COMPARISON OF GROWTH RATE OF SALMONELLA FOR ANTIGEN PRODUCTION

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ABSTRACT. Salmonella stained antigen has been widely used in Malaysia for detection of *Salmonella* infection in poultry. Growth phase of four *Salmonella enterica* serovar Pullorum (SP 9-25, SP 14/11, SP 690/79 and SP 7107/07) used in the antigen production were investigated based on colony enumeration and turbidity. This study aimed to determine the growth curve and the difference between *S.* Pullorum isolates based on turbidity measurement and spread plate technique for optimisation towards biomass production of salmonella antigen using bioreactor. Current production of the antigen used conventional methods and the number of bacterial cells is low and with several other drawbacks. The isolates were cultured in nutrient broth, incubated aerobically with constant shake for 48 hours to determine the lag, exponential, stationary and the death phase of the bacteria. Turbidity of the bacterial cells was measured using spectrophotometer and the colony was counted using total plate count every four hours. Based on the colony forming unit per milliliter, SP 690/79 strain showed the fastest growth where this bacteria achieved its mid-exponential growth at 8 hours. This is followed by SP 14/11 where this strain demonstrated the mid-exponential growth at 12 hours. The other two strains (SP 9-25 and SP 7107-07) are the slowest growth where their mid-exponential growth was measured at 14 hours. However, SP 690/79 also the fastest strain entering the death phase which demonstrates the difference growth of the *S.* Pullorum strains. This study demonstrates that each *S.* Pullorum strains multiplying and dying at different phase though in the same serovar.

Keywords: Salmonella antigen, *Salmonella* Pullorum, growth, turbidity, spread plate

INTRODUCTION

In general, food of poultry origin can be classed to chicken, duck, quail and eggs while poultry meat is almost entirely contributed by chicken broiler meat. According to Agrofood Statistic (2013), percentage of chicken broiler consumption in Peninsular Malaysia is 96% of the total poultry meat consumed in 2012, followed by ducks 3% and other poultry meat around 1%. Based on 2015 data analysis by DVS (2016) in Peninsular Malaysia, total output of broiler chicken is 161 million birds followed by broiler duck with 56 million and quail with two million. On the other hand, total layer chicken is 56 million while layer duck is one million. In total, the number of chicken

and duck eggs produced in the same year is 11,605.6 million eggs. In achieving high levels of self-sufficiency in poultry, Malaysia exports 33,096 metric tons of poultry meat, 46 million of live fowl and 5 million live ducks in 2015 mostly to Singapore and some countries in the Middle East. In the same year, 1,626.3 million chicken and duck eggs were exported. The total revenue for this poultry export sector in year 2015 was MYR1,546.51 million (DVS, 2016).

However, there are several concerns in the poultry industry such as *Salmonella* contamination and infection. *Salmonella* is an important worldwide pathogen causing fowl typhoid and pullorum disease (Barrow and Neto, 2011). Salmonellosis is able to cause heavy economic losses with 12% morbidity and 75% mortality in broiler at the age of 5 weeks (Haider *et al*., 2014). The worst scenario is that *Salmonella* bacteria can easily infect 1-day-old chicks with as low as 10 cells concentration as they lack mature gut microflora or feed in the alimentary tract (Snoeyenbos *et al.,* 1978). However, the susceptibility of the chicks towards salmonellosis tends to decrease with age (Cosby *et al.,* 2015; Milner and Shaffer, 1952).

There are several methods in monitoring or conducting surveillance of *Salmonella* infection in poultry farms. In 2009-2010, Barua *et al.* (2012) conducted a prevalence study on *Salmonella* in commercial layer poultry farms in Bangladesh using faeces samples. Samples were inoculated into enrichment medium Rappaport-Vassiliadis (RV), followed by streaking on XLD agar plate and finalised with biochemical test. However this method is time consuming and laborious as the process required several days to obtain the result. In year 2011 to 2013, *Salmonella* prevalence of poultry in Malaysia is 11.9% (Ong *et al.,* 2014). As the public health issue of salmonellosis is relatively common in Malaysia, it has been one of the necessities for the Department of Veterinary Services (DVS) with the cooperation of farmers to control the disease. Singapore as one of the main countries importing poultry products from Malaysia has issued a policy demanding Salmonella-free certification of the exported poultry products (Republic of Singapore, 2017). Thus, one of the initiatives to control *Salmonella* infection taken by the DVS is by introducing a rapid test kit salmonella antigen into the market in Malaysia. Compared to the *Salmonella* detection using enrichment medium, this rapid test kit requires just a drop of serum or blood sample with results in less than a minute.

Salmonella antigen is a well-known biological product used to detect the presence of *Salmonella* using antigen-antibody binding mechanism. However, current issues in producing salmonella antigen is the conventional production methods with several drawbacks. It is time consuming, with low yield and heavy workload. With poultry production and exports increasing steadily since the 1990s, it is essential for the DVS to improve the production of salmonella antigen. This study to determine the growth pattern of the *Salmonella* is the initial step for a biomass production of the antigen in a bioreactor. One of the factors for biomass production is to determine the optimum time to inoculate the starter culture into the bioreactor. As stated by Zhenqiang Xia and

Wu, (2012), the inoculum age is one of the important variables for the fermentation optimisation process. Based on the study, it is suggested to inoculate the bacteria at its mid-exponential phase to reduce the lag phase time; which consequently will reduce the production time of the bacteria. The other factor is to harvest the bacterial cells before it starts dying to maximise cell production. The stationary phase is the time when growth and death is at an equal rate (Al-Qadiri *et al.,* 2008). Thus, it is essential to know the early stationary phase of the bacteria to maximise the production. The aim of this study is to determine the mid-exponential phase and the early stationary phase time of the *Salmonella* as well as observing the differential growth which is useful in optimising biomass production of the salmonella antigen.

MATERIAL AND METHOD

Microorganism source

Four *Salmonella* Pullorum used in this study were obtained from the biobank in Veterinary Research Institute (VRI), Malaysia. The *Salmonella* Pullorum was isolated from different years of poultry flocks and identified by biochemical tests and serotyping. After confirmation of the species, the strains were used in the production of a rapid test kit of salmonella antigen.

Cultivation method

All strains were grown from stocks maintained at -80 °C in 20% glycerol diluted in brain heart infusion (BHi) with one-to-one ratio. Stocks were defrosted, sub-cultured on blood and McConkey agar. Bacteria was identified using gram stain followed by biochemical tests including oxidase, triple sugar iron (TSI), catalase, indole-methyl red-Voges-Proskauer-citrate (IMViC), motility, urease, malonate broth and control-ornithine-lysine-arginine (COLA). Upon identification of *Salmonella* Pullorum, a single colony was picked up from the blood agar and sub-cultured onto the nutrient agar. On the next day, one to three colonies were chosen and inoculated into 10 ml nutrient broth as preparation for the starter inoculum. All of the cultured bacteria were incubated at 37 °C for 24 hours. The starter inoculum was then inoculated into 200 ml nutrient broth on the following day and incubated on a shaker at 37 °C at 250 rpm.

Bacterial concentration measuring method

The turbidity of 200 ml culture was measured using Eppendorf Biophotometer (Germany) at 600 nm and bacterial spread was plated. Both tests started from 0 hour for 48 hours where sampling was at every 4-hours intervals (Maier *et al.*, 2009). At the initial 4 hours of bacterial growth, the dilution was at 10^5. Spread plate was performed using 10^3, 10^4 and 10^5 dilution and then incubated at 37 °C for 24 hours. In the next following hours, the dilution was until 10^7 where the spread plate was using 10^5, 10^6 and 10^7. The plate was then incubated for 24 hours at the same temperature. The colonies formed were counted using Gallenkamp Colony Counter (United Kingdom). Colonies of more than 250 was considered too numerous to

count (TNTC) and 25 is the lower limit of the countable range of a colony-forming unit (Sutton, 2011). The data obtained was plotted as shown in Figures 1 to 4.

RESULTS AND DISCUSSION

Results were plotted based on the total plate count and OD reading at 600nm for each strain. The entire graph plotted was against hours (h) from 0 until 48 hours for the x-axis. The left y-axis is Log_{10} (cfu/ml) while the y-axis on the right side is OD reading at 600 nm. Based on the figures presented, red line indicated the graph of Log_{10} (cfu/ml) while green line is the OD reading of the bacteria. The results are as in Table 1.

In this observational study, comparison of bacterial growth was made between colony counting method and measurement of light transmission using spectrophotometer as described by Maier *et al.* (2009). A complete bacterial growth curve will include lag phase, exponential phase, stationary phase and decline phase (death) which may extend to several days (Paulton, 1991). Generally, every graph [Log_{10} (cfu/ml) and OD reading) that were plotted showed almost similar starting points of exponential phase which was around 4 hours. This is considered as normal situation for bacteria growth as the lag phase is the physiological adaptation phase of the cell to the culture condition which may require time for induction of specific messenger RNA (mRNA) and protein synthesis to meet new culture requirement (Maier, 2009). The transition from the lag to exponential phase is also the stage of doubling process of the cells (Yates and Smotzer, 2007) which makes all of the graph plotted exponentially increase after the lag phase.

Based on Figure 1, SP 690/79 seems to enter the decline phase (death) rapidly after reaching its maximum growth. This can be observed on both colony forming unit and turbidity graphs. Graphs showed instant decline after 16 hours for the colony forming unit, while based on turbidity, it start to decline after 28 hours. As a consequence of bacterial metabolism, cultures will accumulate waste products. Eventually this situation will lead to death phase where the number of viable cells declined exponentially, reversing the pattern of growth during exponential phase (Finkel, 2006; Navarro *et al.,* 2010). This indicates that SP 690/79 strain die at faster pace compared to the other strains.

Figure 2 showed exponential phase from 4 hours to 16 hours based on colony forming unit and the stationary phase

Table 1. Table shows simplified results based on the graphs plotted in Figures 1 to 4.

Isolates	Mid-Exponential Phase (hours)	Mid Exponential Phase Log_{10} cfu/ml	Mid-Exponential Phase OD 600 nm
SP 690/79	8	7.505	0.075
SP 14/11	12	8.711	0.285
SP 9-25	14	9.359	0.340
SP 7107/07	14	8.665	0.225

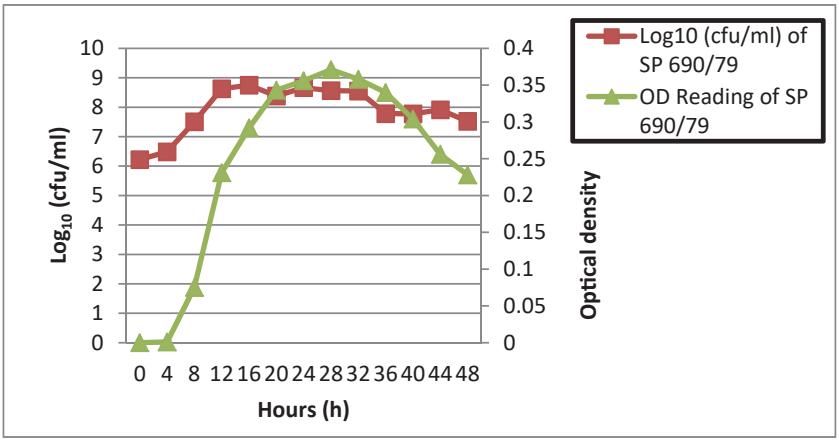

Figure 1. The graph shows growth of SP 690/79 plotted based on total plate count (red line) and OD reading (green line) against 48 hours. Based on the total plate count, the exponential phase was identified starting at 4 hours until 12 hours and the stationary phase start from 12 hours until 32 hours. 32 hours onwards, the graph shows downturn trend until 48 hours. The mid exponential phase was spotted at 8 hours of the growth time. On the other hand, based on the OD reading, the exponential phase was identified at 6 hours until 28 hours and the graph shows downturn trending for the consequence hours. Thus, it was spotted that the mid exponential phase is at 17 hours. At the highest colony population of SP 690/79, the spectrophotometer reading at wavelength 600nm is 0.292.

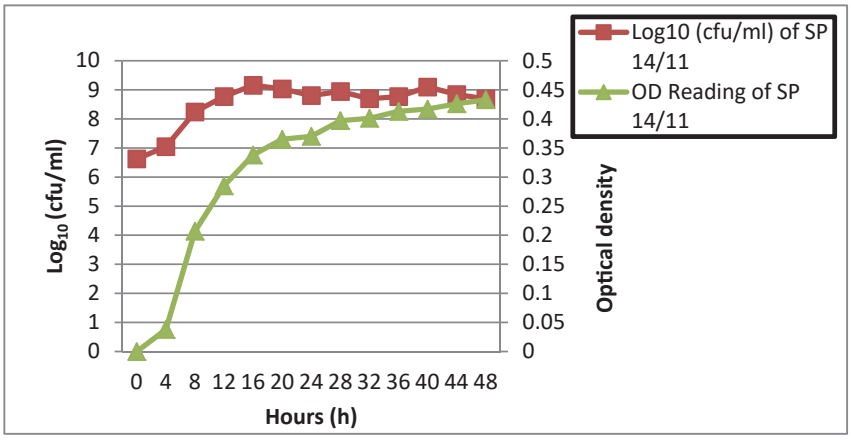

Figure 2. Graph shows growth of SP 14/11 plotted based on total plate count and OD reading for 48 hours. Based on the total plate count, exponential phase was identified starting at 4 hours until 16 hours and the stationary phase start from 16 hours onwards. The mid exponential phase was identified at 10 hours. The exponential phase for OD reading start at the same time which is at 4 hours and the graph shows uptrend for the consequence hours until 48 hours. The mid exponential phase could not be identified in the graph. At maximum population of SP 14/11 which is at 16 hours, the turbidity reading is 0.338.

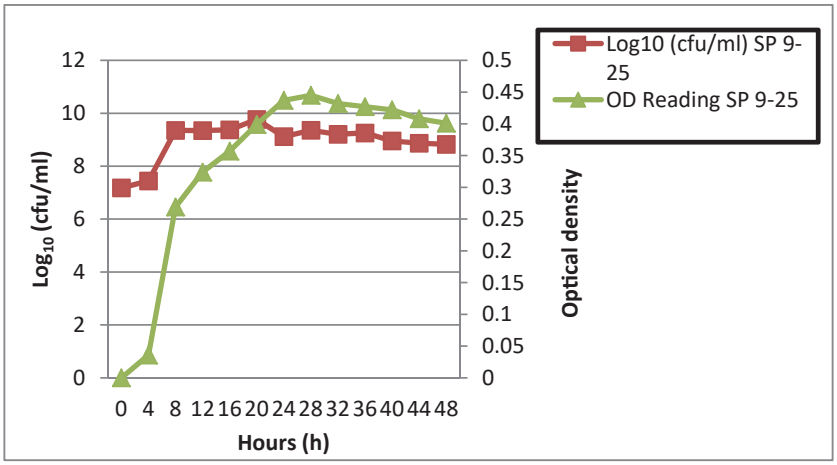

Figure 3. The graph shows the growth of SP 9-25 plotted based on total plate count (red line) and OD reading (green line) for 48 hours. Based on the total plate count, the exponential phase was identified at 4 hours until 20 hours and the stationary phase start from 20 hours onwards. Thus the mid exponential phase was spotted at 14 hours. The exponential phase based on optical density was identified at 4 hours until 28 hours and the graph shows downturn trending for the consequence hours. The mid exponential phase was at 16 hours of the growth. The highest cell number Log10 (cfu/ml) is at 20 hours with value 9.676 and the turbidity reading is 0.399.

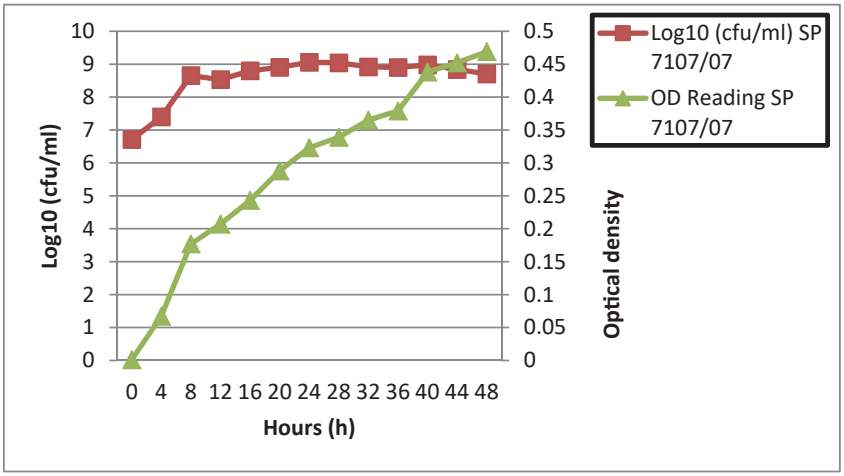

Figure 4. The figure shows growth of SP 7107/07 plotted based on total plate count (red line) and OD reading (green line) for 48 hours. Both graphs show the exponential phase start at 4 hours and for total plate count, the exponential phase continues until 24 hours. However, based on OD reading it is uptrend pattern until 48 hours and the mid exponential phase could not be identified based on the graph. The mid exponential phase based on total plate count is at the 14 hours. The turbidity reading is 0.323 when SP 7107/07 reached maximum population which is at 24 hours.

extend onwards to more than 48 hours with the final cfu/ml taken 10^8. However, based on the optical density, the exponential phase start at the same time but it uptrends until 48 hours incubation. This SP 14/11 strain is expected to die at a slower pace compared to SP 9-25, and at the same time the bacteria kept multiplying at a higher pace, increasing OD although the population is static. The balance between dying and multiplying cells provides a dynamic equilibrium where the final output is the stable viability of the population (Navarro *et al.,* 2010). This situation is where the essential nutrient was completely used up but the cells may continue replicating. This situation may occur because of lysis of the dying cells which provide nutrient to others, called endogenous metabolism (Dawes & Ribbons, 1962).

In Figure 3, growth of SP 9-25 based on colony form and OD reading showed a parallel graph where both graphs started to decline but at slower pace that can still be sustained at 10^9 populations after 20 and 24 hours respectively. It seems bacteria dying is higher compared to bacteria replicating at slower pace, shown in the declining graph. The stationary phase is the stage where there is no longer a net increase in viable bacterial cell numbers and cellular metabolic activity is decreased. This means that the growth rate is equal to the death rate (Akerlund *et al.,* 1995; Al-Qadiri *et al.,* 2008).

Lastly, SP 7107/07 in Figure 4 showed that the graph pattern is almost the same as SP 14/11 in Figure 2. However, the rate of cell multiplication seems faster compared to SP 14/11 as the turbidity graph is steeper than in Figure 2. The turbidity graph shows a steady increase after the culture reached maximum population at 16 hours.

CONCLUSION

In conclusion, both methods of measuring in terms of turbidity and colony counting were reliable in plotting the growth of bacteria. Turbidity measurement shows real time growth and colony formation validate the result on the next day. However, increase OD reading does not indicate increase in amount of bacterial cells. Generally, SP 7107 and SP 14/11 were able to stabilise its population at stationary phase after 48 hours while SP 9-25 showed a slow declining population but still maintained at 10^9 until 48 hours incubation. On the other hand, SP 690/79 is the fastest strain entering the death phase instantly after 16 hours at stationary phase. Based on the results obtained, the mid-exponential phase and early stationary phase was obtained for each strain used. This observational study also shows that different strains of *Salmonella* Pullorum grow at differing periods of time though with the same parameters. Different periods of lag phase, time needed to reach stationary phase and the total number of cells in the population can be observed in this study. It is essential for the researchers to observe the growth timing of their bacterial culture for an optimum bacterial cells production.

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