CHAPTER IV
RESULTS AND DISCUSSION

4.1 Alkali pretreatment of pineapple crown

Pineapple crown (PIC), a top section of pineapple fruit (clustered leaves with stem), is an agro-industrial waste generated during pineapple processing that holds significant potential for conversion into high value-added biofuels and biochemicals through microbial fermentation, owning to its high polymeric sugar contents within its lignocellulosic structure. In this study, dried PIC fiber was found to contain 33.12±0.01% (w/w) cellulose, 13.18±0.01% (w/w) hemicellulose, 18.06±0.17% (w/w) lignin, 0.99±0.00% (w/w) ash, and 65.3±0.01% (w/w) extractives. These results indicate that dried PIC contains lower levels of cellulose and hemicellulose but a higher lignin content compared to pineapple leaves, as reported by Sethupathi et al. (2024), who found that pineapple leaves comprise 68.5-82.0% (w/w) cellulose, 18.0-18.8% (w/w) hemicellulose, 4.4-15.4% (w/w) lignin, and 0.9-2.7% (w/w) ash. Therefore, pretreatment is a crucial preparatory step to facilitate the release of the fermentable sugars from PIC by overcoming its lignocellulosic recalcitrance, enabling efficient conversion into D-(-)-lactic acid. An effective pretreatment process should achieve substantial lignin removal while minimizing the degradation of cellulose and hemicellulose and limiting the generation of inhibitory compounds, thereby improving the substrate's digestibility during enzymatic hydrolysis (Shukla et al., 2023). The application of sodium hydroxide (NaOH) as an alkaline solution to lignocellulosic biomass typically reduces the degree of cellulose crystallinity and polymerization, while expanding the surface area through the disruption of ester-linkage of lignocellulose within the lignocellulosic matrix (Ojo and de Smidt, 2023), thus enhancing accessibility during subsequent hydrolysis. In summary, the pretreatment by NaOH is considered as the effective method conferring low cost cooporation compared to those methods pretreating with other bases.

In this study, alkaline pretreatment of dried PIC was performed using sodium hydroxide (NaOH) at varying concentrations (0.25-1.25 N) performed at 90°C for 90 min. The solid recovery of the NaOH-pretreated PIC decreased significantly with increasing NaOH concentration, yielding 50.5±5.73%, 45.53±6.04%, 37.8±4.02%, 32.11±1.26%, and 30.38±1.54% (w/w) for 0.25, 0.5, 0.75, 1.0, and 1.25 N treatments, respectively, compared to 78.92±3.11% (w/w) in the hot water treatment as a control experiment (Figure 4.1). Thus, pretreatment was investigated with the solid to liquid ratio of 1:7 due to the solid recovery from control condition (78.91±3.11%), resulted in no significant difference to those from with solid to liquid ratio of 1:10 (76.48±1.03%). The highest NaOH concentration (1.25 N) led to the greatest solid loss (69.17±1.54 g per 100 g of the NaOH-pretreated PIC), likely due to the disruption of lignin-cellulose-hemicellulose linkages, swelling of cellulose fibers, and solubilization of lignin into the liquid fraction. These also collectively enhanced biomass surface area and reduced its crystallinity and degree of polymerization (Khan et al., 2021). For comparison, hot water pretreatment (90°C for 90 min) as a control experiment resulted in a 21.08±3.11% (w/w) solid loss, possibly due to autohydrolysis caused by the formation of hydronium ions (H_3O^+) at elevated temperatures. This weak acid effect can catalyze glycosidic bond cleavage in cellulose and hemicellulose and partially fragment lignin. Sun et al. (2022a) similarly reported that hot water pretreatment disrupts the lignocellulosic matrix by solubilizing hemicellulose and modifying lignin structure, thereby reducing solid recovery. Likewise, Chen et al. (2018) observed a marked decrease in solid recovery of hydrothermally pretreated wheat straw from 83.53% to 59.77% when pretreatment temperature was increased from 120°C to 200°C.

To evaluate fermentable sugar yields, crude cellulase was applied at a loading of 40 PCU/g to hydrolyze 20 g/L of the NaOH-pretreated PIC samples from each pretreatment condition. As shown in Figure 4.1, the total sugar concentrations released were 9.7±0.28 g/L (48.5±0.01%, w/w), 10.8±0.60 g/L (54.0±0.03%, w/w), 12.5±1.23 g/L (62.5±0.06%, w/w), 12.3±0.39 g/L (61.5±0.02%, w/w), and 12.7±0.37 g/L (63.5±0.02%, w/w) for the PIC pretreated by NaOH concentrations of 0.25, 0.5, 0.75, 1.0, and 1.25 N,

respectively. In contrast, hot water-pretreated PIC (the control condition) only yielded 8.3±0.39 g/L of total sugars, equivalent to a 41.5% (w/w) sugar recovery. Notably, sugar release reached maximum beyond 0.75 N NaOH, indicating that further increases in alkali concentration did not significantly enhance sugar yield. This observation aligns with the findings of Loow et al. (2016), who reported that excessive NaOH concentration or prolonged pretreatment time led to over-disruption of biomass structure, reducing cellulose retention and sugar conversion efficiency. Similarly, Sawisit et al. (2018) reported a decline in cellulose recovery from rice straw pretreated with NaOH concentrations above 1.0 N, while the total reducing sugar yield remained largely unaffected. Han et al. (2012) also reported a decrease in sugar yield from 350 mg/g to 280 mg/g of pretreated wheat straw when 1.0 N NaOH pretreatment was extended beyond 1.5 hours at 121°C. These results highlight the importance of optimizing pretreatment conditions to balance lignin removal with carbohydrate preservation for effective enzymatic hydrolysis. Surprisingly, Nashiruddin et al. (2020) though demonstrated that optimal pretreatment of pineapple leaves was achieved with 2.43% (w/v) NaOH at 87°C for 57.15 minutes, yielding 17.26 g/L of reducing sugars, a 33% improvement over a nonoptimized condition at 1.5 N NaOH pretreatment. This discrepancy may be attributed to differences in the lignocellulosic biomass source.

Based on the results of this study, the optimal pretreatment condition for pineapple crown (PIC) was achieved using 0.75 N NaOH at 90°C for 90 minutes with a solid-to-liquid ratio of 1:7. Under this condition, the NaOH-pretreated PIC exhibited 50.13±0.00% cellulose, 21.08±0.00% hemicellulose, 12.94±0.24% lignin, and 0.33±0.00% ash (w/w). This pretreatment effectively removed 28.35% of lignin and 66.67% of ash, while increasing cellulose and hemicellulose contents by 51.35% and 59.93%, respectively, compared to untreated PIC. Similarly, Asgher et al. (2013) reported a 48.7% lignin removal from sugarcane bagasse using 5% (w/v) NaOH at 121°C for 30 minutes. Additionally, inhibitors including, fufural, 5-HMF, and acetate were observed less than 1 g/L in total analyzed via the complete hydrolysis with 72% H_2SO_4. These results support the role of hydroxide ions (Na^+) in disrupting lignin structures by

cleaving ester and ether linkages in lignin–carbohydrate complexes (LCCs), thereby promoting lignin solubilization. In contrast, Saini et al. (2022) found that hydrothermal pretreatment of pineapple leaves at 150°C for 20 minutes led to increased xylan (19.7±0.42%, w/w) and lignin (19.7±2.16%, w/w) contents, but decreased glucan (33.55±5.10%, w/w), relative to untreated leaves (10.88±0.35% xylan, 14.2±0.42% lignin, and 56.90±2.10%, w/w glucan). This suggests that hydrothermal pretreatment may result in undesirable degradation of cellulose into glucose. Future studies may focus on refining hydrothermal conditions to maximize lignin removal while preserving cellulosic sugars. In comparison, the 0.75 N NaOH pretreatment method presented here appears to be a more effective preparatory step for subsequent enzymatic hydrolysis, especially when targeting glucose as the primary carbon source (Figure 4.1).

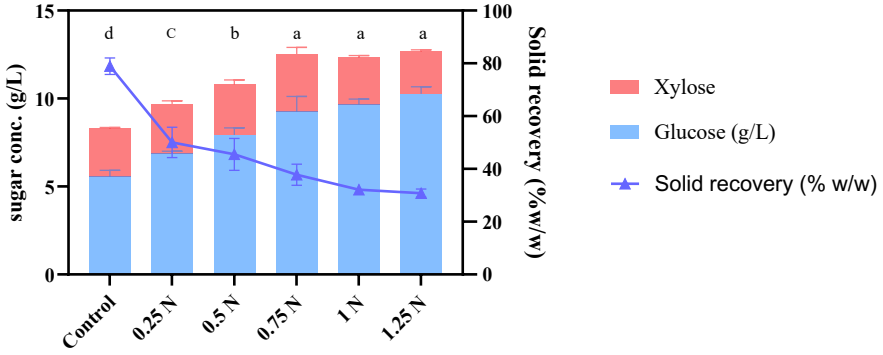

Figure 4.1 Glucose and xylose released from pretreated PIC with different concentration of NaOH (0.25-1.25 N) and hot water pretreatment as a control. Crude cellulase loading at 40 PCU/g was used for enzymatic saccharification of the NaOH-pretreated PIC. The percentage of solid recovery was calculated by dividing gram of the NaOH-pretreated PIC remained after enzymatic hydrolysis with gram of the untreated PIC provided.

4.2 Optimization of enzymatic hydrolysis of pretreated PIC

The enzymatic conversion of NaOH-pretreated PIC (20 g/L) into fermentable sugars was evaluated using varying concentrations (20-100 PCU/g) of crude cellulase

cocktail (VRE P3) at 50°C with 200 rpm agitation over 120 h. Under these conditions, the cellulase complex facilitated the release of glucose and xylose from cellulose and hemicellulose, respectively. Glucose was identified as the predominant sugar released at all enzyme loadings after 120 h of hydrolysis (Figure 4.2A). Total sugar concentrations reached 10.58±0.11 g/L (52.9±0.01%, w/w), 12.50±1.23 g/L (62.5±0.06%, w/w), 13.69±0.36 g/L (68.5±0.02%, w/w), 13.93±0.17 g/L (69.6±0.01%, w/w), and 14.03±0.10 g/L (70.2±0.01%, w/w) at enzyme loadings of 20, 40, 60, 80, and 100 PCU/g, respectively. As shown in Figure 4.2B, the sugar release plateaued after 24 h, with no significant differences at higher enzyme loadings beyond 60 PCU/g. Notably, the total sugar yield at 60 PCU/g was approximately 96.1% of that obtained via complete acid hydrolysis with 72% (w/v) H_2SO_4, which released 14.24±0.07 g/L (71.2±0.00%, w/w). At this optimal enzyme loading, cellulose (%CS) and hemicellulose (%HS) saccharification efficiencies reached 89.77% and 76.81%, respectively. At enzyme loadings of 20, 40, and 60 PCU/g, cellobiose was detected at 12 h hydrolysis and completely hydrolyzed after 72, 36, and 24 h, respectively; suggesting that crude cellulase cocktails contain β-glucosidase activity. However, increasing the cellulase concentration above 60 PCU/g slightly enhanced hemicellulose saccharification but led to a reduction in cellulose saccharification (Table 4.1), likely due to product inhibition, inefficient enzyme utilization, or non-productive enzyme binding.

Kinnarinen and Hakkinen (2014) reported a decline in hydrolysis efficiency when enzyme dosages exceeded the optimal level. Effective cellulose degradation requires the synergistic action of endoglucanase, cellobiohydrolase, and β-glucosidase. While endoglucanase and cellobiohydrolase are prone to feedback inhibition by accumulated cellobiose, β-glucosidase mitigates this by converting cellobiose to glucose. However, elevated glucose concentrations may inhibit β-glucosidase activity (Liu et al., 2023). Furthermore, excessive enzyme loadings can impair substrate accessibility due to enzyme overloading on the biomass surface. Residual lignin in the pretreated PIC may also reduce hydrolytic efficiency by adsorbing enzymes non-productively through hydrophobic or electrostatic interactions (Wu et al., 2023b; Yuan

et al., 2021). Similarly, Sawisit et al. (2018) optimized the enzymatic saccharification of pretreated rice straw using 1–6% (v/w) cellulase complex. Sugar yields significantly increased up to 4% (v/w) enzyme loading, beyond which no further enhancement was observed. The highest total sugar yield of 75.7% (w/w), with 82.0% CS and 35.2% HS, was achieved at 4% (v/w) enzyme loading. Zhu et al. (2023) also demonstrated that enzymatic hydrolysis of pretreated garden waste (grass, leaves, branches) with 10 FPU cellulase/g substrate and 7.5 U β-glucosidase yielded a glucose conversion rate of 60.12% (w/w). However, increasing cellulase loading to 20 FPU/g without adjusting β-glucosidase levels led to a decrease in glucose conversion (56.2% w/w) and a significant drop in cellobiose conversion (39.0% w/w). These findings highlight that cellulase overload, without an adequate β-glucosidase supply, may restrict hydrolysis efficiency due to enzyme overloading, non-productive binding, and progressive inhibition by cellobiose.

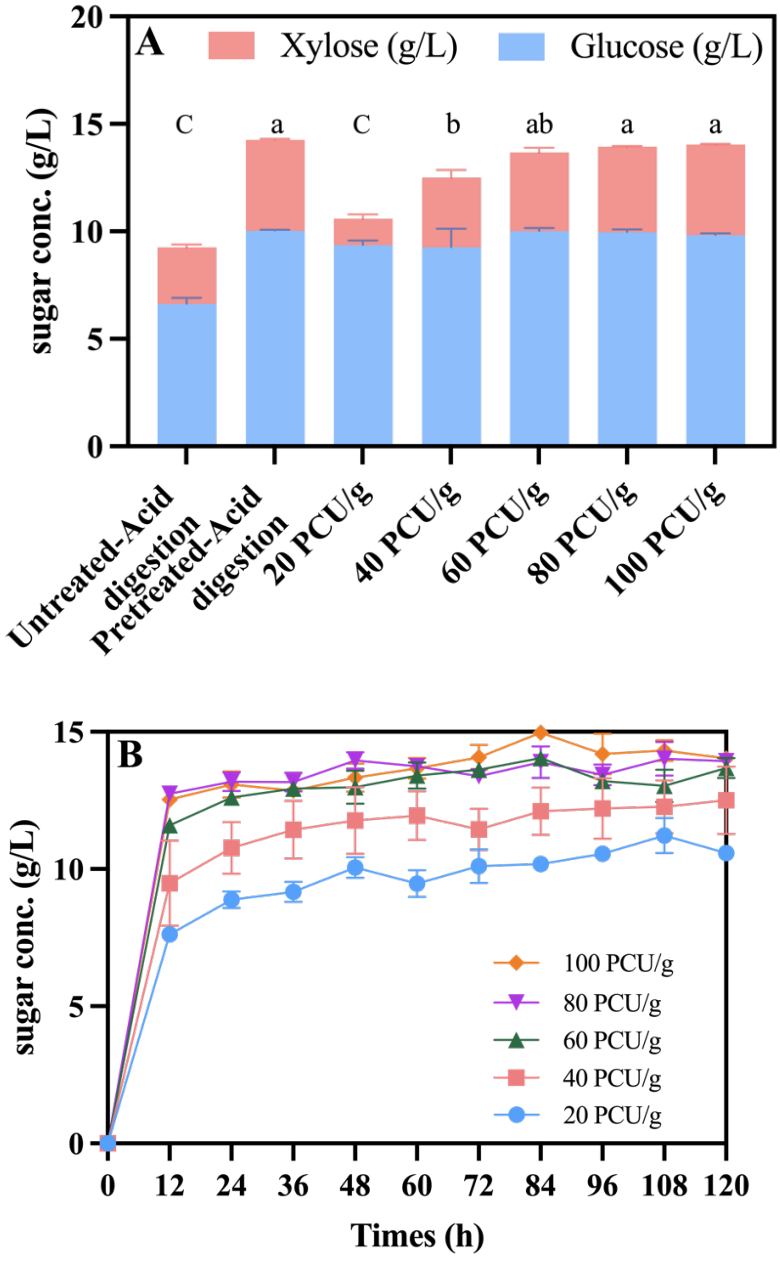

Figure 4.2 **(A)**: Glucose and xylose conversion from 20 g/L pretreated PIC using different crude cellulase loadings (20-100 PCU/g) compared to the acid hydrolysis by 72% H_2SO_4 of untreated and pretreated PIC samples, **(B)** Effect of incubation time on total sugar released from 20 g/L pretreated PIC during hydrolysis with different crude cellulase loadings.

Table 4.1 %CS, %HS, and sugars released from different cellulase concentrations usage during enzymatic saccharification of 20 g/L pretreated-PIC and completed hydrolysis of untreated and pretreated PIC by 72% H_2SO_4

Cellulase cocktail (PCU/g)	%CS	%HS	Glucose (g/L)	Xylose (g/L)	Total sugars (g/L)
20	83.86±2.10 [b]	25.76±4.08 [f]	9.35±0.23 [a]	1.24±0.20 [c]	10.58±0.11 [c]
40	83.01±7.93 [b]	67.83±7.43 [d]	9.25±0.88 [a]	3.25±0.36 [bc]	12.50±1.23 [b]
60	89.77±1.41 [a]	76.81±4.30 [c]	10.0±0.16 [a]	3.68±0.21 [ab]	13.69±0.36 [ab]
80	89.34±1.19 [a]	82.91±0.91 [b]	9.96±0.13 [a]	3.98±0.04 [ab]	13.93±0.17 [a]
100	88.97±0.77 [a]	87.90±0.72 [a]	9.82±0.09 [a]	4.22±0.03 [a]	14.03±0.10 [a]
72% Conc. H_2SO_4					
Untreated-PIC	59.43±2.47 [c]	54.91±2.65 [e]	6.62±0.28 [b]	2.64±0.13 [c]	9.26±0.22 [c]
Pretreated-PIC	89.97±0.42 [a]	87.93±1.23 [a]	10.03±0.05 [a]	4.22±0.66 [a]	14.24±0.07 [a]

Lower-case letters indicate the significant differences between mean values of three replicates ($p \leq 0.05$) in the same column.

4.3 D-(-)-lactic acid fermentation from pretreated PIC under separate hydrolysis and fermentation

The separate hydrolysis and fermentation (SHF) process was employed to evaluate the effect of varying concentrations of NaOH-pretreated PIC (50, 75, 100, 125, and 150 g/L) on D-(-)-lactic acid production by *Klebsiella oxytoca* KIS004-91Tstrain. The pretreated PIC was initially hydrolyzed using a crude cellulase cocktail (60 PCU/g) at 50°C and 400 rpm. After 24 hours of enzymatic saccharification, the temperature was reduced to 37°C, and a pre-culture of *K. oxytoca* KIS004-91T strain was inoculated into the fermenter. At 50 g/L pretreated PIC (corresponding to initial concentrations of 25.06±0.77 g/L glucose and 7.75±0.49 g/L xylose), glucose consumption immediately started within 4 h after inoculation and was completely depleted by 16 h. D-(-)-lactic acid production rapidly increased from 4 to 12 hours and reached a maximum concentration of 25.36±0.36 g/L within 24 hours. This corresponded to a yield of 0.96±0.03 g/g total fermentable sugars consumed (equivalent to 0.51±0.00 g/g pretreated PIC), with a maximum productivity of 2.00±0.04 g/L/h (Figure 4.3A). By the end of fermentation, only trace amounts of by-products were detected, including 2,3-butanediol (0.91 g/L) and acetate (0.42 g/L) (Table 4.2). Despite complete glucose consumption, xylose remained unutilized, suggesting that carbon catabolite repression (CCR) was in effect where glucose is preferentially metabolized over other sugars. Although *K. oxytoca* M5A1, the parent strain of KIS004-91T strain, is capable of metabolizing various sugars (e.g., glucose, xylose, and fructose), Phosriran et al. (2024) demonstrated that CCR remained active in the KIS004-91T strain. In their study, xylose and arabinose were not utilized even after glucose depletion. Genomic analysis revealed spontaneous mutations in genes related to sugar transport and metabolism, including those involved in CCR, glycolysis, and the hexose monophosphate (HMF) pathway. These mutations likely resulted from adaptive evolution to enhance glucose-based D-(-)-lactic acid production under selective pressure from high-glucose (100 g/L) cultivation conditions. Similar result was found when 75 g/L of pretreated PIC was provided. Xylose utilization remained negligible even when fermentation was

extended to 28 hours while glucose consumption was completed at 20 hours incubation (Figure 4.3B). Although D-(-)-lactic acid production increased by 31.3% to a maximum of 33.53±1.53 g/L, the yield (0.76±0.05 g/g sugars consumed) and gross yield (0.43±0.01 g/g pretreated PIC) declined by approximately 20.8% and 15.7%, respectively, compared to the 50 g/L pretreated PIC process. Nonetheless, the maximum productivity of 1.95±0.05 g/L/h was not significantly different (Table 4.2).

For the 100 g/L NaOH-pretreated PIC, D-(-)-lactic acid production was rapid between 4 and 20 hours of fermentation, gradually increasing until 28 hours (Figure 4.3C). This trend was consistent with glucose utilization, which was completely exhausted within 28 hours. As a result, D-(-)-lactic acid reached 45.69±1.16 g/L, with a yield of 0.81±0.02 g/g sugars consumed, a gross yield of 0.46±0.01 g/g pretreated PIC, and a maximum productivity of 1.92±0.35 g/L/h. Only 2,3-butanediol (0.74±0.10 g/L) was detected as a by-product (Table 4.2). Similar to other conditions, after glucose depletion, D-(-)-lactic acid production became negligible, and xylose remained unconsumed, accumulating at 11.65±0.07 g/L by the end of fermentation. This suggests that the cells entered the death phase due to the depletion of glucose, indicating that *K. oxytoca* KIS004-91T strain utilized glucose as its primary energy source, despite the presence of xylose. Although D-(-)-lactic acid production increased by 42.2% compared to the 75 g/L pretreated PIC condition, the yield and maximum productivity were not significantly different. This phenomenon is consistent with the high glycolytic flux, which maintains a high NADH/NAD+ ratio. D-(-)-lactic acid production is prioritized rather than those of 2,3-BDO and acetate productions because it regenerates NAD+ and produces ATP, ensuring cellular redox balance and avoiding overflow metabolism (In et al., 2020; Jain et al., 2012; Wei et al., 2013).

For 125 g/L pretreated PIC, fermentation was extended to 56 hours, and D-(-)-lactic acid production reached 49.25±2.72 g/L, with a yield of 0.69±0.04 g/g sugars consumed, a gross yield of 0.40±0.01 g/g pretreated PIC, and a maximum productivity of 1.63±0.07 g/L/h (Table 4.2). Glucose consumption was also found to be rapid up to 24 hours and was completely exhausted by 48 hours, while xylose remained

throughout the 56-hour fermentation (Figure 4.3D). Compared to the 100 g/L pretreated PIC, D-(-)-lactic acid production increased by 7.8%, but both maximum productivity and conversion yield decreased by 15.1% and 14.8%, respectively. When the initial pretreated PIC concentration was raised to 150 g/L, fermentation was further extended to 76 hours. Glucose was consumed rapidly up to 44 hours and gradually decreased until 76 hours, leaving 2.70±2.08 g/L glucose residue. D-(-)-lactic acid reached 53.26±4.23 g/L, with a yield of 0.64±0.04 g/g sugars consumed, a gross yield of 0.36±0.03 g/g pretreated PIC, and a productivity of 1.21±0.09 g/L/h (Table 4.2). Although D-(-)-lactic acid production increased by 8.1% compared to 125 g/L pretreated PIC, the maximum productivity decreased significantly by 25.8%. No acetate was detected, but 2,3-butanediol production increased to 1.74±0.09 g/L. This shift in by-product formation indicates metabolic overflow, which is likely a result of high glucose concentrations. Excessive pyruvate and NADH may drive the production of 2,3-butanediol to maintain the NAD+/NADH balance, as *K. oxytoca* KIS004-91T strain lacks the pathways for succinate and acetate production (In et al., 2020; Sun et al., 2022b).

Overall, D-(-)-lactic acid production was 2.10 times higher when pretreated PIC concentrations increased from 50 g/L to 150 g/L; however, maximum productivity dropped by 39.5%. This decrease in productivity likely causes from increased broth viscosity, which impairs mixing efficiency and generates osmotic pressure imbalances, thereby reducing substrate-consumption rates and limiting cell growth during fermentation (Lin et al., 2008; Yankov, 2022). Compared to previous studies, Alrumman (2016) reported a maximum of 27.8 g/L D-(-)-lactic acid produced from the date palm hydrolysate (35 g/L initial sugars) by *Lactobacillus delbrueckii* subsp. *lactis*, with a yield of 0.76 g/g glucose and a productivity of 0.39 g/L/h. Bustamante et al. (2020) produced 45 g/L D-(-)-lactic acid from orange peel waste hydrolysate (70 g/L total sugars) using *L. delbrueckii* ssp. *Delbrueckii* CECT 286, achieving a yield of 0.86 g/g and a productivity of 0.63 g/L/h. However, in both studies, glucose was not fully consumed, even after 72 hours of fermentation. In contrast, the results demonstrate that *K. oxytoca* KIS004-91T can efficiently produce D-(-)-lactic acid, reaching 45.7 g/L from 100 g/L pretreated

PIC with significantly higher productivity and comparable yield.

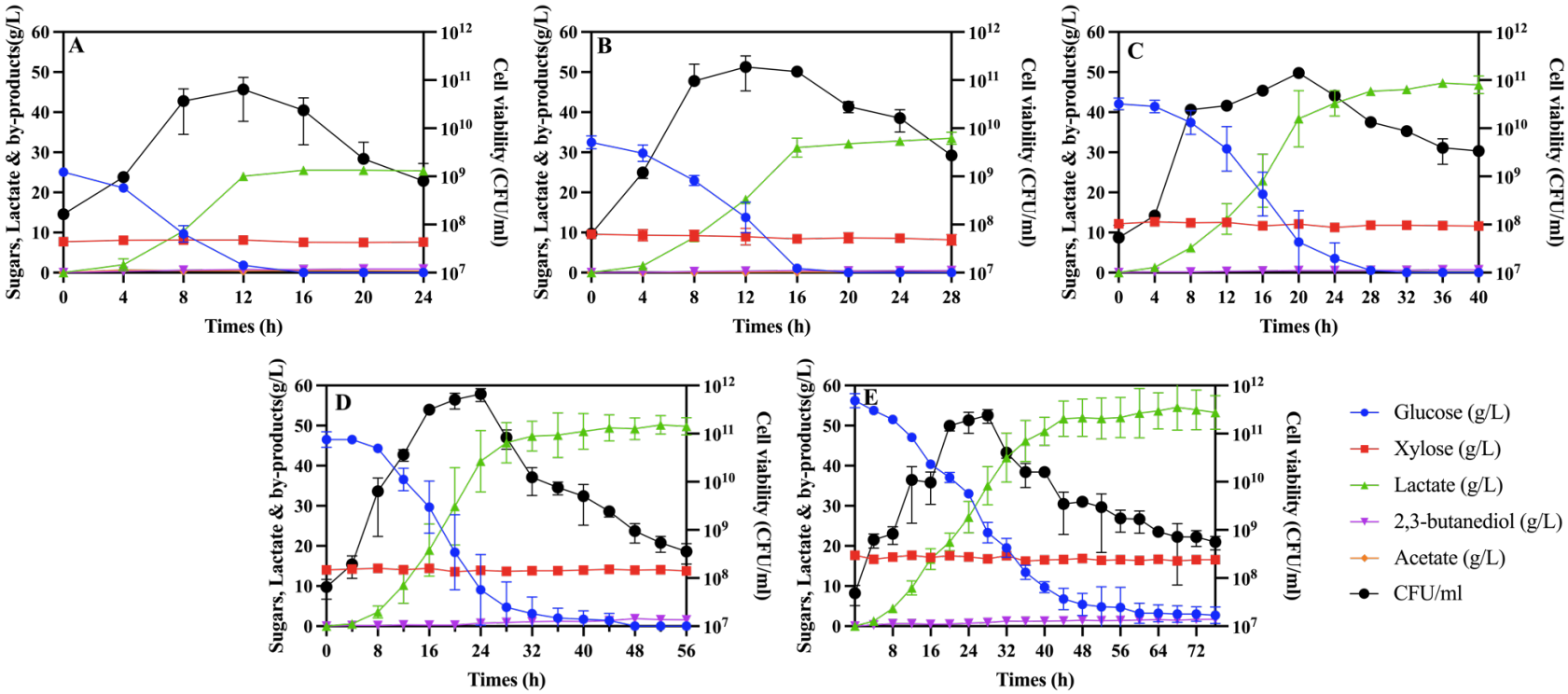

Figure 4.3 D-lactic acid production under SHF with different initial concentration of pretreated PIC. **(A)** 50 g/L, **(B)** 75 g/L, **(C)** 100 g/L, **(D)** 125 g/L, and **(E)** 150 g/L pretreated PIC. Cell viability was determined by spread plate technique.

4.4 D-(-)-lactic acid fermentation from pretreated PIC under simultaneous saccharification and fermentation

Considering production costs, D-(-)-lactic acid production by *K. oxytoca* KIS004-91T strain was evaluated using the simultaneous saccharification and fermentation (SSF) process, aimed at reducing both production time and the impact of glucose inhibition. The process was conducted at 40°C with constant agitation at 200 rpm. The cellulase cocktail and pre-cultured *K. oxytoca* KIS004-91T strain were inoculated simultaneously into the fermentation vessel. The fermentation was maintained at 40°C, and the pH was controlled at 7.0 by automatic addition of 6M KOH. Initial substrate concentrations were varied between 50, 75, and 100 g/L to identify the optimal conditions while minimizing processing time.

As shown in Figure 4.4A, D-(-)-lactic acid was steadily produced from 4 to 16 hours of fermentation, with a slight increase until 36 hours. The final concentration of D-(-)-lactic acid reached 20.73±0.54 g/L, with a yield of 0.67±0.03 g/g sugars consumed (or 0.42±0.01 g/g pretreated PIC) and a productivity of 1.19±0.04 g/L/h (Table 4.2). Glucose was first detected after 4 hours of incubation, rapidly decreasing until 20 hours, while xylose was released and maintained a steady level until 36 hours, reaching 4.53±0.01 g/L. At the end of fermentation, by-products 2,3-BDO and acetate were detected at concentrations of 0.36±0.05 g/L and 0.18±0.04 g/L, respectively. Despite glucose exhaustion, D-(-)-lactic acid production did not significantly increase from 20 to 36 hours, confirming that glucose remained the primary energy source for the strain even in the presence of xylose. For 75 g/L pretreated PIC (Figure 4.4B), both glucose and xylose were detected after 4 hours of incubation. Glucose levels dropped after 8 hours and were completely consumed by 32 hours, while xylose concentration increased rapidly until 60 hours of fermentation. The maximum D-(-)-lactic acid concentration of 34.23±1.01 g/L was reached at 60 hours. By-products, 2,3-BDO (0.57±0.12 g/L) and acetate (0.83±0.25 g/L), were also generated. However, D-(-)-lactic acid production remained stable from 28 to 60 hours, indicating that no further glucose was released after 28 hours. The maximum productivity of 1.07±0.03 g/L/h was

achieved with a yield of 0.70±0.03 g/g sugars consumed and a gross yield of 0.42±0.03 g/g pretreated PIC (Table 4.2). For 100 g/L pretreated PIC (Figure 4.4C), glucose was released at higher levels than in the previous conditions at 4 hours of incubation, but D-(-)-lactic acid production was delayed until 16 hours. D-(-)-lactic acid production sharply increased from 16 to 44 hours, while glucose levels dropped significantly from 24 to 32 hours, indicating simultaneous glucose release and utilization for D-(-)-lactic acid production. After extending fermentation to 60 hours, the maximum D-(-)-lactic acid concentration reached 40.75±3.93 g/L. By-products, acetate (0.68±0.27 g/L) and 2,3-BDO (0.79±0.50 g/L), were also produced. The maximum productivity of D-(-)-lactic acid was 0.87±0.11 g/L/h, with a yield of 0.62±0.08 g/g sugars consumed and a gross yield of 0.38±0.05 g/g pretreated PIC (Table 4.2). In all conditions, the gradual increase of xylose residue (Figure 4.4) indicated that only glucose was utilized for D-(-)-lactic acid production. Feng et al. (2017) suggested that using a mixed sugar source, including xylose or arabinose, leads to decreased D-(-)-lactic acid yield and concentration. Kim et al. (2010) also noted that the carbon catabolite repression (CCR) effect can hinder the co-utilization of sugars, suggesting that glucose released during SSF represses the utilization of other sugars.

In comparison to the SHF process, D-lactic acid concentration in the SSF process was significantly reduced by 18.8%, 2.0%, and 16.4%, while productivity was 40.5%, 45.1%, and 54.7% lower, respectively, for the same initial substrate concentrations. This decrease is attributed to carbon starvation, which results from a lower saccharification rate of cellulose and hemicellulose, causing an imbalance in redox balance and insufficient ATP for cell metabolism and maintenance (Khunnonkwao et al., 2023). The optimal condition for cellulase activity is generally at 50°C and pH 5.0 (Rawoof et al., 2021); however, SSF process was conducted at 40°C and pH 7.0 to support the optimal growth of *K. oxytoca* KIS004-91T strain. This adjustment may have limited the hydrolysis efficiency, thus slowing sugar release and leading to incomplete hydrolysis. The reduction in enzymatic hydrolysis efficiency was reflected in the lower concentration of xylose accumulation at the end of fermentation

in the SSF process (4.53±0.01, 5.53±0.88, and 5.86±2.89 g/L) compared to the SHF process (7.61±0.57, 8.16±1.30, and 11.65±0.07 g/L). Consequently, D-lactic acid production yields were significantly reduced in the SSF process, with decreases of 30.2%, 7.9%, and 23.4% for the initial substrate concentrations of 50, 75, and 100 g/L pretreated PIC, respectively. These results suggest that the increased substrate concentration with simultaneous inoculation of both crude cellulase cocktail and pre-culture in a single step induced high osmotic pressure and shear stress, leading to inefficient mixing due to the high solid-to-liquid ratio (Nguyen et al., 2017; Pratt et al., 2003). Congthai et al. (2025) observed a 33.4%, 66.7%, and 32.6% reduction in succinate production, yield, and productivity, respectively, when acid-pretreated mixed office papers were processed by SSF compared to SHF, due to insufficient sugar availability and inhomogeneous mixing. Even when a 4 h pre-saccharification step was incorporated to reduce shear stress and viscosity, succinate production was still 12.80% lower in SSF compared to SHF, with an initial concentration of 70 g/L of pretreated mixed waste papers, highlighting inefficient hydrolysis. Similarly, the high viscosity of the broth and inefficient saccharification in the biomass slurry contributed to lower SSF process efficiency (Unrean and Khajeeram, 2016). Gosalawit et al. (2024) also reported a decrease of 11.8%, 20.4%, and 12.6% in D-lactic acid concentration, yield, and productivity, respectively, via SSF compared to SHF processes using cassava starch by *Kluveromyces marxianus*. In summary, the results suggest that an initial substrate concentration of 75 g/L pretreated PIC is optimal for the SSF process, as it provided comparable D-lactic acid concentrations and yields to those from SHF, while reducing the total processing time to 32 hours, compared to 44 hours for the SHF process (24 hours for pre-saccharification and 20 hours for fermentation).

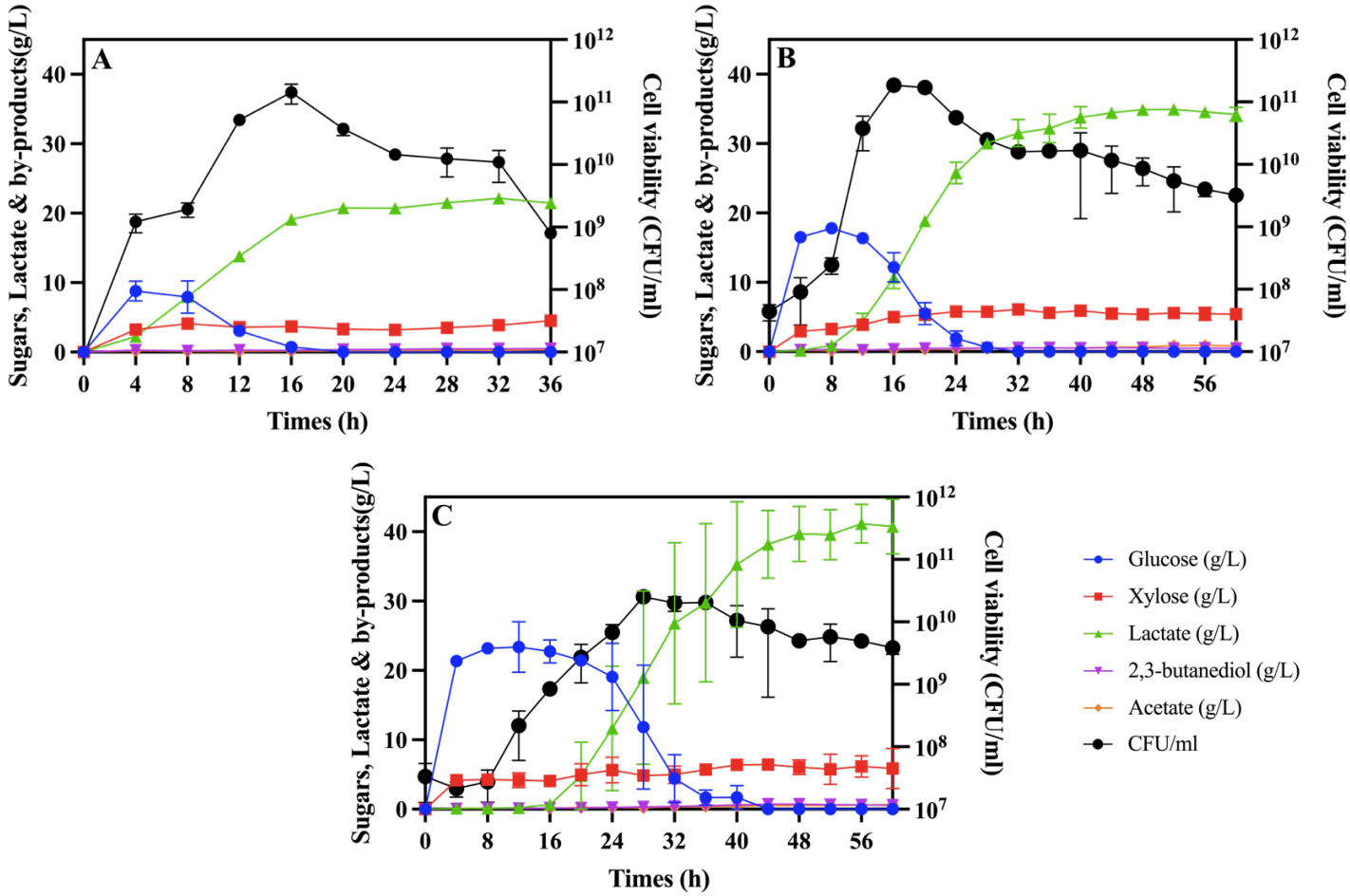

Figure 4.4 D-lactic acid production under SSF with different initial concentration of pretreated PIC. **(A)** 50 g/L, **(B)** 75 g/L, and **(C)** 100 g/L pretreated PIC. Cell viability was determined by spread plate technique.

4.5 D-(-)-lactic acid fermentation from pretreated PIC under Fed-batch SHF

A fed-batch strategy was employed to enhance the concentration, yield, and productivity of D-(-)-lactic acid while mitigating issues related to broth viscosity and substrate inhibition. Initially, 50 g/L NaOH-pretreated PIC was enzymatically hydrolyzed using a cellulase cocktail at 50°C for 24 hours. The resulting hydrolysate was then sterilized at 121°C for 20 minutes and subsequently cooled to 37°C prior to inoculation with the pre-culture strain *K. oxytoca* KIS004-91T strain. During fermentation, the concentrated hydrolysate (120 g/L glucose) was intermittently added to maintain the glucose concentration at approximately 25 g/L, whenever the level dropped to 10 g/L, given glucose served as the principal carbon source for D-(-)-lactic acid production. At the beginning of fermentation, initial sugar concentrations were found at 25.36±0.33 g/L glucose and 8.40±0.32 g/L xylose, totaling 33.75±0.65 g/L. As illustrated in Fig. 4.5, glucose was rapidly consumed, decreasing to 10.90±0.49 g/L within the first 8 hours, allowing the initial feeding to restore glucose levels to 25 g/L. Subsequent feedings were conducted at 12 h and 20 h intervals, contributing a cumulative total sugar addition of 90.28±3.7 g/L. D-(-)-lactic acid production increased sharply between 4 and 20 hours of incubation. However, the production rate gradually declined after 24 hours, reaching a final concentration of 63.14±0.90 g/L at the end of fermentation. Minor by-products were detected, including 2.70±0.69 g/L 2,3-BDO and 0.57±0.01 g/L of acetate. Fig. 4.6 presents an overall mass balance analysis for D-(-)-lactic acid production from NaOH-pretreated PIC using the fed-batch SHF process by *K. oxytoca* KIS004-91T strain. Based on a gross yield of 0.11 g D-(-)-lactic acid per gram of NaOH-pretreated PIC (Table 4.2), approximately 9.1 kg of pretreated PIC is required to produce 1 kg of D-(-)-lactic acid. Given a solid recovery rate of 37.8% following alkaline pretreatment, 24.1 kg of dried PIC is needed to obtain 9.1 kg of NaOH-pretreated PIC. Furthermore, considering that 120 g of dried PIC can be recovered from 1 kg of fresh PIC (equivalent to a yield of 0.12 g/g), an estimated 200.6 kg of fresh PIC is required to produce 1 kg of D-(-)-lactic acid.

Compared to the SSF process, the fed-batch SHF approach demonstrated improved D-(-)-lactic acid production performance, achieving a productivity of 1.31±0.01 g/L/h and a conversion yield of 0.96±0.07 (Table 4.2). When compared to a batch SHF process using 50 g/L of pretreated PIC, the fed-batch mode also achieved a 2.46-fold increase in D-(-)-lactic acid production. These findings are consistent with previous studies. For instance, Vishnu et al. (2020) reported a 3.4-fold enhancement in D-lactic acid production from pretreated rice straw by *Lactobacillus bulgaricus* under fed-batch SSF compared to batch SSF. Similarly, Abdel-Rahman et al. (2021) demonstrated that fed-batch fermentation of sugar beet molasses using *Enterococcus hirae* ds10 resulted in a D-(-)-lactic acid concentration of 61.76 g/L, representing a 1.67-fold increase over batch fermentation. These results collectively support the conclusion that the fed-batch SHF process offers superior performance relative to batch modes of SHF and SSF.

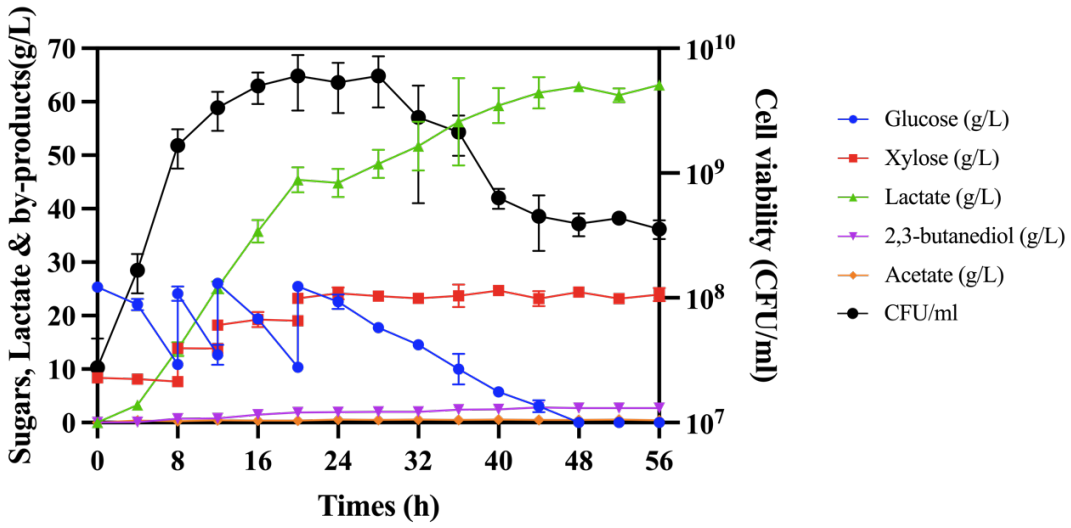

Figure 4.5 D-lactic acid production via fed-batch with initial 50 g/L pretreated PIC. Cell viability was determined by spread plate technique

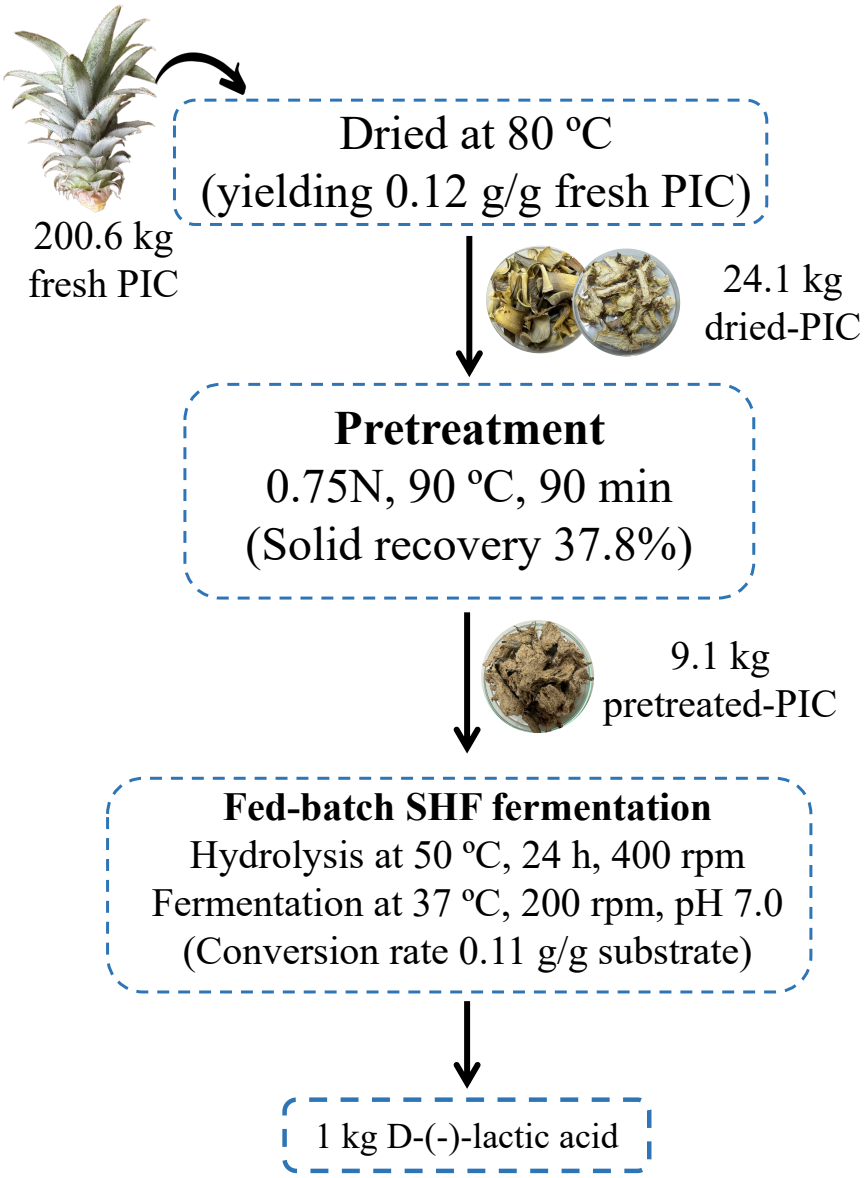

Figure 4.6 Overall mass balance analysis of D-(-)-lactic acid production from fresh pineapple crown using fed-batch SHF.

Table 4.2 The concentration, yield, gross yield, and productivity of D-(-)-lactic acid fermentation from different initial concentration of pretreated PIC via the batch of SHF, SSF and Fed-batch of SHF process.

Substrate Concentration (g/L)	Duration (h)	D-(-)-lactic acid				By-products	
		Concentration (g/L)	Yield (g/g)	Gross Yield (g/g)	Productivity (g/L/h)	2,3-BDO (g/L)	Acetate (g/L)
SHF							
50	16 h	25.53±0.14 ^{ef}	0.96±0.03 ^{a}	0.51±0.00 ^{a}	2.00±0.04 ^{a}	0.79±0.35 ^{ab}	0.41±0.22 ^{a}
75	20 h	32.13±1.04 ^{de}	0.76±0.05 ^{bc}	0.43±0.01 ^{c}	1.95±0.15 ^{a}	0.44±0.16 ^{b}	0.14±0.07 ^{a}
100	28 h	45.69±1.16 ^{bc}	0.81±0.02 ^{ab}	0.46±0.01 ^{b}	1.92±0.35 ^{a}	0.58±0.05 ^{b}	ND
125	48 h	49.25±2.72 ^{b}	0.69±0.04 ^{bc}	0.40±0.01 ^{c}	1.63±0.18 ^{b}	1.49±0.10 ^{ab}	ND
150	76 h	53.26±4.23 ^{ab}	0.64±0.04 ^{bc}	0.36±0.03 ^{cd}	1.21±0.09 ^{c}	1.74±0.09 ^{ab}	ND
SSF							
50	20 h	20.73±0.54 ^{f}	0.67±0.03 ^{bc}	0.42±0.01 ^{c}	1.19±0.04 ^{c}	0.36±0.05 ^{b}	0.18±0.04 ^{a}
75	32 h	31.50±1.97 ^{de}	0.70±0.03 ^{bc}	0.42±0.03 ^{c}	1.07±0.03 ^{d}	0.55±0.06 ^{b}	0.53±0.17 ^{a}
100	44 h	38.19±4.90 ^{ce}	0.62±0.08 ^{c}	0.38±0.05 ^{d}	0.87±0.11 ^{e}	0.74±0.52 ^{ab}	0.51±0.35 ^{a}
Fed batch-SHF							
50	48 h	62.87±0.42 ^{a}	0.96±0.07 ^{a}	0.11±0.00 ^{e}	1.31±0.01 ^{bc}	2.70±0.58 ^{a}	0.52±0.01 ^{a}

- Lower-case letters indicate the significant differences between mean values of three replicates ($p \leq 0.05$) in the same column.
- D-(-)-lactic acid concentration, yield, and gross yield were calculated when glucose was exhausted at the time indicated.
- Productivity was calculated when D-(-)-lactic acid was produced at the maximum rate.
- Yield was calculated based on sugar utilized and gross yield was calculated based on pretreated PIC provided.

Table 4.3 Lactic acid production from different types of substrates and microorganism.

Substrate	Microorganisms	Optical isomer	Fermentation mode	Lactic acid production			References
				Titer (g/g)	Yield (g/g)	Productivity (g/L/h)	
Pineapple crown	*K. oxytoca* KIS004-91T	D-(-)-LA	Fed-SHF	62.9	0.96	1.31	This study
			SHF	45.7	0.81	1.43	
			SSF	31.5	0.70	0.98	
Cassava starch	*K. oxytoca* KIS004-91T	D-(-)-LA	SHF	98.4	0.93	1.43	(In et al., 2020)
Cassava bagasse	*E. coli* JU15	D-(-)-LA	SHF	57.8	1.11	0.98	(Utrilla et al., 2016)
Paper mill sludge	*B. coagulans*	L-(+)-LA	SSCF	82.6	0.83	0.69	(Li et al., 2021)
Wheat straw	*B. coagulans* CC17A	L-(+)-LA	SSCF	26.30	0.71	0.25	(Ouyang et al., 2020)
Spent coffee grounds	*L. rhamnosus* ATCC 10863	N/D	SHF	24.95	0.91	0.54	(Koo et al., 2019)

Table 4.3 Lactic acid production from different types of substrates and microorganism. (Continued)

Cassava bagasse	*L. rhamnosus*	L-(+)-LA	SHF	41.6	0.83	0.87	(Coelho et al., 2010)
Orange peel waste	*L. delbrueckii* ssp. *bulgaricus* CECT 5037	D-(-)-LA	SHF	39	0.84	0.55	(Bustamante et al., 2020)
	L. delbrueckii ssp. *bulgaricus* CECT 286	D-(-)-LA	SHF	45	0.86	0.63	
Broken rice	*L. delbrueckii*	D-(-)-LA	SSF	79	0.81	3.58	(Abdel-Rahman et al., 2013)
Corncob	*R. oryzae* NLX-M-1	L-(+)-LA	SHF	34	0.34	0.71	(Zhang et al., 2016b)

* SHF: Separate hydrolysis and fermentation, SSF: Simultaneous saccharification and fermentation, SSCF: Simultaneous saccharification and co-fermentation.