IMPROVED MUCONIC ACID PRODUCTION FROM GENETICALLY ENGINEERED MICROORGANISMS

CROSS-REFERENCE TO RELATED APPLICATION

(001) This application claims the priority of the U.S. Provisional Application Serial No. 62/302,558 filed on March 2, 2016.

FIELD OF THE INVENTION

(002) The present invention is in the field of producing renewable chemical feedstocks using biocatalysts that have been genetically engineered in the central aromatic biosynthetic pathway. More specifically, the present invention provides processes for improving the production of muconic acid from renewable carbon resources using genetically modified biocatalysts.

BACKGROUND OF THE INVENTION

(003) Adipic acid is a major commodity chemical and is used in the production of nylon 6,6 and polyurethanes. Adipic acid is currently derived from petrochemical feedstocks. Current synthesis of adipic acid is environmentally harmful releasing nitrous acid (Xie et al., 2014). Alternatively, adipic acid can be made from any of the three isomers of muconic acid (cis, cis; cis, trans; trans, trans isomers) by chemical hydrogenation. It would be desirable to produce muconic acid from renewable resources by fermentation with a microorganism, followed by hydrogenation process to yield adipic acid, since such a route to adipic acid would be more environmentally friendly than the traditional petrochemical route (Niu, Draths and Frost, 2002; Frost and Draths, 1997). Many other chemicals can be made by chemical conversion of one or more muconic acid isomers, including, but not limited to 1.6 hexane diol, 3-hexenedicarboxylic acid, 1,6-hexanediamine, and terephthalic acid.

(004) The international patent application publication No. WO 2011/017560 claims biocatalysts having a muconate pathway and a method for producing muconic acid using these biocatalysts. In brief, this published patent application discloses four different pathways for producing muconic acid. The first pathway for muconic acid production starts with succinyl-CoA and

acetyl-CoA. The second pathway for muconic acid production begins with pyruvate and malonate semialdehyde. The third pathway for muconic acid production starts with pyruvate and succinic semialdehyde. The fourth pathway for muconic acid production starts with lysine. All these pathways for muconic acid production proposed in this patent application are based on computer modeling and it is yet to be seen whether such biocatalysts can ever be created with commercially acceptable productivity and yield for muconic acid.

(005) A fermentation route to produce cis, cis-muconic acid using a genetically engineered *E. coli* system has been described in the scientific literature (Niu et al., 2002; Frost and Draths, 1997) and in patent literature (US 5,487,987; US 5,616,496; WO 2011/085311 A1). However, the prior art process for muconic acid production suffered from significant drawbacks such as expensive medium components (aromatic amino acids and vitamins) and chemical inducers, as well as yields lower than required for industrial production. A recent United States Patent Application Publication No. US2015/0044755, which is incorporated herein by reference in its entirety, provides improved biocatalysts for muconic acid production involving constitutive expression of relevant gene, improved heterologous genes and novel "leaky" AroE enzymes. The present invention provides further improvements in the muconic acid biocatalysts described in the United States Patent Application Publication No. US2015/0044755.

SUMMARY OF THE INVENTION

(006) This present invention provides genetically engineered microorganisms that produce cis, cis-muconic acid starting from non-aromatic carbon sources, such as sugars and carbohydrates including, but not limited to glucose, sucrose, glycerol and cellulosic hydrolysate.

(007) In one embodiment of the present invention, the activity of a negative regulator of aromatic amino acid biosynthesis is genetically manipulated. In one aspect of the present invention, the activity of the negative regulator TyrR is substantially reduced by means of controlling the expression of the *tyrR* gene coding for the TyrR protein. In another aspect of the present invention, the activity of the negative regulator TyrR is totally eliminated by means of deleting or inactivating the *tyrR* gene in the chromosomal DNA of the microorganism.

(008) In another embodiment of the present invention, the feedback inhibition of certain enzymes in the aromatic amino acid pathway by certain metabolites is overcome through genetic manipulations. In most wild type *E. coli* strains, deoxyarabino-heptulosonate 7-phosphate synthase ("DAHP synthase") functioning at the beginning of the aromatic amino acid pathway occurs as three different isozymes which are known to be encoded by three different genes namely *aroG, aroF* and *aroH.* The proteins encoded by each of these three genes are subjected to feedback inhibition by one or more metabolites of aromatic amino acid pathway. In one aspect of the present invention, the wild type *aroG* gene is replaced by a modified *aroG* gene which codes for an AroG protein that is resistant to feedback inhibition by one or more metabolites of the aromatic amino acid pathway within the microbial cell. Such a feedback resistant form of AroG protein is referred as "$AroG^{FBR}$". In another aspect of the present invention, the wild type *aroF* gene is replaced by an *aroF* gene which codes for an AroF protein that is resistant to feedback inhibition by one or more metabolites of the aromatic amino acid pathway within the microbial cell ($AroF^{FBR}$). In yet another aspect of the present invention, the wild type *aroH* gene is replaced by an *aroH* gene which codes for an AroH protein that is resistant to feedback inhibition by one or more metabolites of the aromatic amino acid pathway within the microbial cell ($AroH^{FBR}$). In yet another aspect of the present invention the biocatalyst selected for the commercial production of cis, cis-muconic acid may have more than one feedback resistant isozyme for DAHP synthase.

(009) In another embodiment of the present invention, the activity of one or more of the enzymes involved in the central aromatic biosynthetic pathway within the microbial cell is enhanced. In one aspect of the present invention, the enhancement of the activity of one or more enzymes involved in the operation of an aromatic pathway and/or a muconic acid pathway is accomplished through genetic manipulation. In a preferred aspect of the present invention, the expression of one or more of the genes coding for the proteins AroF, AroG, AroH, AroB, TktA, TalB, AroZ, QutC, Qa-4, AsbF, QuiC, AroY, Rpe, Rpi, Pps, CatA and CatX or their homologs or analogs are enhanced leading to the increased activity of said proteins. Rpe is a ribulose-5-phosphate epimerase, Rpi is a ribulose-5-phosphate isomerase, and Pps is a phosphoenol pyruvate synthetase (Neidhardt and Curtiss, 1996). If the host strain is yeast, for example *Saccharomyces cerevisiae*, or a filamentous fungus, for example, *Neurospora crassa*, several of

the enzymes that catalyze reactions in the shikimate pathway can be combined into one large protein or polypeptide, called Aro1p, encoded by the ARO1 gene in the case of *S. cerevisiae*. Aro1p combines the functions of AroB, AroD, AroE, AroK (or AroL), and AroA). As such, for the purposes of this invention, Aro1p, and ARO1, or a portion thereof, can be used as a substitute, or in addition to, AroB, AroD, AroE, AroK, and/or AroA.

(010) In yet another embodiment of the present invention, flux through erythrose-4-phosphate within the bacterial cell is enhanced by means of overexpressing enzymes in the operation of the pentose phosphate pathway. In one aspect of the present invention, the expression of transaldolase enzyme, for example one coded by the *talB* or *talA* gene is enhanced by genetic modification. In another aspect of the present invention, the expression of a gene encoding transketolase enzyme, for example, the *tktA* gene is enhanced by genetic manipulations. In yet another aspect of the present invention, the expression of the genes encoding either or both ribulose-5-phosphate epimerase and ribulose-5-phosphate isomerase are enhanced by genetic manipulations.

(011) In another embodiment of the present invention, the pool of the phosphoenol pyruvate (PEP) necessary for the functioning of the aromatic amino acid pathway is increased through genetic manipulation. In one aspect of the present invention, competition for the use of PEP pool is decreased through elimination and/or complementation of a PEP-dependent phosphotransferase system (PTS) for glucose uptake with a PEP independent system for glucose uptake. In another aspect of the present invention, the GalP based sugar uptake system is inactivated for the purpose of conserving ATP within the microbial cells. In yet another aspect of the present invention, in the microbial cells defective in the functioning of both PTS system and GalP based sugar uptake system (ΔPTS/ΔgalP), the sugar uptake is accomplished by means of introducing an exogenous gene encoding for Glf (protein facilitating the glucose diffusion), or exogenous genes encoding for both Glf and Glk (glucokinase) proteins. In yet another embodiment of the present invention, the availability of PEP is increased by increasing the expression of a gene that encodes a PEP synthetase, such as *pps*.

(012) In yet another embodiment of the present invention, the activity of 3,4-dihydroxybenzoic acid decarboxylase (AroY) is enhanced. In one aspect of this embodiment, the expression of AroY is enhanced by genetic manipulation. In another aspect of the present invention, the expression of a protein acting as an accessory protein to AroY, selected from a group comprising UbiX, KpdB, Elw, Kox, Lpl and homologs thereof, is increased by genetic manipulation leading to an increase in 3,4-dihydroxybenzoic acid decarboxylase activity.

(013) In another embodiment of the present invention, PEP availability is increased by the reduction or elimination of phosphoenolpyruvate carboxylase (Ppc) activity. In one aspect of the present invention, pyruvate carboxylase (Pyc) activity is increased and/or substituted for Ppc activity, particularly when Ppc activity is eliminated.

BRIEF DESCRIPTION OF THE DRAWINGS

(014) FIG. 1. Pathway for aromatic amino acid biosynthesis in *E. coli*.

(015) FIG. 2. Pathway for muconic acid biosynthesis in *E. coli*.

(016) FIG. 3. Reaction steps in the conversion of 3-deoxy-arabino-heptulonate 7-phosphate (DHAP) to muconic acid.

(017) FIG. 4. Chromatograph showing standards used for HPLC analysis of total muconic acid and biochemical intermediates.

(018) FIG. 5. Chromatograph showing standards used for HPLC analysis of muconic acid isomers.

(019) FIG. 6. Titer for the production of DHS in the *E. coli* strain MYR34 transformed with plasmids pCP32AMP, pCP14 and pCP54. MYR34 strain of *E. coli* has a deletion in *aroE* gene. The plasmid pCP32AMP expresses the *aroG* gene coding for DAHP synthase. The plasmid pCP14 expresses the *aroB* gene coding for DHQ synthase. The plasmid pCP54 expresses both the *aroB* and *aroG* genes.

(020) FIG. 7. Titer for the production of DHS in the *E. coli* strains MYR34 and MYR170 transformed with plasmids pCP32AMP and pCP54. The MYR34 strain of *E. coli* has a deletion of the *aroE* gene. The MYR170 strain has a deletion of the *aroE* gene and a second copy of the *aroB* gene under the control of P_{15} promoter integrated at the *ack* locus of the host chromosomal DNA. The plasmid pCP32AMP expresses the *aroG* gene coding for DAHP synthase. The plasmid pCP54 expresses both *aroB* and *aroG* genes.

(021) FIG. 8. Titer for the production of cis, cis-muconic acid in the *E. coli* strains MYR34 and MYR170 transformed with plasmid pMG37 alone or with both pMG37 and pCP32AMP plasmids. The MYR34 strain of *E. coli* has a deletion of the *aroE* gene. The MYR170 strain has a deletion of the *aroE* gene and a second copy of the *aroB* gene under the control of P_{15} promoter integrated at the *ack* locus of the host chromosomal DNA. The plasmid pCP32AMP expresses the *aroG* gene coding for DAHP synthase. The plasmid pMG37 expresses the *aroZ, aroY,* and *catAX,* genes coding for proteins functional in the muconic acid pathway.

(022) FIG. 9. Titer for the production of DHS in MYR170 strain of *E. coli* transformed with a plasmid expressing *aroG* gene alone (pCP32AMP) or *aroG* and *tktA* genes simultaneously (pCP50). The MYR170 strain has a deletion of the *aroE* gene and a second copy of the *aroB* gene under the control of the P_{15} promoter integrated at the *ack* locus of the host chromosomal DNA.

(023) FIG. 10. DHS yield from MYR34 and MYR170 stains of *E. coli* transformed with plasmids pCP32AMP and pCP50. DHS yield is calculated as grams of DHS produced per gram of glucose consumed. The plasmid pCP32AMP expresses the *aroG* gene while pCP50 expresses *aroG* and *tktA*. The bacterial strain MYR34 has a deletion in the *aroE* gene. The MYR170 strain of *E. coli* is derived from MYR34 and has an additional *aroB* gene integrated at the *ack* locus on the chromosomal DNA.

(024) FIG. 11. DHS titer from MYR170 and MYR261 stains of *E. coli* transformed with plasmids pCP32AMP and pCP50. The plasmid pCP32AMP expresses the *aroG* gene while pCP50 expresses the *aroB* and *tktA* genes. The MYR170 strain has a deletion of the *aroE* gene and a second copy of the *aroB* gene under the control of P_{15} promoter integrated at the *ack* locus

of the host chromosomal DNA. MYR261 strain of *E. coli* is derived from the MYR170 strain of *E. coli*. MYR261 strain of *E. coli* has a second copy of the *tktA* gene with its native promoter integrated at the *poxB* locus of the chromosomal DNA.

(025) FIG. 12. Muconic acid and acetic acid production in the *E. coli* strains MYR170, MYR261 and MYR305 transformed with the plasmid pCP32AMP expressing *aroG* coding for DAHP synthase in the shikimic acid biosynthetic pathway and plasmid pMG37 expressing *aroZ*, *aroY* and *catAX* genes coding for proteins functional in the muconic acid pathway. MYR170 strain has a deletion in the *aroE* gene and an additional copy of *aroB* gene under the control of the P_{15} promoter inserted at *ack* locus in the host chromosomal DNA. MYR261 and MYR305 are derivatives of MYR170 strain. MYR261 has an additional copy of *tktA* gene integrated at *poxB* locus on the host chromosomal DNA while MYR305 has a deletion in the *poxB* locus on the host chromosomal DNA.

(026) FIG. 13. Conversion of endogenous DHS produced by *E. coli* strain MYR34 into muconic acid. Strain MYR34 of *E. coli* has a deletion in the *aroE* gene coding of shikimate dehydrogenase. As a result there is an accumulation of DHS. When strain MYR34 is transformed with a plasmid expressing *aroZ, aroY* and *catAX,* genes coding for proteins functional in muconic acid pathway, there is conversion of DHS into muconic acid. However, no conversion of DHS into muconic acid occurs when MYR34 strain is transformed with the empty plasmid vector (pCL1921) without any exogenous genes.

(027) FIG. 14. Comparison of *aroZ* analogs for their ability to divert DHS into the muconic acid pathway. Three different *aroZ* analogs, namely *quiC* from *Acinetobacter sp. ADP1*, *asbF* from *Bacillus thuringiensis*, and *qa-4* from *Neurospora crassa* were cloned under the P_{26} promoter in a low-copy plasmid which also expressed *catAX* and *aroY* genes from the P_{15} and lambda P_R promoters respectively. These three different plasmid constructs were expressed in MYR34 through transformation and the amount of muconic acid produced was measured.

(028) FIG. 15. Single copies of *catAX*, *aroY* and *quiC* were chromosomally integrated into MYR170 strain of *E. coli* (*ΔaroE, Δack::P_{15}-aroB*) resulting in MYR352 (SEQ ID No. 41). MYR170 was also transformed with low copy plasmid pMG37 carrying all genes necessary for

the operation of muconic acid pathway leading to the MYR219 strain. Both MYR352 and MYR219 were transformed with YEp24 (medium-copy empty vector) or pCP32AMP (medium-copy *aroG* expressing plasmid) or pCP50 (medium-copy *aroG* and *tktA* expressing plasmid) and the amount of PCA, catechol and muconic acid produced were quantified using HPLC method.

(029) FIG. 16. Removal of catechol accumulation in MYR352 by means of increasing the expression of *catAX*. MYR352 was transformed with a plasmid expressing *aroY* alone (pMG27) or a plasmid expressing *quiC* alone (pMG39) or a plasmid expressing all three muconic acid pathway genes namely *catAX, aroY* and *quiC* (pMG37) or a plasmid expressing only two genes in the muconic acid pathway namely *catAX* and *aroY* (pMG33). Over expression of *catAX* alone was sufficient to prevent accumulation of catechol.

(030) FIG. 17. Growth of strains using different systems for importing glucose. Deletion of *ptsHI* and *galP* (MYR31) leads to lack of growth in minimal glucose medium, while installation of *glf and glk genes* (MYR217) brings back growth. Control strain MYR34 is Δ*aroE*, but otherwise wild type. The three aromatic amino acids and three aromatic vitamins were added to the medium to allow growth of the auxotrophic strains.

(031) FIG. 18. DHS production in MYR34 and MYR217 strains of *E. coli*. When transformed with plasmids that lead to production of DHS, MYR217, which utilizes *glf-glk* for glucose import, produced a higher titer of DHS than transformants of MYR34, which utilizes the phosphotransferase system (PTS).

(032) FIG. 19. Production of muconic acid by the MYR428 strain of *E. coli* in a 7 Liter fermentor. The MYR261 strain of *E. coli* with a genotype of Δ*aroE* Δ*ackA*::P_{15}-*aroB* Δ*poxB*::*tktA* was transformed with the plasmids pCP32AMP and pMG37 to generate the MYR428 strain of *E. coli*.

(033) FIG. 20. Muconic acid and PCA production in the *E. coli* strain MYR993 genetically engineered to produce muconic acid and two MYR993 derivatives MYR993Δ*ubiX* and MYR993Δ*ubiD*. The MYR993Δ*ubiX E. coli* strain was derived from MYR993 strain by means

of replacing the coding region for *ubiX* gene with a cassette coding for kanamycin resistance. The MYR993Δ*ubiD* *E. coli* strain was similarly derived from MYR933 strain by means of replacing the coding region for *ubiD* gene with a cassette coding for kanamycin resistance.

(034) FIG. 21. Measurement of relative activity of various homologs of UbiX. Measurement of activity of UbiX homologs was carried out from the decarboxylation of PCA as measured by a decrease in the absorbance at 290nm (A290). Five different UbiX homologs namely KpdB, UbiX, Elw, Kok and Lpl were use in this study.

(035) FIG. 22. Muconic acid and PCA production in *E. coli* strains having low or high level of *kpdB* gene expression. *E. coli* strain MYR1305 with no exogenous *kpdB* gene was used as the parent strain and was transformed with a low copy plasmid having the *kpdB* gene expressed either from the P26 promoter or the *E. coli pgi* promoter. The gene expression from the P26 promoter is expected to be at a relatively low level while the gene expression from the *pgi* promoter is expected to be at a relatively high level.

(036) FIG. 23. Muconic acid production in the *E. coli* strain MYR1674 and its derivative MYR1772. MYR1772 was derived from MYR1674 by replacing the coding region and promoter of *ppc* with the P_R-*pyc* gene. P_R is an abbreviation for the strong rightward promoter from the coliphage lambda.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

(037) All the patents, patent applications, publications, sequences, and other published materials are incorporated herein are incorporated by reference.

(038) As used in this patent application, the phrase "for example" or "such as" is meant to indicate that there are more than one method, approach, solution, or composition of matter for the subject at hand, and the example given is not meant to be limiting to that example.

(039) The term "heterologous" refers to a gene or protein that is not naturally or natively found in an organism, but which can be introduced into an organism by genetic engineering, such as by transformation, mating, or transduction. A heterologous gene can be integrated (inserted) into a chromosome or contained on a plasmid. The term "exogenous" refers to a gene or protein that has been introduced into, or altered, in an organism for the purpose of increasing, decreasing, or eliminating an activity, by genetic engineering, such as by transformation, mating, transduction, or mutagenesis. An exogenous gene or protein can be heterologous, or it can be a gene or protein that is native to the host organism, but altered by one or more methods, for example, mutation, deletion, change of promoter, change of terminator, duplication, or insertion of one or more additional copies in the chromosome or in a plasmid. Thus, for example, if a second copy of the *aroB* gene is inserted at a site in the chromosome that is distinct from the native site, the second copy would be exogenous.

(040) The term "microorganism" as used in this present invention includes bacteria, archaea, yeast, algae and filamentous fungi that can be used for the commercial production of cis, cis-muconic acid through a fermentation process. The term "genetically engineered microorganism" refers to microorganisms that are not present in the Nature but generated using one or other genetic modifications as described in this patent application.

(041) For nomenclature, a gene or coding region is usually named with lower case letters in italics, for example "*aroZ*", while the enzyme or protein encoded by a gene can be named with the same letters, but with the first letter in upper case and without italics, for example "AroZ". The enzyme or protein can also be referred to by a more descriptive name, for example, AroZ can also be referred to as 3-dehydroshikimate dehydratase. A gene or coding region that encodes one example of an enzyme that possess a particular catalytic activity can have several different names because of historically different origins, or because the gene comes from different species. For example the gene that encodes 3-dehydroshikimate dehydratase from *Bacillus anthracis* can be named *asbF* instead of *aroZ,* the same gene from *Aspergillus nidulans* can be named *qutC,* the same gene from *Neurospora crassa* can be named *qa-4,* and the same gene from *Acinetobacter baylyi* can be named *quiC.*

(042) A "plasmid" means a circular or linear DNA molecule that is substantially smaller than a chromosome, separate from the chromosome or chromosomes of a microorganism, and that replicates separately from the chromosome or chromosomes. A "plasmid" can be present in about one copy per cell or in more than one copy per cell. Maintenance of a plasmid within a microbial cell in general requires an antibiotic selection, but complementation of an auxotrophy can also be used.

(043) The term "chromosome" or "chromosomal DNA" as used in this invention in the context of a bacterial cell is a circular DNA molecule that is substantially larger than a plasmid and does not require any antibiotics selection.

(044) The term an "expression cassette" or a "cassette" means a DNA sequence that can be part of a chromosome or plasmid that contains at least a promoter and a gene or region that codes for an enzyme or other protein, such that the coding region is expressed by the promoter, and the enzyme or protein is produced by a host cell that contains the DNA sequence. An "expression cassette" can be at least partly synthetic, or constructed by genetic engineering methods, so that the coding region is expressed from a promoter that is not naturally associated with the coding region. Optionally, the "expression cassette" can contain a transcription terminator that may or may not be a terminator that is naturally associated with the coding region. An "expression cassette" can have coding regions for more than one protein, in which case it can be called an operon, or a synthetic operon.

(045) The term "overexpression" of a gene or coding region means causing the enzyme or protein encoded by that gene or coding region to be produced in a host microorganism at a level that is higher than the level found in the wild type version of the host microorganism under the same or similar growth conditions. This can be accomplished by, for example, one or more of the following methods: 1) installing a stronger promoter, 2) installing a stronger ribosome binding site, such as a DNA sequence of 5'-AGGAGG, situated about four to ten bases upstream of the translation start codon, 3) installing a terminator or a stronger terminator, 4) improving the choice of codons at one or more sites in the coding region, 5) improving the mRNA stability, and 6) increasing the copy number of the gene, either by introducing multiple copies in the chromosome or placing the cassette on a multicopy plasmid. An enzyme or protein produced

from a gene that is overexpressed is said to be "overproduced". A gene that is being "overexpressed" or a protein that is being "overproduced" can be one that is native to a host microorganism, or it can be one that has been transplanted by genetic engineering methods from a different organism into a host microorganism, in which case the enzyme or protein and the gene or coding region that encodes the enzyme or protein is called "foreign" or "heterologous". Foreign or heterologous genes and proteins are by definition overexpressed and overproduced, since they are not present in the unengineered host organism.

(046) The term a "homolog" of a first gene, DNA sequence, or protein is a second gene, DNA sequence, or protein that performs a similar biological function to that of said first gene, DNA sequence or protein, and that has at least 25% sequence identity (when comparing protein sequences or comparing the protein sequence derived from gene sequences) with said first gene or protein, as determined by the BLAST computer program for sequence comparison (Altschul et al., 1990; Altschul et al., 1997), using default parameters and allowing for deletions and insertions. An example of a homolog of the *E. coli aroG* gene would be the *aroG* gene from *Salmonella typhimurium*.

(047) Two enzymes or proteins that are very distantly related by homology can carry out the same biochemical function but be only relatively weakly homologous to each other. For example, the FumA fumarase from *E. coli* K-12 (GenBank NP_416129) is about 26.9 % homologous to a fumarase from *Clostridium botulinum* (GenBank GAE03909.1) over their region of overlap, and about 25.1 % homologous to a fumarase beta subunit from *Pyrococcus* sp. ST04 (GenBank AKF23146.1) over their region of overlap. As another example, a *Klebsiella* AroZ and a *Neurospora crassa* Qa-4 enzyme that both function to convert DHS to PCA are 29.3 % identical. Therefore, since for the genetic engineering of a metabolic pathway, the important feature of a heterologous enzyme or protein is the function or reaction carried out by that enzyme or protein, not the source organism or the precise amino acid sequence, we define "homologous" enzymes or proteins or "homologs" to comprise any pair of enzymes or proteins that are 25% or higher in the identity of their amino acid sequences, allowing for gaps, as shown for example by using the default parameters for alignment using the LaserGene 12 (DNAStar, Madison, WI)

MegAlign program with the Lipman-Pearson method (Ktuple = 2, Gap penalty = 4, and Gap length penalty = 12).

(048) The term an "analog" of a first gene, DNA sequence, or protein is a second gene, DNA sequence, or protein that performs a similar biological function to that of said first gene, DNA sequence, or protein, but where there is less than 25% sequence identity (when comparing protein sequences or comparing the protein sequence derived from gene sequences) with said first gene, DNA sequence or protein, as determined by the BLAST computer program for sequence comparison (Altschul et al., 1990; Altschul et al., 1997), and allowing for deletions and insertions. An example of an analog of the *Klebsiella pneumoniae* AroZ protein would be the QutC protein from *Aspergillus nidulans*, since both proteins are enzymes that catalyze the 3-dehydroshikimate dehydratase reaction, but there is no significant sequence homology between the two enzymes or their respective genes. A scientist knowledgeable in the art will know that many enzymes and proteins that have a particular biological function, for example DAHP synthase or 3-dehydroshikimate dehydratase, can be found in many different organisms, either as homologs or analogs, and since members of such families of enzymes or proteins share the same function, although they may be slightly or substantially different in structure, different members of the same family can in many cases be used to perform the same biological function using current methods of genetic engineering. Thus, for example, the AroZ enzyme and the QutC enzyme catalyze the same reaction, DHS dehydratase, so either one will result in production of cis, cis-muconic acid in the proper context, and the choice of which one to use ultimately can be made by choosing the one that leads to a higher titer of cis, cis-muconic acid under similar fermentation conditions.

(049) The terms a "non-aromatic carbon source" or a "non-aromatic compound" means a carbon-containing compound that can be used to feed a microorganism of the invention as a source of carbon and/or energy, in which the compound does not contain a six-membered ring related to benzene. Examples of non-aromatic carbon sources include glucose, xylose, lactose, glycerol, acetate, arabinose, galactose, mannose, maltose, or sucrose. An "aromatic compound" is a compound that contains one or more six-membered rings related to benzene. An example of an aromatic compound is catechol, or 1,2-dihydroxy benzene. A microorganism selected for producing muconic acid using glucose as a source of non-aromatic carbon source can further be

engineered to use other types of non-aromatic carbon sources such as glycerol, sucrose and xylose using the genetic engineering techniques as provided in the patent documents US Patent No. 8,871,489 and US Patent Application Publication Nos. US2013/0337519A1 and US2014/0234923A.

(050) The term a "strong constitutive promoter" means a DNA sequence that typically lies upstream (to the 5' side of a gene when depicted in the conventional 5' to 3' orientation), of a DNA sequence or a gene that is transcribed by an RNA polymerase, and that causes said DNA sequence or gene to be expressed by transcription by an RNA polymerase at a level that is easily detected directly or indirectly by any appropriate assay procedure. Examples of appropriate assay procedures include 1) quantitative reverse transcriptase plus PCR, 2) enzyme assay of an encoded enzyme, 3) Coomassie Blue-stained protein gel, or 4) measurable production of a metabolite that is produced indirectly as a result of said transcription, and such measurable transcription occurring regardless of the presence or absence of a protein that specifically regulates level of transcription, a metabolite, or inducer chemical. An example of a promoter that is not a "strong constitutive promoter" is the P_{lac} promoter of *E. coli*, since it is repressed by a repressor in the absence of lactose or the inducer IPTG. By using well known methods in the art, a "strong constitutive promoter" can be used to replace a native promoter (a promoter that is otherwise naturally existing upstream from a DNA sequence or gene), resulting in an expression cassette that can be placed either in a plasmid or chromosome and that provides a level of expression of a desired DNA sequence or gene at a level that is higher than the level from the native promoter. A strong constitutive promoter can be specific for a species or genus, but often a strong constitutive promoter from a bacterium can function well in a distantly related bacterium. For example, a promoter from *Bacillus subtilis* or a phage that normally grows on *B. subtilis* can function well in *E. coli*. A "strong constitutive promoter" is substantially different from inducible promoters, such as P_{tac}, which have been used in the prior art production of cis, cis-muconic acid and typically require an expensive chemical or other environmental change for the desired level of function (Niu et al., 2002), Examples of strong constitutive promoters are P_{15}, P_{26}, from *Bacillus subtilis* phage SP01, and coli phage lambda P_R.

(051) A "mutation" is any change in a DNA sequence that makes it different from a related wild type sequence. A "mutation" can comprise a single base change, a deletion, an insertion, a replacement, a frameshift, an inversion, a duplication, or any other type of change in a DNA sequence. Usually a "mutation" refers to a change that has a negative effect on function or reduce the activity of a gene or gene product, however, herein, the term "mutation" can also refer to a change that increases the activity of a gene or gene product. For example, a feedback resistant mutation in the *aroG* gene increases the activity of AroG in the presence of an inhibitor such as phenylalanine. Replacement of a promoter with a different, stronger promoter, also result in a mutation that can increase the activity of a gene or gene product. A "null mutation" is a mutation, such as a deletion of most or all of a gene, is a mutation that effectively eliminates the function of a gene. A "mutant" is a strain or isolate that comprises one or more mutations.

(052) The biological production of cis, cis-muconic acid (herein referred to as simply "muconic acid") is based on the redirection of carbon from the aromatic amino acid pathway. Native production of aromatic amino acids and vitamins requires the metabolites erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP). The first committed step in aromatic amino acid synthesis is catalyzed by the enzyme 3-deoxy-arabino-heptulonate 7-phosphate (DAHP) synthase. In *E. coli*, this step can be performed by three different isozymes, AroG, AroF, or AroH. Each of these enzymes is regulated at the transcriptional level by a repressor protein TyrR, as well as at the protein level by inhibition from terminal products of the pathway, phenylalanine, tyrosine, and tryptophan, respectively. The production of muconic acid proceeds from an intermediate in the aromatic amino acid pathway, dehydroshikimic acid (DHS) and requires the expression of three heterologous enzymes, dehydroshikimate dehydratase (AroZ), 3,4-dihydrobenzoate decarboxylase (AroY), and catechol 1,2-dioxygenase (CatA). This pathway is shown in Figures 1 and 2.

(053) A "muconic pathway" or "muconic acid pathway" refers to a biochemical pathway from DHS to PCA to catechol to cis, cis-muconic acid, and a "muconic pathway gene" is a gene that encodes an enzymes that catalyzes a step in a muconic pathway, or encodes an auxiliary function that serves to enhance the activity of one of said enzymes, for example, *aroZ, aroY, catA, catX,* and *qutC* (Figure 3). DHS is an abbreviation for 3-dehydroshikimate, and PCA is an

abbreviation for protocatechuic acid. A"muconic plasmid" is a plasmid that contains one or more muconic pathway genes.

(054) The genetic manipulations used in the present inventions are centered around the common pathway for aromatic amino acid and aromatic vitamin (or vitamin-like) biosynthesis present in many microbial cells as shown in Figure 1. The common pathway for aromatic amino acid biosynthesis as depicted in Figure 1 can be referred to as the "shikimic acid" or "shikimate" pathway, the "chorismic acid" or "chorismate" pathway, or the "central aromatic" or "central aromatic biosynthetic" pathway.

(055) There is a substantial volume of published work on genetic engineering of microorganisms for the production of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan (US 4,681,852, US 4,753,883, US 6,180,373, European Patent Application 86300748.0). The approaches for the production of aromatic amino acids include using various combinations of feedback resistant enzymes (AroF, AroG, PheA, TyrA), deregulation of repression of transcription (*tyrR*), increasing promoter strength (P_{tac}, P_{lac}) and increasing the copy number of one or more genes (*tktA*). Many specific combinations of the above mentioned genetic modifications can be followed to obtain a biocatalyst suitable for muconic acid production.

(056) According to the disclosure in the International Patent Application Publication No. WO2013/116244, incorporated herein in its entirety by reference, the genetically engineered microorganisms do not need to contain any exogenous plasmids in order to produce muconic acid, although they have certain exogenous or heterologous genes necessary to achieve the desired phenotype. In the preferred embodiment of the present invention, the exogenous genes introduced into the microorganisms are stably integrated into the chromosomal DNA. As a result of this chromosomal DNA integration of the exogenous genes, the need for the use of antibiotics or other selective methods to maintain the plasmids carrying exogenous DNA is totally eliminated. In addition, strong promoters that do not require chemical inducers are used to express genes necessary for the operation of the pathway from carbon source, such as glucose, to cis, cis-muconic acid.

(057) When an exogenous coding sequence is integrated into the chromosomal DNA, it is integrated at a locus, the deletion of which has been reported not to cause any adverse effects. For example, the coding region at the physical location 0039 in *E. coli* bacterium, also known as *ydeM*, is annotated as a lipoprotein and has been demonstrated to have no adverse effects upon deletion. Similarly, the coding region at the physical location 2160 in *E. coli* bacterium, also known as *nlpA*, is annotated as a radical SAM domain protein and has been demonstrated to have no adverse effects upon deletion. In the present invention, a copy of P_R-*catAX* was inserted at the physical location 0039 in *E. coli* bacterium and a copy of P_R-$aroG^{FBR}$ was inserted at the physical location 2160 in *E. coli* bacterium. In other examples described herein, an insertion is made in gene that is advantageous to knockout or deleted, such as a gene encoding for an unwanted function, for example a *ptsI* gene or a *tyrR* gene.

(058) The aromatic amino acid biosynthetic pathway is well known for many microorganisms, especially for *E. coli* (Neidhart and Curtiss 1996). In a wild type cell, the pathway is tightly regulated by both feedback inhibition and repression of transcription. The first committed step is catalyzed by deoxy-arabino-heptulosonate 7-phosphate (DAHP) synthase, of which there are three isozymes encoded by *aroF*, *aroG*, and *aroH*. The three isozymes, AroF, AroG, and AroH, are feedback inhibited by the products of aromatic amino biosynthetic pathway namely by tyrosine, phenylalanine, and tryptophan. Feedback resistant mutants of AroF, AroG, and AroH are well known (Hu et al. 2003; Lutke-Eversloh and Stephanopoulos 2007). One aspect of the present invention involves use of feedback resistant alleles of *aroF*, *aroG*, and *aroH* genes in order to express AroF, AroG and AroH enzyme proteins that are resistant to feedback inhibition by the products of aromatic amino acid biosynthetic pathway. The AroF, AroG and AroH enzyme proteins that are resistant to feedback inhibition are referred as $AroF^{FBR}$, $AroG^{FBR}$ and $AroH^{FBR}$.

(059) Transcription of several of the operons involved in the aromatic pathway is regulated by either the repressor encoded by the *tyrR* gene or the repressor encoded by the *trpR* gene, or both (Neidhardt and Curtiss 1996). Of particular importance is the negative regulation of transcription of *aroG* and *aroF* by the TyrR protein when it is bound with one or more of the aromatic amino acids. One aspect of the present invention involves the removal of negative

regulation by *tyrR* or *trpR* genes by means of eliminating these genes from the chromosome of the host bacterial strains.

(060) The present invention teaches certain combinations of genetic elements in the biocatalysts suitable for muconic acid production, for example, but not limited to, various combinations of an overproduced feedback resistant AroG, an overproduced feedback resistant AroF, an overexpressed *tktA*, an overexpressed *talA*, chromosomally integrated cassettes for expressing an *aroZ, aroY*, and a *catAX* (or analogs or homologs thereof) from strong constitutive promoters, and a leaky *aroE* allele, which we define as a gene that encodes an AroE enzyme that confers prototrophy for the aromatic amino acids and vitamins, but without leading to significant secretion of unwanted aromatic compounds.

(061) All specific examples of strain constructions disclosed herein are based on wild type *Escherichia coli* C strain (ATCC 8739), or *Escherichia coli* W strain (ATCC 9637). However, it should be realized at this point that the expression cassettes or appropriate analogs and homologs of the genetic elements disclosed herein can be assembled in any other suitable microorganism, such as any other suitable *E. coli* strains and other species of bacteria, archaea, yeast, algae, and filamentous fungi that can be used for the commercial production of muconic acid through a fermentative process.

(062) In *E. coli*, the aromatic amino acid biosynthesis pathway from glucose starts with the non-oxidative branch of the pentose phosphate pathway (PPP). Four key enzymes in the non-oxidative pentose phosphate pathway are transketolase, transaldolase, ribulose-5-phosphate epimerase and ribulose-5-phosphate isomerase. These enzymes catalyze the reactions that lead to the formation of erythrose 4-phosphate (E4P) from hexose or pentose sugars. To increase the availability of E4P in *E. coli*, the *tktA* gene encoding transketolase can be overexpressed (Niu et al., 2002). Similarly, the overexpression of the transaldolase gene is also expected to increase the availability of E4P in some circumstances (Bongaerts et al., 2001). In yet another aspect of the present invention, the expression of both the transketolase and transaldolase genes are enhanced through genetic manipulations leading to an increase in the activity of transketolase and transaldolase enzymes. In yet another aspect of the invention, flux through the non-

oxidative branch of the PPP is increased by overproducing ribulose-5-phosphate epimerase and ribulose-5-phosphate isomerase.

(063) The first committed step and most tightly regulated reaction in the common aromatic amino acid pathway is the condensation of phosphoenolpyruvate (PEP) and E4P to produce deoxyarabino-heptulosonate 7-phosphate (DAHP) by DAHP synthase (encoded by *aroG, aroF,* and *aroH*). D-glucose consumed by *E. coli* is brought into aromatic biosynthesis partly through the PPP, and partly through glycolysis. The flow of glucose into the aromatic pathway is greatly increased when transketalose (*tktA*) and an isozyme of DAHP synthase (*aroG*) are amplified through transformation with a plasmid that increases their expression by increasing their copy number (Niu et al., 2002). In a preferred aspect of the present invention, the exogenous *aroG* and *tktA* genes are integrated into the chromosomal DNA for the purpose of amplification of activities transketolase and DAHP synthase enzymes.

(064) In another embodiment of the present invention, the flux through PEP within the microbial cell is improved by increasing the PEP available for the synthesis of DAHP by reducing the flux of PEP to other pathways. Many genera of bacterial cells consume PEP in the transport of glucose across the cell membrane using a phosphotransferase system (PTS) in which one PEP molecule is consumed for every molecule of glucose transported across the bacterial outer membrane. By replacing or complementing the PEP-dependent PTS with a non-PEP dependent (PEP independent) glucose uptake mechanisms, it is possible to increase the pool size of the PEP available for the aromatic amino acid biosynthetic pathway within the microbial cell. For example, the PTS system for sugar uptake can be replaced or complemented by a GalP-based sugar uptake system or the sugar transporter system based on Glf/Glk proteins (Chandran et al., 2003; Yi et al., 2003). In a preferred aspect of the present invention besides deleting the PTS system for sugar uptake for the purpose of conserving PEP pool within the microbial cell, the GalP based sugar uptake system is also inactivated for the purpose of conserving ATP within the microbial cell. In a microbial cell which is defective in the functioning of both PTS system and a Gal-P based sugar uptake system (ΔPTS/Δ*galP*), the sugar uptake can be accomplished by means of introducing an exogenous gene coding for Glf (glucose facilitated diffusion protein), or exogenous genes encoding both Glf and Glk (glucokinase) proteins. As used in the present

invention, the term functional glucose-facilitated diffusion protein refers to any Glf protein as well as any other protein which is functionally equivalent to Glf and functions to transport sugars into the microbial cells by facilitated diffusion. In one aspect of the present invention, the gene coding for the glucose facilitator protein Glf is introduced into the microbial cell which is ΔPTS/Δ*galP* and the glucose transported into the microbial cell is phosphorylated by endogenous glucose kinase. In another aspect of the present invention the genes coding for both Glf and Glk proteins are introduced into a microbial cell which is ΔPTS/Δ*galP*. In a preferred aspect of the present invention, the exogenous *glf* and *glk* genes introduced into the microbial cell are integrated into the host chromosomal DNA.

(065) In another embodiment of the present invention, when the carbon source for growth and energy requires gluconeogenesis (for example if the carbon source is acetate or succinate), the PEP pool can be increased by increasing the activity of carboxylating enzymes already present within the cell, for example PEP carboxykinase, which is encoded by *pck* in *E. coli,* or by introducing an exogenous carboxylating enzyme. In a preferred embodiment, the introduced exogenous gene coding for a carboxylating enzyme is stably integrated into the host chromosome. Genes coding for the carboxylating enzyme can be derived from a variety of microbial species. The genes coding for the carboxylating enzymes can further be subjected to genetic manipulations so that the expression of the carboxylating enzyme within the biocatalyst for cis, cis-muconic acid production is significantly enhanced.

(066) PEP is one of two major metabolites for the aromatic pathway and reduction or elimination of Ppc activity preserves PEP for the aromatic pathway and muconic acid production. Ppc catalyzes the anaplerotic reaction forming oxaloacetate, an intermediate in the TCA cycle. Ppc activity is essential for wild type *E. coli* and some other organisms in minimal media, but it is absent in others. Some organisms, such as yeast that lack Ppc, utilize Pyc to replenish oxaloacetate. Lowered or absent Ppc activity can be complemented with Pyc activity, by providing an alternate route to oxaloacetate from pyruvate instead of PEP, which results in increased availability of PEP for muconic acid production as well as for production of other compounds, such as aromatic compounds, that require PEP as an intermediate. In at least one

example disclosed herein, substituting Pyc for Ppc can reduce flux from PEP to OAA, which in turn conserves PEP for the central aromatic pathway.

(067) In yet another embodiment of the present invention, the PEP pool inside the microbial cell is increased by decreasing or eliminating the activity of pyruvate kinase enzymes such as PykA and PykF which use PEP as a substrate.

(068) From DAHP, the aromatic amino acid pathway proceeds via a number of intermediates to chorismate (CHA), a branch point for the biosynthesis of three aromatic amino acids namely L-Tyrosine (L-Tyr), L-Phenylalanine (L-Phe), and L-Tryptophan (L-Trp).

(069) In the initial stages of the common aromatic amino acid pathway, 3-dehydroquinate (DHQ) synthase (AroB) removes the phosphate group from DAHP leading to the formation of DHQ. The enzyme DHQ dehydratase (AroD) removes a water molecule from DHQ leading to the formation of 3-dehydroshikimate (DHS) which is subsequently reduced to shikimate (SHK) by shikimate dehydrogenase (AroE). Shikimate kinase I/II (AroK, AroL) phosphorylates shikimate to shikimate 3-phosphate (S3P). There is a condensation of S3P with PEP leading to the formation of 5 enolpyruvoylshikimate 3-phosphate (EPSP). The formation of EPSP is mediated by EPSP synthase (AroA). A phosphate group from EPSP is removed by chorismate synthase (AroC) leading to the formation of chorismate (CHA).

(070) As shown in the Figure 2, the aromatic amino acid pathway can be blocked at the level of conversion of 3-dehydroshikimate (DHS) to shikimate (SHK) due to a mutation in an *aroE* gene leading to the accumulation of DHS (Niu et al., 2002). Introduction of an exogenous *aroZ* gene functions to convert DHS into protocatechuate (PCA). PCA is subsequently converted into catechol through a decarboxylation reaction mediated by an AroY enzyme. Catechol is ultimately converted into cis-cis-muconic acid (ccMuA) through the action of a *catA* gene product. ccMuA can be acted upon by maleyl acetoacetate isomerase to yield trans-trans muconic acid (ttMuA). The biosynthetic pathway from DHS to ccMuA and/or ttMuA is referred to as a muconic acid pathway. The three different genes responsible for the conversion of DHS to ccMuA can be obtained from various microbial species and introduced into a microorganism selected for muconic acid production such as *Escherichia coli*. In a preferred embodiment of the

present invention, the exogenous genes coding for the proteins involved in muconic acid pathway are integrated into host chromosomal DNA.

(071) In redirecting the aromatic amino acid pathway to the production of cis, cis-muconic acid, the mutation of the *aroE* gene is critical. The *aroE* gene can be completely inactivated leading to a total block in the biosynthesis of aromatic amino acids as was done with the WN1/pWN2.248strain of *E. coli* described for the muconic acid production (Niu et al., 2002). An important drawback with the WN1/pWN2.248 *E. coli* strain and related strains is that due to the complete inactivation of the *aroE* gene, this strain has become auxotrophic for the aromatic acids such as phenylalanine, tyrosine and tryptophan, and aromatic vitamins or vitamin-like compounds mentioned above. As a result, this strain during its growth for the production of cis, cis-muconic acid requires the exogenous addition of these compounds (or a common intermediate such as shikimate), thereby adding substantially to the cost of commercial production of cis, cis-muconic acid using such a strain. A novel approach to overcome this dependency on an exogenous source of aromatic amino acids is to use a strain with a leaky mutation in *aroE*. The leaky *aroE* mutant would allow a limited flow of carbon to shikimic acid while accumulating significant amounts of DHS which is then available for the conversion into PCA by the action of an AroZ enzyme. Thus the use of a leaky mutant form of *aroE* would eliminate the dependence on exogenous aromatic amino acids, while still diverting the flow of carbon to cis, cis muconic acid.

(072) The genes coding for the synthesis of AroZ, AroY and CatA proteins essential for the conversion of DHS into cis, cis-muconic acid can be derived from any one of many microbial species. In one embodiment, these exogenous genes are integrated into the host chromosome of the biocatalyst being developed. In a preferred embodiment, the expression of these exogenous genes within the biocatalyst is driven by a constitutive promoter without the need for any inducers.

(073) The enzyme 3-dehydroshikimate dehydratase (AroZ; EC 4.2.1.118) is required for biosynthesis of the intermediate protocatechuate. In this specification, "AroZ" shall refer to any enzyme that catalyzes the 3-dehydroshikimate dehydratase reaction. In the *prior art*, this

enzyme is expressed from the *aroZ* gene of *Klebsiella pneumoniae* strain A170-40 (ATCC25597) (Niu et al., 2002; Draths and Frost, 1995). However, the specific activity of AroZ varies widely among organisms, from 0.1 to 261 micromoles/min/mg (Wheeler et al, 1996; Fox et al, 2008; Pfleger et al, 2008), so a significant improvement can be had by expressing an *aroZ* gene also known as *asbF* (Fox et al, 2008; Pfleger et al, 2008), *qutC* (Wheeler et al, 1996), *qa-4* (Rutledge, 1984), and *quiC*, from an organism that has a higher specific activity than *K. pneumoniae*, for example *Acinteobacter baylyi, Aspergillus nidulans* (Wheeler et al, 1996), now also known as *Emericella nidulans*, or *Neurospora crassa* (Rutledge, 1984; Stroman et al, 1978), or *Podospora anserina*, also known as *Podospora pauciseta* (Hansen et al, 2009).

(074) As one particular example, the coding sequence for the *qa-4* gene from *N. crassa* that encodes 3-dehydroshikimate dehydratase can be obtained by any of several well-known methods, for example whole gene DNA synthesis, cDNA cloning, or by a combination of genomic DNA cloning and PCR or synthetic DNA linker synthesis. Since there are no introns in the *qa-4* gene, the coding region can be obtained by PCR from genomic DNA (Rutledge, 1984). The protein sequence of the *qa-4* enzyme (SEQ ID No. 4) and the DNA sequence of the native gene (SEQ ID No. 5) are known.

(075) Alternatively, an expression cassette can be constructed for the 3-dehydroshikimate dehydratase from *A. nidulans*. The coding sequence for the QutC enzyme from *A. nidulans* can be obtained by any of several well-known methods, for example whole gene DNA synthesis, cDNA cloning, or by a combination of genomic DNA cloning and PCR or synthetic DNA linker synthesis. The protein sequence of QutC (SEQ ID No. 6) and the DNA sequence of the native gene, containing no introns, are known (SEQ ID No. 7; GenBank accession number M77665.1). An expression cassette can be obtained by DNA synthesis, or by a combination of genomic cloning and PCR, so that the QutC enzyme can be produced accurately in *E. coli*. By expressing a coding sequence for QutC from a strong, constitutive promoter in *E. coli*, sufficient expression can be obtained from one or two copies of the gene integrated in the chromosome, obviating the need for maintaining more than two copies of the expression cassette on a multicopy plasmid as has been disclosed in the prior art (Niu et al., 2002), and which can lead to instability. The method described above can be used in general to obtain a DNA sequence that codes for a

desired enzyme, and that coding sequence can then be used to construct an expression cassette designed to function in *E. coli* or another appropriate microbial host organism.

(076) The specific activity of AroZ can also be improved by using the protein sequence from the prior art (Niu et al., 2002) by constructing an improved expression cassette, for example, in which a stronger promoter and/or ribosome binding site (RBS) has been installed in front of the coding region, as described in Example 4.

(077) The *aroZ* gene encoding AroZ (3-dehydroshikimate dehydratase) from *Klebsiella pneumoniae* strain A170-40 can be obtained as described in the *prior art*. The DNA sequence of the gene and surrounding DNA can be determined by methods well known in the art. A heterologous gene of the invention such as *aroZ* can be built into an expression cassette using a native DNA sequence or it can be synthesized with a codon optimized sequence for the intended host organism. An *aroZ* gene can be cloned as described (Draths and Frost, 1995) from any other microbe that contains an active *aroZ* gene, for example *K. pneumoniae* strain 342, *Acinetobacer Sp. ADP1* (*Acinetobacter baylyi ADP1*), *Bacillus thuringiensis*, *Emericella nidulans*, *Erwinia amylovora*, *Pseudomonas putida* W619, *Neurospora crassa*, *Aspergillus nidulans* and many others.

(078) The enzyme protocatechuate decarboxylase (AroY; EC 4.1.1.63) is required for biosynthesis of the intermediate catechol. In this specification, "AroY" shall refer to any enzyme that catalyzes the protocatechuate decarboxylase reaction. In the *prior art*, this enzyme is expressed from the *aroY* gene of *Klebsiella pneumoniae* strain A170-40 (ATCC25597) on a multicopy plasmid (Niu et al., 2002). However, once again an improvement in the process can be gained by producing enough of the enzyme from one or two copies of an expression cassette integrated in the chromosome of the host organism. This can be accomplished by obtaining an *aroY* gene from an organism that naturally produces an AroY enzyme that has higher specific activity than that of the *K. pneumoniae* AroY enzyme of the prior art, or by increasing the level of expression of the *K. pneumoniae* AroY by constructing an expression cassette that, for example, uses a strong constitutive promoter and/or strong RBS as described above under Example 4. The protein sequence for AroY from *K. pneumoniae* strain A170-40 is given in SEQ ID No. 8. The corresponding gene, *aroY*, can be cloned as described above (Draths and Frost,

1995), or based on the protein sequence, it can be synthesized with optimized codons for the intended host organism.

(079) The *aroY* gene can be obtained from any other microorganism that contains a homolog or analog, for example, *K. pneumoniae* strain NCTC418 (ATCC15380), *Klebsiella pneumoniae 342*, and *Arxula adeninivorans* (Sietmann et al, 2010). The DNA sequence of the *aroY* gene from *Klebsiella pneumoniae* 342 and surrounding DNA is given as SEQ ID No. 9.

(080) The enzyme catechol 1,2-dioxygenase (CatA; EC 1.13.11.1) is required for the last step of cis, cis-muconic acid biosynthesis. In this specification, "CatA" shall refer to any enzyme that catalyzes the catechol 1,2-dioxygenase reaction. In the prior art, this enzyme is expressed from the *catA* gene of *Acinetobacter calcoaceticus* strain ADP1 on a multicopy plasmid (Niu et al., 2002). The source strain, *Acinetobacter calcoaceticus* strain ADP1, apparently has been renamed *Acinetobacter Sp.* ADP1 and *Acinetobacter baylyi* ADP1 (Neidle and Ornston, 1986; Barbe et al, 2004; de Berardinis et al, 2008). In this prior art example, the *catA* gene was expressed from a P_{tac} promoter, which requires either lactose or IPTG (isopropylthiogalactoside) as an inducer. These compounds are too expensive for use in commercial fermentations, so again, significant improvements in the process are needed, both to eliminate the need for an expensive inducer and to create a more stable strain by integrating the expression cassette in the chromosome. This can be accomplished by constructing an expression cassette for the *catA* gene that uses a strong constitutive promoter, strong RBS, and/or more stable mRNA as described above in the other Examples.

(081) The DNA sequence of the *catA* gene and surrounding sequences from *Acinetobacter baylyi* ADP1 is given in SEQ ID No. 10. The protein sequence for CatA from the same strain is given in SEQ ID No. 11. In a preferred embodiment, the expression cassette for *catA* contains one or two additional open reading frames that exist naturally downstream from *catA*, in order to increase the expression level of the *catA* gene (Schirmer and Hillen, 1998). Many other organisms can be a source for a *catA* gene, for example *Pseudomonas arvilla, Pseudomonas fluorescens* (Nakazawa et al, 1967; Kojima et al, 1967), *Streptomyces* Sp. Strain 2065 (Iwagami et al, 2000), *Cupriavidus necator* 335T, and many others (Perez-Pantoja et al, 2008).

(082) In order to improve the flow of carbon towards cis, cis-muconic acid, it is necessary to block certain other pathways branching out of the aromatic amino acid pathway, besides reducing the flow of carbon from DHS to shikimate (SHK) by using a leaky *aroE* mutant. Some bacteria, for example in the genus *Acinetobacter* and *Pseudomonas*, contain a gene named *pobA*, which encodes an enzyme, p-hydroxybenzoate hydroxlase, that converts DHS into gallic acid. Although a PobA homolog or analog has not been found in *E. coli*, strains of *E. coli* engineered to produce DHS secrete measurable amounts of gallic acid (Li and Frost, 1999), so it is likely that such an enzyme does exist in *E. coli*. In addition, the PCA derived from DHS can be converted into gallic acid by the action of p-hydroxybenzoate hydroxlase (PobA) enzyme coded by the *pobA* gene. The gallic acid thus produced can be subsequently converted to pyrogallol. One way to block the carbon flow to gallic acid and pyrogallol in the biocatalyst selected for an improved cis, cis-muconic acid is to block or diminish the activity of p-hydrobenzoate hydroxlase (PobA) protein through genetic manipulations. Similarly, DHQ, the precursor to DHS can also be acted upon by shikimate dehydrogenase coded by *aroE* leading to the production of quinnic acid. In an embodiment of the present invention, the leaky AroE mutant enzyme is additionally selected or screened for its inability or reduced ability to convert DHQ into quinnic acid.

(083) There are several advantages in producing trans, trans-muconic acid in place of cis, cis-muconic acid. Trans, trans-muconic acid is preferred over cis, cis-muconic acid in the Diels Alder reaction with ethylene for the production of terephthalic acid. A biocatalyst with a genetically manipulated aromatic pathway produces cis, cis-muconic acid which can be converted into trans, trans-muconic acid outside the cell using chemical conversion processes. On the other hand by means of introducing a maleylacetoacetate isomerase or similar isomerase enzyme into the biocatalyst, it is possible to convert the cis, cis-muconic acid into trans, trans-muconic acid within the bacterial biocatalyst.

(084) In one embodiment, the present invention provides a genetic approach to enhance the activity of AroY protein involved in the conversion of 3,4-dihydroxybenzoic acid (protocatechuic acid or PCA) to catechol. The activity of AroY protein has been identified as a major limitation and bottleneck in the biological conversion of glucose to muconic acid (Horwitz

et al 2015; Weber et al 2012; Curran et al 2013; Sonoki et al 2014). AroY belongs to a class of non-oxidative decarboxylases that are widespread among many bacteria and utilize a wide-variety of substrates (Lupa et al 2005). Many of these genes encoding non-oxidative decarboxylases are organized into three gene operons, encoding B, C, and D type genes. "C" type genes encode decarboxylases like AroY, and while the specific function of the B and D type genes are not known although they are sometimes shown to be necessary for realizing full activity of the C type decarboxylase (Lupa et al 2005; Jimenez et al 2013; Lin et al 2015; Sonoki et al 2014). Weber et al (2012) cloned the B, C and D genes from *Klebsiella pneumoniae* or *Sedimentibacter hydroxybenzoicus* into a high-copy-number yeast vector pRS4K-HKT7 and performed fermentation in a medium with externally added PCA to determine the presence of PCA decarboxylase activities from these gene clusters; however, neither was any effort made to determine whether the PCA decarboxylase encoded by the C gene was dependent on the B or D gene in these gene clusters, nor was any attempt made to determine whether the expression of these gene clusters comprising B, C and D genes were able to enhance the muconic acid production using a non-aromatic carbon source such as glucose.

(085) The gene encoding AroY that is used in specific examples disclosed herein is from *Klebsiella pneumoniae*, but the local gene structure of this *aroY* gene reveals no transcriptionally linked genes that would encode either a B or D type enzyme. Previous studies have shown that inclusion of another B type gene, *kpdB*, from *Klebsiella pneumoniae* (part of an operon from a 4-hydroxybenzoic acid decarboxylase) can increase AroY activity when producing muconic acid from lignin-related aromatic compounds (Sonoki et al 2014). However, lignin is a complex mixture of chemicals, which requires costly downstream purification processes in order to produce a pure product such as muconic acid. Johnson et al (2016) have shown that in a *Pseudomonas putida* strain expressing AroY (PCA decarboxylase) from *Enterobacter cloaceae*, co-expression of an EcdB protein from *E. cloaceae* having 89.3% sequence identity to KpdB increased the muconic acid production using glucose as a source of carbon from 1.44 g/L to 4.92 g/L at the end of a 54 hour fermentation; however, the muconic acid yield in this *Pseudomonas putida* based system was still found to be extremely (0.077 mol/mol), thus making this *Pseudomonas putida* based system unsuitable for commercial scale applications. Therefore there is still a need for an improved muconic production process from inexpensive, purer, non-

aromatic carbon sources such as carbohydrates and other non-aromatic compounds (see above). Recent studies have shown that UbiX, a homolog of KpdB, produces a prenylated flavin mononucleotide cofactor and this cofactor supports the decarboxylation activity of UbiD in ubiquinone formation (White et al 2015; Payne et al 2015). Although previous characterizations of UbiX and its homologs refer to the proteins as either 4-hydroxy-3-polyprenylbenzoate decarboxylases, hydroxybenzoate decarboxylasse, subunit B proteins, phenolic acid decarboxylases, or phenylacrylic acid decarboxylases, these proteins are now more accurately annotated as flavin prenyltranferases.

(086) Additional limitations in the production of muconic acid can be attributed to the limitations of the precursor metabolites PEP and E4P. Changes that eliminate or lower the consumption of these metabolites have been shown in the present invention to improve product formation. For instance, PEP availability has been improved by replacing the native glucose transport, the phosphotransferase system, (PTS) with an alternate system (Glf-Glk from *Zymomonas mobilis*). *E coli*'s native use of the PTS requires the utilization of PEP for transport and phosphorylation of glucose. The Glf-Glk system utilizes a facilitated diffuser and a glucokinase plus ATP for transport and phosphorylation of glucose, respectively. Use of Glf-Glk in place of a PTS has been shown to improve the yield and titer for several aromatic products. PEP is a substrate for a variety of biological reactions. In addition to glucose transport by the PTS, PEP is a substrate for pyruvate kinase, resulting in the production of ATP and pyruvate providing half of the ATP generated in glycolysis. Inactivation of the genes encoding for pyruvate kinase (*pykA* or *pykF*) has been demonstrated to increase yield of products in the aromatic pathway (Escalante et al. 2010). PEP is also the substrate for the production of oxaloacetate by phosphoenolpyruvate carboxylase (Ppc). In *E. coli*, this is an essential gene (Baba et al. 2006), and strains deleted for *ppc* are unable to grow on minimal media. There are other organisms that do not have phosphoenolpyruvate carboxylase, but instead replenish oxaloacetate from pyruvate with pyruvate carboxylase (Pyc), e.g. *S. cerevisiae*. The addition of pyruvate carboxylase in *E coli* has been investigated, but only for the improvement of succinate production (Lin et al. 2004; Vemuri, Eiteman, and Altman 2002) not for any aromatic products.

(087) The specification in this patent application provides several different aspects of invention related to the construction of a microbial strain for efficient production of muconic acid. A person skilled in the art can compile several different aspects of the present invention to construct a biocatalyst with very high efficiency for the production of muconic acid.

EXPERIMENTAL SECTION

GENERAL REMARKS

(088) Strain and inoculum preparations: A list of the bacterial strains used in the present invention is provided in Tables 1 and 2. A list of the plasmids used in the present invention is provided in Table 3. All specific examples of strain constructions disclosed herein are derived from a wild type *E. coli* C strain (ATCC 8739), or *E. coli* K-12 strains (YMC9 or MM294) but the genetic elements disclosed herein can be assembled in any other suitable *E. coli* strain, and the expression cassettes or appropriate analogs and homologs of the genetic elements disclosed herein can be assembled in any other suitable microorganism, such as other species of bacteria, archaea, yeast, algae, and filamentous fungi that can be used for the commercial production of cis, cis-muconic acid through a fermentative process.

(089) *E. coli* C is capable of fermenting 10% glucose in AM1 mineral media. AM1 medium contains 2.63 g/L $(NH_4)_2HPO_4$, 0.87 g/L $NH_4H_2PO_4$, 1.5 mM $MgSO_4$, 1.0 mM betaine, and 1.5 ml/L trace elements. The trace elements are prepared as a 1000X stock and contained the following components: 1.6 g/L $FeCl_3$, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L $CuCl_2$, 0.2 g/L $ZnCl_2 \cdot 4H_2O$, 0.2 g/L $NaMoO_4$, 0.05 g/L H_3BO_3, and 0.33 g/L $MnCl_2 \cdot 4H_2O$. The pH of the fermentation broth is maintained at 7.0 with 1.0 – 10.0 M KOH or 1.0 – 9.0 M ammonium hydroxide.

(090) Fermentations: Fermentations were started by streaking on a fresh NBS-2% glucose (Jantama et al., 2008a) plate from a 40% glycerol stock of *E. coli* strain genetically engineered and stored in a -80°C freezer. Plasmids, if present, are retained by including the appropriate antibiotic(s) in the agar plates and liquid media. Ampicillin (sodium salt) is used at 150 mg/L, spectinomycin HCL at 100 mg/L, tetracycline HCl at 15 mg/l, and kanamycin sulfate at 50 mg/l. After 24 to 48 hours (37°C), a single colony is picked into 25 ml of the same medium in a shake

flask. After shaking at 200 rpm at 37°C until the cells have grown to an OD_{600} of about 1.0, the culture is cooled on ice and an equal volume of sterile 80% glycerol is added. 2 ml aliquots are then frozen at -80°C to be used as inocula for fermentations. The term "titer" means the amount of fermentation product produced per unit volume of fermentation fluid and the term "yield" means the ratio of fermentation product produced to carbon source consumed (g/g or mol/mol).

(091) Cell growth: Cell mass was estimated by measuring the optical density at 550 nm (OD_{550}) or 600 nm (OD_{600}) using a Thermo Electronic Spectronic 20 spectrophotometer.

(092) Analysis of intermediates in shikimic acid pathway and muconic acid pathways: Total muconic acid produced in fermentation broths, which includes cis, cis-muconic acid and cis, trans-muconic acid, and other biochemical intermediates were assayed by HPLC with a Waters Alliance instrument, and monitoring absorbance at 210 nm or refractive index at 45° C, using standards purchased from Sigma-Aldrich. The column was a BioRad Aminex HPX-87H run at 50°C with 8 mM sulfuric acid as the mobile phase at a flow rate of 0.6 ml/min for 40 minutes. A chromatograph of purchased standards (Sigma-Aldrich) is shown in Figure 4. To prepare for HPLC, fermentation samples are diluted 10 or 100 fold in 0.05 M potassium phosphate buffer, pH 7.0, to preserve the cis, cis- form of muconic acid from isomerizing to the cis, trans-form.

(093) To separate the isomers of muconic acid, the samples prepared as above were run in a second HPLC system. The instrument was an Agilent 1200 HPLC, the column was an Agilent Eclipse XDB-C18, 4.6 x 150 mm run at 30 degrees Centigrade with a mobile phase of 50mM KH_2PO4 in 30% methanol adjusted to pH 3.0 with phosphoric acid. The flow rate was 1 ml/min for 4 minutes, with detection by absorbance at 278 nm. The cis, trans-muconic acid standard was created by dissolving cis, cis-muconic acid in water and allowing it to undergo spontaneous acid catalyzed isomerization for about 2 hours at room temperature, until the HPLC peak had completely shifted to a new position. The other standards were purchased from Sigma-Aldrich. A chromatograph showing standards is shown in Figure 5.

(094) Composition of muconic acid production medium for the fermentation process: Each liter of fermentation medium contains 50 ml /L of 1M KH_2PO_4, 10 ml of 200g/L Citric acid

+ 25 g/L Ferric citrate, 1.2 ml of 98% Sulfuric acid, and a drop of Antifoam 204. These components were mixed with enough water to allow room for addition of other components below. After autoclaving, the following components were added : 10, 20, 30 or 40 ml of 50% glucose (to give 5, 10, 15, or 20 g/l final), 2 ml of 1M MgSO4, 1 ml of 0.1M CaCl2, 10 ml of 1000X Trace elements (Jantama et al. 2008a), and if necessary 1, 2, 4, or 8 ml of 50g/L Phenylalanine + 50 g/L Tyrosine + 50 g/L Tryptophan (to give 0.5, 0.1, 0.2, or 0.4 g/l final), 10 ml of 1 g/L p-hydroxybenzoic acid + 1 g/l p-aminobenzoic acid + 1 g/L 2,3-dihydroxylbenzoic acid (the last three compounds are referred to as the aromatic "vitamins" or "vitamin-like componds", and, as necessary, 1 ml of 150 mg/ml Ampicillin (sodium salt) and/or 1 ml of 100 mg/ml Spectinomycin HCl. Aromatic amino acids and vitamins were not required and were not used for strains expressing functional AroE protein.

(095) For shake flasks, NBS salts (Jantama et al. 2008a) plus 0.2 M MOPS buffer, pH 7.4 was substituted for the pre-autoclave mix described above, but the glucose and other additives were the same. For fed batch fermentation, the feed bottle contained 600g/L of anhydrous glucose and, if necessary, 32 ml/L of 50 g/L phenylalanine + 50 g/L tyrosine + 50 g/L tryptophan. 9 M NH_4OH was used as base to maintain the pH of the fermentation medium. Aromatic amino acids and vitamins were not required and were not used for strains expressing a functional AroE protein.

(096) Fed-batch fermentations were performed in 7 L New Brunswick Scientific Fermentors with pH, DO, temperature, glucose, and feed rate controlled by either DCU controllers or Biocommand Software. The medium was 50 mM K2HPO4, 20 mM K2SO4, 3 mM MgSO4 and trace elements. The trace elements are prepared as a 50X stock and contained the following components: 1.6 g/L $FeCl_3$, 0.1 g/L $CuCl_2$, 0.2 g/L $ZnCl_2 \cdot 4H_2O$, 0.2 g/L $NaMoO_4$, 0.05 g/L H_3BO_3, and 0.55 g/L $MnCl_2 \cdot 4H_2O$. The temperature was maintained at 37°C, the pH was maintained at 7.0 by 9N ammonium water. Aeration was at 0.5 vvm and the dissolved oxygen (DO) was maintained at 30% by automatically increasing the impeller's speed from 750 rpm to 1200rpm. The initial glucose concentration in the medium was around 20 to 25 g/L. Feed glucose solution was added to the fermentor when the concentration dropped to below 5 g/L. The initial glucose feed rate was 4 g/L/hr and was ramped up to a feed rate of 7 g/L/hr by 48 hours after which it was maintained at 7 g/L/hr.

(097) Construction of plasmids expressing muconic acid pathway genes: The three heterologous genes required for conversion of DHS to muconic acid were cloned either singly or in combination into a low-copy plasmid, pCL1921 (Lerner and Inouye, 1990). The DNA sequence of pCL1921 is given in SEQ ID No. 20 in Table 7. Briefly, the coding sequences of *catAX*, *aroY* and *aroZ* analogs or homologs were codon-optimized for expression in *E. coli* and commercially synthesized (GeneArt, Invitrogen). These sequences were then PCR amplified using a forward primer carrying a unique ribosome-binding site and a reverse primer carrying a unique terminator sequence for each gene. The resulting PCR fragment was digested with restriction enzymes and cloned downstream of a unique constitutive promoter sequence by standard molecular cloning procedures. The promoter sequences were cloned by PCR amplification from source DNA sequences previously described (United States Patent Application 20090191610; United States Patent 7244593) followed by restriction digestion and standard molecular cloning. The promoter-RBS-coding sequence-terminator sequence together constituted an expression cassette. Individual expression cassettes were next combined to generate plasmids expressing one, two or all three muconic acid pathway genes.

(098) The present invneiton is further illustrated using the following examples; however the examples provided herein in either alone or in any combination thereof should be construed to limit the scope or the embodiment of the invention. The claims provided at the end define the scope of the invention. A person skilled in the art can clearly understand the scope of the present invention as defined the claims. A person skilled in the art can make modifications or changes to the technical solutions provided by the invention without departing from the spirit and scope of the present invention.

Example 1

Increasing expression of *aroG* and *aroF* genes

(099) The *tyrR* gene of *E. coli* can be mutated by any one of a number of well-known methods, such as chemical or radiation mutagenesis and screening (for example by PCR and DNA sequencing) or selection for analog resistance (for example, resistance to 4-fluorotyrosine),

transposon mutagenesis, bacteriophage Mu mutagenesis, or transformation. In a preferred embodiment, the mutation in *tyrR* gene is a null mutation (a mutation that leaves no detectable activity), and in a more preferable embodiment, at least a portion of the *tyrR* gene is deleted. This can be accomplished, for example, by using a two-step transformation method using linear DNA molecules (Jantama et al, 2008a; Jantama et al, 2008b). In the first step, a cam^R, *sacB* cassette is integrated at the *tyrR* locus to replace most or all of *tyrR* open reading frame by double recombination and selecting for chloramphenicol resistance. In the second step, a linear DNA comprising a deleted version of the *tyrR* gene is integrated by double recombination, selecting for resistance to 5% sucrose in a rich medium such as LB. Correct deletions are identified and confirmed by diagnostic polymerase chain reaction (PCR). The purpose of deleting *tyrR* is to increase expression of *aroG* and *aroF*. An alternative approach that achieves a similar result is to replace the native promoter in front of *aroG* and/or *aroF* with a strong constitutive promoter and add, if necessary, a transcription terminator. More details on how this is accomplished in general are given in Example 4 below.

(0100) The latter of the two approaches described above for overcoming the repression of AroG and AroF activities by TyrR protein is preferable, since deletion of *tyrR* can cause unwanted overexpression of genes such as *aroLM* (Neidhardt and Curtiss, 1996). More detail on how this is accomplished in general is given in Example 4 below.

Example 2

Feedback resistant AroG and AroF

(0101) Mutations in the *aroG* gene that lead to a feedback resistant AroG enzyme (3-deoxy-D-arabinoheptulosonate-7-phosphate synthase or DAHPS) are well known in the art (Shumilin et al, 1999; Kikuchi et al, 1997; Shumilin et al, 2002). Also well known are methods for creating, identifying, and characterizing such mutations (Ger et al., 1994, Hu et al., 2003). A preferable mutation is one that leads to complete resistance to inhibition by phenylalanine. Any of the known published feedback resistant mutations can be introduced into an *aroG* gene contained in the chromosome or on a plasmid by any of a number of well known methods, one example of which is mutagenic PCR in which the desired mutation is synthesized as part of a PCR priming oligonucleotide (Hu et al., 2003). Correct installation of the mutation is confirmed by DNA

sequencing. The sequence of the wild type *aroG* gene from *E. coli* C is given in SEQ ID No. 18. A preferred mutation is a point mutation that changes amino acid 150 of AroG from proline to leucine, for example by changing codon 150 from CCA to CTA (Hu et al, 2003). In a more preferred embodiment, codon 150 is changed from CCA to CTG, which is a preferred codon in *E. coli.* This particular allele of *aroG* is preferred, since the encoded DAHP synthase is completely resistant to inhibition by phenylalanine up to 3 mM, and it has a specific activity similar to the wild type enzyme (Hu et al., 2003).

(0102) Additional feedback resistant *aroG* alleles can be obtained by mutagenesis and selection for resistance to one or more phenylalanine analogs, such as beta-2-thienylalanine, p-fluorophenylalanine, p-chlorophenylalanine, o-fluorophenylalanine, and o-chlorophenylalanine, followed by demonstrating that the mutation causing the resistance is linked to the *aroG* gene (Ger et al., 1994; US Patent 4,681,852). Linkage to *aroG* can be demonstrated directly by DNA sequencing or enzyme assay in the presence and absence of phenylalanine, (Ger et al., 1994) or indirectly by phage mediated transduction and selection for a genetic marker at or near the *aroG* locus that can be selected, either for or against (US Patent 4,681,852). Such a genetic marker can be a deletion or point mutation in the *aroG* gene itself, or a mutation in any suitable closely linked gene such as *nadA* in case of *E. coli*. For an example in *E. coli*, after mutagenesis and selection for phenylalanine analog resistance, individual mutants or pools of mutants can be used as donors for P1 mediated transduction into a naïve recipient that is deleted for all three DAHP synthase genes, *aroG, aroF*, and *aroH*, and selecting for growth on an appropriate minimal medium. The transductants will then be enriched for mutations in the desired gene(s). Alternatively, after mutagenesis and selection for analog resistance, individual mutants or pools of mutants can be used as donors for P1 mediated transduction into a naïve recipient strain that contains a null mutation in the *nadA* gene, again selecting for growth on an appropriate minimal medium lacking nicotinamide. Another approach is to select for resistant mutants in a strain background that contains a transposon, for example Tn10, insertion near the *aroG* gene, such as in the *nadA* gene. P1 transduction from analog resistant mutants into a strain background that does not contain said transposon and selecting for tetracycline or other appropriate antibiotic resistance will enrich for the desired *aroG* mutations. In all such approaches, feedback

resistance is ultimately confirmed by enzyme assay and DNA sequencing of the gene. We shall refer to alleles of *aroG* that are resistant to feedback inhibition as *aroG**.

(0103) Strain WM191 (*ΔtyrR, ΔaroF*) was derived from YMC9 (ATCC 33927). The two step gene replacement method (Jantama et al., 2008a) was used to install clean deletions in both *tyrR* and *aroF*, to give strain WM191. Next, a *nadA::Tn10* allele was transduced in from CAG12147 (CGSC 7351, Coli Genetic Stock Center, Yale University) to give strain WM189 (*ΔtyrR, ΔaroF, nadA::Tn10*). Selection was on LB plus tetracycline HCl (15 mg/l). Strain RY890 (*ΔtyrR::kan, aroF363*) was derived from MM294 (ATCC 33625) in three steps by P1 transduction. The donor strains, in order, were JW1316-1 (CGSC 9179, Coli Genetic Stock Center, Yale University), NK6024 (CGSC 6178, Coli Genetic Stock Center, Yale University), and AB3257 (CGSC 3257, Coli Genetic Stock Center, Yale University), and the three selections, in order, were LB plus kanamycin sulfate (50 mg/l), LB plus tetracycline hydrochloride (15 mg/l), and NBS minimal glucose (Jantama et al., 2008a) with thiamine HCl (5 mg/l).

(0104) WM189 was mutagenized with UV light to about 20% survival and plated on NBS minimal glucose medium (Jantama et al., 2008a) containing o-fluorophenylalanine (1 mM), thiamine (5 mg/l), and nicotinamide (1 mM). Colonies from each of several plates were collected into separate pools, and P1vir lysates were made on each pool. These lysates were used to transduce WM191 to tetracycline resistance (15 mg/l) on LB medium, and the colonies obtained were replica plated to NBS minimal glucose medium containing o-fluorophenylalanine at 1 mM, thiamine (5 mg/l), and nicotinamide (1 mM). Colony replicas that survived both tetracycline and analog were assumed to contain a feedback resistant mutation in *aroG*. Eight individual colonies from 5 independent pools were chosen for DNA sequencing. The *aroG* coding regions were amplified by polymerase chain reaction and sequenced. The results, shown in Table 4, revealed that each of the eight strains contained a point mutation in their *aroG* gene. Some of the alleles were identical to published alleles, but some were novel.

(0105) A P1vir lysate from one of the pools described above was used to transduce RY890 (which has an *aroG* wild type allele) to tetracycline resistance and resistance to o-fluorophenylalanine (0.3 mM) by replica plating as described above. Four colonies, named

RY893, RY897, RY899, and RY901, were picked for DNA sequencing (Table 4), and again, two of the alleles were identical to a published allele, but two were novel. Strain RY902, which is isogenic to the latter four strains, but contains a wild type *aroG* gene, was constructed as a control, by transduction from CAG12147. These five strains were grown overnight in shake flasks in 25 ml NBS minimal glucose (15 g/l) plus thiamine HCl (5 mg/l) and nicotinamide (1 mM). The resulting cells were harvested by centrifugation, resuspended to be rinsed with 10 ml water, re-centrifuged, and resuspended in 0.5 ml of 50 mM potassium phosphate, pH 7.0. The suspended cells were lysed by vortexing with three drops of chloroform, and the crude lysate was assayed for DAHP synthase activity using a method similar to a method described in the literature (Hu et al., 2003), with the following modifications. The phosphate buffer was 50 mM (final concentration), pH 7.0, the final erythrose-4-phosphate concentration was 2 mM, the final phosphoenol pyruvate concentration was 5 mM, the incubation temperature was 30°C, and the reaction was stopped at 10 minutes. We define 1 mU as the activity that produced 1 nMole of DAHP per minute per milligram protein. To test for feedback resistance, each crude lysate was assayed with or without phenylalanine at a final concentration of 18 mM. The assay results are shown in Table 5. The enzymes showed varying specific activity and resistance to phenylalanine, but all of selected mutant versions that were tested were significantly more resistant than the wild type controls.

(0106) The *aroG* alleles from RY893, RY899, RY901, and RY902, described above, were introduced into a muconic acid producing strain background as follows. P1vir lysates from the *aroG** and *aroGwt* donor strains were used to transduce MYR219 (*E. coli* C, Δ*aroE*, Δ*ack::P*$_{15}$-*aroB*, pMG37) to tetracycline HCl resistance (15 mg/l), to give new strains RY903, RY909, RY911, and RY912, respectively. Each of these strains was then transduced to kanamycin sulfate resistance (50 mg/l) using a P1vir lysate of JW1316-1, to introduce the Δ*tyrR::kan* allele, to give strains RY913, RY919, RY921, and RY922, respectively. Spectinomycin selection was maintained throughout to maintain the muconic plasmid. The resulting four strains were grown for 48 hours at 37°C in shake flasks in 25 ml NBS minimal medium (Jantama et al., 2008a) containing supplements of 20 g/l glucose, 0.2 M MOPS buffer, pH 7.4, nicotimamide (1 mM), phenylalanine (100 mg/l), tyrosine (100 mg/l), tryptophan (100 mg/l), p-hydroxybenzoic acid (1 mg/l), p-aminobenzoic acid (1 mg/l), 2,3-dihydroxybenzoic acid (1 mg/l), phenol red (10 mg/l),

and ammonium sulfate (1 g/l). The pH was kept close to 7 as estimated by eye from the color of the phenol red, against a pH 7.0 standard, by manual addition of 1 ml aliquots of 1.0 M KOH as called for to the shake flasks. The muconic acid produced was assayed by HPLC as described above, and the results are shown in Table 6. All three strains that contain a feedback resistant *aroG** allele produced more muconic acid than the isogenic strain containing the wild type *aroG* allele. In a separate experiment disclosed herein, strain MYR205, containing *aroG* on multicopy plasmid pCP32AMP, produced 1.5 g/l muconic acid in a shake flask. Thus, the inventors have shown that the combination of Δ*tyrR* and single copy chromosomal *aroG** can perform well compared to an isogenic *aroG* plasmid containing strain to produce muconic acid in shake flasks. The inherent superior genetic stability of the chromosomal alleles compared to plasmid alleles, plus the alleviation of the need for a selective medium to hold in a plasmid, makes the novel strains described herein more suitable for large scale commercial fermentations. Furthermore, no chemical inducer was required for expression of muconic acid pathway genes. Thus, strains of the instant invention described above are improved over those of the prior art (Niu et al., 2002), all of which contain the gene for overexpression of DAHP synthase on an undesirable multicopy plasmid.

(0107) In a similar fashion to that described above for AroG, a mutation that leads to an AroF or AroH isozyme that is resistant to feedback inhibition by tyrosine can be installed on a plasmid or in the chromosome. A preferred mutation is a point mutation that changes amino acid 148 of AroF from proline to leucine, for example by changing codon 148 from CCG to CTG (Weaver et al., 1990), to give a gene named *aroF**. Other alleles of *aroF** can be isolated by resistance to tyrosine analogs (for example o-fluorotyrosine, m-fluorotyrosine, p-fluorophenylalanine, etc.) in a fashion analogous to that described above for *aroG** alleles. *aroF** alleles can be selected, enriched for, and transduced by linkage to a transposon or a kanamycin resistance insertion, for example in a closely linked Δ*yfiR::kan* as in a strain such as JW2584 (CGSC 10051, Coli Genetic Stock Center, Yale University).

Example 3

Deletion of *aroE* from chromosomal DNA and muconic acid production

(0108) In this example the effect of overexpression of *aroB* and *aroG* on multicopy plasmids as well as the expression of genes coding for proteins functional in the muconic acid pathway was investigated. Strain MYR34 containing a deletion in the *aroE* gene coding for shikimate dehydrogenase was used as parent strain in these studies. The deletion of chromosomal copy of *aroE* was accomplished in a fashion similar to that described above in Example 1. When MYR34 was transformed with the plasmid pCP32AMP overexpressing the *aroG* gene coding for DAHP synthase protein functional in the shikimic acid pathway, there was a significant increase in the accumulation of DHS. When MYR34 was transformed with the plasmid expressing *aroB* from a constitutive promoter, no significant increase in the accumulation of DHS was noticed. However, when the *E. coli* strain MYR34 was transformed with the plasmid expressing both *aroB* and *aroG* genes, there was an increase in the accumulation of DHS than observed with MYR34 transformed with *aroG* alone thus suggesting *aroB* as a secondary bottleneck in DHS production (Figure 6).

(0109) In the experiments presented in Figure 7, the effect of an additional copy of the *aroB* gene integrated into the host chromosomal DNA was examined. In the *E. coli* strain MYR170 derived from MYR34, an additional copy of the *aroB* gene under the control of the P_{15} promoter was integrated into the host chromosome at the *ack* locus. When MYR170 strain was transformed with the pCP32AMP plasmid, there was a slight increase in the DHS accumulation when compared to the DHS accumulation detected in the MYR34 strain transformed with the same plasmid. This slight increase in the accumulation of DHS in the MYR170 can be attributed to an additional copy of *aroB* gene integrated into the host chromosomal DNA. When MYR170 was transformed with pCP54 expressing both *aroB* and *aroG* genes, there was a further increase in the DHS accumulation suggesting *aroB* as a secondary bottleneck in DHS production.

(0110) Figure 8 provides the results on muconic acid production with the *E. coli* strains MYR34 and MYR170. Having established that in the *aroE* deletion strains MYR34 and MYR170, with overexpression of *aroB* and *aroG* genes there is an accumulation of DHS, efforts were made to see whether the expression of "muconic pathway" genes coding for the proteins functional in muconic acid production pathway would lead to conversion of DHS into cis, cis-muconic acid. In these experiments, the *E. coli* stains MYR34 and MYR170 were transformed either with the

plasmid pMG37 alone or with both plasmids pMG37 and pCP32AMP. The plasmid pMG37 expresses *aroZ*, *aroY* and *catAX* genes coding for proteins functional in muconic acid pathway. The muconic acid production in both MYR34 and MYR170 increased when these bacterial strains were transformed with both the plasmids pCP32AMP and pMG37 when compared to the muconic acid production in these two strains transformed only with pMG37 plasmid suggesting that in these strains *aroB* expression is the bottleneck for cis, cis-muconic acid production.

Example 4

Overexpression of *tktA*

(0111) Transketolase encoded by *tktA* is a key enzyme in the pentose phosphate pathway and is thought to be limiting for the production of erythrose-4-phosphate, one of the key intermediates in the production of muconic acid. Overexpression of *tktA*, which encodes transketolase, by installing the gene with its native promoter on a multicopy plasmid (Sprenger et al, 1995, 1995a), is known to improve flux into the aromatic pathway (Draths et al., 1992). However, such plasmids are unstable, and often require antibiotic selection for maintenance. Another approach in the prior art was to add one additional copy of the *tktA* gene to the chromosome of the host strain (Niu et al., 2002). However, one additional copy of *tktA* with its native promoter is not sufficient to saturate the aromatic pathway with erythrose-4-phosphate, since its native promoter is not very close to the ideal. As such, the process needs substantial improvement.

(0112) Improved overexpression of *tktA* can be obtained, for example, by substituting the native *tktA* promoter in the chromosome with a strong constitutive promoter, for example a P_{15} or P_{26} promoter from *Bacillus subtilis* phage SPO1 (SEQ ID No. 1 and SEQ ID No. 2, respectively), or the P_R promoter from bacteriophage lambda (SEQ ID No. 3). This is accomplished in two steps as described in Example 1, except that the *cam*^{R}, *sacB* cassette is used to replace the native chromosomal *tktA* promoter in the first step. In the second step, the strong constitutive promoter is installed by transforming with a linear DNA comprising the strong constitutive promoter, flanked by at least 50 bases of the 5' end of the *tktA* coding region on the downstream side and at least 50 base pairs of homology just upstream of the native *tktA* promoter on the upstream side of the strong constitutive promoter, and selecting for sucrose resistance. Improved expression from such an expression cassette is also accomplished by increasing the stability of the mRNA that is

transcribed from the expression cassette. Improvement of the mRNA stability is accomplished by adding a stem loop structure at either the 5' end of the mRNA, the 3' end of the mRNA, or both. A stem-loop structure is often present at the end of an mRNA that is naturally terminated by a rho-independent transcription terminator, but if it is not, then a rho-independent transcription terminator can be added to the DNA sequence by well known methods of genetic engineering (ligation, PCR, etc.). Such a terminator can be comprised of an inverted repeat of between 4 and 20 bases in each repeat, separated by a "loop" of 3 or more bases, and followed by a region of one or more bases that is enriched for T's (deoxythymidine). The inverted repeats are rich in G's and C's (deoxyguanidine and deoxycytidine). Similarly, a stem-loop can be constructed into the 5' end of an mRNA by inserting a DNA sequence just downstream from the start point of transcription, but before the ribosome binding site, that contains a stem-loop as described above, but without the T-enriched region. An example of this is given in association with the P_{15} promoter (SEQ ID No. 1).

(0113) In the analysis of the effect of overexpression of the *tktA* gene on the flow of carbon through the shikimic acid pathway, *E. coli* strain MYR170 was used as a parental strain. MYR170 has a deletion in the *aroE* gene coding for shikimate dehydrogenase enzyme and an additional copy of the *aroB* gene at the *ack* locus.

(0114) In the experiments described in the Figures 9, 10 and 11 two different plasmids namely pCP32AMP and pCP50 were used. The plasmid pCP32AMP expresses only the DAHP synthase *aroG* gene from its native promoter and the plasmid pCP50 expresses the transketolase gene *tktA* from its native promoter along with *aroG* gene. MYR170, having an *aroE* deletion and an additional copy of *aroB* gene under the control of P_{15} promoter integrated at the *ack* locus of the chromosomal DNA, was transformed individually with pCP32AMP and pCP50 plasmids. As shown in Figure 8 the DHS accumulation was increased further with the expression of *aroG* gene along with *tktA* gene when compared to the *E. coli* cells expressing only *aroG* gene.

(0115) Figure 10 provides data on the DHS yield in two different strains namely MYR34, MYR170 transformed with the plasmid pCP32AMP or pCP50. MYR34 strain having *aroE* gene deletion yielded 0.1 gram of DHS per gram of glucose consumed. The DHS yield in the MYR34 increased to 0.15 gram of DHS per gram of glucose consumed when this strain was transformed

with the pCP32AMP plasmid with *aroG* gene overexpression. MYR170 has an additional copy of *aroB* gene inserted at the *ack* locus. As a result of the presence of this additional copy of the *aroB* gene, the yield for DHS production in the MYR170 strain transformed with pCP32AMP was slightly higher than the DHS yield noted in the MYR34 strain transformed with pCP32AMP. Thus the presence of an additional copy of *aroB* in MYR170 caused an increased carbon flow through shikimic acid pathway. Further increase in the DHS yield was observed when the MYR170 strain was transformed with plasmid pCP50 expressing both *aroG* and *tktA* genes. Thus the presence of additional copy of *tktA* accounted for an increase carbon flow through shikimic acid pathway. More specifically, the effect of presence of additional *aroB* and *tktA* genes caused an additive effect on DHS yield.

(0116) MYR261 used in the experiments described in Figure 11 was engineered to integrate an additional copy of *tktA* gene into the chromosomal DNA of MYR170 at the *poxB* locus. The desired gene replacement (*poxB::tktA*) in the MYR261 strain was confirmed via PCR. MYR261 was transformed either with pCP32AMP (*aroG* overexpression) plasmid or pCP50 (*aroG* and *tktA* over expression) plasmid. As a control, MYR170 was transformed with pCP32AMP plasmid. As the result shown in Figure 11 indicate, the presence of an additional copy of *tktA* gene in the chromosomal DNA of MYR261 increased the titer for DHS production with pCP32AMP plasmid when compared to the titer for DHS production observed in the MYR170 strain transformed with the same plasmid. Further increase in the transketolase level in the MYR261 strain when transformed with the plasmid pCP50 over expressing transketolase led to further increase in the titer for DHS production. The enzyme encoded by *poxB*, PoxB, or pyruvate oxidase, produces acetate as a reaction product. As such, the deletion of *poxB* that results from the insertion of *tktA* as described herein removes a potentially active pathway for acetate production. Similarly, simultaneous insertion of P_{15}*aroB* and deletion of *ackA,* which encodes AckA, or acetate kinase, as described below in Example 12 below, removes another potentially active pathway to acetate. Production of acetate is generally undesirable in fermentations (Jantama et al., 2008b). As such, these deletions can be useful for reducing acetate production.

(0117) Figure 12 provides the titer for muconic acid and acetic acid production in MYR170, MYR261 and MYR305 strains of *E.coli* after transformation with the plasmids pCP32AMP and pMG37. MYR305 is derived from MYR170 by means of deleting *poxB* gene from the chromosomal DNA while MYR261 is a MYR170 derivative wherein the *poxB* gene has been inactivated by means of inserting an additional copy of the *tktA* gene. As mentioned above, the plasmid pCP32AMP expresses the *aroG* gene coding for DAHP synthase protein functioning in the shikimic acid biosynthetic pathway leading to the accumulation of DHS due to the deletion of *aroE* gene in the *E. coli* strains MYR170, MYR261 and MYR305. With the expression of muconic pathway genes namely *aroZ*, *aroY* and *catAX* on the plasmid pMG37, the DHS is converted into cis, cis-muconic acid as illustrated in Figure 2. With the presence of an additional copy of the *aroB* gene and the *tktA* gene in the MYR261 strain, there was a slight increase in the production of muconic acid accompanied by a decrease in the accumulation of acetic acid.

Example 5

Overexpression of *talA* or *talB*

(0118) The *talB* gene encodes the predominant transaldolase in *E. coli*, but the *talA* gene also encodes a minor transaldolase. Overproduction of transaldolase is known to improve flux into the aromatic pathway (Lu and Liao, 1997; Sprenger, 1995; Sprenger et al, 1995b). In the prior art, this was accomplished by overexpression of the *tal* gene (now known to be the *talB* gene) on a multicopy plasmid from its native promoter (Lu et al., 1997, Sprenger et al., 1995b). However, such plasmids are unstable, and require antibiotic selection for maintenance. Thus, there is a need for an improved process. Improved expression of *talB* can be obtained, for example, by substituting the native *talB* promoter in the chromosome with a strong constitutive promoter, for example a P_{15} or P_{26} promoter from *Bacillus subtilis* phage SPO1 (SEQ ID No. 1 and SEQ ID No. 2, respectively), or the P_R promoter from bacteriophage lambda (SEQ ID No. 3). This is accomplished in two steps as described in Example 1, except that the *cam*^{R}, *sacB* cassette is used to replace the native chromosomal *talB* promoter in the first step. In the second step, the strong constitutive promoter is installed by transforming with a linear DNA comprising the strong constitutive promoter, flanked by at least 50 bases of the 5' end of the *talB* coding region on the downstream side and at least 50 base pairs of homology just upstream of the native *talB* promoter on the upstream side of the strong constitutive promoter, and selecting for sucrose

resistance. The *talA* gene can also be overexpressed by a similar method, but it is preferred to over express the *talB* gene, since it encodes the predominant activity (Sprenger, 1995; Sprenger et al, 1995b). See Example 4 for more details on construction of the expression cassette designed for overexpression.

Example 6

Expression of *aroZ*, *aroY* and *catAX* genes

(0119) To demonstrate conversion of endogenous DHS produced by *E. coli* into muconic acid, heterologous genes *catAX* from *Acinetobacter sp. ADP1*, *aroY* from *Klebsiella pneumoniae*, and *quiC* from *Acinetobacter sp. ADP1*, were cloned under strong constitutive promoters (P_{15}, P_R, and P_{26}, respectively) in a low-copy plasmid, pCL1921 (Lerner and Inouye, 1990) to generate a 'muconic plasmid' pMG37. MYR34 strain derivatives carrying the empty vector (pCL1921) or pMG37 were grown at 37°C for 17 hrs. in a shake flask medium (NBS minimal medium supplemented with aromatic amino acids and vitamins) containing 2% glucose. Supernatants were collected and analyzed by HPLC. In contrast to MYR34/pCL1921 which shows accumulation of DHS, MYR34/pMG37 shows production of muconic acid (Figure 13). No significant amount of DHS, or intermediate products such as PCA and catechol were detected from the latter strain, suggesting that the heterologous genes expressed from pMG37 were functional and sufficient.

Example 7

Comparison of *aroZ* homologs

(0120) Three different *aroZ* homologs and analogs were compared (Figure 14) for their ability to divert DHS into the muconic acid production pathway. *quiC* from *Acinetobacter sp. ADP1*, *asbF* from *Bacillus thuringiensis*, and *qa-4* from *Neurospora crassa*, are reported to encode for proteins that have AroZ-like activity (Elsemore and Ornston, 1995; Fox et al, 1995; Rutledge, 1984). Each of these genes was codon-optimized for expression in *E. coli* and synthesized by GeneArt (Invitrogen), and cloned under a strong constitutive P_{26} promoter in low-copy 'muconic plasmid' which also expressed *catAX* and *aroY* genes from the P_{15} and P_R promoters,

respectively. MYR34/pCL1921, MYR34/pMG37 (muconic plasmid with *quiC* as *aroZ*), MYR34/pMG47 (muconic plasmid with *asbF* as *aroZ*), and MYR34/pMG70 (muconic plasmid with *qa-4* as *aroZ*) were grown at 37°C for 48 hrs. in a shake flasks with minimal medium containing 2% glucose, the aromatic amino acids and aromatic vitamins. Supernatants were collected and analyzed by HPLC. As expected, empty vector transformed MYR34 accumulated DHS and produced no muconic acid. The two *aroZ* homologs and the one analog examined were functional in diverting DHS towards muconic acid production, but to a varying degree. The MYR34 derivative expressing *quiC* gene was most robust and showed nearly 100% conversion of DHS to muconic acid with insignificant amount of DHS retention. The MYR34 derivative expressing the fungal *aroZ* homologue, *qa-4*, followed close with about 80% conversion of DHS to muconic acid and 20% DHS retention. Lastly, the MYR34 derivative expressing *asbF* gene showed only 50% conversion of DHS to muconic acid and 50% DHS retention. Taken together, under our shake flask assay conditions, the expression and/or activity of *quiC* gene appeared to be the highest compared to that of other *aroZ* homologs.

Example 8

Chromosomal integration of *catAX, aroY and quiC*

(0121) Muconic acid can be produced by strains that contain only chromosomally integrated single copies of *catA-X*, *aroY* and *quiC* expressed from constitutive promoters at *adhE* locus.

(0122) MYR170 (*ΔaroE, Δack::P_{15}-aroB*), a high DHS producer, was the host strain used for integrating the muconic acid pathway genes at the *adhE* locus in the chromosome (SEQ ID No. 41). The resulting strain MYR352 was transformed with plasmids YEp24 (medium-copy, empty vector), pCP32AMP (medium-copy, *aroG* expressed from native promoter), or pCP50 (medium-copy, *aroG* and *tktA* expressed from their respective native promoters) to generate derivative strains. The latter two plasmids were used to increase DHS production. Strains were grown at 37°C for 72 hrs. in shake flask medium containing 2% glucose as described above. Supernatants were collected at 72 hrs. and analyzed by HPLC. As expected, the *aroG* and *aroG/tktA* transformed MYR352 derivatives showed an overall increase in total product formation compared to an empty vector control (Figure 15). All of the MYR352 transformants produced

measurable titers of muconic acid, demonstrating for the first time that muconic acid can be produced by a strain that contains only integrated "muconic pathway" genes and without a fed chemical inducer of gene expression.

(0123) Not all DHS that was produced in any of these MYR352 derivative strains was converted to the end product muconic acid. Instead, there was a significant amount of catechol accumulation (Figure 15), suggesting that expression or activity of *catAX* is limiting when it is expressed from a single copy on chromosome. Since the major accumulating intermediate was catechol, it is likely that *quiC* and *aroY* gene expression and/or activity is sufficient in the MYR352 strain background for muconic acid synthesis.

(0124) The MYR352 strain derivatives were compared in parallel with analogous MYR219 strain derivatives. MYR219 strain is same as MYR170 strain but contains low-copy plasmid pMG37 expressing muconic acid pathway genes. Thus, the main difference between MYR352 and MYR219 strains is with reference to the dosage of muconic acid pathway genes (1 copy vs. about 5 copies, respectively). In contrast to MYR352 derivative strains, MYR219 derivative strains showed very little accumulation of catechol or other intermediates, and successfully produced the end product muconic acid. Together, these results indicate the need for increasing *catAX* activity in strains such as MYR352.

Example 9

Expression of *catAX*

(0125) Accumulation of catechol and inefficient production of muconic acid in MYR352 strain is due to limiting dosage and/or activity of the *catAX* gene product(s). As described above, MYR352 contains *ΔaroE, Δack::P_{15}-aroB* and chromosomally integrated single copies of *catAX*, *aroY* and *quiC* genes under strong constitutive promoters. This strain was transformed with medium-copy empty vector control (YEp24) or *aroG/tktA* expression plasmid (pCP50) to increase carbon flow into the aromatic amino acid synthesis pathway and produce high amounts of DHS. Growth of transformed strains at 37°C for 72 hrs in shake flask medium supplemented with 2% glucose as described above resulted in accumulation of catechol intermediate. This

result suggested that *catAX* activity may be insufficient in MYR352. To confirm this hypothesis, the ability of one or more muconic acid pathway genes expressed from low-copy plasmid to alleviate catechol accumulation in MYR352/pCP50 was tested (Figure 16). Specifically, MYR352/pCP50 was further transformed with low-copy empty vector control (pCL1921) or plasmids expressing all three genes, two genes, or one gene of the muconic acid production pathway. The derivative strains were assayed in a shake flask experiment as described above. While increasing the dosage of *aroY* alone (from pMG27) or *quiC* alone (from pMG39) did not alleviate catechol accumulation , expression of all of the muconic acid pathway genes (from pMG37) or *catAX* and *aroY* together (from pMG33), resulted in successful conversion of catechol to muconic acid. Further, expression of *catAX* alone (from pMG31) was sufficient for production of muconic acid and preventing accumulation of catechol.

Example 10

Constructing a leaky *aroE* mutation

(0126) In the prior art process for producing cis, cis-muconic acid, the host strain contains a mutation in the *aroE* gene named *aroE353*, which is a null mutation. As a result, the strain requires the feeding of the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and aromatic vitamins made from the shikimate pathway (p-hydroxy benzoic acid, p-amino benzoic acid, and 2,3-dihydroxy benzoic acid). The aromatic amino acids are too expensive to be fed in a commercially attractive process. As such, the *prior art* process needs a substantial improvement. This can be accomplished by installing a leaky version of the *aroE* gene, that we shall call *aroE**. Leaky mutations are obtained by first generating a missense mutation that changes one amino acid in the *aroE* coding sequence that results in a null phenotype. This can be accomplished by any form of mutagenesis and screening for simultaneous auxotrophy for the six aromatic compounds listed above. A preferred method is to create a pool of mutant *aroE* genes by error-prone PCR mutagenesis, using *Taq* DNA polymerase, using wild type *E. coli* C genomic DNA as the template, and using PCR oligonucleotide primers that hybridize about 1000 base pairs upstream and 1000 base pairs downstream of the *aroE* coding region. The resulting pool of linear DNA molecules is used to transform an *E. coli* C derivative that produces cis, cis-muconic acid, and which contains an integrated cam^R, *sacB* cassette that has replaced the *aroE*

coding region (see Example 4 for a related example), and selecting for sucrose resistance. The transformants are then screened for auxotrophs that have lost chloramphenicol resistance and require the six aromatic compounds listed above. Several independent auxotrophs are picked and tested for revertability by plating about 10^7,10^8, or 10^9 cells (rinsed in minimal glucose medium) on a minimal glucose plate without the six aromatic compounds. Revertants that give rise to colonies on the plates are picked and tested for production of cis, cis-muconic acid, but without production of substantial levels of aromatic amino acids. Among such revertants will be strains that carry one or more mutations in the *aroE* gene, such that the AroE enzyme provides enough aromatic amino acids and vitamins for growth, but not a surplus of these aromatic compounds. Another method to obtain a leaky *aroE* mutant is to install one of the classical revertable *aroE* mutants, such as *aroE353* and *aroE24* (both available from the Coli Genetic Stock Center at Yale University, New Haven, CT, USA), into a cis, cis-muconic acid producing strain, and select for revertants as described above.

Example 11

Import of glucose by facilitated diffusion

(0127) One of the substrates in the first committed step of the aromatic pathway is phosphoenolpyruvate (PEP). PEP is also the source of phosphate and energy for importing glucose and some other sugars by the bacterial phosphotransferase system (PTS). Thus, when a bacterium is growing on a PTS-dependent sugar, there is competition between the PTS and the aromatic pathway for PEP. As such, a significant improvement in increasing flux to the aromatic pathway can be achieved by deleting the PTS and providing an alternative pathway for sugar uptake. One solution to this problem is to replace the PTS with the *E. coli* GalP permease, a proton symporter that works reasonably well for glucose uptake (US Patent 6,692,794). However, the proton symporter still uses energy to maintain the proton gradient that is necessary to drive the permease. As such, there is a need for even further improvement in the process.

(0128) Some sugars, such as xylose, can be imported by a transporter protein that derives energy from hydrolysis of ATP (adenosine triphosphate). Once again, if the energy-dependent

transporter can be replaced by a transporter that requires less energy, then an improvement can be made, since the energy inherent in the ATP can be conserved for other beneficial uses.

(0129) A significant improvement can be obtained by using a facilitated diffusion transporter, which expends no energy for the importation of the sugar (Parker et al, 1995; Snoep et al, 1994). For example, the glucose facilitator from *Zymomonas mobilis*, encoded by the *glf* gene, can be used in place of, or in addition to, the PTS in 3-dehydroshikimate (DHS) producing strains (Yi et al., 2003). However, these strains still rely at least partly on GalP for glucose import. Since GalP requires energy in the form of a proton gradient for importation of glucose, there is a need for improvements in the efficiency of glucose import for muconic producing strains.

(0130) A cassette for expression of *glf* plus a glucokinase gene, *glk*, also from *Z. mobilis*, can be assembled with a strong constitutive promoter, for example P_{26}. This cassette can then be integrated into the genome of a host strain at a location that will not interfere with production of the desired compound, which in this case is cis, cis-muconic acid. An example of such a location in the *E. coli* chromosome is the threonine degradation operon, *tdcABCDEFG*. If the growth medium contains no threonine, then this operon is not needed or expressed, so an insertion of an expression cassette in that operon does not interfere with metabolism.

(0131) To achieve the above described improvement, one or more of the genes encoding a PTS function are deleted, using a method similar to that disclosed in Example 1. For example, one or more of *ptsH, ptsI, crr*, or *ptsG* can be deleted. Next *galP* is deleted using the process as decribed in the US Patent No. 8,871,489. The P_{26}-*glf, glk* cassette can then be installed in two steps, similar to those described in Example 1. In the first step, a cam^R, *sacB* cassette is integrated at the *tdc* operon, using a linear DNA derived from pAC21 (SEQ ID No. 15), and selecting for chloramphenicol (30 mg/l) resistance. In the second step, the P_{26}-*glf, glk* cassette is integrated at the *tdc* operon, using a linear DNA derived from pAC19 (SEQ ID No. 15), selecting for sucrose resistance and screening for chloramphenicol sensitivity, and in this case, improved growth on minimal glucose medium.

(0132) To test whether facilitated diffusion of glucose could substitute for the conventional glucose import systems in *E. coli*, the *ptsHI* genes and the *galP* gene were deleted from MYR34 (Δ*aroE*), and then the P_{26}-*glf, glk* cassette was integrated at the *tdc* operon, using a linear DNA derived from pAC19 (SEQ ID No. 15), to give strain MYR217. MYR217 grows reasonably well on a minimal glucose medium supplemented with the required three aromatic amino acids and three aromatic vitamin-like compounds (Figure 17). However, strain MYR31, which contains deletions of *ptsHI* and *galP*, but does not contain the *glf, glk* cassette did not show any measurable growth (Figure 17). Thus, facilitated diffusion is sufficient to replace the two conventional glucose import systems in our strain background.

(0133) To test whether facilitated diffusion is useful for producing compounds derived from the aromatic pathway, MYR34 and MYR217 were transformed with pCP54 (*aroG, aroB*) and pCP55 (*aroG, aroB, tktA)*. Production of the aromatic intermediate 3-dehydroshikimate (DHS) in shake flasks was compared for these two strains (Figure 18). With either pCP54 or pCP55, the strain using facilitated diffusion produced as much or more DHS than the strains using the conventional glucose import systems. Production of DHS is a good proxy for muconic acid production in engineered *E. coli* strains, so we can conclude that facilitated diffusion of glucose is a useful improvement for muconic acid production.

Example 12

Overexpression of the *aroB* gene

(0134) Expression of the *aroB* gene is reported to be rate limiting for cis, cis-muconic acid production (Niu et al., 2002). In the prior art, this was allegedly solved by integrating a second copy of the *aroB* gene with its native promoter. However, this is insufficient to alleviate the *aroB* limitation, since the native promoter and ribosome binding site of the *aroB* gene are far from ideal. As such, the process needs substantial improvement.

(0135) Improved overexpression of *aroB* can be obtained, for example, by replacing the native *aroB* promoter in the chromosome with a strong constitutive promoter, for example a P_{15} or P_{26} promoter from *Bacillus subtilis* phage SPO1 (SEQ ID No. 1 and SEQ ID No. 2, respectively), or

the P_R promoter from bacteriophage lambda (SEQ ID No. 3). This is accomplished in two steps as described in Example 4, except that the *cam*^{R}, *sacB* cassette is used to replace the native chromosomal *aroB* promoter and/or ribosome binding site in the first step. In the second step, the strong constitutive promoter is installed by transforming with a linear DNA comprising the strong constitutive promoter, followed by a ribosome binding site and at least 50 bases from the 5' end of the *aroB* coding sequence, including the ATG start codon, on the downstream side, and at least 50 base pairs of homology just upstream of the native *aroB* promoter on the upstream side of the strong constitutive promoter, and selecting for sucrose resistance. In addition to, or instead of, installing a stronger promoter, using a similar method, a stronger ribosome binding site, for example, AGGAGG, can be installed about 4 to 10 base pairs upstream of the ATG translation start codon of *aroB*. A copy of such a synthetic cassette, for example, a P_{15}-*aroB* cassette, can be integrated in the chromosome at a locus distinct from the native *aroB* locus, for example at the *ack* locus. Simultaneous deletion of the *ack* gene, as well as deleting the *poxB* gene as in Example 4 can help to reduce formation of unwanted acetate during fermentations.

Example 13

Decreasing flux through the oxidative branch of the pentose phosphate pathway

(0136) The erythrose-4-phosphate that is needed for the first committed step in the aromatic pathway is derived from the non-oxidative portion of the pentose phosphate pathway (PPP). There are two different pathways by which carbon can enter the PPP. The first is from glucose-6-phosphate by the enzymes glucose-6-phosphate dehydrogenase (encoded by the *zwf* gene), 6-phosphogluconolactonase (encoded by the *pgl* gene), and 6-phophogluconate dehydrogenase (encoded by the *gnd* gene), to give ribulose-5-phosphate. In the last of these three steps, one carbon is lost as CO_2. This path into the PPP is called the oxidative branch of the PPP. Ribulose-5-phosphate is then converted into a variety of other sugar phosphates by the action of isomerases, epimerases, transketolase, and transaldolase. This group of reversible reactions, starting with ribulose-5-phosphate, is called the non-oxidative branch of the PPP. The second path by which carbon can enter the PPP is through fructose-6-phosphate and glyceraldehye-3-phosphate (both of which come from the Embden-Myerhoff pathway, also known as glycolysis), which are combined and rearranged by transaldolase and transketolase to give the variety of

other sugar phosphates, one of which is erythrose-4-phosphate. If carbon enters the PPP through this second route, then no CO_2 is lost. In order to improve the yield of cis, cis-muconic acid from glucose, the loss of CO_2 can be prevented by blocking the oxidative branch of the PPP, such that all carbon entering the PPP must come through a non-oxidative route from fructose-6-phosphate and glyceraldehye-3-phosphate. The blocking of the oxidative branch of the PPP is accomplished by deleting the *zwf* gene, using a two-step method similar to that disclosed in Example 1 for deleting the *tyrR* gene.

Example 14

Increasing the flux to and through PEP to the aromatic pathway

(0137) It is desirable to ensure that PEP is not a rate limiting intermediate on the pathway to cis, cis-muconic acid. This is accomplished, for example, by increasing the recycling of pyruvate to PEP by the enzyme PEP synthetase, which is accomplished by integrating an overexpression cassette of the *pps* gene as described above in other examples. Another approach is to limit the consumption of PEP by pyruvate kinase, which in *E. coli* is encoded by the *pykA* and *pykF* genes. In this case, the approach is to decrease the activity of the enzyme(s). This is accomplished by deleting one or more genes that encode pyruvate kinase (as described in Example 1 for *tyrR* and in the US Patent No. 9,017,976), or reducing the strength of expression of one or more of these genes, for example, by mutating the promoter, ribosome binding site, or coding sequence, such that the level of pyruvate kinase activity is decreased. For example, the RBS in front of the *E. coli pykA* gene is 5'CGGAGTATTACATG. The ATG translation start codon is underlined. This sequence can be mutated to CaGAGTATTACATG, CaaAGTATTACATG, CaatGTATTACATG, CaataTATTACATG, and so on, such that the RBS sequence is made less like the consensus RBS of AGGAGG by one base change at a time. Each mutated version is then introduced into the chromosome at the *pykA* locus, replacing the wild type, and cis, cis-muconic acid production levels are measured for improvement.

Example 15

Conferring growth on sucrose

(0138) Strains derived from *E. coli* C do not grow on sucrose as a sole carbon source. However, they can be genetically engineered to do so as disclosed in the International Patent Application Publication No. WO2012/082720 and US Patent Application Publication No. US2013/0337519 which are hereby incorporated by reference in its entirety. As such, a cis, cis-muconic acid producing strain can be engineered to grow on sucrose as disclosed in the above mentioned application.

Example 16

An improved producer of cis, cis-muconic acid

(0139) All of the features described in Examples 1 – 15 can be combined in one strain of *E. coli* by installing the features one after another. The resulting strain comprises an improved cis, cis-muconic acid producer. The resulting strain can then be even further improved by integrating a second copy of each overexpression cassette described above, one at a time, at a location separate from the location of the first copy. An example of a convenient and safe location is at a *Bsr*B1 restriction site just downstream from the terminator of *rrfF*, which encodes a ribosomal RNA. The desired cassette is ligated as a blunt linear DNA into the unique *Bsr*B1 site of plasmid pMH17F (SEQ ID No. 17). An example is the ligation of the *catAX* expression cassette to give a plasmid named p*catAX*. In parallel, a cam^R, *sacB* cassette is ligated as a blunt fragment into pMH17F to give pMH28F (SEQ ID No. 19). A linear DNA derived from pMH28 by PCR or by restriction enzyme cutting is used to deposit the cam^R, *sacB* cassette at the *rrfF* site. Next, a linear DNA derived from p*catAX* by PCR or by restriction enzyme cutting is used to install the second copy of the *catAX* cassette at the *rrfF* locus, using selection on sucrose. The resulting strain is then compared with its grandparent strain for cis, cis-muconic acid production to determine that *catAX* was a limiting step. By a similar method, each cassette from Examples 2 – 15 is tested for a rate limiting step. If a step is found to be rate limiting, then one or more additional copies of the relevant cassette is/are integrated at yet other appropriate locations in the chromosome, leading to still further improvements in cis, cis-muconic acid production, without the need for plasmids or inducible promoters.

Example 17

Production of cis, cis-muconic acid by fermentation

(0140) Cis, cis-muconic acid can be produced by genetically engineered microorganisms disclosed in the above Examples 1-15. The growth medium can vary widely and can be any medium that supports adequate growth of the microorganism. A preferred medium is a minimal medium containing mineral salts and a non-aromatic carbon source, such as glucose, xylose, lactose, glycerol, acetate, arabinose, galactose, mannose, maltose, or sucrose (see above for an example of a preferred minimal growth medium). For each combination of engineered microorganism and growth medium, appropriate conditions for producing cis, cis-muconic acid are determined by routine experiments in which fermentation parameters are systematically varied, such as temperature, pH, aeration rate, and compound or compounds used to maintain pH. As cis, cis-muconic acid is produced, one or more compounds must be fed into the fermentor to prevent pH from going too low. Preferred compounds for neutralizing the acid include alkaline salts such as oxides, hydroxides, carbonates, and bicarbonates of ammonium, sodium, potassium, calcium, magnesium, or a combination two or more of such alkaline salts.

(0141) Muconic acid production by MYR428 strain of *E. coli* in a 7 Liter fermentor is shown in Figure 19. MYR261 strain of *E. coli* with a genotype of Δ*aroE* Δ*ackA*::P_{15}-*aroB* Δ*poxB*::*tktA* was transformed with the plasmids pCP32AMP and pMG37 to generate MYR428. MYR428 was grown in a 7 liter fermentor as described above with glucose feeding for 48 hours. The final muconic acid titer was 16 g/l (see Figure 19).

(0142) After fermentation is complete, cells are removed by flocculation, centrifugation, and/or filtration, and the cis, cis-muconic acid is then purified from the clarified broth by a combination of one or more subsequent steps, for example precipitation, crystallization, electrodialysis, chromatography (ion exchange, hydrophobic affinity, and/or size based), microfiltration, nanofiltration, reverse osmosis, and evaporation.

Example 18

Improvement of 3,4-dihydroxybenzoic acid (PCA) decarboxylase (AroY) activity

(0143) *E. coli* strain MYR993 with the genotype as provided in Table 2 was used a parental strain to generate strains with the deletion in either the *ubiX* gene or the *ubiD* gene. In constructing the *E. coli* strains with the deletion in *ubiX*, a kanamycin resistance cassette was amplified using primers MS608 and MS609 having 45 bp of homology to each end of the *ubiX* gene. In constructing the *E. coli* strains with the deletion in *ubiD*, a kanaymcin resistance cassette was amplified using primers MS604 and MS605 having 45 bp of homology to each end of the *ubiD* gene. The PCR products were column purified (QIAquick PCR Purification Kit, Qiagen) and used to transform the *E. coli* strain MYR993 (Table 2) using previously developed methods (Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA97: 6640–6645).to produce the *E. coli* stains with a deletion in either *ubiX* gene or *ubiD* gene (Table 2 - MYR993 ΔubiX and MYR993 ΔubiD). The deletion stains are expected to be impaired in respiration so glucose was added to LB selection plates to provide for fermentative growth.

(0144) The *E. coli* strains MYR993, MYR993 ΔubiX and MYR993 ΔubiD were grown as 25 ml cultures in 250 ml shake flasks at 250 rpm at 37^{O}C for 48 hours in a medium comprised of 5g K_2HPO_4, 3.5g KH_2HPO_4, 3.5g $(NH4)_2HPO_4$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, trace elements (1.6mg $FeCl_3 \cdot 6H_2O$, 0.2mg $CoCl_2 \cdot 6H_2O$, 0.1mg $CuCl_2 \cdot 2H_2O$, 0.2mg $ZnCl_2$, 0.2mg $Na_2MsO_4 \cdot 2H_2O$, 0.05mg H_3BO_3, 0.55mg $MnCl_2 \cdot 4H_2O$), and 0.2 M MOPS buffer (all per liter), using glucose as a carbon source. At the end of 48 hours of growth, the culture supernatants were analyzed for muconic acid and PCA content. As the results shown in Figure 20 indicates, the parental strain MYR993 accumulated primarily muconic acid in the culture medium with very little PCA while the *E. coli* strain MYR993ΔubiX accumulated only PCA and muconic acid was not detectable. On the other hand, the *E. coli* strain MYR993ΔubiD showed the reduced accumulation of both muconic acid and PCA. The conclusion was that UbiX protein is needed for AroY (PCA decarboxylase) activity.

Example 19

Comparison of activities of UbiX homologs in an in vitro assay

(0145) An in vitro assay was followed to compare the activities of UbiX and four of its homologs. In this in vitro assay the lysate from an *E. coli* strain over expressing AroY protein was combined with the lysate from another *E. coli* strain expressing UbiX protein or it homolog and the combined lysate was assayed for its ability to consume PCA as a substrate. In the muconic acid producing *E. coli* strain PCA is decarboxylated by AroY protein to yield catechol which in turn is converted into muconic acid by CatA protein. The decarboxylation activity of AroY protein is expected to be enhanced by the presence of UbiX or one of tis homolog and depending on the efficiency of UbiX or its homologs, the PCA in the assay solution will be consumed at different rate.

(0146) In this in vitro assay UbiX and four of its homologs namely KpdB coded by *kpdB* gene of *Klebsiella pnemoniae (kpdB)* (SEQ ID 42), Elw coded by the *elw* gene of *E. coli W* (SEQ ID 46), Kox coded by the *kox* gene of *Klebsiella oxytoca (kox)* (SEQ ID 48) and Lpl coded by *lpl* gene of *Lactobacillus plantarum (lpl)* (SEQ ID 50). The names for the last three homologs are simply provisional names given for this disclosure. AroY, UbiX, KpdB, Elw, Kox and Lpl were expressed from the strong constitutive Lambda Phage promoter P_R (SEQ ID3) on a low copy plasmid (SC101 origin of replication). The plasmids pCAT350 (SEQ ID 55) and pCP165 (SEQ ID 56) were used for gene cloning. In constructing AroY plasmid, the primers RP712 and RP714 were used to amplify the *aroY* gene and the primers MS461 and MS346 were used to amplify the pCAT350 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing the AroY protein. In constructing a KpdB plasmid, the primers RP731 and RP732 were used to amplify *kpdB* gene and the primers MS461 and MS346 were used to amplify the pCAT350 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing KpdB protein. In constructing a UbiX plasmid, the primers MS669 and MS666 were used to amplify *ubiX* gene and the primers MS461 and RP607 were used to amplify the pCAT350 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing UbiX protein. In constructing an Elw plasmid, the primers MS676 and MS680 were used to amplify *elw* gene and the primers MS461 and MS621 were used to amplify the pCP165 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing the Elw protein. In constructing a Kox plasmid, the primers MS686 and MS684 were used to amplify the *kox* gene and the primers MS461 and MS621 were used to amplify the

pCP165 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing Kox protein. In constructing an Lpl plasmid, the primers MS692 and MS691 were used to amplify the *lpl* gene and the primers MS461 and MS621 were used to amplify the pCP165 plasmid backbone. The resulting PCR products were ligated to obtain a plasmid overexpressing Lpl protein. All fragments contained 20 bp of homology to enable cloning using the NEBuilder HiFi DNA Assembly Cloning Kit and cloned into NEB5α *E. coli* cells (New England Biolabs).

(0147) Following plasmid cloning, an *in vitro* enzyme assay was developed to demonstrate AroY activity. 1 mL of an overnight LB grown culture was spun down and resuspended in 200 µL of Bacterial Protein Extraction Reagent (B-PER) (Thermo Fisher Scientific). After 5 minutes incubation in a rotary mixer, the cell debris was removed by centrifuging the samples at 13,000 rpm in a table top centrifuge. The clarified crude lysate supernatant was transferred to a new tube and stored on ice. 20 µL of an AroY overexpression lysate was combined with 20 µL of UbiX or a homolog lysate into a 150 µL reaction (final volume) containing 100 mM sodium phosphate buffer pH 6.4, 25 mM $MgCl_2$ and 1 mM protocatechuic acid (Sigma-Aldrich). The absorbance at 290 nm was read every minute for 60 minutes. The AroY activity was measured by monitoring disappearance of PCA at A290. The relative activities of the UbiX and its homologs are shown in Figure 21. All UbiX homologs tested improved AroY activity, but a wide variation of enzyme activity was produced depending on the specific homolog tested. The highest AroY activity was achieved using KpdB, while the lowest activity was observed from the *Lactobacillus* homolog. The wide range of activities shown can be employed to improve muconic pathway performance, as the highest activity may not always be optimal.

Example 20

Effect of kpdB expression level on the activity of AroY

(0148) Having established that that the expression of KpdB protein enhance the activity of AroY protein in the in vitro assay, efforts were made to determine if the level of expression of KpdB protein within the muconic acid producing biocatalysts would affect the level of muconic acid production. In this experiment, the muconic acid production strain MYR1305 was transformed

with various plasmids that express KpdB at different levels. Transformation of MYR1305 was conducted with three different plasmids. In the experimental control, MYR1305 was transformed with the control plasmid pCP140 without any genes coding for KpdB protein. The DNA sequence of pCP140 is given in SEQ ID 57. Briefly, pCP140 was constructed to express an *E. coli* codon-optimized *catAX* under the P15 promoter, *E. coli tktA* under native *E. coli* promoter, *E. coli aroB* under the P15 promoter, and *E. coli aroD* under P26 promoter (SEQ ID 2). The second plasmid pCP169 used to transform MYR1305 is a derivative of pCP140 additionally having the kpdB gene under the P26 promoter. The third plasmid pCP170 used to transform MYR1305 is a derivative of pCP140 additionally having kpdB under the *E. coli pgi* promoter (SEQ ID 52). Low level expression was achieved using the P26 promoter while high expression was achieved using the *E. coli pgi* promoter. pCP169 and pCP170 were constructed by first amplifying the pCP140 plasmid in two fragments using two sets of primers (PCR primers RP607 and RP677 for the first fragment and the PCR primers RP671 and RP664 for the second fragment). Two smaller PCR products facilitate easier plasmid construction than a single large PCR product. In constructing the plasmid pCP169, the P26 promoter was amplified using primers RP702 and RP783 and kpdB was amplified using primers RP781 and RP780. In constructing the plasmid pCP170, the *pgi* promoter was amplified using primers RP700 and RP784 and kpdB was amplified using primers RP779 and RP780. All PCR products had 20 bp homology overlaps to enable cloning using the NEBuilder HiFi DNA Assembly Cloning Kit. As shown in Figure 22, strains expressing *kpdB* produced higher levels of muconic acid than did the control strains without any exogenous *kpdB* gene expression. Additionally, the PCA accumulation was eliminated in the strains expressing exogenous *kpdB* gene. The level of muconic acid produced did not increase with the increased expression of *kpdB* gene suggesting a saturating level of activity was achieved even when the exogenous *kpdB* gene is expressed under low level of expression.

Example 21

Complementation of a *ppc* mutant and effect on muconic formation

(0149) The increased availability of PEP for muconic acid formation was investigated using the bacterial strains with the deletion of the phosphoenolpyruvate carboxylase (*ppc*) gene. The E.

coli strain MYR1674 was genetically engineered to use as a biocatalyst for muconioc acid production. MYR1674 is able to grow in minimal medium containing glucose as a source of carbon and energy and produce muconic acid. However, when the ppc gene is deleted from MYR1674, the resulting stain MYR1674 Δ*ppc* is not able to grow in minimal medium containing glucose and is viable only on rich media such as Luria Broth (LB). The loss of the ability of MYR1674 Δ*ppc* to grow on minimal medium can be regained by means of inserting the *pyc* gene coding for pyruvate carboxylase at the original *ppc* locus in the MYR1674 Δ*ppc* strain of E. coli.

(0150) The pyruvate carboxylase (*pyc*) gene from *Saccharomyces cerevisiae* (SEQ ID 53) was cloned using primers MS1383 and MS1384, containing flanking homology to the *E. coli ppc* promoter and terminator. In order to facilitate strong growth on minimal media, the strong constitutive rightward Lambda Phage promoter P_R was required. The P_R promoter was amplified using primers MS1429 and MS1430 and the resulting PCR product was used to replace the endogenous *ppc* promoter. The final nucleotide sequence for the Δ*ppc*:: P_R-*pyc* locus is shown in SEQ ID 58. The *S. cerevisiae pyc* gene was chosen because it is not closely related to any *E. coli* gene, and the expected lower expression due to differing codon usage could be beneficial in preserving PEP for muconic acid production. There are many organisms that contain pyruvate carboxylase, and any homologs or analogs having pyruvate carboxylase activity could be used. New strain MYR1772 derived from MYR1674 by integrating Δ*ppc*:: P_R-*pyc* at the original *ppc* locus was viable on a minimal medium confirming the functionality of the cloned *S. cerevisiae pyc* gene. MYR1772 and its parent MYR1674 strains were compared in shake flask experiments for their ability to produce muconic acid. As the results shown in Figure 23 indicates, MYR1772 strain produced higher titer of muconic acid production than parent MYR1674, demonstrating the advantage in replacing the endogenous phosphoenolpyruvate carboxylase enzyme with the exogenous pyruvate carboxylase enzyme.

Example 22

Measuring muconic acid production

(0151) The bacterial strains MYR814, MYR993, MYR1536, MYR1557, MYR1570, MYR1595, MYR1630, MYR1674 and MYR1772 were grown in shake flask cultures overnight and titer and

yield for muconic acid production were determined. In addition, the growth rate was determined by measuring the absorbance at 600 nm and the relative growth of various bacterial strains are provided in Table 8. The bacterial growth represented by "+++" indicates a growth similar to the growth seen in a wild type *E. coli* strain. The bacterial growth represented by "+" indicates poor growth. An intermediate growth is represented by "++". When a particular strain is showing a poor growth, that strain is subjected to 5 overnight transfers to improve the growth, each transfer produces approximately 10 generations or doublings.

(0152) The bacterial strains MYR814, MYR1570, MYR1630 and MYR1674 were grown in 7 liter fermenters in a fed-batch mode and the titer and yield for muconic acid production were determined (Table 9). The bacterial strains tested for muconic acid titer and yield produced very little byproducts. For example, the bacterial strain after 72 hours of fed batch fermentation showed only 0.08g/L of PCA and 0.07g/L of fumarate as byproducts.

Table 1. Bacterial strains used in the present invention	
Bacterial strain	Genotype / Description
ATCC8739	*Escherichia coli* "C" wild type
MYR34	ATCC8739 Δ*aroE*
MYR170	ATCC8739 Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*
MYR261	ATCC8739 Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, Δ*poxB::tktA*
MYR305	ATCC8739 Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, Δ*poxB*
MYR31	ATCC8739 Δ*ptsHI*, Δ*galP*
MYR217	ATCC8739 Δ*ptsHI*, Δ*galP*, Δ*tdc::glf-glk*, Δ*aroE*
MYR352	ATCC8739 Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, Δ*adhE::P*$_{15}$*-catAX*, P_R*-aroY*, P_{26}*-quiC*
RY903	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-893*
RY909	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-899*
RY911	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-901*
RY912	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroGwt*
RY913	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-893*, Δ*tyrR::kan*
RY919	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-899*, Δ*tyrR::kan*
RY921	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroG*20-901*, Δ*tyrR::kan*
RY922	Δ*aroE*, Δ*ackA::P*$_{15}$*aroB*, pMG37, *aroGwt*, Δ*tyrR::kan*

Table 2. Bacterial used in the present invention		
Strain Name	**Genotype/Description**	**Parent**
MYR802	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP aroE G105M	
MYR814	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP aroE G105M **[P15-catA-catX + tktA + P15-aroB + P26-aroD in pCL1921 backbone]**	MYR802
MYR993	ΔackA::P15-aroB ΔadhE::P_R-aroY + P26-quiC ΔpflB::P_R-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP aroE G105M	MYR802
MYR993 ΔubiX	ΔackA::P15-aroB ΔadhE::P_R-aroY + P26-quiC ΔpflB::P_R-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP aroE G105M ΔubiX::Kan^R	MYR993
MYR993 ΔubiD	ΔackA::P15-aroB ΔadhE::P_R-aroY + P26-quiC ΔpflB::P_R-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP aroE G105M ΔubiD::Kan^R	MYR993
MYR1305	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP ΔaroE Δzwf	MYR802
MYR1305 pCP140	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP ΔaroE Δzwf **[P_R-catA-catX + tktA + P15-aroB + P26-aroD in pCL1921 backbone]**	MYR1305
MYR1305 pCP169	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP ΔaroE Δzwf **[P_R-catA-catX + tktA + P15-aroB + P26-aroD + P26-kpdB in pCL1921 backbone]**	MYR1305
MYR1305 pCP170	ΔackA::P15-aroB ΔadhE::P15-catAX + P_R-aroY + P26-quiC ΔpflB::P15-catAX ΔtyrR aroG^{FBR} ΔpoxB::tktA ΔptsHI Δtdc::P26-glf-glk ΔgalP ΔaroE Δzwf **[P_R-catA-catX + tktA + P15-aroB + P26-aroD + P_{PGI}-kpdB in pCL1921 backbone]**	MYR1305
MYR1536	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX	MYR802
MYR1557	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX (Evolved version of MYR1536 for faster growth)	MYR1536
MYR1570	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX Δzwf (Evolved version of MYR1536 for faster growth)	MYR1536
MYR1595	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI::P_R-AroY Δ0039:: P_R-catAX Δzwf ΔpykF	MYR1557
MYR1630	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX Δzwf ΔpykF Δ2160:: P_R-aroG^{FBR}	MYR1595
MYR1674	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX Δzwf ΔpykF Δ2160:: P_R-aroG^{FBR} (Evolved version of MYR1630 for faster growth)	MYR1630
MYR1772	ΔackA::P_{acpp}-aroB ΔadhE::P15-qa4 ΔtyrR aroG^{FBR} ΔpoxB::P_R-tktA Δtdc:: P26-glf-glk ΔgalP::P15-ubiX ΔpflB:: P_R-CatAX P_{acpp}-aroD ΔmgsA::P_{rplU}-qa4 ΔptsHI:: P_R-aroY Δ0039:: P_R-catAX Δzwf ΔpykF Δ2160:: P_R-aroG^{FBR} Δppc:: P_R-pyc	MYR1674

Table 3. Plasmids used in the present invention	
Bacterial Plasmid	Genotype / Description
YEp24	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R
pCP32AMP	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R, *aroG*
pCP14	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R, P_{15}*aroB*
pCP54	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R, P_{15}*aroB*, *aroG*
pCP50	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R, *aroG*, *tktA*
pCP55	2μ yeast origin, *URA3*, Tc^R, pMB1 replicon, Ap^R, *aroG*, *aroB*, *tktA*
pCL1921	pSC101 replicon, Spc^R
pMG27	pSC101 replicon, Spc^R, P_R-*aroY*
pMG31	pSC101 replicon, Spc^R, P_{15}-*catAX*
pMG33	pSC101 replicon, Spc^R, P_{15}-*catAX*, P_R-*aroY*
pMG37	pSC101 replicon, Spc^R, P_{15}-*catA-CatX*, P_R-*aroY*, P_{26}-*quiC*
pMG39	pSC101 replicon, Spc^R, P_{26}-*quiC*
pMG47	pSC101 replicon, Spc^R, P_{15}-*catAX*, P_R-*aroY*, P_{26}-*asbF*
pMG70	pSC101 replicon, Spc^R, P_{15}-*catAX*, P_R-*aroY*, P_{26}-*qa-4*

Table 4. *aroG**mutant alleles that lead to resistance to phenylalanine feedback inhibition

Strain	Allele number	Nucleotide mutation	Amino acid mutation
RY893	*aroG*20-893*	C449T	Pro150Leu
RY897	*aroG*20-897*	C449T	Pro150Leu
RY899	*aroG*20-899*	T538C	Ser180Pro
RY901	*aroG*20-901*	C438T	Pro150Ser
MYR450	*aroG*111*	C55T	Pro19Ser
MYR451	*aroG*211*	G533A	Gly178Glu
MYR452	*aroG*212*	C540T	Ser180Phe
MYR453	*aroG*311*	Deletion from base pair 36 to 44bp	Deletion of Glu-Ile-Lys
MYR454	*aroG*312*	C632T	Ser211Phe
MYR455	*aroG*411*	T29A	Ile10Asn
MYR456	*aroG*412*	G533A	Gly178Glu
MYR457	*aroG*511*	C448T	Pro150Ser

Table 5. AroG activity measurement in crude extract from various recombinant *E. coli* strains			
Strain	*aroG** allele	Specific activity mU (One mU = one nM product made per milligram protein per minute)	% of activity resistant to phenylalanine
RY893	*aroG*20-893*	62	34
RY897	*aroG*20-897*	55	77
RY899	*aroG*20-899*	92	113
RY901	*aroG*20-901*	78	76
RY902	*aroG wild type*	38	6
RY890	*aroG wild type*	54	7

Table 6. Muconic acid production in shake flasks by strains containing feedback resistant aroG* alleles	
Strain	Muconic acid titer g/l
RY913	3.04
RY919	3.11
RY921	2.99
RY922	1.45

Table 7 Sequence Information		
No.	Name	Description
1	SEQ ID No. 1	The P_{15} promoter from *Bacillus subtilis* phage SP01, with a stem and loop added just downstream from the transcription start site.
2	SEQ ID No. 2	The P_{26} promoter from *Bacillus subtilis* phage SP01
3	SEQ ID No. 3	The P_R promoter from *Escherichia coli* phage
4	SEQ ID No. 4	Protein sequence of 3-dehydroshikimate dehydratase from *Neurospora crassa* encoded by the *qa-4* gene.
5	SEQ ID No. 5	Genomic DNA sequence of the *qa-4* gene from *Neurospora crassa* plus surrounding sequences.
6	SEQ ID No. 6	Protein sequence of 3dehydroshikimate dehydratase from *Aspergillus nidulans.* encoded by the *qutC* gene
7	SEQ ID No. 7	Genomic DNA sequence of the *qutC* gene from *Aspergillus nidulans* plus surrounding sequences
8	SEQ ID No. 8	Protein sequence of protocatechuate decarboxylase (AroY) from *Klebsiella pnemoniae* ATCC25597
9	SEQ ID No. 9	DNA sequence of the *aroY* gene of *Klebsiella pneumoniae* 342 plus 2 kilobases of surrounding DNA sequences
10	SEQ ID No. 10	DNA sequence of the *catA* gene from *Acinetobacter baylyi ADP1*, including 410 bases of upstream sequence and two open reading frames downstream
11	SEQ ID No. 11	Protein sequence of CatA (catechol 1,2-dioxygenase) from *Acinetobacter baylyi ADP1*
12	SEQ ID No. 12	DNA sequence of the *quiC* (3-dehydroshikimate dehydratase)gene from *Acinetobacter sp. ADP1*
13	SEQ ID No. 13	Codon-optimized DNA sequence of the *quiC* (3-dehydroshikimate dehydratase)gene from *Acinetobacter sp. ADP1*
14	SEQ ID No. 14	Protein sequence of QuiC (3-dehydroshikimate dehydrogenase from *Acinetobacter sp. ADP1*
15	SEQ ID No. 15	DNA sequence of the plasmid pAC21
16	SEQ ID No. 16	DNA sequence of the plasmid pAC19
17	SEQ ID No. 17	DNA sequence of the plasmid pMH17F
18	SEQ ID No. 18	DNA sequence of the coding region of the wild type *aroG* gene
19	SEQ ID No. 19	DNA sequence of the plasmid pMH28F

Table 7. Sequence Information – cont.		
No.	Name	Description
20	SEQ ID No. 20	DNA sequence of the plasmid pCL1921
21	SEQ ID No. 21	DNA sequence of the plasmid pMG27
22	SEQ ID No.2 2	DNA sequence of the plasmid pMG31
23	SEQ ID No. 23	DNA sequence of the plasmid pMG33
24	SEQ ID No. 24	DNA sequence of the plasmid pMG37
25	SEQ ID No. 25	DNA sequence of the plasmid pMG39
26	SEQ ID No. 26	DNA sequence of the plasmid pMG47
27	SEQ ID No. 27	DNA sequence of the plasmid pMG70
28	SEQ ID No. 28	DNA sequence of the plasmid pCP32AMP
29	SEQ ID No. 29	DNA sequence of the plasmid pCP14
30	SEQ ID No. 30	DNA sequence of the plasmid pCP50
31	SEQ ID No. 31	DNA sequence of the plasmid pCP54
32	SEQ ID No. 32	DNA sequence of the plasmid pCP55
33	SEQ ID No. 33	DNA sequence of the plasmid YEP24
34	SEQ ID No. 34	DNA sequence of the deleted *aroE* region
35	SEQ ID No. 35	DNA sequence of the integrated cassette $\Delta ack::P_{15}aroB$
36	SEQ ID No. 36	DNA sequence of the $\Delta poxB$ region
37	SEQ ID No. 37	DNA sequence of the integrated cassette $\Delta poxB::tktA$
38	SEQ ID No. 38	DNA sequence of the $\Delta ptsHI$ region
39	SEQ ID No. 9	DNA sequence of the integrated cassette $\Delta tdc::glf\text{-}glk$
40	SEQ ID No. 40	DNA sequence of the $\Delta galP$ region
41	SEQ ID No. 41	MYR352 $\Delta adhE::P_{15}\text{-}catAX$, $P_R\text{-}aroY$, $P_{26}\text{-}quiC$
42	SEQ ID No. 42	Nucleotide sequence of *Klebsiella pneumoniae kpdB* gene.
43	SEQ ID No. 43	Amino acid sequence of KpdB protein of *Klebsiella pneumoniae*

Table 7 Sequence Information – cont.		
No.	Name	Description
44	SEQ ID No. 44	Nucleotide sequence of *Escherichia coli ubiX* gene
45	SEQ ID No. 45	Amino acid sequence of UbiX protein of *Escherichia coli.*
46	SEQ ID No. 46	Nucleotide sequence of *Escherichia coli Wstrain elw* gene
47	SEQ ID No. 47	Amino acid sequence of Elw protein of *Escherichia coli W strain.*
48	SEQ ID No. 48	Nucleotide sequence of *Klebsiella oxytoca kox* gene
49	SEQ ID No. 49	Amino acid sequence of Kox protein of *Klebsiella oxytoca.*
50	SEQ ID No. 50	Nucleotide sequence of *Lactobacillus plantarum lpl* gene
51	SEQ ID No. 51	Amino acid sequence of Lpl protein of *Lactobacillus plantarum.*
52	SEQ ID No. 52	Nucleotide sequence of *Pgi* promoter
53	SEQ ID No. 53	Nucleotide sequence of *Saccharomyces cerevisiae pyc* gene
54	SEQ ID No. 54	Amino acid sequence of Pyc protein of *Saccharomyces cerevisiae.*
55	SEQ ID No. 55	DNA sequence of the plasmid pCAT350
56	SEQ ID No. 56	DNA sequence of the plasmid pCP165
57	SEQ ID No. 57	DNA sequence of the plasmid pCP140
58	SEQ ID No. 58	Nucleotide sequence of Δppc::P_R-*pyc*
59	SEQ ID No. 59	Nucleotide sequence of acpP promoter
60	SEQ ID No. 60	Nuclotide sequence of rplU promoter

Table 7 Sequence Information – cont.		
SEQ ID No.	Primer Name	Sequence
61	MS604	AACGCCGTATAATGGGCGCAGATTAAGAGGCTACAGTGGGCTTACATGGCGATAGCTAGA
62	MS605	TGTCGGATCGATAAATAGGGCAAAACAAACGCGCATCCCGGAAAACGATTCCGAAGCCCA
63	MS608	AAAGTCTGCCTGCAAGTCTGACAGGGCAACTATTTGTGGGCTTACATGGCGATAGCTAGA
64	MS609	TTGCAAAATTGCCCTGAAACAGGGCAACAGCGGAGTCCCGGAAAACGATTCCGAAGCCCA
65	MS461	GGCTATATTCCTTATCTAGATTAGT
66	MS346	GTCTGACAGGTGCCGGATTTCATAT
67	RP712	TCTAGATAAGGAATATAGCCATGACCGCACCGATTCAGGATCTGC
68	RP714	AAATCCGGCACCTGTCAGACTTATTTTGCGCTACCCTGGTTTTTT
69	RP731	CATGTACTAATCTAGATAAGGAATATAGCCATGAAACTGATTATTGGGATGACGGGGGCC
70	RP732	GCCGGATATGAAATCCGGCACCTGTCAGACTTATTCGATCTCCTGTGCAAATTGTTCTGC
71	MS669	TCTAGATAAGGAATATAGCCATGAAACGACTCATTGTAGGCATCA
72	MS666	ACCGAACAGGCTTATGTCCAGATAGCAGGTATAGCGGTTGAATCG
73	RP607	TGGACATAAGCCTGTTCGGTTCGT
74	MS621	TTAGATTTGACTGAAATCGTACAGT
75	MS676	TCTAGATAAGGAATATAGCCATGAAACTGATCGTCGGGATGACAG
76	MS680	ACGATTTCAGTCAAATCTAATTATTCATTCTCCTGAGAAAAATTC
77	MS686	TCTAGATAAGGAATATAGCCATGACGGCACGCATCATCATTGGTA
78	MS684	ACGATTTCAGTCAAATCTAATTAATTAAAACGTAGCTCGCCTTCA
79	MS692	TCTAGATAAGGAATATAGCCATGAAACGAATTGTTGTGGGAATCA
80	MS691	ACGATTTCAGTCAAATCTAATTAATCCCCCTCCCAACGGCGATCA
81	RP677	CGACGTTGTAAAACGACGGCCAGTG
82	RP671	TTAATCGCCTTGCAGCACATCCCCC
83	RP664	ACGAACCGAACAGGCTTATGTCCA
84	RP702	GCCGTCGTTTTACAACGTCGGATCCGCCTACCTAGCTTCCAAGAA
85	RP783	CCTACAATGAGTCGTTTCATTAGGTTTTCCTCAACCCGGGAGCGT
86	RP781	CCCGGGTTGAGGAAAACCTAATGAAACGACTCATTGTAGGCATCA
87	RP780	ATGTGCTGCAAGGCGATTAAGATAGCAGGTATAGCGGTTGAATCG
88	RP700	GCCGTCGTTTTACAACGTCGAGCGGGGCGGTTGTCAACGATGGGG
89	RP784	CCTACAATGAGTCGTTTCATGGCTATATTCCTCCTCTGCATGAGA
90	RP779	TGCAGAGGAGGAATATAGCCATGAAACGACTCATTGTAGGCATCA
91	MS1383	GATGGGGTGTCTGGGGTAATATGTCGCAAAGAAAATTCGCCGGCT
92	MS1384	GGGTTTGCAGAAGAGGAAGATCATGCCTTAGTTTCAACAGGAACT
93	MS1429	ATTCCTGCTATTTATTCGTTCGTTAAATCTATCACCGCAAGGGAT
94	MS1430	GCGAATTTTCTTTGCGACATGGCTATATTCCTTATCTAGATTAGT

Table 8 Titer and yield for muconic acid production and growth of various *E. coli* strains in shake flask cultures			
Strain Name	Titer (grams muconic acid/liter)	Yield (grams muconic acid/gram glucose)	Growth
MYR814	2	0.16	+++
MYR993	1.9	0.15	+++
MYR1536	2.7	0.22	+
MYR1557	3	0.25	+++
MYR1570	4.5	0.38	++
MYR1595	4.5	0.38	++
MYR1630	4.8	0.4	+
MYR1674	3.8	0.32	+++
MYR1772	5	0.42	+++

Table 9 Titer and yield for muconic acid production for various *E. coli* strains in fed batch cultures			
Strain Name	Titer (grams muconic acid/liter)	Yield (grams muconic acid/gram glucose)	Time (hours)
MYR814	30.9	0.20	48
MYR1570	49.0	0.36	48
MYR1630	58.3	0.47	48
MYR1630	69.5	0.42	72
MYR1674	81.5	0.43	72

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What is claimed is:

1. A genetically engineered microorganism that produces muconic acid from a non-aromatic carbon source comprising at least one exogenous gene encoding for 3,4-dihydroxybenzoic acid decarboxylase and at least one gene that codes for a protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase.

2. The genetically engineered microorganism of claim 1, wherein said genetically engineered microorganism produces at least 60 g/L of muconic acid in 72 hours.

3. The genetically engineered microorganism of claim 1 or claim 2, wherein said genetically engineered microorganism is a bacterium.

4. The genetically engineered microorganism of claim 1, wherein said genetically engineered microorganism is *Escherichia coli.*

5. The genetically engineered microorganism of claim 1, wherein said protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase is a KpdB protein encoded by a *kpdB* gene or a homolog thereof, and wherein said KpdB protein or homolog thereof has at least 25% amino acid identity to said KpdB protein.

6. The genetically engineered microorganism of claim 1, wherein said protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase is a UbiX protein encoded by a *ubiX* gene or a homolog thereof, and wherein said UbiX protein or homolog thereof has at least 25% amino acid identity to said UbiX protein.

7. The genetically engineered microorganism of claim 1, further comprising one or more exogenous genes selected from the group consisting of *aroZ*, *qa-4*, *asbF*, *quiC* and *catAX*.

8. The genetically engineered microorganism of claim 1, further comprising one or more exogenous genes selected from the group consisting of *aroB*, *aroD*, *aroF*, *aroG*, *aroH*, *tktA*, *talB*, *rpe*, and *rpi*, wherein said one or more exogenous genes encode one or more proteins that function in a shikimic acid pathway.

9. The genetically engineered microorganism of claim 1, further comprising at least one exogenous gene encoding a pyruvate carboxylase and a mutation of the phosphoenol pyruvate carboxylase gene.

10. A genetically engineered *Escherichia coli* strain that produces muconic acid from a non-aromatic carbon source comprising at least one exogenous gene encoding for 3,4-dihydroxybenzoic acid decarboxylase and at least one gene that codes for a protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase, wherein said genetically engineered *Escherichia coli* strain produces at least 60 g/L of muconic acid in 72 hours.

11. A genetically engineered *Escherichia coli* strain that produces muconic acid from a non-aromatic carbon source comprising at least one exogenous gene encoding for 3,4-dihydroxybenzoic acid decarboxylase and at least one gene that codes for a protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase, wherein said genetically engineered *Escherichia coli* strain produces at least 80 g/L of muconic acid in 72 hours.

12. The genetically engineered *Escherichia coli* strain of claim 10, wherein said protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase is a KpdB protein encoded by a *kpdB* gene or a homolog thereof, and wherein said KpdB protein or homolog thereof has at least 25% amino acid identity to said KpdB protein.

13. The genetically engineered *Escherichia coli* strain of claim 10, wherein said protein that increases the activity of said 3,4-dihydroxybenzoic acid decarboxylase is a UbiX protein encoded by a *ubiX* gene or a homolog thereof, and wherein said UbiX protein or homolog thereof has at least 25% amino acid identity to said UbiX protein.

14. The genetically engineered *Escherichia coli* strain of claim 10, further comprising one or more exogenous genes selected from the group consisting of *aroZ*, *qa-4*, *asbF*, *quiC* and *catAX*.

15. The genetically engineered *Escherichia coli* strain of claim 10, further comprising at least one exogenous gene encoding a pyruvate carboxylase and a mutation of the phosphoenol pyruvate carboxylase gene.