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Optimization of L-arabinose isomerase production from *Lactococcus lactis lactis*: bioconversion of D-galactose to D-tagatose using the enzyme

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Abstract

The present work deals with the enzymatic production of D-tagatose, a low calorie sugar from whey (containing lactose) by biotransformation of galactose to tagatose using the enzyme arabinose isomerase (AI) from *Lactococcus lactis lactis*. The growth of *Lactococcus lactis lactis was* studied and its culture conditions were optimized using response surface methodology (RSM) and artificial neural network (ANN) for enhanced production of arabinose isomerase. It was found that *Lactococcus lactis lactis* reached maximum growth in 24 hours in presence of 4% NaCl. The intracellular arabinose isomerase was obtained using mortar pestle. It was also evident from the HPLC results that tagatose production increased linearly with increased galactose concentration.

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Introduction

Recently, rare sugars have been attracting much attention in terms of a number of uses. The production of rare sugars from cheap and widely available sources using simple methods is our main focus. Rare sugars are usually sweet like the natural sugars, but unlike them, rare sugars are either not metabolized by the body or metabolized to a lesser extent than natural sugars. Due to these features, rare sugars are desirable as low-calorie sweeteners and are well tolerated by diabetics. Other advantage of rare sugars is the absence of an objectionable aftertaste, commonly experienced with artificial sweeteners such as saccharin or cyclamates. However, in spite of the demand for these rare sugars, their commercial availability, application or usefulness is negligible as they are expensive to prepare and unavailable in nature.

Tagatose is one such rare natural ketose sugar and is naturally present in heat-treated dairy products. It has the potential for use as a sugar substitute in food (Bertelsen *et al*., 1999) (Buemann *et al*., 1999) since it has a taste and sweetness similar to sucrose. Essentially it is metabolized differently, has a minimal effect on blood glucose and insulin level and furthermore provides a prebiotic effect. It is especially suitable as a flavor enhancer and as a low carbohydrate sweetener.

Chemically, tagatose is made from lactose in a two-step process. The first step involves hydrolysis of lactose to glucose and galactose. In the second step, galactose is isomerized to tagatose by adding Ca $(OH)_2$. Although, the chemical synthesis is an economical process, it has the disadvantages of high temperature, high pressure and is thus environmentally unclean. As a consequence, enzymatic production of tagatose has gathered much momentum in the recent years. Tagatose production from galactitol using galactitol dehydrogenase is well known (Shimonishi *et al*., 1995). But galactitol is expensive than galactose and seems to have only little potential for commercial application.

Therefore biologically, the isomerization of galactose to tagatose is done by the enzyme arabinose isomerase (AI) (Kim Byoung-Chan *et al*., 2002). This enzyme also catalyses the isomerization of arabinose to ribulose because of similar substrate configuration (Cheetham and Wooton, 1993).

Attempts are being made to produce tagatose from whey (contains about 4.8 % lactose), an easily available dairy waste. Sweet whey, a potent pollutant, is produced in large quantities by cheese industries and in most cases is discharged without any treatment to rivers or streams. Fermentation of whey by microorganisms is one possible way of reducing the pollutant effect.

Recently, a number of statistical experimental designs with response surface methodology (RSM) have been employed for optimizing enzyme production from microorganisms (Ray Dutta *et al*., 2004) Artificial neural network is a more accurate technique than RSM and its application has also been reported in predictive microbiology (Lou and Nakai, 2001) Recently, ANN and RSM had been used to build up a predictive model of the joint effect of NaCl concentration, pH level and storage temperature on kinetic parameters of the growth curve of *Lactobacillus plantarum* (Garcia-Gimeno *et al*., 2004).

Therefore, in this study at first the culture parameters for the enzymatic hydrolysis of lactose (in whey) to glucose and galactose were optimized by response surface methodology (RSM) and artificial neural network (ANN) methods and then the production of intracellular arabinose isomerase was done for the isomerization of galactose to tagatose.

Materials and methods

Microorganism

For the present investigation, the microorganism used was *Lactococcus lactis lactis* (strain MTCC 440, aerobic, best growth in 24 hours) procured

from MTCC Chandigarh, India. This strain was maintained on 2% nutrient agar slants at 4 °C.

Chemicals

Chemicals were all of analytical grade.

Composition of medium

Nutrient agar (2%) was used for maintaining the mother culture; Czapek-dox (Patil and Shastri, 1981) was used for the growth of *Lactococcus sp.* and production of arabinose isomerase by *Lactococcus lactis lactis*, which contained $NaNO_3$ 2.5 g/l; KH_2PO_4 1.0 g/l; $MgSO_4.7H_2O$ 0.5 g/l; KCl 0.5 g/l; Phosphate buffer saline (PBS) pH 7.3 was used for washing the cells of *Lactococcus lactis lactis* which contained NaCl 8.0 g/l; K_2HPO_4 1.21 g/l; KH_2PO_4 0.34 g/l.

Inoculum Preparation

For inoculum preparation, 10 ml sterile distilled water was added to the slant grown on nutrient agar plate for 24 hours at 37 °C and scraped aseptically with an inoculating loop. This suspension having approximately $2x10^7$cells/ml was used as inoculum for subsequent fermentation.

Experimental design and extraction of arabinose isomerase

To optimize the culture parameters (with respect to pH, temperature and inoculum volume) for the optimum growth of the bacteria *Lactococcus lactis lactis,* the organism was grown in 250 ml Erlenmeyer flasks containing 100 ml of sterilized Czapek-dox medium and whey (in 1:1 ratio) supplemented with 4% NaCl (Adriana Molina *et al.*, 2004). The substrate was inoculated with 1ml of freshly prepared bacterial suspension (as discussed earlier) for 24 hours at 37 °C. Fermentation was carried out in BOD incubator-shaker for 24 hours. After 24 hours of incubation the cells were collected by centrifugation at 10,000 x g for 20 minutes and washed in PBS buffer and the O.D was measured at 600nm.

In the next stage, both response surface methodology and the artificial neural network were used to study the interactive effects of three variables, i.e. pH, temperature and inoculum volume for improving total hydrolysis of lactose in whey. All experiments were conducted in triplicate and the results were the average of these three independent trials.

Response surface methodology (RSM)

Using RSM, the relationship among the variables, i.e. initial pH, temperature and inoculum volume were expressed mathematically in the form of a polynomial model, which gave the response as a function of relevant variables. The present work was based on the central composite design (CCD) utilized to obtain the experimental data, which would fit an empirical, full second-order polynomial model representing the response surfaces over a relatively broad range of parameters.

The principle of RSM was described by Khuri and Cornell . An empirical second-order polynomial model for three factors was in the following form:

$$y_i = a_0 + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{3}\sum_{j=i}^{3} a_{ij} x_i x_j \qquad (1)$$

where y_i was the predicted response (lactose hydrolysis) used as a dependent variable; x_i (i=1, 2 and 3) were the input predictors or controlling variables; and a_o, a_i (i=1, 2, 3) and a_{ij} (i=1, 2, 3; j=i,...,3) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least-squares method.

Each factor in the central composite design was studied at three different levels (–1, 0, +1). A 2^3-factorial CCD with three factors leading to a total of 20 sets per experiment with some replication was formulated to optimize the initial pH, temperature and inoculum level. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual and coded form was

listed in Table 1. Upon completion of experiments, the average maximum growth of the bacteria was taken as the dependent variable or response (y_i). A second-order polynomial equation was then fitted to the data by least-squares optimization technique. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

Artificial neural network

ANN model gives a unique representation of the nonlinearity and interdependencies within one framework for different bioprocess (Gonzalo *et al.*, 1998) (Cheroutre-Vialette and Lebert, 2002).

ANN was applied here to provide a nonlinear mapping between input variables (pH, temperature and inoculum volume) and the output variable. Regression-based response surface models require the order of the model to be stated (i.e., second, third or fourth order), while ANN tends to implicitly match the input vector (i.e., culture parameters) to the output vector (lactose hydrolysis). ANN has been applied for the purpose of simulation on the same experimental data used for RSM.

In this study both radial basis function (RBF) network as well as multilayer perceptron (MLP) model have been used. Both the types of ANN chosen here have a feed-forward structure. Radial basis function (RBF) network is very powerful in function optimization modeling. In recent years, radial basis function neural network finds immense application as a modeling tool in bioprocess (Chen *et al.*, 1991). This network consisted of a single hidden layer of locally tuned units that is fully interconnected to an output layer of linear units. If x is an input, the output y is given by

$$y = \omega_0 + \sum_{i=1}^{M} \omega_i z_i(x) \qquad (2)$$

and

$$z_i(x) = \exp\left(-\frac{\|x - \mu_i\|^2}{2\sigma_i^2} \right) \qquad (3)$$

where w_0 and w_i were the weights, z_i was the basis function, μ_i the mean or in general, center of the RBF element and σ_i the standard deviation. The training algorithms tried to find w_i corresponding to a particular σ_i to match the target output (Leang *et al.*, 2003). The main advantage of the RBF network is its speed in training and repeatability of the training results. However, the RBF network requires more number of hidden neurons compared to multilayer perceptron feed-forward networks with sigmoidal response functions.

Each node represented by the box is called a perceptron. However, the most critical part of an ANN-based model is to train the network. The most widely studied and used training algorithm is back-propagation technique, which is robust, reliable. The problem of neural network training is to devise a method of updating the representative weights that minimizes the error. It is essentially an optimization problem. Behaviour of feed-forward network and learning through least square based updating e.g. back propagation rules can be followed more easily corresponding to changes of parameters e.g. number of hidden layers, number of nodes in a hidden layer, change in the type of activation function, learning rate etc. However, the updating of the weights has been done here by Levenberg-Marquardt algorithm. Levenberg-Marquardt algorithm performs much better with some knowledge of the process so that quick convergence is obtained with a very low error. The network used is a feed-forward one with one input layer, one output layer and a hidden layer.

A 3-3-1 feed-forward network has been chosen for the present application with 3 nodes in the hidden layer. It has been found by varying number of nodes in the hidden layer that 3 nodes are sufficient to map the nonlinearity with smooth interpolation. A large number of hidden layer nodes compared to the necessary ones may result into over fitting and undesired high frequency oscillations while interpolating between the training patterns.

Before starting the training, training pattern is normalized by a scale factor to limit the patterns within 1. Three inputs fed to the three nodes are pH, temperature, and inoculum volume. The output taken from the single output node is lactose hydrolysis. Training process has been carried out till the sum of square error is less than 1.e-5. Even such a low error criterion has been satisfied by Levenberg-Marquardt algorithm within 300 number of iterations.

Extraction of arabinose isomerase (AI)

Since AI is an intracellular enzyme, to get the enzyme in soluble form, *Lactococcus lactis lactis* cells were disrupted by crushing in a mortar and pestle. After disruption the crude enzyme was extracted with PBS buffer (pH 7.3). The cell debris was removed by centrifugation for 20 minutes at 10,000 x g and the supernatant containing the crude enzyme was taken for AI assay and protein analysis.

Enzyme assay

L-arabinose isomerase activity was assayed in a reaction mixture containing 0.4 ml of 50 mM glycine NaOH buffer (pH 9), 0.05ml of 0.1M L-arabinose and an appropriate amount of enzyme in a final volume of 0.5ml. The reaction was initiated by the addition of L-arabinose. After incubation for 10 minutes at 30°C, the reaction was terminated by the addition of 0.05 ml of 10% TCA. The formation of L-ribulose was determined by the method of (Dishe and Borenfreund, 1951). To 0.5ml of above, 0.1ml of cysteine hydrochloride and 3ml of a mixture of 190 ml of water and 450ml of conc. H_2SO_4 was added. Immediately after this 0.1ml of 0.12% of alcohol solution of carbazole was added. The mixture was shaken and kept undisturbed at room temperature. After half an hour, the O.D. was measured at 560nm. One unit of enzyme activity is defined as the amount that will convert 1µmol of L-arabinose to L-ribulose in one minute.

Partial purification of extracted arabinose isomerase

After cell disruption the contents were centrifuged at 10,000 x g for 20 minutes. The pellet was discarded and the supernatant, which contained the crude enzyme extract, was taken for partial purification. For salt precipitation a fixed volume of crude enzyme extract was taken to which 60% ammonium sulphate was added slowly and stirred by a magnetic stirrer for 1 hour, within an ice filled container. The precipitate was collected by centrifugation at 10,000 x g for 20 minutes, dissolved in minimum amount of PBS buffer. Then the total protein and AI activity were determined.

For acetone precipitation the crude enzyme extract and acetone were mixed in 1:2 ratio and kept at 4°C. After 1hour the crude enzyme acetone mixture was centrifuged at 10,000 x g for 20 minutes. The supernatant was discarded and pellet dissolved in minimum amount of pre-chilled PBS buffer. The AI activity and protein content were determined.

Transformation reaction and its analysis by HPLC

The kinetics of tagatose production by the biotransformation of galactose to tagatose using arabinose isomerase enzyme was studied by HPLC. Chromatographic separation was undertaken with an isocratic elution mobile phase of acetonitrile-water (75:25, v/v) in an Agilent carbohydrate column of 150 mm length. The flow-rate of this eluent was maintained at 1.8 ml/minute and column temperature at 50°C. The volume of the sample injected was 20 µl (filling the loop completely). Peaks were identified by comparing retention times with sugar standards. The detection of the eluted sugars was made by refractive index (RI) detector.

To 100 µl of 0.2% glucose, 100 µl of 0.2% galactose and 100 µl of AI were added. The reaction mixture was incubated for 20 minutes and thereafter the reaction was stopped using equal volume of methanol:acetic acid in the 1:3 ratio. The reaction mixture was centrifuged at 10,000 x g for 20

minutes and 20 µl of the supernatant was injected into the loop of HPLC column for analyzing the formation of tagatose. Thereafter the concentration of glucose was fixed to 0.2 % and that of galactose varied from 0.1% to 1% to see whether it affected the biotransformation of galactose to tagatose. Tagatose formation in each case was quantified. 20 µl of each of standard tagatose and galactose of the concentration of 1mg/ml was previously injected into the loop of HPLC column to see the standard retention times to compare with the experimental sample.

Table 1. Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors.

Variables	Actual	Coded	Actual	Coded	Actual	Coded
pH	4	-1	6	0	8	+1
Temperature (°C)	25	-1	35	0	45	+1
Inoculum volume (ml)	0.5	-1	1.0	0	2.0	+1

Table 2. Experimental test data using three variables showing observed and predicted values of lactose hydrolysis by RSM and ANN.

pH	Temperature (°C)	Inoculum volume (ml)	Observed response (O.D.at 600nm)	Standard Deviation of O.D.	Predicted response (least squares)	Predicted response (ANN)
4	25	0.5	1.321	0.031	1.2458	1.3207
4	30	1.0	1.701	0.027	1.7955	1.7178
4	35	1.5	1.954	0.035	1.8536	1.8994
4	40	2.0	1.503	0.028	1.4201	1.4154
4	45	2.0	1.101	0.025	1.2051	1.1357
4	40	0.5	1.954	0.034	1.9709	2.0123
5	30	1.0	1.987	0.033	2.1452	1.8983
5	25	0.5	1.525	0.035	1.5679	1.5136
5	35	1.5	2.197	0.032	2.231	2.338
5	40	2.0	1.626	0.031	1.825	1.6863
5	45	1.5	2.197	0.035	1.9931	2.1668
5	40	1.0	2.101	0.033	2.3331	2.0937
6	25	1.0	1.796	0.032	1.752	1.8569
6	30	0.5	2.115	0.033	1.8599	2.0892
6	35	1.5	2.799	0.036	2.2063	2.6758
6	40	2.0	1.970	0.035	1.828	2.0457
6	45	2.0	1.656	0.034	1.5613	1.6354
7	25	1.0	1.026	0.032	1.3235	1.051
7	30	0.5	1.304	0.031	1.3521	1.2852
7	35	1.5	1.536	0.033	1.7796	1.5175
7	40	2.0	1.012	0.031	1.4289	0.99733
7	45	1.5	1.402	0.034	1.4384	1.432
8	25	1.0	0.612	0.031	0.49293	0.60692
8	30	2.0	0.818	0.029	0.71872	0.77647
8	35	1.5	0.987	0.031	0.95079	1.0474
8	40	0.5	0.521	0.028	0.53706	0.51519
8	45	2.0	0.404	0.028	0.30941	0.39409

Results and discussion

L-Arabinose isomerases induced from microorganisms including *Mycobacterium sp.*, *Lactobacillus sp.* and *Lactococcus sp.*, are supposed to catalyze D-galactose to D-tagatose and L-arabinose to L-ribulose isomerization due to similar substrate configuration. In this study *Lactococcus lactis lactis* was grown in whey containing 4.8-5.1% lactose. At first the culture parameters for maximum growth of *Lactococcus sp.* (or optimum hydrolysis of lactose for maximum galactose yield) were optimized using response surface method and artificial neural network model. This bacterium in turn produces arabinose isomerase enzyme intracellularly, which helps in the transformation of galactose to tagatose through isomerization reaction.

The results of CCD experiments and ANN for studying the effects of three independent variables, viz., pH, temperature and inoculum volume, on lactose hydrolysis are presented in Figures 1a, 1b and 1c along with the predicted and observed responses. The coefficients of the model given in Eq. (1) were determined by the Gauss–Newton technique of least-squares optimization. The programs were written in MATLAB using optimization toolbox. The equation with the optimized coefficients is given by

$$Y = -9.7718 + 2.207\,pH + 0.31165\,T + 0.9397\,V - 0.0051665\,pH.T - 0.012388\,T.V + 0.10691\,pH.V - 0.20102\,pH^2 - 0.0036378\,T^2 - 0.49562\,V^2$$

The normalized percentage mean squared error in fitting the factorial data was incidentally very small—less than 3%. The factorial levels were given in Table 1 and the data fitting results in Table 2.

The radial basis function network used for fitting the same factorial data set also produced a normalized percentage mean squared error of less than 3% in fitting the factorial data. The result was very similar to the least square optimized results. In both cases, the error was insignificant due to smooth variation of the response in the experimental range. Both the quadratic form of RSM as well as RBF neural network had been used to generate the response surface and optimize the operating conditions. The results predicted from the ANN model are also given in Table 2.

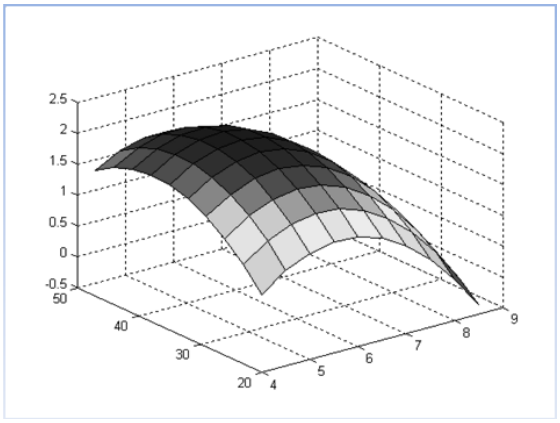

Fig. 1A. Response surface curve showing the interaction between pH and temperature on the growth of *Lactococcus* sp.

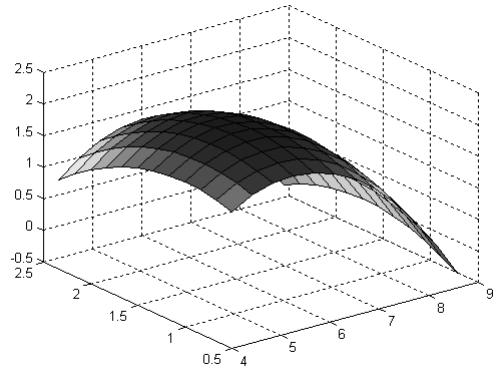

Fig. 1B. Response surface curve showing the interaction between pH and inoculum volume on the growth of *Lactococcus* sp.

However, feed forward multilayer perceptron model trained with Levenberg-Marquardt algorithm produced much accurate but less smooth approximation. Though it was very accurate within the training range, but there are large oscillations just beyond the training data set and also minor oscillations within the training range, when interpolated in between the experimental data.

The three-dimensional response surfaces are plotted in Figs.1A–1C corresponding to the combined effects of pH–temperature, pH–inoculum volume, and temperature–inoculum volume, respectively.

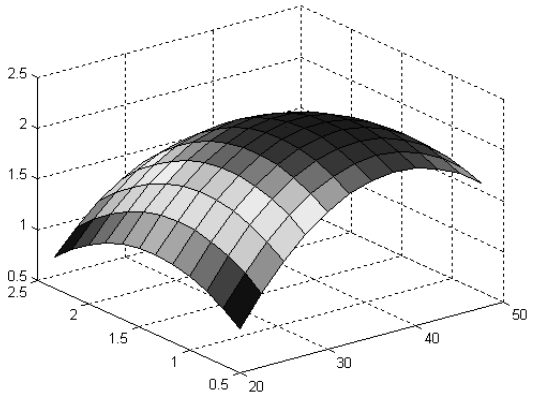

Fig. 1C. Response surface curve showing the interaction between temperature and inoculum volume on the growth of *Lactococcus* sp.

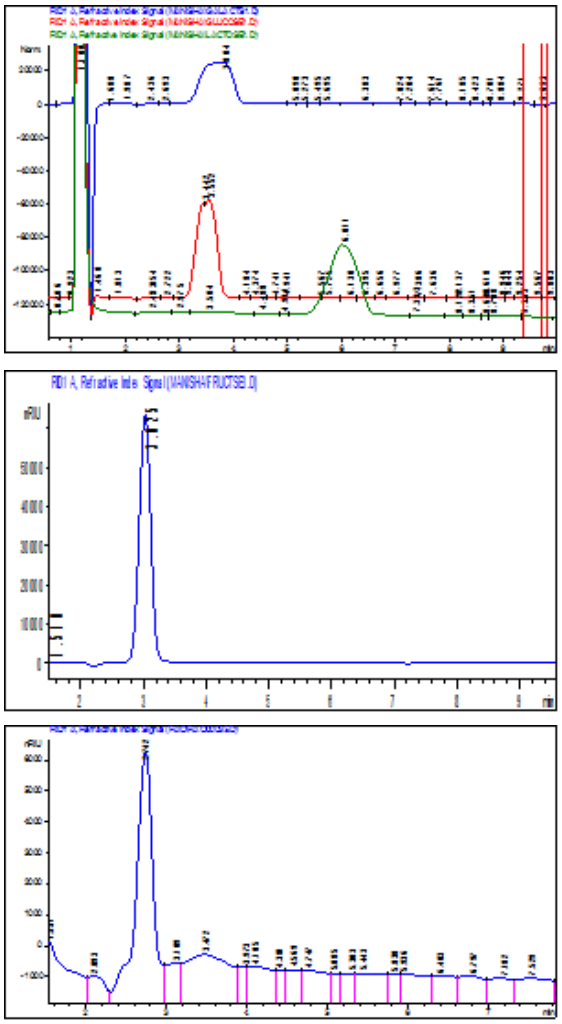

Fig. 2. HPLC Chromatogram of sugar standards (glucose, galactose, lactose, fructose and tagatose) with their retention time (3.5, 3.8, 6.0, 3.0 and 2.742 minutes).

The response surfaces obtained were convex in nature suggesting that there were well-defined optimum operating conditions. Figs. 1B and 1C also revealed that inoculum volume did not show significant variation above 1.0 ml whereas in all the figures pH and temperature showed significant variation both above and below the optimum values. The optimum operating conditions obtained from the quadratic form of the RSM and ANN models were inoculum volume of 1.0 ml having 2×10^7 cells/ml, pH of 6.5 and temperature of 38 $^{\circ}$C. This optimum was close to the single variable optimization results, which corresponded to an inoculum volume of 1.0 ml, pH 6.0 and temperature 35 $^{\circ}$C. Thus by optimizing the culture parameters of the bacteria using RSM and ANN, the optimum bacterial growth as well as optimum lactose hydrolysis was attained. Recently, Lou and Nakai, have reported an error of 14.4% while predicting the effects of pH, temperature and water activity on the thermal inactivation rate of *E. coli* using ANN. There are also reports on the estimation of bio-process states including biomass concentration, lactic acid concentration, etc. with the help of ANN. In this report, the accuracy of prediction varied between 4 and 10%.

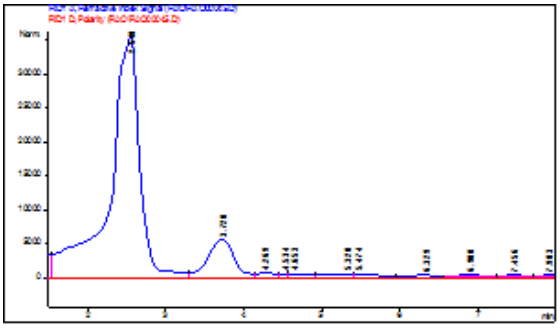

Fig. 3. HPLC chromatogram of the experimental sample showing tagatose and fructose formation at 2.348 and 3.720 minutes respectively.

Thus, both the models based on RSM and ANN performed well and offered stable responses in predicting the combined interactions of the three independent variables, i.e. pH, temperature and inoculum volume with respect to lactose hydrolysis. The ANN based approach was best in fitting to the measured responses in comparison to the RSM model. The chosen neural network has represented the system nonlinearities in an excellent manner.

Every technique has some limitations – neural network based simulation is also not free from it. Though neural network is an excellent tool for representing system nonlinearities, it is very difficult to quantify the order of the nonlinearity in the conventional sense, i.e., whether dependency is quadratic, cubic, exponential or logarithmic. In many nonlinear systems however the dependency is a mixed type and conventional nonlinear models can represent the system often tolerating a large error. In such cases use of neural network is more than justified. This study compared the performance of the central composite design of RSM and radial basis function network in the estimation of fermentation performance parameters (pH, temperature, inoculum volume) for lactose hydrolysis. Both models provided similar quality predictions for the above three independent variables in terms of lactose hydrolysis with ANN showing more accuracy in estimation. Thus, ANN could be a very powerful and flexible tool well suited for modeling the fermentation process due to an implicit corrective action arising from the training methodology and the associated estimation procedure.

Optimization of bacterial cell disruption time and partial purification of arabinose isomerase

Lactococcus lactis lactis biomass after 24 hours of fermentation was harvested by centrifugation and crushed gently in a mortar and pestle for different time intervals. Throughout cell crushing, care was taken that the temperature does not rise and hence was performed in ice bucket. Cell disruption by mortar and pestle was optimized to 4 minutes.

After this the crude extract was partially purified by salt and solvent precipitation. From Table 3 it was found that arabinose isomerase recovery by acetone precipitation was 217.67 % with 1.9 fold purification whereas it was 136.21 % with 0.71 fold in case of ammonium sulphate precipitation.

HPLC results

Lactose hydrolysis was studied by comparing the HPLC peaks of the supernatant obtained after 24 hours of fermentation with that of standard glucose, galactose and lactose (Fig 2). The peak area of lactose decreased while that of glucose and galactose increased in the fermented broth when compared to that of unfermented broth. Product formation increased gradually with the increase in galactose concentration, thus proving the biotransformation of galactose to tagatose. It can be concluded that tagatose production is increasing exponentially with the increase in galactose concentration in presence of a fixed amount of enzyme. Standard tagatose and galactose had retention times of 2.34 and 3.72 minutes respectively (Fig. 3).

Conclusion

Rare sugars, although scarce in nature and expensive, have been shown to be necessary in certain areas of human welfare. Diabetes is a major health ailment affecting major parts of the earth. Treatment of Type II diabetes (non insulin dependant diabetes) serves a major health threat to the human race and a challenge to the ever-evolving medical science. The best part of this work envisages the development of a technology to scale up the process using whey which is a byproduct with negative value generated by cheese manufacturing industries. The studies conducted clearly indicate that significant lactose hydrolysis occurs during fermentation and the galactose yield should be correlated to the amount of tagatose to be produced.

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