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Research Article

COMPARISON OF DIFFERENT FERMENTATION PARAMETERS OF *PASTEURELLA MULTOCIDA* ON THE DRY CELL MASS FOR THE PREPARATION OF FOWL CHOLERA VACCINE

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ABSTRACT

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurellamultocida*. This study was undertaken to investigate the effect of some different parameters as pH and agitation, air with DO (Dissolved oxygen) cascade to increase the production of dry cell mass from the fermentation of *Pasteurellamultocida*. These different kinetic parameters were carried out in three cultures containing two litters of Brain Heart Infusion broth media in 5 L fermentor (New Brunswick). The first culture (A) conditions were ; at 37 ºC, air flow 0.5 L/min, agitation frequency 90 rpm, dissolved oxygen was 80% and adjustment pH with 1 N NaOH, culture (B) was carried out under the same conditions with no pH adjustment. In addition, culture (C) was carried out with Agitation/Air/DO cascade, the set point of agitation was 90- 200 rpm, air flow 0.5-2 L/min. Culture A gave the highest optical density (OD) compared to culture B and culture C. The values of OD were 2.1, 1.7 and 1.5 respectively. Also, the highest dry cell mass (3.1 g/L), (2.7 g/L) were obtained in culture A and culture B respectively, but the lowest dry cell mass (2.2 g/L) was obtained from culture C with the agitation, air with DO cascade. The present study concluded that it adjusting pH, using low air flow and low agitation gives the highest yield of dry cell mass.

INTRODUCTION

Pasteurellamultocida (*P. multocida***)** subspecies *multocida*is an important pathogen that causes fowl cholera (FC) in poultry and wild birds (Xiao *et al.*, 2015). *P.multocida* strains are classified into serogroups (A, B, D, E and F) based on capsule antigens and further classified into 16 serotypes (1-16) based primarily on lipopolysaccharide antigens using the Heddleston scheme (Carter, 1955; Heddleston *et al.*, 1972).

P. multocida is a facultative gram negative short rod, coco bacillus non-motile, capsulated pathogen and is the causative organism of multiple diseases in animals (Carpenter *et al.*, 1989). P. multocida is associated with a wide range of diseases in many species of animals and the major diseases being hemorrhagic septicemia (HS) in ungulates, atrophic rhinitis in swine, and fowl cholera (FC) in wild and domestic birds (Haq and Abdullah, 2015). Fowl cholera caused by P. multocida, is a contagious bacterial disease of domesticated and wild avian species caused by infection with P. multocida. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality in older birds (OIE Terrestrial Manual 2015).

Fowl cholera, occurs sporadically or enzootically in most countries of the world wherever intensive poultry production noccurs, and is known as a bacterial disease with major economic importance due to its high mortality (Glisson *et al.*, 2008). *P. multocida* is a heterogeneous species that pathogenicity of individual strains is highly variable and susceptibility to these bacterial strains varies considerable among avian species (Christensen and Bisgaard, 2000; Mohamed-Wael and Moemen, 2014).

Biomass production of *P.multocida* for hemorrhagic septicemia vaccine preparation is carried out in order to improve animal health and to increase production of livestock. High amount of biomass of *P. multocida* for vaccine preparation can be successfully achieved by using bioreactor. Biofermentor has the ability to provide optimum environmental conditions for the growth and multiplication of microorganisms being in it (Khan *et al.*, 2013). In this study we investigated the effects of varying fermentation condition on the biomass obtained from P. multocida for fowl cholera vaccine preparation.

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MATERIALS AND METHODS

Organism and inoculum preparation

Pasteurellamultocida was kindly supplied from Veterinary vaccine company- VACSERA. Then, the strain was reconstituted and streaked on Brain heart Infusion agar (BHI) media. A loopful from the overnight growth of *Pasteurellamultocida* was transferred to six flasks 100 ml BHI broth medium and incubated at 37°C with vigorous shaking 150 rpm for 18h; cell growth was checked by measuring optical density (OD) of the culture at absorbance 600 nm and purity was checked by Gram stain **(**Cruicksbank *et al.*, 1975).

Culture

A 2% of the primary culture was transferred to two litters of BHI broth medium and steam sterilized at 121°C for 15 min and incubated for 20 h according to Khan *et al.*, 2013.

Fermentation

Fermentation conditions were according to Khan *et al.*, 2013 shown in table (1).

Table 1 Fermentation data sheet

Item	Culture A	Culture B	Culture C
Seed Volume 2%	40 ml	40 ml	40 ml
Culture total volume	2L	2L	2L
Agitation	90 rpm	90 rpm	90-200 rpm
Temperature	37◦C	37◦C	37◦C
Air Flow	0.5 L /h	0.5 L /h	0.5- 2 L /h
Dissolved Oxygen (DO)	80%	80%	80%
pH	7.2 was adjusted by using 1 NNaOH	Not controlled Initial pH 7.2 Final pH 5.6	7.2 was adjusted by using 1 N NaOH
Antifoam	0.5 ml/l was added of antifoam.	0.5 ml/l was added of antifoam.	0.5 ml/l was added of antifoam.
Duration	20 h	20 h	20 h
Agitation/Air / DO Cascade phase	No cascade	No cascade	Agitation/Air/O_2 cascade

Inoculation

The plug was removed from the inoculation port. The inoculum was removed aseptically from its flask using inoculation syringe. The inoculum was injected through the septum in the inoculation port. The plug was reinstalled in the port. When the whole lot of the liquid media is ready about two L for each fermentor, i.e., the pH adjusted to 7.2 with 1 N NaOH for culture A and C, and pH of culture B was not adjusted. Then, 2 % inoculum was added. The growth of the cultures takes place at 37°C with agitation 90 rpm for culture A and B, while culture C was in range 90-200 rpm and incubator period for each fermentor was 20 hrs with a modification in fermentation time than *Khan et al.*,2013 and sampling was collected at 20 h intervals.

Harvesting

The culture had been harvested from late stationary phase culture grown for 20h at 37°C. The cultures were harvested after purity testing of the inoculum is examined by gram stain and centrifuged at 3000 for 30 minutes for clarification. The supernatant was collected and sterilized through Millipore 0.22 µm stericup USA. The cells, on the other hand, were pooled and kept frozen (Collier and Kandel, 1971).

Estimation of dry cell mass

Cell concentration was expressed as dry biomass weight per liter (g/l) after centrifugation of 3 L culture, followed by pellet drying at 60 °C for 48 h (Barugue-Ramos *et al.*, 2005).

RESULTS

The Gram stained smear of *P. multocida*was utilized as indicator for the purity of the culture and demonstrated the morphological characteristic, Gram negative coccobacilli bacteria. Six conical flasks of 100 ml BHI broth were seeded with pure colonies of *P. multocida*and were cultivated as primary culture, the highest three OD cultures 1.6, 1.631, 1.619 were used as seed for culture A, B and C respectively, as shown in table (2).

Fermentation

Three cultures of *P. multocida*were cultivated, the summary of results obtained were described in table 2 below.

Table 2 Summary of experimental results obtained with culture A, B and C

	Culture A	Culture B	Culture C
Gram stain	G. Negative coccobacilli	G. Negative Coccobacilli	G. Negative coccobacilli
OD of primary inoculum	1.6	1.631	1.619
OD after fermentation	2.1	1.759	1.574
The volume taken from 1 NNaOH for neutralization	100 ml	0	70 ml
Final pH	7.2	5.6	7.2
Final Dissolved oxygen	10%	25%	30%

The culture A and B the agitation was fixed at 90 rpm, air flow was about 0.5L/h at all culture time, while culture C the same condition as culture A except a cascade process with air range from 0.5-2 l/h and agitation range 90-200 rpm was applied for 20 h fermentation.

Estimation of bacterial growth

Comparison of bacterial growth for culture A, B and C were estimated, 5 ml samples were taken every two hours and measure optical density using spectrophotometer at 600 nm, growth curve was drawn using optical density versus time. Batch A gave highest OD at 600 nm about 2.1 at the end of

fermentation, while batch B was 1.791 and finally Batch C was 1.574 as shown in figure (1).

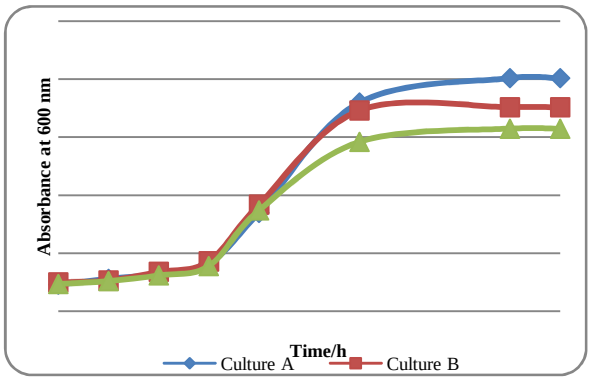

Figure 1 Growth curves comparison of *P. multocida*fermentation for culture A, B and C.

pH pattern curves

The pH value of culture A and C were approximately linear as it adjusted automatically to 7.2 by using about 100 ml and 70 ml of 1NNaOH respectively, while pH value of culture B dropped from 7.2 to 5.6 during fermentation process as it was not adjusted. The following figure (2) represents the comparison for pH pattern of cultures A, B and C.

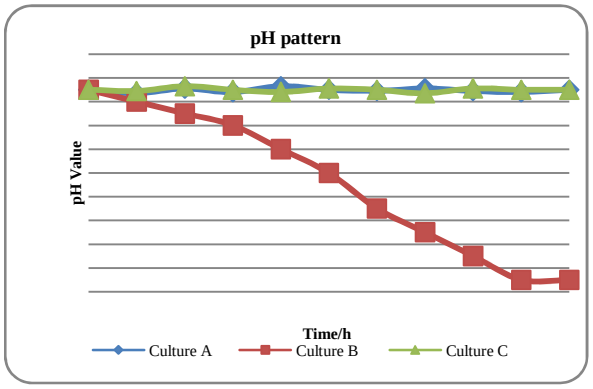

Figure 2 The effect of pH on the growth of *P. multocida*showing the growth occurred from pH 7.2 to 5.6 during the exponential growth.

Dissolved Oxygen comparison of the three cultures

Dissolved Oxygen percent was determined automatically and recorded during fermentation process, the following figure (3) represents comparison of DO in the three cultures. Culture A with lowest DO percent reached to 10%, then culture B with higher value about 25% and culture C with highest one at 30%.

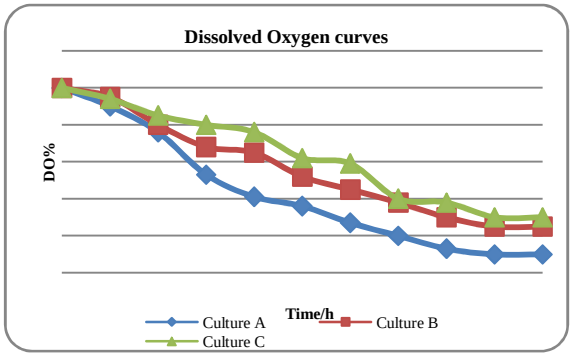

Figure 3 The effect of agitation/ O_2/ DO cascade on DO% during and at the end of cultures A, B and C.

Cell mass estimation

After 20 h of fermentation, the cultures were harvested, centrifuged and pellets of culture A, B and C were dried at 60 ºC for 48 h. The dry cell mass of culture A was 3.1 g/l and the volume of supernatant was 1900 ml, culture B dry cell mass was 2.7g/l while the volume of supernatant was about 1930 ml, the least dry cell mass was found in culture C it was 2.2 g/l and the volume of supernatant was 1875 ml, the results were shown in table (3).

Table 3 Summary of dry cell mass and volume of supernatant results obtained from culture A, B and C.

	culture A	**culture B**	**culture C**
Dry cell mass g/L	3.1 g/L	2.7g/L	2.2 g/L
Volume of supernatant	1900 ml	1930 ml	1875 ml

DISCUSSION

Fowl cholera is a devastating and lethal disease**;** it causes severe economic loss and hampers the development of poultry industry. This study aimed at comparing the effect of some different parameters as pH, agitation and air with DO cascade to increase the production of dry cell mass. In the present study, the bacterial colony morphology was studied on brain heart infusion agar at 37ºC for 18 h were in agreement with (Coetzer and Tustin, 2004; Jabeen *et al.*, 2013) who reported that BHI enriched and supported *P. multocida* growth. Our result showed the highest optical density (2.1) for the growth of *Pasteurella* in culture A than culture B (1.7) and C (1.5) .The OD of culture A was very similar tothose reported by (Khan *et al.*, 2013). The culture fermentation has the ability to maintain the microbial growth and to produce dense biomass. A similar study conducted by (De Alwis, 1992, Imtiaz, 2001) which demonstrated that when the agitation is increased the growth is slower. Our results are concordant with these results as in culture C in which we used increased agitation and air with DO, the dry cell mass decreased when compared to culture A and culture B.

OIE, 2008 reported that 1.5 g/L are sufficient for making one liter of vaccine. Our result show the dry cell mass was 3.1g/L. Our result show the dry cell mass was 3.1g/L inthe present study which is almost similar with (Khan *et al.*, 2013**)** results who reported that the biomass was 3.8 g/L. The present study concluded that the optimum fermentation condition to obtain highest dry cell mass was using low agitation, low air flow and pH adjustment.

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