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Combined effect of inorganic salts with calcium peroxide pretreatment for kenaf core biomass and their utilization for 2,3-butanediol production

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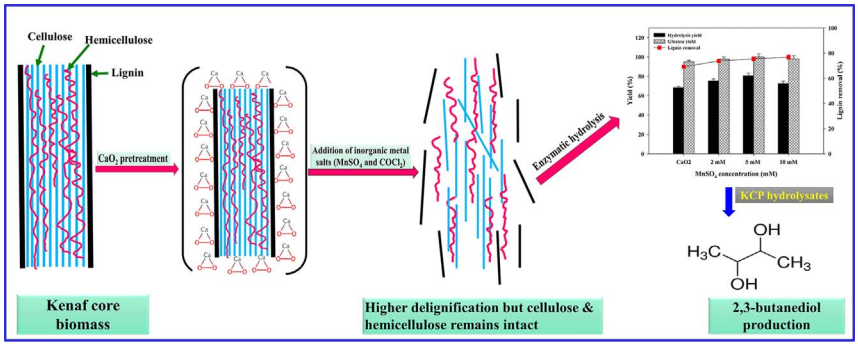
GRAPHICAL ABSTRACT



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ABSTRACT

This study focuses on development of calcium peroxide (CaO_2) pretreatment that removes major part of lignin but retaining most of sugar components of kenaf core powder (KCP) biomass. In chemical pretreatment, usually higher loss of biomass occurs which was less during this pretreatment strategy. Supplementation of inorganic salts; manganese sulfate ($MnSO_4$) and cobalt chloride ($COCl_2$) in CaO_2 pretreatment resulted in maximum delignification of KCP relative to individual CaO_2 pretreatment. Maximum glucose yield (98%) and hydrolysis yield (80.5%) was achieved after enzymatic hydrolysis (30 FPU/g of KCP) under optimized conditions. Analytical results proved effective lignin removal and significant destruction of KCP with this pretreatment strategy. Finally, utilization of KCP enzymatic hydrolysates by developed strain *Klebsiella pneumoniae* KMK05 resulted in maximum 2,3-butanediol (BDO) production (10.42 g/L) and BDO titer (0.385 g/g of sugar). BDO titer achieved with KCP derived sugars were found comparable with the mixture of standard sugars which is notable.

1. Introduction

Worldwide bio-refinery systems deal to generate energy, fuel, and biologically made chemicals from renewable assets are of the main emphasis because of unusual crude oil reserves, steady increases in its value, and adverse effects on the environment (Saratale and Oh,

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2015a). 2,3-Butanediol (BDO) considered as an important chemical which has been widely used as the solvent, food additive, printing inks, perfumes, and in the pharmaceutical and cosmetics industries (Ji et al., 2011; Sun et al., 2009). In addition to this, dehydration of BDO formed into methyl ethyl ketone can be used as a fuel additive to increase the octane rating of gasoline. However, the existing BDO production methods are costly, which confines its wide applications (Wu et al., 2008; Saratale et al., 2016). Microbial BDO production utilizing cheap, renewable lignocellulosic biomass as a carbon source offers an attractive route of economically feasible and sustainable approach (Um et al., 2017; Mazumdar et al., 2013). Moreover, development of new microbial strains using metabolic engineering approaches to increase the concentration and yield of BDO which is an important aspect. Kenaf (*Hibiscus cannabinus* L., *Malvaceae*) is the substantial rapidly growing crops up to 1.5–3.5 m tall, higher photosynthesis rate, and a short time to harvest (2–6 months) (Chen et al., 2013; Batouli et al., 2014). Kenaf produces considerably higher biomass about 15–20 tons dry weight biomass per hectare but still not exploited as a remarkable feedstock for BDO generation which is first time addressed in this study.

In plant biomass, sugar sources are mainly, cellulose and hemicellulose and are protected by the crystallinity of cellulose, lignin, and with a greater degree of polymerization making it more recalcitrant to hydrolysis (Mosier et al., 2005; Saratale and Oh, 2015a). Many investigators have widely studied the various physical, chemical, and combined physic-chemical pretreatment methods to enhance delignification of biomass and its recalcitrant nature by which it enriches enzyme accessibility and can achieve better reducing sugar production. Most of these methods are costly, less effective and comprise the higher removal of lignin and hemicellulose content and thus cellulose content of biomass remains for further enzymatic saccharification which results in low sugar yield (Sivagurunathan et al., 2017). Considering this, some investigators showed that partial removal of lignin from biomass considerably enhances the enzymatic saccharification of cellulose and hemicellulose component to fermentable sugars (Azelee et al., 2014). Thus a suitable method which can partially fractionate the biomass constituents need to be developed. It was observed that addition of inorganic salts with oxidizing agents results in the formation of hydroxyl radicals and superoxide ions that greatly affects the composition of biomass and can remove lignin effectively (Ramadoss and Karuppan, 2015; Monavari et al., 2011). Utilization of lignocellulosic biomass for BDO production makes the process more cost-effective and practically applicable. However, washout of biomass sugar components (mainly hemicellulose) is one of the major limitation of the process. In this study, we have investigated CaO_2 pretreatment in combination with inorganic salts for partial delignification of KCP biomass, to avoid higher washout of hemicellulose content, not only to enhance the hydrolysis yield but also to achieve better saccharification.

Developing the microbial strain using metabolic engineering strategy found to be useful for the efficient conversion of hexose and pentose sugars of renewable biomass into 2,3-butanediol. In this study, the effectiveness of CaO_2 and combination with inorganic salts pretreatment methods on the delignification and enzymatic digestibility of KCP was systematically investigated. The effects of pretreatment on structural features, lignin removal, crystallinity and chemical composition of KCP biomass were evaluated using various analytical techniques. Lastly, we have employed KCP enzymatic hydrolysates for its efficient conversion to 2,3-butanediol (BDO) using an engineered *K. pneumoniae* strain under batch fermentation.

2. Materials and methods

2.1. Materials

The kenaf core biomass was kindly provided by Professor Jeun Joon-Pyo of Korea Atomic Energy Research Institute, South Korea. The kenaf core was milled and the obtained powder was sieved through a 200 μm size mesh. The obtained kenaf core powder (KCP) was dried in a vacuum oven by keeping the temperature at 50 °C for 1 day. The dried sieved biomass was collected in an airtight container, at room temperature for further use. Calcium peroxide, hydrogen peroxide, Whatman filter paper No.1, manganese sulfate monohydrate ($MnSO_4{\cdot}H_2O$), zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), and cobalt chloride hexahydrate ($CoCl_2{\cdot}6H_2O$), were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the enzymatic hydrolysis experiments, cellulase from *Trichoderma reesei* ATCC 26921 (product number C 2730, Enzyme Commission (EC) Number 3.2.1.4, 700 U/g-cellulase), was obtained from Sigma–Aldrich (St. Louis, MO, USA). The other chemicals employed in this study were of the highest purity available and of analytical grade.

2.2. Chemical pretreatment

Initially, chemical pretreatment was carried out by combining KCP biomass individually with CaO_2 and strong oxidizing agent; H_2O_2 by keeping each concentration at 2% (w/v) in an electrically heated water bath at 100 °C and incubated for one hour of incubation. In each pretreatment, the ratio of the solid phase to the liquid phase was maintained at 1:10. After one hour of incubation, the treated biomass samples were washed with tap water till the pH of the solution remains neutral. The samples were then centrifuged well and dried at 60 °C until a constant weight was achieved and further explored to its chemical composition and enzyme hydrolysis analyses.

2.3. Calcium peroxide pretreatment optimization

The effects of calcium peroxide concentrations (1%, 2%, 3%, 4% and 5%) by keeping KCP concentration (10%) constant, the effect of KCP concentration (5%, 8%, 10%, 12% and 15%) by keeping CaO_2 concentration (4%) constant was studied. The KCP (10%) was pretreated using calcium peroxide (4%) in combination with various inorganic metal salts such as; $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, and $CoCl_2{\cdot}6H_2O$ by keeping the concentration of 2 mM were studied. The effects of operational parameters, for instance reaction time (30, 60, 90, 120, and 180 min), temperature (room temperature (RT), 60, 80, 100 °C and autoclave at 121 °C for 15 min) and using the different molar ratio of $MnSO_4$, and $COCl_2$ (1 mM, 2 mM, 5 mM, and 10 mM) with CaO_2 pretreatment were investigated. After the pretreatment, the pretreated KCP was filtered, washed with distilled water to neutral pH and dried at 60 °C until a constant weight was obtained. The pretreated KCP was stored in a vacuum plastic bag for the subsequent analysis and experiments. The delignification ratio (%) was determined according to the following equation:

$$\text{Delignification ratio (\%)} = D_{KCP} - D_{PT-KCP} / D_{KCP} \times 100$$

where, D_{KCP} is the quantity of lignin present in the raw native KCP, and $D_{PT\text{-}KCP}$ is the amount of lignin in the pretreated KCP, measured in (g/g).

2.4. Enzymatic hydrolysis of pretreated KCP biomass

KCP pretreated using individual H_2O_2, CaO_2 and CaO_2 with different metal salts was subjected to enzymatic hydrolysis. The enzymatic hydrolysis of untreated and pretreated KCP was achieved at 2.0% (w/v) in 20 mL of 50 mM citrate buffer (pH 5.0) comprising 0.005% (w/v) sodium azide. An enzyme solution equal to FPU activity of 30 U/g of untreated and pretreated KCP was put into Erlenmeyer flasks and incubated at 50 °C and 150 rpm for 24 h. Samples were taken from the reaction mixture at different time intervals and instantly heated at 100 °C for 15 min to denature the enzymes and then cooled followed by centrifugation for 10 min (at 4 °C; 10,000 rpm). The resulted supernatant was used for the reducing sugar analysis. Experiments were

carried out in triplicates (Saratale and Oh, 2015b). We have assessed the effects of increasing KCP concentration (5, 10, 15 and 20 g/L) while keeping the enzyme concentration constant (30 FPU/g of KCP) along with the effects of increasing enzyme concentration (10, 20, 30 and 40 FPU/g of KCP) by keeping the substrate (KCP) concentration (10 g/L) constant. Filter paperase activity (FPU) was determined to confer IUPAC recommendations by use of Whatman filter paper as the substrate. One unit of enzyme activity can be defined as the amount of an enzyme necessary to release 2 µmol of reducing sugars.

During the two steps of enzymatic hydrolysis, the reducing sugars that get released were combined to determine the overall hydrolysis yield performance (Saratale and Oh, 2015b)

$$\text{Hydrolysis yield (\%)} = \text{Reducing sugar (mg)} \times 0.9 \times 100 \div \text{polysaccharides content of substrate}$$

$$\text{Glucose yield (\%)} = \text{glucose (mg)} \times 0.9 \times 100 \div \text{cellulose content of the substrate}$$

Hydrolysis of polysaccharides involves water. For each mole of reducing sugar released, 1 mol of H_2O is obligatory. During the calculation, a correction factor of 0.9 was included in the amount of polysaccharides hydrolyzed.

2.5. BDO production media and cultivation conditions

Under batch fermentation, we have utilized KCP hydrolysates using the metabolically engineered *K. pneumoniae* KMK-05 strain for BDO production (Jung et al., 2014). The fermentation medium containing (g/L): KH_2PO_4, 3; Na_2HPO_4, 6.8; KCl, 0.75; $(NH_4)_2$ SO_4, 5.35; Na_2SO_4, 0.28; $MgSO_4{\cdot}7H_2O$, 0.26; citric acid, 0.42; yeast extract, 5; casamino acid, 10; and trace element solution (0.3 mL) comprising (g/L) $ZnCl_2$, 34.2; $FeCl_3{\cdot}6H_2O$, 2.7; $MnCl_2{\cdot}4H_2O$, 10; $CuCl_2{\cdot}2H_2O$, 0.85 and H_3BO_3, 0.31 with KCP hydrolysates sugar concentration of 30 g/L was used for BDO production. The cultures, in 50 mL medium in 250-mL flasks closed with silicon stoppers, and further incubated micro-aerobically under shaking condition at 250 rpm and 37 °C. We have also checked the BDO production performance of mixture of standard sugars, which is similar to KCP hydrolysates. The effects of increasing concentration of CaO_2 + $MnSO_4$ pretreated KCP enzymatic hydrolysates (20, 30 and 40 g/L) on BDO production was studied. To attain a neutralized pH condition, 5% $CaCO_3$ was added to the medium before cultivation. One milliliter of culture broth was withdrawn at specific time intervals, transferred to a micro-centrifuge tube and centrifuged for 5 min at 13,000 rpm. These samples were studied for subsequent measurement of pH, amounts of glucose, xylose, arabinose, and 2,3-butanediol, content. Experiments involving the mixture of sugars and KCP hydrolysates were conducted in triplicate.

2.6. Analytical methods

The chemical configuration (cellulose, hemicellulose, and lignin) of raw and pretreated KCP by following the procedure reported by Goering and van Soest (1970). The reducing-sugar content of the KCP biomass hydrolysates was measured by following dinitrosalicylic acid method (Miller, 1959). To determine the functional groups fourier-transform infrared (FTIR) spectroscopy (Cary 630; Agilent, Santa Clara, CA, USA) of raw and pretreated KCP was conducted in the mid-infrared region of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. Scanning electron microscopy (SEM) images of the samples were coated with platinum and obtained using a JEOL JSM-6360A microscope (Tokyo, Japan) and the acceleration voltage and emission current were 20 kV. Samples of the raw and pretreated KCP were analyzed by X-ray diffraction (XRD) was performed using a D2 Phaser model (Bruker, Billerica, MA, USA) operating at 30 kV and 10 mA. The crystallinity index of cellulose before and after treatment were studied considering the peak height method (Kim and Holtzapple, 2006). The residual sugar content glucose, xylose, arabinose in KCP enzymatic hydrolysates and the concentration of BDO in the fermented broth were analyzed using high-performance liquid chromatography (HPLC) with an ACME-9000 instrument (Young-Lin Instrument, Seoul, Korea) and a SH1011 column (Shodex, Tokyo, Japan) with RI detector. The detailed procedure is described in our previous studies (Saratale et al., 2016).

2.7. Statistical analysis

All the statistical results were analyzed and carried out using one-way analysis of variance with Tukey-Kramer multiple comparisons test.

3. Results and discussion

3.1. Effect of H_2O_2 and CaO_2 pretreatment on KCP digestibility

The major chemical composition of the kenaf core is cellulose (45.8%), hemicellulose (21.2%), lignin (22.8%), and ash (9.2%). Generally harsh chemical pretreatment can remove lignin effectively, however there is a loss of hemicellulose sugar and some part of cellulose sugars were also observed. Considering this, the objective of this study is to develop pretreatment strategy which has ability to remove lignin content significantly. However, both sugar components such as hemicellulose and cellulose in the kenaf substrate should remain in large proportion even after pretreatment. Some investigators reported that after partial removal of lignin from biomass the rate of enzymatic accessibility increases which results in better saccharification yield (Yang and Wyman, 2004; Saratale et al., 2016).

Initially, KCP was pretreated with H_2O_2 and CaO_2 (2% each concentration) at 100 °C for one hour. There are certain reports suggesting XRD as a valuable analytical tool to govern the crystallinity index (CrI) of the biomass which directly impacts on enzymatic saccharification (Waghmare et al., 2014). After H_2O_2 (54.5%) and CaO_2 (51.5%) pretreatment there was an increase in the crystallinity index (CrI) than that of the untreated KCP (46.8%) was observed (Table 1). The higher CrI in H_2O_2 biomass indicates higher removal of lignin and hemicellulose components as compared to CaO_2 pretreatment. FTIR is helpful in identification of distinct bands of cellulose, lignin and hemicellulose in

Table 1
Effects of individual H_2O_2 and CaO_2 pretreatments on the chemical composition, biomass recovery, delignification and hydrolysis yield of KCP biomass after enzymatic hydrolysis (30 FPU/g of KCP).

Pretreatment conditions	Biochemical composition (%)			Biomass recovery after pretreatment (%)	Lignin removal (%)	Hydrolysis yield (%)	CrI (%)
	Cellulose	Hemicellulose	Lignin				
Control (No pretreatment)	45.8 ± 2.25	21.2 ± 1.48	22.8 ± 1.88	100	ND	14.9 ± 0.85	46.8
H_2O_2 (2% H_2O_2) at 100 °C for one hour	58.2 ± 2.45	10.8 ± 1.25	9.2 ± 0.95	35–40	59.6 ± 2.25	59.2 ± 2.25	54.5
CaO_2 (2% CaO_2) at 100 °C for one hour	50.1 ± 2.54	18.5 ± 1.28	12.4 ± 0.92	68–72	46.5 ± 1.85	52.5 ± 2.15	51.5

CrI: crystallinity index; ND: not determined; Values are mean of three experiments; (±) standard error (SE) by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test.

the KCP and the results depict the structural changes in KCP after pretreatment with CaO_2 and H_2O_2. The FTIR spectra of untreated KCP and pretreated KCP with CaO_2 and H_2O_2 are depicted as Supplementary Information. The broadening in the absorption peaks around 3200–3300 cm^{-1} can be associated with stretching of O—H bond (Cao and Tan, 2004). After pretreatment with CaO_2 and H_2O_2, it showed a decrease in absorption intensity. A sharp peak at 2920 cm^{-1} was assigned for stretching of C—H vibration (Ramadoss and Karuppan, 2015). Bands in this region were strongly absorbed for control as well for pretreated KCP which suggests distinctive features of cellulose. A minor peak at 1610 cm^{-1} was disappeared, which is assigned for carbonyl group stretching after pretreatment, which might be due to hemicellulose and lignin removal (Jeun et al., 2015). The absorption peak at 1428 cm^{-1} corresponds to symmetric CH_2 bending and the deformation of lignin. The absorbance observed at 1318 cm^{-1} shows the band of hemicellulose from the O—H in-plane bending. The prominent absorption bands at 1029 cm^{-1} was attributed to C═O stretching vibration resulting from the carbohydrate-lignin linkage. The decrease in intensity of the peak specifies the removal of lignin. The region between 700 and 800 cm^{-1} corresponds to (1 → 4) glycosidic bonds of cellulose (Ramadoss and Karuppan, 2015). There was no formation of new peaks from the FTIR was observed. Table 1 shows the detailed results of chemical composition, biomass recovery, delignification and hydrolysis yield of KCP before and after pretreatment. H_2O_2 found more effective for the hydrolysis of KCP biomass and lignin removal, but there is a huge loss of sugar components of biomass was observed as compared to CaO_2 pretreatment. The foregoing results suggest that CaO_2 pretreatment showed good ability to remove lignin and preserved the higher portion of the hemicellulose and cellulose in the KCP. Thus, this pretreatment strategy exhibited cost-effective and a good choice to treat KCP relative to other harsh chemical pretreatment processes.

3.2. Optimization of CaO_2 pretreatment

It was reported that kenaf biomass contains thick lignin layer and carries both hardwood and softwood properties which makes it more resistant for hydrolysis and for delignification (Batouli et al., 2014). Thus, further study was concentrated for optimizing the various operational parameters including; chemical concentration, substrate concentration, reaction temperature and incubation time to improve delignification and saccharification of KCP biomass.

3.2.1. CaO_2 concentration

In the chemical pretreatment of biomass determination of optimum chemical concentration is essential to avoid excess chemical loss and to make the process more cost-effective. The concentration effect of CaO_2 on the hydrolysis of KCP was studied and the results are presented in Fig. 1A. It was observed that from 1% to 4% CaO_2 concentration there is a sharp increase in the hydrolysis yield (62.45%), glucose yield (85%) and delignification ratio (65%), respectively. However, higher chemical concentration (5%) was found effective for lignin removal, but no significant increase in the hydrolysis yield was recorded. These results may be because of higher loss of hemicellulose from KCP and inhibitors formation. Therefore, we have selected 4% CaO_2 as an optimum chemical concentration for further studies. Similar results were observed in the pretreatment of newspaper with H_2O_2 and green liquor pretreatment for rice waste biomass (Takagi, 1987; Saratale et al., 2016).

3.2.2. KCP concentration

We have determined the optimum substrate concentration by taking different KCP concentration; 5%, 8%, 10%, 12% and 15% and keeping 4% CaO_2 concentration constant at 100 °C for one hour of incubation. The obtained results in terms of hydrolysis yield, glucose yield and delignification ratio are shown in Fig. 1B. The results showed that 10% KCP concentration is optimum where maximum hydrolysis yield (64.45%), glucose yield (85%) and delignification ratio (65.8%) was observed. Up to 10% concentration, it was supposed that the better interaction of CaO_2 with KCP leads to destruct the aromatic nuclei and propyl side chains of $C_\alpha—C_\beta$ bonds of complex lignin effectively. However, further increase in KCP concentration sharp decrease in delignification was observed.

3.2.3. Reaction time

Reaction time is one of the vital factor affecting overall pretreatment effectiveness (Gu et al., 2015). The effects of different incubation time keeping 4% CaO_2 and 10% KCP at 100 °C was studied and the results are depicted in Fig. 1C. From 30 min to 90 min there was a sharp increase in hydrolysis yield (from 45 to 68.5%) and delignification (from 48 to 70%) with a significant glucose yield was noticed (Fig. 1C). However, further incubation at 120 and 180 min, no significant difference in hydrolysis yield was observed but slight increase in delignification was noticed. The hydrolysis yield and glucose yield are important to achieve significant fermentable sugar production. Considering these results, we have selected 90 min as the optimum reaction time for the further studies.

3.2.4. Reaction temperature

The pretreatment of KCP with CaO_2 was carried out at different temperatures such as room temperature, 60 °C, 80 °C, 100 °C and autoclaving at 121 °C for 15 min keeping the above-optimized conditions (4% CaO_2, 10% KCP and incubation time 90 min). Temperature elevation in pretreatments has shown to increase in the percentage of delignification of KCP. It was considered that at high temperature, KCP interaction with CaO_2 enhanced which results in swelling of biomass, thereby increase in its surface area, by which it becomes more available to enzymatic hydrolytic treatment (Mosier et al., 2005). The maximum hydrolysis yield, glucose yield and lignin removal was observed at 100 °C. Autoclaving found effective for better lignin removal but reduction in overall hydrolysis yield was observed. This might be due to the loss of hemicellulose fraction or formation of the inhibitors for enzymatic hydrolysis process (Fig. 1D). On the basis of the obtained results, reaction incubation temperature at 100 °C was selected for further optimization studies. The foregoing results suggest that 4% CaO_2, 10% KCP, at incubation temperature 100 °C and incubation time 90 min are the optimum conditions where maximum delignification about 69.2% of KCP was recorded. For enzymatic hydrolysis, initially we have conducted some experiments to determine the optimal cellulase dose (10–40 FPU/g of KCP) and biomass loading (5–20 g/L) for better saccharification of pretreated KCP. The results showed that optimum conditions were 10 g/L biomass loading and 30 FPU cellulase dose for better enzymatic saccharification of pretreated KCP. Under optimized conditions, CaO_2 pretreated KCP gave significant reducing sugar production of about 500 mg/g of KCP with 68.5% hydrolysis yield and 95% glucose yield after enzymatic hydrolysis.

3.3. Effects of supplementation of inorganic metal salts

Due to high lignin layer in KCP it becomes resistant for delignification so there is a need to find alternative strategies to improve the delignification. Some investigators reported that inorganic salts in the pretreatment produce hydroxyl radicals (·OH) which assist the oxidative degradation of complex phenolic structures of lignin. In the preliminary investigation, we have selected $MnSO_4$, $ZnSO_4$, $FeSO_4$ and $COCl_2$ (2 mM concentration each) for the treatment of KCP with CaO_2 under optimized conditions. Among which in the presence of $MnSO_4$, and $COCl_2$, it enhanced lignin removal of about 73.8 and 74.2%, respectively as compared to only CaO_2 pretreatment (69.2%). In addition, it also enhanced hydrolysis yield of KCP (Fig. 2A). Moreover, $ZnSO_4$ and $FeSO_4$ found less effective to enhance delignification as well as hydrolysis yield. Some investigators reported that manganese salts could enhance the degradation of lignin because of its higher catalytic

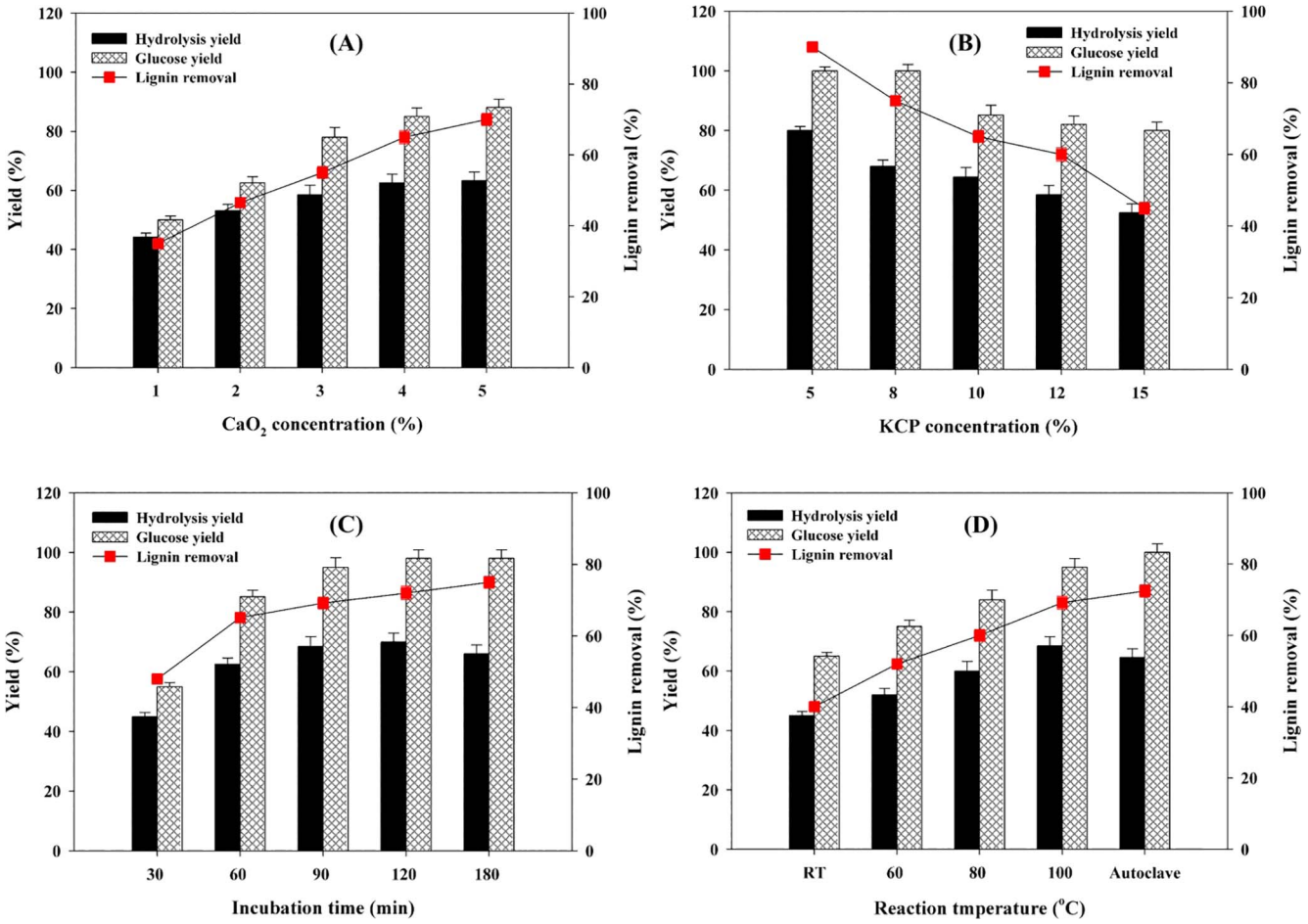

Fig. 1. Effects of (A) CaO_2 concentration, (B) KCP concentration, (C) reaction incubation time and (D) reaction temperature on lignin removal, and hydrolysis yield and glucose yield from KCP after enzymatic hydrolysis (30 FPase/g of KCP).

activity which supports our results (Ramadoss and Karuppan, 2015).

The effect of different molar concentration of $COCl_2$ and $MnSO_4$, (2 mM, 5 mM and 10 mM) along with CaO_2 on the hydrolysis of KCP was investigated and the results obtained are shown in Fig. 2B and C. In case of 5 mM concentration of $MnSO_4$, and $COCl_2$ maximum delignification (75.5% and 76.5%) and hydrolysis yield (80.5 and 78.5%) was recorded which is significantly higher than only CaO_2 pretreatment (68.5 and 69.2%) (Fig. 2B and C). In both studies, there was an increase in delignification and hydrolysis yield of KCP up to 5 mM concentration and further increase in the concentration (at 10 mM) a sharp decrease in the performance was noticed. At higher metal concentration, there is higher formation of increased cations due to the dissociation of metal salts resulting in rapid hydrolysis of hemicellulose and lignin which results in lower hydrolysis yield. However, to understand the exact mechanism further detailed investigation is required (Kamireddy et al., 2013).

The chemical composition of KCP cellulose (45.80%–52.10%), hemicellulose (21.20–18.50%), and lignin (22.80–7.06%) were observed before and after individual CaO_2 pretreatment. Moreover, after supplementation of $MnSO_4$ and $COCl_2$ (each 5 mM) in the reaction mixture showed an increase in cellulose (58.0 and 55.8%) whereas small reduction in hemicellulose (16.5 and 16.8%) and sharp reduction in lignin concentration (5.7 and 6.25%) were observed and the results are depicted in Fig. 3. To understand the effect of individual CaO_2 and in combination with metal salts, we studied KCP biomass surface morphology using SEM analysis. SEM observations of native KCP showed a smooth surface, whereas in CaO_2 pretreated KCP, rough surface and swelling was observed. Moreover, after supplementation of metal additives higher destruction of biomass and swelling can easily be noticed. The results clearly indicated that in the presence of inorganic salts CaO_2 pretreatment significantly removed lignin and a small portion of hemicellulose by which KCP becomes more accessible to the enzymes and gave a better saccharification yield.

In case of CaO_2 + 5 mM $MnSO_4$ pretreatment it gave the maximum 80.5% hydrolysis yield and reducing sugar production about 595 mg/g of KCP after enzymatic hydrolysis. The obtained hydrolysis yield and reducing sugar production was found to be higher than pretreated olive tree wood with combined steam explosion followed by alkaline peroxide (52.6% and 288 mg/g) and lime pretreated rice hull (32% and 154 mg/g) (Cara et al., 2006; Saha and Cotta, 2008). The acquired hydrolysis yield and reducing sugar production performance was found comparable with other chemical pretreatment studies however, in these studies, a major loss of hemicellulose component was observed (Yamashita et al., 2010; Saratale and Oh, 2015a).

3.4. BDO production using CaO_2 and CaO_2 + metal additives pretreated KCP enzymatic hydrolysates

Even though BDO is an important chemical but its production cost is still higher which is mainly depends on the market price of raw materials, and fermentation efficiency (Koutinas et al., 2016). Lignocellulosic biomass could be used as an alternative substrate to pure carbon source for BDO production. However, better yield of sugars (6C and 5C) and development of microbial strain which having the ability to utilize the mixture of sugars present in lignocellulosic biomass hydrolysate is an important aspect to make the process practically

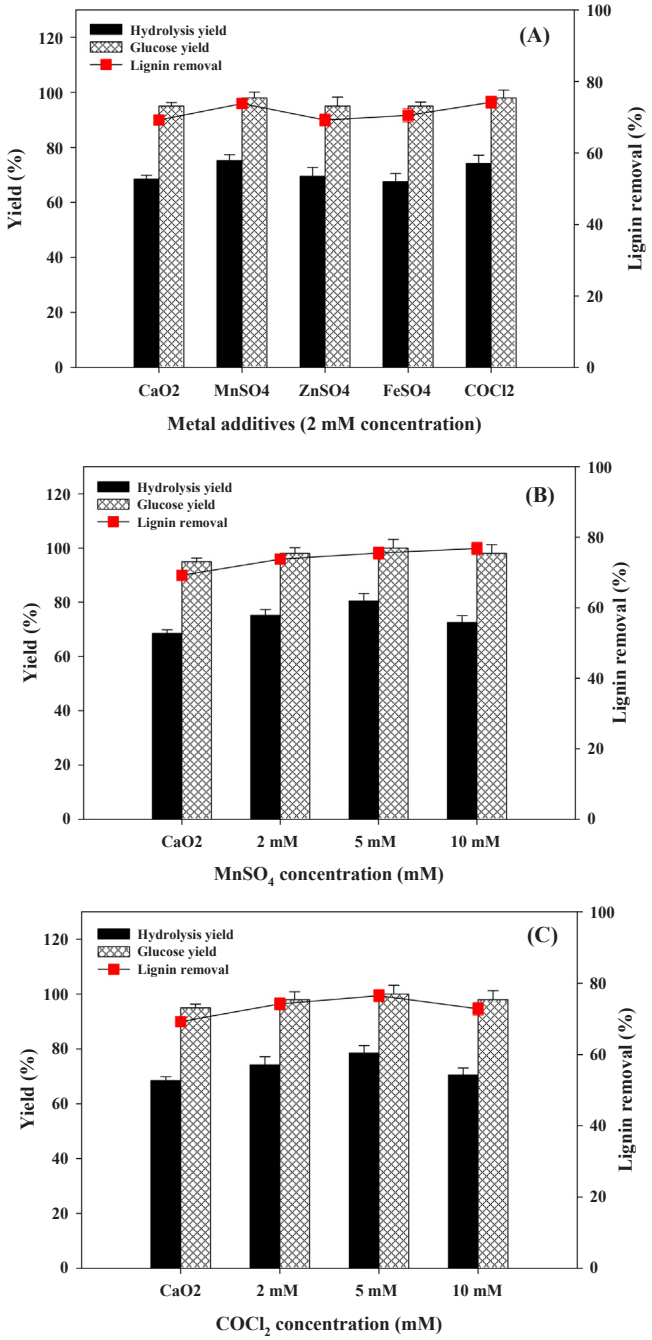

Fig. 2. Effects of (A) supplementation of inorganic metals (2 mM concentration) (B) at different molar concentration of $COCl_2$, and (C) at different molar concentration of $MnSO_4$ with CaO_2 pretreatment on lignin removal, and hydrolysis yield and glucose yield from KCP after enzymatic hydrolysis (30 FPase/g of KCP).

applicable and inexpensive. We have utilized the CaO_2 and CaO_2 + inorganic salt pretreated KCP enzymatic hydrolysates with sugar concentration (30 g/L) for fermentative BDO production using the *K. pneumoniae* KMK-05 strain under batch cultivation. In case of CaO_2 and CaO_2 + $MnSO_4$ KCP hydrolysates the maximum BDO titer was approximately 10.42 and 10.32 g/L and the yield was 0.385 and 0.382 g/g of sugar, respectively (Table 2). Whereas, in case of CaO_2 + $COCl_2$ less BDO productivity was observed. This might be due to the less utilization of sugars, metal inhibition to the fermentative microorganisms (Table 2). We have also studied increasing concentration of CaO_2 + $MnSO_4$ pretreated KCP enzymatic hydrolysates (20–40 g/L) in which at an elevated concentration (40 g/L) there was

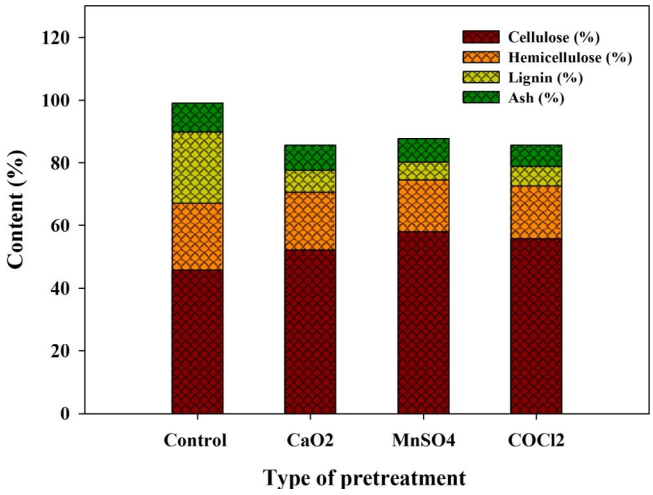

Fig. 3. Biochemical composition of untreated sample and generated KCP biomass after individual CaO_2 and CaO_2 + inorganic salt pretreatment under optimized pretreatment conditions.

no difference in BDO titer and BDO yield was observed (data not shown). In KCP hydrolysates, no complete sugar utilization was observed since after enzymatic hydrolysis along with glucose, xylose it also produces minor quantities of mannose, galactose and arabinose sugar moieties. In all pretreated enzymatic KCP hydrolysates complete utilization (100%) of glucose and xylose takes place however, other sugars mainly arabinose and mannose and galactose were not effectively utilized and may not get converted into BDO. Moreover, the obtained BDO yield was comparable with the reported literature of BDO production using lignocellulosic biomass.

The results proved that CaO_2 with inorganic metal salts pretreatment process is more efficient and effective for the hydrolysis of LC biomass and to make LC-BDO production more cost-effective and practically applicable at commercial scale. Considering these results, this process can be implemented for the bioconversion of carbohydrates available in various lignocellulosic biomasses for sustainable bio-based BDO production. Further studies will be devoted towards modifying the strain for utilization of all sugars of LC hydrolysates and designing small-scale reactor in continuous mode operation to achieve commercial scale BDO production.

4. Conclusion

The developed novel cost-effective pretreatment strategy using calcium peroxide and in combination with inorganic salts showed effective delignification of KCP and preserved the cellulose and hemicellulose components. The pretreated KCP biomass gave a better hydrolysis yield and glucose yield after enzymatic hydrolysis. The maximum delignification about 75.5% and hydrolysis yield of 80.5% was recorded in CaO_2 + $MnSO_4$ pretreatment. This pretreatment strategy could be useful for other lignocellulosic biomass to achieve higher sugar yield. Importantly, the resulted hydrolysates efficiently converts into BDO which increases importance of this work.

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Table 2
Utilization of resulted enzymatic hydrolysates of KCP biomass generated during individual CaO_2, CaO_2 with inorganic salts pretreatment and mixture of standard sugars for 2,3-butanediol production and fermentation parameters.

Substrate	Total sugar concentration (g/L)	Sugar utilization (%)	pH after fermentation	BDO titer (g/L)	Volumetric BDO productivity (g/L/h)	BDO yield (g/g of sugar)
CaO_2	30	90	6.75	10.42 ± 0.55	0.434 ± 0.07	0.385 ± 0.015
CaO_2 + $MnSO_4$	30	90	6.75	10.32 ± 0.54	0.430 ± 0.06	0.382 ± 0.015
CaO_2 + $COCl_2$	30	85	6.75	9.85 ± 0.48	0.410 ± 0.06	0.386 ± 0.012
Mixture of sugar	30	100	6.75	11.52 ± 0.48	0.480 ± 0.05	0.384 ± 0.011

Values are mean of three experiments; (±) standard error (SE) by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.02.115.

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