Bacterial Wax Synthesis

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Abstract

Biological wax esters offer a sustainable, renewable and biodegradable alternative to many fossil fuel derived chemicals including plastics and paraffins. Many species of bacteria accumulate waxes with similar structure and properties to highly desirable animal and plant waxes such as Spermaceti and Jojoba oils, the use of which is limited by resource requirements, high cost and ethical concerns. While bacterial fermentations overcome these issues, a commercially viable bacterial wax production process would require high yields and renewable, affordable feedstock to make it economically competitive and environmentally beneficial. This review describes recent progress in wax ester generation in both wild type and genetically engineered bacteria, with a focus on comparing substrates and quantifying obtained waxes. The full breadth of wax accumulating species is discussed, with emphasis on species generating high yields and utilising renewable substrates. Key areas of the field that have, thus far, received limited attention are highlighted, such as waste stream valorisation, mixed microbial cultures and efficient wax extraction, as, until effectively addressed, these will slow progress in creating commercially viable wax production methods.

Keywords – Wax ester synthesis, bacteria, sustainable, biotechnology, *Acinetobacter*, environment, economic

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1. Introduction

Waxes are lipophilic, organic substances with an enormous range of applications ranging from coatings and lubricants to cosmetics and food additives (Santala et al., 2011). Interest in waxes has increased recently as an alternative to plastics, with many of the same desirable characteristics, including water and chemical resistance and non-toxicity, but with the added advantage of their biodegradability. In 2019 the global wax market was valued at under $10 billion, but non-petroleum, natural waxes from plants and animals constituted just 5% of this (Grand Research View, 2020). With a forecast increase in market size to almost $12.5 billion by 2027, and the finitude and harmful emissions of petrochemical resources becoming ever more of a concern, a shift towards natural waxes is both desirable and inevitable. However, natural plant and animal waxes are currently restricted to small volume, high value products due to high production costs, land limitations (Vanhercke, 2014), intermittent production and prices susceptible to low yields or crop failures (Research Nester, 2019; Rontani, 2010).

Wax esters (WEs), the major component of biological waxes, are esters of fatty acids and alcohols, with typical chain lengths of 20 carbons or greater (Bart et al., 2013). As well as plants and animals, some species of micro-organism produce WEs directly as storage lipids, synthesised and accumulated intracellularly under nutrient, typically nitrogen, limited conditions to preserve excess carbon (Fixter et al., 1986; Santala et al., 2018). The advantages of bacterial WEs over current natural and petroleum waxes are numerous. Oleaginous bacteria grow on a wide range of feedstocks (Salcedo-Vite et al., 2019), including many considered waste streams, thus contributing to closing the carbon cycle. The WE profile of bacteria varies by species (Manilla-Pérez et al., 2011), growth conditions (Russell, 1978; Russell and Volkman, 1980) and feedstock (Salcedo-Vite et al., 2019), offering the opportunity to produce WEs with specific and diverse chemical profiles. Furthermore, employing bacterial WE production reduces pressure on land usage, mitigating 'food-vs-fuel' type debates, whilst decreasing product variability and seasonality and offering potential for greater scalability and cost reduction.

As an area of undoubted growing industrial and environmental interest, it is important that researchers are aware of the suitability of species and substrates, as well as

the current limits in terms of yields, to make informed decisions and optimise production. However, it is equally important that areas of the field which are thus far under-explored, but of vital importance to improving process scalability and viability, are identified and highlighted to encourage further exploration.

This review aims to provide a broad assessment of the successes and progress thus far in WE production by both wild type (WT) and genetically engineered bacteria. WE producing species are discussed in the context of the substrates from which they produce WEs, with substrates classified as structurally-related substrates, renewables and waste streams. Genetic modifications to improve WE synthesis are is divided into modifications of organisms which naturally synthesise WEs and those which do not. The limited reported attempts to utilise waste streams as feedstocks and to employ mixed microbial cultures for WE production are also highlighted, before discussing the three major areas of the field we identified for future research focus; novel substrates, efficient WE extraction and process optimisation and quantification.

2. Bacterial Wax Ester Production Pathway

Neutral lipids, including WEs and triacylglycerols (TAGs), are accumulated by bacteria for storage when carbon is plentiful but other essential nutrients, usually nitrogen, are limited (Alvarez, 2016; Ishige et al., 2003; Rontani, 2010). Stored carbon is subsequently made available for metabolism by the cell during periods of carbon shortage. This behaviour is believed to be an adaptation to extreme environments in which nutrient supplies are variable, with those species able to store carbon better equipped to survive periods of starvation. The use of storage lipids as a store of metabolic water, which is produced during fatty acid oxidation, has also been suggested (Alvarez et al., 2004).

Bacterial WE production occurs in three stages; production of the fatty acid, the fatty alcohol and their esterification (Kalscheuer et al., 2003). Where the carbon source is a small organic molecule, it is taken up into the tricarboxylic acid (TCA) cycle, and converted into acetyl-CoA, then acetyl-acyl carrier protein (ACP). From this, long chain fatty acids are constructed through sequential elongation steps, each adding two carbons to the ACP-bound acyl chain through reaction with malonyl-ACP and elimination of CO_2 (see Figure 1) (Cronan and Thomas, 2009). This results in acyl-ACPs of even carbon chains, commonly C4-C18. Odd chains are achieved if the starting molecule is propionyl-CoA (Stillwell, 2016).

Long chain fatty acids, typically C14 and above (Ishige et al., 2003; Kannisto et al., 2014; Stillwell, 2016), are released from the ACP and form fatty acyl-CoAs, which can be reduced to a primary fatty alcohols if a species possesses reductase enzymes. Reduction is usually catalysed by two enzymes, a fatty acyl-CoA reductase (FAR) and a fatty aldehyde reductase (FAldR), sequentially reducing a fatty acyl-CoA to an aldehyde then a fatty alcohol, although in some species a single reductase completes both steps (Hofvander et al., 2011). While fatty acid biosynthesis occurs in all bacteria, fatty alcohol production is less common, hence WE synthesis is limited to a small number of species. A wax synthase (WS) then catalyses the alcohol condensation with a second acyl-CoA, producing the WE (Bart et al., 2013). The WS is usually a bifunctional wax-synthase/diglyceride acyltransferase

(WS/DGAT), also responsible for TAG synthesis (Kalscheuer and Steinbüchel, 2003). Fatty acid and WE biosynthesis pathways from simple carbon substrates via acetyl-CoA are shown in orange and blue in Figure 1.

If the substrate is a long-chain alkane, the WE synthesis pathway is different. The alkane is oxidised to a fatty alcohol by an alkane hydroxylase (AlkM) (Ratajczak et al., 1998), as shown in green in Figure 1. Further oxidations by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes lead to the fatty acid, which is then converted to an acyl-CoA and condensed with another alcohol molecule to form a WE. Alkanes, and long-chain acids and alcohols, are therefore converted to WEs without entering the central carbon metabolism, with the majority of these WEs exhibiting acid and alcohol moieties of the same chain length as the substrate.

Insert Figure 1 here.

3. Wax Ester Production in Wild Type strains

With bacterial WE production a matter of growing industrial and environmental interest, research in this area is increasingly important. While genetic engineering and synthetic biology offers useful tools to improve production, it is necessary to recognise the advantages of optimising and exploiting WT organisms, to assess what is achievable without genetic modification and to identify desirable hosts and pathways for modification. For these reasons, this section will focus on WE production in WT bacteria.

A summary of all WT species in which WE accumulation has been characterised is given in Table 1, along with the substrates from which WE production has (or, conversely, has not) been detected. The most extensively studied bacterial genus, with six strains that accumulated WEs when grown on a wide range of substrates, including alkanes (Sakai et al., 2014), fatty acids and alcohols (Ishige et al., 2002), sugars (Salcedo-Vite et al., 2019; Santala et al., 2011), simple organic acids (Salmela et al., 2018) and even lignin derivatives (Kannisto et al., 2017), is *Acinetobacter*. However, many other species including *Alcanivorax* (Kalscheuer et al., 2007; Manilla-Pérez et al., 2010), *Marinobacter* (Holtzapple and Schmidt-Dannert, 2007; Lenneman et al., 2013; Nakano et al., 2012), *Mycobacterium* (Sirakova et al., 2012), *Rhodococcus* (Hernandez et al., 2008; Shields-Menard et al., 2015) and *Streptomyces* (Kaddor et al., 2009) have also been demonstrated to accumulate WEs as at least a component of their storage lipids on some substrates.

3.1. Utilisable substrates

Structurally related substrates closely resemble the alcohol and acid residue of the WE, such as alkanes, alcohols or fatty acids of the same chain length. Most common in the literature is hexadecane, which has been identified to yield WEs, predominantly palmityl palmitate, in ten species, with hexadecanol and hexadecenoic acid also widely tested. Bacterial WE production from such substrates was first reported for an unidentified Gram-negative *coccus* (Stewart and Kallio, 1959); since then many bacterial species have been observed to exhibit this trait. More novel alkanes and alcohols, including phytane (Silva et al., 2007) cholesterol, (Holbert et al., 2020), 6,10,14-trimethylpentadecan-2-one (Rontani et al. 1999) and squalene (Rontani et al., 2003) have also been explored as substrates for WE

production, although many were comprised of fragmented derivatives rather than the whole substrate. It is, however, important to note that currently the majority of long chain hydrocarbons and their derivatives are almost entirely derived from petroleum (Rahman et al., 2018). With a growing ambition to transition chemical processes away from fossil-fuel dependence, the exploitation of renewable feedstocks for WE production will inevitably grow.

A broad range of sugars and organic acids can be sourced renewably and employed as feedstock for WE production by microbial processes (Li et al., 2001; Maleki and Eiteman, 2017; McKinlay et al., 2007; Meng et al., 2016). Production on renewable substrates is largely dominated by *A. baylyi* ADP1, with WEs produced from acetate, butyrate, glucose, gluconate and pyruvate (see Table 1). *Marinobacter* species *aquaeolei* VT8, atlanticus CP1 and *hydrocarbonoclasticus* SP17 have also demonstrated WEs as major components of storage lipids, with succinate, citrate and pyruvate all suitable feedstocks.

Conversely, many strains, particularly of the *Alcanivorax*, *Rhodococcus* and *Streptomyces* genii which can produce WEs from structurally-related alkanes and fatty alcohols and acids, fail to do so from short chain acids and simple sugars. This indicates a lack of FAR and/or FAldR enzymes (see Figure 1), with such strains instead accumulating solely TAGs under such conditions. However, despite this reduced selection of suitable chasses, the value of these substrates as renewable feedstocks should not be overlooked.

However, sugars and sugar derived feedstocks can be expensive and problematic in terms of sustainability. High sugar crops must be grown on arable land with fertilisers and plentiful water to attain good yields and are limited by growing seasons (Marzo et al., 2019). The land use raises similar ethical concerns as plant wax production, while large-scale agriculture causes water and land contamination and biodiversity loss (Mohr and Raman, 2013). These issues, along with the massive substrate flexibility of bacteria, has led to growing interest in other sources of renewable carbon sources, including the valorisation of waste streams (see Section 5).

3.2. Wax ester Quantification

WEs have been detected in a small number of bacterial species, but have been characterised and quantified in even fewer. Quantification is extremely important for progress towards commercial technologies, as processes must be economically viable at scale and competitive with established petrochemical alternatives. Three measures of quantification relevant to industrial production are given (Table 2). WE per biomass expresses the quantity of wax each gram of biomass produces. Wax titre expresses the extractable WEs per litre of broth. This is dependent upon both WE per biomass and growth, since in some conditions (high C, low N) WE per biomass is high but biomass production is poor, so the absolute amount of WE is low. Titre is also important in industrial scale reactors, specifically: 1) more concentrated streams require less energy for downstream processing and 2) higher product concentrations allow smaller, lower cost reactors. Finally, yield, how much substrate is converted into the desired product, is vital as feedstock contributes heavily to operational costs. The higher the substrate cost, the more crucial the yield; if waste-streams can be used as feedstock, some yield can be sacrificed.

The maximum achieved WE titre and yield for a WT strain at flask scale was reported to be with *Acinetobacter* NRRL B-14920 grown on soybean oil (comprised of mixed TAGs). A titre of 2.61 g/L WEs accumulated at a yield of 32.2% from the oil, though this figure may be misleading as 4 g/L of glucose and 5 g/L yeast extract were also present in the culture medium (Kaneshiro et al., 1996). *Acinetobacter* M1 achieved the highest WE content, at 17%, when grown on hexadecane, but no other metrics for this production were provided (Ishige et al., 2002). On a non-lipid substrate, *A. baylyi* ADP1 on glucose and casamino acids produced 0.58 g/L of WEs at a yield of 3% g/g, and to a WE content of 9% CDW, the highest achieved across all strains and substrates tested (Luo et al., 2020).

Outside the *Acinetobacter* genus, WE accumulation is generally much lower, with maximum WE content of 4 % CDW (Manilla-Pérez et al., 2011), titre of 0.025 g/L (Bird et al., 2018) and yield of 3% (Bredemeier et al., 2003) for flask grown cultures. *Alcanivorax borkumensis* SK2 was able to produce 8.45 g/L of WEs in a fed batch reactor, the highest WE titre yet recorded, although accumulation of this WE took almost 11 days.

Several strains, including *A. borkumensis* SK2 and *A. jadensis* T9, have been demonstrated to excrete WEs as well as accumulating them intracellularly (Klein et al., 2008; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011; Nakano et al., 2012). In such cases the values presented for quantification are a total of all produced lipids. Both of these strains produced both WEs and TAGs, but the extra-cellular lipids were predominantly WEs (Manilla-Pérez et al., 2011). The ability to export WEs, especially selectively over TAGs, would be advantageous for industrial down-stream processing by reducing extraction costs and increasing efficiency. However, the genes and mechanism responsible have not yet been identified, preventing expression of this phenotype in other species.

4. Wax Ester Production in Modified Organisms

WE production has also been engineered into bacteria and can be divided into two approaches: 1) modifications to improve the breadth of utilisable substrates and 2) alterations to the metabolic pathways through which WEs and their precursors are produced. Attempts at the former have been minimal, largely due to many WE accumulating bacteria already exhibiting high substrate flexibility. Conversely, engineering of the carbon metabolism to divert flux through alternate or non-native pathways to generate WEs has been successful, with many different approaches being taken. Expression of non-native genes can impart new pathways between metabolites not previously accessible, improving efficiency or enabling synthesis of new products (e.g. WEs or fatty alcohols). Gene knock-outs can prevent or limit the flux directed into non-WE products, improving yields and conversion efficiency. In contrast, overexpression of key genes in WE production (see Figure 1) is an effective way to increase flux through a desired pathway, particularly where one reaction is the bottleneck of the process or if there are multiple, competing pathways.

For the purpose of this review, genetic modifications have been divided into two categories: native producing strains with modifications to improve or alter WE production and non-native producing strains with modifications which impart WE production ability.

Here 'native producing strains' includes all strains in which WEs have been observed to accumulate.

4.1. Modifications in Native Producing strains

4.1.1. Acinetobacter baylyi ADP1

Given the success of WE production in WT ADP1, it is unsurprising various engineering approaches have been attempted in this strain. Initial rational engineering involved overexpression of the FAR enzyme acr1, increasing flux through the WE synthesis pathway by increasing fatty alcohol synthesis. WE content increased by 2.7-times, while titre and productivity both doubled (see Table 3) (Lehtinen et al., 2018). When published, this was the highest bacterial WE titre without hydrocarbon supplementation.

Another approach was deletion of *aceA*, the isocitrate lyase in the glyoxylate cycle necessary for growth on non-glycolytic substrates but not on glucose, which directs additional flux towards acetyl-CoA for WE synthesis. Inducible *AceA* mutants were grown on acetate, with high arabinose inducer concentration allowing growth. Gradual arabinose oxidation to non-inducing derivatives caused aceA expression, and hence growth, to decrease, redirecting flux to WE production (Santala et al., 2018). WE content and yield increased compared to the WT, although titre was limited by restricted growth. Acetate was suggested as a more feasible substrate than glucose due to its lower cost and high abundance. However, the set-up used 50 mM acetate and 0.5% arabinose, or 4.1 g/L of sodium acetate and 5 g/L of arabinose, with respective costs of £0.21 and £24.20 per litre of broth (prices from Sigma, most cost-effective quantity). The significant inducer cost makes the process prohibitively expensive, regardless of the improved yield.

Most recently these two modifications have been combined into a single strain with complementary effects. While *AceA* deletion reduced biomass production even in glucose-cultured cells, in combination with *acr1* overexpression it gave similar CDW to the WT but with improved WE content and titre. The yield, whilst lower than just *aceA* deletion, was higher than both WT and *acr1* overexpression and no inducer was required, making the process more economical (Luo et al., 2020). The modified strain also produced WEs on nitrogen rich feedstocks, with amino acids, casamino acids, yeast extract and bakers' yeast hydrolysate all giving greater WE content than the WT.

Modification to alter the WE profile of ADP1 has also been attempted in a mutant possessing an alternative FAR complex LuxCDE in place of the native *acr1*, with hope the new FAR would express different chain length preferences (Santala et al., 2014). A maximum titre of 91 mg/L WE was achieved, a reduction from the WT 162 mg/L. The effect was partly observed, with changes in both chain length and saturation profiles of the acid and alcohol components. However, the average chain length reduced by 2 just carbons, not achieving the 'wider spectrum of alkanol chain lengths' the authors claim is required.

4.1.2. Rhodococcus strains

While successful in producing WEs from structurally-related substrates, *Rhodococcus* strains have been limited in WE production from simple substrates (see Table 1), with WEs from glucose in WT *R. jostii* RHA1 contributing just 0.0002% of CDW. Inducible

overexpression of the native FAR enzyme fcrA, which had very low levels of transcription and expression in the WT, increased the carbon flux into fatty alcohol production, with WE production of up to 13% of CDW (Round et al., 2017). The fcrA performed both stages of the fatty acyl to alcohol reduction, meaning overexpression of a specific FAldR could therefore further increase WE production. The group also tested a WS/DGAT with increased WE activity (Round et al., 2019), utilising both the fcrA and a WS from *Marinobacter hydrocarbonoclasticus* DSM 8798. The resulting species accumulated up to 15% CDW as WEs at yields of 0.05 g/g glucose substrate. A fed batch fermenter was also explored, in which the strain produced 5 g/L (1 g/L per day), with yield of 0.025 g/g.

The *Rhodococcus* strain *R. opacus* PD630, which did not naturally produce WEs on simple substrates, was also engineered for *de novo* WE synthesis by expression of a fatty acyl-CoA reductase from *M. hydrocarbobonclausticus*, which reduced acyl chains to alcohols (Lanfranconi and Alvarez, 2017). Whereas the WT on gluconate produced 100% TAGs, storage lipids in the modified strain were 46% WEs. This strain can also grow and accumulate TAGs on various model and industrial hydrolysates (Wang et al., 2014), hence this modification could facilitate WE production from lignocellulosic biomass waste streams.

4.2. Modifications in Non-native Producing strains

In the first study to isolate and characterise the WS/DGAT in *Acinetobacter* ADP1, the enzyme was expressed heterologously in *E. coli* S17-1 and *Pseudomonas citronellolis* (Kalscheuer and Steinbüchel, 2003). WS activity in the WT strains was negligible, whilst modified cells demonstrated activities comparable to ADP1. Recombinant *E. Coli* XL-1 Blue producing the WS/DGAT enzyme from *M. smegmatis* mc^2155 was also engineered, but WS activity in this strain was only slightly higher than the WT. The production of jojoba-like WEs from *E. coli* was subsequently attempted by co-expression of a fatty-alcohol producing, acyl-CoA reductase from the jojoba plant and the WS/DGAT from ADP1 (Kalscheuer et al., 2006). The mutant produced saturated C_{32} ester palmityl palmitate when grown on oleate, a monosaturated C_{18} fatty acid. WE content in cells was 1%, three times that of cells grown on LB medium, suggesting *de novo* WE synthesis was minimal.

To diversify the chemical characteristics of microbial WEs, *E. coli* was engineered to produce methyl-branched esters (MBEs) using propionate as a substrate (Menendez-Bravo et al., 2014). The group achieved up to 98 mg/L on propionate and octanol, the MBE alcohol component (Murli et al., 2003; Pfeifer et al., 2001). To avoid alcohol supplementation, further engineering with three fatty alcohol biosynthesis pathways was attempted, achieving a maximum titre of 37 mg/L of MBEs from propionate alone. Further exploration using different promoters and plasmid copy numbers achieved maximum titres of 138 mg/L in batch and 790 mg/L in a fed-batch process using an octanol, oleic acid, glucose and propionate mixture as feedstock (Menendez-Bravo et al., 2016). However, the authors' calculated yield of 2 mg MBE/g glucose is low and misleading, as it does not account for the other substrates.

Other *E. coli* modifications have largely focussed on TAG production (Janssen and Steinbuchel, 2014) or short chain esters, e.g. fatty acid ethyl esters, for use in biofuels (Liu

and Khosla, 2010; Rahman et al., 2019; Rottig et al., 2015), which will not be discussed in detail here.

5. Production from Waste Streams

Many waste streams are energetically and chemically rich with a diverse range of molecules and functionalities, and their valorisation improves process efficiency. They are economically attractive due to their inherent low value; since waste processing otherwise incurs significant costs, their price may be negligible, or even negative. However, utilising waste streams presents several challenges for bacterial degradation; 1) in many waste streams few bacterial species are able to process the major components, 2) components or contaminants may be toxic to bacterial cultures and 3) mixtures are complex and can be highly variable, even if taken from the same source or process. Nonetheless, WE production has been achieved using waste stream feedstocks, and the largest future expansion is likely to come from this area (see Section 7.1 for further discussion).

5.1. Lignin Derivatives

Lignocellulosic biomass, the rigid organic material which gives plants their structure resilience, is a common agricultural waste in forms such as wheat stems, corn stover, wood shavings and sugarcane bagasse. It is comprised of three major components; cellulose, the polymer form of glucose, hemicellulose, a polymeric mixture of five and six carbon sugars, and lignin, an irregular aromatic polymer (Sanderson, 2011). Valorisation of this biomass is of interest for fuel, gas and platform chemical production employing the biorefinery concept, the biomass-based equivalent of a fossil-fuel refinery for chemical production. Pretreatment and hydrolysis of the biomass in the refinery hydrolyse the cellulose and hemicellulose into a mixture of C_5 and C_6 sugars, which are directly suitable for many digestion and fermentation processes (Galbe and Wallberg, 2019). However, the complex structure of lignin makes processing this component difficult (Xie et al., 2016), and, once separated from the soluble sugars, it is typically burnt for energy. However, direct utilisation of lignin, which can be depolymerised into a range of aromatic monomers, as a feedstock would provide greater flexibility and potential for higher efficiency.

Lignin can be hydrolysed and depolymerised into a range of aromatic acid and alcohol monomers which often cannot be utilised for fermentation and may act as microbial inhibitors. However, the utilisation of two of these monomers for bacterial WE production has been demonstrated in *A. baylyi* ADP1. Coumarate, a common lignin-related aromatic acid, was reported to be co-consumed with acetate, with maximum combined tolerance of 55 mM acetate and 30 mM coumarate respectively (Salmela et al., 2019). A fed-batch bioreactor provided sequential coumarate additions to avoid exceeding tolerance limits and produced WE titres of up to 202 mg/L, with a yield of 3.7% w/w and cell WE content of 5.8% w/w.

Aromatic acid 4-hydroxy-benzoate (4HB), which is derived from lignocellulosic biomass pre-treatment, also inhibits many bacterial species, but could be utilised by ADP1 as a sole carbon source for growth and WE production for concentrations of up to 4 g/L (Reiser and Somerville, 1997). Genetic modification of ADP1 has since provided an ADP1 mutant which

preferentially utilised 4HB and benzoate even in the presence of better substrates such as glucose and arabinose (Singh et al., 2019).

5.2. Other Waste Stream Utilisation

Another effluent demonstrated to sustain WE production is alkane-rich wastewater from the oil and gas industry. Given its success on hydrocarbons, *A. borkumensis* SK2 was investigated for WE production in a continuous bioreactor using wastewater, achieving a titre of 8.45 g/L, with 6.70 g/L produced extracellularly, over 10 days (Sudmalis et al., 2018). Despite the claim that the process also removed 99% of organics, only 80% of organics were removed at the maximum WE titre. Whilst the long-term viability of the process can also be questioned, since it relies on the oil and gas industry, the progress in converting a classically energy and resource intensive purification into a source of valuable chemical production is nevertheless an impressive landmark, with opportunity for further development and scale-up.

Carbon dioxide, a large and environmentally relevant waste stream, can be reduced to industrially relevant molecules including hydrogen, methane and organic acids and alcohols by microbial electrosynthesis (MES) (Kong et al., 2020). A two-step WE synthesis from CO_2 has been proposed, with acetogenic bacteria producing acetate by MES, then *A. baylyi* ADP1 converting this to WEs (Lehtinen et al., 2017). Acetate concentrations of 38 mM were achieved over 10 days of MES, 13 mM of which was used to produce 18 mg/L of WEs (4.6% C/C yield). While the titre was reduced compared to pure acetate, due to the low substrate concentration, the yield double and the authors suggest significant improvements on the system should be possible (Lehtinen et al., 2017).

6. Wax Ester Production by Mixed Microbial Communities

An area of research attracting a lot of recent interest is the use of mixed natural or synthetic microbial communities for biotechnology (Altermann and Hickey, 2020), as the deployment of monocultures for the generation of products can be problematic and limiting. For instance, the probability of monocultures suffering contamination increases with operation time, meaning processes must be undertaken in batch, which is expensive and often impractical for large scale production. Furthermore, production capacities of monocultures, particularly modified strains, decrease over time because cells evolve to increase their growth rate and reduce metabolic burden as a survival advantage (Rugbjerg and Sommer, 2019). For instance, plasmids or chromosomal DNA encoding for product generation may be lost over many generations.

Mixed communities offer several advantages over single strain production, including generating greater yields, higher growth rates and a wider range of biological transformations (Hesseltine, 1992). Processes can be operated continuously without risk of strain degeneration: 1) conditions are chosen such that product generation, requiring DNA retention, provides an evolutionary advantage (Temudo et al., 2008) and 2) if multiple strains within the culture facilitate the same reaction the metabolic burden on each is reduced, so the driving force to lose genes decreases (Jawed et al., 2019). In addition, multiple products can be obtained simultaneously from the range of species present. Mixed

communities potentially process a broader range of substrates, with mixed cultures utilising complex, impure and typically cheaper feedstocks such lignocellulosic biomass and waste streams (Jawed et al., 2019; Sabra and Zeng, 2014). Microbial communities can also offer protection from contamination, so processes can be carried out in open cultures, making continuous culture processes even cheaper to perform (Sabra and Zeng, 2014).

However, mixed communities, particularly artificial ones, also pose problems, mainly regarding stability. If conditions change, even slightly, any species with a specific growth advantage will rapidly outcompete other members and the balance is lost. Storing mixed cultures also causes issues, as traditional methods such as lyophilisation will not preserve all strains proportionally, so maintaining the balance of species is very difficult (Hesseltine, 1992). Studying and understanding mixed communities is much more challenging than a single species, hence many traditional processes are still carried out with uncharacterised mixed communities.

The simplest form of a bacterial community is a co-culture, with two species cultured simultaneously, which can be used to simultaneously generate WEs and other products. An *Acinetobacter* ADP1, *Clostridium butyricum* co-culture was investigated for WE and 1,3-propanediol production from crude glycerol, a by-product of biodiesel production (Mangayil et al., 2019). ADP1 is unable to grow on glycerol as a sole carbon source (Salcedo-Vite et al., 2019). However *C. butyricum* fermented it anaerobically to produce 1,3-propanediol, with the acetate and butyrate byproducts then utilised aerobically by ADP1. Under optimal conditions, a WE content of 3.4% CDW was achieved, but with titre of just 0.034 g/L, although 1,3-propanediol at 5.5 g/L was also achieved. A modified ADP1 strain deficient in glucose consumption, ADP1g, has also been exploited in co-cultures with *Clostridium butyricum* for WEs and H_2 production from glucose (Salmela et al., 2018). *C. butyricum* consumed glucose in an anaerobic fermentation, producing H_2 gas, acetate and butyrate, the latter two again acting as substrates for ADP1. This approach yielded 1.1% w/w of WE from glucose, at 0.028 g/L, as well as 1.7% mol/mol of H_2. In both cases ADP1 benefits *C. butyricum* growth by removing inhibitors and increases process efficiency by utilising otherwise waste metabolites. These mixed cultures still required sterile growth conditions since they are vulnerable to external contamination and likely to suffer from similar heterogeneity problems as mono-cultures, leading over time to lower production rates.

Alternatively, an open mixed bacterial community has been used to investigate neutral lipid production from organic-rich wastewater, which was inoculated with sludge from a wastewater treatment plant. Selection for hydrocarbonclastic strains was applied by alternating periods of excess carbon and starvation at negligible nitrogen content, with high accumulation rate also encouraged by shortening the 'feast' periods (Castro et al., 2018). The final culture was unsurprisingly dominated by lipid-accumulating species of *Rhodococcus*, *Acinetobacter* and *Pseudomonas.* A factorial experimental design determined the optimal lipid-accumulating conditions as minimal nitrogen, high carbon: nitrogen ratio and the shortest time point (29 hours), and neutral lipids were produced up to 33% of CDW, although just 7% of this was WEs due to the low prevalence of WE-producing bacteria (*Acinetobacter*). Selecting for WE over TAG production would be challenging due to their

similar structure and function, although using substrates on which *Acinetobacter* species, but few others, can grow, such as 4HB, could increase the *Acinetobacter* popularity.

7. Future Perspectives

With the long-term goal to produce WEs as bulk commodity chemicals, greater focus on scalable and affordable technologies must be a priority. As well as improving yields, the focus of most work discussed here, downstream processing and substrate purchase are known to contribute significantly to the cost of a biotechnological process (Straathof, 2011), so these areas will undoubtedly be key targets in readying this process for commercialisation. With this in mind, the current challenges and opportunities for progress in these areas are discussed in more detail below.

7.1. Novel Substrates

With a maximum yield for bacterial processes thus far at 7.5 % per gram of substrate on glucose (Luo et al., 2020), and global sugar prices currently around €240 tonne^{-1} (IndexMundi, 2020), 1 tonne of bacterial WE would require 13 tonnes of glucose to produce, a cost of over €3,100 from substrate alone. By comparison, paraffin wax prices in Europe are around €1,100 per tonne (Trinder, 2018), while beeswax can cost between €3,400-8,500 per tonne depending on quality and origin (Eshete and Eshetie, 2018). Despite this price disparity indicating that, in certain markets, consumers are willing to pay a premium for natural, non-fossil products, the substrate cost alone for bacterial WE production is near treble the refined paraffin wax price, and approaching the lower end cost of beeswax. It is therefore obvious this cost must be decreased in order to make prices competitive with either existing product.

Focus on waste streams as low-cost feedstocks is growing, but the streams of interest must be expanded to determine an optimum balance between price and suitability, both of the sourcing method and substrate success. As very few waste streams have been investigated for WE production thus far, it is appropriate to consider that waste streams explored as feedstocks in other biotechnological processes may be suitable for WE production, particularly streams which have previously been utilised for microbial lipid and biofuel production. As with alkane-rich wastewater, agro-industrial effluent from dairy, olive and confectionary industries have been demonstrated to be appropriate feedstocks for biofuel production by oleaginous yeast *Wickerhamomyces anomalus* EC28, as well as waste cooking oils from meat, fish and potato frying (Arous et al., 2017) These effluent streams have greater longevity than oil and gas wastewater, the availability of which will decrease in the future as fossil fuel use declines. Anaerobic digestate, the nutrient and lignocellulosic-rich residual material after anaerobic degradation of organic matter, has also previously been demonstrated to be suitable feedstock for fungal production of lipid-based biofuels (Zhong et al., 2016). Since digestion can be performed on a wide variety of organic material, including maize and other specific energy crops, sewage and agricultural, animal and food waste, digestate is a widely available and adaptable resource, although the composition can vary greatly, complicating the production.

Raw food-waste has been identified as a feedstock for microbial fermentation and conversion to useful products. Orange peel has been highlighted as a particularly rich in utilisable substrates, with mixed sugars in the peel hydrolysate previously converted to lactic acid by several strains of *Lactobacillus delbrueckii* (Bustamante et al., 2020). Pectin can also be isolated from orange peel and converted to ethanol by microbial consortia (Ojewumi et al., 2018). Although this waste stream has not yet been demonstrated for lipid production, it would likely show less chemical diversity and variation than those above, and hence achieving a reliable WE production pathway may be easier.

These examples highlight the diversity and availability of rich and utilisable waste streams which remain largely unexplored for bacterial WE production. Another approach to increase the breadth of substrates utilisable for this purpose is adaptive laboratory evolution (ALE) (Dragosits and Mattanovich, 2013), which can select for certain behaviours by applying gradually changing external pressures. This technique has previously been used to select for higher lipid accumulation (Castro et al., 2018; Daskalaki et al., 2019), but ALE can also be used to improve utilisation and tolerance of substrates which can act as inhibitors at moderate concentrations, e.g. organic acids (Fernández-Sandoval et al., 2012), alcohols (Horinouchi et al., 2018) and aromatics. This can facilitate bioprocesses with higher concentrations of mixed and unconventional substrates, meaning greater WE titres can be achieved, which is necessary for processes to be viable at industrial scale. If improvements to the desired metrics are observed, sequencing can detect the affected genes, offering insight into approaches subsequent genetic engineering should take to further improve tolerances.

As highlighted in the discussion of Section 6, mixed cultures will undoubtedly play a part in processing complex carbon sources, although their success for WE production specifically may be limited by the preference of many species for TAG accumulation over WEs (Castro et al., 2018). Synthetic consortia have yet to be explored for this purpose beyond two strain co-cultures but could improve this situation.

7.2. Wax Ester Extraction

While not discussed at length here, one area for improvement is extraction of WEs from cells, as downstream processing is a large contributor to the costs of biotechnological products. Regardless of yield and substrate cost, this step is critical to process viability. In recent literature (Holert et al., 2020; Luo et al., 2020), the Bligh and Dyer, and Folch methods first reported in the 1950s are still common for extraction (Bligh and Dyer, 1959; Folch et al., 1957), despite both methods employing toxic solvents chloroform and methanol which are not scalable due to costs and regulations. The limited options in terms of extraction methods, as well as the high cost and inefficiency of many, has been highlighted in a recent review of lipid extraction from bacteria (Hwangbo and Chu, 2020). A method which is both efficient and viable at scale, whilst sufficiently cheap to keep product prices competitive is essential if the area is to have industrial potential. Greener solvent extraction methods employing renewable solvents have been proposed (Breil et al., 2017), but alternatives to centrifugation for cell disruption (e.g. milling, induced lysis etc) should also be investigated to reduce energy consumption and costs (Patel et al., 2018; Peternel, 2013).

Improvement to extraction could also be achieved by exploiting strains that naturally excrete lipids, as demonstrated by several species via a so far uncharacterised mechanism (Bredemeier et al., 2003; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011; Nakano et al., 2012). Species with this trait produce WEs primarily when grown on alkanes and complex alcohols. Research should therefore be undertaken to confirm findings of extracellular WE production on pyruvate (Manilla-Pérez et al., 2011). If reproducible, studies should be expanded to other simple substrates. Alternatively, genetic modification of these species to produce FAR or FAldR enzymes and thus synthesise WEs *de novo* could be explored. A second field of future work around these species would be to identify the genes associated with, and hence better understand, lipid export. These could be expressed in other species already demonstrating high WE yields and employing renewable substrates, e.g. *A. baylyi* ADP1. In yeast *Yarrowia lipolytica,* such an approach combining genetic engineering and *in situ* solvent extraction produced fatty acids above 100% CDW for the first time (Ledesma-Amaro et al., 2016).

7.3. Quantification and Optimisation

Maximisation of WE production by increasing yields, WE content and titres, through either condition optimisation and/or genetic modification, has been the major focus of the literature thus far, particularly in species of *Acinetobacter*, *Marinobacter* and *Rhodococcus*, as emphasised by Table 2 and Table 3. Undoubtedly genetic engineering, directed evolution and ALE will be the key factors in improving these metrics, as has been demonstrated by the near 3-fold increase in all three in *A. baylyi* ADP1 on glucose (Luo et al., 2020).

Alongside this, identification of optimal conditions for different strains and mutants will be essential. To streamline this process, more researchers should consider performing and publishing robust, multi-factor experimental designs for process optimisation (Castro et al., 2018; Rottig et al., 2016a). This would reveal more accurate values of maximum WE output, which are necessary to decide whether pursuing a particular strain, modification and/or feedstock is viable.

A final, yet important factor highlighted by this review as lacking in terms of research effort is quantification of WE production across all species. As evidenced by the small fraction of strains from Table 1 present in Table 2, in many species WEs have been confirmed by TLC and gas chromatography/mass spectrometry, yet no data has been published on the quantity produced. Similarly, several authors publish only limited data (e.g. the WE content, but not CDW, titre or yield) which is unhelpful and potentially misleading. Quantification is fundamental to assess whether optimisation and scale-up are viable, and by omitting this data, key species or substrates may be overlooked by the research community and commercially.

8. Conclusion

This review has assessed a wide range of bacteria for WE production on various substrates. While great success has historically been achieved with respect to alkanes and their derivatives (Ishige et al., 2003), many strains have since been shown to produce WEs on a range of simple, renewable substrates. The main factor differentiating these

behaviours is the presence of fatty acyl reduction enzymes that facilitate *de novo* fatty alcohol synthesis. *A. baylyi* ADP1 has received the most attention in the literature, which is arguably reasonable given the broad range of suitable substrates and high WE yields achieved, although similar achievements in other strains may be made given comparable research attention. Nonetheless, ADP1's natural competence has made it a particularly desirable strain for genetic modifications and this shows no sign of relenting.

Genetic modification can either impart or improve WE synthesis ability in various bacterial strains. Overexpression of FAR-coding genes has been successfully employed to improve production in both ADP1 (Lehtinen et al., 2018) and *Rhodococcus* strains (Lanfranconi and Alvarez, 2017; Round et al., 2017; Round et al., 2019), suggesting a likely biological bottleneck, while redirection of carbon flux can enhance efficiency and yield (Luo et al., 2020). The WE synthesis pathway has also been constructed in *E. coli* but, while interesting as a proof of concept, native producing strains remain more desirable chasses. The few authors to investigate scale up processes found fed-batch reactors to drastically improve WE content and titre over flask scale (Round et al., 2019; Sudmalis et al., 2018), indicating good prospects for future development at pilot and potentially commercial scales.

Future developments in bacterial WE production are likely to focus on increasing efficiency, scalability and economic viability. The deployment of model and real-life waste streams as substrates has already demonstrated the possibility of microbial WE production as a method of waste valorisation, and interest in this area is only expected to grow, accompanied by an expansion of the waste streams identified as suitable substrates. The importance of appropriate substrate choice and efficient downstream processing in the future commercial viability of bacterial WEs are highlighted to be as important as the optimisation of WE production and yields in the bacteria. With sufficient research into these, thus far under-explored, areas of the field, the case for and prospect bacterial WEs becomes ever stronger.

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951Table 1 - Bacterial strains producing WEs and the substrates utilised for production

Genus	Species	WE produced on	WE not produced on	Ref
GRAM-POSITIVE				
Mycobacterium	*ratisbonense* SD4	phytane	gluconate, hexadecane	(Silva et al., 2007)
	tuberculosis	cholesterol		(Holert et al., 2020)
		glycerol		(Deb et al., 2009)
		oleic acid/oleate		(Sirakova et al., 2012)
	smegmatis mc^2155	hexadecanol	glucose	(Kalscheuer and Steinbüchel, 2003)
Rhodococcus	A27	fructose (traces)		(Rottig et al., 2016a)
	jostii RHA1	cholesterol, phytosterol, pyruvate (traces)	glucose, succinate	(Holert et al., 2020)
		hexadecane, hexadecanol	3HB, acetate, gluconate	(Hernandez et al., 2008)
		glucose (traces)		(Round et al., 2017)
	opacus PD630	phenyldecane		(Alvarez et al., 2002)
	rhodochrous	glucose		(Shields-Menard et al., 2015)
Streptomyces	*avermitilis* MA-4680	glucose+hexadecanol	glucose, hexadecane, hexadecanol	(Kaddor et al., 2009)
	coelicolor		glucose, hexadecanol	(Arabolaza et al., 2008)
	sp. strain G25		glucose	(Rottig et al., 2016b)
GRAM-NEGATIVE				
Acinetobacter	ADP1	1,16-hexadecandiol		(Kalscheuer et al., 2003)
		4HB		(Kannisto et al., 2017; Singh et al., 2019)
		acetate		(Kannisto et al., 2017; Lehtinen et al., 2017; Salcedo-Vite et al., 2019; Santala et al., 2018)
		acetate and butyrate mix	glycerol	(Mangayil et al., 2019; Salmela et al., 2018)
				(Salmela et al., 2019)
		acetate and coumerate mix		(Lehtinen et al., 2018; Santala et al., 2011; Santala et al., 2014)
		glucose (with casamino acids)		(Kannisto et al., 2014)
		gluconate		(Manilla-Pérez et al., 2011; Salcedo-Vite et al., 2019)
		pyruvate	ethanol, glycerol, xylose	
	calcoaceticus (various strains)	succinate		(Fixter et al., 1986; Reiser and Somerville, 1997)
	HO1-N	hexadecane, icosane, octadecane		(Dewitt et al., 1982)
	M1	hexadecane, hexadecanol, cis-11-hexadecanal, hexadecenoic acid, octadecane		(Ishige et al., 2002; Sakai et al., 2014)
	NRRLB-14920	oleic acid, oleic alcohol		(Kaneshiro et al., 1996)
	sp. strain PHY9	6,10,14-trimethylpentadecan-2-one	phytol	(Rontani et al., 1999)
Alcanivorax	*borkumensis* SK2	hexadecane, octadecane, pyruvate	acetate	(Kalscheuer et al., 2007; Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011)
	jadensis T9	hexadecane, pyruvate	acetate	(Manilla-Pérez et al., 2010; Manilla-Pérez et al., 2011)
		hexadecane, tetradecane		(Bredemeier et al., 2003)
		pyruvate		(Kalscheuer et al., 2007)
Marinobacter	*aquaeolei* VT8	citrate, pentadecanol, succinate		(Lenneman et al., 2013; Wahlen et al., 2009)
	atlanticus CP1	succinate		(Bird et al., 2018)
	hydrocarbonoclasticus	hexadecane	acetate	(Klein et al., 2008)
	SP17	hexadecane, pyruvate		(Manilla-Pérez et al., 2011)
	hydrocarbonoclasticus DSM 8798	phytol, 6,10,14-trimethylpentadecan-2-one		(Rontani et al., 1999)
	PAD-2	hexadecane, succinate		(Nakano et al., 2012)
	sp. strain CAB	phytol, 6,10,14-trimethylpentadecan-2-one		(Rontani et al., 1999)
	squalenivorans	squalene		(Rontani et al., 2003)
Micrococcus	*cryophilus*	casamino acids		(Russell, 1974, 1978; Russell and Volkman, 1980)
Nocardia	-	fructose		(Rottig et al., 2016a)
	corallina No 724	glucose, hexadecane, valerate (traces)		(Alvarez et al., 1997)
Thalassolitus	*oleivorans*	pyruvate		(Kalscheuer et al., 2007)
		hexadecane	pyruvate	(Manilla-Pérez et al., 2011)

952

953Table 2 - The maximum wax content, titre and yield achieved for bacterial strains on
954different substrates

Genus	Species	Substrate	Maximum WE per biomass (g/g)	Maximum WE titre (g/L)	Maximum yield (g WEs/ g substrate)	Reference
Acinetobacter	*baylyi* ADP1	acetate	0.077	0.094	0.023	(Lehtinen et al., 2017)
		courmerate	0.058	0.202	0.037	(Salmela et al., 2019)
		glucose + casamino acids	0.09[a]	0.58	0.03	(Luo et al., 2020)
	M1	hexadecane	0.17	-	-	(Ishige et al., 2002)
		oleic acid + oleyl alcohol	-	-	0.095	(Kaneshiro et al., 1996)
	calcoaceticus NCIB 10487	succinate	0.141	-	-	(Fixter et al., 1986)
Alcarnivorax	*borkumensis* SK2[b]	hexadecane	0.027	0.005	0.0011	(Manilla-Pérez et al., 2011)
		wastewater (fed-batch)	-	8.45[c]	-	
	jadensis T9[b]	hexadecane	0.014	0.021	0.004	(Manilla-Pérez et al., 2011)
		hexadecane	-	-	0.03	(Bredemeier et al., 2003)
Marinobacter	*atlanticus* CP1	succinate	0.035	0.025	-	(Bird et al., 2018)
	aquaeolei VT8	citrate	0.035	-	-	(Lenneman et al., 2013)
	hydrocarbonoclasticus[b]	hexadecane	0.040	0.004	0.0008	(Manilla-Pérez et al., 2011)
		pyruvate	0.010	0.012	0.0015	
	squalenivorans	squalene	0.011	0.01	-	(Rontani et al., 2003)
Micrococcus	*cryophilus*	casamino acids	0.013	-	-	(Russell, 1974)
Rhodococcus	*josti* RHA1	glucose	0.000002	-	-	(Round et al., 2017)

955[a] Higher wax content was obtained in a different paper(Lehtinen et al., 2018) but with lower
956wax titre and yield

957[b] Combination of intracellular and extracellular waxes

958[c] Fed-batch reactor rather than batch flask experiment

959Table *3* - The maximum wax content, titre and yield achieved for a range of strains and
960genetic modifications

Species	Feedstock	Modification	Maximum WE content (%)	Maximum wax titre (g/ L)	Maximum yield (g WE/ g substrate)	Ref.
Native producing strains						
Acinetobacter baylyi ADP1	Glucose + casamino acids	*aceA* deletion and *acr1* (FAR) overexpression	0.27	1.82	0.075	(Luo et al., 2020)
	Glucose + casamino acids	*acr1* (FAR) overexpression	0.125	0.45	0.04	(Lehtinen et al., 2018)
	Acetate + arabinose	*aceA* deletion	0.19	0.184	0.08	(Santala et al., 2018)
	Glucose, casamino acids + arabinose	*acr1* replaced by *LuxCDE*	0.014	0.091	-	(Santala et al., 2014)
Marinobacter atlanticus CP1	Succinate	Deletion of one FAR(acrB) or FAldR(farA)	0.15	0.035	-	(Bird et al., 2018)
Rhodococcus jostii RHA1	Glucose	*fcrA* and *ws2* (*M*. DSM 8798) expressed constituitively from chromosome	0.157 0.10[a]	- 5.0 (scale up)[a]	0.047 0.025[a]	(Round et al., 2019)
	Glucose	native *fcrA* overexpression on plasmids	0.13	-	-	(Round et al., 2017)
Rhodococcus opacus PD630	Gluconate	Expression of fatty acyl-CoA reductase from *M. hydrocarbonoclausticus*	Total neutral lipids changed from 100% TAG to 46% WE and 54% TAG			(Lanfranconi and Alvarez, 2017)
Non-native producing strains						
Escherichia Coli	Oleate	Co-expression of jojoba plant alcohol-producing, bifunctional FAR/FAldR and ADP1 WS/DGAT	0.01	*De novo* WE synthesis minimal, 1.5% CDW were butyl esters		(Kalscheuer et al., 2006)
	LB Medium + arabinose	Expression of *lpp*β (phosphatidic acid phosphatase gene) and *atfA* (ADP1 WS/DGAT)	WEs and TAGs accumulated, while no WEs present in control strain			(Comba et al., 2014)
`	Glucose + Oleic acid + Octanol + Pyruvate	FadD28 and Mas from *M. Tuberculosis* for methyl-branched fatty acid synthesis, PapA5 for esterification	- -	0.138 0.79[a] (scale up)	- 0.002[a]	(Menendez-Bravo et al., 2016)
Pseudomonas citronellolis	Hexadecanol	Heterologous expression of *atfA* (ADP1 WS/DGAT)	WEs accumulated on hexadecanol, no TAGs detected. No accumulation on hexadecane, hexadecenoic acid or gluconate			(Kalscheuer and Steinbüchel, 2003)

961[a] fed batch process

962

963

964 Figure 1 -The import and synthesis pathways relating to bacterial wax ester
965formation and the associated enzymes: fatty acid and structural lipid biosynthesis pathways
966(orange), fatty alcohol production from fatty acids (blue), alkane uptake and oxidation
967(green) and triacylglycerol production (dark blue) (Cronan and Thomas, 2009; Lehtinen et
968al., 2018).

969