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Statistical Optimization Of Culture Parameters For Lipase Production From Lactococcus lactis And Its Application In Detergent Industry.

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Abstract*:* Optimization of lipase production by *Lactococcus lactis* was carried out using response surface methodology (RSM), artificial neural network (ANN) and genetic algorithm (GA). The influence of various physico-chemical parameters, viz. temperature, oil concentration**,** inoculum volume, pH and incubation period on lipase production was examined. The optimum operating conditions obtained from the quadratic form of the RSM and ANN models with GA were pH 6.7, temperature 35 ^{o}C, and inoculum volume of 1.5, substrate volume 2, with 13 U/ml of predicted lipase activity within 43h of incubation. The results demonstrated a higher prediction accuracy of ANN with GA compared to RSM with GA. This superiority of ANN with GA over other multi factorial approaches could make this estimation technique a very helpful tool for fermentation monitoring and control.
Keywords: Extracellular lipase, *Lactococcus lactis*, Response surface methodology, artificial neural network, Genetic algorithm.

Introduction and Experimental

Lipases (triacylglycerol acylhydrolases, EC no: 3.1.1.3), are a group of biotechnologically relevant enzymes and find immense applications in food, dairy, detergent, pharmaceutical and leather industries[1]. Promising fields for the application of lipases also include the biodegradation of plastics and the resolution of racemic mixtures to produce optically active compounds. Today, lipases stand amongst the most important biocatalysts. They carry out novel reactions in both aqueous and nonaqueous media. Lipases are used to hydrolyze ester bonds of a variety of nonpolar substrates at high activity, chemo-, region- and stereo-selectivity. Moreover, they are used to catalyze the reverse reactions (such as esterification and transesterification) in nonpolar solvents. The interest in microbial lipase production increased in the last decades, because of its large potential in industrial applications[2]. Lipases are available from many sources however; the most suitable sources for lipase production are microbes including bacteria, fungi and yeast. These microorganisms can produce high quality lipases in lower cost and shorter time[3]. Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. Thus to search for new lipases with different characteristic and improve lipase production continue to be important research topics.

Study was done on alkaline lipase production by solid-state fermentation of agro-

industrial wastes by lipase producer strain of Enterobacter aerogenes. Use of such wastes as substrates instead of commercial synthetic media was done to develop a cost-effective method for lipase production[4] . Both extracellular and cell-bound lipases were reported to be produced by *Geotrichum* sp., and its cell-bound lipase production was optimized, and used to synthesize methyl oleate in microaqueous hexane yielding 94% conversion after 24h[5] . The low amount of recombinant lipase was expressed as an active enzyme in *Escherichia coli* harboring a plasmid with the clustered lipase and lipase specific foldase genes[6].

Improving fermentation conditions is the most frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in the microbial system. This approach is not only time consuming, but also ignores the combined interactions between physicochemical parameters. Hence Response surface methodology (RSM) which includes factorial design and regression analysis helps in evaluating the effective factors and in building models to study interaction and select optimum conditions of variables for a desirable response. An artificial neural network (ANN) is superior and more accurate modeling technique when compared to the RSM method as it represents the nonlinearities in much better way[7]. Genetic algorithm (GA) is a stochastic optimization technique that searches for an optimal value of a complex objective function and are used to solve complicated optimization problems by simulation or mimicking a natural evolution. These techniques are often used in predicting optimum process conditions for microbial enzyme production[8].

It is well known that extracellular lipase production in microorganism is greatly influenced by physical factors like pH, temperature, incubation period, substrate volume and inoculum volume. There are several reports on increase in production of lipase by optimization[9]. However, no reports are available on optimization using all the above three techniques using *Lactococcus lactis*. Therefore considering the many industrial applications of lipase, we report here the optimization of extracellular lipase production from *Lactococcus lactis* using response surface methodology, artificial neural network and genetic algorithm.

Materials and Methods

Microorganism

For present investigation, *Lactococcus lactis* producing extracellular lipase was used for these experiments. The strain was maintained on brain heart infusion agar slants at 4 ^{0}C.

Chemicals

Chemicals were all of analytical grade. The oil used as a substrate in the medium was purchased from the local market.

Composition of medium

Brain heart infusion broth was used to maintain mother culture with composition of Brain heart extract (17.5g/l), Peptone (10 g/l), Glucose (2g/l), Sodium chloride (5 g/l), Disodium hydrogen phosphate (2.5g/l).

Lipolytic Activity

For preliminary screening of lipase producing bacteria, tributyrin agar was used. All the isolated cultures were inoculated into tributyrin agar plates and kept for incubation at 37°C for 24 hours and observed for zone formation[10]. A clear zone around the colonies indicates the production of lipase.

Enzyme assay

The lipase assay was performed spectrophoto metrically using p-nitro phenyl palmitate as substrate. P-nitro phenol was liberated from p-nitro phenyl palmitate by lipase mediated hydrolysis[11]. One unit (U) of lipase was defined as the amount of enzyme that liberates one micromole of p-nitro phenol per minute under the assay conditions[12].

Experimental design and Lipase production

Lipase assay medium was used for growth and production of lipase from *Lactococcus* sp. The extracellular lipase production was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of a medium composed of peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), MgSO4 (0.05%) and olive oil (substrate).The optimum levels for lipase production by the *Lactococcus* strain with respect to incubation period, temperature, pH and inoculum volume, substrate volume, were obtained by single factor optimization by conducting the experiments in 250 ml Erlenmeyer flasks containing 50 ml of medium with oil as a substrate inoculated with the freshly prepared bacterial suspension at 37 ^{0}C. After incubation, the cell-free supernatant was obtained by centrifugation at 7860

rpm and the extracellular lipase activity of the fermented broth determined.

In the next stage, both response surface methodology and the artificial neural network along with GA were used to study the interactive effects of five variables, i.e. pH, temperature, inoculum volume, incubation period, substrate volume for improving total lipase production. Experiments were conducted in triplicate and results were the average of these three independent trials.

Response surface methodology

RSM is a combination of mathematical and statistical techniques for empirical model building and optimization. It examines the relationships between one or more response parameters and a set of experimental input parameters. This model is only an approximation, but it is used extensively because such a model is easy to estimate and apply, even when little is known about the process. RSM had not only been used for optimization of culture parameters in the fermentation process[13,14,15] but also for studying the combined effects of medium components. The principle of RSM was described by Khuri and Cornell. Using RSM, the relationship among the variables, i.e. initial pH, temperature, incubation period, inoculum volume, substrate volume were expressed mathematically in the form of a polynomial model, which gave the response as a function of relevant variables. A functional relationship between response and independent process parameters based on a full quadratic model is postulated by:

$$y_i = a_0 + \sum_{i=1}^{5} a_i x_i + \sum_{i=1}^{5}\sum_{j=1}^{5} a_{ij} x_i x_j$$

where y_i was the predicted response (lipase production) used as a dependent variable; x_i (i = 1, 2, 3, 4 and 5) were the input predictors or controlling variables; and a_0, a_i (i = 1, 2, 3, 4, 5) and a_{ij} (i = 1, 2, 3, 4, 5; $j = i, \ldots, 5$) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least-squares method. Designs of this type are usually chosen when there is suspecting curvature in the response surface. These process parameters and their different levels are presented in Table 1. Thus a total of 4*4*5*4*4 = 1280 experiments were conducted to formulate RSM model for lipase production using the pH, Temperature (^{0}C), Incubation period (hours), Inoculum volume (ml) and Substrate volume (ml).

The analysis of the developed RSM models is shown in Table 2. The analysis of variance (ANOVA) and the F-ratio test have been performed to justify the goodness of fit for this RSM model. Both the high adjusted-R^2 value and the close to zero p-value in the analysis of variance (ANOVA) presented in Table 3 show that this RSM model for lipase production has a satisfactory goodness of fit. In Table 2 the second column, Coef, presents the estimated regression coefficients for lipase production.

Table 1 - Process parameters and levels of the experiment

Level	pH	Temperature (^{0}C)	Incubation Period (hours)	Inoculum volume(ml)	Substrate volume(ml)
1	5	25	12	0.5	0.5
2	6	30	24	1	1
3	7	35	36	1.5	1.5
4	8	40	48	2	2
5	-	-	60	-	-

Table 2- Estimated Regression Coefficients for yield

Term	Coef	SE Coef	T	P
Constant	-2.06068	0.066417	-31.026	0.000
Ph	0.43559	0.012695	34.312	0.000
T	0.03587	0.002539	14.129	0.000
Ip	0.00570	0.000529	10.780	0.000
S	0.07126	0.016449	4.332	0.000
I	0.01294	0.016449	0.787	0.432
ph*ph	-0.03518	0.000879	-40.011	0.000
t*t	-0.00062	0.000035	-17.683	0.000

ip*ip	-0.00007	0.000004	-18.331	0.000
s*s	-0.01948	0.003517	-5.540	0.000
i*i	-0.00482	0.003517	-1.369	0.171
ph*t	0.00146	0.000141	10.398	0.000
ph*ip	-0.00000	0.000046	-0.047	0.963
ph*s	-0.00194	0.001407	-1.377	0.169
ph*i	0.00001	0.001407	0.007	0.994
t*ip	-0.00002	0.000009	-1.725	0.085
t*s	-0.00019	0.000281	-0.691	0.490
t*i	0.00003	0.000281	0.122	0.903
ip*s	0.00001	0.000093	0.100	0.921
ip*i	0.00002	0.000093	0.266	0.791
s*i	0.00005	0.002814	0.019	0.985

Coef- coefficient
SE coef- standard coefficient
T-T test
P-probability
S = 0.0314592 PRESS = 1.27980 R-Sq = 76.27% R-Sq (pred) = 75.63% R-Sq (adj) = 75.89%
[2] Response Surface Regression: Yield versus pH (pH), Temperature (T), Incubation period (IP), substrate (S) and Inoculum volume (I).

Artificial Neural Network Model

Artificial neural network is a powerful data information treatment system which tries to simulate the neural networks structure of the human brain. It can represent and capture complex non-linear relationships between inputs and outputs. Each neural network is composed of an input layer, an output layer and one or more hidden layers, which are connected by the processing units called neurons. Each neuron works as an independent processing element, and has an associated transfer function, which describes how the weighted sum of its inputs is converted to the results into an output value. Currently, there are diverse training algorithms available. Among the various kinds of ANN approaches that have existed, the back propagation (BP) learning algorithm has become the most popular in predictive applications. Back propagation algorithm is based on minimization of the quadratic cost function by tuning the network parameters. The mean square error (MSE) is considered as a measurement criterion for a training set. Specially, BP neural network is the most suitable tool for treating non-linear systems.

Hence, a back propagation algorithm was applied to train a feed forward neural network, which is reliable and most commonly utilized. In this investigation, the input variables of ANN include pH, Temperature, Incubation Period, Substrate, Inoculum, while the output variable is Yield. Hence, a feed forward network trained with the back propagation algorithm was developed.

Before training the network, the input and output datasets have been normalized within the range of 0.05 – 0.95 to prevent a specific factor from dominating the learning for the ANN model. The main reason for normalizing the data matrix where the variables have been measured in different units is to recast them in to the dimensionless units to remove the arbitrary effect of similarity between the objects. Thus, using Eq. (1), the experimental data was normalized to make the neural network training more efficient prior to the use of the datasets.

$x_n = 0.05 + 0.90*(x - x_{min})/(x_{max} - x_{min})$… (1)

where x_{min} and x_{max} are the minimum and maximum values of x and x_n is the normalized data of the corresponding x. Once the best trained network is found, all the transformed data returns to their original value using Eq. (2)

$x = x_{min} + (x_n - 0.05)*(x_{max} - x_{min})/0.9$ … (2)

One of the most unresolved questions in the literature on ANN is what architecture should be used for a given problem. Architecture selection requires choosing both the appropriate number of hidden units and the connections thereof. The desirable network architecture contains as minimal as possible hidden units and connections necessary for a good approximation of the true function. In most of the applications of ANN, this selection was done using a trial-and-error procedure. The number of hidden layers determines the complexity of neural network and precision of predicted values. If the architecture is

too complex, it may not converge during training or the trained data may be over fitted. In other way, the trained network might not have sufficient ability to learn the process correctly. Therefore, various network structures with varying number of neurons in hidden layer were examined. The value of mean square error (MSE) is used to check the ability of a particular architecture. It is observed that the mean square error of network decreases to the minimum value when the number of neurons is 21, which indicates that a network with 21 neurons in hidden layer can exhibit the best performance. In addition, based on trial and error procedure, for a double hidden layer ANN, the number of neurons is found to be 13 and 14 respectively for the best predictions. The training data is used to train the network with Levenberg–Marquardt function (trainlm). The neural network toolbox of MATLAB software package is used for training and testing the given data.

Genetic algorithm

Genetic algorithm is a stochastic optimization technique that searches for an optimal value of a complex objective function and are used to solve complicated optimization problems by simulation or mimicking a natural evolution process[16]. GA has been successfully used as a tool in computer programming, artificial intelligence, optimization, neural network training and information technology since its introduction by Holland[17] to improve the performance of simple GA[18]. The major advantage of genetic algorithms over other conventional optimization techniques is the flexibility it provides in providing the objective function and constrains. The application of genetic algorithms in bioprocess optimization had been reported by researches which are more flexible tool had been used here for minimization of reaction time[19,20] while maximizing product concentration.

Partial Purification of Lipase:

The cell-free supernatant containing extra cellular lipase was obtained by centrifugation at 7860 rpm at 4°C for 25-30 mins. The supernatant was collected in a beaker to it chilled acetone is added with continuous stirring, up to 70% (v/v) concentration and kept at 20°C for 4 h to allow protein precipitation. The precipitates were then harvested by centrifugation at 4°C and 7830 rpm for 30 min. The pellet thus obtained was resuspended in 34 mL of 20 mM Tris-HCl buffer (pH 8.0) to allow the solubilization of proteins. Unsolubilized proteins were then removed by centrifugation at 4°C; 7830 rpm for 30 min. Supernatant was dialyzed overnight against same buffer at 4°C. The obtained lipase was studied for its applications.

Application study

Detergents are used for cleaning various types of fabrics and hard surfaces. Enzymes, such as protease, amylase, and lipase may be used as detergent additives to improve cleaning efficiency. Lipase hydrolyzes triglycerides into mono and diglycerides, glycerol, and free fatty acids, which are more soluble than fats thereby increasing their water solubility, particularly at a pH $\geq$8. These more soluble reaction products may be more easily removed from fabrics and surfaces, increasing the cleaning efficiency of the detergent used. Enzymes may be used to improve the cleaning efficiency of detergents, including, for example, liquid, powder, and granular detergents. The performance of enzymes in detergents depends on a number of factors, such as, for example, the composition of the detergent, type of stains to be removed, wash temperature, washing procedure and wash-water hardness. Adding bacterial lipase to detergent and protease additive increases the efficiency of stain removal. In the present study, cotton cloth samples which were immersed in olive oil were placed in a beaker and lipase with Tris-buffer (pH 8) was added. The amount of lipase enzyme was used in different concentrations to the stain (1, 2, 3, 4 and 5 ml respectively). The amount of detergent used here is (0.25, 0.50, 0.75, 1gms). After adding these two they are incubated at different time period (30, 60, 90 and 120 mins respectively) at room temperature.

Fig 1a - ANN showing correlation for training data 5-21-1

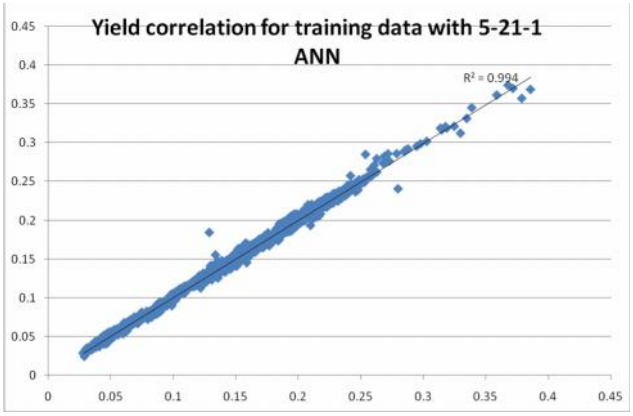


Fig 1b- ANN showing correlation for testing data 5-21-1

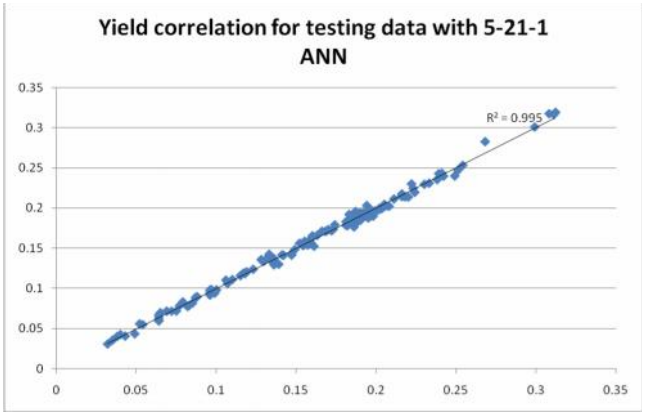
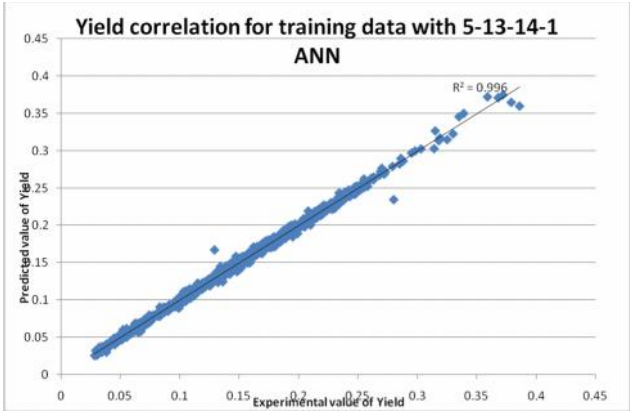


Fig 1c- ANN showing correlation for training data 5-13-14-1



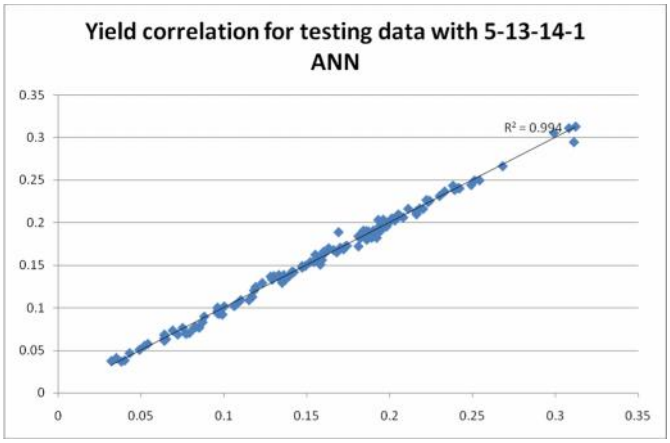


Fig 1d- ANN showing correlation for testing data 5-13-14-1

Fig 2a- Optimum versus Experimental yield for RSM, ANN (5-21-1) and ANN (5-13-14-1)

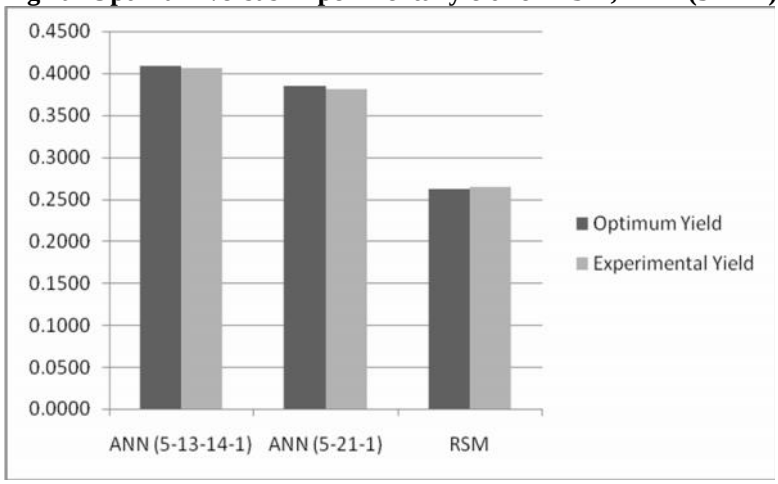


Fig 2b- Specific activity (U/ml) of lipase production for RSM, ANN (5-21-1) and ANN (5-13-14-1)

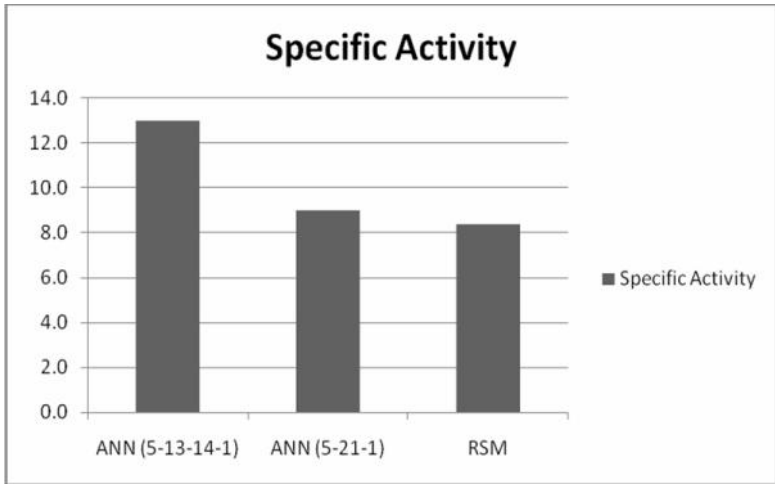


Table 3- Analysis of Variance for Yield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	20	4.00493	4.00493	0.20025	202.34	0.000
Linear	5	1.63394	1.32525	0.26505	267.81	0.000
Ph	1	0.86734	1.16518	1.16518	1177.33	0.000
T	1	0.69829	0.19757	0.19757	199.63	0.000
Ip	1	0.05813	0.11502	0.11502	116.22	0.000
S	1	0.00651	0.01857	0.01857	18.77	0.000
I	1	0.00367	0.00061	0.00061	0.62	0.432
Square	5	2.25860	2.25860	0.45172	456.43	0.000
ph*ph	1	1.58435	1.58435	1.58435	1600.88	0.000
t*t	1	0.30948	0.30948	0.30948	312.70	0.000
ip*ip	1	0.33254	0.33254	0.33254	336.01	0.000
s*s	1	0.03037	0.03037	0.03037	30.69	0.000
i*i	1	0.00186	0.00186	0.00186	1.87	0.171
Interaction	10	0.11239	0.11239	0.01124	11.36	0.000
ph*t	1	0.10700	0.10700	0.10700	108.12	0.000
ph*ip	1	0.00000	0.00000	0.00000	0.00	0.963
ph*s	1	0.00188	0.00188	0.00188	1.90	0.169
ph*i	1	0.00000	0.00000	0.00000	0.00	0.994
t*ip	1	0.00295	0.00295	0.00295	2.98	0.085
t*s	1	0.00047	0.00047	0.00047	0.48	0.490
t*i	1	0.00001	0.00001	0.00001	0.01	0.903
ip*s	1	0.00001	0.00001	0.00001	0.01	0.921
ip*i	1	0.00007	0.00007	0.00007	0.07	0.791
s*i	1	0.00000	0.00000	0.00000	0.00	0.985

DF- Degrees of freedom
Seq SS - sequence of sum of squares
ADJ SS- adjusted sum of square
ADJ MS- adjusted mean square
F-ratio of variance within and between treatment
P- Probability
Residual Error 1259 1.24600 Total 1279 5.25094

Table 4- Parameter setting for optimum yield of lipase production

	Optimum Parameters					Optimum yield	Experimentaly ield	Specific activity
	pH	Temp	I.P	I	S			
ANN(5-13-14-1)	6.17	35.11	43.05	1.57	1.99	0.4083	0.4063	13U/ml
ANN(5-21-1)	6.15	35.34	47.45	1.99	0.53	0.3842	0.3816	9U/ml
RSM	6.90	36.31	38.58	1.32	1.58	0.2622	0.2643	8.4U/ml

pH-pH
Temp-Temperature
I.P-Incubation period
I-Inoculum
S-substrate

Results and Discussion

The correlation coefficient (R) is a commonly used statistic and provides information on the strength of linear relationship between experimental and predicted values. For perfect prediction, all the data points should lie on the line inclined at 45^0 from horizontal. Fig. 1a-1d represent the ANN predicted versus experimental yield values for the training and testing ANN showing correlation for training data 5-21-1 datasets respectively for the two architectures of ANN presented in Fig. 1a & Fig. 1b. For ANN with architecture 5-21-1, Fig. 1a, shows the correlation coefficient to be 0.9943 for the training dataset and similarly Fig. 1b, shows the correlation coefficient to be 0.9953 for the testing dataset, indicating a very good correlation between the experimental and the predicted values. Similarly, for ANN with architecture 5-13-14-1, Fig. 1c shows the correlation coefficient to be 0.9967 for the training dataset and Fig. 1d shows the correlation coefficient to be 0.9944 for the testing dataset, indicating a very good correlation between experimental and predicted values. Using RSM, ANN (5-21-1) and ANN (5-13-14-1), the genetic algorithm is applied to find out the parameter settings for the optimum yield of lipase production, which is presented in Table 4. The optimum operating conditions obtained from the quadratic form of the RSM and ANN models with GA were pH 6.7, temperature 35 ^{o}C, and inoculum volume of 1.5, substrate volume 2, with 13 U/ml of predicted lipase activity within 43h of incubation. Fig**. 2a** shows the graphical representation of optimum versus experimental yield for RSM, ANN (5-21-1) and ANN (5-13-14-1) and similarly Fig.2b shows the specific activity of lipase for the three models developed. Thus, both the models based ANN with GA performed much better than RSM with GA model and offered stable responses in predicting the combined interactions of the five independent variables, i.e. pH, temperature, inoculumvolume, substrate volume, and incubation period with respect to extracellular lipase production. The two intermediate layered ANN (5-13-14-1) with GA based approach is found to be the best for the lipase production characterization and optimization.

Application study results:

After optimizing different combinations of lipase and detergent, the standard combination was addition of 4 ml (52 units) of extracellular supernatant containing lipase to 0.50 gm of detergent at 37^o C for 90 mins which removed 80% of the tough oil stains. Hence we can apply this to detergent industry for removing oil stains.

Conclusion

This study compared the performance of RSM and ANN with GA in estimation of fermentation performance parameters for extracellular lipase production from *Lactococcus lactis.* Though both the models provided good quality predictions in this study, yet the ANN with GA showed a clear superiority over RSM with GA for both data fitting and estimation capabilities. Among the above two ANN models used, the two intermediate layered ANN (5-13-14-1) with GA based approach is found to be the best for the lipase production. The results from these experiments could contribute to the development and use of this system on industrial scale.

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