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Research article

Comprehensive approach to utilize hydrogen peroxide sterilization and urea as nitrogen source for ethanol production from oil palm trunk

Noppawan Danbamrongtrakool[a], Imrana Niaz Sultan[c], Nikhom Laemsak[b], Afrasiab Khan Tareen[a,c], Sarote Sirisansaneeyakul[a], Pramuk Parakulsuksatid[a,*]

[a] *Department of Biotechnology, Kasetsart University, Bangkok 10900, Thailand*

[b] *Department of Wood Technology, Faculty of Forestry, Kasetsart University, Bangkok 10900, Thailand*

[c] *Department of Biotechnology, Faculty of Life Sciences and Informatics, BUITEMS, Quetta 87300, Pakistan*

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Abstract

Importance of the work: Alternative nitrogen sources such as yeast-extract and peptone and a low-cost sterilizing agent are required to replace steam sterilization.

Objectives: To investigate the use of urea as a source of nitrogen and hydrogen peroxide (H_2O_2) as a sterilizing agent for ethanol production.

Materials & Methods: The optimized concentration (1 g/L) of urea as the nitrogen source (an alternative to yeast extract and peptone) was used with *Saccharomyces cerevisiae* SC90. Additionally, the optimized H_2O_2 concentration for the sterilization process was observed for its suitability for controlling microbial contamination in simultaneous saccharification and fermentation (SSF).

Results: The optimized urea concentration produced a mean (± SD) ethanol concentration of 37.41 ± 0.19 g/L. The culture medium sterilization with H_2O_2 concentrations (0 g/L, 0.1 g/L, 0.5 g/L or 1 g/L) was compared to energy intensive steam sterilization (121°C, 15 min). The conditions of an optimized H_2O_2 concentration of 0.5 g/L with both 12 hr and 24 hr of sterilization were suitable for controlling microbial contamination and produced similar maximum ethanol concentrations ($p < 0.05$). The results indicated that after 24 hr of sterilization, the maximum values for ethanol concentration, ethanol productivity, ethanol yield and ethanol theoretical yield were 33.64 ± 0.07 g/L, 0.42 ± 0.05 g/L/h, 0.47 ± 0.01 g/g and 74.35 ± 0.13%, respectively.

Main finding: The utilization of urea with H_2O_2 exhibited potential to reduce the cost of ethanol production using the SSF process.

* Corresponding author.
E-mail address: fagipmp@ku.ac.th (P. Parakulsuksatid)

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Introduction

Bioethanol is a clean energy source that can combust completely compared to petroleum fuels (Anyanwu et al., 2022). Ethanol has various advantages, such as being a direct replacement for gasoline and diesel or in a mixture with gasoline as Gasohol or with diesel as diesohol (Hansen et al., 2005). Ethanol can be applied as an octane enhancer, substituting for methyl tertiary butyl ether additives, which are difficult to decompose and also cause air pollution problems (McCarthy and Tiemann, 2006). Therefore, the use of gasohol can help in the reduction of global warming caused by greenhouse gases. In addition, ethanol can be produced domestically, thus reducing trade imports and saving money for a country. Biological methods or the use of microorganisms to produce ethanol are preferable in turning sugar into ethanol due to large biodegradable compounds present in their composition (Wilaithup et al., 2022). There are three types of raw materials used in producing ethanol: sugar, starch and lignocellulose (Bušić et al., 2018). Even though raw material sources of lignocellulose are readily available in nature (Tareen et al., 2020), most applicable sources are by-products from agriculture, agricultural industries or agricultural waste, such as corncobs, cornstalk, rice straw and palm fiber (Shahirah et al., 2015). Thailand is an agricultural country; therefore, lignocellulose should be a favorable raw material to produce ethanol. Ethanol is produced as the final product from turning cellulose into glucose using simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae* SC90 (Tareen et al., 2021b). In this regard, the composition of the culture medium is an important factor affecting the fermentation efficiency of microorganisms (Shafaghat et al., 2010). Usually, nutrients, such as yeast extract and peptone, are added to the media to act as sources of nitrogen and vitamins; in addition, they reduce the fermentation period and increase the ethanol content (Basu et al., 2015). However, yeast extract and peptone are too expensive to be used at an industrial scale if the investment is to be viable (Tareen et al., 2021a). Consequently, to improve the potential of ethanol production for industrial use, it is necessary to reduce the cost of nutrients in the process by substituting yeast extract and peptone with other nutrients that are lower-priced but similarly effective as a source of nitrogen. After carbon sources, the nitrogen source is the next most important nutrient in fermented liquid (Singh et al., 2017). Arrizon and Gschaedler (2002) found that adding nitrogen to a fermentation tank exerted a positive effect by maximizing the rate of ethanol production and providing high sugar consumption over a long period. Another study reported higher fermentation efficiency and a reduced fermentation period using approximately 300 mg/L to meet the needs of the yeast (O'Connor-Cox et al., 1991). A suitable concentration of urea as a nitrogen source sufficient for yeast growth could increase the rate of fermentation and reduced the formation of by-products (Darvishi and Moghaddami, 2019). Many researchers noted that yeast extract (Bafrncova et al., 1999; Phukoetphim et al., 2017), ammonium (Srichuwong et al., 2009) and urea (Tareen et al., 2021a) helped in the growth of microorganisms, stimulating the fermentation rate and ethanol production. In addition, sterilization is an important step in ethanol production by reducing contamination during fermentation; usually, the sterilization utilizes a steam ingress autoclave, which consumes high energy and causes browning reactions with amino acid proteins or other nitrogen compounds. Therefore, an alternative method to sterilization with heat is necessary. Hydrogen peroxide (H_2O_2) has been classified as a disinfectant with widespread anti-disinfection sensitivity against fungi, yeasts, bacteria, viruses and pathogens in the form of spores (Wallace, 2016; Shen et al., 2017; Noh et al., 2020). H_2O_2, being less energy intensive (Roukas and Kotzekidou, 2020) and a non-contaminating oxidizer (Moreira et al., 2020), has a highly unstable structure which can be broken at any time. During the structural breakage of H_2O_2 based on the Fenton reaction, hydroxyl radicals are formed that initiate the sterilization process (Yoo, 2018). Furthermore, quite a few disinfectants have been investigated by researchers for ethanol fermentations, including anti-*Lactobacillus* bacteriophages, chlorine dioxide, hydrogen peroxide, vaporized hydrogen peroxide sterilizer and potassium metabisulfite (Gibbons and Westby et al., 1986; Narendranath et al., 2000; Skinners and Leathers, 2004; Meneghin et al., 2008; Liu et al., 2015; Noh et al., 2020). In addition, Mitchell and Goen (2012) studied the utilization of sodium metabisulfite ($Na_2S_2O_5$) and H_2O_2, instead of sterilization using heat, of wheat straw with the SFF process. They found that H_2O_2 and sodium metabisulfite could limit the growth of acetic and lactic bacteria without affecting the performance of cellulase enzymes. A sodium metabisulfite concentration of 2.14 g/L affected the inhibition of yeast activities. Similarly, H_2O_2 at a concentration of 5.44 g/L could eliminate acetic bacteria by 52%, completely eliminate lactic bacteria and produce 20% more ethanol.

The current research aimed to use hydrogen peroxide for sterilization and urea as a source of nitrogen for ethanol production through batch SSF with *S. cerevisiae* SC90 as

a potential alternative approach to reduce the cost of ethanol production.

Materials and Methods

Raw material

Dry oil palm trunk (*Elaeis guineensis* Jacq.) was initially chopped into 20 mm × 20 mm × 5 mm pieces suing a wood chipper and then subjected to steam explosion (2.5 L; Nitto Koatsu Corporation; Japan) at 210°C for 4 min. Hot water washing of the steam-exploded fibers was performed for 30 min at 80°C with a total solid-to-liquid ratio of 1:8 (g/mL). These fibers were further treated with 15% NaOH weight per volume (w/v) in a water bath for 60 min at 90°C with a solid-to-liquid ratio of 1:8 (g/mL), according to Tareen et al. (2021c) and stored in sealed bags at 4°C until the next experimental stages.

Preparation of S. cerevisiae SC90 inoculum

An industrial yeast (*S. cerevisiae* SC90) was used for ethanol fermentation with the simultaneous scarification and fermentation (SSF) process. The yeast stock solutions were maintained in yeast, peptone and dextrose (YPD) broth and stored at 4°C. *S. cerevisiae* SC90 was cultured into YPD medium containing 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract (30 mL total volume) at 30°C for 18 hr with continuous shaking at 150 revolutions per minute (rpm).

Utilization of urea as source of nitrogen in ethanol production using simultaneous saccharification and fermentation with S. cerevisiae SC90 yeast

Ethanol production occurred in a 500 ml Erlenmeyer flask containing culture media composed of 1 g/L of urea, 5 g/L of magnesium sulfate ($MgSO_4$•$7H_2O$), and 2 g/L of potassium phosphate (KH_2PO_4) (pH: 4.8, adjusted with 0.05 M citrate buffer and sterilized at 121°C for 15 min) and 10% alkaline pretreated oil palm trunk (OPT) fibers as carbon source. Cellulase enzymes (1.5 L Celluclast) 15 Filter Paper Unit (FPU) per gram of substrate and beta glucosidase enzyme (Novozyme 188) 15 international units (IU) per gram of substrate were added (Tareen et al., 2021a) to flask. A yeast starter was used with 10% concentration by volume and cultured at 40°C and 150 rpm. Samples were collected at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 15 hr, 18 hr, 24 hr, 36 hr, 48 hr, 60 hr, 72 hr, 84 hr and 96 hr to analyze the viability of cells, glucose, cellobiose and ethanol concentration.

Influence of hydrogen peroxide concentration on sterilization of cultured medium for ethanol production.

The culture media (1 g/L urea, 5 g/L $MgSO_4$•$7H_2O$ and 2 g/L KH_2PO_4 (pH of 4.8)) added with 10% alkaline pretreated OPT fibers, varying concentrations of H_2O_2 (0.1 g/L, 0.5 g/L and 1 g/L) according to Barba et al. (2021) and yeast starter culture (10% concentration by volume) was incubated at 40°C and 150 rpm shaking for ethanol production. The samples were collected at 0, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 15 hr, 18 hr, 24 hr, 36 hr, 48 hr, 60 hr, 72 hr, 84 hr and 96 hr to investigate the viability of living cells and the H_2O_2 concentration.

Utilization of hydrogen peroxide sterilization for ethanol production through simultaneous saccharification and fermentation

Effect of hydrogen peroxide on S. serevisiae SC90 inhibition

The effect of H_2O_2 on the inhibition of *S. serevisiae* SC90 cells was observed by pipetting the culture medium (glucose: 20 g/L, urea: 1 g/L, $MgSO_4$•$7H_2O$: 5 g/L, KH_2PO_4: 2 g/L) in a test tube added with varied H_2O_2 concentrations in the range 0.0625–4 g/L and 10% *S. serevisiae* SC90 (by volume) for an incubation period of 96 h. The fermentation conditions applied were 40°C at 150 rpm. A control with no H_2O_2 was comparatively studied for the viability of living cells.

Ethanol production and sampling

For ethanol production 10% alkaline pretreated OPT fibers were used in a culture media consisting of 20 g/L glucose, 1 g/L urea, 5 g/L $MgSO_4$•$7H_2O$ and 2 g/L KH_2PO_4 (pH 4.8). The sterilization process was carried out at the optimized H_2O_2 concentration at periods of 0 hr, 4 hr, 8 hr, 12 hr and 24 hr. Enzymes (cellulase at 15 FPU/g of substrate and β-glucosidase at 15 IU/g of substrate) were added with 10% yeast cells. The samples were incubated at 40°C and 150 rpm. Samples were collected at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 15 hr, 18 hr, 24 hr, 36 hr, 48 hr, 60 hr, 72 hr, 84 hr and 96 hr for analysis of the viability of living cells and the glucose, cellobiose and ethanol contents.

Analytical methods

The chemical compositions of OPT before pretreatment and after pretreatment were determined according to the standard protocols provided by The Technical Association of the Pulp and Paper Industry (TAPPI): TAPPI T264 om-97 (1997): moisture content of OPF before pretreatment and after pretreatment; TAPPI T204 om-97: extractive substances; TAPPI T223om-84 (TAPPI, 1983d): content of pentosan; TAPPI T211om-85 (TAPPI, 1983b): ash; TAPPI T222om-98 (TAPPI, 1983a): acid insoluble lignin; and TAPPI T203om-93 (TAPPI, 1983c): alpha-cellulose content.

Analysis of cellobiose, glucose and ethanol

The viability of living cells was calculated using the plate count method (in duplication) on YPD agar. The kinetic parameters for SSF were calculated in accordance with Selig et al. (2008). The concentrations of cellobiose, glucose and ethanol were analyzed using high performance liquid chromatography with an Aminex HPX 87H column (Bio-Rad; Sunnyvale, CA, USA). Sulfuric acid (0.005 M) was used as the mobile phase at a flow rate of 0.6 mL/min with a maintained column temperature at 50°C (Xue et al., 2015). The H_2O_2 concentration was determined using a calorimetric method (Choleva et al., 2018).

Statistical analysis

The experimental data were subjected to analysis of variance. Significant differences between ethanol concentrations, ethanol productivity, ethanol yield and theoretical ethanol yield were determined using Duncan's new multiple range test in the SAS version 8.01 software (SAS, 2000). Statistical significance was set at 95% ($p < 0.05$). All data were presented as mean ± SD.

Results and Discussion

Utilization of urea in ethanol production using batch simultaneous saccharification and fermentation

Chemical composition of oil palm trunk

The compositional analysis of the OPT fibers before pretreatment indicated 10.99% extractive, 39.86% alpha cellulose, 77.01% holocellulose, 22.45% pentosan, 22.94% lignin and 1.52% ash on a dry weight basis. However, after the combined pretreatment of steam explosion, hot water extraction and alkaline extraction, the OPT fiber analysis was 77.96% alpha cellulose, 90.81% holocellulose, 1.36% pentosan, 6.67% lignin and 0.81% ash on a dry weight basis. The overall content of alpha cellulose increased 51.12% and the lignin content reduced to 70.92% after the combined pretreatment. These results were supported by the findings of Tareen et al. (2021a) who applied a similar pretreatment process to OPT fibers and successfully removed 71.77% of lignin on a dry weight basis. The pretreatment effect current analysis revealed the importance of pretreatment in separating the hemicellulose, cellulose and lignin from fibers for the utilization as value-added chemical products (Overend and Chornet, 1987).

Ethanol production using batch simultaneous saccharification and fermentation

The results of batch SSF indicated that the control (Fig. 1) had increased cellobiose and glucose concentrations at

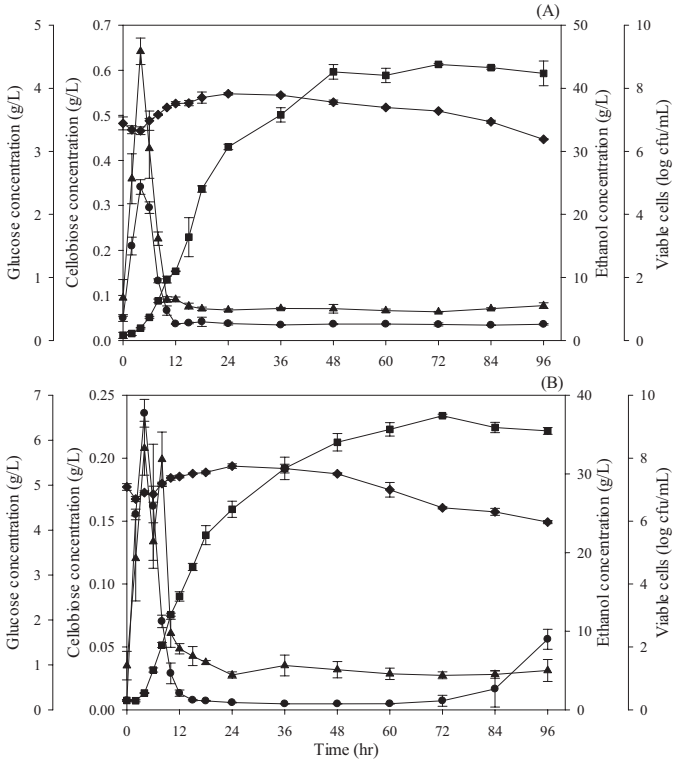

Fig. 1 Batch simultaneous saccharification and fermentation process using 10% (weight per volume) substrate loading with different production media: (A) yeast extract and peptone medium; (B) urea concentration at 1 g/L, where cfu = colony-forming units, ● = glucose, ▲ = cellobiose, ■ = viable cells, ◆ = ethanol production and error bars indicate ± SD

the commencement of fermentation which decreased rapidly after 4 hr of fermentation, reaching the maximum viability of cells at 24 hr of 7.79 ± 0.05 log colony-forming units (cfu)/mL. The ethanol concentration increased steadily up to 48 hr of fermentation, reaching its peak value of 43.83 ± 0.14 g/L at 72 hr.

On the other hand, the results using the urea culture medium indicated increased cellobiose and glucose concentrations at the beginning of fermentation. Nonetheless, the cellobiose concentration decreased swiftly after 8 hr but stabilized after 24 hr. The glucose concentration decreased briskly after 4 hr but stabilized after 12 hr of fermentation. The viability of living cells increased after 6 hr of fermentation and reached its maximum value of 7.81 ± 0.10 log cfu/mL at 24 hr. The ethanol concentration improved progressively until 72 hr when the maximum ethanol concentration was 37.41 ± 0.19 g/L.

The specific growth rate, ethanol productivity, ethanol yield and theoretical ethanol yield of *S. cerevisiae* SC90 at a urea concentration of 1 g/L were compared to the control, utilizing yeast extract and peptone (Table 1). The control medium produced maximum ethanol content of 43.83 ± 0.14 g/L with 0.22 ± 0.05/hr specific growth rate, 0.60 ± 0.09 g/L/h productivity, 0.50 ± 0.01 g/g ethanol yield and 97.24 ± 1.12% theoretical ethanol yield. The overall values for yeast extract and peptone were higher than for the control. For the urea treatment, the maximum values for ethanol concentration, specific growth rate, ethanol productivity, ethanol yield and theoretical ethanol yield were 37.41 ± 0.19 g/L, 0.18 ± 0.03/hr, 0.51 ± 0.01 g/L/hr, 0.52 ± 0.00 g/g and 82.30 ± 0.40%, respectively. Based on both experiments, the control medium produced higher values for ethanol concentration (14.65%), specific growth rate (18.18%), and theoretical yield (15.36%) compared to the medium containing 1 g/L urea because the yeast extract and peptone contain nutrients and complete vitamins, which are essential for the growth of yeast cells (Kayail et al., 2005; Sharma et al., 2018). Conversely, the utilization of urea as a nitrogen source supported its cost-effective potential for ethanol production with possible industrial-scale application.

Table 1 Fermentation kinetics parameters using batch ethanol fermentation simultaneous saccharification and fermentation and different nitrogen sources

Fermentation kinetics	Nitrogen medium (g/L)	
	Yeast extract and peptone	Urea concentration at 1 g/L
μ (h^{-1})	0.22 ± 0.05	0.18 ± 0.03
C_P (g/L)	43.83 ± 0.14	37.41 ± 0.19
Q_P (g/L/h)	0.60 ± 0.09	0.51 ± 0.01
$Y_{P/S}$ (g/g)	0.50 ± 0.01	0.52 ± 0.00
Theoretical yield (%)	97.24 ± 1.12	82.30 ± 0.40

μ = specific growth rate (2–10 hr); C_p = maximum ethanol concentration; Q_p = ethanol productivity; $Y_{P/S}$ = ethanol yield

Influence of hydrogen peroxide concentration on sterile culture medium for ethanol production

The effect of H_2O_2 as a sterilizing agent was studied on pretreated OPT fibers with varying H_2O_2 concentrations (0 g/L, 0.1 g/L, 0.5 g/L and 1 g/L) compared with the control (sterilized by autoclaving at 121°C for 15 min). The control was not contaminated throughout 96 hr of sterilization. In addition, the effects of various concentrations of H_2O_2 (0 g/L, 0.1 g/L, 0.5 g/L and 1 g/L) were investigated in culture medium. At 0 g/L of H_2O_2 concentration (no sterilization), microorganism contamination occurred after 12 hr and continued throughout 96 hr of sterilization, with the maximum total number of aerobic microorganisms and anaerobic microorganisms being 8.79 ± 0.40 log cfu/mL and 9.45 ± 0.02 log cfu/mL, respectively (Fig. 2A). In addition, during 0–10 hr, there were less than 30 colonies of microorganisms counted, apparently due to the long lag phase during which the microorganisms adapted to the environment. This might have been due to the difficulty of accessing the carbon in the culture medium. Thus, contaminated microorganisms needed more time to adapt by producing the necessary substances to be used as a carbon source to increase the cell numbers. At 0.1 g/L concentration of H_2O_2, aerobic and anaerobic microorganism contamination levels occurred at 60 hr and 72 hr, respectively, and lasted throughout the 96 hr sterilization period. The amount of H_2O_2, uninterruptedly decreased from the beginning of the sterilization period. At the end of the sterilization, the H_2O_2 concentration remaining in the culture medium was 0.01 ± 0.01 g/L (Fig. 2B).

Contrariwise, no contamination was observed at 0.5 g/L and 1 g/L of H_2O_2 throughout 96 h (Figs. 2C–2D), because the H_2O_2 acted as a strong oxidizing agent on microorganism cells (Yoo, 2018) by oxidizing the sulfhydryl group which causes protein breakdown. It also affected the lipid content in the cell membrane, leading to peroxidation of fat in the bacterial membrane (Cheignon et al., 2018). Consequently, cell membrane permeability is influenced and facilitates the transportation of substances into and out of cells (Kong and Davison, 1980). Furthermore, the amount of H_2O_2 (0.5 g/L or 1 g/L) slowly decreased at the beginning and the end of the 96 hr of sterilization. The amounts of H_2O_2 from the 0.5 g/L or 1 g/L that remained in the culture medium were

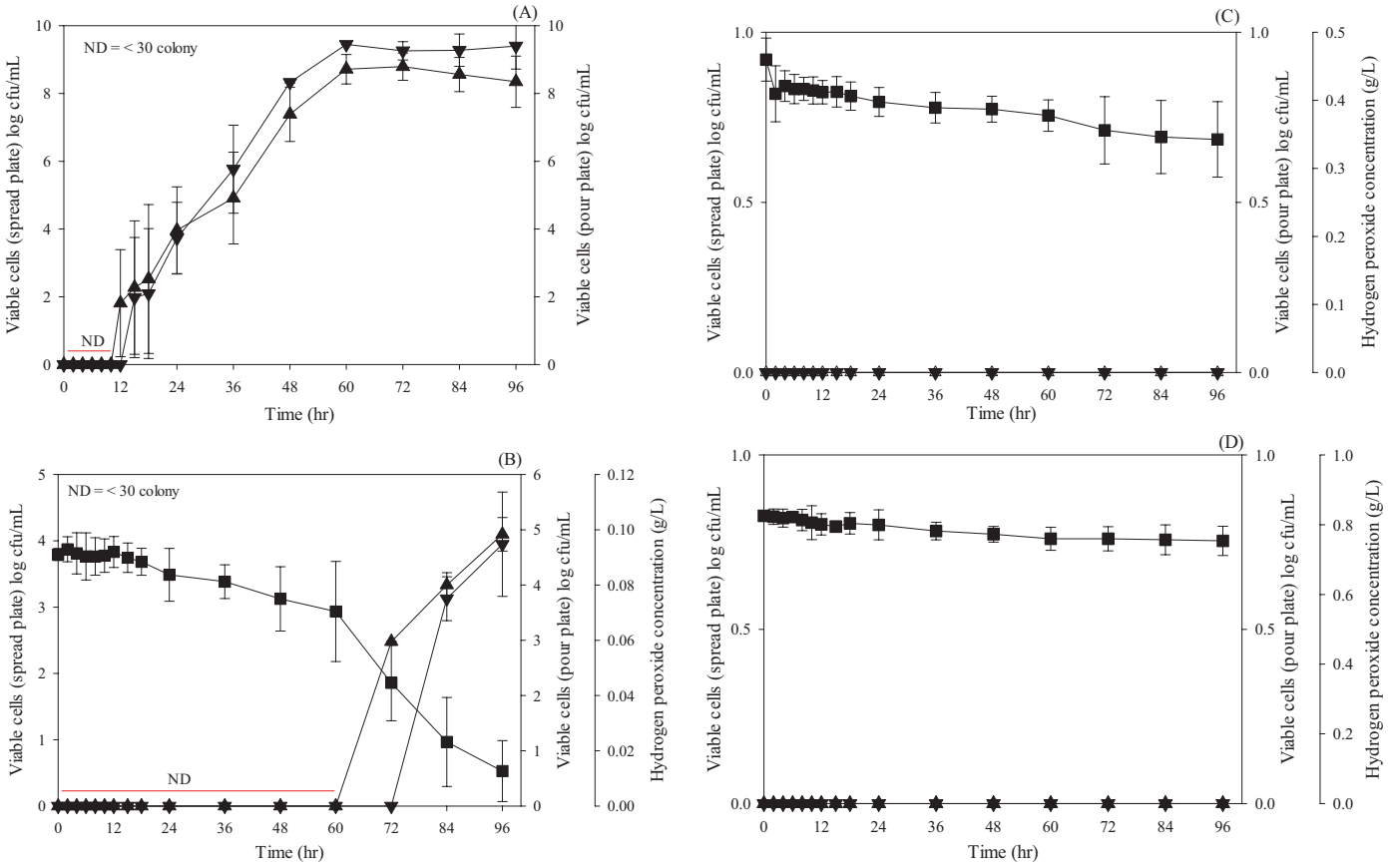

Fig. 2 Effects of medium sterilization at various H_2O_2 concentrations: (A) 0 g/L, (B) 0.1 g/L, (C) 0.5 g/L, (D) 1 g/L, where cfu = colony-forming units, ■ = H_2O_2 concentration, ▲ = viable cells (spread plate), ▼ = viable cells (pour plate) and error bars indicate ± SD

0.34 ± 0.06 g/L and 0.75 ± 0.04 g/L, respectively. An H_2O_2 solution is often unstable because it sensitively reacts with organic matter and subsequently decomposes when heated (Yoo, 2018).

The concentrations of H_2O_2 at 0.5 and 1 g/L could control microbial contamination in the culture medium used for ethanol production. In general, a very high chemical concentration affected the yeast regarding ethanol production by causing cell toxicity and death. Additionally, a high concentration of H_2O_2 can significantly damage the cellulose content (Lee et al., 2020). Consequently, the 0.5 g/L H_2O_2 concentration was more suitable, since this concentration was the lowest that could effectively control contaminants. For that reason, 0.5 g/L H_2O_2 concentration was used for downstream applications. H_2O_2 was more suitable for sterilization than traditional autoclaving with the mechanism of steam sterilization. The sterilization cost per cycle for hydrogen peroxide has been estimated as USD 18.98 while that for steam autoclaving (121–148°C, 103,421 Pa) is $34.29 (Lorson et al., 2020). Steam sterilization is commonly used for surgical items, though it requires water, electricity and steam generation and is damaging to heat-sensitive instruments, with continuous exposure to steam making instruments wet and exposing them to rusting (Mubarak et al., 2019). However, H_2O_2 provides uniform access to the entire exposed surface, whether it has a simple or complex topography and a minute amount is sufficient for sterilization (McEvoy and Rowan, 2019).

Utilization of hydrogen peroxide for sterilization in ethanol production using batch simultaneous saccharification and fermentation with S. cerevisiae SC90

Despite being effective in contaminant control, a large amount of the initial 0.5 g/L H_2O_2 was left in the medium which was further investigated for inhibition of *S. cerevisiae* SC90. The relationship between H_2O_2 concentration (0 g/L, 0.0625 g/L, 0.125 g/L, 0.25 g/L, 0.5 g/L, 1 g/L, 2 g/L and 4 g/L) and the percentage inhibition of yeast is shown in Fig. 3. An H_2O_2 concentration of 0.5 g/L inhibited 12.97% yeast cells.

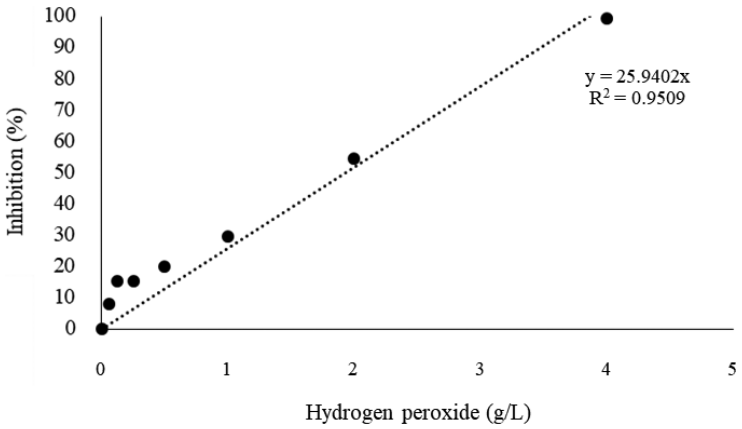

Fig. 3 Correlation between cell inhibition of *Saccharomyces cerevisiae* SC90 and H_2O_2 concentration, where R^2 = coefficient of determination

The H_2O_2 concentration in the culture medium slowly decreased at the beginning of sterilization. Thus, further study was conducted on varying the period of sterilization (0 hr, 4 hr, 8 hr, 12 hr and 24 hr) prior to SSF compared to the control (autoclaving at 121°C for 15 min). During ethanol production using SSF with *S. cerevisiae* SC90, the concentration of cellobiose and glucose rose at the start of fermentation in the control. Then, their concentrations decreased quickly after 4 hr but began to stabilize after 12 hr of fermentation. The viability of cells increased after 2 hr and reached to its maximum viability (7.81 ± 0.10 log cfu/mL) at 24 hr of fermentation. The ethanol concentration increased steadily during 72 hr of fermentation and the maximum ethanol concentration was 37.01 ± 0.19 g/L after 72 hr (Fig. 4).

Sterilization with H_2O_2 at 0 hr initially enhanced the amount of cellobiose and glucose but these rapidly reduced after 6 hr. The maximum viability of living cells at 48 hr was 7.62 ± 0.18 log cfu/mL. Bacterial contamination was evident after 12 hr of fermentation and the maximum ethanol concentration was 26.14 ± 1.94 g/L at 48 hr (Fig. 5). Contamination by microorganisms was countable after 10 hr of fermentation which suggested the utilization of the glucose for the growth of both yeast cells and contaminating bacterial cells, resulting in the growth of yeast cells being slowed and the ethanol concentration reduced. Contaminated bacteria have been reported to cause inhibition of yeast cells and to decrease the ethanol content (Lopes et al., 2016; Seo et al., 2020).

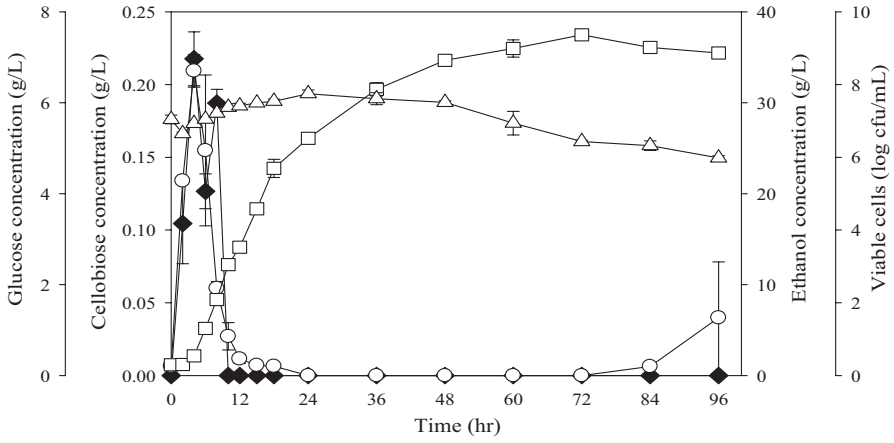

Fig. 4 Profile of batch simultaneous saccharification and fermentation process with 10% (weight per volume) substrate loading and autoclave sterilization (121°C at 103,421 Pa for 15 min) as control, where cfu = colony-forming units, ○ = glucose, ◆ = cellobiose, □ = ethanol, △ = viable cells and error bars indicate ± SD

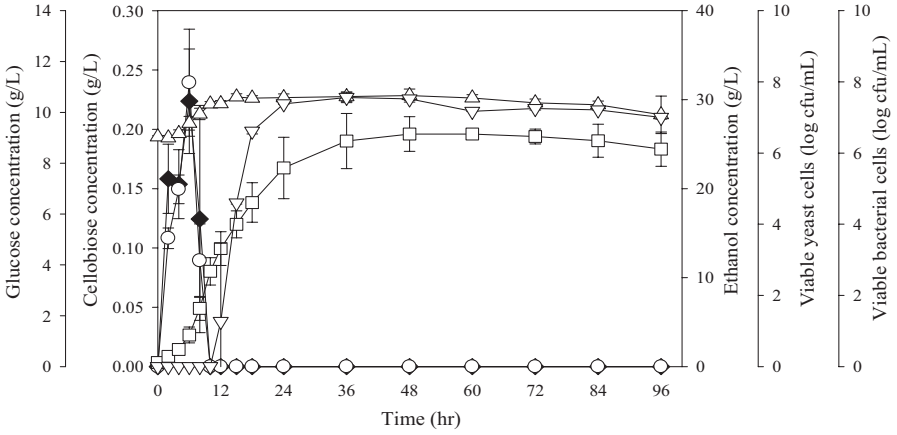

Fig. 5 Profiles of batch simultaneous saccharification and fermentation process with 10% (w/v) substrate loading and medium sterilization using 0.5 g/L H_2O_2 for 0 hr where cfu = colony-forming units, ○ = glucose, ◆ = cellobiose, □ = ethanol, △ = viable bacterial cells, □ = viable yeast cells and error bars indicate ± SD

Varying the period from 4 hr to 8 hr of sterilization increased the cellobiose and glucose contents at the initiation of fermentation, which decreased rapidly after 4 hr (Figs. 6A–B). The maximum viability of yeast cells following 4 hr and 8 hr of sterilization was at 36 hr (7.718 ± 0.05 log cfu/mL and 7.724 ± 0.20 log cfu/mL, respectively) while the maximum ethanol concentrations were at 60 hr and 84 h (28.00 ± 2.71 g/L and 30.05 ± 3.39 g/L, respectively. At 12 hr of fermentation (Fig. 6C), there were escalations in the cellobiose and glucose contents at the beginning, which started decreasing rapidly after 8 hr and 6 hr of fermentation, respectively. The maximum viability of yeast cells was at 36 hr (7.81 ± 0.10 log cfu/mL), whereas the maximum ethanol concentration was at 84 hr (33.48 ± 0.41 g/L). In addition, increasing the sterilization period to 24 hr (Fig. 6D) increased the cellobiose and glucose concentrations when fermentation began and then decreased rapidly after 8 hr and 6 hr, respectively. The maximum values for live yeast cells and ethanol concentration were 7.81 ± 0.10 log cfu/mL at 24 hr and 33.64 ± 0.06 g/L at 72 hr, respectively.

Varying the sterilizing period (12 hr or 24 hr) was suitable for maximum production of ethanol. Compared to other sets of the experiment, increasing the period of sterilization increased the ethanol production due to the complete control of microbial contamination. In addition, a lower H_2O_2 concentration during sterilization reduced the inhibition of yeast cells. Nevertheless, sterilization with H_2O_2 was more likely to produce less ethanol than sterilization using autoclaving, which may be credited to the possible interruption in the growth of the yeast cells by the H_2O_2. The control had the maximum growth of yeast cells at 24 hr but sterilization with H_2O_2 at 0.5 g/L resulted in the highest growth of yeast cells at 36–48 hr. The maximum strength of yeast cells also decreased compared to the control, possibly due to the concentration of H_2O_2 affecting the inhibition of yeast cells.

Additionally, H_2O_2 has been observed to inhibit bacteria in alcohol fermentation, which was attributed to the absence of the catalase enzymes responsible for the breakdown of H_2O_2 into water and oxygen; however, yeast can help reduce the toxicity of H_2O_2 because yeast cells contain catalase enzymes (Chang et al., 1997).

Analysis of the theoretical yield of H_2O_2 at 0.5 g/L with varying sterilization periods compared to the control (autoclaving) indicated that the control had maximum v alues for ethanol concentration of 37.01 ± 0.19 g/L, specific growth rate of 0.18± 0.010/h, ethanol productivity of 0.51 ± 0.01 g/L/h, ethanol yield of 0.52 ± 0.01 g/g and theoretical ethanol yield of 82.30 ± 0.40%. A sterilization period of 24 h produced the maximum ethanol concentration of 33.64 ± 0.07 g/L, specific growth rate of 0.17 ± 0.001/h, ethanol yield of 0.47 ± 0.05 g/g and ethanol theoretical yield of 74.35 ± 0.13%. No sterilization (0 hr) produced the highest ethanol production rate of 0.50 ± 0.18 g/L/hr. However, there were no significant differences between ethanol productivity, ethanol yield and ethanol theoretical yield for sterilization time periods of 12 and 24 hr. Furthermore, the analysis indicated no significant differences among the production rates for all six sets of experiments (Table 2). Therefore, it was clear that the sterilization with H_2O_2 at 0.5 g/L for 12 hr prior to the fermentation process was suitable for ethanol production using the SSF process. This period of sterilization was shorter but produced ethanol content closer to that from the sterilization period of 24 hr, making it more suitable for production of ethanol using batch SSF with *S. cerevisiae* SC90.

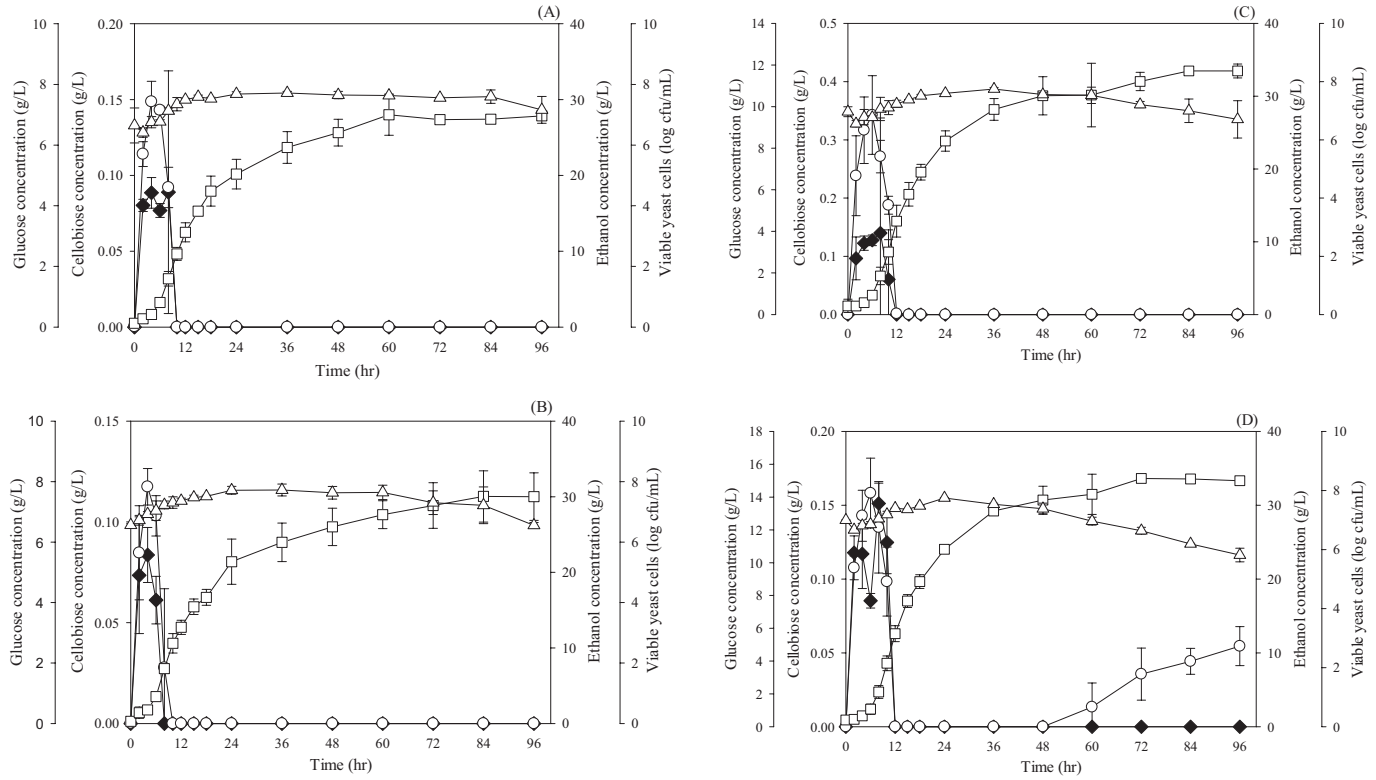

Fig. 6 Profile of batch simultaneous saccharification and fermentation process with 10% (weight per volume) of substrate loading with medium sterilization at 0.5 g/L H_2O_2 for different times: (A) 4 hr; (B) 8 hr; (C) 12 hr; (D) 24 hr, where cfu = colony-forming units, ○ glucose, ◆ = cellobiose, □ = ethanol production, △ = viable cells) and error bars indicate ± SD

Table 2 Fermentation kinetic parameters for batch simultaneous saccharification and fermentation process with *Saccharomyces cerevisiae* SC90 for various sterilization periods of medium

Fermentation kinetics	Sterilization					
	Autoclaving (control)	Sterilization time of media (hr)				
		0	4	8	12	24
μ (h^{-1})	0.18 ± 0.01^{b}	0.24 ± 0.04^{a}	0.24 ± 0.04^{a}	0.18 ± 0.06^{b}	0.17 ± 0.024^{c}	0.017 ± 0.01^{c}
C_P (g/L)	37.01 ± 0.19^{a}	26.14 ± 1.94^{d}	28.00 ± 2.71^{cd}	30.05 ± 3.39^{c}	33.48 ± 0.41^{b}	33.64 ± 0.07^{b}
Q_P (g/L/h)	0.51 ± 0.01^{a}	0.50 ± 0.18^{a}	0.39 ± 0.14^{a}	0.36 ± 0.03^{a}	0.37 ± 0.03^{a}	0.42 ± 0.05^{a}
$Y_{P/S}$ (g/g)	0.52 ± 0.01^{a}	0.38 ± 0.04^{d}	0.40 ± 0.01^{cd}	0.43 ± 0.04^{c}	0.47 ± 0.02^{b}	0.47 ± 0.01^{b}
Theoretical yield (%)	82.30 ± 0.40^{a}	61.02 ± 0.63^{c}	62.39 ± 3.67^{c}	68.87 ± 5.52^{b}	74.23 ± 2.66^{b}	74.35 ± 0.13^{b}

μ = specific growth rate (2–10 hr); C_p = maximum ethanol concentration; Q_p = ethanol productivity; $Y_{P/S}$ = ethanol yield;
Values (mean ± SD) within a row superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Conclusion

The current study investigated using urea instead of YP media as a low-cost nitrogen source for ethanol production from pretreated OPT fibers (10% w/v) based on batch SSF with *S. cerevisiae* SC90. The ethanol concentration of YP media was higher than urea (43.83 ± 0.14 g/L and 37.41 ± 0.19 g/L, respectively. However, urea has the potential to reduce the overall cost of ethanol production and may be best suited to industrial-scale ethanol production.

Sterilization in different culture media by varying the concentration of H_2O_2 (0 g/L, 0.1 g/L, 0.5 g/L and 1 g/L), compared to sterilization using an autoclave as the control, indicated that using an H_2O_2 concentration of 0.5 g/L was suitable to control microbial contamination. Additionally,

H_2O_2 sterilization does not harm the environment since the degradation of H_2O_2 leaves only non-toxic components (oxygen and water) after utilization. For ethanol production using SSF with *S. cerevisiae* SC90 and sterilization with H_2O_2 at 0.5 g/L, the optimized period of sterilization before fermentation was 12 hr to produce the maximum values for ethanol concentration, ethanol productivity, ethanol yield and ethanol theoretical yield of 33.48 ± 0.41 g/L, 0.37 ± 0.03 g/L/hr, 0.47 ± 0.02 g/g and 74.23 ± 2.66%, respectively. Consequently, the cost of ethanol production based on SSF could be reduced through the utilization of urea as an alternative to yeast extract and peptone in conjunction with H_2O_2 as a sterilizing agent (instead of autoclaving).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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