Original Research

Biofilm Formation Of *Staphylococcus Aureus* In Presence Of Sodium Chloride, Ethanol And Ph Different Levels And Application Of Artificial Neural Networks To Describe The Combined Effect Of Them

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Abstract

Background: Some microorganisms have the ability to connect to surfaces and produce biofilms. Bacterial biofilms are a major problem in the food and medical industry. Bacteria in biofilms have higher resistance to environmental adverse conditions and antibiotics than planktonic bacteria. Staphylococcus aureus is a food borne pathogen can form biofilms.

Method: In this research, the main objective of the study was to investigate the effect of three parameters, pH, sodium chloride concentration and ethanol concentration on S.aureus ATCC 33591 biofilm formation after 24 and 48 hours' incubation times (37 °C) by microtiter plate method, furthermore modeling the results with artificial neural network (ANN). For this intention, after both incubation times, the effect of all parameters (separately), and the combined effect of all parameters, it was deliberated.

Results: Results were modeled using ANN. several ANN were compared in terms of MSE and R value. The results showed the strongest biofilm was formed in neutral pH. Increasing the Sodium chloride and ethanol stimulated the biofilm formation, but high concentrations of Sodium chloride and ethanol and highly alkaline or very acidic pH levels had the inhibitory effects. In addition, the biofilm formation increased in more incubation time. Eventually, a kind of multilayer ANN (Feed-Forward Back-Propagation) model with Levenberg–Marquardt (LM) training algorithm was chosen. The topology of this ANN was 4-12-1 with validation MSE=0.0102 and R value=0.989. There was a very high correlation between modeling data and experimental data.

Conclusion: The biofilm formation of S.aureus is affected by Sodium chloride, ethanol, pH and time and the ANN was able to model these parameters with nonlinear relationships.

Keywords: Biofilm; sodium chloride concentration; Ethanol concentration; pH; Artificial Neural Network.

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1. Introduction

Bacterial biofilm is the result of bacterial adhesion to living or non-living surfaces so, it can be found at all surfaces in the nature, medicine and industry (1-3). The first definition of biofilm was presented in 1999 by Costerton, Stewart, and Greenberg (4). In a general definition, adherent microbial cells in a self - produced extracellular polymeric substance (EPS) is called biofilm (4-8). The expansion of biofilm involves four stages, (i) attachment, (ii) micro colony formation, (iii) maturation and (iv) detachment (9-11).

The bacterial cells growing in a biofilm are different from their planktonic counterparts. Therefore, they are more resistant to antimicrobial agents and can tolerate adverse environmental conditions (6, 7, 12, 13). The extracellular polymeric matrix of biofilm as a powerful defensive barrier prevents antimicrobial agents' penetration into biofilms (14, 15). Food pathogenic bacteria, such as *Staphylococcus epidermidis*, *S.aureus*, *Pseudomonas aeruginosa*, proliferate on food processing equipment and form biofilms which provide them with up to 1000 times more effective resistance and tolerance to antibiotics in comparison with their planktonic form. So, it can lead to greater resistance (12, 16-19).

Biofilm formation in the food industry is a major problem and one of the most important causes of secondary contamination (14, 20-22). However, if pathogens exist in biofilms, the problem becomes twofold; because, it leads to the prevalence of food poisoning in the community (6, 23, 24). To date, it has been proven that more than 80% of microbial contaminants in food processing equipment and raw materials are due to the presence of biofilms (10). Microbial food borne diseases are global concern so biofilms that harbor more than one drug tolerant bacterial species, are also a global concern (13, 25, 26).

Staphylococcus aureus, an important food borne pathogen, is a gram positive round shaped bacterium with the ability to form biofilms on different surfaces of foods and food processing plants such as dairy environments, egg, meat products, and fishery products (20, 27-29). In most cases, *Staphylococcal* biofilms lead to gastroenteritis and food poisoning outbreaks due to consumption of raw or processed foods (3, 30, 31).

Since biofilms are resistant to disinfectants, it is very difficult to remove them from surfaces (32, 33). For this reason, the presence of biofilms on food contact surfaces such as glass, stainless steel and etc. is also a serious danger to the health of consumers (34, 35).

Researches have shown that biofilm formation is a multi-stage mechanism which responds to environmental and biochemical factors (36). For example, Korem et al., (2010) announced that in some strains of *S.aureus*, there is a phenomenon called "microbial lymphatic hemolysis" which means that the alcohol increases the hemolytic properties of them (19). This, in turn, increases the formation of biofilms (36). Ganchev (2019) showed that pH is one of the effective environmental factors on biofilm formation ability of bacteria, so that at pH range of 5 - 6, the highest biofilms were formed by *Bacillus subtilis* and *Escherichia coli* bacteria (37). Planchon et al., (2006) stated that the addition of sodium chloride to Tryptic Soy Broth (TSB) would lead to an increase in bacterial growth and survival (38).

The anticipated microbiology is the result of the integration of traditional microbiology knowledge with technological systems and information, statistics and other disciplines of mathematics. Predictive microbiology by finding mathematical equations will be able to describe the behavior of microorganisms under different environmental conditions, whether competitive, chemical or physical (39, 40). Artificial intelligence especially artificial neural network (ANN) is one of the most usable tools of anticipated microbiology; because it is

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capable of modeling nonlinear behaviors (41). The artificial neural network is inspired by the human neural network. The network consists of three layers. Each layer has a number of neurons or nodes, and each node represents one data. The input data is entered into the first layer and then processed in the second layer. Finally, the result as output data is obtained in the last layer (42, 43). Multilayer perceptron neural network (MLPNN) is one of the most widely used ANNs. In the meantime, Feed-forward backpropagation neural network (FFBPNN) is the most widely used MLP. Therefore, in this study, the effect of three environmental factors such as pH, sodium chloride and ethanol concentrations on the biofilm formation of *S.aureus* have been studied.

2. Material and methods

2.1. Bacterial strains

S.aureus ATCC 33591, was purchased from the Persian Type Culture Collection and used for biofilm formation. The stock culture of *S.aureus* was stored frozen at -80°C. In order to activate the bacterial strain, TSB (Merck, Germany) culture medium was used and incubated at 37°C/24-h. For purification, the overnight culture of bacterium was then streaked on the Tryptic Soy Agar (TSA; Merck, Germany) and incubated at 37ºC/24-h (36, 44).

2.2. Experimental conditions

The overall framework of this study was that the effect of three parameters, pH, sodium chloride and ethanol concentrations on the *S.aureus* biofilm formation was investigated. To ensure that the pH, sodium chloride and ethanol concentrations of prepared TSB were not altered during sterilization, they were checked before and after autoclaving.

2.2.1. Sodium chloride concentrations

The TSB media supplemented with sodium chloride (NaCl, Merck, Germany) concentrations from 0.5% to 20% (w/v) in step of 0.5% were prepared to study the effect of sodium chloride concentration on the biofilm formation of *S.aureus*. Each treatment was tested with 8 replicates.

2.2.2. Ethanol concentrations

To investigate the effect of ethanol (Merck, Germany) concentrations on biofilm formation of *S.aureus*, the ethanol concentrations from 0% to 20% (v/v) in step of 0.5% were used in TSB media. For each treatment, 8 replicates were considered.

2.2.3. pH

In order to evaluate the effect of varying pH levels, the pH between 0 and 10.5 in step of 0.5 was used (8 repeats for each treatment). The pH levels of TSB media were adjusted with a digital pH meter (Mettler Toledo, Schwerzenbach, Switzerland) with the help of sodium hydroxide (NaOH, Merck, Germany) or hydrochloric acid (HCl 37%, Merck, Germany).

2.2.4. combination treatment of Ethanol, Sodium chloride and pH

To investigate the combination effects of the Ethanol, Sodium chloride and pH, four treatments were selected (in which the strongest biofilm was observed.) Therefor, 64 (4×4×4) combination treatments were prepared in the TSB culture media to assess the simultaneous effect of all parameters (table 1). All combined treatments were also evaluated at both incubation times (24 and 48 hours).

2.3. Biofilm formation assay

The effect of various concentrations of ethanol, sodium chloride and varying pH levels on biofilm formation ability of *S.aureus* was investigated in 96 well polystyrene flat bottomed micro titer plates (Costar, Corning, NY) using Stepanović et al. Method (45). (With some modifications).

Wells were filled with 180µl of freshly sterilized TSB with different treatments (described in 2.2). Subsequently, 20µl of overnight culture of *S.aureus* ATCC 33591 with the concentration of 1.5×10^8 CFU/ml (0.5 McFarland) was added to each well. Each treatment was tested with four replicates. Also, in each micro titer plate, a column was assigned

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to the control sample (Sterile TSB without culture). All micro titer plates were incubated at 37°C/48-h.

After the incubation period, the wells were evacuated and washed twice with sterile PBS (pH 7.4) and let dry at room temperature. Then, each well was filled with 200µl methanol and kept at room temperature for 15 minutes. In the next step, wells were filled with 200µl crystal violet solution 1% (Merck, Germany) for staining of attached cells. After 15 minutes, the wells were emptied, washed with distilled water and air dried at room temperature. Then 200µl of 33% glacial acetic acid (Merck, Germany) was added to each well to dissolve the color in the solution. Finally, the absorbance (optical density = OD) of each well was read at 630nm using the iMark™ Micro plate Absorbance Reader (Bio Rad Guru gram, India).

To calculate the optical density of each sample, the recorded OD_{630} of control sample (OD_c) was deducted from all the OD_{630} recorded for the treated samples (OD_t) and the difference was obtained (ΔOD). These ΔODs were used to express the effect of treatments on *S.aureus* biofilm formation (36, 46).

$$\Delta OD = OD_t - OD_c \quad (1)$$

2.4. Development of the ANN model

To modeling the effects of these parameters on the biofilm formation of *S.aureus* ATCC 33501, a kind of multilayer ANN model with 3 layers was developed. To build a ANN, MATLAB software (2015) was used. Inputs of this ANN were time, pH, concentrations of sodium chloride and ethanol) and its output was OD. To train this ANN, a 4×330 matrix for inputs and a 1×330 matrix for targets were used which show the static data respectively: 330 samples of 4 elements and 330 samples of 1 element. 75% of data was used for training, 15% of data was used for validation and 15% of data was used for testing. The Levenberg-Marquardt (LM) was its training algorithm. The trial and error method was used for choosing the number of hidden layers and also, the following equation was applied to select the number of neurons (nodes) for the hidden layers:

$$\frac{2(i+o)}{3} < n < i(i+o) - 1 \quad (2)$$

Where, *i* is the number of inputs; *o* is the number of outputs and *n* is the number of nodes in the hidden layer.

Eventually, the FFBPNN which had the least MSE (or MAE and RMSE) and the highest R values (R^2) was selected (Equations (3-6)).

$$R^2 = \frac{\sum_{i=1}^{n}\left(Y_{i.p} - Y_{I.e}\right)}{\sum_{i=1}^{n}\left(Y_{i.p} - Y_e\right)^2} \quad (3)$$

$$MAE = \frac{i}{n}\sum_{i=1}^{n}\left|Y_{i.e\_}Y_{i.p}\right| \quad (4)$$

$$MSE = \frac{i}{n}\sum_{i=1}^{n}\left(Y_{i.e} - Y_{i.p}\right)^2 \quad (5)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_{i.e} - Y_{i.p}\right)^2}{n}} \quad (6)$$

Where: *n* is the total number of experiments; $Y_{i,p}$ is the predicted value of the i^{th} experiment by the model; $Y_{i,e}$ is the experimental value of the i^{th} experiment; Y_e is the mean of experimentally determined values (47, 48).

3. Results

As it can be seen at Fig. 2, in the optimum pHs for *S.aureus* growth, the highest biofilm formation occurred. As a result, the most biofilm was formed at pH 7.5 after 24 h and 48 h. Furthermore, as the incubation time of *S.aureus* increases, the biofilm formation is increased.

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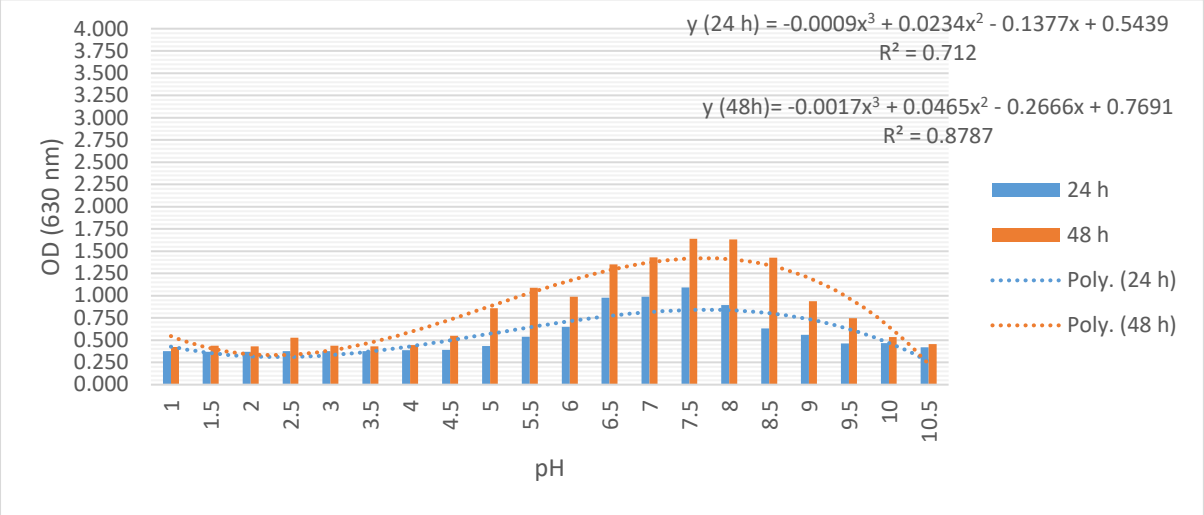

Fig. 2. Effect of pH on biofilm formation of *S.aureus* ATCC 33591.

The results of the Sodium Chloride effect on *S.aureus* biofilm formation are shown at Fig. 3. This indicates that, the strongest biofilm formation occurs at 5% and 5.5% concentrations of Sodium Chloride after 24-h and 48-h incubation, respectively. Subsequently, the addition of sodium leads to a decrease in biofilm formation. So, Sodium Chloride has a deterrent effect on biofilm. The biofilm formation of *S.aureus* ATCC 33591 also increases with incubation time. The effect of ethanol concentration on the biofilm formation is exposed in Fig. 4. The highest biofilm formation occurred after 24-h and 48-h in the presence of 8% and 8.5% ethanol. The biofilm formation of *S.aureus* ATCC 33591 also increases with incubation time. Furthermore, the biofilm was increasing after 48-h then 24-h.

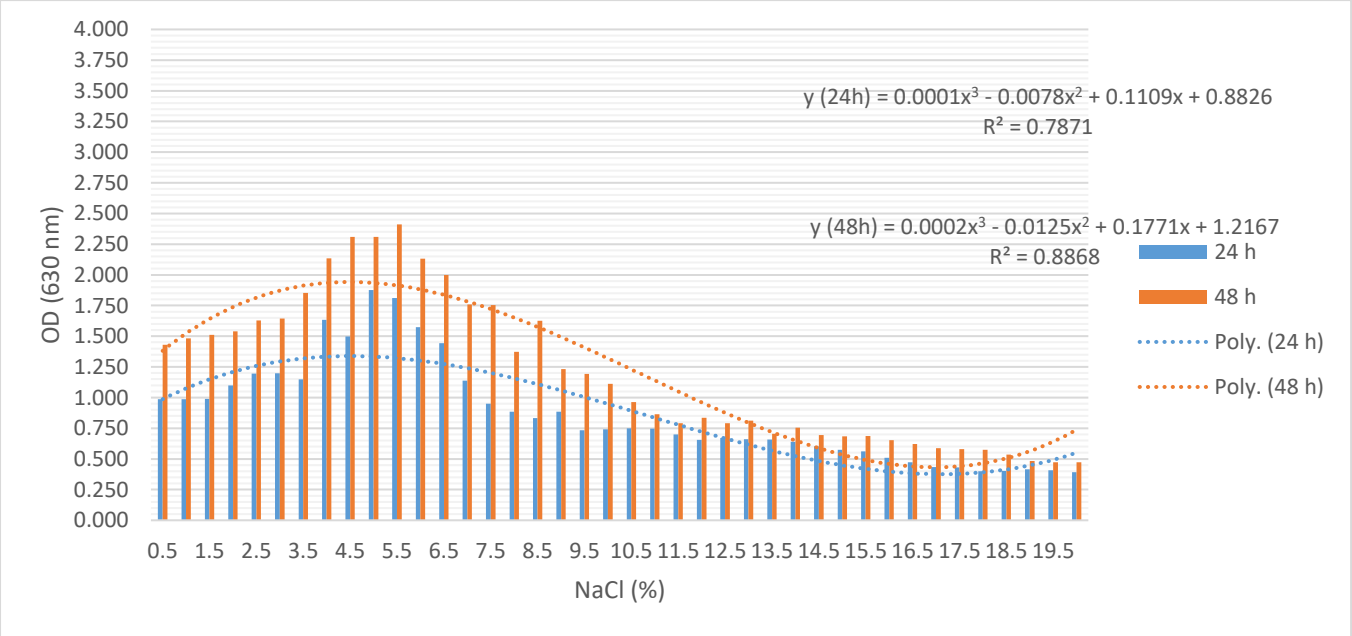

Fig. 3. Effect of Sodium Chloride on biofilm formation of *S.aureus* ATCC 33591.

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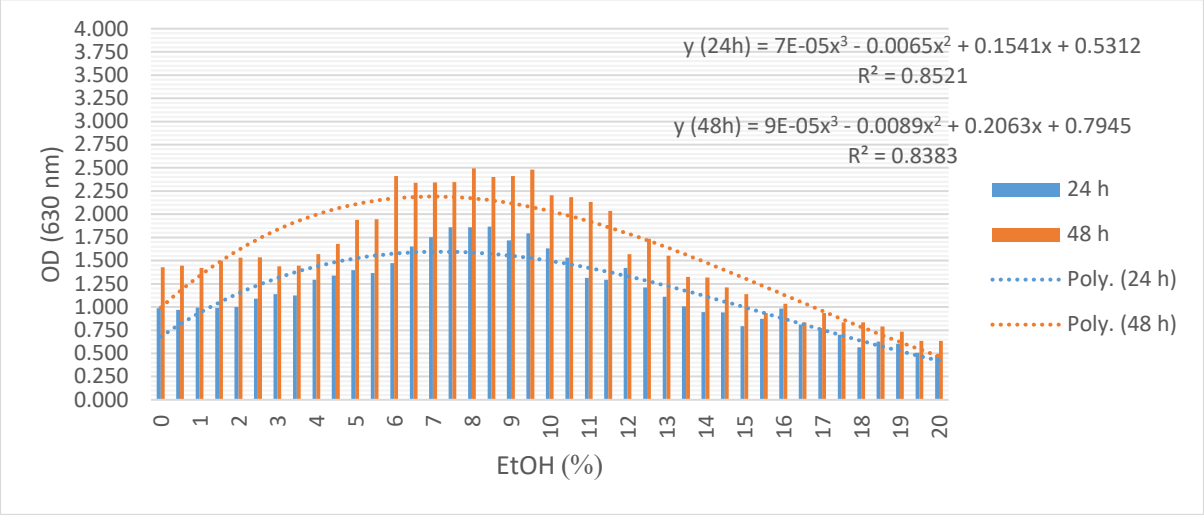

Fig. 4. Effect of Ethanol on biofilm formation of *S.aureus* ATCC 33591.

In Fig. 5, the combination effects of ethanol, sodium chloride, and pH treatments were shown (after 24-h and 48-h). It is seen that there is a nonlinear relationship between these effects. On this basis, the necessity of modeling with ANN is well understood. It is again shown in this graph, the biofilm formation of *S.aureus* ATCC 33591 was increasing after 48-h then 24-h. 64 combination treatments of ethanol, sodium chloride, and pH are presented in Table 1.

Several types of ANNs for modeling of these parameters' effect on biofilm formation of *S.aureus* ATCC 33591 were trained. Finally, it was found that MLP is the best ANN type for this research. Targets of ANN were the ODs. Matching to Equation 1, from 3 to 19 nodes were determined to the hidden layer. In table 2 all R values were shown. Eventually, the FFBPNN with 4-12-1 topology was selected because it had the highest R value (Fig. 6).

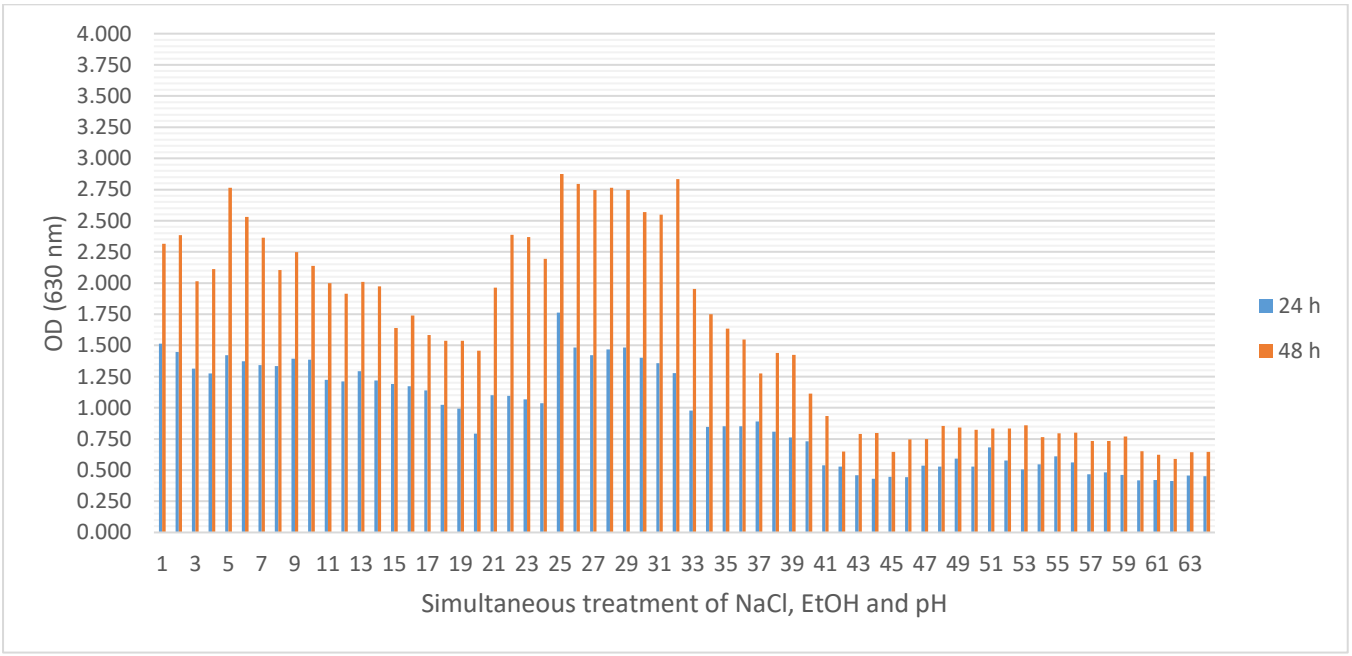

Fig. 5. Simultaneous effect of pH, sodium chloride and ethanol treatments, on biofilm formation of *S.aureus* ATCC 33591.

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Table 1. Simultaneous treatment of pH, sodium chloride and ethanol

#	NaCl (%)	EtOH (%)	pH	#	NaCl (%)	EtOH (%)	pH	#	NaCl (%)	EtOH (%)	pH	#	NaCl (%)	EtOH (%)	pH
1	4.0	6.0	6.5	**17**	5.0	6.0	6.5	**33**	6.0	6.0	6.5	**49**	7.0	6.0	6.5
2	4.0	6.0	7.0	**18**	5.0	6.0	7.0	**34**	6.0	6.0	7.0	**50**	7.0	6.0	7.0
3	4.0	6.0	7.5	**19**	5.0	6.0	7.5	**35**	6.0	6.0	7.5	**51**	7.0	6.0	7.5
4	4.0	6.0	8.0	**20**	5.0	6.0	8.0	**36**	6.0	6.0	8.0	**52**	7.0	6.0	8.0
5	4.0	7.0	6.5	**21**	5.0	7.0	6.5	**37**	6.0	7.0	6.5	**53**	7.0	7.0	6.5
6	4.0	7.0	7.0	**22**	5.0	7.0	7.0	**38**	6.0	7.0	7.0	**54**	7.0	7.0	7.0
7	4.0	7.0	7.5	**23**	5.0	7.0	7.5	**39**	6.0	7.0	7.5	**55**	7.0	7.0	7.5
8	4.0	7.0	8.0	**24**	5.0	7.0	8.0	**40**	6.0	7.0	8.0	**56**	7.0	7.0	8.0
9	4.0	8.0	6.5	**25**	5.0	8.0	6.5	**41**	6.0	8.0	6.5	**57**	7.0	8.0	6.5
10	4.0	8.0	7.0	**26**	5.0	8.0	7.0	**42**	6.0	8.0	7.0	**58**	7.0	8.0	7.0
11	4.0	8.0	7.5	**27**	5.0	8.0	7.5	**43**	6.0	8.0	7.5	**59**	7.0	8.0	7.5
12	4.0	8.0	8.0	**28**	5.0	8.0	8.0	**44**	6.0	8.0	8.0	**60**	7.0	8.0	8.0
13	4.0	9.0	6.5	**29**	5.0	9.0	6.5	**45**	6.0	9.0	6.5	**61**	7.0	9.0	6.5
14	4.0	9.0	7.0	**30**	5.0	9.0	7.0	**46**	6.0	9.0	7.0	**62**	7.0	9.0	7.0
15	4.0	9.0	7.5	**31**	5.0	9.0	7.5	**47**	6.0	9.0	7.5	**63**	7.0	9.0	7.5
16	4.0	9.0	8.0	**32**	5.0	9.0	8.0	**48**	6.0	9.0	8.0	**64**	7.0	9.0	8.0

Table 2. R values for 3-19 nodes for ANN of *S.aureus* ATCC 33591.

Number of nodes	R value	Number of nodes	R value
3	0.74168	12	0.98954
4	0.73428	13	0.98381
5	0.87567	14	0.98814
6	0.90031	15	0.98581
7	0.91285	16	0.95535
8	0.92944	17	0.97659
9	0.97170	18	0.94395
10	0.98353	19	0.94559
11	0.98764		

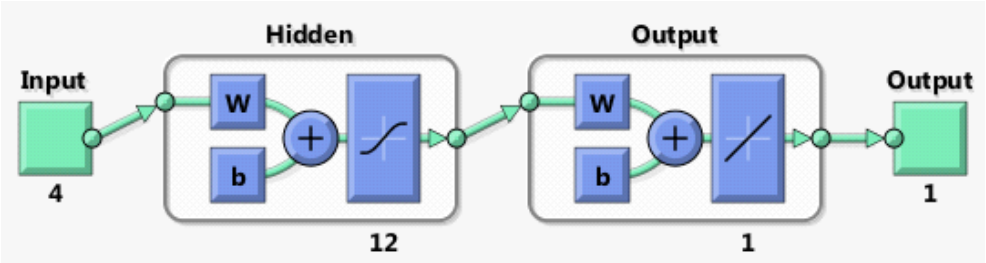

Fig. 6. Topology of FFBPNN for ANN of *S.aureus* ATCC 33591.

All of the R values and MSE for this FFBPNN are placed in the table 3. Tansig function and Levenberg–Marquardt was chosen as transfer function and training algorithm for ANN, respectively. The FFBPNN with LM was more robust and also it was reduced ANN computing time. At this FFBPNN, R^2 and MSEs were the best. (R^2: closer to 1.0 and MSE: closer to 0).

Table 3. R^2 and MSE for 4-12-1 FFBPNN for *S.aureus* ATCC 33591.

	Sample	MSE	R values
Training	230	3.49617e-3	9.92835e-1
Validation	50	1.07224e-2	9.83659e-1
Testing	50	9.76467e-3	9.83814e-1

The MSEs for training (230 data), validation (50 data), and testing (50 data) in this

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FFBPNN is exposed in Fig. 7. The 72th epoch was the final epoch of training, and since the least MSE, for validation data is in epoch 66 (MSE=0.010722), then epoch 66 showed the best validation performance. This graph shows the mean square error on the one hand and the number of iterations on the other. In the highlighted green circle shown in this diagram, four repetitions without progress have been shown to halt network training.

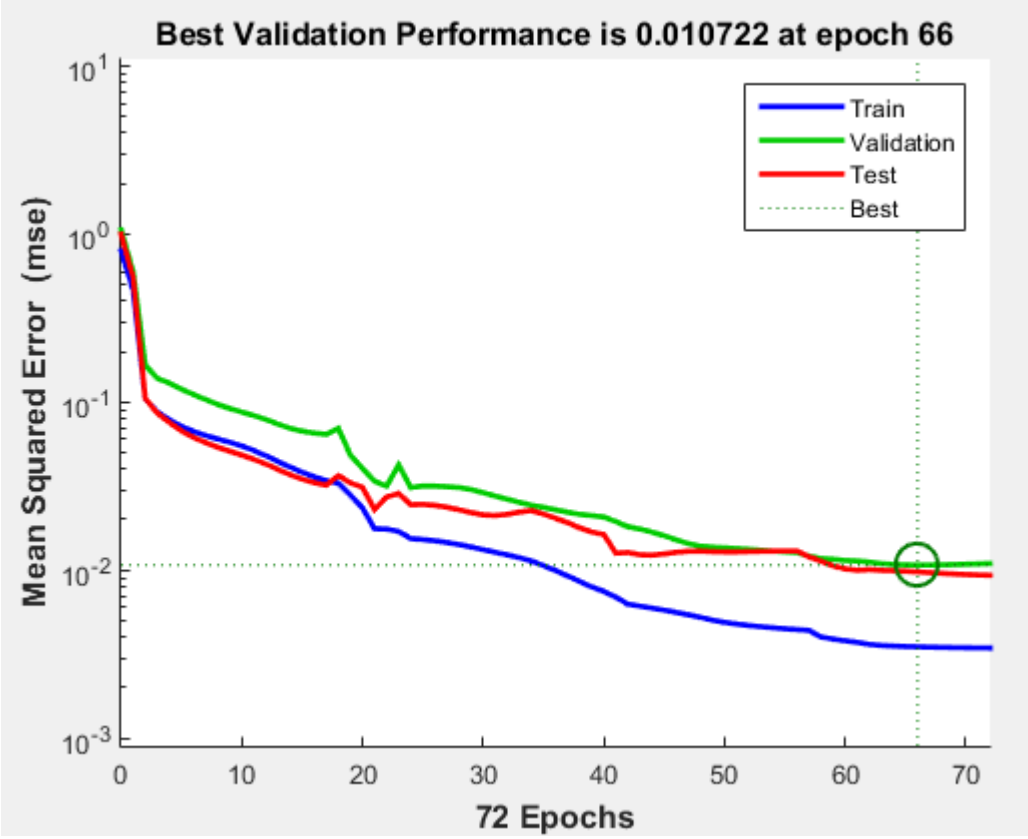

Fig. 7. FFBP ANN training performance (plotperform) for ANN of *S.aureus* ATCC 33591.

In Fig. 8, the error or the difference between the experimental data given to the ANN (target data) and the ANN predictions (output data) is visible. As it can be seen the error is zero at 10th bin. The most data at 10th have an error of approximately zero.

The trend of the R^2 for all data is shown in Figure 9. As it can be seen in Fig. 9, there is a very high correlation between all three data sets (training, validation and testing data).

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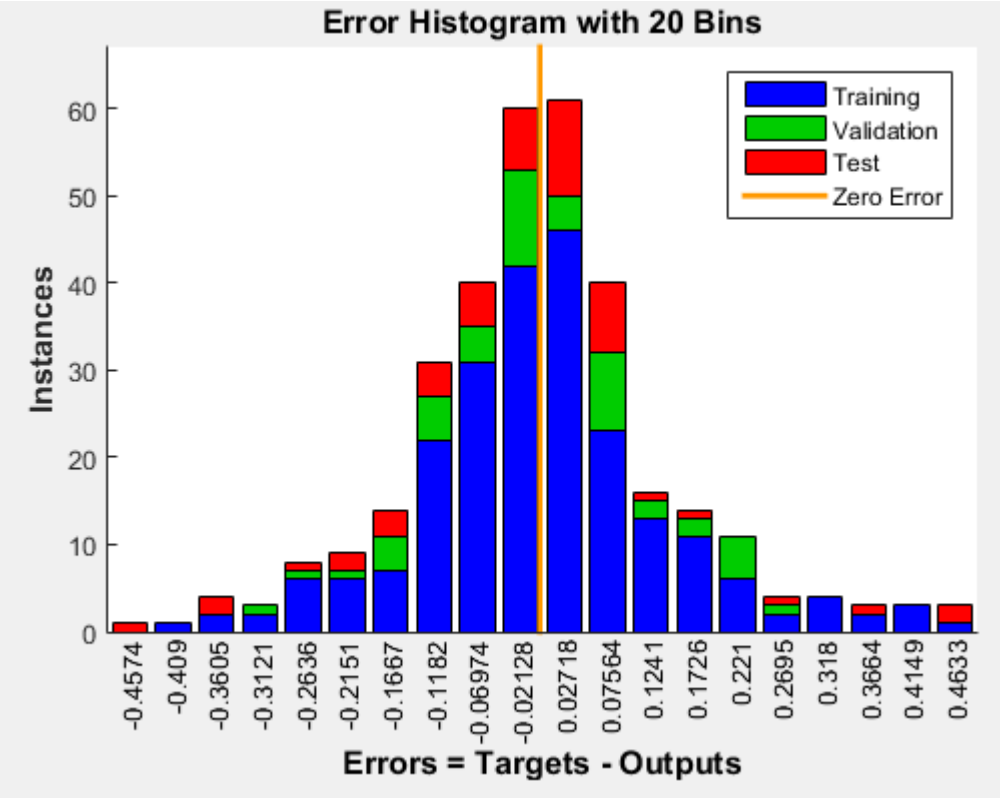

Fig. 8. FFBPNN error histogram (ploterrhist) for ANN of *S.aureus* ATCC 33591.

4. Discussion

Biofilm enables bacteria to survive for long periods of time. Biofilms can be easily bonded to fresh or processed foods such as dairy products, seafood and food processing equipment with stainless steel, glass and plastic. In addition, as biofilms grow and develop, bacterial resistance to antibiotics and conventional disinfectants increases, this is a potential risk for secondary infection and huge economic losses. The process of forming organized structures of biofilms at different surfaces is strongly dependent on the fluctuations of environmental factors (37). Environmental factors affecting biofilm include pH, alcohol and sodium chloride (or aqueous activity). At present, the impact of contaminated surfaces on the spread of pathogens to foods has been well established during food processing processes, food products, and home and medical environments (25, 49).

Environmental conditions in the food industry, such as temperature, nutrient availability, types of surfaces, pH, and humidity, allow for bacterial growth and biofilm formation. In addition, some authors have demonstrated the presence of biofilms on surfaces in contact with food, despite using disinfection (50-52). So that, the growth of *S.aureus* and its biofilm formation is recognized as a serious clinical problem.

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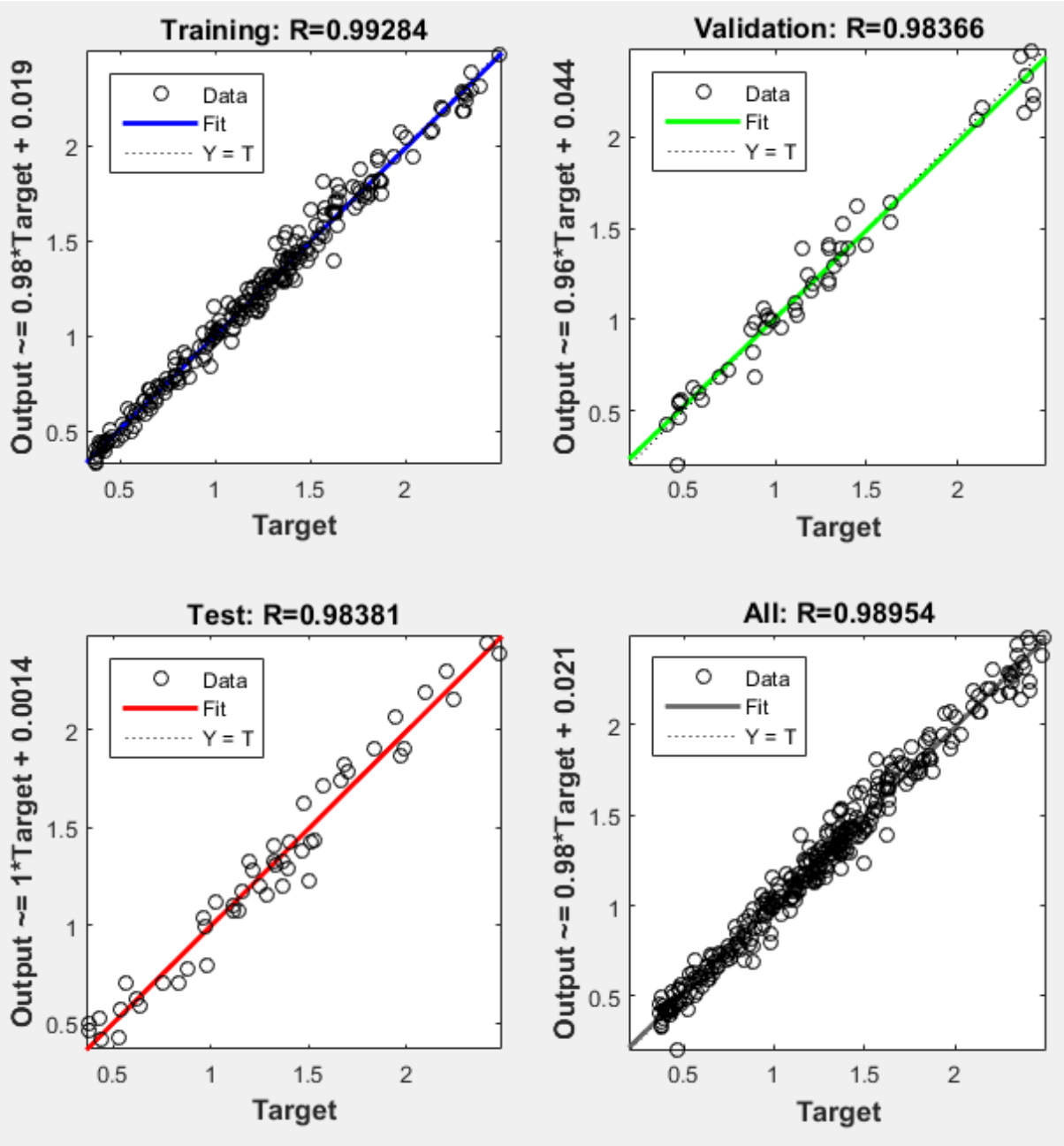

Fig. 9. FFBP ANN regression (plotregression) for ANN of *S.aureus* ATCC 33591.

Vaezi et al. (2020) concluded that *S.aureus* ATCC 25923 under the influence of different pHs showed severe reactions and the highest strength of biofilm formation after 24 hours of incubation, at pH 6.5 and after 48 hours at pH 7 (43). The results obtained from the study of the effect of different pH on *S.aureus* ATCC 33591, in the present study, showed that strain ATCC 33591, unlike strain ATCC 25923, did not have a significant reaction to pH changes. However, at the ideal growth pH, this strain showed maximum biofilm formation strength at both 24-h and 48-h incubation times. The maximum absorption of light during 24-h of incubation belonged to pH= 7.5, which was read at a wavelength of 630nm, equivalent to 1.092. Thus, it was concluded that the pH affects the ability of both strains of *S.aureus* to form biofilms. But, Giaouris et al. (2005) reported that biofilm formation of *Salmonella enteritidis* at 20°C after 7 days of incubation was independent of pH (35).

Chaib et al. (2007) by adding ethanol to the culture medium investigated the formation of *S.epidermidis* biofilm in an environment with

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different pH. They reported the lowest optical density (biofilm formation strength) at pH=3 and pH=12. In addition, their results indicated that the greatest ability of biofilm adhesion was in the presence of 2% ethanol. They attributed this to the stressful pH and ethanol for the bacteria (53). As shown in the present study, with increasing ethanol concentration from 0% to 8%, OD increased; which means an increase in biofilm formation strength. OD from 8% to 8.5% ethanol concentration within 24 hours was approximately constant and equal to 1.86. The results showed that during 48 hours, the light absorption read for ethanol concentration was 8% higher than other concentrations and was equal to 2.495. But then, as the ethanol concentration increases, we experienced a decrease in the strength of the biofilm formation at both incubation times.

In the investigation of the concentration of sodium chloride in the present study, the inhibitory effect of sodium chloride on the formation of *S.aureus* biofilm at concentrations above between 5% and 5.5% had been shown. According to an experiment in which Giaouris et al. (2005) examined the formation of *S.enteritidis* biofilm under the influence of temperature, pH and water activity on stainless steel coupons, it was found that the maximum number of live bacteria in *S.enteritidis* biofilm at 20°C and after 6 days. It had also been observed that when sodium chloride had concentrations above 10.5%, it inhibits bacterial binding to stainless steel surfaces (35). Vaezi el al. (2020) had shown that sodium chloride at lower concentrations of less than 6% reduces the formation of *S.aureus* ATCC 25923 (43). But contrary to the evidence, Planchon et al. (2006) observed that sodium chloride (20g NaCl / L TSB) resulted in increased bacterial growth and viability, and that the efficacy of *S.aureus* biofilm had no significant effect (38). The results of the present study showed that biofilm formation increases at low sodium chloride concentrations and decreases at high sodium chloride concentrations. Numerous studies have reported that biofilm formation increases in the combination of sodium chloride and glucose (32). Møretrø et al. (2003) examined the strength of biofilm formation on *S.aureus* isolated from food processing medium and stated that adding a small amount of sodium chloride or glucose to TSB stimulates biofilm formation (54).

The results of 64 combined treatments (bacteria in the culture medium containing pH, ethanol and sodium chloride were pre-regulated, incubated) for the bacterium *S.aureus* ATCC 33591 during two incubation times of 24 and 48 hours indicate that increasing the incubation time leads, increases the strength of biofilm formation. What is important here is that unlike other treatments that were performed separately and that it was possible to model with a third-order polynomial equation with acceptable regression equations, in the combined treatments the relationships between the parameters were nonlinear. It is not possible to set any equation. So this is where the need for modeling with an ANN becomes tangible. Like the present study, Kote & Wadkar (2019) also used ANN for modeling. Because this type of machine learning has the ability to model nonlinear and complex relationships. They examined separate models of ANN for chlorine doses with radial-based neural networks, feedback neural networks, and general regression neural networks. They similar to the present study used R^2 as a criterion for comparing the function of the neural network (55).

To model this study, different FFBPNN were trained, and finally, according to the characteristics, number and type of data, it was determined that the best topology of ANN for this research is 4-12-1. MSE and R value for chosen ANN with LM training algorithm were equal to 0.0102 and =0.989, respectively. Mittal & Zhang (2000) used FFBPNN to model the effect of temperature and humidity during

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Frankfurter cooking. Their ANN inputs were: lard radius, relative humidity, initial moisture, protein / fat ratio, initial temperature, ambient temperature and processing time. Their ANN outputs were: Frankfurt's average temperature, Frankfurt's center temperature, and Frankfurt's average humidity (56). Idris et al. (2019) as well as Movagharnejad & Nikzad (2007) also indicate that modeling is approved by the ANN (57, 58). In general, the models of ANNs in microbial studies are very diverse and it has been reported that machine learning, and in particular ANNs, is better than standard statistical methods (59, 60). For example, ANN models based on statistical techniques have been used to predict the thermal inactivation of *Escherichia coli* (61, 62), *Salmonella* and *Listeria* (63). ANNs provided better growth predictions for *Shigella flexneri* than the results of regression equations (64).

5. Conclusion

In the present study, three parameters of sodium chloride concentration, ethanol concentration and pH were studied separately and on the strength of *S.aureus* biofilm during two incubation periods of 24 and 48 hours, and finally it was concluded that all of these parameters affect the strength of *S.aureus* biofilm. It was also found that the 4-12-1 FFBPNN with LM training algorithm is well able to model the simultaneous effect of this parameter on biofilm of *S.aureus*. R^2 of this model indicates that the correlation between the results of the prediction data with the ANN and the experimental data in the laboratory is very high.

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