Enzyme Engineering

9. Applications(3): Energy & Environment

9.1 Biodiesel Production

9.2 Bioethanol Production

9.3 Enzymatic Denitrification

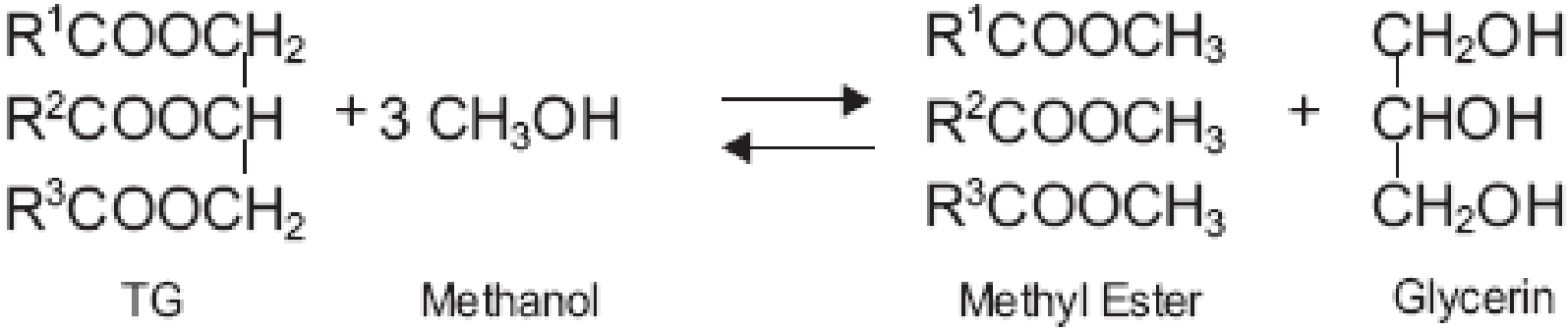
9.1 Biodiesel Production

Biodiesel

- Mixture of mono-alkyl esters (fatty acid alkyl esters)
- Typically made by transesterification of vegetable oils or animal fats.
- Advantages
  - Renewable resource
  - Biodegradable and less toxic
  - Favourable combustion emission profile
    - Low carbon monoxide, sulphur oxide, and nitrogen oxide
  - High flash point
    - Less volatile and safer to transport

Transesterification process

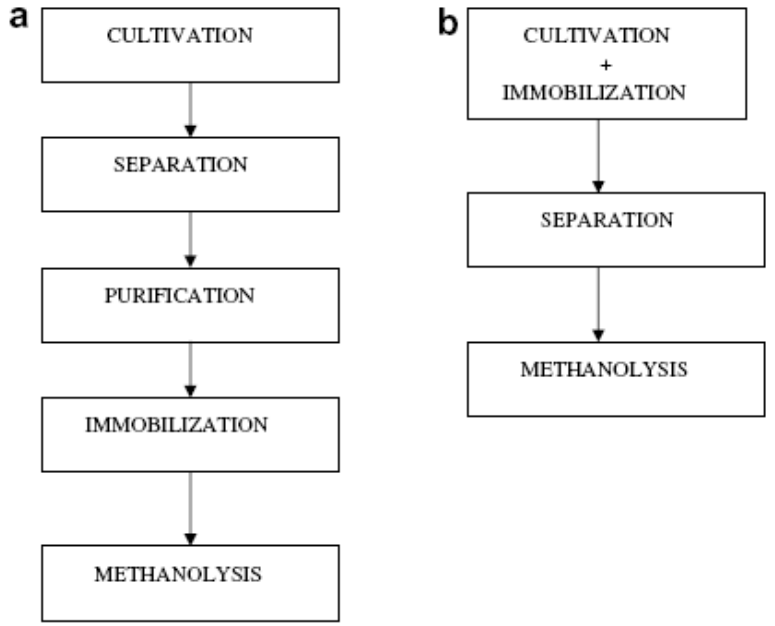
- Conversion of triglyceride to fatty acid alkyl esters



- Bottlenecks for lipase-catalyzed biodiesel production
  - Enzyme inhibitions by methanol and glycerol
  - Decrease of enzyme activity
  - High cost of lipase

Transesterification by lipase

- Enzymatic production is possible using both extracellular and intracellular lipases.
- In both the cases the enzyme is immobilized and used which eliminates downstream operations like separation and recycling.
- Both the processes are reported to be highly efficient compared to using free enzymes.



Biodiesel production

Table 1
Comparison of various works on enzymatic production of biodiesel

S.No	Authors/year	Oil/enzyme	Acyl acceptor	Conversion (%)	Technique employed	Cost of production
1	Watanabe et al. (2000)[a]	Vegetable oil, Novozyme 435	Methanol	90–93	Stepwise addition of methanol	Moderate
2	Samukawa et al. (2000)[a]	Soyabean oil, Novozyme 435	Methanol	97	Stepwise addition methanol and preincubation of enzyme in methyl oleate and soyabean oil	High
3	Ban et al. (2001)[b]	Vegetable oil, *R. oryzae*	Methanol	90	Stepwise addition of methanol and application of glutaraldehyde for stability of enzyme	Low
4	Iso et al. (2001)[a]	Triolein, *P. flourescens*	Butanol	90	Butanol was used as an acyl acceptor and no solvent was used	Moderate
5	Shimada et al. (2002)[a]	Waste cooking oil, Novozyme 435	Methanol	90	Stepwise addition of methanol	Low
6	Bako et al. (2002)[a]	Sunflower oil, Novozyme 435	Methanol	97	Stepwise addition of methanol and removal of glycerol by dialysis	High
7	Du et al. (2004)[a]	Soyabean oil, Novozyme 435	Methyl acetate	92	A novel acyl acceptor, methyl acetate which had no inhibitory effects was used	High
8	Xu et al. (2004)[a]	Soyabean oil, Novozyme 435	Methanol	98	Stepwise addition of methanol and removal of glycerol using the solvent, *iso*-propanol	High
9	Li et al. (2006)[a]	Rapeseed oil, Novozyme 435 & Lipozyme TL IM	Methanol	95	Combined use of Lipozyme TL IM and Novozyme 435 along with *tert*-butanol as solvent	High
10	Royon et al. (2007)[a]	Cotton seed oil, Novozyme 435	Methanol	97	*tert*-Butanol was used as a solvent	High
11	Modi et al. (2007)[a]	Jatropha oil, Novozyme 435	Ethyl acetate	91.3	Ethyl acetate having no inhibitory effects was used	High
12	Hama et al. (2007)[b]	Soyabean oil, *R. oryzae*	Methanol	90	Stepwise addition of methanol in a packed bed reactor	Low

[a] Extracellular lipase.
[b] Intracellular lipase.

Continuous reactor

Author	Oil	Enzyme	Reactor type	Remarks	Conversion	Year
S. Halim et al.	Waste cooking palm oil	Novozyme 435	Packed bed reactor	tert-butanol	80% for 120hr	2009
Royon et al.	Cottonseed oil	Novoayme 435	Fixed bed reactor	tert-butanol	95%	2007
Watanabe et al.	Waste edible oil	Novozyme 435	Fixed bed reactor	Three step reaction	90.9%	2006
Shimada et al.	Vegetable, tuna and waste edible oil	Novozyme 435	Packed bed reactor	3 packed bed reactor in series	90% for 100 days	2002
Watanabe et al.	Vegetable oil	Novozyme 435	Packed bed reactor	2 and 3 packed bed reactors in series	93% for 100 days	2000

Extracellular lipase

- Such enzyme-catalyzed processes in entirely nonaqueous solvents were described by Klibanov et al. (1984).

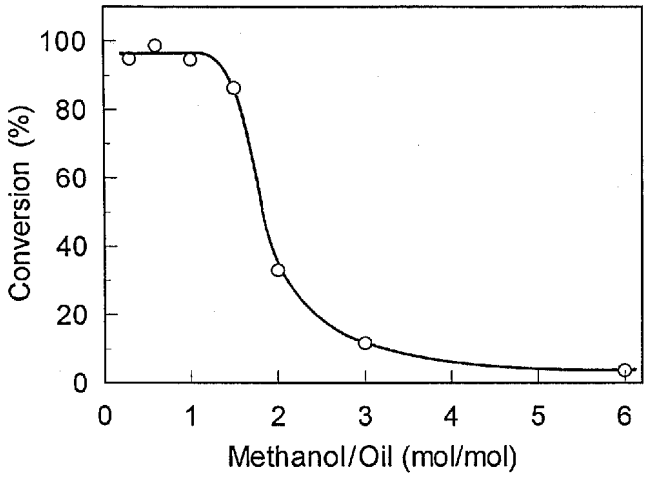
- *Candida antarctica* lipase

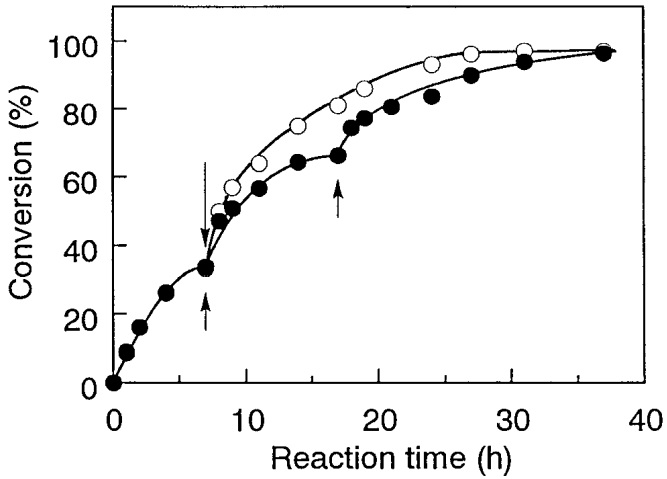
Methanolysis of Vegetable Oil by Immobilized Lipases[a]

	Conversion (%)[b]			
Lipase	First	Second	Third	Fourth
Rhizomucor miehei	19.1	21.8	18.6	19.4
Candida antarctica	16.0	26.6	30.6	33.1
Rhizopus delemar	2.0	0.5	0.3	0.5
Fusarium heterosporum	8.7	1.8	0.5	0.6
Aspergillus niger	0.4	0.5	0.5	0.6

  - Novozyme 435 (CALB immobilized on acrylic resin)
  - The most effective lipase among any of the lipases tested for methanolysis.

Extracellular lipase





Extracellular lipase

Table 2. Collection of studies from which productivity (kg biodiesel/kg enzyme) can be calculated. The data are used to calculate the maximum enzyme cost for breakeven with the cost of chemical catalysts in biodiesel production.

Reference	Oil	Enzyme	Yield [%]	Productivity [kg biodiesel/kg enzyme]	Calculated[§] max. enzyme cost [USD/kg enzyme]
[42]	soy	CALB	>96	1200	30
[43]	soy	CALB	>97	470	12
[40]	cottonseed	CALB	95	2000	50
[44]	soy	CALB	>70	5400	135
[25]	rapeseed	CALB + TLL	95	4250	106
[45]	acid oil	CALB	>71	7400	185
[45]	acid oil	CALB	>90	1700	43

[§] Productivity (kg biodiesel/kg enzyme) multiplied with a catalyst cost of 0.025 USD/kg biodiesel.

- *Candida antarctica B-lipase (CALB);* Novozym 435
- *Thermomyces lanuginosa* (TLL); Lipozyme TL IM

Intracellular lipase

- Bacteria, yeast and filamentous fungi that would serve as whole-cell biocatalysts based on their ability of immobilization and the display of functional proteins of interest on their cell surface.

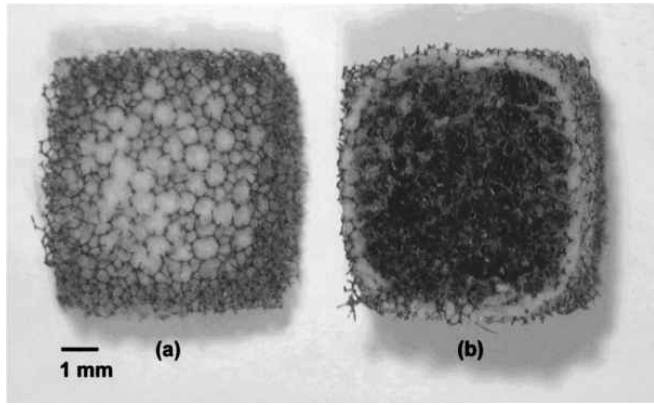
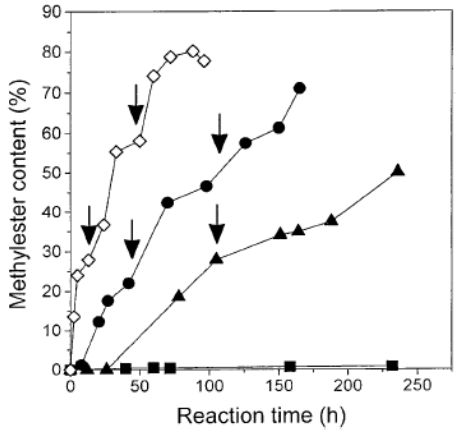


FIG. Micrographs of surface (a) and cross-section (b) of a BSP

- Developed by Matsumoto et al. (2001)
  - *R. oryzae* cell
  - Stepwise methanolysis of plant oil
  - In solvent free and water containing system
  - 71% after a 165 hr at 37℃

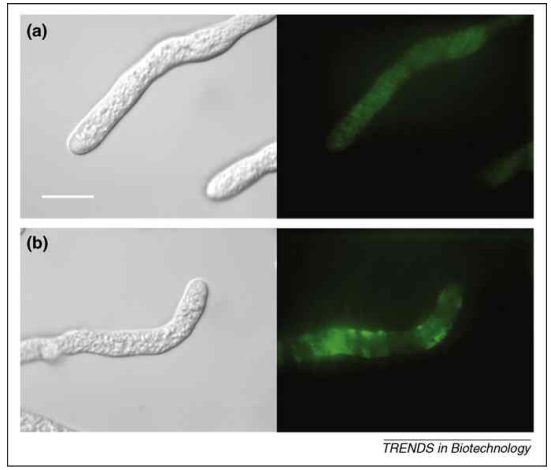


K. Ban et al. / Biochemical Engineering Journal 8 (2001) 39–43

Intracellular lipase

I. Addition of substrate-related compounds to the culture medium improved the lipase activity.

II. Gluteraldehyde cross-linking of membrane bound lipase
   - High lipase activity could be maintained over several cycles.



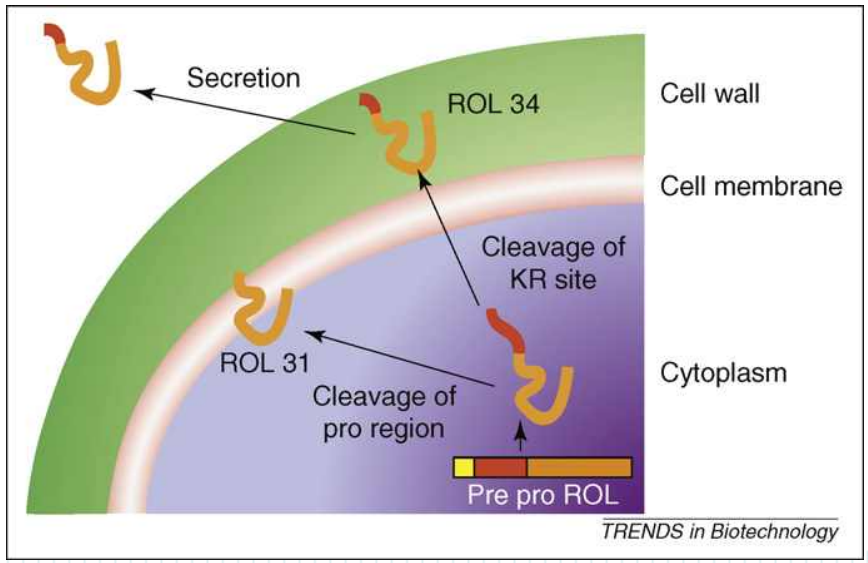
III. Fatty acid membrane composition
   - The membrane permeability and stability is significantly affected by the fatty acid composition.

IV. Yatalase treatment; An enzyme that partially degrades fungal cell wall and allows the exposure of membrane-bound lipase to the substrate, thereby enhancing its activity.

Intracellular lipase

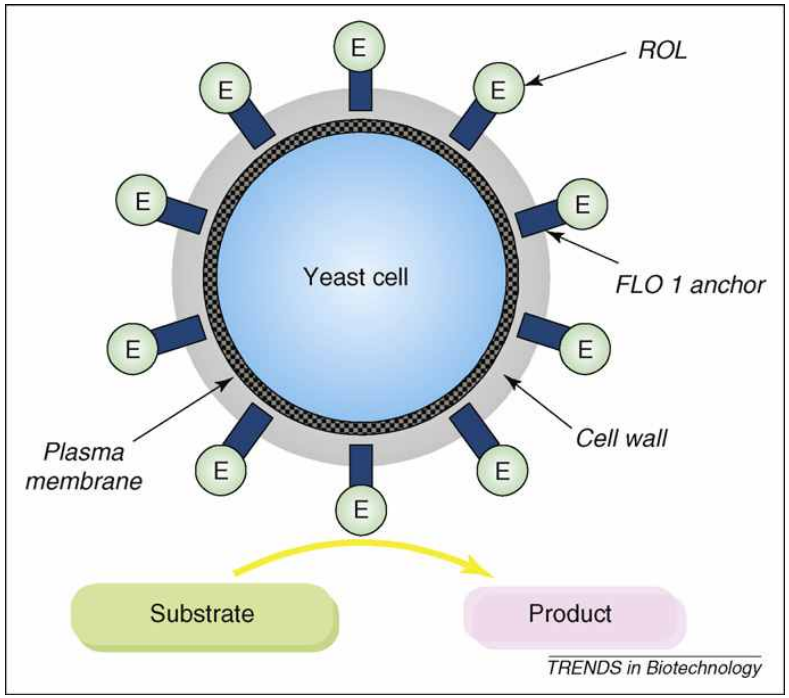
Secretory mechanism and localization

- In the immobilized culture, the fungal mycelium is immobilized into BSPs and large amounts of lipase are retained within the cells.
- Cleavage of pro-region from the precursor lipase Pre-pro ROL gives rise to two lipases.
  a. Cell membrane; ROL 31
  b. Cell wall; ROL 34
- Cell immobilization strongly inhibited the secretion of ROL 31 into the culture medium.



Intracellular lipase

- A yeast cell surface display system for lipase from R. oryzae was developed.
- Schematic diagram of a yeast whole-cell biocatalyst displaying ROL via an FOL 1 anchor.
- Lipase displaying system
  - the N-terminus of ROL including a pro-sequence Pro-ROL has been fused to the flocculation functional domain of FLO p, a lectin-like cell-wall protein of yeast.

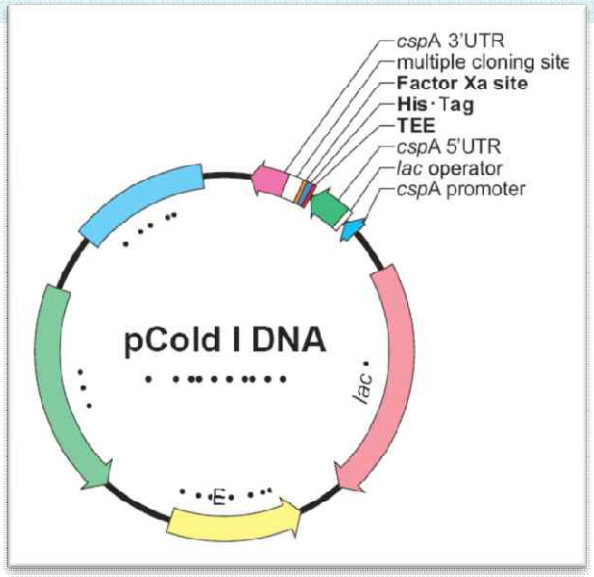


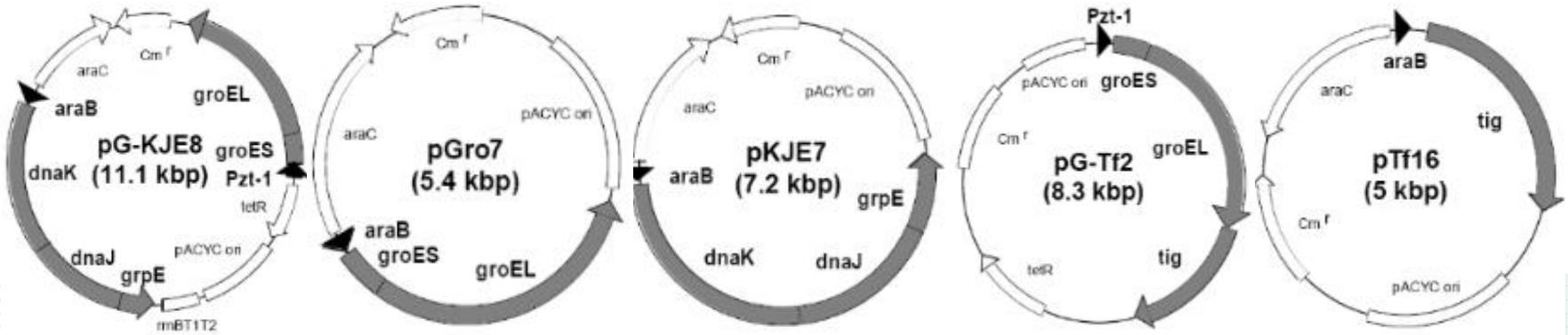
Extracellular lipase vs. Intracellular lipase

- An outstanding reaction rate was obtained with Novozym 435 in continuous operation (7 h with 92–94% ME content) and fed-batch operations (3.5 h with 87% ME content) respectively.
- Considerable reduction in cost can be achieve with intracellular lipase.
- The reaction rate of intracellular lipase is very slow in a batch reaction and more than 70 h are required to obtain 80–90% ME content.

CalB Expression in *E.coli*

- Materials
  - Cell: *E.coli*
  - 1) DH5α
  - 2) Rosettagami
  - 3) Novablue
  - Vector: pCold I
    - Cold shock promoter
    - N-terminal 6-His tag
    - Selection : Ampicillin
- Chaperone plasmid map

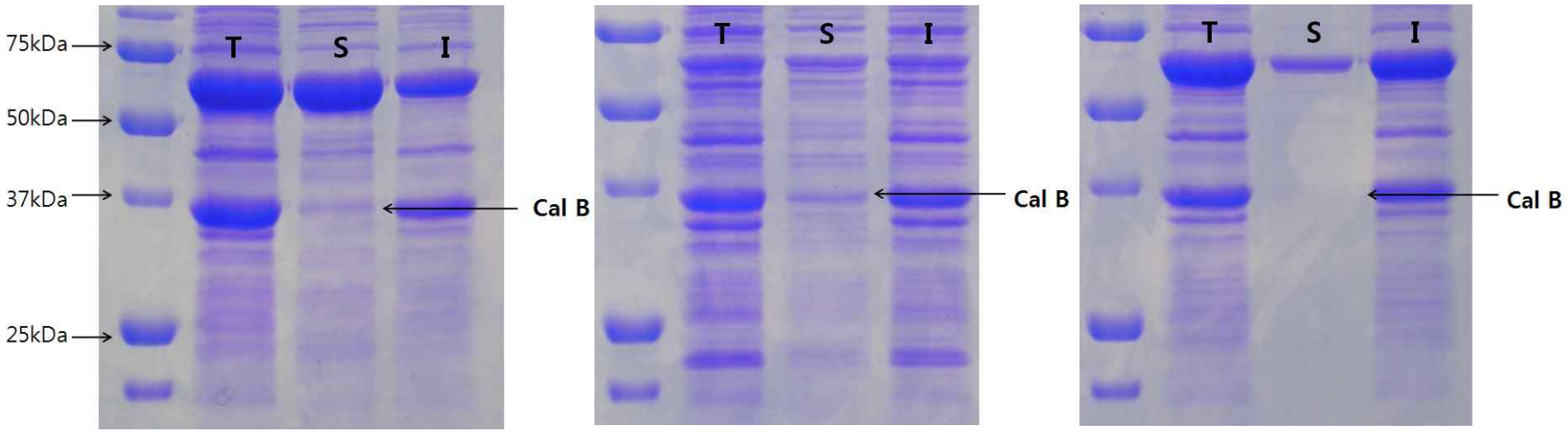




CalB Expression in *E.coli*

- Results
  - (1) Confirmation of lipase activity
  - (2) Halo test
  - (3) SDS-PAGE
    - Co-expression of Cal B and Chaperone

DH5α Rosettagami Novablue



DH5α Rosettagami Novablue

T : Total
S : Soluble
I : Insoluble

CalB Expression in *E.coli*

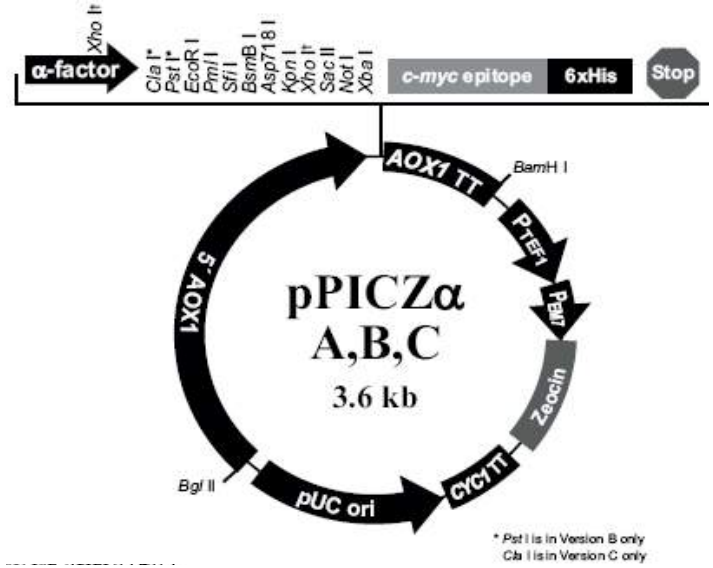
- *E. coli*를 이용하여 *Candida antarctica*에서 유래된 lipase B (Cal B)의 발현여부를 빠르게 확인하고, 특성을 분석하여 효율적인 발현시스템을 구축하며, 대량발현 시스템에 적용이 가능한지를 확인하였다.

- 발현 효율이 높고 활성이 좋은 재조합 lipase 개량에 있어서 빠른 확인을 할 수 있는 시스템을 확보하였다.

- *E. coli*에서 lipase를 효율적으로 발현하기 위해서는 숙주세포 DH5α에 Cal B 유전자가 재조합된 pCold I vector와 pGro7(groES/groEL)을 공동 형질전환 시킨 재조합 균주가 Cal B 발현시스템에 가장 효율이 좋았다.

CalB Expression in *Pichia pastoris*

- Materials
  - Cell: *Pichia pastoris* X-33
  - Vector: pPICZα B
    - α-peptide: protein secretion
    - AOX1 promoter: induction by methanol
    - Selection: Zeocin



Clone gene of interest into the pPICαA

Linearize construct with Sac I

Transform X33 using EasyComp™ method

Plate transformants on medium containing Zeocin

All transformants integrate at 5' AOX1 locus by single crossover

Select 6-10 colonies for small-scale expression

Choose highest expressers for large-scale expression

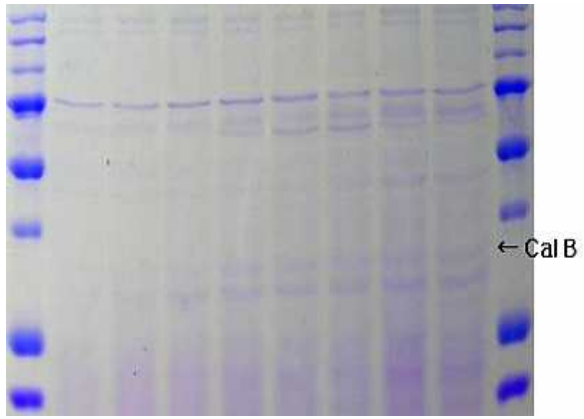
CalB Expression in *Pichia pastoris*

- Results
  - (1) Confirmation of lipase activity

Normal colony

Halo formation by methanol induction

  - (2) Halo test
    - Halo formation around colony by oil degradation due to lipase secretion
  - (3) SDS-PAGE
    - Confirmation of CalB expression and secretion

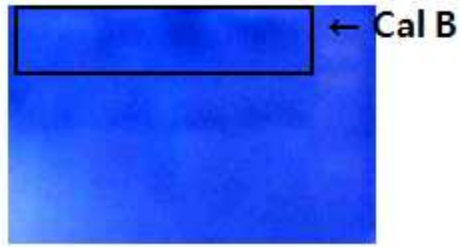


Concentrated media

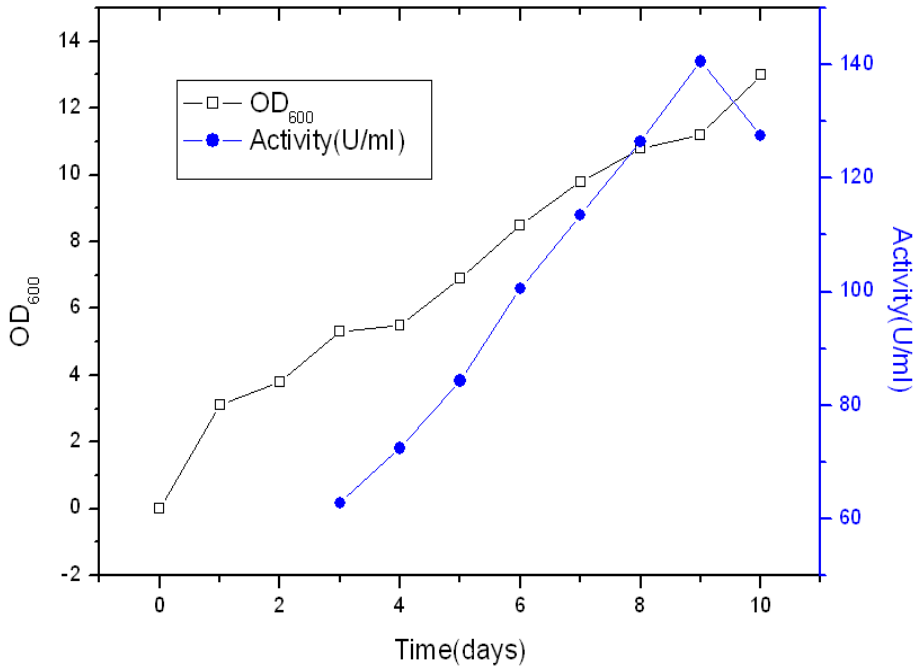
Non-concentrated media

CalB Expression in *Pichia pastoris*

- (4) Cal B Zymogram
- Confirmation of CalB expression and secretion



- (5) Activity test
- Measurement of Cal B activity using p-NPP



Time (days)	OD400	Unit/ml
3	0.057	62.71
4	0.066	72.44
5	0.077	84.34
6	0.092	100.55
7	0.104	113.53
8	0.116	126.50
9	0.129	140.56
10	0.117	127.58

Summary

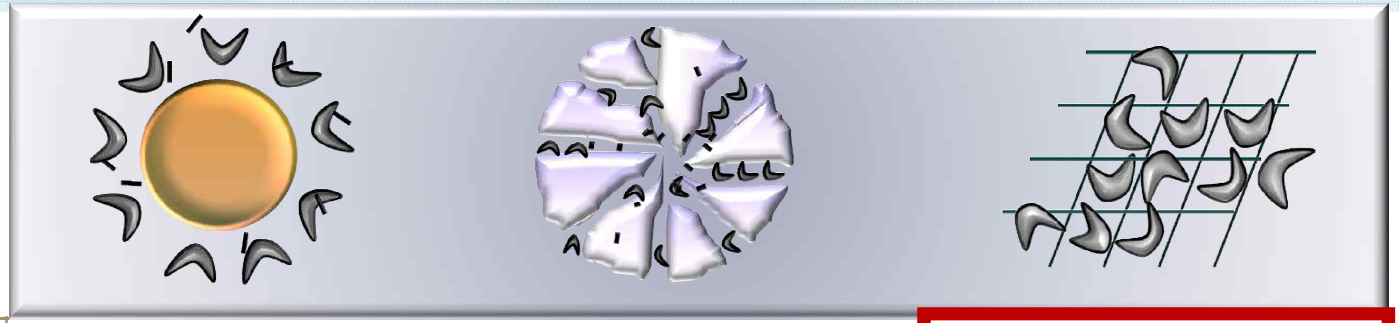
- The pellet sample's band was much clearer than that of the supernatant sample on the SDS-PAGE gel when the CalB expression was checked in the Pichia pastoris X-33 cell culture that was electroporated with the pPICZαB vector recombining a CalB gene.

- From the thickness of the bands the expression level can be confirmed. As the culture time increased, CalB secreted to the media accumulated thereby thickening the bands.

- When the activity of the enzyme was measured using p-NPP, the maximum value of 140,560U/L was reached on the day 9 of the culture. During the cell culture the cell growth reached 57.54~71.64g/L when BMGY→BMMY media was used.

Biocatalyst immobilization methods

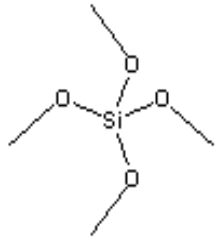
SNU : Sol-gel 방법을 사용



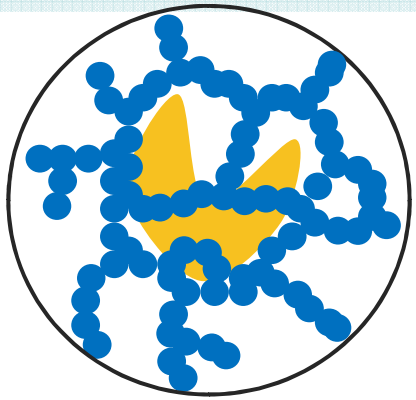
Immobilization methods	Covalent binding on beads	Covalent binding on mesoporous silica	Sol-gel entrapment
Advantages	• Easiness of substrate binding on biocatalyst	• Protection of biocatalyst from solvents • Large surface area to maximize enzyme loading amount	• Protection of biocatalyst from solvents • Maintenance of enzyme native structure • Simple process • Immobilization under mild condition
Disadvantages	• Deactivation by solvent attack	• Diffusion limitation	• Diffusion limitation

Sol-gel entrapment

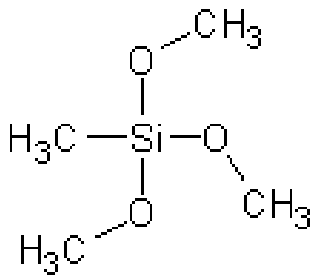
지금까지의 방법 : **Hydrophobic precursor**사용



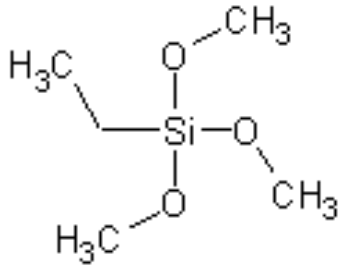
TMOS(Tetramethoxysilane)



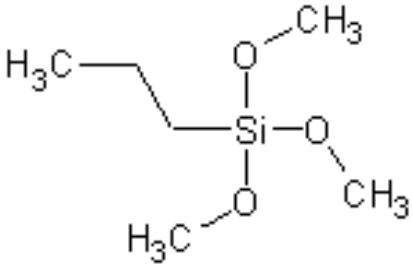
- Use of hydrophobic precursors
  - Known as CalB maintains higher activity in hydrophobic circumstance
  - Easiness of oil feeding to enzyme
  - Prevention of enzyme deactivation by hydrophilic methanol



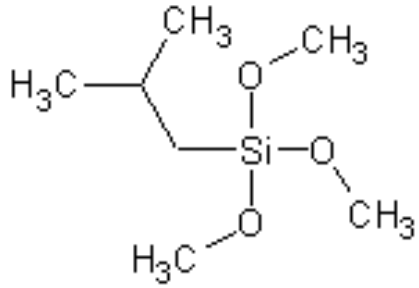
MTMS
(Methyltrimethoxysilane)



ETMS
(Ethyltrimethoxysilane)



PTMS
(Propyltrimethoxysilane)



iso-BTMS
(*iso*-Buthyltrimethoxysilane)

Selection of sol-gel matrix

IDEA :
Hydrophobic-hydrophilic 한 precursor의 비율을 조정 및 최적화

- Comparison of the activity in various matrix
  - TMOS (hydrophilic)
  - MTMS, ETMS, PTMS, *iso*-BTMS (hydrophobic)

matrix	TMOS	MTMS*	ETMS*	PTMS*	*iso*-BTMS*
Activity (U/g $_{support}$)	0.144	0.482	0.579	0.267	0.460
Specific activity (U/g $_{enzyme}$)	9.71	13.4	21.03	7.54	5.13

* Mixed with TMOS (TMOS: hydrophobic precursor= 1:4)

Lipase immobilized in ETMS mixed with TMOS showed the highest activity and specific activity.

ETMS/TMOS

Pretreatment of biocatalyst

IDEA : Oil 전처리 방법 도입

- Activity maintenance and acquisition of stability of CalB
- Methods
  - Olive oil pretreatment for 1hr at 30℃
    - steric hindrance around active site during immobilization
  - GA pretreatment for 1hr at 25 ℃

Matrix	Unpretreated	GA pretreated	Oil pretreated	Oil and GA pretreated	Novozym 435
Activity (U/g support)	0.579	0.498	0.896	0.595	0.916
Specific activity (U/g enzyme)	21.03	17.65	47.23	33.81	-

- Result : Development of new biocatalyst having the same activity compared with Novozym435

Activity of Immobilized lipase

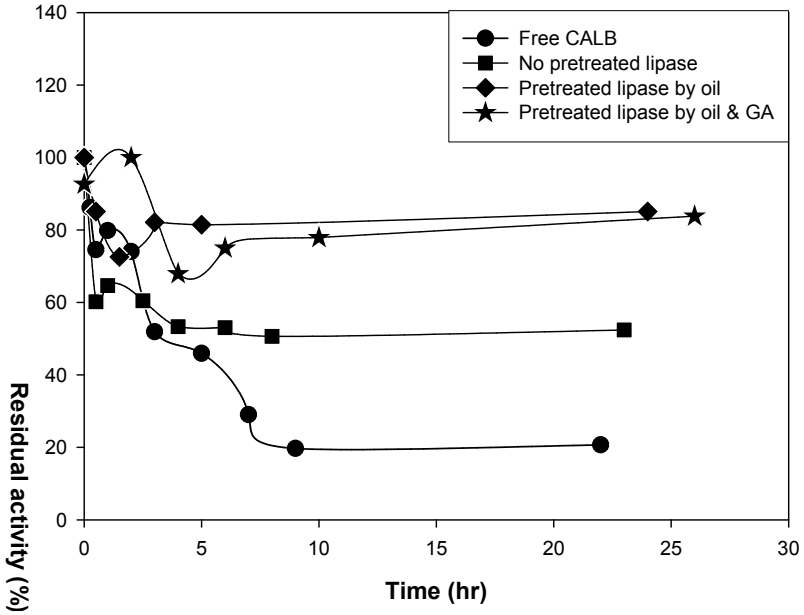
Sol-gel 제조방법 개선 및 **particle** 사이즈 변경 **&** 다른 **lipase**도 고정화

- Comparison of the activity
  - Lipase type
    - *Candida antarctica* lipase B
    - *Rhizomucor miehei* lipase
    - *Thermomyces lanuginosus* lipase
  - Particle size
    - 100-300 μm and 300-500 μm

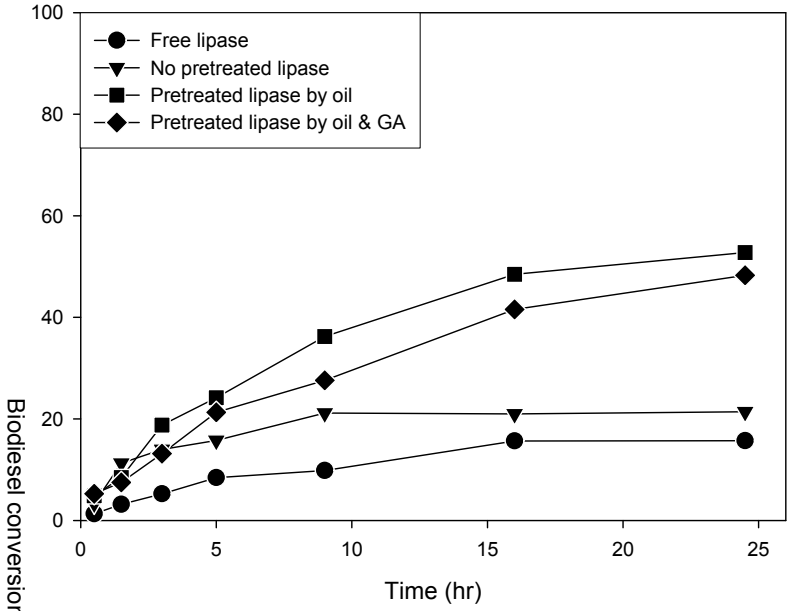
Scale	60 ml				3 ml
Lipase type	*Candida antarctica* lipase B		*Rhizomucor miehei* lipase	*Thermomyces lanuginosus* lipase	*Candida antarctica* lipase B
Particle size (μm)	100-300	300-500	300-500	300-500	300-500
Activity (U/g support)	2.346	1.351	1.822	2.467	0.896

Stability of immobilized enzymes

- Thermostability (60℃)



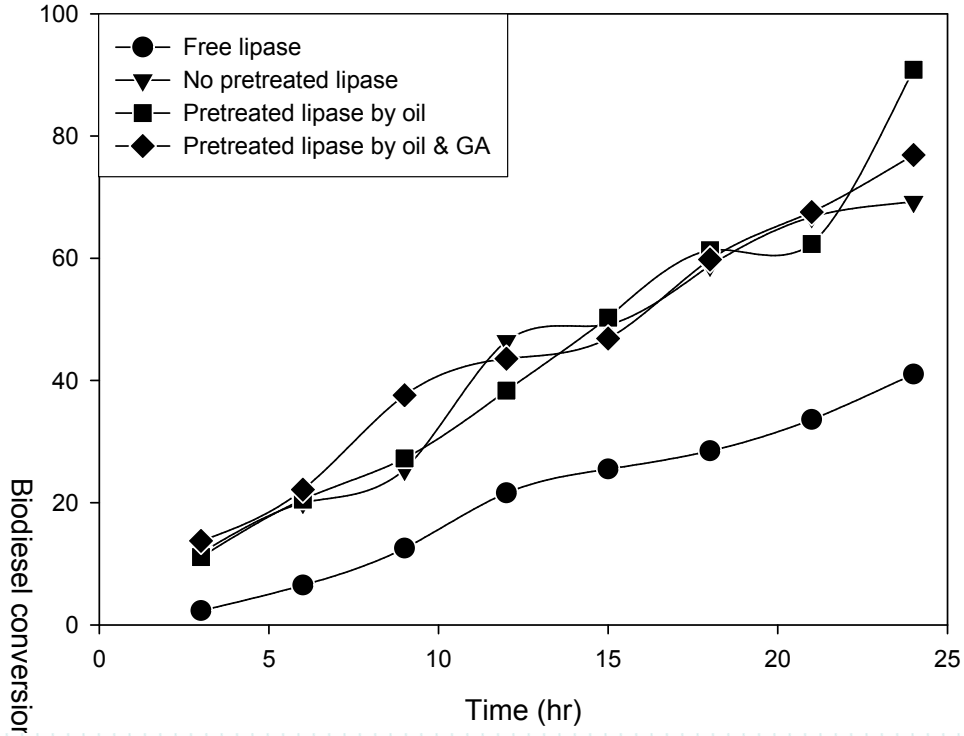
- Methanol tolerance
  (Oil : MeOH = 1:3)



Acquisition of thermostability and methanol tolerance via immobilization of pretreated CALB

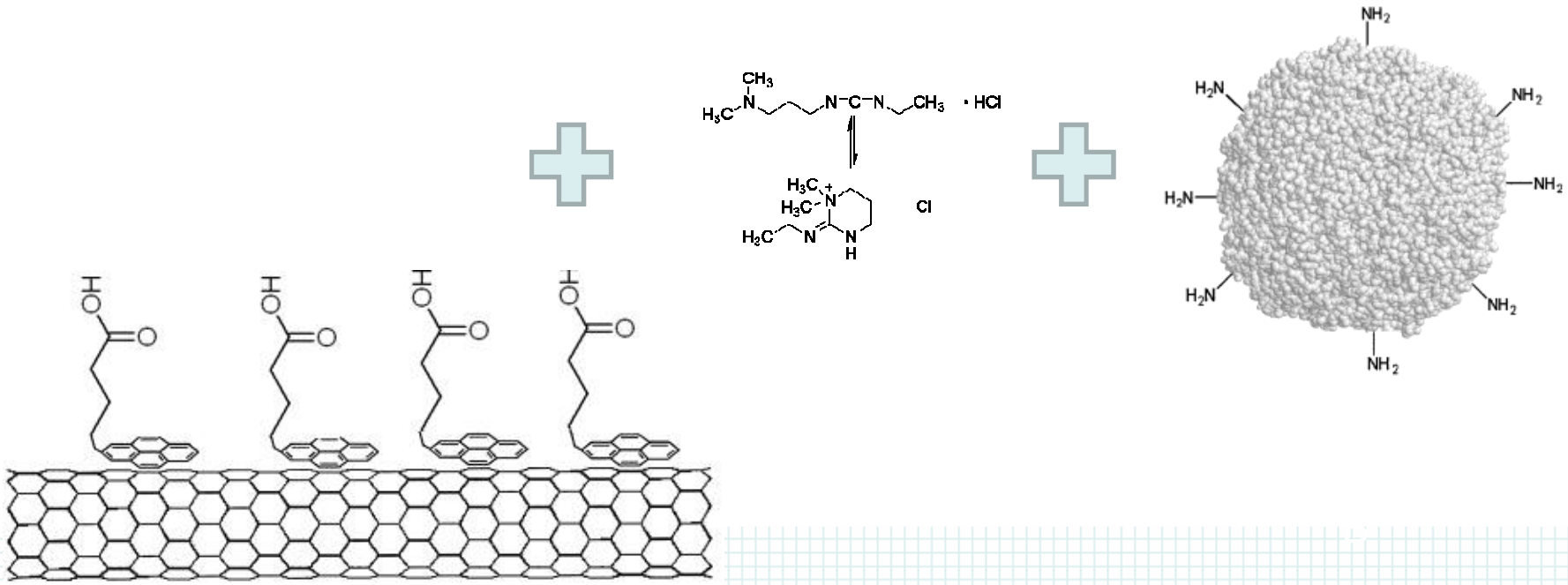
FAME production by immobilized lipases

- FAME conversion using free lipase and immobilized lipase
  - Meterials
    - Free lipase : Lipozyme (CalB)
    - Sol-gel entrapped CalB using ETMS precursor
  - Methods
    - Pre-incubation of oil with methanol for 18 hrs, 300 rpm, at 50℃
    - immobilized lipase: 25%(w/w) of substrate
    - Methanol stepwise addition
  - Results
    - Over 90% of conversion rate in oil pretreated biocatalyst



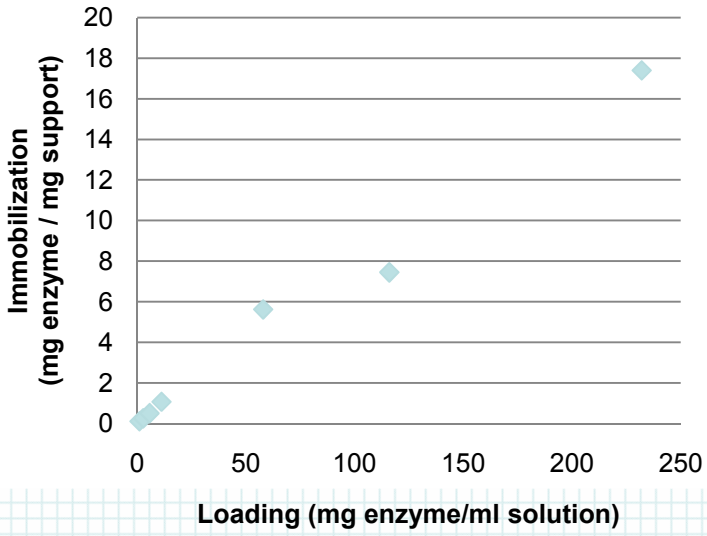
CalB immobilization

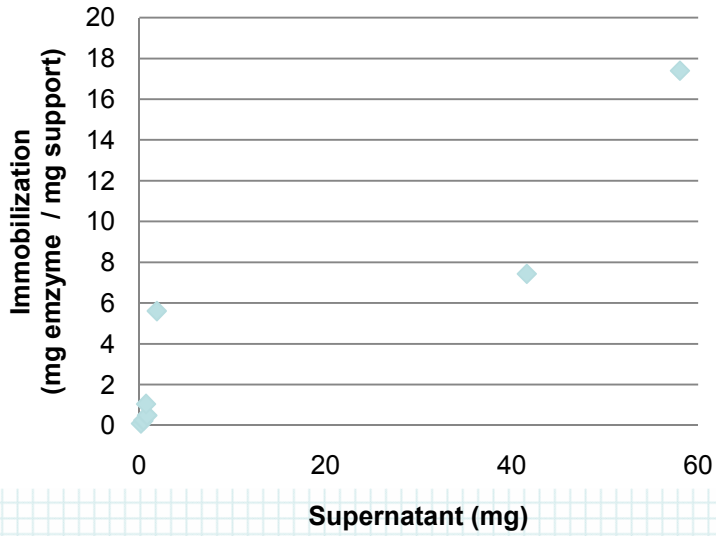
- CalB immobilization onto nanocarbon materials
  - High loading of enzyme
  - Enhancement of CalB stability in organic solvent
  - Support materials: functionalized nanocarbon material



Result_ Immobilization of CalB

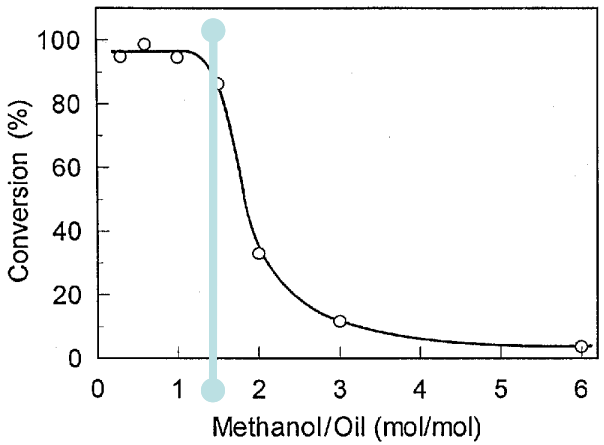
- 1ml of Lipozyme CALB L were immobilized with 10mg of nanoparticle.
- Single layer immobilization was formed around 6mg enzyme/mg support.
  - Maximum ammount : 17.4 mg enzyme/ mg support
  - Unusually higher amount ➔ repeat experiment again.

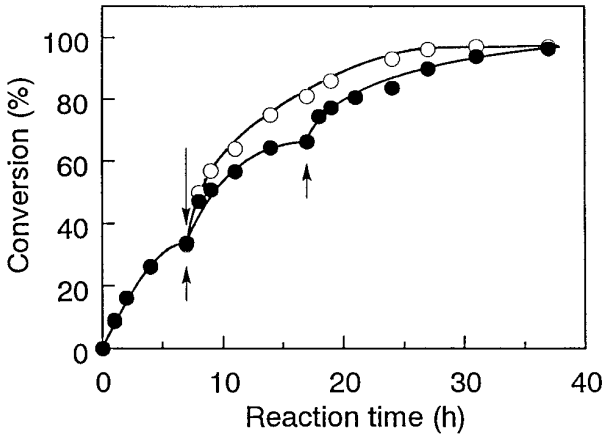




Methanol inhibition

- Methanol has a strong inhibitory effect on the lipase.
  - conversion decreased when methanol and oil molar ratio is more then 1.5.





- To prevent the methanol inhibition, step addition was suggested.

Glycerol inhibition

- A deposit of glycerol coating the immobilized catalyst is formed during the process, which reduces the enzymes activity .

- Glycerol removal by membrane was proposed.
- The two phase is separated by cellulose acetate membrane.
  - Primary phase: oil-biodiesel-glycerol mixture
  - Secondary phase: aqueous phase

  - Hydrophobic molecules(FAME and oil) can not pass this membrane.
  - Only hydrophilic ones, glycerol can pass this membrane.

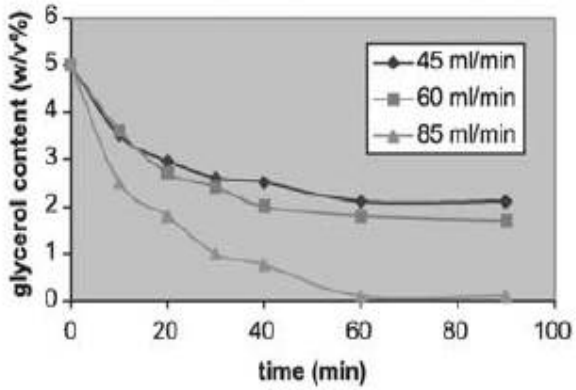


FIGURE 3 The effect of flow rate on removal of glycerol by dialysis.

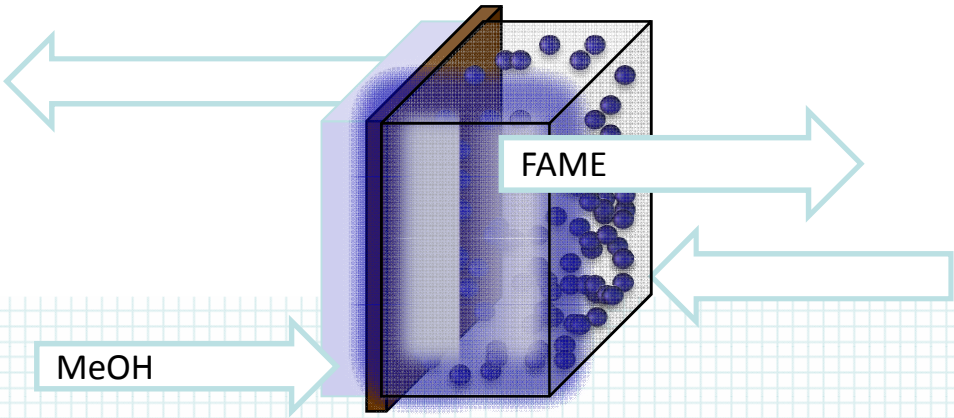
- The continuous inhibition of glycerol can be decreased.

Research objective

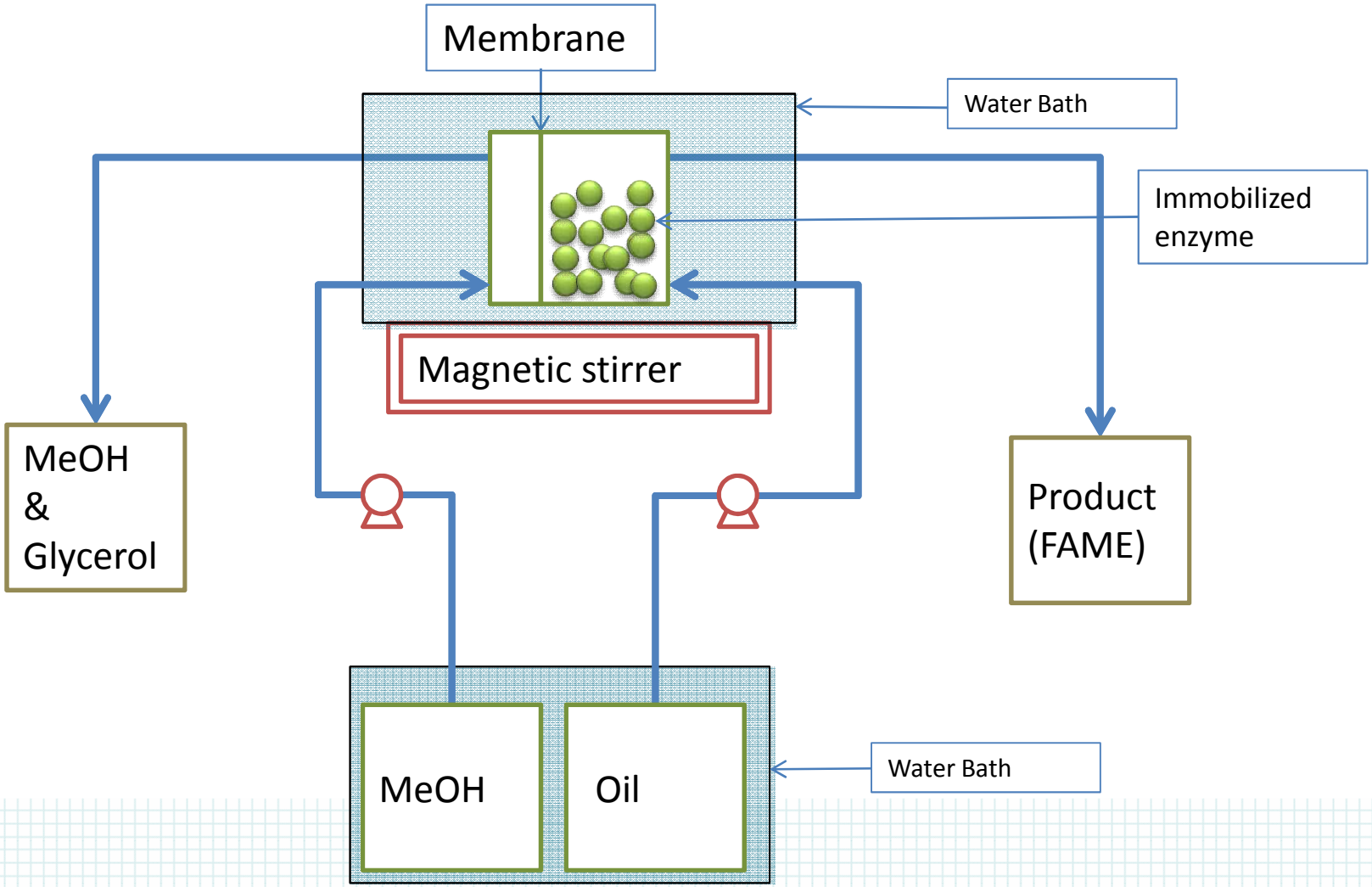
- The packed bed reactor has been investigated by several researchers for biodiesel production.
  - But step addition reactions were needed to prevent methanol inhibition.
  - And it needs down streams for glycerol separation.

- To overcome these disadvantages, flat sheet membrane reactor which was used for glyceride synthesis can be applicable for biodiesel production.

- Using hydrophilic membrane, the inhibition of continuously produced glycerol can be decreased and methanol can be fed continuously to improve the productivity during short operation time.



Continuous system



Experiment without enzyme

Materials

- Hydrophobic phase : Soybean oil 0.6 ml/min (40 ml)
- Hydrophilic phase: Methanol 0.6 ml/min (20 ml)

Reaction Condition

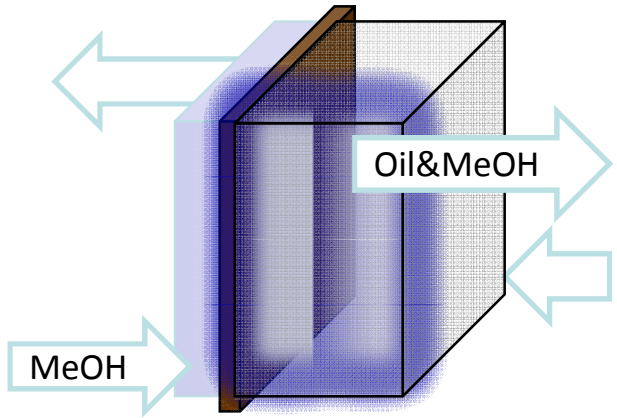
- Temperature: 50℃

Membrane

- Snake Skin Dialysis Tubing
  :Regenerated cellulose (10K MWCO)

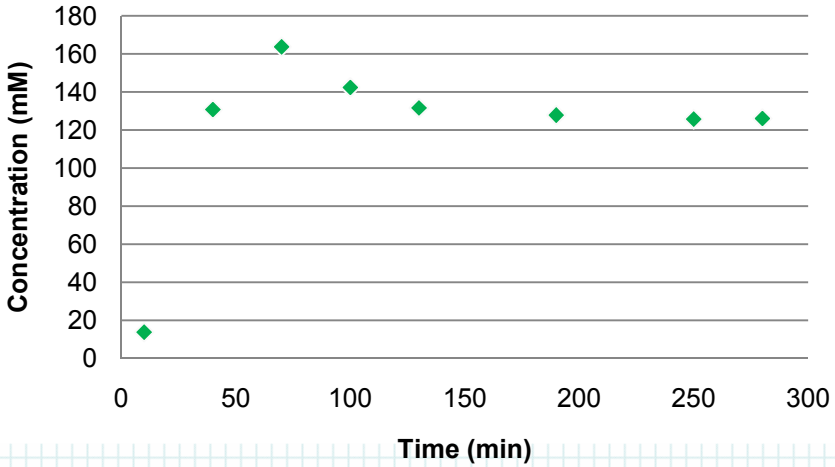
Analysis

- Varian 450-GC
  : HP-5 capillary column 25mx0.32mmx0.17μm

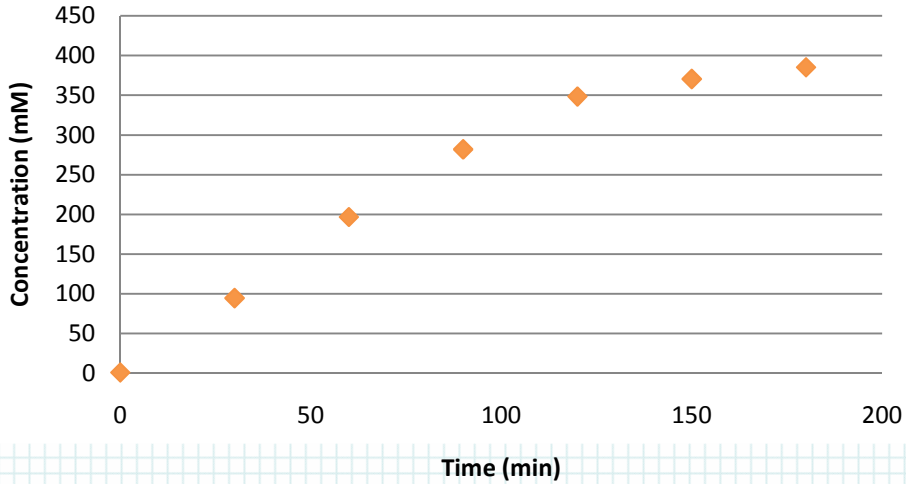


Result_ Methanol permeability

- Methanol permeation was tested without enzyme.
  - Without stirring, 130 mM of methanol was maintained after 2hrs.
  - With stirring;
    - Methanol concentration was increased steadily.
    - Maximum concentration of methanol was 400mM.



Methanol permeability without stirring



Methanol permeability with stirring

Experiment with Novozyme 435

Materials

- Hydrophobic phase : Soybean oil 0.6 ml/min (40 ml)
  Novozyme 4g
- Hydrophilic phase: Methanol 0.6 ml/min (20 ml)

Reaction Condition

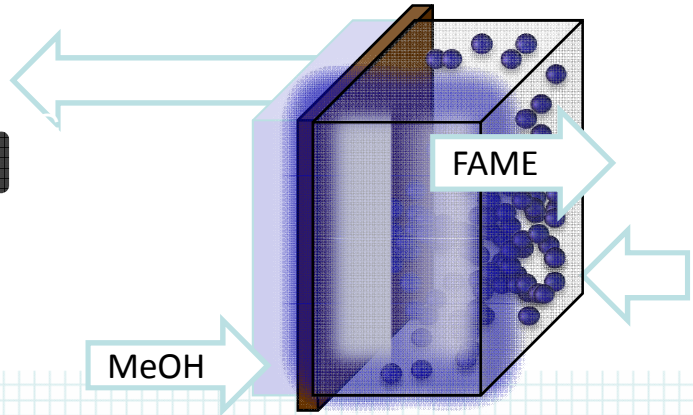
- Temperature: 50℃

Membrane

- Snake Skin Dialysis Tubing
  :Regenerated cellulose (10 K MWCO)

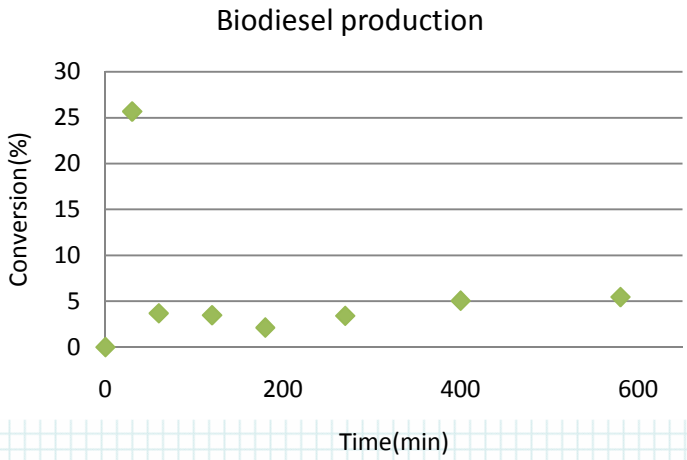
Analysis

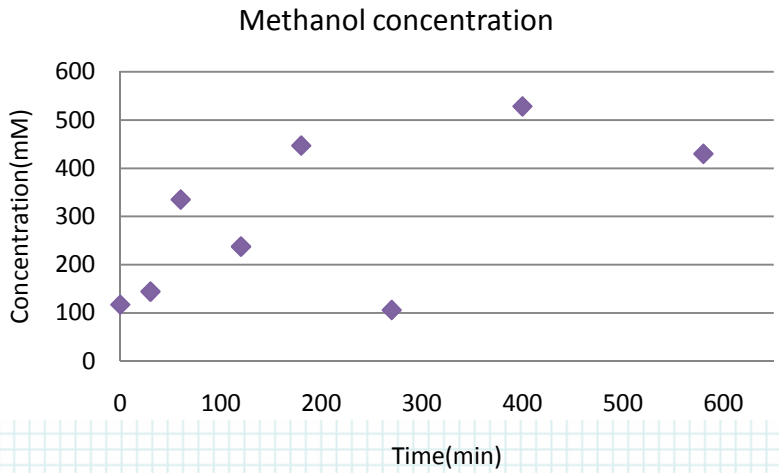
- Varian 450-GC
  : HP-5 capillary column 25mx0.32mmx0.17μm



Result _Biodiesel production

- Biodiesel production was tested with continuous system.
  - 25% conversion was achieved at 30 min.
  - After 1 hr conversion rate was dramatically decreased and this low conversion rate (below 6%) was maintained during 10hr.
  - This low conversion rate was based on enzyme deactivation by the methanol inhibition which is caused by high methanol concentration and mixing problem.





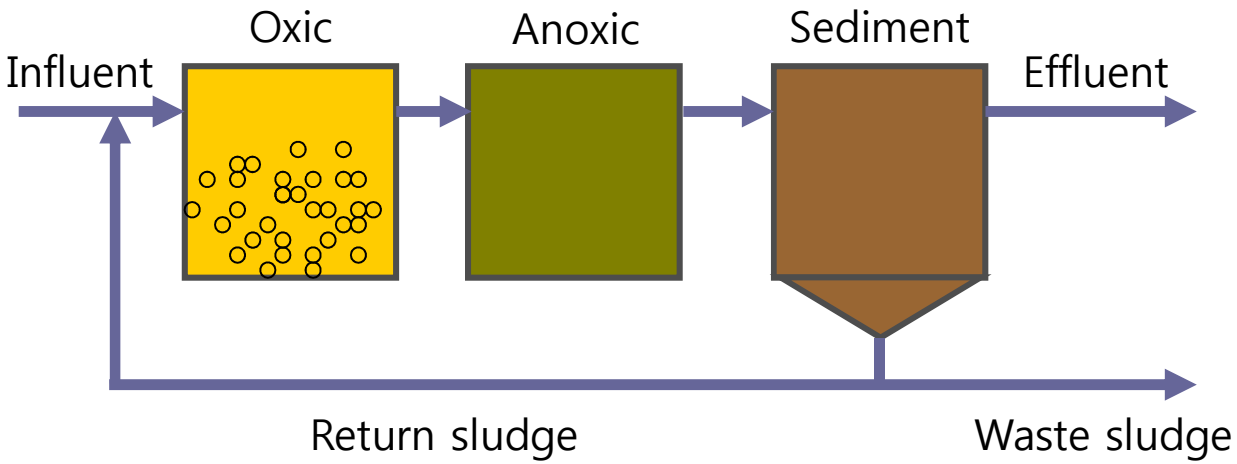
9.2 Bioethanol Production

9.3 Enzymatic Denitrification

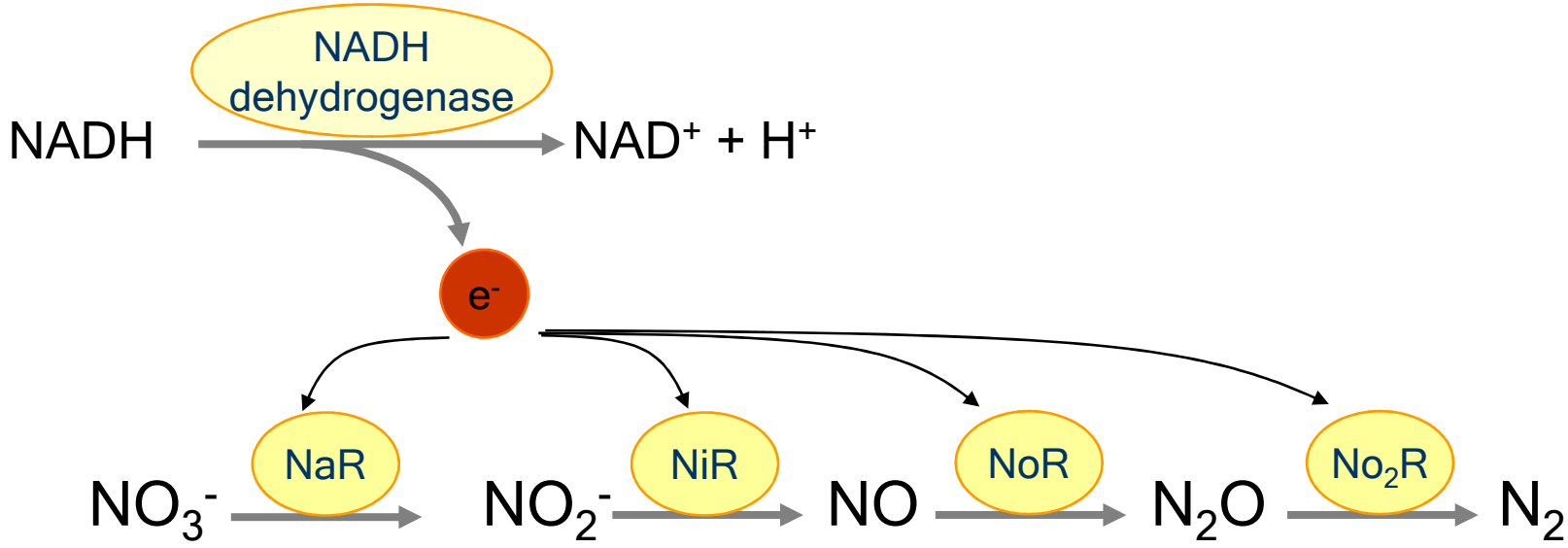
Biological Denitrification

- Economically and environmental friendly
- Microorganisms use nitrate as a final electron acceptor
- Occur under O_2 limitation condition

- General process



Enzyme Reactions



NaR: nitrate reductase

NiR: nitrite reductase

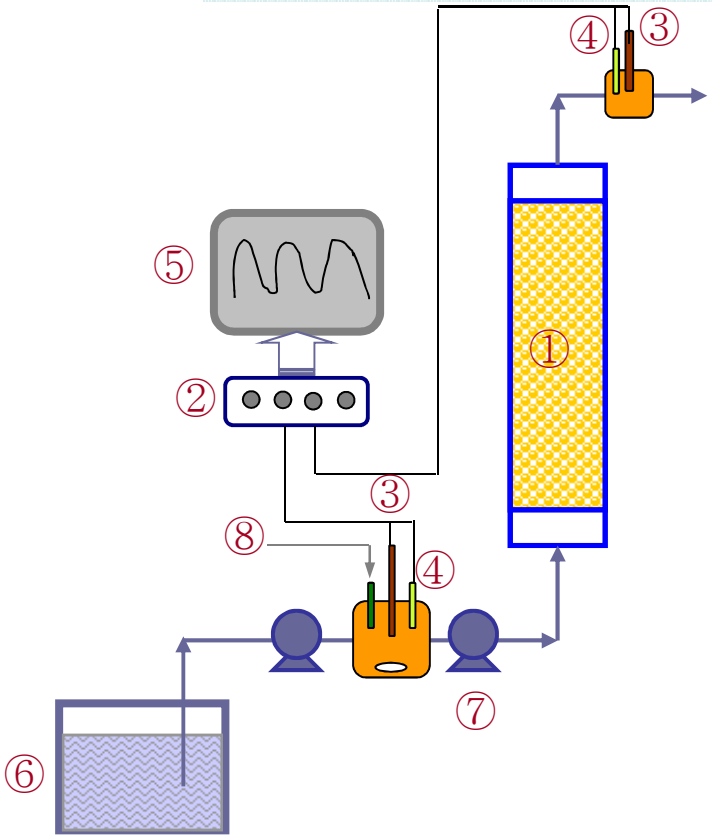
NoR: nitric oxide reductase

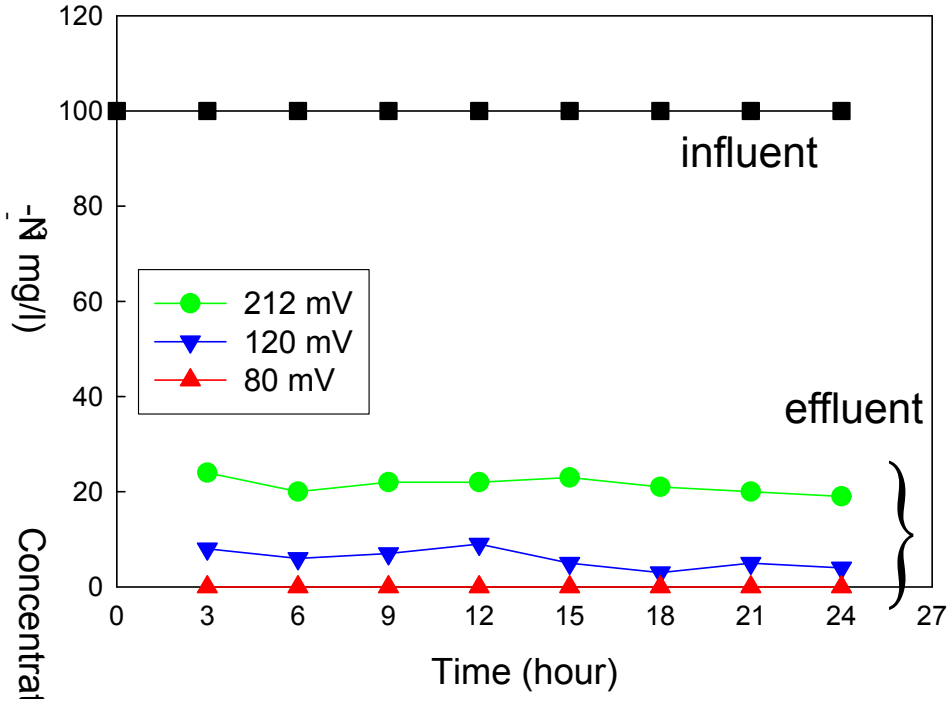
No_2R: nitrous oxide reductase

Screening of Denitrifying Bacteria

Microorganisms	Denitrification activity (nmol/min mg-cell)	References
Pseudomonas stutzeri	90.2	Carlson and Igrshsm (1983)
Pseudomonas aeruginosa	45.2	Carlson and Igrshsm (1983)
Paracoccus denitrificans	34.2	Carlson and Igrshsm (1983)
Flexibacter canadensis	23.4	Wu and Knowles (1994)
Pseudomonas stutzeri	67.2	Nussinovitch et al. (1996)
Shewanella putrefaciens	41.0	Krause et al. (1997)
Ochrobactrum anthropi SY509	104	This study

Packed-bed reactor

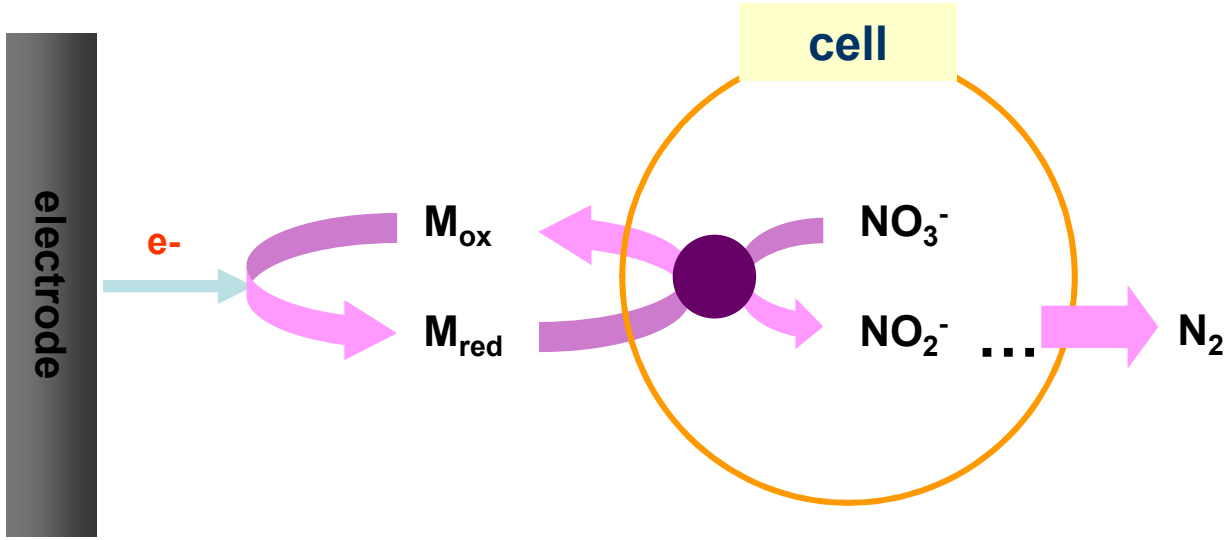




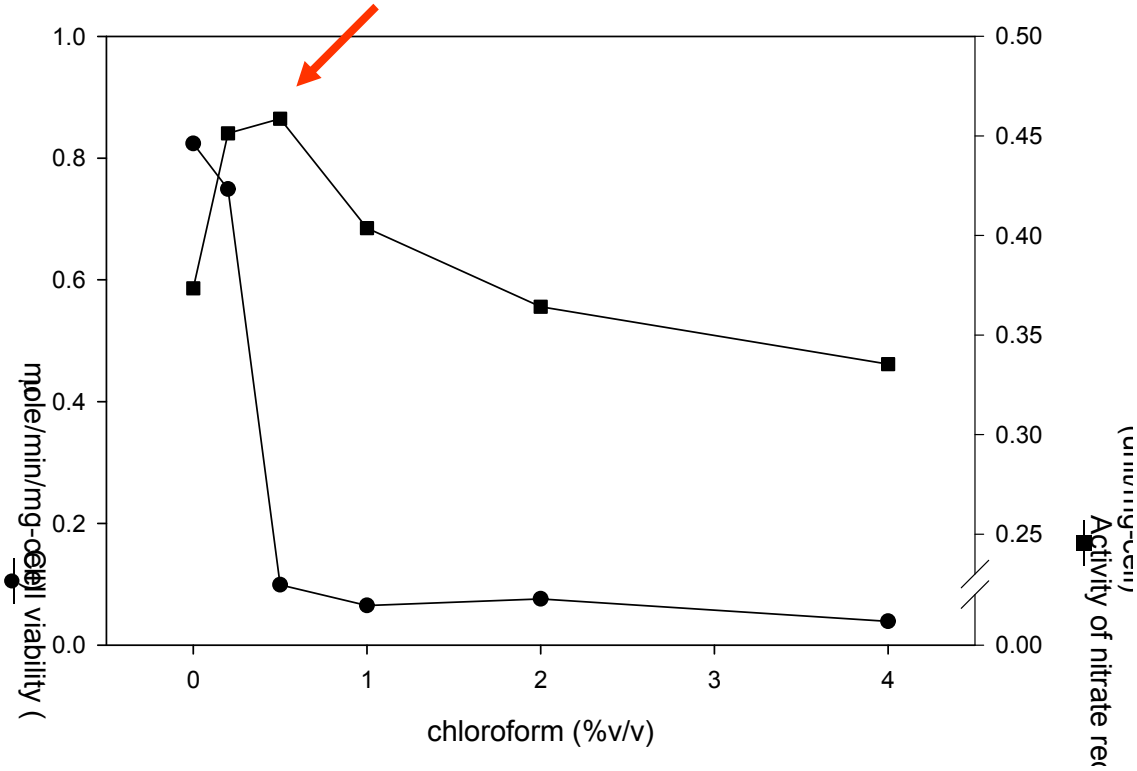
① Packed-bed reactor (100 ml)
② ORP/pH meter ③ ORP probe ④ pH probe
⑤ monitor ⑥ feeding water ⑦ pump
⑧ nitrogen gas purging

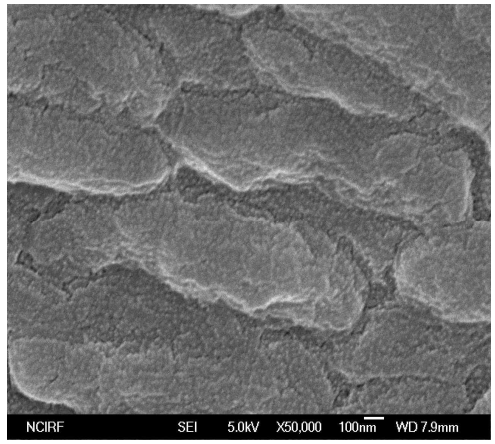
- Flow rate : 5ml/min

Bioelectrochemical Denitrification

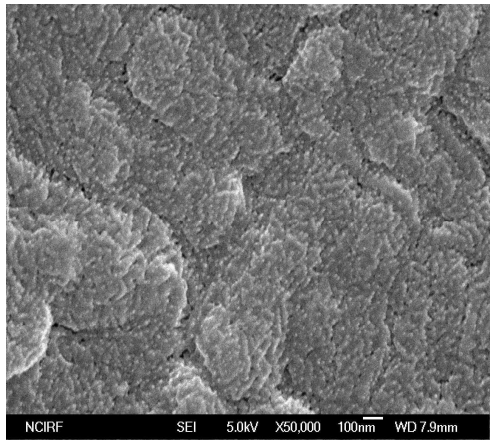


Cell Permeabilization



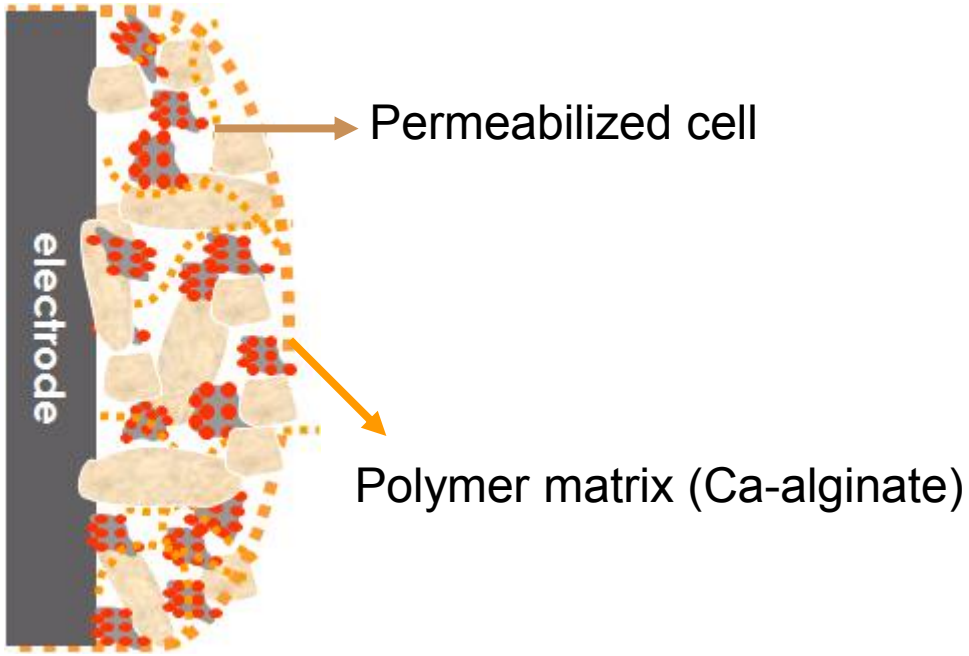


Control



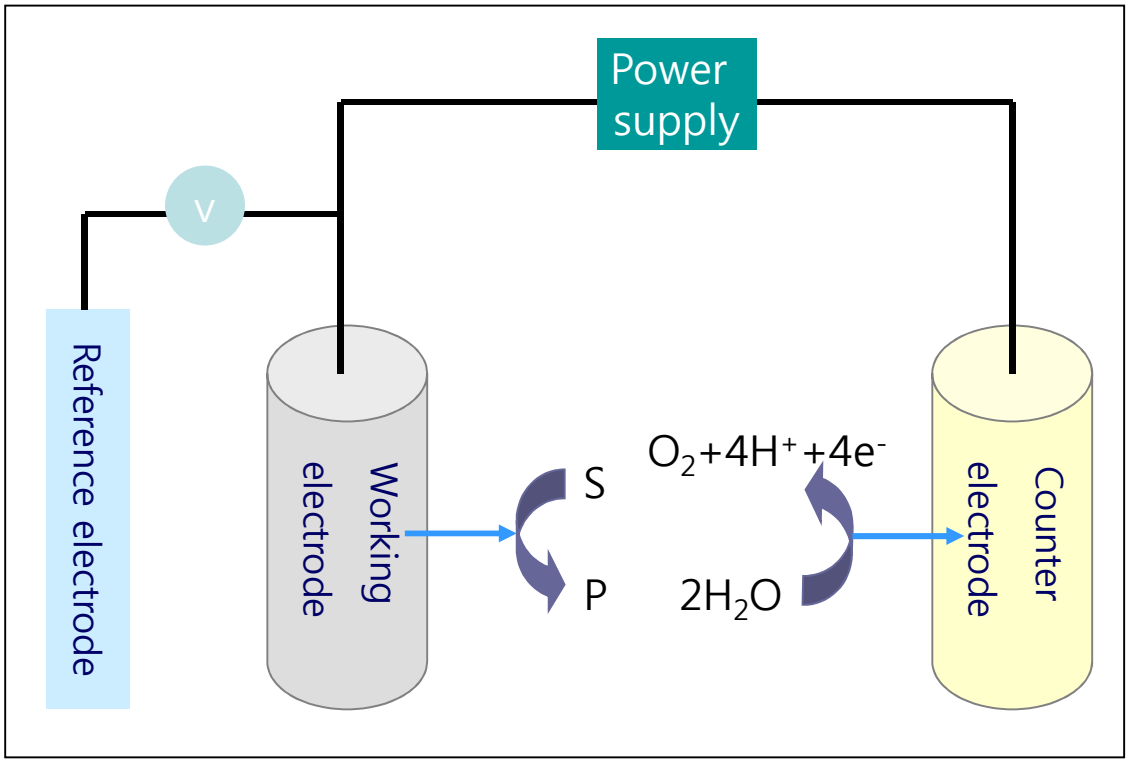
Permeabilized cell

Immobilization of Biocatalyst and Mediator on Electrode Surface



Carbon nanopowder-Neutral red 

Reactor System for Bioelectrochemical Denitrification



Denitrification Efficiency

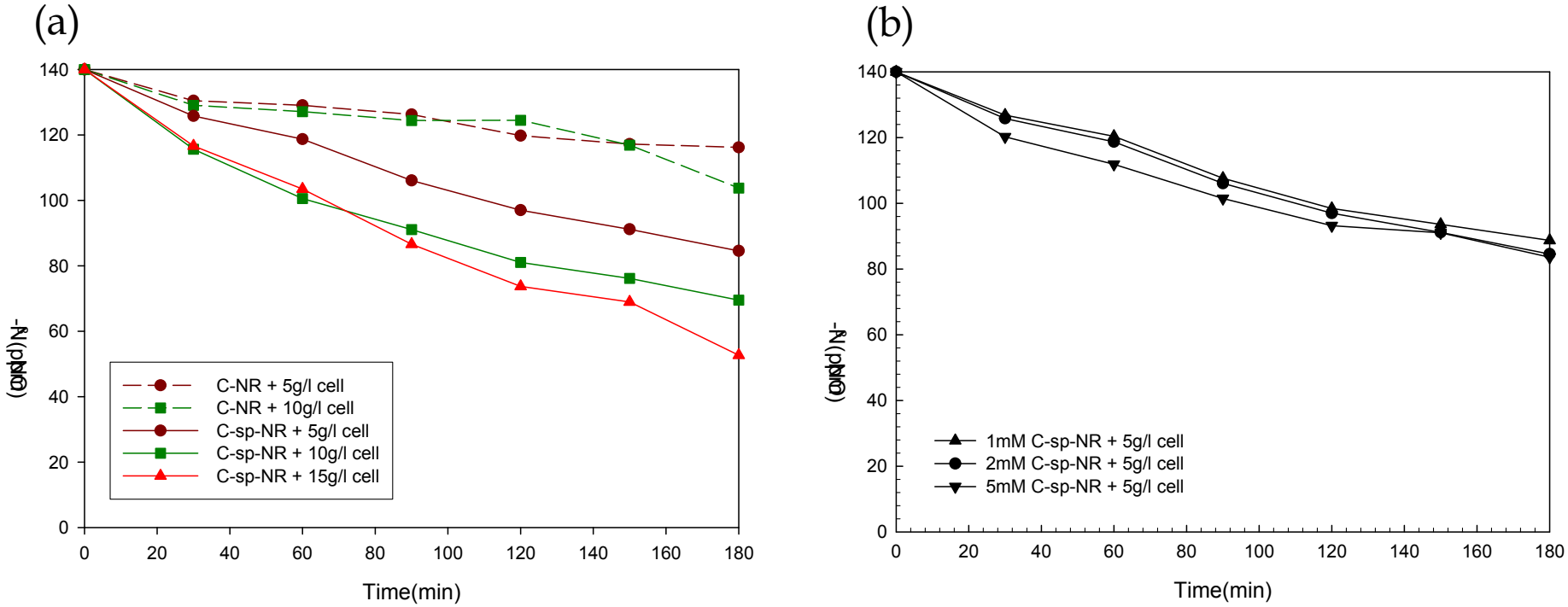
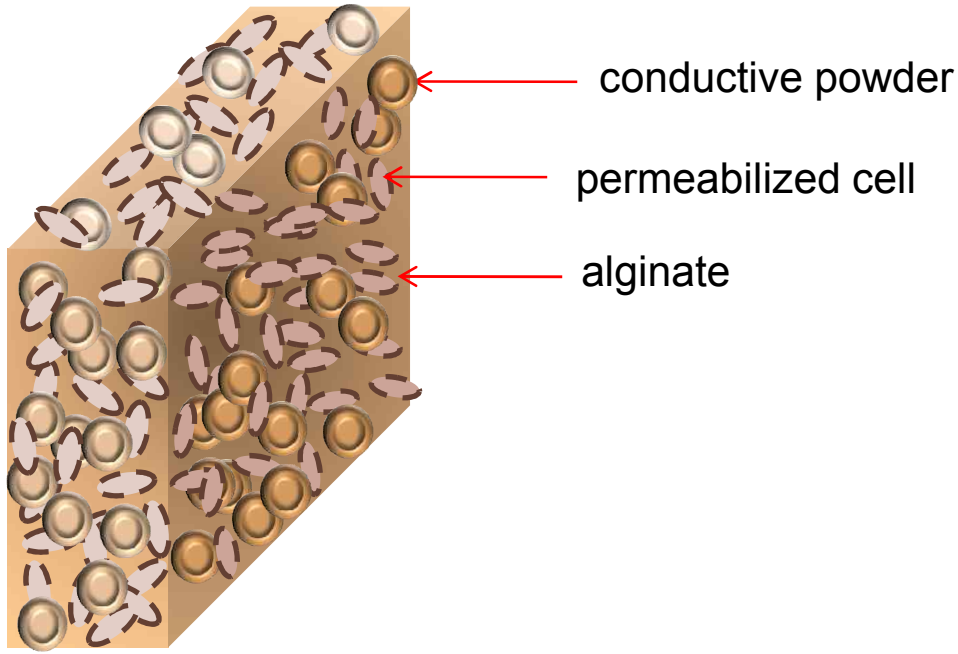


Figure. The residual nitrate level (a)for various cell concentrations and (b)for various concentrations of mediator

⇨ The cell concentration is rate-limiting compared to mediator concentration.

Novel Biocatalytic Electrode

Conductive powder was mixed with the cells and alginate for electrode.



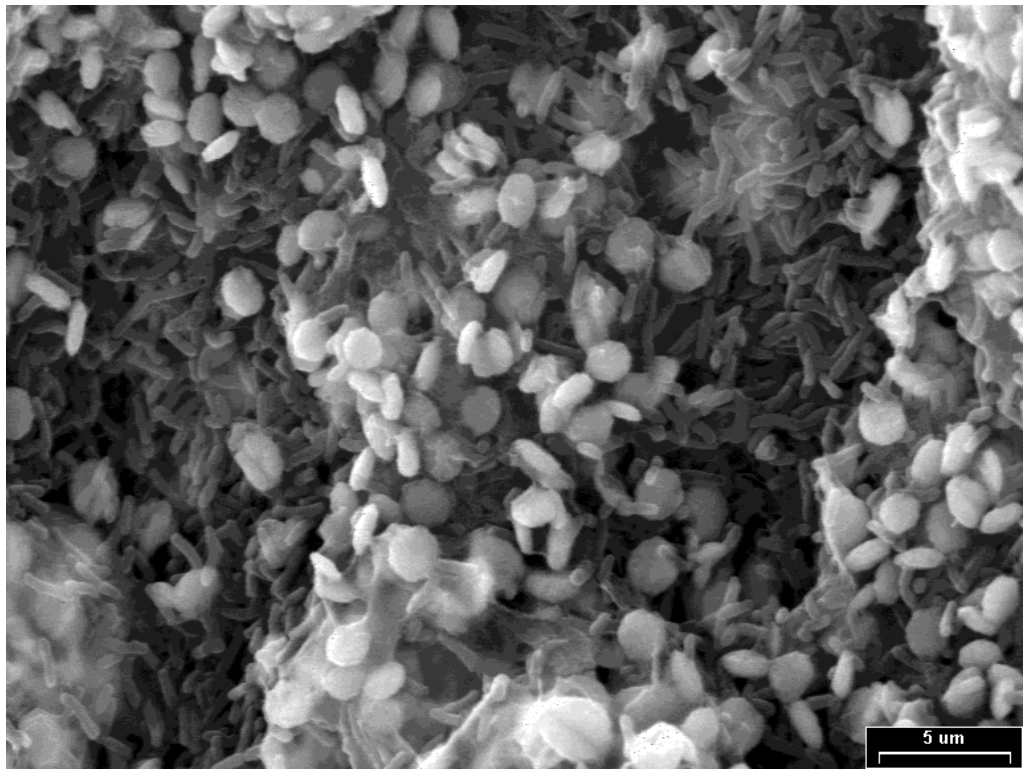
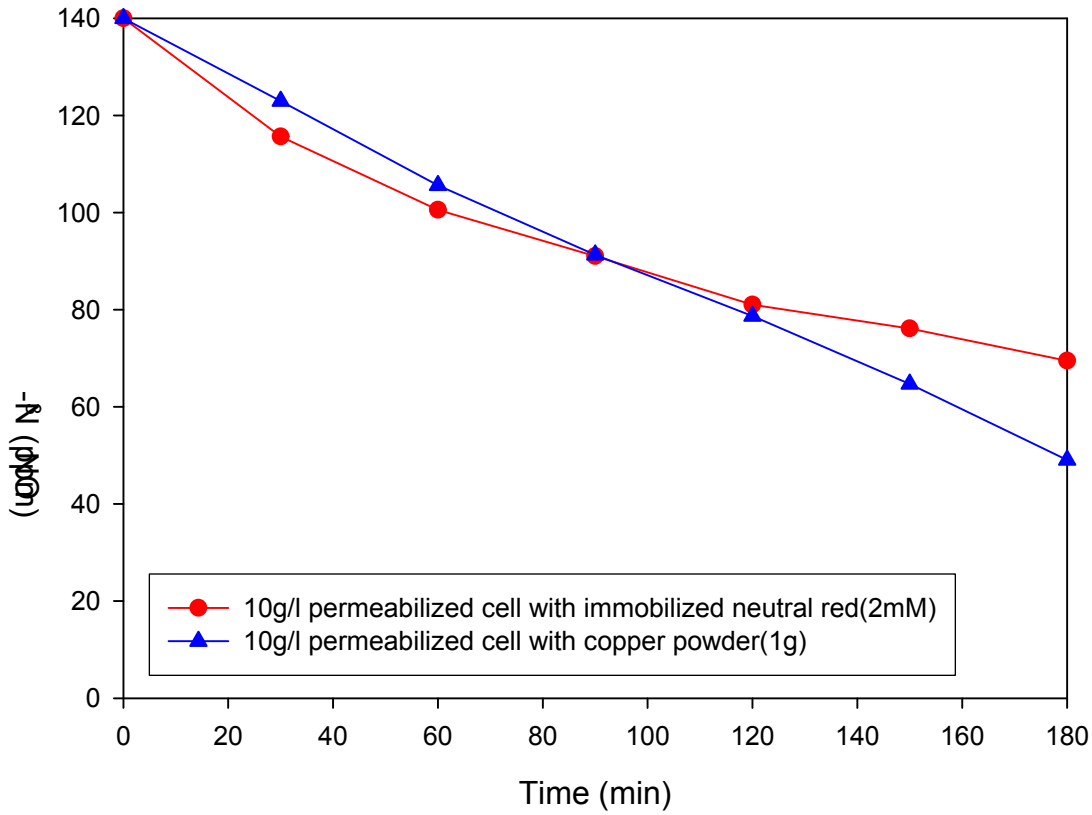
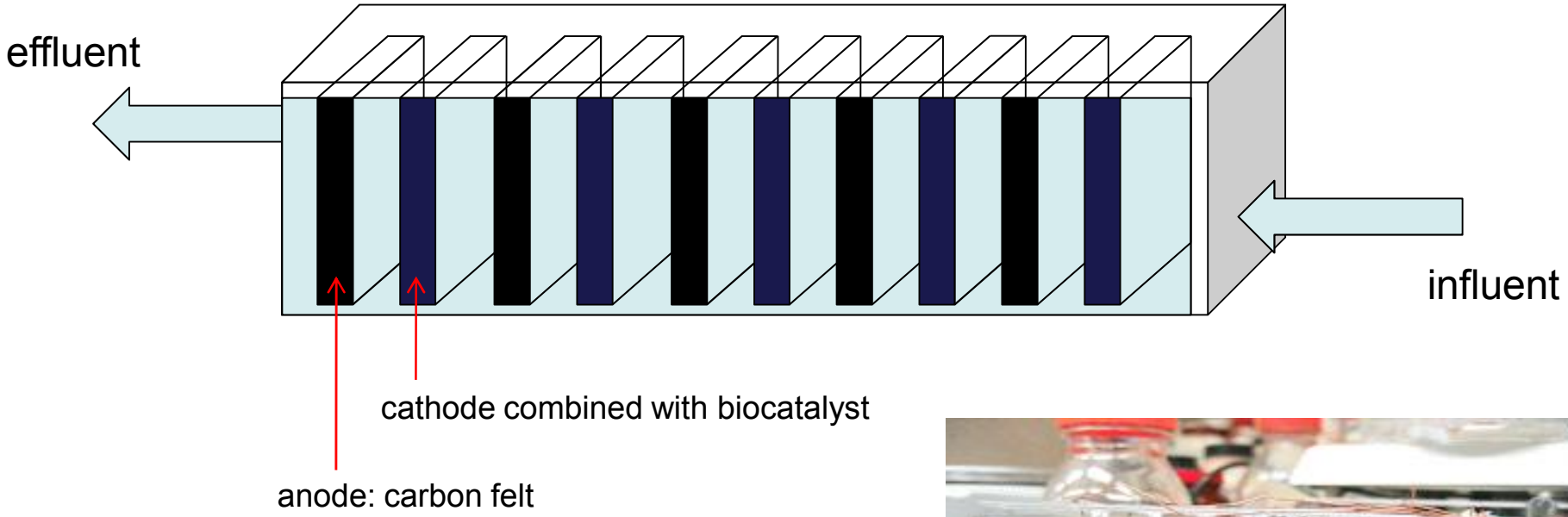


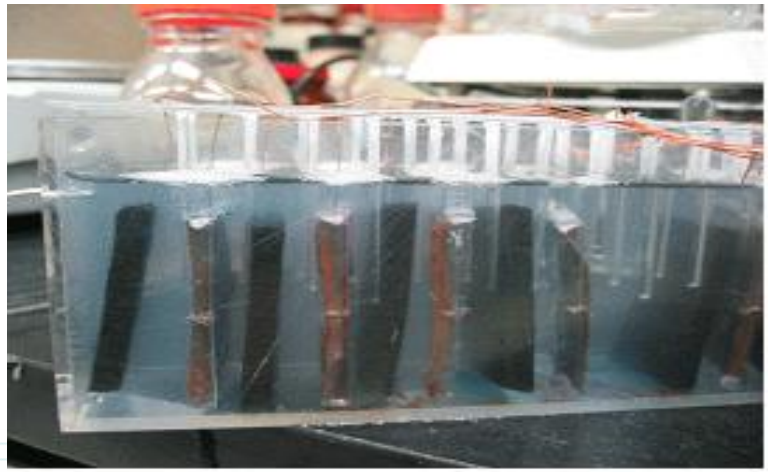
Figure. SEM image (x3500) of novel electrode

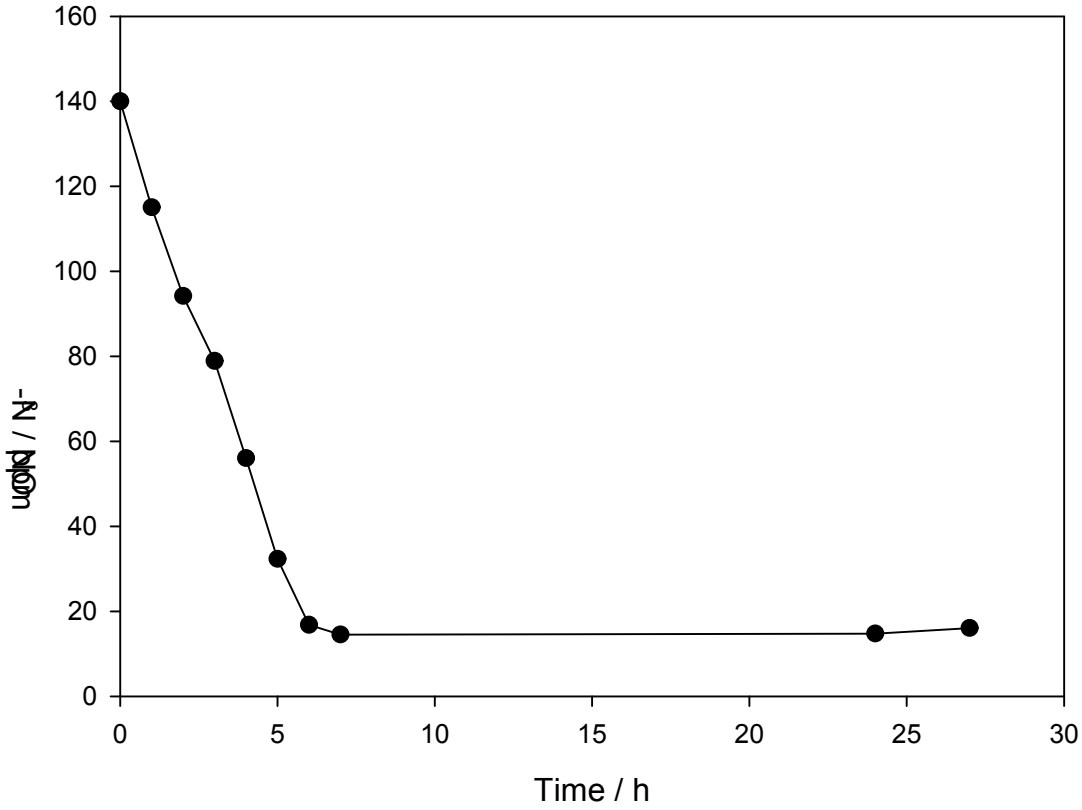
Denitrification Efficiency



Continuous Reactor






160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time / h

Biological Nitrate Removal Technology

- 1st generation: mixed population

  oxic, anoix tanks

- 2nd generation: single microorganism (enzymes)

  immobilized packed-bed reactor

- 3rd generation: whole cell biocatalyst

  enzymatic-electrochemical reactor