redox buffers, *e.g.* glutathione and/or ascorbate, cell-free systems may require engineering to provide for such regulation and are particularly suitable for such engineering and control.

[0008] It is desirable to manipulate parameters that influence the metabolic flux rates of key metabolites and pathways during cell-free biosynthetic reactions in order to optimize conditions that influence system performance, which parameters may reflect a balance between utilization of a carbon source, such as glucose, through different pathways and may also optimize ATP production in relation to NADH, and NADPH production. The present invention addresses this issue.

SUMMARY OF THE INVENTION

[0009] Compositions and methods are provided for monitoring and controlling metabolic flux rates in a cell-free system comprising a complex set of enzymes, during biosynthesis of a desired product of a pathway of interest. Thus, in one aspect the present invention refers to a method of controlling metabolic flux rate in a cell-free system comprising a complex set of enzymes, to produce a desired product of a pathway of interest, the method comprising: taking measurements of at least one metabolic parameter selected from: an adenine metabolite, adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), carbon level, energy level, O_2 consumption rate, dissolved O_2 concentration, and pH value, to measure metabolic performance in the cell free system; and adjusting metabolic flux rate in the cell free system ~~performance~~ based on the measurements by performing one or more steps selected from~~comprising~~: (i) increasing glucose-6 phosphate dehydrogenase activity levels, phosphoglucose isomerase activity~~altering enzyme~~ levels in the cell-free system; (ii) altering feed rate of a substrate that controls redox flux or carbon flux to the cell-free system; (iii) ~~altering~~ increasing O_2 addition to the cell-free system; and (iv) controlling efficiency of electron transport system by altering leakage across a membrane; wherein enzymes present in the pathway of interest catalyze production of the desired product, and wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of (a) NADPH and ribulose-5-phosphate, the enzyme activity of glucose-6 phosphate dehydrogenase is increased; (b) ribulose-5-phosphate without increased NADPH, the enzyme activity of glucose-6 phosphate dehydrogenase and transhydrogenase is increased, O_2 is increased, and proton leakage is increased; (c) pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased, O_2 is increased, and proton leakage is increased; and (d) ATP and pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased; and O_2 is increased. Generally, the methods described herein monitor key metabolic parameters of central metabolism, which parameters may include, without limitation, concentrations of NADP(H); NAD(H); ATP; ribulose-5-phosphate; consumption of a carbon source, such as glucose; and O_2 consumption. For the pathway of interest, desired target levels of one or more of the metabolic parameters are determined, for example through empirical screening methods, or deduction from known metabolic pathway equations. By monitoring the cell-free system for these key metabolic parameters during biosynthesis, and determining the deviation from desired levels, information is obtained regarding the metabolic state of the system. Adjusting metabolic performance based on the measurements is performed by one or more steps comprising: (i) altering enzyme levels in the cell-free system; (ii) altering feed rate of a substrate that controls redox flux or carbon flux to the cell-free system; (iii) altering O_2 addition to the cell-free system; (iv) controlling efficiency of electron transport system by altering leakage across a membrane; wherein enzymes present in the pathway of interest catalyze production of the desired product.

[0010] Some of the key metabolic parameters of interest for the methods described herein relate to central metabolism, including the pathways for glycolysis and pentose shunt; oxidative phosphorylation; and the redox flux, *e.g.* between NAD, NADH, NADP and NADPH, for example as diagrammed in *Figure* 1.

CLAIMS

1. A method of controlling metabolic flux rate in a cell-free system comprising a complex set of enzymes, to produce a desired product of a pathway of interest, the method comprising:

taking measurements of at least one metabolic parameter selected from: an adenine metabolite, adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), carbon level, energy level, O_2 consumption rate, dissolved O_2 concentration, and pH value, to measure metabolic performance in the cell-free system; and

adjusting metabolic flux rate in the cell free-system based on the measurements by performing one or more steps selected from: (i) increasing glucose-6 phosphate dehydrogenase activity levels, phosphoglucose isomerase activity levels and/or transhydrogenase activity levels in the cell-free system; (ii) altering feed rate of a substrate that controls redox flux or carbon flux to the cell-free system; (iii) increasing O_2 addition to the cell-free system; and (iv) controlling efficiency of electron transport system by altering leakage across a membrane;

wherein enzymes present in the pathway of interest catalyze production of the desired product, and

wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of (a) NADPH and ribulose-5-phosphate, the enzyme activity of glucose-6 phosphate dehydrogenase is increased; (b) ribulose-5-phosphate without increased NADPH, the enzyme activity of glucose-6 phosphate dehydrogenase and transhydrogenase is increased, O_2 is increased, and proton leakage is increased; (c) pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased, O_2 is increased, and proton leakage is increased; and (d) ATP and pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased; and O_2 is increased.

2. The method of Claim 1, wherein the adenine metabolite is a nicotinamide adenine dinucleotide.

3. The method of Claim 2, wherein the nicotinamide adenine dinucleotide is one or more of NAD, NADH, NADP and NADPH.

4. The method of Claim 1 wherein dissolved oxygen concentration and pH are continuously monitored and controlled.

5. The method of Claim 1, wherein the step of increasing activity levels comprises addition of the enzyme to the cell-free system.

6. The method of Claim 1, wherein the step of increasing activity levels comprises addition of a coding sequence for said enzyme to the cell-free system, wherein the coding sequence is translated.

7. The method of Claim 1, wherein the step of altering leakage across a membrane comprises addition of dinitrophenol to said cell-free system.

8. The method according to any one of Claims 1-7, wherein the cell-free system comprises a microbial cell lysate.

9. The method of Claim 8, wherein the microbial cell lysate is utilized without fractionation.

10. The method of any one of Claims 1-9, wherein the yield of the product is at least five-fold the basal rate of a native host cell.

11. The method of Claim 10, wherein the yield of the product is at least ten-fold the basal rate of a native host cell.

12. The method of any one of Claims 1-11, wherein the efficiency of converting the substrate into the product is increased by 5% or more relative to the basal conversion efficiency of the native host cell.

13. The method of Claim 12, wherein the efficiency of converting the substrate into the product is increased by 10% or more relative to the basal conversion efficiency of the native host cell.

14. The method of any one of Claims 1-13, wherein a desired target level of the at least one metabolic parameter is determined prior to taking measurements of the at least one metabolic parameter.

15. The method of Claim 14, wherein a deviation from the desired target level is determined after taking measurements of the at least one metabolic parameter and before adjusting metabolic flux rate in the cell free-system.

CLAIMS

1. A method of controlling metabolic flux rate in a cell-free system comprising a complex set of enzymes, to produce a desired product of a pathway of interest, the method comprising:

taking measurements of metabolic performance;

adjusting metabolic performance based on the measurements by performing one or more steps comprising: (i) altering enzyme levels in the cell-free system; (ii) altering feed rate of a substrate that controls redox flux or carbon flux to the cell-free system; (iii) altering O_2 addition to the cell-free system; (iv) controlling efficiency of electron transport system by altering leakage across a membrane;

wherein enzymes present in the pathway of interest catalyze production of the desired product.

2. The method of Claim 1, wherein the measurement of metabolic performance comprises measurement of an adenine metabolite.

3. The method of Claim 2, wherein the adenine metabolite is a nicotinamide adenine dinucleotide.

4. The method of Claim 3, wherein the nicotinamide adenine dinucleotide is one or more of NAD, NADH, NADP and NADPH.

5. The method of Claim 1, wherein the measurement of metabolic performance comprises measurement of ATP or ADP.

6. The method of Claim 1 wherein dissolved oxygen concentration and pH are continuously monitored and controlled.

7. The method of Claim 1, wherein the step of altering enzymes in the cell-free system comprises increasing activity of glucose-6 phosphate dehydrogenase.

8. The method of Claim 1, wherein the step of altering enzymes in the cell-free system comprises increasing activity of phosphoglucose isomerase.

9. The method of Claim 1, wherein the step of altering enzymes in the cell-free system comprises increasing transhydrogenase activity.

10. The method of any one of claims 7-9, wherein the step of increasing activity comprises addition of the enzyme to the cell-free system.

11. The method of any one of claims 7-9, wherein the step of increasing activity comprises addition of a coding sequence for said enzyme to the cell-free system, wherein the coding sequence is translated.

12. The method of Claim 1, wherein O_2 is increased in response to said taking measurements.

13. The method of Claim 1, wherein the step of altering leakage across a membrane comprises addition of dinitrophenol to said cell-free system.

14. The method of Claim 1, wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of NADPH and ribulose-5-phosphate, the enzyme activity of glucose-6 phosphate dehydrogenase is increased.

15. The method of Claim 1, wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of ribulose-5-phosphate without increased NADPH, the enzyme activity of glucose-6 phosphate dehydrogenase and transhydrogenase is increased; O_2 is increased; and proton leakage is increased.

16. The method of Claim 1, wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased; O_2 is increased; and proton leakage is increased.

17. The method of Claim 1, wherein when the measurements indicate the metabolic performance would benefit from increased concentrations of ATP and pyruvate derivatives, the enzyme activity of glucose phosphate isomerase is increased; and O_2 is increased.

18. The method according to any one of Claims 1-17, wherein the cell-free system comprises a microbial cell lysate.

19. The method of Claim 18, wherein the microbial cell lysate is utilized without fractionation.