

## Enhancing SDS-PAGE Detection of Dilute Extracellular Polystyrene Degrading Enzymes Expressed by *Bacillus megaterium* Strain via Centrifugal Freeze Concentration Method

Kian Meng Tan, Noor Akhmazillah Mohd Fauzi\*,  
Angzzas Sari Mohd Kassim and Aisyah Mohd Rehan

Department of Chemical Engineering Technology, Universiti Tun Hussein Onn Malaysia,  
Pagoh Education Hub, Johor 84600, Malaysia

(\*Corresponding author's e-mail: akhma@uthm.edu.my)

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### Abstract

This study aims to enhance the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) detection and visualization of extracellular polystyrene degrading enzyme expressed by *Bacillus megaterium* as a result of utilizing polystyrene as a sole carbon source. Dilute polystyrene degrading enzyme expressed in the culture supernatant could not be directly visualized from SDS-PAGE stained with colloidal Coomassie blue stain as it falls below its sensitivity or detection limit level. To overcome this problem, the crude enzyme extract was concentrated via the centrifugal freeze concentration method (cryoconcentration). The extracellular enzyme profiles were determined by SDS-PAGE. The SDS-PAGE analysis of the concentrated enzyme extract revealed 6 visible protein bands ranging in size from 10 to 60 kDa. Three distinct protein bands at approximately 20, 45 and 60 kDa were observed to be highly expressed in the culture supernatant. Polystyrene degrading enzyme extracted from the culture supernatant was closely related to several polymer degrading enzymes which suggest that the extracted enzyme from *Bacillus megaterium* also belongs to the hydrolase's enzyme family. GC-MS analysis of the extracted samples significantly contains benzene derivatives due to the breaking down of the long-chain aromatic hydrocarbon polymer. Hydroxylation of the aromatic ring formed phenolic substrate took place which suggests being facilitated by intracellular enzyme hydroxylases. Hence, the biodegradation of polystyrene by *Bacillus megaterium* was believed to incorporate both intracellular and extracellular enzymes.

**Keywords:** *Bacillus megaterium*, Extracellular enzyme, Polystyrene, SDS-PAGE

### Introduction

Polystyrene (PS) degrading bacteria were isolated from Superworm (*Zophobas morio*) and identified to belong to the *Bacillus megaterium* strain through the partial 16S rRNA gene sequencing [1]. The biodegradation activity of *Bacillus megaterium* on PS film has been investigated and the identification of PS-degrading bacteria promotes more in-depth research for optimal plastic waste management to switch from conventional landfilling to enzymatic biodegradation.

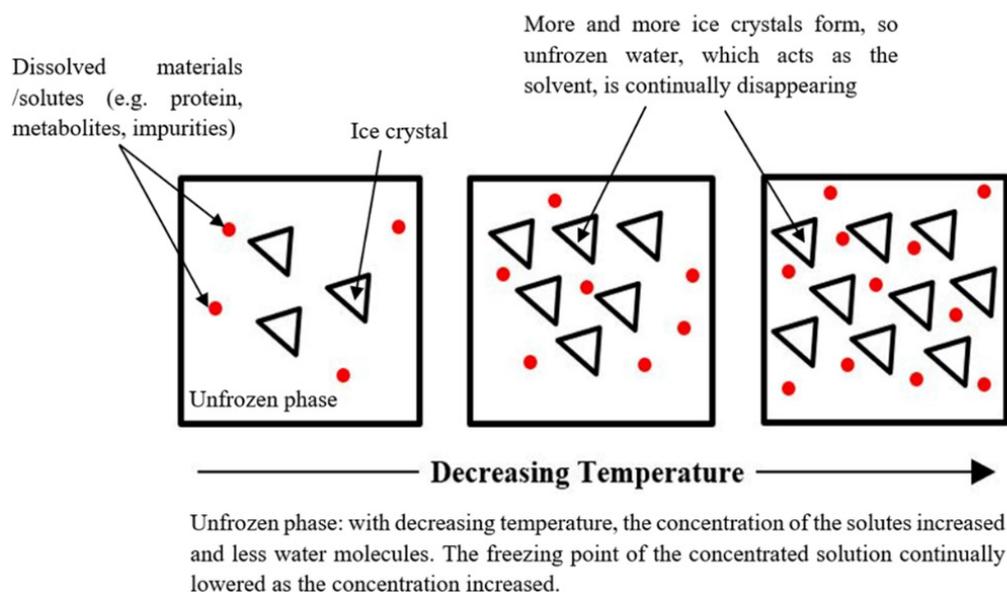
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an essential practice used to provide details on protein molecular weight and purity. Subsequently, the separated proteins retrieved from polyacrylamide gels can be further characterized via various secondary techniques, such as MALDI-TOF or LC/MS mass spectrometry to determine the peptide or amino acid sequence. However, chromatographic analysis of bacterial extracellular protein is often hindered by the dilute concentration of protein secreted into the culture supernatant that is below the detection limit of SDS-PAGE stains. This necessitates concentrating of dilute protein samples prior to SDS-PAGE analysis for their successful detection.

There are several techniques employed to concentrate protein, including lyophilization, ultrafiltration, and chemical precipitation [2]. Nevertheless, there are some drawbacks associated with these general protein concentration techniques as they often involve additional steps for salt or detergents removal [3]. Concentration polarization and membrane fouling are common issues faced by ultrafiltration

due to the formation of the irrevocable bind in some proteins [4]. This may be unfavourable especially when only a few micrograms of proteins are left after a multistep purification. Necessary periodic replacement of membrane will eventually add to the processing cost. Besides, the chemical precipitation of protein is often cumbersome due to partial precipitation resulted in low protein recovery and low solubility of precipitated proteins [5]. Denaturation of proteins or loss of protein bioactivity may occur as a result of organic solvents and isoelectric precipitation [6].

The freezing method has significant use in stabilizing biological and pharmaceutical specimens and compounds for long-term preservation. During the freezing of water, water is separated from the aqueous solution through crystallization, and the solutes are concentrated in the residual solution as shown in **Figure 1** [7]. Physically, freezing involves segregating the protein solution into an ice phase and a freeze concentrated liquid [8]. Proteins like other solutes are isolated from the ice [9]. Spatial partitioning of the protein is thus induced [10,11]. This makes the cryoconcentration system a feasible technology with great potential in recovering and concentrating on bioingredients.

Overall, the purpose of this study was to analyse the extracellular polystyrene degrading enzyme isolated from *Bacillus megaterium*, a new polystyrene degrading *Bacillus* strain. In this study, we show the feasibility of using a cryoconcentration system to aid in concentrating dilute extracellular PS-degrading enzymes secreted in the culture supernatant to enhance SDS-PAGE detection.



**Figure 1** Formation of ice crystal during cryoconcentration process [12].

## Materials and methods

### Production of PS-degrading enzyme

The enzymes production was accomplished through shake flask fermentation using liquid carbon-free basal medium (LCFBM) as production medium. It was prepared by dissolving  $0.7 \text{ gL}^{-1}$  dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ),  $0.7 \text{ gL}^{-1}$  potassium phosphate ( $\text{KH}_2\text{PO}_4$ ),  $0.002 \text{ gL}^{-1}$  sodium chloride ( $\text{NaCl}$ );  $0.005 \text{ gL}^{-1}$  ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ),  $1.0 \text{ gL}^{-1}$  magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ );  $0.001 \text{ gL}^{-1}$  zinc sulphate ( $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ );  $0.001 \text{ gL}^{-1}$  manganate sulphate ( $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ );  $0.002 \text{ gL}^{-1}$  iron sulphate, ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) in deionized water and adjusted to pH 7 which comprising of PS as sole carbon source. 100 mL of sterile production broth was prepared in a 250 mL conical flask and 5 % inoculum (*Bacillus megaterium*) was transferred aseptically into the production medium, incubated at  $30 \text{ }^\circ\text{C}$  for 28 days at 120 rpm. After 28 days of incubation, the crude extracellular enzyme was harvested via centrifugation at 10,000 rpm for 15 min to remove. The supernatant containing crude extracellular enzyme was stored at  $-20 \text{ }^\circ\text{C}$ .

### Centrifugal freeze concentration of PS-degrading enzyme

The centrifugal freeze concentration of protein also known as cryoconcentration protocol was based on the use of 2 commercially available centrifugation tubes: A 15 mL centrifuge tube and a 1.5 mL Eppendorf type microtube, were assembled as illustrated in **Figure 2**.

### Protein content

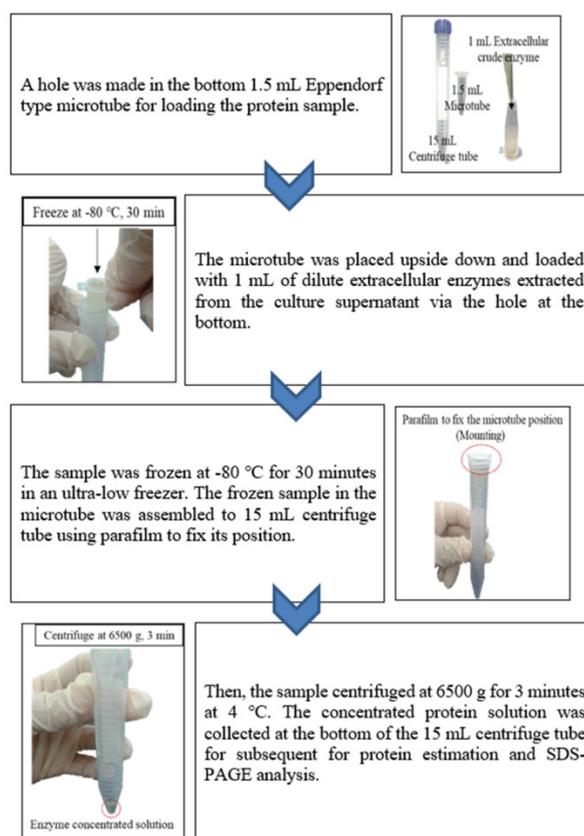
Protein concentration was measured spectrophotometrically at wavelength 595 nm compliance to the Bradford method of protein determination [13]. Bovine serum albumin (BSA) was used as an external standard. All protein determinations were performed in triplicates.

### Protein recovery efficiency

Protein recovery (%) denotes the amount of protein that was concentrated in the permeate with the total amount of protein in the original solution. It was calculated using the equation designated by Ortíz *et al.* [5] as below;

$$\text{Protein recovery (\%)} = (P_c/P_t) \times 100$$

where  $P_c$  is the amount of protein in the concentrated solution and  $P_t$  is the total amount of protein in the original dilute solution.



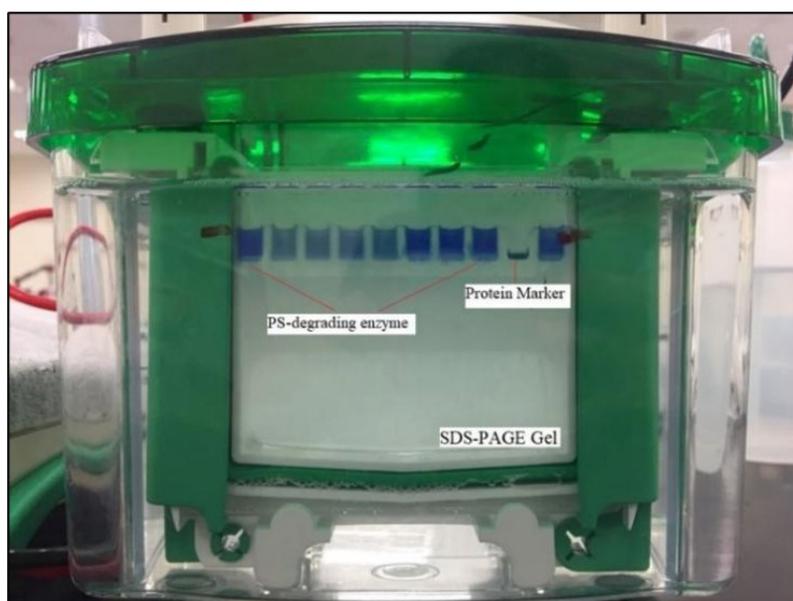
**Figure 2** Flow diagram of apparatus used for concentrating extracellular enzyme via cryoconcentration protocol.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the procedure described by Laemmli [14]. The concentrated protein sample was added to 5× loading buffer (5 % bromophenol blue, 50 % glycerol, 10 % SDS, 25 % β-mercaptoethanol and 20 % 1.5 M Tris-HCl pH 6.8) at a ratio of 4:1. The prepared samples were vortexed and then boiled for 10 min at 95 °C. Then, 24 μL of samples were loaded at the 4 %

stacking gel electrophoresed on 12 % acrylamide gel for 90 min at 150 v using Bio-Rad Mini Protean electrophoresis chamber as shown in **Figure 3**.

The gel was stained overnight using colloidal Coomassie Brilliant Blue G250 stain that consisting of 10 % phosphoric acid, 10 % ammonium sulphate, 0.12 % of Coomassie Brilliant Blue G250 and enhancement of low protein (8 - 10 ng) detecting sensitivity. Then the gel was de-stained overnight in a de-staining solution that consists of methanol, acetic acid and distilled water at a ratio of 2:3:35. The approximate molecular weights of the protein bands were estimated using a standard protein marker SMOBIO PM 2610.



**Figure 3** Bio-Rad Mini Protean electrophoresis chamber.

### Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was carried out to determine the daughter products of PS degradation to give an insight into the enzymatic reaction that has taken place during biodegradation of PS. The culture supernatant was harvested from centrifugation at 10,000 rpm for 15 min and filtered with a 0.22  $\mu$ m membrane filter. The water-soluble daughter products were extracted using the liquid-liquid extraction method. 2 mL of the supernatant sample was placed in a 5 mL vial, and 2 mL of dichloromethane was added. The sample was vigorously shaken for 1 min. 3.6 g of sodium sulfate was introduced by a small portion for binding the aqueous phase, the transparent dichloromethane extract was transferred into a clean vial for further analysis.

The extracted products were identified by a gas chromatograph/mass spectrometer (GC-MS, Agilent 5,975, Palo Alto, CA) equipped with A 30 m of HP-5 MS capillary column for separation of the components. The oven temperature was first held at 50  $^{\circ}$ C for 1 min, then increased to 250  $^{\circ}$ C at 10  $^{\circ}$ C/min and held at 250  $^{\circ}$ C for 5 min. The carrier gas used was helium at 1.2 mL/min, 2  $\mu$ L of the extract was injected into the chromatograph at a split-less mode.

For each GC-MS data file, a set of target components was identified with the aid of the Mass Hunter software. Each of these components was then compared against all compounds comprising the NIST Mass Spectral Library database, the top hit was reported.

### Results and discussion

Crude extracellular enzymes had been undergoing centrifugal freeze concentration and successfully concentrated from  $0.097 \pm 0.005$  to  $0.365 \pm 0.015$  mg/mL, with concentration factor of 3.7 folds. The protein recovery from the culture supernatant was recorded as  $37.79 \pm 2.13$  %. The data obtained were compared to the conventional protein concentration and purification method presented by Shanti [15] in characterizing protease enzyme produced by *Bacillus subtilis* as summarized in **Table 1**.

The data implied that the proposed freeze concentration method shows a higher concentration folds factor and efficiency of protein recovery (%) over ultrafiltration. However, conventional salting-out protein using 60 % ammonium sulphate shows the highest concentration folds factor which is also almost twice the freeze concentration method. Nevertheless, in terms of the efficiency of protein recovery (%), both methods present a percentage below 50 %. The overall data showed that the proposed freeze concentration method made it feasible to concentrate diluted supernatant solution. This practical method has also presented advantages over the conventional methods: low associated cost (no chemicals required and reusable labware), low effective work time (total time taken less than an hour, no overnight treatment needed) and preservation of low molecular weight protein (~10 kDa, refer to **Figure 4**) as well as prevention of loss of enzyme bioactivity due to chemical precipitation.

In addition, the SDS-PAGE analysis was used to visualize the protein profile. Extracellular crude enzymes contained in the 24  $\mu$ L aliquot coming directly from the culture supernatant were dilute and could not be detected and visualized by SDS-PAGE (lanes labelled L1 to L3). In contrast, the cryoconcentrated protein produced sharper bands and could be visualized clearly on the SDS-PAGE (lanes labelled L4 to L6) as shown in **Figure 4**.



**Figure 4** SDS-PAGE analysis of crude extracellular enzyme, (L1 - L3) culture supernatant replicates and (L4 - L6) cryoconcentrated supernatant replicates.

**Table 1** Summary of purification of PS-degrading enzyme.

Purification step	Volume (mL)	Protein concentration (mg/mL)	Concentration factor (folds)	Total protein (mg)	Efficiency of protein recovery (%)	Reference
Crude PS-degrading enzyme by <i>Bacillus megaterium</i>	1.0	0.097 $\pm$ 0.005	1.0	0.097 $\pm$ 0.005	100.00	Current research work
Freezing-out protein	0.1	0.365 $\pm$ 0.015	3.7	0.037 $\pm$ 0.002	37.79 $\pm$ 2.13	
Crude protease enzyme by <i>Bacillus subtilis</i>	20.0	1.600	1.0	32.000	100.00	[15]

Purification step	Volume (mL)	Protein concentration (mg/mL)	Concentration factor (folds)	Total protein (mg)	Efficiency of protein recovery (%)	Reference
Lyophilization	4.5	7.093	4.4	31.920	99.75	
Crude protease enzyme by <i>Bacillus subtilis</i>	1,500.0	1.907	1.0	2,860.700	100.00	
Ultrafiltration	310.0	3.156	2.9	978.500	34.21	
Crude protease enzyme by <i>Bacillus subtilis</i>	300.0	3.280	1.0	984.000	100.00	
60 % ammonium sulphate salt	20.0	24.243	7.4	484.850	49.27	

\*± are standard deviation for triplicate samples

The SDS-PAGE protein profiling results indicated the presence of 6 protein bands with sizes ranged from 10 to 60 kDa as determined by visual assessment of their approximate molecular weights with standard protein marker. Three distinct protein bands visualized at approximately 20, 45 and 60 kDa which were most expressed in the culture supernatant. The SDS-PAGE profiling of the PS-degrading enzyme showed that the estimated enzymes' molecular weights fall within the molecular weight range of several plastic-degrading enzymes such as chitinases, lipase, xylanase, esterase and cutinase as summarized in **Table 2**. These hydrolase enzymes have been reported to be involved in the degradation of recalcitrant polymers.

**Table 2** Summary of plastic-degrading enzymes and their molecular weight.

Enzyme	Molecular weight	Microorganism	Plastic materials	Reference (s)
Lipase	19 - 60 kDa ~20 kDa ( <i>Bacillus</i> sp)	<i>P. protegens</i> BC2-12 <i>Pseudomonas</i> sp.	Polyester, Polyethylene (PET)	[16-21]
Esterase Cutinase	20 - 160 kDa 22 - 25 kDa	<i>Pseudomonas</i> sp. AKS2 <i>R. depolymerans</i> strain TB-87 <i>Leptothrix</i> sp. strain TB-71 <i>Thermobifida alba</i> Est119 <i>P. aeruginosa</i> <i>T. cellulositytica</i> DSM44535 <i>T. fusca</i>	Polyethylene succinate (PES), PET, Polyester PET	[21-27] [28,29]
PETase /α/β hydrolase	~29 kDa	<i>Ideonella sakaiensis</i> 201-F6	PET	[30,31]
Serine hydrolase	~21 kDa	<i>Pestalotiopsis microspora</i>	Polyurethane (PUR)	[32]

Concurrently, *Bacillus megaterium* strains are known as potential hydrolase enzyme-producing bacteria and have been applied widely in industries. For instance, *Bacillus megaterium* strain isolated from the crude oil contaminated soil responded positively to lipases activity which indicated its degradability on crude oil [33]. Polystyrene is synthetic plastic derived from crude oil, the ability of *Bacillus megaterium* in degrading crude oil provide insights into PS degradation aided by lipases activity. The lipolytic system of *Bacillus megaterium* 370 was investigated, showing the existence of secreted lipases and cell-bound esterase [34].

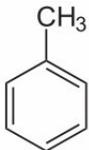
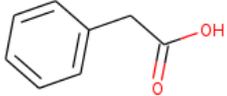
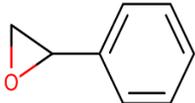
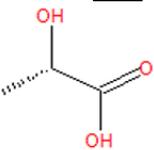
In term of polymer degradation, *Bacillus megaterium* strain has been investigated for the biodegradation of low density polyethylene (LDPE) [35]. This bacterium also has large potential in

degrading natural rubber latex film [36]. *Bacillus megaterium* KIBGE-IB3 was reported proficient in the hyper-production of extracellular dextranase, the synthesized dextranase can be applied for the hydrolysis of the biopolymer [37]. *Bacillus megaterium* N18259 is a known positive poly- $\beta$ -hydroxybutyrate (PHB) degrading bacterial strain which acquires PHB depolymerase gene, PhaZ<sub>Bm</sub> [38]. PHB depolymerase produced has great commercial significance specifically in the bioplastic sector. Lignin peroxidase producing bacteria *Bacillus megaterium* has effectively acted on paper and pulp [39].

Indeed, there is limited or no information on the specific key enzymes that responsible for polystyrene degradation produced by the *Bacillus megaterium* strain. The SDS-PAGE results obtained in the present study serve to give insights into the enzymatic degradation of polystyrene, aligning with literature studies which evinced the isolated extracellular PS-degrading enzymes are presumably susceptible to hydrolases enzyme. Further identification of PS-degrading enzymes needed to be done via protein sequencing to clarify the hypothesis made.

On the other hand, the GC-MS analysis of samples extracted from LCFBM after 28 days incubation with *Bacillus megaterium* strain revealed that the extracted samples significantly contain benzene derivatives as summarised in **Table 3**. The formation of benzene derivatives suggests synthetic processes were activated by the bacteria in addition to the presumably enhanced degradative activity that occurred. The production of lactic acid implies *Bacillus megaterium* strain are capable to derive energy from the inorganic polymer (PS) via anaerobic respiration metabolism activities.

**Table 3** GC-MS identification of water-soluble products released from PS pieces in the presence and absence of the activities of *Bacillus megaterium* strain after 28 days. (Control: in the absence of *Bacillus megaterium* strain).

Potentially proposed chemical	Chemical structure	Mass (g/mol)	Similarity (%)	Control	<i>Bacillus megaterium</i> strain
Toluene, C <sub>7</sub> H <sub>8</sub>		92	84.9	-	+
Benzene acetic acid, C <sub>8</sub> H <sub>8</sub> O <sub>2</sub>		136	83.7	-	+
Styrene oxide C <sub>8</sub> H <sub>8</sub> O		120	79.9	-	+
L-Lactic acid, C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>		90	85	-	+

Research has been reported that *Bacillus megaterium* strains are capable to biodegrade aromatic compounds such as phenol and benzene [40]. It is reasonable to presume the biodegradability of *Bacillus megaterium* on aromatic compounds facilitated biodegradation of polystyrene which is a synthetic aromatic hydrocarbon polymer. The presence of hydroxyl group (-OH) at the sidechain suggested hydroxylase enzymes activity. Adding oxygen atoms to the aliphatic chain in polystyrene is an essential step. The hydroxylation aliphatic chains have been comprehensively investigated in cytochrome P450 BM3 (CYP102), a self-sufficient P450 enzyme from *Bacillus megaterium*. Bacterial enzyme P450 monooxygenase CYP102 can hydroxylate a variety of alkanes, fatty acids and aromatic compounds [41]. Similar to PS, polypropylene (PP) is not a substrate subject to hydrolysis. The enzymatic hydroxylation of PP was investigated using the P450 monooxygenase from *Bacillus megaterium* expressed in *E. coli* DH5 $\alpha$  which led to an increase in hydrophilicity [42] as a result of the insertion of oxygen atoms to the aliphatic backbone. Our previous work has been reported that *Bacillus megaterium* formed biofilm on the PS film surface which triggering biodegradation activity [43]. Hence, it is reasonable to suggest that the

membrane-bound cytochrome P450 enzyme of *Bacillus megaterium* interacted with the substrate PS at the surface and hydroxylation took place within the biofilm.

Overall, it is hypothesized that the enzymatic degradation of PS by *Bacillus megaterium* was first initiated by oxygen insertion in the methylene C-H bonds to alcohols (C-OH) subsequently followed by further oxidation to the carbonyl (C=O) and finally fragmentation to oligomers and monomers via the enzyme-mediated hydrolysis. The findings discussed above remarked that the biodegradation of polystyrene incorporates both intracellular and extracellular enzyme hydroxylases and hydrolases, respectively.

## Conclusions

In this study, the isolated extracellular crude enzyme extracted from *Bacillus megaterium* was successfully concentrated up to 3.7 folds and achieved approximately 40 % of protein recovery efficiency via centrifugal freeze concentration. This method has proven practical in enhancing SDS-PAGE detection and presented advantages over the conventional methods in terms of cost and time consumption. The SDS-PAGE analysis of the cryoconcentrated crude enzyme extract from the culture supernatant with polystyrene as substrate in mineral salt medium showed 6 visible protein bands between 10 to 60 kDa. Three protein bands observed at approximately 20, 45 and 60 kDa were highly expressed in the culture supernatant. The molecular weight range of the PS-degrading enzymes estimated from SDS-PAGE was matched to the molecular weight range of several polymer degrading enzymes such as lipase, esterase and cutinase. Therefore, it is hypothesized extracellular PS-degrading enzyme belongs to the enzyme family of hydrolases. Whereas GC-MS analysis confirmed the enzymatic degradation activity has taken place. Samples extracted from the culture supernatant significantly contained benzene derivatives. Hydroxylation of the aliphatic backbone was noticed, which suggested hydroxylation activity facilitated by intracellular enzyme hydroxylase Cytochrome P450. Overall, the biodegradation of polystyrene by *Bacillus megaterium* is believed to incorporate both intracellular and extracellular enzymes. Further identification of the PS-degrading enzyme will be performed via protein sequencing.

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