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 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. Generally, ~~T~~the methods ~~of the invention~~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 ~~of the invention~~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.

[0011] Various methods may be employed to alter the metabolic flux rate. In some ~~embodiments~~examples, exogenous enzymes involved in redox flux pathways are provided to the reaction mixture as required in order to achieve the desired redox balance, either in the form of protein or in the form of a coding sequence for the protein. In other ~~embodiments~~examples, the

microbial cell utilized in the initial reaction mixture is genetically modified to alter the expression and/or composition of enzymes involved in redox flux pathways in order to provide an optimized initial condition for the reactions. In other ~~embodiments~~examples, targeted enzymes are engineered to comprise a unique recognition sequence for proteolytic cleavage, so that enzyme activity can readily be reduced if necessary. As these enzymes are involved in central metabolism, it may be necessary to modulate expression in a manner that does not affect the growing cells, *e.g.* by relocation or secretion of the enzyme.

[0012] In the methods described herein~~of the invention~~, a microbial cell, which may be genetically modified or may be a wild-type cell, is grown to a desired density, then lysed and the lysate, which may be a crude lysate, is combined with substrate(s) and an energy source if needed during a production phase, and incubated for a period of time sufficient to generate desired product of a pathway of interest. Additional substrate, nutrients, cofactors, buffers, reducing agents, and/or ATP generating systems, may be added to the cell-free system. Genetic modifications of interest to the microbial cell include the introduction of heterologous enzymes to provide for non-native enzymatic activities, and may further include deletion or down-regulation of undesirable enzyme activity; as well as enhancement or upregulation of native enzymes. During the production phase, at least one and preferably two or more key metabolic parameters are monitored, where the monitoring may be continuous or intermittent. Based on the targeted levels of key metabolic parameters, which may be pre-determined target levels, the metabolic performance is adjusted as described above.

[0013] In some ~~embodiments~~examples, methods are provided for producing a product of interest at a high flux rate, the method comprising: growing cells; lysing the cells; and producing the product of the pathway in a cell-free system comprising the lysate, where metabolic flux rates of key parameters are monitored and controlled.

[0014] In some ~~embodiments~~examples, a metabolic parameter for monitoring and adjusting is the concentration of a nicotinamide adenine dinucleotide, for example one or more of NAD, NADH, NADP and NADPH. In some examples~~embodiments~~, a metabolic parameter for monitoring and adjusting is the concentration of ATP or ADP. In some examples~~embodiments~~, a metabolic parameter for monitoring and adjusting is the dissolved O_2 concentration.

[0015] In some examples~~embodiments~~, activity of one or more enzymes selected from glucose-6 phosphate dehydrogenase, glucose phosphate isomerase, and transhydrogenase is adjusted in response to metabolic parameter monitoring.

[0016] In some examples~~embodiments~~ a compound that increases leakage of electrons in added to the cell-free system, *e.g.* a protonophore may be added, such as 2,4-dinitrophenol (DNP);