
# *METABOLIC ENGINEERING OF Escherichia coli CARRYING CATECHINS BIOSYNTHESIS GENES OF Camellia sinensis (L) KUNTZE*

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# METABOLIC ENGINEERING OF *Escherichia coli* CARRYING CATECHINS BIOSYNTHESIS GENES OF *Camellia sinensis* (L) KUNTZE

By

KABIR UMAR MUSTAPHA

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment for the Requirements for the Degree of Doctor of Philosophy**

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## DEDICATION

To my parents and in-laws for their unconditional love and prayers

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

# METABOLIC ENGINEERING OF *Escherichia coli* CARRYING CATECHINS BIOSYNTHESIS GENES OF *Camellia sinensis* (L) KUNTZE

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**July 2012**

**Chair:** **Assoc. Prof. Abdulkarim Sabo Mohammed**

**Faculty:** **Food Science and Technology**

Catechins are the most abundant polyphenolic compounds found in green tea (*Camellia sinensis*) that have been shown to bioactively affect the pathogenesis of several diseases. The sources of these substances are solely from tea leaves, thus relying on agriculture which may result to shortages due to unfavourable environmental conditions. Metabolic engineering strategies have recently been developed for the standardized biosynthesis of flavonoids in recombinant microbial systems through incorporation of genes in the biosynthetic pathway. Nevertheless, none of these strategies have produced any of the major catechins. This study is aimed at metabolically engineering the production of pure catechins in *E. coli* carrying the metabolite genes involved in biosynthesis of catechins in *Camellia sinensis*. DNA from *Camellia sinensis* leaf tissues was extracted and isolated by optimization of extraction parameters. High yield of DNA up to 125µg/ml was obtained from 0.3g of leaf tissue. The optimal extraction parameters were determined to be; precipitation time of 40 minutes, at 50°C incubation temperature and

200/800/100µl (EBA/EBB/SDS) of extraction buffer combinations. Three genes [*CsF3H* (1107bp), *CsDFR* (1044bp) and *CsLCR* (1044bp)] were amplified and cloned into pET expression vectors and transformed into *E. coli* BL21 (DE3) competent cells. Genes were expressed in 1mM IPTG to produce proteins *F3H* (40kDa), *DFR* (45kDa) and *LCR* (38kDa), with corresponding theoretical sizes as analyzed by SDS-PAGE. A mimicked biosynthetic pathway of catechin metabolite genes from *Camellia sinensis* consisting of *CsF3H, CsDFR* and *CsLCR* encoding flavanone 3 hydroxylase, dihydroflavonol 4- reductase and leucoanthocyanidin reductase respectively was designed and arranged in two sets of constructs in the following order: (a) A single promoter upstream of *CsF3H* followed by ribosome binding sequences both upstream of *CsDFR* and *CsLCR*; (b) Three different promoters and ribosome binding sequences each upstream of the three genes. Recombinant *E. coli* BL21 (DE3) harbouring the constructs were cultivated for 65 h at 26°C in M9 medium consisting of 40 g/l glucose, 1 mM IPTG and 3 mM eriodictyol. Compounds produced were extracted from culture medium using ethyl acetate in alkaline conditions after 1 h at room temperature and identified by HPLC. Two of the four major catechins, namely, (-)-epicatechin (0.01 mg/l) and (-)-epicatechin gallate (0.36 mg/l) and two other types; (+)-catechin hydrate (0.13 mg/l) and (-)-catechin gallate (0.04 mg/l) were successfully produced. Optimization of process parameters using a face centred central composite design (FCCCD) consisting of 30 experimental runs involving 6 centre points was designed using MINITAB® software. Glucose concentration had the most significant effect followed by temperature on yield of catechins. The optimum conditions for engineering of catechin production from the recombinant *E. coli* strain was as follows: IPTG (0.9 mM), glucose (10.1 g/l), eriodictyol (1.0 mM) and temperature

(27.9°C) and were predicted to produce a response of 0.97 mg/l with 80.98% desirability. A verification experiment carried out using predicted optimum parameters, produced a yield (0.911 mg/l ) close to the predicted value in which the gallated catechins [(-)-epigallocatechin gallate) and (-)-epicatechin gallate] were metabolically engineered for the first time.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**KEJURUTERAAN METABOLIK *Escherichia coli* YANG MEMBAWA GEN BIOSINTESIS KATEKIN DARIPADA *Camellia sinensis* (L) KUNTZE**

**Oleh**

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**Julai 2012**

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Katekin adalah komponen polypenolik terbanyak yang boleh didapati pada bahawa hijau teh dan terbukti telah memberi kesan bioaktif kepada penyakit berasaskan patogenesis. Sumber utama pada komponen ini adalah dari daun pokok teh dan menyebebkan pergantungan kepada industri pertanian dan memberi impak kepada risiko kekurangan bahan komponen ini disebabkan ke adaan persekitaran yang tidak kondusif. Bidang kejuruteraan metabolic bersepadu telah menghasilkan standad biosintesis berkaitan flavanoid pada rekombinan secara sistem mikrobial melalui cara penyatuan genetik didalam laluan biosisntesis Walau bagaimana pun, tiada hasil yang dapat diperolehi melalui cara ini dalam panghasilan utama katekin. Kajian ini bertujuan untuk menghasilkan catechin tulen melalui kejuteraan metabolisme yang membawa gen metabolit *Camellia sinensis* diekstrak dan diasingkan melalui pengoptimuman parameter pengekstrakan. Ekstrak DNA yang tinggi diperolehi sehingga 125 µg/ml daripada 0.3g tisu daun. Parameter pengekstrakan optimum adalah pada 40 minit masa pemendakan 50°C suhu inkubasi dan 200/800/100 µl

(EBA/EBB/SDS) kombinasi buffer pengekstrakan. Gen yang diasingkan [*CsF3H* (1107bp), *CsDFR* (1044bp) dan *CsLCR* (1044bp)] telah diklonkin dalam vektor ekspresi pET dan berubah menjadi *E. coli* BL21 (DE3) sel-sel. Gen telah diekspresikan dalam 1mM IPTG untuk menghasilkan protein *F3H* (40kDa), *DFR* (45kDa) dan *LCR* (38kDa), dimana sama dengan saiz teon, seperti yang dianalisis oleh SDS-PAGE. Tapak jalan biosintetik yang serupa seperti gen metabolit katekin dari *C. sinensis* yang mengandungi *CsF3H*, *CsDFR* dan *CsLCR* yang mengekodan flavanone 3 hydroxylase, reductase dihydroflavonol dan reductase leucoanthocyanidin, masing-masing telah direka dan disusun dalam dua set susunan yang berikut: (a) promoter tunggal dihadapan *CsF3H* diikuti oleh ribosom binding sekuens (RBS) dihadapan *CsDFR* dan *CsLCR*; (b) promoter dan RBS dihadapan setiap gen pada kedudukan yang sesuai. Rekombinan *E. coli* BL21(DE3) yang membawa set-set telah dibiakkan selama 65 jam pada suhu 26 $^{o}$C di dalam medium M9 yang terdiri daripada 40 g/l glukosa, 1 mM IPTG dan 3 mM eriodictyol. Sebatian yang dihasilkan diekstrak dengan etilasetat dalam keadaan alkali selepas 1 jam pada suhu bilik dan dikenalpasti dengan menggubakan HPLC. Dua daripada empat catechin yang utama, iaitu, (-)-epicatechin (0.01 mg / l) dan (-) epicatechin gallate (0,36 mg / l) dan dua jenis lain; (+) catechin hidrat (0,13 mg / l) dan (-)-catechin gallate (0.04 mg / l) telah berjaya dihasilkan. Pengoptimuman pelbagai parameter yang menggunakan face centred central composite design (FCCCD) terdiri daripada 30 eksperimen yang melibatkan 6 titik tengah telah dirangka deya menggunakan perisian ® MINITAB. Kepekatan glukosa mempunyai kesan paling ketara diikuti dengan suhu terhadap penghasil katekin. Keadaan optimum untuk penghasilan katekin daripada *E. coli* rekombinan adalah seperti berikut: IPTG (0.9 mM), glukosa (10.1 g/l), eriodictyol (1.0 mM) dan suhu (27.9 $^{o}$C) dan telah diramalkan untuk

menghasilkan 0.97 mg/l dengan kebaikan 80.98%. Eksperimen pengesahan telah dijalankan menggunakan parameter optimum yang perolehi, memberikan hasil (0.91 mg/l) yang hampir sam dusu nilai yang diramalkan di mana katekin gallated [(-)-epigallocatechin gallate) dan (-) epicatechin gallate] telah dihasikan melalui kejuruteraan metabolit buat kali pertama.

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## List of abbreviations

| | |
|---|---|
| AAA | aromatic amino acid |
| *CHS* | chalcone synthase |
| *F3H* | flavanone 3-hydroxylase (enzyme) |
| *DFR* | dihydroflavonol 4-reductase (enzyme) |
| *LCR* | leucoanthocyanidin reductase (enzyme) |
| ORF | open reading frame |
| SOPMA | Self-Optimized Prediction Method with Alignment |
| $Fe^{2+}$ | ferrous iron |
| SDRs | short chain dehydrogenase reductase |
| PIP | pheylcoumaran benzylic ether reductase |
| OmpT | outer membrane endoprotease |
| VMP001 | *E. coli* malaria protein 001 |
| LLO | listeriolysin O |
| hPTH | human parathyroid hormone |
| hBD2 | human beta-defensin-2 |
| EC | (-) epicatechin |
| EGC | (-) epigallocatechin |
| EGCG | (-) epigallocatechin-gallate |
| ECG | (-) epicatechin-gallate |
| PTC | polyphenolic tea catechins |
| NF-*k*B | nuclear factor kappaB |
| $p21^{CIP1}$ | cyclin-dependent kinase inhibitor |
| MAPKs | mitogen-activated protein kinases |
| Bcl-2 | B- cell lymphoma 2 |
| Apaf-1 | apoptotic protease activating factor 1 |

| | |
|---|---|
| PARP | poly(ADP-ribose) polymerase |
| PCNA | proliferating cell nuclear antigen |
| MMTV | mouse mammary tumor virus |
| ICAM-1 | intercellular adhesion molecule |
| TNF-alpha | tumor necrosis factor alpha |
| STAT | signal transducers and activators of transcription |
| ROS | reactive oxygen species |
| AMVN | 2,2'-azobis (2,4-dimethylvaleronitrile) |
| AK | arginine kinase |
| PPAR-gamma | peroxisome proliferator-activated receptor-gamma |
| C/EBP-alpha | CCAAT/enhancer-binding protein-alpha |
| SREBP-1c | sterol regulatory element-binding protein-1c |
| aP2 | adipocyte fatty acid-binding protein |
| LPL | lipoprotein lipase |
| FAS | fatty acid synthase |
| CPT-1 | carnitine palmitoyl transferase-1 |
| UCP2 | uncoupling protein 2 |
| HSL | hormone sensitive lipase |
| ATGL | adipose triglyceride lipase |
| Phe | phenylalanine |
| Trp | trptophan |
| Tyr | tyrosine |
| E4P | D-erythrose-4-phosphate |
| PEP | phosphoenol pyruvate |
| DAHP | 3-deoxy-D-arabino-heptulosonate 7-phosphate |
| DHQ | 3-dehydroquinate |

| | |
|---|---|
| DHS | 3-dehydroshikimate |
| S3P | shikimate -3- phosphate |
| EPSP | 5-enolpyruvylshikimate 3-phosphate |
| *CsF3H* | flavanone 3-hydroxylase (gene) |
| *CsDFR* | dihydroflavonol 4-reductase (gene) |
| *CsLCR* | leucoanthocyanidin reductase (gene) |
| ACC | acetyl-CoA carboxylase |
| 4CL-2 | 4-coumarate:CoA ligase |
| FSI | flavones synthase I |
| OMT1A | 7-o-methyltransferase |
| FHT | flavanone 3β-hydroxylase |
| FLS | flavanone synthase |
| C4H | cinnamate 4 hydroxylase |
| DXS | deoxyxylulose 5-phosphate synthase |
| PAL | phenylalanine ammonia lyase |
| 4CL | 4 – coumarate:coenzyme A ligase |
| CHS | chalcone synthase |
| CHI | chalcone isomerase |
| *fcs* | feruloyl co-enzyme A |
| *ech* | enoyl-coA hydratase |
| *vaoA* | vanillyl alcohol oxidase |
| *calA* | coniferyl alcohol dehydrogenase |
| *calB* | coniferyl aldehyde dehydrogenase |
| CUS | curcuminoid synthase |
| PKSs | polyketide synthases |
| STS | stilbene synthase |

| | |
|---|---|
| TAL | tyrosine ammonia lyase |
| IPP | isopentenyl diphosphate |
| DMAPP | dimethylallyl diphosphate |
| G3P | glyceraldehydes 3- phosphate/pyruvate |
| MVA | mevalonic acid |
| DMAPP | dimethylallyl pyrophosphate |
| *idi* | isopentenyl pyrophosphate isomerase |
| GPP | geranyl pyrophosphate |
| OGAB | ordered gene assembly in *Bacillus subtilis* |
| CCD | central composite design |
| CTAB | hexadecyltrimethyl ammonium bromide |
| PVP | polyvinylpyrrolidone |
| EDTA | ethylenediaminetetraacetic acid |
| BME | ß-mercaptoethanol |
| TEMED | N,N,N',N'- tetramethylethylenediamine |
| X-gal | 5-bromo-4-chloro-indolyl-β-D-galactopyranoside |
| IPTG | isopropyl-ß-D-thiogalactopyranoside |
| SDS | sodium dodecyl sulphate |
| $OD_{600}$ | optical density at 600 |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Catechins are synthesized from phenylalanine, an aromatic amino acid (AAA) derived from the shikimate pathway [1]. Phenylalanine, a derivative of the shikimate pathway is the first metabolic node of the phenylpropanoid pathway. The pathway is a metabolic tree with many branches in which a plethora of phenolic compounds including flavonoids are synthesized. The study of Park *et al.* [2] on subtractive cDNA library and EST database in *Camellia sinensis* showed a high level expression of primarily three genes, namely, *CsF3H*, *CsDFR* and *CsLCR* encoding flavanone 3-hydroxylase, dihydroflavonol 4-reductase and leucoanthocyanidin reductase, respectively in young leaves than in mature leaves. The finding further proposes a biosynthetic pathway of catechins. Chalcone synthase (*CHSs*) condenses one molecule of 4-coumaryol-CoA and three molecules of malonyl-CoA to produce a chalcone, which is later converted into dihydroflavonol through a stereospecific hydroxylation by F3H. Then, the dihydroflavonol is stereospecifically reduced by DFR to form leucoanthocyanin that is finally used as a substrate for the production of catechins by the action of LCR [3]. Despite this extensive progress, attempts have not been made to mimic the pathway to prove the production of catechins in other hosts.

The metabolic node leading to the biosynthesis of catechins in tea originates from chalcones and comprises of three enzymes; flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin reductase (LCR). The

respective genes encoding these enzymes have been functionally characterized and their cDNA and coding regions have been made available in GenBank (AY641730.1, AB018686.1 and AY169404.1).

It is important to note that catechins have drawn a lot of attention due to the variety of their properties. Among the most studied properties are antioxidative [4], anti-inflammatory [5], anti-obesity [6], and anti-diabetic [7], just to mention a few.

The aforementioned properties of catechins have made them potential functional ingredients in food stuff and feed stuff that improve the quality of food products with increased shelf life [8]. These potentials have led to the development of food products like tea beverages, cereal bars, as well as ice creams, confectionaries and pet food that contain tea as an active ingredient [9]. The demand for such healthy food products have increased with the increase in awareness of the advantages that comes with consuming of these products. A number of methods have been industrially developed by investigators to maximise the content of catechins in food and drinks [10-11], but the sources of these substances have solely been from tea leaves. One major drawback of these sources is the total reliance on agriculture, which may result in shortages due to unfavourable environmental conditions caused by the ever changing climates. Therefore, there is the need to find an alternative way of producing these compounds to supplement the natural sources, which is from green tea.

To avert this problem, alternative sources like re-direction of metabolic flux in microorganisms that carry biosynthetic genes or other genetic engineering

approaches have to be sought. Nevertheless, the success of these approaches relies on qualitative DNA extracts. Previous studies have also demonstrated the presence of polyphenols in *C. sinensis* [3, 12] which are among the inhibitors of polymerase chain reactions, which makes it difficult for the extraction and isolation of qualitative DNA for biomolecular techniques.

Although a variety of methods are available for DNA extraction in many plant species [13-15] and food substances [16-18], extraction of high quality DNA is almost impossible in plants with high polyphenolic compounds and polysaccharides [19-20]. Moreover, these methods are not applicable to all plants [21]. In this study the method of Keb – Llanes *et al.*,[22] was optimised during the extraction and isolation of the DNA.The resultant high yield of DNA, was then subjected to recombinant DNA techniques from low amounts of starting material.

One of the most significant challenges of metabolic engineering of functional phytochemicals of plant origin has been the functional expression of the genes on the biosynthetic pathway in microorganisms. Nevertheless, development of powerful systems for cloning and expression of selective genes under the control of T7 promoter have been successful in producing high levels of target gene products [23]. In this study, open reading frames of *CsF3H*, *CsDFR* and *CsLCR* were cloned into pET vector and expressed in *E. coli* to produce the respective proteins.

It is worth highlighting that metabolic engineering strategies have recently been developed for the standardized biosynthesis of flavonoids in the recombinant *Saccharomyces cerevisiae* and *Escherichia coli* [24-27] by incorporating the genes in

the biosynthetic pathway. To the best of our knowledge, none of the results has produced any of the four primary catechins, but the potentials for applying the metabolic engineering strategies have been indicated for the synthesis of other natural and non-natural flavonoids. Thus, the production of pure catechins by *E. coli* strains carrying a cluster of *CsF3H*, *CsDFR* and *CsLCR* from *C. sinensis* and cultivated in the presence of eriodictyol, a flavanone is presented in this study.

IPTG is an inducer of the T7 promoter that controls the transcription of genes of interest on a pET vector. Eriodictyol is one of the precursor flavanones on the catechin biosynthetic pathway. Prior studies have shown that low temperature protein expression limits the degradation of recombinant proteins by heat shock proteases [28] and increases their solubility and activity [29-31]. Previous studies have also shown the possibility of synthesizing flavonoids directly from glucose in an effort to eliminate dependence on expensive precursors [32]. Given the aforementioned facts it can be concluded that IPTG, eriodictyol, glucose and temperature are important variables in enhancing the yield of catechin production from the recombinant strain.

Over the years, response surface methodology (RSM) has been widely used in the optimization of culture conditions to enhance the production of enzymes [33], antibiotics [34], biocatalysts [35] and other desired products [36] in microorganisms. Nevertheless, research on the influence of process parameters on the yield of metabolically engineered natural products using a statistically based experimental design is very scanty. In this study, critical process variables in the production of catechins by a recombinant *E. coli* BL21 (DE3) carrying an artificial cluster of

catechin biosynthetic genes will be optimized using a central composite design and response surface methodology.

## 1.2 Hypothesis

1. Recombinant strains of *Escherichia coli* carrying a gene cluster of catechin biosynthetic enzymes will produce catechins when grown in a medium containing appropriate precursors, at optimal conditions.

## 1.3 Objectives

1. To isolate the genes involved in catechin biosysthesis from *Camellia sinensis* leaves.
2. Construction of the expression vectors for isolated genes.
3. Expression of cloned genes in *E. coli* BL21 (DE3).
4. Optimization of culture conditions for the production of catechins using response surface methodology for the recombinant cells.

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