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Production and Kinetics of *Salmonella enterica serovar enteritidis* in Vibrofermentor

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Abstract: Food-borne human Salmonellosis caused by *Salmonella enterica serovar enteritidis* has given a rise to many of economic losses in terms of both poultry and food industries. *Salmonella* has a great importance among the major bacterial pathogens of poultry. In Turkey as well as all over the world prevention of *Salmonella* infection can be achieved by good monitoring and screening programs. More recently immunological test systems are used for diagnosis based on the detection of surface antigens such as lipopolysaccharide (LPS), flagellin etc. As to its production some different methods are used practically. In this work, the lab-scale production of *Salmonella enterica serovar enteritidis* was achieved both in shake-flask and vibrofermentor cultures and a comparative optimization of the process yield and production kinetics was carried out. The microorganisms were cultivated in brain heart infusion broth, at 37°C for 5 h. Specific growth rates and doubling times for both vibrofermentör and shake-flask were estimated as 0.509 and 0.709 h^{-1} and 75.0 and 58.6 min, respectively. LPS and flagella were extracted from lyophilized cultures.

Key words: *Salmonella enterica serovar enteritidis*, vibrofermentor, kinetic modelling

INTRODUCTION

Today, one of the most important problems affecting the poultry industry is the increasing incidence of food-borne human *Salmonellosis* caused by *Salmonella enterica serovar enteritidis* associated with eggs and other poultry products (Carli *et al*., 2001; Keller *et al*., 1993). *Salmonella* infections cause considerable economic losses directly from mortality and poor growth after clinical disease and indirectly from animal carriage and food processing leading to cases of *Salmonella* infections. In many countries, control policies have been introduced with the aim of reducing the prevalence of *Salmonellosis* in poultry and farm animals in order to decrease the outbreaks of human infections. Such control policies are generally based on screening flocks and herds by periodic bacteriological examinations that are laborious and time-consuming and may yield false negative results (Malik *et al*., 2002). More recently immunological methods based on the detection of surface antigens such as lipopolysaccharide (LPS), flagellin and outer membrane proteins have been developed (Ibrahim *et al*., 1985; Iankov *et al*., 2000; Keller *et al*., 1993). Therefore, reliable serological assays employing polyclonal or monoclonal antibodies for use in diagnosis are required (Malik *et al*., 2002). Monoclonal antibodies (MAbs) have great potential for identifying, isolating and purifying bacterial antigens and also in defining the role of specific antigens in diagnosis, classification and immunity to infection. They may also facilitate serotyping and strain differentiation (Malik *et al*., 2002). *Salmonella* are gram negative, non-sporing, sitochrome oxidase negative, usually 1-3 μm long and 0.5-0.7 μm broad and with few exceptions such as *Salmonella enterica serovar Gallinarum*, all *Salmonella* species are motile by peritrichate flagella. It can be easily cultivable on ordinary media aerobically and facultative anaerobicaly (Wilson and Miles, 1955).

Recently, *Salmonella* become among the major bacterial pathogens of poultry in Turkey as well as all over the world. Prevention of *Salmonella* infection has great importance both for poultry health and for the food-industry and it can be achieved only by good monitoring and effective screening programs (Carli *et al*., 2001).

There is a little information about the production of the strain in bioreactors, as well as growth conditions, growth kinetics etc. The strain is used in many companies for the production of test reagents and vaccine. Compared to shake-flask and large scale production of *Salmonella enterica serovar enteritidis* using bioreactor has many advantages: controlling the production parameters like nutrient composition, temperature, pH, aeration, mixing, providing identical and synchronized growth cultures etc. This way is also very useful for isolation of antigenic structure which gives to high yield of exact antigen (Mcneil and Harvey, 1990; www.nbsc.com; Girard *et al*., 1973).

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This study describes the production criteria of *Salmonella enterica serovar Enteritidis* in shake-flask and vibrofermentor systems. The culture produced in vibrofermentor were then used for isolation of LPS and flagella. To our knowledge, this is the first study about in production of *Salmonella enterica serovar enteritidis* in vibrofermentor.

MATERIALS AND METHODS

Strains used: *Salmonella enterica serovar enteritidis* 64K strain was provided kindly by Dr. Tayfun Carli from Uludag University, Faculty of Veterinary Medicine, Bursa, Turkey and stored on slant nutrient agar at +4°C. Cultures were maintained by three replicating subcultures through a single colony to enhance the purity, identity and homogeneity of the bacteria. Those criteria were also checked by Gram stain technique. Streaked single colony was used for stock culture. Before use, fresh BHIB (brain heart infusion broth) was inoculated with a loop-full of stored culture and incubated overnight at 37°C.

Culture medium: The bacteria were grown in BHIB (Becton Dickson, UK) at pH 7.4±0.2 both in shake-flasks and bioreactor. Medium volumes in each shake-flask and in vibrofermentor were 100 mL and 2 L, respectively. The growth medium containing vibrofermentor and shake-flasks were sterilized in an autoclave at 121°C for 45 min and 121°C for 20 min, respectively.

Inoculum: The inoculum was prepared with BHI broth and cultivated overnight in shaker at 37°C and at 100 stroke min^{-1}. The shake-flasks and vibrofermentor were then inoculated at 3% level.

Operation conditions: The vibrofermentor (Chemap AG, Switzerland) has 4 L vessel with 2 L working volume capacity and it was equipped with doubled jacket configurations. The aeration was performed by a diyaphragm pump (Gılmont, USA) and air flow was adjusted to 0.15 vvm. Mixing rate by vibration was amplitude of 180 volts. The flasks were incubated in shake flask at 37°C, 200 rpm.

The following parameters were used in the system: Temperature: 37°C initial pH of the culture medium: 7.4±0.2 and free during production; optical density: measuring at 545 nm (Jenway, UK), cell density determination: in BHI agar as cfu mL^{-1} by pour plate method.

Isolation of LPS and flagella: Lyophilized cells of 1 g were extracted with modified hot-water phenol method (Skultety and Toman, 1998). After the LPS isolation, the quantity of neutral sugar was determined by GC/MS (Q5 5000-GC-17A, column; SP 2330, Supelco, Sigma). Flagella were extracted as described by Ivanova (1986). Flagella extract purified on Sephadex G 200 column was checked by Lowry method for protein contents.

Kinetics: The specific growth rate was calculated from the slope of the line obtained from time-cell concentration graph.

$$\text{Log}_{10}\ \text{cfu mL}^{-1} = A \cdot \mu + B$$

The same graphic can be lined by absorbance values instead of living cell concentration.

Monod kinetics equation as being the simplest one was choosen mathematically to state the microbial kinetics.

$$\mu = \mu_{max}\ S/(S+K_S)$$

K_S values were calculated numerically by trial and error method with the error of smaller than 0.01. Excel 2000 was used as the software for the solution of the equation. The function of the problem to be solved is f(x) and the roots of the function are $x_1 = \mu_{max}$, x_2 = S and $x_3 = K_s$ (Bailey and Ollis, 1986; Kieran and Berovic, 2001; Uysal, 1999).

$$\min_x f(x) \quad \text{roots } x_{1} > x_{2} > x_{3}$$

RESULTS AND DISCUSSION

The optical density and the logarithmic of total viable cell count (cfu mL^{-1}) for both shake flask and the vibrofermentor are shown in Fig. 1 and 2. Estimated specific growth rates and doubling time are also shown in Fig. 1 and 2. The preliminary experiments revealed that

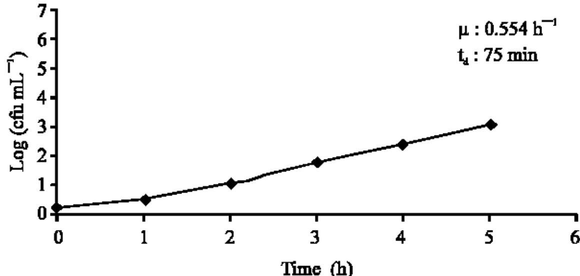

Fig. 1: Growth curve of the microorganisms in 100 mL Erlenmeyer flasks at 200 rpm [Data given are the mean values obtained from four experiments Log (cfu $mL^{-1} \times 10^{-9}$)]

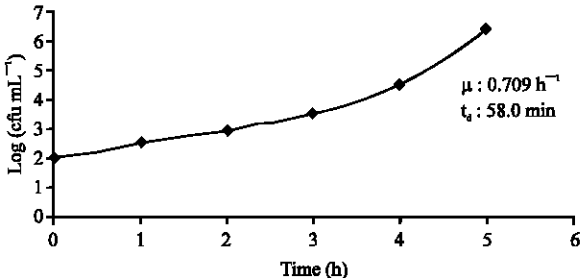

Fig. 2: Growth curve of the microorganisms in the vibrofermentor conditions [Data given are the mean values obtained from four experiments Log (cfu $mL^{-1} \times 10^{-9}$)]

there was no lag phase in this particular microorganism under these conditions. Therefore, the run was conducted at the end of logarithmic phase for total of 5 h and the figures show only logarithmic phase of growth curve of *Salmonella enterica serovar enteritidis* 64K since

After LPS extraction of 1 g lyophilized cells, 60 mg pure LPS was obtained. The neutral sugar contents in 1 mg of LPS determined by GC/MS were as follows: 24.74 μg rhamnose, 4.34 μg rhibose, 59.61 μg mannose, 251.88 μg galactose, 309.66 μg glucose and traces of tyvelose. The lyophilized culture was used for flagella extraction. Purified flagella protein content was 300 μg mL^{-1} determined by Lowry method.

Kinetics: Calculated specific growth rate was 0.709 h^{-1} for the vibrofermentor. Maximum and minimum range of μ_{max}, S and K_S in Monod equation were chosen as 0.25-5 (h) for optimization.

The error in the minimization for calculating of μ_{max}, S and K_s μ was accepted as being smaller than 0.01 and their calculated values by trial and error method are as follows :

$$\mu_{max} : 0.75\ h^{-1},\ S : 0.7\ g\ kg^{-1}\ \text{and}\ K_s : 0.5\ g\ kg^{-1}$$

In this study, the lab-scale production of *Salmonella enterica serovar enteritidis* 64K was performed in both shake-flask and vibrofermentor using by BHI broth at 37°C for 5 h. Then, same kinetic parameters of both systems were calculated. The determined specific growth rates and doubling times were 0.554 h^{-1} and 75.0 min for the shake-flask and 0.7088 h^{-1} and 58.6 min for the vibrofermentor. The comparison of the specific growth rates of the bacteria calculated from the shake-flask and the vibrofermentor showed that the latter one had greater values than the other one. It is considered that the vibration improves the effect of mixing according to that of impeller. Besides, high growth rate due to the quality of the product is also important. Since high initial inoculum concentration affects the growth in vibrofermentor, the inoculum ratio in this particular bioreactor system should be optimized for further experiments. The neutral sugar quantities of LPS and the final flagella amount were also determined. In comparison to the previous study of Weinbaum *et al*. (1971) the same sugars; rhamnose, mannose, galactose and were similarly found in the extracted LPS from bacteria that grown in vibrofermentor. As given by a reference study (Peter *et al*., 1999) the immunodominant sugar tyvelose that determines the seravor designation for group DI Salmonella upon derivatization and thus not used to measure O-chain by GC in this study. On the other hand, the agglutination ratio of cells in O-serum was higher than that of given by Weinbaum *et al*. (1971). These results indicate that under certain cultivation conditions, microorganisms performing rough (R)-like phenotype. Since smooth (S)-form bacteria are more antigenic, if the aim is antigen production, the nutritive value of the media could be decreased. Moreover, instead of BHIB beef extract broth can be suggested and optimization of the process parameters could be directed to this point.

Besides, LPS quality flagella quantity and their structure are also important in antigen production. In this study the quantity of final flagella was lower than the expected values which indicate the occurrence of some flagella destructions during high vibration. However the estimated quantity of flagella was still sufficient for antigen preparation. Since *Salmonella enterica serovar enteritidis* is a pathogenic microorganism, so me other strains having the similar antigenic structure such as *Salmonella enterica serovar gallinarum* and *Salmonella enterica serovar adeyo* could be used for LPS and flagella production.

As final step, accepting the kinetic data to fit Monod kinetic model, the Monod constants were calculated by trial and error method. The constants and maximum specific growth rate according to this model were (S, K_s μ_{max}) 0.7 and 0.5 g kg^{-1} and 0.75 h^{-1}, respectively. Monod kinetic model chosen by this study is based on the expression of specific growth rate for the vibrofermentor. The comparison of the results for specific growth rates in vibrofermentor obtained during this study was not able to be compared with similar works due to the lack of the information in the literature. However, quadratic polynomial models were used to state the cell growth curves for *E. coli* strain BB4 to produce antigen K88 as described by Wong *et al*. (2002).

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NOMENCLATURE

cfu = Colony forming unit (cfu mL^{-1})
K_s = Monod constant
μ = Specific growth rate (h^{-1})
μ_{max} = Maximum specific growth rate (h^{-1})
OD = Optical density
S = Substrate concentration (g kg^{-1})
Td = Doubling time (h)

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