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RESEARCH ARTICLE

Artificial neural network modeling studies to predict the yield of enzymatic synthesis of betulinic acid ester

Mansour Ghaffari Moghaddam
Faculty of Science
University Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia
E-mail: mansghaffari@gmail.com

Faujan Bin H. Ahmad*
Faculty of Science
University Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia
E-mail: faujan@fsas.upm.edu.my

Mahiran Basri
Faculty of Science
University Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia

Mohd Basyaruddin Abdul Rahman
Faculty of Science
University Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia

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Keywords: acylation, artificial neural network, betulinic acid, *Candida antarctica* lipase, enzymatic synthesis, Novozym 435.

Abbreviations: AAD: absolute average deviation
ANN: artificial neural network
BBP: batch backpropagation
GUI: graphical user interface
IBP: incremental backpropagation
LM: Levenberg-Marquardt
MLP: multi-layer percepton
MSE: mean squared error
QP: quick propagation
R^2: coefficient of determination
RMSE: root mean squared error

3β-*O*-phthalic ester of betulinic acid was synthesized from reaction of betulinic acid and phthalic anhydride using lipase as biocatalyst. This ester has clinical potential as an anticancer agent. In this study, artificial neural network (ANN) analysis of *Candida antarctica* lipase (Novozym 435) -catalyzed esterification of betulinic acid with phthalic anhydride was carried out. A multilayer feed-forward neural network trained with an error back-propagation algorithm was incorporated for developing a predictive model. The input parameters of the model are reaction time, reaction temperature, enzyme amount and substrate molar ratio while the percentage isolated yield of ester is the output. Four different training algorithms, belonging to two classes, namely gradient descent and Levenberg-Marquardt (LM), were used to train ANN. The paper makes a robust comparison of the performances of the above four algorithms employing standard statistical indices. The results showed that the quick propagation algorithm (QP) with 4-9-1 arrangement gave the best performances. The root mean squared error (RMSE), coefficient of determination (R^2) and absolute average deviation (AAD) between the actual and predicted yields were determined as 0.0335, 0.9999 and 0.0647 for training set, 0.6279, 0.9961 and 1.4478 for testing set and 0.6626, 0.9488 and 1.0205 for validation set using quick propagation algorithm (QP).

Betulinic acid, 3β-hydroxy-lup-20(29)-ene-28-oic acid (1) is a naturally occurring pentacyclic lupane-type triterpene.

*Corresponding author

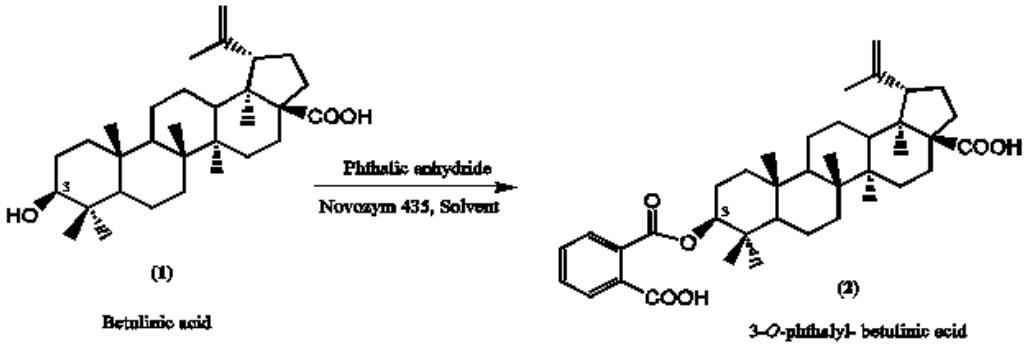

Figure 1. Reaction between betulinic acid and phthalic anhydride using Novozym 435 as biocatalyst.

It shows a broad range of biological and properties such as inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antioxidant and anticancer properties (Yogeeswari and Sriram, 2005). However, the medical uses of betulinic acid in the pharmaceutical industry is strongly limited since it is insoluble in water (0.02 mg/mL), which causes a difficulty in preparation of injectable formulations for biological assays and decreases its bioavailability in the organism. The introduction of polar groups at the C-3 and C-28 positions such as phthalates, amino acids or sugar moieties, in certain cases, increases the hydrosolubility and anticancer activity (Thibeault et al. 2007; Gauthier et al. 2008).

The methods for the synthesis of 3-*O*-acyl-betulinic acid esters based on chemical catalytic esterification have been described (Mukherjee et al. 2004; Kvasnica et al. 2005; Mukherjee et al. 2006; Rajendran et al. 2008), which have a series of disadvantages (*e.g*. formation of many by-products and high energy consumption) (Yasin et al. 2008). In contrast, application of enzymes in organic synthesis provides advantages in comparison with conventional chemical methods such as mild reaction condition, high selectivity, high catalytic efficiency and high product purity and quality (Loughlin, 2000; Zarevuka and Wimmer, 2008).

Artificial neural network (ANN) is a highly simplified model of the structure of a biological network (Mandal et al. 2009).The fundamental processing element of ANN is an artificial neuron (or simply a neuron). A biological neuron receives inputs fromother sources, combines them, performs generally a nonlinear operation on the result, and then outputs the final result (Bas and Boyaci, 2007). The basic advantage of ANN is that it does not need any mathematical model since an ANN learns from examples and recognizes patterns in a series of input and output data without any prior assumptions about their nature and interrelations (Mandal et al. 2009). ANN eliminates the limitations of the classical approaches by extracting the desired information using the input data. Applying ANN to a system needs sufficient input and output data instead of a mathematical equation (Ali Akcayol and Cinar, 2005). ANN is a good alternative to conventional empirical modeling based on polynomial and linear regressions (Kose, 2008).

Recently, ANNs are the most popular artificial learning tool in biotechnology, with applications ranging from pattern recognition in chromatographic spectra and expression profiles, to functional analyses of genomic and proteomic sequences (Manohar and Divakar, 2005). Many applications of the ANN for prediction of the biotechnological processes have been reported in the literatures (Manohar and Divakar, 2005; Szaleniec et al. 2006; Bas et al. 2007; Silva et al. 2008; Abdul Rahman et al. 2009). For example, Manohar and Divakar (2005) reported an analysis of enzymatic esterification of anthranilic acid with methanol using artificial neural network. Methanol concentration, enzyme concentration, period of incubation, buffer volume and log P-values were input parameters, while the percentage yield of ester was the output. The optimized values of network for learning rate and momentum were 0.6 and 0.8, respectively. The learning was completed in 388 cycles with an average absolute deviation of 5.7%. For testing data, absolute deviation of predicted yield was varied between 1.3 and 33%. The average absolute deviation of the predicted values from experimental values was 15%. Abdul Rahman et al. (2009) have presented an artificial neural network (ANN) trained by backpropagation algorithm to predict the yield of enzymatic synthesis of dioctyl adipate. Reaction temperature, reaction time, amount of enzyme, and substrate molar ratio were the four input variables. The best network was found to be composed of seven hidden nodes using a hyperbolic tangent sigmoid transfer function. The correlation coefficient (R^2) and mean absolute error (MAE) values between the actual and predicted responses were 0.9998 and 0.0966 for the training dataset, and 0.9241 and 1.9439 for the validating dataset.

Employing neural network models would lead to saving time and cost by predicting the results of the reactions so

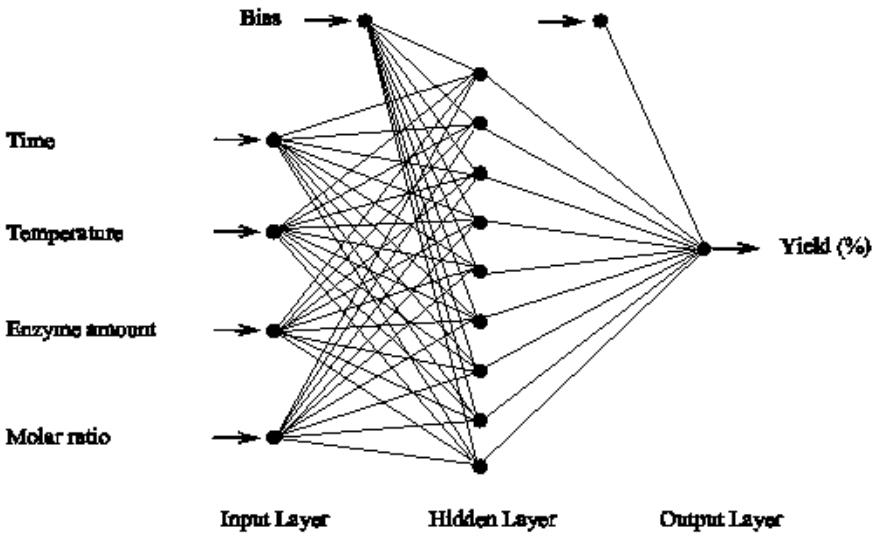

Figure 2. A multilayer feedforward perceptron (MLP) network consisting of four inputs, one hidden layer with nine neurons and one output.

that the most promising conditions can then be verified (Abdul Rahman et al. 2009).

The aim of the present work is to obtain an optimized ANN for predicting the yield of enzymatic acylation of betulinic acid with phthalic anhydride through a proper selection of the training algorithm. To do that, four training algorithms belonging to two broad classes have been evaluated: gradient descent algorithm, and Levenberg-Marquardt algorithm.

MATERIALS AND METHODS

Enzyme

Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435, 10000 PLU/g) from *Candida antarctica*, supported on a macroporous acrylic resin with a water content of 3% (w/w) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Solvents and substrates

Chloroform and *n*-hexane obtained from Fisher chemicals were used as the organic solvents. Betulinic acid was isolated from Malaysian *Callistemon speciosus* as our previous method (Ahmad et al. 1999). Phthalic anhydride was purchased from Acros, Belgium. Ethyl acetate, Celite®545, Na_2SO_4, K_2CO_3 and HCl was purchased from

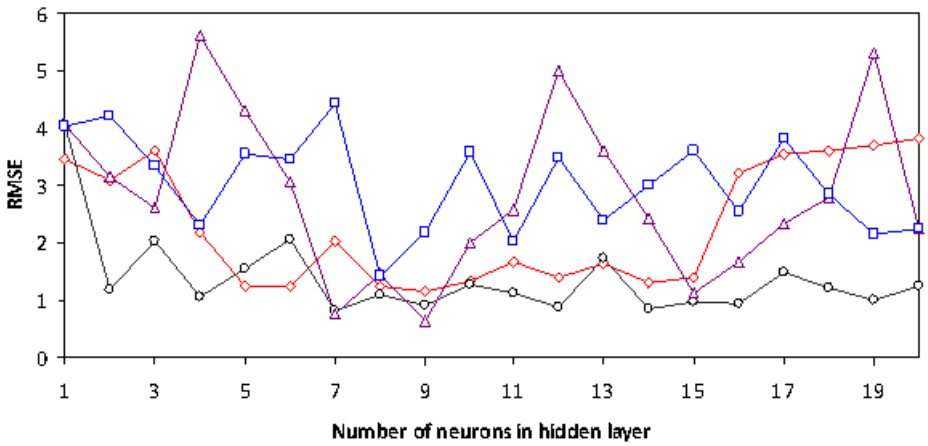

Figure 3. The performance of the network at different hidden neurons using:
(◇) Incremental backpropagation (IBP) algorithm.
(○) Batch backpropagation (BBP) algorithm.
(△) Quick propagation (QP) algorithm.
(□) Levenberg-Marquardt (LM) backpropagation algorithm.

Table 1. Experimental values (training, testing and validation data), actual and model predicated of isolated yield on the enzymatic reaction.

Run No.	Time (h)	Temperature (ºC)	Amount of Enzyme (mg)	Molar ratio[1]	Isolated Yield (%)	
					Actual	predicted
Training Data						
1	8	50	150	0.6	33.3	33.28
2	24	50	150	0.6	58.8	58.85
3	16	40	150	0.6	31.1	31.11
4	16	50	50	0.6	39.8	39.78
5	16	50	250	0.6	43.1	43.15
6	16	50	150	0.2	29.5	29.51
7	12	45	100	0.4	20.2	20.24
8	20	45	100	0.4	36.5	36.49
9	20	55	100	0.4	47.4	47.39
10	12	45	200	0.4	27.6	27.57
11	20	45	200	0.4	43.2	43.15
12	12	45	100	0.8	35.6	35.58
13	20	45	100	0.8	49.1	49.11
14	12	55	100	0.8	55.2	55.22
15	12	45	200	0.8	40.8	40.81
16	20	45	200	0.8	58.6	58.54
17	12	55	200	0.8	52.5	52.44
18	20	55	100	0.8	62.7	62.64
19	16	60	150	0.6	53.3	53.3
20	16	50	150	1.0	58.9	58.94
21	16	50	150	0.6	54.7	54.57
Testing Data						
22	20	55	200	0.4	46.4	46.69
23	12	55	100	0.4	36.2	35.32
24	12	55	200	0.4	35.4	36.2
25	20	55	200	0.8	60.4	60.12
Validation Data						
26	24	45	176	1.0	57.5	58.11
27	24	50	176	1.0	60.5	60.23
28	24	55	176	1.0	61.8	61.34
29	24	60	176	1.0	57.3	58.12
30	20	53	148	0.8	64.3	64.91
31	20	54	145	0.9	64.7	65.65

[1]Molar ratio = mmol betulinic acid/mmol phthalic anhydride

Merck, Germany. All chemicals were of analytical reagent grade.

Enzymatic esterification

The enzymatic reaction performed in this study is shown in Figure 1. To a magnetically stirred solution of betulinic acid (25 mg, 0.0547 mmol), K_2CO_3 (6 mg), Celite®545 (170 mg), different amounts of enzyme (50-250 mg), chloroform (10 ml) and *n*-hexane (10 ml) were added phthalic anhydride with difference molar ratio (mmol betulinic acid /mmol phthalic anhydride; 0.2-1). The reaction mixture was magnetically stirred (150 rpm) at different reaction temperatures (40-60°C) and reaction times (8-24 hrs) as shown in Table 1. Each reaction was repeated in triplicate and results represented were the mean values of three independent experiments. Control experiments were performed in the absence of enzyme. As a result, no chemical acyl transfer reaction was detected.

Analytical procedures

TLC analysis. Qualitative analysis of reaction mixtures was made by thin layer chromatography (TLC) on silica gel plates eluted with system *n*-hexane/ethyl acetate (9:1, v/v). The plates were visualized under UV lamp and/or iodine vapor. Under these conditions, 3-*O*-phthalyl- betulinic acid had an R_f of 0.9.

Determination of 3-*O*-phthalyl- betulinic acid (2). Quantitative analysis of samples was made according to the procedure described by Kvasnica et al. (2005). At predetermined time intervals, flasks were taken and enzyme was removed by filtration and washed twice with chloroform. The filtrate was evaporated to dryness and ethyl acetate was then added and washed twice with aqueous solution of HCl and twice with water. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was chromatographed with

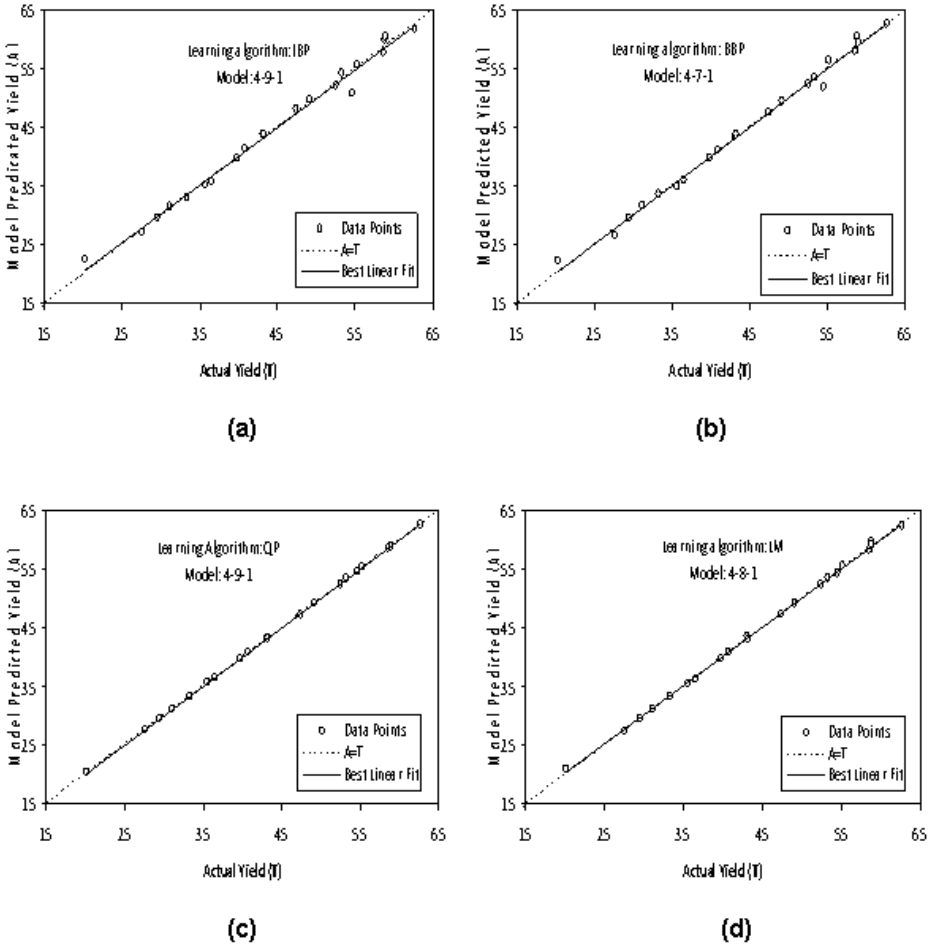

Figure 4. The scatter plots of ANN predicted yield *versus* actual yield for training data set from:
(a) Incremental backpropagation (IBP) algorithm.
(b) Batch backpropagation (BBP) algorithm.
(c) Quick propagation (QP) algorithm.
(d) Levenberg- Marquardt (LM) backpropagation algorithm.

gradient on silica gel 60 (*n*-hexane/ethyl acetate, 9:1-5:1, v/v). The ester fractions were combined and weighed after evaporation of the solvents. The percentage isolated yield of ester (%Yield) is defined as:

$$\%\text{Yield} = \frac{\text{mmol isolated betulinic acid ester}}{\text{mmol used betulinic acid}} \times 100$$

[Equation 1]

Characterization of 3-*O*-phthalyl- betulinic acid (2). The product has been characterized by recording the ^{1}H & ^{13}C-NMR spectra of the compound on a Varian Unity Inova 500 NMR spectrometer operating at 26ºC and matched literature data (Kvasnica et al. 2005).

Experimental design and ANN modelling

Software tool. Commercially available NeuralPower, professional version 2.5 was employed in this study (CPC-X Software, 2004). This software is a Windows®-based package, which supports several types of training algorithms. NeuralPower operates via a graphical user interface (GUI) that enables the user to load the training and test sets, design the network architecture, select the training algorithm and generate the individual models for each output variable in a single operation (Ghaffari et al. 2006).

Data sets. The experimental data used for ANN design are presented in Table 1. The experimental data were randomly divided into two sets using the option available in the software: 21 of data sets were used as training data and four data sets were used as testing data. The training data was used to compute the network parameters. The testing data was used to ensure robustness of the network parameters. If a network ''learns too well'' from the training data, the rules might not fit as well for the rest of the cases in the data. To avoid this ''overfitting'' phenomenon, the testing stage was used to control error; when it increased, the training was stopped (Song et al. 2004). Moreover, six additional experiments were carried out in the range of values given for ANN design. The data from these experiments were excluded from training and testing as unseen or "validation data" to assess the predictive ability

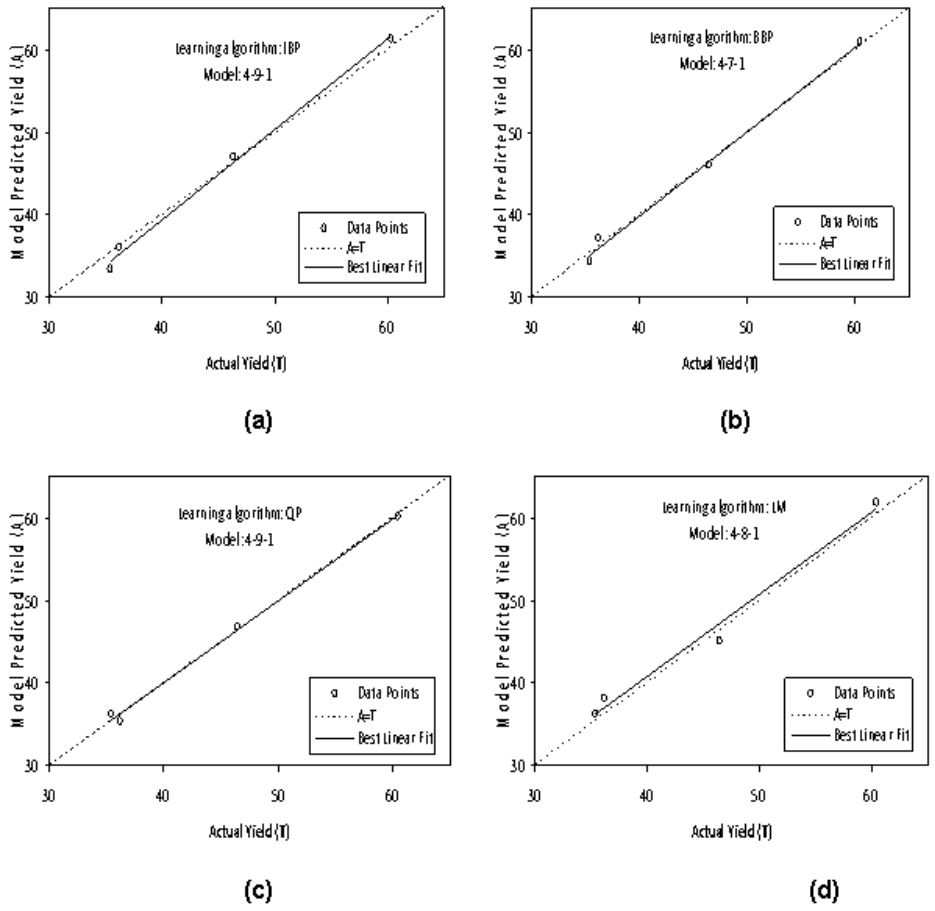

Figure 5. The scatter plots of ANN predicted yield *versus* actual yield for testing data set from:
(a) Incremental backpropagation (IBP) algorithm.
(b) Batch backpropagation (BBP) algorithm.
(c) Quick propagation (QP) algorithm.
(d) Levenberg- Marquardt (LM) backpropagation algorithm.

of the generated model (Amani et al. 2008). The experimental data used for validation data (unseen data) are also presented in Table 1.

ANN description. In this study, a multi-layer perceptron (MLP) based feed-forward ANN which uses back-propagation learning algorithm, was applied for modeling enzymatic synthesis of 3-*O*-phthalyl- betulinic acid. This network is the most popular ANN (Cheng et al. 2008; Jorjani et al. 2008). The network consists of an input layer, hidden layers and an output layer. Inputs for the network are reaction time, reaction temperature, enzyme amount and substrate molar ratio; output is percentage isolated yield of ester. Feed-forward neural network usually has one or more hidden layers, which enable the network to model non-linear and complex functions (Jorjani et al. 2008). The number of hidden layers is difficult to decide (Ghaffari et al. 2006). It was reported in the literature that one hidden layer is normally adequate to provide an accurate prediction and can be the first choice for any practical feed-forward network design (Irie and Miyake, 1988; Cybenko, 1989; Hush and Horne, 1993; Shankar and Bandyopadhyay, 2007). Therefore, a single hidden layer network was used in this study. The structure of proposed ANN is shown in Figure 2.

The determination of number of neurons in hiddenlayers is very important as it affects the training time and generalization property of neural networks. A higher value of neurons in hidden layer may force the network to memorize (as opposed to generalize) the patterns which it has seen during training whereas a lower value of neurons in hidden layer would waste a great deal of training time in finding its optimal representation (Hussain et al. 1992). There is no general rule for selecting the number of neurons in a hidden layer. It depends on the complexity of the system being modelled (Cheng et al. 2008). The most popular approach to finding the optimal number of neurons in hidden layer is by trial and error (Ahmed, 2005). In this study, trial and error approach was used to determine the optimum neurons in hidden layer of the network (examined from 1 to 20 neurons).

Scaled data are passed into the input layer and then is propagated from input layer to hidden layer and finally to the output layer of the network (Hussain et al. 2002). Every node in hidden or output layer firstly acts as a summing junction which combines and modifies the inputs from the previous layer using the following equation (Jorjani et al. 2008):

$$y_i = \sum_{j=1}^{i} x_i w_{ij} + b_j$$

[Equation 2]

where y_i is the net input to node j in hidden or output layer, x_i are the inputs to node j (or outputs of previous layer), w_{ij} are the weights representing the strength of the connection between the ith node and jth node, i is the number of nodes and b_j is the bias associated with node j. Each neuron consists of a transfer function expressing internal activation level. Output from a neuron is determined by transforming its input using a suitable transfer function (Razavi et al. 2003). Generally, the transfer functions for function approximation (regression) are sigmoidal function, hyperbolic tangent and linear function (Jorjani et al. 2008). The most popular transfer function for non-linear relationship is the sigmoidal function (Bowen et al. 1998; Mohanty, 2005; Ghaffari et al. 2006; Shankar and Bandyopadhyay, 2007; Torrecilla et al. 2007). The general form of this function is as follows (Jorjani et al. 2008):

$$z_j = \frac{1}{1+e^{-y_y}}$$

[Equation 3]

z_j, the output of node j, is also an element of the inputs to the nodes in the next layer. In this study, the sigmoidal function was used as the transfer function for the hidden and output layer nodes. The sigmoidal function is bounded between 0 and 1, so the input and output data should be normalized to the range 0 to 1 (Razavi et al. 2003). During

Table 2. Statistical measures and performances of four learning algorithms on the enzymatic synthesis of betulinic acid ester.

Learning algorithm	The best architecture	Training data				Testing data		
		RMSE	R^2	AAD		RMSE	R^2	AAD
Quick Propagation (QP)	4-9-1	0.0335	0.9999	0.0647		0.6279	0.9961	1.4478
Incremental Backpropagation (IBP)	4-9-1	1.1738	0.9900	2.0796		1.1469	0.9871	2.2973
Batch Backpropagation (BBP)	4-7-1	0.9582	0.9933	1.7536		0.8096	0.9935	1.8932
Levenberg-Marquardt (LM)	4-8-1	0.2909	0.9993	0.5402		1.4214	0.9801	3.2160

training initial neural network, weights are chosen randomly. If one input has large number and another has a small number, but both show a similar amount of variance, then the network may ignore the small input due to the large contribution from the other input (Khare and Shiva Nagendra, 2007). Therefore, normalization (scaling) of data within a uniform range (*e.g.*, 0-1) is essential to avoid data with larger magnitude from overriding the smaller ones. Also, it is necessary to prevent premature saturation of hidden nodes, which impedes the learning process (Basheer and Hajmeer, 2000). Scaling of the data to the range 0-1 is carried out automatically within NeuralPower software.

There are many types of learning algorithms in the literature which can be used for training of the network (Krose and Smagt, 1996; Haykin, 1998; Christodoulou and Georgiopoulos, 2001). However, it is so difficult to know which learning algorithm will be more efficient for a given problem (Saracoglu, 2008). The algorithms used to train ANN in this study are standard or incremental backpropagation (IBP), batch backpropagation (BBP), quick propagation (QP) and Levenberg-Marquardt backpropagation (LM). These algorithms are belonging to two classes: gradient descent backpropagation algorithm and Levenberg-Marquardt backpropagation algorithm. The details of the algorithms have been reported elsewhere (Ghaffari et al. 2006).

The learning rate and momentum coefficient for the networks were chosen as the default values of the NeuralPower software. Therefore, the default values of network for learning rate and momentum coefficient are 0.15 and 0.8 using incremental backpropagation (IBP) and 0.1 and 0.4 using batch backpropagation (BBP), respectively. The default value for learning rate using quick propagation (QP) is 0.8 and momentum coefficient is not employed in QP mode (Ghaffari et al. 2006).

Evaluation of model predictability. In order to perform a supervised training, a way in which the ANN output error between the actual and the predicted output could be evaluated is therefore required. A popular measure is the mean squared error (MSE) or root mean squared error (RMSE) (Ghaffari et al. 2006):

$$MSE = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - y_{di}\right)^2 \qquad \textbf{[Equation 4]}$$

$$RMSE = (MSE)^{\frac{1}{2}} \qquad \textbf{[Equation 5]}$$

where nis the number of points, y_i is the predicted value obtained from the neural network model, y_{di} is the actual value.

The coefficient of determination, R^2 reflects the degree of fit for the mathematical model (Nath and Chattopadhyay, 2007). The closer the R^2 value is to 1, the better the model fits to the actual data (Sin et al. 2006):

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(y_i - y_{di})^2}{\sum_{i=1}^{n}(y_{di} - y_m)^2} \qquad \textbf{[Equation 6]}$$

where n is the number of points, y_i is the predicted value obtained from the neural network model, y_{id} is the actual value, and y_m is the average of the actual values.

Absolute average deviation (AAD) is another important index to evaluate the ANN output error between the actual and the predicted output (Bas and Boyaci, 2007):

$$AAD = \left\{\left[\sum_{i=1}^{n}\left(\left|y_i - y_{di}\right| / y_{di}\right)\right] / n\right\} \times 100 \qquad \textbf{[Equation 7]}$$

where y_i and y_{di} are the predicted and actual responses, respectively, and nis the number of the points. The network having minimum RMSE, minimum AAD and maximum R^2 is considered as the best neural network model (Basri et al. 2007; Izadifar and Zolghadri Jahromi, 2007; Wang et al. 2008).

RESULTS AND DISCUSSION

ANN model training with gradient descent backpropagation algorithms

At first, the gradient descent backpropagation algorithms in three versions were used to train the neural networks. In order to determine the optimum number of neurons in hidden layer, a series of topologies was examined, in which the number of neurons was varied from 1 to 20. The root mean square error (RMSE) was used as the error function. Also, the coefficient of determination (R^2) and the absolute average deviation (AAD) were used as a measure of the predictive ability of the network. Decision on the optimum topology was based on the minimum error of testing. Each topology was repeated five times to avoid random correlation due to the random initialization of the weights (Kasiri et al. 2008). After repeated trials, it was found that a network with 9 hidden neurons produced the best performances when IBP algorithm was employed. Similarly, the best results obtained with 9 hidden neurons using QP algorithm. However, a network with 7 hidden neurons produced the best results for BBP algorithm. These topologies have lowest RMSE for the training and testing

sets. Figure 3 illustrates the performance of the network for testing data *versus* the number of neurons in the hidden layer using IBP, BBP and QP algorithms.

ANN model training with Levenberg-Marquardt backpropagation algorithm

Various topologies (from 1 to 20 hidden neurons) using Levenberg-Marquardt (LM) algorithm were examined. The results show that a network with 8 hidden neurons produced the best performances. The performance of the network for testing at different hidden neurons using LM algorithm is also shown in Figure 3.

Selection the best neural network model

The results for various algorithms are summarized and presented in Table 2. As shown in Table 2, the quick propagation algorithm has a better performance relative to incremental backpropagation, batch backpropagation and Levenberg–Marquardt backpropagation algorithms, because the best result derived from QP algorithm with 4-9-1 topology that has minimum RMSE, maximum R^2 and minimum AAD for both training and testing set. Figure 4 and Figure 5 show the scatter plots of ANN predicted yield *versus* actual yield with IBP, BBP, QP and LM algorithms for the training and testing sets, respectively. The predicted model using quick propagation algorithm was fitted so well to the actual values for both training and testing set. Therefore, it could be suggested that model trained with QP algorithm is the most efficient model for this problem; hence this model has been applied for further application. It was reported in literature that the quick propagation learning algorithm can be adopted for the training of all the ANN models (Jain et al. 2008). The predicted values of the best model for training and testing set are presented in Table 1.

Model validation

The predictive ability of generated model was estimated using validation data (unseen data) which were excluded from training. The actual and predicted yields for validation data are also presented in Table 1. The root mean squared error (RMSE) for validation data is 0.6626; the coefficient of determination (R^2) is 0.9488; and the absolute average deviation (AAD) is 1.0205. This results show that the predictive accuracy of the model is high. Figure 6 shows a comparison between actual values and model predicted output values using adopted neural network model for validating data.

CONCLUDING REMARKS

An artificial neural network for enzymatic synthesis of betulinic acid ester has been optimized through a proper selection of the training algorithm. Different ANNs, trained with standard or incremental backpropagation (IBP), batch backpropagation (BBP), quick propagation (QP) and Levenberg-Marquardt backpropagation (LM), have been

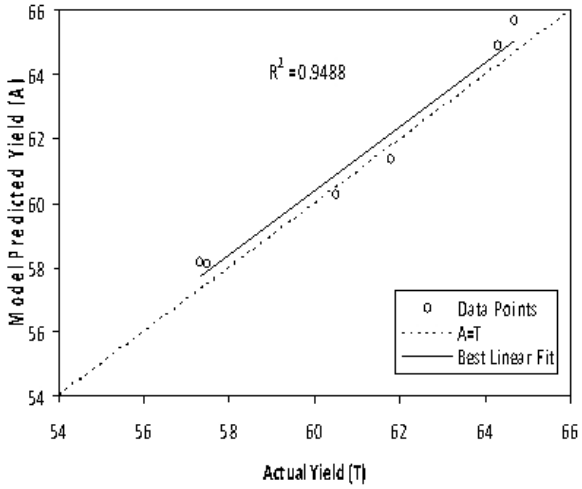

Figure 6. The scatter plot of ANN predicted yield *versus* actual yield for validation data.

evaluated with respect to their predictive ability. A robust comparison of the performances of the above four algorithms was made employing standard statistical indices. The results of this study show that the quick propagation algorithm implemented by NeuralPower software gave the best performances. The optimal configuration of the ANN model using quick propagation algorithm found to be 4-9-1. Therefore, it can be concluded that the ANN model described in this paper is an efficient quantitative tool to predict the isolated yield of ester in the enzymatic synthesis of betulinic acid ester. Finally, it was reported in literature that the appropriate selection of the training algorithm allows maximizing the predictive ability of the artificial neural network (Torrecilla et al. 2007). Thus, the results of this paper show that a correct selection of training algorithm is essential for successful data modeling using artificial neural network.

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