sugars, as expected.

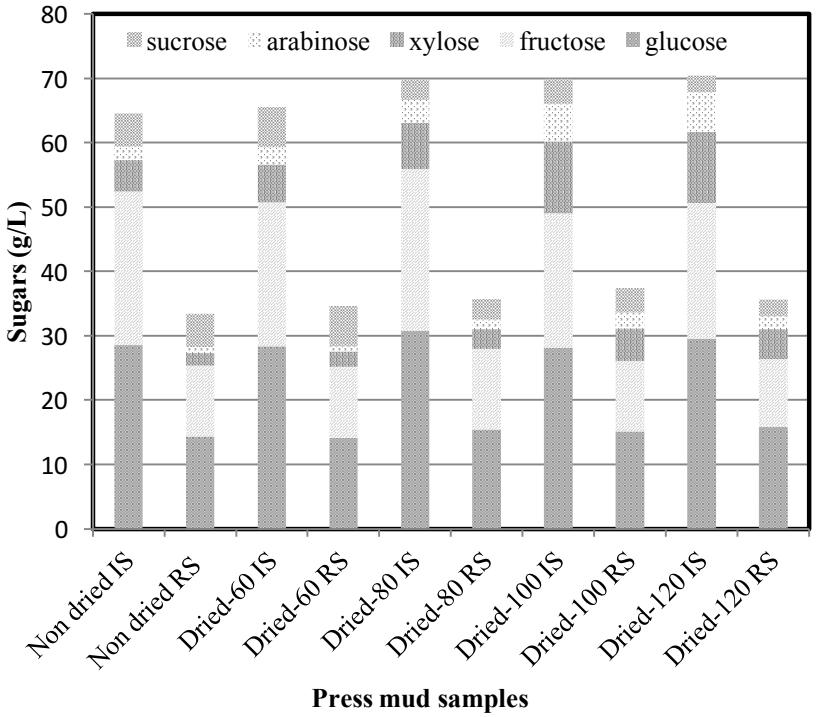

Fig. 5. Individual sugar composition at beginging and after fermentation of detoxified press mud slurries (sample dried and pretreated): IS- initial total sugar, RS- residual total sugar

Initially, microorganisms consume hexose sugars (preferably glucose) in lag and exponential phase. During late exponential phase, when hexose sugar concentration in medium is limiting, microorganisms start to consume pentose sugars as well. This result revealed that *Clostridia* in ABE fermentation exhibit a liking for specific sugars, agreeing with observations made by Bankar et al. [40].

The control P2 medium was able to produced higher total solvents of 13.70 g/L with total ABE yield of 0.27 g/g of sugar consumed. The non-detoxified hydrolysate resulted in poor fermentation process having total solvent of 0.55 g/L with total acids to be 7.60 g/L, leading to acid crash and indicating necessity of detoxification. Table 2 summarizes the batch fermentation results by using detoxified press mud slurries. The total solvents obtained from press mud samples, which were dried at 100 °C, and 120 °C were higher than samples dried at other temperatures.

Table 2

Production of ABE from press mud slurries (sample dried and pretreated): P2: standard production medium

Sample	Butanol (g/L)	Total solvents (g/L)	Total acids (g/L)	Butanol yield (g/g sugar consumed)	ABE yield (g/g sugar consumed)	ABE productivity (g/(L· h))
P2 control	8.26±0.001	13.70±0.01	2.67±0.005	0.16	0.27	0.14
Non-dried	2.85±0.003	5.10±0.004	4.77±0.003	0.09	0.16	0.05

Dried-60 ^{o}C	3.67±0.002	4.94±0.003	4.99±0.001	0.11	0.15	0.05
Dried-80 ^{o}C	3.23±0.001	5.69±0.001	4.98±0.001	0.09	0.16	0.05
Dried-100 ^{o}C	4.43±0.04	6.69±0.002	4.55±0.002	0.13	0.20	0.06
Dried-120 ^{o}C	4.26±0.002	6.30±0.001	4.78±0.003	0.12	0.18	0.06

The presence of lower acid concentration after detoxified slurry (dried at lower temperature and pretreated) might have affected the growth of *Clostridia* and ultimately solvent production, resulted in decreased total solvent titer. Cho et al. [61] reported lower concentrations of weak acids are more toxic to microorganism that affects total solvent production. Furthermore, the fermentation data showed that drying of press mud samples at higher temperature neither affected total sugar release nor the fermentation process. Very few studies from literature are available in line with this report. Avila-Gaxiola et al. [62] showed drying of *Agave tequilana* leaves at higher temperature yields maximum reducing sugars indicating no thermal degradation and also reported minimum inhibitor generation resulting better ethanol fermentation.

Overall, the *C. acetobutylicum* NRRL B-527 was efficiently able to grow and produce solvents in press mud hydrolysate samples. The utilization of approximately 50-53% sugars (Fig. 5) during ABE fermentation process proves the feasibility of press mud samples to be used for large-scale operation after further modification. The results obtained from these experiments are in line with our previous studies [35, 40, 59]. Recently, the press mud was also being utilized for its application in other biofuel production such as bioethanol and volatile fatty acid production [17]. Radjaram and Saravanane, [63] also produced biohydrogen by anaerobic co-digestion of press mud with sewage and water. Further, Kumar and Kesavapillai, [64] used press mud as a substrate for invertase production by solid state fermentation.

The total solvent production, solvent yield and solvent productivity are apparently not so promising from this study. However, we are in a continuous process to improve it. As stated earlier, the overall study of biobutanol production from press mud is divided into three parts. The present paper emphatically highlights the first part of this study while remaining parts such as biobutanol fermentation optimization, material balance, and scale up will be studied in depth and presented in near future papers. Simultaneously, genetic engineering aspects as well as bioprocess modifications will be incorporated to further improve the ABE yields and productivities. In addition, the fed-batch and continuous ABE process development are further target areas to check its commercial feasibility.

4. Conclusions

Press mud was successfully used for the production of biobutanol by *C. acetobutylicum* NRRL B-527. The drying operation considerably favored total sugar release from press mud and in turn aided fermentative productions of ABE. The total sugar yield and total solvents were higher for dried press mud than non-dried press mud waste. The drying kinetics followed zero order with respect to moisture content with a drying rate expression of:

$$-r_M = 2.07 \times 10^3 \times \exp\left(-\frac{3215}{T}\right)$$

The press mud offered highest total sugar release of 19.08 g/L from sample dried at 100°C. The activated charcoal detoxification efficiently removed around 98% furans and 93-97% phenolics from all pretreated hydrolysates. Subsequently, the detoxified hydrolysates were subjected to fermentation using *C. acetobutylicum* NRRL B-527 resulted in total solvent concentration to be in range of 4.94 - 6.69 g/L with the total ABE yield of 0.15-0.20 g/g of sugar consumed. The butanol and ABE yield with respect to press mud was found to be 0.46 and 0.69 g/g treated press mud, respectively for samples dried at 100 °C. Finally, attempts to improve the solvent titer and yield are underway to check the feasibility of this process at large scale.

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