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MEDIA SELECTION, OPTIMIZATION FORTHE EXPRESSION OF DIPHTHERIAE TOXOID IN RECOMBINANT *E. COLI*

SAPAVATU SN[1] AND KAKKERLA A[2*]

1: Professor, Department of Chemical Engineering, University college of Technolgy, Osmania University, Telangana, India

2: Research Scholar, Department of Pharmacy, University college of Technolgy, Osmania University, Telangana, India

***Corresponding Author: Ms. Anitha Kakkerla; E Mail: anithakakkerla.k@gmai.com**

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ABSTRACT

Objective: In the design of recombinant protein expression conditions, a number of essential elements such as type of medium and bioprocess parameters like temperature, agitation, dissolved oxygen, trace elements optimization is considered. As bioprocess is cost effective process, hence production reproducibility issues can be addressed by well designed, medium and protein expression influencing parameters. In this study, rDNA *E. coli* chosen as protein expressive organism. To maximize the expression of protein different types of media and trace elements, temperature and RPM conditions were studied. This study goal was to develop optimized media to maximum expression of protein (Cross reactive Material (CRM197) of Diphtheriae toxoid) in recombinant *E. coli* (*E. coli* Strain-BL21 DE3, Plasmid: PET24a), which is used for conjugation of the polysaccharide vaccines to produce the T dependent immune system in children. **Materials and Methods**: rDNA *E. coli*, LB medium, Trace elements, Probe Sonicator and refrigerated centrifuge. For growth of the rDNA *E. coli*, three different media (Minimal, Medium and Rich Media) were selected, and temperature 25 ºC,37 ºC, RPMs were selected to observe expression of protein. The expression of CRM197 was analyzed by using the SDS-PAGE Gel electrophoresis. Lysis and formation of IBs were purified to get purified protein. **Conclusion:** The result revealed that the Rich media and Trace Element Zinc sulphate combination holds potential for use as a high cell density growth medium and to get high intensity of the

protein expression CRM197 for recombinant *E. coli* (CRM197), when compared with Minimal Medium.

Keywords: CRM197, Diphtheriae toxoid, Rich Media, SDS PAGE, recombinant *E. coli*, polysaccharide vaccine

INTRODUCTION

Capsulated bacteria like pneumococcal, Hib, Typhoid are most polysaccharide restricted to infants and compulsory immunizations at earliest to infants. Polysaccharide variants has less immunity and not T-depend. The absence of efficacy of this vaccine in infants triggered development of conjugate vaccines which are so effective that there is now no room for plain polysaccharide Hib vaccines. Pneumococcal infections pose similar problems to Hib, but are more complex. The immunogenicity of the different pneumococcal serotypes varies considerably in infancy. Although the current CPS vaccine provides limited protection in infancy, the burden of pneumococcal infection is so high that its use could be reconsidered should conjugate vaccines be available later than expected.

The variance between polysaccharide antigen and protein antigen is that the former are T-dependent antigens whereas the later are T-independent. The immaturity of a B-cell population seems to be the reason for the unresponsiveness of young children against Ti-2 antigens. The Hib vaccines, pneumococcal conjugate vaccine contains the capsular polysaccharide chemically conjugated to a carrier protein. This makes the non T-dependent polysaccharide vaccines become T-dependent immune response vaccines for early age infants. CRM197Cross-reactive material 197 (CRM 197) is a non-toxin mutant of *Diphtheria Toxin (DT)* and it is used a carrier protein in several conjugate vaccines. As the starting generation of carrier proteins such as diphtheria toxin and tetanus toxin require detoxification with formaldehyde eliminating part of the lysine residues needed for glycan attachment, thereby limiting conjugation efficacy. One of the most widely used and highly effective carrier protein is Cross-Reactive-Material-197 (CRM_{197}) the carrier protein is covalently linked to poorly immunogenic and T-cell-in-dependent capsular polysaccharides, thus creating T-cell-dependent conjugate antigens that are highly immunogenic in infants [**1, 2]**. As so many Carrier proteins are available to conjugate the polysaccharide vaccines, but the most preferable one is CRM197 because of its nontoxic nature it is favorable for the conjugation of polysaccharide capsule containing Haemophiles influenza and Streptococcus pneumonia (Immunological

principles of polysaccharide-protein conjugate vaccination)

The lack of toxicity in CRM197 has eluded scientists since it was discovered that the difference from DT is the single amino acid substitution G52E. G52 was already known as a non-catalytic residue, and the crystal structures of DT showed that residue G52 is not directly involved in either NAD binding or EF-2 recognition [**3-12**].

Escherichia Coli is a Rod–Shaped gram-negative, nonpathogenic bacterium and favorable host for large scale production of recombinant proteins typical strain will double in number every 20 or 30 min. because of its multiplication capacity also *E. coli* is the best choice for maximum recombinant protein production. It can grow maximum in minimal medium that contains a carbon compound such as glucose (Which serves both as a Carbon and an energy source) and salts that supply Nitrogen, Phosphorus and trace elements. Isolation, characterization, media availability is more for the *E.coli* culture development (Production of Recombinant carrier proteins).

Eventually the cell density increases to a point at which nutrients or oxygen become depleted from the medium, or at which waste products (such as acids) from the cells have built up to a concentration that inhibits rapid growth. At this point, which, under normal laboratory conditions occurs when the culture reaches a density of 1–2 × 109 cells/ml, the cells stop dividing rapidly. This phase is called saturation and a culture that has just reached this density is said to be freshly saturated. The bacterial strains used in recombinant DNA work are derivatives of *E. coli* strain K-12 **[13-14]**.

MATERIALS AND METHODS

Reagents and chemicals

The *E. coli* strain BL21 (DE3) pET23 vector-GENSCRIPT, Luria broth medium (Yeast-Himedia, Tryptone- Himedia, Peptone-Himedia, Nacl -Merck), Zinc Sulphate, Copper Sulphate, IPTG, Tris buffer, Urea, Guanethidine- Manufacture by Merck. Tris Buffer from Finar limited, Q Sepharose Resin, DEAE Resin, Column chromatography – GE health Care.

Equipment/Instruments

Refrigerator Centrifuge– Elekt, Probe Sonicator -PCI analytics, SDS PAGE gel– Bio-Rad, Agarose Gel Electrophoresis – Cleaver (Futurebioscience), Mini Orbital Incubator, Rotary shaker -WAIOMETRA, HPLC – Scemazdu, UV spectrophotometer – Lab India

Methods

Selection and optimization of media in Shake-flask

Seed preparation

From working cell bank of *E. coli* (CRM197) which was stored at -70°C was thawed and was cultured on a streak plate containing antibiotic kanamycin (52mg /L) and was incubated at 37 °C for 24 hrs to obtain single colonies. Well grown colonies were used for the flask studies.

Preparation of Minimal Media [11]

Minimal Media composition Na_2HPO_4, KH_2PO_4, NH_4Cl, NaCl, 15 mg $CaCl_2$ and supplemented with Glucose. The above chemicals are weighed and dissolved in water by heating and stirring until dissolved. Shake well to mix up uniformly.

Two conical flask of 500 ml capacity was taken and poured 250 ml of the above media. With inserting non-absorbent cotton plug wrapped with gauge cloth. All the three flaks are autoclaved under steam at a pressure of 15 lband a temperature of 120°C for 15 minutes. 2 flacks were kept in LAFU to get down the temperatures after getting the media temperature to Room Temperatures required amount of antibiotic Kanamycin (52mg /L) was added in 2 flasks.

Inoculation of Media and Addition of IPTG to minimal Media

A single recombinant colony from streak plate was inoculated in each flask and one flask was incubated in orbital shaker incubator at temperature 37°C, RPM 200. Another flask kept at 25°C, RPM 200. 0.2 mM IPTG was added to culture medium after reaching the OD at 600 nm 1. It was reached after 30 hrs. then process was continued 4 hrs. after 4hrThe OD_{600} nm was checked for both flasks.

Preparation of Medium Media [11]

Medium Media composition $(NH_4)2SO_4$, KH2PO4, $FeSO_4 \cdot 7H_2O$ and Glucose. The above chemicals are weighed and dissolved in water by heating and stirring until dissolved. Shake well to mix up uniformly. Adjust to pH 7 with KOH solution. Prepared Medium media 250ml volume was poured in to 2 conical flask of 500 ml capacity. Capped the conical flasks with non-absorbent cotton plug wrapped with gauge cloth band. All the three flaks are autoclaved under steam at a pressure of 15 lb/in^2 and a temperature of 120°C for 15 minutes. Cool down to room temperature and keep in LAFU. Add antibiotic Kanamycin (52mg /L) in all three flasks.

Inoculation of Media and Addition of IPTG to minimal Media

A single recombinant colony from streak plate was inoculated in each flask and one flask was incubated in orbital shaker incubator at temperature 37°C, RPM 200. Another flask kept at 25°C, RPM 200. 0.2 mM IPTG was added to culture medium after reaching the OD at 600 nm 1. It was reached after 24 hrs.

then process was continued 4 hrs. after 4hrThe OD_{600} nm was checked for both flasks.

Preparation of Rich Media [11, 12]

Tryptone, yeast and NaCl combinedly known as rich media. The name itself indicating it contains maximum source of nutrients supplements to grow the *E.coli* like microorganisms. Along with this 3 ingredients glucose also added and trace elements like Zincsulphate was also added. The above chemicals are weighed and dissolved in water by heating and stirring until dissolved. Pour 250 ml of media in 2 conical flasks and kept for autoclave under steam at a pressure of 15 lb/in2 and a temperature of 120°C for 15 minutes. Cool down to room temperature and keep in LAFU. Add antibiotic Kanamycin (52mg /L) in 2 conical flasks.

Amplification of recombinant *E.coli* (CRM197)

A single recombinant colony from streak plate was inoculated in each flask and one flask was incubated in orbital shaker incubator at temperature 37°C, RPM 200.Another flask kept at 25°C, RPM 200. 0.2 mM IPTG was added to culture medium after reaching the OD at 600 nm 1. OD at 600 nm 1 was reached within 6 hrs in 37°C, RPM 200 parameters maintained conical flask. Another flask the OD was reached 1 after 10 hrs. In both flasks after reaching the OD 1 IPTG was inducted, process continued for 4 hrs. after 4hrs. The OD_{600} nm was checked for both flasks.

Isolation of protein from Minimal, Medium and Rich medium [13]

The cells were harvested using Refrigerated Centrifuge at temperature 4º C, 6000 G for 15 minutes. After centrifugation cycle completed the soup was discarded. The cell pellet is collected, kept at 4º C for further processing.

Lysis of the cell pellet [13]

Lysis Buffer 20 mM Tris buffer was prepared and adjusted to pH 8 by using dil HCl. The cell pellet (1g/10ml) was dissolved in Lysis buffer and kept in probe sonicator for lysis until the color of the solution becomes light and less opaque in nature.

The lysis solution was centrifuged again by using Refrigerated Centrifuge at temperature 4º C, 5000 G for 15 minutes. The cell pellet (1g/10ml) was dissolved in Lysis buffer and kept in probe sonicator. 5 cycle of lysis process was done (20 minuts for each cycle) (20 sec each time, 90% amplitude). The sonication should be performed in ice-water bath, with 2–3 min intervals between rounds to allow the suspension to cool. The same procedure was done for 5 times. The final obtained cell pellet was washed for 3 times. IB pellet was stored at temperature 4º C and soup also collected and stored at temperature 4º C. Same lysis process was repeated for minimal

media, Medium media and Rich media samples. Obtained all medias soups and pellets were performed SDS-PAGE gel electrophoresis analysis to know the CRM197 protein expression intensity.

Solubilization Refolding of formed IBs (Inclusion Bodies -IB) [14, 15]

Solubilized buffer (Urea+ 20 mM Tris pH-8) was prepared and pH was adjusted to 8. Washed and stored cell pellets (only cell pellets, not soup) were solubilized using the above Solubilization buffer. Solubilized samples were analyzed for protein expression under SDS-PAGE gel electrophoresis.

Refolding buffer was prepared by adding 25 mM sodium acetate, 0.3% Tween 20 and 5 µM copper sulphate per 1 liter of water.

The IB pellet was dissolved in Solubilized buffer (1:10 ratio i.e 1g in 10 ml) and kept for 45 min incubation in magnetic stirrer for complete solubilization. The Refolding buffer was added to the sample, adjusted to pH 5.5 by using diluted HCl and kept for 4 hrs under stirring maintaining temperature at 4°C. The solution was then filtered with 3 µm filter and kept for incubation at 4°C for 16 hrs. After incubation the sample was filtered with 0.45 µm filter paper.

SDS-PAGE and Agarose gel electrophoresis [16-18]

SDS/PAGE and Agarose analysis for performed with solubilized rCRM 197. CRM along with the reference CRM 197 was loaded on to 12%, 1% Agarose gel. SDS-PAGE Gels were run at 90-150 V different voltage until blue dye reached the bottom of the gel. SDS/PAGE molecular weight markers (New England Bio labs) were used for molecular weight calibration. After Coomassie Brilliant Blue staining, the image was captured using transilluminator. Nuclease activity by agarose gel electrophoresis and image was captured under UV Transilluminator.

Purification by Q-Sepharose (Ion Exchange chromate Graphy) [19, 20]:

The Acrylic column was packed with Q-sepharose and connected to Bio-Rad Chromatography System. The Resin was packed based on the column height and width. The load sample capacity was calculated based on the protein concentration.

The resin was equilibriated with Equilibration Buffer (20 mM Tris buffer). Pass the Sample solution (Load solution) at 2 ml/min and collect the sample flow through. Then passed Wash Buffer (20 mM Tris buffer), then Elution Buffer (1M Nacl and 20 mM Tris), collect the fractions according to the absorbance. The protein was analyzed by using SDS PAGE Analysis and under UV-Spectroscopy in OD_{280} nm.

RESULTS & DISCUSSION

All flasks were analyzed by SDS-Gel ecletrophoresis and the results were captured in **Table 1**.

Table 1: OD_{600nm}, Biomass with respect to Minimal, Medium and Rich media in lot nos. 1, 2 and 3 along with Expression

S. No	MEDIA (250 ml)	OD at 600	Average Biomass (g)	Expression
1	Minimal a) Flask 1 b) Flask 2	 0.5 0.5	0.2 g	Intensity of expressed protein was negligible
2	Medium a) Flask 1 b) Flask 2	 1 1	0.5g	Intensity of expressed protein was negligible
3	Rich a) Flask 1 b) Flask 2	 3.5 2.5	2g	Protein expression was more than the reference standard

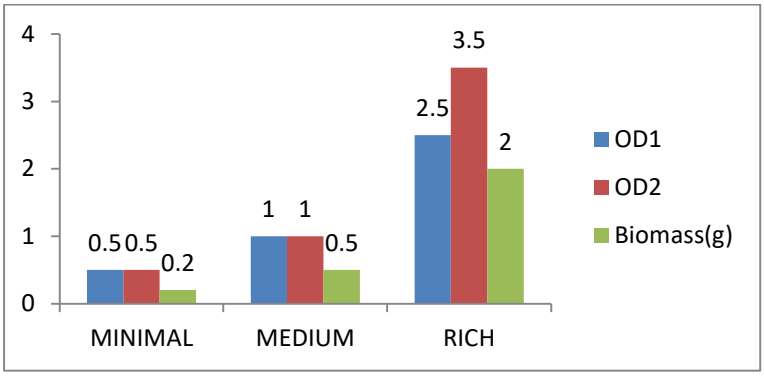

Figure 1: Comparative graph of OD_{600nm} and Biomass with respect to Minimal, Medium and Rich media in lot nos. 1and 2 and 3

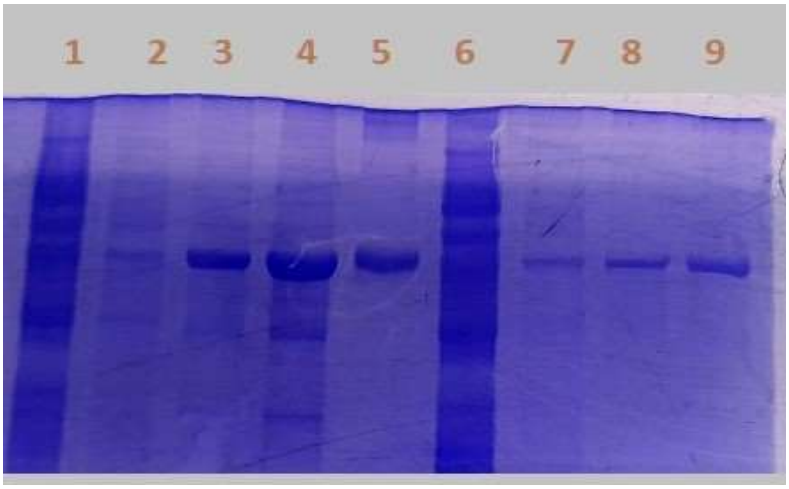

Figure 2: SDS –PAGE gel expression of the purified elution's of minimal, medium and rich medium ($ZnSO_4$, Refolded, nuclease active optimised formula)
Lane 1; 1st wash sample; Lane 2; 2nd wash sample; Lane 3; 3rd wash sample; Lane 4; IB solubilized (37ºC) in pellet; Lane 5; Std BSA; Lane 6; rCRM Protein at 25 ºC in soup; Lane 7; rCRM Protein at 25 ºC in pellet; Lane 8; In minimal media; Lane 9; In Medium media

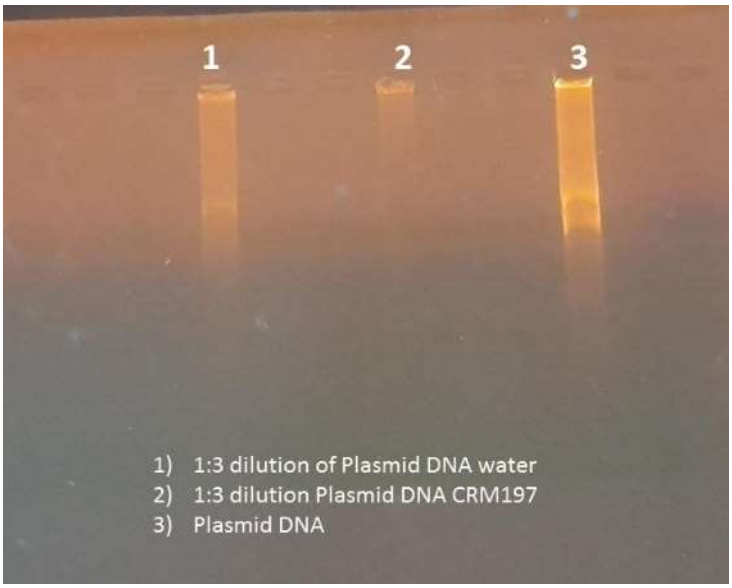

Figure 3: Pellet obtained from rich media were performed the Nuclease activity by Agarose Gel electrophoresis

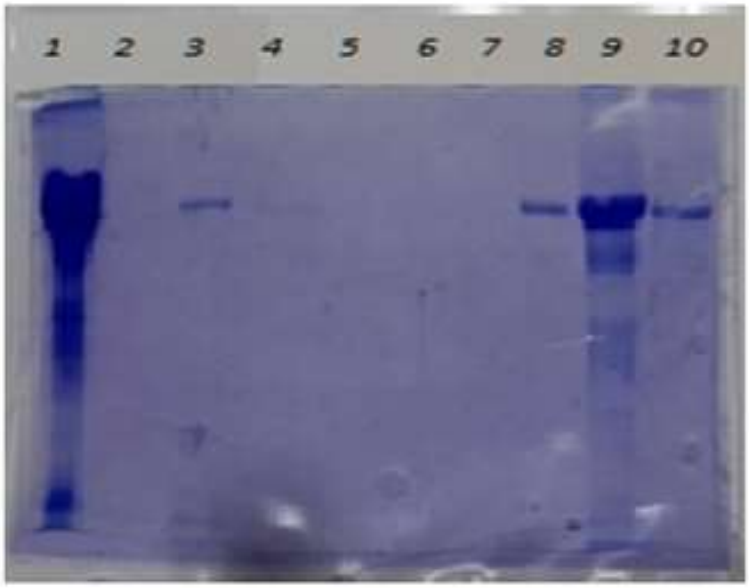

Figure 4: SDS-PAGE gel expression of the purified elution in Rich media
Lane 1: Load sample; Lane 2: Sample Flow Through; Lane 3: Post Load wash; Lane 4: Elution buffer 1; Lane 5: Elution buffer 2; Lane 6: Elution buffer 3; Lane 7: Elution buffer 4; Lane 8: Elution buffer 5; Lane 9: Elution buffer 6; Lane 10: Elution buffer 7;

DISCUSSION

As a proof of concept for production of recombinant protein CRM197, the *E. coli* BL21 (DE3) cells for the protein along with Kanamycin (Antibiotic marker) were cultured in Minimal, Medium and Rich media. The expression of the produced protein was analyzed on SDS PAGE gel and Agarose Gel electrophoresis. It was observed that all the post induction samples i.e. the samples

withdrawn after induction of culture with 0.2 mM IPTG solution showed the expression of the protein differently. Clear bands were seen at approximately 58 kDa molecular weight with respect to the molecular marker and the reference standard in Rich media. Very negligible intensity of the expression was observed with Minimal and Medium Media. In comparison, the OD_{600nm} of *E. coli* reached 0.5,1, and 3. Minimal, Medium and Rich media, respectively, over 48 hours under identical culture conditions (temperature 37°C and RPM 200). Flasks incubated at 25°C, IBs were formed in pellet and soup but the protein expression was less, this makes the purification step tedious.

The Rich media able to show better expression and yield of CRM197 compared to Minimal and Medium compositions. Thus, preliminary data suggested that the Rich medium and Zinc sulphate combination holds potential for use as a high cell density growth medium for recombinant *E. coli* (CRM197).

CONCLUSION:

The result revealed that the Rich media and Trace Element Zinc sulphate combination holds potential for use as a high cell density growth medium and to get high intensity of the protein expression CRM197 for recombinant *E. coli* (CRM197), when compared with Minimal Medium.

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