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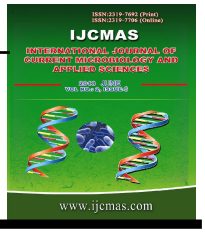

Original Research Article

Optimization of *Mycobacterium tuberculosis* DHFR production from recombinant *Saccharomyces cerevisiae*

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A B S T R A C T

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*Mtb*DHFR, Recombinant yeast; Response Surface Methodology; Sonication; Enzyme Assay.

Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate in a NADPH dependent manner. The production of *Mycobacterium tuberculosis* (*Mtb*) DHFR by a recombinant strain of *Saccharomyces cerevisiae* (Y_{182}) shake-flask culture was optimized by identifying the most significant medium components which affect DHFR production (dextrose, yeast extract, peptone) by Response surface methodology (RSM) to determine the optimal concentrations of these components using Design Expert Version 6.0.10. The *Mtb* DHFR enzyme activity increased from 32.03 ± 0.042 U/l in basal medium to 67.01 ± 0.23 U/l in the optimized medium containing 1% yeast extract, 2.36% peptone and 2.68% dextrose. An enzyme assay was developed to assess the purity of the isolated enzyme by testing for IC_{50} values against known inhibitors.

Introduction

Dihydrofolate reductase (DHFR, EC 1.5.1.3) is an enzyme which is essential for folate metabolism. It is present in both eukaryotic and prokaryotic cells. The enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate and is NADPH dependent. This tetrahydrofolate so formed is a precursor for the synthesis of thymidylate, purine nucleotides, methionine, serine and glycine. This makes it very indispensible for DNA, RNA and protein synthesis (Li *et al*., 2000).

DHFR has been widely explored as a target in treatment of various infectious diseases and also in cancer. However, it is a novel target in case of tuberculosis.. In the quest for development of novel anti TB drugs, the present study was undertaken to standardize the optimum production and isolation of *Mtb*DHFR enzyme which can be used to investigate the activity of newly designed and synthesized inhibitors (Gerum *et al*., 2002, Li *et al*., 2000).

Earlier works reported in this area have shown isolation of *Mtb*DHFR from recombinant *Escherichia coli* (White *et al*., 2004). The present work employs the use of *Saccharomyces cerevisiae*, a simple

eukaryote which is inexpensive and safe to grow under normal laboratory conditions. The advantage of the *Saccharomyces cerevisiae* system is that the cells are easily transformed. Also the *Mycobacterium tuberculosis dfrA* gene integrates preferentially by homologous recombination. Because of this property, targeted disruption of genes is easily accomplished (Sibley *et al*., 1997). Additionally, presence of the *Mtb*DHFR protein in recombinant *Saccharomyces cerevisiae* ensures easy isolation of the enzyme since it does not form inclusion bodies as in case of recombined protein in *Escherichia coli.* Inclusion bodies are known to lower the enzyme activity (Sibley *et al.,* 1997).

DHFR is an intracellular enzyme and hence it is important to produce a high cell biomass during fermentation which can be achieved by optimizing the production media (Kim *et al*., 2007). The first step was to find the right media composition by growth curve studies for maximum production of cell biomass and enzyme (Haltrich *et al*., 1993). The standardization of media components was done with the aid of a statistical method, response surface methodology (RSM). It is also important to isolate the enzyme from the recombinant cell using the right cell disruption method (Engler, 1990). This was achieved by standardizing the use of probe sonicator (Iida *et al*., 2008). The enzyme was purified by a novel chromatographic method (Raju *et al*., 2013). This purified enzyme was assessed by a spectrophotometric assay method (White *et al.,* 2004).

Materials and Methods

Recombinant strain of *Saccharomyces cerevisiae* Y_{182} with *Mtb*DHFR was gifted by Dr. C.H. Sibley from Department of Genetics, Box 357360, University of Washington, Washington. (Sibley *et al*., 1997).

Maintenance of culture

The culture was maintained on YPD (yeast extract dextrose broth) agar slants at 32°C for 3 days. After 3 days the slants were kept at 4 ºC and thereafter subcultured every 30 days.

Medium and Cultivation

For media screening and growth curve studies, the media compositions as shown in **Table 1** were utilised.

Enzyme assay (Hillcoat *et al*., 1967; White *et al*., 2004)

DHFR activity was determined spectrophotometrically by monitoring the decreasing absorbance at 340nm for 2min at 37^{o}C. Standard assay mixture (1.0 ml) contained 50mM KPO_4 (pH 7.4), 5mM DTT, and 60µM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the reaction was started after a 3 min incubation by the addition of 45 µM DHF. The molar extinction coefficient change for the reaction at 340 nm is 12270 $M^{-1}cm^{-1}$. A unit of the enzyme activity is defined as the amount of the enzyme converting 1.0µmol of DHF and NADPH to THF and $NADP^{+}$ per min and was calculated from the change in absorbance at 340nm under specified assay condition. Protein concentrations were determined by Bradford's method using bovine serum albumin (BSA) as the standard.

Fermentative Production

The recombinant strain of *Saccharomyces cerevisiae* Y_{182} with *Mtb*DHFR was cultivated in YPD fermentation medium,

Table.1 Media composition used for screening for maximum production

YPD Broth		Sabouraud Broth (SAB)		Potato Dextrose Broth (PDB)	
Yeast extract	1.0g	-		Potato extract	20g
Peptic digest	3.0g	Peptone	1.0g	-	
Dextrose	2.0g	Dextrose	4.0g	Dextrose	2.0g
Water	100mL	Water	100mL	Water	100mL
pH	7.2	pH	5.4	pH	7.2

which contained 20 g l^{-1} dextrose, 10 g l^{-1} yeast extract and 30 g l^{-1} peptic digest extract in distilled water (Sibley et al, 1997). The initial pH of culture was adjusted to 7.2 before autoclaving. About 2 % cells (with cell density (measured as OD) of 2.8-3.0 at 680 nm) were inoculated from seed culture into 250 ml Erlenmeyer flasks containing 50 ml sterile medium. Fermentation was carried out at 25°C on a rotary shaker (160 rpm) for 32h. The seed medium was composed of 2.0 % (w/v) dextrose, 1.0 % (w/v) yeast extract, and 3.0 % (w/v) peptone. Seed culture was prepared at 25°C in a shaker incubator (160rpm) for 8h.

One factor at a time optimization

With the view of standardization of media components and parameters, one factor optimization was carried out using various concentrations of different constituents.

Probe Sonication studies

Enzyme isolation was carried out by probe sonicator method using Deksin ultraprobe sonicator (frequency 40 KHz, pressure 30psig, diameter of ss tip of horn = $2.1x10^2$ cm, depth of immersion of horn=0.01m) and the whole protein content of the cell was released (Engler, 1990). Probe sonication optimization was carried out for 2 factors which significantly affect the *Mtb*DHFR release, sonication power and irradiation time (Iida *et al*., 2008). The entire process was carried out at 4ºC unless specified otherwise.

Experimental Design for Shake-Flask Cultures

RSM was employed to determine the optimal concentrations of carbon and combined nitrogen sources in the shake-flask cultures. The regression analysis of the experimental data along with the regression equation coefficients were estimated using Design-expert 6.0.10 Stat-Ease, Inc., MN, USA.

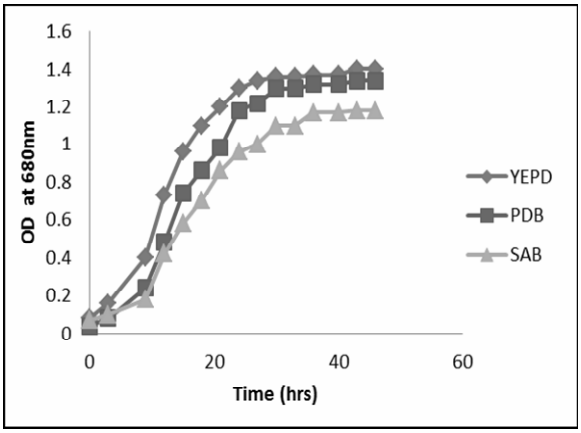
Result and Discussion

Growth curve and media screening

Growth curve study for recombinant strain of *Saccharomyces cerevisiae* containing *Mtb*DHFR was carried out to find out the suitable media for *Mtb*DHFR enzyme production was shown in Table 1.

The growth curve for recombinant strain of *Saccharomyces cerevisiae* containing *Mtb*DHFR showed that YPD gave better growth pattern of the organism as compared to the other media, as depicted in Fig 1.

Figure. 1 Growth curve study for three different media



These media were also screened to find out the maximum production of *Mtb*DHFR. YPD media was found to give the maximum yield of 32.03 ± 0.042 U/l of *Mtb*DHFR as shown in Table 2. Hence, YPD media was finalized for further fermentation optimization.

Probe Sonication and Centrifugation

Cells were harvested using centrifugation. Cell disruption was carried out using probe sonication method. Maximum *Mtb*DHFR activity was found to be 34.0 U/L with sonication time of 12 min. For maximum release of *Mtb*DHFR, the power (W) was optimized in the range of 60-140W with 100W resulting in maximum enzyme release of *Mtb*DHFR.

Table.2 Media screening for maximum production of *Mtb*DHFR

Media	*Mtb*DHFR Production (U/ l)
YPD Broth	**32.03 ± 0.042**
SAB	20.10 ± 0.034
PDB	24.33 ± 0.043

One Factor optimization

From one factor optimization, it was observed that a single nitrogen source was not sufficient for production of the enzyme**.** Thus a combination of yeast extract and peptone was found ideal and this combination was used for further optimization. First, yeast extract concentration was kept constant and peptone concentration was varied in the range of 1-4%w/v. This was followed by keeping peptone concentration constant (2.5%w/v) and yeast extract concentration varying from 0-2.5%w/v. The optimized conditions are shown in Table 3.

Table.3 Concentrations and parameters after one factor optimization for production of *Mtb*DHFR

Media components / parameters	Concentration (%w/v)
Yeast Extract	1.0
Peptone	2.5
Dextrose	2.0
pH	6.5
Temperature	25^{o}C
Fermentation Time	32h
Inoculum size	2%v/v
Seed time	8h

Optimization of screened components by RSM

To examine the combined effect of three

different medium components (independent variables), on *Mtb*DHFR production, a central composite design of 20 experiments was performed (Tsapatsaris and Kotzekidou, 2004). Second order polynomial equation was used to correlate the independent process variables, with *Mtb*DHFR production. The second order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert. The coded values of independent variables are given in Table 4. The design of experiments and respective experimental yields are given in Table 5.

Table.4 Level for central composite design

Factors	Level		
	-1	0	+1
Yeast Extract	0.25	1	1.75
Peptone	1.25	2.25	3.25
Dextrose	1.25	2.25	3.25

The yields were analyzed by using ANOVA *i.e.* analysis of variance suitable for the experimental design, the results of which are shown in Table 6. The Model F-value of 80.02 implies that the model is significant. Model F-value is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob > F) is very low (0.0001**)** which emphasizes the significance of the model.

Interaction effects

The *P* values were used as a tool to check the significance of each of the coefficients which are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.05 indicate model terms to be significant (Kalil *et al*., 2000; Kim *et al.*, 2007). The coefficient estimates and the corresponding *P* values suggests that, among the test variables used in the study, A (yeast extract), B (peptone), C (Dextrose), A×B (yeast extract × peptone), A×C (yeast extract × Dextrose) are significant model terms. Dextrose ($P < 0.0001$) has the largest effect on *Mtb*DHFR production, followed by yeast extract **(**P 0.0041**)** as shown in Table 6. The corresponding second-order response model (Polynomial equation) was found after analysis for the regression was:

$$\textit{Mtb}\text{DHFR (U/l)} = +66.60 + 2.39 * A + 0.32 * B + 6.88 * C - 12.88 * A^2 - 4.66 * B^2 - 7.46 * C^2 + 1.15 * A * B - 3.53 * A * C - 4.91 * B * C$$

This equation can be used to estimate the enzyme activity with respect to the changing concentration of the media components.

Squared effects

Squared terms are used to evaluate whether or not there is a curvature (quadratic) in the response surface. The F-value (Table 6) of 0.0001 for the squared effects is less than 0.05, suggesting a significant quadratic effect. The low coefficient of variation (CV) value of 4.81 indicates a greater degree of precision. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In the present study, a ratio of 24.845 was obtained which indicates an adequate signal. This explains that the model can be used to find the approach of design space. R^2 and adjusted R^2 (R^2adj) represent the proportion of variation in the response that is explained by the model.

Table.5 Experiments runs for central composite design

Run	Yeast Extract (g/l)	Peptone (g/l)	Glucose (g/l)	*Mtb*DHFR enzyme Activity (U/l)	
				Actual value	Predicted value
1	1.00	2.25	0.56	34.12	33.91
2	1.00	2.25	2.25	67.23	66.60
3	0.25	1.25	1.25	25.23	24.72
4	1.75	1.25	3.25	51.23	50.77
5	1.00	2.25	2.25	67.34	66.60
6	1.75	1.25	1.25	34.67	34.26
7	1.00	2.25	2.25	67.12	66.60
8	1.00	3.93	2.25	53.21	53.97
9	0.25	1.25	3.25	58.21	55.35
10	1.00	2.25	2.25	67.45	66.60
11	0.25	3.25	3.25	45.21	43.88
12	1.00	2.25	2.25	68.56	66.60
13	1.00	2.25	3.93	54.41	57.05
14	1.00	0.56	2.25	51.21	52.89
15	2.26	2.25	2.25	34.45	34.18
16	-0.26	2.25	2.25	23.45	26.15
17	1.75	3.25	3.25	45.12	43.90
18	0.25	3.25	1.25	34.15	32.88
19	1.75	3.25	1.25	45.89	47.01
20	1.00	2.25	2.25	62.32	66.60

Table.6 ANOVA for the Experimental Results

Source	Coefficient estimate	Sum of squares	DF	Mean square	F value	Prob > F
Model	66.60014	4092.587	9	454.7319	80.02348	< 0.0001
A	2.387792	77.86522	1	77.86522	13.70268	0.0041
B	0.321713	1.413473	1	1.413473	0.248742	0.6287
C	6.879591	646.3622	1	646.3622	113.7465	< 0.0001
A^2	-12.8795	2390.558	1	2390.558	420.6892	< 0.0001
B^2	-4.65583	312.3893	1	312.3893	54.97411	< 0.0001
C^2	-7.46481	803.0453	1	803.0453	141.3195	< 0.0001
AB	1.14875	10.55701	1	10.55701	1.857817	0.2028
AC	-3.53125	99.75781	1	99.75781	17.55533	0.0019
BC	-4.90625	192.5703	1	192.5703	33.88842	0.0002
Lack of Fit		32.75681	5	6.551363	1.361011	0.3717

The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.986, indicating that 98.0 % of the variability in the response could be explained by the model. The closer the R^2 value is to 1, the better the fit of the model to experimental data. The "R^2" of 0.986 is in reasonable agreement with the "R^2adj" of 0.974 confirming the validity of the model.

Figure.2 Parity plot of Predicted Vs Experimental yield

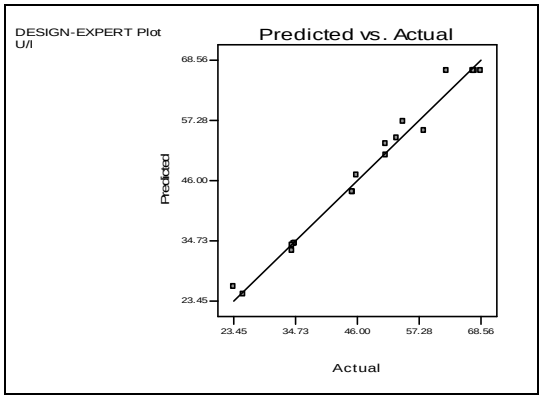


Figure 2 shows the correlation between the actual and the predicted yields of *Mtb*DHFR, suggesting the suitability of the model. Accordingly, three-dimensional graphs were generated for the pair-wise combination of the 3 factors, while keeping the other two at their center point levels. Graphs are given here to highlight the roles played by various factors. From the bump of the 3D plot the optimal composition of medium components were identified. By keeping another variable at its optimal level, three-dimensional plots of two factors versus *Mtb*DHFR production were drawn as shown in Figures 3 a, b, c.

In order to verify the predicted results, an experiment was performed using the optimized nutrient levels. The maximum production of *Mtb*DHFR obtained experimentally using the optimized medium was 67.01 ± 0.23U/l, which is in correlation with the predicted value of 67.89U/l by the RSM regression study. By substituting levels of the factors into the regression equation, the maximum predictable response for *Mtb*DHFR production was calculated and was experimentally verified (Rao *et al*., 2000). The final optimized concentrations of the medium components are given in Table 7.

Table.7 Final optimized medium for production of *Mtb*DHFR

Media components / parameters	Concentration (%w/v)
Yeast Extract	1.00
Peptone	2.36
Dextrose	2.68

Enzyme assay

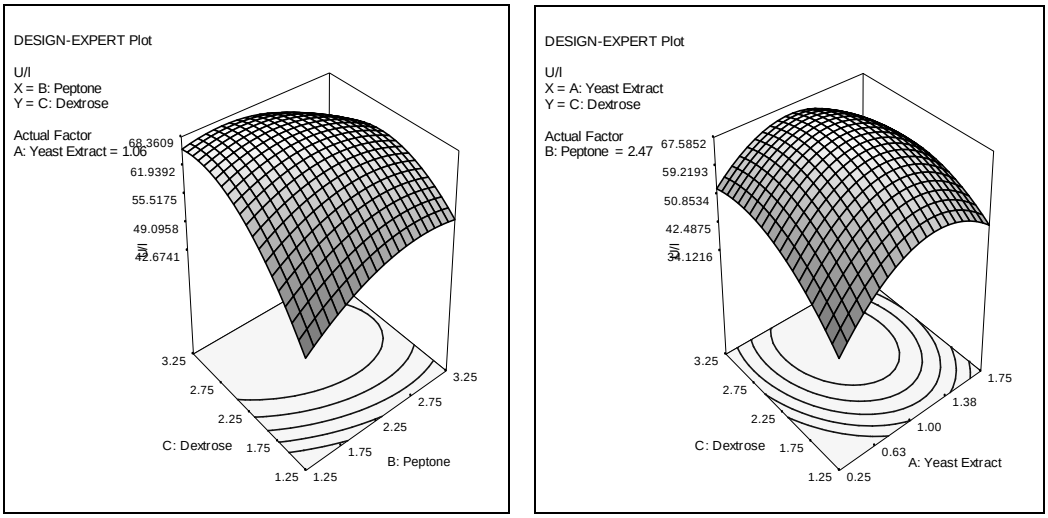
A spectrophotometric assay method was developed and the enzyme was assessed for purity after employing a novel chromatographic purification technique employed in our laboratory (Raju *et al*., 2013). The results obtained for the IC_{50} value with known inhibitors Trimethoprim and Methotrexate were in accordance with the values reported in literature (White *at al*., 2004) and are as shown in Table 8 indicating efficient purification (Raju *et al., 2013).*

In the present study, all the nutrients and the influencing components for enhancing *Mtb*DHFR production by culturing the recombinant strain of *Saccharomyces cerevisiae* Y_{182} and their optimal concentrations were obtained by using RSM.

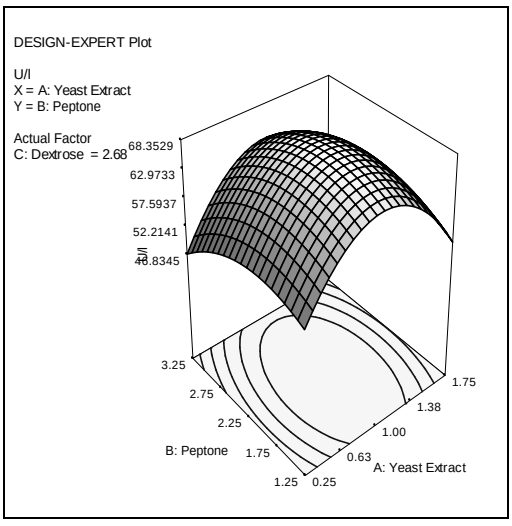
Table.8 Inhibitors of *Mycobacterium tuberculosis*

Inhibitors	IC_{50} values with *Mycobacterium tuberculosis* (µM)	
	Reported from literature (White *et al*., 2004)	Values obtained in our laboratory (Raju *et al*., 2013)
Trimethoprim	16.5 ± 2.5	16.0 ± 3.0
Methotrexate	0.0083 ± 0.0005	0.008 ± 0.0007

Figure.3 3D-Surface plot for human DHFR (U/l)



(a) **(b)**



(c)

a) Response shows effect of peptone and dextrose **b)** Response shows effect of yeast extract and dextrose **c)** Response shows effect of yeast extract and peptone

From growth curve study and media screening YPD was found to be the medium which gave the highest production of *Mtb*DHFR (32.03 ± 0.042U/l). The production of *Mtb*DHFR increased to 67.01 ± 0.23U/l under the RSM optimum conditions, which is a 2 fold increase as compared to the unoptimized medium. It was evident that the systematic methods had the advantage of identifying the most significant medium composition and identifying their optimal levels, and thus was useful for operating the fermentation towards the increased production of the *Mtb*DHFR. Enzyme assay protocol was developed for testing the purity of the *Mtb*DHFR enzyme, purified by employing an inhouse prepared column, by finding IC_{50} values against known inhibitors which were in accordance with reported values.

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