Parametric Optimization of the Purification of Restriction Enzymes with Low Concentration Using Cation-Exchange Chromatography: Model-Based Approach

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Abstract: A model-based approach to investigate the effect of two process parameters was proposed for optimization of the ion-exchange step in the purification of the restriction enzyme PstI .Transport-Dispersive model was used to describe convective and dispersive flow in the column and the proteins transfer to the surface of the resin beads. For protein adsorption step, the well-known Steric Mass Action (SMA) model was coupled with the bulk fluid model. In next step, several experiments designed to estimate model parameters and the resulting model efficiency and ability to predict the elution behavior was tested and verified. Finally, the operating conditions were optimized with changing indicated parameters and study of the objective function (Yield).Gradient slope and feed volume of the sample was selected as the decision variables. Purity was also selected as the constraint and given the value of 99%. The model predicted the yield could become as higher as 52% and this result was validated with the proposed values of decision parameters experimentally. It is also shown that feed volume (on the proposed boundary) has lower effect on the separation efficiency in the defined range and also the effect of molecular size of proteins on band broadening was studied.

Keywords: Protein purification, Ion-exchange columns, Restriction enzymes, Simulation, Model-based optimization, Absorption.

1 Introduction

The purification of proteins has always been the most challenging step in producing and manufacturing different types of proteins, such as biopharmaceuticals or other versatile and highly used proteins [1]. One group of proteins that are widely used in genetic engineering are restriction enzymes. These enzymes, with their unique mechanism in cutting desired nucleotides on the genome of different organisms, are the major tool in designing recombinant proteins or reaching to the best vectors and plasmids for this purpose. This significant property forces the manufacturers of these enzymes to remove other enzymes totally which may lower the efficiency of the products by cutting the nucleotides in different patterns or with no significant pattern, when they've brought to use by scientists and consumers. On of the major impurities existing in processes using wild or recombinant type microorganisms to obtain theses enzymes is deoxyribonucleic (Dnase). This enzyme cut nucleotides following no pattern and should be eliminated from the product (restriction enzyme) in purification steps. Studies have been focused on using existing or more sophisticated procedures, such as heparin-based immuno-affinity or ion exchange chromatography to purify restriction enzymes from this enzyme and other impurities [2, 3]. The cost of producing these proteins (which must be highly pure due to available standards) is highly affected with downstream step and purification procedure used in the process [1]. Because of this, studies are mainly focused on available units to design and chose the proper methods with higher efficiency and lower fixed and operation costs. Even after this step, optimization studies are always conducted to find the best operation parameters. Formerly, this work mainly was being done by designing and running experiments which could made it possible to study parameters role on the yield of the process. This method is being used by different researchers to understand mechanisms and interaction that could not be done by other methods [4, 5]. By developing computer-based methods it was possible to study these effects using related and sophisticated models and solving them that made it possible to simulate the system, experiments and the results as well [6].Cation-exchange chromatography are widely used in purification of proteins because of it's good given

resolution, mild operational condition and high performance in isolation of the proteins [7]. In this work we have focused on ion exchange chromatography and existing models to find better output for this step in purification of restriction enzymes.

2 Theory- Models and optimization

To model chromatographic process we usually describe the process in two main parts. First, the bulk fluid flow equation inside the column which considers convective (and perhaps dispersive) effects on the particles and adsorption mechanism which describe proteins adsorption on the resin. By solving the model, we are able to predict romatograms eluted from the column output and finally we can predict and simulate optimum process parameters by defining our optimization procedure. Defining a suitable model which is applicable for a specific process may have the most effect on the results of the simulation [8]. For chromatography systems, the most comprehensive model is the general rate model which considers all existing and studied mechanisms in the process [9]. This model also describes particle diffusion in resin beads along other mechanisms. However, other models which eliminate this term totally or substitute it with a simpler term are also widely used till recently. A detail review of existing models has been done by Guiochon et al.

2.1 Column Model

A transport-dispersive model was used to describe bulk fluid flow and adsorption in using a kinetic equation. The column model for component i is described by the following equation:

$$\varepsilon_t \frac{\partial C_i}{\partial t} + (1-\varepsilon_t)\frac{\partial Q_i}{\partial t} + u\frac{\partial C_i}{\partial t} = \varepsilon_e D_{ax}\frac{\partial^2 C_i}{\partial x^2} \qquad (1)$$

where x is the axial coordinate along the column(m), ε_t is the total void fraction (m^3mobile phase/ m^3column), ε_e is the external void volume (m3 mobile phase/ m3 column), u is the apparent velocity (m/s), Dax the dispersion coefficient (m^2/s), Ci is the concentration of component i in the mobile phase(mol/m3), Qi is the concentration of component i in the stationary phase(mol/m^3 gel) and t is the time(s).

Dankwerts boundary was considered for the system. It is also considered that the column be as a closed-closed system [10]. The equations for the inlet of the column are as below:

$$D_{ax}\frac{\partial C}{\partial x} = u(C - C_0) \qquad for\ 0 \prec t \le t_f \qquad (2)$$

$$D_{ax}\frac{\partial C}{\partial x} = uC \qquad for\ t_f \le t \qquad (3)$$

In which t_f is the time of injection of the feed sample to the column. At the outlet the condition could be described by a Neumann condition:

2.2 Adsorption model

To describe the adsorption mechanism, we used Steric Mass Action (SMA) formalism which is a three parametric model and model protein adsorption in ion-exchange systems as a stoichiometric exchange. The model's assumption, formulation and characteristics have been described previously in literatures in detail [11-13]. The main equation which states the competition between protein and the salt in occupying available sites on the solid phase is in the form of an equilibrium equation, see Eq.(4):

$$C_i + \nu_i \overline{Q}_s \Leftrightarrow Q_i + \nu_i C_s \qquad (4)$$

where C_i is the protein concentration in the mobile phase and Q_i is the protein concentration in the stationary phase. $\overline{Q}_s$ is the concentration of available sites in the resin and *vi* is the number of interacting sites between protein and bead surface. At equilibrium, Eq. (5) is obtained:

$$K_{eq,i} = \left(\frac{C_i}{Q_i}\right)\left(\frac{\overline{Q}_s}{C_s}\right)^{\nu_i} \qquad (5)$$

The total concentration of sites in the gel can also be described by Eq. (6):

$$\Lambda = \overline{Q}_s + \sum_{i=1}^{N}(\nu_i + \sigma_i)Q_i \qquad (6)$$

In which Λ is the resin capacity (mol /m^3 resin) and σ_i is the steric factor and shows the number of shielded sites on the resin per every bound protein molecule. *s* denotes salt component and N is the number of available proteins in the system. The concentration of the component in fluid phase will be obtained from the equation (7) using Eq. (5) and (6):

$$Q_1 = K_{eq,1}C_1\left(\frac{\Lambda - \sum_{i=1}^{N}(\nu_i + \sigma_i)Q_i}{C_s}\right)^{\nu_i} \qquad (7)$$

This model usually has been used to study chromatographic processes working in high concentration of the solutes [11] because of steric factor parameter which was proposed to describe nonlinear behavior of proteins in the system. But also it is possible to use it when we working in low concentrations [14]. In this work, we used this model instead of simpler models like Longmuir ones to simulate the adsorption mechanism [15].

2.3 Simulation techniques

The overall model was written and calculated for two components (the product and the impurity) coupled with adsorption equation described as follows. The main equation is in the form of parabolic PDE which the solution should be estimated using numerical methods. The method used was Crank-Nicolson finite difference which has higher accuracy among other finite difference methods and also longer calculation time [16]. The column was discretized into a two-dimensional (x and t) grid network and dependent variable (concentration in the fluid phase) was calculated using the values in former grids. In every time step, the values of the concentration calculated for all grid points in x-direction and this will be done for every grid point in tdirection. MATLAB was used for writing the algorithms and solving the model. The number of grid points was set to 50 for both position and time. In practice the two final equations should be solved simultaneously as the concentration in fluid phase *Ci* of each component is related to solid phase concentration of both components. The other point is the existence of term *Qi* in the main equation (that calculates *Ci*) which in return is dependent on the *Ci* and should be calculated using an iterative scheme.

3 Working procedure

To obtain reliable results from our model for optimization, it should be noted that our model should be tested to show its validity in experiments [17] beside methods to estimate model parameters should be implemented correctly and repeated if it was needed. For this, model tested with given parameters and the results was compared with experimental data in the output of the column. To optimize a chromatographic step objective function, decision variables and constraints should be considered. In this work yield was chose as the objective function and it's a very usual option when product has a high value [18]. The yield is calculated as the ratio of the amount of product protein in the output fraction divided by the total amount of available protein (loaded proteins) and could be calculated using Eq. (8):

$$Yield = \frac{\int_{t_1}^{t_2} C_i dt}{C_{i,total} t_f} \quad (8)$$

In which 1 t and 2 t denotes the times were chose as the start and the end of the analyze (s) and t_f is the length of the loading time where feed was injected (s). There are a wide range of decision variables in optimization of a chromatographic process which can alter the objective function [18]. Among different types of these parameters, gradient slope and feed volume were studied in this work which controls the amount of protein injected and also the cycle time of the process. Obviously other variables were set to be constant during the operation. Purity was selected as the only constraint and the optimization process carried out in a frame defined by this parameter. In this work, the purity was set to 99%. Physical constraints could also be chose (like activity in the case of enzymes) but assumed that these parameters are within the desired range.

3.1 Parametric optimization

The objective function was calculated in different values of decision variables and the maximum value was indicated as the optimum operation condition. Different values of gradient slope and feed volume were selected and the resulting yields were measured with the use of chromatograms generated by the model in every value of decision variables. By gradient slope we mean the amount of salt introduced to the column per unit of time. The ability of the model to predict the optimum condition was also tested by experience.

4 Materials and methods

4.1 Chromatography media and column

Whatman P11 Cellulose Phosphate (particle diameter ca. 150 μm, column size 23 mm × 17 mm i.d., total bed volume 5 ml) was used as the cation-exchange chromatography column. Pharmacia UV-MII was also used to detect eluting proteins from the column. The column was packed and equilibrated manually with given data from the manufacturer. Restriction enzyme was from Cinnagen (Tehran, Iran).

4.2 Restriction enzymes

As we need breakthrough and elution experiments using pure enzymes we worked with two different restriction enzyme, *Pst*I as the main product and *EcoR*I as the second one(impurity). To select a restriction enzyme as the impurity, two main characteristic of proteins take into account. Isoelectric pH (theoretical) and size of the protein was selected as much close as possible to the product protein (*Pst*I). These characteristic are two main parameters in ion-exchange chromatography which need to be far enough for proper isolation of the proteins. By choosing these parameters close to each other, we are able to predict more difficult situation, so it may be also possible to simulate other mild conditions. The value of these two parameters is summarized in Table 1. To retain the activity of the proteins, all the experiments were carried out in refrigerator at 4 degree centigrade.

4.3 Methods

4.3.1 Porosity estimation methods

To estimate the porosity in the column, trace materials that do not interact with the resin usually used [19]. For external porosity, one has to use a very large molecule that doesn't penetrate to resin particles. As we have a bead resins with large pores it's not possible to use molecules like Blue dextran 2000 or latex particles. Therefore the column external porosity wasn't measured experimentally and this parameter was set to 0.5. This void fraction is relatively high but it is somehow reasonable as the column was manually packed. Total porosity (internal and external) was measured using a protein that can easily penetrate into resin particles without any interaction. This was done by injecting 200 µl of BSA (Sigma, Germany) diluted in 1 M NaCl buffer to inhibit adsorption of the protein to the resin. The column dead volume was measured in the absence of the column in the process line using Blue dextran 2000 (may use any other detectable molecule that has absorption in 280 nm).

4.3.2 Model parameters

4.3.2.1 Dispersion coefficient

Breakthrough experiments (as described by Persson et al. [19]) were performed and the results were compared with ones obtained from the model. This lumped parameter describes mass transport effects on the proteins which mainly consists of eddy dispersion, diffusion and non-ideality in the movement of the particles (tortuosity and channeling effect for example)[10]. In practice, this parameter depends on the (diffusion coefficient) size of the molecule and should be measured for both proteins. In this work, which both proteins have a close molecular size, we estimate the dispersion coefficient for EcoRI only. A 1M NaCl buffer at a 6.8. The concentration of the protein was 0.5 mg/ml in all breakthrough experiments.

4.3.2.2 Adsorption parameters

Three main parameters of the model ($K_{eq,i}$, ν_i and σ_i) obtained from different experiments. For linear parameters ($K_{eq,i}$, and ν_i), three elution experiments were performed with different salt concentration following known methods mentioned in previous studies [11]. Using Eq. (9), these two parameters are obtainable.

$$\log\left(K_{eq,i}\right) = \log\left(\beta K_{eq,i}\Lambda^{\nu_i}\right) - \nu_i \log\left(C_s\right) \quad (9)$$

The buffer salt concentration in three experiments was set to 100,130 and 150 mM of NaCl respectively.

To estimate the steric factor for both proteins, breakthrough experiments should be implemented using high quantity of the protein until breakthrough occurs, and consequently the value of the parameter may be obtained by calculating the protein bound to the resin and known values of the linear parameters using Eq. (7)[6,12].

As the column used in this work has a large volume, it needs a large quantity of the proteins. We used a procedure proposed by Karlsson et al.[17]. In this method; the parameter could be obtained by measuring it for one molecule and use Eq (10) to calculate it for other protein.

$$\sigma_2 = \sigma_1 \frac{MW_2}{MW_1} \quad (10)$$

This will give an estimate of the steric factor of a protein assuming it is directly proportional to its size. This assumption won't make a great error for proteins with not much high difference in their size.

4.3.2.3 Mass transfer coefficient (k_f)

In the transport-dispersive model, the mass transfer coefficient is responsible for the rate of mass transfer mechanism as the rate limiting step compared to adsorption mechanism [10]. This parameter like dispersion coefficient is mainly influenced with the size of the protein and its molecular weight. In this study, we

calculated this parameter using one protein and considered that it's the same for other protein. To measure this parameter, experiments with adsorption mechanism should be carried out and given simulated data tried to fit the experimental results. Breakthrough experiments data that was used to estimate the steric factor was also studied as the suitable data for this experiment. In this case, this parameter was chose so to minimize the amount of sum of the squares of the errors as a comparison between simulated chromatogram and experimental one.

4.3.3 Linear gradients

Three buffers with different concentration (0.1, 0.5 and 1 M NaCl) were prepared and first two buffers were used to as the primary and final concentration buffers for the gradients in all experiments. The pH of all the buffers was set to 6.8 using dilute forms of two buffers K2HPO4 and KH2PO4. The 1 M NaCl buffer was used for column regeneration. The gradient was performed exactly after the sample loading.

5 Results and discussion

5.1 Estimated parameters

5.1.1 Shape parameters (Dax, ε_l , ε_e and kf)

Single component breakthrough experiments that were indicated before were carried out to obtain the amounts of these parameters. The flow rate in all experiments was set to 0.2 ml/min. These parameters are given in Table 2. The breakthrough curve for the measurement of dispersion coefficient is given in Fig. 1. The mass transfer coefficient was obtained using data given in measuring steric factor in following section (Fig. 2).

To see the ability of the model to predict the effect of different values of axial dispersion imulated breakthrough curves were analyzed and have been shown in Fig. 3. Dispersion coefficient is the main effect when studying band broadening effect when there is no adsorption in the column. By increasing this parameter, peaks will have broader shape and this can be seen clearly by comparing the peaks. The flow was set to 0.2 ml/min for the simulation and was constant for three peaks along with other parameters except the axial dispersion. Usually this parameter is described by two mechanisms of diffusion and eddy diffusion and as an assumption their contribution are additive [11]:

$$D_{ax} = \gamma_1 D_m + \gamma_2 d_p u \quad (11)$$

In which first term in RHS (right hand side) is related to diffusion effect and second term in RHS is contributed to eddy diffusion. γ_1 and γ_2 are geometrical constants, *Dm* is the molecular diffusion, d_p is the particle diameter and *u* is the velocity. The relation of band broadening effect to axial dispersion is important when we use proteins with very different molecular weight and size because of the effect of protein size on the molecular diffusion, specially when system works in very low velocity or resin particles have a very fine size (which causes that first term has larger impact on the axial dispersion).

In cases like this study in which proteins have relatively same size we could measure axial dispersion for one component and assume that it is the same for other component. Otherwise, we have to measure this parameter for each component individually.

The inlet concentration was 0.02 mg/ml for *Pst*I and 0.04 mg/ml for *EcoR*I. Steric factor was measured using curve and simulated data represented in Fig. 2 and estimated for *Pst*I using Eq. (10).

5.1.2 Adsorption parameters

The equilibrium constant (*Keq*) and characteristic charge (*v*) was calculated for both proteins with different salt elution and calculated using Eq. (9).

5.2 Model validation

An experiment was carried out with known values of different variables and the simulated data was compared to experimental results for verification of the model validity(Fig. 4). For this, a sample consisting of both proteins with concentration of 0.02 mg/ml *Pst*I and 0.04 mg/ml *EcoR*I was injected to the column and the elution behavior was recorded and compared with simulated data. The elution was done using 4 CV with the starting buffer of 50 mM. As can be seen prediction of the elution time of the proteins by the program is good. Also it had the ability of simulating broadening effect and maximum concentration relatively good compared with experimental results. One explanation for difference between the maximum concentration from computer and the experiment (and slightly narrower bands in the model) is assuming that axial dispersion is the same for both components. Also the difference between the elution times of the peaks maxima and ones predicted by the model is that adsorption parameters (or

mass transfer coefficient) estimated may not exactly be the real ones in the system. However, the accuracy of the model to predict the resolution and retention time of two peaks is acceptable so the yield (our objective function) is almost the same by what we got by the experiment.

5.3 Optimization

The work that has been done in this study for optimization was mainly a two parameter study (gradient slope and feed volume) and calculating the yield (objective function) at different values of these decision variables. Yield was calculated for different simulation outputs and that the results is shown in Fig. 5. The yield is increasing by increasing the gradient slope from initial conditions used in the experiments carried out for validation of the process. But the objective function becomes flat for values of gradient slope near 2. It is because of increasing band broadening as a function of increasing time of separation that causes the maximum of two peaks separate from each other and from the other hand increasing the width of two peaks that decreases the resolution. Hence, the yield stays relatively constant in this condition. The estimated optimum parameters for the operation were also studied experimentally on the column and the given results shown in Fig. 6.

In comparison to the former condition (Fig. 4), the peaks are wider and maximum of the peaks appeared in the outlet are more far from each other. The feed volume does not affect the yield as much as the gradient slopes. However, by increasing the amount of loaded protein on the column yield slightly decreases especially when it gets higher values. Using this plot, it is also possible to design process space for chromatographic step which save time and number of experiments needed to do so in traditional methods as discussed by Iyer et al.[13].

6 Conclusion

Model-based optimization and study of two restriction enzymes in cation-exchange chromatography was implemented in this work. Use of low concentration samples, simpler formulation and from other side considering the developed method of SMA in the study of adsorption mechanism made it possible to reach to the optimum point for the separation of two restriction enzyme *Pst*I *and EcoR*I with lower experiments and high accuracy. In Transport-dispersive model, the particle description term is not considered in which simpler solution and formulation are obtainable; even this gave acceptable accuracy presented in this study. In the case of low concentration of proteins it may seem not necessary to use more detailed and accurate models to study the behavior of solutes in a two-component system. Nevertheless, considering all the mechanisms take part in the adsorption mechanism leads to better prediction of elution behavior and optimum condition. In the case of large proteins in the systems with lower resin capacities this may affect the adsorption behavior considerably different from ones estimated by simpler models [11] The used Crank-Nicolson Finite Difference Method (FDM), which has simpler formulation in compare to solutions based on Finite Element Method (FEM) showed reasonable results even in not much high number of grid points. However, this method takes longer calculation time and may sometimes show behaviors like oscillation specially in system boundaries or other parts of the system. It is possible to relatively eliminate these phenomena considering correction forms and suggestions given and proposed [20]. This behavior is directly proportional to the system and equations that's been used. In the studied system, this problem doesn't affect the simulated curves very much. The resin which was used in this study is very common in chromatography separation methods in laboratories. Though it doesn't have characteristics that newer resins own (like finer particles, higher capacities, narrow distribution of particle size); which made it possible for them to be used in HPLC systems; but it could be also used for simulation studies and modeling of the column in which it has been used with no significant problem. It is notable that with this column, complete isolation of proteins with similar characteristics (especially pI) is not achievable, but in following steps, the concentration of impurity will decrease which made it possible to increase the total yield of the process. The studied approach is efficient in process development step because of the use of lower experiments and taking less time. In addition, it is possible to use the calculated optimum for modeling of scale-up in large-scale production.

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Table 1. pI and molecular weight for two used enzymes

Protein	pI (Theoretical)	MW (g/mol)
PstI	7.2	37000
EcoRI	7.8	30000

Table 2. Shape parameters of the system

Parameter	value
External dead volume	1.29 ml
External porosity	0.50
Total porosity	0.57
$D_{ax}\left(m^2/s\right)$	$3*10^{-8}$
$k_f\left(s^{-1}\right)$	$1.2*10^{-3}$

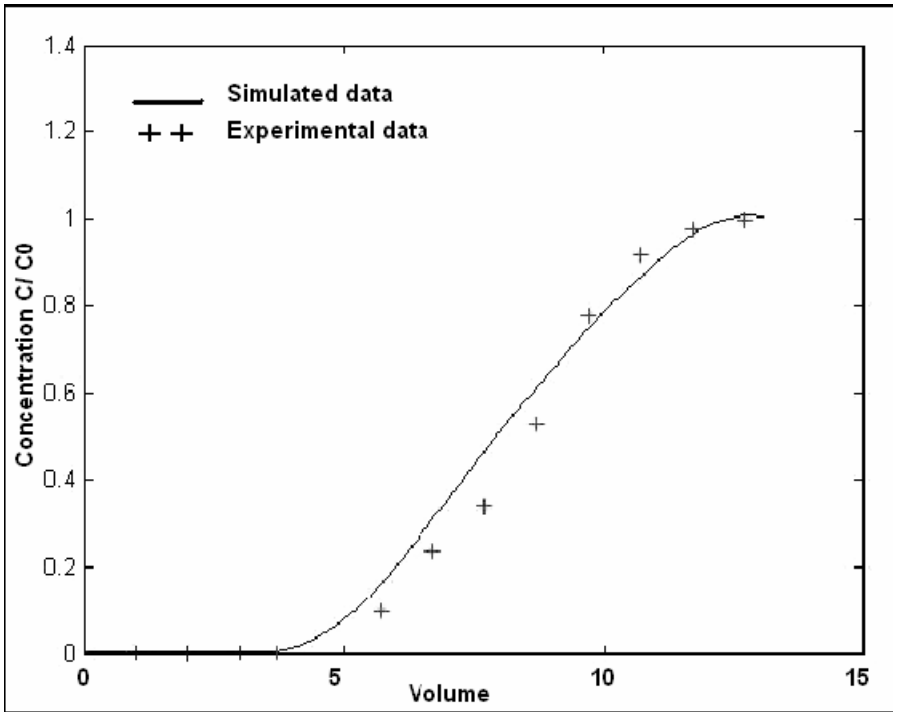

Figure 1. The experimental data and the fitted model data for *EcoR*I protein changing axial dispersion coefficient using the method of minimizing the sum of the squares.

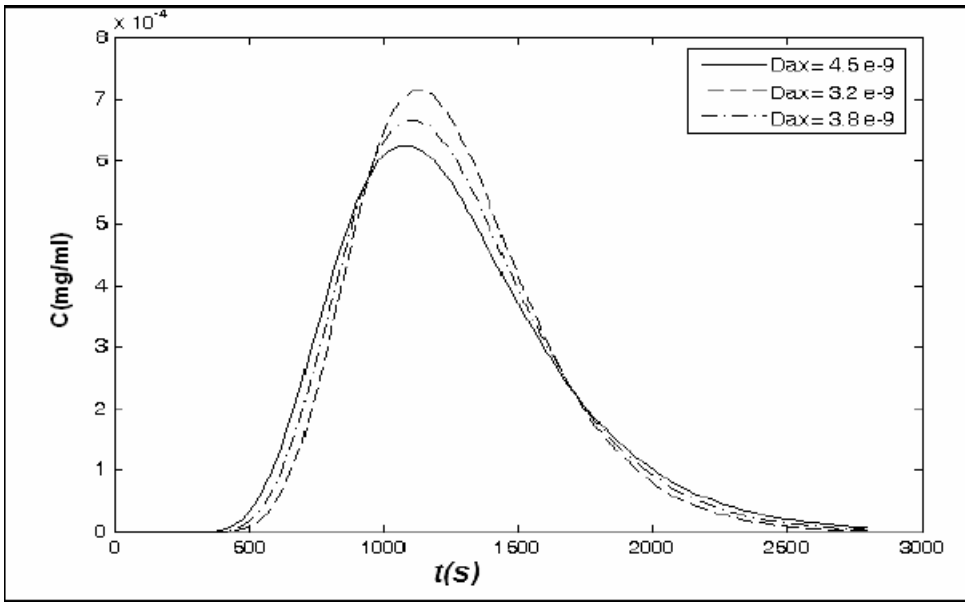

Figure 2. The experimental data and the fitted model data for EcoRI protein used for estimation of mass transfer coefficient and steric factor.

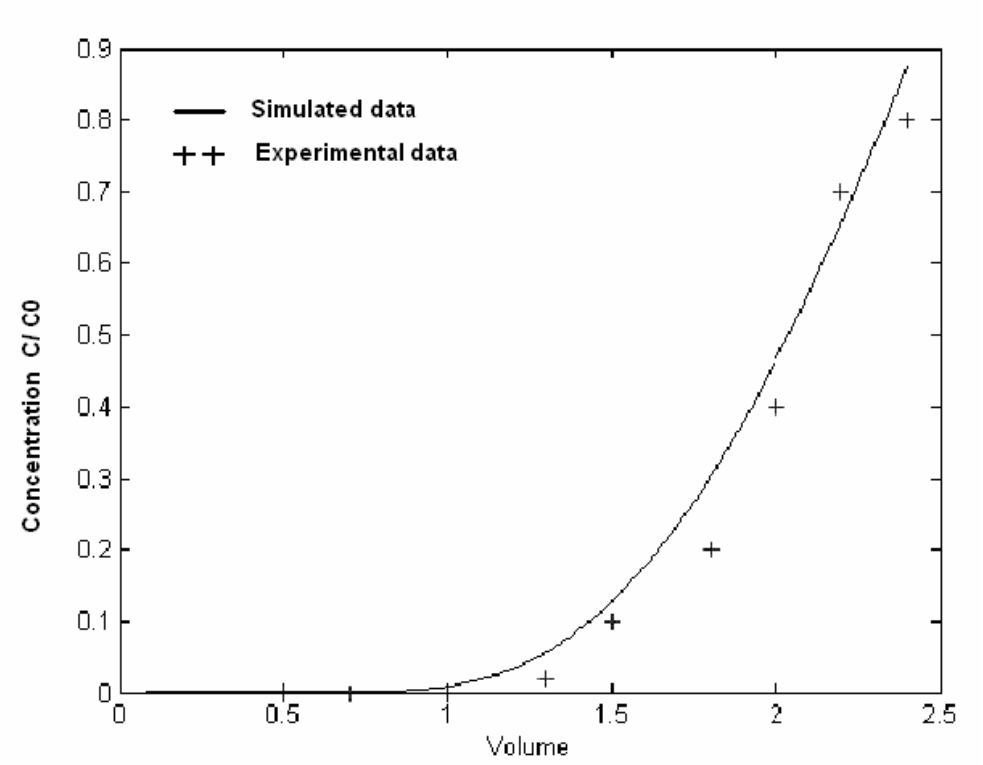

Figure 3. Different values for axial dispersion coefficient and different value for band broadening effect simulated by the model.

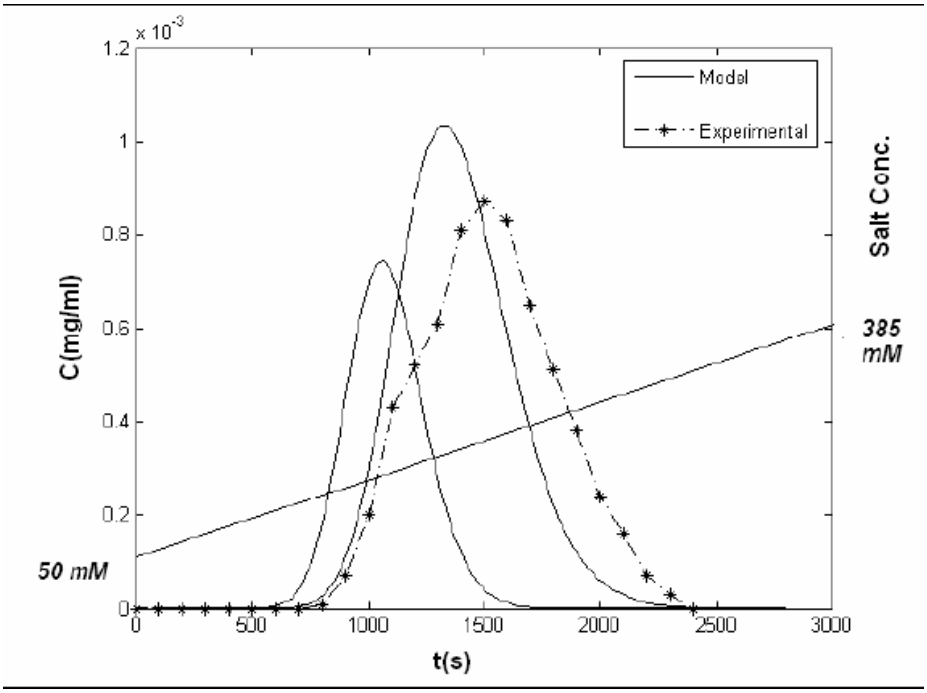

Figure 4. Gradient elution for a sample of two proteins used as the comparison between simulated data and experimental data.

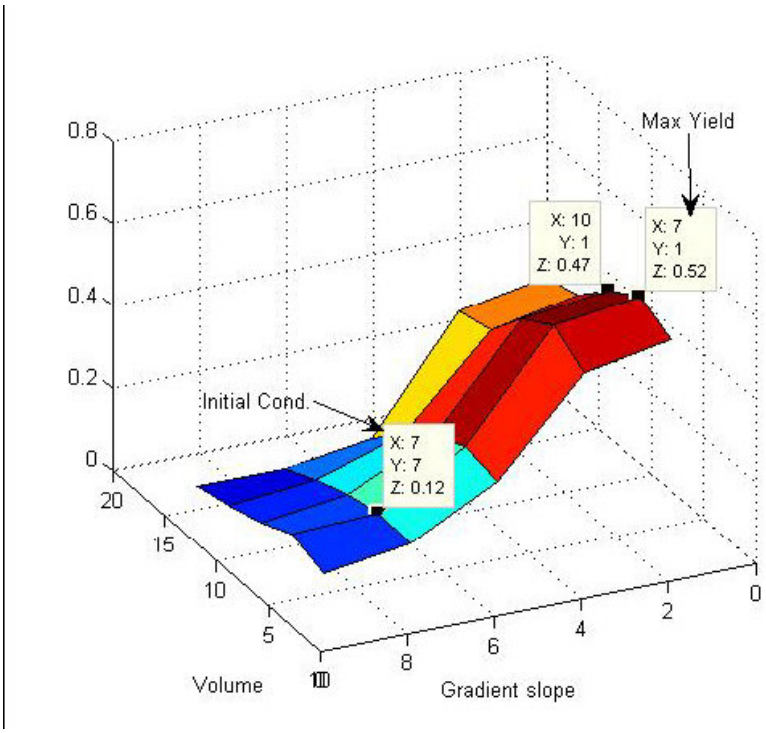

Figure 5. The optimization results plotted against two parameter gradient slope and feed volume. Initial condition is the condition used in validation experiment.

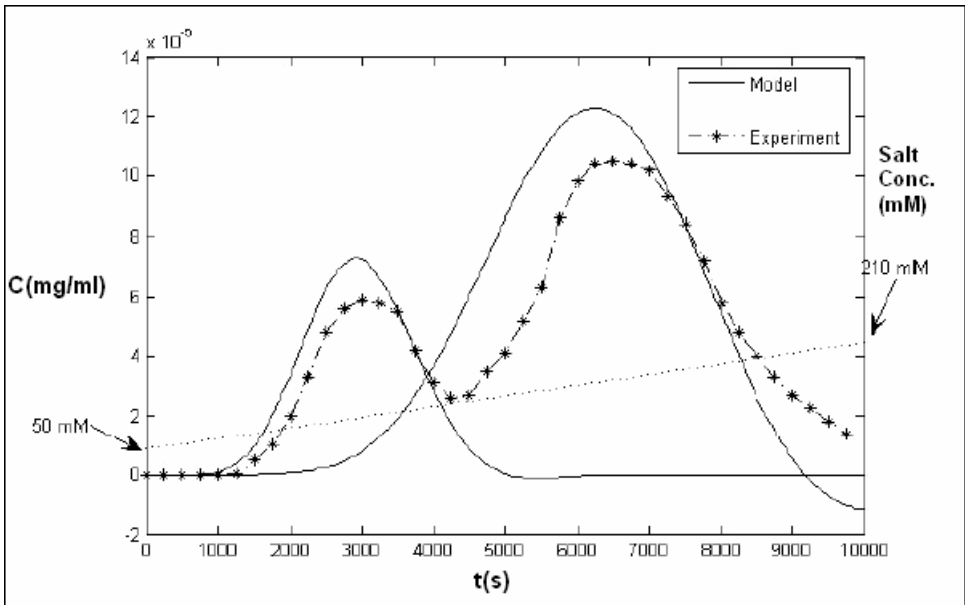

Figure 6. The results obtained in the experiments implemented on the column with optimum condition.