Media Optimization for Production of Recombinant Carrier Protein (CRM\textsubscript{197}) in \textit{Escherichia coli}

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Abstract
Since the advent of vaccines, the mankind has benefited from the same and has been able to curb the mortality rate around the globe. Amongst different types of available vaccines, polysaccharide based vaccines are very widely used against various infectious diseases. The polysaccharide vaccines need to be conjugated with a carrier protein to make the vaccine more immunogenic. Recombinant \textit{Escherichia coli} cells are the organism of choice for large scale production of a carrier protein because of its widely studied scientific aspects. In the present study, for proof of concept, the recombinant \textit{E. coli} cells were cultured in Luria-Bertani media to check the expression of rCRM\textsubscript{197}. At 80L scale, it was observed that when recombinant \textit{E. coli} cells were grown in a chemically defined media, it resulted in inconsistent growth and a long lag phase. When the defined media was supplemented with yeast extract, the lag phase of the culture was substantially reduced and the maximum growth of the culture was achieved. Protein expression was checked using SDS PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) and Western blot technique. The optimized media resulted in a robust fermentation process to achieve high cell density and maximum biomass for the production of recombinant protein.

Keywords: E. coli; rCRM\textsubscript{197}; Fermentation; Media; Vaccine.

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1. Introduction
Vaccine is a biological preparation, administered in body to provide immunity against a specific infectious disease. Vaccines that we know today can be of four major types namely, Live/attenuated, Inactivated/killed, Toxoid and Subunit vaccines [1]. Live attenuated vaccines are those vaccines in which the pathogen is cultured for several generations into a non-human host or under abnormal culture conditions where after

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certain generations it loses its ability to cause disease into human but it can evoke the immune response (MMR-Measles, Mumps, Rubella, Rotavirus, BCG-Bacille Calmette Guerin etc.). Inactivated or killed vaccines are those vaccines in which the vaccine is made from those microorganisms which are killed by heat or chemicals and cannot cause disease (Whole cell pertussis, Polio etc.). Toxoid or Inactivated exotoxins are prepared by inactivating (heat or chemical) the toxoids released by pathogenic bacteria (Diphtheria and Tetanus). Subunit or purified antigen vaccines contains only the antigenic part of the pathogen which is necessary to elicit immune response. If the antigen is not a potent activator of the immune system, it is conjugated with a carrier protein (Hib, Pneumococcal, Hepatitis B etc.) \[2\]. Over the past three decades, many routine childhood and adult vaccines have been developed using conjugation technology.

Out of several carrier proteins in use today, Cross Reacting Material-197 (CRM\textsubscript{197}) is the most researched and widely used carrier protein because of its non-toxic nature. CRM\textsubscript{197} is nontoxic form of diphtheria toxin which is a genetically modified. Single amino acid exchange of glycine in position 52 to a glutamic acid renders the protein non-toxic [3]. Molecular weight of CRM\textsubscript{197} is 58.4 kilo Dalton (kDa). Traditionally, CRM\textsubscript{197} is obtained from supernatant of \textit{Corynebacterium diphtheriae} culture; it is purified using filtration and chromatographic steps. Purification of CRM\textsubscript{197} from \textit{Corynebacterium diphtheriae} suffers from low yield [4].

\textit{E. coli} is a well characterized, safe (nonpathogenic) and suitable host for large scale production of recombinant proteins in a simple culture condition and lesser amount of time with respect to classical fermentation [5-7]. LB (Luria-Bertani) media although used widely for culturing of \textit{E. coli} is not suitable for industrial scale fermentation [8] as the LB media is designed for low density culture and does not match the specific requirement of the recombinant \textit{E. coli} cells for protein production. Each recombinant cell culture requires specific media optimization studies. Although there are various tools available today based on statistical analysis of the variable inputs and the required outputs (Plackett Burman method, Fractional Factorial method, Response surface model etc.) nevertheless extensive research and experiments are required to develop an optimal media for production of specific recombinant protein [9]. The biggest advantage of choosing \textit{E. coli} based model for recombinant protein production is the availability of vast literature and research papers for the culture of \textit{E. coli} [10,11]. The current study was initiated to optimize the \textit{E. coli} fermentation media for production of rCRM\textsubscript{197} protein. The increase in the global demand of polysaccharides conjugate vaccines against encapsulated bacterial pathogens highlight the pressing need for high yield process delivering well characterized carrier protein that meets regulatory and safety requirements. The development of a high yielding process will reduce the ever rising demand of the carrier protein which is often considered as the bottleneck for the development of Glyco-conjugate vaccines and these vaccines can be made available to the common people at a cheaper rate.
2. Material and Methods

To optimize the media for production of rCRM<sub>197</sub> protein, recombinant *E. coli* BL21 (DE3) cells with LAC promoter and Kanamycin resistant strain was used for optimum expression. The sequence for CRM<sub>197</sub> gene was synthesized and cloned in pTWIN 1 vector by New England Biolabs, UK (United Kingdom).

2.1. Proof of concept (POC)

The initial study begin with the Luria-Bertani media [12] as a proof of concept (POC) by streaking loop full culture of cell bank vial on LB agar plates containing Kanamycin (50 mg/L). Streaked plate was incubated at 37 ± 2 °C for 12 to 24 h. After completion of incubation period, a single colony from the streaked plate was inoculated into 5 mL LB media containing Kanamycin (50 mg/L). The Pre-seed culture was incubated at 37 ± 2 °C for 4 to 10 h. Seed culture was prepared by inoculating 0.5 mL of Pre-seed culture into 40 mL of LB media containing Kanamycin (50 mg/L). Seed culture was incubated at 37 ± 2 °C for 10 to 20 h till the OD<sub>600</sub> reached ≥ 1.0. At each stage, the culture was checked for its purity by gram staining. The Seed culture was further inoculated into fermenter (B Braun) containing 5 L sterile LB media and Kanamycin (50 mg/L). Fermentation was carried out at temperature: 30 ± 2 °C, RPM: 200 to 900, pO<sub>2</sub>: ≥ 30 %, pressure: 0.5 ± 0.1 bar and pH: 6.8 ± 0.1 till OD<sub>600</sub> reached 0.8 to 1. At the specified OD<sub>600</sub> the culture was induced with 0.5 mM of IPTG solution to start the expression of the recombinant protein. Fermentation was further carried out till stationary phase of the culture. Samples were withdrawn to analyse the expression of rCRM<sub>197</sub> protein on 12 % SDS PAGE (Reduced). The expression was further confirmed with Western blot.

2.1.1. SDS PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)

Reducing SDS PAGE technique was performed for separation of the expressed protein and analysis as per standard protocol [13]. 12 % tris-glycine gel was used under reducing condition. 80 V voltage was applied for initial 10 min and there after 200 V till completion of gel run. The gel was loaded with the prepared samples (20 µL), Molecular weight marker (22, 32, 58, 80 and 135 kDa; New England Biolabs, cat#P7712S), rCRM standard (10 µL, 58 kDa, GSK). The gel was stained with Coomassie Brilliant Blue R250 dye and image captured on Trans-illuminator (Bio-Rad ChemidocXRS + system).

2.1.2. Western blotting

For Western blotting, the SDS PAGE gel was transferred to Nitrocellulose membrane (Bio-Rad, Cat# 1620115) using standard protocol [14]. The western blot was incubated for 60 min and probed with antidiphtheria polyclonal antibody (Abcam, Cat#ab151222). The probed membrane was incubated with secondary antibody (Rabbit, Make: Santa Cruz
Biotechnology, Cat#SC2054) conjugated with HRP (Horseradish Peroxidase) enzyme. The bands were observed with 3, 3’-diaminobenzidine (DAB) substrate. Further the image was captured on Trans-illuminator.

2.2. Standardization of media for higher yield with chemically defined media

Based on the initial development at 5 L scale fermentation process, 80 L scale fermentation was optimized to achieve higher growth and maximum expression of the protein. A chemically defined fermentation media [15] including: K$_2$HPO$_4$ (11.43 g/L), (NH$_4$)$_2$SO$_4$ (5.71 g/L), citric acid (2.43 g/L), MgSO$_4$·7H$_2$O (0.57 g/L), FeCl$_3$·6H$_2$O (142.86 mg/L), MnSO$_4$ (20 mg/L), ZnSO$_4$·7H$_2$O (17.4 mg/L), EDTA (Ethylenediaminetetraacetic acid) (12 mg/L), H$_3$BO$_3$ (4.29 mg/L), Na$_2$MoO$_4$·2H$_2$O (3.43 mg/L), CoCl$_2$·6H$_2$O (3.43 mg/L), CuCl$_2$·4H$_2$O (2.14 mg/L), glucose (15 g/L), kanamycin (50 mg/L) and antifoam-PPG (25 %) was used. Fermentation was carried out in a 140 L fermenter (Scigenics). The initial glucose concentration in fermentation media was kept low to avoid acetate accumulation. The glucose concentration and the concentration of other trace elements were maintained by Fed batch mode of fermentation [16]. Initially the fermentation was carried out in batch mode and as the growth of culture reached at certain optical density (indicated by depleted glucose level or increased pO$_2$ level), the batch was feed with feeding solution which included: glucose (450 g/L), MgSO$_4$·7H$_2$O (18 g/L), FeCl$_3$·6H$_2$O (36 mg/L), MnSO$_4$ (21 mg/L), ZnSO$_4$·7H$_2$O (18 mg/L), EDTA (13 mg/L), H$_3$BO$_3$ (4.5 mg/L), Na$_2$MoO$_4$·2H$_2$O (3.6 mg/L), CoCl$_2$·6H$_2$O (3.6 mg/L) and CuCl$_2$·4H$_2$O (2.3 mg/mL).

Pre-seed culture was prepared by inoculating 1mL culture from cell bank vial into 200 mL of LB media containing Kanamycin (50 mg/L) and glucose (5 g/L). The culture was incubated at 37 ± 2 °C, 150 to 200 RPM for 4 to 20 h. In the seed culture, 7 flasks containing 600 mL of LB media, Kanamycin (50 mg/L) and glucose (5 g/L) were each inoculated with 5 mL of culture from Pre-Seed and incubated at 37 ± 2 °C, 150 to 200 RPM for 4 to 20 h. After OD$_{600}$ of culture reached ≥ 1, the seed culture was further inoculated into fermenter. At each stage, purity of culture was checked by Gram staining (Rod shaped, gram negative cells).

2.3. Fermentation

56 L of defined media was prepared in fermenter by in-situ sterilization of K$_2$HPO$_4$, (NH$_4$)$_2$SO$_4$, citric acid and MgSO$_4$·7H$_2$O. Glucose and other trace elements along with kanamycin was filter sterilized and added separately into the fermenter after completion of sterilization cycle. Ammonia solution (15 %) was used as base solution for maintaining the pH of the fermentation culture. Polypropylene glycol (PPG) (25 %) was used to control foam during media sterilization and fermentation. Approximately 4.2 L of seed culture was inoculated into fermenter and fermentation was carried out at temperature: 30 ± 0.2 °C, airflow: 80 to 150 SLPM, agitation: 200 to 900 RPM, back pressure: 0.3 bars,
pH: 6.8 ± 0.2 and pO₂ ≥ 30 %. When OD₆₀₀ reached 15 to 20, feeding of glucose solution including the trace elements was started at a flow rate of 18 mL/min. When OD₆₀₀ reached 40 to 50, the culture was induced with 0.5 mM filter sterilized IPTG (Isopropyl ß-D-1-thiogalactopyranoside) solution. After induction, feed flow rate was reduced to 15 mL/min. Once the culture entered in Stationary Phase, RPM and temperature of fermenter was decreased to 200 RPM and 15 ± 5 °C respectively. Culture was harvested and centrifuged at 8000 rpm for 30 min at 5 ± 3 °C. Pellet was stored at -70 °C. During fermentation samples were withdrawn at regular intervals for analysis of OD₆₀₀. Optical density of the withdrawn samples was measured at 600 nm using spectrophotometer. If the sample OD₆₀₀ was above 0.8, the sample was appropriately diluted with WFI (water for injection) to get the OD₆₀₀ less than 0.8. The observed OD₆₀₀ was than multiplied with the dilution factor to get the final OD₆₀₀ [17].

Pre and post induction samples were withdrawn and analysed for expression of protein with SDS PAGE and Western blot.

### 2.4. Fermentation using semi defined media

Based on the results obtained from the batches produced using chemically defined media, it was found that the growth profile of the culture was not robust and also the process time was long (approximately 40 h) for E. coli based system. Hence it was decided to shift from chemically defined media to Semi defined media.

#### 2.4.1. Inclusion of yeast extract in fermentation media

In order to reduce the lag phase of the culture, Yeast extract was included in the fermentation media at a concentration of 7.14 g/L. Final volume of fermentation media was kept same as in chemically defined media i.e. 56 L. Fermentation parameters were kept same as in batches produced using chemically defined media. Filter sterilized yeast extract solution was added into fermenter after completion of sterilization cycle along with glucose, Kanamycin and rest of the trace elements. Feeding, induction and harvesting criteria’s remained same as with chemically defined media batches. Samples were withdrawn at regular intervals for analysis of OD₆₀₀ and protein expression.

#### 2.4.2. Inclusion of yeast extract in feed solution

Based on the results obtained from the batches which included yeast extract in the fermentation media, in the next step, yeast extract was also included in the feed solution to further optimize the fermentation and overcome the sluggish growth of culture in the late phase of growth [18]. Fermentation media was kept same as of semi defined media. In the feeding strategy, a separate 40 g/L filter sterilized solution of yeast extract was used as feed at a flow rate of 8 mL/min. Feeding of glucose solution including the trace elements was kept as same with respect to batches produced using chemically defined media. No changes were made in the fermentation parameters, induction strategy and harvesting
criteria in comparison to the batches produced using chemically defined media. Samples were withdrawn at regular intervals for analysis of OD$_{600}$ and protein expression.

3. Results

3.1. Proof of rCRM protein expression

The initial study was performed using LB media which is widely used media for growth of *E. coli* and other bacteria’s. The streaked LB agar plate showed growth of colonies (Round shaped, off white and translucent) after overnight incubation.

At Pre-seed stage, OD$_{600}$ of 1.6 was achieved after 4 to 20 h of incubation. The Pre-seed culture was checked for its purity by gram staining and *E. coli* cells were observed (Rod shaped, gram negative cells with no contamination) without any contamination.

Seed culture with OD$_{600}$ of 3.7 was achieved after overnight incubation. Seed culture was checked for its purity and 40 mL of culture was inoculated into fermenter containing LB media with Kanamycin. Fermentation was carried out at temperature: 30 ± 2 °C, RPM: 200 to 900, pH: 6.8 ± 0.1, pO$_2$: ≥ 30 %, OD$_{600}$ before induction: 0.9, Induction age: 16 h, Final OD$_{600}$: 9.1

Fermentation samples were withdrawn at various time intervals. The samples were analysed using 12 % SDS PAGE (reduced) electrophoresis method (Fig. 1-A). After the run, the gel was used for Western blot as confirmatory test using CRM specific antibody (Fig. 1-B).

Load pattern for SDS PAGE and Western blot: Lane 1: Marker, Lane 2: Un-induced sample, Lane 3: 2 h post induction, Lane 4: 4 h post induction, Lane 5: 6 h post induction, Lane 6: 8 h post induction, Lane 7: 10 h post induction, Lane 8: Final harvest and Lane 9: rCRM standard.

![Fig. 1. Un-induced and post induction samples of POC lot analysed on A-12 % SDS PAGE (reduced) and B-Western Blot.](image)

Post induction samples appeared at approximately 58 kDa molecular weight in the SDS PAGE analysis. This molecular weight matches with the expected size of rCRM$_{197}$ protein and the reference standard. No band was observed for the un-induced sample. In the western blot analysis, the post induction samples were recognized by the anti CRM
antibodies. Both the SDS PAGE and Western Blot analysis prove that the POC for expression of rCRM\textsubscript{197} protein in recombinant \textit{E. coli} cells is successful.

3.2. \textit{Fermentation with chemically defined media}

In the next phase of development, scale up strategies and the media optimization studies were performed. Two consecutive lots (Lots 1 and 2) were taken. All the parameters were maintained throughout the fermentation lots. However both the lots were not consistent as seen in Fig. 2. A long lag phase and inconsistent growth profile was observed in the fermentation lot 2.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{comparison_graph.png}
\caption{Comparative graph of optical density (600 nm) with respect to fermentation time in lots 1 and 2.}
\end{figure}

\textbf{Lot no. 1}: The withdrawn samples (Un-induced, 16 h, 20 h and final harvest sample post induction) of Lot no. 1 was analysed on 12 \% SDS PAGE (reduced) electrophoresis method (Fig. 3-A) and Western Blot for expression of rCRM\textsubscript{197} protein (Fig. 3-B).

Load pattern for 12 \% SDS PAGE (reduced) and Western Blot: Lane 1: rCRM standard, Lane 2: Marker, Lane 3: 16 h post induction, Lane 4: Un-induced, Lane 5: 20 h post induction, Lane 6: Final harvest and Lane 7: Marker.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{lot1_analysis.png}
\caption{Un-induced and post induction samples of lot 1 analysed on: A- 12 \% SDS PAGE (reduced) and B-Western Blot.}
\end{figure}
Fig. 3-A indicates that the post induction samples of lot no.1 for 16, 20 and 26 h shows clear band at approximately 58 kDa molecular marker and matches with the rCRM standard. The western blot (Fig. 3-B) confirms the presence of rCRM<sub>197</sub> protein as it is recognized by anti CRM antibody probed on the Western blot.

**Lot no.2:** The withdrawn samples (Un-induced-22 and 30 h, Post induction- 5, 8, 10, 12 h and final harvest sample) of Lot no.2 were also analysed on 12 % SDS PAGE (reduced) electrophoresis method (Fig. 4-A) and Western Blot for expression of rCRM<sub>197</sub> protein (Fig. 4-B).

Load pattern for 12 % SDS PAGE (reduced): Lane 1: Marker, Lane 2: rCRM standard, Lane 3: 22 h un-induced, Lane 4: 30 h un-induced, Lane 5: 5 h post induction, Lane 6: 8 h post induction, Lane 7: 10 h post induction, Lane 8: 12 h post induction, Lane 9: Final harvest and Lane 10: Final harvest.

Load pattern for Western blot: Lane 1: Final harvest, Lane 2: Final harvest, Lane 3: 12 hrs post induction, Lane 4: 10 h post induction, Lane 5: 8 h post induction, Lane 6: 5 h post induction, Lane 7: 30 h un-induced, Lane 8: 22 h un-induced, Lane 9: rCRM standard and Lane 10: Marker.

It is evident from Figs. 4-A and 4-B that the post induction samples of lot no. 2 for 5, 8, 10, 12 h and final harvest sample shows clear band with respect to rCRM standard. The western blot confirms the presence of rCRM<sub>197</sub> protein as it is recognized by anti CRM antibody probed on the Western blot.

### 3.2.1. Effect of yeast extract addition in fermentation media on growth of culture

To make the fermentation media more robust and reduce the lag time of culture, chemically defined media was replaced with semi defined media by adding yeast extract in the fermentation media. Yeast extract is a complex media which is a rich source of nitrogen, amino acids, vitamins and several growth factors which when used in combination with chemically defined media can led to optimal growth of the culture [19].
The growth pattern observed for lot no. 3 (Fig. 5) was a clear indication that the addition of Yeast extract in the media has reduced the lag phase of the culture and made the media more robust. Although the maximum $OD_{600}$ obtained in lot no. 3 was lesser as compared to lot nos. 1 and 2; the lag phase was reduced substantially.

![Graph showing comparative optical density (600 nm) with respect to fermentation time in lot nos. 1, 2, and 3.](image)

Fig. 5. Comparative graph of optical density (600 nm) with respect to fermentation time in lot nos. 1, 2, and 3.

**Lot no. 3:** Pre and post induction samples of lot no. 3 were analyzed on 12% SDS PAGE (reduced) for expression of rCRM protein (Fig. 6).

Load pattern for SDS PAGE: Lane 1: Marker, Lane 2: rCRM standard, Lane 3: Un-induced, Lane 4: 18 h post induction, Lane 5: 20 h post induction, Lane 6: 24.5 h post induction, Lane 7: 28 h post induction and Lane 8: Final harvest.

![SDS PAGE gel showing un-induced and post induction samples of lot 3.](image)

Fig. 6. Un-induced and post induction samples of lot 3 analysed on 12% SDS PAGE (reduced).

In Fig. 6 the post induction samples of lot no. 3 for 18 h, 20 h, 24.5 h, 28 h and final harvest sample shows clear band with respect to rCRM standard and molecular weight marker. This indicates that the addition of yeast extract in the media reduced the lag phase of the culture without hindering the protein expression.
3.2.2. *Effect of yeast extract addition in feed solution on growth of culture*

Growth kinetics observed in lot no. 3 suggests that addition of yeast extract in fermentation media has the ability to reduce the lag phase of the culture and enhance process robustness. But since there was sluggish growth observed at the late stage of fermentation, experiment was designed to perform continuous feeding of yeast extract along with glucose and trace element solution in lot no. 4.

Growth profile of all the 4 lots were compared and it was observed that feeding of yeast extract along with glucose and trace element solution resulted in extending the growth of culture to approximately 150 OD<sub>600</sub> (Fig. 7). The combined effect of yeast extract addition in fermentation media as well as in the feed not only reduced the lag phase and the overall fermentation time but also increased the maximum achievable growth of the culture.

![Figure 7](image.png)

**Fig. 7.** Comparative growth profile of all the 4 lots at OD<sub>600</sub> with respect to fermentation time.

**Lot no. 4:** The withdrawn samples (Un-induced-11 h, Post induction- 13, 15, 17, 19, 23 h and Final harvest sample) of Lot no. 4 were also analysed on 12 % SDS PAGE (reduced) electrophoresis method (Fig. 8-A) and Western Blot for expression of rCRM<sub>197</sub> protein (Fig. 8-B).

Load pattern for 12 % SDS PAGE (reduced): Lane 1: Final harvest, Lane 2: 23 h post induction, Lane 3: 19 h post induction, Lane 4: 17 h post induction, Lane 5: 15 h post induction, Lane 6: 13 h post induction, Lane 7: Un-induced, Lane 8: rCRM standard and Lane 9: Marker.

Load pattern for Western Blot was: - Lane 1: Final harvest, Lane 2: 19 h post induction, Lane 3: 15 h post induction, Lane 4: Un-induced and Lane 5: rCRM standard.

It is evident from Figs. 8-A and 8-B that the post induction samples of lot no. 4 shows clear band with respect to rCRM standard. The western blot confirms the presence of rCRM<sub>197</sub> protein as it is recognized by anti CRM antibody probed on the Western blot.
Fig. 8. Un-induced and post induction samples of lot no. 4 analysed on: A- 12 % SDS PAGE (reduced) and B-Western Blot.

3.3. Reproducibility of yeast extract supplementation

To answer to the question whether the data of lot no. 4 was reproducible, another lot with same fermentation and feeding strategy was planned and executed as lot no. 5.

Lot no. 5 proved that the feeding and fermentation strategy was reproducible and in fact the addition of yeast extract in fermentation media and feed solution has made the media more robust and reduced the lag phase of the culture. The maximum OD$_{600}$ achieved in both the lots i.e. lot nos. 4 and 5 was above 140 and the total fermentation time was below 30 h (Fig. 9).

Fig. 9. Comparative growth profile of all the 5 lots at OD$_{600}$ with respect to fermentation time. In lot nos. 4 and 5, the final OD$_{600}$ was above 140 and the final fermentation time was below 30 h.

Lot no. 5: The withdrawn samples (Un-induced-11 h, Post induction- 11, 15, 19, 23 h and Final harvest sample) of Lot no. 5 were analysed on 12 % SDS PAGE (reduced) electrophoresis method (Fig. 10-A) and Western Blot for expression of rCRM$_{197}$ protein (Fig. 10-B).
Load pattern for SDS PAGE and Western Blot: Lane 1: Marker, Lane 2: rCRM standard, Lane 3: Un-induced, Lane 4: 15 h post induction, Lane 5: 19 h post-induction, Lane 6: 23 h post induction and Lane 7: Final harvest.

Fig. 10. Un-induced and post induction samples of lot no. 5 analysed on: A- 12 % SDS PAGE (reduced) and B-Western Blot.

3.4. Biomass

In all the 5 lots, the final harvest was centrifuged at 8000 RPM for 30 min at 5 ± 3 ºC. The collected pellet was stored at -70 ºC. Maximum achieved biomass concentration was 180 g/L in lot no. 4.

Fig. 11. Optical density (600 nm), biomass (g/L) and Fermentation time of all the 5 lots.

It is evident from Fig. 11 that the biomass concentration is directly proportional to the final OD₆₀₀ of the culture. There was approximately 3 fold increase in the final OD₆₀₀ of the culture and hence the biomass concentration. The total fermentation time of the culture was reduced to half which in turn increases the throughput of the production.

4. Discussion

As a proof of concept for production of recombinant protein CRM₁₉⁷, the E. coli BL21 (DE3) cells harboring the gene for the protein along with Kanamycin resistance were
cultured in LB media and the expression of the produced protein was analyzed on SDS PAGE gel and Western blot. It was observed that all the post induction samples i.e. the samples withdrawn after induction of culture with 0.5 mM IPTG solution showed the expression of the protein. Clear bands were seen at approximately 58 kDa molecular weight with respect to the molecular marker and the reference standard. With the success in the POC lot, the scales up studies were initiated with the chemically defined media and 2 lots were executed at 80 L scale. The chemically defined media consisted of Glucose as the carbon source along with other major and minor/trace elements [15]. The feed solution consisted of glucose and trace elements. Fermentation was carried out in Fed batch mode in order to avoid accumulation of Lactate which when accumulated as a result of glucose metabolism results in early stationary phase/sluggish growth of the culture. It was found that the fermentation time in both the lots was above 40 h. The observed lag phase and sluggish growth rate (till 10 h of fermentation, the observed OD\(_{600}\) in both the lots was below 20) can be due to the fact that the recombinant \textit{E. coli} cells into consideration required a long time to acclimatize when grown in chemically defined media. This lag phase and the sluggish growth rate led to prolonged fermentation time of above 40 hours which is unusual for \textit{E. coli} based model of fermentation. Despite extended fermentation time and inconsistent growth, protein expression was observed in both the lots (Lots 1 and 2) as evident from SDS PAGE and Western blot analysis.

In order to reduce the lag phase of the culture and the overall fermentation time, the chemically defined media was supplemented with a complex media i.e. yeast extract. With the inclusion of yeast extract, it was observed in lot no. 3 that the log phase of the culture was reduced without impacting the protein expression profile. The reason for the reduction in the log phase can be attributed to the fact that the initial culture of the cells (Pre-seed and Seed stage) was performed using LB media which is a complex media. Use of Yeast extract in the fermentation media reduced the acclimatization time of the culture at large scale fermentation stage. The analyzed post induction samples on SDS PAGE and Western blot showed bands at approximately 58 kDa molecular weight with respect to the molecular marker and the reference standard. However there was sluggish growth of the culture during late fermentation phase.

To achieve high cell density, in lot no. 4, Yeast extract was included in the Feed solution along with its supplementation in Fermentation media. It was observed that the maximum OD\(_{600}\) was increased to 150 and the overall fermentation time was reduced to less than 30 h. Optical density and the biomass were increased by approximately 3 folds. The results were reproduced in lot no. 5. In both the lots (Lots 4 and 5), the protein analysis on SDS PAGE and Western blot showed expression of protein at approximately 58 kDa molecular weight with respect to the molecular marker and the reference standard. It is evident from the above research that the use of complex media (yeast extract) along with chemically defined media has lead to an optimized media for production of recombinant protein CRM\(_{197}\).
5. Conclusion

Based on POC results and all the fermentation lots, it can be concluded that *E. coli* BL21 (DE3) strain is capable of producing rCRM<sub>197</sub> protein. The chemically defined media did not proved to be robust for the production of the rCRM<sub>197</sub> protein. Chemically defined media when supplemented with yeast extract in the fermentation media and Feed solution (maintaining all the other culture conditions similar to fermentation with chemically defined media) led to the reduction of lag phase of the culture, approximately 3 fold increase in maximum achievable growth and biomass concentration. With the inclusion of yeast extract in fermentation media and feed solution, a robust and reproducible fermentation process was established.

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