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by Astrilia Damayanti

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Citation: AIP Conference Proceedings **1855**, 070006 (2017); doi: 10.1063/1.4985533
View online: http://dx.doi.org/10.1063/1.4985533
View Table of Contents: http://aip.scitation.org/toc/apc/1855/1
Published by the American Institute of Physics

The Influence of Chicken Eggshell Powder as a Buffer on Biohydrogen Production from Rotten Orange (*Citrus nobilis* var. microcarpa) with Immobilized Mixed Culture

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Abstract. This research observed the influence of chicken eggshell on hydrogen production from anaerobic fermentation of rotten orange (*Citrus nobilis* var. microcarpa) using batch method at 36 °C and pH 7. Fermentation material were varied in several types, the first type was meat and peel of oranges with VS of 59.152 g.L^{-1} in A, B, C, and D compositions. The second type was orange meat added with peel (OMP) with VS of 36.852 g.L^{-1}. The immobilized ingredients used in the experiment consisted of 2 % (w/v) alginate and active carbon with the ratio of 1:1. 3.2 g chicken eggshell powder was added to the first type of material (substrates A, B, C, and D). Results showed that pH during fermentation process using chicken eggshell as a buffer was constant at 5.5; however, without the use of chicken eggshell, the pH decreased to 3.8 and increased slightly before it stayed stable at 4.0. The total amount of gas produced in sample using the chicken eggshell was 46,35 mL.mg VS^{-1} and in sample produced without the eggshell, it was 3,4 mL.mg VS^{-1}. The production of hydrogen in substrate that used chicken eggshell was 1,276 mL.gVS^{-1} in average on the first day. Meanwhile, for the substrate with no addition of chicken eggshell, the average production of hydrogen was 0,163 mL.gVS^{-1}. The reduction of volatile solid (VS) in sample that used chicken eggshell was 24 %, while in sample produced without addition of chicken eggshell, the reduction was 12 %. The liquid compounds (VFA) produced in the fermentation using chicken eggshell were acetic acid and butyric acid. Meanwhile, without addition of chicken eggshell, the products were acetic acid, butyric acid, and propionic acid. This study shows that addition of chicken eggshell as a buffer effectively contributed to hydrogen production during fermentation of rotten oranges.

INTRODUCTION

Hydrogen is a clean alternative energy since it contains high energy (143 GJ ton^{-1}) and most of its combustion product is water [1]. Hydrogen can be produced by fermentation using the waste of fruits, like rotten oranges [2]. Orange (*Citrus nobilis* var. microcarpa) is one of the most produced fruits in Indonesia with more than 1.8 million tons production in 2014 [3]. However, some of those fruits go to waste in rotten condition, thus creating environmental problem. Chemical composition of rotten orange (*Citrus nobilis* var. microcarpa) in dry basis was 7.8 % of hemicellulose, 28.9 % of cellulose, 14.23 % of fats, 17.83 % of glucose, 19.43 % of soluble starch, and 11.8 % of others[4]. An alternative use of orange waste is the production of biohydrogen. Biohydrogen production can be performed by using microbial fermentation. Recently, most microbes used for such fermentation is mixed cultures, since the microbes in mixed cultures are not only better to produce hydrogen than the pure culture [5], but the cultures also ease the production, operation, and control of the fermentation [6]. The use of mixed culture microbes from three biodigester sources was different from melon waste substrate in batch method, which produced hydrogen by one or two biodigesters. Moreover, hydrogen production that usually happens on the first day, reached its peak on the third day, and had its lowest level on the fifth day [7].

Green Process, Material, and Energy: A Sustainable Solution for Climate Change
AIP Conf. Proc. 1855, 070006-1–070006-7; doi: 10.1063/1.4985533
Published by AIP Publishing. 978-0-7354-1529-4/$30.00

The main challenge in biohydrogen production from rotten oranges is the existence of limonene in orange peel as an antimicrobial compound. limonene is contained in 90 % essential oil of 2 – 3 % dry basis [8]. One way to overcome this effect is cell immobilization. Mixed culture has an important role in decomposing organic compounds to become volatile fatty acids (VFAs) and hydrogen. The transformation of organic compounds to be VFAs makes the environment acidic. thus decreasingthe production of hydrogen. A study researched hydrogen production from glucose using mixed cultures at 36 °C and pH 4–7 reported an optimum pH of 5.5 [9]. The attempt to maintain pH during fermentation can be performed by adding buffer in the process. The capacity of the buffer should be comparable to the buffer materials or bicarbonate. Many studies have used bicarbonate as a buffer. Bicarbonate can be obtained from synthetic or natural materials. Synthetic bicarbonate ($NaHCO_3$) is used in anaerobic digestion to produce biogas [10] [11]. Natural bicarbonate is produced from the reaction between $CaCO_3$ and an acid [12] and used to neutralize soil [13] as well as to produce biogas from a mixture of fruit waste [14]. Natural bicarbonate can be obtained from chicken eggshell. since it contains 93.6 % of $CaCO_3$ [15]. The eggshell used in this research were waste. which were mainly collected from household. restaurants. and bakery shops [16]. Consumption of chicken eggs in Indonesia is high. which reaches 1.1 million tons in 2014 [3]. The use of chicken eggshell as a buffer in the biogas production of 3.2 g/50 ml cultures was carried out at pH 6 - 7 in 43 days [14].

In a previous research [17]. production of hydrogen used variety of rotten oranges (*Citrus nobilis* var. microcarpa) composition. including the orange meat and peel with mixed culture that was immobilized using alginate and alginate-activated carbon without the addition of the buffer. Thus. this research was aimed to identify the influence of the addition of buffer from chicken eggshell towards pH. amount of hydrogen produced. volatile solid (VS) compounds in rotten oranges. and VFAs produced during fermentation of rotten orange (*Citrus nobilis* var. microcarpa). The research covered several ratios of meat and peel of rotten oranges with mixed culture immobilized using alginate-activated carbon.

MATERIAL AND METHODS

Immobilized material characteristics

Sodium alginate (technical) with 88.88 % of ash content and 47.11 % of water content was used. Activated carbon (Merck) was analyzed using BET method and the analysis showed that it had surface area. porosity total volume and average pore diameter of 738.524 m^2g^{-1}, 0.6365 cm^3g^{-1}, and 1.724 nm, respectively. The pore size was measured by BJH method resulting in values of 86.27 % of mesopores and 13.73 % of micropores.

Composition of substrate and medium

Meat and peel of rotten oranges were separated. Each part of them was calculated for its VS. 15 ml of rotten oranges with similar VS were divided into two parts: firstly. rotten oranges (A. B. C. and D) with 59.152 $g.L^{-1}$VS. and secondly. orange meat added with peel (OMP) with 36.852 $g.L^{-1}$VS (Table 1) [17].

TABLE 1.The substrates

Substrates	Meat (g)	Peel (g)
A	11.912	2.75
B	10.609	3.3
C	9.9	3.6
D	8.953	4
OMP	4.18	1.02

The nutrient composition of enrichment medium for fermentation was similar as in the previous experiment [17].

Preparation of Chicken Eggshell as Buffer

Chicken eggshell was washed with tap water and dried at 110 °C for an hour. Next, the eggshell was mashed using blender and strained with 35 mesh strainer. Fermentor was filled with the substrates A, B, C, and D, and added with 3.2 g of powdered chicken eggshell.

Mixed culture

Mixed cultures were obtained from biodigester such as cow dung, tofu waste, and fruit waste in Yogyakarta, Indonesia, with similar characteristics as those used in the previous experiments [17]. Each mixed culture was acidified and then enriched three times [17]. 2 mL of each digester source was taken from the third enrichment step and mixed. The mixture was then enriched for 24 hours.

Pretreatment and enrichment of mixed culture

Pretreatment and enrichment methods with previous experiments [17]. Next, 2 mL of mixed culture was added into the enrichment medium and left for 24 hours.

Bead Immobilization

50 mL of the mixed culture produced was centrifuged at 4000 rpm for 10 min. Then, it was harvested and washed twice using 0.97 % (w/v) NaCl solution. Active carbon (Merck) was added to the mixture and poured into 2 % (w/v) sodium alginate solution, with the ratio of active carbon to alginate of1:1. The mixture was put into a syringe and injected into 0.1 M $CaCl_2$ form beads and left for 30 min. Beads formed were taken and washed with distilled water.

Batch hydrogen production

Fermentation was carried out following methods explained in the previous experiment [17]. 100 mL bottle was filled with 50 mL culture consisting of 60 % nutrients, 30 % substrate, and 10 % mixed culture. The initial pH of all substrates was 7. The fermentor was flushed with N_2 for 3 min at 36 °C before the incubation process started. The analysis of pH and total gas for both types of substrate were carried out on the first, third, and fifth day, while VFAs for all substrates were analyzed only on the first day. Every experiment was performed in duplicate.

ANALYTICAL METHODS

The gas produced during fermentation was collected with the syringe Terumo. Hydrogen was analyzed using gas chromatography Shimadzu GC 8A equipped with a thermal conductivity detector and 2 m of column MS-5A. Operating temperatures for column, detector, and injector were 60 °C, 70 °C and 70 °C, respectively. Nitrogen was used as a carrier gas with inlet pressure of 100 kPa. Pressure and temperature were 760 mmHg and 0 °C. VFAs (acetic acid, butyric acid, and propionic acid) were analyzed by gas chromatography (HP 5890, Japan) with the temperatures of the column, detector, and injector were 60 °C, 260 °C, and 250 °C, respectively, using a flame ionization detector and 3 m of column packed with FFAP. Helium was used as the carrier gas. pH was measured using a pH meter (Eutfech Instrumen, Cyberscan Waterproof Series, Singapore) and VS was determined according to a standard method [18].

RESULTS AND DISCUSSION

The Influence of Chicken Eggshell Addition to the pH level during the Fermentation

Control of pH level during fermentation process is an important factor in hydrogen production, since pH influences activity of hydrogenase and microbial metabolism pathway [19]. The influence of chicken eggshell addition to pH during fermentation process is presented in Fig. 2.

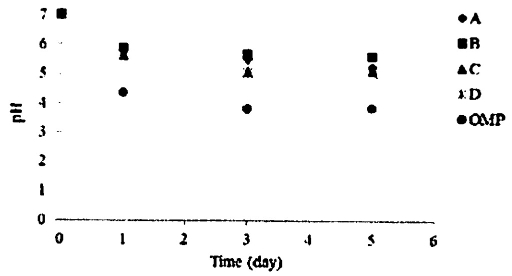

FIGURE 1. pH during fermentation of sample with addition of chicken eggshell (substrates: A, B, C, and D) and without chicken eggshell (substrate: OMP)

Figure 1 shows that during fermentation, the addition of chicken eggshell as a buffer contributes to a decrease in pH to 5.5 on the first day and it stays relatively constant on the following days (substrates A, B, C and D). Meanwhile, the OMP substrate that had no buffer addition had a decrease of pH to 3.8 and the average pH was 4. The buffer system follows Le Chatelier principle [20] as shown in the reaction involving $CaCO_3$ (Eq.1) [12] below:

$$CaCO_3 + H^+ \Leftrightarrow Ca^{2+} + HCO_3^- \quad (1)$$

According to Le Chatelier principles, if an acid is added to calcium bicarbonate, the reaction moves to the right side and forms bicarbonate and calcium ions. In contrast, while forming bicarbonate, H^+ ion is released, and the reaction moves to the left side to form calcium bicarbonate. Consequently, acidity of the solution does not decrease much: instead, it tends to stay constant.

The Influence of Chicken Eggshell Addition to Hydrogen and Total Gas Formation

Total gases formed during the fermentation of substrates both with (A, B, C, and D) and without (OMP) chicken eggshell is provided in Fig. 3, whereas the comparison of hydrogen to the total gases formed on the first day is presented in Table 2.

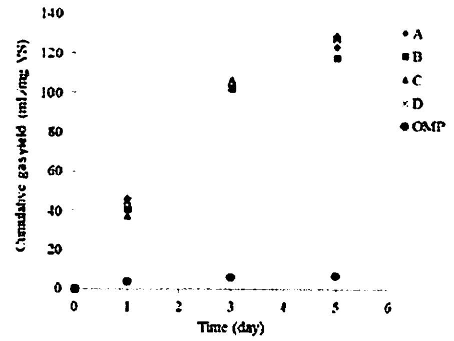

FIGURE 2. Total gas formed during fermentation of substrates, with e (substrates: A, B, C, D) or without eggshell (substrate: OMP)

Figure 2 shows that the total gas formed during fermentation of rotten orange with addition of chicken eggshell as a buffer was 46.35 mL.mg VS^{-1}, while in sample without eggshell addition, the total gas formed was only 3.4

mL.mg VS^{-1}. Addition of eggshell increases the concentration of CO_2 formed during glucose fermentation process [10].

TABLE 2.The comparison of total gas and hydrogen formed in the substrates on the first day

Substrates	Total gas (mL.mg VS^{-1})	Hydrogen concentration	
		Percentage (%)	(mL.mg VS^{-1})
A	46.35	2.583	1.197
B	40.90	2857	1.169
C	37.50	3.895	1.461
D	44.70	9.124	4.078
OMP	3.40	4.498	0.163

Table 2 shows that production of hydrogen (mL.mgVS^{-1}) in the substrates with addition of chicken eggshell (A, B, and C) was1.276 in average, while in substrate (OMP) without chicken eggshell, the value was 0.163. Hydrogen that was formed by the addition of the chicken eggshell was eight times larger than in sample without chicken eggshell. Formation of gases (total gas and hydrogen) was obtained from simple glucose conversion, which exists in starch and as glucose. Moreover, the existence of limonene in the orange's peel makes the fermentation unstable [21].

The Influence of Chicken Eggshell Addition to the Volatile Solid Reduction

The average degradation percentage of organic compound by mixed culture with chicken eggshell addition was 24 %, while without chicken eggshell, the level was 21 % (Table 3).

TABLE 3. Volatile Solid Reduction by Immobilized Mixed Culture during Hydrogen Fermentation of Rotten Oranges

Substrates	Start(mg mL^{-1})	final(mg mL^{-1})	VS reduction (%)
A	59.152	43.06	27.20
B	59.152	44.44	24.87
C	59.152	43.99	25.63
D	59.152	47.51	19.68
OMP	36.852	32.11	12.88

Table 3 shows that the average reduction of VS in the first type of substrate A, B, C, and D) added with chicken eggshell was 24%, while for the second substrate (OMP) without the addition of chicken eggshell, the value was only 12 %. The table also shows that the average comparison of orange meat and peel between the first type of substrate (A, B, C, and D), and the second type (OMP) were 3 times and 4 times respectively. VS reduction in biohydrogen production from melon meat waste was 40% [7] and it was higher than the result found in this study. The more orange used, the more reduction of VC took place. The meat of orange contains glucose that is easier to be degraded by microbes than the peel of the fruit [4].

The Influence of Chicken Eggshell Addition to the VFAs Production

The VFAs production during fermentation process is presented in Figure 3. Figure 3 shows that the most dominant acid formed in the fermentation on the first day was acetic acid. Propionic acid was only found in fermentation of substrate without the addition of chicken eggshell. Acetic acid and butyric acid that were formed in sample with chicken eggshell were 3.7 times and 2.9 times lower than in sample without addition of chicken eggshell. In fermentation process, glucose is turned into acetic acid and 4 mol ATP (Eq. 2), while production of butyric acid used 3 mol ATP (Eq. 3), therefore, when the cell growth rate is high, the cells tend to produce acetic acid than butyric acid [22].

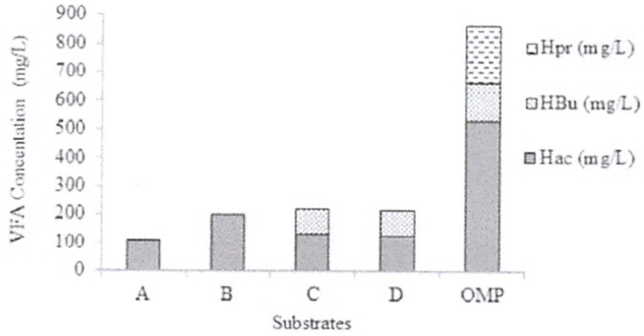

FIGURE 3. Concentration of VFA (mg.L^{-1}) on the first day in substrates with chicken eggshell (A, B, C, and D) and without chicken eggshell (OMP). HAc: acetic acid; HBu: butyric acid; Hpr: Propionic acid

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 + 4ATP \quad (2)$$
$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2CO_2 + 2H_2 + 3ATP \quad (3)$$

Hydrogen is the metabolite produced by microbes during production of acetic acid (Eq.2) and butyric acid (Eq.3). In contrast, propionic acid causes hydrogen to be consumed by microbes (Eq.4) [23].

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (4)$$

CONCLUSION

pH of substrate that used chicken eggshell decreased 1.5 points during fermentation, while the sample without eggshell addition dropped its pH for 3 points. Total gas formed in sample using chicken eggshell was 10 times higher than in sample without chicken eggshell. Moreover, the hydrogen production in sample with addition of chicken eggshell was 8 times greater than in sample without chicken eggshell. Degradation of organic compounds by the mixed culture in the presence of chicken eggshell was twice larger than in sample without chicken eggshell. The acid formed during fermentation were two (acetic and butyric acids) when sample was added with chicken eggshell, whereas without chicken eggshell, there were three acids formed (acetic, butyric, and propionic acids).

ACKNOWLEDGMENT

Authors would like to express our gratitude to the Directorate General of Higher Education, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for financial support of this work through DIPA (PUPT) UGM 2016. Technical assistance of Nurachim Fitri Marshella, Agatha Puput Febrila, Anjar Tri Asmara, and Aquilina Novin Astuti is highly appreciated.

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