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Fermentation of rice bran hydrolysate to ethanol using *Zymomonas mobilis* biofilm immobilization on DEAE-cellulose

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ABSTRACT

Background: The major challenges associated with the fermentation of lignocellulosic hydrolysates are the reduction in the operating cost and minimizing the complexity of the process. *Zymomonas mobilis* biofilm has been emerged to resolve these complexities. Biofilm has been reported to tolerate to the toxic inhibitors and easily manipulated toward the cell recycle through the cell immobilization.
Results: Z. mobilis ZM4 and TISTR 551 were able to develop biofilms on DEAE cellulose under the differences in the morphologies. *Z. mobilis* ZM4 developed homogeneous biofilm that brought DEAE fiber to be crosslinking, while *Z. mobilis* TISTR 551 developed heterogeneous biofilm in which crosslinking was not observed. Ethanol production under batch and repeated batch fermentation of rice bran hydrolysate containing toxic inhibitors were compared between these two biofilms. TISTR 551 biofilm produced the maximum yield ($Y_{P/S}$) of 0.43 ± 0.09 g ethanol/g glucose (83.89% theoretical yield). However the repeated batch could not be proceeded due to the bacterial detachment. *Z. mobilis* ZM4 biofilm produced the maximum yield ($Y_{P/S}$) of 0.177 ± 0.05 g ethanol/g glucose (34.74% theoretical yield) in the batch culture and the biofilm remained intact to proceed along the repeated batch. The highest ethanol yield ($Y_{P/S}$) in the repeated batch of *Z. mobilis* ZM4 was 0.354 ± 0.07 g ethanol/g glucose (69.51% theoretical yield).
Conclusions: Homogeneous biofilm structure of *Z. mobilis* provided more recycle beneficial over the heterogeneous biofilm structure for the ethanol production from lignocellulosic hydrolysate.

1. Introduction

Lignocellulosic biomass (LCB) is an abundant, renewable source of carbohydrates for microbial conversion to value added chemicals and fuels. One of the future prospects is to use lignocellulosic materials for the production of bioethanol and bring the production toward the industrial scale production [1,2]. However, a challenge for the fermentation of lignocellulosic material is the recalcitrance of biomass to breakdown. Pretreatments with either chemical or physic-chemical lead to the production of fermentable sugars together with other toxic by-products [3]. Dilute acid hydrolysis with high temperature is the most cost effective method that has been extensively performed for the pretreatment of lignocellulosic materials. However, relatively high concentrations of inhibitory compounds are formed during the process including furfural hydroxymethylfurfural (HMF), acetic acid, formic acid, levulinic acids and vanillin [4]. These toxic inhibitors are found to have negative effects over microbial growth, metabolism and ethanol production of many ethanologenic microorganisms [5,6,7]. These problems were previously overcome by removing the inhibitors or utilizing inhibitor tolerant microorganisms however, these required extra equipment and time leading to increased production costs [8,9].

Rice bran is an abundant by-product from rice production which can be served as a low cost attractive feedstock for the production of bioethanol [10,11]. Pure rice bran that was dilute acid pretreated enzyme saccharification has been reported as an effective substrate for ethanol production by *Zymomonas mobilis* biofilm. *Z. mobilis* biofilm has illustrated its potential for ethanol production from rice bran hydrolysate than free cells by representing higher survival, higher metabolic maintenance and higher ethanol yield when it is exposed to the toxic inhibitors [12]. Therefore, using biofilm as a biocatalyst represented its feasibility for ethanol production from lignocellulosic material which could lead to the reduction in the operating costs of bioethanol and minimizing the complexity of the process.

Biofilm represents a natural form of cell immobilization by the microbial adsorption or self immobilization on the solid support which is further applicable as biofilm reactor to produce various value added products [13,14,15,16,17,18,19]. The natural process of biofilm formation can be simply constructed and the process of immobilization is economical. Immobilization of microbial cells in the reactor has been found to eliminate the problem of inhibition caused

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by high concentration of substrate and even the product itself as well as enhancing the productivity and yield [13,20,21]. In this reactor, high cell concentrations are achieved by fixing them on various supports [22,23,24,25,26]. *Z. mobilis* biofilm reactor has been effectively designed for ethanol production using glucose rich substrate that was performed in packed bed bioreactor with plastic composite supports [27]. Biofilm reactor of *Z. mobilis* using starch based substrate has also previously developed to improve the fermentation performance using glass bead as a carrier [28]. None of the work has been done to have *Z. mobilis* biofilm form on biotic carrier for the fermentation of lignocellulosic hydrolysates. Biofilm reactor previously represents higher productivity and stability than the suspension reactor as well as facilitating the downstream process [29]. The natural immobilization or biofilm represents the benefit over the forced immobilization via chemical bonding to a substratum or carrier in that the chemical may result in affecting the cell viability. With the cell retention, the biofilm attachment on the carrier can be continuously used by having cell recycle. This technique has the flexibility to be applied in fed batch, repeated batch and continuous fermentations. Repeated batch fermentation process has been considered as a promising method for the cost effective ethanol production since there is a reduction in the time and cost related to the inoculums preparation and adaptation [30].

The purpose of this study was to describe the use of *Z. mobilis* immobilization in the form of biofilm on DEAE-cellulose to enhance the ethanol production from the rice bran hydrolysate. This was studied on two different strains of *Z. mobilis*, ZM4 and TISTR 551. The biofilm developments of these *Z. mobilis* strains on the DEAE carrier were evaluated during the immobilization and fermentation process. The ethanol yields of these immobilized biofilms were analyzed under batch and repeated batch modes in the small scale flask. This work illustrates the potential of using *Z. mobilis* biofilm attaching on biotic supporter to serve an environmental friendly purpose for the production of ethanol from lignocellulosic material. This work also evaluated its capability to be applied under the repeated batch mode.

2. Materials and methods

2.1. Bacterial strains and cultivations

Z. mobilis strain ZM4 (a type strain) and TISTR 551 from Thailand Institute of Scientific and Technological Research (TISTR) were grown in yeast peptone glucose (YPG) medium (peptone 10 g, yeast extract 10 g and glucose 20 g per liter, pH 6.4). The cultures were grown at 30°C in the incubator for approximately 24 h until the optical density at 600 nm (OD_{600}) reached about 1.0 prior the use for each study.

2.2. Preparation of DEAE-cellulose

DEAE-cellulose (Sigma) was used as a supporter for the biofilm formation. The fine particles were largely removed from DEAE-cellulose by repeatedly suspending the resin in water and discarding the unsettled particles. The remaining particles were then repeatedly washed in 1 N NaOH solution on a Buchner funnel until the filtrate was clear. Following equilibration with 0.25 M HCl solution, the resin particles were again treated with 0.25 M NaOH solution and finally washed with water until they were neutralized. The particles were dried at 60°C. Before using for the bacterial adsorption, the resin was first chemically sterilized by soaking them overnight in 0.5 N HCl solution. The resin was then vacuumed and filtered in a sterilized Buchner funnel under a laminar flow hood and washed with sterile water until the filtrate was neutralized [31].

2.3. Biofilm formation on the resin and biofilm evaluation

The biofilm formation was performed in the presence of suspended treated DEAE particles as above. Overnight culture of *Z. mobilis* ZM4 and TISTR 551 (OD_{600} about 1.0) were inoculated 10% v/v into 30 ml of biofilm medium (contains 20 g glucose, 5 g yeast extract, 5 g $(NH_4)_2SO_4$, 0.6 KH_2PO_4, 0.4 g Na_2HPO_4, $12H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.01 g $CaCl_2$ per liter in 10 fold dilution at pH 6.4) in which the biofilm medium in the flask contained 3 g of pretreated DEAE particles (10% w/v of resin to medium). The cultures were grown at 30°C in the incubator for 3 d with the medium replacement every single day. The biofilm developments of these strains were visualized and photographed using bright-field microscopy and a digital camera (dino-eye model AM423×) on d 3. Three day old biofilms of ZM4 and TISTR 551 were collected for the fermentation process. Resin containing 3 d old adsorbed cells (which consisted of 3 g resin with 4.04 ± 0.405 g dry weight of adsorbed cells) was isolated out from the biofilm medium which was ready to introduce into rice bran hydrolysate. The weight of dry adsorbed cells of ZM4 and TISTR 551 was determined separately on 3 d old biofilms by rinsing out the biofilm medium and gently washing cells twice with distilled water. The wet adsorbed cells on DEAE carriers were dried at 60°C for 24 h and placed in the desiccators until the weights were constant; these were performed triplicate.

2.4. Preparation of rice bran hydrolysate

Rice bran was treated with 0.2 M potassium hydroxide (KOH) for 4 h at room temperature (10% w/v). The material was then filtered through cheese cloth and repeatedly washed with tap water until the pH became neutral. The sample was then dried at 85°C until the weight was constant. The rice bran (15% w/v) was then treated with diluted sulfuric acid (H_2SO_4) (2% v/v) at 121°C for 30 min. After cooling down, the pH was adjusted to 6–6.5 with NaOH. The adjusted mixture was treated with cellulase enzyme (Novozyme, 29,950 U/mL) for 72 h at 55°C (2 mL/100 g of solid matter). The sample was filtered through diatomaceous earth and the filtrate was collected. The rice bran hydrolysate was then concentrated by boiling at 80°C to half of the initial volume. Overliming was done using $Ca(OH)_2$ to adjust the pH to 10.5 at 90°C with frequent stirring. The hydrolysate was again filtered through diatomaceous earth and treated with 3.5% w/v activated charcoal for 1 h at room temperature. A total of 7.5 g yeast extract and 10 g peptone were added per liter of hydrolysate obtained. The pH of the hydrolysate was adjusted to 6.0, sterilized, and then used for fermentation processes [12].

2.5. Batch fermentation and repeated batch fermentation

Resin containing adsorbed cells of *Z. mobilis* ZM4 and TISTR 551 was added into the flask containing 30 mL of rice bran hydrolysate. Rice bran hydrolysate contained approximately 18 g/L glucose, furfural 198.68 ppm, 5-hydroxymethyl furfural (5-HMF) 0.095 ppm, vanillin 0.86 ppm, syringaldehyde 3.19 ppm, and 0.168% acetic acid (pH 6.0). The fermentation was preceded in 30°C incubator. Fermentation broths were collected from the batch processes from day 0 toward day 3 to monitor on the presence of glucose and ethanol. Repeated batch fermentation of ZM4 and TISTR 551 was performed by collecting adsorbed cells from the previous batch fermentation process to repeatedly inoculate into the fresh rice bran hydrolysate. The presence of glucose and ethanol was monitored from d 0 toward d 3 of repeated batch fermentation. The yield ($Y_{P/S}$) and percent theoretical yield were calculated from these parameters. The biofilms of *Z. mobilis* ZM4 and TISTR 551 were also visualized on d 1 and d 3 of the batch process.

2.5. Measurement of fermentation products

Fermentation broths were collected from batch and repeated batch culture of *Z. mobilis* ZM4 and TISTR551 that were cultured in the rice bran hydrolysate. The concentration of glucose was measured using a

glucose liquicolor kit manufactured by Human, Germany. Ethanol produced was analyzed by gas chromatography (GC) (HP Innowax Agilent 6890N) using an Innowax column (29.8 m × 0.25 mm × 0.25 μm) with a flame ionization detector (FID). The column temperature was 150°C, program run time 5.5 min, ethanol retention time about 1.9 min and the carrier gas was nitrogen (16 kPa), injector temperature 175°C, detector temperature 250°C, flow rate 40 mL/min, split ratio 1:50, and velocity of H_2 flow 60 mL/min, with a sample quantity of 1 μL. One part of the supernatant was filtered by 0.22 μm cellulose acetate filters prior to GC analysis. Ethanol standard solutions were prepared at 0.1%, 0.3% and 1% (v/v) using absolute ethanol 95%. $Y_{P/S}$ and % theoretical yield were calculated based on these parameters [12].

3. Result and discussion

Z. mobilis has been effectively used for ethanol productions in various glucose rich medium and some lignocellulosic materials [27,30]. It has been extensively studied for its high ethanol production rate, yield and tolerance to inhibitory compounds found in hydrolysates derived from lignocellulosic biomass [32,33]. *Z. mobilis* is a useful ethanologenic bacterium that can form single species of biofilms [34]. The phenomenon of *Z. mobilis* biofilm formation has shown its potential toward various industrial applications by increased cell tolerance to a toxic substrate and allowed continuous biotransformation for the productions [15]. Biofilm is a natural form of cell immobilization or attachment on either biotic or abiotic surfaces [35]. *Z. mobilis* biofilm could enhance the ethanol production from rice bran hydrolysate which consisted of various toxic inhibitors by having the capabilities to maintain the metabolic activity and survived better than free cell suspension [12].

Z. mobilis is effectively attached to plastic composite supports containing up to 25% of various agricultural materials, hydrophobic treated glass materials and polystyrene surface and produces ethanol from either glucose rich medium or lignocellulosic hydrolysates [27,11,36]. However, self-immobilization of *Z. mobilis* in the form of biofilm on pure biotic material for the conversion of lignocellulosic hydrolysate to ethanol has not yet been reported. The application of biotic carrier in bioprocess could provide benefits over abiotic carrier in terms of environmental friendly and more applicable to be used with other available biotic supporters in the nature such as agricultural materials. This work illustrated the potential of using *Z. mobilis* biofilm form on DEAE-cellulose for an ethanol production using pure rice bran hydrolysate as a substrate. Rice bran hydrolysate consisted of 18 g/L glucose, furfural 198.68 ppm, 5-hydroxymethyl furfural (5-HMF) 0.095 ppm, vanillin 0.86 ppm, and syringaldehyde 3.19 ppm, and 0.168% acetic acid was used as a substrate for an ethanol production in this study.

The biofilms formed on the support material were visually determined by bright-field microscopy method (Fig. 1). *Z. mobilis* ZM4 strain was able to form dense biofilm in which the biofilm is homogeneously distributed on the surface of DEAE-cellulose while strain TISTR 551 developed a heterogeneous biofilm on the DEAE-cellulose. *Z. mobilis* ZM4 bound DEAE fiber together like crosslinking with its homogeneous biofilm structure while TISTR 551 biofilm tended to develop on single fiber instead of crosslinking. The mature biofilms appeared on day 3 when cultured in the biofilm medium in which the bacterial cells were covered with an extracellular polymeric substance or EPS. This was believed to provide protective environment by providing a diffusive barrier to any toxic compounds that could harm the cells and also reduced the uptaking of toxic substances [37,38]. *Z. mobilis* biofilm formation on biotic supporter has never been revealed, thus this work firstly revealed the biofilm development phenomenon of *Z. mobilis* on DEAE-cellulose that was observed under the bright-field microscope. The microscopic pictures could explain that different strains of *Z. mobilis* developed different biofilm patterns on DEAE carrier. Biofilm structure of ZM4 biofilm formed on DEAE-cellulose tended to provide more industrial benefit over TISTR 551 since it was speculated to be not easily going through

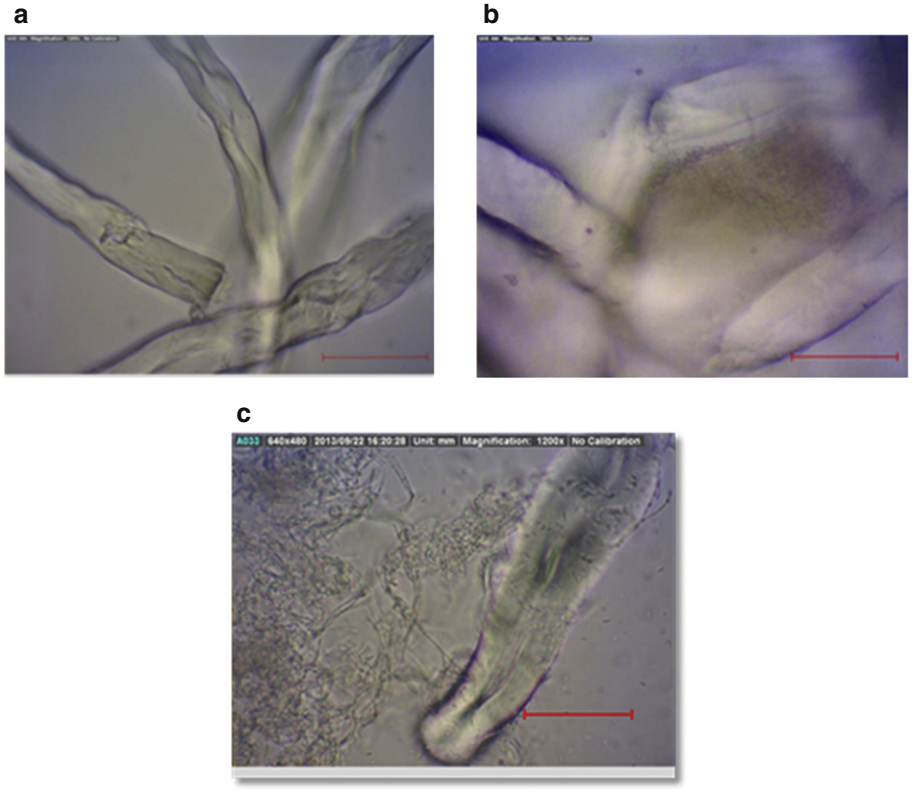

Fig. 1. Bright-field microscopy pictures of *Z. mobilis*, (b) ZM4 biofilm and (c) *Z. mobilis* TISTR 551 biofilm on DEAE cellulose after 3 d cultivation in biofilm medium. (a) DEAE cellulose image was also captured as a control. The bar represents 0.1 mm while the pictures were captured at 1200× using bright-field microscope.

Table 1
Ethanol yield ($Y_{P/S}$, g ethanol/g glucose) and percent theoretical yield from the batch fermentation of rice bran hydrolysate by *Z. mobilis* TISTR 551 from d 0 toward d 3. The values were averaged from three independent experiments with standard deviation ($n = 3$).

Batch		
Day	Ethanol yield ($Y_{P/S}$, g ethanol/g glucose)	% Theoretical yield
0	0	0
1	0.38 ± 0.08	76.14
2	0.43 ± 0.09	83.89
3	0.37 ± 0.15	71.77

Table 2
Ethanol yield ($Y_{P/S}$, g ethanol/g glucose) and percent theoretical yield from the batch and repeated batch fermentation of rice bran hydrolysate by *Z. mobilis* ZM4 from d 0 toward d 3. The values were averaged from three independent experiments with standard deviation ($n = 3$).

Day	Ethanol yield ($Y_{P/S}$, g ethanol/g glucose)	% Theoretical yield
Batch		
0	0	0
1	0.151 ± 0.013	29.70
2	0.114 ± 0.016	22.39
3	0.177 ± 0.05	34.74
Repeated batch		
0	0	0
1	0.354 ± 0.07	69.51
2	0.324 ± 0.059	63.63
3	0.237 ± 0.06	46.43

rounds of attachment and detachment. The biofilms of *Z. mobilis* ZM4 and TISTR 551 strains were formed on DEAE-cellulose with the total weight of approximately 4.04 ± 0.405 g (dry weight of adsorbed cells). Immobilized cells of these strains on the DEAE cellulose supporters were introduced into rice bran hydrolysate for the fermentations under batch and repeated batch modes. Repeated batch is one of the techniques for the cell recycle in the bioprocess. This provides the advantages in terms of reducing in the time and cost associated with inoculum preparation [30,39]. Repeated batch was performed by collecting the adsorbed cells on DEAE-cellulose from the previous batch and reinoculated into a new rice bran hydrolysate. The process efficiencies of batch and repeated batch were evaluated based on the ethanol yield.

Ethanol yield ($Y_{P/S}$) is a measurement of the conversion efficiency of glucose to ethanol. This is defined as ethanol produced divided by glucose consumed. The theoretical yield for ethanol production is 0.51 from glucose as a substrate. Ethanol yield of *Z. mobilis* TISTR 551 was slightly increased toward day 2 and declined on d 3. Day 2 represented the highest percent theoretical yield of 83.89% (Table 1).

The reduction in the ethanol yield of TISTR 551 on d 3 was speculated to be caused by the biofilm detachment that was found on day 3 (Fig. 2b). Therefore, the repeated batch could not be further preceded on TISTR 551 strain. However, the ethanol yield from the fermentation of rice bran hydrolysate by having DEAE-cellulose as a carrier was slightly higher than that previously reported that was carried out on polystyrene surface (48.37 ± 16.64%) and in the circumstance that the free cell suspension produced only 2.046 ± 1.58% [12].

The availability of toxic inhibitors from the rice bran hydrolysate would probably be a problem that led to the biofilm detachment from the carrier when operated under the batch mode since the biofilm represented a long term submerging in the hydrolysate [40]. The bacterial detached from DEAE carrier caused the reduction in the ethanol production. This problem was suggested to be overcome by operating either fed-batch or continuous mode of cultivations.

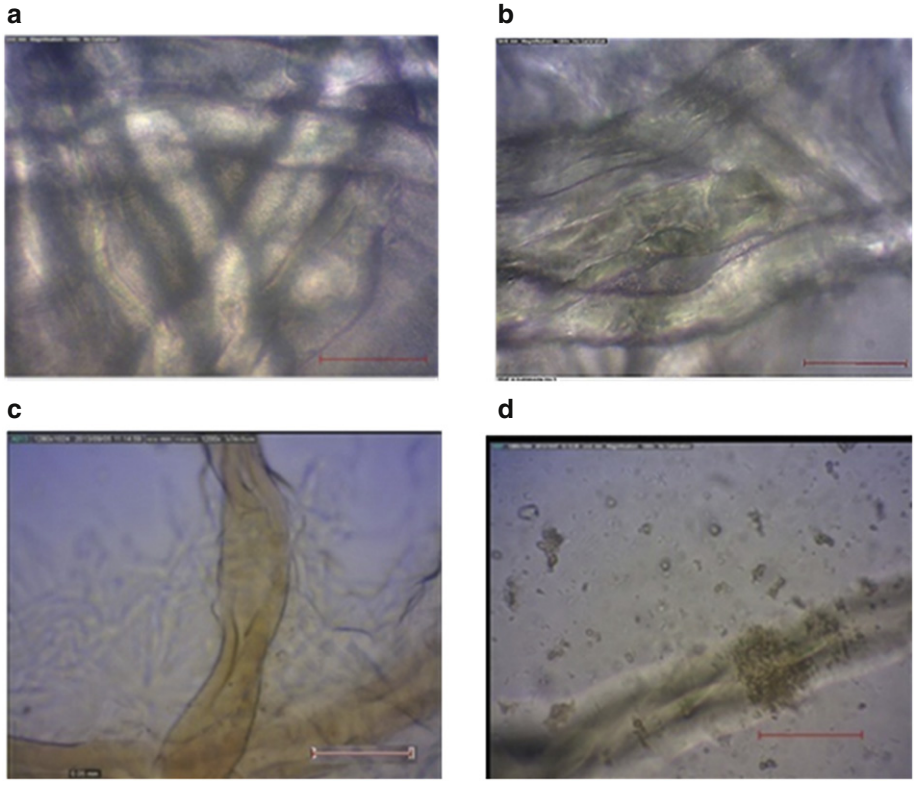

Fig. 2. Biofilms in the rice bran hydrolysate: (a) and (b) *Z. mobilis* biofilms of ZM4 after d 1 and d 3; (c) and (d) TISTR 551 on DEAE cellulose batch fermentation after d 1 and d 3. These pictures were taken under the bright-field microscopy. The bar represents 0.1 mm while the pictures were captured at 1200×. *Z. mobilis* TISTR 551 biofilm detachment was observed on d 3 of fermentation in the hydrolysate.

Continuous and fed batch conversions would be able to keep such inhibitors at lower concentrations than the batch.

Ethanol production from *Z. mobilis* ZM4 biofilm on DEAE-cellulose was less in the first batch in which the maximum theoretical yield was 34.74% (Table 2). However, the biofilm ZM4 remained intact on DEAE cellulose on d 3 of the batch while TISTR 551 appeared to be detached from the carrier (Fig. 2). After 3 d batch fermentation of rice bran hydrolysate, immobilized cells on the DEAE cellulose were collected by rinsing out half of the fermentation broth and replaced with half of the fresh rice bran hydrolysate. The immobilized biofilm of ZM4 could be effectively reintroduced into the fresh rice bran hydrolysate for repeated batch.

The ethanol production in the repeated batch was significantly higher than the first batch with the maximum theoretical yield of 69.51% (Table 2). However, the ethanol yield was declined on d 3. Repeated batch with the cell recycle probably allowed cells to become conditioned and adapted to the fermentation process, hence the ethanol production in the repeated batch was risen up from the first batch [41]. *Z. mobilis* biofilm of ZM4 had shown its potential to be reused in the repeated batch after the adaptation from the previous batch and the biofilm remained intact.

Based on our study, biofilm structure was also speculated to play a significant role to affect the ethanol production from rice bran hydrolysate. Detachment of the *Z. mobilis* biofilm from the supporter could terminate the ethanol production. Therefore, the stabilized biofilm formation could prolong the ethanol production from rice bran hydrolysate containing toxic inhibitors. EPS of *Z. mobilis* TISTR 551 mature biofilm was apparently less than ZM4 mature biofilm that could lead to the reduction in the bacterial tolerant to the toxic inhibitors and sequentially caused the bacterial detachment from the supporter (Fig. 1). Previous studies reported that 50% ethanol yield and 32% ethanol yield were produced as optimal yields when plastic composite (polypropylene 75% and soybean hull 20%) and polypropylene were used as biofilm supports respectively. These were determined when glucose rich medium was used as a fermentation medium under the continuous fermentation [27]. Hence, ethanol production from the biofilm attachment on biotic supporter was comparable to the biofilm attachment on abiotic supporter.

Immobilized cell fermentation by biofilm formation technique represented its potential for the fermentation of rice bran hydrolysate which it can be further applied for other lignocellulosic materials containing toxic inhibitors. This technique can be processed with the cell recycle that is flexible to be applied for fed batch, continuous and repeated batch fermentations with less complexity. However, the detachment of *Z. mobilis* biofilm from the carrier was speculated to be caused by the excessive amount of toxic inhibitors from lignocellulosic materials in combination with the unstable biofilm formation, therefore the suggestion for the improvement is the use of fed batch and continuous mode of cultivation to keep such inhibitors at low concentrations.

4. Conclusion

This study illustrates the potential of using *Z. mobilis* immobilization in the form of biofilm on DEAE cellulose for the production of ethanol from rice bran hydrolysate in which in the future can be applied with other natural biotic carriers together with other lignocellulosic substrates. The efficiency of the repeated batch process directly depended on biofilm immobilization structure. The biofilm detachment caused the significant effect to the process efficiency. With this application, it would lead to a reduction in bioethanol production cost and minimize the complexity of the process in the future.

Conflict of interest

No conflict of interest

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