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Vol. 12(11), pp. 269-274, 21 March, 2018
DOI: 10.5897/AJMR2017.8625
Article Number: AFBEA1B56504
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Physiochemical factors affecting *in vitro* growth of *Pasteurella multocida*

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Received 25 June, 2017; Accepted 1 September, 2017

***Pasteurella multocida* causes fowl cholera (FC), a contagious bacterial disease of domestic and wild avian species. It is a Gram negative coccobacilli causing acute highly fatal septicemia with high morbidity and mortality rate. Control of fowl cholera is primarily performed by good management practice and vaccination in areas where disease is endemic. For a quality vaccine, high density growth of *P. multocida* along with its capsule and outer membrane protein (OMP) is necessary. In the present study, physiochemical factors (growth medium, temperature, pH, incubation time and stirring along with aeration affecting growth density) were evaluated. Growth density was determined by colony forming unit (CFU) and dry mass. Physiochemical factors used in maximum growth density (1.32×10^{12}CFU or 9 mg/ml) of bacteria were between 35 to 40°C, 7.2 to 8.2 pH and 1000 rpm stirring, along with aeration during incubation of 24 h in Brain Heart infusion (BHI) broth. *P. multocida* showed maximum growth (1.32×10^{12} or 9 mg/ml) in BHI broth with continuous stirring and aeration (1000 rpm) at 38°C for 24 h. Thus, there was a positive co-relation between colony forming unit (CFU) and dry mass of bacteria.**

Key words: Brain heart infusion (BHI), aeration, agitation, fermentor, dry mass.

INTRODUCTION

Pasteurella multocida causes fowl cholera (a bacterial disease) in domestic and wild birds (Xiao et al., 2015). The disease is characterized by facial edema, blackening of comb and wattles, diarrhea, dull depressed with high morbidity (up to 50%) and less than 10% mortality in infected flock (Glisson et al., 2003; Choudhury et al., 1985; Kwon and Kang, 2003; Moemen et al., 2012).

Post mortem lesions are enteritis, petechial hemorrhages on the epicardium and serosal membranes, reactive liver and spleen with white to yellow foci (Calnek et al., 1997; Shivachandra et al., 2005; Christensen, 2013). *P. multocida* can be classified into five different capsular serogroups (A, B, D, E and F) and 16 serotypes. It is further divided into 1: A, 5: A, 9: A serotypes (Rimler and Rhoades, 1989; Benkirane and De Alwis, 2002; Glisson et al., 2008; Mohamed et al., 2012). It is a fastidious organism which grows on enrichment medium such as Brain Heart Infusion Broth (BHI), CSY broth, tryptic soya broth and serum broth.

Usually, CSY broth is commonly used in the

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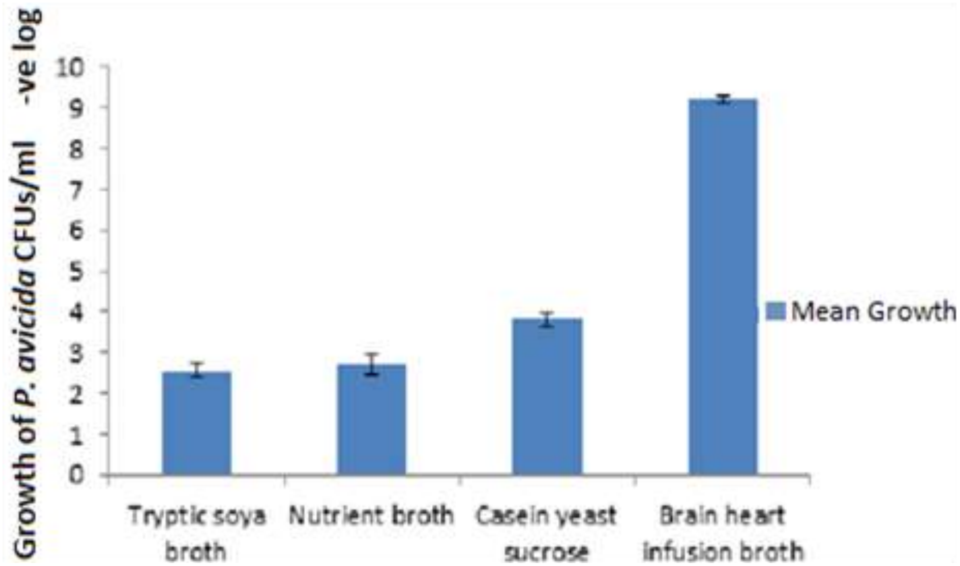

Figure 1. Effect of culture medium on growth of *P. multocida.*

development of vaccine and diagnostics (Sarwar et al., 2013). The causative agent is Gram negative, cocco-bacilli, capsular, non-motile and non-hemolytic on blood agar (De-Alwis, 1999; Boyce et al., 2000; Pedersen et al., 2003; Tabatabaei et al., 2007). It does not grow on MacConkey agar, but show pure growth on media containing potassium cyanide.

Different factors such as culture media, temperature, pH and aeration continuous stirring have effect in *in vitro* growth of bacteria (Imtiaz, 2001; Shah et al., 2008; Sarwar et al., 2013). Limited number of doses has been produced due to insufficient growth of bacteria in still culture, using conventional method of vaccine production. Therefore, this study is designed to investigate the physiochemical factors which enhance growth density of *P. multocida* in semi controlled bio fermentor.

MATERIALS AND METHODS

Activation of *P. multocida*

5 mL fresh growth of capsular serotype *P. multocida* (obtained from Ottoman Pharma 10-km Raiwind Road, Lahore) was transferred aseptically in 50 mL of BHI broth (Oxoid). The inoculated material was incubated at 37°C for 24 h and the activated broth culture was used for further study.

Factors augmenting immunogen/growth

Culture media such as casein sucrose yeast (CSY)- (Oxoid), tryptic soya (TS)- (Oxoid), (BHI) and nutrient broth (NB)- (Oxoid) were prepared according to the instruction of the manufacturer. 50 mL of CSY, TS and NB broths were transferred to conical flat bottomed flask (Pyrex) while 50 mL of BHI broth was transferred to each of the fifteen flasks. Each of the flasks was cotton plugged and autoclaved at 121°C for 15 min. The flasks containing BHI, CSY, TS and NB broth were inoculated aseptically with 5 mL freshly growth culture.

The flasks were incubated at 37°C with aeration for 24 h. Each of the five flasks containing 50 mL of sterile BHI culture broth was incubated at 35, 37, 38, 40 and 42°C with aeration for 24 h. The pH of each of the six flasks containing BHI culture broth was adjusted to 4, 5.2, 6, 7.2, 8 and 9.2 using 4N hydrochloric acid (Sigma) or 4N sodium hydroxide (Sigma). Also, each of the broth was incubated at 37°C with aeration for 24 h.

Each of the four flasks (A, B, C and D) containing BHI culture broth, incubated at 37°C for 24 h was aerated at 500, 750 and 1000 rpm, respectively with stirring during whole period of incubation. The flask D was incubated without aeration and stirring (still culture).

Measurement of growth density

Total viable count in each of the above experiment was determined by Pour plate method (Van Soestbergen and Lee, 1969) with some modifications and dry mass was determined according to the methodology described by Bratbak and Dundas (1984).

RESULTS AND DISCUSSION

Physiochemical factors such as growth media, temperature, pH and agitation affected the growth potential of *P. multocida.* Each parameter has critical significance in augmenting the biomass production *in vitro* (Sawar et al., 2013). BHI broth showed significantly ($P<0.05$) higher growth of the organism than that observed with TS broth, CSY broth and nutrient broth (Figure 1, Table 1). Sucrose at rate 0.1 to 2.0% in culture

Table 1. Effect of culture medium on growth of *P. multocida.*

Growth media	Dry mass (mg/mL)- per flask	M±SD*
Brain heart infusion broth	9, 9.2, 9.2,9.4	9.2 ± 0.163^{c}
Tryptic soya broth	2.5, 2.9, 2.3, 2.5	$2.55\pm.252^{a}$
Nutrient broth	2.5, 2.7, 2.7,2.9	$2.7\pm.163^{a}$
Casein sucrose yeast	3.9, 3.8, 3.8, 3.7	3.8 ± 0.082^{b}

Note: Different letters in the same column show significant difference (P>0.05)*= Mean ± Standard Deviation.

Table 2. Effect of temperature on growth of *P. multocida*

Temperature (°C)	CFU/mL- per flask	Weight	
		Dry mass (mg/ML)	M±SD
35	$1.32x10^{10}$, $1.32x10^{11}$, $1.32x10^{10}$, $1.32x10^{10}$	5.2, 5.2, 5.1, 5.2	$1.33\pm.027^{b}$
37	1.32×10^{11} ,1.32×10^{11}, 1.32×10^{10} ,1.32×10^{11}	7, 7, 6, 7	$1.35\pm.025^{c}$
38	1.32×10^{11}, 1.32×10^{11}, 1.32×10^{10} 1.32×10^{11}	7, 7, 6.8, 7	$1.35\pm.025^{c}$
40	1.32×10^{12}, 1.32×10^{11}, 1.32×10^{12}, 1.32×10^{12}	9.1, 9.0, 8.9, 9.1	$1.40\pm.025^{d}$
42	1.32×10^{10}, 1.32×10^{9}, 1.32×10^{10}, 1.32×10^{10}	5.4, 5.1, 5.2, 5.4	$1.30\pm.035^{a}$

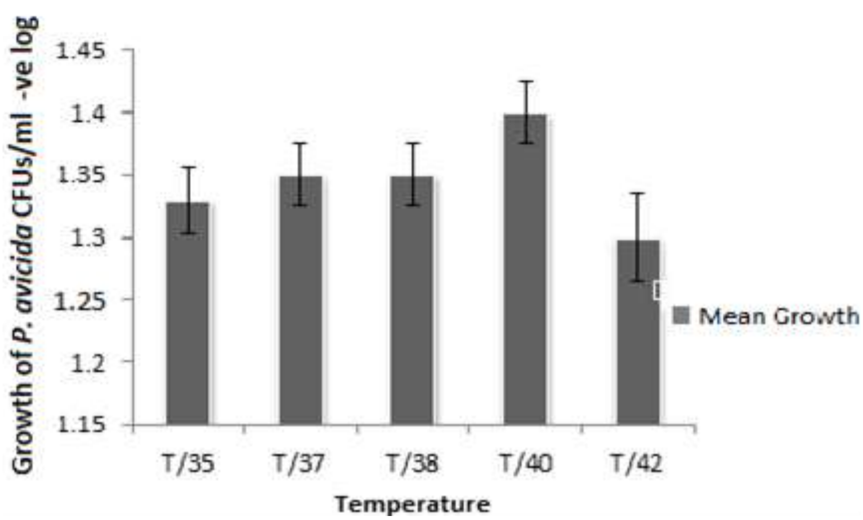

Figure 2. Effect of temperature on growth of *P. multocida.*

medium enhances the biomass of *P. multocida* (Shah et al., 2008) with blood and BHI agar routinely used for the isolation of *P. multocida* (Melody et al., 1994). BHI broth is considered as enrichment medium used for the cultivation of *P. multocida* strains, mammalian and avian origin (Melody et al., 1994).

At temperature range 35 to 40°C, *P. multocida* showed maximum growth in BHI broth (Table 2, Figure 2). However, growth was undetectable when bacteria were incubated at temperature above 40°C. Optimum growth of *P. multocida* was detected when incubated at 35 ± 5°C while no growth was recorded at 25 ± 5°C or above 50°C (Imtiaz, 2001; Shah et al., 2008; Sarwar et al., 2013). At pH range 7.0 to 7.8, the organism showed optimum growth (Table 3, Figure 3). Also, at pH below 3 or above 10, organism shows undetectable growth (Imtiaz, 2001; Shah et al., 2008) which indicated that, the pH of BHI broth is critical in biomass production of *P. multocida.*

Table 3. Effect of pH on growth of *P. multocida.*

pH	CFU/mL-10^{10} –per flask	M±SD
4	$1.6{\times}10^3, 1.6\times10^3, 1.6\times10^3, 1.6\times10^3$	0.76±000[a]
5.2	$1.6{\times}10^4$, $1.6{\times}10^4, 1.6\times10^4, 1.6\times10^4$	0.96±000[b]
6	$1.6{\times}10^9$, $1.6{\times}10^8, 1.6\times10^9, 1.6\times10^9$	1.5±.04[c]
7.2	$1.6{\times}10^9, 1.6\times10^9, 1.6\times10^8 1.6{\times}10^{12}$	1.55±.119[cd]
8	$1.6{\times}10^{10}, 1.6\times10^{10}, 1.6\times10^{10}, 1.6\times10^{10}$	1.6±.000[d]
9.2	$1.6{\times}10^9$, $1.6{\times}10^8, 1.6\times10^9, 1.6\times10^9$	1.5±.04[c]

Note: Different letters in the same column show significant difference (P>0.05).

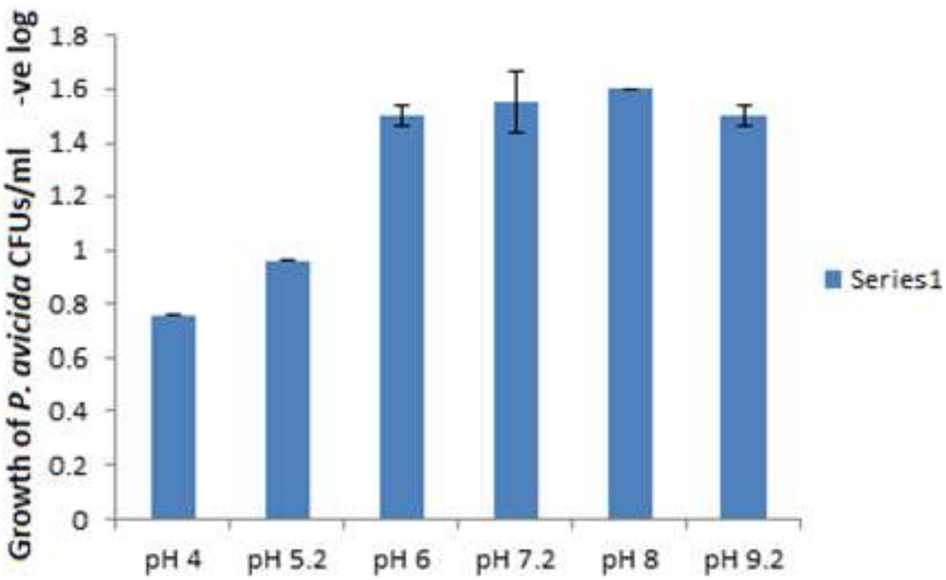

Figure 3. Effect of pH on growth of *P. multocida.*

Aeration influenced the growth of *P. multocida*. Maximum growth of the bacteria was observed when the solution was agitated at 500 to 550 rpm and growth was declined at higher agitation rate (Table 4, Figure 4). *P. multocida* showed optimum growth when broth is agitated at 50 to 500 rpm (Imtiaz, 2001; Shah et al., 2008). In Ali et al. (2000), when the broth was provided with enrichment media and fresh filtered air, it resulted in a bacterial culture with $5{\times}10^9$ CFU/mL and 1.68 mg/mL dry weight. Moreover, when incubated in a flask having BHI broth and agitated at 200 rpm, the bacterial culture showed 10^9 CFU/mL after 3 to 4 h of incubation at 37°C (Tabatabaei et al., 2007). According to Sarwar et al. (2013), dry mass bacterial growth was 8.2 mg/mL measured by centrifugation technique while the bacterial count was 10^{17} CFU/mL.

Repeated culture of *P. multocida in vitro* results in the loss of its capsule. Thus, after every 2nd passage in laboratory medium the bacterial culture, may be injected in susceptible rabbits through intra-muscular route. In this manner, the bacteria regain its capsule and kill the rabbits. Moreover, mice or calves can also be selected for revival of pathogenicity of the bacteria (Muneer et al., 2005). This methodology is used for biomass production of *P. multocida* aimed at biologics (vaccine/diagnostics) production (Sarwar et al., 2013).

Conclusion

From the study, the bacteria showed $1.32{\times}10^{12}$ CFU/ml of growth in brain heart infusion broth (pH 7.2), when incubated at 37°C for 24 h in the presence of aeration and stirring.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 4. Effect o0f aeration on growth of *P. multocida.*

Aeration revolution/min*	CFU/mL-10^{10} – per flask	M±SD
1000	$1.42{\times}10^{12}$, 1.42×10^{11}, 1.42×10^{12}, $1.42{\times}10^{12}$	1.51 ± 0.030
750	$1.42{\times}10^{11}$, $1.42{\times}10^{11}$, $1.42{\times}10^{11}$, $1.42{\times}10^{11}$	1.47 ± 0.000
500	$1.42{\times}10^{10}$, $1.42{\times}10^{10}$, $1.42{\times}10^{10}$, $1.42{\times}10^{9}$	1.40±0.035

*Aquarium pump was used to aerate the culture during incubation time. In one experiment the speed was adjusted to 1000 rpm, in second it was 750 rpm and third it was 500 rpm. Each experiment was repeated four times. At the end of incubation time the Colony forming unit (CFU) of *P. multocida* was measured.

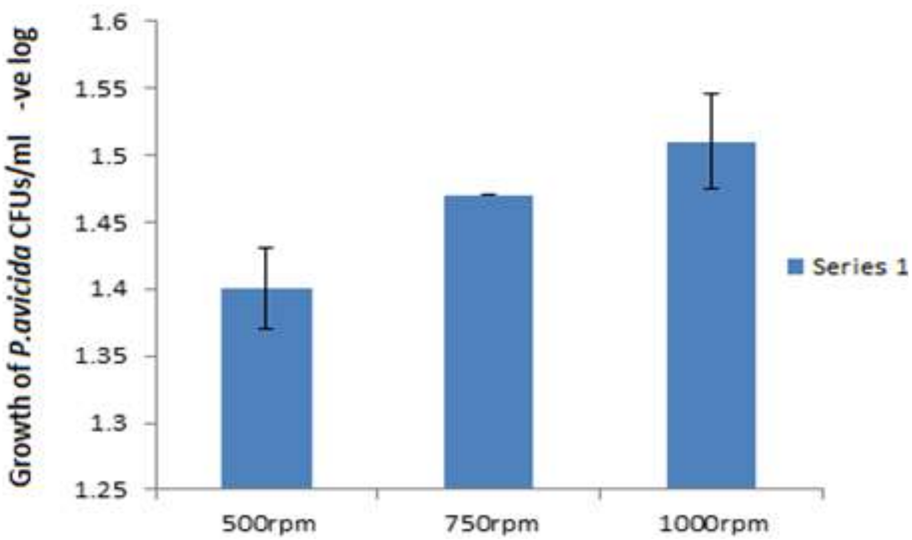

Figure 4. Effect of aeration on growth of *P. multocida.*

ACKNOWLEDGEMENT

The authors are highly grateful to Mr. Usman Farooq Khalid the owner of Ottoman Pharma (Immuno Division), for providing financial support and providing access to laboratory facility in executing the research.

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