

# Modeling of Extracellular Polymeric Substances Production at Different Carbon/Nitrogen Ratio and Solid Retention Time by Artificial Neural Network

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## Abstract

**Aims:** The ability of extracellular polymeric substances (EPS) production was observed in many species of heterotrophic microorganisms through the biological wastewater treatment systems. **Materials and Methods:** The batch experiments at different carbon/nitrogen and solid retention time (SRT) were carried out to investigate the effects of initial nitrogen concentration and SRT on EPS production and chemical oxygen demand (COD) removal efficiency. The artificial neural network (ANN) was developed to modeling of obtained data. **Results:** The results showed that: (i) with increasing SRT, the COD removal improves; (ii) initially, the amount of carbohydrate increases as SRT increases; however, with further increase of SRT, it declines; (iii) the protein/carbohydrate ratio improves as SRT decreases; (iv) the carbohydrate and protein concentration of soluble EPS increased with increasing initial nitrogen concentration from 0 to 10 mg/L; and (v) further increase of initial nitrogen concentration lead to depletion of carbohydrate production. **Conclusion:** The highest yield (Y) value was calculated at low sludge age and deficient initial nitrogen concentration, which may be due to the application of EPS production mechanism. The ANN model moderately predicted effluent COD concentration, carbohydrate, and protein production.

**Keywords:** Artificial neural network model, carbohydrate, extracellular polymeric substances, nitrogen, protein

## INTRODUCTION

The ability of extracellular polymeric substances (EPS) production was observed in many species of heterotrophic microorganisms through the biological wastewater treatment systems.<sup>[1]</sup> The microorganisms can release EPS that consists of some polymers with carbohydrates and proteins as dominant component. The EPS can be categorized as soluble and insoluble that separated by the centrifugation method. The polymers in the supernatant being soluble EPS and those founded in the microbial pellets being insoluble or bound EPS.<sup>[1,2]</sup> As previously mentioned, the presence of EPS depicts the positive effect on the sedimentation properties of sludge, biofilm formation, and bacteria resistance to biocides or other harmful effect, and its presence is essential to strength sludge structure.<sup>[2-4]</sup> The negative effects such as the membrane biofouling, poor

bioflocculation, and greater cell erosion are related to higher EPS content of sludge.<sup>[5,6]</sup>

The EPS production may be influenced by (i) substrate type: the carbon source is the important factor that affect the EPS production, for example, using glucose or soybean oil as carbon source, the highest EPS production was noticed;<sup>[2,7]</sup> (ii) the nutrient content: phosphorus, nitrogen, and carbon/nitrogen (C/N) ratio affects the EPS content of sludge;<sup>[1]</sup> and (iii) other parameters such as the microbial species, growth stage, dissolved oxygen, ionic strength, solid

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retention time (SRT), and food to microorganism (F/M) ratio can also affect the EPS formation.<sup>[1,8,9]</sup>

Previous researches demonstrate many contradictions about EPS effects on the sludge properties and EPS production as a function of SRT and C/N ratio, etc. Despite EPS effects on the sludge settling characteristics, Ye *et al.* reported that the contents of loosely bond protein in EPS is directly related to the flocculation, settleability, and dewaterability of sludge; however, there is no relationship with total amount of EPS.<sup>[10]</sup> Furthermore, a similar contradiction was reported by Xuan *et al.* and Sponza about the effect of EPS content on the sludge volume index (SVI) as the sludge settleability indicator.<sup>[3,4]</sup> Liao *et al.* depict the no-significant correlation between the amount of EPS and SRT, but Sesay *et al.* demonstrate that the total amount of EPS in activated sludge significantly increases as SRT increases.<sup>[11,12]</sup> In respect to C/N ratio effect on the EPS production, Miqueleto *et al.* found that the EPS production is favored by a high C/N ratio,<sup>[2]</sup> although Ye *et al.* reported that the carbohydrate and protein contents in EPS are not influenced by C/N ratio.<sup>[10]</sup>

Considering these contradictions and the effect of other mentioned factors, prediction of EPS production at different situations has been difficult. This study aimed to investigate (i) the influence of SRT and C/N ratio on EPS production; (ii) simulates the results using artificial neural network (ANN) (MATLAB software, R2014a); and (iii) the effect of EPS on sludge sedimentation properties.

## MATERIALS AND METHODS

### Batch experiments

The batch experiments were performed using a cylindrical reactor of 15.5 cm and 16.5 cm (D and H) with a working volume 2 L and aerated with the bottom air stone spray to achieve DO 2–5 mg/L. The sludge inoculum for each batch test was obtained from another sequencing batch reactor. Each set was operated at steady state condition with SRT of 2, 5, 10, and 15 d. At each SRT, the experiment was done in duplicate with C/N ratio of 100:0, 100:1, 100:2.5, 100:5, and 100:10. The initial chemical oxygen demand (COD) and pH for all experiment were maintained to  $1000 \pm 100$  mg/L and 7–8, respectively. The batch experiments were operated for 12 h per cycle at 27°C. During batch experiments, variation of pH was monitored but not controlled.

### Synthetic substrate

The synthetic wastewater by organic concentration with COD  $1000 \pm 100$  mg/L was feed to batch tests. The glucose was used as carbon source and macro and micronutrient elements for microorganism growth were also added to the synthetic substrate.<sup>[13]</sup>

### Analytical procedure

DO, pH, EC, and turbidity were measured using a DO membrane probe (Eutech Cyborscan DO300), precalibrated

glass body pH probe (CG 824 SCHOTT), HACH digital conductivity meter (SESSION 5), and EUTECH instruments turbidimeter (TN-100), respectively. At the end of batch experiments, the supernatant and mixed liquor samples were collected from batches and analyzed for COD, total suspended solids, volatile suspended solids (VSS), and SVI. The standard methods for water and wastewater examination were adopted to measure these parameters.<sup>[14]</sup>

### Extracellular polymeric substances analysis

The EPS extracted using formaldehyde + sodium hydroxide (NaOH) method adapted and the carbohydrate and protein in the sludge and effluent was measured as EPS and soluble EPS (sEPS), respectively.<sup>[15]</sup> To extract EPS and sEPS, the sludge was stored in 4°C for 1.5 h. Then, the supernatant was discarded and residual sludge centrifuged at 6000 rpm for 30 min at 4°C. After this, the supernatant was removed and pellet was resuspended by distilled water and used for EPS extraction using formaldehyde + NaOH method and extracted liquid subjected to EPS analysis. For sEPS analysis, the effluent wastewater sample was filtered using 0.45  $\mu$  cartridge filter then centrifuged (6000 rpm, 30 min) at 4°C and supernatant for sEPS analysis was used. The carbohydrate and protein content in EPS was measured using phenol–sulfuric acid assay procedure with glucose as standard and Lowry method using bovine serum albumin as standard solution, respectively.<sup>[16,17]</sup>

### Statistical analysis

Tests for significant differences between EPS concentration in variable C/N and F/M ratio were determined using one-way ANOVA (or Mann–Whitney). Pearson correlation coefficients were used to examine the relationship between the parameters and were considered significant when  $P < 0.05$ .

### Artificial neural network modeling

As part of this study, the ANN was used to predict carbohydrate, protein production, and effluent COD concentration.

For this purpose, a three-layer ANN with a tangent sigmoid transfer function (tansig) at hidden layer, and a linear transfer function (purelin) at output layer were used. Levenberg–Marquardt back propagation (trainlm) was used to train the designed networks.

To avoid numerical overflows due to very large or small weights, the data are converted to normalized values using Equation 1.

$$x_{norm} = 0.8 \times \left( \frac{x_i - x_{min}}{x_{max} - x_{min}} \right) + 0.1 \quad (1)$$

Where  $x_{norm}$  is the normalized value of  $x_i$ , the  $x_{max}$  and  $x_{min}$  are the maximum and minimum value of  $x_i$ , respectively.

The results of various network structures and training procedures were compared using the mean squared error (MSE) and the

coefficient of determination ( $R^2$ ) defined by Equations 2 and 3, respectively.

$$MSE = \frac{1}{N} \sum_{i=1}^N (y_{pre,i} - y_{exp,i})^2 \tag{2}$$

$$R^2 = 1 - \frac{\sum_{i=1}^N (y_{pre,i} - y_{exp,i})^2}{\sum_{i=1}^N (y_{pre,i} - y_{ave})^2} \tag{3}$$

Where  $y_{pre,i}$  is the predicted value from the ANN model,  $y_{exp,i}$  is the experimental value, N is the number of data, and  $y_{ave}$  is the average value of the experimental value.

## RESULTS AND DISCUSSION

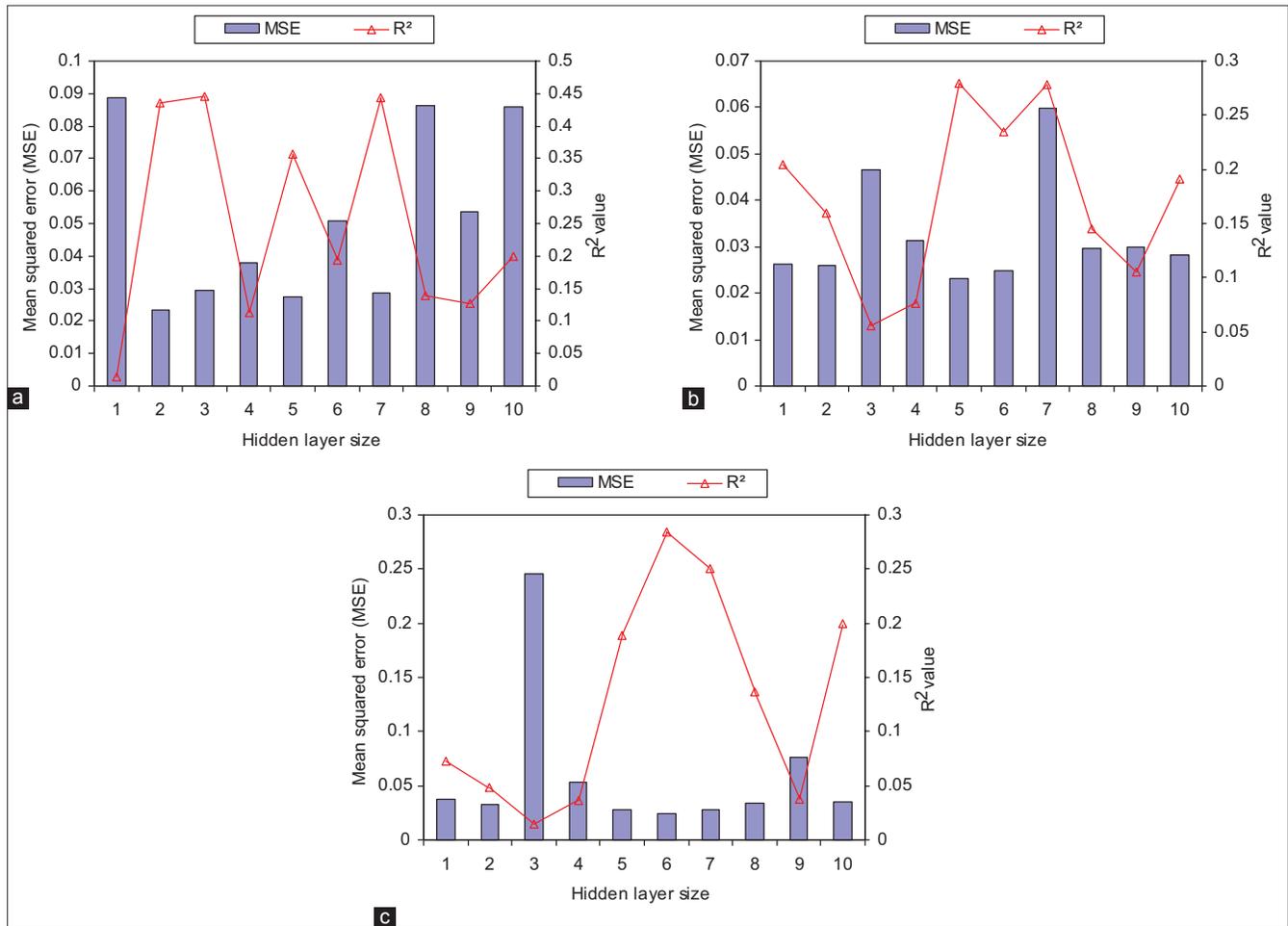
### Artificial neural network modeling

The data obtained from batch experiments were used to train the network. The input layer had three neurons consist of influent COD, SRT, and influent nitrogen. The output layer had one neuron consists of effluent COD concentration (or carbohydrate and protein production). Same approach was adopted to the

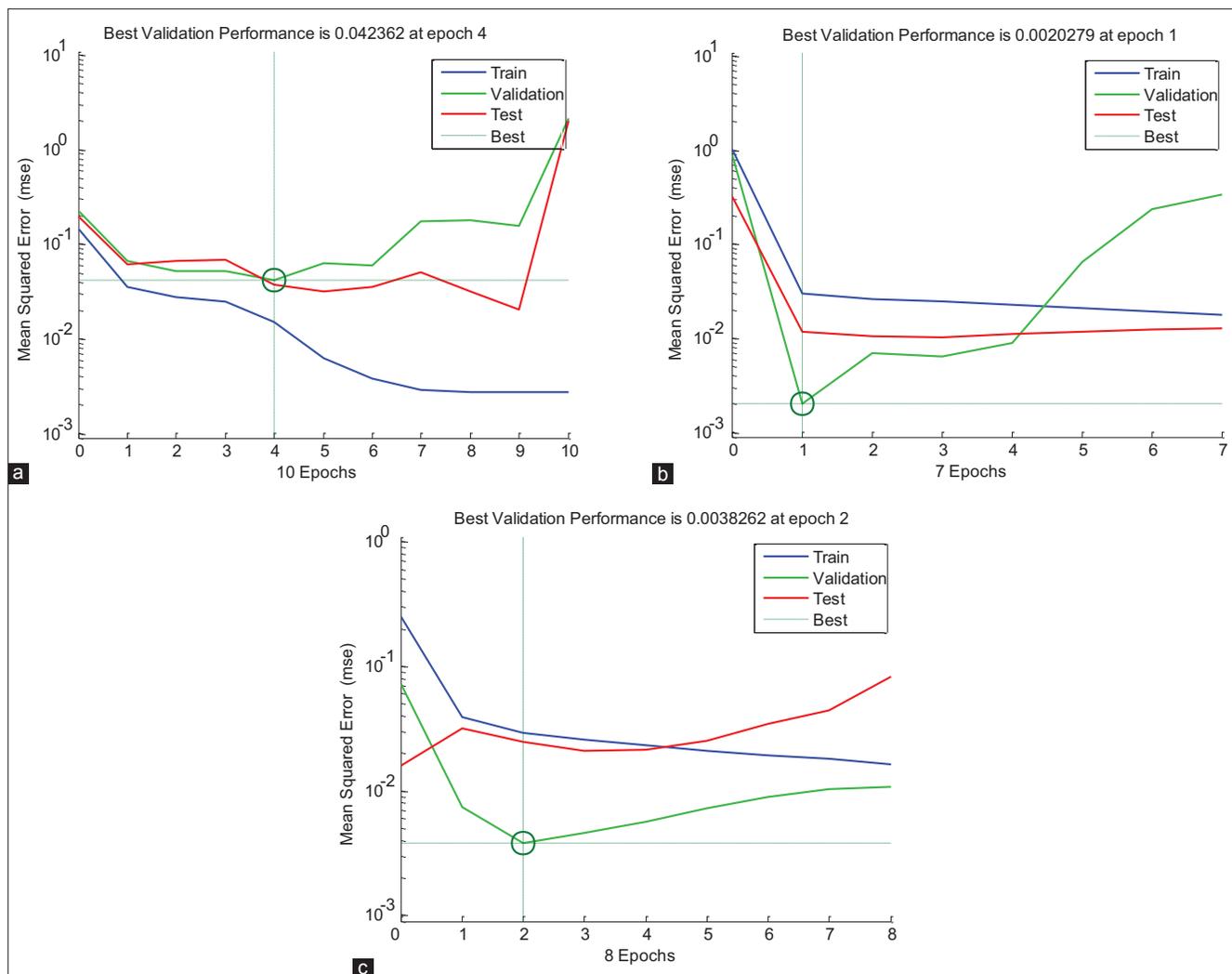
prediction of carbohydrate and protein production. All data were randomly split into three groups (70% for training, 15% for validation, and 15% to test the model). To obtain the optimum number of neurons (N) in the hidden layer, trial and error method was used. Therefore, different number of neurons in the range of 1–10 was tested in the hidden layer. The optimum hidden layer size was determined based on the minimum value of MSE and  $R^2$  of the predicted results. As seen in Figure 1, as the number of neurons in the hidden layer increase, MSE decreases promptly and then rises.

As shown in Figure 1, in this study, three-layered feed forward back propagation neural network including 3:2:1, 3:5:1, and 3:6:1 were used to model the effluent COD concentration, carbohydrate, and protein production, respectively.

Plot of MSE versus the number of iterations for optimal ANN model [Figure 2] show that the training was stopped after 10, 7, and 8 epochs for effluent COD concentration, carbohydrate, and protein production, respectively. Figure 3 showed that the training and testing performance of the ANN model. It shows that the ANN model is able to predict the removal of zinc ions from wastewater using activated almond



**Figure 1.** Comparison of 10 neurons in the hidden layer for value estimating by ANN model development with the Levenberg-Marquardt algorithm; (a) Effluent COD concentration, (b) Carbohydrate production and (c) Protein production



**Figure 2:** MSE versus the number of epochs; (a) Effluent COD concentration, (b) Carbohydrate production and (c) Protein production

shell with  $MSE < 0.05$ . With regard to the fact that many factors involve in EPS production and also the level of uncertainty involves, the proposed modeling methodology can be used to data prediction.

Figure 1 presents the normalized results of ANN model versus normalized experimental data for the effluent COD, carbohydrate, and protein production. The coefficient of determination ( $R^2$ ) was  $>0.5$  for the training, validation, and test set. Although the coefficients of determinations for the testing data are lower than the training values, their difference is acceptable and the proposed model is recommended.

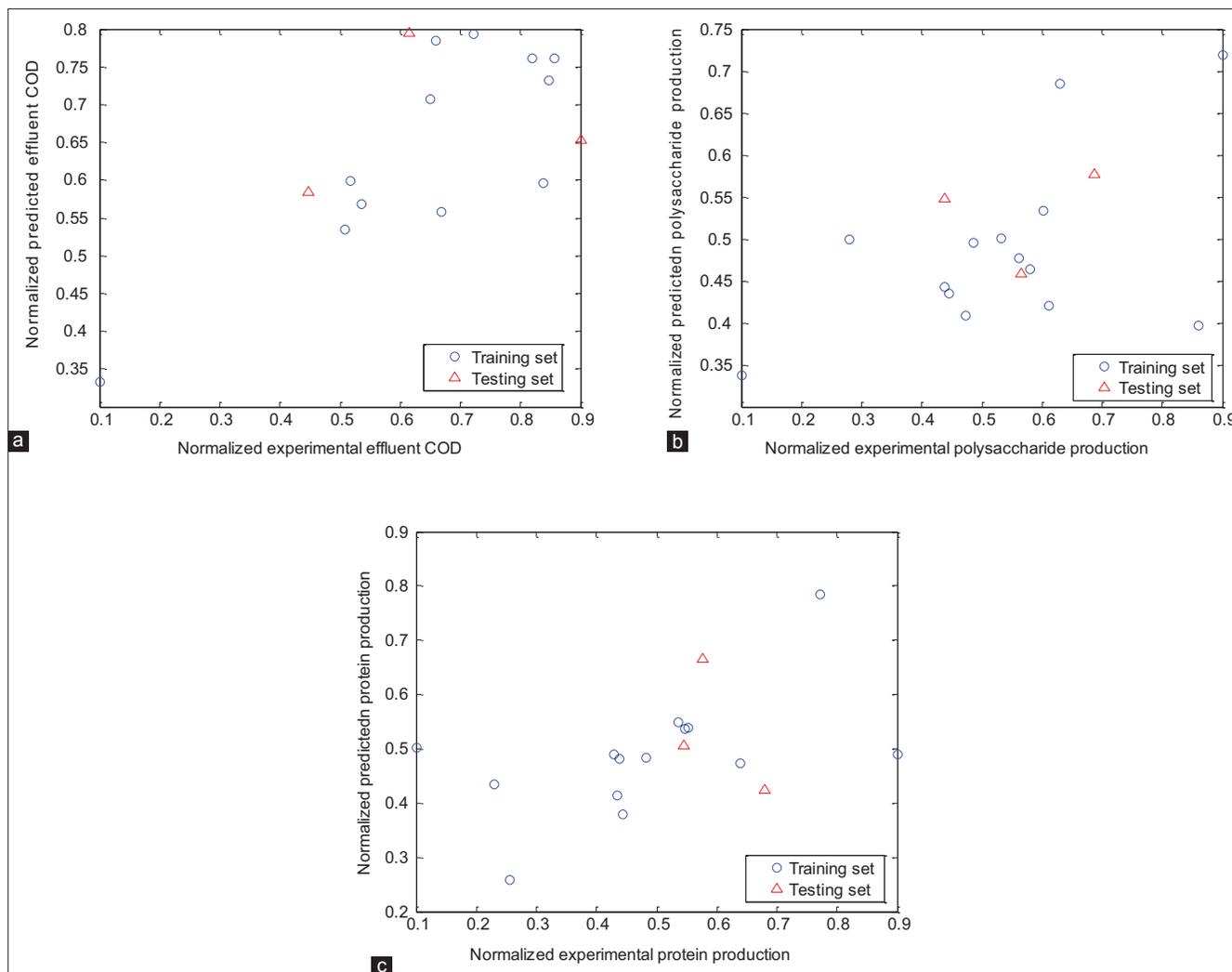
### Chemical oxygen demand removal

The COD removal efficiency throughout all batch experiments is presented in Figure 4a. The results show that with increase of SRT, the COD removal is enhanced. It was expected that the COD removal controlled by two processes including biomass assimilation/accumulation and oxidation, in which these processes were strongly influenced by SRT. The ANOVA test showed that the COD removal efficiency

related to 2-day SRT was significantly lower than those at SRT 5, 10, or 15 days ( $P < 0.05$ ). However, there was no significant difference between COD removal efficiency at other SRTs (5, 10, and 15 days). Furthermore, no significant relationship between COD removal and initial N concentration was observed, and the results demonstrated that by changing of initial N concentration (increasing or decreasing), the COD removal efficiency remains relatively constant. Perhaps, it means that bacteria can uptake carbon as an electron donor in both N deficit and abundance. In these tests, at C/N ratio of 100:0, 100:1, 100:2.5, 100:5, and 100:10, the average of COD removal efficiency was 95.9%, 95.35%, 93.79%, 93.14%, and 94.66%, respectively. The small variation of COD removal at different initial N concentration is in line with Feng *et al.* They reported that as COD/N ratio decreases from 10 to 5, the average efficiency of COD removal fluctuates from 94.3% to 94.5%, respectively.<sup>[18]</sup>

### Extracellular polymeric substances production

The variation of carbohydrate and protein content in the sludge and effluent wastewater as a function of initial N concentration



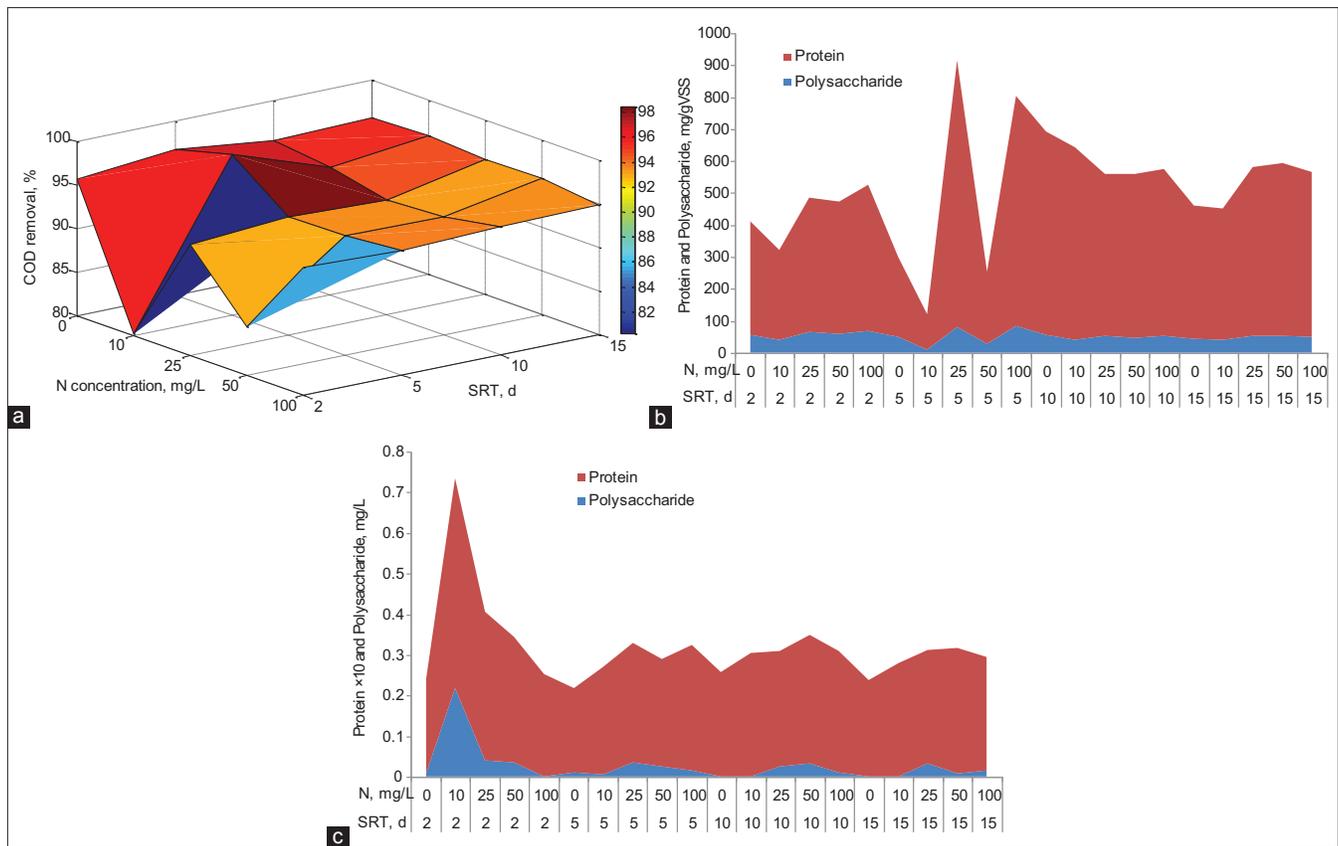
**Figure 3:** Experimental data versus the predicted data of normalized value from ANN: (a) Effluent COD concentration, (b) Carbohydrate production and (c) Protein production

and SRT was evaluated and presented in Figure 4b and c. The results showed that with increasing SRT from 2 days to other studied SRT values (5, 10, and 15 days) led to significant decrease in carbohydrate of sludge (Pearson correlation coefficient:  $-0.685$ ,  $P < 0.05$ ). It may be related to high carbohydrate production by bacteria at low sludge age. The protein/carbohydrate ratio was improved as SRT increases. The results did not prove that as the bacteria age increases, the EPS layer form the exterior of the bacterial cell as demonstrated by a previous study<sup>[19]</sup> and depicted that the EPS production may be controlled by other operating conditions such as nitrogen concentration and electron donor availability. The previous studies report different outcome for EPS production at various SRT. Sesay *et al.* demonstrate the polymers production significantly amplify as SRT increases. With SRT increasing from 4 to 8 days, the carbohydrates amount was constant and also at SRT higher than 8, the carbohydrates concentration was augmented.<sup>[12]</sup> In addition, Ahmed reported that the EPS content and SRT are negatively correlated.<sup>[5]</sup> Other studies

reported that the amount of EPS production is independent of SRT.<sup>[11,20]</sup> Liao *et al.* found that varying SRT from 4 to 12 days, led to increase of proteins/carbohydrates ratio and remain constant for SRTs above 12 days.<sup>[11]</sup> It appears that the SRT variation is an important factor on EPS production.

In this study, statistical analysis demonstrated that the sEPS production has no significant correlation with SRT. These results are in line with the previous study.<sup>[11,20]</sup> Huang reported that as SRT increases, the soluble microbial product content in the matrix raises.<sup>[20]</sup>

Furthermore, the influence of initial N concentration on EPS concentration was surveyed and the results are presented in Figure 4b. As seen in Figure 4b and Mann–Whitney test depicted that with increasing initial N concentration from 0 to 10 mg/L as  $\text{NO}_3^-$ , the carbohydrates concentration diminishes ( $P < 0.05$ ) and then increased ( $P < 0.05$ ). The variation of initial N concentration showed no significant effect on protein content. Amount of EPS production may have been



**Figure 4:** Influence of SRT and initial concentration of N on (a) COD removal, (b) EPS production and (c) sEPS production

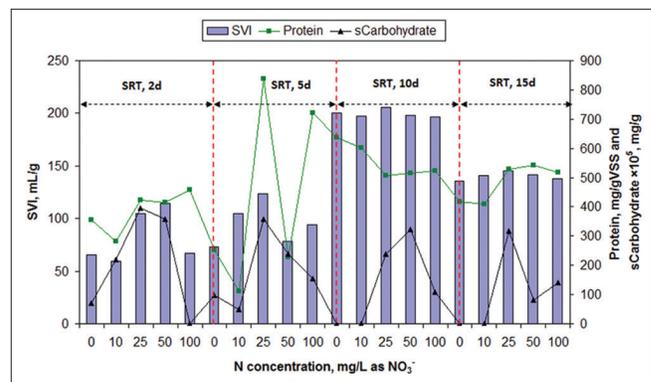
affected by several conditions,<sup>[8]</sup> and presumably, N deficiency influences microbial species to produce more carbohydrate.

According to Figure 4c, the carbohydrate and protein concentration of sEPS was improved by increasing initial N concentration from 0 to 10 mg/L. Furthermore, further increase of initial N concentration (from 10 to 100 mg/L as  $\text{NO}_3^-$ ) led to depletion of carbohydrate production. The statistical analysis showed strong relationship between concentration of protein and COD in effluent. As soluble protein of effluent increases, the amount of COD in the effluent also amplified. This may be due to that the protein content of matrix has influenced on the effluent COD. Therefore, the appropriate level of N in influent wastewater is essential for good quality of effluent.

### Sludge sedimentation properties

The sludge settling characteristics can be examined with microscopic observation of sludge or SVI. For this purpose, the variations of SVI during batch experiments were survived. Following the EPS and sEPS variations by initial nitrogen and SRT fluctuations, SVI was varied as shown in Figure 5. The amount of protein and protein-to-carbohydrate ratio in EPS has positively affected the value of SVI (Pearson correlation coefficient; 0.478 and 0.509,  $P < 0.05$ ).

Furthermore, the carbohydrate concentration in EPS showed negative effect on SVI but statistically insignificant ( $P > 0.05$ ). The value of SVI fluctuated from below 70 mL/g to above



**Figure 5:** Variation of sludge volume index, protein content of extracellular polymeric substances, and carbohydrate of soluble extracellular polymeric substances as function of initial N concentration and solid retention time

70 mL/g as carbohydrate in sEPS was reduced which was statistically significant ( $P < 0.05$ ).

As EPS have high amount of protein and sEPS contain low concentration of carbohydrate, the SVI was increased and resulted in the weak sludge settleability (viscous bulking). With respect to this, the bacteria have negative charge and EPS consist of proteins with positive charge as amino function groups. Furthermore, carbohydrate with carboxyl and phosphate function groups carried negative charges.

[11,19] As EPS contain high level of protein (carried positive charge), the EPS covered bacterial cell and resulted in positive charge on the cell. Furthermore, low concentration of carbohydrate (negative charge) cannot neutralize protein positive charge and resulted in accumulation of positive charge in the environment. Under this circumstance, generation of repulsive force between same charge lead to poor sludge settleability. A similar result was noted by Liao *et al.* Furthermore, Sheng *et al.* reported the negative effect of EPS on SVI.<sup>[1,11]</sup>

### Kinetic coefficients

The different nitrogen concentration resulted in different biological kinetic coefficients. For this purpose, the *Y* coefficient (mg VSS/mg sCOD) was calculated for the studied initial nitrogen concentration base on the previous study.<sup>[21]</sup> The *Y* coefficient at initial N concentration of 0, 10, 25, 50, and 100 mg/L as NO<sub>3</sub><sup>-</sup> was 0.32, 0.11, 0.23, 0.23, and 0.24 gVSS/gCOD, respectively. The highest value of *Y* was obtained at lowest initial concentration of nitrogen (N: 0 mg/L as NO<sub>3</sub><sup>-</sup>).

As mentioned before, the carbohydrate amount of EPS at zero initial nitrogen concentration was higher than other studied initial concentration of nitrogen. The highest *Y* value in lowest N concentration was apparent and not related to cell generation but was due to carbohydrate accumulation in matrix. In fact, the bacteria need all elemental components such as nitrogen for cell generation. In an environment with nitrogen deficiency, the bacteria cell imposed to change its normal metabolism way and produced high amount of compounds including carbohydrate. These results are in line with Eckenfelder.<sup>[22]</sup> Increase of EPS production as nitrogen decreases was observed in a study by Miqueleto *et al.*, who reported that the *Y* value decreases as nitrogen concentration in matrix improve.<sup>[2]</sup>

### CONCLUSIONS

- The results demonstrated that by changing of initial N concentration (increasing or decreasing), the COD removal efficiency remain constant
- The results showed that with increasing SRT from 2 days to other studied SRT values (5, 10, and 15 days) led to significant decrease in carbohydrate of sludge
- Statistical analysis demonstrated that the sEPS production has no significant correlation with SRT
- With increasing initial N concentration from 0 to 10 mg/L as NO<sub>3</sub><sup>-</sup>, the carbohydrates concentration diminishes and then increased. The variation of initial N concentration showed no significant effect on protein content
- The amount of protein and protein to carbohydrate ratio in EPS has positively affected the value of SVI. The carbohydrate concentration in EPS showed negative effect on SVI
- The *Y* coefficient at initial N concentration of 0, 10, 25, 50, and 100 mg/L as NO<sub>3</sub><sup>-</sup> was 0.32, 0.11, 0.23, 0.23, and 0.24 gVSS/gCOD, respectively. The highest value of *Y*

was obtained at lowest initial concentration of nitrogen (N: 0 mg/L as NO<sub>3</sub><sup>-</sup>)

- The proposed ANN modeling methodology can be used to predict the results.

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### Conflicts of interest

There are no conflicts of interest.

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