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Thesis for the Degree of Master of Fisheries Science

Enhanced Production of Bioactive Compounds from Shrimp-shell Waste in a Fed-batch Biodegradation

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February 2018

Enhanced Production of Bioactive Compounds from Shrimp-shell Waste in a Fed-batch Biodegradation

유가식 생물학적 분해에서 새우 컵질

폐기물로부터 생리활성 물질의 향상된 생산

Advisor: Prof. Joong Kyun KIM

by

Harun Ar Rashid

A thesis submitted in partial fulfillment of the requirement

For the degree of

Master of Fisheries Science

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Graduate School of Global Fisheries,

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Enhanced Production of Bioactive Compounds from Shrimp-shell Waste in a Fed-batch Biodegradation

A dissertation

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February 23, 2018

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Enhanced Production of Bioactive Compounds from Shrimp-shell Waste in a Fed-batch Biodegradation

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Abstract

Biodegradation of shrimp shell powder using *Bacillus cereus* EW5 strain was conducted for 96 h in a bioreactor by batch, and fed-batch strategies and the production of bioactive compounds were assayed and compared. In batch degradation, the bioreactor was filled with 1575 ml of productive medium containing 1% shrimp shell powder and 175 ml (10%, v/v) inoculum. In fed-batch strategy, culture broth was fed during 14, 42, and 70 h

of degradation periods with pulse addition at a constant rate of 46.80 ml/h. The final working volume was 1.75 L for batch and 3.0 L for fed-batch operation. The cell dry weight, reducing sugar production, antioxidant activity, TLC analysis, and DNA damage inhibition activity was determined. The result of the fed-batch degradation was better compared to the batch system. The highest amount of reducing sugar (0.297±0.05 mg/ml), antioxidant activity (DPPH, 92.35%, ABTS, 98.16%), was achieved during 48 h of degradation in fed-batch mode. The highest reducing power (at A_{700nm} = 1.55 per ml) was recorded during 96 h of degradation in fed-batch mode. Periodic addition of substrate in fed-batch system leads the higher biomass production (34.57 g/L) which was 61.77% higher than the batch (21.37 g/L) biodegradation, and consequently higher reducing sugar, and higher antioxidant activity.

Keywords: Shrimp-shell waste, *Bacillus cereus* EW5, Bioreactor, Bioactive compounds, Fed-batch biodegradation.

Abbreviations

ABTS: 2, 2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid

BF: Batch Fermentation

CDW: Cell Dry Weight

DO: Dissolved Oxygen

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

DW: Distilled Water

FB: Fed-batch

FBB: Fed-batch Biodegradation

FBF: Fed-batch Fermentation

GlcNAc*: N*-Acetyleglucosamine

LPM: Liter Per Minute

OD: Optical Density

SSP: Shrimp Shell Powder

SSW: Shrimp Shell Waste

TLC: Thin Layer Chromatography

UV: Ultra Violet

1. Introduction

Shrimp-shell waste (SSW), generating in huge quantities from the shrimp processing industries throughout the world, is primarily disposed of into the sea, causing intense environmental pollution (Suresh, 2012). This chitinous waste is considered as a valuable renewable resource. To convert these wastes into useful compounds, various studies have been carried out on environmentally friendly reutilization of SSW using microorganisms. Fermentation productions of chitin (Sorokulova et al., 2009), chitinolytic enzymes, mono-, di- and/or oligosaccharides (Halder et al., 2013; Wang et al., 2012) from SSW using several chitin-degrading bacterial strains have been reported. In addition, diverse functional properties of chitosaccharides, for example antitumor activity (Wang et al. 2008c; Liang et al., 2007), antimicrobial activity (Tsai et al., 2000; Wang et al., 2008a, 2008b) and antioxidant activity (Wang et al., 2010; Annamalai et al., 2011; Azam et al., 2014) have also been reported. In particular, *N*-Acetylglucosamine (GlcNAc), an amino sugar, and unit of chitin, have a great prospect for the treatment of several diseases, such as osteoarthritis (Talent and Gracy, 1996), gastritis and inflammatory bowel disease (Chen et al., 2010).

Bacillus cereus is a chitinase- and protease-producing bacterium (Banik et al. 2004; Wang et al., 2009b). Among studies on microbial reclamation of shrimp processing waste, *B. cereus* has been reported as an efficient microorganism for shrimp waste degradation (Sorokulova et al., 2009; Wang et al., 2009b, 2012; Bellaaj et al., 2012b). In previous study, it was also proven that *B. cereus* EW5 could produce chitinolytic (Azam et al., 2014), proteolytic and lipolytic enzymes (Kim et al., 2010). They (Kim et al., 2010, Azam et al., 2014) also recovered bioactive chitosaccharides like chitobiose and GlcNAc from SSW biodegradation using *B. cereus* EW5. However, scale-up production of these chitosaccharides to improve the productivity using competent bacterial strain in a fed-batch biodegradation has not yet reported. Therefore, it is needed to study the enhanced biodegradation of SSW in a bioreactor level to evaluate its potential for commercial application.

Fed-batch is a method of open system fermentation, in which the culture medium is added continuously or in pulses. In fed-batch system, the substrate is periodically added during the fermentation process up to reach the maximum volume (Hadiyanto et al., 2013). It is commonly used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes, and other compounds (Longobardi et al., 1994; Roukas et al., 1998). A number of feeding strategies have been developed for fed-batch fermentation (FBF) including a constant feeding rate, a pulse feeding rate, and an exponentially increasing feeding rate (Salehmin et al., 2013). In specific growth rate control, the feed rate increases exponentially with time so that the specific growth rate is

maintained at some predetermined value (Salehmin et al., 2014). The advantages of FBF than more conventional batch processes includes the substrate reduction, end-product inhibition, higher dissolved oxygen (DO) in the medium, higher biodegradation rate, higher productivity, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentration (Stanbury et al., 1995; Cheng et al., 2009; Abou-taleb, 2015). Therefore, to scale-up SSW reutilization with optimal performance, improved biodegradation technique is necessary. In FBB, the design of the feeding strategy and feed control is of great importance, as both overfeeding and underfeeding of the nutrient affect the cell growth and the formation of desired products (Bretz and Kabasci, 2012; Salehmin et al., 2013). Therefore, a suitable feeding control strategy is crucial for higher biodegradation of SSW and higher production of bioactive compounds. The objectives of the study were to: (a) enhance the production of bioactive compounds optimizing the feeding strategy in bioreactor level and, (b) compare the kinetic parameters and compounds production level between batch and fed-batch biodegradation.

2. Materials and Methods

2.1 Strain and culture medium

A chitin-degrading strain *B. cereus* EW5 (GenBank accession no. DQ923487) was used in this study, previously isolated from the earthworm viscera and was maintained in our laboratory at -70ºC. The strain EW5 was subcultured in nutrient broth, streaked on nutrient agar plates, incubated for 14 h at 47ºC and then maintained at 4ºC for further use. Davaeifar et al. (2015) reported *B. cereus* SDK2 can grow at a wide range of temperatures and pH values. In a previous study, Azam et al. (2014) identified *B. cereus* EW5 as a promising candidate for SSW degradation at 47ºC.

The medium was composed of (w/v): 1% shrimp-shell powder (SSP) as the sole source of carbon and nitrogen (Halder et al., 2013); 0.1%, K_2HPO_4; and 0.05%, $MgSO_4.7H_2O$ (pH 7.0). Frozen white-leg shrimp (*Litopenaeus vannamei*) was purchased from the local market to prepare SSP. The shell parts were first washed with tap water, boiled for 15 min followed by drying in an oven at 120ºC for 12 h. The dried shells were ground up to

powder form and sieved with a particle size of less than 38 μm and stored at 4ºC until use. Rajdeep and Krishna (2012) reported decreasing in particle size of SSW, increased in demineralization rate.

After sonication of SSP for 1 h, it was treated with an aqueous solution of NaOH at pH 12.5±0.1 on a hot plate at 80±5°C with mild stirring for 5 h for deproteinization. The deproteinized SSP was then treated with HCl at pH 4.0±0.1 at room temperature (25ºC) followed by continuous stirring overnight for demineralization and to increase its solubility. Thereafter, the solution of SSP was autoclaved at 121ºC for 30 min and other components of the culture medium were autoclaved for 15 min before mixing them in a clean bench and thus medium was prepared. Then the pH of the culture medium was adjusted to 7±0.05.

2.2 Optimization of culture conditions

A fresh colony of the strain EW5 from nutrient agar plate was used to inoculate 10-ml tube containing 5 ml nutrient broth. The tubes were incubated in a rotary shaking incubator at 47ºC and 170 rpm for 5 h. A 10% (v/v) inoculum from these tubes was then used to inoculate into 10 ml tubes containing 5 ml of SSP broth, and was incubated for 5 h. Then, viable cells from these tubes was subcultured (10%, v/v) in a conical flask containing 100 ml SSP medium under the same environmental conditions until the mid-

exponential growth phase (for about 8 h) and then the viable cells from this culture was used as inoculum for fed-batch culture.

2.3 Batch and fed-batch biodegradation in bioreactor

In this experiment, 3.8 L sized (maximum working volume of 3 L) Winpact Bench-Top Fermenter (Major Science, U.S.A) was used (Fig. 1). The bioreactor system was equipped with three six-bladed adjustable Rushton-type impellers, four peristaltic pumps, polarographic dissolved oxygen (DO) sensor, pH electrode, temperature control, inlet air flow meter, baffle, condenser, and real-time recording and control system within the vessel. The stirred reactor was aerated through an air pump (LP-40A, Young Nam Yasunaga Co., Ltd., Korea). The airflow rate and rotation speed were 2 LPM and 200 rpm, respectively. By adjusting agitation speed and aeration rate, DO level was maintained at 50–70% of saturation during biodegradation. The vessel temperature and the inoculum size were 47±1ºC and 10% (v/v). During biodegradation, pH was not controlled. Antifoam emulsion (1%) was pumped to the culture vessel to prevent foaming. Besides functioning of condenser to condense the culture vapor in the vessel, some evaporative loss was exist due to high culture temperature and airflow, which was compensated pumping the same amount of sterile distilled water (DW). Samples were

taken periodically during batch and fed-batch biodegradation for analysis of cell density, reducing sugar, antioxidant activity, TLC, and DNA damage inhibition.

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Fig. 1. Biodegradation of SSW in a 3.8 L (maximum working volume of 3 L) Winpact Bench-Top Fermenter (Major Science, U.S.A.).

2.3.1 Batch biodegradation

In batch operation, the above specified degradation conditions were applied. The bioreactor vessel was filled with 1575 ml of productive medium. The pH and DO were calibrated through the touch screen controller and all process set points were entered on the control unit. Once the parameters were at their set points, the inoculum bottle was connected to the addition line in aseptic way using silicon tube (Wang et al., 2009a) and 175 ml of inoculum (10%, v/v) was pumped aseptically into the bioreactor vessel using peristaltic pump. Final working volume was 1.75 L in batch degradation. The concentration of biomass and other parameters was analyzed via periodic sampling.

2.3.2 Fed-batch biodegradation

Fed-batch biodegradation started as a batch operation as above specified conditions and shifted to the fed-batch mode. For fed-batch biodegradation, feeding started just after cellular growth reached an exponential phase. An exponential feeding strategy with pulse additions was followed based on the specific growth rate (μ) calculated in batch biodegradation of SSW. In FB operation, the different amount of fresh SSP broth was pumped to the bioreactor vessel. In this feeding strategy, 1080 ml of active SSP broth medium was inoculated with 120 ml inoculum (10%, v/v) and degradation was run under

the same conditions of batch mode for the first 14 h. Two, three, and four pulsed additions were added during the 14, 42, and 70 h of biodegradation periods with a specific amount of 390 ml, 785 ml, and 625 ml, respectively. Feeding was executed through a peristaltic pump at a constant flow rate of 46.80 ml/h. After that, feeding was stopped and the remaining substrate in the culture vessel was allowed to degrade by batch mode (Lang et al., 1997; Shoemaker and Wright, 2003). Total culture period was 96 h with total working volume of 3.0 L at the end of the feeding period.

2.4 Biomass concentrations:

Samples of 10 ml of culture were harvested and centrifuged at 10,000 rpm for 10 min at 4ºC and the pellet was collected to determine the cell dry weight (CDW). The cell pellet was then washed twice with DW, re-centrifuged and after decanting dried at 80ºC until remains the constant weight, cooled at room temperature and then weighed (Chen et al., 2004). Biomass concentration was determined as the average of triplicate. The supernatant was frozen immediately at -20ºC used to determine reducing sugar, antioxidant activity, TLC, and DNA damage inhibition activity.

2.5 Measurement of reducing sugar

A slightly customized method of Imoto and Yagishita (1971) was used to determine the amount of reducing sugar produced in the degraded broth collected from the fermenting vessel using GlcNAc (Sigma-Aldrich Co., St. Louis, MO, USA) as a reference compound. Four milliliters of the color reagent was mixed with 1 ml of the culture supernatant followed by incubation in boiling water in a glass tube for about 8 min. The absorbance of the mixture was measured using (Optizen Spectrophotometer, Mecasys Co. Ltd., Korea) at 420nm (A_{420}) after cooling at room temperature. The decrease in OD at A_{420} was used to measure the reducing sugar creating a standard curve (Fig. 2).

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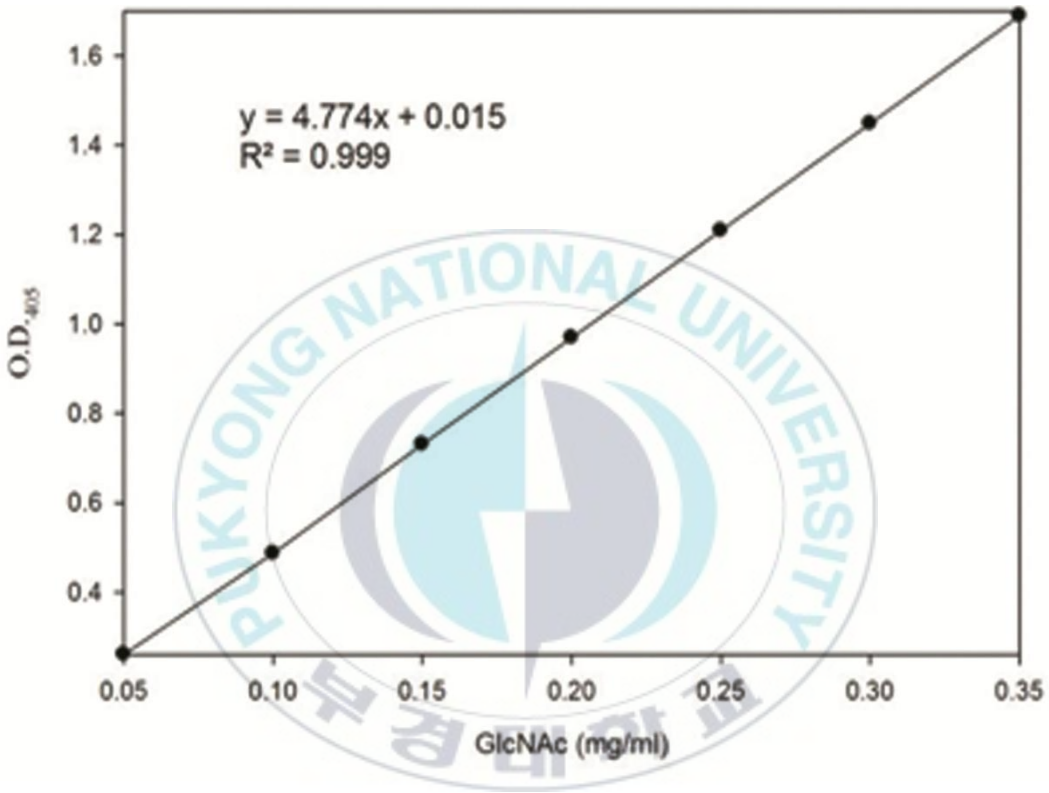

Fig. 2. Standard curve created according to the modified method of Imoto and Yagishita (1971) with GlcNAc as a reference compound.

2.6 Antioxidant activities of biodegraded SSW

2.6.1 DPPH radical scavenging assay

To determine the DPPH *(2, 2-diphenyl-1-picrylhydrazyl)* free radical scavenging activity of the degraded SSW by EW5, Blois (1958) method was applied with some modifications. Two milliliters of 0.1 mM DPPH solution (in 80% ethanol) was added to 1 ml of the culture supernatant. The mixture was placed at room temperature (25ºC) in the dark for 30 min followed by measuring the absorbance at 517nm (Optizen Spectrophotometer, Mecasys Co. Ltd., Korea). The sample blank was prepared by replacing DPPH with 80% ethanol. DPPH radical scavenging activity was calculated as follows:

$$Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ Control} \times 100$$

The control sample was the mixture of 1 ml of 80% ethanol with 2 ml of 0.1 mM DPPH. For positive control, L-Ascorbic acid (0.1 mM) was used under the same conditions. The assay was done in triplicate.

2.6.2 ABTS radical cation decolorization assay

For ABTS radical cation decolorization assay, a slightly modified method of Re et al. (1999) was applied. To prepare the ABTS radical cation (ABTS reagent) 5 ml of 7 mM ABTS (2, 2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) was mixed with 5 ml of 4.9 mM Potassium persulfate ($K_2S_2O_8$) in DW. The mixture was positioned in a dark place at room temperature (25ºC) for 16 h. The absorbance of ABTS reagent at 734nm was then adjusted to 0.720±0.02 with 80% ethanol. Finally, 1.80 ml 0f ABTS reagents was added to 200 μl of culture supernatant followed by measuring the absorbance at 734nm. For positive control, L-Ascorbic acid (0.3 mM) was used. The percentage of inhibition was calculated as follows:

$$Inhibition\,(\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ Control} \times 100$$

For the control sample, DW was used instead of culture supernatant and the sample blank was prepared with 80% ethanol by replacing the ABTS reagent. The assay was done triplicate.

2.6.3 Reducing power assay

A slightly modified method of Wu et al. (2010) was used for reducing power assay. One milliliter of the culture supernatant was mixed with 1.0 ml of 0.2 M phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide followed by incubation at 50ºC for 20 min. After that, the reaction was stunned adding 1.0 ml of 10% (w/v) trichloroacetic acid. The reaction mixture was then centrifuged at 3,000 rpm for 10 min. Two ml of the centrifuged solution was taken from the upper layer, and mixed with 2 ml of DW and 0.4 ml of 0.1% $FeCl_3$ followed by incubation for 10 min at room temperature (25ºC). The absorbance of the solutions was measured at 700nm exactly after 10 min Higher the absorbance indicated higher the reducing power. The control sample was prepared by replacing the supernatant with DW. The assay was conducted in thrice.

2.7 Thin Layer Chromatography (TLC)

For detection of compounds, the collected culture supernatant from the bioreactor was filtered using 0.22 μm membrane filter and concentrated to 1/5 of the original

volume using a vacuum freeze drier (Samwon Freezing Engineering Co., Korea). The samples were then analyzed by thin layer chromatography (TLC). Culture supernatants were applied 10 times (1 μl each) onto TLC Silica Gel 60 plate (Sigma-Aldrich, Germany) and chromatographed in a mobile phase having 5:4:2:1 (v/v/v/v) ratio of n-butanol: methanol: 28% aqueous ammonia solution: water (Songsiriritthigul et al., 2010). The staining of the products were done using a mixture of acetone (4 ml), diphenylamine (80 mg), aniline (80 μl), and 85% orthophosphoric acid (600 μl) (Brunel et al., 2013) followed by baking at 115±2°C for 15 min. As a marker, mixture of GlcNAc and Chitobiose (N, N´-Diacetylchitobiose) solution (0.2%) was also run along with them.

2.8 Determination of DNA damage inhibition

The protective effect of the biodegraded SSW culture supernatant against hydroxyl radical induced oxidative DNA was performed according to the method described by Lee at al. (2002) with some modifications. For this purpose, 2 and 4 μl amount of 48 hour's culture supernatant both from batch and fed-batch degradation was exposed to 2 μl λ DNA (Takara, Cat No.# 3010; 400 μg; 300ng/μl) with 1/4th concentration of freshly prepared Fenton's reagents (20 mM FeCL3, 12.5 mM ascorbic acid and 7.5 mM

hydrogen peroxide). In each case, four different amounts (2.5 μl, 5 μl, 7.5 μl, and 10 μl) of Fenton's reagents were used. Positive control (standard) sample was prepared by mixing DW with 2-μl λ DNA in absence of Fenton's reagents and culture supernatant. Negative control was the mixture of to 2-μl λ DNA, 2 μl Fenton's reagents, and 16 μl DW without culture supernatant. Final volume of each mixture was kept at 20 μl. The mixture was then incubated for 30 min at 37ºC and the DNA was analyzed on 1.5% agarose gel followed by ethidium bromide staining and visualized under UV-transilluminator using Gel Documentation system (Vilber Loumat, France).

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3. Results and discussion

3.1 Batch biodegradation

For optimization of the FBB, batch biodegradation was conducted to study the trend of cell growth, production of reduced sugar and antioxidant compounds. The airflow rate and rotation speed were 2 LPM and 200 rpm, respectively. DO level was maintained at 50–70% of saturation by adjusting agitation speed and aeration rate during biodegradation. The culture temperature and the inoculum size were 47±1ºC and 10% (v/v), respectively. During biodegradation, pH was not controlled. Antifoam emulsion (1%) was pumped to the culture vessel to prevent foaming. The final working volume was 1.75 L for batch biodegradation.

3.1.1 Biomass concentration (g/L)

A typical batch growth pattern presented in Fig. 3 (A) and illustrated that the lag phase last for 0-12 h, exponential phase 12-60 h, declaration phase 60–72 h and stationery phase 72-96 h. In the first hours of degradation period, the strain adapted to grow with physico-chemical parameters (Cheng et al., 2009). After 72 h the growth rate was slowing down due to the shortage of the substrate (Hadiyanto et al., 2013). The CDW reached its maximum value of 21.37 g/L at 96 h of degradation. Zhong et al. (2014) reported 20.37±0.1 g/L of CDW concentrations in batch fermentation in a 5 L fermentor at 18 h of degradation by *Bacillus subtilis* for jiean-peptide production. Fig. 3 (A) signified that, optimum-shifting time from batch to fed-batch degradation was between 12 h to 20 h. Based on this finding, feeding was started at 14^{th} h of degradation for fed-batch.

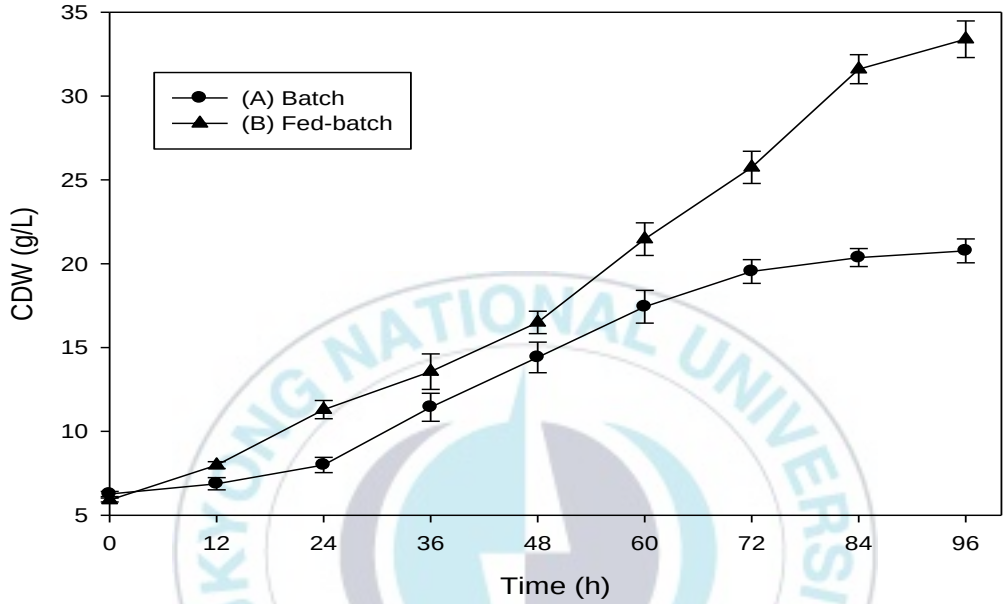

Fig. 3. Growth kinetics of *Bacillus cereus* EW5 with time in batch and fed-batch biodegradation in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.1.2 pH

In batch operation, pH showed first a declining trend (pH, 6.43) and thereafter, it started increasing (pH, 7.09) for the next 12 h of degradation (Fig. 4 A). During fermentation, deproteinization of shrimp waste occurred due to the activity of proteases and demineralization by the acid produced by the microorganisms (Rao et al., 2000). Wang et al. (2009b); Rajdeep and Krisna (2012) report similar findings. This increased possibly due to accumulation of chitosaccharides, which contains an amino group (Halder et al., 2013). After that, the pH value of the broth decreased gradually and reached at the lowest value of 4.57 during 96 h of degradation due to the released of some acidic metabolites accumulated in the broth (Chen et al., 2011). This result was supported by Bellaaj et al. (2012a); they reported that the pH dropped from 7.0 to 4.4 over 7 days of fermentation of SSW using *Pseudomonas aeruginosa*. They also concluded that the demineralization rate was the maximum (92%) when the pH of the culture reached at 4.4. Chen et al. (2011) found that the pH value of the culture broth was decreasing gradually from 7.4 to 5.3 in 14 h in a 5 L fermentor from chitin by chitin degrading factors in *Chitinbacter tainanesis*.

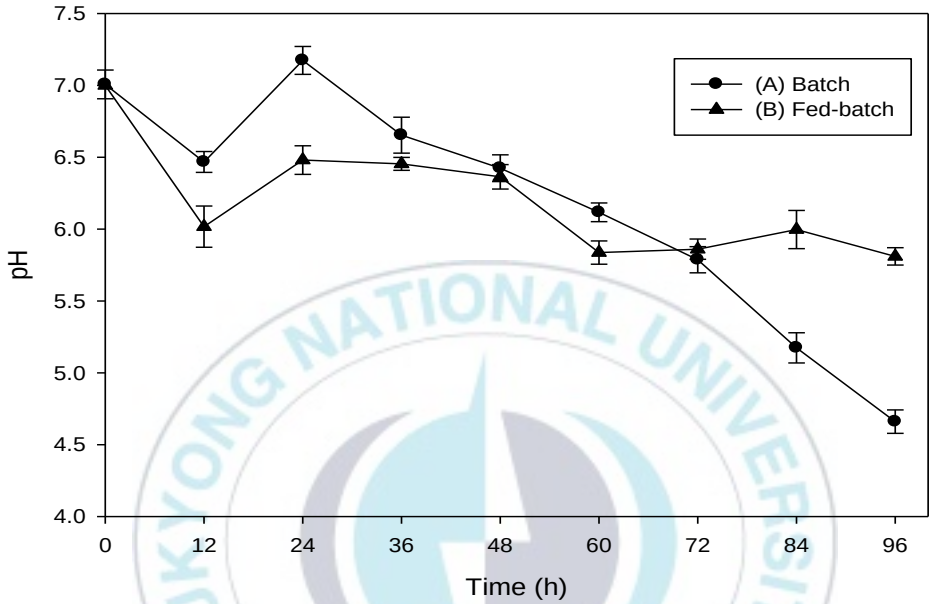

Fig. 4. Changes in pH with time in batch and fed-batch biodegradation of SSW by *Bacillus cereus* EW5 in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.1.3 Production of reducing sugar

In batch feeding strategy, the concentration of reducing sugar showed to increase up to 60 h and reached at the maximum amount of 0.265±0.04 mg/ml followed by 0.252±0.03 mg/ml at 48 h (Fig. 5 A; 6 A). After that, it decreased gradually corresponding to steady increased in the cell growth. This might be due to the consumption of some sugar by the strain for carbon source limitation during the last part of the degradation and cells possibly not used the products much for their energy maintenance. In a previous study, Azam et al. (2014) reported 24 mg of reducing sugar production per gram of dry SSW after 4 days of incubation in a shake flask level. Halder et al. (2013) reported the production of 5.5 mg/g chitosaccharides from SSW fermented by *Aeromonas hydrophila* SBK1 in 5 L fermentor. There was a positive correlation between reducing sugar production and cell growth (Vos et al., 2015). The data clearly illustrated that batch culture in bioreactor level enhances the product formation and simultaneously reduced the degradation time.

Fig. 5. Determination of reducing sugar of culture supernatant collected during biodegradation of SSW by *Bacillus cereus* EW5 in batch and fed-batch operation in a 3 L fermentor. "A" batch; "B" fed-batch. Tubes 1-8: 12 h intervals culture supernatant, Tube 9: Control sample, and Tube 10: Blank sample.

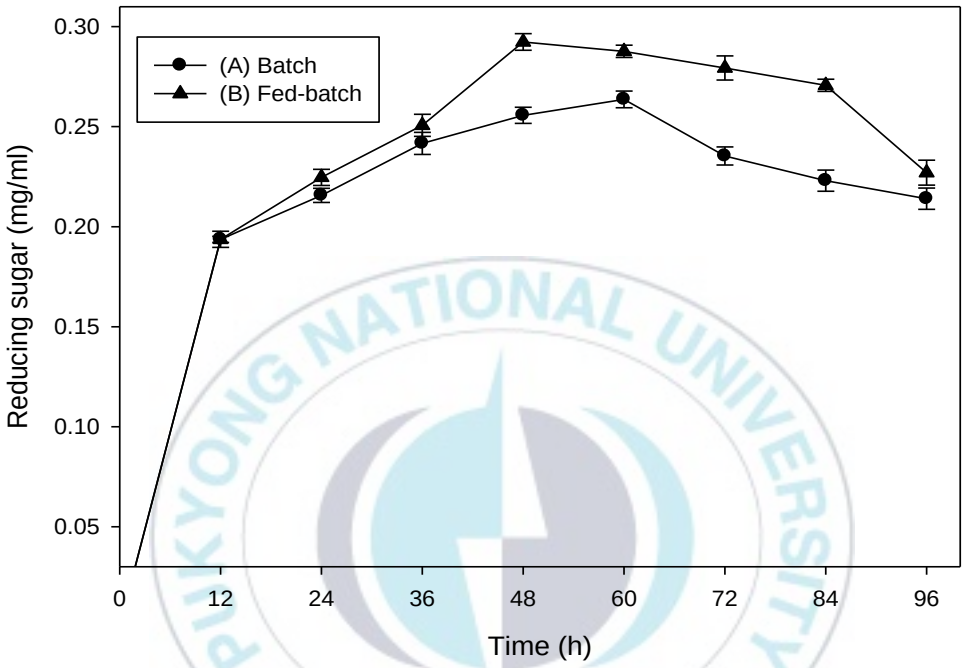

Fig. 6. Production of reducing sugar with time in batch and fed-batch biodegradation of SSW by *Bacillus cereus* EW5 in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.1.4 Antioxidant activity

3.1.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH radical is applied to measure the free-radical scavenging capacity of antioxidants and has been used for more specifically lipophilic antioxidants (Prior et al., 2005). The absorbance is reduced by scavenging the free radicals, when DPPH radicals encounter a proton-donating substance (Bersuder et al., 1998). Fig. 7 (A) and 8 (A), shows the DPPH radical scavenging activity of different time courses. DPPH activity was increasing with degradation time and showed up to 89.33% during 60 h of degradation. After that, decreased gradually and reached to 66.57%, at the last part of the degradation. This activity was comparable to the positive control of 0.1 mM L-Ascorbic acid (81.35%). Wang et al. (2009b) reported 56% DPPH activity of SSP, fermented by *B. cereus* species. Halder et al. (2013) reported 82.5±2.3% scavenging activity after 64 h of biodegradation from SSW by *Aeromonas hydrophila* SBK1 in 5 L fermentor level. Lira et al. (2017) reported 64.86±3.22 and 79.841±4.52% $ABTS^{+}$ activity for cooked and raw shells of shrimp (*Litopenaeus schmitti*) respectively. Therefore, the result of the current study was higher compare to the results of these studies.

Fig. 7. Determination of DPPH free radical scavenging activity of the culture supernatant collected during biodegradation of SSW by *Bacillus cereus* EW5 in batch and fed-batch operation in a 3 L fermentor. "A" batch; "B" fed-batch. Cuvettes 1-8: 12 h intervals culture supernatant, Cuvette 9: control sample, and Cuvette 10: blank sample.

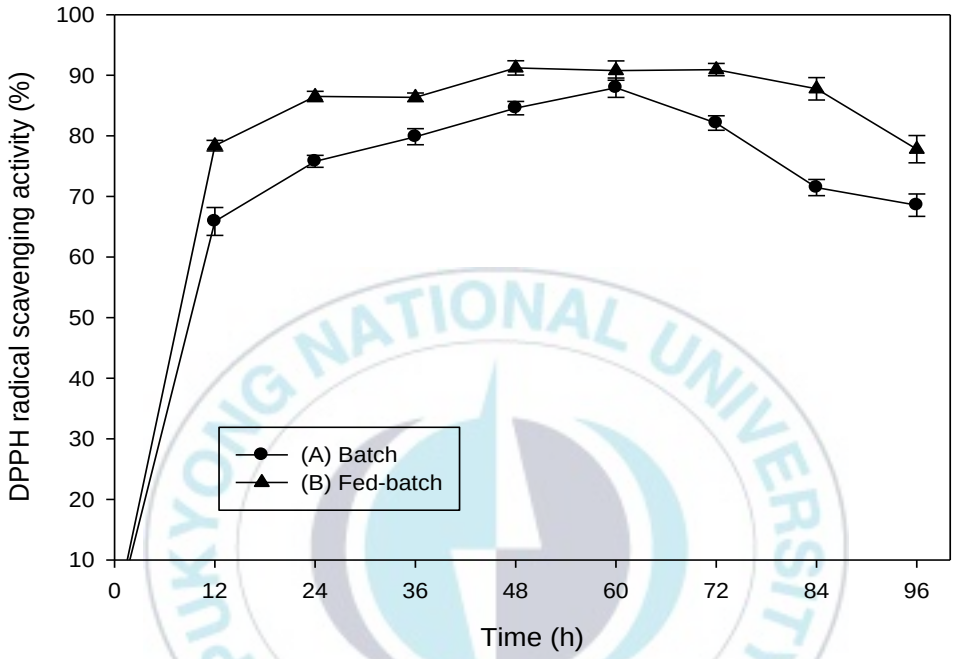

Fig. 8. DPPH free radical scavenging activity of the supernatant with time in batch and fed-batch biodegradation of SSW by *Bacillus cereus* EW5 in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.1.4.2 ABTS radical cation decolorization assay

The ABTS radical cation decolorization assay is often used to evaluate the antioxidant activity of both lipophilic and hydrophilic antioxidant (Prior et al., 2005). The ABTS radical cation scavenging activity of the culture supernatant ranged from 71.42 and 93.33% during 96 h of biodegradation (Fig. 9 A; 10 A). The activity was compared with 0.3mM L-Ascorbic acid, which was used as a positive control displayed 73.2% scavenging activity. Similar findings (94.81% ABTS radical cation decolorization activity) reported by Sachindra and Bhaskar (2008), from fermented SSW. Walke et al. (2014) reported 24% ABTS scavenging activity from shrimp shell sample compared with 0.05 mg/ml Butylated Hydroxyanisole (BHA) as a positive control. Sujeetha et al. (2015) reported 41% ABTS scavenging activity from mud crab, *Scylla serrata* extracts.

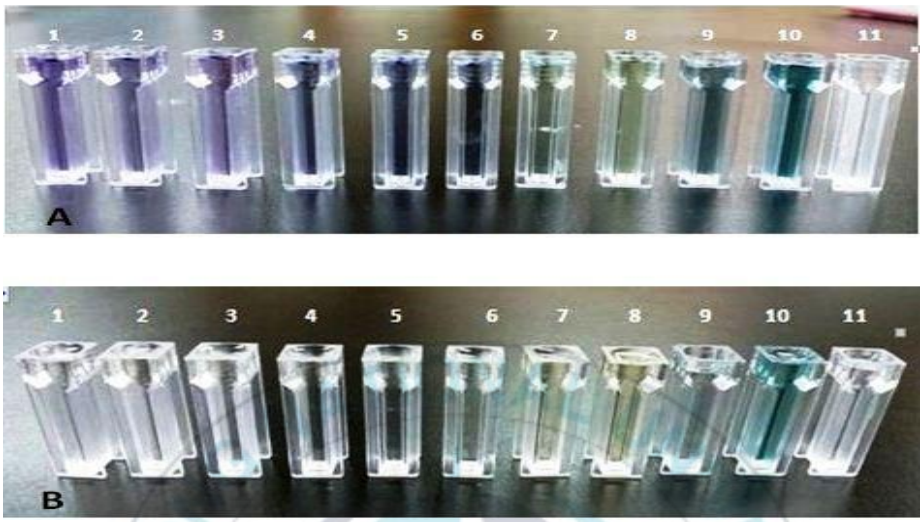

Fig. 9. Determination of ABTS radical scavenging activity of the culture supernatant collected during biodegradation of SSW by *Bacillus cereus* EW5 in batch and fed-batch operation in a 3 L fermentor. "A" batch; "B" fed-batch. Cuvettes 1-8: 12 h intervals culture supernatant, Cuvette 9: positive control sample, Cuvette 10: control, and Cuvette 11: blank sample.

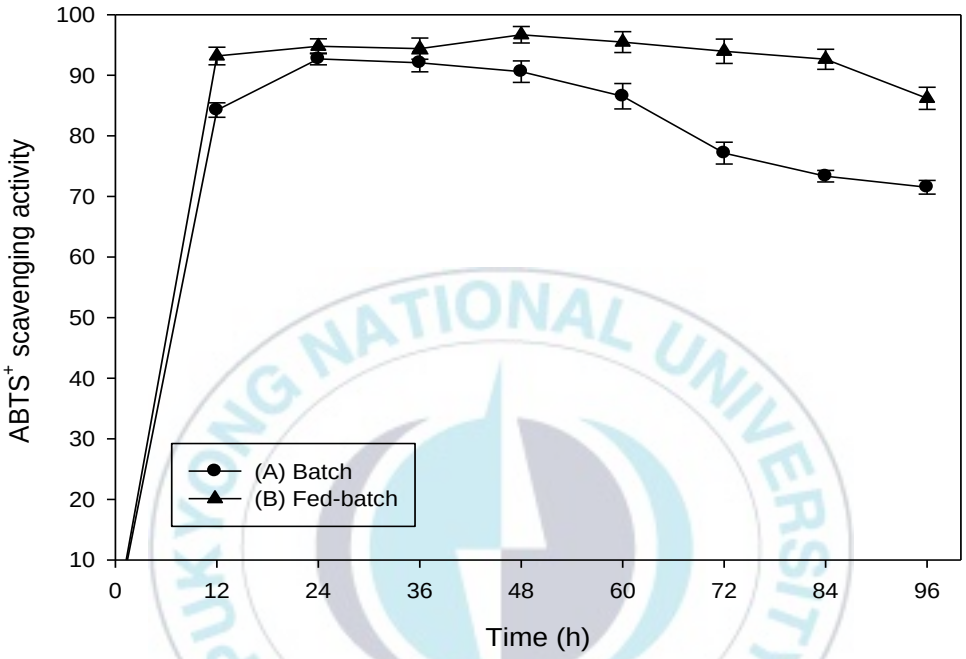

Fig. 10. ABTS radical scavenging activity of the supernatant with time in batch and fed-batch biodegradation of SSW by *Bacillus cereus* EW5 in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.1.4.3 Reducing power assay

The reducing power assay is used to appraise the ability of an antioxidant to donate electron or hydrogen (Gao et al., 2012). Several studies have reported that there is a direct correlation between antioxidant activities and reducing power of certain bioactive compounds (Bahri-Sahloul et al., 2014). The antioxidants compounds in tested sample results in the reduction of ferric cyanide complex in the ferrous form (Bellaaj et al., 2012). In batch biodegradation, the reducing power of the culture supernatant showed a linear increase with time (Fig.11 A; 12 A). The highest reducing power was recorded during at 96 h (OD = 700nm) as 1.430±0.04. The absorbance of the control sample was recorded as 0.025 (OD = 700nm). In a previous study, Azam et al. (2014) reported maximum absorbance of the culture supernatant was reached at 0.34±0.003 (OD = 700 nm) after 5 days of incubation in a shake flask study. Maruthiah (2017) reported that the reducing power of SS hydrolysate showed linear increased with progressive increasing in concentration (0.25-2mg/ml) and recorded reducing power 1.32 (absorbance value) at a concentration of 2.0 mg/ml.

Fig. 11. Determination of reducing power of the culture supernatant collected during biodegradation of SSW by *Bacillus cereus* EW5 in batch and fed-batch operation in a 3 L fermentor. "A" batch; "B" fed-batch. Cuvettes 1-8: 12 h intervals culture supernatant, Cuvette 9: control sample, and Cuvette 10: blank sample.

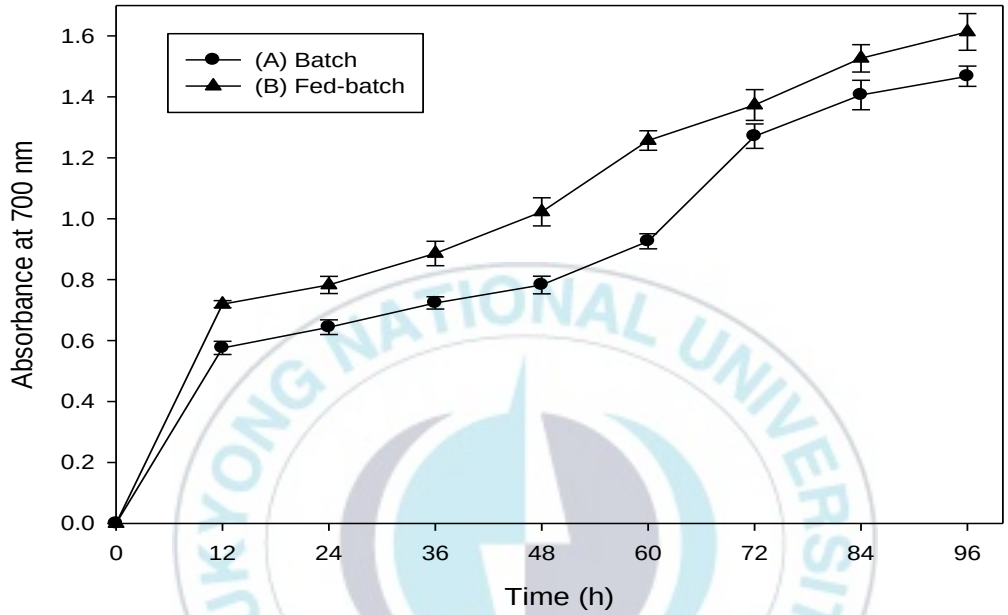

Fig. 12. Reducing power of the supernatant with time in batch and fed-batch biodegradation of SSW by *Bacillus cereus* EW5 in a 3 L fermentor. All data correspond to the mean ±SD (n = 3) of triplicate determination. (A) batch; (B) fed-batch.

3.2 Fed-batch biodegradation

3.2.1 Biomass concentration (g/L)

From the Fig. 3 (B), it was apparent that the cell concentration generally remained constant during 0-12 h of degradation and then increased gradually throughout the time. In general, the cells feds on substrate and started to grow exponentially and there was no long stationary phase. It might be due to the exponential feeding rate that was optimum and had provided a better growth environment to the bacteria. This feeding strategy might also be minimized the substrate inhibition and reduced the catabolic repression, as a result more substrate was contributed to cell growth and consequently the production of other metabolites. As active growing cells were used as inoculum, the production of bioactive compounds started instantly after inoculation. The agitation of the medium clearly improved the specific biomass growth and shortened the time to reach the peak biomass concentration and improve the carbon supply to the cells compare to shake flask culture (Salehmin et al., 2014). Cell biomass showed an increasing trend during feeding and showed a decreasing rate after 84 h might be due to the depletion of carbon source.

At bioreactor level, the maximum cell dry weight was 34.57±0.12 g/L in fed-batch culture at 96 h of SSW, which is 62% higher than batch culture (21.37 g/L). Zhong et al. (2014) reported 77.50 g/L DCW in fed-batch fermentation in 5 L fermentor using *B.*

subtilis. These results indicated that fed-batch strategy was more favorable for cell growth.

3.2.2 pH

In fed-batch degradation, the pH dropped from 7.0 and ranged from 5.74 to 6.50 with slight fluctuation during the experiment (Fig. 4 B), caused by changing alkalinity due to peptide linkages of chitin during enzymatic proteolysis (Bajaj et al., 2016). Bellaaj et al. (2012a) reported *B. cereus* SV1 exhibited high demineralization rate when the pH value was 5.86. They also reported that the decrease of pH could be due to the ability of the strain to use available glucose as a substrate for its growth and simultaneously to produce acids via pyruvate. Chen et al. (2011) reported that the production of GlcNAc from chitin was optimum at pH 5.3 from chitin degrading factors in *Chitinbacter tainanesis*. However, in fed-batch degradation pH was not fallen below 5.74 like batch (lowest pH, 4.57) at the last part of the degradation.

3.2.3 Production of reducing sugar

In fed-batch culture, the production of reducing sugar followed the same pattern as in the batch culture before the feeding was started (Fig. 5 B; 6 B). In batch culture, the amount of reducing sugar was higher at the 60 h of culture and on the other side; in fed-batch, the concentration was higher (0.297±0.05 mg/ml) at the 48 h of culture and maintained more or less stable value up to 84 h. This value was approximately 11% higher compared to the batch and 24%; compare to shake flask study, previously done by Azam et al. (2014). Periodic addition of culture broth supports to maintain the stable production of reducing sugar and found to increase up to 48 h and remain unchanged with a slight deviation until 84 h of degradation. During last part of the degradation, reducing sugar showed decreased value. This was might be due to the utilization of some sugar by the strain due to the shortage of substrate at the later stage (Azam et al., 2014). This suggested that the strain was able to degrade the SSW proficiently and hydrolytic product was used as carbon source for their proliferation (Chen et al., 2011).

3.2.4 Antioxidant activity

3.2.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

In fed-batch degradation, the highest DPPH radical scavenging activity (92.35%) was found during 48 h and remains active with minor change until 84 h (Fig.7 B; 8 B). The lowest activity showed at 96 h (75.63%). Differently, in batch the highest (89.33%) activity showed during 60 h and was not sustained up to the last part of the degradation. Manni et al. (2009) reported nearly 82% radical scavenging activity at a concentration of 2 mg/ml from shrimp waste by treatment with crude protease from *Bacillus cereus* SV1. In a previous study, Azam et al. (2014) reported 68.5 to 83.4% DPPH activity during 8 days of incubation in a 200 ml flask level study. Bellaaj et al. (2012a) reported that the DPPH radical scavenging activity was maximum after 3 days (90%) from SSW using strain *P. aeruginosa*. The obtained result from DPPH radical scavenging activity showed that the culture supernatant may contain peptides and act as electron donors, converted the free radical to more stable products, and terminate the radical chain reaction (Manni et al., 2009). The bacterial enzymatic hydrolysis of protein-astaxanthin complexes of shrimp waste may produce a complex mixture of bioactive compounds like free amino acids, peptides, carotenoid etc. (Manni et al., 2009).

3.2.4.2 ABTS radical cation decolorization assay

In fed-batch biodegradation, the ABTS radical cation decolorization activity ranged from 85.70 to 98.16%.The highest activity was recorded during 48 h of degradation and showed more or less similar activity throughout the time (Fig. 9 B; 10 B.). This result indicated that rational supply of the medium to the bioreactor vessel retained the stable production of antioxidant compounds. Azam et al. (2014) also reported higher (99.6%) ABTS radical cation activity from SSW in a previous study. Higher ABTS activity indicated that the most antioxidant compounds were hydrophilic (Prior et al., 2005). ABTS scavenging activity was higher compared to DPPH, supported by Sachindra and Bhaskar (2008). The production of GlcNAc and chitobiose was the major contributor to the antioxidant activity (Azam et al., 2014). Lira et al. (2017) reported 43.86±3.07 and 45.23±2.21% $ABTS^{+}$ activity for cooked and raw shells of shrimp (*Litopenaeus schmitti*) respectively. Therefore, the result of this study was approximately 55% higher to the above report.

3.2.4.3 Reducing power assay

The highest reducing power (OD = 700nm, 1.55) was found during 96 h of fed-batch degradation (Fig. 11 B; 12 B) which was higher compared to batch (OD = 700nm, 1.43±0.04). This production of antioxidant compounds was higher approximately four and half fold than the result (0.34 at $A_{700\ nm}$) reported by Azam et al. (2014) in a previous study from SSW in a shake flask culture. Bellaaj et al. (2012a) reported that the reducing power was maximum after 1 day (OD 700nm = 1.7) from SSW using *P. aeruginosa.* In

another study by Bellaaj et al. (2012b) reported the highest activity (OD 700nm =1.55 at 1.5 mg/ml) hydrolysate by *B. pumilus* A1.

Some peptides, which exhibited moderate reducing power, are electron donors capable of neutralizing free radicals, converting them into more stable non-reactive species and thus terminating the free radical- initiated chain reactions (Manni et al., 2009).

In fed-batch degradation, the production of bioactive compounds was produced constantly for a longer period of time up to 84 h. and accumulating in the culture broth compared to batch degradation, which was understood by showing the antioxidant activity. Contrary, the batch culture produced the bioactive compounds for a short duration (between 36 and 60 h) and could not sustain for a long time due to the shortage of the substrate. The presented data showed that fed-batch culture performed better than batch culture, which is supported by other researchers who explained that the enzymatic activity and product formation was better in fed-batch culture than batch culture (Cheng et al., 2009; Hadiyanto et al., 2013; Zhong et al., 2014). Therefore, it was found advantageous to optimize the culture conditions in bioreactor level compare to shake flask batch culture to enhance the production of bioactive compounds from SSW.

3.3. Thin Layer Chromatography (TLC)

The production of bioactive compounds was analyzed and identified using TLC (Fig. 13). For TLC, 48 h cultures supernatants both from batch and fed-batch degradation were taken on the basis of antioxidant activity and applied 10 times (1 μl each) onto TLC Silica Gel 60 plate (Sigma-Aldrich, Germany) and chromatographed in a mobile phase. Stronger bands of GlcNAc and chitobiose were appeared. Forty-eight hours culture supernatant from FB degradation showed clearer band than batch degradation. Other Tenuous little spots were also observed. It was possibly due to the production of a wide range of compounds like partially acetylated chitooligsaccharides, oligosaccharides, phenolics, flavonoids, residual chitin or other reactive metabolites produced during SSP hydrolysis (Wang et al., 2011; Halder et al., 2013).

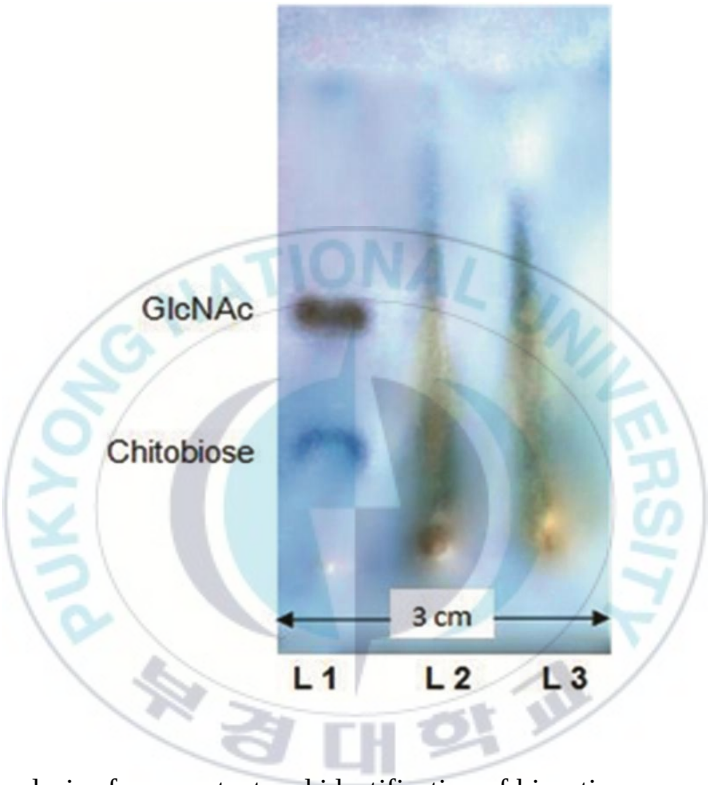

Fig. 13. TLC analysis of supernatant and identification of bioactive compounds produced by *B. cereus* EW5 from SSW in batch and fed-batch degradation in a 3 L fermentor at 48 h. After developing the TLC plate, compounds were visualized. Lane 1: Mixture of GlcNAc and chitobiose (0.2%); Lane 2-3: 48 h culture supernatant from fed-batch and batch operation, respectively.

3.4. DNA damages inhibition activity

Natural antioxidant compounds are proclaimed for their protective ability of cellular components. Saenjum et al. (2010) showed that free hydroxyl radicals cause damage to the DNA and cell death (Kim et al., 2012). To evaluate DNA damage inhibition activity of the culture supernatant, hydroxyl radical-induced DNA was exposed either in the presence or in absence of the supernatant. Significant damage protecting ability of the culture supernatant (sample 4 μl with 2.5 μl Fenton's reagents) from both batch and fed-batch was observed (Fig. 14). Culture supernatant from fed-batch (lane: a-d) showed higher activity than batch (lane: 1-4). Sila et al. (2013) reported DNA protecting activity of astaxanthin from SSW. In a previous study, Azam et al. (2014) also found strong DNA damage inhibition activity of SSW degrading by *B. cereus* EW5. Halder et al. (2014), reported chitosaccharidies prepared from shrimp shell through sequential catalysis, using crude protease and chitinase enzymes, showed reasonable reduction in oxidative damage in DNA and recommended for potential use in gene therapy.

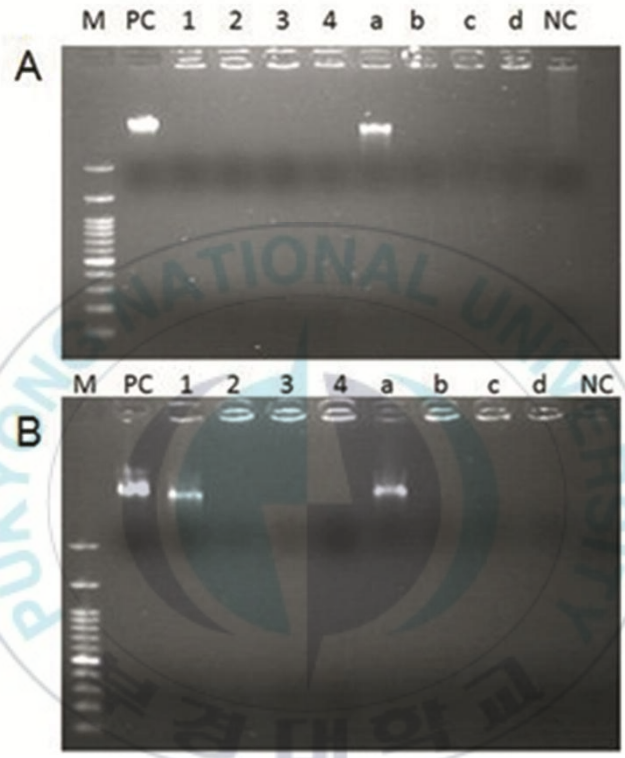

Fig. 14. DNA damage inhibition activity of the culture supernatant of degraded SSW collected during 48 h of degradation by *B. cereus* EW5 in a 3 L Bioreactor. Activity shown in "A" with 2 μl and "B" with 4 μl culture supernatant. Lane: 1-4 for batch and a-b for fed-batch. M: Marker; PC: Positive Control; NC: Negative Control.

4. Conclusion

Based on the data of biomass concentrations, reducing sugar production, antioxidant activities, TLC, and DNA damage inhibition activity, it is concluded that the fed-batch system was better compared to the batch system as the method could provide higher yields, higher antioxidant activity and shortened the degradation periods. Periodic addition of culture broth performed on fed-batch system, assists the maximum cell growth and consequence of higher product formation. Results of this study provide useful information for scale-up production of bioactive compounds from SSW.

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