Applied Environmental Science and Engineering for a Sustainable Future

Zainul Akmar Zakaria
Ramaraj Boopathy
Julian Rafael Dib *Editors*

Valorisation of Agro-industrial Residues – Volume I: Biological Approaches

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Chapter 1
Dark Fermentation and Bioelectrochemical Systems for Enhanced Biohydrogen Production from Palm Oil Mill Effluent: Current Progress, Potentials, and Future Perspectives

Jemilatu Omuwa Audu, Eka Latiffah Nadia Dzulkarnain, Zaharah Ibrahim, Norahim Ibrahim, and Mohd Firdaus Abdul-Wahab

Abstract The global rise in demand for fats and oil has made the palm oil industry grown tremendously over the last decades in countries like Indonesia, Malaysia, and Thailand. Malaysian agro-industrial sector alone accounts for about 51% of the world's palm oil production and 62% of the world's export. The sector has generated billions of dollars in revenues, and tonnes of wastes too. Palm oil mill effluent (POME) is the most abundant waste generated during the crude oil extraction process. Efficient and effective POME treatment technologies are still being actively investigated. POME has great potential as a substrate for biohydrogen production due to the high content of degradable organic matter. Dark fermentation, among the various biological processes for biohydrogen production, is highly favored due to the lower cost and low energy requirement. However, achieving a high biohydrogen yield is the main challenge, due to the co-production of organic acids. Additional treatment steps using bioelectrochemical systems (BES), such as microbial

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electrolysis cell (MEC), can provide the much-needed solution. Enhanced biohydrogen production can potentially be achieved when dark fermentation is coupled with MEC, with better POME treatment. Microbial fuel cell (MFC) can provide additional treatment step, with simultaneous electricity generation. This chapter reviews the various dark fermentation technologies that have been employed in producing biohydrogen from POME, the methods employed to improve biohydrogen yield, the advancements in BES, and the potential integration with dark fermentation for enhanced biohydrogen production.

Keywords Biohydrogen · POME · Dark fermentation · Bioelectrochemical systems

1.1 Introduction

The demand for world's energy is on the increase, and this demand is projected to further increase in the next few decades. Based on the International Energy Outlook 2013 and 2018 reports, energy consumption worldwide is estimated to increase by about 56% between 2010 and 2040, from 524 to 820 quadrillion Btu (Singh et al. 2019). This has led to the search for renewable alternative energy sources. Research on energy production from biomass has been on the rise, in the pursuit to lessen the current dependency on fossil fuels, and to tackle the various environmental problems and health impacts associated with the use (Shahlan et al. 2017). Hydrogen at the moment is touted as the energy carrier of the future, with the highest energy content of 142 kJ/g of any known sources (Lu et al. 2012; Cardoso et al. 2014) and does not produce any greenhouse gases upon combustion (Singh et al. 2013a; Krishnan et al. 2016). The energy obtained from hydrogen can be used directly by direct combustion or used to produce electricity via fuel cells to power electric vehicles (Hames et al. 2018). However, current hydrogen production still relies heavily on thermochemical methods, with fossil fuel as the primary energy source (Kapdan and Kargi 2006; Azwar et al. 2014; Dhar et al. 2015). For hydrogen to fully replace conventional fuels, production has to be from environmentally friendly processes, e.g., via the use of various industrial and environmental wastes as raw material, and produced in large scale (Zhang et al. 2005; O-Thong et al. 2011). Production involving the use of microorganisms (fermentative bacteria, photosynthetic bacteria, cyanobacteria, and algae) has attracted a lot of attention over the last decade, as it requires less energy and potentially environmentally friendly too. At the same time, various organic wastes can be used as substrates (Demirbas 2008).

World's palm oil production is currently dominated by the Southeast Asian region, with countries like Malaysia, Indonesia, and Thailand as the major producers (Mukherjee and Sovacool 2014). Malaysia and Indonesia accounted for about 86% of the world's production in 2011. This industry serves as the key economic driver and the main element of their GDP (Iskandar et al. 2018). In Malaysia alone, the number of hectares of cultivated land increased from 3.79 million in 2003 to 5 million in 2011, resulting in a tremendous increase in the number of operating

mills to about 426 mills (Mohmmed and Chong 2014). The milling process involves the use of a large amount of water that generates a huge amount of wastewater discharge, known as palm oil mill effluent (POME). Malaysian palm oil industry produces tonnes of POME, which accounts for about 60% of the total waste produced. Most of the currently employed treatment systems are the anaerobic–aerobic ponding system and digestion systems for biogas production (mainly methane) (Nasution et al. 2014). Anaerobic digestion are highly favored among other technologies due to the numerous advantages associated with the process, such as less land requirement, reduced sludge production, and easier operation and maintenance (Rizvi et al. 2015). Biogas generated from the treatment process is used to run the daily operations of the mills, which can help decrease the dependency on petroleum fuels, and decrease the impact the palm oil processing has on the environment (Chotwattanasak and Puetpaiboon 2011). It is estimated that from 1 m^3 of POME, around 28 m^3 of biogas can be generated and a net income of MYR3.8 million can be obtained yearly from the electricity generation (Chin et al. 2013). However, the utilization of methane results in the emission of greenhouse gases. Thus, the search for a cleaner, more sustainable, and renewable energy carrier is still ongoing.

The use of POME as a substrate for biohydrogen production is currently under intense investigation, in order to maximize production, from various operational aspects, e.g., different fermentation methods (dark and photo-fermentation), inoculum source, operational temperature, pH, hydraulic retention time, loading rates, and bioreactor types. Commercialization of the process for large-scale production is still farfetched, due to the constraints of low production rate and yield, making it not yet feasible. The low biohydrogen rate is attributed to a number of factors, such as inoculum source, operational mode, bioreactor design, inhibitory substance, hydrogen concentration, soluble metabolites, and concentration of substrates (Dong et al. 2009; Bundhoo and Mohee 2016). This chapter provides a summary of biohydrogen production via dark fermentation using POME, the setbacks associated, and the approaches investigated toward improving biohydrogen production yield. The feasibility of integrating dark fermentation and other fermentation systems is also discussed, focusing on bioelectrochemical systems (BES), toward achieving higher energy recovery and higher pollutant removal. A brief discussion on the challenges facing hydrogen storage and transport is also included, together with the current advancements to address this problem.

1.2 POME as a Substrate for Biohydrogen Production

Malaysia has been the second largest global palm oil producer after Indonesia, which accounts for 39% production and 44% exports in 2016 (Sawe 2018). POME is categorized as extremely high strength wastewater containing an elevated amount of organic material, oil and greases, and suspended solids. It is a mixture of effluents produced and discharged mainly from sterilizer condensate, clarification wastewater,

and hydrocyclone wastewater during crude palm oil extraction process (Lam and Lee 2011). Considering its high content of biodegradable constituents, POME could potentially be the renewable resources for sustainable biohydrogen production through dark fermentation system (Ji et al. 2013). Raw POME is a nontoxic, thick voluminous brownish colloidal suspension of 95–96% water, 0.6–0.7% oil and grease, and 4–5% total solids containing 2–4% suspended solid (Ahmad et al. 2016; Wu et al. 2007). POME generally has a high chemical oxygen demand (COD) and biochemical oxygen demand (BOD), high oil and grease content, high discharge temperature and is acidic in nature with a distinct offensive odor (Ji et al. 2013; Ahmad et al. 2006a). Most of the suspended solids are from palm fruit mesocarp debris composed of a mixture of carbohydrates, ranging from hemicellulose to simple sugars, amino acids, nitrogenous compounds, free organic acids, cell walls, organelles, short fibers, and inorganic nutrients (K, Ca, Mg, Mn, Fe, Zn, Cu, and Co) (Santosa 2008; Ugoji 1997). POME also contains lignin (4700 ppm), phenolics (5800 ppm), pectin (3400 ppm), and carotene (8 ppm) (Sundram et al. 2003). Table 1.1 shows the compositions of POME and its multielement constituents. POME as a substrate for biohydrogen production is still under intense investigation due to its high organic content and its potential for microbial conversion under both mesophilic and thermophilic conditions, using different inoculum sources, reactor configurations, and operating conditions (Atiff et al. 2005; O-Thong et al. 2007, 2008; Chong et al. 2009a; Choi et al. 2013) as summarized in Table 1.2.

1.3 Challenges Associated with Dark Fermentation Process

Biohydrogen production via dark fermentation process is reported to have higher hydrogen production capacities, substrate versatility, low energy requirement, and easier operational conditions compared to other biological methods (Nath and Das 2004; Moreno et al. 2015). However, the feasibility is hindered by the maximum hydrogen yield which is still limited to 33% of the theoretical stoichiometric conversion of glucose (Gomez et al. 2011). Hydrogen yield differs with the fermentation pathway and end-product metabolites production (Dhar et al. 2015; Elbeshbishy et al. 2017), which include propionate, butyrate, lactate, formate, and solvents (butanol, acetone, and ethanol). Metabolite production decreases the overall hydrogen yield (Zong et al. 2009). The main route for producing hydrogen is the acetate–butyrate fermentation pathway of which theoretically, 4 mol of hydrogen can be produced when acetate is the main fermentation product and 2 mol when butyrate is generated, as shown in Eqs. (1.1) and (1.2).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2 \quad (1.1)$$

Table 1.1 POME compositions and the multielement constituents

Parameter	Unit	Range	References	Multielement constituents					
				Elements	Habib et al. (1997)	Wood et al. (1979)	Ahmad et al. (2006b)	Awotoye et al. (2011)	MPOB (2014)
					μg/g dry weight	mg/L			
Temperature	°C	50–90	O-Thong et al. (2007), Ahmad et al. (2011)	Aluminum, Al	16.60 ± 1.44				
pH	–	3.4–6.9	MPOB (2014), Aziz et al. (2019)	Arsenic, As	9.09 ± 0.65				
Biochemical oxygen demand (BOD)	mg/L	10,250–80,400	Ahmad et al. (2006b), Poh et al. (2010)	Boron, B	7.60 ± 0.60		7.6		
Chemical oxygen demand (COD)	mg/L	15,000–100,000	MPOB (2014)	Calcium, Ca	1650.09 ± 160.45	276–405	439	252.41	
Total solids (TS)	mg/L	11,500–79,000	MPOB (2014)	Chromium, Cr	4.02 ± 0.44	0.05–0.43			
Total suspended solids (TSS)	mg/L	5000–54,000	MPOB (2014)	Cobalt, Co	2.40 ± 0.35	0.04–0.06			
Total volatile solids (TVS)	mg/L	9000–72,000	MPOB (2014	Copper, Cu	10.76 ± 1.04	0.8–1.6	0.89		
Total nitrogen	mg/L	180–1400	MPOB (2014	Iron, Fe	11.08 ± 2.20	75–184	46.5	183.49	
Ammoniacal nitrogen	mg/L	4–290	MPOB (2014), Aziz et al. (2019)	Lithium, Li					
Total Kjeldahl nitrogen	mg/L	60–900	Poh et al. 2010, Loh et al. 2013	Magnesium, Mg	911.95 ± 95.50	254–344	615	283.46	345
Oil and grease	mg/L	130–18,000	MPOB (2014)	Manganese, Mn	38.81 ± 3.65	2.1–4.4	2.0	34.25	

(continued)

Table 1.1 (continued)

Parameter	Unit	Range	References	Multielement constituents					
				Elements	Habib et al. (1997)	Wood et al. (1979)	Ahmad et al. (2006b)	Awotoye et al. (2011)	MPOB (2014)
					µg/g dry weight	mg/L			
Total carbohydrates	mg/L	0.02–28,900	Wu et al. (2007), Norfadilah et al. (2016)	Molybdenum, Mo	6.45 ± 0.40				
Total organic carbon	mg/L	21,000–25,000	Poh et al. (2010), Ahmad et al. (2011)	Nickel, Ni	1.31 ± 0.30				
Reducing sugar	mg/L	0.11–1450	Ahmad et al. (2006b), Norfadilah et al. (2016)	Potassium, K	8951.55 ± 256.45	1281–1928	2270	295.74	1958
Total protein	mg/L	2830–12,900	Ahmad et al. (2006b), Wu et al. (2007)	Selenium, Se	12.32 ± 1.35				
Total lignin	mg/L	130–1740	Poh et al. (2010), Ahmad et al. (2011)	Silica, Si	10.50 ± 1.80				
Volatile fatty acids	mg/L	1900	Ahmad et al. (2011)	Sodium, Na					
Acetic acid	mg/L	471–3540	Poh et al. (2010), Poh and Chong (2014)	Sulfur, S	13.32 ± 1.45				
Color	ADMI	>500	Bello et al. (2013)	Tin, Sn	2.30 ± 0.30				
				Vanadium, V	0.12 ± 0.02				
				Zinc, Zn	17.58 ± 2.10	1.2–1.8	2.3	120.95	

Table 1.2 Summary of biohydrogen production from POME via dark fermentation reported in the literature

Inoculum	Reactor type	Inoculum pre-treatment	Operating conditions (pH, temperature)	Maximum H_2 production rate	Maximum H_2 yield	References
POME sludge	Serum bottles	Heated at 100 °C for 1 h	5.5, 50 °C	NA	28.47 mL H_2/g COD	Zainal et al. (2018)
POME digested sludge	UASB-FFR	Heated at 100 °C for 1 h	5.5, 38 °C	N/A	0.31 L H_2/g COD	Mohammadi et al. (2017)
POME pond sludge	UASB	Heated at 90 °C for 60 min	5.5, 55 °C	1.92 L H_2/L/day	N/A	Krishnan et al. (2016)
POME sludge	Serum bottle	N/A	5.5, 37 °C	N/A	5.988 ± 0.5 L H_2/L-medium	Norfadilah et al. (2016)
POME digested sludge	UASB-FFR	Heated at 100 °C for 1 h	5.5, 38 °C	0.514 L H_2/g VSS/day	N/A	Mohammadi et al. (2014)
ASBR sludge	Serum bottle	N/A	5.5, 60 °C	12.12 mmol H_2/L/h	2.53 mol H_2/mol hexose	Nitipan et al. (2014)
Granular sludge seed	UASB	Heated at 100 °C for 30 min	6.0, 37 °C	43.1 mL H_2/h	182.3 mL H_2/g COD	Pisutpaisal et al. (2014)
Clostridium sp. LS2	UASB-PEG	N/A	5.5, 37 °C	336 ml H_2/L/h	0.35 L H_2/g COD	Singh et al. (2013b)
C. butyricum EB6	UASB-PEG	NA	5.5, 37 °C	510 mL H_2/L-POME/h	NA	Singh et al. (2013a)
POME sludge	UASB-PEG	Heated at 80 °C for 50 min	5.5, 37 °C	0.632 L H_2/(L POME h)	N/A	Singh et al. (2013c)
Anaerobic sludge	Serum bottle	N/A	5.5, 60 °C	N/A	5.2 L H_2/L POME	Seengenyoung et al. (2013)
Anaerobic sludge	Serum bottle	Heated at 90–95 °C for 30 min	6.0, 55 °C	75.99 mL H_2/L/h	27.09 mL H_2/g COD	Fernandez et al. (2011)
C. butyricum	Serum bottle	N/A	7.0, 37 °C	N/A	2.18 mol H_2/mol TC	Fatehizadeh et al. (2018)

(continued)

Table 1.2 (continued)

Inoculum	Reactor type	Inoculum pre-treatment	Operating conditions (pH, temperature)	Maximum H_2 production rate	Maximum H_2 yield	References
Anaerobic sludge	Infusion bottle	NA	6.0, 55 °C	4820 mL H_2/L POME	243 mL H_2/g sugar	O-Thong et al. (2011)
POME digested sludge	CSTR	N/A	5.5, 55 °C	2.64 m^3/m^3/day	N/A	Pachapur et al. (2019)
POME sludge	ASBR	N/A	5.5, 60 °C	9.1 L H_2/L/day	0.27 L H_2/g COD	Prasertsan et al. (2009)
POME sludge	N/A	Heated at 70–100 °C for 10 min	5.5, 37 °C	1034 mL H_2/L/h	N/A	Chong et al. (2009a)
C. butyricum EB6	N/A	N/A	5.5, 37 °C	N/A	2.21 mol H_2/mol glucose	Chong et al. (2009b)
POME anaerobic sludge	ASBR	Heated at 100–105 °C for 2 h	5.5, 60 °C	N/A	6.33 ± 0.142 L H_2/L POME	O-Thong et al. (2008)
POME sludge	ASBR	Heated at 100–105 °C for 2 h	5.5, 60 °C	6.1 ± 0.03 L H_2/L	2.24 ± 0.03 mol H_2/mol hexose	O-Thong et al. (2007)
POME sludge	CSTR	N/A	5.5, 60 °C	N/A	4708 mL H_2/L POME	Atiff et al. (2005)

ASBR Anaerobic sludge bed reactor, *UASB* Upflow anaerobic sludge blanket reactor, *UASB-FFR* Upflow anaerobic sludge blanket-fixed film reactor, *CSTR* Continuously stirred tank reactor, *UFA* Upflow anaerobic, *UASB-PEG* Upflow anaerobic sludge blanket-polyethylene glycol reactor, *N/A* Not available

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + 2CH_3CH_2CH_2COOH + 2CO_2 \quad (1.2)$$

The microbial component involved in the biological conversion of substrates to biohydrogen is an integral part of the anaerobic fermentation process, performing various complex series of biochemical reactions. Bacteria with the ability to produce biohydrogen can be obtained from various environments such as soil, compost, wastewater sludge, sediment (Hu and Chen 2007; Mohammadi et al. 2011; Wang and Wan 2009). Biohydrogen production with mixed cultures was found to be easier, faster, more practical to operate and has the ability to degrade a wide range of substrate/mixed substrates compared to processes using pure cultures (Fernandez et al. 2011; Fatehizadeh et al. 2018). Hydrogen evolution rate and yields are higher with mixed cultures, as communities work in synergy using metabolites to ensure rapid degradation of complex substrates (Pachapur et al. 2019). Nonetheless, there are other communities of bacteria (i.e., hydrogen consumers) which naturally coexist with the hydrogen producers, such as the methanogens, nitrate-, and sulfate-reducing bacteria, lactate producers, propionate producers, homoacetogens, all of which adversely affects hydrogen yield (Bundhoo and Mohee 2016; Prasertsan et al. 2009).

Another aspect of concern is the accumulation of fermentation products, both hydrogen gas and volatile fatty acids (VFAs), which is detrimental to hydrogen yield. Continuous operating conditions associated with the removal of gaseous products and influent dilutions coupled with vigorous mixing and gas sparging has the potential to improve process efficiency (Kraemer and Bagley 2006; Gomez et al. 2009). Nguyen et al. (2010), established that the removal of accumulated hydrogen from the reactor headspace during batch fermentation resulted in an increase in hydrogen yield. However, accumulated VFAs were unaffected. Further conversion of these by-products to hydrogen requires the action of hydrogen-consuming microorganisms such as methanogens and sulfate-reducing bacteria to maintain low hydrogen pressure by consuming the hydrogen produced to form hydrogen sulfide or methane (Wang et al. 2011). However, this process also prevents additional hydrogen recovery. Several approaches have been employed to increase hydrogen yield in dark fermentation system, as described below.

1.4 Approaches to Improving Biohydrogen Yield Under Dark Fermentation Process

In order to overcome the drawbacks described above, strategies for improving biohydrogen production yield has focused on various aspects, such as bioreactor configuration and operation, the use of biohydrogen-producing microbial community, substrate preparation and recently, the integration of multiple systems (Krishnan et al. 2016; Chang et al. 2002; Hallenbeck et al. 2012; Kumar et al. 2016) (Fig. 1.1).

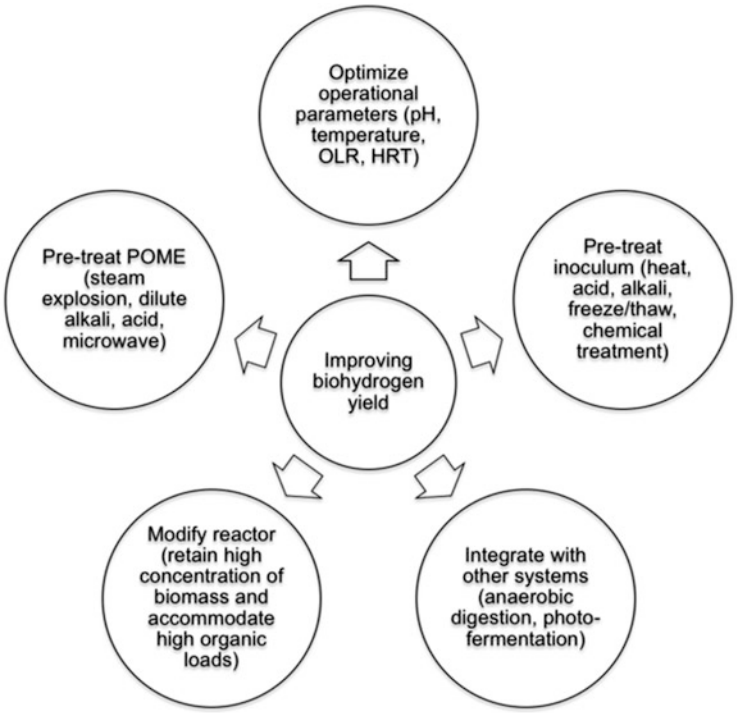

Fig. 1.1 The various strategies employed to improve biohydrogen yield under dark fermentation conditions

1.5 Operational Conditions

1.5.1 Reactor Design

Several anaerobic reactors with different configurations and capacities have been employed to carry out anaerobic digestion/anaerobic fermentation in the treatment of wastewater. Improvements in the reactor designs and configurations have been shown to improve biohydrogen production rate and yield. Continuous stirred tank reactor (CSTR) is the most commonly used reactor system. The reactor has a simple design equipped with an agitator system to enhance the area of contact with biomass (Arriaga et al. 2011). Microbial cells in CSTR are in suspension mode and tend to be sensitive to fluctuation in environmental conditions making it prone to biomass washout and process instability in a continuous operation (Lee et al. 2004; Mu and Yu 2006). Cell immobilization could improve biomass retention and biohydrogen yield (Mu et al. 2006). Cell immobilization matrix is generally made from various supporting materials (both synthetic and natural) and has the ability to create a local anaerobic environment, which is an important requirement for long-term biohydrogen production (Singh et al. 2013c). Higher tolerant to environmental

perturbation, biological activity, and reusability of immobilized cells as a result of higher cell density also makes it attractive (Seol et al. 2011). The use of immobilized cells has led to the development of other reactors such as the upflow anaerobic sludge blanket (UASB) reactor (Abbasi and Abbasi 2012). UASB is able to minimize biomass washout by retaining a high biomass concentration, with short hydraulic retention time (HRT), (Zinatizadeh et al. 2007; Khemkhao et al. 2011) and minimal sludge generation (Abbasi and Abbasi 2012). 69.3% increase in biohydrogen production rate was obtained using cell immobilization of anaerobic sludge using polyethylene glycol (PEG) operated in a UASB reactor, compared to the production using free suspended cells. A further 7% increase in production rate was also observed with the use of heat-treated inoculum (Singh et al. 2013c). Singh and Wahid (2015) investigated the addition of microelements in the immobilization process of *Clostridium* sp. LS2. The maximum biohydrogen production rate of 7.3 L/L POME/day and yield of 0.31 L H_2/g COD was obtained in a continuous operation. Choi et al. (2013) used settleable biomass aggregated and immobilized into granules of the sludge bed. Khemkhao et al. (2011) improved the sludge aggregation process and biogas production by the addition of chitosan a biopolymer. However, UASB reactors have a long start-up time and granules washout due to hydraulic tensions (Mohammadi et al. 2017). Granulated sludge washout was prevented, and a shortened start-up time of 22 days compared to the conventional start-up duration of about 60 days was obtained, with improved biohydrogen production rate of 0.514 H_2/g VSS/d using a modified UASB reactor equipped with a fixed film (Mohammadi et al. 2014). Further modifications include the incorporation of packed bed/fixed bed and membrane-based systems for effective biomass retention in the form of an attached growth system allowing for operation under high organic loading rate (OLR) (Bharathiraja et al. 2016). Upflow anaerobic contact filters (Vijayaraghavan and Ahmad 2006), two-stage UASB (Tahti et al. 2013), and membrane anaerobic system (Fakhrul-Razi and Noor 1999) have all been used to improve biohydrogen yield but will not be discussed further here.

1.5.2 Fermentation Temperature

The activities of biohydrogen-producing enzymes and substrate degradation rate are highly influenced by operating temperatures (Elbeshbishy et al. 2017). Fermentation temperature also influences the production of by-products. Increase in temperature results in a shift in metabolic pathways, by increasing the acetate/butyrate end by-product while decreasing other end product formation (Mu et al. 2006). One way of obtaining such a condition is the use of higher temperatures for the fermentation process (Nitipan et al. 2014). Biohydrogen production at thermophilic or extremely thermophilic (above 60 °C) has the ability to yield more than 2 mol H_2/mol hexose compared to that obtained under mesophilic condition (Ahn et al. 2005). High-temperature biohydrogen production offers several advantages over mesophilic conditions: (1) deactivation of pathogenic microorganisms from waste

materials (Tahti et al. 2013), (2) improvement of the reactions' kinetics, substrates assimilation, and higher hydrolysis rate due to the enhanced thermodynamics (Nguyen et al. 2010). Hydrogen solubility in water decreases with the increase in fermentation temperature (Ismail et al. 2010; Sonne-Hansen et al. 1999). Operation at higher temperatures also avoid the need for seed sludge pre-treatment, since high temperatures can inhibit the activities of hydrogen consumers (methanogen and homoacetogens) (Nath and Das 2004; Saady 2013). Successful biohydrogen production has been obtained with temperatures in the range of 50–80 °C (Kotsopoulos et al. 2009; Boileau et al. 2016), however, the highest temperature used for POME is 55 °C (Krishnan et al. 2016; Ismail et al. 2010; Yossan et al. 2012) and 60 °C (O-Thong et al. 2008; Prasertsan et al. 2009; Nitipan et al. 2014; Seengenyoung et al. 2013) as highlighted in Table 1.2. To obtain an economically sustainable biohydrogen production, energy productivity and yields should be significantly increased. However, production at higher temperatures is not favorable due to the higher energy input, and may also inactivate vital enzymes for cell protein synthesis and growth (Khemkhao et al. 2012). Therefore, to obtain a balance between energy production and energy used, operating the process at mesophilic temperature range seems more favorable (Mu et al. 2006).

1.5.3 Enrichments of Biohydrogen-Producing Microbial Communities

The use of mixed cultures for biohydrogen production offers robustness, and the ability to utilize a wide substrate range (Nitipan et al. 2014). However, enrichment of hydrogen-producing communities while suppressing hydrogen consumers from a mixed environment for increasing hydrogen yield needs to be done via pre-treatment. The reported pre-treatment methods are heat shock treatment (80–100 °C), load shock, acid and alkali treatment, freeze–thaw cycle, aeration, exposure to chemicals (e.g., chloroform, sodium 2-bromoethanesulfonate, and iodopropane) (Mohammadi et al. 2011; Massanet-Nicolau et al. 2008; Wang and Wan 2008), ultraviolet radiation, and ultra-sonication (Fatehizadeh et al. 2018). Majority of hydrogen producers are resistant to extreme environmental conditions (high temperature, acidity, and alkalinity) by forming protective spores. Hydrogen consumers (homoacetogens and methanogens) are mostly obligate anaerobes in nature and are easily affected by aeration (Zhu and Beland 2006). The use of chemical analogs of coenzyme M reductase enzyme complex required for the reduction of methyl-coenzyme M to methanogenesis has been used to inhibit the methanogenesis phase of the degradation process. Analogs such as sodium 2-bromoethanesulfonate (BESA) have been used as methanogen inhibitor (Cheong and Hansen 2006).

The search for the optimum pre-treatment method is still ongoing, as inconsistencies in the outcomes have been obtained without much room for comparison due

Table 1.3 The various pre-treatment methods for enriching biohydrogen-producing bacteria from mixed cultures

Inoculum(s)	Pre-treatment methods	Substrate	Reactor type and operation conditions	Maximum H_2 yield	Optimal method	References
Wastewater anaerobic sludge	HS (70–100 °C, 30 min), UV (150 W high pressure, 20 min) Son (150 W, 20 kHz, 20 min)	Glucose	Batch, pH 7.5 and 37 °C		HS	Fatehizadeh et al. (2018)
POME sludge	HS (100 °C, 1 h), AS (pH 3.0, 24 h), AkS (pH 12.0, 24 h), FT (−10 °C, 30 °C) 24 h, 0.1% (v/v) Cl, 24 h	POME	Batch, pH 5.5 and 35 °C	0.41 mmol H_2/g COD	HS	Mohammadi et al. (2011)
Waste activated sludge	HS (95 °C, 30 min), AS (pH 3.0, 24 h), AkS (pH 10.0, 24 h), Ar for 24 h, 1% Cl for 24 h, and 10 mmol/L of BES for 24 h	Glucose	Batch, pH 7.0 and 35 °C	1.51 mol H_2/mol glucose	AS	Chang et al. (2011)
Anaerobic digested sludge	HS (100 °C, 30 min), AS (pH 3.0, 6 h), FT (−17 °C, 24 h; 25 °C, 12 h), Ar for 2 h, 2% Cl for 24 h. Son at frequency of 20 kHz for 20 min	Rice and lettuce powder	Batch, pH 7.0 and 37 °C	119.7 mL H_2/g VS	HS	Dong et al. (2010)
Digested sludge	HS (100 °C, 15 min), AS (pH 3.0, 24 h), AkS	Glucose	Batch, pH 7.0 and	221.5 mL/g glucose	HS	Wang and Wan (2008)

(continued)

Table 1.3 (continued)

Inoculum(s)	Pre-treatment methods	Substrate	Reactor type and operation conditions	Maximum H_2 yield	Optimal method	References
	(pH 10.0, 24 h), Ar for 24 h, 2% Cl for 24 h		Temp. 35 °C			
Anaerobic sludge from UASB reactor	HS (102 °C, 90 min), AS (pH 3.0–4.0, 24 h), AkS (pH 12.0, 24 h)	Sucrose	Batch, pH 5.5 and Temp. 35 °C	2.00 mol-H_2/mol glucose	HS	Mu et al. (2007)
Cattle manure sludge	HS (dry heat—105 °C, 2 h, wet heat—95 °C, 20 min, AS (pH 2.0, 48 h), FT (−10 °C, 24 h; 30 °C, 6 h), 0.5–1.0 M BESA for 10 min	Glucose	Batch, pH 7.0 and Temp. 35 °C		AS	Cheong and Hansen (2006)
Digested sludge	HS (100 °C, 20 min), AS (pH 3.0, 30 min), AkS (pH 10.0, 30 min), Ar for 30 min, 10 mmol BESA and Io for 30 min	Sucrose	Batch, Temp. 35 °C	5.64 mol-H_2/mol sucrose	Io	Zhu and Beland (2006)
Aerobic activated sludge, Anaerobic digested sludge, soil, lake sediment, aerobic refuse compost	HS (100 °C, 2 h) and AS (pH 3.0, 18 h)	Glucose	Batch, pH 6.0 and Temp. 35 °C	1.4 mol H_2/mol of glucose	HS	Kawagoshi et al. (2005)

HS Heat shock, *AS* Acid shock, *AkS* Alkaline shock, *FT* Freezing and thawing, *Cl* Chloroform, *Ar* Aeration, *BESA* sodium 2-bromoethanesulfonate, *Io* Iodopropane, *Fl* fluvastatin, *Son* Sonication, *UV* Ultraviolent radiation

to the use of different inoculum and substrates, as summarized in Table 1.3. Most studies have shown that heat shock treatment results in the highest biohydrogen yield (Mohammadi et al. 2011; Wang and Wan 2008; Mu et al. 2007; Kawagoshi et al. 2005). A few studies showed different observations, e.g., Zhu and Beland (2006) observed that iodopropane pre-treatment gave the highest biohydrogen yield of 5.64 mol/mol sucrose while heat shock treatment gave 49.9% lower yield. Cheong and Hansen (2006) and Chang et al. (2011) observed that acid shock treatment was the optimum treatment method when cattle manure and waste activated sludge was used. On the other hand, the pre-treatment method can affect the start-up and overall efficiency of the reactors (Fatehizadeh et al. 2018). Thus, manipulation of the fermentation conditions based on the characteristics of biohydrogen-producing communities has been employed in place of pre-treatment methods. Conditions such as hydraulic retention time, pH, loading rate, dilution rates, and biogas circulation have been used to increase yield and avoid the proliferation of hydrogen consumers (Fang and Liu 2002). Zhu and Beland (2006) observed that using untreated control sludge resulted in higher yield than the pre-treated sludge (acid, base, aeration, and heat shock pre-treatment). They concluded that acidic pH developed as a result of the fermentation represses methanogenic activities. Generally, lower yield and traces of methane production are commonly reported, as methanogens could proliferate with time (Wang and Wan 2008). Among the different pre-treatment methods, heat shock and acid treatment have been reported to completely repress methanogenic activities while sonication, freeze/thaw, and aeration and alkaline treatment do not (Dong et al. 2010). Akutsu et al. (2009) and Chang et al. (2011) reported that diversity of the microbial population, fermentation patterns, and the communities enriched differs in different inoculant. Thus, the best pre-treatment method seems to differ with different inoculum used. Nonetheless, heat shock treatment is the widely adopted method of pre-treatment for enriching hydrogen producers using POME (Table 1.3).

1.5.4 POME Pre-treatment Methods

Cellulose and hemicellulose, which are fermentable sugars with monomers linked by β-1,4-glycosidic bonds, are the main components of POME. These polysaccharides are not easily digested and resist hydrolysis when it occurs in a cross-linked matrix with lignin (Baharuddin et al. 2010; Quemeneur et al. 2012). In order to achieve high biohydrogen conversion efficiency, hydrolysis of the complex molecules to simpler molecules through various pre-treatment methods is crucial. This is to allow simpler sugars to be easily accessible to fermentative microorganisms. Pre-treatment methods such as using acid, dilute alkali (Seengenyoung et al. 2013), steam explosion and heat (Kamal et al. 2012), and enzymatic hydrolysis (Silvamany et al. 2015) have been explored.

Exposure of POME to acid and alkali increases its fermentability to produce biohydrogen by catalyzing an efficient hydrolysis process. Mahmod et al. (2017)

obtained a maximum biohydrogen yield of 1.24 mol H_2/mol glucose using POME pre-treated with phosphoric acid, and 1.04 mol H_2/mol glucose when using POME pre-treated with nitric acid. These are equivalent to biohydrogen yield of 97% and 65% higher than using raw POME. Seengenyoung et al. (2013) recorded a 51% increment in biohydrogen yield when using POME pre-treated with 1.5% NaOH. In general, acids decreases the crystallinity of cellulose and catalyze the depolymerization of hemicellulose into xylose and other sugars, and cellulose into glucose (Balat et al. 2008). While alkalis delignify biomass, remove acetyl group and break the intermolecular ester bonds cross-linking lignin and carbohydrates, enabling further reactivity on the released polysaccharides for biohydrogen production (Sun and Cheng 2002).

Meanwhile, Taifor et al. (2017) and Kamal et al. (2012) showed that a combination of chemical and thermal pre-treatments has successfully improved biohydrogen production by elevating soluble sugar concentration. Acids and alkalis rupture the lignin seal whereas heat causes the cellulosic biomass to be converted into simpler sugars such as glucose, fructose, xylose, and arabinose. The study also revealed that microorganisms have different preferences for carbon sources for biohydrogen production, in the following order: glucose, xylose, fructose, arabinose, while no degradation of POME oligomeric sugars was observed (Taifor et al. 2017). Despite the advantages, both strong acid and alkali present safety hazards, and will subsequently need to be neutralized and recovered before disposal. Besides, in addition to the presence of chloride and sulfate ions, acid hydrolysis form by-products such as furfural, phenolics, and hydroxymethylfurfural (HMF) which are inhibitors for fermentative microorganisms (Rodríguez-Chong et al. 2004). Also, excessive heating during thermal pre-treatment will reduce sugar recovery due to sugar degradation into furans (Ruiz et al. 2008). Therefore, considering POME is rich with organic molecules such as triglycerides, carbohydrates, proteins, nitrogenous compounds and minerals, enzymatic hydrolysis present an excellent alternative to heat and chemicals. Rasdi et al. (2012) employed the use of anaerobically treated POME for biohydrogen production. Garritano et al. (2017) demonstrated that POME hydrolysis by plant enzyme preparation improved biohydrogen productivity by 102%. Nevertheless, the high cost of enzymes and low rate of hydrolysis are the potential drawbacks.

In light of the waste disposal issue associated with chemical pre-treatment methods, several physical methods have also been explored. Leaño et al. (2012) reported the feasibility of ultrasonication in pre-treating POME with 38% improved biohydrogen production and 20% higher COD removal. Theoretically, ultrasonicator propagates ultrasound waves in the range of 15–20 kHz in an aqueous milieu which produce cavitation and acoustic streaming. Cavitation generates strong mechanical shear force whereas acoustic streaming increases convection of aqueous slurries (Neis et al. 2008; Nitayavardhana et al. 2008). The combined effects lead to the disintegration of lignin structure, thereby releasing the substrate and increase its bioavailability for subsequent biohydrogen production. Ozonation has also been reported to improve the biodegradability of POME and enhance biohydrogen production with a maximum yield of 182.3 mL/g COD, which is 49% higher than raw

POME (Pisutpaisal et al. 2014). Ozone is a strong oxidant capable of oxidizing a wide range of organics and inorganics into simpler forms suitable for biological conversion (Chaiprapat and Laklam 2011). Álvarez et al. (2005) found that more than 75% of polyphenols were degraded after ozonation and this decreases the toxicity and inhibitory effects of phenolic compound toward the microorganisms in POME. Even though ozonation has the advantages of effectively removing lignin, producing inhibitor-free residues for downstream processes and pre-treatment under room temperature and pressure, large amount of ozone is required thus making the method expensive (Vidal and Molinier 1988).

Various methods are still being investigated in order to eliminate waste products from POME pre-treatments, while at the same time achieve higher efficiency. The effects of several POME pre-treatments on soluble sugar content and biohydrogen yield are summarized in Table 1.4.

1.6 Integration of Dark Fermentation with Other Processes

1.6.1 Dark Fermentation and Anaerobic Digestion

The production and use of methane are desired as it can be used in existing natural gas infrastructures, easily stored, transported, and converted to syngas (Siegert et al. 2015). Biohydrogen and biomethane production share similar biochemical reactions, carried out by hydrolytic and non-hydrolytic fermentative bacteria. Both anaerobic fermentation and anaerobic digestion are initiated by the hydrolysis step, which involves the solubilization and depolymerization of proteins, carbohydrates, lipids to simple sugars, amino acids, long-chain fatty acids, and alcohols (Abbasi and Abbasi 2012). VFAs are utilized by the acetoclastic or hydrogenotrophic methanogens to produce biomethane (Bundhoo 2017). The production of biohydrogen is generally associated with the production of VFAs and electron sink. Accumulation of these VFAs can indirectly affect the productivity by becoming toxic to the hydrogen producers (Rasdi et al. 2012) or by inhibiting the metabolic activity by activating enzymes for solvent production (Khanal et al. 2004), hindering substrate utilization and finally inhibiting microbial growth (Elbeshbishy et al. 2017). Krishnan et al. (2016) used the effluent from a UASB reactor operated under thermophilic condition for biohydrogen production from POME as a substrate for biogas production in a CSTR. 94% COD removal and total energy recovery of 15.43 MJ/kg COD were obtained. Two-stage UASB reactor system operated under thermophilic (70 °C) condition was used for both biohydrogen and biomethane production to give 22% biohydrogen and 78% biomethane (Tahti et al. 2013).

Table 1.4 The effects of different POME pre-treatments on soluble sugar content and hydrogen yield

Pre-treatment conditions	Reactor operation mode	Incubation conditions	Hydrogen yield	References
Ultrasonicated at dose of 195 J/mL	Batch	44 °C, pH 7, 90 rpm	0.7 mmol H_2/g COD (38% higher than raw POME)	Leaño et al. (2012)
Ozonated with ozone loading rate of 300 mg/h	Batch	37 °C, pH 6	182.3 mL H_2/g COD (49% higher than raw POME)	Pisutpaisal et al. (2014)
Dilute acid treatment (a) 0.8% (w/v) H_3PO_4 (b)1% (w/v) HNO_3	Batch	60 °C, pH 5.8, 150 rpm	(a) 1.24 mol H_2/mol glucose (97% increased than raw POME) (b) 1.04 mol H_2/mol glucose (65% increased than raw POME)	Mahmod et al. (2017)
Alkaline treatment 1.5% (w/v) NaOH	Fed-batch	60 °C, pH 5.5	5.2 L H_2/L POME (51% increased than raw POME)	Seengenyoung et al. (2013)
Alkaline treatment autoclaving 10 M NaOH and autoclaved at 121 °C for 20 min	Batch	37 °C, pH 8.5, 120 rpm	0.68 mol H_2/mol total monomeric sugars	Taifor et al. (2017)
Chemicals-heat treatment (a) Added with 10% 1 M NaOH (b) 10% 1 M H_2SO_4 and heated at 80 °C for 1 h	Batch	37 °C, pH 5.5, 300 rpm	(a) 2.18 mol H_2/mol total carbohydrate (56% increased as compared to raw POME[a]) (b) 1.87 mol H_2/mol total carbohydrate (34% increased as compared to raw POME[a])	Kamal et al. (2012)
Enzymatic hydrolysis treatment 0.75% (w/v) of plant enzyme preparation from *Ricinus communis* L. and incubated at 45 °C, 200 rpm for 2 h	Batch	35 °C, pH 6.5, 150 rpm	2.58 mmol H_2/g COD (102% increased than raw POME)	Garritano et al. (2017)

[a]Calculated according to the data presented

1.6.2 Dark Fermentation and Photo-Fermentation

Photo-fermentation has been shown to be able to convert organic residues to more biohydrogen. However, there are difficulties in operating the system due to its

complexity, higher energy demand in the form of light management, low light conversion efficiencies and limitation in scaling up (Zong et al. 2009; Chookaew et al. 2014). VFAs accumulated in single dark fermentation system could be utilized for additional biohydrogen production via the photo-fermentation system. Photo-heterotrophic purple non-sulfur (PNS) bacteria are able to convert short-chain organic acids to hydrogen and carbon dioxide in the presence of light, producing a maximum yield of 4 mol H_2/mol acetate. Theoretically, integrating dark and photo-fermentation systems could yield a maximum of 12 mol H_2/mol glucose, when acetate is the only VFA present (Eroglu and Melis 2011). The yield obtained from the integrated system is higher than 4 mol H_2/mol glucose produced from acetate in a single dark fermentation system. Dark and photo-fermentation systems can be integrated into a sequential two-stage (consecutive operation) or single-stage (simultaneous operation) process. Sequential fermentation was, however, found to be more advantageous and extensively studied than combined fermentation due to higher productivity (Argun and Kargi 2011).

Even though the maximum theoretical yield of biohydrogen production is yet to be achieved practically, the reported overall yields from integrated systems were considerably higher than the single dark or photo-fermentation system (Table 1.5). Lo et al. (2010) used pure sugar (sucrose) to evaluate the performance of sequential dark and photo-fermentation on biohydrogen production using batch and continuous mode of operations. Sucrose was first fermented by *Clostridium butyricum* CGS5 under dark fermentation and subjected to centrifugation and dilution prior to subsequent photo-fermentation by *Rhodopseudomonas palustris* WP3-5. Total biohydrogen yield of sequential dark and photo-fermentation operated under batch mode was 5.45 mol H_2/mol hexose, and 11.61 mol H_2/mol sucrose (equivalent to 5.85 mol H_2/mol hexose) when operated via continuous mode. Interestingly, they further integrated the systems with an autotrophic microalgae reactor to consume carbon dioxide produced from the fermentation process.

Sequential dark and photo-fermentation for biohydrogen production from real wastewater have been reported by Özgür et al. (2010) using sugar beet molasses as feedstock. The dark fermentation was operated under extreme thermophilic condition using *Caldicellulosiruptor saccharolyticus* yielding 4.2 mol H_2/mol sucrose accompanied by acetate and lactate as the main VFAs. The dark fermentation effluent was centrifuged, sterilized, and diluted prior to inoculation of a mutant strain of *Rhodopseudomonas capsulatus* lacking hydrogenase uptake gene (hup-) for photo-fermentation. Cumulative biohydrogen yield of the integrated system was reported to be 13.7 mol H_2/mol sucrose (equivalent to 6.85 mol H_2/mol hexose). Cheng et al. (2011) investigated biohydrogen production from cassava starch in a sequential dark and photo-fermentation operated under batch mode. Heat treated mixed anaerobic bacteria dominated by *C. butyricum* were used as the inoculum in dark fermentation and immobilized mixed photosynthetic bacteria dominated by *R. palustris* in photo-fermentation. Dark fermentation yielded 2.53 mol H_2/mol hexose and VFAs produced were mainly acetate and butyrate. Further conversion of VFAs into biohydrogen in a photo-fermentation system yielded 3.54 mol H_2/mol hexose, resulting in total production of 6.07 mol H_2/mol hexose.

Table 1.5 Integration of dark and photo-fermentation for biohydrogen production

First stage: Dark fermentation						Second stage: Photo-fermentation				Total H_2 yield from sequential dark-photo-fermentation	References
Substrate type	Microbial inoculum	Fermentation conditions	Maximum H_2 production rate	Maximum H_2 yield	Dominant end products	Microbial inoculum	Fermentation condition	Maximum H_2 production rate	Max. H_2 yield		
Sucrose	*Clostridium butyricum* CGS5	37 °C, pH 7.5, Batch	266 mL H_2/L/h	0.98 mol H_2/mol hexose	Lactate, butyrate, formate, acetate and ethanol	*Rhodopseudomonas palustris* WP3-5	N/D, pH 7.1, Batch	12.61 mL H_2/L/h	4.48 mol H_2/mol hexose	5.45 mol H_2/mol hexose	Lo et al. (2010)
	C. butyricum CGS5	37 °C, pH 6.5, CSTR	667.37 mL H_2/L/h	6.56 mol H_2/mol sucrose	Lactate, formate, acetate butyrate and ethanol	*R. palustris* WP3-5	32 °C, pH 7.1, CSTR	6.56 mL H_2/L/h	5.06 mol H_2/mol sucrose	11.61 mol H_2/mol sucrose (5.85 mol H_2/mol hexose)	
Sugar beet molasses	*Caldicellulosiruptor saccharolyticus* DSM 8903	72 °C, pH 6.9, Batch	200 mL H_2/L/h	4.2 mol H_2/mol sucrose	Acetate and lactate	*hup*$^-$ mutant of *Rhodobacter capsulatus*	30–33 °C, pH 6.7, Batch	34.04 mL H_2/L/h	9.5 mol H_2/mol sucrose[a]	13.7 mol H_2/mol sucrose	Özgür et al. (2010)
Cassava starch	Mixed anaerobic bacteria (dominated by *Clostridium* sp.)	31 °C, pH 6.3, Batch	334.8 mL H_2/L/h	2.53 mol H_2/mol hexose	Acetate and butyrate	Mixed photosynthetic bacteria (dominated by *R. palustris* sp.)	31 °C, pH 6.3, Batch	21.1 mL H_2/L/h	3.54 mol H_2/mol hexose	6.07 mol H_2/mol hexose	Cheng et al. (2011)
Cheese whey	Immobilized *Enterobacter aerogenes* MTCC 2822	30 °C, pH 6.8, Batch	1.91 mmol/L/h	3.5 mol H_2/mol lactose	Acetate and butyrate	Immobilized *Rhodopseudomonas* BHU 01	34 °C, pH 6.8, Batch	1.87 mmol/L/h	2.69 mol H_2/mol acetic acid	5.88 mol H_2/mol lactose	Rai et al. (2012)
Sugarcane bagasse	*E. aerogenes* MTCC 2822	30 °C, pH 6.8, Batch	11.90 mL H_2/L/h	1000 mL/L	Acetate and butyrate	*Rhodopseudomonas* BHU 01	34 °C, pH 6.8, Batch	7.86 mLH_2/L/h[a]	755 mL/L	–	Rai et al. (2014)
POME	*C. butyricum* LS2	37 °C, pH 5.5, CSTR	21 mL H_2/h	0.784 mL H_2/mL POME	Butyrate, acetate, ethanol and propionate	*R. palustris*	30 °C, pH 7.0, Batch	26 mL H_2/h	–	3.064 mL H_2/mL POME	Mishra et al. (2016)

CSTR Continuous stirred tank reactor, *N/D* not determined

[a]Calculated according to the data presented

1.6.3 Dark Fermentation and Bioelectrochemical Systems

Bioelectrochemical systems (BES) is an emerging and expanding field of research, with the ability to revolutionize the process of capturing renewable resources by combining biological catalytic redox activity with the typical abiotic electrochemical reactions (Santoro et al. 2017). Energy stored in biodegradable organic substrates can be converted into electricity via the catalytic actions of electrochemically active bacteria, also referred to as electricigens or exoelectrogens (Chookaew et al. 2014; Chae et al. 2009). Microbial fuel cell (MFC) and microbial electrolysis cell (MEC) are the most commonly used BES for bioenergy generation from organic wastes in the form of electricity and biohydrogen, respectively. MEC is similar to MFC in configuration but differs in function with the addition of an electrical power supply and an anaerobic cathode chamber to capture the hydrogen produced, a process referred to as "electrohydrogenesis" (Liu et al. 2005). During the process, bacteria degrade organic substrates, and produce extra electrons, which need to be removed to maintain ionic neutrality as a result of the redox reactions of ferredoxins, and protons. Protons passed through an ion exchange membrane, before hydrogen is formed in the cathode chamber. Biohydrogen and bioelectricity production via MEC and MFC has been demonstrated using a wide range of substrates, such as glucose, fermentation effluents (Logan et al. 2008), bovine serum albumin (BSA), peptone (Lu et al. 2010), hemicellulose, cellulose, and other organic matter (Cheng and Logan 2007; Kadier et al. 2014), industrial wastewater, refinery wastewater, winery wastewater, and dairy manure wastewater (Lu et al. 2012; Logan et al. 2008; Ren et al. 2013) making these systems a highly promising clean energy production technology (Kadier et al. 2016).

In MEC, biohydrogen conversion efficiency using complex fermentable substrates are observed to be lower than that obtained from acetate, a model substrate, which has been extensively used in the investigation of electroactive microorganisms. The highest biohydrogen production yield is close to the theoretical value of 4 mol H_2/mol acetate (Bond et al. 2002). The low conversion efficiency using complex fermentable substrates can be attributed to the slow hydrolysis steps required for the breakdown of the complex polymers to produce electron donors for the exoelectrogenic microbial community (Cheng and Logan 2007).

The use of POME as a substrate in MFC and MEC has been investigated by a few researchers (Jong et al. 2011; Baranitharan et al. 2013; Nor et al. 2015). Jong et al. (2011) evaluated and compared the bioenergy generation from POME and acetate using enriched inoculum in MFC. The maximum power density of 622 mW/m^2 was obtained from the MFC fed with diluted POME having the COD concentration of 200 mg/L. Comparing the efficiency with pure acetate, it showed a 79% decrease as to when the system was operated solely with acetate. Likewise in the anode, a 23% maximum COD removal and 32% coulombic efficiency was obtained when POME was used. As a comparison, 47 and 75% COD removal and coulombic efficiency was achieved when acetate was used. Baranitharan and coworkers observed that different COD concentrations influenced the coulombic efficiency, power density

and COD removal efficiency (Baranitharan et al. 2013). A maximum power density of 45 mW/m^2 was obtained from the raw undiluted POME, resulting in 0.8% coulombic efficiency and 45% COD removal efficiency. Similarly, power density decreased upon dilution at 1:50, while coulombic efficiencies and COD removal increased with maximum values of 24 and 70%, respectively. The power density obtained was, however, lower to that obtained by Jong et al. (2011), using diluted POME. They further concluded that the low coulombic efficiency observed (despite a reasonable percentage of COD removed) was attributed to the complexity of the substrate, and the presence of non-electron transferring bacteria in the community, such as hydrolytic and fermentative bacteria.

To combat the lower electricity production, controlled inoculum was used, which is composed of both fermentative and electrogenic microorganisms isolated from anaerobic sludge and biofilm, respectively (Baranitharan et al. 2015). With the use of the controlled inoculum, a maximum power density of 107.35 mW/m^2 was obtained, which is twice of that obtained from another study (Baranitharan et al. 2013). Likewise, a much higher coulombic efficiency of 50% was also obtained with the controlled inoculum, but COD removal efficiency was found to be lower. The low COD removal was attributed to the possible absence or low abundance of the required fermentative bacterial community in the controlled inoculum. The potential of using pure cultures in the generation of electricity from POME has been attempted (Nor et al. 2015; Islam et al. 2016). Nor et al. (2015) successfully isolated *Pseudomonas aeruginosa* ZH1 from anaerobic POME sludge and compared power productivity between the pure culture and sludge inoculum. The maximum power and current density achieved from the use of POME sludge were 85.12 mW/m^2 and 91.12 mA/m^2, respectively, while the use of *P. aeruginosa* ZH1 yielded 451.21 mW/m^2 and 654.90 mA/m^2, respectively. The power and current density increased by 81 and 86% with the use of pure culture compared to the mixed culture, but showed lower COD removal efficiency. Islam et al. (2016) improved COD removal efficiency to 74.28% by using ultrasonicated POME as the substrate in a single air cathode MFC with *Klebsiella variicola* as the inoculum. Higher power density of 1648.70 mW/m^3 was obtained from the use of the pre-treated POME, compared to untreated POME (1280.56 mW/m^3). Nonetheless, the current density obtained was lower compared to that obtained by others (Nor et al. 2015). These studies have shown that pure electrogenic cultures have the ability to generate much higher power and current density in an MFC, compared to mixed cultures, but are less effective in degrading complex substrates. Thus, utilization of complex substrates such as POME in an MFC, for both electron generation and waste treatment, would require a diverse microbial community of both exoelectrogens and efficient degraders (Lu et al. 2010).

A few researchers have used MEC/MFC to utilize residual organic materials in effluents generated from dark fermentative biohydrogen production as a result of the abundant VFAs content to further improve substrate utilization and energy recovery. Chookaew et al. (2014) investigated the integration of dark fermentation and MFC/MEC using glycerol substrate. The dark fermentation process gave a maximum biohydrogen production rate of 332 mL H_2/L and yield of 0.55 mol/mol

glycerol. Undiluted fermentation effluent gave the maximum current density and COD removal of 50.2% when fed into MFC. However, a 50% dilution effluent showed higher performance than undiluted samples in the MEC reactor with the highest biohydrogen yield of 106.14 mL H_2/g COD consumed, 40.58% COD removal efficiency and 34.8 A/m^3 current density at 1.0 V applied voltage. The studies showed that fermentation effluent resulted in higher power output compared to when raw glycerol was used as a substrate (Nimje et al. 2011).

The possibility of obtaining a self-sufficient system involving the integration of dark fermentation-MFC-MEC was investigated by Wang et al. (2011). Effluent from a continuous dark fermentation system with a maximum biohydrogen yield of 10.1 mmol H_2/g cellulose was used as a substrate in both MFC and MEC. Two MFCs yielding voltage of 0.435 V was used as the source for external power for the MEC setup. The biohydrogen production rate of 0.28 m^3 H_2/m^3 was obtained from the MFC-MEC setup, which is 18 times higher than the rate obtained when acetate was used as a substrate. However, electrohydrogenesis was observed only in the first 28 h of operation resulting in a biohydrogen yield of 33.2 mmol H_2/g COD, no hydrogen recovery was observed upon further reaction time despite substrate availability and current production. This was attributed to COD regeneration as a result of acetogenesis. Moreno and co-workers (2015) explored the use of acidified and enriched cheese whey fermented effluent in a membrane-less MEC as a mean to combat the proliferation of methanogenic or acetogenic activity. They obtained 178 mL biohydrogen production from the fermentation and 1.5 L from the electrohydrogenesis phase, amounting to 2.2 L H_2 which corresponds to 94.2 L H_2/kg versus biohydrogen yield. Similar to the findings of Wang et al. (2011), the total biohydrogen yield of cheese whey through the fermentation-MEC integration was higher than the yield obtained from the use of only fermentation (Islam et al. 2016; Nimje et al. 2011). Babu et al. (2013) studied the effects of different voltage on additional hydrogen recovery via MEC process using acidogenic effluent of dark fermentation. It was observed that the increase in voltage poised resulted in an increase in biohydrogen productivity. However, a further increase beyond 0.6 V resulted in a drop in productivity. Maximum biohydrogen production rate of 0.53 mmol/h and VFA utilization (49.8%) was obtained at 0.6 V. Rivera et al. (2015) supported similar results to those obtained by Babu and co-workers (2013). In addition to the use of high voltage to obtain higher biohydrogen productivity, it was also observed that low COD concentration of UASB effluent used as substrate in a double-chambered MEC setup also influenced biohydrogen production rate. No significant difference between the use of synthetic and dark fermentation effluent was observed. Maximum biohydrogen production rate obtained was 81 mL/L/day with COD consumption of 85%. Table 1.6 summarizes the integration of dark fermentation and bioelectrochemical systems reported in the literatures so far.

Unlike photo-fermentation and anaerobic digestion process for biohydrogen production, MECs are less energy demanding. Nonetheless, for the successful adoption of dark fermentation effluent as feed substrates, feed neutralization, and dilution is critical for obtaining higher productivity. From the economic and environmental point of view, the adoption of MEC as a posttreatment stage of dark

Table 1.6 Research on integrated dark fermentation and bioelectrochemical system (microbial electrolysis cell) reported in the literatures

	Dark fermentation					Bioelectrochemical systems						
Substrate	Seed inoculum	Operational conditions	$BioH_2$ yield	$BioH_2$ rate	% COD removal	Operational conditions	Voltage applied (V)	$BioH_2$ rate	$BioH_2$ yield	% COD removal	% overall energy efficiency	References
Municipal wastewater	N/A	Continuous UASB, 35 °C, pH 4.5	N/A	N/A	N/A	Double chamber		81 mL H_2/L/day		85		Rivera et al. (2015)
Cheese whey	Digested sludge	Batch, 35 °C	N/A	0.7 L H_2/$L_{reactor}$	N/A	Membrane-less chamber, 7.2, 25 °C	1.0	0.5 L H_2/L_a/day	N/A	82	N/A	Moreno et al. (2015)
Sugar beet juice	Anaerobic digested sludge	Batch, 37 °C, 5.5	3.2 mol H_2/mol hexose	N/A	N/A	Double chamber, 7.2, 25 °C	0.4	N.A	12% TCOD	N/A	57	Dhar et al. (2015)
Crude glycerol	Park sediments	Batch, 35 °C, 7.0	0.55 mol H_2/mol glycerol	332 mL/L	20	Double chamber, 7.0		0.050 m^3H_2/m^3/day	106.14 mL H_2/g COD	40.58		Chookaew et al. (2014)
Corn stalk	Cow dung compost	Batch, 36 °C, 7.0	129.8 mL H_2/g-corn stalk	1.73 m^3/m^3 day		Single chamber	0.8	2.43 ± 0.12 m^3/m^3day	257.3 mL H_2/g-corn stalk	44 ± 2	166 ± 10	Li et al. (2014)
Domestic wastewater	USAB anaer-obic consortium	Batch	[a]11.21 mol/kg $COD_{removed}$	N/A	53		0.6	0.53 mmol/h	[a]2.025 mol/kg $VFA_{removed}$	49.8[b]	N/A	Babu et al. (2013)
Cellulose	Rotted wood crumbs	Continuous, 60 °C	10.1 mmol H_2/g cellulose	0.24 m^3 H_2/m^3/day	71[c]	Single cham-ber, 7.0	0.4	0.48 m^3H_2/m^3/day	33.2 mmol H_2/g COD	19	23	Wang et al. (2011)
Corn stover	*Clostridium thermocellum* 27,405	Batch, 55 °C, 6.8	N/A	1.65 L H_2/L/day	N/A					65		Lalaurette et al. (2009)

TCOD total chemical oxygen demand, *N/A* not available, *L* Liter of the anode

[a]Specific hydrogen yield

[b]VFA utilization

[c]Cellulose utilization

fermentation might not be practical. The use of other wastewater streams produced in the same facility or other industrial effluents with a less organic load having similar organic matter composition could be used for the dilution in order to obtain an optimized performance. There has been no report to date of the use of POME dark fermentation effluent as the substrate in an MEC system. However, the potential of adopting the integrated method for improved wastewater degradation and biohydrogen production using POME as the substrate is very attractive.

1.7 Challenges in Using Hydrogen as an Energy Carrier

Despite the environmental benefits using hydrogen to produce electricity or to power vehicles, the issues related to its storage and distribution are still being investigated. Several types of fuel cells can be used for electricity production from hydrogen, e.g., polymer electrolyte membrane fuel cell (PEMFC), alkaline fuel cell (AFC), phosphoric acid fuel cell (PAFC), molten carbonate fuel cell (MCFC), and solid oxide fuel cell (SOFC). If storage and transport of hydrogen are required, there are four main hydrogen storage methods reported: namely (1) as compressed gas, (2) as a cryogenic liquid, (3) physical storage in hydrides and (4) chemical storage in hydrides (Mah et al. 2019). Owing to its physical and chemical properties, the logistics costs for hydrogen are higher than those for other energy sources. Shell in its 2017 Hydrogen Study (Adolf et al. 2017) outlines three different modes of transportation: (1) using compressed gas containers, (2) via liquid transport, and (3) pipeline. Gaseous hydrogen can be transported in small to medium quantities in compressed gas containers by lorry. Hydrogen can also be transported in liquid form, which allows more hydrogen to be carried compared to pressure gas vessels. It is also more cost effective to transport hydrogen in liquid form over longer distances. For comprehensive and longer term use of hydrogen, a pipeline network would be the best option despite being costly. The cost can be offset when larger volumes of hydrogen are used in the future (Adolf et al. 2017).

1.8 Conclusion

Dark fermentation is considered the most feasible among all the methods for biohydrogen production. At the moment, anaerobic digestion is adopted in countries burdened with the treatment of POME for the production of biogas for use in local power generation. At the same time, the utilization of POME for biohydrogen generation has been receiving increasing attention, due to the cleaner nature of hydrogen combustion. Although various technical, operational, and biological improvements have been attempted to increase biohydrogen production yield via dark fermentation, the process still faces several drawbacks. We have described here the potential of integrating bioelectrochemical systems with dark fermentation for

treatment of different waste and wastewater types with simultaneous energy generation, particularly for the treatment of POME. Although the large-scale biohydrogen production might still require further research and development, there is a huge potential in utilizing POME for cleaner energy generation.

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Chapter 2
Spent Mushroom Substrate as Biofertilizer for Agriculture Application

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Abstract In the face of global changes, plants must adapt to a wide range of abiotic and biotic stress combinations such as water stress conditions, soil fertility losses, soil pollutions, drought, pests, and disease that seriously impaired plant growth and development. In addition, the current agriculture practices of worldwide suffering overuse of chemical fertilizers and pesticides since the use of biofertilizer and biocontrol agents produce slow results and expensive. Therefore, there is a need for new biomaterials that are cheap with the characteristics of high bioavailability of nutrients, carriers of biocontrol agents, detoxification, and rehabilitation of toxic materials for an efficient, economical and versatile biofertilizer. Spent mushroom substrate (SMS) is one of the most abundant agricultural wastes produced at the end of mushroom cultivation production. SMS still contains essential nutrients needed for raising a healthy field crop in addition to cultivated mushroom mycelium and large population of heterotropic microbes. Different potential of applying SMS has been discussed nowadays. The application of SMS in agriculture mainly contributes to improveing the soil quality, chemically adsorb the organic and inorganic pollutants, and serves as a good carrier for the Plant Growth Promoting Microbes (PGRM) and shows the best biological efficiency against soil and plant pathogens. This book chapter basically reviews and discusses different scientific research and practical application of SMS especially focusing on the agricultural field to develop a latest insight of SMS as one of the low cost but high productive biomaterials.

Keywords Spent mushroom substrate · Microbial community · Composting · Biofertilizer · Soil health · Biocontrol · Plant nutrients

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2.1 Introduction

In recent years, the spent mushroom substrate (SMS) is becoming a huge problem in environmental pollution issues. SMS still serves as a nutrient-rich organic material with available nutrients and high porosity (Hafifah et al. 2018). Therefore, the value of SMS's has been explored extensively to offer full advantage of agricultural by-product in organic agriculture development. SMS is a type of organic waste produced after several cycles of any type of mushroom cultivation on what initially known as a mushroom substrate. Every 1 kg of mushroom produced will generate approximately 5 kg of SMS (Economou et al. 2017). In Malaysia alone, it is estimated that 120 metric tons of SMS is produced daily (Lam et al. 2019). Various agricultural wastes have been reported as a suitable substrate for various types of mushroom. Different SMS will vary in composition depending on the base substrate and its supplementation (Ibrahim et al. 2015). Ibrahim et al. (2015) investigated a modified combination incorporating oil palm frond with rubber sawdust, rice bran, and hydrated lime for *Pleurotus* sp. Some of the potential types of SMS from different cultivations of mushrooms are shown in Table 2.1. SMS has been reported numerously on its application in agriculture, especially on soil to improve soil physicochemical and fertility (Marín-Benito et al. 2016). Numerous studies have been reported investigating the advantages of supplementing the base substrate with SMS or recompose SMS. Lou et al. (2017a) suggested the use of SMS of *Ploretus eryngii* and *Ploretus ostreatus* in combination with urea improved the mineral nitrogen transformation in amended soil. Meng et al. (2018a) found that SMS in combination with pig manure could positively affect its N, P, and K values. SMS combination with winery sludge was also reported by González-Marcos et al. (2015) makes the mixtures more homogenous concentrated mixtures with negligible contains heavy metals for soil application. During the mushroom cultivation and development period, protein serves as additional nitrogen source while lignin serves as the main source of carbon and energy (Abd Rasib et al. 2015). As the crude protein and fat content increase more than twice, while cellulose decreases more than 50%, lignin decreases by 30% and gossypol by 60%, whereas carbohydrate and lignin also showed reduction in the content (Zhu et al. 2012). Along the cultivation of mushroom, existence of different microbial community in the substrates also contributes to the increase of bioactive compounds in SMS such as polysaccharides, vitamins, macro and micro elements like Fe, Ca, Zn, and Mg (Abd Rasib et al. 2015; Zhu et al. 2012).

2.1.1 Microbial Community in Spent Mushroom Substrate

Treated SMS with suitable beneficial microorganisms has been reported to increase nutritional and biological activities of SMS thus enhancing soil quality, agronomic

Table 2.1 Different components of SMS from different types of mushroom

Types of mushroom	Mushroom substrate	References
Pleurotus sp.	Maize stalk, maize husk, maize cob	Adjapong et al. (2015)
Pleurotus ostreatus	Rubber wood dust, paddy straw, palm empty fruit bunches, palm-pressed fiber	Harith et al. (2014)
Pleurotus ostreatus	Sawdust, rice bran, lime	Omokaro and Ogechi (2013)
Pleurotus eryngii	Sweet potatoes, sawdust, wheat bran, sugarcane bagasse, rice straw	Lou et al. (2017a)
Ganoderma sp.	Sawdust	Kamthan and Tiwari (2017)
Pleurotus ostreatus *Lentinula edodes*	Barley straw Oats or cedar	Bastida et al. (2016)
Pleurotus ostreatus	Barley straw with combination of plant herbs (*Chenopodium ambrosioides* L., *Mentha piperita* L., *Rosmarinus officinalis* L., *Litsea glaucescens Kunth Tagetes lucid Cav*)	Martínez et al. (2015)
Lyophyllum decastes *Pleurotus eryngii*	Japanese cedar, sorghum, soybean pulp, rice bran, and wheat bran	Parada et al. (2012)
Lentinula edodes	Oak sawdust substrate Supplemented with 20% rice bran	Kang et al. (2017)
Lyophyllum decastes	Mixtures of Japanese cedar, sorghum, soybean pulp, rice bran, and wheat bran	Arase et al. (2013)
Agaricus subrufescens	Mixtures of sugarcane bagasse, coast-cross hay, wheat bran, superphosphate fertilizer, limestone, gypsum, ammonium sulfate	Marques et al. (2014)
Pleurotus eryngii, *Flammulina velutipes* *Hypsizigus marmoreus*	Sawdust, cottonseed hull, corncob, sugarcane bagasse, wheat bran, corn powder, gypsum	Zhang et al. (2018)

efficiency, and environmental safety (Hafifah et al. 2018). Ntougias et al. (2004) found that the existence of microbial community in SMS is greatly affected by the origin of the mushroom substrate components. The potential unintended colonization of the mushroom substrate might occur during casing and the rest of the cultivation phase (Ntougias et al. 2004). The nature of mushroom substrates production process also affects microbial community in the SMS either the substrate components mixed with animal manure that undergoes composting or direct use or pasteurization prior to cultivation. The thermotolerant bacteria mainly existing in the mushroom substrate after pasteurization are expected to survive, and then during cultivation of the mushroom they could rapidly colonize the substrate or due to ineffectiveness of the pasteurization process (Omokaro and Ogechi 2013; Ntougias et al. 2004). The changes of the substrate physicochemical properties (e.g., pH, moisture, and nutrient conditions) associate with the growth of mushroom species were likely the direct factor that drove the changes in microbial community composition (Zhang et al. 2018). Most of the identified microbial strains belonged to

mesophilic population that originated from the environment such as from insects, animal, and clinical sources (Ntougias et al. 2004; Bishop et al. 2016). These mesophilic microbial populations normally help to create an internal physicochemical functional characteristic between mycelium of cultivated mushroom and substrates through increase of siderophore production, excretion of enzymes, and antagonistic property against disease-causing microbes (Zhang et al. 2018; Sharma et al. 2013). *Aspergillus, Trichoderma*, and *Bacillus* species had the highest frequency of occurrence for fungi and bacteria in the SMS, which are known as saprophytic microorganisms that are able to break down complex substrates and actively aid nutrient availability in the substrates (Omokaro and Ogechi 2013).

2.1.1.1 Isolation and Identification of Bacteria from SMS

Microbial community in the SMS was investigated revealing the dominance of Gram-positive bacteria have been previously reported to exist in SMS or even in the mushroom substrate during cultivation of mushroom (Ntougias et al. 2004; Watabe et al. 2004; Ivors et al. 2000). Fifty bacterial species were isolated and classified from SMS associated with the genera *Bacillus, Paenibacillus, Exiguobacterium, Staphylococcus, Desemzia, Carnobacterium, Brevibacterium, Arthrobacter*, *Microbacterium, Firmicutes,* and *Actinobacteria* (Ntougias et al. 2004). Majority of the bacterial isolates are lignolytic bacteria that exhibited lignocellulosic biomass enzymes such as amylase, cellulase, and xylanase activity (Ntougias et al. 2004; Sharma et al. 2013; Song et al. 2001). Song et al. (2001) isolated thermophilic *Actinomycetes*, *Streptomyces*, and *Thermoactinomyces* sp. from different mushroom compost prepared from waste cotton and hay. Furthermore, Bishop et al. (2016) have observed existence of four prominent phyla of bacteria population of *Firmicutes, Actinobacteria, Proteobacteria*, and *Chloroflexi* in the different types of mushroom substrate components. According to Szekely et al. (2009), the existence of cellulose degrading bacteria consortium in the mature compost of Agaricus SMS which consists of phylotypes related to Actinobacteria, Thermobifida, and Thermomonospora. The existence of these microbes enhanced turnover of organic matter fractions from lignocellulosic biomass composting during cultivation of mushrooms. Study by Ntougias et al. (2004) showing the identified bacterial group was related to *Comamonas aquatica (β-Proteobacteria*, order *Burkholderiales*) and some of the isolate was affiliated to *Bacteroidetes* (class *Sphingobacteria*) and the closest match was *Sphingobacterium faecium* (98.6%). Furthermore, Watabe et al. (2004) have examined SMS which is produced from the mix of raw materials/chicken manure used for the composting process successfully identified *Bacillus licheniformis, Bacillus subtilis, Klebsiella/Enterobacter* sp., *Microbacterium* sp., *Paenibacillus lentimorbus, Pseudomonas mevalonii, Sphingobacterium multivorum*, and *Stenotrophomonas* sp. in the SMS. Unfortunately, supplementation of animal manure for composting the substrates and unproper composting process may contribute to the presence of fecal pathogens like *Campylobacter* sp., *Salmonella* sp., and *Listeria monocytogenes* which can cause serious implication to animal and human

health (Watabe et al. 2004). In addition, unsuccessful composting of agrowaste materials and poor pasteurization of mushroom substrates may therefore contribute to the presence of fecal pathogens such as *Bacillus, Clostridium, E. coli*, and *Enterobacter* that can cause diseases resulting economic loss (Omokaro and Ogechi 2013).

In the presence of bacterial community in SMS, their biological activities can enhance the production of natural antibiotics or bacteriocins to suppress the competitive effects of neighboring flora (Watabe et al. 2004). Recently, SMS is known as one of the biomaterials that can be used to treat plant and soil pathogens. As the presence of bacterial community such as *Pseudomonas* and *Bacillus* sp. in the SMS contributed to suppressing the growth of root and soil pathogens (Zulfikar et al. 2018; Viji et al. 2003). Furthermore, as studied by Zulfikar et al. (2018), 13 isolates of antagonistic bacteria of 6 genera including *Bacillus* (6 species), *Pseudomonas* (3 species), and *Chryseobacterium, Ochrobactrum, Paraburkholderia*, and *Serratia* were isolated from spent *Pleurotus* mushroom effectively control the growth of *Fusarium solani*. From the isolation, two species of *Bacillus subtilis* subsp. subtilis and *Bacillus* sp. *001* had been identified to boost the efficiency to inhibit the growth of *Fusarium solani* as causal agent of tomato wilt and fruit rot (Zulfikar et al. 2018). Riahi et al. (2012) have been successfully isolated from SMS extracts of three *Bacillus* sp. such as *Bacillus subtilis, Bacillus licheniformis*, and *Bacillus amyloliquefaciens* identified using 16S rRNA, showing an antagonistic effect to *Lecanicillium fungicola* in white button mushroom cultivation. In addition, Pseudomonas aeruginosa also isolated from recompose of SMS inhibited growth of *Pyricularia grisea* as the causal agent of gray leaf spot of perennial ryegrass (*Lolium perenne*) turf (Viji et al. 2003). *Pseudomonas aeruginosa* was also showing inhibition growth of *Rhizoctonia solani, Rhizoctonia cerealis, Sclerotinia homoeocarpa,* and *Fusarium culmorum* (Viji et al. 2003).

2.1.1.2 Isolation and Identification of Fungi from SMS

During cultivation of cultivated mushroom, the growth of unwanted fungimay causedecrease of mushroom yield due to growth inhibition. It is most likely due to the attack of green mold disease by *Trichoderma* sp. during mushroom growth (Colavolpe et al. 2014; Choi et al. 2010). Undesired fungi have been isolated from the compost of oyster mushroom SMS immediately after the pasteurization process and identified as *Aspergillus fumigatus, Chaetomium thermophile, Mucor pusillus*, and *Trichoderma harzianum* that cause unusual discoloration of substrates and inhibit the formation of fruiting bodies in cultivated mushroom (Omokaro and Ogechi 2013; Wickremasinghe et al. 2011). *Penicillium* sp. was also known to compete occupation of green spores in the mushroom substrate (Choi et al. 2010). The SMS from cultivation of *Agrocybe aegerita* is more susceptible to be contaminated by *Trichoderma* sp. that dominate up to 89.65% with 26 isolates of

Trichoderma sp. and the remaining isolates were *Aspergillus* sp., *Mucor* sp., and *Penicillium* sp. (Choi et al. 2010). A similar finding was also observed by Seephueak et al. (2017), as they identified the most frequent types of fungi in SMS were the genus *Trichoderma, Aspergillus*, and *Penicillium* sp.

2.2 Application of SMS in Agriculture

Malaysia is considered one of the blessed countries with a rich agricultural sector and abundance of biomass resources due to its favorable climate resulting in generating a huge amount of solid wastes annually (Chan et al. 2019). With the rapid development in the mushroom industry, the SMS can be categorized as one of the most abundant agricultural waste produced from mushroom harvest. Commonly, the method in handling SMS waste is incineration or landfills which indirectly created serious concern in environmental issues including attracts flies and insects that can carry disease, release a significant amount of greenhouse gases that harmful to human health and lead to water and air pollution if no handling properly (Bong et al. 2017; Lou et al. 2017b). The SMS is still rich in nutrients and organic matter but the residues nutrients are not available anymore for the mushroom uptake (Lou et al. 2017b). Hereby, recycling of SMS gained increased attention by researchers and different potential uses of SMS have been proposed including soil and water remediation (Garcia-Delgado et al. 2013), animal feeds (Kim et al. 2012), and soilless growing medium for horticultural seedling (Medina et al. 2012).

2.2.1 SMS as Composting Material

Among the potential application of SMS for agriculture, composting of SMS is still the most feasible and highly promising strategy to recycle SMS effectively and economically (Lou et al. 2017b). It is a process using natural microorganism to decompose, mineralize the complex organic matter in SMS (proteins, lipids, lignocelluloses, hemicelluloses, cellulose, lignin and other carbohydrate compounds) and produce high level of available soluble substances such as soluble carbon, nitrogen, potassium, phosphorus compounds for farming (Pergola et al. 2018). An SMS-derived compost is also referred as spent mushroom compost (SMC), supposed as an inexpensive and sustainable way in agricultural uses and acts as an alternative way to SMS disposal as waste. Paredes et al. (2016) have reported the uses of SMS-derived compost gave similar lettuce yield as mineral fertilizer and showed an increment of the total content of organic carbon, nitrogen, as well as phosphorus in soil (Paredes et al. 2016). SMS derived compost has served as relatively porous medium and low bulk density that capable to enhance the soil structure and helps to minimize the plant scorch condition. A similar finding by Medina et al. (2012), the uses of SMS as compost proved to improve the soil fertility

and the total available organic carbon, nitrogen, and phosphorus were increased significantly (Medina et al. 2012).

Theoretically, there are four stages involved in a SMW-derived composting process. The first stages namely mesophilic stage involves the pre-breakdown of organic matter of the SMS by mesophilic microorganisms at increased temperature of between 20 and 40 °C. Heat is generated during the population of mesophilic microorganism and the microbial activity is increased. Slowly, the mesophilic microorganism is replaced by thermophilic microorganisms and the number of pathogens is also decreases gradually when the compost temperature raised to 45–70 °C. Throughout the thermophilic stage, the thermophilic microbial degrades the lignocellulose materials like complex fats, lignin, hemicellulose and cellulose. A second phase of mesophilic stage starts after the thermophilic stage during which the temperature of compost material begins to decrease. In this stage, further mineralization of proliferate degraded sugar, amino acid and proteins are performed by mesophilic microbial. The pH and temperature decreased with time until a stable level at pH 7 and room temperature of 25 °C, respectively, also referred as maturation stage due to the completion of decomposition process (Akdeniz 2019). A total of soluble nitrogen, phosphorus, and potassium raised with the reduction of organic matter when achieved the maturation stage and the compost or SMC is considered matured (Akdeniz 2019; El-Haggar 2007; Sánchez et al. 2017). Through the microbial-mediated composting process, SMC minimized the incidence of plant disease by suppressing the pathogenic microorganisms while providing the essential soluble nutrients for plant uptake (Meng et al. 2018a).

The assessment of a matured SMS-derived compost highly dependent on the stability and maturation of an SMC. Generally, stability of a SMC is referred to the extent to which the SMS has been decomposed by the microorganism. Maturation of a SMC depends on the effect of crops' growth. Researchers have established different indicators for a comprehensive evaluation of maturity, including measurement of lignocellulose, as well as the presence of particular microorganisms corresponding to different stages of maturity (Meng et al. 2018a; Paula et al. 2017). Guo et al. (2017) reported aeration rate and C/N ratio are two key factors influencing the SMC stability and maturity, respectively. The recommended aeration rate and C/N ratio to produce matured SMC is estimated to be 0.48 L/kg/min and 18, respectively (Guo et al. 2017). Similarly, Meng et al. (2018a) found that total organic carbon approximately 300 mg/g and C/N ratio at 13.5 are considered a good degree of composting maturity (Meng et al. 2018a).

The primary effect of the SMC is not to enrich and transform the soil quality and structure immediately but to facilitate the generated soluble nutrient mobilization to the plant uptake and leads to achieving the soil equilibrium condition. Although the bioactive compounds obtained by composting are useful and increase the total content of soluble nutrient for plant growth, the obtained concentration of essential nutrients is still not sufficient for plant nutrition compared to chemical fertilizers (Meng et al. 2017). To date, co-composting, which is a simultaneous composting of more than one type of residue raw materials has received increasing attention. Recent studies have discovered the combination of other raw materials either at

Table 2.2 Recent research studies from 2014 to 2019 for co-composting of SMS with other raw materials in agricultural application

Co-composting materials	Impact on the composting process	References
Biogas residues, pig manure, and SMS	Better tomato's seedling quality compared to commercial seedling	Meng et al. (2019)
Rice husks, pig manure, and SMS	Improved nutrition content and increased germination index	Meng et al. (2018a)
Sewage sludge, wheat straw, and SMS	Promoted the degradation of organic matter and reduced the greenhouse gas emission	Meng et al. (2017)
Sucrose and SMS	Enhanced the degradation of organic matter and compost maturity	Meng et al. (2018b)
Green waste, biochar, and SMS	The rate of humification and decomposition of organic waste is increased	Zhang and Sun (2014)

the beginning or during the composting can accelerate the overall composting process, increase the microbial activities, enrich the nutrition for microbial growth and thus, enhance the quality of SMC (Table 2.2).

2.2.2 The Use of SMS as Soil Amendment

Soil amendment with organic residues to preserve and improve soil fertilizer is a popular practice in modern agriculture. An organic amendment contributes to the restoration of eroded and degraded soils, ecological restoration, and remediation of polluted soils (Medina et al. 2015). The amendment of organic residues are reported to increase fulvic acids, humic acids, polysaccharides, water-holding capacity, cation exchange capacity, decrease bulk density, improve or maintain soil's physical and hydrological properties and soil structure, porosity, and aggregation (Marín-Benito et al. 2016; Sharma et al. 2013; Boyle et al. 1989; Abiven et al. 2009). The addition of crop residue can improve soil organic carbon accumulation and contributes to carbon sequestration by capturing carbon from the atmosphere (Follett 2001; Ghimire et al. 2017). Even with a slight increase of soil organic carbon can have large impacts on soil physical properties (Powlson et al. 2011). In addition, organic residues favor soil gas and water exchange, expansion of plant root zone, and population of soil bacteria that reduce soil erosion and runoff (Marín-Benito et al. 2016).

Among the organic residues potentially applicable to soil as organic amendments are those from municipal wastes (food wastes, gardening wastes, and sewage sludge), crop production (crop residues), animal husbandry (manure and sludge), and food processing activities (brewery and mushroom cultivation). Recently, the application of SMS as soil amendments and soil bioremediation, with or without prior composting process is getting widespread due to its high contents of organic matters, low toxic elements, and adsorption ability (Marín-Benito et al. 2016). The SMS is a co-product from mushroom cultivation, thus it is readily available and

inexpensive to obtain. Therefore, it is one of the ideal material for soil amendments and agrichemical control.

The residues from mushroom cultivations are used either fresh or after composting process. This section will discuss the use of SMS directly as soil amendments without any composting process. The substrate from *Pleurotus pulmonarius, Pleurotus ostreatus, Agaricus subrufescens* (syn. *A. blazei*, *A. brasiliensis*), and *Lentinula edodes* are some of the SMS that have been tested as organic amendments in soils (Chiu et al. 1998; Ribas et al. 2009; Nakatsuka et al. 2016). The characteristics of SMS vary depending on the varieties of mushroom and components. Switch grass, rice and wheat straw, cottonseed hulls, oat straw, sawdust, wood sawdust, chicken manure, plant residues, water, ammonium nitrate, urea, gypsum, calcium carbonate, and limestone are some of the typical components found in mushroom spawns (Zhang et al. 2002; Royse et al. 2004; Pathmashini et al. 2009). These residues are used fresh immediately after being discarded from mushroom production, whereas others are used after composting to stabilize them. The application of SMS as soil amendments or conditional for crop production is a favorable strategy for the sustainable recycling of the SMSs. In addition, it increases crop yield. A study reported, the application of SMS to sweetcorn, cabbage, and potato increases yields by 38%, 82–96%, and 26–46%, respectively (Stewart et al. 1998b).

The applications of SMS to soil, especially in low organic matter content, have numerous benefits. It has positive effects on soil microbial populations, increases organic content, improves soil structure and fertilizer. In addition, soil application of SMS is a good source of essential nutrients such as N, P, and K for healthy plant growth (Ribas et al. 2009). Table 2.3 shows various studies on the influence of SMS on soil chemical and physical properties.

2.2.3 Application of SMS for Soil Remediation

The application of organic residues to soil is used as biosorbent of inorganic and organic contaminants, biodegradation, and bioremediation of pesticides and organopollutants (Marín-Benito et al. 2016). Research indicates biosorbents acquired from lignocellulose (main constituents of plant) have high efficiency for removing hydrocarbons such as phenols, PAHs, organic pesticides, and herbicides from water with benefit of good adsorption capacities, ease of alteration, and low cost (Ngo et al. 2015). Plant residues such as wood chips, ryegrass roots, orange peels, bamboo leaves, and pine needle are of great potential as natural biosorbents for polycyclic aromatic hydrocarbons (PAHs) (Chen et al. 2011). Therefore, agricultural wastes are a good biosorbents alternative as it is low-cost and sustainable. Biosorbents made from agriculture wastes are not only sustainable but also divert large quantity of wastes from landfill.

Numerous studies have suggested the potential uses of SMS as biosorbent to bioremediation of pesticide and fungicide contaminated soil. Mushrooms are ligninolytic fungi which produce lignocellulosic enzymes that plays an important

Table 2.3 Influence of SMS on soil chemical and physical properties

Soil physicochemical properties	Changes after application of SMS as soil amendments	References
Structure	Improve soil aggregation	Boyle et al. (1989)
Organic matter (SOM)	Increases soil organic matter content	Medina et al. (2012)
Carbon content (SOC)	Soil organic carbon content significantly increased with the addition of spent mushroom substrates ($P < 0.05$)	Medina et al. (2012)
Aggregation	• Induce development of a granular microstructure at 15–20 cm soil • Increase soil porosity high porosity and a high fractal dimension • Increase spil aggregate stability by 13–16%	Nakatsuka et al. (2016), Stewart et al. (1998a), López Castro et al. (2008)
Bulk density	Decreasing soil bulk density (by 0.05–0.25 g/cm^3 at 100 mm depth)	Nakatsuka et al. (2016), Stewart et al. (1998a), López Castro et al. (2008)
Nutrient source	Good source of N, P, and K from *A. subrufescens* Increases soil available P content for 1.3–1.6 times	Ribas et al. (2009)
Infiltration rate	Increases water infiltration rate by 130–207 mm/h	Stewart et al. (1998b)
Clod and surface crust formation	Reduces clod and surface crust formation by 16–31% and 18–94%, respectively	
Water content	Increases soil water content by 0–7% w/w	
Temperature	Reducing diurnal temperature changes	
pH	No significant changes	Medina et al. (2012)
Electroconductivity (EC)	No significant changes	
Soil respiration	Increases soil respiration rate	
Phosphatase activity	Increases phosphatase activity	
Catalase activity	No significant changes	
Urease activities	No significant changes	

role in natural recycling and bioremediation (Phan and Sabaratnam 2012). A study compared the degradation of pentachlorophenol ability for SMS of *P. pulmonarius* with a number of fungi (*Armillaria gallica, A. mellea, Ganoderma lucidum, Lentinula edodes, Phanerochaete chrysosporium, Pleurotus pulmonarius*, a *Polyporus* sp., *Coprinus cinereus*, and *Volvariella volvacea*) under batch cultivation system and reported better performance in SMS as compared fungi (Chiu et al. 1998).

The SMS is reported to have the ability of retaining a wide number of biocide, fungicide, and pesticide compounds such as azoxystrobin, benalaxyl, carbendazim, cyprodinil, iprovalicarb, mancozeb, metalaxyl, penconazole, pentachlorophenol, pyrimethanil, and tebuconazole via adsorption–desorption mechanism (Chiu et al. 1998; Bechmann et al. 2008; Marín-Benito et al. 2009a, b, 2012). A laboratory test showed, 5% of spent mushroom compost degraded naphthalene completely in PAHs contaminated sandy loam soil in 2 days at 80 °C (Jewell and Kubota 2005). Fresh substrates from *Agaricus bisporus, Pleurotus* spp., shiitake, and also composted *Agaricus bisporus* substrates recorded significant adsorption efficiency for hydrophobic compounds—cyprodinil, penconazole, and tebuconazole (Marín-Benito et al. 2016). An on-site study of soil contaminated with fungicide (100 µg fungicide per g soil) were treated with 10, 20, and 30%, w/w reported the fungicide concentration reduced to half of initial level after 1 month of SMS with the highest degradation of carbendazim and mancozeb in 30% and 20% SMS treatment, respectively (Ahlawat et al. 2010). Therefore, SMS is used as a strategy for the prevention and/or control of pesticide contamination to soil and water by modifying the fate of pesticides in soil.

2.2.4 *Evaluation of SMS as Biocontrol Agents in Agricultural Application*

Currently, overuses of chemical fertilizer and pesticides in agriculture practices since the use of biofertilizer and biocontrol agents produce slow results and expensive. Biocontrol agents can serve as an alternative to chemicals for integrated agricultural disease management systems. Therefore, there is a demand for new bioactive compounds that are cheap and contain high bioactive compounds as biocontrol agents. To achieve that purpose, it does prove that selectivity of the bioactive compounds as biocontrol agents is an effective strategy for disease control. However, this is not a good strategy due to fast growing different types of plant disease in the fields. This is at risk because growers are characterized by lack of patience and make them difficult to prefer biocontrol agents instead of potent pesticides due to income stability. SMS is known to contain bioactive compounds that have antimicrobial properties against pathogens. It has the potential to be developed as biocontrol agents against plant diseases and also treat the unfertile and contaminated soils. A lot of studies have been done to determine SMS rich with antimicrobial properties against fungal and bacterial infections to reduce plant pathogens. Some studies make a correlation of the mushroom substrate with types of mushroom species for production of antimicrobial agents against food borne and clinical pathogens including fungus. Most of SMS showing effectiveness as biocontrol agents either with direct use as soil conditioner or SMS extracts against soilborne and plant pathogens. During growth of the mushroom itself, the growing mycelium of the mushroom excreted nutritious compounds such as high organic matters, bioactive compounds,

and lignocellulosic degrading enzymes after a serial cultivation of mushroom which can contribute to effectiveness of SMS as biocontrol agents against soilborne pathogens causing plant diseases (Zhu et al. 2012; Nidadavolu et al. 2012; Zhao et al. 2017).

Selected edible mushrooms are not only rich in nutritional values but also consists of some therapeutic values such as polysaccharides and antimicrobial peptides that show great antimicrobial properties (Bastida et al. 2016; Shen et al. 2017). The antimicrobial activity was detected during in vitro studies from the extracts of *L. edodes* followed by *Pleurotus ostreatus* and *Hypsizigus tessulatus* against pathogenic bacteria (Chowdhury et al. 2015). As extract of *L. edodes* consists of lentinamicin (octa-2,3-diene-5,7 diyne-1-ol), β-ethyl phenyl alcohol, and lentin that known as an antifungal protein (Komemushi et al. 1996; Ngai and Ng 2003). Polysaccharides from *L. edodes* have been known to be the most potent antitumor and immunomodulating substance (Xu et al. 2014). Water-soluble polysaccharides was also isolated and purified from SMS of *L. edodes*, which showed strongest inhibition against *E. coli* (Zhu et al. 2012). The water extracts of SMS from *Lentinula edodes* consist of oak sawdust substrate supplemented with 20% rice bran inhibited mycelial growth of *Phytophthora capsici* by suppressing blight disease through multiple effects including antifungal activity, plant growth promotion, and defense gene induction (Kang et al. 2017). The presence of oxalic acid was detected as the main organic acid compound in the extract for controlling Phytophthora blight disease of pepper seedlings caused by *Phytophthora capsici* at a minimum concentration of 200 mg/L (Kang et al. 2017). Furthermore, the water extract of SMS from cultivation of *L. edodes* also suppressed the development of lesions caused by rice blast fungus [*Pyricularia oryzae* (syn.: *Magnaporthe grisea*)] (Ishihara et al. 2018).

Several studies have reported the water extracts of SMS from cultivation of *Hericium erinaceus* showing high antibacterial activity against *Pectobacterium carotovorum* subsp. *carotovorum, Agrobacterium tumefaciens, R. solanacearum, Xanthomonas oryzae* pv. *oryzae, X. campestris* pv. *campestris, X. axonopodis* pv. *vesicatoria, X. axonopodis* pv. *citiri*, and *X. axonopodis* pv. *glycine* (Kwak et al. 2015). In some reports, water extracts of SMS from different *Lentinula* species have been used to suppress plant diseases. Martínez et al. (2015) studied the effects of substrate components on the cultivation of *Pleurotus ostreatus*, which contribute to the degree of inhibition against pathogenic bacteria. From their studies, the SMS extracts supplementation with medicinal plants such *Mentha piperita* L. contributed to the largest zone of inhibition against pathogenic bacteria when compared with standard antibiotics. This is also supported by Bastida et al. (2016), where the growth of *L. edodes* and *P. ostreatus* showing a good potential for the production of antimicrobial agents with different inhibition zone under different types of cultivation substrates against *Escherichia coli, Salmonellatyphimurium, Staphylococcus aureus*, and *Micrococcus luteus*.

There is a good opportunity to reuse SMS to produce healthy and high yield of vegetable crops. Further anaerobic recompose of SMS contribute more effectiveness for antagonistic effect against soilborne and foliar simultaneously increase plant yield due to change of materials composition after composting like ascorbic acid,

total soluble solids, and protein content when compared with standard fertilizers (Ahlawat et al. 2010). Further anaerobic recompost of white button SMS showed inhibition of Lecanicillium due to presence of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* that showed an antagonistic effect on the growth of *L. fungicola* (Riahi et al. 2012). Most of the identified fungi in the SMS are those showing effectiveness for composting agrowaste materials. The presence of *Trichoderma* sp. in the SMS not only for composting the substrates but also contributed to antagonist effects against pathogenic microbes in the soil. An antagonistic study of SMS, which contains *T. harzianum* and *T. viride* as biocontrol agents against *Rhizoctonia solani*, showed high germination rate when treated with SMS of seed tomato before planting (Verma et al. 2015).

2.2.5 SMS in Enhancing Accumulation of Health Beneficial Secondary Metabolites in Plants

Inclusion of sufficient fruits and vegetables in daily diet has been associated with a decrease in incidence of cardiovascular diseases (Brat et al. 2006). Health beneficial effects of fruits and vegetables are believed due to the presence of secondary metabolites including vitamins and polyphenolic compounds. Many studies indicated potential health beneficial effect of secondary metabolites in modulating gene expression (Nicholson et al. 2010; Calabriso et al. 2016; Horie et al. 2019). These secondary metabolites exhibit a wide range of biological activities including antioxidant, anti-inflammatory, anti-proliferation, and anti-obesity (Sentkowska and Pyrzyńska 2019; Cao et al. 2019; Yamagata et al. 2018). Therefore, it is favorable to enhance their presence in fruits and vegetables. In response to biotic and abiotic factors, plants synthesize secondary metabolites for a variety of functions such as self-protection mechanism against pathogens, attractants for pollinators, and seed-dispersing animals, allelopathic agents, antioxidant, protection against UV lights, and signaling molecules (Jaganath and Zainal 2016; Agati et al. 2012). With the advancement of current understanding in plant metabolism, researchers have manipulated biotic and abiotic factors to initiate biochemical pathway cascade leading to accumulation of secondary metabolites (Jaganath and Zainal 2016).

Several studies have attempted to increase secondary metabolites through supplementation of SMS and recompose SMS (SMC). The use of SMC can increase the content of essential oil of German chamomile (*Matricaria recutita* L.) flower (Vahid Afagh et al. 2019). The addition of 10–15% of SMC in growth media significantly increased the content of essential oil compared to control (no SMC) (González-Marcos et al. 2015). However, the lower percentage of SMC in growth media has no significant changes in essential oil contents compared to control group (Vahid Afagh et al. 2019). Analysis of GC and GC-MS indicated that the relative level of essential oil composition, namely trans-β-farensene, α-bisabololeoxide B, chamazulen, farnesol, α-bisabolole, increased with increasing the percentage of SMC in growing

media (Vahid Afagh et al. 2019). Such increment in essential oil content and its composition may be due to the effect of SMC on plant's enzyme involved in the synthesis of essential oil (Vahid Afagh et al. 2019). SMC is high in salinity which limits its use in agriculture application. The salinity of SMC can be solved by leaching technique (Gonani et al. 2011). Leached SMC has less salinity and maintain the content of nitrogen, carbon, and other essential elements (Gonani et al. 2011). The addition of leached SMC at 60% of growth media significantly increased essential oil in German chamomile flowers, but not at a lower percentage (Vahid Afagh et al. 2018). In addition, application of leached SMC in growth media also increased total phenolic content, total flavonoids content, and intracellular antioxidant enzymes (peroxidase and superoxide dismutase) content as compared to control group (Vahid Afagh et al. 2018).

The use of SMS of Oyster mushroom and compost of Button mushroom as growth media showed an increase in protein and carotenoid contents in fruits of *Capsicum annuum* L. compared to untreated group (Roy et al. 2015). The increase in carotenoids is favorable since these compounds exhibit various health benefit properties including protection against several types of cancer, cardiovascular, and photosensitive diseases (Fiedor and Burda 2014). In tomato plant model, a combination of SMS compost (2.5 t/ha) and recommended inorganic fertilizer increased produces quality through enhancement of total protein and vitamin C contents as compared to application of recommended inorganic fertilizer alone (Ashrafi et al. 2015). The application of this combination fertilizer also increased in yield (Ashrafi et al. 2015). These findings indicated synergistic effect of both SMS compost and recommended inorganic fertilizer in improving produces quality (Ashrafi et al. 2015). Bio-fortification of SMS with earthworm grazed and *Trichoderma harzianum* (EGTHB-SMS) was found to enhance total phenolic and flavonoids contents in ripe tomato fruit (Singh et al. 2018). Besides the yield, bio-fortified SMS also increases mineral content and antioxidant capacity of tomato fruits as compared to control group (grown on SMS only) (Singh et al. 2018). This study indicated the high potential of EGTHB-SMS in improving fruits' quality through enhancement of bioactive compounds, nutrients, and antioxidant activity which can benefits to human health.

Micronutrients are important for plant growth as well as for human health. SMC contains various micronutrients and the use of this substrate as growth media can increase the content of micronutrients including Fe, Zn, Mn, and Cu in eggplant seedling (Singh et al. 2018). In another study, application of 30% perlite + 70% fresh SMC as growth media increased the content of Fe and Mn in *Capsicum annuum* L. seedling as compared to perlite alone. *Capsicum annuum* L. seedling grown in 100% fresh SMC showed higher Zn content than those grown in 100% perlite and 100% turf (Sonmez et al. 2014). However, the content of micronutrients in fruits is unknown. Although there are limited number of studies, SMS showed potential as elicitors in plants to enhance accumulation of secondary metabolites such as vitamins and polyphenolic compounds. The elicitation of secondary metabolites is important not only for plants, but also for humans to gain health benefits of these metabolites. Therefore, future research should be navigated to explore the effect on

plant's secondary metabolites through manipulation of SMS supplemented with other materials as a part of growth media.

2.3 Conclusion

SMS are a typical by-product of mushroom production, which is rich in nutrient and bioactive compounds such as extracellular enzymes, secondary metabolites, and organic matters for microbial growth. Therefore, there has been considerable discussion recently about the potentials of using SMS for production of value-added products for agriculture application. Detection of bioactive compounds in the SMS due to biological activity of microbial community in the SMS contributed as good biocontrol agents against soil and plant pathogens where biocontrol agents offers an important alternative to synthetic chemicals. Additionally, direct application of SMS also gives a good benefit on soil physicochemical properties such as increases of organic content, improve soil structure, nutrients availability, and potentially useful for the bioremediation of pollutants. The benefits may contribute to increase plants growth and plant's secondary metabolites. Therefore, application of SMS would be significant to develop as new biomaterials for a healthy, safe, and sustainable agriculture practices.

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Chapter 3
Biological Treatment of Agro-Industrial Waste

Nur Izyan Wan Azelee, Nor Hasmaliana Abdul Manas, Daniel Joe Dailin, Aizi Nor Mazila Ramli, and Shalyda Md Shaarani

Abstract Agro-industrial waste is mostly composed of lignocellulosic biomass, which is inexpensive, renewable, and abundant. It provides a unique natural resource for cost-effective bioenergy collection. Agro-industries generate a wide number of waste products either in the form of solid or liquid. The production of agro-industrial waste is growing worldwide and these wastes cannot be disposed of directly on the ground without any treatment, as they will cause serious environmental concerns. The problem of disposal and management of these wastes is a major issue especially in developing countries nowadays. Hence these agro-industrial waste must be treated before discharging or reuse for other purposes by effective methods. The conventional methods require the use of harsh and toxic chemicals with high processing cost and high waste management cost. In serious consideration of the worldwide economic and environmental pollution issues, there has been increasing research interest in the management of the agro-industrial waste proposing value-added green technologies. Biological treatment is seen as one of the promising green

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biotechnologies that gives less harm to the environment while balance out the ecosystem. The biological treatment utilizes microorganisms mainly from the bacterial and fungal species to cope with the issue raised and also act as bioremediation. This chapter begins with an overview of agro-industrial waste and further describes a number of biological treatments performed together with its advantages and disadvantages. This chapter finally deals with the possibility of creating a sustainable practice in industries processing agricultural products. Several suggestions and recommendations for future considerations are also thoroughly highlighted. The ultimate goal of this biological treatment chapter is to prepare the agricultural waste for a cleaner process toward a better and safer product.

Keywords Agro-industrial waste · Biological treatment · Sustainable · Green biotechnology · Bioremediation

3.1 Introduction

Agro-industrial activities could represent one of the primary sectors of the economy of a country, especially those that are characterized by high production rates of agricultural commodities (Pellera and Gidarakos 2017). Agricultural wastes are derived from agricultural production and products' processing, livestock breeding, and rural households while industrial wastes are residues, dust, and other wastes discharged in the process of industrial production (Mo et al. 2018). Therefore, agro-industrial wastes refer to all by-products and residues generated from crops, livestock, forestry, and agro-based industries. Figure 3.1 portrays the sources of these agro-industrial wastes as reported by Andri et al. (2018). Agricultural residues could be distributed into monosaccharide and disaccharide, starch, structural polysaccharide, and protein or lipid-rich sources. As for industrial residues, they could be

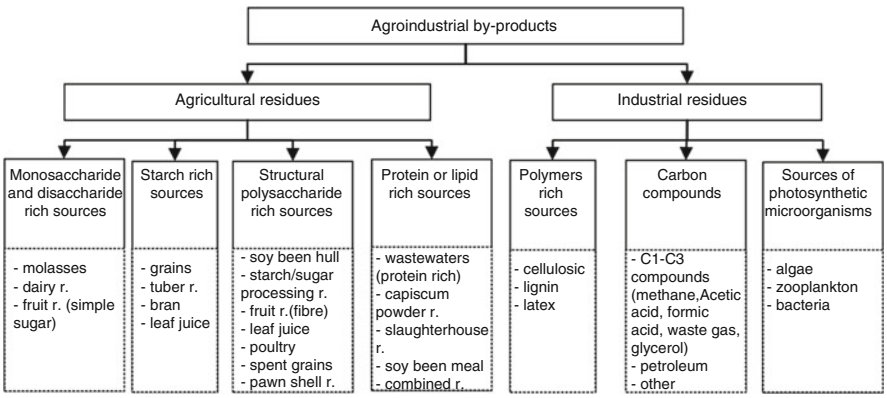

Fig. 3.1 Classifications of agro-industrial wastes according to different sources (Andri et al. 2018)

classified as polymers rich sources, carbon compounds, and photosynthetic microorganisms.

Numerous industries produce massive amounts of wastes, consisting elevated levels of biomass, suspended solids, organic matter, effluent, and sludge which create disposal problems and cause environmental pollution in several ways (air, soil, and water) (Anastopoulos et al. 2017). Common agro-industrial biomass wastes include rice husk, sugarcane bagasse, wheat straw, spent coffee and tea, oil palm wastes, peanut hull, fruits and vegetable peels, seeds, and others. Meanwhile, relevant effluents of agro-industrial activities are wastewaters deposited from mills, winery, and starch industries as well as slaughterhouses. These wastes could be further categorized into solids, liquid, and mixed forms. Table 3.1 demonstrates the agro-industrial wastes from diverse industries according to the respective types.

Most of the agro-industrial wastes are untreated and underutilized, for which majority reports it disposed of either by burning, dumping, or inappropriate landfilling (Sadh et al. 2018). These untreated wastes lead to the release of greenhouse gases that eventually resulted in environmental impact and climate change. The generated wastes might not only be biodegradable in nature but also rich in nutrient components such as carbohydrates, proteins, fibers, and minerals depending upon their sources (Andri et al. 2018; Panesar et al. 2015). Despite being abundantly found and possessing nutritive values, however, the recalcitrant properties of these compounds impede the utilization of the fermentable components in the wastes. In addition, these wastes might also contain organic and/or inorganic (heavy metals and metalloids) pollutants and pathogens that render them unsuitable for further use (Markou et al. 2018). Effective management/treatment is thus necessary to curb these arising problems. Instead of "wastes," these residues should be considered as "raw material" for other industrial processes. The presence of nutrients such as sugars, minerals, and proteins in the agro-industrial wastes offers suitable conditions for the prolific growth of microorganisms. The microorganisms could reuse the waste as raw materials or substrate for their growth via fermentation processes and subsequently produce an array of significant beneficial compounds. The utilization of these agro-industrial wastes would not only contribute to the recycling of waste but also alleviate the production cost and reduce pollution to a great degree (Sadh et al. 2018).

3.2 Biological Treatment

Biological treatment is any kind of treatment, which involves the use of any microorganisms or the enzymes secreted from the particular microorganisms. Biological treatment contradicts conventional treatment which uses harsh and toxic chemicals for their treatment. Biological treatment is more specific and selective in nature and should be able to reduce the loss in carbohydrates significantly (Kusi et al. 2018). The best biological treatment is where it can degrade most of the unwanted compounds while preserving most of the valuable compositions within a short span

Table 3.1 List of industries producing agro-industrial wastes

Types	Industries	Sources	References
Solid	Palm oil milling	Empty fruit bunch (EFB), oil palm frond (OPF), palm press fiber (PPF), decanter cake (DC), and oil palm trunk (OPT)	Mamimin et al. (2019)
Liquid	Palm oil industry	Palm oil mill effluent (POME)	Jayakumar et al. (2017)
Solid	Coconut	Coir dust/pith, shell, and copra cake/meal	Go et al. (2019)
Solid	Paddy rice	Husk, bran	Go et al. (2019)
Solid	Pineapple canning	Core and peel	Sanguanchaipaiwong and Leksawasdi (2018)
Solid	Pineapple juice industry	Pomace along with peels, crown, and core	Banerjee et al. (2018)
Liquid	Pineapple juice industry	Liquid effluents	
Solid	Wine production; produced during the crushing, pressing, and draining stages	Grape marc; mostly composed of stalks, skins, and seeds	Pellera and Gidarakos (2017)
Solid	Olive oil extraction	Olive pomace; olive pulp, peel, pieces of pit and an oil content	Carlini et al. (2015), Batuecas et al. (2019)
Mixture; liquid and solid	Olive oil manufacturing	Olive wet husk, two phases olive mill waste (TPOMW), or alperujo	Navas et al. (2015), Hernández et al. (2019)
Liquid	Olive oil manufacturing	Olive mill wastewater	Batuecas et al. (2019)
Liquid; high amount of total solids and starch	Potato processing	Potato processing wastewater	Singh et al. (2019)
Solid	Cotton processing	Burs, stalks, leaves, and immature cottonseed	Pellera and Gidarakos (2017)
Liquid	Cotton industry	Cotton seed oil	Tacin et al. (2019)
Solid	Sugarcane industry	Bagasse, filter cake	Saha and Ghosh (2019)
Solid	Sugarcane juice filtration	Press mud	Anastopoulos et al. (2017)
Liquid	Sugar manufacturing	Molasses	
Liquid	Sugar manufacturing	Sugar mill wastewater	
Solid	Corn milling	Corn bran (wet), fiber (dry)	Go et al. (2019)
Solid	Soybean oil extraction	Soybean meal	Gerliani et al. (2019)
Liquid	Dairy industry	Milk whey	Martínez-Ruano et al. (2019)
Liquid	Poultry slaughterhouses	Blood and wastewater washed off from poultry	Latifi et al. (2019)

of time. Nowadays, biological treatment is getting more attraction from around the globe, especially from the industries as they have the potential to reduce the wastewater treatment cost and more environmental-friendly in nature.

The increasing volume of agro-industrial waste has become a major problem for the agro-based industries. They are searching for a better solution to solve the issue as the waste tends to spoil easily due to the high moisture content. Moreover, agro-waste is an attractive alternative source for renewable energy without competing with the food sources (Yadav et al. 2019a). Asia region statistically produces the largest amount of rice and wheat straw while America generally produces the largest amount of corn straw and sugarcane bagasse (Kusi et al. 2018). However, the recalcitrance lignin structure of agro-wastes impedes its potential to be utilized directly from the source. They have to undergo a certain treatment process prior to their viability of producing valuable products. This treatment is meant to open up the rigid structure of the cell walls and increase the accessibility of the cellulose and hemicellulose by the enzymes.

There are many types of biological treatment currently been studied and mostly utilizes bacteria and fungus or yeast to treat the agro-waste. Extensive studies have and still ongoing toward achieving the best biological treatment for solving the abundant agro-industrial waste issues while producing valuable products. However, due to the complex and complicated structure of these agro-wastes, it is a real challenge and quite impossible to obtain a single optimized biological treatment for all types of agro-wastes. Even the same type of agro-waste may result in different findings from the same treatment due to the inconsistency in the chemical composition. Factors such as the harvesting age, type of soil used, weather condition, parts of the plant taken, and many more will contribute to the difference in composition and structure. Additionally, improvement of the biological treatment has been carried out by metabolic engineering approach. This approach applies the combination of knowledge between synthetic biology, systems biology, and "omics" which consists of genomics, proteomics, transcriptomics, and metabolomics (Cristobal-Sarramian and Atzmüller 2018).

3.2.1 Fungal Treatment

Fungal treatment is one of widely used biological treatment for agro-waste to replace the conventional chemical treatment (Carrere et al. 2016). The presence of lignin degradation enzymes such as laccase, lignin peroxidase, and manganese peroxidase in the fungi cell naturally helps to overcome the agro-waste recalcitrant structure (Rouches et al. 2016). The well-known fungi that have been used for biomass treatment are the white rot-, brown rot-, and soft rot fungi (Sindhu et al. 2016). Among these three, the white rot fungi are the mostly studied fungi have been reported to produce high enzyme activities for ligninase, laccase, manganese peroxidase, and lignin peroxidase (Pramanik and Sahu 2017). These three types of fungi have their own respective treatments on the biomass, i.e., white rot fungi selectively

degrade lignin, brown rot fungi degrades polysaccharides and lignin while soft rot fungi simultaneously degrades polysaccharides and lignin (van Kuijk et al. 2015). The efficiency of fungal treatment depends on several factors such as particle size of the biomass, temperature, moisture content, pH, inoculum concentration, and treatment time (Meehnian et al. 2016). Prior to the fungal treatment with the biomass, the fungal growth condition, and media nutrient concentrations needs to be optimized first to enable maximum production of targeted hydrolytic enzymes (Chenthamarakshan et al. 2017).

Additionally, during the fungal treatment toward biomass, crude protein may be found due to the incorporation of nitrogen from the biomass into the fungal protein that causes nitrogen enrichment from the degradation of other nutrients. The efficiency of the fungal treatment is very hard to determine and is highly dependent on the type of biomass used. In order to justify the most efficient fungal treatment, several types of fungi must be treated with one similar biomass and evaluate the production yield for each treatment. The highest production yield achieved can then be claimed as the most efficient fungal treatment. Some of the fungal treatments were coupled with bacterial treatment and the result obtained showed an increase in production yield (Yadav et al. 2019b). However, despite the environmental-friendly process of this fungal treatment on agro-waste, it is time consuming and some carbohydrates will be consumed for the metabolism of fungus and thus may lose some of the valuable products after the treatment (van Kuijk et al. 2015). Nevertheless, the researcher is developing some genetically modified microorganisms, especially to enhance biomass degradation. Table 3.2 below shows the list of recent biological treatment using fungal treatment in various biomass. (Table 3.2).

Table 3.2 List of recent biological treatment using fungal treatment on various biomass

Biomass	Type of fungus	Enzyme involved	Product(s) observed	References
Wheat straw and pearl millet straw (India)	*Chaetomium globosporum*	Laccase	Reducing sugar and biogas production	Yadav et al. (2019a)
Pearl millet and wheat straw (India)	*Pleurotus ostreatus*	Laccase	Reducing sugar and biomethanation production	Yadav et al. (2019b)
Hay	*Neocallimastix frontalis*	Lignocellulolytic enzymes	Biogas production	Dollhofer et al. (2018)
Rice straw	*Coprinopsis cinerea*	Laccase, cellulase, and xylanase	Enzymes' activity profile	Zhang et al. (2018)
Copra meal, sugarcane bagasse, and rice straw	*Aspergillus tubingensis* NKBP-55	Cellulase	Fermentable sugars	Prajapati et al. (2018)

3.2.2 Yeast Treatment

Yeast treatment is normally been used for the fermentation process where it converts the sugar-containing hydrolysate from the hydrolysis process in bioethanol. The high carbohydrate and nutritional content make agricultural waste as promising biomass for microbial growth of yeast. Nevertheless, yeast has become one of the pioneer microorganism being used as an essential component for human activities such as in bread making, wine, beer, and other distilled beverages. It is also been used widely as a research model organism to study the molecular mechanisms within a cellular process. The *Saccharomyces cerevisiae* is the most well-known type of yeast among others and is being used industrially for the large-scale production of certain biochemicals. Normally, the utilization of yeast in bioprocessing is carried out subsequently after the pretreatment and hydrolysis process of the biomass. The main function of yeast is to convert the sugar(s) produced in the hydrolysate after the hydrolysis process into biogas in what is known as microbial fermentation process. The efficiency of the pretreatment and the hydrolysis process will determine the success of the yeast fermentation process. There is also a research that utilizes yeast to enhance the nutritive value in some biomass (Kewan et al. 2019). This is one of the broad spectrums of the yeast applications. However, the utilization of yeast in a fermentation technology is not a simple process as it produces inhibitors in the lignocellulosic hydrolysate such as furfural or acetic acid which can interfere the cellular growth and gives diverse metabolic process (Cristobal-Sarramian and Atzmüller 2018). Some approach by using a genetic engineering method has been used to enhance the yeast resistance toward these types of inhibitors and thus may improve the production yield. Table 3.3 shows the recent studies utilizing yeast as a biological treatment for agro-waste. (Table 3.3).

3.2.3 Bacterial Treatment

Due to the massive investigation of fungal treatment on biomass, the ability of bacterial treatment has been overlooked. The longer time taken for fungi to grow

Table 3.3 Recent studies utilizing yeast as a biological treatment for agro-waste

Biomass	Type of yeast	Product observed	References
Sida acuta (Thailand Weed)	*Saccharomyces cerevisiae* K35	Biogas	Siripong et al. (2019)
Wheat straw	*Candida guilliermondii*	Xylitol	Cristobal-Sarramian and Atzmüller (2018)
Moringa tree stalks	*Saccharomyces cerevisiae (yeast) coupled with Trichoderma reesei (fungi)*	Improved nutritive value for lamb feed	Kewan et al. (2019)

and secrete its enzyme is not an industrial interest. Due to this limitation of fungal treatment, the potential of bacteria to be used as a treatment source for agro-waste can be explored further. The most popular bacteria used are from the *Bacillus* sp., *Sphingomonas* sp., *Cellulomonas*, and *Zymomonas* spp. Some bacterial strains are able to secrete lignin-degrading enzymes such as laccase and manganese peroxidase (Zhuo et al. 2018) even though the strength of treatment is lower than the fungal treatment. This is probably due to the low amount of degrading enzyme(s) secreted from the bacteria as compared to fungi and the fragility of the bacteria to the changes in the environment. Bacteria may pose different degradation reactivity toward lignin compared to the fungi. Furthermore, most of the bacterial treatment cannot act independently on the biomass due to the direct interactions occurring between the bacterial enzymes and the lignin of the biomass. However, they can slightly improve enzymatic digestion of the biomass after the outer structure breakdown by other physicochemical treatment. Other strategy to improve the performance of bacteria treatment is to extract out the enzyme(s) inside the bacterial cell and further purified the enzyme(s) prior to the treatment. Reports on the bacterial treatment for agro-waste still remain scarce as researchers did not put more effort toward using bacteria for their treatments. Table 3.4 shows the most recent studies utilizing bacteria for the treatment of agricultural waste. (Table 3.4).

3.2.4 Fermentation Technology

Fermentation is the final process of converting agro-waste or biomass into bioethanol by various microorganisms such as bacteria and fungi. This conversion process of biomass involves metabolic breakdown that releases several compounds which are known as secondary metabolites. The secondary metabolites released can

Table 3.4 The most recent studies utilizing bacteria for the treatment of agricultural waste

Bacteria	Source	Biomass	References
Pandoraea sp. B-6	Bamboo slips	Corn stover	Zhuo et al. (2018)
Paenibacillus chitinolyticus CKS1, *Streptomyces fulvissimus* CKS7	Soil	Tobacco waste	Aneta et al. (2019)
Bacillus subtilis, Bacillus licheniformis, and *Bacillus amyloliquefaciens*	Fermented pig manure	Waste straw consortium	Shen et al. (2019)
Bacillus subtilis	Prawn and fish scales	Straw (wheat and pearl millet)	Yadav et al. (2019b)
Enterococcus, Sporanaerobacter, Para clostridium, and *Clostridium_sensu_stricto_1*	Sludge (a municipal wastewater treatment plants), cow dung, pig manure, and camel dung	Corn straw, rice straw, corncob, and sorghum stalk	Li et al. (2018)

consist of antibiotics, enzymes, and some growth factors. The fermentation process is highly dependent on the type of microorganism's strain used. Some of them are able to one type of sugar, e.g., either glucose or xylose while some are able to ferment both, e.g., glucose and xylose to produce bioethanol. Up to date, several microorganisms have been used for the fermentation process which includes genetically engineered yeast and bacteria, co-culturing and sequential use of yeast strains and also protoplasts fusant strains as glucose and xylose fermenting yeast (Pramanik and Sahu 2017).

3.2.4.1 Submerged Fermentation

Submerged fermentation (SF) is defined as a biological process of producing biomolecules in which microorganisms and enzymes together with other reactive components are submerged in a solution. The liquid solution may be in the form of alcohol, oil, or a nutrient broth. It also involves free-flowing liquid substrates such as molasses and broths. Microorganisms such as fungi will be placed in a closed container or tank containing these rich media. The submerged fermentation process can be divided into two types: (a) aerobic fermentation and (b) anaerobic fermentation with two methods of operation (a) fed-batch fermentation and (b) continuous fermentation. The first critical step in this submerged fermentation is to obtain an adequate number of microorganisms to be used as an inoculum for the subsequent process (Zhang et al. 2019).

3.2.4.2 Solid-State Fermentation

Solid-state fermentation (SSF) is defined as a biological process that involves the growth of microorganism on a dry medium (absence of water) of non-soluble substrate or solid biomass (Sadh et al. 2018). The SSF has already been used by our previous ancestors for natural food processing and currently is regaining more attention due to the increasing number of agro-waste available. These agro-wastes which are rich in valuable composition can be considered as "raw materials" for the SSH instead of "wastes." The existence of these valuable and nutritional compound inside the agro-wastes makes them a suitable platform for the prolific growth of microorganisms. A successful SSF depends on many factors such as microorganism (s) used, biomass, aeration, and water activity. Different biomass may have better characteristics to be used as an immobilization carrier in the SSF because of the high water absorption capacity compared with other biomass. The microorganism used can consist of a single culture, mixed culture, or consortium of a mixed indigenous microorganisms. Molds or fungi are usually been used in the SSF process while bacteria and yeasts may also be used but with lower yield. Table 3.5 shows the comparison between submerge fermentation (SF) and solid-state fermentation (SSF). (Table 3.5).

Table 3.5 Comparison between submerge fermentation (SF) and solid-state fermentation (SSF) (Sadh et al. 2018; Zhu et al. 2011)

Fermentation technology	Submerge fermentation (SF)	Solid-state fermentation (SSF)
Advantages	Can be run in either aerobic or anaerobic condition. Sort period, low cost, high yield easier purification process, and simpler fermentation control	Requires simple fermenter, less effluent produce, better aeration, and less attractive environment for competing bacteria, energy reduction for stirring, and sterilization
Disadvantages	Expensive media	Heat transfer problem, e.g., accumulation of heat inside the solid decreased the productivity by time
Microorganisms involved	Bacteria, fungi, and yeast Best suited for bacteria that require a high moisture content	Bacteria, fungi, and yeast Best suited for fungi that require less moisture content
Applications	Extraction of secondary metabolites that is needed to be in liquid form Production of microbial-derived enzymes The process requires agitation Widely used in the manufacturing industries	Enzymes, organic acids, biopesticides, biofertilizers, biosurfactants, animal feed, pigments, vitamin, and antibiotics The process does not involve agitation

3.3 Advantages and Disadvantages of Biological Treatments

Circular economy is a sustainable concept which is based on minimizing waste generation and making use most of the resources. Biological treatment of agricultural biomass is seen as one of the methods that apply a circular economy concept. Traditionally, abundant agricultural wastes are burned in the field which causes the release of greenhouse gasses. Some are dumped in the landfill which causes foul odor and spread of disease. As science advances, in addition to the energy crisis, the world is facing nowadays, this biomass that was used to be called "waste" and "burden" now seems a blessing in disguise. Biomass is an abundant source of carbohydrate, which means food and energy. Turning the biomass into high-value products minimizes waste, hence in line with the circular economy concept.

Saccharification of lignocellulosic biomass is the main challenge to extract carbohydrates from the lignocellulose complex structure. Research is still on-going to find a feasible lignocellulosic pretreatment method that does not destroy the cellulose and hemicellulose, produces cellulose that is readily available for enzymatic hydrolysis, low in energy usage, less by-product, and does not inhibit the subsequent enzymatic reaction. Single or combination methods of physical pretreatment, chemical pretreatment, and physicochemical pretreatment have been widely used to release the cellulosic materials from biomass. Although it is effective in removing the recalcitrant lignin structure, these methods are expensive, require large inputs of energy, cause pollution, and some release by-products that inhibit the following enzymatic hydrolysis or fermentation process, leading to low yield of final

products (Chaturvedi and Verma 2013). Biological pretreatment is an alternative method that is categorized as safe, eco-friendly and sustainable. Table 3.6 summarizes the advantages and disadvantages of these methods.

Biological pretreatment that includes the use of microorganisms or enzymes provides an alternative for more effective method, eco-friendly, requires low energy and mild operation conditions, and most importantly does not produce inhibitor by-products. Cellulolytic and lignolytic microorganisms such as filamentous fungi are responsible for most of the biodegradation of wood materials in nature. These fungi produce cellulolytic and lignolytic enzymes and degrade cellulose, hemicellulose, and lignin. Therefore, in biological pretreatment of lignocellulosic biomass, various types of fungi were incorporated such as white-rot fungi, brown-rot fungi, and soft-rot fungi (Chaturvedi and Verma 2013). Some bacteria and actinomycetes do not produce lignolytic enzyme but they degrade lignocellulosic biomass by the action of excreted xylanase enzyme. However, the significant side reaction of cellulose degradation by these microorganisms is a major concern (Singh et al. 2008). Hence, the use of purified lignin-degrading enzymes, such as lignin peroxidase, manganese peroxidase, laccase, etc. is another alternative to ensure selective removal of lignin without affecting cellulose content (Kaur et al. 2010). Biological pretreatment of biomass is very specific and selective. In addition, the difference in mechanisms of different species of microorganisms and enzyme reaction specificity offers a broad range of selection to match the desired process goal.

When the biological method is opted for pretreatment of lignocellulosic biomass prior to conversion of cellulose and hemicellulose to the value-added product, it is important to optimize the pretreatment conditions, especially when it involves microorganisms. This is because microorganisms consume carbohydrates. This will lead to reduced yields of cellulose and hemicellulose. Meanwhile, biofertilizer

Table 3.6 Comparison between different types of lignocellulosic biomass pretreatment methods

Treatment	Advantages	Disadvantages
Biological	Eco-friendly Mild operating condition Does not produce inhibitor Specific Low-energy demand	Slow reaction rate Needs deep understanding of the mechanism Needs optimization due to lack of standardized methods Sensitive to inlet feedstock
Chemical	Fast reaction Established standard method	Harsh chemicals Expensive Cause environmental pollution Nonselective reaction; affects cellulose and hemicellulose
Physical	Does not require chemicals	Expensive equipment Limited application
Physicochemical	Fast and established method	High-energy demand Needs special equipment Produces toxic waste

does not require cellulose and hemicellulose retention. Therefore, composting using microorganisms is an advantage in the production of biofertilizer. Composting is seen as a great way to waste management. Composting not only minimizes agricultural waste, but it also helps to improve soil fertility and become a green alternative to chemical fertilizer which often destroys soil microdiversity. Although microbial degradation takes time, composting with right technique at controlled condition and optimum composition shorten the time, especially when fungi are involved. Many research has established the optimized method for composting and this could easily be applied to the field. Another drawback of biological treatment is the microorganisms are sensitive to the inlet feedstock and can be easily inhibited if the feedstock contains substances that are toxic to the microorganisms' growth (Basso et al. 2016).

Biological treatment is considered proper waste management that converts waste into high-end products with a process that produces low greenhouse gases (Basso et al. 2016) and toxic by-products. Conversion of this waste into bioenergy is one of the successful processes that could solve waste accumulation as well as providing sustainable alternatives for solving the energy crisis. Several European countries especially Germany, Denmark, Austria, and Sweden have increased their interest in these renewable energy sources and have invested to promote the development of biogas plants (Weiland 2006). Besides, biological treatment has also been proven effective to convert lignocellulosic biomass into biofuel, biofertilizer, animal feed, and bioactive compounds. Despite the disadvantages of the biological treatment process as mentioned above, research regarding this topic has been progressing and has reach maturity stage, whereby the process drawbacks could actually be catered by proper optimization. And now, it leaves a challenge for the engineers to upscale the process for large-scale application and convinces the policy makers around the world that it is time to change to a greener process for a better world.

3.4 Products from the Biological Treatments of Agro-Industrial Wastes

Commonly, the major constituents of agro-industrial wastes can be accounted for by several elements such as complex polysaccharide/proteins, carbohydrates, and polyphenolic constituents, which is considered as high strength of organic pollutants that could cause an adverse effect on the environment. One of the most efficient means of *lowering* environmental issues in agro-industries is through recycling and clean technology. Execution of this practice targets to constrain environmental pollution and other associated problems in addition to encourage economic benefits, such as the conversion of wastes to added value by-products (Prasertsan et al. 2007). Assuming that the availability and the lavish quantity of nutritious agro-industrial wastes throughout the year, there is a great interest on the recycling of these wastes for the production of other value-added compounds with the expectancy of reducing the production costs. The potentials of agro-industrial wastes are presented in Table 3.7.

Table 3.7 The potentials of agro-industrial wastes

Application	Agro-waste	References
Biofuel	Rice straw, wheat straw, corn straw, and sugarcane bagasse	Kim and Dale (2004), Georgieva et al. (2008), Li et al. (2010)
Enzyme production	Banana peel, wheat bran, rice bran, orange peel, and sugar cane bagasse, corn cob	Ravindran et al. (2018), Bharathiraja et al. (2017)
Bioactive compounds	Tomato, olive, apple, banana, guava, mangosteen, and jackfruit	Kumar et al. (2017), Deng et al. (2012), Mahmoud et al. (2018)
Mushroom cultivation	Rice straw and bran, coffee pulp, sawdust, wheat straw, cotton straw, tea leaves, and banana leaves	Kamthan and Tiwari (2017), Salama et al. (2016), Josephine (2014)

Population and industrialization may lead to an increase in global energy consumption and a shortage of fossil energy sources such as oil, coal, and natural gas. In this regard, many agree that bioethanol is a good *alternative* to fossil energy as a renewable source. As a consequence of *widespread interest* and demand at a worldwide level, the global market share for bioethanol has entered a quick transitional growth phase. In addition, reducing crude oil reserves forced *many countries to shift their focus* from fossil energy to renewable sources for power production (Georgieva et al. 2008; Sarkar et al. 2012). The use of bioethanol in the transport fuel market can *reduce dependency* on costly gasoline and exhaust *greenhouse gas emissions,* in particular, CO_2. Thus, the necessity of using alternative raw materials in addition to conventional crops such as corn and sugarcane, which are currently the main feedstock used for the making of bioethanol, has increased (Sarkar et al. 2012; Gupta and Verma 2015). *Lignocellulosic* biomass contains a wide range of plant-based *waste materials from agriculture industries* such as wheat and rice straw, corn stover, and sugar cane bagasse. These feedstocks, which are cost effective, renewable and abundant, are scientifically tested and proposed for *bioethanol production. Hence,* bioethanol from agro-industrial wastes is very *encouraging* and seem to be an attractive alternative *technology* (Saggi and Dey 2016). Pretreatment process is important to modify the structure of hemicellulose, lignin, and cellulose contain in lignocellulosic biomass for higher accessible to further chemical or biological treatment (enzymatic attack) thus increase the conversion yield of cellulose into monomeric sugars (Demirbas 2005). Production of bioethanol from *lignocellulosic* biomass faces some major challenges and limitations, for example, effective pretreatment methods to enhance the yield of *delignification* and hydrolysis of *lignocellulosic* biomass (Kucharska et al. 2018). Therefore, considerable *effort* in recent years has *been devoted* to investigating efficient and cost-effective methods for hydrolysis and conversion of lignocellulosic biomass into fermentable sugars for biofuels production (Priyanka et al. 2018).

Agro-industrial wastes are the cheapest, lavishly available, and highly nutritious carbon source which can facilitate the growth of a wide range of microorganisms. The large composition of the agro-industrial wastes particularly from lignocellulosic waste is the carbohydrates thus have a great potential to be utilized for the production

of numerous value-added products including industrially important enzymes (Ravindran et al. 2018). wheat bran and straw, rice bran, corn cob, and sugarcane bagasse are the frequently studied agro-industrial waste for the production of numerous enzymes with various industrial processes applications such as food, drug, textile, and agriculture (Bharathiraja et al. 2017). Different fermentation strategies are applied including solid-state fermentation and submerged fermentation methods in which *the former* approach was *preferred* to the latter. Solid-state fermentation becomes an attractive and preferable process due to the direct utilization of crude fermented products as enzyme source which could potentially lessen the production cost as well as *low* amount of *energy required*, high product yield, and simple downstream processing. Recent studies have shown that a number of microorganisms including fungi, bacteria, and actinomycetes have been reported for enzyme production using lignocellulosic waste. Among them, a group of filamentous fungal species including *Aspergillus* (Bhavna and Magar 2010; Kang et al. 2004), *Trichoderma*, *Botrytis*, and *Penicillium* has been widely investigated and commercially employed for cellulase and hemicellulase production using biomass from agro-industrial wastes (Soliman et al. 2013). Several attention *have* also *been* focused on *studying* the production of other industrially important enzymes such as amylases, xylanase, lipases, mannanase, β-glucanase, lactase, invertase, pectinase, and many more by using the action of a wide range of microorganism (Ravindran et al. 2018). Exploitation of agro-industrial wastes has good potentials in *significant* cost reduction and improving the enzymes' demand for industrial purposes.

Many studies have demonstrated the importance of bioactive compounds in human health. Bioactive compounds exhibit numerous therapeutic effects through several cellular mechanisms and are known to possess properties such as anticancer, antioxidant, antidiabetic, and anti-cardiovascular activities. Recently, natural bioactive compounds are being studied for their potential in the prevention and therapy of a number of human diseases/disorders such as diabetic, heart disease, and cancer (Joana Gil-Chávez et al. 2013; Yusuf 2017). Particularly, the *potential* of agro-industrial wastes as inexpensive and readily available resources of *natural bioactive compounds* is considered which could lessen operational cost and reduce environmental impact. The recovery of the bioactive elements, especially the phenolic compounds and vitamins, *offers great potential for* food, cosmetics, and pharmaceutical industries such as for high-value products development (Kumar et al. 2017). The investigation of antioxidant properties and phenolic contents of several fruit residues such as apple, banana, guava, mangosteen, and jackfruit exhibited the different antioxidant level in different fruits and from the analysis, the major bioactive constituents in fruits residues were detected which is composed of catechin, cyanidin 3-glucoside, epicatechin, galangin, gallic acid, homogentisic acid, kaempferol, and chlorogenic acid (Deng et al. 2012). In addition, several therapeutic bioactive compounds extracted from olive and tomato waste exhibited outstanding antimicrobial antioxidant and anticancer efficacy (Kumar et al. 2017; Mahmoud et al. 2018).

Reprocessing *of* agro-industrial wastes with implementations in the agro-food industry is one of the big challenges in the biotechnology field nowadays. Yearly, the

accumulation of massive volumes of lignocellulose wastes resulted from agro-forestry and agro-industrial production causes serious environmental damages. Due to the high nutritional content in them, disposal as a compost is challenging as the leaching process will occur. Thus, most of the time these waste will be disposed by incineration lead to a serious environmental pollution. Hence, recycling of these organic wastes via mushrooms cultivation becomes one of the most efficient solutions to overcome the environmental issues raised by the accumulation of these organic wastes (Kamthan and Tiwari 2017). As a consequence of many investigations performed, the cultivation of edible and medicinal mushrooms was done using both solid-state fermentation and controlled submerged fermentation. These fermentation approaches are using a wide range of lignocellulose by-products which provide a rapid growth together with high biomass yield of the studied mushroom strains (Petre and Teodorescu 2012). Mushroom are capable to breakdown the complex lignincellulosic components in agro-industrial wastes thus this fleshly and spore-bearing fruiting bodies fungi can be generated from lignocellulosic waste materials. Mushrooms are regarded as nutritious food product rich with protein, folic acid along with vitamin B12. The bioconversion of agro-industrial wastes into a value-added products is observed as an environmentally friendly practice with potential economic advantage (Oyedele et al. 2018). Generally, numerous edible mushroom strains are cultivated such as *Agaricus* sp., (button mushroom), *Pleurotus* sp. (oyster mushroom), *Lentinula edodes* (shiitake mushroom), *Volvallella volvacea* (straw mushroom), and *Ganoderma* sp. (Chinese mushroom) using varied agro-industrial wastes cultivation medium including rice straw and bran, coffee pulp, sawdust, wheat straw, cotton straw, tea leaves, and banana leaves (Kamthan and Tiwari 2017; Salama et al. 2016; Josephine 2014).

3.5 Creating a Sustainable Practice for Industries Producing Agricultural Waste

3.5.1 Improve Plant Profitability

Over the years, agro-industrial waste has received huge attention from different researchers worldwide and is being explored as a plentiful source for bioactive compounds and promotes microbial growth. Most of the agricultural wastes have carbohydrates as a major component and lignocellulosic in nature (Ravindran et al. 2018). Production of value-added products using agricultural residues is a promising and smart strategy from an industrial point of view to produce enzymes (Sadh et al. 2018). Utilization of agro-industrial wastes as low-cost raw materials for the production of the value-added product can help to minimize the production cost. Instead of using an expansive substrate as core nutrients, using agro-industrial waste provides a cheaper and more sustainable process in which they can be used as a sole nutrient source during production. In addition, the pollution load from the

environment can be greatly reduced and less cost required to create and maintain a waste management system. Intense research has been carried out in order to investigate different fermentation strategies for the production of enzymes to enable the use of various agro-industrial wastes such as sugarcane bagasse, corn cob, and rice bran during the process (Singh et al. 2012).

Through a comparison of the impact on enzyme production via different fermentation strategies, solid-state fermentation showed greater potential than submerged fermentation utilizing agricultural waste for enzyme production (Ravindran et al. 2018). This is due to the physical–chemical nature of many lignocellulosic substrates naturally lends itself to solid-phase culture, hence, implies a means to procure the acknowledged potential of this fermentation method. Recent studies proved that the enzyme yield could be significantly improved using pretreatment technologies (Ravindran et al. 2018). Currently, starch is the foremost cost element in fermentation to generate bioethanol. Agricultural by-product such as wheat bran, rice bran, sugarcane bagasse, corn cob, and wheat straw can be used to get fermentable starch and sucrose easily (Singh et al. 2012). The use of low-value agricultural wastes in fermentation process provides the reduction of negative impact costing on production (Molina et al. 2018).

3.5.2 Suggestions and Recommendations

Agro-industrial wastes are produced during the industrial processing of agricultural products. These waste products and the by-products are being created in abundance which rising economic losses issue and worsen the low-margin of profitability in the food industry and high costing of raw materials, which should make the caution step to use the residues beneficial to the agricultural industry. Thus, the profitability of the industry could be momentously enhanced. The valorization and following value addition of carbohydrates that obtained easily from lignocellulosic food industry wastes are the cheapest. Numerous enzymes can be generated by both bacterial and fungal species using agricultural wastes. Economics value of enzyme production can be greatly enhanced by pre-treatments that enable high saccharification rates at lower enzyme loadings (Ravindran et al. 2018). The potential of exploiting agricultural by-product is being limited as high host in drying or storage purpose as well as tend to be easily spoiled by the bacteria. The extensively exploiting of agro-industrial coproducts alleviate the environmental problems and add nutritional value to the food products. Due to these coproducts constitute up to 70% of fresh fruit and poor ability to discard these materials. Thus, the pollution of environmental issues is aroused. On the other hand, it is possible to utilize the content of bioactive compounds such as polyphenols, proteases, amd dietary fibers with appropriate processing to improve the nutritional properties in health and pharmaceutical sectors. These nutritional properties can be improved by providing more nutrients such as proteins, carbohydrates, or fats. In addition, it is reported by previous researcher that

chronic diseases can be evaded by consumption of these nutrients with processed products.

Agro-industrial wastes or residues are naturally rich in nutrient composition and bioactive compounds (Beltrán-Ramírez et al. 2019). Sugars, minerals, and proteins are the compositions constituted in agro-industrial waste. Therefore, it presumes to consider as "raw material" rather than "wastes" for use in other industrial processes. The availability of nutrients in these residues contributes to suitable productive growth conditions for the microorganisms. Through the fermentation process, the microorganisms have enormous potential to reutilize the waste as raw materials for growing purpose (Sadh et al. 2018). A wide range of beneficial bioactive compounds can be produced significantly by utilization of agro-industrial wastes as solid support in the solid-state fermentation process (Lizardi-Jimenez and Hernandez-Martinez 2017). Extensively utilizing agricultural and agro-based industrial wastes as raw materials can minimize production cost and contribute in reprocessing of waste. Therefore, environmental pollution problems can be mitigated (Beltrán-Ramírez et al. 2019).

3.6 Conclusion

Biological treatments have immense potential to relieve the alarming issue on the disposal of the escalating amount of agro-industrial wastes. The present study has listed the sources and types of these wastes, along with the various industries producing them. Understanding that most agro-industrial wastes are left untreated or underutilized, it is imperative to seek for effective measures to avoid further environmental damages. A number of possible biological treatments have been highlighted including fermentation, solid-state fermentation, fungal, and bacterial treatments. Any of these methods could be applied according to the suitability of the waste conditions. The advantages and disadvantages have also been discussed for which a comparison between biological treatments and other treatments were deliberated. Aside from being economical, biological treatments are straightforward and easy to implement, for which they will benefit the human and environment simultaneously. This study strengthens the idea that closing the loop by valorizing the agro-industrial wastes through biotransformation significantly contributes to the reduction of waste deposition into the environment. Useful products like biogas, bioactive compounds and mushrooms are prospective outcomes of biological treatments. Lastly, several recommendations on creating a sustainable practice for agro-industries have been outlined. These efforts aim at overcoming probable issues like nutrient imbalance, rapid acidification, and inhibiting compounds, and ultimately ensuring plant profitability. This study provides an important insight into applying a cleaner process on unworthy materials toward a better and safer product.

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Chapter 4
Proteomics of Lignocellulosic Substrates Bioconversion in Anaerobic Digesters to Increase Carbon Recovery as Methane

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Abstract Anaerobic digestion (AD) is a cost-effective treatment for management of lignocellulosic substrates, viz., agricultural wastes and animal manures, which also aids in generation of methane as biofuel. Although the application of AD technology is increasing, one of the major limitations of the process is that the rate of fermentation is higher than the rate of methanogenesis, which significantly affects process stability and methane yield. Normally, the souring of digesters can be observed after 2–4 weeks after the initiation of the volatile fatty acids accumulation, which makes it difficult for early detection and consequently resulting in acidification of digesters. Of late, metagenomic approaches are gaining importance due to their ability to reveal the microbial diversity and their dynamics in a relatively short time. However, their functional nature could not be clearly explained due to the lack of data on their activity. Recent advances in proteomic studies show its potential as a complementary technology to metagenomic studies for efficient management of digesters. Metaproteomic analyses aid in identifying a shift in metabolic paths and in metabolic networks under stress conditions. This provides insights on functionality, microbial interactions, and provides data on spatiotemporal variations and their dynamics of proteins crucial for efficient performance of the digester. Besides, this technique has

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led to identify novel phylotypes with novel functions among the microbial communities of the anaerobic digesters, which suggest the potential of proteomics in bioprospection of novel enzymes for industrial purposes. How proteomics along with metagenomics and transcriptomics data could aid in early detection of disturbances in the digesters helps in formulating recovery strategies as well as to increase the methane content of biogas will be discussed in this chapter.

Keywords Anaerobic digestion · Metabolic networks · Methane · Proteomics

4.1 Introduction

The recent use of lignocellulosic biomass as a renewable energy source has been of increasing interest due to the environmental crisis and alarming decline in fossil fuel reserves. In the next few decades, bioenergy will be considered as one of the potential renewable energy sources, in addition to other renewable energy sources, e.g., wind and solar. Wide-scale practice of these technologies depends on the economics involved in their infrastructure and maintenance. Among all, bioenergy from biomass is constantly investigated due to their easiness in installation and operation. Anaerobic digestion (AD) is one of the processes widely used to recover energy from biomass sources, like animal manures, solid municipal wastes, paper industry wastes, energy crops, or agricultural wastes, in the form of methane (Nallathambi Gunaseelan 1997).

Anaerobic digestion process degrade/oxidize organic matter under anaerobic conditions by several consortia of different metabolic groups of microorganisms, where methane (60–70%) and carbon dioxide (30–40%), and other trace gases (<1% hydrogen, nitrogen, ammonia, and hydrogen sulfide) are major end products. However, methane is the most valuable product because it can be used to generate electricity and heat (Angelidaki et al. 2003). Methane production in AD process involves four different steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

Nevertheless, microorganisms carrying out this degradation/transformation reactions differs in their physiology, nutritional requirement, growth kinetics, and are sensitive to the environment. This characteristics lead to a delicate balance between all group of microorganisms involved in AD, and any modification can cause instability and consequently, low methane yield (Adekunle and Okolie 2015). For this reason, over the last decade, there has been a rapid development in state-of-the-art techniques to understand the microbial community dynamics, interactions, and functionality to achieve a proper digester efficiency and stability. Besides, it is necessary to study the non-culturable microorganisms involved in the process and its proteome to fill the knowledge gaps in our understanding of the complex microbial interplay and functionality in AD process.

The study of microbial diversity and gene expression in AD, through metagenomic and metatranscriptomic analysis, has helped us to reveal “black box” contents and their role to a certain level. Still, there is limited understanding of all the possible metabolic pathways that are active throughout the biomethanation process

and without which, successful operation and maintenance of biodigesters for higher methane yield. Hence, the interest on metaproteomics of digesters is gaining attention. This tool can evaluate growth and activity of different microorganisms in relation to their environment (protein expression and localization), to identify posttranslational modification, to infer certain protein–protein interactions, amino acid sequences, and genotypes, besides protein identification (Vanwonterghem et al. 2014). As well, metaproteomic databases can permit to examine targeted biomarkers from microbial communities for evaluation of the biodigester functioning. In this chapter, we focus on the contribution of metaproteomic approaches to gain an insight on the composition of microorganisms sharing similar metabolic structure, and the shift in their dynamics and functions under certain environmental or induced conditions in biodigesters employing lignocellulosic substrates as main feedstock.

4.2 Anaerobic Digestion of Lignocellulosic Substrates

Global annual production of available lignocellulosic biomass is 181.5 billion tonnes. In USA alone, about 1.25 billion tonnes of lignocellulosic biomass is produced annually, while in Canada, about 69.25 million tonnes are generated (Paul and Dutta 2018). The use of biomass residues as sources of renewable energy has increased. Recently, the lignocellulosic-rich biomass feedstocks such as fibrous food wastes, animal manures, paper industry wastes, agro residues, and energy crops are mostly used as feedstocks for bioenergy production (Sawatdeenarunat et al. 2015). Apart from biogas production, ethanol and butanol production from lignocellulosic biomass, such as wheat straw, corn cob, and sugarcane bagasse is also being studied (Jiang et al. 2017).

The major components of the lignocellulosic biomasses are cellulose, hemicellulose, and lignin, which are hydrolyzed through a series of reactions by microorganisms. Cellulose and hemicellulose are the predominant polysaccharides in these biomass materials. Whereas, lignin is conformed of phenolic polymers, which add recalcitrance to the complex structure of lignocellulose substrates and limits the accessibility of polysaccharides by microbial enzymes (Isikgor and Becer 2015; Liu and Chen 2015). Li et al. (2018) reported the interaction of cellulose, hemicellulose, and lignin components on biodegradability of different lignocellulosic biomasses and observed that methane production was favored and correlated with decomposition of substrates rich in cellulose and hemicellulose, whereas, lignin was not completely digested (Li et al. 2018).

Nevertheless, the lignocellulosic biomass shows low rate of polysaccharide hydrolysis due to the presence of lignin (Cesarino et al. 2012). Therefore, to increase hydrolysis rate, methods of pretreatment have been developed (Ariunbaatar et al. 2014) and becoming crucial to anaerobic digestion process. Pretreatments aid to overcome limitations and eliminate the barriers to access polysaccharides for degradation, augment digestibility, and consequently increase biogas production from lignocellulosic biomass residues (Chen et al. 2014). Pretreatment techniques such as physical (steam explosion, hydro-thermolysis, thermochemical), chemical (alkalis,

acids, oxidants as organic solvents), nonconventional (ionic liquids), and biological processes are mostly applied to polysaccharide decomposition (Singh et al. 2015; Carrere et al. 2016).

Enhancement in methane yield has been reported for several lignocellulosic residues, which reveal the significant improvements on lignin depolymerization. However, type of pretreatment depends on the composition of lignocellulosic feedstocks (Table 4.1), since the type and accumulation of products after pretreatment process can either be beneficial or harmful to the microbial consortium of AD process (Poudel et al. 2012; Ahring et al. 2015).

Additionally, C:N ratio of lignocellulosic-rich substrates is an important parameter as high and low ratios were found to have negative impact on the process by altering pH, and consequently inhibiting growth and activity of microbial communities in the biodigester (Rahman et al. 2017). In general, to overcome the limitations due to C:N ratios, the addition of a co-substrate rich in carbon or nitrogen provide optimum conditions for biomethanation and this process is known as co-digestion. Selection of suitable co-substrate is important, in order to enhance synergisms, dilute detrimental compounds, and optimize the methane yield without affecting digestate quality (Mata-Alvarez et al. 2014; Siddique and Wahid 2018). Hence, research on the AD process evolves continuously to identify optimum operational conditions and their relation to the microbial diversity, their function to increase methane yield.

4.3 Recognizing Important Pathways of AD

In terms of energy, anaerobic digestion is a green technology, where biogas production is a more efficient method for energy generation from biomass than other biological and thermochemical conversion processes (Deublein 2009). AD is an alternative to landfills as a means of organic waste management as AD process generates energy apart from reducing methane emissions. Similarly, traditional management of burning conventional forage residues results in atmospheric pollution and the application of AD process can recover energy from these lignocellulosic biomass (Braun et al. 2008). The conversion of agricultural waste is commonly performed in large parallel or serial biodigester systems of different sizes and designs, known as biogas plants (BGPs). The biodigesters are classified depending on some conditions; such as temperature of the process (psychrophilic, mesophilic, or thermophilic), the type of substrate (e.g., silage, animal manure, or dung), and consistency (e.g., Wet process with low solid content or Dry digestion process with high solids content) (Mcinerney et al. 1979; Weiland 2010; Angelidaki et al. 2005).

AD is a complex multistep process that is performed by a large consortium of microorganisms composed of four major groups as mentioned previously, hydrolytic, fermentative, syntrophic acetogenic bacteria, and methanogenic archaea (Fig. 4.1) (Ferry 1993; Zheng et al. 2014).

First, hydrolytic bacteria hydrolyze biopolymers (lipids, proteins, and carbohydrates) to soluble oligomers and monomers (long-chain fatty acids, glycerol, amino

Table 4.1 Biochemical composition of different lignocellulosic substrates and their potential methane production

Type of substrate	Reactor volume (L)	Cellulose (%)	HC (%)	Lignin (%)	C (%)	N (%)	C:N	TS (%)	VS (%)	Methane production (mL CH_4/g VS)	References
Rice straw	1	35–44	27–34	12–13	39.7	0.9	47–67	92.9	81.6	281	Paul and Dutta (2018), Sawatdeenarunat et al. (2015), Li et al. (2013a)
Wheat straw	1	38–42	20–27	20–22	39.9	0.4	50–60	90.5	77.9	245	
Corn stover	1	40	25–31	14–17	43.2	0.8	50–63	84.9	76.9	241	Paul and Dutta (2018), Sawatdeenarunat et al. (2015), Singh et al. (2015), Li et al. (2013b)
Corn cob	0.575	45	25	15	41.26	0.45	123	[a]	81.22	254.2	Paul and Dutta (2018), Pérez-Rodríguez et al. (2016), Kanwal et al. (2019)
Sugarcane bagasse	2	40–45	20–24	25–30	46.08	0.74	118–150	75–16	73.55	84.75	Paul and Dutta (2018), Inyang et al. (2010), Mustafa et al. (2018)
Switchgrass	1	36–45	28–30	12–26	43.6	0.4	90	91.3	87.4	246	Paul and Dutta (2018), Li et al. (2013b)
Chicken manure	1	20	23.2	1.6	35.9	3.4	10.9	25.9	19.5	295	Paul and Dutta (2018), Li et al. (2013b)
Dairy manure	1	19.5	15.2	17.4	37.6	2.8	13.4	38.5	28.8	51	Li et al. (2013b)
Swine manure	1	11.3	27.7	4.3	34.8	2.2	15.8	30.4	22	322	Li et al. (2013b)
Food waste	1	12	5.9	7.9	43.3	3.3	3–17	3.7	3.3	342	Li et al. (2013b), Divya et al. (2015)

HC Hemicellulose
[a]Not determined

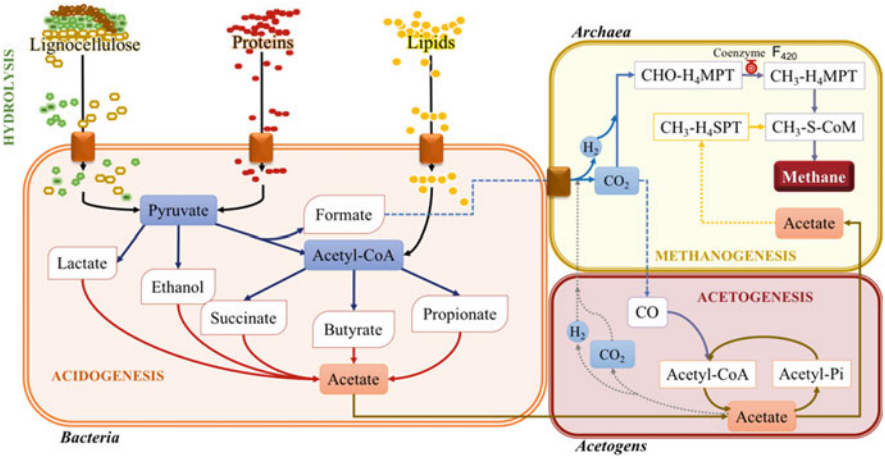

Fig. 4.1 General map of the four main stages of anaerobic digestion with the most abundant intermediates influencing methane yield

acids, and monosaccharides) by extracellular enzymes. These compounds are further converted to volatile fatty acids (VFAs; butyrate, propionate, acetate, among others), alcohol (ethanol and methanol), H_2, and CO_2 by fermentative bacteria. Eventually, VFAs greater than C_2 and alcohols are oxidized to acetate, hydrogen (H_2), formate, and CO_2 by syntrophic acetogens. Finally, the last group of methanogenic archaea converts acetate and CO_2 to methane (Gujer and Zehnder 1983).

In the last AD step (methanogenesis), a complex interplay among different functional microorganisms occurs. Hydrogenotrophic methanogens oxidize H_2 into methane (CH_4) by using CO_2. While the methyl group of acetate or methylamines is reduced to CH_4 by acetoclastic methanogenesis (Schink 1997). Syntrophic acetate oxidation (SAO) (Schnürer et al. 1999) also occurs under anaerobic conditions, yielding CO_2 and H_2, which feed the hydrogenotrophic methanogens (Fig. 4.1). Methanogenic stage is one of the rate-limiting steps as the growth rate of methanogens is low as well as they are sensible to environmental fluctuations such as pH, temperature, and VFAs concentration (Chen et al. 2008). In addition, several other factors influence biogas yield, mainly the recalcitrant nature of the substrate, the binding of bacteria on the substrate during the hydrolysis stage (Angelidaki et al. 2011) and high ammonia concentrations (Appels et al. 2011).

It is worth to mention, methanogenesis involve an optimal organization and interaction among different bacterial and archaeal communities, specific syntrophic interactions, and an imbalance can affect growth and activity of the microbial communities and could cause a deterioration in reactor performance, and thereby decreasing the methane yield (Krause et al. 2008; Rastogi et al. 2008; Akuzawa et al. 2011). Understanding the structure of microbial communities, the possible interactions among different microbial groups, and the active metabolic pathways could help in improving the methane yield. As mentioned earlier, the metaproteomics is a useful

tool that can provide information on transcription and translation, giving discernment between regulation of gene expression, protein synthesis, stability, and turnover of mRNA and proteins synthesized in situ. Of late, this approach has been successfully applied to laboratory- and full-scale anaerobic systems with potential in biogas production from lignocellulose substrates, as seen in Table 4.2.

Laboratory-scale digesters systems are scaled-down models to investigate new different substrate composition, microbial diversity, and its efficiency on organic matter removal and consequently methane yield potential and for testing new reactor configurations (Herrmann et al. 2011). These systems allow us to add control tools to handle several operational parameters and to control a malfunction, if there is any. In contrast, full-scale BGP represents a bigger challenge in operation and maintenance and some disturbances are hard to manipulate (Gerardi 2003).

Table 4.2 Overview of metaproteomic studies on AD of lignocellulosic biomass

Reactor type	Substrate	Major findings	References
Laboratory-scale reactors			
Thermophilic 8 L-stirred tank reactor (55 °C)	Mixture of beet silage (95%) and chopped rye (5%)	Proteins involved in acetoclastic and hydrogenotrophic methanogenesis, energy conservation, and a heat shock protein were identified. Most of them belong to *Methanosarcinales*, and others to *Methanomicrobiales* and *Synergistales*	Hanreich et al. (2012)
Mesophilic 2 L digester under acid stress conditions (35 °C)	Blended Taihu blue algae	Proteins involved in methane production and energy metabolism were identified (MCR: methyl-coenzyme M reductase, alcohol dehydrogenase, coenzyme-B sulfoethyl thiotransferase) from *Methanosarcinales*, *Methanomicrobiales*, and *Clostridiales*	Yan et al. (2012)
Mesophilic 500 mL batch digesters (38 °C)	Cut straw and hay	Members of *Bacteroidetes* were responsible for carbohydrate metabolism, while flagellins from *Firmicutes* showed its prevalence among the community. Otherwise, abundant enzymes from methanogenesis were detected from *Methanobacteriales*, *Methanosarcinales*, and *Methanomicrobiales*	Hanreich et al. (2013)

(continued)

Table 4.2 (continued)

Reactor type	Substrate	Major findings	References
Thermophilic 1 L digester (55 °C)	Unprinted office paper and anaerobic sludge from thermophilic industrial digester fed with municipal solid wastes	Cellulose and hemicellulose hydrolysis and fermentation enzymes were strongly related to *Caldicellulosiruptor* spp. and *Clostridium thermocellum*. Hydrogenotrophic pathway enzymes assigned to *Methanobacteriales*. *Coprothermobacter proteolyticus* recognized to perform proteolysis and fermentation	Lü et al. (2014)
Mesophilic 5 L continuous stirred tank reactors (CSTRs, 37 °C) designed as a "bovid-like" digestive system	Aerobic sludge from a wastewater treatment plant inoculated with cow digestive tract contents (RU) and cow manure (CO)	Glycogen-accumulating microorganisms (*Competibacteraceae* with two-phase metabolism from aerobic to anaerobic) dominated the CSTRs. Hydrogenotrophic methanogenic proteins dominated the RU. While CO reactor was affiliated to subunits from acetyl-CoA decarbonylase/synthase (ACDS) complex and acetate kinase from *Methanosarcinales* and *Methanomicrobiales*	Bize et al. (2015)
Thermophilic digester (2 L; 55 °C)	Fresh and digested swine manure	The most abundant proteins belong to energy production and conversion, carbohydrates, lipids, and amino acid metabolism, followed by information storage and cellular processing proteins. The proteins related to energy production were ATPases subunits, MCR, and acetyl CoA decarbonylase	Lin et al. (2016)
Mesophilic and thermophilic anaerobic digesters (5 L); 37 and 55 °C)	Acidified grass	Both mesophilic and thermophilic reactors contained high abundance of glycolytic proteins, sugar transport systems, and phosphotransferase systems affiliated to *Firmicutes*. While in thermophilic conditions chaperons and heat shock proteins were overexpressed	Abendroth et al. (2017)

(continued)

Table 4.2 (continued)

Reactor type	Substrate	Major findings	References
Mesophilic 2 L batch reactors (37 °C)	Reed straw (pretreated with cellulase) and swine manure sludge	Bacterial proteins (*Firmicutes, Proteobacteria, Actinobacteria*, and *Bacteroidetes*) were mainly affiliated to polymer metabolism. Ferredoxin-NADP reductase for H_2 production was assigned to *Azotobacter.* The most abundant metaproteins were acetyl-CoA decarbonylase, MCR, and acetate pathway from *Methanosarcinales*	Jia et al. (2017a)
Mesophilic 2 L reactors (35 °C)	Food waste with short-term hydrothermal pretreatment	Carbohydrate and energy metabolism were the most active functions during the H_2 production stage of *Firmicutes* and *Bacteroidetes.* Proteins from acetate metabolism, methylotrophic and acetoclastic pathways increased during the methanogenic stage were assigned to *Methanosarcinales, Methanobacteriales,* and *Methanotococcales*	Jia et al. (2017b)
Mesophilic (R1-2), thermophilic (R3-4), and high ammonia levels (R5-6) of multibioreactor system (500 mL)	Sludge from BGP fed with corn, silage, pressed and pulp turnip, chicken dung, liquid manure, and iron sludge	Enzymes of glycolysis and amino acid biosynthesis were found in R1. In reactors 3 and 4 decreased MCR from *Methanosarcinales* and *Methanobacteriales.* While in R5-6 increased the expression of 5,10-ethylene H_4MTP reductase and subunits of ACDS complex from *Methanobacteriales* and *Methanosarinales*	Kohrs et al. (2017)
Mesophilic 500 mL reactor	Cut filter paper	The most upregulated proteins included carbohydrates hydrolases, ABC transporter proteins, outer binding proteins, copper amine oxidases, translation elongation factors, carboxyl transferase, glyceraldehyde 3-phosphate dehydrogenase, and flagellins, mostly belonging to *Firmicutes, Synergistetes*, and *Bacteroidetes*	Speda et al. (2017)

(continued)

Table 4.2 (continued)

Reactor type	Substrate	Major findings	References
Mesophilic 4 L leach bed reactors (37 °C)	Acidified ensiled rye-grass and granular sludge	Proteins related to carbohydrate hydrolysis, glycolysis, and transport proteins were assigned to *Firmicutes, Bacteroidetes, Spirochaetes, and Proteobacteria.* ATPases and oxidoreductases belong mostly to *Firmicutes, Proteobacteria*, and *Bacteroidetes.* Meanwhile, proteins from lipid and amino acid metabolisms and environmental stress were affiliated to *Firmicutes* and *Bacteroidetes*	Joyce et al. (2018)
Mesophilic 10 L stirred tank reactor (37 °C)	Dried distiller grains feedstock under trace element deprivation	Trace element (TE) deprivation causes a decrease of hydrogenotrophic metaproteins from *Methanomicrobiales.* Only coenzyme F_{420}-reducing hydrogenase and methyl-H_4MTP increased its abundance upon the addition of TE. Methylotrophic and acetoclastic metaproteins decreased while formylmethanofuran dehydrogenase from *Methanosarcinales* increased	Wintsche et al. (2018)
Full-scale biogas plant			
Mesophilic biogas plants (BGPs; 270–2280 m^3)	Corn-/grass-/rye whole crop silages Piglet manure/cattle manure/cattle slurry	Peptidases, glycolytic enzymes, glucose transporters, ribosomal proteins, chaperons, amino acid metabolism, and energy conservation proteins were identified from bacteria. No cellulolytic enzymes were detected. For Archaea, hydrogenotrophic and acetoclastic metaproteins from *Methanobacteriales* and *Methanosarcinales* were identified. Changes in protein profiles correlated to MCR decrease upon acidification	Heyer et al. (2013)

(continued)

Table 4.2 (continued)

Reactor type	Substrate	Major findings	References
Mesophilic (43 °C) and thermophilic (52 °C) BGPs (1500–1600 m^3)	M: Whole crop silages of maize, forage rye, cattle manure, and slurry T: Mix of maize whole crop silage and poultry manure	Carbohydrate hydrolases, sugar transporters, glycolytic enzymes, and primary fermentation enzymes were identified. Most of the mesophilic proteins were affiliated to *Methanosarcinales*. Whereas *Firmicutes* and *Thermotogales* were assigned to thermophilic BGP, as well as *Methanobacteriales*	Kohrs et al. (2014)
Mesophilic BGP (43 °C; 1500 m^3)	Whole crop silages of maize and rye, cattle manure and cattle slurry	Proteins as H_4MPT S-methyltransferase, V-type H^+-transporting ATPase and MCR from both acetoclastic and hydrogenotrophic pathways were dominant and belonged to *Methanomicrobiales and Methanosarcinales.* Subunits of ACDS complex were affiliated to *Methanosarcinales*	Theuerl et al. (2015)
35 mesophilic and thermophilic BGPs (min. 33 °C, max. 55 °C; 20–4000 m^3)	Agricultural substrates, industrial wastes, slaughterhouse wastes, sewage sludge, municipal waste, mixed and unknown substrates	The 40 BGPs were dominated by methanogenic enzymes related to nutrient transport and one-carbon metabolism. The most abundant metaproteins (MCR and 5,10-methylene H_4MTP reductase) belonged to *Methanobacteriales* and *Methanosarcinales.* At 33 °C, proteins from short fatty acid metabolism, lipid and one-carbon metabolism were abundant. At 55 °C, proteins from DNA recombination and repair, and amino acid biosynthesis were abundant	Heyer et al. (2016)
Mesophilic and thermophilic BGPs (1–3: 37 and 4: 54 °C; 105 m^3)	BGP1: Maize silage, sugar beet, and poultry manure. BGP2: Maize silage, grass, and pig/cattle manure. BGP3: Maize silage and pig manure. BGP4: Maize silage, grass, and pig manure	ABC transporters, carbon and methane enzymes were assigned to BGP3. ABC transporters were highly expressed and affiliated to *Firmicutes* and *Bacteroidetes*, as well as, hypothetical substrate-binding proteins. Glycolytic enzymes were identified from *Firmicutes* and *Bacteroidetes*. While members of *Methanosarcinales* and	Ortseifen et al. (2016)

(continued)

Table 4.2 (continued)

Reactor type	Substrate	Major findings	References
		Methanomicrobiales were responsible for hydrogenotrophic and methylotrophic methanogenesis	
Thermophilic industrial biogas reactor (60 °C; 2200 m^3)	Food waste with high levels of free ammonia	*Dictyoglomales* and *Planctomycetes* were highly active in polysaccharide hydrolysis. Proteins from obligate hydrogenotrophic methanogens dominated over acetoclastic methanogens. Novel phylotypes of SAO-bacteria (*unFi_c1* and *unFi_c2*) were identified and perform β-oxidation of butyrate and other longer chain fatty acids, as well as in acetate oxidation	Hagen et al. (2017)

4.4 Metaproteomics in AD of Lignocellulosic Substrates

Metaproteomics was first defined by Wilmes and Bond (2004) as "the large-scale characterization of the entire protein complement of environmental microbiota at a given point of time." Through the years several denominations have been used depending on the different experimental procedure, the complexity of the environmental sample or the outcomes. Terms include *environmental proteomics*, *metaproteomics*, *community proteomics*, *proteogenomics*, and *proteotyping*. However, not all are synonyms. Schneider and Riedel (2010) mentioned that *environmental proteomics* refers to the proteome analysis of environmental samples, while *metaproteomics* is the study of highly complex biological systems containing a large number of proteins, which is hard to assign to species within a phylotype. In contrast, *community proteomics* infers that most of the proteins identified are assigned specifically to members of the community. *Proteogenomics* links the gene function to the identified protein, giving the accurate information about a biological system functionality. On the other hand, *proteotyping* refers to a gel-free approach, supported by the rapid protein resolution by mass spectrometers for the characterization of mixed microbial communities (Kohrs et al. 2017). Despite several definitions, proteomics englobes a large-scale study of proteins, which allows the understanding the metabolic networks, syntrophic interactions, carbon and nitrogen fluxes, and novel pathways.

Several analytical methods have been applied to provide an insight into microbial communities in AD, commonly genomic approaches. Cloning and sequencing of

DNA or fingerprint target of 16S rRNA gene have been usually applied to explore communities of *Archaea* and *Bacteria* (Clement et al. 1998; Schlüter et al. 2008). However, metaproteomics emerged as a complementary approach to give a full vision of the physiological and biochemical functions of microbial population. General metaproteomic workflow comprises biogas community sampling, protein extraction, protein gel separation, tryptic digestion of proteins, mass spectrometry of resulting peptides, and database searching of mass spectra (Hassa et al. 2018). However, as mentioned before, new gel-free approaches have led to rapid resolving mass spectrometers (MS) for rapid identification and characterization of microbial communities employing tandem MS and MS/MS-based shotgun proteomics (Karlsson et al. 2015).

4.4.1 Hydrolysis

As mentioned previously, microbial communities degrade polymeric biomass into monomers by hydrolytic enzymes during the first step of AD process in order that simpler compounds are available for the next steps of biomethanation process. The three primary substrates for hydrolysis are polysaccharides, lipids, and proteins, which are generally present in majority of the wastes or feedstocks of anaerobic digesters (Tong et al. 1990). In the case of polysaccharides, there exist two basic types of enzyme system for its hydrolysis: complex systems as cellulosomes, produced by anaerobic bacteria and nonassociated, free enzyme systems produced by aerobic microorganisms (Fig. 4.2) (Felix and Ljungdahl 1993).

The first metaproteomic study conducted by Hanreich et al. (2013), demonstrated the presence of α-amylase and glycoside hydrolase only. α-amylase is an endoamylase, which acts on α-1,4 glycosidic bonds in amylose or amylopectin of starch, releasing oligosaccharides of different length. While, glycoside hydrolases are capable of hydrolyzing cellulose, hemicellulose, and starch. This study employed maize-digestate from a biogas plant fermenting maize and a mix of cut straw and hay as feedstock. The metaproteome was dominated only for few proteins from genus *Thermoanaerobacterum* and *Microscilla* and in less abundance, the proteins of *Cytophaga*, which synthetizes pectate lyase. This enzyme catalyzes the eliminative cleavage of pectate, a main component of cell walls in plants. In contrast, a study of a biogas plant treating silage or whole maize crop showed a set of hydrolytic enzymes performing degradation of high-molecular carbohydrates like cellulose, hemicellulose, xylan, and arabinan (Heyer et al. 2013).

Other nonagricultural substrates, as waste papers, with a composition of about 70% of cellulose and 30% of hemicellulose has led to the identification of structural and catalytic components of *C. themocellum* cellulosome; CelS and CelJ, as well as hydrolytic enzymes degrading high-molecular carbohydrates. Other enzymes as β-mannanase, acetyl xylan esterase, and endoxylanase, specialized in hemicellulose degradation were related to *Caldicellulosiruptor* genus (Lü et al. 2014). On the other hand, food wastes as feedstock, with high levels of free ammonia, indicated that an

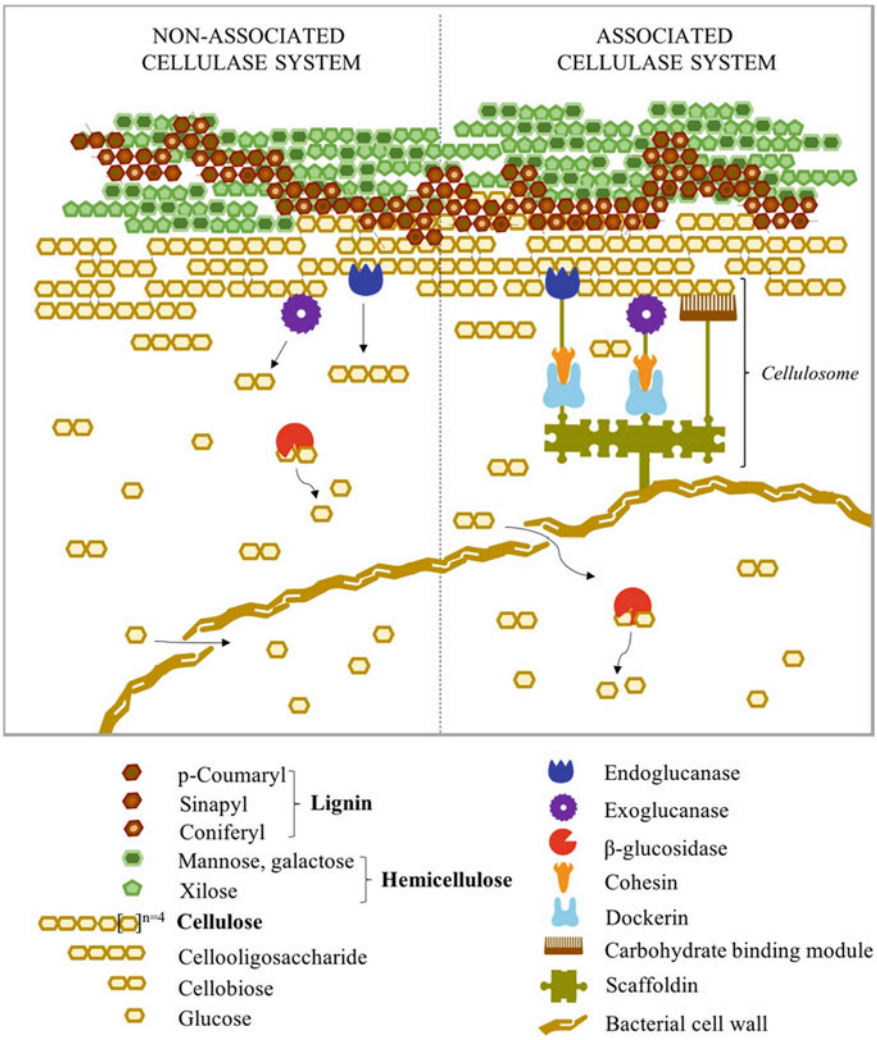

Fig. 4.2 Schematic diagram of the nonassociated and associated cellulase systems. Most aerobic microorganisms degrade cellulose by secreting a set of complex enzymes viz., endoglucanase, exoglucanase, and β-glucosidase. On the contrary, most of the anaerobic microorganisms produce cellulosomes. The cellulosome is an extracellular multienzymatic complex present on the cell wall and binds to the substrate for its hydrolysis. It can incorporate several hydrolases through cohesin–dockerin interaction, while the carbohydrate-binding module keeps the cellulosome attached to the lignocellulosic substrate. This figure was modified from Zhu and McBride (2017)

uncultured *Atribacteria* was mainly responsible for the hydrolysis of polysaccharides synthesizing enzymes as β-glucoside, galactose mutarotase, L-fucose isomerase, and xylose isomerase potentially related with hemicellulose degradation.

For protein hydrolysis, proteases, and endopeptidases belonging to *C. proteolyticus* are reported frequently, when protein-rich biomass is treated in

thermophilic biodigester (Heyer et al. 2013). Other enzymes also related to protein degradation are trypsin-like serine protease and it is reported that *Planctomycetes* and *Atribacteria* groups are metabolically active in carbohydrate and protein degradation (Hagen et al. 2017).

Recently, metaproteomics is employed as a bioprospecting tool for identifying novel enzymes. Speda et al. (2017) showed this tool is useful to identify and select novel enzymes from consortia, that are specifically upregulated upon its induction. Cellulolytic activity was targeted in a defined medium containing filter paper, instead of glucose, and compared with a non-induced sample. Cellulose induction led to the identification of 1,4 β-cellobiosidase, 1,4 β-xylanase, cellobiose phosphorylase, β-glucosidase, and hypothetical Ig domain proteins from several species.

4.4.2 Nutrient Transport

Substrate transporter systems are of great relevance for the following steps of methanogenesis. The main mechanism by which a microorganism can obtain nutrients from the environment is by means of these proteins. Diverse studies have evidenced transport proteins in anaerobic reactors. Two classes of proteins involved in nutrient transport are TonB-dependent receptors and ATP-binding cassette (ABC) transporters. These proteins have been previously reported in AD from several members of *Bacteroidetes* and *Spirochaetes* phylum (Hanreich et al. 2013). The TonB-dependent transporter (TBDT) is a bacterial outer protein that can actively transport siderophores, as well as, vitamin B12, nickel complexes, and carbohydrates. This receptor is part of a starch utilization system that has been recognized by its efficiency to transport oligosaccharides to its further degradation. As mentioned before, TBDT is deployed for more complex substrates and uses a proton motive force for the uptake of oligomers that are too large to diffuse via porins (Lü et al. 2014). Interestingly, TBDT also can degrade polymers as polysaccharides, proteins, proteoglycans and via substrate-binding hydrolytic proteins.

In contrast, ABC proteins are a family of primary transporters that hydrolyzes ATP to transport organic and inorganic compounds. ABC systems are bioenergetically expensive as ATP hydrolysis is needed to translocate the substrate across the membrane. Consequently, investment of ATP in this transport mechanism limits binding and transport, especially when higher concentration metabolites are present. ABC transporters have been reported to be related to peptide transport, maltose, and other metabolites, such as glycerol 3-phosphate (Speda et al. 2017; Kohrs et al. 2014; Hagen et al. 2017). Ortseifen and colleagues (2016) showed in an integrated metagenome-proteome research digesting maize silage and pig manure, that mostly ABC-transporters of peptides, oligopeptides, monosaccharides, and iron of the phylum *Firmicutes* were upregulated, and as well as other translocating proteins from *Spirochaetes, Thermotogae*, and *Thermococcus* phylum. While Jia and coworkers (2017a) made an extensive work of the metaproteome evaluating the four different stages of methanogenesis (peak stage of hydrogen production, late

stage of hydrogen production, peak methanogenic stage, and late methanogenic stage) in a bioreactor fed with cellulase-pretreated reed straw. They found ABC protein expression increased during the peak methanogenic stage where methane production potentials and methane production rate reached 2709.94 mL and 9.71 mL/h.

Otherwise, components of sugar transport systems (like the phosphotransferase system) were identified mainly from *Firmicutes* species, in a biodigester fed with grass, and with a separate acidification step at thermophilic and mesophilic conditions (Abendroth et al. 2017). This active transport is used by bacteria for uptake of carbohydrates, particularly hexoses, hexitols, and disaccharides, where the source of energy is from phosphoenolpyruvate (Roseman 1969).

4.4.3 Acidogenesis

After hydrolysis and nutrient transport, monosaccharides and amino acids are the most abundant substrates for fermentation and a wide range of microorganisms can metabolize both, mostly *Clostridia* and other Gram-positive bacteria (Madigan et al. 2008; Ramsay and Pullammanappallil 2001).

Monosaccharides are channeled to catabolic pathways for the production of pyruvate via the Embden–Meyerhof–Parnas (EMP; glycolysis) or Entner Doudoroff (ED) pathway. During glycolysis, reducing equivalents like NADH and H_2 are produced, and pyruvate is further metabolized to acetate, CO_2, and H_2 (at low partial pressure) or subsequently to C_3 products (lactate or propionate), or C2/C4/C6 products (acetate/butyrate/caproate) via acetyl-CoA (at high partial pressure). At low partial pressure of H_2, the flow of electrons (NADH) lead to H_2 production which leads to pyruvate degradation. As partial pressure increases, the flow of electrons shift to the generation of reduced electron fermentation products (volatile fatty acids, VFAs) such as propionate and long-chain fatty acids, lactate, or ethanol. Thus, in a system where methanogens are effectively consuming H_2, low concentrations of ethanol, lactate, and butyrate are maintained (Bräsen et al. 2014).

At first evaluation of a full-scale agricultural BGP metaproteome, Heyer et al. (2013) demonstrated the identification of metabolic enzymes involved in glycolysis as glyceraldehyde-3-phosphate dehydrogenase (G3PD), enolase, phosphoglycerate kinase, glycerol kinase, and lactate dehydrogenase (LDH), mostly associated to *Clostridia*. Similar results were found in agricultural BGPs in mesophilic and thermophilic conditions assigning proteins from glycolysis as 6-phosphofructokinase, aldolase, G3PD, 3-phosphoglycerate kinase, phosphoglycerate mutase, and enolase, as well as enzymes from the primary fermentation: LDH (assigned to *Lactobacillus*), NADP-dependent isopropanol dehydrogenase, and aldehyde dehydrogenase also related to *Clostridia* (Kohrs et al. 2017). Jia et al. (2017a) also demonstrated the presence of LDH in *Streptococcus* during the hydrogen production stage.

Other BGP digesting maize silage and pig manure revealed highly abundant proteins in fermentation. In spite of the low abundance reported, the use of an

integrated approach for combining metaproteomics and metagenomics tools aid in the identification of glycolysis enzymes (enolase, aldolase, and G3PD) (Ortseifen et al. 2016). Similar results were found in a case study evaluating a proteome from a thermophilic reactor degrading swine manure, where enolase from different species of phyla *Proteobacteria* were identified (Lin et al. 2016).

On the other hand, an industrial biogas reactor predominantly fed with food waste and high levels of free ammonia, few enzymes from glycolysis were identified, and attributed to uncultured phylotypes *Atribacteria, Planctomycetes*, and *Dictyglomus* (Hagen et al. 2017). In contrast, a mesophilic reactor containing pretreated food waste (under short-term hydrothermal) recorded a higher proportion of proteins of carbohydrate metabolism to *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes,* and *Cyanobacteria.* The proteins involved belong to glycolysis, pyruvate, propionate, glyoxylate, and dicarboxylate metabolism (Jia et al. 2017b).

In lab-scale models, anaerobic digestion of office paper, led to the identification and assignation of all glycolytic enzymes to *C. thermocellum*, *C. proteolyticus*, and *Caldicellulosiruptor* genus. Also, proteins involved in the synthesis of fermentation products such as lactate, ethanol, butanol, acetate, formate, and butanoate were also assigned to the same genus (Lü et al. 2014). Corresponding to the metabolism of grass, as lignocellulosic biomass, a set of glycolytic enzymes were identified specifically in the phase of sugar assimilation (Abendroth et al. 2017). Speda et al. (2017) were able to identify carboxyl transferase (gluconeogenesis) and G3PD (glycolysis) in a cellulose-rich (paper filter) biodigester. These findings showed that the EMP is one of the key glycolytic pathways functional during anaerobic digestion, as well as in the formation of intermediary products of fermentation, leading to the next step of methane production (Wilmes and Bond 2009; Abram et al. 2009).

4.4.4 Acetogenesis

During this stage, syntrophic bacteria oxidize VFAs greater than C_2 to produce key intermediates (25% acetate and 11% H_2) of the process. In this acetogenesis step, obligatory hydrogen forming syntrophic bacteria can cause toxic effects by accumulating high hydrogen pressure on the system. Consequently, the microbial consortium is not capable to survive under those conditions. Hence, symbiosis, as a syntrophic relationship, is necessary between acetogenic bacteria and autotrophic methanogens or sulfate-reducing bacteria for hydrogen consumption. The hydrogenotrophic methanogens keep the hydrogen pressure low, which contributes to a thermodynamically controlled condition for the fermentative bacteria to continue oxidizing the organic compounds (e.g., ethanol, propionate, and butyrate into acetate) (Barua and Dhar 2017). This oxidizing activity is related to the genera of *Syntrophomonas* and *Syntrophobacter*, as well to *Chloroflexi*, *Actinobacteria*, and *Spirochaetes*. Nonetheless, more or less abundance is related as well to *Gelria*, *Lachnospiraceae* (uncultured), *Ruminococcaeae*, *Incertae sedis*, *Sporanaerobacter*, and *Petrobacter* (Ziemiński and Frąc 2012; Wang et al. 2017; Jain et al. 2015).

In the case of butyrate, the oxidizing pathways are through the β-oxidation, for propionate oxidation will proceed through the methyl-malonyl-CoA (MMC) pathway and for syntrophic oxidation will be associated to the Wood-Ljungdahl (WL) pathway. According to Hagen et al. (2017), *Syntrophomonas* genus is the major phylotype in AD, where *S. wolfei* required all classes of the β-oxidation enzymes (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase). While for the propionate degradation, *Pelotomaculum thermopropionicum* identification suggests the requirement of the methyl-malonyl-CoA (MMC) cluster and propionate CoA transferase (PCT) cluster in high abundance. Further, this study reported that *Thermoacetogenium phaeum* for acetate oxidation by WL pathway (formyltetrahydrofolate synthase, 5,10-methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase, trimethylamine:corrinoid methyltransferase, carbon monoxide dehydrogenase/acetyl-CoA, phosphotransacetylase, and acetate kinase) (Hagen et al. 2017).

Role of all enzymes participating in AD has been poorly reported, due to the large amounts of proteins and other interfering substances present in the sample. Nevertheless, a relatively high abundance of proteins involved in acetogenesis has been revealed. Mostly, WL-like formyltetrahydrofolate synthase, 5,10-methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase, trimethylamine-corrinoid methyltransferase, carbon monoxide dehydrogenase-acetyl CoA, phosphotransacetylase, and acetate kinase have been identified as crucial for energy transport and microbial interactions performed mainly in syntrophic acetate oxidizers. A protein cluster encoding Fe–S oxidoreductase and an electron transfer flavoprotein were also identified, both related as well as electron transfer mechanisms (Hagen et al. 2017).

4.4.5 Interspecies Hydrogen Transfer in Syntrophs

Interspecies hydrogen transfer mechanism is vital in syntrophic relationships, where the latter groups such as hydrogen consumers are strongly influencing the syntrophic bacteria (Gomez Camacho and Ruggeri 2018). Acetate produced in the process could be converted to methane either directly by methyl reduction or by following a two-step reaction, where acetate is first oxidized to CO_2 and H_2, by syntrophic acetate oxidation (SAO), and then this hydrogen is used to reduce CO_2 into CH_4 (Mulat et al. 2014). Heyer et al. (2019) studied the interactions between microorganisms and the metabolic interchangeability of the different microorganisms. This work suggested that under specific anaerobic digestion conditions the thermodynamic equilibrium of CO_2, H_2, and acetate will decide on the metabolic pathway shift, either between SAO or homoacetogenesis. Understanding this will explain how some archaeal species have major enzyme affinity on acetate and could suppress other acetate-consuming phylotypes.

Thus, competition on the substrate between certain microorganisms such as *Methanosaetaceae* may kill or suppress other species due to the expression of bacteriocins, which inhibit the competitor (Heyer et al. 2019). The H_2 produced by

non-methanogenic syntrophic microorganisms from key fermentation products (ethanol and C_2 and greater than C_2 volatile fatty acids) are reduced to methane by hydrogenotrophic methanogens or to H_2S by sulfate-reducing bacteria. This interspecies microbial exchange of hydrogen suggests that syntrophs are incapable of independently oxidizing alcohols and C_2 and greater than C_2 volatile fatty acids under anaerobic conditions and need a partner that consumes hydrogen to keep the partial pressure of hydrogen under control and facilitate their metabolic activity. Syntrophic interactions consist generally on the intercellular transport of reducing equivalents, like H_2 and/or formate, coupled with H_2/formate consumers, also referred as interspecies hydrogen transfer (IHT) (Shrestha and Rotaru 2014; Summers et al. 2010).

In addition, formate often serves as a substitute for H_2 in interspecies electron transfer. The electron reduced carriers on this type of mechanism are additionally regenerated to an oxidized state (Shrestha and Rotaru 2014; Kouzuma et al. 2015). Westerholm et al. (2016) reported that SAO bacteria are principally classified in the group of homoacetogens, which perform the Wood Ljungdahl (WL) pathway during growth, in presence of autotrophic and/or heterotrophic substrates, and produce acetate as main by-product. In this pathway, they suggest that the gene *fhs* encodes the enzyme formyl tetrahydrofolate synthetase (FTHFS) to catalyze the ATP-dependent activation of formate, postulating as well, the reverse WL performance for acetate oxidation. Interestingly, the *Pseudothermotoga lettingae* acetate oxidizer can combine the methyl branch of the latter pathway with a glycine cleavage system (Westerholm et al. 2016).

Recent discoveries reported that some bacteria could directly transfer electrons to methanogens, as a unique cell-to-cell electron transfer mechanism, in a thermodynamically efficient manner (Cheng and Call 2016). The electron transfer, between microorganisms mediating electron carriers, is referred as direct interspecies electron transport (DIET), where three mechanisms have recently been suggested: (1) the via conductive pili, by association from two bacteria with a conductive pili (conductive nanowires), (2) the membrane-bound mechanism with electron transport proteins, by electron transmission which represents close cell connections by using a multiheme outer surface cytochrome (OmcZ), and finally (3), the more recently studied the magnetite particle which form chains for electrically connecting cells involved in DIET (Park et al. 2018).

4.4.6 Methanogenesis

Methanogenesis is one of the critical steps in the process of AD, characterized by slow reaction rate on the energy workflow. Syntrophic interactions between acetogenic bacteria and methanogens as well as methyl group reduction are essential to CH_4 production. Thus, understanding the microbial community involved in electron transferring dynamics is key to biogas production improvement. Archaeal methanogens are dominant groups in this phase, generally performing the aceticlastic, hydrogenotrophic

and methylotrophic pathways. The microorganisms usually found are the strict hydrogenotrophic *Methanomicrobiales, Methanobacteriales, Methanococcales*, and the acetate-utilizing microorganisms *Methanosarcinales* with specific predominance of *Methanosarcina* and *Methanosaeta*. Methanogens are quite sensitive for changes in environmental and operational conditions of AD process and many factors (i.e., high concentrations of volatile fatty acids, ammonium, sulfide, sodium an heavy metals) could inhibit the process (Al Seadi et al. 2008; Ziganshin et al. 2016).

As mentioned earlier, methane can be produced either by hydrogenotrophic pathway by reducing CO_2 using hydrogen, the methylotrophic pathway where methylated compounds like methanol or methylamine are reduced, and in acetoclastic pathway, methyl group of acetate is directly reduced to methane. Recently, the class of *Thermoplasmata* has been described to be capable to reduce methanol with H_2 and may use methylamines as well, suggesting that methanogenic diversity could be higher (Wintsche et al. 2018).

From the above three pathways, studies were performed using isotope assays to know the metabolic contribution from each one. It was reported that syntrophic acetogenic process and hydrogenotrophic methanogenesis accounted for 41 and 50% of methane formation at 37 °C and 55 °C, respectively (Yin et al. 2018). Another study using isotopes indicated that the non-aceticlastic oxidizers performed, approximately 80% of the pathway of the acetate decomposition in the reactor, which indicated the role of syntrophic acetate-oxidizing bacteria. *Pseudothermotoga lettingae* (previously as *Termotoga lettingae*) strain was reported to show syntrophic acetate oxidizing activity without sulfate ions and under co-culture conditions in relation with hydrogenotrophic methanogens (Sasaki et al. 2011).

Identification of the pathways and enzymes involved in the methanogenesis networks via metaproteomic approach has been carried out (Table 4.2). During the hydrogenotrophic pathway, CO_2 is reduced to methane through the intermediates formyl, methylene, and methyl. These residues are transferred to the coenzyme M, forming a methyl-CoM molecule further reduced to CH_4 by the key methyl coenzyme M reductase (MCR). Meanwhile, the energetically coenzyme F_{420} acts as an electron acceptor for hydrogenase, formate dehydrogenase, and carbon monoxide dehydrogenase, as well as donor electron for reductase $NADP^+$. Moreover, this coenzyme utilize H_2 and formic acid, as electron donor to produce the methane by CO_2 reduction (Jia et al. 2017b). In the aceticlastic pathway, methyl group of acetate is reduced to methane by methyl reductase enzyme. In the case of methylotrophic pathway, the methyl groups are transferred to a methanol-specific corrinoid protein, then reduced by the MCR (Jia et al. 2017b; Guo et al. 2015). It is important to highlight that methyl-coenzyme M (methyl-CoM) reductase is active for all the three pathways (Hanreich et al. 2012).

In a full-scale BGP, CODH/ACS and energy-converting hydrogenase (Ech), proteins, and enzymes involved in metabolism of methanol and methylated amines, as well as, V-type ATP synthase for energy conversion were assigned to *Methanosarcinales*. While F_{420}-dependent N_5,N_{10}-methyleneH_4MPT reductase (Mer), Mtr, and F_{420} reducing hydrogenase (Frh) were assigned to *Methanobacteriales* (Heyer et al. 2016). In the hydrogenotrophic pathway, Lü et al. (2014) found proteins from strains of

Methanothermobacter as H_2-forming methylene H_4MPT dehydrogenase, F_{420}-dependent methyleneH_4MPT dehydrogenase, Mtr, Mer, MCR, Frh, and heterodisulfide reductase. As well as from the methylotrophic pathway, monomethylamine methyltransferase, and a large subunit of the corrinoid/iron–sulfur protein and methylcobamide:CoM metyltransferase were detected. In contrast, none of acetoclastic enzymes were identified in this study.

Furthermore, trace elements on the anaerobic digestion have an impact on the performance process to carry out cell metabolisms and is critical to the final stage of the methane yield. For methanogens, the presence of Fe, Zn, Ni, Cu, Co, Mo, and Mn are essential. First, Fe, is important in stimulant as growth factor and formation of cytochromes and ferroxins vital for energy metabolism. Additionally, trace elements form the active site in metalloproteins, act as a cofactor and give the structure. Enzymes such as Mtr and MCR require Co and a nickel-containing cofactor F_{430} in their active sites, respectively (Choong et al. 2016). Trace element deprivation has shown to decrease hydrogenotrophic metaproteins abundance from *Methanomicrobiales* and methylotrophic and aceticlastic metaproteins from *Methanosarcinales*. However, trace elements may have negative impact when present in high concentrations. It has been demonstrated to cause enzyme disruptions and changes on functional structure, and microbial composition as well. Therefore, effectiveness of the anaerobic digestion performance by using trace elements will further depend on its optimum bioavailability fraction (Bourven et al. 2017).

4.5 Stress Responses and Biomarkers

Although AD is an economic way of waste management combining with renewable energy production in the form of methane, the process has certain thresholds and one of them is high sensitivity to the presence of certain substances at high concentrations during the process. Most frequently, a reactor turns "sour" due to accumulation of volatile fatty acids (VFAs). Further, ammonia, high partial pressure of H_2 have recorded negative effects on AD process, among several other factors (Chen et al. 2014). Therefore, several studies have focused on efforts to overcome stress conditions by detecting different key enzymes to those conditions in the reactor (Table 4.3).

Formerly, when the process contains simple sugars, which are easy to degrade, VFAs are accumulated and decrease in pH results in the imbalance of AD process. Interestingly, under this condition, Theuerl et al. (2015) could detect proteins involved on aceticlastic and hydrogenotrophic methanogenesis from *Methanosarcinales, Methanobacteriales*, *and Methanomicrobiales,* with an abundance ranging from 55 to 77%.

On the other hand, high concentrations of total ammonia nitrogen due to the high rate of protein hydrolysis could negatively affect reactor operation. Nevertheless, lignocellulosic-rich matter is a convenient substrate to slow the rates of process start-up, diminishing the possible ammonia accumulation. Also, trace elements have demonstrated to overcome such problems. It has demonstrated that nickel which is contained in coenzyme F_{430} enhanced methane potential and overcome such

Table 4.3 General overview on protein abundance under varying environmental and operational conditions

Reactor	Key enzyme/ biomarkers	Functional role	RA	Species related to expression	References
Lab-scale anaerobic moving bed reactor at 37 °C	Cofactor F_{430}	Nickel hydrocorrinoid prosthetic group of the methyl-CoM reductase. It decreases the toxic effect of higher VFAs content and increases methane production	++	*Methanococcus jannaschii* *Methanococcus maripaludis* *Methanococcus vaneilii*	Passaris et al. (2018)
Lab-scale mesophilic digester treating starch	Methyl coenzyme-M reductase	It reduces the methyl CoM with hydrogen for methane production	+	*Methanobacterium* *Methanosaeta*	Zhang et al. (2018)
Low-temperature granular sludge reactor operated at 15 °C	[a]Oxygen-sensitive alcohol dehydrogenase	Interconversion from alcohol to acetaldehyde	++	*P. propionicus*	Abram et al. (2011)
	[a]Transketolase	Constitutes the reversible link between glycolysis and pentose phosphate pathways	+ + +		
Sequencing batch reactor with alterations in phosphorus level	[a]Peroxiredoxin	Protects the cell against reactive oxygen species	++ +	*Accumulibacter phosphatis*	Wilmes and Bond (2009)
	[a]Thioredoxins/ chaperon proteins	Responsible to maintain disulfide bonds within the cytoplasmatic proteins in a reduced state	+ + +		

RA Relative abundance
[a]Abundance of proteins depending on the stress condition or inhibitory factor
+ low abundance, ++ medium abundance, +++ high abundance

problems (Capson-Tojo et al. 2017). Additionally, under concentrations of 6–7 g-N/L ammonium, inhibition of *Methanomicrobiales* and *Methanosaetacea* activities was observed along with a shift in microbial community (Lü et al. 2014).

The lignocellulosic-rich feedstocks with high lignin content could also cause inhibitory stress conditions. The negative impacts on these types of substrate, especially due to the by-products of lignin decomposition, inhibitory aromatic compounds. These are mainly composed of furanic acid (5-HMF, fufural) and phenolic compounds leading to a metabolic shift of the H_2-producing pathways to a nonproducing pathway (Monlau et al. 2014). However, several numbers of enzymes are involved in the catabolic pathway of lignocellulosic compounds. The

benzoyl-CoA reductase class II (BamBCDEFGHI) could function as a functional marker, which is expressed and detected when mono-aromatic compounds are degraded (von Netzer et al. 2016).

A well-studied biomarker to understand methane production is the expression of *mcrA* gene, which encodes the key enzyme methyl coenzyme M reductase (MCR). MCR catalyses the last step of methanogenesis and related to methane yield. This biomarker can provide useful information and by monitoring its activity functional performance of a biodigester can be studied (Morris et al. 2014). Expression of *mcr*A has been found related to the presence of concentration of volatile fatty acid such as acetate and propionate (Aguinaga Casañas et al. 2015). However, new protein biomarkers are drawing attention, as proteins can be synthesized and then folded immediately after a stimulus, which could vary under different conditions. Hence, protein identification with potential in biomarker fingerprint could be revealing tool for instant physiological responses (Lacerda et al. 2007).

4.6 Challenges and Future Perspectives

Metaproteomics analysis is the most recently developed tool, which indicates the protein assignment to specific microorganisms and contributes to understand the relationship between phylogenetic analyses and the proteome (Abram et al. 2011). However, its application has not been completely exploited since there is still more "black holes" and methodological challenges to solve the whole metabolic pathways (Wilmes et al. 2015).

Principal problems are related to methodological issues involved in the correct isolation of the proteome, since humic acids and their impurities make the quantification and separation of the proteins very challenging. Humic acids are commonly present in environmental samples and bind to the proteins which hamper protein separation (Wilmes and Bond 2004). Fractioning proteins by gel separation, also involves several difficulties, as poor separation of acidic, alkaline, and hydrophobic proteins and a low load capacity, which affects the resolution and analysis of the gel (Li et al. 2016).

Secondly, peptides' identification is one of the most challenging steps in metaproteomics. Some proteins' sequences are among the most highly conserved across different microorganisms, which entails the same peptide sequences from several different species. These leads to have many proteins identified in the same information storage and processing group. Besides, MS/MS data uses the top of the 10–20 most abundant peptide ions acquired and does not cover all available peptide ions, which misses a plenty of valuable information (Heyer et al. 2017). Thus, metaproteomic approaches have to overcome the cleanup and characterization of peptides and proteins by intensive purification and pre-fractionation methods (Wenzel et al. 2018).

The key to the metaproteomic studies is the resolution level of the peptide identification. In future, considerations on the use of the best algorithm which

could identify the proteins covered on the database to evaluate for de novo results (Heyer et al. 2015). The molecular techniques could provide the microbial information and linking the species on the proteomic data by complementary studies of the microbial consortium, could aid to overcome the challenges encountered in the coverage of the samples. Further, improvement in methodologies to identify proteins along with improvement in methods of data analyses are essential to apply metaproteomics as an effective tool to understand the microbial diversity and their function of biodigesters (Herbst et al. 2016).

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Chapter 5
Circular Economy and Agro-Industrial Wastewater: Potential of Microalgae in Bioremediation Processes

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Abstract Agro-industrial production generates large volumes of effluents with a high content of solids, nutrients, organic matter, and microorganisms. These effluents can negatively modify natural environments that receive them by surface runoff or infiltration through the soil, with possible damage to the population's health. The objective of the circular economy is to maintain—as long as possible—the materials, products, and resources used in the production system to diminish, in this way, contaminating wastes. The "biologization" of industrial processes using the purification capacity of microalgae to decontaminate wastewaters has emerged in recent years. It offers two benefits, the production of biomass for different uses and the production of cleaner effluents. After microalgal treatments, ecotoxicity tests are used to assess the effectiveness of decontamination processes. In addition, bioassays indicate how long it is necessary to continue the decontamination process, i.e., when the concentration with no toxic effects has been reached, thus reducing unnecessary costs.

In this chapter, we will discuss (1) the use of microalgae for the treatment of agro-industrial wastewater derived from dairy, swine, and agrochemicals (fertilizers, pesticides) production. (2) The relevance of a cleaner remediation technology for water contaminated with glyphosate: the advanced oxidation process (AOP), using the microalgae *Chlorella vulgaris* as a test organism. (3) The importance of monitoring environmental pollution in freshwater aquatic ecosystems through ecotoxicology tests using nontarget species.

Keywords Agro-industrial wastewater · Microalgae · Advanced oxidation process (AOP) · Ecotoxicity

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5.1 Introduction

In recent years, many applications of microalgae have been developed, among which the animal and human diet stands out for its high protein values, enhanced by having a good balance of amino acids and low values of nucleic acids, compared to other sources of unicellular proteins (Quevado et al. 2008; Benavides and Rito-Palomares 2008).

It has been found that different species of the genus *Spirulina* sp. have different types of sterols such as clionasterol, which can increase the production of plasminogen activating factors in vascular endothelial cells, facilitating the prevention of vascular diseases. Antioxidant compounds such as β-carotenes and astaxanthin isolated from microalgae have the potential to protect against oxidative stress caused by a broad spectrum of diseases and by aging itself. Studies suggest that the consumption of astaxanthin produced by *Haematococcus pluvialis* could have benefits on human health. Lutein (a yellow pigment found in vegetables and in the group of xanthophylls) is used for the prevention and treatment of degenerative diseases (Stringheta et al. 2006).

Chlorella sp. is also used for these purposes, mainly due to the presence of β-1,3 glucan in its composition. The β-1,3 glucan is an immunostimulator, a free radical scavenger and a blood lipid reducer. In addition, other beneficial health effects have been identified such as preventive antitumor activity and prevention against arteriosclerosis (Lourenço 2006).

Microalgae have also been evaluated with the aim of generating economic resources of various kinds, such as lutein production (Shi et al. 2000, 2002). Other uses include the production of hydrogen for fuel by biophotolysis, the production of methane in biodigesters, the production of biodiesel, the elimination of residual CO_2 emissions, the manufacture of cosmetics, and they are also used as a vector for the production of recombinant proteins (Scragg et al. 2003; Miao and Wu 2004; Sanchez et al. 2008; Plata et al. 2009).

Another line of research is the use of microalgae for bioremediation purposes. This approach arose to respond to the growing problems of contamination of aquatic systems, which created a new demand: the use of microalgae to remove the polluting elements still present in the last phases of effluent treatment, which cannot be done by chemical methods.

Agro-industrial activities produce increasing volumes of wastewater containing high concentrations of inorganic and organic compounds of various kinds. Before being dumped into superficial freshwater courses, the regulations in force in different countries require that the effluents be treated to reduce the concentration of chemical compounds and reach the specified physicochemical parameters for dumping. Phytoremediation is the purification capacity of algae (Rawat et al. 2011; Prajapati et al. 2013). It is the use of macroalgae and/or microalgae for the elimination or biotransformation of pollutants (Rawat et al. 2011; Prajapati et al. 2013; Doušková et al. 2010; León and Chaves 2010; González-López et al. 2011; Wang et al. 2015; Abdel-Raouf et al. 2012; Infante et al. 2012; Maity et al. 2014). Among the

pollutants studied for remediation purposes are petroleum derivatives, heavy metals, detergents, and industrial effluents of very diverse composition, organic and/or inorganic (Rachlin and Grosso 1991; Iannacone and Gutierrez 1999; Chong et al. 2000; Mehta and Gaur 2001; Salomon et al. 2003; Johnstone et al. 2006; Rodrıguez et al. 2007; Vera et al. 2009). In the microalgae *C. vulgaris*, the biotransformation capacity of xenobiotic compounds such as pesticides is due to the participation of monooxygenase enzymes from the cytochrome P450 superfamily (Schocken et al. 1997). Using different pathways, these enzymes oxidize endogenous substrates, such as lipids, steroid hormones, and some xenobiotics, such as pesticides, and particularly herbicides. In the bibliography you can find studies on the ability of microalgae to metabolize different pesticides, such as metflurazon, methyl parathion, parathion, malathion, phorate, quinalphos, monocrotophos, lindane, phenol, chlordimeform, and DDT (Megharaj et al. 1994; Thies et al. 1996; Priyadarshani et al. 2011). In 1960, an alternative to conventional treatments emerged, which consisted of the use of "bacterial-algal" systems. The concept of treatment of effluents using bacteria and algae was proposed in the seminal work of William Oswald (1988). These cultures are of mixotrophic type, in which microalgae obtain energy either from light or from organic compounds. The assimilation of carbon occurs from organic compounds, unlike photoautotrophic cultures in which the assimilation of carbon is from carbon dioxide (CO_2) (Lee et al. 1996; Cho et al. 2017). Although these crops were widely used in the treatment of various effluents, it was in the second half of the 1980s when the interest in investigating the use of algal biomass for different applications was strengthened (Albeliovich 2004).

The use of microalgae for the removal of nutrients has been studied for more than 50 years (Andrade et al. 2009; Hanumantha Rao et al. 2011; Mendez-Suaza et al. 2011; Marchello et al. 2015). These studies proved that the algal biomass obtained has a high content of proteins, lipids, carbohydrates, and other high value-added products. In this way, efforts to cultivate microalgae in wastewater have sought to achieve a double benefit: the production of biomass for different uses and a clean effluent obtained by a relatively simple technology (Rawat et al. 2011).

Data from the United Nations show that in 2017, 54.74% of the world population lived in urban settlements (approximately 4125 million inhabitants) and it is estimated that in 2030 it will increase to 60% (5.1 billion inhabitants). This entails challenges regarding the management of waste and effluents due to the fact that, according to measurements made by the United Nations Environment Program (UNEP), in 2015, between 7 and 10 billion tons of waste from homes, construction, industry, and commerce were generated worldwide. One of the agricultural activities producing the greatest amount of effluents is the intensive breeding of cattle and pigs for the production of milk and meat.

The high concentration of breeding animals per unit area generates environmental problems that require remediation measures. Among them, the high production of nutrients such as ammonium and orthophosphate is of environmental concern. They can be incorporated by different routes to surface aquatic systems or to the layers by infiltration, runoff, or through soils fertilized with nitrogen and phosphate, as well as by discharges of wastewater without any or with insufficient treatment (Vargas et al. 2004).

The effluents of the dairy farms contain excreta, urine, and washing water from the facilities, in addition to the remains of milk or food, detergents, and other chemical products used in the disinfection process of the establishment. As a result, the composition of the effluent has a high content of solids, nutrients, organic matter, and microorganisms that can reduce the quality of the environment that receives this discharge (body of water, underground water, and/or soil). The waste generated can have negative impacts on air, soil, and water, because it is concentrated in small areas and is a source of nutrients, antibiotics—among other veterinary drugs—and pathogens (Herrero and Gil 2008). In addition to the degradation of water and soil resources, this kind of wastewater becomes important due to the proliferation of synanthropic pests (flies, rodents, among others) and the generation of undesirable odors produced when it is not adequately disposed of (United Nations Organization for Agriculture and Food (FAO) and National Institute of Agricultural Technology Argentina (INTA) 2012). Thus, in addition to being a potential source of infection, the sites where solid waste is discharged also represent an important source of air pollution (Cervantes et al. 2007), resulting in *ecosystem disservices* (EDs) (Von Döhren and Haase 2015).

The EDs include negative impacts of natural and anthropic origin. In the latter case, EDs are linked to the contamination of water, soil, and/or air (Sandifer et al. 2015). This term is new and poorly publicized since it does not have the applicability of ecosystem services, but it was imposed by the increase in anthropogenic activities with a negative impact on ecosystems (Von Döhren and Haase 2015). Environmental damage can result in the total or partial loss of the ability to comply with the beneficial functions of ecosystems and consequently, in a decrease in socioeconomic well-being. In this text it is proposed that the term *environmental damage* be used to illustrate the loss of ecosystem services in aquatic systems by enrichment with toxic substances and at the same time by the adverse effect on key ecosystem services, such as the purification of pollutants, the provision of water for irrigation and, in general terms, for the maintenance of the structure and function of natural systems (Sandifer et al. 2015). In order to face this environmental and human health problem, different biotechnological solutions were proposed, among which bioremediation stands out.

Many microorganisms can use a variety of organic pollutants, including pesticides, as an energy source for their growth (Massoud et al. 2008). Recent research shows the potential of *C. vulgaris* to be used as an environmental management tool in the treatment of liquid effluents from the agro-industry.

In this framework, Kim et al. (2007) cultivated *Scenedesmus* sp. in culture medium with 3% of swine effluents, obtaining a biomass rich in chlorophyll and carotenoids, reducing, in addition, the concentration of carbon, nitrogen, and phosphorus by 12.9%, 87%, and 83.2%, respectively.

Regarding each of the aforementioned agro-industries—cattle and swine production—in Argentina, the dairy industry (dairy farms) produces 30–40 L of effluent/day/animal. Works carried out in La Pampa Province show that a dairy farm with 140 milking cows and average milk production of 2500 L^{-1} generates about 4000 L of effluents per day, equivalent to 1.6 L of effluents per liter of milk produced (Diez

2012). In the case of swine production, according to Vicari (2012), if we consider pregnant pigs, lactating pigs, weaned piglets, and pigs weighing 25–100 kg, in Argentina about 26,910 tons of effluents are produced per day.

The excessive incorporation of nutrients in water bodies, especially nitrogen and phosphorus, could cause eutrophication events, altering the environmental quality of aquatic systems (Wu 1999). A third of the decrease in biodiversity in rivers, lakes, and wetlands in the world is attributed to eutrophication (A UN-Water Analytical Brief 2015).

In this scenario, phycoremediation emerges as an environmental management tool that allows reducing the negative impacts of agro-industrial effluents and valorizing by-products obtained from the process.

On the other hand, adding value to commodities is a challenge for countries whose economy rests fundamentally on the resources obtained from agro-industrial raw materials with little or no processing before exporting to European and Asian markets. In this sense, the treatment systems with microalgae are a contribution to the diversification of strategies to increase the added value of agro-industrial products. Bioeconomy indicates the need to promote research in new biological resources that allow the diversification of existing value chains. In addition, in the transition to a circular economy, innovation, and the development of new technologies that make it possible to reduce the generation of waste through its recovery are absolutely necessary, converting waste into resources, as it was proposed by Bayón et al. (2018), for the reuse of agricultural waste for therapeutic and medical purposes. The production of microalgae is currently considered a novel and versatile renewable resource, which not only allows obtaining a wide range of bioproducts with economic value, but is also aligned in an appropriate way with the necessary environmental sustainability.

5.2 Use of *C. vulgaris* for the Treatment of Dairy and Swine Effluents

Large volumes of effluents, generated by intensive livestock production, can enter the water bodies by surface runoff or infiltration through the soil. These effluents can alter the environmental quality of aquatic ecosystems, causing damage to the health of the population that uses the resource (Herrero and Gil 2008).

These production systems, where animals are confined in a small space, increase the amount of effluents to be treated in the facilities, which in many cases do not have adequate infrastructure or planning for their treatment and final disposal, causing socio-environmental and economic problems (De Grandis and Visintini 2015).

There are different technologies for the treatment of effluents from dairy farms, anaerobic lagoons, storage wells, stabilization ponds, series of lagoons, or stabilization ponds. The efficiency of these treatments depends mainly on the quality and

quantity of the effluent generated in each dairy farm (De Grandis and Visintini 2015). On the other hand, the final quality of the effluents treated by the previously affected methods is not always adequate, so it is proposed to complement these processes with microalgae cultures, which eliminate contaminants still present in the last phases of the treatment. In this way, the requirements of effluent quality before discharge into the rivers, can comply with current regulations and minimize the environmental, social, and economic impact of dairy production.

In the last decades and to give answers to the problems of aquatic contamination, phycoremediation was proposed as a new technology that contemplates aspects of sustainability.

In wastewater treatment, the principal challenge is to reduce biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids, nutrients, and coliforms (Abdel-Raouf et al. 2012; Dominic et al. 2009; Park and Craggs Shilton 2011).

Wang et al. (2015) worked with *Chlorella vulgaris* for the bioremediation of swine wastewater, obtaining a COD removal between 60 and 70%. Xie et al. (2019) recorded COD values of 86.72 ± 1.72% in experiments with *Chlorella vulgaris*. These authors concluded that *C. vulgaris* has potential for the treatment of mixed wastewater (anaerobic digestion effluent and rainwater).

Regarding the removal of other compounds, Wang et al. (2010) reported the effectiveness of the use of dairy effluents as a nutrient supplement for *Chlorella* sp. cultivation. They reported elimination values of various compounds: ammonia, total nitrogen, and total phosphorus = 100%, 75.7–82.5%, and 62.5–74.7%, respectively.

Reno et al. (2018) reported the removal of different compounds in culture medium supplemented with 25% effluent from dairy farms (Table 5.1).

The biomass of *C. vulgaris*, obtained in crops with effluents from dairy farms, recorded a chlorophyll-a concentration of 1.68 μg L^{-1} and a protein concentration of 3.75%.

Other works aimed for the production of biodiesel, reported proteins between 49.9 and 55%, after cultivation of *C. vulgaris* in medium with domestic wastewater (Miao et al. 2016; Ma et al. 2016).

Table 5.1 Physical–chemical characterization of the pure effluent and of the supernatant obtained at the beginning and at the end of the trial, after cultivating *C. vulgaris* in medium with 25% of dairy production effluents

	Pure effluent	25% Initial	25% Final	% Reduction
Ammonium (mg L^{-1})	294	67.2	1.9	97.1
Nitrate (mg L^{-1})	43.6	31.1	15.6	49.8
Nitrite (mg L^{-1})	0.064	0.067	0.107	–
Total phosphorus (mg L^{-1})	60.1	48.4	19	60.7
COD (mg L^{-1})	6078	1846	353	80.8
BOD (mg L^{-1})	2981	947	173	81.7
pH (UpH)	7.09	7.58	8.79	–
Conductivity (μS cm^{-1})	4070	1665	1192	28.4

On the other hand, Kim et al. (2007) and Morales-Amaral et al. (2015) cultivated *Scenedesmus* sp. in culture medium with 3% swine effluents and 30% of treatments from urban wastewater, respectively. In both works, it was possible to verify the reduction of phosphorus and nitrogen. In addition, Kim et al. (2007) reported that the biomass obtained contained high amounts of chlorophyll and carotenoids (Pulz and Gross 2004). Moreover, the authors reported that in the algal biomass they obtained a high content of bioproducts (proteins, lipids, carbohydrates, among others) that could be used in different value-added processes. In this way, microalgae cultures in wastewater will seek the production of biomass for different uses, together with a clean effluent using simple technology (Rawat et al. 2011; Pulz and Gross 2004).

5.3 Use of *C. vulgaris* for the Treatment of Agrochemicals Effluents

The toxicity of agrochemicals is permanently under global debate. The environmental pollution caused by pesticides is considered a major problem worldwide. In particular, glyphosate residues are a serious hazard, as these substances can reach the aquatic environment by drifts, surface runoff, drainage, and leaching, which significantly increases the risks to human beings and nontarget organisms.

Given that microalgae are particularly efficient in accumulating nutrients and heavy metals (de-Bashan et al. 2015), they can be used to reduce the concentration of contaminants. Moreover, purification plants can then provide the agro-industry with algal biomass for use as an organic fertilizer or for the production of high value-added derivatives (Rawat et al. 2011).

Baglieri et al. (2016) evaluated the removal capacity of *C. vulgaris* and *Scenedesmus quadricauda* in culture medium supplemented with three fungicides (fenhexamid, iprodione, metalaxyl, pyrimethanil) and a herbicide (triclopyr), reporting removal (%) greater than 50% at 56 days of testing for all active ingredients.

On the other hand, Tang et al. (1998) reported that the absorption of atrazine occurs rapidly, chlorophytes being more efficient than diatoms. In this sense, Regaldo (2013) showed that *C. vulgaris* has low efficiency to remove atrazine, 11.16% and 51.39% when exposed to 43.9 (C1) μg L^{-1} and 131 (C2) μg L^{-1}, respectively. According to Solomon et al. (1996), the low removal capacity of atrazine in freshwater is mainly due to the low octanol–water coefficient (log $K_{ow} = 2.68$ at 25 °C), and its rapid metabolization and excretion. Weiner et al. (2004) also stated that the differences obtained in the removal efficiency of this herbicide by microalgae are explained by a complex interaction between several metabolic factors.

For the case of glyphosate (*N*-phosphonomethylglycine), one of the most widely used pesticides worldwide, Reno (2017) reported the removal capacity of *C. vulgaris* to four commercial glyphosate formulations. The results showed that the removal of glyphosate by *C. vulgaris* reached a maximum of 11.22% at 360 min exposure, for

the commercial formulation Eskoba®, compared to 76% degradation by the advanced oxidation process (AOP-UV/H_2O_2) obtained for the same formulation and evaluation time. These results indicate that it is convenient to increase the reaction times of the AOP-UV/H_2O_2 process and to complete it with a decontamination biological process to obtain concentrations below the guideline levels for the protection of aquatic biota in Argentina (e.g., for glyphosate, $\leq$0.24 mg L^{-1}).

In this same line, Lipok et al. (2009) reported that in media with concentrations higher than 1 mg a.e. L^{-1}, *C. vulgaris* did not remove glyphosate in significant quantities, probably because under the test conditions, the Carbon–phosphorous (P) links were not broken, a necessary previous step, so that the microalga could take the herbicide as source of P. The mentioned limit value (1 mg a.e. L^{-1}) reported by these authors is very close to the minimum concentration tested by Reno (2017) =0.78 (±0.20) mg a.e. L^{-1} for the Sulfosato Touchdown® formulation.

Conversely, with different species of cyanobacteria, bacteria, and fungi, other authors found higher percentages of glyphosate removal, after longer exposure times. For example, Salman and Abdul-Adel (2015) evaluated the ability of *Oscillatoria limnetica* to remove glyphosate, using the commercial formulation Roundup®, and found significant differences in the speed of removal after 3, 5, 7, 14, and 35 days of treatment. They recorded 97.5% after a 7-day removal period, at an initial concentration of 5 mg L^{-1} of glyphosate, 57.9 and 37.1% after 14 days at initial concentrations of 10 and 15 mg L^{-1}, respectively.

Lipok et al. (2007, 2009), using the commercial formulation Roundup 360®, reported that species of *Spirulina* spp. can remove glyphosate. They obtained between 0.2 and 1% of removal at an initial concentration of 10 mg L^{-1}; 20 and 70% at an initial concentration of 0.4 mg L^{-1}, after 4 and 10 days, respectively. In addition, they reported the ability of the cyanobacterium *Spirulina platensis* and the bacterium *Streptomyces lusitanus* to catalyze the molecule of glyphosate and the fungus *Fusarium dimerum* to remove and use this herbicide as a unique source of phosphorus.

One of the greatest difficulties for the management of agro-industrial wastes containing a "cocktail" of pesticides is the presence of more than one active ingredient in the effluents, given that in the agricultural activity pesticides are frequently used in mixtures. In this sense, recently Hussein et al. (2017) reported the potential of *C. vulgaris* to remove pesticides (atrazine, molinate, simazine, isoproturon, propanil, carbofuran, dimethoate, pendimethalin, metolachlor, pyriproxin) in a mixture of all pesticides, with a concentration of 0.1 mg mL^{-1}. The authors reported a removal capacity greater than 80% in live matrices of immobilized *C. vulgaris* and in lyophilized matrices.

As was mentioned above, it is widely accepted to use different species of microalgae to diminish pesticide concentration from an effluent. However, the literature reveals a high variation in the capacity of microalgae to absorb, degrade, and eliminate pollutants, according to different conditions. Among the most important factors are the species used, the possible interaction with other microorganisms, the methodology used in the test, the cultivation system, the chemical parameters of the wastewater to be treated and the environmental conditions (pH, temperature,

salinity, nutrients, quality and intensity of light, available water, oxygen tension, redox potential, binding to the surface, and presence of alternative carbon substrates) (Komolafe et al. 2014).

5.3.1 *Proposal of a Clean Remediation Technology for Water Contaminated with Glyphosate: The Advanced Oxidation Process (AOPs) Using* C. vulgaris *as Test Organism*

In the last decades, the implementation of genetically modified (GM) glyphosate-resistant (GR) cultivars has contributed to increase the agricultural use of glyphosate. The countries with the highest soybean production are the highest consumers of glyphosate. USA, Brazil, and Argentina are the major soybeans producers, according to 2013 statistics. These countries commercialize 89, 81, and 49 million tons of soybean, respectively (United Nations Organization for Agriculture and Food (FAO) 2013). The production of this oleaginous is associated with the use of large quantities of pesticides, especially the glyphosate herbicide. For example, since 1974 in the USA, over 1.6 billion kg of glyphosate active ingredient have been applied, or 19% of estimated global use of glyphosate (8.6 billion kg). Globally, glyphosate use has risen almost 15-fold since so-called "Roundup Ready," genetically engineered glyphosate-tolerant crops were introduced in 1996 (Benbrook 2016).

In commercial glyphosate-based formulations, only the active ingredient is specified, i.e., glyphosate salt, but the formulations also contain surfactants as adjuvant or additives to facilitate the mobility of glyphosate through the waxy cuticle (World Health Organization (WHO) 1994). Although these ingredients are usually classified as "inert," it is well documented that some of them can be even more toxic than glyphosate (Székács et al. 2014).

The AOPs could be an option to reduce the concentration of glyphosate to acceptable limits. Clean technologies, such as AOPs, can greatly reduce pollution. UV radiation combined with hydrogen peroxide has certain advantages over other AOPs; H_2O_2 is commercially available and simple to use. AOPs are based on generating highly oxidizing species, such as hydroxyl radicals (OH), which react with the pollutants and degrade them to harmless products, such as carbon dioxide, water, and mineral acids. In addition, nonselective technologies, such as oxidants, can degrade almost any type of chemical pollutant (Manassero et al. 2010). Vidal et al. (2015) proved that the combination of hydrogen peroxide and UV radiation may become a suitable and very simple process for treating wastewater from glyphosate commercial formulations. However, full mineralization is not always possible at a reasonable price and time. Thus, the intermediate compounds generated during degradation might be equally or more toxic than the parent compound. Bioassays with microalgae or other bisensor specie can then be used to detect when the treated effluent is no longer toxic, which can reduce AOP operating

costs, since complete pollutant degradation is not always necessary to generate a harmless effluent. Because toxicant sensitivity differs among species, organisms from different taxonomic groups must be used as biological models for the assays (Fernández-Alba et al. 2002).

Ecotoxicity tests are a useful tool to assess the decontamination efficiency by AOPs and to determine the most suitable end point and operation time for treated wastewater. Biological assays might reduce operating costs because—as it was previously mentioned—they could indicate that the total mineralization of the pollutant is not necessary. Several toxicity tests have been used to evaluate whether effluent detoxification is effective (Rizzo 2011). The assessment of algal sensitivity to herbicides is very important, as algae are the bases of many aquatic food webs and the primary producers in aquatic ecosystems. Therefore, toxicity tests based on microalgae have also been developed considering their ubiquity and short life cycle (Rizzo 2011). On the other hand, it should be noted that algal species vary widely in their response to pesticide formulations: for example, it was found that *Chlorella pyrenoidosa* was more sensitive than *Scenedesmus obliquus* to 26 herbicides.

In 2016, Reno et al. (2016) evaluated the efficiency of the AOPs (UV/H_2O_2) using as a bioindicator, the freshwater algae *C. vulgaris*. The glyphosate-based formulations were degraded in an annular reactor (V reactor = 870 cm^3) that contains an internal quartz tube with UV radiation passing from a concentrically positioned germicidal lamp (Philips 125 TUV 15 W, low-pressure Hg vapor lamp with peak emission at $\lambda = 253.7$ nm), to the annular reactor containing the glyphosate-based formulations.

This reactor operates in a recirculation batch system, which includes a centrifugal pump and a feed tank with continuous stirring. The total volume of the system is 2500 cm^3. Constant temperature (T = 20 °C) is maintained by a heat exchanger. A more detailed description of the reactor and its operating conditions can be found in Junges et al. (2013) and in Reno et al. (2015) (Fig. 5.1).

For the algal growth inhibition test of the four glyphosate-based formulations, the samples tested were the following: sample M_0: untreated, corresponding to 50 mg L^{-1} a.e. of glyphosate without H_2O_2; and samples M_1, M_2, and M_3, collected at different reaction times of the UV/H_2O_2 process: 120, 240, and 360 min, respectively, with the removal of the remaining H_2O_2. Bovine catalase (2197 U mg^{-1}; Fluka) (1 U decomposes 1 μmol H_2O_2 min^{-1} at pH 7.0 at 25 °C) was used to degrade the remaining H_2O_2 from the samples.

The authors concluded that the results obtained, showed that the bioassay based on *C. vulgaris* is a good tool for evaluating the UV/H_2O_2 process to treat effluents containing glyphosate from agricultural activities.

It was shown that the experimental conditions reached in all the samples treated with UV/H_2O_2 process in a reaction time of 360 min (M_3) are adequate to obtain decontaminated effluents when toxicity is assessed with *C. vulgaris*. The maximum inhibition percentage (1%) was 18.2, lower than the EC_{50} values (Fig. 5.2).

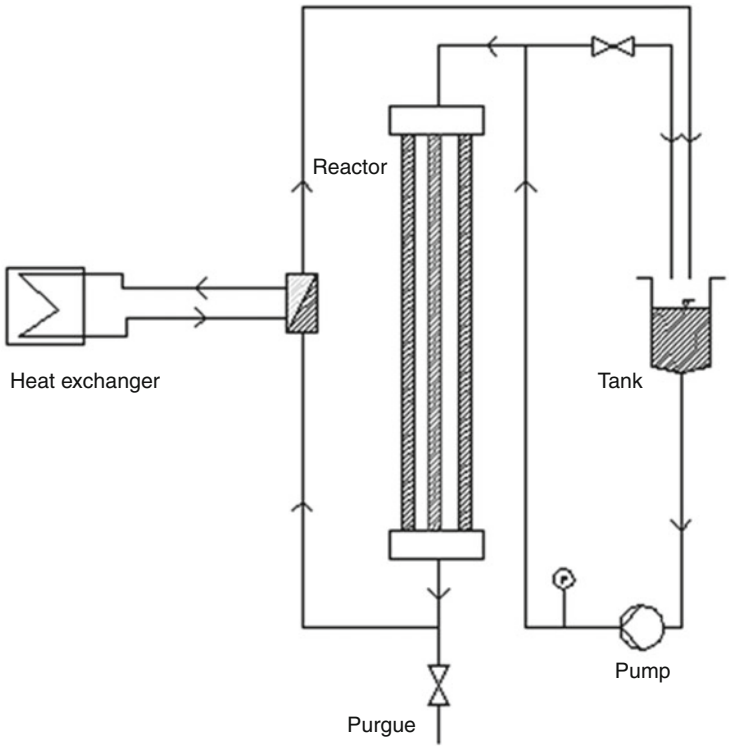

Fig. 5.1 Schematic representation of the laboratory reactor

5.3.2 Added Value to Microalgae Biomass

Microalgal cultivation technology has the potential for the development of new strategies for the valorization of wastes, considering the concept of the circular economy.

A circular economy is an economic system where products and services are traded in closed loops or "cycles." It is characterized as an economy, which is regenerative by design, with the aim to retain as much value as possible of products, parts, and materials. This means that the aim should be to create a system that allows for the long life, optimal reuse, refurbishment, remanufacturing, and recycling of products and materials (Kraaijenhagen et al. 2016).

Recently, Stiles et al. (2018), reported that the application of microalgae to remedy excess nutrients in agricultural effluents has high potential as a circular economy solution, due to the efficiency in the management of organic waste and the production of bioproducts.

The bibliography on different bioproducts obtained from microalgal cultures with effluents of diverse sources is extensive. Jayakumar et al. (2017) concluded that

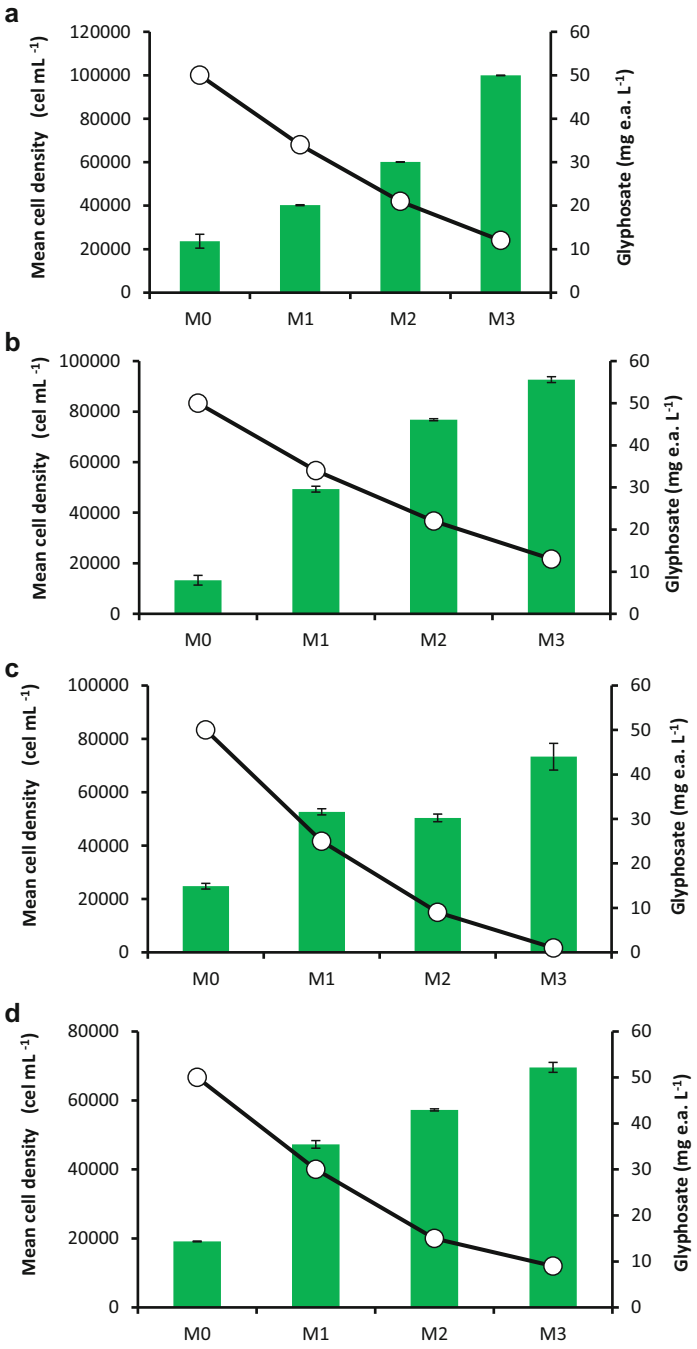

Fig. 5.2 Variations in cell density (cell mL^{-1}) at 96 h regarding glyphosate evolution for the different reaction times (M_0 to M_3, UV/H_2O_2 process). Sample M_0 corresponding to 50 mg L^{-1}

unicellular green microalgae are recommendable as a source of biofuel. Microalgae have high productivity of oils that can be transformed into biodiesel. However, under current technologies, production of this type becomes economically unfeasible. The double role of microalgal cultures for the treatment of wastewater by means of phytoremediation is an option to lower energy and production costs. Moreover, the high biomass generation of microalgae grown in wastewater is a real strategy to reduce greenhouse gas emissions.

In this sense, Cheah et al. (2018) cultivated *Chlorella sorokiniana* in the effluent of palm oil production in photoautotrophic and mixotrophic cultures. The authors reported that mixotrophic conditions were more effective than photoautotrophic ones. Besides, the addition of glycerol showed a higher growth performance of microalgae compared to glucose or urea supplementation. The production of biomass (1.68 g L^{-1}) and lipids (15.07%) was also higher in the effluent medium.

The composition of the ideal fatty acids was reached when effluents were supplemented with urea and glycerol. The efficiency in removing contaminants reached 63.85% COD, 91.54% total nitrogen (TN) and 83.25% of total phosphorus (TP). With these promising results, the authors concluded that the economic and effective supplementation of carbon and nutrients is essential to minimize the economic impact and maximize the yields when cultivating microalgae at commercial scale for the production of biofuels. Besides, they argued, that, at the same time, the technology applied was environmentally sustainable.

Collins Odjadjare et al. (2018) cultivated *Neochloris aquatica* and *Asterarcys quadricellulare* in pre-chlorinated wastewater. They obtained the highest values of reduction in *Asterarcys quadricellulare*: 12.4% in the COD, 48% for the TN, and 50% TP cultivated in water sterile residuals. In the case of *Neochloris aquatica* cultures, the highest lipid values (14.85 $\pm$ 1.63 mg L^{-1}) and carbohydrates (14.84 $\pm$ 0.1 mg L^{-1}) were recorded. The dominant fatty acids in the microalgae were palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1). In the production of biodiesel from oils obtained from crops, the authors stated that the fuel obtained is of good quality with high oxidation stability and low viscosity, conforming to the guidelines of the American Society for Testing and Materials (ASTM).

Another productive activity that provides effluents that can be used as a culture medium for microalgae is aquaculture. Milhazes-Cunha and Otero (2017) explained the importance of microalgal cultures in the processes of integrated multitrophic aquaculture. In addition, to reduce the organic load of the effluents, the biomass obtained can be used to feed low trophic level fish, herbivorous fish, and molluscs,

Fig. 5.2 (continued) a.e. of glyphosate without H_2O_2; M_1, M_2, and M_3: 120, 240, and 360 min, respectively, with the removal of the remaining H_2O_2. (**a**) Eskoba®, (**b**) Panzer Gold®, (**c**) Sulfosato Touchdown®, and (**d**) Roundup Ultramax®. Cell density (cell mL^{-1}) (columns). Glyphosate concentration (white circles). Error bars represent standard error

which in turn has commercial value. In addition, the biomass obtained can be a source of valuable bioproducts.

On the other hand, Xia and Murphy (2016) reported that the treatment of residual liquids by microalgal crops will generate biomass that can be used as a source for the production of biogas. This type of production generates waste—called digestates—that can also be used in microalgal cultures to help optimize bioprocesses and obtain bioproducts, such as biofuels, biofertilizers, proteins, and chemicals with high tagging value (Koutra et al. 2018), such as carotenoids, astaxanthin, lutein, β-carotene, chlorophyll, phycobiliproteins, polyunsaturated fatty acids, β-1,3-glucan, pharmaceutical compounds, and nutraceuticals. It can also be used to feed birds, livestock, fish, and shrimp (Yaakob et al. 2014).

It should also be mentioned that the efficiency of contaminant removal and of obtaining bioproducts in the algal biomass can vary according to the composition of the biomass, since the cellular biochemistry can vary according to the environmental conditions, and to the type and age of the crop (Lourenço et al. 1997; Renaud et al. 1999; Araújo and Garcia 2005).

If we consider that by 2050 it is estimated that the global human population will reach 9.7 billion and there will be an increasing demand for food, energy, and products that must be obtained from the development of renewable and novel sources, microalgal cultures developed in effluents of diverse origin and composition are a possible and promising solution that needs more attention and future research. On the one hand, the environmental impacts could be reduced and on the other, the added value could be obtained using the algal biomass, thus positioning itself as an alternative to the conventional production and remediation systems, in order to contribute to the sustainable development.

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Chapter 6
Utilization of Agro-Waste as Carbon Source for Biohydrogen Production: Prospect and Challenges in Malaysia

Muhd Nazrul Hisham Zainal Alam, Nadia Adrus, Mohd Firdaus Abdul Wahab, Mohd Johari Kamaruddin, and Mohd Helmi Sani

Abstract Hydrogen gas (H_2) is a clean fuel and contained a relatively high energy density which is about 142 kJ g^{-1}. Recently, increasing attention has been given to the production of H_2 from biological route. The biological H_2 (biohydrogen) process is an H_2 production by microorganisms that utilize renewable energy resources as substrates. Possible biohydrogen production technologies include biophotolysis, photo-fermentation processes, and the dark fermentation route. Among these three production processes, the dark fermentation process is often regarded as the most potential route. It generates H_2 by utilizing carbohydrates as the carbon sources whereby glucose was found to be the most commonly used substrate. A high yield of biohydrogen, i.e., about 4 mol of H_2 per mole of glucose consumed can possibly be achieved through this route. Despite a reasonably high yield, industrial-grade glucose (35–50 USD per kg) is expensive and therefore, rendering the process less economical especially considering market value for H_2 typically ranging only between 3 and 5 USD per kg. Obviously, cheaper substrates are needed if dark fermentation process is ever to strive as the potential route for biohydrogen production. In Malaysia, abundance of agricultural waste is disposed into landfills annually and thus, making it free un-tap resources. This chapter reports the prospect and challenges of utilizing agro-waste as the carbon source for biohydrogen production

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in Malaysia. The work will provide basis evaluation on the potential of biohydrogen production where agro-waste is capitalized as main substrates for the process.

Keywords Biohydrogen · Agro-waste · Dark fermentation · Sustainability

6.1 Introduction

H_2 has the highest energy content, i.e., about 142 kJ g^{-1} compared to any other known biofuels such as methane, methanol, and ethanol (Rahman et al. 2016). Main sources for global hydrogen production are still based on natural gas, heavy oil, naphtha, coal, and/or electrolysis of water (Puad et al. 2016). Energy gained from the combustion process is converted to electricity and heat in commercial power plants. Although combustion of H_2 gas for energy generation is considered clean as water is the only by-product, such a process requires high thermal energy (typically operated at 700–1100 °C) and releases greenhouse house gases (CO_2) into the atmosphere; which in this case is not desirable from the environmental point of view. Moreover, carbon-based sources (fossil fuels) are nonrenewable sources and eventually will be depleted (Bharathiraja et al. 2016; Kozłowski et al. 2019).

In recent years, continuing efforts have been given to explore the potential of biohydrogen production processes (Chandrasekhar et al. 2015; Prabakar et al. 2018). Biohydrogen (or biological H_2) production process is a process that involves the use of microbes for the production of H_2. The process is environmentally friendly and consumes low energy as it typically carried out at ambient temperature and pressure. Additionally, a wide variety of biomass feedstock and low-value materials such as food or agricultural wastes can be utilized as substrates for the microbes in biohydrogen production via biochemical pathways (Rahman et al. 2016; Puad et al. 2016). Since, the main carbon sources are mainly from renewable sources, the biohydrogen production is obviously sustainable compared to the classical route via hydrocarbon reforming of fossil fuels.

Main biohydrogen production processes include biophotolysis, photo-fermentation and dark fermentation process. Biophotolysis is a simple process involving the splitting of water into molecular hydrogen and oxygen by *Cyanobacteria* and green microalgae using sunlight. H_2 production takes place by direct absorption of light and electron transfer from water molecules to hydrogenase enzymes under anaerobic conditions (Rahman et al. 2016; Prabakar et al. 2018). In photo-fermentation production process, H_2 is produced through degradation of organic compounds by photosynthetic bacteria with the addition of sunlight. Photo-synthetic bacteria utilized organic acids such as acetic acid as electron donor for H_2 production as the bacteria are incapable of breaking water molecules as in biophotolysis. One of the advantages of this process is that photosynthetic bacteria could also consume a wide variety of organic substrates present in waste streams such as organic agricultural and/or industrial wastes (Rahman et al. 2016; Prabakar et al. 2018). Contrary to biophotolysis and photo-fermentation processes, dark

fermentation process produces H_2 without the need of sunlight and microbes associated with anaerobic dark fermentation process utilize simple carbohydrates (or sugars) such as glucose in their metabolic pathway. Since dark fermentation process is not bound to the need in providing light for H_2 production, it is definitely cheaper. Furthermore, greater H_2 yield can be attained compared to the other two processes, i.e., about 4 mol of H_2 per mole of glucose consumed (Rahman et al. 2016; Prabakar et al. 2018). Despite the potential, large-scale dark fermentation processes are limited by the high cost of glucose compared to the market value of H_2.

Therefore, this chapter reviews the potential of utilizing agricultural waste as potential substrates for dark fermentation process. Agro-wastes are abundant in Malaysia and can be obtained easily without any cost incurred. This justified the reason why agro-wastes are chosen compared to other types of waste streams such as food or industrial wastes. The review will focus on several aspects. These include (1) details on anaerobic dark fermentation process, (2) types of agro-waste that is locally available in Malaysia, (3) suitable strategy to convert agro-waste into simple carbohydrates as carbon source for dark fermentation process, (4) potential of biohydrogen in Malaysia and lastly, (5) the impact of biohydrogen production of the nation economy.

6.2 Biohydrogen Production Via Dark Fermentation Process

Dark fermentation process is a light-independent anaerobic microbial fermentation route for producing biohydrogen. The process does not require any light energy input neither oxygen supply and utilizes sugars such as glucose as the main carbon source (Rahman et al. 2016). Metabolic pathway for the production of H_2 in anaerobic dark fermentation process involved several stages (Prabakar et al. 2018; dos Passos et al. 2019). First step is glycolysis where the carbon source for the process—in this case, glucose is converted into pyruvate and nicotinamide adenine dinucleotide (NADH) (Eq. 6.1).

$$\underset{\text{(glucose)}}{C_6H_{12}O_6} + 2NAD^+ \rightarrow \underset{\text{(pyruvate)}}{2CH_3CO\text{-}COOH} + 2NADH + 2H^+ \tag{6.1}$$

Next, the pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA) and, depending on types of bacteria used, is catalyzed either by ferredoxin oxidoreductase enzyme where it produces ferredoxin (Fd) and carbon dioxide (CO_2) (Eq. 6.2) or by formate lyase where results in a formation of formate (Eq. 6.3).

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \rightarrow \text{acetyl-CoA} + CO_2 + 2\text{Fd (red)} \tag{6.2}$$

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{formate} \tag{6.3}$$

In the production of H_2 through the re-oxidation of ferrodoxin, it is catalyzed by various hydrogenases which releases the hydrogen molecules (Eq. 6.4). On the contrary, H_2 produced from formate is catalyzed by formate H_2 lyase or pathway consists of formate-dependent hydrogenase (Eq. 6.5).

$$2H^+ + Fd\ (red) \rightarrow H_2 + Fd\ (ox) \tag{6.4}$$

$$HCOOH \rightarrow CO_2 + H_2 \tag{6.5}$$

Generally, the yield of biohydrogen production through anaerobic dark fermentation route is depending on the fermentation end products. If acetic acid (CH_3COOH) is the fermentation end product, a theoretical 4 mol of H_2 per mole of glucose consumed are generated (Eq. 6.6).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \tag{6.6}$$

If, however, the reaction pathway shifted and butyric acids ($CH_3CH_2\ CH_2COOH$) are the fermentation end product (Eq. 6.7) the amount of H_2 generated is only 2 mol of H_2 per mole of glucose consumed (Eq. 6.7).

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \tag{6.7}$$

Obviously, these are theoretical approximations. In practice, often actual hydrogen yield is lower than the theoretical yield. This is most probably because substrates are also partially consumed for biomass production (dos Passos et al. 2019). Under some undesirable conditions, different metabolic pathways are followed leading to the production of ethanol and acetate and thus, lowering the stoichiometric hydrogen yield to 2 mol of H_2 per mole of glucose consumed (Eq. 6.8) (Rahman et al. 2016; Prabakar et al. 2018):

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \tag{6.8}$$

Anaerobic bacteria typically used in this type of fermentative hydrogen production include *Clostridium butyricum* and *Enterobacter aerogenes* (Boshagha et al. 2019; Kanchanasuta et al. 2017; Ulhiza et al. 2018; Ziara et al. 2019). *Escherichia coli* are sometimes used as well (Puad et al. 2016; Maeda et al. 2018). *Clostridium butyricum* are strict anaerobes from the Clostridial species that has the capacity to ferment organic compounds such as simple and/or complex carbohydrates in producing hydrogen. It is highly sensitive to dissolved oxygen (inhibitory compounds to its growth) with reporting H_2 yields ranging between 0.73 and 3.1 mol H_2 per mole of sugar (Kanchanasuta et al. 2017; Ziara et al. 2019). *Enterobacter aerogenes* and *Escherichia coli* are both facultative anaerobic bacteria. Facultative anaerobes could grow under both aerobic and anaerobic conditions. These microbes have the advantages of shorter doubling time and faster hydrogen production rates. However, the yield of hydrogen using glucose as carbon source is relatively lower, i.e., between

1 and 2 mol H_2 per mole of sugar (Puad et al. 2016; Boshagha et al. 2019; Ulhiza et al. 2018). Table 6.1 shows the typical operating conditions of dark fermentation processes based on the anaerobic H_2 production bacteria strains.

As shown in Table 6.1, optimal pH and temperature for biohydrogen production vary between pH 6–6.8 and 30–40 °C, respectively. Continuous reactor operation with larger reaction volume (few liters) allows for a high cumulative of biohydrogen production for a much longer fermentation period (>48 h). On the contrary, if the reaction was carried out in batch mode using a milliliter range reactor setup, steady state will most likely occur between 12 and 20 h of fermentation period. Nevertheless, regardless of the type of bacterial strain used, different types of substrates can be utilized as substitutes for glucose in the production of biohydrogen via anaerobic dark fermentation processes. We believed similar outcome, i.e., process yield of about 2–3 mol H_2 per mole of sugar can also be achieved if agro-waste is used as the main carbon source in the dark fermentation process using any of the above mentioned bacterial strains.

Table 6.1 Typical reaction conditions and medium used for biohydrogen production based on types of substrates and bacterial strains used

Conditions and raw materials	*Clostridium butyricum* (Kanchanasuta et al. 2017)	*Enterobacter aerogenes* (Boshagha et al. 2019)	*Escherichia coli* (Puad et al. 2016)
Reaction			
Type of substrates	Synthetic food waste (65% w/w carbohydrate, 15% w/w veggies, and 17% w/w meat)	Glucose	Sago waste
Temperature (°C)	37	30	40
pH	6	6.8	6
Volume	4 L	100 mL	300 mL
Retention time	48 h	8 h	14 h
H_2 yield	0.0315 g H_2/g S	2.56 mol H_2/mol S	2.22 mol H_2/mol S
Medium used			
Sucrose	3 g L^{-1}	–	
Tryptone	5 g L^{-1}	–	10 g L^{-1}
Yeast extract	5 g L^{-1}	5 g L^{-1}	5 g L^{-1}
K_2HPO_4	1 g L^{-1}	7 g L^{-1}	–
Peptone	–	5 g L^{-1}	–
KH_2PO_4	–	5.5 g L^{-1}	–
$(NH_4)_2SO_4$	–	1 g L^{-1}	–
$MgSO_4{\cdot}7H_2O$	–	0.25 g L^{-1}	–
$CaCl_2{\cdot}2H_2O$	–	0.021 g L^{-1}	–
Fe $(NH_4)_2SO_4{\cdot}6H_2O$	–	0.039 g L^{-1}	–
$NiCl_2$	–	0.00002 g L^{-1}	–
NaCl	–	–	10 g L^{-1}

6.3 Agro-Waste in Malaysia: Quantity Versus Quality

6.3.1 Locally Produced Agro-Waste in Malaysia

Agro-waste is described as a waste product derived from agricultural industry including the remaining nonedible crop residue left in the field after harvesting as well as the one that generated after crop processing. It is one of the promising biomass resources and there is an increasing interest in the exploration of this kind of renewable energy for biohydrogen production. Around 998 million tonnes per year of agricultural waste have been generated worldwide (Tahir and Hamid 2012). Malaysia produces about 0.122 tonnes of agricultural wastes per year and the scenario is projected to reach 0.210 tonnes per year by 2025 (Tahir and Hamid 2012).

As shown in Table 6.2, the agricultural waste is abundant, especially from the palm oil industry. With the plantation area of 5.81 million hectares reported in 2017 (Kushairi et al. 2018), palm oil mills produce 80 million tonnes of waste (Hossain et al. 2016) that account for roughly 50% of waste generated from the original fresh fruit bunch processed (Abdullah and Sulaiman 2013; Shamsuddin 2012). Generally, the waste breakdown of this lignocellulosic biomass can be found in the form of fronds (51.3%), empty fruit bunches (EFB—19.7%), oil palm fibers from pericarp/mesocarp (OPF—12.1%), oil palm trunks (OPT—11.9%), and oil palm shells (OPS—4.9%) (Abdullah and Sulaiman 2013). EFB, OPF, OPT, and OPS contain a significant fraction of cellulose which vary from 27 to 42–65 weight % that could be converted into H_2 (Hossain et al. 2016). These palm oil wastes are attractive sources of biomass in terms of energy basis as it contains high specific calorific value that could reach up to 20,108 kJ/kg.

At present, nearly three million tonnes of rice produced in Malaysia are realized from 730,000 ha of area under cultivation (Department of Statistic Malaysia 2018). Typically, between 0.41 and 3.96 kg of rice straw is generated for every kilogram of harvested paddy (Lim et al. 2012). Meanwhile, the paddy residue after milling process so-called rice husk represents approximately 20–33% of paddy weight. Based on this statistic, it is forecasted that this biomass residue will increase to seven million tonnes by 2020 (Shafie 2015). Rice straw and rice husk account for 28–32% of cellulose content as well as comparable calorific (Table 6.2) value which may find a remarkable future for the production of biohydrogen. Like many lignocellulosic biomasses, coconut is the next important crop in terms of cultivation area after palm oil and paddy. From Table 6.2, the annual quantity of coconut waste produced in Malaysia is 273,000 tonnes (Pariatamby 2017). The typical compositions of the coconut waste after harvesting and industrial processing are husks, fronds, leaves, stems, and fibers. Each part of these wastes possesses varying levels of cellulose content (18–22%) (Ding et al. 2012) and calorific value (9690–14,644 kJ/kg) (Jamil 2004) that makes them significant feedstock to generate renewable energy.

With about 700,000 tonnes of sugarcane produced in Malaysia, 203,700 tonnes of sugarcane bagasse are remaining after the recovery of sugar juice via crushing and extraction (Shafie et al. 2012). From Table 6.2, bagasse is considered an alternate

Table 6.2 Source of agro-waste in Malaysia

Source of Agro-waste	Amount (million tonnes)	Sugar content (weight %)	Calorific value (kJ/kg)	References
Palm oil-based biomass Fronds	80			Hossain et al. (2016) and Umar et al. (2014)
EFB		42–65	18,838	
OPF		42–65	19,068	
OPT				
OPS		27	20,108	
Paddy-based biomass	7			Jamil (2004)
Rice husks		28	15,840	Lim et al. (2012)
Rice straws		32	−15,090	Lim et al. (2012)
Coconut-based biomass	0.27	18–22	9690–14,644	Pariatamby (2017) and Jamil (2004)
Sugarcane bagasse	0.2	42–65	9204	Department of Statistic Malaysia (2018), Jamil (2004), and De Araujo Guilherme et al. (2019)
Pineapple waste	0.07	65–85	~18,000	Mansor et al. (2018)
Sago waste	2.5	10	11,363	Ulhiza et al. (2018) and Zulkarnain et al. (2006)

source of biomass as it has acceptable calorific value and cellulose content. On the other hand, pineapple is one of the main commodities in Malaysia of which 80% of its parts including crown, stems, and peels and leaves are discarded as waste. Though pineapple has relatively lesser amount of waste generation, it is able to give high calorific value (~18,000 kJ/kg) (Mansor et al. 2018) due to comparatively high cellulose content (85%). Last but not least, waste derived from sago is also considered worth an effort in producing biohydrogen. This is supported by its abundant wastage production as well as its cellulose component normalizes to the energy basis. In brief, utilization of agricultural waste in Malaysia has greater benefits from the economic and environmental perspectives toward future generations of energy.

6.3.2 *Utilization of Agro-Waste as Carbon Source: How and Challenges*

Researchers have investigated the hydrogen production through dark fermentation using simple sugar from glucose and sucrose as the model substrate. But, fewer

study has looked into the conversion of agro-waste biomass as the cheap substrate sources (Mishra et al. 2019; Guo et al. 2010). In Malaysia, the agro-waste biomass is abundantly available in the form of palm oil-based biomass, paddy-based biomass, coconut-based biomass, and other types of crops (Kushairi et al. 2018; Department of Statistic Malaysia 2018; Pariatamby 2017; Shafie et al. 2012; Mansor et al. 2018). These agro-waste biomass, which gives high contents of substrate for biohydrogen production could be exploited for the sugar content through different pretreatment processes (Guo et al. 2010).

Although the agro-waste is readily available and almost zero cost feedstock, the agro-waste contains complex carbohydrate structure that has cellulose and hemicellulose, which are strongly bonded by lignin complex structure (Ntaikou et al. 2010). The lignin structure contains a cross-linking in the series of polysaccharides of cellulose and hemicellulose and bonded with lignin via ester and ether linkages (Zheng et al. 2014). The complex and varied structure of lignin required pretreatment for the delignification and subsequently loosening the structure of cellulose and hemicellulose to be exploited (Ntaikou et al. 2010; Ghimire et al. 2015). Once the structure is broken, the cellulose and hemicellulose can be hydrolyzed to carbohydrate and converted to simple sugar and further by biological process to biohydrogen via dark fermentation (Ghimire et al. 2015).

Different types of crops has been subjected to different process of pretreatment, which include physical, chemical, and biological (Ntaikou et al. 2010; Ghimire et al. 2015). Generally, the agro-waste biomass will initially pretreat with physical pretreatment, which includes mechanical fractionation (i.e., fractionize to smaller particles through milling, grinding chopping), gamma irradiation, heat by microwaves, hydrothermal treatment, and pyrolysis. The mechanical fractionation function to reduce the size of the particles, ease of enzymatic accessibility and bioconversion affectivity (Ntaikou et al. 2010; Ghimire et al. 2015). Subsequently, the agro-waste biomass will be exposed to acidic, alkaline, or oxidative conditions, at ambient or raised temperature during the chemical pretreatment. Physicochemical methods such as ozonolysis (oxidation of an alkene with ozone), acid or alkaline hydrolysis, solvent extraction, explosion with steam ammonia are effective to increase the specific surface area, delignification, decrease crystallinity, and hydrolyze the hemicellulose (Ghimire et al. 2015; Chandaekar et al. 2015). The biomass that had been fractionized to the specific reduced size, the complex bond of lignin will be loosened and possible change to the chemical structures of cellulose and hemicellulose will increase the chance of lignin breakdown. Combinations of the pretreatment method also have been investigated, either combining two or more physical and chemical treatments (Ntaikou et al. 2010; Zheng et al. 2014). These combinations of methods can be promising for the industrial application as they are quick, however, it demands a high energy and chemical loads.

The biological pretreatment process is considered as the most environmentally friendly and sustainable alternatives for the bioconversion of lignocellulosic substrate to simple sugar (Guo et al. 2010). Biological pretreatment of agro-waste targets for the removal of lignin and thus the structure becomes more accessible to enzymatic attack for the saccharification process where complex sugar will be

broken down to simple sugar (e.g., sucrose being broken down into glucose and fructose). The process can be performed either by whole-cell microorganisms or enzymes that are capable of degrading lignin. Consequently, there are very few species of microbes are capable of performing breakdown of lignin. Among the microbes that can be utilized are white rot fungi, actinomycetes, and some symbiotic bacteria (Zheng et al. 2014). These microbes able to provide the delignification process and partial hydrolysis of cellulose. The most studied white rot fungi, *Phanerochaete chrysosporium* able to selectively degrade lignin. Whereas, the enzymatic degradation of lignin is another complex process that involved specific action of enzymes such as lignin peroxidase, manganese peroxidase, and laccase which produced strong oxidants that subsequently break the lignin framework (Ntaikou et al. 2010). The effect of biological pretreatment methods on a different type of agro-waste lignocellulosic complex structures has a diverse effect on the hydrolysis of soluble sugars which need to be further investigated (Chandaekar et al. 2015). Overall, the selection process of pretreatment for soluble sugar in biohydrogen production should be effective, low energy consumption, economically feasible, and environmentally sustainable (Prabakar et al. 2018).

Table 6.3 summarizes the recent studies on the utilization of agro-waste in Malaysia for biohydrogen production through dark fermentation process. Maarof et al. (2018) utilized palm oil mill effluent (POME) as substrates for biohydrogen production in two-stage anaerobic batch reactor. The raw POME was subjected to heat treatment at 80 °C for 10 min to lysis any methanogenic bacteria that maybe present in the substrates. Methanogenic bacteria is not desirable as it will consume hydrogen and produce methane gas instead. Such heat treatment also helps to increase the cellular biomass content of thermophilic and mesophilic bacteria utilized for the dark fermentation process. Reactions were carried out at various temperatures, i.e., at 55 °C for the reactor containing the thermophilic bacteria and at 37 °C for the reactor consisted of the mesophilic bacteria. Contrary to POME, coconut husk needed to be ground to powder form and underwent acid treatment before utilized as substrates in the dark fermentation process (Arisht et al. 2019). Once treated, the acid–coconut husk mixture was filtered and only the liquid hydrolysate was used as substrates. Similarly, Puad et al. (2015) also employed an acid treatment on sago wastewater at elevated temperature and filtered insoluble particles to isolate liquid hydrolysate retrieved from the pre-treatment phase for further use in the dark fermentation process. Acid treatment is very straight forward to apply but take note, such treatment must be performed using low acid concentrations (<2% v/v). Harsh pre-treatment conditions such as concentrated or high molarity acid solution, long incubation time and very high operating temperature may lead to degradation of sugars into undesirable inhibiting products (Abd Jalil et al. 2018). Abd Jalil et al. (2018) realized an immobilized reactor setup for biohydrogen production via dark fermentation route co-cultured using *E. aerogenes* and *C. sporogenes* bacteria. Microbes were grown on activated carbon material and pineapple waste was used as substrates. Pineapple waste typically consisted of its peel and crown. As agro-waste, pineapple peel carries a significant amount of hemicellulose, cellulose, sugar, and other carbohydrates (Aditiya et al.

Table 6.3 Utilization of agro-waste for the production of biohydrogen via dark fermentation route in Malaysia

Type of agro-waste	Pre-treatment	Reaction conditions	Yield	References
Palm oil mill effluent (POME)	Heat treatment at 80.0 °C for 10 min	Two-stage reactor (in sequence). Batch mode (suspension). Microbe reactor 1: Thermophilic (55 °C). Microbe reactor 2: Mesophilic (37 °C). pH maintained at 6.5–7. Raw POME as substrates.	Reactor 1: 2.99 mol H_2/mol sugar Reactor 2: 1.19 mol H_2/mol sugar	Maarof et al. (2018)
Coconut husk	Ground to powder form before pre-treated with 1% (v/v) phosphoric acid. Treatment carried out at 121 °C for 60 min	Small batch reactor (suspension). Microbe: Mixed culture from municipal wastewater. Reaction carried out at 40 °C at pH 7. Liquid hydrolysate of coconut husk as substrates.	0.68 mol H_2/mol sugar	Arisht et al. (2019)
Pineapple waste	Chopped to small pieces before crushed and mixed with distilled water at ratio 1:2 using a steel blender	Immobilized batch reactor Microbe: *E. aerogenes* and *C. sporogenes* Activated carbon as support material. Reaction carried out at 33 °C at pH 7. Liquid hydrolysate of pineapple waste as substrates.	1.72 mol H_2/L substrate	Abd Jalil et al. (2018)
Sago wastewater	Filtered to remove large particles before pre-treated with 0.5 M sulfuric acid. Treatment carried out at 90 °C for 40 min	Shake flask operation Batch mode (suspension). Microbe: *E. coli*. Reaction carried out at 37 °C at pH 6. Liquid hydrolysate of sago waste as substrates.	2.22 mol H_2/mol sugar	Puad et al. (2015)

2016). In the work by Abd Jalil et al. (2018), the pineapple peels (attained from local market) were first chopped into small pieces before crushed and mixed with distilled water in the ratio of 1:2 in a steel blender. Final mixture was filtered and only the liquid hydrolysate was utilized as substrates. Obviously, the pre-treatment method employed was simpler as neither heat nor acid treatment is required. Despite the differences in the type of pre-treatment method employed, results attained by utilizing these agro-waste are indeed very promising as biohydrogen yield as high as 3 mol H_2 per mol sugar can be achieved.

6.4 Prospect of Biohydrogen Production in Malaysia

6.4.1 Strategy to Purify Biohydrogen Gas Via Chemical Absorption Process

As previously mentioned, the dark fermentation processes produce a mixture of biogas containing essentially H_2 and CO_2. In addition, there is a possibility that a small amount of CO, CH_4, and/or H_2S could also be generated in the biogas mixture. This is highly dependent on the types of substrates and reaction routes taken during the production of biohydrogen via dark fermentation process. A small amount of CO_2 and impurities present in the H_2 fuel may hinder the performance of the fuel cell (Rahman et al. 2016). Thus, in order to utilize H_2 from the fermentation process as a fuel in the fuel cell or gas combustion engine, the gas mixture needs to be separated and purified for high purity H_2. Conceptual idea of a schematic for an industrial-scale biohydrogen process is illustrated in Fig. 6.1.

According to Product Specification Part 2, ISO 14687-2 hydrogen fuel specification required the minimum purity of 99.97 mol% (ISO 14687-2:2012). In order to propose a compatible and efficient method for the separation and purification of the mixed biogas, it is important to highlight the difference between biohydrogen and conventional hydrogen produced from syngas. Generally, there are three main differences between conventional hydrogen and biohydrogen. First, conventional hydrogen is produced at high temperatures between 625 and 950 °C whereas the highest biohydrogen production temperature is below 700 °C. Secondly, conventional hydrogen is produced at elevated pressure between 20 and 40 bars whereas biohydrogen is produced at atmospheric pressure. Lastly, CO_2 composition in a mixed gas of conventional hydrogen is much lower than biohydrogen. Typically, CO_2 composition of conventional hydrogen is around 20 mol% whereas the biohydrogen may contain as high as 50 mol%. In brief, biohydrogen is being produced at much lower temperature and ambient pressure but with higher CO_2 composition. Hence, the biohydrogen purification to be developed must be able to be operated at low temperature and ambient pressure to avoid any additional energy requirement and at the same time is able to reduce higher CO_2 composition. In

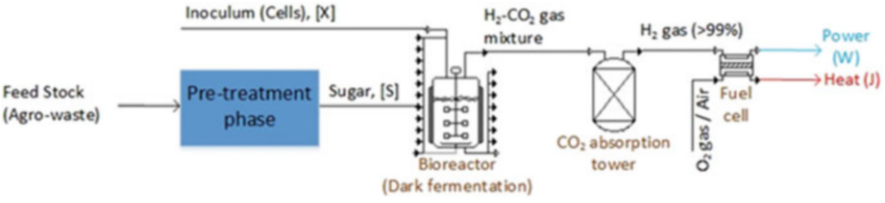

Fig. 6.1 Typical schematic for energy generation via biohydrogen production through dark fermentation process. Schematic included upstream waste conversion into sugar feed for dark fermentation process and downstream processing for separation and utilization of H_2 in a fuel cell for electricity generation (unpublished data)

principle, there are four methods to purify crude hydrogen that are cryogenic separation, pressure swing adsorption, chemical absorption process, and membrane technology (Rahman et al. 2016). These methods have been commercially used to purify conventional hydrogen. Dark fermentation biohydrogen process is normally carried out at the temperature between 30 and 70 °C and atmospheric pressure. Given the circumstances, the chemical absorption route is highly regarded as the most suitable method for biohydrogen purification since it can be operated at low temperature and atmospheric pressure. Hence, in this section, biohydrogen separation and purification systems, namely the chemical absorption process, will be discussed.

In chemical absorption, amine is typically used as the solvent. Amine is a chemical that contains nitrogen atom attached to hydrocarbon chain and/or hydrogen atom. In addition, amine is a molecule which contains amino (−NH) group with at least one hydroxyl (−OH) group attached to it. The general functions of the hydroxyl group are to reduce the vapor pressure of the solvent so that it will not be easily vaporized and to increase solvent solubility in water. Whereas the amino group will create the alkaline environment in the solvent for the extraction of acid gases (e.g., CO, CO_2, and H_2S). In general, amines can be divided into primary, secondary, and tertiary. Primary amines are those amines where a nitrogen atom is directly attached to two hydrogen atoms. As for secondary amines, only one hydrogen atom is directly attached to the nitrogen. Whereas, in tertiary amines, there is no hydrogen atom directly attached to the nitrogen (Smith 2019).

For CO_2 removal, tertiary amines such as methyldiethanolamine (MDEA) is usually used due to its superior to primary and secondary amines in terms of CO_2 loading capacity, less energy requirement for regeneration, thermal and oxidative stability (less degrade), and minimum amines loss due to low vapor pressure. However, the tertiary amines produced a slower absorption rate compared to primary and secondary amines. This is due to the tertiary amines unable to directly react with CO_2. The tertiary amines needed to be hydrolyzed before it can be reacted with CO_2. In order to overcome this drawback is by adding an activator in the form of primary or secondary amines. The most commonly used is activated MDEA or piperazine–MDEA mixture. Amines alone are insufficient to obtain minimum hydrogen purity of 99.7 mol%. Abdeen et al. (2016) reported that various feed gas compositions containing methane (CH_4) between 51 and 79% and carbon dioxide (CO_2) between 21 and 48%, where the CO_2 content was able to reduce to 1.3–10.0% by using amines as a solvent in the absorption process. Based on this study (Abdeen et al. 2016) the single-stage chemical absorption using amines as solvent is not sufficient to achieve minimum hydrogen purity required. Hence, a second polishing stage such as caustic washing as typically used in oil and gas refining application shall be needed.

A two-stage chemical absorption system using MDEA activated with piperazine in first stage and caustic washing (NaOH) in second stage was studied by Abdul and Asli (2019). The experiment was conducted at 1 bar and 29 °C using a ratio of 1:1 of CO_2:H_2 standard gas mixture as the feed. The mixed gas was flown through a packed column consist of activated MDEA (40 wt. %) with piperazine (6 wt. %) in the first stage absorption which resulted in 79% CO_2 removal. To improve CO_2 removal, the

treated gas mixture was flew using a gas distributor and wire mesh packed to create gas bubbles in caustic washing column (NaOH, 20 wt. %) at the second stage. The authors reported this method successfully removed 99.59% of the total CO_2, producing higher than 99 mol% hydrogen gas purity. This type of two stages chemical absorption system was proved able to purify the raw biohydrogen, which has higher CO_2 content at nearly ambient pressure and temperature.

6.5 Biohydrogen in Malaysia: Prospect and Impact

Malaysia's involvements in Kyoto Protocol and the Paris Agreement show the country's commitment to reducing greenhouse gas emissions going forward into 2020 and beyond. In order for us to meet the targets set, extensive and aggressive efforts are required to mitigate current greenhouse gas emissions, including wider adoption of renewable energy. So far, hydrogen for industrial applications is obtained mainly from steam methane reformer (e.g., Air Products Westport facility, for oleochemical industries), with water electrolysis gaining commercial attention too. Other than for industrial applications such as in the hydrogenation process in palm oil industry, feedstock in refinery and petrochemical plant, and glass manufacturing industry, hydrogen can also be used in fuel cell vehicles and electricity generation.

As a comparison, the maximum theoretical yield of hydrogen that can be produced by oil palm biomass via gasification method is 2.16×10^{10} kg H_2 per year with an energy content of 2.59 EJ per year (Kelly-yong et al. 2007). This is based on the annual world oil palm biomass production estimated at 184.6 million tons at that time. Almost 50% of the current worldwide hydrogen demand could be fulfilled by such output. Biomass has been targeted in the Malaysian fifth Fuel Policy, however, progress has been hampered by production cost and financing problems. While hydrogen production via the biological routes is still at the research and development stage, there is a huge potential for its application in Malaysia due to the different agro-waste biomass availabilities. Despite this, there are several limitations that need to be overcome first. Other than the various experimental challenges faced in commercializing biohydrogen production from agro-waste, the cost for transportation and preparation of biomass is also very high. In addition, conversion of the biohydrogen produced in a bioreactor into usable energy must also be taken into consideration. Integration of the bioreactor and fuel cell systems has been proposed (Rahman et al. 2016) using polymer electrolyte membrane fuel cells (PEMFC) with current research focuses on stable and sufficient production, and higher purity required for fuel cell applications. While biophotolysis and dark fermentation have been reported to be able to generate hydrogen continuously (Rahman et al. 2016), transitioning from batch systems to continuous systems is required. Biohydrogen generated via dark fermentation also requires a purification system before it can be used in the PEMFC. These are some of the technical challenges that need to be solved, in addition to the limitations of the biological conversion of the agro-waste itself, before biohydrogen can be sustainably used to generate renewable energy.

Looking at a global scale, the production of hydrogen from agro-waste biomass has been very small. This method is still attractive for the long term though, due to the low-carbon potential that it offers. Currently, market maturity of this method is not yet in sight, since conversion rates and hydrogen yields are still well below a marketable threshold (Shell 2017). It also requires significant volumes of biomass. Thus, a continuous supply of the feedstocks must also be taken into consideration. Notwithstanding, we believe that the large-scale production of biohydrogen from agro-waste can still be developed sustainably, particularly in Malaysia. This is due to the increasing energy demand of a rapidly developing country, and the increasing amount of waste generated. However, more research needs to be done to overcome the technological challenges, and to bring the findings in the lab to the pilot scale, before being commercialized. In the meantime, the economic efficiency of biohydrogen production from agro-waste can also be analyzed, based on the feedstock cost, collection and transportation, technology used, and equipment costs (Rahman et al. 2016).

6.6 Conclusion

Studies on biohydrogen production have been focusing on different biological pathways for hydrogen production, which include direct biophotolysis, indirect biophotolysis photo-fermentation, and dark fermentation. Among these bioprocesses, dark fermentation, which utilizes anaerobic fermentation is more favorable due to the high rate of hydrogen produce and efficient substrate conversion. Dark fermentation has been extensively studied, where agro-waste biomass can be converted to hydrogen and simple organic acids. The process can be accomplished by several fermentative (either obligate or facultative) bacteria where the main substrate (carbon source) is glucose. In the present chapter, we looked at the possibility of utilizing agro-waste in Malaysia as a source for obtainment sugars mainly glucose for such a dark fermentation process. The availability of agro-waste is in abundance in Malaysia and thus, justified the reason for exploring such route. Despite the potential, sugars cannot be directly attained from such agro-waste sources. Obviously, a series of pretreatment steps are needed. Potential agro-waste sources and reasonable choices for pre-treatment steps were reviewed as well. The work also proposed a low cost and sizeable integrated process for biohydrogen fuel cell electricity generation. Interestingly, in this work, in the downstream processing step, the application of chemical absorption process for obtaining hydrogen gas rich stream prior to feeding into the fuel cell system for electricity generation was suggested. With a solid roadmap and sufficient investments in the research, development, and commercialization, biohydrogen generation from agro-waste has the potential to be one of the main renewable energy sources with multiple benefits to the country and the environment.

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Chapter 7
Agro-Industrial Waste as Substrates for the Production of Bacterial Pigment

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Abstract There is worldwide interest in process development for the production of pigments from natural sources due to a serious safety problem with many artificial synthetic colorants, which have widely been used in foodstuff, cosmetic, and pharmaceutical manufacturing processes. Low-cost by-products and residues of agro-industrial origin have shown their potential in production of different pigments by diverse group microorganisms and to explore the possibility of pigment production by different microbial isolates from numerous sources on various substrates. The main applications of recycled wastes are enzyme production, organic acid isolation, pigment extraction, bioactive compound production, etc. Therefore, more regulatory approval and capital investments are required to bring these value-added products in the commercial market. The conversion of agro-industrial residues to important substances may not only provide future dimension to researchers but also reduce the current environmental hazards.

Keywords Agro-industrial waste · Microbes · Fermentation · Pigments · Low-cost substrate

7.1 Introduction

Agro-industrial wastes are those which are generated from the food as well as agricultural industries or from the agricultural practices. Most of the agro-industrial wastes are untreated and underutilized and these untreated wastes produce completely different issues with climate change by increasing the number of

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greenhouse gasses (Bos and Hamelinck 2014). These wastes also cause serious disposal problems (Rodríguez-Couto 2008). However, only a small portion of all waste generated during agro-industrial processes is recovered as by-products, and the vast majority of them are not considered as viable for further use and are discarded directly into the environment or are responsible for major expenses with proper disposal. The environmental damage caused by agricultural waste leads to focus on more research on the utilization of agricultural waste. Even though agricultural residues are produced in large quantities in developing countries, they are mainly utilized as animal feed and landfills (Salihu et al. 2012).

At present, extensive agricultural waste disposal methods not only fail to effectively convert and utilize agricultural resources but also cause serious environmental pollution (Dai et al. 2018). It is a worldwide concern to dictate the improvement of alternative cleaner and renewable bioenergy resources (Okonko et al. 2009). Most of the wastes generated by agro-based food industries are high in nutrients and can form breeding grounds for disease-causing microbes if left unprocessed and inadequately treated. Interestingly, these wastes can serve as raw materials for the production of value-added products or as a source of renewable energy (Ravindran et al. 2018). The utilization of these agro-industrial wastes for the production of useful products emphasizes their biotechnological potentials for efficient value addition (Pandey and Soccol 2000; Pandey et al. 2000a, b, c; Soccol and Vandenberghe 2003).

Agricultural residues contain variability in composition like high amount of proteins, sugars, and minerals. Due to high nutritional composition, these residues not described as "wastes" but considered as raw materials for other product formation and developments (Sadh et al. 2018). The availability of these nutrients in raw materials offers appropriate environments for the growth of microorganisms. Agro-industrial waste and their complex organic contents constitute a significant source of residual nutrients which serve as rich media for microbial growth and production of the enzymes (Martins et al. 2011). In recent times, agricultural wastes have been made to use in biotechnological processes such as production of value-added compounds and substrates for microbial isolation.

7.1.1 Agro-Industrial Waste: A Scenario

The use of agricultural and agro-based industry wastes as raw materials can help to reduce the production cost and contributed in recycling of waste as well to make the environment eco-friendly (Sadh et al. 2018). These wastes may be used as low-cost raw materials for the production of other value-added compounds, with the expectancy of reducing the production costs (Bhatia et al. 2012). The environmental concern is because most of the agro-industrial wastes contain phenolic compounds and/or other compounds of toxic potential; which may cause deterioration of the environment when the waste is discharged to the nature. The agro-industrial wastes may be used in these processes as solid support, carbon, nitrogen, and/or mineral sources, which would allow obtaining more economical fermentation processes

avoiding the use of expensive chemical components in the media formulation (Table 7.1). As a consequence, more economical processes could be established for implementation on an industrial scale (Mussatto et al. 2012).

Agro-industrial wastes are generated during the industrial processing of agricultural products. Those derived from agricultural activities include materials such as

Table 7.1 Agro-industrial waste management

S. No.	Source of waste	Possible ways of utilization	Remarks	References
1.	Fruit wastes	Landfilling or incineration	Emission of methane, carbon-di-oxide, and other toxic substances	Dhillon and Kaur (2016), Deng et al. (2012)
2.	Apple pomace	Animal feed	High sugar content, low digestibility, low vitamin, and mineral content	Vendruscolo et al. (2008)
3.	Rice straw, sweet potato waste, sawdust, potato waste, corn stalks, sugarcane bagasse, and sugar beet waste	Biofuels	Decrease the deforestation by reducing our dependence on *forest* woody biomass	Duhan et al. (2013), Kumar et al. (2014, 2016)
4.	Raw residual coconut milk, raw residual pineapple juice	Bioethanol production by *Saccharomyces cerevisiae*	Alternative to replace fossil fuels is the production of bioethanol from agro-industrial wastes	Domínguez-Bocanegra et al. (2015)
5.	Vegetable's waste like potato peel, carrot peel, and onion peel. Banana stem	Bioethanol—Fermentation technique—*Saccharomyces cerevisiae*	Increases the yield by microbial fermentation	Mushimiyimana and Tallapragada (2016)
6.	Rice straw and corn stalks	Biofuel—*Aspergillus niger* and *Trichoderma viride*	Bioethanol	El-Tayeb et al. (2012)
7.	Sugarcane molasses	Biofuel—*Zymomonas mobilis*	Bioethanol	Cazetta et al. (2007)
8.	Wheat straw, sugarcane bagasse, maize straw, paddy straw	SSF—*Bacillus licheniformis*	α-Amylase	Kaur et al. (2015)
9.	Oil cake—coconut, groundnut, cotton seed, gingelly, or soybean	SSF—*Pseudomonas* sp.	Lipase	Faisal et al. (2014)
10.	Fruit peel waste	*Aspergillus niger*	Invertase	Mehta and Duhan (2014)

straw, stem, stalk, leaves, husk, shell, peel, lint, seeds, pulp, or stubble from fruits, cereals, bagasses or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many others. These wastes are generated in large amounts throughout the year and are the most abundant renewable resources on earth. They are mainly composed by sugars, fibers, proteins, and minerals, which are compounds of industrial interest (Mussatto et al. 2012).

A huge amount of organic residues and related effluents are produced every year through the food processing industries like juice, chips, meat, confectionary, and fruit industries. These organic residues can be utilized for different energy sources. The residue of different fruits and vegetables such as fruit and vegetable peels is commonly known as a waste or no use. But many researches focused on these peels and got good results. So these wastes are considered as a valuable raw material for the production of various pharmaceutical products (Parashar et al. 2014). The maximum percentage of antioxidant activity was observed in pomegranate peel than lemon and orange peel (Singh and Immanuel 2014).

7.2 Microbial Fermentation

Agro-industrial by-products that frequently cause serious environmental problems can be possibly used as inexpensive carbohydrate sources for microbial fermentations, thus decreasing their initial high biological oxygen demand (BOD) while obtaining biochemical compounds like pigments suitable for pharmaceutical, chemical, and food industries (Sarvamangala and Aparna 2016). Low-cost by-products and residues of agro-industrial origin have shown their potential in production of different pigments by diverse group of microorganisms. Researchers have shown a great interest in the processing of agro-industrial wastes for fermentation processes in the development of value-added products (Fig. 7.1).

The utilization of such materials as substrates for microbial cultivation intended to produce cellular proteins, organic acids, mushrooms, biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, and as sources of fermentable sugars in the second generation ethanol production has been reported (Sanchéz 2009). Notably, the microbial enzymes can be the products themselves as well as tools in these bioprocesses. Agro-industrial wastes are valuable sources of lignocellulosic materials. The lignocellulose is the main structural constituent of plants and represents the primary source of renewable organic matter on earth. It can be found at the cellular wall and is composed of cellulose, hemicellulose, and lignin, plus organic acids, salts, and minerals (Pandey et al. 2000a; Hamelinck et al. 2005). Being rich in cellulose, hemicellulose as well as in other nutrients, these wastes are one of the main reasons for pollution and therefore should be used as potential substrates for microbial fermentation rather than considering them as wastes (Rodríguez-Couto 2008).

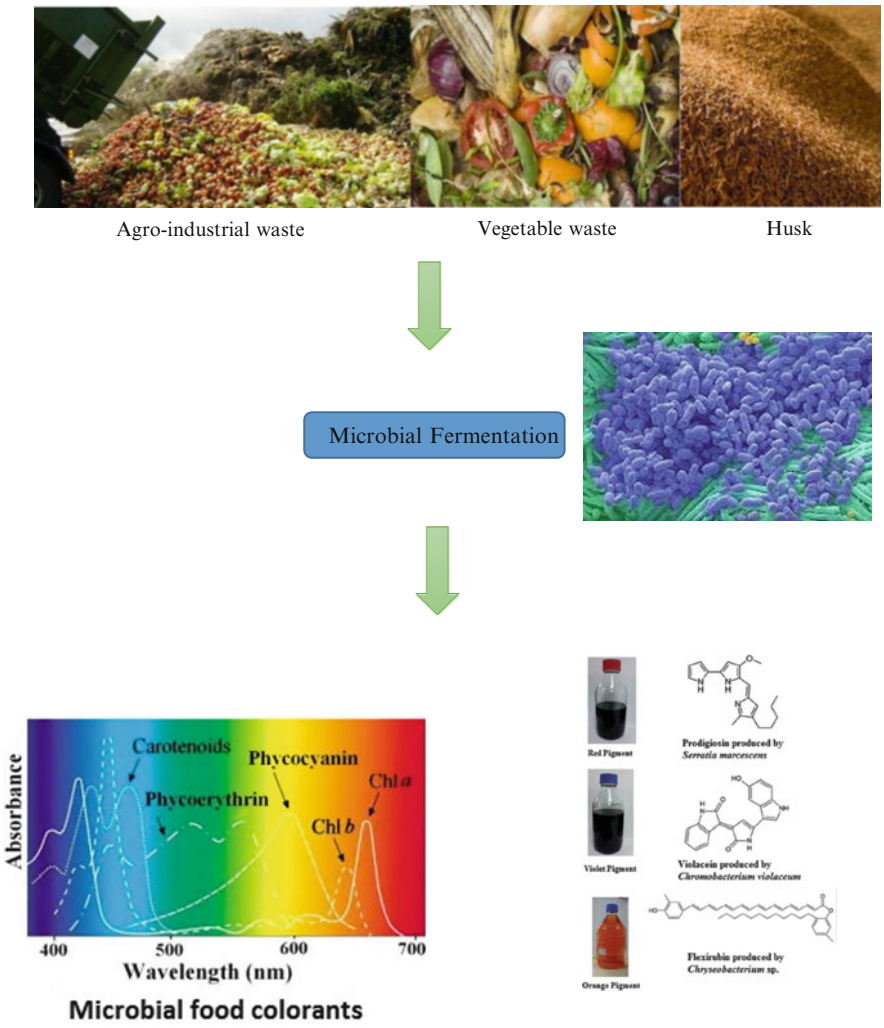

Fig. 7.1 Applications of agro-industrial wastes (Venil et al. 2014)

7.3 Pigment Production

Ever since, natural colors from spices and herbs, fruits, and vegetables have been part of the everyday diet of humans. Fruit by-products have become an important source of those pigments and colors, mainly because they present high color stability and purity. Bio-based pigments have several advantages such as biodegradability, zero or less toxicity, and eco-friendliness with their synthetic counterparts (Yusof 2017). Therefore, a lot of attention is now being undertaken for the synthesis of biocolorants from wastes using the microorganisms (Venil et al. 2013). In the

present scenario, researchers have shown a great interest in the processing of waste for fermentation processes in the development of value-added products like microbial pigments. Utilization of waste not only eliminates the disposal problems but also solves the problem of environment pollution.

Microorganisms such as bacteria, mold, and fungi produce different types of pigments depending on their sources. Some well-studied microbial strains that have potential of bio-pigment production from wastes are belonging to genera *Monascus, Rhodotorula, Aspergillus*, and *Penicillium*. For example, the following species are chiefly reported for bio-pigment production: *Alteromonas rubra, Rugamonas rubra, Streptoverticillium rubrireticuli, Streptomyces longisporus, Serratia marcescens, Pseudomonas magneslorubra, Vibrio psychroerythrous, S. rubidaea, Vibrio gazogenes*, etc. (Dufossé 2006; Méndez et al. 2011; Panesar et al. 2015). Along this line, variety of substrates and microorganisms has been tested. Beta-carotene synthesis by citrus products, carotenoids production using whey ultrafiltrate, sauerkraut brine, and peat extract, riboflavin in concentrated rectified grape must, astaxanthin on grape juice are some promising studies (Korumilli 2014). A wide range of bacterial strains such as *Serratia marcescens, Pseudomonas magneslorubra, Vibrio psychroerythrous, S. rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra*, *Streptoverticillium rubrireticuli*, and *Streptomyces longisporus* have shown their potential in pigment production (Krishna 2008).

Among the natural pigments produced by bacteria reported so far (Table 7.2), most researches have focused on yellow and red pigment production, carotenoid from *Phaffia rhodozyma* (Vazquez et al. 1997), *Micrococcus roseus* (Chattopadhyay et al. 1997), *Brevibacterium linens* (Guyomarc'h et al. 2000) and *Bradyrhizobium* sp. (Lorquin et al. 1997) and xanthomonadin from *Xanthomonas campestris pv.* (Poplawsky et al. 2000). The research concerning violacein has mainly focused on its medical application. In addition to its application in dyeing fabrics (Shirata et al. 2000), violacein has also exhibited cytotoxic activity in human colon cancer cells (de Carvalho et al. 2006), antileishmanial (Leon et al. 2001), antiulcerogenic (Duran et al. 2003), antiviral, antibiotic, antitumoral, and anti-Trypanosomacruzi activities (Andrighetti-Frohner et al. 2003). Recently, prodigiosin has been considered effective as a biological control agent against harmful algae in natural marine environments besides its role in textile dyeing and medicinal uses.

Microbial pigments offer the following benefits and advantages (Hendry and Houghton 1997; Babitha 2009): Easy propagation and wide strain selection; high versatile and productive over other sources; fermentation is inherently faster and more productive production compared with any other chemical process; easy to manipulate genes; simple and fast culturing techniques allowing continuous bioreactor operation; structural complexity suits for industrial needs; microbial pigments extracted using simple liquid–liquid extraction technique minimizing operation cost; cheap substrates used for bulk production.

Many investigations have been performed to reduce the costs and optimize the pigment production (Table 7.3); and factors such as carbon and nitrogen source are very important to consider on the selection of agro-industrial waste as substrates (Panesar and Kennedy 2012). A wide spread natural substrate is milk whey; it

Table 7.2 Natural pigments produced by bacteria (Malik et al. 2012)

Bacteria	Pigments	Color	Applications
Agrobacterium aurantiacum *Paracoccus carotinifaciens* *Xanthophyllomyces dendrorhous*	Astaxanthin	Pink-red	Feed supplement
Rhodococcus maris	Beta-carotene	Bluish-Red	Used to treat various disorders such as erythropoietic protoporphyria Reduces the risk of breast cancer
Bradyrhizobium sp. *Haloferax alexandrinus*	Canthaxanthin	Dark-red	Colorant in food, beverage, and pharmaceutical preparations
Corynebacterium insidiosum	Indigoidine	Blue	Protection from oxidative stress
Rugamonas rubra *Streptoverticillium rubrireticuli* *Vibrio gazogenes* *Alteromonas rubra* *Serratia marcescens* *Serratia rubidaea*	Prodigiosin	Red	Anticancer, immunosuppressant, antifungal, algicidal Dyeing (textile, candles, paper, ink)
Pseudomonas aeruginosa	Pyocyanin	Blue-green	Oxidative metabolism, reducing local inflammation
Chromobacterium violaceum *Janthinobacterium lividum*	Violacein	Purple	Pharmaceutical (antioxidant, Immunomodulatory, antitumoral, antiparasitic activities) Dyeing (textiles) Cosmetics (lotion)
Flavobacterium sp. *Paracoccus zeaxanthinifaciens* *Staphylococcus aureus*	Zeaxanthin	Yellow	Used to treat different disorders, mainly with affecting the eyes
Xanthomonas oryzae	Xanthomonadin	Yellow	Chemotaxonomic and diagnostic markers

contains lactose, proteins, and minerals, principally. Biological wastewater treatment technologies can assist in safe disposal of whey within environmental specifications, but these are expensive (Marova et al. 2012) becoming an attractive low-cost substrate for microbial production of carotenoids.

Table 7.3 Production of microbial pigments utilizing agro-industrial waste (Panesar et al. 2015)

Microbes	Media	Pigment	References
Submerged fermentation			
Rhodotorula rubra	Whey medium containing coconut water	Yellow pigment	Kaur et al. (2008)
Sporidiobolus salmonicolor	Yeast-salt medium	Carotenoids	Valduga et al. (2009)
R. glutinis	Tomato waste-based medium	Carotenoids	Silveira et al. (2008)
Rhodosporium paludigenum	Urea KH2PO4, MgSO4	Carotenoids	Yimyoo et al. (2011)
Solid-state fermentation			
Monascus ruber	Broken rice-based medium, packed bed of long grain rice-based medium, jackfruit seed powder-based medium	Red and yellow pigment	Vidyalaksmi et al. (2009)
Monascus purpureus	Corn meal, coconut residue, peanut meal, soybean meal-based medium	Red pigment	Nimnoi and Lumyong (2011)

7.4 Application of Bacterial Pigments

Bacterial pigments have wide range of applications in the pharmaceutical, food, and textile industry and have been discussed below.

7.4.1 Pharmaceutical Industry

Investigation of most of the pigmented bacteria has shown the efficiency in clinical applications of pigments for treating various diseases such as antibiotic, anticancer, and immune-suppressive properties (Venil et al. 2013). The property of bacteria to produce bio-pigments is used to produce medically important products. Pharmaceutical industry uses many microbial pigments in their products. Many pigmented secondary metabolites have significant potential clinical applications and many research works are going on for treating many diseases such as cancer, leukemia, diabetes mellitus etc. (Kumar et al. 2015). Bacterial pigments with fluorescence are employed in laboratories to label antibodies and indicate the progress of specific reaction. The pigments also play an important role in maintaining the health of human skin; melanin is used in sun block creams to protect the skin from UV radiation (Rao et al. 2017).

Adonirubin and astaxanthin are the xanthophylls, which also act as nutraceuticals. These xanthophylls by the process of antioxidation, anti-free radical or other mechanisms help to prevent carcinogenesis (Kim et al. 2012). The nutraceuticals functions of these xanthophylls and carotenes also help to prevent problems such as heart attacks and strokes (Long 2004). A red pigment, astaxanthin is important carotenoids

which has great commercial value, and is also used as pharmaceuticals feed. A strong therapeutics molecules prodigiosins are known for their immune-suppressive anti-cancer properties (Han et al. 1998). It has high commercial applications as prodigiosin possesses antibacterial, antifungal, anti-protozoal, cytotoxic, and anti-inflammatory properties (Panesar et al. 2015). *Hahella chejuensis* produces a pigment which is also known to have immune suppressant and antitumor properties (Kim et al. 2008).

7.4.2 Food Industry

An important goal of food industry is to produce food with an attractive appearance. Food producers are opting for natural food colors, as artificial ones show many negative impacts on health when consumed. Some fermentation-derived pigments, such as β-carotene, are now in use in the food industry. Various pigments provide a good appearance with additional nutritive and medicinal values such as antibiotic, antioxidants (Yangilar and Yildiz 2016). Demand for natural food colorants is more than its availability in food industry. Many natural colors are available, in which microbial colorants play important role as food coloring agent as their production and down streaming process are easy.

Natural colors are environment friendly and moreover serve as the dual need for visually attractive colors and health benefits in food colorants of probiotic (Nagpal et al. 2011). Therefore, pigments from microbial sources are good alternative. In addition, natural colorants will not only be beneficial to human health but will also be helpful for the maintenance of biodiversity. Some natural food colorants have commercial potential for use as antioxidants (Tuli et al. 2015). Thus bacterial colorants in addition to being environment friendly, can also serve the dual need for visually appealing colors and probiotic health benefits in food products (Venkatasubramanian et al. 2011). They are considered safe and approved by FDA. The successful marketing of pigments derived from bacteria, both as a food color and a nutritional supplement depend on consumer safety and freshness of the products.

7.4.3 Textile Industry

Textile industries use large amount of pigments mostly the synthetic ones. Synthetic dyes are widely available at an economical price and produce a wide variety of colors and have many drawbacks such as toxicity, mutagenicity, and carcinogenicity properties leading to various health problems such as skin cancer and allergies (Srikanlayanukul et al. 2006; Gurav et al. 2011). Hence, consumers demand for dyes of natural origin as colorants. Use of natural pigments in the textile industry is eco-friendly and noncarcinogenic. For successful commercial use of natural dyes for

any particular fiber, the appropriate and standardized techniques for dyeing for that particular fiber natural dye system need to be adopted (Venil et al. 2013). Therefore to obtain newer shade with acceptable color fastness behavior and reproducible color yield, appropriate scientific dyeing techniques/procedures are to be derived.

Microbial pigments are eco-friendly colorants applicable to dyeing textile fabrics (Chadni et al. 2017). Many microbial pigments were used to dye different types of fabric. Prodigiosin from *Vibrio* spp. can dye wool, nylon, acrylics, and silk. By using tamarind as a mordant, pigment from *Serratia marcescens* can color up to five types of fabric, including acrylic, polyester microfiber, polyester, silk, and cotton (Yusof 2008). Ahmad et al. (2012) observed the potentiality of prodigiosin and violacein in batik making. Kumar et al. (2015) reported that red pigment prodigiosin from *Vibrio* sp., *Serrtia* sp., and violet pigment from *Chromobacterium violaceum* are suitable for textile industry for dyeing of all fibers including cotton, wool, silk, nylon, and acrylic fibers.

7.5 Conclusions

Microbes utilize the waste for their growth through fermentation process and produce novel secondary metabolites. Therefore in recent years, various agro-industrial residues have been used as a substrate or additive for pigment production which may represent an added value to the industry thereby reducing the production cost. Extracted bacterial pigments can be used in food, pharmaceuticals, textiles, cosmetics, and also in food research and for the development of functional foods. The use of agricultural and agro-based industry wastes as raw materials not only eliminates the disposal problems but also helps to reduce the production cost contributed in recycling of waste as well to make the environment eco-friendly.

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Chapter 8
Analysis of Termite Microbiome and Biodegradation of Various Phenolic Compounds by a Bacterium Isolated from the Termite gut in Louisiana, USA

Seth Van Dexter and Ramaraj Boopathy

Abstract The eastern subterranean termite (EST) *Reticulitermes flavipes* is an insect pest in the USA. Like all wood-feeding termites (WFT), EST relies on a complex system of microbes to meet its nutritional requirements. The microbiome of WFT is stable, but the relative abundance of bacteria changes depending on diet. The purpose of this study was to explore the microbial diversity within EST collected in Thibodaux and St. Francisville, LA and detect differences based on diet and location to determine if the microbiome has a strict structure. It was found that taxa did not differ much between nearby colonies, but relative abundance is impacted by the wood in the diet. Half of bacteria from the gut of termites on nuttall oak were Bacteroidales, of which 22.7% were members of the family Porphyromonadaceae. 44% of bacteria from termites on red maple were Spirochaetes. All Spirochaetes were members of the genus Treponema. Elusimicrobia, a phylum found exclusively within termites and wood-feeding cockroaches was not abundant in either St. Francisville colony. Taxa differed more between termite colonies from different locations, but the mircobiome of St. Francisville colonies appeared to begin diverging at the family level. Overall, the microbiome was typical of termites, harboring cellulolytic protozoa, nitrogen-fixing bacteria, acetogenic Spirochaetes, and methanogenic archaeans. This has implications in microbial ecology because the organisms are changing, but the function, digestion of lignocellulose, is not. A bacterium was isolated and identified from termite gut as *Acinetobacter tandoii* from our previous studies degraded various phenolics, including phenol, nitrophenol, dinitrophenol, trinitrophenol, and toluene.

Keywords Subterranean termite · Microbiome · Bacteroidales · Spirochaetes · Archaeans · Phenol · Nitrophenol

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8.1 Introduction

Microbes within the gut of termites degrade lignocellulose, fix nitrogen, and produce acetate. In the higher termites (Termitidae), the microbiome consists entirely of bacteria (Warnecke et al. 2007), but all other termite families, including the subterranean termites (Rhinotermitidae), require symbiotic protozoa within the hindgut to degrade the majority of cellulose (Hungate 1943; Yamin 1980) and hemicellulose (Arakawa et al. 2009; Tartar et al. 2009), producing hydrogen gas, carbon dioxide, and acetate. However, termite gut protozoa as well as associated bacteria such as Spirochaetes (Graber et al. 2004) require anaerobic conditions to survive. Growth of *Trichomitopsis termopsidis* isolated from the gut of *Zootermopsis* termites is inhibited by 0.01 atm of O_2 and enhanced by the presence of hydrogen-consuming bacteria (Odelson and Breznak 1985). Oxygenation of the termite gut is an effective method of defaunating the termite gut (Cleveland 1925). Acetogenesis is important to termites because they are incapable of converting pyruvate to acetate following glycolysis (O'Brien and Breznak 1984).

8.2 Bacterial Diversity

Bacterial diversity within the gut of termites can be influenced by geographical location (Boucias et al. 2013), diet (Huang et al. 2013), and vertical inheritance from primary reproductives (Rahman et al. 2015), but some bacteria have coevolved with their termite host and their protozoa and are always present (Tai et al. 2015). The ability of termite gut protozoa to utilize cellulose as a carbon source is independent of bacteria and they can grow axenically when provided with a suitable nutrient medium (Yamin 1981), but within the termite, their symbionts are necessary.

8.3 Stability of Termite Microbiome

The termite microbiome must maintain anaerobic conditions within the interior of the hindgut. Bacteria on the periphery of the gut serve as an oxygen sink to maintain anaerobic conditions on the interior (Brune et al. 1995). Acetogenic bacteria which use hydrogen gas produced by the protozoa are incapable of growth in the presence of oxygen, but are able to tolerate exposure by reducing oxygen to reestablish anaerobic conditions (Boga and Brune 2003).

The stability of the termite microbiome makes it an excellent model system for microbial ecology. In EST, altering the composition of lignin and cellulose does not affect the composition of bacteria (Boucias et al. 2013), but Husseneder et al. (2009) showed that termites switched from a diet of wood to a diet of cellulose experience a change in bacterial composition. Formosan Subterranean Termite (FST) from

colonies on separate continents does not have significantly differing microbiomes (Husseneder et al. 2010). Although the function of the microbiome, the digestion of lignocellulose, and provision of nutrients to the termite host remains unchanged, only some bacteria have clearly defined roles within the microbial ecosystem. Additionally, termites have preferences for different wood types, and prolonged force-feeding trials can lead to termite mortality (Morales-Ramos and Rojas 2001).

The guts of the lower wood-feeding termites (WFT) families (all but Termitidae) harbor diverse bacterial communities and up to about a dozen protozoan species depending on the termite, which in turn harbor endo- and ectosymbiotic bacteria. Protozoa and symbiotic bacteria form close associations and bacteria can be found attached to the cell membrane, in the cytoplasm, on the rough endoplasmic reticulum, and within the nucleus (Smith and Arnott 1974). Husseneder et al. (2010) found that the microbiome of the Formosan subterranean termite, *Coptotermes formosanus*, was not affected by geographic origin. However, the microbiome of the eastern subterranean termite, *Reticulitermes flavipes*, differs based on geographic origin but not diet (Boucias et al. 2013). Several species in the order Bacteroidales only occur within termites and some only associate with a single protist species (Noda et al. 2009). Termites harbor an abundance of Bacteroidales and Spirochaetes (Hongoh et al. 2003; Shinzato et al. 2005). Phylogenetic analysis of gut bacteria from higher and lower termites from two genera shows that termite gut bacteria have coevolved with termites over time rather than being acquired from the environment (Hongoh et al. 2005), and termite microbiomes are vertically inherited between colonies (Rahman et al. 2015). However, the diet of the termite host affects the relative abundance of some bacteria (Huang et al. 2013; Rahman et al. 2015).

8.4 Eusociality in Termites

The most likely explanation for eusociality in termites is that exploiting wood requires symbiosis with microbes capable of degrading lignocellulose and conserving nitrogen. Because gut protozoa die after a termite molts, termites need to reinoculate their gut periodically via proctodeal trophallaxis, requiring termites to stay in family groups (Nalepa 2015). Gut bacteria are also associated with foreign conspecific recognition in *Reticulitermes speratus* (Matsuura 2001). Termites fed gut bacteria of other termite colonies or antibiotics were not recognized by their nestmates and attacked. In Formosan termites, members of colonies feeding on different diets act aggressively toward each other, but when their diet is changed to be identical, they are no longer aggressive (Florane et al. 2004). If relative abundance of certain taxa can be altered by diet in other termites, then it is possible aggression between Formosan termites is indirectly rather than directly mediated by diet.

8.5 Metabolic Function of Termite Gut Bacteria

The metabolic function of termite gut bacteria has been investigated thoroughly. For example, nitrogen fixation is an important role in the termite gut. Symbiotic bacteria of termite gut protozoa harbor bacteria from the order Bacteroidales capable of fixing nitrogen (Lilburn et al. 2001; Desai and Brune 2012) and nitrogen-fixing *Klebsiella* have been isolated from wood-feeding termite guts (Doolittle et al. 2008). Ammonium is also produced by bacterial recycling of uric acid waste of *Reticulitermes flavipes* (Potrikus and Breznak 1981). Bacteria on the periphery of the termite create anoxic conditions necessary for protozoa within the gut by scavenging oxygen (Brune et al. 1995). Protozoa produce acetate (Yamin 1981), the primary carbon and energy source of termites, H_2, and CO_2. Acetogenic bacteria associated with the protozoa utilize H_2 and CO_2 (Leadbetter et al. 1999).

8.6 Termite Gut Microbiome Analysis

Four colonies of *Reticulitermes flavipes* were collected from fallen and decaying wood. Two colonies came from the side of Bayou Lafourche in Thibodaux, LA feeding on tupelo, *Nyssa aquatica*. The other two colonies came from a residence in St. Francisville, LA. One colony was feeding on red maple *Acer rubrum* and the other was feeding on nuttall oak *Quercus texana*. Wood and termites were collected in plastic containers and brought back to the lab. Sampling sites and their GPS coordinates are given in Table 8.1.

Samples were prepared by randomly selecting 10 worker termites, placing them in a 10 mL beaker, and anesthetizing the termites by placing the beaker on an ice pack. When the termites stopped moving, termites were lifted by one antenna or leg using a pair of flame-sterilized forceps and externally sterilized by applying 100% ethanol to their bodies using a sterile cotton applicator. Externally sterilized termites were then placed into a sterile microcentrifuge tube (Eppendorf). After all termites were externally sterilized, the abdomen of each termite was removed and placed into sterile phosphate buffer solution (PBS) using flame-sterilized forceps. PBS was prepared with 1 L deionized H_2O, 7.650 g NaCl, 0.724 g Na_2HPO_4, and 0.210 g

Table 8.1 Sampling sites and their coordinates

Coordinates	Parish	Wood species
N 29°47′42.99″ W 90°47′54.46	Lafourche	*Nyssa aquatic*
N 29°47′37.41″ W 90°47′40.84″	Lafourche	*Nyssa aquatic*
N 30°48′51″ (nearest) W 91°20′42″ (address)	West Feliciana	*Acer rubrum*
N 30°48′51″ (nearest) W 91°20′42″ (address)	West Feliciana	*Quercus texana*

KH_2PO_4. The solution was adjusted to pH 7.4 with concentrated NaOH and HCl. PBS was sterilized in an autoclave at 121 °C and 20 PSI, for 20 min. Abdomens were then homogenized using a sterile plastic pestle.

Frozen samples were shipped to the MR DNA laboratory in Shallowater, TX for microbiome analysis. The V4 variable region of the 16S rRNA gene is useful in identifying bacteria because it is not conserved in bacterial evolution. The V4 variable region of termite gut bacteria was amplified using 515F/806R primers with a DNA barcode on the 515F primer. Samples were prepared using the HotStarTaq Master Mix Kit (Qiagen). The thermocycler was set to run 94 °C for 3 min, 28 cycles at 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. DNA was next-gen sequenced using a MiSeq, according to manufacturer protocol. Observed sequences were classified using BLASTn, and relative abundance of sequences was calculated. All taxa representing less than 0.100% of observed DNA were omitted from comparisons.

An arbitrary frequency threshold may be selected to prevent rare species from skewing community comparisons (Field et al. 1982) and using 0.100% omitted a total of nine phyla (not all unique) from the results.

8.7 Degradation of Phenolic Compounds

A pure culture isolated and identified as *Acinetobacter tandoii* from our previous study (Van Dexter and Boopathy 2018) was subjected to various phenolic compounds including phenol, nitrophenol, dinitrophenol, and toluene as the sole carbon source with basic mineral salt medium as described earlier. The phenolics concentrations used in the study were 100 mg/L. The experiment was conducted in triplicates. Bacterial growth was monitored by optical density using a spectrophotometer set at the wavelength of 600 nm. Phenolic compounds and toluene were analyzed by the method described in Boopathy (1997). Phenol and its metabolites were analyzed by liquid chromatography/mass spectrometry (LC/MS) equipped with two model 600A solvent pumps, a model 990 variable photodiode array multiple wavelength detector set at 220 nm, a data module, and a model 600E system controller. The mobile phase was acetonitrile:methanol:water (21,35,44 vol/vol/vol). Aliquots of 50 µl of samples were injected onto a Waters LC-18 µ Bondopak column (Milford, MA) at room temperature. The flow rate of the solvent was 1.5 ml/min. The relative intensity of the mass spectra of various metabolites was matched with LC/MS library. The retention times of metabolites were also identified using standards.

8.8 Statistics

Analysis of variance (ANOVA) was performed on the data with p value of <0.05 for significance as per the method described by Neter et al. (1990).

8.9 Bacterial Diversity in Termites of Various Geographical Locale and Wood Types

Tables 8.1, 8.2, 8.3, and 8.4 provide a complete list of all bacteria detected within the gut of each termite down to the level of family. The major bacterial phyla detected in every colony were Firmicutes (12.971–24.8%), Spirochaetes (7.241–44.354%), Elusimicrobia (0.406–16.393%, Proteobacteria (8.412–15.985%), and Bacteroidetes (7.384–49.355%) (Tables 8.2, 8.3, 8.4, and 8.5). Actinobacteria made up a significant proportion of the two Bayou Lafourche colonies (6.307 and 5.100%) but not the St. Francisville colonies. Both St. Francisville colonies diverged at the phylum level with each harboring one unique phylum relative to the other (Table 8.6). The Bayou Lafourche colonies contained all bacterial phyla contained by the St. Francisville colonies and also had one unique phylum (Table 8.7) and the nuttall oak colony only harbored one unique class. Nearly half of the bacteria from nuttall oak were Bacteroidetes and 44% of the red maple bacteria were Spirochaetes. Elusimicrobia, a phylum found only with symbiotic gut protozoa of termites and wood-feeding roaches, represented 16.393% of bacteria in the red maple colony, but only 0.406% in the nuttall oak colony. The first Bayou Lafourche colony harbored a substantial amount of Acidobacteria (18.729%), but the second did not (0.277%). There appears to be difference in species based on location and relative abundance based on diet.

More protozoa species were detected in the St. Francisville colony than any of the remaining three colonies and only two protozoan species were detected in the second colony from Bayou Lafourche (Table 8.8). One protozoan species detected in the second Bayou Lafourche colony, *Trichomonas gallinae*, is a Parabasalid, which only exists in birds (Stabler 1954). *Trichomitopsis termopsidis* and a species in the genus *Hypotrichomonas* were detected in each colony, but relative abundances were skewed. The only Archaean detected was a methanogenic species from the genus *Methanobrevibacter*, found in all four colonies. Identity to the species level could not be determined.

In the gut of termites, Spirochaetes are typically ectosymbionts of protozoa and are genetically and morphologically diverse, mostly associated with the genus *Treponema* (Lilburn et al. 1999). All termite gut protozoa harbor ectosymbiotic spirochaetes and symbiosis has evolved multiple times (Noda et al. 2003). All four colonies had positive identification of *Treponema primitia* and *T. azotonutricium*. *T. primitia* is carbon dioxide-reducing acetogen and *T. azotonutricium* is capable of fixing nitrogen into ammonium (Graber et al. 2004). *Endomicrobium* spp. that were found are within the phylum Elusimicrobia, which is only found within termites and

Table 8.2 Relative bacterial abundance (% DNA) in the gut of a *Reticulitermes flavipes* colony collected near Bayou Lafourche in Thibodaux, LA, feeding on tupelo

Phylum	Class	Order	Family
Acidobacteria (18.729)	Holophagae (18.693)	Holophagales (18.693)	Holophagaceae (18.692)
	Actinobacteria (0.699)	Actinomycetales (0.699)	Cellulomonadaceae (0.321)
			Micrococcineae (0.071)
			Microbacteriaceae (0.171)
Bacteroidetes (7.384)	Bacteroidia (7.192)	Bacteroidales (7.192)	Bacteroidaceae (1.127)
			Porphyromonadaceae (2.725)
	Cytophagia (0.100)	Cytophagales (0.100)	Cytophagaceae (0.100)
	Spingobacteriia (0.093)	Sphingobacteriales (0.093)	Chlorobiaceae (0.100)
Chlorobi (0.100)	Chlorobea (0.100)	Chlorobiales (0.100)	Deferribacteraceae (0.271)
Deferribacteres (0.271)	Deferribacteres (0.271)	Deferribacterales (0.271)	Elusimicrobiaceae (13.556)
Elusimicrobia (13.556)	Elusimicrobia (13.556)	Elusimicrobiales (13.556)	Eubacteriaceae (2.255)
Firmicutes (24.800)	Clostridia (15.675)	Clostridiales (15.675)	Symbiobacteriaceae (0.186)
			Prolixibacteraceae (0.521)
			Peptococcaceae (0.214)
			Clostridiaceae (5.915)
			Clostridiales family xiii incertae sedis (0.820)
			Ruminococcaceae (4.830)
	Bacilli (9.054)	Bacillales (1.042)	Bacillaceae (1.027)
		Lactobacillales (8.012)	Leuconostocaceae (4.017)
			Enterococcaceae (1.099)
			Streptococcaceae (2.897)
Planctomycetes (0.200)	Planctomycetacia (0.200)	Planctomycetales (0.200)	Planctomycetaceae (0.200)
Proteobacteria (8.412)	Betaproteobacteria (4.481)	Burkholderiales (0.228)	Oxalobacteraceae (0.107)
		Rhodocyclales (2.932)	Rhodocyclaceae (2.932)
		Neisseriales (1.270)	Neisseriaceae (1.270)
	Gammaproteobacteria (0.421)	Thiotrichales (0.136)	Thiotrichaceae (0.136)
		Xanthomonadales (0.150)	Xanthomonadaceae (0.150)
	Deltaproteobacteria (1.013)	Desulfovibrionales (0.729)	Desulfovibrionaceae (0.729)
		Desulfuromonadales (0.128)	Pelobacteraceae (0.107)
			Rhodospirillaceae (0.728)

(continued)

Table 8.2 (continued)

Phylum	Class	Order	Family
	Alphaproteobacteria (2.105)	Rhodospirillales (0.749)	
		Rickettsiales (0.892)	Rickettsiaceae (0.892)
		Rhizobiales (0.464)	Brucellaceae (0.464)
Spirochaetae (18.828)	Spirochaetes (18.828)	Spirocheatales (18.828)	Spirochaetaceae (18.828)
Synergistetes (0.435)	Synergistia (0.435)	Synergistales (0.435)	Synergistaceae (0.435)
Tenericutes (0.671)	Mollicutes (0.671)	Mycoplasmatales (0.671)	Mycoplasmataceae (0.671)
Verrumicrobia (0.307)	Verrucomicrobiae (0.307)	Verrucomicrobiales (0.307)	Verrucomicrobiaceae (0.307)

wood-feeding cockroaches. *Endomicrobium* within lower termites are obligate anaerobic intracellular symbionts of gut protozoa, and nitrogen fixation has been reported within this genus in the absence of ammonia (Zheng et al. 2016). Nitrogen fixation in termites is also reported in *Desulfovibrio* and *Clostridium* (Okhuma et al. 1999). *Desulfovibrio* was detected in this study. Nitrogen fixation could also be performed by ectosymbiotic Bacteroidales of gut protozoa (Lilburn et al. 2001; Desai and Brune 2012). It is therefore possible that several bacteria are capable of fixing nitrogen within the termite gut, but selection pressures related to the termite diet affect the relative abundance of taxa. Because only hardwoods were used in this study, it is unlikely that competition between species related to nutritional quality of the wood influenced the microbiome.

Actinomycetes are a phylum of fungus-like bacteria and were detected in three of four colonies. Actinomycetales is capable of depolymerizing cellulose within the gut of termites (Pasti et al. 1990; Wenzel et al. 2002). Strains of Actinomycetales isolated from the gut of Formosan termites, *Coptotermes formosanus*, are capable of utilizing cellulose or lignin as a sole carbon source. The presence and relative abundance of strains in Formosan termites is dependent on the geographical location of termites (Watanabe et al. 2003). Actinomycetes isolated from the gut of higher termites are capable of depolymerizing and solubilizing lignin (Pasti et al. 1990). Actinomycetes from higher termites were capable of liberating cellulose from lignin to utilize as a carbon source by producing peroxidase enzymes. Despite the presence of cellulose-degrading bacteria within the termite gut, the majority of cellulase transcripts produced within the termite gut are derived from protozoa, not bacteria (Tartar et al. 2009).

Although DNA abundance was collected for protozoa, it is difficult to directly compare protozoan abundance with bacterial abundance because the size difference between them is so large that the protozoa are capable of serving as habitat for bacteria. It would possibly be helpful to compare relative abundance of bacteria with relative abundance of protozoa, but protozoan metagenomic data in this study must

Table 8.3 Relative bacterial abundance (% DNA) in the gut of a *Reticulitermes flavipes* colony collected near Bayou Lafourche in Thibodaux, LA, feeding on tupelo (Different tree and colony. See Table 8.1)

Phylum	Class	Order	Family
Acidobacteria (0.277)	Holophagae (0.268)	Holophagales (0.268)	Holophagaceae (0.268)
Actinobacteria (5.101)	Actinobacteria (2.07)	Actinomycetales (2.07)	Cellulomonadaceae (1.758)
			Actinomycetaceae (0.139)
Bacteroidetes (10.677)	Coriobacteriia (3.031)	Coriobacteriales (3.031)	Coriobacteriaceae (3.031)
	Bacteroidia (10.512)	Bacteroidales (1.645)	Bacteroidaceae (1.645)
			Porphyromonadaceae (8.322)
			Rikenellaceae (0.260)
		Marinilabiliales (0.285)	Prolixibacteraceae (2.858)
	Spingobacteriia (0.113)	Sphingobacteriales (0.113)	Sphingobacteriaceae (0.113)
	Chlorobea (0.312)	Chlorobiales (0.312)	Chlorobiaceae (0.312)
	Deferribacteres (0.485)	Deferribacterales (0.485)	Deferribacteraceae (0.485)
Firmicutes (21.234)	Bacilli (13.232)	Bacillales (13.232)	Bacillaceae (13.232)
		Lactobacillales (4.780)	Leuconostocaceae (0.554)
			Enterococcaceae (0.468)
			Streptococcaceae (3.758)
	Clostridia (16.315)	Clostridiales (16.315)	Lachnospiraceae (1.559)
			Peptococcaceae (0.511)
			Clostridiaceae (5.248)
			Clostridiales family xiii incertae sedis (0.996)
			Gracilibacteraceae (0.130)
			Eubacteriaceae (3.853)
			Symbiobacteriaceae (0.268)
	Erysipelotrichia (0.113)	Erysipelotrichales (0.113)	Erysipelotrichaceae (0.113)
Elusimicrobia (13.290)	Elusimicrobia (13.290)	Elusimicrobiales (13.290)	Elusimicrobiaceae (13.290)
Proteobacteria (6.391)	Deltaproteobacteria (1.08)	Desulfovibrionales (0.901)	Desulfovibrionaceae (0.901)
	Betaproteobacteria (4.295)	Rhodocyclales (2.537)	Rhodocyclaceae (2.537)
		Burkholderiales (0.104)	
		Neisseriales (1.637)	Neisseriaceae (1.637)

(continued)

Table 8.3 (continued)

Phylum	Class	Order	Family
	Gammaproteobacteria (0.297)	Enterobacteriales (0.156)	Enterobacteriaceae (0.156)
		Xanthomonadales (0.113)	Xanthomonadaceae (0.113)
	Alphaproteobacteria (0.719)	Rhodospirillales (0.268)	Rhodospirillaceae (0.225)
		Rickettsiales (0.416)	Rickettsiaceae (0.416)
Spirochaetae (7.214)	Spirochaetes (7.214)	Spirocheatales (7.214)	Spirochaetaceae (7.214)
Synergistetes (0.182)	Synergistia (0.182)	Synergistales (0.182)	Synergistaceae (0.182)
Tenericutes (0.615)	Mollicutes (0.615)	Mycoplasmatales (0.580)	Mycoplasmataceae (0.580)
Verrumicrobia (0.234)	Verrucomicrobiae (0.191)	Verrucomicrobiales (0.191)	Verrucomicrobiaceae (0.191)

be interpreted with caution. The majority of DNA identified in both Bayou Lafourche colonies was a species in the genus *Hypotrichomonas* and in the St. Francisville colonies, it was *Trichomitopsis termopsidis*. Both are members of the Parabasalid clade of protozoa.

Porphyromonadaceae, the most abundant Bacteroidales family in the nuttall oak colony, is enhanced by a woody diet compared to a grassy diet in EST (Huang et al. 2013). Differences in the digestibility of grass and wood are a possible explanation to differences in the microbiome (Huang et al. 2013), but all termites used in this study were feeding on wood, so some other factor besides digestibility must affect the Porphyromonadaceae. Another study reported a difference in the dominance of bacteria within the gut of *Nasutitermes takasagoensis* (Termitidae) fed on wood (Spirochaetes dominant) and xylan, cellobiose, or glucose (Bacteroidetes dominant) (Miyata et al. 2007). Miyata et al. (2007) suggested that the difference in dominance was due to differences in the fermentation products of digestion of low and high molecular weight carbon sources. However, all colonies used in this study were collected from hardwoods and differences in the microbiome of termite colonies are therefore not likely to be the result of nutritional differences. Two possible explanations are either that they are reliant on location or vertical transmission from other colonies via the colony's primary reproductives (Rahman et al. 2015) or selection pressure exerted by secondary compounds within the wood or a combination of both. Closely related protozoa and associated Elusimicrobia, Tenericutes, Spirochaetes, Syngergistes, and Bacteroidetes are typically found within closely related termites (Tai et al. 2015) and all of the associated bacterial taxa previously listed were detected in this study, but other factors may influence the relative abundance.

In this study, termite colonies were collected from dead wood on the ground, but no distinction was made between sapwood and heartwood. Feeding on bald cypress,

Table 8.4 Relative bacterial abundance (% DNA) in the gut of a *Reticulitermes flavipes* colony collected in St. Francisville, LA, feeding on nuttall oak

Phylum	Class	Order	Family
Acidobacteria (1.143)	Acidobacteriia (1.143)	Acidobacteriales (1.143)	Acidobacteriaceae (1.143)
Actinobacteria (3.031)	Actinobacteria (1.621)	Actinomycetales (0.183)	Actinomycetaceae (0.101)
	Coriobacteriia (1.410)	Coriobacteriales (1.410)	Coriobacteriaceae (1.410)
Bacteroidetes (49.355)	Bacteroidia (48.741)	Bacteroidales (48.604)	Bacteroidaceae (6.886)
			Rikenellaceae (1.965)
			Porphyromonadaceae (22.716)
		Marinilabiliales (0.137)	Prolixibacteraceae (0.137)
	Sphingobacteriia (0.564)	Sphingobacteriales (0.564)	Sphingobacteriaceae (0.564)
	Flavobacteriia (0.101)	Flavobacteriales (0.101)	Chitinophagaceae (0.101)
Deferribacteres (2.682)	Deferribacteres (2.682)	Deferribacterales (2.682)	Deferribacteraceae (2.682)
Elusimicrobia (0.136)	Elusimicrobia (0.136)	Elusimicrobiales (0.136)	Elusimicrobiaceae (0.136)
Firmicutes (24.053)	Clostridia (13.577)	Clostridiales (13.563)	Eubacteriaceae (0.588)
			Lachnospiraceae (4.916)
			Clostridiales family xiii. Incertae sedis (2.608)
			Clostridiaceae (3.318)
			Ruminococcaceae (1.857)
	Bacilli (10.355)	Bacillales (0.118)	
		Lactobacillales (10.237)	Enterococcaceae (8.931)
			Streptococcaceae (1.282)
	Negativicutes (0.115)	Selemonadales (0.115)	Veillonellaceae (0.115)
Planctomycetes (0.179)	Planctomycetacia (0.179)	Planctomycetales (0.179)	Planctomycetaceae (0.179)
Proteobacteria (12.059)	Gammaproteobacteria (4.274)	Enterobacteriales (4.274)	Enterobacteriaceae (4.274)
	Alphaproteobacteria (3.163)	Rhizobiales (0.206)	Brucellaceae (0.120)
		Rhodospirillales (0.347)	Rhodospirillaceae (0.110)
			Acetobacteraceae (0.235)

(continued)

Table 8.4 (continued)

Phylum	Class	Order	Family
		Kopriimonadales (2.433)	Kopriimonadaceae (2.433)
	Betaproteobacteria (3.486)	Burkholderiales (0.969)	Burkholderiaceae (0.852)
		Rhodocyclales (2.537)	Rhodocyclaceae (2.508)
	Deltaproteobacteria (0.994)	Desulfovibrionales (0.975)	Desulfovibrionaceae (0.908)
Spirochaetae (7.241)	Spirochaetes (7.241)	Spirocheatales (7.241)	Spirochaetaceae (7.241)
Synergistetes (0.910)	Synergistia (0.910)	Synergistales (0.910)	Synergistaceae (0.910)
Verrumicrobia (0.298)	Opitutae (0.206)	Opitutales (0.200)	Opitutaceae (0.200)

Taxodium distichum, heartwood and heartwood extract causes Formosan termites to die of starvation because of toxicity to gut protozoa (Scheffrahn et al. 1988). However, termites were not deterred from feeding on bald cypress heartwood. Toxicity of wood types is related to diet choice in Formosan termites (Morales-Ramos and Rojas 2001), but it does not seem to be related to preference in *Reticulitermes* (Grace et al. 1989; Grace 1997), so it is possible that the food source of a colony was disrupting the microbiome of one or more colonies. No assessment of the health of a termite colony was made prior to collecting samples and taking them back to the laboratory.

The greater difference of unique taxa between colonies in different locations and seemingly inexplicable differences in relative abundance observed in this study indicates that geographical location is more likely to predict the microbes found within termites. There is no reason to believe that the essential functions of lignocellulose digestion, nitrogen fixation, detoxification of lignin monomers, or acetogenesis are disrupted within the gut of termites in the environment based on their diet. In the evolution of termites from their cockroach ancestors, microbial functions have been maintained. However, as their ancestors switched to a diet of pure wood, functions related to support of protozoa became more essential, indicating the importance of diet in the termite microbiome (Dietrich et al. 2014). However, the higher termites may have lost their gut protozoa because their gut bacteria began to outcompete protozoa for cellulose, indicating the adaptability of the termite microbiome.

Table 8.5 Relative bacterial abundance (% DNA) in the gut of a *Reticulitermes flavipes* colony collected in St. Francisville, LA, feeding on red maple

Phylum	Class	Order	Family
Actinobacteria (0.780)	Actinobacteria	Micrococcales (0.160)	Micrococcineae (0.160)
		Actinomycetales (0.314)	
	Coriobacteriia(0.466)	Coriobacteriales (0.466)	Coriobacteriaceae (0.466)
Bacteroidetes (8.815)	Bacteroidia (8.760)	Bacteroidales (8.760)	Bacteroidaceae (0.737)
			Porphyromonadaceae (2.969)
			Rikenellaceae (0.149)
Deferribacteres (1.025)	Deferribacteres (1.025)	Deferribacterales (1.025)	Deferribacteraceae (1.025)
Ekusimicrobia	Endomicrobia (16.370)		
Firmicutes (12.971)	Clostridia (11.825)	Clostridiales (11.710)	Eubacteriaceae (0.444)
			Lachnospiraceae (0.975)
			Ruminococcaceae (3.033)
			Clostridiales family xiii. Incertae sedis (1.155)
			Clostridiaceae (5.933)
		Thermoanaerobacterales (0.115)	Thermoanaerobacteraceae (0.115)
	Bacilli (0.991)	Bacillales (0.183)	Bacillaceae (0.160)
		Lactobacillales (0.807)	Streptococcaceae (0.732)
	Negativicutes (0.123)	Veillonellales (0.123)	Veillonellaceae (0.123)
		Selenomonadales (0.123)	
Planctomycetes (0.343)	Planctomycetia (0.343)	Planctomycetales (0.343)	Planctomycetaceae (0.343)
Proteobacteria (13.086)	Deltaproteobacteria (7.283)	Desulfovibrionales (6.698)	Desulfovibrionaceae (6.624)
			Desulfobacteraceae (0.460)
		Desulfobacteriales (0.462)	
	Alphaproteobacteria (2.770)	Rhodospirillales (0.104)	Rhodospirillaceae (1.033)
		Sphingomonadales (0.218)	Erythrobacteraceae (0.211)
		Rhizobiales (0.447)	Brucellaceae (0.399)

(continued)

Table 8.5 (continued)

Phylum	Class	Order	Family
		Rickettsiales (1.004)	Rickettsiaceae (0.626)
	Betaproteobacteria (2.458)	Rhodocyclales (1.701)	Rhodocyclaceae (1.701)
		Burkholderiales (0.756)	Burkholderiaceae (0.748)
	Gammaproteobacteria (0.575)	Enterobacterales (0.518)	Enterobacteriaceae (0.518)
Spirochaetes (44.345)	Spirochaetia (44.345)	Spirochaetales (44.345)	Spirochaetaceae (44.354)
Synergistetes (0.482)	Synergistia (0.482)	Synergistales (0.482)	Synergistaceae (0.482)
Tenericutes (0.256)	Mollicutes (0.256)	Mycoplasmatales (0.256)	Mycoplasmataceae (0.256)
Verrucomicrobia (1.457)	Opitutae (1.268)	Puniceicoccales (0.455)	Puniceicoccaceae (0.455)
		Opitutales (0.814)	Opitutaceae (0.814)
	Verrucomicrobiaceae (0.189)	Verrucomicrobiales (0.189)	Verrucomicrobiaceae (0.185)

Table 8.6 Total and unique bacterial taxa between two *Reticulitermes flavipes* colonies collected from a residence in St. Francisville, LA

Wood source	Total phyla	Unique phyla	Total classes	Unique classes	Total orders	Unique orders	Total families	Unique families
Red maple	11	1	17	2	26	7	34	11
Nuttall oak	11	1	18	3	24	4	31	8

Table 8.7 Total and unique bacterial taxa between *Reticulitermes flavipes* colonies near Bayou Lafourche (two colonies) and St. Francisville, Louisiana (two colonies)

Colony location	Total phyla	Unique phyla	Total classes	Unique classes	Total orders	Unique orders	Total families	Unique families
Bayou Lafourche	13	1	22	5	34	11	44	11
St. Francisville	12	0	20	3	31	6	41	15

Table 8.8 Protozoan species identified in the gut of workers from four *Reticulitermes flavipes* colonies in Louisiana

Bayou Lafourche 1 (tupelo)	Bayou Lafourche 2 (tupelo)	St. Francisville 1 (nuttall oak)	St. Francisville 2 (red maple)
Trichomitopsis termopsidis	*Trichomitopsis termopsidis*	*Trichomitopsis termopsidis*	*Trichomitopsis termopsidis*
Hypotrichomonas sp.	*Hypotrichomonas* sp.	*Hypotrichomonas* sp.	*Hypotrichomonas* sp.
Trichomonas sp.		*Trichonympha campanula*	*Trichonympha campanula*
		Tritrichomonas sp.	*Tritrichomonas* sp.
			Trichonympha collaris

8.10 Phenol Degradation by a Termite Gut Bacterium

Acinetobacter tandoii in our previous study (Van Dexter et al. 2019; Van Dexter and Boopathy 2019) showed growth on various concentrations of phenol as the sole carbon source. The results indicated that this bacterium can grow on phenol up to 600 mg/L. As the concentration increased from 300 mgl/L, the lag phase increased and the longest lag phase was observed with the concentration of 600 mg/L of phenol. At 700 mg/L, there was no growth at all indicating the toxicity limit of phenol for this bacterium is between 600 and 700 mg/L. The ability of several *Acinetobacter* species to degrade phenol and other aromatic compounds was reported by many workers (Beggs and Fewson 1977; Carr et al. 2003; Van Dexter and Boopathy 2018). Phenol-degrading *Acinetobacter* species reported in the literature are from wastewater effluent as these effluents contain many aromatic compounds. The novelty of this study shows termite gut as a potential source for phenol-

degrading organisms. This is because of lignin degradation during wood digestion in the gut of termite produces phenol as the major metabolite (Ke et al. 2011). The bacteria present in the hindgut of termites can break the aromatic ring of phenol in anaerobic conditions, but complete metabolism of phenol requires oxygen (Kuhnigk et al. 1994). Our work agrees with the isolation of strictly aerobic phenol-degrading bacteria by Van Dexter and Boopathy (2018). *A. tandoii* in termite plays a vital function by detoxifying phenol in the gut. The accumulation of phenol from lignin metabolism will be toxic to all microbes in the gut as phenol is the potent antimicrobial chemical (Boopathy 1997; Doolittle et al. 2007, 2008). The isolated bacteria may play a vital function in termite survival from phenol toxicity. The microbiome of termite gut shows distinct role for each species, which includes fixation of free nitrogen, oxygen scavenging to maintain anaerobic condition, methanogenesis, and acetogenesis (Doolittle et al. 2007, 2008).

As previously described in our earlier reports (Van Dexter et al. 2019; Van Dexter and Boopathy 2018), the metabolic pathway of phenol degradation includes various metabolites such as catechol, *cis-cis* muconic acid, succinic acid, oxaloacetic acid, and acetic acid in the culture condition where phenol served as the sole carbon source. Van Dexter and Boopathy (2018) constructed a metabolic pathway for phenol metabolism which is given in Fig. 8.1. This pathway was similar to other published reports in the literature (Stanier and Ornston 1973; Tian et al. 2017; Wang et al. 2007; Shen et al. 2009; Hupert-Kocurek et al. 2012; Sainsbury et al. 2015; Putrins et al. 2007; Yamanashi et al. 2015). Succinate and oxaloacetate are both intermediates in the TCA cycle and acetate is the energy source for the TCA cycle.

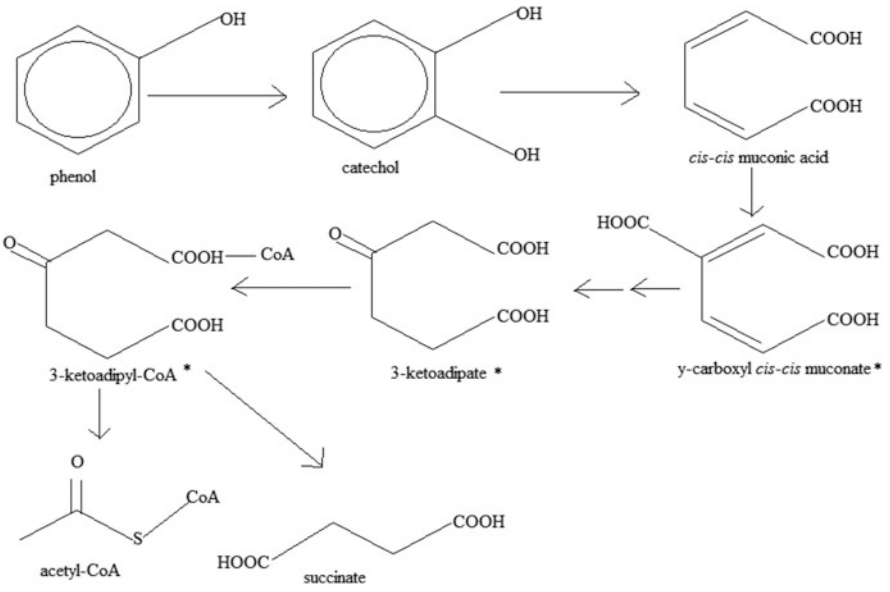

Fig. 8.1 Proposed phenol degradation pathway. Adapted from Van Dexter and Boopathy (2018)

The metabolism of phenol followed beta-ketoadipate pathway with the production following metabolites, catechol, cis-cis muconic acid, cis-cis muconate, lactone, beta-ketoadipate, succinyl coA, and acetyl coA.

8.11 Degradation of Nitroaromatics and Toluene

Acinetobacter species is known to degrade aromatic compounds. Some species of *Acinetobacter* are very hard to differentiate without molecular analysis and analyzing the unique properties of new strains and their phenol-degrading property for each strain will be difficult. We exposed our isolate to various substituted phenolic compounds and forced this organism to use these compounds as the sole carbon source. As shown in Fig. 8.2, the bacteria grew very well in the presence of nitrophenol, dinitrophenol, trinitrophenol, and toluene as the sole carbon source in the culture medium. As the substituents of nitro groups increased, the bacterial growth decreased. The bacteria preferred the compounds in the following order nitrophenol > dinitrophenol > trinitrophenol > toluene. Toluene was relatively hard for this bacterium to degrade as it was never exposed to such compound in the termite gut, from where it was isolated. The concentrations of these compounds in the culture medium are given in Fig. 8.3. Nitrophenol was degraded 98% followed by dinitrophenol (80%), trinitrophenol (67%), and toluene at 49%. This study demonstrated the bacteria present in termite gut could degrade phenolic compounds in vivo, but the application of this organism to clean up phenol contaminated sites is not yet known. Van Dexter et al. (2019) showed phenol concentration at 100 ppm will kill termite microbes residing in the gut and yet we showed the isolated *Acinetobacter* species could degrade phenol up to 600 mg/L. This showed the evolution of this particular bacterial strain to handle

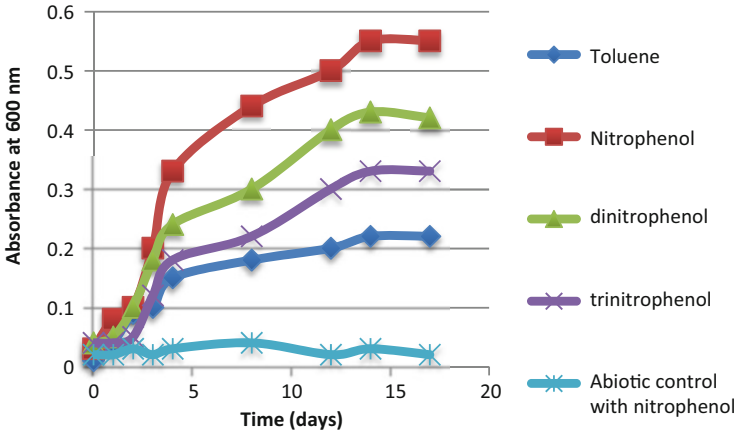

Fig. 8.2 Growth of *A.tandoii* on various substituted phenolics and toluene as the sole source of carbon

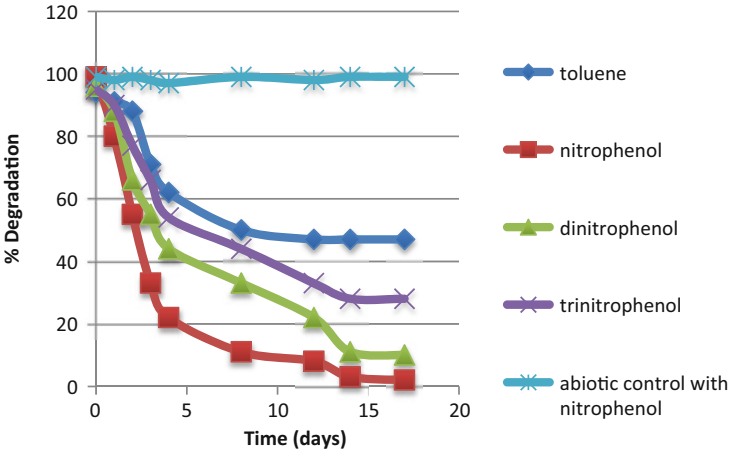

Fig. 8.3 Biodegradation of nitrophenols and toluene by *A. tandoii*

higher concentrations of phenol, which could be commercially exploited in the bioremediation of phenol in the environment.

In a normal ecosystem, termites provide ecosystem services through bioturbation, enriching the soil with nitrogen, and recycling nutrients (Jouquet et al. 2011). Releasing carbon from dead wood is important for the carbon cycle, but the degradation of lignocellulose is chemically demanding and termites cannot digest lignocellulose without protozoa and bacteria within their gut. Endogenous enzymes within the foregut and midgut are capable of degrading cellulose (Zhang et al. 2010) and modifying lignin (Coy et al. 2010), but their digestion is not efficient enough to sustain the termite. In addition, O'Brien and Breznak (1984) reported termites are not capable of utilizing glucose as the carbon source for energy metabolism. All termites require microbes to help degrade wood and meet their nutritional requirements. The role of *Acinetobacter* in termite gut seems to detoxify phenol so the other microbes in the gut can survive and play many vital functions in the survival of termite and thus, this organism is important and has its own niche in the microbial ecology of termite gut.

8.12 Conclusions

This study confirms the stability of the microbial flora of termites on a natural diet. The use of certain natural products can reduce the number of bacteria and protozoa within termite gut. More information is needed regarding the microbial flora of termites feeding on softwoods, but it is possible that the microbial ecosystem is capable of compensating for a partial loss of function of protozoa based on the higher number of cellobiose-utilizing bacteria. The role of *A.tandoii* demonstrated that this bacterium plays a key role in detoxifying phenol from lignin metabolism in the termite gut. This

research shows the potential for termites to be used as a source of organisms capable of detoxifying environmental contaminants such as phenol, various nitrophenols, and toluene to some extent. It might be possible to use this bacterium in bioremediation of various phenolic compounds contaminated soil and water.

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Chapter 9
Compatible Technologies to Anaerobic Digestion for the Integral Valorization of Organic Waste

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Abstract The term "environmental biotechnology" has been coined to describe the use of biological systems, ranging from bacteria to plants, to achieve environmental remediation, pollution prevention, detection, and monitoring of contaminants, and more recently transforming waste to produce energy, biopolymers, and other benefits. Latin-American countries have a privileged location to develop ingenious and sustainable alternatives in environmental biotechnology. An advantage to do innovation in tropics is their biodiversity. Useful compounds can be produced in laboratory settings and/or full-scale operations. However, waste (solid, liquid, or gaseous) released into natural and confined (end of pipes) environments are normally mixtures of different chemical compounds and often microorganisms are part of this waste. Waste valorization can conduce to obtain more rentable by-products in bioremediation. To conduct the bioremediation join to valorization, many processes need to be implemented. Coupled biological processes can increase the efficiency and value to end products. In this chapter, different alternatives to valorize organic wastes under the anaerobic digestion-based biorefinery concept were reviewed. Advantages and challenges of developing countries to use environmental biotechnology and to solve waste problems were also analyzed.

Keywords Environmental biotechnology · Bioprospecting · Anaerobic digestion · Biofactory · Added value

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9.1 Introduction

Anaerobic digestion (AD) is defined as a biological process that converts organic matter into biogas (energy) and nutrient-rich digestate. This process can be seen as a promising alternative for agro-industrial waste management with low environmental impact, and opportunities for value recovering in a simple, economic, and profitable fashion. However, AD does not guarantee a completely safe environmental treatment for products generated in the process (i.e., biogas, solid digestate, and liquid effluent).

The biogas, which is a gas mixture mainly composed of methane (40–70%), has a greenhouse effect equivalent to twenty-five times carbon dioxide effect whether it is emitted directly into the atmosphere (Sawatdeenarunat et al. 2016). Ideally, methane can be used in the process of thermal utilization (e.g., lighting, heating, power generation, etc). Otherwise, burnt it would be an option. Alternatively, biogas might be converted into methanol, which is the source of many high-value industrial chemicals (e.g., anhydride, ethylene, polyols, and other aromatic compounds), under the concept of anaerobic biorefinery (Sawatdeenarunat et al. 2016).

On the other hand, there is a liquid effluent with different amounts of organic matter that depends on the efficiency of AD. This liquid effluent could promote eutrophication in soils and water bodies where is poured. High concentration of nutrients, such as nitrogen, phosphorus, and potassium can be commonly found in liquid effluents that may promote eutrophication. Environmental issues related to ferti-irrigation require regulations and careful handling. Within the safe handling of anaerobic effluents, the use as fertilizer and/or amendment is commonly suggested. In the situation that mentioned uses are not possible, processes of nutrient removal can be considered.

Finally, AD plants generate large amounts of solid digestate that need to be utilized or disposed in an environmental friendly fashion since this matter can be source of contamination due to nutrient runoff (Vasco-Correa et al. 2018). This stream mainly consists of suspended solids and undigested residues. The composition of solid residues varies depending on the substrate type and operating conditions. High concentration of nutrients useful for fertilization can be found. Nevertheless, the concentration of pathogens is the major problem related to this stream. Since AD does not guarantee the elimination of pathogens, the digestate may be converted into the source of contamination for crops that can affect food safety. From this viewpoint, the digestate should be sanitized before its use as fertilizer.

In view of the above, when AD is coupled to other processes, not only the risk of environmental contamination is decreased but also opportunities for recovery and products of added value are generated. This ultimately contributes to the process of sustainability. In this chapter, the current state of AD is presented. In addition, a brief description of compatible technologies with AD and possible models for the integration of processes are included. Thus, a wide variety of high-value bio-based energy and products via biorefinery approach are described. Finally, challenges and perspectives for products of added value around the AD process are considered.

9.2 Current State of Anaerobic Digestion

9.2.1 Worldwide State

Countries that have the greatest AD technology establishment, contexts in which those technologies are used, offering of anaerobic technologies, offering of compatible processes with AD, information from reputable suppliers, and recommended coupling systems are included herein. Additionally, regulations and/or norms from developed countries are considered.

Biogas production has shown a fluctuating behavior over time. It gained a great acceptation in the first half of the twentieth century, followed by a decreasing trend after the 1950s due to the low price of fossil fuels and their accessibility. With the energy crisis in the 1970s, biogas obtained importance as renewable energy. Nonetheless, the high cost of digesters, issues related to design, lack of knowledge, and the inability for proper management were responsible for affecting its growing establishment (Vasco-Correa et al. 2018).

Anaerobic digestion (AD) is a promising biotechnology to convert organic substrates such as high solid feedstocks (animal manure, food wastes, municipal solid wastes, and lignocellulosic biomass), municipal and industrial wastewater to biogas while stabilizing waste (Sawatdeenarunat et al. 2016; Vasco-Correa et al. 2018). The establishment of AD worldwide varies enormously, from small-scale household digesters in developing countries to large farm-scale or centralized digesters in developed countries. Differences in the implementation of this technology are mainly related to a complex set of conditions that include economic and environmental implications, stimulus from polices and incentives in agricultural systems, waste management and renewable energy production (Vasco-Correa et al. 2018).

Over 14,000 commercial AD plants operate in Europe while 9000 plants only work in Germany (Sawatdeenarunat et al. 2016). Europe is a leader in AD technology and its implementation is driven by the establishment of strict environmental regulations for waste disposal (Vasco-Correa et al. 2018). Asia accounts for the largest amount of digesters installed, but most of them are small-scale household digesters used in rural areas for cooking and lighting. China has more than 43 million digesters, and India has about 4.7 million units. The USA, on the other hand, has about 2200 operational AD plants with growing interest in this technology (Vasco-Correa et al. 2018; Serfass 2017).

Two models of digesters are commonly used in Europe; the centralized or joint systems, and the farm-scale digesters. Denmark, for instance, is a pioneer in the development of the first type of digesters with capacities up to 8000 m^3. About 150 biogas plants, containing 20 centralized AD systems, are known in this country. Farm-scale plants have capacities from 200 to 1200 m^3, and they are usually located in swine farms that co-digest animal manure from 1 to 3 farms with agricultural residues and other organic matter. Germany excels with this sort of plants; about 9000 units and great interest to increase the number of these units are highlighted (Vasco-Correa et al. 2018; Wilkinson 2011). The USA has 247 farm-scale anaerobic

digesters, 1269 wastewater treatment plants, 39 industrial AD plants, 645 plants at landfills, and potential for about 13,500 new biogas systems (Serfass 2017). Canada has about 100 biogas plans and various initiatives to reinforce the use of this technology (Vasco-Correa et al. 2018).

Among the renewable energy sources, biomass is used in developing countries. Biomass (e.g., firewood, crop residues, and cattle dung) is considered as a primary energy source for heating and cooking. In developed countries, the use of biogas for combined heat and power generation or transportation fuels is well established (Sawatdeenarunat et al. 2016). Nonetheless, biogas production alone may not be economically competitive due to the volatility of market and price index of fossil fuels, leading to explore alternative products and to adopt other approaches like the biorefinery concept. Many studies have discussed about this topic, but limited studies focused on anaerobic biorefinery have been reported (Sawatdeenarunat et al. 2016; Hagman et al. 2018; MacLellan et al. 2013; Surendra et al. 2015).

9.2.2 Latin America Landscape

In this section, the relationship between AD and agricultural vocation of Latin America countries is included. As well as, regulations and/or norms in these countries are taking into account.

In overall, the application of waste-to-energy (WtE) technologies used to manage and treat organic residues have been developed and implemented in Latin America and Caribbean countries. However, a lot of effort is still needed to significantly contribute and generate impact in the region. Household organic wastes, forest, and agricultural residues, for example, should be seriously recognized as valuable sources of energy since most of them are largely unused (Silva-Martínez and Sanches-Pereira 2018).

As early mentioned, Asia and other developing regions such as Latin America are focused on the use of small-scale digesters (Garfí et al. 2011, 2016, 2019); most household units with capacities between 2 and 10 m^3 are usually located in rural areas. Among the most common digesters used, the following types can be found: Chinese fixed dome digester, Indian floating drum digester, and tube digester. These designs do not have mechanical mixing and heating systems (Vasco-Correa et al. 2018). The latter digester has successfully treated agricultural manure since 1980 when the first plastic tubular digester was introduced in Colombia (Silva-Martínez and Sanches-Pereira 2018; Garfí et al. 2019). In Latin America and Caribbean countries, the first small-scale biodigesters were installed in the 1970s. Most countries in the region implemented this technology for the following decade, and the process had major interest in the 1990s and early in the twenty-first century (Silva-Martínez and Sanches-Pereira 2018).

In countries like Colombia, Mexico, Nicaragua, Caribbean Islands, and Central America (i.e., Costa Rica and Honduras), AD technology has been implemented, and it has also adapted to harsh weather conditions (e.g., Bolivia, Peru, Chile,

Ecuador, and Argentina). Brazil has registered the use of "Sertajeno" digester type based on the Indian model, but it was abandoned for operational problems. In Caribbean countries, the AD technology was introduced by "Deutsch Gesellshaftfur Technische Zusammenarbeit," a German organization (Silva-Martínez and Sanches-Pereira 2018).

Government programs and/or nonprofit international organizations (e.g., Stichting Nederlandse Vrijwilligers-SNV founded in the Netherlands) have supported the implementation of national biogas systems in Latin America (Vasco-Correa et al. 2018). In rural areas of Central and South America, for instance, more small-scale digesters (e.g., tube plastic digesters) have been developed and implemented (Vasco-Correa et al. 2018; Silva-Martínez and Sanches-Pereira 2018; Garfí et al. 2011, 2016, 2019; Cutz et al. 2016). Additionally, the Network for Biodigesters in Latin America and the Caribbean (RedBioLAC) was created in 2009 with the purpose of sharing information and to support this technology (Vasco-Correa et al. 2018; Silva-Martínez and Sanches-Pereira 2018).

Although AD has received less attention, Latin America can benefit from small-scale digesters. Successful implementation has been reported in the last few years for heating and cooking purposes. Interestingly, AD for wastewater treatment has operated for several decades. However, the biogas is not fully used. Mexico has tried to develop projects and to use biogas for energy production (Vasco-Correa et al. 2018). Interest in large-scale biodigesters and second-generation biofuels is gaining ground in the region, seeking out their development and implementation (Silva-Martínez and Sanches-Pereira 2018).

Large-scale anaerobic digesters have not been widely implemented for various reasons. Among these, the following can be mentioned: Related costs, system technical complexity, and maintenance demand. Argentina, Chile, and Brazil, on the other hand, have implemented Continuous Stirred Tank Reactors (CSTR), Upflow Anaerobic Sludge Blanket (UASB), and cover lagoons (Silva-Martínez and Sanches-Pereira 2018). Some reports indicate the construction of large-scale plants in Puerto Rico (Voegele 2018).

9.3 Bioprospecting in Tropical Countries

Several alternatives for valorization of organic wastes are biological processes, therefore it is fundamental to have microbial resources in a sustainable way for their implementation. In this sense, it is interesting to consider the potentialities of bioprospecting in tropical countries characterized by their wide biodiversity, especially (Bull et al. 2000; Sanabria 2014).

The use of pure cultures has allowed researchers to know microorganisms in detail, and to control their harmful effects and use them for beneficial applications (i.e., pharmaceutical medicine and disease control) (Bull et al. 2000). Bioremediation has ever used microbial communities capable of transforming pollutants into less environmentally harmful chemical compounds (Sanabria 2014). Lately, when

combining the need to control pollution and the use of pure cultures, several pieces of research have aimed at searching for useful and specific microorganisms from consortia, focusing on media enrichment. This method consists in successively passing a group of organisms to the same culture media, decreasing the population to gain a pure culture from a single colony. However, when comparing the number of microorganisms and the applications derived from them, the number of results seems out of proportion. This might be related to the chemical complexity of the pollutants and the limited understanding of the ecological relationships between the species involved.

Furthermore, in the case of Latin America and especially Colombia, there is an actual challenge in microbiology and biotechnology laboratories for design, acquisition, and operation of special devices such as reactors. The directed prospection strategy, in continuous or semicontinuous systems, differs in that microorganisms are never taken out of the bioreactor, in such a way that the consortia obtained is the same product of the continuous feed. For this condition, the supplied media must be continuously adjusted to obtain the desired metabolic outcome. This strategy allows the expansion of the microbial diversity range, and to find microorganisms and combined biochemical processes adapted to the stressful conditions of residues. Thus, they can generate products with high-added value. One of the biggest challenges toward the transformation of wastes is their complexity due to the convergence of organic and inorganic pollutants.

Consortia design can be used as separation strategy, focusing on one or more reactors and coupled processes. In the process, it is possible to obtain pure cultures of microorganisms that have been previously described as non-cultivable (Hu et al. 2013). The use of mixed cultures and low-cost substrates such as waste can reduce the production cost, which may generate an actual interest in decreasing the impact of pollutant discharge. Recent studies have shown that cellular content of PHA in mixed cultures from activated sludge can achieve 67 and 78.5% of its dry cell weight (Liu et al. 2011; Serafim et al. 2004), such values flanking those obtained with pure cultures using high-cost raw material.

Mixed Culture Biotechnology (MCB) was firstly defined by Kleerebezem and Loosdrecht (2007). With the use of undefined mixed cultures, process development in MCB can only be based on natural/ecological selection by manipulating the operation of the bioprocess or varying the source of the natural inoculum which is useful for the transformation of substrates into valuable products. This combination of approaches has been used to improve the production of bio-based fuels, chemicals, and materials.

In this approach, energy flows and stoichiometry play an important role (Rodríguez et al. 2006), and genetic changes will no longer be needed (Agapakis et al. 2012). The success of consortia is represented in the naturally established biodiversity and temperature. Surely, if tropical countries possessing the largest diversity on the planet (many of them in conditions of delayed development) could indeed take advantage of this, they will have better options for competitiveness and development.

9.3.1 Processes That Can Be Coupled to Anaerobic Digestion

In this section, the main concepts and current state of technologies that can be coupled to AD for treatment and valorization of the different effluents are described. Alternatives with added value through obtaining of energy, chemical, and materials are included.

9.3.1.1 Biogas

Biogas Upgrading The presence of hydrogen sulfide (H_2S) in biogas due to the digestion of sulfur-rich substrates can be detrimental to combined heat and power units. Higher concentrations than 250 ppm are worrying. Thus, the hydrogen sulfide and other impurities such as CO_2, ammonia, moisture, and particulates should be removed prior to the use of biogas, achieving greater methane content than 95%.

Upgrading processes at commercial-scale plants have been applied (e.g., pressure swing adsorption, water pressure absorption, alkaline absorption, biological filtration, micro-aeration). However, associated costs and economic benefits must be addressed.

Compression and Liquefaction Among the possible uses of the upgraded biogas, energy production (i.e., heat, electricity, or mechanic work) and biogas injection into a natural gas distribution grid can be mentioned. In these processes, the biogas pressure must be increased (Morini et al. 2009), which (including upgrading process) consumes about 20% of the energy contained in raw biogas (Budzianowski et al. 2017).

The natural gas infrastructure might be used for biogas compression. However, some problems can emerge due to the different physical properties of gasses. The surge, for instance, is one of the main problems detected in biogas compression. It is known as compressor instability and is characterized by the time variation of pressure ratio and mass flow rate, which affects the compressor operating range, performance, and reliability. Experimental and modeling strategies have been proposed to study compressor instabilities and to set up devices that avoid compressor operation beyond the surge line (Morini et al. 2009).

The conversion of biogas in liquefied natural biogas (bio-LNG) can be seen as other interesting option. High energy density, in comparison to compressed biogas, guarantees similar ranges to those values obtained with diesel oil (Baccioli et al. 2018).

The liquefaction technology for small-scale systems is recently available in the market but is characterized by an elevated specific energy consumption (0.7 kWh/Stm^3). This highly increases the electric demand of the plant and generates new opportunities in system management (Baccioli et al. 2018). Nonetheless, it is an attractive energy vector with volumes about 600 times less than natural gas volumes at standard conditions, making easy to store and ship (Pasini et al. 2019). Several types of refrigeration cycles can be implemented, but the reverse Joule-Brayton cycle could be the most promising technology for small-scale systems (Baccioli et al. 2018).

Heat and Power Generation Biogas was initially considered as nuisance because of the odor problems and methane emissions. Flaring has always been a cheap and simple solution. For years, biogas has been intentionally produced from biosolids to generate energy in the form of heat and power. Biogas can be converted into CNG through five steps. After each step, the biogas can be used against a high energy efficiency and/or high energy value. The first step is to dry the gas, making it suitable for simple boilers, for instance. The next step is desulphurization, followed by siloxane removal. After this stage, the gas can be used in a CHP or used to produce electricity at the highest efficiency with gas turbines. In a subsequent step, CO_2 can be removed from the biogas, resulting in almost pure methane with the same quality of natural gas. This gas can be injected into a local gas grid after increasing its calorific value with propane. Finally, the gas can be pressurized to 220 bar, allowing to use it as vehicle fuel and being substitute for CNG.

Combined Heat and Power (CHP) is a highly fuel-efficient energy technology, in which usable heat and energy (usually electricity) are simultaneously generated in a single stage. In this process, the excess of heat produced during the conversion of thermal energy into electricity is captured and let available for use. In such a way, CHP can increase the overall efficiency of fuel use up to 89% compared to 55% for the best conventional electricity generation (Coolkeeragh 2007). Most new CHP schemes use natural gas, but a significant portion is dual fuel, and it can burn alternative and renewable fuels, such as biofuels or biogas.

Methane Reforming It is an important industrial process for H_2 and/or syngas production. Fossil natural gas is the main source of methane for the reforming process. However, biomethane might be a renewable option for supplying this demand. Methane reforming can be conducted through three ways (Julio and Barbosa 2013):

(a) *Steam methane reforming*. Almost half of the world's hydrogen demand is produced through the steam methane reforming process (Ewan and Allen 2005), which requires high temperatures (800–900 °C) and pressures (15–30 bar). In this process, two reactions are performed:

$$CH_4 + H_2O \leftrightarrow CO + 3H_2 \tag{9.1}$$

$$CO + H_2O \leftrightarrow CO_2 + H_2 \tag{9.2}$$

The reactor output includes H_2, CO, CO_2, H_2O, and non-reacted CH_4.

(b) *Dry methane reforming*. This process has the advantage of consuming both CH_4 and CO_2 (two intensive greenhouse gasses) by the following reaction:

$$CH_4 + CO_2 \leftrightarrow 2CO + 2H_2 \tag{9.3}$$

Chemical promoters (i.e., Mg, K, and other alkali compounds) are used with catalysts in order to avoid coke formation.

(c) *Partial catalytic methane oxidation.* In this process, an amount of O_2 under stoichiometric ratio is used, allowing the partial oxidation of methane and the production of CO and H_2. The following reaction is carried out:

$$CH_4 + 1/2O_2 \leftrightarrow CO + 2H_2 \tag{9.4}$$

When the process is not controlled, the increase of oxygen may lead to the total oxidation of methane, producing CO_2 and H_2O as shown by the following reaction:

$$CH_4 + 2O_2 \leftrightarrow CO_2 + 2H_2O \tag{9.5}$$

The above reaction is not desirable since it does not produce H_2. However, the released heat may be used as energy source for steam reforming in a coupled configuration based on the following reaction:

$$2CH_4 + H_2O + 1/2O_2 \leftrightarrow 2CO + 5H_2 \tag{9.6}$$

This process is named autothermal reforming.

Biological Processes Biological conversion of methane to methanol using methanotrophic bacteria is a promising alternative since the whole process takes place at ambient pressure and temperature. Large amounts of energy are not required as compared to the thermochemical processes (Ghaz-Jahanian et al. 2018). Other value-added products such as lactic acid, succinic acid, carotenoids, and fatty acids may be obtained by the biological conversion of methane using engineered microorganisms (e.g., methanotrophs, ammonia-oxidizing bacteria, and acetogens) (Hwang et al. 2018; Ge et al. 2014).

9.3.1.2 Liquid Effluent

Valorization of Volatile Fatty Acids (VFAs) In addition to biogas, AD may produce other intermediary and valuable products such as solvents and volatile fatty acids (VFAs), which can be marketed along with methane (Dogan et al. 2009). Moreover, the conversion of organic solid wastes into VFAs can be achieved by the acidogenic step within the AD process. The VFAs can be extracted and turned into valuable products such as methyl or ethyl esters for commercial purposes.

Among the main VFAs produced as a result of hydrolysis and acidification of the OFMSW, acetic acid and butyric acid can be found. As well as, ethanol as the main alcohol is obtained. These variations of by-products formation from acidification depend on the nature of feed and pH variations in the reactor. The pH has been considered as the most important parameter affecting product formation and product spectrum in the acidogenesis phase (Dogan et al. 2009).

Acidogenesis pathways using glucose as substrate can be seen in the Eqs. (9.7–9.10). It is clear that pH and hydrogen partial pressure could determine the choice of acidogenesis pathway. Firstly, ethanol-type acidogenesis is chosen at low operating pH for reducing VFAs production so as to avoid further pH decrease. Secondly, since the ethanol-type acidogenesis does not produce hydrogen, the acidogenesis pathway will shift to ethanol-type from acetic and butyric acid-type in order to avoid further increasing hydrogen partial pressure (Li et al. 2011). This shift easily occurs when pH is lower than 5.7 (Gottwald and Gottschalk 1985).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (9.7)$$

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3(CH_2)2COOH + 2CO_2 + 2H_2 \quad (9.8)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (9.9)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (9.10)$$

A study on mesophilic acidogenesis of protein-rich organic wastes revealed that VFAs were dominant products compared to lactic acid and alcohols. Among all VFA products, acetic and butyric acid accounted for 30–50% and 25–40% of VFA carbon, respectively; followed by propionic acid (10–15%), iso-valeric acid (10–15%), caproic acid (5–10%), and iso-butyric acid (3–6%) (Lü et al. 2008).

A liquid stream rich in VFAs can be effectively used as carbon source to remove nutrients in a biological nutrient removal (BNR) process. Producing a liquid with high VFAs concentration can also be desirable if the goal is, for example, to feed microbial fuel cells for direct bioelectricity generation. Likewise, the production of butanol as clean fuel could soon be of great interest since this alcohol has better energetic properties compared to ethanol. Its production and that of other solvents can also be triggered by a combination of high carboxylic acid concentrations, especially butyric and low pH values (Hüsemann and Papoutsakis 1988).

Algal Production Effluents from AD plants that treat nitrogen-rich substrates (e.g., swine and poultry manure) are rich in nutrients such as nitrogen and phosphorus (Magrí et al. 2017). However, further treatment is needed prior to their disposal to the environment. Alternatively, this nutrient-rich source can be incorporated into an algal production system, providing biomass used for biofuel production (e.g., biodiesel, biomethane, biohydrogen), animal feed, and so on (Sawatdeenarunat et al. 2016; Hagman et al. 2018). Algal bioenergy seems to have a promising future as solution to the global energy crisis and climate change. Algal biomass for bioenergy and biomaterial production offers some benefits, such as no competition with food crops for land or fresh water resources (Daroch et al. 2013), and great potential as AD feedstock in a recycling operation (Hagman et al. 2018).

Nutrient Valorization as Fertilizer Phosphorus as struvite ($MgNH_4PO_4$) can be obtained from anaerobically digested animal manure. This compound has a great potential as biofertilizer (Sawatdeenarunat et al. 2016; Hagman et al. 2018). Large amounts of dairy manure and the global interest in AD of such organic matter

suggest an important market for the production of struvite (Tao et al. 2016). The technical and economic feasibility of recovering nutrients through precipitation of struvite from wastewater treatment plants has been reported (Sawatdeenarunat et al. 2016; Hagman et al. 2018; Shu et al. 2006). Struvite crystals can be directly land-applied for crop cultivation. Among the advantages of its use as solid fertilizer, the following can be mentioned: easy application, low transportation cost, reduced bulky storage, avoided odor, and pathogen contamination issues (Tao et al. 2016).

9.3.1.3 Solid Digestate

Products from AD can vary according to the substrate characteristics. Digestate fiber, for instance, is obtained when lignocellulosic substrates are used. Substrates of nonfibrous nature (e.g., manure, domestic or industrial wastewater) generate digestate sludge which has potential as fertilizer. However, the pathogen content in such stream can limit that use, suggesting a subsequent disinfection step.

Disinfection Solar energy represents a promising alternative for the disinfection of water used for human consumption (Sanabria et al. 2005). This treatment may be applicable to sludges obtained from AD. The Research Group of Advanced Oxidation Processes for Chemical and Biological Treatments from the Universidad del Valle-Colombia, for example, has made important efforts to inactivate *E. coli* through homogeneous (Sambusiti et al. 2016) and heterogeneous (Arancon et al. 2013) catalytic operations that include the use of sun radiation as energy source.

Saccharification When lignocellulosic substrates are processed in AD, the biomass size and hemicellulose content are mainly reduced, resulting in an accumulation of cellulose and lignin (Sambusiti et al. 2016). This action destabilizes the recalcitrant biomass structure, allowing cellulose solubilization and glucose production in downstream processes (e.g., saccharification). However, even if amorphous cellulose is a suitable substrate for fermentation, digested fibers still present recalcitrant physicochemical characteristics (i.e., cellulose crystallinity, and lignin content) that negatively influence their enzymatic/microbial accessibility for further conversions (Sambusiti et al. 2016). From these observations, it is suggested to perform pretreatments (physical and/or chemical) followed by saccharification (chemical or enzymatic).

Cellulose can be used as an initial substrate to synthesize cellulose derivatives such as cellulose esters (e.g., cellulose nitrate, cellulose acetate), which are needed to produce optical media, filtration membranes, food and medical purposes. Cellulose ethers such as methylhydroxyethyl cellulose and carboxymethyl cellulose are commonly used as building materials and milk stabilizers, respectively (Surendra et al. 2015).

Composting Composting is a common and mature biological technology mainly used to stabilize organic waste and to convert such matter into compost for recovery. Notwithstanding, this compost is a relatively low-value product (Sawatdeenarunat

et al. 2016; Arancon et al. 2013). This technology has been considered as option for recycling the surplus of manure in livestock production systems, offering stabilized and sanitized end products for agriculture (Bernal et al. 2009).

Within this aerobic biological degradation technology, organic matter is processed to produce a stabilized material that no longer consumes oxygen or generates toxic metabolites (O'Callaghan 2016). However, management of pathogens has been suggested when animal-derived wastes are used (O'Callaghan 2016; Bezanson et al. 2014).

9.4 Biofactory Model-Based on Anaerobic Digestion

AD can be considered as a centerpiece of the biorefinery approach. This technology is used for waste remediation, biological pretreatment of biomass, and for producing bioenergy and bio-based products (Surendra et al. 2015). Figure 9.1 depicts the scheme of AD-based biorefinery concept.

Operating under optimal conditions (solids retention time, temperature, and pH), plant solubles and hemicellulose can be converted to biogas while cellulosic matter in the digested fiber can be turned into soluble glucose by enzymatic saccharification. Digestate fiber (i.e., solid residue with lignocellulosic matter), which is considered as a low-value product, can be potentially used as feedstock for biofuel and bio-based product generation. This fiber has been mostly used as soil additive or animal bedding. Monomeric sugars can be used as a source of biofuels (e.g., ethanol, butanol), organic acids and biopolymers (e.g., bioplastic). The insoluble solid residue (i.e., lignin and residual fiber) after enzymatic hydrolysis can be combusted for heat and electricity generation. This residue can be also processed into different bio-based products (e.g., lignin-derived products such as lignosulfonates are used as plasticizers in making concrete, binders in animal feeds, and solid fertilizer pelletization).

The liquid phase after separation of the solid residue from digestate or digester effluent is mainly composed of nutrients (i.e., nitrogen and phosphorous) (Sawatdeenarunat et al. 2016). This effluent can be used for macro- and microalgae production, providing nutrient removal and algal biomass that can be further treated for generation of other biofuels and bio-based products (Sawatdeenarunat et al. 2016; Surendra et al. 2015).

For volatile fatty acid (VFAs) production through AD, the operating conditions and substrate type have an important interactive effect on VFAs' yield and composition. Thus, the operating conditions should be adjusted taking into account both substrate type and the VFA of interest (Surendra et al. 2015). These compounds can be further processed into biogas, biologically/chemically converted into alcohols-based fuels, or they can be used in microbial fuel cells to produce electricity. Additionally, VFAs have great potential as carbon source in the biological nutrient removal during wastewater treatment and lipid production with oleaginous microorganism (e.g., microalgae, yeasts, molds) during biodiesel generation (Surendra et al. 2015).

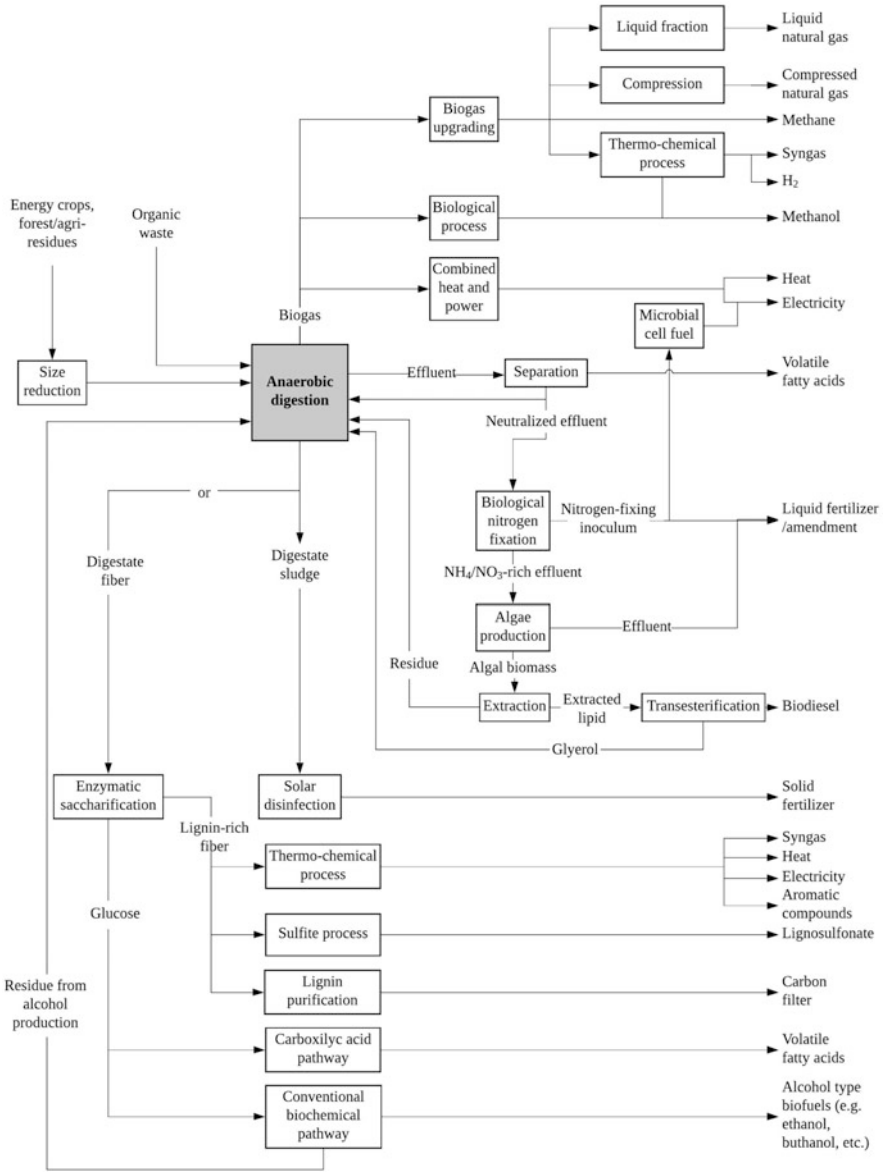

Fig. 9.1 Proposed AD-based biorefinery for producing biofuels and bio-based products modified from Surendra et al. (2015)

To sum up, AD can operate as a biological pretreatment technology coupled to other process units that involve the generation of high-value products (e.g., bioplastics, bioethanol, biobutanol, fungal protein, carboxylates precursors for solvents and/or fuels).

9.5 Challenges and Perspectives

Although the biorefinery concept has received attention in the last decade, much work is still on the road before a coupled system can efficiently prosper. AD system is mostly conceived as a method for reducing organic waste and producing energy. However, fluctuations in energy prices and environmental issues encourage the exploration of other sources of revenue and organic waste treatment. A techno-economic analysis of an anaerobic biorefinery that considers local situations would be worthy before its establishment.

Differences in the implementation of AD worldwide are mostly related to policy drivers, socioeconomic barriers, existing infrastructure, technology availability, and reliability (Vasco-Correa et al. 2018). It is highlighted that AD could be feasible at different scales, from small-scale household digesters to large-centralized biogas plants. The use of biogas at small scale can generate environmental, health, and social benefits related to burn cleaner fuel and stabilizing residues. In addition, the reduction of deforestation and the production of fuels and fertilizers may be simultaneously achieved.

9.6 Opportunities for Tropical and Developing Countries: Colombian Case

There are clear opportunities to implement AD for a developing country such as Colombia:

1. There is a huge amount of organic waste that is not environmentally safe managed and disposed as can be seen in Table 9.1.
2. Tropical conditions benefit biological processes: Climate ~ 24–30 °C, availability of natural and renewable resources (e.g., water, sun, etc.).
3. The necessity of diversification of energy sources due to climate change.
4. Positive social and financial conditions to invest in environmental projects.
5. International commitment to reduce greenhouse gasses. Colombia established an international commitment to reduce 20% of its projected emissions of greenhouse gasses by 2030.

Table 9.1 Energy potential from different organic wastes in Colombia

Sector/Activity	Annual amount of waste (Ton/year)	Energy potential (TJ/year)
Agricultural	71.943.813	331.646
Livestock	105.418.066	117.748
Urban solid waste	120.210	92
Green waste	44.811	318
Total	177.526.900	449.803

Source: Escalante (2011)

9.7 Challenges

Developing countries face some barriers for the implementation of AD, which can be mainly economic, regulatory, and institutional (Beck and Martinot 2004). Strategic actions are required in order to pave the road for a successful implementation.

To facilitate and make attractive the use of biogas as a renewable energy, a well-established policy that supports and promotes the development and use of renewable energy sources is required. As well as, the design, enforcement of instruments, and lines of action to implement unconventional energies are needed. To harness the benefits from biomass, an integral policy for the use and development of biomass is essential. It is also important to make feasible opportunities for technified rural development.

Barriers to overcome in the renewable energy sector (UPME 2015):

1. Wrong incentives, subsidies to conventional sources.
2. High costs and financing difficulties.
3. Market barriers, rules adjusted to conventional sources.
4. Imperfect competition, strong oligopolies based on conventional sources.
5. Externalities those are not valued and internalized.
6. Lack of information on renewable resources.
7. Lack of human capital with knowledge about technologies.
8. Technological prejudice, inclination for conventional technologies.
9. Increased transaction costs, research, negotiation, and execution.
10. Regulatory and institutional factors, schemes around the conventional.

There are also barriers to the biomass sector (UPME 2015):

1. The complexity of the regulatory framework and market mechanisms available to procure surplus delivery and marketing by small producers, even assuming the possibility of accessing the figure of cogenerator.
2. The absence of a tradition or knowledge among agricultural and agro-industrial sectors in order to take advantage of the energy potential they have with special reference to the case of their waste.
3. The high investment costs required to develop new cogeneration projects, and the lack of possible associated financing schemes.
4. The absence of signals from the market to encourage this type of project, as integral projects for the efficient use of waste and mitigation of environmental pollution.
5. The absence of industrial clusters in which the excess of heat produced by some industries could be used by another.
6. The untapped opportunity to generate electricity from some resources that are available in rural areas not interconnected or with deficient provision of electricity.

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Chapter 10
Recycling and Reuse of Ayurvedic Pharma Industry Wastes

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Abstract Ayurvedic medicines are of great importance to the health of individuals. The global market for herbal drugs are growing rapidly due to their less or no side effects, cost-effectiveness, availability, better patient tolerance, and clinical effectiveness. The traditional ayurvedic medicine manufacturing systems combine with the elements of modern technology to improve the production of reasonable drugs for human health care. Globally, ayurvedic pharma industries are among the leading pharmaceutical industries and they generate large volume of biodegradable wastewater, solid waste, and oil waste during processing and production. The waste from herbal pharmaceutical industry is a complex constitute of plant extracts, plant parts, toxic solutes, and heavy metal ions. They also have high BOD and COD concentrations so they can be discharged only after proper treatment otherwise may lead to environmental problems. Integrated microbial-vermifiltration, vermicomposting, and windrow composting are some of the cost-effective methods for the recycling of solid waste from herbal pharmaceutical industry. Microalgae are used for the treatment of waste water due to their potential to reduce the metal contamination and remove toxic substances. The resultant water after the treatment was clean enough to be reused for irrigation process. Biopharmaceutical oil waste and solid waste can be used also as substrate for fermentation process as well as for isolation of beneficial fungal strains for enzyme production.

Keywords Ayurvedic medicines · Herbal pharmaceutical waste · Integrated microbial-vermifiltration · Vermicomposting · Windrow composting

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10.1 Introduction

Ayurveda is a traditional medicinal system which was originated and shaped in the ancient lands of India. The term Ayurveda is a combination of two Sanskrit words: "Ayu," which means life and "veda" means knowledge. Thus the literal meaning of ayurveda is "The science of life." This oldest medicinal system was thought to have originated in the Vedic times around 5000 years ago. The primary focus of ayurveda is to heal and maintain the quality and longevity of life (Kumar et al. 2016; Raj et al. 2011). Various types of ayurvedic medicines are used for the human health care. They are herbal teas, decoctions, infusions, capsules and powders, tinctures, infused oils, ointments, creams, lotions, arishtas (fermented decoction), and asavas (fermented infusions), etc. Arishta, asava, churna, rasayana, and taila are some of the examples of conventional drugs of ayurveda. These products are mainly composed of herbal combinations and minerals. Alcoholic preparations such as arishtas and asavas are made by allowing the herbal juices/decoctions to go through the fermentation process with the addition of sugar. These alcoholic medicaments have several advantages, like enhanced therapeutic effectiveness, better shelf life, and improvement in the productivity of extraction of drug molecules from the herbs (Sekar and Mariappan 2008). Plants are the essence of ayurveda and approximately 90% of herbal preparations are plant based. Therefore, the plant-based formulations play a vital role in the ayurvedic healing process. Most of the ayurvedic medicinal preparations are polyherbal, with a combination of 3–30 plants involved. Ayurvedic plants have a stronger action on the body than either food or spices. Plants have chemical compounds known as phytochemicals, which are naturally occurring and biologically active. These are used as traditional medicines in ayurvedic treatments (Kumar et al. 2017).

Ayurveda is considered as science of healthy life which is based on the principle of maintaining a balance between the interrelated relationships within the body and mind. Ayurvedic medicines aid to interconnect the body's constitution and mind (Kumar et al. 2016). Using these concepts, ayurvedic physicians prescribe individualized treatments, including compounds of herbs, diet, exercise, and lifestyle recommendations. According to World Health Organization (WHO), 65–80% of World's population depends on traditional medicine to promote good health and prevent illness (Sasidharan et al. 2011; Das et al. 2016). Ayurvedic medicine is one of the world's popular and oldest healing system and the demand for herbal drugs was increased many folds at the global level. This is mainly due to the adverse impact of synthetic drugs. Herbal medicines do not have any side effects and proved to be safe for long-term use. Cost-effectiveness and relative ease of availability make herbal medicines a popular choice among treatment for various diseases (Kumar et al. 2016). Therefore, people are now moving toward traditional herbal-based medicinal system. Other factors such as clinical effectiveness, better patient tolerance, and reduction in offensive factors have also helped to gaining the popularity of herbal medicines. The global market for herbal drug is increasing rapidly (Das et al. 2017). Herbal medicines are usually thought to be safe due to its natural origin.

However, the plants used for preparation of herbal drugs should be evaluated by modern scientific methods because case reports indicate that serious side effects and pertinent interactions with other drug can appear altering physiology and these changes can be reflected in abnormal test results. Evaluation of herbal plants helps to demonstrate their usefulness and to avoid the use of useless and toxic herbs (Khan et al. 2016). Ayurveda has two basic aims; first, to preserve the health of healthy person and second is to treat illness and disease. In this sense, there are two types of medicines in Ayurveda; those which promote resistance and vitality of the body and those which cure disease (Shroff 2017).

The ayurvedic traditional preparations comprise medicinal plants, minerals, organic matter, etc. Many drugs are developed with phytochemicals or taking phytochemicals as active principle (Inamdar et al. 2008). In most countries, complex mixtures of one or more plants, which are used for the preparation of standardized herbal medicines for the management of various diseases. These are known as phytotherapeutic agents or phytomedicines. WHO recognized that active ingredients from plant parts or plant materials in the crude or processed state plus certain excipients, i.e., solvents, diluents, or preservatives are the main ingredients of herbal drugs. Usually, the active compounds responsible for their pharmacological action are unknown. One of the main characteristics of phytotherapeutic agents is the fact that they normally do not possess an immediate or strong pharmacological action. Due to this reason, phytotherapeutic agents are not used for emergency treatment. However, herbal medicines possess wide therapeutic use and greater acceptance by the population. The standardized preparations of herbal drugs are normally available in the market as liquid, solid (powdered extract), or viscous preparations. They are prepared by different extraction methods such as decoction, maceration, percolation, or distillation (for volatile oils). Fluid extracts are mainly prepared by using ethanol, water, or mixture of ethanol and water. Solid or powdered extracts are produced by evaporation of the solvents used in the process of extraction of the raw material. The concentrated phytotherapeutic agents have much more better therapeutic efficacy (Calixto 2000).

10.2 Ayurvedic Pharma Industry Wastes

The manufacturing process is one of the key steps in herbal pharmaceutical industry. During the production of herbal drugs using plant parts, such as roots, stems, nuts, barks, seeds, flowers, and fruits, creates large amount of solid wastes. Wastes from ayurvedic pharmacies are mainly organic in nature. Different kinds of wastes are produced from the ayurvedic industries, such as solid waste, wastewater, medicinal oil, etc. Pharmaceutical industries are one of the highly polluting types of industries among the others and they generate strong and high Chemical Oxygen Demand (COD) wastewater along with hazardous waste (Das et al. 2017).

10.3 Solid Wastes

Solid wastes generated during the processing and production of herbal medicines are enormous in volume and need proper treatments before the disposal, otherwise may cause environmental problems. Huge quantities of spent wastes are produced during the manufacturing of ayurvedic products, which are recalcitrant in nature. The manufacturing process of herbal pharmaceutical medicines involves the washing (cleaning) of herbs viz.; flowers, roots, stems, leaves, barks, nuts, fruits, seeds, tubers, leaves, and resins to remove the dust and soil adhered to the material. Herbs are dried after this cleaning and subjected to cutting or powdering through ball mills as per the requirement. In decoction preparation, a portion of the cleaned plant parts is boiled in water for specific time. After decoction preparation plant parts are generated as solid waste. This solid waste is very sharp, hard, and thorny in nature and it cannot be used as animal feed. The dried solid waste also cannot be used as a fodder (like leguminous plants), because of the very sharp and prickly nature. The solid wastes from ayurvedic industries are recalcitrant in nature. So it is important to develop a better technique for reducing the environmental issues associated with these wastes (Das et al. 2017). Ayurvedic pharma industries (processing units and product manufacturing units) produce a large quantity of spent wastes after pre-processing and/or distillation or extraction of active principles from raw materials. So this can be considered as an emerging pollutant for terrestrial environment. If proper treatment and disposal are not made, the solid waste generated from these herbal manufacturing industries create issues of all types of environmental pollution and spoil esthetic sense of local habitats. Different steps involved in production of ayurvedic medicines and generation of both solid and liquid waste are shown in Fig. 10.1.

Inappropriate disposal of plant-origin wastes pose serious environmental effects. In majority of cases these wastes are just dumped openly either at landfill sites or at open space nearby to industrial areas, but this method is not a proper way for disposal. This will lead to the nutrient enrichments in surface water bodies, emission of greenhouse gasses, nitrate leaching to groundwater, proliferation and breeding of disease vectors, etc. The best options for sustainable solid waste management programs are recycling, reuse, and resource.

10.3.1 Vermicomposting

Composting is a decomposition process of organic matter by microorganisms under controlled conditions. Fungi, actinomycetes, protozoa, nematodes, annelids, arthropods, etc. are examples of decomposers or detritus feeders available in nature, which have the capacity to decompose the complex organic substances of wastes as well as enhance the quality of end products. Recycling of organic wastes generated from different sectors of human society can be done with the help of earthworms.

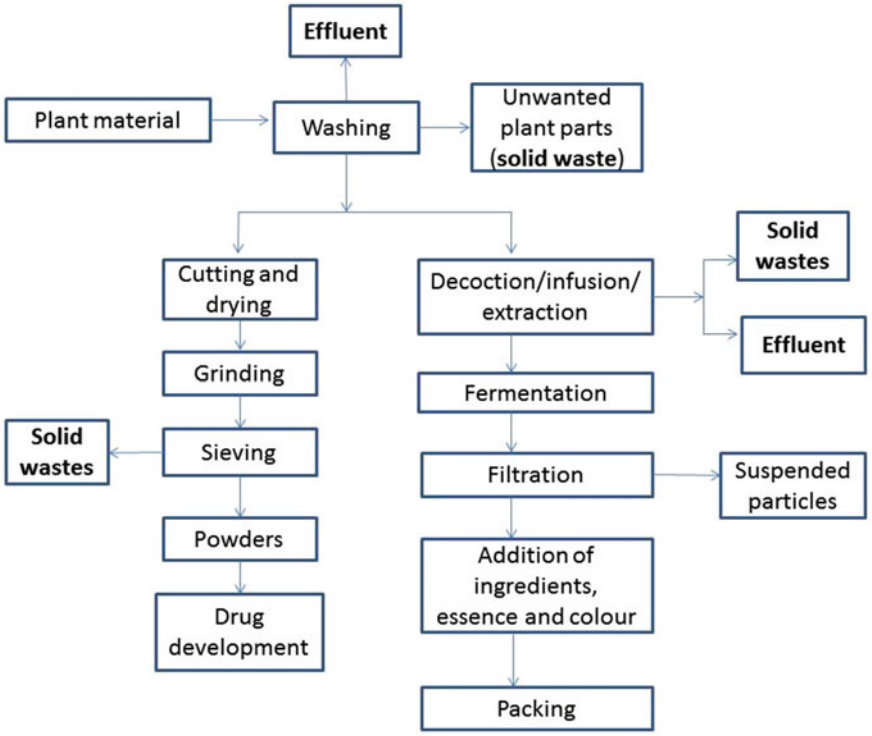

Fig. 10.1 Schematic representation of the production of ayurvedic medicines and generation of waste

Table 10.1 Different categories of earthworms

Earthworm species	Characteristics
Epigeics (humus feeders)	Surface dwelling nature
Anecics (geophytophagous)	Soil dwelling nature and construct vertical tunnels
Endogeics (geophagous)	Soil dwelling organisms which construct horizontal branching burrows

Earthworms are considered as potential decomposer and utilization of earthworms for waste decomposition is called vermicomposting (Singh and Suthar 2012). The growth of earthworms in organic wastes has been termed vermiculture, while the processing of organic wastes by earthworms is known as vermicomposting. Table 10.1 represents the details of three main categories of earthworms.

The epigeics are most suited to vermicomposting (Abbasi et al. 2009). Widely used earthworms for vermicomposting include *Megascolex mauritii*, *Eisenia fetida, Eudrilus eugeniae, Perionyx excavatus, Lampito mauritii, Eisenia andrei, Lampito rubellus*, and *Drawida willis* (Manyuchi and Phiri 2013). In vermicomposting,

stabilization of organic material is achieved through the joint action of earthworms and microorganisms. Microorganisms are involved in the biochemical degradation of organic matter. And the earthworms play an important role, which drives the whole process, preparing the substrate and altering the biological activity (Das et al. 2017). When compared to conventional thermophilic composting system, the vermicomposting has several advantages in terms of process time, nutrients recovery, microbial richness, and phytotoxicity. The operating conditions for traditional composting and vermicomposting methods are different. So the composted products from these composting methods are very different (Singh and Suthar 2012). Nowadays vermicomposting technology is mostly preferred because it accelerates the process and time taken for composting is reduced considerably. While the normal composting method takes longer time for stabilization. Another advantage of vermitechnology is the easy and efficient eradication of pathogens and toxic substances present in the substrates. Vermicomposting is an eco-friendly approach, which is based on soil-based beneficial microorganisms such as lactic acid bacteria, yeast, phototrophic bacteria, and naturally occurring microorganisms in soil and cattle dung, etc. Therefore, the substrate for vermicomposting generally mixed with cattle dung and garden soil and pre-decomposed before vermicomposting. In vermicomposting, cow dung was mainly used as vermibed or feed material for earthworms. In contrast to normal composting method, vermicomposting does not add exothermic reactions; so, there is no measurable rise in temperature in the vermireactors. During vermicomposting, an aerobic condition is maintained with the help of earthworms and the solids in the organic waste are ingest and convert them into vermicast. Vermicomposting increases the manurial value of waste and also reduces the total volume and particle size of the biomass waste. Moreover, the presence of micro- and macronutrients is usually higher in vermicompost than in the traditional compost and inorganic fertilizers. So vermicompost is a better supplement to stimulate plant growth (Das et al. 2017; Abbasi et al. 2009). Vermicompost is an odorless, dark brown bio-fertilizer resultant after the vermicomposting process (Manyuchi and Phiri 2013).

Solid waste from herbal pharmaceutical industry mainly consists of large size stalks, stems, leaves, barks, tubers, nuts, and roots. Hence it chopped before subjecting it to vermicomposting. So the waste material was cut into small pieces is the first step in vermicomposting. However, too fine pieces are not suitable as they get compacted. Size reduction will induce fast vermicomposting, thus the vermiconversion time period is reduced. Das et al. (2017) investigated the vermicomposting of recalcitrant solid waste from herbal pharmaceutical industry. In this study, solid waste collected from a herbal pharmaceutical industry is subjected to routine physicochemical analysis and heavy metal analysis as per the standard methods. Then this recalcitrant solid waste was dried in shade, cut into smaller pieces, and used for the vermicomposting process. Cattle dung was also dried in shade before its use and the earthworm species used was *Eudrilus eugeniae*. Herbal industry solid waste, cattle dung, and soil were mixed in 1:1:1 ratio and kept for pre-decomposition. This pre-decomposed waste was then placed into earthenware vermibeds and 15 numbers of earthworms were introduced in it. The beds were

kept in shade away from direct sunlight and rainwater entry and the beds were sprinkled with water to maintain the moisture content. Das et al. (2017) found that the resultant vermicompost of herbal pharmaceutical solid waste depicted efficient porosity of 80.487%, water holding capacity of 87.5534%, bulk density was around 0.9667 gm/cm^3, and the moisture content was around 18.6%, i.e., well within the applicable range. These results show that the resultant vermicompost is suitable for agricultural activity. The study concluded that herbal pharmaceutical waste is a good substrate with high percentage of volatile solids of total solids demonstrating its degradable nature. Hence it can be easily vermicomposted.

Kumari et al. (2011) selected *Eudrilus eugeniae* species of earthworm, for vermicomposting of herbal industry waste. *Eudrilus eugeniae* is an epigeics or humus feeding earthworm, can tolerate temperatures ranging from 0 to 40 °C. This species of earthworm is extensively used for the vermicomposting because of its voracious appetite, high rate of growth, and reproductive ability. Waste used for this study is a mixture of various remains of medicinally important herbs after industrial operation and the vermibeds were made using a mixture of herbal waste and cow dung (1:1) in comparison with the use of cow dung alone as substrate. Industrial waste used in this study comprises different medicinally important plant species (Table 10.2).

Kumari et al. (2011) found that resultant vermicompost, from the vermibed which contain both herbal waste and cow dung as substrate, strongly influenced the growth of pea (*Pisum sativum*) and marigold plant (*Tagetes erectus*).

Singh and Suthar (2012) used the solid waste from the herbal pharmaceutical industry that was a mixed type and containing spent material after extraction/distillation of herbs and unused part of the plant. They used *Eisenia fetida* for vermicomposting process. In majority of previous studies *E. fetida* was used as candidate species for vermicomposting process because it can tolerate wide range of pH, temperature, and moisture. It can also stand for a wide range of putrescible substances and bio toxic compounds. In this study, the herbal pharmaceutical industrial waste (HPIW) was collected from spent material disposing unit of The Himalaya Drug Company, Dehradun which is one of the leading herbal pharmaceutical product manufacturing or processing unit in India. The waste was of mixed type mainly constituting spent material after extraction/distillation of herbs and unused part of the plant. The waste mixture acts as bedding as well as feed for the composting earthworms. For vermicomposting experimentation, Singh and Suthar

Table 10.2 Important constituents of industrial waste

Plant species	Parts	Percentage (%)
Punica granatum	Fruit	24
Aegle marmelos	Fruit	22
Woodfordia fruticosa	Flowers	14
Berberis aristata	Root	13.5
Symplocus racemosa	Bark	12
Andrographis paniculata	Stem	10
Salmalia malabarica	Bark	3.5

(2012) used 300 g waste mixture (dried material) was filled in plastic circular containers. The waste mixtures were moistened with distilled water to maintain moisture content for initial decomposition of waste mixtures. For the initiation of microbial degradation and softening of waste mixture, these bedding were kept for 1 week. In this study, 20 earthworms were collected from the stock culture and released into each different container containing 300 g of substrate material for vermicomposting. The moisture content (55–65%) was maintained throughout the experiment by periodic sprinkling of sufficient quantity of water. The experimental containers were placed in a humid and dark place at room temperature. The samples (homogenized) were collected at 0, 10, 20, 30, 40, 50, and 60 days from each container and these samples were subjected to measure the changes in chemical characteristics of waste mixture during vermicomposting. Singh and Suthar (2012) found that the earthworm caused significant changes in the chemical characteristics of waste mixtures during vermicomposting process. The resultant worm-worked material was more stabilized, odor free, and dark brown substance with high range of plant available forms of soil nutrients. After the vermicomposting process, there was significant reduction in pH value of all waste mixtures. The results of this study clearly suggest that HPIW may be a valuable source of nutrients for sustainable land restoration program if proper technology is applied to recover the valuable nutrients from such industrial wastes. The product showed significant increase in all chemical characteristics of wastes and C:N also reduced up to its acceptable limit for agronomic uses. Singh and Suthar (2012) suggested that vermicomposting can be an efficient and better tool to convert the noxious community wastes into value-added products for sustainable human development.

Vermicompost and vermiwash are rich in nitrogen (N), phosphorous (P), potassium (K), and trace elements. Their potential use as bio-fertilizers has been investigated in many studies as well as their impact on soil properties. Vermiwash is a leachate that is obtained during the vermicomposting process and is dark brown in color which is also known as liquid-bio fertilizer. So vermiwash can also be used as a foliar spray because it acts as a pesticide in sustainable agriculture. The vermicomposts are also termed as vermicasts because they are expelled as casts from the earthworm gut (Manyuchi and Phiri 2013; Manyuchi 2013). Manyuchi and Phiri (2013) investigated the effects of vermicompost and vermiwash on various plants such as cow pea, soy bean, maize, and marigold. They found that vermicompost and vermiwash influence the growth of plants. Vermicomposting results in earthworms, vermicompost, and vermiwash as products. The vermiproducts can be used as bio-fertilizers while the earthworms can be used for further vermicomposting.

10.3.2 Windrow Composting

One of the most commonly used methods of composting is the windrow process. Windrow composting operation is relatively simple and requires little attention other than monitoring temperature and moisture, which involves stacking organic wastes

into long windrows that are turned periodically. Haq et al. (2016) studied the recycling of herbal pharmaceutical solid waste (HPSW) by windrow composting process for its conversion into a value-added product. Resultant composting material showed a pH 7.4 which was within the suitable limit 6.9–8.0 and hence the application of these products to soil may support the soil microflora. The C: N ratio decreased rapidly and stabilized to 10.42, which indicated the good biological stabilization and the application of the compost to cropping systems. They found that the windrow composting method is an environmental friendly approach, in which the recycling of herbal pharmaceutical solid waste produces an acceptable, nontoxic, and nutrient-rich substrate for agronomic purposes. This process also reduces pollution with respect to the wastes from ayurvedic pharma industry.

10.3.3 Spent Black Pepper: A Thrown Away Residue

Large scale production of ayurvedic herbal drugs generates significant quantities of processed wastes. The yield and purity of piperine isolated from spent black pepper (*Piper nigrum* L.) was studied by Rakesh et al. (2015). Piperine is a major constituent of black pepper and the first amide isolated from genus Piper, possesses diverse pharmacological activities including central nervous system depressant, antipyretic, analgesic, anti-inflammatory, and antioxidant properties. Several studies reported that piperine enhances digestive power and appetite and also play a role in healing cold and cough, dyspnoea, disease of throat, colic, dysentery, etc. (Vyas et al. 2011). Rakesh et al. (2015) used spent black pepper for their study. It is a "thrown away residue" from ayurvedic industry and these spent materials are important to their value-added components. In traditional ayurvedic medicinal system, the fruits of black pepper are commonly used. In this study, the spent pepper collected from a major ayurvedic industry has been screened for the presence of high-value volatiles, active principles, and extracts and they found that these contain 1.0–2.5% of volatile oil, 5–9% of alkaloids. Among which piperine, chavicine, piperidine, and piperetine are major biologically active compounds. In this study, the complete chemical evaluation of essential oil, oleoresin, and piperine recovered from both raw and spent black pepper was performed and the results obtained were lead to the conclusion that piperine content and purity were not much affected by conventional ayurvedic processing. Hence, this spent residue can be further processed for recovering such value-added products. Change in the concentration of aromatic constituents clearly supported that essential oil from spent pepper has commercial value close to that of raw pepper. This study helps to find out a better pathway for the utilization of thrown away residue from ayurvedic industries.

10.4 Liquid Waste

Ayurvedic industries generate a large volume of wastewater during processing and production. The moderately concentrated wastewater from these industries is biodegradable in nature and possesses high value of BOD (Biochemical Oxygen Demand), COD, and total solids. Due to the high strength, and acidic nature of wastewater, it cannot be discharged directly into the surface water, as they putrefy very fast and lead to environmental complications. Untreated pharmaceutical wastewater discharged into the natural environment leads to health hazards to existing flora and fauna. Therefore, treatment of the effluents is required to bring down the concentration of pollutants to suitable limits, before they are finally discharged into the natural systems. Hence, a treatment methodology is required which is easy to operate and can be easily accepted by the local ayurvedic manufacturing units (Vyas et al. 2011; Vanerkar et al. 2015). Herbal pharmaceutical wastewater is moderately strong with COD, BOD, and Suspended Solids (SS) concentration in the range of 21,960–26,000 mg/L, 12,200–15,660 mg/L, and 5460–7370 mg/L, respectively. Physicochemical treatment using conventional coagulants in combination with anionic/cationic/nonionic polyelectrolyte is one of the treatment technologies, which seemed viable for the reduction of organic load in the herbal pharmaceutical wastewater. An attempt has been made to study in detail the treatment of the herbal wastewater using conventional coagulants individually, in combination and also with the addition of anionic/cationic/nonionic polyelectrolyte. The complete treatability study of herbal pharmaceutical wastewater by suitable combination of primary physicochemical, secondary, biological aerobic suspended-growth activated-sludge process, and tertiary advanced oxidation process was done by Vanerkar et al. (2013). They found that Fenton's oxidation method is highly suitable to treat the herbal pharmaceutical wastewater as compared to radiation-induced hydroxyl radical generation processes. In Fenton's oxidation process, hydroxyl radicals can be introduced into the wastewater mixture in any concentration, at any rate, simply by varying the quantity of H_2O_2 and other catalyst, thus making it a much more versatile tool.

10.4.1 Integrated Microbial-Vermifiltration Technique

The use of earthworms as bio-filters in wastewater treatment is known as vermifiltration (Manyuchi and Phiri 2013). Nowadays integrated microbial-vermifiltration technology is used for the treatment of ayurvedic liquid effluents. In this technology, ayurvedic effluent was pretreated with a microbial consortium and later fed to a vermifiltration unit. Das et al. (2015) mainly focused on the development of treatment methods for ayurvedic liquid effluents by integrating microbial pretreatment and vermifiltration. *Eisenia fetida* earthworms were used for this study, which was collected from the composting units of Kerala Agricultural University (KAU), Mannuthy. Liquid effluents were collected from ayurveda industry

and protease and lipase producing strains were isolated from these effluents by using standard plating techniques. A protease (*Bacillus* sp.) and lipase (*Bacillus* sp.) producing strains were selected for their study. Removal of oil portion from the effluent was done by using broken brick pieces of different sizes which were filled in a column and the effluent was passed through the column at a minimum flow rate. In this study, the bedding material for vermicomposting was mainly consisting of cow dung, straw, and vegetable scraps, which are used after suitable sterilization and placed in the uppermost layer of vermicompost. Vermifiltration is an extension of vermicomposting and also known as lumbrifiltration. Dissolved and suspended solids get trapped in the vermifilter bed and then they are digested by complex biodegradation process. Both aerobic and anaerobic microbes present in the soil also promote the degradation of organic and inorganic matter from the waste. Microbial-based degradation process and vermiculture-based process were found to work simultaneously in the treatment of domestic as well as industrial wastewater. In this study, ayurvedic effluent was treated with protease and lipase producing strain (*Bacillus* sp.) and also with a mixture of protease and lipase producing strains. After inoculation, they were incubated at 37 °C at 100 rpm in rotary shaker for 72 h. This pretreatment mainly focused on the protease and lipase action which helps in degradation of proteins and lipids present in the effluent and make it readily available for consumption by microbes and earthworms. The results from their study mentioned that organic matter in liquid effluent was significantly reduced during the treatment with enzymes and microbes. Thus, BOD level for vermifiltration process also decreased. Das et al. (2015) found that organic wastes, solids, and heavy metals are ingested and absorbed through earthworm's body wall and degraded. BOD, COD, total dissolved solids (TDS), and the total suspended solids (TSS) from wastewater were removed by this action and there was no sludge formation in the process. The resultant water was found to be odor free, clean, and disinfected enough to be reused for irrigation. In this study, the final vermifiltered water showed a significant reduction in COD by 98.03%, BOD by 98.43%, TSS by 95.8%, TDS by 78.66%, and oil and grease by 92.58%. Based on these results, they concluded that integrated microbial-vermifiltration technology is a decentralized and cost-effective method which can be applied to treat both domestic and industrial wastewater. A drastic reduction in different effluent parameters, such as BOD, COD, TDS, TSS, Oil and grease, was observed in this study. Pretreatment of effluent with bacterial consortium at optimum conditions showed maximum reduction in the above mentioned parameters. The resulting vermifiltered water is clean and disinfected enough to be reused for irrigation purpose.

10.4.2 Electrocoagulation

Electrochemical techniques are helpful for prevention and remedy pollution problems due to strict environmental regulations. Electrocoagulation (EC) process is a simple and efficient method for the treatments of various industrial wastewaters.

When compared to the conventional methods, the electrocoagulation process possesses some advantages such as easy to operate, less retention time, lower operating costs, absence of adding chemicals, rapid sedimentation of the electrogenerated flocs, and less sludge production and requires a simple equipment. Usually, biological methods are mainly used for the treatment of ayurvedic wastewater. Harshananda and Neera (2017) study the effectiveness of electrocoagulation on ayurvedic pharmaceutical wastewater. In this study, the optimum conditions obtained for EC treatment were pH 6, time 60 min, electrode gap of 0.5 cm, and current density 119.65 A/m^2 and the corresponding COD removal is of 90%. Though the COD removal percentage is high, the effluent COD value does not satisfy disposal standards. The BOD removal rate is also relatively low. Hence they concluded that electrolytic treatments are suitable only as a pretreatment for ayurvedic pharmaceutical wastewater. It can also be observed that the addition of electrolyte to reactor volume increases the treatment efficiency to a certain extent. Harshananda and Neera (2017) concluded that electrocoagulation provides high turbidity removal and also effective in the removal of dissolved matter by charge neutralization and electrostatic interaction.

10.4.3 Treatment of Wastewater Using Algae

Treatment of wastewater using algae for reducing the chemical and organic load has been studied for over 50 years. They are also capable of reducing the metal contamination in aquatic systems. Vanerkar et al. (2015) analysed the various physicochemical characteristics such as pH, COD, BOD, total solids, sodium, potassium, and heavy metals for the judgment of toxicity of herbal pharmaceutical wastewater after its treatment with micro green algae *Scenedesmus quadricauda*. When compared with the conventional tertiary treatment procedures, algal treatment of wastewater can offer an ecologically secure, cheap, and efficient way to remove nutrients and metals. Treatment of effluent by algae is mainly mediated through a combination of nutrient uptake, elevated pH, and high dissolved oxygen concentration. Physicochemically treated effluent (PCTE) and biologically treated effluent (BTE) of herbal pharmaceutical industry were used for this study. Tests for growth, survival rate, and synthesis of metabolites in PCTE and BTE using *S. quadricauda* were conducted. *S. quadricauda* is a common freshwater species of the chlorophycean group, widely distributed in subtropical parts of India. *S. quadricauda* is a good pollution indicator because it is sensitive to polluted wastewater; hence they selected it for their study. These are mainly occurring in canals, rivers, lakes, reservoirs, and other watersheds. In this study, detoxification test was performed for different wastewater concentrations (20, 40, 60, 80, and 100%) of untreated, physically treated, and biologically treated wastewater. Cultured *S. quadricauda* algae were added into different wastewater concentrations and incubated for 21 days. After completion of the incubation period, optical density (OD) was measured at 660 nm using a spectrophotometer to find biomass. The growth rate of *S. quadricauda* in

PCTE gradually increased throughout the incubation in 10, 20, and 30% dilution and no growth of *S. quadricauda* was found in 40–100% dilution of PCTE. Results showed that algae can remove toxic substances by accumulation, absorption, extracellular secretion, and enzymatic degradation. Algae can slowly utilize the herbal pharmaceutical wastewater as nutrient source and make it available for reuse. Vanerkar et al. (2015) concluded that, if a suitable algae like *S. quadricauda* is added to natural discharge of pharmaceutical wastewater, it will help in reducing the toxicity and facilitate recycling and reutilization of polluted water. They also highlighted that, differential tolerance of this microalga to the effluents shows that there is great scope for industrial wastewater treatments.

10.5 Medicinal Oil Waste

The biopharmaceutical oil waste is used as a substrate for fermentation and it also acts as a source of microorganisms for enzyme production. These roles help to minimize the environmental problems associated with biopharmaceutical oil waste. The usage of biopharmaceutical oil waste, on the one hand, provides good and alternative substrate for enzyme production and, on the other hand, helps in solving pollution problems. Oil rich waste from biopharmaceutical industry can use as substrate for lipase production. A comparative study of lipase enzyme yields by solid state fermentation (SSF) and submerged fermentation (SmF) was performed by Mohanasrinivasan et al. (2009). Lipases, one of the prominent industrial enzymes, act over a wide range of pH and temperature. It possesses high specificity, does not require cofactors, and can catalyze a wide range of reactions. In this study, three fungal colonies were isolated from biopharmaceutical oil waste. These wastes were collected from "Oushadhi" (The Pharmaceutical Corporation (IM) Kerala Ltd). More than 450 products were manufactured from this industry and which include asavas, arishttas, dhravams, choornams, khashayas, thailas (medicated oil), and chyavanaprasams, etc. The manufacturing steps of these ayurvedic products produce a vast amount of waste materials both oily and non-oily. The present study utilized biopharmaceutical oil waste as substrate for SSF which consisted of plant matter containing cellulose, medicinal oil, etc. The fungal strains were also isolated from this substrate (oil waste). After incubating the substrate in the open air environment for a week, the substrate containing plate was showed the growth of fungal strains. The pure colonies from these oil containing wastes were then used as inoculums for both solid state and submerged fermentation of lipase enzyme. The three fungal strains were identified as *Aspergillus* sp., *Trichoderma* sp., and *Penicillium* sp. In SSF, the oil waste itself was used as a substrate and it was enriched with $(NH_4)_2SO_4$ 5.0 g/l; Na_2HPO_4 6.0 g/l; KH_2PO_4 2.0 g/l; $MgSO_4.7H_2O$ 3.0 g/l; and $CaCl_2$ 3.0 g/l at pH 6. The production media (pH 6) used for SmF manily includes glucose-10, peptone-20, NaCl-5, and yeast extract-5 (g/l). Mohanasrinivasan et al. (2009) found that enzyme yields were higher in SSF when compared with SmF. They concluded

that utilization of biopharmaceutical oil waste, on the one hand, provides alternative substrate and, on the other hand, helps in solving pollution problems.

10.6 Conclusion

Ayurveda is a comprehensive science and the ayurvedic medicines are important to the human health care system. The rapidly growing trends in herbal drugs lead to the development of ayurvedic industries. Ayurvedic pharmaceutical industries have become the major contributors to water pollution nowadays. Biological as well as chemical methods are available for reducing the negative effects of ayurvedic pharma industrial wastes in environment. Vermicomposting, vermifiltration, and windrow composting can be used as herbal pharmaceutical waste management strategy and at the same time these techniques access bio-fertilizers which are environmentally friendly. Vermicomposting results in earthworms, vermicompost, and vermiwash as products. The vermiproducts can be used as bio-fertilizers while the earthworms can be used for further vermicomposting. The resulting effluent from vermifiltration becomes highly nutritious and can be reused for irrigation purpose. Biopharmaceutical oil waste can be used as substrate as well as for isolation of beneficial fungal strains for enzyme production. Wastewater treated by electrocoagulation gives clear, colorless, and odorless water.

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Chapter 11
Production of Unicellular Biomass as a Food Ingredient from Agro-Industrial Waste

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Abstract Humanity has been used microbial biomass for food production and now; for biofuels, drugs, and other useful compounds. Different microorganisms are employed in the production of biomass ranging from bacteria, yeast, fungi, and algae which are used to produce food, bioactive compounds, enzymes, hydrolysates, among others. Due to the accelerated population growth in the world and the need to meet its nutritional requirements, the search for alternatives that help to solve this social problem is one of the most pressing tasks.

Several studies have demonstrated the nutritional value of microbial biomass related to a high protein content, an excellent source of vitamins and minerals necessary for a quality diet. The production of unicellular biomass has been carried out through submerged and solid-state fermentations. For the production of biomass, the design of various culture media has been considered, where different sources of carbon, nitrogen, pH, and aeration level have been some of the evaluated variables that favor the yields of protein production. The use of various agro-industrial waste

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as carbon source in the biomass production could contribute in solving a problem of accumulation of waste causing soil contamination. This chapter describes the state of the art of unicellular biomass production, microorganisms used, types of fermentation, carbon sources used, agro-industrial residues used as substrate, characteristics of biomass produced and other related topics.

Keywords Unicellular biomass · Microbial protein · Agro-industrial waste · Fermentation

11.1 Introduction

The use of biomass has been reported since ancient times. There are records of the use of yeast to produce food since 7000 BC. Currently the use of this material has different purposes, besides the production of food it can be used to produce biofuels as a good option to replace fossil fuels (which are one of the main causes of global warming), and other useful compounds and medicines as well (Zabed et al. 2019; Pérez-Torrado et al. 2015).

Biomass is a product derived from biological material such as plants, animals, and microorganisms, which is composed mainly of carbon, hydrogen, oxygen, and nitrogen (Balaman 2019; Houghton 2008). From another perspective, bioactivity is the process of catalysis to transform a raw material into a desired product, either a protein or some other useful compounds. Microorganisms are employed to produce a specific product or products from macromolecules from the culture medium (Lübbert 2017).

The biomass production by non-photosynthetic microorganisms is affected by several factors; the substrate used in the fermentative process, treatments to modify the availability of the substrate to the microorganism, the bioreactor utilized, operational conditions, and downstream processing for separation and purification (Moo-Young and Gregory 1986; Reihani and Khosravi-Darani 2018).

The microbial biomass has different uses in a wide range of applications, for example; in food industry can be used as a Single-Cell Protein (SCP), which is expected to become an auxiliary source of food in the near future. Henceforth; SCP can help to suffice the food demand related to the accelerated growth of the population. It is therefore necessary a twofold increase in the amount of food produced, since by 2050, it is estimated that the number of inhabitants on the planet will increase from 7 billion to 9 billion (Spalvins et al. 2018; Rasouli et al. 2018; Objetivo 2011). Another application is the extraction of various compounds, for example, lipids, carotenoids, oligosaccharides, free polyphenols, and folates which are added to foods to increase their nutritional value (Catchpole et al. 2010; Rai et al. 2018).

Moreover, biorefinery is one of the fields where the use of biomass has had a great boom, promoting the research in the development of analogous fuels, which are expected to replace fossil fuels, one of the main factors that related to the climate

change on the planet. For example, different yeasts use carbon sources to modify the composition of fatty acids as well as the accumulation of triglycerides present in the cell (Faife-Pérez et al. 2012; Shields-Menard et al. 2018). Oleaginous yeasts can reach between 40% to 70% of lipids in their cellular structure, allowing be a good raw material for biodiesel conversion and its implementation as a fuel.

11.2 Fermentation

One of the main forms to produce biomass is through fermentation. Biotransformation via fermentation is the main biological procedure for biomass production. Under optimal conditions, microorganism can double the amount of biomass in a short period of time (Schulz and Oslage 1976). Fermentation is one of the oldest methods in food processing which helps for their conservation for longer time. From the biochemistry point of view; it is a metabolic process where energy is generated from organic compounds without the involvement of exogenous oxidizing agents (Bourdichon et al. 2012). Furthermore, the fermentation can be defined as a process where decontamination and detoxification phenomena, of hazardous compounds obtained from agro-industrial waste, are possible (Farinas 2015).

There are two types of fermentation, liquid/submerged or solid-state fermentation. Each one is described below.

11.2.1 Liquid or Submerged Fermentation

Submerged fermentation is a process which utilizes substrates containing free water; such as broths and molasses. The substrate is quickly consumed, so, it is necessary a constant replacement of the culture medium to provide new nutrients. In these fermentation methodologies, the fermentation products may or may not be released in the fermentation broth, it depends if they are extracellular or intracellular. (This type of fermentation is used mainly in microorganisms that need a high moisture content in the medium to be able to develop at an optimum level Subramaniyam and Vimala 2012).

11.2.2 Solid-State Fermentation

Solid-state fermentation unlike submerged state fermentation, is the process whereby a microorganism growing in a non-soluble material, serves as a physical support and as a nutrient source, in the absence of a large amount of free water (Couto and Sanromán 2006).

A very important aspect of solid-state fermentation is the choice of microorganism that will be used in this type of process. The microorganisms which are most suitable for this type of fermentation are fungi due they do not require the presence of free water in the substrate. The second group of microorganisms are some yeasts that, like filamentous fungi, do not require a large amount of water in the medium. Moreover, there are certain bacteria that can develop in a solid-state medium (Farinas 2015; Soccol et al. 2017).

In addition to energy sources, microorganisms require different nutrients to satisfy their energetic needs. Some microorganisms use compounds with simple structures, while others use very complex compounds. However, four types of compounds predominate in solid medium: lipids, carbohydrates, nucleic acids, and proteins (Kampen 2014).

To maximize the production of biomass, it is necessary to use different sources of carbon together with a balance with nitrogen sources, minor elements, trace elements, and vitamins (García-Garibay et al. 2014).

11.3 Carbon Sources

In order to survive the microorganisms, they require different nutrients to obtain energy and produce different organic compounds through fermentation. Carbon sources are considered a factor with great importance in the formulation of the culture medium. Usually at laboratory scale pure sugars are utilized as carbon sources to achieve a better performance (Yatmaz and Turhan 2018) and have a better bioprocess control.

Historically, different materials have been used as carbon sources in substitution of pure substrates to produce biomass. However, the use of these materials brings the disadvantage that there may be variation in the production yield (Stanbury et al. 2017). Despite this, the usage of different agroindustry residues, as carbon source, has been carried out giving added value to these residues (Reihani and Khosravi-Darani 2018) and to obtain microbial biomass.

The waste generated in agriculture after harvest and sequential processing creates a severe problem of pollution. However, it is known that these residues contain a high amount of hemicellulosic and/or lignocellulosic compounds, which can be used as carbon sources for different fermentation processes (Patel and Shukla 2017).

11.4 Agro-Industrial Waste Used as Carbon Source

Agro-industrial waste is the most abundant renewable resource and is produced in huge amounts every year, causing pollution, and represents an economic challenge for companies. It is estimated that about five billion tons of this type of waste are generated annually. So, it is necessary to use them efficiently for the development of new products

and applications in order to reduce their impact on the environment (Bharathiraja et al. 2017; Motaung and Linganiso 2018). Agro-industrial residues are those that are not commercialized after the harvest process, among them are mainly sugar cane bagasse, corn residues (stems, leaves, husks, and cobs), wheat and rice straw, rice bran, nutshells, and others. However, they can have different uses, these residues are widely used as cattle feed and compost material and in recent years biorefinery and microbial biomass production are the main trends to consider (Popa 2018).

These residues are also used as fodder for livestock, fuel for stoves, and water heaters (Sarkar et al. 2012). To dispose of waste in an easy and inexpensive way, these are often incinerated outdoors. This practice ends up being more harmful due to air pollution and consequently soil erosion. On the other hand, waste may also be buried avoiding air pollution. The incorporation of such residues in the soil significantly increases its quality and health; however, it is usually an expensive way, because of the required effort and time (Singh and Singh 2012).

11.5 Composition of Agro-Industrial Waste

Agro-industrial waste consists mainly of carbohydrates, proteins, and fibers. Carbohydrates are the most abundant components in these residues and within this category are cellulose, starches, pectins, among others.

Lignocellulose, which is formed by cellulose, hemicellulose, and lignin, is one of the main components of agro-industrial waste. Different residues come to be composed of 20–30% lignocellulose, which can have a great potential for bioconversion and fermentation (Singh et al. 2015). The composition of lignocellulose can vary according to the species from which it is obtained, in addition to the age of the plant, the state of maturation, or growth conditions (Sharma et al. 2019).

11.5.1 Cellulose

It is one of the most abundant biopolymers on the planet; it is a fibrous substance insoluble in water which is found in the cell walls of plants, fungi, and algae as structural material (Kushwaha et al. 2016). Structurally it is formed by a chain of hundreds or even thousands of glucoses linked by a bond β 1-4 (Ummartyotin and Manuspiya 2015) (Fig. 11.1).

11.5.2 Hemicellulose

Hemicellulose is a group of compounds found in most plants which is a renewable resource that can be used for different purposes. Some studies of these compounds

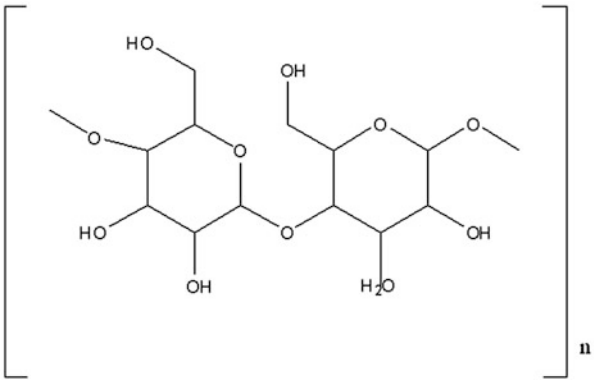

Fig. 11.1 Cellulose structure

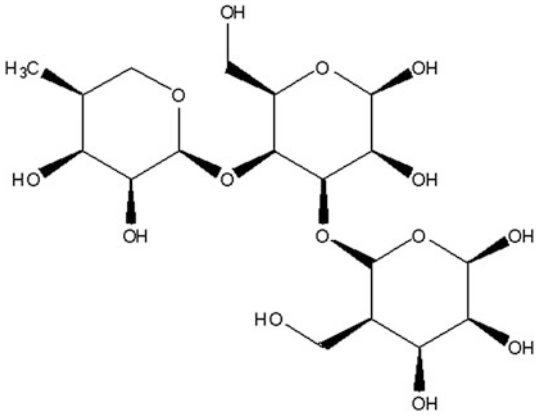

Fig. 11.2 Chemical structure of hemicellulose

have been focused on the release of carbohydrate monomers through different ways, for example, by means of chemical or enzymatic hydrolysis during production of new biofuels as an alternative of fossil fuels (Ebringerova et al. 2005). These polysaccharides are structurally formed by hexoses (galactose, mannose, and glucose) and pentoses (arabinose and xyloses) linked by covalent bonds (Fig. 11.2). Hemicellulose is a hydrophilic polymer and its degree of polymerization (80–200) is small compared to the polymerization degree of cellulose (10,000). As a result of this, they show greater chemical and thermal stability, so they are more soluble in water and the hydrolysis process is simpler (Ummartyotin and Manuspiya 2015; Ajao et al. 2018).

11.5.3 Lignin

Lignin is one of the most abundant phenolic polymers in the world. It has a complex structure as it combines aliphatic chains as well as aromatic compounds. The three

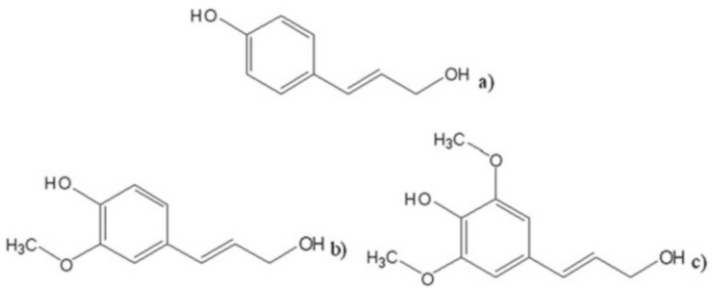

Fig. 11.3 Precursor compounds of lignin: (**a**) *P*-coumaryl alcohol; (**b**) coniferyl alcohol; (**c**) Sinapyl alcohol

main compounds of lignin are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 11.3). Lignin forms esters and ethers with hemicellulose for the formation of various compounds including lignocellulose (Bajpai 2017a; Chio et al. 2019).

11.6 Sources of Nitrogen

To grow properly, microorganisms have specific nutritional requirements such as nitrogen which is present in various molecules mainly as amino and amide groups. It is common to find nitrogen in microorganisms in its reduced form as primary, secondary, or tertiary amines groups, whereas the oxidized form of this element, such as nitro or nitrous groups, is very rare (Egli 2009).

The relationship between carbon and nitrogen content in nutrient source is an important factor that affects the microorganism growth. A deficiency in the nitrogen content can affect the microbial biomass production yield. There are different sources of organic and inorganic nitrogen which microorganisms can use in their development (Li et al. 2017).

Like the carbon sources behavior, nitrogen sources have the function of stabilizing the pH in the fermentation processes. Nitrogen sources applied in fermentation may be either inorganic or organic. In the first case, inorganic or mineral sources include ammonium salts, ammonia, nitrates, and within the organic can be used urea, yeast extract, corn liquor, or peptone. In addition, it is sometimes necessary to use different mineral sources for optimal biomass growth. In the case of ammonia, which is the nitrogen source most used by autotrophs, nitrogen is in its reduced form, which makes it easier to assimilate (Reihani and Khosravi-Darani 2018; Ohkouchi and Takano 2014).

From the different types of nitrogen source; ammonium salts are one of the preferred. They are the compounds in which inorganic nitrogen can be used for the amino acids formation, unless the same microorganism possesses enzymes or enzymatic complexes that can form ammonium ions from different sources of inorganic nitrogen (Atkins 2016).

11.7 Types of Biomass

11.7.1 Biomass from Microalgae

Microalgae are microorganisms which can convert solar energy into chemical energy through photosynthesis, have been used mainly to produce biofuels. Recently, it is highlighted their use to produce food and pharmaceuticals. It has been shown that algae have a large amount of mainly unsaturated lipids, proteins, and carbohydrates, which can be used in a wide variety of markets (Priyadarshani and Rath 2012; Vanthoor-koopmans et al. 2013; Ramanna et al. 2017). Due to the variety of species, culture conditions and post-cultivation, there is a great potential of biomass from algae for the production of food and biofuels (Walsh et al. 2018). Moreover only a few microalgae species have been included in the "generally regarded as safe" classification for food sources categorized by the US Food and Drug Administration, with examples including *Haematococcus* spp., *Dunaliella* spp., *Chlorella* spp., *Arthrospira* spp., and *Schizochytrium* spp. (Hashemian et al. 2019).

Similar to plants, the chemical composition of algae is not a constant factor since it can be affected by various factors such as temperature, pH, salinity, lighting, CO_2, growth phase and physiological state (Paula et al. 2013).

11.7.2 Biomass of Algae as a Food Supplement

Different microalgae and macroalgae stand out for their high protein content, which can be compared with the content of other foods such as milk, meat, eggs, and soy. In addition, protein production yield is much higher than traditional sources, so, seaweed and microalgae have a production at 2.5–7.5 ton/Ha/year and 4–15 ton/Ha/year, respectively, while other protein sources, such as soy and other known legumes, have a production at around of 1–2 ton/Ha/year (Bleakley et al. 2017).

Nowadays, technological developments and research focus on the use of algae as micro-ingredients, obtaining very specific benefits. Moreover, the growth of algae biomass production suggests that the use of algae as a micro-ingredient can become the basis of different products for livestock feed, as well as in aquaculture (Shields and Lupatsch 2012).

Although there are different data on the use of algae as an animal supplement, only the use of microalgae supplements had been studied. Different genera of microalgae, such as *Chlorella* and *Scenedesmus* sp., attracted the attention of the researchers, because they had a large amount of carotenoid compounds and crude protein (Table 11.1). It was observed that the animals gained weight by consuming algal biomass (Lum et al. 2013).

However, the use of Algae as biomass has some disadvantages, such as non-absorption by the human digestive system due to the cellulose content in the

Table 11.1 Main compounds present in the microalgal biomass (Priyadarshani and Rath 2012)

Group of compounds	Examples
Pigments/ carotenoids	Beta-carotene, astaxanthin, lutein, canthaxanthin, chlorophyll, phycocyanin, phycoerythrin, fucoxanthin
Polyunsaturated fatty acids	DHA (C22: 6), EPA (C20: 5), ARA (C20: 4), GAL (C18: 3)
Vitamins	A, B1, B6, B12, C, E, biotin, riboflavin, nicotinic acid, pantothenate, folic acid
Antioxidants	Catalases, polyphenols, superoxide dismutase, tocopherols
Others	Antifungal activity, antibacterial, antiviral, proteins, sterols

cell wall of the biomass. Secondly, they also concentrate heavy metals and phycotoxins which can be a health problem (Nasseri et al. 2011; Kay and Barton 1991). Finally the incorporation of microalgal biomass into food products has the challenge of sensory profiles to address to enable future food products to become mainstream in the wider marketplace (Birch and review 2019).

In the human diet, various algae are consumed, including *Spirulina* and *Chlorella*, which have been commercialized since the 1960s. Despite the nutritional characteristics of algae, the quality of this material is also measured by the content of compounds such as heavy metals, aromatic polycyclic hydrocarbons, toxins, pathogens, and pesticides that can become harmful when consumed (Muys et al. 2018). Despite the nutrients offered by algae as food, the organoleptic characteristics are not favorable, making it difficult for potential consumers to accept it. So, it is necessary to develop new products that are pleasing to the general public (Becker 2007).

11.7.3 Biomass of Algae as Fuel

As a raw material for fuel production, algae biomass shows as an advantage the impact to counteract the greenhouse effect on the planet: due to the high yields of biomass production, algae are able to absorb large amounts of CO_2, which is one of the main causes of the problem of climate change (Ambat et al. 2019).

Microalgae can use the nutrients found in wastewater and carbon dioxide to achieve biomass growth which is rich in lipids that can be used in biodiesel production. There are several methods by which it is possible to obtain fuel from the lipids of microalgae; one of these is transesterification catalysis (acid/base) or another is enzymatic biocatalysis (He et al. 2018; Liu et al. 2016).

11.7.4 Microalgae Biomass Production

Regardless of the great versatility of algae as food and as a biofuel, the cost of production is very high, due to the complexity of their collection. It is estimated that in the biofuels production between 20% and 30% of cost corresponds to the collection process and algae biomass production (de Assis et al. 2019; Molina Grima et al. 2003).

In addition to this, the spread of algae for food additives could solve the problem of bioremediation of wastewater mainly in control of nitrogen and phosphorus levels. Therefore, they are looking for new ways to produce them more effectively and efficiently. It has been shown that algae are able to better assimilate the nutrients contained in waters of this type. Several studies have shown that microalgae can reduce between 82–92% nitrogen and 58–98% phosphorus, in addition to reducing the chemical oxygen demand (Ambat et al. 2019; Zhou et al. 2012; Marin-Batista et al. 2019).

In addition to the optimal parameters for the algae growth (temperature, inorganic nutrients, such as nitrogen, phosphorus, sulfur, and water), the production of the biomass may require control of conditions of two types: (1) the use of CO_2, and (2) the use of light (Ramanna et al. 2017). It has been studied that the limitation of nitrogen, in addition to the combination with levels of other metals, leads to algae stress, which is reflected in the reduction of biomass production (Singh et al. 2016).

To separate different biomass fractions, it is necessary to degrade the cell wall of the algae. This wall protects the algae from biodegradation related to enzymatic attacks caused by different microorganisms. Different methods have been used to achieve the cell wall break, including physical and mechanical treatments which, despite being able to degrade the cell wall, often get to negative yields in energy terms: the loss of energy is greater than for biomass production. Kavitha et al. (2017) performed pretreatments using bacteria for the degradation of cell walls in microalgae, obtaining better yields compared to physical and mechanical pretreatments, which require higher energy consumption.

11.8 Fungal Biomass Production

Currently, fungi are used for the production of secondary metabolites, which have medicinal and industrial importance, since medicines and foods can be made with these metabolites (Nigam and Singh 2014). In addition, they are sources of different pigments such as carotenoids, melanin, among others (Dufossé 2018).

One of the uses of fungus is for the production of mycoprotein, which is a food product derived from the continuous fermentation of the fungus *Fusarium venenatum*, which has been noted for its high protein yield and a low amount of fats (Denny et al. 2008), being a very interesting nutritional source compared with other sources (Table 11.2).

Table 11.2 Nutritional comparison of fungi biomass with other foods (Nigam and Singh 2014)

Food	Amount (g)	Energy (kcal)	Proteins (g)	Carbohydrates (g)	Dietary fiber (g)	Total fat (g)
Mycoprotein	85	72	9.4	7.7	5.1	2.6
Cheddar cheese	30	120	7.5	0.38	0	10
Eggs	50	75	6.3	0.6	0	5
Meat	85	245	19.6	0	0	17.8
Chicken	85	130	23.1	0	0	3.5
Fish	85	89	19.4	0	0	0.7
Soya flour	30	131	10.4	10.6	2.9	6.2
Chickpeas	100	364	19.30	60.65	17.4	6.04
Green peas	100	343	21.70	62.78	15.0	1.49

The chemical analysis of mycoprotein demonstrates a wide variety of nutrients. This food is also combined with low-energy and high-fiber content. Mycoprotein has a low fat content which consists mainly of unsaturated fatty acids predominantly linoleic and linolenic acid. The fiber consists of a mixture of chitin and β-glucan that performs physiologically as dietary fiber and does not have an adverse effect on mineral status (Finnigan et al. 2017)

For the production of this type of food, it is possible to use different cellulosic materials (straw, sugarcane bagasse, corn cob, etc.) as a source of carbon in the fermentation (Moo-Young et al. 1993). However, to make use of these materials rich in cellulose, it is necessary to implement a pretreatment for this type of material to facilitate access of microorganisms to the available glucose.

Different substrates can be used for biomass production of these organisms. Oshoma et al. (Marin-Batista et al. 2019) demonstrated that banana peel is a good substrate for the production of *Aspergillus niger* biomass obtaining better yields than with other substrates, like orange peel among others (Oshoma and Eguakun-Owie 2018). Alemu (Singh et al. 2016) indicates that the best process for a better yield of biomass of filamentous fungus is the solid-state fermentation (Alemu 2013) using agro-industrial wastes (Table 11.3).

Ugalde (Kavitha et al. 2017) lists certain filamentous fungi which can be used in the production of biomass and thus generate foodstuffs where the genera stand out: *Aspergillus, Fusarium, Rhizopus, Trichoderma, Cephalosporium, Mucor, Monilia, Penicillium, Spicaria, Paecilomyces*, *and Cunninghamella* (Ugalde and Castrillo 2002) (Table 11.3).

The presence of mycotoxins in certain fungal species especially *Aspergillus parasiticus* and *Aspergillus flavus* is a major hindrance in their use. These toxins are known to produce many allergic reactions, diseases, and liver cancer in humans as well in animals. Hence, it is required to eliminate contamination (Anupama 2000).

Although fungi usually grow more slowly than yeasts, the yields in biomass production are usually similar. However, as shown in Table 11.3, they can grow in different agro-industrial residues under very similar temperature conditions.

Table 11.3 Examples of fungi and yeasts grown in agro-industrial wastes

Microorganisms	Waste used to produce biomass	Growth conditions	References
Aspergillus niger	Orange peel	32 °C, 144 h	Azam et al. (2014)
Fusarium venenatum	Date Juice	26 °C 72 h	Fatemeh et al. (2018)
Trichoderma ressei	Rice Straw	30 °C, 21 days	Zaki and Said (2018)
Candida tropicalis	Pineapple waste	28 °C, 168 h	Dharumadurai et al. (2011)
Candida sp.	Orange, Plantain, Banana	25 °C, 24–36 h	Adoki (2008)
Candida parapsilosis	Vinasse	28 °C 48 h	Dos Reis et al. (2018)

11.9 Yeast Biomass

Yeasts are considered fungi; they reproduce by fission or by budding. It has been documented that yeasts can cause some deterioration in food and beverages. It is also possible to apply yeasts in fermentative processes for food generation. It has been applied in agriculture, production of biofuels, medicines, chemical industry, and even environmental protection (Jach and Serefko 2018; Fleet 2011). They are divided into two families: Ascomycota and Basidiomycota.

Yeasts can be used for the treatment of different residues generated in various industries; an example of this is the treatment of vinasse to produce unicellular protein (Table 11.3). Different studies have shown that it is possible to use this waste from the wine industry as a substrate for yeast fermentation, to reduce the pollution. Dos Reis et al. in 2018 reported that *Candida parapsilosis* was grown in medium with 5 g/L of peptone and 70% v/v of vinasse (Dos Reis et al. 2018).

It have been shown that yeasts have a resistance to different factors such as acid and alkaline pH, antibiotics, different sterilizing agents, and they can grow on different carbon sources ranging from carbohydrates (glucose, sucrose, and maltose), biopolymers (starch, cellulose, hemicellulose, and pectin), alcohols, hydrocarbons, fatty acids, and organic acids (Yang and Zheng 2014).

One of the uses of yeast biomass is the obtention of different oligosaccharides due to the presence of β-glucan in the yeast cell wall. The β-glucan is composed of glucoses linked by β-1-6 and β-1 bonds, which gives to the cell wall some stability and rigidity. This structure is similar to that of cellulose because, like this one, it is also has a crystalline region and an amorphous region (Bychkov et al. 2010).

11.9.1 Yeast Biomass as Food

Yeasts are applied to produce different functional foods and are considered a fundamental part in obtaining of nutraceuticals. Yeasts have different applications in the functional food industry, since they can be used as probiotics. Several studies show that the cell wall of yeasts has a large amount of nutrients such as proteins and B vitamins. Extracts obtained from these microorganisms are a source of metabolites that have bioactive compounds (carotenoids, folates, and γ-aminobutyric acid). Also yeast are producers of enzymes and that the products of their viability can be metabolites with high nutraceutical value (Rai et al. 2018).

One of the applications of yeast biomass is the production of unicellular protein, which can be employed as a substitute for other protein sources such as soy and fish protein used for animal feed. However, traditional production of this type of feed requires pure strains of certain types of yeasts and crop optimization processes (Yang and Zheng 2014). Due to the growing interest in production of unicellular protein from raw material, i.e., from agro-industrial waste, it has become a less expensive process (Kieliszek et al. 2017).

Nigam (Nigam 2000) demonstrated that for yeast growth in sugar cane bagasse, the pretreatment of support by hydrolysis is necessary for a greater availability of glucose to obtain higher biomass yield. In addition, the presence of a high amount of amino acids such as lysine, arginine, and isoleucine is reported in the yeast biomass (Nigam 2000).

A wide variety of yeasts has been used in the production of unicellular protein (Table 11.4). The composition of biomass in nutritional terms is variable and rich (Table 11.5). So, the production of unicellular protein from yeast is a viable alternative for obtaining nutritionally rich foods.

Table 11.4 Yeasts applied for the unicellular protein production (Bajpai 2017b)

Yeasts	Raw material	Fermentation conditions	References
Pichia guilliermondii	Waste brine of kimchi production	12% substrate (w/v), 30 °C, 72 h	Choi and Park (1999)
Candida tropicalis	Pineapple peel	1–5% of substrate, 28 ° C, 7 days	Dharumadurai et al. (2011)
Candida utilis	Corn stover	20 g/L, 35 ° C, 72 h	Ahmed et al. (2010)
Hansenula polymorpha	Sugar beet stillage	30 °C, 48 h	Shojaosadati et al. (1998)
Kluyveromyces marxianus	Cheese Whey	34 °C, 28 h	Anvari and Khayati (2011)
Rhodotorula sp.	Lettuce brine	30 °C, 96 h	Suntornsuk (2000)
Saccharomyces sp.	Papaw and Banana juice	100 mL/L of substrate, 72 h, 30 °C	Rajendran et al. (2018)

Table 11.5 Nutritional composition of yeast biomass (Martínez et al. 2018)

Composition	%	Vitamins	(mg/100 g)	Minerals	(mg/100 g)
Proteins	52	Thiamin	0.80	Phosphorous	2100
Carbohydrates	22	Riboflavin	4.50	Potassium	2000
Minerals	8	Niacin	55.00	Magnesium	300
Fat	7	Folic Acid	0.40	Sulfur	200
Humidity	6	Pyridoxine	8.30	Sodium	100
Raw fiber	5	Pantothenic acid	9.40	Calcium	15
		Biotin	0.08	Iron	9.5
		P-aminobenzoic acid	1.40	Zinc	9.3
		Choline	780	Fluoride	1.2
		Inositol	460	Manganese	0.7
		B12 vitamin	0.0004		

11.9.2 Biofuels Obtained by Yeast Fermentation

Many yeasts are known for their enzymatic complexes capable of producing ethanol (Adelabu et al. 2019) In the production of this biofuel, it is preferable to use yeasts instead of bacteria, due to the tolerance that they have against to different inhibitory factors, such as, ethanol, and other organic acids.

For the production of biodiesel, from oleaginous biomass, using yeasts; three main steps are required: (1) The culture of the microorganism, (2) The breaking of the cell wall, and (3) The extraction of the lipids of the microorganism for their subsequent transesterification (Yellapu et al. 2016).

11.10 Biomass of Bacteria

The biomass of certain bacteria, such as photosynthetic bacteria, is not only rich in proteins, but also in other compounds such as, carotenoids, biological cofactors, vitamins, among others (Shipman et al. 1975).

11.10.1 Biomass of Bacteria as Food

One of the main uses given to the biomass of these organisms is mainly for aquaculture. Due to its high demand, the implementation of new protocols to enhance the biomass production has been required to satisfy the consumer demand (Kiessling and Askbrandt 1993). Different methods are used to produce unicellular protein from photosynthetic bacteria. One of them is the use of bovine animal waste. This type of bacteria is found mainly in wells, dykes, or other types of water sources. Also, this type of bacteria is used in the biogas industry due to the high biogas

production. Some residues generated are a good source of nutrients, various studies reported that in addition to proteins, a large amount of carotenoids and vitamins should be obtained as valuable subproduct (Vrati 1984).

To produce biomass, it is possible to use heterotrophic bacteria which can convert phosphorus and nitrogen from waste obtained from fish farming. This type of bacteria requires a C:N ratio between 12:15 for optimum biomass production (Jach and Serefko 2018). Few hydrogen oxidizing bacteria can obtain good yields in the production of unicellular protein. The production of these bacteria is carried out via autotrophic biochemical route, in environments with CO_2. Dou Junei in 2018 demonstrated that this type of bacteria has good yields at a temperature of 30 °C, with pH 7 and using NH_4HCO_3 as a source of nitrogen (Fleet 2011).

Use of bacterial biomass is limited due to high cost. Harvesting is difficult due to small size of bacteria and hence the cells must be flocculated in order to ease a higher solid slurry obtention, prior centrifugation. Bacterial cells also have a high concentration of nucleic acids (Anupama 2000).

11.11 Conclusion

The production of microbial biomass has been an alternative that has helped humanity to meet various needs, including food and fuel production. While there is a large amount of biomass that can be used to meet the demands of these two sectors, it is necessary to develop new bioprocesses or to optimize some existing ones to increase the yields of the products of interest. In addition, the development of new technologies will allow us to contribute to the solution of pollution problems caused by large amount of agro-industrial waste, which are not used. Currently, our working group (Bioprocesses & Bioproducts Group) has carried out research based on the use of agro-industrial waste to obtain compounds of commercial interest, including the production of single-cell protein hoping to contribute to meeting food needs.

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Chapter 12
Cyanobacterial Degradation of Organophosphorus Pesticides

Nimisha Vijayan P and Sabu Abdulhameed

Abstract Pesticides are chemicals which are widely used for the protection of crops from the attack of pathogens. Pesticides include fungicides, insecticides, herbicides, and bactericides. Among the different classes of pesticides used, organophosphorus class of pesticides is commonly used for agricultural applications. Indiscriminate use of such chemicals causes many environmental problems. It also poses high risk to other organisms such as birds, fishes, other beneficial insects, and humans. So it is highly desirable to remove these harmful chemicals from the environment in a proper way. Biodegradation is one of the best available methods and is the breakdown of toxic chemicals into nontoxic compounds through the use of microorganisms. It is a cost-effective, eco-friendly, and efficient method for the detoxification of pesticides. Different microorganisms are involved in the biodegradation process such as bacteria, fungi, and cyanobacteria. Among these organisms, cyanobacteria play an important role in the degradation of pesticides. They can be found in a wide variety of habitats. They are photoautotrophic organisms and hence the use of cyanobacteria for degradation process would overcome the need to supply heterotrophs with organic nutrients. Studies have proved the efficiency of cyanobacteria in the degradation of organophosphorus pesticides. The widespread appearance of cyanobacteria in the polluted area is also a contributing factor for making them a better candidate for biodegradation.

Keywords Pesticides · Organophosphorus pesticides · Biodegradation · Microorganisms · Cyanobacteria

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12.1 Introduction

Modern agriculture practices make use of synthetic chemicals called pesticides for the protection of crops from the attack of pests, insects, etc. They are extensively used in agricultural fields to meet increased agricultural yield and product quality (Parte et al. 2017). The use of pesticides in agriculture has become very essential because of the reduced crop yield due to pests. Pesticides has caused an increase in product yield and prevented the spread of insect-borne diseases (Verma et al. 2014). With the increasing demand for pesticides, the problems associated with the use of these chemicals have also increased. It affects the stability of the ecosystem and their continuous use causes development of resistance in the pests. It has become a major cause of environmental pollution because their extensive use causes the accumulation of these pollutants in soil and water (Rani and Dhania 2014).

Considering the health hazards associated with the use of these pesticides, it is necessary to remove the pollutants from the environment in a proper way. Many of the methods that are in use for the removal of pesticides, such as landfilling, recycling, pyrolysis, and incineration produces toxic intermediates which makes these methods less suitable for pesticide detoxification (Paul et al. 2005). So biological methods are more preferred over these costly conventional methods. Biodegradation is the safest and cheap method for the removal of these contaminants from the environment since it ensures the complete detoxification of these chemicals. It is the breakdown of toxic chemicals into nontoxic compound through the action of microorganisms or their enzymes (Aislabie and Lloyd-Jones 1995). This method is minimally hazardous and environment friendly (Verma et al. 2014). This technique exploits the natural ability of the microorganisms to degrade the pesticides. Researchers are more interested to discover new species of microorganisms in the pesticide polluted areas to study the mechanism of degradation and to develop methods to increase the efficiency of the microorganisms to degrade the pesticides.

Microorganisms such as bacteria, fungi, and blue-green algae have proved to be efficient degraders of pesticides. Compared to other microorganism, cyanobacteria is considered to be a better organism in the field of bioremediation. They are widely distributed in the environment and can grow at any place where moisture and sunlight are available. Most of the species can combine photosynthesis and nitrogen fixation (Vijayakumar 2012). They are applied as biofertilizers in paddy filed (Singh et al. 2011). Their minimal growth requirements and their abundance in polluted areas are important contributing factors for the use of cyanobacteria in the field of biodegradation.

12.2 Pesticides

Pesticides are chemicals which are used against the attack of pests, weeds, and other plant pathogens to reduce the loss of crop yield and to increase the product quality (Damalas and Eleftherohorinos 2011). In today's world, over 500 compounds are in use as pesticides or as their metabolites (Parte et al. 2017). They are able to persist in

the environment for a long time and their activity in the environment depends upon the physicochemical properties of the pesticide (Gavrilescu 2005). They are a diverse group of chemicals and it includes insecticides, herbicides, fungicides, etc. (Verma et al. 2014). They cause disturbance in the physiological activity of the target organism. Pesticides possess certain characteristics such as high lipophilicity, bioaccumulation, and long half-life. All these factors contribute to the increased chances of contamination due to these chemicals (Jayaraj et al. 2016) Pesticides mainly fall into two categories, persistent or nonpersistent (Verma et al. 2014). Based on their chemical nature, they are classified into organochlorines, organophosphates, carbamates, etc. (Jayaraj et al. 2016) The possible reasons for the persistence of a pesticide in the environment are i) unfavorable conditions for the biodegradation ii) absence of pesticide degrading microorganisms in the contaminated site iii) resistance of the pesticide toward biodegradation or their inability to cross the cell membrane for degradation by intracellular microbial enzymes (Gavrilescu 2005). Degradation of the pesticide involves three phases. In the first phase, the pesticide undergoes oxidation, reduction, or hydrolysis reactions which convert the parent compound into more water-soluble and less toxic compounds. In most cases, oxygenation is the first step in the biotransformation of the pesticide and many oxidative enzymes such as cytochrome P-450, peroxidases, and polyphenol oxidases play an important role in this phase. The second phase involves the conjugation of the pesticide or their metabolites with a sugar, amino acid, or glutathione. It results in the increase in water solubility of the parent compound. The toxicity gets reduced. The third phase is the conversion of metabolites in the second phase into secondary conjugates (Palanisami et al. 2009).

A pesticide must be only lethal to the target species. But in most of the cases, it is not the case and it brings out the harmful effects of these chemicals. In reality, only 10% of the applied pesticides reach the target organism, remaining gets deposited in soil, water, and other nontarget areas (Parte et al. 2017). Pesticides pose a risk to the living organisms. It is also harmful to the useful organisms present in the soil (Rani and Dhania 2014). Adverse effects of using these pesticides in soil are decreased soil fertility, nitrate leaching, soil acidification, groundwater and surface water pollution and contamination of agricultural soils (Kumar et al. 2018). It will also affect nontarget vegetation. Volatilization of the applied pesticide may contaminate air also (Aktar et al. 2009). Demand for agro products has increased over these years which in turn caused an increase in the use and application rate of pesticides (Shetty et al. 2008). Indiscriminate use of pesticides is detrimental to humans and it also affects the biodiversity (Gavrilescu 2005; Hussain et al. 2009). Some pesticides may undergo biomagnification which will cause more problems in the environment.

12.3 Organophosphorus Pesticides

Among the different classes of pesticides used, organophosphorus class of pesticides is the commonly used group of pesticides and constitutes approximately 38% of the total pesticide used globally. Organophosphorus pesticides mainly involve

dialkylarylphosphates, phosphorothioates, and phosphorodithioates (Ragnarsdottir 2000). Organophosphorus pesticides are actually introduced in replacement of the more toxic organochlorine pesticides. Compared to organochlorine compounds, they are less persistent in the environment but they are detected in air and water due to their wide use (Kuritz 2010). They are broad-spectrum insecticides and are applied on crops such as vegetables, fruits, grains, and ornamentals. They are derived forms of phosphoric, phosphonic, phosphinic, thiophosphoric acids that are usually in the form of esters, amides, and thiols. The general structure involves two organic groups and an additional side chain consisting of cyanide, thiocyanate, or phenoxy groups (Kumar et al. 2018) (Fig. 12.1). Since they are esters, they have many sites that are exposed to the hydrolytic reactions. Hydrolysis of these compounds reduces their mammalian toxicity (Singh and Walker 2006). They are soluble in water which makes them more prone to humans causing serious health hazards. The fate of OP pesticides in the environment is determined by its transport, volatilization, hydrolysis, oxidation/reduction reactions, photolysis, and biodegradation (Ragnarsdottir 2000). Some of the important organophosphorous pesticides along with their half-life in soil are listed (Table 12.1).

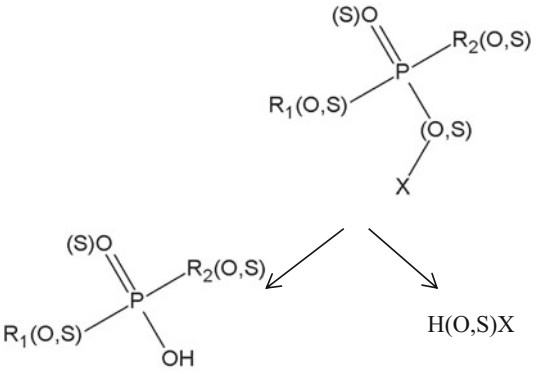


Fig. 12.1 General structure of OP pesticides and their transformation (Kumar et al. 2018)

Table 12.1 Some of the commonly using organophosphorus pesticides and their half-life in soil (Singh and Walker 2006)

Pesticide	Type of pesticide	Half-life (days)
Chlorpyrifos	Insecticide	10–120
Methyl parathion	Insecticide	25–130
Fenamiphos	Nematicide	28–90
Parathion	Insecticide	30–180
Monocrotophos	Insecticide	40–60
Diazinon	Insecticide	11–21
Dimethoate	Insecticide	3–30
Dicrotophos	Insecticide	45–60

12.3.1 Toxicology of Organophosphorus Pesticides

They possess high mammalian toxicity (Singh and Walker 2006). It interferes with the action of the acetylcholine esterase enzyme thereby preventing the hydrolysis of acetylcholine into choline and acetic acid. Some organophosphorus pesticides also inhibit some important enzymes in the central nervous system. It causes trouble in the transmission of nerve impulses (Rani and Dhania 2014). Exposure to organophosphorus compounds may cause long-term neurological effects (Rani and Dhania 2014). Symptoms of OP poisoning include headache, weakness, excessive sweating, nausea, vomiting, diarrhea, abdominal cramps, and tremors (Kanekar et al. 2004). It will cause adverse effects in the central nervous system, autonomic nervous system, and neuromuscular junctions. The aftereffects are convulsions, paralysis, and death. Continuous use of these compounds cause problems in the ecosystem also. Chlorpyrifos, fenamiphos, methyl parathion, coumaphos, and diazinon are some of the commonly used organophosphorus pesticides (Ragnarsdottir 2000).

12.3.2 Conventional Techniques to Remediate Organophosphorus Pollution

Conventional methods for the removal of organophosphorus pesticides mainly involve various physical and chemical methods. But these methods are expensive and have various disadvantages (Parte et al. 2017). The following table illustrates some of the commonly used physical and chemical techniques for the removal of organophosphorus pesticides (Marican and Durán-lara 2018; Mary et al. 2015; Table 12.2).

Table 12.2 Conventional techniques for the remediation of organophosphorus pesticides

Type of remediation process	Methods		Pesticides
Physical process	Adsorption	Commonly using materials	Methyl parathion, Dimethoate, Dichlorvos
		Clays Activated carbon Zeolites Polymeric materials (cyclodextrins, dendrimers, hyper crosslinked polymers)	
Chemical process	Advanced oxidation process	UV-H_2O_2 and UV-ozone	Chlorpyrifos
		Zero valent iron	Chlorpyrifos, methyl parathion, malathion
		Photocatalysis	Chlorpyrifos
		Fenton reaction	Diazinon, dichlorvos
		Photodegradation	Chlorpyrifos-methyl
		Ultrasound assisted remediation	Diazinon

12.3.3 Biodegradation Organophosphorus Pesticides

Microorganisms are capable of degrading organic compounds and the microbial processes involved in biodegradation are mineralization, detoxification, co-metabolism, and activation. Biodegradation takes place when the microorganisms use these pesticides as a source of carbon or energy or they consume the pesticide along with the other sources of food (Gavrilescu 2005). They can interact with the pesticide both physically and chemically bringing a structural change to the compound or complete degradation (Briceño et al. 2007). The rate of biodegradation varies depending upon the microbial community present and environmental factors such as pH, temperature, moisture, and sunlight (Ragnarsdottir 2000). The ability of the microorganism to degrade the pesticide depends upon the presence of microbial enzymes and the availability of favorable conditions for the reaction to occur (Gavrilescu 2005). Through biodegradation one of the following changes may take place, (1) least biodegradation may take place which will change the identity of the compound, (2) complete biodegradation to water, carbon dioxide, and inorganic compounds, (3) detoxification or biotransformation (Gavrilescu 2005). In soil, biodegradation may take place either aerobically or anaerobically (Briceño et al. 2007). Rate of biodegradation depends upon the availability of the pesticide to the microorganism, physiological status of the microorganism, survival of pesticide degrading organisms at the polluted site, and sustainable population of these organisms (Rani and Dhania 2014). In some cases, the microorganisms present in the polluted site may not be able to degrade the pesticide. In such cases external supply of degrading microbes is needed (Singh 2008). Fungi mainly degrade pesticide by introducing some minor structural changes to the compound making it nontoxic. It is then released into the soil where further degradation is carried out by bacteria (Briceño et al. 2007). In some cases, the intermediates produced during the transformation are more toxic than the parent compound and also they may show high resistance toward microbial attack (Golovleva et al. 1990).

Most microorganisms use organophosphorus compounds as a source of phosphorous and carbon and the degradation process involves chemical reactions such as oxidation, hydrolysis, alkylation, and dealkylation (Singh and Walker 2006). Biodegradation of these pesticides mainly proceeds through the hydrolysis of P-O alkyl and P-O aryl bonds. Enzymes having a major role in the degradation process are hydrolase, phosphotriesterase, and phosphatase (Kumar et al. 2018). Phosphotriesterase activity is an important step in detoxification (Parte et al. 2017). In some cases the biotransformation takes place only when there is another organic compound to supply the carbon source. This involves the cometabolic removal of the pesticides (Ragnarsdottir 2000). The first microorganism reported to degrade organophosphorus pesticides was *Flavobacterium* sp. (Singh and Walker 2006). The degradation pattern of organophosphorus pesticides by most of the bacteria occurs in a similar way involving a structurally related enzyme, organophosphorus hydrolase (OPH), or phosphotriesterase. OPH encoding gene (opd) can be isolated from different organisms and can be cloned in suitable organisms for

increased activity and for the enhanced degradation of pesticides. *Aspergillus niger, Aspergillus fumigatus, Penicillium raistrickii,* and *Cladosporium cladosporioides* are some of the fungal species involved in the biodegradation of organophosphorus pesticides (Kumar et al. 2018).

12.4 Cyanobacteria

Cyanobacteria are photoautotrophic organisms and are found in a wide variety of environments. They are one of the oldest life forms on the earth. They can form mats often called blooms and some of them are able to fix atmospheric nitrogen. They have a specialized structure for nitrogen fixation called heterocyst and nitrogenase is the enzyme that is involved in the nitrogen fixation (Singh et al. 2016). These organisms increase the availability of nitrogen to plants. The ability to fix nitrogen, production of growth-promoting substances, and soil conditioning properties promotes their use as bio-fertilizers in soil (Subramanian et al. 1994). They are involved in decomposing the organic waste and catalyze nutrient cycling. They produce bioactive compounds such as vitamins, hormones, and enzymes which helps to promote the plant growth (Singh et al. 2016). Their presence in polluted areas initiated research on using this organism as a tool for bioremediation (Kuritz 2010). Compared to the other microorganisms, the photoautotrophic nature, the ability to fix atmospheric nitrogen and to survive in polluted environments makes them more suitable for biodegradation (Sorkhoh et al. 1995). They have minimum requirements for growth so that the removal of pollutants using cyanobacteria is an inexpensive method (Chungjatupornchai and Fa-Aroonsawat 2008). Because of these special characteristics, they are considered as an important organism in both terrestrial and aquatic ecosystems (Palanisami et al. 2009). Nitrogen-fixing cyanobacteria are commonly seen in paddy fields where they occur naturally or introduced as biofertilizers (Kuritz 2010). They are involved in enhanced solubility and mobility of limited nutrients. They take part in an important role in the solubilization and mobilization of insoluble inorganic phosphates with the help of phosphatase enzymes. It helps to increase the bioavailability of phosphorous to the plants (Singh et al. 2016). Cyanobacterial strains that are used as biofertilizers in soil are found to tolerate pesticides to a level recommended for field application and these organisms are able to accumulate high concentrations of pesticide (Vijayakumar 2012).

Cyanobacteria have antagonistic properties against different plant pathogens. They are capable of producing bioactive compounds having antibacterial, antiviral, and antifungal potential. These compounds belong to the group of polyketides, amides, fatty acids, indoles, alkaloids, and lipopeptides (Singh et al. 2016).

The presence of cyanobacteria in paddy fields is affected by phosphate content, dissolved inorganic nitrogen, oxygen content, and light (Kuritz 2010). Cyanobacteria release extracellular polymeric substances which have an important role in mat formation and stress tolerance under adverse conditions. They are highly

heterogeneous polymers including polysaccharides and noncarbohydrate constituents such as protein, phospholipids, and nucleic acids (Chug and Mathur 2013). These extracellular substances help to modulate the pH, redox activity, temperature, and also play a role in the volatilization of ammonia and methane generation. It also increases the stability of the soil through excretion of polysaccharides (Prasanna et al. 2008). The other beneficial effects of cyanobacteria include curbing of ammonia volatilization, reducing methane emission, transformation of P, Fe, Zn, Cu, Mn, pesticide degradation, and reclamation of wastelands (Mandal et al. 1999; Kumar et al. 2005). Because of these properties they find application in agriculture, industry. These organisms help to maintain environmental sustainability. They have the potential to increase agricultural yield by acting as a biofertilizer and at the same time they can be involved in the biodegradation of pollutants.

12.4.1 Cyanobacteria as a Tool for Bioremediation

Cyanobacteria are capable of degrading various pollutants such as dyes, crude oil, heavy metals, xenobiotics, etc. They can complex with the heavy metals and xenobiotics, thereby limiting their mobility and transport in plants. They have been in use for wastewater treatment for removing phosphorous and nitrogen (Singh et al. 2016). Cyanobacteria are more tolerant to heavy metals. Cu uptake was reported in the diazotrophic cyanobacterium *Nostoc calcicola* (Verma and Singh 1990). Uptake of metal ions such as Cu, Pb, Ni, Cd, and Cr was reported in *Spirulina platensis* (Prasanna et al. 2008). *Synechococcus elongatus*, *Microcystis aeruginosa*, and *Anacystis nidulans* are found to degrade organophosphorus and organochlorine insecticides in the polluted aquatic system (Vijayakumar 2012). Cyanobacteria are also capable of degrading the naturally occurring aromatic compounds such as naphthalene, phenanthrene, phenol, and catechol (Kuritz 2010). They play an important role in the detoxification of effluents from various industries such as brewery, oil refinery, paper mill, sugar mill, dye, and pharmaceutical industries (Singh et al. 2016).

Anabaena sp. was shown to remove linear alkylbenzene sulfonate, an anionic surfactant from culture medium (Yan et al. 1998). Cyanobacteria were found to get involved in the degradation of glyphosate. Usually the degradation of glyphosate is carried out by soil microorganisms, but once it gets their entry into the aquatic system cyanobacteria are found to be involved in the degradation process. They are found to tolerate high levels of the glyphosate. The resistance mechanism in cyanobacteria is suggested by the carrier independent uptake of glyphosate and the presence of the resistant form of the target enzyme 5-enolpyruvylshikimate-3-phosate (EPSP) (Arunakumara et al. 2013). The cyanobacterial species *Anabaena azotic*a was able to degrade γ-hexachlorocyclohexane (lindane) an organochlorine pesticide. The metabolite of lindane detected was γ-pentachlorocyclohexane (Zhang et al. 2012).

12.4.2 Organophosphorus Pesticide Degradation by Cyanobacteria

Cyanobacteria play an important role in the degradation of organophosphorus pesticides most of them having the ability to tolerate the pesticides to a higher level. Some of the commonly used organophosphorus pesticides and their degradation by cyanobacteria are explained below.

12.4.2.1 Chlorpyrifos

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro 2 pyridyl phosphorothioate) is a widely used broad-spectrum insecticide. It is a moderately toxic compound which is sparingly soluble in water. Microbial degradation and chemical hydrolysis are the main route of degradation in soil (Kumar et al. 2018). Half-life of chlorpyrifos is 10–120 days. Rate of chlorpyrifos degradation is slow in acidic soil. Degradation rate increases with an increase in soil pH (Singh and Walker 2006). Aerobic bacteria degrades chlorpyrifos into 3,5,6 trichloro 2 pyridinol (TCP) and diethyl thiophosphoric acid (DETP) and this reaction removes the mammalian toxicity of the pesticide (Singh and Walker 2006). Exposure to chlorpyrifos causes oxidative stress in animals and is also associated with bladder cancer and chromosomal damage (Chishti et al. 2013).

The unicellular cyanobacterium *Synechocystis* sp. strain PUPCCC 64 was reported to degrade chlorpyrifos. It removed 3.78 and 4.69 mg/L of chlorpyrifos from media containing 5 mg chlorpyrifos /L. At the initial stage, the pesticide was found to be adsorbed on the surface of the biomass followed by slow intracellular uptake. The uptake of the pesticide was increased with the increase in biomass. The organism metabolized the pesticide. One of the degradation products identified was 3,5,6 trichloro 2 pyridinol (TCP) (Fig. 12.2). Cell extract, medium, and biomass wash showed the presence of TCP which indicated both intracellular and extracellular degradation of the pesticide. Depletion of the pesticide from the culture

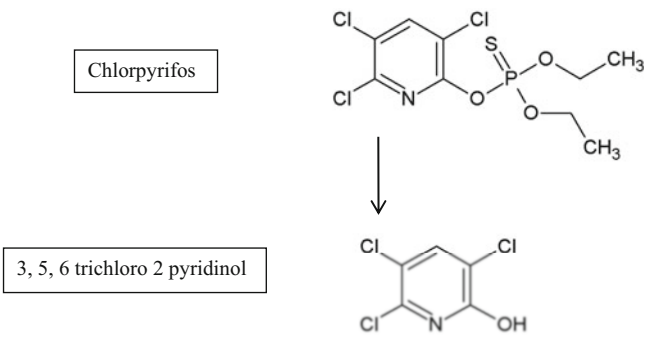
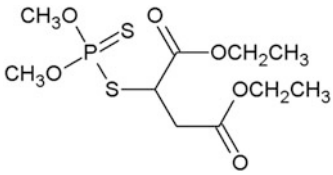


Fig. 12.2 Chlorpyrifos degradation by *Synechocystis* sp.

Fig. 12.3 Malathion



medium was suggested to be the evidence for extracellular degradation of the pesticide (Singh et al. 2011).

An alkaline phosphatase isolated from *Spirulina platensis* was found to be involved in the degradation of chlorpyrifos. The organism was able to grow in medium containing 80 ppm chlorpyrifos which was due to the activity alkaline phosphatase present in these cultures. When the organism was grown in media containing varying concentrations of chlorpyrifos (10–120 ppm), it was found that below 40 ppm, only TCP could be detected and above 40 ppm both TCP and chlorpyrifos were detected. It indicates the tolerance of the organism toward this pesticide and its ability to degrade the pesticide. The purified enzyme degraded 100 ppm chlorpyrifos to 20 ppm within 1 hour. The primary metabolite was 3,5,6 trichloro 2 pyridinol (Thengodkar and Sivakami 2010).

The marine cyanobacterium *Phormidium valderianum* was found to be able to grow in 45 ppm chlorpyrifos. 48 hour exposure to chlorpyrifos showed an increase in the activity of pesticide metabolizing enzymes such as polyphenol oxidase, catalase, superoxide dismutase, and esterase and glutathione S transferase. The organism used esterase A for metabolizing chlorpyrifos (Palanisami et al. 2009).

12.4.2.2 Malathion

Malathion (S-(1,2-dicarbethoxyethyl)-O, O-dimethyldithiophosphate) (Fig. 12.3) is a nonsystemic broad-spectrum organophosphorus pesticide. It was the first identified organophosphorus insecticide with high selective toxicity and is classified as the toxicity class III pesticide (Singh et al. 2014) It is used on crops, fruits, and vegetables to control sucking and chewing insects. It causes hepatotoxicity, human breast carcinoma, and genetic damage and affects normal hormonal activity (Ibrahim et al. 2014). Malathion has toxic effects in the immune system of higher vertebrates, adrenal gland of vertebrates, and tissues of fishes. Their degradation in soil and water mainly involve the hydrolysis of the P-S bond (Singh et al. 2014).

Ibrahim et al. (2014) studied three strains of filamentous cyanobacteria for their growth and utilization of malathion. Among the strains tested, *Nostoc muscorum* was shown to be efficient in degrading the pesticide malathion. The protein and carbohydrate content of this strain were also found to be higher when compared with the other tested strains. The strains were also tested for their growth in phosphorous deficient medium both in the presence and absence of malathion. The growth of the strain was found to be low in phosphorous deficient medium, whereas increased growth was seen in phosphorous deficient medium supplemented with malathion.

The study proved the efficiency of *Nostoc muscorum* to utilize malathion as a source of phosphorous.

Subramanian et al. (1994) reported the biodegradation and utilization of organophosphorus pesticide by cyanobacteria. Ten strains of heterocystous filamentous cyanobacteria were tested for their growth and tolerance in the presence of organophosphorus pesticides malathion and monocrotophos. Among the tested strains, two strains *Aulosira fertilissima* ARM 68 and *Nostoc muscorum* ARM 221 showed maximum growth in pesticide containing medium even in the absence of inorganic phosphorous and they are again studied for their growth in the presence of varying concentrations of monocrotophos, malathion, dichlorvos, phosphamidon, and quinalphos. Increased growth of *A. fertilissima* in the absence of inorganic phosphate indicates that the strain could use all the five tested pesticides as a source of phosphorous. The presence of pesticides induced acid phosphatase activity in the organism.

12.4.2.3 Fenamiphos

Fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidite) is a widely used pesticide for the control of soil nematodes. It is applied on a variety of plants such as banana, pineapple, citrus, vegetables, and grains (Cáceres et al. 2007). It is effective against ecto- and endoparasitic root-knot nematodes (Singh and Walker 2006). It is a toxic and persistent pesticide. Its activity in soil lasts for 3–4 months and for years in groundwater (Megharaj et al. 2003). Under environmental conditions, fenamiphos is oxidized into fenamiphos sulfoxide (FSO), which was later oxidized into fenamiphos sulfone (FSO_2). They have high solubility in water which causes surface water and groundwater contamination by leaching off the pesticide from the applied area (Caceres et al. 2008).

Caceres et al. (2008) reported the biodegradation of fenamiphos by cyanobacteria (Fig. 12.4). Oxidation and hydrolysis were the major steps in biodegradation. They tested five different species of cyanobacteria namely *Nostoc* sp. MM1, *Nostoc* sp. MM2, *Nostoc* sp. MM3, *Nostoc muscorum*, and *Anabaena* sp. Among the tested species three strains, *Nostoc muscorum*, *Nostoc* sp. MM2, and *Anabaena* sp. hydrolyzed fenamiphos into fenamiphos phenol (FP). All the tested species transformed fenamiphos into fenamiphos sulfoxide (FSO). It was then hydrolyzed into fenamiphos sulfoxide phenol (FSOP). *Anabaena* sp. was found to be having the highest potential in degrading the pesticide.

12.4.2.4 Methyl Parathion

Methyl parathion (O, O dimethyl O-*p*-nitrophenyl phosphorothioate) (Fig. 12.5) is applied as an insecticide in paddy fields. It is used on crops such as cotton, rice, melon, corn, and soy. It has a short half-life in aquatic environments (30–100 days). It is used to control insects such as aphids, mealybugs, and mites (Pino and Peñuela

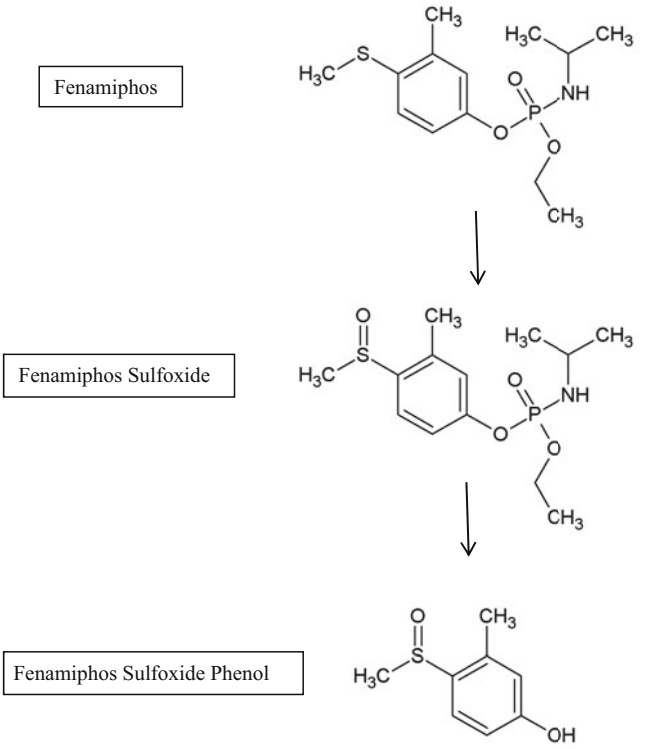


Fig. 12.4 Biodegradation of fenamiphos by cyanobacteria

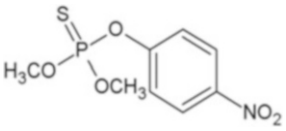


Fig. 12.5 Structure of methyl parathion

2011). On hydrolysis, it gives *p*-nitrophenol and dimethyl orthophosphoric acid. On exposure, it can cause mutagenesis, carcinogenesis, and teratogenesis (Fioravante et al. 2010).

Megharaj et al. (1994) studied the biodegradation of methyl parathion by soil isolates of cyanobacteria. Their ability to use this pesticide as a source of phosphorous and nitrogen was checked by growing them in the presence of methyl parathion. All four species showed good growth indicating their ability to use the pesticide as a nutrient source. Biodegradation was tested by measuring the disappearance of the pesticide from the culture media. Hydrolysis of the pesticide was indicated by the appearance of *p*-nitrophenol in the medium. *Nostoc muscorum*, *Oscillatoria animalis*, *Phormidium foveolarum* completely hydrolyzed the pesticide by 20 days. Further degradation of *p*-nitrophenol was indicated by the accumulation

of nitrite in the culture medium. The study proved biodegradation of methyl parathion to *p*-nitrophenol which undergone nitro group reduction forming nitrite.

Fioravante et al. (2010) investigated the removal of methyl parathion by the cyanobacteria *Microcystis novacekki* under culture conditions. Complete removal of methyl parathion was occurred and no metabolites were found in the culture medium which was explained by the mechanism of bioaccumulation and mineralization.

Fischerella is a filamentous, branched, and heterocystous cyanobacterium capable of degrading methyl parathion. The organism utilized methyl parathion (MP) as a source of phosphorous. Adsorption of the pesticide on to the cell surface was a major event in the process of degradation. Interaction of MP with -OH group on the cell surface was revealed by Fourier transform infrared analysis (FTIR). Oxidative stress was created as a result of interaction of MP with cellular components which resulted in decreased growth and pigment content. The organism responded to this condition by elevating the level of antioxidative enzyme activity and modulating the fatty acid and hydrocarbon profile. The presence of *p*-nitrophenol in the medium and the physiological activity of the organism clearly indicated the degradation of MP. When the cyanobacteria was exposed to MP under phosphorous deficient conditions, intracellular alkaline phosphatase activity was induced in the organism, which indicates the use of MP as a phosphorous source by the organism (Tiwari et al. 2017)

Barton et al. (2004) reported the reductive transformation of methyl parathion (O, O-dimethyl O-*p*-nitrophenyl phosphorothioate) by *Anabaena* sp. (Fig. 12.6). The reaction took place under aerobic, photosynthetic conditions. Methyl parathion was transformed first to *o, o*-dimethyl *o-p* nitrosophenyl thiophosphate, and then to *o, o*-

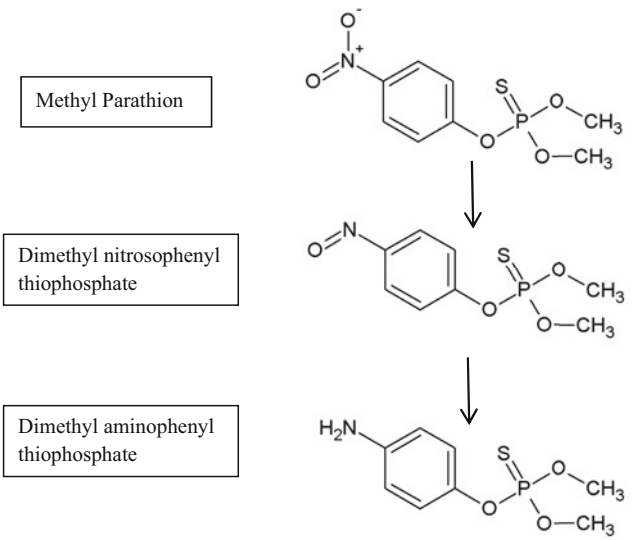


Fig. 12.6 Biotransformation of methyl parathion by *Anabaena* sp. (Barton et al. 2004)

dimethyl *o-p* aminophenyl thiophosphate. The process of transformation was occurred in the light. The pesticide was toxic to the cyanobacteria in the dark but it has no effect on the viability of cyanobacteria in light. The nitro group of methyl parathion was reduced to an amino group by the *Anabaena* sp. through the formation of nitroso group intermediate. There was no accumulation of the intermediate in the medium and it was converted to amino compound. Mutation in the gene required for nitrate reduction had no effect on methyl parathion transformation.

12.4.3 Recombinant Cyanobacteria for Biodegradation

Recombinant studies have also performed in cyanobacteria. Biodegradation of organophosphorus pesticide using recombinant cyanobacteria with surface and intracellular expressed organophosphorus hydrolase enzyme was found to be successful by Chungjatupornchai and Fa-Aroonsawat (Chungjatupornchai and Fa-Aroonsawat 2008).

Organophosphorus hydrolase (OPH) is an important enzyme involved in the degradation of organophosphorus pesticides. It is a homodimeric organophosphotriesterase. *Escherichia coli* can be used for expressing intracellular OPH. But the cell membrane acts as substrate diffusion barrier for the substrate, which affects biodegradation efficiency (Rainina et al. 1996). This enzyme from *Flavobacterium* was surface and intracellular expressed in *Synechococcus* PCC7942. A small fraction was expressed on the outermost surface of the cyanobacterial cells. A significant fraction was buried into the cell wall. Activity of OPH was tested for paraoxon degradation. Cells with intracellular expressed OPH showed higher activity than cells with surface-expressed OPH. This study is helpful in developing low-cost and low-maintenance biocatalyst for the biodegradation of organophosphorus pesticides.

All the above reports prove the potential of cyanobacteria in biodegradation of organophosphorus pesticides.

12.5 Conclusion

The use of pesticides in agricultural fields has become a necessary evil. Indiscriminate use of these pesticides is a major cause of environmental pollution. Among the various classes of pesticides used, organophosphorous pesticides are the most commonly used one. Removal of these pollutants from the environment is necessary since these compounds cause many adverse effects to living organisms. These compounds interfere with the normal functioning of the central nervous system causing various neurological disorders in humans. Various physicochemical methods exist for the removal of these contaminants from the environment. But these methods are expensive and less effective compared to biodegradation. Biodegradation is an eco-friendly method. Many microorganisms are capable of

degrading these pesticides. Cyanobacterium is a promising organism in the field of biodegradation and an excellent model system. Although other microorganisms are in use for biodegradation, cyanobacterium is considered to be a better organism because of their photosynthetic nature and nitrogen-fixing capacity. Compared to other microorganisms, cyanobacteria are less exploited for the biodegradation studies. More research is required to explore the potential of cyanobacteria for pesticide degradation. Genetic engineering can be employed to increase the degradative ability of cyanobacteria. Many researches are being carried out in this area and the main aim of these studies is the identification and characterization of new strains of cyanobacteria for the efficient biodegradation of pesticides.

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Chapter 13
Microbial Identification and Extracellular Polymeric Substances Characterization of Aerobic Granules Developed in Treating Rubber Processing Wastewater

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Abstract The goal of this study was to investigate the species of the abundance microbial in seed sludge and aerobic granular sludge. Experiments were carried out in a sequencing batch reactor with a working volume of 1.6 L. During the start-up period, the reactor was inoculated with 800 mL of sludge from a municipal sewage treatment plant plus 800 mL of rubber processing wastewater. Further investigation by Illumina high-throughput sequencing was performed to analyze the microbial diversity and phylogenetic structures during the granulation of seed sludge to aerobic granules. A diversity of microorganisms was identified from the seed sludge and aerobic granular sludge. Seed sludge consisted of 96.4% bacteria, 1.7% eukaryote, 1.2% archaea, and 0.7% viruses. Aerobic granular sludge consisted of 97.8% bacteria, 1.0% eukaryote, 0.8% archaea, and 0.4% viruses. As the granulation process succeeded in SBR, distinct differences of the microbial community in the seed sludge and aerobic granular sludge were observed, which suggested that there was high microbial selection pressure during granulation in the system. The most

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abundance species in seed sludge was *Dechloromonas aromatica*, while *Pseudomonas fluorescens* was the most abundance species in aerobic granular sludge.

Keywords Aerobic granular sludge · Sequencing batch reactor · Microbial community · Metagenomics · Rubber processing wastewater · Wastewater treatment

13.1 Introduction

The efficiency of biological wastewater treatment system depends on the selection and growth of microorganisms. Microorganisms required a specific environment to grow since the microbial populations change as the environment changes (Sun et al. 2015). The wastewater treatment system operation could be manipulated to develop a favorable environment for growth of granule aggregation species in the system. The sequencing batch reactor (SBR) condition is conducive to a pressure selection of excellent aggregation bacteria population.

The granulation process is affected by environmental conditions, which changes the physiology of the microbes to transform from a dispersed sludge to a dense aerobic granular sludge. The greater community of hydrophobic bacteria in activated sludge allows the granular sludge to develop into dense granules with excellent settling properties. Meanwhile, hydrophilic bacteria in the system do not anticipate in the aggregation process (Wilen et al. 2004). Thus, it was emphasized that the development of aerobic granular sludge from seed sludge involved different physicochemical characteristics bacteria with different capabilities in cultivating aerobic granular sludge. The evolution of microbial community in the sludge is essential for successful aerobic granular sludge formation (Zhang et al. 2011; Zhao et al. 2013).

The identification and characterization of microbial population and diversity in biological wastewater treatment system such as granulation SBR has been increasingly studied based on deoxyribonucleic acid (DNA) molecular biology techniques (Zhao et al. 2013; Li et al. 2008, 2010; Weissbrodt et al. 2014; Aqeel et al. 2016). The purpose of microbial communities' studies is to discover species composition, structure, spatial activity, and the bacterial distribution within an environment, as well as the function of microbial (Cardenas and Tiedje 2008). In this study, metagenomics sequencing analysis was used to determine the microbial diversity in both samples of seed sludge and aerobic granular sludge in order to understand the compositions and changes of bacterial communities during granulation process. Thus, this study provided further understanding on evolution of bacteria in the formation of aerobic granular sludge in treating rubber processing wastewater.

Extracellular polymeric substances (EPS) are sticky materials secreted by cells and highly involved in the formation of matrix structure and improvement of long-term stability of aerobic granular sludge. It is thought that EPS act as an effective bioglue to cross-link bacteria into an aerobic granule and the EPS matrix of the aerobic granule can protect bacteria from harsh environmental conditions. Besides, EPS facilitate cell-to-cell interaction and further strengthen microbial structure by

forming a polymeric matrix. It has been reported that sludge cells have a double-layered EPS structure which are loosely bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) (Liu and Fang 2003). EPS produced in aerobic granules contain variable proportions of proteins, polysaccharides, nucleic acids, humic-like substances, lipids, and heteropolymers-like glycoprotein (McSwain et al. 2005; Adav et al. 2008).

The production of EPS in aerobic granules is induced by some so-called stressful culture conditions (Nichols et al. 2004; Qin et al. 2004). A number of operating parameters such as substrate composition, substrate loading rate, hydrodynamic shear force, settling time, feast-famine regime, culture temperature, and cycle time may stimulate bacteria to secrete more EPS. The composition of EPS is also related to the characteristics of the feed wastewater. For example, EPS in anaerobic granules grown on protein-rich wastewater had high protein and DNA levels, whereas high polysaccharides content was found in anaerobic granules fed by other types of organic wastewaters (Batstone and Keller 2001).

There is no need for microorganisms to secrete excessive EPS under normal culture conditions. Cycle time in sequencing batch reactor (SBR) is one of the operating conditions that may enhance the production of EPS in aerobic granules. In this study, the production and composition of EPS in aerobic granular sludge was investigated at different cycle times (3, 6, and 12 h). Furthermore, 3D-EEM spectroscopic technology was used for component analysis of extracellular polymeric substances (EPS) in aerobic granules. It is hopeful for understanding the characteristics of EPS from aerobic granular sludge.

13.2 Materials and Methods

13.2.1 Wastewater Collection

Real wastewater was used throughout the experimental period which is the Standard Malaysian Rubber (SMR) process wastewater, obtained from a local rubber processing factory located at Kota Tinggi, Johor, Malaysia. The existing wastewater treatment plant applied for treating rubber wastewater is the pond system. The treatment system comprises settling tank (to trap large debris or particle), one anaerobic pond (with size of 140 m $\times$ 60 m $\times$ 3.4 m), and five facultative ponds (with different size, capacity, and retention time). Raw SMR process wastewater which was used as feed was sampled directly from the settling compartment. The collected rubber processing wastewater was kept in cold storage room at a temperature of 4 °C to prevent the wastewater from undergoing biodegradation due to microbial activity.

13.2.2 Seed Sludge Sampling

A fresh activated sludge was taken from an aeration tank of a local, domestic wastewater treatment plant which is located at Taman Angkasa (JKI039), Senai, Johor. The sludge was sieved with a mesh of 1.0 mm twice to remove large debris and particles before inoculation into the reactor. Figure 13.5 shows several pictures taken during the seed sludge sampling at the site. The domestic wastewater treatment plant was operated with an extended aeration type.

13.2.3 Experimental Setup

Experiments were carried out in an SBR with a working volume of 1.6 L. During the start-up period, the reactor was inoculated with 800 mL of sludge from a municipal sewage treatment plant plus 800 mL of rubber processing wastewater. The rubber processing wastewater entered through port located at the bottom of the reactor column. The organic loading rate (OLR) was varied throughout the experiments since real rubber processing wastewater was fed into the reactor as influent. While fine air bubbles for aeration stage were supplied by means of a porous air stone placed at the bottom at a volumetric flow rate of 3.5 L/min (1.16 cm/s superficial upflow air velocity) through an aerator pump. A mass-flow controller was used to keep the air flow constant.

After the aeration process stopped, the activated sludge suspension was left for solids settlement about 10 min. The effluent of 500 mL was discharged through the outlet port positioned at 22 cm height from the bottom of column reactor, resulting in volumetric exchange ratio (VER) of 30%. The reactor was operated at room temperature (27 ± 2 °C) and without oxygen and pH control. The dissolved oxygen (DO) in the reactor during the aeration process was within the range of 6.0–7.5 mg/L.

The cycle time was varied between 3 and 12 h to study the influences of cycle time on the characteristics of aerobic granular sludge and reactor removal performances. The experiment was conducted separately for three different cycle times with the same reactor dimension, working volume (1.6 L), and volumetric exchange ratio (30%). Details of the experimental conditions are shown in Table 13.1. The organic loading rate (OLR) was changed due to the different cycle times.

For extracellular polymeric substances (EPS) characterization analysis, the aerobic granules were harvested from the reactor at all cycle times of 3, 6, and 12 h during the end of each operation period. The compositions of EPS of aerobic granular sludge were measured including protein, polysaccharide, and carbohydrate.

Table 13.1 Detailed experimental conditions of the reactor system

	Cycle time (h)		
Operational conditions	3	6	12
Feeding (min)	5	5	5
Aeration (min)	150	330	690
Settling (min)	10	10	10
Decanting (min)	5	5	5
Idling (min)	10	10	10
Cycles per day (cycle/d)	8	4	2
Exchange ratio (%)	30	30	30
OLR (kg COD/m^3 day)	2.52–3.31	1.26–1.67	0.63–0.83
SUAV (cm/s)	1.16	1.16	1.16

13.2.4 Characteristics of Extracellular Polymeric Substances

A heat extraction method was modified to extract extracellular polymeric substances (EPS) from seed sludge and aerobic granules suggested by Li and Yang (2007). The measurement for EPS characteristics included protein, polysaccharide, carbohydrate, and excitation-emission matrix (EEM) fluorescence spectroscopy.

13.3 Results and Discussion

13.3.1 Microbial Diversity in Seed Sludge and Aerobic Granular Sludge

Since the functions and behaviors of microbial diversity influenced the performance of aerobic granular sludge in SBR, further investigation by Illumina high-throughput sequencing was performed to analyze the microbial diversity and phylogenetic structures during the granulation of seed sludge to aerobic granules. From the analysis, microbial distribution of both samples demonstrated similar diversity. Table 13.2 and Fig. 13.1 show the microbial diversity of seed sludge and aerobic granular sludge in term of the relative abundance percentages. There are four main microorganisms' domains that existed in both seed sludge and aerobic granular sludge which were bacteria, eukaryote, archaea, and viruses. This is in agreement with Yu and Zhang (2012), who reported a complex microbial system, consists of bacteria, eukaryote, archaea, and viruses which are highly exist in activated sludge as well as granular sludge.

In the seed sludge, bacteria was accounting for 96.4%, followed by eukaryote with 1.7%. Meanwhile, archaea and viruses were about 1.2% and 0.7%, respectively. However, as the granules developed in rubber processing wastewater, the percentage of microbial domains was varied. The abundance of bacteria was increased to 97.8%,

Table 13.2 Top 15 of the identified bacteria species from seed sludge and aerobic granular sludge (AGS)

No.	Bacteria species in seed sludge	Bacteria species in AGS
1	*Dechloromonas aromatica*	*Pseudomonas fluorescens*
2	*Acidovorax* sp. JS42	*Rhodobacter* sp. SW2
3	*Albidiferax ferrireducens*	*Brucella canis*
4	*Polaromonas* sp. JS666	*Shewanella* sp. ANA-3
5	*Acidovorax citrulli*	*Stenotrophomonas maltophilia*
6	*Comamonas testosteroni*	*Clostridium subterminale*
7	*Variovorax paradoxus*	*Acidovorax* sp. JS42
8	*Delftia acidovorans*	*Candidatus Accumulibacter phosphatis*
9	*Alicycliphilus denitrificans*	*Thauera* sp. MZ1T
10	*Rhodopseudomonas palustris*	*Alicycliphilus denitrificans*
11	*Methylococcus capsulatus*	*Acidovorax delafieldii*
12	*Pseudomonas aeruginosa*	*Thiobacillus denitrificans*
13	*Acidovorax ebreus*	*Pseudomonas aeruginosa*
14	*Anaeromyxobacter dehalogenans*	*Nitrosococcus oceani*
15	*Pedosphaera parvula*	*Bradyrhizobium* sp. BTAi1

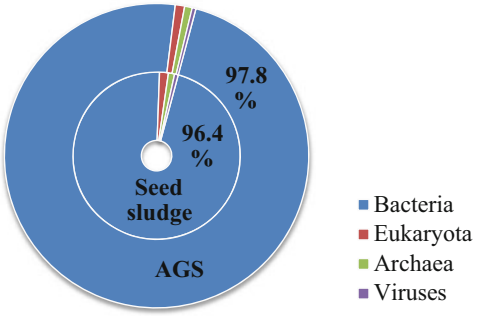

Fig. 13.1 Percentage distribution of microbial in seed sludge and aerobic granular sludge (AGS)

eukaryote was decreased to 1.0%, archaea was 0.8%, and viruses were 0.4% in aerobic granular sludge. The difference in the domains distribution showed the changes of microbial communities during the granulation process. As claimed by Yadav et al. (2014), shifting of present microbial community was due to change of the operational parameter in the treatment system such as settling, flocculation, and floc formation characteristics. Those operational parameters also control the microbial diversity. For example, the diversity of microorganisms in a system that operated with short settling time would retain fast settling microorganisms, while, others with slow settling properties were being washed out from the system. So, the diversity of slow settling microorganisms in the system was affected by the operational parameter of the short settling time.

Bacteria were the dominant domain in both seed sludge and aerobic granular sludge compare with other domains. The percentage abundance of bacteria in seed sludge was 96.4% and increased to 97.8% in aerobic granules. Basically, bacteria

form a large percentage of microbial community since they are the most versatile of all organisms in terms of their nutrient requirements, catabolic and metabolic activities (Di Iaconi et al. 2010). Bacteria played an important role in degrading organic and nutrient of pollutant. Bacterial community that resided in seed sludge was important for aerobic granulation process (Lee et al. 2010). Almost all bacteria excrete extracellular polymeric substances (EPS), as they are surface-attached and favor the formation of aerobic granules (Liu et al. 2004a).

Within the bacteria domain, there are several common types of microbial that were found in seed sludge, for example, floc formers, filamentous bacteria, and denitrifiers. Comparing the microbial population between seed sludge and aerobic granular sludge, it is revealed there is a relative activity level of different populations in a microbial community. Determination of abundance of bacteria from the aerobic granules has provided significant information that the involvement of these bacteria is important in the granulation process. Significantly, the performances of aerobic granular sludge in biodegradation of organic and nutrients were also in consequence to the bacterial community that occupied in aerobic granules.

Eukaryote is an organism that feeds on bacterial cells and detritus. It has a minority population of all living things (Whitman et al. 1998). Even though not directly contributing to granular structure, eukaryote can provide selective pressure for floc and granule formation as a defense mechanism to avoid predation (Williams and De los Reyes 2006). Several studies on fungi and protozoa in activated sludge system demonstrated that these eukaryotic organisms fulfill a wide variety of important tasks in the biomass conversion and water clarification processes (Nicolau et al. 2001; Weber et al. 2007) revealed that eukaryotes are involved in the formation, structure, and function of biofilms for several biofilm systems besides wastewater treatment. Meanwhile, in aerobic granulation system, eukaryotic organisms play a crucial role in the formation of granules. Protozoa with stalked ciliates were always involved in the process of granule development, whereas fungi were found only in some cases (Weber et al. 2007).

The abundance percentage of eukaryote during initial stage of the experiment was 1.7% and decreased to 1.0% when aerobic granular sludge was developed. According to Li et al. (2010), the high COD loading condition in a system as the sequencing batch reactor (SBR) system used in this study was unfavorable to enhance the growth of eukaryote species. Eukaryote is a microbial that could lead to the formation of filamentous structure of granules (Weber et al. 2007). Aerobic granular sludge developed in the SBR had a clear boundary outline with nonfilamentous growths. This is because the abundance of eukaryote species was in a small amount which not capable to lead the formation of filamentous sludge. The filamentous organisms are generally undesirable in wastewater treatment processes as they contribute to detrimental effects, like bulking (Martins et al. 2004). However, filamentous fungi can aid in strengthening sludge flocs by acting as the floc backbone, as well as immobilizing particulate sludge during settlement, thus enhancing the separation process (More et al. 2010).

In the SBR, 1.2% of archaea were accounting in seed sludge and 0.8% in aerobic granular sludge. The abundance of archaea population became smaller as the aerobic

granules developed in SBR. Basically, archaea that involved in sewage treatment works are methanogens species that lived in anaerobic environments (Kundu et al. 2014). Therefore, in the deeper parts of aerobic granular sludge, anaerobic micro-environments were existed which may allow growth of the methanogens organisms (Gao et al. 2014). As proved by Kundu et al. (2014), the archaea community was present in the inner layer with limited oxygen concentration, while, bacteria were present at the outer layer of granules.

Very little in a number is known about archaeal communities in seed sludge and aerobic granules compared with bacteria community. Although present, archaea seem to be minor importance for both nitrogen and carbon removal. However, it is still possible that the archaea have other functions which affected the properties of aerobic granules. Archaea can constitute a small but constant as integral part of activated sludge and that it can therefore be useful to include archaea in future studies of sludge and granules properties (Fredriksson et al. 2012). In aerobic granular sludge, archaea are important for the structure and stability as they form dense aggregates which act as nuclei for granule formation (Zheng et al. 2006). However, in activated sludge, archaea did not appear to have this function since they were mostly detected as small colonies or single cells and there was no apparent difference in structure between flocs with high or low numbers of archaea (Fredriksson et al. 2012).

The detection of pathogenic viruses circulating in the environment is a fundamental component of health monitoring. It is important to monitor viruses in wastewater (both raw and treated) to protect the environment from being exposed to wastewater discharges from biological contamination and prevent further spread of pathogens. Significantly, it is vital to reduce and remove the pollutants including viruses and pathogenic organisms in wastewater treatment. The abundance of viruses at the initial treatment of SBR was 0.7% and being reduced to 0.4% at the end of the experiment. The virus community reduction was achieved up to 97% using activated sludge process (Arraj et al. 2005). Nonetheless, biological treatment processes are not always successful in removing pathogens and viruses, hence chemical disinfectants are required to be added to the treatment process.

13.3.2 Comparison of Bacterial Community in Seed Sludge and Aerobic Granules

In order to analyze the shift in the bacteria community during granulation process, the phylogenetic profiling of seed sludge and aerobic granules samples were compared. The relative abundance of bacteria diversity is demonstrated in Fig. 13.2. The figure shows abundance percentage from phylum level of bacteria population in seed sludge and aerobic granular sludge. The top 20 taxonomic categories at phylum level have been summarized. Bacterial population distributions from both samples were significantly different at the phylum level. This showed that there was immense

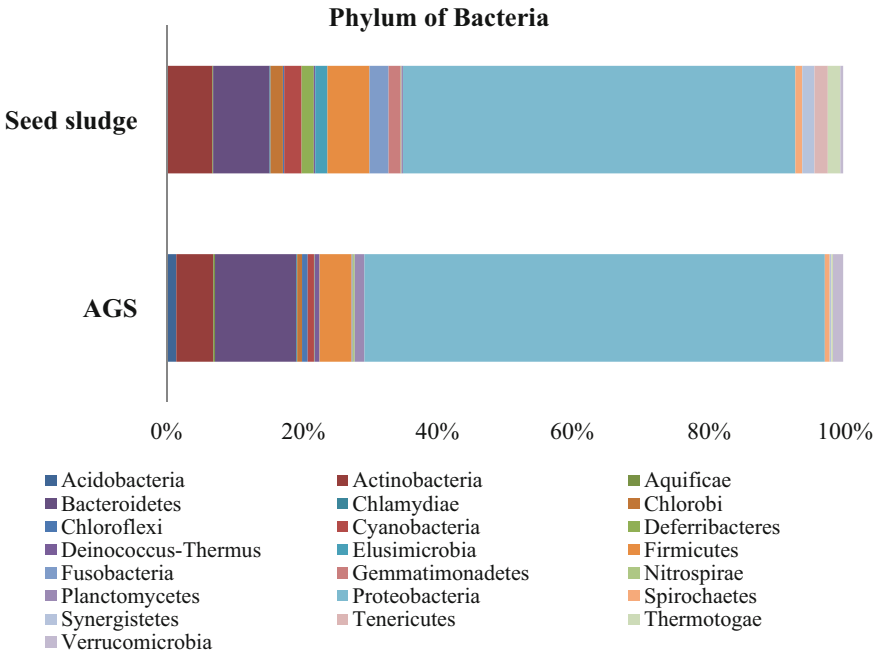

Fig. 13.2 Classification of top 20 bacterial taxonomic at phylum level of seed sludge and aerobic granular sludge (AGS)

diversity of bacteria in both samples. Additionally, some evidence of conserved differences between the samples sets indicates that these differences may be inherent to the granulation. In phylum levels, Proteobacteria was the dominant taxonomy in both seed sludge and aerobic granular sludge followed by Bacteroidetes and Actinobacteria. The varieties of bacteria had assured that they could contribute to the characteristics of aerobic granular sludge (Winkler et al. 2013).

There were 71.0% and 77.0% of annotated phylum belonging to Proteobacteria in seed sludge and aerobic granular sludge, respectively. The high abundance of Proteobacteria in activated sludge was also reported by Yu and Zhang (2012) as well as the analysis of bacteria communities in sewage influent in which Proteobacteria was the most dominant community. The dominant of Proteobacteria is an advantage to the wastewater treatment system for enhancing the biological phosphorus removal (Lee et al. 2002). In this study, Proteobacteria was the dominant taxonomy observed in both seed sludge and aerobic granular sludge. Proteobacteria phylum has the highest abundance compared with other phylum and followed by Bacteroidetes (8.3% in seed sludge and 12.0% in aerobic granules) and Actinobacteria (6.7% in seed sludge and 5.5% in aerobic granules). Therefore, two major phylum that have been identified from the phylum level in both samples were Proteobacteria and Bacteroidetes. Most of detected bacteria in the seed sludge in this study had also been reported in the treatment of municipal wastewater (Bolhuis and

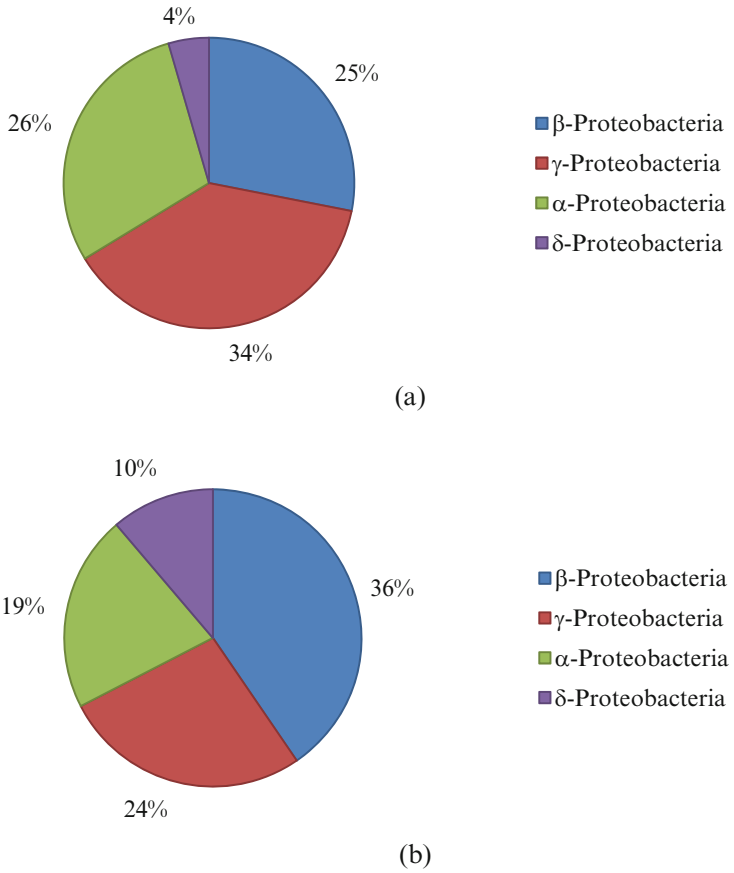

Fig. 13.3 Distribution of Proteobacteria at class level in (**a**) seed sludge and (**b**) aerobic granular sludge

Stal 2011). This is because seed sludge used in this study was also from the municipal wastewater treatment plant that contributed to a high total dissolved solid, which favors the growth of bacteria in the activated sludge.

In class level, Betaproteobacteria (β-Proteobacteria), Gammaproteobacteria (-γ-Proteobacteria), and Alphaproteobacteria (α-Proteobacteria) were dominant in the seed sludge and aerobic granular sludge as shown in Fig. 13.3. The domination of those bacteria class was due to the highest percentage of Proteobacteria phylum in the system. There are many studies on the phylogenetic groups of bacteria in activated sludge reported that the most predominant group found were from β-Proteobacteria class (Bond et al. 1998; Jiang et al. 2008). The same goes with this study as the β-Proteobacteria were dominant class of bacteria in aerobic granular sludge. Etterer (2006) discovered the bacteria under β-Proteobacteria level were able to grow and dominated the microbial structure without destabilizing the granule structure.

Previous studies showed that β-Proteobacteria were often dominant both in aerobic biofloc and aerobic granular sludge (Bond et al. 1999; Williams and De los Reyes 2006; Thomsen et al. 2007). The most known aerobic nitrogen-cycling bacteria are mainly β-Proteobacteria, including nitrifying and denitrifying bacteria. Langone et al. (2014), claimed that highly diversified denitrifiers from the β-Proteobacteria were detected in nitritation-anammox biomass, showing the importance of denitrification in nitritation-anammox systems when treating ammonium-rich wastewaters containing organic substrate. The importance of denitrifying bacteria in a nitritation-anammox process is related to the overall nitrogen removal efficiency by contributing to the removal of nitrate produced by the anammox process (Desloover et al. 2011), and also other aspects, such as aggregates formation. β-Proteobacteria were also reported to be dominant in aerobic granules by Li and Li (2009).

The bacteria under class γ-Proteobacteria have been reported capable of heterotrophic nitrification and aerobic denitrification (Zhang et al. 2011; Kim et al. 2008 and Su et al, 2006). γ-Proteobacteria were also mostly present in aerobic granular sludge. Bacteria under γ-Proteobacteria class can grow heterotrophically mainly on low substrate and store carbon source as intracellular polyhydroxyalkanoates (PHA) (Yan et al. 2007). They are versatile microorganisms which can grow aerobically to reduce carbon and nitrogen compounds. As proved in previous studies (Di Iaconi et al. 2006; De Sanctis et al. 2010), the occurrence of simultaneous aerobic and anaerobic conditions in granular sludge leads to optimal carbon and nitrogen removals, which confirm the flexibility and applicability of this system for treating a wide range of complex wastewaters.

Significantly, α-Proteobacteria played important role in the function of aerobic granular sludge since they are often associated with granules development and influenced the settling properties of aerobic granules (Levantesi et al. 2004; Van Der Waarde et al. 2002). According to Kragelund et al. (2006), the α-Proteobacteria has the ability to form granules. α-Proteobacteria is a large and robust filamentous bacterium which were found to be dominant in many industrial activated sludge systems (Martins et al. 2004). Therefore, it is essential for the filamentous bacteria to stay attached to the granules in order to settle in the clarifier and not to be washed out with the effluent. Moreover, α-Proteobacteria had proved the capabilities in degrading nitrate and nitrite in substrate (Kragelund et al. 2006).

In the present study, another bacterial phylum, i.e., Bacteroidetes were detected in both seed sludge and aerobic granular sludge with percentages of 8.3% and 12.0%, respectively. According to Shah and Gharbia (2010) and Dahalan (2012), Bacteroidetes are normally present in diverse habitats, such as human body system, soil, and fresh water. Significantly, the presence of Bacteroidetes phylum which commonly associated with the human's oral cavity and gastrointestinal tract were able to play a significant role in the formation of aerobic granules (Dahalan 2012). Bacteroidetes were observed to be one of the dominant bacterial populations commonly found in aerobic granules as investigated in various studies (Zhang et al. 2011; Adav et al. 2009; De Sanctis et al. 2010). Interestingly, some obligated anaerobic Bacteroidetes had been found deep inside aerobic granular sludge (Tay

et al. 2002). Guo et al. (2011) confirmed that a number of bacteria classified under the phylum Bacteroidetes were the main EPS inducer to increase cell hydrophobicity during flocculation and granulation of aerobic granules. Bacteroidetes in the granules produce EPS or slime layers, which extend beyond the outer cell wall due to metabolism and cell lysis (Wingender et al. 1999). EPS have been reported as major granular sludge components to keep the granules together in a three-dimensional matrix and granulation key in a treatment system (Liu et al. 2004a). Hence, EPS might be responsible in determining biomass production and the formation of aerobic granular sludge.

The presence of bacteria from Actinobacteria phylum with the percentages of 6.7% and 5.5% were detected in seed sludge and aerobic granular sludge, respectively. This signified with the aerobic condition used to develop aerobic granules which are beneficial to the growth of Actinobacteria in SBR. Actinobacteria's habitat is generally observed to be in soil, which plays important roles in decomposition and humus formation. Song et al. (2010) reported that Actinobacteria have important roles in the formation of aerobic granular sludge when all the bacteria extracted from aerobic granules belonged to the class of Actinobacteria. However, microbial aggregation is probably not restricted to a single organism, and specific process conditions may select other organisms with similar functions (Bossier and Verstraete 1996; Beun et al. 1999; Wang et al. 2009).

13.3.2.1 Abundant Bacterial Species of Seed Sludge and Aerobic Granular Sludge

This study was further investigated the species of the abundance microbial in seed sludge and aerobic granular sludge. Table 13.2 shows the most abundance species from seed sludge and aerobic granular sludge. The bacteria species were dominated the top 15 of microbial abundance rather than eukaryote, archaea, and viruses in the seed sludge and aerobic granular sludge due to more than 70% of microbial species in the SBR system were from bacteria domain. Wastewater treatment plant provided a suitable condition to enhance the growth of bacteria to degrade organic and nutrient. There are some physicochemical and ecological factors that favor the growth of certain types of bacteria, but they may be detrimental to the growth of some other bacteria (Dahalan 2012). This is due to maintaining the biodiversity balance on the microbial ecosystem (Nielsen et al. 2010).

From the bacterial species listed in Table 13.2, the most abundant species in the seed sludge was *Dechloromonas aromatica*. These species have been shown to degrade several aromatic compounds and to be involved in biofilm formation (Langone et al. 2014). Moreover, *Dechloromonas aromatica* strain RCB appears to support a highly complex lifestyle, which may involve granules formation and interaction with eukaryotic host (Salinero et al. 2009). Dechloromonas spp. has been described as an accompanying guild of Accumulibacter, and has been proposed as putative polyphosphate-accumulating organisms (PAO) as well (Kong et al. 2007; Oehmen et al. 2010).

Meanwhile, the presence of polyphosphate accumulating organism (PAO), *Candidatus Accumulibacter phosphatis* was noticed in the aerobic granular sludge in which these bacteria favoring the phosphorus release/uptake and hence, indicates that phosphorus removal could play a role in the granular sequencing batch reactor (SBR). Another bacterial species, Thauera sp. MZ1T was found in abundance in the aerobic granular sludge. It is unique among the Thauera spp. based on its abundant production of extracellular polymeric substances (EPS), which contributes to sludge granulation (Allen et al. 2004; Heylen et al. 2006). Liu et al. (2010) described that the genus Thauera was found to be a highly abundant species in aerobic granular sludge fed with acetates. Thauera and Nitrospira are found in nitrifying–denitrifying activated sludge and conventional activated sludge systems (Zhang et al. 2011). These genera have also been reported as major populations in other single-aerobic granules (Li et al. 2008; De Sanctis et al. 2010).

Additionally, some bacterial species such as *Thiobacillus denitrificans*, *Nitrosococcus oceani*, and *Bradyrhizobium* sp. BTAi1 were present in the aerobic granular sludge. Each bacterial species has its own unique characteristics and different roles, such as being involved directly or indirectly in the biodegradation of organics and nutrients and, successful formation of aerobic granular sludge. For example, *Thiobacillus denitrificans*, a Proteobacteria from beta-subclass, can remediate engineered wastewater treatment systems by removing excess nitrate. Moreover, *Nitrosococcus oceani*, a Proteobacteria from gamma-subdivision, is a Gram-negative, ammonia-oxidizing bacterium (AOB). Its main source of energy is from oxidized ammonia, and in return, the cells release nitrogen and nitrogen oxide. More importantly, *N. oceani* performs both nitrification and denitrification activities. In addition, *Bradyrhizobium* sp. BTAi1, a Proteobacteria from alpha-subclass, is a Gram-negative, nonspore-forming bacterium. These types of species can produce large amount of water-soluble extracellular polysaccharide.

The determination of various types of bacteria from seed sludge and aerobic granular sludge signified that the involvement of these bacteria is important in the granulation process. The composition of different genus and species of bacteria found in seed sludge and aerobic granular sludge has confirmed that these bacteria contributed to the granular sludge formation scheme, morphology, and physical and chemical characteristics of the aerobic granules. Furthermore, the performance of aerobic granular sludge in the biodegradation of organic and nutrient was based on the bacterial community occupying the aerobic granules. As suggested by Wang et al. (2005), the relationships between different genera of bacteria are complex due to the existence of diverse and large quantity of microorganisms in the compacted form of sludge flocs, i.e., aerobic granules. It is necessary for the diverse community of bacteria and other possible microorganisms to coexist and contribute to the sludge granulation process.

13.3.2.2 Role of Bacteria in Sludge Granulation

The formation mechanism of aerobic granular sludge is basically influenced by many physical and chemical factors that trigger the microbial aggregation in the

system to form stable and dense microbial community under the stressful operational conditions (Erşan and Erguder 2014). EPS is one of the important factors that enhanced the aggregation of bacterial cells during the granulation process, which secreted by microbes as the general attributes in the bacteria's natural environment (More et al. 2014). The higher amount of microorganisms in an aerobic granular sludge provides higher amount of EPS secretion, which gives an advantageous in improvement of granules density, structural properties, and settling velocities. The higher amount of effective microorganisms in an aerobic granular sludge could also increase the efficiency in treating high strength wastewaters (Chen et al. 2010). EPS is a sticky material that secreted by microorganisms as metabolic products that facilitate the cell-to-cell adhesion to aggregate the microbial biomass, which is crucial for start-up of granulation process (Sheng et al. 2010; Wang et al. 2014; Kim et al. 2014; Schmidt and Ahring 1996; Veiga et al. 1997) also supported that microbial population and EPS play important roles in the formation of granular sludge. Therefore, the bacteria that potentially produced EPS, which led to the granulation of aerobic granular sludge in SBR system need to be identified.

Table 13.3 lists the top 30 of EPS producing bacteria that had been identified in the seed sludge and aerobic granular sludge. The total percentage abundance of EPS producing bacteria in aerobic granular sludge was 70.71%, which is higher than seed sludge (25.29%). The higher percentage of EPS bacterial population in aerobic granules lightened the assumption of higher EPS bacterial existence in SBR, which supported the granulation of aerobic granular sludge. This may indicate that the EPS bacteria population is related to the physiological state of bacteria affected by the operating conditions to develop aerobic granules (Liu et al. 2004a). As the EPS producing microbial, Pseudomonas, Bacteroides, Clostridium, Citrobacter, Flavobacterium, Agrobacterium, Escherichia, and Thauera were the most abundance bacteria identified in aerobic granular sludge and the abundance was higher compared with seed sludge.

A Rhodocyclales-affiliated organism such as Thauera was found higher in aerobic granular sludge with 1.28% of the bacteria species. The Rhodocyclales-affiliated organisms share some physiological properties in biological nutrient removal system of wastewater treatment plants (Hesselsoe et al. 2009). They produce EPS and store poly-β-hydroxyalcanoates (PHA) when high organic load is present under aerobic condition and forms flocs or biofilms (Sich and Van Rijn 1997; Allen et al. 2004; Dugan et al. 2006; Oshiki et al. 2008; Seviour et al. 2003). Feast-famine regimes and high shear stress applied on the reactor system would also trigger and stimulate EPS production during granulation (Seviour et al. 2003; Liu and Tay 2002; Dulekgurgen et al. 2008). *Thauera* sp. has been described as a floc-forming bacterium and could produce copious quantities of EPS from relatively simple short chain fatty acids. Guo et al. (2011) reported that there was high abundance of Betaproteobacteria such as *Thauera*, *Zoogloea*, and other unidentified *Rhodocyclaceae* are present in hydrophobic population of aerobic granular sludge.

Firmicutes that had found in aerobic granular sludge, which was producing EPS for the microbial aggregation purposes are Bacillus, Clostridium, and Staphylococcus with 0.60%, 2.68%, and 0.11% of the abundance in aerobic granules, respectively. Hence, the existence of EPS producing bacteria from the phylum Firmicutes

Table 13.3 EPS producing bacteria involved in granulation process identified from the seed sludge (SS) and aerobic granular sludge (AGS)

No.	EPS Microbial	% Abundance	
		Seed Sludge	AGS
1	*Pseudomonas*	9.39	34.05
2	*Bacteroides*	2.52	10.27
3	*Clostridium*	1.42	2.68
4	*Citrobacter*	0.24	2.19
5	*Flavobacterium*	0.35	1.92
6	*Agrobacterium*	0.66	1.70
7	*Escherichia*	0.51	1.52
8	*Rhizobium*	0.54	1.33
9	*Thauera*	0.31	1.28
10	*Azotobacter*	0.28	1.08
11	*Vibrio*	1.50	1.27
12	*Sphingomonas*	0.71	1.11
13	*Serratia*	0.73	1.01
14	*Yersinia*	0.52	1.08
15	*Rhodococcus*	0.39	0.96
16	*Enterobacter*	0.25	0.85
17	*Proteus*	0.23	0.32
18	*Hyphomonas*	0.21	0.88
19	*Klebsiella*	0.28	0.87
20	*Acinetobacter*	0.65	0.83
21	*Aeromonas*	1.07	0.62
22	*Bacillus*	0.62	0.60
23	*Chryseobacterium*	0.18	0.59
24	*Arthrobacter*	0.29	0.42
25	*Pantoea*	0.24	0.39
26	*Corynebacterium*	0.27	0.37
27	*Bifidobacterium*	0.28	0.16
28	*Legionella*	0.23	0.15
29	*Staphylococcus*	0.22	0.11
30	*Lactobacillus*	0.20	0.10
Total % abundance of EPS bacteria		25.29	70.71

in aerobic granular sludge is beneficial for granulation. Meanwhile, according to Dumitriu (2004), prokaryotes with Gram-positive rods bacteria such as *Bacillus* and *Clostridium* with rigid cell wall have the ability to secrete EPS that widespread among the microorganism. Zhang et al. (2007) proposed that a mixed culture consortium of *Staphylococcus* and *Pseudomonas* were able to produce highly amount of EPS.

13.3.2.3 Role of Bacteria in Organic and Nutrients Biodegradation

The most important organisms in biological wastewater treatment plants are bacteria. Bacteria at wastewater treatment plants can be classified according to the roles that it performs. Table 13.4 lists the top 30 of potential bacteria for biodegradation process in SBR which identified from the seed sludge and aerobic granular sludge. From the table, the total amounts of biodegradation bacteria in aerobic granular sludge were 65.08%, which were higher than seed sludge (25.32%).

From the analysis, some denitrifying bacteria were detected higher in the aerobic granular sludge compared with seed sludge. In this study, the denitrifying bacteria that existed in SBR were *Pseudomonas*, *Alicycliphilus*, *Rhodobacter*, *Clostridium*, *Agrobacterium*, *Escherichia*, *Thauera*, *Comamonas*, *Enterobacter*, *Acinetobacter*, and others. The denitrifying bacteria are facultative anaerobic bacteria that use nitrate (NO_3) in the absence of oxygen to degrade soluble COD. The use of NO_3 results in the return of nitrogen to the atmosphere as molecular nitrogen (N_2) and nitrous oxide (N_2O) (Wei et al. 2012). Denitrification is important to reduce concentration of total nitrogen (TN) in the discharged effluent of wastewater treatment plant Gerardi (2006). Although there are numerous denitrifying bacteria, there are three important bacteria in denitrifying process, which are *Alcaligenes*, *Bacillus*, and *Pseudomonas*. Fortunately, those three denitrifying bacteria had been retained in SBR during granulation process which promising the efficiency of denitrification process in SBR.

In this study, nitrifying bacteria were also detected in the SBR and the percentage abundance in aerobic granular sludge was higher than seed sludge. Nitrifying bacteria are strictly aerobes and inhabit at the outer layer of aerobic granules Bassin et al. (2012). These bacteria are very efficient in oxidizing ammonia (NH_4^+) into NO_2 and NO_2 into NO_3. The nitrifying bacteria that oxidize NH_4 were detected in SBR, which were *Nitrosomonas* and *Nitrosospira*. Meanwhile, bacteria that involved in oxidized NO_2 were *Nitrobacter*. Nitrifying bacteria was oxidized to NH_4 and NO_2 in order to obtain energy for cellular activity including reproduction (Khan et al. 2013). Due to the oxidation process of NH_4 and NO_2, small amount of energy was produced and was used by the nitrifying bacteria. Therefore, the bacterial growth rates are relatively small and slow (Gerardi 2006). Thus, high SRT are required to establish a population of nitrifying bacteria that are capable for effective nitrification process (Winkler et al. 2013).

Polyphosphate bacteria or phosphorus accumulating organisms (PAO) are used in biological phosphorus removal units. Phosphorus exists in inorganic and organic forms. Inorganic forms of phosphorus are orthophosphates and polyphosphates (Gerardi 2006) Orthophosphates are available for biological metabolism without further breakdown and are considered to be the readily available nutrients for bacterial use in SBR (Podedworna and Żubrowska-Sudoł 2012). In this study, polyphosphate bacteria that retain in SBR were *Enterobacter*, *Thauera*, *Klebsiella*, *Acinobacter*, *Aeromonas,* and *Arthrobacter*.

Table 13.4 Potential bacteria for biodegradation process identified from the seed sludge (SS) and aerobic granular sludge (AGS)

No.	Microbial	Roles	% Abundance	
			SS	AGS
1	*Pseudomonas*	COD and phosphorus degrading bacteria Heterotrophic nitrifier and aerobic denitrifier Denitrifying bacteria	9.39	34.05
2	*Alicycliphilus*	Denitrifying bacteria Degrade NH_4	1.62	5.01
3	*Rhodobacter*	Denitrifying bacteria Degraded dyes and organic chemicals	1.27	3.21
4	*Desulfovibrio*	Sulfate-reducing bacteria Nitrogen-degrading bacteria	1.03	3.09
5	*Clostridium*	Hydrogen-producing bacteria Sulfur-reducing bacteria Denitrifying bacteria	1.42	2.68
6	*Agrobacterium*	Denitrifying bacteria Involved in NH_4 removal Heterotrophic nitrifier and aerobic denitrifier	0.66	1.70
7	*Escherichia*	COD-degrading bacteria Denitrifying bacteria	0.51	1.52
8	*Thauera*	Denitrifying and PAO bacteria	0.31	1.28
9	*Sphingomonas*	COD-degrading bacteria Aromatic compounds degrader	0.71	1.11
10	*Azotobacter*	COD and phenols degrading bacteria	0.28	1.08
11	*Comamonas*	Denitrifying bacteria Aromatic compounds degrader Heterotrophic ammonium oxidation	1.72	0.97
12	*Enterobacter*	Organic substrates degrading bacteria PAO bacteria Denitrifying bacteria	0.25	0.85
13	*Cytophaga*	Aromatic compounds degrader Phosphorus- and nitrogen-degrading bacteria	0.29	0.89
14	*Klebsiella*	PAO bacteria	0.28	0.87
15	*Acinetobacter*	PAO under aerobic conditions Denitrifying bacteria Converted NH_4 to N_2 aerobically	0.65	0.83
16	*Arcobacter*	Heterotrophic-nitrifying bacteria	0.11	0.73
17	*Aeromonas*	COD-degrading bacteria PAO bacteria Denitrifying bacteria	1.07	0.62
18	*Nitrobacter*	Oxidation of NO_2 into NO_3	0.68	0.61
19	*Bacillus*	COD-degrading bacteria Denitrifying bacteria Converted NH_4 to N_2 aerobically Heterotrophic nitrifier and aerobic denitrifier	0.62	0.60

(continued)

Table 13.4 (continued)

No.	Microbial	Roles	% Abundance	
			SS	AGS
20	*Dyadobacter*	Oxidation of N_2 Ammonia-oxidizing bacteria Oxidation of NH_4 into NO_2	0.29	0.58
21	*Sphingobium*	Aromatic compounds degrader	0.28	0.44
22	*Arthrobacter*	COD-degrading bacteria PAO bacteria	0.29	0.42
23	*Corynebacterium*	Heterotrophic-nitrifying bacteria Denitrifying bacteria	0.27	0.37
24	*Gordonia*	Capable of metabolizing a wide range of environmental pollutants	0.11	0.35
25	*Nitrosomonas*	Ammonia-oxidizing bacteria Oxidation of NH_4 into NO_2	0.49	0.34
26	*Nitrosospira*	Nitrifying and PAO bacteria	0.39	0.32
27	*Riemerella*	Oxidation of N_2	0.17	0.25
28	*Alcanivorax*	Denitrifying bacteria by reducing NO_3 to NO_2	0.13	0.16
29	*Lactococcus*	Degradation of sucrose substrate	0.02	0.11
30	*Alcaligenes*	Denitrifying bacteria COD-degrading bacteria Converted NH_4 to N_2 aerobically	0.01	0.04
Total % abundance of bacteria			25.32	65.08

Table 13.5 Content and composition of extracted sludge EPS at different cycle times

Content	EPS composition (mg/g VSS)	Sludge			
			Aerobic granular sludge		
			Cycle time		
		Seed sludge	3 h	6 h	12 h
LB-EPS	Polysaccharides (PS)	3.2 ± 0.5	8.9 ± 0.6	7.3 ± 0.6	5.8 ± 0.7
	Proteins (PN)	4.8 ± 0.7	25.0 ± 0.6	17.5 ± 0.8	11.9 ± 0.1
	Carbohydrates	6.0 ± 0.3	16.3 ± 0.4	9.8 ± 0.9	7.7 ± 0.3
TB-EPS	Polysaccharides (PS)	4.1 ± 0.1	6.4 ± 0.5	5.9 ± 0.5	6.4 ± 0.4
	Proteins (PN)	4.4 ± 0.4	22.7 ± 0.4	12.2 ± 0.4	9.1 ± 0.2
	Carbohydrates	6.4 ± 0.1	11.7 ± 0.6	7.6 ± 0.4	8.8 ± 0.5

13.3.3 EPS Contents of Aerobic Granular Sludge

The content distribution of LB-EPS and TB-EPS in seed sludge as well as aerobic granular sludge at three different cycle times (3, 6, and 12 h) was listed in Table 13.5. The extracted proteins, carbohydrates, and polysaccharides contents of the seed sludge were analyzed at the beginning of the reactor start-up, while, the aerobic granules were analyzed at the end of the operational period of each cycle times. Proteins and carbohydrates were considered to represent the extracted EPS,

assuming that these are likely to be the dominant components in extracted EPS (Yu et al. 2006).

The results suggested that no matter in LB-EPS or TB-EPS, protein contents in aerobic granules exceeded the value in seed sludge which indicated an increment of proteins at all three cycle times compared to the seed sludge. Furthermore, the proteins fraction of the EPS was higher than the polysaccharides and carbohydrates fractions at all three cycle times. According to McSwain et al. (2005), the formation and stability of aerobic granules are dependent on a noncellular, protein core. Meanwhile, the carbohydrates fraction of both EPS (i.e., LB-EPS and TB-EPS) was higher than the polysaccharides fraction but lower than the protein fraction in aerobic granular sludge at all three cycle times.

Polysaccharides content of the EPS was increased in aerobic granular sludge at all three cycle times as compared to the seed sludge. However, the protein fraction was the predominant component of the EPS in aerobic granules. Polysaccharides are hydrophilic polymers, which reversibly absorb and exude water or biological fluids, and contribute to high water retention Seviour et al. (2003). Increases in bound water content of granules resulted in poor sludge settling and dewatering. Van Dierdonck et al. (2012) observed deterioration in floc structure owing to an increase in water-soluble EPS of flocs grown under low organic loading conditions in a laboratory-scale activated sludge system.

Proteins and the amino acid composition of proteins contribute to the hydrophobic character of granules (Dignac et al. 1998; Raszka et al. 2006). A strong correlation exists between the protein content in the EPS fraction hydrophobicity and good settling sludge. Since protein has a high content of negatively charged amino acids, it is more involved than sugars in electrostatic bonds with multivalent cations, a key factor in stabilizing the aggregate structure (Laspidou and Rittmann 2002). The functions of protein include the aggregation of bacterial cells, and the formation of an active gel-like matrix that maintains cell cohesion (Dogsa et al. 2005).

It has been generally believed that extracellular polymeric substances (EPS) can mediate both bacterial cohesion and adhesion. Hence, EPS, especially the ratio of the content of cell protein (PN) to the content of cell polysaccharide (PS), has a decisive role in building and keeping the structural integrity of a microbial community (Liu et al. 2004a, b). The variations in the PN/PS ratio of both LB-EPS and TB-EPS in aerobic granular sludge at three different cycle times (3, 6, and 12 h) as well as in the seed sludge were also shown in Fig. 13.4. The results showed that the PN/PS ratio of both LB-EPS and TB-EPS in aerobic granules at all three cycle times was quite higher compared to seed sludge which indicated that the PN/PS value rises during granulation. Some previous studies also implied that a higher PN/PS was beneficial to the formation and stability of granules (Zhu et al. 2012, 2015). Consequently, PN was the predominant component of EPS in aerobic granular sludge.

In this study, the PN/PS ratio of LB-EPS was 2.82, 2.40, and 2.07 for aerobic granules from the sequencing batch reactor (SBR) operated at 3, 6, and 12 h cycle times, respectively. Meanwhile, the ratio of PN/PS of TB-EPS in aerobic granules was 3.57, 2.06, and 1.42 at cycle time of 3, 6, and 12 h, respectively. It was found that the highest PN/PS ratio of both LB-EPS and TB-EPS was correspond to the aerobic

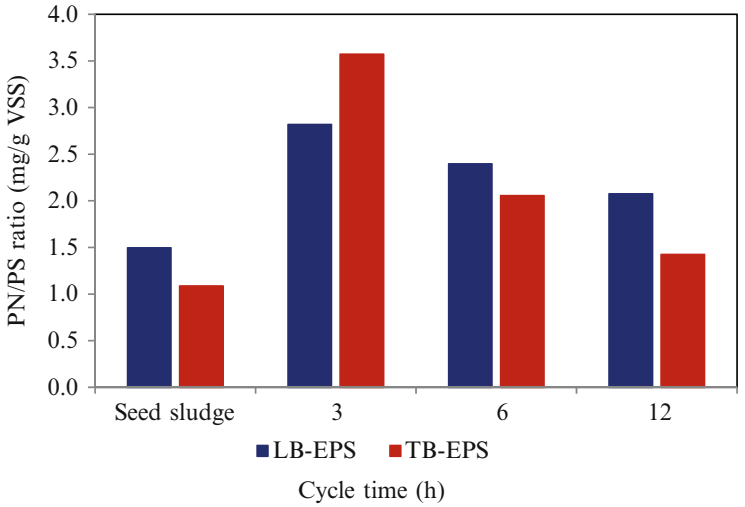

Fig. 13.4 Ratio of protein to polysaccharide content of aerobic granules from the three different cycle times with inoculums sludge is presented for comparison

granules from SBR operated at the shortest cycle time (i.e., 3 h) which suggested that a short cycle time would stimulate the production of cell proteins over polysaccharides in aerobic granules. Therefore, a higher PN/PS ratio of EPS in aerobic granular sludge shows that the stressful operating condition in terms of a short cycle time or a higher selection pressure applied in SBR were significantly excite the bacteria to overproduce PN. This result was consistent with the previous researches (Adav et al. 2008; Nichols et al. 2004). Pan et al. (2004) reported that the amounts of extracellular proteins (PN) and extracellular polysaccharides (PS) averaged 6.1, 6.5, 5.3 and 2.4 mg PN per mg PS, at cycle times of 1, 3, 6, and 12 h, respectively which indicated the granules associated with shorter cycle times possessed higher PN/PS ratios. In addition, according to Pan et al. (2004), the higher PN content facilitated the existence of stable aerobic granules at the relative short cycle times and contributed to the structural strength of the granules, as evidenced by the relatively higher values of specific gravity and integrated coefficient.

It appears that the content of cellular proteins is higher than the content of cellular polysaccharides in the aerobic granules, which suggested that the cellular proteins contribute more to the formation, structure, and stability of granular sludge. Normally, dispersed bacteria are negatively charged at usual pH values, and electrostatic repulsion exists between cells. It had been proposed that extracellular polymers could change the surface negative charge of bacteria, and thereby bridge two neighboring bacterial cells physically to each other as well as other inert particulate matter. Di Iaconi et al. (2006) claimed that the main component of EPS in aerobic granular biomass in a sequencing batch biofilter reactor was made of proteins, and protein-rich EPS would improve the stability of granular biomass structure. As the main component of proteins is amino acids, which often carry negative charges, it will contribute more than carbohydrates to electrostatic bonds, with consequent

increase of biomass structure stability (Di Iaconi et al. 2006). Adav and Lee (2008) reported that the mass ratio of PN to PS for sludge flocs was approximately 0.9, while the PN content was significantly enriched in the EPS of aerobic granules, producing a mass ratio of PN to PS being 3.4–6.2.

13.3.4 EEM Fluorescence Spectra of the EPS of Aerobic Granular Sludge

Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy can provide spectral information about the chemical compositions of EPS samples. In this study, 3D-EEM fluorescence spectroscopy was further applied for characterizing the LB-EPS and TB-EPS extracted from both seed sludge and aerobic granular sludge (that were formed in the sequencing batch reactor under three different cycle times of 3, 6, and 12 h). The EEM spectra of LB-EPS and TB-EPS fractions are displayed in Fig. 13.5a–h.

Three main peaks could be identified from the 3D-EEM fluorescence spectra of both LB-EPS and TB-EPS. The first peak (Peak A) was located at the excitation/emission wavelengths (Ex/Em) of 200–230/335–355 nm, while the second peak (Peak B) was observed at the Ex/Em of 245–270/340–355 nm. The two peaks were reported as protein-like peaks, which were associated with the aromatic protein-like substances such as tyrosine (Peak A) and tryptophan protein-like substances (Peak B) (Chen et al. 2003). Moreover, the third peak (Peak C) was occurred at the Ex/Em of 230–255/480–495 nm which was regarded as visible humic acid-like fluorescence (Coble 1996). EEM spectra can also be employed for quantitative analysis. Fluorescence parameters, such as peak location and fluorescence intensity, were extracted from EEM fluorescence spectra and listed in Table 13.6.

Results showed that the locations of peaks of aromatic protein-like substances (represented as peaks A and B) and humic acid-like substances (represented as peak C) were quite consistent in both LB-EPS and TB-EPS of seed sludge and aerobic granules except for a little difference in peak A, which indicated the components and physical structures in LB-EPS and TB-EPS of all the sludge samples were quite similar. However, the EPS samples of seed sludge and aerobic granular sludge have different fluorescence intensities. It was observed that the fluorescence intensities in EPS of aerobic granules at all three fluorescence peaks were much higher as compared to the seed sludge, implying that EPS played an important role during the granulation process.

Furthermore, the different values of peak fluorescence intensity of the two EPS may imply that the structural differences in the compounds are responsible for the fluorescence characteristics of the EPS of seed sludge and aerobic granular sludge. It was reported that intensity shifts of the fluorescence peaks could provide spectral information on the chemical structural changes of sludge EPS, which would indicate the functional group change of sludge EPS during aerobic sludge granulation (Liu et al. 2011).

In this study, the EEM fluorescence spectra in both LB-EPS and TB-EPS of aerobic granular sludge formed at varied cycle times (3, 6 and 12 h) were also investigated. The peak locations are independent of the EPS concentration, but the peak intensity depended on the EPS concentration. It was found that the intensities of the three peaks decreased with increasing cycle time from 3 to 12 h. As the cycle time was increased to 12 h, the intensities of peaks A, B, and C were all reduced. Therefore, it appears that when the hydraulic selection pressure imposed on the microbial community is decreased, the production of EPS secreted by cells became less. EPS are produced by most bacteria out of cell wall with the purpose of providing cells with the ability to compete in a variety of environments, providing a mode for adhesion to surface or self-immobilization. In addition, it was noted the

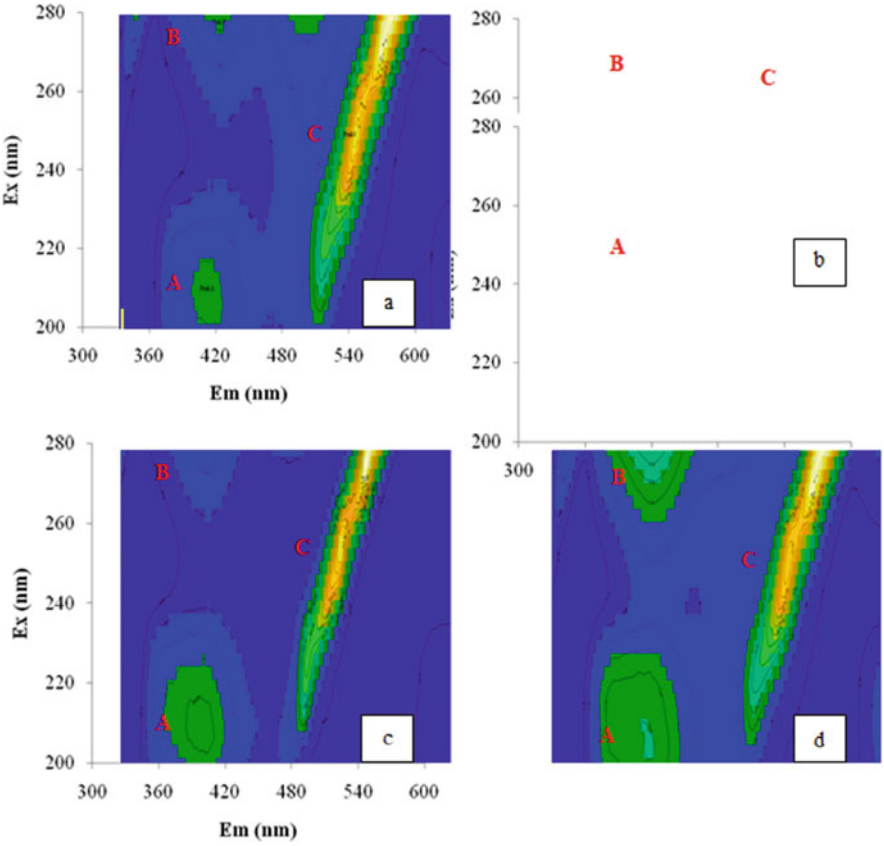

Fig. 13.5 EEM fluorescence spectra of (**a**) LB-EPS of seed sludge, (**b**) TB-EPS of seed sludge, (**c**) LB-EPS of aerobic granular sludge from 3 h cycle time, (**d**) TB-EPS of aerobic granular sludge from 3 h cycle time, (**e**) LB-EPS of aerobic granular sludge from 6 h cycle time, (**f**) TB-EPS of aerobic granular sludge from 6 h cycle time, (**g**) LB-EPS of aerobic granular sludge from 12 h cycle time and (**h**) TB-EPS of aerobic granular sludge from 12 h cycle time

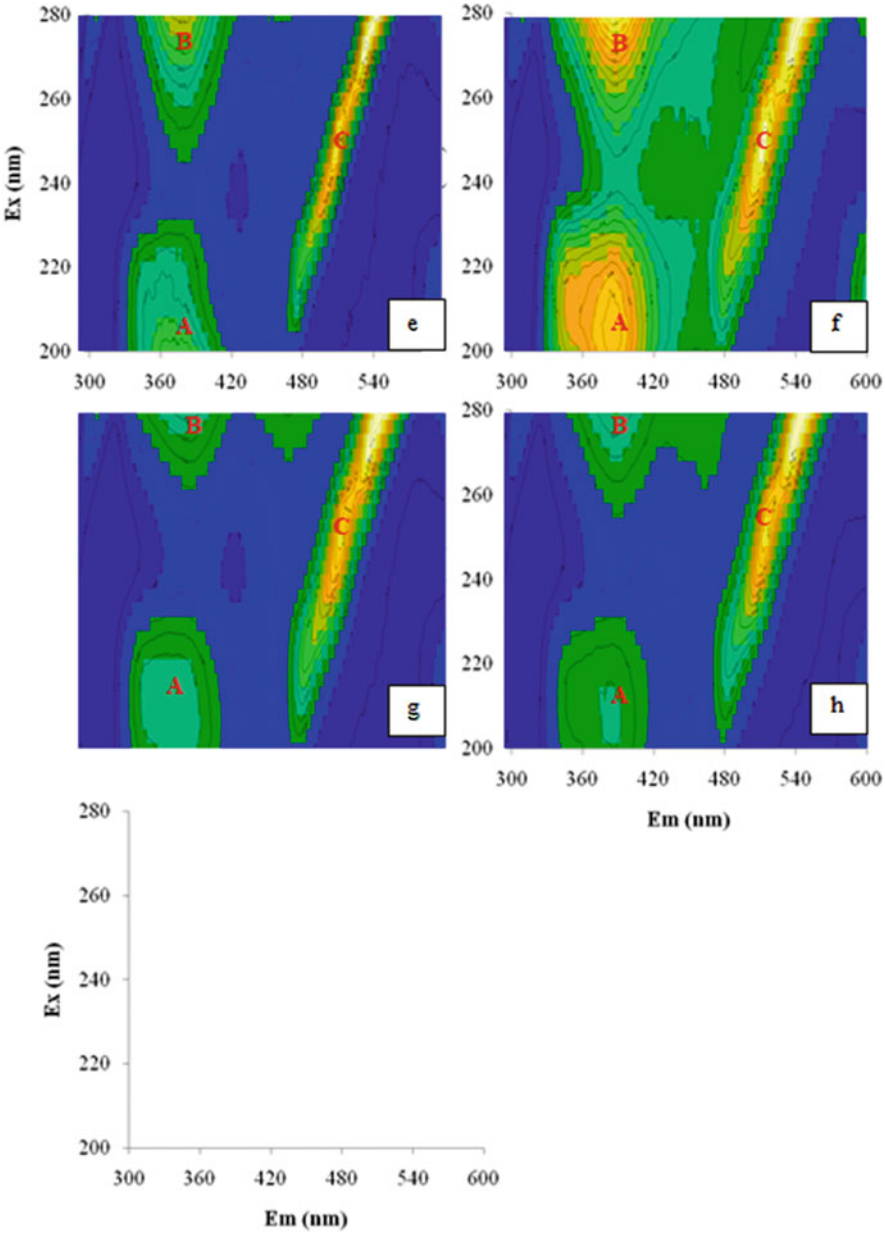

Fig. 13.5 (continued)

Table 13.6 Fluorescence spectral parameters of the sludge EPS samples (seed sludge and AGS: aerobic granular sludge) at various cycle times

Sample	EPS fractions	Peak A		Peak B		Peak C	
		Ex/Em	Intensity	Ex/Em	Intensity	Ex/Em	Intensity
Seed sludge	LB-EPS	225/341	11.85	268/352	11.85	250/495	47.39
	TB-EPS	226/350	10.06	268/355	13.43	250/495	26.9
AGS (3 h)	LB-EPS	205/335	65.31	248/340	97.64	233/480	187.52
	TB-EPS	223/354	13.26	268/354	15.48	251/495	33.24
AGS (6 h)	LB-EPS	220/342	37.74	268/350	46.19	250/495	71.56
	TB-EPS	224/352	11.32	268/354	12.83	250/495	15.10
AGS (12 h)	LB-EPS	226/340	13.07	268/352	15.25	250/495	34.87
	TB-EPS	224/350	11.17	268/355	13.03	250/495	29.82

peak intensity was peak C > peak B > peak A, which suggested that humic acid-like substances may be an important component of EPS during the stable granular sludge system. Besides that, the aromatic protein-like substances played an important role in the aggregation, favoring the formation and stability of aerobic granules. The study of Zhu et al. (2012) also showed the importance of aromatic protein-like substances in maintaining the stable structure of the granular sludge.

13.4 Conclusion

A diversity of microorganisms was identified from the seed sludge and aerobic granular sludge. Seed sludge consisted of 96.4% bacteria, 1.7% eukaryote, 1.2% archaea, and 0.7% viruses. Aerobic granular sludge consisted of 97.8% bacteria, 1.0% eukaryote, 0.8% archaea, and 0.4% viruses. As the granulation process was succeed in SBR, distinct differences of the microbial community in the seed sludge and aerobic granular sludge were observed, which suggested that there was high microbial selection pressure during granulation in the system. The most abundance species in seed sludge was Dechloromonas aromatica, while *Pseudomonas fluorescens* was the most abundance species in aerobic granular sludge. Furthermore, the potential bacteria for biodegradation process were found higher in aerobic granular sludge compared with seed sludge with 65.08% and dominated by *Pseudomonas* and *Alicycliphilus*. The aerobic granules were also occupied with aerobic, anaerobic, and facultative microorganisms. The role of bacteria that involved in biodegradation process such as denitrifying bacteria, nitrifying bacteria, and polyphosphate bacteria was also discussed.

Seed sludge and aerobic granular sludge formed at three different cycle times (3, 6, and 12 h) in the SBR system was selected for understanding the characteristics of sludge EPS. Proteins (PN) were found to be the predominant component in the sludge EPS, and its content in aerobic granular sludge was higher than that in seed sludge. Meanwhile, the PN/PS ratio of the granules at 3 h cycle time was the highest

which suggested that a short cycle time would enhance the production of cellular proteins due to the high hydraulic selection pressure. Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy was further used to elucidate the characteristics of EPS in the aerobic granules. Three individual fluorescence peaks (Peak A at 200–230/335–355 nm, Peak B at 245–270/340–355 nm, and Peak C at 230–255/480–495 nm) were identified in 3D-EEM fluorescence spectra for the EPS of the aerobic granules. Peaks A and B were attributed to the protein-like fluorophores, and Peak C to the humic acid-like fluorophore. The EEM results indicated the importance of aromatic protein-like substances, especially tryptophan, which may contribute in maintaining the stable structure of the granular sludge.

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Chapter 14
Granulation and Biodegradation by Microbial Species in Granular Sequencing Batch Reactor for Soy Sauce Wastewater Treatment

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Abstract At least 80,000 tonnes of the condiments were produced in Malaysia and estimated to increase in volume by 5% by the following year. In addition, one tonne of soy sauce generates about 7–9 tonnes of high strength wastewater. Aerobic granules are known to be regular, smooth, and nearly round in shape with excellent settling ability. They also have dense and strong microbial structure and high biomass retention with the ability to withstand high organic loading. These advantages encouraged recent development of aerobic granulation technology to treat high strength wastewaters such as soy sauce wastewater. Therefore, an efficient Granular Sequencing Batch Reactor (GSBR) treatment system ought to be in place to treat the high strength wastewater. The metagenome sequencing analysis revealed an abundance of microbial diversity accommodating in aerobic granular sludge cultivated with soy sauce wastewater. Existence of 77.52% exopolysaccharides substances (EPS)-producing bacteria such as *Pseudomonas* and *Bacteroides* which had the capability in biodegraded waste in wastewater biological treatment were found in

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aerobic granular sludge. Thus, the performances of aerobic granular sludge in biodegraded organic and nutrient from soy sauce wastewater were in consequence to the bacterial community that occupied in aerobic granules.

Keywords Soy sauce wastewater · Aerobic granular sludge · EPS bacteria · *Pseudomonas*

14.1 Introduction

Soy sauce is widely used and known products made from the soybean. It is a traditional all-purpose fermented food present in the form of a brown liquid with a meaty taste, which is used for food flavoring and often as a part of the marinade and a table condiment. Traditionally, soy sauce has been extensively used in Japan and several Southeast Asian countries, such as China, Thailand, Korea, Indonesia, and also Malaysia. China becomes the largest producer of the sauce and condiments in Asia since the origin of the fermented soy is considered from Chinese food, which called "miso" in Japan. These traditional products were also consumed throughout Thailand since the soy sauce was widely known and most important in terms of the largest market share (Mongkolwai et al. 1997). Thus, Thailand becomes the second largest of the sauce and condiment producers in Asian countries. Meanwhile, Malaysia becomes the top ten countries among the Asian countries, which exported sauce and condiments. Malaysia contributed 6% of the sauce and condiment in Asia. Since its demand remains all-time high, its production has undergone quite some changes to allow mass production (Hui et al. 2004). However, this has also produced a large volume of high load effluent that needs better and more responsible discharge management to preserve our environment.

Today, strict legislation to discharge wastewater from industrial areas exists to handle the wastewater in treatment plant using built facilities or new designs with reduced surface areas. Most often than not, the sequencing batch reactor (SBR) is the chosen biological method since it produces a relatively small footprint. In addition, the SBR system is extremely easy to adapt to the regulatory changes of effluent parameters such as nutrient removal. However, a compact system is required rather than an SBR system to overcome space limitation while treating high loading wastewater. Biological treatment using aerobic granular sludge in SBR has been well practiced since 1991 (Mishima and Nakamura 1991). The technology has also been known as an efficient biological treatment for municipal wastewater capable of removing nutrient and organic compounds in one single reactor (Cirik et al. 2013). Recent times have seen more rigorous research efforts poured into developing the technology. For one, it has remained attractive because of the dense and compact aerobic granular sludge structure that has been proven to have good settling ability. This has resulted in high biomass retention in the reactor to degrade organic matters. The aerobic granulation technology in SBR has been successfully implemented to treat industrial wastewater, such as dairy (Schwarzenbeck et al. 2005), textile (Muda

et al. 2010), livestock (Kishida et al. 2009; Othman et al. 2013), abattoir (Pijuan et al. 2009; Verawaty et al. 2013), and synthetic wastewater (Filali et al. 2012; Wu et al. 2012). However, its performance in treating high strength and real wastewaters remains largely unexplored. Moreover, to date, there has been no reported study on the use of this highly beneficial technology in treating soy sauce wastewater.

Aerobic granular sludge has been introduced in recent years as an alternative for biological wastewater treatment (Adav et al. 2008). This is due to the unique attribute of the self-immobilized microorganism—it can accumulate a large amount of active microorganisms and transform into a dense granule with strong structure and excellent settling capability. As such, the aerobic granulation technology has become a reliable alternative to reduce the space of the treatment plant and also increase treatment efficiency even with high organic loading rates. However, despite various substrates used in cultivating aerobic granular sludge, there is still a lack of knowledge about the cultivation of the sludge in real wastewater and this has become the focus of this study for the treatment of soy sauce wastewater.

Aerobic granulation is a novel gradual transformation process of fluffy seed sludge into compact granules without carrier materials. The granulation involves aggregation of microorganisms of activated sludge as the bacteria from that environment are significant for the granulation process (Lee et al. 2008). The granulation process is affected by the environmental conditions, which changes the physiology of the microbes to transform from a dispersed sludge to a dense aerobic granular sludge. The development of aerobic granular sludge from the seed sludge involved different physicochemical characteristics from bacteria with different capabilities in cultivating aerobic granular sludge. The aerobic granular sludge contains diverse microbial communities that contribute to the biological treatment of wastewater. The type and characteristics of the microbial communities together with its morphotypes' distribution depend very much on the substrate type used for its cultivation as well as the operational SBR setup. The purpose of the microbial communities' studies is to discover species composition, structure, spatial activity, and the bacterial distribution within an environment, as well as the function of the microorganisms (Cardenas and Tiedje 2008). The granulation involves aggregation of microorganisms of activated sludge as bacteria from that environment are significant for the granulation process (Lee et al. 2008). The granulation process is affected by the environmental conditions, which changes the physiology of the microbes to transform from a dispersed sludge to a dense aerobic granular sludge. Therefore, this study provided further understanding of the evolution of bacteria in the formation of aerobic granular sludge in treating soy sauce wastewater.

14.1.1 Wastewater and Sludge Collection

The raw soy sauce wastewater was collected from a wastewater treatment plant (WTP) of a local soy sauce processing company located in Johor Bahru, Malaysia. The wastewater was collected once every 2 weeks to maintain microbial activity and

then kept at 4 °C to prevent the wastewater from undergoing biodegradation due to microbial action (Vijayaraghavan et al. 2007). The existing WTP comprises preliminary treatment (oil trap slump and pH stabilizer); secondary treatment (two aeration tanks and a clarifier) to reduce COD, nitrogen, and phosphorus; and tertiary treatment (chemical reaction tank) with the wastewater production rate 62,000 L/day. Fresh activated sludge was collected from a local municipal wastewater treatment plant that operates the municipal wastewater treatment plant with an aerobic biological nutrient removal configuration. The activated sludge was collected five times during the study and then fed to the lab-scale reactor for the aerobic granular sludge development. The sludge contained very high concentrations of microbial populations useful for granule development and degradation of organic and nutrient processes (Winkler et al. 2013) in treating soy sauce wastewater. Thus, it encourages granules development and degradation of organic and nutrient processes in treating soy sauce wastewater. The sludge was sieved with a 1.0-mm sieve to remove large debris and inert impurities.

14.1.2 Laboratory-Scale Reactor Design

Granular Sequencing Batch Reactor (GSBR) is a 3 L working volume of lab-scale reactor with 100 cm in height and 6.5 cm in diameter. The operation of GSBR was based on the standard SBR system. The shape of the GSBR was customized to be high and slender to give maximum aeration surface. Wastewater was feed into GSBR via peristaltic pump at the bottom of the reactor to allow maximum nutrient distribution within the biomass. During the circulation phase, the wastewater was pumped out at higher elevation and then circulated back to the bottom of the reactor to promote adequate content mixing. Controlled air supply was given through a porous air stone from an air pump with an airflow meter at the bottom of the reactor to encourage the formation of small bubbles so that the sludge would remain homogeneous. The effluent discharge outlet is located at the midpoint of the reactor to collect the supernatant of the treated wastewater. The reactor was attached to timers connected to feeding, circulation, aeration, and effluent pumps to control and continuously operate the system. The GSBR was operated continuously for 24 h. All the operations of peristaltic pumps, circulation of influent, air pump, and decanting processes were controlled by timers. The first stage of GSBR system was the feeding stage of soy sauce wastewater into the reactor. The GSBR was acclimatized with 1.5 L of activated sludge and 1.5 L of soy sauce wastewater. The food to microorganism ratio (F/M) for this study was 0.3–1.8 kg BOD/kg MLVSS/day. GSBR system was set up for an intermittent anaerobic/anoxic and aerobic reaction phase. The reaction phase started with an anaerobic/anoxic phase and followed by an aerobic phase. During the anaerobic/anoxic reaction phase, the wastewater in the reactor system was allowed to circulate where it was pumped out from the upper level of the reactor system and then circulated back to the bottom. A peristaltic pump was used to carry out the circulation process until it had stopped completely,

indicating the end of the anaerobic/anoxic phase. This circulation process was required to homogeneously distribute the substrate as well as uniformly distribute the granular biomass while restricting the concentration gradient. Aeration was started right after the circulation process where a porous air stone was used to provide aeration at a volumetric flow rate of 2 L/min through an air pump. After the aeration process stopped, the activated sludge suspension was left for solids settlement for 10 min. Then, 1.5 L of the supernatant of the treated wastewater was discharged from the reactor within 5 min. After that, the SBR system would start all over again. The reactors were operated at room temperature (28 $\pm$ 2 °C).

14.1.3 Microbial Identification

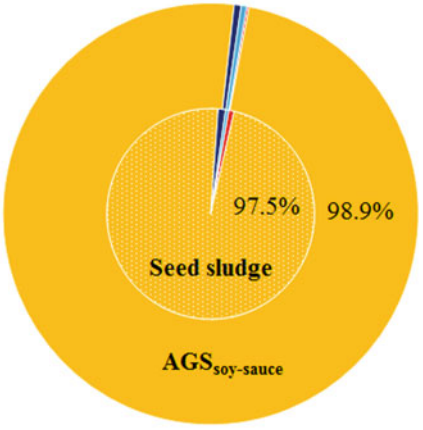
The presence and abundance of specific microorganisms found during the development of aerobic granular sludge from the seed sludge were studied using metagenomic analysis, specifically through the sequencing deoxyribonucleic acid (DNA) fragments extracted from the microbial population. The sample of seed sludge was collected during the first day of treatment. Meanwhile, the aerobic granular sludge sample was collected when the system became stable. Several steps were involved in microbial identification in the seed sludge and aerobic granular sludge sample, which included DNA preparation, DNA sequencing, and statistical analysis. DNA in the seed sludge and aerobic granular sludge sample was extracted by alkaline lysis method using Vivantis GF-1 Soil DNA Extraction kit according to the manufacturer's protocol. The extracted DNA was first purified by electrophoresis where its solution was processed into a 1% (w/v) agarose gel and the DNA was visualized under UV light (Weiss et al. 2007). After that, the isolated DNA was evaluated via spectrophotometry to measure the concentration and for further purification using NanoDrop 1000 V3.7 Spectrophotometer. The prepared DNA of seed sludge and aerobic granular sludge was sequenced using Illumina high-throughput sequencing on HiSeq 2000 platform. The high-throughput sequencing was performed using an Illumina Hiseq 2000 through paired-end sequencing on one lane to generate 100 bp paired-end DNA fragment sequences in fastq format. The raw sequences contaminated by adapter or containing three or more unknown nucleotides ("N") were removed via the quality control pipeline using a modified Dynamic Trim (Cox et al. 2010). A Phred score of 15 was taken as the high-quality base and the sequences were trimmed when the Phred score was less than five. This was to ensure that all the datasets generated during the next generation sequencing were of high quality. The quality-filtered Illumina reads of the seed sludge and aerobic granular sludge samples were submitted to the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST), v3.3.7 server (Meyer et al. 2008). For functional annotation, the reads were searched against GenBank database with the default settings on MG-RAST (e-value cutoff at 10–5), 60% minimum identity cutoff, and 15 bp minimum alignment length cutoff.

In this study, the bacterial community DNA was extracted from both seed sludge and aerobic granular sludge samples. In order to understand the compositions and changes of microbial communities during the granulation process. The next generation sequencing was used to identify the microbial consortium that developed in GSBR. It has the ability in identified low abundance of microorganism population in bacterial communities, the existing microbial communities in the samples would be well discovered in their natural environments with high resolution (Cardenas and Tiedje 2008). Therefore, this study provided further understanding of the evolution of bacteria in the formation of aerobic granular sludge in treating soy sauce wastewater.

14.1.4 Microbial Community Shift During Granulation

Since the functions and behaviors of microorganisms influenced the performance of aerobic granular sludge in SBR, further investigation by Illumina high-throughput sequencing was performed to analyze the microbial community diversities and phylogenetic structures during the granulation of seed sludge to aerobic granular sludge. From the analysis, microbial distribution of both samples demonstrated similar diversities but different abundance. Figure 14.1 shows the taxonomic result of seed sludge and aerobic granular sludge in terms of the relative abundance percentage of microorganisms existed in both samples. The result shows the four main microorganisms domains present in both samples: bacteria, viruses, eukaryota, and archaea, which is in agreement to Yu and Zhang (2012) who reported a similar microbial composition in both activated and granular sludge samples.

In the seed sludge, bacteria was accounting for 97.5%, followed by eukaryota with 1.2%. Meanwhile, archaea and viruses comprised 0.8% and 0.5%, respectively.



Domain	Seed sludge	AGS soy-sauce
Bacteria	97.5%	98.9%
Eukaryota	1.2%	0.5%
Archaea	0.8%	0.2%
Virus	0.5%	0.4%

Fig. 14.1 Percentage distribution of microorganisms in seed sludge and aerobic granular sludge

However, as the granules developed in soy sauce wastewater, the microbial taxonomic in GSBR were largely shifted. The abundance of bacteria was increased to 98.9%, eukaryota was decreased to 0.5%, viruses 0.4%, and archaea was 0.2% in aerobic granular sludge. From the domain distribution analysis, it is revealed that seed sludge contained greater microbial diversity than aerobic granular sludge because the seed sludge consists of 6718 of microbial species, which is more than aerobic granular sludge. This is because the seed sludge was enriched with the microorganisms that would initiate the formation of granules (Zhang et al. 2011). As a complicated ecological system, the microbial community residing in seed sludge was important for aerobic granulation process (Song et al. 2010). Meanwhile, during the granulation, microorganisms in aerobic granular sludge grew and evolved into 5668 species, which is less than 19% than seed sludge's species.

The shift of the distribution of the domains shows the changes in microbial communities throughout the granulation process. As claimed by Yadav et al. (2014), shifting of microbial community presence was due to changes in the operational parameter in the treatment system such as settling, flocculation, and floc formation characteristics. Those operational parameters also control microbial diversity. For example, the diversity of microorganisms in a system that operated with fast settling time would retain fast settling microorganisms meanwhile others with slow settling properties are being washed out from the system. So, the diversity of slow settling microorganisms in the system was affected by the operational parameter of the fast settling time.

14.1.5 Shifting of Bacterial Community in Seed Sludge to Aerobic Granular Sludge

Bacteria were the dominant domain in both seed sludge and aerobic granular sludge compared to other domains. Table 14.1 shows the percentage abundance of bacteria in seed sludge which was 97.5% and the percentage was increased to 98.9% in aerobic granular sludge. Basically, bacteria form a large percentage of the microbial community since they are the most versatile of all organisms in terms of their nutrient requirements, catabolic and metabolic activities (Di Iaconi et al. 2010). They played an important role in degrading the organic and nutrient of pollutants. The bacterial community that resided in the seed sludge was important for the aerobic granulation process (Lee et al. 2010). Almost all bacteria produce

Table 14.1 Abundance of bacteria in seed sludge and aerobic granular sludge

Sample	Seed sludge	Aerobic granular sludge
Bacterial abundance	1,959,495	2,984,334
Percentage abundance (%)	97.5	98.9
No. of species	4673	4331

exopolysaccharides (EPS) as they are surface-attached and favor the formation of aerobic granules (Liu et al. 2004).

Therefore, the existence of the abundance of bacteria in a granulation system is important to produce more EPS. Within the bacterial domain, there are several common types of microbes that were found in seed sludge, for example, floc formers, filamentous bacteria and denitrifiers. Comparing the microbial population between seed sludge and aerobic granular sludge, it is revealed there is a relative activity level of different populations in a microbial community. Determination of abundance of bacteria from the aerobic granules has provided significant information that the involvement of these bacteria is important in the granulation process. Significantly, the performances of the aerobic granular sludge in biodegradation of organic and nutrient were also a consequence of the bacterial community that occupied the aerobic granules.

14.1.6 Abundance of Bacterial Population in GSBR

In order to analyze the shifting of the microbial community during the granulation, the phylogenetic profiling of the seed sludge and aerobic granular sludge samples were compared. Only the top 20 taxonomic categories at phylum, class, order, and family levels have been summarized. Bacterial populations' distribution from both samples was significantly different at all taxonomy levels. This shows, there was immense diversity of bacteria in both samples. Additionally, some evidence of conserved differences between the sample sets indicate that these differences may be inherent to the granulation. In phylum levels, *Proteobacteria* was the dominant taxonomy in both seed sludge and aerobic granular sludge followed by *Bacteroidetes* and *Actinobacteria*. Meanwhile, in class level of bacteria, *Betaproteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria* were dominant in both samples. *Burkholderiales*, *Alteromonadales*, and *Rhizobiales* order were dominant in samples as well as *Comamonadaceae*, *Shewanellaceae*, and *Pseudomonadaceae* family. The varieties of bacteria had assured that they could contribute to the characteristics of the aerobic granules (Winkler et al. 2013).

In phylum levels, *Proteobacteria* was the dominant taxonomy observed in both seed sludge and aerobic granular sludge which were 76% and 71%, respectively. The high abundance of *Proteobacteria* in activated sludge was also reported by Yu and Zhang (2012) as well as the analysis of bacterial communities in sewage influent in which *Proteobacteria* was the most dominant community. The dominant of *Proteobacteria* is an advantage to a wastewater treatment system for enhancing the biological phosphorus removal, with and without nitrogen removal (Lee et al. 2002). Figure 14.2 shows the overview of all bacterial phylum detected in the seed sludge and aerobic granular sludge. The tree profile represents the bacteria domain level which further divided into constituent phylum. The tree demonstrates the bacterial abundance in seed sludge and aerobic granular sludge at each phylum. The tree profile was assigned to 28 phyla, including *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*,

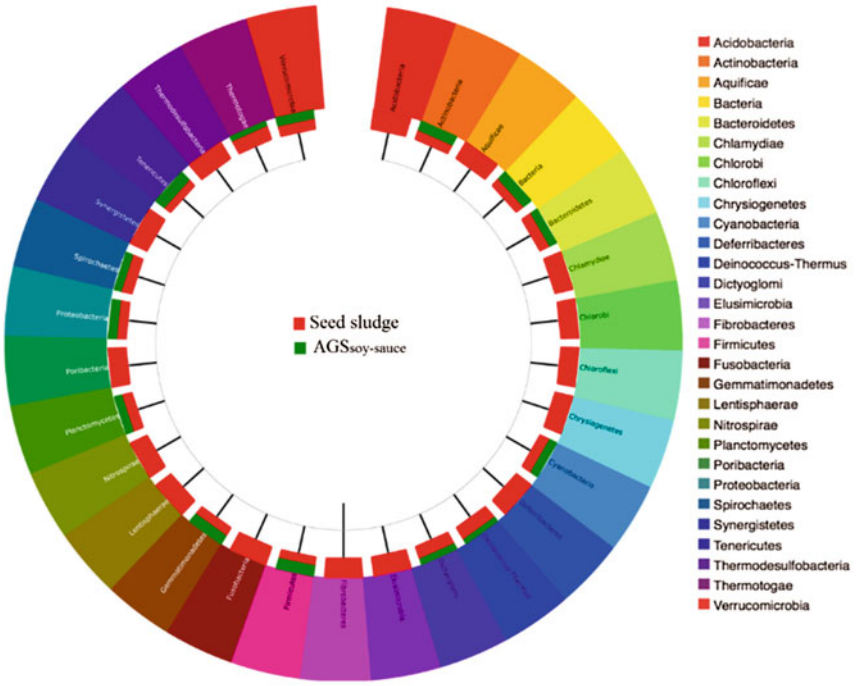


Fig. 14.2 Tree profiles of overall bacterial phylum from the seed sludge (red) and aerobic granular sludge (green)

Cyanobacteria, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, *Thermotogae*, and *Verrucomicrobia*.

From the tree profile, the overview of shifted bacterial community from seed sludge into aerobic granular sludge was presented. The major populations of bacteria in the seed sludge were noticeably slightly different from those in aerobic granular sludge. The seed sludge consisted of 28 phyla of bacteria but after the granulation, aerobic granular sludge only consisted of 14 bacterial phylum. The bacteria found in the seed sludge were mostly preserved throughout the granulation process. According to Jiang et al. (2004), this phenomenon occurs due to the adaptation process of the microbial community toward the aerobic granular sludge selection pressures as the seed sludge aggregated into matured granules in the system. The bacteria that preserved throughout the granulation process which success in the adaptation process in the GSBR including *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Thermotogae*, *Verrucomicrobia*, *Spirochaetes*, *Streptophyta*, *Fusobacteria*, *Euryarchaeota*, *Deinococcus-Thermus*, *Chlorobi*, *Ascomycota*, and *Arthropoda*. There were 71.5% and 75.7% of annotated phylum belonging to *Proteobacteria* in seed sludge and aerobic granular sludge, respectively. It shows the highest abundance of *Proteobacteria* phylum compared to other phylum and followed by *Bacteroidetes* (6.8% in seed sludge and 13.3% in

aerobic granular sludge) and *Actinobacteria* (5.82% in seed sludge and 4.4% in aerobic granular sludge). Therefore, two major phyla that have been identified from the phylum level in both samples were *Proteobacteria* and *Bacteroidetes*. Most of the detected bacteria in the seed sludge in this study had also been reported in the treatment of municipal wastewater (Bolhuis and Stal 2011). This is because seed sludge used in this study was also from the municipal wastewater treatment plant that contributed to high total dissolved solids, which favored the growth of bacteria in the activated sludge.

In class level, *Betaproteobacteria* (*β-Proteobacteria*), *Alphaproteobacteria* (*α-Proteobacteria*), and *Gammaproteobacteria* (*γ-Proteobacteria*) were dominant in the seed sludge and aerobic granular sludge (Fig. 14.3). The domination of those bacterial class was due to the highest percentage of the *Proteobacteria* phylum in the system. There are many studies on the phylogenetic groups of bacteria in activated sludge reported that the most predominant group found were from *β-Proteobacteria* class (Bond et al. 1998; Jiang et al. 2008). Same goes with this study as the *β-Proteobacteria* were dominant class of bacteria in the seed sludge. In the study done by Etterer (2006), he found that the bacteria under *β-Proteobacteria* level was able to grow and dominated the microbial structure without destabilizing the granule structure. In addition, the *β-Proteobacteria* have presented in steady-state conditions were responsible for a very compact granule structure.

Significantly, *α-Proteobacteria* played an important role in the function of aerobic granular sludge since they are often associated with granules development and influenced the settling properties of the granules (Levantesi et al. 2004; Van Der Waarde et al. 2002). According to Kragelund et al. (2006), the filamentous *α-Proteobacteria* have the ability to form granules. *α-Proteobacteria* is a large and robust filamentous bacteria that were found to be dominant in many industrial activated sludge systems (Martins et al. 2004). Therefore, it is essential for the filamentous bacteria to stay attached to the granules in order to settle in the clarifier and not to be washed out with the effluent. Besides, *α-Proteobacteria* had proved the capabilities in degrading nitrate and nitrite in the substrate (Kragelund et al. 2006).

The bacteria underclass *γ-Proteobacteria* have been reported capable in heterotrophic nitrification and aerobic denitrification (Zhang et al. 2011; Su et al. 2006; Kim et al. 2008). *γ-Proteobacteria* are mainly filamentous bacteria that were mostly present in the aerobic granular sludge. Bacteria under *γ-Proteobacteria* class can grow heterotrophically mainly on a low substrate and store carbon source as intracellular polyhydroxyalkanoates (PHA) (Yan et al. 2007). They are versatile microorganisms that can grow aerobically to reduced carbon and nitrogen compounds. As proved in previous studies (Di Iaconi et al. 2006; De Sanctis et al. 2010), the occurrence of simultaneous aerobic and anaerobic conditions in granular sludge leads to optimal carbon, nitrogen, and sulfur removals, which confirm the flexibility and the applicability of this technology for treating a wide range of complex wastewaters.

Bacteroidetes were detected in both seed sludge and aerobic granular sludge with percentages of 6.78% and 13.29%, respectively. According to Shah and Gharbia (2010), *Bacteroidetes* are normally present in diverse habitats, such as the human

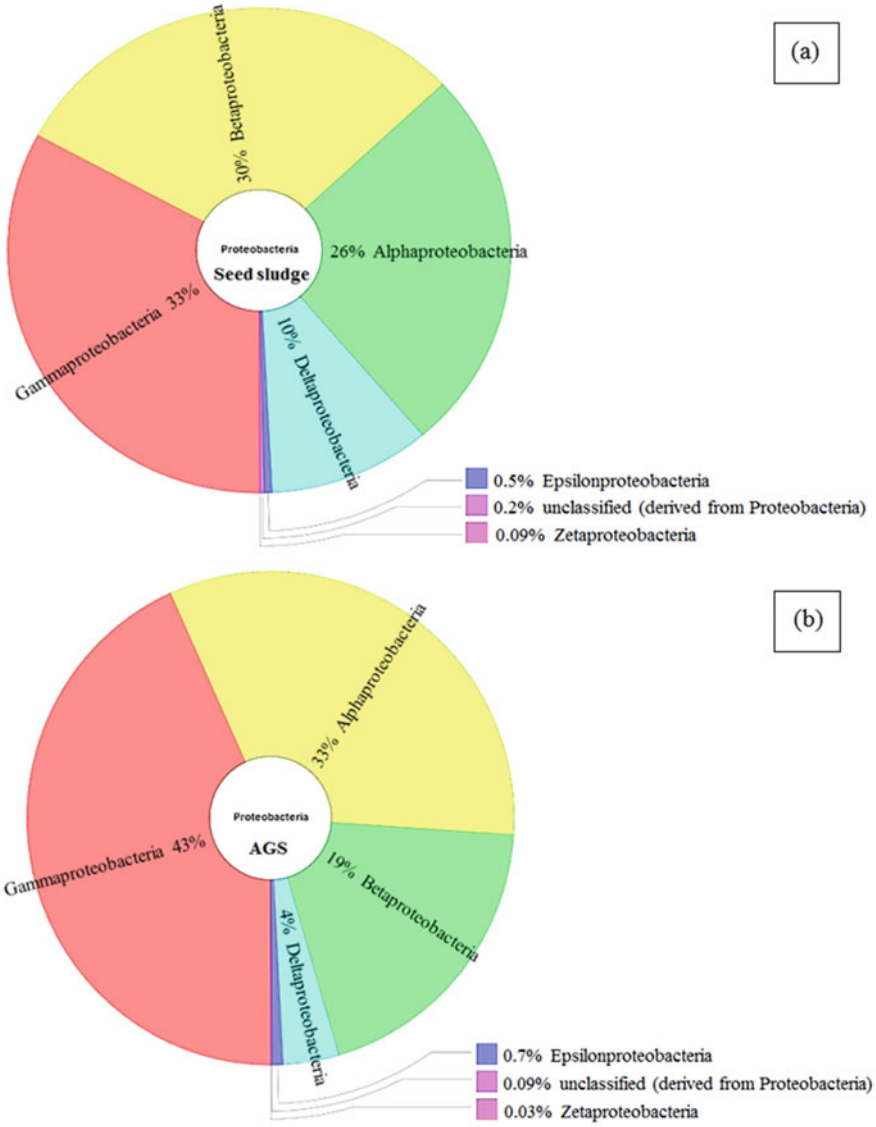


Fig. 14.3 Distribution of *Proteobacteria* at class level in (**a**) seed sludge and (**b**) aerobic granular sludge

body system, soil, and fresh water. Significantly, the presence of the *Bacteroidetes* phylum which commonly associated with human's oral cavity and gastrointestinal tract were able to play a significant role in the formation of aerobic granules (Dahalan 2012). Lately, *Bacteroidetes* were observed to be the dominant bacterial population found in aerobic granules as investigated in various studies (Adav et al.

2009; Fernández-Nava et al. 2008). Guo et al. (2011) confirmed that a number of bacteria classified under the phylum of *Bacteroidetes* were the main EPS inducer to increase cell hydrophobicity during the granulation processes of aerobic granules. *Bacteroidetes* in the granules produce EPS or slime layers, which extend beyond the outer cell wall due to metabolism and cell lysis (Wingender et al. 1999). EPS have been reported as major granular sludge components to keep the granules together in a three-dimensional matrix and granulation key in a treatment system (Nagaoka et al. 1996). Hence, EPS might be responsible for determining biomass production and the formation of aerobic granular sludge.

The presence of bacteria from Actinobacteria phylum with the percentages of 5.82% and 4.43% were detected in the seed sludge and aerobic granular sludge, respectively. This signified with the aerobic condition used to develop aerobic granular sludge that is beneficial to the growth of Actinobacteria in GSBR. Actinobacteria's habitat is generally observed to be in the soil, which plays important role in decomposition and humus formation. Song et al. (2010) reported that Actinobacteria have important roles in the formation of aerobic granular sludge when all the bacteria extracted from the aerobic granular sludge belonged to the class of Actinobacteria.

From the bacterial species listed, they are Gram-negative bacteria in rod shaped. According to Tay et al. (2004), EPS are polymeric structures of bacteria that origin lying outside the outer membranes of Gram-negative cells. Therefore, microbes in the system would enhance the formation of the granules since the EPS function is known to facilitate the adhesion of bacterial cells. Interestingly, a few rod-shaped bacteria were observed in aerobic granular sludge proven the existence of those listed species in the system. The existence of rod-shaped bacteria serves as the supporting structures for the granules to grow in the GSBR system (Zhang et al. 2011). While the high efficiencies of the organic and nutrient removals may suggest the presence of the high capability species in the GSBR to degrade a wide range of compounds including toxic pollutants. It is reasonable to conclude that the organics and nutrients (i.e., COD, NH_4, TN, and TP) in the GSBR had been successfully degraded.

14.1.7 Role of Bacteria in Sludge Granulation

The formation mechanism of aerobic granular sludge is basically influenced by many physical and chemical factors that trigger the microbial aggregation in the system to form stable and dense microbial community under the stressful operational conditions (Erşan and Erguder 2014). EPS is one of the important factors that enhanced the aggregation of bacterial cells during the granulation process, which is secreted by microbes as the general attributes in the bacteria's natural environment (More et al. 2014). The high amount of microorganisms in an aerobic granular sludge provides a high amount of EPS production, which gives an advantage in the improvement of granules density, structural properties, and settling velocities in

order to increase the efficiency in treating high strength wastewaters (Chen et al. 2010). EPS is a sticky material that is secreted by microorganisms as metabolic products that facilitate the cell-to-cell adhesion to aggregate the microbial biomass, which is crucial for the start-up of the granulation process (Kim et al. 2014). Veiga et al. (1997) also supported that the microbial population and EPS play important roles in the formation of granular sludge. Therefore, the bacteria that potentially produced EPS, which led to the granulation of aerobic granular sludge in the GSBR system need to be identified.

28.5% and 12.6% from the total abundance bacteria in aerobic granular sludge and seed sludge, respectively, were sorted out for the top 30 of EPS-producing bacteria. Table 14.2 lists the top 30 of EPS-producing bacteria that had been identified in the

Table 14.2 EPS-producing bacteria involved in the GSBR granulation process isolated from the seed sludge (SS) and aerobic granular sludge (AGS)

No.	EPS microorganisms	% Abundance	
		SS	AGS
1	*Pseudomonas*	6.26	39.06
2	*Bacteroides*	2.91	10.26
3	*Clostridium*	1.47	2.46
4	*Citrobacter*	0.14	2.00
5	*Flavobacterium*	0.35	1.93
7	*Agrobacterium*	0.62	1.61
8	*Escherichia*	0.51	1.58
9	*Rhizobium*	0.59	1.34
10	*Lactobacillus*	0.31	1.31
11	*Azotobacter*	0.31	1.08
12	*Vibrio*	1.62	1.29
13	*Sphingomonas*	0.81	1.10
14	*Serratia*	0.31	1.00
15	*Yersinia*	0.52	0.99
16	*Rhodococcus*	0.39	0.99
17	*Enterobacter*	0.24	0.87
18	*Proteus*	0.22	0.12
19	*Hyphomonas*	0.22	0.83
20	*Klebsiella*	0.27	0.82
21	*Acinetobacter*	0.55	0.74
22	*Aeromonas*	0.64	0.66
23	*Bacillus*	1.04	0.60
24	*Chryseobacterium*	0.13	0.60
25	*Arthrobacter*	0.27	0.42
26	*Pantoea*	0.24	0.39
27	*Corynebacterium*	0.29	0.38
28	*Bifidobacterium*	0.28	0.17
29	*Legionella*	0.28	0.16
30	*Staphylococcus*	0.12	0.08
Total % abundance of EPS bacteria		22.48	77.52

seed sludge and aerobic granular sludge. The total percentage abundance of the EPS-producing bacteria in aerobic granular sludge was 77.52%, which is higher than the seed sludge (22.48%). The higher percentage of the EPS bacterial population in aerobic granular sludge lightened the assumption of higher EPS bacterial existence in GSBR which supported the granulation of aerobic granular sludge. This may indicate that the EPS bacterial population is related to the physiological state of the bacteria, which affected by the operating conditions to develop aerobic granules (Liu et al. 2004). As the EPS-producing microbes—*Pseudomonas*, *Bacteroides*, *Clostridium*, *Flavobacterium*, and *Agrobacterium*—were the most abundant bacteria identified in aerobic granular sludge and the abundance was higher compared to the seed sludge.

Lactobacillus was found higher in aerobic granular sludge with 1.31% of the bacterial species. *Lactobacillus* belongs to the phylum *Firmicutes* and it is a saprophytic bacterium, which feeds on organic matter such as BOD and recognized as granule-forming bacteria (Gerardi 2006). According to Klein (2011), *Lactobacillus* basically found in fermenting food and in the intestinal tract of humans and animals. As the GSBR system is filled by activated sludge and soy sauce fermentation wastewater, the environment has favored the cultivation of *Lactobacillus*. According to Vu et al. (2009), Gram-positive Firmicutes are well known as EPS producer. Therefore, *Lactobacillus* that belongs to the phylum Firmicutes is an EPS producer. In addition, in a recent study by Yadav et al. (2011), isolated *Lactobacillus* was cultured in synthetic media to measure the potential in producing EPS. The EPS produced by *Lactobacillus* was found to be in a complex and dense structure. Torino (Yadav et al. 2011) previously reported *Lactobacillus* is a lactic acid bacterium that has the ability to produce more EPS in low pH environment conditions.

Firmicutes that had found in aerobic granular sludge, which was producing EPS for the microbial aggregation purposes are *Bacillus*, *Clostridium*, and *Staphylococcus* with 0.60%, 2.46%, and 0.08% of abundance in aerobic granular sludge, respectively. Hence, the existence of EPS-producing bacteria from the phylum *Firmicutes* in aerobic granular sludge is beneficial for granulation. Meanwhile, according to Dumitriu (2004), Gram-positive bacteria such as *Bacillus* and *Clostridium* with rigid cell walls have the ability to secrete EPS. Zhang et al. (2007) proposed that a mixed culture consortium produces a high amount of EPS. The EPS produced by the mixed culture of *Staphylococcus* and *Pseudomonas* was successfully applied for the treatment of dyeing wastewater with 80% of COD removal.

From the metagenomics analysis, it shows that the heterogeneous growth microorganisms are mainly found in aerobic granular sludge. Based on the identified microbial metabolism properties, the structure of aerobic granules was suggested to build with different layers of biomass regions, which are aerobic, anaerobic, and facultative anaerobic as illustrated in Fig. 14.4. The bacterial distribution in every biomass layers were expressed in percentage. The typical bacteria identified in aerobic granular sludge coexisted in the aerobic granular sludge, which developed in proportion to the different biomass layers of aerobic, facultative, and anaerobic regions formed. The structured layers of aerobic granules consisted of aerobic, anaerobic, and facultative microorganisms that illustrated in this study was clearly

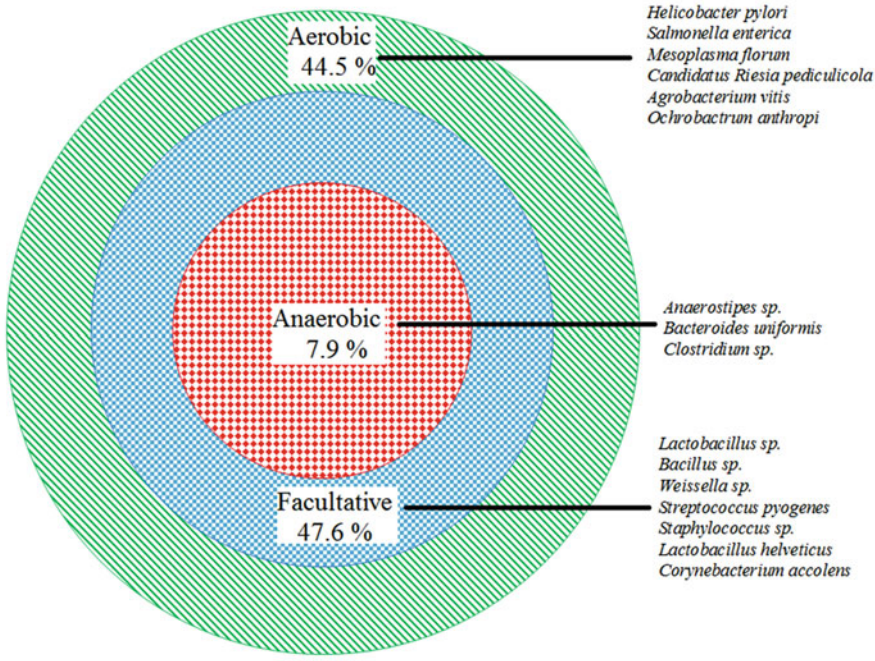


Fig. 14.4 Distribution of identified bacteria in aerobic granular sludge

discussed by Li et al. (2006). Basically, the typical depth of the aerobic zone is between 50 and 200 μm while the anaerobic zone could be detected at 800–900 μm from the granule surface (Tay et al. 2001). However, the depth of each zone depends on the oxygen penetration into the granule and the microbial metabolism activity (Pijuan et al. 2009). Concurrently, in aerobic granular sludge, microorganisms association in every layer is important for the development of the granules in order to obtain compact, dense, regular, and stable granular sludge for the improvement of soy sauce wastewater treatment.

From the illustration of layered granules, 44.5% of the identified bacteria in aerobic granular sludge were aerobic bacteria that dominated the outer layer of the aerobic granules. These bacteria are the fast-growing heterotrophs, where both substrates of oxygen and ammonia concentration and detachment rate are high (Ni and Yu 2008). Chiu et al. (2007) suggested that these bacteria dominated the outer layer of the aerobic granules, which were in close proximity to high oxygen supply as suggested. Meanwhile, 47.6% of the identified bacteria were facultative bacteria. Gao et al. (2011) described those facultative and anaerobic bacteria grown in the core of the granules. The anaerobic condition is beneficial for the survival of facultative bacteria and strengthened the removal capacity of nitrogen and phosphorus in a single SBR cycle (Zhang et al. 2011). According to Qin and Liu (2006), most of the denitrifying bacteria are facultative and they use organic carbon as the electron

donor for energy generation and growth. In this study, it was also found that 7.9% of the bacteria identified were anaerobic bacteria. Significantly, anaerobic bacteria have been found in various aerobic granulation studies (Adav et al. 2009; Gao et al. 2011). Based on their growth preference, it is suggested that the inner part of the aerobic granular sludge was favorable for the growth of these types of bacteria.

14.1.8 Role of Bacteria in Organic and Nutrients Biodegradation

The most important organisms in biological wastewater treatment plants are the bacteria. Bacteria at wastewater treatment plants can be classified according to the roles that they perform. Some bacteria perform positive roles in the treatment of wastewater that contribute to the efficient treatment of wastewater. The bacteria are able to biodegrade organic and nutrient upon the operational conditions. 23.44% and 69.45% from the total abundance bacteria in aerobic granular sludge and seed sludge, respectively, were sorted out for the top 30 of potential bacteria for biodegradation process. Table 14.3 lists the top 30 potential bacteria for biodegradation process in GSBR which isolated from the seed sludge and aerobic granular sludge. From the table, the total amounts of biodegradation bacteria in aerobic granular sludge were 69.45%, which were higher than the seed sludge (23.44%).

From the analysis, some denitrifying bacteria were detected higher in aerobic granular sludge compared to the seed sludge. The denitrifying bacteria that existed in the GSBR were *Pseudomonas*, *Alicycliphilus*, *Rhodobacter*, *Clostridium*, *Agrobacterium*, *Escherichia*, *Comamonas*, *Enterobacter*, *Thauera*, *Acinetobacter*, and others. The denitrifying bacteria are facultative anaerobic bacteria that use nitrate (NO_3) in the absence of oxygen to degrade soluble COD. The use of NO_3 results in the return of nitrogen to the atmosphere as molecular nitrogen (N_2) and nitrous oxide (N_2O) (Wei et al. 2012). Denitrification is important to reduce the concentration of TN in the discharged effluent of wastewater treatment plant (Gerardi 2006). Although there are numerous denitrifying bacteria, there are three important bacteria in the denitrifying process, which are *Alcaligenes*, *Bacillus*, and *Pseudomonas*. Fortunately, these three denitrifying bacteria had been retained in GSBR during the granulation process which promise the efficiency of the denitrifying process in GSBR.

In this study, nitrifying bacteria were also detected in the GSBR and the percentage abundance in aerobic granular sludge was higher than the seed sludge. Nitrifying bacteria are strict aerobes and inhabit at the outer layer of aerobic granules (Bassin et al. 2012). They are very efficient in oxidizing ammonia (NH_4^+) into NO_2 and NO_2 into NO_3. The nitrifying bacteria that oxidize NH_4 were detected in GSBR, which were *Nitrosomonas* and *Nitrosospira*. Meanwhile, bacteria that involved in the oxidation of NO_2 were *Nitrobacter*. Nitrifying bacteria oxidized NH_4 and NO_2 in order to obtain energy for cellular activity including reproduction (Khan et al. 2013).

Table 14.3 Potential bacteria for biodegradation process isolated from the seed sludge (SS) and aerobic granular sludge

No.	Microorganisms	Roles	% Abundance	
			SS	AGS
1	*Pseudomonas*	COD and phosphorus degrading bacteria Heterotrophic nitrifier and aerobic denitrifier Denitrifying bacteria	6.26	39.06
2	*Alicycliphilus*	Denitrifying bacteria Degrade NH_4	1.73	4.96
3	*Rhodobacter*	Denitrifying bacteria Degraded dyes and organic chemicals	1.34	3.39
4	*Desulfovibrio*	Sulfate-reducing bacteria Nitrogen degrading bacteria	1.01	3.12
5	*Clostridium*	Hydrogen-producing bacteria Sulfur-reducing bacteria Denitrifying bacteria	1.47	2.46
6	*Agrobacterium*	Denitrifying bacteria Involved in NH_4 removal Heterotrophic nitrifier and aerobic denitrifier	0.62	1.61
7	*Escherichia*	COD degrading bacteria Denitrifying bacteria	0.51	1.58
8	*Sphingomonas*	COD degrading bacteria Aromatic compounds degrader	0.81	1.10
9	*Azotobacter*	COD and phenols degrading bacteria	0.31	1.07
10	*Comamonas*	Denitrifying bacteria Aromatic compounds degrader Heterotrophic ammonium oxidation	1.88	0.99
11	*Enterobacter*	Organic substrates degrading bacteria PAO bacteria Denitrifying bacteria	0.24	0.87
12	*Thauera*	Denitrifying and PAO bacteria	1.32	0.87
13	*Cytophaga*	Aromatic compounds degrader Phosphorus and nitrogen degrading bacteria	0.28	0.85
14	*Klebsiella*	PAO bacteria	0.27	0.82
15	*Acinetobacter*	PAO under aerobic conditions Denitrifying bacteria Converted NH_4 to N_2 aerobically	0.55	0.74
16	*Arcobacter*	Heterotrophic nitrifying bacteria	0.07	0.70
17	*Aeromonas*	COD degrading bacteria PAO bacteria Denitrifying bacteria	0.64	0.66
18	*Nitrobacter*	Oxidation of NO_2 into NO_3	0.69	0.65
19	*Bacillus*	COD degrading bacteria Denitrifying bacteria Converted NH_4 to N_2 aerobically Heterotrophic nitrifier and aerobic denitrifier	1.04	0.60
20	*Dyadobacter*	Oxidation of N_2 Ammonia oxidizing bacteria Oxidation of NH_4 into NO_2	0.3	0.59

(continued)

Table 14.3 (continued)

No.	Microorganisms	Roles	% Abundance	
			SS	AGS
21	*Sphingobium*	Aromatic compounds degrader	0.28	0.43
22	*Arthrobacter*	COD degrading bacteria PAO bacteria	0.27	0.42
23	*Corynebacterium*	Heterotrophic nitrifying bacteria Denitrifying bacteria	0.29	0.38
24	*Gordonia*	Capable of metabolizing a wide range of environmental pollutants	0.09	0.36
25	*Nitrosomonas*	Ammonia oxidizing bacteria Oxidation of NH_4 into NO_2	0.50	0.33
26	*Nitrosospira*	Nitrifying and PAO bacteria	0.40	0.27
27	*Riemerella*	Oxidation of N_2	0.07	0.27
28	*Alcanivorax*	Denitrifying bacteria by reducing NO_3 to NO_2	0.14	0.18
29	*Lactococcus*	Degradation of the sucrose substrate	0.03	0.10
30	*Alcaligenes*	Denitrifying bacteria COD degrading bacteria Converted NH_4 to N_2 aerobically	0.01	0.02
Total % abundance of bacteria			23.44	69.45

Due to the oxidation process of NH_4 and NO_2, a small amount of energy was produced and was used by the nitrifying bacteria. Therefore, the bacterial growth rates are relatively small and slow (Gerardi 2006). Thus, high SRT are required to establish a population of nitrifying bacteria that are capable of effective nitrification process (Winkler et al. 2013).

Polyphosphate bacteria or phosphorus accumulating organisms (PAO) are used in biological phosphorus removal units. Phosphorus exists in inorganic and organic forms. Inorganic forms of phosphorus are orthophosphates and polyphosphates (Gerardi 2006). Orthophosphates are available for biological metabolism without further breakdown and are considered to be the readily available nutrients for bacterial use in SBR. By recycling the polyphosphate bacteria through an anaerobic and aerobic phase in a system, the removal of the orthophosphate from the wastewater was greater than a normal cell (Bassin et al. 2012). Polyphosphate bacteria that retain in GSBR were *Acinetobacter*, *Enterobacter*, *Klebsiella*, *Arthrobacter*, *Aeromonas*, and *Thauera.* According to Nielsen et al. (2012), bacterial communities that cultivated under anaerobic and aerobic conditions in the granular sludge system were composed of the main population of essential microorganisms from activated sludge. The dynamics of the microbial population was due to the alternating anaerobic and aerobic conditions due to the selected and versatile microbial populations that become active at any conditions in GSBR (Nielsen et al. 2012).

14.2 Conclusions

The metagenome sequencing analysis of the DNA revealed an abundance of microbial diversity accommodating in both seed sludge and aerobic granular sludge cultivated with soy sauce wastewater. There were distinct differences of the microbial community in the seed sludge and aerobic granular sludge, which suggested that there was high microbial selection pressure during the granulation in the system. *Pseudomonas putida* was found to be the most abundant species in aerobic granular sludge meanwhile, *Acidovorax* sp. JS42 was the most abundant species in seed sludge. 77.52% of the EPS-producing bacteria involved in aerobic granular sludge granulation were determined and the higher EPS bacteria that exist in aerobic granular sludge were *Pseudomonas* and *Bacteroides*. Meanwhile, potential bacteria for biodegradation process were also found higher in aerobic granular sludge compared to the seed sludge with 69.45% and dominated by *Pseudomonas* and *Alicycliphilus*. The role of bacteria involved in biodegradation processes such as denitrifying bacteria, nitrifying bacteria, and polyphosphate bacteria were also discussed. Therefore, the higher amount of potential bacteria producing EPS and degrading organic and nutrient which retained in aerobic granular sludge proved that aerobic granular sludge contains a large mixture of microbial species to synthesize various kinds of EPS.

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