

Isolation and statistical optimization of rhodanese (a thiosulphate sulphur transferase) production potential of *Klebsiella oxytoca* JCM 1665 using response surface methodology

Babamotemi Oluwasola Itakorode^{1,2,✉}, Raphael Emuebie Okonji²

¹Department of Chemical Sciences, Oduduwa University Ipetumodu, Ile-Ife, Osun State, Nigeria

²Department of Biochemistry and Molecular Biology, Faculty of Science, Obafemi Awolowo University Ile-Ife, Osun State, Nigeria

✉ Corresponding author: itakorgsoli29@gmail.com

Article info

Article history:

Received: 2nd August 2021

Accepted: 20th December 2021

Keywords:

Klebsiella oxytoca

Optimization

Polymerase chain reaction

Response surface methodology

Rhodanese

Abstract

Microorganisms are increasingly being used in cyanide bioremediation. Several organisms have been reported to thrive in cyanide contaminated wastewater due to their ability to produce cyanide detoxifying enzymes. However, to improve the production efficiency of these enzymes combinations of process variables need to be optimized. In this study, *Klebsiella oxytoca* JCM 1665 was isolated from industrial wastewater, identified by sequencing its 16S rRNA gene and subjected to rhodanese production using submerged fermentation. The conditions for production were optimized using response surface methodology (RSM). Central composite design was employed to evaluate the effects of three production parameters – peptone (1 – 5 %), KCN (0.1 – 0.5 %), and time of incubation (1 – 24 h). Second-order polynomial model was used to predict the response. Rhodanese activity in the experiments varied from 0.05 to 7.5 RU.mg⁻¹. Under the optimum conditions of 4.35 % peptone, 0.4 % KCN and incubation time of 13 hr., the value for rhodanese yield was 7.810 U.mL⁻¹. The R² value for the model was 0.9925 (R² = 0.9925). Also, the experimental values are in accordance with those predicted, indicating the suitability of the employed model and the success of RSM in optimizing the production conditions.

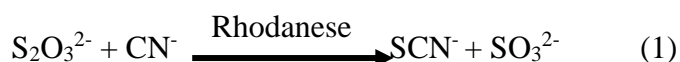
Introduction

Cyanides are toxic chemicals that are widely present in both the abiotic and biotic components of an ecosystem. It acts as a defense mechanism in various organisms such as fungi, bacteria, algae and plants. However, the amount of cyanide generated by these organisms is insignificant when compared to the ones generated through anthropogenic activities (Luque-Almagro *et al.* 2016). In industries, cyanide is used for gold extraction and in the synthesis of various

agrochemicals such as fertilizers, liming and pesticides. Cyanide toxicity is due to its high binding affinity to metal ions; it inhibits metalloenzymes especially the cytochrome c oxidase in the electron transport chain (Luque-Almagro *et al.* 2016). The amount of cyanide in the environment has been observed to have increased greatly due to rapid industrialization and cyanide leaching methods have been the major techniques in gold extraction. The demand for gold jewellery in countries like the United States, Russia and most African countries has led to the increase

in mining activities (Mudder *et al.* 2000). Industrial processes such as mining and electroplating have contributed significantly to the cyanide contamination in water bodies. To minimize environmental disasters, cyanide-containing liquid must be properly treated before releasing into the water bodies. The removal of cyanide from the effluent is one of the challenges in the cyanide-based industries to fulfil their standards and recycling of the water (Kuyucak and Akcil 2013). Despite the toxicity of cyanide, cyanotrophic microorganisms such as *Pseudomonas sp.* (Akciil and Mudder 2003; Oyedeji *et al.* 2013), *Bacillus pumilus* (Kandasamy *et al.* 2015), and *Bacillus cereus* (Itakorode *et al.* 2019) has been reported to survive in the presence of cyanide due to their ability to synthesize cyanide metabolizing enzyme such as rhodanese.

Rhodanese catalyzes cyanide detoxification by transferring sulfur from a suitable substrate such as thiosulfate to cyanide. This leads to the formation of a less toxic compound (thiocyanate) that can be excreted in the urine (Eq. 1):



where $\text{S}_2\text{O}_3^{2-}$ – thiosulfate, CN^- – cyanide ion, SCN^- – thiocyanate ion, SO_3^{2-} – sulfite.

Its role as a detoxification enzyme is supported by its mitochondria and cytosolic localization (Cipollone *et al.* 2007; Steiner *et al.* 2018). Although the use of microorganisms has been proven to be viable in cyanide bioremediation, enzymatic proteins may be of good alternative (Gianfreda *et al.* 2004; 2010). Enzymes can retain its activity at extreme conditions that limit microbial activities. Also, they are not inhibited by inhibitors of microbial metabolism and the technique is eco-friendly. Therefore, the importance of producing cyanide metabolizing enzymes for industrial application cannot be overemphasized.

Many factors including incubation time, the temperature, the pH, carbon and nitrogen sources contribute to the efficacy of metabolite production by organisms (Adekunle *et al.* 2017; Itakorode *et al.* 2019). Optimization of metabolites production

may be achieved by either one factor at a time or statistical means (Rodrigues *et al.* 2008; Annegowda *et al.* 2012). Response surface methodology (RSM) is a statistical experimental protocol used in mathematical modelling (Triveni *et al.* 2001; Gong *et al.* 2012). RSM technique reduces the experimental assays and improves the statistical interpretation and interaction between variables (Tsapatsaris *et al.* 2004; Yim *et al.* 2012). The RSM can give a mathematical equation. It is also helpful to calculate the response value when different levels of variables are set. Central Composite Design is a widely used protocol in response surface methodology (Yang *et al.* 2008; Rao 2010). The impact of industrialization on the quality of the environment is evident and there is a need to have eco-friendly strategies to treat effluent for the sustainable development of industries. Hence, this study aims to investigate optimal production conditions of rhodanese by *Klebsiella oxytoca*.

Experimental

Wastewater was collected from iron and steel smelting company in sterile plastic bottles and stored at 4 °C. It was centrifuged at 5,000 × g for 10 min. The clarified supernatant was collected and used for further analysis. Physicochemical parameters such as pH, turbidity, chemical oxygen demand, total dissolved solids, dissolved oxygen, cadmium and Lead were determined using the methods described by Ademoroti (1996).

Isolation and screening for rhodanese production

A loopful of diluted sample was spread on a modified Bushnell Hass agar plate and incubated at 37 °C for 96 h to select for cyanide degrading bacteria. Rhodanese producing potential of the isolates was done by employed the method described by Zlosnik and Williams (2004). Production medium consists of NaCl (0.5 g/100 mL), bacteriological peptone (1 g/100 mL) and KCN (0.3 g/100 mL) at pH of 6.0. The growth media prepared were inoculated with standardized cells suspension. The culture media were checked for rhodanese activity for 24 h at an interval of 2 h.

Isolates identification and characterization

The most productive strain was identified by sequencing its 16S rRNA gene. This was carried out at the Bioscience Centre of the International Institute of Tropical Agriculture (Ibadan, Oyo State, Nigeria). DNA extraction was carried out using modified method of [Trindade et al. \(2007\)](#). The assay mixture for Polymerase Chain Reaction (PCR) amplification consists of 4 μL of the DNA solution, 0.4 μL of 10 mM dNTPs, 2 μL of 25 mM MgCl_2 , 1 μL of 10 pmol each of primer (Forward 5'-CCAGCAGCCGCGGTAATACG-3' and Reverse 5'-TCGGCTACCTTGTTACGACTTC-3') ([Yamamoto and Harayama 1995](#)), 0.24 μL of Taq polymerase (1 $\text{U}\cdot\mu\text{L}^{-1}$) (Promega USA) and the 5 μL of 5 \times PCR buffer. Sterile DNase free water was added to make a volume of 25 μL . PCR was conducted in an automated thermal cycler. The thermal conditions were as follows: denaturation at 94 $^\circ\text{C}$ for 1 min, annealing at 56 $^\circ\text{C}$ for 1 min and 1 min extension at 72 $^\circ\text{C}$. The PCR amplicons were visualized using 1.5 % agarose gel electrophoresis.

Enzyme assay and experimental design

Sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) and potassium cyanide (KCN) were used as substrates for rhodanese activity. 1 mL of the assay mixture consists of 50 mM borate buffer (pH 9.4), 0.25 M KCN, and $\text{Na}_2\text{S}_2\text{O}_3$ and 0.1 mL of the enzyme. The enzyme reaction was terminated with 0.5 mL 38 % formaldehyde after 1 min of incubation at 25 $^\circ\text{C}$ ([Lee et al. 1995](#)). Concentration of thiocyanate produced was quantified by the addition of 1.5 mL Sorbo reagent (which is made up of 10 g $\text{Fe}(\text{NO}_3)_2\cdot 9\text{H}_2\text{O}$, 20 mL HNO_3 and 80 mL distilled water) ([Sorbo 1953](#)). The absorbance of the reaction medium was taken at 460 nm. The unit of rhodanese activity (RU) is defined as the micromoles of the product (thiocyanate) formed in one minute. The protein concentration was determined by the method of [Bradford \(1976\)](#), bovine serum albumin (BSA) was used as standard. The extracellular production of rhodanese was established by RSM which was employed to determine the best combination of variables for optimum rhodanese production by *K. oxytoca*. The independent variables used in this study were

peptone concentration (A – % (w/v)), potassium cyanide (B – % (w/v)), and time (C – h) while response was rhodanese activity ($\text{RU}\cdot\text{mg}^{-1}$). The coded and uncoded levels of the independent variables are presented in [Table 1](#).

Table 1. Coded and decoded levels of independent variables used in the RSM design.

Run	A: Peptone	B: KCN	C: Time
1	-1 (0.36)	0 (0.30)	0 (12.50)
2	-1 (1.00)	+1 (0.50)	-1 (1.00)
3	+1 (6.30)	0 (0.30)	0 (12.50)
4	0 (3.00)	0 (0.30)	0 (12.50)
5	-1 (1.00)	-1 (0.10)	+1 (24.00)
6	+1 (5.00)	+1 (0.50)	-1 (1.00)
7	0 (3.00)	0 (0.30)	0 (12.50)
8	-1 (1.00)	+1 (0.50)	+1 (24.00)
9	+1 (5.00)	-1 (0.10)	+1 (24.00)
10	0 (3.00)	-1 (0.03)	0 (12.50)
11	0 (3.00)	0 (0.30)	+1 (31.84)
12	0 (3.00)	0 (0.30)	0 (12.50)
13	0 (3.00)	+1 (0.60)	0 (12.50)
14	0 (3.00)	0 (0.30)	0 (12.50)
15	0 (3.00)	0 (0.30)	0 (12.50)
16	+1 (5.00)	+1 (0.50)	+1 (24.00)
17	0 (3.00)	0 (0.30)	-1 (6.84)
18	+1 (5.00)	-1 (0.10)	-1 (1.00)
19	0 (3.00)	0 (0.30)	0 (12.50)
20	-1 (1.00)	-1 (0.10)	-1 (1.00)

Statistical analysis

The experimental data were analyzed using JMP 11's response surface regression algorithm (statistical analysis system Inc., SAS). P-values under 0.05 ($P < 0.05$) were considered significant. ANOVA was used to assess the quality of the mathematical models fitted by RSM, based on the F-test and the percentage of total explained variance (R), as well as the adjusted determination coefficient (R^2_{adj}), which provide a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their linear and quadratic interactions ([Granato et al. 2012](#)). To fit the data, a second-order polynomial quadratic equation ([Eq. 2](#)) was used.

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i < j} \beta_{ij} x_i x_j + \sum_{i=1}^k \beta_{ii} x_i^2 + \epsilon. \quad (2)$$

where Y is the predicted response, β_0 , β_i , β_{ii} , β_{ij} , are the correlation coefficients for intercept, linear, quadratic and interaction terms, respectively and x_i and x_j are the levels of the independent variables. Experimental data were fitted to the chosen regression model to have a better understanding of the relationship between each variable and response. The predictive equation of RSM was used to find the optimal conditions for the production of rhodanese. The validity of the model was determined by comparing the experimental and predicted response values.

Results

Isolation and identification of the bacteria isolate

Table 2 shows the physicochemical properties of the wastewater such as the chemical oxygen demand, pH, turbidity, total dissolved solids, dissolved oxygen, lead, and cadmium. The selection of microorganisms from the wastewater led to the isolation of nine gram-positive and two-gram negative bacteria. One strain was chosen for further study due to its appreciable rhodanese production. Molecular analysis based on 16S rRNA gene sequencing revealed that isolates JCM 1665 shared 99.65 % homology with *K. oxytoca* AY873801, 99.72 % homology with *K. oxytoca* MF144436 and 99.79 % homology with *K. oxytoca* MT568561 strain (Table 3).

Table 2. Physicochemical analysis of the wastewater.

Parameter	
pH	6.0 ± 1
	Concentration of parameter [mg.L⁻¹]
Turbidity	8.23 ± 2.74
Chemical Oxygen Demand	74.38 ± 5.19
Total dissolved solids	536 ± 10.6
Lead	0.02 ± 0.002
Cyanide	67.49 ± 9.2
Cadmium	0.024 ± 0.006
Dissolved Oxygen	3.94 ± 0.88

Table 3. Sequence identity of *K. oxytoca* (MN590525) with other *Klebsiella oxytoca*.

<i>Klebsiella oxytoca</i>	% Identity	Accession in the GenBank database
MT568561	99.79	MT568561

MF144436	99.72	MF144436
AY873801	99.65	AY873801

The sequence was deposited in the GenBank database of the NCBI as accession MN590525 (<https://www.ncbi.nlm.nih.gov/nucleotide/>).

Analysis of the model

The result for the analysis of the model for the production optimization is shown in Table 4. In this model, two linear (B and C) and five quadratic models (AB, AC, A², B², and C²) were found to be significant at the level of $P < 0.05$.

Table 4. Experimental and predicted result for rhodanese activity.

Run	Response (activity U.mg ⁻¹)	Actual value	Predicted value
1	5.00	5.00	5.30
2	2.50	2.50	2.13
3	6.00	6.00	5.69
4	7.10	7.10	7.25
5	5.70	5.70	5.50
6	5.50	5.50	5.71
7	7.20	7.20	7.25
8	5.70	5.70	5.71
9	2.00	2.00	2.38
10	3.50	3.50	3.35
11	3.50	3.50	3.43
12	7.10	7.10	7.25
13	6.50	6.50	6.64
14	7.50	7.50	7.25
15	7.20	7.20	7.25
16	6.00	6.00	5.94
17	0.05	0.05	0.10
18	2.00	2.00	2.00
19	7.40	7.40	7.25
20	1.70	1.70	1.77

The result for the fitting quadratic model is listed in Table 5. The result of the analysis of variance (ANOVA) indicates that the model was significant ($P < 0.05$) for the response of the dependent variables (rhodanese activity). The result also indicates a good model performance with correlation coefficient (R^2) values of 0.9925. The fitted quadratic model for rhodanese production is shown in Eq. 3.

$$\text{Activity} = +7.25 + 0.1158A + 0.9772B + 0.98887C + 0.8375AB - 0.8375AC - 0.0375BC - 0.6214A^2 - 0.7982B^2 - 1.94C^2 \quad (3)$$

Table 5. Analysis of Variance (ANOVA) for Response Surface Quadratic Model for the production of Rhodanese.

Source	SS	df	MS	F-value	p-value
Model	99.35	9	11.04	147.52	< 0.0001*
A-peptone	0.1832	1	0.1832	2.45	0.1487
B-KCN	13.04	1	13.04	174.28	< 0.0001*
C-Time	13.35	1	13.35	178.40	< 0.0001*
AB	5.61	1	5.61	74.99	< 0.0001*
AC	5.61	1	5.61	74.99	< 0.0001*
BC	0.0112	1	0.0112	0.1503	0.7063
A ²	5.56	1	5.56	74.36	< 0.0001*
B ²	9.18	1	9.18	122.69	< 0.0001*
C ²	54.15	1	54.15	723.62	< 0.0001*

SS – sum of squares; df – degrees of freedom; MS – mean square. R² = 0.9925; R² adj = 0.9858; CV = 5.52 %. (*values statistically significant at P < 0.05).

Analysis of response surface

The best way of expressing the relationship between the independent variables and dependent variables is to graphically plot the response surface plots generated by the model (Fig. 1, 2, 3). Fig. 1 showed the interaction between potassium cyanide (KCN) and incubation time while holding peptone at the center point (0). Fig. 2 showed the interaction between potassium cyanide (KCN) and peptone while incubation time is held constant. Fig. 3 showed the interaction between incubation time and peptone while KCN is held constant.

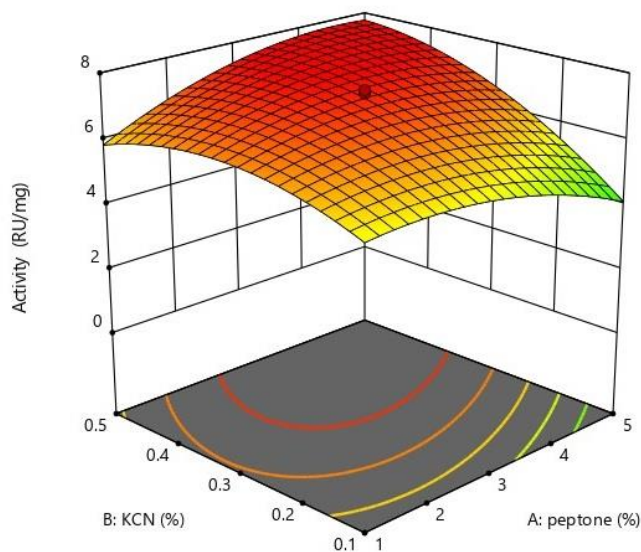


Fig. 1. Response surface plots showing the effects of KCN (% w/v) and peptone (% w/v) on rhodanese production.

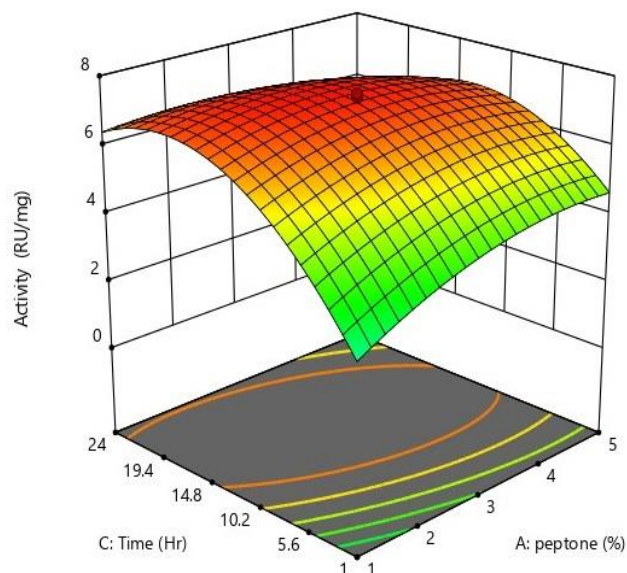


Fig. 2. Response surface plots showing the effects of incubation time (hr) and peptone (% w/v) on rhodanese production.

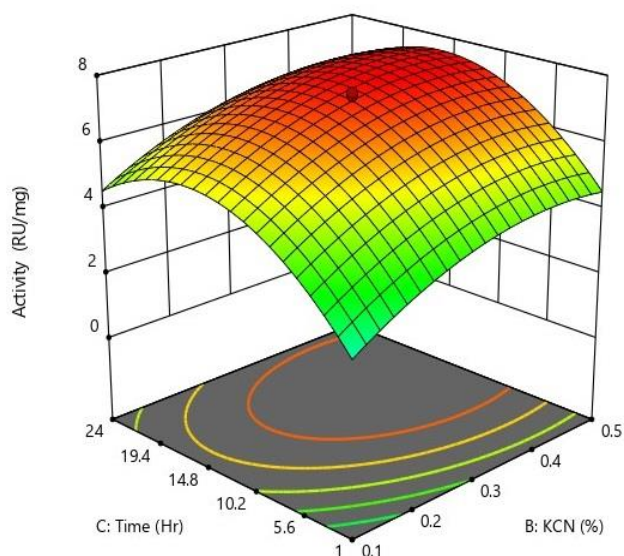


Fig. 3. Response surface plots showing the effects of incubation time (hr) and KCN (% w/v) on rhodanese production.

Discussion

Klebsiella oxytoca was isolated from an industrial effluent, identified and subjected to extracellular rhodanese production. Rhodanese production ability has been identified in a variety of bacteria species such as *E. coli* (Ray *et al.* 2000), *P. aeruginosa* (Cipollone *et al.* 2008), *Azotobacter vinelandii* (Kaewkannetra *et al.* 2009), *Bacillus brevis* (Oyediji *et al.* 2013) and *Bacillus cereus* (Itakorode *et al.* 2019). Enzyme production by an organism is often dependent on the growth of the bacterium in the appropriate media composition. The optimization of culture media and environmental conditions is essential for effective production as it tends to reduce cost of production by increasing yield (George-Okafor *et al.* 2012). The optimum nutritional requirement for rhodanese production was determined to obtain maximum enzyme production by *K. oxytoca*. A central composite design (20 runs) was chosen with start and center points. The design was rotatable; this means that the designs have points that are equidistant from the center. Experiments at the center were carried out to obtain an estimation of the experimental error. The designed experiment matrix and the experimental results are presented in Tables 1 and 4. The rhodanese yield varied (from

0.05 to 7.5 RU.mg⁻¹). The rhodanese production yield reached its maximum value (7.5 RU.mg⁻¹) at 3 % of peptone, 0.3 % of KCN and incubation time of 12.5 h (run 17). As noted, rhodanese producing ability of *K. oxytoca* was affected by the time of incubation. The analysis of the overall data set indicated that incubation time, KCN and interaction AC had the most pronounced effects on the response (Table 5). Also, from Eq. 3, it is evidence that KCN (B) concentration and incubation time (C) had the highest positive effect on the rhodanese production while interaction (AC) and (C²) had the highest negative effect on production.

Analysis of variance (ANOVA) was important in determining the adequacy and significance of the quadratic model. ANOVA summary is shown in Table 5. The fitness of the model was expressed by the R² value, which is 0.9925, indicating that 99.25 % of the variability in the response can be explained by the model. The adjusted R² value of 0.9858 suggested that the model was significant. A very low value of coefficient of the variation (CV) 5.52 % indicated a very high degree of precision and a good deal of reliability of the experimental values.

The interaction between KCN and peptone concentration at a constant time of incubation is shown in Fig. 1. The result showed that rhodanese production increases as the concentration of both variables increases. At high concentrations, a gradual fall in production was observed. Research had shown KCN and peptone to influence the production of cyanide detoxifying enzymes (Adekunle *et al.* 2017). Itakorode *et al.* (2019) reported KCN as the best carbon source for *B. cereus*. The ability of *P. putida* and *B. pumilus* to make use of cyanide as carbon source was also reported by Kandasamy *et al.* (2015). Okonji *et al.* (2018) also reported the ability of *Pseudomonas straminea* to make use of peptone as nitrogen source. The high cyanide tolerance of *K. oxytoca* observed was probably due to its ability to synthesize rhodanese as a means of surviving in cyanide contaminated environment. Fig. 2 showed the interaction effect of peptone concentration and incubation time while keeping KCN concentration constant. In this interaction, minimal rhodanese production was observed at a low concentration of peptone. However, as concentration and incubation

time increases, an increase in rhodanese activity was recorded. The same trend was also observed in the interaction between incubation time and KCN. However, a decline in production was noted at high time of incubation. The decline in rhodanese production may be due to exhaustion of the nutrients or accumulation of other products or metabolites which are both inhibitory to the growth of the bacterium and rhodanese production. This result agrees with the findings of Okonji *et al.* (2018) who observed a decrease in metabolite production after 15 hours of incubation. Linawati *et al.* (2002) also reported the influence of environmental conditions on the production of pigment by *Serratia marcescens*.

The optimal value of the independent variables for rhodanese production was examined using the maximum desirability. The result of optimal conditions used to obtain the highest rhodanese production by *K. oxytoca* were 4.35 % peptone, 0.4 % KCN and incubation time of 13 hours, at which rhodanese yield was 7.810 RU.mg⁻¹.

Conclusion

It can be concluded that the regression equations obtained in this study can be used to maximize the rhodanese production by *K. oxytoca*. Also, further study is required to improve the production of the enzyme through genetic engineering and to examine the cyanide bioremediation potential of the rhodanese synthesized by the organism.

Conflict of interest

The authors declare that they have no conflict of interest.

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