CANDIDA ANTARCTICA AS CATALYST FOR POLYCAPROLACTONE SYNTHESIS: EFFECT OF TEMPERATURE AND SOLVENTS

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ABSTRACT

The Effects of temperature on ring-opening bulk polymerizations of ε-caprolactone was studied by using lipase Novozym 435 (immobilized form of lipase B from *Candida antarctica*), as biocatalyst. The polymerization of ε-caprolactone was carried out at 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C. For Novozym 435 the results showed that increasing the reaction time of the polymerization system resulted in an increased rate of monomer consumption and hence increased the molecular weight. For an increase in reaction time the conversion increases steadily and after a gradual increase there is a decrease which is found uniform for all the temperature showing a uniform trend. For a temperature of 70°C and 4 hours molecular weight was found to be 8.4 x 10^4 which was the highest of all the readings that were obtained. A series of solvents including chloroform, isopropyl ether, isooctane, and toluene were evaluated at 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C. The modeling approach which had the objective to model the molecular weight distribution for different solvents used in the experiment that is necessary for various parameters namely the temperature that can be obtained by continuous training, testing and validating the model was carried out.

Keywords: Enzymatic Polymerization; Ring opening polymerization; Polycaprolactone Synthesis, Neural Network Modeling.

1. INTRODUCTION: RING OPENING POLYMERIZATION

Recently, Enzymatic polymerization has been receiving more and more attention as a new environmentally friendly method of polymer synthesis, in contrast to the chemical methods, which generally need harsh conditions and metallic catalysts that must be completely removed especially for medical applications. Furthermore, enzymatic polymerization can offer a novel method to produce polymers that are difficult to be synthesized by conventional Polymerization (ROP) [1]. Among the various polymerization methods, Ring-Opening polymerization is an important alternative route because leaving groups that can limit monomer conversion or degree of polymerization are not generated during polymerization [2]. Biodegradable polyesters, one of the most important synthetic polymers, are always synthesized by ring-opening polymerization of cyclic lactones. Poly (ε-caprolactone) and polylactides are synthetic polymers with quite unusual properties of biodegradability and biocompatibility. They have thus great potential as biomaterials and environmentally friendly thermoplastics. This explains why attention was paid quite early to the macromolecular engineering of these aliphatic polyesters [3].

For this purpose, living, or at least controlled, polymerization is a prerequisite, which nowadays is met whenever the ring opening polymerization (ROP) of lactones, lactides, and glycolide (Fig. 1) proceeds through a coordination–insertion mechanism. Then, aliphatic

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polyesters can be prepared with a predictable degree of polymerization, a narrow molecular weight distribution, and well-defined end groups at one or both ends. Synthesis of block and graft copolymers of lactones and lactides has also been reported. In all these instances, lactones of different ring sizes, substituted or not by functional groups, have been involved [4] .

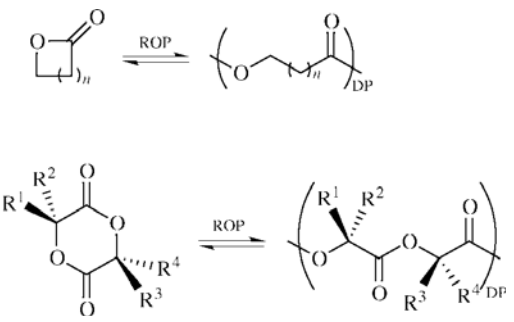

Fig. 1. Ring opening polymerization of unsubstituted lactones, lactides, and glycolide. n= 1: β-propionolactone (β-PL); n = 2: γ -butyrolactone (γ -BL); n = 3: δ-valerolactone (δ-VL); n = 4: ε-caprolactone (ε-CL); DP = degree of polymerization. R1 = R2 = R3 = R4 = H: glycolide; R1 = R4 = CH3 and R2 = R3 = H: L-lactide (L-LA); R1 = R4 = H and R2 = R3 = CH3: D-lactide (D-LA); R1 = R3 = CH3 and R2 = R4 = H: meso-lactide (meso-LA); D-LA/L-LA = 50/50: racemic-lactide (D,L-LA). (Herman F. Mark, Encyclopedia of Polymer Science and Technology, Volumes 11, pp-547-565, 2004)

1.1 Lipases as catalyst for Ring-Opening Polymerization

It is known that lipases can accept a wide range of substrates, including cyclic lactones, to produce a wide range of esters. Lipase-catalyzed polymerization may be one of the most attractive applications in the industrial field for the next generation, because enzymatically polymerized polyesters are expected to be potentially biodegradable. In recent years, some efforts have been made to develop a novel polymer synthesis using enzymes, such as polyesters by condensation, by transesterification, or ring-opening polymerization.

Lipase-catalyzed ROP of lactones was first presented by two independent groups, Kobayashi et al. [5, 6] and Knani et al. [7] in 1993 and the technique has rapidly developed as a novel methodology for polymer synthesis since then. Lately the ring opening polymerization of ε-CL have been carried out with several enzymes as catalysts namely various lipases of different origin, lipase CA (Candida Antartica), lipase CC (*Candida cylindracea*), lipase PF (*Pseudomonas fluorescens)*, PPL (porcine pancreas lipase), lipase PC (*Pseudomonas cepacia*), and *Rhizopus japonicus* lipase (lipase RJ) and also by various other non lipase enzymes like Humicola Insolens Cutinase (HIC). But of all Novozyme-435, lipase B from Candida antartica immobilized on macroporous acrylic resin, has been proven effective to catalyze the ring-opening polymerization of ε-CL [8]. Also lipase PF(*Pseudomonas fluorescens*) have provided good results. Lately Humicola Insolens Cutinase (HIC) have been proving good results. Also Lactones, lactides, cyclic carbonates and depsipeptides of ring-size from 4 to 17 have been polymerized using lipases from various sources. Despite significant advancements in recent years, a number of problems still exist in transferring the protocols of in vitro enzyme-catalyzed polyester synthesis from the

laboratory to an industrial scale. Of particular concern is the relatively low catalytic activities displayed by enzymes when used in nonaqueous media.

The problem is well illustrated in studies by Kobayashi et al. [9] and Dong et al. [10] that describe low reaction rates and molecular weight values for lipase catalyzed ε-CL polymerizations. This necessitates the use of comparatively large amounts of enzymes to ensure that the desired extent of polymerization occurs within a reasonable time scale. The productivity of enzymatic catalysis, in terms of units of polymer obtained per units of enzyme used, will to a large extent determine the feasibility of employing an enzymatic process in commercial processes. Surprisingly, limited effort has been directed at enhancing the kinetics of in vitro enzyme-catalyzed ring opening lactone polymerization. Such work would transform these new polymerization routes from academic curiosities to methods with reaction kinetics that are worthy of attention by industrial scientists. In that spirit, gross et al. [11] described Novozyme-435 (immobilized *Candida Antarctica* lipase B) catalyzed ring-opening polymerizations of ε-CL. They explored how engineering of the reaction media, by manipulating the solvent structure and concentration can be used to expand the range of temperatures at which lipase catalyzed polymerizations can be conducted. Furthermore, systematic regulation of these variables led to new knowledge on how to control the propagation kinetics, molecular weight, and "living" character of these polymerizations.

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2. MATERIAL AND METHODS

2.1 Novozyme 435 (Candida Antartica lipase):

Polymerization grade ε-caprolactone purchased from Merck Pte. Ltd, was first dried over calcium hydride and then distilled under reduced pressure in nitrogen atmosphere. Chloroform and toluene, were purchased from Merck Pte. Ltd. Toluene was dried over calcium hydride, and distilled under nitrogen atmosphere. Novozyme-435 (specified activity 7000 PLU/g) was purchased from Science Technics Pte. Ltd. All liquid chemical transfers were performed by syringe through rubber septum caps under nitrogen atmosphere.

ε-CL (10 g), toluene (20 mL), and Novozyme-435 (1 g) were added to a round-bottom flask (250 mL) and the reaction was maintained at 70°C, for 4 h, with magnetic stirring. After a desired monomer conversion, the reactions were terminated by adding an excess of cold chloroform and removing the enzymes by filtration (glass-fritted filter, medium pore porosity). The chloroform in the filtrate was in large part removed by rotary evaporation and the polymer in the concentrated solution was precipitated in methanol. The precipitate was

isolated by filtration and dried (0.1 mmHg, 50 °C, 24 h). The molecular weights of the samples were determined by gel permeation chromatography (GPC).

2.2 Measurements:

GPC analysis was performed using a Waters 2487 apparatus (USA) with a Dual λ Absorbance detector with Styragel HR5E THF 7.8 x 300 mm column or Styragel HR4 THF 7.8 x 300 mm column and chloroform eluent at a flow rate of 1.0 mL/min. The calibration curves for GPC analysis were obtained using polystyrene standard.

3. RESULTS AND DISCUSSION

3.1 Novozyme 435 (Candida Antartica lipase):

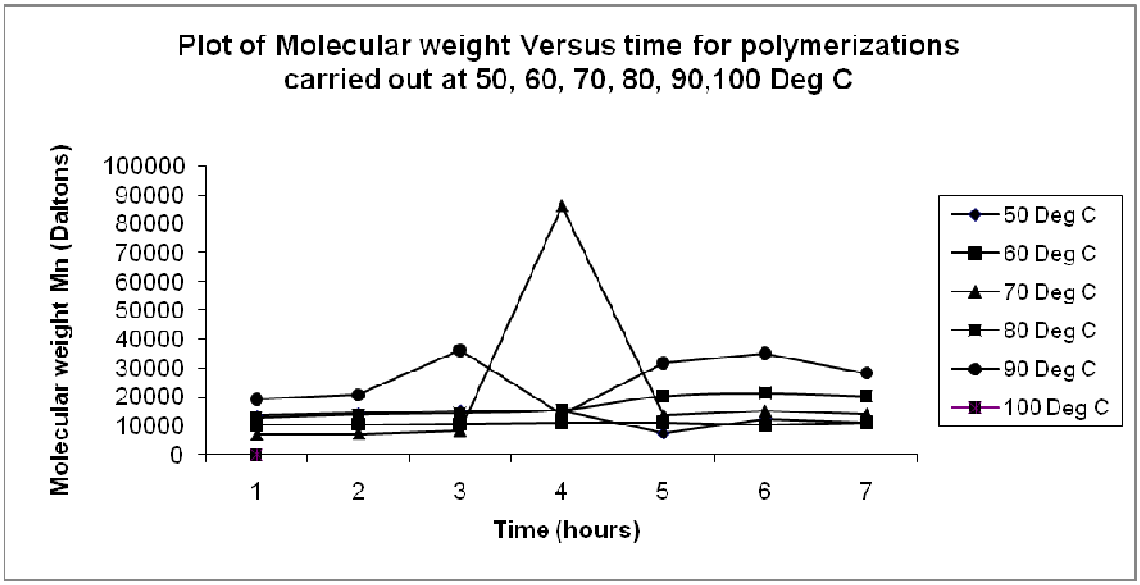

Figure 1: Effect of Temperature For Toluene As Solvent

Temperature Effects:

Figure 1 shows the variation of molecular weight with reaction time at various temperatures (50-100°C) with a volume ratio of 1:2 of ε-caprolactone:/toluene being taken. The polymerization rates increase with an increase of time up to the 4 hours after which there has been a steep decrease for all temperature from 50-100°C. Hence the maximum molecular weight is found to be 8.4 x 10^4 Daltons at a temperature of 70°C for the 4^{th} hour.

Effect of Solvents:

Polymerizations are being conducted at various temperatures while using different organic media. The concentration of the catalytic protein relative to monomer was maintained at about 1% (wt/wt) throughout this work. Reactions were performed by transferring ε-CL (0.3 ml) and Novozyme-435 (30 mg) into Pyrex tubes stoppered with rubber septa under a nitrogen atmosphere. Solvents (0.6 mL) including chloroform, dibutyl ether, isopropyl ether, isooctane, and toluene were transferred by syringe into reactions that were stirred for 4 h and maintained at 70°C. According to the literature, the following observations have been taken

into account and our experiments have been in progress to achieve maximum molecular weight. A better understanding of how solvent geometry, dipole moments, solubilization of substrates, and other factors influence the physicochemical and catalytic properties of enzymes is needed, but since our research focus is on obtaining maximum molecular weight, we are dealing with only those parameters which would enhance our results.

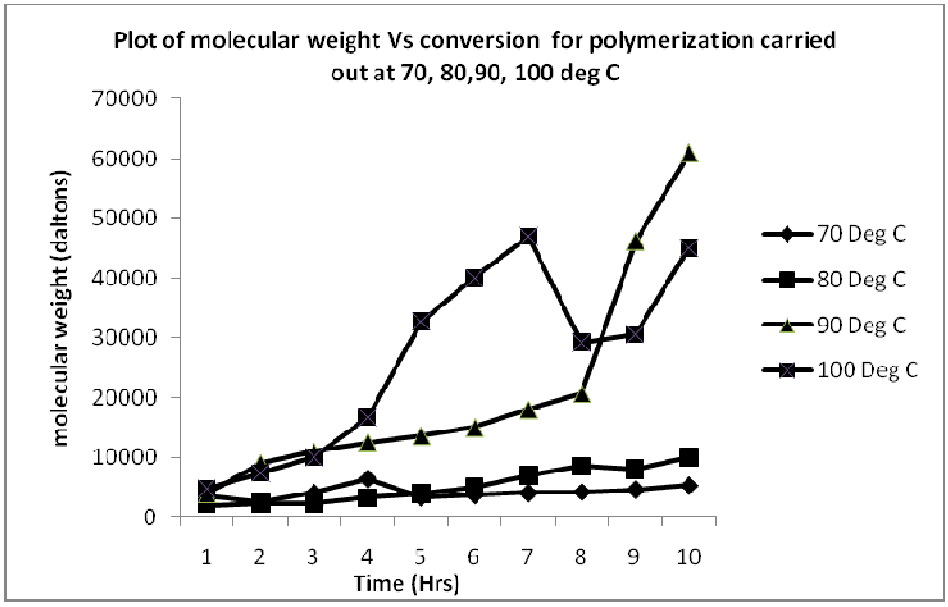

Figure 2: Effect of Temperature For Isoctane As Solvent

3.2 Comparison of the two plots

The plot obtained with toluene as the solvent results in a molecular weight that is found to be the maximum at a temperature of 70°C for the 4th hour, where as in the case of isoctane as solvent there was complete change in the trend of the plot obtained,where in it is seen that for the temperature of 70°C there is an increase of molecular weight as time progresses and is found to be maximum at 7th hr and followed by a sudden decrease in molecular weight with an unexpected trend to follow. For the temperatures of 80, 90, and 100°C, the trend seems to be uniform with an increase in molecular weight till 7th hour.Hence the maximum molecular weight is found to be 4×10^4 Daltons.On comparing with the previous plot where in toluene was used as the solvent the result was that the maximum molecular weight was found to be 8.6×10^4 Daltons. Clearly indicating that toluene if more suitable for the polymerization of lactone to polyester using novozyme 435 as catalyst.

3.3 Inferential estimation of biopolymerisation

This section is intended to explain the necessity of the current experimental work that is being carried out in our laboratory. The main objective of the experimental work is to carry out a series of experiments where in the conversion of lactone to polyester using ring opening polymerization using the enzyme Novozyme 435, as the catalyst is intended to produce a product of higher molecular weight. Hence the experiments involving solvents namely toluene and isoctane is carries out at the temperature range between 50 deg C and 100 deg C for toluene and between 70 deg C and 100 deg C for isocatane.

As indicated earlier the main objective is to obtain the results from these experiments and to create a prediction model using neural network modeling technique. In this paper the prediction of the molecular weight using the neural network modelling approach is only subjected to the toluene solvent that has been used in the set of experiments involving different temperature range from 50 to 100 deg C. and not for isoctane solvent the reason being that the amount of data obtained from the experimental work is quite insufficient for dividing the data for training, testing and validation.

Therefore in this section, the objective is to model the molecular weight necessary for any particular condition namely the temperature can be obtained by continuous training, testing and validating the model that is obtained. Hence the series of steps lies in splitting the available set of datas into 3 different parts. First set for the training of the model, followed by the set of datas for testing and finally the last part for the validation which is intended to check the accurate prediction capability of the model. The final fine tuning of the model results in the best possible outcome of the model which would be to have a model that can achieve a closest prediction of results in comparion to the experimental data ie. the molecular weight obtained from the results.

In this modeling approach, neural networks with fixed identical structure, which consist of 3 input, 1 ouput and 20 hidden nodes were developed from boostrap re-samples technique of the original training and testing data of the biopolymerization process in flask level. The data from the flask level are taken for every 1 hour for 7 hours. Bootstrap re-sampling is then employed to resample the data. In re-sampling the training and testing data using bootstrap re-sampling techniques, the training and testing data was already in the discrete time function, therefore by re-sampling discrete time function it does not affect the sequence of input-output mapping of the prediction. Then, the neural networks were trained by the Levenberg-Marquardt optimization algorithm with regularization and "early stopping". All weights and biases were randomly initialized in the range from –0.1 to 0.1. The structure of networks are single hidden layer feed forward neural networks.

Hidden neurons use the logarithmic sigmoid activation function whereas output layer neurons use the linear activation function. To cope with different magnitudes in the input and output data, all the data were scaled to zero mean and unit standard deviation. The data for neural network model building need to be divided into: 1) Training data (for network training); 2) Testing data (for cross-validation based network structure selection and early stopping); and 3) Unseen validation data (for evaluation of the final selected model). The data for training and testing are resampled before the processes are executed whereas the validation data are taken as fresh data from the experimental processes. In networks with fixed structure, the network structures, i.e. the number of hidden neurons, were determined through cross validation. Single hidden layer neural networks with different numbers of hidden neurons were trained on the training data and tested on the testing data. The network with the lowest sum of squared errors (SSE) on the testing data was considered as having the best network topology. In assessing the developed models, SSE on the unseen validation data is used as the performance criterion. In this case study, one-step-ahead predictions process was proposed where the objective is to predict or model the molecular weight of polycaprolactone.

The model is shown as follows:

$$\hat{y}(t) = f[y(t-1), u1(t), u2(t-)] \qquad (1)$$

where *u1*(*t*) is the reaction temperature at time *t*, *u2*(*t*) is the reaction time at time *t*. $\hat{y}(t)$ is the predicted process output which is molecular weight at time *t(7)* while *y(t-1)* is the molecular weight at 1 hour until *y(t-7)* which is at 7 hours respectively. In this work, bootstrap resampling technique has been employed to resample the data. The input for the neural networks comprises of the data from reaction temperature and biopolymer molecular weight.

To create the model, the data was split into two sections where the last two batches were taken as a validation data or unseen data and the rest of the data were resampled based on the bootstrap method into 722 data. Then, the resampled data were divided into another two sections for training and testing. Fig. 3 shows the plot of actual and predicted validation data for moleculat weight using toluene as a solvent. It is shown that the actual and predicted value is more or less the same or in good agreement. On the other hand, correlation coefficient value, *r* for training, testing and validation plots are as in Table 1. The performance of neural network modeling capability is generally assessed by *r* value for validation data. From Table 1, *r* value for validation data is 0.996. It is shown that neural network with bootstrap re-sampling method successfully captured the entire inferential estimation process for biopolymer molecular weight.

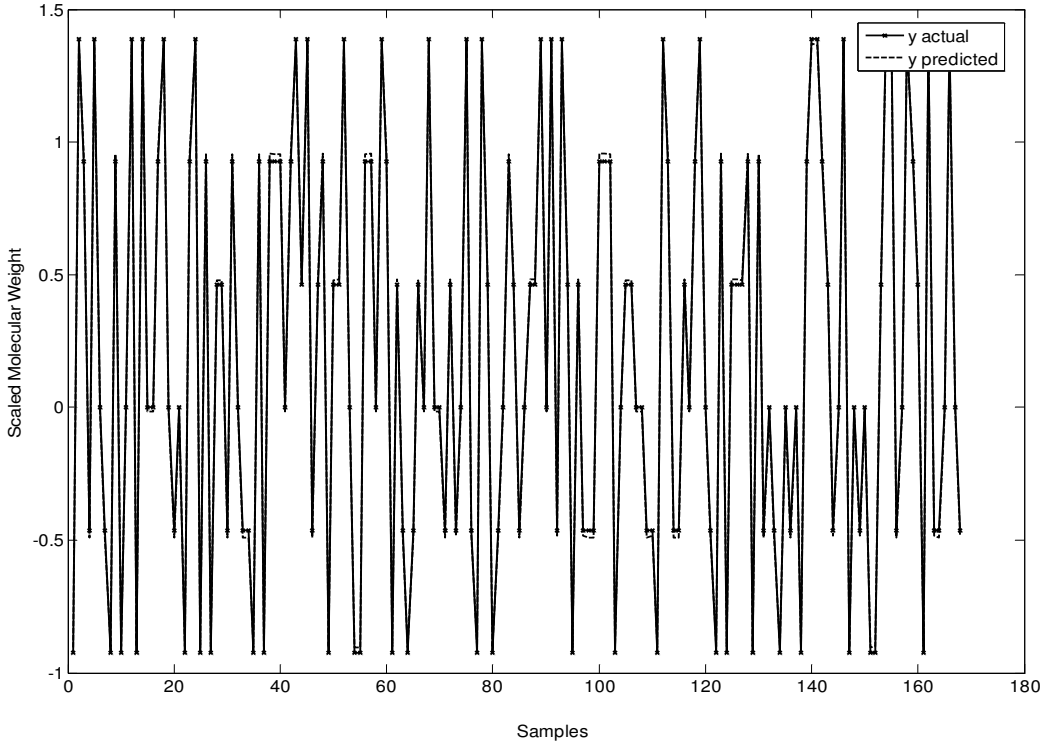

Figure 3: Actual and predicted data for validation

Table 1: Correlation Coefficient Value

Data	Correlation Coefficient, *r*
Training	0.996
Testing	0.996
Validation	0.996

4. CONCLUSION

The result of the enzyme *Candida Antarctica* lipase has lead us to the conclusion that the maximum molecular weight of the polymer ie. polycaprolactone is found to be 8.6 x 10^4 Daltons for Novozyme 435 (Candida Antartica lipase) proving that *Candida Antarctica* lipase is the best option of being the enzyme catalyst. In conclusion, the enzymatic ring-opening copolymerization of ε-CL with lactones using lipase P as catalyst afforded the copolyesters. On top of that the molecular weight distribution is varies from minimum 2 x 10^3 Daltons to maximum 8.6 x 10^4 Daltons for toluene as solvent and for isoctane molecular weight varies from minimum of 2 x 10^3 Daltons to 4 x 10^4 Daltons. Thus clearly illustrating that the molecular weight distribution is clearly dependent on the temperature and time of various samples that have been taken up for the ring opening polymerization of lactone to polyester.

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