Comparison Between INT and TTC Assay to Determine the Dehydrogenase Activity of Flocs

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***Abstract*—In order to evaluate the efficiency of anaerobic activated sludge in hydrogen-producing system, iodonitrotetrazolium chloride (INT) and triphenyl tetrazolium (TTC) assay were optimized for detecting the specific dehydrogenase activity of the flocs. The results showed that both INT and TTC assay under the optimum conditions could be used to determine dehydrogenase activity. In comparison with TTC assay, INT assay was more sensitive and precise. In the range between 3.5-12.5 g/L of sludge concentration (MLVSS), the INT assay are 2.8 mL reactant, 0.3 mL sludge sample, 1 mL 19.8 μmol/L INT solution, pH 5.0, an oscillating reaction at 45°C for 30 min, anhydrous ethanol as the extraction solvent and wavelength of 478 nm. The correlation coefficient between specific dehydrogenase activity and specific hydrogen production rate, R, was above 0.93, excellently reflecting the hydrogen-producing activity of flocs.**

Keywords-Anaerobi; Fermentation; Biohydrogen production; Dehydrogenase

I. INTRODUCTION

Hydrogen offers tremendous potential as a clean, renewable energy and helps to reduce the consumption of fossil fuels. Bio-hydrogen production techniques, which are energy-saving, have been drawn increasingly attention nowadays [1].Particularly fermentative bio-hydrogen production has widespread use in the future. Furthermore, it is important to evaluate the efficiency of anaerobic activated sludge in hydrogen-producing system.

The traditional methods to evaluate sludge activity are by the ratio of volatile suspended solids and total suspended solids (MLVSS/MLSS), the specific hydrogen production rate of activated sludge (L/g MLVSS/d) or chemical oxygen demand (COD) removal [2]. However, all these methods have drawbacks. For instance, MLVSS does not only contain the active microbial cells, but also contains inactive microbes, thus the MLVSS/MLSS is higher than the practical value and the specific hydrogen-producing rate is lower [3]. In addition, because organic matters are mainly degraded into volatile fatty acids (VFAs) and alcohols by anaerobic sludge, COD removal is low, which can not reflect the sludge activity in the hydrogen-producing system [4].

In contrast, DHA serves as an ideal indicator of sludge activity. DHA determination is simple, rapid and without expensive equipment. Although DHA determination has been reported a lot before, there are seldom researches introducing DHA in biohydrogen production system [5]. Currently, DHA determination mainly involves iodonitrotetrazolium chloride (INT) and Chloride Triphenyl Tetrazolium (TTC) assay. Some scientists compared INT with TTC assay and drew different conclusion on which assay was superior because they adopted different samples and conditions. Therefore, it is necessary to estimate the DHA with both assays under optimum conditions in bio-hydrogen production system.

This study is aimed to optimize DHA determination in biohydrogen production system. The comparison of INT and TTC assay under the optimum conditions was made to instruct the reactor's operation timely.

II. EXPERIMENTAL MATERIALS

The flocs was collected from an Anaerobic Baffled Reactor (ABR) hydrogen production system [6].

Analytical items such as pH, sludge concentration (MLVSS) etc. were analyzed according to standard methods [7]. The biogas yield of the ABR was measured using a wet gas meter, and the H_2 were analyzed by gas chromatography. The operations of the two methods were under anaerobic conditions (flushing the tubes with N_2 gas). Besides, three replicates were measured and the mean value for each assay was obtained. The basic operations of the INT assay and the basic operations of the TTC assay were seen the reference [8].

III. PREPARE YOUR PAPER BEFORE STYLING

A. Operating conditions

A single factor experimental method was used in the research. The factors consist of substrate (INT or TTC) concentration, reaction time, temperature and pH, etc [9]. The results (Table 1) showed that the absorbance of INF rose from 0.18 to 0.51 when the INT concentration was increased (1.9~19.8 μmol/L). However, the gradual increase stopped with a higher concentration. The absorbance of INF rose from

978-1-61284-340-7/11/$26.00 ©2011 IEEE

0.12 to 0.24 when the enzyme reaction time was increased from 10 min to 30 min. With longer reaction time, the increasing trend was not significant. The absorbance of INF was observed highest (0.37) at 45°C. The maximum absorbance (0.58) of INF was observed when pH was 5.0. The results above showed that the optimum conditions of INT assay were: INT concentration of 19.8μmol/L, reaction for 30 min at 45°C and pH 5.0.

TABLE 1. INFLUENCE TO THE ABSORBANCE ON DIFFERENT CONDITIONS IN INT ASSAY

INT/μmoL/L		**Time/min**		**Temperature/°C**		**pH**	
vel	A	level	A	level	A	level	A
1.9	0.18	10	0.12	25	0.19	4.0	0.28
9.9	0.41	20	0.18	35	0.25	5.0	0.58
19.8	0.51	30	0.24	40	0.36	6.0	0.13
39.6	0.55	40	0.25	45	0.37	7.0	0.17
79.2	0.57	50	0.27	55	0.36	8.0	0.18
158.4	0.58	—	—	65	0.28	9.0	0.20

Similarly, the optimum conditions of TTC (Table 2) assay were TTC concentration of 17.8 mmol/L, reaction time of 20 min, temperature of 35°C and pH 8.0.

TABLE 2. INFLUENCE TO THE ABSORBANCE ON DIFFERENT CONDITIONS IN TTC ASSAY

TTC/mmoL/L		**Time/min**		**Temperature/°C**		**pH**	
level	A	level	A	level	A	level	A
3.0	0.13	10	0.31	25	0.31	4.0	0.10
5.9	0.14	20	0.49	35	0.41	5.0	0.12
11.9	0.28	30	0.33	40	0.32	6.0	0.28
17.8	0.35	40	0.24	45	0.13	7.0	0.17
23.8	0.27	50	0.15	55	0.11	8.0	0.36
29.7	0.28	—	—	65	0.17	9.0	0.28

In order to achieve a better extraction purpose, different extraction agents were tested. The Fig.1 demonstrates the absorbance of extractant reached maximum values, 0.53 (INT) and 0.63 (TTC) when alcohol was the extraction agent. In addition, alcohol is innoxious, so anhydrous alcohol was the appropriate extraction agent.

The INT-A calibration curve was made as follows: 1 mL INT solution, whose concentrations were 9.8, 19.8, 39.5, 79.0, 118.5 and 158.1 μmol/L were respectively

added into 18 centrifuge tubes. The 18 tubes were divided into 6 groups and each group contained three replicates. 2.5 mL sodium acetate solution and 0.2 mL ascorbic acid (7%) were added into each tube. The resulting mixture was then stirred at 45 °C in the dark for 30 min. 1 mL sulfuric acid was added to terminate enzyme reaction. The samples were centrifuged at 5000rpm for 5 min. The supernatant was discarded and 5 mL ethanol was added. Then the mixtures were stirred at 45°C in the dark for 5 min and centrifuged at 5000rpm for 5 min. The INF was measured at 478 nm to draw an INT-A calibration curve (Fig. 2). The INT-A calibration curve equation is as follow:

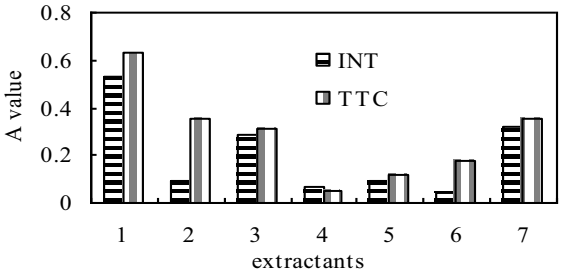

Figure 1. Effects of extractants on absorbance of the extraction liquid

1.anhydrous alcohol, 2.chloroform, 3.ethyl acetate, 4.hexane, 5.toluene, 6.ethyl ether, 7acetone

$$y=0.0109x-0.0459 \quad (2)$$

In the equation (2), x stands for INT concentration (μmol/L), y for the corresponding absorbance.

Similarly, The TTC-A calibration curve was made as follows: 1mL TTC solution, whose concentrations were 59.1, 118.2, 177.3, 236.4, 295.5 and 354.6 μmol/L. 2.5mL Tris-HC1 buffer, 0.5 mL Na_2SO_3 (0.36%) and 1 mL fresh Na_2S (10%) were added into each tube. The resulting mixture was then stirred at 35°C in the dark for 20 min. 2 mL sulfuric acid was added to terminate enzyme reaction. The samples were centrifuged at 5000rpm for 5 min. The supernatant was discarded and 5 mL anhydrous alcohol was added. Then the samples were shaken and extracted at 35°C in the dark for 10 min; they were centrifuged at 5000 rpm for 5 min. The TF was

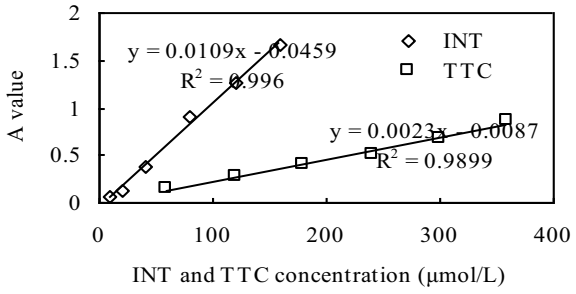

Figure 2. The calibration curve curve of INT and TTC concentration and absorbance of the extraction liquid

measured at 475 nm in the spectrophotometer. The TTC-A calibration curve equation is as follow:

$$y =0.0023x-0.0087 \quad (4)$$

By the equation, x was the TTC concentration, y was absorbance of TF.

Sludge samples with different concentrations (2.66-26.64 g MLVSS /L) were collected from the ABR hydrogen production system. The determination of DHA using the two assays was carried out under optimum conditions. The results (Fig.3) showed that the sludge concentration (2.66~26.64 g MLVSS/L) and absorbance of extractants had a favorable correlation. Both the assays could be used to determine the DHA of sludge in the biohydrogen production system. To

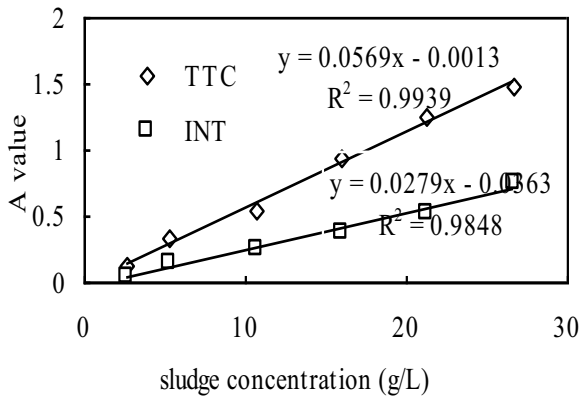

Figure 3. Relationship between sludge concentration and absorbance of the extraction liquid liquid

obtain a more accurate value, the absorbance of the extractant should be kept at 0.2-0.7. Therefore, the ranges of sludge concentration were 3.5-12.5 g MLVSS /L (INT) and 8.5-26.4 g MLVSS /L (TTC).

When the ABR kept at HRT 30 h and influent COD of 8000 mg/L, the specific hydrogen production rate and specific DHA of the activated sludge were correlation analysed [10]. The results were shown in table 3 and table 4.

A correlation analysis of the data from specific hydrogen production rate and specific DHA of sludge were conducted separately using the CORREL of Microsoft Excel.

The correlation coefficient between specific dehydrogenase activity and specific hydrogen production rate, R, of the first compartment in ABR were 0.96 (INT) and 0.89 (TTC), and of the second compartment in ABR were 0.93 (INT) and 0.87 (TTC), indicating that the specific dehydrogenase activity and the specific hydrogen production rate were closely correlation. Therefore, INT and TTC assay could be used to reflect the hydrogen production activity of anaerobic sludge.

Many reporters found INT suitable for detecting DHA of anaerobic sludge, while TTC was more suitable for that of aerobic sludge. However, the results showed that the specific dehydrogenase activity and the specific hydrogen production rate have a close correlation in both the assays. Therefore, under the anaerobic operation, both the INT and TTC assay could be applied to characterize the anaerobic sludge in biohydrogen production system. INT and TTC, as hydrogen acceptors, contain different functional groups, which enable them to have different characteristics to detect DHA. The results indicated that the optimum conditions of the two assays such as pH, sludge concentration, substrate concentration, were different.

TABLE 3. SPECIFIC HYDROGEN PRODUCTION RATE AND SPECIFIC INT-DHA OF THE ACTIVATED SLUDGE IN ABR

Active parameter		**Time / day**				
		1	*3*	*5*	*7*	*9*
Room 1	SHPR (mol/kg/d)	2.84	2.93	2.71	2.14	2.27
	DHA (μmol /g/min)	26.3	26.2	24.6	22.1	23.7
Room 2	SHPR (mol/kg/d)	2.03	2.02	2.15	1.67	2.07
	DHA (μmol /g/min)	21.31	20.48	21.26	18.49	21.87

The results show that the INT assay is superior to TTC assay for testing the DHA of sludge in a hydrogen-producing system. The reasons are: (1) INT assay has a better correlation. The correlation coefficient of INT ($R \geq 0.93$) is obviously above that of TTC ($R \geq 0.87$). (2) INT has better sensitivity. The slope of the calibration curve of INT-A (0.0109) was greater than that of TTC-A (0.0023). (3) INT assay showed higher reproducibility. The standard errors of INT assay was 5% while that of TTC assay was 9%. (4) INT required less amount of sludge. To ensure the absorbance ≥0.2, INT assay requires 3.5 g MLVSS/L, while TTC assay requires 8.5 g MLVSS/L at least.

TABLE 4. SPECIFIC HYDROGEN PRODUCTION RATE AND SPECIFIC TTC-DHA OF THE ACTIVATED SLUDGE IN ABR

Active parameter		Time / day				
		1	3	5	7	9
Room 1	SHPR (mol/kg/d)	2.84	2.93	2.71	2.14	2.27
	DHA (μmol/g/min)	10.84	11.71	10.25	9.17	10.16
Room 2	SHPR (mol/kg/d)	2.03	2.02	2.15	1.67	2.07
	DHA (μmol/g/min)	9.32	9.13	9.98	8.75	9.76

An ultimate conclusion could only be drawn concerning the comparison between the INT and TTC assay. The report sets out the finding of a study that both the INT and TTC assay can be applied to measure the DHA of anaerobic activated sludge in hydrogen-producing system. Furthermore, the INT assay has the advantage of increased sensitivity over the TTC procedure under the optimal conditions. INT assay,

compared to TTC assay, requires less sludge and shorter time and turns out to be a more sensitive determination. Therefore, INT assay is more suitable to measure DHA in biohydrogen production system.

IV. CONCLUSION

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INT assay excellently reflect the hydrogen-producing activity in the biohydrogen production system. INT-DHA may be an ideal indicator to evaluate the potential hydrogen-producing activity of sludge. TTC and INT assay were under largely anaerobic condtions (flushing the tubes with N2 gas). Nevertheless, it requires more sensitive and safe substrates than TTC and INT for analyzing DHA of sludge without the necessity of anaerobic operation.

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