ii) culturing the transformed *E. coli* cell under conditions that permit the overexpression of the DsbC protein to an intracellular concentration of at least 1 mg/ml.

[0010a] In a sixth aspect, there is provided a method of improving the expression levels of biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial S30 extract prepared from *E.coli* that expresses an exogenous disulfide isomerase and an exogenous prolyl isomerase at a total concentration of at least 1 g/liter of extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

ii) incubating the bacterial cell free synthesis system under conditions permitting the expression of the protein of interest to a concentration of at least 100 mg/L,

wherein the bacterial extract has an active oxidative phosphorylation system and comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, and the exogenous disulfide isomerase and the exogenous prolyl isomerase, wherein the bacteria from which the extract is prepared expresses at least one of the exogenous disulfide isomerase and the exogenous prolyl isomerase from a gene operably linked to a constitutive or inducible promoter, where the exogenous disulfide isomerase is DsbC, and the exogenous prolyl isomerase is FkpA.

[0010b]In a seventh aspect, there is provided a bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous disulfide isomerase and an exogenous prolyl isomerase, wherein the exogenous disulfide isomerase and the exogenous prolyl isomerase were expressed in the bacteria from which the extract was prepared at a concentration of at least 1 g/liter of extract; and

ii) a nucleic acid encoding a protein of interest,

where said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L,

wherein the bacteria from which the extract is prepared expresses at least one of the exogenous disulfide isomerase and the exogenous prolyl isomerase from a gene operably linked to a constitutive or inducible promoter, and

where the exogenous disulfide isomerase is DsbC, and the exogenous prolyl isomerase is FkpA.

[0010c] In an eight aspect, there is provided a method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial extract with a nucleic acid encoding a protein of interest; and

ii) incubating the bacterial extract with the nucleic acid under conditions permitting the expression and proper folding of the protein of interest,

wherein the bacterial extract comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, wherein the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least about 1 g/liter in the extract, and wherein the bacteria from which the extract is prepared expresses at least one of the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and

the exogenous protein disulfide isomerase is DsbC, and the exogenous peptidyl-prolyl cis-trans isomerase is FkpA.

[0010d] In a ninth aspect, there is provided a bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least 1 g /liter in the extract;

wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter; and

ii) a nucleic acid encoding a protein of interest,

wherein said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L and

the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

[0010e] In a tenth aspect, there is provided a method for preparing a bacterial extract, comprising:

i) culturing a bacteria that expresses an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, and

ii) preparing an extract having an active oxidative phosphorylation system, and comprising biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, the exogenous protein disulfide isomerase, and the exogenous peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract, wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and
the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

[0010f] In an eleventh aspect, there is provided a bacterial cell free extract for expressing biologically active proteins comprising an active oxidative phosphorylation system containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase were expressed in the bacteria from which the extract was prepared at a total concentration of at least 1 g/liter in the extract,

wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and

where the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **Figure 1** shows that eukaryotic PDI and bacterial DsbC are functionally interchangeable.

[0012] **Figure 2A** shows a schematic illustration of the chaperone sequential expression screen described in the Examples.

CLAIMS

1. A method of improving the expression levels of biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial S30 extract prepared from *E.coli* that expresses an exogenous disulfide isomerase and an exogenous prolyl isomerase at a total concentration of at least 1 g/liter of extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

ii) incubating the bacterial cell free synthesis system under conditions permitting the expression of the protein of interest to a concentration of at least 100 mg/L,

wherein the bacterial extract has an active oxidative phosphorylation system and comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, and the exogenous disulfide isomerase and the exogenous prolyl isomerase,

wherein the bacteria from which the extract is prepared expresses at least one of the exogenous disulfide isomerase and the exogenous prolyl isomerase from a gene operably linked to a constitutive or inducible promoter,

where the exogenous disulfide isomerase is DsbC, and the exogenous prolyl isomerase is FkpA.

2. The method of claim 1, wherein the extract is prepared from a bacteria that further expresses and comprises an exogenous deaggregase.

3. The method of claim 2, wherein the bacteria from which the extract is prepared was co-transformed with genes encoding the disulfide isomerase and the prolyl isomerase, or the deaggregase.

4. The method of claims 2 or 3, wherein the exogenous deaggregase is Skp.

5. The method of any one of claims 2-4, wherein the bacteria from which the extract is prepared expresses the exogenous deaggregase from a gene operably linked to a constitutive promoter.

6. The method of claim 1 wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

7. The method of claim 1 wherein the protein of interest has at least two proline residues.

8. The method of claim 1 wherein the protein of interest is an antibody or antibody fragment.

9. The method of claim 1 wherein the bacterial cell free synthesis system has a volume of between 0.5 liter and 500 liters and the incubation is of a time period lasting from 1-36 hours.

10. A bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous disulfide isomerase and an exogenous prolyl isomerase, wherein the exogenous disulfide isomerase and the exogenous prolyl isomerase were expressed in the bacteria from which the extract was prepared at a concentration of at least 1 g/liter of extract; and

ii) a nucleic acid encoding a protein of interest,

where said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L,

wherein the bacteria from which the extract is prepared expresses at least one of the exogenous disulfide isomerase and the exogenous prolyl isomerase from a gene operably linked to a constitutive or inducible promoter, and

where the exogenous disulfide isomerase is DsbC, and the exogenous prolyl isomerase is FkpA.

11. The system of claim 10, wherein the bacteria from which the extract is prepared was co-transformed with genes encoding the disulfide isomerase and prolyl isomerase.

12. The system of claim 10 or 11, wherein the bacteria is Escherichia coli.

13. The system of claim 10 or 12, wherein the extract is an S30 extract of E. coli.

14. A method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial extract with a nucleic acid encoding a protein of interest; and

ii) incubating the bacterial extract with the nucleic acid under conditions permitting the expression and proper folding of the protein of interest,

wherein the bacterial extract comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, wherein the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least about 1 g/liter in the extract, and wherein the bacteria from which the extract is prepared expresses at least one of the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and

the exogenous protein disulfide isomerase is DsbC, and the exogenous peptidyl-prolyl cis-trans isomerase is FkpA.

15. The method of claim 14, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of between 1 g /liter and 14 g /liter in the extract.

16. The method of claim 14, wherein the expression of the protein of interest is improved to a concentration above that concentration where one but not both of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present, and the incubation conditions are otherwise the same.

17. The method of claim 14, wherein the bacteria is Escherichia coli.

18. The method of claim 14, wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

19. The method of claim 14, wherein the protein of interest has at least two proline residues.

20. The method of claim 14, wherein the protein of interest is an antibody or antibody fragment.

21. A bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least 1 g /liter in the extract;

wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter; and

ii) a nucleic acid encoding a protein of interest,

wherein said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L and

the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

22. The system of claim 21, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of between 1 g /liter and 14 g /liter in the extract.

23. The system of claim 21, wherein the expression of the protein of interest is improved to a concentration above that concentration where one but not both of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present, and the incubation conditions are otherwise the same.

24. The system of claim 21, wherein the bacteria is Escherichia coli.

25. The system of claim 21, wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

26. The system of claim 21, wherein the protein of interest has at least two proline residues.

27. The system of claim 21, wherein the protein of interest is an antibody or antibody fragment.

28. A method for preparing a bacterial extract, comprising:

i) culturing a bacteria that expresses an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, and

ii) preparing an extract having an active oxidative phosphorylation system, and comprising biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, the exogenous protein disulfide isomerase, and the exogenous peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract, wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and

the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

29. A bacterial cell free extract for expressing biologically active proteins comprising an active oxidative phosphorylation system containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase were expressed in the bacteria from which the extract was prepared at a total concentration of at least 1 g/liter in the extract,

wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive or inducible promoter, and

where the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA.

30. The method of claims 1-9, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

31. The method of claims 1-9, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

32. The method of claims 1-9, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a total concentration of 1 g/liter to 20 g/liter of extract.

33. The system of claim 10, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

34. The system of claim 10, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

35. The system of claim 10, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

36. The method of claim 14, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

37. The method of claim 14, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

38. The method of claim 14, wherein said protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

39. The system of claim 21, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

40. The system of claim 21, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

41. The system of claim 21, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

42. The method of claims 1-9, where the growth rate of the bacteria used to prepare the extract is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.

43. The system of claim 10, where the growth rate of the bacteria that express the exogenous disulfide isomerase and the exogenous prolyl isomerase is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.

44. The method of claim 14, wherein the growth rate of the bacteria from which the extract is prepared is similar to the growth rate of bacteria that do not express at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

45. The system of claim 21, wherein the growth rate of the bacteria from which the extract is prepared is similar to the growth rate of bacteria that do not express at least one of the disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

46. The method of claim 28, wherein the growth rate of the bacteria that expresses the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans isomerase is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.