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Assessment of *Bordetella pertussis* Strain 509 Cell Mass Yield in Baffle and Vortex Mode of Agitation during Large Scale Industrial Fermentor Cultivation

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Abstract

The effect of baffle and vortex agitation system on B. pertussis vaccine strain 509 growth yield was examined in industrial fermentor cultivation used single turbine impeller fixed at the bottom of vessel, which has the provision of growth of the organism under controlled conditions with regard to temperature, pH, aeration and agitation. From this study it was found that the use of baffled system shows lesser the growth yield of B. pertussis (strain 509) due to the excessive froth accumulation on the surface of the culture which deplete the oxygen supply could minimizes the cell biomass/growth yield. Whereas remarkable increased in the biomass yield was observed in the vortex mode of agitation due to vortex flow supports homogenous mixing and good aeration for bacterial growth could enhance final growth yield at the end of 48 hours of cultivation as a result of vortex mode, the effective yield of the fermentor culture was increased up to two fold times, whereas the remarkable increased in the growth yield was observed, due to swirl flow pattern of culture agitation supports homogenous mixing and good aeration for bacterial growth. Therefore vortex system of agitation can be industrially used for the production of whole cell B. Pertussis (strain 509) vaccine to obtain good yield.

Keywords: Fermentor; Baffle; Vortex; Pertussis; Growth rate

Abbreviations:

wP: Whole Cell Pertussis; BGM: Bordet Gangou Media; lpm: Liter Per Minute; rpm: Rotation Per Minute; DO2: Dissolved Oxygen; IOU: International Opacity Unit; IRP- International Reference Pertussis

Introduction

Pertussis is a highly contagious disease caused by the bacterial pathogen Bordetella pertussis. World-wide there are 30-50 million pertussis cases and about 300000 deaths per year [1]. Most of deaths occurs in young infants who are either unvaccinated or incompletely vaccinated. Widespread vaccination of children reduced the incidence of illness and deaths caused by pertussis [2]. However globally pertussis remains one of the top 10 causes of death in children [3]. Further, in the 1990s a resurgence of pertussis was observed in several countries with highly vaccinated populations [2,4] and pertussis has become the most prevalent vaccine-preventable disease in industrialized countries. Use of killed whole-cell Bordetella pertussis vaccines has been a major factor in control of symptomatic whooping cough.

Whole cell pertussis (wP) vaccines have been used worldwide as part of combined DTP vaccine in national childhood immunization programmes for 4 decades. Although concerns about possible adverse events following their administration have led to adoption of acellular pertussis vaccines (ACPV) in some countries, whole cell pertussis vaccines are still widely produced and used globally in both developed and developing countries. Whole cell pertussis vaccine that comply with WHO requirements, administered in an optimal schedule have a long and successful record in the control of whooping cough. ACPV vaccines consist of 1-8 B. pertussis protein virulence factors such as Pertussis Toxin (PT) Filamentous Heamagglutinin (FHA) Fimbriae, Pertactin (PRN) Tracheal Colonization Factor (TCF) Adenylate cyclase toxin (ACT) Heat-labile toxin (HLT) and Tracheal cytotoxin (TCT), in order to produce an acellular vaccine, the cultivation step is followed by extensive purification procedures. For both vaccine types a high yield of virulence factors per unit biomass produced is desirable, since more vaccine doses per production run can be obtained leading to lower costs. For cellular vaccines a higher yield of virulence factors per unit biomass is even more important, since the amount of Lypopolysacharides (LPS) per dose can be lowered, which is likely to result in less adverse reactions. In addition to a high yield, it is important for cellular vaccines (wP) all virulence factors produced remain cell associated, since the culture supernatant is discarded.

The production of whole cell pertussis vaccine in large scale cultivation method using fermentor depends on the standardization of fermentor parameters such as temperature, pH, dissolved oxygen, aeration, and agitation. The growth, virulence property and metabolism of pertussis bacteria in fermentor culture vessel depends on various designed parameters of fermentor used for pertussis cultivation [5].

The aim of the present study is to examine growth yield and virulence property of B. pertussis vaccine strain 509 using baffle and vortex mode of agitation system using large scale fermentor.

Materials and Method

Strain of *Bordetella pertussis*

Bordetella pertussis vaccine strain 509 used in this study was obtained from Rijks institute, Bilthovan, Holland. The strain is maintained in lyophilized state at 4°C.

Bordet-Gengou (BG) Medium

Bordet-Gengou (BG) Medium was prepared as per [6] the composition and followed by, Potato slice 250 g, NaCl 9 g, Proteose peptone 20 g, Glycerol 20 g. The potatoes were cleaned, peeled and cut into slices, the slices with NaCl and deionised water were added to a flask and solution is boiled, after boiled the potato was macerated and filtered through muslin cloth which was squeezed to extract all the fluid. The pH was adjusted to 7.0 and the volume of the filtrate was made upto 500 ml. Protease peptone and glycerol were added and mixed. Separately 60 gram of agar was dissolved in 1500 ml deionised water by steaming for 30 min and filtered. When still hot it was mixed with the above solution and distributed in 500 ml flasks, each flask containing 200 ml of the medium. The medium was sterilized by autoclaving, and stored at 4°C till used. For the preparation of BG slant, the base medium was cooled to 40-45°C and sheep bloods were added at the ratio of 1:2 and gently mix without any frothing and distributed 2 ml into each test tube and kept the test tubes in slant position till media solidified. Later the slants were incubated at 35°C for 24 hrs and then stored at 4-8°C.

Preparation of Preliminary seed

One ampoule of freeze dried working seed copy of B. pertussis 509 was taken and the contents were resuspended in 2 ml of sterile normal saline. The suspension was then inoculated onto the slopes of BG medium. After inoculation the BG tubes were incubated at 35°C for 72 hrs. After 72 hrs the growth and purity was checked by gram staining.

Preparation of B2 culture medium

The B2 culture medium was prepared with the following compositions. Bactocasamino acid (BCA) 1800 g, L-glutamic acid 1500 g, NaCl 750 g, KH_2PO_4 150 g, $MgSO_4$ 30g, $CaCl_2$ 3g, $FeSO_4$ 3.74 g, $CuSO_4$ 0.15 g, Glutathione 3.05 g, Yeast extract 1500 g, Soluble starch 450 g. Starch solution was prepared by dissolving starch in cold water. The suspension was then added to 20 L of hot distilled water and steaming in autoclave at 118°C about 20 minutes separately. The remaining chemicals were dissolved in serial order in 50 L of warmed distilled water in a separate vessel.

L-glutamic acid solution was prepared by dissolving in 50% NaOH still get amorphous solution hot distilled water. BCA was dissolved in 10L of distilled water and yeast extract was added to this solution. Finally L-glutamic acid solution and other chemicals were added and made upto 300L and mixed properly. Finally the media was transferred to fermentor vessel and sterilized it at 121°C for 30 minutes.

Sterility Media

Preparation of starter culture

One ampoule freeze dried working seed stock (509 strains) was opened under sterile environment and resuspended in 2 ml of sterile B2 medium. The suspension was then inoculated BG slope and incubated at 35°C ± 1°C for 72 hours, ensured the purity by Gram staining. Furthermore the culture was scraped aseptically and inoculated into a 1ltr flask containing 400 ml of B2 medium. The flasks were loaded on seed shaker at 140 rpm for 24-hours at 35°C ± 1°C. After 24 hours stop the shaker and the growth was checked for purity, pH and opacity before inoculation in to the fermentor.

Purity and sterility test

The purity and morphology was studied by Gram staining method. The purity of seed samples, fermentor culture was also checked by taking 1 ml of seed sample and inoculated into 3-nutrient agar slopes and other three slopes were kept as control without adding any sample and incubated both control and test sample at 35°C ± 1°C for 24 hours and observed for its sterility.

Similarly the Samples (1 ml) of vaccines were inoculated into 4 bottles (100 ml each) of each thioglycolate medium and Soya bean Caesin Digestive medium. 4 bottles of Thioglycolate medium were incubated at 35°C and other 4 bottles are incubated at 20-22°C for 14 days ensure its final sterility.

Cell mass determination by (opacity test)

Opacity Reference standard 10 IOU WHO 5th IRP [7], was used for opacity control. The cell mass concentration during fermentation process was determined by measuring opacity using opacity standard tube. The fermentor samples for cell mass concentration (broth) were collected aseptically through sampling port periodically at different time intervals like, 24, 36 and 48 hours for all the batches checked the opacity (Figure 1).

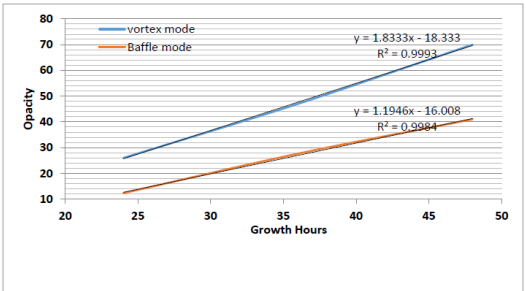

Figure 1: Comparative growth yield (opacity IOU organisms/ml) analysis of B. pertussis strain 509 in vortex and baffle modes during industrial fermentor cultivation.

Method of Fermentor cultivation

The pertussis cell cultivation was done using large scale batch fermentor (Sartorious India 500 liter) with a working volume of 300 L. Initially for experimental study 4 baffles (Figure 2b) were fixed to the fermentor vessel and set all the required parameters such as

temperature, pH, dissolved oxygen, stirrer speed and aeration (surface) at 35°C ± 1°C, 7.2, 100%, 500 rpm and 16 lpm respectively. The process duration for the cultivation time was 48 hours. For all experimental batches the pH, sterility, purity and opacity were checked at every, 24, 36 and 48 hours for both vortex and baffle culture during the study period.

Toxicity /MWGT test

The toxicity test was performed by mouse weight gain test (MWGT) described by pitman and cox was performed according to the WHO guidelines of [8]. The MWGT was executed for each harvested sample of different cultivation hours after heat inactivation at 56°C/30 minutes.

14-15 gram of healthy male Lacca mice were weighed in groups (n=10) and injected intra peritoneally with 0.5 ml of diluted culture sample. Control group of 10 mice were injected with an equal volume of normal saline. During the assessment the animals are properly fed, both groups are weighed in the first, third and seventh day after injection.

Results and Discussion

The B. pertussis seed strain 509 was revived on BG medium and seed growth is free from contaminants/purity was ensured in Grams staining. In order to the production of whole cell pertussis vaccine from seed level, it was propagated from BG medium, the BG medium was prepared from using fresh good quality potatoes. Because potato based BG medium was optimized at this institute for more than four decades with higher opacity yields, but not in the other commercial available dehydrated media.

Subsequently the final seed is inoculated aseptically into the sterilized fermentor vessel containing sterilized B2 medium (300l) and fermentor cultivation has been carried out in Sartorius (India) fermentor.

In this study the fermentor cultivation system was modified with baffle mode (insertion of four baffles into fermentor vessel) and without baffle (vortex mode) and culture run was setup accordingly with suitable environment for B. pertussis strain 509. In each mode (baffle/vortex) the following fermentor culture parameters were maintained constantly i.e. aeration 14-16 LPM, Agitation 500 RMP, cultivation temperature 35.5°C and Growth duration 48 hours. For all the experiments the effect of baffle and vortex system on cell mass yield of B. pertussis strain 509 as evidenced by purity, sterility, pH and opacity, were studied to each and every experiments and the results obtained were analyzed and tabulated (Table1a and 1b).

The result indicates that, the average growth yield of B. pertussis varies substantially in the presence, absence of baffle. The lesser biomass yield was obtained in 24 and 36 hours culture in baffle mode cultivation and the average opacity was found to be 12.33 IOU organisms/ml and 27.66 IOU organisms/ml respectively. Whereas profound increases in bacterial growth yield was noticed in 24, 36 hours cultivation in vortex mode agitation, and the average opacity yield was found to be 26 IOU organisms/ml and 47 IOU organisms/ml respectively.

In presence of baffles mode it was observed that the insertion of baffles were prevented the vortex formation, and increased turbulence in the media fluid [9]. Optimum air flow rate and vortex agitation preferable for large scale cultivation which enhance the growth yield due to optimum oxygen could be transferred to the actively growing bacterial cells increases respiration rate, enzymes activity and substrate utilization create homogenous condition [10].

Similarly in presence of baffle system, the lower opacity of pertussis culture was also observed when harvested at 48 hours and mean average opacity was found to be 41 IOU organisms/ml when compared with vortex mode (without baffle) culture the average opacity was 70 IOU organisms/ml in 48 hours. In agreement with the findings of Bellalou and Relyveld [11] and Munoz et al. [12] the vortex aeration is the preferred method employed for large scale cultivation. Furthermore in their studies showed that the yield of B. pertussis showed 60 to 80 IOU organisms/ml. The decreased in the growth yield of strain 509 in baffle system was due to prevention of vortex formation. In presence of the baffles the kinetic behavior of the cells are affected in different directions, and circulation of culture media is restricted in limited area between the inclined blades of the baffles, which disturbs the free circulating cells by scouring action in vessel results slower growth [13] (Figure 2).

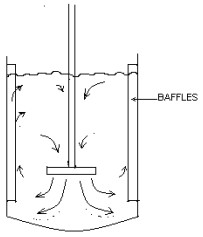

Figure 2a: Axial flow culture pattern of Baffle mode

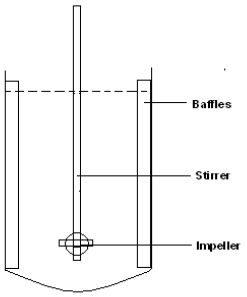

Figure 2b: Fermentor vessel with four baffles.

Whereas increased biomass yield in vortex agitation system was due to the uniform dispersion of air in vortex circulation which enhanced the organism growth yield. Higher agitation rate is not preferred for cultivation of B. pertussis strain 509 for lab scale cultivation [14]. However the improvement of bacterial cell mass depends on the uniform uptake of dissolved oxygen and nutrients as facilitated by direction of airflow in vortex mode resulted in rapid multiplication. In the present study it was observed that the axial broth flow (Figure 2a) in the baffled system of cultivation cause decreased and eventual cessation of growth. And there by potentially the metabolic state of whole organism reduced steadily. When compare to vortex mode

which it influences the homogeneous mixing of culture throughout the vessel from surface to base, it enhance superior air circulation provide during cultivation.

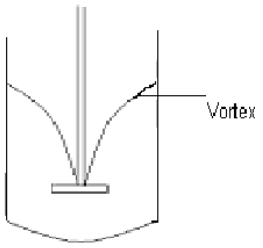

Figure 2c: Swirl flow culture pattern of vortex mode.

The present study showed that in absence of baffles the culture flow pattern swirl (vortex form) the broth radially progressed to the base of the vessel entraining fluid adjacent to the impeller. From the impeller the flow moved radially along the base towards the wall of the vessel at which it turned upward in a well-defined wall of the fermentor vessel and extended up to more than 2/3rd of the vessel height before it turned back almost completely towards the impeller and formed a large egg- shaped ring formed called vortex as illustrated in the Figure 2c.

The results of the culture pH, in baffle mode at the 24 hrs average values of the samples were 7.48, in case of 36 and 48 hrs it was 7,82 and 8.03 respectively, these pH range was not deviated neutral, and it reveals the culture status. All the baffle experiments were passed the purity and sterility (Table 1a). In case of pH during vortex mode, at the 24 hrs average pH of the samples was 7.44, in case of the 36 and 48 hrs it was 7.78 and 8.11 respectively.

Media used	Growth Hours	Culture pH			Opacity IOU (x109 org / ml)			Average yield (Opacity IOU/ml)	Purity	Sterility
		Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3			
B2	24	7.48	7.52	7.44	15	10	12	12.33	Passed	Passed
B2	36	7.81	7.88	7.79	30	25	28	27.66	Passed	Passed
B2	48	8.11	8	7.98	45	40	38	41	Passed	Passed

Table 1a: Growth yield analysis of B. pertussis strain 509 at different growth hours in baffle mode Fermentor cultivation. Ex: Experiment, Org: Organisms, IOU: International opacity units.

The culture pH was higher in vortex culture mode *B. pertussis*. In general pH is crucial function in a metabolism. If it is diverse, it leads to the improper metabolic cycle of the bacteria. In case of B. Pertussis strain 509 is a genetically modified vaccine strain, when the increasing pH is not affecting its metabolic cycle. This pH range was not deviated neutral pH, it reveals the culture status. All the vortex mode experiments were passed the purity and sterility (Table 1b).

The results of the toxicity test (MWGT) in mice, for the inoculation of baffled and vortex culture sample strain 509 is as shown in Table 2. The results indicated that baffled samples showed weight gain of more than 4.9 g/mouse and vortex samples 5.2 g/mouse respectively after seven days of inoculation, compared with standard batch which was found to be 5.4 g/mouse weight gain. Whereas saline injected (control sample) showed weight gain of 6.5 g/mouse. No death of mice were observed in both baffle and vortex sample whose percentage of weight gain meet the criteria of ≥ 60% [8].

As per this studies of toxicity in mice, those vaccine was produced from vortex mode was showed above than the WHO prescribed standard vaccine. In case of baffle mode the toxicity value was matching with WHO. When compare with baffle mode derived vaccine and vortex mode vaccine the toxicity effective ratios was very higher in vortex mode pertussis vaccine than the baffle mode.

Media used	Growth Hours	Culture pH			Opacity IOU (x109 org / ml)			Average yield (Opacity IOU/ml)	Purity	Sterility
		Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3			
B2	24	7.51	7.41	7.41	30	25	23	26	Passed	Passed

B2	36	7.9	7.82	7.62	50	48	45	47.66	Passed	Passed
B2	48	8.1	8.15	8.1	75	70	65	70	Passed	Passed

Table 1b: Growth yield analysis of B. pertussis strain 509 at different growth hours in vortex mode Fermentor cultivation. Ex: Experiment, Org: Organisms, IOU: International opacity units.

Vaccine Sample	Average weight gain gram(g)per mouse Days after Injection			Mortality	% of Control	Test Results
	Day 1	Day 3	Day 7			
Baffle culture	-1	1.8	4.9	0	65	Passed
Vortex Culture	-0.7	2	5.2	0	75	Passed
Saline Control	1	1.4	6.1	0	NA	NA
Standard Batch	-1	1.6	5.4	0	65	Passed

Table 2: Toxicity/Mouse weight gain analysis of B. Pertussis 509 strain culture of Baffle/Vortex mode. g-Grams, NA-Not applicable, Number of Mice used per test: 10; Weight of Mice: 14-16 grams Dilution: half of a single human dose, Route of inoculation: Inraperitonial.

However the MWGT of vortex and baffle mode cultivated harvest samples passed the toxicity, when inoculated in the mice produced a weight gain results in the acceptable value of 3 g/mouse weight gain as required according to WHO [15].

All the results adhered to the regulatory norms. Bellalou and Relyveld [11] reported that the mouse lethal toxin of B. pertussis is increasingly labile at pH values diverging from neutral. However it seemed unlikely that the higher pH alone in the vortex aerated culture would have caused the loss of toxicity.

Hall et al. [16] pointed out that Waldhof and Baffle type fermentors were particularly affected due to the bubbles becoming entrapped in the continuously recirculation foam, resulting in high bubble residence times and, therefore, oxygen depleted bubbles. The presence of foam in a conventional agitator baffled fermentor may also increase the residence time of bubbles and therefore result in there being depleted of oxygen.

Conclusion

Commercial production of whole cell pertussis vaccine is cumbersome process, Bordetella pertussis is fastidious organism which has proved difficult to grow in large scale fermentor. Currently the B. pertussis strain 509 was used in the of large scale fermentor method. In this study the B. pertussis strain 509 was cultivated in two different culture modes like vortex and baffle. During baffle mode culture condition there was excess of froth was accumulated on the surface, it influence for the formation of smaller air bubbles were circulate along the axial flow of culture surface. During fermentor cultivation the overlay supply of air interacted with the small air bubbles those was formed due to insertion of baffle, which effect depletion of the oxygen and concomitant increases in CO_2 partial pressure could stimulate the respiration shift and reduction rate of the metabolic cycle. And also the axial broth flow pattern was observed in baffled mode of cultivation, because decreased growth rate and eventual cessation of B. pertussis final biomass yield.

The improvement of B. pertussis biomass yield during fermentor cultivation depends on the uniform uptake of dissolved oxygen as facilitated by direction of airflow in vortex mode resulted in rapid multiplication. The vortex culture mode condition, the formation of swirl pattern from the base of the impeller enhanced the homogenous culture condition of B. pertussis, it enhance superior air circulation provide during entire culture period. While large scale fermentor cultivation the B. pertussis organism growth requires vortex stirring and surface aeration. As a result of vortex agitation, the effective growth yield of the fermentor culture was increased up to two fold times, within 48 hours of cultivation without affecting virulence property of B. pertussis. This study indicated that vortex mode of large scale cultivation system is more significant compare with baffle mode cultivation system for commercial scale production of whole cell pertussis vaccine which reduced the duration of fermentation period would minimize energy cost and maximize production output/yield.

Basic aspects of the production of whole cell B. pertussis vaccine are the biomass yield but also the virulence state of the bacteria is important. When the production of acellular pertussis vaccine the virulence factors such as Pertussis Toxin (PT) Filamentous Hem agglutinin (FHA) Fimbriae, Pertactin (PRN) Tracheal Colonization Factor (TCF) Adenylate cyclase toxin (ACT) Heat-labile toxin (HLT) and Tracheal cytotoxin (TCT) are very important. But in this study we focused only in the different culture mode conditions for B. pertussis biomass yield, for the whole cell B. pertussis vaccine production.

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