

Optimization of Polystyrene Biodegradation using Response Surface Methodology (RSM) Measured by Simple Colorimetric Method

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Abstract

Nearly 280 kilotons of polystyrene (PS) waste being discarded yearly in Malaysia. Banning usage of PS food packaging aims to minimize this “white pollution”. However, the existing PS waste volume remains the same despite the termination of PS production. Therefore, this study has investigated the optimization of biodegradation of PS using isolated strain *Bacillus aryabhatai* as an alternative solution to the biodegradation of recalcitrant plastic. The effect of pH and temperature as independent variables were studied at three levels each (pH: 6, 7.5 and 9, temperature: 25 °C, 35 °C and 45 °C) under the designation by three level-factorial and analysed using response surface methodology (RSM) by Design Expert Version 10.0. The biodegradation activity of *Bacillus aryabhatai* was quantified using simple colorimetric method. *Bacillus aryabhatai* was inoculated on the dye PS-film and incubated under designed conditions. As a result of biodegradation, the entrapped methylene blue dye was released and measured photometrically. Optimum conditions for the highest reading of blue dye absorbance were obtained at pH 9 and temperature of 25°C with the desirability of 75.9%. This study provides significant information on the biodegradation activity of *Bacillus aryabhatai* for future research that can be applied in waste management.

Keywords: *Bacillus aryabhatai*; biodegradation; polystyrene; optimization; response surface methodology.

1. Introduction

Globally, the demand for plastic has increased and is expected to reach 375 billion dollars by the year 2020 [1] Polystyrene (PS) is a type of plastic that has many uses in electrical appliances and also as a storage for materials. It is estimated that the global demand for PS to rise up till an average of 2.3% per annum [2]. In Malaysia, nearly 280 kilotons of PS waste being discarded yearly [3]. Due to the nature of PS to be non-biodegradable due to the polymer chains of styrene, majority of PS end up in our landfills and incinerators. Incineration is a globally accepted PS wastes treatment process that produces only carbon dioxide and water, and that because of its petroleum content it burns at a very high temperature and can burn out impurities in the incinerator. Excessive of carbon contributes to global warming. However, Hawley-Fedder et al [4] reported that when PS was burned at temperatures of 800-900 °C (the typical range of a modern incinerator), more than 90 different compounds were identified in its combustion effluent such as dioxins, furans, hexachlorobenzene and chlorophenols which are toxic and apparently hazardous to both environment and human health. Currently, the world is moving toward to minimize pollution and reduce environmental impacts. Hence, biological approach in PS waste management has become the existing focus globally for the past decade.

Traditionally, it has been argued that PS non-biodegradable polymer due to its slow rate or insignificant sign of biological

degradation. Nevertheless, biodegradation is not restricted by time. It may be very slow or very rapid and dependent on many various factors. Sielicki et al. [5] performed a study proved that mixture of bacteria was able to degrade PS but to a slow extend where up to 3% was degraded over 16 weeks. Some researches that had been done on the effect of few strains of pure bacteria isolated from soils, the application of these isolates has not been proven to be effective in the PS biodegradation [6] [7]. Recently, Yang *et al* [8] reported that the bacteria found in the guts of *Tenebrio molitor* also known as mealworm was able to degrade PS. Similarly, bacteria strain *Bacillus aryabhatai* isolated from *Zophobas morio*'s guts was proved to be able to thrive on PS at the early stage of the study by the authors. However, a fundamental study and further investigation on the feasibilities of plastic eating bacteria (from worm) in the PS biodegradation is still unexplored.

This research aims to investigate the effects of culture conditions on PS biodegradation by isolating strain *Bacillus aryabhatai* prior to optimisation the biodegradation of PS via response surface methodology (RSM). This study applied a rapid and simple colorimetric method for evaluating the degradation of PS-film. Handmade dye-containing PS-films were used as substrates and a UV spectrometer was used to measure the concentration of dye leaked out after treatment in reflection to biodegradation activity...

2. Material

2.1. Polystyrene (PS)

The Styrofoam feedstock tested for biodegradation was collected as recycle waste from local electrical sellers, Parit Raja, Batu Pahat, Johor, Malaysia.

2.2. Liquid carbon free basal medium (LCFBM)

In this study, LCFBM was used as the enrichment medium for PS-degrading microorganisms and characterization of PS-film degradation. It was prepared with deionized water and containing 0.7 g/L dipotassium phosphate (K_2HPO_4), 0.7 g/L potassium phosphate (KH_2PO_4), 0.002 g/L sodium chloride (NaCl); 0.005 g/L ammonium nitrate (NH_4NO_3), 1.0 g/L magnesium sulphate ($MgSO_4 \cdot 7H_2O$); 0.001 g/L zinc sulphate ($ZnSO_4 \cdot H_2O$); 0.001 g/L manganate sulphate ($MnSO_4 \cdot H_2O$); 0.002 g/L ferum sulphate, ($FeSO_4 \cdot 7H_2O$) following the ASTM standard for identifying the resistance of plastics to bacteria (ASTM G22-76) [9].

3. Methodology

3.1 Preparation of dye-containing polystyrene film

The method applied for dye PS film preparation was adapted from [10] with some modifications. Dichloromethane was used as the solvent to dissolve PS film at 3 weight percent (wt. %), and then in the ratio of 3:1 the solution was mixed with Methylene blue staining reagent at 7% weight per volume (w/v). Each dye-containing PS was cast onto water repellent well with 20 μ L in each well (\varnothing 7 mm) and was air-dried in a fume hood at room temperature for 24 hours as shown in Figure 1. The weight of dye PS films was approximately between 0.0020 to 0.0030 g, based on the 1.33 g/mL density of dichloromethane density, 1.33 g/mL). Handmade dye-containing PS films were acted as substrates. Each dye-containing PS film on the glass slide was rinsed twice with 80 μ L of distilled water to remove extra methylene blue.

3.2 Simple colorimetric method for biodegradation using *Bacillus aryabhatai*.

Bacteria suspension (10^8 cells) of 50 μ L in LCFBM (pH 6, 7.5 and 9) were separately dropped onto the PS films as shown in Figure. 1 (A). The 96 wells plate was placed into a small container lined with wet paper, sealed and incubated at temperature 25, 35 and 45 $^{\circ}$ C for 24 hours to avoid the inoculum on PS-film from drying. After the microbial biodegradation with the dye-containing PS films, blue colour solutions were observed (refer to Figure. 1 (B) and (C)). The wavelength used by microplate reader was OD 485 nm for determining the absorbance of blue dye released by dye-containing PS film in the microplate wells. The measured reading indicated biodegradation activity of isolated strain.

3.3 Optimization of PS Biodegradation

Temperature and pH are known as physical factors that affect the bacterial growth and enzyme activity. In this study, temperature and pH were using Design Expert Version 10.0.8 software based on response surface methodology (RSM). Three levels for each factor i.e. temperature and pH, which are (25, 35 and 45 $^{\circ}$ C) and (pH 6, 7.5 and 9), respectively were chosen to determine the maximum absorbance which correlates with the best PS biodegradation via PEB, as the optimized parameters. The number of trials was determined from the software. LCFBM used for assays was prepared to be at the respective pH of 6, 7.5 and 9 with the addition of acid or caustic. Absorbance of dye released was used as a response function Table 1 summarises the three levels

and range of the independent variables used in this experiment. Each level of independent values was represented by “-1”, “0” and “1”; which equivalent to a minimum value, a middle value and a maximum value, respectively.

Table 1: Independent variable values of the process and their corresponding levels for dye-PS assays

Independent variable	Range and levels		
	-1	0	1
Temperature ($^{\circ}$ C)	25	35	45
pH	6	7.5	9

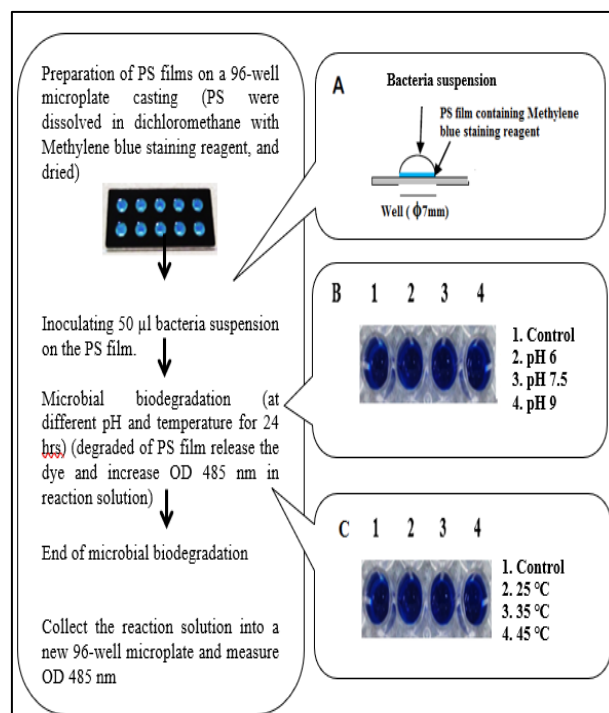


Fig 1: Evaluation of the relative PS-degradation activity using dye-containing PS films (dye-PS assay). (A) Cross-sectional view of a PS film and *Bacillus aryabhatai* suspension in 96-well microplate (B) Photograph of the microplate with PS films and *Bacillus aryabhatai* after the reaction (pH 6, 7.5 and 9 for 24 hours). (C) Photograph of the microplate with PS films and *Bacillus aryabhatai* after the reaction (25, 35 and 45 $^{\circ}$ C for 24 hours) (developed from [10]).

4 Results and Discussion

4.1 Enzyme activity of PS biodegradation under designation of response surface methodology (RSM)

The simple colorimetric method measured enzymatic activity during PS biodegradation by quantifying the intensity of dye released. Enzyme activity results in embrittlement and loss of polymer stability, as a consequence of entrapped dye leak out into the culture medium.

The enzyme activity from 13 individual runs was presented in Table 2. The results indicate that under all 13 runs possess positive enzyme activities. The highest enzyme activity is achieved at absorbance of 0.631 at 25 $^{\circ}$ C and pH 7.5, whereas the lowest enzyme activity occurs at 45 $^{\circ}$ C and pH 9 giving absorbance of 0.196.

Table 2: Absorbance measured at OD 485 nm of dye released at different conditions designed by 3-level factorial.

Run	Culture conditions		Response
	Temperature ($^{\circ}$ C)	pH	Absorbance (A)
1	45.0	7.5	0.212
2	35.0	7.5	0.408
3	25.0	7.5	0.631
4	35.0	7.5	0.317

5	35.0	6.0	0.329
6	35.0	9.0	0.357
7	35.0	7.5	0.387
8	25.0	9.0	0.497
9	45.0	6.0	0.201
10	35.0	7.5	0.423
11	25.0	6.0	0.245
12	35.0	7.5	0.388
13	45.0	9.0	0.196

It can be noticed that at pH 7.5, the fluctuation of temperature by 10 °C the rate of enzymatic activity experienced nearly 50% reduction found in Run 1, 2 and 3. Whereas, at temperature 35 °C, the effect of pH seem to be less notable as compare to effect of temperature where there were less than 10% of difference in enzymatic activity rate as the pH changes. This can be observed in Run 4, 5, 6, 7 and 12. The effect of the independent variables was evaluated using Response Surface Methodology (RSM). Data validity was verified using ANOVA analysis. Numerical method modelling was applied to study the optimum enzyme activity under optimal conditions.

4.2 Analysis of variance and model validation

Analysis of variance (ANOVA) was used to validate the results data. Table 3 summarizes the results for the regression analysis. The sum of squares is the deviations of all the observation from their mean whereas the degree of freedom is the value that the data are free to vary. The considerable R^2 and adjusted R^2 show that the data are in acceptable range to the chosen model for the absorbance for PS biodegradation (0.6051 and 0.5261). In addition, the model has F value and p-value of 7.66 and 0.0096 respectively, which implies that the model was very much significant. Hence, the linear model appeared to be suitable and reliable for the PS biodegradation at different conditions prior to temperature.

Table 3: ANOVA analysis for linear model of experimental data of biodegradation activity

Source	Sum of squares	Degree of freedom (df)	Mean square	F value	p-value Prob > F
Model	0.12	2	0.060	7.66	0.0096
Temperature (°C)	0.097	1	0.097	12.35	0.0056
pH	0.023	1	0.023	2.97	0.1153
Std. dev.	0.089		R-squared	0.6051	
Mean	0.36		Adj R-squared	0.5261	
C. V. %	24.60		Pred R-squared	0.0745	
			Adeq precision	8.904	

Therefore, a mathematical model for enzymatic activity measured in term of absorbance is developed by Design- Expert Software as Eq 1:

$$\text{Absorbance} = 0.5697 - 0.0127 * \text{Temperature} + 0.0306 * \text{pH} \quad (1)$$

4.3 Effect of single factor temperature on *Bacillus aryabhatai* strain in polystyrene biodegradation

The effect of temperature on *Bacillus aryabhatai*'s polystyrene biodegradation activity under a constant pH 7.5 is studied using dye PS-film as substrate and the amount of dye released in term of absorbance as a measurement of enzyme activity. The optimum temperature for bacterial enzymatic activity was identified at 25 °C. However, it can be observed that the absorbance value of the blue colour dye released as a result of bacterial enzyme

activity decreased as the temperature increased from the 25 to 45 °C as shown in Figure 2.

In many areas of biology, it is fundamentally rely on the way enzymes respond to temperature [11][12]. Prescott et al. [11] suggested that the bacterial growth temperature requirements are largely determined by the temperature requirements of its enzymes. *Bacillus aryabhatai* is strain isolated from *Zophobas morio*'s gut which has temperature range from 21 to 27 °C [13]. Therefore, it is reasonable that the bacterial activity was the highest at 25 °C which is within the range of its habitat temperature. High enzyme activity was observed as a result of active metabolism in bacterial activity.

Increasing temperature caused enhancement of enzyme activity however, resulted in enzyme activity to be lost by denaturation at high temperature [12]. Moreover, high temperatures cause the destroy of microbial cell membrane as destruction and decomposition of lipid bilayer coating occurring at above 40 °C [14]. Hence, enzyme activity reduced as temperature increased. The lowest enzyme activity has taken place at 45 °C due to inhibited growth of *Bacillus aryabhatai* under high temperature. Therefore, it was verified that at temperature 25 °C is an ideal temperature for maximum enzyme activity of *Bacillus aryabhatai* in PS biodegradation.

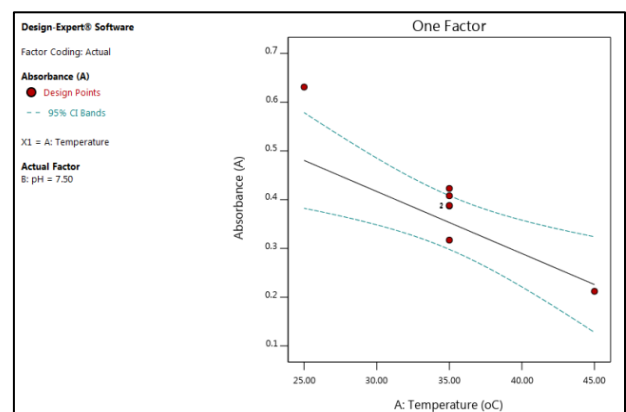


Fig 2: Effect of temperature on the *Bacillus aryabhatai* strain in PS biodegradation.

4.4 Effect of single factor pH on *Bacillus aryabhatai* strain in polystyrene biodegradation

The influence of pH on *Bacillus aryabhatai*'s PS biodegradation activity under a constant temperature was studied using dye PS-film as substrate. Enzyme activity was determined by measuring the amount of dye released in term of absorbance.. The results demonstrated a linear trend that enzyme activity increased with an increase of pH as shown in Figure 3.

However, the linear trend of enzyme activity seem to deviate from previous studies done on the effect of pH on enzyme activity which shows a bell-shaped curve with optimum pH ranging from pH 7 to 7.5 [13],[14]. This is because microbes of different species have different optimum pH as stated by Dutton et al.[17] that some microbes that produce enzyme oxalate decarboxylase work in vary pH optimum such as pH 3 in *Collybia velutipes*, pH 5.2 in *Aspergillus niger* and pH 4 in *Myrothecium verrucaria*. In addition, Okanlawon et al [18] studied the influence of pH on the growth of *Bacillus cereus* revealed that most of the strains tested had their optimum growth at pH 9. The optimum pH for enzyme activity of extracellular protease by *Bacillus sp* occurred at pH 9 [19]. Hence, abovementioned literatures clearly verify that *Bacillus aryabhatai* have an optimal activity at pH 9.

In addition to the different data in the experimental result and abovementioned literature, this difference could be also because of the nutrient availability for bacterial growth [20]. Apparently, PS is not a ready carbon source that could be easily utilized by *Bacillus aryabhatai*. Under starved condition may result in

intraspecies competition. Cultures grown in the normal way with an initial excess of food may react to pH differently from those developing on a restricted but regular food supply [21]. Therefore, these evidences provide a concrete justification that at pH 9 *Bacillus aryabhatai* could produce a maximum enzyme activity on PS biodegradation.

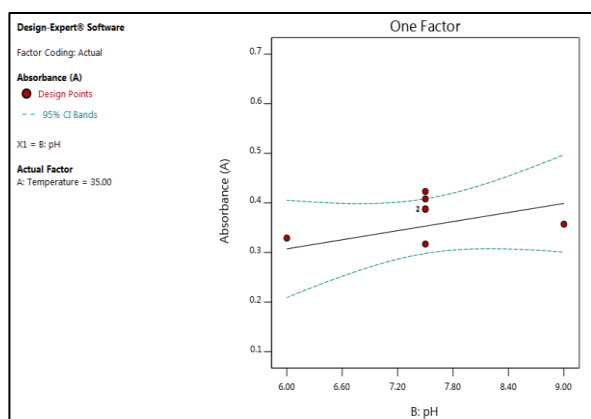


Fig 3: Effect of pH on the *Bacillus aryabhatai* strain in PS biodegradation

4.5 Response Surface Methodology (RSM) for optimization

The combined effect of temperature and pH on the enzymatic activity was illustrated in a response surface plot as shown in Figure 4. The result showed that the enzyme activity was more pronounced at low temperature (25 °C) and high alkaline environment (pH 9), respectively. There were two design points (circled in red) that were obvious outliers from the predicted values which were Run 3 (25 °C, pH 7.5) and 11 (25 °C, pH 6) that contributed to imprecision of data (Figure 4). However, the finding in combined effect of the two factors was in line with the results on single effect (pH or temperature), which was discussed in the previous sections.

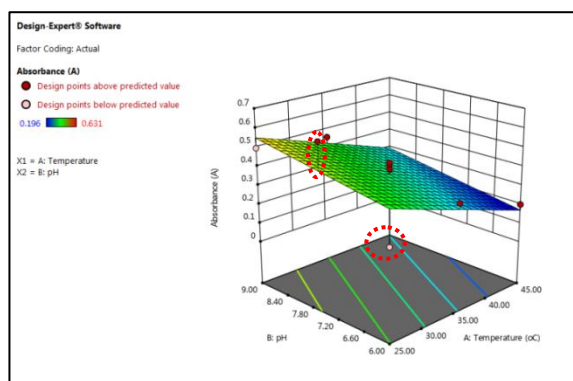


Fig 4: Response surface plot for temperature and pH on enzymatic activity (Note: Red dotted circle represents the outlier)

As for optimization modelling, a numerical method was applied in modelling the optimum condition for enzyme activity based on two independent variables which were temperature and pH was set according to the constraints as presented in Table 4.

Table 4: Constraints for numerical modelling of optimization of enzyme activity

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
A: Temperature	In range	25	45	1	1
B: pH	In range	6	9	1	1
Absorbance	Maximize	0.196	0.631	1	1

The main purpose of the study is to achieve the highest enzyme activity. Hence the constraints were set to give emphasis on the maximum absorbance of dye released. Optimum conditions for the highest enzymatic activity were temperature of 25 °C and pH of 9 which achieved desirability of 75.9% as presented in Figure 5.

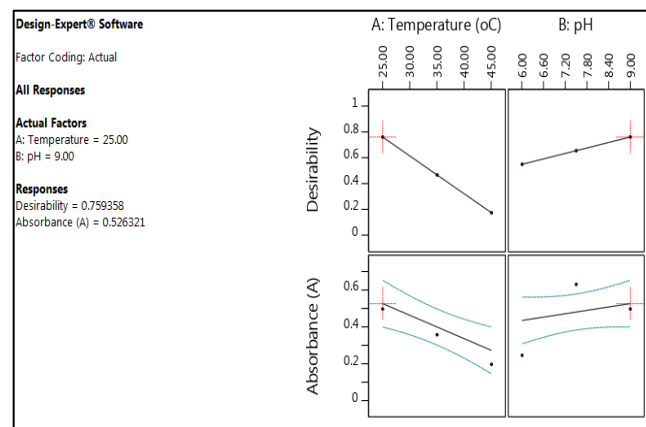


Fig 5: Numerical method modeling of optimization conditions (pH and temperature) for enzyme activity

It clearly indicates that the optimization result for experimental differs from the statistical software. In dye colorimetric experiment, the optimum conditions of highest enzyme activity with absorbance of 0.631 occur at 25 °C and pH of 7.5. This could be due to the selection of data for optimization process using statistical software. However, the design point which given highest absorbance was identified as outlier as discussed earlier and showed in Figure 4 which again highlighted its invalidity.

5 Conclusion

Based on RSM supported with previous literatures, it was suggested that at temperature of 25 °C and pH of 9 could produce the highest enzyme activity (0.526) in PS biodegradation. The simple approach of using dye PS-film as colorimetric methods has successfully measured the enzyme activity of *Bacillus aryabhatai* on biodegradation of polystyrene which has verified by valid data for optimization modelling. This preliminary study discovered the optimum medium conditions (25 °C and pH 9) for *Bacillus aryabhatai* to produce PS-degrading enzyme. This research finding would be a beneficial reference in producing enzyme to degrade plastic wastes, mainly PS. It could be also helpful for further research particularly in environmental science industry.

6 Recommendation

Future works can be conducted with extraction of targeted PS-degrading enzyme from *Bacillus aryabhatai* strain to give a deep insight on enzymatic degradation. It is recommended a more thorough and deeper analysis involving other conditions like cofactors that could probably enhance enzymatic PS biodegradation.

Acknowledgement

The authors would like to thank the Postgraduate Research Grants (GPPS U769) and Research Fund Universiti Tun Hussein Onn Malaysia (UTHM). Authors also gratefully appreciate to laboratory representatives from Department of Chemical Engineering Technology, Faculty of Engineering Technology i.e. Mohd Redzuan Mohd Nor, Masayu Maslan and Aziah Abu Samah for their helps and technical supports in this project.

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