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Chapter 7

Anaerobic fermentation technologies for the production of chemical building blocks and bio-based products from wastewater

Pieter Candry[1,2], José Maria Carvajal-Arroyo[1,2], Steven Pratt[3], João Sousa[4], Çağrı Akyol[5,6], Francesco Fatone[5] and Ramon Ganigué[1,2]

[1]*Centre for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, 9000 Ghent, Belgium*
[2]*Centre for Advanced Process Technology for Urban REsource Recovery, Frieda Saeysstraat 1, 9052 Gent, Belgium*
[3]*School of Chemical Engineering, The University of Queensland, St Lucia, QLD 4072, Australia*
[4]*Paques Technology B.V., T. de Boerstraat 24, 8561 EL, Balk, The Netherlands*
[5]*Department of Science and Engineering of Materials, Environment and Urban Planning-SIMAU, Marche Polytechnic University, Via Brecce Bianche 12, 60100 Ancona, Italy*
[6]*Department of Green Chemistry & Technology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium*

7.1 INTRODUCTION

Anaerobic digestion (AD) is currently one of the most widely applied technologies for the treatment and valorization of organic waste streams as electrical and/or thermal energy (as illustrated in Chapter 5). Yet, the economic value of the produced biogas is rather low (Table 7.1), which has led to an increasing interest in developing anaerobic based technologies that can produce high(er)-value products – beyond biogas – to increase the economic profits from organic waste streams. Within this context, the production of short-chain carboxylic acids (SCCA) and their derivative products have gained significant interest in recent years.

As discussed in detail in Chapter 5, AD is a multi-step biochemical process, breaking down the complex organic matter into, ultimately, CH_4 and CO_2. In the acidogenic step, organic monomers are fermented to SCCA, organic acids with one carboxyl group and a saturated chain of 2–5 carbon atoms. SCCA hold a higher economic value than CH_4 (Table 7.1) and can be further upgraded to a wide variety of consumer products through chemical and/or biological processes. Some of these applications include, but are not limited to, the production of polyhydroxyalkanoates (PHAs, a type of bioplastics), esters (fragrances and flavours), and solvents (Albuquerque *et al.*, 2011; Zacharof & Lovitt, 2013). SCCA can also be converted to medium chain carboxylic acids (MCCA) microbially. These can be used as such as anticorrosion agent or antimicrobial agent in, for example, animal rearing, but can also be processed into biofuels, bioplastics and other consumer goods (Angenent *et al.*, 2016). Halting anaerobic digestion at the level of SCCA formation would allow for the redirection of the AD process from a low-value energy recovery to a higher-value chemical production platform. In this way, the economics of the process could be substantially improved.

Table 7.1 Economic values for various products that can be obtained through anaerobic treatment of waste streams with mixed cultures.

Product	Market Price (€·tonne^{-1})	Market Price (€·tonne C^{-1})	Market Price (€·tonne COD^{-1})
CH_4	90–200	120–267	20–50
Acetic acid	330–670	825–1675	310–630
Propionic acid	1250–1380	2570–2840	830–910
Butyric acid	1670–2090	3060–3830	920–1150
Lactic acid	840–1510	2100–3775	790–1410
Caproic acid	1880–2090	3030–3370	850–950

Prices were obtained from Moscoviz *et al.* (2018).

This chapter will first introduce the microbiology and biochemistry of carboxylic acid production (both SCCA and MCCA), knowledge not only relevant to the chapter at hand, but also to preceding chapters that discuss anaerobic digestion (Chapters 5 and 6). Gaining a fundamental understanding of the microbiology and biochemistry is essential prior to describing the more engineering orientated design considerations and applications of this platform. A product is only valuable if it can be recovered and separated. As such, the general principles of the main downstream approaches to recover carboxylates from fermentation broths and/or upgrade them to value-added products are subsequently discussed. Last, the chapter will touch upon some of the research needs as well as the key challenges and opportunities for SCCA and MCCA production to become viable technologies that can be implemented by the water industry at full-scale.

7.2 LEARNING OBJECTIVES

At the completion of this chapter you should be able to:

- Understand what the carboxylate platform is and why it is an interesting emerging approach to produce chemical building blocks from wastewater (and other waste streams).
- Gain a fundamental understanding of the different metabolic steps in the production of carboxylic acids, their microbiology and biochemistry and understand the implications for practice.
- Outline the main physico-chemical routes to recover carboxylates/carboxylic acids from fermentation broths and understand their basic engineering principles.
- Explain how the intermediates of the acidogenic fermentation can be biologically converted to alternative higher-value end-products.
- Describe the current status of SCCA and MCCA production from wastewater (and derivatives) and outline the key challenges and opportunities.
- Discuss the economic rationale driving the development of carboxylic acid-producing technologies, their inherent advantages – and disadvantages – compared to AD, and how are these connected to the technical challenges ahead of it.
- Synthesize all the knowledge in this chapter and describe how you would turn anaerobic digesters into reactors for the production of carboxylic acids.

7.3 MICROBIOLOGY AND BIOCHEMISTRY OF CARBOXYLIC ACID PRODUCTION

Organic waste streams – such as waste activated sludge – are a complex mixture of carbohydrates, proteins and fats. As explained in Chapter 5, AD can break down this complex organic matter in a four-step microbial process: (i) hydrolysis of the complex organic matter to release the biochemical building blocks, that is monosaccharides, amino acids and fatty acids; (ii) acidogenesis, in which these

building blocks are fermented to a mixture of SCCA; (iii) acetogenesis, that is a secondary fermentation converting the mixture of SCCA to acetic acid, H_2 and CO_2; and lastly, (iv) methanogenesis, converting the acetic acid, H_2 and CO_2 to CH_4. This metabolic network changes when the target is shifted from CH_4 to carboxylic acids; complex organic matter is still hydrolyzed and fermented to a mixture of SCCA, but acetogenesis and methanogenesis are suppressed. Potentially, under certain conditions the end-products of acidogenesis can be further converted to, for example, other SCCA or MCCA in secondary microbial processes. The following sections will introduce the main processes involved in the transformation of complex organic material (e.g., sludge) into mixtures of SCCA, lactic acid and ethanol (i.e., hydrolysis and acidogenic fermentation), and their potential further conversion to MCCA.

7.3.1 Hydrolysis

Hydrolysis is the process of breaking up polymers, for example polysaccharides, proteins and lipids, into their monomeric building blocks – respectively monosaccharides, amino acids and fatty acids. During this process, a molecule of water is used to break the bonds between the monomer units (Figure 7.1).

Although these reactions are catalyzed enzymatically, organisms do not receive energy from this process. It is, however, necessary to gain access to the monomer units, which can be then used for energy generation. With most substrates – also waste activated sludge – hydrolysis is the rate limiting step in AD. It is usually modelled by a first order degradation kinetic, that is the hydrolysis rate depends on the concentration of degradable particulate organic matter (Equation (7.1)) (Batstone *et al.*, 2002; Pavlostathis & Giraldo-Gomez, 2009):

$$\frac{dF}{dt} = -k_h \cdot F \tag{7.1}$$

where F is the concentration of degradable particulate organic matter ($g \cdot L^{-1}$) and k_{hyd} is the hydrolysis constant (d^{-1}).

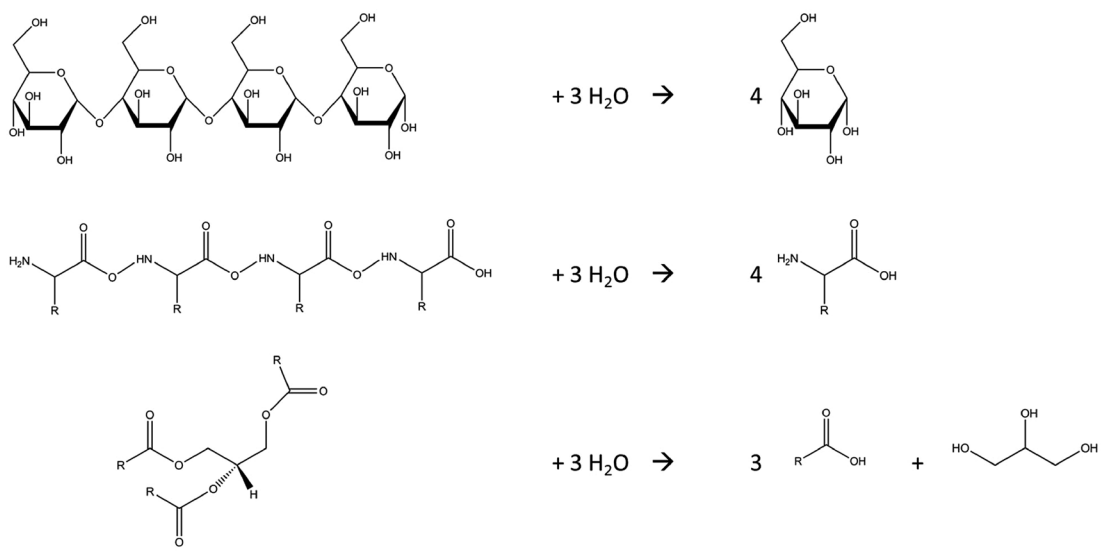


Figure 7.1 Schematic representation of hydrolysis for polysaccharides, amino acids and lipids.

Hydrolysis constants are mostly dependent on: (i) the nature of the material to be degraded (i.e., carbohydrate, protein, lipid); (ii) the temperature; (iii) pH; and (iv) the particle size and its surface area (Vavilin *et al.*, 2008). As such a broad range of constants can be found in literature. For instance, k_{hyd} in the order of 0.005–0.01, 0.015–0.075 and 0.025–0.2 d^{-1} have been reported for the hydrolysis of lipids, proteins and carbohydrates in solid organic waste at 55°C. The anaerobic mesophilic hydrolysis constants of protein-rich waste activated sludge from municipal WWTPs are usually in the order of 0.1–1 d^{-1} (Christ *et al.*, 2000; Sanders, 2001; Tomei *et al.*, 2009; Vavilin *et al.*, 2008). It should be mentioned that feedstocks can be highly varied in their composition (i.e., proportions of carbohydrates, protein, lipids), and by extension also in the rate of hydrolysis and the rate of the process.

7.3.1.1 Hydrolysis of polysaccharides

Carbohydrates may be an important constituent in industrial wastewater but are usually not present at high concentrations in waste activated sludge, due to the aeration steps removing most of these compounds. However, co-digestion of waste activated sludge with more degradable carbohydrate-rich feedstocks has been suggested as a means to improve digestibility (Sosnowski *et al.*, 2003). Carbohydrates in such organic waste streams are often present in the form of polysaccharides such as starches and (hemi)-celluloses. These compounds are essentially chains of saccharides, often high in D-glucose, but other saccharides can be present as well. As a first step, these chains need to be broken up into mono- or oligosaccharides before they can be fermented. In the case of starches, this can be done by a group of enzymes called amylases, ubiquitous throughout all domains of nature (Tester *et al.*, 2006). Even though cellulose chains are also predominantly made up of D-glucose molecules, like starches, the cellulase enzymes necessary to break up these chains are less widespread throughout nature. Fungi are well-known cellulose degraders, and cellulolytic activity is widespread throughout the family. However, only few genera in Bacteria contain cellulolytic organisms, and generally only a few species within one genus are active cellulose degraders (Lynd *et al.*, 2002). This is partly due to the different configuration of the cellulose chain – glucose units are linked with β(1-4)-bond unlike the α(1-4) bond in starches – as well as the insoluble nature of celluloses, which require cellulolytic enzymes to be functional outside the cell. Some examples of anaerobic cellulose degraders are *Clostridium thermocellum*, *Clostridium cellulolyticum* and *Butyrivibrio fibrosolvens* (Koeck *et al.*, 2014). Despite the apparent phylogenetic sparseness of cellulose-hydrolyzing organisms, there is usually no need to add hydrolyzing organisms or enzymes to waste activated sludge: the native community always contains some hydrolytic organism that can take up this role to liberate the solid organic matter for further conversion.

7.3.1.2 Hydrolysis of proteins

Proteins are essentially a chain of different amino acids, which then assemble into a 3D structure. During the hydrolysis, protease enzymes break the peptide bonds in the protein, releasing the individual amino acids, or short chains of amino acids, called peptides. Commercial production of proteases has been demonstrated with *Bacillus* and *Clostridium* genera (Rao *et al.*, 1998; Siebert & Toerien, 1969), yet, again, there is usually no need to add specific hydrolytic organisms or enzymes to waste feedstocks, as protease-producing organism can be readily found in nearly all environments.

7.3.1.3 Hydrolysis of fats

Wastewater and microbial biomass contain fats, which are made up of triglycerides, composed of a glycerol backbone linked to three fatty acid molecules via ester bonds. Fats can be hydrolyzed by lipases, which cut the ester bonds, resulting in the production of one molecule of glycerol and three of fatty acid, the length of which is dependent on the type of fat (Madigan *et al.*, 2012). These lipase enzymes are ubiquitous throughout all domains of life, and a wide range of bacteria have been employed to produce these enzymes on an industrial scale for enzymatically catalyzed processes (Javed *et al.*, 2018), but for (anaerobic) degradation of waste sludge, lipase producing organisms are commonly present in mixed communities.

7.3.2 Primary fermentations

Monomers generated during the hydrolysis step can subsequently be utilized by microbes. Under anaerobic conditions the lack of external electron acceptors forces microorganisms to metabolize these monomers via fermentation. Fermentation is an anaerobic redox process, in which the oxidation of the substrate is coupled to the reduction of another substrate or an intermediate derived from the oxidation, with the difference in redox potential of the substrate and the end product providing energy for ATP synthesis (Müller, 2008). Some special cases exist where the oxidation of one substrate is linked to the reduction of another (organic) substrate, for instance Stickland reactions involved in protein degradation.

7.3.2.1 Primary fermentation pathways for saccharides

Monosaccharides can be utilized in a wide range of anaerobic fermentative metabolisms. Monosaccharides contain either five carbon atoms (C5 monosaccharides, e.g., xylose, arabinose, etc.) or six carbon atoms (C6 monosaccharides, e.g., glucose, fructose, etc.). Different metabolisms are involved in the fermentation of C5 and C6 monosaccharides, affecting product and energy yields for fermentative bacteria. During fermentation of C6 monosaccharides, glycolysis first converts glucose through glyceraldehyde-3-phosphate to pyruvic acid, as depicted in Figure 7.2. This can in turn be: (i) reduced to succinic acid and/or propionic acid; (ii) reduced to lactic acid; or (iii) decarboxylated to acetyl-CoA resulting in the release of either a formic acid molecule or CO_2 and H_2 molecules, depending on the pH. The formed acetyl-CoA can subsequently be converted to various products such as ethanol, acetic acid, or reduced to butyryl-CoA, followed by conversion to butyric acid (Temudo *et al.*, 2007). Which of these reactions will occur depends on the fermentative pathway(s) present in the organism in the system.

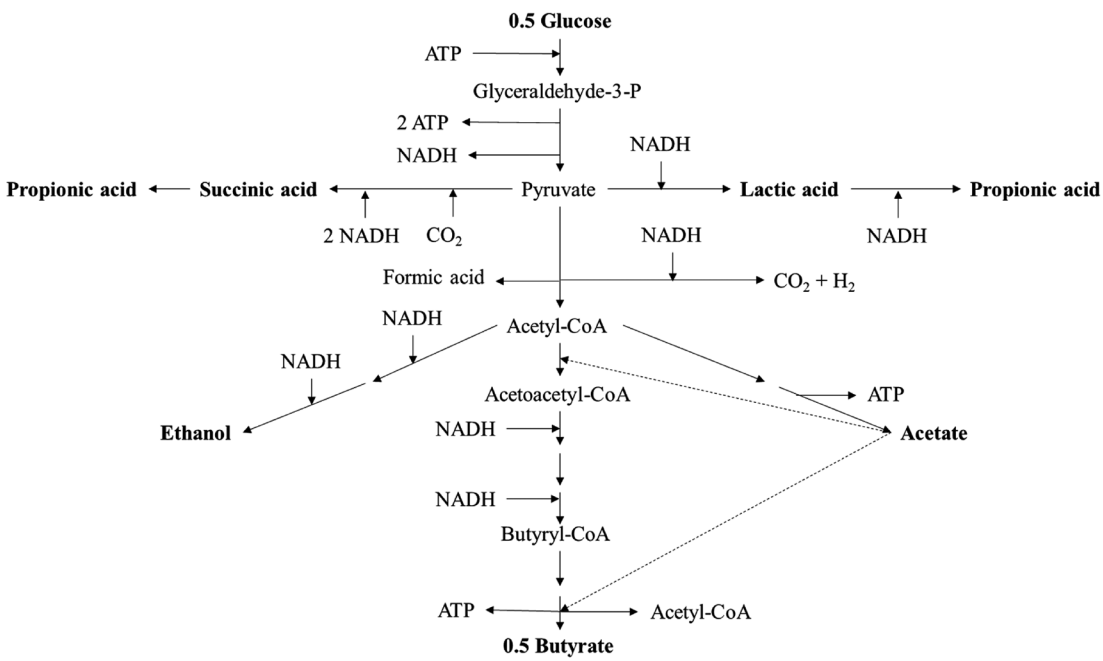


Figure 7.2 Overview of potential fermentation pathways during mixed culture fermentation of C6 saccharides, using glucose as a model compound. Adapted from Temudo *et al.* (2007).

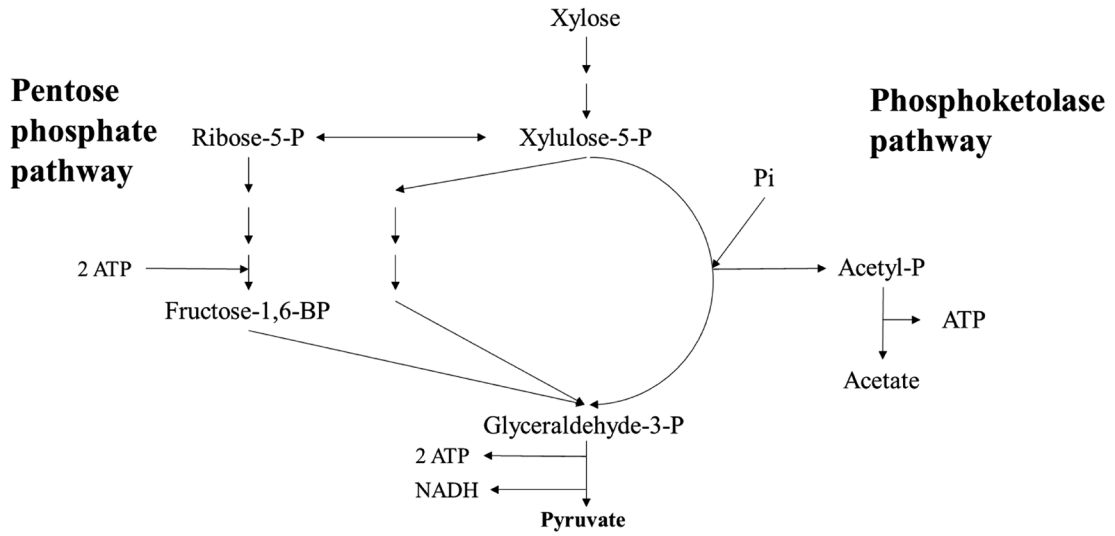


Figure 7.3 Initial conversion steps of C5 saccharides to pyruvate, with xylose as a model compound. After pyruvate, the same pathways as for C6 saccharides are possible. Figure adapted from Temudo *et al.* (2009).

Fermentation of C5 monosaccharides differs from that of C6 mostly in the initial steps of the fermentation process. While they are also converted to glyceraldehyde-3-P and subsequently pyruvate, this conversion goes through specific pathways for C5 monosaccharides, as depicted in Figure 7.3 for xylose. In short, the pentose phosphate pathway (PPP) converts part of the xylose to ribose-5-P and part to xylulose-5-P. These are then used to form fructose-1,6-BP, followed by conversion into glyceraldehyde-3-P. Ultimately, for every three molecules of xylose converted, five molecules of pyruvate are produced, with production of five ATP and five NADH. An alternative pathway, the phosphoketolase pathway (PKP), also goes through xylulose-5-P. However, conversion of xylulose-5-P to glyceraldehyde-3-P in the PKP also generates acetyl-P, which will in turn be used to produce acetic acid and ATP. This means that every molecule of C5 monosaccharides converted through the PKP will always yield one molecule of acetic acid on top of the product obtained from pyruvate. Since both the PPP and PKP yield pyruvate, the same products can be obtained as from C6 monosaccharides (Temudo *et al.*, 2009).

An intermediate version of these two metabolisms is heterolactic fermentation, as performed by *Leuconostoc* bacteria. In this fermentation process, glucose is converted to xylulose-5-P by liberating one molecule of CO_2. The xylulose-5-P is then further converted over the phosphoketolase-pathway as mentioned above. The pyruvate obtained is converted to lactate, while the acetyl-CoA can be converted to ethanol. This yields equimolar amounts of lactic acid and ethanol from glucose (Demoss *et al.*, 1951; Dols *et al.*, 1997).

Ultimately, the metabolic potential of an open, undefined microbial community will enable conversion of the saccharide building blocks to a mixture of the products mentioned above. The product profile will be determined by: (i) the microbial community present; (ii) operational parameters, for example pH, temperature, substrate concentration; and (iii) type of saccharides present after hydrolysis.

7.3.2.2 Primary fermentation pathways for amino acids

The amino acids released during hydrolysis of proteins can be further fermented towards a range of products, yielding one molecule of carboxylic acid, NH_3 and CO_2 per amino acid fermented.

The carboxylic acid produced is amino acid-specific, although it is in most cases acetic acid. The most common mechanism for amino acid fermentation is through Stickland reactions (coupled fermentations). In this pathway, two amino acids are concomitantly degraded, where one of the acids is oxidized and acts as electron donor and the other is reduced by accepting these electrons. The electron-donating amino acid is deaminated (i.e., release of NH_3) and decarboxylated (i.e., release of CO_2), and subsequently oxidized to an SCCA with a carbon chain shortened by one carbon atom. During this process, electrons are released, which are used to reduce the deaminated electron accepting amino acid to a SCCA with a carbon chain of the same length as the original amino acid. Besides SCCA, also branched carboxylic acids (e.g., iso-valeric acid or iso-caproic acid), alcohols and aromatic compounds (e.g., phenol, cresol) can be released during fermentation of amino acids, depending on the initial structure of the amino acid (Madigan *et al.*, 2012). Alternatively, amino acids can be fermented individually, although not all amino acids can be converted this way. Again, the products obtained depend on the amino acid substrate (Elsden & Hilton, 1978). In a wastewater context, the range of amino acids present in waste sludge is usually very broad, and steering protein fermentations is highly challenging. A last pathway for conversion of amino acids is the Ehrlich pathway, found in yeasts (e.g., *Saccharomyces cerevisiae*). Here, proteins are first converted to an aldehyde, which is then used to produce an acid or ester, and an (amyl) alcohol (Hazelwood *et al.*, 2008). It needs to be noted that these yeasts are usually not present in wastewater settings, and the contribution of the Ehrlich pathway to protein degradation from waste sludge will be negligible.

In general, amino acid fermentations are mostly carried out by gram-positive bacteria, principally from the genus Clostridia. These organisms are key members of nearly all anaerobic, fermenting communities, so amino acids in waste streams can be readily degraded in most applications. The fermentation stoichiometry of each specific amino acid and the microbiology of amino acid fermentation are extensively reviewed elsewhere and will not be further discussed in this chapter (Mead, 1971; Ramsay & Pullammanappallil, 2001).

7.3.2.3 Primary fermentation pathways for long-chain fatty acids (LCFA)

Breakdown of LCFA happens through a cyclical pathway called beta-oxidation. In this pathway, a coenzyme A-group is attached to the carboxylic group, forming an acyl-CoA. This acyl-CoA is then degraded in a cyclical process, releasing one molecule of acetyl-CoA and one NADH molecule per turn of the cycle, resulting in an acyl-chain shortened by two carbon units (Madigan *et al.*, 2012). The intracellular pool of $NADH/NAD^+$ is limited, which means the generated NADH needs to be quickly re-oxidized back to NAD^+ to balance the $NADH/NAD^+$-pool. Under aerobic conditions, NADH oxidation can be combined with the reduction of O_2 to H_2O. Due to the lack of oxygen under anaerobic conditions, this can only be achieved through reduction of H^+ to H_2. This process becomes thermodynamically unfeasible at H_2 partial pressures over approximately 100 Pa (equal to 0.01% H_2 in the headspace of the system), implying H_2 has to be removed immediately for this reaction to continue. In anaerobic digestion, it has been shown that a syntrophy between an anaerobic fatty acid oxidizer and hydrogenotrophic methanogens provides the low H_2 partial pressures required for this process (Conrad *et al.*, 1986). However, in fermentations aimed at carboxylic acid production no H_2-scavenger can sufficiently lower H_2 partial pressures to enable continued LCFA-degradation. This means lipids-rich wastes are challenging feedstocks for the production of high-value products. So far, no reports have targeted such wastes for bioproduction processes beyond lower-value applications such as biogas production through anaerobic digestion.

7.3.2.4 Practical implications

In the previous sections, we offered an overview of the metabolic pathways involved in the primary fermentations from different substrates commonly found in organic waste feedstocks such as waste sludge. While many products can be formed, the heterogeneity and complexity of the feedstocks relevant in wastewater settings means that steering these processes is highly challenging. The large

volumes and complexity of the substrate excludes the application of pure cultures for bioproduction, and technologies rely on mixed consortia to provide us with high-value products. Yet, obtaining a pure product profile made up of only one product from real wastes has not yet been demonstrated. Even finding general trends in operational parameters (e.g., pH, organic loading rate, organics concentration, etc.) to steer the fermentation process has proven highly challenging (Arslan *et al.*, 2016). Consequently, most applications developed so far address this by upgrading the mixed product profile, an approach that will be discussed in section 7.4.

7.3.3 Secondary anaerobic conversions

The products of a primary fermentation can be further transformed anaerobically into other compounds. An example found in AD is acetogenesis, where the mixture of products (e.g., SCCA, lactic acid, ethanol) from acidogenesis are converted to acetic acid through anaerobic oxidation. On the other hand, in the context of resource recovery and bioproduction from waste streams, other anaerobic biotransformations aim to increase the value of the primary fermentation products. Depending on the target product, it can be necessary to add carbon or electron sources, such as ethanol or lactic acid, to stimulate these secondary processes and/or physically separate primary and secondary biotransformations.

7.3.3.1 Secondary fermentations to SCCA from lactic acid

Lactic acid is a common product formed in fermentations of carbohydrates, as discussed in section 7.3.2.1. Lactic acid bacteria are renowned for their relatively high growth rates and tolerance to low pH values, giving them a competitive advantage over other fermentative bacteria (Castillo Martinez *et al.*, 2013). Lactic acid can in turn be further fermented to other SCCA. Lactic acid can either be: (i) converted to acetyl-CoA, after which it used to produce acetic acid, or butyric acid (cf. Figure 7.2); or (ii) converted to propionic acid with either acrylyl-CoA – via the acrylate pathway – or succinate – Wood–Werkman cycle – as key intermediates (Parizzi *et al.*, 2012; Prabhu *et al.*, 2012).

7.3.3.2 Reverse beta-oxidation with ethanol as electron donor

As mentioned earlier in this chapter, the production of MCCA has gained interest as a way to recover high-value chemicals from waste streams. The microbial production of MCCA in itself is not new and has been known since the early 1940s (Barker, 1941), when a mesophilic anaerobic bacterium capable of using ethanol and acetic acid to produce caproic acid (C6) (*Clostridium kluyveri*) was isolated from canal mud. For a long time, *C. kluyveri* was of special interest to microbiologists due to its unique metabolism, named reverse beta-oxidation, by which it adds two carbon units to the acyl chain of a monocarboxylic acid per turn of the cycle. This metabolism has been elucidated over the years, its reaction stoichiometry is shown in Table 7.2, and is schematically represented in Figure 7.4. The process starts by the oxidation of six molecules of ethanol to acetyl-CoA, generating 12 molecules of NADH. One of the six molecules of acetyl-CoA is further converted into acetic acid, generating one ATP from substrate-level phosphorylation. The other five molecules of acetyl-CoA go into the reverse beta-oxidation cycle, combining with five molecules of acetyl-CoA to form acetoacetyl-CoA, which are further transformed into butyryl-CoA with the consumption of 15 NADH molecules. In a final step, these five molecules of butyryl-CoA then combine with five molecules of acetic acid, releasing five molecules of butyric acid, and five molecules of acetyl-CoA that then go back to the start of the cycle. This process can also be repeated by combining acetyl-CoA from ethanol oxidation with butyryl-CoA from the first cycle, which will ultimately yield hexanoyl-CoA and subsequently caproic acid (Angenent *et al.*, 2016).

During this process, an imbalance is created in the $NADH/NAD^+$ pool, because 15 NADH molecules are consumed while only 12 are produced. Because of the limited size of the $NADH/NAD^+$ pool, the process needs to be balanced. This is achieved by reducing three NAD^+ to NADH with

Table 7.2 Overview of common metabolic reactions in fermentation from mixed organic feedstocks, for example industrial wastewater, waste sludge or co-digestion feedstocks, and examples of organisms responsible for these pathways.

Substrate	Product	Stoichiometry	Example Organisms
Glucose	Ethanol	$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ H_2$	*Sacharromyces cerevisiae*, *Zymomonas mobilis*
	Lactic acid	$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$ $C_6H_{12}O_6 \rightarrow C_3H_6O + C_2H_5OH + CO_2 + H_2$	*Lactobacillus* *Leuconostoc*
	Acetic acid	$C_6H_{12}O_6 \rightarrow 2\ CH_3COOH + 2\ CO_2 + 2\ H_2$	*Clostridium*, *Acetobacterium*
	Butyric acid	$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2\ CO_2 + H_2$	*Clostridium*, *Faecalibacterium*
	Succinic acid	$C_6H_{12}O_6 + CO_2 \rightarrow 2\ C_4H_6O_4$	*Actinobacillus succinogenes*
Ethanol	Acetic acid	$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2\ H_2$	*Acetobacter*
Ethanol, acetic acid	Butyric acid	$6\ C_2H_5OH + 4\ CH_3COOH \rightarrow 5\ C_3H_7COOH + 2\ H_2 + 5\ H_2O$	*Clostridium kluyveri*
Ethanol, butyric acid	Caproic acid	$6\ C_2H_5OH + 5\ C_3H_7COOH \rightarrow 5\ C_5H_{11}COOH + 2\ H_2 + 5\ H_2O$	*Clostridium kluyveri*
Ethanol, acetic acid		$12\ C_2H_5OH + 3\ CH_3COOH \rightarrow 5\ C_5H_{11}COOH + 4\ H_2 + 10\ H_2O$	*Clostridium kluyveri*
Lactic acid	Propionic acid	$C_3H_6O_3 \rightarrow C_2H_5COOH$	*Propionibacterium*
	Acetic acid	$C_3H_6O_3 + H_2O \rightarrow CH_3COOH + CO_2 + 2\ H_2$	*Acetobacter*
	Butyric acid	$2\ C_3H_6O_3 \rightarrow C_3H_7COOH + 2\ CO_2 + 2\ H_2$	*Megasphaera elsdenii*
	Caproic acid	$3\ C_3H_6O_3 \rightarrow C_5H_{11}COOH + 3\ CO_2 + 2\ H_2$	*Ruminococcaceae* CPB6

ferredoxin – originating from the penultimate step of the reverse beta-oxidation cycle. This is done at the membrane-bound RnF-enzyme complex. During this process, six protons or Na^+ molecules are pumped outside the cell. This creates a proton, or sodium, motive force over the membrane that can be used to generate an additional 1.5 ATP by a membrane-bound ATPase complex. In this way, balancing the NADH/NAD^+ pool is a crucial part of the energy generation in the metabolism of *C. kluyveri* (Angenent *et al.*, 2016).

7.3.3.3 Chain elongation using alternative electron donors

Due to the diverse nature of wastewater and organic waste streams, converting them into a mixture of ethanol and acetic acid suitable for subsequent chain elongation can be challenging. Alternatively, ethanol could be supplemented after primary fermentation to convert the short-chain products to MCCA, however, this supplementation reduces the potential profits, and increases the environmental impacts of the process (Chen *et al.*, 2017). Other substrates have been shown to allow production of MCCA as well, often through metabolisms similar to the reverse beta-oxidation described earlier, such as saccharides, lactic acid, proteins, and pyruvate (Angenent *et al.*, 2016).

Saccharides in waste streams can originate from the hydrolysis of starches and celluloses and are often present in a mix of mono- and oligosaccharides. Several chain elongating bacteria have been isolated capable of using saccharides, for example *Megasphaera hexanoica* using fructose, and *Caproiciproducens galactitolivorans* using D-galactitol, a sugar alcohol. Both isolates were capable of producing over 7 g caproic acid·L^{-1} (Jeon *et al.*, 2017; Kim *et al.*, 2015).

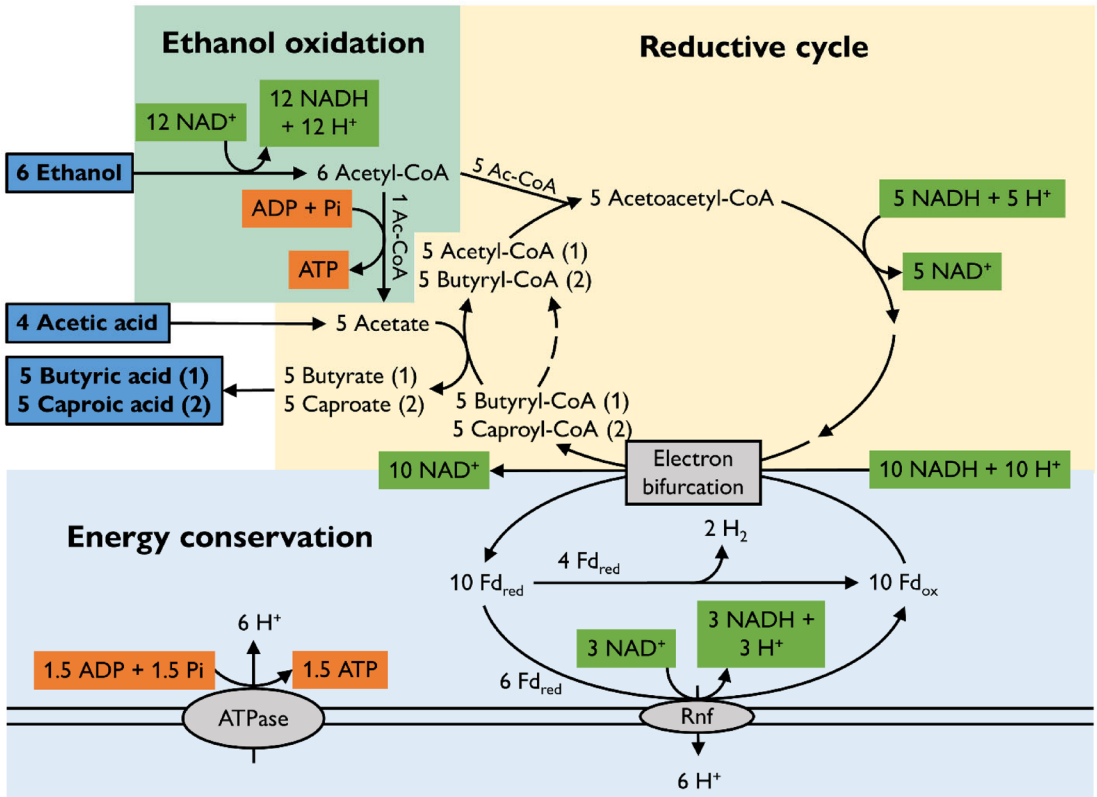


Figure 7.4 Simplified representation of the reverse beta-oxidation metabolism (adapted from Angenent *et al.*, 2016).

Lactic acid, which can easily be obtained from saccharides by lactic acid bacteria such as *Lactobacillus*, can also be used as electron donor for MCCA production. This metabolism was first reported in *Megasphaera elsdenii*, although MCCA production from lactic acid is negligible (Elsden *et al.*, 1956; Weimer & Moen, 2013). In a series of studies carried out with mixed cultures fed with thin stillage – a side-stream from the bio-EtOH production process – it was hypothesized that saccharides in the stillage could first be converted to lactic acid, which was then used for MCCA production (Andersen *et al.*, 2015, 2017). The first conclusive observation of lactic acid-based chain elongation came from a study investigating caproic acid production in Chinese liquor pit fermentations. Of the potential electron donors present in these fermentations – ethanol, lactic acid and glucose – lactic acid was the only substrate that enabled production of caproic acid (Zhu *et al.*, 2015). In a follow-up study, the responsible bacterium (*Ruminococcaceae* sp. CPB6) was isolated and characterized (Zhu *et al.*, 2017).

Peptides are the product of protein hydrolysis and can in turn be further fermented. While fermentations to SCCA from peptides were described in section 7.3.2.2, other metabolisms are also possible. For instance, one isolate (*Eubacterium pyruvativorans*) has been shown to convert peptides into caproic acid, likely through a pathway similar to reverse beta-oxidation, while using the nitrogen to produce ammonia (Wallace *et al.*, 2003).

Towards practice, the above substrates are rarely found at sufficiently high concentrations in waste activated sludge – or other wastewater-derived feedstocks – to enable direct MCCA-production. To

achieve this, co-digestion of waste with sugar- or lactic acid-rich feedstocks could be a potential approach for the valorization of waste activated sludge. Industrial wastewater from the dairy, food and beverage industry may allow for MCCA production via lactic acid or saccharides.

7.3.4 The reason behind it all: energy maximization and redox balancing

Over the last section, a range of anaerobic metabolic pathways have been discussed, some of which can be performed by a large group of bacteria, such as carbohydrate fermentation, while other metabolisms belong to a phylogenetically narrow group, or even to unique species (e.g., ethanol chain elongation by *Clostridium kluyveri*). Some metabolisms are enzymatically very simple (for instance, lactic acid fermentation), while others require many enzymes (for instance, reverse beta-oxidation). In any system, organisms will aim to maximize the net energy generation rate (Großkopf & Soyer, 2016). This net energy includes both energy yield, that is ATP, but also energy consumption, for example enzyme synthesis and cell maintenance (Kleerebezem & Van Loosdrecht, 2010). Considering the energy yield of glucose as substrate, Table 7.3 shows the ATP yields for different metabolic end products. When producing lactic acid, ethanol, or a mixture of the two (i.e., heterolactic fermentation), this will yield two ATP per molecule of glucose, originating from substrate-level phosphorylation (SLP) during the conversion of glucose to pyruvate over glyceraldehyde 3-phosphate. Fermentation to acetic acid yields an additional two ATP per molecule of glucose, by SLP when acetyl-CoA is converted to acetate over acetyl-phosphate. However, fermentation to lactic acid is a shorter pathway than acetic acid fermentation, requiring lower energy investment in enzymes, and enabling higher rates (Kreft *et al.*, 2020).

In parallel, every metabolic pathway needs to achieve a redox balance. This means that every electron going into the system must go out, for instance glucose as electron donor coming into the cell and being excreted as an extracellular carboxylic acid. The first step in glucose fermentation is the oxidation to pyruvate. Any oxidation reaction needs a reduction to balance the electrons in the system, which is why cells use intracellular electron carriers to store these electrons. Examples of such carriers are NADH, NADPH or ferredoxin. For instance, in the oxidation of glucose to pyruvate, two molecules of NAD^+ are reduced to NADH (Temudo *et al.*, 2007). However, a cell cannot accumulate electrons infinitely, due to the limited $NAD(P)H/NAD(P)^+$ pool, and the NAD(P)H generated must be converted back to $NAD(P)^+$. In fermentations, lacking any other electron acceptors, two options exist for this regeneration. A first option is the use of NADH to reduce an organic electron acceptor (Müller, 2008). This is what happens during lactic acid fermentation, reducing pyruvate to lactic acid. Lacking this, NADH can also transfer its electrons to H^+ as an electron acceptor, producing H_2. The latter happens during fermentation of glucose to acetic acid, or for instance, during anaerobic

Table 7.3 Overview of product, ATP and NADH yields for fermentation of glucose and xylose as model compounds for C6 and C5 saccharides.

Product	Glucose			Xylose (PPP)			Xylose (PKP)		
	Y_{prod}	Y_{ATP}	Y_{NADH}	Y_{prod}	Y_{ATP}	Y_{NADH}	Y_{prod}	Y_{ATP}	Y_{NADH}
Succinic acid	2	2	−2	1.67	1.67	−1.67	1 + 1 Ac	2	−1
Lactic acid	2	2	0	1.67	1.67	0.00	1 + 1 Ac	2	0
Ethanol	2	2	−2	1.67	1.67	−1.67	1 + 1 Ac	2	−1
Acetic acid	2	4	2	1.67	3.33	1.67	2	3	1
Propionic acid	2	2	−2	1.67	1.67	−1.67	1 + 1 Ac	2	−1
Butyric acid	1	3	0	0.83	3.33	0.00	0.5 + 1 Ac	3	0

Xylose can be fermented through the pentose phosphate pathway (PPP) and phosphoketolase pathway (PKP). The latter yields one acetic acid (Ac) and one ATP during conversion of xylose to pyruvate (see Figure 7.3).

oxidation of carboxylic acids. In processes were NAD^+ is in excess, for example reverse beta-oxidation or ethanol fermentation, ferredoxin is used as a high energy electron carrier. At the membrane bound Rnf complex, this ferredoxin is oxidized, reducing NADH to NAD^+, as well as pumping protons or sodium ions out of the cell to generate a chemical energy gradient. This energy gradient can in turn be used to generate additional ATP through an ATPase complex (Biegel *et al.*, 2011).

There is, however, a last consideration to be made: the thermodynamics of the chemical reactions. Ethanol can be converted by two pathways in the absence of electron acceptors: (i) anaerobic ethanol oxidation; or (ii) reverse beta-oxidation. The former yields 1 mol ATP per mol ethanol (cf. Figure 7.2; ethanol to acetyl-CoA to acetate), while the latter yields only 0.42 mol ATP per mol ethanol (Seedorf *et al.*, 2008). On top of that, the reverse beta-oxidation is a complex pathway, requiring synthesis of a large number of enzymes. Why then, would any organism go through the effort of setting up such a complex pathway? The answer to this can be found in thermodynamics and energy generation. Converting ethanol to acetate under anaerobic conditions yields H_2. When the partial pressure of H_2 increases, the amount of energy generated per molecule of ethanol decreases. Eventually, the reaction becomes thermodynamically infeasible at partial pressures of approx. 0.1 bar (Cavalcante *et al.*, 2017). However, this does not mean that all available substrate has been depleted. Under these specific conditions (high organic load, high H_2 partial pressures) reverse beta-oxidation provides a way to generate more energy from the available substrates, even if it requires a larger number of enzymes. This knowledge can help in the design of systems targeting specific product profiles, by engineering operational conditions to stimulate the targeted metabolisms. In the case of chain elongation, this means it is crucial to maintain sufficiently high partial pressures of H_2 to prevent anaerobic oxidation of ethanol (Steinbusch *et al.*, 2011). In the end, it becomes clear that organisms have developed an array of ways to maintain the redox balance under the niche conditions they thrive in in nature. Understanding these conditions is crucial to harness the metabolic potential of these organisms and stimulate these metabolic pathways in any bioproduction system.

7.4 CHEMICAL AND BIOLOGICAL DOWNSTREAM/UPGRADING ROUTES FOR THE RECOVERY OF CARBOXYLIC ACIDS

7.4.1 Solid–liquid separation before product recovery

The organic acids generated through fermentation can be further upgraded via additional bioprocessing, referred to as biological upgrading, or via physicochemical processes, as schematized in Figure 7.5. For both of these trajectories, cell separation is the first step after fermentation. Cell separation is needed to protect downstream processes, for example preventing fouling, clogging in physicochemical unit operations, or microbial contamination of bioprocesses. Different methods, based on different principles, can be applied for separation of cell biomass with different effluent qualities. Additionally, the separation technique used will depend on the objective, generating a clear effluent, or dehydrating the solid biomass fraction. Here we will shortly discuss two of the most commonly used technologies that aim at generating clear streams for further downstream processing, namely centrifugation and filtration.

Centrifugation is widely applied in food processing, bioprocessing and wastewater treatment and is based on the differential density between solid particles and liquids (Svarovsky, 2001a, 2001b). Depending on scale and application, centrifugation can be done in batch or continuous mode. On the other hand, centrifugation often leads to only partial solid separation producing a liquid fraction that still contains suspended solids.

Filtration can be applied to retain biomass in the fermenter, or to clarify a fermentation effluent, as a stand-alone unit, or downstream of a centrifugation step. Given the size of bacterial cells, pore sizes between 0.1 and 0.5 μm are often enough to ensure complete biomass retention. Microfiltration (0.1–10 μm) and ultrafiltration (0.001–0.1 μm) are applied in processes that require a clear stream devoid of

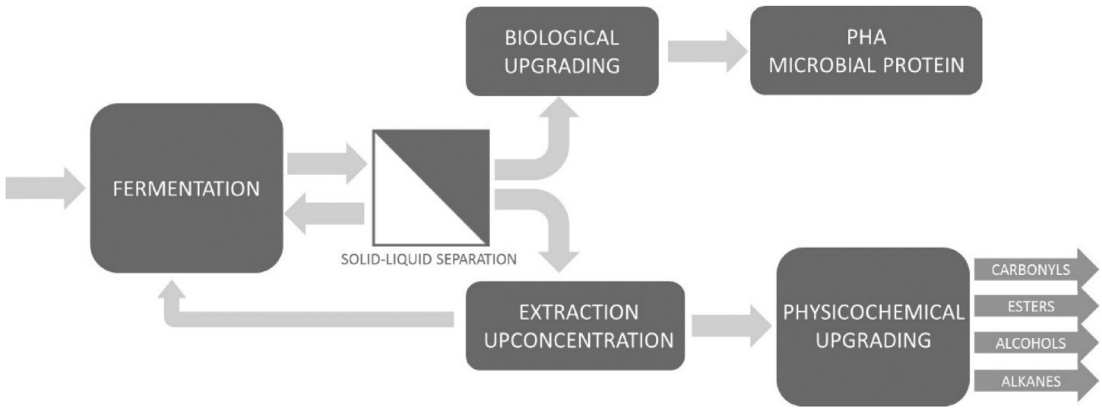


Figure 7.5 Combination of fermentation with downstream processing options.

suspended solids. The filtration can be carried in different configurations, that is internally, by using membranes submerged in the reactor broth, or externally, by recirculating the broth tangentially to the membrane, also known as cross flow filtration or tangential flow filtration. Membranes are built in different shapes and materials. Polymeric membranes (PVDF, PP, PE, etc.) are built as flat sheet, tubular or hollow fibre membranes. Ceramic membranes are built in flat sheets or in tubular shapes (Lin *et al.*, 2013). The type of membrane, material and configuration is determined by the process conditions, suspended solid concentration and composition of the broth. Especially when broths contain medium chain fatty acids at pH <6, ceramic membranes are preferred over polymeric membranes, due to the degradation of the polymers by the organic acids. One key issue to be considered for filtration processes in waste-contexts is fouling, where solids in the stream build up on the membrane, leading to increased pressure drops, and increased energy requirements for filtration. Membrane cleaning, back-washing and other fouling-mitigating strategies have been developed and are routine practice for these systems, as described elsewhere (Judd & Judd, 2011).

7.4.2 Physicochemical product upgrading

This sub-section briefly summarizes some of the most promising physicochemical approaches for the recovery and upgrading of carboxylic acids from fermentation broths. These are, in most cases, based on existing technologies applied in other settings that can be bought off-the-shelf. Therefore, the focus of this sub-section will lay on introducing the general principals of each technology rather than discussing how to engineer them, which has been extensively described elsewhere (Green & Perry, 2008). The latter will be dependent on many factors such as the carboxylic acid composition, concentration, final target product, and so on.

7.4.2.1 Product extraction and up-concentration

Traditional anaerobic digestion generates methane, a gaseous product that spontaneously separates from the broth. Open culture acidogenic fermentations produce organic acids with variable, but generally, high water solubility. In this context, acetic, propionic, butyric and valeric acids are water miscible, and only acids with carbon length of five carbon atoms or longer form an oil phase when the concentration of their undissociated form reaches the solubility limit (e.g., 49.7 g L^{-1} for valeric acid, 10.8 g L^{-1} for caproic acid, etc. (Saboe *et al.*, 2018)). Due to the high solubility of organic acids, and their low concentration achieved in mixed culture fermentations, extraction, concentration and purification methods are often needed. For instance, the addition of $CaCO_3$ or NH_4HCO_3 to

counteract acidification may lead to the production of calcium or ammonium carboxylate salts that will precipitate when their concentration in the fermentation broth is high enough (e.g., >320 g L^{-1} for Ca acetate, at 25°C, >61 g L^{-1} for Ca lactate, at 25°C, etc.) (López-Garzón & Straathof, 2014). On the other hand, when fermentations are operated at low pH, the concentration of organic acids in the broth are seldom higher than 10–20 g L^{-1}, too low to enable their direct recovery as salts, requiring other approaches. In general, the choice of downstream route (i.e., extraction, concentration and purification) depend on the physicochemical characteristics of the acid and the final application. Here we will describe methods for extracting and up-concentrating SCCA and MCCA. Extraction methods include gas stripping, adsorption, pressure-driven membrane processes, liquid-liquid extraction, and electrochemical membrane processes.

7.4.2.1.1 Gas stripping combined with absorption

Air and gas stripping applications aim to remove volatile compounds from a solution by contacting clean air with the aqueous solution (i.e., fermentation broth) across a high surface area. This process is governed by Henry's law (Equation (7.2)), which describes the partial pressure of a volatile component (i.e., SCCA) in a gas phase (P_{SCCA}) in equilibrium with a dilute solution of that component at a concentration $C_{SCCA,aq}$ where H_{cp} is Henry's law constant (mol·m^{-3}·Pa^{-1}):

$$H_{cp} = \frac{C_{SCCA,aq}}{P_{SCCA}} \tag{7.2}$$

The maximum transfer rate (TR) of a specific SCCA that can be achieved at a stripping gas volumetric flow rate, Q, is given by Equation (7.3):

$$TR = QC_{SCCA(g)} = Qx\frac{C_{SCCA(aq)}}{H_{cp}RT} \tag{7.3}$$

Since only the undissociated species of the acids are volatile, the process is pH dependent. The carboxylic acids in open culture fermentations have similar pKa values (4.87–4.89) but differ in their Henry's law constants that range between 23 and 70 mol·m^{-3}·Pa^{-1} (Sander, 2015).

A scrubber can be used to simultaneously regenerate the stripping gas and quantitatively recover the SCCA. The absorption of the SCCA can be done in a $CaCO_3$ solution that enables recovering the products as carboxylate calcium salts. The stripping gas remains water saturated at constant temperature (Li *et al.*, 2015).

Pervaporation is a modified version of gas stripping, where clean gas is not stripped through the liquid, but instead contacts the liquid via a membrane. The SCCA can diffuse through the membrane and transfer to the gas phase, after which the SCCA can be recovered as calcium salts, as described above.

7.4.2.1.2 Adsorption

Carboxylic acids in gas or liquid phase can be sorbed onto a solid surface. This process is called adsorption and depends on the interaction between the solid surface moieties and the carboxyl group and hydrophobic tail of the carboxylic acids. Adsorption is typically carried out in a column packed with a sorbent, which is often a resin with positively charged amine functional groups on the surface (López-Garzón & Straathof, 2014). The ion exchange mechanism of an acid can be described with the following equilibria (Equations (7.4)–(7.6)), in which HA is the carboxylic acid, A^- is the carboxylate anion, R_xNH_{3-x} is the non-protonated amine (for a primary amine x = 1, for a secondary amine x = 2, and for tertiary amine x = 3), $R_xNH_{4-x}^+$ is the protonated amine and $RxNH_{4x}^+A$ is the acid-resin salt:

$$HA \leftrightarrow A^- + H^+ \tag{7.4}$$

$$R_xNH_{3-x} + H_2O \leftrightarrow R_xNH_{4-x} + OH^- \tag{7.5}$$

$$R_xNH_{4-x} + A^- \leftrightarrow R_xNH_{4-x}A \tag{7.6}$$

Additionally, van der Waals forces occur between the hydrophobic tail of the acids and the matrix of the resin (Rebecchi *et al.*, 2016). The adsorption efficiency depends on the adsorption capacity of the resin, that is mass of adsorbate per kilogram of adsorbent (Rebecchi *et al.*, 2016). Once the resin is saturated, the carboxylic acids can be recovered by using a suitable solvent. The overall efficiency of the process depends on the type of resin used, the adsorption capacity of the resin, operating pH and temperature, type of desorption and the desorbing chemical used, presence of other competing anions and the adsorption/contact time (Reyhanitash *et al.*, 2017).

7.4.2.1.3 Pressure-driven membrane processes

Reverse osmosis and nanofiltration are used to separate solutes from solvents by semi-permeable membranes. These can be used to recover SCCA from streams after solids-removal as described in section 7.4.1. In a second filtration step for product recovery, a membrane is used to concentrate the SCCA in solution, by allowing the transfer of water, but rejecting the SCCA (Zacharof & Lovitt, 2013). Several factors affect the recovery rate, including solution pH, temperature, product concentration and hydraulic pressure. While pH and pressure are positively correlated with the organic acid recovery, the increase in temperature decreases its concentration. This approach is established for industrial fermentations and there is a growing literature on these methods for SCCA recovery from waste streams (Atasoy *et al.*, 2018). As a final note, it is important to highlight that issues such as biofouling or inorganic scaling can occur, resulting in increased energy input and the need for regular membrane cleaning.

7.4.2.1.4 Liquid–liquid extraction

In liquid–liquid extraction systems, an aqueous phase containing the organic acids is put in contact with an organic solvent. The organic acids in their undissociated form are hydrophobic and partition into the organic phase. The longer the chain, the higher is the partition coefficient (Table 7.4). Since only the undissociated form dissolves in the organic solvent, it is evident from the equations listed below (Equations (7.7)–(7.9)) that the efficiency of the extraction step is pH dependent:

$$K_{isw} = \frac{C_{is}}{C_{Hiw}} \tag{7.7}$$

$$C_{Hiw} = \frac{C_{Tiw}}{1 + 10^{(pH - pK_{ia})}} \tag{7.8}$$

Table 7.4 Standard octanol – water partition coefficients for different organic acids (Hansch *et al.*, 1995).

Carboxylic Acid	Log K_{ow}
Acetic acid	−0.17
Propionic acid	0.33
Butyric acid	0.79
Valeric acid	1.39
Caproic acid	1.92
Caprylic acid	3.05

$$C_{is} = K_{isw} \cdot \frac{C_{Tiw}}{1 + 10^{(pH - pK_{ia})}} \tag{7.9}$$

where K_{isw} is the partition coefficient of the compound i between the solvent s and the aqueous phase at equilibrium, C_{is} is the concentration of i in the organic phase, C_{Hiw} is the concentration of the protonated species of the acid in the aqueous phase, C_{Tiw} is the total concentration of the carboxylic acid in the aqueous phase, and pK_{ia} is the pH at which the concentration of the protonated and dissociated species of a monoprotic acid are equal. Table 7.4 lists the log of the partition coefficient of different carboxylic acids between octanol and water under standard conditions.

Alkylphosphine oxides, trialkylamines, apolar solvents and combinations thereof are the most investigated solvents for the extraction of carboxylic acids from fermentation broths. Kerosene and paraffin oil have been successfully used as organic solvents in combination with trioctylphosphine oxide (TOPO) in concentrations in the range of 3–20% (Agler *et al.*, 2012; Levy *et al.*, 1981). Higher concentrations of TOPO in the solvent enhance the extraction of shorter chain carboxylic acids. The main advantage of the extraction with solvents is that it is more selective than other extraction techniques and enables selective extraction of medium chain over short chain carboxylic acids. Since the separation between the organic and liquid phases is slow, these can be indirectly contacted across a hydrophobic membrane, which enables the diffusion of the undissociated acids to the organic solvent (Agler *et al.*, 2012, 2014). This allows for a very significant reduction in equipment size, but membranes are easily fouled and wet, requiring periodical cleaning and regeneration of the hydrophobic surface.

7.4.2.1.5 Membrane electrochemical processes

Technologies like electrodialysis (Qian-Zhu *et al.*, 2016) or membrane electrolysis (Andersen *et al.*, 2014) allow extracting and concentrating the dissociated carboxylates to an aqueous extract by using the potential difference between two electrodes. In electrodialysis, cations are transported across a cation exchange membrane to the cathode compartment while anions are transported across an anion exchange membrane to the anode compartment. Carboxylic acids can be recovered in their undissociated form in the acidic anode compartment, while reduction of water at the cathode generates a caustic stream that can be used to control the pH in the fermenter. In membrane electrolysis, the fermentation broth is fed to the cathode compartment and the carboxylates extracted to the anode compartment where they become protonated. Additionally, the broth in the cathode compartment becomes alkaline due to the OH^- generation from water reduction and the acidification of the fermentation can be counteracted without the addition of chemicals. The main disadvantage of these membrane electrochemical technologies is the low selectivity of the membranes for organic acids which cause low power efficiencies due to co-extraction of smaller inorganic ions. Smaller molecules have higher electrophoretic mobility and therefore electrochemical techniques are more suitable for extraction of SCCA.

7.4.2.1.6 Hybrid downstream trains

Combinations of the techniques described above can be applied. For example, if MCCA are to be produced in acid form, liquid–liquid extraction can be combined with membrane electrolysis (Xu *et al.*, 2015). The organic acids in their protonated form are extracted from the fermentation broth to an organic solvent. Once in the organic phase they can be recovered in a second extraction step with an alkaline aqueous solution. The difference in pH between the broth and the alkaline side generates a gradient in the concentration of undissociated organic acids that drives the extraction. In order to sustain the driving force, the pH of the aqueous extract needs to be maintained alkaline. This can be done by circulating it through the cathode compartment of a membrane electrolysis cell. The OH^- generated in the cathode maintains the alkaline conditions. Additionally, if the electrolysis cell is fitted with an anion exchange membrane, the dissociated organic acids will be transported to the anode compartment, where they become concentrated and acidified by the H^+ generated by anodic

water oxidation. Once the organic acids accumulate over their solubility limit, they form an organic phase composed of MCCA. This extraction pipeline requires the fermentation to be run at pH values in the range of 5–5.5, since only undissociated MCCA are extracted in the solvent extraction part of the system.

Liquid–liquid extraction can be combined with distillation, a well-established technology in the chemical industry. A first step of extraction with a solvent can be followed by distillation, which enables separating the organic acids from the solvent. For instance, it has been calculated that acetic acid can be recovered by using a pipeline including liquid–liquid extraction followed by a heat-integrated two-stage distillation, at 2.6 MJ Kg^{-1} acetic acid (Saboe *et al.*, 2018).

7.4.2.2 Chemical conversions

Organic acids derived from fermentations can be used as substrates for the production of multiple chemical derivatives. Organic acids can be esterified with alcohols under acidic conditions to generate alkyl esters. Alternatively, after pre-concentration, acids can be converted into ketones by thermochemical reduction. Ketones can be further hydrogenated to alcohols (Holtzapple *et al.*, 1999). Additionally, organic acids can be used to produce alkanes by an electrochemical hydrolytic process known as Kolbe electrolysis (Agler *et al.*, 2011; Levy *et al.*, 1981; Urban *et al.*, 2017).

The list of possible transformation products of organic acids is larger than their current applications and the progressive substitution of petroleum based feedstocks is likely to promote the generation of novel pipelines and applications.

7.4.3 Biological product upgrading

This sub-section will introduce two potential biological routes for the upgrading of carboxylic acid and their recovery as a marketable product: (i) polyhydroxyalkanoates as bioplastics; and (ii) microbial protein as feed. Both routes are further discussed in upcoming chapters, which also cover the more engineering-related aspects of such processes.

7.4.3.1 Polyhydroxyalkanoates for bioplastics

Polyhydroxyalkanoates (PHA) have been proposed as bio-based and bio-degradable alternative polymers to current petrochemical thermoplastics (Reddy *et al.*, 2003). Their mechanical and physical properties, for example insoluble in water, resistant to hydrolytic degradation, biocompatible and easier to process than traditional polymers (Bugnicourt *et al.*, 2014), are of particular interest because they can be tailored. They are also truly biodegradable in ambient conditions in soil and marine environments, which is not the case for some alternatives (like poly-lactic acid) that require specific conditions for biodegradation, such as higher temperatures (Tansengco & Tokiwa, 1998). PHA consist of esterified chains of hydroxyalkanoic acids (HA), that is carboxylic acids with an additional alcohol group, with most biologically produced PHA being made up of either polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), or a copolymer of the two (PHBV). However, many variations of PHA have been demonstrated, and the monomers present in the PHA will determine their physical properties as a plastic.

Microbial synthesis of PHA has been long known (Lemoigne, 1927; Weibull, 1953), with PHA functioning as an energy storage molecule during aerobic growth of organisms under nutrient limited conditions (Doudoroff & Stanier, 1959). SCCA are often used as substrates for PHA production. These polymers are stored intracellularly, presenting themselves as intracellular granules. PHA production can be achieved in pure culture systems where over 90% of the final dry biomass weight is PHA. However, these processes require sterile culturing conditions and are limited to the use of defined substrates, mostly crop based. Alternatively, mixed cultures can be enriched in PHA-accumulating bacteria by applying selective strategies such as feast-famine and/or nitrogen limitation. These strategies select for organisms that can take up the carbon quickly, and store it internally for later use, for exmaple when nitrogen becomes available in the environment. With such a process, up to 90%

of the final dry biomass weight is PHA when using a defined feedstock (acetic acid), while a product with up to 80% PHA can be obtained when using waste-based SCCA (Johnson *et al.*, 2009; Korkakaki *et al.*, 2016). A case study on the PHA production from wastewater is presented in Chapter 8.

7.4.3.2 Microbial protein for feed and food

Microbial protein, that is microbial biomass with high protein content, has been suggested to create feed and food with high nutritional value from low-value or waste streams (see Chapter 9) (Matassa *et al.*, 2016). The strength of this concept is the direct recycling of carbon, nitrogen and energy from wastes to food. This can happen indirectly, by using it as animal feed, or directly using it for human consumption. Both methods have been shown to significantly reduce the pressure on the environment associated with food production. In a recent study, it was estimated that by 2050, microbial protein could make up 10–19% (175–307 Mton) of protein fed to livestock. This substitution could result in up to a 13% reduction in land use for crops, up to 8% reduction in nitrogen losses from croplands and up to 9% reduction in greenhouse gas emissions when comparing to a full crop-based livestock diet (Pikaar *et al.*, 2018). While the core of the microbial protein platform is the recovery of nitrogen into a valuable product, microorganisms also need a carbon and electron source to grow. Several pipelines have already been explored to supply both nitrogen and carbon to processes producing microbial protein (see Chapter 10). One example is the conversion of low-value industrial side or process streams to microbial protein, for instance, wastewater from a potato processing facility. Another route that has been suggested is the use of carboxylic acids produced through fermentation processes as a sustainable carbon source to grow biomass. Two types of bacteria have been proposed for the production of microbial protein with carboxylic acids as substrates: aerobic heterotrophic bacteria and purple non-sulphur bacteria (PNSB). The former group uses the carboxylic acids as a conventional aerobe, using them as energy source as well as carbon source. PNSB on the other hand are phototrophic and use light as a source for energy. This means that carboxylic acids are solely directed to biomass growth, resulting in a higher carbon yield. This coupling of fermentation and microbial protein production can provide a sustainable source of feed and food. However, this technology is still in development, and several technological and policy barriers need to be tackled before it can move forward (Alloul *et al.*, 2018).

7.5 CONCEPTUAL OVERVIEW OF THE PRODUCTION OF SHORT-CHAIN CARBOXYLIC ACIDS (SCCA) FROM WASTEWATER

7.5.1 Technological principles

SCCA are considered high value-added products, which can be converted into biofuels, bioplastics, and so on. or used as chemicals in various industries, including as a supplementary electron donor in wastewater treatment. Currently, most SCCA are petrochemically produced, but there is a growing research and industrial interest in the development of alternative biological routes that are potentially more sustainable (Reyhanitash *et al.*, 2017). Organic-rich industrial wastewater or sewage sludge can be hydrolyzed and fermented to SCCA in acidogenic fermentation. In the case of waste activated sludge, it has been estimated that if organic matter in sewage is first captured as sludge using, for example, chemically enhanced primary treatment, and subsequently fermented, about 20–30 g SCCA-COD·IE^{-1}·d^{-1} can be produced (Alloul *et al.*, 2018). The separation of the produced SCCA is a crucial process to obtain value-added products that can be sold (see section 7.4 for a summary of various separation technologies). It is important to highlight that the recovery of SCCA may only be economically viable at medium- and large-scale WWTPs due to the large capital costs required. An alternative to separation and commercialization of these SCCA as chemicals, is their use either directly in a WWTP as electron donor or as feedstock to produce other valuable compounds, such as polyhydroxyalkanoate (PHA) bioplastics. Therefore, the specific processes that are necessary

to implement will vary according to the intended application(s). The benefit of direct use on-site is that the WWTP becomes its 'own client' and, as such, further purification, transformation or commercialization efforts are not needed.

7.5.2 Fundamental principles

Over the past 20 years much research has been devoted to the production of SCCA from complex organic wastes by mixed cultures, instead of methane. The main fundamentals of open mixed culture fermentation and product recovery have been introduced in sections 7.3 and 7.4. The following further builds on this knowledge by summarizing the key process parameters and conditions to achieve SCCA production.

First and foremost, SCCA accumulation in an open mixed culture requires the inhibition/suppression of organisms consuming them as substrate, for instance methanogenic archaea. The fermentation pH is one of the most important parameters to steer the process, and SCCA accumulation is usually achieved by inhibiting/suppressing methanogenesis at pH above 8.0 or decreasing the fermentation pH below 6.0 (Atasoy *et al.*, 2018). The fermentation pH also has a direct effect on the hydrolysis and acidogenesis steps, and pH deviating from the neutrality tend to negatively affect the rates of hydrolysis and/or acidogenesis. Temperature has also a great impact on enzymatic activities, growth of microorganisms, and hydrolysis rate. Within the mesophilic range, reaction rates increase with temperature. Shifting from mesophilic to thermophilic conditions may also result in higher SCCA concentrations and changes in the SCCA product spectrum, but at the expense of increasing heating cost (Hao & Wang, 2014; Zhang *et al.* 2009). Short biomass retention (<5 days) times can also contribute to SCCA accumulation by resulting in a partial/complete wash-out of methanogens, which have doubling times often in the order of 4–5 days.

The quantity of SCCA produced is dependent on the degree of acidification of the input substrates, which is reflected in the percentage of initial COD converted to organic acids and other fermentation products (Atasoy *et al.*, 2018). More reduced substrates such as cheese whey or energy crops will result in higher SCCA yields than, for instance, waste activated sludge. A membrane filtration unit (Longo *et al.*, 2015) or centrifuge (Morgan-Sagastume *et al.*, 2014) can be installed after the fermentation tank to separate the SCCA-rich fermentation liquid from the sludge, as discussed in section 7.4. The SCCA recovery rate is highly dependent on both the recovery approach and fermentation conditions (as the latter determine the fraction of SCCA that are in their (un)dissociated form and can be extracted by a certain method).

7.5.3 Applications

SCCA have various potential applications. Organic acids are used to generate very diverse chemical commodities. Acetic acid is used in the production of vinyl acetate, ketene and acetic anhydride (Sneeden, 1982). Although organic acids generally have an unpleasant smell, their ester products are often used in fragrances. Esters like methyl-, ethyl- or isobutyl-acetate, are commonly used as solvents. Esters of valeric acid are primarily used as food additives due to their fruity smell. Acetic, propionic and butyric acids are used in the preparation of cellulose (-propionate, -butyrate) acetates, which are thermoplastics used in the production of films and fibres (Jiang *et al.*, 2018). Typical market prices, applications, and production methods associated with common SCCA are shown in Table 7.5.

SCCA can also be used as feedstocks in the production of renewable plastics and bio textiles. The most paradigmatic example is their use as carbon and energy source in PHA production (see Chapter 9 for further information). The required types of SCCA are different depending on the polymers to be produced. Whereas acetic acid and butyric acid are necessary for polyhydroxybutyrate (PHB) production, propionic acid is favoured for polyhydroxy valerate (PHV) (Peces *et al.*, 2016).

SCCA-containing fermentation liquids can also be used on-site for the optimization of biological nutrient removal (BNR) in WTTPS, as they contain easily biodegradable carbon sources (Kehrein

Table 7.5 SCCA features (adapted from Atasoy *et al.*, 2018).

SCCA	Market Price (€/ton)	Application	Production Methods	Reference
Acetic acid	400–800	Vinyl acetate monomer (polymers, adhesives, dyes), ester production, chemicals, food additive, vinegar, solvent	Chemical synthesis and microbial fermentation	Bhatia and Yang (2017)
Butyric acid	1500–1650	Animal and human food additive, chemical intermediate, solvent, flavouring agent	Chemical synthesis, microbial fermentation, extraction from butter	Zigová and Šturdík (2000)
Propionic acid	2000–2500	Esters used for food industry as aroma additive, food additive, flavouring, pharmaceuticals, animal feed supplement, fishing bait additive	Chemical synthesis and microbial fermentation, by-product of acetic acid production	Cheryan (2009)

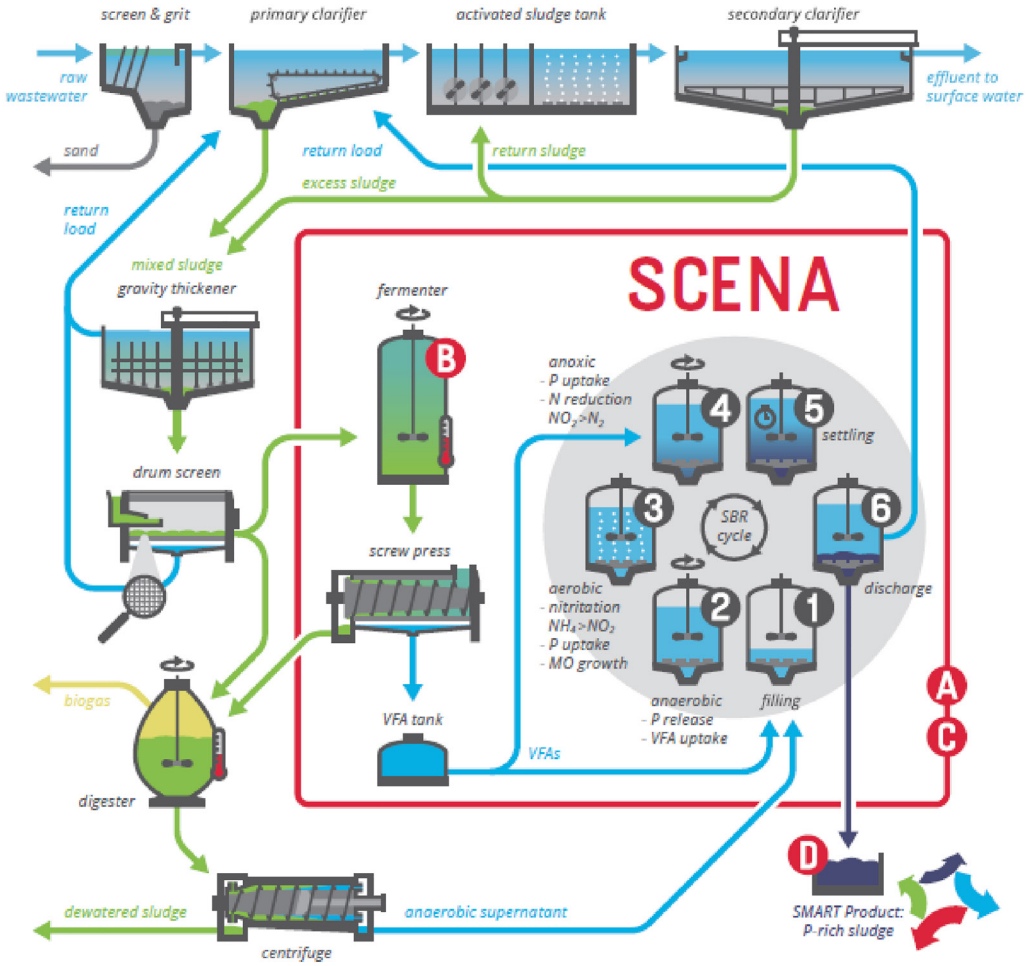
et al., 2020). In many cases, wastewater does not include a sufficient amount of biodegradable soluble organic carbon for denitrification and hence external electron donors such as methanol, glucose or acetic acid, are supplied externally for efficient BNR (Hu *et al.*, 2018). In this case, SCCA recovered from wastewater can act as a source of electrons in biological nutrient removal units, replacing methanol or other external substrates. Acetic acid is the most preferred SCCA for denitrification since it is an easily degradable compound by many bacteria, followed by butyric and propionic acid. Propionic acid has been found to enhance denitrifying biological phosphorus removal via nitrite due to the increase in the amount of phosphorus-accumulating organisms (Frison *et al.*, 2016).

7.5.4 Case studies

Despite the research interest in SCCA production from wastewater or its derivatives, to the best of the authors' knowledge there are no pilot-scale installations or case studies demonstrating the production of SCCA from wastewater and their recovery as organic acids. However, several case studies have explored the production of SCCA and its use in secondary biological processes.

First, the PHARIO project (www.phario.eu) demonstrated the production of PHA from SCCA derived from waste activated sludge. One of the focuses of the project was to demonstrate the production of SCCA from waste streams (i.e., carbohydrate-rich process effluent delivered from a local candy factory and waste activated sludge). Experiments were conducted in a pilot-scale (1200 L) well-stirred batch anaerobic fermentation vessel temperature-controlled at 37°C. The process water from the candy factory was fermented for in batch for 7 days with a pH controlled between 5.5 and 6.0. The SRT of the biomass was between 7 and 10 days. The fermentation product contained 16 g readily biodegradable COD·L^{-1} and the SCCA composition of the different batches was composed of mixtures of C2–C7 carboxylic acids. The fermentation experiments with the primary sludge from the Waterschap de Dommel were conducted in batches with continuous mechanical stirring for 6 days at 37°C, with pH monitoring but with no pH control. The sludge matrix was self-buffering and generally the pH was inherently maintained between 4.8 and 5.5. As opposed to the candy factory process water, no biomass was retained between batches since the WAS contained also anaerobic fermenters (STOWA, 2017). The PHARIO project has achieved SCCA yields around 0.25 g SCCA·g^{-1} VSS and SCCA concentrations in the fermentate in the order of 8–10 g COD·L^{-1}, mostly mixtures of acetic, propionic, butyric and valeric acids (STOWA, 2014).

Another example of this is the production of SCCA and their in-situ use in a WWTP for enhanced nutrient removal is the Short-Cut Enhanced Nutrients Abatement (SCENA). This process aims at biological nitrogen removal and P-bioaccumulation via nitrite during the treatment of anaerobic supernatant (Figure 7.6). Sludge liquor from dewatering of digested sludge is heavily loaded with



Unique Selling Points

- **A** Low-energy nutrient removal from sludge liquor
- **B** Biological N and P elimination without chemicals or external carbon source
- **C** Stable and robust operation compared to other biological processes
- **D** P-rich sludge can be valorized as organic fertilizer

	Nitrification Denitrification	Deammoni-fication	SCENA
External C Source	Yes	No	Bio-based VFAs from sewage sludge
Type of inocolum	conventional activated sludge	Deammoni-fication inocolum	conventional activated sludge
Cost and Energy	High	Medium	Low

Figure 7.6 Schematic diagram of the SCENA process. The SCENA process was demonstrated at full-scale in the WWTP of Carbonera (Italy) within the H2020 SMART-Plant project.

nitrogen and phosphorus, especially when the sludge is pre-treated by thermal pressure hydrolysis (THP) before digestion, putting an additional load on the mainline and limiting the overall treatment capacity of the plant. Sludge liquor after THP also contains a low fraction of biodegradable organic carbon, so that conventional biological processes for nutrient removal in sludge liquor have to be operated with the costly addition of an external carbon source. The SCENA process can remove nitrogen and phosphorus from sludge liquors with low energy demand using an internal carbon source such as supernatant from primary sludge thickening or SCCA from fermentation of sludge. The SCENA system integrates the following processes: (i) optional upstream concentration of cellulosic sludge; (ii) fermentation of dynamic thickened sewage sludge to produce SCCA as carbon source; and (iii) via nitrite nitrogen and phosphorus removal (by P-bioaccumulation) from sludge reject water using an SBR. In this configuration, nitrogen is removed through the bioprocesses of nitritation/denitritation and enhanced biological phosphorus removal (EBPR) via nitrite using the SCCA from sludge fermentation liquid as carbon source.

The targeted recovery of SCCA from municipal wastewater using the SCENA process was achieved by controlled fermentation in the Carbonera WWTP at pilot-scale (Longo *et al*., 2015) and was the basis of a subsequent scale-up carried out in 2017 within the Horizon2020 SMART-Plant innovation action (Longo *et al*., 2017). Currently, a sequencing batch fermentation reactor (SBFR) (total volume of 3 m^3) is in operation for the production of SCCA by acidogenic fermentation (Conca *et al*., 2020). The SBFR is operated at 37°C and the mixed liquor is kept under continuous agitation by a mixer. High SCCA concentrations are obtained for the main species as acetic acid (2000–3000 mg L^{-1}), propionic acid (4000–5000 mg L^{-1}) and valeric acid (700–800 mg L^{-1}). The environmental life cycle assessment and life cycle costing analysis revealed that the implementation of the SCENA process in the WWTP side-stream is an economic and environmentally friendly solution compared to the traditional plant layout with no side-stream treatment, due to the reduction of energy and chemicals for N and P removal, respectively, allowing the production of BioP (biological phosphorus removal) biosolids in the sludge line. Based on the results of the pilot operation, it was estimated that the installation of the SCENA process on an existing plant would have an associated additional CAPEX investment of €6/PE and provide an OPEX saving of €1.3/PE/y. The payoff time of such installation would be approximately five years. The application of SCENA affects the overall WWTP because it reduces the nitrogen load to the mainstream biological reactor. Implementation of a SCENA system for side-stream nutrient removal does not have a positive energy balance when operated with an external carbon source.

7.6 CONCEPTUAL OVERVIEW OF THE PRODUCTION OF MEDIUM-CHAIN CARBOXYLIC ACIDS (MCCA) FROM WASTEWATER

MCCA have been suggested as interesting bioproducts due to their market value and interesting chemical properties. Their production requires coupling SCCA production to a secondary anaerobic biotransformation (i.e., chain elongation). In this section, we will explore the fundamentals, applications and state of technology development for production of MCCA from waste streams.

7.6.1 Technological principles

Since MCCA production requires, in most cases, two distinct metabolic processes, an MCCA production process can be conceived as a one- or two-stage system depending on the wastewater properties and chosen route. In a two-stage process, the wastewater (or sludge) would be first fermented to SCCA in a first acidification step, and subsequently converted to MCCA in a chain elongation step. The latter could proceed via ethanol chain elongation if ethanol is added externally, or via lactic acid if this is in-situ generated in the first step. Alternatively, these processes could be engineered in a one-stage system, decreasing the capital costs (only one reactor is needed instead of two), but making process

optimization more challenging as it is not possible to optimize process conditions (e.g., organic loading rate, retention time, pH, temperature, etc.) for each metabolic step individually.

A second important consideration from a process design perspective is whether or not MCCA are extracted in-line or not, and using what approach. Accumulation of MCCA and their associated toxicity is a strong driver to recover the product directly from the reactor broth (i.e., in-line product recovery). The choice of product recovery strategy will highly depend on the process pH. When a neutrophilic chain elongation process is chosen, recovery has been mostly undertaken via adsorption processes. When targeting in-line product recovery, mildly acidic chain elongation coupled to liquid-liquid extraction (i.e., pertraction, see section 7.4.2.1.4) is, at present, the best recovery approach because: (i) it is selective to more hydrophobic compounds such as MCCA over SCCA; and (ii) does not extract anions and cations.

7.6.2 Fundamental principles

Microbial MCCA production entails SCCA production by an acidogenic fermentation coupled to a biological upgrading step (i.e., chain elongation). Exceptions to that are the production of iso-caproic acid via leucine fermentation and the direct production of MCCA from carbohydrates by *Megasphaera hexanoica* or *Caproiciproducens galactitolivorans* (Jeon *et al.*, 2010, 2016, 2017; Kim *et al.*, 2015). In general, however, MCCA production in mixed microbial communities requires the availability of SCCA and either ethanol or lactic acid as electron donors.

MCCA production via ethanol chain elongation is, to date, the most studied route (although not necessarily the most relevant for their production from wastewater since ethanol is very seldom produced at relevant concentrations in mixed culture fermentations (Chen *et al.*, 2017). As such, this approach requires the addition of external ethanol as substrate. Using this ethanol as electron donor, ethanol chain elongators can convert the SCCA produced in an acidogenic fermentation from carbohydrates or proteins into MCCA (see section 7.3.3.2 for further details).

In contrast, lactic acid can be produced from carbohydrate-rich wastewater by lactic acid bacteria, even in open cultures (Kim *et al.*, 2016). This lactic acid can then be used as electron donor to elongate SCCA present in the broth (or derived from lactic acid oxidation) in processes analogous to ethanol chain elongation from a biochemical standpoint (Zhu *et al.*, 2017). Other compounds often present in wastewater such as peptides or carbohydrates (e.g., fructose or galactitol) have also been shown to be potential substrates for MCCA production (see section 7.3.3.3 for further details), although their relevance in the wastewater context is, at this point, unclear.

Usually, molar ratios of electron donor (either ethanol or lactic acid) to SCCA of four are needed to elongate, that is acetic acid to caproic acid (Barker *et al.*, 1945). In practice, most reports use ratios $\geq$4 to enhance the production of MCCA (mostly targeting higher fractions of C8) (Spirito *et al.*, 2018). However, this approach comes at the expense of excess electron donor oxidation, decreasing the overall process selectivity (Candry *et al.*, 2020; Roghair *et al.*, 2018a, 2018b).

7.6.3 Applications

Caproic (C6), enanthic (C7) and caprylic (C8) acid(s) are some of the most target MCCA for bioproduction processes. These compounds are currently sourced from natural oils (e.g., palm oil, coconut oil), have small markets (<10 000 tonnes/year) and high market values (around €2000/tonne) (Moscoviz *et al.*, 2018). This market opportunity has triggered the recent interest in the microbial production of MCCA from waste streams. MCCA are recovered as an oil, very rarely pure, but rather containing variable fraction of SCCA (usually below 30% w/w (Agler *et al.*, 2012)). Depending on the application, for example feed additive, antibiotic substitute and corrosion inhibitor as well as feedstock for the production of fragrances, flavours, fuels and plasticizers (Angenent *et al.*, 2016), it may be possible to directly use the bio-oils as raw material, although in most cases they will need to be fractionated via, that is, distillation.

7.6.4 Case studies

First and foremost, it is important to start this sub-section by emphasizing that microbial MCCA production is still an emerging concept and at the moment this chapter is being written, no full-scale installations are yet operational. There are however a couple of full- or demo-scale installations currently being constructed that are worth mentioning to highlight the industrial relevance of the topic at hand. It should be emphasized that these cases do not start from wastewater or wastewater-derived feedstocks. The first one to be considered is a semi-commercial demonstrator at the port of Amsterdam built by Chaincraft, a Dutch company that targets the production of MCCA from food waste by acidification and chain elongation with external ethanol addition (ChainCraft, 2019). As discussed later in this sub-section, several of the reports on the production of MCCA from waste activated sludge follow a similar approach, combining waste acidification with chain elongation using external ethanol as electron donor. Secondly, Afyren is a French company focused on the production of carboxylic acids (among which is caproic acid) from co-products from the sugar industry. Afyren is currently constructing a first plant with a capacity of 16 000 tons mixed carboxylic acids per annum (Sofinnova, 2020).

Beyond these two large-scale endeavours, a last commercial effort worth mentioning is that of Capro-X, an agritech spin-off from Cornell University. Capro-X aims to produce MCCA from acid whey, a dairy industry wastewater that requires treatment before discharge (Capro-X, 2020; Carvalho *et al.*, 2013; Duber *et al.*, 2018). This process is currently being tested at a 5600 L demonstration-scale plant at a dairy plant. Although specific information on the Capro-X process is currently not available, recent publications from the company founders suggest a temperature-phased two-stage process via lactic acid, coupling thermophilic acid whey fermentation to lactic acid, and subsequent mesophilic conversion of the lactic acid to MCCA. In that particular study both steps were operated at a pH of 5, and MCCAs were extracted using pertraction. MCCA production rates of 1.7 g L^{-1} d^{-1} were reported at an MCCA selectivity of 66% on a carbon basis (Xu *et al.*, 2018). Similar production rates and selectivity (2.6 g L^{-1} d^{-1}, 58–83%) were obtained in another lab-scale study by Duber and co-workers converting acid whey to MCCA in a one-stage system operated at 30°C and pH 5.5 without in-line product recovery (Duber *et al.*, 2018). Following a similar approach, the production of MCCA from thin stillage (an organic-rich watery stream generated as a side-stream of the bioethanol industry) has been demonstrated in a one-stage 60 L semi-pilot operated at pH 5.0–5.5 with product extraction (both pertraction coupled to membrane electrolysis and direct membrane electrolysis). The system, fed at an organic loading rate of 5 kg COD m^{-3} d^{-1} achieved a production of 1.7 ± 0.6 g MCCA L^{-1} d^{-1}. Using pertraction, the n-caproic acid was extracted at an efficiency of 73% (Carvajal-Arroyo *et al.*, 2020). Besides, successful MCCA production (7 g MCCA acid L^{-1} – mostly caproic acid, 81% MCCA selectivity) has been reported from Chinese liquor-making wastewater, which contains both ethanol and lactic acid (Wu *et al.*, 2018) and wine lees (Kucek *et al.*, 2016), and so on. proving that the production of MCCA from carbohydrate-rich industrial wastewater is a promising concept to generate value of an organics-rich wastewater.

Comparatively, the production of MCCA from domestic wastewater (mostly from waste activated sludge) is in a more preliminary stage and low technology readiness level. Low concentrations of caproic acid (representing less than 5% of the SCCA chemical oxygen demand) have been reported in fermentation broths derived from the fermentation of thermally hydrolyzed waste activated sludge (WAS) (Morgan-Sagastume *et al.*, 2011). Recently, several researchers have attempted to boost MCCA production in WAS fermentations by adding external ethanol, in a similar approach as that of the company Chaincraft. Wu and co-workers fermented WAS in alkaline conditions and with the external addition of ethanol, produced about 2–2.5 g L^{-1} caproic acid (Wu *et al.*, 2020). Similarly, Wang and co-workers fermented WAS (8% TS) and further converted the SCCA produced to MCCA in a second step using a chain elongation inoculum and with external ethanol addition (ratio 1:3). In that case, they reached final caproic acid concentrations of 5 g L^{-1} (Wang *et al.*, 2020). It is important to mention that these two studies were small-scale laboratory proofs of concept, far from what one would consider

realistic case studies. Finally, a last report worth mentioning is the co-fermentation of waste activated sludge with organic waste consisting of alcohol and soda beverage, food, dairy, fruit, fat and oil wastes. By operating a 15-L bio-reactor at pH 5.0 semi-continuously, using as feed a mixture of primary sewage sludge to organic waste at ratio of 1:1, it was possible to achieve an MCCA concentration of 4 g L^{-1} at a selectivity of around 55% (Owusu-Agyeman *et al.*, 2020). Despite not considering product recovery (the focus of the study was the production of SCCA to aid denitrification in the WRRF), this latter study represents a more realistic take on the production of MCCA from sludge as it avoided the need for external addition of expensive ethanol and was conducted in a bio-reactor. However, while promising, this MCCA production avenue is still in its infancy.

7.7 CHALLENGES, OPPORTUNITIES AND RESEARCH NEEDS

Bioproduction of carboxylic acids from wastewater and industrial side-streams is, as mentioned before, still in its early stages. There are several key challenges that need to be overcome to further develop and mature these technologies. In what follows, we give a brief overview into these challenges, where research can help overcome these challenges, and the opportunities these systems offer for the future.

7.7.1 Bioprocess engineering

An efficient bioproduction process should aim to convert the feedstock at high production rates, with high product selectivity and substrate consumption efficiency. Optimizing these targets simultaneously may not be possible, and the best bioprocess may be obtained by balancing these optimization goals.

Volumetric production rates (g product·L^{-1} reactor·h^{-1}) are the result of the biomass concentration (g biomass·L^{-1}) and biomass-specific production rates (g product·g^{-1} biomass·h^{-1}). High production rates could be achieved by engineering ways to keep these two simultaneously high. For instance, biomass concentrations can be strongly increased by uncoupling hydraulic retention time (HRT) and sludge retention time (SRT), that is by retaining biomass in the reactor. This can be achieved through membrane retention (Pan *et al.*, 2020), anaerobic filter reactors (Grootscholten *et al.*, 2013) or granular reactors (Carvajal-Arroyo *et al.*, 2019; Roghair *et al.*, 2016). Granules are self-aggregating biofilms with high settling velocities that enable their retention in a reactor system, an approach already used in full-scale anaerobic digestions, for example UASB, EGSB or IC reactor configurations (see Chapter 5 for more details). Reduced biological activity can be the result of low substrate concentration – which should be avoided by amending feedstocks with more concentrated streams or engineering the feeding strategy – or product toxicity. Carboxylic acids can be toxic through different mechanisms, such as acidification of cell cytoplasm, disruption of cell membranes or interactions with intracellular enzymes (Desbois & Smith, 2010; Palmqvist & Hahn-Hägerdal, 2000; Royce *et al.*, 2013). One of the main approaches to alleviate product toxicity in carboxylic acid producing bioprocesses has been in-situ product recovery (see sections 7.4.2 and 7.6.2). Future research should aid in developing strategies to overcome both the low biomass concentration and low biomass activity challenges and pave the way to high-rate production processes.

Another key challenge to take into account is product selectivity, and associated with this, substrate conversion efficiency. Mixed culture fermentations rarely yield a single product, but usually result in a mixture of several SCCA and/or MCCA. Steering fermentations towards a target product is important to achieve an economically feasible process, as the substrate utilization and its conversion to the product of interest should be maximized. How to steer this depends heavily on what your target product is, and whether the goal is a single product or a mixture of products. For instance, for the production of polyhydroxyalkanoate (PHA) bioplastics, a mix of SCCA is actually desirable. Choosing an appropriate strategy to steer fermentation towards a product requires information about the substrate(s) used and good control over the process. Future research could aid in defining process-specific approaches to optimize product selectivity. A point that is associated with this, and the maximization of substrate conversion efficiency, is the need to inhibit methanogenesis in carboxylic

acid-producing systems. Generation of methane implies a loss of substrate to a low-value and, in these processes, undesirable product. Several feasible strategies to suppress methanogenesis have been developed, and usually a combination of these is applied to prevent incursion of methanogens in the system. For instance, (non)-specific chemical inhibitors can be used, such as 2-bromoethanesulfonate (BES) or iodoform (Holtzapple *et al.*, 1999; Zinder *et al.*, 1984). Methanogens can also be removed from the process inoculum by heat treatment, retaining only spore-forming organisms. Both of these approaches have the downside that they do not prevent methanogens from re-entering the system through the feedstock, and, they may be too costly to apply on a frequent basis. Alternatively, operation at low or high pH has been applied to keep methanogens out (Agler *et al.*, 2012; Zhang *et al.*, 2009) and using a low SRT can lead to the wash out of slower growing methanogens (Appels *et al.*, 2008), yet both strategies may have negative impacts on the activity and/or composition of the community. Realistically, a 'silver bullet' to address the presence of methanogens without affecting process performance has yet to be developed.

A last challenge that is worth mentioning is the development of realistic co-fermentation approaches for valorization of waste sludge as MCCA. A first research hurdle to overcome is identifying substrates that could be used to amend waste sludge with. Likely targets would be carbohydrate-rich wastes such as food waste, vinasse from sugar refineries, or side-streams from bio-ethanol production. However, a factor that is at least as important as 'which streams are suitable?' is 'which streams are available?'. Developing industrial symbioses for chemicals recovery from wastes is a context-dependent challenge and restricting suitable co-fermentation wastes may result in foregoing high-potential, but only locally suitable, approaches.

7.7.2 SCCA/MCCA product recovery

In many bioprocesses, downstream processing represents a key component of the process economics and environmental footprint, often constraining their full-scale application (Woodley *et al.*, 2008). The product recovery impacts the economics of the overall processes in several ways. An ideal downstream process has high extraction capacity, high selectivity and requires a low energy and chemical input, although these are often opposed concepts.

A high extraction capacity minimizes the size of the downstream processing units, but often results in poor selectivity. The selectivity impacts the overall production train in different ways: it ensures that only the final target products are extracted, enabling fermentation intermediates and unwanted compounds to remain in the fermentation broth and minimizes the cost of product purification. For instance, in a bioprocess targeting caprylic acid, the extraction of shorter elongation intermediates, that is acetic acid, butyric acid and caproic acid, would impact the selectivity of the bioprocess, since those extracted intermediates cannot be converted to the final target product. Additionally, the presence of unwanted by-products in the extract means that further fractionation is necessary. When the extract is a mixture of salt carboxylates, purification of the salts would require recrystallization (Kim *et al.*, 2018), and when the extract is an oily mixture of free acids, distillation would be needed to fractionate and purify the final products, at an additional energetic cost (Saboe *et al.*, 2018).

When using liquid–liquid extraction, the selection of the solvent is crucial, for example Saboe *et al.* (2018) showed that Cyanex 923, a solvent composed mostly of trialkyphosphine oxides, could extract four times more caproic acid than trioctylamine, but the latter was two times more selective for caproic acid with respect to valeric acid. Similar results were obtained when comparing mineral oil with ketone-based solvents. Mixtures of phosphine based extractants with an organic apolar solvent provided better selectivity towards longer carboxylic acids than the phosphine-based solvents alone, but butyric and valeric acids are co-extracted (Naert, 2020).

Membrane processes enable extracting carboxylic acids from fermentation broths while minimizing or dispensing the need for chemical dosage, although they suffer from low selectivity and are generally energy intensive. Further development of membrane processes should improve their selectivity and their fouling potential, which dramatically reduces their life.

Even though there has been a vast effort in developing microbial processes for the production of carboxylic acids as resource recovery products, there is a need for the development of economically sound downstream processing pipelines. Interdisciplinary research crossing the boundary between microbiology, chemistry and process engineering will be crucial for the future of these technologies.

7.7.3 From lab to real life

For several decades, biogas production from organic waste streams and wastewater has been considered one of the most efficient technologies for resource recovery, together with land application as organic fertilizer. However, as stated in this chapter, technologies to produce higher value products from organic-rich wastes such as SCCA and MCCA are breaking through. Although several pilot and demonstration scale projects are underway, most technologies for the production of carboxylic acids from waste and industrial streams are currently at laboratory scale.

Bridging the gap from lab to real life remains challenging and requires, beyond the technological and scaling up challenges, careful consideration of market development so that supply and demand match. Using caproic acid as an example: its current global market is 25 000 tonnes per year (Moscoviz *et al.*, 2018). Even if caproic acid can be supplied at a more attractive price and necessary quality, its mid-users need time to build-up capacity and/or develop new processes and product applications that will result in an increased demand. As a result, production will need to be adjusted to market development to ensure price stability. Another important aspect is whether or not the mid- and final-user will accept a product derived from wastes, which has an impact not only on the acceptance, but also on the selling price of the wastewater-derived carboxylic acids (and hence on the overall economics and feasibility of the process).

Securing partners to cover all links of the supply chain (i.e., feedstock, production, recovery, mid-user, distributor of final product and consumer of final product) is also critical. All these components of the chain need to be secured by agreements that will ensure that once the SCCA/MCCA production plant starts, its product will be taken up along the chain. Only with such agreements in place will it be possible to guarantee a return on investment for the feedstock/producer and attract the necessary investment. However, getting product off-take agreements can prove itself a challenge. One needs to consider that mid-users would only commit themselves after successful preliminary tests or prototypes. To conduct preliminary tests at such industrial scale, high volumes of product may be required (up to a ton of SCCA/MCCA), which can only be achieved through demonstration-scale projects. The investment needed for such large-scale projects that do not result in short-term profits is a key challenge of the technology development, a stage commonly known as ‘the innovation valley of death’ in which promising research cannot reach the market due to a lack of investment. It is thus essential to find partners willing to invest in this step and take the risk associated with it. For industry, this can only be achieved if the company has a long-term vision connected to their investment. A demonstration project for SCCA/MCCA production is the final step towards commercialization, being the gains from the investment expected at this later stage. However, it is challenging for companies to accept that it may take five years or more until profits can be obtained. Hence, finding the right supply chain partners to bridge the gap from lab to real life will be crucial for the development of full-scale SCCA/MCCA bioproduction applications.

7.8 CHAPTER SUMMARY

In this chapter, some emerging concepts for the production of biobased products from organics present in wastewater and/or derivatives (i.e., waste activated sludge) have been discussed, with SCCA as central intermediate. The chapter focuses first on the microbial fundamentals of the process (hydrolysis and acidogenesis), describing how organic compounds can be converted to various SCCA as a function of the type of substrate, microbiology and operational conditions. Subsequently, various physicochemical and biological recovery and/or upgrading routes of these SCCA are briefly illustrated,

including their fundamental principles and basic engineering concepts. Based on this knowledge, this chapter then describes how SCCA and MCCA could be produced from wastewater and the status of development of such technology. Last but not least, the key challenges and shortcomings with regards to bioprocess engineering, product recovery and market development are briefly touched upon.

7.9 EXERCISES

Exercise 7.1: Hydrolysis is the rate-limiting step in anaerobic conversions, and is usually described using a first order kinetic (see Equation (7.1)). Assuming a substrate with an initial solids concentration of 30 g COD·L^{-1} and an anaerobic hydrolysis constant of 0.1 d^{-1}, how long should the HRT of a reactor be to ensure at least 85% substrate conversion?

Exercise 7.2: Calculate the ATP yield of a lactic acid fermentation of 1 kg glucose [mol ATP·kg glucose^{-1}], according to the information provided in Table 7.3. If a wastewater contains 30 g glucose·L^{-1}, how much energy can be potentially harvested by microorganisms performing such fermentation [mol ATP·L^{-1}]?

Exercise 7.3: A continuous stirred tank reactor (CSTR) of 50 m^3 of volume treating industrial wastewater is operated at a hydraulic retention time of 4 days. If the concentration of organic matter in the wastewater is 45 g COD·L^{-1}, what is the organic loading rate of the system [g COD·L^{-1}·d^{-1}]? If the biomass concentration in your reactor is 5 g VSS·L^{-1}, what is then your F/M ratio [kg COD·Kg VSS^{-1}·d^{-1}]?

Exercise 7.4: In a given fementation process, carbohydrate-rich wastewater is fermented to either: (i) lactic acid; or (ii) propionic acid. Calculate the NaOH requirements as meq OH^-·mol $^{-1}$ acid produced and kg NaOH·kg^{-1} acid produced for each scenario at two operational pHs, 5.5 and 7. The *pka* of lactic acid and propionic acid are 3.85 and 4.87 respectively.

Exercise 7.5: A food and beverage industry produces a wastewater flow of 250 m^3 daily, containing 27 g·L^{-1} COD, of which 87% is biodegradable. Calculate what would be the maximum annual caproic acid production if all organic material can be selectively converted to it. Assume that 5% of the bCOD ends up in biomass.

Exercise 7.6: Caproic acid is produced at a rate of 0.7 g·L^{-1}·h^{-1} in a CSTR operated at an HRT of 2 days. What should be the caproic acid extraction rate [g·L^{-1}·h^{-1}] to ensure a concentration at the effluent below 1 g caproic acid·L^{-1}?

Exercise 7.7: A fermentation broth contains 10 g butyric acid·L^{-1} and 5 g caproic acid·L^{-1}. Assuming pka of 4.82 and 4.88, respectively, calculate the concentration of their undissociated form at a pH of 5.5 and 7. By which factor should we concentrate this stream to allow their recovery by phase separation?

Exercise 7.8: A dairy industry has invested in a process to convert their wastewater streams (200 m^3·d^{-1}) into MCCA, which consists of a continuous stirred bioreactor coupled to an in-line extraction system (V = 100 m^3). Caproic acid is the dominant end product and it is produced at a rate of 0.5 g·L^{-1}·h^{-1}. The reactor pH is controlled at 5.5 and extraction is set in a way that the caproic acid concentration in solution is always equal or below 1 g·L^{-1}. A process issue results in a failure in the extraction system but does not affect the bioreactor operation. As a result, caproic acid accumulates in the system. If we assume that the impact of caproic acid toxicity can be described using a non-competitive inhibition term and the K_I for this specific microbial community is 0.15 g·L^{-1}, how would activity be reduced if extraction is stopped for 24 hours. For the sake of simplicity, assume that the production rate will remain unaffected during that period.

Exercise 7.9: A bio-production plant consist of fermentation and downstream processing. The fermentation step has a yield of 4 mmol propionic acid·g COD^{-1}. The propionic acid in the fermentation broth can be recovered with a 75% efficiency. If we want to produce 100 tonne propionic acid·d^{-1}, what should be the substrate loading [tonne COD·d^{-1}]?

Exercise 7.10: The size of an installation determines the investment needed, although often not in a linear manner. Larger installations are comparatively cheaper than smaller plants when one takes into consideration the cost per unit of volume. This is usually described using a scaling law of the formula: $Cost_2 = Cost_1 \cdot (Size_2/Size_1)^a$, where $Cost_1$ is the cost to build a certain installation of $Size_1$, and $Cost_2$ is the cost to build it at a different scale, $Size_2$. These costs are related by a parameter a, which reflects the linearity in scale-up cost. Imagine that constructing a 1 m^3 installation to produce and extract a mixture of SCCA (acetic, butyric and propionic acids) costs €20 000. Assuming an a of 0.7, a unit production of 1 tonne SCCA·m^{-3}·d^{-1}, and a selling price of €1700·$tonne^{-1}$, what should the plant size be to reach break-even in five years.

7.10 DISCUSSION QUESTIONS

Question 7.1 (*process design*, *choice of wastewater*): You are an R&D engineer working for a company that has developed a process to convert aqueous waste and side-streams into MCCA. You are currently exploring different possible applications for your technology, including wastewater from a potato factory rich in starch, secondary sludge from a local wastewater treatment facility and an aqueous waste stream from a food-processing company specialized in the production of oil and margarine. The chief technical officer of your company has asked you to select the most suitable of these streams based on your a-priori knowledge. Which one do you think is more suitable to your technology and why?

Question 7.2 (*process design*, *dimensioning*, *capital costs*): When constructing a new plant or facility, the initial investment needed for the purchase and equipment can represent a large part of the total costs. Discuss how key feedstock constraints and design and operational factors (e.g., organic loading rate, substrate degradability, product concentration, production rate, etc.) can influence the CAPEX. Take into consideration not only the bio-reactor, but also other equipment needed for, for example, solid–liquid separation or downstream processing.

Question 7.3 (*fermentation products*, *biochemistry*): According to Table 7.3, the fermentation of glucose to acetic acid results in the highest energy yields [ATP per mol glucose fermented]. Is the fermentation of glucose to purely acetic acid possible and if so can you explain why?

Question 7.4 (*fermentation products*, *biochemistry*): Does the fermentation of glucose to carboxylic acids always result in the production of CO_2? If so, why does this happen? Discuss whether the production of this CO_2 could be considered an issue in terms of climate change.

Question 7.5 (*process design*, *selectivity*): High product selectivity is important to ensure the inputs resources are efficiently transformed into the product of choice. What are the critical factors to take into consideration when designing a bio-process to ensure a selective production process. Take into consideration aspects such as inputs, microbiology, fermentation and downstream. Moreover, discuss the difficulties in order achieving selectivity with a special focus on the complexity and often fluctuating nature of wastewater streams in terms of varying flows, composition and concentrations.

Question 7.6 (*wastewater properties*, *product quality*): Product recovery is one of the critical steps of a technology to convert wastewater into raw materials. How does the input wastewater affect

downstream processing, both from a technical performance, economic, product quality as well as from a consumer acceptance standpoint? Based on these points, compare the use of sludge from municipal wastewater treatment plants versus a carbohydrate-rich industrial wastewater.

Question 7.7 (*product quality*, *chemical industry and economy-of-scale*): SCCA, such as acetic acid and propionic acid, are produced through chemical process. Considering that chemical process can produce these chemicals at a large scale and of constant quality, discuss the potential impact of the latter in terms of market potential. In your answer, include the following aspects: (i) quality and quantity aspects; (ii) logistics (i.e. means of storage, transportation and distribution); and (iii) ultimate end-user.

Question 7.8 (*product properties*, *product recovery*): MCCA are seen as a more desirable product than SCCA. Discuss what are the key technical and economic drivers for their production over SCCA. Are there downsides to be considered? On what basis do you think that a company will make the end-product choice?

Question 7.9 (*technology uptake*, *new business models*): Traditionally, the first and foremost task of municipal wastewater treatment plants is to remove pollutants as a means to safeguard human health and protect the receiving aquatic environment. Likewise, industries in the food and beverage sector, dairy, and so on. focus on the manufacturing of certain goods, with wastewater being an unavoidable, yet undesired, side-product containing pollutants (but thus also resources) that require treatment prior to disposal similar to municipal WWTPs. The development of novel technologies for resource recovery from wastewater should never conflict with is 'protecting' task, yet it requires more than that, including the development of new business models and management approaches. Reflect on how the need for the latter can potentially (negatively) impact the development and acceptance of new processes and consider what type of business model would encourage their implementation in real life installations.

Question 7.10 (*environmental impact*, *externalities*): Beyond economics, are there other drivers for the development of novel routes to valorize organic materials in wastewater? Discuss how different stakeholders can positively/negatively affect this development effort. In your discussion include terms such as green image, sustainability, social license to operate, regulatory and political drivers.

Question 7.11 (*scaling-up technologies*, *technology development and technology readiness level*): 'The valley of death' is the stage of technology development between academic-based innovations and their commercial application in the marketplace. The production of SCCA and MCCA is (or will soon reach) this stage. Identify the key shortcomings of the different routes to convert wastewater into carboxylic acids and consider what sort of technical advances are required to convince potential investors. In your opinion, what role (if any) should policymakers play in the effort to bridge the gap of 'the valley of death'?

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