In contrast to other studies with *E. coli* (Table 1), the studies reported herein have focused on the development of strains that are able to convert high level of sugars into succinate using mineral salts medium to minimize the costs of materials, succinate purification, and waste disposal.

(011) One aspect of the invention provides various strains of *E. coli*, that produce succinate at high titers and yields in mineral salts media during simple, pH-controlled, batch fermentations without the need for heterologous genes or plasmids. The inventors have surprisingly identified a number of target genes useful in genetic manipulation of biocatalysts for achieving high efficiency for succinic acid production.

BRIEF SUMMARY ~~OF THE INVENTION~~

(012) It is an objective of the present ~~invention~~embodiments disclosed herein to provide a method for obtaining a biocatalyst for manufacturing succinic acid using biological feedstocks. The method for obtaining the biocatalysts for succinic acid production from biological feedstocks combines rational genetic manipulations and the process of metabolic evolution.

(013) In generating a biocatalysts for succinic acid production from biological feedstocks, using a rational design derived from our existing knowledge about the microbial metabolic pathways, a set of genes within the bacterial chromosome are inactivated followed by the process of metabolic evolution to select a strain with desirable phenotype.

(013a) In one aspect, there is provided an *Escherichia coli* bacterial cell comprising increased levels of phosphoenol pyruvate carboxykinase (*pck*) gene transcripts and one or more genetic modifications in one or more genes selected from the group consisting of *ptsG*, *ptsH* and *ptsI*, said transcripts encoding active phosphoenol pyruvate carboxykinase (PCK), said cell exhibiting increased PCK activity and wherein said one or more genetic modifications inactivate the function of the gene product encoded by *ptsG*, *ptsH* and *ptsI* and is selected from deleting the entire coding region of the gene, deleting a portion of the coding region of the gene, introducing a frame shift mutation in the coding region of the gene, introducing a missense mutation in the gene, insertion or introducing a stop codon in the coding region of the gene or combinations thereof.

(013b) In another aspect, there is provided a genetically modified *Escherichia coli* bacterial cell, wherein said genetically modified bacterial cell is NRRL B-50279, NRRL B-50280, or NRRL B-50281.

(014) In one ~~aspect~~embodiment of the present ~~invention~~disclosure, the mutation of the genes in the bacterial chromosome is accomplished without introducing any exogenous genetic material.

(015) In another ~~aspect~~embodiment of the present ~~invention~~disclosure the mutation of the endogenous genes are accomplished either by introducing point mutation or by introducing a stop codon in the open reading frame of the endogenous gene.

(016) In another ~~aspect~~embodiment of the present ~~invention~~disclosure the entire open reading frame of the endogenous gene is deleted from the chromosomal DNA.

(017) In certain preferred embodiments of the ~~invention~~disclosure, the expression of certain endogenous genes is significantly increased. In one ~~aspect~~embodiment of the present ~~invention~~disclosure, the transcription of the endogenous gene is increased by means of introducing certain mutations in the promoter region of the endogenous genes. In other ~~aspect~~embodiment of the ~~invention~~disclosure, the transcription of the endogenous gene is enhanced by means of reliving the repressive control of the target gene.

(018) In certain ~~aspect~~embodiment of the present ~~invention~~disclosure, an exogenous nucleotide sequence may be introduced to inactivate a target gene for the purpose of selecting a bacterial strain with a mutated gene with desirable phenotype. In the most preferred ~~aspect~~embodiment of the present ~~invention~~disclosure, the exogenous nucleotide sequence introduced into the microbial genome is subsequently removed in a seamless fashion without leaving behind any residual exogenous nucleotide sequence.

(019) The rationally designed genetic manipulations can all be done in a single stage or in multiple stages in which a single genetic change is accomplished at one time. The microbial strain resulting from genetic manipulations may be subjected to metabolic evolution in order to improve the yield, titer and volumetric productivity of the desired organic acid. In the most preferred ~~aspect~~embodiment of the present ~~invention~~disclosure, the rational genetic manipulations are done in stages and the process of metabolic evolution is carried out in between the stages of genetic manipulation.

(020) In one embodiment of the present ~~invention~~disclosure, the spontaneous mutations that occur during the metabolic evolution are identified through sequencing appropriate regions of the chromosomal DNA. In yet another embodiment of the present ~~invention~~disclosure, the mutations that occur during the metabolic evolution are identified by measuring the activities of suspect enzyme.

(021) In one embodiment of the present ~~invention~~disclosure, based on rational designing, one or more of the genes coding for the proteins known to function in the fermentative pathways are inactivated through one or more mutations.

(022) It yet another ~~aspect~~embodiment of the present ~~invention~~disclosure, the genes which are functional homologues of the genes coding for the proteins functioning in the fermentative pathway are inactivated beside the genes coding for the proteins directly involved in the fermentative pathway.

(023) In one embodiment of the present ~~invention~~disclosure the genes functioning within the TCA cycle are genetically manipulated so that there is an increased flow of carbon towards succinic acid production. In one ~~aspect~~embodiment of the present ~~invention~~disclosure, the carbon flow through reductive arm of the TCA cycle is enhanced. In another ~~aspect~~embodiment of the present ~~invention~~disclosure, the carbon flow through oxidative arm of the TCA cycle is genetically manipulated. In another ~~aspect~~embodiment of the present ~~invention~~disclosure, the carbon flow to succinic acid is improved through genetic manipulation of glyoxalate bypass pathway closely associated with TCA cycle.

(024) In yet another embodiment of the present ~~invention~~disclosure, the carbon flow from TCA to other metabolic pathways within the cell is blocked so that the carbon pool within the cell is funneled towards the succinic acid production.

(025) In another embodiment of the present ~~invention~~disclosure, the carbon flow into the TCA cycle is enhanced through genetic manipulation leading to one of more carboxylating enzymes within the cell. In one ~~aspect~~embodiment of the present ~~invention~~disclosure, the expression of one or more carboxylating enzyme is achieved through the genetic manipulation of the promoter region or by relieving the repression of the gene expression.

(026) In another embodiment of the present ~~invention~~disclosure, the phosphoenol pyruvate pool within the cell is conserved by mutating those genes involved in the carbon uptake pathway requiring phosphoenol pyruvate. In one ~~aspect~~embodiment of the present ~~invention~~disclosure, the phosphotransferase system for carbon uptake is inactivated in order to conserve the phosphoenol pyruvate available for the operation of the TCA cycle.

(027) The present ~~invention~~disclosure illustrates a number of targets that can be genetically manipulated to achieve an increased succinic acid production. All these various targets described in the present ~~invention~~disclosure can be genetically manipulated to achieve an improved

succinic acid production. In the most preferred embodiment, a minimum number of targets are selected for genetic manipulation to achieve a desirable rate of succinic acid production.

(028) In the preferred embodiment of the present ~~invention~~disclosure, the biocatalysts are selected for their ability to produce succinic acid at high titer, yield and volumetric productivity. In the most preferred ~~aspect~~embodiment of the present ~~invention~~disclosure, a biocatalyst capable of producing at least 1.0 mole of succinic acid for every one mole of carbon source consumed is preferred.

(029) In another most preferred embodiment of the present ~~invention~~disclosure, the biocatalyst is selected during metabolic evolution for its ability to produce at least 1.0 mole of succinic acid for every mole of carbon source consumed in a mineral salt medium and coupling the succinic acid production to microbial growth.

(030) In yet another embodiment of the present ~~invention~~disclosure, biocatalysts capable of producing succinic acid using glycerol as a feed stock are provided.

(031) Additional advantage of this ~~invention~~disclosure will become readily apparent from the ensuing detailed description of the ~~invention~~disclosure and the examples provided.

BRIEF DESCRIPTION OF THE DRAWINGS

(032) **Figures 1A-1B.** Fermentation of glucose to succinate. **Fig. 1A** shows the standard pathway for fermentation of glucose by *E. coli*. This pathway has been redrawn from Unden and Kleefeld (2004). Bold arrows represent central fermentative pathways. Crosses represent the gene deletions performed in this study to engineer KJ012 (*ldhA, adhE, ackA*). Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate-formate lyase; *focA*, formate transporter; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *adhE*, alcohol dehydrogenase; *ppc*, phosphoenolpyruvate carboxylase; *pdh*, pyruvate dehydrogenase complex; *gltA*, citrate synthase; *mdh*, malate dehydrogenase; *fumA*, *fumB*, and *fumC*, fumarase isozymes; *frdABCD*, fumarate reductase; *fdh*, formate dehydrogenase; *icd*, isocitrate dehydrogenase; *acs*, acetyl~CoA synthetase; *mgsA*, methylglyoxal synthase; *poxB*, pyruvate oxidase; *aldA*, aldehyde dehydrogenase; and *aldB*, aldehyde dehydrogenase. **Fig. 1B** shows the coupling of ATP production and growth to succinate production in engineered strains of