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**Paper:**
Zhang, W., Alvarez-Gaitan, J., Dastyar, W., Saint, C., Zhao, M. & Short, M. (2018). Value-Added Products Derived from Waste Activated Sludge: A Biorefinery Perspective. *Water, 10*(5), 545
http://dx.doi.org/10.3390/w10050545

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# Value-Added Products Derived from Waste Activated Sludge: A Biorefinery Perspective

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Received: 29 March 2018; Accepted: 18 April 2018; Published: 25 April 2018

**Abstract:** Substantial research has been carried out on sustainable waste activated sludge (WAS) management in the last decade. In addition to the traditional approach to reduce its production volume, considering WAS as a feedstock to produce bio-products such as amino acids, proteins, short chain fatty acids, enzymes, bio-pesticides, bio-plastics, bio-flocculants and bio-surfactants represents a key component in the transformation of wastewater treatment plants into biorefineries. The quality of these bio-products is a key factor with respect to the feasibility of non-conventional WAS-based production processes. This review provides a critical assessment of the production process routes of a wide range of value-added products from WAS, their current limitations, and recommendations for future research to help promote more sustainable management of this under-utilised and ever-growing waste stream.

**Keywords:** bioplastics; biopolymers; biosolids; biorefinery; resource recovery; waste activated sludge

## 1. Introduction

Waste activated sludge (WAS) is a by-product of today's activated sludge-based wastewater treatment and presents a major ongoing disposal challenge for water management authorities worldwide. For example, based on 2015 United Nations global population data, reported rates of population sewerage and wastewater treatment [1] combined with per capita biosolids production rates for the United States [2], Europe and Asia [3], current global biosolids production rates are estimated at 25–60 Mt dry solids/year. With appreciable future expansion in global wastewater collection and treatment expected over the coming decades in line with global development and sanitation objectives [4] and in the face of burgeoning advanced wastewater treatment rollout across Asia, the burden of WAS as a waste product is set to increase, such that more sustainable alternate management paradigms are urgently needed.

Conventional WAS has long been reused in agriculture, such as land application or conditioning, however, concerns about offensive odours and disease risks from pathogens and toxic chemicals has restricted the scale and public acceptance of such practice [5]. Sustainable WAS management

involves the recovery and reuse of valuable products and the minimisation of the possible adverse impact of sewage sludge on both environmental and human health. Because of its high organic matter and nutrient content, sustainable WAS management has traditionally been applied in agricultural land or as the feedstock to produce methane via anaerobic digestion, in particular when mixed with primary sludge. Despite its high organic content and potentially valuable constituents, WAS remains an under-utilised resource and is often disposed of in landfills or given low value end-uses like mine site rehabilitation [6]. The presence of detectable amounts of contaminants in treated sewage sludge (concentrations of μg/kg to mg/kg) has led to the concerns that land application of WAS derived from urban wastewater treatment activities may result in an accumulation of contaminants in the soil and groundwater as secondary pollution and their subsequent translocation through the food chain may impact public health [7]. Another popular sustainable route for WAS management is the generation of electricity via incineration and thermal energy recovery [8]; however, due to high energy consumption owing to high moisture content and low heating value of biosolids, additional fuel is required for maintenance of incineration facilities [9]. Post-incineration carbon residues can also be utilised as alternative construction materials (e.g., brick manufacture and turf production) or adsorbents for environmental applications [10,11].

The composition of WAS can be typically characterised by six groups of components: (1) non-toxic organic carbon compounds (approximately 60% on dry weight basis), including extracellular proteins and polysaccharides (carbohydrates), either in pure form or in conjugation with other compounds, such as glycoproteins, rhamnolipids, lipoproteins Kjeldahl-N and phosphorus containing components; (2) heavy metals, such as Zn, Pb, Cu, Cr, Ni, Cd, Hg, and As (concentrations vary from <1 ppm to >1000 ppm); (3) toxic organic compounds, PCBs, PAHs, dioxins, pesticides, endocrine disrupters, linear-alkyl-sulfonates, nonyl-phenols, etc.; (4) bacteria and other higher organisms, microbial pathogens and associated microbiological metabolites; (5) inorganic compounds such as silicates, aluminates, calcium and magnesium containing compounds; and (6) water, varying from a few percent to >95%.

The fundamental challenge to any successful biorefinery approach involving WAS is attributed to the fact that all these compounds are present in a single complex and heterogeneous mixture. Therefore, the aim of this review is to highlight the potential for recovering a range of value-added products from WAS which we have categorised into six groups: (1) amino acids and protein; (2) short chain fatty acids; (3) enzymes; (4) bio-pesticides; (5) bio-plastics; (6) bio-flocculants; and bio-surfactants.

## 2. Amino Acids and Proteins

WAS can be harvested as a protein or amino acid source, since the principal constituents of its organic matter consist of complex compounds in the form of lipids, polysaccharides and proteins (70–80% combined), of which the protein fraction accounts for around 50% of the dry weight for bacterial cells [12]. Therefore, WAS-derived protein has significant potential to be utilised as the main constituent for the production of animal feeds compared with traditional protein sources (see Table 1), although effective detoxifications of sludge (i.e., sterilisation, removal of heavy metals and other toxicants) should necessarily be carried out [13].

The key step in recovering protein from WAS is the effective solubilisation of intracellular materials, on which a variety of extraction methods found in the literatures are based. One of the simplest methods is thermal digestion followed by centrifugal separation. Thermal digestion can increase WAS dewaterability, which results from breaking sludge flocs and lysing of microbial cell walls. The most notable benefits are: (1) low treatment cost, particularly effective heat exchange; (2) no additional reagents required; and (3) no additional waste stream generated. However, thermal extraction has the inherent disadvantage of not being able to remove heavy metals and some toxic organics present in WAS [14].

**Table 1.** Comparison of amino acid distribution in WAS with the content in single cell proteins and conventional feed meals (adapted from [15]).

| Amino Acid | WAS | Soybean Meal | White Fish Meal | FAO Ref. Protein | Wheat | Whole Egg |
|---|---|---|---|---|---|---|
| Alanine | 7.3 | - | - | - | - | - |
| Glycine | 4.9 | - | - | - | - | - |
| Valine | 4.1 | 5.2 | 4.7 | 4.2 | 4.4 | 7.3 |
| Threonine | 4.2 | 4.4 | 3.8 | 2.8 | 2.9 | 5.1 |
| Serine | 3.4 | - | - | - | - | - |
| Leucine | 5.6 | 7.6 | 6.5 | 4.8 | 6.7 | 8.9 |
| Isoleucine | 2.7 | 5.8 | 3.9 | 4.2 | 3.3 | 6.7 |
| Proline | 3.1 | - | - | - | - | - |
| Methionine | 1.45 | 1.3 | 2.9 | 2.2 | 1.5 | 3.2 |
| Aspatic acid | 8.3 | - | - | - | - | - |
| Phenylalanine | 3.1 | 5.3 | 3.5 | 2.8 | 4.5 | 5.8 |
| Glutamic acid | 8.1 | - | - | - | - | - |
| Lysine | 3.3 | 6.6 | 7.6 | 4.2 | 2.8 | 6.5 |
| Tyrosine | 2.4 | 4.1 | 3 | 2.8 | - | - |
| Arginine | 2.9 | 7.3 | 6.8 | - | - | - |
| Histidine | 0.6 | 2.7 | 2 | - | - | - |
| Cystine | 2.1 | 1.2 | 0.7 | 2 | 2.5 | 2.4 |
| Tryptophan | 0.8 | 1.3 | 0.9 | 1.4 | 1.1 | 1.6 |

Accordingly, more chemically-sophisticated processes have been developed—to accommodate increasingly stringent feed health and safety criteria. Early work by Chishti et al. studied alkali solubilisation process using NaOH and NaCl followed by precipitation for protein extraction from WAS [16]. Hydrochloric acid, sodium lignosulphate, sulphuric acid, acetic acid and ammonium sulphate were tested as precipitants, among which ammonium sulphate (40%) was observed to be most effective, providing maximum protein recovery (around 91%). Hwang et al. later applied a hybrid process of ultrasonic-alkaline pretreatment followed by precipitation and drying to release and subsequently recover the intracellular protein from WAS [17]. The supernatant protein concentration was observed to be 3178 mg/L after pretreatment of WAS (5330 mg/L TSS) after treating the substrate with ultrasound ($1.65 \times 10^{10}$ kJ/kg VSS) at pH 12 for 2 h. Zhang et al. reported a method of enzyme (i.e., papain)-assisted hydrolysis to extract protein from WAS in the Shanghai Qingpu sewage treatment plant, China [18]. Optimal conditions of the *papain*-assisted hydrolysis process were described as having an enzyme/substrate ratio of 6%, pH 6.5, liquid-to-solid ratio 4:1, hydrolysis time of 5.5 h and a hydrolysis temperature of 55 °C. These studies demonstrated that the recovered products contained all the necessary amino acids as compared with commercially-available protein feeds and thus could serve as an animal feed supplement. More importantly, all methods reported in these studies lowered the heavy metal content of the original WAS considerably through alkaline precipitation. With the ultrasonic-alkaline method, Hwang et al. even achieved non-detectable levels of other contaminants of concern such as aflatoxin B1, ochratoxin A and *Salmonella* D group organisms [17]. Ras et al. investigated the efficiency of mechanical (i.e., ultraturax or sonication) and chemical (i.e., cationic exchange resin and triton) treatments on protein extraction from two activated sludges, as well as their compatibility with usual quantification methods [19]. Comparison of various extraction protocols, combining mechanical and/or chemical treatments, demonstrated that. by repeating a similar approach or by employing a prior mechanical treatment, the efficiency can be increased. Proteins were preferably extracted by triton treatments, which showed the importance of hydrophobic interactions linking proteins to the extracellular polymer substance (EPS) matrix. The amount of extracted proteins was, respectively, up to 182 and 148 mg eq. BSA $g^{-1}$ VSS using triton/triton and ultraturax/triton extractions. In addition, depending on extraction protocols, protease activity/extracted protein ratios vary widely. Protease observed to be preferably extracted by ultrasound and triton treatments (150–220 U $mg^{-1}$ protein).

Detergents have also been observed to be effective protein extraction agents, but large amounts are usually required, limiting their feasibility. For example, sodium dodecylsulfate (SDS) can extract up to 3 mg protein/mL from sludge samples with 5% solids; however, the SDS requirement is >2% wt/vol and SDS is also notoriously difficult to remove from protein preparations post-extraction. Sodium

palmitate is another detergent that, unlike SDS, can be readily removed by organic solvent extraction at low pH. Combined with alkali (e.g., 0.15 mM NaOH), sodium palmitate can achieve up to 15 mg protein/mL from WAS with 5% solids solubilised [20].

Protein recovered from WAS has also reportedly been used to produce amino acid-chelated trace element (AACTE) fertilizers, which at the moment is recognised as an environmentally friendly fertilizer for cotton, fruits and other cash crops in China [18]; however, its production is restricted by other limited protein sources such as animal hair, hoof, horn and leather [21]. Liu et al. reported a novel process for bacterial protein extraction from sewage sludge to generate AACTE fertilizer involving five critical steps: acidic hydrolysis; purification through activated carbon decolourisation and glacial acetic acid dissolution; chelating with trace elements (i.e., Zn, Cu, Fe, Mn, Mo and B); centrifugal separation; and evaporation [22]. Optimal conditions were obtained for protein extraction from WAS at pH 0.5, a hydrolysis temperature of 121 °C for 5 h, while that for protein hydrolysis into amino acids was 121 °C hydrolysis temperature for 10 h and the ratio of 6 mol/L HCl solution to 8 g dry sludge. Under optimum hydrolysis conditions, 78.5% of protein was extracted from the sewage sludge and the amino acids yield was 10–13 g/100 g of dry sludge.

One reportedly novel use of WAS-derived amino acids is as an environmentally-friendly corrosion inhibitor for industrial pickling, due to their amphiphilic molecular structure containing amino and carboxyl groups. Application of amino acids as effective inhibitors to control corrosive reactions in several different metals in acidic media has been verified by a series of investigations [23–26]. Using an ultrasonic-assisted hydrochloric acid hydrothermal method, Su et al. reported a total of 50 different amino acids in the obtained hydrolysate, which can be readily adsorbed onto steel surfaces through hydrophilic (-COOH) and hydrophobic (-$NH_2$) groups and result in the stable formation of protective film [27]. Corrosion testing of steel surfaces with and without amino acid surface coating pre-treatment showed a reduction in surface roughness and a six-fold lower corrosion rate (Figure 1).

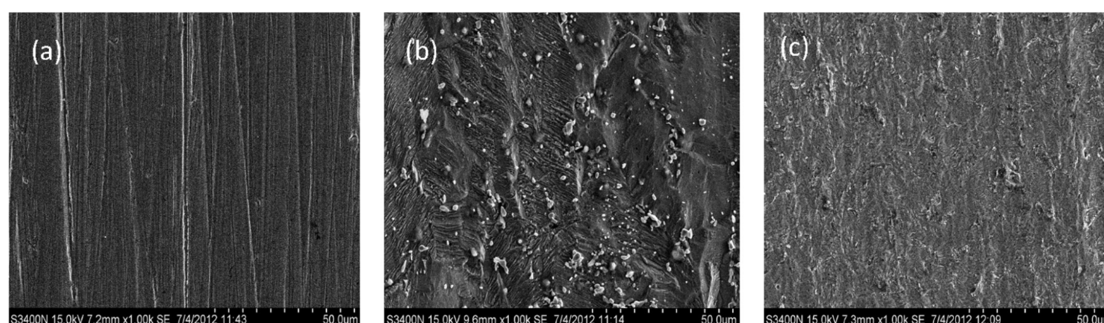


**Figure 1.** SEM images of steel surfaces under different corrosion testing conditions: (**a**) polished sample before testing; (**b**) sample immersed in the corrosion solution (HCl, 10 wt.%) without amino acid pre-treatment; and (**c**) sample immersed in the corrosion solution with 0.090 g $L^{-1}$ amino acid pre-treatment for 2 h [27].

Protein recovery from secondary WAS from a paper mill can also be used as an eco-friendly source of wood adhesive. For example, Pervaiz and Sain used an alkaline cell-disruptive technique—similar to that of Hwang et al. (2008)—to extract protein, with the end product achieving up to 41% the shear strength of traditional protein-based wood adhesive such as soy protein isolate and phenol formaldehyde resin [28].

## 3. Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) such as acetic acid, formic acid and propionic acids can be recovered from WAS using anaerobic digestion, or thermal processes such as wet air oxidation and hydrothermal treatment [29]. The recovered SCFAs can serve as a chemical feedstock and be converted to a range of chemicals (e.g., ketones, carboxylic acids, esters, and ethers) and fuels (e.g., primary

alcohols, secondary alcohols, and hydrocarbons). Research on SCFAs production from WAS-derived media is summarised in Table 2.

During the anaerobic digestion of WAS, the following occur in sequence: (1) hydrolysis; (2) acidogenesis; (3) acetogenesis; and (4) methanogenesis. The accumulation of SCFAs can be increased if the first two steps are accelerated; temperature, sludge retention time and pH are the main factors influencing the first two steps. Alkaline conditions are also beneficial by way of increased sludge solubilisation (i.e., chemical effect), as well as increasing the abundance of major bacteria involved in sludge hydrolysis and acidification (i.e., biological effect) [30,31], although no consensus has been reached on which of these effects is the leading cause [32]. Liu et al. studied the effect of ultrasonic pre-treatment of WAS and the impact of digestion temperature on SCFAs accumulation during anaerobic digestion and examined during anaerobic digestion [33]. The authors reported that a moderate temperature (35 °C) was optimal under alkaline conditions (pH 10) and that the changes to protease activity showed that, in the initial phase, ultrasonic pre-treatment and alkaline adjustment rather than biotic effects was the leading cause of SCFAs improvement in alkaline conditions. *Clostridium* and *Bacillus* (phylum Firmicutes) and *Gammaproteobacteria* (phylum Proteobacteria) were found to be the dominant functional bacteria for high SCFAs accumulation. The last step of methanogenesis usually should be inhibited during the anaerobic digestion of WAS to enhance the SCFAs production, which can be achieved by adding a methane analogue, for example, iodoform [34]. Li et al. reported on the effect of $CaO_2$ as an additive for enhanced SCFAs production during anaerobic digestion of WAS. The promoting effect of $CaO_2$ addition was reported to be two-fold: (1) alkalinity increased by $Ca(OH)_2$ formation; and (2) extra oxygen released through its slow decomposition in aqueous environments, which can stimulate beneficial bacterial growth and enhance their microbiological processes, while also potentially inhibiting anaerobic methanogens [35]. For example, members of the phylum Firmicutes were found to increase significantly with the addition of $CaO_2$ and the fraction of acetic acids was observed to increase relative to the total SCFAs produced (from 45.1 to 60.2%). In addition to the promoting effects on SCFAs production, hydroxyl radicals released by $CaO_2$ can also effectively remove refractory organic contaminants (e.g., endocrine-disrupting chemicals [36]), which could further enhance its usefulness as a feedstock for livestock production.

Jiang et al. examined a biological process for SCFAs production from WAS in the presence of chemical surfactant (i.e., sodium dodecylbenzene sulfonate (SDBS)) [37]. They demonstrated the impact of SDBS on SCFA generation from WAS, and consequently the potential of using fermentative SCFAs to promote enhanced biological phosphorus removal. Results revealed that, in the presence of SDBS at room temperature and a fermentation time of sox days, total SCFA production considerably increased to 2599 mg COD/L compared to 339 mg COD/L without SDBS addition. The authors suggested that during WAS fermentation, solubilisation and hydrolysis of sludge particulate organic carbon as well as acidification of hydrolysed products were all enhanced in the presence of SDBS. Thereafter, and because of reduced methanogenesis, the production of SCFAs was markedly enhanced. The authors also verified that the observed improvement in SCFA generation with SDBS addition was mainly due to biological effects, rather than by chemical effects and SDBS decomposition.

Hydrothermal recovery of SCFAs from WAS generally proceeds in two critical steps: (1) hydrolysis, which results in the formation and accumulation of dissolved organics; and (2) oxidation (e.g., by hydrogen peroxide), which can facilitate carbon abstraction and oxygenation involving the hydroxyl radicals [38]. As a modification, the use of microwave radiation as an alternate heating source combined with hydrogen peroxide and sulfuric acid as the oxidants for SCFAs recovery has also been reported [39]. Microwave radiation can lead to a combination of thermal and non-thermal effects on cell disruption, and possible release of cellular organic matter [40,41]. Intracellular liquid heating to boiling point leads to the thermal effect, increasing kinetic energy as molecular rotation [42], while the non-thermal effect causes the disruption of hydrogen bonds due to the polarisation of macromolecule

chains aligning with electromagnetic poles [43]. The addition of hydrogen peroxide and acids during this treatment can further help solubilize WAS solids, and release ammonia and phosphorus.

Lipid production from WAS has also attracted attention for biodiesel production. Dufreche et al. (2007) emphasised the potential of sludge as a cheap feedstock for biodiesel due to its high amount of lipids [44]. They have used solvents (i.e., *n*-hexane, methanol, acetone, and supercritical $CO_2$) for lipid extraction. They showed that use of polar solvents gave a considerably increased yield while the gravimetric yield of oil was low for nonpolar solvents. Extraction of lipids with a mixture of *n*-hexane, methanol, and acetone (i.e., polar solvents) gave the largest conversion to biodiesel (around 4.41% based on total dry weight of sludge) compared with other nonpolar solvents. In their study, in situ transesterification of dried sludge resulted in a yield of 6.23%. Based on data acquired, they claim that outfitting 50% of municipal wastewater treatment plants for lipid extraction from sludge and transesterification could contributed to replacement of 0.5% of the national petroleum diesel demand in US ($0.7 \times 10^6$ $m^3$ $year^{-1}$) by biodiesel produced. Huynh et al. (2010) profiled the amount of neutral lipids and fatty acid in neutral lipids extracted from activated sludge pretreated with or without sub-critical water (SCW) treatment [45]. Results indicated that the amount of neutral lipid extracted from SCW-treated activated sludge was nearly four times more than the process without SCW treatment. At least 14 kinds of fatty acids were identified in the neutral lipids of sludge oil, although there was no considerable difference between the fatty acid profiles of neutral lipids obtained from activated sludge with and without SCW treatment. Kwon et al. (2012), following a laboratory assessment of biodiesel production from WAS using a thermochemical process under ambient pressure, further demonstrated the high lipid yields and viability of WAS-based biodiesel production, with yields in the order of 980,000 L $ha^{-1}$ $year^{-1}$ possible, some 100 to 1000-fold greater than other feedstocks such as microalgal and soybean oil [46]. Based on the remarkably high oil yield and low cost of WAS-derived oil (US$ 0.03 $L^{-1}$) compared to conventional biofuel feedstocks as well as petroleum diesel (US$ 0.80 $L^{-1}$), the authors highlighted the considerable potential for sustainable WAS-based biodiesel production [47].

**Table 2.** Reported laboratory-scale studies for SCFAs production from WAS-derived media.

| SCFAs Composition | Yield | Fermentation Conditions | Medium | References |
|---|---|---|---|---|
| Acetic, butyric, valeric and propionic acid | Max 520.1 mg/g VSS | 21 ± 1 °C, pH 8, 8 days | Wastewater sludge | [48] |
| Acetic, butyric, valeric and propionic acid | Max 2599.1 mg/L | 21 ± 1 °C, pH 7.2, 6 days | Wastewater sludge | [37] |
| Acetic, butyric, valeric and propionic acid | Max 319.8 mg/g VSS | 15–55 °C, pH 10 ± 0.3, 48 days | Wastewater sludge | [49] |
| Acetic, butyric, valeric and propionic acid | 450–790 mg/g VSS | 55 °C, pH 7, 1 rpm, 28 days | Wastewater and bagasse | [34] |
| Acetic, butyric, valeric and propionic acid | Max 284 mg/g VSS | 35 ± 1 °C, pH 7, 200 rpm, 7 days | Wastewater sludge | [35] |
| Acetic, butyric, valeric and propionic acid | 345.49–2708.02 mg/L | 21 ± 1 °C, pH 4–11, 80 rpm, 20 days | Wastewater sludge | [31] |
| Acetic, butyric, valeric and propionic acid | Max 256.2 mg/g VSS | 20–22 °C, pH 4–11, 20 days | Wastewater sludge | [30] |
| Acetic, butyric, valeric and propionic acid | Max 368.71 g/kg TS | 35 ± 1 °C, C/N ratio 26 to 7, 24 days | Wastewater sludge and perennial ryegrass | [50] |
| Acetic, butyric, valeric and propionic acid | 704.9–1994.7 mg/L | 20 ± 2 °C, pH 7–10, 70 rpm, 15 days | Wastewater sludge | [33] |
| Acetic, butyric, valeric and propionic acid | 18.1–539.4 mg/L | 21 ± 2°C, 2 days | Wastewater sludge and potato-processing wastewater | [51] |

## 4. Enzymes

A huge fraction of the organic matter in WAS (30–85%) is formed by particles larger than 0.1 µm that cannot directly be assimilated by microorganisms [52]. Therefore, domestic WAS contains a diversity of microbial enzymes (e.g., protease, lipases glycosidase, galactosidases, aminopeptidases, dehydrogenase, catalase, peroxidase, phosphatases, α-amylase, and α-glucosidase) responsible for the

breakdown of complex organic matter (i.e., lipids, proteins and carbohydrates) into smaller compounds which are suitable substrates for biogas generation during anaerobic digestion [53]. If WAS-derived enzymes could cost-effectively substitute the commercial medium ingredients in industries such as detergents, food, pulp and paper, dairy, agrochemistry, cosmetics, pharmaceutical, diagnostics, and fine chemicals, the economic benefits could be significant because 30–40% of the production costs in these industries relate to the industrial enzymes [54]. Reported laboratory-scale studies for enzymes production from WAS-derived media are summarised in Table 3.

To date, ultrasonication—either alone or combined with detergents, or ion exchange resins—is considered one of the most widely reported methods allowing for the recovery of the abovementioned enzymes, while maintaining their activity [55]. Ultrasonication enhances enzymatic activities and promotes the shifts of extracellular proteins, polysaccharides and enzymes from inner layers of sludge flocs (i.e., pellet and tightly-bound extracellular polymeric substances), to outer slime layers, to increase the contact and interaction among extracellular proteins, polysaccharides and enzymes that were originally embedded within sludge flocs, resulting in improved volatile solids destruction efficiency during aerobic digestion [32]. Yu et al. (2007) compared five different extraction methods (i.e., ultrasonication, cation exchange resin, ethylenediaminetetraacetic acid (EDTA), formaldehyde (36.5% $w/w$ at 4 °C for 1 h) and formaldehyde + NaOH (1 mol $L^{-1}$ at 4 °C for 3 h)) and confirmed ultrasonication to be the most effective [55]. Yu et al. (2009) later demonstrated an optimal ultrasonication condition (i.e., 20 kHz and intensity of 552 W/g total suspended solids for 10 min) for the recovery of α-amylase from WAS with protease, α-glucosidase, alkaline and acid phosphatase in minor proportions [56]. Nabarlatz et al. (2010) reported on a more sophisticated extraction method that consisted of ultrasonication, dialysis, precipitation with ammonium sulphate and lyophilisation to recover the hydrolytic enzymes (i.e., protease and lipase) from WAS [57]. The effects of different parameters, such as extraction time, concentration of additives (detergent Triton X100, Cation Exchange Resin and Tris buffer), stirring velocity, ultrasonic power and sludge source, were investigated. The authors subsequently reported that a power intensity of 3.9 W/$cm^2$ and sonication time of 10–20 min was able to achieve a higher rate of enzyme recovery [58].

Due to the presence of salts (≈0.4–1.5 g/L) in urban wastewater, salinity effects on hydrolytic enzymatic activities (i.e., acid phosphatase, alkaline phosphatase, glucosidase, protease and esterase) in WAS have been reported in the literature, whereby increased salinity correlates negatively with enzyme activities in the range of 0–44.1 g/L NaCl [59]. To obtain enzymes suitable for certain industrial applications (e.g., as an additive for detergents or biocatalyst in esterification reactions) requires high degree of purity and high specific enzymatic activity, and therefore further purification and concentration procedure are usually required. The establishment of a protocol to purify and concentrate all the enzymatic fractions present in WAS remains a key challenge to be overcome and one that is frustrated by the inherent temporal variations in activated sludge composition (daily and seasonally) and its relatively low enzyme concentration.

**Table 3.** Representative studies for enzymes extraction from WAS-derived media.

| Enzymes Type and Activities | Extraction Techniques and Conditions | Medium | References |
| --- | --- | --- | --- |
| Protease: max. ≈3 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-amylase: max. ≈20 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-glucosidase: max. >1 μmol $min^{-1}$ $g^{-1}$ VSS<br>Alkaline phosphatase: max. ≈9 μmol $min^{-1}$ $g^{-1}$ VSS<br>Acid phosphatase: max. ≈3 μmol $min^{-1}$ $g^{-1}$ VSS | Ultrasound 20–40 kHz, 2–20 min, 138–690 W/g VSS, pH 7 and EDTA 2% at 4 °C for 3 h | Wastewater sludge | [56] |
| Protease: 1.06–28.2 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-amylase: 10.2–14.9 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-glucosidase: 188–319 μmol $min^{-1}$ $g^{-1}$ VSS | Ultrasound 40 kHz, 120 W, 2 min, 0–15 kW/L, pH 8 and 2% EDTA, 36.5% formaldehyde, CER 70 g/g VSS at 4 °C for 1 h | Wastewater sludge | [55] |
| Protease: max. ≈37 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-amylase: max. ≈34 μmol $min^{-1}$ $g^{-1}$ VSS<br>α-glucosidase: max. ≈3 μmol $min^{-1}$ $g^{-1}$ VSS<br>Alkaline phosphatase: max. ≈16 μmol $min^{-1}$ $g^{-1}$ VSS<br>Acid phosphatase: max. ≈16 μmol $min^{-1}$ $g^{-1}$ VSS | Ultrasound 20 kHz, 0–20 min, 0–15 kW/L, pH 7, 4 °C | Wastewater sludge | [32] |

**Table 3.** *Cont.*

| Enzymes Type and Activities | Extraction Techniques and Conditions | Medium | References |
|---|---|---|---|
| Protease: max 52 units/g VSS<br>Lipase: max 22.9 ± 0.8 units/g VSS | Ultrasound 24 kHz, 3.9 W/cm$^2$, 20 min, 5 ± 1 °C, and Triton X100 0.1 to 2% ($v/v$) | Wastewater sludge | [58] |
| Protease: max 57.4 units/g VSS<br>Lipase: max 21.4 units/g VSS | Ultrasound 20–24 kHz, 3–8 W/cm$^2$, 1–60 min, pH 7–8, 5 ± 1 °C, TX 100 0.1 to 10% ($v/v$), CER 0.24–0.8 g/mL | Wastewater sludge | [57] |
| Protease: max. ≈4,000 units/g VSS<br>Lipase: 108.4–335 units/g VSS | 1 h 900 rpm stirring followed by 30 min sonication (200 W + 15 kHz), pH 8, 0–2% Triton X-100 and EDTA, CER 60–70 g/g VSS | Wastewater sludge | [60] |
| Protease: 3450 ± 124 units/g mixed liquor suspended solids (MLSS)<br>Amylase: 111 ± 5 units/g MLSS<br>Glucosidase: 59.5 ± 2.9 units/g MLSS<br>Lipase: 8.8 ± 1.4 × $10^{-2}$ units/g MLSS<br>Dehydrogenase: 36.6 ± 3.8 × $10^{-3}$ units/g MLSS | Agitation 200–4200 rpm, 4 °C, 1–10 min and ammonium sulphate | Wastewater and laboratory-cultivated sludge | [61] |

## 5. Bio-Pesticides

Owing to an enriched makeup of carbon, nitrogen, phosphorus and other nutrients, WAS has the potential to be an attractive alternative growth medium for industrial microorganisms to produce valuable metabolic products. Organisms such as the aerobic spore-former *Bacillus thuringiensis* (*Bt*) have long been known to possess the ability to produce a proteinaceous parasporal crystal inclusion during sporulation called δ-endotoxin—by far the most popular bio-pesticide worldwide and used extensively in agronomy, forestry and the public health sectors [62]. The production of these entomopathogenic bacteria is conventionally carried out in synthetic medium containing carbon, nitrogen, yeast extracts and protein sources (e.g., soybean meal, fishmeal, glucose, peptone and trace elements), of which the respective cost represents 35–59% of the total production cost, depending on the plant production capacity [63]. Reuse of WAS sludge as a low-cost "eco-alternative" medium for *Bt* production followed by its utilisation in agricultural crops and forests for pest control appears economical and compatible with existing sludge disposal exercises [53]. Typical reported studies for biopesticide production from WAS-derived media are summarised in Table 4.

*Bt* production process from WAS consists of three key stages: (a) sludge fermentation; (b) product recovery/harvesting; and (c) product formulation. During fermentation, several factors including pH, C/N ratio, dissolved oxygen (DO) concentration, foaming, solids concentration and inoculum sludge type can affect the process of bio-pesticide production [64,65]. Sachdeva et al. (2000) reported on the effects of sludge solids and inoculum concentration on the fermentation production of *Bt* (subsp *kurstaki* HD-1) [66]. Cell and spore counts were comparable to the industry standards, as well as the production time. Sludge samples with lower solids concentration were observed to produce considerably higher entomotoxicity while having no significant effect on cell and spore count. Lachhab et al. (2001) further optimised two process parameters (i.e., sludge solids and inoculum concentration) of the same process, based on cell, spore counts and entomotoxicity [67]. They concluded that an inferior entomotoxicity under higher solids concentration resulted from the oxygen transfer limitation; the optimum total solids and inoculum concentration were found to be 20–26 g/L and 2% ($v/v$), which resulted in an improved potency of 12,970 IU/µL, cell and spore concentrations of 5.0 × $10^9$ and 4.8 × $10^9$ CFU/mL, respectively, and a sporulation rate of 96%. Vidyarthi et al. (2002) studied the effect of C/N ratio in sludge medium on the sporulation and entomotoxicity yield of *Bt* subsp *kurstaki*, with the optimal C/N ratio recommended as within 7.9–9.9 [68]. Montiel et al. (2003) examined the effect of temperature, pH and agitation speed during the fermentation on *Bt* entomotoxicity, cell and spore counts [69]. Higher agitation speed in the range of 50–350 rpm was observed to have positive effects on all three parameters by increasing the oxygen transfer rate, and the optimum pH for *Bt* production was reported to be around 7.

Sufficient aeration during fermentation is essential to maximize cell growth, sporulation and δ-endotoxin production by *Bt*, which at the same time can cause severe foaming depending on the solid contents. Therefore, investigations of foaming effects on *Bt* fermentation production from WAS

and its subsequent control are required to optimize *Bt* productivity. Vidyarthi et al. (2000) investigated the antifoaming effects of both synthetic (i.e., polypropylene glycol) and natural antifoaming agents (i.e., corn, canola, olive, peanut, sesame, soybean and sunflower oils) on *Bt* productivity [70]. While all agents provided effective foaming control, synthetic antifoaming agents influenced cell respiratory activity and growth adversely by affecting transportation of nutrients though the cell walls, including oxygen transport. On the other hand, foaming control using natural oils proved more favourable, with less severe impacts on the cell growth and entomotoxicity yield.

The effect of sludge pretreatment schemes on the entomotoxicity production by *Bt* in secondary and mixed sludge has also been investigated. Barnabe et al. (2006), for example, observed a significant improvement of about 50% in the entomotoxicity for mixed and/or secondary sludge after alkaline and thermo-alkaline pretreatment alone, or in combination with partial oxidation using $H_2O_2$ [71]. Montiel et al. (2001) suggested that an acid-hydrolysis pretreatment step improved the sludge performance in sustaining the growth, sporulation and δ-endotoxin production of *Bt* (subsp *kurstaki* HD-1) [72].

**Table 4.** Reported laboratory-scale studies for biopesticide production from WAS-derived media.

| Type | Entomotoxicity | Fermentation Conditions | Medium | References |
|---|---|---|---|---|
| *Bacillus thuringiensis* | 9534–13,020 IU/μL | 30 °C, pH 6.9 ± 0.1, 400 rpm, inoculum concentration 2% (*v*/*v*) | Tryptic soy yeast broth and sludge | [70] |
| *Bacillus thuringiensis* | 8300–10,813 IU/μL | 30 °C, pH 7.0 ± 0.1, 300 rpm, inoculum concentration 2% (*v*/*v*), C/N ratio 7.9–9.9, total solids up to 33 g/L | Tryptic soy yeast broth and sludge | [68] |
| *Bacillus thuringiensis* | 8115–12,970 IU/μL | 31 °C, 250 rpm, inoculum concentration 1–5% (*v*/*v*), total solids 10–46 g/L | Tryptic soy yeast broth and sludge | [67] |
| *Bacillus thuringiensis* | 900–4100 IU/μL | 30 °C, pH 7.0, 250 rpm | Wastewater sludge | [72] |
| *Bacillus thuringiensis* | 3400–10,000 IU/μL | 30–36 °C, pH 7.0, 50–350 rpm, inoculum concentration 5% (*v*/*v*), total solids up to 13 g/L | Wastewater sludge | [69] |
| *Bacillus thuringiensis* | 4500–10,840 IU/μL | 31 °C, pH 7.0, 250 rpm, inoculum concentration 1% and 5% (*v*/*v*) | Wastewater sludge | [66] |
| *Trichoderma* sp. | 6278–15,036 SBU/μL | 28 ± 1 °C, pH 6.0 ± 0.01, 250 ± 5 rpm, total solids 10–50 g/L | Wastewater sludge | [73] |

The potential of WAS regarding *Bt* production has the capability to be extended to generate other beneficial metabolic products, such as endotoxins, spores and some other compounds (vegetative insecticidal proteins, hemolysins, enterotoxins, chitinases, proteases, phospholypases and others) which are insecticidal in nature and display entomotoxicity. For example, Verma et al. (2005) demonstrated the use of WAS as a raw material for the production of *Trichoderma* sp.-based bioherbicides/biopesticides, which have a broader spectrum activity in comparison to *Bt* [73]. Similar to *Bt* fermentation production, thermal alkaline pretreatment was found to be beneficial by enhancing solubilisation of organic matter; optimal solid concentration under conditions of the study was reportedly to be 30 g/L.

## 6. Bio-Plastics

Polyhydroxyalkanoates (PHAs) (see Figure 2) are a group of biodegradable polyesters and have received a lot of attention as an environmentally-friendly alternative to petroleum-based plastics in applications such as packaging films, disposable utensils, diapers, cosmetic containers, bottles, cups, etc. [74–76], and also innovative technologies such as tissue engineering, drug delivery, and medical device implants [77]. Poly-β-hydroxybutyric acid (PHB) and its copolymer poly (3-hydroxybu-tyrate-co-hydroxyvalerate [P(3HB-*co*-HV)]) are the most widely used PHAs, while other forms have potential future uses [78]. PHB has the characteristics of increasing crystallinity, stiffness and brittleness, while polyhydroxyvalerate (PHV) increases softness and flexibility. PHAs can be synthesised by over 75 different bacterial genera, as an intracellular carbon and energy reserve and/or as sink for reducing redundant power consumption or electrons under unfavourable environmental and nutritional conditions [79]. Microbial PHAs can be produced in nature by bacterial fermentation of sugar or lipids; however, their widespread application has so far been restricted due to the relatively high production costs (i.e., ≈US$ 4–6/kg compared to US$ 0.6–0.9/kg for conventional

petroleum-derived plastics) [74,78]. Since the organic carbon growth substrate (i.e., carbohydrate) accounts for around 50% of the overall microbial PHA production cost [80], future changes to sugar production and associated commodity price fluctuations will directly impact the future cost-effectiveness and viability of microbial PHA production. For example, increasing feedstock competition from the bio-ethanol industry is likely to have a dominant influence, with global biofuel ethanol production and consumption increasing by around 25% between 2009 and 2014 [81]. Such competitive changes may alter the future economics of bio-plastics production to make them more cost-competitive.

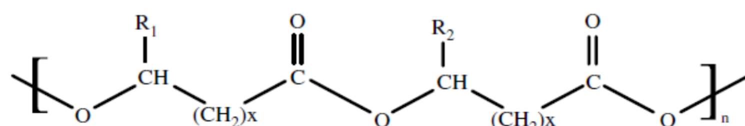


**Figure 2.** General structure of polyhydroxyalkanoates ($R_1/R_2$ = alkyl groups $C_1$–$C_{13}$, X = 4, $n$ = 100–30,000) [76].

PHA-accumulating microorganisms are found naturally in WAS and can accumulate PHAs at concentrations of 0.3–22.7 mg polymer/g sludge [82]. Polyphosphate- and glycogen-accumulating organisms accumulate PHAs by taking up SCFAs under anaerobic conditions, which can be readily sourced from agricultural, industrial and domestic WAS [83]. Microbial PHA production from WAS is dependent on several process parameters, such as temperature, C/N ratio, pH, sludge retention time (SRT) and DO concentration, with higher pH (≈8–9) and shorter SRT (three days) known to favour higher PHA production rates [84]. A study by Krishna and Van Loosdrecht (1999) suggested that PHA accumulation in WAS increased with a decrease in the temperature [85]. The effect of C/N ratio on PHA production from different types of WAS derived medium has also been investigated [78,86,87], with higher C/N ratio shown to restrict cell growth but favour PHA accumulation. Therefore, optimal C/N ratio in a specific WAS-derived medium should be obtained with consideration of these contradictory factors. Anaerobic–aerobic WAS processes have further been investigated for their accumulation capacity of PHAs, which was ascertained to improve greatly by increasing the DO level in anaerobic zones [88–90]. Literature-reported experimental (laboratory-scale) conditions for PHA production from WAS-derived media are summarised in Table 5.

**Table 5.** Reported experimental (laboratory-scale) conditions for PHA production from WAS-derived media.

| Organism | PHA Yield (mg/g Biomass) | Fermentation Conditions | Medium | Additional Carbon Source | References |
|---|---|---|---|---|---|
| Unspecified | - | pH 7, 25 °C, solids concentration 15 g/L, 0.3–0.5 vvm aeration, 60 h SRT | Paper and pulp wastewater | Acetate | [91] |
| Unspecified | 111 | pH 7, 30 °C, C/N ratio 20–140, 300 rpm shaking, 48 h SRT | Domestic WAS | Valeric acid | [78,86] |
| *Bacillus* | - | 37 °C, 250 rpm shaking, 18 h SRT | Beer brewery WAS | None | [80] |
| Unspecified | 792 | Solids concentration 3 g/L, C/N ratio 144, 30 °C, 150 rpm shaking, 48 and 96 h SRT | Dairy and food processing WAS | Acetic acids | [87,92] |
| *Alcaligenes eutrophus* | - | pH 7, 30 °C, 50 rpm shaking, 0.15 vvm aeration | Domestic WAS | None | [93] |
| Unspecified | - | pH 7.5, 15–35 °C, 450 rpm shaking, 0.29 vvm aeration, 60 h SRT | Domestic WAS | Acetate | [85] |
| *Halomonas boliviensis* | - | pH 7.5, inoculum size 2%, 35 °C, 850–1300 rpm stirring | Bakery WAS and seawater | None | [94] |

At present, sequencing batch reactors are reported to be promising for enhanced PHA production, due to their highly flexible operation, ease of process control and biomass growth under transient

conditions [95,96]; however, full- or pilot-scale studies are rarely reported in the literature [50]. The major technical challenge associated with bioplastics production from WAS remains the economically lower yields [97], which requires extensive studies on the optimisation of organic loading rate and efficacy of utilising mixed substrates (e.g., sewage sludge and agroindustry wastewater sludge) with mixed cultures.

## 7. Bio-Flocculants and Bio-Surfactants

Another group of valuable metabolic by-products during microbial transformation of organic substrates are bio-surfactants and bio-flocculants (i.e., extracellular macromolecules). Bio-flocculants (i.e., extracellular bio-polymers such as functional proteins), polysaccharides, cellulose derivatives, lipids, etc. secreted by microorganisms in synthetic growth media have been widely used in chemical and mineral industrial fields such as wastewater treatment, dredging, downstream processing, fermentation and food industries [98,99]. Efficient flocculating activities comparable to synthetic flocculants have been demonstrated and they are also expected to be readily biodegradable and non-toxic to both the environment and humans [100–102]. However, the wider use of these by-products is currently limited by the high production cost, largely associated with the supply of organic substrates (i.e., glucose and sucrose) as alluded to earlier.

Morgan et al. (1990) undertook a comparative study of the nature of biopolymers extracted from anaerobic sludge (including UASB granules) and activated sludge [103]. They ascertained considerable differences in terms of the total yield of ECP/(g SS), with activated sludge samples generating 70–90 mg ECP/(g SS), much higher than granular sludge with 10–20 mg ECP/(g SS). Therefore, activated sludge (i.e., WAS) was recommended as a potential source for biopolymer production even in comparison to other biological wastewater streams like anaerobic granules. Their investigation also revealed that protein was the most dominant fraction of the extracted polymers in anaerobic samples compared with carbohydrate in the activated sludge. Therefore, utilising WAS as a cheap source of carbon, nitrogen and phosphorus for microorganism growth and production of bio-flocculants has been explored as an innovative strategy [104].

Frølund et al. (1996) studied the extraction of water soluble EPS from activated sludge using a cation exchange resin (CER) implemented on activated sludge harvested from two different wastewater treatment plants [105]. By increasing the stirring intensity and extraction time, EPS yield was found to be enhanced to levels twice as high as other common procedures. The extracted EPS consisted mainly of protein, even though, depending on how the extraction was performed, the extracted amounts and relative fraction of the individual compounds varied considerably. Sesay et al. (2006) showed enzyme hydrolysis to be a mild but effective method for EPS extraction from mixed culture activated sludge flocs [106]. Using two carbohydrate-specific enzymes ($\alpha$-amylase and cellulase), a protein-specific enzyme (proteinase) and a consistent CER extraction method, enzymatic EPS extraction was relatively quick, reaching equilibrium within a few hours. By increasing the doses of enzymes, extracted polymer quantities were also enhanced.

WAS is known to be one of the best environmental reservoirs for the isolation of bio-flocculant-producing microorganisms, since bio-flocculation naturally occurs in WAS during aerobic processes. Various strains of bio-flocculants producing microorganisms have successfully been isolated from WAS, including *Achromobacter* sp., *Agrobacterium* sp., *Bacillus cereus, Exiguobacterium acetylicum, Enterobacter* sp., *Acinetobacter* sp., *Haemophilus* sp., *Citrobacter* sp., *Galactomyces* sp., *Klebsiella* sp., *Ochrobactium cicero*, *Pichia membranifaciens*, *Rhodococcus erythropolis*, *Saccharomycete* spp., *Solibacillus silverstris*, etc. [107–111].

Reported experimental conditions for bio-flocculants production from WAS-derived medium in the literature are summarised in Table 6. Comparable or superior performance to synthetic flocculants has generally been demonstrated among these studies based on flocculation tests using solutions containing different dyes and suspended particles, such as kaolin, diatomite, cellulose powder, bentonite, soil, dry yeast, activated sludge, etc. Some authors have even indicated high flocculation

activity using digested WAS liquor (5.5 g/L acetic acid and 0.7 g/L propionic acid) and derived chitosan-like polymers without adding any cations enhancement (e.g., $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Al^{3+}$, and $Fe^{3+}$) [112]. More et al. (2012) studied the pretreatment effect (i.e., sterilisation, alkaline-thermal and acid thermal treatment) on bio-flocculants production in *Serratia* sp., with alkaline-thermal pretreatment achieving the highest concentration (3.4 g/L) of bio-flocculants through improved sludge solubilisation [104].

Sponza (2003) assessed the impact of different wastewater compositions on the EPS (protein, polysaccharide and DNA) production and physicochemical properties (surface charge, bound water and contact angle) in five lab-scale stirred reactors under steady-state conditions [113]. Lower levels of protein (≈38–42 mg $g^{-1}$ VSS) and higher DNA content (≈11–17 mg $g^{-1}$ VSS) were measured in the pulp-paper, textile (cotton knit fabrics) and petrochemical floc EPS under steady-state conditions. Contrastingly, higher protein levels (≈70 mg $g^{-1}$ VSS) and a lower DNA content (≈6 mg $g^{-1}$ VSS) were measured in the EPS of the winery industry and municipal activated sludge. The work of Sponza (2003) demonstrated that EPS composition is very dependent on wastewater type, something also supported by the more recent findings [114].

**Table 6.** Reported experimental (laboratory-scale) conditions for bio-flocculants production from WAS-derived media.

| Bio-Flocculants Composition and Yield | Organisms | Medium | Fermentation Conditions | Reference |
|---|---|---|---|---|
| Polysaccharide, 2.58 g/L | *Klebsiella mobilis* | Dairy wastewater | Inoculum size 5% (*v*/*v*), pH 6.0, 30 °C | [115] |
| Polysaccharide and protein, 15 g/L | *Staphylococcus* sp. and *Pseudomonas* sp. | Brewery wastewater | Inoculum size 2% (*v*/*v*), pH 6.0, 30 °C and shaking speed 160 rpm | [116] |
| 91.2% polysaccharide, 7.6% protein and 1.2% DNA, 1.6 g/L | *Rhodococcus erythropolis* | Livestock wastewater | Inoculum size 2% (*v*/*v*), pH 7, $Na_2HPO_4$ 0.5 g/L | [117] |
| Unspecified, 1.5–3.4 g/L | *Serratia* sp. | Pretreated WAS | Inoculum size 3% (*v*/*v*), 25 °C, shaking speeding 250 rpm, 72 h | [104] |
| Carbohydrates (91% *w*/*w*), 2.1 g/L | *Bacillus subtilis, Bacillus fusiformis, Bacillus flexus* | WAS digestion liquor | Inoculum size 10% (*v*/*v*), pH 4–5, 24 h | [118] |
| Chitosan-like polymer, 4.6 g/L | *Citrobacter* sp. | WAS digestion liquor | pH 8.5, 30 °C, shaking speed 120 rpm | [112] |

Microorganisms release surface-active bio-surfactants to emulsify the hydrophobic substrates of organic carbon sources during growth, at the expense of water-insoluble substrates. For example, *Gordonia amarae*, a filamentous actinomycete found in foaming WAS, is well-known for its bio-surfactant production capability [119]. Compared with chemically-produced surfactants, bio-surfactants are more effective and efficient, and their critical micelle concentration is about 10–40 times lower, i.e., less surfactant is necessary to get a maximal decrease in surface tension [120]. In addition, they are also readily biodegradable, less toxic, antimicrobial and highly tolerant to physicochemical changes (e.g., pH, temperature and ionic strength) [121]. Consequently, bio-surfactants have for some time seen ever-increasing applications in food, personal-care products, and household/laundry detergents as emulsifiers, humectants, dispersants and detergents [122]. The molecular structure of bio-surfactants commonly features two portions: the hydrophobic end of long-chain fatty acids or their derivatives; and the hydrophilic end of carbohydrate, amino acid, phosphate and cyclic peptide. Bio-surfactants can further be divided into two sub-groups: low-molecular-weight substances including glycolipids, lipopeptides and phospholipids; and high-molecular-weight substances including polymers (e.g., emulsan, alsasan, liposan and lipomanan) and particulates (e.g., vesicles and whole microbial cells) [123,124].

Similar to bio-flocculant production, expensive components of pure culture media (accounting for 10–30% of overall process costs) historically limited further development and widespread application of bio-surfactants [125]. Therefore, the potential use of WAS-derived media as an economical alternative to produce bio-surfactants by various routes has been the subject of interest for some time [126].

The possibility of direct extraction through cell lysis and subsequent bio-surfactant release in domestic WAS by alkaline treatment, has been demonstrated [127–129]; however, it should be noted that the pathogenicity of certain microbes commonly found in domestic and industrial wastewaters may affect its practicality. For example, mycobacterial glycolipids, produced by pathogenic mycobacteria, such as *M. avium intracellure*, *M. scrofulaceum* and *M. fortulitum* [130], as well as methylrhamnolipids from *Pseudomonas aeroginosa* [131], are well-known for their toxicity and antigenic properties. Instead, the application of isolated non-pathogenic bacterial strains (*Bacillus licheniformis* and *Bacillus subtilis*) may be preferred due to the mitigation of public health concerns. Nitschke and Pastore (2003) reported that the performance of bio-surfactant produced by *Bacillus* sp. from cassava flour wastewater as a culture medium was comparable to that produced using pure cultures under aseptic conditions and with expensive high-purity organic substrates [132]. More recently, others have sought to optimize and increase production of the bioflocculant MSBF17 during submerged fermentation using more economical substrates (i.e., palm jaggery, $NH_4NO_2$, $K_2HPO_4$, NaCl, etc.) using marine sponge-associated *Bacillus subtilis* MSBN17 [133].

After microbial cultivation in WAS-derived medium, bio-surfactants and bio-flocculants produced by different microorganisms can be recovered using various physical (centrifugation, CER, heating and sonication) and chemical methods (alkaline-NaOH, acid-$H_2SO_4$, trichloroacetic acid, boiling benzene, crown ether, EDTA, ethanol precipitation, enzymes, glutaraldehyde, sulphide and NaCl treatments). Combined physical–chemical methods have also been investigated (e.g., alkaline + heating, ion exchange + stirring, formaldehyde + heating, CER + sonication) [134–136], with some authors reporting optimum contaminant-free EPS extraction from WAS using sonication followed by chemical treatment (formamide and NaOH) [137]. To date, reported yields and final concentrations of bio-flocculants and bio-surfactants, cultivated from various WAS types, or its derivatives, remain relatively low, even under optimal conditions. Therefore, further optimisation of cultivation processes with the consideration of key process parameters is required to tackle future scale-up production issues. Additionally, each bio-flocculant and bio-surfactant obtained should be characterised based on their ionic properties and structure, as this should serve as the basis for selecting these operational parameters to realize full process optimisations.

## 8. Current Challenges

Sewage sludge is today a growing waste stream, and one that is both costly to manage and largely undervalued in terms of its constituents. For example, approximately 30~60% of sewage treatment plant operating costs are estimated to relate to sludge treatment activities [138]. Biorefinery production from WAS offers several key advantages for future sludge management, including: providing an alternate treatment pathway for what is an ever-growing and problematic urban waste stream; and recovering valuable resources and value-added bio-products which can potentially find application as sustainable green product alternatives in a time when humanity is increasingly looking toward a circular economy future. The technical feasibility, risks, costs and benefits all need to be assessed to determine viability of each biorefinery development involving WAS, and this has been the subject of much research to date. The quality of WAS-derived products and their market values are important factors with respect to the future feasibility of these processes. At present, issues and challenges associated with biorefinery production from WAS include:

(1) Biorefinery production of protein and enzymes are still considered to be higher cost routes compared with agro-industry residues and animal health in terms of pathogenic and metals toxicity, which has effectively stifled developments in this area [139].
(2) Recovery of metabolic WAS products (e.g., bio-plastics, bio-pesticides, bio-flocculants and bio-surfactants) remains in its infancy and further optimisation of operational parameters and selection of non-toxic strains are needed to progress this research area.

(3) Important factors such as optimum growth environment or wastewater matrix for harvesting WAS with the highest concentration of specific bio-products need to be further investigated and refined.
(4) WAS bio-product production through biological processes such as fermentation, bioleaching or enzymatic extraction, while an evolving area of research, still requires further optimisation.
(5) Anaerobic digestion as a potential option for enhancing the production of SCFAs and their extraction from WAS through fermentation in the presence of key chemical surfactants deserves further exploration.
(6) Simple and efficient purification procedures for certain bio-products recovered from WAS (in line with the required purity for specific applications) need further development and would contribute to improving the overall economics of biorefinery approaches.
(7) Scale of WAS-based biorefinery production is an important future challenge, since much of the research so far has been at laboratory scale. To scale up the biorefinery approach using WAS, further research must be carried out at larger scales (pilot- and ultimately full-scale) to optimize each biorefinery process. Only then can the full techno-economic performance of these processes be fully investigated and understood.

**Acknowledgments:** The authors acknowledge the financial support of the Cooperative Research Centre for Low Carbon Living Ltd. (grants RP2017 and RP2008) whose activities are supported by the Cooperative Research Centres program, an Australian Government initiative. W.Z. would also like to acknowledge the support from European Commission Horizon 2020 Marie Skłodowska-Curie Actions (Grant Agreement No.743993).

**Author Contributions:** W.Z. and M.D.S. contributed equally to this work; J.P.A.G. and W.D. revised the manuscript; C.P.S. and M.Z. proof-read the final manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the writing of the manuscript, and in the decision to publish the results.

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