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Fermentation of Apple Pomace Using Mixed Microbial Culture to Organic Acids

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Abstract

The potential of apple pomace (AP) as carbon source for microbial production of biochemicals, via the carboxylate platform, was investigated. The physical and chemical properties of AP were characterized before fermentation by FTIR spectroscopy, TGA, XRD, and compositional (lipids, carbohydrate, lignin and ash) analyses. AP was fermented using a mixed microbial culture (MMC) from wastewater treatment activated sludge. Organic loading (35, 55 and 75 g L^{-1}), temperature (27, 35 and 40°C), gelatinization of feedstock, and a fermentation time of 13 days were examined. The maximum yield of lactic acid was 0.81 g L^{-1} after 6 days at 35°C and 75 g L^{-1} feed concentration. However, no significant effect of gelatinization of AP was found to improve fermentation yields. AP fermentation also generated methanol (9.57 g L^{-1}), citric acid (16.8 g L^{-1}), and oxalic acid (24.8 g L^{-1}) with minor amounts of ethanol (0.54 g L^{-1}) and acetic acid (3.42 g L^{-1}). The results of characterization of physical properties, chemical composition and fermentation characteristics of AP waste as source material indicated that AP can be used as potential fermentation substrate using MMC for the synthesis of biochemical products.

Keywords: *Apple Pomace; Alcohols; Fermentation; Physicochemical Properties; Organic Acids; Mixed Microbial Culture*

Abbreviation

E_α: Activation Energy; AP: Apple Pomace; ASTM: American Standard Test Method; CrI%: Cellulose Crystallinity Index; DTG: Differential Thermogravimetric Analysis; ESI-MS: Electrospray Ionization-Mass Spectrometry; FAME: Fatty Acid Methyl Esters; FC: Fixed Carbon; FTIR: Fourier Transform Infrared; FWO: Flynn-Wall-Ozawa's; GCMS: Gas Chromatography-Mass Spectrometry; HPLC: High Performance Liquid Chromatography; MMC: Mixed Microbial Culture; NIST: National Institute of Standard and Technology; TCI: Total Crystallinity Index; TGA: Thermogravimetric Analysis; TMS: Trimethylsilyl; VM: Volatile Matter; XRD: X-Ray Diffraction

Introduction

In the United States 40% of food is thrown away as waste, ends up in landfills, and accounts for a large portion of greenhouse gas emissions [1]. Furthermore, food processors also generate solid wastes (20% for fruit and vegetables) [2]. For example, 5.2 x 10^6 tons (2.45 x 10^8 bushels) of apples were produced in the United States in 2018 and about 33% of the crop is processed into juices and sauces [3]. Conversion of apples into juice or cider generates AP, a solid residue (1/3rd of apple weight), which consists of peel, fibers, seeds and core [4]. Currently, AP is a low value resource and is used as either as animal feed or fertilizer [5]. Therefore, AP is a good candidate feedstock for producing value added products.

AP is composed primarily of carbohydrate (56.1%), protein (4.6%), lignin (16.8%) and ash (2.1%) on a dry basis [6]. This carbohydrate rich material is suitable for fermentation to organic acids and ethanol via the carboxylate platform [7]. Organic acids such as lactic acid, acetic, oxalic acids are of value and are used in many applications in the food, pharmaceutical, cosmetic, and chemical industries [8]. Fermentable sugars in AP such as glucose, fructose and sucrose can be converted to lactic acid and ethanol using bacteria [9]. In industrial fermentation processes, specific microbial cultures are commonly used to produce specific desired products. Although there are many positive sides of this pathway, there were also some technical and economic challenges such as it requires sterile conditions and expensive pure substrates. In MMC fermentation, the type and yield of products depend on the type of forming organisms and environmental conditions [10]. A number of studies on AP fermentation have been employed using single strain organisms [9]. However, the utilization of MMC have recently attracted considerable attention as potential culture media using plant substrates such as potato peel, rice, wheat, corn, soybeans, and peanuts in field of biotechnology in food industry [11]. This is mainly due to their ability to utilize various carbon sources (carbohydrates, proteins, and fats) as substrates for fermentation, low operating costs of purification and sterilization of culture media [11,12].

Objective of the Study

The objective of the current study was to evaluate AP fermentation with MMC to produce organic acids. AP was also characterized for its physical and thermal properties and chemical composition. Fermentation products were characterized by HPLC and GCMS. Fermentation conditions such as, AP solid loading, temperature and AP gelatinization, on product yield were also investigated.

Materials and Methods

Sample preparation and extraction

AP was provided frozen from Tree Top (Selah, WA) which was comprised of several desert apple varieties in June 2018. AP was used as received for fermentation studies. For characterization studies, the AP was freeze dried and ball milled (IKA ULTRA-TURRAX Tube Drive).

Characterization of AP

The moisture content of the AP samples was determined, in duplicate, using a moisture analyzer (HB 43-S, Mettler Toledo). C and N contents were determined, in duplicate, on a Costech ESC 4010 elemental analyzer. The calorific value was determined by oxygen bomb calorimetry (Model 1341, Parr Instruments) according to ASTM D5865-04. The proximate analysis (ash, volatile matter (VM), and fixed carbon (FC)) on was performed on AP according to ASTM E870-82. Ash content was determined after furnacing at 600°C for >16 h. VM and FC were determined after combustion at 950°C in a muffle furnace for 7 minutes. FTIR spectra were recorded on a ThermoNicolet iS5 spectrometer with an attenuated total reflection accessory (ZnSe, iD5).

XRD analysis of AP was performed on a Siemens D500 powder diffractometer with Cu/kα radiation (λ = 1.54 Å). The diffraction intensities were recorded from 2θ (10°- 80°) with 0.01° steps. The crystallinity indices (CrI) of the samples were estimated from the peak 002 (I_{002}) and the peaks 101 and 10^{-1} (I_{am}) using Origin pro 8.5 software with a Gaussian-in fitting function to obtain amorphous and crystalline diffraction peaks [13,14].

Crystallinity index (CrI%) = $((I_{002})) / ((I_{002} + I_{am})) \times 100$ (2)

Where I_{002} represents the area crystalline material, while I_{am} represents the area amorphous region.

In order to determine the E_α and study thermal degradation behavior of AP, TGA and DTG) were performed (5 mg dried sample on a Perkin Elmer TGA-7 instrument from 30 to 700°C at heating rates (β) of 15, 20, 25, 35, and 45°C min^{-1} under N_2 (30 mL min^{-1}). TGA and

DTG data were analyzed using Pyris v8 software. The E_α and pre-exponential factor (A) were obtained through TGA using the following equations by the Flynn-Wall-Ozawa's (FWO) method [15,16].

$$\log \beta = \log (AE/g(\alpha)R) - 2.315 - 0.457 (E_\alpha/RT) \quad (3)$$

Where g(α) is constant at a given value of conversion and R is the gas consent.

The AP (4g) was Soxhlet extracted with CH_2Cl_2 (150 mL) for at least 16h, in duplicate, to obtain lipid (extractives) content gravimetrically according to ASTM D 1108-96. The fatty acids were converted to their fatty acid methyl esters (FAME) derivatives and analyzed by GCMS. Extract (5.0 mg, in duplicate) was weighed into 5 mL reacti-vial to which $CH_3OH/H_2SO_4/CHCl_3$ (1.7:0.3:2.0 v/v/v, 2 mL) was added and heated at 90°C for 90 minutes. $CHCl_3$ contained 1-naphthaleneacetic acid as an internal standard (50 µg mL^{-1}). Water (1 mL) was added to the mixture, the $CHCl_3$ layer removed and analyzed by GCMS (FOCUS-ISQ, ThermoScientific; temperature profile- 40°C (1 minute) to 320°C at 5°C min^{-1}; ZB5 capillary column (30m x 0.25 mm Ø, Phenomenex). The eluted compounds were identified with authentic standards (C_{12} to C_{20} fatty acids) and by spectral matching with the 2017 NIST mass spectral library.

Total carbohydrate content (TCC) was determined by the modified phenol-sulfuric acid colorimetric method for cellulosic material [17]. Briefly, extractive free AP (10 mg) was hydrolyzed with 77% H_2SO_4 for 10 min, then mixed with phenol (5%, 1 mL) and then added concentrated H_2SO_4 (5 mL) and mixed. The absorbance at 490 nm was measured using the Bio-mate 5 spectrophotometer (ThermoElectron. Corp). Total starch content was determined using the amyloglucosidase/α-amylase method according to Association of Official Agricultural Chemists - Method 996.11 using a commercial assay kit from Megazyme International (Ireland). Total lignin content (Klason lignin + acid insoluble lignin) were determined from extractives free AP according to ASTM D 1106 [16]. Extractives free AP (200 mg, in duplicate) was incubated in 72% H_2SO_4 at 30°C for 1h, diluted to 4% H_2SO_4 then subjected to a secondary hydrolysis (121°C and 117 kPa) for 30 minutes. Klason lignin was determined gravimetrically. Whereas, acid soluble lignin was quantitated spectrophotometrically at 205 nm using an absorption coefficient of 110 L g^{-1} cm^{-1} (Biomate 5 spectrometer, Thermo Electron Corp). The hydrolysate was analyzed for neutral sugars by HPLC using two Rezex RPM columns in series (7.8 mm x 30 cm, Phenomenex, Torrance, CA) and a Waters HPLC equipped with differential refractive index detector (Waters 2414), on elution with water (0.5 mL min^{-1}) at 85°C [15].

Fermentation of AP

Fermentation experiments were carried out in 250 mL flasks equipped with fermentation locks in a temperature controlled (27, 35 and 40°C) environmental chamber (Lab-Line 846 Biotronette Mark III) for 13 d. The flasks contained 150 mL of AP at feed solids content (SC) levels of 35, 55 and 75 g L^{-1}, inoculated with 4.5% (v/v) mixed microbial cultures (MMC) from activated sludge (from a wastewater treatment system in Dr. Erik Coats laboratory, University of Idaho), and shaken (150 rpm). The AP was either used as is or gelatinized at 100°C for 30 minutes prior to fermentation at 35°C. All fermentation experiments were carried out in triplicate. The pH and dissolved oxygen content in the fermenters were measured using an Orion 3 Star pH/RDO meter (ThermoScientific).

The filtered (0.45 µm, nylon) fermentation products were analyzed, in duplicate, by HPLC using a Rezex ROA organic acid column (7.8 mm × 300 mm, Phenomenex) at 65°C on elution with 0.01M H_2SO_4 at 0.5 mL min^{-1} and detected by refractive index (Shodex SE-61). Quantitation of the fermentation products were determined using an external calibration method with authentic standards. The products were analyzed by positive and negative ESI-MS (ThermoQuest LCQ-deca instrument) from their $[M+H]^+$ and $[M-H]^-$ ions, respectively [2]. An aliquot portion (10 mL) of the centrifuged AP fermentation broth (35°C at 75 g L^{-1} solids content) supernatant was freeze dried. The freeze dried sample (1 mg) was dispersed in pyridine (50 µL) and then silylated with addition of N,O-bis(trimethylsilyl)-trifluoro-acetamide containing 1% trimethylchlorosilane (50 µL) after heating at 70 °C for 30 min. The prepared TMS derivatives were analyzed by GCMS (ISQ7000/Trace1300 instrument, ThermoScientific) using a temperature profile of 40°C (1 minute) to 305°C (10 minutes) at 5°C

min^{-1} and ZB1ms capillary column (30 x 0.25 mm Φ, Phenomenex). The eluted compounds were identified by spectral matching with the 2017 NIST spectral library and known standards.

Results and Discussion

Characterization of AP

The chemical composition and properties of dry AP were determined and given in table 1. It can be seen that the moisture content of AP was 5.38 ± 0.05% and comparable to that reported by Guerrero [18]. AP had an initial pH of 5.24 ± 0.4. The ash content was 1.52 ± 0.02% and is in agreement with the literature [18,19]. An FC of 8.03 ± 1.21% and VM of 85.1 ± 1.4% were obtained and are close to those previously reported at 6.4% and 81.3%, respectively [20]. Elemental composition analysis showed high C (53.8%) and H (7.33%) contents with a small amount of N (0.70%) and were similar to literature values [6]. A calorific value of 22.7 MJ kg^{-1} was obtained and was higher than that obtained by Verma., *et al*. (20.3 MJ kg^{-1}) [21]. Protein content of 4.37% was calculated from N content and shown to be 15% lower than that reported in the literature [20]. AP contained a small amount of starch (1.10 g/100g), and this result was consistent with previous findings (2.11 g/100g) [22]. The total carbohydrate content of the dried AP was 35.5 ± 0.8% and comparable to reported values [23,24]. Hydrolysis of AP afforded the following neutral sugars: glucose, xylose, galactose, arabinose and mannose giving at total neutral sugar content of 21 ± 1% (Table 1). This value is in agreement with some previously reported data [6] however, slightly lower to other reports [19].

Parameters/unit	Dry APP
Proximal analysis Moisture (%)	5.38 ± 0.05
Volatile matter (VM) (%)	85.1 ± 1.4
Fixed carbon (FC) (%)	8.0 ± 1.2
Calorific value (MJ kg^{-1})	22.7 ± 0.3
CH_2Cl_2 extractives (%)	9.52 ± 1.10
Total carbohydrate	35.46 ± 0.78
Starch (g/100g)	1.10
Total (Klason + acid soluble) lignin (%)	19.5 ± 0.7
Ash (%)	1.52 ± 0.02
Elemental analysis C (%)	53.75 ± 0.28
H (%)	7.33 ± 0.05
N (%)	0.70 ± 0.02
Protein (N tot 6.25) (%)	4.37 ± 0.12
Neutral sugar content (NSC) (%)	
Glucose (%)	10.5 ± 0.2
Xylose (%)	1.9 ± 0.1
Galactose (%)	1.3 ± 0.1
Arabinose (%)	3.1 ± 0.1
Mannose (%)	4.3 ± 0.5
Total Neutral sugar (%)	21.0 ± 1

Table 1*: Physico-chemical characterization of apple pomace.*

AP had a total (Klason + acid soluble) lignin content of 19.5% and was slightly higher that obtained by Nikolic., *et al*. [23] and Kosmala., *et a*l. [25] at 16.1 - 18.5%, while Guerrero., *et al*. [20] and Dhillon., *et al*. [24] reported higher lignin values of 22.4% and 23.5%, respectively. The lipid content was 9.52% and was of slightly higher value than reported by Nikolic., *et al*. between 4 - 7% [23]. The fatty acid composition of the extract was determined as their FAME derivatives (Table 2). The fatty acids in AP ranged between C_{10} and C_{24} and were mainly saturated. The main fatty acids were palmitic, oleic, tricosanoic and tetracosanoic acids. Ke., *et al*. [26] found fatty acids ranging from C_4 to C_{24} with the main fatty acids being linolenic acid (44%), oleic acid (26%) and palmitic acid (15%). While, Waila., *et al*. found mainly palmitic (7.3%), linoleic (44%), and oleic (47%) acids in oils from AP with minor amounts of stearic and arachidonic acids [27]. Generally, it was noted that the composition of AP in this work was different to those values of other studies and this could be attributable to different apple cultivars, ripeness, juice/cider extraction technology and various enzymes in juice production [9,18,23].

Peak #	Compound	RT (min)	M^+ (m/z)	AP (µg/mg extract)
1	Decanoic acid (C10:0)	21.98	186	34.8 ± 1.7
2	Lauric acid (C12:0)	24.14	214	15.3 ± 0.7
3	Tridecanoic acid (C13:0)	25.88	228	181 ± 9
4	Myristic acid (C14:0)	28.69	242	12.5 ± 0.6
5	Palmitic acid (C16:0)	32.78	270	440 ± 22
6	Heptadecanoic acid (C17:0)	33.58	284	190 ± 9
7	Linolenic acid (C18:2)	36.08	294	35.5 ± 1.7
8	Oleic acid (C18:1)	36.56	296	230 ± 11
9	Stearic acid (C18:0)	37.66	298	45.2 ± 2.2
10	Eicosanoic acid (C20:0)	40.01	326	153 ± 8
11	Docosanoic acid (C22:0)	43.21	354	139 ± 7
12	Tricosanoic acid (C23:0)	47.18	368	427 ± 31
13	Tetracosanic acid (C24:0)	49.45	382	685 ± 58

Table 2: *Fatty acid analysis of apple pomace CH_2Cl_2 extract as FAME derivatives.*

FTIR spectroscopy was employed to examine chemical functional groups present in the AP (Figure 1). The major peak assignments in AP are summarized in table 3. A strong and broad adsorption band at 3352 cm^{-1} was assigned to O-H stretching [28-30]. The broad bands at 2925 and 2856 cm^{-1} were assigned to C-H symmetric and asymmetric stretching of $-CH_3$ and $-CH_2$ groups, which is attributed to lipids, lignin and polysaccharides [28,31]. The absorption band at 1738 cm^{-1} and shoulder at 1710 cm^{-1} were assigned to C=O stretching of an ester and carboxylic acid, respectively [32]. The bands at 1635 and 1433 cm^{-1} were assigned to COO- (carboxylate) asymmetric and symmetric stretching in pectin, respectively [32,33]. The small band at 1520 cm^{-1} was assigned to lignin [34]. Cellulose was identified in AP by the characteristic bands at 892 (C_1-O-C_4), 1030 (C-O stretching), 1161 (C-O-C vibration), 1375 (C-H bending) and 1433 cm^{-1} (C-H

wagging) [25,29,34]. Cellulose crystallinity in AP was estimated using the total crystallinity index (TCI) method [35] from the intensity ratios at 1376 cm^{-1} and 2900 cm^{-1}. The TCI value for AP was 0.22 and were comparable to those obtained for banana (0.20) and plantain (0.18) peels [36].

Wavenumber (cm^{-1})	Band assignment
3352	O–H stretching vibration
2925	C–H (CH_3, CH_2) stretching vibration
2856	C-H symmetric stretching
1738	–C=O stretching vibrations in the ester groups
1635	–C=O stretching of carbonyl group
1433	Carboxylate group stretching
1520	Semicircle ring stretching – aromatic lignin
1418	-CH_2 cellulose wagging
1375	C–H bending
1161	C–O–C asymmetrical stretching cellulose
1030	C-O stretch cellulose
892	β-(1-4) Glycosidic linkage in cellulose

Table 3: *FTIR spectral band assignments and absorption regions of AP [28-34].*

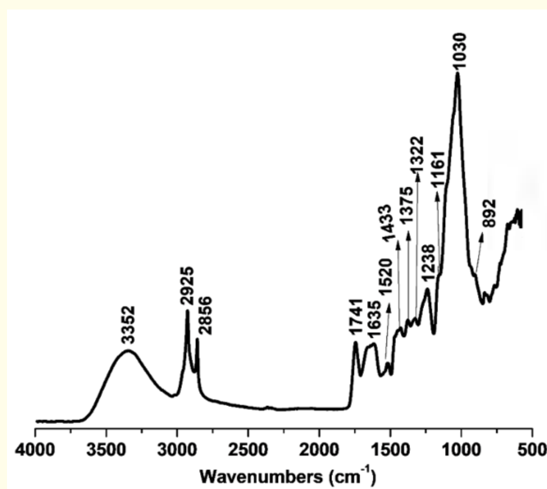

Figure 1: *FTIR spectrum of apple pumice.*

XRD analysis was used to confirm the presence of crystalline cellulose in AP (Figure 2). The AP sample showed peaks located at 2θ values of 12.3°, 15.4° and 21.5, and 12.5°, 15.3° and 21.2 indexed at (110), (1$\bar{1}$0), (002) and (004), respectively for lattice planes of cellulose [37]. Cellulose crystallinity index (CrI%) was determined at 43%. This value was lower than that reported by Szymanska-Chargot., *et al*. for cellulose from AP at 51% [38].

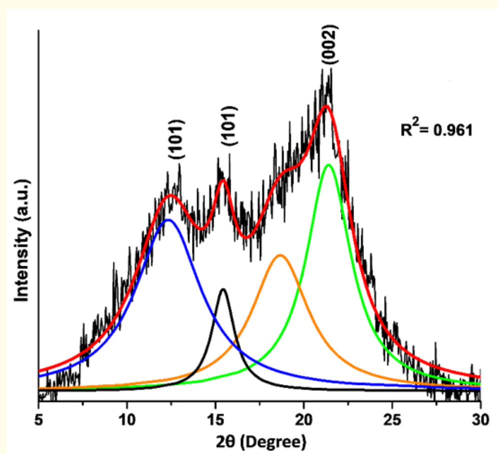

Figure 2: *X-ray diffractogram (XRD) of apple pomace.*

Thermogravimetric analysis of AP

The thermal degradation behavior of AP was assessed by TGA (Figure. 3). According to the TGA and DTG thermograms, AP shows three weight loss stages between 30 to 700°C. An initial weight loss (3%) between 72 and 120°C was attributed to moisture loss and some volatile organic components [39]. The second stage (193 - 400°C) of decomposition had a significant weight loss (61%), which can be associated with degradation of polysaccharides and lipids [20,39,40]. The third stage (400 - 700°C) resulted (29%) weight loss, which can be attributed to the degradation of cellulose [20,39]. Similar results have been found in previous study [20]. Degradation of lignin occurs over a wide temperature range (250 - 700°C), due to different thermal stability of oxygen functional groups (e.g. carbonyl, phenolic hydroxyl, benzylic hydroxyl functional groups) on its complex structure [20,40,41].

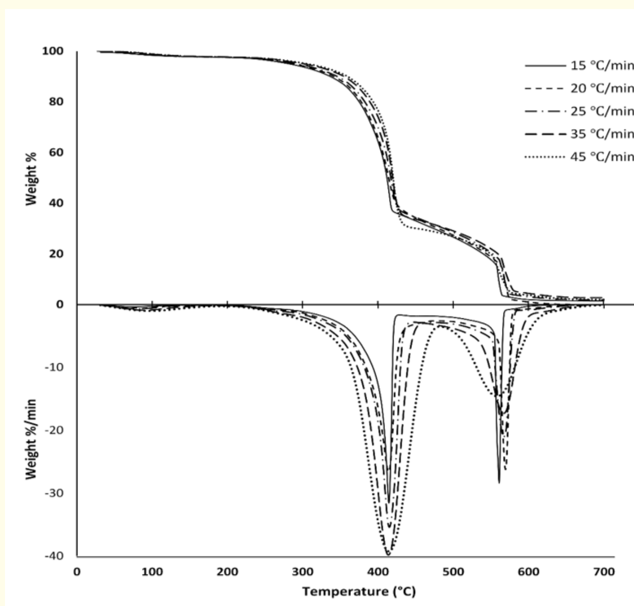

Figure 3: *Thermogravimetric analysis (TGA) (above) and differential thermogravimetric (DTG) analysis (below) of apple pomace under N2 at different heating rates (β = 15 to 45°C min−1).*

E_α was determined (Table 4) according to the FWO method from a plots of iso-conversional lines log β against 1/T corresponding to the different conversion degrees (α) (Figure 4). The average E_α calculated from the FWO method was 299 kJ mol^{-1} and was higher than that reported by Guerrero., *et al*. (201 - 213 kJ mol^{-1}) [20]. Differences in E_α could be attributable to AP compositional differences [9,18,23].

Conversion(α)	E_α (kJ mol^{-1})	R^2
0.1	163	0.9799
0.2	211	0.9795
0.3	249	0.9792
0.4	357	0.9754
0.5	426	0.9533
0.6	275	0.9851
0.7	311	0.9850
0.8	343	0.7772
0.9	360	0.9948
Average	299	

Table 4: *Activation energy (E_α) as a function of conversion (α) for apple pomace using the FWO method.*

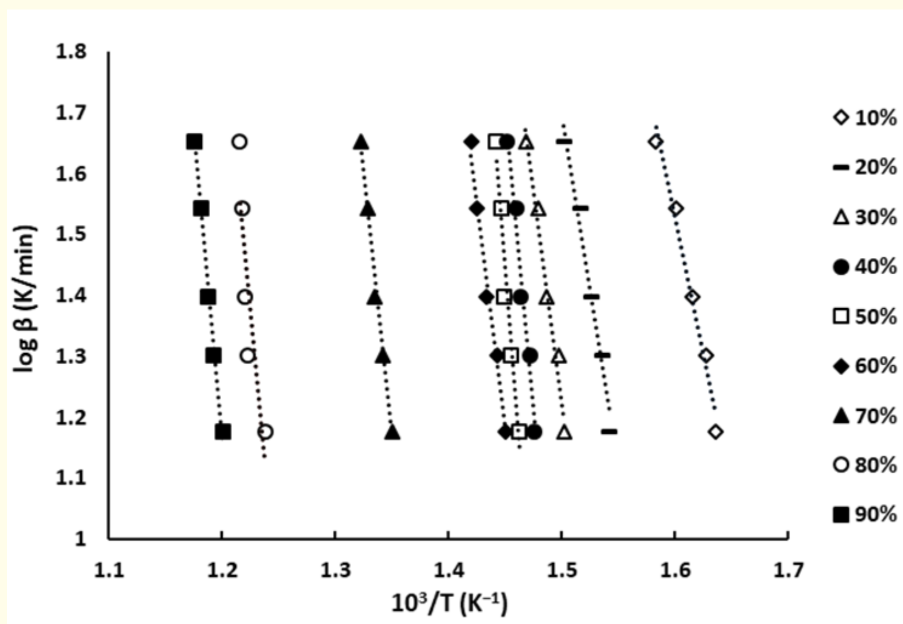

Figure 4: *Plot of heating rate (β) versus 1/T to determine activation energy (Eα) by FWO method for apple pomace.*

Fermentation of AP

Fermenter operating conditions ((solid loading of 35, 55 and 75 g L^{-1}), gelatinization of feedstock, and temperature (27, 35 and 40°C)) were based on previous research on potato, orange and banana peel fermentation to lactic acid [2,42]. The main fermentation products identified by HPLC were lactic acid, acetic acid, oxalic acid, citric acid, ethanol and methanol. Lactic acid [43], acetic acid [44], ethanol [44,45] and citric acid [46] have been previously observed as fermentations products from AP. The identity of lactic (and/or oxalic) and citric acids were also supported by ESI-MS as their (i) $[M+H]^+$ ions at m/z 91 and 193 and (ii) $[M-H]^-$ ions at m/z 89 and 191, respectively [2]. The identity of lactic and oxalic acids were confirmed by GCMS analysis of the fermentation broth as their TMS derivatives (Table 5). However, citric acid was not detected. A variety of other compounds were present in the fermentation broth and are not discussed. The presence of methanol is most likely arises from pectin esterase activity cleaving methyl-ester groups from pectin [47].

RT (min)	Compound	Molecular formula	M^+(m/z)
6.83	2-Thiophenethiol-TMS	$C_7H_{12}S_2Si$	188
7.44	Oxalic acid-2TMS	$C_8H_{18}O_4Si_2$	234
9.15	Propylamine, 2TMS	$C_9H_{25}NSi_2$	203
12.11	1,5-Pentanediol-2TMS	$C_{11}H_{28}O_2Si_2$	248
12.33	Lactic acid-2TMS	$C_9H_{22}O_3Si_2$	234
13.60	Linolool oxide-TMS	$C_{13}H_{26}O_2Si$	242
28.78	Undecenoic acid-TMS	$C_{14}H_{28}O_2Si$	254
30.74	Jasmonic acid-TMS	$C_{15}H_{26}O_3Si$	282
35.38	Myristic acid-TMS	$C_{17}H_{36}O_2Si$	300
38.28	Traumatic acid-2TMS	$C_{18}H_{36}O_4Si$	372
38.35	Petroselinic acid-TMS	$C_{21}H_{42}O_2Si$	354
38.87	Stearic acid-TMS	$C_{21}H_{44}O_2Si$	356
49.33	1-Hexacosanol-TMS	$C_{29}H_{62}OSi$	454
50.99	1-Heneicosanol-TMS	$C_{24}H_{52}OSi$	384
51.80	1-Octacosanol-TMS	$C_{31}H_{66}OS_i$	482
54.11	β-Sitosterol-TMS	$C_{32}H5_8OSi$	486
56.67	Oleanolic acid-2TMS	$C_{36}H_{64}O_3Si_2$	600
57.17	Ursolic acid-2TMS	$C_{36}H_{64}O_3Si_2$	600

Table 5: *GCMS analysis of apple pomace fermentation products as their TMS derivatives.*

Effect of temperature

Temperature has been shown to be a significant controlling factor in organics conversion [2] and AP fermentation was investigated at 27, 35 and 40°C, with a solids loading of 55 g L^{-1} without gelatinization (Table 6). AP fermentation at 35°C showed significantly higher levels of lactic acid, acetic acid, oxalic acid, citric acid, ethanol and methanol production than at 27 or 40°C. At 27°C, AP fermentation showed lower concentrations of acetic acid which reached 0.34 g L^{-1} on day 3, oxalic acid which reached 7.60 g L^{-1} on day 8 and methanol which reached 4.32 g L^{-1} on day 7. However, lactic and citric acids were not observed at this fermentation temperature. However, ethanol production at 27°C was the highest compared to 35 and 40°C. To note, fermentation at 27°C increased the time required for acetic acid and ethanol to be produced. While, AP fermentation at 40°C produced 0.43 g L^{-1} of lactic acid, 1.22 g L^{-1} of acetic acid, 17.2 g L^{-1} of oxalic

acid, 11.0 g L^{-1} of citric acid and 5.66 g L^{-1} of methanol. Ethanol was not detected at 40°C fermentation of AP [2]. Liang., *et al*. [2] found that lactic acid, acetic acid and ethanol production was optimized at solid loading of 60 g L^{-1} at 35°C for potato peel waste. However, increasing the fermenter temperature from 15 to 35°C had a negative effect on ethanol production [48].

Temperature °C	Maximum product conc. (g L^{-1})											
	Lactic acid		Oxalic acid		Acetic acid		Citric acid		Ethanol		Methanol	
	g L^{-1}	Time(d)	g L^{-1}	Time (d)	g L^{-1}	Time (d)	g L^{-1}	Time (d)	g L^{-1}	Time (d)	g L^{-1}	Time (d)
27	--	--	7.60	8	0.34	8	--	8	1.18	1	4.32	4
35	0.63	9	21.8	8	1.73	8	12.82	8	0.37	1	6.81	4
40	0.43	8	17.1	8	1.22	9	11.01	8	--	1	5.66	7

Table 6. Effect of temperature on apple pomace fermentation product concentrations.

Effect of gelatinization

Gelatinization of AP (55 g L^{-1}) was carried out in order to make the starch and other polysaccharides more susceptible to fermentation at 35°C. The effect of gelatinization of AP fermentation product was clearly observed giving lower yields of products (Figure 5a-5f). The gelatinized AP had lower oxalic acid (10.5 g L^{-1}), citric acid (0.69 g L^{-1}), ethanol (0.33 g L^{-1}) and methanol (5.38 g L^{-1}) concentration compared to AP under the same fermentation conditions. Gelatinization of AP resulted in no lactic and acetic acids being produced.

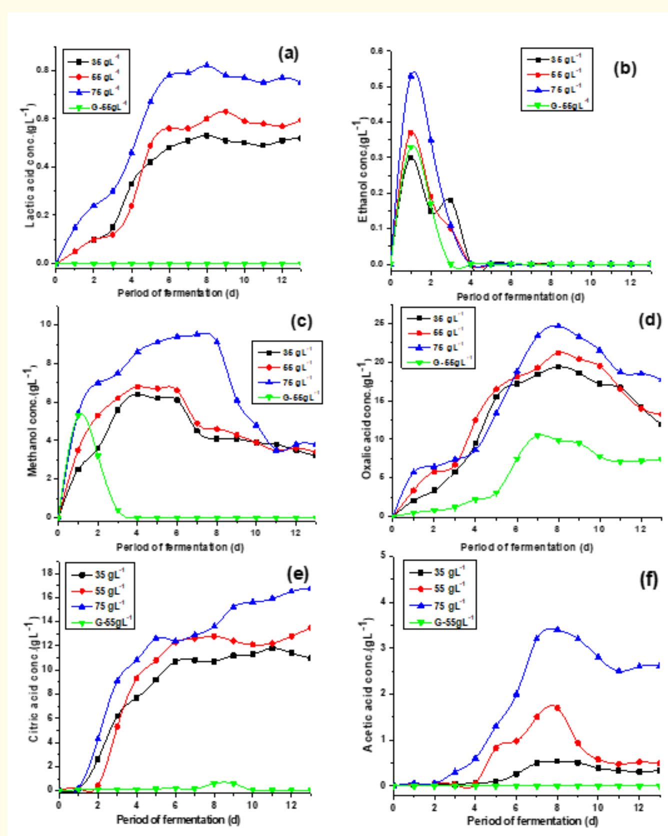

Figure 5: *Influence of apple pomace solids loading (35, 55, 75 g L^{-1}) and gelatinization (G-55 g L^{-1}) at 35°C on fermentation to produce (a) lactic acid; (b) ethanol; (c) methanol; (d) oxalic acid; (e) citric acid and (f) acetic acid.*

Effect of solids loading

A fermentation temperature of 35°C was selected to investigate the effect of AP solids loading (Table 6 and Figure 5a-5f). The fermentation products (lactic, oxalic, acetic, citric, ethanol, and methanol) yields were shown to increase with AP solids loading from 35 to 75 g L^{-1}. Production of lactic acid was shown to increase with time results showed that 0.81 g L^{-1} (3 mg/g of AP) lactic acid was obtained after 6 d at 75 g L^{-1} (Figure 5a). Similar results (lactic acid at 14.7 g L^{-1}) were observed by Liang., *et al*. on potato peel fermentation using MMC [2]. Lactic acid yield were low compared to those reported by Chatterjee., *et al.* on AP fermentation with lactic acid producing bacteria [49]. Methanol production increased by 34% going from 35 to 75 g L^{-1} solids loadings to give a concentration of 9.57 g L^{-1} (33 mg/g of AP) (Figure 5c).

Ethanol was produced rapidly (4 h) to 0.54 g L^{-1} (2 mg/g of AP) at 75 g L^{-1} (Figure 5b) and then consumed. The decrease in ethanol concentration is likely due to MMC metabolizing it to organic acids [50,51]. Furthermore, AP fermentation also gave rise to oxalic acid, acetic acid and citric acid (Figure 5d-5f), the Interestingly, oxalic acid was the highest yielding product at 24.8 g L^{-1} (83 mg/g of AP) at 75 g L^{-1} AP loading on day 8 and then subsequently decreased. The presence of methanol, as a stimulating agent in the fermentation medium, possibly contributed to a marked increase in organic acids such as citric and oxalic acids [52,53]. High levels of citric acid (16.8 g L^{-1}, 60 mg/g of AP) and acetic acid (3.42 g L^{-1}, 20 mg/g of AP) were obtained during fermentation at 75 g L^{-1} (Figures 5e and 5f). Acetic acid production peaked around day 8 (Figure 5f). Citric acid yield increased with AP solids loading (Figure 5e). Other studies on AP fermentation have found that citric acid production peaks on day 7 [54].

Solids loading (g L^{-1})	Maximum product yields yield (mg/g of AP)											
	Lactic acid		Oxalic acid		Acetic acid		Citric acid		Ethanol		Methanol	
	mg/g	Time (d)	mg/g	Time (d)	mg/g	Time (d)	mg/g	Time (d)	mg/g	Time (d)	mg/g	Time (d)
35	1.8	8	135	8	11.5	8	82.5	12	3.8	1	38.4	4
55	2.7	9	96	8	8.2	8	59.4	13	1.7	1	29.2	4
75	3.0	8	83	8	3.9	8	55.2	13	2.2	1	33.0	7
G-55	--	--	46	7	--	--	2.8	8	1.5	1	21.5	1

Table 7: *Yields of apple pomace fermentation products generated at different solids loading (35, 55, 75 g L-1) and gelatinization (G-55g L^{-1}) at 35°C.*

Conclusion

Apple pomace (AP) is a byproduct of apple juice/cider production and is considered a waste. AP was shown to be a suitable feedstock, based on its composition (carbohydrates and lipids), for conversion into chemicals. Upcycling of AP using a mixed microbial culture (MMC) fermentation process was utilized evaluated. AP fermentation was optimized using a solids loading of 75 g L^{-1} and at 35°C. AP fermentation produced lactic, citric, acetic, and oxalic acids as well as ethanol and methanol. The major products were oxalic acid (83 mg/g of AP), citric acid (60 mg/g of AP) and methanol (33 mg/g of AP) which are all valuable chemicals. Lactic acid production was unexpectedly low (3 mg/g of AP). This biorefinery approach can potentially lower the cost of chemical production by using zero value food processing waste stream, without the need of using a refined sugar feedstock, and sterile bioreactors with pure cultures.

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Conflict of Interest

The authors declare no financial interest or any conflict of interests.

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