Enzyme Engineering

7. Applications(1): Chemicals Synthesis

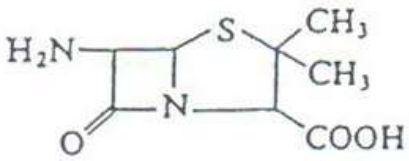
7.1 6-APA Synthesis

7.2 L-DOPA Synthesis

7.1 6-APA Synthesis

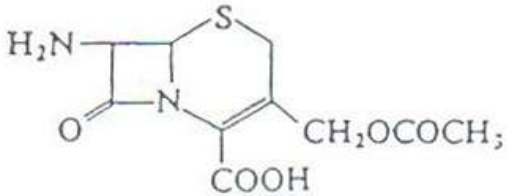
Production of Penicillin/Cephalosporin Antibiotics

1. Penicillin Nucleus

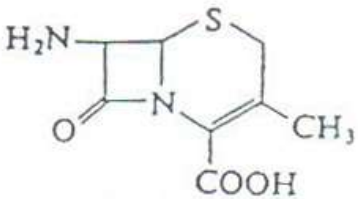


6–APA

2. Cephalosporin Nucleus

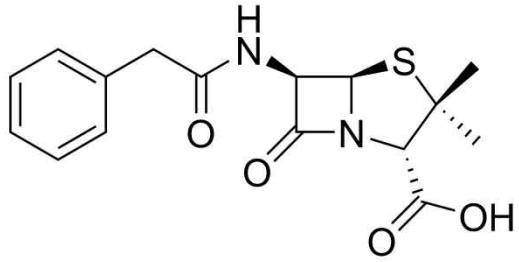


7–ACA

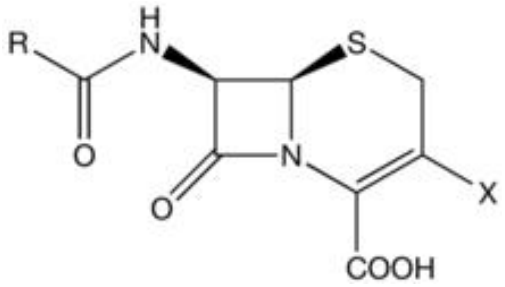


7–ADCA

Structures of 6-APA, 7-ACA, and 7-ADCA.

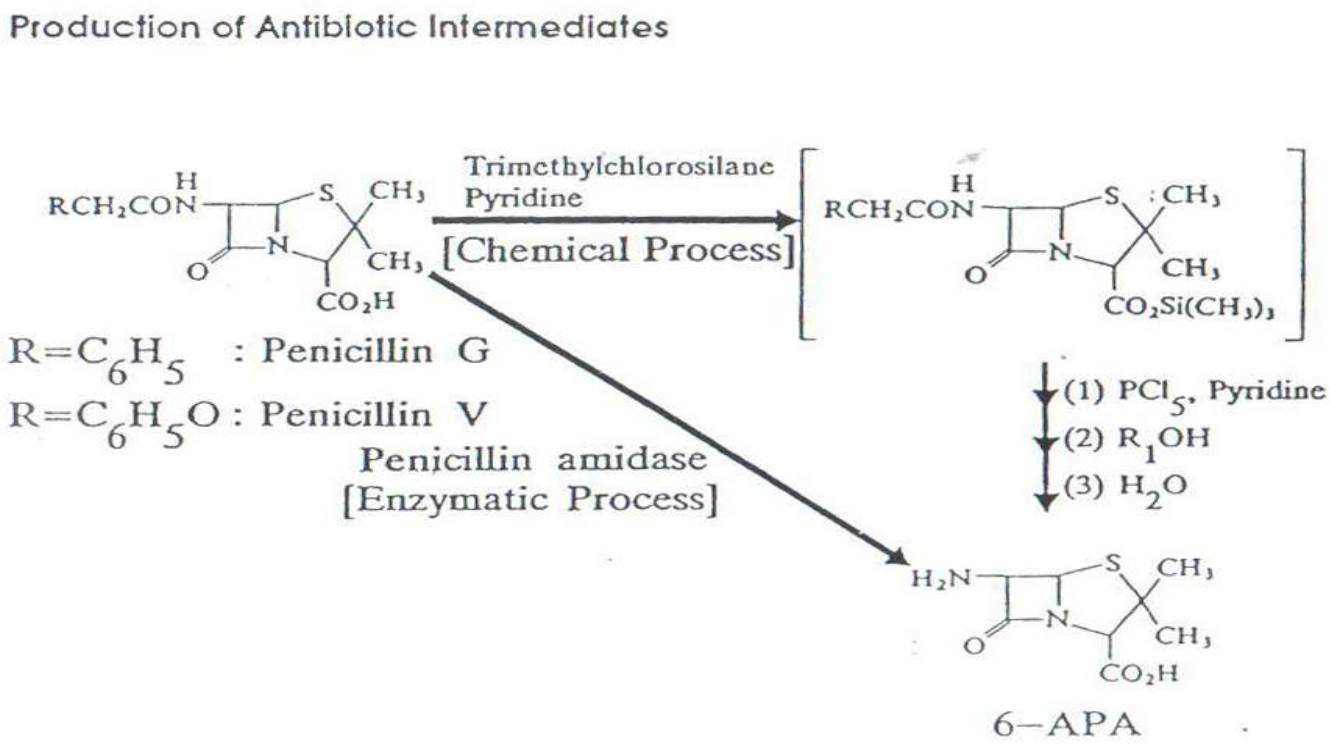


Penicillin G



Cephalosporin

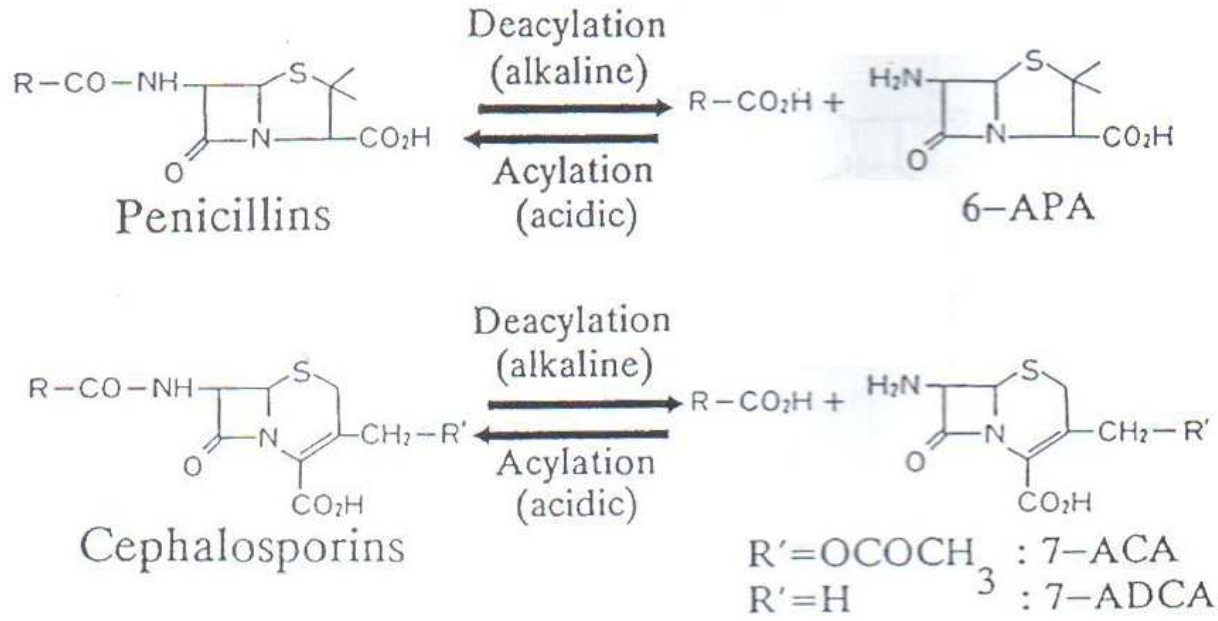
6-APA (6-aminopenicillanic acid)



6-APA production by chemical and enzymatic deacylation.

* Chemical process : toxic chemicals, by-products

Penicillin Amidase



Reaction formula for penicillin amidase.

6-APA

Pen-G → 6-APA + phenylacetic acid

- Phenylacetic acid formation

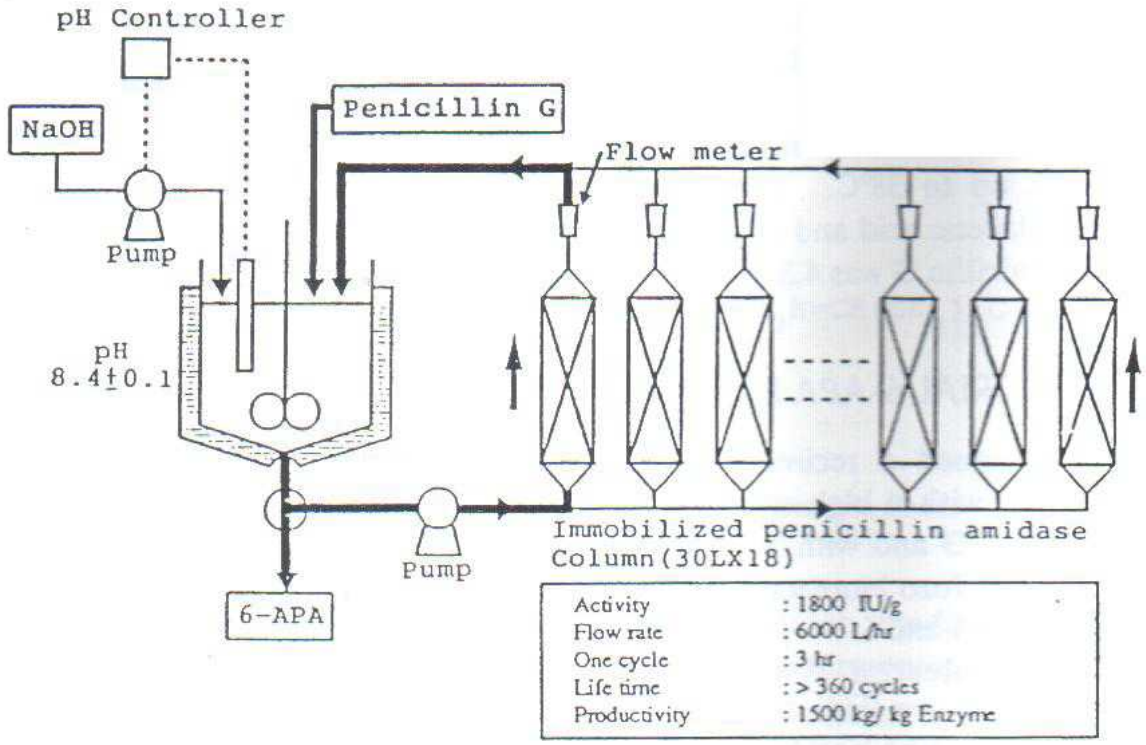
  → pH become low

  → Enzyme inhibition, reversible reaction

- Therefore pH control is very important

  → Recycled reaction is better than packed-bed reaction system

6-APA



Toyo Jozo Bioreactor

-18 parallel columns

-30 L/column

-10% circulation, 6000 L/hr

-T = 30 - 36℃

-pH = 8.4 ± 0.1

-Life time = 360 cycles

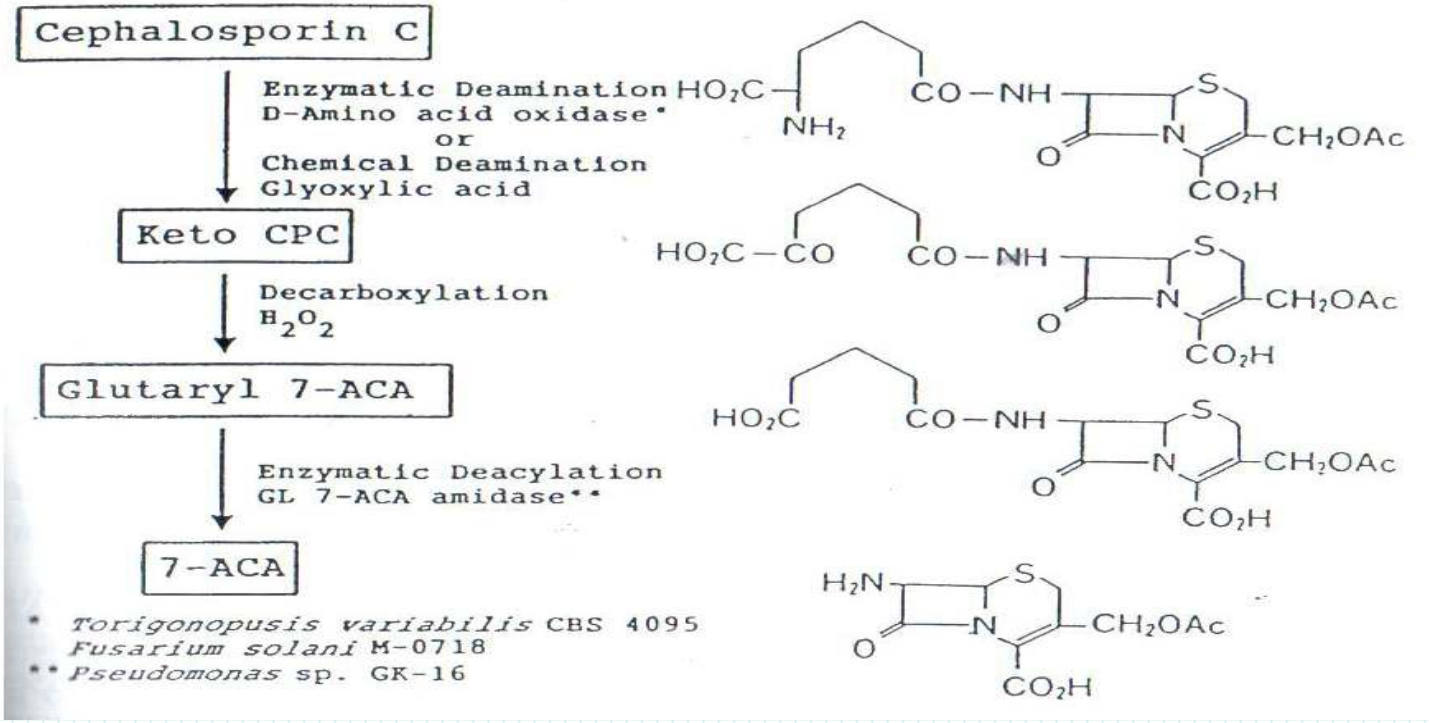
(1 cycle = 3 hrs)

- Enzyme make-up/replace after enzyme deactivation
- 6-APA precipitation after reaction
- Batch reactor for 6-APA production

7-ACA (7-amino cephalosporanic acid)

Cephalosporin-C → 7-ACA

- 1979 Toyo Jozo : 2-step process (chemical + enzymatic)
- 1990 2-step enzymatic process
- 2009 1-step enzymatic process (Amicogen)



7-ACA Amidase

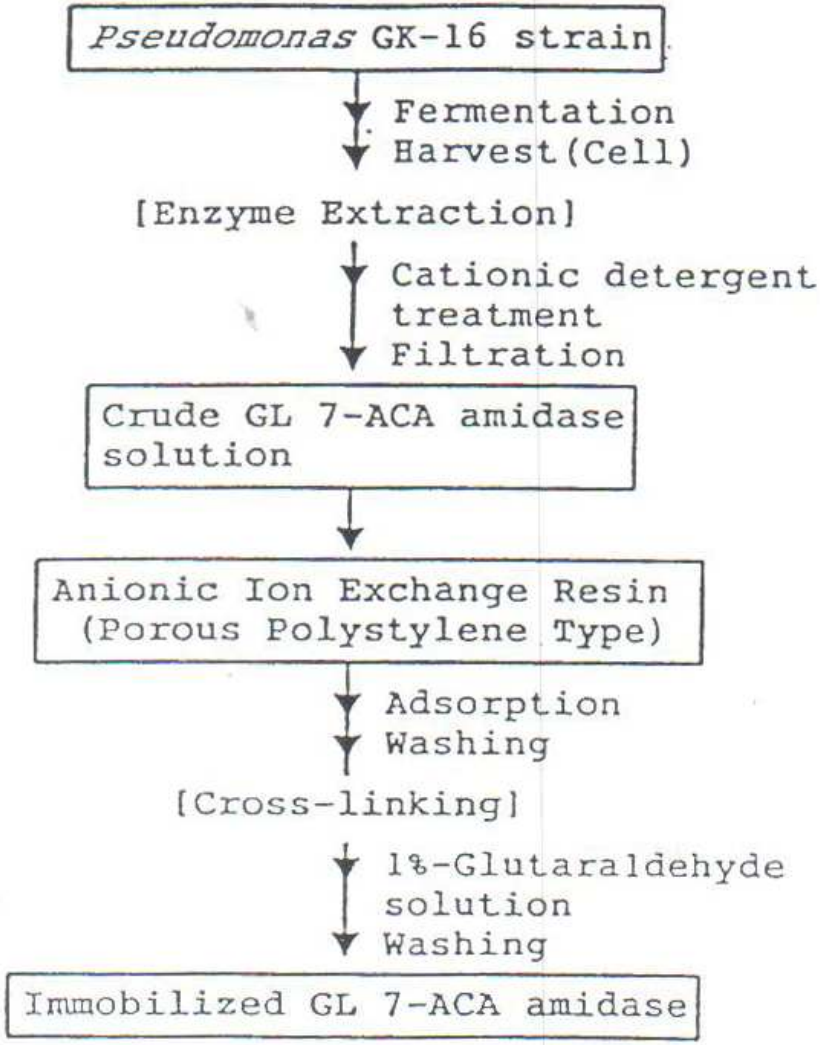
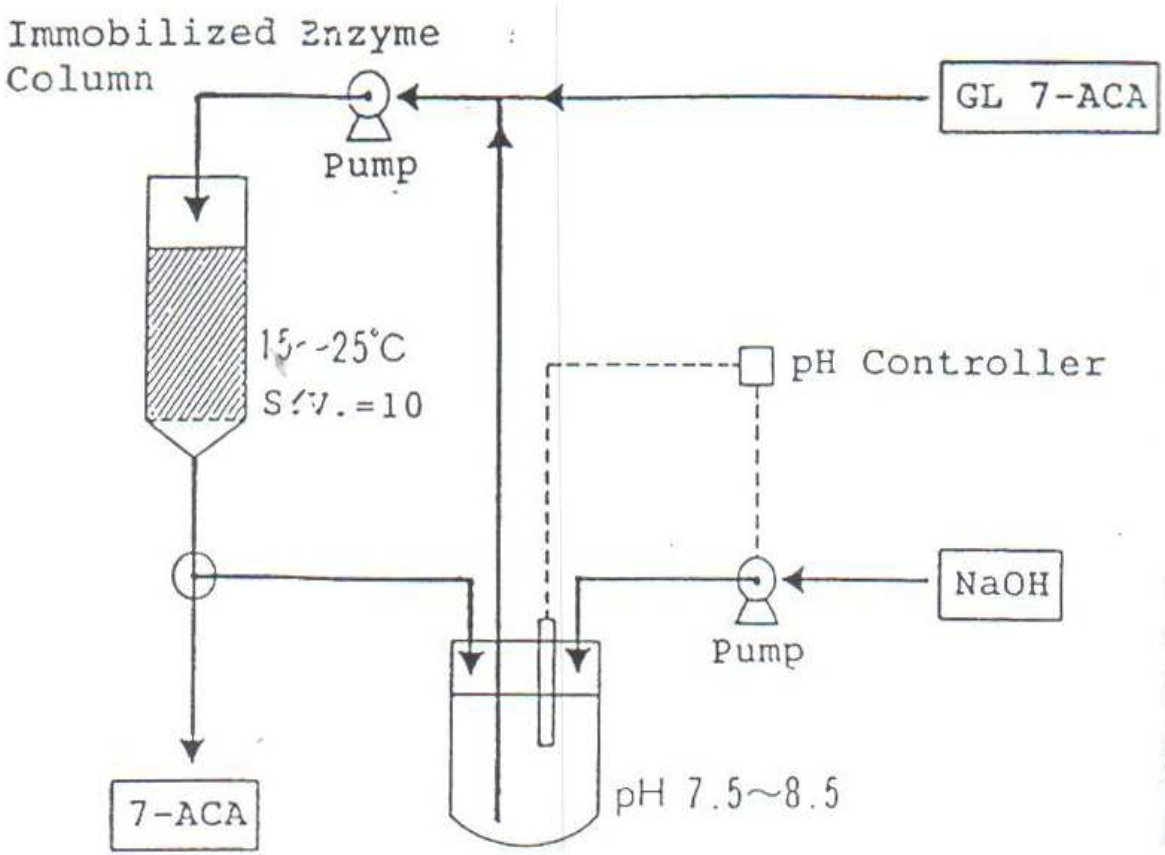


Table 2 Properties of Ion-Exchange Resins for Immobilization

Ion-exchange residues	Specific surface area (m^2/g)	Maximum frequency pore size (Å)	Efficiency of immobilization (%)
$-N(CH_3)_3X$	7-60	$300\text{-}8\times10^4$	70-90
$-N(CH_3)_3X$	0.01-9.16	<40	9-16
$-NH(CH_2CH_2NH)_nH$	14	250	4
$-N(CH_3)_2$	20-30	450-550	18-21

7-ACA Bioreactor



- Reactor = 1000 L
- Flow rate = 10,000 L/hr
- Temp. : 15℃(initial) → 25℃

(* To compensate the decay of Enzyme)

- Cycle = 4 hours
- $T_{1/2}$ (life time) = 70 cycles

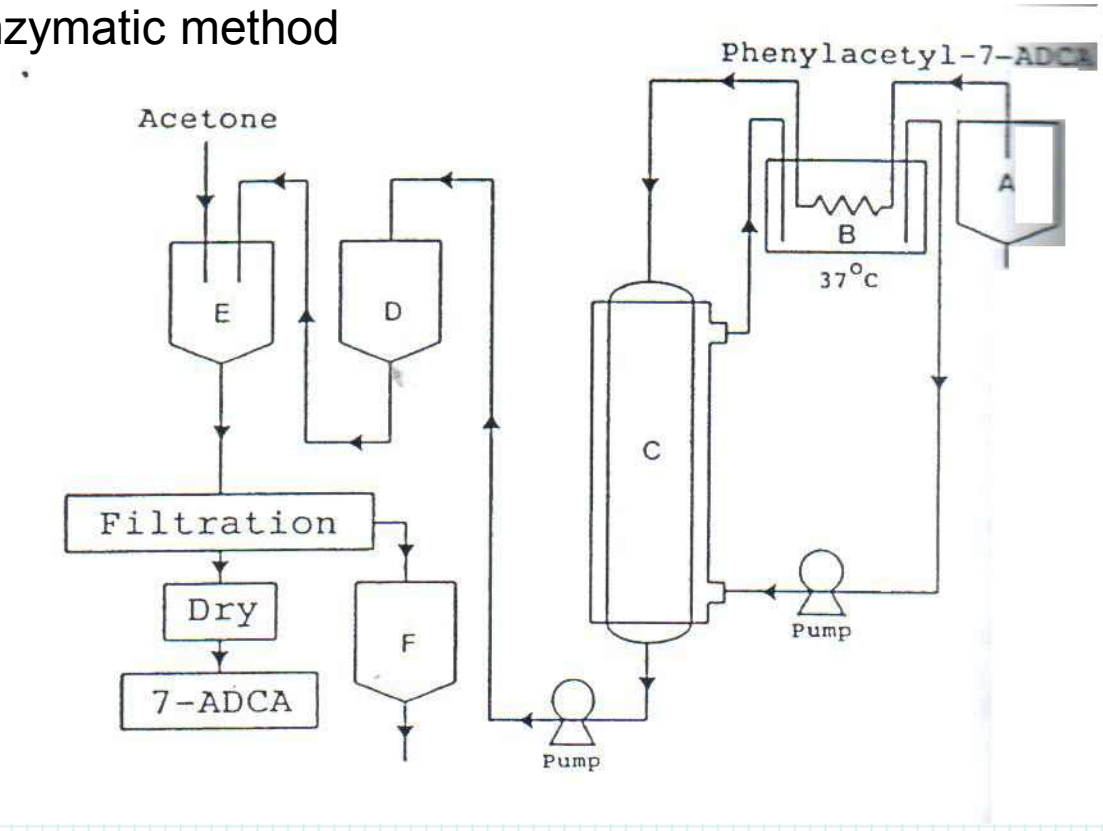
7-ADCA

Pen-G → phenylacetyl 7-ADCA → 7-ADCA

(Ring expansion)

7-ADCA for semisynthetic cephalosporins

Chemical → enzymatic method



Acrylamide

- Monomer for polyacrylamide
- Made from acrylnitrile

  $CH_2=CHCN + H_2O \rightarrow CH_2=CHCONH_2$

- Before 1970

  Acrylamide sulfate → ammonia → polymerization reaction

- Recently

  Raney copper catalyst at 100℃

- Currently

  Nitto Chemical(Japan)

  •Prof. Hideaki Yamada(Kyoto Univ.)

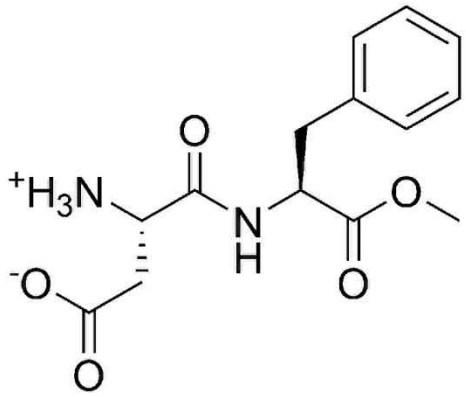
  •Dongsuh Chem,Yongsan Chem(Korea)

- Low temperature reaction to retard enzyme deactivation

  * Enzyme : nitrile hydratase

Aspartame

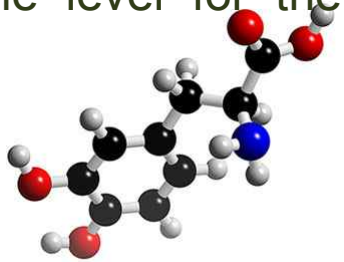
- α-L-aspartyl-L-phenylalanine –OMe (dipeptide)
- Aspartic acid + phenylalanine → aspartame
- 200 times as sweet as sucrose
- 1965 discovered
- Low-calorie sweetener
- Reversible reaction : product insoluble



7.2 L-DOPA Synthesis

What is L-DOPA?

- L-DOPA (L-3,4-dihydroxyphenylalanine) has been used **drug for Parkinson disease**, neurological disorder which afflicts one out of every 1700 individuals and is caused by deficiency of neurotransmitter dopamine. L-DOPA is a precursor of dopamine, and since it is able to pass across the blood brain barrier while dopamine itself cannot, it is used to increase dopamine level for the treatment of Parkinson's disease

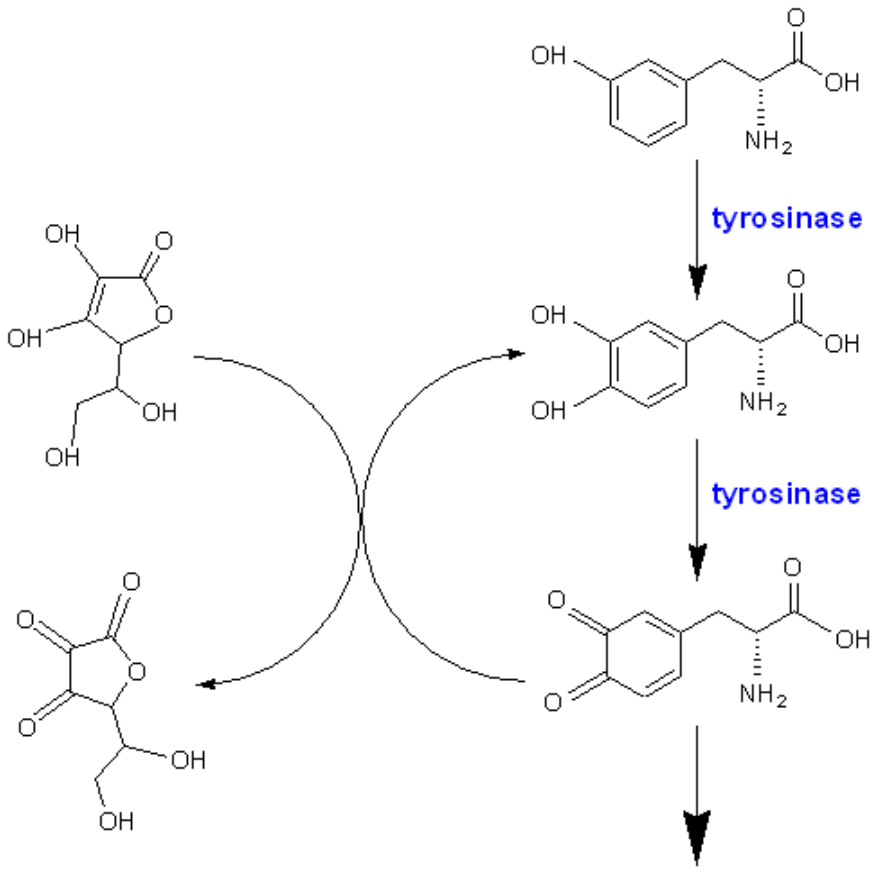


- About 250 tons of L-DOPA is now supplied per year and most of the current supply is produced by chemical method. Because of the high production cost and its high commercial value, the alternative production of L-DOPA has been investigated

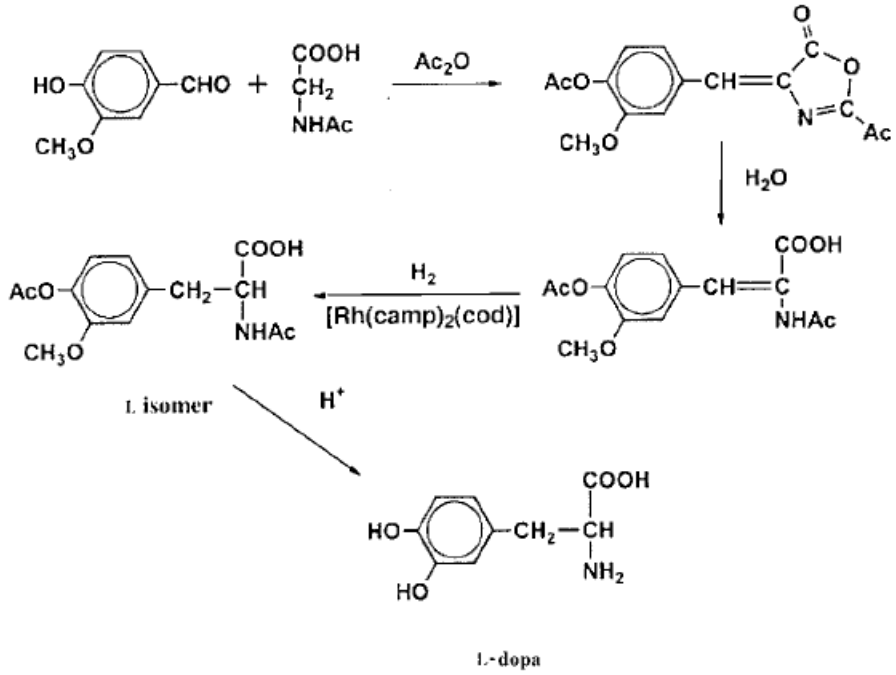
; **microbial or enzymatic production.**

Approaches for L-DOPA Production

Chemical	Microbial	Enzymatic
▪ Chiral pool, enantioselective homo-geneous hydrogenation, asymetirical hydrogenation	▪ Whole cell with Tpl activity ; *Erwinia herbicola* cell *Stizolobium hassjoo* cell	▪ Tyrosinase (E.C.1.14.18.1)
▪ Complex process Metal catalyst	▪ Carbon source feeding ▪ Separation and purification from culture media	▪ Two enzymatic activity ; creasolase/catecholase
▪ Low overall yield Low enantiomeric excess	▪ Long operation time Low conversion rate	▪ Subsequent oxidation ▪ Reducing reagent



Dehydroascorbic acid



Objective

- Electroenzymatic production of L-DOPA without reducing reagent.

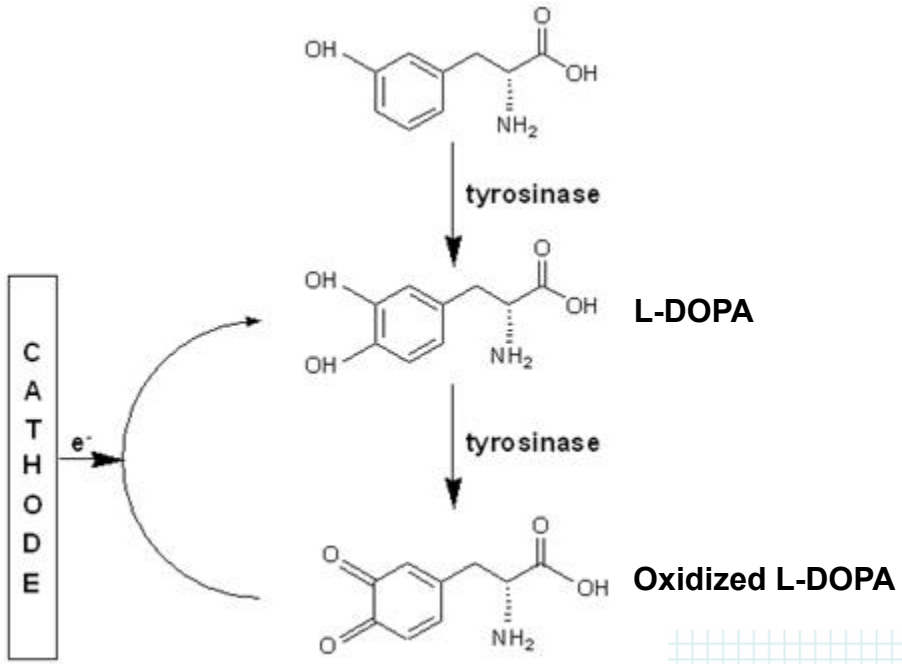
  Instead of reducing reagent such as ascorbate,

  DOPAquinone, which is a product of subsequent reaction of tyrosinase,

  is re-converted to L-DOPA again at reduction potential.

- Reduced production cost

  Improved productivity



Cyclic Voltammogram

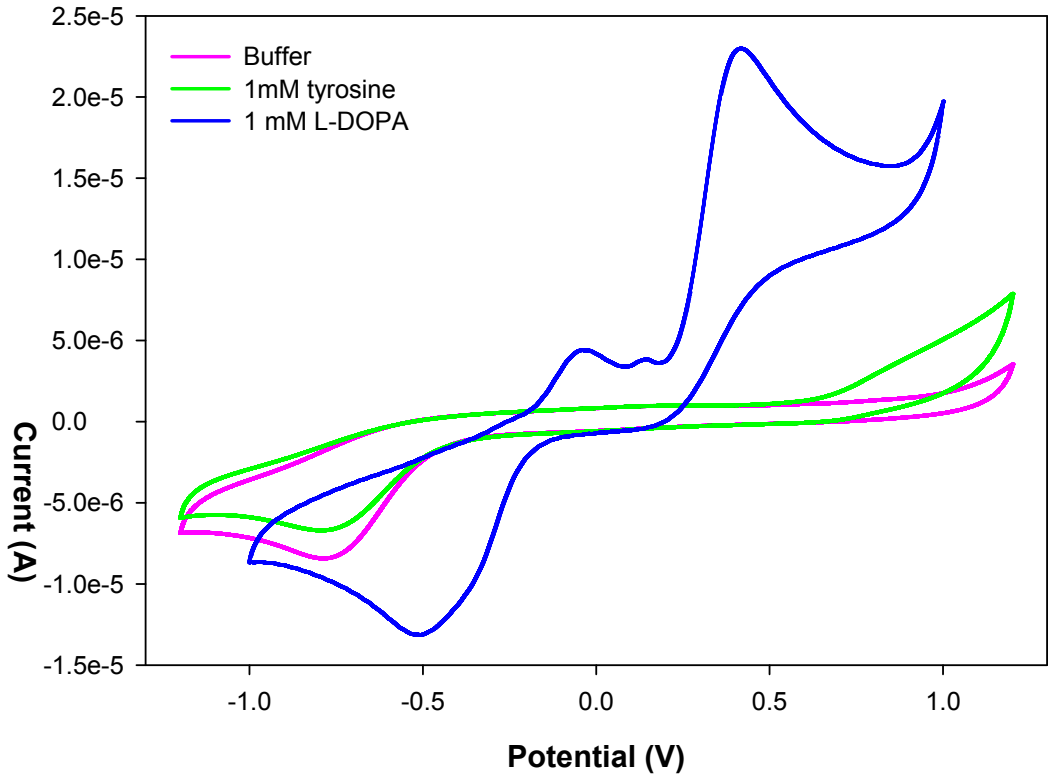


Figure. **Cyclic voltammogram of L-DOPA** (WE:glassy carbon electrode, CE: Pt wire, RE: Ag/AgCl electrode) in 50mM phosphate buffer (pH 6.5) at 20℃. DOPA was oxidized to DOPAquinone at 0.40V and DOPAquinone was oxidized at -0.06V and reduced to DOPA again at -0.53V

Electroenzymatic Production of L-DOPA

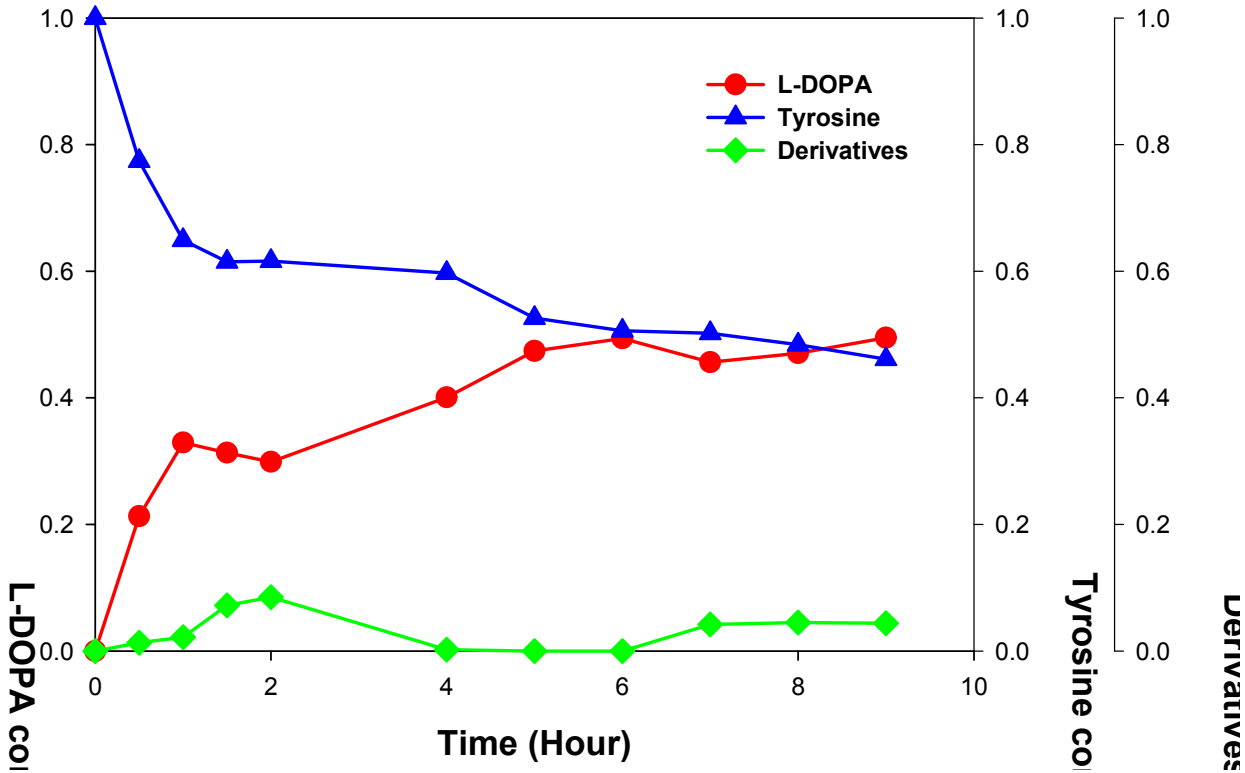


Figure. **Electroenzymatically synthesized L-DOPA concentration with 250 unit free tyrosinase** (WE:carbon felt, CE: ELAT, RE: Ag/AgCl electrode) in 30 ml L-tyrosine solution (1mM, in 50mM phosphate buffer (pH 6.5) at 20℃) at -0.53V

Effect of Reducing Power

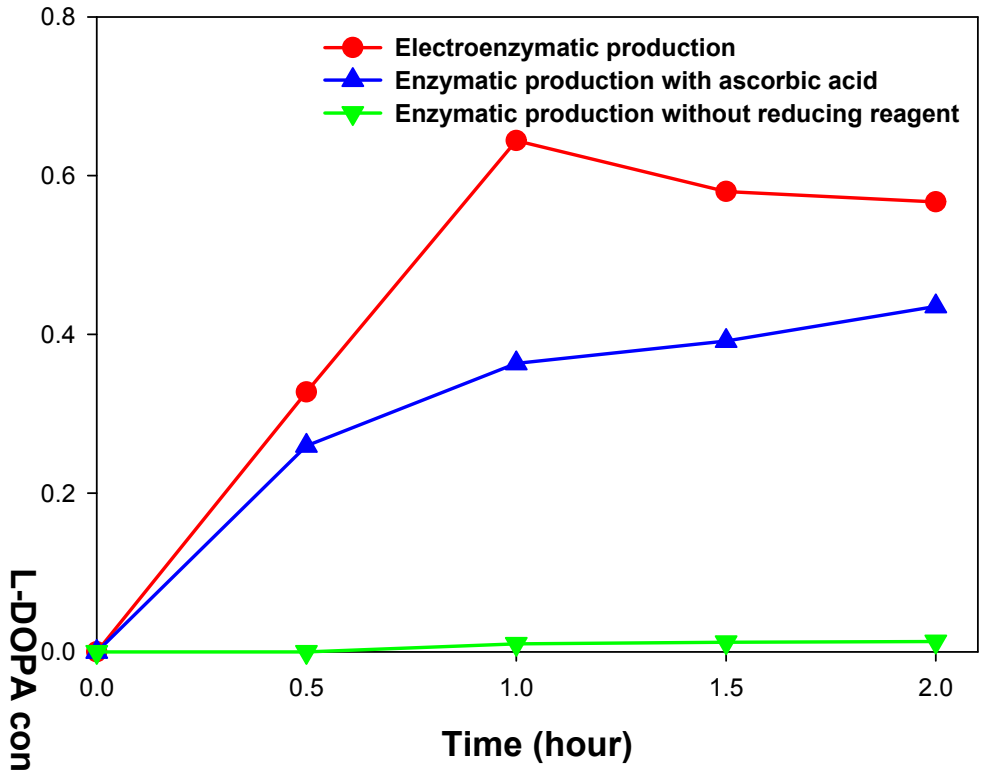


Figure. **The effect of reducing power on the electroenzymatic L-DOPA synthesis** (WE:carbon felt, CE: ELAT, RE: Ag/AgCl electrode) in 30 ml L-tyrosine solution (1mM, in 50mM phosphate buffer (pH 6.5) at 20℃) at -0.53V with 2000 unit free tyrosinase

Effect of Electrode Size

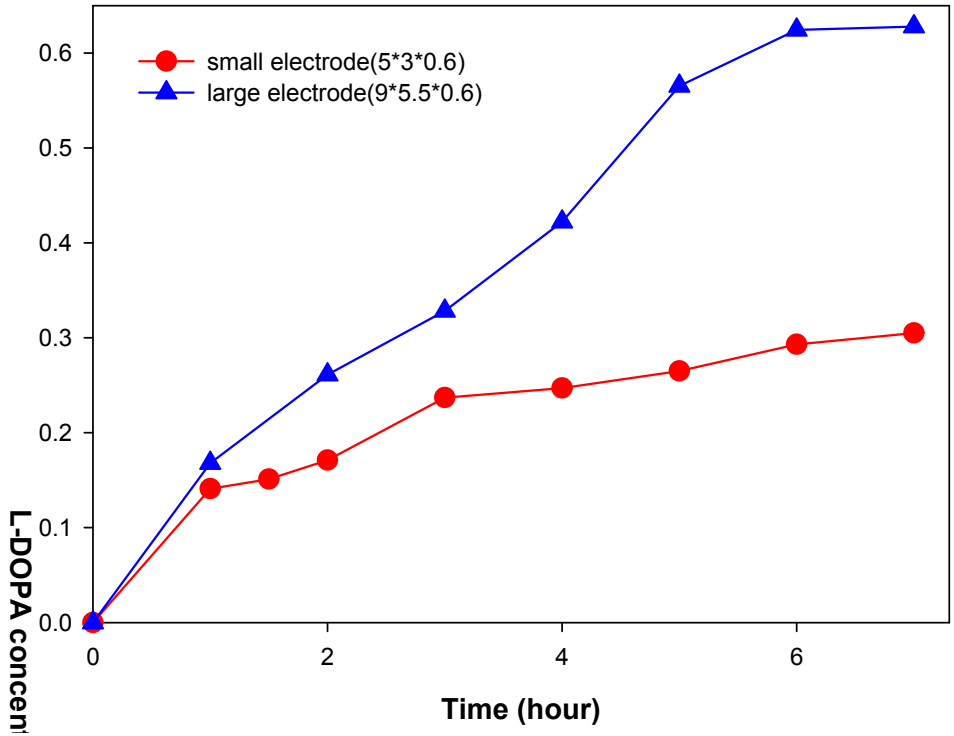


Figure. **The effect of electrode size on the electroenzymatic L-DOPA synthesis** (WE:carbon felt, CE: ELAT, RE: Ag/AgCl electrode) in 30 ml L-tyrosine solution (1mM, in 50mM phosphate buffer (pH 6.5) at 20℃) at -0.53V with 200 unit free tyrosinase

Tyrosinase Immobilized Electrode

◆ Tyrosinase adsorbed carbon felt electrode

- Adsorption of tyrosinase into the carbon felt electrode (2000 unit tyrosinase)
- Dry at room temperature
- Coated by Nafion® solution (5 wt% in water and alcohol)

◆Tyrosinase/CNPs/Polypyrrole composite

- Functionalization of CNP by 1-pyrenebutyric acid
- Tyrosinase immobilization on fuctionalized CNP by EDC activation, covalently
- Mix the monomer pyrrole and $LiClO_4$ for chemical polymerization
- Preparation of 3-dimensional composite(1.5*1.5*0.1) by mixing the chemically polymerizing polypyrrole and tyrosinase immobilized CNPs

Electroenzymatic L-DOPA Synthesis with Tyrosinase-Immobilized Electrodes

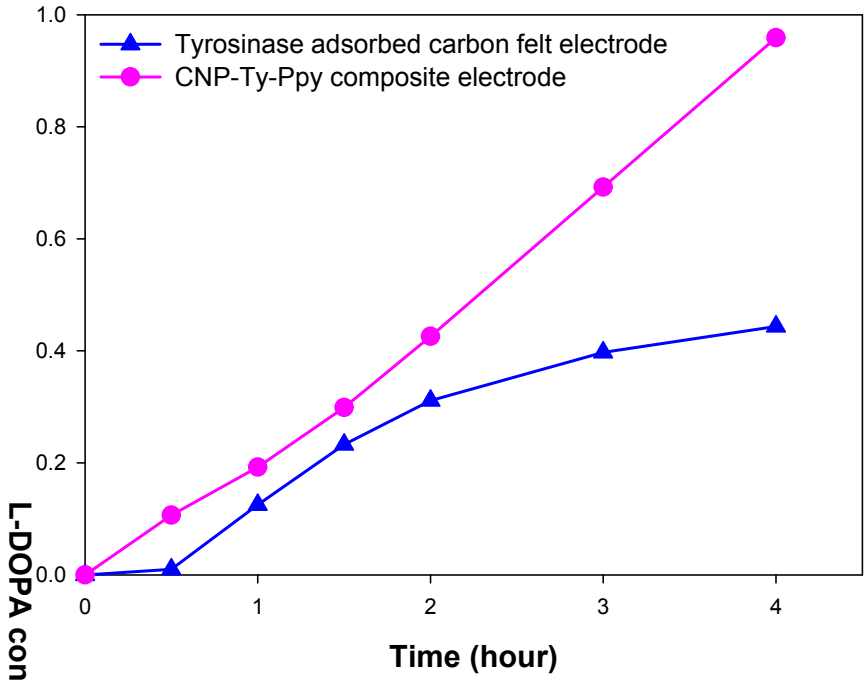


Figure. **Electroenzymatically produced L-DOPA concentration with immobilized tyrosinase** (WE:tyrosinase immobilized electrode, CE: ELAT, RE: Ag/AgCl electrode) in 30 ml L-tyrosine solution (1mM, in 50mM phosphate buffer (pH 6.5) at 20℃) at -0.53V, **2000 unit tyrosinase** was immobilized.

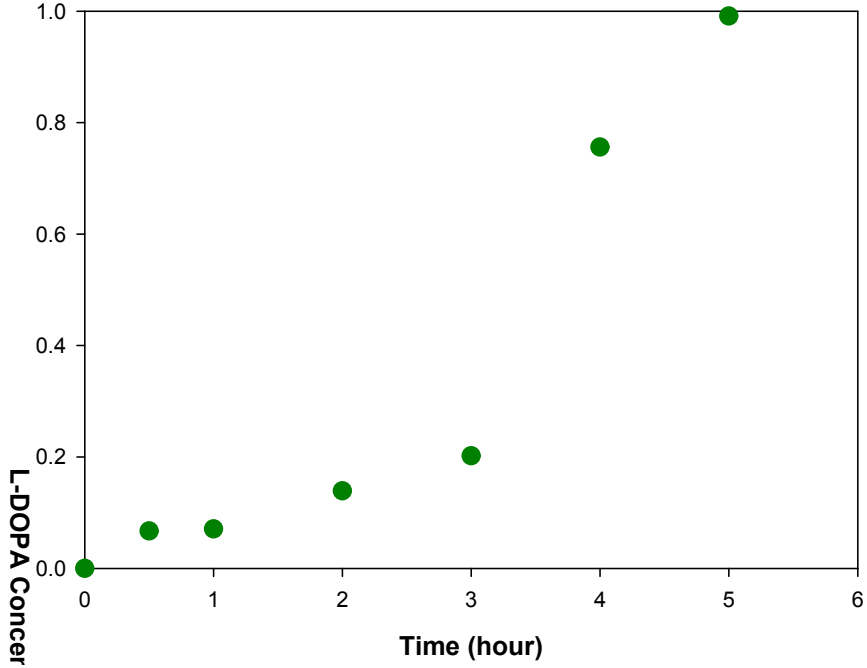


Figure. **Electroenzymatically produced L-DOPA concentration (WE:CNP/Ty/Ppy composite electrode**, CE: ELAT, RE: Ag/AgCl electrode) in **300 ml L-tyrosine** solution (1mM, in 50mM phosphate buffer (pH 6.5) at 20℃) at -0.53V, **2000 unit tyrosinase** was immobilized.

Operational Stability ;Tyrosinase Immobilized Electrode

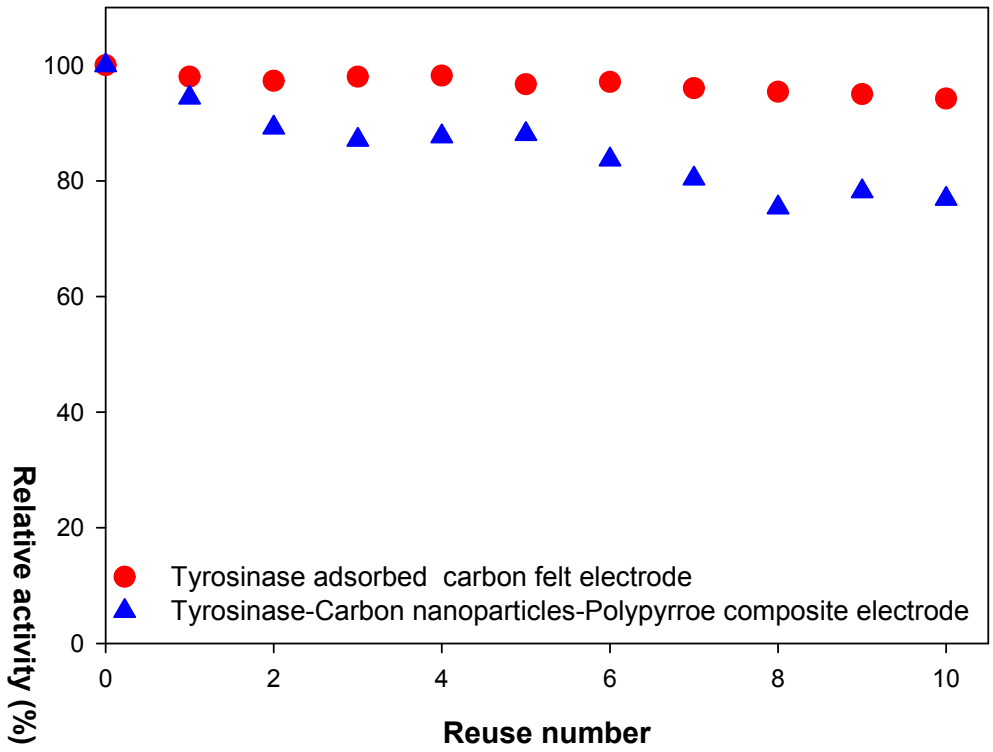


Figure. **Operational stability of tyrosinase immobilized electrodes** as function of reuse number. Relative activity was determined from the synthesized L-DOPA concentration in 30ml L-tyrosine solution (1mM, in 50mM phosphate buffer (pH 6.5)) at -0.53V for 4 hours .

Table. L-DOPA production on different reaction type

Reaction Type	Productivity (mg/Lh)	Conversion rate (%)	Remark	Ref.
Chemical synthesis		44	▪ Several reaction steps	1
Immobilized tyrosinase Batch reactor	1.7		▪ Long time operation (170hrs) ▪ Low operational stability	2
Immobilized tyrosinase Batch reactor	4.5	1.8	▪ Low conversion rate	3
Erwinia herbicola culture	1800	7.34	▪ Low conversion rate ▪ Substrate mixture feeding (pyruvate,ammonia, catechol)	4
Stizolobium hassjoo culture	3.13		▪ Long time operation (over 10days)	5
Free tyrosinase in batch Electroenzymatic	**134.66**	**68.3**	**▪ High conversion rate ▪ Short operation time (1hr)**	**This study**
Immobilized tyrosinase Batch, Electroenzymatic	**39.08**	**99.1**	**▪ High conversion rate ▪ Short operation time (4hr) ▪ Good operational stability**	**This study**

Ref.) [1] Catalysis Comm. 5; 631, [2] Biotechnol. Bioeng. 51; 141, [3] EMT 40; 683, [4] J. Biotech. 115; 303, [5] EMT 30; 779

The reason why the electroenzymatic system can show the high conversion rate and productivity?

- **Hypothesis**

; The reason for the enhanced conversion rate and productivity was **efficient electron transfer** from electrode to DOPAquinone. In terms of reaction rate, the electrical reduction of DOPAquinone to L-DOPA predominated over the oxidation of L-DOPA by catecholase activity in tyrosinase/CNPs/Ppy composite.

(1) The tyrosinase was covalently attached on the carbon nanoparticles which play roles as not only an immobilization support but also electron carriers in the composite electrode.

(2) The tyrosinase, which was immobilized on the electron carrier, converted L-DOPA to DOPAquinone by its catecholase activity, and the DOPAquinone was directly reduced to L-DOPA by electrons from the electrode.

(3) Therefore, by-product DOPAquinone did not accumulate in the reactor and the conversion rate increased up to 99.1%.

Summary

- L-DOPA can be synthesized in electroenzymatic system. In electroenzymatic system for L-DOPA production, by-product DOPAquinone was reduced to L-DOPA by electrons from cathode. The electrical reducing power was more efficient to enzymatic L-DOPA synthesis than reducing reagent, ascorbic acid.

- In electroenzymatic L-DOPA synthesis, the conversion rate and productivity by tyrosinase/CNPs/Ppy composite electrode was 95.9 % and 134.7 mg/Lh, respectively. When the reactor was scaled up to 10 times, the conversion rate was maintained.

- Based on the kinetic constants k_1, k_2, k_e electrical reduction of DOPAquinone to L-DOPA was faster than oxidation of L-DOPA by catecholase activity.