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Research article

Utilization of urea as a nitrogen source for ethanol production from oil palm trunk using simultaneous saccharification and fermentation

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Abstract

The utilization of urea as a low cost nitrogen source to replace yeast extract and peptone was investigated for ethanol production using yeast *Saccharomyces cerevisiae* SC90 at 40°C. The effect of urea concentration (1 g/L, 2 g/L, 4 g/L and 7 g/L) was determined for ethanol production in medium consisting of 220 g/L glucose, 2 g/L KH_2PO_4 and 5 g/L $MgSO_4$. The optimum urea concentration of 1 g/L produced the maximum mean (± SD) ethanol concentration of 70.53 ± 5.81 g/L. The optimized urea concentration and mixtures of yeast extract and peptone (YP) were compared for ethanol production from alkaline-extracted oil palm trunk fibers using simultaneous saccharification and fermentation (SSF). The maximum ethanol concentration, productivity, yield and theoretical ethanol yield attained with urea were 37.00 ± 0.18 g/L, 0.51 ± 0.06 g/L/hr, 0.414 ± 0.02 g/g and 81.24 ± 0.39%, respectively which were comparatively lower than those obtained when YP was used as a nitrogen source for which the ethanol concentration, ethanol productivity and theoretical ethanol yield were lowered by 14.23%, 16.70%, and 16.56%, respectively. Conversely, using urea concentration of 1 g/L as a nitrogen source could replace the YP medium for ethanol fermentation using SSF. The results showed that ethanol production using urea as a nitrogen source was a better alternative to reduce the production medium cost on an industrial scale.

Introduction

Global demand for natural energy resources such as fossil fuel, gas and coal have considerably increased over the last few decades which have augmented the risk of their depletion (Martins et al., 2019). Consequently, alternative energy resources are becoming more important such as bioethanol which is a renewable energy source, an environmental-friendly fuel and helps reduce the detrimental environmental impacts resulting from fossil fuel consumption (Cardona and Sanchez, 2007).

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The oil palm trunk (OPT) is a valuable and abundant lignocellulosic biomass in Thailand as the palm oil industry is the backbone of Thailand's economy, particularly in the southern region (Noparata et al., 2015). Generally, in the first 2.5 yr post planting, fruiting commences in palm trees though productivity becomes lower within 25–30 yr (Maluin et al., 2020). Hence, the old palm trees are cut down and replaced by new seedling (Woodham et al., 2019). The OPT fibers are an agricultural byproduct that accumulates in large amounts and causes severe environmental pollution problems (Shahirah et al., 2015). However, it can play a vital complementary role, being a source of cellulosic pulp with an alpha-cellulose component with possible consequent applications in ethanol fermentation (Noparata et al., 2015).

Simultaneous saccharification and fermentation (SSF) accomplishes enzyme hydrolysis and fermentation in one step which helps in minimizing the production of inhibitory enzymes, as the released sugars during fermentation are instantaneously utilized by microorganisms (Brethauer and Wyman, 2010). Furthermore, SSF reduces the number of reactors (Hazeena et al., 2019) the contaminants and the ethanol production cost (Kadar et al., 2004; Martín et al., 2002).

The media formulation, supplementation mechanism and optimization have huge influences on the expenses of fermentation on an industrial level, amounting to more than 30% of overall production charges (Miller and Churchill, 2005). The growth factor and robustness of microorganisms depend on the appropriate nutritional composition of a medium (Basu et al., 2015). In fermentation media, microorganism can utilize organic or inorganic sources of nitrogen and their concentrations can play a vital role in desired metabolite production (Singh et al., 2017). On the smaller scale such as laboratory experimentation, the media used are mostly appended with yeast extract and peptone but this is unlikely done on the industrial level due to its high production costs. However, there is a need to identify cost-effective nutrient sources to comply with all nutritional supplies for the growth of yeast and fermentation processes. In the current research, urea was used as a nitrogen source to produce ethanol from oil palm trunk using SSF.

Materials and Methods

Raw material

Oil palm trunk (OPT) samples of *Elaeis guineensis* Jacq. aged 25–26 yr were obtained from a native agriculturist in Plai Phraya district, Krabi province, Thailand. Initially a wood chipper was used to chop the OPT into 20 mm × 20 mm × 5 mm pieces that were later subjected to steam explosion for 4 min at 210°C. The steam-exploded fibers were treated using hot water extraction for 30 min at 80°C with a total solid-to-liquid ratio of 1:8 g/mL. Afterwards, the fibers were treated with sodium hydroxide, generally known as alkaline extraction using 15% weight per volume (w/v) NaOH for 60 min at 90°C with a total solid-to-liquid ratio of 1:8 (g/mL), according to Tareen et al. (2021). The chemical composition of the fibers was analyzed using the standard protocols of the Technical Association of the Pulp and Paper Industry (TAPPI). T264 om-97, (1997) for moisture content; T204 om-97 for extractives; T222 om-98 for acid insoluble lignin; T223 om-84 for pentosan (TAPPI, 1983d); TAPPI T211 om-85 (TAPPI, 1983b) for ash. The alpha-cellulose was determined by TAPPI T203 om-93 (TAPPI, 1983c). The holocellulose was analysed by acid – chloride method of Browning.

Microorganism and culture media

The yeast strain *Saccharomyces cerevisiae* SC90 used in this study was obtained from the Liquor Distillery Organization, Bang Khla district Chachoengsao province, Thailand. The strain SC90 was grown at 30°C for 48–60 hr on yeast extract, peptone and dextrose (YPD) agar plates containing 20 g/L each of agar, peptone and glucose and 10 g/L yeast extract.

Preparation of inoculum

The inoculum was prepared by scraping the yeast cells (two loops) from the YPD plate and immersing in a liquid YPD medium flask containing 10 g/L yeast extract and 20 g/L each of peptone and glucose. The inoculated flasks were incubated in a rotary shaker for 18 hr at 30°C and 150 rpm.

Effect of urea concentration on ethanol production by S. cerevisiae SC90

The experiment was performed in a 500 mL Erlenmeyer flask holding 300 mL of production medium containing 10 g/L yeast extract, 220 g/L glucose and 20 g/L peptone as control (medium 1). The production medium constituted 220 g/L glucose, 2 g/L KH_2PO_4, 5 g/L $MgSO_4$ and various urea concentrations of 1, 2, 4 or 7 g/L as a nitrogen source (media 2–5). Each medium pH was adjusted to 4.8 using 50 mM sodium citrate buffer. After sterilization at 121°C for 15 min, 10% starter culture was transferred to the Erlenmeyer flask. The fermentation was

conducted in a rotary shaker for 96 hr at 40°C and 150 rpm. The treatments were assigned to experimental units at random.

Utilization of urea for ethanol production from oil palm trunk using simultaneous saccharification and fermentation

The fermentation process was accomplished in a 500 mL Erlenmeyer flask containing 300 mL of 10% alkaline extracted oil palm trunk (OPT) fibers, 2 g/L KH_2PO_4, 5 g/L $MgSO_4$ and the appropriate concentration of urea and compared with YP medium (10 g/L yeast extract and 20 g/L peptone). Each medium's pH was adjusted to 4.8 using 50 mM sodium citrate buffer followed by sterilization for 15 min at 121°C. Then, 10% starter culture, 15 Filter paper unit/g substrate of cellulose (Celluclast 1.5 L) enzyme and 15 IU/g substrate of β-glucosidase (Novozyme 188) were simultaneously added into the Erlenmeyer flask. The SSF process was continued in a rotary shaker for 96 hr at 40°C and 150 rpm.

Analytical methods

The number of viable yeast cells was evaluated using the plate count method (in duplication) on YPD agar. The cellobiose, glucose and ethanol concentrations were determined using high performance liquid chromatography (HPLC) with an Aminex HPX 87H column (Bio-Rad; Sunnyvale, CA, USA). The temperature of the column was maintained at 50°C with a mobile phase of 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min (Xue et al., 2015). The concentration of urea was determined using the colorimetric method with diacetyl monoxime as the color-developing agent (Reay et al., 2019).

Statistical Analysis

All data were presented as mean values ± SD. Data were analyzed using analysis of variance. Then significant differences between production media were determined using Duncan's new multiple range test facilitated by the SAS version 8.01 software (SAS, 2000). The level of statistical significance was set at 95% ($p < 0.05$).

Results and Discussion

Effect of urea concentration on ethanol production by S. cerevisiae SC90

The effects were compared of ethanol batch fermentation with *S. cerevisiae* SC90 in the ethanol production medium containing 220 g/L initial glucose concentration and various urea concentrations (1 g/L, 2 g/L, 4 g/L and 7 g/L) with modified YPD medium as control. The results showed that in the control medium, the glucose concentration decreased slowly at the beginning of fermentation but started decreasing rapidly after 18 hr. The viable cells increased after 4 hr but decreased after 48 hr of fermentation, whereas the maximum strength of viable cells was at 12 hr (8.32 ± 0.05 log colony forming units (cfu)/mL). The ethanol concentration continuously increased at 18 hr; conversely the final ethanol concentration achieved at 96 hr of fermentation was 74.14 ± 0.95 g/L (Fig. 1).

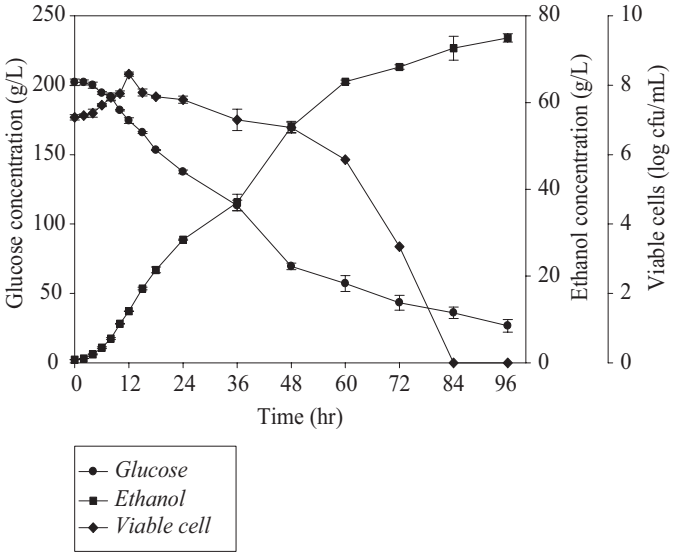

Fig. 1 Profiles of ethanol production of *Saccharomyces cerevisiae* SC90 in modified yeast extract peptone dextrose medium as control, for (●) viable cells, (◆) glucose and (■) ethanol concentrations and cfu = colony forming units

The profiles were extensively studied of residual glucose, viable cells and ethanol concentration during the batch fermentation at a urea concentration of 1 g/L as nitrogen source. The glucose concentration decreased slowly at the beginning of fermentation but a rapid decrease was observed after 18 hr. The number of viable cells increased after 4 hr but started decreasing after 48 hr, which was similar when the urea concentration increased to 2 g/L. The maximum number of viable cells at urea concentrations of 1 g/L and 2 g/L were at 18 hr (7.84 ± 0.07 log cfu/mL and 7.84 ± 0.10 log cfu/mL, respectively). The maximum ethanol concentrations of 70.53 ± 5.81 and 69.41 ± 6.33 g/L were obtained at urea concentrations of 1 g/L and 2 g/L, respectively (Figs. 2A–B). The profiles of residual glucose, number of viable cells and ethanol concentration at urea concentrations of 4 g/L and 7 g/L

as the nitrogen source are shown in (Figs. 2C–D). The glucose concentration which started decreasing slowly at the start of fermentation, rapidly decreased after 18 hr. The number of viable cells increased after 4 hr but started decreasing after 36 hr; however the maximum number of viable cells (7.82 ± 0.05 log cfu/mL) was observed at 24 hr. The concentration of ethanol also increased in the first 48 hr, whereas the maximum ethanol concentrations from urea concentrations of 4 g/L and 7 g/L were 47.80 ± 1.37 g/L and 46.07 ± 6.09 g/L, respectively.

The residual concentration of sugar in modified YPD medium was 26.63 ± 4.56 g/L. The sugar consumption was similar for the 1 g/L and 2 g/L urea concentrations but higher than those for the 4 g/L and 7 g/L urea concentrations. The total remaining sugar in the urea concentrations of 1 g/L and 2 g/L were 33.38 ± 9.36 and 35.36 ± 9.23 g/L, respectively while the total remaining sugar in the urea concentrations of 4 g/L and 7 g/L were 69.91 ± 14.17 and 85.18 ± 6.08 g/L, respectively. Accordingly, the number of viable cells in urea concentrations of 1 g/L and 2 g/L were similar but higher from the urea concentrations of 4 g/L and 7 g/L. The amount of remaining sugar was also dependent on the source of nitrogen and the various concentrations of urea. It was clearly observed that sugars present in the media were not entirely consumed by the yeast. At the end of fermentation, none of the viable cells were alive which could have been due to high pressure of the ethanol concentration and the high initial sugar concentration under elevated temperature (40°C) during ethanol fermentation. It has been reported that a high glucose concentration is particularly vulnerable to partial fermentation because the yeast cells are exposed to numerous stresses (Wang et al., 2013). A higher concentration of dissolved sugar escalates external osmotic pressure (Stanley et al., 2010). However, this might have been due to thermal stress as an increase in the fermentation temperature above 25°C may reduce the amount of sugar that could be fermented (Jones and Ingledew, 1994) but a lower temperature might result in low ethanol productivity. Similar observations were reported by Bai et al. (2008) who reported that the effect of high temperature on ethanol production worsened with high sugar conditions in the fermentation process. In addition, the concentration of ethanol increased to a toxic level for yeast cells during high glucose fermentation which led to ethanol and osmotic stress by affecting the structure of the cell membrane of the yeast (Gibson et al., 2007; Lahtvee et al., 2016).

The results showed that the urea concentrations with 1 g/L and 2 g/L had higher numbers of viable cells, specific growth rates (μ) and ethanol concentrations compared to the urea concentrations at 4 g/L and 7 g/L. Such results indicated that an increase in the urea concentration can significantly decrease the number of viable cells, specific growth rate and overall ethanol efficiency (Chan-u-tit et al., 2013). An appropriate concentration of urea as a nitrogen source reduces the formation

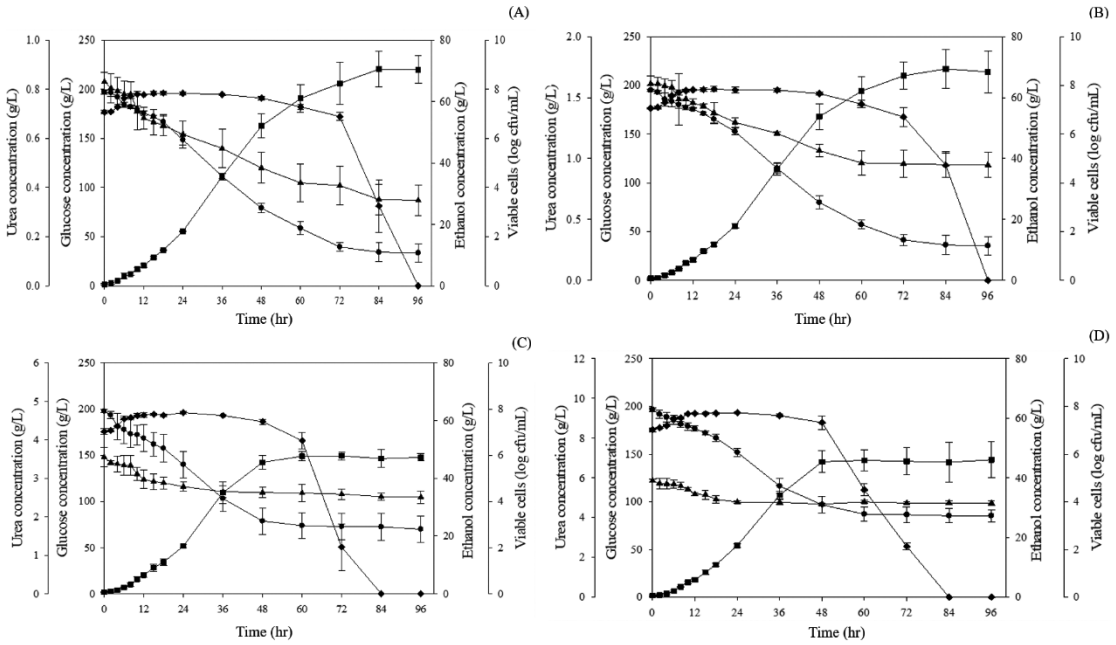

Fig. 2 Profiles of ethanol production of *Saccharomyces cerevisiae* SC90 using various urea media as nitrogen source: (A) urea concentration 1 g/L; (B) urea concentration 2 g/L; (C) urea concentration 4 g/L; (D) urea concentration 7 g/L, for (●) glucose, (▲) urea, (◆) viable cells and (■) ethanol concentration and cfu = colony forming units

of by-products (Darvishi and Moghaddami, 2019) but an increase in the urea concentration enhances the formation of by-products such as glycerol which is an osmoprotectant that causes osmotic stress (Kutyna et al., 2010). Other by-products such as acetic acid, acetaldehyde and acetone are overproduced during this osmotic stress, resulting in reduced yeast viability and ethanol production (Pratt et al., 2003).

The optimized urea concentration of 1 g/L was compared with modified YPD medium as the control which produced a maximum ethanol concentration of 70.14 ± 5.81 g/L. However, the ethanol concentration (4.86%) was lower than the control medium. The yeast extract plays a vital role in optimum fermentation by having a protective effect on growth and viability during ethanol fermentation (Bafrncova et al., 1999; Cao and Liu, 2013; Sharma et al., 2018). Furthermore, the quantity of yeast extract and peptone in a high glucose medium would allow the yeast to tolerate osmotic pressure and high temperature (Laopaiboon et al., 2009).

Table 1 shows the kinetic parameters of ethanol concentration (C_p), ethanol productivity (Q_p), ethanol yield ($Y_{p/s}$) and theoretical yield of ethanol fermentation under various nitrogen sources and urea concentrations for *S. cerevisiae* SC90. The various urea concentrations of 1 g/L, 2 g/L, 4 g/L and 7 g/L and modified YPD medium as the control were investigated to produce efficient ethanol. The maximum values obtained from the control medium were 74.14 g/L for C_p, 0.77 g/L/hr for Q_p, 0.42 g/g for $Y_{p/s}$ and 82.74% for the theoretical ethanol yield. In comparison with different urea concentrations, 1 g/L of urea produced 70.53 g/L and 0.83 g/L/hr as the highest values for ethanol concentration and ethanol productivity, respectively. The ethanol yield and theoretical ethanol yield were slightly lower for 2 g/L (0.43 g/g and 84.77%, respectively) which were not significantly different from the control medium and the urea concentration of 1 g/L. Therefore, using a urea concentration at 1 g/L or 2 g/L as the nitrogen source could replace yeast extract and peptone for cost reduction in ethanol fermentation.

Compositional analysis of oil palm trunk

Prior to ethanol fermentation, the chemical composition of OPT was analyzed before pretreatment (raw material) and after pretreatment (Table 2). The chemical composition of the raw material contained 11.49% extractive substances, 39.73% cellulose, 76.98% holocellulose, 22.86% pentosan, 23.64% lignin and 1.46% ash on a dry weight basis. After pretreatment with steam explosion, hot water and alkaline extraction, the percentage dry weight of cellulose and holocellulose increased to 49.04% and 15.22%, respectively and the percentage dry weight of pentosan, lignin and ash decreased to 94.07%, 71.77% and 44.73%, respectively. Despite lack of statistical analysis, it was apparent that after steam explosion, hot water and alkali extraction, sufficient cellulose remained that could be used as substrate for second generation bioethanol production which was similar to the findings of Tareen et al. (2021).

Table 2 Chemical composition (mean ± SD) of oil palm trunk before and after pretreatment

Chemical composition	Oil-palm trunk (% dry weight)	
	Before pretreatment	After pretreatment
Extractives	11.49 ± 0.13	-
Cellulose	39.73 ± 0.59	77.96 ± 0.80
Holo-celluose	76.98 ± 0.21	90.80 ± 0.36
Pentosan	22.86 ± 0.16	1.35 ± 0.12
Lignin	23.64 ± 0.23	6.67 ± 0.13
Ash	1.46 ± 0.46	0.80 ± 0.08

values are presented as mean ± SD

Table 1 Fermentation kinetic parameters for batch ethanol fermentation using various nitrogen sources (yeast extract and peptone) and urea concentrations from *Saccharomyces cerevisiae* SC90

Fermentation kinetics	Nitrogen source (g/L)				
	Yeast extract and peptone(control)	Urea concentration (g/L)			
		1	2	4	7
μ (hr^{-1})	0.26 ± 0.02^{a}	0.21±0.01^{a}	0.20 ± 0.01^{a}	0.19 ± 0.04^{a}	0.17 ± 0.05^{a}
C_P (g/L)	74.14 ± 0.95^{a}	70.53±5.81^{a}	69.41 ± 6.33^{a}	47.80 ± 1.37^{b}	46.07 ± 6.09^{b}
Q_P (g/L/hr)	0.77 ± 0.01^{ab}	0.83±0.18^{a}	0.81 ± 0.08^{ab}	0.65 ± 0.14^{b}	0.47 ± 0.09^{b}
$Y_{P/S}$ (g/g)	0.42 ± 0.01^{a}	0.43±0.02^{a}	0.43 ± 0.08^{a}	0.37 ± 0.04^{b}	0.40 ± 0.02^{a}
Theoretical yield (%)	82.74 ± 3.09^{a}	84.17±5.36^{a}	84.77 ± 1.54^{a}	73.98 ± 9.67^{b}	79.91 ± 4.28^{a}

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

Utilization of urea for ethanol production from oil palm trunk using simultaneous saccharification and fermentation

The pretreated oil palm trunk (OPT) fibers with 10% (w/v) substrate loading at 40°C were used in SSF processes to study the effect of different nitrogen sources (yeast extract and peptone and urea concentration at 1 g/L) on ethanol production.

The medium of yeast extract and peptone (YP) with glucose and cellobiose concentrations increased at the beginning and decreased after 4 hr, as shown in Fig. 3A. The maximum number of viable cells at 24 hr was 7.82 ± 0.03 log cfu/mL. The ethanol concentration rapidly increased up to 48 hr, whereas the maximum concentration of ethanol (43.79 ± 0.17 g/L) was observed at 72 hr of fermentation. The cellobiose and glucose concentrations in the 1 g/L urea medium increased at the beginning of fermentation, although their concentrations rapidly decreased after 8 and 4 hr, respectively. The maximum number of viable cells (7.75 ± 0.07 log cfu/mL) occurred at 24 hr (Fig. 3B). The ethanol concentration continuously increased until 72 hr of fermentation and the maximum concentration was 37.09 ± 0.18 g/L.

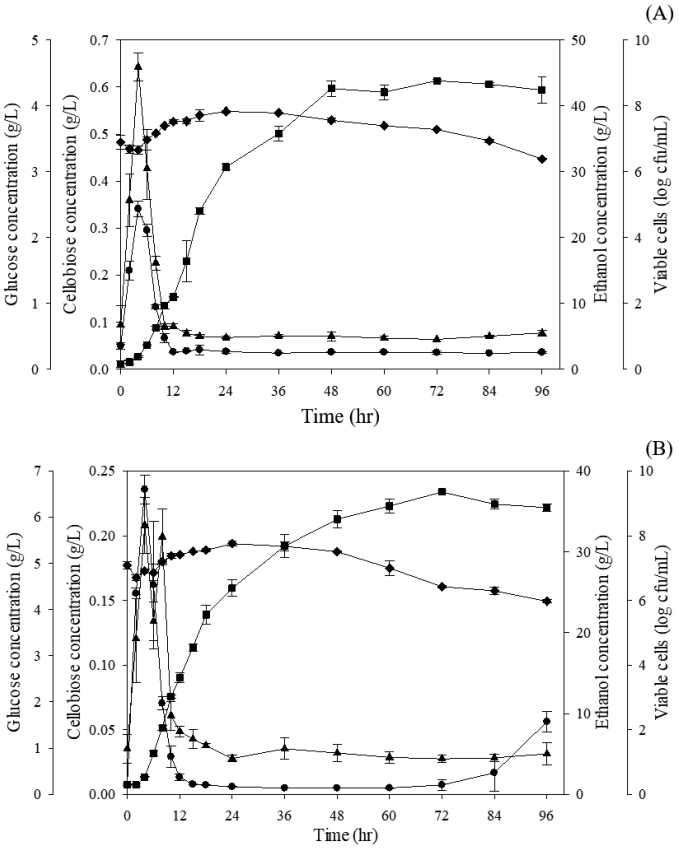

Fig. 3 Batch simultaneous saccharification and fermentation process carried out with 10% (weight per volume) of substrate loading using different production media: (A) yeast extract and peptone, (B) urea concentration at 1 g/L, for (●) glucose, (▲) cellobiose, (◆) viable cells and (■) ethanol concentration and cfu = colony forming units

The different nitrogen sources (YP and urea concentration at 1 g/L) were investigated regarding the efficient production of ethanol (Table 3). YP as the nitrogen source had the highest ethanol concentration (43.79 ± 0.17 g/L), ethanol productivity (0.59 ± 0.09 g/L/hr), ethanol yield (0.49 ± 0.05 g/g) and theoretical ethanol yield (97.37 ± 1.07%). The urea concentration at 1 g/L produced an ethanol concentration of 37.09 ± 0.18 g/L, ethanol productivity of 0.51 ± 0.06 g/L/hr, ethanol yield of 0.41 ± 0.02 g/g and a theoretical ethanol yield of 81.24 ± 0.39%. These results were analogous to those reported by Adela and SohKheang (2015) who could produce 38.08 g/L ethanol from OPT sap using urea as a nitrogen source. Additionally, Mongkolchaiarunya et al. (2016) performed fermentations using small-flowered umbrella sedge and obtained maximum ethanol concentrations of 16.54 g/L and 20.00 g/L with urea and peptone, respectively. These values were less than obtained in the current work. Compared to the YP media, urea as a nitrogen source produced a lower ethanol concentration (15.49%), ethanol productivity (15.38%), ethanol yield (14.23%) and theoretical ethanol yield (16.56%). It has been reported that nutrients in the fermentation medium are the key factors restricting the rate of fermentation (Jones and Ingledew, 1994; Petelenz-Kurdziel et al., 2013; Seguinot et al., 2020). To tolerate osmotic stress, yeast cells require adequate quantities of nutrients to grow and sustain their metabolic functions (Święciło, 2016).

The current study utilized urea as a low cost nitrogen source to replace yeast extract and peptone for ethanol production from glucose with an initial concentration of 220 g/L using *S. cerevisiae* SC90. The highest ethanol concentration of 70.53 g/L was obtained using 1 g/L urea concentration. The optimized concentration of urea as a nitrogen source for ethanol production based on using oil-palm trunk fibers in the SSF process produced an ethanol concentration of 37.09 ± 0.18 g/L, ethanol productivity

Table 3 Fermentation kinetics parameters (mean ± SD) in batch ethanol fermentation using simultaneous saccharification and fermentation under different nitrogen sources from *Saccharomyces cerevisiae* SC90

Fermentation kinetics	Nitrogen source (g/L)	
	Yeast extract and peptone	Urea concentration at 1 g/L
μ (hr^{-1})	0.22 ± 0.04	0.18 ± 0.02
C_P (g/L)	43.79 ± 0.17	37.09 ± 0.18
Q_P (g/L/hr)	0.59 ± 0.09	0.51 ± 0.06
$Y_{P/S}$ (g/g)	0.49 ± 0.05	0.41 ± 0.02
Theoretical yield (%)	97.37 ± 1.07	81.24 ± 0.39

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

of 0.51 ± 0.06 g/L/h, ethanol yield of 0.41 ± 0.02 g/g and a theoretical ethanol yield of 81.24 ± 0.39%. Urea as an alternate low cost nitrogen source instead of yeast extract and peptone may be used for batch ethanol fermentation in the SSF process.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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