Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Isolation and screening of bacteria with biofilm formation ability and characterization with hydrolytic enzyme production for enhanced biogas production

Nurul Alia Fazil, Md Zahangir Alam*, Azlin Suhaida Azmi, Mariatul Fadzillah Mansor, Nurul Hidayah Mohd Zubairi

Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, P.O. Box 10 50728, Kuala Lumpur, Malaysia.
Email: zahangir@iium.edu.my

ABSTRACT

Aims: Biofilm is a complex structure that provides protection towards the bacteria within the barrier. Enhanced biogas production from Palm Oil Mill Effluent (POME) can be achieved by applying biofilm based anaerobic digestion system.
Methodology and results: Bacteria that produces biofilm were isolated and tested on its hydrolytic enzyme secretion. The biofilm produced were also characterized. Out of 120 strains isolated from POME, PKC and food waste compost, only 33 strains were producing biofilm and only 11 of them exhibited significant amount of biofilm produced at optical density of wavelength 595 nm (>0.01). In hydrolysis enzyme assay test, all strains were not able to secrete protease enzyme. The biofilms were extracted and characterized to show similar characteristic for all strains. Strain numbers of 11, 9C, 23C and 30C showed positive result for cellulase, amylase and lipase enzymes, to be tested as single strain bacteria and also mixed with other isolated bacterium for prospect research on effective hydrolysis towards enhanced biogas production. The composition of biofilms from different bacteria mixture also similar under the same incubation condition.
Conclusion, significance and impact of study: Bacteria producing biofilm are very limited and does not secrete the same hydrolytic enzymes. Utilization of these bacteria may eliminate the problem of microbial instability in a system.

Keywords: Biofilm, isolation, hydrolytic enzyme

INTRODUCTION

Malaysia is one of the biggest palm oil producers in the world. Parallel to palm oil production, high organic waste, also known as palm oil mill effluent (POME) is also produced. Due to the high organic content, POME needs to be treated prior to discharge to any water body. On the other hand, due to its high organic content treatment of POME can be applied to produce biogas, a type of renewable energy (Langer *et al*., 2014). This opens the opportunity of turning waste to wealth.

Biogas production using POME as a feedstock has been studied by many researchers. Biogas production can also be increased with better pre-treatment (digestion) (Sapci, 2013; Tedesco *et al*., 2013; Taherdanak and Zilouei, 2014). However, biofilm mediated anaerobic digestion of POME has yet to be explored deeply.

Slow growth and process instability of anaerobic digestion were overcome by the high-rate anaerobic concept based on anaerobic granular sludge and biofilm systems (Chai *et al*., 2014). More efficient degradation of organic substrates can be achieved in biofilm system (Langer *et al*., 2014). Biofilm based system may be able to solve the problem of biomass washout because of the continuous mixing of effluent during anaerobic treatment of POME (Basri *et al*., 2010). However, biofilm structure is very complicated and complex, extreme thoroughness is needed to design the system (Takriff *et al*., 2013).

Other than preventing washout of biomass, biofilm have other advantage such as synthrophism. Synthrophism is “special case of cooperation between two metabolically different types of microorganisms, which depend on each other for degradation of a certain substrate” (Bengelsdorf *et al*., 2015). Moreover, extracellular polymerase substances (EPS) formed after cell attachment to any surface offers protection to the cells within it, and it also helps with mechanical stability and act as the barrier for diffusion of small molecules (Flemming and Wingender, 2010).

A study done by previous researcher have resulted in 28% increase in biogas production using biofilm mediated digestion from mixed culture of anaerobic sludge digester with liquid manure as the feedstock (Szentgyörgyi *et al.*, 2010). This study aims to isolate biofilm producing bacteria from POME and palm kernel cake (PKC) as the source of bacteria that is most related to the nature of POME and food waste compost as additional source of bacteria. The isolated bacteria were studied for their hydrolysis enzyme secretion in order to understand their role in pre-treating POME for future research.

***Corresponding author**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

MATERIALS AND METHODS

Sample collection

Palm oil mill effluent (POME) and palm kernel cake (PKC) sample were collected at Sime Darby Sdn. Bhd. Banting, Selangor. Food waste compost was collected from composter in International Islamic University Malaysia (IIUM).

Microbial isolation

One mL of POME sample was cultivated overnight in 10 mL of Luria Bertani (LB) broth at temperature of 37 °C and agitation speed of 150 rpm. PKC and food compost waste were incubated overnight by adding 0.1 g of the sample into 100 mL LB broth and incubated at temperature of 37 °C with agitation at 150 rpm. The culture was then diluted in LB broth up to 10^{-6} dilution factor and plated on LB agar (10^{-3} to 10^{-6}) and then incubated at temperature 37 °C for duration between 24 to 48 h. Any visible colony was transferred on new fresh plate. Isolates from POME was labelled with numbers, isolates from PKC was labelled with numbers and letter P and isolates from food waste compost was labelled with numbers and letter C.

Biofilm assay test

Method of biofilm assay test was adapted from Djordjevic (2002). Five mL of overnight cultures were prepared for each isolate and incubated at temperature of 37 °C with agitation at speed 150 rpm. The overnight cultures were diluted by adding 0.1 mL of culture into 10 mL LB broth. The diluted culture was then aliquot 100 µL into wells of sterile 96 wells microtiter plate, triplicated for each culture. After 24 h and 48 h of incubation, the growth of bacteria was measured at its optical density at wavelength of 595 nm with 96 wells microtiter plate reader. After that, the plate was washed with sterile distilled water for 5 times, and dried for 45 min. Each well of the plate was then stained with 150 µL of 1% crystal violet solution for 45 min followed by washing them again with sterile distilled water for 5 times consecutively. After drying for 45 min, the wells of the plate were destained with 200 µL of 95% ethanol solution at OD_{595nm}. Positive biofilm producing bacteria was used for hydrolysis enzyme assay test.

Cellulolytic enzyme assay test

Carboxymethylcellulose (CMC) agar was prepared to detect cellulase enzyme. The formula of the CMC agar was adapted from Gohel *et al*., (2014). A 1% of CMC and 2% of bacteriological agar was prepared. The isolated bacteria then streaked on CMC agar plate and incubated for 24 h. After 24 h, the plates were stained with two mL Gram's iodine solution and then washed with distilled water after 5 min of staining.

Lipolytic enzyme assay test

Lipase enzyme was detected using phenol red agar with olive oil as the indicator (0.01% w/v phenol red, 0.1% v/v olive oil, 0.1% w/v calcium chloride, and 2% w/v agar). The bacteria were streaked on agar plate and incubated at 37 °C for 24 h and 48 h (Lee *et al*., 2015). Colour changes were observed.

Amylolytic enzyme assay test

Amylase was detected using starch medium. The formulation of the agar is as follow; 2 g soluble starch, 1 g peptone, 1 g yeast extract, 20 g agar, and 1 L distilled water (Abdel-Raheem and Shearer, 2002). After 24 h to 48 h of incubation, the plates were stained with 2 mL Gram's iodine solution and after 5 min the plates were washed with distilled water.

Proteolytic enzyme assay test

The method of detecting the presence of protease enzyme was adopted from Vijayaraghavan *et. al*, (2013). For 1 L of distilled water, 5 g of peptic digest of animal tissue, 1.5 g of beef extract, 1.5 g of yeast extract, 5 g of calcium chloride, 15 g of agar and 0.0015% w/v of bromocresol green (BCG) reagent were added. BCG dye was prepared by adding 0.56% w/v succinic acid, 0.1% w/v sodium hydroxide, 0.028% w/v BCG dye and 1% Brij-35. The pH then adjusted to pH 4.15 and the solution was stored at 2-8 °C. Protease producing bacteria will be screened on by streaking on the prepared plate and incubated at temperature of 37 °C for 48 h. Any colour changes were recorded.

Biofilm formation assay of screened bacteria mixture

Bacteria mixtures were tested for biofilm assay. Bacteria that are positive with two or more hydrolytic enzymes after screening were randomly mixed and the list is shown in Table 1. The inoculum cell density of each bacterium was adjusted to 0.1 at OD_{595nm}. It was then mixed in equal volume ratio. The procedure of biofilm assay then is the same as described in Djordjevic *et al.*, (2002).

EPS extraction and characterization

The EPS compositions of bacteria mixture with promising amount of biofilm produced ($OD_{595} \geq 0.3$) were studied. EPS extraction was carried out by adapting the protocol from Bales *et al*., (2013). The extracted EPS then studied for its composition of total carbohydrate, total protein and total lipids. The total carbohydrate were analysed using phenol - sulphuric acid method (Dubois *et al.*, 1956). Total protein assay protocol was adapted from modified Lowry's method (Hartree, 1972) and the method to analyse total lipid was adapted from Bligh and Dyer (1959) using liquid – liquid extraction method. The moisture content was studied by drying the biofilm for 24 h at 105 °C.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 1: Codes and its bacteria mixture.

Code	Bacteria Mixture	Code	Bacteria Mixture
A	7, 11	K	7, 9C
B	11, 36	L	19P, 23C
C	11, 39	M	11, 9C
D	11, 3P	N	23C, 30C
E	11, 19P	O	11, 9C, 23C, 30C
F	7C, 9C	P	19P, 23C, 30C
G	9C, 10C	Q	7, 19P, 9C
H	3P, 19P	R	7, 19P, 23C
I	10C, 23C	S	7, 19P, 30C
J	10C, 30C	T	7, 11, 19P

RESULTS AND DISCUSSION

Biofilm assay

A total of 120 isolates were obtained from 60 microbial isolates from POME, and 30 microbial isolates from PKC and another 30 isolates from food waste compost. 33 positive biofilm producing bacteria were obtained after initial screening. After confirmation test, only 11 strains (7, 11, 36, 39, 3P, 19P, 7C, 9C, 10 C, 23C, and 30C) were confirmed for hydrolysis enzyme assay test. These strains exhibited significant amount of biofilm by spectrophotometer reading at optical density (OD) of 595 nm (<0.01). A total of 20 randomly mixed bacteria were done and Figure 1 (a) and (b) shows the bar graph of bacterial growth and the biofilm amount at 24 h and 48 h.

From Figure 1 (a) and (b), there are no conclusive relationship between the growth of bacteria and the amount of biofilm produced by the bacteria. Several patterns of growth and biofilm formation have been identified. For some strains, such as 19P and 10C the biofilms formation increased as the growth increase. For strain 7 and 36, the biofilm formation was increased although the growth decreased. This result is in agreement with previous study that concludes growth rate of bacteria does not correlate with biofilm formation when grown under the same environmental condition (Sapci, 2013). A study done on *Salmonella* strains also found that different strain have different strength of producing biofilm (Agarwal *et al*., 2011). The highest biofilm formed are from strain 10C at 48 h. From the result for strain 11, we can see that as the bacteria grow, the biofilm produced has lessened. However, biofilm produced by strain 19P increased as the growth of the bacteria increased. In contrast with strain 19P, strain 39 has decreased in growth and also decreased in amount of biofilm produced.

On the other hand, at a glance, the productions of biofilm from mixed culture are generally higher than single culture. The stability obtained from the syntrophic activity that can only be achieved with the presence of other bacteria contributes to higher biofilm production (Flemming and Wingender, 2010). Different bacteria have different way of producing biofilm, and the time of producing biofilm differ for each bacterium.

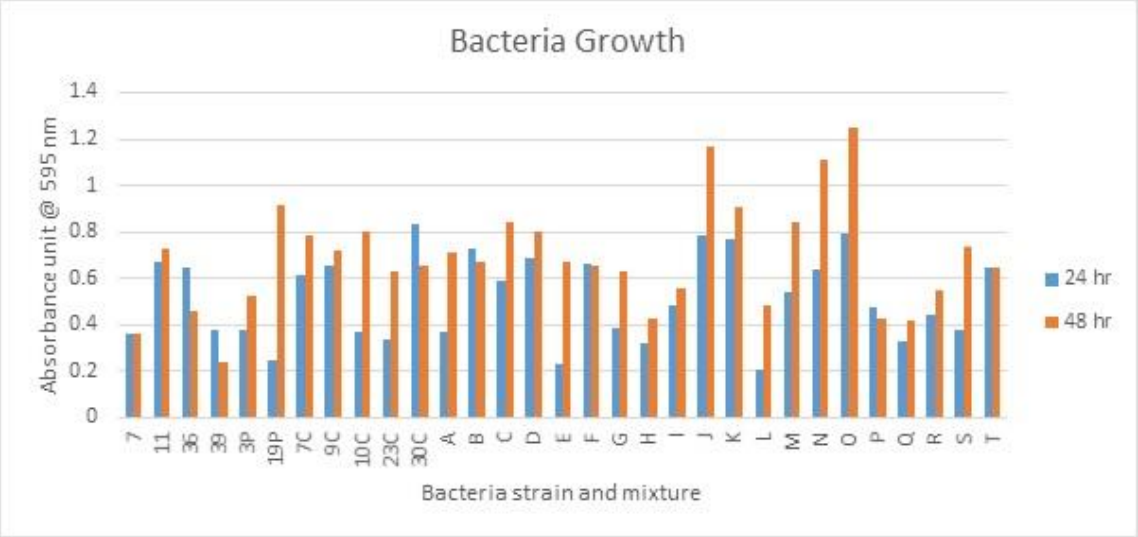

Figure 1(a): Cell density of bacteria producing biofilm from optical density at wavelength of 595 nm.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

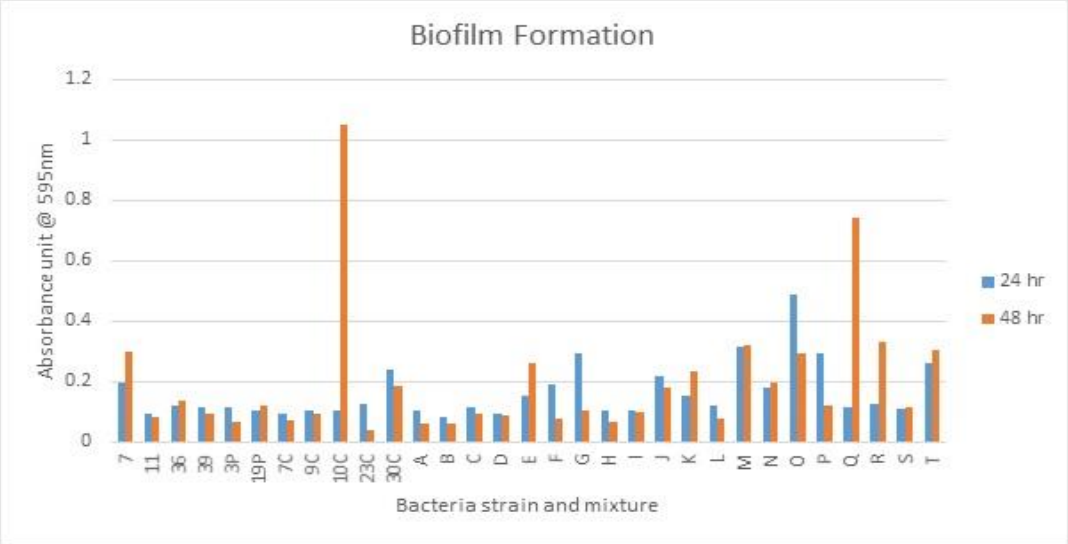

Figure 1(b): Biofilm formation from isolated bacteria and bacteria mixture from optical density at wavelength of 595 nm.

Hydrolytic enzyme screening

Table 2: Summary for the response of hydrolysis enzyme assay test.

Label	Cellulase	Amylase	Protease	Lipase
7	Positive	Positive	Negative	Negative
11	Positive	Positive	Negative	Positive
23	Negative	Negative	Negative	Negative
36	Negative	Positive	Negative	Negative
39	Negative	Positive	Negative	Negative
3P	Negative	Positive	Negative	Negative
19P	Positive	Positive	Negative	Negative
1C	Negative	Negative	Negative	Negative
7C	Negative	Positive	Negative	Negative
9C	Positive	Positive	Negative	Positive
10C	Negative	Positive	Negative	Negative
23C	Positive	Positive	Negative	Positive
30C	Positive	Positive	Negative	Positive

Table 2 shows the result of hydrolysis test for 13 isolated bacteria. For cellulase and amylase assay test, after the plate was stained with Gram's iodine, bacteria with positive enzyme assay will have clear zone around the colony on the plate. The result can be seen as shown in Figure 2. For lipase enzyme assay test, the reddish – orange colour of the agar will change to yellow in the presence of lipase enzyme as shown in Figure 3. Positive proteolytic enzyme assay test will also show clear zone around the colony on the agar when compared to the milky colour of the agar without staining the agar due to the presence of BCG dye in the agar.

All isolated bacteria are negative for protease enzyme assay. This result is good for hydrolysis process, where the absence of protease means that it cannot degrade other enzymes. In a previous research, it was stated that degradation process was slowed down due to the protease breaking down lipase enzyme (Bhumibhamon *et al*., 2002).

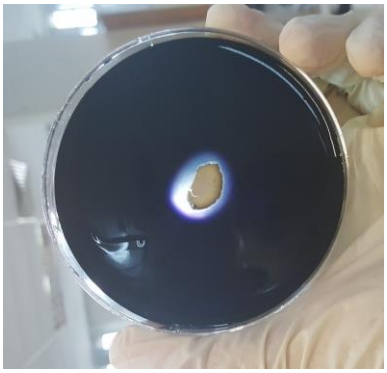

Figure 2: Example for cellulase/amylase enzyme test result after staining with iodine. Clear zone surrounding the colony indicates positive enzyme production after stained.

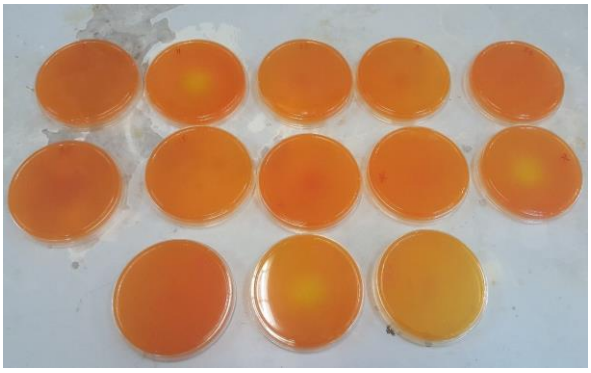

Figure 3: Visual result of lipase enzyme assay test. Positive lipase enzyme secretion will change the plate colour from red to yellow due to difference in pH.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Strain number 11, 9C, 23C and 30C shows positive results for all cellulase, amylase, and lipase enzyme. Since the production of biofilms are better in mixed culture than in single culture, these four strains were present in every random mixture of bacterial strains in order to achieve better biofilm production and to ensure the application of these mixed culture for pre-treatment of POME contains all the enzymes required for hydrolysis.

EPS composition

The composition of biofilm content was summarized in Table 3. The composition of biofilms of mixed culture of isolated bacteria is similar for all extracted EPS under the same growing condition. There are no differences in the content of biofilms produced, the difference may be in the duration of biofilm produced.

Table 3: EPS compositions.

Content	Percentage (%)
Water	74-85
Lipid	8-14
Carbohydrate	6-10
Protein	2-4

CONCLUSION

Biofilm producing bacteria are very limited compared to the total bacteria isolated. The biofilm producing bacteria also does not secrete the same enzymes. The composition of each biofilm extracted was similar of each other. In thorough study is required to understand the biofilm application on pre-treatment for enhanced biogas production of POME.

ACKNOWLEDGEMENT

This research project was supported by a grant from Fundamental Research Grant (FRG) FRGS15-257-0498. The authors are grateful to the Department of Biotechnology, Kulliyyah of Engineering IIUM for providing the laboratory facilities. The authors would also be very grateful for the supply of food waste compost from IIUM Biolizer team and to West Mill Sdn. Bhd., Sime Darby Research Centre for providing POME and PKC.

REFERENCES

Abdel-Raheem, A. and Shearer, C. A. (2002). Extracellular enzyme production by freshwater ascomycetes. *Fungal Diversity* **11, 1-19.**

Agarwal, R. K., Singh, S., Bhilegaonkar, K. N. and Singh, V. P. (2011). Optimization of microtitre plate assay for the testing of biofilm formation ability in different *Salmonella* serotypes. *International Food Research Journal* **18(4), 1493-1498.**

Bales, P. M., Renke, E. M., May, S. L., Shen, Y. and Nelson, D. C. (2013). Purification and characterization of biofilm-associated EPS exopolysaccharides from ESKAPE organisms and other pathogens. *PLoS ONE* **8(6).**

Basri, M. F., Yacob, S., Hassan, M. A., Shirai, Y., Wakisaka, M., Zakaria, M. R. and Phang, L. Y. (2010). Improved biogas production from palm oil mill effluent by a scaled-down anaerobic treatment process. *World Journal of Microbiology and Biotechnology* **26(3), 505-514.**

Bengelsdorf, F. R., Gabris, C., Michel, L., Zak, M. and Kazda, M. (2015). Syntrophic microbial communities on straw as biofilm carrier increase the methane yield of a biowaste-digesting biogas reactor. *AIMS Bioengineering* **2(3), 264-276.**

Bhumibhamon, O., Koprasertsak, A. and Funthong, S. (2002). Biotreatment of high fat and oil wastewater by lipase producing microorganisms. *The Kasetsart Journal (Natural Science)* **36(3), 261-267.**

Bligh, E. J. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37(8), 911-917.**

Chai, S., Guo, J., Chai, Y., Cai, J. and Gao, L. (2014). Anaerobic treatment of winery wastewater in fixed bed reactors. *Desalination and Water Treatment* **52(10-12), 1841-1849.**

Djordjevic, D. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology* **68(6), 2950-2958.**

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28(3), 350-356.**

Flemming, H. C. and Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology* **8(9), 623.**

Gohel, H. R., Contractor, C. N., Ghosh, S. K. and Braganza, V. J. (2014). A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. *International Journal of Current Microbiology and Applied Sciences* **3(5), 261-266.**

Hartree, E. F. (1972). Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry* **48(2), 422-427.**

Langer, S., Schropp, D., Bengelsdorf, F. R., Othman, M. and Kazda, M. (2014). Dynamics of biofilm formation during anaerobic digestion of organic waste. *Anaerobe* **29, 44-51.**

Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C. B., Lakshmipriya, T. and Tang, T. H. (2015). Lipase-secreting *Bacillus* species in an oil-contaminated habitat: Promising strains to alleviate oil pollution. *BioMed Research International* **2015**, **1-9.**

Sapci, Z. (2013). The effect of microwave pretreatment on biogas production from agricultural straws. *Bioresource technology* **128, 487-494.**

Szentgyörgyi, E., Nemestóthy, N. and Bélafi-Bakó, K. (2010). Anaerobic moving bed biofilm fermenter for biogas production. *Environment Protection Engineering* **36(4), 117-125.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Taherdanak, M. and Zilouei, H. (2014). Improving biogas production from wheat plant using alkaline pretreatment. *Fuel* **115, 714-719.**

Takriff, M., Jaafar, N. and Abdullah, S. R. (2013). A review of biofilm treatment systems in treating downstream palm oil mill effluent (POME). *Journal of Applied Sciences* **14, 1334-1338.**

Tedesco, S., Benyounis, K. Y. and Olabi, A. G. (2013). Mechanical pretreatment effects on macroalgae-derived biogas production in co-digestion with sludge in Ireland. *Energy* **61, 27-33.**

Vijayaraghavan, P., Gnana, S. and Vincent, P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Fuel* **4, 628-630.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538