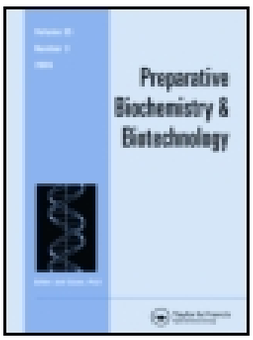

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Application of recurrent neural network for online prediction of cell density of recombinant *Pichia pastoris* producing HBsAg

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ABSTRACT

Artificial neural networking (ANN) seems to be a promising soft sensor for implementing current approaches of quality by design (QbD) and process analytical technologies (PAT) in the biopharmaceutical industry. In this study, we aimed to implement best-fitted ANN architecture for online prediction of the biomass amount of recombinant *Pichia pastoris* (*P. pastoris*) – expressing intracellular hepatitis B surface antigen (HBsAg) – during the fed-batch fermentation process using methanol as a sole carbon source. For this purpose, at the induction phase of methanol fed-batch fermentation, carbon evolution rate (CER), dissolved oxygen (DO), and methanol feed rate were selected as input vectors and total wet cell weight (WCW) was considered as output vector for the ANN. The obtained results indicated that after training recurrent ANN with data sets of four fed-batch runs, this toolbox could predict the WCW of the next fed-batch fermentation process at each specified time point with high accuracy. The *R*-squared and root-mean-square error between actual and predicted values were found to be 0.9985 and 13.73, respectively. This verified toolbox could have major importance in the biopharmaceutical industry since recombinant *P. pastoris* is widely used for the large-scale production of HBsAg.

KEYWORDS

Artificial neural networking; soft sensor; online biomass measurement; fermentation; recombinant *Pichia pastoris*; HBsAg

Introduction

Production of recombinant therapeutic proteins by using host cells such as bacteria, yeast, and animal cells is one of the key processes in the pharmaceutical industry.[1–4] *Pichia pastoris (P. pastoris)* as a member of methylotrophic yeasts is one of the host cells which has gained attention for biopharmaceuticals production.[5,6] High-expression level, post-translational processing, low cost, protein-free growth medium, and simple scalability are considered as the main advantages of this lower eukaryotic system.[7–10] Recombinant hepatitis B surface antigen (r-HBsAg) is one of the well-known approved biomedicine for human use which has been successfully produced by *P. pastoris*.[11–13] Immunization with HBsAg is the most effective way of preventing HBV infection which may lead to chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC).[14–16]

In recombinant *P. pastoris*, Mut^+ and Mut^s phenotypes are usually employed for the production of rHBsAg.[14,17–19] In both phenotypes, methanol induction is responsible for the expression of the antigen; however, in the Mut^s phenotype, the lack of Aox1 as a primary promotor for methanol induction leads to lower methanol consumption rate.[7,14,19] Although the advantages and disadvantages of each of these two phenotypes for production of various recombinant proteins still is a hot debate, the Mut^+ *P. pastoris* has already been established for the production of r-HBsAg on a commercial scale.[12,20] In this production method, at first, glycerol is used as a sole carbon source for increasing cell density of *P. pastoris*.[21,22] After reaching the maximum biomass concentration and glycerol depletion at the same time, methanol is added as a sole carbon source in the limited rate for both the purposes of rHBsAg expression and biomass growth.[14,15] During the methanol induction phase, two major points must be taken into consideration. Firstly, during methanol addition, it is critical that glycerol concentration is significantly low since the presence of higher concentration of this carbon source represses methanol induction.[23,24] Secondly, although methanol consumption rate is higher in Mut^+ phenotype, its concentration in the broth must not exceed from the specific value; otherwise, substrate inhibition occurs which prevents biomass growth and consequently r-HBsAg expression.[25–29]

Generally, the fermentation process of recombinant *P. pastoris* producing r-HBsAg is carried out in fed-batch operation for both glycerol and methanol as limiting nutrients.[30,31] In this operation mode, high-cell density fermentation is the primary approach for increasing the productivity and titer of rHBsAg.[30,32,33] However, depending on the scale of the process and design characteristic of the

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fermenter, the cell density must not exceed from a certain level as insufficient oxygen supply and heat dissipation cause a detrimental effect on physiology and metabolic reactions resulting in significant decrease of process efficiency.[5,27,34,35] For this reason, during fed-batch fermentation, the feed rate must be controlled according to the cell density. In this way, not only the cell density of *P. pastoris* will be optimized, but also excessive methanol accumulation in the fermenter will be prevented.[25,36]

Similar to many other biotechnology products, efficient fermentation process of *P. pastoris* – producing HbsAg – requires implementation of advanced automated control strategies to maintain the process under optimal condition.[31,37] Thus, during the fermentation process, the presence of robust and reliable sensors are necessary in order to examine online critical process parameters including dissolved oxygen (DO) concentration, temperature, pH, carbon source concentration, and biomass concentration.[38] For online measurement of cell density, so far, significant improvements have been made for the development of hardware sensors based on both optic and biochemical mechanisms for shake flasks, microbioreactors, and pilot-scale bioreactors.[39–44] However, for the commercial productions with large bioreactors and long fermentation time, these techniques still are not fully and globally developed due to the concerns related to the range of applicability, costs, and reliability.[40–42,45,46]

Given these constraints, special attention has been made to develop online software sensors.[47,48] Software sensors are monitoring systems, which find a correlation between less accessible and rarely measured process variables with accessible and measurable online variables.[39] Soft sensors are considered a great alternative measurement approach and since the 1980s, it has gained significant attention among scientists in the field of bioprocess engineering.[49] In the case of determination biomass amount, this unknown variable parameter is predicted based on the values of online measured parameters.[50] Mechanistic modeling is one of the main tools which is used as a soft sensor. However, due to the complex nature of the metabolic mechanism of microorganisms and non-linearity of biological processes over time, the construction of mechanistic models has always been a difficult subject, especially when the side-effect synthesis mechanisms are not well defined.[40,51]

To complete and resolve this problem in the dynamic model, the artificial neural network (ANN) is currently used to simulate and control the dynamics of biological processes.[52] So far, not enough adequate research has been done on the use of the ANN with various architectures and training processes to simulate and design biological processes, particularly for the intracellular production of r-HBsAg. Therefore, the purpose of this study was to design a best-fitted ANN to simulate and predict the biomass amount in the fed-batch methanol fermentation of recombinant *P. pastoris* producing r-HBsAg by using the previous experimental data obtained by off-line analyses. Carbon evolution rate (CER), dissolved oxygen (DO) percentage, and methanol feed rate were selected as accessible and measurable online variables for ANN input vectors.

Materials and methods

Materials

Chemicals used to prepare buffers and culture media were of analytical grade and obtained from Merck company (Germany). The Mut^+ strain of *P. pastoris* GS115 (His4) was used for expression of HBsAg under the control of inducible AOX1 promoter.[53–55] The programing and computing were conducted on a Laptop (Intel® Core™ i7-2630QM @ 2.00 GHz, 4 GB RAM, MS-Windows 10 Home) connected to 10 L fermenter (model Winpact FS01-V-B -L), shown in Fig. 1, using Matlab R2016b (ver. 9.1.0).

Methods

Using Mut^+ strain of *P. pastoris* for the biomass growth and expression of rHBsAg,[18,33,36,56] in this study, ANN was only used for wet cell weight (WCW) prediction in the final stage of fed-batch fermentation, which uses methanol as a sole carbon source. To have enough biomass at the starting point, shake flask cultivation and glycerol batch fermentation – the pre-induction phase – were performed.[18,53,57,58] Reaching the WCW of 212 g/L and depletion of glycerol in the broth, which is indicated by a sharp increase in DO,[24,56,59] methanol adaptation stage was started.[53] After adaptation of recombinant *P. pastoris* to the methanol, which was detected by the sharp decline in the DO, the initial point for collecting data and training ANN was provided.[5,53,60]

The fed-batch fermentation process was started by using methanol as a sole carbon source and limiting nutrient. The broth volume at this stage was about 4 L, and the fermentation process was performed at 29 °C and pH around 4.7–5 via adding 20% *v/v* ammonia under agitation/oxygen cascade operation (20–30%).[53] The feeding rate was based on predefined values according to large-scale production protocol aiming to have a negligible concentration of methanol residue during the fed-batch fermentation.[5,12,53] The methanol inhibition effect was tried to be minimized not only by using the selected feeding rate but also by monitoring DO and agitation/oxygen cascade profile (Fig. 1).[5]

Off-line and online analyses

The WCW was determined after separation of the biomasses (cell pellets) via centrifugation at 3000 g for 20 min.[61,62] Gas analyzer and weighing scale were used during fermentation for determination exhaust CO2 percentage and methanol feed rate, respectively.

Artificial neural network architecture

Supervised ANN was used for identifying the non-linear relationship between three input vectors including CER,

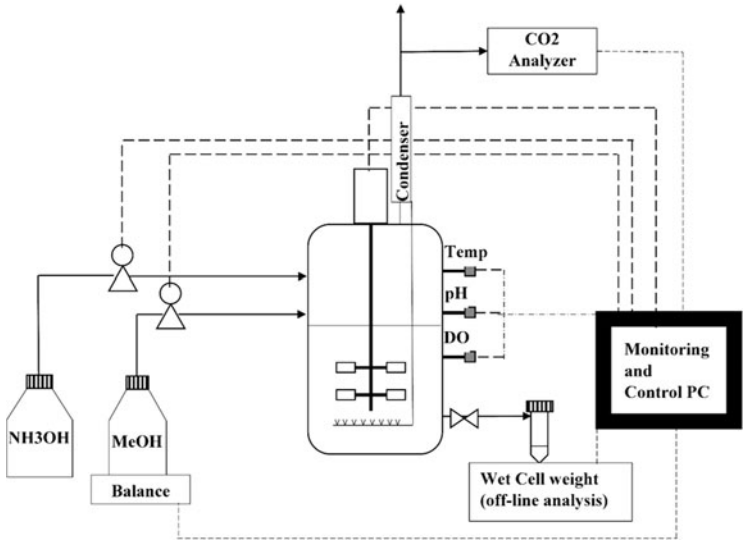

Figure 1. Schematic diagram of the experimental setup of 10 L fermenter and on-line control system with Winpact SF01 software. The H/D ratio of the vessel was 2.25 ratio containing an agitator with three Rushton impellers and it was equipped with temperature, pH, DO sensors, and CO2 gas analyzer.

DO, and methanol feed rate and an output parameter which was WCW. Various ANN topology of 3:x:1 was applied to build a predictive model in this study. As shown in Fig. 2A, the input and output layers contain 3 and 1 nodes signifying independent and dependent variables, respectively. Between input and output layers, one hidden layer existed which connected these layers by transfer weights, bias, and transfer function. In this study, we selected the number of hidden layers' variable between 2 and 8, for which tansig transfer function was used for each. The hidden layer range was chosen based on previous research studies which reported the approximate optimal range for fermentation processes.[36,40,49,63]

Training, validating, and self-testing procedure

As a training algorithm, the back propagation method based on a Levenberg Marquardt algorithm was used (Fig. 2B). In this training algorithm, the error between the predicted biomass value and the experimentally measured biomass values were calculated and propagated backward from the output layer to the hidden layer and finally to the input layer by the weights of each layer. During training, the weights and biases were adjusted by the algorithm with the goal of fitting the predicted biomass response closer to the experimental biomass response (Fig. 2B). Of 30%, given data for ANN were used for validating and self-testing. Validation tests were used during training in order to minimize overfitting problem meaning poor generalization. The ANN switched to a validation test when the mean square error was smaller than the pre-set value. The training is stopped when validation and training errors were sufficiently close. In the self-testing step, the prediction capability of the trained ANN was evaluated by data sets which were not used in the training stage (Fig. 2B).

Results and discussion

Fed-batch fermentation in contrast to chemostat fermentation does not achieve to the steady-state condition; thus, due to the complexity of the system, the prediction of fermentation behavior and key variables such as biomass concentration is a more challenging task in this operation mode. In this study, we selected three parameters of methanol feed rate, CER, and DO as input vectors for ANN. The CER value has already been identified as one of the critical factors in predicting biomass concentration in previous studies.[47,63,64] In this study, the methanol feed rate is considered another input parameter since its feed rate was variable among different fed-batch fermentation processes depending on the biomass concentration at each time point.[45,65] The fermentation profile of *P. pastoris*, specifically Mut^+, is highly dependent on the methanol concentration in the broth. Although increasing the methanol concentration until a certain level accelerates the biomass growth of *P. pastoris* after exceeding its concentration from the limit in the broth, methanol inhibition occurs which prevents biomass growth and product formation.[25] The DO was selected as another input parameter in this study. Although the dissolved oxygen DO was almost kept constant, 20% according to the protocols, during the fermentation process, we assumed that its fluctuation – in particular, in the large-scale process – might affect the error between the actual and predicted biomass growth.[40,65,66] For the fermentation process, other parameters such as initiative biomass concentration, temperature, and pH were tightly controlled and kept constant as the increase of data variation could adversely affect the quality of estimation by ANN.[36,66] Nonetheless, in the presence of such fluctuations, ANN still would be able to estimate biomass value in a high degree of precision and accuracy, given that a higher number of experimental data were provided.

The results of methanol fed-batch fermentation of four experiments are shown in Fig. 3. This figure only illustrates

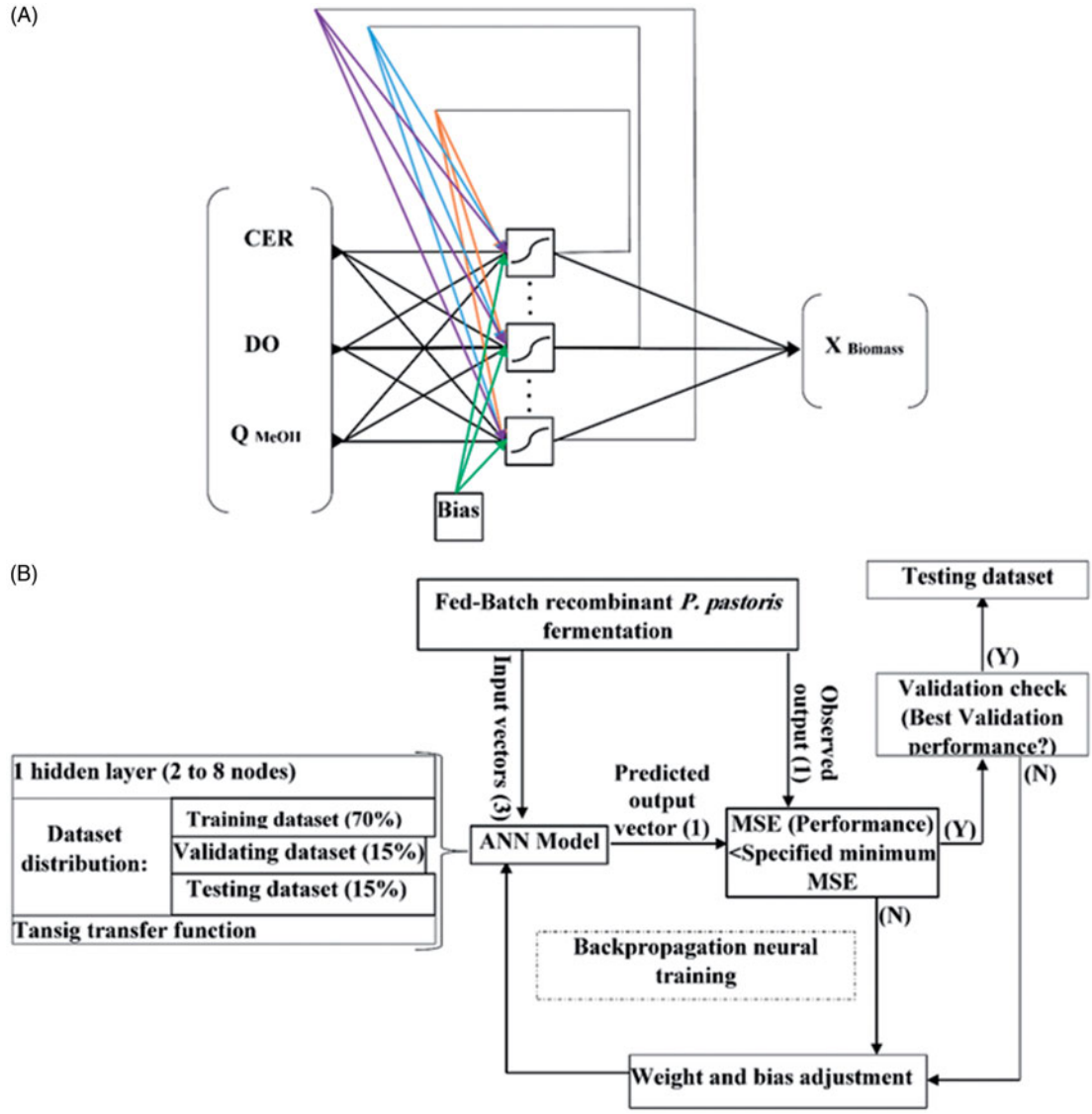

Figure 2. (A) Architecture of the neural network – recurrent – with one hidden layer. Three input vectors of DO, CER, and MeOH feed rate were used to predict the output vector biomass. The number of nodes in the single hidden layer was variable. (B) Flowchart of the model-based training procedure to train, validate, and test neural network to predict biomass amount. The loop was repeated until the network converged to a threshold value of the error function.

fed-batch fermentation using methanol as a sole carbon source – no glycerol was present in the broth. The methanol adaptation stage as well as the glycerol phase are not presented in this figure since the data from these stages were not used for ANN training and testing. Various reasons made us come to the conclusion that using such data would complicate ANN training. First of all, during shake flask and batch fermentation using glycerol – as a sole carbon source – no induction and rHBsAg expression occur. Due to the significant difference, considering both fermentation operation and *P. pastoris* metabolism, between these stages and fed-batch methanol fermentation,[67–69] ANN performance in predicting WCW could be adversely affected. Thus, it would be more practical to train separate ANN for predicting WCW in different phases. As fermentation complexity during glycerol fermentation was less than methanol induction and rHBsAg expression stage, this study only focused on the latter stage.[36,46]

Regarding methanol adaptation stage, we used an institute-specific protocol which might not be employed in other rHBsAg production methods using Mut + strain of *P. pastoris*. Hence, we tried to minimize variations related to local protocols and did not use the data obtained in this phase for training and testing ANN. In this way, we could obtain the best general ANN structure for methanol fed-batch fermentation of recombinant *P. pastoris* producing rHBsAg.

To find the best adapted neural network topology for our study, by trial and error, we pre-examined the performance accuracy and precision of various architectures of neural networks, having a hidden layer with 2–8 nodes for predicting *P. pastoris* WCW. For this purpose, the obtained data from each of the four fed-batch fermentation experiments were used separately for training these neural networks. Training, validating, and self-testing of these neural networks, the recurrent artificial neural network (RNN) with four nodes in the hidden layer demonstrated the highest

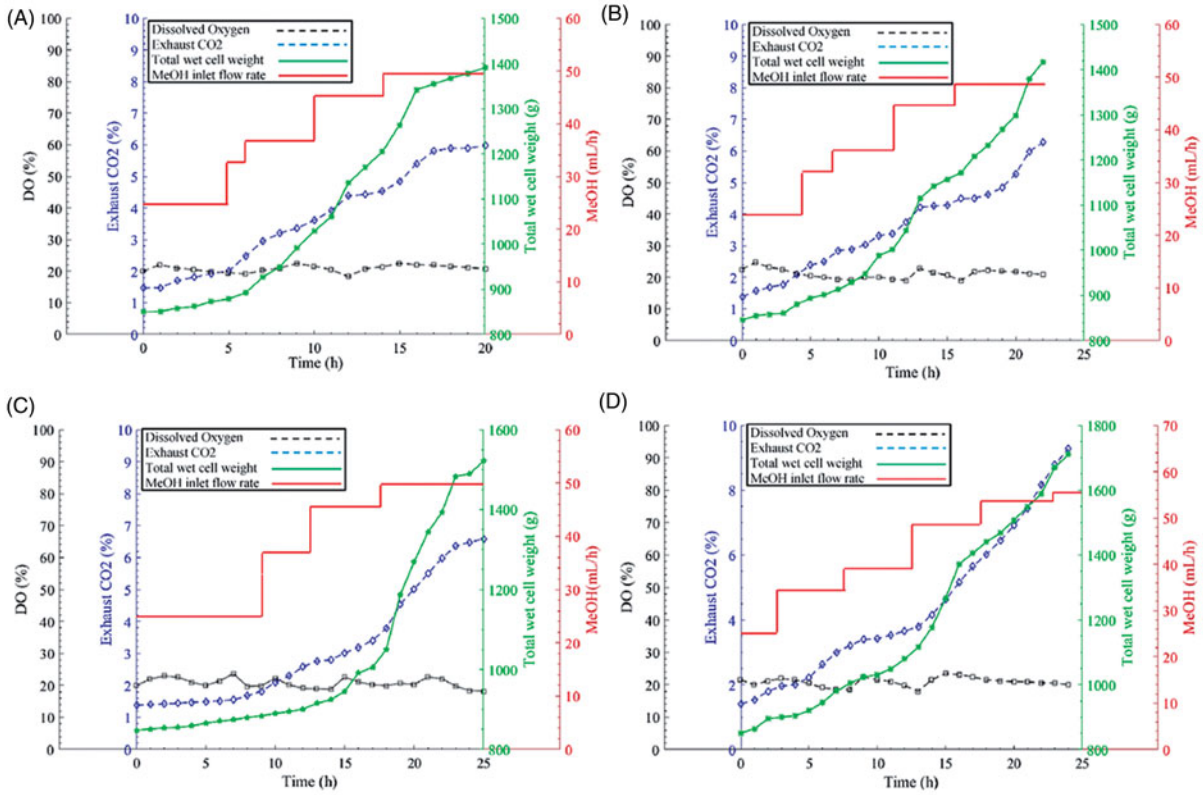

Figure 3. Four fed-batch fermentation experiments for training the artificial neural network (A–D).

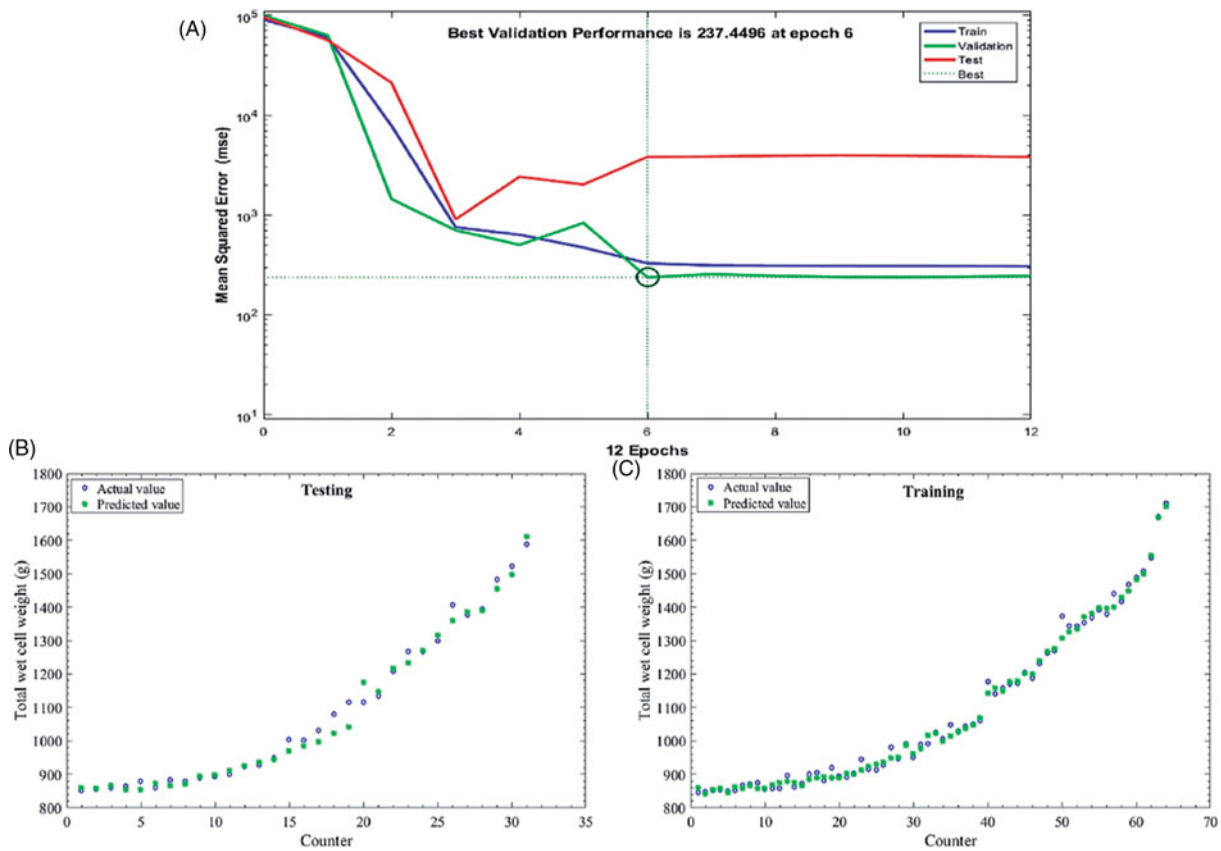

Figure 4. The results of performance (A), training (B), and self-testing (C) of ANN from the given all data sets of four methanol fed-batch fermentation experiments.

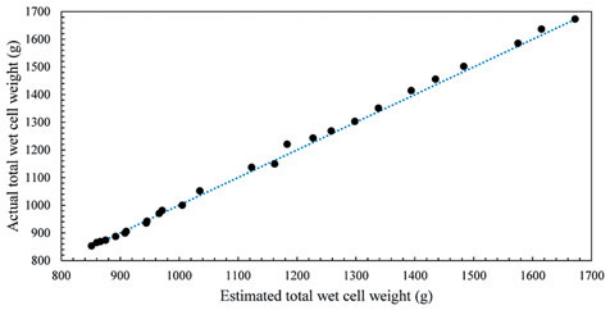

Figure 5. Comparison between predicted values by ANN and actual (off-line analyzing) values about total wet cell weight for the 5th fed-batch fermentation, non-given datasets for ANN training.

capability for providing accurate approximations. For training the RNN, the back propagation learning algorithm was used to correct the synaptic weights connecting the neurons in the adjacent layers until the desired degree of convergence was achieved. Many previous studies in the field of bioprocess engineering have used feed-forward ANN for the modeling of nonlinear systems; however, according to our datasets, the RNN demonstrated better estimation with rapid convergence.

Obtaining the best-fitted ANN topology, all four datasets of past time fed-batch processes were used to train the neural network. The results of training and testing the neural network is presented in Fig. 4. As shown in Fig. 4A, the best validation performance was at epoch 6 with root mean square error (RMSE) of 15.39. The calculated RMSE of both training and self-testing stages, Fig. 4B and Fig. 4C were 12.38 and 19.22, respectively, meaning the high performance of ANN in the learning process.

In the final and the most critical step, we examined the accuracy and precision of the trained ANN for real-time estimation of *P. pastoris* WCW for a new fed-batch fermentation experiment using the three online parameters as ANN input vectors. As shown in Fig. 5, it was found that the experimental results were in good agreement with the computed ones. The predicted values were very close to the actual values from the off-line analysis; the R-squared and RMSE were found to be 0.9985 and 13.73, respectively. The current result indicates that the ANN with the selected topology and input variable could predict WCW of recombinant *P. pastoris* – expressing intracellular rHBsAg – in a high-degree of accuracy during fed-batch methanol fermentation. In other words, it could be possible to establish the defined ANN for estimating and controlling the biomass amount in production plants of rHBsAg using recombinant *P. pastoris* as a host cell.

For the better understanding of the importance of ANN usage as an online biomass prediction tool for the robust rHBsAg production process, in another study, *P. pastoris* fed-batch methanol fermentation process will be investigated under controlled condition by linking ANN and methanol feed rate. In this way, the yield, titer, and productivity of this newly designed system will be compared with the conventional fermentation strategy using predefined methanol feed rate.

Conclusion

In this study, for the first time, recurrent ANN (3:4:1) was used for the online prediction of WCW of recombinant *P. pastoris* – producing intracellular rHBsAg. Comparing the actual biomass values and the predicted value, based on ANN, which indicated that we could successfully construct a reliable soft sensor for estimating and controlling the WCW of this recombinant methylotrophic yeast. This verified toolbox could have vital importance in the biopharmaceutical industry, as the large-scale production of rHBsAg based on *P. pastoris* has already been established in various parts of the world.

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