Optimization of culture medium for production of recombinant dengue protein in *Escherichia coli*

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ABBREVIATIONS
BCA, bicinchoninic acid; E, recombinant dengue envelope protein; ELISA, enyzme-linked immunosorbent assay; GE, glucose-enriched; LB, Luria Bertani; NS1, recombinant dengue nonstructural protein; rDME-M protein, recombinant dengue multiepitope protein; SB, Super broth; TB, Terrific broth; TY, tryptone yeast

Abstract

Enhanced production of recombinant dengue multi-epitope protein in Escherichia coli was achieved by optimization of culture medium. Complex media (Luria Bertani broth, Terrific broth, Super broth, M9 minimal media, five times Luria Bertani broth, semi-defined medium enriched with tryptone and yeast extract, and semi-defined medium enriched with glucose) were evaluated for production of recombinant dengue multi-epitope protein in shake flasks. The recombinant protein was further produced by fed-batch fermentation using 5 L bioreactor. Cells were grown in optimized semi-defined medium, and feeding was carried out with 5X medium and glycerol. When growth reached 14.35 g/L of dry cell weight, culture was induced with 0.5 mM IPTG and further grown for 4 h to reach 18.37 g/L dry cell weight. The recombinant dengue multi-epitope protein was purified from inclusion bodies under denaturing conditions using metal affinity chromatography, which yielded 96.43 mg/L of protein. The purified protein was found to be reactive with dengue-infected human serum samples.

Introduction

Dengue virus infection is recognized as a major mosquito-borne human infection of the 21st century. Recombinant dengue envelope (E) and nonstructural (NS1) protein have been well studied as antigens in diagnostic assays for detection of dengue infection[1-3] and as reagents for vaccine studies.[4-5] *Escherichia coli* is commonly used to produce recombinant proteins because it can be grown to high densities on inexpensive media and its genetics are well understood. Recombinant *E. coli* can be grown to high densities in common media such as Luria Bertani broth (LB),[6-7] the synthetic M9 minimal salt medium, Terrific broth (TB),[8-9] and Super broth (SB).[10] The optimal growth temperature for *E. coli* is 37 °C, and pH between 6.4 and 7.2. The bacterium is generally grown under aerobic conditions since anaerobic growth provides less energy for metabolic processes such as protein synthesis.[11]

To ensure that oxygen supply does not become limiting, fed-batch operations are used extensively.[6,12] This improves biomass and recombinant protein yield, over batch culture. Fed-batch operation overcomes possible limitations due to high concentrations of substrate and enables growth to be prolonged compared to traditional batch fermentations. High-cell-density fed-batch fermentation using glucose as the main carbon and energy source is a standard technique in *E. coli* cultivation.[13,14] However, accumulation of acetate and other acidic by-products during fed-batch cultivation diminishes cell growth as well

as recombinant protein production.[15] Supplementation with tryptone and yeast extract with glycerol have helped to decrease the accumulation of acetate.[16] In a previous paper,[17] we reported batch fermentation for production of IgM-specific recombinant dengue multi-epitope (rDME-M) protein using SB medium and its evaluation as an antigen in a diagnostic assay for detection of dengue infection. This paper describes optimization of culture medium as well as a simple fed-batch fermentation strategy using tryptone- and yeast extract-enriched semi-defined medium, and glycerol as carbon source, in order to achieve high yield of recombinant dengue multi-epitope protein in *E. coli*.

Materials & methods

MEDIA, CHEMICALS, & MICROORGANISMS

Luria Bertani broth (LB), Terrific broth (TB), Super broth (SB), M9 minimal salt (M9), five times Luria Bertani broth with 1% glycerol (5X LB), tryptone yeast (TY) medium, and GE (glucose-enriched) medium were used for media optimization in shake flasks (*Table 1*). Tryptone yeast (TY) medium was used in batch fermentation, and five times (5X) of TY medium with glycerol (300 g/L) was used as feeding media during fed-batch fermentation. Ampicillin (100 µg/ml) was added to all media for shake flask culture and fermentation. Media and chemicals were purchased from Sigma (St Louis, Missouri, USA), Difco (Sparks, Maryland, USA), Qiagen (Valencia, California, USA), Promega (Madison, Wisconsin, USA), and Bio-Rad (Hercules, California, USA). *E. coli* DH5α harboring recombinant plasmid pMAL-c2X containing IgM-specific dengue multi-epitope gene[2] was obtained from Navin Khanna of International Centre for Genetic Engineering and Biotechnology (New Delhi, India).

OPTIMIZATION OF MEDIA

Optimization of media was performed for the yield of rDME-M protein in seven media (*Table 1*) in shake flask culture. Seed culture was prepared by growing the organisms in 50 ml of LB medium in 250 ml Erlenmeyer flasks shaken overnight at 200 rpm, 37°C. For shake flask culture, 2 ml of the seed culture was inoculated into 200 ml of the respective medium in a 1 L Erlenmeyer flask and incubated at 37°C, 200 rpm, for 8 h. When cultures were in logarithmic growth phase (after ~4 h), they were induced with 0.5 mM IPTG and further grown for 4 h. Samples were collected for measurement of optical density at 600 nm (OD_{600}), determination of dry cell weight, and rDME-M protein yield.

Table 1. Composition of media used for shake flask culture and fermentation

Media	Composition (g/L)
LB	Tryptone, 10.0 g; Yeast extract, 5.0 g; NaCl, 5.0 g
5x LB	Tryptone, 50.0 g; Yeast extract, 25.0 g; NaCl, 25.0 g; Glycerol, 10.0 g
TB	Pancreatic digest of casein, 12.0 g; Yeast Extract, 24.0 g; Di-potassium phosphate, 9.4 g; Monopotassium phosphate, 2.2 g; Glycerol, 4 g
SB	Soy Hydrolysate, 12.0 g; Yeast Extract, 24.0 g; Di-potassium phosphate, 11.4 g; Monopotassium phosphate, 1.7 g; Glycerol, 5 g
M9	Di-sodium phosphate, 6.78 g; Sodium Chloride, 0.5 g; Ammonium chloride, 1.0 g; Monopotassium phosphate, 3.0 g
TY medium	Tryptone, 26.8 g; Yeast extract, 21.4 g; Monopotassium phosphate, 5.4 g; Di-ammonium hydrogen phosphate, 1.6 g; Magnesium sulphate, 1.2 g; NaCl, 8.5 g; Glycerol, 10 g
GE medium	Glucose, 28 g; Di-ammonium hydrogen phosphate, 4 g; Monopotassium phosphate, 13.3 g; Citric acid, 1.7 g; EDTA, 8.4 mg; Magnesium sulphate, 1.2 g

EXPRESSION OF rDME-M PROTEIN IN FED-BATCH CULTIVATION

The rDME-M protein was produced in 4.0 L of TY medium in fed-batch mode in a 5 L bioreactor (BioFlo 3000, New Brunswick Scientific, Edison, New Jersey, USA). For primary seed culture, a 1.5 ml aliquot of frozen cells of *E. coli* containing rDME-M gene in LB medium containing 30% (v/v) glycerol was inoculated in 50 ml of TY medium and grown at 37°C, 200 rpm, for 8 h. Primary seed culture (2 ml) was then inoculated into 200 ml of TY medium and further grown at 37°C, 200 rpm, overnight. The initial batch culture was started by aseptic addition of adding 1% (v/v) of overnight-grown culture into the bioreactor; pH was adjusted to 6.8 with addition of 25% (v/v) NH_4OH. Aeration occurred through a perforated pipe sparger ring. Dissolved oxygen (DO) was kept at 30%–40% of air saturation by variation of agitation between 100 and 600 rpm, and aeration, between 4 and 10 L/min, with 5% to 50% oxygen mixed into the sparged air at high cell densities. Oxygen was supplied to the culture by using the Gasmix function of the fermentor. Antifoam A (Sigma) was added automatically to control foaming; foam levels were monitored by use of a conductivity probe mounted 5 cm above the culture level. The feed medium contained 5X of batch (TY) medium and 300 g/L glycerol. The DO-stat and pH-stat were controlled by the automated BioCommand Plus control software (New Brunswick Scientific). After depletion of initial nutrient in the batch medium, feeding was initiated. Feed rate was set initially at 12 ml/h, increasing by 0.12 ml/h for every minute after a preset period. Temperature was controlled at 37°C. After ~8 h of inoculation, IPTG was added to a final concentration of 0.5 mM to induce expression of rDME-M protein, and cultures were further grown for 4 h. Cultures were also sampled from the bioreactor to measure optical density (OD_{600}) and dry cell weight.

ANALYSIS OF rDME-M PROTEIN EXPRESSION

After centrifugation, the cell pellet was dissolved in Ni-NTA buffer containing 8 M urea by stirring for 1 h at room temperature. This suspension was then disrupted using ultrasonic disintegrator (Sonics, Newtown, Connecticut, USA) and centrifuged at 10,000 rpm, 4°C for 40 min, to remove insoluble material or debris. The clear supernatant was used in Ni-NTA affinity chromatography for purification of recombinant dengue protein. Yield of purified rDME-M protein was estimated by the bicinchoninic acid (BCA) method as well as by measuring absorbance at 280 nm with a theoretical extinction coefficient (1.783 $mg^{-1} \cdot ml$) based on amino acid sequence.[2]

ANALYTICAL METHODS

In order to determine the dry cell weight, 2 ml broth was centrifuged at 8,000 rpm for 10 min, washed twice with deionized water, and dried at 105°C for 16 h to a constant weight. Elutes were separated by SDS-PAGE gel to verify purity of the recombinant protein. Expression of rDME-M protein was determined by SDS-PAGE on 10% polyacrylamide gels stained with Coomassie Brilliant Blue R250 and quantified by densitometry. Total protein was also estimated by the BCA method (Pierce, Rockford, Illinois, USA). rDME-M protein was characterized by Western blot analysis. Functionality of rDME-M protein was tested by an enzyme-linked immunosorbent assay (ELISA) using dengue-positive and dengue-negative human serum samples as described previously.[17]

Results & discussion

MEDIA OPTIMIZATION

In shake flask culture, final cell concentration was found to depend upon the media used. TY medium achieved higher cell density, of about 2.42 g/L dry cell weight. Terrific broth and Super broth produced final cell densities of 1.98 g/L and 2.13 g/L dry cell weight, respectively. GE medium did not support culture growth. This may be attributed to the presence of higher concentrations of glucose. M9 medium and 5X LB medium also did not support growth (due to higher concentrations of yeast extract, tryptone, and sodium chloride, in the 5X LB medium, and the absence of yeast extract, tryptone, and other carbon/nitrogen source, in M9 medium). Comparison of dry cell weight and yield of purified rDME-M protein in different media is shown in *Table 2*. The use of chemically defined media in producing recombinant proteins is a common practice[18-20] as these media attain more consistent titers, allow easier process control and monitoring, and simplify downstream recovery of the target protein. The high cell densities attained with TY medium are due to the medium being rich in tryptone, yeast extract, and phosphate salts as compared to the other media used. Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during synthesis of recombinant protein.[8] Similarly, a high concentration of phosphate is known to be important for attaining high cell densities of *E. coli*, as phosphate can easily become a limiting nutrient when provided in low doses.[16] In addition to providing a source of phosphate, the phosphate salts in the media provided a buffering capacity against pH fluctuations that could adversely affect normal metabolic activity.

Table 2. Comparison of dry cell weight and yield of purified rDME-M protein in different media

Media	Culture Condition	Dry cell weight (g/L)	rDME-M protein (mg/L)
LB	Shake flask	1.12	10.37
TB	Shake flask	1.98	16.53
SB	Shake flask	2.13	17.32
TY medium	Shake flask	2.42	21.65
TY medium	Bioreactor	18.37	96.43

GE , 5X LB, and M9 media did not support culture growth.

LB medium, commonly used in expression of recombinant proteins in *E. coli,* produced the lowest final cell density: 1.12 g/L dry cell weight, and recombinant protein yield (10.37 mg/L). The relatively poor performance of LB versus those of complex media such as TB and SB has been discussed for other recombinant proteins[9-10] and is attributed to lower amounts of readily accessible carbon and nitrogen in LB.[9] Because TY medium produced the highest cell density and yielded more protein (*Table 2*), this was used for fed-batch fermentation.

FED-BATCH CULTIVATION TO PRODUCE rDME-M PROTEIN

Based on the above shake flask cultivation results, the overnight-grown culture in TY medium was further grown in a 5 L bioreactor by fed-batch cultivation to achieve higher cell densities. The feeding strategy used in the present experiments was a combination of both the pH-stat and DO-stat methods. Simultaneous monitoring of both these parameters gives better control over the growth conditions of the growing culture. In bioreactor cultivation prior to induction (up to ~8 h), the dry cell weight was 14.35 g/L. During this period, the nutrients in the medium were mainly utilized for the bacterial growth. After induction, optical density increased slowly as expression levels increased. The expression level was determined by SDS-PAGE analysis of samples taken at 1 h interval for 1–4 h after induction (results not shown). Thus, the additional nutrients fed were ascertained to be used primarily for the protein production. After approximately 12 h of bioreactor cultivation (~4 after induction), final biomass yield was 18.37 g/L dry cell weight. The bioreactor provides for control of culture pH, effective aeration, feeding with a carbon source, antifoam, and a nitrogen source, and also maintains culture sterility. Recombinant dengue multi-epitope protein was cultivated by fed-batch fermentation using completely defined medium. The method adopted for nutrient feeding is also crucial for success of fed-batch fermentation as it affects both cell density and cell productivity. Feeding medium with glycerol was added at a predetermined rate (12 ml/h). Cells were induced with 0.5 mM IPTG at 14.35 g/L dry cell weight and grown further for 4 h. With the increase of biomass, O_2 demand also increased in fed-batch cultivation phase. The feeding rate must be controlled such that it does not exceed the feed consumption rate; this is achieved by maintaining the pH and dissolved oxygen (DO) around their set values. Decreases in pH and DO are indications of substrate overdosing; increases in pH and DO values indicate that the carbon source or one of the substrates is limiting and that feed is thus required. The real time profile of fed-batch fermentation for production of rDME-M protein is shown in *Figure 1*.

PURIFICATION & CHARACTERIZATION OF rDME-M PROTEIN

The rDME-M protein was purified by Ni–NTA affinity chromatography under denaturing conditions.[2] The protein was bound to the column at pH 7.5, washed with Ni-NTA buffer, pH 6.3, and eluted with

Ni-NTA buffer pH 4.3. Yield of rDME-M protein after Ni-NTA chromatography was 96.43 mg/L, which corresponds to 18.37 g/L dry cell weight. SDS-PAGE analysis of rDME-M protein is shown in *Figure 2A*. The final yield of rDME-M protein after fermentation was increased to more than nine times in comparison with commonly used shake flask culture using LB media (*Table 2*). The lower specific yield in the bioreactor versus that with shake flask culture may be attributed to the presence of greater amounts of inhibitory substances (acetate, lactate,

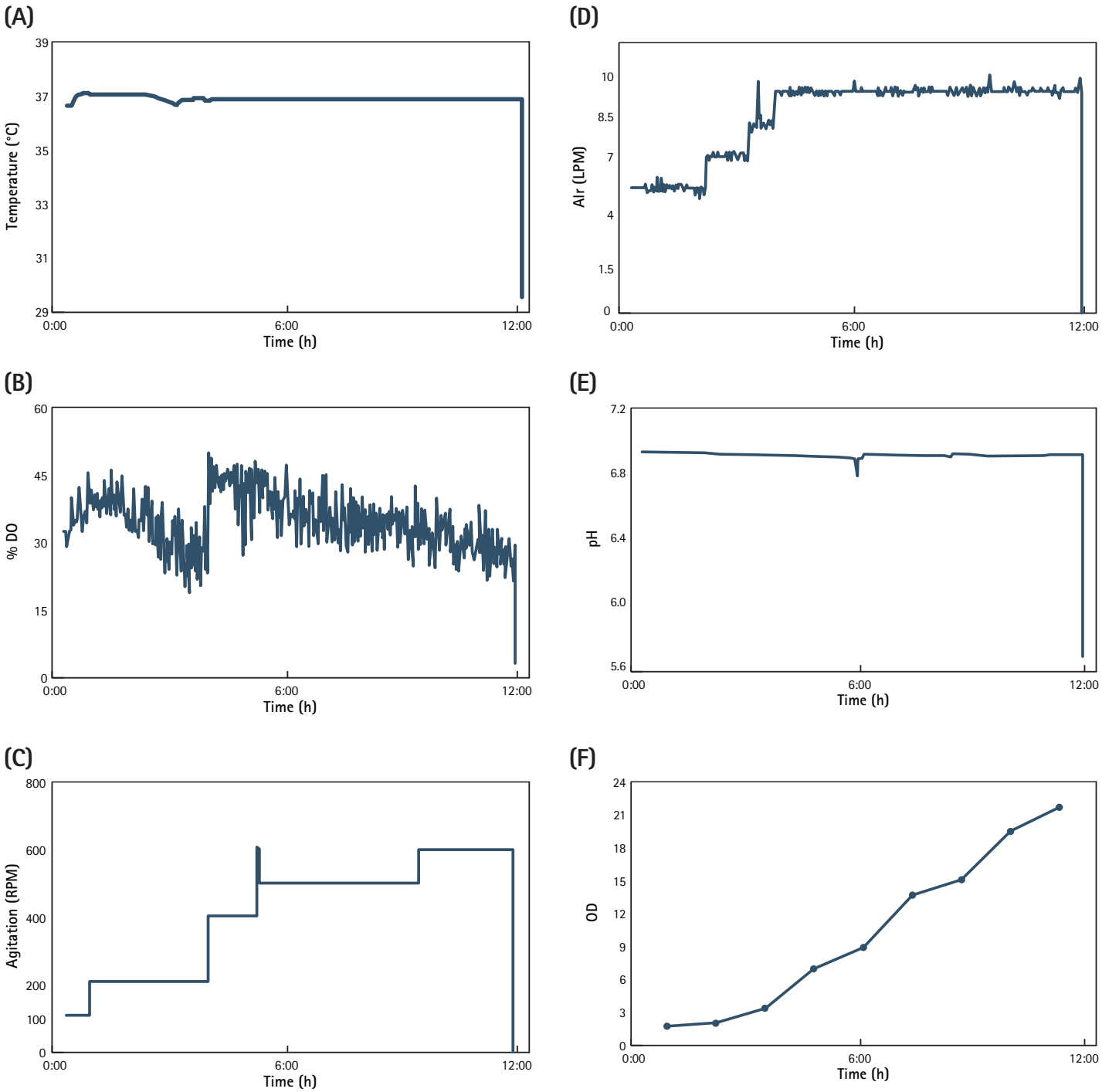

Figure 1. Real-time profile of fed-batch fermentation. (A) Temperature vs. time; (B) Dissolved oxygen vs. time; (C) Agitation vs. time; (D) Air vs. time; (E) pH vs. time; (F) OD vs. time.

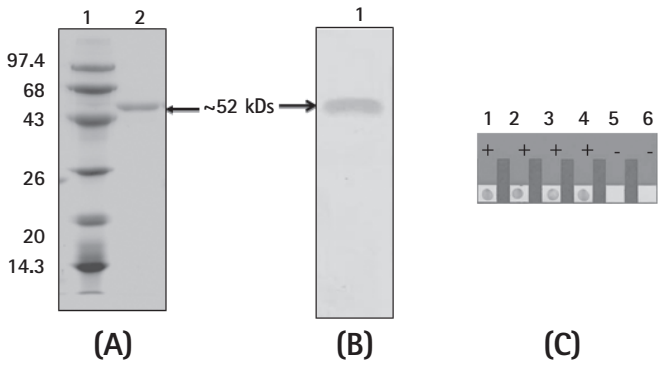

Figure 2. (A) SDS–PAGE analysis. Lane 1, Molecular weight marker (kDa); Lane 2, Ni-NTA purified protein. (B) Western blot analysis. Lane 1, dengue-positive (IgM) human serum sample. (C) Dipstick ELISA. Lanes 1–4, dengue-positive (IgM) and Lanes 5–6, dengue-negative serum sample.

etc.) as well as to complex conditions in the bioreactor (higher agitation rate, dissolved oxygen tension, etc.). Western blot analysis was performed with dengue-positive and -negative human sera. These results revealed that the dengue-positive human sera reacted and bound with the rDME-M protein in the blot (*Figure 2B*) whereas the negative sera did not react (result not shown). Reactivity of this protein with dengue-positive and -negative sera was also tested by dipstick ELISA (*Figure 2C*) for evaluation of its diagnostic potential.[17] These results suggest that the rDME-M protein could indeed be used to detect the presence of anti-dengue antibodies in patient serum.

In a previous report,[17] we described production of IgM-specific recombinant dengue multi-epitope protein for early diagnosis of dengue infection. In order to increase the potential of rDME-M protein for its application as a diagnostic reagent, enhancement in production of the protein is necessary. Optimization of the cultivation medium and fed-batch fermentation strategy, as described in this report, should make this application more feasible.

Conclusions

In this work we have optimized the culture medium for production of rDME-M protein. TY medium was found to support best growth and produce more recombinant protein than other media used. We have also developed a simple fed-batch fermentation strategy to produce biologically active rDME-M protein in *E. coli*. Fed-batch fermentations resulted in approximately 96.43 mg/L pure, biologically active recombinant protein. The large quantity of pure and antigenically active recombinant antigen produced by these methods may be utilized for early diagnosis of dengue infection. This process may also be suitable for high-yield production of other recombinant proteins.

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