Strategies for recovery of inhibited commercial biogas reactor feeding with palm oil mill effluent

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Background: The commercial biogas reactor feeding with palm oil mill effluent (POME) was inhibited by low pH and high volatile fatty acid (VFA). The strategies for recovering inhibited biogas reactor with economically feasible such as dilution, pH adjustment, and re-inoculation strategies were investigated.

Results: The inhibited biogas reactor was recovered within 33 days by pH adjustment strategy with 0.50% w/v $Ca(OH)_2$ and 8.0% w/v oil palm ash. The dilution with 20% v/v biogas effluent strategy was considered as more economically feasible than other strategies with a recovery time of 36 days. The recovered biogas reactor has a higher methane yield of 25-45% than self-recovery with a significantly increased hydrolysis constant and specific methanogenic activity. *Clostridiales* sp., *Bacilli* sp., and *Methanosarcina* sp. was increased in a recovered biogas reactor.

Conclusion: The dilution with 20% v/v of biogas effluent recovered the inhibited POME feeding commercial biogas plant with economically feasible and high biogas production performance.

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2 **palm oil mill effluent**

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23   **Abstract**

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25   inhibited by low pH and high volatile fatty acid (VFA). The strategies for recovering inhibited

26   biogas reactor with economically feasible such as dilution, pH adjustment, and re-inoculation

27   strategies were investigated.

28   **Results:** The inhibited biogas reactor was recovered within 33 days by pH adjustment strategy

29   with 0.50% w/v $Ca(OH)_2$ and 8.0% w/v oil palm ash. The dilution with 20% v/v biogas effluent

30   strategy was considered as more economically feasible than other strategies with a recovery time

31   of 36 days. The recovered biogas reactor has a higher methane yield of 25-45%than self-

32   recovery with a significantly increased hydrolysis constant and specific methanogenic activity.

33   *Clostridiales* sp., *Bacilli* sp., and *Methanosarcina* sp. was increased in a recovered biogas

34   reactor.

35   **Conclusion:** The dilution with 20% v/v of biogas effluent recovered the inhibited POME feeding

36   commercial biogas plant with economically feasible and high biogas production performance.

37   **Keywords** Palm oil mill effluent, Commercial biogas plant, Organic overloading, Acidified

38   inhibition, Recovery strategy, Microbial community

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50   **Introduction**

51   Palm oil mill effluent (POME) is the primary wastewater generated from the palm oil

52   extraction process, which mostly treated through anaerobic treatment (*Wu et al., 2010*) with

53   energy production in terms of biogas (*Stamatelatou et al., 2011*). Biogas production from POME

54   has been successfully reported by many researchers (*Fang et al., 2011*; *O-Thong et al., 2012*;

55   *Mamimin et al., 2019*; *Tanikkul et al., 2019*). However, long-term operation of commercial

56   biogas reactor feeding with POME was confronted with process imbalance such as volatile fatty

57   acid (VFA) inhibition, long-chain fatty acids (LCFAs) inhibition, low pH inhibition, and

58   foaming. The imbalanced biogas reactor resulted in poor performance and instability, even the

59   failure of the anaerobic digestion (AD) process (*Joo-Hwa & Xiyue, 2000*; Menardo et al., *2011*).

60   The imbalanced biogas reactor feeding with POME was mainly caused by fluctuating in the

61   composition and volume of POME. POME composition and volume were varied depending on

62   the qualities of fresh fruit bunches, season, time of harvest, and oil palm extraction processing.

63   The feedstock composition and organic loading rate (OLR) were affected in both bacterial and

64   archaeal communities in the AD process (*Supaphol et al., 2011*; *Xia et al., 2012*). The fluctuation

65   in feedstock composition and organic loading rate (OLR) always caused the process imbalanced

66   in case of high strength feedstock leading to unstable biogas production (*Joo-Hwa & Xiyue,*

67   *2000*). Moreover, Fotidis et al. (*Fotidis et al., 2014*) reported that the organic overloading was

68   inhibited the AD process resulting in losses of methane yield up to 30%. Reactor acidification by

69   organic overload is one of the most common reasons for the AD process imbalanced (*Akuzawa et*

70   *al., 2011*) due to the rapid build-up of VFAs accumulation from uncoupling between the acid

71   producers and consumers (*Ahring, 1995*). The consequences of the AD process imbalanced are

72   financial losses due to reducing biogas yield as well as increase deployment of staff and costs for

73   the chemical. Thus, the inhibition of commercial AD reactor feeding with POME is necessary for

74   recovery within the short-time.

75   The typical recovery strategy for the AD process imbalanced is stopped substrate feeding

76   to the digester to restore the ecological function of the AD system via the self-recovery of

77   microorganisms. However, this strategy requires a long time and is not economically feasible.

78   The stop feeding strategy combining with trace elements addition could accelerate the process

79   recovery of the VFA-inhibition AD reactor but still required a long feeding-stop period

80   (*Voelklein et al., 2017*). The recovery of VFA and low pH inhibition in real-time without stopped

81   feeding POME is still a significant challenge. The adjusting pH in the reactor to a near-neutral

82   was often applied to enhance the buffering capacity of the AD system against VFA disturbance

83   due to their low cost and smooth operation. Zhang et al. (*Zhang et al., 2013*) proved that the pH

84   adjustment in digester could be able to recover the AD process from low pH inhibition with

85   stability reactor operation. Alkalinity addition directly improves the buffering capacity of the AD

86   process to meet the requirements of the microbial populations (*Zhang et al., 2016*) and enhance

87   activities of the acidogenic bacteria and methanogenic archaea (*Zhang et al., 2012*). Alkaline

88   substances such as Na_2CO_3 and $NaHCO_3$ were more pronounced effects on the stability of the

89   anaerobic process of municipal solid waste than $NaOH$ due to CO_3^{2-} and HCO_3^- have a higher

90   buffering capacity than OH^- (*Jun et al., 2009*). Also, wood ash was used to adjust the pH of the

91   AD process instead of alkaline chemicals as a cheap material (*Saritpongteeraka & Chaiprapat,*

92   *2008*). The palm ash was used to adjust the pH of POME with high biogas production than raw

93   POME due to ash releasing buffer capacity and micronutrient (*Gómez et al., 2006*). However,

94   the pH adjustment strategy could not reverse the AD process imbalanced but only delayed the

95   AD process failure (*Gómez et al., 2006*). Nevertheless, the strategy of inoculum addition with

96   micronutrient supplementation has been used to recover the AD process imbalanced (*Lee &*

97   *Shoda, 2008*; *Qiang et al., 2013*). The re-inoculation (*Wu et al., 2015*) or bioaugmentation (*Li et*

98   *al., 2018*) of high-activity anaerobic microorganism was used to restart the out of order AD

99   reactor. The re-inoculation was significantly effective in the short-term, but instability for the

100   long-term. The combination of the pH adjustment, trace elements, and re-inoculation was always

101   useful but was costly (*Zhang et al., 2018*). All strategies above have proved to be effective

102   methods to recover the AD process imbalanced. However, a systematic and comprehensive

103   evaluation of recovery strategies from the imbalanced of the POME biogas reactor has not been

104   reported.

105   This work aimed to recover the imbalanced of POME biogas reactor by pH adjustment

106   with an alkaline substance, the dilution of the toxic compounds, and re-inoculation strategies. All

107   strategies were combining with 20% v/v POME addition as low flow ate feeding instead of stop

108   feeding. The microbial community responsible for each recovery strategy was investigated. The

109   knowledge from our research can provide economically feasible and quick recovery methods for

110   imbalanced commercial biogas reactor with microbiological communities relevant to information

111   to stable biogas production.

Materials & Methods

Inhibited AD sludge samples and inoculum

114   Raw palm oil mill effluent (POME), inhibited sludge samples, and active methane-

115   producing sludge was collected from mesophilic biogas plant (40±2 °C), Krabi province,

116   Thailand. POME is brownish liquid effluent discharged from the palm oil at a temperature

117   between 80-90°C and acidic pH typically between 4.0-5.0. An inhibited sludge sample was

118   collected from the mesophilic AD digester, which was operated continuously with POME at high

119   organic loading and consequently acidification on the process. Both the samples were measured

120   for alkalinity, total volatile fatty acids (tVFA), total nitrogen (TN), lipid content, total solids

121   (TS), and volatile solids (VS) (*APHA, AWWA, 2012*). Table 1 summarizes the characteristics of

122   the raw POME, inhibited sludge samples, and inoculum used in this study. The active methane-

123   producing sludge had a pH, suspended solids (SS), and volatile suspended solids (VSS) content

124   of 7.80±0.15, 9.8±0.1 g/L, and 37.2±0.3 g/L, respectively.

Recovery of inhibited AD sludge

126   An inhibited sludge sample was recovered by pH adjustment strategy, dilution strategy,

127   and re-inoculation strategy. The experiments were carried out in a batch reactor. 40 mL of

128   inhibited sludge sample was diluted with tap water (TW) and biogas effluent (BE) at

129   concentrations of 10, 20, 30, 40, and 50%v/v, respectively, as dilution strategy. 40 mL of

130   inhibited sludge samples were added with active methane-producing sludge at concentrations 5,

131   10, 15, 20, 25, 30, 35, 40, 45, and 50 %v/v, respectively, as a re-inoculation strategy. 40 mL of

132   inhibited sludge samples was added alkaline chemicals in the form of sodium hydrogen

133   carbonate ($NaHCO_3$), sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) and palm oil ash

134   at concentrations of 0.85-1.5% w/v, 0.1-0.14% w/v, 0.1-0.5% w/v, and 6-10% w/v, respectively,

135   as adjusting pH strategy. All strategies were combining with 20% v/v POME addition as low

136   flow ate feeding instead of stop feeding. The self-recovery was used as a control. All

137   experiments were flushed with N_2: CO_2 mixed at 80:20 ratios to create the anaerobic condition

138 and tightly with a butyl-rubber septum and aluminum cap. The experiment was carried out in

139 duplicate and placed at ambient temperature (38±3ºC) for 45 days. The biogas production in the

140 headspace was measured via water displacement method, and biogas content was analyzed by a

141 gas chromatograph equipped with thermal conductivity detectors (GC-TCD). Sludge samples in

142 the AD system were analyzed for the microbial community structure using polymerase chain

143 reaction denaturing gradient gel electrophoresis (PCR-DGGE) techniques. The specific

144 methanogenic activity (SMA) of the sludge was investigated before and after recovery strategies

145 applied.

Microbial activity and community analysis

147   The methanogenic activities from inhibited sludge and sludge after recovery were

148 evaluated by the SMA test using acetic acid, glucose, crystalline cellulose, and gelatin as

149 substrate. The sludge sample without a substrate was used as a negative control to eliminate the

150 probable influence of residual biodegradable organic substances in the sludge *(Liu et al., 2016)*.

151 The SMA value was calculated by the slope of methane production (based on g COD of CH_4)

152 against incubation time and divided with VSS added of sludge sample *(Hussain & Dubey, 2017)*.

153 The microbial community structure was analyzed by PCR-DGGE techniques, according to

154 Prasertsan et al. *(Prasertsan et al., 2009)*. 0.2 g of sludge sample was extracted for genomic

155 DNA using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA). The 16S rDNA gene of

156 bacteria was amplified by the first PCR with universal primer 27f

157 (GAGTTTGATCCTTGGCTCAG) and 1525r (AAGGAGGTGWTCCARCC). 16S rDNA gene

158 for archaea was amplified using Arch21f primers (TTCCGGGTTGATCCYGCCGGA) and

159 Arch958r (YCCGGCGTTGAMTCCAATT). The V3 region of bacteria was amplified in second

160 PCR by primer 357f (CTCCTACGGGAGGCAGCAG) with CG clamp and 518r

161 (GTATTACCGCGGCTGCTGG) with the product of first bacteria PCR as a template. The V3

162 region of archaea was amplified in second PCR by primer 340f (CCTACGGG-GYGCASCAG)

163 with CG clamp and 519r (TTACCGCGGCKGCTG) with the product of first archaea PCR as a

164 template. Second PCR products were performed the DGGE analysis by using electrophoresis

165 with 6% polyacrylamide gel for bacteria and 8% polyacrylamide gel for archaea contained a

166 linear the urea/formamide gradient of denaturant ranging from 40% to 70% in 0.5 TAE buffer at

167 20 volts for 20 min and 70 volts for 15 hours at a constant temperature of 60 °C. Sybr-Gold was

168   stained in the DGGE gels for 60 min and photographed on the Gel Doc XR system (Bio-Rad

169   Laboratories). Predominant DGGE bands were excised with a sterile tip and suspended in 30 µL

170   sterilized Milli-Q water. Excised DGGE band was incubated at 4 °C overnight and re-amplified

171   by PCR using the same primers without the GC clamp. PCR products were purified and

172   sequenced by Macrogen Inc. (Seoul, Korea). Closest matches for partial 16S rRNA gene

173   sequences were identified by database searches in Gene Bank using BLAST

174   (http://blast.ncbi.nlm.nih.gov) (*Muyzer & Smalla, 1998*).

Analytical methods and calculation

176   The biogas composition (H_2, N_2, CH_4, and CO_2) was measured by gas chromatograph

177   GC-8A (Shimadzu, Kyoto, Japan) with a 1-meter stainless steel column packed with Shin

178   Carbon (60/80 mesh) equipped with thermal conductivity detectors (TCD). The argon at a flow

179   rate of 14 mL/min was used as the carrier gas. The temperatures of the oven, detector, and

180   injection port were at 40°C, 100°C, and 120°C, respectively. The 0.5 mL of the biogas sample

181   was injected in duplicate for each reactor. The daily biogas production for each reactor was

182   counted using the water displacement method (*Yan et al., 2015*). The chemical and physical

183   composition of POME, active methane-producing sludge, and inhibited sludge samples were

184   measured of pH, lipid content, TS, VS, VSS, TN, tVFA, and alkalinity according to standard

185   methods for the examination of water and wastewater (*APHA, AWWA, 2012*). POME is acidic

186   wastewater, the determination of TS was performed at a temperature of 90°C instead of 105°C,

187   till constant weight in order to avoid decreasing of volatile fatty acids (*Angelidaki et al., 2009*).

188   The liquid samples were collected by syringe (1 mL) and filtered through a nylon membrane (0.2

189   µm). The filtrated samples were acidified to pH 3.0–3.2 with 30% (v/v) phosphoric acid for

190   VFAs analysis (*Raposo et al., 2015*). VFAs were determined through a gas chromatograph GC-

191   17A (Shimadzu, Kyoto, Japan) with a Stabilwax®-DA fused silica column (30 m of length, 0.53

192   mm of diameter, 85°C) connected to a flame ionization detector (FID) at 240°C. The helium at

193   30 mL/min was used as the carrier gas. Buswell's equation was used to calculation theoretical

194   methane yield, assuming the total stoichiometry conversion of the organic matter to methane and

195   carbon dioxide (*Buswell & Mueller, 1952*). The hydrolysis constants (k_h) were determined by

196   using the first-order kinetic model in equation (1) according to the protocol of Raposo et al.

197   (*Raposo et al., 2006*).

198 $\ln = \dfrac{Bo}{Bo - Bt} k_h t$ (1)

199 Where B_t is the cumulative methane yield (mL-CH$_4$/g-VS$_{added}$) for time t, and B_0 is the ultimate

200 methane yield of the substrate. The first-order model is usually used to describe the hydrolysis

201 progress and determine the rate of biodegradation. The hydrolysis rate depends on the nature of

202 the substrate and microbial community function. The recovery time is 70% of maximum

203 methane production in the recovery experiment obtained by simulation of the results with the

204 modified Gompertz equation (2). The Gompertz equation was determined the lag-phase before

205 starting of the methane production (*Lay et al., 1997*; *Nopharatana et al., 2007*) as follows in

206 equation (2).

207 $M = P \cdot \exp\left\{ -\exp\left[\dfrac{Rm \cdot e}{P}(\lambda - t) + 1\right]\right\}$ (2)

208   Where M is the cumulative methane production (mL-CH$_4$), P is the methane production

209 potential (mL), R_m is the maximum methane production rate (mL-CH$_4$/day), λ is the duration of

210 the lag phase (d), and t is the duration of the assay. The parameters P, λ, and R_m were estimated

211 by applying the least-squares fit of the above equation to the experimental data set.

212 **Results**

213 **Recovery of AD process imbalanced**

214   This biogas production process failed due to system overload or less efficiency by

215 underload, which results in an inhibition system. Inhibited sludge sample was acidic with a pH of

216 3.9±0.1. High tVFA of 4.8±0.1 g/L was observed with containing butyric acid (2.5±0.1 g/L) and

217 acetic acid (1.8±0.2 g/L) as main products (Table 1). The liquid in the digester still has a high

218 COD content of 13.9±0.2 g/L. The imbalanced AD process was caused by low pH and VFA

219 accumulation. It is possible to recover the imbalanced AD process by adjusting pH to natural or

220 dilute intermediate inhibitors. The biogas production rate and methane yield, pH, lag phase, and

221 recovery time of all strategies compare with self-recovery were summarized in Table 2. The self-

222 recovery has methane yield and methane production rate of 209 mL-CH$_4$/g-VS$_{added}$ and 40.77

223 mL-CH$_4$/day, respectively. The low pH was observed in self-recovery at initial and final pH of

224 5.70 and 5.20, respectively. The imbalanced AD process caused by extreme consumption of

225 alkalinity and high VFA accumulation leading to low pH. Suitable pH for AD should maintain at

226 6.6-6.8. It indicates that the pH was directly affected by microbial activity, leading to low

227 biodegradability and methane production. The low hydrolysis constant (k_h) of 0.005 day^{-1} and

228 prolonging the lag phase of 21.2 days was observed in self-recovery.

229   The dilution strategy with tap water (TW) was lowering of the inhibitor concentration.

230 All dilution rates (10%-50%) have a low methane yield (173.09-190.42 mL-CH$_4$/g-VS$_{added}$) and

231 methane production rate (40.58-62.49 mL-CH$_4$/day) with the two times shorter lag phase (9.9-

232 15.1 days) than self-recovery. The recovery time of TW dilution was 33-42 days. It was evident

233 that all dilution ratios with TW cannot improve the biogas production due to the stronger

234 desorption capability on LCFA or its inhibitor. Since dilution with TW can dilute inhibitors, but

235 this strategy can also dilute the importance of microbe and substrate in the AD system at the

236 same time. The dilution with BE obtained the methane yield (213-281 mL-CH$_4$/g-VS$_{added}$) and

237 methane production rate (53-108 mL-CH$_4$/day) higher than TW at all dilution. However, the

238 methane production rate tends to decrease when the dilution of BE was increased. Besides, the

239 lag phase of the dilution with BE (7.1-9.9 days) was significantly shorter than the dilution with

240 TW (9.9-15.1 days) and self-recovery (21.2 days). It was indicated that the dilution with BE at

241 dilution rate over 10% v/v has a short lag phase, which could directly interact with large amounts

242 of organic acids degraded in inhibited sludge. The inhibition was recovery by providing

243 alkalinity sources for methane-producing bacteria (*Chen et al., 2015*) which the BE was

244 alkalinity 4.9±0.2 g/L as CaCO$_3$ that can see in Table 1.

245   The adjusting pH with NaOH addition at a concentration of 0.10-0.14 %w/v showed

246 significant improvement in methane yield of 217.98.-383.33 mL-CH$_4$/g-VS$_{added}$ and methane

247 production rate 86-112 mL-CH$_4$/day. The hydrolysis constant (k_h) of adjusting pH with NaOH

248 was ranged of 0.006-0.007 day $^{-1}$ for all concentrations, indicating that the biodegradability did

249 not depend on the concentration of NaOH addition. The adjusting pH with NaOH has a lag phase

250 of 8.90-9.50 days and recovery time of 30-35 days that shorter than self- recovery. Lens et al.

251 (*Lens et al., 2003*) reported that alkalinity addition has higher methane productions than non-

252 alkalinity addition reactors. The adjusting pH with NaHCO$_3$ at a concentration of 0.85-1.25

253 %w/v had improved the biogas production. However, increasing the concentration of NaHCO$_3$ to

254 1.45 and 1.50 %w/v resulted in decreasing methane production. The addition of NaHCO$_3$ at

255 1.25%w/v was enhanced the methane yield for 22.06% comparing with self-recovery. The lag

256 phase time of this strategy was more prolonged than another strategy except for dilution with

257 TW, which showed in the range of 12-14 days and relatively long recovery time (45-62 days). It

258 has been reported that the addition of $NaHCO_3$ resulted in a relatively high level of alkalinity and

259 pH in the digester, which gradually causes specific inhibition on the metabolism and growth of

260 methanogens. The adjusting pH with $Ca(OH)_2$ at a concentration of 0.20-0.50 %w/v has the

261 methane yield of 318.96-372.65 mL-CH_4/g-VS_{added} and methane production rate of 103.82-

262 151.41 mL-CH_4/day. The addition of $Ca(OH)_2$ at a concentration of 0.20-0.50 %w/v can improve

263 methane production in the range of 34.47-43.92% compared with self-recovery. The maximum

264 methane yield of 372.65 mL-CH_4/g-VS_{added} was achieved at a concentration of 0.40 %w/v of

265 $Ca(OH)_2$ with enhancing methane yield of 43.92% compared to self-recovery with the methane

266 production rate of 151.41 mL-CH_4/day. The results similar to the previous reported that lime

267 mud ($Ca(OH)_2$) addition at a concentration of 0.6 and 1.0% w/v could maintain an active and

268 stable state of the AD process (*Zhang et al., 2014*). An adjusting pH with oil palm ash can

269 improve the buffer capacity and methane production of the inhibited sludge with methane yield

270 and the methane production rate of 211.43-346.54 mL-CH_4/g-VS_{added} and147.49-226.27 mL-

271 CH_4/day, respectively. All concentrations of oil palm ash can recover the AD process with a

272 shorter lag phase of 7.90-9.20 days and recovery time 32.14-33.19 days than self-recovery and

273 dilution with TW. The maximum methane yield and methane production rate of 347 mL-CH_4/g-

274 VS_{added} and 226 mL-CH_4/day, respectively, were achieved at 8.00 %w/v oil palm ash addition.

275 The addition of 1.18% w/v oil palm ash into POME has a high methane yield of 218.79 mL-

276 CH_4/g-COD $_{removed}$ and adjusts the pH in the suitable range (*Jijai et al., 2017*).

277   The results of the re-inoculation strategy were shown in Table 3. The addition of active

278 methane-producing sludge at 5-50% to inhibited sludge was able to accelerate the recovery

279 process. It was observed that methane production increased with increasing the concentration of

280 active methane-producing sludge. The biodegradable of LCFAs or VFAs in POME could be

281 complete by re-inoculation at a concentration of 40-45% v/v, which has a high methane yield of

282 226.66 – 237.05 mL-CH_4/g-VS_{added} and methane production rate of 80.92-83.45 mL-CH_4/day. In

283 our test found that at 50%, re-inoculation achieved high methane yield (Table 3) due to it can

284 improve the amount of the biomass in the mixed liquid and reduce inhibiting intermediate

285 compound. The previous report also showed that a re-inoculum size of 80% could recover the

286   inhibition of mesophilic anaerobic sludge treating the de-oiled grease trap waste (*Wu et al.,*

287   *2015*).

Economic evaluation

289   The cost of various strategies for recovery imbalanced biogas reactor was investigated.

290   The cost estimation was listed in Table 4. This evaluation of chemical costs in the recovery

291   process was considered, whereas the human resources, transporting, and energy costs were not

292   discussed. The best concentration of NaOH for recovery of the imbalanced biogas reactor was

293   0.14 %w/v corresponding to 1.40 kg/m^3-inhibited sludge with the cost for recovery of 0.41 $/m^3-

294   inhibited sludge. The best concentration of NaHCO$_3$ for recovery was 1.25 %w/v corresponding

295   to 12.5 kg/m^3-inhibited sludge with the cost to restore the system about 2.63 $/m^3-inhibited

296   sludge. Meanwhile, the best concentration of Ca(OH)$_2$ for recovery was 0.4 %w/v corresponding

297   to 4.0 kg/m^3-inhibited sludge with the cost to restore the system about 0.76 $/m^3-inhibited

298   sludge. The best concentration of BE for recovery was 20%v/v corresponding to 60-100 kg/m^3-

299   inhibited sludge and100-500 L/m^3-inhibited sludge with no cost considering in the calculation.

300   The best concentration of oil palm ash for recovery was 8%v/v corresponding to 60-100 kg/m^3-

301   inhibited sludge with no cost considering in the calculation. Because the oil palm ash and BE

302   used in the experiments are by-products from the process of palm oil mill industry and POME

303   biogas reactor. Oil palm ash is by-product obtained by burning of fibers, shells, and empty fruit

304   bunches as fuel in palm oil mill boilers while BE is effluent from POME AD digester with free

305   of charge. Hence, we suggest that the recovering imbalanced AD reactor with alkali substances

306   that existing in a biogas plant, e.g., biogas effluent (BE) and oil palm ash could be economically

307   feasible.

Microbial activity and microbial community

309   The best-recovered system was selected based on the maximum biogas production, short

310   lag time, and high hydrolysis constant. Successful methods were 20%v/v BE, 0.14% w/v NaOH,

311   0.50% w/v Ca(OH)$_2$ and 8.0% w/v ash. SMA test was used for evaluating the performance of the

312   inhibited sludge (*Hussain & Dubey, 2017*). The original inhibited sludge had SMA values of

313   0.40 g CH$_4$-COD/g-VSS/day (Fig. 1). Certainly, the recovery sludge has SMA value higher than

314   the self-recovery strategy. The recovered system by 0.50%w/v Ca(OH)$_2$ and 8.00%w/v oil palm

ash has a high activity of hydrolytic bacteria and acidogenic bacteria with SMA value between
0.69-0.84 g CH_4-COD/g-VSS/day and 0.44-0.60 g CH_4–COD/g-VSS/day, respectively. The
higher microbial diversity encountered in this strategy, indicating that more opportunities for
microorganisms for degrading within the microbial consortium. The heat map of the dynamics of
the bacterial and archaeal communities of self-recovery and recovered systems analyzed by
DGGE was shown in Fig. 2. The bacterial communities were dominated by phyla of
Bacteroidetes and *Firmicutes,* which appeared all strategies during the 5th-20th days (Fig.2A).
The phylum *Firmicutes* are the major bacteria commonly found in the AD process (*Sundberg et
al., 2013)*. The order *Clostridiales* and *Bacilli* were observed as main bacteria in the recovered
system. A major genus of *Clostridiales* order was observed in the recovered system was
Desulfotomaculum sp., *Blautia* sp., and *Clostridium* sp., which were fermentative bacteria and
acetogenic bacteria that could convert soluble organics to VFAs. The *Clostridiales* has high
abundance in the stable AD digester (*Li et al., 2015). Desulfotomaculum* sp. was found in all
strategies while *Blautia* sp., *Clostridium* sp., and *Anaerostipes* sp. were remarkably abundant
during 12-20 days in adjustment pH with 0.50%w/v of $Ca(OH)_2$ and 8.00 %w/v of oil palm ash
addition. During the third week, *Bacilli*, *Lactobacillus helveticus* (98 % similarity,
Supplementary data, Table S1), *Staphylococcus intermedius* (100% similarity), *Kurthia gibsonii*
(99 % similarity), *Lysinibacillus* sp., *Exiguobacterium* sp., and *Bacillus* sp were observed in all
strategies. The strategy of 0.50 %w/v $Ca(OH_2)$ and 8.0 %w/v oil palm ash addition were
predominant by bacteria belonging to the class of *Bacilli* consists of *Kurthia gibsonii*,
Exiguobacterium sp., and *Bacillus* sp. These bacteria can produce fatty acids from monomers
after hydrolysis. Especially*, Exiguobacterium* sp. was a high number in the third week. The
member of *Bacteroides* sp. and *Selenomonas* sp. was a low number in self-recovery but abundant
in the recovered system with 0.14% of NaOH, 0.5% of $Ca(OH)_2$, and 8.0% of oil palm ash
addition. *Bacteroidetes* sp. has been shown in other studies to be the major microbial
components of anaerobic reactors (*Levén et al., 2007*; *Trzcinski et al., 2010)*. The distribution of
the exclusive bacterial in different groups clearly shown that recovery strategy significantly
influences the bacterial community structure and selectively enriches specific acidogenic bacteria
during recovery. Meanwhile, self-recovery was observed as a low member of *Bacilli* and
Clostridium resulting in the lowest SMA activity in the self-recovery (0.11- 0.12 g CH_4–COD/g-
VSS/day). The dilution with 20%v/v BE and adjustment pH with 0.14%w/v NaOH also had

346 relatively low SMA activity. While, the adjustment pH with 0.5%Ca(OH)$_2$ and 8.0% of oil palm

347 ash addition had a high SMA activity and high microbial diversity resulting in higher redundant

348 functions that being able to operate under stable conditions, which results in good AD

349 performances *(Carballa et al., 2015)*. The acetoclastic methanogen (*Methanosaeta concilii*, 99%

350 similarity, Supplementary data, Table S2) was more abundant than hydrogenotrophic

351 methanogens in the recovered system (Fig. 2B). *Methanosaeta* sp. and *Methanosarcina* sp. were

352 predominant in the recovered system with 0.50%w/v Ca(OH)$_2$ and 8.00 %w/v oil palm ash

353 addition. These strategies also have a high SMA activity of 0.761-0.767 g CH$_4$–COD/g-VSS/day

354 that higher than 20%v/v BE and 0.14%w/v NaOH follow by self-recovery (Fig. 1). The

355 dominant of *Methanosarcina* sp. (at 20th day) of adjusting pH by 0.50%w/v Ca(OH)$_2$ and 8.00

356 %w/v oil palm ash addition was most important during the recovery process, which utilization

357 acetate to produce CH$_4$.

Discussion

359   The inhibited AD sludge was caused by the extreme consumption of alkalinity from high

360 VFA accumulation in the AD reactor. It indicates that the pH was directly affected the microbial

361 activity leading to low biodegradability and methane production. The long lag phase was

362 observed self-recovery, indicating the adaptation and initiating bacterial multiplication is

363 required due to the loss of a dynamic balance between acetogens and methanogens. The dilution

364 with water was not significant to accelerate the recovery process, but this strategy could delay

365 the time of inhibition *(Wu et al., 2015)*. The dilution with 20%v/v BE could enhance methane

366 yield of 25.78% comparing with self-recovery with shorter recovery time and high k_h, indicating

367 a high conversion rate of the recovered AD sludge *(Sosnowski et al., 2008)*. The adjusting pH

368 with 0.14 %w/v NaOH addition could adjust the pH of inhibited sludge to 7.30 due to NaOH can

369 capture CO$_2$. The pH recovered AD was relatively stable (6.80-7.50) with enhancing the methane

370 yield of 45.48% comparing with self-recovery. The results of this study agreement with Zhang et

371 al. *(Zhang et al., 2018)*, who reported that the adjustment pH by 0.013% NaOH could delay the

372 time of process failure by enhancing the tolerance of methanogens to the high concentration of

373 VFA via reducing the ratio of un-dissociated. NaOH is one of the most popular alkaline

374 chemicals used in AD due to the potential to buffer the pH (*Gáspár et al., 2007*). The adjusting

375 pH with NaHCO$_3$ at a concentration of 1.25%w/v was enhanced the methane yield. However,

376 increasing the concentration of NaHCO$_3$ at 1.45 and 1.50 %w/v resulted in decreasing methane

377 production. The Na$^+$ concentration at a NaHCO$_3$ addition higher than 1.25 %w/v was started to

378 inhibit the methanogens and resulted in decrease biogas production. The Na$^+$concentration at

379 NaHCO$_3$ of 0.31%w/v were slightly inhibitory to methanogens at mesophilic temperatures has

380 been reported by Chen et al. *(Chen et al., 2008).* The adjusting pH with Ca (OH)$_2$ showed

381 functional buffering capacity for the AD system with a stable pH in the recovered AD reactor.

382 The results agree with Li et al. *(Li et al., 2009),* who indicated that the pH adjustment with Ca

383 (OH)$_2$ was improved methanogenic activity via maintaining a stable near-neutral environment

384 for methanogens. The Ca(OH)$_2$ at concentrations of 0.40 %w/v exhibited remarkable

385 performance as indicated by suitable pH (7.00-7.30), shorted lag phase (9.10 days), and short

386 recovery time of 33.76 days. However, the accumulation of Ca^{2+} maybe leads to the precipitation

387 of calcium salt and accumulation onto reactor walls leading to loss of nutrition and lower buffer

388 ability in the digestion system *(Zhu et al., 2010)*. The adjusting pH with oil palm ash can

389 improve the buffer capacity and methane production of the inhibited sludge. All concentrations

390 of oil palm ash can recover the AD process imbalanced with a shorter time of the lag phase and

391 recovery time. An optimal concentration of 8.00 %w/v of oil palm ash is the best concentration

392 for improving the inhibited sludge with raising the pH, supply trace elements, and enhancing the

393 methane yield of 39.69% comparing with self-recovery. The addition of ash showed functional

394 buffering capacity corresponding with Bunrung et al. *(Bunrung et al., 2011),* who reported that

395 15% (w/v) palm ash addition increasing the pH from 7.5 to 9.1. The pH of ash addition was the

396 same as the effluent of POME anaerobically digestion with a pH range of 8.25-9.14

397 *(Tangchirapat et al., 2009)*. The oil palm ash composed of silicon dioxide (58-65%), calcium

398 oxide (6-7%), and potassium oxide (7-8%) could result in balance or enhance of trace element in

399 the digester. Re-inoculation in our test can decrease the lag phase and can improve biogas

400 production higher than self-recovery. The results in line with previous research *(Salminen &*

401 *Rintala, 2002*; *Cirne et al., 2007*) found that an increase of active methane-producing sludge

402 proportion can reduce the recovery time from 45 to 28 days. The re-inoculation at 30%-50% was

403 applicable range with shorter lag time and increased tolerance of microorganism to low pH, high

404 VFA or LCFA and greater methane yield than the self-recovery. However, the re-inoculation

405 strategies showed methane production and efficiency for recovery less than alkali substance

406 addition due to lacking buffering capacity.

407   The results indicated that the best strategy of 0.50 %w/v $Ca(OH_2)$ and 8.0 %w/v oil palm

408   ash addition were dominated by bacteria belonging to the class of *Bacilli* consists of *Kurthia*

409   *gibsonii*, *Exiguobacterium* sp., and *Bacillus* sp. It has been identified as being involved in biogas

410   production, especially in hydrolysis and acidogenesis stages (*Wirth et al., 2012*). Mainly,

411   *Exiguobacterium* sp. was dominated when the system reached three weeks. This microbe has

412   been confirmed as amylase and protease producing bacterium (*Kumar et al., 2014*) and produces

413   highly effective proteolytic enzymes (*Oh et al., 2018*). The member of *Bacteroides* sp. have been

414   shown as major microbial components of anaerobic reactors and abundant in recovered systems

415   with 0.14% of NaOH, 0.5% of $Ca(OH)_2$, and 8.0% of oil palm ash addition. Addition alkaline in

416   the form of $Ca(OH)_2$ and oil palm ash can enrich *Clostridium*, *Bacillus,* and *Bacteroides*. The

417   acetoclastic methanogens were more abundant than hydrogenotrophic methanogens in the

418   recovered system. The inhibited sludge commonly induced hydrogen production and

419   consequently facilitating the growth of hydrogenotrophic methanogens (*Liu et al., 2016*). The

420   hydrogenotrophic methanogens were decreased in the recovered system. The recovered system

421   by 0.50%w/v $Ca(OH)_2$ and 8.00 %w/v oil palm ash addition enhanced acetoclastic methanogens

422   resulting in the highest methane yield. The dominant *Methanosarcina* sp. was most important in

423   the recovered system, which utilization acetate to produce CH_4. Maintenance of

424   *Methanosarcinales* during the anaerobic process is critical for the stability of performance (*Yang*

425   *et al., 2016*).

426   **Conclusions**

427   The recovery strategies with $Ca(OH)_2$ and oil palm ash addition have a short time for

428   recovery of the AD process imbalanced with a recovery time of 33 days without stop feeding.

429   The dilution strategy with 20% biogas effluent was considered a more economical strategy with

430   a recovery time of 36 days. The pH and dilution have two times shorter recovery time capering

431   to self-recovery time (50 days). The recovered system can increase methane yield by 25-45%

432   more than self-recovery and significantly high kinetics, the SMA activity, and the multiplication

433   of initial microorganism leading to short lag time. The *Clostridiales* sp., *Bacilli* sp., and

434   *Methanosarcina* sp. were dominated in the recovered system. The pH adjustment with $Ca(OH)_2$

435   and dilution with 20% v/v of biogas effluent can recovery the acidified and low pH inhibition of

436   POME feeding commercial biogas plant with economically feasible.

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641

642   **Figure Captions**

643   Figure 1. Specific methanogenic activity (SMA) of inhibited microbial sludge, self

644   recovery sludge and the recovered sludge with various methods.

645

646   Figure 2. Dynamic diversity of bacteria (A) and archaea (B) during recovery by selfrecovery,

647   20%v/v BE 0.14% w/v NaOH, 0.5% w/v Ca(OH)$_2$ and 8.0% w/v ash. The size of

648   the rectangle respect to the dominance of microorganisms is represented from long-size to

649   short-size for strong dominant to low decrease dominant of microorganisms, respectively.

Figure 1

Specific methanogenic activity (SMA) of inhibited microbial sludge, self recovery sludge and the recovered sludge with various methods.

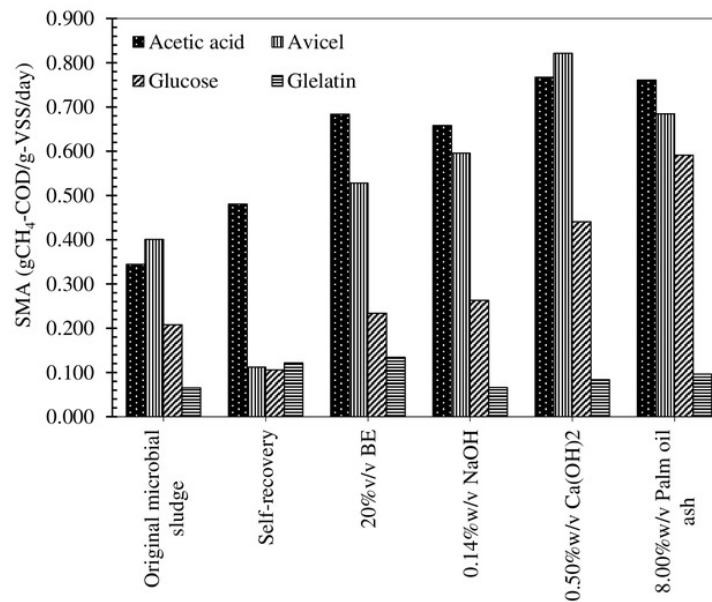
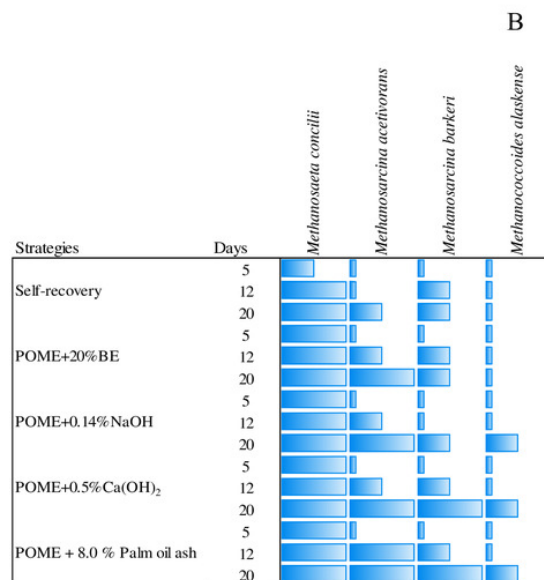
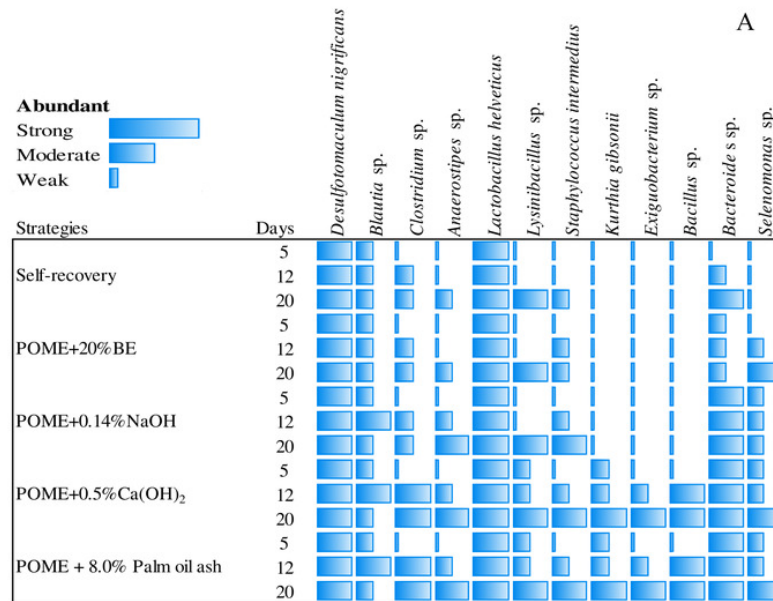



Figure 2

Dynamic diversity of bacteria (A) and archaea (B) during recovery by selfrecovery, 20%v/v BE 0.14% w/v NaOH, 0.5% w/v Ca(OH)$_2$ and 8.0% w/v ash. The size of the rectangle respect to the dominance of microorganisms is represented from long-size to

The characteristics of POME, inoculum and inhibited sludge samples.

1 **Table 1** The characteristics of POME, inoculum and inhibited sludge samples.

Parameter	Unit	POME	Biogas effluent	Active methane producing sludge	Inhibited sludge sample
pH	-	4.1±0.1	7.8±0.1	7.5±0.1	3.9±0.1
Total solids (TS)	g/L	45.4±0.2	13.4±0.3	48.4±0.3	24.0±0.2
Volatile solids (VS)	g/L	36.1±0.3	4.8±0.2	41.5±0.2	7.0±0.1
Suspended solids (SS)	g/L	N.D.	N.D.	59.8±0.1	N.D.
Volatile suspended solids (VSS)	g/L	N.D.	N.D.	37.2±0.3	N.D.
Total nitrogen (TN)	g/L	1.2±0.2	0.2±0.3	2.5±0.4	0.7±0.2
Alkalinity	g/L as $CaCO_3$	2.9±0.2	4.9±0.2	6.1±0.1	2.4±0.2
Lipid	g/L	6.5±0.3	1.1±0.2	4.5±0.1	4.4±0.1
Total chemical oxygen demand (tCOD)	g/L	59.0±0.1	7.5±0.1	N.D.	33.9±0.2
Soluble chemical oxygen demand (sCOD)	g/L	38.2±0.3	N.D.	12.5±0.2	23.9±0.3
Total volatile fatty acids (tVFA)	g/L	1.1±0.1	0.12±0.2	0.92±0.3	4.8±0.1
Acetic acid	g/L	0.4±0.1	N.D.	0.3±0.3	1.8±0.2
Propionic acid	g/L	0.06±0.01	N.D.	0.07±0.3	1.4±0.1
Isobutyric acid	g/L	0.03±0.03	N.D.	0.05±0.3	0.3±0.1
Butyric acid	g/L	0.6±0.1	N.D.	0.5±0.1	2.5±0.1

4 Note: N.D. = Not determined.

Process performance of recovery reactor by by self-recovery, 20%v/v BE 0.14% w/v NaOH, 0.5% w/v Ca(OH)$_2$ and 8.0% w/v ash, the colors respect to the high performance to low performance for recovering inhibited system is represented from dark red

The colors respect to the high performance to low performance for recovering inhibited system is represented from dark red to light red, respectively.

1 **Table 2** Process performance of recovery reactor by by self-recovery, 20%v/v BE 0.14% w/v NaOH, 0.5% w/v Ca(OH)$_2$ and 8.0% w/v ash, the colors respect to the

2 high performance to low performance for recovering inhibited system is represented from dark red to light red, respectively.

Strategies	Initial pH	Final pH	Methane yield (mL-CH$_4$/g-VS$_{added}$)	Methane Production rate (mL-CH$_4$/day)	k_h (d^{-1})	Lag time (day)	Recovery time (day)
Self-recovery	5.70	5.20	209.00	40.77	0.005	21.20	49.42
10%v/v TW	6.20	6.30	190.42	42.72	0.007	15.10	41.72
20%v/v TW	6.30	6.40	186.79	40.58	0.006	12.40	39.23
30%v/v TW	6.30	6.40	178.37	46.02	0.007	14.30	38.11
40%v/v TW	6.50	6.50	173.09	46.43	0.007	10.90	36.53
50%v/v TW	6.50	6.50	176.57	62.49	0.006	9.90	33.21
10%v/v BE	6.70	6.30	213.71	53.41	0.008	12.30	41.99
20%v/v BE	6.80	6.40	281.59	91.38	0.009	7.10	36.36
30%v/v BE	6.90	6.50	253.04	108.04	0.008	7.70	30.77
40%v/v BE	6.50	6.60	229.20	100.42	0.009	7.20	31.23
50 %v/v BE	6.50	6.80	230.10	92.81	0.008	9.90	32.79
0.10%w/v NaOH	6.50	6.60	217.98	85.89	0.007	9.50	30.79
0.11%w/v NaOH	6.60	6.80	237.98	87.25	0.007	9.50	29.97
0.12%w/v NaOH	6.80	6.80	277.68	87.02	0.006	8.90	34.98
0.13%w/v NaOH	7.00	7.20	263.39	85.88	0.006	9.10	34.07
0.14%w/v NaOH	7.30	7.50	383.33	111.65	0.006	9.00	34.96
0.85%w/v NaHCO$_3$	6.70	6.80	222.74	31.80	0.008	12.00	45.75
1.00%w/v NaHCO$_3$	6.80	6.80	224.01	24.22	0.008	12.00	58.83
1.25%w/v NaHCO$_3$	6.90	6.90	268.15	27.22	0.009	12.00	61.57
1.45%w/v NaHCO$_3$	6.90	7.00	167.93	20.82	0.008	14.00	58.45
1.50%w/v NaHCO$_3$	7.00	7.10	158.01	22.01	0.008	14.00	53.35
0.10%w/v Ca(OH)$_2$	6.70	7.10	318.96	103.82	0.008	7.60	36.70
0.20%w/v Ca(OH)$_2$	6.80	7.10	333.26	129.46	0.007	9.00	35.30
0.30%w/v Ca(OH)$_2$	6.80	7.00	367.87	112.01	0.007	8.30	35.33
0.40%w/v Ca(OH)$_2$	7.00	7.30	372.65	151.41	0.008	9.10	33.76
0.50%w/v Ca(OH)$_2$	7.10	7.60	359.87	137.66	0.006	9.40	33.85
6.0%w/v Palm oil ash	6.50	7.00	239.00	155.44	0.007	7.90	33.19
7.0%w/v Palm oil ash	6.90	7.00	264.80	155.22	0.006	8.60	32.14
8.0%w/v Palm oil ash	6.90	7.00	346.54	226.27	0.007	8.20	32.38
9.0%w/v Palm oil ash	6.90	7.00	217.99	147.49	0.006	9.20	33.04
10.0%w/v Palm oil ash	6.90	7.00	211.43	152.15	0.006	9.10	32.48

4   **Notes**.

5   The colors respect to the high performance to low performance for recovering inhibited system is represented from dark red to light red, respectively.

Process performance of recovery reactor by reinoculation strategy

The colors respect to the high performance to low performance for recovering inhibited system is represented from dark red to light red, respectively.

1   **Table 3** Process performance of recovery reactor by reinoculation strategy

Strategies	Initial pH	Final	Methane yield (mL-CH$_4$/g-VS$_{added}$)	Methane production rate(mL-CH$_4$/day) pH	k_h (d^{-1})	Lag time (day)	Recovery time (day)
5% reinoculation	6.40	6.50	211.70	40.42	0.007	15.20	45.75
10% reinoculation	6.40	6.50	216.25	47.22	0.007	15.10	46.11
15% reinoculation	6.40	6.50	214.35	60.76	0.007	15.00	47.21
20% reinoculation	6.40	6.50	221.74	77.04	0.006	15.00	45.23
25% reinoculation	6.40	6.50	222.07	70.65	0.006	15.00	44.21
30% reinoculation	6.40	6.60	223.74	65.94	0.006	13.20	43.56
35% reinoculation	6.40	6.60	222.29	67.87	0.006	12.00	43.92
40% reinoculation	6.40	6.60	226.66	80.92	0.006	12.00	44.00
45% reinoculation	6.40	6.90	229.92	80.12	0.007	11.00	44.45
50% reinoculation	6.40	6.95	237.05	83.45	0.008	11.00	45.75

3   **Notes.**

4   The colors respect to the high performance to low performance for recovering inhibited system is represented from dark red to light red, respectively.

The economic analysis of strategy for recovery the commercial failure biogas.

1 **Table 4** The economic analysis of strategy for recovery the commercial failure biogas.

Chemical or alkali substance	Market price ($/kg or $/L)	Cost of the proposed process ($/m³-reactor)
Tap water (TW)	0.0005	0.02-0.05
Biogas effluent (BE)	-	-
NaOH	0.29	0.29-0.41
$Ca(OH)_2$	0.19	0.19-0.95
$NaHCO_3$	0.21	1.79-3.15
Palm oil ash	-	-

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