Volatile fatty acids production from waste streams by anaerobic digestion: a critical review of the roles and application of enzymes

Siran Feng[a], Huu Hao Ngo[a,f,*], Wenshan Guo[a], Soon Woong Chang[b], Dinh Duc Nguyen[b], Yi Liu[c], Shicheng Zhang[c], Hoang Nhat Phong Vo[d], Xuan Thanh Bui[e], Bich Ngoc Hoang[f]

[a]*School of Civil and Environmental Engineering, University of Technology Sydney, Sydney, NWS 2007, Australia*
[b]*Department of Environmental Energy Engineering, Kyonggi University, 442-760, Republic of Korea*
[c]*Department of Environmental Science and Engineering, Fudan University, 2205 Songhu Road, Shanghai 200438, China*
[d]*Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Cornwall Street, Woolloongabba, Queensland 4102, Australia*
[e]*Key Laboratory of Advanced Waste Treatment Technology, Faculty of Environment & Natural Resources, Ho Chi Minh City University of Technology (HCMUT), Vietnam National University Ho Chi Minh (VNU-HCM), Ho Chi Minh city 70000, Vietnam*
[f]*Institute of Environmental Sciences, Nguyen Tat Thanh University, Ho Chi Minh City, Viet Nam*

* Corresponding author: *E–mail address:ngohuuhao121@gmail.com*

Abstract Volatile fatty acids (VFAs) produced from organic-rich wastewater by anaerobic digestion attract attention due to the increasing volatile fatty acids market, sustainability and environmentally friendly characteristics. This review aims to give an overview of the roles and applications of enzymes, a biocatalyst which plays a significant role in anaerobic digestion, to enhance volatile fatty acids production. This paper systematically overviewed: (i) the enzymatic pathways of VFAs formation, competition, and consumption; (ii) the applications of enzymes in VFAs production; and (iii) feasible measures to boost the enzymatic processes. Furthermore, this review presents a critical evaluation on the major obstacles and feasible future research directions for the better applications of enzymatic processes to promote VFAs production from wastewater.

Keywords Volatile fatty acids, anaerobic digestion, enzyme, wastewater

1. Introduction

The world is currently experiencing a huge amount of wastewater production and discharge due to geological and anthropogenic activities (Deshpande et al., 2020), leading to a great threat to humans, flora, fauna and the environment. Traditional wastewater treatment is costly and energy-intensive, mostly due to the disposal of activated sludge produced during this process (Appels et al., 2008). Since waste streams such as brewery wastewater (Arantes et al., 2017; Chen et al., 2022a), diary wastewater (Hemalatha & Mohan, 2022), aquaculture wastewater (Saxena et al., 2022), slaughterhouse wastewater (Hilares et al., 2021), and food industry wastewater (Cheng et al., 2020) are rich in organics, how to recover those organic wastes by transforming them into valuable products to reduce treatment costs or create a new kind of economy is attractive. In recent decades, bioenergy production from organic-rich waste streams via anaerobic digestion has emerged as a 'hot' topic, the aim being to diminish activated sludge production, reduce pathogens, ease the world energy crisis, and benefit downstream wastewater purification (Khan et al., 2016; Nabaterega et al., 2021; Serrano León

et al., 2018). Biomethane (Pakkaew & Polprasert, 2021; Kang et al., 2020), biohydrogen (Lin et al., 2016; Liu et al., 2021), bioethanol (Katsimpouras et al., 2017), and bioplastic (Yan et al., 2010a) are feasible bioenergy forms produced by anaerobic digestion from waste streams, and with volatile fatty acids (VFAs) a good option.

VFAs, also called short-chain fatty acids, are saturated or unsaturated carboxylic acids with six or fewer carbon atoms, such as formic, acetic, propionic, butyric, valeric, caproic acid (Atasoy et al., 2018). VFAs are not only important products for food, pharmaceuticals, cosmetics, tanning, and chemical industries (Atasoy et al., 2018) but also inexpensive raw materials for making biodiesel, bioplastic, biogas, and other valuable products (Fortela et al., 2016; Fradinho et al., 2014; Sydney et al., 2018). VFAs constitute a promising market size and have wide applications (Table 1). It has been reported that the Asian-Pacific acetic acid and butyric acid markets currently register a compound annual growth rate (CAGR) of over 5% during the forecast periods 2022-2027 (https://www.mordorintelligence.com/industry-reports/acetic-acid-market) and 2021-2026 (https://www.mordorintelligence.com/industry-reports/butyric-acid-market#), respectively. Meanwhile an over 6% CAGR of propionic acid Asian-Pacific market was predicted during the forecast period of 2022-2027 (https://www.mordorintelligence.com/industry-reports/propionic-acid-market). At present, VFAs are mainly produced by petrochemical processes that are not eco-friendly since these processes are linked to a large amount of greenhouse gas emissions and non-renewable feedstocks. In the meantime, bio-based VFAs production from waste streams is more cost-effective, sustainable, and environmentally friendly (Atasoy et al., 2018). Compared to biogas production, generating VFAs during anaerobic digestion is more promising since VFAs production needs much less retention time, smaller reactor unit, and nearly all substrates can be transformed into VFAs (Fang et al., 2018).

[Insert Table 1]

Anaerobic digestion is a bioprocess requiring the cooperation of various enzymes. Thus, bio-based VFAs production is closely related to enzymes. VFAs production by anaerobic digestion includes the processes of hydrolysis, acidogenesis, and acetogenesis. During the process of hydrolysis, substrates such as carbohydrates, proteins, and lipids are biodegraded by hydrolases secreted by hydrolysis microorganisms into monomers or oligomers. Then, those monomers or oligomers are converted into VFAs by acidifying enzymes such as dehydrogenase, acetate kinase (AK), butyrate kinase (BK), and acetyl-CoA synthase (Tian et al., 2020). Referring to acetogenesis, the by-products of acidogenesis such as alcohols and other VFAs are further converted into acetate as well as carbon dioxide (CO_2) and hydrogen (H_2) under the cooperation of enzymes in acetogens (Appels et al., 2008; Wu et al., 2016). Enzymes play a significant role in bio-based VFAs production and good applications of enzymes will definitely benefit bio-VFAs production. Any application of enzymes should be sustainable, eco-friendly, and energy-saving (Cheng et al., 2020). However, current application of enzymes in bio-based VFAs production is restricted in enzymatic pre-treatment when substrates contain cellulose, lipase, and other resistant pollutants. Furthermore the enzymatic mechanisms of VFAs fermentation have not been well-learned, which does not encourage the application of enzymes.

Organic-rich wastewater has great potential in bio-based VFAs production, and enzymes play a crucial role in this process. The main aim of this review is to reveal the roles and applications of enzymes in VFAs production from waste streams. Enzymatic processes of volatile fatty acids formation, competition, consuming pathways, and current applications of enzymes in anaerobic digestion are well described. Furthermore, factors that affect bio-based VFAs production and feasible measures such as ideal conditions, additives, and genetic engineering to enhance these enzymatic processes were summarized. Finally, perspectives on the obstacles of enzyme applications in VFAs production and relative solutions were discussed.

2. Enzymatic processes during VFAs production

The process of acidogenesis involves numerous distinct reaction pathways catalysed by various enzymes under anaerobic conditions. During the creation of these by-products, pyruvate is a vital intermediate product, the flow directions of which are determined by substrates, fermentation conditions, and strains (Zhou et al., 2018) wield a great influence on by-products composition. There are various by-products such as VFAs, H_2, CO_2, lactic acid, and alcohols during the acidogenesis process (Appels et al., 2008; Bhatia & Yang, 2017). Some of those intermediates would be further converted into acetate through the process of acetogenesis (Appels et al., 2008; Ragsdale & Pierce, 2008). To improve VFAs production, understanding the metabolism pathways of VFAs formation, competition, and consumption are of great importance. In this scenario the relative enzymatic processes are reviewed.

2.1 Enzymatic processes with VFAs production

During acidogenesis, VFAs formation started with pyruvate, the ultimate product of the Embden-Meyerhof pathway (EMP). According to the types of produced VFA, bio-VFAs production pathways could be divided into acetate-ethanol type fermentation, acetone-butanol-ethanol fermentation, propionate-type fermentation, butyrate-type metabolic pathway, mixed-acid metabolic pathway, and homoacetogenic fermentation pathway (Zhou et al., 2018). Since acetic acid, propionic acid, and butyric acid are the main components of bio-based VFAs, the enzymatic pathways of these fatty acids are emphasized (Fig. 1). Firstly, pyruvate formate lyase (PFL) catalyses pyruvate into formate and acetyl-CoA (Oh et al., 2011). It is worth noting that PFL is not the only enzyme that can catalyse the formation of acetyl-CoA. Pyruvate dehydrogenase complex, pyruvate ferredoxin/flavodoxin oxidoreductase, acetyl-CoA synthase, phosphotransacetylase (PTA) and other enzymes are all feasible options responsible for acetyl-CoA synthesis directly or indirectly (Zhu et al., 2022). Then, acetyl-CoA will be further converted into acetic acid, propionic acid, and butyrate acid

by key enzymes AK, propionyl-CoA transferase (PCT), and BK, respectively (Zhou et al., 2018). Besides, pyruvate can be transformed into lactate by lactate dehydrogenase (LDH) (Sikora et al., 2013), and the produced lactate, acetyl-CoA, as well as succinate are all feasible intermediates for propionic acid formation (Bhatia & Yang, 2017; Zhou et al., 2018). Pyruvate can be converted into 2-acetolactate under the condition of acetolactate synthase, and 2-acetolactate would be further transformed into isobutyric acid and isovaleric acid by acidifying enzymes. These enzymes include keto-acid reductoisomerase, 2-keto acid decarboxylase, dihydroxy acid dehydratase, and alcohol dehydrogenase (Bhatia & Yang, 2017).

[Insert Fig. 1]

During acetogenesis, those higher organic acids and alcohols produced during acidogenesis can be further converted into acetate by acetogens (Appels et al., 2008). Besides, CO_2, H_2, and formate could also be converted into acetate by homoacetogens via acetyl-CoA reduction or Wood Ljungdahl pathway (WLP) (Drake, 1994; Ragsdale & Pierce, 2008). WLP consists of two branches, namely the carbonyl branch and methyl branch (Fig. 2). In the methyl branch, CO_2 is first reduced to formate by formate dehydrogenase (Fdh). Then, the produced formyl group combines with co-factor tetrahydrofolate (THF) to form formyl-THF under the conditions of formyl-THF synthetase and ATP. After that, the formyl-THF is transformed into methenyl-THF, then methylene-THF, followed by methyl-THF, and finally methyl-corrinoid-iron-sulfur protein (methyl-CoFeSP) by a series of enzymes (Fig. 2). For the carbonyl branch, CO_2 is first reduced to carbon monoxide (CO), followed by the formation of acetyl-CoA from CO, methyl-CoFeSP, and coenzyme A (CoA) via the catalysis of CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). Finally, acetyl-CoA is converted into acetate by enzymes PTA and AK (Bhatia & Yang, 2017).

[Insert Fig. 2]

2.2 Pathways competing with or consuming VFAs

It is worth noting the flow directions of pyruvate and acetyl-CoA are not only transformed into VFAs but are also involved in the formation of lactic acid, alcohols, acetone, and other by-products (Fig. 3) (Yang et al., 2021; Zhou et al., 2018). Specifically, pyruvate is able to be converted into lactate by enzyme LDH (Sikora et al., 2013). It is possible for acetyl-CoA to be catalysed into acetaldehyde and then ethanol by enzyme aldehyde/alcohol dehydrogenase (AdhE) (Yang et al., 2021). Also, acetyl-CoA can be transformed into acetone under the cooperation of CoA-transferase subunit A and B (CTFA/B) and acetone dehydrogenase (ADC). Under the enzymes of crotonase (CRT), hydroxybutyryl-CoA dehydrogenase (HBD), butyryl-CoA dehydrogenase (BCD) and butanol dehydrogenase, acetyl-CoA can be converted into butanol. In this scenario, measures such as genetic engineering to inhibit those competition pathways or switch the pathways towards VFAs production are of great significance

It is well-known that acetate, CO_2 and H_2 are feasible feedstock for producing biomethane (Niu et al., 2018). The methyl group of acetate could be catalysed into biomethane through aceticlastic pathways (Liu & Whitman, 2008). During this process, acetate is first transformed into CH_3-CO-S-CoA, then the methyl group of CH_3-CO-S-CoA is transferred to tetrahydromethanopterin (H_4SPT) and followed by HS-CoM to form CH_3-SCoM. Finally, the CH_3-SCoM is converted into biomethane. The enzymes that are part of this process are shown in Fig. 3. As for the reduction of carbon dioxide, CO_2 is reduced to biomethane through formyl, methylene, and methyl levels (Liu & Whitman, 2008) (Fig. 3). Exactly, CO_2 is first reduced to formyl level (formyl-H_4MPT) by binding to methanofuran and then H_4MPT with ferredoxin as a direct electron donor. Then under the condition of reduced F_{420} as a direct electron donor, the formyl group is further catalysed into methylene level in the form of methylene-H_4MPT, an intermediate which would be further dehydrated to

methyl-H_4MPT. Next, the methyl group of methyl-H_4MPT is transferred to coenzyme M (CoM), transforming into CH_3-SCoM. The methyl group of CH_3-SCoM is finally transformed into methane by a key methanogenesis enzyme methyl-CoM reductase (Mcr). It is worth noting that formate is also a feasible substrate during this process since: firstly, it can be oxidized into CO_2 by enzyme Fdh; and secondly, the produced CO_2 could be further reduced to biomethane (Liu & Whitman, 2008).

[Insert Fig. 3]

During acetogenesis, other organic fatty acids would be transformed into acetate, which curtails the value of by-products (Zacharof and Lovitt, 2013). If the targeted products are propionic acid, butyrate acid, or other high organic fatty acids, acetogenesis is not conducive to by-products accumulation. So the process of acetogenesis will be inhibited in this situation. However, if the targeted VFA is acetic acid, acetogenesis could enrich acetate, which will definitely benefit this process since it is the VFAs mixture rather than pure VFA that is obtained from anaerobic digestion, and typical VFAs purification methods such as reverse osmosis, membrane distillation, high voltage electrodialysis, and pervaporation; these are costly and energy-intensive (Aghapour Aktij et al., 2020). For this reason, it is necessary to explore low-cost purification methods or switch enzymatic processes to a specific VFA production.

3. Enzyme applications in VFAs production

Currently, studies have concentrated on applying enzymes to improve VFAs production from wastewater. Enzymatic pre-treatment is the main application of enzymes in VFAs production. Hydrolases like cellulase, lipase, and amylase are typical enzymes used for increasing the hydrolysis rate of cellulose, lipids, and other resistant substrates, which will further contribute to VFAs production. For example, a study which used immobilized

Candida Rugosa lipase for the degradation of oil and grease from pet food industries demonstrated that about 50% of oil and grease decomposition worked due to enzyme activity, and an obvious increase in VFAs yield was achieved (Jeganathan et al., 2006). The production of long-chain fatty acids increased by 155% and 85%, respectively, when free lipase and immobilized lipase were used for the hydrolysis of high oil and grease containing wastewater from the pet food industry (Jeganathan et al., 2007).

It is worth noting that solid waste is an important part of wastewater and employing solid waste for fermentation is much easier since they are organic intensive and have smaller volumes. However, solid waste is also rich in resistant pollutants, which means pre-treatment before anaerobic digestion is necessary. There are various methods for solid wastes collection and the addition of chemical flocculant is one of them. However, the flocculant typical contains Al^{3+}, which would inhibit the hydrolysis process (Chen et al., 2018). Fortunately, it has been proved that the chemically enhanced primary sedimentation sludge is still feasible for VFAs production (Lin & Li, 2018a; Lin & Li, 2018b). Many cases have proved the positive effects of enzyme applications on VFAs production from sludge. For instance, when employing primary sludge and organic waste from municipal wastewater, the direct addition of enzyme cocktail enhanced VFAs yield by 37 - 43% under the pH of 5.0 (Owusu-Agyeman et al., 2021). Bahreini et al. (2020) employed cellulase for the pre-treatment of primary and rotating belt filter sludges derived from wastewater at 25 °C; they indicated that the VFAs yields rose from 78 - 192 to 87 - 202 mg COD/g volatile solids (VS) and 52 - 103 mg COD/g VS to 93 - 188 mg COD/g VS, respectively. The usage of endogenous amylase increased by 129.6% VFAs concentration when anaerobic sludge served as the substrate for VFAs production (Yu et al., 2013). More examples are shown in Table 2.

[Insert Table 2]

Despite the significant effects of enzyme application, it still has limitations. For one thing, enzyme-based pre-treatment is not always the best option. Enzymatic pre-treatment is not perfect, and chemical and physical methods have better hydrolysis efficiency than enzymes in some cases (Gonzalez et al., 2018). For this reason, it is necessary to take measures such as the combination of enzymes with other pre-treatment strategies (Li et al., 2021; Yan et al., 2010b) to remove the drawbacks of enzymatic pre-treatment and achieve better VFAs production. For another, it is important to select appropriate enzymes for pre-treating a specific substrate. Fang et al. (2018) discovered the white-rot fungi *P. sajor-caju* exhibited better delignification ability than that of *T. versicolor*. This may because the dominant ligninolytic enzymes produced by *T. versicolor* was laccase, which has poorer redox potential and can only decompose substrates with low redox potential (Fang et al., 2018).

Conversely, manganese peroxidase, the dominant ligninolytic enzymes in *P. sajor-caju*, has higher redox potential and the ~~produced~~ formation of alkylitaconic acids and other low molecular acids in *P. sajor-caju* can protect cellulose from being attacked by hydroxyl radicals produced with lignin degradation (Fang et al., 2018; Kishi et al., 1994; Rahmawati et al., 2005). Compared to endogenous protease and the mixture of endogenous protease and amylase, endogenous amylase had the best solubilization and acidification performance on sludge pre-treatment. After pre-treatment lasting 7 h using endogenous amylase, the VFAs concentration in supernatant increased by 129.6% (Yu et al., 2013).

4. Measures for enhancing VFAs enzymatic processes

4.1 Fermentation conditions

4.1.1 Temperature and pH

Despite the feasible applications and crucial roles of enzymes in VFAs production, the performance of enzymes is affected by temperatures, pH, trace elements, and other parameters (Hendriks et al., 2018; Lee et al., 2014). Temperature is an important factor that would affect enzyme activity by modifying the tertiary structure of enzymes. Relative research showed that mesophilic (35 ℃) conditions are the most efficient and economic temperature for VFAs production (Jiang et al., 2013). pH, a parameter that influences hydrolysis rate and acidogenesis process, is important for microbial survival and enzyme activity (Chen et al., 2017; Huang et al., 2018; Lin & Li, 2018a). It has been reported that extreme pH conditions like pH < 3 or pH >12 will inhibit the activity of acidogenic bacteria (Liu et al., 2012). As for the hydrolases like protease and α-glucosidase, they exhibited higher activities with the pH increase in the pH range of 2.0 - 10.0 when employing Al-sludge for VFAs fermentation under the conditions of temperature 37 ± 1℃ (Lin & Li, 2018a). Besides, pH affects the components of produced VFAs when exploring the effects of enzyme cocktail addition on VFAs fermentation (Owusu-Agyeman et al., 2021). Thus, finding suitable pH is important for VFAs production and its composition.

4.1.2 Hydrolysis time and enzyme dose

Despite the high efficiency of enzyme, it still needs enough time for the hydrolysis of substrates. Meng et al. (2017) observed that the hydrolysis rate of lipid by lipase increased with the extension of hydrolysis time. Yet the hydrolysis speed decreased with the prolonged of hydrolysis time and eventually levelled off in a certain range. From this perspective, an appropriate hydrolysis time could improve enzymatic pre-treatment efficiency and benefit the industrialization of enzyme application. Enzyme dose also affects the hydrolysis of substrates and VFAs production. One study employing 0.5%, 1.0%, and 1.5% enzyme dose for the pre-treatment of cellulose in primary sludge revealed a positive relationship between enzyme dose

and cellulose removal efficiency, and the VFAs production was increased from 78 - 192 to 87 - 202 mg COD/ g VS after enzyme addition (Bahreini et al., 2020).

This research also showed there was a small increase in cellulose removal when the enzyme dose increased from 1% to 1.5% (Bahreini et al., 2020). When using lipase Z for the hydrolysis of fat from dairy wastewater under the conditions of temperature at approximately 30 °C, agitation of 200 rpm, power of ultrasound at 125 W, and 66% duty cycle, an improved hydrolysis rate was observed when enzyme dose increased from 0 to 0.4% w/v. There was, however, a marginal difference in the enzyme content range of 0.2% to 0.4% w/v (Adulkar & Rathod, 2014). A higher enzyme dose benefits the hydrolysis of substrates but will remain stable after the enzyme dose exceeds a certain value. Based on this finding, it is necessary to find a suitable enzyme dose that is cost-effective.

4.1.3 Enzyme addition methods

Enzymatic pre-treatment and direct addition are the two main methods for enzyme application in VFAs production (Parawira, 2012), and both perform well in promoting VFAs production. Endogenous amylase, endogenous protease, and their mixture pre-treatment promoted the solubilization and acidification of sludge (Yu et al., 2013). Luo et al. (2021) introduced cellulase to enhance the co-fermentation of WAS and paper waste, the results illustrated that the highest VFAs yield of 3014 mg COD/L was achieved when 60 mg cellulase/g total suspended solid (TSS) was added to the fermentation system directly, while the VFAs yield was 1512 mg COD/L in the group without cellulase addition. Interestingly, this research also pointed out that adding cellulase enhanced the activities of key acid-forming enzymes, such as AK, PTA, oxaloacetate transcarboxylase (OAAT), and CoA transferase (CoAT).

The effects of the two enzyme addition methods vary from fermentation systems and enzyme types. It has been reported that sometimes the direct addition of enzymes does not

have a significant promotion on VFAs yield since the enzymes might be biodegraded in the fermentation system (Sutaryo et al., 2014). Conversely, the direct addition of enzymes wields a better influence on VFAs production than enzymatic pre-treatment in some cases. A study that co-digest primary sludge and external organic waste under the condition of initial pH 10.0 illustrated that the direct addition of enzyme cocktail elevated VFAs production by 29-39%, whilst 24-h enzymatic pre-treatment did not benefit VFAs yield (Owusu-Agyeman et al., 2021). Regarding this, suitable enzyme addition methods should be selected when using different fermentation systems for VFAs production.

4.2 Substrates and inoculum

The components of substrates also affect enzymatic performance. Some reports showed that carbohydrate-rich waste streams are typically converted into butyric acid and propionic acid, whilst protein-rich waste streams support the production of valeric and iso-valeric acids (Garcia-Aguirre et al., 2017). It can be concluded from these results that the types of substrates could affect the pathways and enzymes involved in VFAs production. Besides, other compounds in substrates may also influence enzyme activities (Table 3). As a typical pollutant in petroleum wastewater, trichloroacetaldehyde could reduce the activity of key enzymes like dehydrogenase, acetate kinase, butyrate kinase, and acetyl-CoA during VFAs production because of its toxic effect on microbes in anaerobic granular sludge (Tian et al., 2020). It is difficult for antibiotics in wastewater to be biodegraded by activated sludge (Watkinson et al., 2007), and recent studies showed antibiotics could affect VFAs fermentation significantly. For instance, 10 mg/ kg total suspended solids antibiotic chlortetracycline could increase the activity of acetate kinase and 21.1% VFAs production during the anaerobic fermentation of WAS (Tang et al., 2021). Adding sulfadiazine (SDZ) could improve the activity of protease, α-glucosidase, and acetate kinase (Xie et al., 2019).

[Insert Table 3]

As for inoculum, it may contain resistant compounds that influences VFAs production by affecting relative enzyme activities. For example, humic acid has a high potential to boost the activities of amylase, protease, acetate kinase, butyrate kinase, and PTA, whilst inhibiting the key enzyme activity in methanogenesis (Wang et al., 2022a). Besides, since VFAs can be easily converted into biomethane during anaerobic digestion, it is necessary to suppress the activities of methanogens, more specifically, methanogen enzymes. Thermal pre-treatment is one feasible option for inhibiting the activities of methanogens because some acidogens could form spores to protect themselves under adverse conditions (Wang & Yin, 2017). Typically, the inoculum is preheated at 105 ℃ for 30 min, and this measure is more common in biohydrogen fermentation (Moreno-Dávila et al., 2019). However, the temperature and pre-treatment time are highly flexible, which could range from 65 to 121 ℃ and 10 min to 10 h, respectively (Wang & Yin, 2017). The addition of 2-bromoethanesulfonate (BES) is a feasible approach to inhibit methanogens. One employed waste activated sludge for VFAs production and here the used 5 mmol/L BES prohibited methanogens (Li et al., 2021). In fact, BES belongs to methanogen inhibitors, chemicals such as long-chain fatty acids, and chloroform comprises methanogens inhibitors that could suppress relative methanogen enzymes (Hu & Chen, 2007; Ray et al., 2010).

Since the desired pH for methane production is typically under neutral conditions (Khan et al., 2016), acid or alkaline pre-treatment of inoculum will inhibit the activities of methanogens (Moosbrugger et al., 1993), while acidogens could work well under acid (pH >3) and alkaline (pH<12) conditions (Liu et al., 2012). It is worth noting that some acidogens are facultative anaerobic bacteria, whereas methanogens are obligate anaerobes. Therefore, aeration also works for methanogens inhibition (Moosbrugger et al., 1993; Wang & Yin, 2017). The components of substrates and inoculum do greatly influence the mechanisms and enzymes involved in VFAs production. More studies are urgently required to explore the

VFAs formation mechanisms from different substrates and selecting or pre-treating inoculum for its better application.

4.3 Trace elements

Regarding trace elements like Fe, Cu, Ca, and Mn, they are required in minute amounts but have great significance on the production and function of enzymes and co-factors. As reported, calcium could stimulate lipases activity, although this trace metal is not necessary for their function (Gupta et al., 2004). Trace elements like zinc, cobalt, manganese, and/ or calcium are essential to proteases, whereas elements Cu, Ni, and Fe are important to acetyl-CoA synthase (Hendriks et al., 2018). Despite the significant roles of trace elements to enzymes, a trace amount of trace metals is meaningful to enzyme activity, and high concentrations of metals affect enzyme activity negatively. For instance, as co-factors in the catalytic centre of cellulase, Cu^{2+} and Cd^{2+} could stimulate enzyme activity at low concentrations, while a large concentration of these two metals inhibited enzyme activity by disrupting protein structures (Guo et al., 2019). Low Cu^{2+} content is able to boost VFAs production (Facchin et al., 2013; Karlsson et al., 2012), whereas the acidogenesis process will be inhibited when Cu^{2+} content was higher than 100 mg/L (Hao et al., 2017). Providing fermentation systems with appropriate trace elements will contribute to enhancing enzyme activity and VFAs production, but more studies are needed.

4.4 Genetic engineering

Genetic engineering is a strategy with tremendous potential to enhance specific VFA production. Basically, there are several approaches to achieving this strategy. The first pathway is to mutate microbial strains or induce metabolic pathways favorable for VFAs production under extreme conditions (Varghese et al., 2022). More propionate production of *Megasphaera elsdenii* H6 was observed at pH 5.5 after being treated with UV irradiation and nitrosoguanidine (Long et al., 2012). High acidic (Long et al., 2012), modifying certain genes like *uvrA* (Zheng et al., 2018), *groESL*, and *htpG* (Suo et al., 2017) to increase specific VFA

production by improving the acid tolerance of the strains or relative enzymes are used for suitable strain selection or construction. Deleting genes for competition pathways is another feasible option. Lactate dehydrogenase and pyruvate oxidase (*poxB*) genes are viable targeted genes that be inactivated or deleted to enhance propionic acid production (Liu et al., 2016). Some researchers set out to improve propionic acid production by inactive or knocking out the acetate kinase gene (*ack*) and they actually achieved great success (Suwannakham et al., 2006; Zhang & Yang, 2009). Conversely, the overexpression of enzymes that favors acids production is a typical route for enhancing the VFAs yield. Suo et al. (2018) observed an increased glucose tolerance of *C. tyrobutyricum* ATCC 25755 after overexpressing the enzymes phosphofructokinase (*pfkA*) and pyruvate kinase (*pykA*). A 64% increase of butyrate productivity was achieved under the conditions of batch fermentation and 120 g/L glucose. The combination of gene overexpression and deletion is also feasible. A study that aimed to enhance propionic acid production demonstrated that the *P. jensenii* with phosphoenolpyruvate carboxylase gene (*ppc*) overexpression and lactate dehydrogenase gene (*ldh*) deletion increased propionic acid titer from 26.95 ± 1.21 g/L to 34.93 ± 2.99 g/L (Liu et al., 2016). Despite the effectiveness of genetic engineering on VFAs yield improvement, it should combine with appropriate optimization processes to pick up suitable strains. More studies on VFAs metabolisms during anaerobic digestion and new acidogenic bacteria should be conducted for improved VFAs production. It is also possible to lower VFA recovery and purification costs by redirecting the metabolic pathways to specific VFA production to enrich the targeted VFAs.

4.5 Additives

Sometimes, the addition of additives will benefit VFAs production (Table 4). For instance, bio-surfactant could benefit VFAs production by promoting the solubilization of extracellular polymeric substances (EPS) and releasing the hydrolases contained in EPS (Huang et al., 2015). Research reported that the presence of bisphenol A encouraged VFAs

production by enhancing PTA, AK, and other enzymes related to VFAs production during waste activated sludge anaerobic fermentation, and the maximum VFAs production of 2095 mg COD/L was achieved with a 50 mg/kg BPA (Jiang et al., 2021b). The addition of 200 mg/kg TSS triclosan reached the maximum VFAs production of 15083 mg COD/L since triclosan could enhance waste activated sludge solubilization and promote the activity of key enzymes among all stages during waste activated sludge anaerobic fermentation (Zou et al., 2021). Fenton sludge has a positive effect on hydrolysis acidification, one of the mechanisms is the Fe^{2+} in Fenton sludge could promote the activity of dehydrogenase and increase extracellular polymeric substances production (Wang et al., 2022b). Additives not only benefit VFAs production but also promote specific VFA formation. It is noted that the addition of Fe^0 powder was conducive to transforming propionic acid into acetate by increasing the abundance of propionic-utilizing bacteria and homoacetogenic bacteria as well as the activities of enzymes related to acetogenesis (Meng et al., 2013). As discussed above, the mechanisms of different additives varied. Therefore, working out the mechanism of specific additives is important for its better application to promote VFAs production.

[Insert Table 4]

4.6 Others

Not only the chemical but some physical methods benefit enzyme activities. Voltage supplementation enhanced secondary acidogenic fermentation of activated sludge promoted VFAs production since this measure could increase the hydrolysis rate of activated sludge, loosen sludge structure, and improve the activities of protease and α-glucosidase significantly (Li et al., 2021). One analysis which employed waste activated sludge for VFAs production under the conditions of pH 10.0 and ultrasonic pre-treatment illustrated that the maximum VFAs production was achieved when the ultrasonic energy density and reaction time was 1.0 kW/L and 72 h, respectively. Under the ultrasonic energy density range of 0 to 4.0 kW/L, the

key acid-forming enzymes like AK, PTA, PTB, BK, OAAT, and CoAT exhibited the highest activity at the energy density of 1.0 kW/L (Yan et al., 2010b). Trace air or oxygen can promote the secretion of hydrolases like cellulase, amylase, and protease from facultative microorganisms, increasing the hydrolysis of corresponding substrates and VFAs production (Zhang et al., 2021). Many methods can stimulate enzyme activities to enhance VFAs production. This would be a good topic of research to enhance the production of VFAs.

Typically, enzyme activity/ability enhancement will benefit VFAs production during anaerobic fermentation, but it also should be acknowledged that sometimes high enzyme activity may result in less VFAs production. For instance, one study illustrated that the VFAs yield was 2 to 34 times under the conditions of pH 10.0 compared to that of pH 5.5 during activated sludge anaerobic fermentation for VFAs production. This is despite the activities of protease, a-amylase, alkaline phosphatase and acid phosphatase at pH 10.0 all being lower than that of pH 5.5. The research also pointed out that pH 10.0 helped the hydrolysis of sludge matrix and improved the contact efficiency of enzymes and substrates (Yu et al., 2008). Therefore, it is important to enhance the efficiency of enzymes while increasing enzyme activities/abilities.

5. Future Perspectives

Wastewater is a feasible substrate for biological VFAs production and enzymes play a significant role in this process. The application of enzymes is conducive to enhancing VFAs production. However, there are some problems that exist with this strategy. During hydrolysis, enzyme selection is important since the hydrolysis performance on the same substrate varies according to the type of enzyme. Enzyme is not the only option although it is the most commonly used approach in nature. However, compared to physical and chemical methods, an enzyme needs longer hydrolysis time when degrading cellulose and other resistant substrates. Thus, the combination of enzymatic processes with other methods such as ultrasonic which could improve hydrolysis efficiency is a good option. Apart from this, there

may exist some unexpected intermediates which have a negative effect on VFAs production when using the same method for the decomposition of different substrates. In this scenario, the hydrolysis mechanisms of different methods should be well analysed.

During anaerobic digestion, enzymatic processes are heavily influenced by operation conditions, wastewater types, inoculum, trace elements and other factors. Working out the best fermentation conditions and the key mechanisms of possible parameters will benefit VFAs production. Acidogenesis is a vital process in VFAs formation. To improve VFAs production, how to enhance the enzymatic processes of acid-producing processes and inhibit VFAs competition or consumption pathways are important. There are many strategies for VFAs production enhancement from the perspectives of enzymes. However, they are based on well-studied mechanisms, while many studies failed to reveal the mechanisms from the enzyme level. Each enhancement approach has its disadvantages. For example, the effects of enzyme addition methods vary from fermentation systems; genetic engineering is complex and should be combined with appropriate optimization methods; the addition of additives may cause environmental problems. For this reason the application of those enzymatic enhancement methods should be well-matched with fermentation systems and needs to combine with other methods to eliminate the negative effects. Sometimes, an enhanced enzyme activity/ability does not mean better performance, so enzymatic efficiency should also be considered.

Last but not the least, it is the pure VFAs that are more valuable, while the VFAs obtained by anaerobic digestion are typically a mixture with low concentration, and existing separation and purification technologies are costly. In this scenario, what will help are measures like inoculum control, genetic engineering, and suitable substrates selection to enrich specific VFA production and exploring low cost VFAs separation and purification methods.

6. Summary and Conclusion

Organic-rich wastewater is a great feedstock for biological VFAs production. During this process, enzymes play an irreplaceable role in VFAs formation. Yet, enzymes are not always the best option to improve VFAs production. Operational conditions, trace elements, substrate, and inoculum are all feasible parameters that affect the enzymatic performance in VFAs production. In this scenario, measures to enhance enzymatic processes like exploring the ideal fermentation conditions, genetic engineering, adding additives, and blocking VFAs competition or consumption pathways are necessary. More studies on enzymatic mechanisms of VFAs formation, competition, and consumption during anaerobic digestion are essential.

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Table 1 The global market value and their usage/applications of typical bio-VFAs

Global market value	References	Usage/applications	References
USD 620 million in 2019	(Chen et al., 2020)	Wide applications in agricultures, industries such as rubbery, animal feeds, pharmaceuticals, leathers, and textile. Involved in reductant, green solvent, and various chemical synthesis; act as a key energy carrier/medium	(Chen et al., 2020)
USD 7.07 billion in 2020	https://www.verifiedmarketresearch.com/product/acetic-acid-market/	Vinegar, food additive and preservative; terephthalic acid and acetate esters production; Alternative feedstock for biofuel production	(Kim et al., 2021; Vázquez-Fernández et al., 2022)
USD 1.1 billion in 2020	https://www.verifiedmarketresearch.com/product/propionic-acid-market/	Wide applications in food, feed, pharmaceutical, plastic, and cosmetic industries	(Chen et al., 2021)
USD 244.35 million in 2019	https://www.verifiedmarketresearch.com/product/butyric-acid-	Flavouring; materials for the synthesis of cellulose acetate butyrate thermoplastics; pharmaceutical industries	(Vázquez-Fernández et al., 2022)

VFAs	Chemical formula
Formic acid	HCOOH
Acetic acid	CH_3COOH
Propionic acid	CH_3CH_2COOH
Butyric acid	$CH_3(CH_2)_2COOH$

Table 2 Enzymatic pre-treatment enhanced bio-VFAs production

Wastewater	Enzymes	Operating conditions	Results	References
Poultry wastewater	Lipase from oleaginous seeds of *Pachira aquatica*	40 °C for 90 min; pH 8.0-9.0.	Free fatty acids release increased by 7.4 times; promoted about 10% fats hydrolysis	(Polizelli et al., 2013)
Dairy and poultry slaughterhouse wastewater	Commercial lipase	30 °C for 12 or 24 hours, pH 7.0; enzyme dose 0.1% w/w	Increased the content of long-chain free fatty acids	(Pascale et al., 2019)
Slaughterhouse wastewater	Lipase	37 °C; pH 7.0; stirred at 200 rpm over 6 days	Lipid content decreased from 17 g/L to 1.12 g/L	(Affes et al., 2017)
Coconut mill effluent	Lipase from *Staphylococcus pasteuri* COM-4A	50 °C; pH 9.0; One in four effluent dilutions; Contact time of 30 hours; 1% (w/v) immobilized lipase beads	46% and 24% increase in VFAs and long-chain fatty acids; 52% decrease in oil and grease	(Kanmani et al., 2015)
Dairy wastewater	Lipase-rich fungal enzymatic preparation	0.1% (w/v) of solid enzymatic preparation at 30 °C for 24 hours, pH 7.0	Free acid concentration increased 8 folds	(Rosa et al., 2009)
Synthetic dairy wastewater	Lipase from *Candida rugosa*-free form	0.2% enzyme loading (w/v), 30 °C, 165 W of ultrasound power at 25 kHz and 66% duty cycle	Maximum 78% fat hydrolysis was achieved	(Adulkar & Rathod, 2014)
Primary sludge	Cellulase enzyme	25 °C, solid retention time of 1, 2, and 4 days, enzyme dose	VFAs production increase from 78–192 to	(Bahreini et al., 2020)

		0.5%, 1%, and 1.5% of the total solids in the feed	87-202 mg COD/g VS	
Waste activated sludge (WAS)	Protease	Initial pH 10.0, 37 °C, stirring speed about 100 r/min	VFAs yields increased by over 40% in anaerobic dynamic membrane bioreactor	(Liu et al., 2018)
Primary sludge and extra organic waste	Enzyme cocktail	Initial pH 10.0, 24 h enzymatic pretreatment	Direct enzyme addition enhanced VFAs production by 29-39%	(Owusu-Agyeman et al., 2021)
WAS	Hydrolytic enzymes, including lysozyme, protease, α-amylases and cellulase	37 ± 0.2 °C for 3 h	Enzymatic pretreatment enhanced VFAs production by improving WAS solubilization, with 3580 mg COD/L VFAs production after 10-d fermentation	(Xin et al., 2019)
WAS	The combination of alkaline protease and pH 10	WAS was pretreated by 5% alkaline protease at 200 rpm, 35 ± 1 °C for 6 h. The pH was maintained at 10.0 ± 0.5	Short-chain fatty acids yield of 607 mg COD/g VSS was achieved after three days fermentation, which is 5.4 times higher than the control	(Pang et al., 2020)
WAS	Alkaline hydrolase blend	WAS was pretreated under the conditions of pH 10.0, 200 rpm, 35 ± 1 °C for 2 h	528.9 mg COD/g VSS was achieved after 3 days fermentation, which is higher than other groups	(Pang et al., 2022)

Table 3 Effects of substrates composition on enzymatic processes and VFAs

Substrate	Compounds	Effects on enzymes	Results	References
WAS	Polystyrene (30 particles/g $_{\text{total solid}}$)	Enhanced solubilization and enzyme activity	VFAs production increased to 112.8 ± 2.4%	(Zheng et al., 2021)
WAS	Polystyrene (90 particles/g $_{\text{total solid}}$)	Improved organic matter releasement but inactivated relative microbial activity	VFAs production decreased to 83.01 ± 0.76%	(Zheng et al., 2021)
WAS	Diclofenac (DCF)	DCF promoted the processes of acidogenesis, acetogenesis, and homoacetogenesis, while methanogenesis was severely inhibited	VFAs yield increased from 559 to 1113 mg COD/L when DCF concentration increased from 2.5 to 25 mg/kg TSS, but decreased to 896 mg COD/L when further increased DCF concentration to 47.5 mg/kg TSS	(Hu et al., 2018)
WAS	Sulfadiazine (SDZ)	Extracellular polymeric substances (EPS) increased, the activities of protease, α-glucosidase, and acetate kinase were promoted	VFAs production increased from 1540.2 to 2032.8 mg COD/L when the SDC content was 50 mg/kg $_{\text{dry sludge}}$	(Xie et al., 2019)
Food waste (FW) and WAS	Thiosulfinate	The activities of butyryl-CoA and NADH were inhibited	VFAs yield reduced from 765.7 ± 21.1 to 376.4 ± 21.7 mg COD/g VSS when the dosage of thiosulfinate increased from 0 to 12.5 μg/g VSS	(Tao et al., 2020)
Activated sludge	Poly aluminium chloride (PAC)	Hydrolysis, acidogenesis, and methanogenesis were all inhibited	Short-chain fatty acids production declined from 212.2 to 138.4 mg COD/g	(Chen et al., 2018)

			VSS when PAC addition increased from 0 to 40 mg AL per gram TSS	
WAS	Bisphenol A	The activities of enzymes related to the pathways for amino acid metabolism, fatty acid biosynthesis, ATP-binding cassette transporters and quorum sensing, as well as the abundance of carbohydrate-active enzymes were increased	The addition of bisphenol A benefited VFAs production in the range of 0-200 mg/kg dry sludge	(Jiang et al., 2021b)
FW and WAS	FW mainly consisted of rice, vegetables, and meat; rice residues (RS)	The key metabolic capacity of different substrates and the expression of VFAs formation genes were related to food waste composition	The VFAs production of WAS+FW and WAS+RS were 4000.78 ± 172.9 and 1563.4 ± 58.8 mg COD/L	(Zhang et al., 2020)
Synthetic wastewater	Crotonaldehyde (from 0 to1000 mg/L)	The activities of acetyl-CoA, acetic kinase, butyrate kinase, and aldehyde dehydrogenase all decreased with in increasing concentration of crotonaldehyde	The VFAs production decreased with the increasing concentration of crotonaldehyde	(Liu et al., 2022)
Different ratio of protein and carbohydrate	Bovine serum albumin and dextran	The activities of protease and α-glucosidase increased and decreased with the carbohydrate/protein ratio in the range of 0.25-1 and 1-3, respectively	Carbohydrate/protein ratio of 1 had the highest VFAs yield. The processes of hydrolysis and acidogenesis were inhibited when the carbohydrate/protein ratio was 3 and 0.25, respectively	(Wang et al., 2022)

Table 4 Enzymatic processes enhanced by additives to improve VFAs production

Substrate	Compounds	Effects on enzymes	Results	References
WAS	Alkylethoxyglucoside (AEG) (0.4g/g TSS)	AEG promoted sludge hydrolysis and enzyme-substrate interaction, while supressed methanation	VFAs production improved by about 6.15 times than the blank	(Wu et al., 2021)
WAS	Superfine sand	Superfine sand greatly promoted the activity of AK and the quantity of AK encoding gene	VFAs production increased from 2513 to 3002 mg COD/L	(Jiang et al., 2021a)
FW, WAS and rice residues (RS)	zero-valent iron	The key metabolic capacity of different substrates and the expression of VFAs formation genes were related to food waste composition were improved by zero-valent iron	VFAs production of WAS+FW and WAS+RS were increased from 4000.78 ± 172.9 and 1563.4 ± 58.8 to 21711.6 ± 798.8 and 11952.4 ± 436.3 mg COD/L, respectively	(Zhang et al., 2020)
WAS	Copper nanoparticles	Acid-consuming microbial was enrich while enzyme activities of acid-forming bacteria were inhibited	When the content of copper nanoparticle was 25, 50, and 100 mg/g TSS, the VFAs production was inhibited by 11.1%, 56.0%, and 83.1%, respectively	(Chen et al., 2022b)
WAS	Rhamnolipid (0.05 g/g dry sludge)	The activities of protease and α-glucosidase were promoted significantly, while the activities of dehydrogenase, acetate kinase, and coenzyme F_{420} were supressed	VFAs production increased about 4-fold than blank	(Huang et al., 2015)
WAS	Surfactin	The activities of protease, α-	VFAs production	(Huang et al.,

	(0.05 g/g dry sludge)	glucosidase, and acetate kinase were increased, whereas the activity of dehydrogenase was decreased by 24%	increased about 4-fold than blank	2015)
Kitchen wastewater	Surfactant linear alkylbenzene sulfonate	The addition of surfactant increased the diversity of Proteobacteria and Firmicutes	VFAs yield increased by 10-25% when surfactant concentration increased from 0.03-0.12 g/L	(Bose et al., 2022)

Fig. 1 Enzymatic processes of bio-based VFAs production during acidogenesis (modified from Bhatia & Yang, 2017; Chen et al., 2013; Yang et al., 2021)

(*acul*: acrylyl-CoA reductase; *AK*: acetate kinase; *BCD*: butyryl-CoA dehydrogenase; *BK*: butyrate kinase; *CoAT*: CoA transferase; *CRT*: crotonase; *HBD*: 3-hydroxybutyryl-CoA dehydrogenase; *lcdA*: lactoyl-CoA dehydrogenase; *LDH*: lactate dehydrogenase; *OAAT*: oxaloacetate transcarboxylase; *PCT*: propionyl-CoA transferase; *PCT*: propionyl-CoA transferase; *PFL*: pyruvate formate lyase; *PTA*: phosphotransacetylase; *PTB*: phosphotransbutyrylase; *THL*: thiolase)

Fig. 2 Enzymatic processes of WLP in homoacetogens (modified from Ragsdale & Pierce, 2008; Yang et al., 2021)

Note: [H]: reducing equivalent; THF: tetrahydrofolate; CoFeSP: corrinoid-iron-sulfur protein; a: Formate dehydrogenase; b: formyl-THF synthetase; c: Formyl-THF cyclohydrolase; d: Methylene-THF dehydrogenase; e: Methylene-THF reductase; f: Methyltransferase; g: Codehydrogenase/acetyl-CoA synthase; h: Phosphotransacetylase; i: Acetate kinase

Fig. 3 Enzymatic processes of feasible by-products during VFAs production (modified from Liu & Whitman, 2008; Niu et al., 2018; Yang et al., 2021)

The enzymes involved in these pathways are *2,3-BDH*: 2,3-butanediol dehydrogenase; *ADC*: Acetoacetate decarboxylase; *AdhE*: aldehyde/alcohol dehydrogenase; *AK*: acetate kinase; *ALDC*: acetolactate dehydrogenase; *ALS*: acetolactate synthase; *AOR*: aldehyde:ferridoxin oxidoreductase; *BCD*: butyryl-CoA dehydrogenase; *BdhA*: butanol dehydrogenase A; *BdhB*: butanol dehydrogenase B; *CODH/ACS*: CO dehydrogenase/acetyl-CoA synthase; *CRT*: crotonase; *CTFA/B*: CoA-transferase subunit A and B; *Fdh*: formate dehydrogenase; *Fmd*: formyl-MFR dehydrogenase; *Ftr*: formyl-MFR:H_4MPT formyltransferase; *HBD*: hydroxybutyryl-CoA dehydrogenase; *Hmd*: methylene-H_4MTP dehydrogenase; *LDH*: lactate dehydrogenase; *Mch*: methenyl-H_4MPT cyclohydrolase; *Mcr*: methyl-CoM reductase; *Mer*: methylene-H_4MPT reductase; *Mtr*: methyl-H_4MPT:HS-CoM methyltransferase; *PFL*: pyruvate formate lyase; *PTA*: phosphotransacetylase; *THL*: thiolase

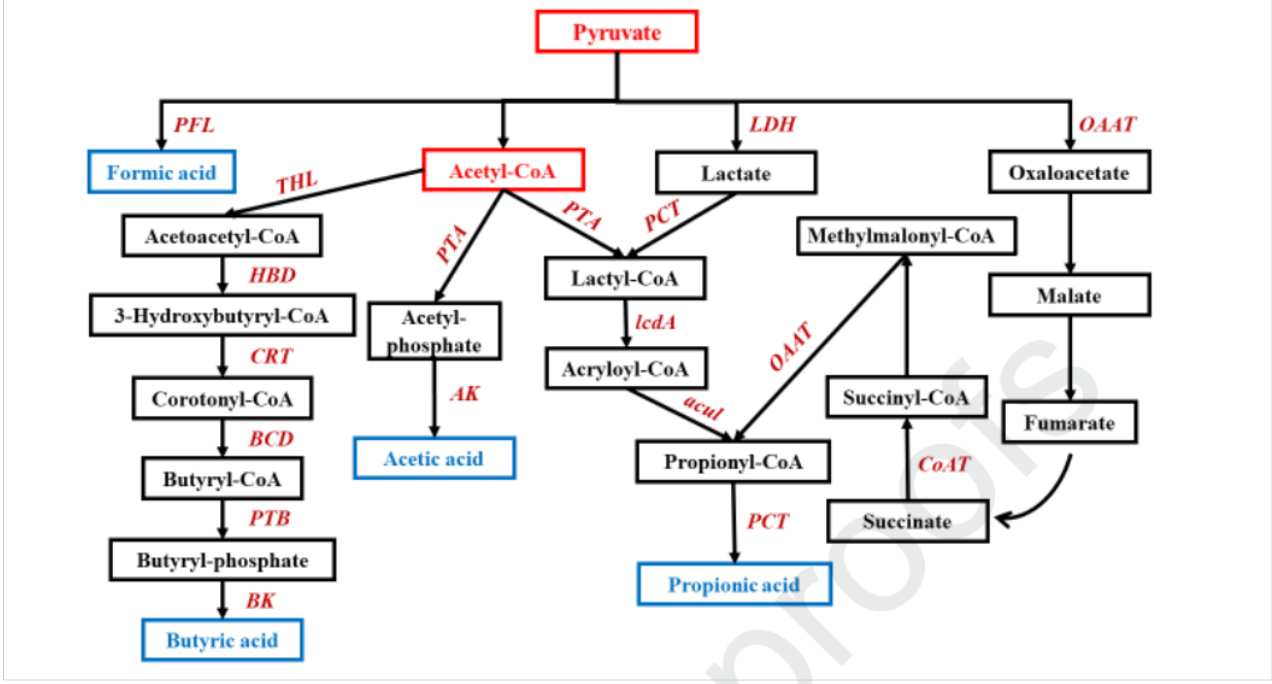
Pyruvate
PFL
LDH
OAAT
Formic acid
THL
Acetyl-CoA
Lactate
Oxaloacetate
Acetoacetyl-CoA
PTA
PTA
PCT
Methylmalonyl-CoA
HBD
Lactyl-CoA
Malate
3-Hydroxybutyryl-CoA
Acetyl-phosphate
lcdA
CRT
Acryloyl-CoA
OAAT
Succinyl-CoA
AK
Fumarate
Corotonyl-CoA
acuI
BCD
Acetic acid
Propionyl-CoA
CoAT
Butyryl-CoA
PCT
PTB
Succinate
Butyryl-phosphate
Propionic acid
BK
Butyric acid

Fig. 1

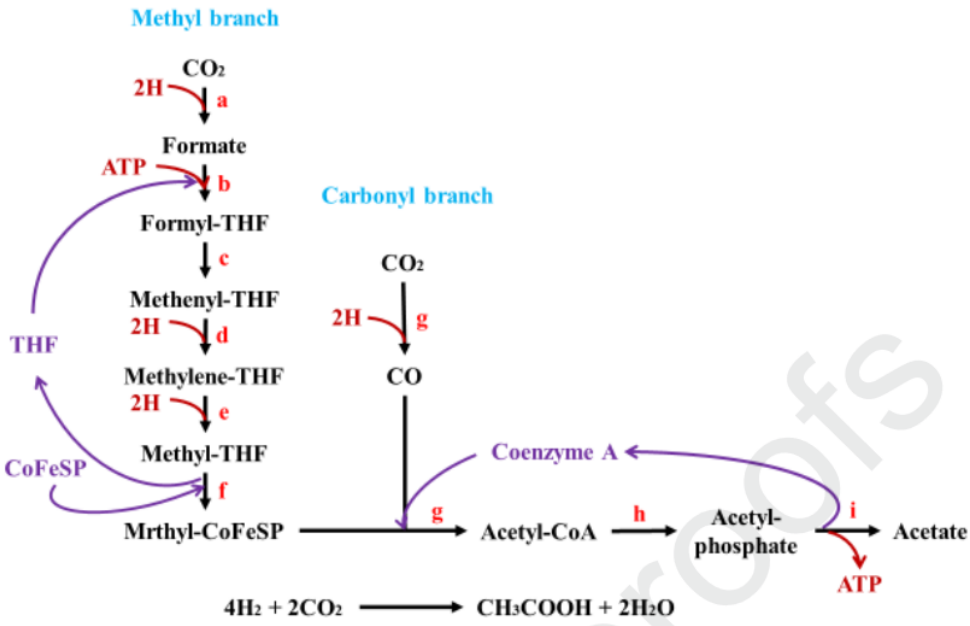
Methyl branch
CO2
2H
a
Formate
ATP
b
Formyl-THF
c
Methenyl-THF
2H
d
Methylene-THF
2H
e
Methyl-THF
f
THF
CoFeSP
Mrthyl-CoFeSP
Carbonyl branch
CO2
2H
g
CO
g
Coenzyme A
Acetyl-CoA
h
Acetyl-phosphate
i
Acetate
ATP
4H2 + 2CO2
CH3COOH + 2H2O

Fig. 2

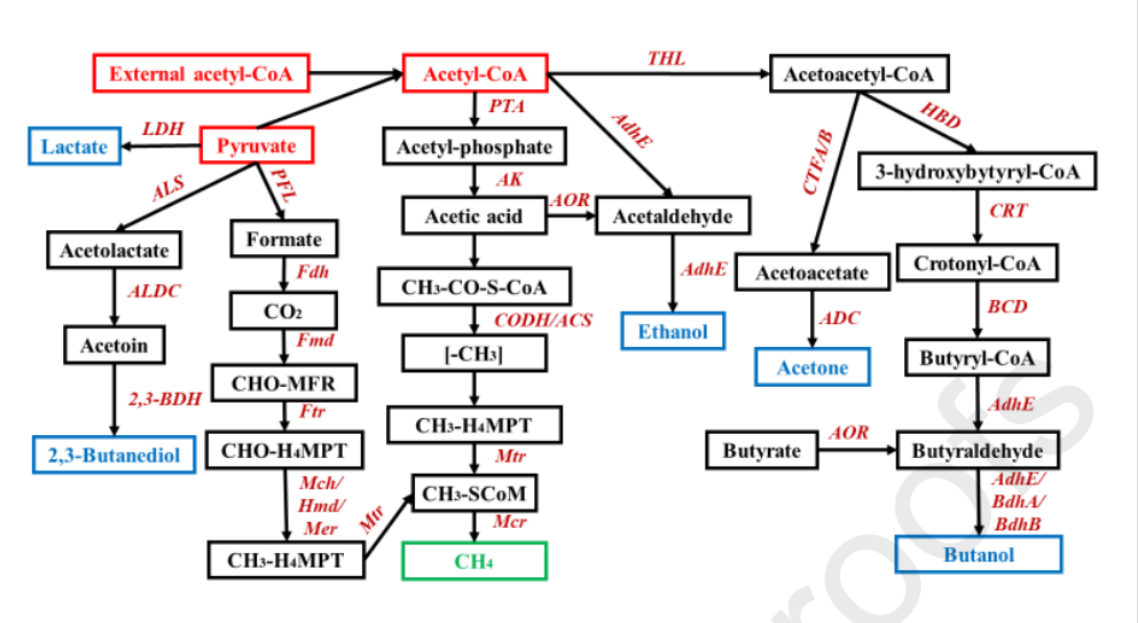

Fig. 3

Highlights

Enzymatic processes are essential in bio-VFAs production

Bio-VFAs enzymatic processes and related impact factors were reviewed

VFAs competition and consumption pathways should be discouraged

Enzymatic pre-treatment improves VFAs yields but needs further refinement

Enzyme ability/activity and efficiency enhancements benefit VFAs production