Sustainable enzymatic technologies in waste animal fat and protein management

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Abstract

Waste animal fats and proteins (WAFP) are rich in various animal by-products from food industries. On one hand, increasing production of huge amounts of WAFP brings a great challenge to their appropriate disposal, and raises severe risks to environment and life health. On the other hand, the high fat and protein contents in these animal wastes are valuable resources which can be reutilized in an eco-friendly and renewable way. Sustainable enzymatic technologies are promising methods for WAFP management. This review discussed the application of various enzymes in the conversion of WSFP to value-added biodiesel and bioactivate hydrolysates. New biotechnologies to discover novel enzymes with robust properties were proposed as well. This paper also presented the bio-utilization strategy of animal fat and protein wastes as alternative nutrient media for microorganism growth activities to yield important industrial enzymes cost-effectively.

Keywords: waste animal fat and protein, bioconversion, biodiesel, bioactivate hydrolysates, enzyme production

1. Introduction

Huge amounts of wastes from animal-related industries were generated due to year after year population and consumption growth which have aroused widespread environmental and public health concerns (Jayathilakan et al., 2012). During meat processing, the edible part of any livestock which are mainly the animal muscle are made as food, whereas the non-edible parts are produced as low value by-products

which are regarded as animal wastes (Cristina and César., 2019). Annually, million tons of animal giblets, fat, bones, blood, feather, skin and shell from cattle, pigs, sheep, goats, poultry, fish and seafood are generated worldwide through slaughter house, butcher shop and meat packing factories (Charles and David., 2016; Jayathilakan et al., 2012). Approximately, the materials not consumed by humans account for 49%, 44%, 37% and 57% of the live weight of cattle, pigs, broilers and most fish species, respectively. The animal wastes mainly contain excreta, carcasses, feathers, hair, skin, bone, tissue, blood, processing wastewater and other wastes (Hibbard et al., 1996). Martínez-Alvarez et al. (2015) stated that blood and feathers are the main by-products from poultry processing, which account for 2-6% and 10% of the total chicken weight, respectively. For fish processing industries, wastes (including muscle, skin and fins, bones, heads, viscera and scales) represent 57% (w/w) of the total weight after filleting.

The disposal of large amount animal wastes has significant impact on environment security and life health. They are not only highly perishable and smelly unbearable, but rich in nutrients and pathogens that result in potential pollution to soil, surface water and groundwater (Ndiaye et al., 2020). However, nutrients in animal wastes are the valuable resource which could be recovered and utilized. Apart from 60% water, fat and proteins are the main valuable and recoverable contents of those waste materials (Meeker, 2009; Jayathilakan et al., 2012). The fats derived from waste skin of fish, pig, cattle, sheep and chicken contain rich free fatty acids (FFAs) and have good combustion properties with intensive net calorific value (around 37.91MJ/kg) which was slightly lower than that of petroleum fuels (Kirubakaran & Selvan, 2018; Lazaroiu et al., 2017).

As well, the protein level in liver, tail, ears and feet of cattle is close to that in lean meat tissue (Jayathilakan et al., 2012). As reported earlier, the protein content in feathers, blood meal, bones, heads and feet, viscera and intestines of chicken was 85-99%, 60-80%, 23-24%, 16%, 11-12% and 53-60%, respectively (Lasekan et al., 2013).

Waste animal fats, generated from many animal meat-processing facilities, refining industries and large food processing, are sustainable feedstock for the synthesis of biodiesel (Mata et al., 2010; Adewale et al., 2015;). Biodiesel is a renewable biofuel which is biodegradable, sulfur free, oxygenated and non-toxic, and now is also a potential reproducible energy for replacing current diesel derived from petroleum (Basha et al., 2009). As reported earlier, the feedstock generally accounts for around 70% of the total production cost of biodiesel (Tan et al., 2010). Biodiesel production from high available animal fat wastes instead of vegetable oils is a promising and sustainable method, which can eliminate the need for waste disposal and the debate of the food vs. fuel simultaneously. For instance, beef tallow is a by-product of cattle slaughter plants, which has become the second most important raw material for biodiesel production after soybeans in many countries (da Cunha et al., 2009; Moraes et al., 2008). Based on the characterization of waste animal fats, the biodiesel produced from them has greater oxidative stability compared with methyl esters from vegetable oils (Mata et al., 2011). Whereas, the saturated share of animal based biodiesel results in a high cold filter clogging point, which can cause engine blockage at low temperature choking when used as engine fuel. This issue can be solved by separating the saturated fraction from the unsaturated fatty acid methyl ester (FAME) via simple

precipitation and filtration method, and further transforming to valuable polyhydroxyalkanoate (PHA) biopolymers through microorganisms (Koller et al., 2018; Shahzad et al., 2017)

In addition, animal proteins can be reused in animal feed ingredients, fertilizers and even materials associated with cosmetics, artificial organs, medicine, etc. (Ferraro et al., 2016; Morimura et al., 2002; Oro et al., 2018). A large amount of proteins in animal by-products are in the form of keratin and collagen whilst collagen and keratin are inactive in their original sequence due to their stable protein structure. Thus, large-scale use of these proteins has not been developed to any great degree. Thermochemical treatment is an effective method that makes the protein more soluble and digestible, but it is energy intensive and economically unfeasible. Moreover, some important amino acids can be destroyed during thermochemical treatment (Cai & Zheng, 2009; Papadopoulos, 1989). However, enzymatic hydrolysis is preferred due to its advantages of controllable, reproducible, as well as retaining the nutritional value of the product and resulting in fewer unwanted side-effects and by-products (Halim et al., 2016; Hathwar et al., 2011; Tarté, 2009). Furthermore, enzyme catalysis has more advantages in degrading fats and protein than physicochemical catalysis because of its mild, highly efficient, eco-friendly reaction and catalytic specificity as well, without affecting other nutrients in the sample (Brandelli et al., 2015; Yao et al., 2020).

The degradation and re-utilization of animal fat and protein wastes to reduce environment pollution and produce green energy and high-valued chemicals have great significance to build a sustainable environment and more promising researches. Hence,

this review focused on the sustainable enzymatic processes in two main animal waste components fats and proteins management. The technological parameters in transformation of waste animal fats into biodiesel by lipase and conversion of waste proteins into valuable hydrolysis products by protease were critically discussed. Novel biotechnologies in improving relevant enzymes properties were proposed to promote biodegradation efficiency. Furthermore, the bio-utilization strategy of animal wastes as nutrient sources for microbial life activities to generate important industrial enzymes towards enhancing the economic benefits and relief environmental pollution was also discussed.

2. Enzyme application in animal wastes management

2.1 Enzyme application in waste animal fats management

Transesterification is the most commonly used method for the conversion of waste animal fats to biodiesel, specifically, fats react with a short chain alcohol (methanol and ethanol) under the presence of catalysts (Meher et al., 2006). For animal fat wastes, high levels of high free fatty acids (FFAs) are the main factors that determine the viability of the biodiesel generation by the transesterification process. In general, conventional chemical catalysts are commonly utilized for biodiesel production, but they are sensitive to the high FFA level in waste animal fats. The reaction of alkaline catalysts and FFA can form soap and result in serious emulsification and separation problems (Antonio et al., 2018). To reduce FFA level in waste fats, a pretreatment step by using acid esterification is normally required, leading to high energy consumption

and costs (Ching-Velasquez et al., 2020). In contrast, the application of enzymes for transesterification is an emerging approach due to the advantages of insensitivity to high FFA oil, not required for pre-treatment, moderate operating conditions, easy recovery of glycerol, minimal requirement for wastewater treatment, reusability and enhancing biodiesel yield (Cesarini et al., 2014; Kumar et al., 2015; Wancura et al., 2020). The biodiesel production process is displayed in Fig. 1 (Gumba et al., 2016). In addition, the content of glycerol produced in transesterification via enzymes is much higher and commercially favorable in comparison with that in conventional transesterification via chemical catalysts (Van Gerpen, 2005).

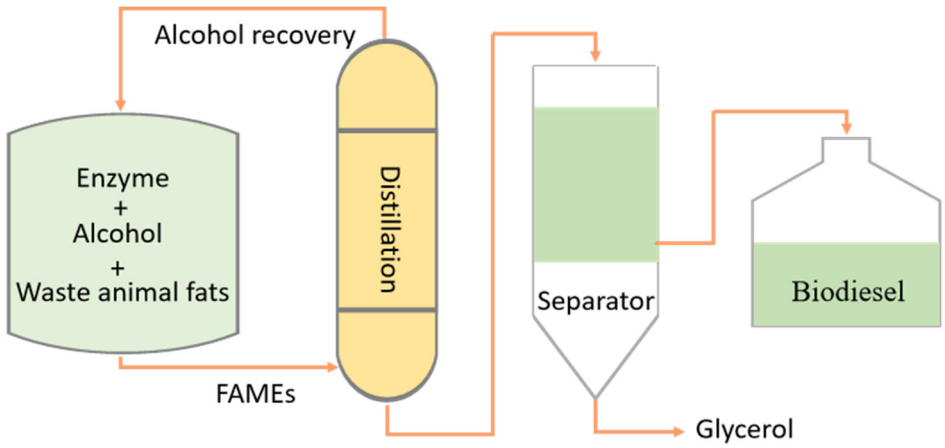

Fig.1 Flow diagram of biodiesel production process.

Lipases are the most commonly used enzymes in transesterification reaction of waste animal fats. Lipase is useful to modify the structure of fat by catalyzing the release of free fatty acids from long-chain triacylglycerols (C>10) (Hitch and Clavel, 2019; Zhu et al, 2011). Yao et al (2020) investigated that a commercial lipase could change effectively the structure of bovine bone and improve a degree of 8.19% of subsequent enzymatic hydrolysis efficiency

than control, indicating significant decreasing of lipid content. Additionally, in transesterification process of animal fats, the triglyceride reacts with alcohol (as acyl receptors) in the presence of lipase, could form glycerol and esters or biodiesel (methyl or ethyl fatty acid ester) (Singh et al, 2020; Aarthy et al, 2014). Under the optimal conditions, various lipases, especially the supported ones are capable of producing up to 90% or more fatty acid methyl ester (FAME). However, the maximum yield, enzyme life time and reaction time are affected by several conditions such as temperature of the reaction, type of alcohol, molar ratios of alcohol to oil, source of lipase, optimal water activities and application of immobilized or free lipase (Wancura et al., 2019). Different impact factors for the lipase application in transesterification reaction of waste animal fats has been given in table 1.

17 Table 1 Examples of the application of lipases in the convention of waste animal fats to biodiesel.

Entry	Waste animal fats	Lipase source	Lipase loading to the fat (wt%)	Type of alcohol	Alcohol: fat molar ratio	Reaction conditions	Biodiesel yield (conversion) (%)
1	Lard (Lee et al., 2002)	*Candida antractica* (Chirazyme L-2)	10	Methanol	1:01	30℃, 24h, immobilized lipase Chirazyme L-2	74
2	Lard (Lu et al., 2007)	*Candida* sp. 99–125	20	Methanol	-	40℃, 30 h, immobilized lipase catalyzed	87.4
		Candida antractica (Novozym435)	3	-	-	-	90.5
3	Lard (Huang et al., 2010)	*Thermomyces lanuginosus* (Lipozyme TLIM)	8	Methanol	5:01	50 ℃, 20 h, immobilized lipases Novozym435 and Lipozyme TLIM	72.8
		Novozym 435 and Lipozyme TLIM combine	4 (1.96% of Novozym435 and 2.04% of Lipozyme TLIM)				97.2
4	Lard (Adewale et al., 2016)	*Candida Antarctica* Lipase B (CALB)	6	Methanol	4:1	20 min, ultrasound assisted transesterification	96.8
5	Beef tallow (Kumar et al., 2013)	Enzyme catalyst NS88001	25	Methanol	4:1	45 ℃, 16 h	95.75

6	Beef tallow (Da Rós et al., 2010)	*Burkholderia cepacia* (Lipase PS)	20	Ethanol	/	50 ℃, 48 h, lipase immobilized on Nb2O5 or SiO2–PVA	40.2 (Lipase immobilized on SiO2–PVA)-89.7 (Lipase immobilized on Nb2O5)
7	Beef tallow (Da Rós et al., 2012)	*Burkholderia cepacia*	/	Ethanol	6:1	50 ℃, 8 h, lipase immobilized on silica-PVA and microwave-assisted enzymatic synthesis of biodiesel	100
8	Beef tallow (Wancura et al., 2018)	*Thermomyces lanuginosus*	1.45	Methanol	4.5:1	35 °C, 8 h	84.6
9	Waste Chicken Fat (Antonio et al., 2018)	*Candida antarctica*	/	Ethanol	/	/	96
10	Animal fats and fish oil (Aryee et al., 2011)	Lipozyme-IM	/	/	1:1	45 ℃, 96 h	50
11	Porcine fat waste (Skoronski et al., 2016)	Lipozyme TL IM	20	Methanol Ethanol	3:1	55 ℃, 2 h, immobilized enzymes	99.77 76.04
12	Waste animal fat (Lee et al., 2017)	*Candida antarctica* lipase B variant	10	Methanol	0.14:1	40 °C, 6 h, immobilized, carried out in supercritical CO_2	87

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Lipase can be produced from animals, plants and microorganisms. Comparatively, lipases produced from microbial sources have benefits of high yield production, more available catalytic activities, ease of genetic manipulation, no seasonal fluctuations, more stable and regular supply (Chandra et al., 2020). As shown in Table 1, lipases from *Candida antractica, Thermomyces lanuginosus* or *Burkholderia cepacia* have already been proved effective for conversion of waste animal fats into biodiesel. The lipase from *Candida antarctica* is the most widely used biocatalyst for the production of biodiesel. The *Candida antarctica* lipase can catalyze acyl transfer reactions between oils and various acyl acceptors, and it shows high activity in organic solvents and has a wide range of reaction selectivity (Subhedar & Gogate, 2017).

Biodiesel production yield might be increased by elevating the lipase concentration due to the increased abundance of activation sites and sufficient mass (Huang et al., 2010). Skoronski et al. (2016) found that overall biodiesel yields increased from 33.12 to 36.84% by raising the enzyme concentration from 1.25 to 2.5% to the fat. However, Huang et al. (2010) discovered that the increase of the biodiesel yield by raising the lipase concentration only happened initially, which stopped increasing when the amount of lipase also increased. Lu et al. (2007) found that 20% lipase based on the fat was the optimal amount for biodiesel yield, since no obvious influence was found more than 20% of lipase used was. Moreover, the combined use of different lipases did better than using single lipase. As observed in Table 1, the highest biodiesel yields were 90.5% and 72.8% using single Novozym435 and Liposome TLIM, respectively, which increased to 97.2% with the combination of 1.96% of Novozym 435 and 2.04% of Lipozyme TLIM.

Referring to the alcohol used for the transesterification, methanol is more popular than ethanol since more biodiesel yields could be produced when methanol is used as a substrate. Skoronski et al. (2016) observed that the biodiesel yield from porcine fat waste by enzymatic

conversion was 99.77% for methanol and 76.04% for ethanol. It was found that increasing the alcohol: fat molar ratio might promote the reaction equilibrium to produce biodiesel, so more biodiesel might be produced with higher alcohol concentration (Lee et al., 2017). Lee et al. (2017) observed that the biodiesel yields increased from 81% to 87% by rising the methanol/feedstock ratio from 14% to 16%. Lu et al. (2007) also reported there were 16% and 14% increases in the biodiesel yield when the alcohol: oil molar ratio ranged from 1:1 to 3:1 and 4:1, respectively. However, the formation of biodiesel might decline if the alcohol concentration was too high to inhibit the enzyme activity. For example, the research by Kumar et al. (2013) stated that the biodiesel conversion yield from beef tallow increased by raising the alcohol : oil molar ratio from 1:1 to 4:1, which fell if further rising the alcohol : oil molar ratio from 4:1 to 5:1. One possible reason is that excessive amounts of methanol could inactivate the lipase in the reaction by forming a barrier around the active sites on the surface of lipase, hindering its contact with the acyl donor (Lee et al., 2002).

Temperature is a main impact factor to affect lipase activity. As observed in Table 1, the temperature used for enzymatic transesterification of fat is in the range of 30-55 ℃. Increasing the temperature in this range could increase the solvent solubility, reduce the oil viscosity and enhance the mass transfer between substrates and enzyme catalyst, resulting in the improved conversion yield of biodiesel (Aryee et al., 2011; Kumar et al., 2013). However, the irreversible deactivation of the enzyme might happen if continuing to increase the temperature, leading to a fall in biodiesel production (Antonio et al., 2018). Under a certain alcohol molar ratio and temperature scenario, the production of biodiesel could be increased by extending the reaction time appropriately. Conversely, the yield was then decreased by further increasing the reaction time due to the backward reaction of transesterification (Aryee et al., 2011; Kumar et al., 2013).

Although enzyme transesterification of waste animal fats has its own advantages, the large-scale application is still limited by high costs of the enzyme in free form that is impossible

for recovery (Adewale et al., 2015; Bajaj et al., 2010). However, enzyme immobilization techniques are promising technologies to reduce costs of enzymatic biodiesel production and have been successfully applied in various field. The reason is that immobilized lipases are more stable and can separate from the product and then reuse them to reduce biodiesel production costs (Nielsen et al., 2008). Huang et al. (2010) and Pollardo et al. (2018) found that the immobilized lipases could be regenerated through the wash of organic solvent after each reaction cycle and continuously reused without any loss of activity. Adsorption is the most widely used technology for lipase immobilization due to its easy operation and low cost. This process is the attachment of lipase on the carrier surface by relative week van der Walls, hydrophobic interactions or dispersion forces (Tan et al., 2010). By contrast, immobilization technologies including covalent bonding and cross-linking can offer more strong binding between the lipase and the support matrix, but the lipase activity might low (Tan et al., 2010). Carriers, such as acrylic resin, textile membrane, hydrotalcite, silica gel, acrylic resin, and diatomaceous earth have been explored for lipase immobilization to produce biodiesel (Jegannathan et al., 2008).The choice of the carrier mainly depends on the biocompatibility, chemical and thermal stability, insolubility under reaction conditions, ability to be easily regenerated and reusable, and cost efficiency (Chandra et al., 2020). It is significant to continuously explore the immobilized lipase technology to achieve a high efficiency, low cost and robust catalyst, as well as applying it to the industrial scale.

Additionally, assisted transesterification technologies including microwave and ultrasound-assisted technologies are helpful for improving the production of biodiesel, reducing the reaction time and costs of production (Adewale et al., 2015). For instance, the microwave assistance could speed up the enzyme-catalyzed reactions through decreasing the damaging effect of operating conditions on enzymes and allowing the whole reaction medium heated evenly. Da Rós et al. (2012) found that the productivity of biodiesel with microwave

assistance was six times higher than the process under conventional heating conditions. Ultrasound-assisted transesterification is also novel method for the enzymatic synthesis of biodiesel, since cavitation during the ultrasound improves enzyme activities and achieves the highest conversion even at the lower level of enzyme (Michelin et al., 2015). Adewale et al. (2016) remarked that the reaction time was significantly reduced by ultrasound compared with traditional method; 96.8% yield was achieved within 20 min. In addition, the application of supercritical CO_2 in enzymatic transesterification of waste animal fats to biodiesel is also a promising method to enhance enzyme activity and reaction rate by increasing the mass transfer rate and further reducing the toxicity of methanol to the enzyme (Lee et al., 2017; Nyari et al., 2018). Nyari et al. (2018) demonstrated that the enzymatic activity of immobilized *Candida antarctica* lipase B increased from 189 U/g to 2486 U/g after activation in pressurized CO_2, and the activity could maintain >80% after 13 cycles of utilization. In spite of this, further investigation on these assisted technologies for biodiesel production are still required.

2.2 Enzyme application in waste animal proteins

In addition to the transesterification of waste animal fats, the conversion of waste animal proteins into valuable products would reduce environmental pollution and create additional economic value as well. The two key structural fibrous proteins able to be recovered are collagen and keratin, of which collagen is the most abundant (Ferraro et al., 2016; Tarté, 2009). However, collagen and keratin are inactive in their original sequence due to their stable protein structure. Specifically, collagen in animal bone and skin is stable because of the hard-fiber structure and consists of α- and β-amino acids linked by peptide bonds (Shoulders & Raines, 2009). Keratin collected mainly from feathers contains high levels of disulphide bonds, hydrogen bonds and salt bridges, which are highly resistant to proteolytic degradation (Sangali & Brandelli, 2000; Gómez-Guillén et al., 2011). For this reason, waste animal proteins must

119 be converted into hydrolysates (including low molecular weight peptides and free amino acids)
120 before they can be effectively utilized. During the enzymatic hydrolysis process, protein-
121 digesting enzymes can break down protein into smaller peptides and amino acids which could
122 also function as sources for protein biosynthesis (see Fig. 2) (Dey & Dora, 2014). Examples
123 for the enzymatic hydrolysis of animal protein wastes reported by previous researchers are
124 summarized in Table 2.

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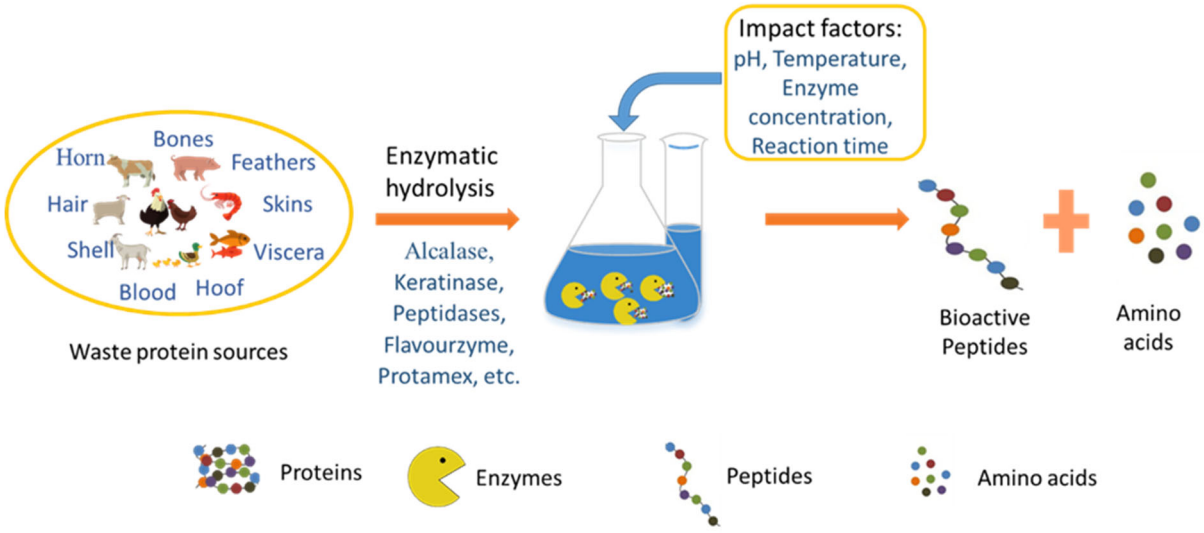

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127 Fig. 2 Enzymatic hydrolysis of protein from waste animal sources

128 Table 2 Examples for the enzymatic hydrolysis of waste animal proteins.

Waste animal proteins	Waste sources	Enzymes	Source of enzymes	Hydrolyzed conditions	Degree of hydrolysis (degradation efficiency) (%)	Hydrolytes property
Collagen-rich waste (Halim et al., 2016; Huo and Zheng, 2009; Morimura et al., 2002; Nurilmala et al., 2019; Ohba et al., 2003; See et al., 2011; Sole et al., 2019; Vasileva-Tonkova et al., 2007)	Animal bone and skin	Enzymes	*Bacillus subtillus*	pH 8 and 60 ℃ for 1h	/	Strong angiotensin-I-converting enzyme (ACE) inhibiting activity
	Pretreated fish bone	Alcalase	*Bacillus* sp.	pH 8 and 60 ℃ for 1h, enzyme concentration 5%	75-80	High anti-radical activity and a high potential for decreasing blood pressure
	De-fatted pig skin			pH 8 and 60 ℃ for 1 h, enzyme concentration 10%	90-95	
	Calf skin	Alcalase	*Bacillus subtilis*	pH 8 and 50 ℃ for 30 min, enzyme concentration 5%	65	Use as peptone for bacterial growth
	Bovine hides byproduct	Combination of proteolytic enzymes	Non-genetically modified organisms	50-60 ℃ for up to 12 h	9.2	Use for the synthesis of a new acrylic biopolymer (retaining agent)

	Salmon skin	Alcalase	*Bacillus licheniformis*	pH 8.39 and 55.30 °C, enzyme concentration 2.5%	77.03	Serve as a good source of desirable peptide and amino acids
	Tuna skin	Alcalase	/	pH 7.0 and 53 °C for 8 h, enzyme concentration 2%	/	High antioxidant and antiglycation activities
	Gadus morrhua Skin	Alcalase	/	pH 10 and 50 ℃ for 3 h, enzyme concentration 3%	25.74	Small soluble peptides below 1200 Da
	Fish protein (Skin, bone, Viscer)	Alkaline protease, protamex, papain, bromelain, flavourzyme	/	pH 6-11, 37-60 ℃ for 60-600 min, enzyme concentration 0.04-3 %	10.22-40.2	Used as food ingredients and additives
	Animal horn, hoof, feather, snails and beaks	Protease	*Bacillus subtillus*	pH 8.3 and 50 ℃ for 1 h	/	High antioxidative activity
Keratin-rich materials (Jaouadi etal., 2013; Mokrejs et al., 2011; ohba et al., 2003; Paul et al., 2013; Stiborova	Feather	Keratinase	*Bacillus* spp.	pH 8.0, 50 °C for 30 min.	/	Used as biological fertilizer
	Feather	Keratinase	*Pseudomonas* sp. P5	pH 7.5 and 50 °C for 24 / 48 h	/	Amino acids and Peptides

et al., 2016; Villa et al., 2013; Thankaswamy et al., 2018)	Feather	Peptidases and keratinases	*Bacillus subtilis* AMR	28 °C for 5 d	90-95%	Used for hair care products
	Feather	/	/	pH 9 and 70 °C for 8 h, enzyme concentration 5%	91	Applied in packaging technology
	Waste animal hair	Keratinase	*Brevibacterium luteoum* MTCC 5982	pH 10.0 and 30 °C for 72 h	80	Valuable amino acids
	Goat hair				77	/
	Cattle hair	Keratinase	*Brevibacillusbrevis* US575	pH 8 and 40 °C	66	/
	Sheep wool				12	/
Miscellaneous by-products (Dey and Dora, 2014; Gbogouri et al., 2004; Pérez- Gálvez et al., 2011; Sowmya et al., 2014)	Salmon heads	Alcalase	*Bacillus licheniformi*	pH 8.0 and 57 °C for 2 h	17.2	/
	Blood meal	Alcalase	/	pH 6.24 and 54.2 °C, enzyme concentration 10%	28.89	Used as organic fertilizer
	Shrimp waste (head and shell)	Alcalase	/	pH 8.25 and 59.37 °C for 84.42 min, enzyme concentration 1.84%	33.13	protein content (72.3%) and amino acid (529.93 mg/gm)

	Shrimp waste	Alcalase	/	25–30 °C for 4 h, enzyme concentration 0.3 %; 50 °C, enzyme concentration 0.5 %	/	High antioxidant activity	129 130

Generally, the process of enzymatic hydrolysis mainly includes pretreatment of animal protein wastes, selection of the enzyme, controlling of the hydrolysis process for a period of time, measuring the extent of enzymatic reaction, and terminating the reaction. Various enzymes including alcalase, protamex, protease, bromelain and flavourzyme have been used in hydrolyzing protein-rich wastes (Di Bernardini et al., 2011). The study by Dey and Dora (2014) compared the hydrolysis of shrimp waste (head and shell) by several microbial proteases (alcalase, neutrase, protamex and flavoursome) and concluded that alcalase revealed the highest degree of enzymatic hydrolysis and recovery of protein. As shown in Table 2, alcalase with higher hydrolytic activity is preferred to other enzymes for the hydrolysis of collagen-rich wastes. Keratinase is usually chosen for enzymatic hydrolysis of keratin-rich materials considering its better ability to treat compact substrates than other proteases (Brandelli, 2008; Thankaswamy et al., 2018). Virtually, the origin of enzymes such as bacteria, yeast and fungi would affect their activities. Morimura et al. (2002) compared the efficiency of sixteen commercial enzymes for fish bone and pig skin degradation. They found that the alkaline protease originated from *Bacillus* species performed better than those originating from fungi. From Table 2, it is observed that most of the enzymes used for animal by-products hydrolysis are originated from bacteria. *Bacillus* species seems to represent a major source of alcalase and keratinase for animal by-products hydrolysis (Pant et al., 2015). Hug et al. (2020) also discussed that a deeper understanding of the underlying biosynthetic logic of secondary metabolites in recent years, in turn, boosts the unprecedented use of bacteria as programmable as biological factories for the production of valuable bioactive compounds.

In addition to the substrate source and the employed enzyme, the degree of hydrolysis (DH) is another parameter affecting the physicochemical properties of enzymatic hydrolysates (Lasekan et al., 2013). High DH hydrolysates had relatively high solubility, while lower DH resulted in greater capacity of emulsion, stability of emulsion, and absorption of fat (Gbogouri

et al., 2004). To obtain the optimal DH, hydrolyzed conditions, including pH of the reaction medium, temperature, time of hydrolysis and enzyme concentration, had to be well controlled during the hydrolysis (Lasekan et al., 2013). Moreover, at higher reaction temperatures, the peptide bonds are unfolded to facilitate access to peptide bonds to increase the initial reaction rate. Conversely, too high temperatures provoke protein denaturation where the proteolytic activity is lost (Prieto et al., 2008). Therefore, the optimal temperature should consider both enzyme reaction kinetics and enzyme inactivation (Pérez-Gálvez et al., 2011). As for the enzyme-substrate ratio, the DH increased by increasing the enzyme concentrations (Halim et al., 2016). A combination of the above impact parameters is important for hydrolysis, and response surface methodology (RSM) has been considered an effective tool that can optimize the parameters (Diniz and Martin, 1996). The optimized combination of the hydrolyzed parameters reported in other analyses and the corresponding DH value are summarized in Table 2.

For products application, hydrolysates from animal wastes could be used as bio-fertilizers and food ingredients and additives (Kim & Patterson, 2000; Paul et al., 2013). Darah et al. (2013) indicated that enzymatic hydrolysate of feather wastes can be used as nitrogen-rich fertilizers and animal feed ingredients with high nutritional quality. However, the added value of those hydrolysates as fertilizers or additives was relatively low and might not meet economic feasibility standard. Nevertheless, the more cost-effective option is to shift to a molecular level of utilization where the nature protein hydrolysates with bioactive properties would be applied in highly profitable areas such as cosmetics and biomedicine industries (Alahyaribeik and Ullah, 2020). Enzymatic hydrolysates of waste animal proteins could be a source of bioactive peptides and free amino acids, which showed strong antioxidative activity and angiotensin-I-converting enzyme (ACE) inhibiting activity (Halim et al., 2016; Kim & Mendis, 2006; Paul et al., 2013; Pérez-Gálvez et al., 2011). For example, the study by Nurilmala et al. (2019) and

Bougatef et al. (2010) indicated that the antioxidant activities of hydrolyzed proteins from animal by-products were higher than their original form. Compared with the synthetic antioxidants and antihypertensive drugs, natural antioxidant and antihypertensive drugs from animal wastes are safer, which cause fewer side effects and damage to health (Centenaro et al., 2011; Ghassem et al., 2011). According to previous reports, keratinous hydrolysates could offer stronger antioxidant activities as shown by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activities, while collagen hydrolysates exhibited a higher antihypertensive activity as shown by higher ACE inhibitory activities due to the higher concentration of proline and hydroxylproline in collagen waste compared with keratin waste (Cheng et al., 2009; Karamać et al., 2005; Alahyaribeik et al., 2020). The study by Cheng et al. (2009) found that the hydrolysate from chicken leg bone protein hydrolyzed by alcalase for 4 h had several peptides with strong ACE inhibitory activities (above 50%). Karamać et al. (2005) indicated that the inhibition of ACE by chicken feathers was up to 49.6%. Further, the antioxidant and ACE inhibitory activity of protein hydrolysates could be influenced by the protein substrate, enzyme type, degree of hydrolysis and molecular weight of peptides (Lasekan et al., 2013). Noteworthily, keratin hydrolysates exhibit a higher antioxidant activity than collagen hydrolysates and peptides, because of its high resistance to proteolysis (Alahyaribeik and Ullah, 2020).

Hence, enzymatic hydrolysis is a promising technology for converting waste animal proteins to high-valued products, but further studies are important to optimize the hydrolysis process, and to investigate the bioactivity of protein hydrolytes and reduce the operational costs.

2.3 Potential of enzymes for the commercial application

In most of previous studies, the application of enzymes in animal fat and protein wastes management was controlled under the optimized conditions. However, to date, there are not adequate studies about the enzyme application under harsh industries conditions. The

development of thermo- and solvent-stable lipases is important for improving the catalysis efficiency in the industrial application. The novel lipase with robust activity could be isolated from special spots like thermophilic region and organic solution outlet (Sahoo et al., 2020; Zhu et al., 2011). Nehal et al. (2019) isolated a new thermophilic non-induced lipase from oil waste in Algeria with a high organic solvents tolerance and 75% retaining activity the presence of 10 mM Fe^{2+}, K^{+}, and Na^{+} ions. The half-time of this novel lipase could reach 22 hours and 90 minutes at 50℃ and 60℃, respectively. Ktata et al. (2020) discovered a newly thermostable lipase from *Aeribacillus pallidus* (GPL) and applied it in oil wastewater treatment. The GPL showed maximum activity at 65℃ and pH10 and exhibited a 96% oil removal efficiency.

Modern biotechnology has developed many powerful tools to explore novel enzymes from the larger uncultured microorganism group that are suitable to harsh industrial conditions. Rapidly developed in recent years, the combination of molecular biological technique like high-throughput sequencing and bioinformatics such as metagenomics or proteomics has helped to discover massive uncultivable microorganisms and their metabolic enzymes in nature (Bilal et al., 2018; DeCastro et al., 2016;). The mass sequencing data of samples directly collected from nature or some reactors could be organized, assembled and binned to genomes in *silico* (Stewart et al., 2019; Uritskiy et al., 2018). After gene sequence alignment to existing DNA and protein databases, the targeted enzyme genes were obtained and the property of enzymes could be identified and characterized after recombinant expression by gene engineering (von Meijenfeldt et al., 2019; Paek et al., 2020). Nonetheless, the comprehensive computer knowledge and skills are very crucial for environmental scientists and biologists to discover novel enzymes. For example, Verma et al. (2019) screened a total of 21 unique sequences of new alkaliphilic lipases from the public gene databases of archaeal and environmental metagenomic proteins according to homologous relationship. The putative lipases shared a significantly conserved pentapeptide sequence similarity [X-His-Ser-X-Gly]

motif, which is the key feature of the lipase family, to the bacterial alkaliphilic lipases and thermostable ones. Gong et al. (2020) constructed a more efficient enzyme expression system that ten promoters were evaluated and the aprE promoter showed an excellent promotion of keratinase. The optimized promoter screening system significantly improved the keratinase activity by 16-fold which increase from 165 U/mL to 2605 U/mL in *Bacillus subtilis*. Su et al. (2017) successfully expressed a novel keratinase coding gene mined via function-driven screening with fosmid library in *Escherichia coli* BL21 (DE3). The purified recombinant keratinase was stable in various surfactants and had potential in biodegradation of keratin wastes.

Moreover, enzyme directed evolution is another powerful method to develop enzymes with bioactivity withstanding any harsh and unfavorable conditions (Romero et al., 2009; Morrison et al., 2020). Artificial selection or screening from the successive generations of random mutation could optimize protein function. Druteika et al. (2019) harvested three mutants of lipase isolated from *Geobacillus* sp. using site-directed mutagenesis. The lipase mutants successfully obtained thermo-activity, thermostability and activity in organic solvents. Furthermore, the adaptive evolution could enhance the productivity of lipase, reducing the application costs. The activity of lipase2, an 80℃ thermostable mutant of *Yarrowia lipolytica* and overexpressed in *Pichia pastoris*, improved from 482 to 1465 U/mL by the optimization of the shaking flask culture conditions (Zhou et al., 2020). Yuan et al. (2019) obtained 1.9-fold higher activity of lipase than that of original strain SPZ1 after an adaptive evolution over 1000 generations of growth-based selection and acquired a 556% increase in lipase flux.

Although new biotechnologies have been developed generation by generation, just a few novel relevant enzymes with superior properties were applied in animal fat and protein wastes treatment and reutilization. Therefore, further studies are urgently required for this area to improve biodegradation efficiency and achieve sustainable and renewable energy goals.

3. Bio-utilization of waste fats and proteins for enzymes production

One major challenge in enzyme production and application industries is the cost of culture media for microorganism growth. Nowadays, frequently-used ingredients in culture media (e.g. peptones) contain essential components like organic nitrogen, peptides and amino acids as nitrogen sources. Generally, the producing cost of these valuable components from animal and plant materials are relatively high (Taskin et al., 2011). Thus, enzymes producing by microorganism need to be carried out in a low-cost way for not only more extensive biotechnological applications but more large-scale commercial purposes. Hence, some biowastes like protein hydrolysates of discarded feathers and wool would be interesting alternatives as the sources of organic nitrogen of culture media to commercial casein, fish, meat and soy peptones. For example, feather hydrolysates (feather peptones) contain the essential nutrients (such as C, N, S, Ca, Fe) that are needed for microbial growth activities (Callegaro et al., 2019). More importantly, the production of enzymes (i.e. proteases) and the biodegradation of nitrogen-rich residual biowastes both occur at the same stage of the biological process and simultaneously under suboptimal conditions (Falco et al., 2019). For example, Altun et al. (2018) used feathers as the sole carbon/nitrogen source for keratinase production by *S.pactum* 40530 at 30L fermentation scale, following that the enzymatically produced feather hydrolysate (concentration of 60 g/L) was used successfully as a low-cost alternative peptone to produce cyanophycin. Rebah and Miled (2013) noted that by-products including fish meat wastes and chitinous materials created during fish processing could act as efficient nutrient sources for microbial life activities to generate the important industrial enzymes such as protease, lipase, chitinolytic enzymes and even ligninolytic enzymes. The defatted pre-treatment of fish-based medium could enhance protease production from 124.90 U/ml (using commercial peptone) to134.57 U/ml as the lipid-free by-product could support protease

281 synthesis by the microbial species more efficiently than other nitrogen sources. Furthermore,
282 the comparative trials of lipase production could confirm that the presence of lipids wielded an
283 inhibitory effect on microorganisms' growth (Esakkiraj et al., 2010). Feathers could act as the
284 single carbon or nitrogen source for protease production by *Rhodococcus erythropolis*,
285 *Geobacillus stearothermophilus* and two *Bacillus* species (Alahyaribeik et al., 2020). A
286 thermostable protease was produced by a mesophilic feather-degrading species-*Bacillus* sp.
287 CL33A where the optimal temperature and pH conditions for proteolytic activity were,
288 respectively, 48 - 62 °C and pH 7.2 - 9.2 (de Oliveira et al., 2017). Pernicova et al. (2019) also
289 demonstrated that waste chicken feather was used as sole carbon source by *Pseudomonas*
290 *putida* KT2440 for the production of keratinase and medium-chain length
291 polyhydroxyalkanoate (mcl-PHA). For wider-range applications, Liu et al. (2020) attempted
292 firstly to use feather wastes as cheap nitrogen source for alkaliphilic *Bacillus agaradhaerens*
293 C9 growth. This strain C9 produced keratinase and bio-flocculants at same time and the latter
294 were applied in treating straw ash-washing wastewater.

295 It is worth noting that the growth of microorganisms depended strongly on medium
296 composition, therefore the majorization of substrate component proportion is the key factor for
297 enzymes production improving. Biologists have traditionally employed response surface
298 methodology (RSM) to design the optimal variable factors. RSM is a helpful model to reduce
299 number of experiments by checking the effect of factors influencing the responses when
300 varying them simultaneously (Hajji et al., 2008). Fakhfakh-Zouari et al. (2010) isolated a
301 keratinolytic activity producing strain *Bacillus pumilus* A1. They firstly used Plackett–Burman
302 design to identify five major variables: feathers meal, soy peptone, NaCl, KCl, and KH_2PO_4
303 influencing keratinolytic enzymes production. Then they further optimized significant
304 variables central composite design (CCD). Finally, a 3.4-fold increase in keratinase production
305 (87.73U/ml) was achieved whereas the production was 25.9U/ml in initial medium.

306 Kalaikumari et al. (2019) carried out statistical analyses to enhance the activity of keratinase
307 production and results showed that maximum enzyme activity was 1872.5 U/ml, achieving a
308 6-fold yield. They even did this on a large scale from 5L to 500L production of keratinase and
309 observed that the production rate of keratinase improved greatly together with increased
310 volume of bioreactor. Meanwhile, the production time was greatly reduced.

311 In a word, the transformation of by-products of meat processing into valued hydrolysates
312 and those by-products function as substrates to produce enzymes have not only commercial but
313 environmental significance. Recently, researchers have obtained those hydrolysates through
314 biological activities driven by protein-rich waste. While this has been successful, more efforts
315 are needed to research large-scale enzymes production using very large amounts of animal
316 wastes.

317

318 **4. Future perspectives**

319 Enzyme technology is a sustainable and promising strategy for waste animal fats and
320 proteins reduction and reutilization, which solves the issue of environmental pollution and
321 brings the economic benefits simultaneously. However, the large-scale enzymatic utilization
322 in either animal fat or protein wastes biodegradation is still limited due to the high enzyme
323 costs and low reaction rate at room temperature. The discovery of novel lipase with robust
324 activities should be developed simultaneously with optimization of enzymatic biodiesel
325 production process to realize cost-efficient production. The wider range and higher profitable
326 application of bioactivate hydrolysates (e.g. cosmetics and bio-pharmaceutical) originated
327 from protein wastes would in turn, promote the progress of technologies, efficiency and
328 capacity of animal protein wastes biodegradation. It is a promising strategy that combine the
329 biodegradation and the production of enzymes from carbon/nitrogen-rich residual biowastes at
330 the same stage and under suboptimal conditions. Meanwhile, the products and enzymes could

be harvest separately. Numerous advanced studies are therefore expected to be done in this mentioned area for the future sustainable development.

5. Conclusions

Enzymatic hydrolysis has been found to be a better efficient and sustainable way in the management of waste animal fat and protein. It can achieve dual benefits for environmental protection and valuable products generation. Biodiesel and bioactivate hydrolysates as the major bioconversion products of animal fat and protein wastes could be used as green biofuel in engine and nature bio-additives in animal food, soil, cosmetics and pharmaceuticals. Moreover, the nutrient-rich animal fat and protein wastes could act as carbon/nitrogen source for the growth of enzyme-producing microorganisms to reduce industrial enzyme production costs. Therefore, it is necessary to develop an integrated process involving wastes biodegradation and enzyme production at same stage and harvest products separately in the future to maximize the benefits.

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