Achieving High Volatile Fatty Acid Production from Raw Henna (*Lawsonia inermis*) Biomass at Mild Alkaline Conditions

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Strong alkaline hydrolysis is usually employed to enhance fermentative production of volatile fatty acid (VFA) from plant biomass. However, this method is not ecofriendly due to the large consumption of energy/chemicals. In this study, a special henna plant biomass (*Lawsonia inermis*) containing the chemical compound lawsone, served as a fermentation substrate (without any pretreatment) for high VFA production under mild alkaline conditions (pH 9). The results indicated that the fermentative VFA production was 0.31 g per gram of henna biomass, which was comparable to other plant biomass pretreated with hydrothermal, strong acidic, and/or alkaline methods. Acetate was the main VFA component, and accounted for 69% to 82% of the COD fraction. The soluble lawsone concentration in the fermentation system ranged from 8.5 mg/L to 31.2 mg/L, which mediated the potential fermentation process. Thus, in this study henna was established as a feasible plant biomass for high fermentative VFA production in mild alkaline conditions.

Keywords: Henna plant biomass; Volatile fatty acid (VFA); Mild alkaline conditions; Fermentation; Lawsone

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INTRODUCTION

Henna (*Lawsonia inermis*) is a source of natural dye (lawsone: 2-hydroxy-1,4-naphthoquinone), which is popularly used for tattooing, hair, and textile dyeing (Bhuiyan *et al.* 2017). Like other plant biomass, henna contains several organic polymers such as cellulose, hemicellulose, and lignocelluloses. For the utilization of plant biomass, the fermentative production of volatile fatty acids (VFA) is usually appreciated. This process includes hydrolysis of carbohydrates and proteins into glucose and amino acids, fermentative production of propionate, butyrate and valerate, acetogenic generation of acetate and H_2/CO_2, and methanogenic production of methane (Huang 2019). The VFA is a good precursor component for the synthesis of biodegradable materials, such as polyhydroxyalkanoates (PHAs) (Tu *et al.* 2019). Furthermore, it is also an excellent electron donor and carbon source for the bio-reductive removal of refractory contaminants such as oxidized organics, nitrates, and heavy metals (Xu *et al.* 2019).

The complex structure of plant biomass limits the fermentative VFA production. Strong alkaline hydrolysis (under high pH) is required for breaking the chemical bonds present in biomass components such as lignocelluloses. Thus, high pH can serve as a pretreatment to enhance the fermentation process (Hsu 2018; Stachowiak-Wencek *et al.*

2019; Han *et al.* 2019). Although this operation has merits, the demerit is that this process is not an ecofriendly technology due to the large energy/chemical consumption involved. Although biological pretreatments using special bacteria, fungi, and/or enzyme cocktails (mixture) seemed a promising approach with less operation fees and secondary pollution, their longer incubation has still limited their wide applications (Ummalyma *et al.* 2019). Thus, the fermentative VFA production at mild pH condition by normal microbes is of vital important for the efficient utilization of plant biomass.

Some redox mediators containing quinone functional groups have been considered to enhance the fermentative VFA production by transferring electrons *via* its oxidized state and reduced state. Yang *et al.* (2012) reported that anthraquinone-2,6-disulfonate (AQDS) could enhance VFA production from sewage sludge by up to 2.7-fold. Humic acid played a positive role in VFA accumulation by promoting the acidification step, reducing the acetoclastic methanation, and enriching the fermentative community responsible for VFA production (Liu *et al.* 2015; Luo *et al.* 2018). However, the external supplement of artificial redox mediator increased the treatment costs as well as the environmental risks.

Henna is a special plant biomass containing the lawsone-type compound, which is an active quinone group functioned redox mediator (Baskaran *et al.* 2017). The authors' previous studies have found that lawsone released from henna benefited VFA production in the co-fermentation system with waste activated sludge and raw henna biomass as substrates (Huang *et al.* 2016a). Further study, indicated that lawsone mediated redox reaction of protein/amino acids degradation enhanced the protease activities and triggered an increased abundance of bacteria responsible with VFA fermentation (Huang *et al.* 2019).

Thus, henna plant biomass seems a feasible raw material for high VFA production. In this study, the effect of ambient pH on fermentative VFA production from natural raw henna plant biomass was studied. Through investigating the release of redox mediators of lawsone and various organic compounds, the authors aimed to achieve high VFA accumulation from raw henna at mild alkaline condition.

EXPERIMENTAL

Materials

Henna plant biomass and inoculated sludge

Henna plant biomass was purchased from Kaihangzhongyi Trading Co., Ltd. (Xinjiang, China). No pretreatment was performed before use except for oven-drying at 45 °C. The Brunauer-Emmett-Teller (BET) specific surface area of raw henna was measured as 5.619 m^2/g, and the particle size (Malvern Mastersizer 2000 system; Malvern Panalytical, Westborough, MA, USA) was mainly distributed between 3 μm to 25 μm.

The functional groups were identified using Fourier transform infrared (FTIR) spectroscopy (Nicolet 6700; Thermo Fisher Scientific, Waltham, MA, USA). The resulting spectra showed two specific bands at 1625 cm^{-1} and 1725 cm^{-1}, indicating the presence of 2-hydroxy-1,4-naphthoquinone (lawsone) in henna biomass. The inoculated sludge used was from an up-flow anaerobic sludge bed (UASB) from StarPro Starch Co., Ltd. (Hangzhou, China).

Experimental Set-up

The fermentation using henna biomass as substrate was performed in a 5-L airtight anaerobic sequential batch reactor (ASBR). The ASBR was continuously stirred by a force mixer (HD 2004W; Sile Co., Shanghai, China) at a speed of 60 rpm to 70 rpm. Seven ASBR series (P1, P2, P3, P4, P4, P6, and P7) controlled at various pHs (5, 6, 7, 8, 9, 10, and 11) were respectively set up. The fermentation was performed at 35 °C using a water bath. The designed pH in each ASBR was adjusted daily with 0.1 M HCl or 0.1 M NaOH. The concentration of fermentation substrate of henna in each ASBR was 20 g/L, and the inoculated sludge was kept at 1.0 g of Volatile Suspended Solids (VSS) per liter. The entire fermentation period lasted for 30 days.

In each fermentation system, the following chemicals were used as nutrients, vitamins, and supplemental metals: NH_4Cl (75 mg/L), KH_2PO_4 (15 mg/L), $MgCl_2{\cdot}6H_2O$ (10 mg/L), $CaCl_2{\cdot}2H_2O$ (7 mg/L), $FeSO_4{\cdot}7H_2O$ (1.4 mg/L), H_3BO_3 (0.25 mg/L), $ZnCl_2{\cdot}7H_2O$ (0.25 mg/L), $MnCl_2{\cdot}7H_2O$ (0.2 mg/L), calcium D(+) pantothenate (0.2 mg/L), pyridoxine- HCl (0.25 mg/L), thiamine VB1 (0.25 mg/L), nicotinic acid (0.25 mg/ L), *p*-aminobenzoic acid (0.1 mg/L); and $CoCl_2{\cdot}6H_2O$, $CuCl_2{\cdot}7H_2O$, $NiCl_2{\cdot}6H_2O$, and $Na_2WO_4{\cdot}2H_2O$ were all added at 0.025 mg/L.

Fermentation Product Analysis

During the 30-day operation period, the fermentation mixture was collected at 1, 3, 6, 9, 12, 15, 20, 25, and 30 days. The samples withdrawn were centrifuged at 8000 × g for 20 min, and the supernatant was used for analyzing the chemical oxygen demand (COD), carbohydrates, protein, VFA, and soluble lawsone levels.

The COD was analyzed according to standard methods (APHA/AWWA/WEF, 2005). Soluble carbohydrates and protein contents were spectrophotometrically measured using the anthrone-sulfuric acid colorimetric method and Bradford method, respectively, and with glucose and bovine serum albumin as the respective standards.

The VFA contained C2~C5 short-chain fatty acids, *i.e.*, acetate, propionate, *n*- and *iso*-butyrate, and *n*- and *iso*-valerate. The VFA compounds and lawsone were separated and quantified by an Agilent 1200 high-performance liquid chromatography (HPLC) unit (Agilent Technologies, Santa Clara, CA, USA). The HPLC was equipped with an auto-sampler and an ultraviolet (UV) detector. For separating each VFA compound, an organic acid analytical column (8 mm I. D. × 300 mm, Shodex RSpak KC-811; Showa Denko, Kanagawa, Japan) combined with a guard column (6 mm I.D. × 50 mm, Shodex RSpak KC-G; Showa Denko, Kanagawa, Japan) were used. The mobile phase was 0.05% H_3PO_4 solution at a flow rate of 0.7 mL/min, and the wavelength of the UV detector was 210 nm. The temperature in the column compartment was set at 50 °C. For separating lawsone, an Agilent XDB-C18 column (4.6 I.D. × 150 mm, Agilent Technologies, Santa Clara, CA, USA) was equipped, and the temperature was set at 35 °C. The mobile phase for lawsone separation was a mixture of 100% methanol and 0.05% H_3PO_4 (1:1), and the flow rate used was 0.6 mL/min. The wavelength used for lawsone detection was at 278 nm.

To make the results be more comparable, the concentrations of all soluble organics were converted into the equivalent values of COD with special coefficients as follows: carbohydrate (1.070), protein (1.500), acetate (1.067), propionate (1.512), butyrate (1.818), and valerate (2.039).

RESULTS AND DISCUSSION

High VFA Production Using Untreated Raw Henna Plant Biomass

Under anaerobic conditions, the polymeric compounds in henna biomass could be hydrolyzed to soluble intermediates of carbohydrates, proteins, and then be fermented to VFA and other products. The concentrations of these soluble compounds and the calculated ratio of VFA/COD in each pH series are shown in Fig. 1.

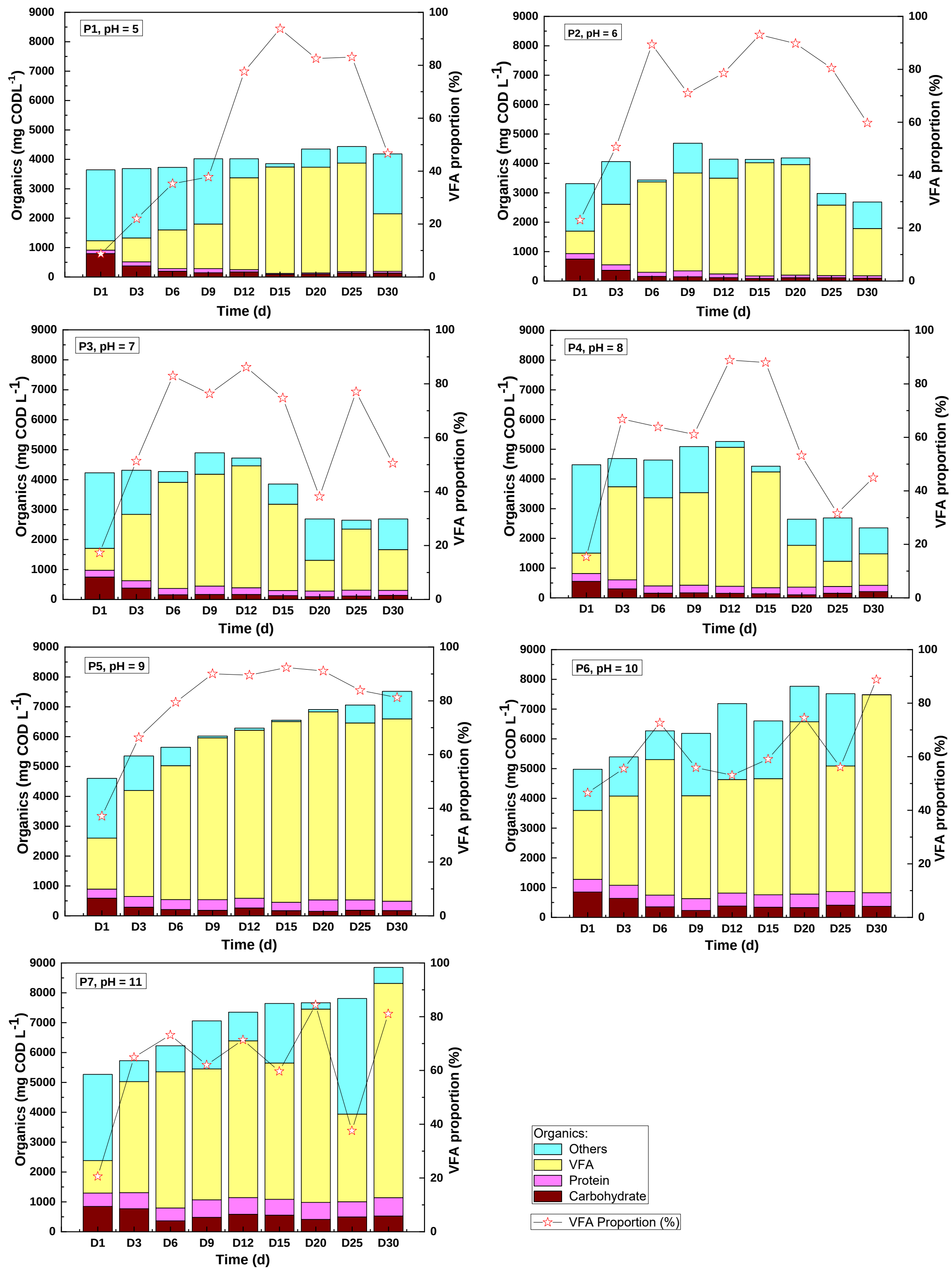

Fig. 1. Distribution of organics and VFA proportions in fermentation liquid at different pH values

Under weak acidic conditions (pH 5), the total soluble COD in the entire fermentation period was relatively low (3600 to 4400 COD mg/L). Under nearly neutral conditions (pH 6, 7, and 8), the total COD decreased in the latter periods after Day 15. However, it was found that the alkaline conditions (pH 9, 10, and 11) benefited the release of organic compounds, and the COD still increased in the latter fermentation period. This may be because under alkaline condition, the physical properties of lignin and hemicellulose compounds in henna biomass could be changed by de-esterification and dissolution processes (Kim *et al.* 2016).

During the initial period, especially under acidic conditions (pH 5, until Day 9), the VFA accounted for only a small fraction of the soluble COD. However, in the succeeding days, the VFA contributed to most soluble COD. During the middle stage (Day 12 to 20), the VFA/COD ratio in each ASBR achieved to the maximum from 74% to 94%. Notably, under weak alkaline conditions (pH 9), the VFA concentrations always increased during the entire fermentation period and stabilized at 6100 ± 200 mg COD/L, *i.e.*, 0.31 g COD / g biomass was obtained. During the latter period (after Day 20), the VFA fraction under pH 9 was between 81% and 91%; while those in acidic conditions (pH 5) and nearly-neutral (pH 6, 7 and 8) decreased. However, in stronger alkaline conditions (pH 10 and 11), the VFA production and its COD fraction were not stable either and showed a wave-like accumulation. This result was similar to a previous study on excess sludge alkaline fermentation (pH 10), suggesting that VFA's concentrations reached maximum at the Day 8 (Li *et al.* 2018).

The comparison of VFA yield from various plant biomass (raw or pretreated) is shown in Table 1. It implied that other plant biomass, such as rice straw, corn stover, waste wood, and sugarcane filter cake, could fermentatively produce comparable VFA (0.27 to 0.35 g COD / g biomass), but was hydrothermally, strong acidic, and/or alkaline pretreated. These pretreatments were not an ecofriendly method because of large amounts of the chemical/energy consumption involved. However, henna biomass used in this study could serve as an excellent raw material for high VFA production (0.31 g COD / g biomass) at mild condition of pH 9 without any pretreatment.

Table 1. Comparison of VFA Yield from Various Plant Biomass

Plant Biomass	Pretreatment	Temperature (°C)	VFA Yield (g COD / g Biomass)	References
Rice straw	0.5% HNO_3 at 150 °C for 20 min	35	0.30	Park *et al.* (2015)
Rice straw	2% NaOH at 150 °C for 20 min	35	0.35	Park *et al.* (2015)
Corn stover	2% ammonia at 90 °C for 7 h	35	0.09	Yuan *et al.* (2019)
Waste wood	Hydrothermal at 200 °C for 40 min	35	0.32	Li *et al.* (2019)
Sugarcane filter cake	6% NaOH at 45 °C for 30 min	--	0.27	Janke *et al*. (2016)
Henna (*Lawsonia inermis*)	No pretreatment	35	0.31	This study

The Main VFA Components

It was identified that VFA was mainly composed of acetate, propionate, *n*-isobutyrate, and *n*-isovalerate as the main soluble organics. Among these, the acetate and propionate were the two most efficient carbon sources/electron donors for the process of denitrification, decolorization, detoxification, dehalogenation, and so on (Huang *et al.* 2012; Lefevre *et al.* 2019; Li *et al.* 2020). Furthermore, the acetate is a "final" fermentation product, which is more stable and is suitable for the storage of fermentation product. The individual VFA components in each pH series obtained during the entire fermentation period are shown in Fig. 2.

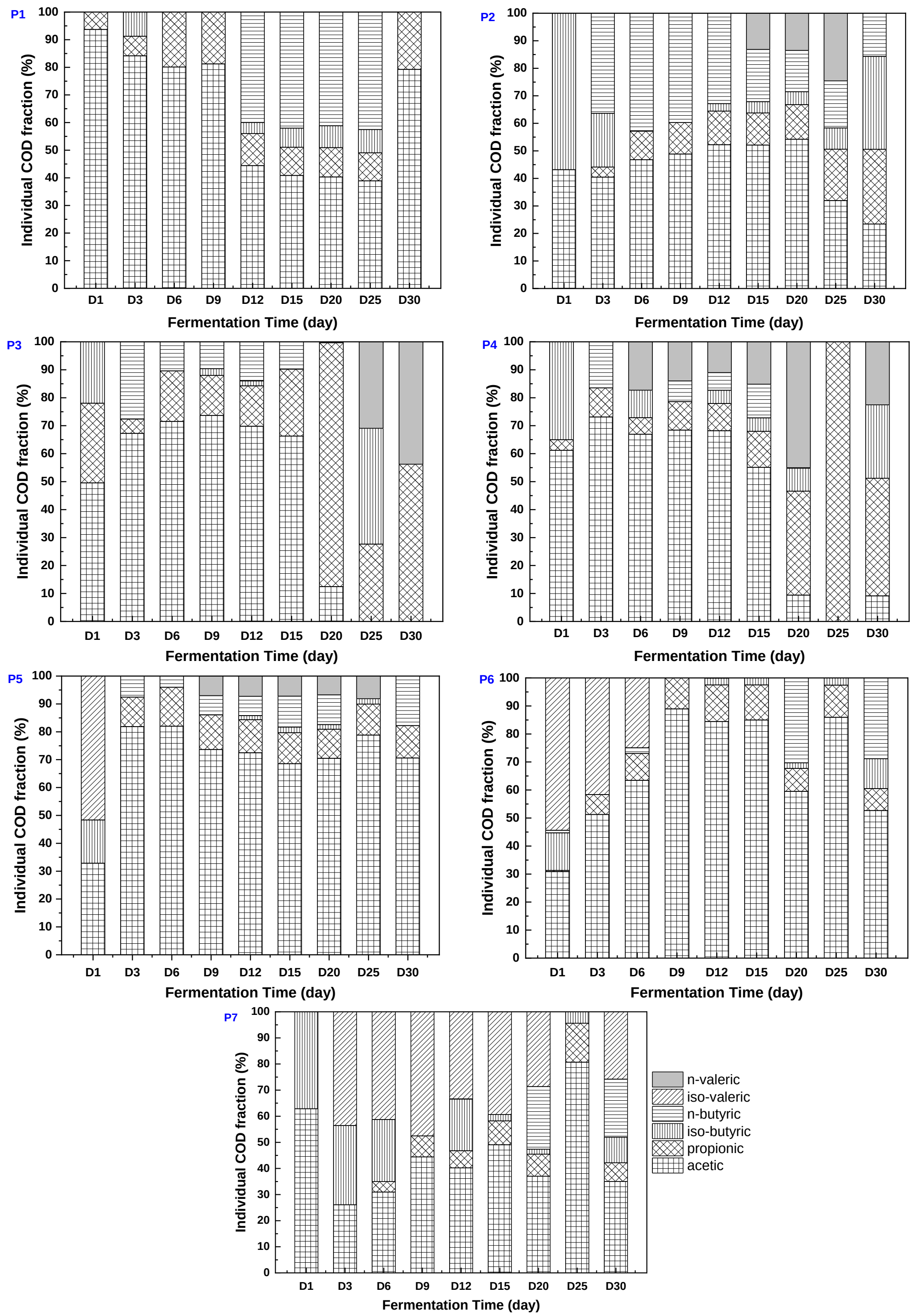

Fig. 2. COD fraction of individual VFA compounds under different pH values

The results implied that the COD fraction of "acetate + propionate" accounted for 26% to 100% of the total COD. Under acidic conditions (pH 5), although the acetate-COD fraction accounted for 93%, the concentration was lower than for the other series. Under weak alkaline conditions (pH 9), the "acetate + propionate" fractions (except on Day 1) were all above 80%, and the acetate fraction ranged between 69% and 82%. Thus, henna biomass, without any pretreatment, was feasible to produce acetate at mild alkaline condition of pH 9. This rationally explained the improved bio-reduction of azo dyes and hexavalent chromium (Cr(VI)) in the authors' previous studies, because the yield of acetate and propionate from henna plant biomass served as efficient electron donors (Huang *et al.* 2014, 2016b).

Presence of Lawsone in the Fermentation System

Henna plant biomass has been demonstrated as natural source for chemical lawsone, which triggered the increased abundances of microbes responsible for VFA fermentation (Huang *et al.* 2019). Under anaerbic condition, such as in this study, the lawsone was prone to be released from its fixed state in henna tissue. The soluble lawsone concentration in fermentation systems with varied pHs are shown in Fig. 3. It was observed that lawsone could be quickly released to the fermentation liquid. On Day 1, the soluble lawsone achieved in each pH series were 8.1 mg/L (pH 5), 7.6 mg/L (pH 6), 22.0 mg/L (pH 7), 17.8 mg/L (pH 8), 31.2 mg/L (pH 9), 49.4 mg/L (pH 10), and 45.3 mg/L (pH 11). In the succeeding days, the soluble lawsone decreased to some extent. Generally speaking, higher pH accelerated the release of lawsone from henna biomass. This was attributed to the fact that components in plant biomass were easier to be dissolved in alkaline condition (Zheng *et al.* 2009). According to the literature, henna usually contains approximately 1.8% of lawsone (Aktas Sukuroglu *et al.* 2017). Thus, a maximum of 72 mg/L of lawsone could be theoreticaly released from solid henna. This value was much higher than the observed concentrations. In this study, the released lawsone under mild alkaline condition (pH 9) ranged from 8.5 mg/L to 31.2 mg/L, with the potential to promote the higher fermentative VFA and acetate productions.

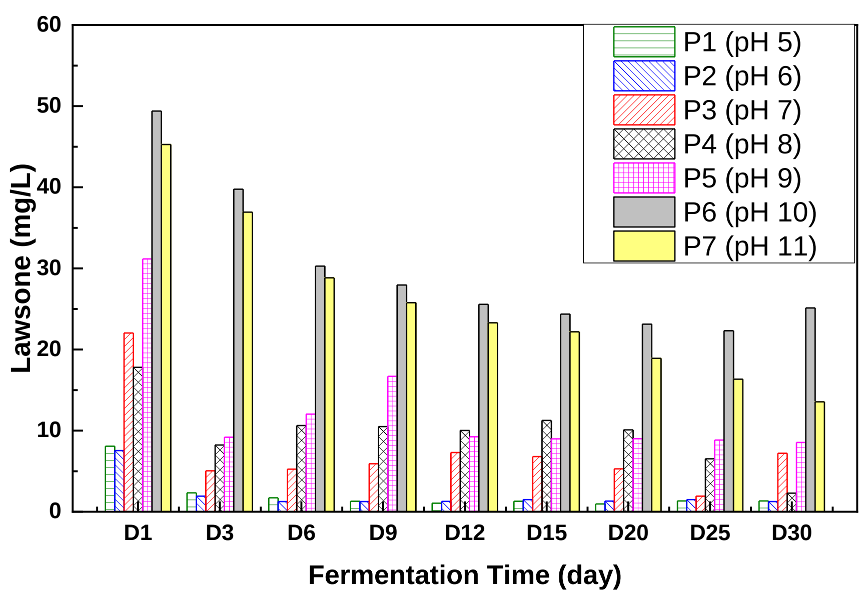

Fig. 3. Soluble lawsone produced in the fermentation systems with different pH values

CONCLUSIONS

1. High VFA production could be achieved by using raw henna (*Lawsonia inermis*) as a fermentation substrate.
2. Under ambient pH 9, high fermentative yield for VFA (0.31 g COD / g biomass) was achieved, which was comparable to other plant biomass pretreated using hydrothermal, strong acidic, and/or alkaline methods.
3. Acetate was the main VFA component, and its COD fraction was 69% to 82%.
4. The soluble lawsone formed in the fermentation systems ranged from 8.5 mg/L to 31.2 mg/L, with a potential electron mediating ability to enhance the fermentation process for higher VFA and acetate production.

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