# OPTIMIZATION OF MONOLITHIC ENZYMATIC MEMBRANES ACTIVITY AS A FUNCTION OF THEIR COMPOSITION BY USING DESIGN OF EXPERIMENTS (DOE)

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# OUTLINE

## Introduction

## Experimental

Design of experiments

Membrane preparation

Enzymatic activity assays

## Results and discussion

Analysis of the model

Effects of membrane composition on the enzymatic activity of membranes

Predictive capacity of the model and optimization

## Conclusions

# INTRODUCTION

**Advantages**

1. Increase of enzyme stability
2. Reduction of cost and complexities of the process REUSE
3. Obtention of products of higher purity than the obtained with enzyme in solution

## ENZYME IMMOBILIZATION

Obtention of insoluble forms of the enzyme that allow its reutilization while maintaining the catalytic activity

**Disadvantages**

1. Alteration of enzyme conformation with regard to the native form
2. Loss of activity due to the blocking of active sites

# INTRODUCTION

## ENZYME IMMOBILIZATION IN MEMBRANES

### PHYSICAL IMMOBILIZATION

- Adsorption
- Entrapment:

  incorporation of the enzyme with the monomers and further polymerization

  introduction of the enzyme in a polymeric matrix

### CHEMICAL IMMOBILIZATION

- Covalent linkage between functional groups of the enzyme and the support
- *cross-linking:* use of bifunctional reagents

# INTRODUCTION

## ENZYME IMMOBILIZATION IN MEMBRANES

### Carbodiimide coupling assisted by *N*-Hydroxysuccinimide (NHS) [1]

### PHYSICAL IMMOBILIZATION

### CHEMICAL IMMOBILIZATION

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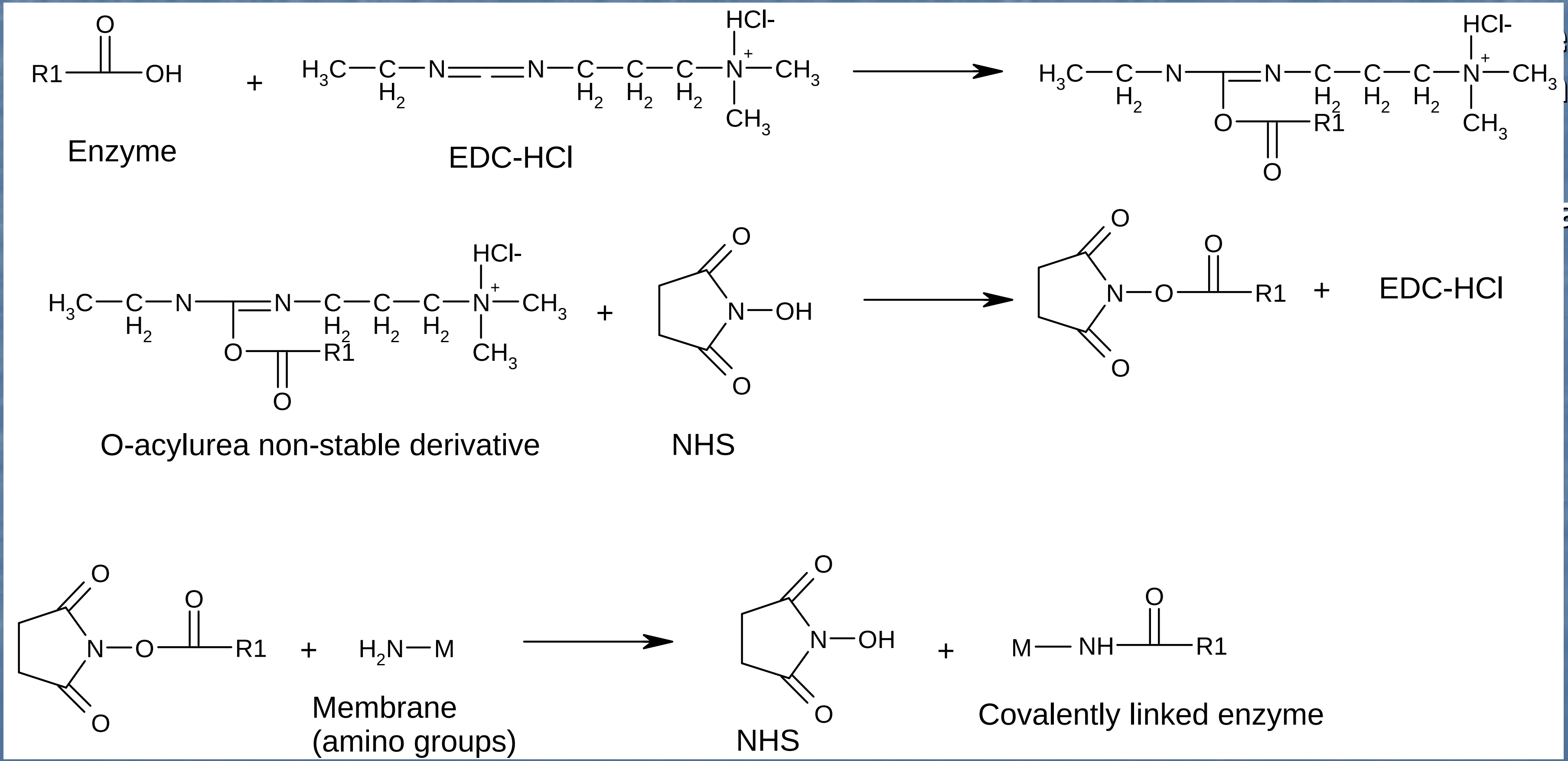


[1] Staros, J.V., Wright, R.W.,Swingle, D.M. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal. Biochem.* 156 (1986) 220-222.

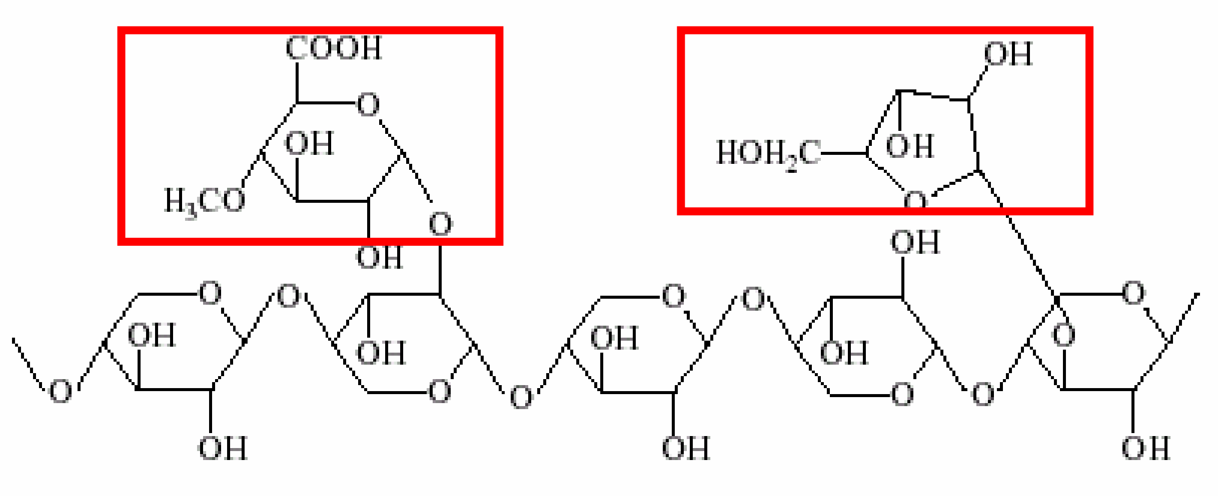
# INTRODUCTION

## ENDO-1,4-B-XYLANASE AND XYLOOLIGOSACCHARIDES

**Endo-1,4-β-xylanase II (E.C. 3.2.1.8) from *Trichoderma reesei***
**Hydrolyzes the xylan 1,4- β-glycosidic linkages**

**Xylan**
**Structural functions/ source of carbohydrates.**
**Natural origin: forestry and agricultural materials.**
**Heteropolysaccharide: xylose backbone + ramifications and substituents.**

**Chemical/enzymatic hydrolysis of xylan ⟶ Xylooligosaccharides**



**Applications**

- **Food ind** … **ts.** [2]
- **Pharmac** … **rocesses.** [4]
- **Producti**

[2] M. Okazaki, S. F
[3] A. L. Stone, D. J
[4] K. Hisado, K. Mi … (1985).
[5] M. Godliving, Y. Nakamura. *Biodegradation* 16 (2005) 493-499.

# INTRODUCTION

## MONOLITHIC POLYMERS

- Highly crosslinked and porous materials with numerous interconnected cavities
- Their internal structure remains rigid even in the dry state
- Low mass transfer resistance
- Chemical characteristics (i.e. functionality) and physical properties (i.e. porosity, mechanical stability) depend on the selection of the appropriate constituents and their content in the precursor solution.

Monomer mixture + crosslinker + porogenic solvents

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Monomer mixture + crosslinker + porogenic solvents

↓

Phase separation during polymerization

**Applications**

separation media in HPLC
supports for solid phase chemistry
gas chromatography
immobilization of enzymes [6]

[6] M. Petro, F. Svec, J.M.J. Fréchet *Biotech. Bioeng*, (1996) 49: 355.

# INTRODUCTION

## CHEMOMETRICS AND DESIGN OF EXPERIMENTS (DOE)

DOE: plan of experiments suitably arranged so that with the observed results it is possible to obtain the desired information.

Three major steps:

1. Estimation of the coefficients in the mathematical model.
2. Response prediction.
3. Evaluation of the model predictive capacity (additional experiments).

Screening experiments: study of which of the experimental variables (factors) have real influences on the results.

- Identification of the potentially significant variables.
- Known experimental results: fitting of data.

**Polynomial model (response surface model)**

# INTRODUCTION

## CHEMOMETRICS AND DESIGN OF EXPERIMENTS (DOE)

### Response surface model

- Influence of all experimental variables on the measured response.
- Analyze how sensitive is the response to variations in the settings of the experimental values.
- Locate the optimum conditions.

Scaled and centred variables ($x_i$) instead of the original variables ($u_i$)

- Normalize the influence of all the variables considered.
- The estimated model parameters can be directly compared to each other.
- Linear transformation into a variation of $x_i$ centred around zero between the interval [$-1 \leq x_i \leq +1$]:

$$x_i = (u_i - u_0) / \delta u$$

$$u_0 = (u_{high} + u_{low}) / 2$$

$$\delta u = u_{high} - u_0$$

# INTRODUCTION

## OBJECTIVES

- Development of enzymatic monolithic membranes with high catalytic activity for the degradation of xylan into xylooligosaccharides.
- Study of the effect of three variables in the membrane composition on the enzymatic activity of the resulted membranes by using design of experiments (DOE).
- Evaluation of the predictive capacity of the model with additional experiments.
- Optimization of the suggested model to provide the best membrane composition to achieve maximum enzymatic activity.

# EXPERIMENTAL

## DESIGN OF EXPERIMENTS (DOE)

**Software *Modde©* 6.0, *Umetrics©* (Norway)**

**Process variables (factors) :**

1. **Porogenic solvents ratio $X_1$**
2. **Amount of the crosslinker EDMA (ethylene dimethacrylate) $X_2$**
3. **Amount of the monomer AEM (2-aminoethylaminomethane) $X_3$**

**Response: enzymatic activity of the membranes.**

### a) Two-level full factorial design

- **Variables can take two fixed values (two-level): low and high.**
- **Full design: all possible settings of the experimental variables (k factors at least $2^k$ experiments).**
- **Response: lineal combination** {
  - **3 independent variables**
  - **cross products (interaction effects)**
  - **3 variables product term**

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{23}X_2X_3 + A_{13}X_1X_3 + A_{123}X_1X_2X_3$$

**Total exp: 8 + 3 central points (orthogonality)= 11**

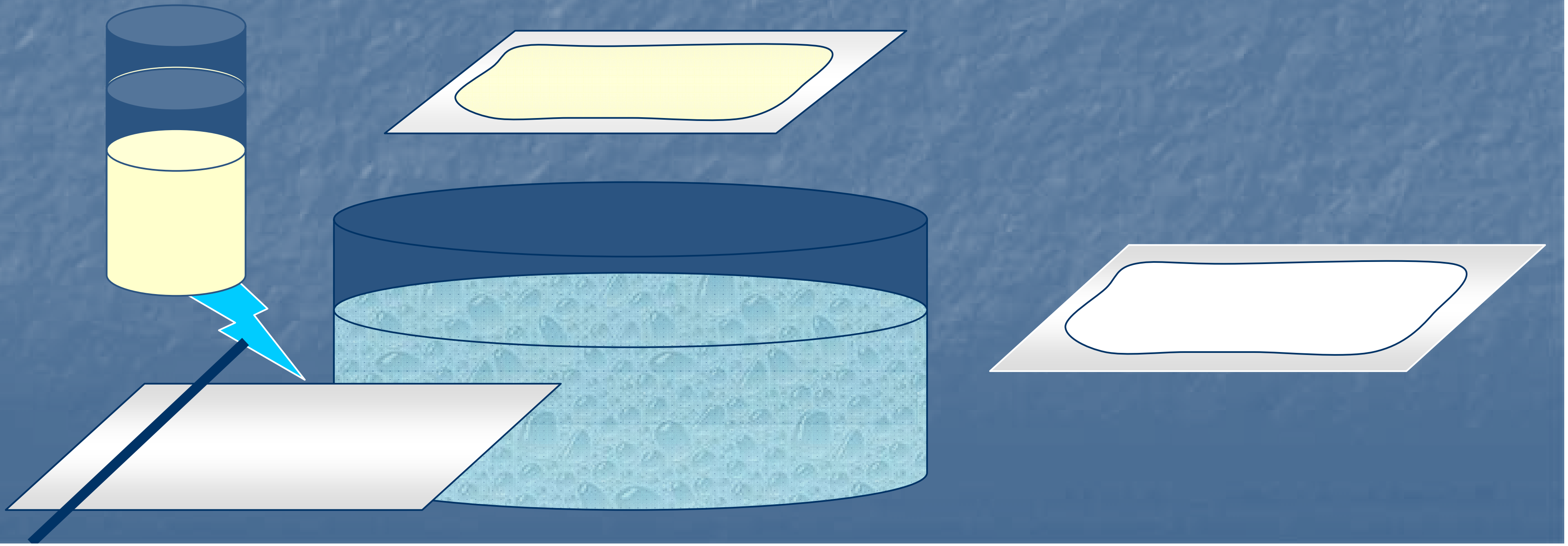
# EXPERIMENTAL

## MEMBRANE PREPARATION

- Mixture of the monomers
  2-aminoethylaminomethane (AEM)
  Hydroxymethyl methacrylate (HEMA)
  Ethylenedimethacrylate (EDMA)
- HMPP (free radical initiator)

} porogenic solvents (cyclohexanol & dodecanol)

- Cast of membrane solution on a non-woven fabric
- Polymerization through UV irradiation (254 nm)
- Wash membranes with ethanol to remove excess of porogenic solvents



# EXPERIMENTAL

## MEMBRANE PREPARATION

| Membrane | EDMA (mg) | AEM (mg) | HEMA (mg) | Molar ratio of solvents | Cyclohexanol (mg) | Dodecanol (mg) | % monomer | % solvents |
|---|---|---|---|---|---|---|---|---|
| N1 | 315 | 70 | 1115 | 2.54 | 4416 | 3280 | 15.94 | 83.69 |
| N2 | 315 | 70 | 1115 | 35.29 | 7776 | 410 | 15.15 | 84.51 |
| N3 | 750 | 70 | 680 | 2.54 | 4416 | 3280 | 15.94 | 83.69 |
| N4 | 750 | 70 | 680 | 35.29 | 7776 | 410 | 15.15 | 84.51 |
| N5 | 315 | 100 | 1085 | 2.54 | 4416 | 3280 | 15.94 | 83.69 |
| N6 | 315 | 100 | 1085 | 35.29 | 7776 | 410 | 15.15 | 84.51 |
| N7 | 750 | 100 | 650 | 2.54 | 4416 | 3280 | 15.94 | 83.69 |
| N8 | 750 | 100 | 650 | 35.29 | 7776 | 410 | 15.15 | 84.51 |
| N9 | 532 | 85 | 883 | 18.92 | 7504 | 739 | 15.06 | 84.60 |
| N10 | 532 | 85 | 883 | 18.92 | 7504 | 739 | 15.06 | 84.60 |
| N11 | 532 | 85 | 883 | 18.92 | 7504 | 739 | 15.06 | 84.60 |

| Experiment | Porogenic solvents ratio | Amount of crosslinker EDMA | Amount of AEM |
|---|---|---|---|
| N1 | -1 | -1 | -1 |
| N2 | 1 | -1 | -1 |
| N3 | -1 | +1 | -1 |
| N4 | +1 | +1 | -1 |
| N5 | -1 | -1 | +1 |
| N6 | +1 | -1 | +1 |
| N7 | -1 | +1 | +1 |
| N8 | +1 | +1 | +1 |
| N9 | 0 | 0 | 0 |
| N10 | 0 | 0 | 0 |
| N11 | 0 | 0 | 0 |

| Porogenic solvents molar ratio | Amount of crosslinker EDMA (µl) | Amount of AEM (mg) |
|---|---|---|
| 2.54 (-1) | 300 (-1) | 70 (-1) |
| 18.915 (0) | 507 (0) | 85 (0) |
| 35.29 (+1) | 714 (+1) | 100 (+1) |

# EXPERIMENTAL

## MEMBRANE PREPARATION

### Enzyme immobilization in monolithic polymeric membranes

- Xylanase solution (0.5 mg/ml).
- 5 mM N-Hydroxysuccinimide (NHS) + 2 mM N-(3-Dimethylaminopropyl) N’-ethyl-carbodiimide hydrochloride (EDC).
- Room temperature, 2h phosphate buffer (pH 7.4) + 0.15 M NaCl.
- The membranes are washed with distilled water and stored at 4 ºC prior to activity test.

## ENZYMATIC ACTIVITY ASSAYS

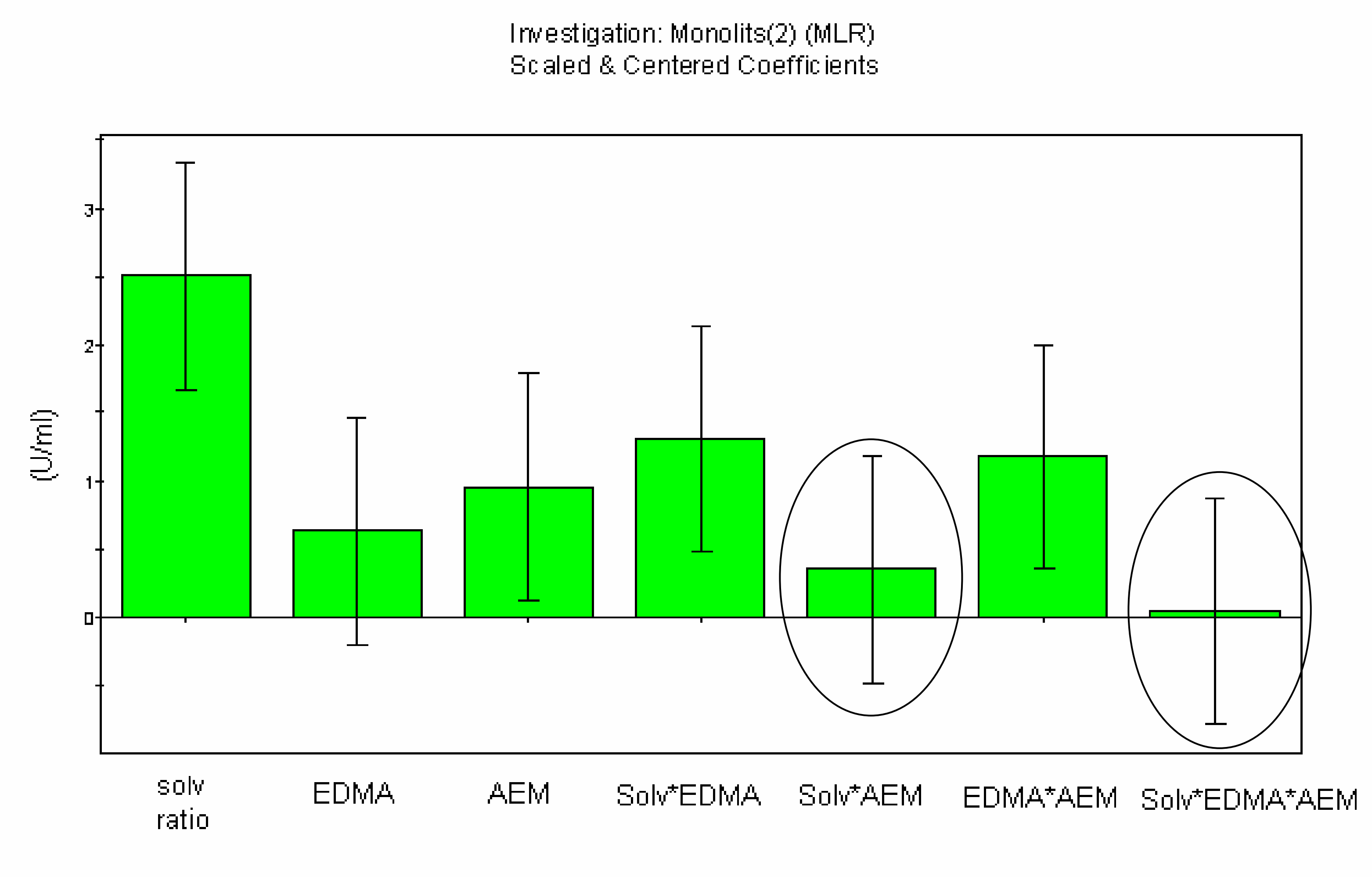
*Sigma quality control test procedure, enzymatic activity assay for endoxylanase* (Colorimetric assay)

- Incubate 1 $cm^2$ of enzymatic membrane at a fixed temperature (50 ºC).
- Substrate: Birchwood xylan in acetic acid/sodium acetate 0.05 M pH 5.
- Concentration of reducing sugars released is related to the enzymatic activity (U) per ml of membrane.

# RESULTS

## ANALYSIS OF THE MODEL

**Enzymatic activity results fitted with a multiple linear regression (MLR).**



**RESULTS**

## ANALYSIS OF THE MODEL

**Enzymatic activity results fitted with a multiple linear regression (MLR).**

**New model: all the terms considered are significant, except the EDMA term but its interaction with the other variables is significant.**

**Scaled centred coefficients of the proper model:**

| *Term* | *Coefficient* | *Std. Err.* | *Conf. int(±)* |
|---|---|---|---|
| Constant ($A_0$) | 7.66346 | 0.255781 | 0.560724 |
| Solvent ($A_1$) | 2.5085 | 0.255781 | 0.657507 |
| EDMA ($A_2$) | 0.6275 | 0.255781 | 0.657507 |
| AEM ($A_3$) | 0.954251 | 0.255781 | 0.657507 |
| solvent*EDMA ($A_{12}$) | 1.30575 | 0.255781 | 0.657507 |
| EDMA*AEM ($A_{23}$) | 1.1745 | 0.255781 | 0.657507 |

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{23}X_2X_3$$

## RESULTS

## ANALYSIS OF THE MODEL

ANOVA:

No lack of fit of the experimental data to the linear model

$R^2_{adj}$ = 0.941 (fraction of variation of the response explained by the model adjusted for degrees of freedom)

$Q^2$ = 0.837 (fraction of variation of the response explained in predicted data)

There are not outliers among the data for building the model

- No additional experiments are needed to describe the system.
- Data can be fitted by a Multiple Linear Regression (MLR) (most common data fitting method based in the data matrix inversion)
- No need to include quadratic terms to describe a non-linear influence of the variables (i. e: CFC, 20 exp) [7].

[7] A. Cano, E.A. Moschou, S. Daunert, J. Coello, C. Palet. *Bioprocess and Biosystems Engineering* (2006) in press.

# RESULTS

## EFFECT OF MEMBRANE COMPOSITION ON THE ENZYMATIC ACTIVITY OF MEMBRANES

### Effect of EDMA

**Affects:** porous properties of the polymeric monoliths prepared.
membrane composition.

Maximum : EDMA moles = (AEM + HEMA) moles.

EDMA (crosslinker) ↑
- polymeric surface area ↑
- pore size ↓
- xylanase immobilized ↑

Enzymatic activity ↑

EDMA is the variable with less influence (lowest coefficient).

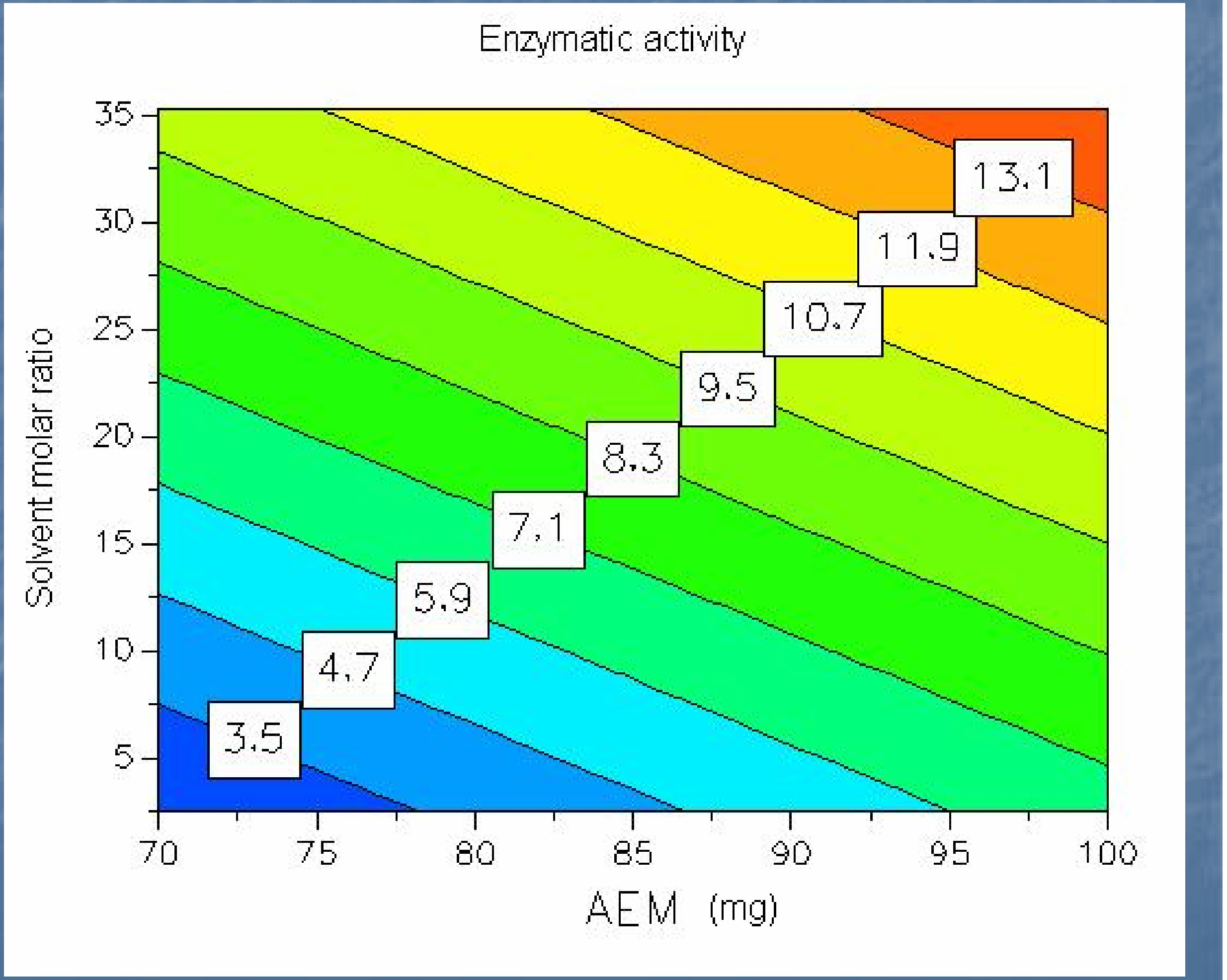
EDMA is selected at a constant level to observe the effect of the other variables.

# RESULTS

## EFFECT OF MEMBRANE COMPOSITION ON THE ENZYMATIC ACTIVITY OF MEMBRANES

**Response Surface Plot**

**EDMA at a constant level**
**High: +1 (714 µl)**



# RESULTS

## EFFECT OF MEMBRANE COMPOSITION ON THE ENZYMATIC ACTIVITY OF MEMBRANES

### Effect of porogenic solvents

Control of the final porosity of the polymeric structure.
Do not affect the final membrane composition.

At any level of EDMA:

Molar ratio (cyclohexanol) ↑ Polymer surface ↑ Immobilized enzyme ↑

Enzymatic activity ↑

Cyclohexanol is a better solvent for the monomers.

- Decreases phase separation rate during polymerization.
- Leads to smaller pore sizes.

## RESULTS

## EFFECT OF MEMBRANE COMPOSITION ON THE ENZYMATIC ACTIVITY OF MEMBRANES

### Effect of AEM

**Provides amino groups: react with the EDC activated enzyme.**

**AEM ↑ Amino groups in the membrane ↑ Immobilized enzyme ↑**

**↑ Enzymatic activity**

**Response surface plot: at AEM content lower than the optimum**

- **Membranes are less robust.**
- **Changes in composition → Higher changes in the enzymatic activity.**

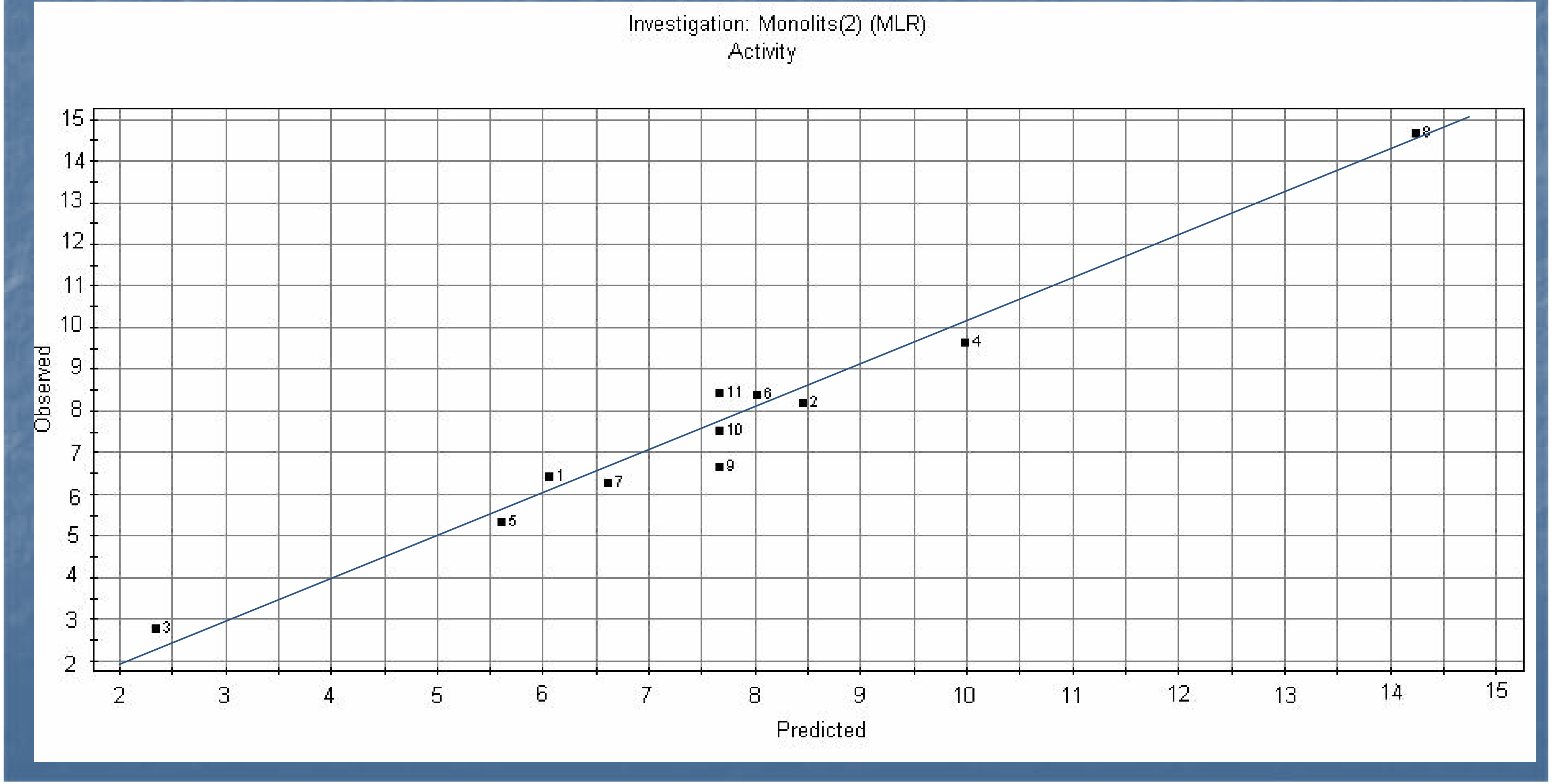
# PREDICTIVE CAPACITY OF THE MODEL

## RESULTS

**Good fitting of the data and $Q^2$ value**

**Good correlation among the observed (experimental) activity and the predicted by the model**

**Observed (y) = predicted (x) + $2*10^{-5}$ $r^2$= 0.9703**



## RESULTS

## PREDICTIVE CAPACITY OF THE MODEL

3 New membranes composition:

central point {
solvent ratio 18.915
EDMA 507 µl
AEM 85 mg

| | *Enzymatic activity (U/ml)* |
|---|---|
| Experimental | 8.2 ± 0.7 |
| Predicted | 7.7 ± 0.2 |

Confirmation of the lack of need of including quadratic terms

## OPTIMIZATION

EDMA (714 µl) and AEM (100 mg) fixed, solvents ratio optimized.
Enzymatic activity: target value of 16 U/ml.
Limits solvents ratio: 35.29 -100.
Optimum solvents ratio suggested: 100.

| | *Enzymatic activity (U/ml)* |
|---|---|
| Experimental | 29.1 ± 0.8 |
| Predicted | 29.3 ± 0.2 |

Membrane: EDMA (714 µl), AEM (100 mg), porogenic solvent ratio (748).

Enzymatic activity 28.6 ± 0.7 U/ml.

# CONCLUSIONS

- The two-level full factorial design is a simple and easy tool to study the effect of three components in the enzymatic membrane, leading to the same conclusions as obtained with more complicated designs.
- The enzymatic activity of the membranes can be expressed, with a good fitting of data, as a linear combination of the factors.
- The proposed model enables results prediction for new membranes and this predictive capacity has been demonstrated with additional experiments.
- Moreover it is possible to estimate the proper membrane composition to optimize the response selected.
- The mathematical conclusions related to each variable/factor , which correspond to membrane components, correspond to chemical conclusions in each case (EDMA, AEM, solvents molar ratio).
- The selection of the membrane composition depends on criteria such as chemical significance, economical viability or reagent availability.

# ACKNOWLEDGMENTS

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THANK YOU FOR YOUR ATTENTION

EUROMEMBRANE 2006, Taormina, Italy, 26th September 2006

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