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RESEARCH ARTICLE

BIOMASS PRODUCTION OF *PASTEURELLA MULTOCIDA* USING BIOFERMENTER

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A study on biomass production of *Pasteurella multocida* was carried out in the department of Microbiology Kohat University. *P.multocida* is a Gram negative short rod bacterium responsible for causing hemorrhagic septicemia in cattle's, buffaloes and many wild animals characterized by an acute, highly fatal septicemia with high rate of morbidity and mortality. Master seed of the *P. multocida* B: 2 strainswere collected from VRI Peshawar in freeze dried form. After reactivation in BHI it's morphological and biochemical characteristics were studied. It was inoculated in rabbit for re-isolation. Death of rabbit occurred in thirteen hours and the isolate was identified by standard cultural and biochemical methods. Biomass production of the bacterium was conducted by simple conventional and biofermenter methods in order to check efficacy of the two methods. On conventional method at 37 ºC after 14 hours, highest dry mass on BHI was 0.38 g/10 ml, while it was 0.13 and 0.098 on PB and NB respectively. When the Optical density (OD) was checked of the three Media, it also showed that BHI has highest OD which was 1.577 at pH 7 as compared to other two Media. This investigation showed that BHI is highly enriched media that supports *P.multocida* as compared to others. The result showed that biofermenter gave 2.1329 g/10 ml and it was much higher than conventional method. The present study concluded that high biomass for HS vaccine preparation can be best obtained using biofermenter and large number of vaccine doses can be prepared in short duration.

Introduction

Hemorrhagic septicemia (HS) is an acute and fatal disease caused by *Pasteurella multocida*, primarily occurring in cattle and buffaloes, but occasionally other domesticated and wild mammals can be affected (De-Alwis, 1992). *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). It is normal flora of the nasopharynx of many species of domestic and wild animals. Human infection usually follows animal bites or droplet transmission from animals (Connell, K.O et al., 2009).

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvante vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV. A live HS vaccine prepared using an avirulent*P. Multocida* strain B: 3, 4 (Fallow deer strain) has been used for control of the disease in cattle and buffaloes over 6 months of age in Myanmar since 1989. The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in

other countries and killed vaccines are the only preparations in use by the countries affected with HS (Priadia and Natalia 2001).

Vaccination is internationally acknowledged as a best way of controlling hemorrhagic septicemia in animals. Therefore good quality vaccine, occupies most important place in management of HS. An important problem associated with the production of quality vaccine against HS is its high cost of production. This is due to low yield of biomass of *P. multocida* when grown in ordinary or routine media. *Pasteurella multocida* when grown in various media yielded moderate to dense growth. Good growth is obtained in BHI and CYS and poor growth is obtained in RCM and in NB media. Maximum growth of *P. multocida* is supported by 1 Percent casein hydrolysate, 1 % yeast extract, 0.5 sodium chloride and in 1 percent sucrose in the medium. Increased aeration resulted with proportional increase in biomass of *P. multocida*. When the agitation rate increased at an increment of 100 rpm up to 700 rpm, a steady and slow increase of growth occurs (Mahmood, 2001). There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (Thomas J, 1968).

For vaccine production, dense suspensions of bacteria are necessary. The biomass should have a minimum bacterial content of 1.5 g dry weight per liter of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinized physiological saline. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports *P. multocida*. Dense cultures are also obtained using fermentor, where heat sterilization of the tanks and culture can be carried out *in situ*, with automatic temperature, pH and aeration control devices. Liquid sterilization systems by filtration, for heat-labile components, can also be built into the fermentor (OIE Terrestrial Manual,2008).

Biomass production of *P.multocida* for hemorrhagic septicemia (HS) vaccine preparation is carried out in order to improve animal health and to increase production of livestock. Being an agricultural country, livestock is our main source of importing and also to fulfill our own need. High amount of biomass of *P. multocida* for vaccine preparation can be successfully achieved by using bioreactor. Biofermenter has the ability to provide optimum environmental conditions for the growth and multiplication of microorganisms being inoculated in it.

The present study was aimed at isolation and biochemical characterization of *P. multocida* vaccine strain (B: 2), to produce biomass of *P.multocida* using biofermenterand to compare the efficacy of biofermenter biomass growth with conventional method of biomass production.

METHODOLOGY

Pasteurella multocida seed and Inoculation

P. multocida B: 2 strain freeze dried culture was obtained from Veterinary Research Institute (VRI) Peshawar in one ml vial. The seed was kept at 4 °C and further processing was carried out at Department of Microbiology Kohat University of Science and Technology, Pakistan.The seed was inoculated into previously sterilized 100 ml of Brain Heart Infusion (BHI) broth and was incubated at 37 °C for 24 hours to achieve bacterial growth from freeze dried culture.Fresh 24 hours growth obtained in BHI was further inoculated in three different agar media includingTrypticase soya agar (TSA), Nutrient Agar (NA) and MacConkey Agar to observe the growth and cultural characteristics of *P.multocida*. All the three media were streaked separately from BHI broth containing *P. multocida* and were incubated at 37 °C for 24 hours.Colonies were observed on these plates. The cultural characteristics were studied and recorded. Morphology was studied using standard Gram staining and Capsular staining procedures.

BIOCHEMICAL TESTS

The catalase test, oxidase test, growth in the absence of CO_2, H_2S Production Test,Nitrate Reduction Test, Motility testwere employed for the biochemical characterization of *Pasteurella multocida* (Cheesbrough 1984).

ANIMAL INOCULATION

Rabbits were used to determine the pathogenicity and re-isolation of *P. multocida*strain. Two healthy rabbits about six month's age were purchased from the local market. These rabbits were kept in cage and were fed ad libidum.

Preparation of inoculum dose

With the help of sterile wire loop few colonies from TSA agar plate were taken and mixed in sterilized normal saline solution (0.9%). 0.1 ml of this bacterial suspension was injected aseptically in the ear vein of one rabbit with the help of one cc insulin sterilized syringe. The other rabbit was injected only with normal saline solution and it was kept as negative control. Both the rabbits were observed for signs and symptoms.

Animal dissection

Death of rabbit was observed in 13 hours post injection. Dissection of the rabbit was performed in sterilized environment (Biosafety Cabinet). A mid line ventral longitudinal incision was given to open the abdomen. Liver, spleen and heart were taken out with the help of sterilized forceps and were stored in sterilized 100 ml beaker. These organs were obtained to reisolate the *P. multocida* strain. The bones were also dissected out and were kept in – 20 ºC for future re-isolation of the pathogen.

ISOLATION FROM HEART, SPLEEN AND LIVER

For proper isolation of bacteria from rabbit three types of Media i.e. Nutrient agar, Trypticase Soya agar (TSA) and Brain Heart Infusion (BHI) agar were prepared. The superficial as well as deep tissue samples were taken with the help of sterile wire loop from heart, spleen and from liver and inoculate each sample aseptically and separately into different media and labeled them. All plates were incubated aerobically at 37 ºC for 24 hours.

Preparation of Stock culture

For maintaining stock culture, 200 ml BHI broth was prepared in a 500 ml graduated flask and autoclaved. Fine colonies of *P.multocida* from TSA agar plate were inoculated in it. All the process was carried out under aseptic condition and incubated it for 24 hours at 37 ºC and it was further checked for dense growth of *P.multocida*. Then the stock culture was kept at 4 ºC for biomass production using conventional method as well as through biofermenter.

BIOMASS PRODUCTION THROUGH CONVENTIONAL METHOD

Preparation of BHI, Plain and Nutrient broth

All the three broth media were prepared (500 ml) each in separate flask according to the standard manufacturer protocol and labeled these flasks properly. From stock culture 10 ml broth was inoculated in each of the three flasks. They were kept in a shaking incubator at increment of 100 rpm at 37 ºC. Spectrophotometer was used for OD calibration at 600 filters, pH meter for pH determination and centrifuge machine for dry mass at 2000 rpm for 10 minutes. Initial OD and pH was checked. After each reading 10 ml sample was regularly examined for OD calibration, dry mass and pH until eight samples were examined and results were noted. The initial sample was not tested for dry mass.

BIOMASS PRODUCTION USING BIOFERMENTER

Sterilization of biofermenter vessel and media preparation

Biofermenter vessel along with acid, base and chilling bottles were autoclaved at 121 ºC for 20 minutes along with special sterilizable pipes. 2.5 Liter BHI broth was prepared in a separate flask and was autoclaved. Then the media was transferred to biofermenter vessel in safety cabinet under aseptic condition through the help of sterilized funnel. All the screws were firmly tight to stop entry of contaminate air.

Parameters setting and taking readings of the biofermenter

All the parameters were set at optimum level. pH was set at 7.2, agitation rate was set at 90 rpm, and dO_2 was set at 1.6 gram/ 100 ml (80 %) and temperature was set at 37 °C. Initial reading was note at zero hour and then after each two hours 10 ml sample was withdrawn with the help of 50 ml syringe. The sample was checked for its OD and microbial dry mass/10 ml. Gram staining procedure was also performed for the four samples in order to check *P.multocida* morphology and other bacterial contamination.

INACTIVATION OF BIOMASS

After the period of 16 hours, the biomass was inactivated by using 0.25% formalin to stop further bacterial growth. For this purpose 4 ml formalin

(absolute) was added in the media flask, mixed well and was allowed to stand for 4-6 hours.

Safety checking

For safety checking, formalin treated killed microbes were once again inoculated on TSA agar plate to see if any growth of formalin treated microbes appears on the media or not.

RESULTS

Pasteurella multocida stock culture was inoculated in three different types of media was performed in order to confirm it's morphological, cultural and biochemical characteristics.

Cultural characteristics

After 24 hours aerobic incubation at 37 ºC on TSA agar showed small non convex rounded and pale colonies which were about 2-3 mm in diameter (Table 1). White small colonies were observed on nutrient agar plates while no growth was observed on MacConkey agar plate.

Morphological identification of *Pasteurella multocida*

Morphological identification of *P.multocida* was performed using Gram staining, Capsular staining.Gram staining showed small rods red to pink color when observed under 100X oil immersion lens.Capsular staining microscopic examination using 100X oil immersion lens showed that a fine capsule was around the cell wall (Table 1).

Biochemical characterization of the stock culture

For confirmation of *P.multocida* B: 2 strain, biochemical tests were performed. *P. multocida* was Catalase positive, Indole positive, Oxidase positive, H_2S positive, Urease negative, Nitrate production positive and non-motile (Table 2).

Animal inoculation

Rabbit was regularly examined after every hour for appearance of symptoms. After eight hour signs of dullness were observed as compared to the healthy one. Death of rabbit was observed after thirteen hours. It was immediately kept at 4 ºC in refrigerator till further use.

Isolation of *P. multocida* from heart, spleen and liver of rabbit on different media

In biosafety cabinet samples from heart, spleen and liver of rabbit were aseptically taken with the help of sterilized platinum loop and were inoculated on TSA, BHI and NA media.

Biochemical and Morphological analysis of the propagated organism

Analytical profile index 20 E (API E20) kit was used to confirm the *P. multocida* reisolated from rabbit. Morphological confirmation of *P. multocida* on Gram staining and Capsular staining showed that these cells were short rods, red to pink in color and a fine capsule was around them (Table 2).

Conventional method used for Biomass production

Three types of broth media were used and results of each were recorded after two hours interval. The first reading of microbial biomass in BHI broth, Plain broth and Nutrient broth was 0.024, 0.017 and 0.02 g/10 ml respectively. Second reading result was 0.056, 0.023 and 0.026 g/10 ml. Third reading was 0.087, 0.045 and 0.053 g/10 ml respectively. Fourth reading was 0.094, 0.064 and 0.067 g/10 ml respectively. Fifth reading was 0.12, 0.077 and 0.083 g/10 g/10 ml respectively. Sixth reading was 0.27, 0.095 and 0.087 g/10 ml. Seventh reading was 0.38, 0.13 and 0.098 g/10 ml. Results showed that BHI medium is the best medium that supports growth and multiplication of *P.multocida* as compared to other media (Table 3).

OD Values When Conventional Media Used

Optical density of each sample was also recorded using 600 Filter. Initial reading from BHI, Plain and Nutrient broth gave 0.303, 0.260 and 0.282 respectively. This reading was considered blank as it contained no microbial growth. Highest OD of BHI, Plain and Nutrient broth was recorded after seventh reading which was 1.577, 0.824 and 1.082 respectively. It was observed that BHI medium is the best medium that gave higher optical density due to more biomass as compared to others.

Dry mass Using biofermenter

*P. multocida*when grown in BHI broth using biofermenter, gave higher growth as compared to the

conventional method. Highest growth/ dry mass were recorded after 18 hours which was 2.6401 g/10 ml. After fourteen hours *P. multocida* dry weight was 2.1329 (Table 4).

OD Values Using biofermenter

Spectrophotometric analysis of each sample showed that a clear increase in OD of each sample occurred with increase in processing time. Initial reading was 0.444 and it was considered zero as it was before stock culture inoculation. Highest reading was obtained after 18 hours which was 3.692.

Comparison of Conventional Method and Biofermenter (Dry Mass)

Biomass produced by conventional method in BHI medium was 0.38 g/10 ml and it was 2.1329 g/10 ml using biofermenter. It clearly indicated that Biofermenter has the ability to produce high biomass as compared to conventional method.

Comparison of Conventional Method and Biofermenter (OD values)

Using conventional method the highest OD obtained was 1.577 while it was 3.368 when used biofermenter. It shows that biofermenter provides an optimal environment for growth and multiplication of *P. multocida*. The process of aeration and agitation along with cooling and heating ensures microbial growth.

Inactivation of *Pasteurella multocida* for HS vaccine

P.multocida biomass obtained in biofermenter was finally inactivated using 0.125% formalin (37% formaldehyde solution) in order to stop further growth after 16 hours. The biomass was then ready to be used in further processing of manufacturing of HS inactivated vaccine.

Table 1:Observations of Cultural and Morphological Characteristics of *P. multocida*

Test Performed	Results	Comments
Colonial morphology	Whitish gray and opaque	On BHI agar plates it shows whitish grey and on TSA plates it shows whitish colonies
Colony diameter	1-3 mm	Colonies diameter were 1-3 mm
Mortality in rabbit	Killed	Mouse killed within 13 hours
Growth on MacConkey agar	No Growth	No growth occur on MacConkey plates
Gram stain	Negative	Pink/ Red short rods with bipolar
Capsule stain	Present	Capsule was observed around cell wall

Table 2:Biochemical properties of the animal isolates of *P.multocida*

Biochemical property	Reaction positive (+ve) negative (–ve)	Inference
Catalase production	+ve	Active bubbling observed
Haemolysis	–ve	No haemolysis on blood agar observed
Hydrogen sulphideprodution	+ve	Blackening of the media observed
Urease production	–ve	Red–pink medium observed
Oxidase Prodution	+ve	Blue color observed
Nitrate reduction	+ve	Red color observed
Motility	–ve	No motility
Growth on MacConkey media	–ve	No growth on MacConkey media observed
Growth in the absence of CO_2	+ve	Luxuriant growth in the absence of CO_2 observed

Table 3:*P. multocida* growth in different media using conventional technique

Time (Hours)	Parameters	BHI	PM	NB
0	pH	7.67	7.34	7.46
	OD	0.303	0.260	0.282
	Dry mass	-----	-----	----
2	pH	6.72	6.97	6.35
	OD	1.082	0.583	0.463
	Dry mass	0.024	0.017	0.02
4	pH	6.5	7.6	7.64
	OD	1.345	0.483	0.586
	Dry mass	0.056	0.023	0.026
6	pH	6.92	7.84	7.92
	OD	1.36	0.47	0.634
	Dry mass	0.087	0.045	0.053
8	pH	6.63	7.85	7.78
	OD	1.414	0.474	0.756
	Dry mass	0.094	0.064	0.067
10	pH	7.00	7.95	7.92
	OD	1.457	0.489	0.863
	Dry mass	0.12	0.077	0.083
12	pH	7.06	8.00	7.98
	OD	1.523	0.511	0.923
	Dry mass	0.27	0.095	0.087
14	pH	7.44	8.24	8.31
	OD	1.577	0.824	1.082
	Dry mass	0.38	0.13	0.098

Table 4: *P.multocida* Biomass production and OD results on biofermenter

Time (hour)	pH	OD/ 600 filter	Dry mass gram/10 ml
0	7.2	0.444	N/T
2	7.2	0.740	0.0857
4	7.2	1.640	0.4918
6	7.2	2.297	0.8130
8	7.2	2.541	1.2434
10	7.2	2.821	1.7243
12	7.2	3.115	1.9795
14	7.2	3.368	2.1329
16	7.2	3.564	2.4914
18	7.2	3.692	2.6401

DISCUSSION

Pasteurella multocida is a gram negative, aerobic, chemoorganotrophic organism that causes hemorrhagic septicemia in cattle and buffaloes and results in serious economic losses every year. *P. multocida* B: 2strains used in the study were obtained from the seed bank VRI Peshawar. The colony morphology was best studied on common laboratory media such as nutrient agar and brain heart infusion agar. Special media such as casein-sucrose-yeast supported abundant growth. The optimum growth temperature was 37 °C after 18-24 hours culture. Similar results have been reported by Coetzer J.A.W. and R.C. Tustin (2004). He stated that BHI and CYS media are the medium of choice as these are enriched and supports *Pasteurella* growth for vaccine preparation.

P. multocida has five serogroups (A, B, D, E and F) and sixteen serotypes. *Pasteurella multocida* B: 2 strain is responsible for causing H.S in buffalos. *P. multocida* was inoculated intravenously in rabbit and death of rabbit observed after about 13 hours, the organism was recovered from its heart, liver and spleen. These samples were inoculated on TSA, BHI and on NA media. Gram's staining and capsule staining method and examined under oil immersion objective for the morphological characteristics of the organism. Biochemical analysis was conducted by using API E20 kit and it corresponded to *P. multocida*with 93% identification. Similar work was also conducted by Atta Husain Shah et al., 2008 they introduce *P. multocida* suspension into mice for recovery of the organism. On death of mice they recovered organism from its heart blood. They performed Gram's staining method for the morphological characteristics of the organism. They cultured heart blood aseptically in brain heart infusion agar (BHIA) and also on the MacConkey agar plates. The pure colonies were transferred on BHI plates and no growth on MacConkey medium. Jabeen et al., (2013) also isolated the *P. multocida* B: 2 from the heart of mice.

Similar study was conducted by Atta Hussain Shah et al., (2008) and Munir et al., (2007). API 20 identification system is an effective and rapid method of biochemical characterization of isolates. They stated that biochemical confirmation provides 93% identification and 96 % were recorded by Jabeen et al., 2013.

For vaccines preparation high biomass is needed in order to make more doses. Biomass was obtained by conventional method and by using biofermenter. Biofermenter has the ability to produce dense biomass as that of conventional method. It has the ability to maintain the environment for microbial growth at its optimum level. OIE Manual for terrestrial vaccines (2008) has also recommended use of biofermenter to achieve maximum bacterial biomass and quality vaccines production. According to the Manual 1.5 gram dry weight are sufficient for making one liter of vaccine.

P. multocida biomass was produced by conventional and biofermenter. Dry weight and OD was compared of conventional method using BHIB, NB and PB. BHI broth gave higher biomass i.e. 0.38 g/10 ml as compared to others. Similarly BHI broth gave highest

OD (1.577) as compared to other media. Dry weight and OD was compared of the conventional and biofermenter methods using BHIB. It was found that biofermenter gave 2.1329 g/10 ml dry mass which was much higher that obtained on conventional method. Similarly biofermenter gave 3.368 OD which was also higher than obtained on conventional method using same media. Similar study was conducted by De Alwis, M.C., (1992), Imtiaz (2001) conducted a study to observe the influence of agitation and stir on the growth of the cultures of *P. multocida*. He demonstrated in his investigation that when the agitation was increased at an increment of 100 rpm (rotation per minute) up to 700 rpm, a steady and slow increase in growth with the increase in agitation was recorded. At 600 rpm and with a vessel volume of one liter, maximum growth occurred, which dropped when agitation of culture rose to 600 rpm onward.

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