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Biohydrogen production from oil palm frond juice and sewage sludge by a metabolically engineered *Escherichia coli* strain

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ABSTRACT

Biohydrogen is considered a promising and environmentally friendly energy source. *Escherichia coli* BW25113 *hyaB hybC hycA fdoG frdc ldhA aceE* has been previously engineered for elevated biohydrogen production from glucose. In this study, we show that this strain can also use biomass from oil palm frond (OPF) juice and sewage sludge as substrates. Substrate improvement was accomplished when hydrogen productivity increased 8-fold after enzymatic treatment of the sludge with a mixture of amylase and cellulase. The OPF juice with sewage sludge provided an optimum carbon/nitrogen ratio since the yield of biohydrogen increased to 1.5 from 1.3 mol H_2/mol glucose compared to our previous study. In this study, we also reveal that our engineered strain improved 200-fold biohydrogen productivity from biomass sources compared to the unmodified host. In conclusion, we determined that our engineered strain can use biomass as an alternative substrate for enhanced biohydrogen production.

1. Introduction

The demand for petroleum fuels impacts economic development; researchers have tried various methods to produce an alternative energy source which minimizes environmental pollution and greenhouse gases (GHG) [1–3]. Biohydrogen is an attractive energy source due to its high energy value and since it produces only water instead of GHG during combustion [4]. Biological production is the cheapest and most environmental friendly way to produce hydrogen compared to other methods such as coal gasification, water electrolysis and the water-gas shift reaction [1].

Escherichia coli is well-known and widely used due to the availability of its complete known genome sequence [1,5]. During glucose metabolism in *E. coli*, hydrogen can be produced by the formate hydrogen lyase (FHL) system that requires hydrogenase 3 and formate dehydrogenase H (FdhF) [6,7]. In our previous study [5], *E. coli* BW25113 *hyaB hybC*

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hycA fdoG ldhA frdc aceE was metabolically engineered to direct glucose utilization pathways toward hydrogen production. This strain was constructed by deletion of hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*) to prevent hydrogen uptake activity; inactivation of the FHL complex repressor inhibitor (*hycA*); inactivation of formate and pyruvate consumption by deletion of formate dehydrogenase (*fdoG*) and pyruvate dehydrogenase (*aceE*); inactivation of fumarate reductase (*frdC*) and lactate dehydrogenase (*ldhA*) to prevent glucose shifting to succinate- and lactate-producing pathways [5]. Thus, the total of seven deletions from the parent strain was successful in improving hydrogen production from *E. coli* [5].

The palm oil industry is one of the biggest industries in Malaysia and generates different types of biomass during oil processing such as frond, palm oil mill effluent, empty fruit bunch, mesocarp fiber, trunk, and shell [8,9]. Oil palm frond (OPF) juice from palm oil plantations contains a large amount of sugars [10]. OPF derivatives were used in the poly-3-hydroxybutyrate production [10] and animal feed [11] but no reports have been published for biohydrogen related applications. Additionally, sewage sludge is the most abundant waste from wastewater treatment plants worldwide. The production of sewage sludge in Japan has increased annually and is expected to increase [12]. Reported, sewage sludge has been consumed for hydrogen and/or methane production [13,14]. Most prior work used the sewage sludge as an inoculum or nutrient additive for biohydrogen production [2,14]. Sewage sludge contains complex structures that require treatment to improve the hydrolysis process for biohydrogen production [15]. Many research projects have shown that heat, physical, chemical and biological treatment with an enzyme to sludge improve soluble substrate for biogas production [15]. Thus in this study, we use enzymes such as amylase and cellulase to degrade starch, cellulose and lignocellulase readily available in sewage sludge for biohydrogen production. In our previous report, there were no applications for renewable substrates using our septuple-engineered strain [5]. Thus in this study, we investigated the use of oil palm frond (OPF) juice and sewage sludge in place of glucose to ascertain whether this engineered strain can utilize less expensive biomass for higher biohydrogen production.

We demonstrate here that our metabolically engineered *E. coli* uses OPF juice and sewage sludge as substrates that enhance biohydrogen productivity. We also show that sewage sludge may be used as an alternative feedstock after enzyme treatment and as a supplementary nitrogen source. This report is important for future research for both strain and substrate improvement and for a better understanding of metabolic engineering research for enhanced biohydrogen production.

2. Materials and methods

2.1. Bacterial strain

E. coli BW25113 and BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* were initially streaked on Luria–Bertani (LB) (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) agar and LB agar with 100 μg/ml kanamycin, respectively and grown overnight at 37 °C. One single colony was picked and cultured on complex glucose and complex glucose with 100 μg/ml kanamycin at 37 °C with shaking at 120 rpm [5]. Each strain was harvested and washed with autoclaved 0.85% NaCl, and the turbidity was adjusted to the same initial value of 0.5 prior to the inoculation to the substrate. The cell turbidity was measured by a UV/VIS spectrophotometer (JASCO V-530) at 600 nm.

2.2. Preparation of substrate

2.2.1. Sludge preparation and enzyme treatment

Sewage sludge was obtained from the Hiagari wastewater treatment plant, Kitakyushu, Japan. Sewage sludge was centrifuged at 8000 g, 10 min at 4 °C using a TOMY-GRX 250 High Speed Refrigerated Centrifuge. The supernatant was discarded and the solid pellet was washed and re-suspended three times with autoclaved distilled water by centrifugation. The final solid pellet was adjusted to 50% (wet-w/v) of sludge concentration with autoclaved distilled water [16]. Then, the sludge was autoclaved at 121 °C for 40 min, and checked for contamination by spreading on LB agar plates prior to the subsequent experiments. The mixture of amylase and cellulase enzymes were added up to the concentration of 10 U/ml to the autoclaved sludge, and the enzyme treatment was conducted for 2 h at 37 °C, 120 rpm followed by enzyme deactivation for 30 min at 60 °C, 120 rpm. Sterile water was used instead of enzymes for the sludge with no enzyme treatment as a negative control. The characteristics of sludge with and without enzyme treatment are indicated in Table 1.

Table 1 – Characteristics of substrates used in this study.

Parameters	Unit	Sludge		OPF juice	OPF juice with sludge
		Without enzyme treatment	With enzyme treatment		
pH	–	6.3 ± 0.1	6.0 ± 0.1	4.3 ± 0.1	5.8 ± 0.1
Protein	g/l	2.0 ± 0.4	3 ± 1	2.7 ± 0.2	5 ± 2
Total sugar	g/l	3.25 ± 0.71	4.7 ± 0.7	28.8 ± 5.8	20.3 ± 1.3
Glucose	g/l	2.2 ± 0.5	3.3 ± 0.3	23 ± 3	17.0 ± 1.9
Fructose	g/l	0.82 ± 0.01	1.0 ± 0.2	3.5 ± 1.9	1.7 ± 0.1
Sucrose	g/l	0.23 ± 0.2	0.4 ± 0.2	2.3 ± 0.9	1.6 ± 0.3
Total nitrogen	mg/l	14 ± 1	16 ± 1	0.30 ± 0.02	6.1 ± 0.5
Total solid	g/l	80 ± 2	62 ± 2	nd	35 ± 1

nd: not detected.

2.2.2. *Oil palm frond (OPF) juice preparation*

OPF juice was obtained from Universiti Putra Malaysia, Serdang, Selangor, Malaysia and the juice was extracted from the petioles and pressed by a sugarcane pressing machine as described by Zahari et al. [10]. The juice was centrifuged and the supernatant was filtered to obtain a sterile substrate and to prevent sugar degradation by bacteria contaminated. The sterile OPF juice was stored in polyethylene plastic containers and was kept at 4 °C. The characteristics of the OPF juice used in this study are indicated in Table 1.

2.2.3. *Oil palm frond (OPF) juice and sewage sludge*

OPF juice as described above was mixed with sterile 50% (w/v) sewage sludge at a ratio of 7:3. The characteristics of the mixture of OPF juice and sewage sludge are indicated in Table 1.

2.3. *Biohydrogen assay*

One ml of overnight culture with an adjusted turbidity of 0.5 was inoculated into 9 g of substrate in 34 ml serum vials. The vials were tightly crimped and sparged with nitrogen gas for 5 min to create anaerobic conditions [4]. Biohydrogen assay was conducted in an incubator shaker at 37 °C, 120 rpm.

2.4. *Low hydrogen partial pressure assay*

Five ml of each strain with an adjusted turbidity of 0.5 were inoculated into 45 g of substrate in 125 ml serum vials and stirred with a magnetic stirrer. The vials were tightly crimped and sparged as described above. Hydrogen gas was allowed to leave the headspace of the vials through a tube connected with a needle in the rubber septa. Additional details of the assay have been reported previously [17].

2.5. *Analytical methods*

Fifty μl of gas generated from the headspace of vials was analyzed by a 6890-N gas chromatograph (Agilent Technologies, Glastonbury, CT) as described previously [18]. The organic acids measurement was analyzed by high performance liquid chromatography (Shimadzu LC-10AD) [1]. The sugar component was measured by high performance liquid chromatography (Shimadzu LC-20A) equipped with a column Rezex RCM monosaccharite Cazt (8%) 00h-0130-K0 (300 × 75 mm) with a reflective index detector at 80 °C. Filtered distilled water was used as a mobile phase at a flow rate of 0.6 ml/min. pH was measured by an AS ONE compact pH meter, AS-211 (Horiba Ltd, Kyoto, Japan). The protein measurement was according to Lowry method [19] while total nitrogen was measured via the alkaline persulfate oxidation method [20]. Total solids were measured according to the Standard Method for Water and Wastewater, APHA [21].

2.6. *Scanning electron microscope (SEM)*

A 5 μl of sludge samples were directly dropped and spread on the SEM mount without any special pre-treatment. The surface of the samples were compared by visualization using a scanning electron microscope (Hitachi S-3500N) equipped with a chemical composition analyzer (EMAX 7021-H, Horiba, England).

3. Results and discussion

3.1. *Biohydrogen from sewage sludge with and without enzyme treatment*

Sludge samples of 50% (wet-w/v) were used throughout this study by washing and re-suspending the sludge with sterilized water to ensure the consistency of sludge samples in the initial conditions. In our preliminary study, sludge was treated with different enzymes (amylase, cellulase and protease) as a pre-treatment to investigate the effects of starch, cellulose and protein hydrolysis on biohydrogen production. We found that the amylase and cellulase enzyme treatments resulted in higher biohydrogen production when compared to protease treatment. This might be because the protein degradation to amino acids did not affect biohydrogen production as much as when compared to sugars [22]. Amylase treatment degraded the starch into sugars to enhance the hydrolysis process during the anaerobic degradation pathway. This result was supported by Wang et al. [23] when they added amylases to a kitchen-waste-feed into a hydrogen fermenter to increase the efficiency of starch hydrolysis due to the high molecular weight of starch. Cellulose and lignocelluloses are the most abundant biopolymers from plants present in sewage sludge. However, cellulose is not degraded much due to its crystalline and rigid structure [24]. Thus, many groups have applied physical treatments such as steam-explosion or chemical treatment using acid or alkaline treatment to disrupt the rigid structure of cellulosic and lignocellulosic materials for cellulose chain hydrolysis [25]. Thus in this report we only consider the mixture of amylase and cellulase enzymes as a pre-treatment to the sludge for biohydrogen production. We showed that the mixture of enzymes (amylase and cellulase) to sludge increased biohydrogen productivity roughly 8-fold (Fig. 1). Sludge without enzyme treatment produced 53 μmol

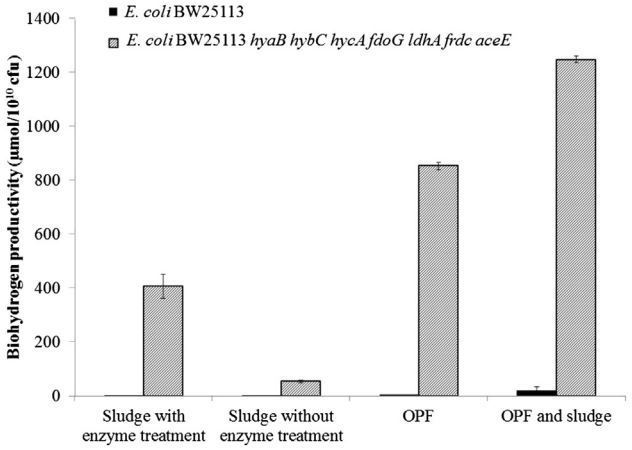

Fig. 1 – Biohydrogen productivity by *E. coli* BW25113 and *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* from different substrate at 24 h fermentation.

$H_2/10^{10}$cfu from fermentation with our engineered strain while biohydrogen productivity was increased to 407 μmol $H_2/10^{10}$cfu when the sludge was treated with the mixture of amylase and cellulase enzymes. In our previous study, the hydrogen productivity was calculated in μmol H_2/mg protein based on the turbidity of *E. coli* [5]. However, in this study the growth of *E. coli* was measured by colony forming unit (cfu) to eliminate the interruption of sludge during turbidity measurement. Thus, hydrogen productivity was reported in μmol H_2/cfu in this study.

Glucose has been considered as a sole substrate for microbial utilization in biohydrogen production, and sewage sludge is usually considered as a dead-end product in sewage treatment plants since it does not contain many sugars (Table 1). However, we found that after treatment with a mixture of amylase and cellulase, sugar and protein content increased by 31% and 23%, respectively (Table 1). These results indicate that enzyme pretreatment made available smaller compounds of the complex sugars. Table 1 shows that the total nitrogen content also increased by 13% after enzyme treatment. The total solid was low (62 ± 2 g/l) after enzyme treatment when compared to the total solid without enzyme treatment (80 ± 2 g/l) due to the degradation of cellulosic and lignocellulosic structure in the sewage sludge [25]. This result was supported by scanning electron microscope (SEM) micrographs which showed the degradation of the complex structure of the sludge when it was treated with the mixture of amylase and cellulase (Fig. 2a). Sludge treated with enzymes shows a hole in the sludge structure (Fig. 2a). On the other hand, the non-degraded structure of sludge can be seen when sludge was not treated with amylase and cellulase enzymes (Fig. 2b). Fig. 2c shows the degradation of sludge to smaller structures after fermentation of sludge with enzyme treatment while Fig. 2d shows that the complex structure of the sludge is still present after fermentation without enzyme treatment.

These results proved that enzyme treatment increases the hydrolysis of complex structures in the sludge as indicated by the increase in monosaccharides (glucose and fructose) and disaccharides (sucrose) in Table 1.

3.2. *Biohydrogen from OPF juice and OPF juice mixed with sewage sludge*

Fig. 1 shows biohydrogen productivity was 854 μmol/10^{10}cfu when OPF juice was used as a substrate by our engineered strain. The enhancement of biohydrogen productivity to 1249 μmol/10^{10}cfu was observed when sewage sludge was added with OPF juice from our engineered strain. Table 1 indicates that OPF juice contains a high amount of sugars mainly composed of glucose as reported by Zahari et al. [10]. The glucose concentration was important in glucose metabolism for biohydrogen production from *E. coli*. However, addition of sewage sludge to OPF juice increases the total nitrogen content in the substrate to 6.1 ± 0.5 from 0.30 ± 0.02 mg/l in OPF juice. The combination of glucose and nitrogen source from OPF juice and sewage sludge, respectively provide a proper combination of carbon to nitrogen (C/N) ratio [2]. The addition of sewage sludge to OPF juice increase protein concentration from 2.7 ± 0.2 to 5 ± 2 g/l. The increment of protein content might also contribute to nutrient supplementation for cell growth in *E. coli* [26].

The addition of sewage sludge also increases the pH of the substrate to pH 5.8 from 4.3. In addition, sewage sludge mixed along with OPF juice makes the pH suitable for biohydrogen production without requiring pH control since pH was a crucial

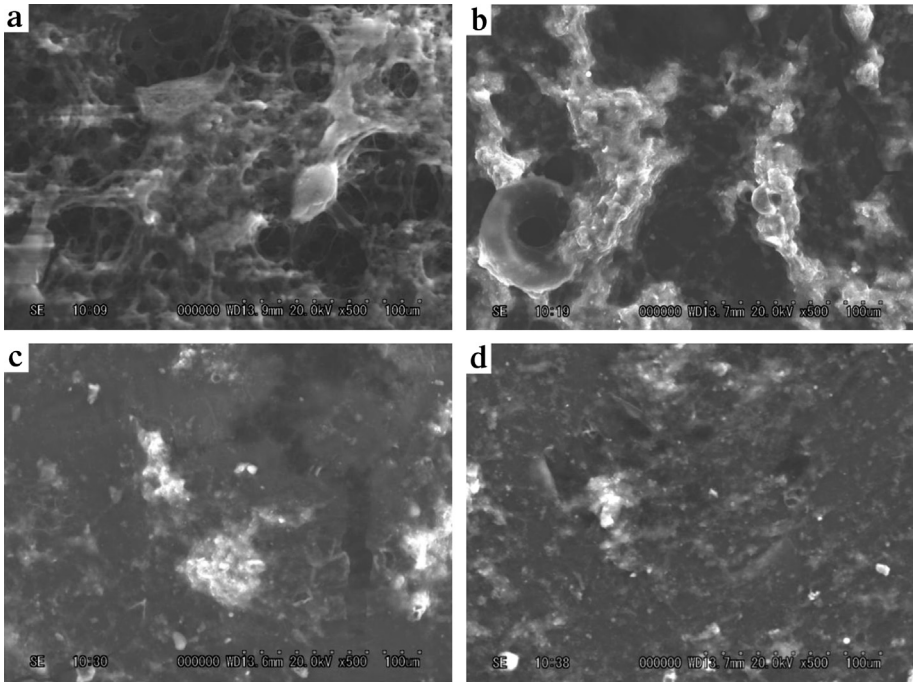

Fig. 2 – Scanning electron microscope (SEM) micrograph under 500× magnification of (a) sludge treated with enzymes at 0 h, (b) sludge without enzyme treatment at 0 h, (c) sludge treated with enzymes at 24 h, and (d) sludge without enzyme treatment at 24 h from fermentation with *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE*.

factor in influencing biohydrogen production [4]. Thus, higher biohydrogen productivity which resulted when sewage sludge was added along with OPF juice as indicated in Fig. 1 was due to the following two factors. The first factor was protein and nitrogen supplementation to OPF juice and the second factor was due to the suitable pH which provides favorable conditions for the growth of *E. coli* for hydrogen evolution.

Inexpensive feedstock and higher biohydrogen productivity from fermentation of OPF juice and sludge without requirement of pH adjustment can decrease the cost of energy production and can be a promising strategy for renewable energy production [6]. Thus, OPF juice mixed with sludge can be an alternative feedstock for biohydrogen production.

3.3. *Biohydrogen enhancement by* E. coli BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE*

Fig. 1 shows hydrogen productivity from all the conditions tested by *E. coli* BW25113 and our engineered *E. coli* strain. Compared with the parent strain, the enhancement of biohydrogen productivity from our engineered strain was 35-fold for sludge without amylase and cellulase treatment, 254-fold for sludge with amylase and cellulase treatment, 193-fold for OPF juice, and 64-fold for OPF juice with sludge. Fig. 1 shows low biohydrogen productivity was observed in all conditions from the unmodified *E. coli* BW25113. However, outstanding biohydrogen productivity was observed from *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* due to the elimination of related genes that can reduce hydrogen production [5].

Table 2 indicates the yield comparison from our study with previous studies. The low hydrogen partial pressure assay was carried out in this study to compare the yield with our previous study. The yield obtained from this study with the engineered strain was 1.5 mol H_2/mol glucose from OPF juice and sludge. Meanwhile, the yield from our previous study was 1.3 mol H_2/mol glucose from complex glucose [5]. However, only 0.8 mol H_2/mol glucose was produced from the parent strain from fermentation with OPF juice and sewage sludge. The same yield was also achieved by our engineered strain when OPF juice was used as a substrate. The yield of 1.5 mol H_2/mol glucose from our study indicates that our engineered strain can produce more hydrogen since the theoretical yield of hydrogen from glucose is 2 mol H_2/mol glucose [27].

Compared to other studies, Cheng and Chang achieved 0.96 mol H_2/mol glucose by *Pseudomonas* sp. CL3 which secretes cellulase for saccharification of bagasses followed by separate hydrolysis and fermentation process by *Clostridium pasteurianum* CH4 [28]. In addition, Zhao and colleagues achieved 1.97 mol H_2/mol glucose from fermentation with *Clostridium beijeirinckii* RZF-1108 from PYG medium [29]. The theoretical yield of 4 mol H_2/mol glucose from strict anaerobes such as *Clostridium* sp. indicates that the yield from other studies was significantly less from the theoretical yield compared to our engineered strain [27].

Thus, our *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* has been shown here to generate an outstanding improvement in biohydrogen production in the new substrate. This strain can be further tested for advanced molecular study for improvement of hydrogen production.

3.4. *Organic acids profile*

Biohydrogen production was also accompanied by organic acids production during glucose metabolism by *E. coli*. In glycolysis, glucose degradation by *E. coli* can follow succinate- and lactate- producing pathways [27]. Glucose can be degraded to phosphoenol pyruvate and pyruvate to produce succinic acid and lactic acid, respectively. However, phosphoenol pyruvate and pyruvate are needed for formate synthesis to be used by *E. coli* for biohydrogen production through the active formate hydrogen lyase (FHL) complex [6]. Thus, succinic acid and lactic acid production reduce the efficiency of glucose metabolism for hydrogen evolution. In this study, we observed lactic acid as the main metabolite produced from fermentation of *E. coli* BW25113 (Fig. 3a). The highest lactic acid production was detected from fermentation of OPF juice and OPF juice with sludge due to higher glucose concentration in both sources as indicated in Table 1. As expected, lactic acid was not produced from our engineered strain when grown on sludge with enzyme treatment due to knock-out of the lactate dehydrogenase (*ldhA*) gene that is related to lactate-producing pathway [5] (Fig. 3b). Lactic acid can be found in sludge samples without enzyme treatment by our engineered strain. This might be due to another lactate dehydrogenase isoenzyme that may trigger lactic acid production in glycolysis pathway [30].

Fig. 3b shows the main metabolites produced by our engineered strain were acetic acid and succinic acid from fermentation of sludge with and without enzyme treatment. Meanwhile, only acetic acid was observed as the main metabolites from fermentation of OPF juice and OPF juice mixed along with sludge. More acetic acid production was observed from fermentation by our engineered strain due to the degradation of sludge to hydrogen which parallels the theoretical yield of 4 mol of hydrogen produced when the final product is acetic acid [4]. Both samples from sludge with and

Table 2 – Comparison of biohydrogen yield.

Strain	Substrate	Yield (mol H_2/mol glucose)	References
E. coli BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE*	Complex glucose	1.3	[5]
Pseudomonas sp. CL3 and *Clostridium pasteurianum* CH4	Bagass	0.96	[28]
Clostridium beijerinckii RZF-1108	PYG medium	1.97	[29]
E. coli BW25113	OPF juice	0.04	This study
E. coli BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE*	OPF juice	0.8	This study
E. coli BW25113	OPF juice with sewage sludge	0.8	This study
E. coli BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE*	OPF juice with sewage sludge	1.5	This study

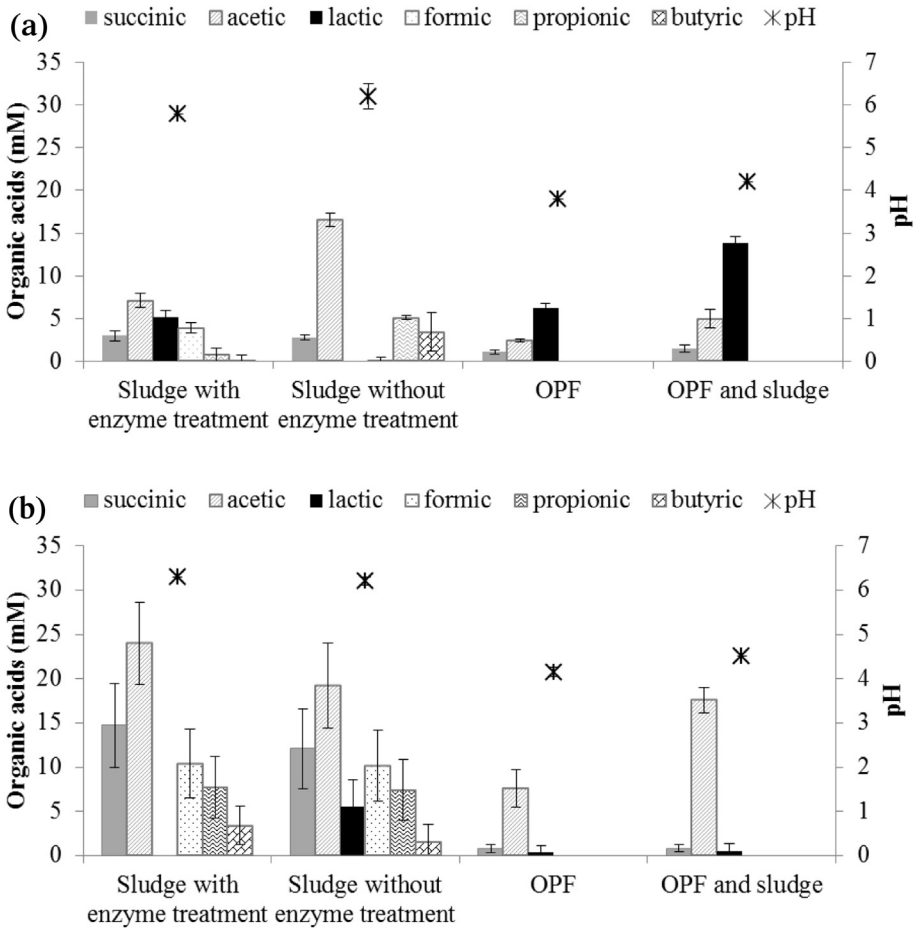

Fig. 3 – Organic acids and pH at 24 h of incubation with (a) *E. coli* BW25113 and (b) *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* from different substrates.

without enzyme treatment produced more formic acid in the fermentation with our engineered strain than with the parent strain. These result reveal that our engineered strain enhanced production of formic acid but not all of the formate was fully utilized for production of biohydrogen [1] when sludge is used without OPF. No formic acid was observed from both strains in OPF juice and OPF juice mixed along with sewage sludge due to the complete utilization of formate to hydrogen through FHL activity [1].

4. Conclusions

This study revealed that using our metabolically engineered strain, *E. coli* BW25113 *hyaB hybC hycA fdoG ldhA frdc aceE* biohydrogen productivity from biomass sources such as OPF juice and sewage sludge was improved 200-fold compared to the unmodified host. Sludge with enzyme treatment (amylase and cellulase) accomplished 8-fold hydrogen productivity when compared to without enzymes treatment from our engineered strain. The metabolically engineered strain also allowed us to obtain one of the highest yields, to 1.5 mol H_2/mol glucose, with the mixture of OPF juice and sewage sludge as a substrate compared to the reported yield of 1.3 mol H_2/mol glucose. The results demonstrate the feasibility of our engineered strain for utilizing industrial biomass with an outstanding improvement towards hydrogen production when compared to the unmodified host.

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