INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A. C.

POSGRADO EN CIENCIAS AMBIENTALES

Effect of operational parameters on methanol biofiltration coupled with Endochitinase 42 production

Tesis que presenta:

Rodolfo Palomo Briones

Para obtener el grado de

Maestro en Ciencias Ambientales

Directora de tesis:

Dra. Sonia Lorena Arriaga García

San Luis Potosí, San Luis Potosí. Septiembre, 2013

IPICYT

Constancia de aprobación de la tesis

La tesis ***"Effect of operational parameters on methanol biofiltration coupled with Ech42 production"*** presentada para obtener el Grado de Maestro en Ciencias Ambientales fue elaborada por **Rodolfo Palomo Briones** y aprobada el **seis de agosto del dos mil trece** por los suscritos, designados por el Colegio de Profesores de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Dra. Sonia Lorena Arriaga García
Director de la tesis

Dra. Ana Paulina Barba de la Rosa
Miembro del Comité Tutoral

Dr. Francisco Javier Cervantes Carrillo
Miembro del Comité Tutoral

Dr. Armando González Sánchez
Miembro del Comité Tutoral

INSTITUTIONAL ACKNOWLEDGEMENT

The present work was mainly developed in the laboratories of the Environmental Sciences Department at the *Instituto Potosino de Investigación Científica y Tecnológica, A.C.* The research work was funded by the *Consejo Nacional de Ciencia y Tecnología* through the project grant SEP-CONACYT-CB-2009-133930, which was assigned to Dr. Sonia Lorena Arriaga García.

The Scanning Electron Microscopy study presented in chapter III was developed in the Laboratorio Nacional de Investigaciones de Nanociencias y Nanotecnología (LINAN).

During the accomplishment of this work, the author received a scholarship from the *Consejo Nacional de Ciencia y Tecnología* (CONACYT-423853).

As part of this study, there was a research stay in *Pratt School of Engineering* at *Duke University* under the supervision of Dr. Marc Deshusses. The academic stay was funded by the *Consejo Nacional de Ciencia y Tecnología* thought a complement scholarship. The results of such stay are shown in chapter 4 of this work.

CRÉDITOS INSTITUCIONALES

El presente trabajo de tesis se desarrolló principalmente en los Laboratorios de la División de Ciencias Ambientales del Instituto Potosino de Investigación Científica y Tecnológica, A.C. y fue financiado por el Consejo Nacional de Ciencia y Tecnología a través del proyecto SEP-CONACYT-CB-2009-133930 asignado a la Dra. Sonia Lorena Arriaga García.

Los estudios de microscopia electrónica que se presentan en el capítulo III se realizaron en las instalaciones del Laboratorio Nacional de Investigaciones de Nanociencias y Nanotecnología (LINAN).

Durante su realización, el autor recibió una beca académica del Consejo Nacional de Ciencia y Tecnología (CONACYT-423853).

Como parte de este trabajo se realizó una estancia de investigación en *Pratt School of Engineering at Duke University* bajo la supervisión del Dr. Marc Deshusses. La estancia fue financiada por CONACYT a través de una beca mixta. Los resultados de la estancia se presentan en el capítulo 4 de la presente tesis.

IPICYT

Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Acta de Examen de Grado

El Secretario Académico del Instituto Potosino de Investigación Científica y Tecnológica, A.C., certifica que en el Acta 007 del Libro Primero de Actas de Exámenes de Grado del Programa de Maestría en Ciencias Ambientales está asentado lo siguiente:

En la ciudad de San Luis Potosí a los 6 días del mes de septiembre del año 2013, se reunió a las 11:00 horas en las instalaciones del Instituto Potosino de Investigación Científica y Tecnológica, A.C., el Jurado integrado por:

Dra. Ana Paulina Barba de la Rosa	**Presidenta**	**IPICYT**
Dr. Francisco Javier Cervantes Carrillo	**Secretario**	**IPICYT**
Dr. Armando González Sánchez	**Sinodal externo**	**UNAM**
Dra. Sonia Lorena Arriaga García	**Sinodal**	**IPICYT**

a fin de efectuar el examen, que para obtener el Grado de:

MAESTRO EN CIENCIAS AMBIENTALES

sustentó el C.

Rodolfo Palomo Briones

sobre la Tesis intitulada:

Effect of operational parameters on methanol biofiltration coupled with Ech42 production

que se desarrolló bajo la dirección de

Dra. Sonia Lorena Arriaga García

El Jurado, después de deliberar, determinó

APROBARLO

Dándose por terminado el acto a las 13:00 horas, procediendo a la firma del Acta los integrantes del Jurado. Dando fe el Secretario Académico del Instituto.

A petición del interesado y para los fines que al mismo convengan, se extiende el presente documento en la ciudad de San Luis Potosí, S.L.P., México, a los 6 días del mes de septiembre de 2013.

Dr. Marcial Bonilla Marín
Secretario Académico

Mtra. Ivonne Lizette Cuevas Vélez
Jefa del Departamento del Posgrado

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.
IPICYT
SECRETARIA ACADEMICA

Para mi familia

Porque, a pesar de la distancia, siempre los tuve a mi lado.

Para Gaby

Por darle sentido a mi vida.

PERSONAL ACKNOWLEDGEMENTS

First of all, I am grateful to Dr. Sonia Lorena Arriaga García for her constant commitment with this research. For her lessons, trust, and support.

I wish to express my sincere thanks to the committee members, Dra. Ana Paulina Barba de la Rosa, Dr. Francisco Javier Cervantes Carrillo, Dr. Armando González Sánchez, and Dr. Sergio Revah Moiseev, for their invaluable comments and attentions.

I place on record, my sincere gratitude to academic technicians, MC Dulce Isela de Fátima Partida Gutiérrez, MC Juan Pablo Rodas Ortiz, and MC Guillermo Vidriales Escobar, for their friendship and support.

I also thank to IQ Ma. del Carmen Rocha Medina and MC Gladis Judith Labrada Delgado, for their time and important contributions to this thesis.

I take this opportunity to record my sincere thanks to all the faculty members of the Deparment of Environmental Sciences at the *Instituto Potosino de Investigación Científica y Tecnológica,* for their help and encourage during this journey.

AGRADECIMIENTOS

A la Dra. Sonia Lorena Arriaga García por su compromiso siempre constante con la presente investigación. Por sus lecciones, su confianza y su apoyo.

A los miembros del comité tutelar, Dra. Ana Paulina Barba de la Rosa, Dr. Francisco Javier Cervantes Carrillo, Dr. Armando González Sánchez y Dr. Sergio Revah Moiseev. Por sus invaluables comentarios y atenciones.

A los técnicos académicos, MC Dulce Isela de Fátima Partida Gutiérrez, MC Juan Pablo Rodas Ortiz y MC Guillermo Vidriales Escobar, por su amistad y apoyo.

IQ Ma. del Carmen Rocha Medina y MC Gladis Judith Labrada Delgado por su tiempo y sus importantes aportes.

A todos los académicos de la División de Ciencias Ambientales, por orientarme en esta gran aventura. Por sus enseñanzas, consejos y cuestionamientos.

TABLE OF CONTENTS

Constancia de aprobación de tesis ii

Institutional acknowledgment iii

Créditos Institucionales iv

Dedication v

Personal acknowledgments vi

Agradecimientos vii

Table of contents viii

List of figures xii

List of tables xv

Abbreviations xvi

Resumen xviii

Abstract xix

CHAPTER I

INTRODUCTION 1

ATMOSPHERIC POLLUTION 1

STRATEGIES FOR AIR POLLUTION CONTROL 2

Physicochemical methods 3

Biological methods 4

BIOFILTRATION 6

METHANOL BIOFILTRATION 7

A novel microorganism for methanol biofiltration: *Pichia pastoris* 12

Methanol biofiltration coupled with heterologous proteins production:

state of the art and current challenges 15

OBJECTIVES AND STRUCTURE 15

REFERENCES 16

CHAPTER II

EFFECT OF OPERATIONAL PARAMETERS ON METHANOL BIOFILTRATION COUPLED WITH ECH42 PRODUCTION 22

ABSTRACT 22

INTRODUCTION 22

METHODOLOGY 24

Inoculum and growth medium 24

Biofilters 24

Experimental setup 24

Performance evaluation 25

Analytical methods 26

Total proteins 27

Ech42 activity 27

RESULTS AND DISCUSSION 27

Methanol load effect 27

Nitrogen concentration effect 30

pH effect 33

EBRT effects 34

Carbon balance 35

SUMMARY AND PERSPECTIVES 35

CONCLUSIONS 36

REFERENCES 36

CHAPTER III

OPERATIONAL DIVERGENCE IN BIOFILTERS TREATING METHANOL VAPORS 41

ABSTRACT 41

INTRODUCTION 41

MATERIALS AND METHODS....42
Inoculum....42
Analytical methods....43
Statistics....43
Monod kinetics....43
RESULTS AND DISCUSSION....44
Biofilter performance....44
SEM analysis....47
Monod kinetics....49
CONCLUSIONS....50
REFERENCES....50

CHAPTER IV

MATHEMATICAL MODEL OF METHANOL BIOFILTRATION COUPLED WITH PROTEIN PRODUCTION....54
ABSTRACT....54
INTRODUCTION....54
METHODOLOGY....55
Model design....55
Assumptions....56
Mass balances....57
Growth and degradation kinetics....57
Protein production....58
Frontier equations....59
System setup....59
Biomass fraction and growth kinetics evaluation....59
Validation....61
RESULTS AND DISCUSSION....61
Biomass fraction....61

Y_A effect 62
ECmax effects 62
Ks effects 63
Decay coefficient affects 63
Sensitivity 69
Validation 69
CONCLUSIONS 72
REFERENCES 72

CHAPTER V

PERSPECTIVES AND CONCLUSIONS 76
CONCLUSIONS 76
CONCLUSIONS 76

ANNEXES

ANNEX I: ENDOCHITINASE 42 78
ANNEX II: CALIBRATION CURVES 80
ANNEX III: CARBON TO NITROGEN RATIO CALCULUS METHOD 83
ANNEX IV: BIOMASS/EC CORRELATION 83

LIST OF FIGURES

CHAPTER I

Figure 1. Treatment methods for atmospheric pollutants based on flow rate and concentration 3

Figure 2. Biofiltration scheme 5

Figure 3. Methylotrophic metabolism in yeasts 14

CHAPTER II

Figure 1. Diagram of the experimental biofilters. 1.- rotameter, 2.- needle valve, 3.- humidifier, 4.- methanol reservoir, 5.- biofilter, 6.- mineral solution, 7.- recirculation of leachate, 8.- sprinkler.....25

Figure 2. Biofiltration performance. A) Biofilter I, B) Biofilter II, C) Biofilter III 29

Figure 3. Biomass density in A) biofilter I, B) biofilter II, and C) biofilter III 32

CHAPTER III

Figure 1. Profiles of methanol concentrations along biofilters for stages A) 1 1-28 days 1, B) 2 29-52 days, and C) 3 53-82 days.. 45

Figure 2. SEM images from the first stage of three biofilters. A) Biofilter I - stage 1, B) Biofilter II - stage 1, C) Biofilter III - stage 1, D) Biofilter I - stage 2, E) Biofilter II - stage 2, F) Biofilter III - stage 2, G) Biofilter I - stage 3, H) Biofilter II - stage 3, I) Biofilter III - stage 3.....48

Figure 3. Monod kinetics. A) Biofilter I; B) Biofilter II; C) Biofilter III. Dotted line represents 100% efficiency.....49

CHAPTER IV

Figure 1. Conceptual representation of the biofiltration process. M1, Module 1; M2, Module 2; M3, Module 3; W: number of divisions along biofilter height; N: number of divisions along biofilm depth; [C]: Methanol concentration in gas phase; [S]: Methanol concentration in the biofilm. 56

Figure 2. Model behavior under different BiomassA fractions at the start of operation. A) Heterologous protein production. B) Biofilm thickness along the biofilter. C) Methanol concentration. Parameters: Ks=3280 g/m^3 Y=0.032 gC/gMetOH, ECmax=5269 g/m^3*h, b=0.0028/h 64

Figure 3. Model behavior under different Y_A at the start of operation. A)Ech42 production, B) Methanol degradation, C) Biofilm volume, D) Biofilm thickness. Parameters: Xaf = 0.95, Ks=3280 g/m^3, ECmax=5269 g/m^3*h,b=0.0028/h, Yp/s=0.2 65

Figure 4. Model behavior under different ECmax at the start of operation. A) Ech42 production, B) Methano degradation, C)*P. pastoris* biofilm volume, D) Others volume. Parameters: Xaf = 0.95, Y=0.032gC/gMetOH, Ks=3280 g/m^3*h, b=0.0028/h, Yp/s=0.2 66

Figure 5. Model behavior under different Ks at the start of operation. A)Ech42 production, B)Methanol degradation, C) Biofilm volume, D)Biofilm thickness. Parameters: Xaf = 0.95, Y=0.032 g_C/g_{MetOH}, ECmax=5269g/m^3*h , b=0.0028/h, Yp/s=0.2 67

Figure 6. Model behavior under different b at the start of operation. A)Methanol degradation, B) Ech42 production, C)Biofilm thickness, D)Biofilm volume. Parameters: Xaf = 0.95, YA= 0.032gC/gMetOH, Ks=3280g/m^3, ECmax=5269 g/m^3*h, b=0.0028/h, Yp/s=0.2 68

Figure 7. Model validation. A)Methanol degradation, B) Ech42 production, C)Biofilm volume. Parameters: $ECmax_A$=1887.5g/m^3*h, $ECmax_B$=1156.79 g/m^3*h, Ks_A=300.3g/m^3, Ks_B=300.4 g/m^3, Y_A=0.06g_C/g_{MetOH}, Y_B=.022 g_C/g_{MetOH}, b_A=0.0017/h, bB=0.0001/h, Yp_A=0.2$g_{Protein}/g_{MetOH}$, Yp_B=15.52$g_{Protein}/g_{MetOH}$ 70

ANNEX II

Figure A1. Methanol calibration curve 80

Figure A2. CO_2 calibration curve 80

Figure A3. Total proteins calibration curve 81

Figure A4. 4MUQT calibration curve 81

Figure A5. COT calibration curve 82

ANNEX IV

Figure A6. EC and biomass correlation during the whole operation time in biofilter I 84

Figure A7. Biomass based elimination capacity in biofilter I 84

Figure A8. EC and biomass correlation during the whole operation time in biofilter II 85

Figure A9. Biomass based elimination capacity in biofilter II.....85

Figure A10. EC and biomass correlation during the whole operation time in biofilter III.....86

Figure A11. Biomass based elimination capacity in biofilter III.....86

LIST OF TABLES

CHAPTER I

Table 1. Volatile organic compounds and their effects on the human health derived from its consumption by breathing 2

Table 2. Advantages and drawbacks of gas treatment biosystems. Adapted from Utkin et al. (1992); Groenestijn and Hesselink (1993) 4

Table 3. Recent methanol biofiltration studies 10

Table 4. Heterologous proteins expressed in *P. pastoris* 14

CHAPTER II

Table 1. Experimental setup 26

Table 2. Summary of the protein production and Ech42 activity 31

Table 3. Carbon distribution 35

CHAPTER III

Table 1. Summary of pseudo steady state methanol removal 44

Table 2. ANOVA analysis on the pseudo steady state efficiencies. P values lower than 0.05 indicate significant difference among biofilters 46

CHAPTER IV

Table 1. Start up parameters used in the model 60

Table 2. Estimation of sensitivity ratios as a parameter to assign a relative importance 69

Table 3. Parameters comparison between this study and literature 71

ANNEX I

Table A1. Principal areas for chitin application. (Dutta et al., 2004; Lárez et al., 2006, Tröger y Niranjan, 2010) .78

ABBREVIATIONS

In order of appearance

g: gram

m: meter

h: hour

L: liter

U: Enzymatic unit

C: Celcius

Ech42: Endochitinase 42

CSTR: Continuous Stirred Tank Reactor

P. pastoris: *Pichia pastoris*

EBRT: Empty Bed Residence Time

SOx: sulfur oxides

NOx: nitrogen oxides

PM10: Particulate Matter of 10μm or less in size

PM2.5: Particulate Matter of 2.5 μm or less in size

VOC: Volatile Organic Compound

C_L: Concentration in liquid phase (g/m3)

C_G: Concentration in gas phase (g/m3)

H: Henry's constant

J: diffusion flux (kg/s·m^2)

D: diffusion coefficient (m^2/s)

x: diffusion direction (m)

$\frac{dC}{dx}$: local gradient in the biofilm (kg/m^4)

f_s^0: Cell synthesis fraction coefficient

EC: Elimination Capacity

PVC: Polyvinyl chloride

pAOX1: Alcohol oxidase 1 promoter

AOX: Alcohol Oxidase

$C_{MetOH(in)}$: Gas methanol concentration at inlet

$C_{MetOH(out)}$: Gas methanol concentration at outlet

Q: Air flow rate

V_f: Effective volume under evaluation

$C_{CO_2(in)}$: CO2 concentration at inlet

$C_{CO_2(out)}$: CO_2 concentrations at outlet

FID: Flame Ionization Detector

TCD: Thermal Conductivity Detector

4MUQT: 4-methyl-ubenylferyl quitotriose

ECmax: Maximum Elimination Capacity

ILR: Inlet Loading Rate

EPS: Exopolysacharides

SEM: Scanning Electron Microscopy

Yx/s: Biomass yield

B: biomass decay

Ks: Half saturation constant

W: number of divisions along biofilter height

N: number of divisions along biofilm depth

[C]: Methanol concentration in gas phase

[S]: Methanol concentration in the biofilm

C_{i-1}: Methanol concentration at inlet of gas element i

C_i: Methanol concentration at outlet of gas element i

$J_{i,j}$: Flux of methanol to the biofilm element i,j

A: Transfer area

D: Effective methanol diffusivity

$S_{i,j}$: Methanol concentration in the biofilm element i,j

$S_{i,surface}$: Methanol concentration in the biofilm element i,surface

$S_{i,1}$: Methanol concentration in the biofilm element i,1.

δ: thickness of one biofilm element

V^{bf} is the total biofilm volume

Y: biomass yield

q:Maximum specific substrate utilization rate

X: Density of biomass

$Y_{p/s}$: Protein productivity

$Ra_{i,j}$: Methanol degradation rate due to *Biomass A* in the biofilm element i,j

$Rb_{i,j}$: Methanol degradation rate due to *Biomass B* in the biofilm element i,j

$R_{i,j}$: Total methanol degradation rate in the biofilm element i,j

RESUMEN

Efecto de los parámetros operacionales sobre la biofiltración de metanol acoplada a la producción de Endoquitinasa 42

Palabras clave: Biofiltración, Metanol, Proteína heteróloga, Divergencia, *P. pastoris.*

En la presente tesis se estudió la biofiltración de metanol acoplada con la producción de proteínas heterólogas. Para ello, se utilizó un cultivo de *P. pastoris* transformado con el gen Ech42 responsable de producir la Endoquitinasa 42 de *Trichoderma Atroviride* (Pérez-Martínez et al., 2007). Se diseñaron tres biofiltros idénticos y se operaron bajo diferentes condiciones de carga de metanol, concentración de nitrógeno, pH y tiempo de residencia. La máxima capacidad de eliminación de metanol observada fue de 643.5 g/m^3*h a una carga de metanol de 800 g/m^3*h, 5 g/L $(NH_4)_2SO_4$, pH 5 y 60 segundos de tiempo de residencia. La actividad de Ech42 varió entre 578 y 1172 U/L siendo el pH el parámetro más importante para el proceso. Además, se encontró que la capacidad de eliminación del sistema fue muy sensible a los cambios de condiciones manejadas. Sin embargo, la actividad de la proteína heteróloga fue notablemente estable.

Por otro lado, también se estudió la divergencia del sistema a través de un análisis estadístico simple, microscopía electrónica de barrido y análisis cinético, durante las primeras tres etapas de operación. Se observó que dos o más sistemas de biofiltración pueden comportarse de distinta manera con el tiempo, aún y cuando son operados bajo las mismas condiciones. Algunas explicaciones probables son los procesos aleatorios de colonización de otras especias así como la impredecible evolución de comunidades microbianas. En cualquier caso, los resultados mostrados en esta tesis son el primer reporte de divergencia en biofiltros.

Finalmente, se desarrolló un modelo matemático dinámico para describir el desempeño de los sistemas de biofiltración acoplados a la producción de proteínas. Éste se basó en ecuaciones para reactores CSTR (Completely Stirred Tank Reactor) pero en una estructura discretizada del sistema. Para la degradación de metanol se utilizó el modelo de Monod. Se consideró la presencia de dos grupos de microorganismos creciendo en los sistemas: *P. pastoris* y otros microorganismos. El modelo mostró un buen ajuste en relación con las capacidades de eliminación experimentales y la producción proteica. Sin embargo, fue difícil ajustar los valores experimentales de biomasa total presente (volumen). Tal discrepancia puede deberse a que el modelo no considera biomasa inerte. Por lo anterior, es recomendable que el modelo matemático sea modificado o replanteado.

ABSTRACT

Effect of operational parameters on methanol biofiltration coupled with Endochitinase 42 production

Key words: Biofiltration, Methanol, Heterologous protein, Divergence, *P. pastoris.*

In this thesis, methanol biofiltration coupled with heterologous protein production was studied. A *P. pastoris* strain transformed with the gen Ech42 responsible for producing Endochitinase 42 (Ech42) was used (Pérez-Martínez et al., 2007). Three different biofilters were designed and tested under different methanol loads, nitrogen concentrations, pH and EBRT (Empty Bed Residence Time) conditions. The maximum elimination capacity observed was 643.5 g/m^3*h under a methanol load of 800 g/m^3*h, 5 g/L $(NH_4)_2SO_4$, pH 5 and 60 seconds of EBRT. Also, the Ech42 activity ranged from 578 to 1172 U/L and was mainly affected by pH changes. Additionally, it was observed that EC was very sensitive to changes under the mentioned operational conditions. However, the heterologous protein activity was quite stable.

On the other hand, divergence in the system was approached. It was noted that two or more systems operating under identical conditions can behave differently over time. Thus, repeatability in biofiltration shouldn't be taken for granted. Some probable explanations for such divergence are the random colonization of foreign species as well as the unpredictable evolution of microbial communities. In any case, this is the first report of operational divergence in biofilters.

Finally, a dynamic mathematical model was developed based on CSTR equations. A Monod kinetic was used in order to describe methanol consumption behavior. Also, microbial growth was considered for two different kinds of microorganisms: *P. pastoris* and others. The model fit well with experimental elimination capacities and protein production. However, total biomass volume was not well described. The source of the model discrepancy could be the failure to consider inert biomass. Therefore, the mathematical model must be further explored.

The world is a dangerous place to live; not because of the people who are evil, but because of the people who don't do anything about it. (Albert Einstein)

INTRODUCTION

ATMOSPHERIC POLLUTION

Since the origin of mankind, science and technology have improved human welfare; however, it has been accompanied by increased industrial activity and excessive exploitation and use of natural resources (e.g. wood, water, petroleum, gas, coal, etc.). The consequence is that huge amounts of pollutants have been released into bodies of water, soil, and the atmosphere, jeopardizing the future of life on Earth.

Nowadays, atmospheric air pollution, the focus of this work, causes thousands of deaths around the world. In our country, more than 10,800 people die annually because of poor air quality. Actually, air pollution in Mexico is one of the top ten causes of death (Secretaría de Salud, 2005).

According to the latest national emissions inventory, 40.5 million tons of pollutants (Carbon monoxide, CO; sulfur oxides, SOx; nitrogen oxides, NOx; particulate matter, PM10 y PM2.5; volatile organic compounds, VOCs; and ammonia) were emitted into the atmosphere in 1999 (SEMARNAT, 2005). Such research also revealed that VOCs represent 15% of the total emissions behind only carbon monoxide and sulfur oxides.

VOCs, substances volatile at room temperature (EPA, 2012), are important not only from the environmental standpoint but also from that of human health. It is well known that breathing VOCs may result in many health problems ranging from headaches and nausea to heart disorders and cancer (Table 1). Therefore, their treatment and control must be a priority in healthcare systems.

Methanol is a colorless VOC with an annual worldwide production of around 45.6 million tons. It is used and emitted by different industries such as construction, plastics, automotive, home and paper production (IMPCA, 2011; MI, 2011). Moreover, it is important for the synthesis of acetic acid, formaldehyde, and biodiesel.

For many years, methanol (boiling point (1 atm.) 65.15°C; density (20°C) = 0.7914g/cm^3) has been considered as an alternative automotive fuel to reduce pollution in the urban environment (Gordon and Austine, 1992). Nevertheless, its characteristics, such as hydrophobicity, make it a highly toxic compound. When inhaled, methanol

can lead to blindness, neurobehavioral disorders, shorter gestational length in pregnant women and cancer (Ikeda 1992; Chuwers et al., 1995; Health Effects Institute, 1999). These reasons and others have led methanol be considered a Hazardous Air Pollutant by the Environmental Protection Agency (USA) . In any case, it seems that methanol treatment is preferred in order to safeguard human health and avoid any other undesirable scenarios.

Table 1. Volatile organic compounds and their effects on the human health derived from its consumption by breathing.

VOC	**Human Health Effects**	**Reference**
1,3-Butadiene	Carcinogenic	Health Effects Institute (2000)
Benzene	Leukemia and blood disorders	Health Effects Institute (2003)
Toluene	Central nervous system encumbrances, neurotoxicity	IRIS EPA (2005)
Methanol	Decreased gestational length Neurobehavioral negative effects in kids	Health Effects institute (1999)
Styrene	Myocardial ischemia	Health Effects Institute (2002)
Formaldehyde	Carcinogenic	International Agency for Research on Cancer (2006)
Xylene	Leukopenia, cyanosis, migraine, abnormalities of the heart	International Agency for Research on Cancer (1989)

STRATEGIES FOR AIR POLLUTION CONTROL

Different technologies are available for the treatment and control of air pollutants. In general, selection is guided by availability, cost, required efficiency, physicochemical properties, concentration, and flow rate. Figure 1 shows a widely known selection guide made on the basis of the pollutant flow rate and its concentration (Devinny et al., 1999). In general, the different treatment technologies can be classified as physicochemical and biological.

Physicochemical methods

Condensation: It is achieved by decreasing stream temperature or increasing stream pressure to exceed the saturated vapor state. This is an economical method that is viable for highly concentrated streams, especially when the recovery of the main component represents an economical benefit. Otherwise, when the stream is a mixture, recovery is difficult and the condensation process is useless and economically unviable (Devinny et al., 1999).

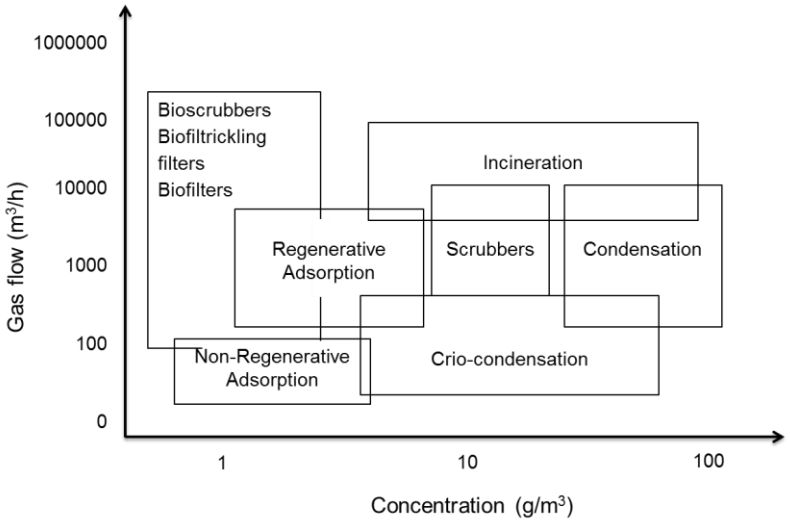

Figure 1. Treatment methods for atmospheric pollutants based on flow rate and concentration

Incineration: It is the conversion of organic compounds into carbon dioxide and water through combustion. Commonly used combustion methods are: a) direct flame combustion, b) thermal combustion and c) catalytic combustion. Regardless of the method, it is important to achieve complete pollutant combustion to avoid the synthesis of aldehydes, organic acids, and carbon monoxide, which are, in some cases, more dangerous than the original pollutant (Green and Perry, 2007).

Adsorption: It is a selective transfer of pollutants from a gaseous phase to a surface of solid particles (adsorption column). At ordinary temperatures, adsorption occurs due to intermolecular forces (especially Van der Waal forces) and it is known as physical adsorption. At higher temperatures (greater than 200 ºC), system energy is high enough to overcome the activation energy, required to form bonds (covalent or ionic) between pollutants and adsorption surface. In the latter case, adsorption is called chemisorption (Green and Perry, 2007).

Selective capacity of adsorbent materials allows recovering different waste that is interesting from an economic standpoint. In fact, selective capacity is probably the most important thing that should be addressed when designing adsorption processes.

Absorption: It is based on the gas pollutant inclusion into an absorbent liquid phase. In this process the most important parameters are the pollutant solubility and solvent absorption capacity (Green and Perry, 2007). The

principal disadvantage of absorption is the need to have some other method for recovering or treating the pollutant (Devinny et al., 1999).

Biological methods

Biological methods take advantage of metabolic reactions to degrade a wide range of organic substances producing carbon dioxide, water, and biomass (Devinny et al., 1999). One of the most important advantages of biological treatment is that it can be carried at room temperature (10-40 C). Also, biological methods are inexpensive, easy to operate, and environmentally friendly.

Biological reactors can be classified as bioscrubbers, biotrickling filters, or biofilters. Their advantages and drawbacks are described in Table 2.

Table 2. Advantages and drawbacks of gas treatment biosystems. Adapted from Utkin *et al*. (1992); Groenestijn and Hesselink (1993).

System	Advantages	Drawbacks
Bioscrubber	Better control of reactions Avoids byproducts accumulation Compact Low pressure loss	Small gas-liquid contact surface Perceptible to feeding changes Generates waste sludge Complicated start Requires more aeration High investment, operation, and maintenance costs Requires nutrients supply
Biotrickling filter	Similar to bioscrubbers	Small gas-liquid contact area Generates waste sludge Perceptible to feeding changes Requires nutrients supply Complicated start High investment, operation, and maintenance costs
Biofilter	High gas-liquid contact area Easy start and operation Low investment and operation costs Resist feeding changes	Little control over reaction phenomena Poorly adaptation to changes in gas flow rate Needs more area

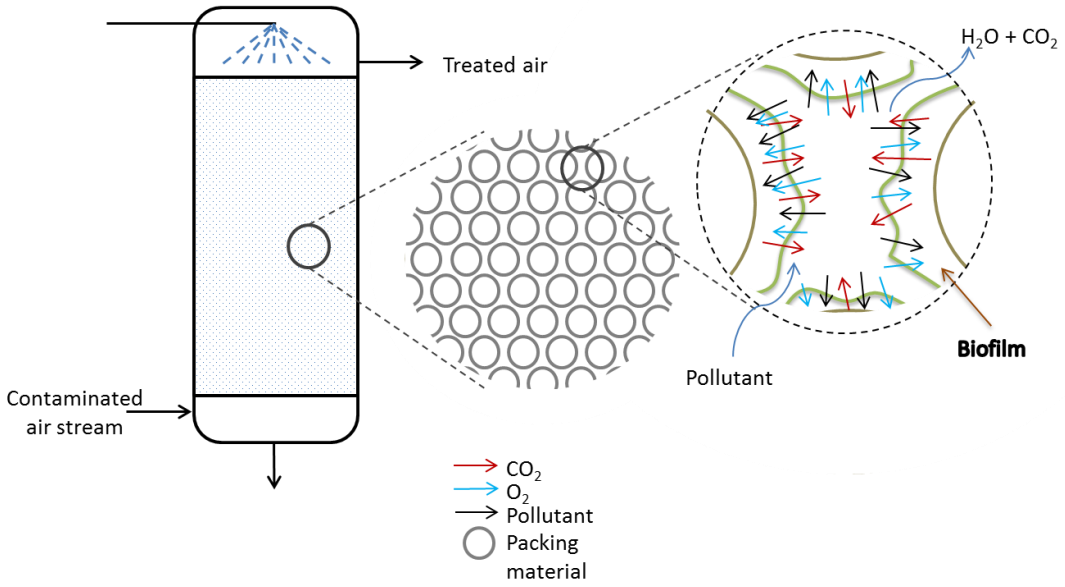

Figure 2. Biofiltration scheme.

Bioscrubbers. This kind of system is characterized by a pollutant gas phase that is absorbed to an aqueous continuously re-circulated phase. The treatment is reached with the use of an absorption column. Later, water and the absorbed pollutant are aerobically treated in an activated sludge system. The control of parameters (pH, temperature, oxygen concentration, nutrients concentration, etc.) is simple, but the system is limited by gas-liquid contact area, and so it is recommended for soluble pollutants with Henry's coefficients below 5 or 10 (Groenestijn and Hesselink, 1993).

Biotrickling filters. In these systems, the pollutant gas flows through an absorption column in which microorganisms grow. They develop a biofilm where the contaminant can be degraded. Water is continuously trickling in order to maintain moisture, and at the same time, pollutant is absorbed to the biofilm and then degraded. Furthermore, everything occurs in only one system (Groenestijn and Hesselink, 1993). The transfer area is again an important limiting step. Nonetheless, water offers an excellent way to control important parameters to enhance the biotrickling filter performance, for instance, pH, nutrients availability, temperature, biomass growth rate, and biomass accumulation (Cox and Dehusses, 1998; Yang et al., 2010).

Biofilters. In biofiltration, a waste airstream flows through a column packed with a porous medium where different microorganisms are fixed and growing. Those microorganisms are responsible for gas pollutant degradation, at

the same time releasing CO_2, water and likely some byproducts. As noted, biofilters are very similar to biotrickling filters, but biofilters are not constantly sprinkled with liquid, so their control is a bit more difficult. This technology in particular will be approached in greater detail in the next section because it has been of great importance in the abatement of different pollutants, including methanol, at an industrial scale.

BIOFILTRATION

Biofiltration (Figure 2) is a technology for the treatment of atmospheric pollutants. It has been employed to treat multiple organic and inorganic air contaminants such as hydrogen sulfide, dimethyl sulfide, methanol, acetaldehyde, formaldehyde, ethylene, toluene, xylenes, ethyl benzene, trimethyl benzene, butyl acetate, methyl ethyl ketone, etc. (Ralebitso-Senior et al., 2012). Its proven functionality and efficiency make it a frequent option for treating odors and VOCs from waste air streams.

This process involves several transfer and reaction phenomena. Convection, gas-biofilm equilibrium, diffusion, biodegradation, and microbial growth are the most important. Understanding these phenomena is essential for a better design, evaluation and improvement of biofiltration systems; therefore, they will be briefly described below and addressed in detail in Chapter IV.

a) **Convective mass transfer** is the mechanism that brings the pollutant and oxygen molecules to the proximity of microbial surface. Also, it carries CO_2 and byproducts out of the biofilter. The quality of convection is reflected on treatment efficiency; if pollutant and oxygen do not reach every site in the bed because of poor convection, some space in the column will be wasted and the process performance will fall. In practice, convection is not usually a problem, but it is always suggested to keep a clean bed in order to avoid clogging.

b) When the gas phase approaches the **gas-biofilm boundary**, its components are transferred. The pollutant, oxygen and any other compound of airflow is transported to the biofilm until a specific equilibrium concentration is reached. The concentrations at both sides of the gas-liquid boundary are well correlated through Henry's law.

$$C_L = C_G / H \qquad (1)$$

where C_L and C_G are the concentrations of pollutant in the liquid and gas phase, respectively; and H is the proportionality constant well known as Henry's constant (Green and Perry, 2007).

The pollutant capacity to be transported toward the biofilm phase is directly related to its hydrophilicity. The more hydrophilic the more difficult its transfer and consequent degradation are.

c) Once the pollutant is in the biofilm phase, it diffuses in the direction of the lower concentration. **Diffusive transport** is driven by the concentration gradient between the surface of the biofilm and the interior thereof. The higher the gradient, the higher the transfer rate is. This phenomenon is accurately described by first Fick's law:

$$J = -D\frac{dC}{dx} \qquad (2)$$

where J is the diffusion flux (kg/s·m^2), D is the diffusion coefficient (m^2/s), C is the pollutant concentration, x is the diffusion direction (m) and $\frac{dC}{dx}$ (kg/m^4) is the local gradient in the biofilm.

d) **Biodegradation** is generally referred to as pollutant oxidation although this is not a rule. Throughout this process, microorganisms take the pollutant and use it as a carbon and/or energy source. Derived from that pollutant abatement, CO_2 and H_2O are produced as well as biomass and some byproducts. The ratios of such products depend on numerous factors, for instance, microbial species in the biofilm, pollutant availability, oxygen and nutrients concentration, temperature, pH, *etcetera*.

e) **Microbial growth** is the increase of cells in the biofilm and consequently its volume. It is linked to biodegradation through the f_s^0 coefficient which indicates the amount of electrons, from the energy source, used for cell synthesis (Rittman, 2002). The way microbial growth affects biofiltration performance is well known. On the one hand, growth is necessary to increase the system biodegradation capacity, while on the other, an excessive growth can drive the system to poor efficiencies due to dead zones and clogging problems.

In summary, biofiltration is a quite complex technology which is yet useful and effective to control air pollution. More details and discussion about the biofiltration mechanism are given in Chapter IV.

METHANOL BIOFILTRATION

Methanol vapor treatment has been recently approached by different researchers around the world (Table 3). As can be observed, many packing materials and inocula as well as nitrogen sources, pH values, and methanol

loading rates, have been studied. Accordingly, the elimination capacity (EC) values, which are the ratio between the amount of pollutant degraded and methanol fed, are also heterogeneous.

Sologar et al. (2003) studied methanol and hydrogen sulfide biofiltration as well as its kinetic aspects. They found a maximum methanol removal rate of 380 g/m^3*h. The steady states were reached within the first two days of operation. Moreover, the methanol removal rate increased linearly with the loading rate throughout the study. These results were expected from a pollutant with such a high solubility and relatively low microbial toxicity.

Prado et al., (2004) compared the efficiencies of four bioreactors for the removal of formaldehyde and methanol. Three of them were operated as convectional biofilters while the fourth was operated as a biotrickling filter. The biofilters were also packed with different materials: lava rock, perlite and activated carbon. The results showed that lava rock was the most suitable material for effective methanol and formaldehyde biofiltration while perlite was the worst. Further, biofiltration must be preferred rather than biotrickling biofiltration. The maximum methanol EC reached was 619.4 g/m^3*h.

Chetpattananondh et al., (2005) investigated the biofiltration of methanol, toluene and its mixture using palm shells and activated sludge as packing materials. With such system, they observed a maximum elimination capacity of 188 g/m^3*h with a removal efficiency of 100%. It was possible to increase the EC until 380 g/m^3*h, however the efficiency was affected and dropped to 62%.

Moreover they remarked, as did many others, that removal efficiency does not reflect the real performance of a biofilter. This is because removal efficiency can be simply modified by increasing or decreasing the reactor volume despite its capacity. Therefore, the best way to compare two or more biofilters is through their EC. Actually, EC is by definition normalized on the reactor volume as well as on the empty bed retention time (EBRT).

Media composition and pH effects have been also tested (Prado et al., 2006). For the specific case of methanol degradation, it was found that efficiency is affected at low pH values (near 4). In contrast, it is improved at pH values close to 7.5. Such findings are coherently linked with the optimal pH of 8 reported for formaldehyde dehydrogenase which is an important enzyme in methanol metabolic pathway (Kato et al., 1983).

On the other hand, they observed that the nitrogen source is not important in the performance of the system, although it was slightly better when using ammonia than when nitrate is applied. Furthermore the maximum elimination capacity was 552 g/m^3*h.

Prado et al., (2008) studied the removal of a mixture of formaldehyde, methanol, dimethylether and carbon monoxide from waste gases using a multistage reactor (made up of a biotrickling filter and a biofilter). Among pollutants tested, methanol and formaldehyde were the most readily eliminated, followed by carbon monoxide and dimethylether. Concerning methanol biofiltration, a maximum EC of 533 g/m3*h was observed. It was also proven that the use of combined bioreactors can overcome problems frequently presented in biofilters or biotrickling filters alone.

Dastous et al., (2008) have also done research on methanol biofiltration. They reported a maximum EC of 82 g/m^3*h at an optimal nitrogen concentration of 3.8 g-N/L. At lower nitrogen concentrations, the EC drops because nitrogen becomes the limiting substrate. On the other hand, at higher concentrations, nitrogen had negative effects in the biofiltration performance indicating it becomes increasingly toxic for the microbial community. Therefore, a high nitrogen supply was not necessary to improve the biofilter performance, but it had to be enough in order to support adequate microbial growth.

Avalos-Ramirez et al., (2008) studied growth kinetics and methanol-toluene biodegradation. They compared two kinds of packing materials, clay spheres against polypropylene spheres. In addition, they also studied the effects of nitrogen concentration on system performance. Their research focused on the kinetics of microbial growth and degradation. Haldane and Michaelis-Menten kinetics were used to fit experimental data.

As a result of fitting with Michaelis-Menten kinetics (the most suitable for the set of results), a theoretical maximum EC of 2230 g/m^3*h was determined. Nevertheless, the experimental maximum EC obtained was 650 g/m^3*h.

Furthermore, the effects of packing material and nitrogen concentration were evaluated through microbial growth estimates. It was clearly demonstrated that clay spheres promote major growth as does higher nitrogen concentration. However, it was also observed that there was no relation between the biomass concentration and the elimination capacity.

Babbitt et al., (2009) showed that a biofilter packed with activated carbon and inoculated either with or without an adapted consortium is efficient when removing methanol from an air stream. However, when the inlet loading rate was modified, the biofilter not inoculated presented dramatic changes in performance. This phenomenon was clearly related with a low robustness on the second case that could be a consequence of the start-up strategy. Regardless of the inoculation strategy, the maximum elimination capacity was 20 g/m^3*h.

Table 3. Recent methanol biofiltration studies.

Packing material	Pollutants*	Nitrogen source	Inoculum	Inlet concentration	EBRT	pH	Pressure drop	MEC	RE	Reference
PVC tubes	MetOH	$(NH_4)_2SO_4$ - 1 g/L	Methanol biofilter	2.14 g/m^3	35 s **	6.7	-	172.91 g/m^3*h	78%	Thalasso et al., 2000
Nova Inert (glass)	MetOH	-	-	2.1 g/m^3	16 s	7	-	380 g/m^3*h	80%	Sologar et al. 2003
Palm shells and Activated sludge	MetOH	$(NH_4)_2SO_4$ - 1.97 g/L	Indigenous	1.53 g/m^3	9 s	7	< 78 mm H_2O /m	380 g/m^3*h	62%	Chetpattananondh et al. 2005
Palm shells and Activated sludge	MetOH	$(NH_4)_2SO_4$ - 1.97 g/L	Indigenous	1.83 g/m^3	35 s	7	< 78 mm H_2O /m	188 g/m^3*h	100%	Chetpattananondh et al. 2005
Lava rock	MetOH-CH_2O	NH_4Cl - 2g/L	Aerobic sludge	14.31 g/m^3	80 s	-	-	619.4 g/m^3*h	96%	Prado et al., 2004
Lava rock	MetOH-CH_2O	NH_4Cl - 2g/L	Aerobic sludge	0.10 g/m^3	81.6 s	-	< 17.8 mm H_2O /m	4.7 g/m^3*h	96.2%	Prado et al., 2004
Lava rock	MetOH-CH_2O	NH_4Cl - 2g/L	Aerobic sludge	6.6 g/m^3	36 s	-	< 4 mm H_2O /m	552 g/m^3*h	83%	Prado et al., 2006
Lava rock	MetOH-CH_2O	NH_4Cl - 2g/L	Aerobic sludge	26.6 g/m^3	80 s	7.5	-	1000 g/m^3*h	83%	Prado et al., 2008
Compost	MetOH-EtOH	$(NH_4)_2SO_4$ - 3.8 g/L	Indigenous	3.2 g/m^3 **	85 s **	-	-	82 g/m^3*h	63%	Dastous et al. 2008
Polypropylene spheres	MetOH	Urea - 0.005 gN/L	Biofilter lixiviate	9 g/m^3	30 s	-	-	650 g/m^3*h	60%	Avalos Ramirez et al., 2008
Clay spheres	MetOH	Urea - 0.6 gN/L	Biofilter lixiviate	2.45 g/m^3 **	30 s **	-	-	80 g/m^3*h	26% **	Avalos Ramirez et al., 2008
Granular activated carbon	MetOH	-	Pulp and paper-board wastewater	1.41 g/m^3	300 s	-	-	17 g/m^3*h	100%	Babbitt et al 2009
Peanut shells	MetOH	$(NH_4)_2SO_4$ - 0.87 g/L	Indigenous	0.66 g/m^3	19 s	7	< 200 Pa/m	120.41 g/m^3*h	96%	Ramirez-Lopez et al. 2010
Peanut shells	MetOH	$(NH_4)_2SO_4$ - 0.87 g/L	Indigenous	1.35 g/m^3	19 s	7	-	232.9 g/m^3*h	92%	Ramirez-Lopez et al. 2010
Peanut shells	MetOH	$(NH_4)_2SO_4$ - 0.87 g/L	Indigenous	10.64 g/m^3	19 s	7	-	1438 g/m^3*h	69%	Ramirez-Lopez et al. 2010
Polyvinyl chloride	MetOH	$(NH_4)_2SO_4$ - 0.5 g/L	Pesticides and VOCs consortium	0.76 g/m^3	25 s	-	-	99 g/m^3*h	90%	Balasubramanian et al., 2012
Diatomaceous hearth	MetOH-Hexane	-	-	2.16 g/m^3	120 s	7	-	63.7 g/m^3*h	98%	Zehraoui et al., 2012
Wood pine bark chips	MetOH	$(NH_4)_2SO_4$ - 1 g/L	-	2.2 g/m^3	82 s **	-	< 100 mm H_2O /m	86.4 g/m^3*h	90%	Barcón et al., 2012.
PVC	MetOH	$(NH_4)_2SO_4$ - 1 g/L	-	2.2 g/m^3	82 s **	6.5 - 7	< 10 mm H_2O /m	96 g/m^3*h	100%	Barcón et al., 2012.

EBRT: Empty Bed Residence Time MEC: Methanol Elimination Capacity RE: Removal Eficiency
*All reports are about methanol treatment but in some cases it was treated as part of a mixture.
** Information not reported explicitly.

Ramirez-Lopez et al., used a biofilter packed with peanut shells to evaluate methanol biofiltration under different methanol loads. The best average removal efficiencies reported (96%) were obtained under a loading rate of 125 g/m^3*h. Nevertheless, the maximum EC was 1438 g/m^3*h, which is one of the highest EC values reported ever, if not the highest.

Additionally, they noted increased pressure drop across the biofilter. The shift was attributed to an increase in biomass on the support. However, during the 180 days of the experiment no operational problems came about.

Balasubramanian et al., (2012) investigated the treatment of VOCs from pharmaceutical industries. A mixture of methanol, ethanol, acetone, and toluene was forced to flow through a biotrickling filter. A maximum VOC EC of around 335 g/m^3*h was observed. Particularly, the maximum methanol EC was approximately 99 g/m^3*h.

Also, Zehraoui et al., (2012) used methanol in order to increase the bioavailability of n-hexane in a biotrickling filter. The study revealed that using methanol is an interesting strategy for the effective treatment of hydrophobic pollutants. Furthermore, a maximum methanol EC of 63.7 g/m^3*h was observed.

Finally, Barcón et al., (2012) studied methanol biofiltration using two different packing materials: wood bark chips and PVC rings. The results revealed better performance of the biofilter packed with the inorganic material. One of the main reasons was that the biofilter packed with wood bark chips was characterized by changing pH conditions (no pH control was set up), while the other system, the biofilter packed with PVC rings, did have pH control which enabled reaching a higher removal capacity(EC = 96g/m^3*h).

As noted in preceding paragraphs, biofiltration is a technology briefly used to treat methanol air-streams. On average, the maximum methanol EC from the studies is around 347 g/m^3*h. Therefore, methanol EC is higher than typical elimination capacities commonly reported for VOCs such as styrene, ethanol, xylenes, hexane, toluene, phenol, etc. (Iranpour et al., 2005).

A novel microorganism for methanol biofiltration: *Pichia pastoris*.

As it was reviewed, methanol biofiltration is a highly efficient technology in which different microbial populations are involved (either directly or indirectly). Although few studies have been focused on this topic, an enormous number of microbial genera such as *Pseudomonas*, *Sphingomonas*, *Rhodotorula*, *Methylobacterium*, *Methylophylus, Methylocella,* Methylocystis, Methylococcus, Methylomonasand, *Candida, Nectria* and *Scytalidinum,* have been identified (Babbit, et al., 2009; Moreno-Terrazas et al., 2010, Barcón et al., 2012).

Pichia pastoris is a methylotrophic (meaning it can degrade methanol) yeast recently used in methanol biofiltration. In contrast to the rest of microorganisms commonly inoculated or found in biofilters, *P. pastoris* is a suitable expression system for heterologous proteins. The main advantages of using *P. pastoris,* not only in biofilters but also in other kinds of bioreactors, for proteins production are the following (Potvin et al., 2012):

1. Highly efficient Alcohol Oxidase 1 promoter (pAOX1), which is induced by methanol.
2. Low levels of endogenous protein secretion, which simplifies heterologous protein recovery.
3. Performance of post-translational modifications, including glycosylation and disulfide bonds.
4. Its respiratory metabolism which dominates over a fermentative pathway.

AOX promoter.

Alcohol oxidase (AOX) is the first enzyme involved on the methanol degradation pathway. It catalyzes the oxidation of methanol to formaldehyde. Two different genes in *P. pastoris* are responsible for its expression: AOX1 and AOX2. The first one produces between 90 and 95% of total AOX expressed by *P. pastoris*, while the second affords the rest (Macauley-Patrick et al., 2005).

Due to such efficiency, the promoter of the AOX1 gene is used for constructions of recombinant protein expressing cassettes, which are integrated into the genome of the yeast. The resulting *P. pastoris* strains are capable of producing the peptide of interest under the control of methanol availability. (Kupcsulik and Sevella, 2004)

In the framework of biofiltration, *P. pastoris* represents in theory a remarkable opportunity to make methanol biofiltration less expensive and more interesting for industry.

Methanol metabolism

The *P. pastoris* methanol metabolism is comprised of two different routes: dissimilative and assimilative (Figure 3). The first one is focused on energy production and the second on biomass production.

The dissimilatory pathway starts with methanol oxidation to formaldehyde through an alcohol oxidase in peroxisomes. Simultaneously, hydrogen peroxide is produced and immediately transformed to oxygen and water through a catalase. Formaldehyde diffuses from peroxisomas to cytosol, where it is finally oxidized to CO_2 by formaldehyde dehydrogenase, S-formyl glutathione hydrolase and formate dehydrogenase (Negruta et al., 2010; Yurimoto et al., 2011).

Alternatively, the methanol degradation pathway can lead to the synthesis of acetic acid. It starts with the synthesis of aldehyde from a methanol molecule and a subsequent combination with another in order to produce methyl hydroxymethyl ether. Afterwards, the ether oxidation takes place through an alcohol dehydrogenase leading to acetic acid formation (Yurimoto et al., 2011).

On the other hand, the assimilatory pathway starts with the conversion of methanol to formaldehyde. It's followed by a reaction between formaldehyde and xylulosa-5-fosfato to synthesize glyceraldehyde-3-phosphate and dihydroxyacetone. This last reaction is catalyzed by a dihydroxyacetone synthase. Newly formed Dihydroxyacetone diffuses to cytosol and is phosphorylated by a dihydroxyacetone kinase to produce dihydroxyacetone phosphate. This compound and glyceraldehyde-3-phosphate, which also diffuses to cytosol, are combined to form fructose-1,6-biphosphate. Subsequently, fructose-1,6-biphosphate is transformed in fructose-6-phosphate by a phosphatase.

Finally, xylulose-5-phosphate is regenerated from two molecules of fructose-6-phosphate and one molecule of triose phosphate in non-oxidative part of pentose cycle (Santana, 2004; Negruta et al., 2010; Yurimoto et al., 2011).

Clearly, the use of *P. pastoris* in biofiltration is a promising concept. Nevertheless, scarce research has been developed on the topic. In the next section, a review of biofiltration coupled with protein production is approached as well as the future challenges.

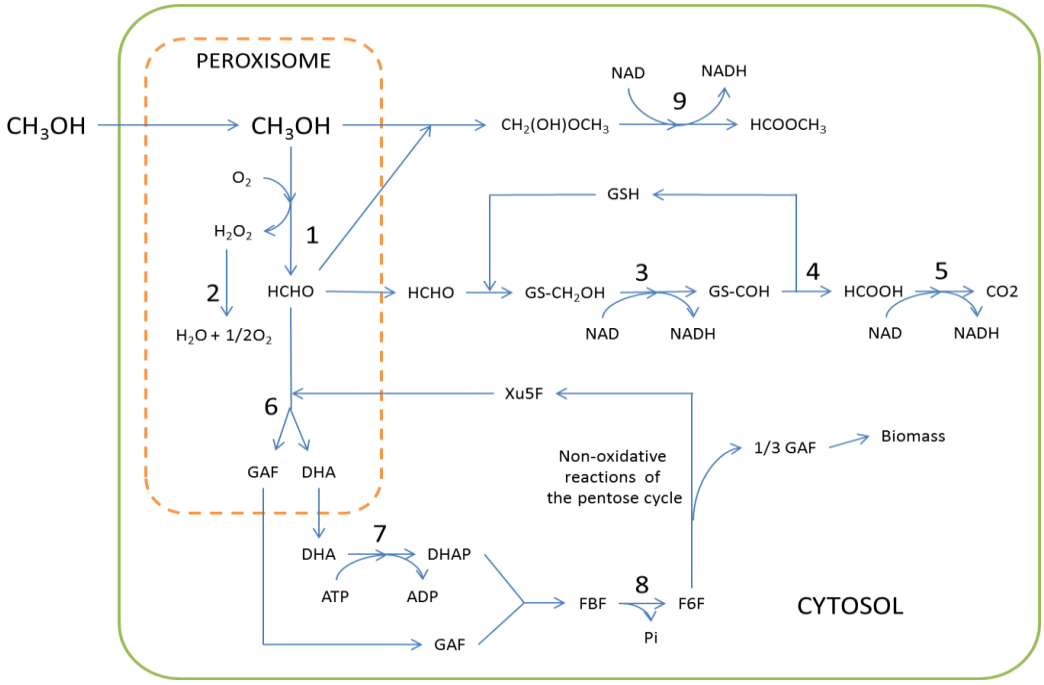

Figure 3. Methylotrophic yeast metabolism. 1 - Alcohol oxidase, 2 - catalase, 3 - formaldehyde dehydrogenase, 4 - S-formyl glutathione hydrolase, 5 - formate dehydrogenase, 6 - dihydroxyacetone synthase, 7 - dihydroxyacetone kinase, 8 - fructose-2 ,6-bisphosphatase, 9 - alcohol dehydrogenase, GSH - glutathione, GAP - glyceraldehyde phosphate, DHA - dihydroxyacetone, DHAP - dihydroxyacetone phosphate, FBP - fructose-1 ,6-bisphosphate, F6P - fructose-6-phosphate, Xu5P - xylulose-5-phosphate. (Negruta et al., 2010; Yurimoto et al., 2011)

Table 4. Heterologous proteins expressed in *P. pastoris*.

Heterologous protein	Reference
Xylanase	Jun-Qing et al., 2013
Glycoside hydrolase of 7-b-xylosyltaxanes	Wen-Bo et al., 2013
Haemagglutinin protein	Murugan et al., 2013
Human heteromeric amino acid transporters	Costa et al.,2013
vMIP-II-IgG3-TfN	Wang et al., 2013
Cellobiohydrolase	Oliveira et al., 2013
Enhanced green fluorescent protein	Liang et al., 2013
Lipasa	Liang et al., 2013

Methanol biofiltration coupled with heterologous proteins production

Cornabé et al., (2002) found that it is possible to employ *P. pastoris* to remove methanol from air streams and to produce a heterologous protein at the same time. Cornabé's work was focused on the production of laccase with a genetically modified *P. pastoris* strain. They achieved elimination capacities as high as 175 g/m^3*h and correlated them with the highest laccase activity of 916 U/L (Cornabé et al., 2002). Laccase activities ranged from 283 to 916 U/L during the 90 days of operation.

Arriaga et al., (2010) reported the biodegradation of methanol coupled with the expression of Endochitinase (Ech42) from *Trichoderma atroviride* in *P. pastoris*. Maximal volumetric methanol consumption rates ranging from 23.6 to 88.7 g/m^3*h were determined from the experiments conducted. Although the experiments were not conducted on biofilter columns, it was observed that Ech42 recovered was active at all methanol concentrations.

Lately, methanol biofiltration coupled with the production of Ech42 has been successfully carried within a biofilter (Arriaga et al., 2012). The system showed an elimination capacity of 165 g/m^3*h at an inlet loading rate of 170 g/m^3*h. From a loading shock study a maximum EC of 1320 g/m^3*h was obtained (loading rate of 1465 g/m^3*h). Certainly, this EC is one of the highest reported for methanol biofiltration, but the loading shock experiments were very brief. Therefore, it's unknown if the newly discovered process can support such loads under long times.

On the other hand, the Ech42 activity fluctuated between 82 and 125 U/L throughout the study. Based on those results, it was calculated that 50,000 $USD/m^3_{reactor}$ could be obtained after a year of biofilter operation. Hence, it was demonstrated that the system is not only a suitable option for methanol treatment but also for heterologous protein production.

OBJECTIVES AND STRUCTURE

Although important progress has been made by testing the efficiency of *P. pastoris* in lab-scale biofilters (Arriaga et al., 2012), there is still poor knowledge concerning responses under different operational strategies. Therefore, the objective of this thesis was to evaluate methanol biofiltration

coupled with heterologous protein production when operated under different methanol loads, pH values, nitrogen concentrations, and EBRTs. Additionally, based on the experimental results found, we aimed to study the divergence in biofilters, especially those inoculated with only one strain. Finally, we targeted developing a mathematical model that fits the behavior of biofiltration coupled with protein production.

In Chapter II of this thesis, the behavior of methanol biofiltration using *P. pastoris* was shown under different conditions of methanol load, nitrogen concentration, pH and EBRT. In Chapter III, repeatability in biofiltration systems was questioned and discussed. In Chapter IV, a mathematical model based on simple CSTR equations and Monod kinetics is shown. Lastly, in Chapter V we give a general discussion about the experimental findings as well as some suggestions for a better develop of the technology.

REFERENCES

Arriaga, S., Acosta-Munguía, A., Pérez-Martínez, A.S., De León-Rodríguez, A., Barba de la Rosa, A.P. (2010). Coupling aerobic biodegradation of methanol vapors with heterologous protein expression of Endochitinase Ech42 from *Trichoderma atroviride* in *Pichia pastoris*. *Bioresource Technology*,101 9661-9665.

Arriaga, S., Serrano, M.A., Barba de la Rosa, A.P. (2012). Methanol vapor biofiltration coupled with continuous production of recombinant endochitinase Ech42 by *P. pastoris*. *Process Biochemistry*, 47 2311-2316.

Ávalos-Ramirez, A., Benard, S., Giroir-Fendler, A., Peter, J., Heitz, M. (2008). Kinetics of microbial growth and biodegradation of methanol and toluene in biofilters and an analysis of the energetic indicators. *Journal of Biotechnology*, 138 88-95.

Babbitt, C.W., Pacheco, A., Lindner, A.S. (2009). Methanol removal efficiency and bacterial diversity of an activated carbon biofilter. *Bioresource Technology*, 100 6207-6216.

Balasubramanian, P., Philip, L., Bhallamudi, S. (2012). Biotrickling filtration of complex pharmaceutical VOC emissions along with chloroform. *Bioresource Technology*, 114 149-159.

Barcón T, Alonso-Gutiérrez J, Omil F. (2012). Molecular and physiological approaches to understand the ecology of methanol degradation during the biofiltration of air streams. *Chemosphere* 87 1179-1185.

Chetpattananondh P, Nitipavachon Y, Bunyacan C. (2005). Biofiltration of air contaminated with methanol and toluene. *Journal of Science and Technology*, 27 761-773.

Chuwers, P., Osterloh, J., Kelly, T., D'Alessandro, A., Quinlan, P., Becker, C. (1995). Neurobehavioral effects of low level methanol level exposure in healthy human volunteers. *Environmental Research*, 71 141-150.

Cornabé T, Auria R, Christen P, Ferrer H, Revah S, Moukha S. (2002) Biofiltration of methanol vapors by a recombinant *Pichia pastoris* strain. In: *USC-TRG Conference on Biofiltration,* p. 1–6.

Costa, M., Rosell, A., Álvarez-Marimon, E., Zorzano, A., Fotiadis, D., Palacin, M. (2013). Expression of human heteromeric amino acid transporters in the yeast *Pichia pastoris*. *Protein Expression and Purification*, 87 35-40.

Cox, H., Deshusses, M.A. (1998). Biological air treatment in biotrickling filters. *Current Opinion in Biotechnology*, 9 256-262.

Dastous PA, Nikiema J, Soreanu G. (2008) Elimination of methanol and ethanol by biofiltration: an experimental study. *Water Air and Soil Pollution: Focus*, 8 275-285.

Devinny J. S., Deshusses M. A., Webster T. S. (1999) Biofiltration for air pollution control. Lewis Publishers.

EPA (2012) http://ofmpub.epa.gov/sor_internet/registry/termreg/searchandretrieve/glossariesandkeywordlists/search.do?details=&glossaryName=Improving%20Air%20Quality Webpage viewed

Gordon, A.S., Austin, T.C. (1992). Alternative fuels for mobile transport. *Progress in Energy and Combustion Science*, 18 493-512.

Green, D.W., Perry, R. (2007). Perry's chemical engineers' handbook. 8th edition, McGraw-Hill.

Groenestijn J. W., Hesselink P. (1993). Biotechniques for air pollution control. *Biodegradation*, 4 283-301.

Health Effects Institute (1999). Reproductive and offspring developmental effects following maternal inhalation exposure to methanol in non-human primates. Report #89, *HEI Reports*.

Health Effects Institute (2000). 1,3-Butadiene: cancer, mutations, and adducts. Report #92, *HEI reports.*

Health Effects Institute (2002). Case-cohort study of styrene exposure and ischemic heart disease. Report #108, *HEI Reports*.

Health Effects Institute (2003). Benzene metabolism in rodents at doses relevant to human exposure from urban air. Report #113, *HEI reports*.

Ikeda, M. (1992). Public health problems of organic solvents. *Toxicology Letters*, 64/65 191-201.

IMPCA. International Methanol Producers and Consumers Association http://www.impca.be/en/about_methanol/ (Accessed 11-03-12).

International Agency for Research on Cancer (1989). Xylene exposure data. www.monographs.iarc.fr/ENG/Monographs/vol71/mono71-58.pdf (Accessed 11-03-12).

International Agency for Research on Cancer (2006). Formaldehyde exposure data. www.monographs.iarc.fr/ENG/Monographs/vol88/mono88-6.pdf (Accessed 11-03-12).

Iranpour, R., Cox, H., Deshusses, M., Schroeder, E. (2005). Literature review of air pollution control biofilters and biotrickling filters for odor and volatile organic compound removal. *Environmental Progress*, 24 254-267.

IRIS EPA (2005). Toxicological review of toluene. USA.

Jun-Qing, W., Yin, X., Min-Chen, W., Hui-Min, Z., Shu-Juan, G., Jing-Tu, W., Cun-Duo, T., Jian-Fang, L. (2013). Expression of a family 10 xylanase gene from *Aspergillus usamii* E001 in *Pichia pastoris* and characterization of the recombinant enzyme. *Journal of Industrial Microbiology and Biotechnology,* 40 75-83.

Kato N., Shirakawa K., Kobayashi H. and Sakazawa C. (1983) The Dismutation of Aldehydes by a Bacterial Enzyme. *Agricultural and Biological Chemistry*, 47 39-46.

Kupcsulik, B., Sevella, B. (2004). Effect of methanol concentration on the recombinant *Pichia pastoris* Muts fermentation. *Periodica Polytechnica: Chemical Engineering*, 48 73-87.

Liang, S., Cheng, L., Yanrui, Y., Ying, L. (2013). Endogenous signal peptides efficiently mediate the secretion of recombinant proteins in *Pichia pastoris*. *Biotechnology Letters*, 35 97-105.

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*, 22 249–270.

MI. Methanol Institute. The methanol industry. http://www.methanol.org/Methanol-Basics/The-Methanol-Industry.aspx (Accessed 11-03-12)

Moreno-Terrazas R, Flores-Tena FJ, Barbara-Avila MD, Guerrero-Barrera AL, Avelar-Gonzalez FJ, Ramirez-Lopez EM. A comparative analysis of microfloro during biofilm development on grape seeds exposed to methanol in a biofilter. *World Journal of Microbiology and Biotechnology*, 2010 26 657-664.

Murugan, S., Ponsekaran, S., Kannivel, L., Mangamoori, L.N., Chandran, D., Alwar, S.V., Chakravarty, C., Lal, S.K. (2013). Recombinant haemagglutinin protein of highly pathogenic avian influenza A (H5N1) virus expressed in *Pichia pastoris* elicits a neutralizing antibody response in mice. *Journal of Virological Methods*, 187 20-25.

Negruta O., Csutak O., Stoica I., Rusu E., Vassu T. (2010). Methylotrophic yeasts: diversity and methanol metabolism. *Romanian Biotechnological Letters*, 15 5369-5375.

Oliveira, G.S., Ulhoa, C.J., Silveira, M., Andreaus, J., Silva-Pereira, I., Pocas-Fonseca, M.J., Faria, F.P. (2013). An alkaline thermostable recombinant *Humicola grisea* var. *thermoidea* cellobiohydrolase presents bifunctional (endo/exoglucanase) activity on cellulosic substrates. *World Journal of Microbiology and Biotechnology*, 29 19-26.

Potvin, G., Ahmad, A., Zhang, Z. 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochemical Engineering Journal*, 64 91-105.

Prado, O.J., Veiga, M.C., Kennes, C. (2004). Biofiltration of waste gases containing a mixture of formaldehyde and methanol. *Environmental Biotechnology*, 65 235-242.

Prado, O.J., Veiga, M.C., Kennes, C. (2006). Effect of key parameters on the removal of formaldehyde and methanol in gas phase biotrckling filters. *Journal of Hazardous Materials*, B138 543-548.

Prado, O.J., Veiga, M.C., Kennes, C. (2008). Removal of formaldehyde, methanol, dimethylether and carbon monoxide from waste gases of synthetic resin-producing industries. *Chemosphere*, 70 1357-1365.

Ralebitso-Senior, T.K., Senior, E., Di Felice, R., Jarvis, K. (2012). Waste gas biofiltration: Advances and limitations of current approaches in microbiology. *Environmental Science and Technology*, 46, 8542-8573.

Ramirez-López, E.M., Corona-Hernandez, J., Avelar-Gonzalez, F.J., Omil, F., Thalasso, F. (2010). Biofiltration of methanol in an organic biofilter using peanut shells as medium. *Bioresource Technology*, 101 87-91.

Rittman B.E., McCarty P.L. (2002). Environmental Technology: Principles and applications. McGraw Hill.

Santana A. (2004). Tesis: Biofiltración de metanol por *Pichia methanolica*. UAM.

Secretaría de Salud. Mexico Health Metric 2005 Report. México 2005.

SEMARNAT 2006. Inventario Nacional de Emisiones de Mexico 1999. México.

Sologar VS, Lu Z, Allen DG. (2003). Biofiltration of concentrated mixtures of hydrogen sulfide and methanol. *Environmental Progress*, 22 129-136.

Thalasso, F., Omil, F., Otero, J.O., Lema, J.M. (2000). Treatment of methanol in a dry biofilm reactor using tubular carrier. *Water Science and Technology*, 42 419–427.

Utkin, J.B., Yakimov, M.M., Kozlyak, E.I., Rogozhin J.S. (1992).Biological air purification, *BioCycle*, 613-620.

Wen-Bo, Y., Xiao, L., Ping, Z. (2013). High-cell-density fermentation and pilot-scale biocatalytic studies of an engineered yeast expressing the heterologous glycoside hydrolase of 7-b-xylosyltaxanes. *Fermentation, Cell Culture and Bioengineering*, 40 133-140.

Wuang, F., Xiu-Ying, L., Xue-mei, M., Guang, Z., Han-Xiao, S. (2013). A biologically active vMIP-II-IgG3-TfN fusion protein, secreted from methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification*, 87 47-54.

Yang, C., Cheng, H., Zeng G., Yu, G., Luo, S. (2010). Biomass accumulation and control strategies in gas biofiltration. *Biotechnology Advances*, 28 531-540.

Yurimoto, H., Oku, M., Sakai, Y. (2011). Yeast methylotrophy: metabolism, gene regulation and peroxisome homeostasis. *International Journal of Microbiology*, 8 pages.

Zehraoui, A., Hassan, A., Sorial, S. (2012). Effect of methanol on the biofiltration of n-hexane. *Journal of Hazardous Materials*, 219/220 176-182.

EFFECT OF OPERATIONAL PARAMETERS ON METHANOL BIOFILTRATION COUPLED WITH ENDOCHITINASE 42 PRODUCTION

Palomo-Briones Rodolfo, Arriaga Sonia

IPICYT, Instituto Potosino de Investigación Científica y Tecnológica, División de Ciencias Ambientales. Camino a la Presa San José 2055, Lomas 4ta. Sección, C. P. 78216, San Luis Potosí, México. Tel.: +52 444 834 2000; fax: +52 444 834 2010.

E-mail address: sonia@ipicyt.edu.mx (S. Arriaga).

ABSTRACT

A novel methanol biofiltration process coupled with Ech42 protein production using a genetically modified *Pichia pastoris* strain was investigated. Four important parameters: methanol loading rate, pH, nitrogen source concentration and empty bed residence time (EBRT) were tested employing three identical biofilters. The maximum elimination capacity observed was 643.5 g/m^3*h under a methanol load of 800 g/m^3*h, 5 g/L $(NH_4)_2SO_4$, pH 5 and 60 seconds of EBRT. In general, the novel biofiltration system showed a fragile equilibrium which was easily perturbed through changes in the different parameters aforementioned.

Also, the Ech42 activity ranged from 578 to 1172 U/L being pH the most important parameter in the process. Methanol loading rate and EBRT did not have an important effect on the Ech42 production.

INTRODUCTION

Methanol is a volatile organic compound highly soluble in water (1000g/L) which is commonly released by several industries (e.g. paper, automotive, paint, etc.). If inhaled, methanol can cause headache and nauseas, cause shorter gestational length in pregnant women and alter the neurobehavioral development in children (Health Effects Institute, 1999). Due to these and other potential effects, methanol is considered one of the most hazardous air pollutants by the Environmental Protection Agency (Clean Air Act, 1990).

Among the different technologies available for methanol treatment, biofiltration is the most widely used (Arulneyam and Swaminathan, 2003; Sologar et al., 2003; Chetpattananondh et al., 2005; Dastous et al., 2008; Babbit et al., 2009; Ramirez-Lopez et al., 2010). Biofiltration is characterized by the use of microorganisms capable of degrading pollutants. Such microorganisms are fixed and growing on the surface of a particulate material packed in a biofilter column. When the air flows through the bed, the pollutant is decomposed as a result of the microbial catalytic activity.

Microorganisms with the ability to degrade methanol are called methylotrophs. In methanol biofiltration, a wide variety of that kind of microorganisms has been found, e.g. *Pseudomonas* sp., *Methylobacterium* sp., *Methylococcus* sp. and *Scytalidinum* sp. (Babbit et al., 2010; Moreno-Terrazas et al., 2010; Barcon et al., 2012). Recently, *Pichia pastoris* was used in methanol biofiltration in order to simultaneously obtain a valuable product: a heterologous protein (Cornabé et al., 2002; Arriaga et al., 2010; Arriaga et al., 2012).

P. pastoris is a methylotrophic yeast well known for its efficiency in heterologous protein expression (Potvin et al., 2012). Among its multiple features, three advantages can be highlighted: its highly efficient methanol inducible promoter (pAOX1), its low endogenous protein secretion, and its capacity for post-translational modifications (Negruta et al., 2010; Potvin et al., 2012). All of these together make *P. pastoris* one of the best options for protein production.

So far, methanol elimination capacities (EC), reached with the novel *P. pastoris* biofiltration system, have been as high as those obtained in conventional methanol biofiltration (Cornabè et al., 2002; Arriaga et al., 2012). Also, according to the recovered protein, the system has proven its economic profitability (Arriaga et al., 2012). However, the process is still little known and more research is needed for its industrial implementation.

In this study we evaluate the effect of operational parameters such as methanol load, nitrogen concentration, pH and empty bed retention time (EBRT), on the performance of the coupled process.

METHODOLOGY

Inoculum and growth medium

P. pastoris GS115 transformed with the plasmid pPIC-ech42 (Perez-Matínez et al, 2007), which contains the 42 kDa endochitinase gene from *T. atroviride* was used as a model of study. The strain was grown in 5 mL of YPD growth medium (10 g/L yeast extract, 20 g/L dextrose, 20 g/L peptone, and 10 g/L bacto-agar) plus 250 µg/mL geneticin (G418 sulfate, Phyto Technologies Laboratories, USA) at 28 C overnight (Arriaga et al., 2010). Cultures were centrifuged for 5 min at 13000 rpm and the resulting pellets were re-suspended in 500 mL of mineral medium (1.7g/L YNB w/o amino acids and ammonium sulphate; 2.5 g/L $(NH_4)_2SO_4$; 100mM Citrate-Phosphate Buffer pH5) and methanol (1%, v/v). The flasks were incubated for 48 h at 28 C. The resulting cultures were recirculated through the biofilters during 2 h.

Biofilters

Three identical glass biofilters were designed and packed with perlite. Each reactor was comprised of three sections with gas and biomass sampling ports. The internal diameter and efficient height in all sections were 8.89 cm and 18 cm, respectively. Therefore, the total volume for each reactor was approximately 3.3 L. The arrangement of the systems is presented in Figure 1.

Experimental setup

In order to evaluate the effects of methanol loading rate, nitrogen concentration, pH and EBRT, the experimental operation was comprised of 6 different stages (Table 1). In Stages 1, 2 and 3, biofilters were identically operated under three different inlet methanol concentrations. Furthermore, in Stages 4, 5 and 6, biofilters were used to evaluate nitrogen concentration, pH and EBRT effects.

The methanol loading rate and EBRT were adjusted by changing the airflow through the methanol reservoir as well as the total air entering the system. Nitrogen supply and pH regulation were accomplished by the addition of 500 mL of mineral solution, which was sprinkled on the top of the column and recirculated for one hour every two days.

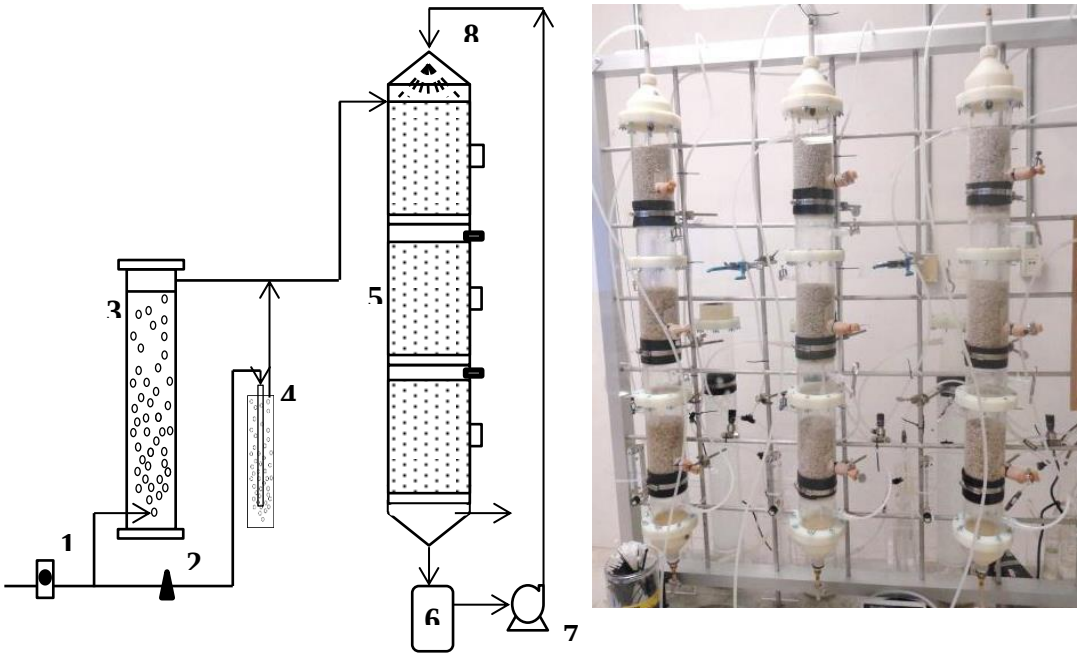

Figure 1. Diagram of the experimental biofilters. 1.- rotameter, 2.- needle valve, 3.- humidifier, 4.- methanol reservoir, 5.- biofilter, 6.- mineral solution, 7.- recirculation of leachate, 8.- sprinkler.

Performance evaluation

Methanol and CO_2 concentrations were tested every other day at three different heights along the biofilters (18, 36, and 54 cm from inlet). Thereafter, the performance in every section and the whole system was evaluated through the elimination capacity (EC), removal efficiency (RE) and mineralization, according to the following equations:

$$EC = \frac{(C_{MetOH(in)} - C_{MetOH(out)}) * Q}{V_f} * 100\% \quad (1)$$

$$EE = \frac{C_{MetOH(in)} - C_{MetOH(out)}}{C_{MetOH(in)}} * 100\% \quad (2)$$

$$Mineralization = \frac{\left(C_{CO_2(out)} - C_{CO_2(in)}\right)}{(C_{MetOH(in)} - C_{MetOH(out)}) * 1{,}375} * 100\% \quad (3)$$

Table 1. Experimental setup.

	BIOFILTER I				BIOFILTER II				BIOFILTER III			
STAGE	ILR (g/m^3*h)	$(NH_4)_2SO_4$ (g/L)	pH	EBRT (s)	ILR (g/m^3*h)	$(NH_4)_2SO_4$ (g/L)	pH	EBRT (s)	ILR (g/m^3*h)	$(NH_4)_2SO_4$ (g/L)	pH	EBRT (s)
1	330	5	5	60	330	5	5	60	330	5	5	60
2	600	5	5	60	600	5	5	60	600	5	5	60
3	800	5	5	60	800	5	5	60	800	5	5	60
4	400	7.5	5	60	400	5	3.5	60	400	5	5	60
5	400	2.5	5	60	400	5	6.5	60	400	5	5	120
6									400	5	5	45

ILR: Inlet Loading Rate

EBRT: Empty Bed Residence Time

Where $C_{MetOH(in)}$ and $C_{MetOH(out)}$ are gas methanol concentrations at inlet and outlet, respectively. Q is airflow and V_f is the biofiltration effective volume under evaluation. Similarly, $C_{CO_2(in)}$and $C_{CO_2(out)}$are CO_2 concentrations at inlet and outlet of control volume.

Mineralization percentage is the ratio between methanol biodegraded to CO_2 and total methanol removed from inflow. The biochemical mineralization reaction for methanol is:

$$CH_3OH + \frac{3}{2}O_2 \xrightarrow{Microorganisms} CO_2 + 2H_2O$$

In this way, 1.375g of CO_2 are produced per each methanol gram consumed.

Analytical methods

Methanol concentrations were determined by injecting 200µL gas samples into a 6890 series gas chromatograph (Agilent Technologies, CA, USA) with a flame ionization detector (FID) and a capillary column DB-624. Nitrogen was used as a carrier gas at a flow rate of 25 mL/min. The temperatures of the injector, oven and detector were 150, 80 and 250 C, respectively. On the other hand, CO_2 production was measured with a gas chromatograph (Agilent Technologies, CA, USA) equipped with a thermal conductivity detector (TCD) and capillary column HP-PLOT Q. Helium was used as a carrier gas at a flow rate of 10.1 mL/min. The temperatures of the injector, column, and

detector were 200, 50, and 250 C, respectively. The volume of injection for CO_2 determinations was 400µL.

Moreover, biomass in the support was determined through a total organic carbon analyzer (Shimadzu, Kyoto, Japan). Also, biomass in the leachates was measured by total solids methodology according to the standard method.

Total proteins

Total proteins were tested in filtered leachate samples through Bradford methodology. Bovine serum albumin was used as standard. For each determination, 10 µL of sample was mixed with 200µL of Bradford solution (1X), the mixture was shaken and given 5 minutes to react. After that, absorbance at 595 nm was determined and compared with a calibration curve.

Enzymatic activity

The enzymatic activity assay was determined with 4-methyl-ubenylferyl quitotriose (4MUQT) at a final concentration of 1 µM with 100 µl of sample. Reactions were incubated at 40 C for 10 min and then stopped by the addition of 1.9 mL of 500mM calcium carbonate addition. Fluorescence data were measured with VersaFluor equipment (BioRad, Hercules, CA, USA) at 360 nm and 460nm for excitation and emission, respectively.

RESULTS AND DISCUSSION

Methanol load effect

Three identical biofilters were operated 82 days in order to investigate the methanol load effects on biofiltration performance and protein production efficiency. During the first stage (28 days), methanol was fed at a loading rate of 330 g/m^3*h. Within seven days, the biofilters reached ECs of 305±18, 304±20 and 273±44 g/m^3*h, respectively (Figure 1). The performance was comparable with the highest ECs reported so far (Sologar et al., 2003; Ramirez-Lopez et al., 2010).

Around the twelfth day of operation, the EC was negatively affected by a delay of 24 hours in the mineral medium addition. This was a first indicator of the system's high sensitivity. This particular response may be caused by the very low microbial diversity in the biofilter (only one species) which

impacts its resilience. Such ecological theory has been extensively studied (e.g. Goodman, 1975; Diaz and Cabido, 2001; Tscharntke et al., 2005; Huang et al., 2013); however, the link is neither simple nor universal, and every ecosystem should be studied separately.

With time and adequate irrigation, the biofilters recovered almost all the EC previously shown. By the end of the first stage, the ECs were 273±24, 280±27 and 280±28 g/m^3*h, in Biofilters I, II and III, respectively (Figure 1). In the second stage, the biofilters were operated at a methanol loading rate of 600 g/m^3*h. Under such conditions, the biofilters performances were slightly increased. The ECs obtained in Biofilters I, II and III, were 297±9, 338±7 and 341±2 g/m^3*h, respectively. Finally, in the third stage (30 days), the methanol loading rate was increased to 800 g/m^3*h. In said stage the performance of the systems was notably affected. The ECs in the Biofilters I, II and III, were 330±2, 461±1 and 643±5 g/m^3*h, respectively.

Clearly, the behavior of the three biofilters varied widely, especially in Stage 3. If biomass is taken into consideration (Figure 2), it can be observed that it is not a determinant factor on the irregular performance. Thus, the possible explanations are reduced to unpredictable natural phenomena such as microbial colonization and evolution. Naturally, more research is needed to completely understand the aforementioned findings.

Protein production rate similarly increased from one stage to another among the three biofilters (Table 2). Nonetheless, the Ech42 enzymatic activity did not follow the same pattern. In the Biofilter I, the Ech42 activity at the end of the first stage was 1020 U/L, while 802 U/L was observed at the end of Stage 2 and 934 U/L at the end of Stage 3. This behavior reflects that in Biofilter I the total protein production was not linked to Ech42 activity. In contrast, Ech42 activity seemed to be quite stable indicating that Ech42 secretion was not highly affected by the different feeding strategies.

In Biofilter II, the enzymatic activity decreased from 965 U/L in Stage 1, to 811 U/L in Stage 2. Additionally, a slight difference was observed between the enzymatic activities in Stages 2 and 3. So, in this particular biofilter, the total protein production and enzymatic activity were also difficult to bind.

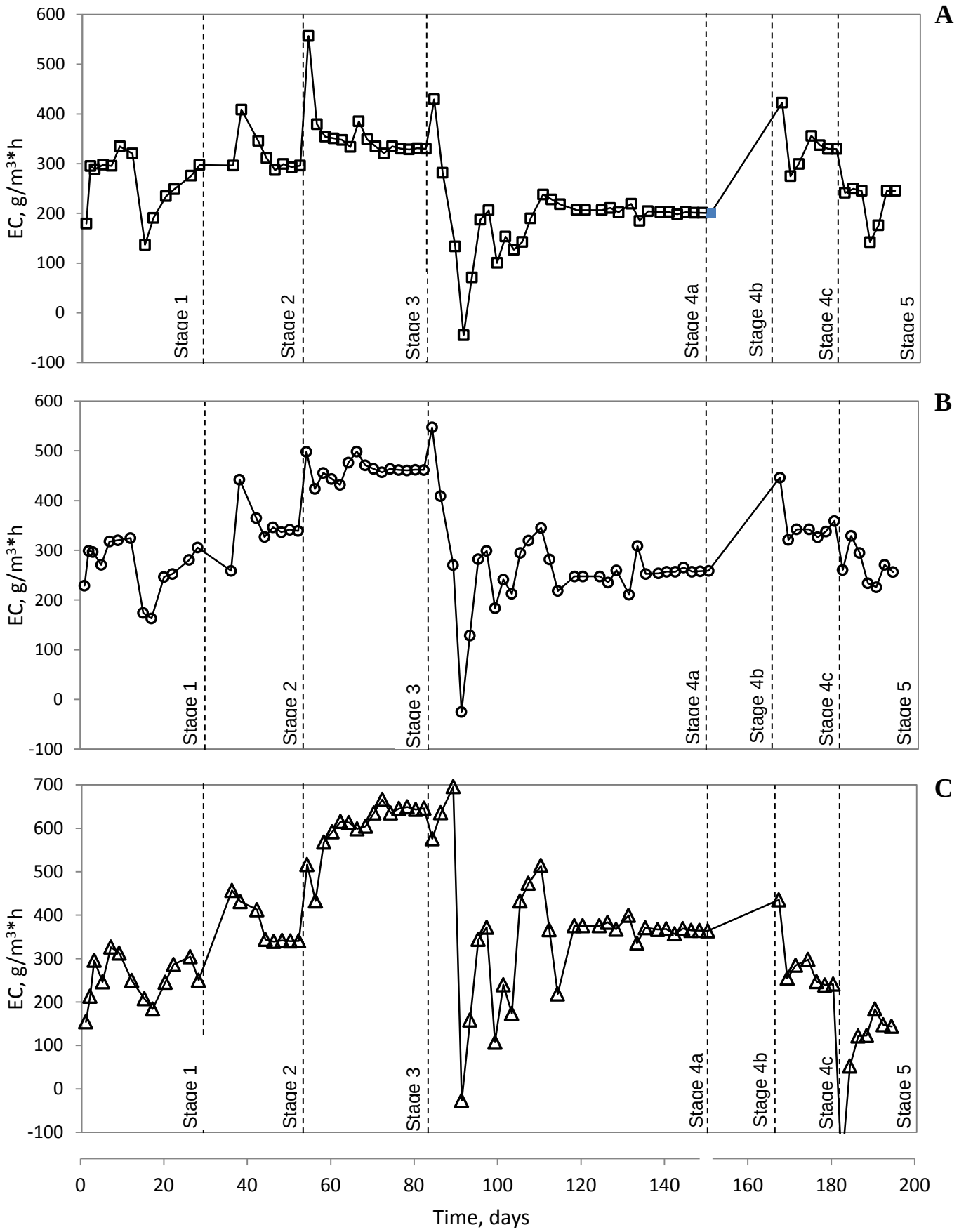

Figure 2. Biofiltration performance. A) Biofilter I, B) Biofilter II, C) Biofilter III.

Finally, in Biofilter III, it was observed that enzymatic activity increased from 762 U/L in the first stage to 813 U/L in the second stage, and 1020 U/L in the third stage. Apparently, the total protein and Ech42 productivities were better correlated.

In general, it was observed that the protein production rate increased over time (Table 2). However, Ech42 activity was stable along the three stages mentioned above. The results of Ech42 activity were between 6.5 and 8.3 times higher than the previous report in a biofiltration system (Arriaga et al., 2012). Still, the Ech42 activity was between 28 and 44% lower than those obtained from batch experiments (Perez-Martinez et al., 2007). A likely reason for this last observation is that in batch systems, methanol, nutrients and other conditions are uniform due to mechanical mixing. This leads the system to avoid mass transfer limitations which are commonly present in biofilters. Additionally, the batch systems are characterized by a well-controlled growth conditions which keep other species away. In contrast, biofilters are opened units where other microorganisms can easily enter.

Nitrogen concentration effect

In Stages 4 and 5, Biofilter I was used to evaluate the nitrogen concentration effects on the biofiltration efficiency and Ech42 production. For such purpose, two sets of conditions were tested (Stages 4 and 5). In the first one, $(NH_4)_2SO_4$ concentration was set at 7.5 g/L while the methanol loading rate was 400 g/m^3*h, which is equivalent to a carbon to nitrogen concentration of 3 gC/gN, approximately (see Annex 4). During the first days, the efficiency of the system dropped to very low values which indicated desorption of methanol from the biofilm (Marek et al., 2000; Fazaelipoor et al., 2006). With time (around 30 days), the system efficiency reached a steady state of 203 ± 12 g/m^3*h.

In this study, the steady state EC of 4th stage was negatively impacted at the high nitrogen concentration tested. As observed in Figure 2A (day 126), the biomass was diminished, which is consistent with the EC value found. This behavior could be related to reported nitrogen transformation to ammonia, which is toxic to microorganisms and can completely halt the fermentation (Rughoonundun et al., 2012). Nevertheless, its presence was not confirmed in this work.

Table 2. Summary of protein production and Ech42 activity.

	Operational conditions					Protein concentration (g/L)	Protein production rate (mg/h)	Ech42 Activity	
	Stage	MLR (g/m^3*h)	$(NH_4)_2SO_4$ (g/L)	pH	EBRT (s)			nmol/min*mg	U/L
Biofilter I	1	330	5	5	60	0.33 ± 0.18	3.53 ± 1.87	4081 ± 815	1020 ± 203
	2	600	5	5	60	1.26 ± 0.24	13.13 ± 2.5	3210 ± 950	802 ± 237
	3	800	5	5	60	2.08 ± 0.21	21.74 ± 2.18	3740 ± 739	934 ± 184
	4	400	7.5	5	60	2.55 ± 0.16	26.57 ± 1.66	4688 ± 605	1172 ± 151
	5	400	2.5	5	60	2.20 ± 0.34	22.92 ± 3.54	3780 ± 723	945 ± 180
Biofilter II	1	330	5	5	60	0.37 ± 0.22	3.87 ± 2.29	3863 ± 722	965 ± 180
	2	600	5	5	60	1.29 ± 0.41	13.52 ± 4.27	3247 ± 461	811 ± 115
	3	800	5	5	60	2.17 ± 0.33	22.63 ± 3.43	3274 ± 651	818 ± 162
	4	400	5	3.5	60	2.37 ± 0.18	24.72 ± 1.87	2641 ± 306	660 ± 76
	5	400	5	6.5	60	2.47 ± 0.29	25.75 ± 3.02	2312 ± 498	578 ± 124
Biofilter III	1	330	5	5	60	0.38 ± 0.16	3.96 ± 1.66	3049 ± 733	762 ± 183
	2	600	5	5	60	1.36 ± 0.20	14.20 ± 2.08	3255 ± 534	813 ± 133
	3	800	5	5	60	2.25 ± 0.27	23.51 ± 2.81	4081 ± 697	1020 ± 174
	4	400	5	5	60	2.37 ± 0.19	24.68 ± 1.97	4210 ± 648	1052 ± 162
	5	400	5	5	120	1.28 ± 0.29	13.36 ± 3.02	3300 ± 422	825 ± 105
	6	400	5	5	45	1.00 ± 0.21	10.42 ± 2.18	3240 ± 501	810 ± 125

MLR: Methanol Loading Rate
EBRT: Empty Bed Residence Time
U/L : Enzymatic Units per Liter

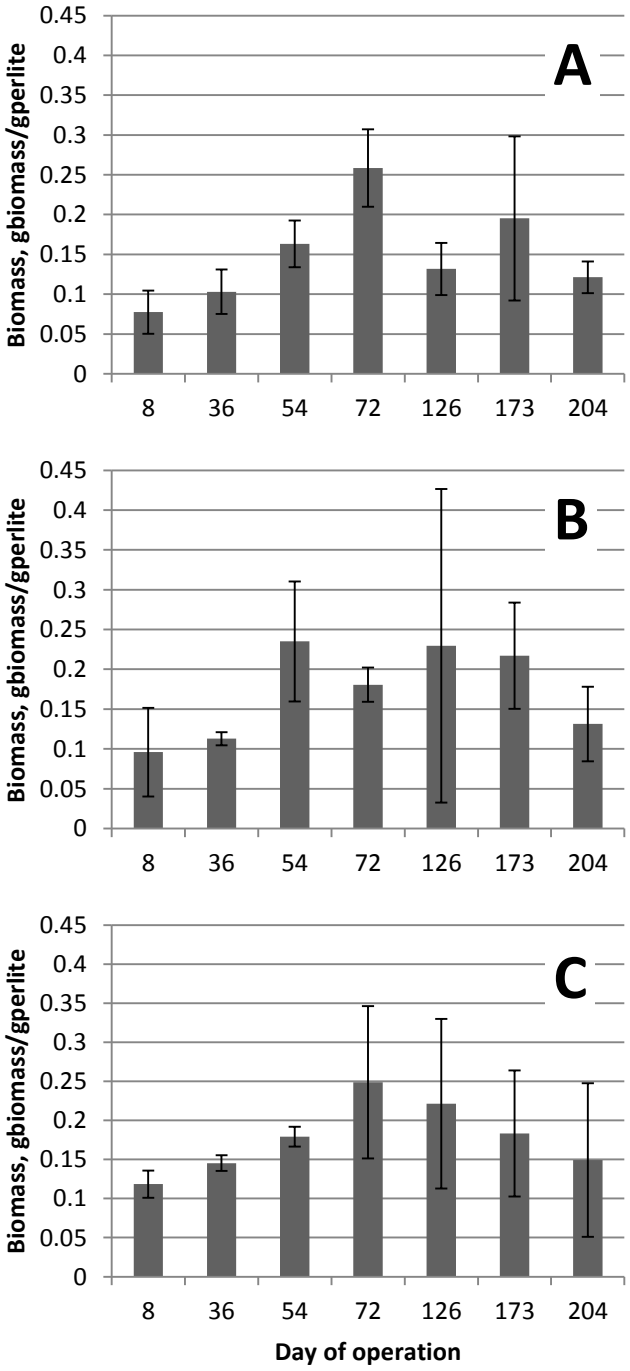

Figure 3. Biomass density in A) biofilter I, B) biofilter II, and C) biofilter III.

Alternatively, if biomass is considered, it is possible to calculate the microbial methanol elimination capacity, which is the amount of methanol degraded per gram of biomass per hour. In this case, it was possible to see that such specific capacity was not affected by the increase of nitrogen (see Annex IV).

Between Days 150 and 166 (Stage 4b), the pattern of nutrients addition was modified. During such stage, the biofilter was sprinkled every 4 days instead of every other day. The original irrigation strategy was restored by the 167th day (Stage 4c). In this latter stage, the EC values showed by the biofilter were notably increased. At the end of Stage 4c, the EC of the system was 330±20 g/m^3*h which is quite similar to the EC obtained in stage three. As observed in Figure 2A (Day 173), such EC increase was consistent with a higher amount of biomass. Additionally, the EC based on biomass was quite higher (See Annex A7).

In the last stage, the nitrogen concentration fed into the Biofilter I was decreased to 2.5 g/L, which is equivalent to a carbon to nitrogen ratio of 37 gC/gN, approximately (see Annex III). The change was immediately reflected on the biofilter performance (Figure 1). By the end of the stage, the biofilter reached an EC of 237 g/m^3*h which was 30% lower than the EC from previous stage. However, when the amount of biomass was considered, the EC turned into 0.014 g/gC*h which is 16% higher than in the fourth stage. This behavior indicates that a higher carbon to nitrogen ratio was beneficial to the system. Also, the lower

biomass amount (Figure 2A, day 204) caused a better gas flow. Thus, methanol, nitrogen, and other nutrients were better distributed and more accessible for microorganisms.

Particularly, the optimum ammonium sulphate concentration for *P. pastoris* is 5 g/L (Plantz et al., 2006) or 7gC/gN. The values tested in this work were higher and lower than such optimum, respectively. Thus, it is logical to suggest that an ammonium concentration of 5 g/L was the most suitable condition for high EC in this study.

On the other hand, according to Table 2, the nitrogen concentration impacted on the total protein and Ech42 production. At high nitrogen loads, the protein was produced at a rate of 26.5 mg/h with 1172 U/L of enzymatic activity. On the other hand, at low nitrogen concentrations, the protein productivity dropped to 22.9 mg/h with an enzymatic activity of 945 U/L. The low nitrogen system behaviour was totally expected since nitrogen is well known as a limiting factor of protein synthesis (Song et al., 2003; Hartner and Glieder, 2006). However, when nitrogen concentration was increased, the system became limited not by nitrogen concentration but by cellular capacity, i.e. although more nitrogen is available, the cells reached a limit of production. Moreover, such results were coherent with the optimum nitrogen concentration of 5 g/L previously reported for *P. pastoris* in batch cultures (Yu et al., 2011).

pH effect

Biofilter II was operated under two different pH conditions. In Stage 4, it was operated with a pH of 3.5 while in Stage 5, the pH was 6.5. Also, methanol loading rate was 400 g/m^3*h. Beyond that, all other conditions remained constant.

As shown in Figure 1b, the shift caused a very unstable period of approximately 30 days. This phenomenon was also presented in the other two systems, indicating that the effect was not caused by the pH but probably by methanol desorption as has been identified in biofilters that decrease their inlet loads (Marek et al., 2000; Fazaelipoor et al., 2006). With time, the biofilter reached a stable EC of 263 ± 32 g/m^3*h. It means that the low pH affected negatively the performance of the system.

Moreover, the irrigation pattern was also modified. From Days 150 to 167, the biofilter was sprinkled with a frequency of 4 days instead of 2 days. Afterwards, the performance was improved reaching an EC of 341 ± 11 g/m^3*h. As in Biofilter I, the performance reached a value very close to that obtained in the first three stages. In this case, as well as described in the nitrogen effects section, the specific microbial degradation rate was stimulated. We hypothesized that the change in irrigation pattern acted as a stress factor that triggered the strength-

ening of microbial community, which was reflected when suitable conditions were restored. Such phenomenon has been previously described for microbial populations subjected to starvation periods (Finkel and Kolter, 1999; Gresham, et al., 2008). Nevertheless, this field has been poorly explored in biofilters and more evidence should be generated.

In Stage 5, the biofilter showed a decrease on the elimination capacity (EC=269g/m^3*h). However, the microbial activity was increased (Figure A9). Thus, the higher pH represents a more suitable environment not only for *P. pastoris* but also for other microorganisms that could be present in the system. This finding is consistent with a previous report of biofiltration of formaldehyde and methanol (Prado et al., 2006) where it was found that better results could be obtained at pH values around 7.5. It's also known that formaldehyde dehydrogenase, which is a key enzyme in methanol degradation, is more active at circumneutral pH values (Kato et al., 1983).

On other hand, pH value was the most important parameter affecting heterologous protein production. Protein activity in stages 4 and 5 decreased to values of 660 U/L and 578 U/L, respectively. Thus pH of 5.5 was the most favorable for enzymatic synthesis. An optimum pH close to 5.5 has been also found for the expression of other proteins in *P. pastoris* (Thiruvengadam et al., 2011; Gao et al., 2012). Nevertheless, reasons for such behavior are still unclear and should be approached in future studies.

EBRT effect

The third biofilter was used to evaluate the EBRT effects on methanol biofiltration performance as well as on protein productivity. During the fifth stage, the biofilter was fed with a methanol loading rate of 400 g/m^3*h and at an EBRT of 120s. Under such conditions, the EC was 261±27 g/m^3*h, which was equivalent to 70% of the EC reached in stage 4 (Methanol load =400 g/m3*h; EC=367±15 g/m^3*h).

At high EBRT values, microorganisms get more time to degrade the pollutant, which is reflected as an increase of the EC and efficiency. However, the system reported in this study was not improved under such conditions. A probably cause is anew the low resilience of the system and also the higher methanol concentration, which is necessary to maintain the inlet load in 400 g/m^3*h.

Moreover, in the last stage, the biofilter performance dropped until reaching an EC of 143±26 g/m^3*h. This efficiency was the lowest among all stages in all biofilters. The main reason is the abrupt decrease on time for methanol degradation.

On the other hand, protein production was highly impacted by the EBRT of the system. As shown in Table 2, protein productivity at an EBRT of 120s was 13.36mg/h. Enzymatic activity was also lower (825 U/L) but still close to the average registered in this same biofilter during the first three stages (865 g/m^3*h) . In the last stage (EBRT=45s) the system showed similar response, the protein productivity was 10.42mg/h and the enzymatic activity 810 U/L. This behavior is probably linked to the quite lower EC values reported.

Carbon balance

Carbon balance was developed based on total organic carbon fed to the biofilters in first three stages. Biomass growth, CO_2 production, and carbon in leachates were considered. The results showed that around 70% of carbon degraded in the biofilters was transformed into CO_2. Also around 10% went into biomass, 2.5% into the leachate and the rest was lost in gas phase. A previous study developed by Arriaga et al., (2012) showed similar carbon distribution; nevertheless, our study reached a higher performance.

Table 3. Carbon distribution.

Total Methanol Eliminated, gC	217.3	222.6	209.8
CO_2 (%)	69.0	69.3	72.0
Biomass in the support (%)	10.1	10.7	14.7
Dissolved carbon (%)	1.7	1.6	2.3
Biomass detached (%)	0.6	0.5	0.6
By-products in gas phase (%)	18.7	17.6	10.4

gC= grams of carbon

SUMMARY AND PERSPECTIVES

It was showed that methanol EC of the coupled system was affected by four operational parameters in the following order: methanol load, pH, EBRT and ammonium sulfate concentration, being methanol load the most important while ammonium sulfate concentration being the lowest.

On the other hand, aforementioned operational conditions affected the Ech42 production in the following order: pH, methanol load, EBRT, and ammonium sulfate concentration, being pH the most important parameter and ammonium sulfate concentration being the lowest.

It is worth to remark that EC and Ech42 production were not directly related each other, and therefore, they were not affected in the same way by the experimented changes. For instance, pH importance was higher for Ech42 recovery than for methanol EC. Particularly, this work was developed from an environmental perspective, where

methanol EC was prioritized above Ech42 production. However, industrial perspective requires the profit to be as high as possible, sometimes regardless the environmental impact. Thereon, two different alternatives are suggested to maximize the Ech42 production without jeopardizing methanol degradation. The first one includes sterilization of the nutrimental solution fed to the reactor, sanitization of the air flow entering the system, and keeping the biofilter column closed to foreign microorganisms. The second option implies the separation of the processes into a bio-scrubber and a biofilter. In the bio-scrubber, the growth of *P. pastoris* would be well controlled prioritizing the Ech42 production. On the other hand, the biofilter operation would be focused on the complete degradation of methanol.

Furthermore, it is well known that biofiltration coupled with heterologous protein production using *P. pastoris* is a promising alternative for methanol treatment. However several tasks must be addressed before an industrial implementation. A couple of examples are the study of the effects of hydrogen sulfide (commonly issued with methanol) on the performance of the system, and the robustness evaluation of the *P. pastoris* biofilm.

CONCLUSIONS

A whole analysis of the EC in all biofilters let us to note that biofiltration coupled with heterologous protein production is a very sensitive process. Nonetheless, from the protein production point of view, the system was quite stable. This singular behavior makes this technology a promising alternative for damping air treatment costs through the synthesis of valuable proteins. However, more research should be done in order to prevent it decreases in methanol degradation capacity.

In this report it was observed that an optimal nitrogen concentration of 5g $(NH_4)_2SO_4$/L should be maintained in order to avoid operational problems. Also, it was found that a pH of 5 is suitable for high ECs as well as high enzymatic activities. Moreover, the EBRT of 60 second was appropriate to reach a good performance. Finally, the biofilters showed that the methanol inlet loading rate does not play an important role in the process.

REFERENCES

Aizemberg, R., Terrazas, W., Ferreira-Dias, S., Valentini, S., Gattas, E. (2011). Optimal conditions for biomass and recombinant glycerol kinase production using the yeast *Pichia pastoris. Food Technology and Biotechnology,* 49 329-335.

Arriaga, S., Acosta-Munguía, A., Barba de la Rosa, A.P., Pérez-Martínez, A.S., De León-Rodríguez, A. (2010). Coupling aerobic biodegradation of methanol vapors with heterologous protein expression of endochitinase Ech42 from Trichoderma atroviride in *Pichia pastoris*. *Bioresource Technology,* 101, 9661–9665.

Arriaga, S., Serrano, M., Barba de la Rosa, A. (2012). Methanol vapor biofiltration coupled with continuous production of recombinant endochitinase Ech42 by *Pichia pastoris*. *Process biochemistry*, 47, 2311-2316.

Arulneyam D., Swaminathan T. (2003). Biodegradation of methanol in a biofilter. *Journal of Environmental Sciences*, 15 691-696.

Auria, R., Aycaguer, AC., Devinny, J. (1998). Influence of water content on degradation rates for ethanol in biofiltration. *Journal of the Air and Waste Management Association*, 48 65-70.

Babbitt, C.W., Lindner, A.S. (2011). Effect of nitrogen source on methanol oxidation and genetic diversity of methylotrophic mixed cultures enriched from pulp and paper mill biofilms. *Bodegradation*, 22 309-320.

Babbitt, C.W., Pacheco, A., Lindner, A.S. (2009). Methanol removal efficiency and bacterial diversity of an activated carbon biofilter. *Bioresource Technology*, 100, 6207-6216.

Barcón T, Alonso-Gutiérrez J, Omil F. (2012). Molecular and physiological approaches to understand the ecology of methanol degradation during the biofiltration of air streams. *Chemosphere*, 87 1179-1185.

Bohn, H., Bohn, K. (1999). Moisture in biofilters. Environmental progress, 18 156-161.

Chetpattananondh, P., Nitipavachon, Y., Bunyacan, C. (2005). Biofiltration of air contaminated with methanol and toluene. *Journal of Science and Technology*, 27 761-773.

Clean Air Act, 1990. Signed into law by President Lyndon B. Johnson on December 17, 1963. USA. Available on http://www.epw.senate.gov/envlaws/cleanair.pdf.

Cornabé, T., Auria, R., Christen, P., Ferrer, H., Revah, S., Moukha, S.,(2002). *USC-TRG Conference on Biofiltration*. Los Angeles CA, EU.

Dastous, P.A., Nikiema, J., Soreanu, G. (2008). Elimination of methanol and ethanol by biofiltration: an experimental study. *Water Air and Soil Pollution: Focus*, 8, 275-285.

Diaz, S., Cabido, M. (2001). Vive la différence: plant functional diversity matters to ecosystem processes. *TRENDS in Ecology and Evolution*, 16 646-655.

Fazaelipoor, M., Shojaosadati, S., Farahani, E. (2006). Two liquid phase biofiltration for removal of n-hexane from polluted air. *Environmental Engineering Science*, 23 954-959.

Finkel, S., Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation. *Proccedings of the National Academy of Sciences*, 96 4023-4027.

Gao, Z., Li, Z., Zhang, Y., Huang, H., Li, M., Zhou, L., Tang, Y., Yao, B., Zhang, W. (2012). High-level expression of the *Penicillium notatum* glucose oxidase gene in *Pichia pastoris* using codon optimization. *Biotechnollogy Letters*, 34 507-514.

Goodman, D. (1975). The theory of diversity-stability relationship in ecology. *The Quarterly Review of Biology*, 50 237-266.

Gresham, D., Desai, M., Tucker, C., Jenq, H., Pai, D., Ward, A., DeSevo, C., Botstein, D., Dunham, M. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLOS Genetics*, 4 19pp.

Gribbins MJ, Loehr RC (1998) Effect of media nitrogen concentration on biofilter performance. *Journal of Air Waste Management Association*, 48 216–226

Hartner, F., Glieder, A. 2006. Regulation of methanol utilization pathway genes in yeast. *Microbial cell factories*, 5 39-60.

Health Effects Institute (1999). Reproductive and offspring developmental effects following maternal inhalation exposure to methanol in non-human primates. Report #89, *HEI Reports.*

Huang, Y., Martin, L.M., Isbell, F.I., Wilsey, B.J. (2013). Is community persistence related to diversity? A test with prairie species in a long-term experiment. *Basic and Applied Ecology*, 14 199-207.

Kato N., Shirakawa K., Kobayashi H. and Sakazawa C. (1983). The Dismutation of Aldehydes by a Bacterial Enzyme. *Agricultural and Biological Chemistry*, 47 39-46.

Marek, J., Paca, J., Gerrard, A. (2000). Dynamic responses of biofilters to changes in the operating conditions in the process of removing toluene and xylene from air. *Acta Biotechnologica,* 20 17-29.

Morales, M., Hernández, S., Cornabé, T., Revah, S., Auria, R. (2003). Effect of drying on biofilter performance : modeling and experimental approach. *Environmental Science and Technology*, 37 985-992.

Moreno-Terrazas, R., Flores-Tena, F. J., Barbara-Avila, M. D., Guerrero-Barrera, A. L., Avelar-Gonzalez, F. J., Ramirez-Lopez, E. M. (2010). *World Journal of Microbiology and Biotechnology*, 26, 657-664.

Negruta O., Csutak O., Stoica I., Rusu E., Vassu T. (2010). Methylotrophic yeasts: diversity and methanol metabolism. *Romanian Biotechnological Letters*, 15 5369-5375.

Pérez-Martínez, A.S., León-Rodriguez, A., Harris, L.J., Herrera-Estrella, A., Barba de la Rosa, A.P. (2007). Overexpression, purification and characterization of the trichoderma atroviride endochitinase, Ech42, in *Pichia pastoris*. *Protein Expression and Purification*, 55 183-188.

Plantz, B., Sinha, J., Villarete, L., Nickerson, K., Schlegel, V. (2006). *Applied Microbiology and Biotechnology*, 72 297-305.

Potvin, G., Ahmad, A., Zhang, Z. (2012). Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochemical Engineering Journal*, 64 91-105.

Prado, O.J., Veiga, M.C., Kennes, C. (2006). Effect of key parameters on the removal of formaldehyde and methanol in gas phase biotrckling filters. *Journal of Hazardous Materials*, B138 543-548.

Ramirez-López, E. M., Corona-Hernandez, J., Avelar-Gonzalez, F.J., Omil, F., Thalasso, F. (2010). *Bioresource Technology*, 101, 87-91.

Rughoonundun, H., Mohee, R., Hotzapple, M. (2012). Influence of carbon-to-nitrogen ratio on the mixed-acid fermentation of wastewater sludge and pretreated bagasse. *Bioresource Technology*, 112 91-97

Rumjantsev, A., Padkina, M., Sambuk, E. 2013. Effect of nitrogen source on gene expression of first steps of methanol utilization pathway in P. pastoris. *Russian Journal of Microorganisms*, 49 394-400.

Sakuma, T., Hattori, T., Deshusses, M. (2009). The effects of a lower irrigation system on pollutant removal and on the microflora of a biofilter. *Environmental Technology*, 30 621-627.

Sologar VS, Lu Z, Allen DG. (2003). Biofiltration of concentrated mixtures of hydrogen sulfide and methanol. *Environmental Progress*, 22 129-136.

Thiruvengadam, G., Init, I., Fong, M., Lau, Y. (2011). Optimization of the expression of surface antigen SAG1/2 of *Toxoplasma gondii* in the yeast *Pichia pastoris*. *Tropical Biomedicine,* 28 506-513.

Tscharntke, T., Klein, A., Kruess, A., Steffan-Dewenter, I., Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity – ecosystem service management. *Ecology letters*, 8 857-874.

Yu, P., Yan, Y., Tang, Y. (2011). Medium optimization for Endochitinase production by recombinant *Pichia pastoris* ZJGSU02 using response surface methodology. *African Journal of Biotechnology*, 10 75-84.

Zilli, M., Nicolella, C. (2011). Removal of monochlorobenzene from air in a trickling biofilter at high loading rates. *Journal of chemical technology and biotechnology*, 87 1141-1149.

OPERATIONAL DIVERGENCE IN BIOFILTERS TREATING METHANOL VAPORS

Palomo-Briones Rodolfo, Arriaga Sonia

IPICYT, Instituto Potosino de Investigación Científica y Tecnológica, División de Ciencias Ambientales. Camino a la Presa San José 2055, Lomas 4ta. Sección, C. P. 78216, San Luis Potosí, México. Tel.: +52 444 834 2000; fax: +52 444 834 2010.

ABSTRACT

Biofiltration is a technology widely used to remove organic and inorganic pollutants from waste airstreams. It consists of a packed column containing a microbial community immobilized on a porous material. When the air flows through the column, the pollutant is taken by the microorganisms and CO_2, H_2O and biomass are produced. Several studies have been conducted on the biofiltration field and due to multiple reasons system's repeatability is rarely considered. In this study we report the performance differentiation of three identically operated biofilters inoculated with a pure strain. The statistics indicates that biofilters became different with time. Furthermore, microscopic analysis suggests that the phenomenon could be due to random colonization of airborne microbial species. The primary reasons are still unknown; nevertheless, it's clear that the system of study is divergent rather than repeatable.

INTRODUCTION

Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces (Costerton et al., 1995). The structure of a biofilm constitutes a protected mode of growth that allows survival under hostile conditions. It provides a widely spectrum of suitable microenvironments where microorganisms can live and interact (Costerton et al., 1999, Poltak and Cooper, 2011).

Biofilms are very dynamic structures where several interactions can take place (Costerton et al., 1999; Stoodley et al., 2002; Poltak and Cooper, 2011; Moormeier et al., 2013; Wright et al., 2012; Schlisselberg and Yaron, 2013). In addition, its evolution is a randomly divergent process that is difficult to predict (Lensky et al., 1991; Lenski and Travisano, 1994). So, neither repeatability nor stability is a characteristic of biofilms and must not be taken for granted. However, in biofiltration it seems to be the opposite.

Biofiltration is a technology widely investigated for the treatment of numerous atmospheric pollutants. It is a biological process where the contaminant flows through a column packed with a porous material. Different microor-

ganisms are attached and growing on the material surface. Consequently, when the pollutant is transferred from the airstream to the biofilm, microorganisms can degrade it. As a result of such biological activity, CO_2, H_2O and biomass are produced. CO_2 is transported back to the airflow while the newly biomass is retained inside the biofilter increasing the total degradation capacity of pollutants.

Biofiltration is considered to be a repeatable process that reaches stability within few days. Apparently, the dynamism of biofilms in biofilters is not reflected on its efficiency. In fact, it has been shown that different biofiltration microbial communities are able to reach equal degradation capacities (Cabrol et al., 2012). Thus, microbial dynamics as well as its repeatability have been poorly explored.

Recently, a new kind of biofiltration has been reported (Arriaga et al., 2012). The novel system couples biofiltration with the production of heterologous proteins. An important feature of this process is that, at the very beginning of the operation, there is only one strain in the biofilter. Eventually, the packing material could be colonized by airborne species creating a completely new ecosystem with its particular networks of interaction. It finally leads us to the principal question posed on this work: would it be repeatable?

In this study, we focus on the evaluation of system divergence. We examine the behavior of three identically operated biofiltration columns in order to determine whether their evolution and performance are identical. Otherwise, it should be necessary to propose new strategies for its operation.

MATERIALS AND METHODS

Inoculum

A *P. pastoris* strain transformed with the plasmid pPIC-ech42, which contains the 42 kDa endochitinase gene from *T. atroviride*, was previously obtained (Perez-Martinez et al., 2007). Recombinant *P. pastoris* was grown in 5 mL of yeast extract, peptone, dextrose medium (YPD) plus 250 μg/mL of Geneticin (G418 sulfate, PhytoTechnologies Laboratories, USA) at 28 C overnight (Arriaga et al., 2010).

Culture was centrifuged for 5 min at 13000g and the resulting cell pellet was resuspended in 500 mL of fresh mineral medium supplemented with methanol. The flasks were incubated during 48h at 28 C and 250 rpm. Afterwards, the biofilters were sprinkled with the resulting media.

Biofilter setup

Three identical biofilters were manufactured with an internal diameter of 8.9 cm and an effective height of 54 cm, thus the total volume was 3.3L. The columns were equipped with multiple sampling ports to measure gas and biomass. Perlite was selected as the packing material in order to avoid bed compression as well as to eliminate an additional nutrimental source (Arriaga et al., 2012).

Methanol feeding strategy was integrated by three consecutive stages. In the first stage methanol was fed at a concentration of 5.5 g/m^3*h. During the second stage methanol inlet concentration was 10 g/m^3. Finally, in the third stage, methanol concentration was increased to 13.3 g/m^3. An empty bed retention time (EBRT) of 1 min was kept during the whole experiment.

Analytical Methods

Gas samples were taken at 0, 18, 36 and 54 cm from the inlet every other day. Methanol concentration was determined through gas chromatography (Agilent Technologies, CA, USA) coupled with a flame ionization detector. Nitrogen was used as carrier gas at a flow of 25 mL/min. Further, the temperatures of the injector, oven and detector were 150, 80 and 250 C, respectively.

SEM Images were taken from biomass samples. Such samples were dried and fixed onto a conductive support using a carbon adhesive tape. Also, the fixed samples were covered with gold layer according to standard procedures. The scanning electron microscope (XL 30 SFEG, Philips, Ámsterdam, Netherlands) was operated at 86 pA and 5 kV.

Statistics

Pseudo steady states were compared each other among the different stages operated. One-way ANOVA was used to compare the ECs among the different biofilters along the three stages reported on this work. Furthermore, t-test was carried out in order to contrast biofilters one by one. Specialized statistical software was employed with such purposes (STATISTICA, Statsoft, USA).

Monod Kinetics

In order to predict the EC behavior of the systems, data were adjusted to a Monod kinetic equation where the term of biomass decay was neglected:

$$EC = EC_{max} \frac{ILR}{k_s + ILR}$$

where EC_{max} is the theoretical maximum EC, ILR the inlet loading rate and k_S the half saturation constant. Monod equation has shown to be an adequately description of biofiltration systems (Gallastegui et al., 2011; Ávalos-Ramirez et al., 2008; Romero et al., 2013).

RESULTS AND DISCUSSION

Biofilter performance

Three identical biofilters inoculated with *Pichia pastoris* were operated 82 days (Table 1). During the first 28 days, the Biofilters I, II, and III reached ECs of 274±24, 280±27, and 280±28 g/m^3*h, respectively. As shown by the concentration profile (Figure 1A), the performance of the three systems was quite similar. Statistical analysis confirmed that biofiltration performances were technically identic (Table 2). Such fact was actually expected given that the startup procedures as well as the operation were the same in all biofilters.

Table 1. Summary of pseudo steady state methanol removal.

			Section 1	Section 2	Section 3	Total
BIOFILTER I	First Stage	Average	93	132	49	274
		SD	34	47	12	24
	Second Stage	Average	98	122	78	297
		SD	9	10	10	9
	Third Stage	Average	44	160	127	331
		SD	8	7	4	2
BIOFILTER II	First Stage	Average	110	123	47	280
		SD	36	28	17	27
	Second Stage	Average	108	123	106	338
		SD	6	2	11	7
	Third Stage	Average	177	161	125	462
		SD	6	4	3	1
BIOFILTER III	First Stage	Average	97	141	42	280
		SD	22	41	20	28
	Second Stage	Average	103	143	95	341
		SD	6	3	6	2
	Third Stage	Average	396	103	144	644
		SD	24	15	6	5

First stage: Methanol loading rate, 330 g/m^3*h.
Second stage: Methanol loading rate, 600 g/m^3*h.
First stage: Methanol loading rate, 800 g/m^3*h.
SD: Standard deviation
Values are expressed in g/m^3*h

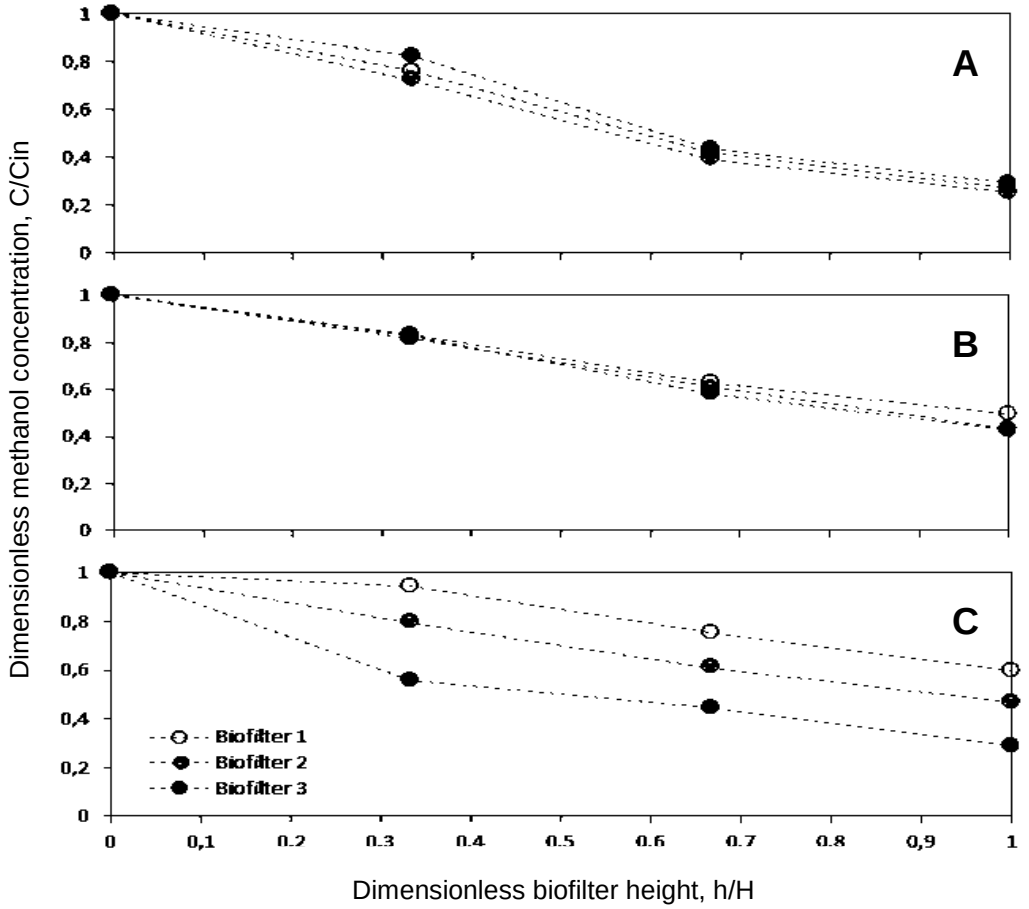

Figure 1. Profiles of methanol concentrations along biofilters for stages A) 1 *1-28 days* 1, B) 2 *29-52 days*, and C) 3 *53-82 days*.

In this first stage, 24, 28 and 18% of methanol was degraded within the first 18cm of the columns in Biofilters I, II, and III, respectively. In the second section (18-36 cm), 35, 33 and 39% of methanol was eliminated. Finally, in the last third (36-54cm) 15, 14 and 14% of methanol was degraded.

The profile showed that biofilters experienced a stratification phenomenon along the biofilter height. As observed, methanol degradation capacity was higher in the middle, while the bottom section had the lowest efficiency. This phenomenon was probably originated by the low methanol inlet concentration of this section which impacted negatively in methanol degradation. Nevertheless the upper section, which has the highest methanol inlet concentration, did not present the maximum efficiency. This is probably because of poor moisture conditions close to airflow inlet. Indeed, drying of the packing material in the upper section is a commonly found problem in biofiltration systems that operated in a down flow mode (Auria et al., 1998; Bohn and Bohn, 1999; Morales, et

al., 2003; Sakuma et al., 2009). The moisture degree was not determined in this study but it was visually confirmed.

After day 29 and until day 52, methanol inlet concentration was increased. The system capacities in this second stage were markedly higher but the efficiencies were much lower (Table 1). The methanol profile seemed to be pretty similar (Figure 1B). Nevertheless, statistical analysis showed clearly evidence of divergent performances. t-test confirmed that the Biofilter I was statistically different from the others ($p<0.05$)

The degradation percentages reached by the first section were 17, 19 and 18%, in Biofilters I, II and III, respectively. In the middle sections (18-36 cm), 20, 20 and 24% of methanol degradation was achieved. Lastly, 14, 18 and 16% of methanol was degraded in the last third of biofiltration columns. The total ECs found in Biofilters I, II and III were 297±9, 338±7, 341±2 g/m^3*h, respectively.

From day 53 until the end of the experiment, methanol concentration was increased to 13.3 g/m^3. Methanol profiles clearly show that the performance among biofilters was completely different. Within the first 18 cm of the biofilter, the efficiency was around only 6% in reactor I, while 21% and 44% were observed in Biofilters II and III, respectively. From this data, it's possible to indicate that the first sections of biofilters were greatly affected by the change on inlet concentration.

Table 2. ANOVA analysis on the pseudo steady state efficiencies. *P* values lower than 0.05 indicate significant difference among biofilters.

		SS	Degrees of freedom	MS	F	P
Stage 1	Biofilter	97,1	2	48,5	0,0217	0,97
	Error	26813,8	12	2234,5		
Stage 2	Biofilter	5967	2	2983	65,89	3 E-07
	Error	543	12	45		
Stage 3	Biofilter	246959	2	123480	10957,7	0,00
	Error	135	12	11		

Stage 1: Methanol loading rate, 330 g/m^3*h.
Stage 2: Methanol loading rate, 600 g/m^3*h.
Stage 3: Methanol loading rate, 800 g/m^3*h.

Furthermore, in the second section of the biofilters the efficiencies were 19, 18 and 12% while in the third section, 16, 15 and 16% of methanol degraded. Total methanol EC in this last stage was equal to 331±2, 462±1, 644±5 g/m^3, in Biofilters I, II and III, respectively. Statistical analysis proved that biofilters' behaviors became completely divergent from each other ($p<0.05$).

Multiple reasons could be given to explain such phenomena. Some of them are related to the manipulation of biofilters, the composition of mineral media, the bed structure, etc. However, assuming that those experimental errors could not lead the systems to such different scenarios, the most logical explanation is focused on the colonization and evolution processes.

The colonization (microbial contamination) is a process poorly understood and difficult to predict (Lensky et al., 1991; Lenski and Travisano, 1994; Cabrol et al., 2012). Notwithstanding, it is known that two principal conditions should be reached for a microorganism to colonize: attachment and persistence.

Following the Yao's granular filtration model (Yao et al., 1971), there are three different mechanisms by which microorganisms can be attached to a packing particle: interception, inertia and diffusion. On the other hand, microorganisms also need to find the appropriate micro environmental conditions (e.g. pH, temperature, nutrients availability, etc) in order to subsist and successfully establish a colony. If one of the requirements is not achieved, then the colonization will fail.

Once the colony is established, it can evolve in different ways. All depends on changes of the local environment and interactions with other species. Unfortunately, such micro-environmental changes as well as microbial interactions are, up today, unpredictable. Therefore, the evolution of a biofilm is by the moment a randomly divergent process.

SEM analysis

SEM images were obtained from the support samples of the first section in all different biofilters. At the end of first stage (Figure 2A,B,C) the majority of microorganisms found in the support were morphologically similar to *P. pastoris* with a cell size between 2 and 4 μm (Terpitz et al., 2012). Such visible predominance of *P. pastoris* could be due to the inoculation strategy. When *P. pastoris* cells were added to the biofilters, they rapidly colonized the material surface and no opportunity to other microorganisms was given. Moreover, biofilm structure was noticeable composed by cells as well as adherent material (exopolysacharides, EPS).

After the second stage, biofilm structure became more complex in Biofilters II and III (Figure 2E,F). The biofilm was visually wider and some foreign microorganisms were distinguished. Nonetheless, the biofilm from Biofilter I (Figure 2D) was apparently very similar to first stage.

The biofilms after third stage were completely different from those of the first and second phases. Their structures were highly complex and wide. In all cases, abundant EPS was found as well as foreign species. Additionally, interesting differences were observable among the biofilters.

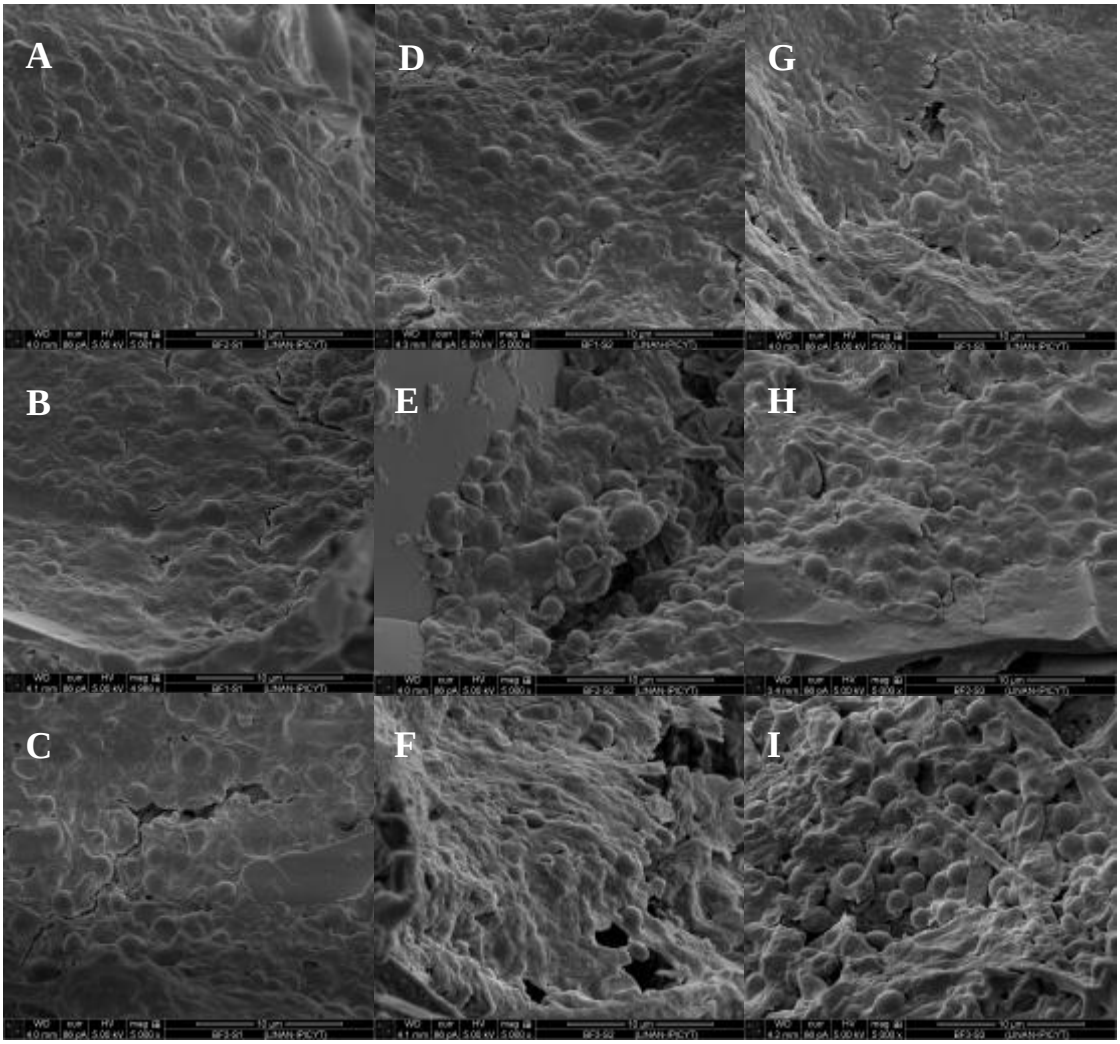

Figure 2. SEM images from the first stage of three biofilters. A) Biofilter I - stage 1, B) Biofilter II - stage 1, C) Biofilter III - stage 1, D) Biofilter I - stage 2, E) Biofilter II - stage 2, F) Biofilter III - stage 2, G) Biofilter I - st*age 3, H) Biofilter II - stage 3, I) Biofilter III - stage 3.*

The biofilm sample taken from Biofilter I was the most uniform and the least microbially invaded (Figure 2G). Such feature could be responsible of its low resistance at high methanol concentrations. Without a strong biofilm layer protecting the cells, the microenvironment could become hostile and poor performance would result. On

the other hand, the biofilm taken from Biofilter II was markedly wider (Figure 2H) and no other relevant change was noted. This confirms that width is an important characteristic of resistant biofilms. However, experiments should be performed in order to validate such hypothesis. Moreover, the biofilm taken from Biofilter III showed the highest complexity (Figure 2I). Plenty EPS and biomass as well as different foreign microorganisms were observed. Probably, the presence of other different microorganisms was ligated to the higher removal efficiency reported. Nonetheless, more studies such as microbial identification are needed.

Monod Kinetics

The steady-state experimental data were adjusted to a Monod kinetic in order to identify kinetic differences among biofilters (Figure 3). Estimated parameters showed that the system with the maximum EC was Biofilter III, which EC increase according to the inlet loading rate up to a maximum EC of 2172 g/m^3*h. Similar predictions has been obtained for methanol biofiltration (Avalos-Ramirez et al., 2008). In such study as well as in ours, methanol degradation was not inhibited even at very high loading rates. Additionally, Ks values obtained for Biofilter I and II were markedly higher than to those reported in literature (Avalos-Ramirez et al., 2009). Additionally, there is not any report with a Ks value as high as the obtained in the Biofilter III.

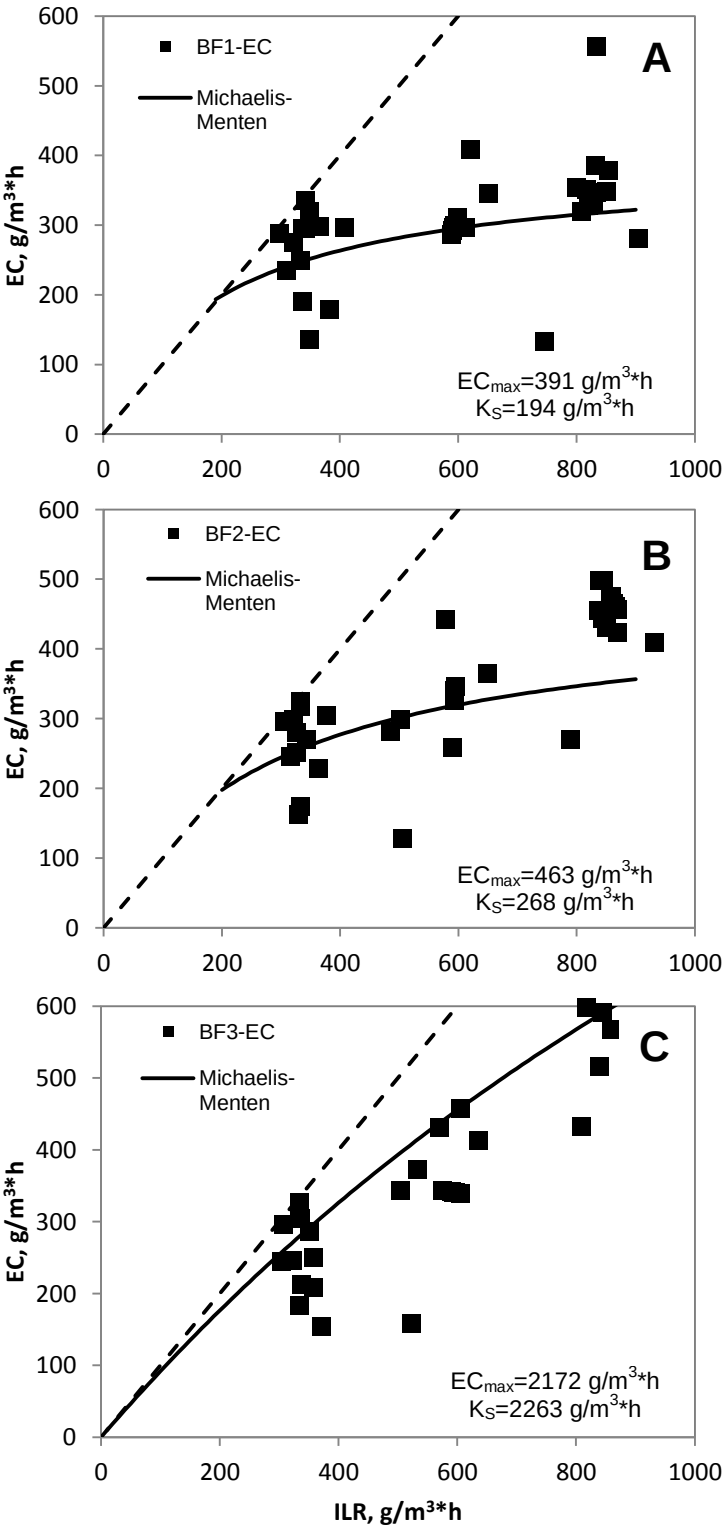

Figure 3. Monod kinetics. A) Biofilter I; B) Biofilter II; C) Biofilter III. Dotted line represents 100% efficiency.

The magnitude of aforementioned ECmax can be used for designing, but only if diffusional problems caused by biomass accumulation remain negligible. The model also predicted a maximum EC of 391 g/m^3*h for Biofilter I and 462 g/m^3*h for Biofilter II. Those results are in the range of previously reported maximum ECs for methanol biofiltration systems (Shareefden et al., 1993; Mohseni and Allen, 2000; Arulneyam and Swaminathan, 2003; Chetpattananondh et al., 2005; Ramirez-Lopez et al., 2010). However, they are very low compared with predictions in Biofilter III showing that biofilters differences could have originated by kinetic differences.

Moreover, the experimental data fitted quite well with the model indicating that Monod equations were appropriate for describing the systems. In addition, according to Figure 3, all biofilters were limited by the microbial degradation rate which means that no diffusional problem was presented. Such finding is consistent with the high methanol concentrations used.

CONCLUSIONS

Three biofilters were operated under identical conditions, and it was demonstrated that they became different with time. The mechanism of such divergence is still poorly understood. Here we suggested that the key of this phenomenon could be related to microbial evolution and foreign microorganisms' colonization. Such phenomena are also a cause of different degradation kinetics, which were approached through Monod equations.

Nowadays, aforementioned phenomena seem to be unnoticed in the biofiltration researches. In most cases because the target of the systems is to effectively degrade some pollutant regardless of the microbial species involved. However, research in community evolution in biofiltration processes would be useful to have a better control over the system. Also, the investigation of colonization mechanisms would give tools needed to avoid microbial contamination in biofilters. Therefore, it is necessary to begin exploring deeper in the biofiltration black box.

REFERENCES

Arriaga, S., Acosta-Munguía, J., Pérez-Martínez, S., De León-Rodríguez, A., Barba de la Rosa, A. (2010) Coupling aerobic biodegradation of methanol vapors with heterologous protein expression of endochitinase Ech42 from *Trichoderma atroviride* in *Pichia pastoris Bioresource Technology*, 101 9661-9665.

Arriaga, S., Serrano, M., Barba de la Rosa, A. (2012) Methanol vapor biofiltration coupled with continuous production of recombinant endochitinase Ech42 by *Pichia pastoris*. *Process Biochemistry*, 47 2311-2316.

Arulneyam D., Swaminathan T. (2003). Biodegradation of methanol in a biofilter. *Journal of Environmental Sciences*, 15, 691-696.

Auria, R., Aycaguer, AC., Devinny, J. (1998). Influence of water content on degradation rates for ethanol in biofiltration. Jo*urnal of the Air and Waste Management Association*, 48 65-70.

Ávalos-Ramirez, A., Benard, S., Giroir-Fendler, A., Peter, J., Heitz, M. (2008). Kinetics of microbial growth and biodegradation of methanol and toluene in biofilters and an analysis of the energetic indicators. *Journal of Biotechnology*, 138 88-95.

Avalos-Ramirez, A., Benard, S., Giroir-Fendler, Al, Jones, J., Heitz, M. (2008). Kinetics of microbial growth and biodegradation of methanol and toluene in biofilters and an analysis of the energetic indicators. *Journal of Biotechnology*, 138 88-95.

Bohn, H., Bohn, K. (1999). Moisture in biofilters. *Environmental progress*, 18 156-161.

Cabrol, L., Malhautier, L., Poly, F., Lepeuple, A.S., Fanlo, J.L. (2012). Bacterial dynamics in steady-state biofilters: beyond functional stability. *FEMS Microbiology Ecology*, 79 260-271.

Chetpattananondh, P., Nitipavachon, Y., Bunyacan, C. (2005). Biofiltration of air contaminated with methanol and toluene. *Journal of Science and Technology*, 27, 761-773.

Costerton, J., Stewart, P., Greenberg, E. (1999) Bacterial biofilms: A common cause of persistent infections. *Science*, 284 1318-1322.

Gallastegui, G., Ávalos-Ramirez, A.,Elías, A., Jones, J., Heitz, M. (2011). Performance and macrokinetic analysis of biofiltration of toluene and p-xylene mixtures in a conventional biofilter packed with inert material. *Bioresource Technology*, 102 7657-7665.

Hartner, F., Glieder, A. 2006. Regulation of methanol utilization pathway genes in yeast. Microbial cell factories, 5

Lenski, R., Rose, M., Simpson, S., Tadler, S. (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *The American Naturalist*, 138 1315-1341.

Lenski, R., Travisano, M. (1994). Dynamics of adaptation and diversification: A 10,000-generation experiment with bacterial populations. *Proceedings of the National Academy of Sciences*, 91 6808-6814.

Mohseni, M., Allen, D.G. (2000). Biofiltration of mixtures of hydropilic and hydrophobic volatile organic compounds. *Chemical Engineering Science*, 55, 1545–1558.

Moormeier, D., Endres, J., Mann, E., Sadykov, M., Horswill, A., Rice, K., Fey, P., Baylesa, K. (2013) Use of microfluidic technology to analyze gene expression during *Staphylococcus aureus* biofilm formation reveals distinct physiological niches. *Applied and Environmental Microbiology*, 79 3413-3424.

Morales, M., Hernández, S., Cornabé, T., Revah, S., Auria, R. (2003). Effect of drying on biofilter performance : modeling and experimental approach. *Environmental Science and Technology*, 37 985-992.

Morgenroth, E., Schroeder, E., Chang, D., Scow, K. (1996). Nutrient limitation in a compost biofilter degrading hexane. *Journal of Air and Waste Management*, 46 300-308.

Pérez-Martínez, A., De León-Rodríguez, A., Harris, L., Herrera-Estrella, A., Barba de la Rosa, A. (2007) Overexpression, purification and characterization of the *Trichoderma atroviride* endochitinase, Ech42, in *Pichia pastoris. Protein Expression and Purification,* 55 183-188.

Poltak, S., Cooper, V. (2011) Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *The International Society for Microbial Ecology Journal*, 5 369-378.

Romero, A., Rodríguez, M., Andrés, Y., Dumont, E. (2013). Steady- and transient-state H2S biofiltration using expanded schist as packing material. *New Biotechnology*, 30 210-218.

Rumjantsev, A., Padkina, M., Sambuk, E. 2013. Effect of nitrogen source on gene expression of first steps of methanol utilization pathway in *P. pastoris*. *Russian Journal of Microorganisms*, 49 394-400.

Sakuma, T., Hattori, T., Deshusses, M. (2009). The effects of a lower irrigation system on pollutant removal and on the microflora of a biofilter. *Environmental Technology*, 30 621-627.

Schlisselberg, D., Yaron, S. (2013). The effects of stainless steel finish on Salmonella Typhimurium attachment, biofilm formation and sensitivity to chlorine. *Food Microbiology*, 35 65-72.

Shareefdeen, Z., Baltzis, B.C., Oh, Y-S., Bartha, R. (1993). Biofiltration of methanol vapor. *Biotechnology and Bioengineering*, 41, 512–524.

Song, J., Kinney, K., John, P. (2003). Influence of nitrogen supply and substrate interactions on the removal of paint VOC mixtures in a hybrid reactor. *Environmental Progress*, 22 137-144.

Stoodley, P., Sauer, K., Davies, D., Costerton, J. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology,* 56 187-209.

Terpitz, U., Letschert, S., Bonda, U., Spahn, C., Guan, C., Sauer, M., Zimmermann, U., Bamberg, E., Zimmermann, D., Sukhorukov, V. (2012). Dielectric analysis and multi-cell electrofusion of the yeast *Pichia pastoris* for electrophysiological studies. *The Journal of Membrane Biology*, 245 815-826.

Wright, C., Burns, L., Jack, A., Back, C., Dutton, L., Nobbs, A., Lamont, R., Jenkinson, H. (2013) Microbial interactions in building of communities. *Molecular Oral Microbiology*, 28 83-101.

Yao, K.-M., Habibian, M. T., and O'Melia, C. R. (1971) Water and Waste Water Filtration: Concepts and Applications. *Environmental Science and Technology*, 5 1105–1112.

Yu, P., Yan, Y., Tang, Y. (2011). Medium optimization for endochitinase production by recombinant *Pichia pastoris* ZJGSU02 using response surface methodology. *African Journal of Biotechnology*, 10 75-84.

Zilli, M., Nicolella, C. (2011). Removal of monochlorobenzene from air in a trickling biofilter at high loading rates. *Journal of Chemical Technology and Biotechnology*, 87 1141-1149.

MATHEMATICAL MODEL OF THE METHANOL BIOFILTRATION COUPLED WITH PROTEIN PRODUCTION

Palomo-Briones Rodolfo[a], Deshussess Marc[b], Arriaga Sonia[a]

[a]IPICyT, Instituto Potosino de Investigación Científica y Tecnológica, División de Ciencias Ambientales. Camino a la Presa San José 2055, Lomas 4ta. Sección, C. P. 78216, San Luis Potosí, México. Tel.: +52 444 834 2000; fax: +52 444 834 2010.

[b]Duke University, Dept. of Civil and Environmental Engineering, 127C Hudson Hall; Box 90287. Durham, NC 27708

ABSTRACT

A dynamic mathematical model for biofiltration systems coupled with the production of a heterologous protein was developed. The model was constructed from mass balances as well as growth and consumption kinetics. A set of simulations under different scenarios showed that the parameters with higher importance in the protein production are Biomass yield (Y), Maximum elimination capacity (ECmax) and biomass decay (b) while a lower importance is ascribed to half saturation constant (Ks). When compared with experimental data, the model accurately describes the elimination capacity of the system as well as its protein production. However, important differences were observed between experimental measurements and predictions of the total volume of the system. The results suggest that such differences are attributable to the presence of inactive biomass.

INTRODUCTION

Biofiltration has been studied as an effective technology for the treatment of volatile organic compounds (VOCs). In particular, biofiltration of hydrophilic pollutants like methanol has achieved very acceptable results (Arulneyam and Swaminathan, 2003; Sologar et al., 2003; Chetpattananondh et al., 2005; Dastous et al., 2008; Babbit et al., 2009; Ramirez-Lopez et al., 2010).

Recently, the future and potential of VOCs treatment has been widened due to the emergence of a novel conception of the biofiltration process (Cornabé et al., 2002; Arriaga et al., 2010; Arriaga et al., 2012). In the innovative biotechnique, an air stream contaminated with methanol flows through a porous media which supports a *P. pastoris* biofilm. The methanol in gas phase diffuses into the biofilm layer where it becomes vulnerable to biological activity. The presence of methanol inside a properly transformed *P. pastoris* cell triggers the synthesis of enzymes needed for its consumption and also for the production of a heterologous peptide. So then, it is possible to recover the valuable protein in the leachate when the biofilter is sprinkled.

To describe and predict the behavior of the methanol biofiltration coupled with protein production is necessary to develop a mathematical model able to capture all different phenomena occurring in the system. Following similar aims, many models have been conceived to describe biofiltration.

Ottengraf and Van den Oever employed a model with diffusion and first and zero-order kinetics to model a biological filter treating a mixture of VOCs (Ottengraf and Van den Oever, 1983). An extension of this model was proposed to describe methanol biofiltration (Shareefdeen et al., 1993). In this model, simplified expressions for biological degradation were replaced with those obtained from shake-flask experiments. Afterwards, Shareefdeen and Baltzis (1994), and Deshusses et al., (1995a-b) worked on a mathematical model to describe the treatment of toluene and a mixture of pollutants under transient conditions which is a more detailed and accurate representation of a real biofiltration system.

Alonso et al., (1996, 1998) showed that biomass growth is an important phenomenon when modeling such systems, especially for long-term operations. Shareefdeen et al., (1998) improved the model considering dispersion and then, Spigno et al., (2004) showed that such effects are important just for some values of the Peclet number.

However, in spite of the significant advances in the field, none of the current models would be useful to predict the biofiltration behavior. The principal reason is that they do not consider the presence of multiple species neither the different kinetics they could have. In addition, growth under transient conditions has never been approached in such systems.

Therefore, the objective of this work was to develop a mathematical model able to represent the methanol biofiltration coupled with protein production over time. Several simulations were carried out in order to show the effects of different model parameters (Yx/s, ECmax, b and Ks). The effectiveness of the model was validated using experimental data.

METHODOLOGY

Model design

The column height of a biofilter was divided into W layers. Each section integrated by three subdivisions: the gas phase, the biofilm and the packing material (Figure 1). The gas phase carries the methanol throughout the biofilter and each of its layers is considered ideally mixed. The biofilm subdivision is in time sliced into N well mixed elements where biological activity takes place. The packing material was considered inert, being the support of

cells its unique role. Further, two different kinds of microorganisms are considered since the very beginning of the operation. *Biomass A* has the capacity to degrade methanol and produce a heterologous protein at the same time. *Biomass B* is also able to degrade methanol, but the proteins produced by this group are not considered a valuable product.

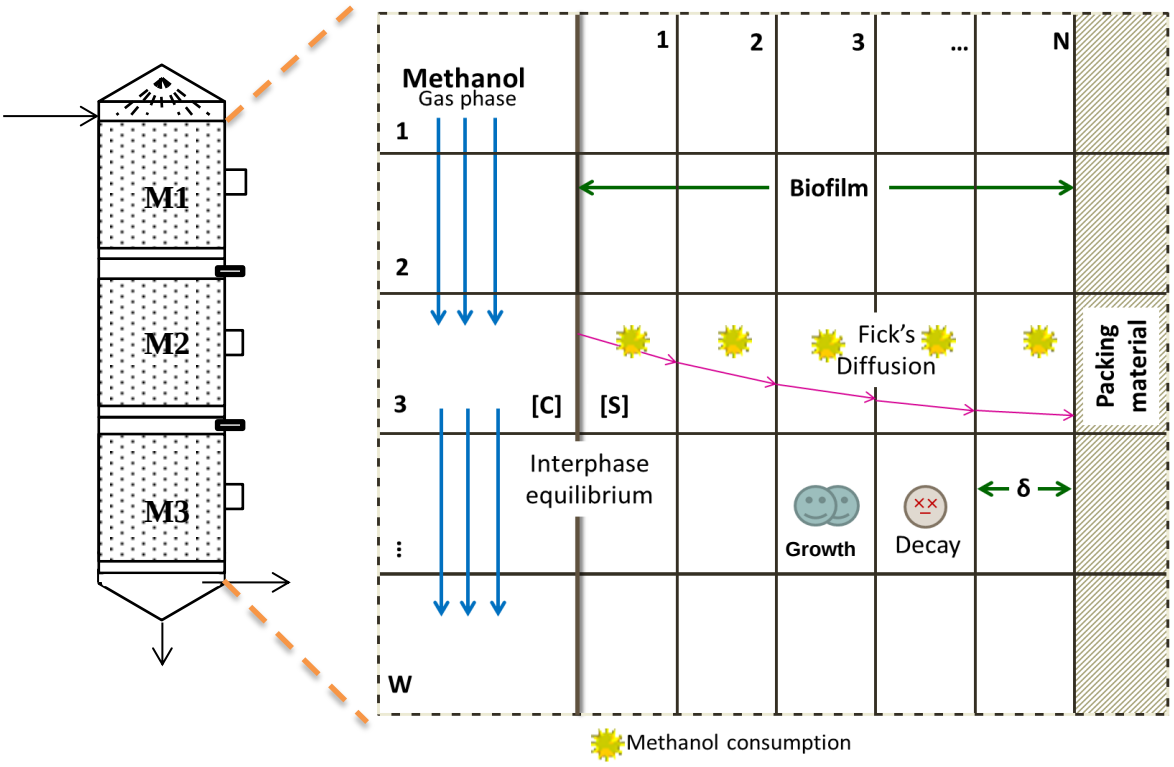

Figure 1. Conceptual representation of the biofiltration process. M1, Module 1; M2, Module 2; M3, Module 3; W: number of divisions along biofilter height; N: number of divisions along biofilm depth; [C]: Methanol concentration in gas phase; [S]: Methanol concentration in the biofilm.

Assumptions

1. Methanol is the unique carbon source and microorganisms can grow only if they can degrade methanol.
2. Methanol is the limiting substrate.
3. All microorganisms are able to produce protein, but only *Biomass A* can produce the heterologous protein.
4. Protein production is proportional to methanol degradation.
5. The effect of increase the transfer area is not significant.
6. Active biomass is only considered.
7. Mass transport in the biofilm can be described by Fick's diffusion.
8. Concentration in liquid phase can be estimated with a partition coefficient.
9. There is no reaction in the gas phase.
10. Biomass density is constant and it is the same for all species.

11. There is no direct interaction among different species.

Mass balances

Following the nomenclature given by the grid, an appropriate balance over any element will indicate the change of mass over time. For the gas phase, the change of methanol in the element i can be described by the following equation:

$$\frac{d(Methanol)_{Gas}}{dt} = Q(C_{i-1} - C_i) - J_{i,j}A \quad (1)$$

where Q is the gas flow, C_{i-1} is the methanol concentration at inlet, C_i is the methanol concentration at outlet, $J_{i,j}$ is the flux of methanol to the biofilm layer and A is the transfer area. Furthermore, $J_{i,j}$ can be defined with the following expression:

$$J_{i,j} = D\frac{dS_{i,j}}{dz}\Big|_{z=0} = \frac{D(S_{i,surface} - S_{i,1})}{\delta_{i,1}} = \frac{D\left(\frac{C_i}{H} - S_{i,1}\right)}{\delta_{i,1}} \quad (2)$$

Where D is the effective methanol diffusivity, ($S_{i,surface}$ - $S_{i,1}$) is the concentration difference between surface and first biofilm layer while δ is thickness of the *i,1* biofilm element. In accordance, the methanol concentration in the surface, $S_{i,surface}$, can be calculated with the concentration in gas phase (Ci) divided by a partition coefficient, *H*.

Concerning to the biofilm phase, the mass balance can be illustrated with the following equation:

$$\frac{d(Methanol)}{dt} = \frac{DA(S_{i,j-1} - S_{i,j})}{(\delta_{i,j-1} + \delta_{i,j})/2} - \frac{DA(S_{i,j} - S_{i,j+1})}{(\delta_{i,j} + \delta_{i,j+1})/2} - R_{i,j}V_{i,j}^{bf} \quad (3)$$

Where *S* is the methanol concentration in the element *i,j*, R is the methanol degradation rate and V^{bf} is the biofilm volume. Note that for the first layer of the biofilm, the term for mass entering the element, $\frac{DA(S_{i,j-1} - S_{i,j})}{(\delta_{i,j-1} + \delta_{i,j})/2}$, needs to be substituted with: $\frac{D(S_{i,surface} - S_{i,1})}{\delta_{i,1}}$.

Growth and degradation kinetics

Growth balance in both *Biomass A* and *Biomass B* is given by the following expressions:

$$\frac{d(BiomassA)}{dt} = Y_A\hat{q}_A X_A V_{i,j}^{bf}\frac{S_{i,j}}{Km_A + S_{i,j}} - b_A X_A V_{i,j}^{bf} \quad (4)$$

$$\frac{d(BiomassB)}{dt} = Y_B\hat{q}_B X_B V_{i,j}^{bf}\frac{S_{i,j}}{Km_B + S_{i,j}} - b_B X_B V_{i,j}^{bf} \quad (5)$$

where *Y* is the biomass yield, *q* is the maximum specific substrate utilization rate, *X* is the biomass density, *Ks* is the half saturation constant, and *b* is the decay coefficient.

From the biomass amounts, it is possible to calculate partial ($V_{i,j}^{bfA}\ and\ V_{i,j}^{bfB}$) and total ($V_{i,j}^{bf}$) volumes in a biofilm element *i,j* according with the next expressions:

$$V_{i,j}^{bfA} = \frac{BiomassA_{i,j}}{X_A} \quad (6)$$

$$V_{i,j}^{bfB} = \frac{BiomassB_{i,j}}{X_B} \quad (7)$$

$$V_{i,j}^{bf} = \frac{BiomassA_{i,j}}{X_A} + \frac{BiomassB_{i,j}}{X_B} \quad (8)$$

In addition, methanol degradation rate (*R*) can be calculated with the following Michaelis-Menten type equations:

$$Ra_{i,j} = X_A \hat{q}_A \frac{S_{i,j}}{Km_A + S_{i,j}} \quad (9)$$

$$Rb_{i,j} = X_B \hat{q}_B \frac{S_{i,j}}{Km_B + S_{i,j}} \quad (10)$$

$$R_{i,j} = Ra_{i,j} + Rb_{i,j} \quad (11)$$

Protein production

Protein production was linked with methanol consumption by a simple parameter called protein productivity, $Y_{p/s}$. This parameter is specific for each specie or group presented in the biofilter. Thus, the protein production of the system can be expressed with the next equation:

$$\frac{dP_{i,j}}{dt} = Ra_{i,j} V_{i,j}^{bfA} Y_{\frac{P}{s},A} + Rb_{i,j} V_{i,j}^{bfB} Y_{\frac{P}{s},B} \quad (12)$$

Additionally, the equation below can be used to calculate the amount of a specific protein produced by microorganisms in the group A.

$$\frac{d\check{P}_{i,j}}{dt} = Ra_{i,j} V_{i,j}^{bfA} Y_{\frac{P}{s},A} Y_{p/p} \quad (13)$$

where $\check{P}$ is the mass of the specific protein and $Y_{p/p}$ the fraction of heterologous protein produced by the group A.

Frontier equations

Frontier equations need to be added in order to solve the system. The first one corresponds to the top layer of gas phase where the methanol concentration entering the element is given by the air quality to be treated with the biofilter. Thus, the equation can be re-written as follows:

$$\frac{d(Methanol)_{Gas}}{dt} = Q(C_{Inlet} - C_i) - J_{i,j}A \quad (14)$$

The second equation corresponds to the last layer of the biofilm. Here, the term of mass leaving the element needs to be eliminated because there is no transfer to the packing material. Accordingly, the equation is rewritten as follows:

$$\frac{d(Methanol)}{dt} = \frac{DA(S_{i,j-1} - S_{i,j})}{(\delta_{i,j-1} + \delta_{i,j})/2} - R_{i,j}V_{i,j}^{bf} \quad (15)$$

System setup

The biofilter characteristics were set similar to those from our previous experimental work. Therefore, the system was a glass column packed with 3.3x10^{-3} m^3 of perlite. Every particle was considered as a perfect sphere of 3.35x10^{-3} m in diameter. Void fraction (VF) before the start-up of the system was 0.43. However, after the inoculation with the Ech42 - *P. pastoris* strain and along time, VF became slightly lower due to the presence and growth of biomass. Furthermore, the initial volume of biomass was calculated to be 2.5x10^{-4} m^3 and was assumed to be homogenously distributed along the biofilter.

From the top of the column, methanol was fed at concentrations of 5.5, 10 and 13.3 g/m^3 during 28, 24 and 30 days, respectively. The air and methanol mixture flowed at a rate of 3.3x10^{-3} m^3/min. Moreover, a mineral solution containing $(NH_4)_2SO_4$, vitamins and minerals, was sprinkled every two days to harvest the heterologous peptide. More details about characteristics of the system are presented in Table 1.

Biomass fraction and growth kinetics evaluation

Simulations were developed in the software Berkeley-Madonna using Rosenbrock algorithm. The code was written based in a system with two different biomass groups. The first group is constituted by biomass able to produce a specific heterologous protein, henceforth called *Ech42*. The second group, also named *Biomass B*, includes biomass that is not desirable but it is present because of the typical aseptic limitations in industrial scale.

Different scenarios were devised to evaluate consistency and sensibility of the model. During the first one, both biomass groups were considered to have similar growth kinetics coefficients (Yx/s, Qsx, Ks, b), but the fraction of *P. pastoris* at the start of operation was varied between 0 and 1. Elsewhere, four more scenarios were conceived to evaluate the growth kinetics coefficients effects of *Biomass B* on the model behavior. With such purpose, it was assumed that the presence of *P. pastoris* was equal to 95% of initial biomass volume and its parameters remained constant. Details of these five simulations are presented in Table 2.

Table 1. Start up parameters used in the model.

Parameter	Value	Description	Source
W	6	Number of vertical layers of the biofilter	-
N	10	Number of horizontal layer in the biofilm	-
D	0.000005904	Methanol diffusivity, m^2/h	Lee and Li, 1991
Q	0.198	Gas flowrate, m^3/h	Experimental
VF	0.5	Void fraction, dimensionless	Experimental
H	0.00025	Henry's law coefficient, dimensionless	Guota et al., 2000
A	1020	Specific area, m^2/m^3	Experimental
V	0.0033	Reactor volume, m^3	Experimental
X_A	51930	Biomass density, gC/m^3	Experimental
X_B	51930	Biomass density, gC/m^3	Experimental
Y_A	0.0322	Biomass yield, $g_{Biomass}/g_{MetOH}$	Presetting
Y_B	0.0322	Biomass yield, $g_{Biomass}/g_{MetOH}$	Presetting
$ECmax_A$	5269	Maximum elimination capacity based in biofilm volume, g/m^3*h	Presetting
$ECmax_B$	5269	Maximum elimination capacity based in biofilm volume, g/m^3*h	Presetting
Ks_A	3280	Half saturation coefficient, g/m^3	Presetting
Ks_B	3280	Half saturation coefficient, g/m^3	Presetting
b_A	0.0028	Decay coefficient, 1/h	Presetting
b_B	0.0028	Decay coefficient, 1/h	Presetting
Yp/s_A	0.2	Protein yield, $g_{protein}/g_{MethOH}$	Presetting
Yp/s_B	0.2	Protein yield, $g_{protein}/g_{MethOH}$	Presetting
q_A	$q=EC_{max}/X_A$	Specifi methanol consumption rate, $g_{MetOH}/g_{Biomass}*h$	Presetting
q_A	$q=EC_{max}/X_B$	Specifi methanol consumption rate, $g_{MetOH}/g_{Biomass}*h$	Presetting

Presetting refers to previous simulations where the model was adjusted to experimental data. The values obtained were taken as starting point.

Y_A, Y_B, $ECmax_A$, $ECmax_B$, Ks_A, Ks_B, b_A and b_B were first considered to be equal among the two species considered, but then they were adjusted to the best fitting.

Validation

Experimental data from chapter II was used to validate the model. In order to achieve it, fitting function of Berkeley-Madonna software was employed. The purpose of such validation was to find values for Y_A, Y_B, $ECmax_A$, $ECmax_B$, Ks_A, Ks_B, b_A and b_B that best fits with the experimental information.

RESULTS AND DISCUSSION

Biomass fraction

Biomass fraction effects were tested by varying the *Biomass A* fraction, ($\frac{Biomass_A}{Biomass_A+Biomass_B}$), and fixing all other model parameters (Ks_A, Ks_B, $ECmax_A$, $ECmax_B$, X_A, X_B, Y_A, Y_B, b_A, b_B, $Y_{p/s}$) assuming that both groups of microorganisms shared identical kinetics. The results showed that the heterologous protein production was reduced with the increase in the Biomass A fraction value. However, no other response was affected (methanol degradation, biomass growth, or total protein production).

The main reason for this behavior is that both kinds of microorganisms grow, decay and produce proteins at identical rates. The only difference is the kind of protein they produce. As defined previously, *Biomass A* is responsible of the heterologous protein production while *Biomass B* produces proteins without any economic interest. Therefore, as shown in Figure 2A, the higher the fraction of *Biomass A*, the higher is the cumulative valuable protein produced.

Protein is produced apparently at a constant rate along all operational time simulated. However, it is foreknown that the protein production is actually linked to methanol concentration in every element in the biofilm and thus, the slope must change accordingly. Also, since biomass is increasing with time, it is expected to have an increase on the protein production caused by a higher biomass in the system.

Moreover, the model showed an appropriate representation of biomass growth and stratification phenomena. As noted in Figure 2B, the growth was dependent of time and methanol concentration at inlet. Also, it can be observed (Figure 2C) that growth was not uniform along the height of the column. In fact, the biomass growth was stratified according to the methanol availability in each section of the biofilter. Thereby, by the end of the operation time simulated, the thickness of the biofilm at the top of the column (205 μm) was around twice the thickness estimated for the biofilm at the bottom (126 μm). Experimentally, the biomass stratification phenomenon is usual-

ly attributed to the existence of a concentration profile but also to hydrodynamic differences along the biofilter (Stewart and Franklin, 2008; Schreyer and Coughlin, 1999; Song and Kinney, 2000).

Y_B effect

In order to evaluate the effects of the biomass yield on the model behavior, *Biomass B* yield (Y_B) was tested from 0.005 to 0.05 gC/gMetOH. As shown in Figure 3A, Y_B changes gave an answer spectrum in the cumulative Ech42 production ranging from 532 to 544 mg of cumulative Ech42 protein. According to model structure, the increase of the Y_B coefficient triggers an increase of *BiomassB*, which is not able to produce Ech42 and thus the Ech42 production drops.

On the other side, the effect of Y_B value over methanol was remarkable (Figure 3B). The increase of Y_B value leaded to a lower outlet concentration. An appropriate explanation for the observed behavior is that every increase of Y_B will be accompanied with an increase of growth rates of the second group. This is also reflected in an increase of volume and thickness (Figure 3C, D). However, the amount of *Biomass A* remains much higher than the *Biomass B* group and therefore the Ech42 production is slightly different from one condition to another.

Concerning to the increase of methanol degradation, it seems to be clear that an increase in the parameter Y_A leads to the increase of total biomass which means the presence of more microorganisms capable to carry out methanol degradation.

ECmax effects

The effect of the maximum elimination capacity coefficient was evaluated by running the model with several values ranging from 1000 g/m^3*h to 8000 g/m^3*h. The responses showed that this parameter was of big importance for the degradation efficiency and cumulative Ech42 production. As observed in Figure 4A, when the $ECmax_B$ was increased there was also a decrease of methanol concentration and *vice versa*. To better illustrate it, in one boundary it was possible to reach an efficiency of 100%, while in the other only 50% of methanol was degraded.

Furthermore, Ech42 cumulative production was greatly affected by the change of ECmax in *Biomass B*. The higher the ECmax the less Ech42 obtained (Figure 4B). A logical explanation for this phenomenon is that *P. pastoris* is displaced by the other biomass group. In other words, species with higher capacity for methanol degradation compete better for the resources and its growth is enhanced. As a consequence, *P. pastoris* growth is limited and displaced. Such shift in the ecology of the system can be observed in Figure 4 (C, D, E) where *P. pastoris*

biomass started to decay when the $ECmax_B$ value was over 6000g/m^3*h. Simultaneously, the amount of *Biomass B* increased and became dominant.

Ks effects

As can be observed in Figure 5, the half saturation constant has minimal effects over the elimination efficiency and cumulative Ech42 production. Based on Figure 5A it is possible to aim that the capacity for methanol elimination of the system was not important even if the parameter was modified up to 2 orders of magnitude. Nevertheless, the lower the Ks value, the higher the removal efficiency is. This behavior is consistent with the shape of the monod equation which dictates that Ks is the inlet load at which the half of the maximum elimination capacity is reached. Therefore, the lower the Ks value, the sooner the ECmax is achieved.

On the other hand, cumulative Ech42 production became lower at low Ks_B values (Figure 5B). In this case, the low Ks_B values increased the methanol degradation rate (gMetOH/h) of *Biomass B*. This fact triggered the growth of *Biomass B* and a consequently displacement of *Biomass A*. Therefore, the proportion of protein without value was also increased.

Decay coefficient effects

The decay coefficient effect over the model responses was studied ranging the b_B values from 0.0005 to 0.0027 1/h. As observed in Figure 6, decay coefficient can have very different consequences in the elimination efficiency. Under high b_B values, the elimination efficiency of the system was 50% while under low b_B values the efficiency was boosted until 80%. Clearly, a lower decay coefficient allows the development of a wider biofilm and therefore, the capacity of the biofilter is increased.

The drawback of a higher growth of *Biomass B* reached at low values of b_B is that *Biomass B* became dominant in the system. Consequently, the amount of Ech42 produced by the system is negatively affected. It can be observed in Figure 6 that the cumulative Ech42 produced was 3% lower at low b_B values in comparison with the Ech42 produced at high b_B values.

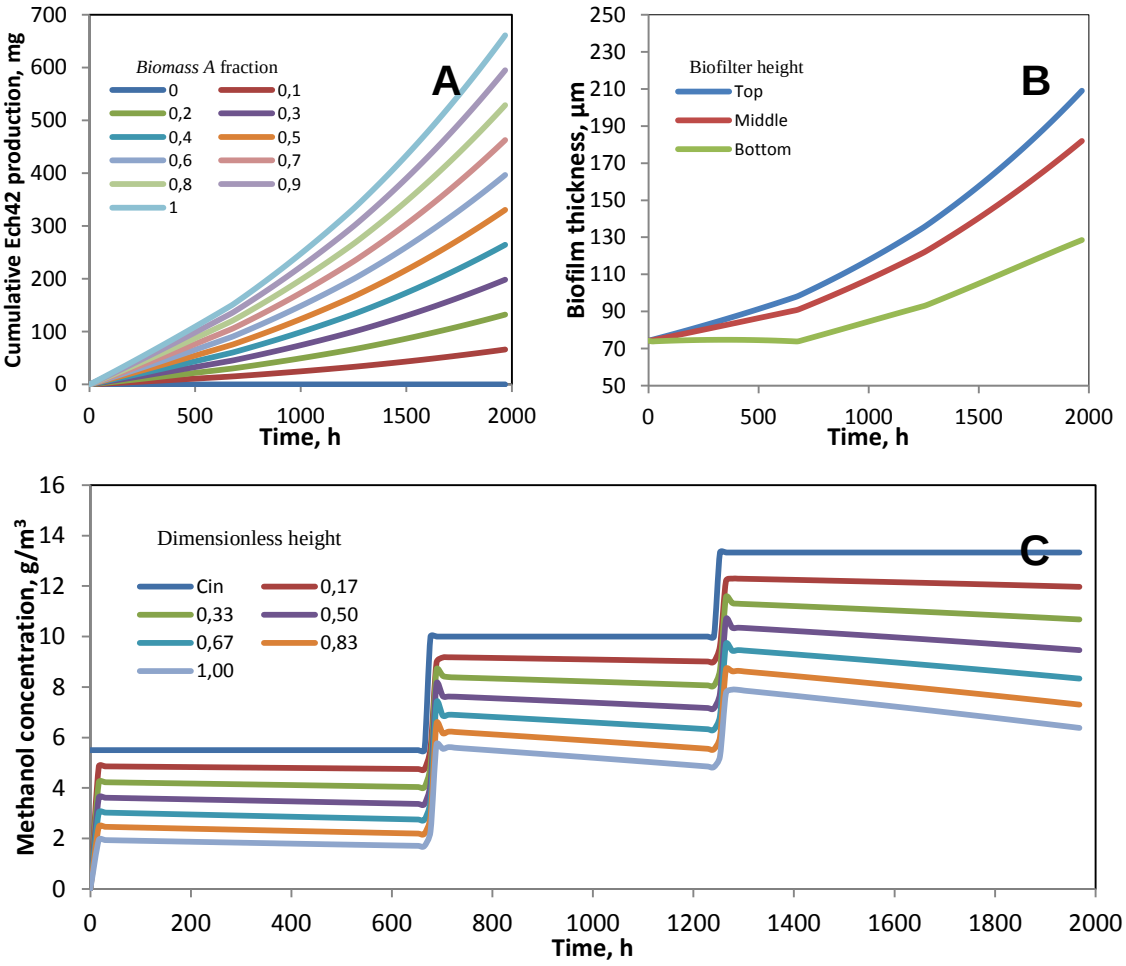

Figure 2. Model behavior under different *BiomassA* fractions at the start of operation. A) Heterologous protein production. B) Biofilm thickness along the biofilter. C) Methanol concentration. Parameters: Ks=3280 g/m^3 Y=0.032 gC/gMetOH, ECmax=5269 g/m^3*h, b=0.0028/h

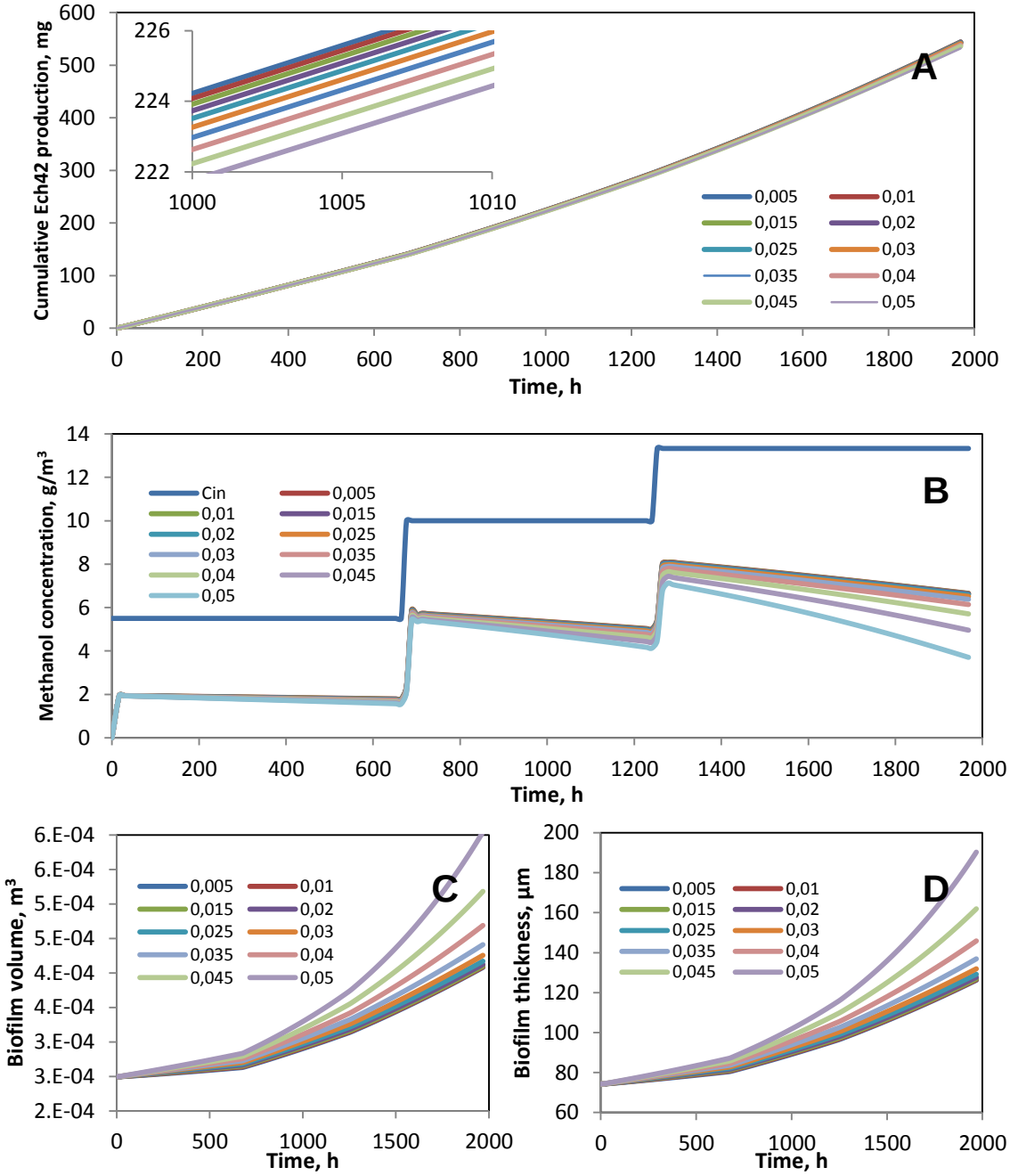

Figure 3. Model behavior under different Y_A at the start of operation. A)Ech42 production, B) Methanol degradation, C) Biofilm volume, D) Biofilm thickness. Parameters: Xaf = 0.95, Ks=3280 g/m^3, ECmax=5269 g/m^3*h, b=0.0028/h, Yp/s=0.2

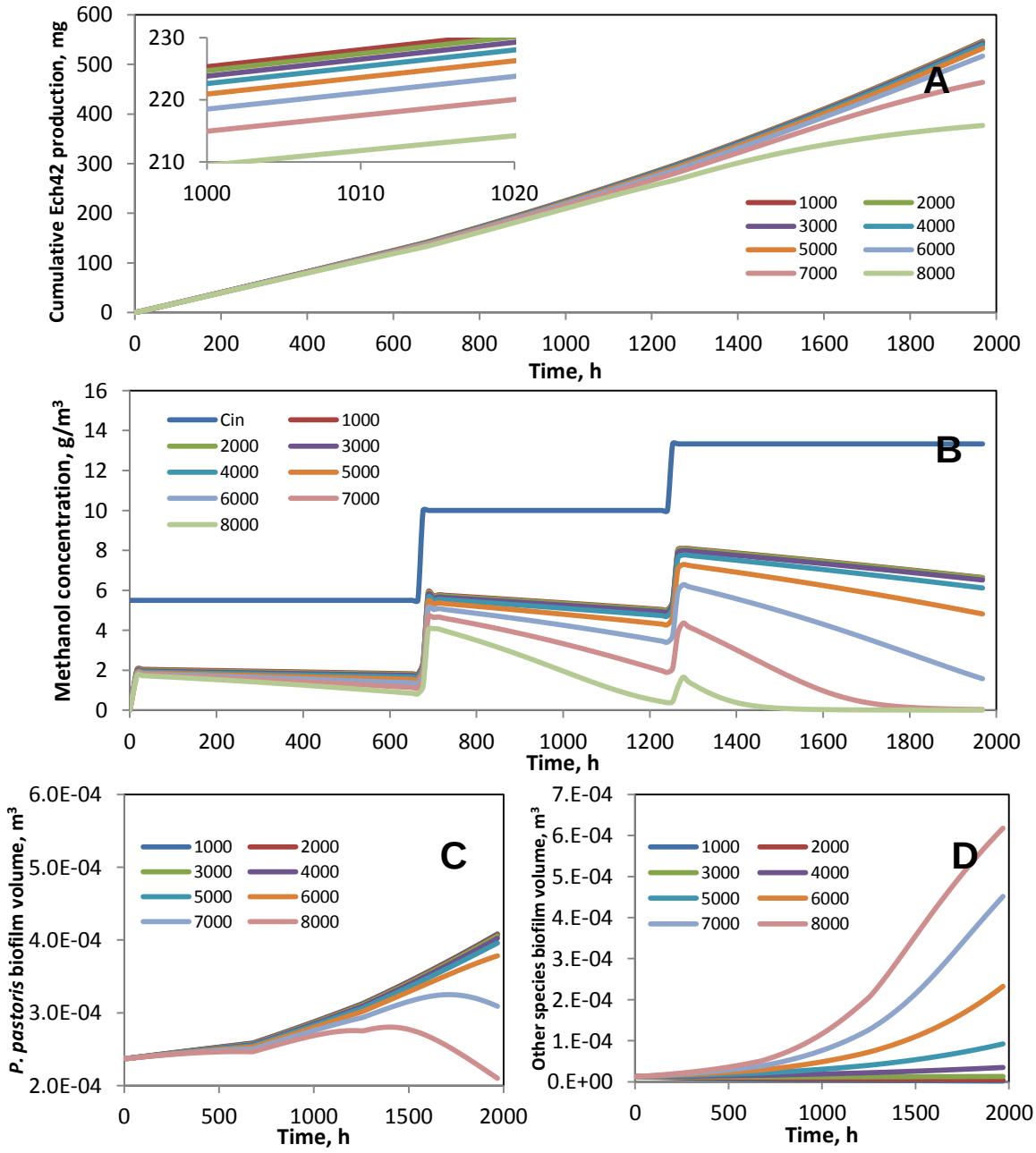

Figure 4. Model behavior under different ECmax at the start of operation. A) Ech42 production, B) Methano degradation, C)*P. pastoris* biofilm volume, D) Others volume. Parameters: Xaf = 0.95, Y=0.032gC/gMetOH, Ks=3280 g/m^3*h, b=0.0028/h, Yp/s=0.2

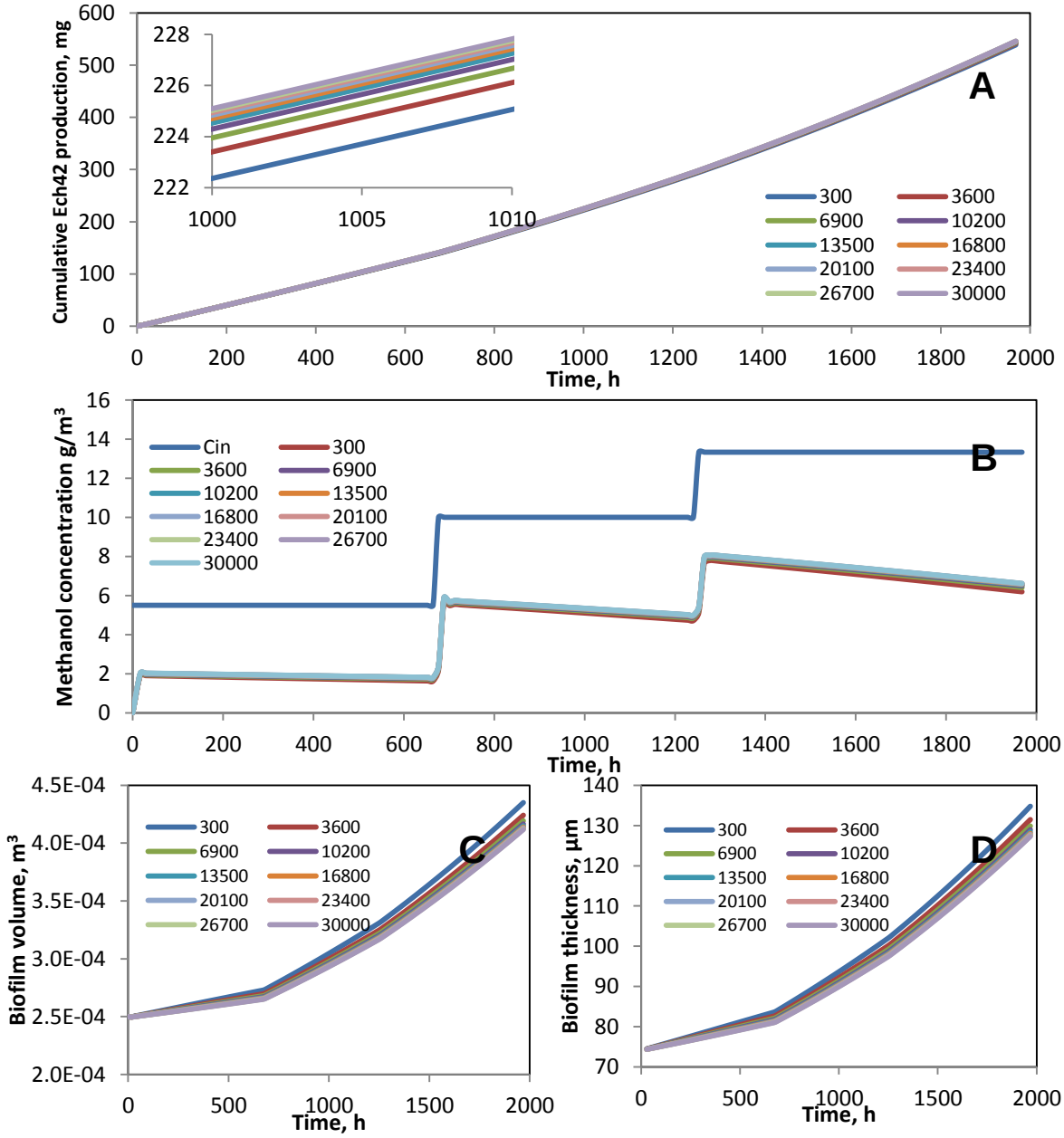

Figure 5. Model behavior under different Ks at the start of operation. A)Ech42 production, B)Methanol degradation, C) Biofilm volume, D)Biofilm thickness. Parameters: Xaf = 0.95, Y=0.032 g_C/g_{MetOH}, ECmax=5269g/m^3*h , b=0.0028/h, Yp/s=0.2

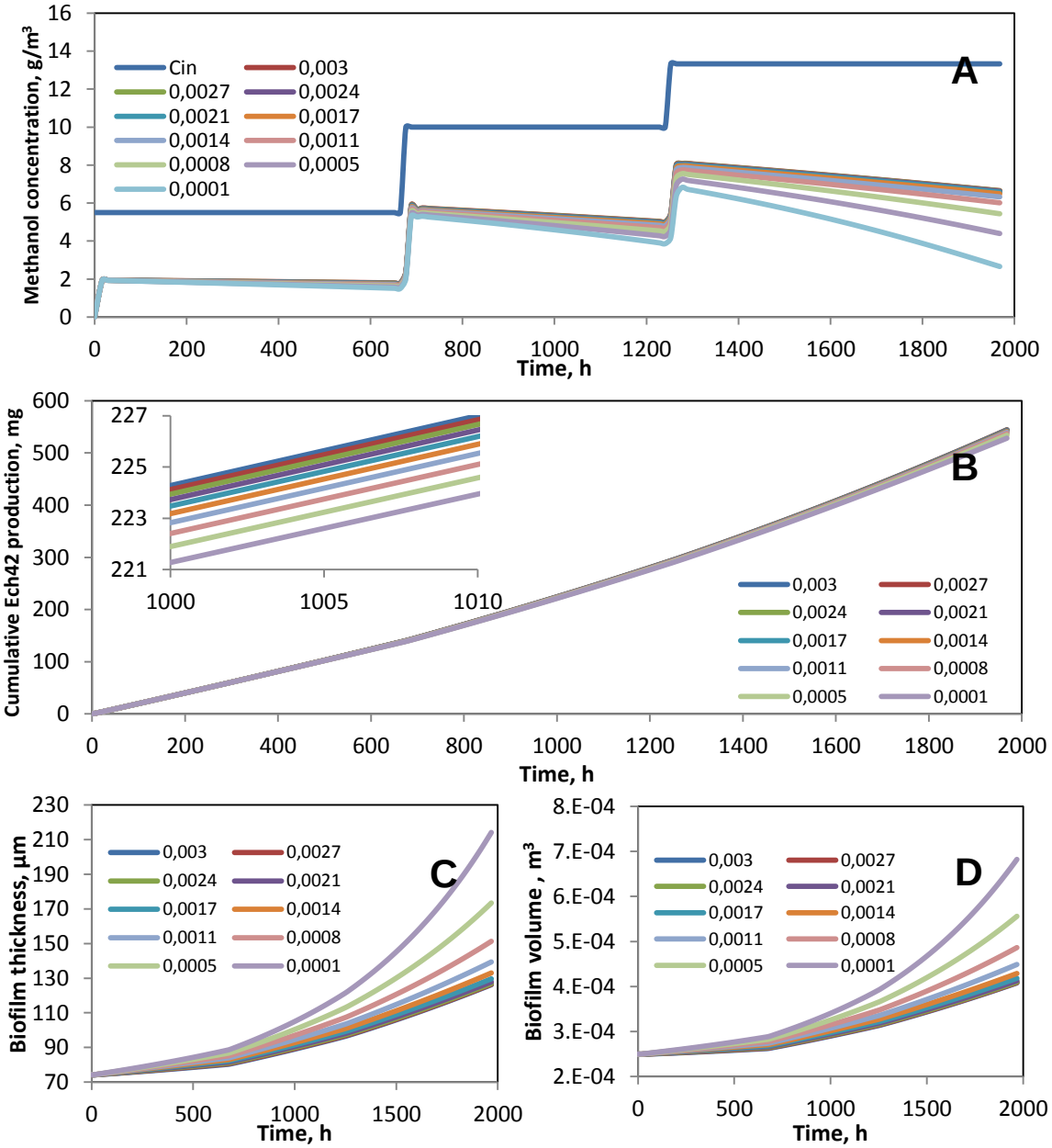

Figure 6. Model behavior under different b at the start of operation. A)Methanol degradation, B) Ech42 production, C)Biofilm thickness, D)Biofilm volume. Parameters: Xaf = 0.95, YA= 0.032gC/gMetOH, Ks=3280g/m^3, ECmax=5269 g/m^3*h, b=0.0028/h, Yp/s=0.2

Sensitivity

Multiple simulations of the model have shown that Y_B, $EC_{B\text{-}max}$ and b_B, play an important role in the system. In addition, Ks parameter seems to be not relevant under the conditions tested. In order to assign an appropriate relative importance to aforementioned parameters, a sensitivity analysis was developed.

As shown in Table 2, the parameters were classified according to its relative importance, which was defined as the ratio between the variation in the input and output. The results showed that the most important parameter in the model was the maximum elimination capacity (ECmax), followed by the decay coefficient (b), the biomass yield (Y), and the half saturation constant (Ks).

Validation

Data obtained from our experimental work was used to validate the model proposed. For such objective, *P. pastoris* fraction at the beginning of operation was considered to be 0.95. Biofilm volume, cumulative protein production, cumulative Ech42 production and methanol outlet concentration were fit with the model system by changing values of the key parameters. Results summarized in Figure 7 show that the model cannot converge well with experimental determinations.

Particularly, the model predicts with acceptable accuracy the methanol degradation behavior. However, the slopes of the model are progressively decreasing and the predicted removal efficiency tends to be higher than it actually is. Such change is one of the consequences of an increasing biofilm volume. Nonetheless, in the experimental data such effect is apparently balanced with another.

Table 2. Estimation of sensitivity ratios to assign a relative importance.

Parameter	Input amplitude	Response amplitude			Sensitivity ratio		
		Cumulative Ech42 production	Methanol concentration	Biofilm Volume	Cumulative Ech42 production	Methanol concentration	Biofilm Volume
Y_B	0.9	0.0227	0.443	0.3248	0.025	0.492	0.360
$EC_{B\text{-}max}$	0.9993	0.875	0.3108	0.505	0.875	0.311	0.505
K_{Bm}	0.99	0.0133	0.064	0.0534	0.013	0.064	0.053
b_B	0.9666	0.0297	0.6001	0.402	0.030	0.620	0.415

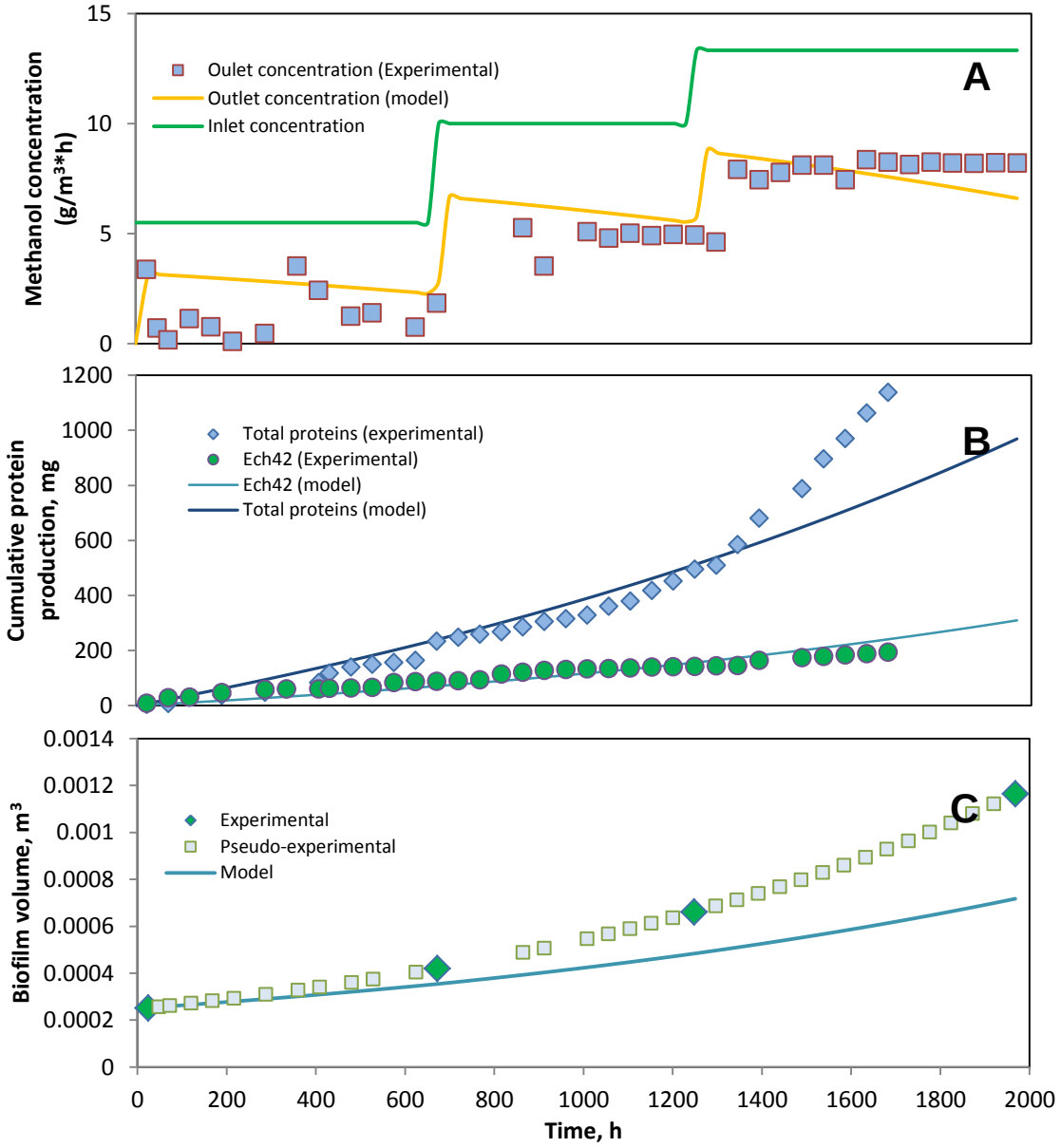

Figure 7. Model validation. A)Methanol degradation, B) Ech42 production, C)Biofilm volume. Parameters: $ECmax_A$=1887.5g/m^3*h, $ECmax_B$=1156.79 g/m^3*h, Ks_A=300.3g/m^3, Ks_B=300.4 g/m^3, Y_A=0.06g_C/g_{MetOH}, Y_B=.022 g_C/g_{MetOH}, b_A=0.0017/h, bB=0.0001/h, Yp_A=0.2$g_{Protein}$/g_{MetOH}, Yp_B=15.52$g_{Protein}$/g_{MetOH}.

Elsewhere, cumulative protein production fitted well with the model. Notwithstanding, in the last days of operation, total protein suddenly increased and then the model was not able to follow that shape anymore. That's probably because at some point in the experiment, new airborne species were introduced to the system and the model is not conceived to predict that random scenarios.

Table 3. Parameters comparison between this study and literature.

Estimated parameter	This study	Literature	Source
ECmax	1887.5g/m^3*h	2230 g/m^3*h	Ávalos-Ramirez et al., 2008
	1156.79 g/m^3*h	-	-
Ks	300.3g/m^3	34.23 g/L – 72.77 g/m^3	Ávalos-Ramirez et al., 2009
	300.4 g/m^3	1 g/m^3	Alonso et al., 2000
Y	0.06 g_C/g_{MetOH}	0.015 - 0.217 g_C/g_{MetOH}	Ávalos-Ramirez et al., 2009
	0.022 g_C/g_{MetOH}	0.108 g_C/g_{VOC}	Alonso et al., 2000
b	0.0017/h	0.004/h	Alonso et al., 2000
	0.0001/h		
Yp	0.2 $mg_{Proteina}/g_{MetOH}$	-	-
	15.52 $mg_{Proteina}/g_{MetOH}$	148 $mg_{proteina}/g_{MetOH}$	Arriaga et al, 2010. (Batch)
Biofilm thickness	89 µm - 267µm	4-210 µm	Schreyer and Coughlin, 1999.
		50-100 µm	Álvarez-Hornos et al., 2009.

ECmax: Maximum Methanol Elimination Capacity
Ks: Monod's half saturation constant
Y: Biomass yield coefficient
B: Biomass decay coefficient
Yp: Protein yield coefficient

Moreover, the set of equations shown in this work failed to predict the volume changes associates with growth. The model predicted a volume that is close to the half of experimental volume. A possible explanation is the presence of another kind of biomass that was not considered in the model, namely inactive biomass. In the frame of this work, inactive biomass could be considered biomass that is not involved in the degradation process neither considered in the decay coefficient. Typical examples of that kind of biomass are exopolysaccharides.

Finally, it is important to give the parameters values an appropriate context. Table 3 shows the different values of the model parameters that were found through model fitting. ECmax predicted values for both groups of micro-organisms considered in the model were close not only to kinetic parameters reported (Ávalos-Ramirez et al., 2008), but also to different ECmax values that are commonly found in biofilters treating methanol (Prado et al., 2008; Ramirez-Lopez et al., 2010). On the other hand, Ks value predicted in this study was markedly different from values reported in literature. However, it is consistent with the ECmax predicted.

Moreover, Y values predicted in this report were also well correlated with reports in literature. It has been report-ed that biomass yield values in methanol biofiltration ranged from 0.015 to 0.217 gC/gMetOH (Avalos-Ramirez et

al., 2009). The estimated values of both groups of biomass, *Biomass A* and *Biomass B*, were within the mentioned values.

In addition, the b value predicted was slightly higher than other b values found in biofiltration systems (Alonso et al., 2000). However, they are lower than results commonly found in other biological treatment systems (Akhbari et al., 2012; El-Kamah and Mahmoud, 2012; Emerald et al., 2012). This last fact was actually expented since biofiltration technology is a fixed biomass system.

CONCLUSIONS

The model described on this work showed to be a simple, but useful tool for prediction, control and design of bifiltration systems. Different important phenomena, such mass transfer, mass diffusion, degradation kinetics, microbial growth, protein production, and evolution of a simple community, were included in the structure of the model and analyzed under transient conditions.

Aforementioned features make this model a very complete approximation of the behavior of a biofilter coupled with production of heterologous proteins. However, some details can be added in order to improve the model (e.g. non-active biomass, competition, etc). In fact, non-active biomass seems to be a promising consideration that can be helpful to get a better fit with biomass volume and degradation efficiency.

REFERENCES

Akhbari, A ., Zinatizadeh, A., Mohammadi, P., Mansouri, Y., Irandoust, M., Isa, M. (2012). Kinetic modeling of carbon and nutrients removal in an integrated rotating biological contactor-activated sludge system. *International Journal of Environmental Science and Technology*, 9 371-378.

Alonso, C., Suidan, M.T., Kim, B.R., Kim, B.J. (1998). Dynamic mathematical model for the biodegradation of VOCs in a biofilter: biomass accumulation study. *Environmental Science and Technology*, 32, 3118.

Alonso, C., Suidan, M.T., Sorial, G.A., Smith, F.L. Biswas, P., Smith, P.J., Brenner, R.C. (1996). Gas treatment in trickle-bed biofilters: biomass, how much is enough? *Biotechnology and Bioengineering*, 54, 583.

Alonso, C., Zhu X., Makram S. (2000). Parameter estimation in biofilter systems. *Environmental Science and Technology*, 34 2318-2323.

Álvarez-Hornos, F., Gabaldón, C., Martínez-Soria, V., Marzal, P., Penya-Roja, J. (2009). Mathematical modeling of the biofiltration of ethyl acetate and toluene and their mixture. *Biochemical Engineering Journal*, 43 169-177.

Arriaga, S., Acosta-Munguía, J.A., Pérez-Martínez, A.S., De León-Rodríguez, A., Barba de la Rosa, A.P. (2010). Coupling aerobic biodegradation of metanol vapors with heterologous protein expression of endochitinase Ech42 from *Trichoderma atroviride* in *Pichia pastoris*. *Bioresource Technology*, 101, 9661.

Arriaga, S., Serrano, M.A., Barba de la Rosa, A. (2012). Methanol vapor biofiltration coupled with continuous production of recombinant endochitinase Ech42 by *Pichia pastoris*. *Process biochemistry*, In press. http://dx.doi.org/10.1016/j.procbio.2012.09.008

Arulneyam D., Swaminathan T. (2003). Biodegradation of methanol vapor in a biofilter. *Journal of Environmental Sciences*, 15 691.

Avalos-Ramirez, A., Bénard, S., Giroir, A., Jones, J., Heitz, M. (2009) Treatment of air polluted with methanol vapours in biofilters with and without percolation. *Canadian Journal of Civil Engineering,* 36 1911-1918

Ávalos-Ramirez, A., Benard, S., Giroir-Fendler, A., Peter, J., Heitz, M. (2008). Kinetics of microbial growth and biodegradation of methanol and toluene in biofilters and an analysis of the energetic indicators. *Journal of Biotechnology*, 138 88-95.

Babbit, C. W., Pacheco, A., Lindner, A. (2009). Methanol removal efficiency and bacterial diversity of an activated carbon biofilter. *Bioresource Technology*, 100 6207.

Chetpattananondh, P., Nitipavachon Y., Bunyacan C. (2005). Biofiltration of air contaminated with methanol and toluene. *Journal of Science and Technology*, 27 761.

Cornabé, T., Auria, R., Christen, P., Ferrer, H., Revah, S., Moukha, S. (2002). Biofiltration of methanol vapors by a recombinant *Pichia pastoris* strain. *USC-TRG Conference on Biofiltration*. Los Angeles CA, EU.

Dastous P. A., Nikiema J., Soreanu G., Bibeau, L., Heitz, M. (2008). Elimination of methanol and ethanol by biofiltration: An experimental study. *Water, Air, and Soil Pollution: Focus*, 8 275.

Deshusses, M.A., Hamer, G., Dunn, I.J. (1995a). Behavior of biofilters for waste air biotreatment. 1. Dynamic model development. *Environmental Science and Technology*, 29, 1048.

Deshusses, M.A., Hamer, G., Dunn, I.J. (1995b). Behavior of biofilters for waste air biotreatment. 2. Experimental evaluation of a dynamic model. *Environmental Science and Technology*, 29, 1059.

El-Kamah, H., Mahmoud, M. (2012). Performance evaluation of sequencing batch reactor for beverage industrial wastewater treatment. *Water Environmental Research*, 84 155-161.

Emerald, F., Prasad, D., Ravindra, M., Pushpadass, H. (2012). Performance and biomass kinetics of activated sludge system treating dairy wastewater. *International Journal of Dairy Technology*, 65 609-614.

Gupta, A., Teja, A., Chai, X., Zhu, J. (2000). Henry's constants of n-alkanols (methanol through n-hexanol) In water at temperatures between 40 C and 90 C. Fluid Phase Equilibria, 170 183-192.

Lee, Y., Li, S. (1991). Binary diffusion coefficients of the methanol/water system in the temperature range 30-40 C. Journal of Chemical and Engineering Data, 36 240-243.

Ottengraf, S.P.P. and Van Den Oever, A.H.C. (1983). Kinetics of organic compound removal from waste gases with a biological filter. *Biotechnology and Bioengineering*, 25 3089.

Pérez-Martínez, A.S., De León-Rodríguez, A., Harris, L.J., Herrera-Estrella, A., Barba, A. (2007). Overexpression, purification and characterization of the Trichoderma atroviride endochitinase, Ech42, in *Pichia pastoris*. *Protein Expression and Purification*, 55 183.

Ramirez-López, E. M., Corona-Hernandez, J., Avelar-Gonzalez, F.J., Omil, F., Thalasso, F., (2010). Biofiltration of methanol in an organic biofilter using peanut shells as médium. *Bioresource Technology*, 101.

Schreyer, H., Coughlin, R. (1999). Effects of stratification in a fluidized bed bioreactor during treatment of metal-working wastewater. Biotechnology and Bioengineering, 63 129-140.

Shareefdeen S.M. Shaikh, A.A., Azam, S.M. (1998). Axial dispersion in biofilters. *Biochemical Engineering Journal*, 1, 77.

Shareefdeen, Z., Baltzis, B.C. (1994) Biofiltration of toluene vapor under steady-state and transient conditions: theory and experimental results. *Chemical Engineering Science*, 49 4347-4360.

Shareefdeen, Z., Baltzis, B.C., Young-Sook, O., Bartha, R. (1993). Biofiltration of methanol vapor. *Biotechnology and Bioengineering*, 41 512.

Silva, J., Morales, M., Cáceres, M., San Martín, R., Gentina, J.C., Aroca, G. (2010). Effect of the biomass in the modelling and simulation of the biofiltration of hydrogen sulphide: simulation and experimental validation. *Journal of Chemical Technology and Biotechnology,* 85 1374-1379.

Sologar V.S., Lu Z., Allen D.G. (2003). Biofiltration of concentrated mixtures of hydrogen sulfide and methanol. *Environmental Progress*, 22 129.

Song, J., Kinney, K. (2000). Effect of vapor-phase bioreactor operation on biomass accumulation, distribution, and activity: linking biofilm properties to bioreactor performance. Biotechnology and Bioengineering, 68 508-516.

Spigno, G., Zilli, M., Nicolella, C. (2004). Mathematical modelling and simulation of phenol degradation in biofilters. *Biochemical Engineering Journal*, 19, 267.

Stewart, P., Franklin, M. (2008). Physiological heterogeneity in biofilms. *Nature Reviews Microbiology*, 6 199–210.

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS

In the present thesis it was showed the performance of three biofilters inoculated with a *P. pastoris* strain. Such microorganism was previously transformed with a gene from *Trichoderma atroviride* in order to produce a valuable heterologous protein: Endochitinase 42. Results showed that 5 g/L of $(NH_4)_2SO_4$, pH 5 and an EBRT of 60s are suitable conditions to reach an acceptable elimination capacity. Minor changes on such conditions can dramatically change the performance of the system. In contrast, the Ech42 activity was principally impacted by pH changes, being the pH 5.5 the most suitable. In the rest of conditions, the enzymatic activity ranged from 762 to 1172 U/L.

Beyond that, it was found that two or more biofiltration systems inoculated with only one species of microorganisms (in this case *P. pastoris*) diverge from each other even if they are operated under identical conditions. Thereon, it was theorized that the population inoculated into each biofilter was affected in unequaled ways by external factors such as foreign microorganisms. Thus, repeatability in biofilters should not be taken for granted.

Furthermore, a simple mathematical model was developed to describe the methanol biofiltration coupled with heterologous protein production. It was assumed that two different groups of microorganisms were growing in the biofilter in order to approach divergence phenomena observed. Results showed that the model describes well the EC and protein production of the process. Nevertheless, some characteristics in the model can be improved in order to do a better prediction of volume changes.

PERSPECTIVES

Industrial perspective requires the profit of the methanol biofiltration coupled with Ech42 production to be as high as possible. On the other hand, methanol is a hazardous compound which requires to be completely eliminated. According to the results showed in this thesis, EC and Ech42 production were not directly related each other, making difficult to achieve a high Ech42 production with efficient methanol degradation. Hence, it is necessary to develop a novel strategy that allows obtaining both aforementioned benefits.

Thereon, two different alternatives are suggested. The first one includes the sterilization of nutrimental solution fed to the reactor, sanitization of the air flow entering the system, and keeping the biofilter column closed to foreign microorganisms. The second option implies the separation of the processes into a bio-scrubber and a biofil-

ter. In the bio-scrubber, the growth of *P. pastoris* would be well controlled prioritizing the Ech42 production. On the other hand, the biofilter operation would be focused on the complete degradation of methanol.

Also, it is important to note that there are multiple biofilter conditions that were out of the analysis of this work, for example: moisture, temperature, packing material, etc. Moreover, it is needed to take into consideration (in the way to a practical application) the effects of other gases issued with methanol, such as hydrogen sulfide which is also emitted by the paper industry.

On other side, it was assumed that airborne microorganisms randomly colonized the biofilters along the time they were operated. Afterwards, many interactions between *P. pastoris* and the others microorganisms could have taken place. However, since in this thesis the microbial composition evolution was not followed, it is difficult to conclude anything. Therefore, it is crucial to study the microbial evolution in the system. Additionally, it is important to start researching the specific interactions that *P. pastoris* could have with other microorganisms in order to better understand possible inhibition or enhancement of the yeast population.

Finally, the model proposed in this work considered two groups of microorganisms existing since the very beginning of operation, but such assumption is not completely right. Rather, it is more likely, due to the inoculation strategy, that only *P. pastoris* was present then. Eventually, another microorganism could enter the system and colonize it. The difficulty to describe such phenomenon rests on the unpredictable nature of it. A first approach suggested is to implement a timer in order to simulate colonization events. On the other hand, the model failed to predict the biomass volume. Thereon, it is suggested to consider the existence of inert biomass. As well as active biomass, inert one is increasing with time and creating volume that has to be considered, but no additional methanol is degraded.

ANNEX I

ENDOCHITINASE 42

Chitine is the second most abundant polysaccharide in the planet. Its natural production is estimated to be 10^{11} tons. This polymer of N-acetyl-glucosamine is the principal component of exoskeleton of insects, crustacean, and arachnids as well as the principal component of fungi cell wall.

Chitin, discovered by Henri Braconnot in 1811, is an important product for different industries such as pharmaceutical, cosmetics, paper, textiles, and wastewater treatment

Table A1. Principal areas for chitin application. (Dutta et al., 2004; Lárez et al., 2006, Tröger y Niranjan, 2010)

Area	**Application**
Agriculture	Seed coating for preservation
	Fertilizers releasing systems
	Bactericides and fungicides
Medicine	Surgical sutures
	Gauze and bandages
	Burns treatment creams
	Contact lens manufacture
	Drug dosing
Wastewater treatment	Coagulant
	Flocculants
	Heavy metals removal
	Dyes removal
Cosmetics	Help in the control of obesity
	Bactericidal additive in soaps, skin creams, toothpaste
	Skin moisturizing agent
Biosensors	Blood glucose sensor
	Phenol detection sensor

Enzymes associated to enzymatic extraction are chitinases, cellulases, deacetylases, and proteases (Trôger and Niranjan, 2010). chitinases, particularly, are a group of glycosyl-hydrolases enzymes which includes exoquitinases, endoquitinasas, and N-acetylglucosaminidases. In particular, the endochitinases cuts in β-1,4 bonds and the resulting pieces vary in size (3 to 8 residues of N-acetylglucosamine) depending on reaction conditions. Enzymatic quitine extraction is preferred over the chemical and physical extraction because, enzymes do not generate hazardous pollutants and the environmental impact is very low.

Recently, a *Pichia pastoris* strain which is a methylotrophic yeast was transformed with an endochitinase gene from *T. atroviride (Perez-Matínez et al., 2007)*. Afterwards, the strain was inoculated in a biofilter in order to obtain such protein from the degradation of methanol vapors. The process showed pretty decent efficiencies not only in methanol elimination but also in Ech42 production. Therefore, it is expected to boost companies to treat their methanol emissions by obtaining at the same time *Chitinase*, and then recovery part of the money spent.

However, it would be a mistake does not realize that the process is not limited to produce that specific enzyme. In theory, every protein which gene code can be introduced into the genome of *P. pastoris* could be produced at the same time of degrading methanol vapors.

ANNEX II

CALIBRATION CURVES

Methanol

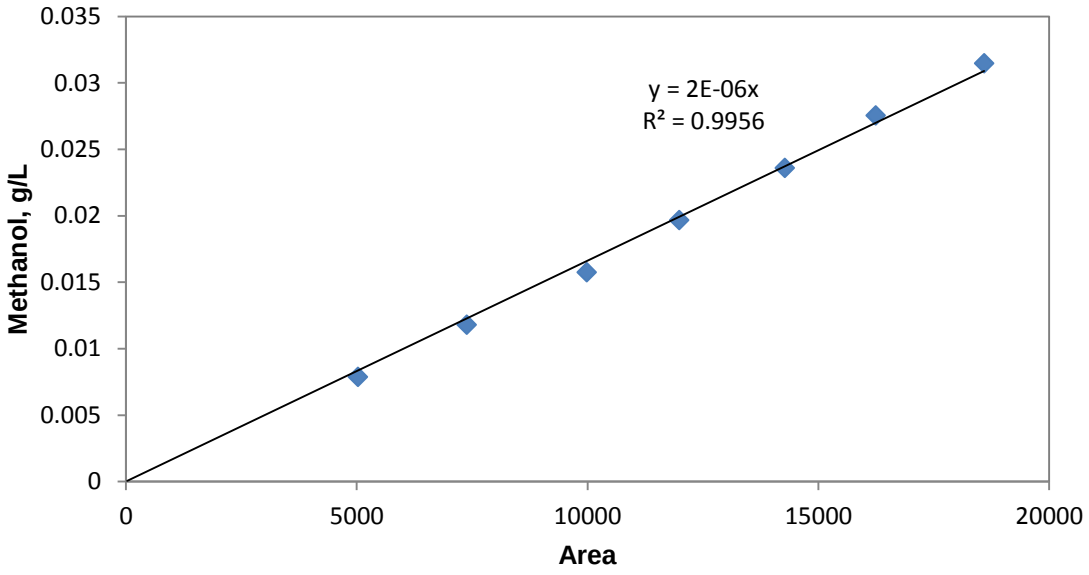

Figure A1. Methanol calibration curve.

CO_2

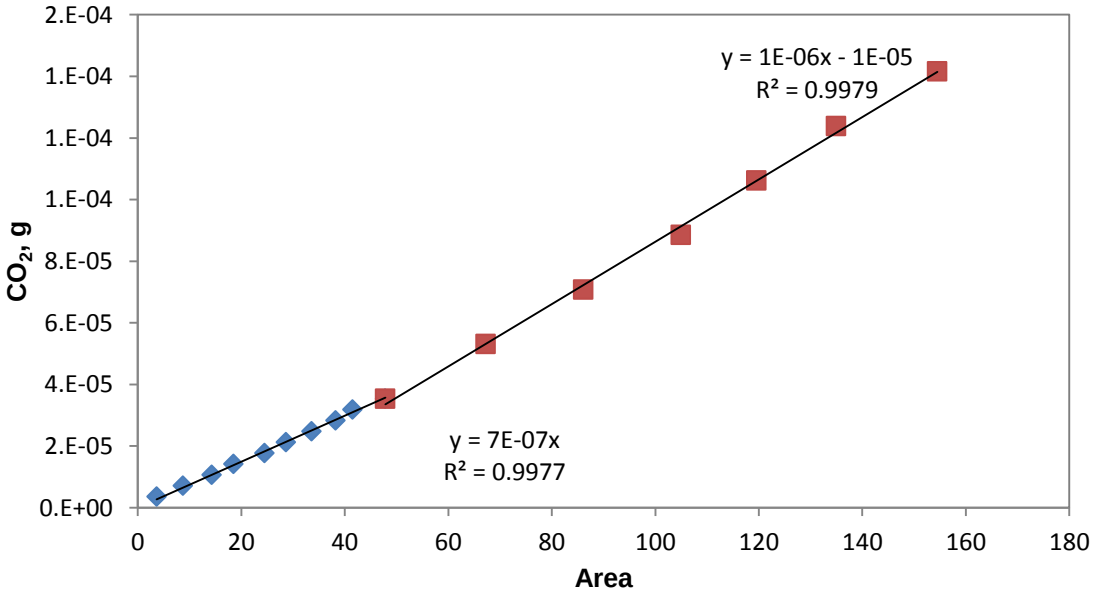

Figure A2. CO_2 calibration curve.

Bradford proteins

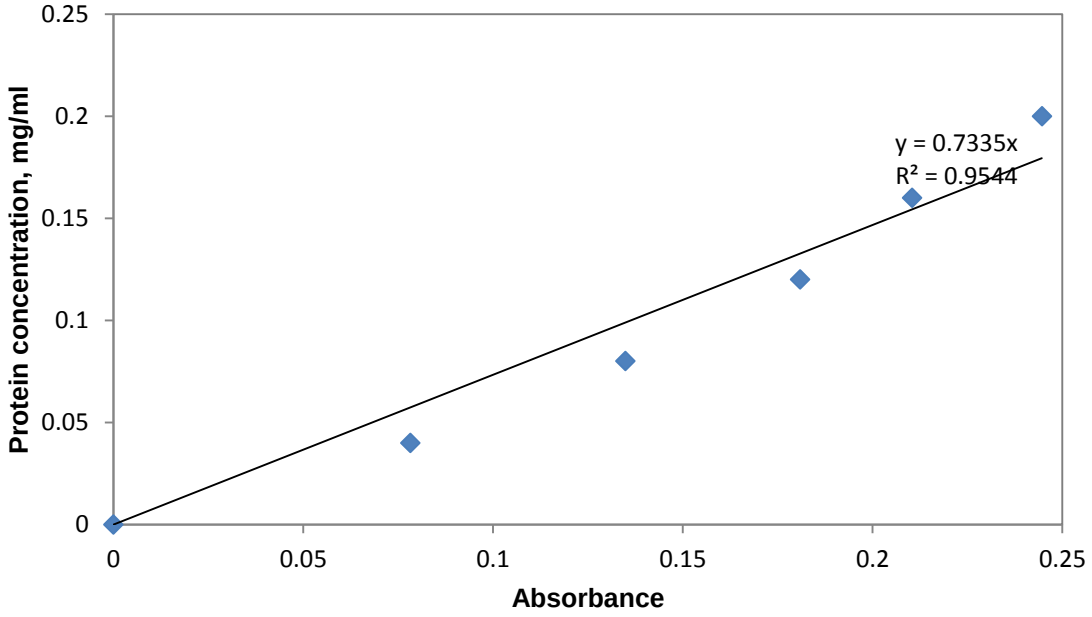

Figure A3. Total proteins calibration curve.

Ech42 activity

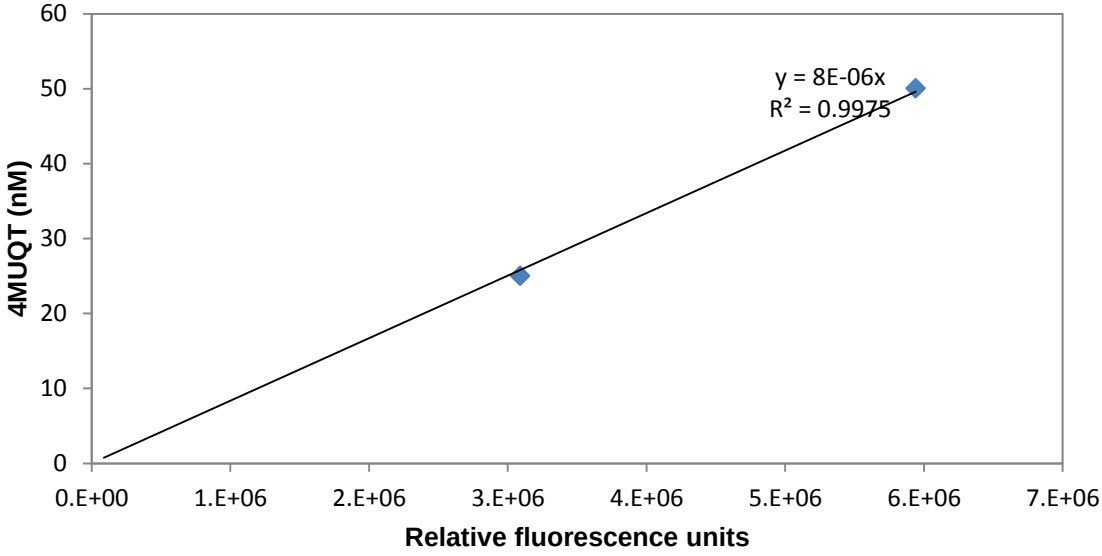

Figure A4. 4MUQT calibration curve

Total Organic Carbon (TOC)

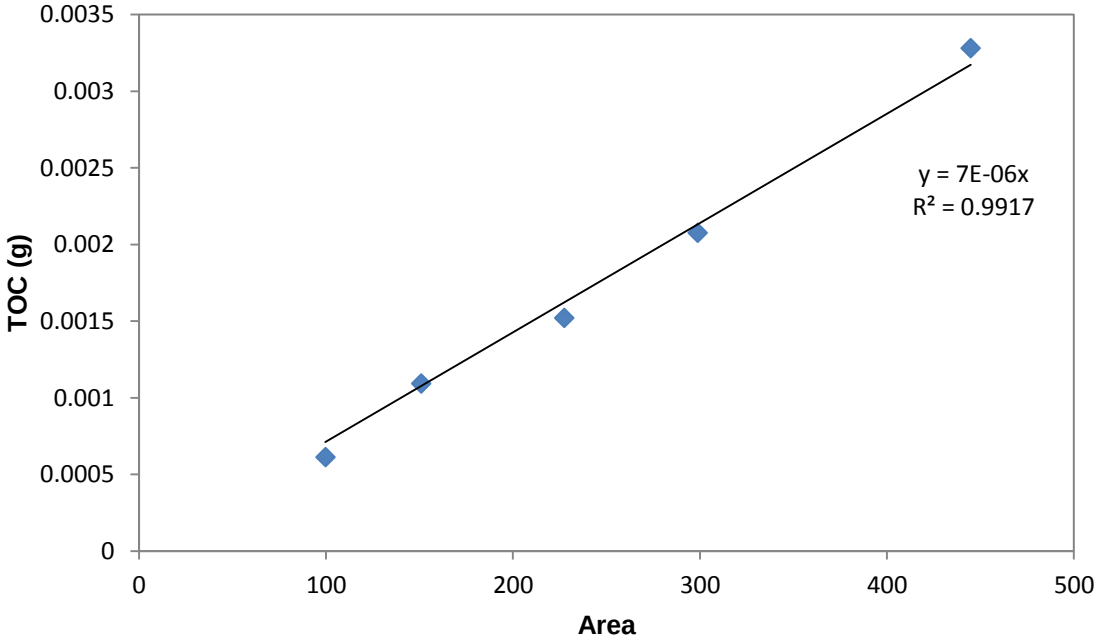

Figure A5. TOC calibration curve.

ANNEX III

CARBON TO NITROGEN RATIO CALCULUS METHOD

Assumptions:

The biofilm reaches the same nitrogen concentration than in the nutrimental solution.

The partition coefficient biofilm-gas is the same as in water-gas ($2.5*10^{-4}$ Cgas/Cliq)

Calculus method:

The calculus method is based on the volume of biofilm at the end of the stage to be analized

1. Biomass density is previously determined through experimental analysis. In this study it was equal to 51930 gC/m^3.
2. Total biomass volume is calculated from biomass (determined through TOC) mass data and biomass density.
3. Liquid methanol concentration is calculated from gas concentration (experimentally determined) and the henry partition coefficient.
4. Methanol mass is calculated from biofilm methanol concentration and biomass volume.
5. Ammonium mass is calculated from ammonium concentration and biomass volume.
6. Carbon mass is calculated from methanol mass and the carbon mass fraction for methanol. This last is equal to 0.375 (gC/gMetOH).
7. Nitrogen mass is calculated from ammonium sulfate mass and the nitrogen mass fraction for ammonium sulfate. This last is equal to 0.21 ($gC/g(NH_4)_2SO_4$).
8. Finally, the carbon to nitrogen ratio is calculated dividing carbon mass over nitrogen mass.

ANNEX IV

Biomass/EC correlation

Biofilter I

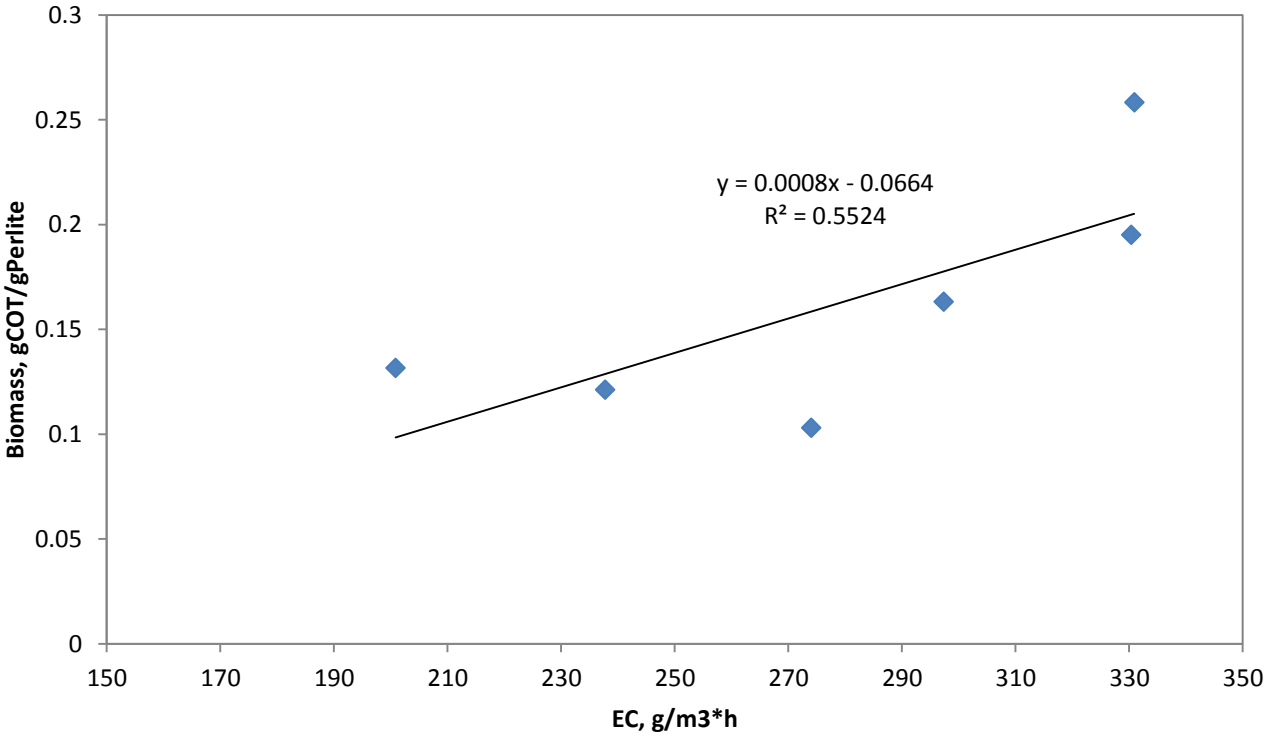

Figure A6. EC and biomass correlation during the whole operation time in biofilter I.

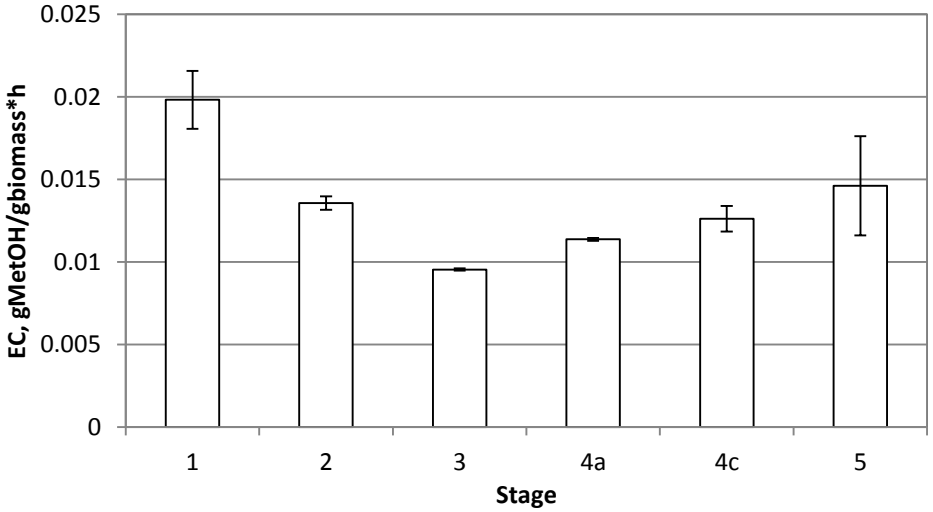

Figure A7. Biomass based elimination capacity in biofilter I.

Biofilter II

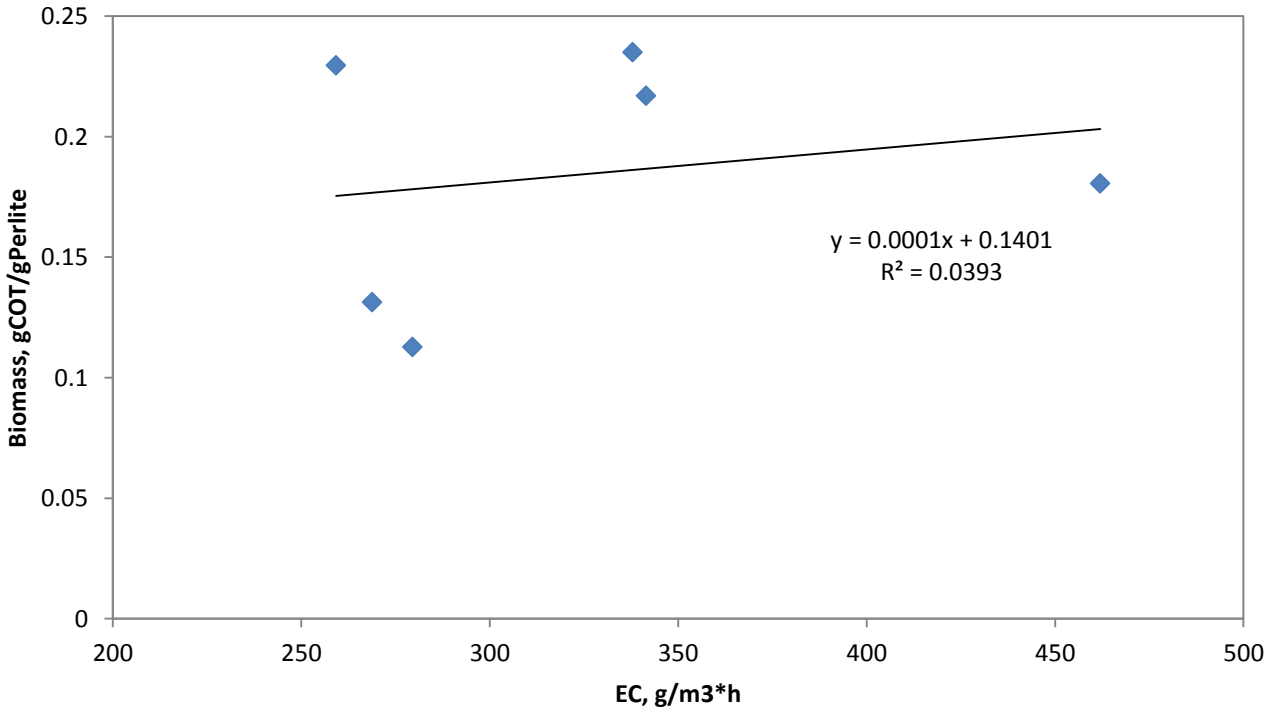

Figure A8. EC and biomass correlation during the whole operation time in biofilter II.

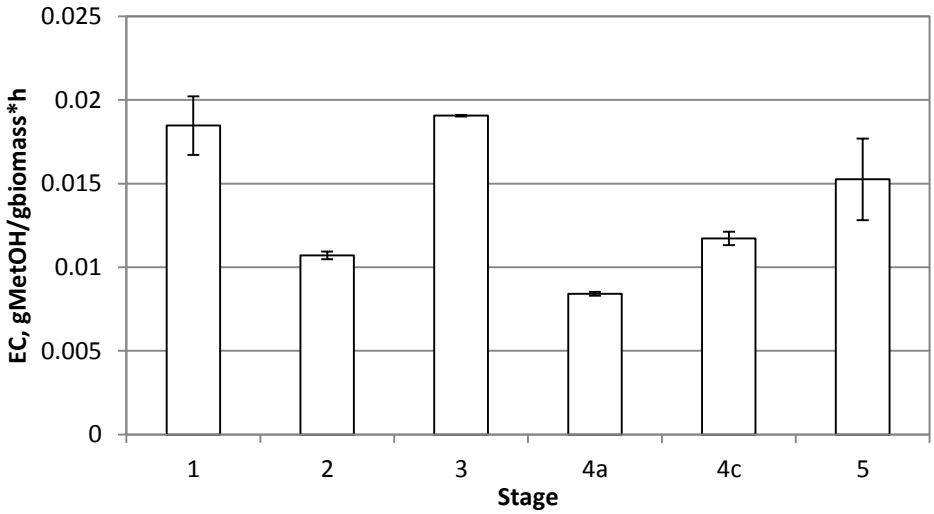

Figure A9. Biomass based elimination capacity in biofilter II.

Biofilter III

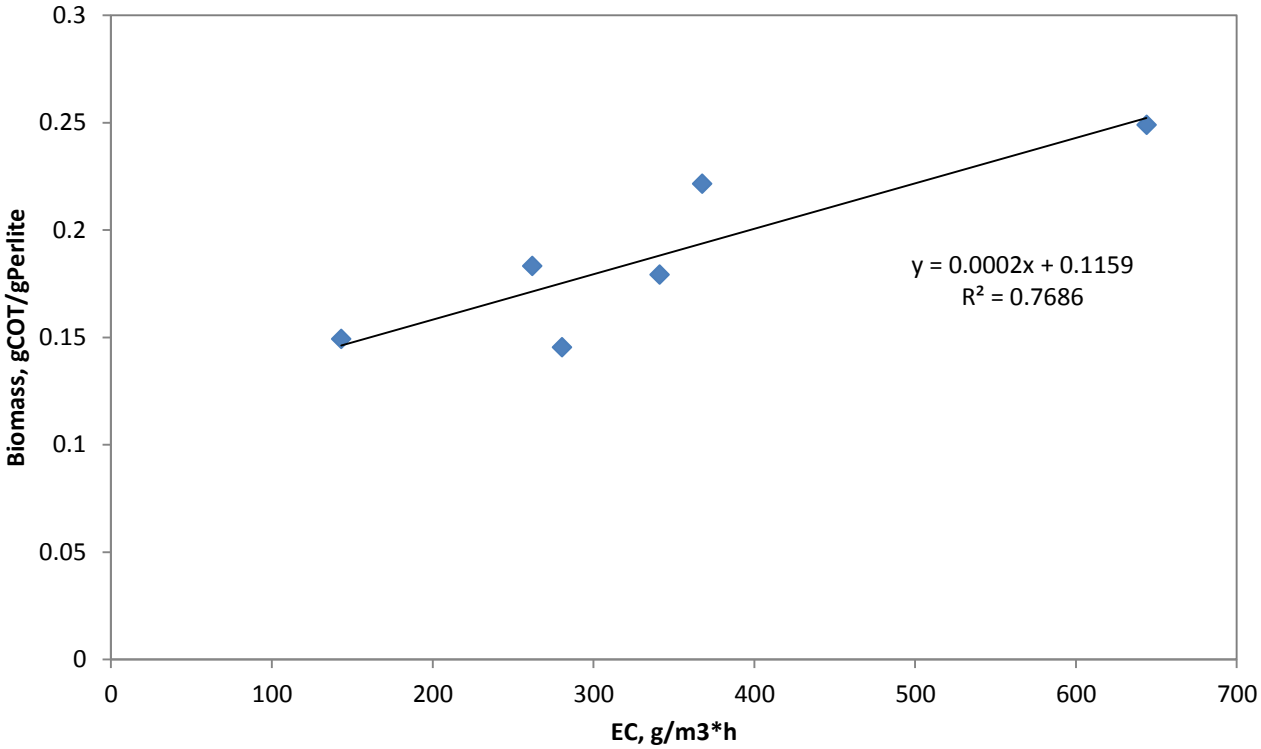

Figure A10. EC and biomass correlation during the whole operation time in biofilter III.

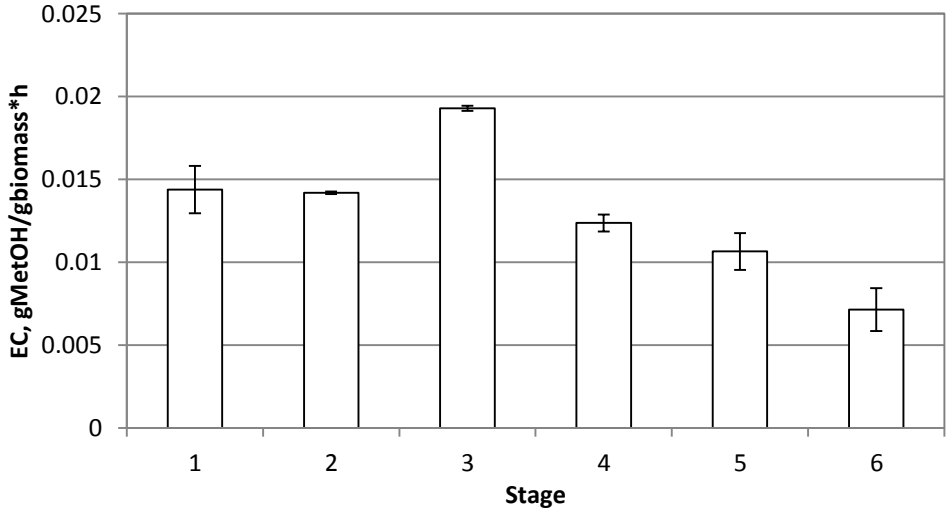

Figure A11. Biomass based elimination capacity in biofilter III.