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Optimization of Semisynthetic Media Components for Intensive *Corynebacterium Diphtheriae* Growth and Toxin Production

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Abstract

Diphtheria is considered a serious infection occurred due to *Corynebacterium diphtheriae* which can able to produce toxins. This disease cause difficulty in breathing, paralysis, heart failure, and even death. The Centre for disease control suggested vaccines for adults and children for preventing the disease. The production of such vaccinesdepends on meat-based media that possess several complications. This study was motivated by the fact that the usage of semisynthetic media will prevent the side effects of meat-based media. Further few parameters like calcium chloride, iron, and iron-free L-Cystine were not deeply investigated in previous studies. In this research, diphtheria toxin has been synthesized by growing *Corynebacterium diphtheriae* ATCC *strain* in a semisynthetic medium during the shake flask stage fermentation. Hence the study attempted to optimize the parameters by measuring the optical density value through a UV spectrophotometer thereby determining the toxin expression by the Limes flocculation (Lf/mL) test. The semisynthetic medium is found to be free from animal-derived products and includes carbohydrates, water, nitrogen source, free amino acids, and iron source in initial concentration. Iron plays a vital role in affecting the production of toxins during *C.diphtheria* cultivation in the medium. The study obtained a high yield of diphtheria toxin with a Fe2+ concentration of 0.1g/L and a calcium chloride concentration of 11.1 g/L. Under these optimized parameters, this research could obtain effective production of diphtheria toxin.

Keywords: Diphtheria, *Corynebacterium diphtheria*, Calcium Chloride, Iron, L-Cystine.

Introduction

Diphtheria is an acute disease caused due to exotoxin synthesizing *Corynebacterium diphtheriae*. Further diphtheria shows a declining trend because of effective childhood vaccination schemes(20). A considerable portion of the worldwide burden of the disease has been observed in India. Diphtheria outbreaks and several hospital-based surveillance studies that have been published in the past twenty years represent that diphtheria cases are frequently found among adolescents and school children. As per the various national survey report, three-dose coverage of the diphtheria vaccine was nearly 80% and few other studies represent the low immunity prevalent among children and adults(5). The methods for diphtheria prevention have to be focused on improving the coverage of preliminary and booster vaccines that are administered bythe Universal Immunization Program.

Diphtheria toxin (DT) is a single-chained 62 kilodalton protein comprising 535 amino acid residues and lysogenic beta-phage. The toxin mediates the cyto-lethal effects by inhib-

iting the synthesis of protein in the susceptible cells. DT is Y shaped- molecule comprising two kinds of functionally different A and B regions in which a fragment situated at the N-terminus comprises the catalytic (C) domain that prohibits the synthesis of protein within eukaryotic cells. B fragment which is located at the C terminus possesses a receptor binding domain and trans-membrane domain. DT is an extracellular matrix that induces considerable health issues like sub-acute and acute cardiac, neurological, and renal complications. Since it is a life-threatening and devasting disease, various pharmacological and biological industries produce diphtheria vaccines and antibodies(21). After the DT vaccine development, diphtheria cases decreased to a great extent. But the increasing diphtheria cases were still found to be reported in poor and developing countries and hence it is mandatory to prevent the disease by various techniques for the production of DT with high immunity. Since the introduction of mass immunization in the 1920s, the vaccines against diphtheria are said to be the safest vaccines. However, in addition to diphtheria toxin, various proteins might be secreted during cultivation by *Corynebacterium diphtheriae*. It is assumed that diphtheria toxoid is not the only component that is present in the vaccine. Respiratory diphtheria is considered a critically fatal toxin disease, which does not affect the vaccinated population. Further, cutaneous infections with toxic C. diphtheriae are connected to travel to endemic regions. In recent years, public health agencies from various vaccinated regions have extended their guidelines for investigating toxic cutaneous diphtheria irrespective of the travel history. As diphtheria toxin testing is considerably increasing over the last 10 years, this could result in a significant increase in public health investigations(12). To address this, Moller et al(13)developed a protocol for reversing the formaldehyde cross-linking and analysed it through mass spectroscopy. And the results detected diverse secreted, cytoplasmic, and membrane-associated C. diphtheriae proteins in various preparations globally. Based on these outcomes, the authors performed western blot as well as bioinformatics analyses for characterizing the proteins as immunogenic or non-immunogenic. Additionally, the authors could deliberate that C. diphtheriae toxoid vaccine induces antibodies against various C. diphtheriae proteins. This toxoid acts as the immunizing antigen for providing against the serious impacts of Diphtheria toxin. It is synthesized by producing toxigenic *C.diphtheriae* in a Pope L in good meat extract medium. This media has been found to be rich in animal proteins and blood-group components and could be standardized hardly. The utilization of semisynthetic culture media possesses more benefits than meat-based media concerning WHO(15).

But the substances have to be optimized and standardized for supporting the intense bacterial growth of selected Diphtheria and toxin production. Further along with several physiological parameters during the growth of a bacterium, the concentration of casein hydrolysate, calcium chloride, and iron in the semisynthetic media are important equally for high toxoid purity and yield. The present study attempted to investigate and optimize the significant components of semisynthetic media like iron, calcium chloride, and L cystine that influence the intensive growth and toxin production of C. diphtheriae in shake flask stage fermentation. The following are the existing studies focused on in accordance with the research undertaken.

Studies associated with the production of diphtheria toxin with various media

Several kinds of transport medium have been suggested by (17) and observed that the best transport medium for C. diphtheria to be used at room temperature and equivalent to silica gel is serum telluride. This medium is also suitable for use as a transportation medium for cold temperatures (2-8 ºC) which is equivalent to amies as commercial media and silica gel.

Corynebacterium diphtheriae PW8's (Parke Williams 8) toxin production has been comprehensively examined by (19)Further,

several kinds of literature on amino acids in NZ medium, as well as BD (Beef digest medium), have recommended that an inadequate amino acid supply wasn't responsible for low yield of toxin in NZ medium. Growth promoting nutrients as well as amino acid supplementation into NZ-medium has enhanced cell growth, but it did not enhance toxin production. Therefore, the Beef digest medium has been selected by the study as a suitable medium for the production of toxins since it produced a significantly high flocculation limit (93±0 Lf per ml) than that of (46±0 Lf per ml) NZ medium. Moreover, supplementing 0.2% of YE in beef digest medium has led to a substantial increase in toxin production and cell growth (235±5 Lf per ml). Finally, the study concluded that higher toxin titre can be obtained from beef digest medium at 5-L scale production if 0.2% YE was supplemented if the protein content was at least 24 grams per litre, and the iron content was less than 0.15ppm.

Suwanpatcharakul et al (19) focused on the composition of amino acids of the medium synthesized from beef digest and casein hydrolysate medium. The study compared the two kinds of media for increasing the titre of toxin. Various factors affecting growth have been investigated and evaluated (19). Generally, Td-based integrated vaccines comprise only a very small amount of diphtheria toxoid antigen. But an increased purity level is required for the antigen. This toxin is synthesized by the growth of *C.diphtheriae* in a fermenter in the semisynthetic medium containing casein. For obtaining pure diphtheria toxoid, this study (4) determined the optimal conditions for toxin expression at 300 Lf/mL in the fermenter. During the cultivation of *C.diphtheriae* in a fermenter and obtaining the high toxin concentration, particular patterns for pH and dissolved O_2 levels were identified. This study divided the cultivation process into four in accordance with the pH variations. The particular range of KLa has been needed for producing high expression levels of diphtheria toxin. Previous studies (1) reported that the important factors associated with media synthesis are agitation and aeration during microorganism cultivation. Further mass production of diphtheria toxoid with high purity was achieved by detoxification and purification from the process.(Kamzolova et al., 2018)investigated the impacts of pH, aeration, and agitation for ensuring the better growth of *Yarrowia lipolytica*. This study also suggested the addition of an increased amount of zinc and, itaconic acid into the corresponding nutrition medium. Ko et. al., (2021) (11) produced *Corynebacterium* capable of oversynthesis of iron and membrane surface modification. The integration of two precursor pathways in accordance with the thermodynamic data raised the carbon flux of heme in the intermediate biosynthesis.

Other existing research associated with C. diphtheriae toxin

Immunization programs in several developing countries have not been optimized yet causing the outbreak of the disease. Hence it is vital to investigate the optimization of the existing immunization program. This study (8) suggested a mathematical model for describing the spread of diphtheria and formulated an optimized control for minimizing the spread by adopting quarantine and increasing the proportion of vaccinated persons by conducting camps. This study applied Pontryagin minimum principle to determine the characteristics of the problems in an analytical manner and suggested DOTcvpSB solve the issues numerically. With this optimized solution, the constructed research could be able to reduce the spread of the outbreak and the corresponding cost function. It has been reported that Diphtheria was considered an outbreak in Indonesia, particularly in East Java Province. Further, resistance to erythromycin, penicillin, and various other antibiotics was reported in prevailing articles. Such that, (7) aimed to evaluate first-line antibiotic susceptibility of the corresponding toxic isolates. The study suggested a method, in which it performed a descriptive observational study from Aug 2018 to Nov 2018. Further, the study collected C. diphtheriae isolates from diphthe-

ria patients between the years 2012 and 2017. The study conducted an antimicrobial sensitivity test (ETEST) of 5 antibiotics such as clarithromycin, azithromycin, erythromycin, oxacillin as well as penicillin for determining MIC (minimum inhibitory concentration). From the experimental analysis, it was observed that, from 114 isolates, a total number of 108 isolates were toxic and viable, and the ETEST was conducted on viable isolates. Which, the study noticed that most of the hosts (58.3%) were males who were aged between 1 and 14 years. Moreover, the ETEST results for penicillin were found to be 31.48% intermediate, 68.52% susceptible as well as 0% resistant. Finally, they concluded that the rate of susceptibility of *Corynebacterium diphtheria* to erythromycin was significantly higher than penicillin. The further study deals with the requirement of formulating MIC reference standards for clarithromycin as well as azithromycin.

Host-pathogen interactions were commonly studied in vitro by utilizing immortal or primary cell lines. Such that, this has significantly reduced costs and also avoided ethical issues of animal testing. Nevertheless, the influence of cell-culture media on metabolism and bacterial growth was not investigated or even considered in the majority of the cases. To address this, (14) investigated proteome adaptation of C. Diphtheria strain ISS3319. Further, the study cultured bacteria in foetal calf serum, a cell culture medium as well as a standard growth medium. Mass spectrometric analysis for quantifying protein at a high level of pathogenicity has been investigated during cell growth.

Research gap

Despite the diphtheria vaccine has been directed, the process of immunization has not been expected to inhibit the *C. diphtheriae* carriage on the skin and epithelia(16). Diphtheria toxoid vaccines could also prevent bacterial infections because of the existence of immunogenic proteins (3, 6)T</author><author>Tahoori, F</author><author>Nazari, A</author><author>Salehi Najafabadi, Z</author><author>Samianifard, M</author><author>Faramarzi, A</author><author>Soleimani, M</author></authors></contributors><titles><title>Investigation of the Effect of PEG Detoxification on Diphtheria Vaccine</title><secondary-title>Archives of Razi Institute</secondary-title></titles><periodical><full-title>Archives of Razi Institute</full-title></periodical><dates><year>2021</year></dates><isbn>0365-3439</isbn><urls></urls></record></Cite></EndNote> after the production of the most commonly utilized toxigenic vaccine strain. Existing studies revealed dissatisfactory results due to the poor optimization of the addition of calcium chloride, iron, and iron-free amino acids. These failures in the pre-optimization lead to frequent expenditure on bioinformatics and western blotting analysis for the elucidation of immunity protection against *C. diphtheriae.* Further, the selected parameters such as iron, iron-free L-Cystine, and Calcium chloride have not been evaluated briefly by the existing studies.

Materials and Methods

C. diphtheriae strain, ATCC 13812, obtained from American type culture collection Strain related ATCC 13812 https://www.atcc.org/products/13812, https://pubmed.ncbi.nlm.nih.gov/8784575/, for the present study followed by maintaining as in frozen form at -80^{o}C in 15% (v/v) glycerol. Further *C. diphtheriae* culture has been cultivated in the semisynthetic medium.

The following are the chemicals needed for the one litre preparation of semisynthetic media: NZ Amine, A Solution comprising of N-Z Amine A 100g, Anhydrous KH_2PO_4 1.3 g, Anhydrous Na_2HPO_4 3.9 g, $CaCl_2$dihydrate 11.1 g, Sodium hydroxide 400 g, Glacial acetic acid; 50% Maltose Solution comprising of Maltose powder 500 g, $Na_2HPO_4.12H_2O$ 10 g, $Cacl_2.2H_2O$ 5.0 g, Sodium carbonate 200 g; Mueller Growth Factor solution comprising of $MgSo_4.7H_2O$ 225 g, Pimelic acid 0.15 g, β – Alanine 2.30 g, $ZnSo_4.7H_2O$ 0.8 g, Nicotinic acid 4.60 g, $CuSo_4.5H_2O$ 0.5 g, $MnCl_2.4H_2O$ 0.24 g,

Conc. HCl 30 mL; 20% L-Cystine Solution comprising of L-Cystine 200 g, Conc. HCl 200 mL; 50% Sodium Lactate solution 500 mL; Ferrous sulphate heptahydrate 10 g.

Preparation of N-Z amine stock solution

100g of NZ-Amine powder was dissolved in 500 mL distilled water and heated to 80±1°C. Consequently, the study added anhydrous Na_2HPO_4 (3.9g) and anhydrous KH_2PO_4 (1.3g). The pH was set to 9.3 with a 50% NaOH Solution. Add 1.11g/100mL of $CaCl_2$solution to the NZ Amine solution and heat for half an hour to 85±1°C to carry out the precipitation reaction. The mixture was cooled and centrifuged for the collection of supernatants. The final volume of the supernatant solution was adjusted to 1.0 L with distilled water. The stock solution was stored at 2-8°C.

Semisynthetic media was used for the growth of C. diphtheriae with the following composition: 1.0 mL of 20% L-Cystine hydrochloride, 2.0 mL of 0.01% Ferrous sulphate solution, 1.7 mL of 25% sodium lactate, 50.0mL of 50% Maltose solution, 9.0mL of Muller's Growth Factor, 333.4 mL of NZ amine stock solution

Further, the study added L-Cystine hydrochloride as well as Mueller's Growth Factor to NZ amine solution, sterilized at 121°C for half an hour, and cooled them to 35±1°*C*. Sodium lactate solution, Maltose solution, and ferrous sulphate solution have been filtered and added to pre-sterilized and cooled media in flasks. This study undertook to find the optimum ferrous sulphate concentration as well as the preparation method of iron-free L-Cystine hydrochloride and was standardized. The Fe2+ concentrations were experimentally varied at flask levels. In every experiment, the same set of ferrous sulphate and NZ amine were utilized. The results exhibiting an optimum concentration of Fe^{2+} and iron-free L-Cystine were expressed in Table 2 and Table 3.

Method of cultivation

The freeze-dried *C. diphtheriae* culture was suspended in a semisynthetic medium, inoculated on Loeffler's serum slant, and cultured at35±1°*C*for 48 to 72 h. Culture from Loeffler's slant was then inoculated to 2'L flasks containing 400 mL of the semi-synthetic medium and incubated in a shaker (New Brunswick Scientific Co. Edison, New Jersey) having a throw length of 25 mm at 35±1°C and 200 RPM for 48 h. During cultivation, samples were taken for spectro-photometrical determination of bacterial density and determination of the concentration of diphtheria toxin in the culture medium with the flocculation test (flocculation units per millilitre). Cultivation was stopped when the toxin concentration in the cultivation medium reached a plateau.

The inoculation with subcultures with a density of 75-100 international opacity units (IOU) was carried out in a ratio of 1:20-1:40 to the final volume of the fermenter medium. The cultivation was carried out in a fermenter with a volume of 2.0 L (New Brunswick Scientific Co. Edison, New Jersey). The pH level was automatically regulated by the addition of 20% NaOH. Cultivation was carried out for 40 h at 35.5 °*C*. During cultivation, samples were taken for spectro-photometrical determination of bacterial density and determination of the concentration of diphtheria toxin in the culture medium with the flocculation test. Cultivation was stopped when the toxin concentration in the cultivation medium reached a plateau.

Results and Discussion

This section provides the results and discussion of the experiment. Here the effects of calcium chloride in NZ Amine stock for the toxin production, the effects of iron concentration, and optical density, as well as Lf/mL of diphtheria toxin after cultivating in the flask with optimized media concentration, were briefly discussed. The study observed that 1.11g/100ml of $CaCl_2$ had significantly higher optical density (32.5 and 34.2) and toxin concentration (140 Lf/

mL and 150 Lf/mL) in flask 1 and flask 2. Further, it is noticed that 0.20mL/100mL ferrous sulphate concentration had high toxin concentration (220 Lf/mL) and optical density (34.8).

Table 1 illustrated the effects of Calcium chloride concentration in NZ Amine stock solution on diphtheria toxin production. From this table, it is noticed that optical density (OD), as well as concentration of toxin (Lf/ml), is calculated for the given Calcium chloride concentration (g/100 mL) in flask 1 and flask 2. The study observed that 1.11g/100ml of $CaCl_2$ had significantly higher optical density (32.5 and 34.2) and toxin concentration (140 Lf/mL and 150 Lf/mL) in flask 1 and flask 2. Moreover, 100 grams of NZ Amine powder have been dissolved in distilled water (500mL), and they are heated up to 80±1°C. Consequently, the study added anhydrous Na_2HPO_4 (3.9grams) and anhydrous KH_2PO_4(1.3g). The pH was set to 9.3. This mixture and 1.11g/100ml of $CaCl_2$ were heated for half an hour to 85±1°C. Finally, the mixture has been cooled and it was centrifuged. The study collected supernatant and adjusted the final volume with distilled water to 1.0L.

Table 1. Effects of Calcium chloride concentration in the NZ Amine stock solution on the production of diphtheria toxin at the shake flask experiments (48 hours of incubation)

S.No.	Calcium chloride Conc. (g/100 mL)	OD at 600 nm		Lf/mL	
		Flask 1	Flask 2	Flask 1	Flask 2
1	0.41	23.6	22.3	60	70
2	0.51	23.8	23.5	70	60
3	0.61	22.5	22.9	60	70
4	0.71	24.8	23.8	80	80
5	0.81	23.7	24.7	70	70
6	0.91	25.2	24.2	50	60
7	1.01	24.7	25.7	80	70
8	1.11	32.5	34.2	140	150
9	1.21	25.8	24.9	70	60
10	1.31	23.9	23.5	60	70

Table 2 demonstrated the effects of Iron concentration in media on diphtheria toxin production. The study measured the optical density at 600nm as well as the concentration of toxin (Lf/ml) for the given Ferrous sulphate concentration. Further, it is noticed that 0.20mL/100mL ferrous sulphate concentration had high toxin concentration (220 Lf/mL) and optical density (34.8).

The study standardized the preparation method of iron-free L-Cystine solution. The study prepared the first set of iron-free L-cystine (10%) by utilizing the deferration method as well as adding flask 1 and flask 1A. Also, the study added a second set of iron-free L-Cystine without the deferration method and it was added in flask 2 and flask 2A. The outcomes are demonstrated in the following figure 1a and figure 1b.

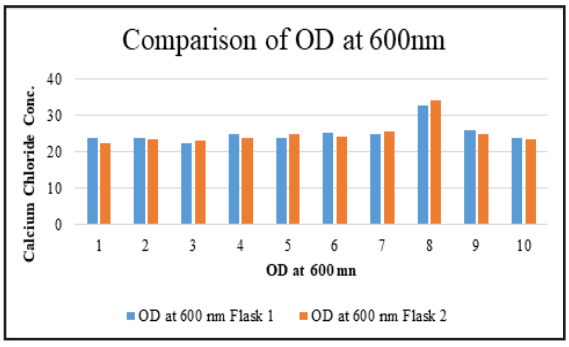

Figure1a. Comparison of OD at 600nm

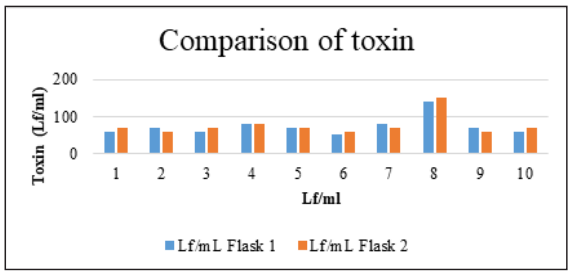

Figure 1b. Comparison of toxins between Flask 1 and 2

Table 2. Effects of Iron concentration in the media on the production of diphtheria toxin at the shake flask experiments (48 hours of incubation)

S/No.	Ferrous sulphate Conc. (mL/100 mL)	OD at 600 nm	Lf/mL
1	0.05	26.4	70
2	0.1.	25.2	80
3	0.15	24.8	90
4	0.20	34.8	220
5	0.25	25.0	90

Table 3 depicted the effects of preparing Iron free L-cystine solution on diphtheria toxin production. From this table 3 and figure 2, it is observed that the study measured optical density and toxin concentration. When comparing Flask 2, flask 1A had a higher optical density (34.6) but a lower concentration of toxin (220 Lf/mL).

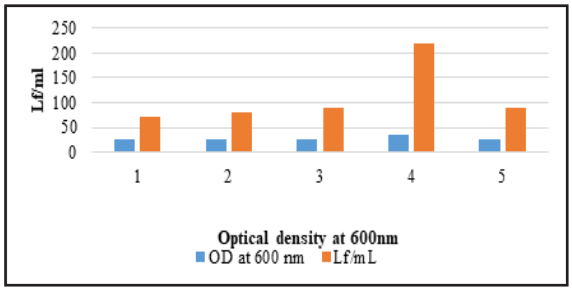

Figure 2: OD at 600nm versus toxin concentration

Table 3. Effects of method of preparation of Iron-free L-cystine solution on the production of diphtheria toxin at the shake flask experiments (48 hours of incubation)

S/No.	Flask No.	OD at 600 nm	Lf/Ml
1	Flask 1	34.0	230
2	Flask 1A	34.6	220
3	Flask 2	32.6	220
4	Flask 2A	34.2	230

Growth in shake flask

The study prepared semi-synthetic media as per optimized composition, as well as distributed them in the Erlenmeyer flask. Further, the study inoculated C. diphtheriae in the media, and it was kept at 35±1°C for44-48 hours. By Gram staining, the study confirmed the culture's bacterial purity. The sample was considered for determining the culture medium's cellular density by observing optical density at 600nm as well as toxin concentration by performing a flocculation test.

Table 4: Results of Lf/mL and OD of diphtheria toxin after cultivation in a flask with optimized conc. of media:

Flask No.	OD at 600 nm	Lf/mL
1	34.8	220
2	34.2	210
3	34.6	220
4	33.9	190
5	35.2	230

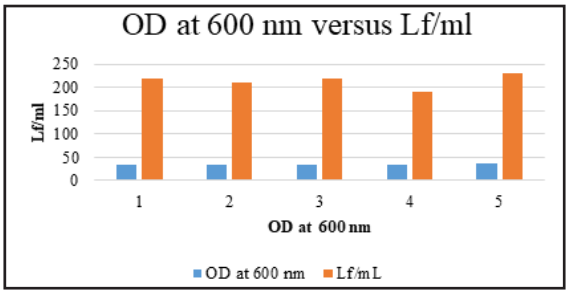

Figure 3: Results of Lf/mL and OD of diphtheria toxin after cultivation in a flask with optimized conc. of media

Table 4 and figure 3 illustrated the outcomes of Lf/mL as well as the optical density of diphtheria toxin after cultivation in the flask with optimized media concentration. From this table, it is observed that flask number 5 had a higher optical density (35.2) and toxin concentration (230 Lf/mL). But flask 4 had significantly less concentration of toxin (190 Lf/mL) and optical density (33.9).

Discussion

From this experimental analysis, the study observed the effects of the concentration of $CaCl_2$ in NZ Amine stock solution, the concentration of Iron as well as preparation of iron-free L-Cystine hydrochloride solution, which were added to the semi-synthetic medium in diphtheria toxin growth as well as the production of toxin.Moreover, in initial experiments, the study identified that a concentration of 11.1 grams per literof $CaCl_2$ was considered to be good for the growth of bacteria since it had higher optical density and Lf/mL (32.5 and 140) after 44-48 hours of incubation in the flask. Further, to determine the $CaCl_2$ optimal concentration in NZ Amine stock solution for the production of the toxin, the study tested the media with the concentration of $CaCl_2$, which ranges between 4.1 to 13.1 g/L. The outcomes of table 1 illustrated that 11.1g/L calcium chloride concentration showed increased production of toxin and cellular density in the bacterial suspension. The calcium acts as an extra-cellular matrix by binding Fe, thereby ensuring unavailability to organisms. Thus, the $CaCl_2$ optimum concentration in the culture medium is essential for toxin production.(2, 9) also investigated the growth of *Corynebacterium diphtheriae* (13) deliberated the significance of diphtheria toxoid vaccines (18) have investigated the microbial susceptibility patterns of the *C. diphtheriae* strain. Iron concentration in the cultivation medium has a significant effect on the production of toxins. Further, the prevalence of higher iron concentration in the growth medium activates the protein repressor synthesis as well as inhibits the production of toxins. Consequently, diphtheria toxin production relies upon the concentration of iron in growth media, thus the optimum iron concentration is needed in the growth medium for toxin production. In this study, the concentration of iron in semi-synthetic media has been varied from 0.5 to 2.5mL/L. The outcomes of this study revealed that the production of the toxin was higher in the concentration of 2.0ml/L (0.1g/L iron).

The study optimized the preparation method of Iron free L-Cystine hydrochloride solution. The study prepared this solution by two methods. Iron-free L-Cystine hydrochloride (10%) with deferration method, and Iron free L-Cystine hydrochloride (20%) without deferration method. Further, it has been established that the change in preparation method did not impact the toxin production as well as cellular density as mentioned in table 3. The study preferred 2nd method (20%Iron free L-Cystine hydrochloride without deferration as it facilitated the ease of preparation.

Conclusion

In conclusion, Diphtheria Vaccine is found to be more effective for preventing the disease, but animal product-based medium cause few complexities. Hence, the research aimed to grow *Corynebacterium diphtheriae* ATCC strain in a shake flask to be followed by cultivation in the fermenter with optimized parameters to obtain a higher yield. The parameters selected for the optimization of the semi-synthetic media are calcium chloride, iron, and L-cystine. A high yield of diphtheria toxin has been obtained with the optimized parameters. As the chemical composition of semisynthetic media is exactly known it can be easily and quickly prepared, and can always be duplicated. Results obtained by its use are always uniform and comparable. By the use of such a medium some light may be thrown on bacterial metabolism.

Declaration

Conflict of Interest: The author reports that there is no conflict of Interest.

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