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Bacillus Licheniformis Coated Bioparticles for Hydrogen Peroxide Degradation

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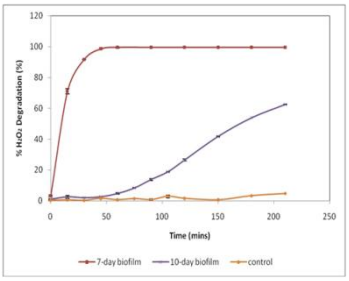
Graphical abstract



Abstract

The potential use of *Bacillus licheniformis* coated bioparticles for hydrogen peroxide (H_2O_2) degradation was assessed in this study. Bioparticles were made by mixing zeolite, activated carbon and cement in ratio 20:5:6 for attachment of biofilm. The efficiency of H_2O_2 degradation was examined in the presence and absence of biofilm (control) on bioparticles. Optimisation of biofilm development (7 and 10 days) and reusability were also investigated for H_2O_2 degradation. Actively growing bacterial suspension (late exponential phase) of *B.licheniformis* was used in development of pure culture biofilm. The 7-day biofilm coated bioparticles system successfully achieved complete H_2O_2 degradation within an hour (highest rate = 1.17 % H_2O_2 degraded per minute) while the control showed no significant H_2O_2 degradation. After repeated use of biofilm coated bioparticles, the rate of H_2O_2 degradation declined to 0.654 % H_2O_2 degraded per minute, and second use, the rate of H_2O_2 degradation was 0.166 % H_2O_2 degraded per minute. Field Emission Scanning Electron Microscope (FESEM) images of the biofilm coated bioparticles showed the attachment of cells and formation of extracellular polymeric substances (EPS), whereas the control showed no biofilm formed.

Keywords: Bacillus licheniformis; biofilm; bioparticles; hydrogen peroxide; degradation

Abstrak

Potensi Bacillus licheniformis biofilem meliputi biopartikel untuk penguraian hidrogen peroxida dikaji. Biopartikel adalah dibuat daripada mineral zeolit, karbon aktif dan simen dalam nisbah 20:5:6 untuk pelekatan biofilem. Kecekapan penguraian H2O2 dikaji dengan kehadiran dan ketiadaan biofilem (kawalan) pada biopartikel. Pengoptimuman umur biofilem (7 dan 10 hari) dan penggunaan semula juga dikaji untuk penguraian H2O2. Bakteria Bacillus licheniformis dari phasa aktif pertumbuhan digunakan dalam penghasilan biofilem kultur bacteria tulen. Biofilem berjangka 7 hari meliputi biopartikel dapat mencapai keseluruhan penguraian H2O2 dalam masa satu jam (kadar tertinggi= 1.17 % H2O2 terurai per minit) manakala tiada penguraian H2O2 yang signifikan pada kawalan. Selepas penggunaan balik biopartikel diliputi biofilem, kadar penguraian H2O2 turun ke 0.654 % H2O2 terurai per minit, dan kali kedua penggunaan semula biopartikel diliputi biofilem, kadar penguraian adalah 0.166 % H2O2 terurai per minit. Gambar Field Emission Scanning Electron Microscope (FESEM) biofilem pada biopartikel menunjukkan perlekatan bakteria dan penghasilan bahan berpolimer ektrasel (EPS) manakala tiada penghasilan biofilem pada kawalan.

Kata kunci: *Bacillus licheniformis*; biofilem; biopartikel; hidrogen peroxide; penguraian

1.0 INTRODUCTION

In textile processing industries, most of the processes involved are water-based process due to the nature of their operations, for example dyeing and bleaching. This leads to high energy and water cost in textile waste processing [15]. For processing 1 kg of fabric requires about 100 L of water [8]. Nowadays, as water availability is getting scarce, wastewater treatment and reusability of the water is a major concern for all.

Different types of wastewater are contributed by textile industries, one of which commonly found is textile bleaching effluent. Bleaching of textiles is conducted at temperatures above 50ºC and under alkaline conditions (pH 9 and above). The concentration of H_2O_2 used or bleaching agent is about 4.4 mM as previously reported by Oluoch *et al*. [12].

Hydrogen peroxide is a type of bleaching agent that is widely used in textile processing [6]. It is more environmentally benign compared to chlorine-based bleaching agent and also less costly. Due to the use of hydrogen peroxide in textile bleaching process,

high volume of water is needed to wash off hydrogen peroxide from the fabric prior to the dyeing process. This is to avoid subsequent problems related to O_2-sensitive dye to arise, such as unevenness in color, insufficient dyeing and shade changes between batches [12].

Other methods to remove H_2O_2 involve chemical treatment using sodium bisulphate or hydrosulphite [12]. However, addition of these chemicals will contribute to high salt concentration in the degradation process.

Thus, the naturally occurring enzyme from microorganism, catalase has been explored for degradation of H_2O_2 in textile bleaching effluent as it has specific affinity towards H_2O_2 and able to degrade H_2O_2 into one molecule oxygen and water. It is one of the most effective enzymes with highest turn-over number [2].

However, free catalase may interact with dyeing process [1] as they are prone to inactivation and unfolding. This leads to unacceptable colour changes on dyed fabrics [16]. Hence, immobilization of catalase or whole cells is essential for efficient removal of hydrogen peroxide and enables the recycling of bleaching effluent.

In industrial processes, numerous carrier materials have been used to immobilize enzyme catalase. The matrixes are gelatine, polyacrylamide and hen egg shell [5], artificial membranes [7], carbon materials [9], alumina pellet [13] and magnetic particles [10]. As for immobilization of cells, Oluoch *et al*. [12] used calcium alginate to be the matrix.

B.licheniformis coated bioparticles is a new and different approach to be used in hydrogen peroxide degradation. With biofilm formation on bioparticles, bacterial cells can be protected and withstand shear forces. The bacteria produce its own extracellular polymeric substances (EPS) to entrap the cells inside the matrix. This method utilizes economical and easily available materials which contribute to more sustainable way for hydrogen peroxide degradation in textile bleaching effluent. These biofilm coated bioparticles are suitable for the application to large volume of bleaching effluent as only minimal maintenance is required. Thus, in this study biofilm coated bioparticles were explored for their use in hydrogen peroxide degradation, where the optimization of the biofilm age and reusability of the biofilm coated bioparticles were tested.

2.0 MATERIALS AND METHODS

The thermoalkaliphilic Bacillus sp. used in this project was isolated from textile bleaching effluents [11] and has been identified via 16S rRNA as *B. licheniformis*.

The culture medium components, yeast extract and peptone were purchased from Scharlau and BactoTM respectively, while D-glucose, sodium carbonate anhydrous and potassium dihydrogen phosphate were purchased from Fischer. In addition, magnesium sulfate heptahydrate was purchased from Sigma-Aldrich. Sodium chloride and 30% hydrogen peroxide were purchased from QReC used for rinsing the biofilm and hydrogen peroxide degradation tests respectively. The components needed for preparing buffers such as glycine and potassium dihydrogen phosphate were purchased from Fischer, dipotassium hydrogen phosphate anhydrous was purchased from QReC. Chemicals for determination of hydrogen peroxide degradation such as horseradish peroxidase were purchase from Sigma and 4-aminoantipyrine from Fluka. All other chemicals used are reagents of analytical grade.

3.0 CULTIVATION OF BACTERIA

The pure culture of *B. licheniformis* was cultivated in Horikoshi broth medium which consisted of D-glucose 10.0 g, Na_2CO_3 5.0 g, peptone 5.0 g, yeast extract 5.0 g, KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g and distilled water 1000 mL at pH 10. Both D-glucose and Na_2CO_3 were autoclaved separately and added to the medium.

Cultivation was carried out in 500 mL conical flask with temperature at 55^0C and agitation at 200 rpm. Growth of bacteria was measured indirectly using spectrophotometric method. The growth was then allowed to proceed until late exponential phase was achieved (OD=5.8-6). The inoculum was then used in development of biofilm.

4.0 METHODOLOGY

4.1 Preparation of Bioparticles

The bioparticles were developed using zeolite, activated carbon and cement in the ratio of 20:5:6. Zeolite 40 g, activated carbon 10 g, cement 12 g was mixed with 12 mL of distilled water. After mixing, the mixture was placed into moulds and allowed to dry for at least 1 day. The bioparticles which had been developed were then immersed in distilled water to strengthen its structure, which was known as curing of bioparticles. The cured bioparticles were kept prior to use.

4.2 Development of Biofilm using Bioparticles

B.licheniformis (OD 5.8-6.0), obtained from late exponential phase, was used to develop biofilm on bioparticles. The bioparticles with suitable ratio of components as described were used as a support matrix for the biofilm development. One litre beaker was used for the biofilm formation on bioparticles. Five cubes of bioparticles (2 cm x 2 cm x 2 cm) were immersed together with 500 mL Horikoshi broth medium, pH 10, inoculated with 10% (v/v) pure bacterial cultures. The bioparticles system was left for a duration of 7 days at room temperature with sufficient aeration to allow for bacterial attachment and biofilm formation. The formation of biofilm was subsequently observed using Field Emission Scanning Electron Microscope (FESEM JSM-6701F) for visualization of the extracellular polymeric substances (EPS) and bacterial cells.

4.3 Optimization of Biofilm Age

Biofilm of *B.licheniformis* was cultivated over a period of time in batch system supplied with sufficient aeration. Development of biofilm for different days possessed different rate of hydrogen peroxide degradation. Optimization of cultivation time for biofilm formation was 7 days and 10 days. Comparison between the catalase activity of biofilm of different ages was done by determining the hydrogen peroxide concentration left in the sample after a certain period of time. Rate of hydrogen peroxide degradation was then determined.

4.4 Hydrogen Peroxide Degradation Using Biofilm Coated Bioparticles

Hydrogen peroxide degradation was studied by using the biofilm coated bioparticles and non-biofilm coated bioparticles for comparison. The rate of H_2O_2 degradation was calculated to compare the efficiency of the system. First, the excess Horikoshi broth for immersing the biofilm coated bioparticles was removed using pipette. Then, the biofilm coated bioparticles were rinsed

once with 0.9 % (w/v) NaCl solution to remove residual Horikoshi broth. After the removal of NaCl solution, 500 mL of 7.5 mM H_2O_2 solution in 50 mM Glycine-NaOH buffer at pH 11 was added slowly into the biofilm coated bioparticles. Non biofilm coated bioparticles were used as control. The samples were withdrawn periodically for at least 1 hour for determination of residual H_2O_2 concentration.

4.5 Determination of Hydrogen Peroxide Concentration

The concentration of H_2O_2 in the sample was determined by using horseradish peroxidase [4]. Previously prepared sample (0.2 mL) was mixed with 0.8 mL of Horseradish reagent consisting of 10 mM phenol, 0.74 mM 4-aminoantipyrine, 0.5 mg mL-1 of horseradish peroxidase in 100 mM potassium phosphate buffer, pH 7.0. After incubation for 15 minutes at 37^{0}C water bath, the absorbance of colored complex was measured at 550 nm by UV-visible spectrophotometer (Shimadzu). Thus, percentage of H_2O_2 degradation was calculated by using formula as shown below:

$$\% H_2O_2 \text{ degradation} = \frac{Abs_{550}initial - Abs_{550}final}{Abs_{550}initial} \times 100\%$$

4.6 Reusability of Biofilm Coated Bioparticles

The reusability of the bioparticles was assessed. First, the previously used biofilm coated bioparticles were rinsed with saline solution, NaCl, 0.9% (w/v). The rinsed biofilm coated bioparticles were then re-incubated in a solution of H_2O_2 (7.5 mM H_2O_2 solution in 50 mM Glycine-NaOH buffer at pH 11). Hydrogen peroxide degradation was determined. This step was repeated until no significant H_2O_2 degradation was observed.

5.0 RESULTS AND DISCUSSION

5.1 Hydrogen Peroxide Degradation

Textile bleaching effluent consists of large volume of alkaline water, which is made up of H_2O_2 with concentration more than 4.4 mM, pH more than 9 and temperatures ranging from 45^{0}C and 60^{0}C [12]. The use of enzyme catalase has been extensively studied for application in treatment of textile bleaching effluent for removal of residual H_2O_2. However, the use of free catalase is not recommended as the enzyme will subsequently interrupt the textile dyeing process, resulting in reduced dye uptake by fabric [16]. Thus, immobilisation is a better option to prevent interference from free enzyme. However, due to involvement of treatment of large volume of wastewater, the system has to be cost effective and would require minimal maintenance to ensure sustainability of the system. Biofilm coated bioparticles involve the use of whole cells where the cells self-attached to surface of bioparticles by secretion of EPS. Without the use of purified enzyme and cheap material used to make bioparticles, the cost of the system is relatively low. The source of enzyme catalase comes from the biofilm. Retainment of enzymes in their physiological system can reduce possibility of enzyme deactivation caused by environmental disturbance. The rate of H_2O_2 degradation was investigated in terms of cultivation days of biofilm and the reusability of the biofilm coated bioparticles.

5.2 Comparison Between 7-Day Biofilm and 10-Day Biofilm Coated Bioparticles

Degradation of H_2O_2 was assessed between biofilm coated bioparticles and non-biofilm coated bioparticles which act as the control of this experiment. Based on Figure 1, degradation of H_2O_2, biofilm coated bioparticles achieved better H_2O_2 degradation, as compared with non-biofilm coated bioparticles. 7-day biofilm coated bioparticles achieved complete degradation of H_2O_2 in an hour (1.17 % H_2O_2 degraded/min), while non-biofilm coated bioparticles showed no significant degradation of H_2O_2 (only 1.54 % H_2O_2 degraded in 4 hours).

Biofilm coated bioparticles consisted of self-immobilized cells adhering to each other onto the bioparticles surfaces entrapped by extracellular polymeric substances (EPS). These whole cells in biofilm produce catalase as catalase is an essential enzyme in response to oxidative stress in aerobic and facultative anaerobic cells metabolism. Thus in 7-day biofilm, there was significant amount of catalase activity with high cell density on the bioparticles that lead to high rate of H_2O_2 degradation, which was 1.17 % H_2O_2 degraded/ min.

Bioparticles without biofilm are just mixture of zeolites, activated carbon and cement that are inert and do not contribute to significant decomposition of H_2O_2 (1.54 % H_2O_2 degraded in 4 hours). The slight degradation of H_2O_2 may be due to the spontaneous chemical decomposition of H_2O_2. Bioparticles only act as a binding material for attachment of the biofilm, so that the *B.licheniformis* biofilm can be formed on the surface of bioparticles. The 3-dimension of bioparticles with the size of 2 cm x 2 cm x 2 cm provides large surface area for attachment of biofilm forming cells.

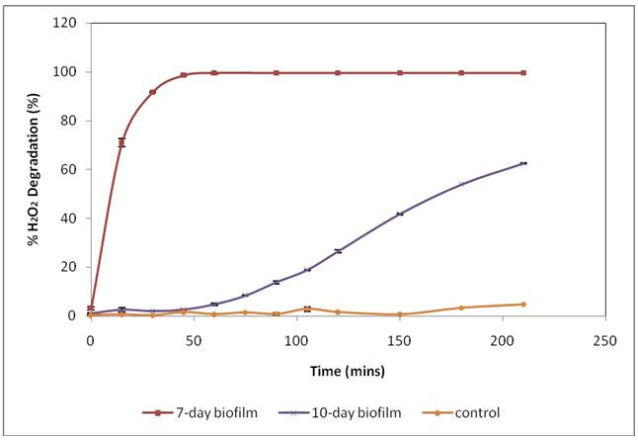


Figure 1 Percentage degradation of 7.5 mM H_2O_2 in 50 mM Glycine-NaOH buffer, pH 11 at room temperature (28^{0}C), using 7-day and 10-day biofilm coated bioparticles

In addition as shown in Figure 1, 7-day biofilm coated bioparticles showed better rate of H_2O_2 degradation (rate = 1.17 % H_2O_2 degraded/min) as compared with 10-day biofilm coated bioparticles (rate = 0.39 % H_2O_2 degraded/min). The total rate in H_2O_2 degradation of 10-day biofilm coated bioparticles reduced by 66.5% as compared with 7-day biofilm coated bioparticles.

Seven-day biofilm coated bioparticles showed a better rate of H_2O_2 degradation. This is due to the buildup of biomass on the bioparticles with high catalase activity. Catalases are produced in aerobic or facultative anaerobic cells to scavenge oxygen radical produced during metabolism of cells.

For 7-day biofilm coated bioparticles, the biofilm metabolism might be more active compared with 10-day biofilm coated bioparticles, as the system was set up as batch system. There was limitation in terms of nutrient availability, toxin accumulation and pH changes in batch system as the medium was not replaced. The longer the system operates, more toxin

accumulates that will cause the detachment of biofilm and loss of catalase activity.

With increase in cell biomass over time within the biofilm, more diffusion limitation may occur [14]. Hence the lack of substrate to be transported to cells within biofilm might contribute to lower rate of H_2O_2 degradation in 10-day biofilm.

5.3 Analysis of Reusability of Biofilm Coated Bioparticles

Reusability of 7-day biofilm coated bioparticles was investigated by calculating the rate of H_2O_2 degradation after reuse. Figure 2 shows the percentage degradation of 7.5 mM H_2O_2 in 50 mM Glycine-NaOH buffer using 7-day biofilm coated bioparticles with first and second reuse. After first reuse of the biofilm coated bioparticles, the rate of degradation was 0.65 % H_2O_2 degraded/min, where total of 86.1 % H_2O_2 degraded in 180 min (3 hours). The rate of H_2O_2 degradation decreased by about 44.1 % as compared with the first used 7-day biofilm coated bioparticles, which rate was 1.17 % H_2O_2 degraded/min.

Second reuse of the biofilm coated bioparticles achieved 0.2 % H_2O_2 degraded/min, where total H_2O_2 degraded in 180 mins (3 hours) was only 26.5 %. Significant reduction in rate of H_2O_2 degradation was noticed from Figure 2, as compared with the first used 7-day biofilm coated bioparticles, the rate of H_2O_2 degradation was reduced by 85.8 % in second reuse.

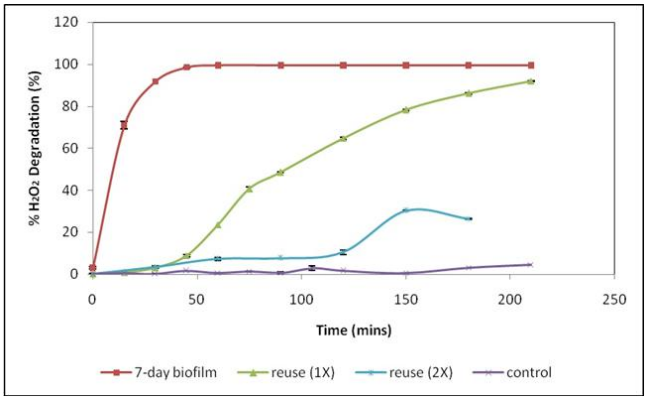


Figure 2 Degradation of H_2O_2 with time using 7-day biofilm coated bioparticles with first and second reuse

From the result obtained, the rate of H_2O_2 degradation decreased with increase usage, which suggests the lack of reusability of biofilm coated bioparticles. This might be due to the detachment of biofilm during washing of the biofilm coated bioparticles and replacement of new H_2O_2 solution to the biofilm coated bioparticles. Environmental perturbation can contribute to biofilm detachment [14].

5.4 Microscopic Analysis of Biofilm on Bioparticles

Attachment of cells and biofilm on bioparticles were confirmed by observing the biofilm coated bioparticles under Field Emission Scanning Electron Microscope (FESEM). Both the bioparticles without biofilm (control) and 7-day biofilm coated bioparticles were crushed to smaller pieces for observation under FESEM.

Bioparticles which acted as the binding matrix of biofilm were added to cultured medium for 7 days for development of biofilm. Biofilms are sessile microbial communities which can anchor to various materials at the interface to an aqueous phase [3]. The bacterial cells would first adhere on to the surface of the bioparticles. Slimy material would be formed by the cells to hold the cells together where the cells are living within the matrix, which is called extracellular polymeric substance.

Figure 3 represents the FESEM micrograph of the surface of bioparticle without biofilm (control), under magnification of 1000X. The bioparticle was made up of zeolite, activated carbon and cement, where porous and uneven surface can be observed. No cells were observed on the surface of the bioparticle.

Figure 4 and 5 represent the FESEM micrograph of the 7-day biofilm attached on the surface of bioparticle, under magnification of 5,000X and 15,000X respectively. Biofilm can be noticed with the presence of cells and slimy matrix of EPS, where the rod-shaped cells were entrapped within the matrix.

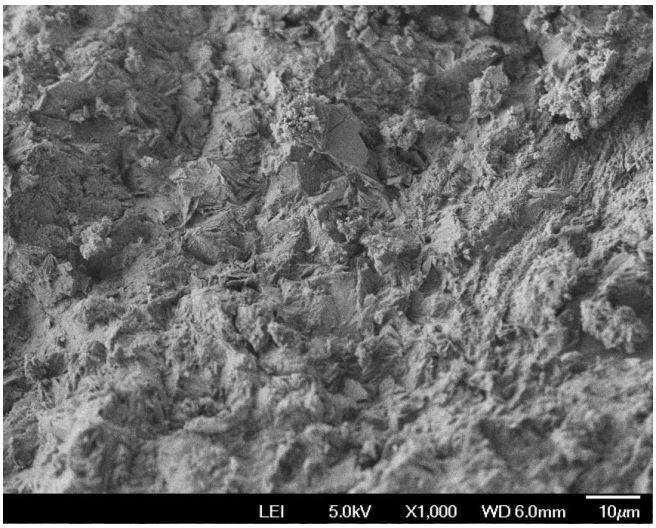


Figure 3 FESEM image of bioparticles without cells (control) under magnification of 1,000X

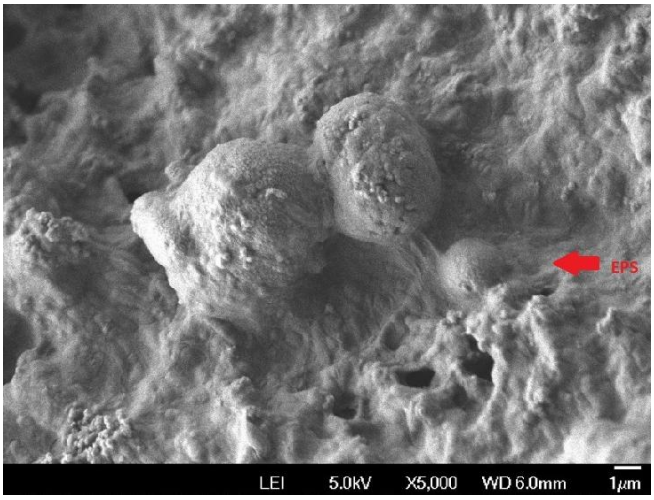


Figure 4 FESEM image of 7-day biofilm coated bioparticle showing EPS formation under magnification of 5,000X

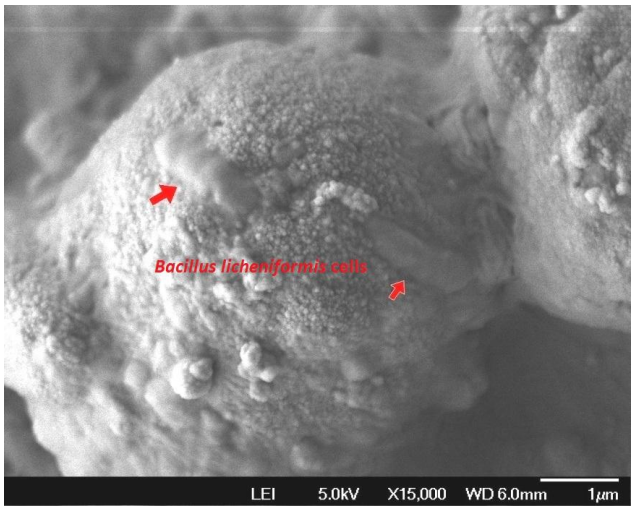


Figure 5 FESEM image of 7-day biofilm coated bioparticle observed under magnification of 15,000X.

6.0 CONCLUSION

In this study, 7-day *B.licheniformis* coated bioparticles showed better rate of H_2O_2 degradation with rate of 1.17 % H_2O_2 degraded/min, where complete degradation of H_2O_2 was achieved within 1 hour. However there was minimal reusability of the biofilm coated bioparticles as with increase usage, the efficiency of H_2O_2 degradation declined. First reuse of biofilm coated bioparticles achieved rate of 0.65 % H_2O_2 degraded/min, while second reuse achieved rate of 0.2 % H_2O_2 degraded/min. Significant decline in the rate of H_2O_2 degraded was noticed. Based on current work achieved in this study, this project can be extended to compare the rate of H_2O_2 degradation for freely suspended cells and biofilm coated bioparticles. The biofilm will be washed out, resuspended and centrifuged to be compared with freely suspended cells for the efficiency of H_2O_2 degradation. In addition, the development of biofilm in this system can be carried out in a column in future. This can minimize the detachment of biofilm during removal of medium and rinsing of biofilm. Other than that, the development of biofilm onto bioparticles can be done in continuous system rather than in batch system, as nutrient depletion and toxic accumulation of by products in culture medium can be prevented.

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