공학석사학위논문

Production of *p*-Hydroxybenzaldehyde from Phenol Using Engineered *Escherichia coli*

재조합 대장균을 이용한 페놀로부터 파라-하이드록시벤즈알데하이드의 생산

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주성연

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이 논문을 공학석사 학위논문으로 제출함

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주 성 연

주성연의 공학석사 학위논문을 인준함

2019년 8월

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Abstract

p-Hydroxybenzaldehyde (*p*HBAL) is versatile intermediate for various applications such as perfumes, medicines, liquid crystals as well as food ingredients. Along with great attentions for *p*HBAL, its chemical synthetic routes have been well established. However, relatively harsh condition and removal of large excess amount of base is still problematic.

For these reasons, successful biosynthetic routes using engineered *E.coli*, from L-tyrosine and *p*-coumaric acid (*p*CA), have been done. Nevertheless, the use of relatively expensive precursor provides the necessity of new biosynthetic route from more simple and available precursor.

To produce *p*HBAL from more cheap and available phenol, two modules were separately applied and combined into *E.coli*. In the module 1, tyrosine phenol lyase (TPL) from *Citrobacter freundii* and tyrosine ammonia lyase (TAL) from *Saccharothrix espanaensis* were heterogously expressed in *E.coli* respectively. The expressions and activity were confirmed through SDS-PAGE and biotransformation in which 10 mM of phenol converted into 0.8 mM of *p*CA in 12h (8 %). In the module 2, 4.5 mM of *p*HBAL (90 %) was formed from 5 mM of *p*CA using heterogously expressed feruloyl CoA synthase (FCS) and enoyl CoA hydratase/aldolase (ECH) in 12h.

Finally, the module 1 and module 2 were combined, showing to produce 0.06 mM of *p*HBAL from 5 mM of phenol (1.2 %) in 12h. Despite of the low

conversion, this result demonstrated de-novo synthesis of *p*HBAL from phenol in *E.coli* for the first time.

Keywords: pHBAL, TPL, TAL, FCS, ECH, de-novo synthesis.

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1. Introduction

p-Hydroxybenzaldehyde (*p*HBAL) is one of the valuable phenolic aldehydes for its various applications as synthetic intermediate of perfumes, medicines and liquid crystals.[1, 2] Also, it can be used as food ingredients owing to the health beneficial effects.[3, 4] In keeping with gradually increasing demand for *p*HBAL around industries, various chemical synthetic routes have been reported. (i) Reimer-Tiemann process in which phenol reacts with carbene derived from chloroform under aqueous alkaline condition to give benzal-chlorides which are rapidly hydrolyzed by the alkaline medium to give salicylaldehyde as a major product and *p*HBAL as a byproduct.[5](ii) Direct oxidation of alkyl benzenes by classical oxidants such as chromic acid or potassium permanganate.[6]

However, these methods have several disadvantages including use of comparably expensive reagents producing huge amounts of inorganic salts which cause serious effluent problems and inefficiency of these of excessive toxic inorganic waste.[7] Although, lately, heterogeneous cobalt catalysts have shown to minimize the waste disposal and improving the overall process availability, relatively harsh condition and the removal of large excess amount of base is still problematic. [8, 9]

For this point of view, production of *p*HBAL via biosynthetic route have been spotlighted in which key enzymes, involved in the phenyl-propanoid pathway and lignin metabolism in plants, are exploited to generate microorganisms producing *p*HBAL starting from L-tyrosine or *p*-coumaric acid. [10, 11] More

specifically, tyrosine ammonia lyase (TAL) gives deamination of L-tyrosine into *p*-coumaric acid, in which Fefuloyl-CoA synthase (FCS) generates *p*-coumaroyl CoA using ATP and CoA, further hydration and retro aldol α, β carbon bond cleavage of *p*-coumaroyl CoA yielding *p*HBAL by enoyl CoA hydratase/aldolase (ECH). [11, 12]

Jun Ni et al, recently have described biotransformation of *p*CA into *p*HBAL using FCS and ECH co-expressing *E.coli* system with the productivity from 0.47 to 6.41 mM/h^{-1} in which the endogenous competition of Alcohol dehydrogenases (ADHs) were inactivated by relatively higher temperature.[13] Apart from the coenzyme dependent platform where ATP and CoA should be regenerated continuously from cells, they have introduced coenzyme free system comprising aromatic dioxygenase and phenolic acid dioxygenase from thermophilic *Thermothelomyces thermophile* and *Bacillus coagulans* (TtADO & BcPAD) resulting 8.57 mM/h of *p*HBAL from *p*CA. [14] Also, Seyoung Jang et al have obtained 0.14 mM of *p*HBAL from 1.1 mM of L-tyrosine using FCS and ECH from *Burkholderia glumae* BGR1 (BgFCS & BgECH) and TAL from *Rhodotorula glutinis* (RgTAL) co-expressing *E.coli*. [15]

However, above mentioned approaches have a disadvantage as the use of relatively expensive precursor, providing the necessity of biosynthetic route from more simple and available precursor.

In this study, two modular systems were constructed and applied into *E.coli*. For the module 1, tyrosine phenol lyase from *Citrobacter fresundii* (CfTPL) and tyrosine ammonia lyase from *Saccharothrix espanaensis* (SeTAL) were

heterologously expressed in *E.coli*. This system could convert 5mM of phenol into 0.8 mM of *p*CA in 12h (8 %).

For the mdoule 2, coenzyme dependent system (BgFCS & BgECH) and independent system (TtADO & BcPAD) were constructed by heterologous expression of corresponding enzymes in separated cells. Each cell was applied for biotransformation of *p*CA into *p*HBAL, showing the coenzyme dependent system could produce 4.4 mM of *p*HBAL from 5 mM of *p*CA, over the coenzyme independent system. Further optimization, including glucose feeding and replacing host strain with engineered reduced aromatic aldehyde reduction strain showed enhanced initial product forming rate (4 mM/h), as well as lowered product degradation.

Finally, module 1 and module 2 were combined into single cell, introducing each component of two modules. The combined system could produce *0*.15 mM of *p*HBAL from 5 mM of phenol (3%). Despite of the low conversion, this result demonstrated de-novo synthesis of *p*HBAL from phenol for the first time.

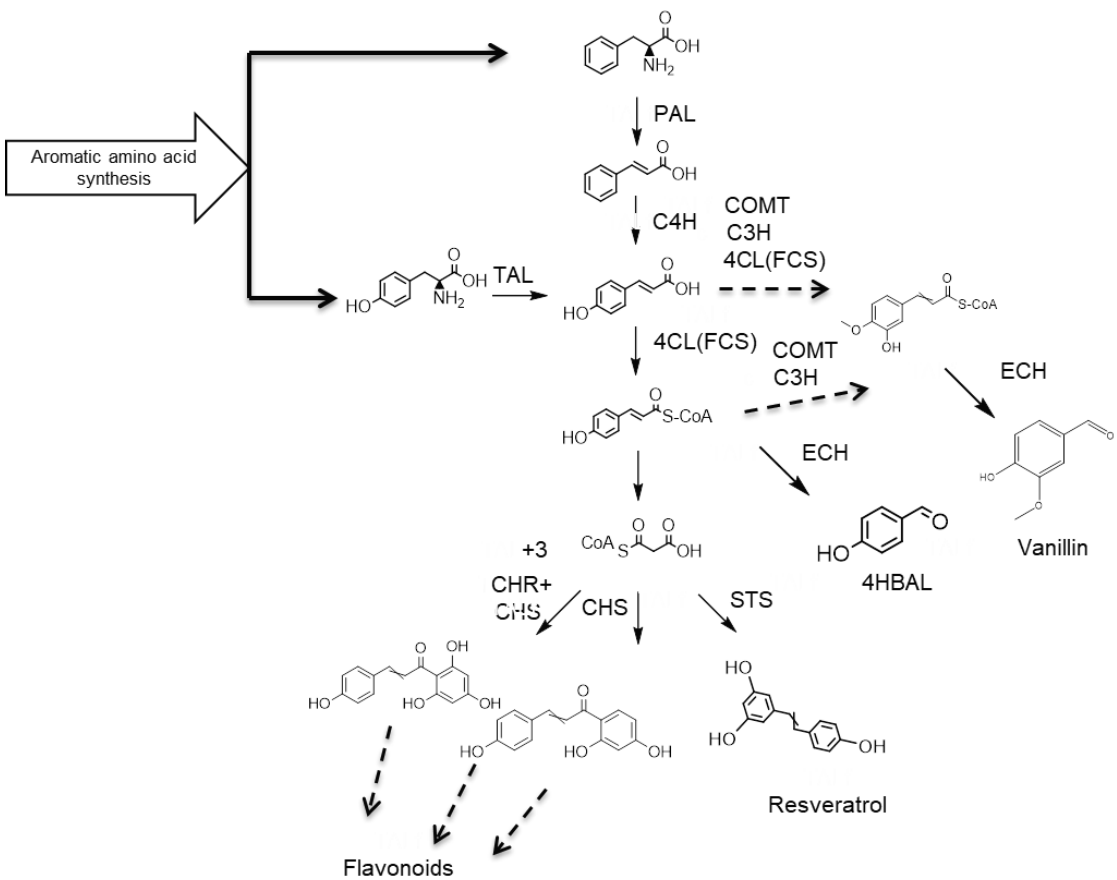


Figure 1. Phenyl-propanoid pathway including key enzymes.

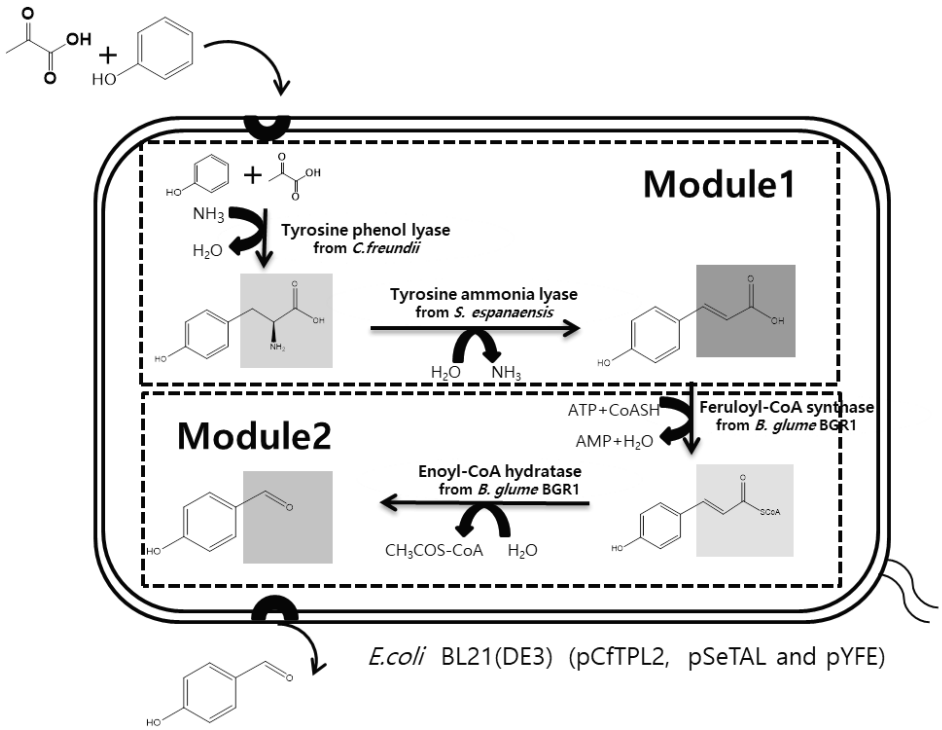


Figure 2. Schematic diagram of enzymatic synthesis of *p*HBAL from phenol.

2. Materials and methods

2.1. Chemicals and media

All chemicals, such as phenol, *p*CA, *p*HBAL, *p*-hydroxybenzyl alcohol (*p*HBAOL), *p*-hydroxybenzoate (*p*HBA), sodium pyruvate, dimethyl sulfoxide (DMSO), ethanol, methanol, isopropyl-thio- β -D-galactopyranoside (IPTG), purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacteriological agar, Luria Bertani (LB) broth, and terrific broth (TB) media were purchased from BD Difco (Franklin Lakes, NJ, USA). All chemicals used in this study were of analytical grade.

2. 2. Plasmids construction

For the construction of module 1, pHCII vector having codon optimized tyrosine phenol lyase from *Citrobacter. freundii* (CfTPL) was previously cloned using *KpnI* & *BamHI* and provided by Prof. Yun's lab from Konkuk university, Seoul, South Korea. The gene was further subcloned into pETDuet-1 using circular polymerase extension cloning protocol [16] with DH5α as host strain. Tyrosine ammonia lyase from *S. espanaensis* and *R. sphaeroides* (SeTAL and RsTAL, respectively) were codon optimized and synthesized by Bionics (Seoul, Korea). Each TAL gene was inserted into pET24m using *EcoRI* and *HindIII.*. For the module 2, feruloyl CoA synthase (FCS) and enoyl CoA hydratase/aldolase from *B. glumae* BGR1 were previously cloned into pET_duet1 vector using

BamHI and *XbaI*.[17] And aromatic dioxygenase (ADO) from *T. thermophile* and phenolic acid decarboxylase (PAD) from *B.coagulans,* they were codon optimized and synthesized by Bionics (Seoul, Korea) and inserted into the pETDuet-1 using the same cut site as Jun Ni described. [14]

Table 1. Plasmids and strains used in this study

Plasmids/Strains	Description	Refference
Plasmids		
pET24ma	P15A ori lacI T7 promoter, KmR	[18]
pETDuet-1	pBR322 ori lacI T7 promoter, AmpR	*Novagen*
pCDFDuet-1	CDF ori lacI T7 promoter, SmR	*Novagen*
pACYCDuet-1	P15A ori lacI T7 promoter, CmR	*Novagen*
pHCII-2B	pMB1 ori HCII constitutive promotor	*KRIBB*
pHCIICfTPL	pHCII-2B encoding TPL from *C. Freundii*	This study
pETDuetCfTPL	pETDuet-1 encoding TPL from *C. freundii*	This study
pET24mRsTAL	pET24ma encoding TAL from *R. Sphaeroides*	This study
pET24mSeTAL	pET24ma encoding TAL from *S. espanaensis*	This study
pETDuetFE	pETDuet-1 encoding FCS&ECH from *B. glumae* BGR1	[17]
pACYCDuetFE	pACYCDuet-1 encoding FCS&ECH from *B. glumae* BGR1	[17]
pETDuetAP	pETDuet-1 encoding ADO from *T. thermophile* and PAD from *B. Coagulans*	[14]
Strains		
DH5α	F- *80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+) phoAsupE44 -thi-1 gyrA96 relA1*	*NEB*
MG1655 (DE3)	F- *λ- ilvG- rfb-50 rph-1* (DE3)	*[19]*
BL21 (DE3)	F- *ompT lon hsdSB (*rB-mB-*) gal dcm* (DE3)	*NEB*
RARE	MG1655(DE3) *ΔdkgB ΔyeaE Δ(yqhC-dkgA) ΔyahK ΔyjgB ΔendA ΔrecA*	*[20]*

BCf1	BL21(DE3) carrying pHCIICfTPL	This study
BCf2	BL21(DE3) carrying pETDuetCfTPL	This study
BRsTAL	BL21(DE3) carrying pET24mRsTAL	This study
BSeTAL	BL21(DE3) carrying pET24mSeTAL	This study
BTT1	BL21(DE3) carrying pHCIICfTPL and pET24mSeTAL	This study
BTT2	BL21(DE3) carrying pETDuetCfTPL and pET24mSeTAL	This study
BFE1	BL21(DE3) carrying pETDuetFE	This study
BFE2	BL21(DE3) carrying pACYCDuetFE	This study
BAP	BL21(DE3) carrying pETDuetAP	This study
RFE1	RARE carrying pETDuetFE	This study
RFE2	RARE carrying pACYCDuetFE	This study
RTTFE	BL21(DE3) carrying pETDuetCfTPL, pET24mSeTAL andpACYCDuetFE	This study

Table 2. Primers used in this study

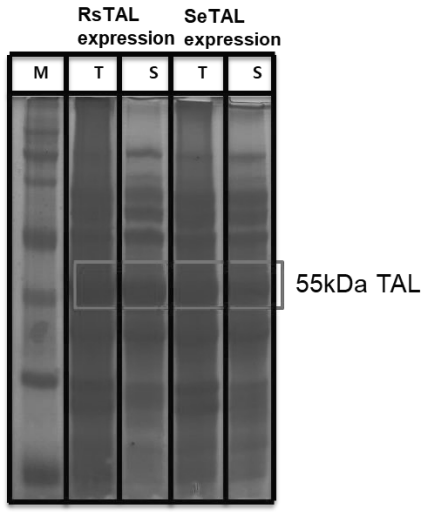
Primer Name	Sequence
TPL-F	**ACATATGGCAGATCTCATGAACTACCCTGCCG**AA
TPL-R	**CAGACTCGAGGGTACCTTATATATAATCAAAC**CTAGCCG
pETDuet-F	**GTTTGATTATATATAAGGTACCCTCGAGTCTG**GT
pETDuet-R	**CGGCAGGGTAGTTCATGAGATCTGCCATATGT**AT

2. 3. Heterologous expression of proteins

E. coli BL21 (DE3) strain was utilized for the biotransformation studies. Plasmid DNAs were transformed into host strains using standard heat shock method. For the expression of proteins, fresh colonies from agar plates of transformants were cultured in 2 mL of LB medium containing appropriate antibiotics at 37 °C overnight. The seed-cultured cells were inoculated into 50ml Terrific-Broth (TB) in 250 mL baffle flask and cultured at 37 °C until the cell concentration reached an optical density at 600 nm (OD_{600}) of 0.6-0.8 for IPTG induction.

The induction was performed by adding 0.1 mM IPTG at 30 °C for 16 h. The cells having each enzyme were harvested by centrifugation at 4°C 4400 *g* in 15 min, washed and re-suspended in the 20 mL Tris buffer (100 mM, pH 8.0), and disrupted by sonication. The soluble fraction of each lysates was collected after centrifugation at 16,000 rpm for 30 min and subjected to SDS-PAGE analysis where 5x loading dye was mixed with the soluble& total fractions and heated at 100°C for 5 minutes. After preparation, samples were loaded onto 10% PAGE gel, followed by coomassie staining, confirmed expression of each protein. (Fig. 3 and Fig 4)

(a)



(b)

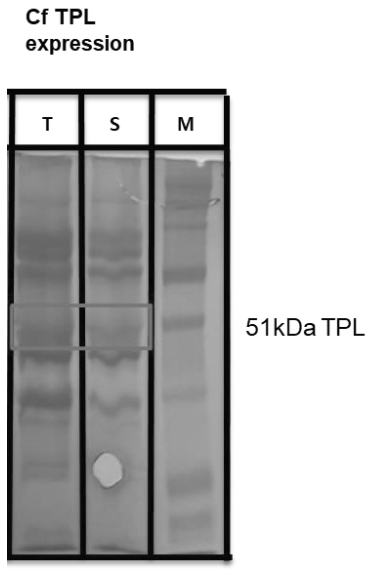
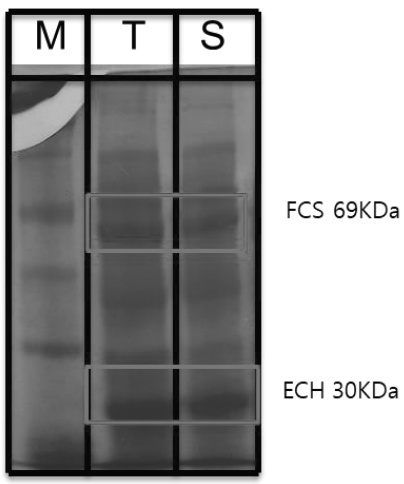


Figure 3. SDS-PAGE gels of TALs and TPL expression cells. (a), RsTAL and SeTAL(55 kDa each) and (b), CfTPL(51 kDa) and SeTAL (55 kDa) were expressed in BL21(DE3). M: marker, T: total fraction and S: soluble fraction, respectively.

(a)



(b)

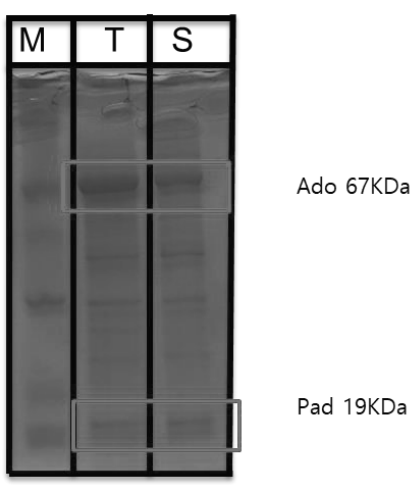


Figure 4. SDS-PAGE gels of module 2 expression cells. (a) BgFCS (69 kDa) & BgECH (30kDa) and TtADO (67 kDa) & BcPAD (19 kDa) were co-expressed in BL21 (DE3). M: marker, T: total fraction and S: soluble fraction, respectively.

2. 4. Whole cell biotransformation

Cells were harvested by centrifugation and washed with 100 mM Tris HCl buffer (pH 8.0), followed by resuspension in the same buffer. The cell buffer resuspension was taken into 100 mL shake flask or test tube, with the cell density adjusted to 30 OD_{600} in final 10 ml or 2 ml volume. In the module 1, reaction was initiated by adding phenol (5 or 10 mM) with two equivalent of sodium pyruvate or L-tyrosine (3 or 10 mM) and 100mM of ammonium chloride. In the module 2, 5 mM of *p*CA was added into the shake flask or 2ml test tube. reactants were incubated at 30 °C and 200 rpm, and 0.5 mL sample aliquots were collected with 0.5 mL methanol in corresponding interval hours. The samples were centrifuged and filtered through a 0.2 μm membrane filter for HPLC analysis.

2. 5. Product identification and quantification

Quantitative analysis was performed by HPLC YL9100 having a dual-wavelength UV detector from Young Lin Chromass (Anyang, Gyeonggi, South Korea) equipped with Waters symmetry C18 column (4.6_150mm, 5.0mM particle size, Waters). A gradient scheme of solvent A (water, 0.1% TFA) and solvent B (acetonitrile, 0.1% TFA) were applied as followings: 0-5 min, 5% solvent B at start: 5-12 min, 50% solvent B linear gradient, 12-15 min, holding at 50% solvent B: 15-20 min 5% solvent B (flow rate, 0.7 mL/min). Eluted peaks were detected at 270nm and the retention times of phenol, L-tyrosine, *p*-

hydroxybenzoate (*p*HBA), *p*HBAL and *p*CA were 6.2, 2.7, 10.6, 18.2 and 21.3 min respectively. Each peak was identified by comparison of the HPLC chromatogram with that of an authentic sample.

3. Results and Discussion

3. 1. Construction of module I: *p*CA from phenol

Tyrosine phenol lyase is a pyridoxal 5-phosphate (PLP) dependent enzyme catalyzing β elimination of L-tyrosine, producing one equivalent phenol, pyruvate and ammonia.[21] Under high amount of ammonium condition, it has been well known that CfTPL (tyrosine phenol lyase from *C.freundii*) can catalyse reversible β elimination in which phenol and pyruvate are linked into L-tyrosine[21, 22]. Also, the CfTPL has been widely used for synthesis of various L-tyrosine analogues, *p*CA derivatives, vinylation and hydroxyl ethyl functionalization of corresponding phenol.[23-26]. For these reasons, CfTPL was selected in the module 1.

The two TALs from different strains were transformed into *E.coli* and their activities were confirmed by biotransformation as described above. In 3mM of tyrosine reactions, *E. coli* with RsTAL has shown to produce 0.5 mM of *p*CA while 2 mM of *p*CA was formed from that of SeTAL in 24h. (Fig 5) Based on the higher conversion, SeTAL was chosen for the module 1

To make *p*CA from phenol, *E.coli* with CfTPL and SeTAL were tested to biotransformation where 100mM of ammonia was added together with 10mM of L-tyrosine for the ammonia compatibility and feasibility of TPL and TAL combining reaction. In 10mM of phenol and tyrosine reaction, each cell expressing CfTPL or SeTAL has shown to produce 9.7 and 8.2mM of L-

tyrosine and *p*CA, respectively in 12h. (Fig. 6) The performance of CfTPL and SeTAL showed the feasibility of combining two separate reactions as the presence of 100mM ammonia didn't seem to retardate the TAL reaction in which TPL reaction is favorable but not deamination with same range of substrate.

Previously, the CfTPL and SeTAL expressing cells were mixed into one pot biotransformation in which optical density at 600 nm (OD_{600}) of each cell was 15. However, only 0.7 mM of *p*CA was formed providing, cell to cell transport of L-tyrosine might be problem. For this reason, single cell was used to the biotransformation for favorable volumetric effect. Both vector having CfTPL and SeTAL were co-transformed into single cell, and the performance of the cell was tested with 10mM of phenol, 20mM of pyruvate and 100mM ammonia. The most of products was L-tyrosine, while 0.5 mM of *p*CA was formed (5 %) in 12h. (Fig. 6a) Compared to the expression levels of TAL with and without TPL from the SDS-PAGE (Fig 7), the relatively high amounts of TPL over TAL might result this low *p*CA conversion yield.

It might be envisioned that the expression of CfTPL in the pHCII vector could lead to this unbalance in which constitutive promoter with high copy number origin (500~700) pre-express CfTPL while SeTAL was not expressed until the addition of IPTG. For the same expression system, the CfTPL was sub-coloned into the pETDuet-1 vector and co-transformed with pET24mSeTAL into single cell. The cell was subjected to biotransformation as the same condition where pHCIICfTPL vector used. (Fig 6b) When the CfTPL & SeTAL were co-

expressed under IPTG induction system, it was noting worth that the expression levels of two enzymes were similar, showed in SDS-PAGE (Fig 9) while the conversion of *p*CA was slightly higher than that of reaction with pHCIICfTPL used (8 %). This could demonstrate generally lower activity of TAL compared to TPL. For more efficient system the expression balance should be adjusted.

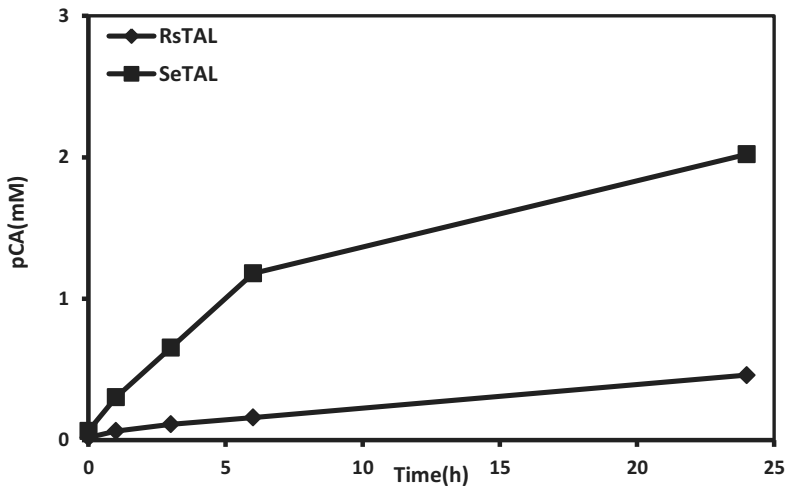
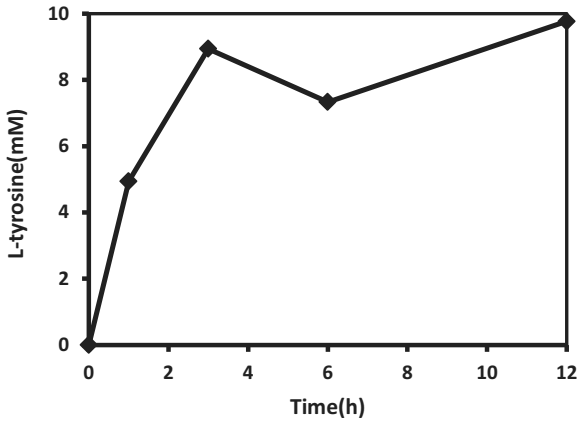


Figure 5. Biotransformation of L-tyrosine with RsTAL and SeTAL expressing cells. Reaction conditions: Substrate concentration, 3mM tyrosine (DMSO 10%); Volume, 2mL; Temp, 30°C; Cell type, BL21 (DE3) containing pET24mRsTAL or pET24mSeTAL.Cell OD600, 30; Tris buffer, 100 mM; pH, 8.0.

(a)



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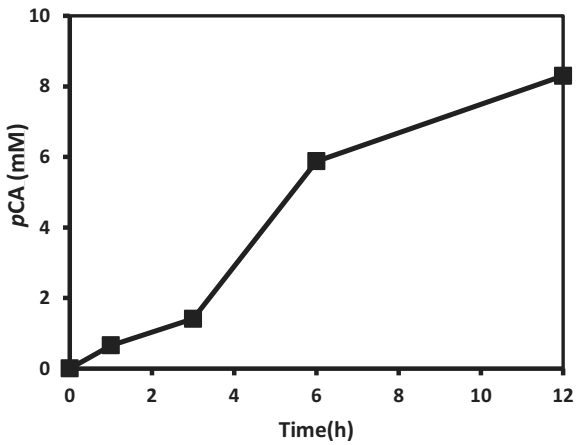
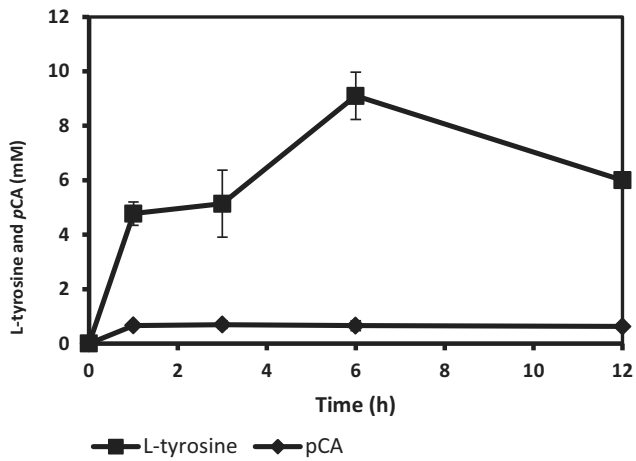


Figure 6. Biotransformation of phenol and L-tyrosine with CfTPL or RsTAL expressing cells. Reaction conditions: Substrate concentration, 10mM phenol or tyrosine (DMSO 10%), 100mM ammonia and 20mM pyruvate for TPL; Volume, 2mL; Temp, 30 ° C; Cell type, BL21 (DE3) containing pHCIICfTPL and pET24mSeTAL respectively, Cell OD600, 30; Tris buffer, 100 mM; pH, 8.0.

(a)



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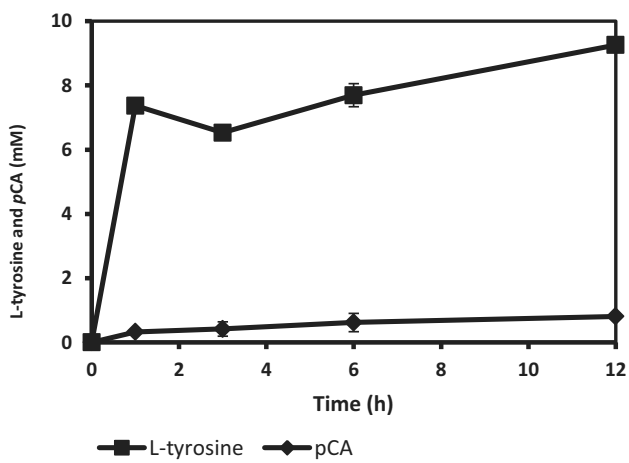
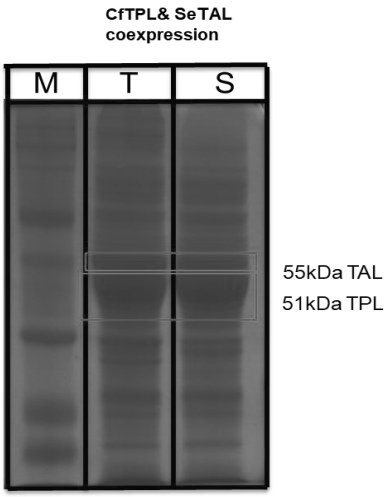


Figure 7. Biotransformation of phenol with CfTPL & SeTAL co-expressing cell. Reaction conditions: Substrate concentration, phenol 10mM, pyruvate 20mM, ammonia 100mM; Volume, 2 mL; Temp, 30°C; Cell type, BL21 (DE3) containing (a) pHCIICfTPL & pET24mSeTAL and (b)) pETduetCfTPL & pET24mSeTAL, Cell OD600, 30; Tris buffer, 100 mM; pH, 8.0.

(a)



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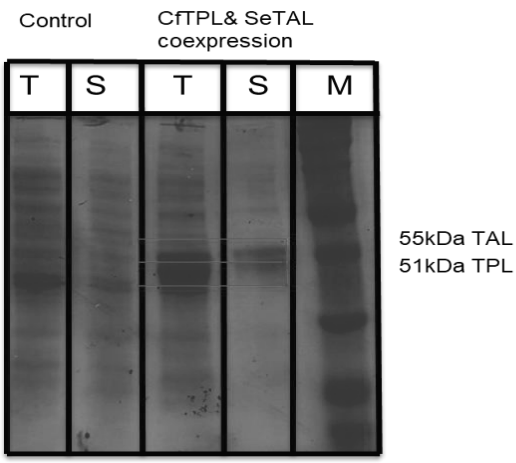

Figure 8. SDS-PAGE gel of CfTPL & SeTAL co-expressing cell. CfTPL(51 kDa) and SeTAL (55 kDa) were co-expressed in BL21(DE3) containing (a) pHCIICfTPL & pET24mSeTAL and (b) pETDuetCfTPL & pET24mSeTAL. M: marker, T: total fraction and S: soluble fraction, respectively.

3. 2. Construction of module 2: *p*HBAL from *p*CA

In the module 2, two systems, in which genes encoding coenzyme dependent (BgFCS & BgECH) and independent (TtADO & BcPAD) enzymes were transformed into *E.coli*, were subjected to biotransformation of *p*CA as described above.

With *E.coli* having TtADO & BcPAD, 1.1 mM of *p*HBAL was formed in 12h along with 3.6 mM of *p*-hydroxystyrene (*p*HST) which is decarboxylated product of BcPAD. In case of BgFCS & BgECH co-expressing cell, 4.4 mM of *p*HBAL was formed. (Fig 7 and Fig 8). Previously, the performance of coenzyme free catalysis showed, 146 mM of *p*HBAL from 160 mM of *p*CA with relatively high amount of cells and higher temperature. When the same amount of cell was used, the BgFCS& BgECH has shown to better performance over that of TtADO& BcPAD. Plausibly, the lower temperature might impair their inherent thermophilic characteristics and give the low conversion in this study. Although, compensating the activity loss (approximately, up to 40%) as reaction temperature decreased from 50°C to 30°C in the literature[14], BgFCS & BgECH system showed better performance over TtADO& BcPAD system. Therefore, BgFCS & BgECH were exploited to further studies.

Because of the characteristics for BgFCS using ATP and CoA, whole cell biotransformation was carried out with BgFCS& BgECH under presence of glucose owing to regenerate co-substrates. As expected, the initial rate was increased from 1.8mM/h to 3.7mM/h with 0.25% of glucose, while the formation

of *p*-hydroxybenzalcol (pHBALCOL) was also increased from 1.1 mM to 1.6 mM. The presence of glucose in the reaction mixture might regenerate not only ATP and CoA but also NADH which is responsible substrate for endogenous Alcohol dehydrogenases (ADHs).[27]

To obtain high initial reaction rate with low byproduct, it is essential that endogenous ADHs are inactivated. Recently Aditya M. Kunjapur et al reported *E.coli* MG1655 strain with reduced aromatic aldehyde reduction (RARE) in which endogenous three ADHs and three aldo-keto reductases (AKRs) were deleted.[20] Along with the same purpose, RARE strain was exploited to produce *p*HBAL.

BgFCS & BgECH containg pETduet was successfully transformed into RARE and subject to whole cell biotransformation of *p*CA in the presence of glucose. The initial rate was nearly 4mM/h in the presence of glucose from 0.25 to 0.5% while, 2.9mM/h in the absence of glucose. The amounts of *p*HBAL and *p*HBALCOL formed in 12h were nearly same (4.3~4.5 mM for *p*HBAL and 0.7~0.9mM for *p*HBALCOL). As the relatively higher initial product forming rate with lowered byproduct formation from RARE expressing BgFCS & BgECH, the cell was subject to combining with module 1.

3. 3. Combination of module 1 and module 2: *p*HBAL from phenol

As module 1 seemed to be rate limiting step, it can be envisioned that combining of module 1 and module 2 might give better conversion in which relatively more active module 2 draws reaction forward equilibria. Genes having

module 1 and module 2 were co-transformed into RARE and subject to biotransformation.

In reaction with 5mM of phenol, most of products were 3.8 mM of L-tyrosine, while 0.06 mM of *p*HBAL and 1.16 mM of *p*CA were formed in 12h. Because of three plasmids used in a single cell, the expression levels of BgFCS and BgECH were dramatically decreased, comparing the only BgFCS & BgECH only expressing cells. (Fig. 13) Modulating appropriate protein expression level balance could increase the overall titer in the future work. Despite of the low conversion, this result demonstrated de-novo synthesis of *p*HBAL from phenol in *E.coli* for the first time.

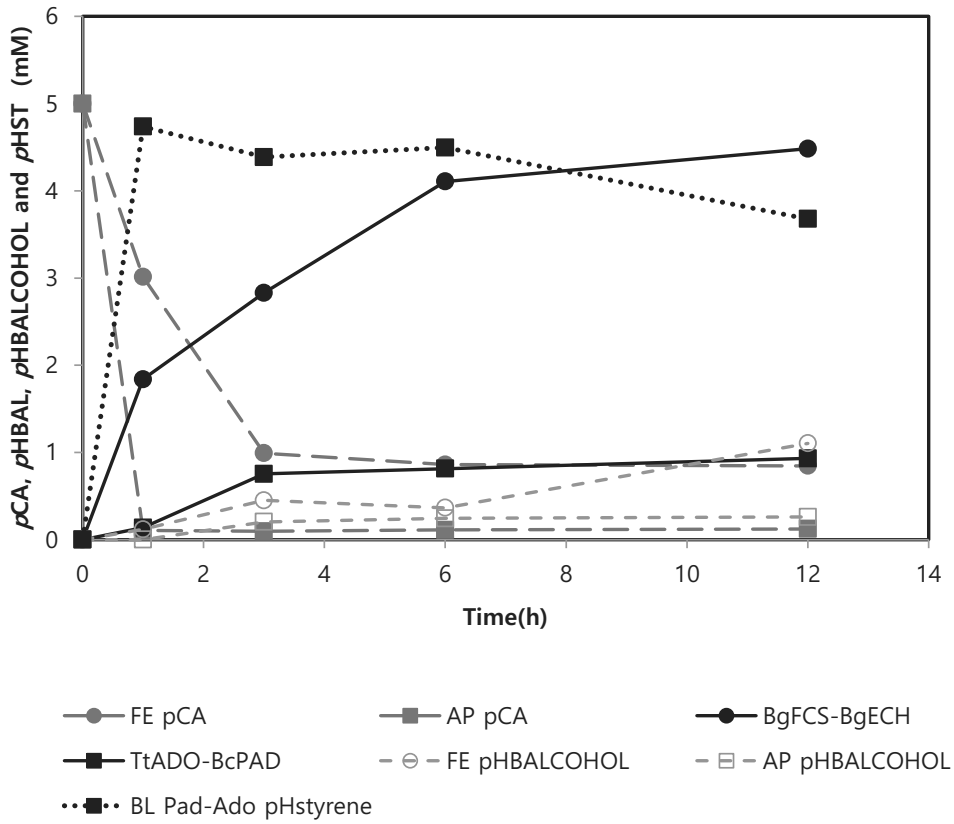

Figure 9. Biotransformation of *p*CA with FCS & ECH and Ado & Pad co-expressing cells. Reaction conditions: Substrate concentration, 5 mM *p*CA; Volume, 10 mL in 100 mL flask; Temp, 30°C; Cell type, BL21 (DE3) containing pETduet FCS-ECH and ADO-PAD, respectively; Cell OD_{600}, 30; Tris buffer, 100 mM; pH, 8.0.

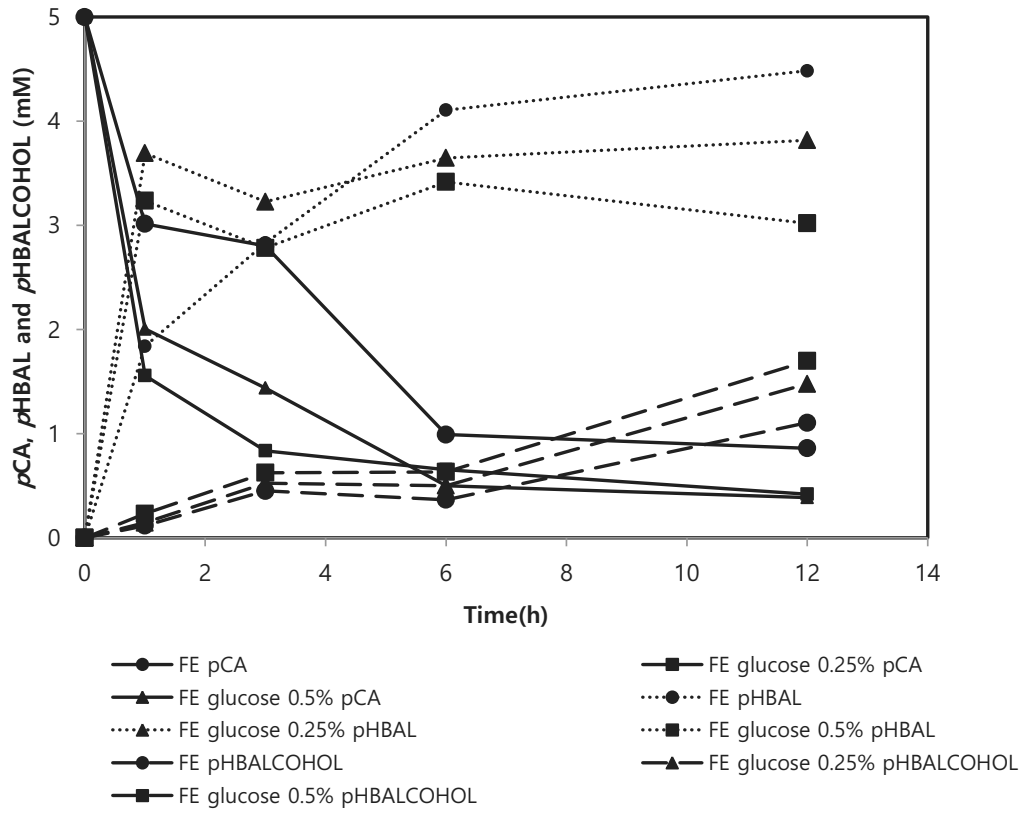

Figure 10. Biotransformation of *p*CA with FCS & ECH co-expressing cell in the presence of glucose. Reaction conditions: Substrate concentration, 5 mM *p*CA; Volume, 10 mL in 100 mL flask; Temp, 30°C; Cell type, BL21 (DE3) containing pETduet FCS-ECH; Cell OD_{600}, 30; Tris buffer, 100 mM; pH, 8.0; glucose was added into reaction mixture from 0 to 0.5% (w/v) using 80% glucose stock.

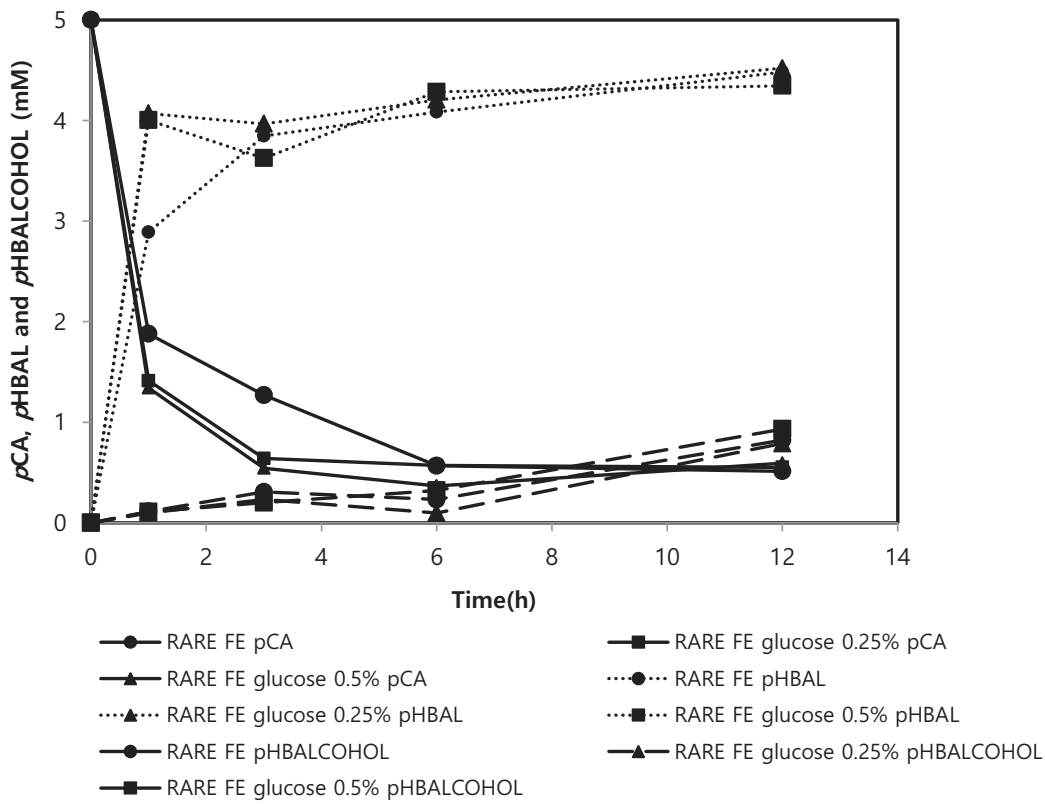

Figure 11. Biotransformation of *p*CA with RARE expressing BgFCS & BgECH in the presence of glucose. Reaction conditions: Substrate concentration, 5 mM *p*CA; Volume, 10 mL in 100 mL flask; Temp, 30°C; Cell type, BL21 (DE3) containing pETduet FCS-ECH; Cell OD_{600}, 30; Tris buffer, 100 mM; pH, 8.0; glucose was added into reaction mixture from 0 to 0.5% (w/v) using 80% glucose stock.

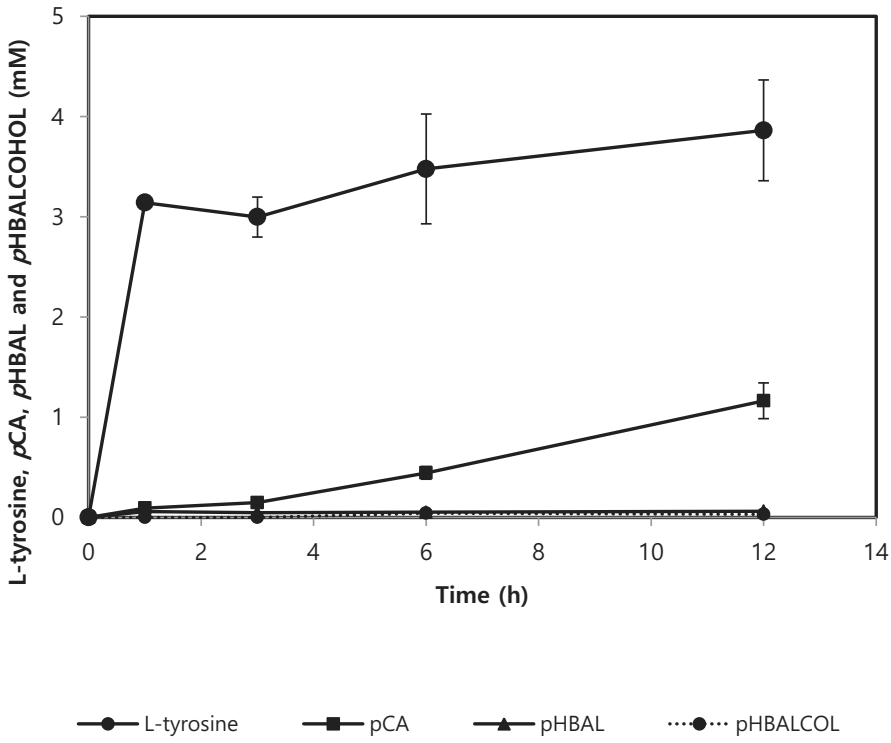

Figure 12. Biotransformation of phenol with module 1 and module 2 combined cell. Reaction conditions: Substrate concentration, 5mM of phenol, pyruvate 7.5mM, ammonia 100mM, glucose 0.5% w/v, and Ethanol 5% v/v; Volume, 10 mL; Temp, 30°C; Cell type, RARE containing pHCIICfTPL, pET24mRsTAL pRSFduet FCS-ECH Cell OD600, 30; Tris buffer, 100 mM; pH, 8.0.

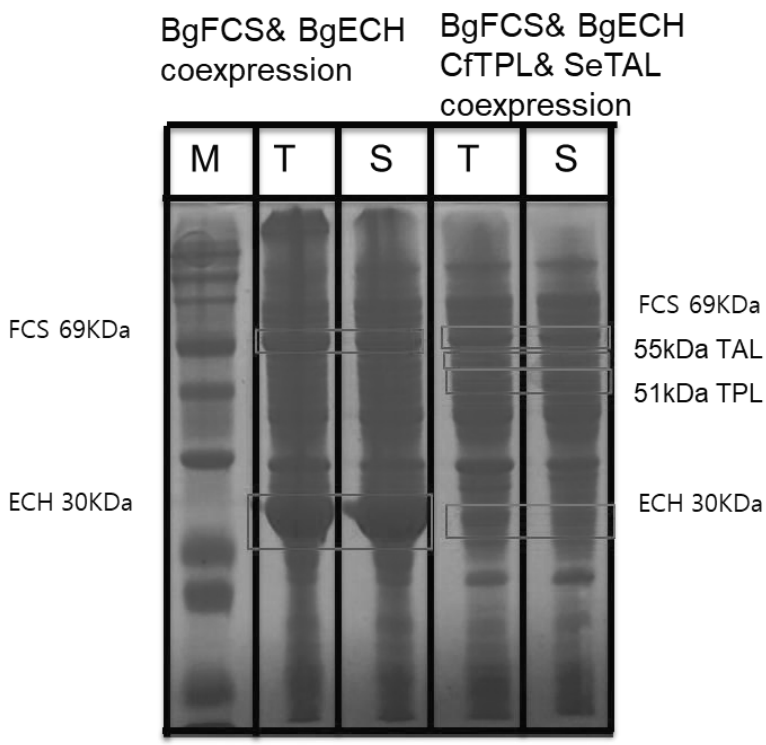

Figure 13. SDS-PAGE gel of BgFCS & BgECH with and without CfTPL & SeTAL co-expressing cells. BgFCS (69 kDa) & BgECH (30kDa) and CfTPL (51 kDa) & SeTAL (55 kDa) were co-expressed in BL21 (DE3). M: marker, T: total fraction and S: soluble fraction, respectively.

4. Conclusion.

p-hydroxybenzaldehyde (*p*HBAL) is versatile intermediate for various applications such as perfumes, medicines, liquid crystals as well as food ingredients. To produce *p*HBAL from more cheap and available phenol, two modules were separately applied and combined into *E.coli.*

In the module 1, tyrosine phenol lyase (TPL) from *Citrobacter freundii* and tyrosine ammonia lyase (TAL) from *Saccharothrix espanaensis* were heterogously expressed in *E.coli* respectively. The expressions and activity were confirmed through SDS-PAGE and biotransformation in which 5 mM of phenol converted into 0.8 mM of *p*-coumaric acid (*p*CA) in 12h (8 %).

In the module 2, feruloyl CoA synthase (FCS) and enoyl CoA hydratase/aldolase were expressed in engineered reduced aromatic aldehyde reduction strain. The 4.5 mM of *p*HBAL was formed from 5 mM of *p*CA with initial product forming rate 4 mM/h introducing glucose feeding.

Finally, the module 1 and module 2 were combined, showing to produce 0.06 mM of *p*HBAL from 5 mM of phenol (1.2 %) in 12h. Modulating appropriate protein expression level balance could increase the overall titer in future work. Despite of the low conversion, this result demonstrated de-novo synthesis of *p*HBAL from phenol in *E.coli* for the first time.

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국문초록

파라-하이드록시벤즈알데하이드는 향수, 의약품, 액정 및 식품 성분과 같은 다양한 용도의 다기능 중간체로써, 이를 제조하기 위한 화학 합성 경로가 잘 확립되었지만, 상대적으로 어려운 고온 고압의 조건 및 반응상 넣어주는 과량의 염기의 제거는 아직 문제로 남아있다. 이러한 이유로, L-타이로신과 파라-쿠마레이트로부터 재조합 대장균을 이용한 성공적인 생합성 경로가 보도되고 있다. 그러나 상대적으로 비싼 전구체의 사용은 보다 저렴하며 접근성이 높은 전구체를 사용하는 생합성의 필요성을 제공한다.

본 연구에서는 페놀로부터 파라-하이드록시벤즈알데하이드를 생산하기 위해 두 개의 모듈을 각각 대장균에서 적용하여 합쳤다. 모듈 1 에서 *C. freundii* 유래의 tyrosine phenol-lyase (TPL)와 *S. espanaensis* 유래의 tyrosine ammonia-lyase (TAL)가 각각 *E.coli* 에서 이형 적으로 발현되었다. 발현과 반응성은 SDS-PAGE 와 세포 생전환을 통해 확인했으며, 10 mM 의 페놀이 12 시간 동안 0.8 mM 의 파라-쿠마레이트로 전환되었다 (전환율 8 %). 모듈 2 에서는 *B.glumae* 유래의 feruloyl coa synthase (FCS)와 enoyl coa hydratase/aldolase (ECH)를 이형 적으로 발현하였다. 5 mM 의 파라방향족 알데하이드의 환원이 감소된 균주를 도입하고 포도당을 반응상에 넣어줌으로써, 12 시간 동안 5 mM 의 파라 쿠마레이트로부터 4.4 mM 의 파라히드록시벤즈알데히드를 얻을 수 있었다. (전환율 90 %)

최종적으로 하나의 세포에서 모듈 1 과 모듈 2 를 구성하는 유전자를 발현하였고, 이 세포를 통해 12 시간 동안 5 mM 의 페놀이 0.06 mM 의 파라-히드록시벤즈알데히드로 전환될 수 있었다. 낮은 전환율에도 불구하고, 이 결과는 처음으로 *E.coli* 의 페놀로부터 파라-히드록시벤즈알데히드의 생전환을 보도하는데 그 의의가 있다.

주요어: 파라-히드록시벤즈알데히드, 생전환.

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