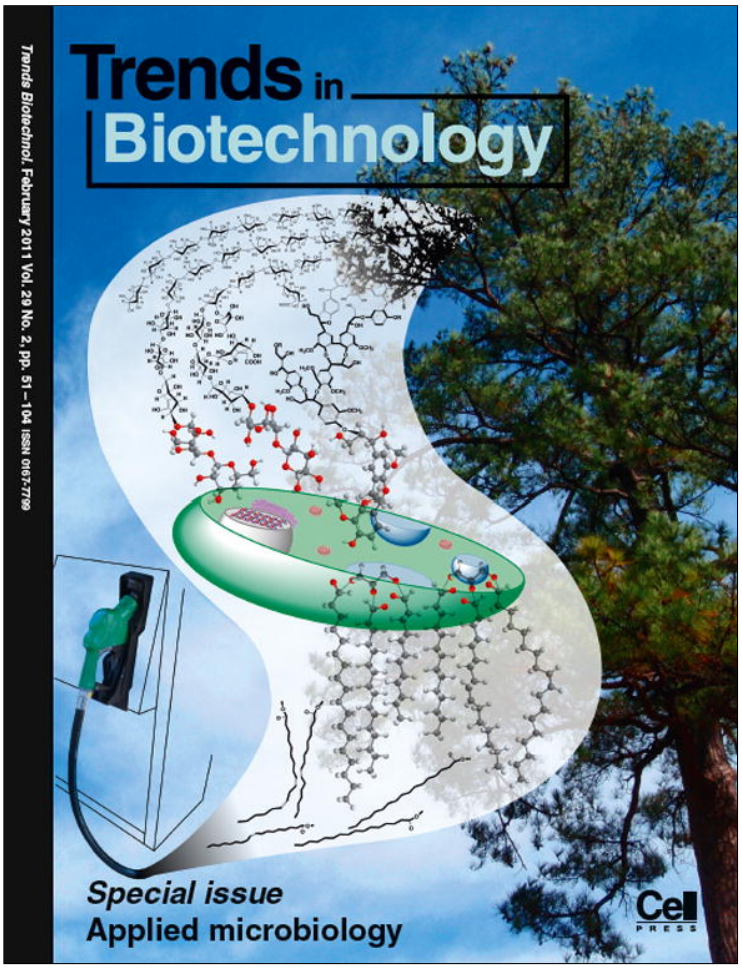

Review

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Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform

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Our societies generate increasing volumes of organic wastes. Considering that we also need alternatives to oil, an opportunity exists to extract liquid fuels or even industrial solvents from these abundant wastes. Anaerobic undefined mixed cultures can handle the complexity and variability of organic wastes, which produces carboxylates that can be efficiently converted to useful bioproducts. However, to date, barriers, such as inefficient liquid product separation and persistence of methanogens, have prevented the production of bioproducts other than methane. Here, we discuss combinations of biological and chemical pathways that comprise the 'carboxylate platform', which is used to convert waste to bioproducts. To develop the carboxylate platform into an important system within biorefineries, we must understand the kinetic and thermodynamic possibilities of anaerobic pathways, understand the ecological principles underlying pathway alternatives, and develop superior separation technologies.

Introduction

The discrepancy between the rates of discovery of new oil reserves and consumption will undoubtedly lead to a future oil crisis. Our societies are also generating an increasing quantity of organic wastes, such as industrial and agricultural wastewater. An opportunity therefore exists to shift the view of these waste streams from pollutant to renewable resource. In the biorefinery concept, the value of each stream must be maximized (similar to oil refineries) [1], and such waste treatment creates an opportunity to generate additional fuels or chemicals (i.e. bioproducts), while simultaneously recycling nutrients and water. Processing steps within biorefineries, such as chemical/physical pretreatment, enzyme production, and fermentation and extraction steps, all create large volumes of wastewater that must be treated. The two best-known biorefinery platforms are the sugar platform, in which purified enzymes convert biomass into five- and six-carbon sugars as intermediate feedstock chemicals that are converted further by, for example, fermentation to fuels; and the syngas platform, in which thermochemical systems convert biomass into syngas (i.e. synthesis gas, such as CO, H_2, and CO_2) as feedstock chemicals that are converted further by, for example, catalysis to fuels (National Renewal Energy Laboratory: www.nrel.gov/biomass/biorefinery.html). We envision a third important platform – the carboxylate platform – to convert organic feedstocks, which are often derived from industrial and agricultural wastes, to short-chain carboxylates as intermediate feedstock chemicals, using hydrolysis and fermentation with undefined mixed cultures in engineered systems under anaerobic conditions. The differences in platforms are essentially based on the method of biomass conversion and its resultant chemicals (e.g. sugar, syngas and carboxylates), because the subsequent conversion step into bioproducts is interchangeable between platforms. The use of undefined mixed cultures in waste treatment systems is vital, because they can tolerate the complexity and variability of substrates owing to the metabolic flexibility conferred by the many members of the community [2,3]. Furthermore, they are open and anaerobic systems, which makes energetically unfavorable sterilization and aeration superfluous [3].

The terminology 'carboxylate platform' is not new, and has been used to describe an undefined-mixed-culture process to generate a mixture of carboxylates as intermediate platform chemicals towards generation of complex fuels [4]. Carboxylates are dissociated organic acids that are characterized by the presence of at least one carboxyl group. The short-chain carboxylates – acetate, propionate, lactate and *n*-butyrate – are the main organic products of undefined mixed cultures through primary fermentation reactions (Figure 1a,b). They are themselves valuable products when separated from the culture broth, but often they are substrates for further fermentation in the same undefined mixed culture through secondary fermentation reactions (Figure 1c–j) or in separate bioprocesses. The carboxylates from primary fermentation can also be further processed with separate pure-culture biochemical, electrochemical, and thermochemical steps (chemical post-processing step in Figure 2). An important carboxylate flux occurs within the undefined mixed culture; as such, anaerobic digestion is included within the carboxylate platform because short-chain carboxylates are the (pen)ultimate intermediate platform products for gaseous methane formation

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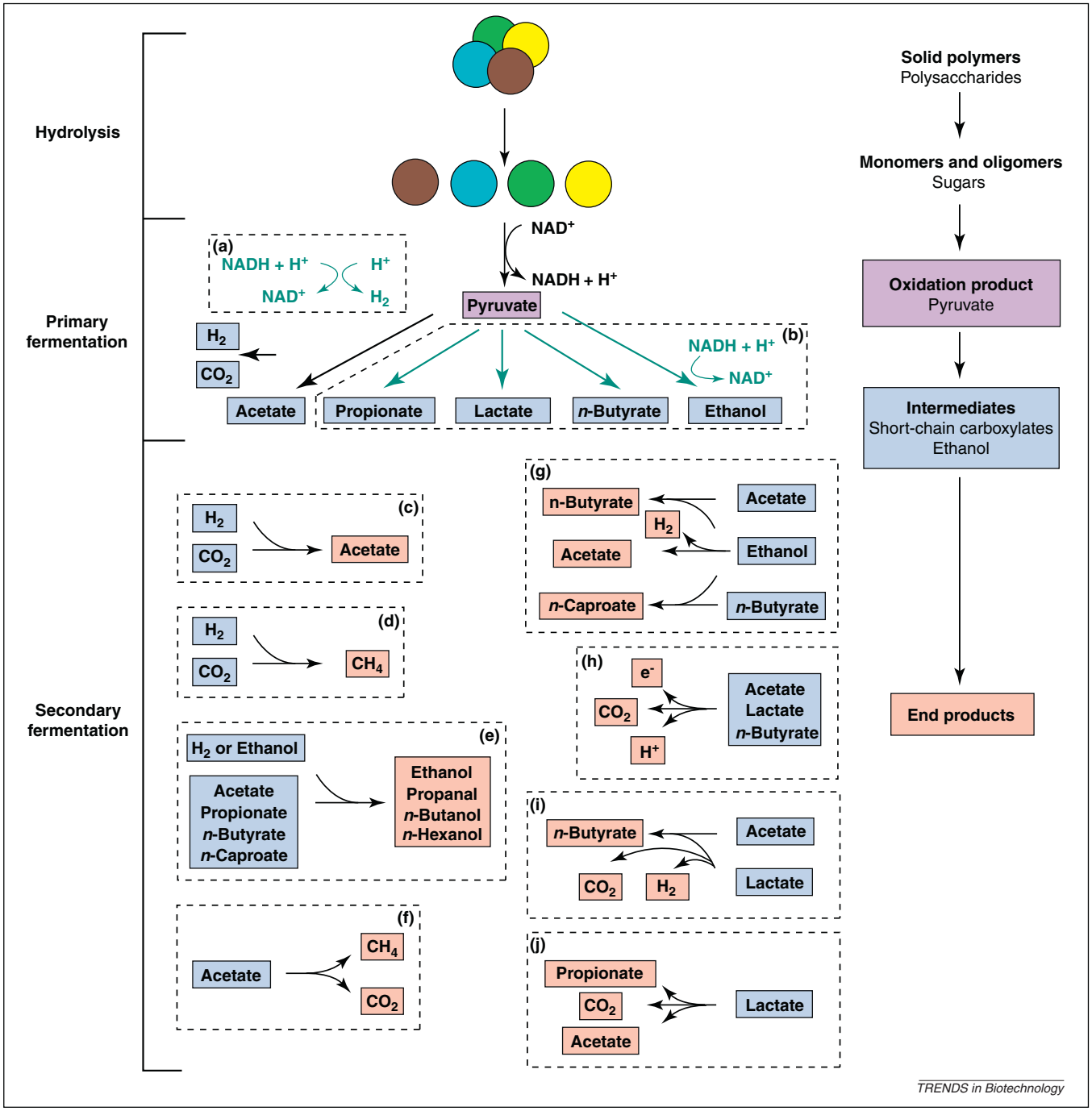


Figure 1. Hydrolysis of solid polymers to monomers and oligomers (e.g. insoluble polysaccharides, such as cellulose and hemicellulose) and subsequent conversion by primary and fermentation reactions with undefined mixed cultures. During primary fermentation of sugars, substrates are converted to pyruvate, which results in the production of NADH and H^+. All equivalents must be re-oxidized via H^+ reduction by: **(a)** NADH oxidation; or **(b)** NADH oxidation via reduction of pyruvate or its oxidized organic derivatives, depending upon the hydrogen partial pressure [8]. At increasing hydrogen partial pressures, the flow of electrons from NADH shifts from H_2, acetate and CO_2 production towards formation of increasingly reduced fermentation products [70]. CO_2 and H_2 are produced in the pyruvate oxidation reaction that is catalyzed by pyruvate:ferredoxin oxidoreductase. The products of primary fermentation can react further within undefined mixed cultures through several secondary fermentation reactions: **(c)** autotrophic homoacetogenesis; **(d)** hydrogenotrophic methanogenesis; **(e)** carboxylate reduction to alcohols with hydrogen or ethanol; **(f)** aceticlastic methanogenesis; **(g)** chain elongation of carboxylates with ethanol; **(h)** electricigenesis **(i)** lactate oxidation to *n*-butyrate (acetate and H^+ as electron acceptor); and **(j)** lactate reduction to propionate (oxidation to acetate for energy conservation).

(Figure 1). Even though pure culture and defined mixed culture studies are performed to understand the underlying ecological principles of undefined mixed communities [5–7], the commercial process to convert biomass into carboxylates must be an undefined microbial processing step to handle the complexity of the organic waste stream.

This review is a much-needed update to previous reviews [2,8], because several additional bioprocessing schemes have been developed in the interim, to generate energy-rich chemicals with undefined mixed cultures, rather than with pure or defined cultures. The organization of this review is based on the production and subsequent conversion of the

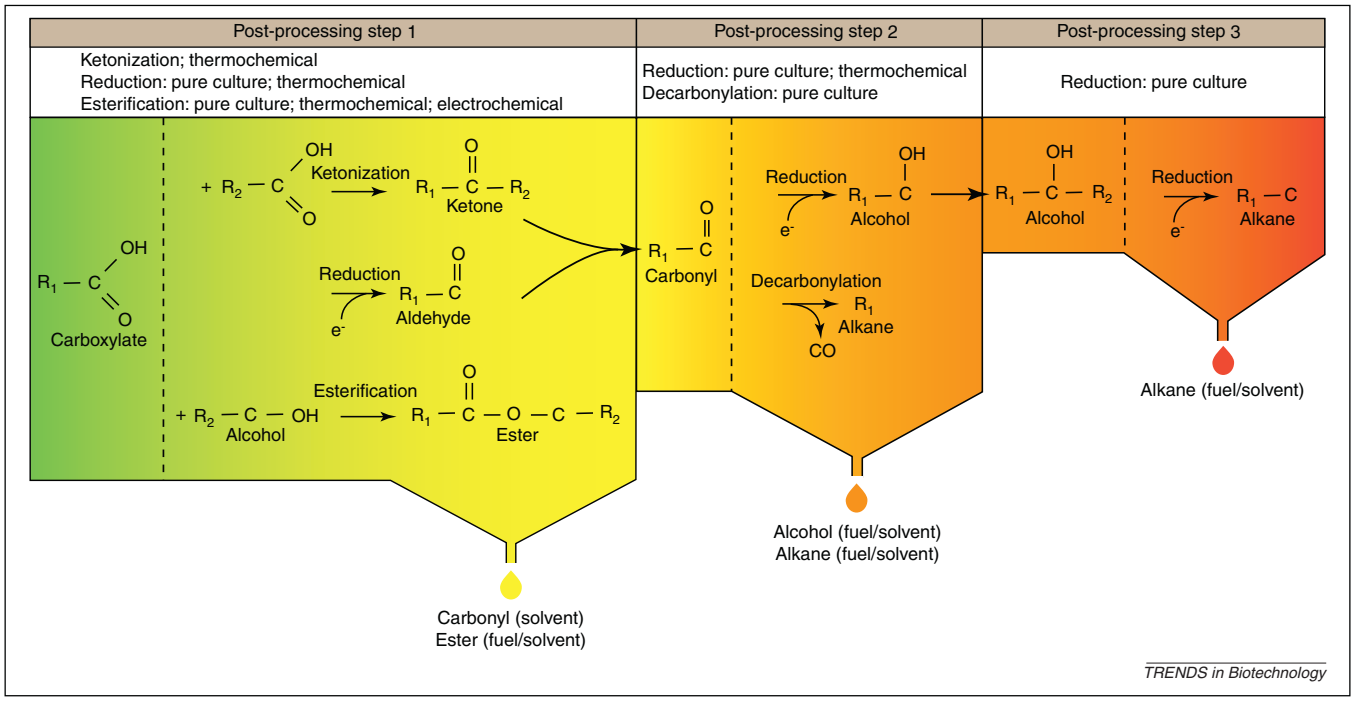


Figure 2. Chemical post-processes that convert carboxylates to bulk fuels or solvents with pure-culture biochemical, electrochemical, and thermochemical steps, or a combination thereof. In post-processing step 1, carboxylates are converted to esters via esterification [50,51,71]; are reduced to carbonyls [72,73]; or ketonized to carbonyls [29]. In post-processing step 2, the carbonyl intermediates are converted to alkanes via decarbonylation [72]; or reduced to alcohols [47,51]. Finally, in post-processing step 3, the alcohol intermediates are converted to alkanes via reduction [74]. Other conversions (not shown here) are possible.

primary fermentation end products, the carboxylate feedstock chemicals: acetate (C2), propionate and lactate (C3), *n*-butyrate (C4), and mixed carboxylates. In this review, we discuss how these chemicals can be further processed into high-volume fuels or industrial solvents because these bulk bioproducts would have the largest impact in an integrated biorefinery. Table 1 shows balanced chemical equations and their thermodynamic values under standard biological conditions for the reactions and processes that we discuss in this review. Other chemicals that might also be generated within a carboxylate platform concept, but are not discussed here, are *iso*-butyrate, long-chain fatty acids, and biopolymers, such as poly(lactic acid).

Acetate

When the hydrogen partial pressure is maintained at low levels in stable anaerobic digesters by scavenging hydrogenotrophic methanogens (secondary fermentation reaction in Figure 1d), a maximum acetate flux is maintained (mainly the primary fermentation pathway in Figure 1a). This maximum acetate flux explains the superiority of anaerobic digestion as an efficient biomass-to-energy conversion process because primary fermentation is directed towards acetate and hydrogen, both of which are then converted to the end product methane with secondary fermentation reactions (Figure 1d,f). Propionate and *n*-butyrate are formed during protein hydrolysis and subsequent fermentation of amino acids, regardless of the H_2 partial pressure [9]. On the other hand, propionate and *n*-butyrate can only be converted to the intermediate products acetate and hydrogen by secondary syntrophic carboxylate-oxidation reactions, if these products are removed by methanogens (low H_2 partial pressure) (Box 1; Table I). This guarantees the maximum carbon and electron flux towards methane because almost no side-products that decrease efficiency are released from the anaerobic food web [10]; or, in other words, the microbial process is directed to the final product (i.e. methane) with the lowest available free energy content per electron [11]. Anaerobic digestion is a mature technology and methane freely bubbles out without requiring additional separation processes, therefore, it is easy to understand why anaerobic digestion is the most popular waste-to-energy conversion technology worldwide. However, methane has a low monetary value, and therefore, we focus here on the promises and challenges of producing liquid fuels and high-value chemicals with the carboxylate platform.

Acetate by itself is a useful chemical; or as a feedstock chemical, acetate can be processed into bulk bioproducts via secondary fermentation reactions (Figure 1e–i) and chemical post-processing reactions (Figure 2). Although a variety of chemical and biological processes can be used to transform carboxylate intermediates to valuable products, three biological processes in particular might integrate well with the production of carboxylates from complex waste streams by undefined mixed cultures. These processes are biological reduction of carboxylates to the corresponding alcohols (Figure 1e); biological elongation of short-chain carboxylates to longer chain products (Figure 1g); and bioelectrochemical systems (BESs), in which biological reactions are coupled to reactions at solid electrodes to produce electric power or valuable chemicals (Figure 1h). Regardless of the conversion method, further processing of acetate relies on being able to separate it from the undefined mixed culture broth, because consolidated bioprocesses in which the primary and secondary fermentation reactions occur in the same reactor are

Table 1. Secondary fermentation reactions and processes[a]

Reaction	Microbe	Carboxylate conversion reactions	Coupled repetitions[d]	$\Delta G_r^{o'}$ (kJ/mol at 37 °C)[e]	$\Delta G_r^{o'}$ (kJ/mol at 55 °C)[e]
(c) Carbon dioxide reduction to acetate	*Acetobacterium woodii*	$4H_2 + 2CO_2 \rightarrow acetate^- + H^+ + 2H_2O$		−86.78	−74.56
(d) Hydrogenotrophic methanogenesis	*Methanospirillum hungatei*	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$		−125.84	−118.47
(e) Carboxylate reduction with molecular hydrogen	[b]	$acetate^- + H^+ + 2H_2 \rightarrow ethanol + H_2O$		−7.22	−4.37
		$propionate^- + H^+ + 2H_2 \rightarrow propanol + H_2O$		−7.49	−4.59
		$n\text{-butyrate}^- + H^+ + 2H_2 \rightarrow n\text{-butanol} + H_2O$		−3.58	−0.73
		$n\text{-caproate}^- + H^+ + 2H_2 \rightarrow n\text{-hexanol} + H_2O$		−7.55	−3.63
(e) Propionate reduction with ethanol	[c]	$ethanol + H_2O \rightarrow acetate^- + H^+ + 2H_2$	×1	7.22	4.37
		$propionate^- + H^+ + 2H_2 \rightarrow propanol + H_2O$	×1	−7.49	−4.59
				Total = −0.27	**Total =** −0.22
(f) Aceticlastic methanogenesis	*Methanosaeta soehngenii*	$acetate^- + H^+ \rightarrow CH_4 + CO_2$		−39.06	−43.91
(g) Chain elongation of acetate	*Clostridium kluyveri*	$ethanol + H_2O \rightarrow acetate^- + H^+ + 2H_2$	×1	7.22	4.37
		$ethanol + acetate^- \rightarrow n\text{-butyrate}^- + H_2O$	×5	−201.68	−198.50
				Total = −194.46	**Total =** −194.13
(g) Chain elongation of *n*-butyrate	*C. kluyveri*	$ethanol + H_2O \rightarrow acetate^- + H^+ + 2H_2$	×1	7.22	4.37
		$ethanol + n\text{-butyrate}^- \rightarrow n\text{-caproate}^- + H_2O$	×5	−190.00	−195.20
				Total = −182.78	**Total =** −190.83
(i) Lactate oxidation to *n*-butyrate	*Clostridium acetobutylicum*	$2\ acetate^- + H^+ + 2H_2 \rightarrow n\text{-butyrate}^- + 2H_2O$	×1	−47.55	−44.10
		$2\ lactate^- + H^+ \rightarrow n\text{-butyrate}^- + 2CO_2 + 2H_2$	×2.5	−209.35	−232.55
				Total = −256.90	**Total =** −276.65
(j) Lactate reduction to propionate	*Selenomonas ruminantium*	$lactate^- + H_2O \rightarrow acetate^- + CO_2 + 2H_2$	×1	28.51	25.96
		$lactate^- + H_2 \rightarrow propionate^- + H_2O$	×2	−86.63	−85.21
				Total = −58.12	**Total =** −59.25

[a]Secondary fermentation reactions correspond to those in Figure 1 [(h) is not shown here].
[b]Carboxylate reduction to alcohol with H_2 as the electron donor has been observed in undefined mixed cultures [23].
[c]Propionate reduction with ethanol has been observed in undefined mixed cultures [32].
[d]For reactions that are coupled within microbes, number of repetitions of individual reactions to achieve the total reaction.
[e]All $\Delta G_r^{o'}$ values are calculated considering all reactants and products to be in the aqueous phase except for H_2, CO_2 and CH_4, which are gaseous at 1 atm. $\Delta G_r^{o'}$ values are at biological standard state (pH = 6.82 at 37 °C; 6.58 at 55 °C). Reactions that are coupled by microbes are shown individually with individual and coupled (total) $\Delta G_r^{o'}$ values. $\Delta G_r^{o'}$ quantities were calculated from ΔG_f^{o} values from [67], except for *n*-caproate$^-$ and *n*-hexanol, which were calculated using the HKF equations of state [68] and thermodynamic parameters [69].

often precluded by incompatible optimal conditions. One of the main barriers for large-scale liquid fuel and chemical production with the carboxylate platform is limitations with separation (Box 2). The other barrier is that hydrogenotrophic methanogenesis must be ceased.

Bioelectrochemical oxidation to electrons

Most BES research initially focused on production of electric power by bioelectrochemical oxidation of organic substrates in microbial fuel cells (MFCs) in which a solid electrode served as the electron acceptor (Figure 1h) [12]. Electric power is generated in MFCs by developing a natural potential difference between an anaerobic anode and an aerobic cathode. Although more complex substrates have been used, only acetate and other short-chain carboxylates result in coulombic efficiencies appropriate for scale-up [13]. Recent studies, however, suggest that converting organic substrates, such as short-chain carboxylates, into chemical products by applying an electric potential across the electrodes has greater economic and environmental benefits than the production of electric power [13,14]. This approach overcomes the thermodynamic limitations of the cathodic reactions by using a potentiostat to apply an electric potential between the electrodes in microbial electrolysis cells (MECs). Separation of product formation at the cathode from substrate oxidation at the anode is an inherent advantage of using a membrane-based BES to generate valuable products from complex organic wastes. Abiotic cathodes have been used to produce hydrogen [15,16], hydrogen peroxide [17], and sodium hydroxide [18], and undefined mixed cultures have been used to produce methane without the addition of mediators at potentiostatically poised cathodes (i.e. biocathodes) [19,20]. Recent studies have demonstrated reduction of CO_2 in biocathode-based and potentiostatically poised BESs [21,22]; and reduction of carbon dioxide into multi-carbon products (i.e. microbial electrosynthesis), such as alcohols, with all electrons originating from carboxylate oxidation at the anode, is a focus of ongoing research.

Biological reduction to alcohols

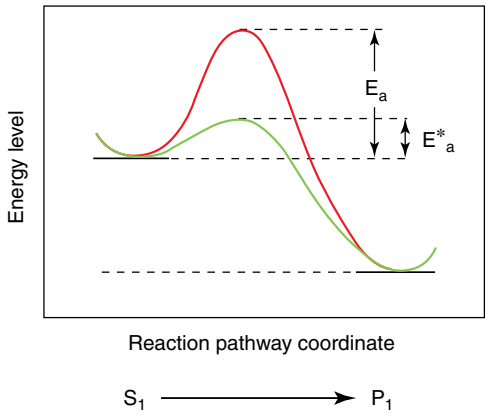
Biological reduction of carboxylates, such as acetate, *n*-butyrate and *n*-caproate, to the corresponding alcohols has been observed in separate secondary fermentation bioprocesses (Figure 1e) [23,24]. The thermodynamic free energy under biological standard conditions (Table 1) is smaller than the biological limit for cellular functions (Box 1) [25,26]; therefore, the hydrogen partial pressure must be maintained at elevated levels (~1.5 atm). Hydrogen would be available in a biorefinery that includes the carboxylate platform, therefore, it is an attractive reducing agent. Unfortunately, very low rates of alcohol production from acetate ($0.07\ g\ l^{-1}\ day^{-1}$ ethanol) have been observed under these conditions [27]. Alternatively, acetate can also be reduced to ethanol with an artificial mediator and a mixed culture at the cathode of a BES [28], where electrons donated from the cathode provide the required reducing power.

High-rate consolidated bioprocesses for producing ethanol from acetate cannot be envisioned because high

Box 1. Predicting reaction occurrence

The food web of the undefined mixed culture is made up of a network of reactions in which microorganisms convert one or more substrates (S_1) into products (P_1) (Figure I). Modeling of the sequence of reactions that occurs for a specific set of operating conditions might help engineers optimize the product spectrum. In any biological system, there are two prerequisites for a reaction to proceed in space and time: **(i)** a catalyst is present to drive the reaction; **(ii)** the reaction is thermodynamically favorable. The microorganisms in the undefined mixed culture are the catalysts (i.e. their enzymes) that decrease the activation energy (E_a) of the transition state, which results in increased kinetic rate constants (Equation 1) and reaction rates (Equation 2) (Figure I). The second requirement dictates that the available free energy of reaction, which is a function of the temperature, pressure, and substrate and product activities (Equation 3), is below an energetic threshold (ΔG_{thr}). Thus, if the required microorganism is available in a culture, the environmental conditions (e.g. pH, temperature, and pressure) must be maintained to favor the reaction of interest ($\Delta G_r < \Delta G_{thr}$).

The energetic threshold is the amount of energy that must be conserved in a reaction pathway. Here, we show an example of how this threshold is calculated for the specific oxidation reaction of *n*-butyrate to acetate. In this reaction, one ATP is produced by substrate-level phosphorylation; however, up to two-thirds of one ATP molecule are reinvested in a proton gradient with a combined $\Delta G_{thr} \approx -23.3$ kJ/mol [60]. For many reactions throughout our review for which the biochemistry is poorly defined, we have used this ΔG_{thr} value as a conservative approximation. Note that, in the text, we have used the free energy nomenclature for standard biological conditions ($\Delta G_r^{0\prime}$) to compare ΔG_{thr} to free energy numbers of specific reactions in Table 1. Research has shown, however, that certain reactions (including *n*-butyrate oxidation) have occurred when ΔG_r was above −10 kJ/mol (Table I). Indeed, modelers have been able to explain biological metabolic activity under these conditions ($\Delta G_r > \Delta G_{thr}$), by allowing the energy required to produce ATP and pump protons to be variable, depending on environmental conditions [26,61]; but for such modeling efforts, a detailed knowledge of the biochemical pathways is required.



$$k = k_o \cdot e^{-Ea/RT} \quad \text{Eqn (1)}$$

$$r = k \cdot [S_1] \quad \text{Eqn (2)}$$

$$\Delta G_r = \Delta G^\circ_r + RT \ln \frac{[P_1]}{[S_1]} \quad \text{Eqn (3)}$$

ΔG°_r is a function of environmental conditions, such as pH, temperature (T) and pressure.

[ ] denotes concentration, which is often used in practice in place of activity.

TRENDS in Biotechnology

Figure I. Reaction coordinate diagram and equations to calculate reaction kinetics and thermodynamics. The presence of microbial catalysts decreases the activation energy E_a to E^*_a so that the rate of reaction is increased with increasing rate constants (Equations 1 and 2). Environmental conditions, microbial biochemistry, and substrate (S_1) and product (P_1) activities affect the thermodynamic feasibility of a reaction (Equation 3).

Table I. Thermodynamic considerations for secondary oxidation reactions

Microbe	Carboxylate oxidation reactions	$\Delta G_r^{o\prime}$ (kJ/mol at 37 °C)[a]	$\Delta G_r^{o\prime}$ (kJ/mol at 55 °C)[a]
Syntrophospora bryantii or *Syntrophomonas wolfei*	$acetate^- + H^+ + 2H_2O \rightarrow 4H_2 + 2CO_2$	86.96	74.56
	$propionate^- + 2H_2O \rightarrow acetate^- + CO_2 + 3H_2$	68.53	60.74
	$n\text{-butyrate}^- + 2H_2O \rightarrow 2\ acetate^- + H^+ + 2H_2$	47.55	44.07

[a]All $\Delta G_r^{o\prime}$ values are calculated considering all reactants and products to be in the aqueous phase except for H_2, CO_2 and CH_4, which are gaseous at 1 atm. $\Delta G_r^{o\prime}$ values are at biological standard state (pH = 6.82 at 37 °C; 6.58 at 55 °C). $\Delta G_r^{o\prime}$ quantities were calculated from ΔG_f^o values from [67].

hydrogen partial pressures are required to drive acetate reduction, and very low partial pressures are required to sustain high rates of acetate production. Thus, a secondary-fermentation bioprocess with two separate streams of acetate and hydrogen would be required. The ethanol concentrations obtained in batch experiments with an undefined mixed culture (0.17 g/l at an acetate conversion efficiency of ~55%) [23] are too low for economical recovery of the product by *ex situ* distillation, and the maximum concentration that can be achieved (<1.1 g/l at pH 4.5) is limited by the required threshold free energy for this reaction [27]. Therefore, it might be necessary to remove ethanol continuously during the acetate reduction process to maintain sufficiently low product concentrations and high fluxes. Ethanol is highly polar, therefore, continuous extraction of ethanol would be difficult, and further conversion to longer-chain chemicals that are easier to separate from the aqueous medium (e.g. medium-chain carboxylates, described below) might be a solution.

Chain elongation to medium-chain carboxylates

An undefined mixed culture that is capable of reducing acetate to ethanol (Figure 1e) can also produce *n*-butyrate (0.61 g/l) by further reaction of ethanol with acetate (Figure 1g; Box 3) [27]. Thus, these two secondary fermentation processes allow one undefined mixed culture to convert acetate and hydrogen to *n*-butyrate by elongation of the acetate carbon chain. Further optimization is needed

Box 2. Liquid/liquid extraction to separate acetate

Currently, acetate is primarily produced petrochemically or via microbial fermentations that convert yeast-derived ethanol to acetate, but it is also possible to develop an undefined-mixed-culture process with high acetate yields. To do this, it is useful to consider two systems with a high acetate flux: a termite gut and an anaerobic digester. In both systems, a maximum acetate flux from polysaccharide breakdown (Figure 1a) occurs because a low hydrogen partial pressure is maintained by scavenging homoacetogens (Figure 1c) [62,63] and hydrogenotrophic methanogens (Figure 1d) [41]. However, accumulating the product acetate would quickly inhibit these hydrogenotrophs, which results in rising hydrogen partial pressures and a shift towards increasingly reduced fermentation products. Anaerobic digesters can maintain a sustainably high acetate flux because acetate is converted and removed by aceticlastic methanogens (Figure 1f). Termites have another strategy: they take up acetate. Thus, to produce acetate at high yields, both hydrogen and acetate should be maintained at low concentrations in the mixed culture.

One possibility to remove acetate from our engineered systems is with continuous liquid/liquid extraction by using membranes (i.e. perstraction) [31]. This separation technology has been shown to improve carboxylate yields and selectivity for pure cultures and might hold promise for undefined mixed cultures [38,46]. Liquid/liquid extraction of organic acids is traditionally achieved by contacting the fermentation broth with an organic solvent phase (often a substituted amine or phosphine, such as trioctylphosphine oxide, dissolved in an alkane), followed by contact of the organic phase with an aqueous alkaline phase. In this way, undissociated acids move along a pH gradient, and the dissociated form of the carboxylate concentrates in the alkaline phase. Dissolution in the organic phase is particularly effective for relatively hydrophobic acids, such as *n*-butyrate and *n*-caproate (although it is much easier to separate the C6 *n*-caproate than the C4 *n*-butyrate), whereas shorter chain molecules, such as the C2 carboxylate acetate and the C3 carboxylate propionate, have always presented greater challenges [44]. Extraction of acetate has become more feasible recently by using ionic liquids as the organic phase [64].

to accelerate the production rates, but conversion of the intermediate ethanol to *n*-butyrate is logical because, as discussed above, ethanol accumulation renders the biological reduction reaction thermodynamically unfeasible. It is important to understand that hydrogenotrophic and aceticlastic methanogens must be completely inhibited by heat-shocking the inoculum, lowering the pH, or by adding a methanogenic inhibitor (e.g. 2-bromoethanosulfonic acid), because methanogenesis competes with the desired reaction.

Box 3. Biological chain elongation

Biological chain elongation includes reversed β-oxidation of carboxylates – the addition of a two-carbon acetyl-CoA derived from ethanol, which has been described for a pure culture of *Clostridium kluyveri* [65]. Although reversed β-oxidation of acetate with ethanol is thermodynamically feasible ($\Delta G_r^{0'} < -23.3$ kJ/mol), it cannot gain ATP, and the oxidation of ethanol to acetate is required to conserve the necessary energy. The latter reaction has a positive free energy in acidogenic systems with high hydrogen partial pressures, and thus the ethanol oxidation and reversed β-oxidation reactions must be coupled (Table 1). The combined process has a sufficiently negative free energy under biological standard conditions ($\Delta G_r^{0'} = -194.5$ kJ/mol at 1 atm and 37 °C) (Table 1). Through this process, 5 mol ethanol and 5 mol acetate are converted to 5 mol *n*-butyrate for every 1 mol ethanol oxidized to acetate [66]. It has been known for several decades that *C. kluyveri* can also generate *n*-caproate [65], which is a C6 carboxylate. The process is analogous to *n*-butyrate formation through chain elongation, and results in the same two-carbon-atom elongation, but now through reversed β-oxidation of *n*-butyrate with ethanol ($\Delta G_r^{0'} = -182.8$ kJ/mol for *n*-butyrate at 1 atm and 37 °C) (Table 1).

Further chain elongation reactions can also occur, ultimately converting acetate to *n*-caproate (C6) (Figure 1g) and even *n*-caprylate (C8). *n*-Caproate concentrations of 8.27 g/l and *n*-caprylate concentrations of 0.32 g/l have been observed when acetate and hydrogen (or ethanol) are provided to undefined mixed cultures [24]. These concentrations approach the solubility limits for these carboxylates (*n*-caproate: 10.19 g/l; *n*-caprylate: 0.79 g/l). The rate of *n*-caproate formation is promising ($0.49\ g\ l^{-1}\ day^{-1}$) at neutral pH [24]. Both of these compounds have excellent energy densities, might be relatively easy to separate from the fermentation broth, and both can be precursors for production of biodiesel and fuel alkanes by chemical post-processing reactions (Figure 2) [29]. These chain-elongation reactions proceed most effectively when ethanol is available as the reducing agent and when reduction of acetate to ethanol occurs at a relatively slow rate; however, it might be necessary to add a separate stream of ethanol to produce *n*-caproate and *n*-caprylate economically. This ethanol might have to come from the sugar platform in the biorefinery concept because of the cumbersome production and extraction of ethanol from undefined mixed cultures. An in-depth economic analysis is needed to investigate if the higher energy density of a fuel derived from medium-chain carboxylates and the superior separation characteristics warrant the use of ethanol in an integrated biorefinery.

Propionate and lactate

Propionate

Propionate is one of the reduced products of primary fermentation at elevated levels of hydrogen (Figure 1). Under anaerobic conditions, propionate can only be oxidized when the hydrogen partial pressure is extremely low (Box 1; Table I). Microbial production of propionate from industrial waste has been studied primarily with pure cultures and has been plagued by microbial toxicity of the accumulating undissociated propionic acid at low pH values [30]. To circumvent propionate accumulation, *in situ* extraction provides some improvement over traditional fermentation, but the short chain length of propionate makes it relatively difficult to extract [31].

Undefined mixed cultures can reduce propionate to propanol (Figure 1e). Relatively high rates of propionate reduction to propanol ($0.49\ g\ l^{-1}\ day^{-1}$) have been observed in a continuous-flow bioreactor when propionate and ethanol are provided (Figure 1e) [32], even though the calculated free energy of the coupled reaction is below the expected threshold value (Table 1; Box 1). Others have shown propionate-to-propanol reduction in the absence of ethanol oxidation at elevated hydrogen partial pressure, but the rates are lower ($0.03\ g\ l^{-1}\ day^{-1}$), even though the calculated free energy change is sufficient (Box 1) [23]. The preference for ethanol as a reducing agent might be attributed to the availability of a simple mechanism for energy conservation during ethanol oxidation.

Biological chain elongation can be used to convert propionate into *n*-valerate (i.e. addition of two carbon atoms)

[32,33]. The process is similar to that described for elongation of the acetate carbon chain (C2), except that uneven-chain-length carboxylates, such as *n*-valerate (C5) and *n*-heptanoate (C7), are produced. *n*-Butyrate (C4) and *n*-caproate (C6) are also produced in this process because ethanol is oxidized to acetate, which can start the even-chain carboxylate elongation process, to provide energy for microbial growth (Table 1).

Lactate

Lactate fermentation (Figure 1) dominates primary fermentation in undefined mixed cultures when high concentrations of easily degradable substrate are available, because the lactate pathway enables rapid disposal of reducing equivalents [34]. This phenomenon of lactate accumulation occurs in the rumen with diets that are high in grain [35] and in fermentation processes used to preserve plant material, such as in silage and sauerkraut fermentation [36]. In fact, bio-energy crops, such as maize, are sometimes ensiled as a pretreatment and storage step before anaerobic digestion at full-scale installations (primary and secondary fermentations in different processes). To the best of our knowledge, mixed cultures have not been used specifically to produce useful quantities of pure lactate, but pure cultures have been used to produce lactate in an optically pure (only D- or L-isomer) form [37] aided by *in situ* lactate extraction [38].

Lactate can be oxidized and reduced by secondary fermentation reactions to other carboxylates with undefined mixed cultures, such as in the gut [39]. For example, lactate oxidation to *n*-butyrate (Figure 1i) is catalyzed by *Clostridium acetobutylicum* [40]. However, this reaction must be coupled to acetate reduction to become energetically feasible (Table 1), effectively converting both acetate and lactate to *n*-butyrate. Another secondary fermentation pathway is lactate reduction to propionate (Figure 1j), which is catalyzed by *Selenomonas ruminantium* [41]. In this pathway, energy is conserved as ATP during acetate production while it is coupled to lactate reduction (Table 1), which results in the conversion of three molecules of lactate into one of acetate and two of propionate. The former pathway to convert lactate into *n*-butyrate might actually add more value than the latter pathway, because chemicals with a higher carbon chain are generally energetically superior and easier to separate.

n-Butyrate

n-Butyrate is usually a product in undefined-mixed-culture acidogenic systems, from both primary and secondary fermentation pathways. It has often been found to be the most important side product during biological hydrogen production with dark fermentation [42]. Similar to propionate and lactate, *n*-butyrate production with undefined mixed cultures has been largely ignored. Problems with bacterial production with pure cultures include low yields owing to product toxicity at lower pH levels and product streams contaminated with co-products, such as acetate and propionate [43]. As a result of the longer carbon chain for *n*-butyrate compared with acetate and propionate, the extraction of *n*-butyrate might become feasible in the future, especially by using ionic liquids [44,45]. Pure culture fermentation studies have already reported improved *n*-butyrate yields and product purity with *in situ* liquid/liquid extraction, and this technology should considerably improve the prospects of producing *n*-butyrate with undefined mixed cultures [46].

n-Butyrate is an excellent feedstock for the production of *n*-butanol with pure cultures of *Clostridium* spp., using organic electron donors (Figure 2) [47]. Therefore, we have proposed coupling *n*-butyrate production by an undefined mixed culture with a pure culture bioprocess that would reduce *n*-butyrate to *n*-butanol (sugars are necessary for reducing equivalents) [3]. Recently, the feasibility of reducing *n*-butyrate to *n*-butanol with an undefined mixed culture has been demonstrated by using high partial pressures of H_2 (Figure 1e), although this biological reduction is slow. Others have also found chain elongation of *n*-butyrate with undefined mixed cultures [32,33,48,49]; but, to the best of our knowledge, never by feeding a separate stream of *n*-butyrate.

Mixed carboxylates

Rather than optimizing the production and separation of a single carboxylate as a bulk feedstock, researchers have developed systems for which the product spectrum is mixed and variable. In some cases, the carboxylate products are converted into a blend of liquid fuels and organic chemicals by chemical post-processing (Figure 2). For example, one system has converted wastes into carboxylates using an undefined mixed culture, followed by electrochemical conversion into either esters (Figure 2) or a mixture of alkanes and alcohols [50]. The authors have proposed using liquid/liquid extraction to remove and concentrate the carboxylates before post-processing. Another system, the MixAlco process, has been tested for many different feedstocks [51]. For example, fermentation studies with pretreated municipal solid waste and sugarcane bagasse as substrate have achieved up to 69% and 60% degradation of volatile solids, with maximum total carboxylate concentrations of 20.5 g/l and 18.7 g/l, respectively [52,53]. The MixAlco system concentrates the carboxylates by drying and calcium precipitation. The carboxylate mixture is thermally decomposed to ketones, and the ketones are catalytically hydrogenated to a mixture of alcohols (Figure 2).

Outlook

The volume of organic waste will drastically rise when crude lignocellulosic or algal biomass is converted to liquid biofuels in biorefineries using the sugar and syngas platforms. Integration of the carboxylate platform into the biorefinery concept could increase bioproduct formation and recover nutrients and water that can be recycled within the biorefinery, thereby serving as a crucial component of biorefineries. Anaerobic digestion is the only bioprocess within the carboxylate platform that is currently utilized for complex waste treatment on a large scale, because production of liquid chemicals presents important scientific and technical challenges that production of gaseous methane does not. Three major barriers must be overcome: (i) the separation barrier (efficient separation of carboxylates from fermentation broth); (ii) the methanogen barrier (economic inhibition

of hydrogenotropic methanogens); and (iii) the ecology barrier (directing the microbial process to generate the target carboxylates at sufficient rates).

Two promising research directions are being pursued to overcome the separation barrier. First, laboratory-scale BESs have used ion-exchange membranes to separate production of organic chemicals at cathodes from anaerobic microbial processes that occur at anodes [22,28]. Second, medium-chain carboxylates, such as *n*-caproate and *n*-caprylate, have been produced by undefined mixed cultures at promising rates [24]. For the first time, this would allow undefined mixed cultures to produce liquid chemicals with an energetic value that is equivalent to *n*-butanol, and with chemical characteristics that ensure superior extraction.

These developments provide incentives to address seriously the methanogen barrier. The *n*-caproate and *n*-caprylate experiments described in this review were all performed by adding bromoethane sulfonic acid [24,27], which is too expensive for use on a large scale. If a cheaper chemical that selectively inhibits methanogens cannot be developed, two alternative methods must be pursued: periodic heat shock, or low pH (~5.5). The former might be possible when enough waste heat is available in the biorefinery to perform this periodic activity to enrich for spore-forming bacteria while inhibiting methanogens. The latter might be especially effective at a combination of low pH and high concentrations of carboxylic acids, owing to a relatively high abundance of the inhibiting undissociated form. These methods could be aided by managing trace elements carefully; for example, cobalt is not available in corn-derived waste, and its continued absence severely limits methanogenic activity [54].

We must realize, however, that these methods, such as the slightly acidic conditions (pH ~5.5), might be incompatible with the conditions needed to overcome the ecology barrier. Microbial ecology cannot be uncoupled from thermodynamic considerations, and this interplay is important to predict and manage the behavior of complex communities. The environmental conditions, in turn, determine which reactions are thermodynamically feasible and which microbes are selected [11]. Indeed, the relative product composition of sugar-fed acidogenic reactors with high hydrogen partial pressures has been affected by environmental conditions [48,55,56]. In a full-scale system, several environmental conditions will be varying constantly owing to the complexity and variability of organic wastes. It is therefore important to perform experiments to predict which environmental conditions have the largest effects on community composition. A combined approach of high-throughput metagenomics and massive environmental data monitoring is necessary to find correlations between environment and community [57]. In addition, ecological principles can aid in selecting for superior communities that, for example, are rich in parallel metabolic pathways [58], have high evenness [5], and are either resistant, resilient, or redundant [59] to sustain a stable bioprocess. Based on all this knowledge, the engineer must make decisions on how to design, inoculate, and operate the full-scale system to obtain the sufficient kinetic rates and yields for a viable bioproduct. We realize, however, that some breakthroughs, such as better separation technologies, still need to be made before success can be achieved.

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