CHAPTER III
MATERIALS AND METHODS

3.1 Strains, media, and growth condition

K. oxytoca KIS004-91T strain was previously engineered to enhance D-(-)-lactic acid production by deleting genes responsible for fumarate reductase (*frdABCD*) and pyruvate formate lyase (*pflB*) activities (In et al., 2020). Cultures were maintained on LB agar composed of 10 g tryptone, 5 g yeast extract, 20 g agar, and 5 g sodium chloride per 1 L distilled water. Seed cultures were prepared by inoculating a full loop of fresh colonies into LB broth supplemented with 2% (w/w) glucose and incubating overnight at 37°C with shaking at 200 rpm until reaching an optical density of approximately $OD_{550} \approx 3.0$. Throughout the fermentation experiments, a low-salts medium known as, Alfredo Martinez medium version 1 (AM1) (Martinez et al., 2007), was used. The AM1 components are shown in Table 3.1.

Table 3.1 Components of AM1 medium and trace metal

AM1	Concentration (mmol/L)
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.93
Total K	1.00
$MgSO_4$. $7H_2O$	1.50
Betaine-HCl	1.00
Trace metal	**Concentration (μmol/L)**
$FeCl_3$.$6H_2O$	8.88
$CoCl_2$.$6H_2O$	1.26
$CuCl_2$.$2H_2O$	0.88
$ZnCl_2$	2.20
Na_2MoO_4. $2H_2O$	1.24
H_3BO_3	1.21
$MnCl_2$. $4H_2O$	2.25
Total salts	**4.1 g/L**

KOH will be used to neutralize betaine-HCl stock

Trace metal stock (1000x) was prepared in 120 mM HCl

3.2 Alkaline pretreatment of pineapple crown

Pineapple crown (PIC) was obtained from Chin Huay Public Company Limited, Thailand. It was cut into small pieces (3–5 cm) and dried overnight in an air oven at 80°C. The dried PIC was then soaked in sodium hydroxide (NaOH) solutions of varying concentrations (0.25 N, 0.5 N, 0.75 N, 1 N, and 1.25 N) at 90°C for 90 minutes, using a solid-to-liquid ratio of 1:7 with periodically mixed every 30-minutes interval. Following the alkali treatment, the liquid fraction was discarded, and the solid fraction was thoroughly washed with tap water until the pH of the rinse water reached 7. The washed, pretreated PIC was subsequently dried in an air oven at 65°C until a constant moisture content of less than 10% (w/w) was achieved. The dried, pretreated PIC was then stored in an airtight bag at ambient temperature until further use.

3.3 Enzymatic hydrolysis of pretreated pineapple crown

The dried pretreated-PIC was milled into particle size less than 40 µm. Therefore, the milled and pretreated PIC at the concentration of 2% (w/w, on dried basis) in AM1 medium was enzymatically hydrolyzed by a sterilized VRE P3 crude cellulase enzyme purchased from Siam Victory Chemicals Co., Ltd (Thailand) with a working volume of 50 ml at 50°C with the agitation speed of 200 rpm for 120 hours at different enzyme loadings (20, 40, 60, 80, and 100 PCU/g). Samples were collected every 12 hours for analyzing released fermentable sugars.

3.4 Total sugar hydrolysis using concentrated acid

The cellulose, hemicellulose, lignin, and ash contents of untreated and pretreated pineapple crown (PIC) were determined according to the method of Sluiter (2012). For total reducing sugar analysis, 4 g of substrate were hydrolyzed with 7 mL of 72% (w/w) sulfuric acid (H_2SO_4) at 55°C for 10 minutes with continuous agitation at 200 rpm. The acid mixture was subsequently diluted with deionized water to a final volume of 200 mL. The resulting solution was autoclaved at 121°C for 35 minutes to complete hydrolysis. After cooling to room temperature, samples were collected for the quantification of total sugars.

3.5 Lactic acid production from pretreated pineapple in mineral salts medium

3.5.1 Lactic acid production using separate hydrolysis and fermentation (SHF) technique

D-(-)-lactic acid fermentation was carried out in a 5 L bioreactor (Infors, Basel, Switzerland) with a working volume of 2 L. Batch separate hydrolysis and fermentation

(SHF) was performed by dissolving pretreated PIC in AM1 medium, followed by sterilization at 121°C for 20 minutes. Hydrolysis was initiated by adding crude cellulase at an enzyme loading of 60 PCU/g (equivalent to 3.75 mL/g substrate) and incubating the mixture at 50°C with agitation at 400 rpm for 24 h. After hydrolysis, the slurry was cooled to 37°C, and inoculation was performed at an initial optical density (OD_{550}) of 0.3 at pH 7.0. Fermentation was carried out at 37°C with agitation at 200 rpm, and pH was maintained at 7.0 by the automatic addition of 6 M KOH. The effect of varying concentrations of pretreated PIC (50, 70, 100, 125, and 150 g/L) on D-(-)-lactic acid production was also evaluated. All experiments were in triplicate.

3.5.2 Lactic acid production using simultaneous saccharification and fermentation (SSF) technique

D-(-)-lactic acid production was assessed under batch simultaneous saccharification and fermentation (SSF) conditions using pretreated PIC concentrations of 50, 75, and 100 g/L. In SSF, crude cellulase and inoculum were simultaneously added to the sterile PIC mixture, and fermentation was conducted at 40°C under the same conditions as SHF. All experiments were in triplicate.

3.5.3 Lactic acid production by fed-batch fermentation

Fed-batch fermentation under SHF conditions was performed to improve D-(-)-lactic acid concentration, yield, and productivity. An initial concentration of 50 g/L of pretreated PIC was used. During fermentation, a concentrated hydrolysate was fed into the bioreactor when the residual glucose concentration in the fermentation broth dropped to 10 g/L, raising the glucose concentration to approximately 25 g/L after feeding. All experiments were conducted in triplicate.

3.6 Analytical methods

3.6.1 Concentration of organic acids, sugars and by-products

Fermentation broth samples were collected every 4 hours to measure cell biomass, organic acid concentrations, and sugar levels. The samples were analyzed using high-performance liquid chromatography (HPLC) with an ion exchange column (Aminex® HPX-87H, 7.8×300 mm, BioRad) and a refractive index detector (RI-1206, Thermo Spectra System, USA) at 65°C. A 4 mM sulfuric acid solution was used as the mobile phase at a flow rate of 0.6 mL/min. After fermentation, cultures were centrifuged to separate the cells from the supernatant, which was then filtered through a 0.2 μm nylon filter prior to HPLC analysis.

3.6.2 Cell biomass and cell viability

Cell dry weight (CDW) or biomass was determined by measuring the optical density at 550 nm (OD_{550}) using a Bausch & Lomb Spectronic 70 spectrophotometer. The relationship between OD_{550} and biomass was 1 OD_{550} = 0.333 g CDW/L (In et al., 2020). During fermentation, the viable cell count was determined by the spread plate technique on LB agar plates, which were incubated at 37°C for 24 hours. Colonies were counted and expressed as colony-forming units per milliliter (CFU/mL) of culture.

3.6.3 Calculations and statistical analysis

Statistical analysis was performed using GraphPad Prism 10.3 (GraphPad Software, Boston, MA, USA). Data were analyzed by one-way analysis of variance (ANOVA), and all values presented in the figures represent the averages ± standard deviations. Tukey's multiple range test at a 95% confidence level ($p < 0.05$) was used to identify significant differences among the mean values. The percentages of cellulose (%CS) and hemicellulose (%HS) saccharifications of the NaOH-pretreated PIC after the enzymatic hydrolysis were calculated according to equations below, where $C_{cellulose}$ and $C_{hemicellulose}$ are concentrations of cellulose and hemicellulose derived from the NaOH-pretreated PIC, and C_{glu} and $C_{xyl+ara}$ are concentrations of glucose and combined xylose and arabinose released from the NaOH-pretreated PIC during its hydrolysis.

$$\%CS = \frac{0.90 \times C_{glu}}{C_{cellulose}} \times 100 \quad \text{(Eq. 2)}$$

$$\%HS = \frac{0.88 \times C_{xyl+ara}}{C_{hemicellulose}} \times 100 \quad \text{(Eq. 3)}$$

D-(-)-lactic acid production yield (D-LA yield), D-(-)-lactic acid production gross yield (D-LA gross yield), and D-(-)-lactic acid productivity (D-LA productivity) were calculated using equations below, where C_{LA}, $C_{max.\ sugars\ released}$, $C_{sugar\ residue}$, and $C_{substrate}$ were concentrations of D-(-)-lactic acid produced, total sugars released from the NaOH-pretreated PIC digested with 60 PCU/g crude cellulase, sugars residue remained after fermentation, and the NaOH-pretreated PIC provided during fermentation, respectively.

$$\text{D-LA yield} = \frac{C_{LA}}{C_{max.\ sugar\ released} - C_{sugar\ residue}} \qquad \text{(Eq. 4)}$$

$$\text{D-LA gross yield} = \frac{C_{LA}}{C_{Substrate}} \qquad \text{(Eq. 5)}$$

$$\text{D-LA productivity} = \frac{C_{LA}}{\text{Fermentation duration}} \qquad \text{(Eq. 6)}$$