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Lipid-based biofuel production from wastewater☆

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Increasing world population, urbanization and industrialization are driving global increases in wastewater production. Wastewater comprises significant amounts of chemical energy primarily in the form of organic molecules (in particular lipids), which are currently not being recovered comprehensively. Within biological wastewater treatment (BWWT) systems, specialized microorganisms assimilate and store lipids anaerobically. These intracellular stores represent interesting feedstocks for biofuel synthesis. Here, we review our current understanding of the genetic and functional basis for bacterial lipid accumulation and processing, and relate this to lipid accumulating bacterial populations which occur naturally in BWWT plants. A grand challenge for microbial ecologists and engineers now lies in translating this knowledge into the design of new BWWT processes for the comprehensive recovery of lipids from wastewater streams and their subsequent conversion into biofuel.

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Introduction

Microorganisms predominate Earth's biota. They encode the majority of genetic diversity on the planet and underpin nearly all biogeochemical processes. Advances in molecular methods are now allowing us to uncover the extensive gene pool contained with microbial ecosystems, and this resource will ultimately drive and sustain our societal and environmental needs. Among the renewable commodities of immediate interest which can be produced by microbes in significant quantities are biofuels. It is projected that microbially produced biofuels will eventually replace petroleum-based fuels as well as first-generation and second-generation biofuels [1]. First-generation biofuels synthesized from edible plant material and second-generation biofuels derived from non-food vegetable feedstocks (e.g. lignocellulosic material) are often considered unsustainable due to their competition with arable land and their insignificant impact in terms of reducing anthropogenic greenhouse gas emissions [1]. Nonetheless, second-generation biofuels may have a significant impact in specific spatiotemporal contexts in which enhanced feedstock availability may for example turn the tide in their favor [2]. Third-generation biofuels that are based on oleaginous material derived from microorganisms (microalgae, yeasts, bacteria) capable of growing (photo-) heterotrophically on organic waste or phototrophically on inorganic carbon, do not share these limitations [1]. Although research and development has mainly focused on algae-derived and yeast-derived lipids (for recent reviews see [3–5]), certain wastewater-borne microorganisms exhibit exciting phenotypic traits which may be harnessed for biofuel production, especially since increasing world population, urbanization and industrialization are leading to an increasing production of wastewater globally. In particular, oleaginous biomass from biological wastewater treatment (BWWT) plants represents a potentially important feedstock for the production of third-generation liquid biofuels such as biodiesel (fatty acid alkyl ester) as well as other high-value organic and inorganic resources [6••,7].

Current BWWT systems are based on the activated sludge process and this primarily relies on the microbial oxidation of organic molecules to CO_2. Only a small proportion of the wastewater organic fraction is assimilated into the sludge biomass. Subsequent anaerobic digestion of primary (mainly floating solids and greases collected from primary clarifier tanks) and secondary (activated sludge) sludge to biogas only allows limited chemical energy recovery.

In municipal wastewater, lipids can represent in excess of 40% of the total organic fraction [8], with the vast majority consisting of triacylglycerols (TAGs) and a minor part of free long-chain fatty acids [9]. In order to potentially facilitate significant chemical energy recovery from wastewater, for example through direct biodiesel synthesis from lipid-rich biomass, specialized bacteria which occur within BWWT plants and accumulate copious amounts of intracellular lipids may be exploited. These oleaginous microorganisms either assimilate lipids from the wastewater or synthesize them *de novo* from other carbon sources, and store them intracellularly as neutral

lipids, for example, TAGs, wax esters (WEs) or polyhydroxyalkanoates (PHAs) [10].

Preliminary studies have demonstrated that lipid-rich bacterial biomass from biological wastewater treatment plants can be directly used for biofuel synthesis. The predominant fatty acids in wastewater are in the range of C14–C18 which are ideal chain lengths for the production of biodiesel [11,12]. Mondala *et al.* [11] were able to produce fatty acid methyl esters (FAMEs) — a biodiesel form — from primary and secondary sludge by chemical transesterification. On the basis of their method, they estimated a yield of 10% FAMEs per dry weight of sludge resulting in an estimated production cost of $ 3.23 per gallon (approximately 0.20 € per liter), which is lower than current consumer prices for petroleum-based diesel and alternative biodiesels [11]. Furthermore, they estimated that the integration of dedicated lipid extraction and transesterification processes in 50% of all existing municipal BWWT plants in the United States could produce an equivalent of 0.5% of the US yearly petroleum diesel demand [12]. However, the future utilization of oleaginous biomass feedstocks could dramatically improve the yield and cost-effectiveness of wastewater-derived biodiesel production. Therefore, together with projected improvements in engine efficiency, wastewater biodiesel could become an important part of any future renewable energy portfolio.

Knowing and understanding the function of the genes involved in microbial lipid assimilation, accumulation and processing are critical considerations for the optimization and large-scale production of biofuels from wastewater as well as from other complex lipid-rich feedstocks. The present review discusses our current knowledge of bacterial lipid metabolism in relation to representative lipid accumulating organisms in BWWT plants, that is the long-chain fatty acid accumulating organism '*Candidatus* Microthrix parvicella' (henceforth referred to as *M. parvicella*) and the PHA accumulating organism '*Candidatus* Accumulibacter phosphatis' (henceforth referred to as *A. phosphatis*), as well as in relation to metagenomic sequence data obtained from BWWT plants. In addition, lipidic wastewater-derived biofuels such as bio-oils and hydrocarbons produced using the Fischer-Tropsch process are also succinctly presented. Finally, a 'wastewater biorefinery column' concept for future recovery of energy-rich lipids from wastewater is also briefly discussed.

Bacterial lipid accumulation

Triacylglycerols and wax esters

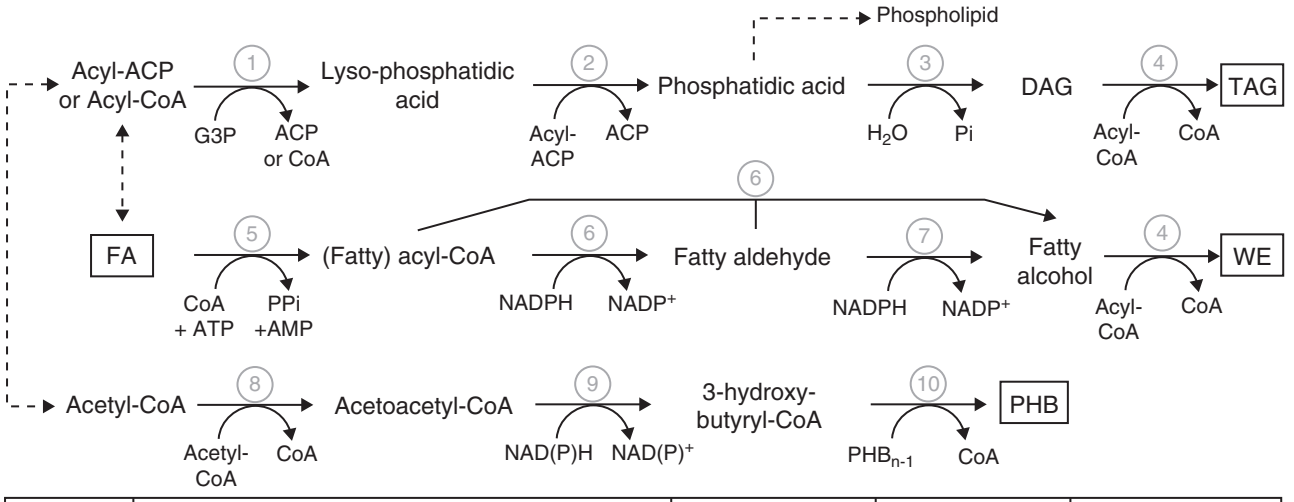
In bacteria, the last step in the biosynthesis of TAGs and WEs is catalyzed by the same enzyme, wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT), which assures the transfer of an acyl-CoA onto a fatty alcohol to produce a WE or onto a diacylglycerol (DAG) to yield a TAG [13] (Figure 1). Recent studies have demonstrated the general broad substrate specificity of the WS/DGAT [14•,15•,16•] and have highlighted specific amino acid residues which determine its substrate spectrum [17•,18,19]. The analysis of a recently published activated sludge metagenome [20] and the genome sequence of *M. parvicella* strain Bio17-1 [21] highlights the importance of the WS/DGAT gene in activated sludge biomass. Interestingly, *M. parvicella* strain Bio17-1 [21] encodes 4 homologs of WS/DGAT (Figure 1) suggesting that this enzymatic redundancy plays a role in this organism's ability to accumulate large amounts of lipids intracellularly.

Our understanding of the structure and dynamics of lipid bodies formed during TAG or WE accumulation has been greatly improved by recent work. The current model posits that WS/DGAT docks to the cytoplasmic membrane, where it catalyzes the synthesis of lipid microdroplets. These microdroplets are then released into the cytoplasm after conglomeration and surrounded by a phospholipid monolayer decorated with proteins [22]. The recent isolation of lipid bodies has led to the identification of many associated proteins, including a highly abundant protein referred to as TadA, MLDS or LPD06283 [23,24,25••]. This protein is involved in controlling the size of lipid droplets [23,24,25••] and therefore potentially represents a key gene which regulates overall lipid accumulation phenotypes. The search for *Rhodococcus*' TadA (HM625859.1) or LPD06283 (4218150) homologs in *M. parvicella* did not result in any significant hits suggesting that the discovery of additional sequences of TadA homologs experimentally validated in phylogenetically unrelated organisms will be necessary to unravel the distribution of this gene in the bacterial domain. Additionally, the mechanism of action of this protein needs to be clarified in order to exploit its biotechnological application.

Our latest understanding of bacterial lipid accumulation is already being used to bioengineer laboratory strains to accumulate greater quantities of TAGs or WEs (for recent reviews see [26,27]). Strikingly, the complete list of bacterial genes involved in WE and TAG biosynthesis was not known until quite recently. These genes include the fatty aldehyde reductase necessary for fatty alcohol synthesis before WE synthesis [28–30] and the bacterial phosphatidic acid phosphatase (PAP) [31•] which catalyzes the biosynthesis of DAG, a first dedicated reaction for the synthesis of TAGs or WEs rather than phospholipids in bacteria (Figure 1). Moreover, other studies have suggested the existence of an alternative TAG biosynthesis pathway involving a different enzyme to WS/DGAT [32,33] but details so far remain elusive.

Bacterial populations known to accumulate TAGs and WEs (mainly actinobacterial genera such as *Mycobacterium*, *Rhodococcus*, *Nocardia* or *Microthrix*, but also from other taxa such as *Acinetobacter*) are present in the biomass

Figure 1



Enzyme #	Enzyme name	Metagenomic EBPR Aalborg	*Candidatus* Microthrix parvicella	*Candidatus* Accumulibacter phosphatis
1	glycerol-3-phosphate acyltransferase (GPAT)	9	1	2
2	lyso-phosphatidic acid acyltransferase (LPAT)	25	1	2
3	phosphatidic acid phosphatase (PAP)	nd[a]	0[b]	0[b]
4	wax ester synthase/ acyl-CoA:diacylgycerol acyltransferase (WS/DGAT)	3 19[c]	4	2
5	fatty acid-CoA synthase	143	28	16
6	fatty acyl-CoA reductase (FAR)	6	0[e]	0[e]
7	fatty aldehyde reductase (FALDR)	nd[a]	0[b]	0[b]
8	β-ketothiolase (BKD)	135	13	6
9	acetoacetyl CoA reductase (AAR)	18	1	1
10	PHB polymerase	5	1	4

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The main bacterial metabolic pathways involved in lipid accumulation. The frequencies of specific homologous genes are reported in the corresponding table for (*i*) a metagenomic dataset generated from the biomass of a BWWT plant operated for enhanced biological phosphorus removal (EBPR) [20], (*ii*) the genome of the lipid accumulating filamentous actinobacterium *M. parvicella* Bio17-1 [21] and (*iii*) the polyphosphate accumulating betaproteobacterium *A. phosphatis* UW-1 [63], based on their annotation (for details on annotations, see Supplementary Tables). Abbreviations: ACP: acyl carrier protein; DAG: diacylglycerol; FA: fatty acid; G3P: glycerol-3-phosphate; PHB: polyhydroxybutyrate; Pi: inorganic phosphate; PPi: pyrophosphate; TAG: triacylglycerol; WE: wax ester. [a]not determined; [b]not found by BLAST search of nucleotide sequences of the 3 characterized PAP sequences (*Streptomyces coelicolor* A3(2) genes SCO1102 and SCO1753 [31•] as well as gene EF535727.1 from *Geobacillus toebii* strain T85 [64]) or FALDR of *Marinobacter aquaeolei* VT8 (accession number yp_959486 [30]); [c]annotated as diacylglycerol acyltransferase only.

of activated sludge [34,35]. These organisms typically assimilate long-chain fatty acids under anaerobic conditions, can become very abundant [36] and are often discernible as foam on the surface of anoxic BWWT tanks [34]. Therefore, we suggest that the recuperation of this lipid-rich biomass by simply skimming the surface could provide the feedstock for subsequent high-yield biodiesel production.

In activated sludge-based BWWT processes, specific TAG accumulating organisms excrete extracellular lipases, catalyzing the lipid hydrolysis present in the surrounding wastewater before its assimilation (Figure 1) [37]. *M. parvicella* possesses 8 lipases and due to the complexity of lipid mixtures in wastewater and extensive inter-organismal competition, these must have broad substrate specificity and high enzymatic efficiency. These enzyme characteristics should prove very useful for transesterification reactions involved in biodiesel production using a range of different lipid feedstocks (see also section 'From oleaginous biomass to biofuels via biocatalysis').

Polyhydroxyalkanoates

The most common bacterial lipid inclusions are so-called carbonosomes consisting of PHAs. PHAs have typically been promoted as replacement for petroleum-derived plastics [38]. However, an alternative use of PHAs includes the production by esterification of hydroxyalkanoate methyl esters (HAMEs), which can serve as biofuels or as fuel additives [39,40] (see also Box 1).

Polyhydroxybutyrate (PHB) typically is the most abundant PHA. The genes encoding the key enzymes involved in the synthesis of the monomeric precursor of PHB as well as its polymerization and depolymerization are well established [10] (Figure 1). Our current understanding based on work in model organisms suggests that PHB granule formation follows a 'scaffold model' in which PHB synthases attach first to a scaffold molecule to produce the initiation complex for PHA biosynthesis. These scaffold molecules likely are DNA and PHA granule-associate protein PhaM, which also aids in the dimerization of PHA synthases [41•,42••]. Other proteins coating PHA granules have also been identified and recently reviewed [43]. Due to the industrial interest in PHA for the production of bioplastics, extensive work has been carried out to enhance the production of this polymer through for example translating it into heterologous contexts [44].

In the bacterial communities underpinning BWWT, PHA accumulation can be very rapid and pronounced (e.g. up to 77% of cell dry weight in five hours [45]). Two major categories of organisms, namely phosphorus accumulating organisms and glycogen accumulating organisms accumulate PHA when exposed to anaerobic conditions. These organisms encode homologs of the genes implicated in PHA synthesis (Figure 1).

From oleaginous biomass to biofuels via biocatalysis

Apart from the WEs of ethanol or methanol, in order to be used in common internal combustion engines, the content of lipid granules has to be converted into a less viscous form. To achieve this, transesterification of TAGs is most commonly carried out to produce fatty acid alkyl esters (FAAEs), as well as the esterification of PHAs into HAMEs.

TAG and PHA (trans-)esterification reactions require an alcohol (methanol for FAMEs and HAMEs, or ethanol for fatty acid ethyl esters — FAEE) and a catalyst. The TAG

Box 1 Fuel blending.

Crude oil is a complex mixture of hydrocarbons composed of branched-chain alkanes, alkenes and aromatics ranging from 4 to 23 carbons in length. Therefore, the hydrocarbon composition of crude oil has to be modified by distillation and refining processes to achieve distinct cetane numbers, which reflect ignition quality in diesel engines. Although biodiesel in general satisfies standard fuel specifications, variations in oxidative stability, cold-flow properties and exhaust emissions can cause quality concerns [65]. However, the use of additives and cetane enhancers as well as the blending of various fuels can minimize these limitations, thereby improving overall fuel properties and ensuring consistent quality [66]. Given the chemical differences of biodiesel when compared to petroleum-based diesel and short-chain alcohols, blending allows fine-tuning of fuel characteristics. Preliminary studies suggest that blends of ethanol–diesel and of ethanol–biodiesel–diesel exhibit increased fuel energy recovery due to increased fuel oxygenation as well as reduced exhaust emissions. Mixed feedstock biodiesel production has recently been used to improve the physical properties of fuels. Such blending strategies can also be used in the context of wastewater biodiesel production.

Box 2 Alternative strategies for using wastewater and microorganisms to produce energy.

Alternative strategies for exploiting wastewater or waste sludge have involved eukaryotic photoheterotrophic (e.g. micro-algae [67]) or heterotrophic organisms (e.g. oleaginous yeast [68]).

Additionally, anaerobic digestion of sludge to biogas is an established bioenergy recovery strategy from wastewater, but few notable limitations exist such as the limited recovery of chemical energy, quality of gas generated, storage problems and overall capital investment [69].

Apart from lipid-based biofuels, electrochemical energy production represents another interesting avenue for energy recovery from wastewater. When microorganisms oxidize substrates, electrons are transferred to an electron acceptor. Microbial fuel cells (MFCs) contain anodes and cathodes to generate electricity by harnessing this flow of electrons. Generally, microorganisms involved in the oxidation of substrates are located at the anode and electron accepting microorganisms at the cathode of MFCs. The ensuing difference in potential between the anode and a cathode leads to the generation of an electrical current. The power produced is a key factor for evaluating the performance of MFCs. At present, the yields of MFCs fed with wastewater are far below what would be required to make them economically sustainable. However, intense research in the past five years suggests that energy output of MFCs is far from reaching its full potential (overall we have witnessed a 10-fold increase in power generation from MFCs compared to preliminary outputs) and, consequently, this technology shows great potential as a future means of recovering chemical energy from wastewater streams [70].

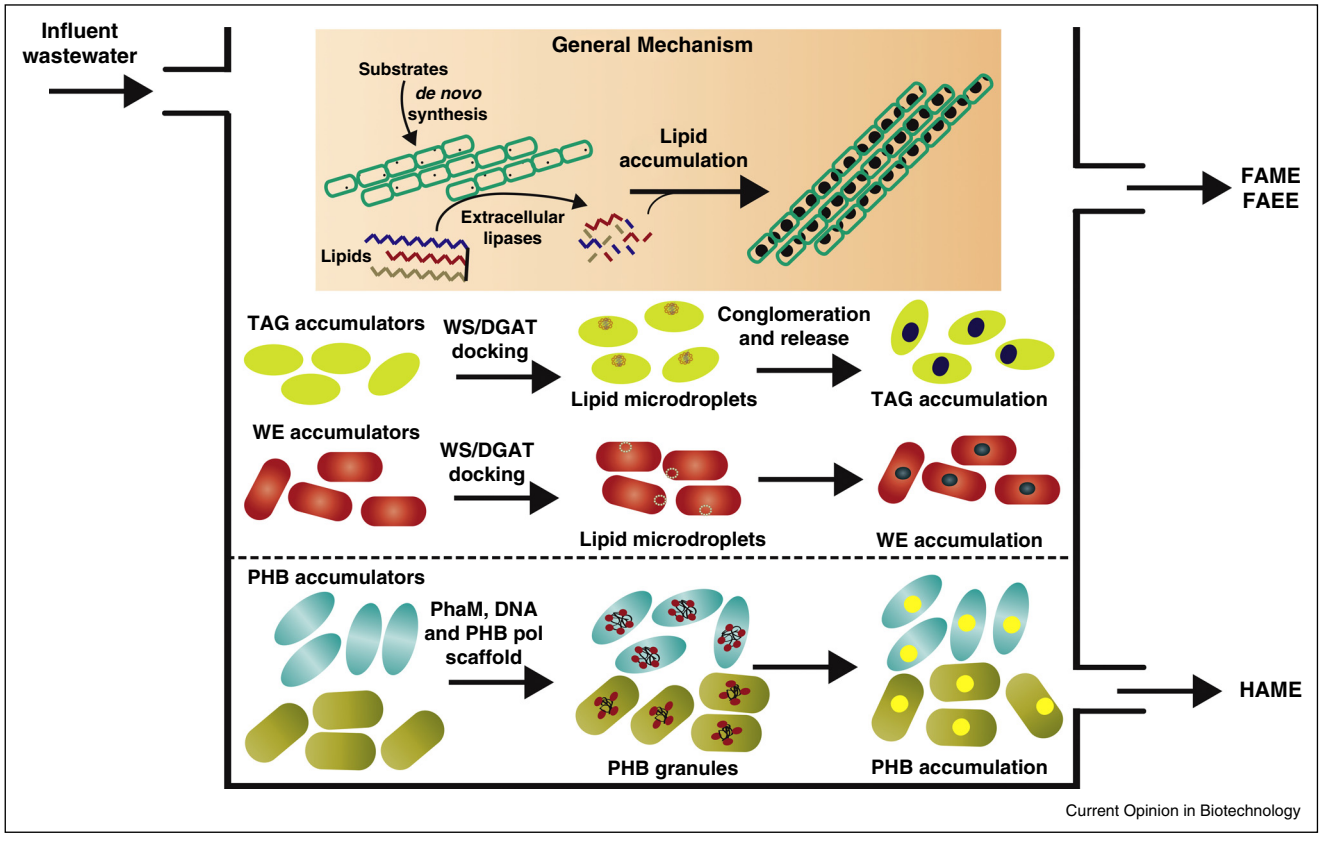
transesterification catalyst can be a lipase or a chemical such as an acid or a base (e.g. of catalyst-free methods, see [46,47]). The current industrial production of biodiesel by chemical transesterification of TAGs has several disadvantages including high energy consumption, the need for salt and water removal to avoid saponification of free fatty acids and the requirement for downstream processing, for example, glycerol removal. Recently, enzymatic transesterification (recently reviewed in [48•]), catalyzed by intracellular or extracellular lipases, has been suggested as a future alternative. The *in vivo* enzymatic method appears to be the most convenient path to produce biodiesel, but in many cases requires bioengineering of strains and such strains may not be able to compete with other organisms in open bioreactor systems such as BWWT plants. On the other hand, *in vitro* processes may be used involving for example enzyme immobilization or encapsulation [48•]. Interestingly, glycerol, the byproduct of biodiesel production via transesterification of TAGs, can also be reused to synthesize other commodities [49], notably biodiesel [50] and bioethanol [51] (for short-chain alcoholic biofuel production using waste biomass, see the next section).

Alternative routes of liquid biofuel production from wastewater

The production of bio-oils (also called pyrolysis oils), short-chain alcoholic biofuels or complex hydrocarbons by the Fischer-Tropsch process represent alternative methods for the production of liquid biofuels from wastewater biomass. Other strategies to produce bioenergy (biogas, electricity) from wastewater are also highlighted in Box 2.

The production of bio-oils from waste sludge by pyrolysis has gained significant attention in the past few years [52]. Biomass feedstock quality majorly affects pyrolysis yield, but recent advances in accurately defining reaction conditions according to sludge composition have demonstrated the feasibility of cost-effective and commercial production of bio-oils from wastewater biomass [53,54]. For the sustainable full-scale production of sludge-derived bio-oils, the composition and combustion quality of produced fuel should provide the required performance without causing engine and infrastructure damages (drop-in fuels) [55] (Box 1). The calorific value of pyrolysis-derived sludge bio-oil (36 MJ kg^{-1}) is comparable

Figure 2



Conceptual scheme of a 'biorefinery column' for biofuel production from wastewater under anaerobic conditions using specifically enriched lipid accumulating bacterial populations. Abbreviations: FAEE: fatty acid ethyl ester; FAME: fatty acid methyl ester; HAME: hydroxyalkanoate methyl ester; PHB: polyhydroxybutyrate; PHB pol: PHB polymerase; TAG: triacylglycerol; WE: wax ester.

with that of commercial diesel (45 MJ kg^{-1}) [56•]. Furthermore, the bio-oil from BWWT sludge contains various hydrocarbons ranging from C6 to C20 [54], including isoprenoid lipids such as farnesene [57] with substantial calorific values. By monitoring the hydrocarbon composition of sludge-based bio-oils and their further targeted refinement, the conversion of sludge-derived bio-oils into a variety of specific products opens up exciting commercial prospects.

A wide range of residues from wastewater can be used to produce short-chain alcoholic biofuels but these are typically confined to small-scale plants [58]. Although poorly studied, preliminary studies have highlighted the fermentation capabilities of microorganisms producing industrial relevant organic acids within BWWT biomass [59]. Additional research on identifying and cultivating microorganisms that are capable of producing significant quantities of short-chain alcohols from wastewater may improve large-scale short-chain alcoholic biofuel production in the future.

Another alternative process to produce liquid biofuels such as biodiesel and short-chain alcohols using wastewater-derived gasified biomass involves the Fischer-Tropsch process or microbial syngas fermentation [60,61]. Both processes involve the conversion of CO and H_2 into hydrocarbons. Although still in their infancy when applied to wastewater biomass, large-scale production is currently being piloted (e.g. SYNPOL project, URL: http://www.synpol.org/).

Towards a biorefinery column for biofuel production from wastewater

In the context of increasing global population growth coupled to environmental deterioration due to human activity, future sustainable development scenarios should not only include the recovery of energy (biofuel or electricity) from wastewater to reduce our overall fossil fuel footprint, but also include provisions to meet other commodity needs. In this context, we have recently proposed the concept of a 'wastewater biorefinery column' which would leverage the existing and future wealth of information concerning the genetic reservoir of microorganisms and their functional capacity for sustainable production of bioenergy (Figure 2), in addition to other commodities such as bioplastics and fertilizers [6••]. However, in order to make this concept come to fruition, it is essential that we first obtain detailed descriptions of the niches of the individual community members, using global *in situ* monitoring methods ('meta-omics' [62]). Once detailed knowledge has been obtained, BWWT processes may be (re-)engineered using bottom-up design principles which take into account for example the ecological niches of the individual organismal groups. The optimization of processes may then involve a discovery-driven planning approach [62], rather the top-down strategies pursued so far. We still have a long way to go to bring this vision to fruition but it may represent a grand challenge for microbial ecologists and engineers alike to tackle at the centenary of Ardern & Lockett's discovery of the activated sludge wastewater treatment process.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.copbio.2014.03.007.

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