Optimizing the Conditions for Devulcanization of Rubber Waste by Thiobacillus Ferroxidans

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ABSTRACT

Vulcanized rubber is chemically stable. It is difficult to combine it into new material because bonding between the new material and the vulcanized rubber is weak. Present devulcanization process is costly, hence only a small percentage of waste rubber can be recycles, thus a need for a cost effective devulcanization process would permit a substantially greater portion of waste vulcanized rubber to be recycled. Enzymatic devulcanization has been successfully attempted by using bacteria Thiobacillus ferroxidans. This bacteria releases tetrathionate hydrolase that helps to alter the sulphur chains that crosslinked rubber molecules in vulcanized rubber products, after which the rubber surface is now ready to accept virgin rubber during recycling process. Factorial design was applied to determine the optimal physical condition and medium composition for minimum doubling time and maximum protein secretion and accumulation by Thiobacillus ferroxidans. Six independent variables, temperature, initial pH, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ and $CaCl_2$, in the growth medium were tested. KH_2PO_4, pH, $(NH_4)_2SO_4$, $MgSO_4$ was found to promote positive correlation with the minimum doubling time and maximum protein secretion and accumulation. The optimal composition of the growth medium to achieve the minimum doubling time and maximum protein secretion and accumulation was determined as follows (g/l): KH_2PO_4 = 5, $(NH_4)_2SO_4$ = 7.5, $MgSO_4$ = 0.25, $CaCl_2$= 0.25 at initial pH of 4 and 25°C. The corresponding doubling time and protein concentration were 25.92 hours and 67.5μg/ml, which are about 2.0-fold decrease and 2.88-fold increase, respectively, compared with those using the conventional T. ferroxidans growth medium

Keywords: *Enzymatic devulcanization, optimization, rubber waste, Thiobacillus ferroxidans, tetrathionate hydrolase*

Introduction

Malaysia has successfully established itself as a major producer and exporter of rubber and rubber products globally. Malaysian finished rubber products include rubber gloves, footwear, tyres, condoms and prophylactic sheaths. Malaysian rubber is also employed in the building and automotive industry for variety of uses.

Despite the high achievement in rubber production, Malaysia is facing problem as it continually produces million tons of vulcanized rubber waste from rubber containing products such as tyre, gloves, thread and medical product. Vulcanized rubber wastes gave significant health and environmental threat if they are not properly treated. The vulcanized rubber wastes consist mainly of scrap tyre (Tan Cheng Li, 2006). The only known treatment of rubber consist of processes called devulcanization which transform the stable form of rubber into reclaim rubber, rubber which can be added to the virgin rubber or remoulded into other household material (CIWMB, 2004).

Up until now, the world has taken serious effort into developing method of devulcanization. Such methods are using chemical, microwave, ultrasonic, pyrolysis and biological material to devulcanize the rubber (CIWMB, 2004). However, these existing processes have their own constraints and weaknesses. For example, the process of incineration, contrary to the process of

vulcanizing rubber, needed a lot more of energy. To be exact 32 kWh/kg of power is needed to manufacture a tyre, while only 9 kWh/kg is release when incinerating scrap tyre (Reschner, 2006). To add to that the process of incineration require a temperature of 400°C to 600°C for it to decompose vulcanized rubber (CIWMB, 2004). This is not economical as the as the quantity of tyres the public dispose is not small, a total up of 19.7 million tyres or 157,000 tons annually, according to a 2003 study by the Economic Planning Unit (EPU) and Danish International Development Agency (DANIDA). Advanced Pyrotech estimated that 180,000 tons are tossed out each year, or about 500 tons a day (Tan Cheng Li, 2006).

Microbial devulcanization using *Thiobacillus ferroxidans* provide some avenue of solving problem of the other conventional processes. This is due to the characteristic of the microbe, which secretes enzyme in order to utilise elemental sulphur, organic sulphur and iron available in environment for its growth (Rawlings, 2005). Tetrathionate hydrolase was proposed to be enzyme that degrades the sulphur crosslink in the devulcanization process as it catalyzes the metabolism of tetrathionate to sulphate (Ramírez et al., 2004). This rare characteristic of the microbe has made it the excellent choice of microorganism for the process. The devulcanization using *Thiobacillus ferroxidans* process has been proven successful by researchers (Christiansson et al., 1998).

In order to obtain larger biomass and large protein secretion and accumulation in *Thiobacillus ferroxidans* for the devulcanization process, the media composition and its condition will need to be carefully formulated and controlled. The media composition and its condition will affect the fission cycle of the microbe. The cycle is called generation or doubling time, which is a measure of growth rate in a batch process (Todar, 2002).

It is primarily known that the protein or enzyme secreted by the cell is sensitive to the change of pH and temperature. Therefore, temperature and pH is also need to be carefully controlled. Rawling suggested an empirical approach to solve both problem through the optimization of fermentation conditions and medium formulation such as the rate of aeration, the concentration of CO_2 and O_2 in the air, temperature, pH, and the addition of nutrients such as NH_4^+ and PO_4^{3-} (Rawlings, 2005).

Therefore, this study proposes the optimization of process condition (pH, temperature, KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration, $CaCl_2$ concentration) of the growth of *Thiobacillus ferroxidans* to facilitate the process of rubber waste devulcanization. Thorough observations and deep studies of the process conditions will successfully improve and optimize the devulcanization process. The objectives of the study are to optimize cultivation condition to minimize the doubling time and to maximize the protein secretion and accumulation of *Thiobacillus ferroxidans*. Biochemical changes resulting of devulcanization were monitored. The surface changes on the devulcanised rubber were observed physically via electron microscopy.

Materials and Methods

Microorganism

A bacterium, *Thiobacillus ferroxidans* (ATCC #: 19377) was secured from American Type Culture Collection (ATCC), USA.

Propagation of Bacteria
Thiobacillus ferroxidans was first propagated to increase its amount, using the method provided by ATCC.

Cultivation of Bacteria
Thiobacillus ferroxidans was cultivated in a sulphur based media containing KH_2PO_4 (3.0g), $MgSO_4.7H_2O$ (0.5g), $(NH_4)_2SO_4$ (3.0g), $CaCl.2H_2O$ (0.25g), $Na_2S_2O_3.5H_2O$ (5.0g) and distilled water (1.0L). The medium was prepared without thiosulphate, the pH was adjusted to pH 4.4-4.7 and autoclaved at 121°C for 15 minute. Thiosulphate was filter sterilized and aseptically added

after autoclaving.

200ml of sulphur medium was transferred into shake flasks. A flask of the propagated bacteria were shaken gently to dislodge the iron and then 2.0ml was aseptically withdrawn and transferred into the 200ml broth. This medium was used as a basis for the optimization experiment.

Rubber Material

Automobile rubber particles were obtained from automotive workshop of Kulliyyah of Engineering, IIUM. The particles were produced by mechanically grinding of used tyres. They were mixtures of particles with different sizes and had irregular shapes with rough surfaces.

Analytical Analysis

Optical Density

Optical density of the growth culture was measured at 440nm. The UV–spectrophotometer was calibrated to zero by a blank consisting of 1ml media without inoculation. Absorbance reading was taken starting from day one till the reading consistently decrease which can be up to 2 weeks.

Protein Assay by Bradford Method

The BSA standard solution and samples were prepared for Bradford assay according to the Bradford method (Bradford, 1976). 30 minutes after the addition of Bradford reagent, the samples and standard solution were transferred into 1ml cuvette and the absorbance was measured at 595nm. The standard curve for optical density versus BSA concentration was plotted. The optical densities values for the samples were measured and the protein concentrations were determined through the extrapolation from the standard curve of BSA.

Field Emission Scanning Electron Microscope (FESEM)

Rubber samples were dried to preserve the surface structure and prevent collapse of the samples when they are exposed to the FESEM's high vacuum. Before viewing, dried sample was mounted and coated with a thin layer of metal to prevent the build-up of electrical charges on the surface and to give better image. Platinum was used to coat the rubber sample (Prescott et al., 2004).

Experimental Design and Statistical Analysis

Design of the experiment and statistical analysis in this study was done using statistical software, STATISTICA (Version 6.0; Stat-Soft Inc., Oklahoma City, OK, USA). The optimization was done using ¼ factorial designs with five factors: pH, temperature, KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration in the first phase. In the second phase, four factors are used: KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration and $CaCl_2.2H_2O$ concentration.

All design was at two levels, two replications, two centre points and one block for all parameters. In analyzing the results of the experiment, regression analysis was done where multiple regression equation was developed and followed by analysis of the regression equation by statistical tools; ANOVA (analysis of variance), P and T test. The doubling time (day) and maximum protein concentration (μg/ml) was taken as the dependent variables of response (Y). A second order polynomial equation was then fitted to the data by multiple regression procedure. This result in empirical model that related the response measured in the independent variables to the experiment.

The experiments were set to be in three (3) phases so that a thorough optimization of the conditions and media can be achieved.

Phase 1 - Optimizing the Cultivation Condition to Minimize the Doubling Time of Thiobacillus Ferroxidans

Phase 1 is designed to minimize the doubling time of the *T. ferroxidans* through media optimization. The major problem with bacterial devulcanization is that longer time is needed to obtain enough biomass to perform the process. In order to achieve higher growth rate and thus the increase of biomass formation, lower doubling time is necessary (Talaro, 2007). An experiment was designed by using quarter fractional factorial design with 2-centre point to study the effect of five factors; namely temperature, initial pH, KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration on the doubling time. The values used at set point in the optimization were obtained from the conventional media formulation and condition (Table 11). Samples were analyzed everyday for optical density. From this analysis, the specific growth rate was determined from optical density reading and then the doubling time was calculated. The run with lowest doubling time was selected to be the optimum value for phase 2 experiment.

Phase 2 - Optimizing the Cultivation Condition to Maximize Protein Secretion and Accumulation

The aim of this phase is to validate the optimum value obtained from phase 1 by using the maximum protein as response. It was assumed that tetrathionate hydrolase was the key enzyme in the devulcanization of the rubber waste as it oxidizes the sulphur-containing compound (Haaland, 1989). The increase in protein content in shake flask resulting from secretion of enzyme and protein accumulation from *T. ferroxidans* was assumed to have positive effect in increasing quantity of tetrathionate hydrolase. After the best run for minimum doubling time from Phase 1 has been identified, an experiment was designed by using half fractional factorial design with two-centre point. The effects of four factors are used; KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration and $CaCl_2.2H_2O$ concentration on maximum protein secretion and accumulation in a 100 ml shake flask in static condition were studied. By utilizing the best run, these four factors were optimized for maximum protein secretion and accumulation by testing for high and low value with two-centre point. The samples were analyzed everyday for protein content in the pellet and supernatant of the *T. ferroxidans* broth using Bradford method of protein assay. From these data, statistical analysis was used to ascertain the validity of the empirical model derived from multiple regression of the run.

Phase 3 - Devulcanization of Rubber Waste with Optimal Cultivation Condition for Thiobacillus Ferroxidans

The final experiment was done using optimum values for every parameter (optimum temperature, pH and salts concentration) obtained from the optimization experiments. These values were expected to give optimum conditions for *T. ferroxidans* to perform devulcanization of rubber waste. In this final experiment, the grounded tyre rubber was used as substrate in the devulcanization process using *T. ferroxidans*. Samples were analyzed everyday for optical density and protein content. The treated rubber sample were analysed using the Field Emission Scanning Electron Microscope (FESEM) to observe the surface change due to the treatment of both conventional and optimized medium.

Table 1: Experimental Range and Levels of the Independent Test Variables for Phase 1. (g/100ml)

Parameter	Coded	Unit	Levels		
			-1	0	1
Initial pH	pH	-	2	3	4
Temperature	Temp	°C	25°C	30°C	35°C
KH_2PO_4	P	g	0.1	0.3	0.5
$(NH_4)_2SO_4$	NH3	g	0.1	0.3	0.5
$MgSO_4.7H_2O$	Mg	g	0.025	0.05	0.075

Results and Discussion

Phase 1: Optimizing the Cultivation Condition to Minimize the Doubling Time of *Thiobacillus ferroxidans*

By the aid of STATISTICA software, the regression equation was generated based on the obtained experimental results. The generated polynomial regression model relating the doubling time (tdouble) with independent variables initial pH (pH), temperature (Temp), KH_2PO_4 concentration (P), $(NH_4)_2SO_4$ concentration (NH3), $MgSO_4.7H_2O$ concentration (Mg) is as follows:

$$t_{double} = -1.41288 + 1.56542\ pH - 0.39422\ pH*Temp*P*Mg - 0.37477\ pH*NH3 \quad (1)$$

pH*Temp, pH*P, pH*Mg, pH*Temp*P, pH*Temp*NH3, pH*Temp*Mg, pH*Temp*P*NH3 are highly correlated with other variables and has been removed from the equation. Therefore, the model indicates that no interactions between these variables.

The doubling time of *T. ferroxidans* in every run obtained was referred as dependent variables or response of Y. From the statistical software, the regression equation and determination coefficient (R^2) were evaluated as to test the fitness of the design of experiment or model. The model resulted to a high determination coefficient of R^2 (0.935) and adjusted determination coefficient R^2 (adj.) (0.922) which indicate that the model is highly significant (Cochran & Cox, 1957).

The corresponding of analysis of variance (ANOVA) is presented in Table 2. The ANOVA of quadratic regression model demonstrates that the model is highly significant. The computed *F-value* (67.70256) indicates that the model is highly significant and at high confidence level. This is also supported by a very low probability value (*P*=0.00000).

Table 2: ANOVA for the Selected Quadratic Model

Source	Degree of freedom	Sum of Squares	Mean Squares	*F-value*	*P*
Regression	6	1797.268	299.55	99.02956	0.000000
Residual Error	11	33.95900	3.0871		
Total	17	1831.227			

The *T-values* and *P-values* for the linear and quadratic interaction are summarized in Table 3. The significance of each coefficient or factor was determined by the *T-values* and *P-values*. The pattern of interactions between the variables is presented by these coefficients. The variables with low probability levels contribute to the model, whereas others with high probability level can be neglected and eliminated from the model. The low values of *P* of less than 0.05 and the larger magnitude of *T-value* indicates the more significant correlation of coefficients.

Table 3: Statistical Analysis showing Coefficient of *T-value* and *P-value*

Predictor	Coefficient	Standard error coefficient	Computed *T-value*	*P-value*
Constant	-1.41288	0.294319	-4.80051	0.000
pH	1.56542	0.111015	14.1009	0.000
pH*Temp*P*Mg	-0.39422	0.05960	-6.6143	0.000
pH*NH3	-0.37477	0.144931	-2.58583	0.021

Table 3 shows that all *P-values* are less than 0.05 which (*P-values* < *T-values*). This indicates that variable pH, pH*Temp*P*Mg and pH*NH3 have significant effect on doubling time. *T-value* with larger magnitude and smaller *P-values* indicated the high significance of the corresponding coefficient or factor. The value of computed *T-value* determines the level of significance of the variables on doubling time. Thus, it can be evaluated that the variable with the largest effect was the linear term of pH, cubic term of 4 way relation of initial pH, temperature and $MgSO_4$ (pH*Temp*P*Mg) and quadratic term of 2 way relation of initial pH and $(NH_4)_2SO_4$ (pH*NH3) accordingly.

Phase 2: Optimizing the Cultivation Condition to Maximize Protein Secretion and Accumulation

The optimum value obtained in Phase 1 was used as the minimum value in design of experiment of Phase 2. The result obtained in Phase 1 does not point to the optimum value as desired. The uncertainty in determining the specific optimum value rose to the fact that the experimental design used in this optimization uses only two levels. For example, the KH_2PO_4 optimum value obtained from Phase 1 is 0.5g, but there are possibilities that the value may be larger than 0.5g. Therefore, another experiment was set up to determine if the values obtained in the Phase 1 is the optimum.

The analysis used here is the protein concentration by protein assay by Bradford method of protein assay in the pellet and supernatant of the broth of *T. ferroxidans*. Since the protein concentration in the pellet and supernatant increases with the growth (growth associated production), there is no dire need to measure the optical density.

After the potential run was selected from Phase 1, four factors, namely: KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ concentration and $CaCl_2.2H_2O$ concentration were observed to study the maximum protein secretion and accumulation. The two level quarter fractional factorial design was used to obtain the possible conditions for maximum protein secretion and accumulation.

The initial pH factor (pH) was very significant due to low *P-value* and high *T-value* found in Phase 1. Therefore, it was kept constant at initial pH of 4 since any change in initial pH would result in greater positive change in doubling time. This was due to the large effect of initial pH on doubling time as shown by large positive *T-value* (14.1009) compared to other effect. Since temperature factor (Temp) has been removed from the equation, operation at any value of temperature would be favourable for minimum doubling time. The temperature of developed condition was set to be at minimum point: 25°C.

Table 4: Experimental Range and Levels of the Independent Test Variables for Phase 2 (In g/100ml)

Component	Coded	Unit	Levels		
			-1	0	1
KH_2PO_4	P	g	0.5	0.625	0.75
$(NH_4)_2SO_4$	NH3	g	0.5	0.625	0.75
$MgSO_4.7H_2O$	Mg	g	0.025	0.0375	0.05
$CaCl_2.2H_2O$	Ca	g	0.01	0.025	0.04

In order to search for the optimum value of process conditions, a total of 18 treatments for each optimization were established by using statistical software applying factorial design with different range of the said parameters (Table 4). The polynomial regression model relating to the maximum protein secretion and accumulation (MaxProt) with independent variables KH_2PO_4 concentration (P), $(NH_4)_2SO_4$ concentration (NH3), $MgSO_4.7H_2O$ concentration (Mg), $CaCl_2.2H_2O$ concentration (Ca) is as follows:

$$\text{MaxProt} = 88.682 - 82.0\ \text{P} + 46.618\ \text{NH3} - 474.208\ \text{Mg} \qquad (2)$$

P*Ca, P*NH3, P*Mg, P*NH3*Mg and P*NH3*Ca is highly correlated with other variables and has been removed from the equation. Therefore, the model indicates that there are interactions between these variables.

The maximum protein secretion and accumulation of *T. ferroxidans* in every run obtained was referred as dependent variables or response of Y. From the statistical software, the regression equation and determination coefficient (R^2) were evaluated as to test the fitness of the design of experiment or model. The model resulted in a high determination coefficient of R^2 (0.981) which means 98.1% of the factors correlated with each other. The value of the adjusted determination coefficient (R^2 (adj.)) was also shown very high value (96.713%) to indicate the high significance of the model (Haaland, 1989).

The corresponding of analysis of variance (ANOVA) is presented in Table 5. The ANOVA of quadratic regression model demonstrates that the model is highly significant. The computed *F-value* (99.02956) indicates that in overall, the model is highly significant and at high confidence level. This is also supported by very low probability value (*P*=0.000000).

Table 5: ANOVA for the Selected Quadratic Model

Source	Degree of freedom	Sum of Squares	Mean Squares	*F-value*	*P*
Regression	6	1797.268	299.55	99.02956	0.000000
Residual Error	11	33.95900	3.0871		
Total	17	1831.227			

The *T-values* and the corresponding *P-values* of the variable estimation were evaluated using STATISTICA software. The significance of each coefficient or factor was determined by *T-values* and *P-values*. The pattern of interactions between the variables is indicated by these coefficients. The variables with low probability levels contribute to the model, whereas others with high probability level can be neglected and eliminated from the model. *T-value* with larger magnitude

and smaller *p-value* indicated the high significance of the corresponding coefficient or factor (Haaland, 1989). The *T-values* and *P-values* for the linear, quadratic and the iterative terms are presented in Table 6

Table 6: Statistical Analysis showing Coefficient of *T-value* and *P-value*

Predictor	Coefficient	Standard error coefficient	Computed *T-value*	*P-value*
Constant	88.682	13.2499	6.69306	0.000
P	-82.001	20.8211	-3.93833	0.003
NH3	46.618	17.9182	2.60172	0.024
Mg	-474.208	179.1824	-2.64651	0.023

Table 6 shows that all *P-values* are less than 0.05 which (*P-values*< *T-value*). This indicates that variable P, NH3 and Mg have significant effect on maximum protein secretion and accumulation. The value of computed *T-value* determines the level of significance of the variables on maximum protein secretion and accumulation. Thus, it can be evaluated that the variable with the largest effect was the linear term of KH_2PO_4 (P) followed by the linear term of $MgSO_4.7H_2O$ (Mg) and linear term of $(NH_4)_2SO_4$ (NH3). The factor $CaCl_2$ (Ca) has been discarded since the *P-value* of the factor was too high to be included in the model.

Phase 3: Devulcanization of Rubber Waste with Optimal Cultivation Condition for *Thiobacillus ferroxidans*.

A final experiment was done using optimum values obtained from the phase 2. The developed process condition was obtained by using the regression equation generated by STATISTICA software. Since factor $CaCl_2.2H_2O$ (Ca) has been removed from the equation, changing the value of Ca will not result in any changes in the expected maximum protein secretion and accumulation. The $CaCl_2.2H_2O$ concentration of developed condition was set to be at centre point; 0.250g/l

Table 7: Conventional and Optimum Parameter Conditions for Propagation, Maximum Protein Secretion and Accumulation in 100 ml Shake Flask (g/100ml) (DSMZ, 2005)

Parameters	KH_2PO_4	$(NH_4)_2SO_4$	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	pH	Temperature
Conventional Value	0.3g	0.3g	0.05g	0.025g	4.4	25
Optimum Value	0.500g	0.750g	0.0250g	0.025g	4	25

One gram of grounded rubber tyre is washed with ethanol 95%, suspended in 100 ml medium and inoculated with 1 ml of *T. ferroxidans* and was grown in shake flask. Two media were used, the optimized media and conventional media (see Table 7). A shake flask, not inoculated, but otherwise treated as in optimum value parameters, was included as a reference experiment. After 11 days of cultivation, the rubber flakes were withdrawn from the shake flask. It was rinsed with distilled water and dried in oven at 60°C. Rubber flakes surfaces were investigated using a JSM-6700F Field Emission Scanning Electron Microscope (FESEM).

The result in Table 8 shows the different on specific growth rate, doubling time and maximum protein secretion and accumulation between the conventional and optimized media.

Table 8: Comparison on Specific Growth Rate, Doubling Time and Maximum Protein Secretion and Accumulation between the Conventional and Optimized Media.

Media Parameter	Conventional Media	Optimized Media
Specific Growth rate, μ (day^{-1})	0.584	0.626
Doubling Time, t_d (day)	2.226	1.1
Maximum Protein secretion and accumulation, (µg/ml)	23.4138	67.5072

Conclusion

The developed process condition was obtained by using the multiple regression equation generated by STATISTICA software. The developed condition was found to be at initial pH of 4 and 25°C and with concentration in (g/l) of KH_2PO_4 = 5g, $(NH_4)_2SO_4$ = 7.5g, $MgSO_4$ = 0.25g. Since factor $CaCl_2$ (Ca) has been removed from the equation; operation at any value of $CaCl_2$ would be favourable for minimum doubling time and maximum protein production. The $CaCl_2$ of developed condition was set to be at the centre point: 0.250g/l. The predicted doubling time by the optimized media is 0.98-day ≈ 23.52 hours. The measured doubling time achieve by the optimized media is 1.1 day ≈ 26.4 hours. The measured maximum protein production was achieved on day 11 of fermentation with the amount of 67.9µg/ml. This was lower than the phase 2 protein production of 69.7939µg/ml. In conclusion, there are many factors identified to give significant effects on the growth of *T. ferroxidans* in a shake flask. However, results show the absence of interactions between pH, temperature, KH_2PO_4 concentration, $(NH_4)_2SO_4$ concentration, $MgSO_4$ concentration and $CaCl_2$ concentration.

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