doi: 10.2166/9781780409566

Chapter 9

Producing microbial-based protein from reactive nitrogen recovered from wastewater

Ilje Pikaar[1], Heidy Cruz[1], Tim Hülsen[2], Damien Batstone[2], Korneel Rabaey[3,4], Paul Jensen[2], Yoram Avnimelech[5] and Willy Verstraete[3]

[1]*The School of Civil Engineering, The University of Queensland, St Lucia, QLD 4072, Australia*
[2]*The Advanced Water Management Centre, The University of Queensland, St Lucia, QLD 4072, Australia*
[3]*Centre for Microbial Ecology and Technology (CMET), Ghent University, 9000 Gent, Belgium*
[4]*Centre for Advanced Process Technology for Urban Resource Recovery, Frieda Saeysstraat 1, 9052 Gent, Belgium*
[5]*Israel Institute of Technology, Faculty of Civil and Environmental Engineering, Haifa, Israel*

9.1 INTRODUCTION

Recent estimates of the UN indicate that the global population will grow to 9–10 billion people by the year 2050 (United Nations, 2015). For physical and mental health, it is important that on average each person can consume some 0.66 g protein/kg body weight per day (World Health Organization, 2002). The current route to provide this protein is mainly through agricultural plant production, using massive amounts of mineral nitrogen fertilizer fabricated by means of the Haber–Bosch process (Bodirsky *et al.*, 2014). This mineral nitrogen is generated as ammonium and may be converted to nitrate prior to use. These forms of reactive nitrogen are, when applied to the soil as fertilizer, taken up by the plants to produce plant proteins. The plant proteins can be directly used as food for the human population, but are, to a large extent, used as feed to produce animal protein. In the latter case, approximately 4% of the Haber–Bosch nitrogen is ultimately consumed as high-value animal proteins. The overall nitrogen efficiency for plant proteins for human consumption is substantially higher, albeit still low, with an efficiency of 14% (Galloway & Cowling, 2002).

At present, the need for animal (and hence vegetable) protein containing essential amino acids is increasing because a growing part of the world desires to, and can afford to, consume more protein products and higher quality protein products (Bodirsky *et al.*, 2015; Godfray *et al.*, 2010). The current major supply routes for high quality protein are agri-crops (for about 50% of food protein inputs, particularly wheat, rice and pulses such as soy beans), animal proteins based on agri-crops (another 40%) and finally fish (some 5–10%). These routes are facing limitations. Limitations of the agri-crop route include the massive amount of nitrogen fertilizer used worldwide, that is ~100 Mton of nitrogen fertilizer, and this is expected to increase to about 150 Mton of by 2050. Indeed the Haber–Bosch process consumes about 1–2% of the total world industrial energy (Erisman *et al.*, 2008) and moreover – due to the fact that agriculture is subjected to losses by leaching, run-off and denitrification – nitrogen pollution is of major environmental concern (Erisman *et al.*, 2013; Galloway & Leach, 2016), often

referred to as the nitrogen cascade (Galloway *et al.*, 2003). The last route is very much struggling with the international limits set to prevent overfishing of the world seas.

Ultimately, a substantial fraction of proteins consumed by humans ends up as wastewater ammonium, often referred to as reactive nitrogen; currently about 20 million tons, which is expected to increase to over 35 million tons by 2050. For more than 100 years now, emphasis has been placed on removing the reactive nitrogen from wastewater by oxidizing it back into its elemental, non-reactive form (N_2) by means of biological oxidation, predominantly by the conventional activated sludge process (Jenkins & Wanner, 2014). Recent innovations such as anammox (Kartal *et al.*, 2010) and aerobic granular sludge (Pronk *et al.*, 2017) decrease the energy requirements of nitrogen removal and may even result in energy self-sufficient wastewater treatment plants. While these innovations can be considered a step forward, the working principle remains unchanged: the dissipation of reactive nitrogen to N_2. Yet generally, the concept that there is sufficient nitrogen in the air (i.e., about 80% of air is nitrogen gas – N_2) has dominated the mindset in urban water management. The requirement for 2–3 L of fossil fuel equivalent to produce 1 kg of reactive Haber–Bosch nitrogen, and another 2 L of fossil fuel per kg wastewater nitrogen is needed to subsequently dissipate that nitrogen and return it to the atmosphere, has largely been disregarded.

The time has come to recover reactive nitrogen embedded within the wastewater matrix as microbial biomass rich in proteinaceous components. This chapter examines the possibility to recover nitrogen from wastewater in its reactive form, coupled with upgrading the recovered reactive nitrogen into microbial proteins. This microbial-based protein can, depending on the quality and type of the treated wastewater, subsequently be used as a food, as a feed or as an organic nitrogen fertilizer, contributing to a more sustainable nitrogen cycle. Alternatively, the recovered nitrogen can also be used as fertilizer as an alternative to Haber–Bosch nitrogen fertilizer, or as feedstock in the chemical industry (e.g., in the Denox process).

9.2 LEARNING OBJECTIVES

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

- Describe the current status of nitrogen management in urban water management and its relevance in relation to the global nitrogen cycle.
- Understand the limitations of conventional nitrogen dissipation technologies in wastewater treatment.
- Explain the fundamental principles of microbial-based protein production by means of autotrophic, organotrophic, and phototrophic microbial growth.
- Describe the key design criteria and performance parameters for aerobic growth of microbial cells using recovered nitrogen in different wastewater matrices.
- Characterize the process and explain the key technological challenges and limitations for the production of microbial protein using recovered reactive nitrogen.
- Evaluate the different microbial protein production processes used to recover the nitrogen from various wastewater streams against conventional nitrogen removal processes in terms of process economy, process robustness and economy of scale of the produced microbial protein.

9.3 CONCEPTUAL OVERVIEW PRODUCTION OF MICROBIAL PROTEIN USING RECOVERED NITROGEN

9.3.1 Fundamental principles of the microbial conversion

In general, microbial growth involves chemical transformation coupled to energy generation (catabolism), with energy stored chemically (generally as ATP) to enable cell synthesis (anabolism). The dominant mode is coupled chemical oxidation and reduction with electrons transferred from one

molecule (i.e., the electron donor) and this molecule becomes oxidized. These electrons are transferred to an acceptor (i.e., electron acceptor) which becomes reduced. The process of anabolism may be oxidative or reductive, depending on the substrate. Specifically, the electron state of the substrate needs to change to match that of the microbial biomass. There are different types of microorganisms that can use different types of electron donors. In this chapter, two key microbial synthesis routes most relevant to production of microbial protein using recovered nitrogen from wastewater are described in detail. These are the *organotrophic* and *autotrophic* microbial cell synthesis through aerobic fermentation.

Very importantly, independent of the synthesis route, catabolism must be reduced to the minimum necessary for energy generation, since anabolism leads to generation of the desired product and catabolism is a cost factor. At increasing sludge ages, additional oxygen consumption is required for endogenous respiration (Van Haandel & Van Der Lubbe, 2012). It is therefore vitally important that the system is operated at the shortest possible sludge age in order to minimize catabolism and maximize cell yields.

9.3.1.1 Organotrophic microbial cell synthesis through aerobic fermentation

The most straightforward and easiest way to produce microbial protein from an engineering perspective is the use of organotrophic bacteria. Organotrophic bacteria directly use soluble organic compounds present in the growth medium, in this case wastewater. To make chemistry more transparent, the conversions are written at the level of the electron. Since no bacterium can be 'charged', one must always come to electron neutrality. As an example, consider that a bacterium is supplied with acetate, a common organic compound present in wastewater, as an energy source. This gives the following equations:

The electron donor reaction:

$$CH_3COO^-(aq) + 3H_2O(aq) \rightarrow CO_2(g) + HCO_3^-(aq) + 8H^+(aq) + 8e^- \tag{9.1}$$

The electron acceptor reactions:

For energy generation:

$$2O_2(g) + 8H^+(aq) + 8e^- \rightarrow 4H_2O(aq) \tag{9.2}$$

For cell biosynthesis using ammonia as nitrogen source (Rittmann & MacCarty, 2001):

$$5CO_2(g) + 1NH_3(aq) + 20H^+(aq) + 20e^- \rightarrow C_5H_7O_2N(s) + 8H_2O(aq) \tag{9.3}$$

According to Equation (9.3), the microbial biomass formed (approximated by $C_5H_7O_2N$) has a molecular weight of 113 g. The nitrogen present in this microbial biomass is considered to be present in the form of protein. Protein contains 16% nitrogen on weight basis (known as the Jones factor). Hence if the nitrogen content is known, the protein content can be calculated: protein content = nitrogen-N × 6.25 (100 divided by 16). If one mole of microbial biomass is produced at a molecular weight of 113 g, the biomass will contain 14 g of nitrogen, therefore the amount of protein produced equals 14 × 6.25 = 87.5 g of protein. This represents 77% of the dry weight in this example. Let us consider the aerobic production of 'young' bacterial cells at very low sludge ages (i.e., 1–2 days maximum). Under these conditions, the cell maintenance metabolism becomes negligible. Consequently, under such conditions for every electron equivalent generated approximately half goes to producing energy and the other half goes to growth of cellular biomass (Park *et al.*, 2015), one then obtains the following overall balance:

$$5CH_3COO^-(aq) + 5O_2(g) + 1NH_3(aq) \rightarrow C_5H_7O_2N(s) + 5HCO_3^-(aq) + 3H_2O(aq) \tag{9.4}$$

At both sides of the equation, the ions and all chemical elements are equated. In this example five moles of acetate (MW 59), representing $5 \times 59 = 295$ g of organic matter, are used to generate 1 mole of biomass (MW 113). Thus, a cell yield of 113/295~0.38 or about 40% on a dry matter basis is obtained. Under conditions of stress (insufficient oxygen levels, limitation of essential nutrients or at long(er) cell residence times (i.e., in wastewater treatment this is referred to as sludge age)), this yield factor may become lower. It should be noted that different values are obtained in case one uses a more oxidized substrate (e.g., formic acid) or a more reduced substrate (e.g., ethanol). As a rule of thumb however, 0.4–0.5 is a good value for produced microbial biomass at short sludge ages (i.e., 1–2 days).

Organotrophic microorganisms in general can use a wide variety of electron donors. Besides a very common product such as acetate used in the example of above, they use all kinds of carbohydrates as energy source: sugars, starches, cellulose but also lipids and hydrocarbons. They can also use proteins and resynthesize them to their own cellular proteins. The provided oxygen functions as electron acceptor. All these substrates result in a cell synthesis efficiency of about 50% under optimal growth conditions and short sludge ages. The fact that microbes can use a wide range of organic compounds is important as in wastewater a large variety of organics can be present. In this respect, microbes are extremely effective compared to, for instance, insects or higher animals that achieve only some 10–25% of conversion efficiency. Indeed, microbes have no such complex instruments such as eyes and ears to make and can focus on their rather straightforward cellular components, that is a cell wall, a cell membrane, a nucleus and the cytoplasm. The latter is the major protein component of the microbial cell. It should be emphasized that the above described cell yields considers only exogenic respiration and no endogenic respiration. In practice, this can only be achieved when the sludge age is very low (i.e., 1–2 days). At higher sludge ages, the endogenic respiration is generally greater than the exogenic respiration, which can substantially lower the overall cell yield (Van Haandel & Van Der Lubbe, 2012).

Besides using ammonia as a nitrogen source, bacteria can also use nitrogen gas. Under such conditions of nitrogen fixation, they must spend a lot of energy to convert the N_2 to ammonia. Actually, microbial nitrogen fixation is far less efficient than the Haber–Bosch process. Therefore, it makes sense to use the industrial process to fix atmospheric nitrogen to reactive nitrogen and 'keep the unused' reactive nitrogen (that ultimately ends up in wastewater) in its reactive form and use the latter in a microbial fermenter to produce microbial protein. Microbes can also use nitrate and nitrite as electron acceptors instead of oxygen. However, this form of electron acceptor is not suitable for microbial protein production due to cost, and it is only used where nitrate or nitrite are to be removed to produce nitrogen gas rather than assimilation of nitrogen into cell biomass.

9.3.1.2 Autotrophic microbial cell synthesis

Plants can fix CO_2 by using sunlight, and thereby convert the inorganic carbon to plant biomass. In Equation (9.3), it appears that the microbial cell synthesis also starts from CO_2, but this is not the case for the so-called organotrophic microorganisms. In their biochemistry, organotrophic microorganisms do not break down the organic compounds fully to CO_2 but rather they recover small units of organics and integrate them in their own complex building units. However, there is a group of microorganisms capable of growth without utilizing organic molecules. These organisms are autotrophic – that is, can generate their own organics (mainly from CO_2). Two groups of autotrophic bacteria can be distinguished, namely (i) the chemo-lithotrophic organisms and (ii) photo-lithotrophic organisms.

9.3.1.2.1 The chemo-lithotrophic organisms

Chemo-lithotrophic microorganisms use a chemical reaction to generate energy. The most prominent example is hydrogenotrophic bacteria, which utilize the reaction shown in Equation (9.5):

$$H_2(g) \rightarrow +2H^+(aq) + 2e^- \tag{9.5}$$

Hydrogenotrophic bacteria are of particular interest since the current trend is evolving towards a hydrogen driven economy. The hydrogen, for instance generated by means of water electrolysis using renewable energy (i.e., green electricity), can be used to split water by electrolysis into hydrogen and oxygen. These two gases thus represent the major part of the energy present in the electricity (at a conversion efficiency of approximately 70% of the electrolysis process using state-of-the-art fuel cell technology). Moreover, one electron equivalent in hydrogen combustion releases 143 kJ, which is about 30% higher than that of methane (i.e., 111 kJ per electron equivalent). The energy present in the combination of oxygen and hydrogen, when offered in a reactor system to hydrogenotrophic bacteria, allows the latter to grow by using CO_2 and NH_3 as given in Equation (9.6):

$$\frac{1}{4}CO_2(g) + \frac{1}{20}NH_3(aq) + H^+(aq) + e^- \rightarrow \frac{1}{20}C_5H_7O_2N(s) + \frac{2}{5}H_2O(aq) \quad (9.6)$$

Actually, they grow, expressed in terms of electron equivalents handled as indicated above, with yields, which are of the same order of organotrophs. Indeed 1 mole of hydrogen requires half a mole of oxygen:

$$H_2(g) + \frac{1}{2}O_2(g) \rightarrow H_2O(aq) \quad (9.7)$$

Thus, 2 g of hydrogen equals to 16 g of oxygen, in other words 16 g of COD equivalents. On the basis of hydrogen expressed as COD, the hydrogenotrophic bacteria, although they indeed have to build up their biomass starting from CO_2, attain yields in the order of 0.3 g of biomass per gram COD converted (expressed in 100% dry weight) (Matassa *et al.*, 2015b). The overall reaction equates to (Ishizaki & Tanaka, 1990):

$$21.36\,H_2 + 6.21\,O_2 + 4.09\,CO_2 + 0.76\,NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.70\,H_2O \quad (9.8)$$

9.3.1.2.2 Photo-lithotrophic organisms

Purple phototrophic bacteria (PPB) are a diverse group of anoxygenic phototrophs that are mostly Gram-negative facultative anaerobes spread throughout the phylogenetic tree of bacteria with many subdivisions, particularly within the *Proteobacteria*. This group of bacteria is characterized by an extremely diverse metabolism including anaerobic photoheterotrophic and photoautotrophy with light, aerobic chemoheterotrophy or chemoautotrophy in the dark as well as fermentation (Tabita, 1995). As opposed to oxygenic photosynthesis, these bacteria cannot utilize H_2O as electron donor to reduce CO_2 to cell materials. Instead, they utilize a range of lower potentials electron donors such as hydrogen sulphide (H_2S), thiosulphate ($S_2O_3^{2-}$) ferrous iron (Fe^{2+}) and several others for photoautotrophy, and organics such as volatile fatty acids (e.g., acetic, propionic, butyric acid), alcohols and some sugars for photoheterotropy. Consequently, PPB do not generate oxygen during photosynthesis and this process is therefore referred to as anoxygenic photosynthesis. This process is further differentiated from oxygenic photosynthesis by the absorption maxima at which photons from sunlight are harvested. While photosynthetic active radiation (PAR), for example trees and algae, designates the spectral range of solar radiation from 400 to 700 nm, absorbed by chlorophyll, PPB utilize bacteriochlorophyll (BChl) and can absorb photons in the near infra-red >800 nm (and at lower wavelengths, e.g. 375 and 590 nm for BChl a) (Overmann & Garcia-Pichel, 1998) to drive a proton motive force to produce energy in the form of ATP. The generation of energy via photons in combination with photoheterotrophic growth makes PPB an interesting mediator for wastewater treatment. In fact, the organics and nitrogen can in theory be completely recovered as microbial biomass in case one has optimal COD/N ratios (Suhaimi *et al.*, 1987). Due to the high yield, nitrogen and phosphorous are required as macronutrients to support growth which allows for simultaneous and non-destructive removal of organics, nitrogen and phosphorous from wastewater and the option to recover these resources as PPB biomass.

For cell biosynthesis using different organic donors and ammonia as nitrogen source the following reactions can be written for acetic (Equation (9.9)) and butyric acid (Equation (9.10)):

$$1.5\,CH_3COOH(aq) + 0.48\,NH_3(aq) \rightarrow 2.67\,CH_{1.8}O_{0.38}N_{0.18}(s) + 0.33\,CO_2(g) + 1.32\,H_2O(aq) \quad (9.9)$$

$$1.5\,C_4H_8O_2(aq) + 0.6\,CO_2(g) + 1.19\,NH_3 \rightarrow 6.6\,CH_{1.8}O_{0.38}N_{0.18}(s) + 1.84\,H_2O(aq) \quad (9.10)$$

Based on Equation (9.9), 1 kg of acetic acid could generate approximately 0.66 kg of PPB biomass. The formed biomass contains approximately 70% protein (~11% N on a dry weight basis), which is similar to the protein content for organotrophic microbial cell synthesis. The latter implies that more nitrogen can be recovered as microbial protein than the organotrophic route. Photoautotrophic PPB growth can occur through diverse metabolic pathways, including through inorganic electron donors such as hydrogen sulphide (H_2S) (Equation (9.11)). Elemental sulphur can also serve as electron donor with sulphate or thiosulphate as oxidized products (Equation (9.12)). In these reactions, an overall cell composition of $CH_{1.6}N_{0.2}O_{0.4}$ was assumed (Van Gemerden, 1968):

$$CO_2(g) + 2.1H_2S + 0.2\,NH_3 \rightarrow CH_{1.6}O_{0.4}N_{0.2} + 2.1S^0 + 1.6H_2O \quad (9.11)$$

$$CO_2(g) + 0.5H_2S + 0.2\,NH_3 + 0.4H_2O \rightarrow CH_{1.6}O_{0.4}N_{0.2} + 0.5H_2SO_4 \quad (9.12)$$

Oxidation of H_2S to elemental sulphur results in biomass yields of 0.31 g cell dry matter (CDM) per g H_2S (or 0.25 g COD g COD^{-1}), while oxidation of H_2S through to sulphate results in biomass yields of 1.3 g CDM per gram H_2S (or 1.0 g COD g COD^{-1}). The latter is because the oxidation of H_2S to elemental sulphur (Equation (9.13)) and sulphate (Equation (9.14)) involves two and eight electrons, respectively:

$$H_2S \rightarrow S^0 + 2H^+ + 2e^- \quad (9.13)$$

$$H_2S \rightarrow 4H_2O \rightarrow SO_4^{2-} + 10H^+ + 8e^- \quad (9.14)$$

The processes described above can occur simultaneously in a single environment, such as a bioreactor treating wastewater. This is one reason why anaerobic cultures with mixed culture PPB do not generate the characteristic rotten egg smell of H_2S. When diverse mixed culture microbial populations are present, such as those consortia commonly in industrial bioreactors, there are bacteria that can provide CO_2 for VFA assimilation which enables direct CO_2 fixation rather than CO_2 generation from the VFA itself, as described in Equation (9.9).

The most critical process parameter (and bottleneck hindering practical implementation) of the production of PPB is the inherent light requirement of phototrophic bioreactors. Light penetration is generally very poor through aqueous cell suspensions (particularly long wavelengths), limiting the active reactor volumes to very narrow fields around the illuminated surface. This represents a major challenge and is, for example, one of the most critical bottlenecks hindering successful commercialization of algae-based, photobioreactor wastewater treatment technologies (Posten, 2009). The same issues arise for PPBs. Another critical challenge to address is the energy requirement when applying artificially illumination, which are in the order of ~10 kWh per m^3 wastewater treated (Hülsen *et al.*, 2018). In order to become viable in the future, energy requirements need to be reduced, which in terms of radiation can potentially be achieved by the utilization of sunlight. However, natural light/dark cycles are challenging for continuous removal performance. Another major cost factor is the harvesting and drying of the biomass, which has been reported to be up to 20–30% of the operational costs for algal systems (Molina Grima *et al.*, 2003). This is mainly caused by rather diluted cultures

(~1.0 g/L) and the small size of algal cells (5–50 μm). Considering the above-described challenges, it is evident that it remains uncertain whether PPB can become a viable concept for the production of microbial protein from wastewater.

9.3.2 Application and design

The processes to produce microbial protein from recovered nitrogen has to focus on the following factors: the origin of the wastewater (i.e., is the nitrogen 'mixed' with, e.g., fecal matter, pathogens, metals or other unwanted compounds), the yield of the protein, the amount generated per unit reactor volume per day (i.e., volumetric production rate), the harvesting and downscale processing of the biomass and finally the quality and end-use of the biomass produced.

9.3.2.1 The working microbiome

Ideally, one would aim to work only under very specific conditions, for instance in a sterile reactor with sterile input materials (purchased chemical grade reactive nitrogen and electron donors) and well-defined microbial strains. Such conditions are implemented in conventional industrial microbiology. However, in the case of upgrading recovered nitrogen from wastewater at the wastewater treatment plant, at best one can work under conditions which resemble those of making artisanal cheese. Thus, the incoming material is seeded with a starter culture which has been selected over time for the fact that it provides a good end-product and subsequently the environmental conditions are controlled so that the proper mixed association of microbes (also called the microbiome) brings about the conversion in a desired way. In the following sections, the most promising microbial protein production pathways are described in more detail. For every kg of nitrogen to be upgraded, a readily biodegradable organic carbon source is needed. The overall stoichiometry is approximately 20–25 kg COD equivalent per kg nitrogen. The conversion is based on aerobic microbial metabolism (see Equations (9.1)–(9.4)). Of every kg of COD supplied, approximately half is oxidized to CO_2 and H_2O. Hence the supply of oxygen is critical (about 0.5 kg oxygen needs to be transferred to the cells per kg carbohydrate converted). The energy generated by these catabolic processes is used for anabolic processes, that is to synthesize new microbial cells which will have a typical composition of young microbial cells (i.e., a dry matter consisting of 70% protein, 5% minerals (of which some 2% are phosphate) with the remainder being exo and endo polymers with a composition relating to sugars and lipids.

Besides the supply of an appropriate electron donor and oxygen as an electron acceptor, the ambient conditions for the microbiome should also be appropriate. Normally, one operates at temperatures in the 20–40°C range; higher is possible but the biomass yields tend to be lower outside that range. Normally, the pH is controlled within the range of 5–8 but more extreme ranges can used to exclude certain microbial species. Indeed, by imposing very acidic or alkaline conditions one can (even in non-sterile open reactor systems) disfavour unwanted microbes, such as for instance enteric bacteria. Similarly, light of specific wavelength can be utilized to select for desired bacteria, for example the supply of infrared radiation to outcompete algae and favour purple phototrophic bacteria in a photobioreactor. As described above, PPB biomass yields are higher (e.g., 66% for acetic acid based on stoichiometry) compared to organotrophic organisms and the reactive nitrogen upgrade is enhanced to around 1 kg N for every 13 kg of COD.

9.3.2.2 The volumetric production rate

The key feature of industrial production is thus to achieve a maximal conversion of electron donor per unit volume and per unit time. This determines the volumetric reactor substrate loading rate and relates to the kg biomass (CDM) generated per m^3 reactor per day, that is the volumetric production rate. In conventional treatment systems, the focus is on removing the last bit of organic matter (or at least to below discharge limits) from the wastewater and to obtain a minimal amount of residual microbial biomass, that is excess waste activated sludge. Hence, in waste treatment systems, one

operates at low loading rates (of the order of approximately 1 kg organic matter per m^3 per day) and long reactor cell residence times of 20 days or more. Microbial biomass which has been maintained at a low food supply level for a residence time of several weeks is characterized by old cells which are low in protein and exopolysaccharides and hence can be harvested relatively easily because they dewater quite well. However, in the case of nutrient recovery by means of microbial protein production, high volumetric loading rates of at least 10 kg organic matter (or COD equivalent) per m^3 per day and cell residence times of only a few days need to be used to obtain young cells high in protein. The latter, however, can be quite difficult to harvest and to dewater. This is a key feature in the downstream processing of microbial protein, as at present it incurs high operational costs because the produced microbial biomass needs to be almost completely dewatered (i.e., >95%) in order to become a viable product with a market value.

9.3.2.3 Oxygen supply

Aerobic production is normally based upon oxygen as the electron acceptor. Indeed, oxygen is the most economical of all electron acceptors. One kilogram of oxygen dissolved in water has a cost of approximately €0.05. A principal factor in the reactor design is the transfer of oxygen to the water phase. As in used water treatment, a variety of aeration mechanisms can be used such as propellers, fine bubble distributors, jets, membranes, high-pressure reactors. According to Equation (9.4), to generate 1 kg of microbial cell dry weight, about 1 kg of oxygen input is generally required. This cost factor is not excessive, but a good supply of oxygen is connected with a second factor, namely the intensity at which the reactor is performing. It is important to realize that the aerobic oxygen uptake rates in these biomass production processes are much higher than those commonly found in wastewater treatment processes, which are aimed at 'cleaning up' and typically in the order of 40–60 mg/L.h (Van Haandel & Van Der Lubbe, 2012). In fact, in pure culture industrial fermentation often industrial grade pure oxygen is used, which incurs higher operational costs, albeit still only representing a relatively small part of the overall production cost.

9.3.2.4 Infrared irradation

Note that in case phototrophic bacteria are used, these have the benefit that they work under anaerobic conditions. However, they require substantial energy input in the form of light where the growth can be achieved with infra-red light only, but additional wavelength can be harvested, for example 375 and 590 nm for bacteriochlorophyll a (Overmann & Garcia-Pichel, 1998), which is relevant when growing cultures outside with sunlight (or filtered sunlight). A maximum light conversion efficiency (LCE) between 3.7 and 25.6% has been given by several authors and summarized by Adessi and De Philippis (2014), where LCE tend to be at the higher end when calculated using a specific wavelength, for example 860 nm, rather than integrating spectral ranges. This rather broad range shows that the real efficiency has to be determined specifically for given conditions, substrates and wavelengths. In any case the illuminated surface to volume ration is a crucial design parameter where typical volume ratios are 80–100 m^2/m^3 for algal systems (Posten, 2009), and in order to guarantee economic production estimated capital and operational costs may not exceed €40/m^2, which is currently several times higher.

9.3.2.5 Pumping and mixing

Fermentation reactors require energy for sufficient mixing and pumping as well as to dissolve the oxygen (and hydrogen and methane in case these gaseous compounds are used as electron donors). The use of these gaseous substrate requires substantial mixing in order to create high gas-to-liquid transfer conditions, which is a crucial factor in obtaining high volumetric productivity and also increases the absorption of these gaseous compounds. The latter is very important in terms of process economics considering that they represent a large fraction of the operational expenditure. Energy for pumping and mixing can be assumed to be on the order of 0.2–3 kWh per cubic meter of water handled (Niazi & Brown, 2015).

9.3.2.6 Dewatering, drying and conservation

After the fermentation step, the produced microbial biomass can undergo heat treatment, in case it is destined to be used as animal feed. Heat treatment can be carried out for multiple reasons, namely: (i) lyse cells thereby increasing the protein accessibility; (ii) decrease the nucleic acid content of the microbial biomass; and (iii) obtain a dry and quality assured end-product (Anupama & Ravindra, 2000). Prior to this, the microbial biomass is first dewatered to lower the water content by centrifugation (or other conventional dewatering methods) to lower the energy consumption for the heat-treatment step. Normally, dry solids contents of maximum 25 wt% can be achieved in this way. Spray-drying with integrated fluidized bed, a common practice in the food processing industry (Chen & Mujumdar, 2009), can be used for the drying step. The energy costs for this step are quite substantial, that is about 3500 MJ per cubic meter of water (Chen & Mujumdar, 2009).

9.3.3 Direct assimilation of recovered nitrogen into microbial biomass

In most practical situations, the recovered nitrogen is upgraded by supplying the bacteria with low value organic carbon sources like starch and organic acids that are present in various wastewater streams from the food and beverage industry and distillery side-streams. It is of crucial importance to select for a 'microbial team' (i.e., a microbiome). To the degree possible, rather than processing one type of molecule, a suitable microbiome converts the overall assembly of organics present in the wastewater to cellular biomass. The microbiome must consist of several members that complement one another so that the metabolites of one species are further used by the other species. Indeed, the final mixed liquor, when subjected to cell harvesting, should give rise to water which is low in residual nutrients (i.e., N, P) and organic carbon (cells and soluble organics) on the one hand and a harvest of biomass rich in nutritious microbial protein on the other hand.

Today, the major challenges in this respect are issues relating to the type of microbiome needed and the ways to maintain and support the microbiome in order to produce a microbial biomass of consistent quality and composition. Indeed, first a selection of species must be assembled. The focus must be on the fact that the team members should grow at high rates (with doubling times of only a couple of hours) and consume the N and electron donor down to low residual levels. The residual level of mineral nitrogen in the medium is easy to monitor; also the amount of residual soluble organics can be easily quantified (through COD or solids analysis). In addition, the oxygen uptake rate is a helpful tool to control the oxidation capacity of the microbiome. The constitution of the generated microbial biomass is more difficult to select for. It is highly advantageous for the further processing of the biomass if one can obtain a coagulation/flocculation of the single microbial cells into flocs that readily separate by sedimentation, filtration, or centrifugation forces. Indeed, one should try to obtain a harvested biomass at a level of some 5–10% dry matter content to achieve further handling in the form of a thick slurry of biomass. The latter can then be either preserved as such (e.g., by bringing the pH below 4.0 as done in the pickling of foods or ensiling of feeds) or by further drying to 85% dry matter or more. In this respect, the current R&D is focusing on the development of proper coagulating agents which are effective in the harvesting process and, moreover, which are of food/feed grade quality.

Wastewater from the food and beverage industry has one distinct advantage over domestic wastewater in terms of production of microbial protein, namely, it is free of fecal matter, pathogens and toxic metals. Consequently, there is no need for a nitrogen separation and purification step, allowing direct assimilation into microbial biomass. Moreover, wastewater streams from the food and beverage industry usually also have higher concentrations of ammonium and organics. The engineering is thus less challenging. Crucial will be to find the optimum COD/N/P ratio in order to stimulate microbial biomass high in protein. In fact, it turns out that in many cases, many waste streams are ammonium deficient, as shown in Table 9.1 below. As such, although it seems counterintuitive, one needs to add nitrogen to the wastewater!

Table 9.1 Overview of industrial wastewater streams free of fecal matter from the food and beverage industry and their COD to nitrogen ratio, adapted from Pikaar *et al.* (2017b).

Type of Industrial Wastewater	COD (mg/L)	Total N (mg/L)	COD/N*	Maximum Attainable Protein Content[a] (wt%)	Ref.
Dairy	4000	55	100:1.37	17	Kasapgil *et al.* (1994)
Dairy	4500	60	100:1.33	16	Koyuncu *et al.* (2000)
Dairy	4000	60	100:1.50	18	Koyuncu *et al.* (2000)
Dairy	1745	75	100:4.30	53	Koyuncu *et al.* (2000)
Dairy	18 045	329	100:1.82	22	Arbeli *et al.* (2006)
Dairy	4000	55	100:1.37	17	Ince (1996)
Dairy	2800	140	100:5	62	Schwarzenbeck *et al.* (2005)
Cheese	4430	18	100:0.41	5	Monroy *et al.* (1996)
Yoghurt and buttermilk	1500	63	100:4.2	52	Koyuncu *et al.* (2000)
Beverage	1750	28.4	100:1.62	20	Amuda and Amoo (2007)
Distillery	150 000	6000	100:4	50	Mohana *et al.* (2009)
Distillery (raisins)	57 500	750	100:1.30	16	Vlissidis and Zouboulis (1993)
Distillery (wines)	27 500	650	100:2.36	29	Vlissidis and Zouboulis (1993)
Distillery (figs)	35 400	880	100:2.48	31	Vlissidis and Zouboulis (1993)
Brewery	4000	52.5	100:1.31	16	Driessen and Vereijken (2003)
Sugar industry (beet)	6300	53.23	100:0.84	10	Güven *et al.* (2009)
Olive oil mill	40 000–220 000	300–1200	100:0.54–100:0.75	6–9	Azbar *et al.* (2004)
Olive oil mill	40 000–195 000	500–15 000	100:0.77–100:1.25	9–15	Sierra *et al.* (2001)
Palm oil mill	50 000	750	100:1.5	18	Ahmad *et al.* (2006)

[a]COD/N ratio where no external nitrogen is added to the wastewater.

9.3.4 Case studies and implementation

9.3.4.1 Industrial production of microbial protein

Microorganisms have always been central in basic feed and food processing techniques, for instance converting fibres into edible food when fermenting dough to produce bread, or milk into cheese, allowing its long-term preservation. They have often been used as a direct food source, as is the case for yeast or algae. The latter, together with bacteria, constitute the microbial actors involved in processing food. They can also be used directly as a feed or food source. The term microbe is used here in the broad connotation of bacteria, fungi, yeast and algae. Some 40 years ago, Imperial Chemical Industries (London, UK) developed a single cell protein (SCP) generated from methanol using the bacterium *Methylophilus methylotrophus*. The product received the name Pruteen and contained up to 70% protein and was used in pig feed. Pruteen, however, could not compete with cheaper animal feeds that were available at the end of the 1970s and production was discontinued. In recent years, methane is gaining interest as a substrate for the producing of microbial biomass. UniBio A/S (utilizing knowledge

gained by Dansk BioProtein A/S (Odense, Denmark)) has developed fermentation technology to convert natural gas to animal feed protein by using methanotrophic bacteria. UniBio A/S UniBio A/S uses a U-loop fermenter to achieve a productivity of 4 kg m^{-3} h^{-1}, producing UniProtein® with ~70% protein, which has been approved for use in animal feed. The U-loop fermenter is designed to enhance mass transfer rates of methane from the gas to the liquid phase, making more methane available for the biomass. Another industrial producer using methane is Calysta Inc. (California, USA). It recently opened a production facility for their product, FeedKind®, in the UK in 2016 and is partnering with Cargill (Minnesota, USA) to build a larger production facility in the USA. The fact that natural gas is used means that ultimately such a production pathway does not provide a long-term sustainable solution. However, the use of methane generated in digesters at WWTPs seems a promising route that warrants further exploration. Clearly, in recent years, research and development around microbial protein is regaining momentum both in the scientific and industrial domains. The steep increase in the prices of fishmeal (from about €500 per ton in the 1990s to €1500–2500 in recent years), together with the environmental pressure of soybean production on land and water use in the tropical areas of the globe, justify the examination of the production of microbial protein in general and, in particular, in combination with using recovered nitrogen as a more sustainable and climate independent alternative to fishmeal. Indeed, at present, there are several ongoing initiatives at pilot and demonstration, which are described in more detail below.

9.3.4.2 Microbial protein production from used water

There are two main lines of emerging concepts that produce microbial protein from water containing organic waste materials enriched in respect to nitrogen:

(1) The direct assimilation of inorganic nitrogen into microbial protein and its on-site subsequent use as feed in aquaculture ponds, so-called the Biofloc technology (Avnimelech, 2015) or the ValPromic concept (http://avecom.be/product/promic-microbial-protein).
(2) The selective stripping of ammonia from the water and upgrading the latter to microbial biomass in a separate reactor. The latter, so-called power-to-protein, is a recent initiative for production of microbial protein from domestic wastewater (https://www.powertoprotein.eu/).

Biofloc technology – Water quality control and direct assimilation of nitrogen in aquaculture. Intensive, high-density fish (shrimp, other animals) production leads to the release of high concentrations of waste products (i.e., feed residues, fish excretions and dead algae). Unlike terrestrial animals, aquatic animals are totally immersed in this waste stream that often contains toxic components, a situation leading to the collapse of the system. One means to solve this situation included the replacement of the used water by fresh clean water. However, this solution is presently not acceptable due to the waste of clean water, introduction of pathogens and environmental pollution by the disposed water. The biofloc technology, in a way, can be seen as the ultimate circular nitrogen approach (Figure 9.1). This technology is based on a minimal to zero water exchange which leads to the build-up of organic substrates in the water and intensive development of microbial population (10^6–10^9 cells/mL). This population develops in a way that degrades and metabolizes the organic matter, including deleterious organic metabolytes (Avnimelech, 2015). Under aerobic conditions, approximately 50% of organic carbon metabolyzed is converted to CO_2 and eventually dissipated to the atmosphere. However, most of the mineralized nitrogen accumulate in the water, since under conditions existing in the pond, only a small portion of this nitrogen is converted into volatile nitrogenous species and released to the atmosphere. A large percentage of the excess nitrogen left in the water is made of ammonium, a highly toxic component to animals in the pond, nitrite, another toxic component, and nitrate. The biofloc process further deals with the use of the excess nitrogen to produce microbial proteins. The inorganic nitrogen excreted by the fish (or, e.g., shrimps) are directly assimilated into microbial protein by heterotrophic bacteria and are being used in-situ as protein rich feed for the fish, thereby creating a closed-loop system. This is controlled through the addition of carbohydrates (molasses, flour of

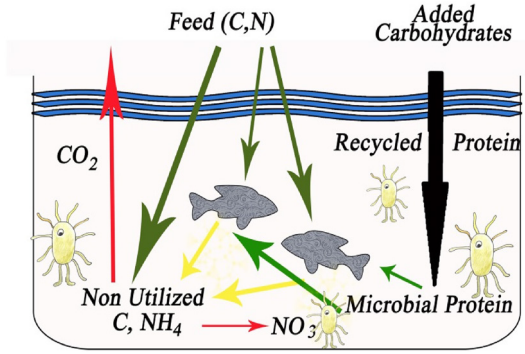

Figure 9.1 Schematic overview of the working principle of the biofloc technology.

wheat, rice or corn, and others) to the water. About 20 g carbohydrate are needed for the assimilation of 1 g nitrogen in protein (about 6.25 g carbohydrate to produce 1 g protein) (Avnimelech, 1999). The carbohydrates can be added separately to conventional feed provided to the pond (traditionally in the range of 30–50% protein), or by using a feed containing less protein. It is possible to reliably control the level of inorganic nitrogen through the control of the C/N ratio of feed and feed additives added to the pond (normally to a C/N ratio of 15–20). The microbial protein produced, present in the water in the form of bioflocs that can be easily harvested, serves as a good source of protein to important commercially-grown fishes (tilapia, carps, catfish) and shrimps. Using ^{15}N as a tracer to the microbial protein, it was found that tilapia and shrimp can consume up to 50 and 25% of their protein requirement, respectively (Avnimelech & Kochba, 2009; Burford *et al.*, 2004). This can be an important means to reduce production costs to the farmers, by saving both protein and feed amounts.

The assimilation of nitrogen into microbial protein, essentially representing a recycling of non-utilized feed protein, are being practiced in a commercial scale in ponds of varying sizes (ranging from 100 to ~10 000 m^2). The present development of biofloc technology systems all over the world may contribute to very significant nitrogen recycling in aquatic feed production (Avnimelech, 2015).

ValPromic Technology. The promic concept, a patented concept by Avecom (Ghent, Belgium) (http://avecom.be/product/promic-microbial-protein), is based on direct assimilation of nitrogen (and phosphate) present in the wastewater by a mixed culture of heterotrophic bacteria (Figure 9.2). A key difference with the biofloc concept is that wastewater from the food and beverage industry is treated without the opportunity to directly use the produced microbial protein on-site. As such, as explained in more detail in section 3.3, it requires a dewatering, drying and sterilization step. Moreover, it requires the use of a fermentation reactor, further adding complexity and capital investment. By the end of 2018, the first full-size demonstration plant of Valpromic is expected to commence operation at a total capacity of about 5000 ton microbial protein per year from potato process water (see Figure 9.3).

Power-to-Protein. For treatment of domestic wastewater, a very interesting route for upgrading recovered nitrogen is to combine it with hydrogen and oxygen generated by water electrolysis and with CO_2 from biogas, the so-called power to protein concept (https://www.powertoprotein.eu/). A schematic overview of the concept is provided in the figure below.

The key difference is the need for a 'bullet-proof' barrier between the domestic wastewater (and associated pathogens), from which the reactive nitrogen is recovered, and the microbial production step. The latter can be achieved by ammonia stripping, and in some instances one could even add a membrane step to further increase regulator and consumer confidence. The organics present in the wastewater are used to produce biogas. The produced biogas can subsequently be used to produce a clean hydrogen gas streams by means of a Combined Cooling Heating and Power unit (CCHP) fed to the fermentation reactor. As the amount of hydrogen gas produced in this way at best can only account

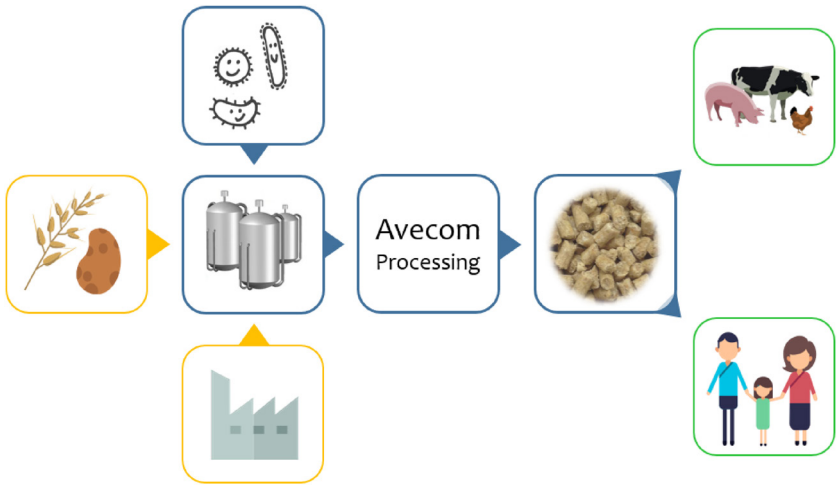

Figure 9.2 Schematic representation of Valpromic (Source: Personal communication Avecom).

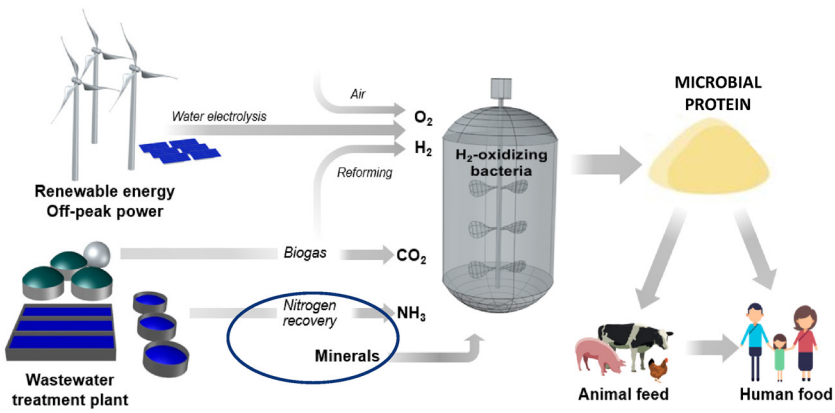

Figure 9.3 Schematic representation of the production of microbial protein using hydrogenotrophic bacteria from domestic wastewater using recovered nitrogen.

for some 12% of the total hydrogen requirements, in order to upgrade all the nitrogen that theoretically can be recovered from the wastewater, additional hydrogen is produced by means of water electrolysis driven by renewable energy. The upgrading of stripped nitrogen is currently at pilot scale explored by the association of KWR (Nieuwegein, The Netherlands) and Avecom (Ghent, Belgium) (https://www.kwrwater.nl/en/projecten/power-to-protein-pilot-phase-2/). Clearly this domain has quite a way to go before it becomes of comparative quantitative significance.

9.4 CHALLENGES, OPPORTUNITIES AND RESEARCH NEEDS

Processes to recover nitrogen from wastewater are not new at all. In fact, several mature technologies with a proven track record have been available on the market for several decades, such as struvite precipitation (http://ostara.com/nutrient-management-solutions/) and ammonia stripping (http://

www.nijhuisindustries.com). However, the economic competitiveness of such technologies are restricted to higher ammonium concentrations such as side-stream process treating digestate. Technologies that can recover reactive nitrogen from domestic wastewater directly from the mainstream at an economic competitive price are not available yet. Moreover, it is evident that technologies to recover nitrogen should always have energy requirements that are lower than the energy requirement to generate the reactive nitrogen via the Haber–Bosch process. The latter, however, is in most circumstances not the case with the energy consumption of current and emerging concepts being higher compared to the Haber–Bosch process (Matassa *et al.*, 2015a). As mentioned before, production of microbial protein from wastewater free of fecal matter does not require a separation step and the challenges are mainly related to the microbial production step, which is described in more detail below.

9.4.1 The microbial production step

Two aspects deserve special attention, namely: (i) the management of the functional microbiome; and (ii) the harvesting and downstream processing of the biomass.

The management of the functional microbiome. With respect to the management of the functional microbiome, the following aspects are considered important (Pikaar *et al.*, 2017b):

- *Steering the process towards creating suitable microbiomes*: the key feature in producing microbial protein from recovered nitrogen from wastewater is the use of proper microbiomes, in contrast to using 'pure' cultures as practiced in industrial biotechnology. While pure cultures are ideal from a product stability and quality point of view, such conditions are very hard to maintain. Therefore, it is important to gain a fundamental understanding about the dynamics and relative abundances of the different species within the microbiome.
- *Overcoming the process limitations of microbiomes*: the disadvantages of working with microbiomes and recovered nitrogen that originates from wastewater are considerable and must be addressed with an open mindset. First, the operator must create a set of conditions in terms of pH, dissolved oxygen, temperature, hydraulic and cell residence time, and specific biomass loading rate so that the microbiome achieves good conversion yields.
- *Creating a stable end-product of high quality*: it is of crucial importance that the end-product, that is the microbial biomass which is harvested, has a constant composition in terms of microbial protein content, crude amino acid composition and quality (i.e., no unwanted microbes present). Albeit the microbiome does not have to be homogenous and constant in terms of microbial composition, the overall quality of the end-product in terms of parameters such as percentage of protein, digestibility, amino acid composition and nucleic acid content has to be stable. At present, there is a lack of fundamental understanding on how to steer the microbiome in time so that the mixed culture aerobic fermentation reactors can operate in such a stable way that the overall quality of the end-product is stable. Obviously, this quality also implies that the final end-product microbiome should have metabolites (e.g., colour, odour, taste) that are attractive to the consumer, yet free of allergens at all times. Moreover, at all times, the microbiome needs to contain only species that comply with the status of GRAS (generally regarded as safe). Normally, the regulator will impose a set of detection methods for unwanted microorganisms, which are based on specific targeting by plate cultivation or quantitative PCR. For more insight, in-depth genomic analysis is also possible. However, currently, there is very little knowledge on how to install advanced and rapid monitoring of such dynamic microbiomes.

9.4.2 The harvesting of the microbial produced microbial cells

Microbial biomass needs optimal growth conditions for rapid growth, that is doubling times of hours to a couple of days. Only under such conditions is the cellular biomass very rich in digestible protein. The matrix in which the microbes are suspended has to have a low density and salt content

(i.e., conductivity of 10–25 mS/cm). This results in quite dilute suspensions in the order of 5–10 kg CDM per m^3. Therefore, the cells have to concentrate with a factor of at least 10 and the water that is separated should be of such quality that it can be re-used or discharged at very low cost. Clearly, the separation is a critical factor and requires considerable development, for instance applying food grade flocculants can improve this step. Once the thick slurry is obtained, it can be further processed in several ways. It can be stabilized by decreasing the pH and used in liquid feeding. It can also be dried to a final product to be used for food, feed or as an organic fertilizer. Yet, the handling of the 5–10% slurry should be of such a nature that the material is not allowed to deteriorate in quality and it must furthermore preserve the quality of the proteinaceous compounds. Drying can cause the conversion of the proteins to Maillard products which can alter the taste, odour and digestibility. The major challenge is to remove the water at low costs. The use of techniques to rapidly and safely dry the harvested microbial biomass by using low value (waste) heat is an issue of critical importance.

9.4.3 Economic and environmental competitiveness

The aerobic fermentation process used to recover the nitrogen has to be evaluated against other processes in terms of economy and environmental impact. Normally, to remove 1 kg of reactive nitrogen from domestic wastewater by means of the conventional nitrification, the overall costs for aeration and electron donor to denitrify are of the order of €2–4 per kg reactive nitrogen. In the case of microbial production, it is clear that the overall costs will be substantially higher. Hence, the key feature for microbial protein to become a viable alternative in the future is to produce an end-product of high-quality that has a high market value similar to fish meal. Due to the microbial growth, 1 kg of nitrogen recovered from the wastewater becomes about 6.25 kg of microbial protein (or 8.9 kg CDM at a crude protein content of 70%).

Considering a WWTP with a capacity of 250 000 PE, this equals to almost 30 tons of microbial protein per day. This microbial biomass can subsequently enter the value chain. The value of food protein (100% active substance such as Quorn(TM)) is at present of the order of €3–30 per kg. That of feed protein in the form of fish meal is of the order of €1–2 Euro per kg. That of organic fertilizer is of the order of some €0.2 per kg. Hence, in the case of food and feed, upgrading of nitrogen could potentially be rewarding. However, as the costs for reactive nitrogen only represent a small fraction in the overall production costs for microbial protein, one needs to consider very carefully in which case the implementation of recovered nitrogen will be beneficial. Another key feature is of course that the microbial-based protein is indeed fully accepted in the market and can be valorized at such prices relative to other sources of quality protein. In the case of slow-release organic fertilizer, the overall economy appears negative. Yet, the use of slow-release organic fertilizer is expected to represent environmental benefits compared to the use of inorganic fertilizer such as a reduction in nitrogen emissions, increased soil organic carbon content and water holding capacity. In case the microbial is used as animal feed, it is expected to come with several environmental benefits, reduction in greenhouse gas emissions due to land use change (LULUC), pesticides, water use, soil erosion, nitrogen and phosphorus pollution (Pikaar *et al.*, 2017a). Life Cycle Analysis allows to scout for these potential unexpected environmental impacts.

9.5 CHAPTER SUMMARY

In this chapter, we first described the environmental limitations of nitrogen dissipation pathways in wastewater management. Subsequently, we indicated the need to recover the ammonium from wastewater in its reactive form. In particular, we have examined the opportunities to recover ammonium from industrial and domestic wastewater streams and to upgrade it to microbial biomass, rich in protein (i.e., microbial protein) through the implementation of an aerobic fermentation process. The microbial biomass generated in such aerobic conversion can, depending on the quality,

be used as a protein rich supplement in livestock husbandry as well as aquaculture or as slow-release organic fertilizer. Such forms of upgraded mineral N have can help to alleviate the global nitrogen pollution burden. It should be noted that provided the microbial protein is produced from clean food grade inputs, it can be used as a meat replacement in human consumption, similar to other microbial biomass products such as Quorn™. Clearly, such upgrade requires a careful dialogue with the consumer and regulator in order to create sufficient consumer confidence and regulatory acceptance.

There are different aerobic fermentation approaches that can be used using different types of microorganisms. In this chapter, we have described aerobic microbial fermentation using organotrophic, autotrophic bacteria, chemo-lithotrophic and anaerobic fermentation using photo-lithotrophic bacteria, all of which are relevant for various used water treatment applications. We have also discussed the key differences in terms of design criteria, operation and maintenance and challenges for each of these microbial protein production pathways. An important difference between the various approaches described is the wastewater origin in terms of presence or absence of fecal matter, pathogens and waterborne viruses. In case the wastewater matrix is considered 'clean', which is the case for specific process waters originating from the food and beverage industry, one can adopt direct assimilation approaches. In case of fecal contaminated wastewater like domestic waster, a strict barrier, for example via a gas phase, between the wastewater from which the reactive nitrogen is recovered and the microbial production step needs to be implemented. Finally, we have described the main challenges, opportunities and research needs to achieve a widespread implementation of microbial protein production from various process and used waters.

9.6 EXERCISES

Exercise 9.1: Consider the following stoichiometry for the oxidation of methane:

$$CH_4 + 2H_2O \rightarrow CO_2 + 8H^+ + 8e^-$$

Methane is a very stable molecule that provides little energy for cell growth; typically only 20% of available energy is utilized for cell growth. Develop overall mass balance equations for a process where methane is used as an electron donor for biomass production and anomia is used as a nitrogen source and a negligible decay rat. Note: assume biomass stoichiometry of $C_5H_7O_2$ N (s).

Exercise 9.2: Consider the example where bacteria grow using starch as an electron donor and ammonium as a nitrogen source. The cell yield is 0.5 in terms of electrons going respectively to energy (i.e., catabolism) and to cell synthesis (i.e., anabolism). Develop overall mass balance equations for this process. Given the mass balance equations, calculate the amount of starch needed in order to produce 10 tons per day of microbial protein (on a dry solids basis). Note: assume biomass stoichiometry of $C_5H_7O_2$ N (s).

Exercise 9.3: Consider the following:

(a) If the typical composition of hydrogenotrophic biomass is $CH_{1.74}O_{0.46}N_{0.19}$. Present the elemental compositions as mass fractions. If all nitrogen is present as protein, determine the protein content as a fraction of CDM.
(b) Hydrogen gas is to be generated using electrolysis at a rate of 20 kg H_2 gas per MWh electricity. If a 50 MW hydrolysis cell is used to generate feed for the production of protein biomass, determine the maximum rate of biomass production per hour for a cell yield of 0.3 kg CDM per kg COD-H_2.
(c) For the process in (b), determine the mass of nitrogen that must be supplied for complete uptake of H_2 gas.
(d) If (i) hydrogen at a rate of 20 kg H_2 gas per MWh electricity and nitrogen is produced using Haber–Bosch at an energy cost of 10 MWh/ton N and energy costs are €50/MWh, determine if this process is feasible when bulk microbial protein is valued at €1/kg CDM.

Exercise 9.4: Consider the following:

(a) The typical composition of PPB is given as $CH_{1.8}O_{0.38}N_{0.18}$. Write a balanced chemical equation for the oxidation of PPB and use this equation to determine the COD to CDM ratio of PPB.
(b) If the typical composition of purple phototrophic biomass is $CH_{1.8}N_{0.18}O_{0.38}P_{0.02}$. Present the elemental compositions as mass fractions. If all nitrogen is present as protein, determine the protein content as a fraction of CDM.
(c) Research shows that a photobioreactor with a light intensity of 50 W m^{-2} will produce purple phototrophic bacteria at an average areal productivity of 20 g CDM/m^2.d. Estimate the illuminated surface of the photobioreactor required to produce enough protein to sustain 600 average people (assume 70 kg/person). What is the energy requirement to light this reactor?

Exercise 9.5: Assume that 1 kg of organic matter dry weight represents approximately 1 kg chemical oxygen demand (COD) (in reality this differs somewhat depending on the type of organics, e.g., fats or sugars), which represents the amount of oxygen needed to oxidize the organic matter present. Generally 1 kg of COD, such as sugar or starch, when consumed by rapidly growing microbial cells, gives rise to 0.4 kg CDM. The latter, when consisting out of young cells (1–5 days cell residence time), contains approximately 70% protein. Considering the above, how much carbohydrates are required to tie up 1 kg of reactive nitrogen in microbial biomass.

Exercise 9.6: Assume that 1 kg of organic matter dry weight represents approximately 1 kg COD (in reality this differs somewhat depending on the type of organics, e.g., fats, acetate or sugars), which represents the amount of oxygen needed to oxidize the organic matter present. Considering that 1 kg of acetate gives rise to 0.66 kg CDM using PPB. The latter contains approximately 70% protein. Considering the above, how much acetate is required to tie up 1 kg of reactive nitrogen in microbial biomass using PPB.

Exercise 9.7: A soft drink manufacturing process produces 0.5 ML/day of concentrated industrial wastewater with the composition shown in the table below:

Component	Concentration
COD	120 g/L
VFA	70 g/L
Alcohols	20 g/L
TKN	94 mg/L
NH_4-N	30 mg/L
Total P	104 mg/L
PO_4-P	92 mg/L

(a) If no external nitrogen is added to the wastewater (i.e., only nitrogen in the wastewater can be used for growth), calculate: (i) the maximum rate of biomass production; and (ii) the maximum rate of protein production that can be generated each day using fast growing organotrophic biomass (cell yield of 0.38 kg CDM per kg COD and protein content of 70%).
(b) Estimate the dose rate of external nitrogen that must be supplied in order to convert all COD in the wastewater into fast growing organ tropic biomass. Calculate the biomass production under these conditions.
(c) If no external nitrogen is added to the wastewater, calculate: (i) the maximum rate of biomass production; and (ii) the maximum rate of protein production that can be generated each day using purple phototrophic biomass (cell yield of 0.62 kg CDM per kg COD and protein content of 70%).
(d) Estimate the dose rate of external nitrogen that must be supplied in order to convert all COD in the wastewater into PPB. Calculate the biomass production under these conditions.

Exercise 9.8: A meat processing plant produces 3 ML/day of industrial wastewater with an average daily composition shown in the table below:

Component	Concentration
COD	5500 mg/L
VFA	1200 g/L
TKN	460 mg/L
NH_4-N	230 mg/L
TP	120 mg/L
PO_4-P	80 mg/L

(a) If wastewater discharge costs are €1.0/kg COD, €2.0/kg N and €2.5/kg P, calculate the discharge costs without wastewater treatment.
(b) If the wastewater is to be treated using PPB, calculate: (i) the maximum rate of biomass production; and (ii) the maximum rate of protein production that can be generated each day (cell yield of 0.62 kg CDM per kg COD and protein content of 70%).
(c) Using a biomass composition of $CH_{1.8}N_{0.18}O_{0.38}P_{0.02}$, assume complete conversion of the limiting component and estimate the COD, N and P composition of treated wastewater after production and harvesting of PPB.
(d) Using an areal productivity of 20 g CDM/m^2.d, calculate the required photobioreactor area for this process.
(e) Initial cost benefit assessments estimate the capital costs for the PPB process at €40/m^2, biomass production costs are estimated at €1/kg CDM and costs to harvest and process the biomass are estimated at €2/kg CDM. The biomass product is valued at €2/kg CDM. If the plant life is 20 years, and the hurdle return on investment is 15%, determine the preliminary economic feasibility of the process.

Exercise 9.9: The wastewater generated in a potato factory has the following typical composition: 10 g/L of starch, NH_4-N = 0.5 g/L and ortho-P = 0.1 g/L. This water, subjected to inoculation with a proper seed culture and aeration in a reactor will in a time period of 2–3 days convert the starch, ammonium and phosphate present in the water to microbial biomass. Actually, some 4 g CDM per L will be obtained (yield: 0.4 kg CDM per kg starch converted). Assume a starch removal efficiency of the process of 96%. Considering the above-described COD/N/P ratio, yield and typical biomass composition of young cells, calculate the expected effluent concentrations in terms of N and P that can be achieved through assimilation of the N and P into microbial biomass.

9.7 DISCUSSION QUESTIONS

Question 9.1: What is the large scale potential of microbial protein? (palm oil industry): Consider the case for which palm oil waste is upgraded by adding recovered ammonium sulphate N. The latter is obtained at zero cost because it is the result of a stripping process of gases and the level of the nitrogen in the recovered product is variable which makes it difficult to directly apply as a crop fertilizer. Consider that the reactor operates at a loading rate of 10 kg COD per m^3 reactor per day. Also take into account that the palm oil waste has a tipping fee of €100 per ton. What is the cost of microbial biomass-based fertilizer under conditions where downstream processing of the biomass (separation/drying) can be considered to be covered by heat generated by other processes within the factory.

Question 9.2: The public acceptance of microbial protein from recovered nitrogen? (public acceptance, cultural differences): Recovered nitrogen will with some part of the public raise questions in terms of acceptability, depending on its final use, food, animal food or slow release fertilizer. To

convince the consumer, discuss the following number of other processes in which used nitrogen is recycled in the food chain. Examples are crop fertilized with manure or compost, mushrooms grown on chicken manure, the biofloc technology used to upgrade fish feces to microbial protein, for example.

Question 9.3: Worth the trouble? (sustainability on a planetary level). Of the total amount of nitrogen entering the biosphere (soil/water) every year by means of the Haber–Bosch process, percentage wise only a relative small fraction ultimately ends up in domestic wastewater. We can develop technology to recover this amount and upgrade it into a valuable protein source or slow release fertilizer, but considering the complexity of this concept compared to the conventional activated sludge process and/or recent emerging technologies, such as anammox and Nereda, is it worthwhile to invest in creating such a paradigm shift in urban wastewater management? In your discussion, take into account that worldwide a substantial part of the wastewater is discharged untreated back into the environment. Should we invest in those problems first?

Question 9.4: Worth the trouble of taking such a risk? (utility management, technology): As the innovation manager of a large water utility, you are in charge of reorganizing the existing water infrastructure from its current situation to a more circular approach within a timeframe of 15 years. You have heard of the production of microbial protein using recovered nitrogen (as schematized in Figure 9.1). You are enthusiastic about such an approach but you are wondering why you would take such a risk? Your job is to ensure that the wastewater is treated to below discharge limits, thereby protecting the environment and the community, which is difficult enough. You are asked to give a presentation to the board of directors in which you evaluate the current status and justify your masterplan. Would you focus on the microbial production as the key innovative process or would you focus on less break-through technology concepts. What are your key considerations/motivations with respect to your decision?

Question 9.5: What is the economic potential (economics, market potential): As described in this chapter, a key factor governing production performance is the rate by which the bacteria consume the electron donor and use it to grow. The microbiome must at all instances assure a good rate of conversion because the capital investment on the hardware is quite high. If, for instance, a reactor costs in capital investment some €5000 per m^3 installed reactor capacity, and it generates some 10 kg of end-product (microbial protein cell dry weight) per day at a gross margin of €1 per kg protein, then the payback for the capex at 220 production days per year is in the order of 2200: $(220 \times 10 \times 1) = 2.3$ year. Industrial investors often prefer payback periods of that order or less than that. Note that this is a rough estimate, not taking into account the operational cost and also assuming a very high gross margin per kg protein produced. Assume you are the business developer of an international company in the food and beverage industry that wants to improve the sustainability of their wastewater facilities worldwide. Evaluate the economic potential and practical feasibility of the full-scale implementation of: (i) organotrophic production; (ii) photo-lithotrophic; or (iii) chemo-lithotrophic microorganisms. Clearly indicate the key design criteria, the limitations of each of the routes, and estimate the expected operational costs.

FURTHER READING

Hülsen T., Barry E. M. and Lu Y. (2016). Low temperature treatment of domestic wastewater by purple phototrophic bacteria: performance, activity, and community. *Water Research*, **100**, 537–545.

Liu B., Song J. and Li Y. (2013). Towards industrially feasible treatment of potato starch processing waste by mixed cultures. *Applied Biochemistry and Biotechnology*, **171**(4), 1001–1010.

Matassa S., Batstone D. J. and Hülsen T. (2015a). Can direct conversion of used nitrogen to new feed and protein help feed the world? *Environmental Science & Technology*, **49**(9), 5247–5254.

Matassa S., Boon N. and Verstraete, W. (2015b). Resource recovery from used water: the manufacturing abilities of hydrogen-oxidizing bacteria. *Water Research*, **68**, 467–478.

Matassa S., Boon N. and Pikaar I. (2016a). Microbial protein: future sustainable food supply route with low environmental footprint. *Microbial Biotechnology*, **9**(5), 568–575.

Matassa S., Verstraete W. and Pikaar I. (2016b). Autotrophic nitrogen assimilation and carbon capture for microbial protein production by a novel enrichment of hydrogen-oxidizing bacteria. *Water Research*, **101**, 137–146.

Pikaar I., Matassa S. and Rabaey K. (2017a). Microbes and the next nitrogen revolution. *Environmental Science & Technology*, **51**(13), 7297–7303.

Pikaar I., Matassa S. and Rabaey K. (2017b). *The Urgent Need to Re-Engineer Nitrogen-Efficient Food Production for the Planet*. DNC2017 Position Paper. UNU-FLORES, Dresden.

Ritala A., Häkkinen S. T., Toivari M. and Wiebe M. G. (2017). Single cell protein-state-of-the-art, industrial landscape and patents 2001–2016. *Frontiers in Microbiology*, **8**, 1–18.

Verstraete W., Clauwaert P. and Vlaeminck S. E. (2016). Used water and nutrients: Recovery perspectives in a 'panta rhei' context. *Bioresource Technology*, **215**, 199–208.

REFERENCES

Adessi A. and De Philippis R. (2014). Photobioreactor design and illumination systems for H2 production with anoxygenic photosynthetic bacteria: a review. *International Journal of Hydrogen Energy*, **39**(7), 3127–3141.

Ahmad A. L., Sumathi S. and Hameed B. H. (2006). Coagulation of residue oil and suspended solid in palm oil mill effluent by chitosan, alum and PAC. *Chemical Engineering Journal*, **118**(1–2), 99–105.

Amuda O. S. and Amoo I. A. (2007). Coagulation/flocculation process and sludge conditioning in beverage industrial wastewater treatment. *Journal of Hazardous Materials*, **141**(3), 778–783.

Anupama and Ravindra P. (2000). Value-added food: single cell protein. *Biotechnology Advances*, **18**(6), 459–479.

Arbeli Z., Brenner A. and Abeliovich A. (2006). Treatment of high-strength dairy wastewater in an anaerobic deep reservoir: analysis of the methanogenic fermentation pathway and the rate-limiting step. *Water Research*, **40**(19), 3653–3659.

Avnimelech Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, **176**(3–4), 227–235.

Avnimelech Y. (2015). *Biofloc Technology: A Practical Guidebook*. World Aquaculture Society, Baton Rouge, LA, USA.

Avnimelech Y. and Kochba M. (2009). Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using 15N tracing. *Aquaculture*, **287**(1–2), 163–168.

Azbar N., Bayram A., Filibeli A., Muezzinoglu A., Sengul F. and Ozer A. (2004). A review of waste management options in olive oil production. *Critical Reviews in Environmental Science and Technology*, **34**(3), 209–247.

Bodirsky B. L., Popp A., Lotze-Campen H., Dietrich J. P., Rolinski S., Weindl I., Schmitz C., Müller C., Bonsch M., Humpenöder F., Biewald A. and Stevanovic M. (2014). Reactive nitrogen requirements to feed the world in 2050 and potential to mitigate nitrogen pollution. *Nature Communications*, **5**, 1–7.

Bodirsky B. L., Rolinski S., Biewald A., Weindl I., Popp A. and Lotze-Campen H. (2015). Global food demand scenarios for the 21th century. *PLoS ONE*, **10**(11), 1–27.

Burford M. A., Thompson P. J., McIntosh R. P., Bauman R. H. and Pearson D. C. (2004). The contribution of flocculated material to shrimp (litopenaeus vannamei) nutrition in a high-intensity, zero-exchange system. *Aquaculture*, **232**(1–4), 525–537.

Chen X. D. and Mujumdar A. S. (2009). *Drying Technologies in Food Processing*. Wiley, Hoboken.

Driessen W. and Vereijken T. (2003). *Recent Developments in Biological Treatment of Brewery Effluent*. Livingstone, Zambia.

Erisman J. W., Sutton M. A., Galloway J., Klimont Z. and Winiwarter W. (2008). How a century of ammonia synthesis changed the world. *Nature Geoscience*, **1**(10), 636–639.

Erisman J. W., Galloway J. N., Seitzinger S., Bleeker A., Dise N. B., Petrescu A. M., Leach A. M. and de Vries W. (2013). Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **368**(1621), 1–9.

Galloway J. N. and Cowling E. B. (2002). Reactive nitrogen and the world: 200 years of change. *Ambio*, **31**(2), 64–71.

Galloway J. N. and Leach A. M. (2016). Your feet's too big. *Nature Geoscience*, **9**, 97–98.

Galloway J. N., Aber J. D., Erisman J. W., Seitzinger S. P., Howarth R. W., Cowling E. B. and Cosby B. J. (2003). The nitrogen cascade. *BioScience*, **53**(4), 341–356.

Godfray H. C. J., Beddington J. R., Crute I. R., Haddad L., Lawrence D., Muir J. F., Pretty J., Robinson S., Thomas S. M. and Toulmin C. (2010). Food security: the challenge of feeding 9 billion people. *Science*, **327**(5967), 812–818.

Güven G., Perendeci A. and Tanyolaç A. (2009). Electrochemical treatment of simulated beet sugar factory wastewater. *Chemical Engineering Journal*, **151**(1–3), 149–159.

Hülsen T., Hsieh K., Tait S., Barry E. M., Puyol D. and Batstone D. J. (2018). White and infrared light continuous photobioreactors for resource recovery from poultry processing wastewater – a comparison. *Water Research*, **144**, 665–676.

Ince O. (1996). Performance of a two-phase anaerobic digestion system when treating dairy wastewater. *Water Research*, **32**(9), 2707–2713.

Ishizaki A. and Tanaka K. (1990). Batch culture of Alcaligenes eutrophus ATCC 17697T using recycled gas closed circuit culture system. *Journal of Fermentation and Bioengineering*, **69**(3), 170–174.

Jenkins D. and Wanner J. (2014). *Activated Sludge – 100 Years and Counting*. IWA Publishing, London, UK.

Kartal B., Kuenen J. G. and Van Loosdrecht M. C. M. (2010). Sewage treatment with anammox. *Science*, **328**(5979), 702–703.

Kasapgil B., Anderson G. K. and Ince O. (1994). An investigation into the pre-treatment of dairy wastewater prior to aerobic biological treatment. *Water Science and Technology*, **29**(9), 205–212.

Koyuncu I., Turan M., Topacik D. and Ates A. (2000). Application of low pressure nanofiltration membranes for the recovery and reuse of dairy industry effluents. *Water Science and Technology*, **41**(1), 213–221.

Matassa S., Batstone D. J., Huelsen T., Schnoor J. L. and Verstraete W. (2015a). Can direct conversion of used nitrogen to new feed and protein help feed the world? *Environmental Science & Technology*, **49**(9), 5247–5254.

Matassa S., Boon N. and Verstraete W. (2015b). Resource recovery from used water: the manufacturing abilities of hydrogen-oxidizing bacteria. *Water Research*, **68**, 467–478.

Mohana S., Acharya B. K. and Madamwar D. (2009). Distillery spent wash: treatment technologies and potential applications. *Journal of Hazardous Materials*, **163**(1), 12–25.

Molina Grima E., Belarbi E. H., Acién Fernández F. G., Robles Medina A. and Chisti Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances*, **20**(7–8), 491–515.

Monroy O. H., Vazquez M. F., Derramadero J. C. and Guyot J. P. (1996). Anaerobic-aerobic treatment of cheese wastewater with national technology in Mexico: the case of 'El Sauz'. *Water Science and Technology*, **32**(12), 149–156.

Niazi S. K. and Brown J. L. (2015). *Fundamentals of Modern Bioprocessing*. CRC Press, Boca Raton, Florida, USA.

Overmann J. and Garcia-Pichel F. (1998). The phototrophic way of life. In: *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt & F. Thompson (eds), Springer, New York, pp. 203–257.

Park D. H.-D., Chang I.-S. and Lee K.-W. (2015). *Principles of Membrane Bioreactors for Wastewater Treatment*. CRC Press, Boca Raton, Florida, USA.

Pikaar I., Matassa S., Rabaey K., Bodirsky B. L., Popp A., Herrero M. and Verstraete W. (2017a). Microbes and the next nitrogen revolution. *Environmental Science & Technology*, **51**(13), 7297–7303.

Pikaar I., Matassa S., Rabaey K., Laycock B., Boon N. and Verstraete W. (2017b). *The Urgent Need to Re-Engineer Nitrogen-Efficient Food Production for the Planet*. DNC2017 Position Paper. UNU-FLORES, Dresden.

Posten C. (2009). Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences*, **9**(3), 165–177.

Pronk M., Giesen A., Thompson A., Robertson S. and Van Loosdrecht M. (2017). Aerobic granular biomass technology: advancements in design, applications and further developments. *Water Practice and Technology*, **12**(4), 987–996.

Rittmann B. E. and MacCarty P. L. (2001). *Environmental Biotechnology: Principles and Applications*. McGraw-Hill, Boston, Mass.

Schwarzenbeck N., Borges J. M. and Wilderer P. A. (2005). Treatment of dairy effluents in an aerobic granular sludge sequencing batch reactor. *Applied Microbiology and Biotechnology*, **66**(6), 711–718.

Sierra J., Martı E., Montserrat G., Cruanas R. and Garau M. A. (2001). Characterization and evolution of a soil affected by olive oil mill wastewater disposal. *The Science of the Total Environment*, **279**, 207–214.

Suhaimi M., Liessens J. and Verstraete W. (1987). NH+/4-N assimilation by Rhodobacter capsulatus ATCC 23782 grown axenically and non-axenically in N and C rich media. *Journal of Applied Microbiology*, **62**(1), 53–64.

Tabita F. R. (1995). The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. In: *Anoxygenic Photosynthetic Bacteria*, R. Blankenship, M. Madigan and C. Bauer (eds), Springer, the Netherlands, pp. 885–914.

United Nations. (2015). *World Population Prospects: The 2015 Revision, Key Findings and Advance Tables*. Department of Economic and Social Affairs, The United Nations, New York.

Van Gemerden H. (1968). Utilization of reducing power in growing cultures of Chromatium. *Archiv für Mikrobiologie*, **64**(2), 111–117.

Van Haandel A. and Van Der Lubbe J. (2012). *Handbook of Biological Wastewater Treatment*. IWA Publishing, London, UK.

Vlissidis A. and Zouboulis A. I. (1993). Thermophilic anaerobic digestion of alcohol distillery wastewaters. *Bioresource Technology*, **43**, 131–140.

World Health Organization. (2002). *Report of A Joint WHO/FAO/UNU Expert Consultation. Protein and Amino Acid Requirements in Human Nutrition*. United Nations, Geneva, Switzerland.