

CLAIMS

What is claimed is:

1. A thermostable cell-free protein expression system comprising:

- a reaction mixture containing a cell extract derived from a thermophilic microorganism culture having all cell-free reaction components necessary for *in vitro* macromolecule synthesis wherein said thermophilic microorganism is genetically modified for:
 - knock-out expression of OmpT-homologue;
 - knock-out expression of RNaseI;
 - knock-out expression of DNA-methylation dependent DNase;
 - reduce expression of culture density-dependent sporulation operon;
 - overexpress sigma factor RpoD; and
 - overexpress RNA polymerase (RNAP), or any combination thereof
- at least one nucleic acid synthesis template;
- a quantity of purified thermostable RNAP;
- a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of purified Adenosyl Kinase (AdK) enzyme; and
 - a quantity of purified Polyphosphate Kinase (PPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and
 - a quantity of adenosine monophosphate (AMP).
- wherein said AdK and PPK enzymes work synergistically to regenerate cellular ATP energy from PPi and AMP.

2. A cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of thermostable Adenosyl Kinase (Gst AdK) enzyme; and
 - a quantity of thermostable Polyphosphate Kinase (TaqPPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and

- a quantity of adenosine monophosphate (AMP).
- wherein said AdK and PPK enzymes work synergistically to regenerate cellular ATP energy from PPi and AMP.

3. A cell-free protein expression system as described in claim 2 wherein said thermostable Gst Adk enzyme comprises SEQ ID NO. 8.

4. A cell-free protein expression system as described in claim 2 further comprising a quantity of Sorbitol-dehydrogenase (SDH).

5. A cell-free protein expression system as described in claim 2 further comprising a quantity of tRNAs isolated from same type of microorganism used to generate said cell extract.

6. A cell-free protein expression system as described in claim 2 wherein said at least one nucleic acid synthesis template comprises a linear DNA template.

7. A cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of purified Adenosyl Kinase (AdK) enzyme; and
 - a quantity of purified Polyphosphate Kinase (PPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and
 - a quantity of adenosine monophosphate (AMP).
 - wherein said AdK and PPK enzymes work synergistically to regenerate cellular ATP energy from PPi and AMP.

8. A cell-free protein expression system as described in claim 30 wherein said cell extract comprises a thermophilic bacterial cell extract and/or a thermostable bacterial cell extract.

9. A cell-free protein expression system as described in claim 30 further comprising a quantity of thermostable RNA polymerase (RNAP).

10. A cell-free protein expression system as described in claim 30 wherein said nucleic acid synthesis template comprises a linear DNA template.

11. A bacterial cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract derived from a bacterial culture having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of Adenosyl Kinase (AdK) enzyme; and
 - a quantity of Polyphosphate Kinase (PPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and
 - a quantity of adenosine monophosphate (AMP).
 - wherein said AdK and PPK work synergistically to regenerate cellular ATP energy from PPi and AMP.

12. An *in vitro* cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract derived from a culture having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of purified Adenosyl Kinase (AdK) enzyme; and
 - a quantity of purified Polyphosphate Kinase (PPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and
 - a quantity of adenosine monophosphate (AMP).
 - wherein said AdK and PPK work synergistically to regenerate cellular ATP energy from PPi and AMP.

13. An energy supplemented cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a quantity of Sorbitol-dehydrogenase (SDH); and
 - a quantity of sorbitol.

14. A cell-free expression system comprising:

- a cell-free expression system having:
 - a reaction mixture containing a cell extract having all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - a nucleic acid synthesis template;
 - a cellular adenosine triphosphate (ATP) energy regeneration system comprising:
 - a quantity of purified thermostable Adenosyl Kinase (Gst AdK) enzyme; and
 - a quantity of purified thermostable Polyphosphate Kinase (TaqPPK) enzyme.

15. A thermostable energy supplemented cell-free protein expression system comprising:

- a cell-free expression bioreactor having:
 - a reaction mixture containing a cell extract all cell-free reaction components necessary for *in vitro* macromolecule synthesis;
 - at least one nucleic acid synthesis template;
 - a quantity of thermostable Sorbitol-dehydrogenase (tSDH); and
 - a quantity of sorbitol.

16. An inorganic polyphosphate-based energy regeneration system comprising:

- an ATP-dependent system supplemented with the following:
 - a quantity of purified Adenosyl Kinase (AdK) enzyme; and
 - a quantity of purified Polyphosphate Kinase (PPK) enzyme;
 - a quantity of inorganic polyphosphate (PPi); and
 - a quantity of adenosine monophosphate (AMP).

17. An inorganic polyphosphate-based energy regeneration system comprising:

- an ATP-dependent system supplemented with the following:

- a quantity of purified thermostable Adenosyl Kinase (Gst AdK) enzyme; and
- a quantity of purified thermostable Polyphosphate Kinase (TaqPPK) enzyme;
- a quantity of inorganic polyphosphate (PPi); and
- a quantity of adenosine monophosphate (AMP).

18. A method of cell-free protein expression incorporating an inorganic polyphosphate-based energy regeneration system comprising the steps:

- establishing a thermophilic *Geobacillus* microorganism, wherein said thermophilic *Geobacillus* is genetically modified for:
 - knock-out expression of OmpT-homologue;
 - knock-out expression of RNaseI;
 - knock-out expression of DNA-methylation dependent DNase;
 - reduce expression of culture density-dependent sporulation operon;
 - overexpress sigma factor RpoD; and
 - overexpress RNA polymerase (RNAP), or any combination thereof.
- generating a cell culture of said genetically engineered a thermophilic *Geobacillus* microorganism;
- generating a cell lysate of said cell culture;
- generating a cell extract derived from said cell lysate;
- supplementing said cell extract with one or more cell-free reaction components forming a reaction mixture having components necessary for *in vitro* macromolecule synthesis;
- supplementing said reaction mixture with purified tRNAs derived from the same strain as said thermophilic *Geobacillus* microorganism;
- supplementing said reaction mixture with purified tRNAs derived from *E. coli*;
- supplementing said reaction mixture with a quantity of thermostable Sorbitol-dehydrogenase (Gst SDH);
- supplementing said reaction mixture with a quantity of sorbitol;
- supplementing said reaction mixture with a quantity of thermostable Adenosyl Kinase (AdK) enzyme;
- supplementing said reaction mixture with a quantity of thermostable Polyphosphate Kinase (PPK) enzyme;

- supplementing said reaction mixture with a quantity of inorganic polyphosphate (PPi);
- supplementing said reaction mixture with a quantity of adenosine monophosphate (AMP);
- supplementing said reaction mixture with a quantity of purified thermostable RNAP (Gst RNAP);
- introducing a linear DNA synthesis template configured to generate one or more expression products;
- incubating said reaction mixture in a cell-free expression bioreactor;
- generating said one or more expression products in said cell-free expression bioreactor; and
- isolating and purifying one or more of said expression products from said reaction mixture.

19. A method of generating a protein through a cell-free expression system comprising the steps:

- generating a cell culture of a thermophilic *Geobacillus* microorganism;
- generating a cell lysate of said cell culture;
- generating a cell extract derived from said cell lysate;
- supplementing said cell extract with one or more cell-free reaction components forming a reaction mixture having components necessary for *in vitro* macromolecule synthesis;
- supplementing said reaction mixture with a quantity of thermostable Adenosyl Kinase (AdK) enzyme;
- supplementing said reaction mixture with a quantity of thermostable Polyphosphate Kinase (PPK) enzyme;
- supplementing said reaction mixture with a quantity of inorganic polyphosphate (PPi);
- supplementing said reaction mixture with a quantity of adenosine monophosphate (AMP);
- introducing a nucleic acid synthesis template configured to generate one or more expression products;
- incubating said reaction mixture in a cell-free expression bioreactor;

- generating said one or more expression products in said cell-free expression bioreactor; and
- isolating and purifying one or more of said expression products from said reaction mixture, wherein in said expression product is a protein.

20. A genetically modified thermophilic microorganism for use in a cell-free expression system wherein said thermophilic *Geobacillus* strain is genetically modified for:

- knock-out expression of OmpT-homologue;
- knock-out expression of RNaseI;
- knock-out expression of DNA-methylation dependent DNase;
- reduce expression of culture density-dependent sporulation operon;
- overexpress sigma factor RpoD; and
- overexpress RNA polymerase (RNAP), or any combination thereof.