INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–0357/2018/20–6–1419–1423
DOI: 10.17957/IJAB/15.0676
http://www.fspublishers.org

Full Length Article

Evaluation of Lipopeptide Biosurfactants Produced from Native Strains of *Bacillus cereus* as Adjuvant in Inactivated Low Pathogenicity Avian Influenza H9N2 Vaccine

Madiha Basit[1], Muhammad Hidayat Rasool[1*], Muhammad Farooque Hassan[2], Mohsin Khurshid[3], Nosheen Aslam[4], Muhammad Farooq Tahir[5], Muhammad Waseem[1] and Bilal Aslam[1]

[1]*Department of Microbiology, Government College University Faisalabad, Pakistan*
[2]*Shaheed Benazir Bhutto University of Veterinary & Animal Sciences, Sakrand, Sindh, Pakistan*
[3]*College of Allied Health Professionals, Directorate of Medical Sciences, Government College University Faisalabad, Pakistan*
[4]*Department of Biochemistry, Government College University, Faisalabad, Pakistan*
[5]*Poultry Research Institute (PRI) Rawalpindi, Pakistan*
[*]For correspondence: drmhrasool@gcuf.edu.pk

Abstract

Biosurfactants were produced from native strains of *Bacillus cereus* isolated from soil samples and characterized as cationic lipopeptide. The cytotoxicity assay was performed in BHK-21 cell line and found safe with 63% cell viability up to of 5 mg/mL concentration. Two types of inactivated vaccines i.e., biosurfactant based and oil adjuvanted were prepared using low pathogenicity AIV-H9N2 virus. Experimental trials were conducted in broiler and layer birds and the humoral immune response was measured through haemagglutination inhibition (HI) test in all groups at different days post vaccination. Results revealed that biosurfactant based vaccine considerably increased the titer of antibodies in both broilers and layers at 14th and 21stdays of vaccination and showed comparable immunogenicity to oil-based vaccine. GMTs were higher and prolonged in layers as compared to broilers. On challenge, 100% protection was achieved in both vaccinated groups. It was concluded that lipopeptide biosurfactants produced from *B. cereus* were safe and immunogenic as an adjuvant in inactivated AIV-H9N2 vaccine and may be recommended as are placement of synthetic adjuvants in future.

Keywords: Biosurfactants; Adjuvant; Avian influenza; Vaccine

Introduction

The poultry sector is playing a significant role in connecting the supply and demand of protein. The commercial poultry farming in Pakistan was initiated during the early 1960s and exhibited rapid growth over the decades and becomes the second largest industry. Poultry production is one of the well organized and most dynamic sectors that contribute much i.e., 1.26, 5.76 and 26.8%, respectively to the overall GDP, agriculture area and meat production in Pakistan (Memon, 2012; Hussain *et al.,* 2015). Despite the efforts, the poultry population has faced several hitches and diseases especially the infectious diseases.

A viral infection caused by Avian Influenza Virus (AIV) has emerged and leads to significant losses in commercial poultry production (Alexander, 2017). In 1998, an outbreak caused by AIV-H9N2 was reported from Abbottabad and Mansehra districts of Northern Pakistan. Later, different outbreaks of highly pathogenic strains of influenza Virus and relatively less pathogenic Avian Influenza strains (H9N2) occurred in different districts of Pakistan. The mortality was 50% and morbidity was 100 percent (Naeem *et al.,* 1999). Due to the continuous spread of AIV-H9N2 among the broiler and layer birds, it has attained significant importance in the recent years. The virus leads to respiratory tract infection in birds at 14 days of age (Iqbal *et al.,* 2009). During production, it also involved in reproductive tract infection in laying birds, thus, affecting the egg quality and hatchability (Rafique *et al.,* 2015).

Consequently, a viral infection can only be controlled by mass vaccination program or bio-security measures. The effective vaccination is a prerequisite to protect the birds from AIV-H9N2. An effective vaccine not only depends on the existence of suitable antigen but also the adjuvants such as mineral compounds, oil based emulsions, immunostimulating complexes (ISCOM) and microbial derivatives to boost the immune response. Moreover, the adjuvants were found to increase the immunogenicity as well as the life of vaccine (Biswas *et al.,* 2008).

To cite this paper: Basit, M., M.H. Rasool, M.F. Hassan, M. Khurshid, N. Aslam, M.F. Tahir, M. Waseem and B. Aslam, 2018. Evaluation of lipopeptide biosurfactants produced from native strains of *Bacillus cereus* as adjuvant in inactivated low pathogenicity avian influenza H9N2 vaccine. *Int. J. Agric. Biol.*, 20: 1419–1423

Economical and effective vaccines which can provide long-term protection against AIV-H9N2 are the need of the hour. Biosurfactants are the secondary metabolites produced by the different microorganism. Among them, lipopeptides are the most effective class of biosurfactants produced by *Bacillus* spp. Lipopeptide biosurfactants enhance the humoral immune response, additionally, they are non-toxic and non-pyrogenic thus, they have prospective application as adjuvants in vaccines. Therefore, the present study was designed to prepare an effective biosurfactant based vaccine using indigenous low pathogenicity AIV-H9N2 virus and to evaluate its immunogenicity in broiler and layer birds in comparison to the conventionaloil-based vaccine.

Materials and Methods

Isolation and Molecular Characterization of *B. cereus*

Three native strains of *Bacillus cereus* named as MMIC1 (MF613976), MMIC2 (MF613977) and MMIC3 (MF613978) were obtained from the soil samples and initially identified by routine microbiological techniques. Further, the molecular characterization was done through amplification of 16s rDNA gene using Polymerase Chain Reaction (PCR). The sequencing was performed by the commercial DNA Sequencing Service of Macrogen (Seoul, South Korea). The obtained sequences of 16s rDNA from these three strains were compared by using the BLAST (NCBI) and were aligned by using ClustalW. The phylogenetic tree was constructed based on maximum likelihood using phylogeny.fr.

Biosurfactant Production and Characterization

Production of biosurfactants from these native strains was performed using the mineral salt medium (MSM) that was supplemented with different agro-industrial wastes i.e. molasses, corn steep liquor, potato peels and waste frying oil (Al-Wahaibi *et al.,* 2014). The growth conditions including pH, temperature and incubation time were optimized for maximum yield. Biosurfactants were extracted from culture supernatant by acid precipitation and chloroform: methanol (2:1) method. Characterization of chemical nature of biosurfactants was done through Biuret test, agar double diffusion test, TLC (Thin Layer Chromatography) FT-IR (Fourier Transform Infrared Spectroscopy) (Makkar *et al.*, 2011; Gudiña *et al.,* 2015).

Cytotoxicity Assay

The MTT colorimetric was used to assess the viability of cells upon the exposure of baby hamster kidney cell line (BHK-21) to the varying concentrations of biosurfactants. Briefly, the deionized water was used to prepare the serial dilutions (2-fold) of biosurfactants (5 mg/mL) and cell viability was determined for each dilution. Any concentration with greater than 50 percent cell survival was considered as safe/non-cytotoxic.

Virus Cultivation and Inactivation

The indigenous low pathogenicity AIV-H9N2 virus was procured from Poultry Research Institute (PRI) Rawalpindi Pakistan and inoculated into a chicken embryo (9 days old) through the allantoic sac and incubated at 37°C in the presence of 70 to 80 % humidity. The allantoic-amniotic fluid was obtained following 48 h of viral inoculation to calculate the egg infectious dose 50 (EID_{50}). The hemagglutination (HA) test was used to confirm the presence of AIV-H9N2, which was further validated by hemagglutination inhibition and the assays for virus neutralization. For the inactivation of virus, the samples were treated with formalin (0.1%) and incubated at 37°C for 2 days. The inactivation of the virus was confirmed by hemagglutination assays following at least three passages in the embryonated eggs (Khalili *et al.,* 2015).

Preparation of Vaccines

In the present study, a total of 2 different types of vaccines were prepared. The biosurfactant based vaccine that was prepared after the desolvation of pure and characterized biosurfactant into the deionized water and further diluted to obtain a concentration of 50%. This solution was further mixed with inactivated viral antigen (10%). The second oil-based vaccine was prepared following the protocol as described in a previous study (Riaz *et al.,* 2017). The sterility testing for the both prepared vaccines was analyzed using various culture media to ensure the absence of bacteria as well as fungi prior to their trials in the birds (Elseify *et al.,* 2017). The stability was tested by storing these vaccines at4°C, as well as room temperature and 37°C for a period of 6 months and the results, were recorded (Riaz *et al.,* 2017).

Evaluation of Vaccines

The immunogenicity of these two vaccines was compared in the poultry birds including the broiler and layers at PRI, Rawalpindi, Pakistan. The standard hygienic conditions were maintained for the housing of birds in addition to the watering, recommended feeding, routinely used medication and vaccination.

The experiments for the evaluation of vaccines were as follows:

Experiment 1: A total of three groups namely A_1, B_1 and C_1were designed for the broiler chickens with each group comprising of 12 birds. The birds included in both A_1 and B_1 groups were subcutaneously vaccinated by injecting with 0.3 mL (10^5 $TCID_{50}$/dose) in the neck of both biosurfactant and oil-based vaccines on the 5^{th} day. The birds included in the C_1 group were not vaccinated and served as non-vaccinated controls.

Experiment 2: A total of thirty-six (36) layer chickens (desi

mixed cross) were obtained from PRI hatchery and were divided into 3 groups (12 bird each) i.e., A_2, B_2,and C_2. On the day 5, the each of the birds in A_2 and B_2groups were subcutaneously vaccinated by injecting biosurfactant based and oil based vaccines in a similar way and dosage as in experiment 1 in the neck region with 0.3 mL (10^5 $TCID_{50}$/dose) of biosurfactant based and oil based vaccines at day 5, respectively. The birds included in theC_2group were not vaccinated and served as non-vaccinated controls.

The blood samples from each bird were collected aseptically prior to the vaccination as well as on 7, 14, 21 28 and 35 days following the vaccination. The serum was separated following centrifugation and was inactivated by using heat treatment and hemagglutination tests were performed. The immunogenicity of both vaccines was evaluated and compared by determining the antibody titers and calculating Geometric Mean Titers (GMTs). Each bird in vaccinated groups was challenged intranasally with a dose of 0.2 mL 10^5 EID_{50} AIV-H9N2 virus at 35-days post-vaccination. Afterward, the birds were observed for clinical signs up to 10 days post-challenge. The swabs from the cloaca and oropharyngeal region were collected at day 1, 5 and 10 following a challenge from all birds and transferred to 2 mL of virus transport medium. Subsequently, samples were inoculated into 9–10 days old SPF chicks, later evaluated for viral growth from the allantoic fluid (OIE, 2012). Data were analyzed by calculating GMTs, mean ± SD. The Duncan's Multiple Range (DMR) test and analysis of variance (ANOVA) were also performed.

Results

Biosurfactants were successfully produced from the 3 native *Bacillus cereus* strains i.e., MMIC1 (MF613976), MMIC2 (MF613977) & MMIC3 (MF613978) which were isolated and characterized from the soil specimens (Fig. 1). These were extracted from culture supernatants as grey-white precipitates by acid precipitation technique and the chloroform-methanol based extraction method. The optimum conditions to produce the biosurfactants were 37°C at the 7^{th} day of incubation at pH 6.5 using 0.5% NaCl. Furthermore, the corn steep liquor was found as the best source of carbon. Chemical nature revealed the peptide bond as detected by Biuret test whereas the presence of lipopeptides was confirmed by TLC. The agar double diffusion technique indicated the cationic properties of biosurfactants. The FT-IR spectra identified the functional groups and indicated that the lipopeptide biosurfactants contains both alkyl and amino groups. The MTT assays have shown the cell survival percentage in the BHK-21 cell line as 63% by using the biosurfactants (5 mg/mL). However, it was increased as the concentration decreased (92% at 0.04 mg/mL).

The sterility testing experiments was shown that the H9N2 biosurfactant based vaccine was sterile and no growth of bacteria or fungi was observed in the various culture media. Moreover, the vaccine was found stable at the different temperature conditions used in this study. In experimental trials, the immunogenicity was comparable to the oil-based vaccine as evident by the antibody titers in both types of birds (broiler and layer) on different days after vaccination.

GMTs in all three groups before vaccination were same i.e., 4 which indicated the maternal antibodies. The GMTs were significantly different $P<0.05$ (16 and 32) in group A_1 and B_1 at day 7 post-vaccination. However, the GMTs were same at 14 and 21 days post-vaccination in both vaccinated groups i.e., (64 and 16, respectively) which showed that biosurfactant based vaccine had immunogenicity comparable to the oil-based vaccine. The GMTs were significantly ($P<0.05$) lower in the non-vaccinated control group than vaccinated groups during the study period (Table 1).

GMTs in all three groups before vaccination were same i.e., 2 which indicated the maternal antibodies. The same trend in the increase of GMTs was observed at day 7 post-vaccination in layers as was seen in broilers. Gradually increase in GMT was seen in both vaccinated groups from day 14 post-vaccination through day 28 post-vaccination. The results revealed that biosurfactant based vaccine was equally immunogenic as was an oil-based vaccine. However, GMTs were significantly ($P<0.05$) lower in un-vaccinated control group throughout the study period (Table 2). The overall comparison between broilers and layers revealed that both vaccinations raised GMTs of antibodies in layers at higher levels and for extended period after vaccination as compared to broilers (Fig. 2). The challenge protection test revealed 100% protection with biosurfactant based as well as oil-based vaccine with no apparent signs and zero lesions.

Discussion

Biosurfactants are biologically produced surfactants that reduce the surface tension or interfacial tension between two liquids or a liquid along with a solid. These are also shown to possess excellent detergent, emulsification, dispersing and foaming properties (Shoeb *et al.,* 2013). The biosurfactants are preferred as compared to the synthetic ones as the lateris derived chemically from the petroleum sources and are generally considered as non-biodegradable. Moreover, the processes for the manufacturing and their by-products are not friendly to the environment. The biosurfactants production got considerable interest that is increasingly being common owing to their diversity, biodegradability, safety and their eco-friendly nature. Further, the production process of biosurfactants is relatively easier to the synthetic surfactants as evident from the various studies (Fakruddin, 2012; Kapadia and Yagnik, 2013).

Table 1: Geometric Mean Titers (GMTs) before and at 7, 14, 21, 28 and 35 days post-vaccination in broilers (Experiment 1)

Group	Days post-vaccination					
	before vaccination	7	14	21	28	35
A_1	4 ± 0.03	16a ± 0.09	64b ± 1.03	16b ± 0.13	8a ± 0.18	4b ± 0.09
B_1	4 ± 0.07	32b ± 0.10	64 b± 1.12	16b ± 0.17	16b ± 1.03	4b ± 0.15
C_1	4 ± 0.05	2c ± 0.15	1c ± 0.06	1c ± 0.02	1c ± 0.09	1c ± 0.03

Each GMT value is Mean ±SD HI antibody titers of 12 birds. Values written a column followed by different lower case letters are significantly different (P<0.05), A_1, biosurfactant based vaccine; B_1, oil based vaccine; C_1, un-vaccinated control

Table 2: Geometric Mean Titers (GMTs) before and at 7, 14, 21, 28 and 35 days post-vaccination in layers (Experiment 2)

Group	Days post-vaccination					
	before vaccination	7	14	21	28	35
A_2	2 ± 0.10	16a ± 0.15	32a ± 1.17	64b ± 1.62	64b ± 1.05	16b ± 1.02
B_2	2 ± 0.12	32b ± 1.06	64b ± 1.24	64b ± 1.30	64b ± 1.26	16b ± 0.90
C_2	2 ± 0.09	1c ± 0.04	1c ± 0.06	1c ± 0.02	1c ± 0.04	1c ± 0.02

Each GMT value is Mean ±SD HI antibody titers of 12 birds. Values written a column followed by different lower case letters are significantly different (P<0.05), A_1, biosurfactant based vaccine; B_1, oil based vaccine; C_1, un-vaccinated control

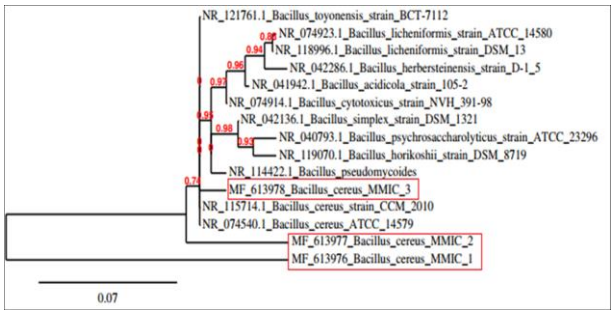

Fig. 1: Phylogenetic tree constructed by Maximum Likelihood based on 16s rRNA gene sequencing

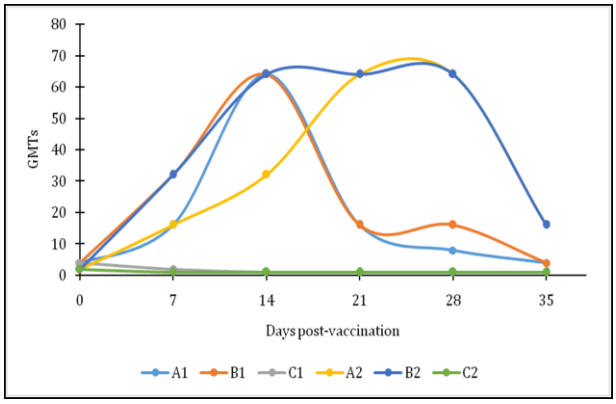

Fig. 2: Comparative immunogenicity of biosurfactant and oil based vaccines in broilers and layers at different days post-vaccination

Broilers (A_1biosurfactant based vaccine; B_1 oil based vaccine; C_1 un-vaccinated control)

Layers (A_2biosurfactant based vaccine; B_2 oil based vaccine; C_2 un-vaccinated control)

The lipopeptides especially have gained significant attention among the biosurfactants, therefore, the present study was aimed to explore the immune-adjuvant property of lipopeptide biosurfactants that were produced from the indigenous *B. cereus* strains.

The AIV-H9N2 is comparatively a less virulent influenza virus strain that is a substantial threat in the zoonotic settings. The effective vaccination of poultry birds is essential to protect the birds from AIV infection. The effective vaccination is not only dependent on the selection of suitable antigens but also the delivery systems for these antigens. The adjuvants are indispensable to boost the immune response and a long-term memory for the particular vaccine. The inactivated AIV vaccines (non-adjuvant) are possibly absorbed from the site of inoculation and are unable to provide appropriate stimuli to the immune cells (Awate *et al.*, 2013). The lipopeptide biosurfactants which were obtained from the *B. cereus* strains and its implications in the vaccination of AIV-H9N2 vaccine as an adjuvant were compared to the oil-based vaccine in the present study among the poultry birds.

In 1stexperiment, the broiler birds vaccinated with the biosurfactant based vaccine (A_1) showed GMT of 32 on the14th day after the vaccination while the oil-based vaccine revealed GMT of 64 at 14th day. The GMT was same i.e. 16 on day 28 after the vaccination using both types of vaccines. Therefore, it is concluded that the immunogenicity of both vaccines was found comparable at different days post-vaccination in broiler birds.

In experiment 2, no significant differences in GMT were observed for the vaccinated and non-vaccinated birds before the start of vaccination; however, the differences were quite significant in both groups at the 7th and 14th days after vaccination. The GMT was increased up to 64 among the birds who received the biosurfactant based vaccine on 21th and 28th following vaccination in that is comparable to the oil-based vaccine. However, the immunogenicity in terms of antibody titers was more pronounced and high titers persisted for a longer period in layers than broilers. Thus, the overall results suggest the efficacy of

biosurfactant based AIV vaccine in the poultry birds for against the AI virus. Various studies have highlighted the potential of biosurfactants in adjuvant for the vaccination of poultry birds. The *B. amyloliquefaciens* WH1 was found to produce the Surfactin lipopeptide that was a potent adjuvant for immunization through oral route (Gao *et al.*, 2013). In a study, the lipopeptides were found to increase the humoral immunity to the tetanus toxoid without a decrease in the serum IgG levels in the mouse model (Mittenbuhler *et al.*, 2003). The lipopeptides are potent adjuvants that can be used for a wide range of antigens. Furthermore, the lipopeptides are degraded into amino acids and fatty acids and do not possess side effects as are not cytotoxic in comparison with the synthetic adjuvants.

Based on the results, it was confirmed that the lipopeptide biosurfactants in the present study isolated from the *B. cereus* strains were stable, non-cytotoxic and therefore safe to use in the poultry industry. Furthermore, their immune-adjuvant potential was very good and comparable to a conventional oil-based vaccine for boosting the humoral immune response of broiler and layer birds against theAIV-H9N2 virus. Due to the additional advantages of biosurfactants over synthetic agents, their role as adjuvant needs to be further explored in other human and veterinary vaccine formulations which may be greatly beneficial to vaccine production industry in near future.

References

Alexander, D.J., 2017. An overview of the epidemiology of avian influenza. *Vaccine*, 25: 5637–5644

Al-Wahaibi, Y.M., S.N. Al-Bahry, A.E. Elshafie, A.S. Al-Bemani, S.J. Joshi and A.K. Al-Bahri, 2014. Screening of Minimal Salt Media for Biosurfactant Production by *Bacillus* spp. *Int. J. Environ. Chem. Ecol. Geol. Geo. Eng.*, 8: 80–85

Awate, S., L.A. Babiuk and G. Mutwiri, 2013. Mechanisms of action of adjuvants. *Front. Immunol.*, 4: 114

Biswas, P.K., J.P. Christensen, S.S.U. Ahmed, H. Barua, A. Das, M.H. Rahman, M. Giasuddin, A.S.M.A. Hannan, M.A. Habib, A. Ahad, A.S.M.S. Rahman, R. Faruque and N.C. Debnath, 2008. Avian influenza outbreaks in chickens, Bangladesh. *Emerg. Infect. Dis.*, 14: 1909–1912

Elseify, R.M., G.F. El-Bagoury, O.G.A. Salman and A.F. Noura, 2017. Efficacy of a tetravalent oil emulsion vaccine against Avian Infectious Bronchitis and Avian Influenza subtype H9N2 viruses. *Life Sci. J.*, 14: 95–101

Fakruddin, M.D., 2012. Biosurfactant: production and application. *J. Petrol. Environ. Biotechnol.*, 3: 1–5

Gao, Z., X. Zhao, S. Lee, J. Li, H. Liao, X. Zhou, J. Wu and G. Qi, 2013. WH1fungin a surfactin cyclic lipopeptide is a novel oral immuno adjuvant. *Vaccine*, 31: 2796–2803

Gudiña, E.J., E.C. Fernandes, A.I. Rodrigues, J.A. Teixeira and L.R. Rodrigues, 2015. Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. *Front. Microbiol.*, 6: 1–7

Hussain, J., I. Rabbani, S. Aslam and H.A. Ahmad, 2015. An overview of poultry industry in Pakistan. *World Poult. Sci. J.*, 71: 689–700

Iqbal, M., T. Yaqub, K. Reddy and J.W. McCauley, 2009. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. *PLoS One*, 4: 5788

Kapadia, S.G. and B.N. Yagnik, 2013. Current trend and potential for microbial biosurfactants. *Asian J. Exp. Biol. Sci.*, 4: 1–8

Khalili, I., R. Ghadimipour, S.S. Eteghad, M.F. Najafi, M.M. Ebrahimi, N. Godsian, Y.S. Heris and M.T. Khalili, 2015. Evaluation of immune response against inactivated avian influenza (H9N2) vaccine, by using chitosan nanoparticles. *Jundishapur J. Microbiol.*, 8: 12

Makkar, R.S., S.S. Cameotra and I.M. Banat, 2011. Advances in utilization of renewable substrates for biosurfactant production. *AMB Express*, 1: 5

Memon, N.A., 2012. Poultry: Country's second-largest industry. *Food J.*, 2: 27–30

Mittenbuhler, K., L. Heinevetter, W.G. Bessler and M. Huber, 2003. Lipopeptides: adjuvanticity in conventional and genetic immunization. *FEMS Immunol. Med. Microbiol.*, 37: 193–200

Naeem, K., A. Ullah, R. Manvell and D.J. Alexander, 1999. Avian influenza: A subtype H9N2 in poultry in Pakistan. *Vet. Rec.*, 145: 560

Office International des Epizooties (OIE), 2012. *In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.*, pp: 259–260, 7th edition. Office International des Epizooties, Paris, France

Rafique, S., N. Siddique, M. Qayyum, M.A. Abbas, A. Ali, S. Yasmeen and K. Naeem, 2015. *In Ovo* vaccination against avian influenza virus subtype H9N2. *Pak. Vet. J.*, 35: 299–302

Riaz, A., M.A. Shah, M.U. Hasan, M. Chaudhry, A. Malik, M.A. Zafar, S.U. Rehman and E. Gawri, 2017. Comparative efficacy of different adjuvant containing inactivated vaccines against low-pathogenicity Avian Influenza H9N2 virus. *Adv. Life Sci.*, 4: 66–71

Shoeb, E., F. Akhlaq, U. Badar, J. Akhter and S. Imtiaz, 2013. Classification and industrial applications of biosurfactants. *Acad. Res. Int.*, 4: 243–252

(Received 07 March 2018; Accept 12-March 2018)