An expression construct for sensing cell density and substrate availability and its use in conversion of hydroxycinnamic acids

Cross-Reference to Related Application

[0001] This application claims the benefit of priority of United States of America Provisional Patent Application No. 61/661,593 filed June 19, 2012, the contents of which being hereby incorporated by reference in its entirety for all purposes.

Field of the invention

Prokaryotic recombinant systems designed to operate at a high cell density.

Background of the invention

[0002] Hydroxycinnamic acids are a class of polyphenols having a C6-C3 skeleton. These compounds are hydroxy derivative of cinnamic acid. Hydroxycinnamic acids are phenolic phytochemicals found in a wide range of plants and fungi and are found abundantly in nature. For example, hydroxycinnamic acid such as caffeic acid and ferulic acid (4-hydroxy-3-methoxy-trans-cinnamic acid) can constitute about 3% of the dry weight of graminaceous plants (*1*) such as flax seed with the highest concentrations found in seeds and the bran of graminaceous plants.

[0003] Ferulic acid links arabinoxylans to lignin, the lignin-ferulate-xylan complex (Figure 3) can contribute to the recalcitrance of plant tissues, thereby lowering the efficiency of the biomass conversion in biofuel production (*2*). As such, to reduce substrate complexity and increasing cellulose accessibility, biomass can be pretreated with hemicellulases such as xylanase and feruloyl esterase (*3-5*), with ferulic acid released as a major by-product (*6*). With the

espousal of using switchgrass (a rich source of ferulic acid) (*7*) as the renewable source for biofuel production, the by-product, ferulic acid is currently a waste product.

[0004] Another type of hydroxycinnamic acid found in graminaceous plants is p-coumaric acid (4-hydroxybenzaldehyde). Oxidizing 4-hydroxybenzaldehyde can lead to the production of p-hydroxybenzoate which is used as a monomer for synthesizing liquid crystal polymers. The aromatic compound *p*-hydroxybenzoate is a building block of liquid crystal polymers, high performance plastics that are employed in electronic devices such as mobile phones electronic devices for telecommunication and aerospace applications. p-hydroxybenzoate is also used in chemical and food packaging applications. The cascade bio-synthesis pathway of p-coumaric acid to p-hydroxybenzoate is realized through the biological pathway of 4-hydroxybenzaldehyde formation by feruloyl-CoA synthetase (Fcs), followed by oxidation to p-hydroxybenzoate by enoyl-CoA hydrotase (Ech).

[0005] Hydroxycinnamic acid can provide a subtrate for the production of high value chemicals. Another such economic interest is the production of vanillin (4-hydroxy-3-methoxybenzaldehyde), the key flavor component of vanilla from hydroxycinnamic acids such as ferulic acid. Vanillin is used extensively in food, cosmetic, and pharmaceutical industries (*8*). Due to popular demand, vanillin derived from natural sources such as vanilla pod (*Vanilla planifolia*) can reach as high as US$4000/kg (*9*). The bio-synthesis of vanillin from ferulic acid can be realized through the established biological pathway of feruloyl-CoA thioester formation by feruloyl-CoA synthetase (Fcs), followed by hydration to β-hydroxy derivative and then cleavage to give vanillin and acetyl-CoA by enoyl-CoA hydrotase (Ech) (*10*) (See Figure 4). Many studies had reported the use of these

enzymes from various microorganisms for the bio-production of vanillin (*11-16*). Microorganisms such as *Pseudomonas fluorescens* BF13 (*11*), *Pseudomonas sp.* HR199 (*12*) , *Amycolatopsis sp.* HR167(*13*), *Pseudomonas putida* (*14*), *Bacillus subtilis* (*15*), *Delftia acidovorans* (*16*), and *Streptomyces setonii* (*17*) had been proposed as candidates for the bioconversion of ferulic acid to vanillin. However, these organisms produce natural ferulic acid degraders that are capable of using vanillin as a source of carbon and energy. Thus, to avoid any reduction in the yield, downstream genes such as vanillin dehydrogenase (Vdh) which converts vanillin to vanillic acid may have to be knocked out (*12*).

[0006] An alternative approach has been developed to use a non-native vanillin producer such as *Escherichia coli* (*14, 18-21*) to host the genes responsible for converting ferulic acid to vanillin. Among bacteria, recombinant *E. coli* has been touted as the most efficient biocatalyst for vanillin production (*11*).

[0007] However, in many of the studies where *E. coli* was used as the host for bio-vanillin production, artificial induction with common inducers such as isopropyl-b-D-thiogalactoside (IPTG) and arabinose were performed. Although generally, artificial induction offers the control over protein expression, artificial induction in many cases is less favorable due to high economic cost of inducers, inducer toxicity, incompatibilities with industrial scale-up and detrimental growth conditions (*22*). Thus, these issues often limit the usage of the artificial inducible systems in industrial scale protein production. Using recombinant *E. coli* as a biocatalyst for vanillin production with and without the inhibiter IPTG Lee at al. (19) demonstrated that the addition of IPTG only further decreased production of vanillin.

[0008] A substitute to the artificial inducible systems is the use of constitutive promoters which can initiate protein expression in the absence of the inducers. Though constitutive promoters may offer comparative economic advantage, strong constitutive expression of recombinant proteins may divert the cellular resources away from essential metabolic activities to overproduction of unnecessary RNAs, and proteins. This may subsequently leads to growth retardation or adaptive responses from the host cells that may reduce yield and productivity (*23*). To overcome such problem, previous work by Barghini et al. (2007) (*20*) had tried to use low-copy number vector in vanillin synthesis. Nonetheless, the instability issue brought by strong constitutive promoter still remains.

[0009] One strategy for that has been seen in recent studies for avoiding instability is to use the Vibrio fischeri's quorum sensing system for cell density regulated protein production (*22*), fatty acid bio-sensor for biodiesel production (*23*) and pathogen detection for anti-microbial peptide production (*24*). This strategy uses the lux regulon. It is unclear if the lux regulon would work in the complex control of hydroxycinnamic acid catabolism.

[0010] Gamma-aminobutyrate (GAB) expression is activated under carbon or nitrogen difficient cell stress. Expression of GAB is activated by the sigma factor RpoS a stress induced transcription factor often transcribed in the stationary phase of bacterial cell growth. There are about 70 stress genes having RpoS dependent expression.

Summary

[0011] A first aspect of the invention includes an expression system comprising:

(i) A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter;

(ii) A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter, wherein the second promoter is inducible by the product generated by the first enzyme;

(iii) Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene.

[0012] Another aspect of the invention includes an isolated nucleic acid molecule comprising (i) A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter; (ii)A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter, wherein the second promoter is inducible by the product generated by the first enzyme; (iii) Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene.

[0013] Another aspect of the invention includes a host cell comprising the expression system or the isolated nucleic acid molecule of the invention.

[0014] Another aspect of the invention includes a method of converting a substrate to a product comprising the steps of: (a) subjecting the expression system or the host cell according the invention under conditions that allow the

expression of the first and second enzyme; and (b) isolating the product from said host cell.

[0015] Other aspects of the invention would be apparent to a person skilled in the art with reference to the following drawings and discription of various non-limiting embodiments.

Brief Description of the Drawings

[0016] The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

Figure 1. A schematic of the construct having a Tandem switch design indicated as switch 1 ① and switch 2 ②, that resembles a logic "AND" gate **A**; and a schematic Block diagram of our design indicating the positive feedback loop for Ferylic-CoA synthase (Fcs) expression is included **B**. This design reduces the response time for production.

Figure 2. Our designed genetic system responds to a wide variety of hydroxycinnamic acids, namely caffeic, ferulic and p-coumaric acids, which can be derived from lignin.

Figure 3. A depiction of a lignin-ferulate-xylan complex often constitutes a high amount of ferulic acid in plants especially graminaceous plants such as Switch grass. During pre-treatment process, ferulic acid can be released as a by-product.

Figure 4 Biological pathway for conversion of ferulic acid to vanillin.

Figure 5. A schematic overview of our genetic circuit. The genetic circuit is described in terms of the production of vanillin from ferulic acid implemented in E. coli BL21 (DE3)

Figure. 6 The comparison of growth profiles of our designed construct ▓ in comparison with other constructs such as those that are artificially induced (pBAD) induced at t=0 ▉ and induced at OD_{600} 1.0 □ as well as constructs designed using the quorum sensing lux regulon systems gltA ▒ and Vibrio fischeri ▒.

Figure 7. The comparison of ferulic acid uptake rate and time to saturation of for vanillin production between our designed construct and other constructs. Our designed construct ▲ has the fastest ferulic acid uptake rate and the shortest time to saturation for vanillin production in comparison to pBAD induced at t=0 ◊; pBAD induced at OD_{600} 1.0 □; gltA ●; and Vibrio fischeri system ■.

Detailed description

[0017] We have constructed a recombinant expression system to improve biological system stability for economical bio-production.

[0018] Accordingly a first aspect of the invention includes an expression system comprising: (i) A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter; (ii) A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter, wherein the second promoter is inducible by the product generated by the first enzyme; (iii) Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene.

[0019] The expression system may be designed to operate in vitro. Preferably the expression system is a cell based expression system, more preferably a prokaryotic cell based system.

[0020] Preferably the time delay promotor is activatable by a transcription factor comprising amino acid sequence set forth in SEQ ID NO. 2. preferably, the transcription factor is RpoS sigma factor also known as E sigma 38. While this expression product is primarily known to activate stress related genes in the stationary phase of bacterial cell growth the inventors have been able to use the onset of transcription of the RpoS sigma factor after the exponential growth phase of a bacterial population as a time delay promoter to activate non-stress genes at a time that cell density is stable. In this way expression system is activatable by RpoS and can be switched on in a procaryotic cell when cell population density is optimal for production.

[0021] The sequence set forth in SEQ ID NO. 2 includes any functional varient of Rpos. This may vary from the sequence by 1, 2, or more amino acids. In the context of the present invention, a functional varient is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 20, 50, 100, 200, 300 or 400 amino acids with the amino acid sequences set out in SEQ ID. NO 2. In particular, functional varient should typically be considered with respect to initiation or activation of expression of stress genes known to be activatable by RpoS or initiation or activation of expression of the first heterogonous nucleic acid of at least 2 fold. Preferably, expression inceases 2-100 fold, or 10 to 50 fold.

[0022] Preferred polypeptides of the invention comprise a contiguous sequence having greater than 50, 60 or 70% homology, more preferably greater than 80 or 90% homology, to one or more of amino acids of SEQ ID NO: 2.

[0023] We espoused a new method for gene expression. It is an autonomous regulated system of induction with sensors and feedback loops. Not only does this

system independent of costly artificial inducers, it is capable of regulating the protein expression level based on desired features such as substrate concentration and cell density. By introducing sensors and feedback loops, we are implementing the "control theory" which is widely used in the mature engineering fields (such as mechanical and electrical engineering) into the biological systems. The control theory provides the means for attaining optimal performance in dynamic biological system and achieving system stability through corrective action. By implementing control and self -monitoring in recombinant protein expression, it allows the biological systems to produce what is needed, thereby preventing the wastage of precious cellular resources and compromising key metabolic activities that may affect cell growth.

[0024] The control theory can be applied in our genetic circuit through introducing other bio-sensors. The second nucleic acid promoter sequence activatable by a substrate acts as a substrate sensor and the first nucleic acid promotor sequence acts as a time-delay sensor element. The substrate sensor allows regulation of protein expression. The system would only trigger the protein production when the substrate, is available. Further, in order for the cells to grow well, a time delay is needed before activating the system through the bio-sensor of the first nucleic acid promotor sequence. This is to ensure that in the presence of substrate inducers, cells growing from low cell density (e.g. single colony inoculation) would not experience overwhelming metabolic stress that may lead to growth retardation. The time-delay element would provide the cells some time to reach a higher cell density before activating the bio-sensor of the second nucleic acid promoter sequence, so that the amount of substrate inducers per cell would be lower and thus less detrimental to growth.

[0025] Our mode of gene induction in bio-catalysts is more economically attractive. Compared to existing methods which use costly inducers such as IPTG, our method for vanillin production eliminates the need for external inducers and can bring considerable material cost savings in the industrial conversion.

[0026] Preferably, the time delay promoter sequence comprises a consensus sequence set forth in SEQ ID NO: 1. This sequence $nCTAn_3Tn_6$ is conserved in the promotor region of operons where expression is dependent on the presence of RpoS. Usually the consensis sequence is located in the -10 region of the operon where the CTA is at -13 to -11 and T is at -7 variations may exist provided the the first nucleic acid promoter sequence is activatable by RpoS in that the nucleic acid sequence adjacent the time delaypromoter has increased expression of at least 2 fold when RpoS is present. Preferably, expression inceases 2-100 fold, or 10 to 50 fold.

[0027] There are many stress genes known to have RpoS dependent expression. Many of these genes have been shown to have a promotor region comprising the consensus sequence mentioned above. The promotor region of any such gene may be separated from its natural structural genes that express a stress peptide and placed 5' adjacent the first gene expressing the first enzyme being a heterogonous nucleic acid able to express the first enzyme thereby forming a time delay promotor. The promotor regoin of any stress genes known to have RpoS dependent expression can be used as the time delay promotor. Preferably, the time delaypromoter is selected from gabDT promoter; otsA promoter; katE promoter; osmY promoter; yhiUV promoter; ecnB promoter; dps promoter; osmE promopter; sodC promoter; rpsV promoter; yahoo promoter and

tnaA promoter.In one embodiment the time delaypromoter comprises a gabDT promoter set out in SEQ ID NO: 3.

[0028] The second nucleic acid promoter sequence is activatable by the substrate interfering with an inhibitor of the second nucleic acid promoter sequence thereby allowing expression of the second gene, a heterogonous nucleic acid. There are many procaryotic operons that consists of the repressor/inhibitor, promoter, operator and the structural genes. Any such operon may be used in the isolated nucleic acid expression system described herein.

[0029] In a prefered embodiment the second gene is able to express a enoyl-CoA hydrolase. Preferably the gene encoding the enoyl-CoA hydrolase may comprise the sequence set forth in SEQ ID NO. 4 or a functional variant. In this embodiment the second promoter operably linked to the second gene comprises a sequence set forth in SEQ ID NO. 6 activatable by the product expressed by the first gene.

[0030] In a prefered embodiment the optional third gene encodes the transcription factor PP3359 that represses expression of the second gene enoyl-CoA hydrolase in the absence of the product Ferulic-Co-A generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene. Preferably the third gene comprise the sequence set forth in SEQ ID NO. 7 encoding the transcription factor comprise the sequence set forth in SEQ ID NO. 8 or a functional variant.

[0031] Another aspect of the invention includes an isolated nucleic acid molecule comprising (i) A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter; (ii)A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter,

wherein the second promoter is inducible by the product generated by the first enzyme; (iii) Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene.

[0032] The term "isolated nucleic acid" as used herein refers to any nucleic acid molecule in any possible configuration, such as single stranded, double stranded or a combination thereof. Isolated nucleic acids include for instance DNA molecules, RNA molecules, analogues of the DNA or RNA generated using nucleotide analogues or using nucleic acid chemistry, locked nucleic acid molecules (LNA), peptide nucleic acid molecules (PNA) and tecto-RNA molecules. DNA or RNA may be of genomic or synthetic origin and may be single or double stranded. Such nucleic acid can be e.g. mRNA, cRNA, synthetic RNA, genomic DNA, cDNA, synthetic DNA, a copolymer of DNA and RNA, oligonucleotides, etc.

[0033] In a prefered embodiment the first gene of the isolated nucleic acid molecule is a heterogonous nucleic acid that is able to express the first enzyme comprising feruloyl-CoA synthase and the second gene is a heterogonous nucleic acid that is able to express the second enzyme comprising enoyl-CoA hydrolase. The first gene able to express feruloyl-CoA synthase may comprise the sequence set forth in SEQ ID NO. 4. The second gene able to express enoyl-CoA hydrolase may comprise the sequence set forth in SEQ ID NO. 5. In a prefered embodiment the optional third gene encodes the transcription factor pp3359 that represses expression of the second gene enoyl-CoA hydrolase in the absence of the product Ferulic-Co-A generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene. Preferably

the third gene comprise the sequence set forth in SEQ ID NO. 7 encoding the transcription factor comprise the sequence set forth in SEQ ID NO. 8 or a functional variant.

[0034] In this embodiment the prefered substrate may be a hydoxycinnamic acid or a derivative thereof. Preferably, the hydoxycinnamic acid or derivative thereof may include caffeic acid; ferulic acid; p-coumaric acid; and cinnamic acid. In one embodiment the hydoxycinnamic acid is ferulic acid. In another embodiment the hydoxycinnamic acid is p-coumaric acid. In various embodiments the nucleic acid expression system may convert ferulic acid or caffeic acid to vanillin. In various embodiments the nucleic acid expression system may convert p-coumaric acid, to 4-hydroxybenzaldehyde. Oxidizing 4-hydroxybenzaldehyde can lead to the production of p-hydroxybenzoate. Thus, our system is versatile in generating different products depending on the type of substrate present.

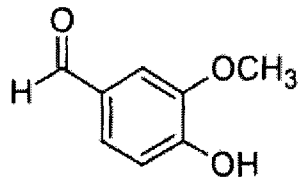
[0035] The isolated nucleic acid molecule may be comprised in a vector such as a plasmid as any vectors or expression systems known in the art.

[0036] Another aspect of the invention comprises a host cell comprising the expression system or the isolated nucleic acid molecule described herein.The host cell is a prokaryotic cell, preferably a bacterial cell. Preferably the host cell is a cell that does not naturally express polypeptides involved in the breakdown of the product. In one embodiment the host cell is an E.coli cell.

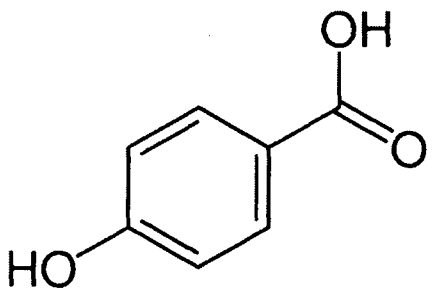
[0037] Another aspect of the invention includes a method of converting a substrate to a product comprising the steps of: (a) subjecting the expression system or the host cell according the invention under conditions that allow the expression of the first and second enzyme; and (b) isolating the product from said host cell.

[0038] In one embodiment step a) may include cultivating the host cell under conditions that allow the expression of the first and second enzyme.

[0039] Preferably the product is a hydroxybenzaldehyde. In one embodiment the product is vanillin having formula 1



[0040] In one embodiment the product is p-hydroxybenzoate having formula 2



[0041]

[0042] **Examples**

[0043] In the design of our production system, we have created a tandem switch for the activation of the hydroxycinnamic acid metabolism system. This tandem switch can be regarded as a logic AND gate device. Only when both hydroxycinnamic acid and threshold cell density are present, a product such as vanillin would be produced. This tandem switch is illustrated in Figure 1A.

[0044] To construct the switches and the vanillin production device, the list of biological parts which were used is stated in Table 2. Using these parts, we have developed a ferulic acid sensor coupled with a time-delay element for the regulation of the vanillin genes, *fcs* and *ech*. To develop the ferulic acid sensor,

we studied and implemented the ferulic acid sensing mechanism in *Pseudomonas. putida* KT2440 in *Escherichia. coli* BL21(DE3). However, for system stability and fast response time to ferulic acid, the design of the genetic circuit is important.

Table 1. List of biological parts used in the genetic circuit for vanillin production

No.	Name	Type	Function
1	P_{gabDT}	Promoter	Time-Delay
2	P_{PP3359}	Promoter	Regulates PP3359
3	P_{ech}	Promoter	Essential component of ferulic acid sensor
4	PP3359	Transcription Factor	Represses Pech. For ferulic acid sensing
5	fcs	Gene	Converts ferulic acid to feruloyl-CoA
6	ech	Gene	Converts feruloyl-CoA to vanillin

[0045] To shorten the response time to the ferulic acid, we had modeled our genetic circuit layout after the ferulic catabolic operon in *P. putida* KT2440. *fcs* together with its time-delay promoter are placed downstream of *ech* gene. Hence, once activated, P_{ech} would lead to the transcription of *ech* and *fcs*. Additional *fcs* transcripts together with the ones transcribed from the time-delay promoter may lead to higher Fcs expression. We expected higher Fcs concentration to shorten the response time of P_{ech} activation and increase ferulic acid uptake. Increase ferulic acid uptake leads to higher formation of feruloyl-CoA, and this drives a faster activation of P_{ech}, which in turn transcribes *ech* and *fcs* genes. Thus, a positive feedback loop is created (Figure 1B), and the system behaves as an amplifier for vanillin enzymes expression, especially for Fcs. Figure 1B describes the signal flow in the block diagram of the vanillin production system. The chief signal in the system is the feruloyl-CoA. Not only does it increases *fcs* transcripts

and activates Ech expression, it is involved in the next step to vanillin production. Once all the feruloyl-CoA has been converted to vanillin, P_{ech} is expected to be repressed by PP3359, and the amplifier behavior to be reduced to naught, leaving only the constitutive expression of Fcs.

[0046] Our engineered E. coli may convert ferulic acid or caffeic acid to vanillin or p-coumaric acid, to 4-hydroxybenzaldehyde. Oxidizing 4-hydroxybenzaldehyde can lead to the production of p-hydroxybenzoate. Thus, our engineered E. coli is versatile in generating different products depending on the type of substrate present.

[0047] Our engineered E. coli can sense a variety of lignin-derived hydroxycinnamic acids. Our designed genetic system not only allowed host cells to auto-regulate enzyme expression based on substrate availability, it also enabled host cells to respond to a wide range of lignin-derived hydroxycinnamic acids such as ferulic, p-coumaric and caffeic acid, making them highly useful and flexible for hydroxycinnamic acid conversion (Figure 2).

[0048] Our system improves biological system stability for economical production of enzymes for vanillin conversion. We strongly believe that in the whole cell bioconversion of ferulic acid to vanillin, bacteria growth is closely related to the efficacy of the bio-catalysis. By having a large bio-catalytic population in the shortest time, it leads to a faster production of vanillin. Thus, here we demonstrated that by improving host cell viability, the time to reach saturation for the vanillin bioconversion would be reduced. To achieve robust cell growth in *E. coli*, we engineered stability and auto-regulation in the biological vanillin production system by applying the control theory and bacteria growth

model in the design of the genetic circuit expressing the vanillin enzymes, Fcs and Ech.

[0049] The control theory can be applied in our genetic circuit through introducing bio-sensors such as the substrate sensor for ferulic acid and a time-delay element. The ferulic acid sensor is crucial for regulating the protein expression; it would only trigger the protein production when the substrate, ferulic acid is available. Further, in order for the cells to grow well, there must have a time delay before activating the bio-sensor. This is to ensure that in the presence of substrate inducers, cells growing from low cell density (e.g. single colony inoculation) would not experience overwhelming metabolic stress that may lead to growth retardation. The time-delay element would provide the cells some time to reach a higher cell density before activating the bio-sensor, so that the amount of substrate inducers per cell would be lower and thus less detrimental to growth.

[0050] Our mode of gene induction in bio-catalysts is more economically attractive. Compared to existing methods which use costly inducers such as IPTG, our method for vanillin production eliminates the need for external inducers and can bring considerable material cost savings in the industrial conversion of ferulic acid to vanillin. Our system auto-regulates the enzyme production based on the presence of ferulic acid as well as cell density.

[0051] An overview of the entire vanillin production system can be portrayed in Figure 5. Implementation of P_{gabDT} introduces the time-delay for the cells to reach a higher cell density such that the extra-cellular carbon source would be depleted due to increased utilization. Depletion of carbon source increases transcription of RpoS which activates P_{gabDT} which transcribes *fcs*, thereby triggering the ferulic acid sensor. The ferulic acid sensor functions by detecting feruloyl-CoA, product

of Fcs and activates the transcription of *ech*. Ech produced leads to conversion of feruloyl-CoA to vanillin.

[0052] Our engineered E. coli is more stable and robust. In industrial bio-conversion, the size of bio-catalytic population is an important factor. We have created a genetic circuit that has minimal impact on cell growth. Implementation of a ferulic acid sensor helps in saving precious cellular metabolic resources, which in turn aids in the growth rate. Together with a time-delay element, the ferulic acid sensor is not activated until the cells reach a considerable density. This is so that the cells would not experience an overwhelming metabolic stress during early phase of growth where the substrate inducer per cell is high. Having a robust growth rate allows a quicker vanillin production. Our engineered E. coli certainly has good growth profile over other types of genetic constructs (Figure 6).

[0053] Our engineered E. coli has the fastest ferulic acid uptake rate and vanillin production rate. Compared to other genetic constructs such as the arabinose inducible system (pBAD), our designed construct has the best performance in terms of ferulic acid uptake and vanillin production (Figure 7). This can be attributed to robust growth of the bio-catalytic cells. Our engineered E. coli also offers the fastest turnover rate (grams per liters per hour) compared to previously reported methods (Table 1). Having a quick turnover allows more vanillin to be produced in a fed-batch reaction per day.

Table 2. Compared to previous works, our construct (highlighted) has the fastest turnover rate.

Strain	Mode of Induction	Media	Ferulic Acid added (g/L)	Vanillin produced (g/L)	Molar Conversion Efficiency (%)	Time Taken (hr)	Turnover rate (g/L per hr)	Source or Reference

E. coli XL1-Blue (pSKechE/Hfcs)	Artificial	MM	0.74	0.27	47.37	> 12	N.A.	Overhage et al. (1999)
E. coli XL1-Blue (pSKEHechfcs)	Artificial	MM	1	0.26	32.50	> 12	N.A.	Plaggenborg et al. (2001)
E. coli strain DH5α (pDAHEF)	Artificial	LB	1	0.58	72.50	18	0.03	Yoon et al. (2005)
E. coli JM109 (pBB1)	Constitutive	M9	0.64	0.35	69.70	>12	N.A.	Barghini et al. (2007)
E. coli DH5a (pTAHEF-gltA)	Leaky (No IPTG)	2YT	2	1.60	100	36	0.04	Lee et al. (2009)
E.coli BL21(DE3) (pBbE8k-ech/fcs)	Artificial	M9	1	0.68	84.91	12	0.06	This study
E.coli BL21(DE3) (Design construct)	Auto	M9	1	0.61	75.47	**8**	**0.08**	This study
E.coli BL21(DE3) (Design construct-gltA)	Auto	M9	1	0.62	77.36	11	0.06	This study
E.coli BL21(DE3) (V. fischeri's QS)	Auto	M9	1	0.53	66.04	12	0.04	This study

[0054] We envisioned that our invention is highly applicable in the industrial production of vanillin from agro-industrial wastes. Agro-industrial wastes such as food and wood waste may serve as a source of ferulic acid. Given that ferulic acid rich bio-energy crops such as switchgrass are gaining support in global green energy industries, we anticipated ferulic acid source to be more available when ferulic acid is released as a by-product during biomass pre-treatment. With the increase availability in ferulic acid in near future, bio-production of vanillin from ferulic acid seems highly probable. Further, our invention may also value add to

the biomass conversion process since the existing ferulic acid is often neglected and remains in the bio-compost which is to be served as fertilizer.

[0055] In addition, our invention is highly applicable in the economic context of industrial microbial bio-catalysis. Not only is our system free of costly inducers, our engineered E. coli can treat the substrate, ferulic acid as a signal for triggering the enzyme expression. This mode of auto-induction lowers the overall material cost. Towards the aim of industrial applicability, we have also engineered our system for stability and growth. By improving the viability of the engineered cells, we can achieve a large bio-catalytic population in the shortest time, thereby leading to a quicker vanillin production.

[0056] By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0057] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[0058] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within

the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0059] By "about" in relation to a given numberical value, such as for temperature and period of time, it is meant to include numerical values within 10% of the specified value.

[0060] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0061] Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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SEQUENCE LISTING

<110> Nanyang Technological University
CHANG, Mathew W
LO, Tat-Ming S
POH, Chueh-Loo

<120> An expression construct for sensing cell density and substrate availability and its use in conversion of hydroxycinnamic acids

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Claims

1. Expression system comprising:

(iv) A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter;

(v) A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter, wherein the second promoter is inducible by the product generated by the first enzyme;

(vi) Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is operably linked to a third promoter that regulates expression of the third gene.

2. The expression system of claim 1, wherein the expression system is a cell-based expression system.

3. The expression system of claim 1, wherein the expression system is a prokaryotic, heterologous expression system.

4. The expression system of any one of claims 1 to 3 wherein the time delay promoter sequence comprises a consensus sequence set forth in SEQ ID NO: 1.

5. The expression system of claim 4 wherein the time delay promoter is selected from gabDT promoter; otsA promoter; katE promoter; osmY promoter; yhiUV promoter; ecnB promoter; dps promoter; osmE promopter; sodC promoter; rpsV promoter; yahoo promoter and tnaA promoter.

6. The expression system of claim 4 wherein the time delay promoter comprises a gabDT promoter.

7. The expression system of claim 6 wherein the time delay promoter has the nucleotide sequence set forth in SEQ ID NO:3.

8. The expression system of any one of claims 1 to 7 wherein the time delay promoter is activatable by a transcription factor comprising amino acid sequence set forth in SEQ ID NO. 2 or a functional variant;

9. The expression system of any one of claims 1-8, wherein the substrate of the enzyme encoded by the first gene is a hydroxycinnamic acid or derivative thereof selected from the group consisting of ferulic acid, caffeic acid, and p-coumaric acid.

10. The expression system of any one of claims 1-9, wherein the first gene encodes a feruloyl CoA synthetase.

11. The expression system of any one of claims 1-10, wherein the first gene has the nucleotide sequence set forth in SEQ ID NO:4.

12. The expression system of any one of claims 1-11, wherein the second promoter has the nucleotide sequence set forth in SEQ ID NO:6.

13. The expression system of any one of claims 1-12, wherein the second gene has the nucleotide sequence set forth in SEQ ID NO:5.

14. An isolated nucleic acid molecule comprising
    a. A first gene encoding for a first enzyme linked to a first promoter, wherein the first promoter is a time delay promoter;
    b. A second gene encoding for a second enzyme capable of using the product generated by the first enzyme as a substrate, wherein the second gene is operably linked to a second promoter, wherein the second promoter is inducible by the product generated by the first enzyme;
    c. Optionally, a third gene encoding a transcription factor that represses expression of the second gene in the absence of the product generated by the first enzyme, wherein the third gene is

operably linked to a third promoter that regulates expression of the third gene.

15. The isolated nucleic acid molecule of claim 14, wherein the nucleic acid molecule is comprised in a plasmid.
16. The isolated nucleic acid molecule of claim 15, wherein the plasmid is a cell-based.

17. The isolated nucleic acid molecule of claim 16, wherein the cell is a prokaryotic cell and the plasmid is, heterologous.

18. The isolated nucleic acid molecule of any one of claims 14 to 17 wherein the time delay promoter sequence comprises a consensus sequence set forth in SEQ ID NO: 1.
19. The isolated nucleic acid molecule of claim 18 wherein the time delay promoter is selected from gabDT promoter; otsA promoter; katE promoter; osmY promoter; yhiUV promoter; ecnB promoter; dps promoter; osmE promopter; sodC promoter; rpsV promoter; yahoo promoter and tnaA promoter.
20. The isolated nucleic acid molecule of claim 18 wherein the time delay promoter comprises a gabDT promoter.
21. The isolated nucleic acid molecule of claim 20 wherein the time delay promoter has the nucleotide sequence set forth in SEQ ID NO:3.
22. The isolated nucleic acid molecule of any one of claims 14 to 21 wherein the time delay promoter is activatable by a transcription factor comprising amino acid sequence set forth in SEQ ID NO. 2 or a functional variant;
23. The isolated nucleic acid molecule of any one of claims 14-22, wherein the substrate of the enzyme encoded by the first gene is a hydroxycinnamic acid or derivative thereof selected from the group consisting of ferulic acid, caffeic acid, and p-coumaric acid.

24. The isolated nucleic acid molecule of any one of claims 14-23, wherein the first gene encodes a feruloyl CoA synthetase.

25. The isolated nucleic acid molecule of any one of claims 14-24, wherein the first gene has the nucleotide sequence set forth in SEQ ID NO:4.

26. The isolated nucleic acid molecule of any one of claims 14-25, wherein the second promoter has the nucleotide sequence set forth in SEQ ID NO:6.

27. The isolated nucleic acid molecule of any one of claims 14-26, wherein the second gene has the nucleotide sequence set forth in SEQ ID NO:5.

28. A Host cell comprising the expression system of claims 1-13 or the isolated nucleic acid molecule of any one of claims 14 to 27.

29. The host cell of claim 28, wherein the host cell is a prokaryotic cell, preferably a bacterial cell.

30. The host cell of claim 28 or 29, wherein the host cell is an E.coli cell.

31. A method of converting a substrate to a product comprising the steps of:

a. subjecting the expression system of any one of claims 1-13 or the host cell according to any one of claims 28 to 30 under conditions that allow the expression of the first and second enzyme; and
b. isolating the product from said host cell.

32. The method of claim 31, wherein the method uses the host cell of claim 28 and further comprises the step of cultivating the host cells under conditions that allow the growth of the host cells, wherein said step is conducted prior to or simultaneously with step (a).

33. The method of claim 31 wherein the product is a hydroxybenzaldehyde

34. The method of claim 31 wherein the product is vanillin

35. The method of claim 31 wherein the product is p-hydroxybenzoate