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Effect of Impeller Number and Position on Growth Yield and Virulence of *Bordetella pertussis* Strain 509 During Large Scale Batch Fermentor Cultivation

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ABSTRACT

Studies have been undertaken to investigate, the effect of impeller number and its position upon the growth yield of *Bordetella pertussis* strain 509 during large scale batch cultivation. It was shown that, the maximum growth yield opacity was found to be 70 IOU mL^{-1} at 48 h cultivation in single impeller fixed at the bottom position of fermentor vessel. Whereas the optimal and moderate culture opacity 60 and 50 IOU mL^{-1} was observed during the two impellers mounted at two different positions on the shaft. Similarly the least growth yield was obtained during three impeller located at top, middle and bottom region of the shaft and the final opacity was found to be 30 IOU mL^{-1} at the end of 48 h cultivation with an increasing of culture pH 8.02. However, in two impeller combination experimental study also showed moderate growth yield at 48 h could give to homogenized mixing and good aeration. Thus, the two different location of the impeller combination influences two vortex flows, enhances optimal growth rate, viz; the lower impeller act as gas disperser and creates radial velocity with higher rates of air dispersion, whereas the upper impeller pumps the broth towards down thus the combined agitation of the broth leads to good mixing and even transfer of Dissolved Oxygen (DO_2) to the growing cells and found moderate bacterial growth. In the combination of three impellers mounted at three different positions experimental study shown that, lesser growth yield at the end of 48 h cultivation, due to higher forth accumulation.

Key words: Impeller, stirrer, agitation, *Bordetella pertussis*, opacity

INTRODUCTION

Whooping cough, otherwise called pertussis is an irresistible illness of the upper respiratory tract, caused by *Bordetella pertussis*. As per the World Health Organization (WHO), this sickness assaults around 60 million kids consistently, being in charge of 6,00,000 passings, basically in poor or developing countries, with problematic sterile conditions or absence of inoculation projects. Babies, especially those under 6 months of age keep on being the more powerless gathering in the infection burden and mortality (CDC., 2002). Despite the fact that pertussis has been the most ordinarily reported immunization preventable infection among kids under 5 years old, it is the one and only with an expanding reported occurrence (Greenberg *et al*., 2005).

The most commonly used vaccine against whooping cough is whole cell pertussis (wept) vaccine. The wept consists of the suspension of heat inactivated pertussis cells. The causative agent of

whooping cough is *Bordetella pertussis,* a gram negative aerobic bacterium and it is difficult to cultivate in a large scale fermentor without optimized growth parameters. Before mass vaccination, Whooping cough was a major cause of child morbidity and mortality. The introduction of *B. pertussis* vaccines in the 1940s dramatically reduced the health burden caused by whooping cough and virtually eliminated infant mortality. The wcPv, is a component of the Diphtheria-Pertussis-Tetanus Toxoid (DPT) vaccine has been shown to be very effective in protecting humans against *Bordetella pertussis* infection (Galit *et al*., 2015; Wendelboe *et al*., 2005). Most developing countries using inactivated wcPv (cellular vaccine) for their immunization programme. The production process of such a cellular vaccine is straightforward and low-cost, cells are cultivated, concentrated and inactivated by exposure to heat and/or chemicals. However, to obtain good yield of *B. pertussis* during large scale cultivation is difficult without optimization of growth parameters, such as cultivation temperature, pH, aeration and agitation and stirrer speed are crucial. The mixing is one of the essential parameter of fermentor. The use of impeller (agitator) is required to achieve good mixing process of fermentation broth, air dispersion, oxygen transfer and maintaining uniform environment throughout the process (Nasrallah *et al*., 2008).

With a specific end goal to keep up legitimate mass exchange of all supplements in the vessel, some type of blending is normally needed. By and large this blending is accomplished by one or a few impellers which blend the liquid in the tank. The impeller is generally mounted on a stirrer shaft which is controlled by an engine. This design goes about as a pump to move liquid in a customary example all through the vessel, consequently giving intends to sufficient mass exchange of supplements to cells. Unsettling of aging vessels cleaves up the air stream into little air pockets, courses the fluid and builds gas hold-up and makes turbulent shear, which diminishes the fluid film thickness (Finn, 1954).

Different fermentative growth in different fermentor shows different growth rate, different rate of biomass depending on type of impeller used. The good agitation achieved during fermentation process is mainly dependant on the choice of good impeller. The number of impellers and geometry of the impeller employed during fermentation process and the position of an impeller on the shaft which ensures thorough broth flow pattern and good mixing in stirrer fermentor tanks (Kumaresan and Joshi, 2006). Adler and Fiechter (1986) evaluated different types of bioreactors with biological system of microbial growth depends on different type of impeller used in stirred fermentor (Adler and Fiechter, 1986). The quality of good mixing in fluid media depends upon mainly on the type of configuration of impeller and number of impeller and obviously the real flow of mass transfer is in between the two extremes and depends on the impeller design and diameter, number of impeller and location of impeller in fermentation vessel (Kumaresan and Joshi, 2006).

However, the good growth rate obtained during batch fermentation depends on the stirrer rotation, sufficient aeration which leads to mixing efficacies of fluid broth (Nasrallah *et al*., 2008). Patwardhan and Jhosi (1999) have indicated an enormous scope for improvement in the mixing efficiency (mixing time per unit power consumption) by achieving desired combination of impeller at various positions in the tank. In view of these facts this study was undertaken to investigate the effect of impeller number and position on growth rate of *B. pertussis* during large scale cultivation in batch fermentor (Patwardhan and Joshi, 1999).

MATERIALS AND METHODS

Strain of *Bordetella pertussis:* *Bordetella pertussis* vaccine strain 509 was obtained from RJKS 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 Cruickshank *et al*. (1965) the composition was followed, Potato slice 250, NaCl 9, Proteose peptone 20 and Glycerol 20 g. The potatoes were cleaned, peeled and cut into slices, the slices with NaCl and demonized water were added to a flask and boiled, after cooking, potato was macerated and filtered through muslin 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. Proteose peptone and glycerol were added and mixed. Separately 60 g of agar was dissolved in 1500 mL demonized 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 blood was added at the ratio of 1:2 and gently mixed 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 h and then stored at 4-8°C (Cruickshank *et al*., 1965).

Preparation of preliminary seed: One ampule of freeze dried working seed copy of *B. pertussis* 509 was taken and the contents were resuspended in 2 mL of normal saline. The suspension was then inoculated onto the slopes of BG medium. After inoculation it was incubated at 35°C for 72 h. After 72 h of the growth the purity was checked by gram stain.

B2 culture medium: The B2 culture medium was prepared with the following compositions; Bactocasamino acid (BCA) 1800, L-glutamic acid 1500 g, NaCl 750 g, KH_2PO_4 150 g, $MgSO_4$ 30 g, $CaCl_2$ 3 g, $FeSO_4$ 3.74 g, $CuSO_4$ 0.15 g, glutathione 3.5 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 min separately. The remaining chemicals were dissolved in serial order 50 L of warmed distilled water in separate vessel. L-glutamic acid solution was prepared by dissolving in 50% NaOH still get amorphous solution hot distilled water. The BCA was dissolved 10 L of distilled water and yeast extract was added to this solution. Finally L-glutamic acid solution and other chemicals were added and made upto 300 L and mixed properly, transferred to fermentor and sterilized the medium at 121°C for 30 min (Shivanandappa *et al*., 2015b).

Sterility media: Nutrient agar medium, Soybean Casein Digest Medium (SCDM), Alternate Thio Glycolate Medium (ATGM) were used to study the purity and sterility of the culture at appropriate stage to ensure its purity and safety (Shivanandappa *et al*., 2015a).

Preparation of starter culture: One ampule freeze dried working seed stock *Bordetella pertussis* (509) was opened under sterile environment and resuspended in 2 mL of sterile B2 medium. The suspension was then inoculated BG slope and incubated at 35C±1°C for 72 h, ensured the purity by Gram staining. Furthermore the culture was scraped aseptically and inoculated into a 1 L flask containing 400 mL of B2 medium. The flasks were loaded on seed shaker for 24 h at 35±1°C and checked for purity, pH and opacity before inoculation in to the fermentor.

Purity and sterility test: The purity, 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±1°C for 24 h and observed for its sterility.

Samples (1 mL) of vaccines are inoculated into 4 bottles (100 mL each) of each thioglycolate medium and Soya bean Caesin Digestive medium. 4 bottles of thioglycolate medium are incubated at 35ºC and other 4 bottles are incubated at 20-22ºC for 14 days (Shivanandappa *et al*., 2015b).

Cell mass determination by opacity test: Opacity Reference standard 10 IOU WHO 5th IRP 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 h for all the batches checked the opacity (Pittman, 1979).

About 0.5 mL of the test vaccine was taken in the opacity tube and diluted the sample with normal saline until the opacity is identical with 5th International reference preparation of opacity (5th IRP) when compared by eye under uniform background, taking the dilution factor into account, opacity of the sample is calculated using the formula:

$$\text{Opacity} = \text{Sample+saline taken} \times 10$$

Method of Fermentor cultivation: The pertussis cell cultivation was done using large scale batch fermentor (Sartorious India 500 L) with a working volume of 300 L. Intially the fermontor was charged with 300 L of B2 media and sterilized at 121°C for 30 min, then cooled upto 35°C. Later the fermontor was inoculated with 3% inoculums and set all the required parameters such as temperature, pH, dissolved oxygen, stirrer speed and aeration (surface) at 35±1°C, 7.2, 100%, 500 rpm and 14-16 lpm, respectively. The process duration for the cultivation time was 48 h. For all batches the pH, sterility, purity and opacity were checked at every 24, 36 and 48 h (Table 1).

Impellers: For experimental study, Two types of impellers used, namely Ruston turbine type impeller (Dia-150 mm), 6 bladed was fixed at the bottom region of the shaft for all the four experiments and secondly the Marine type impeller (Dia-175 mm) 4 bladed two numbers were fixed in different position on the shaft were tested at optimum agitation speed 500 rpm for all the experiments and growth yield was assessed in different cultivation hours in a fermentor (Fig. 1).

Cell shearing effect: One milliliter of sample taken from the each intravals of 24, 36 and 48 h of cultvation which was aseptically inoculated on to the surface of fresh BG plates and incubated at 35°C for 48 h. After 48 h incubation the bacterial growth was observed visually and microscopically to find cell shearing and record the results of each sample.

Toxicity/MWGT test: The Toxicity test was performed by Mouse Weight Gain Test (MWGT) described by Pitman and Cox. The MWGT was executed for each harvested sample of different cultivation hours after heat inactivation at 56°C/30 min (Pittman, 1979; Shivanandappa *et al*., 2015c).

Table 1: Effect of growth yield upon *Bordetella pertussis* strain 509 in single impeller mounted at bottom of fermentor vessel

Cultivation time (h)	pH*	Opacity* (IOU mL^{-1})	Purity	Sterility	Shearing effect
24	7.52	25	Coccobacilli	Passed	Viable
36	7.88	40	Coccobacilli	Passed	Viable
48	8.02	70	Coccobacilli	Passed	Viable

*Mean value of three consecutive tests, condition; Temperature: 35°C, Agitation: 500 rpm, Aeration: 14-16 lpm, Ruston turbine impeller (diameter 150 mm), 6 vertical blades

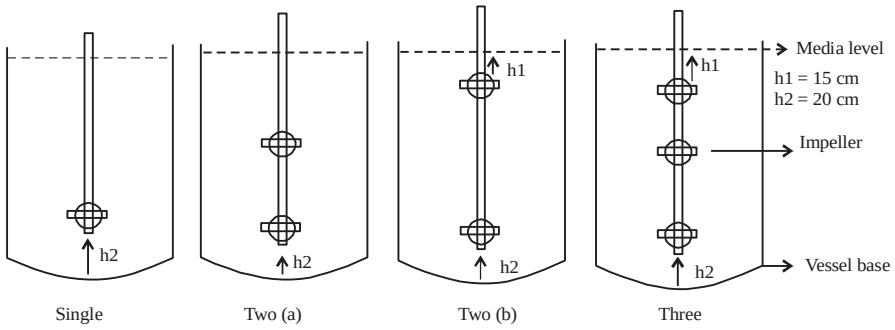

Fig. 1: Schematic representation of impeller positions in fermentor vessel, Single: Single impeller fixed at the base of the vessel , Two (a): Two impellers are set at 90ºC to each other, Two (b): Two impellers are fixed at different positions on same shaft, (One at bottom of the vessel and other one at just underneath the media surface), Three: Three impellers are fixed at different positions on same shaft (One at bottom of the vessel, second one at the middle zone of the vessel and third one at the underneath of the media surface)

About 14-15 g of healthy male Lacca mice are weighed in groups (n = 10) and injected intraperitoneally with 0.5 mL. Control group of 10 mice was injected with an equal volume of 0.9% saline. During the assessment the animals are properly feeded, both groups are weighed in the 1st, 3rd and 7th day after injection. A vaccine is considered non-toxic if, at the end of 72 h the weight of the group is not less than that at the time of injection and at the end of 7 days the average weight gained per mouse is no less than 60% of that of the control group of mice: In addition, no vaccine-related deaths should occur. If any mice die the test may be repeated but the aggregated deaths may not exceed 5%.

RESULTS AND DISCUSSION

In the present study the growth rate of *Bordetella pertussis* strain 509 was investigated in large scale batch fermentor (300 Lit) employed two types of impellers mounted at three different positions on the shaft of fermentor vessel. The result was obtained, the growth yield in terms of Opacity unit (IOU mL^{-1}) is depicted Table 2-4. It was observed that, the variation in the growth rate which depends upon the position and the number of impellers used.

In the first experimental study, single impeller (disc Ruston turbine) was fixed on the shaft at the bottom region i.e., 20 cm above the fermentor vessel base. In order to use with single impeller condition of the *B. pertussis* fermentor culture, the in process quality tests of the samples are assessed culture pH, opacity, purity by gram staining method and sterility in nutrient agar method. The samples were collected at different intervals 24, 36 and 48 h aseptically and the results obtained are shown in the Table 2. Indicated that, the maximum growth yield opacity was found to be 70 IOU mL^{-1} at the end of 48 h cultivation with an increasing of culture pH 8.02 and there is an optimal culture opacity 40 IOU mL^{-1} was noticed after 36 h cultivation with culture pH 7.88. Whereas, lower the growth opacity was found to be 25 IOU mL^{-1} with culture pH 7.52 observed in 24 h cultivation.

As far as the purity of the culture was concerned, in all three tests there were no changed in the morphology of the bacterium appeared as coccobacilli. However, in all three tests there is no deviation in sterility test observed and passes the sterility. In case of shearing effect, the bacterial cells are viable in entire culture period.

Table 2: Effect of growth yield upon *Bordetella pertussis* strain 509 in two impeller located one at bottom and other one at center region on the shaft of fermentor vessel

Cultivation time (h)	pH*	Opacity* (IOU mL^{-1})	Purity	Sterility	Shearing effect
24	7.45	16	Coccobacilli	Passed	Viable
36	7.75	30	Coccobacilli	Passed	Viable
48	8.13	60	Coccobacilli	Passed	Viable

*Mean value of three consecutive tests, condition, Temperature: 35°C, Agitation: 500 rpm, Aeration: 14-1 6 l pm, Bottom mounted impeller Ruston turbine type, (diameter 150 mm), 06 vertical blades and Middle mounted impeller Marine type (diameter 175 mm),04 vertical blades

Table 3: Effect of growth yield upon *Bordetella pertussis* strain 509 in Two impeller mounted at bottom of shaft and 15 cm below the media surface of fermentor vessel

Cultivation time (h)	pH*	Opacity* (IOU mL^{-1})	Purity	Sterility	Shearing effect
24	7.44	10	Coccobacilli	Passed	Viable
36	7.65	23	Coccobacilli	Passed	Viable
48	8.10	50	Coccobacilli	Passed	Viable

*Mean value of three consecutive tests, condition; Temperature: 35°C, Agitation: 500 rpm, Aeration: 14-1 6 l pm Bottom mounted impeller Ruston turbine type, diameter 150 mm, 06 vertical blades and top impeller Marine type (diameter 175 mm), 4 vertical blades

Table 4: Effect of growth yield upon *B. pertussis* strain 509 in three impeller mounted at three different region on the shaft of fermentor vessel

Cultivation time (h)	pH*	Opacity* (IOU mL^{-1})	Purity	Sterility	Shearing effect
24	7.34	0	Coccobacilli	Passed	Viable
36	7.56	20	Coccobacilli	Passed	Viable
48	7.93	35	Coccobacilli	Passed	Viable

*Mean value of three consecutive tests, condition; Temperature: 35°C, Agitation: 500 rpm, Aeration: 14-6 l pm, Bottom impeller Ruston turbine impeller, diameter 150 mm, 6 vertical blades, Middle impeller marine type (diameter 175 mm), 4 vertical blades and top impeller marine type (diameter 175 mm), 4 vertical blades

The higher growth yield 70 IOU mL^{-1} achieved at 48 h cultivation during the use of single impeller experiment could be due to the influence of homogenous mixing throughout the fermentor vessel. The point of the impeller at different position on the shaft also induce broth flow direction during bulk circulation of the culture, thus single turbine impeller fixed at the bottom position forms vortex flow which influence free air circulation to the cells thought the fermentor vessel (Shivanandappa *et al*., 2015c). During the cultivation period optimal agitation rate 500 rpm was maintained, it influenced uniform suspension of pertussis cells to achieve equal intake rates of nutrients would results in increasing of biomass concentration. As per the WHO the single disc turbine type impeller (width 0.3 cm) was fixed at 15 cm above the bottom of the fermentor vessel the agitation begins from the tip of the impeller zone, therefore the position of impeller also play major role during cultivation (WHO., 2007). Hence, the single impeller was fixed at 20 cm above from of the vessel bottom supports higher opacity without any cell damage.

In the second experimental study, the use of combination of two impellers at different position on the same shaft, one was mounted on exactly at the bottom region of the vessel as similar to as previous experiment (Ruston turbine) and second impeller which was fixed exactly at the centre region on the shaft (Marine impeller). Two impellers are set at 90ºC to each other. In order to use with double impeller condition of the *B. pertussis* fermentor culture, the in process quality tests of the samples are assessed pH, opacity, purity in gram staining method and sterility in nutrient agar method, the samples are collected 24, 36 and 48 h aseptically and the results are shown in the Table 1. In case of culture pH during cultivation of 24, 36 and 48 h showed, gradual increases and the range was found in between 7.45-8.13. Similarly the culture growth opacity was also increases optimally and it was found to be 16, 30 and 60 IOU mL^{-1}, respectively, at an end of 24, 36 and 48 h of the cultivation time. There is a two-fold increasing in the growth rate opacity during cultivation at 36 and 48 h was noticed during this study. As far as the purity of the culture was concerned, in all three tests there was no changed in the morphology of the

bacterium appeared coccobacili. However, in all three tests, there is no deviation observed in sterility test and passes the sterility. And also there is no shearing effect noticed, the bacterial cells are viable in entire culture period.

The optimal increased in the growth yield opacity during in this experiment, could be due to the use of two impeller combination provided higher dispersion of air throughout the media in radial flow and better mass transfer of fluid media was achieved. The bottom impeller created radial velocity with higher rate of air dispersion whereas the top impeller circulated the media downwards radially. This study is in accordance with the findings of Neinow and Ulbrecht (1985) in his study reported that, good mixing and aeration in broth can be achieved through dual combination, where the lower impeller act as the gas disperser and the upper impeller act primarily as device for aiding circulation of vessel contents. The good growth of *B. pertussis* depends on the optimum mass transfer rate and utilization of dissolved oxygen content during the process. It was also interesting to note that the down flow impeller caused reversible radial flow of broth near the corner of the vessel base (Neinow and Ulbrecht, 1985).

Whereas the impeller fixed at the upper position assist in bulk movement of upward and inward radial flow of broth in the vessel core region. The broth flow generated by two impeller depends on the degree of interaction that occurs between the impeller streams. If the spacing between the impeller is such that the two impeller positions cause combined positive effect on flow direction pattern. The impeller spacing must be adjusted in such a way that some positive interaction occurs between impellers and one gets beneficial results from the system of good mixing of broth (Kuzmanic *et al*., 2008).

In the third experimental study was performed by the use of combination of the two impellers at different position on the same shaft, one was fixed (Ruston turbine type) on at the bottom region (20 cm above) from the bottom of the fermentor vessel base and another marine type impeller was fixed (15 cm below) from the media surface. In order to use with this combination of impeller experiment of the *B. pertussis* fermentor culture, the in process quality tests of the samples are assessed pH, Opacity, purity in gram staining method and sterility in nutrient agar method, the samples are collected 24, 36 and 48 h and the results are shown in the Table 3. In this experimental study it was observed that, the culture pH was increased slowly and found to be 7.44 at 24 h, 7.65 at 36 h and pH 8.10 at 48 h. Whereas, growth yield is concerned, the culture opacity was slightly increased at 24 h and found to be 10 IOU mL^{-1}. Similarly the opacity 23 IOU mL^{-1} was observed at 36 h cultivation and moderate growth opacity was noticed i.e., 50 IOU mL^{-1} at the end of 48 h of cultivation. Higher agitation rate is not preferred for cultivation of *B. pertussis* strain 509 for lab scale cultivation (Jayaraj *et al*., 2011).

As far as the purity of the culture was concerned, in all the all three tests there was no changed in the morphology of the bacterium appeared coccobacilli. However, in all three tests there is no deviation in sterility test observed and passes the sterility. In case of shearing effect, the bacterial cells are viable in entire culture period.

The good growth of *B. pertussis* depends on the optimum mass transfer rate and utilization of dissolved oxygen content during the process. The use of two impeller combination provided higher dispersion of air throughout the media in radial flow and better mass transfer of fluid media was achieved.

The quality of mixing mainly depends upon the relative distribution of mean and turbulent kinetic energy exists in the form of mean kinetic velocity. The other extreme is that the flow is turbulent at all location of impeller and the mean velocity is zero. Obviously the real broth flow is

in between the two extremes and it depends upon impeller design, diameter and location of impeller position in fermentor tank in stirrer vessel. The quality of the broth flow is generated by the impeller position in vessel and it mainly depends upon impeller size and shape. When two impeller were fixed, the impellers generated radial flow where as the three impellers generated more turbulent kinetic energy, as the flow proceeds from the impeller and circulation of the media depends on stirrer rate (Kumaresan and Joshi, 2006). The mixing efficiency is also important during any fermentation process as it is dependent on the factors such as impeller geometry, agitator speed, agitation time and position of the impeller. The process of fermentation varies on the degree of agitation and the size of the impeller (Thalen *et al*., 2006).

The fourth experiment was performed using combination of three impellers at different positions on the shaft, one was fixed (Ruston turbine) on the shaft at the bottom region (20 cm above the bottom of the fermentor vessel base) and another impeller marine type was fixed (15 cm below from the media surface) and the third impeller marine type was fixed at the exact center region on shaft in between the two impeller. In order to use with three impeller condition of the *B. pertussis* fermentor culture, the in process quality tests of the samples are assessed pH, opacity, purity in gram staining method and sterility in nutrient agar method, the samples are collected 24, 36 and 48 h and the results are shown in the Table 4. In this course of experimental study it was observed that, there was no bacterial growth found at 24 h of cultivation and also no increased in culture pH but there was slightly turbid color changes noticed at an end of 24 h. Further the incubation period was extended, showed there was gradual increased in the opacity, which was found to be 20 IOU mL^{-1} at the end of 36 h of cultivation, similarly lesser in the growth rate was noticed and opacity was found to be only 35.0 IOU mL^{-1} with an moderate increased of culture pH 7.93 at 48 h of cultivation (Soons *et al*., 2006).

As far as the purity of the culture was concerned, in all the all three tests there was no changed in the morphology of the bacterium appeared coccobacilli. However, in all three tests there is no deviation in sterility test observed and passes the sterility. In case of shearing effect, the bacterial cells are viable in entire culture period.

There is a drastic reduction of the growth yield opacity i.e. 35 IOU mL^{-1} after 48 h was noticed, during this experiment could be due to the non ideal mixing of broth by three impellers combination. The flow circulation of broth was observed in three different directions and there was no vortex flow formation because the free circulation of the fluid was disturbed by the location of three impellers located at different position which created inadequate space between the impeller and vessel, which may effects the degree of flow angle and results more turbulence and changes in kinetic behavior of the cells in the media fluid. These different impeller positions can cause reduction in velocity of media flow and also affect swirl formation. Zhou and Kresta (1996) observed that, the fluctuation in directions of the principal flow of three impellers contributes more towards the turbulent kinetic energy. The high degree of agitation at three impeller position inside the vessel viz; top, middle and bottom disturbs the radial flow of broth and it was observed that there was more foam accumulation throughout the fluid media which increases the chance of contamination of culture at later stage during fermentation (Zhou and Kresta, 1996). Vardar-Sukan (1986) findings suggested that, the bottom impeller is the one most concerned with dispersing air throughout the vessel while the top impellers promote gas hold-up of the air bubbles. The spacing of impellers is also crucial. If impellers are spaced too closely, the result will be poor overall mixing of the fluid. However, during the course of these experiments we found that, bacterial cells grown under aerobic conditions with limited mixing effect did not support good growth of bacterial cells.

Table 5: Toxicity/(MWGT) analysis of four different impeller experimental fermentor culture samples

	Average weight gain (g) per mouse days after injection					
Culture sample	Day 1	Day 3	Day 7	Mortality	Control (%)	Test results
Single impeller	-6.0	1.4	4.1	0	78.8	Passed
Two (a) impeller	-0.8	1.7	3.9	0	75.0	Passed
Two (b) impeller	-0.4	1.0	3.0	0	65.0	Passed
Three impeller	0.9	1.8	3.8	0	73.0	Passed
Saline control	1.1	2.7	5.2	0	100.0	Passed

Because the froth formation during cultivation reduces the oxygen supply which could be restrict oxidation which in turn effect the enzyme activities during culture process hence the slower growth rate resulted minimal growth yield (Vardar-Sukan, 1986).

To check the virulence property of *B. pertussis* strain 509 during the course of different experiment, the 48 h of fermentor culture sample such as single, double and triple impeller was accessed by Mouse Weight Gain Test (MWGT) used laboratory healthy Swiss albino mice weighing 14-16 g and the results are shown in Table 5. At the end of the day 7, the average mouse weight (n = 10)/experiment, found to be variation between 4.1 g per mouse (78.8%) in the single impeller and 3.9 g per mouse (75%) and 3.0 g per mouse (65%) in twin impeller culture sample a and b, respectively and in case of three impeller experiment the average weight gain of mice found to be 73% after 7 days. Whereas average mouse weight gain observed in saline control was found to be 5.2 g per mouse. In this research study no death of any mouse was observed for all the culture samples. However, all the four experiment samples were passed the mouse weight gain test (Mouse toxicity test) as per (WHO., 2007). Which indicates that, there was no changes in the virulence property of *B. pertussis* strain 509 was noticed during the course of all the four different impeller experiments. From this study, the number and position of the impeller during large scale cultivation does not affected toxicity rather than variation in growth yield, of bacteria.

CONCLUSION

Mixing is an essential and crucial process for the productivity in microbial fermentation, in batch fermentation process. In this study, position and number of impeller experiments reveals that single and combination of two impeller system yield more opacity unit when compared with others.

When using with single disc turbine impeller leads vortex circulation and radial flow of broth utilization of DO_2 by the *B. pertussis* bacterial cells during fermentation process in single impeller increased growth rate, in two impeller combination, the radial flow was affected but forms vortex flow at certain distance of lower impeller zone, the flow structure generation was significantly increases of mixing velocity and mass transfer. Whereas, three impeller, there was significant decreased in growth rate could due to profound effect of oxygen depletion by excess froth formation. Furthermore, it was noticed that, the more turbulent flow in the region of impeller disturbed mixing efficiency and the bottom clearance of the lower impeller were found to have slighter effect on flow pattern that diverted axially towards upper region mingled with froth. The better agitation supports homogeneous blending which balances physical and chemical condition of the bacterial culture (no shearing effect). It concluded that single impeller condition has improved growth yield of *B. pertussis* strain 509. Whereas two and three impellers located at various positions shown decreased growth rate due to turbulence in flow directions as well as non-ideal mixing. When accomplishment of single impeller in industrial fermentor it could minimize the energy cost and maximize the production output.

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REFERENCES

Adler, I. and A. Fiechter, 1986. Valuation of bioreactors for low viscous media and high oxygen transfer demand. Bioprocess Eng., 1: 51-59.

CDC., 2002. Pertussis-United States, 1997-2000. Morbidity Mortality Weekly Rep., 51: 73-76.

Cruickshank, R., J.P. Duguid and R.H.A. Swain, 1965. Medical Microbiology: A Guide to the Laboratory Diagnosis and Control of Infection. 11th Edn., Churchill Livingstone, Edinburgh, UK., Pages: 1067.

Finn, R.K., 1954. Agitation-aeration in the laboratory and in industry. Bacteriol. Rev., 18: 254-274.

Galit, S.R.L., N. Otsuka, Y. Furuse, D.J.V. Almonia and L.T. Sombrero *et al*., 2015. Molecular epidemiology of *Bordetella pertussis* in the Philippines in 2012-2014. Int. J. Infect. Dis., 35: 24-26.

Greenberg, D.P., C.H.W. von Konig and U. Heininger, 2005. Health burden of pertussis in infants and children. Pediatr. Infect. Dis. J., 24: S39-S43.

Jayaraj, R., K. Vidhyacharan, K.C. Shivanandappa and J. Jayaprakash, 2011. Effect of agitation rate on growth yield and virulence of *Bordetella pertussis* strain 509 in lab scale cultivation. Res Bioscientia, 2: 22-27.

Kumaresan, T. and J.B. Joshi, 2006. Effect of impeller design on the flow pattern and mixing in stirred tanks. Chem. Eng. J., 115: 173-193.

Kuzmanic, N., R. Zanetic and M. Akrap, 2008. Impact of floating suspended solids on the homogenisation of the liquid phase in dual-impeller agitated vessel. Chem. Eng. Process.: Process Intensif., 47: 663-669.

Nasrallah, N., J. Legrand, A. Bensmaili and L. Nouri, 2008. Effect of impeller type on the mixing in torus reactors. Chem. Eng. Process.: Process Intensif., 47: 2175-2183.

Neinow, A.W. and J.J. Ulbrecht, 1985. Gas-Liquid Mixing and Mass Transfer in High Viscosity Liquids. In: Mixing of Liquids by Mechanical Agitation, Ulbrecht, J.J. and G.K. Patterson (Eds.). Chapter 6, Taylor and Francis, USA., ISBN-13: 9782881241123, pp: 203-237.

Patwardhan, A.W. and J.B. Joshi, 1999. Relation between flow pattern and blending in stirred tanks. Ind. Eng. Chem. Res., 38: 3131-3143.

Pittman, M., 1979. Pertussis toxin: The cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. Rev. Infect. Dis., 1: 401-412.

Shivanandappa, K.C., S. Jagannathan, S. Pandiyarajan, P. Tamilvanan, T. Umadevi, J. Kalaiselvan and B. Seker, 2015a. Purification of heat labile toxin from *Bordetella pertussis* vaccine strain 134 employed indigenous technology. Alexandria J. Med., (In Press). 10.1016/j.ajme.2015.06.001

Shivanandappa, K.C., S. Jagannathan, S. Pandiyarajan, R. Vijayakumar, T. Umadevi, J. Kalaiselvan and B. Sekar, 2015b. Assessment of toxic activity of heat labile toxin from *Bordetella pertussis* vaccine strain 134. Biochem. Physiol., Vol. 4. 10.4172/2168-9652.1000168

Shivanandappa, K.C., K.R. Mani, S. Jagannathan and R. Vijayakumar, 2015c. Assessment of *Bordetella pertussis* strain 509 cell mass yield in baffle and vortex mode of agitation during large scale industrial fermentor cultivation. J. Bacteriol. Parasitol., Vol. 6. 10.4172/2155-9597.1000214

Soons, Z.I.T.A., J.A. Voogt, G. van Straten and A.J.B. van Boxtel, 2006. Constant specific growth rate in fed-batch cultivation of *Bordetella pertussis* using adaptive control. J. Biotechnol., 125: 252-268.

Thalen, M., M. Venema, A. Dekker, L. Berwald and J. van den IJssel *et al*., 2006. Fed-batch cultivation of *Bordetella pertussis*: Metabolism and pertussis toxin production. Biologicals, 34: 289-297.

Vardar-Sukan, F., 1986. Dynamics of oxygen mass transfer in bioreactors. II: Design variables. Process Biochem., 21: 40-44.

WHO., 2007. Recommendations for whole-cell pertussis vaccine. WHO Technical Report Series No. 941, World Health Organization, Geneva, Switzerland, pp: 301-333.

Wendelboe, A.M., A. Van Rie, S. Salmaso and J.A. Englund, 2005. Duration of immunity against pertussis after natural infection or vaccination. Pediatr. Infect. Dis. J., 24: S58-S61.

Zhou, G. and S.M. Kresta, 1996. Impact of tank geometry on the maximum turbulence energy dissipation rate for impellers. AIChE J., 42: 2476-2490.