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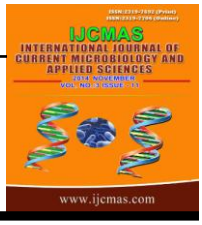

Original Research Article

Effect of culture media and their ingredients on PRP production by *Haemophilus influenzae*

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Keywords

Haemophilus influenzae, poly ribosyl ribitol phosphate, Vaccine, soy-peptone and, Casitone Yeast extract (CY) medium

A B S T R A C T

The production of capsular polysaccharide, poly ribosyl ribitol phosphate (PRP), of *Haemophilus influenzae* type b (Hib) differs quantitatively in different growth media. PRP is important for the production of conjugate vaccine. The present study was carried out in order to study the effect of growth medium components like dextrose and yeast extract on the amount of PRP production by Hib and also to compare PRP production in two growth media i.e. modified Soy-Peptone Yeast extract (MP) and Casitone Yeast extract (CY). After determining the optimum concentrations of dextrose and yeast extract in Erlenmeyer flasks containing one liter of each medium, the seed cultures were inoculated into 50 liter fermentors and the amount of cell mass and PRP produced in either media were compared.Maximum PRP was produced when concentrations of dextrose and yeast extract in the both the media were 6 and 2.5 g/l respectively.Bacterial cultivation in 50 liter fermentors revealed PRP produced in MP medium was significantly more than PRP produced in CY medium ($P<0.05$).Our findings indicate PRP is greatly dependent on not only the kind of growth medium of *H. influenzae* but also on various medium components. Selecting an ideal medium with optimal amount of constituents increases the production of PRP; thereby, reduces the price of PRP protein conjugate vaccines.

Introduction

Haemophilus influenzae is a gram negative bacterium responsible for severe pneumonia, meningitis, sinusitis, otitis media (Yeruva et al., 2010; Mawas et al. 2007), and other invasive diseases, almost exclusively in children aged less than 5 years (Yeruva et al., 2010), approximately two thirds of all cases occurred among children <18 months of age (Morbidity and Mortality Weekly Report. CDC, Atlanta, Georgia,1993). It is estimated that *H. influenzae* causes 2 to 3 million cases of disease annually; majority of them in developing countries (Hamidi et al., 2009). Meningitis occurs in approximately two third of children with invasive Hib disease, resulting in hearing impairment or neurologic sequelae in 15%–30% cases. The case-fatality rate is 2%–5% (Morbidity and Mortality Weekly Report. CDC, Atlanta, Georgia,1993).

H. influenzae isolates are usually classified according to their polysaccharide capsule into six capsular types, a to f. *H. influenzae* type b populations are often heterogeneous; capsulated bacteria coexist with non-capsulated bacteria. The capsule is the major factor of virulence of Hib strain. The polysaccharide consists of a succession of repeating units of ribosyl ribitol phosphate (Maitre-Wilmotte G. et al., 2008). Like other polysaccharides, the PRP of the Hib capsule is a T-independent antigen and not immunogenic when administered as a vaccine in infancy.

The efficacious Hib vaccines have been designed by covalently linking the PRP to a carrier protein that recruits T-cell help for the polysaccharide immune response and induces anti-PRP antibody production even in the first 6 months of life. Introduction of Hib protein-polysaccharide conjugate vaccines into many industrialized countries has resulted in the virtual elimination of invasive Hib disease (Dominic, 2004).

Studies have confirmed the effectiveness of these vaccines in low-income countries. Hib vaccine is one of the most under-utilized vaccines because of its relatively high cost. In order to contribute to the goal of the World Health Organization (WHO) to make Hib conjugate vaccine available for all children in the world and in order to give people in developing countries a chance to get access to Hib technology, a relatively simple and easily up-scalable production process has to be developed. An optimized cultivation conditions that could lead to an increase in PRP production would be of great interest for mass vaccination programs (Hamidi et al., 2009).

To manufacture these vaccines, it is necessary to produce large quantities of bacteria in large volumes of culture medium from which PRP is extracted and purified (Maitre-Wilmotte G., 2008). Since PRP is a growth related product, low culture density limited the PRP concentration (Hamidi et al., 2009).

The present study was carried out in order to study the effect of growth medium components like dextrose and yeast extract on PRP production by *H. influenzae* type b and also to compare PRP production in two growth media i.e. modified Soy-Peptone Yeast extract (MP) and Casitone Yeast extract (CY).

In this research, modifications to culture medium components were made in 1 liter flasks and the mass cultivations were carried out in 50 liter fermentors.

Materials and Methods

Bacterial strain - The bacterium used in this study was *Haemophilus influenzae* type b ATCC No. 10211.

Growth Media –Two growth media i.e. Casitone Yeast extract (CY) and modified Soy-Peptone and Yeast-extract (MP) were used for cultivation of *H. influenzae* type b. Both MP and CY media contain yeast extract, Na_2HPO4, NaH_2PO4, K_2HPO4, dextrose, Nicotinamide Adenine dinucleotide (NAD) and hemin. In addition to the common ingredients, MP medium contains Soy Peptone while CY medium contains casamino acid (Atlas, 2004).

Ingredients assay- In order to study the effect of growth medium components like yeast extract and dextrose on PRP production, and to identify the optimum concentrations of these constituents, 10mL of *H. influenzae* suspension was cultivated in Erlenmeyer flasks containing one liter of each medium and incubated in a shaker at 200 rpm at 37 ◦c for 15h.

Shake flask experiments were carried out using increased initial concentrations of yeast extract and dextrose in each medium. Since the inoculation of the seed cultures into each medium, time was measured to determine the cell density and PRP produced, on the basis of bacterial growth phase as discussed by Takagi et al (Takagi et al., 2006).

Different concentrations of yeast extract and dextrose and their outcome on PRP production are presented in Tables 1 & 2.
The seed cultures were also inoculated into fermentors**.** The experiments were conducted in a contact flow Bilthoven unit fermentor (Roestvrijstaal Bilthoven unit-Netherlands) containing 50 liters of either media for mass cultivation. Optical density (O.D) of each medium was measured at 550 nm using a Beckman spectrophotometer (Beckman co.) and the cell density and PRP produced in either media were measured and compared.

Dry cell weight (DCW) was determined by centrifuging 10ml of broth in pre-weighed tubes collected at time intervals. After washing the pellets with 10 ml saline, the cell pellets were vacuum dried at 80°c for 16 h and the mass of the cell pellets were determined.

PRP production- To determine PRP concentration, 1.5 ml of each cultivation media collected at different time intervals was centrifuged at 6000×g at 10˚c for 10 min to remove the cells (the exponential growth phase was considered at the first 10 h of cultivation while stationary growth phase reached after 15 h of cultivation), 50 µl of 100 g/l of hexadecyl tri methyl ammonium bromide solution were added to 1ml of the supernatant. After 5 min. centrifugation at 8000×g, the pellets were washed with distilled water. Following the next centrifugation, the pellets were solubilized in 1ml of 0.25M NaCl;.200 µl of which was diluted by adding 1 ml water. 100 µl of Orcinol (100 g/l) solution and 1 ml of $FeCl_3$ (5 g/l) in 12N HCl were added and the mixture was incubated at 100˚c for 40 minutes in sealed tubes (Merrit et al., 2000).

Results and Discussion

The effect of different concentrations of yeast extract and dextrose in each medium on PRP production are presented in Tables 1 & 2. Optical density of both media showed a linear relationship with concentrations of yeast extract. Increasing concentrations of yeast extract, more than 2.5 g/l, led to decrease in total PRP production in both the media. Increase in dextrose concentrations, more than 6 g/l, increased the cell mass but decreased the PRP production.

At yeast extract concentration of 7.5 g/l and also 10g/l of dextrose, a significant decrease in total PRP was observed compared to yeast extract concentration of 2.5 g/l and 6 g/l of dextrose in both the media ($P<0.005$). 20 h after cultivation of the bacteria in CY and MP media release of PRP in supernatant occurred in both logarithmic and mid stationary phases of the bacterial growth.
Following cultivation of bacteria in one liter flasks containing either media, maximum PRP was produced when dextrose and yeast extract concentrations were 6 and 2.5 g/l respectively.

Furthermore, we studied the effect of various concentrations of dextrose and yeast extract on PRP production and DCW in 50L fermentors. The results obtained are summarized in Table 3. As is clear from Table 3, PRP production in MP medium was more than the amount of PRP produced in CY medium.

An optimally balanced culture medium is mandatory for maximum production of the secondary metabolites (Yeruva et al., 2010). Methods for cultivation of *H. influenzae* type b are well-known (Hamidi et al., 2009). For highest PRP yield, many factors are to be considered during cultivation of *H. influenzae*. The need therefore exists to improve the methods for PRP production, particularly when the culture volume is large (Maitre-Wilmotte,G., 2008). Designing a fermentation medium is also a critical and important process as the medium composition can significantly affect the product yield (Yeruva et al., 2010).

MP medium containing soy peptone and yeast extract described by Carty and collaborators is used for the production of *H. influenzae* polysaccharide (Marburg et al., 1989). The major ingredients of both CY and MP media are yeast extract, dextrose, hemin chloride and Nicotinamide Adenine Dinucleotide (Atlas, 2004).

Takagi *et al*.(2007) used MP medium for batch cultures as proposed by Carty *et al*. They improved the *H. influenzae* culture medium by studying the effect of hemin (X factor) and Isovitale X supplement containing V factor, on PRP production (Takagi et al., 2007). In the present study like Esmailey et al. (2011), we also used MP medium, in addition to CY medium, for bacterial growth and we compared PRP production in these media.

Several groups have attempted to increase PRP production by varying growth media and increasing key medium components (Hamidi et al., 2009; Esmaily et al., 2011). PRP is a growth related and medium dependent product; different growth media and also various medium components may affect its production. PRP production is not simply a function of medium cell density, but a sufficient supply of nutrients, especially dextrose and yeast extract are critical to PRP biosynthesis (Marburg et al., 1989). Some cultivation conditions could affect regulation or expression of genes involved in biosynthesis of PRP (Zhongwu et al., 2009). Yeruva et al. (2010) studied on 11 medium components and concluded that yeast extract, dextrose, Nicotin amide adenine dinucleotide (NAD) and Na_2HPO4 contribute to a large extent for PRP production .

Our findings are in accordance with Maitre-Wilmotte et al (2008) who stated that yeast extract concentration in the culture medium is to be between 0.2 to 5 g/l of medium for efficient RPR production by bacteria. The results of present work indicate that PRP production is maximum at yeast extract concentration of 2.5 g/l in both CY) and MP (453±5 g/l PRP) media.

Dextrose is an important source of energy and carbon for *H. influenzae* metabolism and its concentration in the *H. influenzae* culture media is generally between 2 to 20 g/l. But recently it has been reported that the PRP production in the range of 0.2 to 5g/l of yeast extract is stimulated (Merritt et al., 2000).

According to our findings 6 g/l dextrose yielded the highest amount of PRP in either media. In the present study we obtained highest amount of PRP when the dextrose concentration was 6 g/l in either media. Like Takagi et al. (2006) we also obtained the best production of capsular polysaccharide in the modified MP medium.

Authors of the present study noticed that at higher concentration of dextrose and yeast extract, higher than 6 g/l and 2.5 g/l respectively, of both media the cell mass was increased but the PRP production was

decreased. The biomass production was adjusted on the basis of time required for growth phases as is given by Takagi et al. (2006).

Following the modifications applied in the present work, we obtained slightly higher DCW in MP medium at an industrial scale production. In spite of slightly higher DCW, we obtained PRP higher than the amount of PRP obtained by others (Takagi et al., 2006; Maitre-Wilmotte G. et al., 2008). Takagi et al. (2006) by improving the cultivation conditions of the modified MP medium could attain the overall PRP production as 529–574mg/ l at an industrial scale production of PRP while in the present study we obtained 524 mg/l PRP which is close to Takagi et al 's findings.

Table.1 Effect of different concentrations of yeast extract on PRP production by *H. influenza*

Yeast extract (g/l)		**PRP (mg/l)**		**OD (550 nm)**	
CY medium	MP medium	CY medium	MP medium	CY medium	MP medium
0	0	151±2	155±2	1.1± 0.1	1.2±0.1
2.5	2.5	398±3	453±5	3.5±0.2	4.1±0.2
5	5	387±3	420±4	5.2±0.3	5.0±0.4
7.5	7.5	321±5	368±5	6.0±0.1	6.3±0.2

Table.2 Effect of different concentrations of dextrose on PRP production by *H. influenza*

Yeast extract (g/l)		**PRP (mg/l)**		**OD (550 nm)**	
CY medium	MP medium	CY medium	MP medium	CY medium	MP medium
0	0	110±2	113±2	2.4±0.2	2.4±0.1
2	2	280±5	296±4	3.6±0.3	3.8±0.2
4	4	378±2	421±3	4.4±0.2	4.7±0.1
6	6	439±2	511±3	5.0±0.2	5.6±0.2
8	8	426±3	466±4	5.2±0.2	6.0±0.4
10	10	402±5	429±3	5.8±0.2	6.4±0.3

Table.3 50L fermentation results

Exp. No	**Medium**	**Dextrose (g/l)**	**Yeast extract (g/l)**	**DCW (g/l)**	**PRP produced**
1	control CY medium	10	5	2.7±0.1	302±3
2	modified CY medium	6	2.5	2.8±0.2	431±5
3	control MP medium	5	2.5	3.4±0.3	511±4
4	modifiedMP medium	6	2.5	3.2±0.1	524±3

P (1:2)<0.05=S, P(1:3)<0.05=S, (P2:4)<0.05 =S,

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