JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4465-4469

Optimization of Media Components for Tetrathionate Hydrolase Production from *Thiobacillus ferrooxidans*

Azratul Ashimah Nur Mohd Dom and Faridah Yusof*

Department of Biotechnology Engineering, Faculty of Engineering,
International Islamic University Malaysia, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.

(Received: 20 August 2014; accepted: 27 October 2014)

Tetrathionate hydrolase produced by *Thiobacillus ferrooxidans* has been known to be the enzyme responsible in the devulcanization of used rubber prior to recycling. The aim of this study was to statistically obtain a model that would yield an optimized media concentrations for the best production of this bacterial enzyme. One-factor-at-a-time (OFAT) experimental design was applied to find the possible optimum range of five media components. Based on the OFAT results, a face centered central composite design (FCCCD) by Design Expert® 6.0.8 was applied using a 4-factors, 3-center points to find the optimum media concentrations to yield the highest tetrathionate hydrolase. Response Surface Methodology (RSM) predicted the level of factors for maximized response. In this study, it is concluded that the medium containing 0.4g/L $MgSO_4.7H_2O$, 4.0g/L $(NH_4)_2SO_4$, 2.0g/L $Na_2S_2O_3.5H_2O$ and 3.0 g/L KH_2PO_4 results in the production of the highest specific activity of tetrathionate hydrolase (295.7 U/mg Protein). Other parametric conditions used were 0.25g/L for $CaCl_2.2H_2O$ with an initial pH buffer of 4, incubated at 25°C while agitating at a speed of 125 rpm.

Key words: *Thiobacillusferrooxidans*, tetrathionate hydrolase, OFAT, FCCCD, devulcanization of rubber.

Thiobacillus ferrooxidans are autotrophic sulfur-oxidizing bacteria belonging to the family of *Thiobacteriaceae* and are commonly termed as colourless sulfur bacteria[1]. These microorganisms whose energy metabolism is uniquely adapted to obtain all the energy required for growth from oxidation of inorganic sulfur compounds to sulfate and utilize carbon dioxide for the synthesis of cellular materials[2]. The ability of these sulfur-oxidizing bacteria to oxidize sulfide and reduced inorganic sulfur compounds are attributed to an enzyme system present in the cell[3] by which sulfide or the reduced inorganic sulfur compounds are biologically oxidize to sulfate[4].

* To whom all correspondence should be addressed.
Tel.: +6017-3669840; Fax:+603-6196 4442;
E-mail: yfaridah@iium.edu.my

This mentioned enzyme is known as tetrathionate hydrolase, reported to be responsible for the enzymatic devulcanization of waste rubber products where it breaks the sulfur cross-linking in vulcanized tires. Until now, the exact cell location of this enzyme is still unclear. Buonfiglio *et al*., described this enzyme as an outer membrane protein[5]. However, de Jong *et al*., and Chi *et al*., described it as a soluble and probably periplasmic protein[6,7] while Kanao *et al*. reported it to be a membrane-associated protein[8].

The aim of this study was to statistically obtain a model equation that would yield an optimized media concentrations for the highest production of this bacterial enzyme. Since the location of the enzyme is still unclear, in this study, tetrathionate hydrolase was extracted from the incubation media as well as the cell lysate.

MATERIALS AND METHODS

One-factor-at-a-time analysis was employed to evaluate the possible optimum levels of the medium components that showed positive effects. The five media components of nutrients studied for tetrathionate hydrolase production were KH_2PO_4, where the range of concentration studied were from 0.0 - 0.5% (w/v), $MgSO_4.7H_2O$ from 0.0 – 0.05% (w/v), while $(NH_4)_2SO_4$, from 0.0 – 0.5% (w/v). In the cases of $Na_2S_2O_3.5H_2O$ and $CaCl_2.2H_2O$, the ranges studied were from 0.0 – 0.5% and 0.0 – 0.03% respectively. Face centered central composite design (FCCCD) was used to optimize the four screened variables for enhancing tetrathionate hydrolase production. The four significant factors were chosen based on the results obtained from the OFAT experiments and they were KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and $Na_2S_2O_3.5H_2O$. Using Design Expert software, the 4 independent factors and 3 center points yielded a set of 27 experimental runs.Also, based on the OFAT results, $CaCl_2.2H_2O$ was maintained at a fixed concentration. The initial pH buffer of 4, agitation speed of 125 rpm and incubation temperature at 25°C were maintained in the incubation shaker based on results of a previous study[9]. Fermentation was carried out in a batch process using shake-flask culture technique and samples were collected at 24 and 48 hours for enzyme and protein assays.

RESULTS AND DISCUSSION

One-factor-at-a-time (OFAT) Analysis

Figure 1 and 2 represents the responses obtained in the OFAT analysis, in terms of tetrathionate hydrolase activity and specific activity respectively, as a function of $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, $Na_2S_2O_3.5H_2O$, $CaCl_2.2H_2O$ and KH_2PO_4 concentration in % (w/v). Yusof and Ahmad have

Table 1. Results of specific activity of tetrathionate hydrolase using four selected media components

Run	Fact. A $MgSO_4.7H_2O$(g/L)	Fact. B $(NH_4)_2SO_4$(g/L)	Fact. C $Na_2S_2O_3$(g/L)	Fact. D KH_2PO_4(g/L)	Resp. Specific Activity (U/mg)
1	0.70	1.00	0.50	5.50	6.25
2	0.40	4.00	0.50	3.00	103.96
3	0.70	4.00	2.00	3.00	12.61
4	0.70	7.00	0.50	0.50	9.30
5	0.40	7.00	2.00	3.00	2.75
6	0.10	1.00	0.50	5.50	69.57
7	0.10	7.00	0.50	0.50	9.37
8	0.70	7.00	0.50	5.50	61.07
9	0.10	7.00	0.50	5.50	45.32
10	0.40	4.00	2.00	0.50	132.41
11	0.70	7.00	3.50	0.50	79.89
12	0.40	1.00	2.00	3.00	48.31
13	0.70	1.00	3.50	5.50	14.76
14	0.70	1.00	0.50	0.50	50.35
15	0.10	1.00	3.50	5.50	49.99
16	0.10	1.00	3.50	0.50	1.94
17	0.40	4.00	2.00	3.00	295.70
18	0.70	1.00	3.50	0.50	2.03
19	0.10	1.00	0.50	0.50	153.80
20	0.40	4.00	2.00	3.00	98.84
21	0.10	7.00	3.50	0.50	44.59
22	0.10	7.00	3.50	5.50	116.52
23	0.40	4.00	2.00	5.50	247.95
24	0.40	4.00	2.00	3.00	211.77
25	0.10	4.00	2.00	3.00	3.69
26	0.40	4.00	3.50	3.00	111.91
27	0.70	7.00	3.50	5.50	13.26

optimized the process conditions to promote the growth of *Thiobacillus ferooxidans* and the accumulation of protein during the growing process[9]. According to their findings, the highest bacteria growth and highest protein accumulation was achieved when the optimized concentration of $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and $Na_2S_2O_3.5H_2O$ were 0.03g, 0.4g and 0.5g respectively. While $CaCl_2.2H_2O$ and KH_2PO_4 were 0.025g and 0.4g respectively in

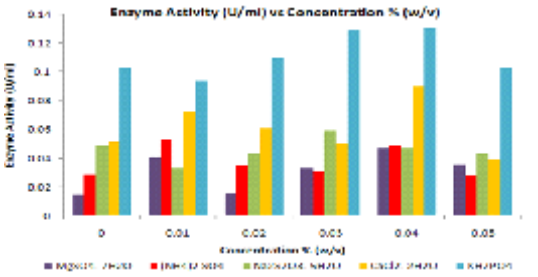

Fig. 1. OFAT for tetrathionate hydrolase activity were done at temperature 25°C and pH incubation of 4. The absorbance was measured at 290nm after 48 hours

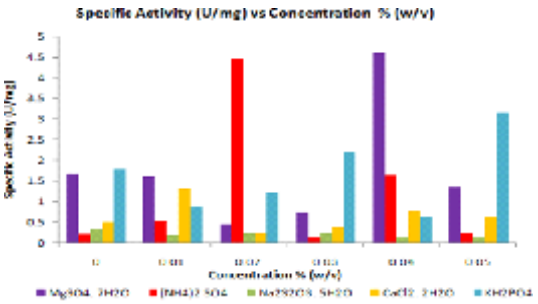

Fig. 2. OFAT for specific activity of tetrathionate hydrolase were done at temperature 25°C and pH incubation of 4. The absorbance was measured at 290nm after 48 hours

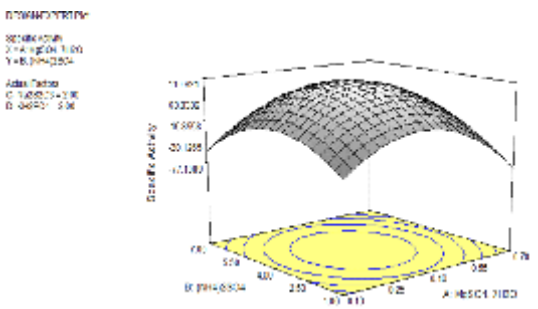

Fig. 3. Response surface for specific activity of tetrathionate hydrolase after 48 hrs incubation at pH 4, 25°C.

100 mL dH_2O with initial pH buffer 4, agitation speed of 125 rpm and incubation temperature at 25°C.

However, the present study focused on the optimizing the media components to improve the production of tetrathionate hydrolase. Based on the optimized growth conditions reported by Yusof and Ahmad, the production of tetrathionate hydrolase was screened and measured by first employing OFAT analysis on five media components.

The results show that the effect of $MgSO_4.7H_2O$ to the activity of tetrathionate hydrolase was highest at the concentration of 0.04% (w/v) which was 0.047 U/ml. This concentration was found to be adequate for enzyme activity since Mg^{2+} plays some regulatory functions through the increase of ATP metabolism and nucleic acid synthesis[10]. For nitrogen sources, inorganic source was used which was $(NH_4)_2SO_4$. The highest tetrathionate hydrolase activity was 0.053 U/ml at 0.1% (w/v). Most enzymes such as amylase and protease are repressed by ammonium salts[11]. This is also applicable to tetrathionate hydrolase where at concentration of 0.5% (w/v), the enzyme activity was low.

$Na_2S_2O_3.5H_2O$ was supplemented to the media as food and carbon source for the bacteria. At the concentration of 0.3% (w/v), the activity of enzyme was maximal (0.059 U/ml). Schook and Berk reported that the increasing amount of thiosulfate in the growth medium did not stimulate bacterial growth[12]. Thus, further addition of more than 0.3% (w/v) resulted in a decrease in tetrathionate hydrolase production.

Phosphate is a key element in the metabolic currencies of known life forms and provides the connecting bridge between the nucleobases in RNA and DNA[13]. Various concentrations of KH_2PO_4 were found to have positive effect on tetrathionate hydrolase activity. The highest enzyme activity was 0.131 U/ml at a concentration 0.4% (w/v). While for $CaCl_2.2H_2O$, the highest enzyme activity was 0.090 U/ml at concentration 0.025% (w/v).

Response Surface Methodology

Table 1 presents the responses obtained in the experimental design, in terms of tetrathionate hydrolase specific activity, as a function of $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, $Na_2S_2O_3.5H_2O$ and KH_2PO_4 concentration in (% w/v), where one can

observe that the highest tetrathionate hydrolase specific activity (Run 17, 295.70 U/mg) was obtained when all the factors used were at the center point.

The analysis of variance (ANOVA) permitted us to validate an empirical model (Equation 1) for tetrathionate hydrolase specific activity, with a correlation coefficient of 0.50 ($p<0.05$)and to build the response surface presented in Figure 3.

$$Y_{Sp} = 110.37-13.63 *A-0.83 *B-4.12 *C + 7.83*D-91.36 *A2-73.97*B2 + 8.43 * C2 + 90.67 *D2 + 9.35*AB+ 3.00 *AC-7.37 *AD +21.28 *BC+ 10.04 *BD +6.67* CD \quad ...(1)$$

where Y_{Sp} is tetrathionate hydrolase specific activity, A is $MgSO_4.7H_2O$, B is $(NH_4)_2SO_4$, C is $Na_2S_2O_3.5H_2O$, and D is KH_2PO_4 concentration.

The response surface in Figure 3 was described by the model equation to estimate specific activity over the independent variables $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$. This measurement refers to the activity of enzyme per milligram of total protein (µmol/ min. mg) at specific pH and temperature. It is seen that the specific activity is optimal when both salts are at 0.40g/L and 4.00g/L respectively. Harahuc *et al*. reported that low magnesium ion requirement can be attributed to the slow growth of bacteria culture[14]. Thus, specific activity is low when magnesium ion is low. *Thiobacillus ferrooxidans* acquire nitrogen in the form of NH^{4+}. As mentioned earlier in the above text, tetrathionate hydrolase production usually repressed by ammonium salts. Thus, at 4.00g/L of $(NH_4)_2SO_4$ is an adequate amount for this enzyme to function. This model will be validated in the future for further analysis.

CONCLUSIONS

In this study, it is concluded that the concentration of 0.4g/L $MgSO_4.7H_2O$, 4.0g/L $(NH_4)_2SO_4$, 2.0g/L $Na_2S_2O_3.5H_2O$ and 3.0 g/L KH_2PO_4 results in the production of the highest specific activity of tetrathionate hydrolase (295.7 U/mg Protein). Other parametric conditions were maintained as previously found. They were $CaCl_2.2H_2O$ at 0.25g/L with an initial pH buffer of 4, incubated at 25°C while agitating at a speed of 125 rpm.

ACKNOWLEDGEMENTS

This work is supported by the Ministry of Science, Technology and Innovation (MOSTI), Malaysia through the E-Sciencefund scheme (Grant No: SF12-020-0049) and we gratefully acknowledge the support.

REFERENCES

1. Kuenen, J. G. Colourless sulfur bacteria and their role in the sulfur cycle. *Plant Soil*., 1975; **43**: 49-76.
2. Peck, H. D. Jr. Comparative metabolism of inorganic sulfur compounds in microorganisms. *In:* Symposium on Metabolism of Inorganic Compounds. April 26, 1961; Chicago, Illinois. 67-94.
3. Kurosawa, H. Endo, S., Hirano, T., Nakamura, K., Amano, Y. Stabilization of freeze-dried *Thiobacillusthiooxidans*cells as a bacterial deodorant for removal of hydrogen sulfide. *J. Ferment. Bioeng.*,1997; **83**(2): 213-215.
4. Das, S. K., Mishra, A. K., Tindall, B. J. Rainey, F. A., Stackerbrandt, E. (1996). Oxidation of thiosulfate by a new bacterium, *Boseathiooxidans* (strain BI-42) gen. nov., sp. nov.: Analysis of phylogeny based on chemotaxonomy and 16S Ribosomal DNA sequencing. *Int J. Sys. Bacteriol.*, 1996; **46**(4): 981-987.
5. Buonfiglio, V., Polidoro, M., Soyer, F., Valenti, P., Shively, J. A novel gene encoding a sulfur-regulated outer membrane protein in *Thiobacillusferrooxidans*. *J. Biotechnol.*, 1999; **72**: 85–93.
6. de Jong, G. A. H., Hazeu, W., Bos, P., Kuenen, J. G. Polythionate degradation by tetrathionate hydrolase of *Thiobacillusferrooxidans*. *Microbiology*., 1997; **143**: 499–504.
7. Chi, A., Valenzuela, L., Beard, S., Mackey, A. J., Shabanowitz, J., Hunt, D. F., Jerez, C. A. Periplasmic proteins of the extremophile *Acidithiobacillusferrooxidans:* A high throughput proteomics analysis. *Mol. Cell. Proteomics.*, 2007; **6**: 2239–2251.
8. Kanao, T., Kamimura, K., Sugio, T. Identification of a gene encoding a tetrathionate hydrolase in *Acidithiobacillusferrooxidans*. *J. Biotechnol.*, 2007; **132**: 16–22.
9. Yusof, F., Ahmad, A. A. Enzymatic devulcanization of waste rubber, In *Current Research and Development in Biotechnology Engineering at IIUM;* 2011; **2**: 144-153. IIUM

Press, ISBN No: 978-967-418-151-2.
10. Bankar, S. B., Bule, M. V., Singhal, R., Ananthanarayan, L. Optimization of *Aspergillus niger* fermentation for the production of glucose oxidase. *Food Bioprocess Technol.,* 2009; **2**: 344 -352.
11. Saxena, R. K., Dutt, K., Agarwal, L., Nayyar, P. Highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. *Biores. Technol.,* 2007; **98**: 260-265.
12. Schook, L., Berk, R. S. Nutritional Studies with *Pseudomonas aeruginosa* grown on inorganic sulfur sources. *J. Bacteriol.,* 1978;**133**(3): 1377-1382.
13. Tawfik, D. S., Viola, R. E. Arsenate replacing phosphate: Alternative life chemistry and ion promiscuity. *Biochemistry.,* 2011; **50**: 1128–1134.
14. Harahuc, L., Lizama, H. M., Suzuki, I. Selective inhibition of the oxidation of ferrous iron or sulfur in*Thiobacillusferrooxidans.Applied and environmental microbiology.,* 1999; **66**(3): 1031–1037.